Peanut sensitization during the first five years of life is associated with elevated levels of peanut-specific IgG

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38   **Abstract**

39   Background: Allergen-specific IgE antibodies are implicated in allergic diseases while
40   allergen-specific IgG antibodies have been proposed to prevent allergic reactions. The
41   objective for this study was to study if the immune response (IgG and IgG4) to peanut
42   differs in IgE sensitized and non-sensitized young children.

43   Methods: A total of 239 children have been followed prospectively from birth to 5 years of
44   age. Levels of IgG and IgG4 to peanut, Ara h 2 and Ara h 8 were analyzed at 2 and 5 years
45   of age and related to IgE-sensitization and peanut consumption.

46   Results: Levels of peanut-specific IgG and IgG4 were significantly higher in peanut-
47   sensitized children at 2 and 5 years of age when compared to non-sensitized children and
48   children sensitized to other food/inhalant allergens. A strong correlation was seen between
49   levels of peanut-specific IgG/IgG4-ratios and peanut-specific IgE at 5 years of age.
50   Children avoiding peanuts, a subgroup of the peanut sensitized, had statistically significant
51   higher levels of IgE to peanut and a tendency of higher IgG and IgG4 levels to peanut. In
52   the avoidance-group significant correlations between IgE and IgG/IgG4 to peanut was
53   found compared to children eating peanuts.

54   Conclusion: Peanut-specific IgG or IgG4 levels were elevated in peanut-sensitized children
55   especially those avoiding peanuts. In our study, IgG and IgG4 do not seem to indicate
56   tolerance or protection from sensitization.

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58   **Introduction**

59   Allergen-specific IgE antibodies are implicated in the pathogenesis of allergic diseases, by
60   mediating the activation of mast cells and basophils upon allergen cross-linking. Peanut-
61   specific IgE antibodies develop early (1) and quantification seems to be useful in predicting
62   peanut allergy, especially to the peanut component Ara h 2 (2, 3). Allergen specific IgG
63   antibodies, on the other hand, have been proposed to prevent allergic reactions (4) but have
64   also been proposed to be associated with atopy and allergic sensitization (5). Indeed,
65   extended exposure of airborne allergens either naturally (cat allergen exposure, beekeepers,
66   animal handlers) or during immunotherapy is associated with increasing IgG levels, and
67   IgG4 in particular, indicating a role for IgG and IgG4 in tolerance or protection (6, 7).

68   In food allergies, previous studies have found conflicting results concerning the role of IgG
69   and IgG4. One recent study reports that children sensitized to egg and/or milk and who
70   could eat and drink the offending food at 4½ years had higher levels of IgG4 to ovalbumin
71   and β -lactoglobulin than those who could not consume cow's milk and/or hen's egg (8).
72   Besides, high IgG4 levels to cow's milk were found to be associated with maintenance of
73   tolerance to cow's milk in atopic children and adults (9). In contrast, Tay et al reports
74   increased levels of peanut-specific IgG4 in peanut-sensitized children, while there were no
75   differences in ovalbumin-specific IgG4 levels between subjects sensitized and non-
76   sensitized to hen's egg (10).

Today it is possible to analyze IgE and IgG/IgG4 to different proteins in foods, amongst others Ara h 2 and 8 from peanuts where IgE to Ara h 2 is associated with more severe reaction than IgE to Ara h 8 which is a birch-homolog protein (2, 11, 12).

The aim of this study was to investigate if the immune response to peanuts differs in IgE sensitized and non-IgE sensitized young children in relation to avoidance/consumption of peanuts. The levels of IgG and IgG4 to peanut, Ara h 2 and Ara h 8 were analyzed in relation to IgE-sensitization to peanut until 5 years of age.

Material and methods

Subjects

Two hundred and thirty-nine children were followed prospectively from birth to 5 years of age. They are part of a larger study (n= 281) presented elsewhere (13, 14). All infants were born full term (> 35 weeks of gestation, mean: 40 weeks) at hospitals in Stockholm, and had birth weights within the normal range (mean: 3600 g). Children in this study were selected based upon complete sensitization data (SPT and/or specific IgE) at 6, 12 and 18 months, 2 and 5 years of age. The 42 children with incomplete data and therefore not included did not differ statistically from children in the cohort (data not shown). Furthermore, there was no statistically significant difference between sensitized children (n=93) and non-sensitized children (n=146) concerning gestation week, delivery mode, birth weight, maternal age, duration of exclusive breastfeeding, age at daycare start or attending daycare at 2 years, furred pets at home or parental smoking (data not shown). However, the IgE-sensitized children statistically significant more often, had two allergic parents and born between October to March, than non-sensitized children, OR = 3.68; 95% CI 1.83 – 7.39 and 1.89; 95% CI 1.11 – 3.24 respectively.

This study was approved by the Human Ethics Committee at Huddinge University Hospital, Stockholm (Dnr 75/97, 113/97, 331/02), and the parents provided informed consent.

Skin prick testing

Skin prick tests (SPT) were performed at 6, 12, 18 months and 2 and 5 years of age against food allergens such as egg white (Soluprick weight to volume ratio 1/100), peanut (Soluprick 1/20), cow's milk (3% fat, standard milk) and soy bean protein (Soja Semp® Semper AB, Stockholm, Sweden), in accordance with the manufacturer's recommendations (ALK, Copenhagen, Denmark). SPTs were also performed against inhalant allergens for cat, dog, *Dermatophagoides farinae*, birch and timothy (Soluprick 10 HEP). Histamine chloride (10 mg/ml) was the positive control and allergen diluent the negative control. The SPT was considered positive if the wheal diameter was ≥ 3 mm after 15 minutes.

Blood sampling and specific IgE, IgG and IgG4

Peripheral venous blood samples were collected (at 6 and 12 months and 2 and 5 years of age) using an aspiration technique as previously described (13).

Circulating IgE antibodies (ab) against cow's milk, egg white, peanut, Ara h 2, Ara h 8, cod fish, soy bean, cat dander, dog dander, birch pollen, timothy pollen and *Dermatophagoides farinae* were determined in plasma (ImmunoCAP1000®, Phadia AB, Uppsala, Sweden) at 2 and 5 years of age. A positive test was defined as an IgE ab level ≥ 0.35 kU_A/l.

Circulating IgG and IgG4 ab specific to peanut, Ara h 2, Ara h 8, were determined in plasma at 2 and 5 years of age (ImmunoCAP® 1000, Phadia AB, Uppsala, Sweden). The detection limits for IgG was 0.02 mg_A/l and for IgG4 0.07 mg_A/l. Allergen-specific IgG and

IgG4 under the detection limit was in the statistical analysis given the values 0.01 mg_A/l and 0.000001 mg_A/l, respectively.

Classification of the children

In accordance with Johansson et al (15), each child was classified as IgE-sensitized to peanut if at least one SPT was positive (≥ 3 mm) and/or if the specific IgE for at least one of the selected allergens was ≥ 0.35 kU_A/l. Twenty-nine children were identified.

According to parental report at 5 years of age, the peanut-sensitized children were classified as avoiding (n=19) or eating (n=10) peanuts.

Statistics

Statistical analyses were performed using STATISTICA 7.1 software (Statsoft Inc., Tulsa, OK, USA) and IBM SPSS statistics 20. Differences in demographic data between sensitized and non-sensitized children were analyzed using logistic regression (Odds Ratios) and the Mann-Whitney U-test when applicable. Since levels of specific IgG and IgG4 to the selected allergens were not normally distributed, overall differences between groups were evaluated using a Kruskal Wallis test for non-parametric independent samples. Where overall significant differences were found, differences between groups were further evaluated using pair-wise Mann Whitney tests. To evaluate if levels of IgG and IgG4

correlated with levels of IgE with the same specificities, Spearman's rank correlation coefficient was used. A p-value < 0.01 was considered as significant.

Results

The children were divided into three groups depending on overall sensitization as well as sensitization type. In detail; for statistical analyses, the groups were as follows: group 1 (n=31) including peanut-sensitized children (cumulative sensitization 0-5 years); group 2 (n=62) including children sensitized to one or more of the selected allergens, but not to peanut (cumulative sensitization 0-5 years); group 3 (n=146) including the children who were non-sensitized at all time-points tested.

The number of children IgE sensitized to peanut was three at 6 months, eight at 12, and seven children at 18 months of age. At 2 and 5 years of age the number of children was 13 and 29 respectively. The cumulative number of children sensitized (0-5 years) was 31.

Specific IgG and IgG4 to peanut in sensitized children

Peanut-specific IgG and IgG4 levels were investigated at 2 and 5 years of age. Levels of peanut-specific IgG were significantly higher in peanut-sensitized children (group 1) compared to non-sensitized children (group 3) at both 2 ($p < 0.001$) and 5 ($p < 0.001$) years of age (Table 1). Peanut-specific IgG were also higher in peanut-sensitized children (group 1) compared to children sensitized to other allergens, but not to peanut (group 2), at both ages ($p = 0.009$ and $p < 0.001$).

The same pattern was observed for peanut-specific IgG4. Thus, levels of peanut-specific IgG4 were significantly higher in children in group 1 compared to children in group 3 at

both 2 ($p < 0.001$) and 5 ($p < 0.001$) years of age (Fig. 1b, d). Furthermore, levels of peanut-specific IgG4 in children in group 1 were significantly higher than in children in group 2 at both ages ($p = 0.001$ and $p < 0.001$). Peanut-specific IgG levels were rather stable between 2 and 5 years of age in all study groups (Fig 1a, c), while IgG4 levels increased approximately 5 times in the peanut-sensitized children between 2 and 5 years (Table 1).

Ratios of peanut-specific IgG4 and IgG were significantly higher in children in group 1 as compared to children in group 3 ($p < 0.001$) at 5 years of age. Besides, there was a tendency of an increased ratio (IgG4/IgG) to peanut in children in group 1 compared to children in group 3 at 2 years of age (Table 1).

In peanut-sensitized children, levels of peanut-specific IgG and IgG4 correlated with levels of peanut-specific IgE at 5 years of age ($\rho = 0.57$, $p = 0.001$ and $\rho = 0.49$, $p = 0.007$ respectively).

Specific IgG and IgG4 to peanut allergen components

IgG4 levels to Ara h 2 and to Ara h 8 were significantly higher among children in group 1 than among children in group 3 ($p < 0.001$ and $p < 0.001$) as well as children in group 2 ($p < 0.001$ and $p = 0.004$) at five years of age (Table 1). IgG levels to Ara h 8 were only observed at low levels irrespective of study group while IgG levels to Ara h 2 had a tendency to be elevated in children in group 1 when compared to children in groups 2 and 3 at 5 years (data not shown). At two years, we could not detect any component-specific IgG-

or IgG4 levels in any of the study groups, apart from low levels of Ara h2-specific IgG4 in peanut-sensitized children (data not shown).

Furthermore, Ara h 2-specific IgG and IgG4 levels strongly correlated with levels of Ara h 2-specific IgE at 5 years of age ($\rho = 0.69$, $p < 0.001$ and $\rho = 0.73$, $p < 0.001$ respectively). Such a correlation could not be observed for Ara h 8-specific IgG, IgG4 and IgE levels. Of all peanut-sensitized children in this study, 11 had Ara h 8-specific IgE levels above $0.35 \text{ kU}_A/\text{l}$. All of these 11 children were also sensitized to birch and there was a tendency of Ara h 8-specific IgG4 to correlate with birch-specific IgE at 5 years ($\rho = 0.53$, $p = 0.08$).

Specific IgG and IgG4 to peanut and Ara h 2 in peanut-sensitized children eating or avoiding peanuts

Children IgE-sensitized to peanut were subdivided according to parental report on peanut consumption; children avoiding peanuts ($n=19$) and children eating peanuts ($n=10$) at the age of five. The levels of IgE, IgG and IgG4 to peanut and Ara h 2 at five years were compared between children eating or avoiding peanuts (Table 2). Children avoiding peanuts had statistically significantly higher levels of IgE to peanut compared to children eating peanuts ($p=0.009$). The levels for IgG and IgG4 to peanut did not differ statistically significant between children eating or avoiding peanuts, $p=0.082$ and $p=0.157$ respectively. However, IgG and IgG4 to Ara h 2 had a tendency of higher levels in the group avoiding peanuts, $p=0.066$ and $p=0.04$ respectively. Interestingly, in peanut-sensitized children

avoiding peanuts, the levels of peanut-specific IgG and IgG4 to peanut and Ara h 2 correlated statistically significant with levels of peanut-specific IgE ($r = 0.48 - 0.64$, $p = < 0.001 - 0.002$) but this was not seen in children eating peanuts (shown for peanut-specific IgE versus peanut-specific IgG in Fig. 1).

Discussion

The role of IgG and IgG4 in food allergies is still a matter of debate. In this study we report that peanut-sensitized children have significantly elevated levels of peanut-specific IgG and IgG4 compared with children sensitized to other selected allergens and non-sensitized children, at both 2 and 5 years of age. We also report that IgG and IgG4 to peanut and Ara h 2, in peanut-sensitized children avoiding peanuts, statistically significant correlate to peanut- and Ara h 2-IgE in contrast to peanut-sensitized children eating peanuts.

Previous studies have reported that high levels of allergen-specific IgG4 and/or IgG are more common in allergic than in non-allergic patients (16-18). In specific immunotherapy studies, IgG1 and IgG4 levels increase (19-22) and this is thought to mirror a change from a Th2-biased immune profile to a more T-regulatory immune profile (23). The production of both IgG4 and IgE is described to be enhanced by Th2-cytokines (24-26), while IL-10 from T-regulatory cells inhibits IgE production and promotes the secretion of IgG4 (27). This suggests different ways to control IgE and IgG4 production, and that elevated IgG4 indicates an ongoing regulatory response (27-29). Indeed, IgG4 could have a protective role, as allergen specific IgG4 is believed to compete with allergen specific IgE for the binding of allergens and thereby inhibit FcεRI cross-linking and allergic reactions (30, 31).

In our study, increased levels of peanut-specific IgG and IgG4 were associated with peanut sensitization, as these levels strongly correlated to peanut-specific IgE both at 2 and 5 years of age. Peanut-specific IgG4 constituted a larger part of total peanut-specific IgG in peanut-sensitized children than in their non-sensitized peers, exemplified by higher ratios of

peanut-specific IgG4 and IgG in peanut-sensitized children than in non-sensitized children. When dividing peanut-sensitized children in those avoiding or eating peanuts we demonstrate that it is especially the children avoiding peanuts who have higher levels of IgE and IgG/IgG4 to peanut. The strong significant correlation between IgE and IgG/IgG4 to peanut and Ara h 2 at five years of age is mainly due to the IgG/IgG4 levels in children avoiding peanuts. This might indicate an attempt to regulate peanut responses in the peanut-sensitized children avoiding peanuts, who probably are peanut allergic. Our results are in line with a recent publication also showing that peanut allergic children had higher levels of IgG to peanut compared with their non-allergic siblings (18). These authors also reported a strong correlation between IgE and IgG to peanut in peanut allergic children but not in their non-allergic siblings.

However, there is a possibility of misclassification of our outcome since some of the children avoiding peanuts may do so for other reasons than being truly peanut allergic. Still, if this is true, it would not strengthen our finding, this would rather lead to a dilution of our results. Interestingly, another recent study showed a simultaneous decrease in peanut-specific IgE and increase in peanut-specific IgG and IgG4 in subjects undergoing oral immunotherapy, further supporting a regulatory role of peanut-specific IgG and IgG4 (21).

A longitudinal analysis of our data revealed that peanut-specific IgG4 levels increased approximately 5 times in the peanut-sensitized group between 2 and 5 years. This is to be expected, and in line with the normal development and maturation of the child's immune system to react on foreign proteins.

Previous studies have linked IgG4 not only to sensitization but also to allergen exposure. In a recent report, investigating levels of food-specific IgG4 in healthy laboratory workers, several positive results for food-specific IgG4 was observed, despite absent sensitization in the subjects (32).

IgG4 responses to Ara h 2 and Ara h 8 were elevated in peanut-sensitized children, in contrast to non-sensitized children, at 5 years of age. Interestingly, of these specificities, only Ara h 2-specific IgG4 levels correlate with Ara h 2-specific IgE at 5 years. Indeed, Ara h 2 is a major peanut allergen (33-35) and IgE responses to Ara h 2 is strongly correlated to more severe symptoms (11). Furthermore, a recent study in a population-based cohort demonstrated that Ara h 2 was the most important predictor of clinical allergy (2, 12). In addition, Ara h 2 is an efficient inducer of basophil activation *in vitro* (3, 36). On the other hand, reactivity to the Bet v 1-homolog Ara h 8 is associated with reported mild to modest symptoms and IgE reactivity to Ara h 8 is most frequently seen in Bet v 1-sensitized individuals (11). One third of the peanut sensitized children in our study were IgE sensitized to Ara h 8 and all of them were sensitized to birch. This could explain the lack of a correlation between Ara h 8-specific IgG4 and IgE.

Our study comprised a relatively large sample size and should be considered as sufficiently powered. It was further strengthened by the lowering of the significance level from $p < 0.05$ to $p < 0.01$. However, the study participants did not undertake food challenges, why we can relate our findings to sensitization or parental reported avoidance and not to true food allergy.

In summary, our results shows that specific IgG and IgG4 to peanut is increased in peanut sensitized children and correlates well with peanut IgE especially in children avoiding peanuts. Elevated levels of peanut-specific IgG4 do not seem to be connected with protection from sensitization but could be a mechanism for the immune system to counteract IgE responses.

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Figure Legends

1. Correlation between peanut-specific IgG levels and peanut-specific IgE levels at 5 years of age in children eating and avoiding peanuts.

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	Group 1, n = 31	Group 2, = 62	Group 3, n = 146	Gr 1 vs Gr 2, p	Gr 1 vs Gr 3, p
2 years of age					
Peanut-specific IgG, mean (min-max)	3.34 (0.01-36.0)	0.79 (0.01-5.7)	0.18 (0.01-7.1)	0.009	< 0.001
Peanut-specific IgG4, mean (min-max)	0.11 (<0.01-0.95)	0.03 (<0.01-0.44)	0.01 (<0.01-0.21)	0.001	< 0.001
Ara h 2-specific IgG, mean (min-max)	0.72 (0.01-12.0)	0.30 (0.01-5.2)	0.12 (0.01-3.1)	0.404	0.04
Ara h 2-specific IgG4, mean (min-max)	0.03 (<0.01-0.32)	0.01 (<0.01-0.03)	0.01 (<0.01-0.06)	0.015	0.003
Ratio peanut-specific IgG4/IgG, mean (min-max)	1.41 (0.0-10.0)	1.01 (0.0- 20.0)	0.44 (0.0-10.0)	0.028	< 0.001
5 years of age					
Peanut-specific IgG, mean (min-max)	4.2 (0.0-23.0)	0.37 (0.0-5.7)	0.28 (0.0-5.8)	< 0.001	< 0.001
Peanut-specific IgG4, mean (min-max)	0.34 (<0.01-2.1)	0.08 (<0.01-0.99)	0.05 (<0.01-1.3)	< 0.001	< 0.001
Ara h 2-specific IgG, mean (min-max)	1.31 (0.0-7.0)	0.2 (0.0-2.8)	0.3 (0.0-6.4)	0.003	0.001
Ara h 2-specific IgG4, mean (min-max)	0.09 (<0.01-0.91)	0.01 (<0.01-0.4)	0.01 (<0.01-1.0)	< 0.001	< 0.001
Ratio peanut-specific IgG4/IgG, mean (min-max)	7.81 (0.0-98.0)	2.83 (0.0-33.0)	2.5 (0.0-48)	0.183	< 0.001

Table 1. Mean levels of peanut-specific and Ara h 2-specific IgG and IgG4 levels at 2 and 5 years of age.
Group 1 = Peanut-sensitized children 0-5 y, group 2 = Children sensitized, but not to peanut, group 3 = Non-sensitized children.

Peanut sensitized at 5 years	Peanut sensitized All children, n=29	Peanut sensitized and avoiding peanuts, n=19	Peanut sensitized but eating peanuts, n=10
IgE to peanut, kU _A /l, median (min-max)	50,5 (0,1 – 399)	0,87 (0,1 – 2,0)	79,8 (0,1 – 399)
IgG to peanut, mg _A /l, median (min-max)	3,8 (0,01 – 23)	5,6 (0,01 – 23)	0,77 (0,01 – 5,2)
IgG4 to peanut, mg _A /l, median (min-max)	0,34 (0,0 – 2,1)	0,39 (<0,0001 – 1,4)	0,26 (<0,0001 – 2,1)
IgG to Ara h 2, mg _A /l, median (min-max)	1,16 (0,01 – 7,0)	1,7 (0,01 – 7,0)	0,24 (0,01 – 2,3)
IgG4 to Ara h 2, g _A /l, median (min-max)	0,09 (<0,0001 – 0,91)	0,14 (<0,0001 – 0,91)	0,016 (<0,0001 – 0,08)

Table 2. IgE, IgG and IgG4 sensitization to peanut and Ara h 2 in 5 year olds divided into children eating or avoiding peanuts.

Figure 1