

Production of Leukotrienes in Human Skin and Conjunctival Mucosa after Specific Allergen Challenge

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The production of leukotrienes has been monitored in tear fluids from subjects following a conjunctival provocation test, and skin blister fluids following initiation of a Prausnitz-Kustner reaction. In tear fluids elevated levels of leukotriene C₄ (LTC₄)-immunoreactive material were measured following allergen challenge as compared to control tear fluid obtained by mechanical or reflex stimulation. Analysis by high performance liquid chromatography indicated the presence of LTC₄, LTD₄ and LTE₄. In the skin, significantly elevated levels of LTC₄-immunoreactive material were measured following allergen challenge in the Prausnitz-Kustner reaction. HPLC analysis indicated the presence of both LTC₄ and LTD₄. LTB₄ immunoreactive material was detected both in the tear fluid and the skin tissue fluid. However, no significant increase occurred in either tissue after the allergic reactions. These results indicate that the SRS-A leukotrienes are released *in vivo* in man following allergen challenge, and indicate these mediators may be important in human allergic diseases.

Key words: allergic conjunctivitis; allergic skin reaction; leukotriene B₄; leukotriene C₄; leukotriene D₄; leukotriene E₄.

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CLINICAL ASPECTS

The present results demonstrated the production of peptidolipid leukotrienes in man following allergen challenge in the skin or the conjunctiva. These results indicate that SRS-A leukotrienes may be important mediators of allergic disease in man.

The pathophysiology of human hypersensitivity disorders is thought to involve the release of chemical mediators from tissue-bound mast cells. The release may be triggered by physical stimuli or by the interaction of allergen with specific IgE on the surface of the mast cell. Slow-reacting substance of anaphylaxis (SRS-A) has long been thought to be one of the more important of these mediators released after specific allergen challenge (11). SRS-A is now known to be a group of mediators identified as leukotrienes (LT) C₄, D₄, and E₄ (16). After the identification (13) and subsequent synthesis (15) of LTC₄, LTD₄, and LTE₄, their biologic

effects in humans were found to mimic many of the pathophysiological events seen in specific allergic reactions. Thus, they were potent bronchoconstrictors in non-atopics (18), and, more so in asthmatics (3), they were potent vasodilators in the human skin (4) and mucosa (5), and they induced edema in the skin (6). LTB₄ is another product of this pathway of arachidonic acid metabolism, and this mediator is a potent chemotactic and chemokinetic agent for polymorphonuclear leukocytes (9, 17).

The identification of leukotrienes in body fluids during a specific allergic reaction has relied on the development of high-performance

liquid chromatographic (HPLC) and radioimmunoassay (RIA) techniques. Preliminary studies, in which the levels of LTC₄-immunoreactivity were determined in venous as well as arterial plasma from either human asthmatic patients or rat plasma following antigen challenge in the lung, failed to show increases in the level of LTC₄-immunoreactive material (unpublished observations). These studies suggested that if LTC₄ is released during antigen provocation *in vivo*, then mechanisms exist for the rapid removal and/or metabolism of leukotrienes. It was the purpose of the present study to identify the leukotrienes in fluids other than plasma, where specific challenge of mucosal mast cells could be achieved.

In vitro experiments have pointed to the existence of different lines of mast cells in mucosa and connective tissue with different abilities for leukotriene production (1). A secondary purpose of the present study was to identify leukotrienes in the skin after specific allergic challenge.

SUBJECTS, MATERIAL AND METHODS

All subjects volunteered fully informed, and the study was approved by the local ethical committee. No anti-allergic medication was taken by any of the test persons within 1 month prior to the test.

Conjunctival provocation test

The study group comprised 23 allergics (9 females, 14 males, aged 18–32) with conjunctivitis due to allergy to grass. The diagnosis was established through a positive case history, a positive skin prick test, at least 2+ according to Aas et al. (19), and a positive conjunctival provocation test, as defined below. All subjects were free of symptoms during the test period. Where any subject was included in the study twice, 1 week was interposed.

Prausnitz-Kustner reaction

The study group comprised 17 volunteers (9 female, 8 male, aged 18–52) without any history of allergic reaction towards peanuts.

Grass allergen: The lyophilised alpha-fraction of *Pleum pratense* and *Dactylis glomerata* (Dome-Hollister-Stier).

Peanut allergen: fresh peanuts. Serum containing IgE antibodies against peanuts was obtained from a young boy allergic to peanuts, verified by a skin prick test and a blinded provocation test. Occurrence of HbAg was excluded and the serum frozen.

Conjunctival Provocation Test (CPT)

Grass allergen was diluted on the day of the test, and kept at +4°C. Saline with 0.3 mg/ml (w/v) of human serum albumin and 4 mg/ml (w/v) phenol was used as diluent. The diluent factor was 10^{0.5}, from 300 to 1 AUR (Activity Units by RAST).

The test was performed by administering one drop of the diluent in the conjunctival sac. If no reaction occurred within 10 min, one drop of allergen dilution at the lower concentration was applied. If the subject failed to react to the challenge, increasing doses were given in the same eye at 10-min intervals until a positive reaction was found. If a patient gave a positive reaction to the first allergen challenge (1 AUR) he was to be excluded. The criterium for positive CPT was congestion of the conjunctival mucosa and itching of the eye within 10 min. The congestion was evaluated by an experienced investigator who specifically asked if itching occurred after each challenge. Tear fluid was collected in siliconized capillary tubes over a period of 5 min, and frozen immediately in liquid nitrogen.

Prausnitz-Kustner reaction

Peanut antiserum (50 µl) from the patient allergic to peanuts was given intracutaneously on the forearm of the test subject. After 24 h a suction blister was raised at the site of injection using a negative pressure of 200 mmHg. After 3–4 h a blister had developed covering a circular skin window with a diameter of 16 mm. The blister was reinforced by Nobecutan® and left undisturbed for 6 h for the suction trauma to wear off. At that time a 27-gauge needle was

introduced through the base of the blister, making it possible to empty the blister without damaging it. The blister fluid was used as control. Subsequently, the subjects ate approximately 20 g of peanuts. Thirty minutes after the intake of peanuts the blister was emptied again. All tissue fluid samples were immediately frozen in liquid nitrogen.

Radioimmunoassay of leukotrienes

The levels of LTC₄- and LTB₄-immunoreactive material were determined by double antibody radioimmunoassay (RIA) as previously described (2, 10). Cross-reactivity data with related lipids for the antisera used have been previously published (2, 10).

Extraction of leukotrienes and analysis of HPLC

Pooled tear or blister fluids were applied to C₁₈ SepPak cartridges (Waters) previously treated with 20 ml MeOH followed by 20 ml H₂O. The cartridges were then washed with 10 ml H₂O and the leukotrienes eluted with 10 ml MeOH. 200 µl of 5 % (w/v) NaHCO₃ was added to the methanol wash and the sample taken to dryness under vacuo. The residue was taken up in 200 µl of H₂O followed by a wash of 50 µl of H₂O for application (150 µl) to a Bondapak C₁₈ column (Waters) and eluted with CH₃CH₂H₂O:acetic acid (35:65:0.1) pH 5.8 at a flow rate of 2 ml/min. Retention times of standard leukotrienes were determined before and after application of the sample. Fractions were collected before and after application of the sample at 1- or 2-min intervals (2 ml) into tubes containing 200 µl of 5 % (w/v) NaHCO₃ and the contents lyophilised. Water (250 µl) was added to each tube and 100 µl aliquots removed for RIA.

Statistics

Group comparisons were performed by the Kruskal-Wallis test. Paired tests were done by Wilcoxon or Pratt's test.

The central tendency of the figures was expressed by the median values and the variation by the quartiles.

Procedure

Conjunctival provocation test. In one set of experiments, 16 patients with allergic conjunctivitis were challenged in the eye and tear fluid collected as described for detection of LTC₄-immunoreactive material.

In a second study tear fluid was obtained from 16 patients from the study group after application of the diluent. Tears were obtained either through the mechanical stimulation caused by the capillary tube (9 patients), or non-traumatically through NH₃ sniffing (7 patients), which induced lacrimation through a trigeminal reflex. In the latter instance the tear fluid could be collected without touching the conjunctival mucosa. The samples were tested for the presence of LTC₄-immunoreactive material.

In a third experiment, tear fluids from seven patients in the study group, allergen-challenged in both eyes, were pooled for verification of the presence of sulphido-leukotrienes by HPLC and RIA.

In a fourth series of experiments on 12 persons from the study group, tear fluid was collected from one eye after application of the diluent, and, subsequently, from the other eye after an allergic challenge. These samples were tested for the presence of LTB₄-immunoreactive material.

Prausnitz-Kustner reaction. Skin tissue fluid before and after allergen challenge was obtained from 14 non-atopic individuals. The LTC₄-immunoreactive material were determined in eight, and the level of LTB₄-immunoreactive material in 12 of the tests. In another set of experiments, three control and three allergen challenge fluids were pooled separately for verification of the presence of leukotrienes by HPLC and RIA.

RESULTS

Specificity of radioimmunoassay

The specificity of the anti-sera used in the present experiments has been described previously (2, 10). Of particular importance is the fact that

the antibody directed against LTC₄ cross-reacts significantly with LTD₄ and LTE₄. The reactivity against LTC₄, LTD₄ and LTE₄ respectively was 0.25, 0.3 and 5.0 expressed in pmol as the amount of ligand required for 50 % inhibition of the binding of (³H)-LTC₄, (IC₅₀).

Conjunctival provocation test

All patients gave a clinically positive reaction to one of the allergen concentrations applied. None reacted to the first concentration applied, nor to the diluent.

LTC₄-immunoreactive material was detected in 12 of 16 tear fluids from subjects following allergen challenge. In contrast, low levels of LTC₄-immunoreactive material were detected in only two of nine tear fluids induced by mechanical stimulation and two of seven tear fluids induced through the trigeminus reflex following inhalation of NH₃ (Table 1). The levels in allergen-challenged tear fluid were significantly greater than those obtained following mechanical stimulation or exposure to NH₃ (*P* < 0.05, Kruskal-Wallis test). A comparison

Table 1

Levels of LTC₄-immunoreactive material (ng/ml) in tear fluid samples obtained following lacrimation induced by either allergen challenge, mechanical stimulation or inhalation of NH₃

Allergen challenge	Control stimulation	
	NH ₃	Mechanical
1.5	0	0.1
0	0	0
0	0	0
1.5	0	0.5
0.7	0	0
0	0.2	0
1.2	0.4	0
2.0		0
0.3		0
0.5		
0.5		
0		
0.3		
0.3		
0.1		
0.2		
Median: 0.34	0.07	0.03
Kruskal-Wallis test: * <i>P</i> < 0.05.		

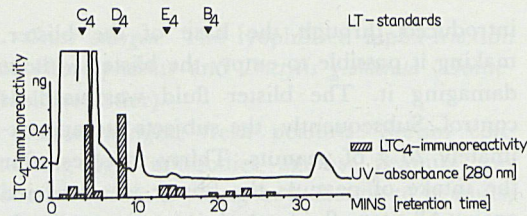


Fig. 1. Pooled tear fluid from seven patients were collected after allergen challenge in the eye, extracted and separated by reverse phase HPLC. Fractions were sampled every minute and analysed by RIA for the presence of LTC₄-immunoreactive material (expressed as ng/ml). The retention time for authentic leukotrienes is indicated.

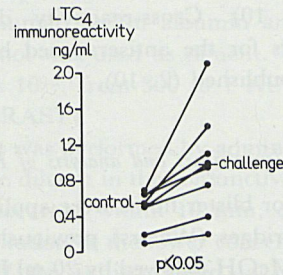


Fig. 2. The levels of LTC₄-immunoreactive material in the skin tissue fluids of eight patients before (control) and after (challenge) the initiation of a Prausnitz-Kustner reaction.

between the threshold allergen concentration and the concentration of LTC₄-immunoreactive material in the tear fluid showed no correlation.

LTB₄-immunoreactive material was found in detectable amounts (median: 0.55 ng/ml, range: 0.0–3.6 ng/ml) before challenge, yet no consistent changes were seen after allergen challenge.

To verify the presence of sulphidopeptide leukotrienes in the tear fluid obtained during the specific allergic reaction, a sample of pooled tear fluids was extracted and separated by HPLC. All HPLC fractions were subsequently analysed by RIA for the presence of LTC₄-immunoreactive material. Within the LTC₄-immunoreactive material recovered from the column, 35, 37, and 13 % was associated with the known retention times of synthetic LTC₄, LTD₄ and LTE₄, respectively (Fig. 1). The recovery of LTC₄-immunoreactive material following extraction and HPLC was estimated as 87 %.

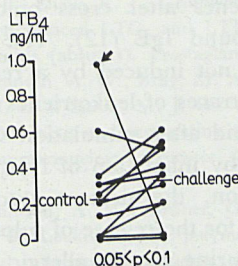


Fig. 3. The levels of LTB_4 -immunoreactive material in the skin tissue fluids of 12 patients before (control) and after (challenge) the initiation of a Prausnitz-Kustner reaction. The arrow indicates a possible outlier.

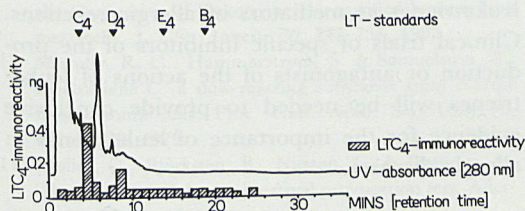


Fig. 4. Pooled skin tissue fluids from three patients after initiation of a Prausnitz-Kustner reaction were extracted and separated by reverse phase HPLC. Fractions were sampled every minute and analysed by RIA for the presence of LTC_4 -immunoreactive material (expressed as ng/ml). The retention time for authentic leukotrienes is indicated.

Prausnitz-Kustner reaction

In all of the eight tests, the levels of LTC_4 -immunoreactive material were greater after allergen challenge than before ($P < 0.01$, Wilcoxon test) (Fig. 2).

The levels of LTB_4 -immunoreactive material were determined in 13 of the paired test samples. The levels were increased in nine cases, unchanged in two, and decreased in another two (Fig. 3). The difference was not significant: $0.05 < P < 0.1$ (Pratt's test), although one of the paired observations might be considered an outlier, indicated by an arrow on the figure. In this test the control showed a high level of LTB_4 -immunoreactive material, whereas the concentration after challenge was 0. If this result is left out of the calculations a significant increase in the level of LTB_4 immunoreactive material was found after allergen challenge ($P < 0.01$, Pratt's test).

Three pre- and post-challenge samples were pooled, extracted, and analysed by RIA after separation by HPLC. In the post-challenge sample two prominent peaks of LTC_4 -immunoreactive material were recovered from the HPLC, with retention times corresponding to the known retention times of LTC_4 and LTD_4 (Fig. 4). Due to the small amounts of material it was not possible to determine recoveries. In the pre-challenge sample one very small peak of LTC_4 -immunoreactive material was recovered with retention time corresponding to that of LTC_4 (results not shown).

DISCUSSION

The identification of leukotrienes in specific allergic reactions in man has been hampered because these mediators are not preformed, are only produced in small amounts, and are rapidly eliminated from or degraded within the circulatory system. The conjunctival provocation test is a useful model in man for controlled allergic reactions and has good reproducibility (14). Previous studies using this model have provided the first evidence for the release of peptidolipid leukotrienes following allergen challenge in man (2). The obvious disadvantage is that the CPT is not feasible for individual dose-response studies, as the procedure of tear collection is likely to interfere with subsequent challenges in the same eye.

The P.-K. reaction is a useful model for allergic reactions in human skin, allowing for strong but limited allergic reactions without the possibility of anaphylactic shock. As for the CPT the P.-K. model has the disadvantage of not allowing for dose-response studies. Other recent studies have demonstrated the release of LTC_4 in nasal secretions in patients with allergic rhinitis (7, 8).

The present results using RIA demonstrate release of LTC_4 -immunoreactive material in the Prausnitz-Kustner reaction in the skin and confirm the previous results obtained following allergen challenge in the eye (2). The antisera used in the present study for detection of LTC_4 -immunoreactive material cross-reacted significantly with other sulphidopeptide leukotrienes

and could, in theory, cross-react with substances unrelated to leukotrienes. To verify the presence of the leukotrienes, pooled test samples of tear fluid were extracted, subjected to HPLC, and all HPLC fractions were analysed by RIA. Within the LTC₄-immunoreactive material from tear fluid recovered from the column, 35, 37 and 13 % was associated with the known retention times of LTC₄, LTD and LTE. As the LTC antisera had an IC₅₀ almost similar to LTD₄, the results indicated that LTC₄ and LTD₄ occurred in approximately similar amounts. In contrast, the radioimmunoassay was 20-fold less sensitive for LTE₄, indicating that this was probably the major sulphidopeptide leukotriene present in the samples from tear fluids. This suggested that metabolism of LTC₄ had occurred and that the RIA may have given a considerable underestimate of the actual amounts of leukotrienes released in these reactions. HPLC analysis of allergen-challenged fluids from the Prausnitz-Kustner reaction indicated the presence of both LTC₄ and LTD₄, suggesting that metabolism of LTC₄ also occurred in the skin. Lower levels of LT-immunoreactive material were detected in control blister fluids, and HPLC analysis indicated that LTC₄, albeit in much smaller amounts, was present. These observations suggest that the ongoing aseptic inflammation induced by the suction trauma is sufficient in itself to induce a limited production of leukotrienes.

LTB₄-immunoreactive material was detected in nine of 13 control blister fluids, suggesting that aseptic inflammation could induce the production of LTB₄ as well. After allergen challenge a trend was found which could indicate an increased production of LTB₄. In the tear fluid no consistent change was found in the levels of LTB₄-immunoreactive material after allergen challenge. These preliminary results, which were not confirmed by HPLC analysis, do not indicate a role for LTB₄ in immediate allergic reactions.

The study does not provide any direct indications of the cellular source of the leukotriene production. Tissue bound mast cells are a likely source as human mast cells are known to pro-

duce leukotrienes after cross binding of the membrane bound IgE (12). The leukotriene production is not induced by a reflex mechanism as only traces of leukotrienes were found in the tear fluid after stimulation via the trigeminal nerve by inhalation of NH₃.

In conclusion, the present study provides firm evidence for the release of sulphidopeptide leukotrienes during specific allergic reactions in both the human conjunctival mucosa and in human skin *in vivo*. The identification of leukotrienes in such body fluids during allergic reactions provides further evidence for the role of leukotrienes as mediators of allergic reactions. Clinical trials of specific inhibitors of the production or antagonists of the actions of leukotrienes will be needed to provide conclusive evidence for the importance of leukotrienes in allergic diseases.

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