

Influence of bronchial allergen challenge on histamine release by human basophils

W. J. LIE*†, M. J. VAN DER VEEN†, E. F. KNOL*‡, F. P. J. MUL*,
H. M. JANSEN†, D. ROOS* and J. S. VAN DER ZEE†

*Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical and Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, †Department of Pulmonology, Academic Medical Center, Amsterdam and ‡Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands

Summary

Background Basophils can be primed by cytokines such as interleukin (IL) -3, IL-5 or granulocyte macrophage-colony stimulating factor (GM-CSF). It has been described that the concentrations of these cytokines are enhanced at sites of allergic inflammation as well as systemic in allergic asthma.

Objective To investigate the priming status of basophils as detected by thapsigargin-induced histamine release during bronchial allergen challenge.

Methods Ten subjects allergic to house dust mite were challenged via an aerosol delivery system. Spontaneous leucocyte histamine release as well as histamine release induced by various stimuli was measured *in vitro* at several time points. In addition, lung function parameters, serum IL-5 and blood eosinophil counts were evaluated.

Results We found no effect of bronchial allergen challenge upon spontaneous leucocyte histamine release, nor upon histamine release induced by anti-immunoglobulin (Ig) E, house dust mite extract, C5a, fMLP, IL-3, PMA+ thapsigargin or IL-3+ thapsigargin. However, the priming status of basophils as measured by thapsigargin-induced histamine release was enhanced at 24 h after bronchial allergen challenge. Analysis of the individual data showed a heterogeneous initial response (30 min, 6 h) followed by a predominant increase at 24 h after allergen challenge. This increase in the thapsigargin-induced histamine release correlated with the increase in serum IL-5 levels at 24 h after allergen challenge.

Conclusion The priming status of human basophils as measured by thapsigargin-induced histamine release is enhanced 24 h after allergen challenge.

Keywords: allergen challenge, allergy, asthma, basophils, histamine release

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Introduction

Basophils have been demonstrated in organs affected by allergic disorders. In the lung, basophils have been found in the airway lumen, in the bronchial epithelium and in the submucosa [1–3]. Because basophils can be a source of

interleukin (IL) -4 and IL-13 [4–8], and because they can directly interact with B cells via CD40–CD40L, basophils can form a T-cell-independent route of signalling for immunoglobulin (Ig) E synthesis [9–11]. Furthermore, basophils are able to release potent allergic inflammatory mediators such as LTC₄, major basic protein, elastase, β -glucuronidase and histamine [12]. Degranulation of basophils *in vitro* can be achieved by stimulation with various agents. Several cytokines that are detected at sites of allergic inflammation, such as RANTES, eotaxin,

Correspondence: D. Roos, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

eotaxin-2, MCP-1, MCP-2, MCP-3, and MIP-1 α have been described to induce histamine release from human basophils [13–17]. Cytokines such as IL-3, IL-5 or GM-CSF, for which basophils express receptors, do not induce histamine release by themselves under physiological conditions. However, IL-3, IL-5 and GM-CSF are able to sensitize basophils in a way that they show an increased response to subsequent stimuli, such as anti-IgE, C5a or fMLP [13,18–20]. This phenomenon is called priming. Previously, we have shown that interfering with the intracellular Ca²⁺ homeostasis by blocking the Ca²⁺ re-uptake into the intracellular stores with thapsigargin acts as a strong stimulus for histamine release when basophils have been exposed before to picomoles of IL-3, IL-5 or GM-CSF [21]. Thus, by measuring leucocyte histamine release induced by thapsigargin one has a tool to detect previous (that is *in vivo*) exposure of basophils to IL-3, IL-5 or GM-CSF. Previously, we have shown an increase in serum IL-5 levels at 6 and 24 h after bronchial allergen challenge [22]. Furthermore, we have shown that basophils from patients with allergic asthma show a significantly higher release of histamine upon stimulation with thapsigargin compared with basophils from patients with nonallergic, nonasthmatic lung disorders or basophils from healthy controls (Lie *et al.* J. Allergy Clin Immunol, in press). Because the spontaneous histamine release, as well as the histamine release induced by anti-IgE or fMLP did not differ among the four groups, we consider the thapsigargin-induced histamine release as a sensitive tool to investigate the priming status of the basophils. We therefore investigated whether thapsigargin can be used to predict the inflammatory response induced by bronchial allergen challenge. In this study we evaluated the amount of histamine released by basophils from asthmatic patients sensitized to house dust mite, after challenge by inhalation of house dust mite extract. In addition to the thapsigargin-induced histamine release, spontaneous histamine release and release induced by various other stimuli were measured at several time points. Serum IL-5, as well as eosinophil counts were determined to confirm the effect of the challenge.

Materials and methods

Study subjects

Ten subjects with mild to moderate asthma [23] (five female; mean age 25.7 years; range 19–35 years) were investigated. All patients were sensitized to house dust mite (*Dermatophagoides pteronyssinus*) as determined by skin prick test and RAST. All subjects were investigated in a stable phase of their disease and had a forced expiratory volume in 1 second (FEV₁) $\geq$ 70% of the predicted value

on all study days. The geometric mean of baseline bronchial hyperresponsiveness expressed as PC₂₀ histamine was 2.8 mg/mL (GSD 5.9). Medication of oral antihistamines, inhalation therapy with short acting β_2 -adrenoceptor agonists and corticosteroids was ceased 1 week, 12 h and 6 weeks prior to allergen challenge, respectively. None of the patients had received house dust mite-specific immunotherapy. The patients did not have a history of respiratory tract infection or obvious changes in allergen exposure for at least 6 weeks prior to allergen challenge. All patients were life-long non-smokers.

Materials

An acetone-precipitated house dust mite (HDM) extract was derived from a *D. pteronyssinus* culture (ALK, Hørsholm, Denmark). The freeze-dried HDM extract was diluted in sterile PBS and stored at -70°C . The content of HDM major allergen was determined by radio-immunoassays as previously described [24] and found to be 65.2 mg Der p 1/mg and 17.0 mg Der p 2/mg. The concentration of endotoxin was measured with a Limulus amoebocyte lysate assay (Endosafe, Charleston, SC, USA) [25] and found to be <1 pg/mg. For use in allergen challenge, the HDM extract was diluted to a 100 mg/mL stock solution in PBS, 0.03% HSA, 0.5% phenol (ALK Benelux, Groningen, The Netherlands). For use as a stimulus for histamine release the stock solution was diluted to a 1:5, 1:500 and 1:50 000 solution in incubation medium. Recombinant human IL-3 (Pepro Tech Inc., Rocky Hill, NJ, USA) and polyclonal anti-IgE (Instruchemie Hilversum BV, Hilversum, The Netherlands) were obtained from the manufacturers. Thapsigargin, C5a, fMLP and PMA, all purchased from Sigma Chemicals (St. Louis, MO, USA), were dissolved in DMSO and were stored at -20°C . These agents were diluted at least 300-fold in the cell incubation media. The final concentration of DMSO ($<0.3\%$, v/v) had no effect on cell viability or histamine release.

Bronchial allergen challenge

On the day before each allergen challenge, series of twofold dilutions of the allergen preparations were made from the stock solution in the same buffer. Bronchial allergen challenge was performed as described by Sterk *et al.* [26] by using an aerosol delivery system with a large-volume aerosol reservoir [27]. An allergen aerosol was generated from a 0.5 mL sample with a highly efficient nebulizer (Mallinckrodt Diagnostica, Petten, The Netherlands) into a collapsible reservoir of approximately 30 L, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA). Subsequently, the entire volume of the reservoir was inhaled by tidal breathing in

approximately 3 min through a three-way valve system with the nose clipped. The day before allergen challenge, a control bronchial challenge was performed with three volumes of diluent (PBS, 0.03% HSA, 0.5% phenol; ALK Benelux, Groningen, The Netherlands) and diurnal variation in FEV₁ was established.

The starting dose for allergen challenge was determined by the relation between the skin prick test threshold and the PC₂₀ histamine, as described by Cockcroft *et al.* [28]. Doubling doses of allergen were inhaled with 10 min intervals until a fall in FEV₁ $\geq$ 20% (best of two measurements) relative to baseline FEV₁ (mean of three measurements within 5%) occurred, or the highest concentration of HDM (50 mg/mL) was reached. Lung function was measured 10 and 20 min after each inhalation step with a dry rolling-seal spirometer (SensorMedics BV, Bilthoven, The Netherlands). If the FEV₁ dropped between 15 and 20%, the last dose of allergen was administered again. After the final dose of allergen, FEV₁ was measured every 10 min until 60 min and at 90 min. Hereafter, FEV₁ was measured hourly until 13 h after allergen inhalation with a portable spirometer (Micromedical Diarycard, SensorMedics BV, Bilthoven, The Netherlands). The spirometers were mutually calibrated. The temporal bronchial response to allergen was expressed as the percentage change from baseline FEV₁ as measured with the corresponding spirometer, and was corrected for diurnal variation in FEV₁ as found after control challenge.

Preparations of leucocyte suspensions

Human blood (20 mL) was obtained from the donors after informed consent. Five blood samples per subject were drawn; 30 min after control bronchial challenge with diluent, 30 min after allergen challenge, 6 h, 24 h and 7 days after allergen challenge. The blood was anticoagulated with trisodium citrate. The erythrocytes were lysed with an ice-cold buffer containing 155 mmol/L NH₄Cl, 10 mmol/L KHCO₃ and 0.1 mmol/L EDTA (pH 7.4). The leucocytes were spun down. The pellet was resuspended in fresh lysis buffer for 5 min. The cells were washed once in PBS containing 1% v/v trisodium citrate and once in 'incubation medium' containing 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L MgSO₄, 1.2 mmol/L potassium phosphate, 20 mmol/L HEPES, 5.5 mmol/L glucose, 1 mmol/L CaCl₂ and 0.5% (w/v) human albumin (pH 7.4).

Histamine release

Prior to the addition of stimuli, the leucocyte suspensions were warmed for 5 min at 37°C. The incubation volumes were 450 μ L. After 30 min of incubation the cells were pelleted for 10 s at 12 000 g, and 350 μ L of the supernatant

was collected. The supernatants were mixed with 200 μ L of 12% (w/v) perchloric acid and 750 μ L of saline (0.9% NaCl w/v). The samples were stored at 4°C. For measurement of the total amount of histamine the same amount of cells as used for the incubations was lysed in the perchloric acid. Histamine was measured by fluorometric analysis as described by Siraganian [29]. Histamine release was calculated as percentage of the total amount of histamine in the cells.

Serum IL-5, blood eosinophil counts, total and specific IgE levels

At the same time points, blood was obtained for IL-5 measurements and eosinophil count. Total and differential leucocyte counts were performed directly in EDTA blood by using an automated fluorometric method (H₃-RTX, Bayer-Technicon, Tarrytown, NY, USA). Filtered serum samples were obtained after 1 h of clotting at room temperature and centrifugation for 10 min at 1700 g, and were stored at -20°C. Measurement of levels of IL-5 in the serum samples was performed by a specific solid-phase sandwich enzyme-linked immunosorbent assay as described before (detection level, 0.5 pg/mL) [30]. Total IgE and specific IgE in serum samples obtained 24 h before allergen challenge were measured as described before [24]. In the RAST, 20 mg HDM extract was bound to the solid phase (CNBr-activated Sepharose 4B; Pharmacia, Uppsala, Sweden).

Ethical consent

This study was approved by the local ethics committee and performed after written informed consent of the patients.

Statistical analysis

All graphic data are presented as mean $\pm$ SEM. Statistical significance of differences was calculated by the Student-*t*-test or Wilcoxon signed rank test. Correlation between two variables was calculated by linear regression or Spearman rank correlation. All *P*-values correspond with two-tailed tests.

Results

Influence of bronchial allergen challenge on lung function and bronchial hyperresponsiveness

The magnitude of the early asthmatic response (EAR) after bronchial allergen challenge showed a change in FEV₁ with a median of -23.0% (range -37.2 to -3.5%). The late asthmatic response (LAR) showed a change in FEV₁ with a median of -16.3 (range -64.3 to -9.5%). Six out of 10

patients developed a LAR with a $\Delta FEV_1 \geq 15\%$. The allergen-induced increase in bronchial hyperresponsiveness expressed as ΔPC_{20} histamine (doubling dose) was $1.9 \pm SD 1.1$.

Effect of bronchial allergen challenge on spontaneous histamine release and histamine release induced by various stimuli

Spontaneous histamine release

The spontaneous histamine release was low (maximum at 6 h: mean $\pm SD$: $7.0 \pm 2.4\%$) and was not significantly influenced by allergen challenge (see Fig. 1a [no stimuli used: spontaneous]).

C5a and fMLP-induced histamine release

As depicted in Fig. 1a,b the histamine release induced by C5a or fMLP, i.e. the activation via a G-protein-coupled receptor, was not affected by the bronchial challenge with house dust mite extract.

Anti-IgE and HDM-induced histamine release

Another main route of basophil activation, via cross-linking of the IgE receptor appeared to be affected slightly by allergen challenge (Fig. 1d,e). However, the enhancement at 6 h after allergen challenge did not reach statistical significance (anti-IgE $P = 0.06$). The mean response to HDM was higher than histamine release induced by anti-IgE. For anti-IgE as well as for mite extract, three concentrations were used. A shift in optimal concentration for histamine release induced by the allergen challenge was not observed (data not shown).

PMA/thapsigargin-induced histamine release

Previously, we have shown that emptying the intracellular Ca^{2+} stores with thapsigargin accelerates and enhances the histamine release induced by direct stimulation of PKC with PMA [21]. In our hands, this combination is a very potent stimulus and can be considered as a standard of maximal degranulation. This maximal response of about 80% release was not affected by the allergen challenge (Fig. 1f).

IL-3

We investigated the effect of IL-3 (Fig. 1b), because it has been described that this cytokine can induce histamine

release in allergic patients [31]. IL-3 (500 pmol/L) did increase the histamine release compared with spontaneous histamine release slightly at all time points (paired Student t -test, $P = 0.04$ at 6 h, all other time points $P \leq 0.01$). There was no effect of the allergen challenge (Fig. 1g).

IL-3/thapsigargin-induced histamine release

To examine the response induced by thapsigargin after maximal priming, we measured the histamine release induced by thapsigargin in combination with IL-3 (500 pmol/L). The histamine release induced by this combination (Fig. 1h) was only slightly less than the maximal histamine release induced by PMA/thapsigargin (Fig. 1f) and not influenced by allergen challenge.

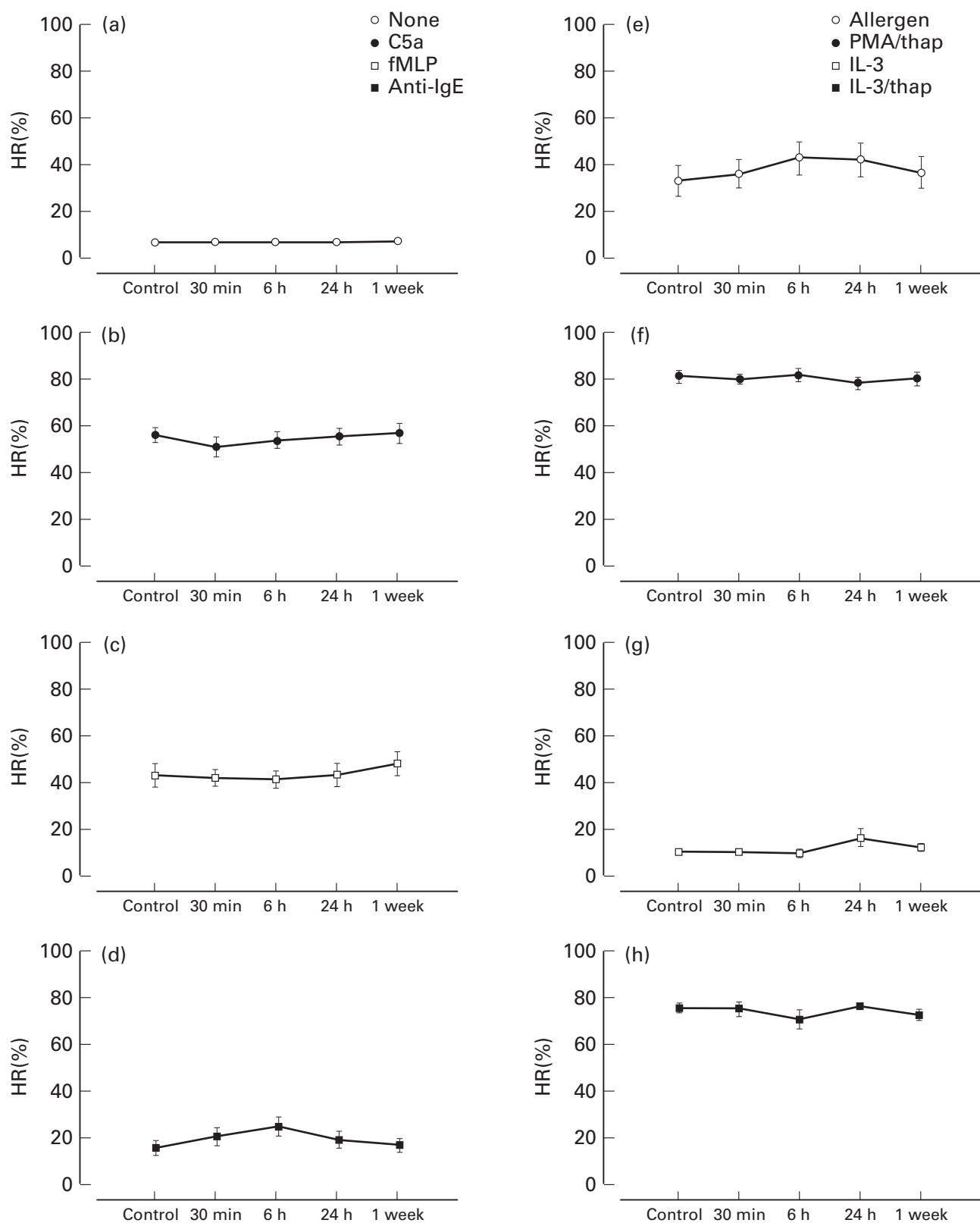
Thapsigargin-induced histamine release

At 24 h after bronchial allergen challenge there was a significant increase in thapsigargin-induced histamine release (Fig. 2a). Analysis of individual data showed that the five subjects with the highest baseline priming status showed a decrease of the thapsigargin-induced histamine release at 30 min after allergen challenge (Fig. 2b). In the five subjects with the lowest priming status at baseline the thapsigargin-induced histamine release was increased at 30 min and 6 h after allergen challenge. These opposite initial responses of the thapsigargin-induced histamine release appeared to be reproducible in the six subjects who were re-challenged on a different occasion (see Fig. 3). Moreover, the thapsigargin-induced histamine release of all patients had returned to base-line 1 week after allergen challenge.

Influence of allergen challenge on blood eosinophil count and serum IL-5

After an early decrease at 30 min and 6 h after allergen challenge the eosinophil count in the blood peaked at 24 h (control, $262 \pm SD 118 \times 10^6/L$ vs 24 h, $398 \pm SD 214 \times 10^6/L$; paired t -test $P = 0.03$). The serum levels of IL-5 were significantly raised at 6 h ($7.0 \pm SD 9.1$ pg/mL) and 24 h after allergen challenge ($17.3 \pm SD 25.5$ pg/mL) vs control ($2.0 \pm SD 4.0$ pg/mL) (Wilcoxon signed rank test $P = 0.01$ and $P = 0.02$). The increase in serum levels of IL-5 at 24 h were positively correlated with the change of histamine release induced by thapsigargin at 24 h (Fig. 4).

Fig. 1. Histamine release induced by various stimuli at time points: saline challenge (control), 30 min after final bronchial allergen challenge (30 min), 6 h, 24 h and 1 week after allergen challenge. Leucocyte suspensions were incubated for 30 min at 37°C. Histamine was determined in the cell-free supernatant. Results are the mean and SEM, $n = 10$. Stimuli used: (a) none, (b) C5a (10 nmol/L), (c) fMLP (1 μ mol/L), (d) anti-IgE (50, 100 or 200 ng/mL) max response, (e) HDM (1:5, 1:500, 1:50,000) max response, (f) PMA 100 ng/mL + thapsigargin 2 mmol/L, (g) 500 pmol/L IL-3, (h) 500 pmol/L IL-3 + thapsigargin 2 μ mol/L



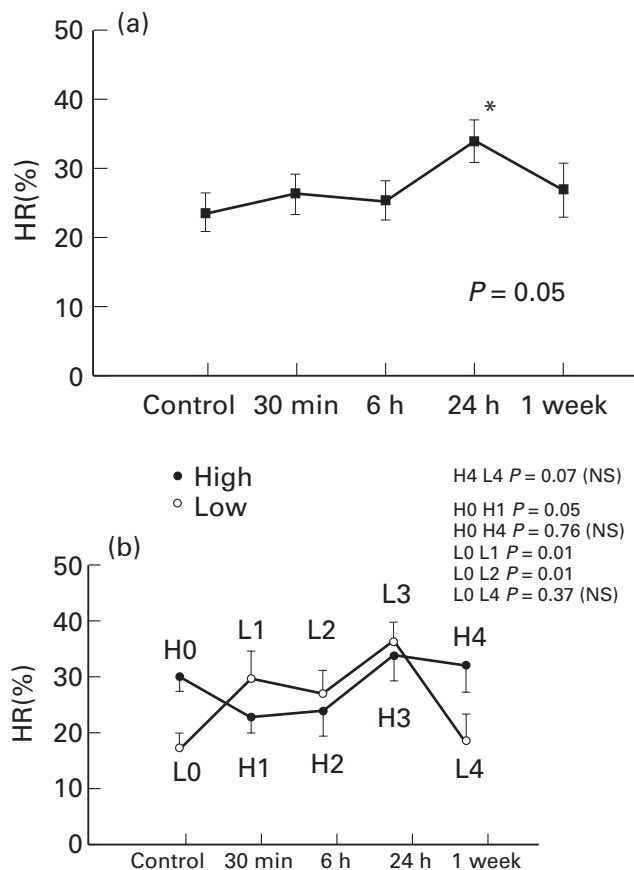


Fig. 2(a) Histamine release after addition of thapsigargin ($2 \mu\text{mol/L}$) at time points: saline challenge (control), 30 min after final bronchial allergen challenge (30 min), 6 h, 24 h and 1 week after allergen challenge. Leucocyte suspensions were incubated for 30 min with thapsigargin at 37°C . Histamine was determined in the cell-free supernatant. (b) Histamine release after addition of thapsigargin ($2 \mu\text{mol/L}$) at various time points. Individuals divided in two groups by thapsigargin-induced histamine release at baseline, five highest (H) and five lowest (L). Statistical significance of difference between H and L: unpaired *t*-test, between H and H or L and L: paired *t*-test

Discussion

We have previously reported on the priming effect of Ca^{2+} -ATPase blockers such as thapsigargin on basophils. We found that thapsigargin in combination with picomolar concentrations of the cytokines IL-3, IL-5 or GM-CSF stimulated basophils to release considerable amounts of histamine [21]. Furthermore, we have shown that in patients with allergic asthma the thapsigargin-induced histamine release is enhanced compared with controls (Lie *et al.* J Allergy Clin Immunol, in press). This is in concordance with the described increase in levels of cytokines (IL-3, IL-5 and GM-CSF) at sites of allergic inflammation after allergen

challenge [32,33]. In addition, we have shown an increase in serum IL-5 levels at 6 and 24 h after bronchial allergen challenge [22]. This was in accordance with a raised level of IL-5 in induced sputum after bronchial allergen challenge as found by others [34]. We now examined whether within a group of asthmatic patients, allergen challenge affected the priming status of basophils by measuring thapsigargin-induced histamine release.

Inhalation of house dust mite extract resulted in a LAR in six patients. It also resulted in an increase in the number of circulating eosinophils and the serum level of IL-5, which was most pronounced at 24 h after challenge.

Spontaneous histamine release was not affected by allergen challenge, nor were the responses to established stimuli of different activation routes of basophil degranulation. We were not able to confirm the small increase in spontaneous histamine release after allergen challenge as found by others [35]. The small number of patients in our study may have provided insufficient statistical power to detect small changes in spontaneous release.

Allergen challenge neither affected histamine release induced by C5a or fMLP. It has been reported that IL-5 enhances LTC₄ production after C5a incubation. Therefore, it would be interesting to investigate whether allergen challenge does affect other responses of basophils to C5a or fMLP, such as production of LTC₄, PAF, IL-4 or IL-13. IL-4 and IL-13 have been detected to be raised in broncho-alveolar lavage (BAL) at 18 h after segmental allergen challenge [36]. Cross-linking of the IgE on the Fcε receptor by anti-IgE or mite extract was not influenced by allergen challenge, as was recently reported for metachromatic cells in BAL [37]. PMA in combination with thapsigargin is a very strong stimulus and can be considered as a standard for maximal degranulation; its effect was not significantly affected by allergen challenge. IL-3 alone (500 pmol/L) induced a significant increase in histamine release above spontaneous release (paired *t*-test $P = 0.01$, $P < 0.001$, $P = 0.04$ and $P = 0.01$ at control, 30 min, 6 h and 24 h, respectively). However, no influence of allergen challenge was noticeable. Also, the histamine release induced by thapsigargin in combination with additional 'priming' by IL-3 was not influenced by allergen challenge.

Despite the small number of patients studied, thapsigargin-induced histamine release changed significantly after allergen challenge. At 24 h after allergen challenge the histamine release induced by thapsigargin was significantly increased (Fig. 2a). When the individual data were studied (Fig. 2b), it appeared that in the five subjects with the highest thapsigargin-induced histamine release at baseline, the histamine release initially (at 30 min) decreased. This was followed by a general increase (eight out of 10) in the thapsigargin-induced histamine release at 24 h after allergen challenge. One week after allergen challenge the

thapsigargin-induced histamine release had returned to baseline levels. The variable initial response (30 min, 6 h) does not appear to be just regression to the mean since the individual responses appeared to be reproducible in the six patients who were challenged a second time (Fig. 3). Moreover, the initial heterogeneity of the response is limited to the thapsigargin stimulus and is not found with other stimuli of basophil degranulation (data not shown). Difference in diurnal variation between patients is also an unlikely cause for the initial heterogeneity of the response as we did not find significant diurnal variation in the parameters studied (Lie *et al.*, J Allergy Clin Immunol, in press). Patients with an initial increase or decrease in the thapsigargin-induced response did not differ in baseline lung function, extent of the EAR or LAR, bronchial hyperresponsiveness, allergen dose, eosinophil counts or total and specific IgE. None of the patients had atopic dermatitis, which has been described to affect the LAR after bronchial allergen challenge [38]. The heterogeneity of the initial response may be caused by a different outcome of two competing mechanisms, i.e. recruitment of activated basophils from the circulation to the airways and recruitment and priming of basophils from the bone marrow. It has been found that bone marrow of asthmatic patients responds within 6 h to allergen challenge [39] with increase in eosinophil/basophil colony-forming units [40]. Furthermore, levels of adhesion molecules like ICAM-1 have been described to be affected by allergen challenge, leading to raised levels in serum and BAL [41] and change in proportion of T cells expressing adhesion molecules [42]. Thus, the initial decrease in thapsigargin-induced histamine release of basophils in the circulation after allergen challenge seen in the group with the highest baseline priming status could be due to extravasation of basophils that are

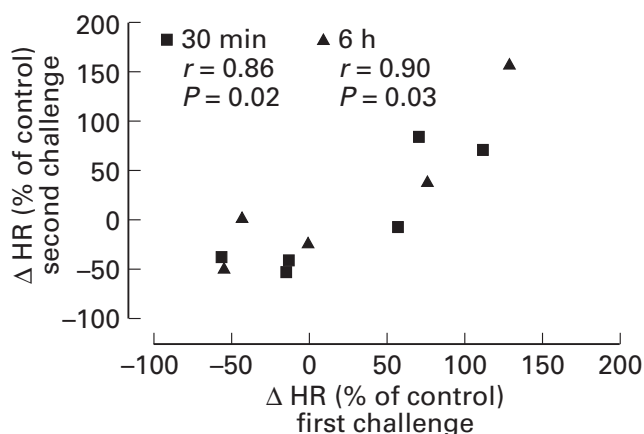


Fig. 3. Thapsigargin-induced change in histamine release 30 min and 6 h after allergen challenge; correlation between first and identical second allergen challenge of same subject at different occasions. (r = Pearson's r , n = 6)

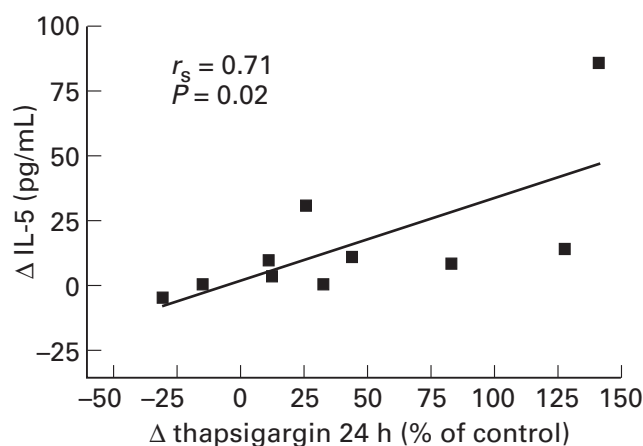


Fig. 4. Thapsigargin ($2 \mu\text{mol/L}$)-induced change in histamine release at 24 h vs change of serum IL-5 at 24 h. (r = Spearman r , n = 10)

activated above a certain level and migration to the airways. However, evidence for such a disappearance of (a subpopulation of) basophils is extremely difficult to obtain because there is no specific marker for establishing the 'age' of basophils. We hypothesize that the uniformly enhanced thapsigargin-induced histamine release 24 h after allergen challenge is explained by the production and systemic release of cytokines that lead to priming of basophils. This is in accordance with the correlation between the increase in serum levels of IL-5 at 24 h and the change in thapsigargin-induced histamine release (Fig. 4).

In conclusion, we have investigated the effect of bronchial allergen challenge on histamine release by human basophils induced by thapsigargin and several other stimuli. Histamine release was measured before allergen challenge, during the early allergic response (30 min), and 6 h, 24 h and 1 week after allergen challenge. Spontaneous histamine release as well as histamine release induced by various stimuli of basophils was not significantly affected by the allergen challenge. However, the priming status of the basophils as measured by thapsigargin-induced histamine release, was enhanced 24 h after allergen challenge. This priming correlated with enhanced serum levels of IL-5 at 24 h.

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