

Total and allergen-specific immunoglobulin A levels in saliva in relation to the development of allergy in infants up to 2 years of age

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Summary

Background The association between salivary IgA levels and development of allergy is controversial and the employed methodology has been questioned.

Objective The aim of the study was to relate the levels of total IgA, SIgA and allergen-specific IgA antibodies in saliva to the development of allergy in infants during the first 2 years of life.

Methods Saliva samples from 80 infants participating in a prospective study regarding the development of allergy were collected at 3 or 6, and 12 and 24 months of age. Total IgA, SIgA and Fel d 1 and β -lactoglobulin specific IgA levels were analysed with ELISA.

Results The levels of total IgA and SIgA increased with age. The number of samples with detectable IgA to Fel d 1 tended to increase with age, whereas the opposite was observed for IgA to β -lactoglobulin. Infants who developed allergy tended to have higher levels of total IgA, and allergen-specific IgA was more commonly detected than in non-allergic children. In contrast, non-allergic children tended to have higher levels of SIgA. Furthermore, the levels of SIgA were higher in sensitized infants with no allergic symptoms than in sensitized children with symptoms. Infants with allergic parents had lower SIgA levels than infants without. Direct exposure to cat and cow's milk did not influence the levels of allergen-specific IgA levels, nor was there any association between breast-feeding and IgA production.

Conclusion The kinetics of food and inhalant allergen-specific IgA in saliva during the first 2 years of life is similar to what has earlier been shown for IgG in serum. Development of allergy tended to be associated with high levels of total and allergen-specific IgA antibodies, but low levels of SIgA. Furthermore, high levels of SIgA seemed to protect sensitized children from developing allergic symptoms during the first 2 years of life, supporting a possible protective role of SIgA against development of allergy.

Keywords allergy, children, IgA, saliva

Submitted 29 October 2001; revised 17 May 2002; accepted 10 June 2002

Introduction

Prospective studies have shown that IgE and IgG antibodies to foods are commonly detected both in atopic and non-atopic infants, although the magnitude of the responses is higher and of longer duration in the former group [1, 2]. The IgE and IgG responses to inhaled allergens appear later in life and are mostly down-regulated in non-atopic individuals, whereas they reach higher and more persistent levels in atopic children [1, 2]. Less is known about the kinetics of IgA antibodies to allergens in atopic and non-atopic children.

Immunoglobulin A is the predominant immunoglobulin isotype in humans. The bone marrow is the major source of serum IgA, which is predominantly present in a monomeric form [3]. Secretory IgA (SIgA), produced locally in mucosal tissue and

glands by gut- and bronchus-associated lymphoid tissue activated B-cells, is mostly in a dimer form. Attached to the dimer is a secretory component (SC), which makes SIgA more resistant to cleavage by proteolytic enzymes. Antigen-specific SIgA prevents adherence and penetration of antigens, capable of provoking immune inflammatory responses, through the mucosal epithelium. Thus, high levels of allergen-specific SIgA could, theoretically, prevent allergen absorption and thereby sensitization and subsequent development of allergy. Supporting this possibility, low levels of total SIgA and transient IgA deficiency have been associated with an increased risk for allergy [4, 5]. This is controversial, however, as a tendency towards the opposite has also been reported [6]. Furthermore, Renz et al. reported higher levels of SIgA to casein, but not to β -lactoglobulin (BLG), in saliva at birth in children who developed atopic disease during the first 3 years of life, as compared with those who did not [7].

In order to analyse the possible relationship between allergen-specific and total IgA production and atopic disease, we performed a prospective 2-year study of new-born infants.

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Materials and methods

Subjects

Saliva samples were collected at 3 or 6 and at 12 and 24 months of age from 80 infants participating with their families in a prospective study regarding the development of allergy. At the same time-points, blood samples were drawn, skin prick tests (SPT) to common food and inhalant allergens were performed, and questionnaires regarding atopic symptoms, breast feeding, introduction of cow's milk and solid foods, presence of pets and smoking habits were answered. In addition, a clinical examination regarding allergic symptoms was performed at 2 years. Atopic dermatitis was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as three or more episodes of bronchial obstruction, at least once verified by a physician. Allergic rhinoconjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. Skin prick tests were done in duplicate on the volar aspects of the forearms with thawed egg white, fresh skimmed cow's milk (lipid concentration 0.5%) (3, 6, 12 and 24 months), cat (12 and 24 months), birch and timothy (24 months) extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/mL) was used as a positive control, and albumin diluent was included as a negative control. The test was regarded as positive if the mean diameter was > 3 mm.

A typical clinical history, e.g. allergic rhinoconjunctivitis, allergic asthma or flexural itchy dermatitis, established the atopic status of the parents.

Saliva sampling and preparation

The same person collected all saliva samples in a standardized way. The saliva was collected from the buccal cavity using a hand-pump connected to a thin plastic tube and immediately frozen at -20°C . Before analysis, the samples were heated at 56°C for 30 min and then centrifuged at $5000g$ for 15 min. The supernatants were stored in aliquots at -20°C . Long-term storage has earlier been reported to not have any effect on the antibody affinity [8], nor does long-term storage and repeated cycles of freezing and thawing alter the molecular weight of IgA [9].

ELISA for detection of salivary IgA antibodies

For detection of BLG and cat allergen (Fel d 1) specific IgA in saliva, high-binding ELISA plates (Costar 3590, Life Technologies, Täby, Sweden) were coated with 200 μL /well of 20 $\mu\text{g}/\text{mL}$ Fel d extract (ALK), or with 10 $\mu\text{g}/\text{mL}$ BLG (Sigma Immunochemicals, Stockholm, Sweden), diluted in PBS. Every second row was left uncoated as control of unspecific binding. The plates were incubated overnight at room temperature (21°C). The plates were blocked for 90 min at 37°C using 200 μL /well of 2% human serum albumin (HSA) (Behring Diagnostics, Marburg, Germany) diluted in PBS. Saliva samples were diluted 1 : 50 in PBS-Tween with 0.5% HSA (PHT) and 100 μL /well was added in quadruplicate to the plates (two coated and two uncoated wells). Saliva samples containing high titres of specific IgA to cat and BLG, respectively, were diluted in eight steps in PHT and used as standard curve. After incubation for 1 h in 37°C , 100 μL /well of a biotinylated mouse anti-human IgA (Sigma Immunochemicals), diluted 1 : 10 000 in PHT was added. After incubation for 1 h,

100 μL /well of streptavidin-poly HRP conjugate (CLB, Amsterdam, Netherlands), diluted 1 : 10 000 in 1% casein buffer (art. no. M2052, CLB) was added, and the plates were incubated for 30 min. As substrate, 50 μL /well of TMB (Sigma Immunochemicals) was used and after 30 min, addition of 50 μL /well of 0.46 M H_2SO_4 stopped the reaction.

Values for the samples were calculated from the optical density (OD) of the reference curve and expressed as arbitrary units (AU). The OD of the blanks and the OD values for the individuals controls were subtracted from the OD values of the saliva samples. The specificity of the methods was verified by blocking experiments, in which saliva samples were incubated overnight with BLG, Fel d 1 extract or ovalbumin to final concentrations of 0, 1, 10 and 100 $\mu\text{g}/\text{mL}$. The incubated samples were centrifuged at $10\,000g$ for 15 min and the antibody levels in the supernatants were analysed on plates coated with BLG or Fel d 1. Incubation with 100 $\mu\text{g}/\text{mL}$ of the relevant antigen abolished 90% of the values, whereas no inhibition was seen with a non-relevant antigen (ovalbumin).

For analysis of total IgA, an anti-human IgA antibody directed against the alpha chain (Dakopatts AB, Täby, Sweden) was used as coating antibody, enabling detection of all IgA, including monomeric, polymeric and secretory IgA. For detection of SIgA, an anti-human secretory component antibody (Dakopatts AB) was used, enabling detection of only SIgA. High-binding plates were incubated at 37°C for 2 h and thereafter for at least 3 h at 4°C with 200 μL /well of anti-human IgA diluted 1 : 1000, or anti-secretory component diluted 1 : 500 in PBS. Plates were blocked with 200 μL /well PHT at 37°C for 2 h and then at 4°C overnight. Samples, 100 μL /well, were diluted 1 : 100 to 1 : 400 in PHT. Human IgA (Sigma Immunochemicals) was diluted in seven steps for standard curve. Antibody binding was detected by 100 μL /well of alkaline phosphatase conjugated goat anti-human IgA antibodies (Sigma Immunochemicals) diluted 1 : 10 000 in PHT and 200 μL /well of FAST-pNPP substrate. The detection intervals were 40 to 2500 ($\mu\text{g}/\text{L}$) for total IgA and 16 to 1000 ($\mu\text{g}/\text{L}$) for SIgA.

Saliva samples from five patients with total IgA deficiency (kindly provided by Professor Lennart Hammarström, Huddinge University Hospital, Stockholm, Sweden) were used to verify the specificity of the methods. No IgA or SIgA could be detected in these samples, even at a 1 : 2 dilution.

Statistics

As the concentrations of antibodies were not normally distributed, comparisons between unpaired groups were analysed using the Mann-Whitney *U*-test and paired groups were analysed using Wilcoxon signed rank test. Trends over time were analysed using ANOVA for repeated measurements. The chi-squared test was employed for categorical variables, and Fisher's exact test was used when the expected frequency for any cell was less than five. Although the study had a prospective design, the Bonferroni correction was employed to minimize the risk for Type I error. A probability level of < 5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA).

Ethics

The Regional Ethics Committee for Human Research at the University Hospital of Linköping approved the study.

Results

The cumulative incidence of SPT-positivity at 2 years was 23% ($n = 18/80$). Sixteen of the children developed a positive SPT to egg white and four of them were also SPT positive to cow's milk, one to birch and one to cat. One child developed only a positive SPT to birch and one to cat. Seventeen infants developed atopic dermatitis during the first 2 years of life, two children developed only asthmatic symptoms and one child developed only symptoms of allergic rhinoconjunctivitis after exposure to cat. Eleven of the children with allergic symptoms had positive SPT. The child who developed symptoms of allergic rhinoconjunctivitis after exposure to cat was sensitized to egg and cat. The two children who were sensitized to birch developed atopic dermatitis but showed no allergic symptoms associated with the birch pollen season. Fifty-seven (71%) of the children had at least one parent with a history of allergic symptoms.

Forty-three saliva samples were collected at 3 months, 37 at 6 months, 78 at 12 months and 60 at 24 months. At 24 months, many children protested heavily when the saliva samples were to be collected, and mostly only small amounts could be collected.

Both IgA and SIgA were present in all the samples, and the levels increased with age (Table 1). The IgA levels were significantly higher than the SIgA levels at all ages (Table 1). Antibodies to Fel d 1 were found in 30% of the samples at 3 months (Table 2). The number of positive samples increased with age, although the increase did not reach statistical significance (chi-squared test) (Table 2). In contrast, the number of samples with detectable BLG-specific IgA antibodies decreased with age (chi-squared test) (Table 2). The frequency of samples with detectable IgA to Fel d 1 at 12 months was higher in infants with, than without, allergic symptoms at 24 months (12/19 vs. 18/59, chi-squared test, $P = 0.011$) and in children with, than without, positive SPT (11/18 vs. 19/61, chi-squared test, $P = 0.022$). These differences were statistically significant also after the Bonferroni correction ($P = 0.022$ and $P = 0.044$, respectively). A similar trend was observed for IgA to BLG at 6 months, comparing SPT-positive and SPT-negative children (5/7 vs. 9/31 infants had detectable IgA to BLG, Fisher's exact test, $P = 0.077$). As compared with SPT-negative children, those with positive SPT had higher levels of IgA to Fel d 1 at

12 months (median value (ranges), 0.5 (0.3–2.4) vs. 0.3 (0.3–3.4), Mann–Whitney U -test, $P = 0.024$) and IgA to BLG at 6 months (median value (ranges), 0.4 (0.2–2.4) vs. 0.2 (0.2–2.5) Mann–Whitney U -test, $P = 0.041$). Similar trends were seen after the Bonferroni correction ($P = 0.048$ and $P = 0.082$, respectively). No differences were observed at other ages (data not shown).

Although the median levels of total IgA were higher at all ages in infants with allergic symptoms than in those without (Table 1), and in SPT-positive, as compared with SPT-negative infants, this only reached statistical significance for the latter comparison at 6 and 12 months (Fig. 1), and this was the case also after the Bonferroni correction at 12 months ($P = 0.036$) but not at 6 months ($P = 0.12$). In contrast, SIgA tended to be higher in infants without allergic symptoms at 6, 12 and 24 months (Table 1), although this was only statistically significant at 24 months (Mann–Whitney U -test, $P = 0.024$). A similar trend was seen after the Bonferroni correction ($P = 0.072$). Among the SPT-positive infants, those who developed allergic symptoms had lower levels of total SIgA in saliva at 12 months than those who did not (Fig. 2). Similar results were observed at 6 and 24 months (Mann–Whitney U -test, $P = 0.034$ and

Table 2. The number of saliva samples with detectable levels of Fel d 1 and BLG-specific IgA. Median values and ranges (AU) are indicated for samples with detectable levels. The number of samples with detectable IgA to BLG was significantly higher at 3 than at 6 months ($P = 0.009$, chi-squared test), and a similar trend was seen between 12 and 24 months ($P = 0.084$, chi-squared test)

	Number of positive samples	Median (AU)	Range (AU)
IgA to Fel d 1			
3 months	13/43 (30%)	1.2	0.4–1.9
6 months	12/36 (33%)	0.9	0.4–3.4
12 months	30/78 (38%)	0.9	0.3–3.4
24 months	25/60 (42%)	0.9	0.4–4.2
IgA to BLG			
3 months	28/40 (70%)	0.5	0.3–1.8
6 months	14/38 (37%)	0.5	0.4–2.5
12 months	23/76 (30%)	0.5	0.3–1.6
24 months	10/59 (17%)	0.4	0.3–2.1

Table 1. The median levels and ranges (mg/L) of total IgA and SIgA in saliva samples at 3, 6, 12 and 24 months. The levels of total IgA were higher than the levels of SIgA at all ages, $P < 0.001$ (Wilcoxon signed rank test) and the levels of total IgA and SIgA increased with age, $P < 0.001$ (ANOVA for repeated measurements). Non-allergic children had higher levels of SIgA at 24 months than allergic children, $P = 0.024$ (Mann–Whitney)

	Number of samples		Median (mg/L)		Range (mg/L)	
	Allergic	Non-allergic	Allergic	Non-allergic	Allergic	Non-allergic
Total IgA						
3 months	8	28	35	29	2–113	4–132
6 months	2	30	82	37	25–140	10–245
12 months	10	58	107	64	23–235	2–444
24 months	7	48	164	109	64–402	9–250
SIgA						
3 months	12	28	16	12	0.8–40	0.8–59
6 months	7	30	8	13	3–34	0.8–67
12 months	17	58	11	16	2.8–25	0.8–75
24 months	11	47	12	19	2–31	3–48

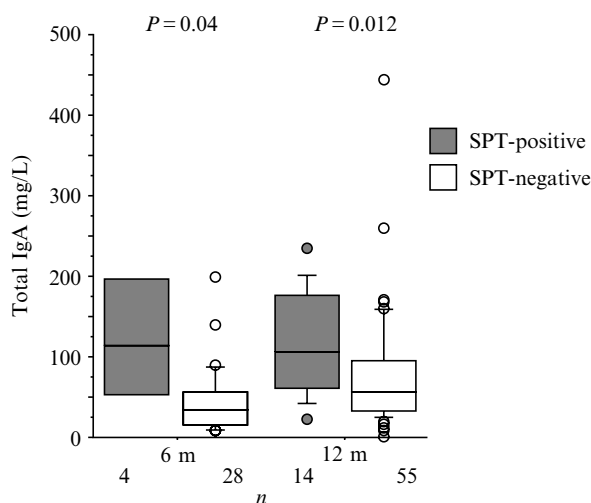


Fig. 1. Total IgA levels (mg/L) were higher at 6 and 12 months in infants with a positive SPT (grey box) than in children with negative SPT (white box) during the first 24 months of life (Mann–Whitney *U*-test). The 10th, 25th, 50th, 75th and 90th percentiles, as well as outliers, and the number (*n*) of children are indicated.

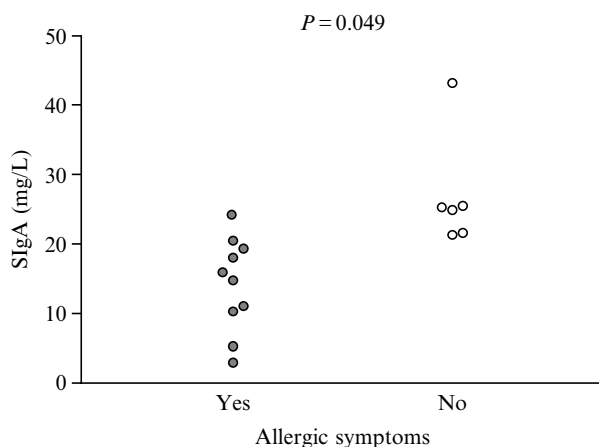


Fig. 2. Secretory IgA levels (mg/L) in saliva at 12 months in SPT-positive infants who did (*n* = 10, ●) and did not develop clinical allergy (*n* = 6, ○).

$P = 0.060$, respectively) (data not shown). A positive family history of allergy was associated with low levels of SIgA in the saliva, median values and ranges (mg/L), 12 (0.8–27) vs. 24 (5–59), $P = 0.0053$ at 3 months, 9 (0.8–67) vs. 17 (6–51), $P = 0.021$ at six months, 14 (0.8–75) vs. 19 (10–39), $P = 0.013$ at 12 months, and 18 (2–48) vs. 22 (12–40), $P = 0.049$ at 24 months. The differences were still statistically significant at 3 ($P = 0.016$) and 12 months ($P = 0.039$) and tended to be so at 6 and 24 months ($P = 0.063$ and 0.15, respectively) after the Bonferroni correction.

The levels of Fel d 1 specific IgA were similar in children of families with and without a cat (data not shown). Similarly, there was no correlation between length of exclusive or partial breast-feeding and either BLG-specific or total IgA levels at any age (data not shown).

Discussion

Total IgA and total SIgA were detected in all saliva samples, and the levels increased during the first 2 years of life. The

specificity of our assay was confirmed by the lack of demonstrable IgA in five samples from IgA-deficient individuals. Other investigators have reported transient absence of IgA in the saliva in 18% of the population during the first year of life [6]. The higher sensitivity of the method used in our study would explain the difference. The levels of total IgA in saliva were higher than the levels of SIgA at all ages. Previous studies have shown that both monomeric and secretory IgA are present in the saliva in children at least up to 12 months of age, and thereafter a switch to only SIgA production has been proposed [9]. This might be explained by an immature secretory component mediated IgA transport in children. Our results extend these findings, suggesting that the transport mechanism is not fully developed even at 24 months of age.

The number of saliva samples with detectable levels of IgA to Fel d 1 tended to increase with age, while the opposite was observed for BLG-specific IgA. The latter finding supports the results of a previous study [10], while the kinetics of IgA production in saliva to an inhalant allergen has not been studied previously. The different kinetics of IgA responses to BLG and Fel d 1 is similar to the development of IgG subclass responses, showing decreasing levels of food allergen-specific IgG subclass antibodies [2, 11] and increasing levels of inhalant-specific IgG with age [2].

It has been proposed that low levels of salivary SIgA are associated with the development of allergy [4, 5], but it has also been suggested that a transient SIgA deficiency is associated with a decreased risk for allergy [6]. In our study, development of allergy during the first 2 years of life tended to be associated with low secretory IgA levels but high levels of total and allergen-specific IgA. High levels of casein-specific IgA in saliva have earlier been found at birth in babies who later developed allergy [12]. The Th2 cytokine IL-4 is important in regulating antibody production, as it activates and induces proliferation of B cells, protects B cells from apoptosis (for review see [13]), and may up-regulate IgA production [14]. An up-regulated production of IL-4 in children who later develop allergy [15] could explain, not only an increased IgE production, but also the higher levels of salivary IgA in allergic individuals observed in this study.

The levels of total SIgA were lower at all ages in children with at least one allergic parent than in infants with a negative family history, which is in agreement with earlier studies [5]. Interferon- γ [16], IL-4 [17] and TNF- α [18] all up-regulate the expression of SC and the release of SIgA [19]. Even if IL-4 and IFN- γ synergistically up-regulate SC-expression [17, 19], the effect of IFN- γ may be more important *in vivo* [17, 20]. Decreased IFN- γ production has been reported in infants with subsequent development of atopy [21] and in children with at least one allergic parent [22–24]. This might explain the lower levels of SIgA in children who develop allergy within the first 2 years of life and in children with a family history of atopic disease and, furthermore, could indicate that the production of secretory component or secretory component-mediated IgA transport is impaired in these children.

Among the SPT-positive infants the levels of SIgA were lower at 12 months in those with, as compared to without, allergic symptoms. Although the number of infants with allergic symptoms included in this analysis was rather low (*n* = 17), our findings may support the notion that low levels of SIgA are involved in the development of allergy. Immunoglobulin

A antibodies are able to suppress chemotaxis of neutrophils, eosinophils and monocytes [3], and inhibit IgE-induced histamine release [25]. Thus, high levels of SIgA in the mucosa could possibly interfere with the interaction between allergen and IgE antibodies and thereby prevent the development of allergic symptoms.

There was no correlation between the levels of allergen-specific salivary IgA levels and exposure to either cat or cow's milk. It has previously been suggested that early exposure to cow's milk is associated with higher levels of IgA antibodies to casein, but not to BLG [10]. However, immunotherapy with birch pollen did not change the levels of birch-specific IgA in saliva [26], indicating that the degree of exposure to inhalants allergens does not affect mucosal IgA production in a major way. Furthermore, there seems to be no association between exposure to food [27] or inhalant allergens [28], and specific IgA levels at other mucosal sites. In contrast to this, however, the levels of avian-specific IgA antibodies are elevated in saliva from pigeon breeders, indicating that avian exposure stimulates IgA production [29]. High levels of cat allergen are found in all public areas [30] and BLG is readily detectable in breast milk [31]. The universal exposure to these allergens might explain the similar levels of Fel d 1 and BLG-specific IgA levels in saliva of infants who were and were not directly exposed to cat or cow's milk. Possibly, exposure to avian allergens is less general, which would explain the differences in antibody production between pigeon breeders and unexposed controls.

In conclusion, the kinetics of food and inhalant allergen-specific IgA in saliva during the first 2 years of life is similar to what has earlier been shown for IgG in serum. Development of allergy tended to be associated with high levels of total and allergen-specific IgA antibodies, but low levels of SIgA. Furthermore, high levels of SIgA may protect sensitized children from developing allergic symptoms up to 2 years of age, supporting a possible protective role of SIgA against development of allergy.

Acknowledgements

We thank Lena Lindell and Ulrika Bengtsson for excellent technical assistance and Mats Fredriksson for statistical assistance.

The Swedish Medical Research Council (grant no. 7510), the The Swedish Foundation For Health Care Sciences and Allergy Research, the National Swedish Association against Allergic Diseases, the National Heart and Lung Association and the First May Flower Annual Campaign for Children's Health and Glaxo-Wellcome are acknowledged for financial support.

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