

## Humoral response to food antigens

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The diagnosis of food allergy or food intolerance is generally based on the anamnesis, avoidance diets and oral double-blind challenges, while laboratory tests are less useful (1, 2). The presence of circulating antibodies to food antigens, in fact, is a general phenomenon and it is clear that their presence is the consequence of antigenic stimulation (3-5). The finding of detectable serum levels of such antibodies does not necessarily mean that the patient is allergic to the food, nor that specific antibodies represent the damaging mechanism. Between different possible pathogenetic mechanisms, those that are IgE-mediated have been extensively studied. Several food-induced symptoms do not appear to belong to the immediate-type reaction and usually are detectable at sites distant from the gut (i.e. skin, lungs etc.). Moreover, specific IgE may persist long after a clinical reaction has disappeared and the significance is less clear than that of IgE antibodies to inhalant allergens. The significance of specific IgG and IgM is more unclear: these antibodies have been reported to occur frequently also in healthy persons, and in several conditions, including IgA deficiency, chronic inflammatory bowel diseases, malnutrition, food allergy and atopic dermatitis (6-8). In 1970, Parish described the short-term sensitizing (STS) antibodies belonging to the IgG class, with reaginic activity (9). More recently, a potential role in mediating anaphylactoid reactions and other atopic manifestations has been associated with the IgG<sub>4</sub> subclass: both "sensitizing" and "blocking" activities on mediator release by basophils have been demonstrated by different groups (10, 11). Moreover, specific IgG<sub>4</sub> increases during hypo-sensitizing immunotherapy and prolonged antigenic exposure (12).

In this study we analyzed the IgG subclasses to 2 protein antigens, ovalbumin (OVA) and beta-lactoglobulin (BLG), in normal, intolerant and atopic subjects on a free or restriction diet for milk and/or egg, with different ELISA assays.

### MATERIAL AND METHODS

#### *Sera*

Sera obtained from 68 patients with atopic dermatitis (AD) (aged 1-12 y) and 40 age-matched controls were tested for the presence of IgG subclasses to ovalbumin (OVA) and beta-lactoglobulin (BLG) by ELISA, of specific IgG<sub>4</sub> to OVA and BLG by FAST, and of specific IgE to OVA and BLG by RAST.

*Antigens:* Purified OVA and BLG were obtained from Sigma, St. Louis, MO, USA.

*Specific IgE determination* was made as already described (13).

*Specific IgG subclasses determination.* We set up an ELISA test using monoclonal antibodies (Abs) to human IgG subclasses (14). Briefly, polyvinyl microtiter plates were coated overnight with purified antigen (OVA or BLG) at a concentration of 10 µg/ml in carbonate-bicarbonate buffer 0.1 M, pH 9.6. After 3 washes with phosphate-buffer saline (PBS) plates were blocked with gelatin 0.1% in carbonate buffer for 1 h at RT. After 3 washes, with PBS-Tween, 100 µl of diluted samples in duplicate were added to plates and incubated overnight at RT. After washes, monoclonal

SPECIFIC IgG SUBCLASSES TO OVA: COMPARISON OF 8 ELISA PROTOCOLS IN 6 PATIENTS

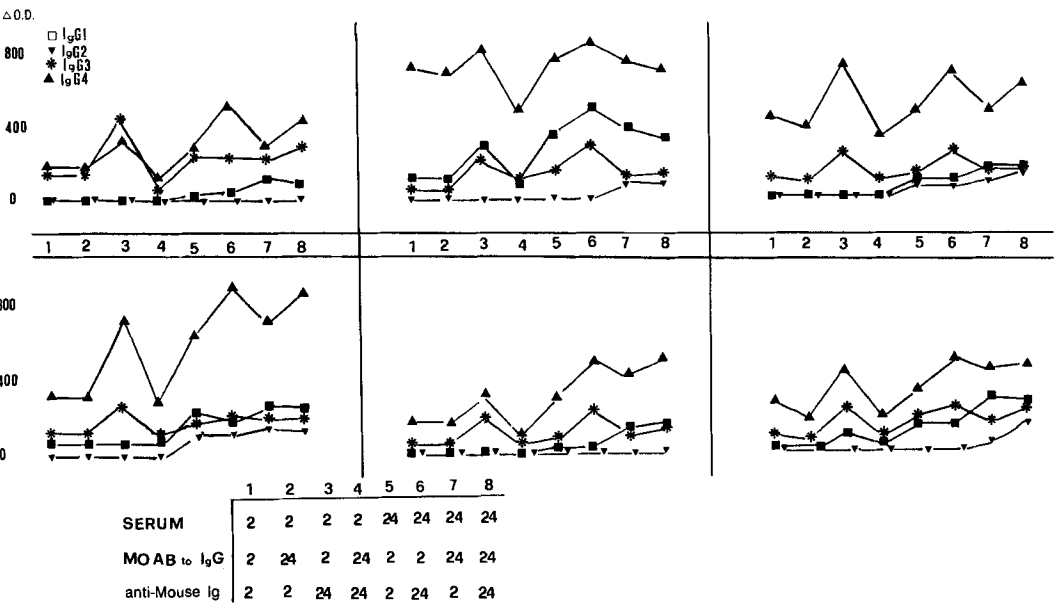


Fig. 1. IgG<sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>4</sub> to OVA in 6 patients according to 8 different ELISA protocols; see insert with incubation times in hours.

Abs to human IgG subclasses were added to plates and incubated overnight at RT. 100 µl of goat anti-mouse IgG, alkaline phosphatase-conjugated, diluted 1:1000, were added to plates and again incubated overnight at RT. After 3 washes with PBS-Tween, 100 µl of paranitrophenilphosphate, 1 mg/ml, diluted in Tris HCl buffer 0.1 M pH 8.6 were added

and the reaction allowed to develop for 30 min. The optical density (OD) was read at 405 nm using a Titertek MultiScan reader; the results were expressed as the difference between samples OD and controls OD.

Specific IgG<sub>4</sub> determination (FAST)

We compared results obtained with the described ELISA procedure with an *in vitro* immunoassay that measures circulating allergen-specific IgG<sub>4</sub> (3M Diagnostic Systems (IgG<sub>4</sub> FAST), St. Clara, Ca, USA). The procedure used was according to the package insert. In each plate a calibration curve was obtained using different dilutions of honey bee venom-specific IgG<sub>4</sub> that react with specific allergen-coated wells. IgG<sub>4</sub> levels of each sample were determined comparing the OD of the serum with those of the standard curve and expressed as µg/ml equivalents. The sensitivity of the method was 2 µg/ml of specific IgG<sub>4</sub> to either antigen.

Table 1

Correlation coefficient between different methods

| Correlation coefficient                          | r     |                 |
|--------------------------------------------------|-------|-----------------|
| <i>OVA</i>                                       |       |                 |
| IgG <sub>4</sub> (FAST)/IgG <sub>4</sub> (ELISA) | 0.334 | NS              |
| IgG <sub>4</sub> (FAST)/IgE (RAST)               | 0.221 | NS              |
| IgG <sub>4</sub> (FAST)/Prick test               | 0.311 | NS              |
| IgG <sub>4</sub> (ELISA)/Prick test              | 0.125 | NS              |
| <i>BLG</i>                                       |       |                 |
| IgG <sub>4</sub> (FAST)/IgG <sub>4</sub> (ELISA) | 0.548 | <i>P</i> < 0.05 |
| IgG <sub>4</sub> (FAST)/IgE (RAST)               | 0.260 | NS              |
| IgG <sub>4</sub> (FAST)/Prick test               | 0.1   | NS              |
| IgG <sub>4</sub> (ELISA)/Prick test              | 0.1   | NS              |

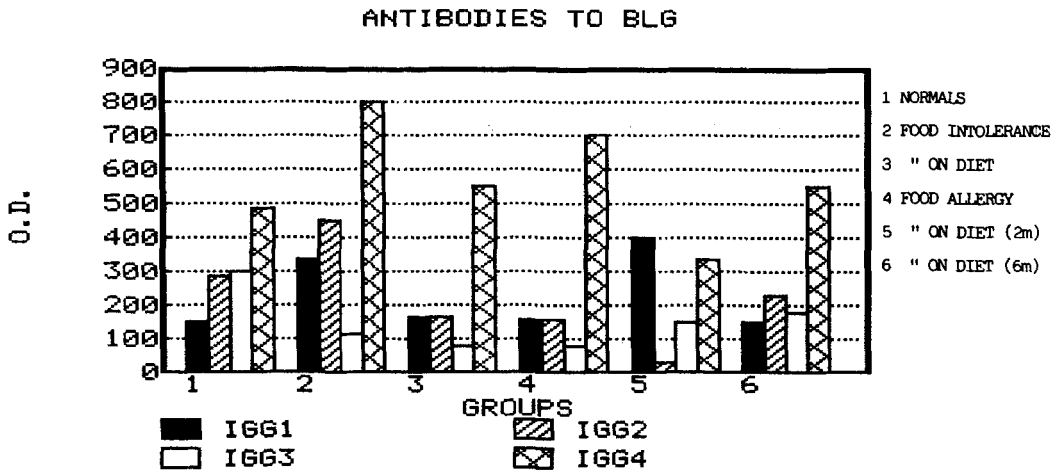


Fig. 2. IgG<sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>4</sub> to BLG (mean OD) in different groups.

#### Statistical analysis

We compared groups with the non-parametric Mann-Whitney U test for unpaired samples, as a two-tailed test, and the Fisher test. For comparison between different methods, we used linear regression analysis. The level of significance was chosen as  $P = 0.05$ .

#### RESULTS AND DISCUSSION

Because of the discrepancy of results obtained by different groups using the same reagents and

comparable patient samples, we postulated that technical problems could interfere with the conclusions drawn in different studies. When a purified antigen is available, an antigen-binding immunoassay could be set up that allows simultaneously measurement of antibodies of several immunoglobulin classes and subclasses: owing to the high sensitivity of the ELISA test, low concentrations of specific antibodies can be detected. However, slight modification of the parameters of the method may change the outcome. Kilshaw et al. for example (15), demonstrated that the antigen molecule underwent

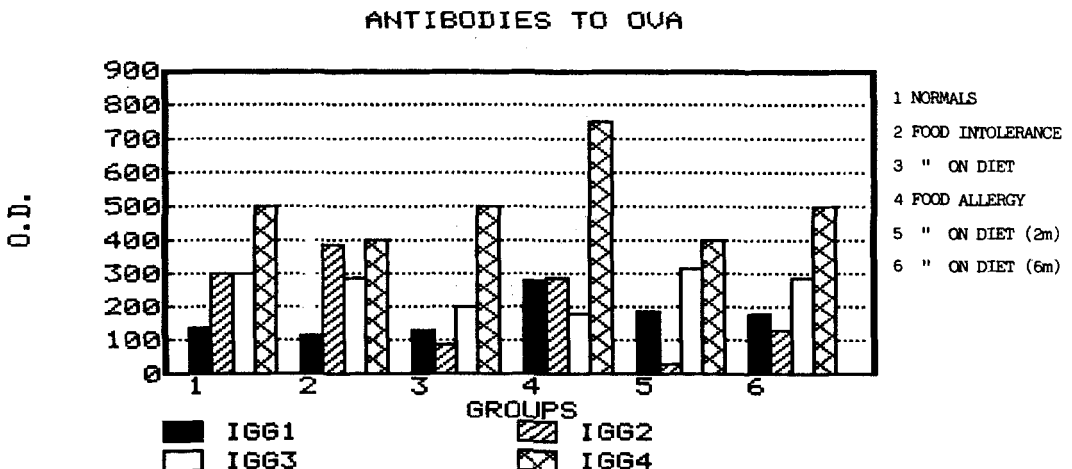


Fig. 3. IgG<sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>4</sub> to OVA (mean OD) in different groups.

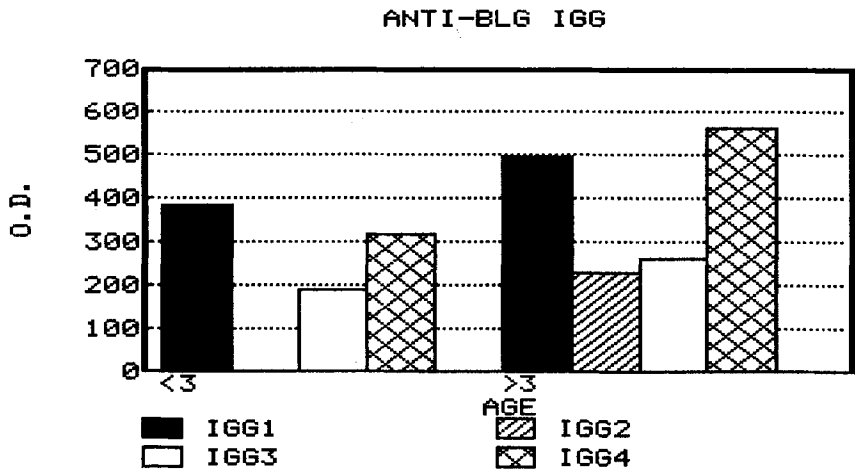


Fig. 4. IgG<sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>4</sub> to BLG (mean OD) in children with AD aged ≤ 3 years or ≥ 3 years.

conformational changes on adsorption to the plastic surface, and that the results obtained with native or denatured antigens were different. In our test, we used OVA and BLG directly coated to the plastic surface, since both antigens in the diet are substantially denatured, and we mainly focused our attention on incubation times. The monoclonal Abs to IgG subclasses and the IgG subclasses to both antigens may differ in affinity, as already demonstrated for other antigens (16). Our results showed that specific antibody profile changed,

in the same sample, modifying the reaction times (Fig. 1). All six samples studied showed the same behaviour: with short incubation (2 h) we detected only specific IgG<sub>4</sub>, while incubating samples longer (24 h) we detected also specific IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>3</sub>. In particular, a low avidity and/or concentration of IgG<sub>2</sub> Abs prevented the detection of such antibodies, if incubation times were not prolonged for at least 18 h. Therefore, specific IgG subclasses present in sera in low amounts or with a lower affinity than specific IgG<sub>4</sub> could bind the antigen and

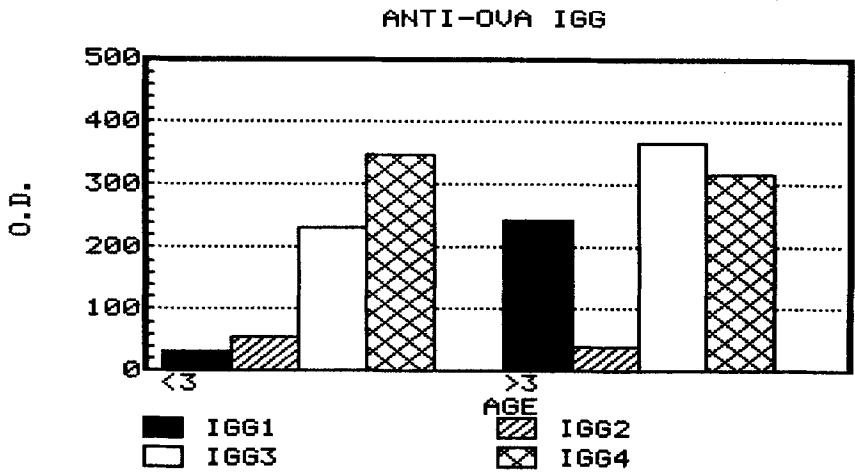


Fig. 5. IgG<sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>4</sub> to OVA (mean OD) in children with AD aged ≤ 3 years or ≥ 3 years.

then could be revealed in the ELISA assay only when prolonging incubation times. After these results, we decided to use 24-h incubation times in our assay.

The correlation coefficients between different tests are shown in Table 1. No significant correlation was found comparing the specific IgG<sub>4</sub> with specific IgE or results of prick test or comparing specific IgG<sub>4</sub> determined using the ELISA and the FAST methods for the OVA antigen. However, a significant correlation was found comparing the specific IgG<sub>4</sub> (FAST) with specific IgG<sub>4</sub> (ELISA) for the BLG antigen ( $r = 0.548$ ).

Figs. 2 and 3 show that antigen-specific Abs to BLG and OVA belong to all four subclasses both in the patient groups and in controls. In particular, no statistically significant differences have been observed between values obtained in the groups analyzed:

- 1) normal controls (n.40);
- 2) intolerants to OVA or BLG (n.5);
- 3) intolerants on a dietary treatment specific for egg or milk (n.5);
- 4) patients with food allergy (n.44);
- 5) patients with food allergy on a diet treatment for egg or milk for 2 months (n.8);
- 6) patients with food allergy on a diet without egg or milk for 6 months (n.6).

Anti-OVA-specific IgG<sub>4</sub> are present in all groups, with higher values in the food allergy group; specific diet causes a decrease in specific IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> in the food allergy group and in the intolerants. Anti-BLG-specific IgG<sub>4</sub> are very high in both intolerant and food allergy groups. Specific avoidance diet causes a decrease in specific IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub> in the intolerants and in specific IgG<sub>2</sub> and IgG<sub>4</sub> in the food allergy group. However, differences observed are not significant, and our results indicate that IgG<sub>4</sub> Abs against food antigens cannot serve as markers of food allergy, nor indicate a risk of developing atopic disease. Only a partial subclass restriction of these antibodies was observed.

The physiological significance of such restriction, already observed for a variety of antigens, has not until now been clarified (17). Partic-

ularly interesting seems to be the biological properties of IgG<sub>4</sub> of poor complement-fixing activity and Fc receptor-binding ability (18). IgG<sub>4</sub> has been shown to inhibit immune precipitation and complement activation by IgG<sub>1</sub> Ab; moreover, IgG<sub>4</sub> has been demonstrated to be unable to cross-link the antigen and behaves as a monovalent Ab (19). Therefore it is possible that IgG<sub>4</sub> Abs that appear after prolonged antigenic stimulation, protect against complement-induced damage.

In Figs. 4 and 5, specific IgG subclasses to both antigens were compared in subjects aged  $\leq 3$  or  $\geq 3$  years. No IgG<sub>2</sub> was detectable in the  $\leq 3$  group, probably because of the transient IgG<sub>2</sub> deficiency in the first years of life (20). Also IgG<sub>1</sub> to OVA was very low in the  $\leq 3$  group. This could be explained with the glycoprotein nature of the OVA. It is well-known, in fact, that small children do not respond to polysaccharide antigens (21), but that a response comes with age and parallels the IgG<sub>2</sub> increase. Variations of the antibody response with time could probably be a good measure of antigen exposure, and our data seem to indicate that the IgG antibody response to dietary antigens increases during the first years of life.

## CONCLUSIONS

This report on the antibody subclass response to OVA and BLG demonstrates that:

- Conflicting results in the literature are possibly due to different methods applied in different laboratories. Also in our experience, results obtained using different tests do not correlate;
- the antibody response to food antigens belongs to all IgG subclasses, with a prevalence of IgG<sub>4</sub>;
- the antibody response increases with age, and is decreased by exclusion diet;
- IgG<sub>4</sub> restriction of the response is similar to the restriction observed for other subclasses in response to different antigens.

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