

Leukotriene B₄ in atopic dermatitis: increased skin levels and altered sensitivity of peripheral blood T-cells

S. THORSEN,¹ K. FOGH,² U. BROBY-JOHANSEN¹ & J. SØNDERGAARD¹

¹Dept. of Dermatology, University of Copenhagen, Bispebjerg Hospital, Copenhagen, and ²Dept. of Dermatology, University of Aarhus, Marselisborg Hospital, Aarhus, Denmark

Employing a radioimmunoassay, de-proteinated suction blister fluid from 12 patients with active atopic dermatitis appeared to contain higher levels of the pro-inflammatory and immunomodulatory mediator leukotriene B₄ (LTB₄) than suction blister fluid from 12 non-atopic individuals. Indirect support for the identity of the mediator was obtained by HPLC of pooled samples. Nylon wool enriched T cells from six patients with atopic dermatitis and six non-atopic people preincubated with LTB₄ (10⁻¹⁰ M - 10⁻⁸ M) expressed no statistically significant suppression in co-culture with mitogen stimulated autologous mononuclear cells, and there was no difference between atopic and non-atopic T cells in this respect. In contrast, LTB₄ induced a dose-dependent reduction in the percentage of phenotypic Leu 2a (suppressor) cells leading to an increased helper/suppressor ratio in five atopic patients that was not observed in five non-atopics. Elevated skin levels of LTB₄ may initiate or amplify dermal inflammation, and abnormal T cell response to the mediator may account for the increased helper/suppressor ratio characteristic of patients with atopic dermatitis.

Key words: leukotriene B₄; atopic dermatitis.

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CLINICAL ASPECTS

Delineation of the possible role of leukotriene B₄ in the pathogenesis of atopic dermatitis may lead to alternative therapeutic approaches. It is conceivable that drugs inhibiting relevant enzymes or blocking leukotriene receptors, when available for clinical use, may exhibit the beneficial effect of glucocorticosteroids, without harbouring the well-known steroidal side effects.

For more than two decades altered production or releasability of inflammatory substances and abnormal regulation of immune functions have been incriminated in the pathogenesis of atopic disorders. Endogenous mediators that can be formed in the skin, induce local inflammatory changes, and modulate immune reactions without causing systemic disturbances, attract particular interest in the study of atopy of the skin. Leukotriene B₄ (LTB₄) (5-(S), 12-(R)-dihydroxy-6-cis-8, 10-trans-14-cis-eicosatetra-

noic acid) is a 5-lipoxygenase metabolite of arachidonic acid (AA). AA is ubiquitously present esterified in the phospholipid bilayers of mammalian cells and must be released intracellularly to act as substrate for the enzymatic production of prostaglandins and leukotrienes (28). LTB₄ can be formed and released from various cell types, in particular neutrophils, but also keratinocytes have this capacity (11). The mediator is potently pro-inflammatory (28) and interacts with lymphocytes in a manner sug-

gesting a role in immunoregulation (22). With regard to the biological profile of LTB₄, we decided to investigate the levels of the mediator in the inflamed skin of patients with atopic dermatitis, and further to test the sensitivity of atopic T lymphocytes to LTB₄.

MATERIAL AND METHOD

In the first part of the study 12 patients with atopic dermatitis (age range: 19–33 years) and 12 non-atopic volunteers (age range: 20–34) were included. Six patients with atopic dermatitis (AD) (age range: 18–29) and six non-atopics (age range: 24–34) entered the second substudy. At the time of investigation all patients presented with moderate signs of disease according to the criteria of Hanifin & Rajka (13). None of the patients was taking oral glucocorticosteroid or non-steroidal antiinflammatory drugs, and none of the tested skin areas had received topical treatment other than moisturizing cream for at least 3 days prior to the study. Consent was obtained following written and verbal information.

In the first substudy suction blister fluid (SBF) was drawn from areas on the flexor aspects of forearms or abdomen of clinically moderately erythematous skin. We find that well-defined suction blisters cannot consistently be elicited on more severely involved skin. Similar suction blisters were developed on the skin of the non-atopic controls.

200 µl SBF samples were precipitated with 1 vol. methanol and 3 vol. acetonitrile. The supernatants were lyophilised and resuspended. Recovery in this procedure is 73%. The samples were tested in duplicate for LTB₄ immunoreactivity, using a radioimmunoassay (RIA) designed by Salmon et al. (26). ³H-LTB₄ was obtained from Amersham, and LTB₄-standard and rabbit anti LTB₄ was purchased from Wellcome Diagnostics. According to the manufacturer the antibody crossreactivity with other eicosanoids is low; 50% displacement of radioactivity requires 30 times more 6-trans-LTB₄, 50 times more 12-HETE and more than 3000 times more SRS-A leukotrienes, 15-HETE or prostaglandins than LTB₄.

Protein-precipitated aqueous samples were pooled and applied to prewashed Sep-Pak silica cartridges (Waters Millipore), washed successively with hexane and dichloromethane and the polar lipids eluted with methanol in a modification of the scheme outline by Metz et al. (19). The extracted lipids were separated by reversed phase high-performance liquid chromatography (RP-HPLC) using a system from Waters equipped with a Hypersil C₁₈-column (5 µm, 100 × 4.6 mm. ID) (9). The column was eluted isocratically using methanol/water/acetic acid, 70:30:0.01 (by vol.) as the mobile phase. The eluent was collected by a fraction collector, programmed to collect 1 min fractions.

The fraction corresponding to the retention time of authentic LTB₄ was assayed for biological activity using a microdroplet chemokinesis assay as described elsewhere (10). To further explore the identity of the immunoreactivity, HPLC eluates from another pool of SBF were evaporated, resuspended and tested in the RIA.

In the second substudy all washing procedures were performed using RPMI with 1% L-glutamine, 10% fetal calf serum and penicillin/streptomycin. All incubations were performed at 37°C and 5% CO₂ in RPMI with 1% L-glutamine, 15% mixed human AB serum plus penicillin/streptomycin. Peripheral blood mononuclear cells (MNC) were isolated from heparinized venous blood by gradient centrifugation (Ficoll-Hypaque). The majority of monocytes and B cells were removed by incubation in nylon wool for 30 min, leaving mainly T cells in the eluate. The T cells were incubated in the absence (control) or presence of LTB₄ (Wellcome Diagnostics) at a final concentration of 10⁻¹⁰ and 10⁻⁸ M for 18 h. Some of the cells were frozen in liquid nitrogen in 5% DMSO for later subset analysis. The remainder were treated with mitomycin (final concentration 50 µg/ml) for 30 min, washed and resuspended to a concentration of 2 × 10⁶/ml (effector cells). Fresh autologous MNC were adjusted to 1 × 10⁶/ml (responder cells). In microtiter plates equal volumes of effector and responder cells were mixed in triplicate without or with concanavalin A (Con A) at suboptimal (2 µg/ml) or optimal (20

µg/ml) final concentrations. The plates were incubated for 72 h and incorporation of tritiated thymidine during the last 24 h was determined by liquid scintillation counting. The T cells that had been frozen after incubation with LTB₄ were thawed, washed and resuspended in microtiter plate wells in RPMI, NaN₃ and 10 % fetal calf serum. Sufficient cells for this substudy were only available from five of the AD patients and five of the non-atopic controls. Monoclonal antibodies for lymphocyte surface markers, anti-human Leu 3a or Leu 2a (Becton-Dickinson) were added for 30 min at 0°C. After washing, fluorescein-coupled rabbit anti-mouse F(ab')₂ fragments (Dako) were added, the cells washed, resuspended in glycerol buffer and counted in a fluorescence microscope.

Nonparametric statistical methods were applied. In the first substudy the Mann-Whitney test was used and results in the second substudy were analysed using the Wilcoxon test for paired data.

RESULTS

SBF from the AD group contained more LTB₄ immunoreactivity (median: 1035 pg/ml, interquartile range: 750–1200 pg/ml) than SBF from

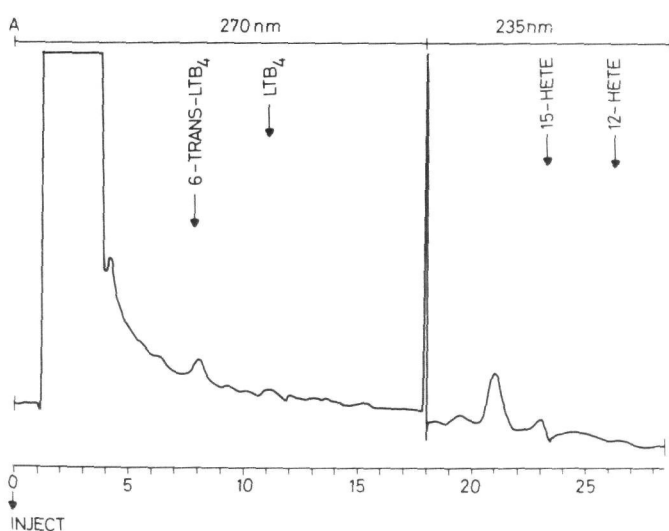


Fig. 1. Reversed-phase high-performance liquid chromatogram of lipids extracted from pooled samples of SBF from patients with atopic dermatitis. Column: hypersil, C₁₈, 5 µm (100 × 4.6 mm I.D.) Flow rate: 0.7 ml/min (0–10 min) and 1.2 ml/min (10–30 min). The UV detector was set at 270 nm from 0 to 18 min and at 235 nm from 18 to 30 min. Retention times of authentic eicosanoids are indicated by arrows.

the non-atopic control group (median: 635 pg/ml, interquartile range 240–790 pg/ml). The difference was significant with $P < 0.05$.

To find whether what was measured in the RIA did indeed represent LTB₄, small fractions of each sample were pooled, Sep-Pak extracted and subjected to HPLC analysis. Fig. 1 shows the chromatogram of pooled samples from AD patients. A peak, albeit small, is seen with the retention time of authentic LTB₄. Small peaks are also seen coeluting with the non-enzymatically formed isomers of LTB₄, 6-trans-LTB₄, and the 15-lipoxygenase metabolite 15-HETE, representing quantities of these eicosanoids insufficient to cause interference in the radioimmunoassay. No peak corresponding to the retention time of 12-HETE is identified.

Since the HPLC procedure does not separate 5(S), 12(S)diHETE from LTB₄ (10) this eluate was tested for biological activity. In contrast to LTB₄ the former does not induce chemokinesis. We found chemokinetic activity in the eluate precluding 5(S), 12(S)diHETE as the origin of the peak (data not shown). Immunoreactivity was only recovered in the HPLC eluate corresponding to authentic LTB₄, supporting, though not proving, the notion that LTB₄-immunoreactivity in the unextracted samples may in fact reflect mediator presence.

In the co-culture experiment LTB₄ induced only a modest and not statistically significant suppressor cell activity in T cells (median: 11 % at 10⁻¹⁰ and 5 % at 10⁻⁸ M). No difference was observed between atopic and non-atopic T cells in this respect, irrespective of whether optimal or suboptimal concentrations of mitogen were used (data not shown). In contrast, atopic T cells preincubated with LTB₄ demonstrated a reduction in the percentage of cells bearing the Leu-2a (suppressor cell) marker, leading to a dose-dependent increase in helper/suppressor ratio. LTB₄ induced no phenotypic changes in non-atopic T cells (Table 1).

DISCUSSION

Due to its unique properties as chemoattractant, particularly towards neutrophils, LTB₄ was early suspected of playing a role in psoriasis, a

Table 1

Median (and range) helper/suppressor ratio of peripheral blood T cells incubated without or with LTB₄ at different concentrations for 18 h.

	Without LTB ₄	10 ⁻¹⁰ M	10 ⁻⁸
Non-atopic controls: (n = 5)	1.2 (0.9-2.8)	1.0 (0.9-1.7)	1.4 (1.0-2.3)
AD patients: (n = 5)	2.1 (1.5-3.7)	2.5 (1.6-4.6)	3.2 (1.9-5.2)

histological hallmark of which is neutrophil infiltration of the skin (5). Elevated skin levels of the mediator have subsequently been identified in allergic contact dermatitis (2). We here report on increased levels of LTB₄-immunoreactivity in SBF from patients with AD. Obviously, measurements of trace amounts of mediators in biological fluids by radioimmunoassay is disputable. Other lipoxygenase metabolites of arachidonic acid as well as unrelated substances may interfere. The 12-lipoxygenase product, 12-HETE has previously been identified in SBF from inflamed human skin, and recently keratome-obtained biopsies from atopic skin were shown to contain rather high – though not biologically active – concentrations of 12-HETE (9). With a cross-reactivity of 2% in the RIA, high concentrations of 12-HETE could account for part of the immunoreactivity observed in our study. However, when extracted lipids from pooled samples were separated by RP-HPLC, we found no peak corresponding to the retention time of 12-HETE, and when all HPLC fractions were tested in the RIA, immunoreactivity was found exclusively in the fraction co-eluting with authentic LTB₄, which also displayed chemokinetic activity. Evidently this may only be taken as circumstantial evidence, that our assay actually measured LTB₄.

We cannot from the present study determine the cellular source of LTB₄. Keratinocytes, stimulated *in vitro*, have been shown able to generate LTB₄ (11), but it is conceivable that the monocyte/macrophage component of the mononuclear infiltrate, once established, will contribute to the local production of LTB₄, hereby perpetuating the process.

Our data are in agreement with an earlier report by Ruzicka et al. (25) although our mea-

surements are generally higher, perhaps because we tested protein-precipitated samples, whereas they assayed SBF directly. In the recent report by Fogh et al. (9) lesional skin from AD patients obtained by keratome showed LTB₄ levels at least 100 times higher than uninvolved atopic skin and non-atopic normal skin. The difference reflects the difficulties in comparing concentrations of mediators obtained from different tissues by different methods.

Elevated LBT₄ levels in atopic skin could account for the dermal infiltrate of mononuclear cells, characteristic of AD. Epicutaneous application of this mediator will elicit an infiltrate in normal human skin, which initially is dominated by neutrophils but after 48 h predominantly consists of mononuclear cells (6). Repeated epicutaneous applications produce a state of tachyphylaxis with respect to neutrophil accumulation, whereas mononuclear infiltration becomes more pronounced (31).

Atopic dermatitis is often linked with evidence of impaired cell-mediated immunity in the skin, as demonstrated clinically by the increased incidence of fungal and viral skin infections, anergy towards recall-antigens and a low incidence of allergic contact dermatitis (14). Numerous *in vitro* studies have demonstrated defects in T cell functions associated with active disease, though the pathogenic importance hereof has been disputed (16). Since atopic individuals obviously do not show symptoms of generalized suppression of CMI it would seem relevant to try to identify local mediators that may modulate immune functions in the target organ, i.e. the skin. LTB₄ would seem a likely candidate. It is known to inhibit lymphocyte LIF production (27) and T cells preincubated with LTB₄ inhibit PWM-induced production of

IgG by B cells (1). Both release of LIF and T cell dependent IgG production is reduced in patients with AD (15, 7).

Peripheral blood T cells from patients with active AD typically display an increased T4/T8 ratio compared with non-atopics (17), and the dermal mononuclear infiltrate seen in AD is characterized by an even more pronounced increase in the T4/T8 ratio (29). Since LTB₄ has been reported to induce appearance of suppressor cell surface markers as well as suppressor cell activity in normal T cells (1, 20, 23, 24) we investigated the possibility that atopic T cells, in analogy to what has been demonstrated with PGE₂ (3), show altered responsiveness to this immunosuppressive effect of LTB₄. In our co-culture set-up no such difference was detected regarding proliferation. In fact, the overall suppressive effect induced in T cells was 2-3 times lower than that reported by Rola-Pleszczynski (23, 24) and failed to reach statistical significance. No further suppression was demasked using suboptimal concentrations of Con A. Other investigators using direct addition of LTB₄ to proliferation assays rather than a co-culture approach, have likewise been unable to demonstrate significant immunosuppressive effect of LTB₄ (12).

On a phenotypic level, however, we observed a difference between atopic and non-atopic T cells. The increased helper/suppressor ratio previously described in peripheral blood from AD patients was confirmed in our study and it was found that preincubation with LTB₄ further increased this ratio almost to a level seen in atopic dermal infiltrates. This increase was dose-dependent and a consequence of a reduced percentage of cells bearing the CD8 marker. In contrast, the T cell phenotype distribution of non-atopics was not influenced by LTB₄. These data are at variance with other studies using normal T cells where LTB₄ is reported to increase the percentage of CD8 cells (1, 24). If confirmed, our observations could provide an explanation of the typical T cell phenotype distribution in the skin of patients with AD. A number of observations suggest that a relative lack of suppres-

sor cells may lead to impaired regulation of IgE production in human atopic diseases (18). Direct correlation between the number of phenotypic suppressor cells and serum levels of IgE has been demonstrated (8), but was not found in another study (30).

In attempting to explain the differential induction by LTB₄ of surfacemarkers on atopic and normal T cells it is worth noting that the suppressive effect on proliferation reported earlier is dependent on intact prostaglandin production in the responding cell population; PGE₂ probably acting as second messenger. The suppression is actually reversed to enhancement if indomethacin is added (24). Since atopic monocytes produce less PGE₂ when stimulated with histamine-induced suppressor-factor (21) it is conceivable that elevated levels of LTB₄ in the microenvironment of atopic mononuclear cells may exert a predominantly stimulating action; in our *in vitro* setting apparent only in the phenotypic composition.

With the demonstration of increased skin levels of LTB₄-immunoreactivity, mimicry of certain hallmarks of AD by LTB₄, e.g. mononuclear dermal infiltrate and increased helper/suppressor ratio, a role for the mediator in the multifactorial pathogenesis of AD is possible. In favour of such a hypothesis is the therapeutic benefit of interventions leading to reduced production of LTB₄. Glucocorticosteroids, widely used in the management of AD, are known to induce production of the endogenous phospholipase A₂-inhibitor lipocortin, hereby reducing availability of unesterified arachidonic acid for 5-lipoxygenation. Recently, it was shown in a double blind, placebo-controlled study that supplementing AD patients' diet with eicosapentaenoic acid, which competes with AA for 5-lipoxygenase, leading to production of the biologically much weaker 5-series of leukotrienes, ameliorates some of the patient-reported symptoms (4).

Direct evidence, however, of the possible pathogenic significance of this potent mediator will rely on the outcome of future clinical trials with specific enzyme inhibitors or receptor antagonists.

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Address:
S. Thorsen, MD.
Dept. of Infectious Diseases
Hvidovre Hospital
Copenhagen, Denmark

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