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**PEDIATRIC ALLERGY AND IMMUNOLOGY**

# High levels of $IgG_4$ antibodies to foods during infancy are associated with tolerance to corresponding foods later in life

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Children with eczema and sensitization to foods are recommended skin care and, if food allergy is proven by challenge, an elimination diet. For most children the diet period is transient, but the process behind tolerance development and the influence of decreased allergen exposure is not fully known. The aim of the study was to investigate the effect of elimination diet on serum and salivary antibodies and to identify immunological parameters related to the ability to tolerate foods. Eighty-nine children, below 2 yr of age, with eczema and suspected food allergy were included. Recommended treatment was skin care to all children, and 60 children had a period of elimination diet. At 4½ yr of age, the children were divided into two groups, based on if they had been able to introduce the eliminated foods, or not. Serum and salivary antibodies were analyzed with enzyme-linked immunosorbent assay and UniCAP® before and after a 6-wk treatment period and at 4½ yr of age. Children sensitized to egg and/or milk that could eat and drink the offending foods at 4½ yr of age, had higher levels of Immunoglobulin $G_4$ antibodies to ovalbumin and β-lactoglobulin and also higher $IgG_4$/Immunoglobulin E ratios on inclusion in the study, than those who had to eliminate egg and/or milk from their diet, beyond 4½ yr of age. The highest $IgG_4$/IgE ratios were found in children with circulating IgE antibodies to egg and/or milk but negative skin prick test on inclusion. The 6-wk treatment period did not significantly affect the levels of serum and salivary antibodies. In conclusion, eczematous, food sensitized infants with high levels of $IgG_4$ and high ratios of $IgG_4$/IgE antibodies to food allergens are more likely to consume these foods at 4½ yr than infants with low levels and ratios.

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Chronic, relapsing eczema (1), which is often the first manifestation of an atopic phenotype in small children, is commonly associated with sensitization to food allergens. β-Lactoglobulin (BLG) in cow's milk and ovalbumin (OVA) in hen's egg are common allergenic food proteins encountered during infancy. These food items are also important sources of nutrients in childhood. When sensitization to a food allergen is documented in an eczematous child, e.g. by a positive skin prick test (SPT), a temporary elimination of the suspected allergen from the diet is often considered because of food allergy and this should be confirmed by a food challenge (2, 3). Most children will outgrow their food allergy, i.e. develop clinical tolerance before the age of five. However, the benefit of dietary strategies on allergy preventive effects has recently been questioned (4). Moreover, recent studies have suggested that administration of increasing oral

doses of the offending food can promote faster tolerance induction in food-allergic children [reviewed in Ref.(5)].

Prospective studies have shown that Immunoglobulin E antibodies to foods are commonly detected both in atopic and non-atopic infants during their first year of life, although the magnitude of the responses is higher and of longer duration in the former group (6). In most cases, the initial IgE responses to foods are downregulated at the age of 2–4 yr (6). The allergic reaction is triggered when allergens cross-link specific IgE antibodies on mast cells and basophils, but other immunoglobulin isotypes than IgE have also been linked to atopic disease.

Immunoglobulin G antibodies to food allergens are produced in both atopic and non-atopic children. IgG antibody responses to food allergens are observed already at birth and are at that time maternally derived. The children's own IgG antibody production to food allergens peak in early childhood and decline by 8 yr of age (7, 8). Allergic symptoms and atopic sensitization are associated with high levels of specific IgG subclass antibodies to allergens, particularly $IgG_4$ (7). The production of IgE and $IgG_4$ antibodies is regulated by similar mechanisms, e.g. Interleukin (IL)-4 from Th2 cells induces both IgE and $IgG_4$ switching in B-cells (9). In contrast, IL-10 inhibits IgE production but up-regulates the secretion of $IgG_4$, suggesting different ways to control the IgE and $IgG_4$ production (10). High exposure to some airborne allergens may favor immunological tolerance development to such allergens through a modified Th2 response characterized by a high $IgG_4$/IgE ratio (11). However, less is known about food allergens. The IgG antibody responses to BLG have been shown to develop more slowly in infants if the introduction of cow's milk is delayed (8). Furthermore, even a temporary neonatal exposure to cow's milk induces high IgG production to cow's milk up to 2 yr of age (12).

The pre dominant mucosal immunoglobulin in humans is IgA, outlining the inner surfaces of the body and preventing adherence and penetration of antigens through the mucosal epithelium. Theoretically, high levels of IgA antibodies to allergens could prevent allergen absorption and thereby sensitization and subsequent development of allergy. In line with this, low levels of IgA and transient IgA deficiency have been associated with an increased risk of allergy (13), although this is controversial (14). The levels of total secretory IgA (SIgA) in saliva increase with age (15), a development that has been suggested to occur more slowly in allergic than in non-allergic children. IgA antibodies to allergens are found in both allergic and non-allergic children, and the levels of IgA antibodies to foods are downregulated with age (15).

The aim of this study was to identify immunological differences between infants with clinical signs of eczema and sensitization to food allergens, who at 4½ yr of age could consume the food or not. The objective was further to investigate the effect of an elimination diet on serum and saliva antibodies.

## Methods

### Subjects

Between June 1999 and September 2001, 123 children were invited to participate in a prospective multicenter study of clinical and immunological development in young children with eczema. A comprehensive description of the cohort and clinical aspects during the first treatment period has been described in detail elsewhere (16). All children were under 2 yr of age on inclusion [mean and range: 8.3 (2–23) months], and they were examined three times: at inclusion, after a treatment period of 6 wk and at 4½ yr of age [mean and range: 4.5 (3.1–6.6) yr)]. The diagnosis of eczema on inclusion was established using the Hanifin–Rajka criteria, and the eczema severity was assessed at each visit using the severity scoring of atopic dermatitis (SCORAD) system. All children were recommended treatment with emollients and/or topical steroids. SPT to fresh hen's egg white and cow's milk were performed on inclusion and at the 4½ yr examination. The present study included 89 children, based on the availability of serum samples obtained on inclusion, and 29 of them had negative SPT to both egg and milk. Twenty-four children had positive SPT (≥3 mm) to egg only, 11 were positive to milk only, and 25 children had positive SPT to both egg and milk. Median SPT diameters for egg were 8.0 mm (range 4.0–17.0) and 6.3 mm (range 3.0–11.0) for milk. Children with eczema and sizeable SPT were recommended a transient elimination diet. As reported previously, the treatments lead to improvement of the eczema in both SPT positive and negative children (16). Thirty-seven of the initially egg SPT positive children consumed egg at 4½ yr of age, while 28 of the initially milk SPT positive children consumed milk, introduced into their diet either by the parents at home or after a negative, standardized food challenge test (17). These groups will be referred to as food tolerant at 4½ yr. Thirteen of the initially SPT

positive children had not been able to discontinue the egg and/or milk elimination diet at 4½ yr of age because of a positive standardized food challenge test (17) (n = 3) or recent allergic reactions after an accidental exposure to the offending foods during the last 6 months (n = 10). Seven out of 13 children reacted to egg only, two children reacted to milk only and four children reacted to both egg and milk. Reported symptoms at accidental exposure to egg or milk were reactions from the skin (n = 9) combined with anaphylactic symptoms (n = 1), gastro-intestinal symptoms (n = 3), and asthma symptoms (n = 3). One child reported throat itching and difficulties to swallow. These children, reacting at food challenges or at recent accidental exposure, will be referred to as non-tolerant. Data from the 4½ yr follow-up were missing in one and two of the initially egg and milk SPT positive children, respectively. There were no differences between the two groups of children regarding heredity or SCORAD values, neither on inclusion, nor the 4½ yr follow-up. All children were not included in all analysis because of missing samples, or inadequate sample size.

### Collection of samples

Venous blood and saliva samples were obtained on inclusion and at the 6-wk and 4½ yr follow-ups. Sera were collected after allowing the blood to clot at room temperature and were then stored at −20°C until analysis. Unstimulated saliva was collected from the oral cavity of the infants using a hand-pump connected to a thin plastic tube and immediately frozen and stored at −20°C. Before analysis, the saliva samples were heated in water at 51°C for 30 min and then centrifuged at 5000 ***g*** for 15 min.

### Total and specific IgE levels

Serum levels of total and egg- and milk-specific IgE antibodies were analyzed with UniCAP®, according to the recommendations of the manufacturer (Phadia, Uppsala, Sweden). The cut-off level for total IgE was 2 kU/l and test results at values ≥0.35 kUA/l were considered positive for specific IgE.

### $IgG_1$ and $IgG_4$ antibody levels to food allergens

$IgG_1$ and $IgG_4$ antibodies to BLG and OVA in serum were determined as described earlier (18), except that blocking was performed with bovine serum albumin (BSA) (Fraction V; Sigma-Aldrich Stockholm, Sweden) instead of human serum albumin (HSA). The effect of BSA as a blocking agent has been extensively examined with the same or better results in blanks and individual controls when compared with HSA. The serum samples were diluted 1:25 to 1:10,000. Values were expressed in arbitrary units (AU)/ml deduced from the optical density (OD) of a standard curve after subtracting the blanks. The standard was obtained from an individual with high $IgG_1$ or $IgG_4$ antibody titers to BLG and OVA. A coefficient of variation (CV) below 15% was accepted for duplicate samples. A control sample was included in every analysis, and the interassay CV was 21% for $IgG_4$ to OVA and BLG, 11% for $IgG_1$ to OVA and 4% for $IgG_1$ to BLG.

### Total IgA and total SIgA antibody levels

Total IgA and total SIgA antibodies in saliva were analyzed as described earlier (19). A CV below 15% was accepted for duplicate samples. A control sample was included in every analysis, and the interassay was 10% for total IgA and 14% for total SIgA.

### IgA antibody levels to food allergens

An enzyme amplification system was used to detect salivary IgA antibodies to OVA and BLG, as described earlier (20), with the exception that all samples were referred to a reference saliva sample with high levels of IgA antibodies to OVA and BLG and low background. Both the reference sample and saliva samples were diluted 1:25. Uncoated rows were used for individual controls. Antibody levels in the samples were calculated as a ratio between the OD of the sample and the OD of the reference, after subtracting the OD of the blanks and the OD values for the individual controls. The ratio was then expressed in AU. A CV below 15% was accepted for duplicate samples. Two control samples were included in every analysis, and the interassay CV was 25% for IgA to both OVA and BLG.

### Statistics

As the antibody levels were not distributed normally, non-parametric tests were used. Paired analyses were performed with the Wilcoxon signed-rank test and unpaired analyses with the Mann–Whitney *U*-test. A probability level of $< 5\%$ was considered to be statistically significant. The calculations were performed with a statistical package, StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA).

To enable statistical analysis, samples with concentrations below the limit of detection were assigned a value equivalent to half the cutoff value.

### Ethics

The study was approved by the Human Research Ethics Committee at the Faculty of Health Science in Linköping and at the Medical Faculty at Uppsala University.

Informed consent was obtained from the children's parents. To minimize the discomfort for the children, topical analgesic cream was used prior to the collection of blood samples.

## Results

Children, initially SPT positive to egg and/or milk, who at 4½ yr of age could consume the offending food, had higher levels of $IgG_4$ antibodies to both OVA and BLG on inclusion, compared with children who could not (Table 1). The levels of IgE antibodies to egg and milk on inclusion were similar in the groups (Table 1), and high inclusion ratios between $IgG_4$ and IgE antibodies to OVA and egg or BLG and milk were strongly associated with ability to consume these items at 4 ½ yr of age (Fig. 1). A subgroup of infants was milk SPT negative on inclusion, but had circulating specific IgE antibodies to milk, and they showed the highest BLG $IgG_4$/IgE ratio on inclusion (Fig. 1). A similar pattern, although not significant, was observed for the OVA $IgG_4$/IgE ratio in egg SPT negative children with circulating IgE to egg (Fig. 1). The inclusion levels of egg- and milk specific IgE antibodies were similar in the two subgroups when compared with the egg and milk SPT-positive children (data not shown).

Neither total IgE levels, serum $IgG_1$ or $IgG_1$/IgE ratio to OVA nor salivary IgA antibodies on inclusion differed between children who could, or could not consume egg and milk at 4½ yr (Table 1), but the levels of $IgG_1$ to BLG and the ratio of $IgG_1$/IgE to BLG tended to be higher on inclusion in the children who were later classified as tolerant compared with non-tolerant (Table 1).

At 4½ yr of age the levels of IgE antibodies to both milk and egg were lower in the children who could eat egg and milk, than in those who could not, whereas the opposite results were found for both OVA and BLG $IgG_1$/IgE and $IgG_4$/IgE ratios (Table 2). Otherwise the antibody levels in serum and saliva at 4 ½ yr of age did not differ between the groups (Table 2).

Food-allergen elimination during 6 wk had no impact on the levels of antibodies in serum and saliva, as total and allergen-specific IgE, IgG and IgA antibody levels did not change over the 6-wk treatment period neither in children on a food-allergen-free diet nor in children treated only with emollients (data not shown).

Table 1. Levels (median and range) of serum and salivary antibodies on inclusion in skin prick test positive children who had (tolerant) or had not (non-tolerant) been able to introduce the offending foods before the age of 4½

| | Tolerant | Non-tolerant |
|---|---|---|
| **To egg and/or milk** | | |
| | n = 47 | n = 13 |
| Tot IgE (kU/l) | 20.2 (2.07–434) | 38.3 (8.09–272) |
| SIgA (ng/ml) | 18.8 (5.99–58.5) | 17.3 (8.72–123) |
| Tot IgA (ng/ml) | 36.4 (10.3–106) | 28.6 (12.2–118) |
| **To egg** | | |
| | n = 37 | n = 11 |
| Egg IgE (kUA/l) | 1.260 (0.175–22.2) | 4.67 (0.175–38.4) |
| OVA IgA (AU) | 0.41 (0.08–0.94) | 0.29 (0.08–1.2) |
| OVA $IgG_1$(AU/l) | 152 (11.2–8650) | 74.0 (1.57–11300) |
| OVA $IgG_1$/egg IgE | 168 (4.81–7800) | 47.4 (3.90–11200) |
| OVA $IgG_4$ (AU/l) | **279 (14.9–42700)**** | **62.0 (9.03–572)**** |
| **To milk** | | |
| | n = 28 | n = 6 |
| Milk IgE (kUA/l) | 0.725 (0.175–33.1) | 0.175 (0.175–23.4) |
| BLG IgA (AU) | 0.19 (0.05–0.55) | 0.36 (0.05–0.68) |
| BLG $IgG_1$ (AU/l) | 161 (1.63–12000)p = 0.05 | 15.4 (1.57–238)p = 0.05 |
| BLG $IgG_1$/milk IgE | 129 (3.16–68600)p = 0.07 | 19.7 (0.365–107)p = 0.07 |
| BLG $IgG_4$ (AU/l) | **2210 (98.5–90400)***** | **46.2 (13.2–172)***** |

Significant differences are in bold. **p < 0.01; ***p < 0.001.
SIgA, secretory IgA; OVA, ovalbumin; BLG, β-lactoglobulin.

## Discussion

The present study indicates that sensitized, eczematous children with high ratios of $IgG_4$/IgE to food allergens at an early age are more likely to consume the offending foods at 4½ yr of age, than children with low ratios. This could not be explained by different antigen exposure in the two groups of children on inclusion, as the differences were noted before any elimination diet of the infants had been instituted. High levels of IgE to food allergens have earlier been shown to predict persistent food allergy (21, 22), but in the present study this association could not be verified, possibly due to the low numbers of children in the non-tolerant group. Instead high $IgG_4$ antibody levels to food allergens early in life foretell the ability for initially food SPT positive children to consume the food at 4½ yr of age.

Previous studies on immunotherapy with inhalant allergens indicate a protective role of $IgG_4$ antibodies, as the levels increase during treatment (23, 24). $IgG_4$ antibodies to allergens have been proposed to act as blocking antibodies by competing with IgE for allergen binding to

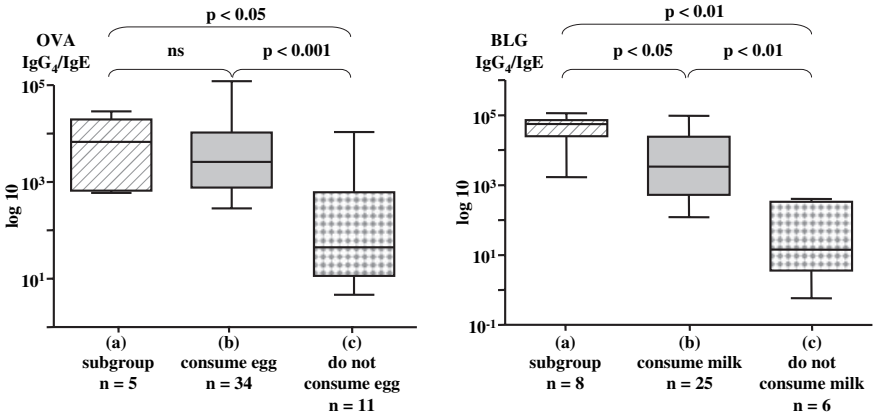


*Fig. 1.* Ovalbumin- (OVA) and β-lactoglobulin- (BLG) specific $IgG_4$/IgE ratios on inclusion in egg- and/or milk-sensitized children (a) with negative skin prick test (SPT) to egg and/or milk but with circulating IgE to corresponding antigen (b) who consume the corresponding food at the age of 4½ and (c) who did not consume the corresponding food at the age of 4½. The 10th, 25th, 50th, 75th, and 90th percentiles are indicated.The y-axis is logarithmic.

Table 2. Levels (median and range) of serum and salivary antibodies in 4½ yr old children who can (tolerant) or can not (intolerant) consume egg and/or milk

| | Tolerant | Intolerant |
|---|---|---|
| **To egg and/or milk** | | |
| | n = 46 | n = 13 |
| Tot IgE (kU/l) | 111 (2.15–1100) | 153 (12.8–1460) |
| SIgA (ng/ml) | 54.1 (31.9–96.4) | 67.3 (32.8–88.3) |
| Tot IgA (ng/ml) | 89.4 (45.6–422) | 76.1 (49.3–280) |
| **To egg** | | |
| | n = 36 | n = 11 |
| Egg IgE (kUA/l) | **0.175 (0.175–36.0)**** | **2.22 (0.780–18.8)**** |
| OVA IgA (AU) | 0.46 (0.05–0.94) | 0.20 (0.05–0.86) |
| OVA $IgG_1$(AU/l) | 83.3 (36.2–192) | 86.8 (46.8–204) |
| OVA $IgG_1$/egg IgE | **350 (50.3–1010)*** | **24.1 (10.9–51.1)*** |
| OVA $IgG_4$ (AU/l) | 21000 (257–165000) | 4320 (145–41900) |
| OVA $IgG_4$/egg IgE | **54300 (463–941000)*** | **1950 (7.69–20400)*** |
| **To milk** | | |
| | n = 26 | n = 6 |
| Milk IgE (kUA/l) | **0.175 (0.175–6.83)*** | **1.81 (0.175–150)*** |
| BLG IgA (AU) | 0.13 (0.08–0.69) | 0.25 (0.05–0.40) |
| BLG $IgG_1$ (AU/l) | 18.3 (0.210–917) | 13.3 (0.470–326) |
| BLG $IgG_1$/milk IgE | **73.2 (1.20–5240)**** | **2.17 (0.088–11.6)**** |
| BLG $IgG_4$ (AU/l) | 3500 (9.38–101000) | 1290 (0.593–41900) |
| BLG $IgG_4$/milk IgE | **20000 (53.6–462000)*** | **343 (2.02–1680)*** |

Significant differences are in bold. *p < 0.05; **p < 0.01.
SIgA, secretory IgA; OVA, ovalbumin; BLG, β-lactoglobulin.

IgE receptor expressing cells, such as mast cells and basophils (23) and competition between IgE and $IgG_4$ antibodies at the level of antigen-presenting cells has been demonstrated *in vitro* as well (23). The production of both IgE and $IgG_4$ antibodies is upregulated by IL-4 from activated Th2 cells (9). IL-10, however, which is secreted by e.g. regulatory T cells during immunotherapy (23), potently suppresses IgE production, and simultaneously increases $IgG_4$ production (10). Another immune response associated with protection from development of allergic disease is a proposed modified Th2 immune response that includes high levels of $IgG_4$ antibodies in combination with a lack of IgE antibodies (11). Clinical and immunological outcomes after immunotherapy with food allergens are less investigated, but it has been suggested that tolerance may be induced after oral administration of food allergens (5). Recently, it was shown that in food allergic children, 64% could at least tolerate daily doses of actual food after a specific oral tolerance induction scheme, compared with 35% of food allergic children on an elimination diet (25). Our study may indicate that $IgG_4$ may be a valuable parameter to foretell which children that could benefit from administration of food allergens instead of elimination diet, although this needs further evaluation.

The levels of $IgG_1$ antibodies have also been shown to increase during immunotherapy, at least during the beginning of treatment. However, other investigators have observed increased levels of $IgG_1$ to OVA (26) and BLG (27) in children with persisting sensitization to food. In our study the levels of $IgG_1$ antibodies to BLG and the ratios of $IgG_1$/IgE to BLG, tended to be higher on inclusion in children who could drink milk at 4½ yr of age compared with those children who could not. The expression of $IgG_1$ is promoted by IFNγ, and the antibody can form immune complexes with antigens, bind to Fc receptors on lymphocytes and activate complement (28). The favored $IgG_1$ antibody production in these children may depend on an immune response that is balanced towards a Th1 milieu, possibly in combination with $IgG_4$-inducing factors, e.g. IL-10. High doses of allergens might favor the induction of Th1-type responses (29), and some studies on airborne allergens suggest that exposure to the allergen may favor immunological tolerance (30).

Serum antibodies to cow's milk decrease during the first year of life in healthy children, indicating immunological tolerance development to these proteins during continued ingestion (8). Furthermore, salivary IgA and serum IgG antibody responses to BLG normally decrease with age in children, as previously reported by our

group (7, 8, 15). However, in this study we found no clear changes in antibody levels in eczematous, sensitized children during their 6-wk treatment period with allergen elimination, which is in line with a previous study reporting constant levels of serum antibodies during an elimination diet for 3 wk (31). It can be speculated whether a treatment period of 3–6 wk is sufficient to ensure any changes in antibody levels. The half-life of antibodies fluctuates, but may be up to several weeks, and antibody-producing plasma cells may be much more long-lived than has been proposed earlier [reviewed in Ref.(32)].

In the present study, we report that a small subgroup of children with negative food-allergen SPT but with circulating IgE antibodies to egg and/or milk had higher $IgG_4$/IgE ratios than children with positive SPT to egg and/or milk. The negative outcome in SPT in this sensitized subgroup may be explained by their high levels of $IgG_4$ antibodies to allergens competing with IgE for allergen binding to IgE receptor expressing cells and thereby inhibiting mast cell degranulation (23). Moreover, this response may be associated with high levels of anti-inflammatory IL-10, which has been observed to correlate negatively with the SPT wheal diameter (33).

A definite limitation of our study is that double-blind, placebo-controlled food challenges (DBPCFC) were not routinely performed on inclusion, but were performed later. Consequently, we can not prove that the sensitized children with eczema had food allergy on inclusion. We can only state whether they tolerated the food items at 4½ yr or not. The children classified as non-tolerant at 4½ yr had either had a positive DBPCFC or a recent reaction at accidental exposure. However, it is worth to report that when analysing the three children with positive DBPCFC separately, we still could observe lower levels of $IgG_4$ and $IgG_4$/IgE ratios in them, compared with children who had introduced the eliminated food without problems (data not shown). Incidentally, our results coincide with a recent study by Noh et al. (34), reporting the outcome of DBPCFC in older children, mean age 13.9 yr. They found that the mean IgE/$IgG_4$ ratio was significantly higher in children with a positive challenge test, compared with those with a negative test. Also, results from a recent study show that elevated levels of cow's milk specific $IgG_4$ was associated with tolerance to milk in atopic children and adults (35).

In summary, eczematous, food sensitized children with high levels of $IgG_4$ and high ratios of $IgG_4$/IgE antibodies to food allergens early in life are more likely to consume the offending food at 4½ yr than children with low levels and ratios. Treatment with allergen exclusion and/or skin care for 6 wk did not significantly affect the levels of serum IgE, IgG or salivary IgA antibodies in eczematous children.

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