

In vitro antigen-specific sulphidoleukotriene production in patients allergic to *Dermatophagoides pteronyssinus*

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Summary

Background Sulphidoleukotrienes (slt) are important mediators in allergic diseases that are synthesized after allergen-specific stimulation.

Objectives The aim of this study is to determine *in vitro* slt production after allergen-specific (*Dermatophagoides pteronyssinus*) stimulus of peripheral blood leucocytes and to observe whether histamine release in whole blood with the same allergen correlates with slt production. We also wanted to evaluate whether a correlation exists between the release of slt and histamine and other diagnostic procedures as well as various clinical situations.

Methods We studied 62 patients sensitive to *Dermatophagoides pteronyssinus* (Der p), 30 atopic controls and 12 healthy donors. We determined slt production using the CAST-ELISA technique and histamine release using two concentrations of Der p extract (20 and 2 ng/mL). We also carried out quantification of specific and total IgE levels, skin tests and pulmonary function test on each patient.

Results We observed a significantly increased slt release after *in vitro* stimulation with Der p. There was a significant difference in the slt release between controls and sensitive patients ($P < 0.001$) and between atopic controls and sensitive patients ($P < 0.001$). The data are similar to those obtained with histamine release. We noted a positive correlation ($P < 0.001$) between slt and histamine release ($r = 0.71$, at 2 ng/mL and $r = 0.83$ at 20 ng/mL). We also found a positive ($P < 0.001$), although weak ($r = 0.4$ with at 2 ng/mL, and $r = 0.34$ with $P = 0.003$ at 20 ng/mL) correlation between slt release and specific IgE levels as well as between slt release and skin-test reactivity ($r = 0.49$ at 2 ng/mL and $r = 0.45$ at 20 ng/mL; $P < 0.001$). No significant correlation between slt release and asthma severity was observed, although a trend toward higher slt production in severe and moderate asthma was detected. We found a significant ($P < 0.001$) but weak ($r = -0.3$) negative correlation between age and slt release. With respect to sex-related differences, we found significant differences ($P < 0.05$) in slt release between the sexes with a higher slt release in men than in women.

Conclusion We conclude that CAST-ELISA for quantification of slt production is a useful *in vitro* method for diagnosing sensitization to Der p. There also exists a close correlation between slt release and other parameters of allergic sensitization *in vitro* as well as *in vivo*.

Keywords: asthma, CAST-ELISA, *D. pteronyssinus*, diagnosis, histamine, histamine release test, sulphidoleukotrienes

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Introduction

The basophil is one of the important cells in the allergic inflammatory response. It is implicated in both the

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immediate phase and late phase and releases several mediators such as histamine and sulphidoleukotrienes (slt). Histamine is stored in cytoplasmic granules and released after cell stimulation by allergen-specific IgE, while leukotrienes are synthesized *de novo*. Leukotrienes are derived from the oxidative metabolism of arachidonic acid by action of 5-lipoxygenase [1]. Leukotrienes, LTC₄, LTD₄, and LTE₄ (sulphidoleukotrienes), possess potent bronchoconstrictor activity; they also increase vascular permeability and stimulate mucus secretion, while LTB₄ has chemotactic activity and attracts blood leucocytes to sites of tissue inflammation. The basophil also secretes cytokines such as IL4 [2] which contributes to the recruitment of additional cells responsible for the inflammatory response.

The functions of slt have been demonstrated *in vitro* [3–5] as well as *in vivo* [6] and they have been quantified during allergic disease exacerbation [7], after allergenic stimulation [8–10] or after exercise [11]. The pathophysiological role of slt is also inferred by the successful employment of inhibitors or receptor antagonists which attenuate allergen-induced effects *in vivo* [12].

The aim of this study is to evaluate slt production after allergen-specific (*D. pteronyssinus* [Der p]) stimulation of peripheral blood leucocytes (CAST assay) obtained from patients sensitive to this allergen who have allergic rhinosinusitis and/or asthma. The relationship of leukotriene production to the degree of sensitization, severity, and specific diagnosis was determined as well as any relationship to histamine secretion.

Materials and methods

Patients

We studied 62 patients sensitive to *D. pteronyssinus* (age: 24.3 years $\pm$ 10.9 [mean $\pm$ SD]). Thirty-eight were monosensitized and 24 had other concomitant sensitizations. A group of 30 atopic subjects, non-sensitive to Der p, and 12 healthy donors were used as controls. The diagnosis was dependent on the patient's history, skin tests with the suspected allergens, total and specific IgE, and pulmonary function tests. Of the total, 63 were male and 41 female. With respect to their allergic disease, 15 suffered only from rhinitis, five suffered from asthma, 39 from both diseases, and three from asthma and atopic dermatitis. The asthma severity stage was scored according to the International Consensus paper [13]. Ten had asymptomatic asthma (i.e. symptom free intervals of at least 1 month), 16 mild, 12 moderate and six severe asthma.

None of the patients took medication for at least 24 h prior to the time of the venipuncture.

Sulphidoleukotriene determination in cell supernatants

SlT quantification was carried out by means of the CAST

(Cellular Stimulation Allergen Test)-ELISA [14] (Bühlmann Laboratories, Allschwil, Switzerland). Leucocytes were washed with dextran and resuspended in a buffer solution containing IL3. Two hundred microliters cell suspension was then incubated for 40 min at 37°C (in order to incubate the same time as the histamine release test) with 50 μ L of allergen (*D. pteronyssinus*, [Bühlmann] at a concentration of 2 and 20 ng/mL), buffer or anti-IgE, respectively (Le27, Bühlmann, at a concentration of 0.2 ng/mL). The optimal antigen dose for *in vitro* production of slt by this technique as well as for histamine release was 20 ng/mL of *D. pteronyssinus* extract. Lower doses (e.g. 0.2 ng/mL) or higher ones (e.g. 200 ng/mL) did not cause slt release.

After incubation the samples were centrifuged, and the amount of slt released in the supernatant was measured by an ELISA assay. The anti-slt monoclonal antibody possesses the same affinity for LTC₄ and its two metabolites LTD₄ and LTE₄, but does not crossreact with LTB₄, arachidonic acid or other compounds with L-cysteine or glutamine side chains.

We used washed leucocytes instead of isolated basophils in order to approximate the physiological conditions of an allergic reaction.

Histamine release test

The quantification of antigen-specific histamine release was done in whole blood samples using the fluorometric method of Shore [15], automatized by Siraganian [16–18] and modified by us [19–21]. We used the Analyzer II (Bran & Luebbe, Madrid, Spain), with the same allergens at the same final concentrations as was used for CAST-ELISA as employed by us previously [22]. The data between slt production and histamine release are comparable since the histamine release from washed leucocytes and whole blood is reproducible [23].

Pulmonary function tests

Volume-flow curves were performed with each patient, evaluating the parameters FEV₁ and MEF₅₀, with Jaeger-Masterlab (v3.3) equipment (Würzburg, Germany).

Statistical analysis

For correlation studies, Pearson's coefficient was employed. To compare means, the Student's *t*-test was used. For the comparison of means from more than two independent groups (e.g. clinical diagnosis), we used the Student-Newman-Keuls test.

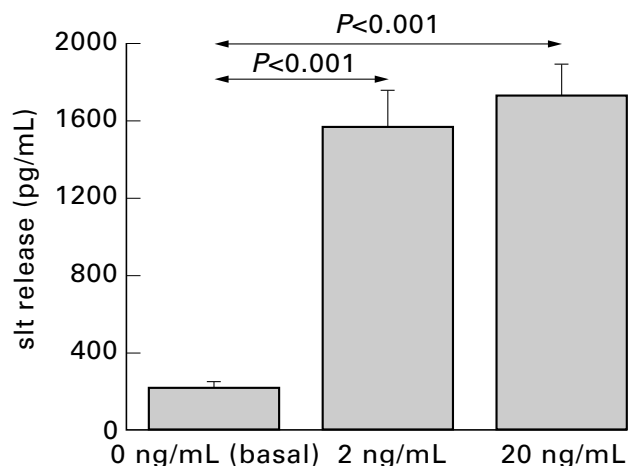


Fig. 1. Sulphidoleukotriene production after leucocyte incubation with buffer (basal production) and *Dermatophagoides pteronyssinus* at two different concentrations.

Results

Mediator release

A highly significant ($P < 0.001$) slt release after *in vitro* antigenic stimulation (1568.6 ± 191.4 pg/mL with 2 ng/mL Der p concentration and 1731.6 ± 162 pg/mL with 20 ng/mL Der p concentration) with Der p extract was observed with respect to the basal release (215.3 ± 32.6 pg/mL) with no significant differences between the two allergen concentrations (Fig. 1). The optimal allergen concentration for slt release was the same as for the histamine release. When stimulated with anti-IgE the release was 1772 ± 130 pg/mL. We found a significant difference ($P < 0.001$) in the slt release between controls (55.3 ± 16.7 pg/mL with 2 ng/mL Der p concentration and 54.4 ± 17.3 with 20 ng/mL Der p concentration) and patients sensitive to Der p (1568 ± 191 with 2 ng/mL Der p concentration and 1731 ± 162 pg/mL

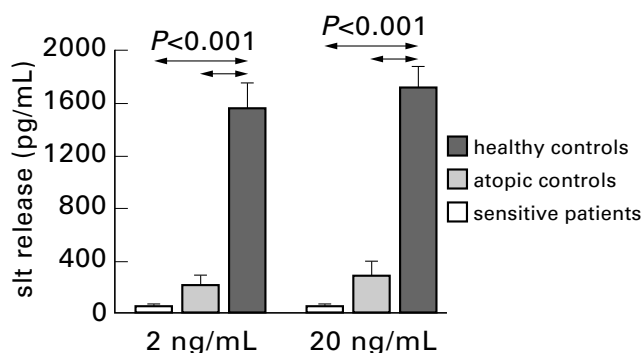


Fig. 2. Differences in slt production after leucocyte incubation with *Dermatophagoides pteronyssinus* among healthy donors, atopic controls and patients sensitive to the mite.

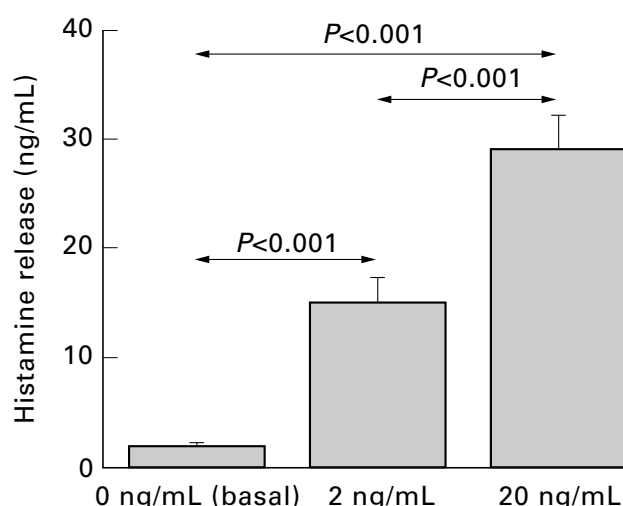


Fig. 3. Histamine release after incubation with buffer (basal release) and *Dermatophagoides pteronyssinus* at two different concentrations.

with 20 ng/mL Der p concentration), as well as between non-Der p sensitive atopic controls (226.7 pg/mL with 2 ng/mL Der p concentration and 297 ± 102.2 with 20 ng/mL Der p concentration) and patients, with no statistical difference between healthy and non-Der p sensitive atopic controls (Fig. 2).

These data are similar to those obtained with histamine release. Basal histamine release was 2.1 ± 0.1 ng/mL, after allergen stimulus histamine release was 15.3 ± 2.1 ng/mL (2 ng/mL Der p concentration) and 29.4 ± 2.9 ng/mL (20 ng/mL Der p concentration) with a significant difference between the two concentrations ($P < 0.001$) (Fig. 3). In the healthy donor group the histamine release was 2.6 ± 0.2 ng/mL (2 ng/mL Der p concentration) and 2.9 ± 3.4 ng/mL (20 ng/mL Der p concentration). In the group of atopic controls histamine release was 3 ± 0.2 ng/mL (2 ng/mL

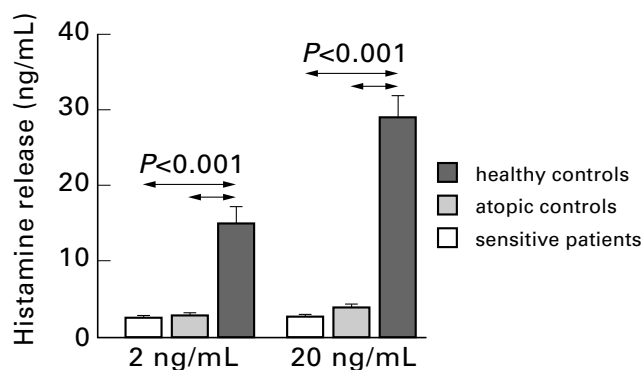


Fig. 4. Differences in histamine release after incubation with *Dermatophagoides pteronyssinus* among healthy donors, atopic controls and patients sensitive to the mite.

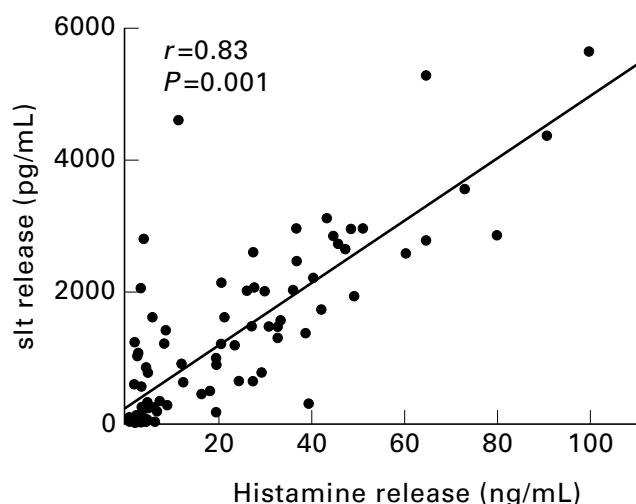


Fig. 5. Correlation between histamine release and silt production after stimuli with *Dermatophagoides pteronyssinus* at a concentration of 20 ng/mL.

Der p concentration) and 4.1 ± 0.6 ng/mL (20 ng/mL Der p concentration) (Fig. 4).

Correlation with other diagnostic methods

We noted a positive and significant correlation between allergen-specific histamine release and silt production ($r = 0.71$, at Der p 2 ng/mL concentration; and $r = 0.83$, at Der p 20 ng/mL concentration, $P < 0.001$) (Fig. 5). Quite interestingly, we also found a positive and significant correlation between skin test (weal size measured in mm²) and antigen-specific silt production ($r = 0.49$, at Der p 2 ng/mL; and $r = 0.45$, at Der p 20 ng/mL, $P < 0.001$) (Fig. 6). Similar results were observed between histamine release and skin test. No significant correlation between specific IgE and skin test was assessed. A significant ($P < 0.001$) though weak positive correlation was found between antigen-specific silt production and specific IgE levels ($r = 0.4$, at Der p 2 ng/mL; and $r = 0.34$, at Der p 20 ng/mL). There was no correlation between total IgE levels and antigen-specific silt release nor between spirometric parameters (FEV₁, MEF₅₀) and silt release.

Correlation with clinical parameters

No significant correlation between levels of released silt and asthma severity (asymptomatic, mild, moderate and severe) was observed; however, there was a trend toward greater production in severe and moderate asthmatic patients than in mild or asymptomatic patients.

With respect to clinical diagnosis, we observed at the Der p concentration of 20 ng/mL a significantly ($P < 0.05$)

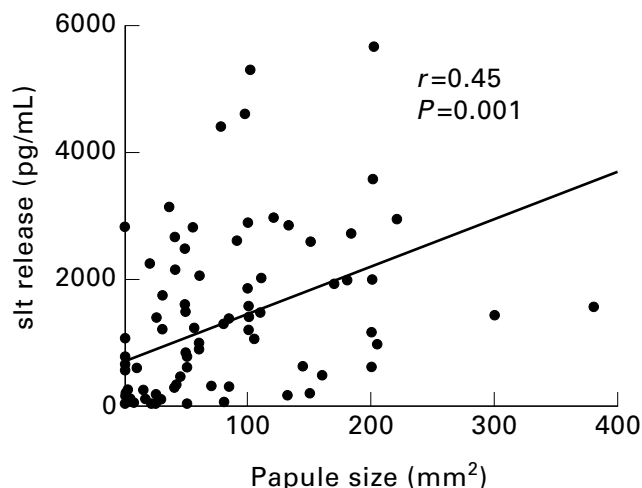


Fig. 6. Correlation between skin test with *Dermatophagoides pteronyssinus* extract and silt production after leucocyte stimuli with the same allergen at a concentration of 20 ng/mL.

higher silt production in the patients' group suffering from rhinitis (2764 ± 401 pg/mL) than the group of patients with asthma and rhinitis (1357 ± 134 pg/mL). There was also a significantly ($P < 0.05$) higher silt production in patients with rhinitis than the group of patients suffering from asthma and atopic dermatitis (511 ± 343 pg/mL). Interestingly, the basal silt production was significantly higher ($P < 0.05$) in atopic patients than in healthy donors although no significant differences were observed among atopic patients according to the clinical diagnosis in basal silt production.

Age-related differences

After a global analysis of all patients, we found a significant ($P < 0.001$) and weak ($r = -0.3$) negative correlation between age and allergen-specific silt release. When we assessed patients within three age groupings (<20, 20–40 and >40 years), no significant differences were observed in IgE levels, asthma severity or skin-test results among the three.

Sex-related differences

There is a significantly ($P < 0.05$) higher silt release in men than in women at the two mite concentrations tested. In the case of allergen-specific histamine release, however, this effect was only observed at a concentration of 20 ng/mL ($P < 0.001$). No significant differences were observed between two sexes in the other parameters studied.

Discussion

It is well known that there is an allergen-specific silt release within 40 min of allergenic stimulation. In this manuscript

we have related slt release to other parameters of allergic sensitization *in vitro* and *in vivo* in a large study population. We demonstrate that this slt release is allergen-dependent, since it is not observed in dust mite sensitive patients under basal conditions (without antigen) and is not observed after challenging atopic controls with Der p who are not sensitive to this allergen.

The variability of mediator release in slt production (from 10 pg/mL to 7842 pg/mL) is much greater than in histamine release (from 1.77 ng/mL to 99.2 pg/mL). This agrees with previous work from McGlashan [24], Mita [25], and Shichijo [26] using blood basophils as well as MacGlashan *et al.* who found similar variability using lung mast cells [27]. It may be interesting to evaluate the ability of the new leukotrienes inhibitor to dampen the slt release in different patients after allergen stimulus, in order to assess whether this variability in the release of slt relates to the leukotriene inhibitor response. The same authors found, in agreement with our results, a significant positive correlation between slt and histamine release, which is higher in our case ($r=0.7$, $P<0.001$ for Der p 2 ng/mL; and $r=0.83$, $P<0.001$ for Der p 20 ng/mL) than reported by McGlashan ($r=0.3$, $P=0.032$) and Mita ($r=0.47$, $P<0.01$). Shichijo, on the other hand, found an exponential correlation.

We found a significant correlation between IgE-dependent mediator releasability and clinical sensitivity to Der p as did Casolaro *et al.* [28]; moreover, we found a correlation between sulphidoleukotriene and histamine release *in vitro* and *in vivo* reactivity measured by skin test. According to our results, antigen-specific slt and histamine release both correlate with allergen-specific IgE levels present in patients' sera. Thus basophil releasability may correlate with releasability of tissue cells such as mast cells [14].

We observed that allergen-induced slt release *in vitro* is higher in the group of patients suffering only from rhinitis than the group with asthma and rhinitis or asthma and atopic dermatitis. Casolaro *et al.* [28], measuring histamine release, did not find such differences. Several factors, however, may complicate this interpretation. Slt production and releasability may be better correlated with controlled experimental allergen provocation [29] than with spontaneous clinical evolution, which is dependent upon unpredictable allergen exposure. In addition, continuously stimulated cells *in vivo* may be down regulated or desensitized with regard to mediator release than cells stimulated intermittently. This could explain the apparent paradox of lower slt production in patients with asthma or asthma and rhinitis than in patients with rhinitis alone. A similar situation has been described with regard to basophil histamine release in patients with chronic urticaria [30]. However, it must be pointed out that these differences could be due to different basophil counts among patients. For that reason will be necessary to perform future studies based on mediator release per million cells to reach definitive conclusions.

It is interesting that basal slt production is higher in atopic patients than in healthy donors. It might mean that atopics possess some intrinsic mechanism that facilitates releasability; another hypothesis could be that atopic cells lack some inhibiting factors such as cytokines, chemokines, etc. However, since repeated exposure to allergens in sensitive patients increases both IgE levels and density of IgE cellular receptors [31], increased reactivity of atopic cells may reflect the intensity of allergen exposure.

In agreement with Mita *et al.* [25], the trend of slt release was to be higher in the severe asthma cases, although some asymptomatic or mild asthmatic patients possess a great capacity to produce slt. In addition slt may be released from mast cells and monocytes, eosinophils as well as from basophils and differences seen with severity may relate to these infiltrating cells. Further studies are needed to elucidate if slt determination, which is useful to detect sensitization to an allergen, is also useful for monitoring disease severity because it correlates with inflammation.

With respect to sex-related differences found in our study, they might be related to the fact that slt are implicated in the ovulation process. For example, it has been demonstrated that leukotriene inhibitors cause follicular rupture while hormones such as LH and hCG may exert a negative feedback role [32,33]. On the other hand, the negative trend between age and mediators' release could be explained by a physiological decrease of receptor number and/or cellular function with ageing [32]. These results are in agreement with previous data from our department with regard to histamine release [34]. Other authors, however, observed a positive correlation between histamine release and age [35].

We conclude that studies on cellular releasability by quantification of mediator production allow a better evaluation of allergic patients. Our data stress the value of making a diagnosis, based not only on history, skin tests and total or specific IgE levels, but also on other *in vitro* parameters. The CAST-ELISA assay for detecting slt release is a useful *in vitro* method for diagnosing allergic diseases mediated by type I hypersensitivity. This diagnostic procedure offers several advantages [16]: it is absolutely safe for the patient; there is no danger of sensitizing a patient; it can be performed in those patients that cannot be skin-tested, and it allows quantitative study reflecting sensitization. Because the CAST-ELISA test measures mediators that are produced *de novo* by leucocytes, it reproduces closely a physiological parameter of the allergic reaction.

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