

ORIGINAL ARTICLE

Egg-white-specific IgA and IgA2 antibodies in egg-allergic children: Is there a role in tolerance induction?

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Abstract

Background: Decreased serum food-specific IgA antibodies have been associated with allergic disease in cross-sectional, case-control studies. The purpose of this study was to prospectively compare egg-white-(EW)-specific IgA and IgA2 levels between egg-allergic children and children tolerating egg.

Methods: Seventeen egg-allergic children were followed prospectively. Total IgA, EW-specific IgA, and EW-specific IgA2 levels were measured in their sera with a sensitive ELISA. As negative controls were used children with no previous history of egg allergy. Egg-allergic children with or without concomitant milk allergy were evaluated as additional controls with measurement of casein-specific IgA.

Results: After 2.5 ± 0.9 yrs, nine out of the 17 allergic children became tolerant and eight remained allergic to baked egg. Baseline EW-specific IgA2 levels were significantly lower in the egg-allergic subjects (median 23.9 ng/ml) compared with the negative control subjects (99.4 ng/ml) and increased significantly by 28% over the study time period in eight out of the nine allergic children that became tolerant to baked egg. There was no significant change over time in EW-specific IgA in any of the study groups. Non-milk-allergic subjects with concomitant egg allergy had almost threefold higher casein-specific IgA levels than the milk- and egg-allergic subjects ($p = 0.025$).

Conclusions: These results suggest a potential role for allergen-specific IgA2 antibodies in the induction of food tolerance. Furthermore, they support the hypothesis that immature or impaired production of allergen-specific IgA2 may be associated with the pathophysiology of food allergy, a defect that seems to be selective for the culprit allergen.

Immunoglobulin A (IgA) in its secretory form has a fundamental role in the immune response at mucosal surfaces. It provides a first-line defense by aggregating, immobilizing, and neutralizing pathogenic microbes and harmful molecules that interact with mucosal surfaces, a process that is known as immune exclusion (1).

Food proteins are among the molecules that reach human mucosal tissues and may induce allergic responses; these proteins may also interact with IgA. A defective IgA response has been hypothesized to be associated with having IgE responses to foods (2). However, the contribution of IgA to the clinical expression of allergy has not been elucidated.

Patients with selective (3), partial IgA (4), or transient (5) IgA deficiency, or even patients with IgA levels at the lowest normal limit for their age (6), have a relatively high prevalence

of allergies, including food allergy. In addition, IgA production may be associated with oral tolerance (7). This evidence suggests that IgA insufficiency, not only in mucosa but also in serum, may be associated with allergies.

In grass-allergic patients with respiratory allergies, serum grass-specific IgA and IgA2 levels have been shown to increase in parallel with an improvement in their respiratory symptoms after they have been treated with subcutaneous (8, 9) or sublingual immunotherapy (10). This suggests that an immaturity in the regulation of IgA class and subclass switching may contribute to the clinical expression of allergy.

In this study, we sought to prospectively investigate two hypotheses relating specific IgA and food-allergic responses. Our first hypothesis is that egg white (EW) allergy is related to a deficient EW-specific IgA response. To investigate this

hypothesis, we compared EW-specific IgA and EW-specific IgA2 levels in EW-allergic children, who developed tolerance to baked egg, and children with no previous history of egg allergy. We additionally hypothesized that there is allergen specificity to the relationship of IgA deficiency and allergy. To evaluate this hypothesis, casein-specific IgA levels were determined in egg-allergic children with or without concomitant milk allergy.

Patients and methods

Subjects

In a study conducted previously by our group (11), children with IgE-mediated allergy to baked egg were followed prospectively until they became tolerant (confirmed by an oral food challenge) to foods containing baked egg (muffin and waffle) (*experimental group*). All patients with available baseline (1st measurement corresponding to the challenge they reacted) and follow-up (2nd measurement corresponding to the first challenge they did not react) serum samples were included (samples were obtained just before oral food challenges). Samples were tested for total IgA, EW-specific IgE, IgG4, IgA and IgA2. Controls consisted of two groups: (i) egg-allergic children that remained reactive to egg in baked goods (*positive controls*) and (ii) atopic children that regularly consume eggs, without a previous history of egg allergy and at least two serum samples from two different time points similar to the study group (*negative controls*).

To investigate whether there is a selective EW-specific impairment or a universal defect, we measured casein-specific IgA levels in children allergic to egg with or without concomitant milk allergy. These serum samples were selected randomly from our serum repository.

The study was approved by the Mount Sinai Institutional Review Board, and informed consent was obtained from the study subjects' parents or guardians.

Serum EW-specific IgE and IgG4 levels

Egg white -specific IgE (lower level of detection 0.1 IU/ml) and IgG4 levels (lower level of detection 0.001 µg/l) were measured with the UniCAP system (ThermoFisher Scientific, Portage, MI, USA).

Serum total IgA and EW-specific IgA and IgA2 ELISA

Total and allergen-specific IgA concentrations were determined by sandwich ELISA. 96-well Immulon 4HBX plates (Fisher Scientific, Pittsburgh, PA, USA) were coated overnight at 4°C with 100 µl of the following: (i) an α -chain-specific goat F(ab)² anti-human IgA (InvivoGen, San Diego, CA, USA) antibody (primary antibody) at 2 µg/ml for generating the standard curve (range 0.391–50 ng/ml) and for total IgA measurements, (ii) hen's EW (Eminent Services Corporation, Frederick, MD, USA) at 5 µg/ml (500 ng/well) for detection and measurement of EW-specific IgA, and (iii) casein (Sigma Aldrich, St Louis, MO, USA) at 2 µg/ml (200 ng/well) for detection and

measurement of casein-specific IgA, all diluted in 0.05 mol/l carbonate-bicarbonate buffer (pH 9.6). After washing ($\times 3$) with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), the plates were blocked with 200 µl/well of 2% BSA in PBS-T (blocking buffer) at 31°C for 60 min. Standard [native monomeric IgA1 antibodies isolated from human plasma (GenWay Biotech Inc, San Diego, CA, USA)] and serum samples (prepared in a series of twofold dilutions in blocking buffer starting from 1/5 for IgA2 measurements, 1/40 for specific IgA and $1/16 \times 10^4$ for total IgA) were pipetted in triplicates of 100 µl and incubated at 31°C for 2 h. After washing ($\times 3$) with PBS-T, 100 µl of IgA1 protease (Boca Scientific Inc, Boca Raton, FL, USA), diluted to 0.8 µg/ml in blocking buffer, was added to appropriate wells for EW-specific IgA2 measurement and incubated at 37°C for 2 h. All other wells were filled with blocking buffer. Following washing as before, 100 µl/well of goat anti-human IgA – HRP Fc specific (Antibodies Online, Atlanta, GA, USA) diluted 1/2000 in blocking buffer was applied for 60 min at 31°C. After thorough washing ($\times 6$) with PBS-T, 100 µl/well of 2,2'-azino-bis (3-ethylbenzthiazolinesulfonic acid) peroxidase substrate (ABTS, KPL, Inc, Gaithersburg, MD, USA) was added and allowed to develop at 31°C for 60 min. Absorbance values were read at 405 nm using SOFTMAX PRO© software (for experiment optimization conditions see online repository). This indirect IgA2 measurement strategy from the proteolytic cleavage of IgA1 was adopted to produce comparable measurements for IgA and IgA2 and assess in parallel their potential longitudinal increase or decrease trend, as the absorbance values for both these variables were read from the same standard curve.

Statistical analysis

Descriptive statistics are presented as median (interquartile range) for non-normally distributed continuous variables and as mean $\pm$ standard deviation for normally distributed variables. The distribution of the variables of interest was assessed with the Shapiro–Wilk's test. The Wilcoxon rank-sum test and Kruskal–Wallis tests were used to compare continuous variables and the Fisher's exact test was used to compare categorical variables among studied groups. The Wilcoxon matched-pairs rank-sum test was used for comparing continuous variables within the same individual between the first and second measurement. All correlations were evaluated by calculating the Spearman's rho correlation coefficient. All reported p-values are based on 2-sided tests and compared with a significance level of 5%. STATA 9.1 for Windows (Stata Corp LP, College Station, TX, USA) and GRAPHPAD PRISM version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical calculations and plots.

Results

Baseline and demographic characteristics of the subjects

Seventeen subjects with both baseline and follow-up serum samples were available. Nine of these subjects became tolerant

(*experimental group*) and eight remained reactive to baked egg (*positive controls*) over a mean $\pm$ s.d. follow-up period of 2.5 ± 0.9 yrs. Fourteen children without egg allergy served as *negative controls*. In addition, six egg-allergic children with and six without concomitant milk allergy were used to measure casein-specific IgA levels.

Subjects from the two control groups were gender- and age-matched and had serum samples available from time points comparable with that of the experimental group to account for age-dependent IgA fluctuations expected in the age range of the current study (Table 1) (12, 13).

There were no differences between the experimental group and the positive controls in baseline: EW-specific IgE ($p = 0.387$), EW-specific IgG4 ($p = 0.164$), total IgA ($p = 0.923$), EW-specific IgA ($p = 0.441$), and EW-specific IgA2 ($p = 0.165$) levels. In contrast, EW-specific IgG4 and EW-specific IgA2 levels were significantly higher in negative controls as opposed to either the experimental group or the positive controls (Table 1).

Humoral changes over time

Total serum IgA and EW-specific IgA did not change significantly over time in any of the groups. In contrast, EW-specific IgA2 levels increased significantly, over 2.6 ± 0.9 yrs, only in the experimental group from median (IQR)

23.9 (18.9–44) ng/ml to 30.6 (22.8–45.3) ng/ml, $p = 0.038$ corresponding to a median increase of 28% per child (Table 1). EW-specific IgA2 increased in eight of the nine subjects from the experimental group but only in three out of the eight positive control ($p = 0.027$) (Fig. 1a,b). A negligible but statistically significant increase of 3.6% per child was observed for EW-specific IgG4 in the experimental group [from 0.28 (0.01–0.29) μ g/ml to 0.29 (0.03–0.68) μ g/ml] (Table 1).

Egg white -specific IgG4 and EW-specific IgA2 levels were significantly correlated in the subjects from the experimental group both before and after they became tolerant (Fig. 2a,b). This correlation was even stronger when assessed in all studied subjects (1st measurement: Spearman's $\rho = 0.935$, $p < 0.001$; 2nd measurement: Spearman's $\rho = 0.834$, $p < 0.001$) but non-significant between EW-specific IgG4 and EW-specific IgA in both measurements (1st measurement: Spearman's $\rho = 0.325$, $p = 0.203$; 2nd measurement: Spearman's $\rho = 0.362$, $p = 0.187$). No significant correlation was found between EW-specific IgA2 or EW-specific IgA with EW-specific IgE and total IgA.

Low specific IgA levels appear to be allergen specific

Casein-specific IgA antibodies in the milk-tolerant but egg-allergic subjects were almost three times higher than in the milk- and egg-allergic subjects ($p = 0.025$) (Table 2).

Table 1 Baseline characteristics of study subjects, total serum IgA, and egg-white-(EW)-specific IgE, IgG4, IgA, and IgA2 levels: between- and within-groups comparisons at two different time points (1st and 2nd measurement). Experimental group: subjects who developed tolerance to baked egg, positive controls: subjects allergic to baked egg in both measurements and negative controls: egg-tolerant subjects in both measurements

	Measurements	Experimental group (n = 9)	Positive controls (n = 8)	Negative controls (n = 14)	p-value*
Age (yrs)		8.5 ± 2.6	6.4 ± 4.3	8.9 ± 3.2	0.188
Time† (yrs)		$t = 2.6 \pm 0.9$	$t' = 2.3 \pm 1$	$t'' = 2.5 \pm 1.3$	0.538
Gender (male)		7	7	9	0.585‡
EW-specific IgE (IU/ml)	1st	6.1 (1.99–8.76)	15.45 (1.42–32.6)	<0.35	0.387§
	2nd	6.3 (2.9–8.3)	10.6 (1.27–39.3)	<0.35	0.491§
p-value¶		0.953	0.398	–	
EW-specific IgG4 (μ g/l)	1st	0.28 (0.01–0.29)	0.78 (0.35–0.91)	6.02 (3.53–10.5)	0.007
	2nd	0.29 (0.03–0.68)	0.23 (0.12–0.7)	6.57 (3.46–9.86)	0.025
p-value¶		0.012	0.465	0.853	
Total IgA (mg/ml)	1st	1 (0.5–1.7)	1 (0.6–1.7)	0.8 (0.6–1.3)	0.892
	2nd	1.1 (0.5–2)	0.9 (0.9–2.5)	0.9 (0.7–1.4)	0.780
p-value¶		0.575	0.269	0.679	
EW-specific IgA (ng/ml)	1st	134.7 (113–189.1)	111.3 (67.9–196.8)	182.1 (109.8–213.7)	0.437
	2nd	176.1 (101.1–208.6)	152.3 (67.5–244)	132.5 (89–198.2)	0.665
p-value¶		0.086	0.208	0.198	
EW-specific IgA2 (ng/ml)	1st	23.9 (18.9–44)	35.5 (29.7–54.7)	99.4 (66–156.2)	<0.001
	2nd	30.6 (22.8–45.3)	45.2 (34.8–65.4)	95.8 (62.3–160.5)	0.002
p-value¶		0.038	0.124	0.877	

Values are presented as mean $\pm$ s.d. or medians (interquartile range).

*p-values based on Kruskal–Wallis test (comparison among all groups) unless otherwise indicated. In bold are the values contributing to the significant p-values.

†From 1st to 2nd measurement.

‡Fisher's exact test.

§Wilcoxon rank-sum test (comparison between experimental group and positive controls).

¶Wilcoxon matched-pairs signed-rank test (comparison between 1st and 2nd measurement for each of the studied group).

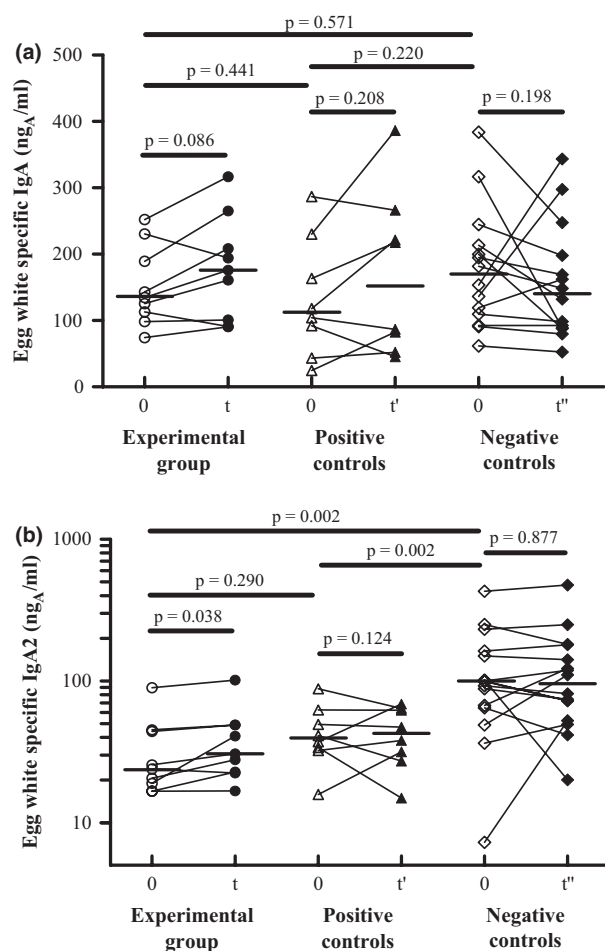


Figure 1 Comparisons between first (open symbols) and second (filled symbols) measurements in the studied groups (experimental group: subjects who developed tolerance to baked egg, positive controls: subjects allergic to baked egg in both measurements and negative controls: egg-tolerant subjects in both measurements). (a) for egg-white-specific IgA and (b) for egg-white-specific IgA2. (Lines in between correspond to medians. t , t' , and t'' time points are defined in Table 1).

Because of the significantly different casein-specific IgA levels between milk and non-milk-allergic subjects, casein-specific IgA2 levels were not measured.

Discussion

This study demonstrates a qualitative difference of the serum EW-specific IgA2 in egg-allergic children as opposed to children consuming regular egg and products with baked-in egg. We also report a significant and time-independent increase in serum EW-specific IgA2 in almost all the children who developed tolerance to baked egg (*experimental group*), while this was not the case in most of the children who remained egg intolerant.

Total serum EW-specific IgA level was not different among the groups. This is in agreement with previous

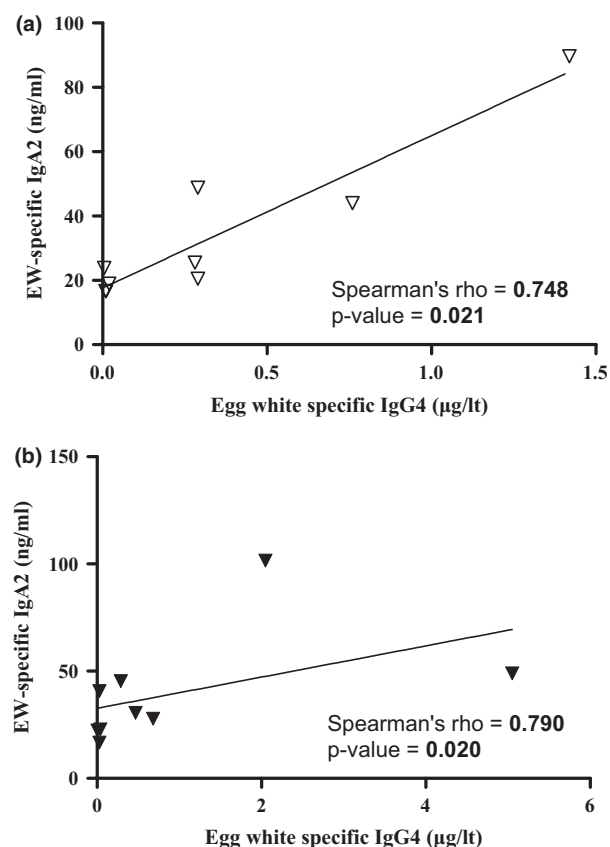


Figure 2 Correlation scatter plots between serum egg-white (EW)-specific IgG4 and serum egg-white-specific IgA2 in the experimental group (subjects who developed tolerance to baked egg) at the 1st measurement (subjects were allergic to baked egg, graph a) until they became tolerant (2nd measurement, graph b).

Table 2 Total and casein-specific IgA levels: comparison between milk-allergic and tolerant children. All subjects were allergic to egg white.

	Milk tolerant n = 6	Milk allergic n = 6	p-values*
Age (yrs)	4 ± 2.6	5.5 ± 2.9	0.389
Gender (boys)	4	5	>0.999
Serum total IgA (mg/ml)	0.4 (0.3–1.1)	1.4 (0.4–1.9)	0.262
Casein- specific IgA (ng/ml)	836.1 (510.9–1013.5)	312.9 (134.7–497.1)	0.025

Values presented as median (interquartile range).

*p-values based on Wilcoxon rank-sum test.

studies examining specific IgA to various food allergens. IgA antibodies to cow's milk proteins, for example, were found to be similar in healthy and in allergic individuals in almost all studies (14–20). One study demonstrated higher levels of

casein-specific IgA antibodies in milk-allergic individuals than in controls (21), and another study showed lower casein- and β -lactoglobulin-specific IgA levels in children with allergy to cow's milk than in non-allergic children (22). Moreover, peanut-specific IgA antibodies did not differ between peanut-sensitized and peanut-allergic patients and control subjects (23). A clinically irrelevant increase in serum peanut-specific IgA antibodies was observed in peanut-allergic patients after 12 months of sublingual immunotherapy (24).

House-dust-mite-(HDM)-allergic children had significantly lower HDM-specific IgA antibodies than the controls, while sublingual immunotherapy appeared to stimulate allergen-specific IgA production (25). A significant increase in HDM-specific IgA level was also observed in another cohort of HDM-allergic individuals, 70 days after they started subcutaneous immunotherapy (26). A similar effect was shown in grass-allergic patients (8–10) with the very interesting finding that IgA2 increased in association with clinical improvement (9).

To our knowledge, the current study is the first to simultaneously evaluate IgA and IgA2 humoral responses in serum of egg-allergic and egg-tolerant children. The induction of EW-specific IgA2 in parallel with EW-specific IgG4 increase deserves attention for several reasons. First, in keeping with the aeroallergen immunotherapy studies (8–10), increases in IgA2 were more pronounced (28%) compared with the negligible increases in IgG4 (3.6%) in children that became tolerant to baked egg. It was also evident that these significant increases occurred in all but one of the egg-allergic children that became egg tolerant mostly in the experimental group rather than the children who remained egg intolerant, while it was a non-significant, random phenomenon in the negative controls.

Second, both IgG4 and IgA have been shown to exhibit allergen-specific inhibitory activity demonstrated *ex vivo* and *in vitro* in biologic assays such as basophil histamine release assays (27, 28). There are also *in vivo* studies in which this inhibitory activity has been shown directly or indirectly (8, 29–31). It has been shown that lesser amounts of neutralizing-specific IgG4 antibodies are needed to inhibit immunologic reactivity compared with greater amounts of neutralizing-specific IgA antibodies on mucosal surfaces (30). The distribution of the two IgA subclasses varies between serum (80–85% IgA1 monomers) and mucosal surfaces (50–60% IgA2 dimers or polymers). The disproportionate increase in specific IgG4 and IgA2 found in the current study may reflect these different site-specific needs in the regulation of what is expected as a normal immunologic response. It could be hypothesized that an intestinal origin of IgA2 may account for this increase. Such an increase has been shown in patients with celiac disease in which jejunal IgA2 immunocytes were significantly increased in both untreated and treated individuals, as compared to healthy controls, and were highly correlated with serum levels of gluten-specific IgA (32). Moreover, it has been shown that there might be a mucosal induction of regulatory T cells or a general activation and expansion of these cells in response to cow's milk proteins in children with outgrown milk allergy

(33). It could be assumed that oral tolerance induction to EW involves an active immune response in duodenal mucosa, with stimulation of both regulatory T cells and IgA plasma cells. It would be of great interest to further investigate this hypothesis by determining the origin and the proportion of the different subclasses (IgA1 and IgA2) and forms (monomeric and dimeric IgA) of EW-specific IgA antibodies.

Third, the negligible increase of only 3.6% in serum EW-specific IgG4 was significant only in the individualized longitudinal approach and did not differ significantly in the unpaired comparison. This finding might explain why the absolute numbers of specific IgG4 levels do not seem to be predictive of tolerance and are not recommended for the diagnostic evaluation of food allergy (34). Nevertheless, studies with more subjects are needed to substantiate this assumption.

Increases in EW-specific IgG4 levels parallel EW-specific IgA2 levels. Allergen-specific IgG4 antibodies increase with exposure to the particular allergen (35). Taking into account the high correlation of IgG4 and IgA2 (Spearman's rho coefficients > 0.8), we could speculate that IgA2 may also increase with exposure. Interestingly, EW-specific IgA2 increased in most of the children who finally became baked egg tolerant, while this was not the case in the children who remained intolerant. This could happen either due to the lack of adherence to the suggested egg-free diet or due to the exposure to small quantities of eggs that were unable to induce allergic symptoms and signs, but were sufficient to stimulate the production of inhibitory specific-IgA2 antibodies and raises the question whether adherence *per se* could be responsible for the outcomes observed.

A significant IgA2 antibody increase has been shown to be an allergen-immunotherapy-specific-induced phenomenon (9). This subclass switching regulation appears to be complex (36). The chronological evolution of the sequential IgA1-to-IgA2 class switch recombination can explain why this process needs more time to mature. In addition, it reflects the need for serum EW-specific IgA2 production or EW-specific IgA2 secretion to induce tolerance taking into account the fact that secretory IgA2 dimers are functionally more resistant to proteolytic cleavage of the mucosal proteases than secretory IgA1 (37) and thus more efficient.

It is not clear if these decreased levels of EW-specific IgA reflect an EW-specific B-cell deficiency, impaired B-cell-dependent IgA production, a T-helper activation impairment, or simply a finding explained by lack of exposure to egg white. In an *ex vivo* study with peripheral blood mononuclear cells from egg-allergic children 0–6 yrs of age (38), it was shown that cells producing IgA specific to ovalbumin (OVA) were significantly less in egg-allergic subjects, while *ex vivo* production of OVA-specific IgA levels from the patients' cells was significantly lower. However, patients' B cells cultured with supernatants from OVA-stimulated normal T cells were able to produce OVA-specific IgA levels comparable with those of normal B cells, which was not the case with patients' T cells and normal B cells. These findings indicate that the impairment may lie at the T-cell cytokine production level, rather than reflecting an exposure effect.

The decreased levels of specific IgA seem to be allergen specific. Casein-specific IgA was significantly higher in EW-allergic children without milk allergy as opposed to children with concomitant EW and cow's milk allergy. Exposure itself does not seem enough to explain this finding, taking into consideration the findings from the previous study (38) and the fact that EW-specific IgA levels in the current study were comparable in all groups examined, no matter the level of exposure (avoidance in the experimental group, consumption of regular egg in negative control subjects).

In conclusion, our findings suggest a potentially important role of allergen-specific IgA and especially allergen-specific IgA2 antibodies in the induction of food tolerance. Furthermore, they support an immaturity or impairment of allergen-specific IgA2 production associated with food allergy pathophysiology, a defect that seems to be selective for the culprit allergen. Further studies are needed to validate these findings and elucidate IgA and IgA2 role in pathophysiology of food allergy.

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Conflict of interest

All authors do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Online repository.

Figure S1. Eight standard curves fitted with a five-parameters logistic model: four of them were run the same day and four 2 days after (concentration range 0.391–50 ng/ml).

Online Repository

Optimization of the experimental conditions

The concentrations of the coated antibodies/antigens were optimized by checkerboard titration. The primary antibody was evaluated for binding performance at 1, 2, 4, 5 and 8 $\mu\text{g/ml}$. Similar concentrations were compared for the EW and casein binding performance. A concentration of 2 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$, respectively were found to be enough for the coating step.

The different blocking buffers tested included i) 1% human serum albumin (HSA) in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T)~~PBS~~ and 5% goat serum albumin (~~NGS~~A), ii) 1% HSA in PBS and 10% goat serum albumin (~~NGS~~A), iii) 1% HSA in PBS-T, iv) 1% BSA in PBS and 10% ~~NGS~~A, v) 2% ~~NGS~~A in PBS-T, vi) 1% normal sheep serum (NSS) in PBS-T, vii) 2% NSS in PBS-T, viii) 1% BSA in PBS-T and ix) 2% BSA in PBS-T. The comparison of the nonspecific binding, defined by the blank wells used, showed that 2% BSA in PBS-T was the best blocking buffer (wells with primary and secondary antibodies without serum, optical density (OD): 0.072-0.081). There was no nonspecific binding for the wells coated with EW (OD=0).

Human IgA exists in two subclasses: IgA1 and IgA2. The major difference between these two subclasses is the resistance of IgA2 to IgA1-specific proteases that are released from several bacteria. This feature is based on structural differences in the hinge region of IgA (12). The specificity of IgA1 protease and the appropriate concentrations of IgA1 protease to cleave all the existing IgA1 antibodies has been previously examined (13). However, the appropriate concentration was reassessed in this new assay based on the manufacturer's recommendations. The final ODs after

applying different IgA1 protease concentrations (0.1, 0.2, 0.4, 0.8 and 1.6 µg/ml) to the same serum samples were compared. A plateau was achieved with at least 0.8 µg/ml of IgA1 protease, suggesting complete cleavage of existing EW-specific IgA1 antibodies. Increasing the IgA1 protease incubation period from 2 hours to 4 or 8 hours did not change the final ODs measured. Thus a 2 hour incubation period was used. Serum total IgA, EW-specific IgA and EW-specific IgA2 were measured on the same plate utilizing the same standard curve for every subject. This was the reason the IgA1 protease approach was followed, instead of directly measuring specific IgA2 with an anti-human IgA2 as a secondary antibody. Whether applying blocking buffer for 2 extra hours in wells that were not incubated with IgA1 protease at 37°C affected the estimated concentrations of total and specific IgA was tested, and there was no difference.

Standard curves were generated using eight different fitting mathematical models (semi-log, log-log, fully specified log-logit, exponential, quadratic, 4-parameter logistic-log and 5-parameter logistic-log). Based on the coefficient of determination R^2 , which provides the proportion of variability in a data set that is accounted for by the statistical model, the 5-parameter logistic model¹ was most accurate with values $R^2=1$ in all cases. **Figure E1** presents eight standard curves: four of them were run on the same date in different plates and four of them two days later. Mean OD values, after adjusting for the unspecific binding (blank wells), ranged from 1.308 to 1.407 for the upper limit of the standard curve (50 ng/ml) and from 0.006 to 0.009 for the lower part of the standard curve (0.391 ng/ml). The coefficients of

$$^1 y = \frac{A-D}{[1+(\frac{x}{C})^B]^G} + D$$

variation ranged from 0.7 to 6 and from 1 to 10.7, respectively. Unknown serum samples coefficients of variation followed the same distribution (data not shown).

Figure E1. Eight standard curves fitted with a 5-parameters logistic model: four of them were run the same day and four two days after (concentration range 0.391-50 ng/ml).

