

Mite antigen-induced IL-4 and IL-13 production by basophils derived from atopic asthma patients

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Summary

Background There is increasing evidence for the role of basophils in the pathogenesis of atopic diseases such as bronchial asthma, atopic dermatitis and atopic rhinitis. Recently, it has been reported that basophils derived from healthy donors produce the immunoregulatory cytokines interleukin (IL)-4 and IL-13 after cross-linking of cell surface IgE. In addition to well-known inflammatory mediators, such as histamine and leukotriene C₄, these cytokines produced by basophils are also considered to be associated with atopic diseases.

Objective Our first objective was to determine whether or not mite-sensitive atopic asthmatic basophils produce IL-4 and IL-13 in response to mite antigens. Our second objective was to investigate the relationship between antigen-specific or nonspecific IgE in the serum and the production of these cytokines in order to determine the association of basophil-derived cytokines with the pathogenesis of atopic asthma. Our final objective was to study how production of these cytokines could be regulated by some anti-asthma drugs.

Methods Basophils were purified from peripheral venous blood of 67 atopic asthma patients with elevated RAST for the house dust mite. Cells were stimulated with mite antigens for 6 hours and then IL-4 and IL-13 levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA).

Results Mite-sensitive asthmatic basophils produced IL-4 and IL-13 when stimulated with mite antigens. Mite-induced IL-4 production peaked at 6 hours after the stimulation, whereas IL-13 production continued up to 24 hours. The higher the concentration of mite-specific IgE but not total IgE released in the serum, the more IL-4 and IL-13 were produced by basophils in response to mite antigens. The production of these cytokines was significantly suppressed by the anti-asthma drugs theophylline (IL-4, $p < 0.001$, $n = 6$; IL-13, $p < 0.001$, $n = 10$) and dexamethasone (IL-4, $p < 0.001$, $n = 15$; IL-13, $p < 0.001$, $n = 10$).

Conclusion Mite-antigen-induced IL-4 and IL-13 production by basophils derived from mite-sensitive asthma patients was associated with the concentration of mite-specific IgE and may play an important role in the pathogenesis of atopic asthma. The inhibitory effect of dexamethasone and theophylline on allergic inflammation may be due to the inhibition of IL-4 and IL-13 production not only by T cells but also by basophils.

Keywords: basophil, atopic asthma, mite antigens, RAST, IL-4, IL-13

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Introduction

Allergic inflammatory reactions involve the participation of many different types of cells. Basophils are known to participate in allergic inflammation of the skin and the nose during the late phase response (LPR) [1–4]. Recent studies have reported the existence of basophils in the airways of bronchoalveolar lavage fluids (BALF) during the LPR following antigen challenge [5,6]. These reports suggest that the histamine detected in the LPR derives from basophils but not from mast cells. Basophils release pro-inflammatory and bronchoconstricting mediators such as histamine and leukotriene C_4 in response to IgE-mediated stimuli. Therefore, basophils seem to relate to the pathogenesis of allergic disease, especially the LPR, via the release of mediators.

Allergen-specific IgE immunoglobulins from B cells are important factors in the allergic inflammatory reaction and IL-4 has an important role in regulating the production of IgE. Recently, IL-13 was found to have characteristics similar to those of IL-4. Like IL-4, IL-13 is known to induce an Ig class switch to IgE [7–10], to up-regulate the expression of MHC class II [9] and the low-affinity receptor for IgE (CD23) on B cells [8], and to induce the expression of vascular cell adhesion molecule-1 on the endothelium [11]. It is also known that IL-4, but not IL-13, promotes the differentiation of Th0 cells into Th2 cells [12,13]. Moreover, clinical studies demonstrate that the protein and mRNA expression of IL-4 and IL-13 increase in the allergen-challenged BALF of atopic asthma and rhinitis patients [14,15]. Both IL-4 and IL-13 are therefore essential factors in the development of allergic diseases. Although the main source of IL-4 and IL-13 has been considered to be T cells, recent studies showed that basophils also produce these cytokines [16–22] and the quantity of cytokine production per basophil cell is much larger than that of the T cell [16–19]. Basophils therefore play an important role in allergic disease as a source of inflammatory cytokines.

Recent studies have shown that peripheral blood basophils derived from healthy donors produced IL-4 and IL-13 in response to anti-IgE. However, it is not clear whether or not basophils produce them during actual allergic inflammation. We therefore obtained basophils from mite-antigen-sensitive atopic asthma patients and challenged them with mite antigens. We also examined the relationship between the production of these cytokine and the pathogenesis of atopic asthma. Finally, we investigated the pharmacological regulation of IL-4 and IL-13 production by certain anti-asthma drugs.

Materials and methods

Reagents and media

The following were used: PIPES buffer; RPMI 1640 (Sigma

Immunochemicals, St. Louis, MO, USA); fetal bovine serum (FBS: Filtron Pty Ltd., Brooklyn, Australia); Percoll (Pharmacia, Uppsala, Sweden); crude mite antigens (Torii Medical Co., Tokyo, Japan); theophylline, dexamethasone (Sigma). The 10×PIPES buffer contained 250 mM PIPES, 1.1 M NaCl, 50 mM KCl, 0.1% glucose, and 0.003% HSA, pH 7.47. 1×PIPES consisted of 10% 10×PIPES and 90% distilled water. Isotonic Percoll consisted of one part 10×PIPES and nine parts Percoll. The culture medium of peripheral blood basophils was RPMI 1640 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM non-essential amino acids.

Patients

We obtained basophils from 67 adult atopic asthma patients (48 men, 19 women, 46.8 ± 2.1 years of age). All patients had mild to moderate bronchial asthma with elevated serum IgE specific for the house dust mite (*Dermatophagoides pteronyssinus*: Der p). The patients' total serum IgE and mite (Der p)-specific IgE were measured by outside commercial laboratories. Mite-specific IgE levels were expressed by RAST scores (score 0: <0.35 U/ml, score 1: 0.35–0.7 U/ml, score 2: 0.7–3.5 U/ml, score 3: 3.5–17.5 U/ml, score 4: 17.5–50 U/ml, score 5: 50–100 U/ml, score 6: >100 U/ml). The mean mite RAST score was 3.8 ± 0.2 and the total IgE in the serum derived from patients was 457.8 ± 101.2 U/ml. At the time of the study the patients' symptoms were controlled using appropriate therapy, including occasionally inhaled β -agonist, regularly inhaled corticosteroid (beclomethasone dipropionate), and oral theophylline. Patients who were taking systemic corticosteroids were excluded from this study. Informed consent was obtained from each patient for donation of blood.

Preparation of basophils and mononuclear cells

Peripheral blood from each donor was anticoagulated with 10 mM ethylene diamine tetraacetic acid (EDTA: Nakarai Chem. Ltd., Kyoto, Japan) and mixed with the same amount of physiologic salt solution. Then it was layered onto a two-step gradient solution (1.068/1.079, 10 ml each) made by mixing appropriate amounts of 90% Percoll (1.068/55%, 1.079/64%) and 1×PIPES solution in clear 50-ml polypropylene tubes. The cells were centrifuged ($700 \times g$, for 20 minutes at room temperature) and the basophil-containing interface between the densities 1.068 and 1.079 was harvested. For control experiments, the upper cell layer, containing lymphocytes, monocytes, and <0.1% basophils (mononuclear cells; MNC), was harvested as well and used in parallel under the same conditions. Basophil-enriched and MNC fractions were washed and centrifuged ($700 \times g$ for 6 minutes at 4°C) twice with RPMI 1640 and

resuspended in RPMI 1640 containing 10% FBS, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM non-essential amino acids. The mean basophil purity of the basophil-enriched fraction was 13.3% with a range of 3.1 to 37.0% when counted in a Burkner chamber using Kimura's solution. For convenience, we describe these basophil-enriched fractions simply as basophils.

In some experiments basophils were further purified by negative selection using mouse anti-human monoclonal antibody (anti-CD4, anti-CD8, anti-CD19: PharMingen, San Diego, CA, USA), anti-mouse immunoglobulin magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany) and magnetic beads conjugated to anti-human monoclonal antibody (anti-CD14, anti-CD16: Milteny Biotec). Negative selection was conducted by exposing these cells to a magnetic cell sorting system (MACS: Milteny Biotec). The basophil purity of this fraction was 44.9 to 77% and the remainder of the cells primarily consisted of MNC and a small number of monocytes.

Basophil depletion

In order to evaluate the influence of other contaminating cells, basophils were completely depleted by positive selection using MACS. The basophil-enriched preparation was incubated with FITC-labeled anti-human IgE antibody (Organon Teknika Co., West Chester, PA, USA) for 30 minutes at 4°C and washed with phosphate buffer solution (PBS) containing 2 mM EDTA and 0.5% bovine serum albumin (BSA: Seikagaku Corp., Tokyo, Japan). Then 2 ml of anti-FITC microbeads (Milteny Biotec) for 2×10^6 total cells were added to the cell preparation. Labeled cells were depleted by MACS. We confirmed that no basophils existed in the basophil-depleted preparation as monitored by a flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA) and by Kimura's solution in the Burkner chamber.

Culture conditions

In this study, basophils were cultured at cell densities of 0.6 to 2.0×10^6 cells/ml and the number of basophils was 12 000 to 75 000 cells per well. After stimulation with mite antigens (10 ng/ml), the cells were incubated in sterile 96-well culture plates (200 µl/well) (Becton Dickinson & Co., Lincoln Park, NJ, USA) at 37°C in a humidified atmosphere with 5% CO₂ for 6 hours. For pharmacological experiments each drug was preincubated 10 minutes before stimulation with mite antigens. Cell-free supernatants were then harvested after high-speed centrifugation and stored at -30°C.

Measurement of IL-4 and IL-13

The amounts of IL-4 and IL-13 in the supernatants were

measured by an ELISA kit (IL-4: Biosource International, Camarillo, CA, USA; IL-13: CLB, Amsterdam, Netherlands) according to the manufacturer's instructions. The assay sensitivity for IL-4 and IL-13 was less than 65 fg/ml and 0.5 pg/ml, respectively.

Statistical evaluation

The data are shown as mean and range or mean $\pm$ SEM, and the means of each of the two groups were compared by the paired Student *t*-test. Differences associated with a probability of $P < 0.05$ were considered significant. Correlation coefficients and statistical significance were determined by Bartlett analysis.

Results

Dose response of mite-antigen-induced production of IL-4 and IL-13 by basophils

We first examined the dose-response of the production of IL-4 and IL-13 by stimulation with mite antigens (0.1–1000 ng/ml). As shown in Fig. 1(a) and 1(b), both IL-4 and IL-13 produced by basophils peaked at 10 ng/ml of mite antigens while higher concentrations of mite antigens suppressed the production of these cytokines. At lower concentrations (0.1–1 ng/ml), we observed almost the optimal production of IL-13 but not IL-4, suggesting that the sensitivity of basophils to mite antigen might be different between IL-4 and IL-13 production. Moreover, the antigen concentration that induces maximum release of both cytokines was 100–1000 times lower than for MNC ($n = 3$, data not shown). This result suggests that the sensitivity of basophils to mite antigens for cytokine production is higher than that of MNC. A previous study has already shown this phenomenon [19].

Kinetics of mite-antigen-induced production of IL-4 and IL-13 by basophils

The kinetics of the production by basophils of IL-4 and IL-13 induced by mite antigens (10 ng/ml) showed that both cytokines were detected up to 3 h after the stimulation (IL-4: 22.0 ± 5.0 pg/ 10^6 basophils, $n = 4$; IL-13: 12.3 ± 5.1 pg/ 10^6 basophils, $n = 4$; both cytokines were not detected 1 hour after the stimulation.) Although IL-4 production peaked at 6 h after the stimulation (6 h: 67.2 ± 12.4 pg/ 10^6 basophils, $n = 4$; 12 h: 58.2 ± 10.2 pg/ 10^6 basophils, $n = 4$), IL-13 production increased up to 24 h (6 h: 60.4 ± 20.2 pg/ 10^6 basophils, $n = 4$; 24 h: 110.3 ± 24.5 pg/ 10^6 basophils, $n = 4$). In one of four experiments, IL-13 production was recognized in basophil-depleted preparations at 24 h of incubation. The source of the IL-13 was probably

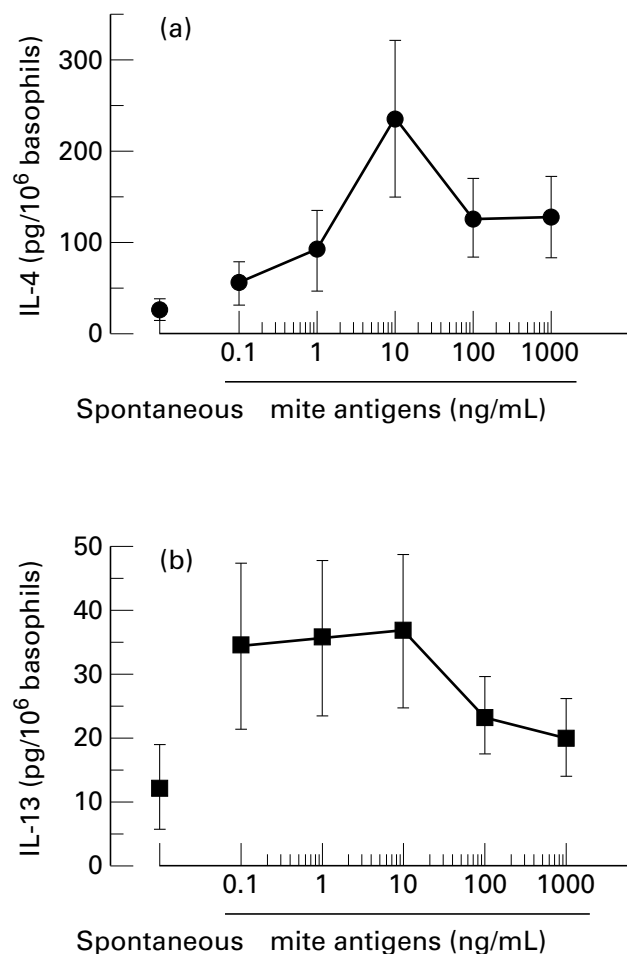


Fig. 1. Dose responses of mite-antigen-induced productions of IL-4 (a) and IL-13 (b) by basophils. Cells were stimulated with various concentrations of mite antigens (0.1–1000 ng/ml) for 6 hours. The spontaneous IL-4 and IL-13 production values were 20.2 ± 5.2 and 12.0 ± 6.6 pg/10⁶ basophils, respectively. Each result is the mean $\pm$ SEM of 5 (IL-4) and 12 (IL-13) experiments.

lymphocytes. In the present study, in order to focus on basophil cytokine release, the cells were challenged with 10 ng/ml of mite antigens for 6 hours.

Mite-antigen-induced IL-4 and IL-13 production by purified basophils and basophil-depleted suspensions

In order to evaluate the influence of other contaminating cells, we prepared purified basophils (the basophil purity was 44.9–77%) and basophil-depleted preparations (no basophils, $n = 4$), and then challenged them with 10 ng/ml of mite antigens for 6 hours. Highly purified basophils produced IL-4 (spontaneous 19.4 ± 11.4 pg/10⁶ basophils, $n = 4$; mite antigen 49.7 ± 36.1 pg/10⁶ basophils, $n = 4$) and IL-13 (spontaneous 16.3 ± 5.9 pg/10⁶ basophils, $n = 7$; mite antigen 25.2 ± 9.2 pg/10⁶ basophils, $n = 7$), whereas

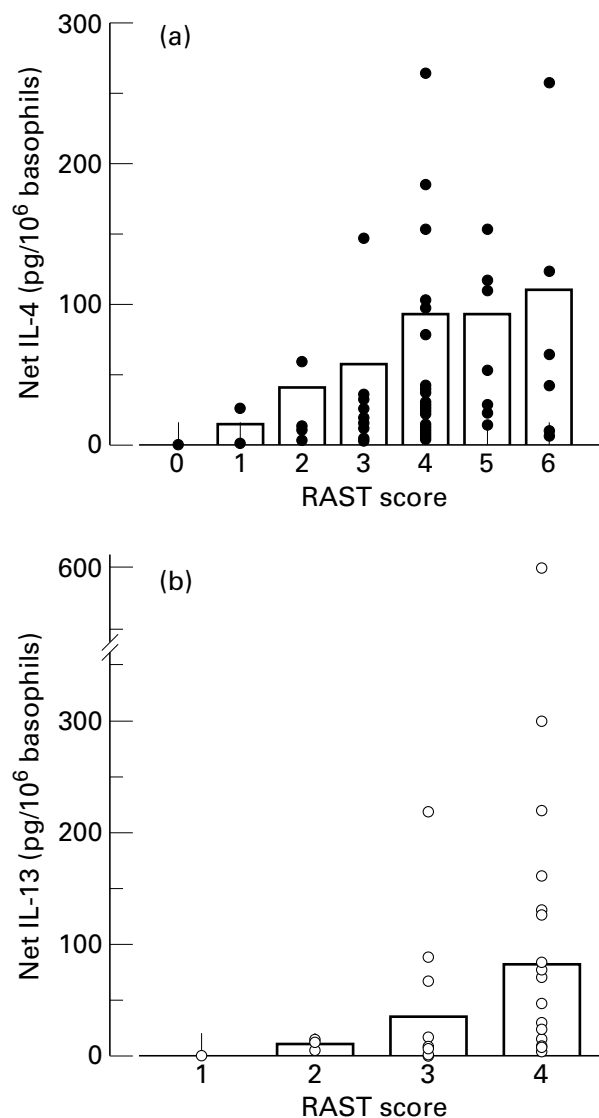


Fig. 2. Relationship between mite-specific IgE value (mite RAST score) in serum and mite-antigen-induced production of IL-4 ((a) $n = 52$) and IL-13 ((b) $n = 43$) by basophils. Cells were stimulated with 10 ng/ml of mite antigens for 6 hours. The sera were obtained at the same time as the preparation of cells. Data are expressed as net values: (antigen-induced value)–(spontaneous value).

basophil-depleted preparations did not produce these cytokines (data not shown). This mite-antigen-induced cytokine production by purified basophils was blocked by a 10-minute preincubation with 10 μ g/ml cycloheximide (IL-4: 9.9 ± 3.3 pg/10⁶ basophils, $n = 4$; IL-13: 0.2 ± 0.2 pg/10⁶ basophils, $n = 5$). These results suggest that basophils, but not the contaminating cells, produce IL-4 and IL-13 when stimulated with specific antigen in the present experimental condition. However, the amount of both cytokines produced by purified basophils was lower than that from basophil-enriched preparations (net IL-4: 57.0 ± 16.3 pg/10⁶ basophils,

net IL-13: 58.0 ± 30.0 pg/ 10^6 basophils). The methods used for the purification of basophils may decrease their responsiveness to stimulation. The method for purification also induces a loss of total cells. We therefore used basophil-enriched preparations obtained rapidly from whole blood for the following studies.

Relationship between mite-specific or total IgE values in serum and mite-antigen-induced IL-4 and IL-13 production by basophils

In order to evaluate the role of basophil-derived cytokines from the clinical point of view, we examined the relationship between mite RAST scores or the total IgE value in serum and cytokine production by basophils. Figure 2(A) shows that the higher the RAST score was, the more IL-4 was produced. The amount of IL-4 produced by patients with RAST scores of 3 to 6 was significantly higher than for those with RAST scores of 0 to 2 ($p < 0.001$).

With regard to IL-13 production, although we could not obtain sufficient data for RAST scores of 5 and 6, the same tendency was shown in the relationship between the IL-13 production and mite RAST scores of 1 to 4 (Fig. 2(B)).

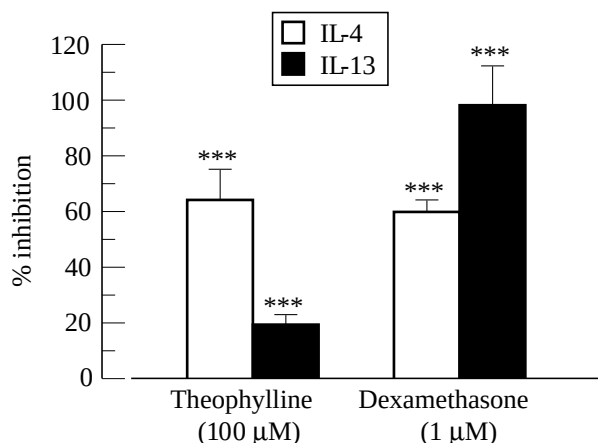


Fig. 3. The effects of theophylline (100 µM) and dexamethasone (1 µM) on IL-4 and IL-13 production by basophils. Cells were incubated with the drugs for 10 minutes and then stimulated with 10 ng/ml of mite antigens for 6 hours. Values are expressed as the percent inhibition of the control cytokine production. The mean net control IL-4 value reflecting the effect of theophylline was 64.8 ± 39.6 and that for dexamethasone was 51.2 ± 18.3 pg/ 10^6 basophils (spontaneous production values were 0.7 ± 0.7 and 4.2 ± 1.7 pg/ 10^6 basophils, respectively). The mean net control IL-13 value for the effect of theophylline was 11.3 ± 2.2 and that of dexamethasone was 7.8 ± 2.3 pg/ 10^6 basophils (spontaneous production values were 1.4 ± 0.4 and 1.2 ± 0.4 pg/ 10^6 basophils, respectively). There is a significant difference between the net control production of cytokines and the net drug-treated production: *** $p < 0.001$.

Since most donors for the IL-13 assay have a RAST score of 3 or 4, we selected donors with RAST scores of 3 and 4 and compared IL-13 production for the two categories. It was shown that mite-antigen-induced IL-13 production by basophils of donors with a RAST score of 4 was significantly higher than that for donors with a RAST score of 3 ($p < 0.05$).

In contrast to the case of mite-specific IgE, total IgE values had no significant correlation with the production of IL-4 and IL-13 (data not shown).

Effects of theophylline and dexamethasone on IL-4 and IL-13 production by basophils

We finally examined the effect of certain drugs on the basophil cytokine production. Figure 3 shows that the anti-asthma drugs theophylline and dexamethasone, which have been shown to suppress histamine release from basophils, also suppress IL-4 and IL-13 production by basophils. Theophylline at 100 µM significantly inhibited the production of IL-4 ($77.9 \pm 8.2\%$ inhibition, $p < 0.001$, $n = 6$) and IL-13 ($19.5 \pm 3.8\%$ inhibition, $p < 0.01$, $n = 10$) by basophils. Dexamethasone (1 µM) also inhibited the production of IL-4 ($51.1 \pm 7.0\%$ inhibition, $p < 0.001$, $n = 15$) and IL-13 ($97.7 \pm 14.3\%$ inhibition, $p < 0.001$, $n = 10$) by basophils. Interestingly, the effect of theophylline on IL-4 production was significantly higher than that on IL-13 production ($p < 0.01$), whereas the effect of dexamethasone on IL-4 production was lower than that on IL-13 production ($p < 0.001$).

Discussion

Our results have shown that IL-4 and IL-13 were produced by basophils in response to specific antigens. We speculate that basophils produce these cytokines by specific antigen stimulation *in vivo* and the produced cytokines have important roles in the pathogenesis of atopic asthma. We previously reported that serum IL-4 in atopic asthmatics was elevated compared with non-atopic asthmatics [23]. Although the total number of basophils in peripheral blood is small, the amount of IL-4 production per basophil cell is much larger than that of the T cell [16–19]. We therefore suggest that specific IgE-mediated IL-4 production by basophils is involved as a source of serum IL-4 in atopic asthmatics. A study of the endobronchial secretion of IL-4 and IL-13 in atopic asthmatics found that high concentrations of both cytokines were measured in bronchoalveolar lavage fluid (BALF) 18 hours after endobronchial allergen challenge [15]. Although the authors discussed that the potential sources of IL-4 and IL-13 are most likely lymphocytes, we suggest that basophils also could be a source of these cytokines. It is known that allergic inflammation in

the lung is initiated by the release of multiple mediators from mast cells and that basophils infiltrate into the airways during the late phase response [5,6,24]. The main source of histamine in the LPR to nasal antigen challenge is basophils, and not mast cells [1,2]. In the lower airways, increased numbers of basophils are seen in the BALF during the LPR [5], and most of the cells containing histamine and bearing IgE, which are found in the late-phase BALF are basophils [6]. We therefore speculate that IL-4 and IL-13 production by basophils also plays an important role in the LPR of atopic asthma.

Ochi *et al.* [19] have reported that basophils from patients with elevated serum IgE specific to mite allergens (RAST scores >3) could produce detectable levels of IL-4 in response to mite antigens, and that there is no significant correlation between mite-induced IL-4 production and the concentration of total IgE in the serum. In the present study, we also showed that the mite-antigen-induced IL-4 from patients with mite RAST scores <3 was significantly higher than that from donors with RAST scores <2. In the case of IL-13 production, the cytokine production by basophils of donors with a RAST score of 4 was significantly higher than that of donors with a RAST score of 3. Furthermore, we showed that mite-induced IL-4 and IL-13 production by basophils had no significant correlation with the concentration of total IgE. These results suggest that antigen-specific IgE elevation is associated with the increase in antigen-induced IL-4 and IL-13 production. The higher concentration of specific IgE on circulating basophils may induce more mediator release from basophils in response to specific stimulation. Two groups demonstrated that the level of FcεRI expression on basophils can exhibit a positive correlation with the serum concentration of IgE [25–27]. Moreover, a recent study showed that the down-regulation of FcεRI expression on basophils during *in-vivo* treatment of atopic patients with anti-IgE antibodies decreased histamine release from basophils in response to antigen [28]. Therefore, the inhibition of mediator release, including IL-4 and IL-13 production, by prevention of binding of IgE to FcεRI may lead to a therapy for atopic asthma.

In the present study, the production of IL-4 reached a plateau at 6 hours after the stimulation, whereas IL-13 production increased up to 24 hours. The kinetics of antigen-induced production of the two cytokines are almost the same with anti-IgE-induced cytokine production, as previously reported [16–22]. On the other hand, the cytokine production from peripheral blood mononuclear cells (PBMNC) required a longer period (48 to 72 hours) for antigen-induced IL-4 and IL-13 production [29]. The role of IL-4 and IL-13 produced by basophils in the pathogenesis of allergic diseases may differ from that of these cytokines produced by PBMNC.

Pharmacological studies showed that theophylline and

dexamethasone inhibit IL-4 and IL-13 production by basophils. Although the inhibitory mechanism of these agents is unclear, the inhibitory effect on allergic inflammation may be due to inhibition of the IL-4 and IL-13 production not only by T cells, but also by basophils. Moreover, theophylline has a greater effect on IL-4 than on IL-13 production, whereas dexamethasone's effect was greater on IL-13 production, suggesting that the production mechanisms of the two cytokines were different.

In conclusion, we showed that basophils produce IL-4 and IL-13 in response to specific antigen, and these cytokines may be associated with the pathogenesis of atopic asthma. Moreover the inhibition of basophil activation may contribute to the treatment of atopic asthma.

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