

Humoral Immunity to Dietary Antigens in Atopic Dermatitis

I. Biotin-Avidin Amplified ELISA-Analysis for Serum IgG Antibodies

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Enzyme-linked immunosorbent assays (ELISA) employing a biotin-avidin amplification step are described for the quantification of human serum IgG antibodies to the dietary antigens ovalbumin (OA) and beta-lactoglobulin (BLG). The analytical quality of these assays was acceptable. Antibodies were measured in 16 patients with mild or moderate atopic dermatitis (AD), in 31 patients with a history of AD, and in closely matched controls. Levels of serum anti-OA antibodies did not differ in patients and controls, whereas anti-BLG antibodies tended to be higher in patients with mild or moderate AD than in controls ($P < 0.05$).

Key words: atopic dermatitis; beta-lactoglobulin; ELISA; IgG antibodies; ovalbumin.

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CLINICAL ASPECTS

The two sensitive ELISA's for determination of antibodies to dietary antigens represent a useful alternative to radioimmunological techniques. The level of serum IgG antibodies against beta-lactoglobulin was higher in a group of patients with atopic dermatitis than in controls. This finding may be of pathogenic importance, but has little diagnostic value, as there was a considerable overlap between the two groups.

Humoral immunity to dietary antigens has been studied with a variety of techniques, such as radioimmunoassays (21, 26) and enzyme immunoassays (12). Antibodies to dietary antigens were demonstrable in sera from a majority of healthy adults and children (14, 18, 21). Clinically, interest has centered especially on IgE antibodies as the offending antibodies in food allergy, although non-IgE mediated allergic mechanisms may also be of importance, particularly in late reactions (6, 9, 10).

The present report describes sensitive ELISA-techniques for the quantification of IgG antibodies to ovalbumin (OA) and beta-lactoglobulin (BLG), employing a biotin-avidin amplification step. Sera from patients with atopic dermatitis

(AD), and sera from closely matched controls were analysed by use of these assays.

MATERIAL AND METHODS

Patients and controls

The patients comprised a part of an epidemiological study of AD (23, 24), conducted in the County of Funen, Denmark. A total of 47 unrelated patients with AD (31 with a history of AD, 10 with overt but mild AD and 6 with moderate AD) were found and tested in parallel with 47 non-atopic individually age- and sex-matched controls. The clinical examination was performed by one of us (F.S.L.). Peripheral

blood samples were collected, and serum stored in aliquots at -80°C. Clinical data for the patients are given in Table 1. Three of the patients, two with a history of AD and one with moderate AD, had experienced worsening of their eczema after the ingestion of food, but none reacted after the intake of milk or egg.

Reagents

Ovalbumin (OA, crystalline, grade V) and beta-lactoglobulin (BLG, crystalline, A + B) were purchased from Sigma (St. Louis, MO., USA). Human serum albumin (HSA, purified) was from Behringwerke (Marburg, FRG). Tris buffered saline (TBS) was prepared as 10 mM Tris, 0.14 M NaCl, 15 mM NaN₃, pH 7.4. TBS was also used with 0.05 % Tween 20 (TBS-Tween). Avidin (Sigma) was coupled to beta-galactosidase (*E. coli*, EC 3.2.1.23, Boehringer, Mannheim, FRG) by means of the one-step glutaraldehyde method (3).

Antibodies and sera

Affinity-purified goat anti-human IgG antibody was purchased from Tago (Burlingame, CA., USA). The antibody preparation was coupled with biotin-hydroxysuccinimide as described by Guesdon et al. (11).

Serum from a healthy adult with a high level of IgG anti-OA antibody was chosen for the calibration of the IgG anti-OA assay, and

serum from a healthy person with high level of IgG anti-BLG antibody for the IgG anti-BLG antibody assay. Each calibration serum was assigned an antibody activity of 100 U/ml of undiluted serum.

ELISA for the determination of serum IgG antibody

The wells of microplates (Immunoplate II, Nunc, Kamstrup, Denmark) were incubated overnight with OA or BLG at 4 µg/ml of 0.1 M bicarbonate buffer, pH 9.6 using 200 µl per well. One antigen preparation was used per plate. Free binding sites were blocked with 0.1 % HSA for 1 h, followed by three times washing with TBS-Tween.

Test sera were diluted 1:100 in TBS-Tween and further 1:5 in TBS-Tween containing 1 % human serum without detectable antibody (NS₀). Corresponding patient and control samples were analysed in the same run. Calibration sera were diluted 1:100 in TBS-Tween, and in order to avoid carry-over effects three separate 10-fold dilution protocols (in 1 % NS₀) were used further: a) 1:10, 1:100 and 1:1000, b) 1:1.66, 1:16.6, 1:166 and c) 1:3.33, 1:33.3 and 1:333.

Samples (200 µl) were applied to the wells in duplicates and incubated overnight. The plates were washed three times, and 200 µl of biotinylated goat anti-human IgG antibody (1:2000) in TBS-Tween was added and the plates were incubated for 4 h. After three times washing, avidin-beta-galactosidase (1:2000 in TBS-Tween with

Table 1
Clinical characteristics of 47 patients with atopic dermatitis and 47 controls

		History of AD (n = 31)	Mild AD (n = 10)	Moderate AD (n = 6)	Controls (n = 47)
Sex	male (n)	14	6	2	22
	female (n)	17	4	4	25
Age	median	10	11	12	11
	range	6-21	7-19	8-19	6-20
Respiratory atopy	yes	2	1	3	0
	no	29	9	3	47
Single parental history of AD	yes	1	3	1	1
	no	30	7	6	46
Actual disease extent (0-16)*	median	0	3	5	0
	range	0	2-3	4-10	0

* Number of skin areas affected, ranging from 0-16. AD: Atopic dermatitis.

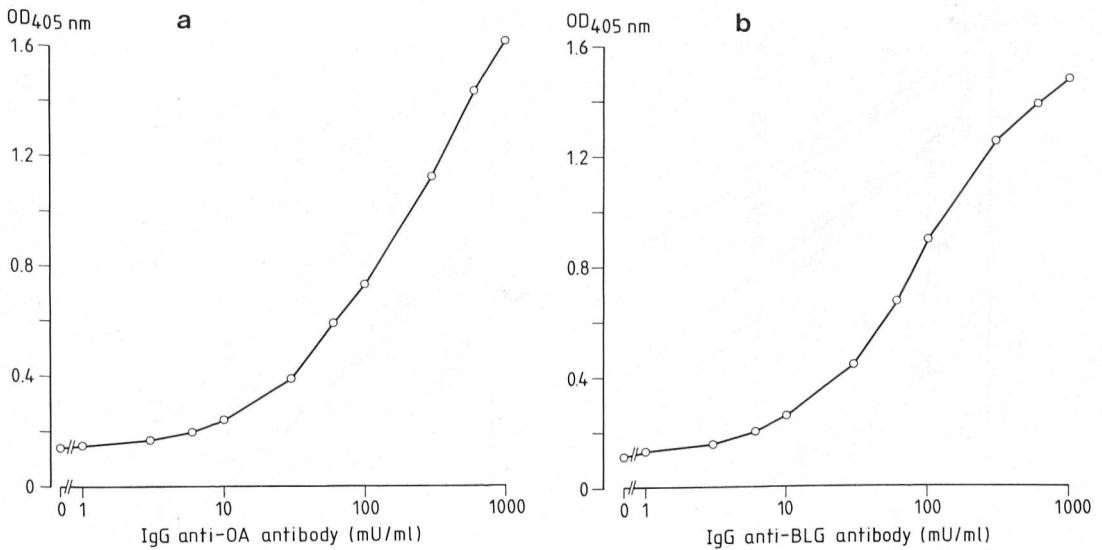


Fig. 1. Typical calibration curves for the determination of serum antibodies of the IgG class: (a) IgG anti-OA antibodies, (b) IgG anti-BLG antibodies. Each standard serum was arbitrarily assigned to contain 1000 mU/ml at a 1:100 dilution of serum.

0.1% normal rabbit serum) was added and the plates were incubated for 2 h. The enzyme reaction was developed with o-nitrophenyl-beta-D-galactopyranoside (Sigma) at 1 mg/ml of 0.05 M phosphate buffer, 1 mM MgCl₂, pH 8.2 at 37°C. Other incubation steps were at room temperature, and all incubations were performed in a humidified chamber. The colour intensity of the wells was recorded at 405 nm (620 nm absorbance subtracted as a background) in a multichannel spectrophotometer (Immunoreader NJ-2000, Nippon Intermed, Tokyo, Japan). Before reading, the samples were shaken for 10 s on a Dynatech microshaker (Billinghurst, Sussex, U.K.) to reduce variability (17). No edging effect was noted in these ELISAs.

The assays were initially performed with sera diluted in TBS-Tween only. However, since a matrix effect was observed it was decided to use a 1% NS₀ solution, when diluting beyond 1:100. In preliminary experiments HSA-coated wells were used as controls of unspecific binding to the microwell surface. But, as no absorbance values for 20 normal sera exceeded those obtained with NS₀ in the antigen-coated wells (max. OD = 0.250), this HSA-control was omitted.

Antibody avidity index

Antibody levels were measured at 1:100 and 1:500 dilutions of the sera. The ratio between the concentrations directly read off the calibration curve was calculated as the "antibody avidity index" for sera with antibody levels less than 1000 mU/ml (the maximum of the calibration curve). This ratio reflects the slope of the antibody dilution curve, supposedly related to the antibody avidity (8).

Statistics

The non-parametric Mann-Whitney U-test for unpaired samples was used as a two-tailed test. A significance probability level of 0.05 was chosen.

RESULTS

Method characterization and evaluation

Typical calibration curves of the standard sera are shown for IgG anti-OA and IgG anti-BLG antibody in Figs. 1 a + b, respectively.

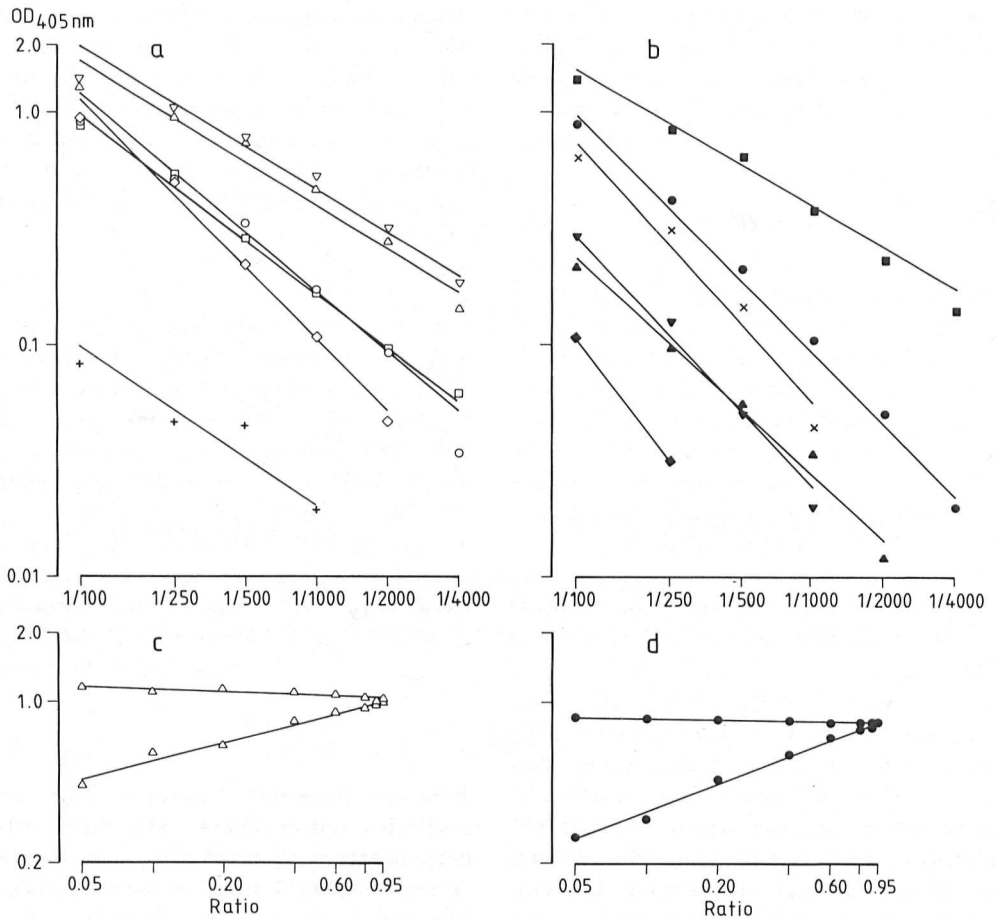


Fig. 2. Dilution curves for IgG anti-OA antibodies (a) and IgG anti-BLG antibodies (b) in six normal human sera. A reasonable degree of parallelism between the sera was seen in both assays. Mixing experiments (1:20–20:1) of normal human sera with high and low, and high and high antibody levels, respectively, were performed for IgG anti-OA antibodies (c) and IgG anti-BLG antibodies (d). Mean variation of duplicate determinations was 0.03.

Dilution curves for six normal human sera diluted 1:100–1:4000 are shown in Figs. 2 a + b. The curves were nearly linear and parallel in the logarithmic plots. When mixing two sera with high and low antibody levels, and two sera, both with high antibody levels, the absorbance readings showed a closely linear dependency on the ratios of the sera (log-log scale) (Figs. 2 c + d).

Reproducibility was tested for each assay by means of three control sera, representing a fairly wide range of antibody levels (Table 2).

Antibody determination

The serum IgG anti-OA and IgG anti-BLG antibody levels from patients with mild or moderate AD and their corresponding controls are shown in Fig. 3. The distribution of the anti-OA antibody levels was very similar for the AD patient group and the controls, whereas some AD patients had slightly higher anti-BLG IgG levels than the controls. The latter difference is statistically significant ($z = 2.75$, $P < 0.0125$). Still, the degree of overlapping is

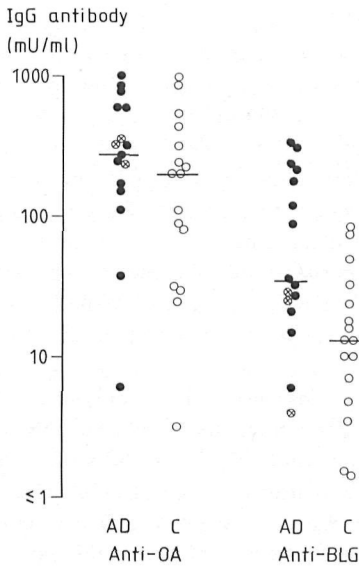


Fig. 3. Serum IgG antibodies to OA and BLG in patients with mild or moderate AD (●) and in controls (○). The logarithmic ordinate scale is expressed in arbitrary units (see text). AD-patients with concomitant asthma/rhinitis are denoted as (⊕). Bars indicate median values.

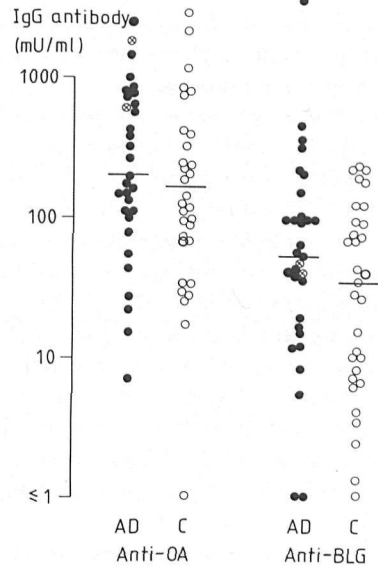


Fig. 4. Serum IgG-antibodies to OA and BLG in patients with a history of AD (●) and in the corresponding controls (○). AD-patients with asthma/rhinitis are denoted as (⊕). Bars indicate median values.

considerable. Patients with a history of AD and their controls (Fig. 4) did not differ significantly in either anti-OA antibody levels or anti-BLG antibodies. No significant differences in antibody avidity indices were seen when testing sera from AD-patients and the controls (data not shown).

DISCUSSION

The present study describes ELISA techniques for the determination of the IgG antibodies to

two dietary antigens, OA and BLG. The assays utilize the strong interaction ($K_a \sim 10^{-17} \text{ M}^{-1}$) between biotin and avidin as an amplification step (for review, see 28). This procedure eliminates the polymer formation, which may occur during the direct conjugation of indicator antibodies to the enzyme. Characterization of the two ELISAs showed an analytical quality considered acceptable for most antibody assays. However, the between-day coefficients of variation (17–27%) were considerably larger than the within-run coefficients of variation (9–

Table 2

Reproducibility of ELISA for IgG anti-OA and IgG anti-BLG antibody determinations, estimated by the use of three control sera included in each run and assay ($n = 28$)

Anti-OA antibodies	CV _w	CV _B	Anti-BLG antibodies	CV _w	CV _B
Serum I (460 mU/ml)	0.11	0.27	Serum I (96 mU/ml)	0.13	0.20
Serum II (150 mU/ml)	0.09	0.24	Serum II (15 mU/ml)	0.07	0.18
Serum III (47 mU/ml)	0.10	0.17	Serum III (5 mU/ml)	0.17	0.24

CV_w denotes within-run coefficient of variation and CV_B between-run coefficient of variation.

17%). This could be caused by several factors, such as variability in the non-covalent binding of antigen to the solid-phase, or the numerous washing steps in the assays.

IgG antibodies to the two dietary antigens were observed in most of the 94 6-21-year-old persons studied, comprising both AD-patients and controls. This finding is not surprising in view of the high frequency of antibodies to dietary antigens in normal sera detected by non isotype-specific assays (14, 18, 21). The somewhat varying frequencies of the antibodies observed in these studies probably reflect the different detection limits of the assays used. Interestingly, the quantitative assays in this study revealed up to 1000-fold differences in antibody levels both in AD-patients and controls.

The slope of the dilution curve reflects the antibody avidity, and determination of antibody levels at two serum dilutions (1:100 and 1:500) allows for the estimation of an avidity index. No differences in avidity indices were observed between patients and controls. Direct calculation of antibody avidities by Scatchard plot analysis has been carried out previously on sera from a few atopic patients (1, 20) and no high avidity IgE and IgG antibodies were found.

The distribution of the IgG anti-OA antibody levels was very similar for AD-patients and controls (Figs. 3, 4). The same was basically true also for the IgG anti-BLG antibodies, but in the AD-patients with overt disease these antibody levels tended to be higher than in the controls. The persons included in the present study were part of a population study of AD, and no patients with severe AD were found at this survey. Such patient sera might have shown more extreme deviations from the normal range.

An increased frequency of precipitating antibodies to foods has previously been reported in food-allergic AD-patients (4). Increased IgG anti-food antibody levels were also found in children with food allergy and AD, as measured by radioimmunological techniques (6, 7).

An increased gut permeability has been indicated in AD-patients with or without clinical

food allergy, by use of polyethylene glycol probes (16). However, conflicting results were obtained with techniques employing mono- and disaccharides, as increased lactulose/mannitol excretion was found in adults with AD (27) whereas no differences were observed between eczematous children and controls in lactulose/rhamnose excretion (19). Among other possibilities a mucosal defect may cause increased macromolecular passage into the blood resulting in enhanced antibody formation, without being causally related to the specific food allergy.

BLG seems to be more readily degraded than OA by gastrointestinal enzymes, and we could in normal adults only detect OA, but not BLG, in the circulation after a test meal (15). Thus, a putative mucosal defect in AD may lead to an increased passage of BLG into the blood, resulting in the increased levels of anti-BLG antibodies found in this study, whereas the systemic immune system is stimulated by OA to the same degree in AD-patients and healthy controls.

Several studies have indicated a beneficial effect of the avoidance of certain antigens in the diet of AD-patients (2, 13, 22) pointing to dietary antigens as potential offending allergens in AD. Also, the incidence of AD in Denmark and in the U.K. has increased from approximately 3% to 10% during the last decades (24, 25), obviating a search for specific environmental factors, including foods, which may trigger the disease.

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