

Chemical mediators in atopic dermatitis: Involvement of leukotriene B₄ released by a type I allergic reaction in the pathogenesis of atopic dermatitis

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Background: The mediators produced from a type I allergic reaction have not yet been able to explain the pathogenesis of atopic dermatitis (AD).

Objective: The purpose of this study was to elucidate the involvement of leukotriene (LT) B₄ produced from a type I allergic reaction in the pathogenesis of AD.

Method: The release of LTB₄ was measured both in vitro, in passively sensitized and antigen-challenged human skin slices, as well as in vivo, in skin chambers on patients with AD.

Results: LTB₄ was released from in vitro human skin by stimulation of the antigen (54.9 ± 14.6 pg/g wet weight of skin by antigen challenge and 28.0 ± 11.1 pg/g in control skin, $P < .002$). Antigen-specific release of LTB₄ and histamine was also observed in vivo in nonlesional skin from the patients with AD by using the skin chamber technique.

Conclusion: LTB₄ release during type I allergic reaction in human skin has been determined in vitro. The released LTB₄ possibly contributes to cellular response at the acute inflammatory lesion of AD. (*J Allergy Clin Immunol* 1999;103:663-70.)

Key words: Atopic dermatitis, type I allergy, late-phase response, histamine, leukotriene B₄, neutrophil chemotactic activity

Atopic dermatitis (AD) is a chronic inflammatory skin disease that frequently occurs in patients with a personal or family history of atopy. Although the pathogenesis of AD is still unknown, many observations suggest that an IgE-mediated reaction contributes to the pathogenesis of AD. Serum IgE levels are elevated in 80% of patients with AD.^{1,2} Antigen-specific IgE antibodies are also frequently positive against many environmental allergens, and skin tests for various allergens show positive reactions in the immediate phase.^{3,4} These findings suggest that many patients with AD are sensitized by various allergens and thus may react with an inflammatory response.

Abbreviations used

AD:	Atopic dermatitis
HPF:	High-power field
LPR:	Late-phase response
LT:	Leukotriene
NCA:	Neutrophil chemotactic activity
PG:	Prostaglandin

Histopathologic changes at the inflammatory lesions of AD are nonspecific but consist of acanthosis, spongiosis in the epidermis, and a dermal infiltrate mainly composed of mononuclear leukocytes, with a lesser degree of neutrophils and eosinophils. These granulocytes are observed mainly in acute eczematous lesions.⁵ The cellular response in this disease is comparable to urticaria, another representative cutaneous allergic disease.

The mast cell is known to play an important role in type I allergic reactions and to release many inflammatory mediators, such as histamine,⁶ prostaglandin (PG) D₂,⁷ and leukotriene (LT) C₄.⁸ Among the mediators, histamine plays an important role by evoking a vascular response and probably itching, which are important clinical symptoms in AD, although the cellular response in lesions of AD cannot be explained by histamine.

A type I allergic reaction is sometimes accompanied by a late-phase response (LPR). Histologically, the LPR is characterized by cellular infiltrate, especially neutrophils and eosinophils, as well as mononuclear cells.⁹ This histologic feature suggests that some chemotactic factors are involved in the LPR. Among many chemotactic compounds, LTB₄ is known to be a potent chemoattractant for leukocytes,¹⁰ although its cellular source in cutaneous allergic reactions has not yet been identified. Intradermal injection of LTB₄ provokes transient erythema and persistent induration with the histologic change of neutrophil infiltration.¹¹ These changes suggest that LTB₄ is possibly involved in the development of LPR.

In this study we have investigated the release of mediators, particularly the release of LTB₄, during type I allergic reactions to explain the inflammatory cell infiltration of LPR. We have also investigated the involve-

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TABLE I. Skin reactions against 4 environmental allergens and 3 food allergens in patients with AD

Antigen	WFR (%)	LPR (%)
House dust mite	68.5	72.2
<i>Candida</i> spp	59.3	66.7
Japanese cedar	27.8	31.5
Ragweed	7.5	17.0
Egg	5.9	5.9
Cow's milk	6.0	12.0
Soybean	2.0	4.0

Data are expressed as percentage of positive reaction against each allergen. WFR, Wheal-and-flare response.

ment of LTB₄ produced by allergen stimulation in the pathogenesis of AD.

METHODS

Patients

Fifty-four patients with AD who were 8 to 48 years of age (mean $\pm$ SD, 21.8 ± 7.2 years; 28 men and 26 women) were involved in this study. The patients were classified as having moderate to severe AD according to the grading of severity by Rajka and Langeland.¹² The patients had received no topical treatment except for white petrolatum on the examined area and no systemic treatment for more than 3 days before the study. The study was performed during a period of clinical remission in each patient.

Skin reactions

Four environmental allergens and 3 food allergens were injected intradermally on the volar side of the forearm or on the back. The volume of each injection was 0.02 mL. Antigens used in the skin reactions were *Candida* spp (1:10000), Japanese cedar (1:1000), ragweed (1:1000), egg (1:1000), cow's milk (1:1000), and soybean (1:1000), which were purchased from Torii Co, Ltd (Tokyo, Japan). House dust mite antigen (*Dermatophagoides farinae*, 300 AU/mL) was purchased from Hollister-Stier, Miles Inc (West Haven, Conn).

The diameters of erythema, wheal, and edema were determined over the time course (15 minutes, 4 hours, 6 hours, and 8 hours after injection) at each injection site.

For the evaluation of the immediate-phase response, we used the criteria reported by Norman.¹³ However, no criteria were available for LPR at that time, and therefore we established criteria of our own in which we defined a positive reaction as an erythematous and edematous change of more than 5 mm in diameter at any points appearing 4, 6, or 8 hours after injection.

Histologic examination

To investigate the involvement of LPR in the pathogenesis of AD, biopsy specimens were obtained from the lesional skin of 27 patients with AD and from the site of intradermal injection of the antigens in nonlesional skin at 15 minutes and 4 hours after injection, as well as from the saline-injected site in 5 patients with AD. The specimen was stained by hematoxylin-eosin, and the concentrations of infiltrating neutrophils and eosinophils in each high-power field (HPF) were calculated with the aid of a microscope.

Sera for sensitization of human skin in vitro

Sera for passive sensitization of human skin for in vitro study were obtained from the patients with AD who showed high titers of specific IgE antibody against house dust mite allergen. The levels of IgE antibodies in sera exceeded 100 UA/mL, as measured by the

CAP-RAST method with a commercial kit (Pharmacia CAP System; Kabi Pharmacia Diagnostics, Uppsala, Sweden).

Release of mediators in vitro from human skin

Tyrode solution (NaCl, 137 mmol⁻¹; KCl, 2.7 mmol⁻¹; NaH₂PO₄, 0.42 mmol⁻¹; NaHCO₃, 11.9 mmol⁻¹; MgCl₂, 0.5 mmol⁻¹, CaCl₂, 1.8 mmol⁻¹, and glucose, 5.6 mmol⁻¹) was used as a buffer solution for all experiments in this study.

To demonstrate the release of inflammatory mediators from cutaneous type I allergic reactions, we performed an in vitro experiment. This experiment has been used as a model of cutaneous type I allergic reaction in vitro.⁷

Mastectomized human skin was used. Adipose tissue was gently removed, and the skin was cut into small 5 mm $\times$ 0.5 mm pieces by using a hand microtome. The sliced skin was passively sensitized for 90 minutes, with 20% of the serum containing a high titer of IgE antibody against house dust mite antigen in RPMI-1640. After sensitization, the skin was washed with Ca⁺⁺- and Mg⁺⁺-free Tyrode solutions 3 times to remove the serum and incubated for 20 minutes at 37°C with 100 μ g/mL mite antigen in Tyrode solution. House dust mite antigen was donated as the lyophilized crude extract of *D farinae* from Torii Co, Ltd. The supernatant and skin slices were separated immediately after discontinuation of the reaction by means of an ice-cold bath.

For the assay of histamine, the supernatant of the reaction mixture was stored directly. The residual skin was boiled with Tyrode solution to extract residual histamine. The extracted residual histamine was also stored at -70°C until the assay was performed.

LTB₄ was immediately extracted from the supernatant of reaction mixtures as previously reported.¹⁴ Briefly, a supernatant was acidified by addition of an equal volume of 0.1 mol/L sodium acetate buffer (pH 3.5), and lipids were extracted by partitioning twice with 2 volumes of ethyl acetate. The ethyl acetate phases were evaporated by nitrogen gas, reconstituted with absolute ethanol, and stored at -70°C sealed with argon gas until the assay was performed.

In a representative experiment the supernatant of the reaction mixture was fractionated by a reversed-phase HPLC. Immediately before HPLC separation, ethanol was evaporated, and each sample was reconstituted with a carrier buffer described below. Separations were achieved with a packed column A-311 (YMC, A-311; ODS, S-5 μ m, pore size 120Å; YMC Co, Ltd, Kyoto, Japan) containing a mobile phase of distilled water, methanol, and acetic acid (75:25:0.01 in volume, pH 5.6) at a flow rate of 1 mL/min. The detection was performed at a wavelength of 280 nm by a UV detector (intelligent UV/VIS detector, 875-UV; JASCO Corp, Tokyo, Japan). Neutrophil chemotactic activity (NCA) and LTB₄ in each fraction were determined by the method described below.

Release of mediators in vivo from human skin (skin chamber technique)

Experimental protocols were approved by the ethics committee of Hiroshima University School of Medicine. Informed consent was obtained from all patients.

Patients with positive skin reactions to house dust mite antigen by intradermal injection in the immediate phase and in the late phase were involved in the skin chamber study, 8 for the detection of histamine, 4 for PGD₂, and 6 for LTB₄. Samples were obtained by the skin chamber method, which we have previously reported.⁷ Briefly, 2 areas of uninvolved skin on the back were abraded with the edge of a scalpel blade to remove the superficial scale and upper layers of the epidermis, revealing a smooth glistening surface without bleeding. A funnel-shaped and silanized glass chamber with an

TABLE II. Histologic examination in lesions of patients with AD

	Neutrophils	Eosinophils	Total cells
Acute	2.7 ± 3.5	2.6 ± 2.7	41.7 ± 4.2
Subacute	3.4 ± 5.3	4.1 ± 3.7	53.9 ± 13.9
Chronic	1.9 ± 2.8	1.8 ± 2.7	43.7 ± 11.5

Data are expressed as numbers of neutrophils, eosinophils, and total infiltrating leukocytes in the HPF (mean ± SEM).

Acute, Erythema without induration (n = 3); *subacute*, erythema with induration (n = 16); *chronic*, papular and lichenified (n = 8).

inner diameter of 12 mm connected to a short silicon tube at its tail was placed upside down over each abrasion and held in place with cyanoacrylate adhesive. The abraded sites were initially washed twice with Tyrode solution to remove exudate induced by the preparation. Each chamber was filled with 0.7 mL of Tyrode solution sealed with forceps to avoid contact with air and incubated for 10 minutes to obtain a prechallenged sample from each site. One abraded site was then challenged for 1 hour by 0.7 mL of 150 AU/mL mite antigen solution, which was the same solution as used in the skin reaction study. The whole fluid was collected, and 0.7 mL of antigen solution was replaced. Three more samples of chamber fluid were collected for another 3 hours at 1-hour intervals. The other abraded site was incubated with Tyrode solution alone as a control in the same time course. The chamber fluid was kept at -70°C for the assay of histamine and LTB₄ after the extraction as previously described.

Assay for neutrophil chemotactic activity

NCA in the reaction mixture of the in vitro study and each fraction after HPLC separation was determined by Boyden's chamber method,¹⁵ with human blood neutrophils of more than 80% purity used as indicator cells. The neutrophils were purified from human peripheral venous blood obtained from normal control subjects by Lymphoprep (Difco Laboratories) centrifugation. Neutrophils were suspended at a concentration of 3×10^6 /mL in Tyrode solution with 0.5% BSA. Two hundred microliters of cell suspension was loaded into the upper well on a cellulose acetate filter (Millipore filter SS, pore size 3 mm; Nihon Millipore, Kogyo, Japan), and 0.1 mL of sample aliquot was loaded into the lower well below the filter. Duplicated chambers were incubated for 90 minutes at 37°C in a 5% CO₂ incubator. Filters stained with hematoxylin were mounted, and cells in 5 random HPFs were enumerated microscopically at a depth of 60 or 70 μm from the surface of the filter. The magnitude of neutrophil chemotaxis was expressed as neutrophil counts at the specified depth in an HPF. Harris' hematoxylin for neutrophil stain in chemotaxis assay contained 5.0 g of hematoxylin, 50 mL of ethanol, 100 g of ammonium alum, and 2.5 g of mercuric oxide in 1000 mL of distilled water.

LTB₄ radioimmunoassay

The amount of LTB₄ extracted from the reaction mixture in the in vitro study and from chamber fluid in the in vivo study was determined by an LTB₄-tritiated assay system (Amersham International plc, Amersham, UK). Percent cross-reactivity of the antibody to several arachidonate derivatives was as follows: 0.4% for 20-OH-LTB₄, 0.4% for 6-trans LTB₄, and less than 0.05% for other substances. The detection limit of this assay was 31 pg/mL.

PGD₂ radioimmunoassay

PGD₂ in the reaction mixture from the chamber fluid in the in vivo study was determined by a PGD₂-tritiated assay system

TABLE III. Histologic examination in LPRs in patients with AD

	Neutrophils	Eosinophils	Total cells
Antigen/15 min	1.1 ± 0.3	1.1 ± 0.7	11.6 ± 2.2
Antigen/4 hr	19.8 ± 8.6	13.8 ± 4.0	48.1 ± 9.9
Buffer/4 hr	4.9 ± 2.9	0.3 ± 0.2	12.9 ± 3.0

Biopsy specimens were obtained from 3 sites: 15 minutes (*Antigen/15 min*) and 4 hours (*Antigen/4 hr*) after intradermal injection of antigen and 4 hours after intradermal injection of saline (*Buffer/4 hr*). Data are expressed as numbers of neutrophils, eosinophils, and total infiltrating leukocytes in the HPF (mean ± SEM, n = 5).

(Amersham International plc). Percent cross-reactivity of the antibody to several arachidonate derivatives was as follows: 7% for PGJ₂, 0.3% for thromboxane B₂, 0.04% for PGF_{2α}, and less than 0.02% for other substances. The detection limit of this assay was 31 pg/mL.

Histamine assay by HPLC

Histamine was extracted and measured by reversed-phase HPLC as described previously.¹⁶ In brief, 0.5 mL from each sample was mixed with 0.5 mL of 0.1 N HCl and 1.4 mL of 0.5 N HClO₄ followed by centrifugation to obtain a supernatant. Two milliliters of the supernatant was mixed with 1.0 g of NaCl, 0.8 mL of 5 N NaOH, and 5.0 mL of *n*-butanol followed by centrifugation to obtain the organic phase. Four milliliters of the organic phase was mixed with 4.0 mL of *n*-heptane and 0.4 mL of 0.1 N HCl followed by centrifugation to obtain the acidic phase. The acidic phase was stored at -20°C until the HPLC assay was performed. Immediately before the HPLC assay, 0.26 mL of the sample in the acidic phase was mixed with 0.04 mL of 1 N NaOH and incubated with 0.02 mL of 0.5% *o*-phthalaldehyde (in methanol) for 45 minutes in an ice-cold bath. After incubation, the reaction was stopped by adding 0.12 mL of 0.5 N H₂SO₄. The reaction mixture was injected into the HPLC system.

The HPLC system consisted of an LC-4A (Shimadzu Co, Kyoto, Japan) with an RF-535 fluorescence monitor (Shimadzu Co) operating at an emission wavelength of 450 nm and an excitation wavelength of 350 nm. The HPLC column was a Crestpak C18T-5 (4.6 mm inner diameter and 250 mm length) purchased from JASCO Corporation. The assay was performed by using a mobile phase of 55% distilled water and 45% methanol in volume with 0.11 mol/L NaCl (pH 3.0) with a flow rate of 1 mL/min. Histamine released in the supernatant was expressed as a percentage of the total histamine content in the skin.

RESULTS

Skin reactions

In all 54 patients with AD, intradermal injections of 4 environmental allergens and 3 food allergens were examined. To evaluate skin reactions in the immediate phase, we used the criteria reported by Norman.¹³ However, there were no suitable criteria for cutaneous LPR at this time, and therefore we established our own by defining a positive reaction as an erythematous and edematous change exceeding 5 mm in diameter at 4, 6, and 8 hours after injection. Table I shows the results of the skin reactions. The positive rates of immediate responses and LPRs against environmental allergens are higher than those against food allergens. In all sites with LPR, a clin-

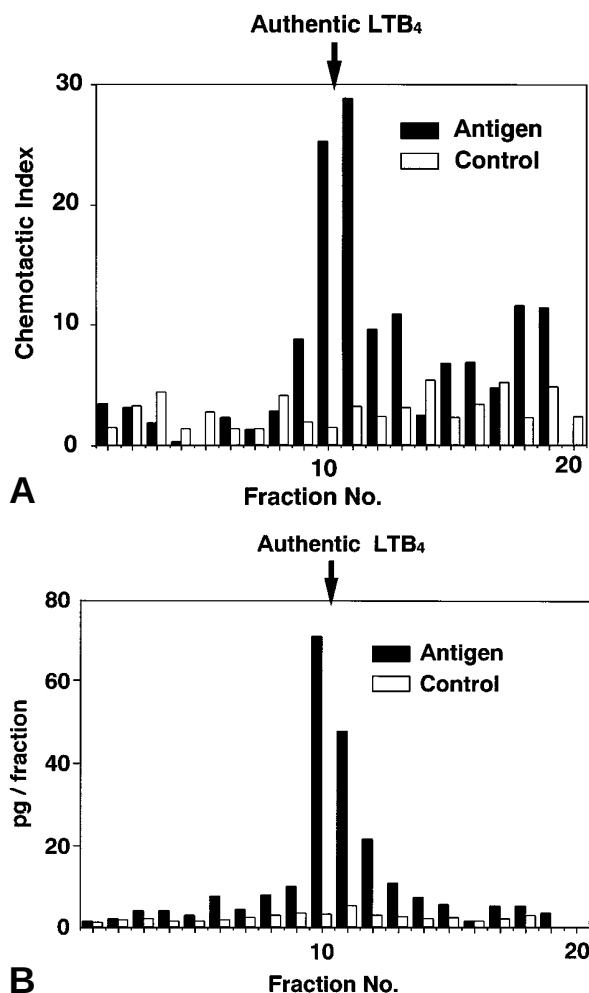


FIG 1. **A**, NCA in fractions of HPLC. Reaction mixture of the in vitro study was fractionated by reversed-phase HPLC. NCA is expressed by a chemotactic index (number of neutrophils infiltrated by sample/number of neutrophils infiltrated by corresponding buffer). **B**, LTB_4 in the fractions of HPLC. LTB_4 is expressed as picograms per fraction.

ical response to intradermal injection of the antigen is observed continuously from the immediate wheal-and-flare response to the late-phase, erythematous, edematous response.

To compare the rate of LPR between the patients and normal healthy control subjects against house dust mite antigen, which caused LPR in 72.2% of the patients, we also performed skin tests against house dust mite antigen in 40 normal healthy control subjects (25 to 41 years of age, 35 men and 5 women) who had not had any atopic diseases. Eighteen of 40 subjects had positive immediate responses, although only 1 subject had a positive LPR.

Although the criteria for the immediate wheal-and-flare response was different from that for the LPR, these figures were similar at both phases. These results show that many patients with AD are sensitized by some environmental allergens and that the patients are capable of inducing LPR after an immediate response to some environmental allergens.

TABLE IV. Release of mediators from passively sensitized human skin by stimulation with 100 μ g/mL house dust mite antigen in vitro

	Antigen	Control	P values
Histamine	18.1 $\pm$ 6.2	1.8 $\pm$ 1.3	.001
NCA	36.8 $\pm$ 8.2	15.2 $\pm$ 5.5	.001
LTB_4	54.9 $\pm$ 14.6	28.0 $\pm$ 11.1	.002

Data are expressed as mean $\pm$ SD. Releases of mediators are expressed as follows: *histamine*, percent release of total histamine; *LTB₄*, picograms per wet weight of skin; *NCA*, neutrophil counts at depth of 60 to 70 μ m of chemotaxis filter. The significance of the difference was examined by Wilcoxon's signed-rank test ($n = 6$).

Histologic study

Biopsy specimens were obtained in 27 patients with AD. Table II shows the results of the histologic examinations. The clinical features at the examined sites were classified into the following 3 groups: acute erythematous lesions without induration ($n = 3$), subacute erythematous and indurative lesions ($n = 16$), and chronic papular and lichenified lesions ($n = 8$). The number of infiltrating cells was determined microscopically in 5 HPFs for each specimen. Although the populations of neutrophils and eosinophils were less than 10%, these cells were observed at the perivascular area in any clinical feature.

We also performed histologic examination in the LPR lesion in 5 patients with AD. Antigen solution was injected in clinically nonlesional skin, and biopsy specimens were obtained at 15 minutes and 4 hours after injection, as well as 4 hours after injection from the saline-injected site. As shown in Table III, neutrophils and eosinophils were predominantly infiltrating at the site of cutaneous LPR; however, at the site of immediate reaction, infiltration of neutrophils and eosinophils was not observed.

Release of mediators in vitro

In all 6 experiments significant amounts of NCA and LTB_4 were released from the human skin in vitro samples in addition to histamine over the first 20 minutes after antigen stimulation (Table IV). This result showed that NCA and LTB_4 were released from type 1 allergic reaction, as well as histamine.

HPLC study

In a representative experiment the supernatant of the reaction mixture in vitro was fractionated by reversed-phase HPLC. NCA in each fraction is shown in Fig 1, A. There were 2 peaks of NCA in all fractions obtained from the antigen-stimulated sample. The main peak was observed in the tenth and eleventh fractions, in which authentic LTB_4 was eluted under the same conditions. To confirm this result, the amount of LTB_4 in each fraction was determined by RIA. LTB_4 was detected in the same fractions for which the main peak of NCA was observed (Fig 1, B).

Release of mediators in vivo

Antigen-stimulated release of histamine was observed during the first hour after antigen challenge (mean $\pm$

TABLE V. Release of histamine from abraded human skin of patients with AD stimulated by house dust mite antigen in vivo

	Patient	1	2	3	4	5	6	7	8	Mean $\pm$ SEM
Before challenge	Antigen	0	0	0	0.2	0.8	0	0	0	0.1 $\pm$ 0.1
	Control	0	3.2	0	0.4	0	0	0	0	0.5 $\pm$ 0.2
0-1 hr	Antigen	3.1	12.5	7.8	8.2	11.4	5	25.4	25.4	13.4 $\pm$ 5.1*
	Control	0	3.4	0.4	0.6	1.1	0	0	0	5.1 $\pm$ 0.3
1-2 hr	Antigen	3.0	11.8	4.2	11.6	10.2	3.5	21.8	17.7	11.5 $\pm$ 4.4*
	Control	0	0	2.5	0	1.1	1.2	0	8.3	1.6 $\pm$ 0.6
2-3 hr	Antigen	3.0	11.7	4.4	13.5	11.1	4.7	16.8	15.3	11.1 $\pm$ 4.2*
	Control	0	3.1	0.9	0	1.1	0.9	8.4	8.7	2.9 $\pm$ 1.1
3-4 hr	Antigen	0	10.1	4.0	13.6	14.6	4.7	16.8	13.8	10.5 $\pm$ 4.0*
	Control	0	3.1	0	0	0	0.8	0	12.5	2.1 $\pm$ 0.8

Data are expressed as nanograms per site.

* $P < .02$, antigen-challenged sites versus corresponding buffer sites (Wilcoxon's signed-rank test).

TABLE VI. Release of PGD₂ from abraded human skin of patients with AD stimulated by house dust mite antigen in vivo

	Patient	1	2	3	4	Mean $\pm$ SEM
Before challenge	Antigen	90	101	204	153	137 $\pm$ 26
	Control	173	219	158	0	137 $\pm$ 48
0-1 hr	Antigen	74	99	192	225	147 $\pm$ 36
	Control	131	102	0	0	58 $\pm$ 34
1-2 hr	Antigen	375	113	173	297	239 $\pm$ 59
	Control	0	122	0	0	30 $\pm$ 30
2-3 hr	Antigen	225	0	164	179	142 $\pm$ 49
	Control	0	0	0	0	0
3-4 hr	Antigen	173	99	132	156	140 $\pm$ 16*
	Control	0	0	0	0	0

Data are expressed as picograms per site.

* $P < .005$, antigen-challenged sites versus corresponding buffer sites (paired t test).

TABLE VII. Release of LTB₄ from abraded human skin of patients with AD stimulated by house dust mite antigen in vivo

	Patient	1	2	3	4	5	6	Mean $\pm$ SEM
Before challenge	Antigen	ND	ND	17.6	10.5	35.9	30.6	23.7 $\pm$ 5.8
	Control	ND	ND	26.8	12.7	26.9	34.8	25.3 $\pm$ 4.6
0-1 hr	Antigen	242.7	759.6	152.8	117.3	139.9	85.5	282.5 $\pm$ 121.2*
	Control	63.0	98.6	47.2	35.4	31.5	47.0	53.8 $\pm$ 11.0
1-2 hr	Antigen	66.6	168.3	63.7	24.4	52.9	38.8	69.6 $\pm$ 25.5
	Control	72.0	103.6	24.0	63.0	28.0	28.4	53.2 $\pm$ 14.3
2-3 hr	Antigen	101.7	84.6	52.1	34.5	44.9	38.7	51.0 $\pm$ 8.9*
	Control	71.0	61.4	22.4	33.2	21.4	30.4	40.0 $\pm$ 9.4
3-4 hr	Antigen	65.8	121.3	54.9	37.4	66.5	50.6	68.4 $\pm$ 14.3*
	Control	58.0	64.0	44.4	18.2	22.3	26.5	38.9 $\pm$ 8.7

Data are expressed as picograms per site.

* $P < .05$, antigen-challenged sites versus corresponding buffer sites (Wilcoxon's signed-rank test).

SEM, 13.4 $\pm$ 5.1 ng/site; $n = 8$). The release was significantly higher than at the control site (0.7 $\pm$ 0.3 ng/site, $P < .02$). The average level of histamine in the chamber at the challenged site was over 10 ng/site per hour up to the fourth hour, whereas the average level at the control site was less than 3 ng/site (Table V). The average level of PGD₂ in the chamber at the challenged site was also higher than that at the control site from the first hour up to the fourth hour, although the statistical significance between

the challenged site and the control site was observed only in the fourth hour (Table VI). To examine the release of LTB₄ by the antigen challenge in vivo, as well as in vitro, we determined the amount of LTB₄ elicited by the antigen challenge in a skin chamber. Antigen-challenged release of LTB₄ was observed during the first hour after the antigen challenge (mean $\pm$ SEM, 282.5 $\pm$ 121.2 pg/site; $n = 6$), whereas release at the control site was 53.8 $\pm$ 11.0 pg/site ($P < .05$). Release at the challenged site was also

significantly higher than at the control site during the third and fourth hour after antigen challenge (Table VII).

DISCUSSION

In this study we have demonstrated a release of LTB₄ from human skin during type 1 allergic reaction *in vitro*. We are unaware of any previous reports of such a phenomenon. We have also demonstrated the release of LTB₄, as well as that of histamine, from the nonlesional skin of patients with AD by antigen stimulation.

Atkins et al¹⁷ demonstrated that NCA can appear during anaphylactic reactions of either IgG- or IgE-sensitized guinea pig lung fragments in an antigen-specific fashion. They identified the NCA as that of LTB₄. In humans LTB₄ is a mediator in early and late reactions from the nasal mucosa *in vivo*¹⁸ and from purified human lung mast cells *in vitro*.¹⁹ On the other hand, Robinson et al²⁰ demonstrated IgE-mediated PGD₂ and LTC₄ release from purified human cutaneous mast cells *in vitro*, but they mentioned nothing about release of LTB₄. In this study antigen-induced NCA was shown in human skin *in vitro* and identified as LTB₄ by HPLC. The human skin that we used in this *in vitro* study was successfully sensitized by a reaginic serum, and mediators, including LTB₄, were released by corresponding antigens within 20 minutes. The cellular source of LTB₄ has not been identified. In our *in vitro* study mast cells were one possible source of LTB₄ after antigen challenge because of the absence of inflammatory cell infiltration in the experimental system. Macrophages could also be a possible source of LTB₄. Jacobsson et al²¹ demonstrated that activated monocytes from peripheral venous blood release LTA₄, which is converted into LTB₄ by monoclonal lymphocytic cells. Freeland et al¹⁹ speculated that a large spontaneous release of LTB₄ may come from cells other than mast cells, perhaps macrophages, which have been shown to be a potent source of LTB₄. However, they also speculated that mast cells could respond to an IgE-mediated stimulus by generating LTB₄ because anti-IgE activation of lung fragments leads to an increase in LTB₄ levels. Although it is now controversial whether human mast cells can produce LTB₄, murine mast cells clearly can produce LTB₄.^{22,23}

Neutrophils are also potential sources of LTB₄ in addition to mast cells and macrophages. However, skin slices from normal healthy subjects used for *in vitro* study contained only a small number of neutrophils. Therefore mast cells and macrophages are potential sources of LTB₄, especially in the early process of inflammation in LPRs.

A contaminant, especially LPS, in the antigen solution could activate mast cells or macrophages in our *in vitro* study. Because the antigen purchased from Torii Co was a crude one prepared by an extraction procedure, we determined a concentration of LPS in the antigen solution by a new endotoxin assay procedure with recombinant limulus coagulation enzymes.²⁴ The antigen solution (100 µg/mL) contained 228 pg/mL LPS. LPS has no

effect on the activation of mast cells in serum-free conditions because anaphylatoxin is not produced without serum.²⁵ However, LPS itself possibly acts on macrophages.²⁶ Therefore we examined antigen-induced release of LTB₄ from nonsensitized human skin slices. In a representative experiment *in vitro*, antigen-induced release of LTB₄ from nonsensitized human skin slices was not observed. The result showed that LPS contained in antigen solution used for *in vitro* study had no effect on mediator release.

We hypothesized that the release of LTB₄ in a type 1 allergic reaction may cause persistent inflammatory reaction with leukocyte infiltration at the lesional site of AD. Although AD is a typical allergic disorder in skin, a number of investigators have been reluctant to accept the concept that a mechanism mediated by IgE plays a role in the pathogenesis of AD because histology of the skin lesions found in AD more closely resembles a type IV delayed-type hypersensitivity reaction than a type 1 immediate hypersensitivity reaction.

We have demonstrated that LPR after the intradermal injection of common environmental allergens is frequently observed in patients with AD. The incidence is as high as that found in patients with ragweed allergy as previously reported.^{27,28}

Since Dolovich et al²⁸ first described the cutaneous LPR of IgE-mediated hypersensitivity, cellular infiltration in the type 1 allergic reaction has been explained by LPR. Very few studies, however, have investigated involvement of the LPR in the pathogenesis of AD. Mitchell et al²⁹ demonstrated a novel mechanism that produces eczema involving the chronic interaction of antigen with specific IgE. They showed that eczema could be elicited at a patch testing site if an allergen was applied to abraded skin continuously for 48 hours only in patients with immediate prick test reactions to the specific antigen. They concluded that the chronic interaction of antigens with specific IgE in the skin can induce eczematous reactions, perhaps by eliciting LPR. Ring and Dorsch³⁰ suggested that the vasoactive mediators released from mast cells or basophils after allergen stimulation (eg, histamine, eicosanoids, or platelet-activating factor) induce itch, contact urticaria, or LPR, which when combined with scratching, induce eczematous skin changes.

Mihm et al⁵ described the histologic features of acute vesicular lesions in AD as dominance of lymphocyte and monocyte/macrophage infiltration with occasional infiltration of neutrophils, eosinophils, and basophils. In this study, to show the involvement of LPR in the pathogenesis of AD mainly by the nature of the cellular response, the histologic change at the inflammatory site was also observed. In the chronic lesion, clinically characterized by papules and lichenification, histologic findings were thickening of the epidermal layer and mononuclear cell infiltration. In the acute and subacute lesions, clinically characterized by erythema, seropapules, and edema, histologic findings were infiltration of granulocytes and mononuclear cells.

On the other hand, some investigators^{31,32} have reported the histologic changes of LPR in human skin as perivascular infiltrates of lymphocytes, monocytes/macrophages, neutrophils eosinophils, and basophils at 6 to 8 hours after allergen injection. Histologic studies revealed similarities of histologic features between cutaneous LPR and inflammatory lesions of AD, especially granulocyte infiltration accompanied by mononuclear cells. The histologic findings suggest that some chemoattractant for leukocytes may play an important role in inducing inflammatory changes in AD through LPR. On the other hand, injection of LTB₄ (1.6 nmol/site) into human skin is followed 6 hours later by infiltration of neutrophil granulocytes.¹¹ Although these histologic features are not completely specific to AD, high levels of LTB₄-like immunoreactivity with biologically active concentrations have been detected in suction blister fluid³³ and in biopsy specimens³⁴ from lesional skin from patients with AD. These findings suggest that LTB₄ may play an important role in the inflammatory changes in AD.

Reshef et al⁸ demonstrated, by the skin chamber technique, the production of eicosanoids (PGD₂ and LTC₄) and histamine during cutaneous LPR, but they did not mention production of LTB₄. We have likewise previously demonstrated the production of histamine and PGD₂ during the immediate reaction by stimulation of specific antigens by using the skin chamber technique.⁷

In this in vivo study, antigen-specific release of histamine and PGD₂ were observed from the first to the fourth hour. Antigen-specific release of LTB₄ was also continuously observed from the first to the fourth hour. The amount of antigen-induced LTB₄, 283 pg/site (about 400 pg/mL), in our results means that 10⁻⁹ mol/L LTB₄ was contained in the chamber fluid. This concentration of LTB₄ is enough to evoke chemotactic movement of granulocytes in tissue.

Shalit et al³⁵ demonstrated antigen-stimulated release of LTB₄ in skin chambers on patients with allergic rhinitis. They described a different pattern of release of LTB₄ from that of histamine and emphasized the release of LTB₄ in a late (3 to 9 hours) period. In our skin chamber study LTB₄ reached its peak of release 1 hour after antigen stimulation, along with histamine and PGD₂, suggesting that eicosanoids from mast cells can be released during the immediate phase by antigen stimulation.

Granulocytes have been shown to be the main reactants in LPR.⁹ Recently, however, T cells have also been identified as important for the development of an LPR in mice and human skin.^{36,37} Recently, some other compounds that may be involved in LPR have been reported. IL-1,³⁸ IL-6,³⁹ GM-CSF,⁴⁰ IL-8, RANTES, and MCP-1⁴¹ have been reported to be involved in cutaneous LPRs in atopic subjects. Intradermal injection of MIP-1α and MCP-1, which have histamine-releasing activity, caused immediate and late reactions with neutrophil and mononuclear infiltration, as well as mast cell degranulation.⁴² Although these cytokines have leukocyte chemotactic activities and may play some role in LPRs, the time course of the release of LTB₄ is different from that of

these cytokines. In the early phase of the LPR, LTB₄ mainly acts as a chemoattractant to prolong the inflammatory reaction.

REFERENCES

1. Jones HE, Inouye JC, McGerity JL, Lewis CW. Atopic disease and serum immunoglobulin-E. *Br J Dermatol* 1975;92:17-25.
2. Ogawa M, Berger PA, McIntyre OR, Clendenning WE, Ishizaka K. IgE in atopic dermatitis. *Arch Dermatol* 1971;103:575-80.
3. Champion MD, Rowntree S, Mitchell EB, DiPrisco de Fuenmajor MC, Platts-Mills TAE. Quantitative assessments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitis. *J Allergy Clin Immunol* 1983;72:27-33.
4. Carswell F, Thompson S. Does natural sensitization in eczema occur through the skin? *Lancet* 1986;2:13-5.
5. Mihm MC, Soter NA, Dvorak HF, Frank Austen K. The structure of normal skin and the morphology of atopic eczema. *J Invest Dermatol* 1976;67:305-12.
6. Greaves MW, Yamamoto S, Fairley VM. IgE mediated hypersensitivity in human skin studied using a new in vitro method. *Immunology* 1972;23:239-48.
7. Barr RM, Koro O, Francis DM, Kobza Black A, Numata T, Greaves MW. The release of prostaglandin D₂ from human skin in vivo and in vitro during immediate allergic reactions. *Br J Pharmacol* 1988;94:773-80.
8. Reshef A, Kagey-Sobotka A, Franklin Adkinson N Jr, Lichtenstein LM, Norman PS. The pattern and kinetics in human skin of erythema and mediators during the acute and late-phase response (LPR). *J Allergy Clin Immunol* 1989;84:678-87.
9. Gleich GJ. The late phase of the immunoglobulin E-mediated reaction: A link between anaphylaxis and common allergic disease? *J Allergy Clin Immunol* 1982;70:160-9.
10. Udén A-M, Palmblad J, Lindgren J-Å, Malmsten C. Effects of novel lipoxygenase products on migration of eosinophils and neutrophils in vitro. *Int Arch Allergy Appl Immunol* 1983;72:91-3.
11. Soter NA, Lewis RA, Corey EJ, Frank Austen K. Local effects of synthetic leukotrienes (LTC₄, LTD₄, LTE₄, and LTB₄) in human skin. *J Invest Dermatol* 1983;80:115-9.
12. Rajka G, Langeland T. Grading of the severity of atopic dermatitis. *Acta Derm Venereol (Stockh) Suppl* 1989;144:13-4.
13. Norman PS. In vivo methods of study of allergy. Skin and mucosal tests, techniques, and interpretation. In: Middleton E, Reed CE, Ellis E, editors. *Allergy: principles and practice*. 2nd ed. Vol II. St. Louis: Mosby; 1983. p. 295-302.
14. Brain S, Camp RDR, Dowd P, Kobza Black A, Greaves MW. The release of leukotriene B₄-like material in biologically active amounts from the lesional skin of patients with psoriasis. *J Invest Dermatol* 1984;83:70-3.
15. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* 1962;115:453-66.
16. Tsuruta Y, Kohashi K, Ohkura Y. Simultaneous determination of histamine and N-methyl-histamine in human urine and rat brain by high-performance liquid chromatography with fluorescence detection. *J Chromatogr* 1981;224:105-10.
17. Atkins PC, Valenzano M, Goetzel EJ, Ratnoff WD, Graziano FM, Zweiman B. Identification of leukotriene B₄ as the neutrophil chemotactic factor released by antigen challenge from passively sensitized guinea pig lungs. *J Allergy Clin Immunol* 1989;83:136-43.
18. Freeland HS, Pipkorn U, Schleimer RP, Bascom R, Lichtenstein LM, Naclerio RM, et al. Leukotriene B₄ as a mediator of early and late reactions to antigen in humans. The effect of systemic glucocorticoid treatment in vivo. *J Allergy Clin Immunol* 1989;83:634-42.
19. Freeland HS, Schleimer RP, Schulman ES, Lichtenstein LM, Peters SP. Generation of leukotriene B₄ by human lung fragments and purified human lung mast cells. *Am Rev Respir Dis* 1988;138:389-94.
20. Robinson C, Benyon C, Holgate ST, Church MK. IgE- and calcium-dependent release of eicosanoids and histamine from human purified cutaneous mast cells. *J Invest Dermatol* 1989;93:397-404.
21. Jacobsson P-J, Odlander B, Claesson H-E. Effect of monocyte-lymphocyte interaction on the synthesis of leukotriene B₄. *Eur J Biochem* 1991;196:395-400.
22. Fonteh AN, Bass DA, Marshall LA, Seeds M, Samet JM, Chilton FH. Evidence that secretory phospholipase A2 plays a role in arachidonic acid

- release and eicosanoid biosynthesis by mast cells. *J Immunol* 1994;152:5438-46.
23. Wershil BK, Wang ZS, Gordon JR, Galli SJ. Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor- α . *J Clin Invest* 1991;87:446-53.
 24. Obayashi T, Tamura H, Tanaka S, Ohki M, Takahashi S, Arai M. A new chromogenic endotoxin-specific assay using recombinant limulus coagulation enzymes and its clinical applications. *Clin Chim Acta* 1985;149:55-66.
 25. Glauser FL, Palmer J, Cecconi S, Schoolcraft W, Wells I, Novey H, et al. The effect of endotoxin on the mast cell cAMP system. *Ann Allergy* 1977;38:104-6.
 26. Aderem A, Cohen DS, Wright SD, Cohn ZA. Bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites. *J Exp Med* 1986;164:165-79.
 27. Gleich GJ. The late phase of the immunoglobulin E mediated reaction: A link between anaphylaxis and common allergic disease? *J Allergy Clin Immunol* 1982;70:160-9.
 28. Dolovich J, Hargreave FE, Chalmers R, Shier KJ, Gauldie J, Bienenstock J. Late cutaneous allergic responses in isolated IgE-dependent reactions. *J Allergy Clin Immunol* 1973;52:38-46.
 29. Mitchell EB, Crow J, Chapman MD, Jouhal SS, Pope FM, Platts-Mills TAE. Basophils in allergen-induced patch test sites in atopic dermatitis. *Lancet* 1982;1:127-30.
 30. Ring J, Dorsch W. Altered releasability of vasoactive mediator secreting cells in atopic dermatitis. *Acta Derm Venereol (Stockh)* 1985;114:9-23.
 31. Bernhisel-Broadbent J, Pampson HA. Role of food allergy in atopic eczema. In: Ruzicka T, Ring J, Przybilla B, editors. *Handbook of atopic eczema*. Berlin: Springer-Verlag; 1991. p. 204-11.
 32. Charlesworth EN, Hood AF, Soter NA, Kagey-Sobotka A, Norman PS, Lichtenstein LM. Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. *J Clin Invest* 1989;83:1519-26.
 33. Ruzicka T, Simmet T, Peskar BA, Ring J. Skin levels of arachidonic acid-derived inflammatory mediators and histamine in atopic dermatitis and psoriasis. *J Invest Dermatol* 1986;86:105-8.
 34. Fogh K, Herlin T, Kragballe K. Eicosanoids in skin of patients with atopic dermatitis: prostaglandin E₂ and leukotriene B₄ are present in biologically active concentrations. *J Allergy Clin Immunol* 1989;83:450-5.
 35. Shalit M, Valone FH, Atkins PC, Ratnoff WD, Goetzl EJ, Zweiman B. Late appearance of phospholipid platelet-activating factor and leukotriene B₄ in human skin after repeated antigen challenge. *J Allergy Clin Immunol* 1989;83:691-6.
 36. Werfel S, Massey W, Lichtenstein LM, Bochner BS. Preferential recruitment of activated, memory T lymphocytes into skin chamber fluids during human cutaneous late-phase allergic reactions. *J Allergy Clin Immunol* 1995;96:57-65.
 37. Sawada K, Nagai H, Basaki Y, Yamaya H, Ishizawa K, Watanabe M, et al. The expression of murine cutaneous late phase reaction requires both antibodies and CD4 T cells. *Clin Exp Allergy* 1996;27:225-31.
 38. Bochner BS, Charlesworth EN, Lichtenstein LM, Derse CP, Gillis S, Dinarello CA, et al. Interleukin-1 is released at sites of human cutaneous allergic reaction. *J Allergy Clin Immunol* 1990;86:830-9.
 39. Lee CE, Neuland ME, Teaford HG, Villacis BF, Dixon PS, Valtier S, et al. Interleukin-6 is released in the cutaneous response to allergen challenge in atopic individuals. *J Allergy Clin Immunol* 1992;89:1010-20.
 40. Massey W, Friedman B, Kat M, Cooper P, Kagey-Sobotka A, Lichtenstein LM, et al. Appearance of granulocyte-macrophage colony-stimulating factor activity at allergen-challenged cutaneous late-phase reaction sites. *J Immunol* 1993;150:1084-92.
 41. Zweiman B, Kaplan AP, Tong L, Moskovitz AR. Cytokine levels and inflammatory responses in developing late-phase allergic reactions in the skin. *J Allergy Clin Immunol* 1997;100:104-9.
 42. Alam R, Kumar D, Anderson-Walters D, Forsythe PA. Macrophage inflammatory protein-1 α and monocyte chemoattractant peptide-1 elicit immediate and late cutaneous reactions and activate murine mast cells in vivo. *J Immunol* 1994;152:1298-303.