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ORIGINAL PAPER

# Patterns of immunoglobulin G responses to egg and peanut allergens are distinct: ovalbumin-specific immunoglobulin responses are ubiquitous, but peanut-specific immunoglobulin responses are up-regulated in peanut allergy

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Clinical and Experimental Allergy

**Summary**

*Background* The clinical significance of food-specific IgG subclasses in food allergy and tolerance remains unclear. Specific IgG titres are often reported in non-standardized units, which do not allow comparisons between studies or allergens.

*Objective* To quantify, in absolute units, ovalbumin (OVA)- and peanut-specific IgG levels in children with peanut or egg allergy (active or resolved) and in non-allergic controls.

*Methods* Children aged 1–15 years were recruited. Peanut allergy was diagnosed by convincing history and a 95% predictive level of specific IgE; egg allergy or resolution was confirmed by oral challenge. Serum IgG, IgG1 and IgG4 levels (μg/mL) to OVA and peanut extract were quantified by ELISA.

*Results* OVA- and peanut-specific IgG was detected in all subjects. In non-allergic controls ($n = 18$), OVA-specific IgG levels were significantly higher than peanut-specific IgG (median μg/mL IgG = 15.9 vs. 2.2, IgG1 = 1.3 vs. 0.6, IgG4 = 7.9 vs. 0.7; $P < 0.01$). There were no differences in OVA-specific IgG, IgG1 and IgG4 between egg-allergic ($n = 40$), egg-resolved ($n = 22$) and control ($n = 18$) subjects. In contrast, peanut-specific IgG (median μg/mL IgG = 17.0, IgG1 = 3.3, IgG4 = 5.2) were significantly higher in peanut-allergic subjects ($n = 59$) compared with controls and with non-peanut-sensitized but egg-allergic subjects ($n = 26$). Overall, the range of IgG4 was greater than IgG1, and IgG4 was the dominant subclass in >60% of all subjects.

*Conclusion* OVA-specific IgG levels of egg-allergic, egg-resolved or control groups are not distinguishable. Higher peanut-specific IgG levels are associated with clinical allergy, but the range of IgG titres of the allergic and control groups overlapped. Hence, OVA and peanut-specific IgG measurements do not appear to be of diagnostic value. Strong IgG responses to OVA may be a normal physiological response to a protein frequently ingested from infancy, whereas up-regulated IgG responses in peanut allergy may be indicative of a dysregulated immune response to peanut allergens.

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## Introduction

Food allergies are a significant health problem, and allergy to peanut and egg is common in children [1, 2]. Peanut allergy appears to be more persistent than egg allergy, which resolves in the majority of children [3–5]. Food-specific IgE is implicated in the pathogenesis of food allergy and specific IgE titres or skin prick test (SPT) weal diameters have been reported to be predictive of clinical reactivities [6–10]. On the other hand, the clinical significance of food-specific IgG is not very well understood.

Studies investigating the role of specific IgG in food sensitization, allergy and tolerance have reported different findings. In two studies, peanut-specific and cow milk protein-specific IgG were higher in allergic patients compared with healthy controls [11, 12]. Increased ovalbumin

(OVA)-specific IgG is reported in patients with persistent sensitization and allergy compared with transient sensitization [13, 14]. Sletten et al. [15] found higher levels of IgG4 to β-lactoglobulin in patients with milk allergy and reduced levels in tolerant subjects. In contrast, some authors report no differences in specific IgG between allergic patients and controls [16, 17] or even lower levels in allergic subjects [18], while Hill et al. [19] did not show any association of specific IgG with tolerance to cow's milk. As most of the measurements made of specific IgG titres have been reported in non-standardized units, it has not been possible to compare data from different studies and examine the conflicting data more closely.

The four IgG subclasses are functionally diverse and differentially regulated. Studies have shown that the IgG4 (together with IgE) are induced in T-helper type 2 (Th2)-type responses, e.g. responses to helminth infections and in allergic disease [20]. High levels of allergen-specific IgG4 are associated with asymptomatic sensitization and successful immunotherapy, suggesting protective or blocking functions for this subclass [21, 22], whereas the significance of the IgG1 subclass is not often examined in allergy.

The aim of this study was to examine levels of specific IgG, IgG1 and IgG4 in children with peanut allergy, egg allergy, resolved egg allergy and non-allergic controls using an ELISA optimized for quantifying antibody levels in micrograms per millilitre. This would allow comparisons to be made between antibodies of different subclasses and allergen specificities.

## Materials and methods

### *Study populations*

*Peanut-allergic population*. Peanut-allergy was diagnosed by (a) a recent convincing history of a clinical reaction to peanut with typical symptoms and (b) supported by the 95% predictive value for SPT weal diameter $\geqslant$8 mm to peanut (ALK-Abello, Horsholm, Denmark) or peanut-specific IgE$\geqslant$15 kU/L (ImmunoCap FEIA, Phadia, Uppsala, Sweden) on study entry. Challenges were not performed.

*Egg-allergic and egg-resolved populations*. Children with a confirmed history of a typical type-1 hypersensitivity reaction to egg and an SPT weal diameter $\geqslant$3 mm to whole egg (Allergy Therapeutics, West Sussex, UK) at the time of diagnosis were enrolled for an open oral challenge to determine their current clinical reactivity to egg. Antihistamines were stopped 72 h before challenge. Incremental doses of cooked or uncooked egg (cumulative doses 12 and 21.5 g, respectively, the maximum practicable dose for each challenge type) were given at 10-min intervals. The challenge was stopped when either all doses were tolerated or an objective reaction [development of two or more of erythema, urticaria (distant to the mouth) or angiooedema, rhinoconjunctivitis, wheeze, abdominal pain or vomiting] occurred. Assessments were made by two clinical observers experienced in allergy. SPT and egg-specific IgE measurements were repeated on the day of the challenge. Children who reacted to *either* cooked or uncooked egg were egg-allergic; children who tolerated *both* cooked and uncooked egg had resolved egg allergy. We did not measure specific IgE or oral challenge reactivity to OVA during this study, but relied on clinical testing with whole egg.

*Non-allergic population*. These were children attending hospital for orthopaedic surgery. Parental questionnaires reported no history of allergy-related illness and that children were not actively avoiding egg or peanut. Egg- and peanut-specific IgE were not detected ($<$0.35 kU/L) in these controls. We further identified children with no history of sensitization to peanut (specific IgE $<$0.35 kU/L) but were clinically allergic to egg (by oral challenge). The study was approved by the local Research Ethics Committee, and informed consent was obtained.

### *Preparation of crude peanut extract and ovalbumin proteins*

Crude peanut extract (CPE) was prepared in house. In all, 50 g of crushed peanuts were defatted by hexane extraction for 2 h at 4 °C and air drying the supernatant overnight. The defatted extract was dialysed into 50 mM ammonium bicarbonate and freeze dried for 48 h before reconstitution in phosphate-buffered saline (PBS) at 10 mg/mL. Aliquots were stored at −20 °C until use. OVA of grade V purity (Sigma, Poole, UK) was reconstituted in PBS at 10 mg/mL. CPE and OVA concentrations were measured by the bichinchoninic acid assay (Pierce Biotechnology, Rockford, USA).

### *Quantification of specific antibody titres in reference anti-sera*

Sera from 27 peanut-allergic and 23 egg-allergic subjects with high specific IgE titres against each food allergen (specific IgE $>$5.0 kU/L) were used to create two reference pools. Specific IgG, IgG1 and IgG4 titres in each pool were quantified by a competitive ELISA method modified from Rieben & Blaser [23, 24]. Multibind microtitre plates (Greiner Bio-one, Gloucester, UK) were coated for 2 h at room temperature (RT) with CPE (2 μg/mL) or OVA (5 μg/mL) in 100 μL carbonate/bicarbonate buffer (pH 9.6). Plates were washed three times in PBS-containing 0.1% Tween 20 (PBST) and blocked with 5% heat-inactivated fetal calf serum (Sigma) in PBST for 1 h at RT. One dilution of reference anti-sera was added to all wells and incubated overnight at 4 °C. Serial twofold dilutions

of the monoclonal anti-human IgG, anti-IgG1 or anti-IgG4 (WHO/IUIS clones HP6064/8a4, HP6012/NL16 or HP6011/RJ4, respectively) antibodies were added in triplicate overnight at 4 °C. Plates were washed three times in PBST, and the bound monoclonal antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, 1 : 1000) incubated for 3 h at RT. After two PBST washes and a final wash in distilled water, 100 μL of *p*-nitrophenyl phosphate (Sigma, 1 mg/mL in diethanolamine buffer pH 9.8) was added. Optical densities at 405 nm were read using BioRad Model 550 (Bio-Rad, Hemel Hempstead, UK). An optimal concentration of the monoclonal antibody was selected based on the titration curve, and the assay was repeated in the presence of pre-titrated amounts of inhibiting purified human IgG (Calbiochem, San Diego, CA, USA), IgG1κ (Sigma) or IgG4κ (Sigma). The CPE- or OVA-specific IgG titres of each reference pool were calculated from the derived inhibition curves. Five independent measurements were made for each pool. The antibody titres (mean ± standard deviation in μg/mL) of the CPE-specific pool were 96.66 ± 2.13 (IgG), 19.93 ± 1.49 (IgG1) and 35.58 ± 1.68 (IgG4). The sensitivity limits (ng/mL) were 2.5 (IgG), 4.0 (IgG1) and 7.0 (IgG4). The inter-assay coefficients of variation (CV) were 3.8% (IgG), 6.2% (IgG1) and 9.4% (IgG4). The antibody titres (μg/mL) of the OVA-specific pool were 92.18 ± 0.69 (IgG), 7.47 ± 0.33 (IgG1) and 83.6 ± 4.14 (IgG4). The sensitivity limits (ng/mL) were 4.0 (IgG), 6.0 (IgG1) and 4.0 (IgG4). Inter-assay coefficient of variations (CVs) were 6.0% (IgG), 8.8% (IgG1) and 9.1% (IgG4).

### *Quantification of specific antibody titres in patient sera*

Serial dilutions of reference anti-sera and at least three dilutions of patient sera (1 : 20 to 1 : 1600) were incubated with CPE- or OVA-coated plates in duplicate overnight at 4 °C. Each subclass of bound anti-sera was detected with monoclonal anti-human IgG (8a4), anti-IgG1 (NL16) or anti-IgG4 (RJ4) used at previously selected concentrations, overnight at 4 °C. Bound monoclonal antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, 1 : 1000) for 3 h at RT, followed by *p*-nitrophenyl phosphate substrate addition and reading of optical densities at 405 nm. Washes and incubation conditions were performed as for quantification of reference anti-sera pools. Specific antibody titres in micrograms per millilitre were extrapolated from the standard curve of reference anti-sera.

### *Statistical analysis*

IgG values were not normally distributed and so non-parametric tests were applied. The Mann–Whitney *U*-test was used to compare medians between groups. The Kruskal–Wallis test was used to compare medians in three groups. The Spearman rank test was used to assess the significance of correlations (ρ) between the different parameters measured, e.g. age, antibody titres, SPT weal diameters. All tests were performed using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). Graphs were generated in Graphpad Prism (version 3.02, GraphPad Software Inc., San Diego, CA, USA).

## Results

### *Egg-specific immunoglobulin E and ovalbumin-specific immunoglobulin G responses in children*

Oral challenges with cooked or uncooked egg were performed on 63 children, who all had a confirmed history of egg allergy. Children who could tolerate cooked egg were challenged with uncooked egg. Twenty-three children passed the challenge with uncooked egg and have resolved egg allergy. Children who reacted to either cooked egg ($n = 17$) or uncooked egg ($n = 23$) were still allergic (total $n = 40$). Age, total and specific IgE, and SPT weal diameters of children who were challenged and of 18 age-matched non-allergic controls are presented in Table 1. Total IgE was higher in children with active and resolved egg allergy compared with controls ($P < 0.01$), with no significant differences between the allergic and resolved groups. In contrast, egg-specific IgE and SPT weal diameters were significantly higher in the allergic group compared with the resolved group ($P < 0.05$). Egg-specific IgE titres were positively correlated to total

**Table 1.** Characteristics of groups where OVA-specific IgG levels were compared ($n = 81$)

| | Controls | Egg allergic | Egg resolved |
|---|---|---|---|
| Total number (% male) | 18 (16.8) | 40 (37.4) | 23 (21.5) |
| Age (years) | 7.7 (8.5) | 5.6 (6.8) | 5.8 (7.5) |
| | 0.8–15.4 | 0.9–15.3 | 2.2–14.1 |
| Total IgE (kU/L) | 23 (86) | 270* (796) | 394* (666) |
| | 2–243 | 10–2828 | 2–2040 |
| Egg-specific IgE (kU/L) | <0.35 | 2.4*† (5.8) | 0–4.4*§ |
| | | 0.35‡–39 | 0.35–4.38 |
| Egg SPT weal diameter (mm) | ND | 4*†¶ (3.5) | 0*‖ (2) |
| | | 0–12 | 0–4 |

Data expressed as median (interquartile range) in top row, range in bottom row.

*Elevated compared with controls (Mann–Whitney *U*-test $P < 0.05$).

†Elevated in egg-allergic compared with the egg-resolved group (Mann–Whitney *U*-test $P < 0.05$).

‡1/40 subject had specific IgE <0.35 kU/L.

§10/23 subjects had IgE >0.35 kU/L.

¶2/40 subjects has SPT = 0 and 10/40 subjects had SPT <4 mm.

‖6/23 subjects had SPT >0 mm but ⩽4 mm.

ND, not determined; OVA, ovalbumin; SPT, skin prick test.

 

IgE ($\rho = 0.58$; $P < 0.01$) but not to SPT weal diameters ($\rho = 0.3$; $P = 0.9$). Age was significantly correlated to total IgE (but not egg-specific IgE) in egg-allergic ($\rho = 0.63$; $P < 0.01$) and -resolved ($\rho = 0.79$; $P < 0.01$) children but not controls.

OVA-specific IgG, IgG1 and IgG4 levels are presented in Figs 1a–c. Age was not correlated to IgG subclass. Significant levels of specific IgG, IgG1 and IgG4 (medians of 12.7, 2.1 and 3.3 μg/mL, respectively; $n = 81$) were detected in all subjects. There were no significant differences in specific IgG, IgG1 and IgG4 levels across the non-allergic controls, egg-allergic or egg-resolved groups, nor were there significant inter-group differences. The median OVA-specific IgG, IgG1 and IgG4 levels were 15.9, 1.3 and 7.9 μg/mL, respectively, in controls ($n = 18$); 10.1, 2.3 and 1.8 μg/mL, respectively, in the egg-allergic group ($n = 40$), and 15.2, 1.9 and 3.8 μg/mL, respectively, in egg-resolved subjects ($n = 23$). The ratios of specific IgG4 : IgG1 were also not significantly different between these three groups. In subjects with egg-specific IgE > 0.35 kU/L, positive correlations existed between specific IgE and specific IgG ($\rho = 0.54$; $P < 0.01$), IgG1 ($\rho = 0.49$; $P < 0.01$) and IgG4 ($\rho = 0.38$; $P < 0.05$).

The IgG4 subclass titres were spread over a wider range (0.03–488 μg/mL) compared with IgG1 (0.2–17 μg/mL) and IgG4 was the predominant subclass in 78% (14/18) of the controls, 47% (19/40) of egg-allergic and 57% (13/23) of the egg-resolved subjects. IgG1 and IgG4 were both strongly correlated ($\rho > 0.6$; $P < 0.01$) to total specific IgG in all groups, but correlations between the two subclasses were seen only in the control ($\rho = 0.60$; $P < 0.01$) and egg-allergic ($\rho = 0.42$; $P < 0.01$) groups.

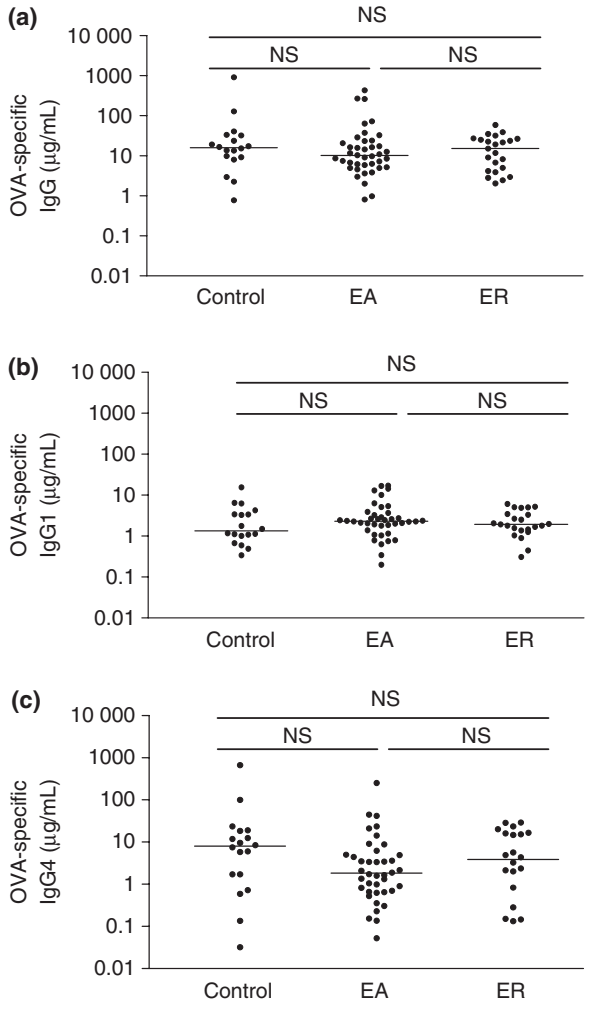


**Fig. 1.** Ovalbumin (OVA)-specific IgG (a), IgG1 (b) and IgG4 (c) levels in controls ($n = 18$), egg-allergic (EA) ($n = 40$) and egg-resolved (ER) ($n = 23$) groups. Median bar and individual titres (dots) are indicated. Comparisons of medians between groups by a Mann–Whitney *U*-test marked by bars and *P*-values. NS, not significant.

### *Peanut-specific immunoglobulin E and immunoglobulin G responses*

CPE-specific IgG antibody levels were measured in 92 children. The populations compared included the same non-allergic controls ($n = 18$), children with peanut allergy ($n = 59$) and 22 children who were not sensitized to peanuts but were egg-allergic (NPS). Peanut-specific IgE was correlated to total IgE ($\rho = 0.54$ $P < 0.01$) but not to SPT. These three groups were age matched and there were no significant correlations of age with total IgE, peanut-specific IgE or CPE-specific IgG subclasses in any of the groups (Table 2).

CPE-specific IgG, IgG1 and IgG4 levels are presented in Figs 2a–c. CPE-specific IgG1 was detected in all subjects in this study, including all non-allergic controls and all

**Table 2.** Characteristics of groups where CPE-specific IgG levels were compared ($n = 103$)

| | Controls | Peanut allergic | NPS |
|---|---|---|---|
| Total number (% male) | 18 (17.5) | 59 (57.3) | 26 (25.2) |
| Age (years) | 7.7 (8.5) | 10.5 (5.8) | 4.5 (3.0) |
| | 0.8–15.4 | 1.9–15.1 | 1.8–15.3 |
| Total IgE (kU/L) | 23 (86) | 600* (1503) | 176† (343) |
| | 2–243 | 22–6690 | 2–983 |
| Peanut-specific IgE (kU/L) | <0.35 | 23.7* (80.4) | <0.35 |
| | | 0.48–100 | |
| Peanut SPT weal diameter (mm) | ND | 10* (4) | ND |
| | | 3–12 | |

Data expressed as median (interquartile range) in top row, range in bottom row.

*Elevated compared with controls and with NPS groups (Kruskal–Wallis test for all three groups, $P < 0.05$; Mann–Whitney *U*-test for each paired group compared, $P < 0.05$).

†Lower levels compared with the peanut-allergic group (Mann–Whitney *U*-test, $P < 0.05$).

CPE, crude peanut extract; SPT, skin prick test; ND, not determined; NPS, non-peanut-sensitized but egg-allergic.

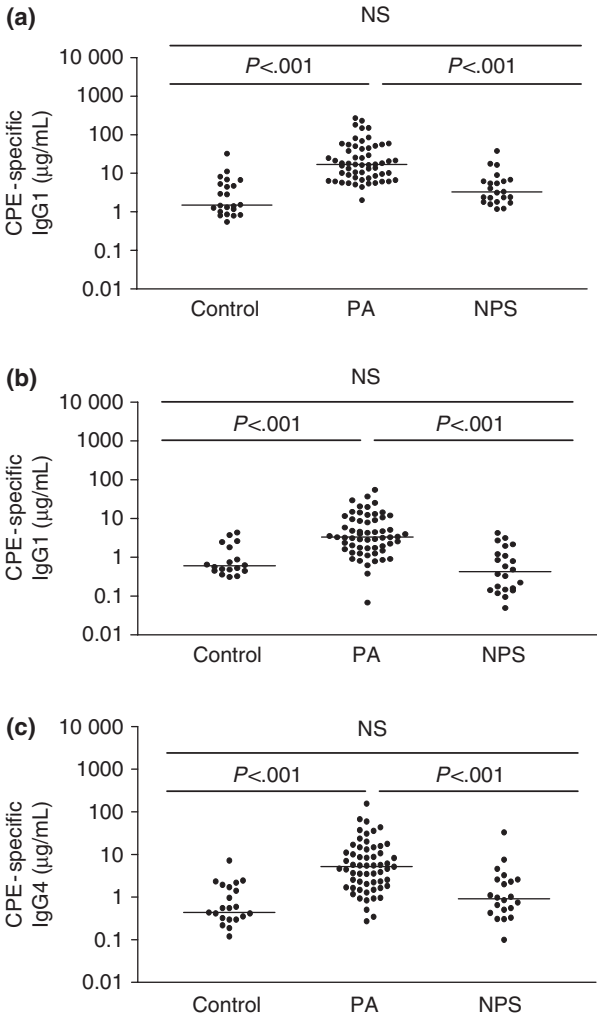


Fig. 2. Crude peanut extract (CPE)-specific IgG (a), IgG1 (b) and IgG4 (c) levels in controls ($n = 18$), peanut-allergic ($n = 59$) and NPS ($n = 26$) groups. Median bar and individual titres (dots) are indicated. Comparisons of medians between groups by a Mann–Whitney *U*-test marked by bars and *P*-values. PA, peanut allergic; NPS, non-peanut-sensitized but egg-allergic; NS, not significant.

NPS subjects. CPE-specific IgG4 was detected in all but three subjects (two controls and one NPS). CPE-specific IgG, IgG1 and IgG4 levels were significantly higher in children with peanut allergy (medians of 17.2, 3.3 and 5.2 μg/mL, respectively; $n = 59$) compared with controls (medians of 2.2, 0.6 and 0.8 μg/mL, respectively; $n = 18$) and NPS subjects (medians of 3.3, 0.4 and 0.9 μg/mL, respectively; $n = 22$). The CPE-specific IgG, IgG1 and IgG4 levels of NPS subjects were not significantly different from control levels. The ratio of specific IgG4 : IgG1 did not differ between the three groups. In children who had detectable peanut-specific IgE, significant positive correlations existed between specific IgE and specific IgG ($\rho = 0.72$; $P < 0.01$), IgG1 ($\rho = 0.67$; $P < 0.01$) and IgG4 ($\rho = 0.49$; $P < 0.01$).

In the 18 non-allergic controls, both CPE- and OVA-specific IgG levels were measured. OVA-specific IgG, IgG1 and IgG4 (medians of 15.9, 7.3 and 7.9 μg/mL, respectively) were significantly higher than CPE-specific IgG, IgG1 and IgG4 (medians of 2.2, 0.6, 0.8 μg/mL, respectively) levels ($P < 0.01$). Of interest, in peanut allergy, CPE-specific IgG, IgG1 and IgG4 were increased to levels comparable with OVA-specific IgG levels.

The IgG4 subclass titres were spread over a wider range (0–157 μg/mL) than IgG1 (0.5–55 μg/mL). IgG4 was the predominant subclass in 44% (8/18) controls, 69% (41/59) peanut allergic and 65% (17/26) of the NPS subjects. Both subclasses were correlated to total specific IgG ($\rho = 0.75$, $P < 0.01$ for IgG1; $\rho = 0.61$, $P < 0.01$ for IgG4), and positive correlations existed between IgG1 and IgG4 ($\rho > 0.49$; $P < 0.05$) in all subject groups.

## Discussion

We have developed a sensitive and robust ELISA system for measuring serum CPE- and OVA-specific IgG, IgG1 and IgG4 in absolute units. The subject groups were well characterized and included non-allergic controls, patients with active allergy, resolved allergy and those who were allergic to another food. By determination of specific antibody titres in micrograms per millilitre, we were able to reveal differences between IgG responses to two different food allergens. We also observed quantitative differences in IgG1 and IgG4 subclass expression.

An interesting finding of this study was the distinct pattern of IgG responses to peanut and egg proteins in children. OVA-specific IgG levels were significantly higher than CPE-specific IgG in non-allergics. Significant OVA-specific IgG responses were detected in all children including controls, with no differences between egg allergy and resolution. In contrast, CPE-specific IgG, while also detected in all children, were at low levels in controls and non-peanut-sensitized subjects. Increased IgG responses to peanut were specifically associated with peanut allergy, with CPE-specific IgG levels reaching that comparable with OVA-specific IgG levels.

The finding of significant IgG responses to OVA in all groups, including non-allergics, supports the notion that IgG production can be a normal physiological response to frequently ingested proteins. Others have also reported the presence of IgG antibodies to dietary antigens, in particular egg and milk, in a large proportion of healthy subjects [25–27]. CPE-specific IgG was also detected in all subjects in the study.

The larger IgG response to OVA compared with peanut (in controls) may be due to earlier introduction of egg and/or a more frequent consumption of larger amounts of egg in these children. Indeed, exposure to cow's milk

during the first 3 months of life is associated with high levels of IgG to β-lactoglobulin [28], and OVA-specific IgG levels can reflect dietary intake of egg in healthy adults over a 20-week period [29].

On the other hand, our findings also suggest that food-specific IgG levels are not solely related to dietary exposure. IgG responses to peanut were significantly elevated in children with peanut allergy, despite their current peanut avoidance. Although detailed consumption records were not available, it is known that 80% of peanut-allergic children report reactions to their first apparent peanut ingestion [30]. Therefore, it is unlikely that they would have eaten substantial amounts of peanut prior to diagnosis. In this setting, CPE-specific IgG up-regulation is associated with the allergic state rather than dietary exposure. This enhanced IgG response to peanut that is specific to peanut-allergics (and not found in NPS subjects) extends the findings of Kolopp-Sarda et al. [11], who reported higher peanut-specific IgG in peanut-allergic adults compared with controls.

Platts-Mills et al. [22] proposed that high-allergen exposure results in a protective response characterized by high levels of specific IgG and IgG4 and absence of specific IgE. Sletten's [15] findings that high β-lactoglobulin-specific IgG4 is associated with tolerance to milk also seems to support the protective roles for IgG4 in allergy. However, our results suggest that this might not be true of peanut allergy, where the association of high-IgG1 and IgG4 levels with clinical reactivity argues against protective or blocking functions for IgG1 and IgG4. Although the IgG1 subclass has been reported to make the largest contribution to total IgG, and IgG4 the smallest [31], we found a wide range of IgG4 : IgG1 ratios in all subjects, which were not associated with allergic status. Therefore, increased food-specific IgG4 subclass is not necessarily associated with Th2-type allergic responses. The OVA-specific and CPE-specific IgG4 predominates over IgG1 in two-thirds of the subjects. While it is not known whether IgG4 predominance extends to all food allergens, or indeed to other allergens, preliminary findings in our laboratory showed that IgG4 to a systemic antigen (tetanus toxoid) predominates in only 25% of the subjects (data not shown).

It is thus clear that different allergens, route, dosage and timing of exposure can induce varied magnitudes and distinct patterns of IgG responses. In this study, the patterns of IgG responses to two food allergens are shown to be very distinct. For egg allergens, IgG responses may reflect previous exposure and may not be indicative of disease, whereas for the peanut allergen, a higher IgG response is associated with clinical allergy, despite low exposure. Thus, tests for specific IgG to food, especially those designed to a panel of foods, may not be of value in diagnosing clinical allergy.

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