

Increased leukotriene production by food additives in patients with atopic dermatitis and proven food intolerance

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Summary

Background Recently, we identified a subgroup of patients with atopic dermatitis (AD) with a clinical relevant food intolerance proven by double blind placebo controlled challenge. In search of possible pathomechanisms involved in this food intolerance, which leads to aggravation of the disease, the aim of the present study was to determine sulfidoleukotriene production in these patients using isolated leucocytes from the peripheral blood after stimulation with different food additives.

Methods Leukotriene production of peripheral leucocytes was detected by incubation of isolated cells with the food additives at different concentrations ranging from 0.2 to 200 µg/mL after pre-stimulation with IL-3. Ten non-atopic donors (A), nine AD patients of the diet responder group with negative oral provocation test against food additives (B) and nine patients of the responder group with positive reactions after the oral provocation test (C) were investigated.

Results In the non-atopic group (A), no increased sulfidoleukotriene (sLT) release was observed for all food additives tested. In group B, increased sLT production was determined using tartrazine in one patient (1/9) and using nitrite in two patients (2/9), whereas sLT production remained below the cut-off range in all patients of group B (9/9) using benzoate, metabisulfite and salicylate. By contrast, in group C increased sLT production was observed with food colour mix in 1/9, with tartrazine in 3/9, with benzoate in 4/9, with nitrite in 5/9, with salicylate in 2/9 and with metabisulfite in 1/9. However, no increased sLT concentration was determined in the presence of the tested food additives in two patients of group C.

Conclusions Increased sLT production by peripheral leucocytes in the presence of single food additives was observed in the majority of patients with a proven food intolerance towards food additives proven by double-blind-placebo-controlled challenges. These food additives were particularly tartrazine, benzoate and nitrite. These findings indicate that single food additives as aggravating factors in AD patients may trigger the disease through increased sLT production as a pathophysiological mechanism.

Keywords: sulfidoleukotriene production, atopic dermatitis, food additives

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Introduction

Atopic dermatitis (AD) is a chronic remittent, pruritic skin disease with typical clinical appearance and distribution of skin lesions [1]. Several immune deviations occur in

patients suffering from AD and play an important role for the multifactorial pathogenesis of this disease. Besides elevated total IgE levels and sensitizations towards several environmental allergens, abnormal T cell functions are involved in the chronic skin inflammation [2]. Allergen specific T cells, which infiltrate lesional skin are activated after antigen exposure and produce various proinflammatory cytokines. Type I food allergy with occurrence of allergen specific T cells in affected skin lesions has been implicated

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as playing an important role for AD in infancy. By strict avoidance of the allergen(s) the skin status can be controlled in these patients [3]. Apart from allergic mechanisms, also non-allergic reactions (pseudoallergic reactions) against food have been previously reported to cause an exacerbation of AD [4,5,6]. These pseudoallergic reactions, as has been shown for a subgroup suffering from chronic urticaria, are induced by food additives, vasoactive substances (e.g. histamine) and naturally occurring substances such as salicylates, benzoates and other compounds in fruits, vegetables and spices [7]. We have recently shown that > 50% of AD patients improve on a low-pseudoallergen diet and that a subgroup of these patients respond to double-blind placebo controlled oral provocation with food additives [8].

Previous studies have indicated that cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ are potent inflammatory mediators. Biologically active amounts of LTB₄ and LTC₄ can be found in the skin of AD patients [9]. Also increased urinary excretion of leukotriene E₄ in AD patients has been shown, indicating increased whole-body cysteinyl leukotriene synthesis [10]. Therefore, in search of possible mechanisms involved in these food intolerance reactions as aggravating factors for the disease, and acknowledging the fact that increased sLT production has not only been seen after allergenic stimulation [11] but also after stimulation with pseudoallergens such as salicylates or antibiotics [12,13], we measured the sLT production of peripheral leucocytes in AD patients with proven food intolerance. SLT production was determined by using the cellular antigen stimulation test which has been introduced by de Weck *et al.* [14].

Materials and methods

Non-atopic donors and patients

Twenty-eight individuals from three different groups (A-C) were evaluated (Table 1).

Group A represents non-atopic donors ($n = 10$), defined by a negative history for allergic diseases or intolerance reactions and normal total serum IgE levels. Group B and C are patients suffering from AD proven by the criteria from Hanifin and Raika [1]. These patients were 11 females and seven males, ranging from 17 to 72 years (median 29), one was an asthmatic as well, 12 had hay fever. None of these patients were on systemic medication or were known to be intolerant of NSAIDs. Group B includes patients ($n = 9$), who had improved Costa skin score and eosinophil cationic protein (ECP) serum levels, which are useful as an *in vitro* follow-up parameter in AD patients [15], after following a low pseudoallergen diet for 6 weeks (Table 2), but did not show a worsening of the eczema after a double blind placebo controlled food challenge (DBPCFC) using food

Table 1. Characterization of the study groups. Group A are non-atopic donors, group B are patients suffering from AD who had improved by Costa skin score and ECP serum levels after following a low pseudoallergen diet for 6 weeks, but negative DBPCFC using food additives. Group C are AD patients, who had improved after a low pseudoallergen diet for 6 weeks and reacted to food additives proven by DBPCFC

	<i>n</i>	Total IgE KU/mL	Costa score (mean points)		Serum ECP (mean µg/mL)	
			Before	After	Before	After
Group A	10	< 100	0	0	n.d.	n.d.
Group B	9	1854	28	11.5	14	8
Group C	9	2002	33	15.2	41	15

additives. Group C includes patients ($n = 9$), who had improved Costa skin score and ECP serum levels after following a low pseudoallergen diet for 6 weeks and reacted to food additives proven by DBPCFC with worsening of the eczema. The food additives were given in capsules to guarantee double-blind, placebo-controlled provocation tests. Dosages were based on former provocation tests in patients with suspected pseudoallergy [7]. Capsules containing all food additives were given in one single administration of challenge, the identical amount of placebo capsules were given at another time point. The order was randomized and the time of observation was 48 h after each provocation; the skin status was documented before and after each provocation test.

Analysis of sulfidoleukotriene production *in vitro*

For the detection of the sulfidoleukotriene production *in vitro* the cellular antigen stimulation test was used according to the manufacturer's instructions (DPC, Biermann GmbH, Bad Nauheim Germany). Briefly, from each patient 10 mL blood was drawn into an EDTA tube after informed consent. Cells were then mixed with dextran solution. After 90 min the upper phase was transferred into another tube and centrifuged. Afterwards, the platelet rich supernatant was removed and the cell pellet was resuspended into stimulation buffer containing IL-3 (20 ng/mL). The presence of IL-3 induces priming of the basophils and is used to increase the mediator release *in vitro* [16]. The cell suspension was transferred to separate tubes and the food additives were added to final concentrations ranging from 0.2 to 200 µg/mL. For each patient, spontaneous sulfidoleukotriene production was determined using unstimulated cells in the presence of IL-3 as a negative control and with anti-IgE (2,75 µg/mL) stimulated

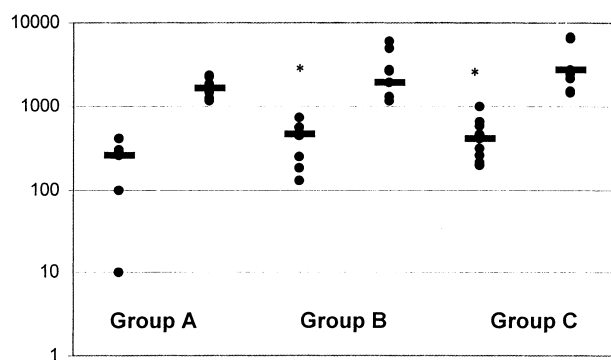


Fig. 1. Spontaneous and anti-IgE induced sLT production (pg/ml) in the different groups. The dots indicate different donors, the horizontal bar indicates the median of each group. Baseline sLT production in groups B and C, which represent AD patients ($n = 18$) ranged from 12 to 1007 pg/ml (median 392 pg/ml) and was significantly ($P < 0.05$) higher than baseline sLT production from normal donors, which ranged from 0 to 411 pg/ml (median 263 pg/ml).

cells as a positive control. Cells were incubated 40 min at 37 °C and were finally centrifuged. The supernatants were subsequently measured for sulfidoleukotriene production by an ELISA using antileukotriene pre-coated plates. After adding the enzyme conjugate and antibody solution, samples were incubated for 4 h at 4 °C. After a washing step, substrate solution was added and incubated for another 30 min at room temperature. Finally, stop solution was added and the absorbance was measured at 405 nm in an ELISA-reader. Sulfidoleukotriene production is expressed in pg/mL after subtracting spontaneous leukotriene production.

Reactions were considered as positive if the sulfido-leukotriene (sLT) concentrations were greater than the mean of three standard deviation ranges of sLT release generated by the corresponding substance concentration in non-atopic donors from group A (cut-off-range).

Statistical analysis

The Mann–Whitney *U*-test was used for statistical comparisons between the groups. Sensitivity, specificity and predictive values were calculated by using a 2×2 contingency table.

Results

Increased baseline and anti-IgE induced leukotriene production in AD patients

As depicted in Fig. 1, spontaneous sLT release from 10 non-atopic donors ranged from 0 to 411 pg/mL (median 263 pg/mL) and anti-IgE induced sLT production from 1263 to 2335 pg/mL (median 1689 pg/mL). By contrast, baseline sLT production in groups B and C, which represent AD patients ($n = 18$), was significantly ($P < 0.05$) higher and ranged from 127 to 1007 pg/mL (median 392 pg/mL). In these groups anti-IgE induced sLT production ranged from 1188 to 6890 pg/mL, respectively. Within the different AD groups (B and C), no significant differences of baseline and anti-IgE induced sLT production was observed. In group B baseline sLT production ($n = 9$) ranged from 127 to 739 pg/mL

Table 2. Low-pseudoallergen diet. Strictly forbidden: all food containing preservatives, dyes or antioxidants; all industrially processed food should be carefully checked regarding food additives (Zuberbier et al. 1995)

	Forbidden	Allowed
Basic food	Preservative-free bread, potatoes, rice, unprocessed cereals, flour (not self-raising!), rice cakes, durum wheat pasta (without egg)	All others (e.g. pasta with eggs, cake, biscuits potato chips, crisps)
Fats	Butter, cold pressed plant oils	All others (e.g. margarine, mayonnaise)
Milk products	Fresh milk, cream without stabilisers, white cheese, fromage frais, a small amount of mild Gouda	All others
Food from animals	Fresh meat without seasoning	All others including eggs, sea-food, smoked meat
Vegetables	All (e.g. lettuce, carrots, zucchini, cabbage, broccoli, asparagus) except those listed as forbidden	Artichokes, peas, mushrooms, spinach, rhubarb, tomatoes and tomato products, olives, sweet peppers, spinach
Fruit	None	All including dried fruits or fruit juices
Herbs, spices	Salt, sugar, chives, onions	All others including garlic and herbs
Sweets	None	All including chewing gum
Beverages	Milk, mineral water, coffee, black tea	All others including beer, wine, spirits and herbal teas
Spreadings	Honey	All others

Table 3. Increased sLT production at least at one concentration of the tested food additives (0.2–200 µg/mL) related to the studied numbers of patients from the different groups (A normal donors, B patients with AD and negative oral provocation test and C patients with AD and positive oral provocation test)

	Group A	Group B	Group C
Food colour mix	0/9	0/9	1/9
Tartrazine	0/9	1/9	3/9
Nitrite	0/9	2/9	5/9
Benzoate	0/9	0/9	4/9
Metabisulfite	0/9	0/9	1/9
Salicylate	0/9	0/9	2/9

(median 392 pg/mL) and anti-IgE induced sLT production from 1188 to 6031 pg/mL (median 1981 pg/mL). In group C, baseline sLT production ($n = 9$) ranged from 201 to 1007 pg/mL (median 413 pg/mL) and anti-IgE induced sLT production from 1464 to 6890 pg/mL (median 2826 pg/mL).

Leukotriene production by basophils of AD patients using food additives

In normal non-atopic controls (group A, $n = 10$), no increased sulfidoleukotriene production was observed in the presence of any of the used food additives including food colour mix, benzoate, tartrazine, nitrite, metabisulfite and salicylate.

The mean values of sLT production in this non-atopic group using food colour mix, benzoate, tartrazine, nitrite, metabisulfite and salicylate were below 100 pg/mL for all concentrations analysed.

In AD patients with negative oral provocation tests (group B, $n = 9$), sLT production was not induced in the presence of the food colour mix, benzoate, metabisulfite and salicylate. However, in this group a modest induction of sulfidoleukotriene production was determined in 1/9 patient using tartrazine and in 2/9 patients using nitrite (Table 3). In group C ($n = 9$), which represent patients who had improved after following a low pseudoallergen diet for 6 weeks and reacted to food additives proven by DBPCFC, 7/9 patients showed increased sLT production in the presence of the different food additives (Table 3). The most frequent food additives, which induced sLT production were nitrite (5/9), benzoate (4/9) and tartrazine (3/9), whereas food colour mix and metabisulfite induced sLT release in only 1/9 patients and salicylate induced sLT release in 2/9 patients (data not shown). However, in 2/9 patients from group C no increased sLT production

was observed in the presence of any of the tested food additives.

Induction of leukotriene production by basophils from AD patients with positive DBPCFC using nitrite, benzoate and tartrazine

The most frequently increased sLT production in AD donors with positive oral provocation test towards food additives was observed when nitrite, benzoate and tartrazine were used (Fig. 2a-c). These food additives showed also the highest increase of sLT production ranging from 352 to 2072 pg/mL for nitrite, from 687 to 1502 for benzoate and from 362 to 2195 pg/mL for tartrazine. However, the maximum of sLT production for one particular food additive was not always observed at the highest concentration of 200 µg/mL (Fig. 2a-c).

Nitrite

The highest mean sLT production in group C was determined when nitrite was used (Fig. 2a); here mean sLT production was 202 pg/mL at 0.2 µg/mL, 375 pg/mL at 2 µg/mL, 623 pg/mL at 20 µg/mL and 492 pg/mL at 200 µg/mL, whereas in group B and also group A (not shown) the mean values of sLT production remained below the cut-off levels at any concentration tested. However, two patients from group B also showed a slight increased sLT production in the presence of nitrite (762 and 398 pg/mL).

Benzoate

In the presence of benzoate the mean values of sLT production at the different concentrations (0.2–200 µg/mL) were in group C 344 pg/mL, 325 pg/mL, 426 pg/mL and 442 pg/mL (Fig. 2b) and in group B as well as in normal donors (not shown) again in any case with 33 pg/mL, 34 pg/mL, 36 pg/mL and 23 pg/mL below the cut-off level.

Tartrazine

Mean values of sLT production using tartrazine in group C were 291 pg/mL at 0.2 µg/mL, 556 pg/mL at 2 µg/mL, 284 pg/mL at 20 µg/mL and 246 pg/mL at 200 µg/mL, whereas the mean values in group B were 89 pg/mL, 50 pg/mL, 50 pg/mL and 43 pg/mL, respectively, and therefore always below the cut-off levels for tartrazine (Fig. 2c). A similar result was observed also in the non-atopic donor group (data not shown). In one patient from group B a slight increase of sLT production in the presence of tartrazine was observed (250 pg/mL).

(a) sLT pg/ml

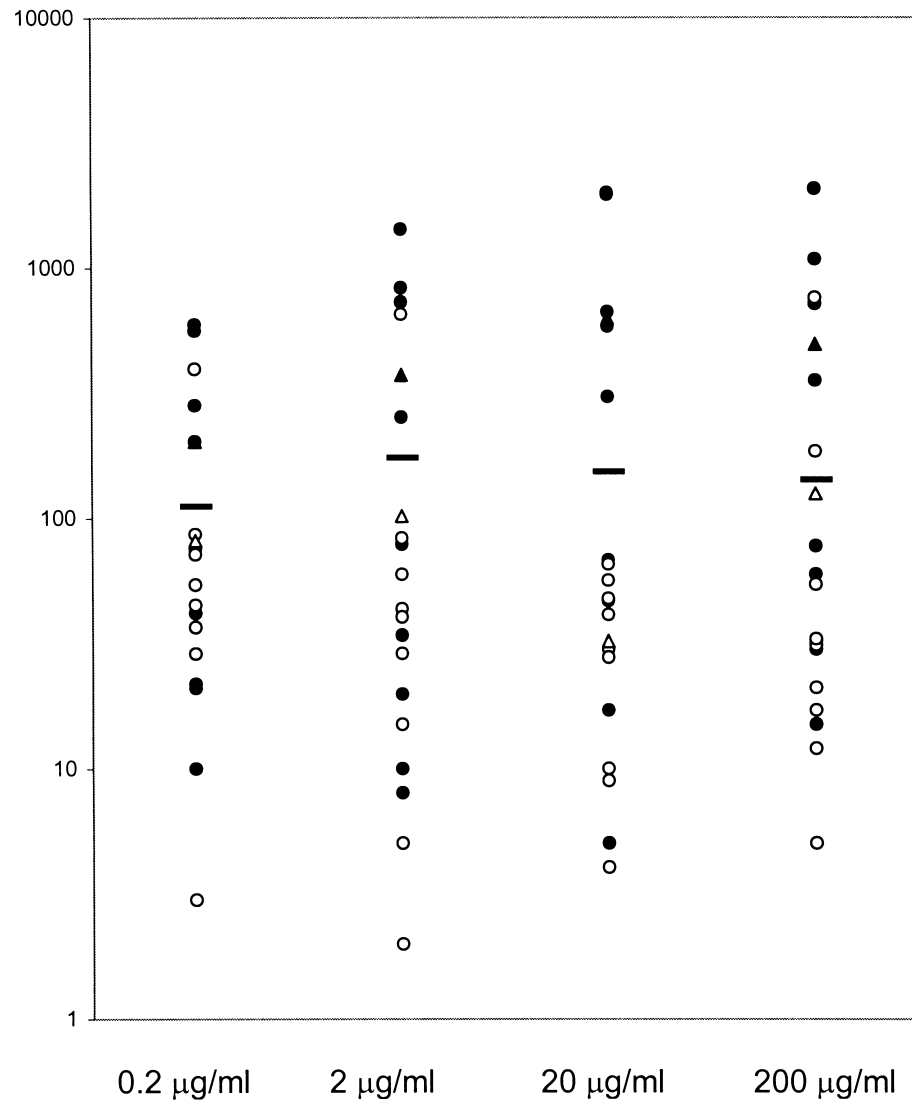


Fig. 2. sLT production of basophils in the presence of nitrite (0.2–200 µg/ml) from the different AD groups. Patients from group B are indicated by unfilled dots, mean values of group B by unfilled triangles and patients from group C are indicated by filled dots, mean values of group C by filled triangles; the cutoff levels of each food additive are shown by black bars. Mean values in group A were 71, 5, 31 and 2 pg/ml in the presence of nitrite 0.2–200 µg/ml respectively (data not shown).

Predictive value of the sLT production for determining food intolerance in diet-responder AD patients

In order to assess the predictive value of sLT production for determining food intolerance reactions in patients suffering from AD with positive DBPCFC compared to patients suffering from AD with negative DBPCFC we determined first the sensitivity and specificity and thereafter the positive predictive value in the different groups by using

a 2×2 contingency table. When pooling data from all the patients showing positive *in vitro* tests within groups B and C the calculation reveals a sensitivity of 78% and a specificity of 67% using sLT production by basophils in the presence of food additives for identification of a clinically relevant food intolerance. Consecutively, the positive predictive value reaches only 70%. However, these calculations must be regarded on the basis that the total numbers of tested individuals are low.

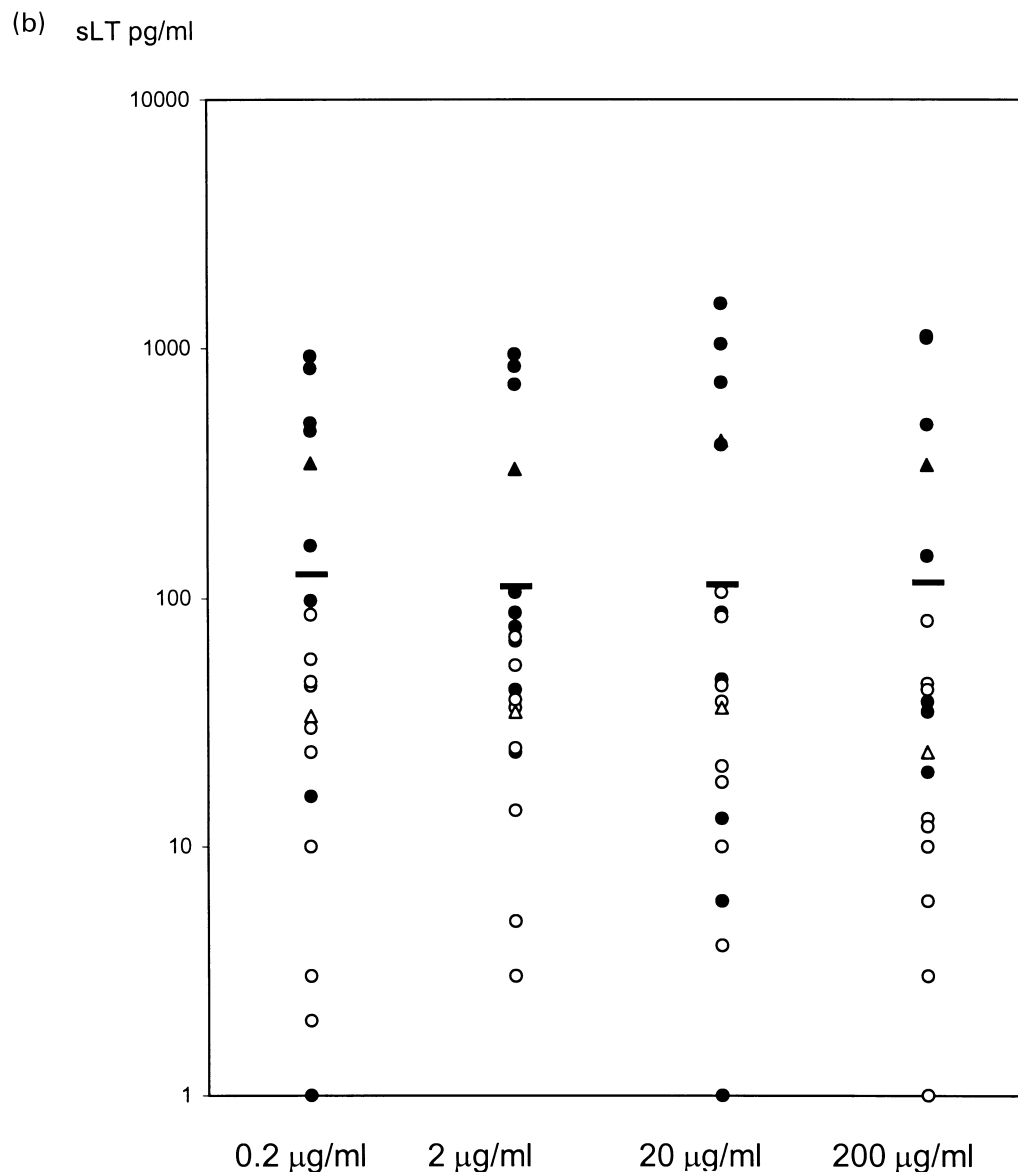


Fig. 2. cont. sLT production of basophils in the presence of benzoate (0.2–200 µg/ml) from the different AD groups. Symbols were used as indicated in (a). Mean values in group A were 42, 102, 88 and 20 pg/ml in the presence of benzoate 0.2–200 µg/ml respectively (data not shown).

Discussion

The major role of leukotrienes for the inflammatory reaction in allergic diseases has been clearly demonstrated in the past [17]. These newly formed mediators show powerful pro-inflammatory activities and their release from various cell types may be induced by agonists including allergens and pseudoallergens [18]. Our data first show that spontaneous sLT production from peripheral leucocytes is significantly higher in atopic individuals compared to normal donors. This increased spontaneous releasability of mediators such as histamine and leukotrienes from

basophils of AD patients has been reported before by other groups [19,20] and indicates that circulating basophils are pre-activated in these individuals. However, although we observed increased anti-IgE-induced sLT production within the different groups (1689 pg/mL in group A, 1981 pg/mL in group B and 2826 pg/mL in group C) statistical significance was not reached. Besides basophils, other granulocytes such as neutrophils or eosinophils have been reported to produce leukotrienes [21,22]. However their contribution to the measured sLT production in this study is not clear, but may only be of minor importance.

Recently we could show that a subgroup of patients will

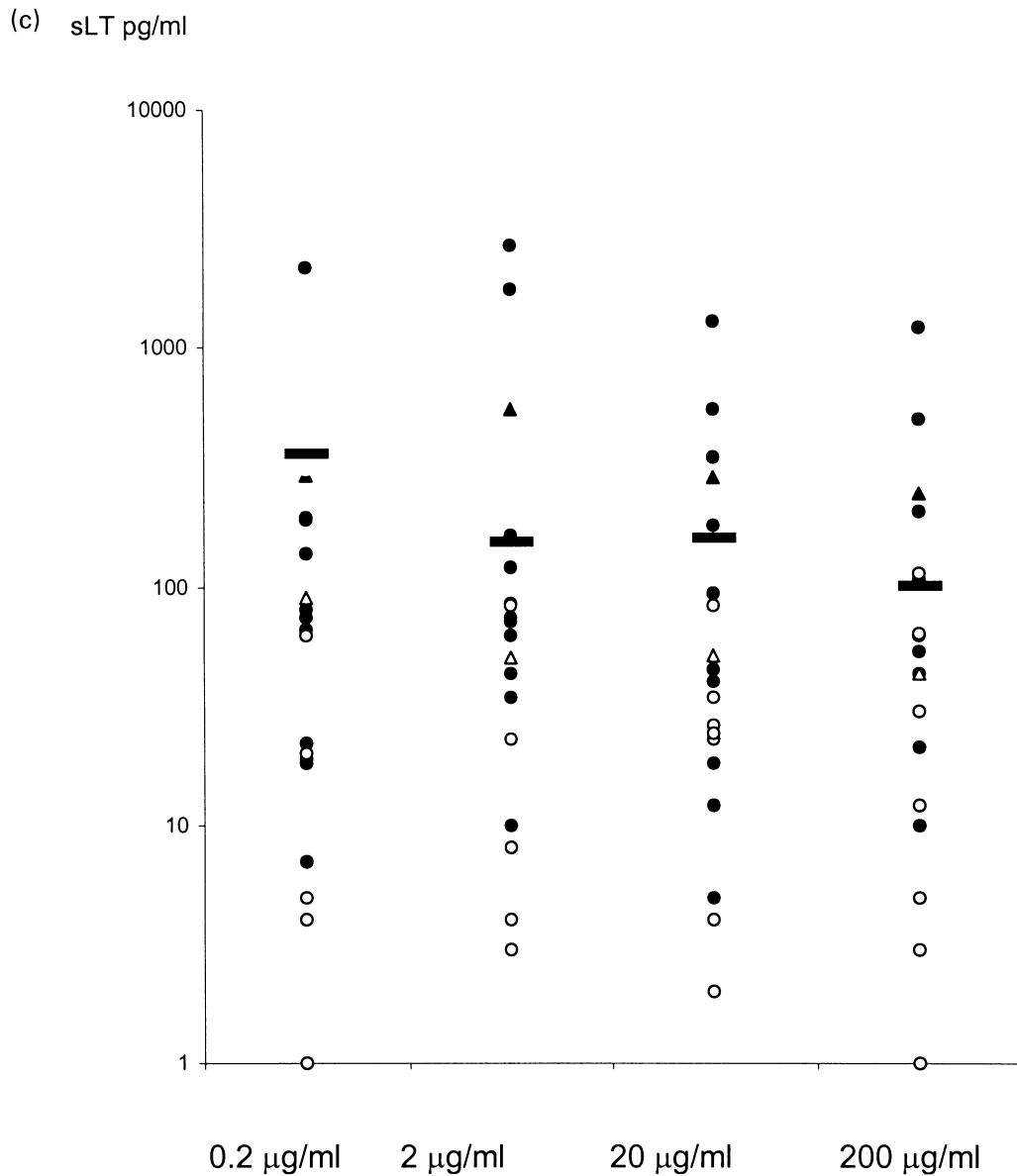


Fig. 2. *cont.* sLT production of basophils in the presence of tartrazine (0.2–200 µg/ml) from the different AD groups. Symbols were used as indicated in (a). Mean values in group A were < 5 pg/ml in the presence of tartrazine 0.2–200 µg/ml respectively (data not shown).

profit from a low pseudoallergen diet over 6 weeks and show worsening of the eczema by DBPCFC using food additives [8]. In this study we demonstrate that in these investigated AD patients with a proven food intolerance as an aggravating factor for their disease an increased sLT production in the presence of single food additives by peripheral leucocytes is determined in a majority of patients (7/9). Increased sLT production was determined most frequently in the presence of tartrazine (3/9), benzoate (4/9) and nitrite (5/9). These results indicate that food additives induce sLT production in a subgroup of AD patients and

suggest a possible role of enhanced sLT production for worsening of the eczema in these patients.

That food additives are capable of inducing histamine, tryptase and other proinflammatory mediators by mast cells *in vivo* has been shown previously [23]. However, the mechanisms by which food additives affect the immune system are only partly understood. Apparently, they are not IgE-mediated and are based on direct activation of mast cells. An indirect T cell activation is feasible, because mast cell numbers are increased in lesional skin of AD patients and due to the close vicinity of infiltrating T cells [24].

Thus, it has been shown that mast cell derived mediators and cytokines such as IL-4, IL-10 and IL-13 are capable of activating a TH2 cell response [25]. Whether abnormal T cell functions, which are known to play an important role in the pathogenesis of AD, are involved in triggering skin inflammation by food additives needs to be determined.

Not only mast cells, but also basophils of the peripheral blood release several mediators including histamine and leukotrienes after antigenic or pseudoallergenic stimulation [14]. Leukotriene metabolism has been suggested to play a role for food intolerance and in spite of the fact that the exact mechanism involved is still unknown, we can demonstrate here leukotriene release *in vitro* by food additives in a well-defined subgroup of AD patients. So far, increased sLT production in well-defined study groups has only been reported for aspirin [12,26]. However, in these aspirin intolerant patients, increased sLT production was dependent on the presence of C5a. However, our attempts to increase sLT production by the addition of C5a were not successful.

Our results also show that in normal non-atopic donors no induction of sLT production was observed by any of the food additives used. By contrast, increased sLT production against at least one food additive was observed in 7/9 patients with a proven food intolerance confirmed by DBPCFC. In a few AD patients with a negative oral provocation test (2/9), a modest increased sLT production was observed in the presence of single food additives. Taken together, the calculated sensitivity, specificity and positive predictive values of sLT production for the food additives were low and suggest that *in vitro* measurement of sulfidoleukotriene production may not be helpful for identifying AD patients, who would profit from a particular diet. Therefore an elimination diet followed by DBPCFC is still indispensable for diagnosing a food intolerance. However, as mentioned above, these calculations must be regarded on the basis that the total numbers of tested individuals are low.

Taken together our findings indicate that nitrite, benzoate and tartrazine are capable of inducing sLT release in patients with a food intolerance proven by DBPCFC and did so compared to other food additives more frequently. This suggests that these food additives are of major importance if food additives play a role as aggravating factors in AD patients. By contrast, food colour mix, metabisulfite and salicylate induced increased sLT release in only a few patients, suggesting that they play only a minor role in these patients or act through other mechanisms than induction of sLT production.

Food additives and especially benzoate and tartrazine as aggravating factors in AD patients have been reported before [27,28]. However, it needs to be considered that the overall frequency in AD patients is low. Based on different studies, including our own, it can be estimated as between 2% and 10% in children and adults [8,29], nevertheless they should be kept

in mind as possible aggravating factors of this multifactorial disease. That sLT may play an important role triggering the inflammation in the skin resulting in clinical symptoms like eczema and itching has been suggested by the observation that leukotriene receptor antagonists are effective in AD patients [30]. However, larger double-blind-placebo-controlled studies are necessary to prove these preliminary observations.

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