

Specific humoral response to cows' milk proteins and ovalbumin in children with atopic dermatitis

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Summary. Serum antibodies to four common food antigens, three cows' milk proteins (casein, α -lactalbumin and β -lactoglobulin) and ovalbumin, were investigated in 21 children with atopic dermatitis (aged 3 months to 3 years) and in 15 age-matched healthy controls. Specific IgE was measured by radioallergosorbent test; an ELISA was developed to detect specific IgG, IgG subclasses and IgA. Specific IgE was found in 76% of patients, while antigen-directed IgG and IgA were present both in patients and healthy controls; IgG to ovalbumin and IgA to α -lactalbumin were significantly higher in children with atopic dermatitis. The analysis of the IgG subclass distribution showed different patterns of response, IgG1 and IgG4 being higher in patients (even though statistically significant only for ovalbumin), and IgG2 and IgG3 being lower in this group. The presence of food-specific IgE in the majority of atopic children and the different specific IgG subclass patterns observed in patients and controls may reflect an alteration in the immune response to dietary proteins in atopic dermatitis.

Key words: Atopic dermatitis – Dietary antigens – Enzyme-linked immunosorbent assay – IgG – IgG subclasses

Introduction

Atopic dermatitis (AD) is often associated with elevated circulating levels of IgE, mainly directed against food antigens [2]. However, a role for other immunoglobulin classes in allergic diseases has recently been proposed [5, 17, 20]; various studies have investigated the correlation of AD symptoms with the presence of food-directed antibodies of the IgG, IgA and IgM classes, with discordant results [9, 13, 15, 16, 18].

In this study we investigated the presence of IgE, IgG, IgG subclasses and IgA against four widely encountered

dietary antigens, three cows' milk proteins (CMP) and ovalbumin (OA), in children with AD less than 3 years of age; the specific response was analyzed in relation to age-matched healthy controls.

Patients and methods

Study population. The patient group consisted of 21 children, 16 males and 5 females, aged from 3 months to 3 years (median 18.5 months); 9 patients (43%) were less than 1 year; all had been breast-fed, for between 2 weeks and 5 months. All fulfilled the criteria of Hanifin and Rajka [7] for the diagnosis of AD. In all patients, avoidance of cows' milk led to clinical improvement, while its reintroduction repeatedly resulted in exacerbation of the disease. In children more than 8 months, introduction of egg to the diet did not cause worsening of symptoms. On the basis of the extent and severity of cutaneous lesions, symptoms were defined as mild (4/21 patients), moderate (10/21) or severe (7/21).

Patients were compared with 15 unrelated non-atopic children, hospitalized for neurological and orthopedic diseases; these were matched for sex, age and diet: 11 were male and 4 female; their ages ranged from 3 months to 3 years (median 18.2 months) and 6 (40%) were less than 1 year. All had been breast-fed for 1–6 months and their current food patterns were similar to those of the patients.

Total IgE, IgG and IgG4 determination. Total serum IgE was measured by the paper radioimmunosorbent test procedure (Phadebas PRIST kit, Pharmacia, Uppsala, Sweden); age-related normal IgE levels were determined in 120 healthy children, subdivided into four age-groups.

Total serum IgG and IgG4 were determined by single radial immunodiffusion (immunodiffusion plates from the Binding Site Ltd.) Manufacturer's age-related normal values were employed. Other IgG subclasses were not measured because of shortage of serum, due to the young age of the children.

Specific IgE determination. Specific IgE against the following CMP were measured by radioallergosorbent test (Phadebas IgE RAST, Pharmacia) according to the manufacturer's instructions: α -lactalbumin (ALA), β -lactoglobulin (BLG) and casein (CA), and to an apparently irrelevant food protein, OA. Results were expressed as Phadebas RAST units per milliliter (PRU).

Specific IgG and IgG subclass determination. Allergen-specific IgG and IgG subclasses were determined in sera from patients and controls by an ELISA, according to the method of Engvall and Perl-

mann [6], modified as follows. Microtiter plates (Immunoplate II, Nunc) were coated overnight at 4°C with ALA, BLG, CA or OA (Sigma) and with human serum albumin (HSA, Behringwerke) at 120 µg/ml in 0.067 M phosphate-buffered saline (PBS) pH 7.4. Non-specific binding was then blocked with 0.1% HSA in PBS for 1 h and plates were then washed three times in PBS with 0.05% Tween 20 (PBS-T).

Sera were diluted 1:10 in PBS-T and incubated for 2 h in antigen-coated wells at room temperature; plates were subsequently washed and then a 1:1,000 dilution of alkaline phosphatase-labelled sheep anti-human IgG (Sigma) was added and plates were incubated for 4 h at room temperature. For determination of IgG subclasses, subclass-specific monoclonal antibodies (anti-IgG1, HP6091, diluted 1:400; anti-IgG2, HP6014, diluted 1:500; anti-IgG3, HP6050, diluted 1:250; anti-IgG4, HP6023, diluted 1:200; The Binding Site Ltd.) were added [12] and plates were incubated overnight at 4°C. After washing a 1:1,000 dilution of alkaline phosphatase labelled goat anti-mouse IgG (Sigma) was added and plates were incubated for 2 h at room temperature. A separate plate was used for the determination of each subclass. Bound enzyme was visualized with *p*-nitrophenylphosphate (Sigma 104 phosphatase substrate tablets) in 1 M diethanolamine buffer pH 9.8. Samples from the AD patients and the corresponding controls were always analyzed at the same time on the same plate. The absorbance was read at 405 nm and the results expressed as absorbance units (Abs-U) after subtraction of the absorbance of HSA-coated wells. Binding to HSA did not differ significantly between specific total or subclass IgG assays and was typically less than 0.15 Abs-U. Values greater than the mean plus two standard deviations (SD) of the control group were considered abnormal.

Specific IgA determination. Sera from patients and controls, diluted 1:10 in PBS, were incubated for 1 h at 37°C in antigen-coated wells prepared as described above. After washing, the plates were incubated for 1 h at 37°C with β -galactosidase-labelled rabbit anti-human IgA (Pharmacia IgA Gluten EIA kit), and the enzyme reaction was then developed according to the Gluten EIA kit instructions. Results were expressed as Abs-U; values greater than the mean plus 2 SD of the control group were considered abnormal.

Statistical analysis. Statistical analysis was performed by the non-parametric Mann-Whitney U-test. The concentrations of the immunoglobulin classes and subclasses were compared using Spearman's rank correlation test.

Results

Total immunoglobulin determination

Increased total IgE levels were observed in 14 of 21 (67%) AD patients and in 3 of 15 (20%) controls; total IgE levels were significantly higher in patients than in controls ($P < 0.01$). Total IgG values were normal in patients and in 14 of 15 controls; slightly decreased IgG was found in 1 control subject. Total IgG4 levels were normal in 15 of 21 patients, decreased in 5 of 21 and elevated in 1 of 21 patients. In the control group, normal and decreased levels were found in 10 of 15 and 5 of 15 individuals, respectively. No significant differences between patients and controls were found for either total IgG or IgG4.

Specific IgE determination

Detectable levels of IgE against CMP and/or OA were found only in the patient group (Fig. 1). The majority of

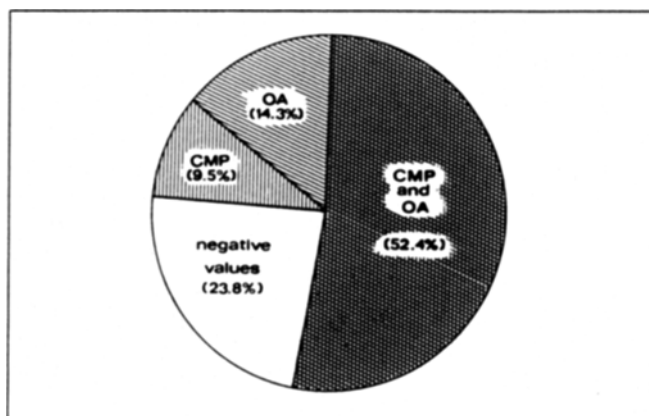


Fig. 1. Incidence of specific IgE to cows' milk proteins (CMP) and/or ovalbumin (OA) in 21 children with atopic dermatitis (AD)

patients (16/21, 76%) were positive to at least one antigen. Eleven children (52%) had specific IgE reactive with at least one CMP and with OA; 4 of these children, with elevated IgE to CMP (> 3.5 PRU) also had elevated RAST values for OA. Specific IgE against CMP was present in 2 of 21 (9.5%) subjects, while 3 of 21 (14%) reacted only with OA. Highly significant differences were observed between the antibody levels to ALA ($P = 0.005$), BLG ($P = 0.008$), CA ($P = 0.007$) and OA ($P = 0.009$) of patients and controls.

Children giving positive reactions in RAST with all antigens tested had the highest total IgE levels, while subjects without specific IgE had normal or slightly elevated total IgE. Children with severe symptoms had RAST values greater than 3.5 PRU in 5 of 7 cases and reacted with at least two antigens; 2 of 10 subjects with moderate disease had elevated specific IgE directed against three antigens, while mild symptoms were associated with elevated PRU in 1 subject.

Specific IgG and IgG subclasses

OA-directed IgG was significantly higher in children with AD than in controls ($P = 0.002$), while no significant differences were found for CMP (Fig. 2). Similar results were obtained for specific IgG1 and IgG4 subclasses (Fig. 3a, d); OA-directed antibodies were significantly higher in the AD group ($P = 0.04$ both for IgG1 and IgG4), while no differences were found for CMP.

A different pattern was observed for IgG2 and IgG3 subclasses (Fig. 3b, c): patients' values tended to be lower for all the allergens tested; in particular, IgG3 levels were significantly lower for all the antigens examined (CA $P = 0.00003$; ALA $P = 0.0002$; BLG $P = 0.0001$; OA $P = 0.00005$), while levels of specific IgG2 were significantly different only for BLG ($P = 0.05$).

Correlations between the levels of specific IgE and IgG/IgG subclasses were analyzed in the patient group (Table 1). A positive correlation was found between specific IgE and IgG/IgG1/IgG4, with the following significant differences: IgE versus IgG directed against CA ($P = 0.05$), IgE versus IgG1 against ALA ($P = 0.01$) and

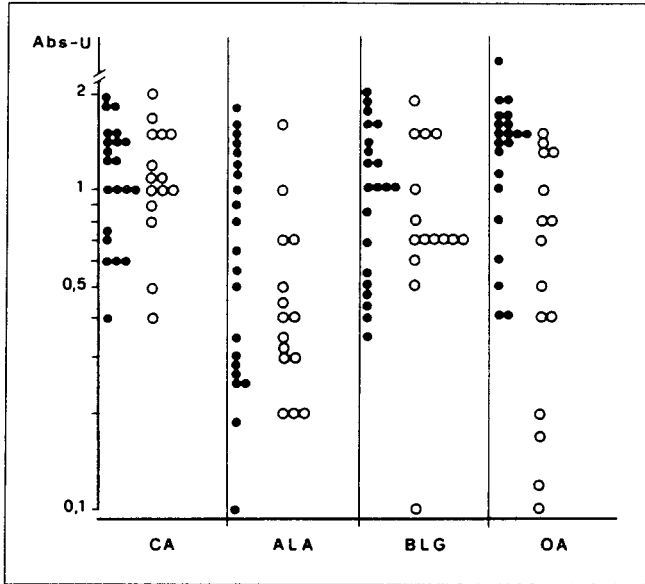


Fig. 2. Specific serum IgG antibodies to ALA, BLG, CA and OA in 21 children with AD (●) and in 15 age-matched controls (○)

IgE versus IgG4 against CA ($P=0.05$). There was a negative correlation between IgE and IgG3; this was significant for BLG antibodies ($P=0.03$).

Disease severity was associated with elevated (>0.700 Abs-U) specific IgG, IgG1, IgG2 and IgG4 to at least three antigens. Moderate and severe symptoms were associated with elevated specific IgG in 6 of 10 and 4 of 7 subjects, respectively. One patient with mild symptoms, however, showed high values of specific IgG/IgG subclasses.

Specific IgA

Specific IgA levels were comparable in patients and controls, except for anti-ALA antibodies which were significantly higher ($P=0.03$) in children with AD (Fig. 4). There was a positive correlation between OA-specific IgA and OA-specific IgE levels in the patient group ($P=0.01$, Table 1). There were also correlations between specific IgA and CA-specific IgG ($P=0.05$), BLG-specific

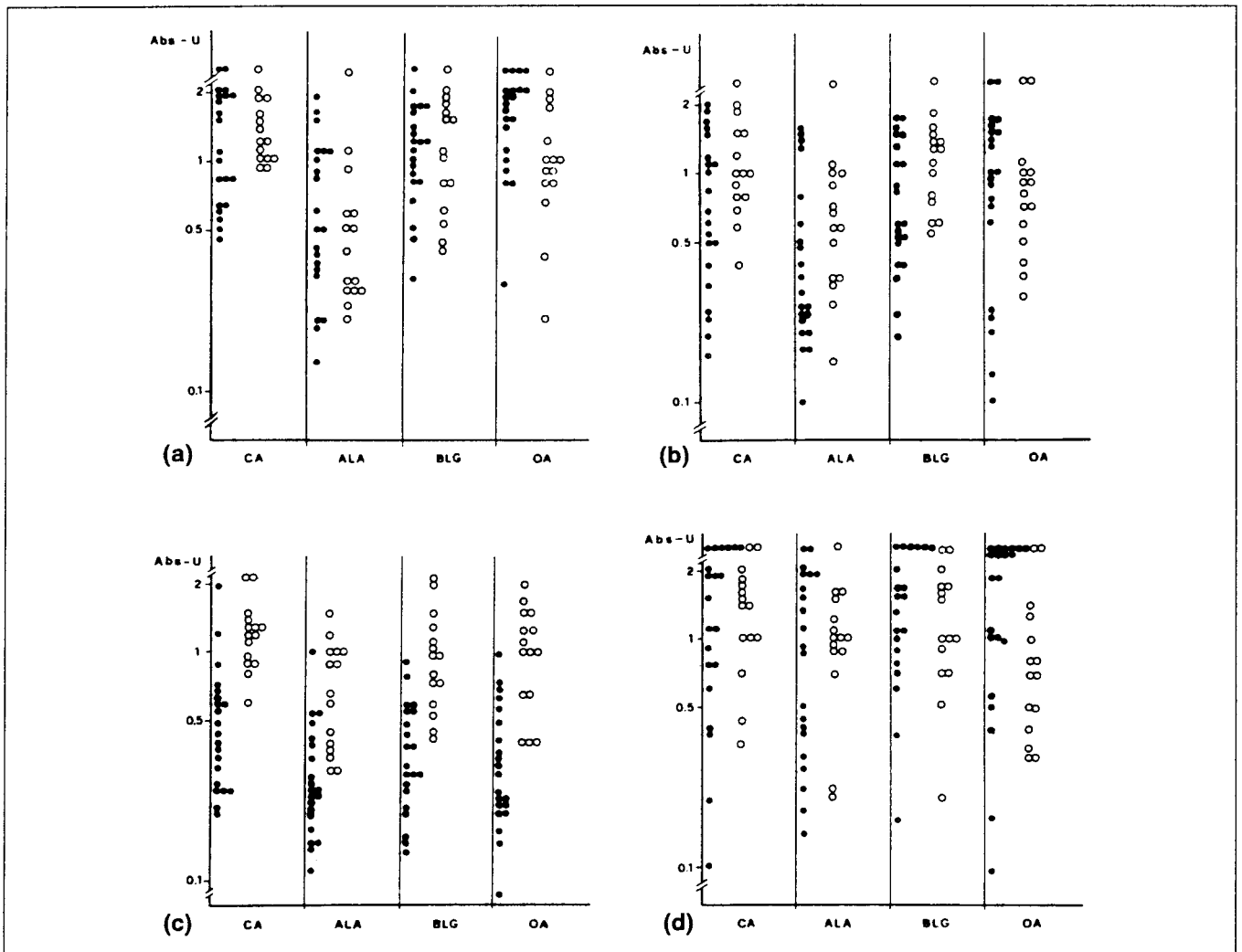


Fig. 3. Specific serum IgG1 (a), IgG2 (b), IgG3 (c) and IgG4 (d) to ALA, BLG, CA and OA in 21 children with AD (●) and in 15 age-matched controls (○)

Table 1. Correlations of specific IgE and IgA with specific IgG and IgG subclasses^a

Antigen	Specific antibodies							
	IgE	IgA	IgG	IgG1	IgG2	IgG3	IgG4	IgA
ALA	IgE —	IgA —	IgG $P=0.01$ $r=0.62$	IgG1 —	IgG2 —	IgG3 —	IgG4 —	IgA —
		IgA —	IgG —	IgG1 —	IgG2 —	IgG3 $P=0.03$ $r=-0.52$	IgG4 $P=0.04$ $r=0.45$	IgA —
BLG	IgE —	IgA —	IgG —	IgG1 $P=0.03$ $r=0.56$	IgG2 $P=0.02$ $r=0.51$	IgG3 —	IgG4 —	IgA —
		IgA —	IgG —	IgG1 —	IgG2 —	IgG3 —	IgG4 $P=0.05$ $r=0.42$	IgA —
CA	IgE —	IgA —	IgG $P=0.05$ $r=0.43$	IgG1 —	IgG2 —	IgG3 —	IgG4 —	IgA —
		IgA —	IgG —	IgG1 —	IgG2 —	IgG3 —	IgG4 —	IgA —
OA	IgE —	IgA —	IgG —	IgG1 —	IgG2 —	IgG3 —	IgG4 —	IgA $P=0.01$ $r=0.78$
		IgA —	IgG —	IgG1 —	IgG2 —	IgG3 —	IgG4 —	IgA —

ALA, Lactalbumin; BLG, β -lactoglobulin; CA, casein; OA, ovalbumin
^a Spearman's rank correlation test

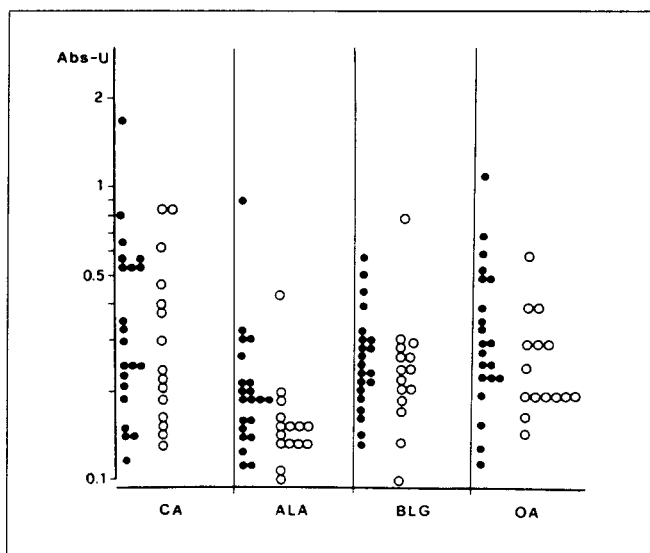


Fig. 4. Specific serum IgA antibodies to ALA, BLG, CA and OA in 21 children with AD (●) and in 15 age-matched controls (○)

IgG1 ($P=0.03$), BLG-specific IgG2 ($P=0.02$) and ALA-specific IgG4 ($P=0.04$).

Discussion

The antibody response to four dietary proteins was analyzed in a group of children with AD and cows' milk-related symptoms and in normal age-matched controls. Specific IgE to three CMP and to OA were detected in 16 of 21 (76%) children with AD; this is in agreement with other reports [2, 21]. Specific IgG and IgA were found both in both patients and controls, without significant differences, except for anti-OA IgG and anti-ALA IgA, which were elevated in the AD group. However, IgG subclass analysis showed different response patterns: IgG1 and IgG4 were higher in patients (even if significance was reached only for OA), while IgG2 and IgG3 levels tended to be lower in the AD group. Therefore, IgG subclass determination may be of some importance, as variations in minor subclass antibodies (e.g., IgG3 and IgG4) may not be detected when whole IgG is measured [20].

It is not always easy to compare the results of different studies, since not only are different clinical sampling criteria used, such as symptoms or patients' age, but differences in technical procedures may also influence the results. Quinti et al. [17] found that minor modifications of methodology, such as incubation times, could interfere with the results. The coating efficiency of some antigens [20] and the binding affinity of monoclonal anti-subclass antibodies [17] should also be taken into account.

Unfortunately, many authors examined only the IgG4 subclass [5, 15, 18]. Our results, with all four subclasses, agree partially with other studies. In particular, Okahata et al. [16] found an elevated IgG1 and IgG4 response to OA in children with AD without food-related symptoms up to 2 years of age; this is in agreement with our findings in children aged 3 months to 3 years. In contrast, Husby

et al. [9] did not find any difference in the anti-OA response between AD patients and healthy controls, while anti-BLG IgG4 was higher in patients. However, the age-range of patients, which is not reported by the authors, may have influenced the results. Moreover, these authors could not demonstrate an IgG3 response to OA and BLG, either in children with AD [9] or in healthy adults [8]; only a few patients and controls had specific IgG2 to these food antigens. However, a relatively high detection threshold for these subclasses may have prevented the detection of low levels of antibodies. Quinti et al. [17], who examined anti-OA and anti-BLG IgG subclasses in children with AD and food-related symptoms, found no statistical difference with healthy controls, even though IgG4 antibodies seemed to be higher in patients, while the IgG2 and IgG3 responses to OA and BLG tended to be lower in patients than in controls.

The biological and clinical significance of the food-directed IgG response in AD is still under discussion [9, 13, 15–18]. Analysis of IgG subclass distribution may help to clarify a possible pathological role of such antibodies. In accordance with other studies [9, 16, 17], our data suggest an increase in the IgG1 and IgG4 responses to milk and egg antigens, although this increase reaches significance only for the symptom-unrelated OA response. Such findings may reflect an overall increased gut permeability of AD patients [11], which may lead to the formation of antibodies (symptom-related or not) to all sorts of foods, particularly to those which are highly antigenic and heat stable, such as OA. This has also been suggested by Calkhoven et al. [3]; such views are consistent with our findings of a marked immune response to food antigens (both IgE and IgG) in subjects with more active disease, and probably with increased gut permeability. A common regulatory mechanism triggered by interleukin-4 (recently observed in vitro by Lundgren et al. [14]) should also be considered; in fact, concomitant specific IgG1 and IgG4 responses are also observed in pollen- or dust mite-sensitized individuals [4, 10]. Moreover, the increase in IgG4 could be part of a protective mechanism, since IgG4 is a poor complement-fixing subclass and may therefore interfere with complement fixation by other antibody subclasses [1].

In contrast, IgG2 and IgG3 subclasses were markedly decreased in the patient group for all antigens tested. IgG3 is an efficient complement-fixing subclass [19]; the decrease in IgG3 serum levels could be due to local consumption of this subclass in immune complexes containing food antigens. Complement activation with resulting tissue damage could play a part in the development of food-related symptoms. IgG2 is also lower in the AD group; even IgG2 directed against the glycoprotein OA is somewhat depressed, although this subclass is primarily involved in the anti-carbohydrate immune response [22]. This finding may reflect a biological, as yet unknown, role of this subclass.

In conclusion, our results confirm the frequently reported findings of food-directed IgE in AD children, while specific IgA and IgG determinations reveal considerable overlap with controls and appear of limited diagnostic value. IgG subclass analysis suggests a different

role for the four subclasses; this could indicate a pathogenetic role of some subclasses, particularly the complement-fixing IgG3, and a protective role of other subclasses. Further studies are required to clarify the biological role of food-directed antibodies in AD.

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