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Correlation of allergen-specific IgG subclass antibodies and T lymphocyte cytokine responses in children with multiple food allergies

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Cytokines can affect the quantity and class of allergen-specific immunoglobulins through the T cell polarization that accompanies atopy. Antigen-specific IgG subclasses and IgE antibodies were compared with intracellular T cell cytokine changes to sensitizing antigens in 23 children with multiple food allergies and 20 healthy controls. Allergic children showed higher levels of total and food-specific IgE, IgG1 and IgG4 to peanut, milk and egg than non-atopic children or adults, coinciding with a TH2 cytokine response to sensitizing antigens. IgG1 and IgG4 antibodies specific to milk and egg and peanut protein were elevated relative to age-matched healthy children ($p \leq 0.05$) and, in milk- and egg-sensitized children, correlated with cytokine responses ($p < 0.05$). Peanut-sensitized children additionally had elevated levels of IgG2 and IgG3 also which correlated inversely ($p < 0.003$ and $p < 0.04$, respectively) with IFNγ production. Elevated allergen-specific IgG subclass antibodies in sensitized children correlated with total IgE levels ($p \leq 0.05$) in all three food allergen groups. The ratio of specific IgG1 to IgG4 was highest in those with high IgE, inverted with resolution of allergy, and correlated with total IgE levels ($p \leq 0.01$) in milk- and egg-sensitized children. The correlation of TH2 responses with allergen-specific antibodies would implicate polarized T cells in food allergic children in IgE hypersensitivity and overproduction of particular IgG subclasses alike. IgG1:IgG4 ratio declines with allergy sensitization and may denote emerging tolerance.

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The central role of IgE in food-related allergy has been well described, much less is known about the role of other immunoglobulin classes. Specific IgG antibody production appears to reflect antigen exposure and occurs in healthy individuals as part of the normal immune response to foods (1). The humoral response to common dietary antigens in early infancy in both atopic and non-atopic children is principally IgG1, and the ranges of IgG concentrations in sensitized and healthy children frequently overlap (1). The higher levels of IgA, IgG1 or IgG4 in atopic children (2, 3) have not always been consistent, and some studies have found no difference (4, 5) or even lower levels of IgG in children with food allergy (6).

However, levels of certain IgG subclasses have been associated both with allergic sensitization and with atopic disease. Elevated maternal IgG4 antibodies are associated with the development of atopy in children, and IgG of all subclasses are acquired earlier in children of atopic mothers (7). The titer of maternal food-specific IgG4 antibody was directly related to allergy in children with

atopic dermatitis (8) and high levels of food-specific IgG4 are frequently found in children with eczema (9). Peak IgG4 levels occur in healthy children before 2 yr of age and thereafter decline, whereas in children sensitized to foods, specific IgG4 peaks earlier and persists for 8 yr and longer. Specific antibodies to dietary and inhalant antigens are mostly IgG1 in normal individuals, while IgG4 predominates in allergy suffers (10). It is very likely that the relative quantities of IgG subclass antibodies can have prognostic and symptomatic significance in allergic sensitization.

The T cell polarization that is a cardinal feature of atopy (11) could directly influence the quantity and subclass of allergen-specific antibody by production of TH2 cytokines. IL4 and other TH2 cytokines have additive effects on B cell production of antibody, promoting both IgE and IgG *in vitro*, and production of both antibody classes is down-regulated by IFNγ (12). Additionally, TH1 and TH2 polarizaton promote IgG subclasses differentially, with IgG4 subclass switching preferentially promoted in sensitized B cells by IL4 treatment (3). TH2 cytokines stimulate transcription from the CE locus, coding for the constant regions of the IgE heavy chain, while IL4 promotes the expression of both IL4 receptors and CD40 ligand, the second requisite B cell factor for class switching (11). In atopic children, persistent TH2 cytokine responses to sensitizing antigens could promote IgE hypersensitivity and overproduction of certain IgG subclasses alike.

A distinction in the relative concentration of IgG subclass antibodies may also denote changes in the degree of allergic sensitization. Differences in shrimp-specific IgG2 and IgG4 subclass antibody levels were found in sensitized fishermen (14), and elevated IgG4 to wheat flour correlated with duration of allergic rhinitis, whereas IgG1 levels correlated with lower IgE in bakers (15). Elevated IgG4 to birch correlated with increased production of IL4 in sensitized children, while high birch IgG1 subclass antibodies corresponded with IFNγ production in non-sensitized children (16). Distinctions in the quantity and persistence of the subclass of IgG antibodies between atopic and healthy children may indicate allergic sensitization and may denote when clinical sensitivity has altered in favor of developing tolerance (17).

In this study, therefore, we have examined cytokine production in atopic children in conjunction with immunoglobulin concentration to see whether persistent TH2 cytokine responses could promote IgE hypersensitivity and overproduction of certain IgG subclasses. We previously documented a persistent population of CD4+ CD45RO+ cells in a panel of food-sensitized children producing TH2 cytokines to exposure with common dietary allergens (18). Correlation of the concentrations of IgG subclass antibodies specific to food antigens with total and specific IgE and antigen-specific IL4 and IFNγ production in response to peanut, β-lactoglobulin (BLG) and ovalbumin (OVA) in this cohort would support the hypothesis that TH2 cytokine polarization of T lymphocytes affects allergen-specific humoral responses.

Materials and methods

Blood was obtained from children attending allergy outpatient services of Great Ormond Street Hospital NHS Trust in London, from 10 age- and sex-matched children without any history of allergy and 10 healthy adults, with prior local Medical Ethics Committee approval and informed consent. Total serum IgE and specific IgE to egg, cow's milk and peanut were measured with Pharmacia Cap® RAST, according to the manufacturer's recommendations. Results above 0.35 kU/l were considered positive. Clinical symptoms of the allergic children have been displayed in Table 1 with IgE values. No IgE specific to food antigens was detected in the sera of the healthy control children.

Monoclonal antibody hybridomas and reagents

Hybridoma OKT3 to human CD3 [American Type Culture Collection (ATCC)], ATCC HB-247 (anti-human CD14), L242 (anti-HLA DR, ATCC HB-171) and BU12 (anti-human CD19, from Dr Debbie Hardie, Division of Infection and Immunity, University of Birmingham) were cultured to high density in 20% FCS/Hybricare medium (ATCC, cat. No. 46-X), supernatant collected, clarified and filtered (0.4 um) and frozen. Peanut protein antigen (defatted peanut protein; SPF Inc., Austin, TX, USA), BSA, BLG, OVA (Sigma B6917, A5503, L3908) were tested for LPS content by Limulus Amoeba Lysate kit (Quadratech Ltd, PO Box 167, Epsom, Surrey).

Serum IgG immunoglobulins

Nunc Maxisorp plates were coated with BLG, OVA, BSA and peanut at concentrations of 25, 10, 20 μg/ml and 10 μg/well, respectively, in coating buffer (0.016 M $NaHCO_4$/0.034 M Na_2CO_3, pH 9.6) at 4°C overnight. Washing

Table 1. Major clinical features and IgE values of food sensitized children

Patient	Age (yr, month)	Total IgE (kU)	Specific peanut	IgEs milk	(kU) egg	Major symptoms & allergies
S1	8,5	14600	>100	>100	>100	Eczema; autism: M, E, P
S2	6,8	6768	>100	>100	>100	Eczema;GI;C18 del; M, P*, E*
S3	10,6	5748	>100	12.4	4.97	Eczema; P, mT
S4	6,7	4647	54.0	>100	>100	Eczema; C18 del; M, E, P*
S5	11,4	3319	9.4	10.7	26.4	Asthma; E, P*
S6	9,7	3099	29.9	10.2	7.27	Eczema; E, P*
S7	7,5	1949	14.6	6.92	>100	Eczema; E,P*
S8	9,7	1081	2.1	2.91	10.8	Food allergy (GI); M, E, P
S9	8,0	1010	46.5	8.1	42.0	Asthma; M, E, P*
S10	6,8	1000	52.9	3.84	7.31	Food allergy (GI); M, E, P*
S11	3,4	868	<0.4	23.6	<0.35	Eczema; E, S*
S12	9,2	692	9.8	1.87	0.94	Asthma; P
S13	11,5	569	35.9	4.7	<0.35	Eczema; P, E, mr
S14	12,11	478	>100	<0.35	<0.35	Asthma; P
S15	6,8	444	<0.35	0.72	<0.35	Food allergy (GI); B*
S16	3,7	325	<0.35	0.57	<0.35	asthma, E
S17	3,5	286	3.44	5.17	4.99	Food allergy (GI); M, E, P
S18	5,8	212	5.06	3.82	1.1	Asthma; E, P
S19	8,2	146	1.8	0.79	<0.35	Food allergy (GI); mR
S20	11,3	128	3.9	0.71	<0.35	Eczema; mR
S21	4,6	122	28.6	6.1	4.9	Food allergy (GI); M, E
S22	5,3	11.6	0.8	<0.35	<0.35	Atopy; no food allergy
S23	7,0	11.4	<0.35	<0.35	<0.35	Urticaria; no food allergy

ID, immunodeficiency; GI, Gastrointestinal symptoms such as diarrhea, vomiting and abdominal pain; C18q del, chromosome 18q deletion syndrome; E, egg; P, peanut; M, milk; mT, milk tolerated; mr, resolving milk allergy; mR, resolved milk allergy; S, sesame; B, Brazil nut. *anaphylaxis.

was performed three times between each step with PBS/0.05% Tween 20 and plates were blocked with 1% normal mouse serum in PBS/Tween for 1 h at 37°C. Sera from five patients with high food-specific IgE titers were pooled to prepare a standard curve. Buffer or serially diluted pooled serum standard and patient sera or plasma samples applied to each plate in triplicate at two dilutions, 1/10 and 1/50, overnight at room temperature. Directly conjugated murine antibodies against human total IgG or IgG subclasses were applied in PBS/0.05% Tween 20 containing 0.5% normal mouse serum for 90 min at 37°C. Anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 (clones HP6014, 80/6-39, HP6050 and HP6025, The Binding Site, PO Box 11712, Birmingham) were applied at dilutions 1/500, 1/200, 1/250 and 1/1000, respectively, and 3,3′,5,5′,-tetra methyl benzidine (TMB) hydrochloride substrate (Sigma, cat No. T-3405) used in 0.05 M phosphate citrate buffer, pH 5. Anti-human IgG conjugated to alkaline phosphatase (Sigma A-9544) was added at a concentration of 1/15,000 for 1 h at 37°C and used with O phenylene diamine substrate (Sigma P-8287) in 0.05 M phosphate citrate buffer. Absorbance was read after an hour in the dark at room temperature at 450 nm for TMB and 405 nm for O phenylene diamine substrates, in a Dynatech Ltd. MRX plate reader. Immunoglobulin concentration was expressed in arbitrary units (AU/ml), generated from standard curves with the reference sera, subtracting buffer absorbance values.

Cell culture and treatment

Peripheral blood monocytes (PBMC) were collected from heparinized blood over a cushion of Lymphoprep™ (Axis-Shield, UK, Kimbolton, Cambridgeshire) after 30 min centrifugation at 280 ***g***. PBMC were washed twice with RPMI 1640 for 10 min at 220 × g to reduce platelets. Functional antigen-presenting cells were generated from adherent PBMC cultured for 7–12 days in 20% autologous plasma/RPMI supplemented with 100 ng/ml human GM CSF (Insight Biotech JR-1900) and 50 ng/ml human IL-4, (Insight Biotech, IB-1016, Wembley). Dendritic cells (DCs) were depleted of contaminating cells by magnet with 10 μl of sheep anti-mouse IgG-coated beads (M101, Dynal) per 1×10^6 cells after incubation for 15 min at 4°C on a tube rotator with 350 μl BU12, 200 μl OKT3 and 450 μl HB-247 hybridoma supernatants in 2 ml of RPMI. DCs were cultured with food antigens (100, 10 and 1 μg/ml) for 24 h and mixed by pipetting with T cells.

Non-adherent PBMC were suspended for 15 min in 2 ml of RPMI with 350 μl of BU12 hybridoma supernatant, 300 μl per ml of HB-247 hybridoma supernatant and 300 μl of HB-171 supernatant. Non-T cells were depleted with a magnet with 10 μl of sheep anti-mouse IgG-coated beads per 5×10^6 cells, cell concentration adjusted to 3×10^6/ml in 20% autologous plasma/RPMI and 100 μl aliquots (300,000 per well) dispensed in 96-well plates. T cells were pulsed with BSA, BLG, OVA and peanut at a final concentrations of 100, 10 and 1 μg/ml or incubated with two successive aliquots of 30,000 cells per well of antigen-pulsed dendritic cells in a 10:1 T:DC ratio, 72 h apart. Brefeldin A (Sigma B6751), Ionomycin (Sigma IO634) and phorbol myristate acetate (PMA, Sigma P8139) were added at final concentrations of 10, 1 μg/ml and 10 ng/ml, respectively, for the last 2 h of incubation.

Antibody staining and flow cytometry

Cytokines were detected intracellularly in fixed, permeabilized cells with and without the addition of food antigen. Cells in 96-well plates were suspended in 25 μl of fixative solution (reagent A, Serotec BUFO9B) and the plate incubated at room temperature for 15 min. Cells were stained with three antibodies simultaneously in permeabilizing agent (Leukoperm, Serotec BUFO9B) for 1 h at room temperature. Mouse anti-human antibodies CD3 FITC (Pharmingen 30108X) and CD8 Cy-Chrome (Serotec MCA1226F) or CD45 RO Cy-Chrome (31308X Pharmingen), CD45 RA APC (Pharmingen 557015), and one of four anti-human cytokine reagents IL-2 (BD 340450), IL-4 (BD 340451), IL-10 (Serotec MCA1531PE) or IFNγ (BD 340452) conjugated to phycoerythrin. Antibodies were premixed in a replicate 96-well plate diluted 1:5 in 50 of Leukoperm and applied simultaneously to pelleted cells in the test plate. Controls were stained with mouse isotype control antibodies IgG1 FITC, IgG1 PE and IgG2 Cy-Chrome (BD 349041, 349043 and 349047), anti-CD8 FITC, anti-IFNγ PE, and anti-CD45 RO or CD3 Cy-Chrome. Finally, cells were washed twice with 200 μl RPMI/0.01% sodium azide, resuspended in 100 μl PBS and transferred to LP2 tubes (Life Sciences International, LK00023) and acquired with a Becton Dickinson FACSCalibur. The measurement of the percentage change of intracellular cytokine expression in response to food antigen exposure was derived by the following formula: (% of cytokine positive cells after addition of antigen divided by the % of cytokine positive values without antigens × 100)−100.

Statistical analyses

Statistical analyses were performed using SSPS for Windows (version 8.0) and Microsoft Excel 07. Results were tested using an independent sample two-tailed *t*-test. Mean and standard deviation of group stimulation indices were compared by *F* test. Correlation was assessed using Spearman's rank correlation coefficient. p Values < 0.05 were considered significant.

Results

The age range of 23 allergic patients (11 female, 12 male, Table 1) studied was 3 yr 4 months to 13 yr 11 months. Total serum IgE varied from 11.4 to 14600 kU/l with a median of 2386 kU/l. Five children were sensitized to only one of the three foods in the study, five children to two foods in the study and 12 children were allergic to egg, milk and peanut.

Food antigen-specific total and subclass IgG levels

β-lactoglobulin-, OVA- and peanut protein-specific antibodies are summarized as complete range, 25–75% interquartile ranges and median values of allergic children, non-allergic children and non-allergic adults (Fig. 1). BLG-specific IgG1 and IgG4 antibodies were only elevated in milk-sensitized children (Fig. 1a). Both IgG1 and IgG4 values of milk-sensitized children were significantly increased when compared to non-sensitized children ($p = 0.04$ and $p = 0.05$, respectively) and adults ($p \leq 0.01$), and BLG-specific total IgG values differed between sensitized children and non-sensitized adults ($p = 0.03$) but did not reach statistical significance between sensitized and non-sensitized children ($p = 0.06$). In addition, adult BLG-specific IgG1 values differed from those of non-sensitized children ($p = 0.03$) and adult IgG3 from non-sensitized children ($p = 0.03$).

OVA- and peanut-specific Ig subclasses

A similar pattern to that of milk was observed in egg-allergic children. OVA-specific IgG1 and IgG4 levels were elevated in egg-sensitized children (Fig. 1b) compared with both non-sensitized children ($p < 0.01$ and $p = 0.04$ for IgG1 and IgG4) and non-sensitized adults ($p < 0.01$ and $p = 0.03$ for IgG1 and IgG4). IgG2, IgG3 and total IgG values did not differ between

sensitized and non-sensitized children. Higher levels of OVA IgG2 were observed in non-sensitized adults compared with normal ($p = 0.04$) or egg-sensitized children ($p = 0.03$).

Peanut-sensitized children (Fig. 1c) demonstrated a different pattern, exhibiting a higher levels of peanut-specific total IgG and elevated levels of all IgG subclasses compared with peanut non-sensitized children and non-sensitized adults ($p \leq 0.03$ for all groups). There were no differences between non-sensitized children and adults for any peanut IgG subclass or total IgG. A single peanut-sensitized adult tested (age 25) had specific IgG1, IgG2, IgG3, IgG4 and total IgG antibody levels comparable to those of sensitized children (data not shown).

Correlation of antibody levels with *in vitro* antigen-specific cytokine responses

In circulating peripheral CD3+ cells (Fig. 2a), the proportion of IL4-producing cells was

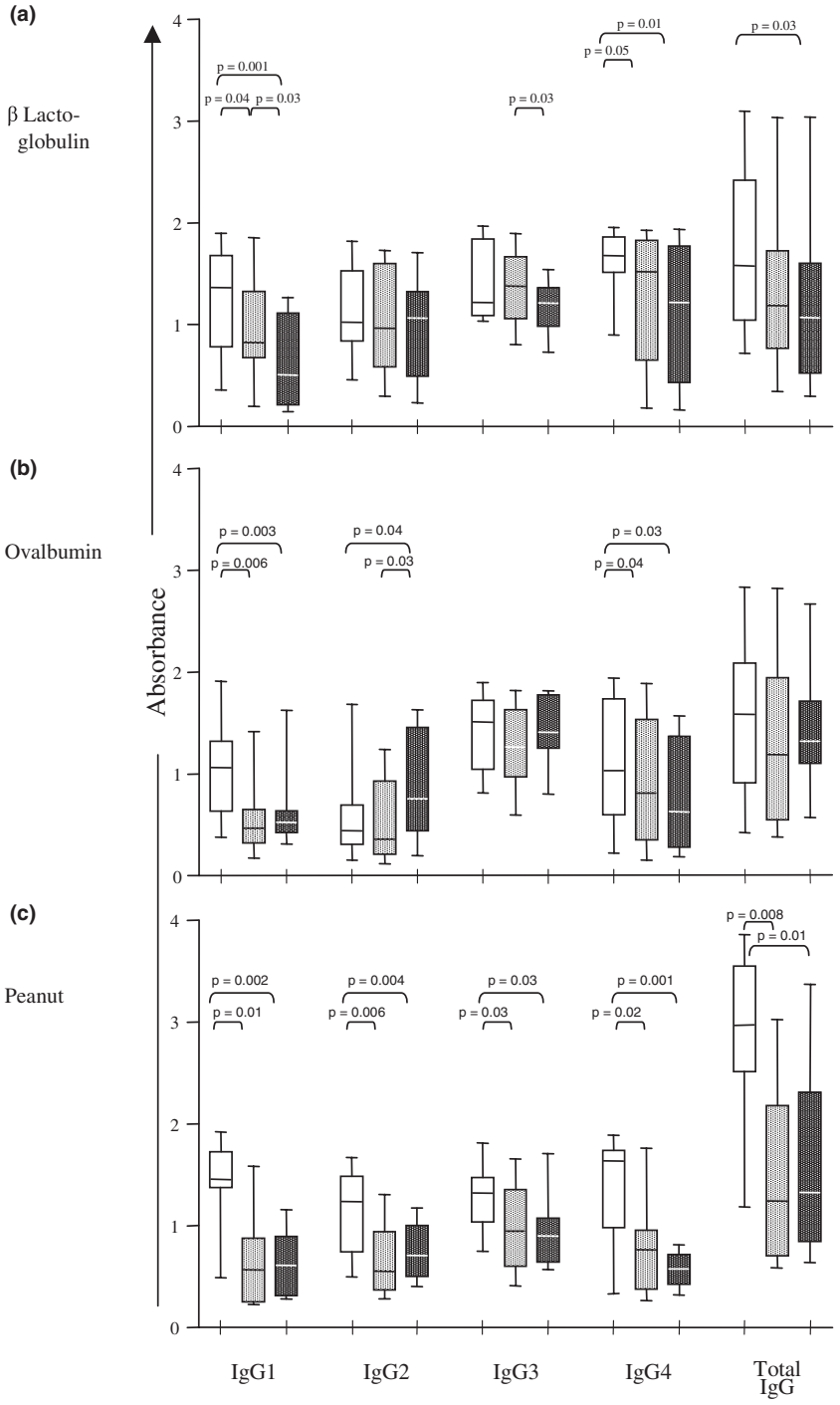

Fig. 1. Ovalbumin (OVA)-, β-lactoglobulin (BLG)- and peanut-specific total subclass IgG antibody concentrations. Antibodies were evaluated in egg-, milk- and peanut-sensitized children □ and non-sensitized children ▨ and non-sensitized adults ■ by ELISA and displayed as median, total and 25–75 percentile ranges (box). Total immunoglobulin G and subclasses specific to (a) BLG in milk-sensitized ($n = 12$), non-sensitized ($n = 18$) children and non-sensitized adults ($n = 15$). (b) OVA in sensitized ($n = 17$), non-sensitized ($n = 14$) children and non-sensitized ($n = 15$) adults. (c) Peanut in sensitized ($n = 19$), non-sensitized ($n = 13$) children and non-sensitized adults ($n = 14$). Groups were compared with a non-parametric Mann–Whitney *U* test and p values < 0.05 indicated.

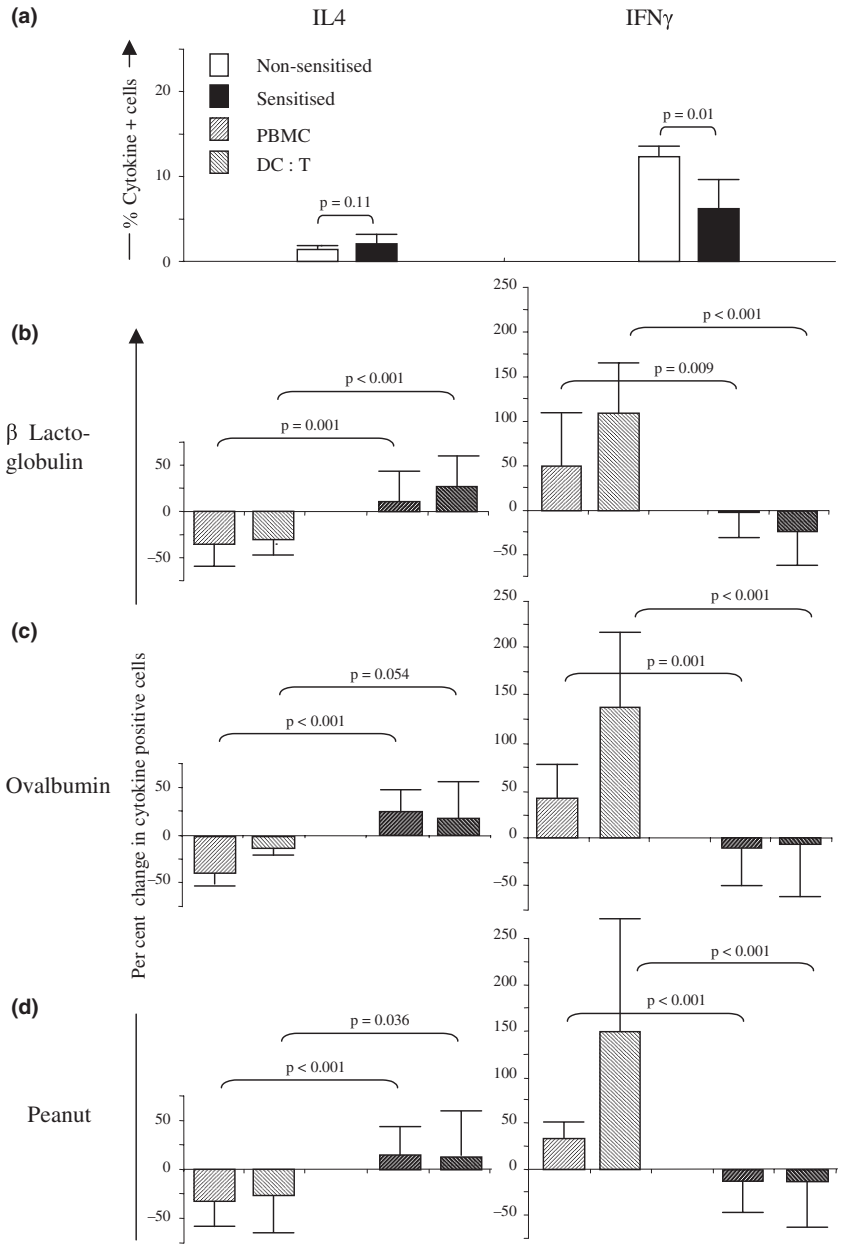

Fig. 2. Cytokine production to food antigen in sensitized and non-sensitized children. Mean + standard deviation (error bars) in IL4 and IFNγ production in (a) circulating T cells of non-sensitized □ and sensitized ■ children. (b) Percent change in cytokine production in CD3+ cells of antigen-exposed peripheral blood monocytes ▨ and antigen-pulsed dendritic cell cocultured CD3+ cells ▧ in milk-sensitized and non-sensitized children, (c) in egg-sensitized and non-sensitized children, (d) in peanut-sensitized and non-sensitized children.

slightly raised (non-significantly, $p = 0.11$) and IFNγ-producing cells were substantially reduced ($p < 0.01$) in sensitized children. Cytokine responses of PBMCs incubated in four log dilutions of BLG, OVA and peanut protein and in CD3+ T cells exposed to DCs pulsed with same antigen dilutions are shown as the mean percentage change in the proportion of IL4- and IFNγ-producing cells from untreated cells (Fig. 2b–d). Dendritic cell presentation of antigen to CD3+ cells induced cytokine responses similar to those of PBMC and were additive for up to three additions of pulsed dendritic cells. IL4 and IFNγ responses differed between sensitized and non-atopic children ($p \leq 0.05$) for all conditions. Cells from milk-, egg- and peanut-sensitized children responded to presentation of each sensitizing antigen with an increase in IL4 and a minor reduction in IFNγ-producing cells, in contrast to the responses of non-sensitized children in which IL4-producing cells were reduced ($p \leq 0.05$) and numbers of IFNγ-producing cells substantially increased ($p < 0.01$) for milk, egg and peanut PBMC and DC-presented antigen. After two incubations with antigen-pulsed cells in a 1:10 ratio, DC-induced T cell changes exceeded those found in treated PBMC in the majority of cases, with greater reduction of IFNγ and further promotion of IL4 production. IL4 and IFNγ responses of

sensitized children were highly inversely related, an increase in IL4 correlating closely with reduced IFNγ production ($p < 0.001$) for each food antigen in sensitized children.

To relate IgG subclass antibodies to cytokine responses, absorbance units for total IgG and antibodies of each IgG subclass specific to OVA were plotted against the individual changes in the percentage of IL4- and IFNγ-producing CD3+ cells after incubation with two successive aliquots of OVA-pulsed DCs (Fig. 3). Regression analysis trend lines approaching a 45° angle from bottom left to top right indicate a positive association between an increase in cytokine production in response to *in vitro* exposure to OVA and a high level of IgG subclass antibodies specific to OVA. Children sensitized to egg (unfilled shapes) increased IL4 and decreased IFNγ production to OVA in contrast to decreased IL4 and increased IFNγ production in cells of non-atopic children (filled shapes). For egg-sensitized children, the IL4-producing T cell percentage correlated with OVA-specific IgG1 (◇) ($p = 0.04$), IgG2 (□) ($p = 0.05$), IgG4 (○) ($p = 0.01$), as well as total IgG antibodies (x) ($p = 0.004$). Additionally, OVA-specific subclass antibodies of IgG1 ($p = 0.06$), IgG4 ($p = 0.05$) as well as total IgG ($p = 0.01$) correlated with reduced proportion of IFNγ-producing cells after OVA exposure.

Neither milk nor peanut-specific IgG antibodies were as closely associated with cytokine responses as found for egg-sensitized children (Table 2). BLG-specific IgG4 subclass antibodies only correlated weakly with IL4 ($r = 0.47$, $p = 0.05$), and IgG2 inversely correlated ($r = 0.51$, $p = 0.045$) with change in IFNγ production to β lactoglobulin. No peanut-specific IgG subclass correlated with IL4 production but peanut-specific IgG2 and IgG3 levels correlated with the reduction in IFNγ-producing cells ($r = 0.58$, $p = 0.003$ and $r = 0.57$, $p = 0.004$, respectively) in peanut-sensitized children. Specific IgG values of non-sensitized children or adults did not correlate with cytokine responses.

Table 2. Correlation (p) of IgG subclasses and total IgG with IL4, IFNγ, total and specific IgE

Children	IgG Subclass	IL4	IFNγ	Total IgE	Specific IgE
Egg-sensitized children	IgG1	0.04*	0.06	0.31	0.09
	IgG2	0.05*	0.76	0.91	1.0
	IgG3	0.41	0.41	0.02*	0.86
	IgG4	0.01*	0.05◆	0.08	0.43
	Total IgG	0.004*	0.01◆	0.17	0.45
Milk-sensitized children	IgG1	0.05*	0.88	0.06	0.33
	IgG2	0.86	0.05◆	0.90	0.50
	IgG3	0.21	0.14	0.56	0.40
	IgG4	0.04*	0.51	0.05*	0.31
	Total IgG	0.22	0.26	0.07	0.07
Peanut-sensitized children	IgG1	0.66	0.47	0.05*	0.43
	IgG2	0.07	0.003◆	0.48	0.96
	IgG3	0.35	0.04◆	0.52	0.31
	IgG4	0.09	0.14	0.08	0.43
	Total IgG	0.82	0.22	0.17	0.45

*Correlation $p \leq 0.05$. ◆inverse correlation $p \leq 0.05$.

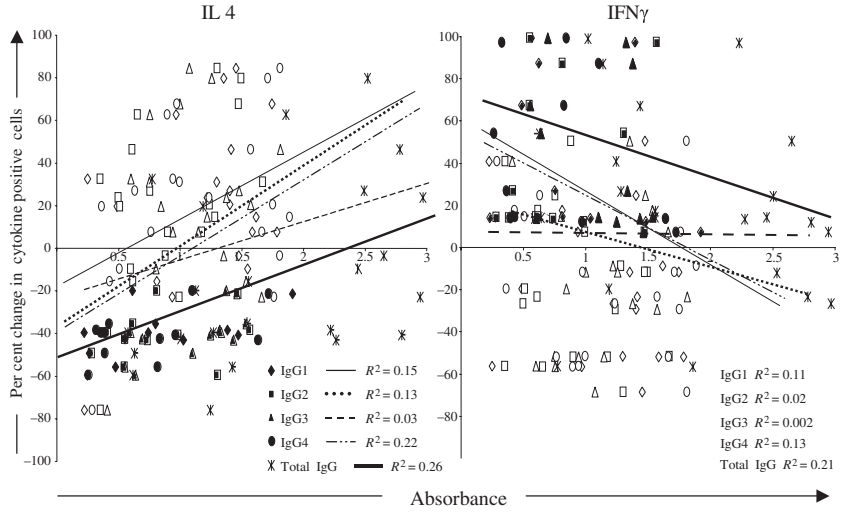

Fig. 3. Association of ovalbumin (OVA)-specific IgG subclass antibodies with IL4 and IFNγ responses in egg-sensitized (empty shapes) and non-atopic (filled shapes) children. Subclass and total IgG absorbance values measured by ELISA were plotted against % change in cytokine positive T cells to OVA antigen presentation. A line of 45° from bottom left to top right would indicate perfect correlation of IgG subclass absorbance and cytokine production. Correlation (r) values for trend lines for IgG1 (◇, ——), IgG2(□, ·····), IgG3 (△, - - - -), IgG4 (○, – ··) and total IgG (X, ——) are indicated.

IgG subclass ratios and correlation with IgE

A close association between IgE and particular IgG subclass antibody levels was found for each of the food antigens (Table 2). IgG4 of milk-sensitized- ($r = 0.46$, $p = 0.05$), IgG1 of peanut-sensitized- ($r = 0.44$, $p = 0.05$), and IgG3 of egg-sensitized children ($r = 0.52$, $p = 0.02$) correlated with total IgE values. There was no relation between food antigen-specific IgE and IgG subclass antibodies for egg, milk or peanut. IgG1 and IgG4 antibodies specific to peanut, milk and egg were equally raised in the older sensitized children, and there were no differences in the relative amounts of specific IgG subclass antibodies in sensitized children with age. Compared with non-atopic children, BLG-specific IgG4 antibodies were elevated in children with reducing milk sensitization, remained raised in children who had attained tolerance to milk, while BLG-specific IgG1 antibodies of milk-tolerant children were reduced. The ratio of IgG1:IgG4 absorbance values for non-sensitized children (mean IgG1:IgG4 ratio = 1.24) was higher than that for milk-sensitized children (mean ratio = 0.80, $p = 0.04$), decrease with resolving sensitization and inverted in milk-tolerant children (mean ratio = 0.47). Mean IgG1:IgG4 ratios for egg- and peanut-specific antibodies of sensitized children (0.83 and 1.01, respectively) were also lower than those of non-sensitized children (1.17 and 1.37, respectively, $p = 0.01$ and $p = 0.11$). Additionally, the IgG1:IgG4 ratios for BLG and OVA correlated with total IgE absorbance values in milk- and egg-sensitized children ($r = 0.68$ and 0.53, respectively, $p = 0.04$ and $p = 0.02$). Peanut-specific IgG subclass ratios were not associated with IgE, and the patterns of immunoglobulin association and cytokine production varied with each food sensitization.

Discussion

In keeping with previous studies (eg. 16, 19, 22), atopic children sensitized to multiple foods consistently had elevated total IgE and food-specific IgE levels combined with increased IL4 cytokine production *in vitro* to sensitizing antigens. Additionally, TH2 cytokine responses to sensitizing antigens were associated with an increased level of particular IgG subclass antibodies. Both IgG1 and IgG4 antibodies specific to OVA, BLG and peanut were elevated in multiallergic children compared with both non-atopic, age-matched children and non-atopic adults. The distinctive elevation of all four subclasses of peanut-specific IgG, previously observed in sensitized children (20), could in part be because of complexity of the antigen. All of the predominant peanut proteins are immunogenic to both atopic and healthy children, generating antibodies of diverse IgG subclasses (21), which may contribute to the unique severity as well as the low frequency of resolution of the allergy. Peanut-specific IgG subclass antibodies do not seem to have a protective effect in peanut immunotherapy (22) and their activation of complement and opsonization (23) increase the likelihood of acute reactions in peanut allergy.

Allergen-specific antibodies are thought to be generated in parallel in atopic individuals primarily by IL4-induced B cell class switching (13). We have previously demonstrated a persistent TH2 CD4+ T cell subset in this cohort of children and a close relation between total IgE levels and IL4 responses to allergens (18). This report extends this data to show a consistent association between TH2 cytokine responses to specific OVA, BLG and peanut antigens and the presence of particular IgG subclasses in sensitized children but not age-matched healthy controls. OVA-specific IgG1 and IgG4 and total IgG correlated with an increase in IL4 production as well as a decrease in IFNγ production, indicating that in egg sensitization at least, the immediate reactivity of T cells to allergen matched the specificity of circulating B cells. BLG-specific IgG antibodies in food-sensitized children in this study did correlate with IL4 production but were less closely associated with TH2 T cell cytokine response than antibodies to OVA and the cytokine response to peanut antigen presentation was associated with different IgG subclass antibodies than found for milk or egg. The elevated peanut-specific IgG2 and IgG3 antibodies associated with the reduction of the proportion of IFNγ-producing cells are associated with immunity to helminths. Thus, TH2 cytokine responses to food antigen presentation did correlate with total IgE and with IgG subclass concentrations specific for each food but the associated subclasses of IgG varied and there was no consistent pattern of specific antibody associated with all food sensitizations. Proportional IgG and IgE to milk proteins (19), to peanut (21) and to aero-allergens have previously been reported, supporting a strong connection between TH2 cytokine polarization and antibody production in atopy.

A rise in IgG4 subclass antibodies has often been, as in our study, associated with allergic sensitization (e.g., 2, 3, 10). The direct relation of IgG4 concentration to clinical symptoms has led to the presumption that IgG4 may have a

pathological role in allergy (24). However, IgG4, unlike IgG1, is non-compliment fixing, binds poorly to Fc receptors and is TH2 cytokine regulated (25). IgG4 has reduced inter-heavy chain disulfide bond stability, resulting in a high proportion of monovalent molecules, intermolecular exchange and bispecific antibodies unable to form large immune complexes, and a low capacity for inducing inflammation. IgG4 antibodies may instead compete of for antigenic sites, interfering with inflammation induced by complement-fixing antibodies and operate by blocking anti-helminthic immunity and the sensitization of IgE antibodies in allergy (26).

A link between cytokine polarization and antibody production also operates in the development of clinical tolerance. Resolution of milk allergy was accompanied in this cohort of children by a shift toward TH1 cytokine responses by T cells (18) and by antibody subclass modulation, as observed in children who had outgrown peanut allergy (27). The shift in T cell cytokine profile accompanying IgG subclass redistribution in immunotherapy has been suggested as the mechanism underlying successful desensitization. A change in the ratio of IgG subclasses can be indicative of the status of allergy and has denoted when clinical sensitivity altered in favor of developing tolerance. In this study, the BLG-specific IgG1:IgG4 ratio was higher in non-atopic than in milk-sensitized children, decreased with resolution of allergy and correlated with IgE levels in two of three groups of sensitized children. Similarly, casein- and BLG-specific IgG1:IgG4 ratios were lower initially and decreased further in children with decreasing clinical reaction to challenge with milk (16). Higher levels of food-specific IgG4 were associated with tolerance in atopic children (28) as well as a reduction in response to a previously sensitizing meal in clinical challenge. Allergic children able to consume sensitizing foods have higher IgG4 levels and children with higher specific IgG4 levels in infancy (29) are more likely to become tolerant in early childhood (30). The decline in IgG1 antibodies to egg and milk proteins even in atopic children (1, 3) can disturb the usual proportions of IgG subclasses with IgG4 increasing to constitute the highest proportion of total immunoglobulin (1, 7). The association of reducing IgG1:IgG4 ratio with IgE in two groups of sensitized children would indicate closely linked isotype switching in atopy and a decreasing IgG1:IgG4 ratio may be a good indicator of emerging clinical tolerance. Correlation of food-specific IgG levels of sensitized children with the proportion of T cells producing TH2 cytokine would support a direct connection between cytokine polarization and antibody stimulation as operating in food sensitization. However, variation in isotype level and subclass switching dependent on the allergen in different sensitizations limit the diagnostic significance of specific IgG subclass evaluation in atopy.

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