

Development of IgG1 and IgG4 antibodies against β -lactoglobulin and ovalbumin in healthy and atopic children

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Background: The IgG responses to food antigens are preferentially restricted to the IgG1 and IgG4 subclasses. Increased levels of IgG1 and IgG4 antibodies against food allergens have been reported in girls and adults with current atopic eczema.

Objective: To study the relation between the levels of IgG1 and IgG4 antibodies against β -lactoglobulin and ovalbumin and the development of atopic disease.

Material and methods: Atopic symptoms were recorded in 36 girls from birth to 7 years of age. Blood samples were taken at 3 and 8 months and at 2, 4, and 7 years. IgG1 and IgG4 antibodies were measured by ELISA.

Results: Anti- β -lactoglobulin IgG1 was detected at all ages, peaking at 8 months. Anti- β -lactoglobulin IgG4 antibodies were detected in 18 to 29 girls at different ages and the antibody levels peaked at 2 years. The levels of anti- β -lactoglobulin IgG1 were lower in atopic, as compared with healthy individuals at 4 and 7 years ($P < .01$ and $P < .05$) and lower anti- β -lactoglobulin IgG4 antibody levels were found in atopic individuals ($P < .05$) at 4 years.

Anti-ovalbumin IgG1 antibodies were detected in 3/35 girls at 3 months and in 16/35 to 26/35 girls later in life. The number of positive samples and antibody levels peaked at 2 years. Anti-ovalbumin IgG4 positive samples increased from 4/33 at 8 months to 30/32 at 7 years. The levels increased up to 2 years and then remained stable. The anti-ovalbumin IgG1 antibody levels were lower in atopic girls at 4 years ($P < .05$), while the anti-ovalbumin IgG4 antibody levels were similar at all ages.

Conclusion: An early IgG1 response and later appearing IgG4 antibodies to the two food antigens β -lactoglobulin and ovalbumin are common during the first years of life. The levels were similar in the nonatopic and the atopic girls up to four years; then they tended to be lower in the first group.

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INTRODUCTION

The IgG responses to food antigens are preferentially restricted to the IgG1 and IgG4 subclasses.^{1,2} Increased levels of IgG1^{3–5} and IgG4 antibodies^{3,6,7} against food allergens have been reported in girls and adults with current

atopic eczema and against inhalants in individuals undergoing successful immunotherapy.^{8,9} Children and adults with inflammatory bowel disease also have higher levels of IgG4 antibodies against food proteins than healthy controls.¹⁰ The clinical significance of these antibodies in atopic diseases is controversial as the levels of IgG4 antibodies against allergens after immunotherapy are not well correlated to clinical improvement.¹¹ There are also several indications that atopic individuals have a reduced capacity to suppress certain immune responses, *ie*, the formation of IgE and IgG antibodies to ingested and inhaled common anti-^{8,12–14}

To shed further light on the development of immune responses to ubiquitous antigens, we studied prospectively the appearance of antibody responses from birth. As food proteins are the most important allergens in infancy and early childhood, we studied the natural development of IgG1 and IgG4 antibodies against β -lactoglobulin and ovalbumin over the first 7 years of life and the relation between these antibodies and atopic disease.

MATERIAL AND METHODS

Patients

The study group has been described in previous studies.^{15,16} Briefly, 86 Rh-negative girls of Rh-positive mothers, born at the central Hospital in Skövde after uncomplicated pregnancies and deliveries between August 1977 and August 1978 were included. All the girls were clinically examined at 3, 8, and 25 months of age and at 4 and 7 years. Forty six (56%) of the girls were exclusively breast fed for more than 3 months. The figure was similar in atopic (58%) and nonatopic girls (56%). Venous blood samples were obtained at the same time. As the girls have previously been subject to other studies, serum samples from all five occasions were available from only 36 of the originally 86 girls. These 36 girls did not differ from the original 86 with regard to allergy, family history of allergy, duration of breast feeding, nor time of introduction of other foods. The samples were analyzed without any knowledge regarding the clinical conditions in the girls.

Infant feeding was done according to general recommendations in Sweden. Briefly, breast feeding was en-

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couraged for at least 4 to 6 months, introduction of solid foods was recommended at 4 months, and gluten at 6 months of age.

Atopy in the girls was defined as current or past clinical symptoms of atopic eczema, allergic rhinoconjunctivitis, bronchial asthma, and allergic urticaria at 7 years of age. Atopic eczema was defined as pruritic chronic or chronically relapsing dermatitis with typical morphology and distribution verified by a pediatrician. Allergic rhinitis was defined as a rhinitis or conjunctivitis appearing at least twice after exposure to an allergen and unrelated to infections. Asthma was defined as bronchial obstruction three times or more and at least once diagnosed by a pediatrician. Allergic urticaria was defined as generalized urticaria, occurring at least twice within an hour of exposure to an allergen. Food allergy was defined as a skin prick test ≥ 3 mm combined with positive radioallergen sorbent test (RAST) against food allergens in blood samples and a positive clinical or open challenge reaction to food allergens. Positive challenge to an allergen was defined as an obvious reaction occurring within one hour after exposure on at least two occasions. Definite exacerbation of eczema, bronchial obstruction, generalized urticaria, rhinoconjunctivitis, and anaphylactic shock were classified as obvious reactions, whereas minor manifestations such as perioral exanthema were not included.¹⁵

Laboratory Tests

The anti- β -lactoglobulin IgG1 and IgG4 antibody levels were determined by a modified ELISA assay for total anti- β -lactoglobulin IgG antibodies.¹⁷ Briefly, microtiter plates (Dynatech Lab Inc, Alexandria, Va, USA) were coated overnight at 4°C with 100 μ L of 100 μ g/mL β -lactoglobulin in TRIS-HCl buffer, pH 8.2, and then stored at -70°C for less than 1 month. The plates were initially washed twice in phosphate buffered saline (PBS) containing 0.5% Tween 20 (pH 7.4) before blocking the wells with 200 μ L 0.5% human serum albumin (HSA) to

avoid nonspecific binding. Incubation was done for one hour in a humid chamber with 100 μ L of patient serum diluted 1:10 to 1:150 (one or two dilution steps) in 0.5% HSA in PBS buffer (pH 7.4). The plates were washed four times in PBS-Tween 20 before incubation with HRP-conjugated antibodies, anti-IgG1 (MH 1715, Caltag Labs., San Francisco), and anti-IgG4 (Zymed, California, USA) for one hour (dilution 1:1000 for IgG1 and 1:2500 for IgG4, 100 μ L/well) at room temperature. After washing, the wells were incubated with substrate buffer o-phenylenediamine dihydrochloride (OPD, Dakopatts A/S, Denmark) and 30% H_2O_2 in citric phosphate buffer, pH 5.0 (according to the manufacturer's instruction). The reaction was stopped at exactly ten minutes by adding 0.1 M H_2SO_4 (100 μ L/well). The plates were read in Dynatech ELISA Minireader and the absorbance was measured at 490 nm (Dynatech Lab Inc, Alexandria, Va, USA).

Serum from a blood donor with high levels of IgG1 and IgG4 antibodies against β -lactoglobulin was used as reference. It was diluted 1:5 to 1:500 for IgG1 and 1:50 to 1:500 for IgG4 to construct a standard curve with seven standard points. The total concentration of antibodies against β -lactoglobulin and ovalbumin in the reference serum was measured by Dr L Hammarström, Huddinge Hospital, Stockholm, Sweden. The level of antibodies against β -lactoglobulin was 5.2 mg/L for IgG1 and 4.9 mg/L for IgG4. The detection limit was 100 μ g/L for IgG1 and 24 μ g/L for IgG4, at an absorbance value at three times above the background. The interassay CV was less than 10% for IgG1 and less than 15% for IgG4. Intra-assay CV was less than 5% for both IgG-subclasses.

The anti-ovalbumin IgG1 and IgG4 antibodies were measured by modifying the ELISA for β -lactoglobulin in the following manner; microtiter plates (Costar, High Binding 3950) were coated overnight at 4°C with 200 μ L/well of 25 μ g/mL ovalbumin in PBS-buffer (pH = 7.4). The plates were then stored at -20°C for not more than

1 week. Incubation with patient sera were performed at 37°C for one hour. The washing solutions, the HRP-conjugated antibodies, the substrate solution, the time for the substrate reaction, and the stop solution were exactly the same as described above.

The total concentrations of IgG1 and IgG4 against ovalbumin were 3.2 mg/L and 4.9 mg/L, respectively, in the reference serum. The reference serum was then diluted 1:5 to 1:320 for IgG1 and 1:50 to 1:320 for IgG4 to construct a standard curve with seven standard points. The detection limit was 40 μ g/L for IgG1 and 3 μ g/L for IgG4, corresponding to an absorbance value at three times above the background. The interassay CV was less than 10% for IgG1 and less than 20% for IgG4. Intra-assay CV was less than 5% for both IgG-subclasses.

A modified RAST (Pharmacia Diagnostics, Uppsala, Sweden) was used to measure specific IgE antibodies against β -lactoglobulin and egg protein.^{7,9,15,16} Levels above 0.1 PRU/mL (Phadebas RAST Units) were regarded as positive (twice the level of the detection limit of the method).

Statistical Methods

As the levels of antibodies were not normally distributed, the data were transformed to log values for the statistical calculations and nonparametrical statistical methods were then applied. Wilcoxon signed-rank test was used to compare the antibody levels and Mann Whitney U test to compare atopic and nonatopic subjects. The levels of antibodies at the different ages were given as medians with the 10th and 90th percentiles. *P* values < .05 were considered as significant.

RESULTS

The cumulative incidence of allergic disease at 7 years was 42% (15/36) and the prevalence at 7 years was 25% (9/36). A family history of atopy was present in 34% (12/36) of the girls. In one child, IgE antibodies against β -lactoglobulin were detected once. This child did not develop cow milk allergy. In 8 of the girls, IgE antibodies

against egg were detected, but only two of them had positive reactions upon open egg challenge. One of these girls was breast fed for more than 3 months and one for a shorter period.

Immunoglobulin G1 (IgG1) antibodies to β -lactoglobulin were detected in all the samples up to 2 years and in all but one at 4 and 7 years (Fig 1A). The IgG1 levels increased from 3 months to 8 months and then gradually declined up to 7 years of age. Anti- β -lactoglobulin IgG1 antibody levels tended to be lower in atopic individuals at all ages, however, significantly so only at 4 and 7 years (median = 0.6 mg/L versus 1.32 mg/L, $P < .01$ and .62 mg/L versus 1.0 mg/L, $P < .05$, respectively, at 4 and 7 years). Girls with and without a family history of atopy had similar levels (data not shown).

Immunoglobulin G4 (IgG4) antibodies against β -lactoglobulin were less frequently detected than IgG1 (Fig 1B). The highest levels were recorded at 2 years and then the levels remained constant. Three girls did not develop any detectable IgG4 antibodies against β -lactoglobulin at any age and in seven girls only low levels were recorded, *ie*, just above the detection limit (24 μ g/L). The levels of anti- β -lactoglobulin IgG1 in these ten girls were slightly, but not significantly, lower than in the 26 girls with more pronounced IgG4 antibody responses. The levels of IgG4 antibodies against β -lactoglobulin were similar in atopic and nonatopic girls at all ages except at 4 years (median 0.04 mg/L and 0.61 mg/L, respectively, $P < .05$).

Anti-ovalbumin antibodies of the IgG1 subclass were found in only three girls (3/35) at 3 months of age and then only at low levels (0.4 to 1.2 mg/L) (Fig 2A). At 8 months, 16/35 girls had detectable levels of anti-ovalbumin IgG1. The frequency of positive samples increased to 23/34 (68%) at 2 years and 26/36 (72%) at 4 years of age. Anti-ovalbumin IgG1 antibody levels increased up to 2 years (median 1.3 mg/L) and then the levels declined (median 0.3 mg/L at 7 years) ($P < .001$). The anti-ovalbumin IgG1 anti-

body levels were similar in atopic and nonatopic girls from 3 months to 2 years. At 4 years, however, lower levels were recorded in atopic girls (median = 0.17 mg/L versus 0.86 mg/L, $P < .05$) but not at 7 years. Anti-ovalbumin IgG1 antibodies were not detected at any age in seven girls and only transient low anti-ovalbumin IgG1 levels were measured in three individuals. Seven of those ten girls developed atopic manifestations, as compared with 42% of the entire study population (ns). Furthermore, all the three girls with the highest levels of anti-ovalbumin IgG1 antibodies, *ie*, outliers above the 90th percentile at 4 and 7 years were nonatopic.

Anti-ovalbumin IgG4 antibodies were detected in 12/34 girls at 3 months but only in 4/33 at 8 months (Fig 2B). At 2 years, the number of positive samples was 23/31, at 4 years 30/33, and at 7 years 30/32. The levels also increased significantly from 8 months to 2 years and then, similarly to anti- β -lactoglobulin IgG4 antibody levels, remained relatively constant up to 7 years of age. Anti-ovalbumin IgG4 antibody levels were similar in atopic and nonatopic girls at all ages.

The levels of IgG1 against β -lactoglobulin were closely related at the different ages in the individual girls (Fig 3A). This was also true for antibodies against ovalbumin; thus, the

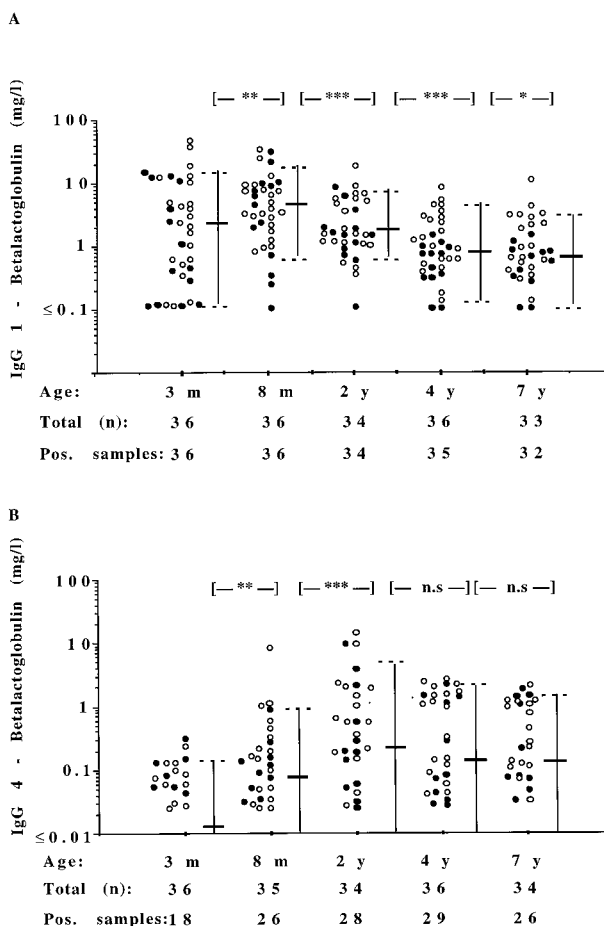


Figure 1. Levels of IgG1 (A) and IgG4 (B) antibodies against β -lactoglobulin during the first 7 years of life in 36 girls. Nonatopic girls are indicated by open circles and atopic individuals by closed circles. The values are presented in mg/L. The median values (line) and the 10th and 90th percentiles (hatched line) are shown. * $P < .05$, ** $P < .01$, *** $P < .001$.

IgG1 anti-ovalbumin antibody levels at 2 years correlated with the anti-ovalbumin IgG1 levels at 4 ($r = .72$, $P < .001$) and 7 years ($r = .46$, $P < .01$). There was also a significant correlation between anti-ovalbumin IgG1 antibody levels at 4 and 7 years of age ($r = .75$, $P < .001$).

Similarly to the IgG1 antibodies, the levels of IgG4 antibodies against β -lactoglobulin were closely related at the different ages in the individual girls. The same was true for ovalbumin, thus indicating that high and low responders were the same individuals at all ages (Fig 3B).

DISCUSSION

All the girls developed an IgG1 antibody response to the major milk protein β -lactoglobulin. The antibody levels peaked within the first year and then significantly declined. The kinetics of the anti-ovalbumin IgG1 antibody responses were similar to that for anti- β -lactoglobulin although the number of infants with detectable levels were lower and the levels peaked at 2 years. The lower prevalence of IgG1 and IgG4 to ovalbumin than to β -lactoglobulin early in life may be explained by the time of introduction of milk and egg in the food *ie*, milk during the first 3 to 4 months and egg from 8 to 12 months of life.

The IgG1 and IgG4 antibody response to subcutaneous administered allergens during immunotherapy^{8,9} are similar to the pattern we observed for primary immune responses to foods, *ie*, an early IgG1 response and a later appearing IgG4 response. This supports that the IgG4 response is part of the natural immune response to prolonged exposure to antigens.

Eight girls developed IgE antibodies against egg white and one against β -lactoglobulin. An early production of IgE antibodies to common allergens is common in children.¹⁵ The transient nature of IgE antibody response, combined with a continuous IgG4 antibody response against food proteins may indicate separate down regulation of the two immunoglobulins. This notion is supported by the observation that

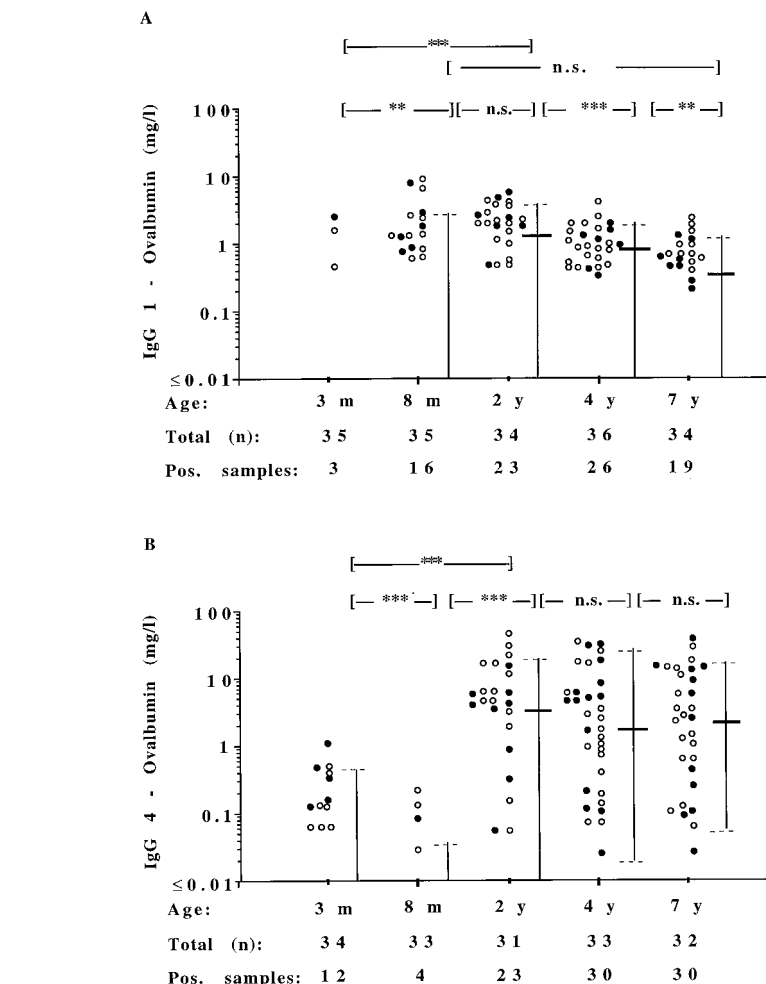


Figure 2. Levels of IgG1 (A) and IgG4 (B) antibodies against ovalbumin during the first 7 years of life in 36 girls. Nonatopic individuals are indicated by open and atopic girls by closed circles. The values are presented in mg/L. The median values (line) and the 10th and 90th percentiles (hatched line) are shown. * $P < .05$, ** $P < .01$, and *** $P < .001$.

nerve growth factor (NGF) specifically induces production of human IgG4 in vitro, without any influence on IgE production¹⁸ and that disodium cromoglycate (DSCG) inhibits IgE production in a IFN γ -independent way, while enhancing IgG4 production.¹⁹

Most previous studies on IgG antibody responses to foods in infancy and early childhood did not include an analysis of the subclasses and if this was done, the studies were not longitudinal but limited to single blood samples from small groups of children with a widely varying age.^{2,20,21} With one exception,² the levels were expressed in arbitrary units rather than

mg/L. There are, however, two previous longitudinal studies similar to the present.^{22,23} Rowntree et al²³ observed similar anti- β -lactoglobulin IgG4 and anti-ovalbumin IgG4 kinetics over the first 5 years of life as in this study, in a small group of children. The latter study and a prospective study,²² in which 39 children with cow milk protein allergy and 39 healthy children were followed over the first 12 months of life, support our findings both for IgG1 and IgG4 antibodies against β -lactoglobulin and ovalbumin.

In a few previously reported studies, total IgG4 antibody levels were found to be higher in adults with atopic ec-

A

IgG1 antibodies

B-LACTOGLOBULIN					
Age	3 m	8 m	2 yrs	4 yrs	7 yrs
3 m					
8 m			0.61 ***	0.49 **	0.44 *
2 yrs				0.80 ***	0.80 ***
4 yrs					0.98 ***
7 yrs					

OVALBUMIN					
Age	3 m	8 m	2 yrs	4 yrs	7 yrs
3 m					
8 m					
2 yrs					
4 yrs			0.72 **		
7 yrs			0.46 **	0.75 **	

B

IgG4 antibodies

B-LACTOGLOBULIN					
Age	3 m	8 m	2 yrs	4 yrs	7 yrs
3 m					
8 m			0.59 **	0.39 *	0.39 *
2 yrs				0.81 ***	0.83 ***
4 yrs			0.76 **		0.97 ***
7 yrs			0.69 **	0.86 **	

OVALBUMIN					
Age	3 m	8 m	2 yrs	4 yrs	7 yrs
3 m					
8 m					
2 yrs					
4 yrs					
7 yrs					

Figure 3. Correlation between IgG1 (A) and IgG4 (B) antibody levels against β -lactoglobulin (upper part of the figure) and ovalbumin (ovalbumin) (lower part of the figure) at different ages in 36 girls up to 7 years of age. Only r values with a statistically significant correlation are shown with corresponding P values. [* $P < .05$, ** $P < .01$, and *** $P < .001$].

zema than in healthy individuals.³⁻⁵ Husby et al⁴ found higher levels of anti- β -lactoglobulin IgG and anti- β -lactoglobulin IgG4 but not of anti- β -lactoglobulin IgG1 antibodies in children with active atopic eczema than in healthy children, but not in those with only a previous history of atopic eczema. IgG, IgG1, and IgG4 antibodies against ovalbumin were similar in children with and without atopic eczema. These authors, however, only determined the total IgG and IgG-subclass antibody responses to food proteins on a single occasion in each child and the study population comprised adults or children with clinical symptoms of atopic eczema.

Maternal antibodies in the babies²⁴⁻²⁶ against β -lactoglobulin could in principle explain the wide range of antibody levels at 3 months. We found though that IgG1 and IgG4 antibodies against ovalbumin, a protein not ingested in the food until 6 to 8 months of age, were detected in only 3/35 and 12/34 girls respectively at 3 months and 3/33 at 8 months for IgG4. This suggests that maternal antibodies of IgG1 isotype have little influence on infant antibody levels at 3 months of age.

Feeding pattern has an obvious influence on the early IgG antibody levels against foods. The diet of the breast feeding mothers could also possibly

explain the large individual variations in antibody levels. Feeding habits do not, however, explain the lower anti- β -lactoglobulin and anti-ovalbumin antibody levels in atopics than in nonatopics at 4 and 7 years of age. Atopic individuals are known to be better IgE responders and there is a relationship between specific IgE and IgG4 antibodies via IL-4.²⁷⁻³¹ A possible explanation could be regulatory differences in the IgE/IgG4 ratio between atopic and nonatopic subjects.

In conclusion, there is an early, high and relatively transient IgG1 response and a late appearing IgG4 response in infants to common foods like milk and egg. The fact that almost all the girls showed an IgG4 response, but only few of them developed specific IgE responses to the antigens suggest different down regulation after the initial IL-4-dependent antibody switch to IgE and IgG4. The IgG1 and IgG4 antibody responses to β -lactoglobulin and ovalbumin were similar in healthy and atopic girls during the first 2 years of life; then the levels tended to be lower in atopic girls.

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