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### Research Article

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# Accumulation of Leukotriene C4 and Histamine in Human Allergic Skin Reactions

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## Abstract

To determine whether lipoxygenase products of arachidonic acid metabolism are released *in vivo* during human allergic cutaneous reactions, we serially assayed chamber fluid placed over denuded skin sites for the presence of both C-6 peptide leukotrienes (e.g., LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), using radioimmune assay and HPLC separation, and compared it to histamine (assayed radioenzymatically) in 13 atopic and two nonatopic volunteers. Skin chamber sites challenged with ragweed or grass pollen antigen (250–750 protein nitrogen units/ml) for the first hour and phosphate-buffered saline (PBS) for the next 3 h were assayed hourly and compared to sites challenged with PBS alone. As assessed by HPLC, LTC<sub>4</sub> composed >85% of the C-6 peptide leukotriene released at any skin site, whereas little LTD<sub>4</sub> or LTE<sub>4</sub> was detected. LTC<sub>4</sub> was present in significantly greater concentrations at antigen sites as compared to PBS-challenged sites throughout the 4-h period. Minimal concentrations of LTB<sub>4</sub> were found throughout this time period and were not different at antigen or PBS sites. Histamine was present in significantly greater concentrations at antigen rather than PBS sites, but the pattern of release was different from that of LTC<sub>4</sub>. Peak histamine release invariably occurred during the first hour and decreased progressively thereafter, whereas the greatest amounts of LTC<sub>4</sub> were detected during the 2nd to 4th hours. The amount of LTC<sub>4</sub> accumulating at the site was dependent upon the dosage of antigen used in the epicutaneous challenge. We have demonstrated in this study that of the leukotrienes assessed LTC<sub>4</sub> is released in the greatest quantity *in situ* during *in vivo* allergic cutaneous reactions and that it is present at such sites for at least 4 h after antigen challenge. Since intradermal injection of LTC<sub>4</sub> in humans induces wheal and flare responses that persist for hours, our findings support the hypothesis that LTC<sub>4</sub> is an important mediator of human allergic skin reactions.

## Introduction

The C<sub>6</sub>-peptide leukotrienes LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, formerly defined by their biologic activity as slow reacting substance of anaphylaxis (SRS-A), are lipoxygenase metabolites of arachidonic acid that are thought to play an important role in

human mast cell-mediated reactions. They are potent mediators of bronchoconstriction, vascular and nonvascular smooth muscle constriction, increased vascular permeability, and epithelial mucus secretion (1). Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) may also be an important allergic mediator, as it stimulates neutrophil and eosinophil chemotactic activity, as well as their aggregation, secretion of lysosomal constituents, and adherence to vascular endothelium (1). *In vitro* studies have demonstrated that many cells, including mast cells, neutrophils, eosinophils, basophils, and alveolar macrophages, are capable of generating leukotrienes (LTs)<sup>1</sup> (2). Mast cells and Langerhans cells are among the resident cell population in the skin. Our laboratory has found a prominent accumulation of neutrophils, eosinophils, and mononuclear cells during the course of cutaneous allergic reactions (3). Therefore, the skin provides an ideal site to investigate the profile and temporal pattern of LTs released during ongoing allergic reactions. We have studied the release of the C<sub>6</sub> peptide LTs and LTB<sub>4</sub> in the skin of atopic subjects during challenge with ragweed or grass pollen extract. We found that LTC<sub>4</sub> is the LT present in largest amounts during such reactions; here, we compare its release to that of histamine.

## Methods

**Patients.** 13 atopic and two nonatopic healthy volunteers gave informed consent before study. Atopic subjects fulfilled the following criteria: (a) a history of allergic rhinitis during the fall and/or spring seasons; (b) a positive skin test, defined as a wheal of at least 10-mm diameter in response to an intradermal injection of 0.02 ml of 10 protein nitrogen units (PNU) per milliliter grass or ragweed pollen extract (Greer Laboratories, Lenoir, NC); and (c) no history of previous immunotherapy. Nonatopic subjects had no history of allergic rhinitis and had negative skin tests in response to intradermal injections of 0.02 ml of 1,000 PNU/ml grass and ragweed pollen extracts. No subject had urticaria or atopic dermatitis and no medicines were ingested for at least 72 h before study. The degree of antigen sensitivity of individual subjects was assessed by the extinction dilution skin test titer (EDST). EDST was defined as the greatest 10-fold dilution of relevant pollen extract that would elicit a positive skin test (4). Seven subjects were sensitive to an EDST of 10 PNU/ml, three to an EDST of 1 PNU/ml, and three to an EDST of 0.1 PNU/ml.

**Epicutaneous antigen challenge.** 20 epicutaneous antigen challenges were performed on 13 atopic and two nonatopic subjects according to methods described previously (4). Using mild heat and suction, two 1-cm diameter blisters were raised on each forearm. The blisters were aseptically unroofed and a sterile plastic collection chamber was taped in place over each blister base. To wash away any mediators released by induction of the blister itself, each chamber was filled with sterile isotonic phosphate-buffered saline (PBS); after 5 min, the PBS was removed and discarded. Incubations were then performed in the

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1. *Abbreviations used in this paper:* EDST, extinction dilution skin test titer; HPLC, high-performance liquid chromatography; LT, leukotriene; and PNU, protein nitrogen units.

appended chambers according to the following protocols. Protocol I: simultaneously, two blister bases were incubated for 1 h with 0.3 ml of 500 PNU/ml grass or ragweed pollen extract and two control sites were incubated with PBS. The chambers were removed at the end of the 1-h incubation period; the blister bases were aspirated dry; fresh chambers were taped in place; and all chambers were filled with 0.3 ml PBS. The contents were removed after 1 h and the procedure was repeated in this way, a total of four serial 1-h incubations was performed at each site. Protocol II: chambers were removed and replaced at hourly intervals as described above. During the first hour, one site each was incubated with 0.3 ml of 250 PNU/ml pollen extract, 500 PNU/ml pollen extract, 750 PNU/ml pollen extract, or PBS. For the subsequent three incubation periods, PBS was placed in all four chambers. In protocol I, paired antigen-challenged sites and paired PBS-challenged sites, respectively, were pooled for subsequent mediator assay. In both protocols, fluids were aliquoted and frozen at  $-20^{\circ}\text{C}$  for histamine assay and at  $-70^{\circ}\text{C}$  after being gassed with nitrogen for LT assays.

A recovery experiment was performed to determine whether loss of chamber fluid LTB<sub>4</sub>, LTC<sub>4</sub>, or LTD<sub>4</sub> was excessive in our model either by means of nonspecific sticking to the chamber or blister base, by degradation in the chamber fluids during incubation, or during storage before assay. In two of the atopic subjects, the washed blister bases were incubated for 1 h with synthetic LTC<sub>4</sub>, LTD<sub>4</sub>, LTB<sub>4</sub>, and PBS. Specific radioimmunoassays were performed on fluids before and after incubation; percent recovery was defined as recovered immunoreactive LT concentration/immunoreactive LT concentration before incubation. In these two subjects, 35% (5.5:16 pmol/ml) and 24% (3.3:14 pmol/ml) of incubated LTC<sub>4</sub> was recovered; 56% (36:64 pmol/ml) and 71% (51:72 pmol/ml) of incubated LTD<sub>4</sub> was recovered; 62% (34:56 pmol/ml) and 58% (32:55 pmol/ml) of incubated LTB<sub>4</sub> was recovered. None of the LTs was present at the PBS site.

**Mediator assays.** Histamine: histamine concentrations were measured in triplicate by a radioenzymatic technique as previously described (5). This assay reproducibly detects histamine levels of at least 2 ng/ml in skin chamber fluids. Leukotrienes: [14,15-<sup>3</sup>H(N)]LTC<sub>4</sub> (34.0 Ci/mmol) and [14,15-<sup>3</sup>H(N)]LTD<sub>4</sub> (36.0 Ci/mmol) (New England Nuclear, Boston, MA), [5,6,8,9,11,12,14,15-<sup>3</sup>H(N)]LTB<sub>4</sub> (180–221 Ci/mmol) (Amersham Corp., Arlington Heights, IL), and Sep-Pak columns (Waters Associates, Milford, MA) were obtained. Synthetic LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> were provided generously by Dr. J. Rokach of Merck Frosst Laboratories, Dorval, Canada. Chamber fluids were acidified to pH 3.5 with 0.1 M acetic acid and applied to prewashed Sep-Pak columns; the columns were developed with 2 ml distilled water, 2 ml of 0.001 M acetic acid, and 2 ml of methanol and 0.001 M acetic acid (1:4, vol/vol), and the LTs were eluted in 2 ml of methanol. In selected samples, one-half of each of the eluates was dried *in vacuo* and redissolved for reverse-phase high-performance liquid chromatography (HPLC) on a 4.6 mm × 25 cm column of 10 μm octadecylsilane (Ultrasil, Altex Division of SmithKline Beckman, Inc., Berkeley, CA) that was developed isocratically with methanol: 0.03 g phosphoric acid per 100 ml of distilled water (pH 3.5 with ammonium hydroxide) (70:30, vol/vol) at a flow rate of 1 ml/min in a dual metered pump system (Beckman Instruments, Inc., San Jose, CA) (6). The elution times of synthetic LTC<sub>4</sub>, LTB<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, as assessed optically, were 14.1–15.6, 19.3–20.7, and 22.4–23.8 and 26.2–27.5 min, respectively (range,  $n = 8$ ).

Radioimmunoassays for LTs were performed as described with rabbit antisera of high specificity for LTC<sub>4</sub> and LTB<sub>4</sub> (7, 8) and a rabbit antiserum that recognizes all of the C-6 peptide LTs (Drs. E. Hayes and A. Rosenthal, Merck Laboratories, Inc., Rahway, NJ) (6), using [<sup>3</sup>H]LTC<sub>4</sub> and synthetic LTC<sub>4</sub>, H<sup>3</sup> of LTB<sub>4</sub> and synthetic LTB<sub>4</sub> and H<sup>3</sup> of LTD<sub>4</sub> and synthetic LTC<sub>4</sub>, LTD<sub>4</sub>, or LTE<sub>4</sub> as the respective pairs of ligands. Each value was corrected for the recovery of radiolabeled compound. The lowest limit of reliable quantification and the amount required for 50% inhibition of the radioligand, respectively, were 0.08 pmol and 0.35 pmol for the LTC<sub>4</sub> assay (7) and 0.03 pmol and 0.15 pmol for the LTB<sub>4</sub> assay (8). The same values for the C-6 peptide LT

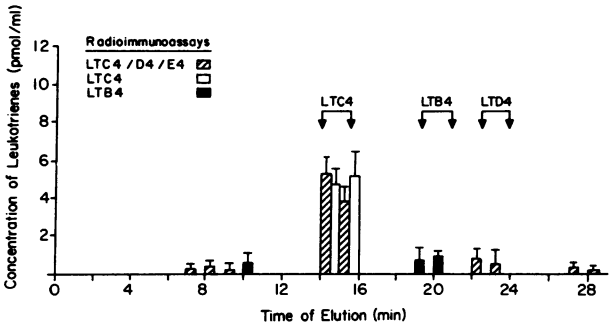
assay were dependent on the unlabeled LT employed to displace [<sup>3</sup>H]LTD<sub>4</sub> and were 0.1 and 0.7 pmol for LTC<sub>4</sub>, 0.2 and 1.3 pmol for LTD<sub>4</sub>, and 0.5 and 6.8 pmol for LTE<sub>4</sub>. LTC<sub>4</sub> was used as the competing ligand to develop the standard curve from which values were calculated for analyses of samples before HPLC. Three separate standard curves appropriate for the different regions of the eluate from HPLC were developed using LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, respectively, to displace [<sup>3</sup>H]LTD<sub>4</sub> (Fig. 1). In all cases in which sufficient fluid was available (31 of 100 fluids from antigen challenged sites and 25 of 52 fluids from PBS-challenged sites), chamber fluids were assayed for C-6 peptide LT, LTC<sub>4</sub>, and LTB<sub>4</sub> both before and after HPLC coelution with synthetic standard LTB<sub>4</sub>, LTC<sub>4</sub>, and LTD<sub>4</sub>. A mean of 95% of the [<sup>3</sup>H]LTC<sub>4</sub> was recovered after HPLC with a correlation coefficient of 0.91 ( $P < 0.001$ ) between LTC<sub>4</sub> values before and after HPLC.

LT and histamine accumulations were expressed as concentrations (pmol/ml and ng/ml, respectively). The absolute amount of accumulated mediator over the 4-h period was determined by multiplying the sum of the concentrations in the hourly collections by the volume of fluid in the chambers, 0.3 ml.

## Results

Seven atopic and two nonatopic subjects were studied with Protocol I to determine whether radioimmunoactive C-6 peptide LTs or LTB<sub>4</sub> were released in response to cutaneous antigen challenge. Assays for immunoreactive C-6 peptide LTs were performed on all chamber fluids, while assays for immunoreactive LTB<sub>4</sub> were performed only on fluids obtained from atopic subjects. More total (Table I) and hourly (data not shown) immunoreactive C-6 peptide LTs were recovered from chamber fluids at antigen than at PBS-challenged sites ( $12.24 \pm 5.93$  pmol vs.  $2.73 \pm 0.36$  pmol, respectively). Analysis of histamine in the chamber fluid (Table I) revealed significantly greater release at antigen compared to PBS sites. Chamber fluids from subject 6, which did not contain elevated concentrations of histamine at antigen-challenged sites, also did not contain elevated concentrations of immunoreactive C-6 peptide LTs.

Chamber fluid obtained from nonatopic subjects contained equivalent amounts of immunoreactive C-6 peptide LT at



**Figure 1.** Reverse-phase HPLC resolution of LTs in cutaneous chamber fluid. 1-ml aliquots of the eluate from HPLC purification of three replicate extracts of cutaneous fluid from an antigen challenge site of one allergic subject were dried *in vacuo*, reconstituted in 0.2 ml of 0.05 M Tris-HCl (pH 8.0), and analyzed by radioimmunoassays. The values for LTC<sub>4</sub> and LTD<sub>4</sub> determined by the LTC<sub>4</sub>/D4/E4 assay were derived from two different standard curves of displacement of [<sup>3</sup>H]LTD<sub>4</sub> by LTC<sub>4</sub> and LTD<sub>4</sub>, respectively. Each bar depicts the mean  $\pm$  SD of the results of duplicate assays of every 1-min pool of the three HPLC eluates. The arrows and brackets above represent the range of elution times of the synthetic LTs.

Table I. C-6 Peptide LTs and Histamine at Antigen and PBS-Challenged Skin Sites

C-6 Peptide LTs*			Histamine†	
Atopic subjects	Antigen sites	PBS sites	Antigen sites	PBS sites
1	2.94	1.27	35	3
2	47.31	2.07	81	2
3	6.75	3.03	9	3
4	10.89	2.94	26	7
5	8.37	3.63	26	7
6	3.99	2.94	5	5
7	5.46	3.24	24	6
Mean±SEM	12.24±5.93§	2.73±0.36	29.5±9.5	4.5±0.8

\* Total picomoles of chamber fluid C-6 peptide LT collected over 4 h of incubation (summed concentration in pmol/ml × 0.3 ml).

† Total nanograms of chamber fluid histamine collected over 4 h of incubation (summed concentration in ng/ml × 0.3 ml).

§  $P < 0.02$  compared to PBS sites (Wilcoxon's test for paired differences).

||  $P < 0.025$  compared to PBS sites (Student's  $t$  test for paired values).

antigen (mean = 0.45 pmol) and PBS (mean = 0.42 pmol) challenged sites. The low concentrations of immunoreactive LTB4 found (range < 0.05–1.60 pmol/ml), in fluids of antigen-challenged sites was not significantly different from that in PBS-challenged sites.

Having ascertained that epicutaneous antigen challenge caused the release of immunoreactive C-6 peptide LTs, we next performed HPLC on selected fluids from antigen-challenged sites followed by specific radioimmunoassays (RIA) for LTB4, LTC4, and a combination, LTC4-D4-E4. As shown in one representative analysis of the eluate from HPLC (Fig. 1), most of the immunoreactive C-6 peptide LT consisted of LTC4 with little LTD4; the quantity of LTB4 also was small. The values for the LTC4-D4-E4 immunoreactivities eluting at 7–9 min and 27–28 min were calculated using the LTD4 standard curve, but probably represent sulfur oxidation products of the C-6 peptide LTs and LTE4, respectively, based on the times of elution relative to synthetic standards. Using an LTE4 standard curve, the quantity of presumptive LTE4 detected in HPLC eluates of these samples and of singlicate samples from four other patients were  $0.65 \pm 0.17$  pmol/ml (mean±SD) relative to  $7.7 \pm 2.2$  pmol/ml of LTC4. RIA of eluted LTC4 and LTD4 peaks demonstrated that a mean of 93% (range = 67–100%) of the C-6 peptide LTs consisted of LTC4 and 7% (range = 0–33%) consisted of LTD4 (Table II). In all cases involving protocols I and II in which sufficient fluid was available (31 of 100 fluids from antigen-challenged sites and 25 of 52 fluids from PBS challenged sites) assays were performed for C-6 peptide LT, LTC4, and LTB4 both before and after HPLC coelution with synthetic standard LTB4, LTC4, and LTD4. In this larger group of samples >85% of the immunoreactive C-6 peptide LT was LTC4 and 10% was LTD4, minimal amounts of LTB4 were detected.

Having determined that LTC4 is the predominant C-6 peptide LT present in chamber fluids following epicutaneous antigen challenge, we examined the time-course of LTC4 release during the 4-h study period. In five of the seven subjects in protocol I, for whom the quantity of fluid was sufficient, and in all six subjects in protocol II, the levels of specific

LTC4 were assayed by a monospecific RIA in chamber fluids collected hourly at sites incubated with antigen or PBS during the first hour followed by PBS incubation at each site for the subsequent 3 h. The LTC4 levels (Table III) were significantly higher in fluids collected each hour at sites challenged with antigen than in those challenged with PBS. Although the temporal patterns of release varied among subjects, release was generally sustained throughout the 4-h collection period, with higher levels tending to occur during the second to fourth hours of incubation. At PBS challenged sites, a similar temporal pattern of LTC4 release was noted for the group as a whole, with the values at 2 h being significantly higher than at 1 h.

Chamber fluid histamine was present in significantly greater concentrations at antigen than PBS-challenged sites (Table IV). For all subjects, the highest concentration was present in the first hour with decreasing concentrations thereafter. This temporal pattern was quite different than the pattern of sustained release over 4 h of LTC4 at the same sites.

Protocol II was designed to determine whether LTC4 release is antigen dose-dependent. Six atopic subjects (numbers 8–13) were studied. As shown in Fig. 2, greater total and hourly concentrations of LTC4 were measured at antigen-challenged rather than PBS-challenged sites. The greatest LTC4 accumulations were seen at sites challenged with 500 PNU/ml antigen solutions (mean =  $3.0 \pm 0.30$ ), with significantly less total LTC4 at sites challenged with 250 PNU/ml of antigen (mean =  $1.4 \pm 0.55$ ,  $P \leq 0.05$ ). The dose-response relationship was most apparent during hours 2–4 of collection of chamber fluids, whereas concentrations of LTC4 were similar during the first hour at all antigen-challenged sites. Unlike LTC4, histamine was not released into chamber fluids in a dose-dependent response manner within this range of antigen concentrations (Fig. 3).

At sites challenged with 500 PNU/ml antigen where accumulated LTC4 was at peak concentrations and histamine was at a plateau level, there was no correlation between hourly or total fluid histamine and LTC4 concentrations in individual chamber fluids. In addition, no correlations were found between hourly or total chamber LTC4 or histamine accumulation and subject sensitivity as measured by EDST.

## Discussion

The findings described have indicated that significantly greater amounts of C-6 peptide LT are released into skin chambers

Table II. Concentration of LTC4 and LTD4 Determined by Radioimmunoassay after HPLC Separation

Subject	Hour	LTC4*	LTD4*
3	1	6.3 (100)	<0.25 (0)
4	2	7.6 (100)	<0.25 (0)
4	3	6.7 (74)	2.4 (26)
4	4	3.5 (100)	<0.25 (0)
5	4	6.3 (100)	<0.25 (0)
6	4	5.1 (100)	<0.25 (0)
7	2	5.2 (100)	<0.25 (0)
7	3	2.9 (67)	1.4 (33)
Mean		(93%)	(7%)

\* pmol/ml (%).

Table III. LTC<sub>4</sub>\* Release at Antigen (500 PNU/ml) and PBS-challenged Sites

Antigen sites		PBS sites									
		Hours				Hours					
Subject		1	2	3	4	1	2	3	4	Total‡	Total‡
3	6.2*	2.3	2.3	3.1	2.0	1.2*	2.7	0.6	1.3	4.08	1.74
4	4.6	11.0	8.1	8.1	4.0	1.9	1.7	0.9	1.2	8.31	1.72
5	3.7	4.3	3.9	3.9	6.0	1.0	3.0	1.2	3.0	5.37	2.46
6	4.1	5.0	2.7	2.7	2.7	1.2	3.3	2.3	2.7	4.35	2.85
7	3.5	6.2	2.4	2.4	3.0	1.6	2.5	1.5	3.7	4.53	2.79
8	0.8	3.9	6.0	6.0	3.7	0.7	0.9	0.2	0.2	4.32	0.60
9	1.7	4.7	2.3	2.3	0.3	0.9	1.7	0.8	0.2	2.70	1.08
10	0.8	2.9	2.0	2.0	5.4	0	0	1.4	1.2	3.33	0.78
11	0.2	1.6	2.8	2.8	4.0	0	0.6	0.3	0.2	2.58	0.33
12	0.2	0.5	3.9	3.9	2.7	0	0.6	0	0	2.19	0.18
13	1.9	ND	4.8	4.8	0.5	0	0	0.7	0.7	2.88	0.42
Mean±SE	2.51±0.60 <sup>  </sup>	4.24±0.92 <sup>††</sup>	3.81±0.92 <sup>††</sup>	3.81±0.56 <sup>  </sup>	3.11±0.53 <sup>  </sup>	0.77±0.20	1.54±0.36 <sup>**</sup>	0.90±0.20	1.30±0.38	4.05±0.51 <sup>§</sup>	1.35±0.30

\* pmol/ml. ‡ pmoles (sum of all four hourly concentrations × 0.30 ml). §  $P < 0.001$  from PBS site (Student's  $t$  test for paired values). †  $P < 0.02$  from PBS site (Student's  $t$  test for paired values). \*\*  $P < 0.01$  from 1-h PBS site (Student's  $t$  test for paired values). ††  $P < 0.01$  from 1-h PBS site (Student's  $t$  test for paired values).

Table IV. Hourly Chamber Fluid Histamine Concentrations after Epicutaneous Antigen (500 PNU/ml) and PBS Challenge in 11 Atopic Subjects

	500 PNU/ml antigen-challenged sites					PBS-challenged sites				
	Hour					Hour				
	1	2*	3	4	Total	1	2	3	4	Total
	(ng)					(ng)				
Mean	49‡	14‡	9	5	23‡	4	4	6	4	5
SEM	9	3	2	1	3	1	1	3	2	2

\*  $n = 10$  (one specimen not available). ‡  $P < 0.001$  compared to PBS sites, Student's  $t$  test for paired values.

for at least 4 h after epicutaneous antigen challenge as compared with the amounts released at control skin sites. LTC<sub>4</sub> accounted for >85% of the C-6 peptide LT with little LTD<sub>4</sub> or LTE<sub>4</sub> detected. Essentially, no chamber fluid LTB<sub>4</sub> was found at either antigen- or control-challenged sites. Chamber fluid concentrations of LTC<sub>4</sub> were significantly elevated during the entire 4 h of incubation; and although temporal patterns varied among individuals for the group as a whole, the greatest amounts were detected during the second, third, and fourth hours of incubation. This pattern of release differed from that of histamine, in which the greatest concentrations were obtained during the first hour with progressively decreasing concentrations in fluids collected during hours 2–4.

Many cell types have been demonstrated to be capable of generating C-6 peptide LTs and LTB<sub>4</sub>: human lung mast cells (9) and eosinophils (10–12) release predominantly LTC<sub>4</sub> and little LTB<sub>4</sub>, whereas neutrophils (10–13) and alveolar macrophages (14, 15) produce a substantial quantity of LTB<sub>4</sub>. Human monocytes generate up to 3.5 times more LTB<sub>4</sub> than LTC<sub>4</sub> (16). Using skin biopsies, our laboratory has previously documented the timing of progressive cellular infiltration

during ongoing cutaneous allergic reactions (3). Neutrophilic leukocytes begin to appear at the reaction site at 20 min after antigen challenge and continue to accumulate for a number of hours. Eosinophils do not begin to accumulate in the interstitial areas until 2 to 4 h after antigen administration. Mononuclear cells progressively infiltrate the site beginning at ~2 h, and increase in concentration over the next several hours. Basophils are not seen in significant quantities during the first 4 h of cutaneous allergic reactions. This would suggest that in our model, resident skin mast cells and perhaps accumulating eosinophils are the source of the LTC<sub>4</sub> collected in the epicutaneous chamber fluids. Although neutrophils are able to generate LTC<sub>4</sub>, their predominant lipoxygenase pathway product is LTB<sub>4</sub>. It is possible that LTB<sub>4</sub> is released in significant quantities during cutaneous allergic reactions but is not detected in our collection chamber model. The recovery of LTs described in the Methods section would suggest that if LTB<sub>4</sub> diffuses into chamber fluids in concentrations greater than several picomoles per milliliter it would have been detectable. Since the oxidative degradation of LTB<sub>4</sub> is intracellular and that of the C6-peptide LTs is extracellular (2), it

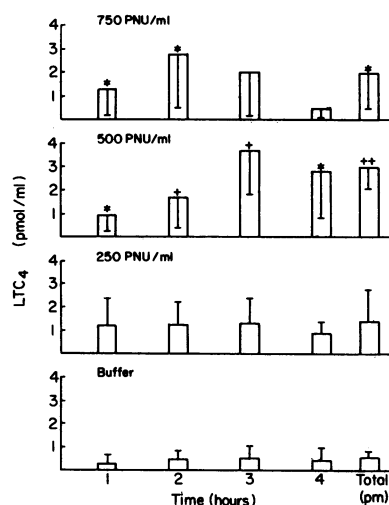


Figure 2. Hourly chamber fluid LTC<sub>4</sub>: dose response characteristics. Mean and standard deviation of chamber fluid LTC<sub>4</sub> (pmol/ml) of six subjects is represented on the ordinate, the time of collection after 750, 500, and 250 PNU/ml of antigen or buffer (PBS) on the abscissa. The total LTC<sub>4</sub> release (sum of all hours  $\times$  0.3 ml) is represented by the 5th bar. \* represents  $P < 0.05$  +  $P < 0.01$  ++  $P < 0.001$  from buffer sites.

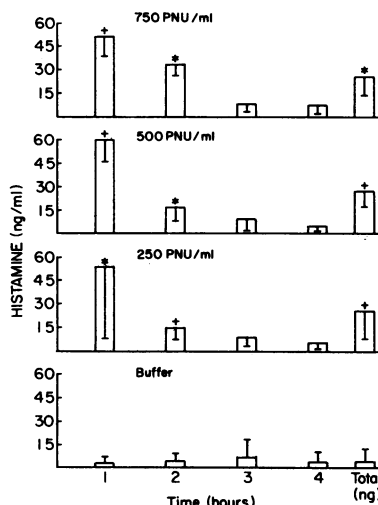


Figure 3. Hourly chamber fluid histamine levels: dose response characteristics. Mean and standard deviation of chamber fluid histamine levels (ng/ml) of six subjects is represented on the ordinate, the time of collection after 750, 500, and 250 PNU/ml of antigen or buffer (PBS) on the abscissa. The total histamine release (sum of all hours  $\times$  0.3 ml) is represented by the fifth bar. \* represents  $P < 0.01$  +  $P < 0.001$  from buffer sites.

is conceivable that LTB<sub>4</sub> might be catabolized effectively before it could diffuse into the chamber fluids in measurable amounts. Eosinophils might contribute to LTC<sub>4</sub> detected during the third and fourth hours, but are probably not present in sufficient numbers in the first 2 h to account for the earlier accumulation of LTC<sub>4</sub>. Our laboratory has previously reported the release of histamine in progressively decreasing amounts during a 4-h period in ongoing allergic reactions (4). In that report, we suggested that the prolonged release of histamine represented continuing mast cell activity and that other mast cell-derived mediators might also be released for prolonged periods during ongoing allergic reactions. The findings reported here are additional, although circumstantial, evidence that mast cells might be the primary source of the LTC<sub>4</sub> detected at antigen-challenged sites in the present study.

The amount of chamber fluid LTC<sub>4</sub> that was recovered was related to the amount of antigen incubated with a peak response following the 500 PNU/ml epicutaneous antigen challenge. Previous studies in our laboratory have demonstrated that chamber fluid histamine accumulation and the degree of neutrophilic leukocyte infiltration into epicutaneous antigen-challenged skin sites are antigen dose-dependent (17). In that study, the antigen doses examined were 0, 100, 500, and 1,000 PNU/ml. Perhaps the broader dose range and larger number of subjects used in the former study detected a dose-dependent response for histamine release, not apparent with the use of narrower antigen dose range in the study reported here.

However, the temporal pattern of release of LTC<sub>4</sub> was different from that of histamine. Whereas peak histamine release invariably occurs during the first hour and progressively decreases thereafter, LTC<sub>4</sub> release was relatively constant after the first hour. This difference is most readily explained by the fact that histamine is present within mast cell granules as a pre-formed mediator, whereas LTC<sub>4</sub> must be generated after perturbation of the cell membrane and release of arachidonic acid by lipoxygenase metabolism.

A substantial amount of in vitro evidence has been accumulated that suggests a major role for the lipoxygenase products of arachidonic acid metabolism in allergic as well as nonallergic inflammatory diseases. However, to clearly demonstrate a pathophysiologic role for the LTs, Koch's postulates must be fulfilled. The availability of synthetic LTs has permitted experiments which indicate that the LTs can reproduce many features of allergic disease. Inhalation of LTC<sub>4</sub> (18, 19) and LTD<sub>4</sub> (19, 20) by humans causes bronchoconstriction. Intracutaneous injections of LTC<sub>4</sub>, LTD<sub>4</sub>, or LTE<sub>4</sub> cause a wheal and a flare that persists for as long as 2 and 6 h, respectively (21, 22). In addition, intracutaneous injections of LTC<sub>4</sub> and LTD<sub>4</sub> have been shown to be as potent as histamine in causing an increase in the microvascular cutaneous blood flow (22). Intracutaneous injection of LTB<sub>4</sub> (21) causes a transient wheal and flare that is followed in 3–4 h by tender induration with neutrophil infiltration and fibrin deposition, histologically resembling a late cutaneous allergic reaction. Although what may have been slow reacting substance of anaphylaxis in the sputums of asthmatics was first detected by means of a bioassay using guinea pig tissue in 1930 (23), definitive proof of the presence of LTs during ongoing allergic reactions has been difficult to obtain. Reports such as ours have only become possible since the development of sensitive radioimmunoassays for the C-6 peptide LTs and LTB<sub>4</sub>. Since our experiments were completed, there have been reports that have demonstrated

the presence of C-6 peptide LTs in body fluids during ongoing allergic reactions. LTC<sub>4</sub>-like immunoreactivity has been detected in human tear fluid following in vivo conjunctival antigen challenge (24). LTC<sub>4</sub>, -D<sub>4</sub>, and -E<sub>4</sub> have been detected in nasal washings in a dose-dependent manner following intranasal pollen challenge in atopic humans (25). As suggested by the authors of this report, the presence of LTD<sub>4</sub> and LTE<sub>4</sub> in nasal washings may represent the metabolism by tissue or nasal secretory enzymes of mast cell-derived LTC<sub>4</sub>. In our skin model, we detected little LTD<sub>4</sub> and no LTE<sub>4</sub>. It is possible that the LTC<sub>4</sub> we collected in skin chamber fluids originated from cells located superficially in the denuded blister base allowing rapid diffusion into the epicutaneous fluids, where it was relatively protected from further enzymatic metabolism.

Thus, our findings, which conclusively demonstrate the presence of LTC<sub>4</sub> in situ during ongoing cutaneous allergic reactions, as well as the other reports (24, 25) mentioned above, fulfill the second criterion of Koch's postulates. It now remains only for clinical trials of pharmacologic LTs inhibitors to demonstrate definitively the role of C-6 peptide LTs in in vivo allergic reactions in humans.

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