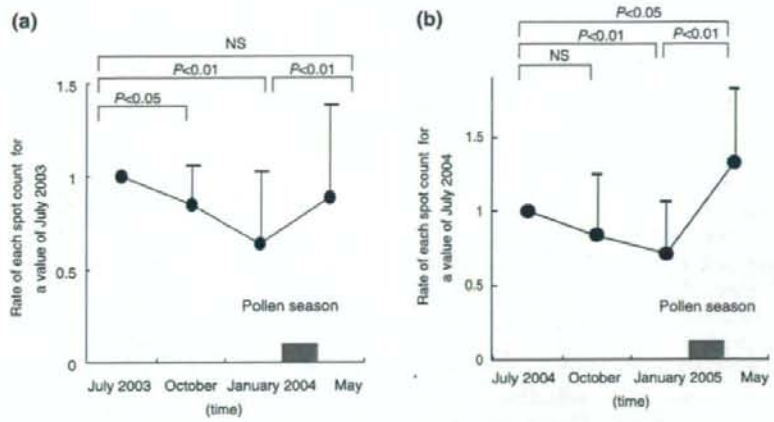

Fig. 3. The samples of each time-point were analysed simultaneously. The Cry j 1 and 2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique. The rate of each spot count for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

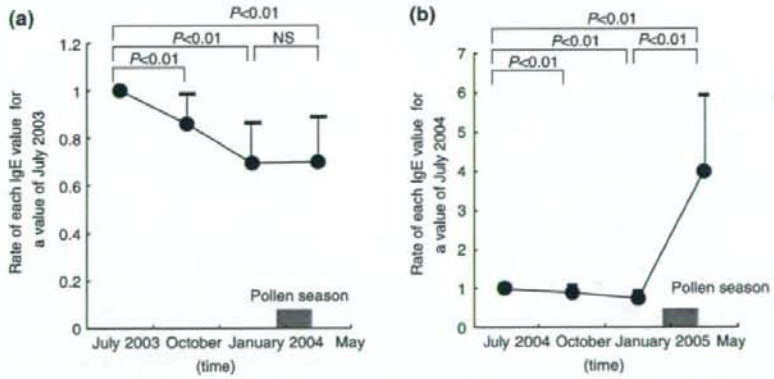

Fig. 4. The samples of each time-point were analysed simultaneously. Cedar-specific IgE were analysed using the RAST technique. The rate of each IgE for the value in July is shown. (a) Twenty-three patients were enrolled during the 2004 season, which was characterized by very little pollen scattering. (b) Eighteen patients were enrolled during the 2005 season, which was characterized by massive pollen scattering. NS, not significant.

2004 (Fig. 4a). The specific IL-4 spots decreased during the pollen season in some patients, and the cedar-specific IgE levels decreased during the pollen season in all patients. During the 2004–2005 season, the cedar-specific IgE levels decreased after the pollen season in 2004, but increased fivefold during the pollen season in 2005 (Fig. 4b). The number of cedar-specific IL-4 spots did not show a correlation with the cedar-specific IgE levels.

The yearly changes in the mite-specific IgE levels and Der f peptide-specific IL-4 spots were also examined in the samples from 22 patients with mite allergy. The yearly changes in mite-specific IgE and mite-specific memory Th2 clone size could not be clearly obtained (Fig. 5).

The patients with mite AR had persistent nasal symptoms all year around. Although the symptom scores varied among the patients as well as the seasons, the deviation was occasionally large; overall, no significant difference was observed. The pollinosis only demonstrated significant symptoms during the pollen dispersal season; however, the patients' nasal symptom scores during the pollen season were higher than those of the mite AR patients as shown in Fig. 6.

Discussion

We examined the Japanese cedar-specific IL-4-producing memory T cells in the peripheral blood of patients with

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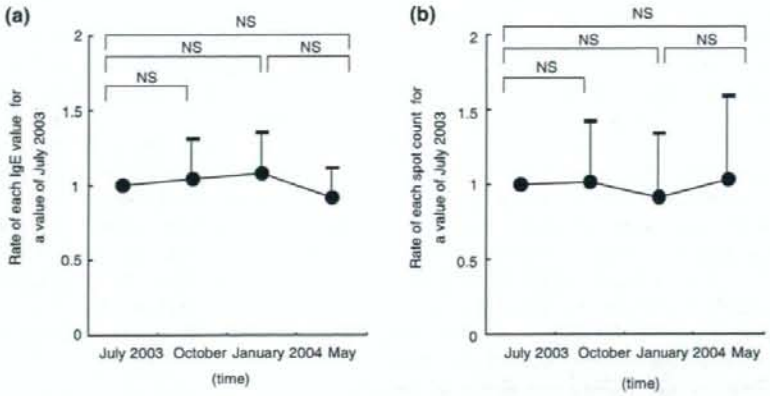

Fig. 5. The samples of each time-point were analysed simultaneously. Mite-specific IgE were analysed using the RAST technique (a). The Der f 1/2 peptide-specific IL-4-producing cells were counted using the ELISPOT technique (b). The rate of each value for the value in July is shown. NS, not significant.

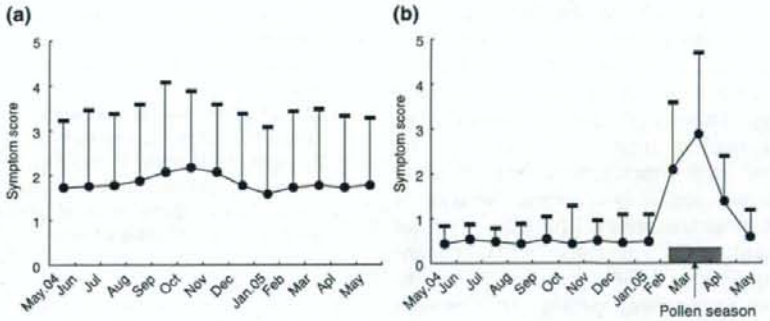

Fig. 6. The mean monthly symptom scores (mean+SD) from May 2004 to May 2005 are shown. (a) Mean nasal symptom scores of 22 patients with mite allergy. (b) Mean nasal symptom scores of 18 patients with Japanese cedar pollinosis.

Japanese cedar pollinosis by an ELISPOT assay using Japanese cedar-specific peptides. Although the number of cedar peptide-specific IL-4 T cells was low, the samples from all the patients examined had 10–100 spots/10^5 PBMC. In contrast, the healthy subjects had no positive IL-4 spot. These IL-4 T cells were thought to be cedar-specific Th2. The cedar peptide-specific IFN-γ T cells, which were thought to be cedar-specific Th1 cells, were detected in several patients. The cedar-specific peptides used in the ELISPOT assay were hybrid peptides that would only react with the T cells of those patients with Japanese cedar pollinosis, but not with the T cells of subjects not suffering from cedar pollinosis. Consequently, these findings could explain the low incidence and detection rates of cedar peptide-specific Th1 cells. The number of cedar peptide-specific IL-4 T cells (Th2 cells) did not correlate with the numbers of specific IFN-γ T cells (Th1 cells). More specifically, no relationship was observed between the cell types, and the cell numbers appeared to be independent of each other (although cedar-specific serum IgE levels did not correlate with the number of cedar-specific IL-4 T cells, the patients who had specific IFN-γ T cells had lower specific IgE levels than those without specific IFN-γ T cells).

IgE production is likely to be controlled by many factors. IFN-γ T cells inhibit IgE production independently of the number of specific IL-4 T cells [21, 22]. Similarly, pollen-specific IgE is known to exhibit seasonal changes [23, 24], thus increasing during the pollen season and decreasing during the off-season. However, the amounts of cedar and cypress pollen in 2004 were extraordinarily low and the pollen counts, which were less than 1/20 of the annual average for the last 10 years, did not contribute to the enhancement of IgE production in the patients enrolled in this study. Conversely, the cedar peptide-specific IL-4 T cells increased by 30% despite the low levels of pollen exposure. The levels of Th2 clone size increased to levels equivalent to that in July 2003.

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Although IL-4 is indispensable for IgE production, the cedar peptide-specific Th cells are thought to be very sensitive to exposure to cedar pollen.

In contrast, because the amount of pollen in 2005 was large, the cedar-specific IgE levels increased to five times after the pollen season. The specific Th2 clone size increased considerably after the pollen season, too, and the clone size increased by 70% after the pollen season. The levels of clone size increased to 1.4 times more than those observed in July 2004. A memory clone size might be affected by allergen exposition considerably. While cedar peptide-specific IL-4 Th2 cells decreased in number during the off-season, 8 months after the cessation of the pollen season, more than 60% of these memory Th2 cells were still found. Such annual drifting was absent in the perennial-allergic subjects. Therefore, pollinosis that undergoes exposition for a very limited period is different from mite perennial allergies.

Indeed, it is difficult to estimate the half-life of allergen-specific Th2. Generally, memory T cells are thought to have a long life-span, considering the duration of the vaccine effect of viral infections. Almost all reports of antigen-specific memory T cell have so far been about infectious diseases, while only a few reports of memory T cell are about type I allergy. The simple theory that an antigen creates a pool of long-lived antigen-specific memory T cells has been surprisingly difficult to prove and it is also still not completely accepted. The issue of longevity is much more controversial for $CD4^+$ than for $CD8^+$ T cells. Regarding $CD4^+$ memory T cells, lymphocytic choriomeningitis virus (LCMV)-specific $CD4^+$ memory T cells [25] and Sendai virus-specific $CD4^+$ memory T cells [26] at first disappeared dramatically, whereas virus-specific $CD8^+$ T cells were fairly stable in numbers over the same period. In humans, however, there have been several reports of vaccinia virus-specific $CD4^+$ memory T cells with a long life-span. These cells were found to decline with a half-life of approximately 10 years, conferring long-term protection [27]. The question remains, however, as to whether these memory Th cells are maintained in the absence of an antigen or by contact with a persistent antigen, bound in the immune complexes on follicular dendritic cells, or by contact with other environmental cross-reactive antigens. Based on our results on memory Th2 cells from cedar pollinosis in comparison with those from perennial mite allergy, the clone size maintenance requires antigen irritation of repetition and clonal mitosis by it. Therefore, the half-life of allergen-specific Th2 might be estimated to be less than 1 year.

The mechanism wherein a daughter Th2 cell discharges Th2 cytokines such as IL-4 in the same way after mitosis from the original Th2 has not been well understood. The opening and closing of chromatin helps to determine the cellular characteristics. It is believed that acetylation and methylation of the chromatin are very stable. Therefore, it is thought that the characteristics of a parent cell are transferred to the daughter cells after mitosis. However, a splitting enzyme for the acetylation and methylation has been discovered in a recent study, and it does not always seem to be stable [28]. It has recently been proposed that the opening and closing of chromatin is induced by an antigen, and that this is due to the Th cells' response [29, 30]. These changes in chromatin may therefore play an important role in retaining the memory functions of Th cells. Further studies need to be conducted on memory retention in order to clarify how these mechanisms could be applied to the development of an effective and fundamental solution for the treatment of allergic diseases.

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Ortho-phthalaldehyde–induced anaphylaxis after laryngoscopy

To the Editor:

In August 2005, a 25-year-old woman was referred to our outpatient clinic for evaluation of a surgical indication for a vocal cord papilloma. She had undergone once-a-month check-ups by laryngoscopy for the previous 5 months. On the day of her first visit to our hospital, an anaphylactic reaction occurred, with dyspnea, runny nose, systemic urticaria, tachycardia, and a mild decrease in the blood pressure at the end of a laryngoscopic examination. The anaphylaxis was brought under control by administration of epinephrine, hydrocortisone, and chlorpheniramine.

Two weeks after the anaphylaxis, a search for the causal agent was performed. She had cedar pollen rhinitis but no history of asthma or atopic eczema. Her serum IgE was mildly elevated (340 IU/mL). After informed consent was obtained, skin tests and challenge tests were performed for latex and the local anesthetics used during the laryngoscopic procedure, but no local or systemic allergic reactions were observed. We next focused on a disinfectant solution containing 0.55% ortho-phthalaldehyde (OPA) that was routinely used for pretreatment of the laryngoscope at both the previous hospital and our hospital. The solution showed a positive reaction in an intracutaneous test at a 1:1000 dilution (wheal of 6 × 6 mm and flare of 22 × 15 mm) at 15 minutes, which lasted for more than 24 hours. Four healthy volunteers had a negative result to this test. These results suggested that the disinfectant was the cause of her symptoms, via either an immunologic or, with a remote possibility, an irritant reaction. Accordingly, subsequent laryngoscopic examinations were performed using an OPA-disinfected, thoroughly rinsed fiberscope, and the patient showed no allergic symptoms.

A histamine release test using the patient's basophils was performed. Briefly, basophils were exposed to serial dilutions of the OPA-containing disinfectant and pure OPA solution (original concentration of OPA in both solutions: 0.55%) in a buffer containing 0.03% human serum albumin. It revealed that both the disinfectant and pure OPA solution evoked obvious release of histamine (Fig 1, *A*), but no release was observed for basophils from 3 healthy control donors. Next, sensitization experiments were performed; basophils from healthy subjects were treated at 4°C for 5 minutes with acetate buffer (pH 3.7) to remove surface-bound IgE, sensitized at 37°C for 2 hours with the patient's serum and then exposed to OPA. As a result, after sensitization with the patient's serum, basophils from the control donors released histamine in response to OPA, although they did not release histamine when the patient's serum was heated for 2 hours at 56°C before use (Fig 1, *B*). These results clearly demonstrated that the patient's anaphylaxis was mediated by OPA and IgE in her serum.

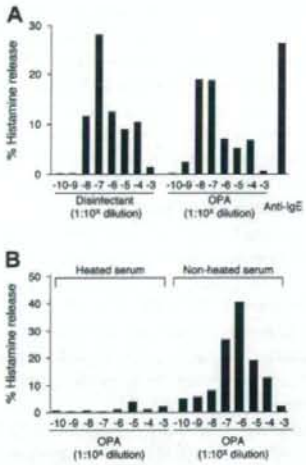

FIG 1. A, Histamine-release tests using the patient's basophils. The histamine in the supernatant was measured. Anti-IgE antibody at 14 μg/mL was used as a positive control stimulus. Data are representative of 2 separate experiments. **B,** Histamine release tests of basophils sensitized with the patient's serum. Data are the mean values of 3 separate experiments.

This is the first report of anaphylaxis following OPA-disinfected laryngoscopy. Skin tests and *in vitro* histamine release tests were useful for identifying the allergen in this case. Based on our *in vitro* findings, the participation of OPA-specific IgE antibody in the pathogenesis of the anaphylaxis is strongly indicated. This patient had been sensitized before referral to our hospital, presumably by repeated exposure to trace amounts of OPA remaining on the fiberscope. Importantly, extensive rinsing (>15 minutes) of the fiberscope decreased the amount of residual OPA below the threshold level capable of triggering an anaphylactic response in this patient.

Ortho-phthalaldehyde is a recently developed disinfectant.[1] This compound has several advantages compared with the commonly used glutaraldehyde, including high stability over a wide pH range, and a negligible level of evaporation, leading to a lower risk of mucosal irritation in medical workers.[2] However, OPA is a low-molecular-weight substance and can probably act as a hapten after conjugation to carrier proteins, presumably in a similar fashion to glutaraldehyde.[3] In our histamine release test, we assume that OPA was haptenated to a constituent protein (eg, albumin) in the test system, enabling it to elicit histamine release from the basophils. Recently, Sokol[4] reported 4 patients demonstrating anaphylaxis after cystoscopy and positive skin prick tests for OPA. That report and the present case collectively suggest that repeated exposure to OPA-disinfected equipment can sensitize some patients, and that OPA can rarely cause IgE-mediated anaphylaxis in

sensitized subjects. Allergists thus need to be aware of such potential allergens to which patients may be exposed in routine medical procedures.

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Obesity and airway inflammation in asthma

To the Editor:

The concurrent increase in the prevalence of obesity and asthma leads to the interest in potential mechanisms linking these 2 epidemics.[1] Obesity has been recently suggested as a proinflammatory state,[2] but the links with airway inflammation are still scarce. Our study aimed to investigate the association between overweight or obesity assessed by the body mass index (BMI) and asthma airway inflammation assessed by exhaled nitric oxide (F_{ENO}), a simple, quick, noninvasive, and highly reproducible surrogate marker of airway inflammation that could enhance diagnosis and management of asthma.

After informed consent, 297 nonsmoking patients, age 15 to 73 years, with a medical diagnosis of asthma were recruited from an asthma and allergy outpatient clinic at University Central Hospital in Porto. The study protocol included F_{ENO} measurement using the online technique with the NIOX system (Aerocrine, Stockholm, Sweden)[3]; FEV_1 determination using PIKO-1 (Ferraris Respiratory, Hertford, United Kingdom)[4]; and body weight and height anthropometric measurements using a mechanical balance with stadiometer (Seca model 700; Seca, Hamburg, Germany) followed by BMI determination (weight/height^2). The World Health Organization BMI Classification was used to define underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25.0-29.9 kg/m^2), and obesity (≥30.0 kg/m^2).[5] Atopy, defined by skin prick test to common aeroallergens, and inhaled corticosteroid use were registered. The data analysis was performed by using the statistical package SPSS, 12.0 version (SPSS Inc, Chicago, Ill). F_{ENO} was logarithmically transformed to attain normal distribution. Spearman correlation, linear regression, and general linear model were fitted to estimate the association between BMI and F_{ENO} and the effect after adjusting for age, FEV_1, sex, atopy, and inhaled corticosteroid use. A .05 level of significance and 95% CIs were considered.

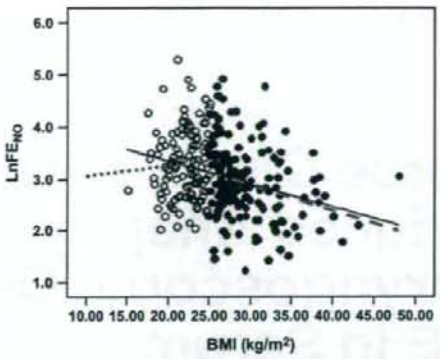

FIG 1. Association between BMI (kg/m^2) and F_{ENO} (ppb) in patients with asthma. (...) Normal/underweight regression line (R^2 = 0.004); (_ _) overweight/obese regression line (R^2 = 0.074); (__) all patients regression line (R^2 = 0.094). (○) Normal/underweight patients with asthma; (●) overweight/obese patients with asthma. *LnF_{ENO}*, F_{ENO} logarithmically transformed.

Subjects' mean (SD) age was 39 (15.1) years, 76% (n = 226) were female, 55% were atopic, and the mean (SD) percent predicted FEV_1 was 85.7 (20.5). Geometric mean (95% CI) of F_{ENO} was 30.6 (27.9-33.6) and of BMI was 26.0 (25.4-26.5). One in 5 (20%) of the patients was obese, 38% were overweight, 40% were normal, and 2% underweight. Spearman correlation and linear regression analysis showed a negative association between mean BMI and mean F_{ENO} ($r_s = -0.329$, $P < .001$; B = −0.044; 95% CI, −0.060 to −0.028; P < .001). A different relationship was noted between overweight or obese patients with asthma with the others. In normal weight/underweight patients with asthma, no association between BMI and F_{ENO} ($r_s = -0.061$, $P = .499$; B = 0.019; 95% CI, −0.039 to 0.077; $P = .511$) was found. In overweight or obese patients with asthma, a significant negative association with F_{ENO} ($r_s = -0.307$, $P < .001$; B = −0.049; 95% CI, −0.075 to −0.023; $P < .001$) was observed (Fig 1). By general linear model analysis, BMI maintained the negative association with F_{ENO} (B = −0.032, 95% CI, −0.051 to −0.014; $F = 11.75$, $P = .001$) after adjustment for age, FEV_1, sex, atopy, and inhaled corticosteroid use. Age was also negatively associated with F_{ENO} (B = −0.014; 95% CI, −0.020 to −0.008; $F = 18.10$, P < .001). In overweight or obese patients with asthma, BMI (B = −0.047, 95% CI, −0.077 to −0.017; $F = 9.38$, $P = .003$) and age (B = −0.013, 95% CI, −0.022 to −0.003; $F = 6.23$, $P = .014$) maintained a significant

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CASE REPORT

Awarded Article Annual Meeting of JSA

Three Cases of Ortho-phthalaldehyde-induced Anaphylaxis after Laryngoscopy: Detection of Specific IgE in Serum

Maho Suzukawa[1], Akiko Komiya[1], Rikiya Koketsu[1], Ayako Kawakami[1], Miwako Kimura[2], Takaharu Nito[2], Kazuhiko Yamamoto[1] and Masao Yamaguchi[1]

ABSTRACT

Background: Ortho-phthalaldehyde (OPA) has recently been used as a disinfectant for various medical apparatuses. OPA is not generally recognized as a potential allergen.

Case Summary: Subsequent to our recent report describing a patient presenting with OPA-induced anaphylaxis following laryngoscopy, we experienced two more such cases. In all three cases, the basophil histamine release test was useful for identifying the allergen as OPA. OPA-specific IgE was successfully detected in the serum of the patients by ELISA.

Discussion: Physicians and co-medical workers need to be aware of potential allergens to which patients may be exposed during routine medical procedures.

KEY WORDS

basophils, ELISA, histamine release, IgE-mediated anaphylaxis, skin test

INTRODUCTION

Ortho-phthalaldehyde (OPA) is a recently developed disinfectant.[1] Although glutaraldehyde is still widely used for disinfection of various medical apparatuses, increasing numbers of hospitals are adopting OPA, since this compound is not volatile and is much less irritable to medical workers.[2,3] Here we report three cases of OPA-induced anaphylaxis after laryngoscopy. Our *in vitro* analyses clearly demonstrated that OPA-specific IgE was present in the serum of all three patients.

CLINICAL SUMMARY

CASE 1

The clinical course of this patient was already described elsewhere.[4] In brief, a 25-year-old woman developed an anaphylactic reaction with dyspnea, runny nose, systemic urticaria, tachycardia and a mild decrease in the blood pressure following a laryngoscopic procedure for assessment of a vocal cord tumor in August 2005. She had undergone once-a-month checkups by laryngoscopy for the previous five months. Two weeks after her anaphylaxis had disappeared, skin tests and challenge tests were performed for latex and local anesthetics, since they had been used during the laryngoscopic procedure, but no allergic reactions were observed. However, a disinfectant solution containing 0.55% OPA, which was routinely used for pretreatment of the laryngoscope, showed a positive reaction in an intracutaneous test at a 1 : 1000 dilution. Her serum IgE level was 340 IU/ml.

CASE 2

In February 2006, a 36-year-old otolaryngologist de-

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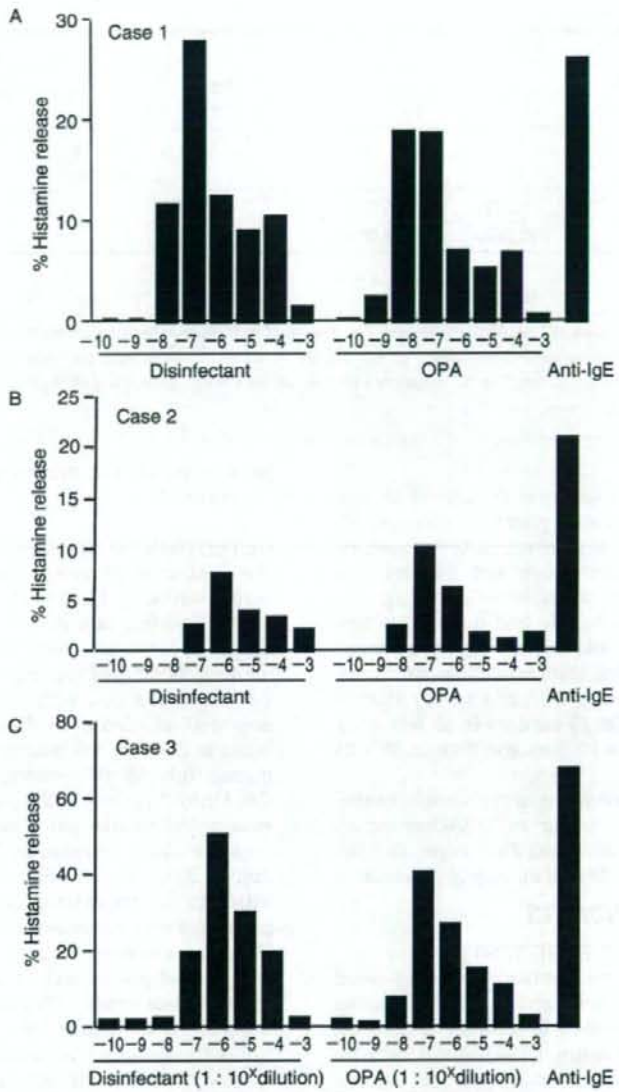

Fig. 1 Results of histamine release tests using basophils from patients 1 (**A**), 2 (**B**) and 3 (**C**). Basophils were exposed to serial dilutions of the OPA-containing disinfectant and pure OPA solution (original concentration of OPA in both solutions: 0.55%) for 45 minutes at 37℃, and histamine released in the supernatant was assayed. Anti-IgE antibody at 14 μg/ml was used as a positive control stimulus. Data are mean values of duplicate determinations. The result for patient 1 was already reported.[4]

veloped an anaphylactic reaction including tachycardia, sneezing, runny nose and general urticaria immediately after a laryngoscopic demonstration of his vocal cord. Treatment with chlorpheniramine and hydrocortisone was effective in relieving his symptoms. He had previously performed such laryngoscopic procedures many times. His serum IgE level was 36 IU/ml.

Table 1 ELISA for OPA-specific IgE.

	Patient 1	Patient 2	Patient 3	Control 1	Control 2	Control 3	Control 4
Exp 1	0.034 (0.007)			0.008 (0.008)			
Exp 2	0.032 (0.012)				0.008 (0.008)		
Exp 3		0.272 (0.097)	0.206 (0.089)			0.087 (0.078)	
Exp 4		0.142 (0.051)					0.058 (0.074)

The detailed assay method was as described in the text. The results of four representative experiments are presented. Other experiments using case 1 serum samples showed essentially similar OD levels. All experiments were performed in duplicate, and the data are the mean OD values measured at 450 nm. The OD values of samples loaded in wells without any HSA-precoating step prior to OPA treatment are shown in parentheses.

CASE 3

In March 2006, a 58-year-old man developed an anaphylactic reaction immediately after laryngoscopic examination of a pharyngeal tumor. His symptoms, including tachycardia, runny nose and general urticaria, improved following treatment with chlorpheniramine and hydrocortisone. He had undergone several laryngoscopic examinations during the previous 10 months. His serum IgE level was 73 IU/ml. A disinfectant solution containing OPA at a 1 : 100 dilution gave a positive reaction at 15 minutes in an intracutaneous test (wheal of 14 × 10 mm and flare of 28 × 24 mm).

In all three cases, subsequent laryngoscopic examinations were performed using an OPA-disinfected, thoroughly rinsed (>15 minutes) fiberscope, and the patients never again manifested allergic symptoms.

PATHOLOGICAL FINDINGS

IN VITRO HISTMINE RELEASE TESTS

After informed consent was obtained, venous blood was drawn from the patients, and *in vitro* histamine release tests were performed in a buffer containing 0.03% human serum albumin (HSA).[5,6] All *in vitro* tests and skin tests were performed after an interval of at least two weeks after the anaphylactic episode. Also, basophils obtained from at least one or two control subjects were included in each experiment using the patients' basophils. Importantly, the tests demonstrated that both the disinfectant and pure OPA (purchased from Wako Pure Chemical, Osaka, Japan) evoked obvious release of histamine not only from the basophils of case 1 (Fig. 1A),[4] but also from the basophils of case 2 (Fig. 1B) and case 3 (Fig. 1C). On the other hand, the basophils from healthy control donors did not show histamine release in response to OPA. As already noted elsewhere, passive sensitization experiments revealed that the serum of case 1 contained an OPA-specific, heat-sensitive component capable of sensitizing basophils from control subjects, implying the presence of OPA-specific IgE in the serum.[4]

DETECTION OF SPECIFIC IGE BY ELISA

We next tried to detect the specific IgE in the patients' serum by ELISA. The procedures were as follows: 1) coating of a 96-well polystyrene plate (Nunc, Roskilde, Denmark) with 1% HSA in PBS at 4℃ overnight; 2) additional coating with 0.55% OPA at 4℃ for 1 hour; 3) blocking with 1% HSA in PBS at 4℃ for 3 hours; 4) addition of 2× diluted serum samples, incubated at 37℃ for 2.5 hours; 5) addition of biotin-anti-human IgE Ab (Biosource International, Camarillo, CA, USA) 2 μg/ml at 37℃ for 1 hour; 6) addition of streptoavidin-horseradish peroxidase (Amersham Bioscience Corp, Piscataway, NJ, USA) at 2 × 10^5 dilution at 37℃ for 30 minutes; 7) color development with 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) at room temperature for 15 minutes; 8) addition of 3 N phosphate; and 9) measurement with a microtiter plate reader (Bio-Rad, Hercules, CA, USA). The tests demonstrated the presence of OPA-specific IgE in the sera from all three patients (Table 1), as indicated by higher OD levels compared to the control serum samples. In addition, it was possible to detect the difference in OD values between the patients and normal controls even if the serum samples were diluted up to 10-fold. Precoating of the plates with albumin prior to treatment with OPA was essential; when the precoating step was omitted, detection of OPA-specific IgE was unsuccessful. These results showed that specific IgE in the serum of OPA-induced anaphylactic subjects can be detected by our ELISA system, and that conjugation of OPA to proteins is critical for this assay method.

DISCUSSION

After we first reported a case of anaphylaxis following an OPA-disinfected laryngoscopic procedure,[4] we ex-

perienced two additional similar patients, described here as cases 2 and 3. Both *in vitro* histamine-release tests and ELISA were useful methods for identifying the allergen and the specific IgE. The interaction of OPA and OPA-specific IgE antibody may be directly involved in the pathogenesis of anaphylaxis in the patients. Presumably these patients had been sensitized by repeated exposure to OPA remaining on the fiberscope.

Demonstration of the involvement of specific IgE is often difficult for anaphylactic reactions induced by low-molecular-weight substances. In fact, immobilization of such small substances often destroys their antigenicity, making it difficult to detect specific IgE binding. The Prausnitz-Küstner reaction is a traditional method for definitively demonstrating the presence of specific IgE, but it is not feasible in many situations since infection by unknown pathogens may occur. *In vitro* assay methods such as ELISA depend on the binding property of the molecule.[7,8] In this regard, OPA (molecular weight 134.1) may be an ideal molecule for establishing such an *in vitro* test system, since this molecule binds to carrier proteins. It could be said that OPA acts as a hapten when it induces an anaphylactic reaction *in vivo*, and that *in vitro* haptenated OPA may be antigenically potent, capable of eliciting basophil histamine release and ELISA detection. The present cases, in addition to Sokol's report,[9] collectively suggest that repeated exposure to OPA-disinfected equipment can sensitize some patients due to production of specific IgE, and that OPA may be a clinically important allergen that induces type I allergic reactions. Thus, it is important for us to be aware of such potential allergens to which patients may be exposed during routine medical procedures.

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IgE- and FcεRI-Mediated Enhancement of Surface CD69 Expression in Basophils: Role of Low-Level Stimulation

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Key Words
Basophils, CD69 · Der f 2 · Interleukin-3

Abstract
Surface-expressed CD69 is a recently recognized activation marker for basophils and is reported to be strongly induced in vitro by IL-3. In this study, we investigated whether IgE- and high-affinity receptor for IgE (FcεRI)-dependent stimuli can affect basophil CD69 expression. Highly purified basophils were cultured for 24 h in the presence of anti-FcεRI α-chain mAb, CRA-1 and IL-3, and surface CD69 expression was analyzed by flow cytometry. CRA-1 mAb at 1 ng/ml or lower concentrations, levels too low to provoke direct histamine release, dose-dependently enhanced surface CD69 expression in the presence of IL-3, although low-dose CRA-1 mAb failed to induce CD69 expression in the absence of IL-3. Recombinant Der f 2 at 10 to 100 pg/ml enhanced CD69 levels in the presence of IL-3 in basophils from mite-sensitive subjects. These results suggest that allergens may influence basophil CD69 expression even when the levels of the antigens are too low to trigger direct degranulation. Upregulated CD69 expression on locally accumulated basophils in bronchial asthma may be attributed at least in part to a combination of local cytokines, especially IL-3, plus exposure to low levels of IgE-crosslinking allergens.

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Introduction

Basophils comprise the least abundant circulating leukocytes, but they demonstrate abundant expression of high-affinity receptor for IgE (FcεRI) on their cell surface. Cross-linking of IgE by antigen induces activation of basophils, and various mediators secreted by activated basophils are thought to be important participants in the pathogenesis of IgE-mediated allergic diseases, such as asthma [1].

Accumulation of basophils occurs in local tissues during antigen-induced late-phase reactions and chronic allergic diseases [2, 3]. Both basophils and basophil-derived mediators have been identified in late-phase reactions induced by experimental antigen challenge of various organs [4, 5]. Based on the findings of previous reports, it is thought that locally migrated basophils possess an acti-

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vated phenotype, as suggested by basophil surface marker analysis including CD69 [6, 7]. For example, we recently reported that basophils, retrieved by bronchoalveolar lavage from patients with asthma, showed elevated levels of surface CD69 compared to their peripheral blood basophils, and that strong induction of surface CD69 expression occurs in vitro in the presence of relatively high doses of IL-3 [7]. Thus, CD69 was thought to be a useful marker of basophil activation with potentially clinical relevance, although we failed to observe any functional significance for basophil CD69. However, in that study, there was no apparent induction of basophil CD69 expression by IgE-cross-linking stimuli. In this study, we investigated whether IgE- and FcεRI-dependent stimuli can modulate basophil CD69 expression.

Materials and Methods

Reagents

The following reagents were purchased: FITC-conjugated anti-CD69 mAb (IgG1, clone FN50, Pharmingen, San Diego, Calif., USA), FITC-conjugated mouse IgG_1 mAb (Coulter Immunotech, Marseille, France), recombinant Der f 2 (Asahi Breweries, Tokyo, Japan), and IL-3 (PeproTech, London, UK). Anti-FcεRI α-chain mAb (CRA-1) was prepared as previously described.

Determination of Basophil Surface CD69 in vitro

CD69 expression on basophils was analyzed as previously described [7]. Briefly, basophils were purified from venous blood obtained from consenting volunteers with no history of atopic diseases. Briefly, basophils were semipurified by means of Percoll density gradient centrifugation; this was followed by negative selection through use of a MACS Basophil Isolation Kit (Miltenyi BioTech, Bergisch-Gladbach, Germany). The mean purity of the basophil preparations was 97.4%. Cells (2×10^4/200 μl) were cultured in RPMI 1640 (Gibco, Grand Island, N.Y., USA) supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 for 24 h in flat-bottomed 96-well culture plates (Iwaki, Chiba, Japan). IL-3 and/or CRA-1 mAb were included in the medium throughout the culture period.

For flow cytometry, cells were incubated first with human IgG (5 mg/ml) and then with 10 μg/ml of FITC-labeled anti-CD69 mAb. An isotype-matched FITC-labeled mouse IgG was used as a negative control. Stained cells were analyzed through use of an EPICS XL SYSTEM II (Coulter, Miami, Fla., USA). The median values of fluorescence intensity were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as described previously. Surface receptor levels were calculated with the following formula: (MESF of cells stained with anti-CD69 mAb) – (MESF of cells stained with control IgG).

In a part of the experiments, basophils were purified from mite-sensitive subjects with allergic asthma after obtaining informed consent. Cells were then cultured in the presence of IL-3 and/or various concentrations of Der f 2.

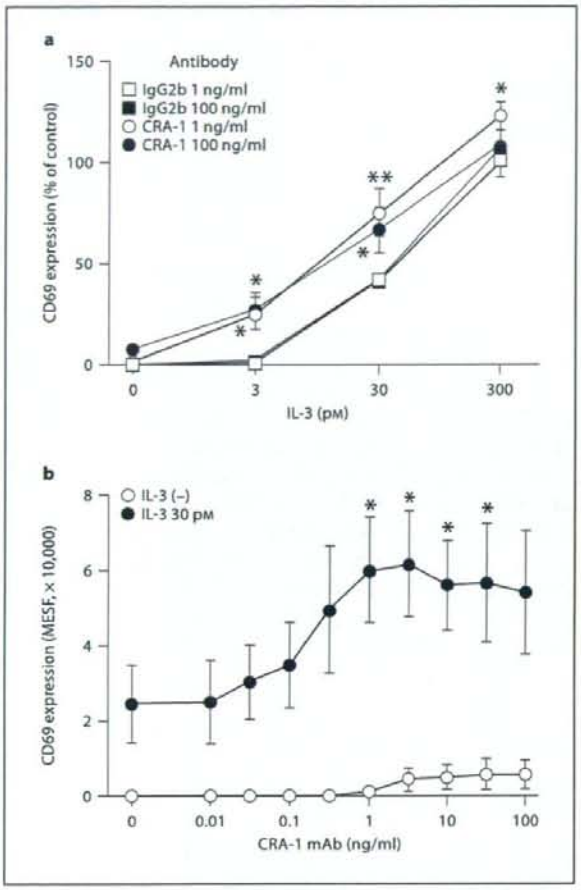
Statistics

All data are expressed as mean ± SEM. Differences between values in the in vitro experiments were analyzed by means of one-way ANOVA. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

Results

Highly purified basophils were cultured for 24 h in the presence of mouse IgG2b anti-FcεRI α-chain mAb (CRA-1) and IL-3, and surface CD69 expression was analyzed by flow cytometry. In the absence of IL-3, CD69 was induced weakly by relatively high concentrations of CRA-1 (fig. 1a, b); CD69 levels of basophils cultured with CRA-1 at 100 ng/ml were significantly higher than those of cells cultured with the same concentration of control IgG_{2b} (CD69 levels expressed in MESF units were 4,630 ± 1,730 and 150 ± 70, respectively ($n = 10$); $p < 0.05$). As shown in figure 1a, CD69 expression was obviously enhanced by this mAb in the presence of IL-3. IL-3 at as low as 3 pM, too low to induce CD69 by itself, induced CD69 when the cells were cultured in the presence of CRA-1 mAb. Interestingly, CRA-1 mAb at a relatively low dose (1 ng/ml) showed no less enhancement of IL-3-induced CD69 expression than at a much higher dose (100 ng/ml). We next analyzed the dependency of basophil CD69 expression on CRA-1 mAb in the presence or absence of IL-3. In the presence of IL-3 at 30 pM, CRA-1 at 1 ng/ml or lower concentrations, levels too low to provoke direct histamine release, dose-dependently enhanced surface CD69 expression (fig. 1b). Flow cytometric analysis suggested that CRA-1 mAb at 1 ng/ml demonstrated a low level of binding, only ~5% of the maximal binding capacity on basophils. These results indicate that low doses of CRA-1 mAb can potently affect basophil CD69 expression, for which the presence of IL-3 in the culture medium is essential.

Next, purified basophils from mite-sensitive asthmatics were cultured with recombinant Der f 2 and IL-3, and CD69 expression on basophils was analyzed. The results were essentially similar to those for basophils cultured with CRA-1 mAb; Der f 2 at 10 to 100 pg/ml (note that concentrations of Der f 2 at or above 30 pg/ml are usually necessary to provoke direct degranulation) enhanced CD69 levels in the presence of IL-3, whereas nearly no induction of CD69 was observed with Der f 2 alone (fig. 2).



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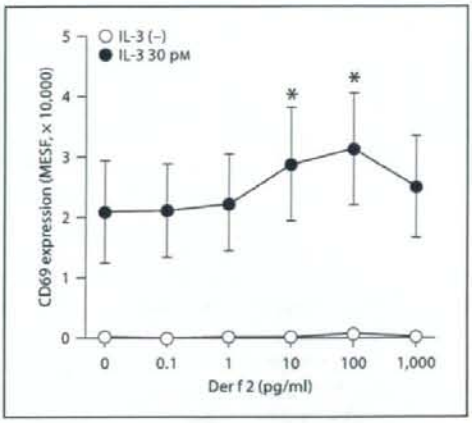

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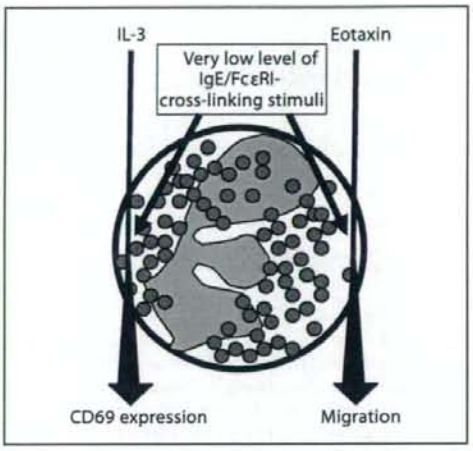

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Fig. 1. Modulation of basophil CD69 expression by anti-FcεRI α mAb. **a** The indicated concentrations of IL-3 and CRA-1 mAb were included in the medium. After culture for 24 h, CD69 expression on the basophils was analyzed by flow cytometry. The percentages of MESF were calculated based on the MESF of cells cultured with control IgG2b 1 ng/ml plus IL-3 300 pM; the actual MESF level of the control cells was 124,000 ± 9,000. Bars represent the SEM (n = 4). * $p < 0.05$, ** $p < 0.01$ versus corresponding values of cells cultured with control IgG2b. **b** Basophils were incubated with various concentrations of CRA-1 mAb in the presence and absence of IL-3 at 30 pM. Levels of surface CD69 expression are expressed using MESF units. Bars represent the SEM (n = 3). * $p < 0.05$ versus corresponding values of basophils cultured without CRA-1 mAb.

Fig. 2. Modulation of basophil CD69 expression by an antigen. Basophils obtained from mite-sensitive asthmatics were incubated with the indicated concentrations of Der f 2, with or without IL-3 at 30 pM. Data are mean ± SEM (n = 4). * $p < 0.05$ versus corresponding values of basophils cultured without Der f 2.

Fig. 3. Low level of IgE/FcεRI-cross-linking stimuli enhances both IL-3-induced CD69 expression and eotaxin-induced migration in basophils.

Discussion

Activation of basophils and mast cells is a hallmark aspect of IgE-mediated allergic reactions and clinical allergic diseases. And antigen- and IgE-dependent stimulation is thought to play a central role as the trigger of basophil and mast cell activation in allergic reactions. It is widely known that cross-linkage of IgE directly activates many changes in basophils, resulting in not only mediator release but also potently enhanced expression of surface integrin CD11b and other activation markers [6–8]. On the other hand, IL-3 is generally conceived as another

type of basophil-activating substance, and it potentiates the cellular activation profiles induced by IgE-cross-linkage or other stimuli [9, 10]. Our present results revealed a curious situation for regulation of basophil CD69 expression: IL-3 is a central inducer of CD69 expression, while antigen is an enhancing factor acting on basophils cooperatively with IL-3. Interestingly, very low doses of antigen or CRA-1 mAb maximally enhanced basophil CD69 expression in the presence of IL-3; such concentrations of antigen or CRA-1 mAb correspond to threshold or subthreshold doses for triggering degranulation of basophils.

The results of our present study bring to light a new aspect of IgE- and FcεRI-dependent events occurring in basophils. Recently, we demonstrated that similarly low concentrations of CRA-1 mAb (1 ng/ml) significantly enhance basophil migration towards eotaxin [11]. And a previous study by Bochner et al. [12] demonstrated that threshold doses of IgE-cross-linking stimuli can enhance CD11b expression on basophils. However, IgE-dependent weak stimulation does not affect all events in basophils; our preliminary experiments using pure basophils indicate that threshold doses of CRA-1 mAb do not suppress apoptosis or enhance the anti-apoptotic effect of IL-3 [13]. These results suggest that antigens may influence some, if not all, basophil functions even when the levels of the antigens are too low to provoke direct degranulation (fig. 3). The findings of the present study and our previous report collectively suggest that upregulated CD69 expression on locally accumulated basophils in bronchial asthma may be attributed at least in part to a combination of local cytokines, especially IL-3, plus IgE-cross-linking allergens. It thus may be important to elucidate the delicate actions of low-dose allergens on basophils, and probably on mast cells as well, in order to fully understand the pathogenesis of chronic asthma associated with continual exposure to low levels of environmental allergens.

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REVIEW ARTICLE

Human Basophils and Cytokines/ Chemokines

Masao Yamaguchi[1], Rikiya Koketsu[1], Maho Suzukawa[1,2], Ayako Kawakami[1] and Motoyasu Iikura[3]

ABSTRACT

Basophils comprise the smallest population in human peripheral blood leukocytes. The role of basophils in the pathogenesis of allergic diseases has long been obscure, although their accumulation and activation in tissues have suggested their potential importance. Recent advances in the field of basophil biology have indicated that cytokines and chemokines are the primary regulators of basophil functions. In addition, various functions of these cells seem differently modulated. The evidence strongly supports the notion that basophils exposed to these substances and allergens will behave as unique effector cells that presumably play proinflammatory roles in type I allergic reactions.

KEY WORDS

allergy, basophils, cell activation, chemokines, cytokines, human

INTRODUCTION

Basophils are the least abundant cell type among human peripheral blood leukocytes. They have a high affinity receptor for IgE (FcεRI) on their surface, and cross-linking of IgE molecules by specific antigens or anti-IgE antibodies, or ligation of FcεRI molecules by anti-FcεRI antibodies, leads to the liberation of preformed mediators such as vasoactive amines. Basophil activation also results in *de novo* synthesis of lipid mediators such as LTC4, in addition to cytokines including IL-4 and IL-13. Through release of these mediators and cytokines, basophils are thought to be active participants in the pathogenesis of IgE-mediated allergic inflammation.[1,2]

Several lines of evidence have suggested that basophils represent important effector cells in the pathogenesis of allergic late-phase reactions. Local accumulation of these cells is observed at sites of nasal and cutaneous late-phase reactions,[3,4] and it is reported that late-phase nasal secretions and bronchoalveolar lavage fluid contain a cell population which is morphologically identified as basophils.[5,6] In addition, analysis of the local chemical mediator profile in nasal secretions has indicated that basophils may be a significant source of the mediators in late-phase reactions.[4,7] Recently, Karasuyama *et al.* elegantly showed that basophils are a critically important player in the pathogenesis of IgE-mediated very-late-phase allergic responses and IgG-mediated anaphylactic reactions in mice[8-10] (also see the article by Karasuyama appearing in this issue).

Although the precise mechanisms of basophil accumulation and activation in local tissues of antigen-induced allergic inflammation and chronic allergic diseases such as asthma remain unclear, it is thought that there must be an active pathway that attracts and stimulates basophils, since those cells usually reside in circulating blood. Basophils are motile cells, and they seem to have a sophisticated sensor system relative to their surrounding environment.[11,12] In a series of analyses of basophils' biological functions, we have identified certain cytokines and chemokines as potentially important regulators that act on them. Interestingly, these biologically potent regulators modulate various arrays of basophil functions. In this article, we discuss how each function of basophils is regulated by cytokines and chemokines. And, in the last section, we describe our recent findings that a new cytokine of the IL-1 family, IL-33, is able to regulate diverse basophil functions.

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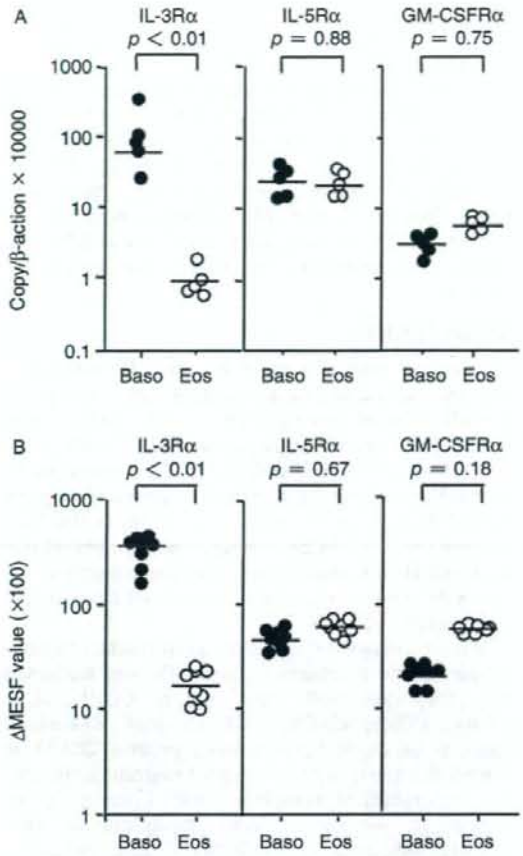

Fig. 1 Expression profiles of IL-3Rα, IL-5Rα, and GM-CSFRα in basophils vs. eosinophils.[20] (**A**) The expression levels of mRNA of IL-3Rα, IL-5Rα, and GM-CSFRα in basophils ($n = 5$) and eosinophils ($n = 5$). Data are expressed as the copy number ratio against β-actin. (**B**) The surface protein expression levels of IL-3Rα, IL-5Rα, and GM-CSFRα on basophils ($n = 8$) and eosinophils ($n = 8$). Data are expressed as ΔMESF values calculated using the following formula: (MESF of cells stained with specific mAb) - (MESF of cells stained with control IgG). MESF: molecules of equivalent soluble fluorochrome unit.

ACTIVATION

Accumulated evidence indicates that IL-3 is the most potent activator of mature human basophils. Although IL-3 is capable of inducing direct histamine release from basophils in a small and selected subset of atopic patients, it potentiates basophil degranulation in almost all normal as well as atopic individuals. Short-term (15 min) pretreatment of basophils with IL-3 results in marked enhancement of histamine release initiated by anti-IgE, formylmethionyl-leucyl-phenylalanine (FMLP), calcium ionophore A23187 and phorbol ester.[13-15] IL-3 also renders basophils susceptible to stimulation with secretory IgA, complement C3a or platelet-activating factor (PAF), which alone are unable to transduce signals sufficient for basophil degranulation.[16-18]

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 also enhance histamine releasability from basophils. Based on inspection of the ED_{50} values, IL-3 exerted the most potent priming effects (ED_{50}: 0.3 and 1 pM for anti-IgE- and FMLP-induced degranulation, respectively), while IL-5 and GM-CSF were ~30-fold less active compared with IL-3.[13,19,20] The difference in basophil sensitivity to the cytokines may reflect the varied receptor levels (Fig. 1A, B).[20] However, the maximal responses at the optimal doses of IL-5 and GM-CSF were equivalent to those induced by IL-3.

Insulin-like growth factor (IGF)-I enhanced histamine release initiated by anti-IgE, calcium ionophore A23187 and phorbor ester.[21] Stem cell factor (SCF) also weakly enhanced degranulation triggered by some, but not all, secretagogues.[22-24] Interestingly, IGF-I and SCF co-operatively enhanced surface expression of an activation marker, CD69, on basophils.[24] IL-1, interferon (IFN)-γ, and nerve growth factor (NGF), but not IL-4, tumor necrosis factor (TNF)-α, or transforming growth factor (TGF)-β, can also enhance basophil degranulation.[11]

Several studies, including ours, have shown that prolonged incubation with IL-3 profoundly affects basophil releasability. For example, basophils from 10 to 20% of donors completely fail to release histamine in response to anti-IgE, and such donors are called nonreleasers.[25,26] Long-term culturing (>3 d) with IL-3 results in these basophils becoming able to release histamine in response to anti-IgE or specific antigen.[27,28] Detailed intracellular signal analysis indicated that a defect in the tyrosine kinase Syk accounts for the lack of basophil response to IgE crosslinking stimulus in those donors, and that the defect is corrected after >3 days of culture with IL-3.[29]

IL-3 is also known to enhance the secretion of *de novo* synthesized mediators such as LTC4. At picomolar concentrations, short-term preincubation with IL-3 primes basophils for enhanced LTC4 secretion after challenge with anti-IgE or FMLP.[14] Basophils are reported to be an important source of Th2 cytokines, including IL-4 and IL-13; [30-32] although short-term pretreatment with IL-3 (30 min) does not affect IL-4 release from basophils, long-term incubation with IL-3 (18 h) results in a marked increase in IL-4 secretion.

In addition to their chemotactic activities on leukocytes, chemokines are able to directly activate basophils. As demonstrated by us and others, MCP-1/CCL2 was the most potent secretagogue among chemokines.[33,34] MCP-1 induced approximately 20%

of histamine release from freshly isolated basophils, which was apparently amplified by priming with IL-3.[35] IL-8/CXCL8 elicited weak degranulation from basophils, which was also enhanced by IL-3 pretreatment. On the other hand, freshly isolated basophils did not degranulate significantly in response to SDF-1/CXCL12, MIP-1α/CCL3 or MIP-1β/CCL4 in our study.[35]

We recently reported that basophils, retrieved by bronchoalveolar lavage from patients with asthma, showed elevated levels of surface CD69 compared to the patients' peripheral blood basophils, and that strong induction of surface CD69 expression occurs *in vitro* in the presence of relatively high doses of IL-3.[20,36] This was in clear contrast to the finding that other surface molecules on basophils such as CD44 or CD54 failed to demonstrate differences between healthy and asthmatic subjects. Thus, CD69 was thought to be a useful marker for basophil activation, with potential clinical relevance, although we failed to observe any functional significance for basophil CD69. We previously reported that expression of CD69 by basophils was preferentially upregulated by IL-3. CD69 analysis using basophils cultured for 24 h with various concentrations of cytokines showed that expression of CD69 required considerably large amounts of IL-3 (ED_{50}: 50 pM), whereas even as large as nanomolar orders of either IL-5 or GM-CSF induced only marginal levels of CD69 expression.[20] Interestingly, very low doses of antigen or anti-FcεRIα chain mAb (CRA-1) (1 ng/ml) clearly enhanced basophil CD69 expression in the presence of IL-3; such concentrations of antigen or CRA-1 mAb correspond to subthreshold doses for triggering degranulation of basophils.[37]

RT-PCR analysis demonstrated that basophils possessed transcripts for several Toll-like receptors (TLR), such as TLR4, TLR2, TLR9 and TLR10. Various TLR ligands were tested on freshly isolated basophils, but no functional changes were detected. After incubation of basophils with IFN-γ, a TLR4 ligand lipopolysaccharide slightly upregulated CD11b expression on basophils.[38,39]

ADHESION

Several lines of evidence indicate that IL-3 is involved in the regulation of basophil adherence to vascular endothelial cells. As reported by Bochner *et al.*, IL-3 stimulates *in vitro* adherence of basophils to vascular endothelial cells.[40] And their enhanced adherence was thought to be mediated by upregulation of surface β2 integrin (CD11b/CD18) expression. We found that not only IL-3 but also other hemopoietic growth factors, i.e., IL-5 and GM-CSF, up-regulated CD11b expression on basophils.[20] The receptors for these cytokines are known to share a common β subunit as a signal-transducing apparatus, but the potency of the three cytokines in enhancing CD11b expression was not equal. IL-3 was the strongest inducer of surface CD11b, and half-maximal induction was obtained at as low as 6 pM of IL-3. IL-5 and GM-CSF were less potent compared to IL-3, and ~10-fold higher doses of these two cytokines were necessary for significant induction of CD11b expression on basophils. In a recent study analyzing basophil rolling and adhesion under physiological shear flow conditions, P-selectin and β1 integrins (CD49d and CD49e/CD29) on basophils were involved in both rolling and adhesion on IL-3-treated endothelial cells.[41]

MIGRATION

In previous studies using a modified Boyden chamber and polycarbonate membrane with 5-μm pores, serially diluted hemopoietic growth factors were tested for basophil migratory activity.[42,43] We found that GM-CSF, IL-3 and IL-5 had marked effects on basophil locomotion: the maximal numbers of migrated basophils increased to more than 10 times the background levels. GM-CSF was slightly more potent than IL-3 and IL-5. Checkerboard analyses indicated that these cytokines had mainly chemokinetic rather than chemotactic effects.

We next examined the expression profile of a panel of chemokine receptors in basophils, and found that basophils expressed transcripts of CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR2 and CXCR4.[35,44] Among 16 chemokines tested, eotaxin/CCL11 induced the most potent basophil migration. In addition, migration of basophils toward eotaxin was enhanced by very weak FcεRI-crosslinking stimulus, CRA-1 mAb at 1 ng/ml.[45] SDF-1 also induced a strong, migratory response comparable to that induced by eotaxin in 24-h cultured basophils, reflecting CXCR4 expression induced during culture.[35,46] SDF-1 did not elicit any calcium influx in freshly isolated basophils but caused strong influx in 24-h cultured basophils. Similar to as we already demonstrated for eosinophils,[47,48] expression of CXCR4 on basophils was also regulated by several cytokines: it was markedly suppressed by IL-3 and slightly suppressed by GM-CSF, IL-5 and IL-4. However, IFN-γ, TGF-β and TNF-α each had no effect on basophil CXCR4 expression. CXCR4 expression on basophils was highly sensitive to IL-3, a femtomolar order of which was sufficient for inhibition of the expression.[35]

TRANSENDOTHELIAL MIGRATION (TEM)

We have established a basophil TEM assay using human basophils and Transwell systems (Costar, Cambridge, MA, USA) with cultivated human umbilical vein endothelial cells (HUVEC) on the surface.[49] HUVEC were activated by stimulation with IL-1β for 4 h before performing assays.

Figure 2 depicts the effects of various chemokines

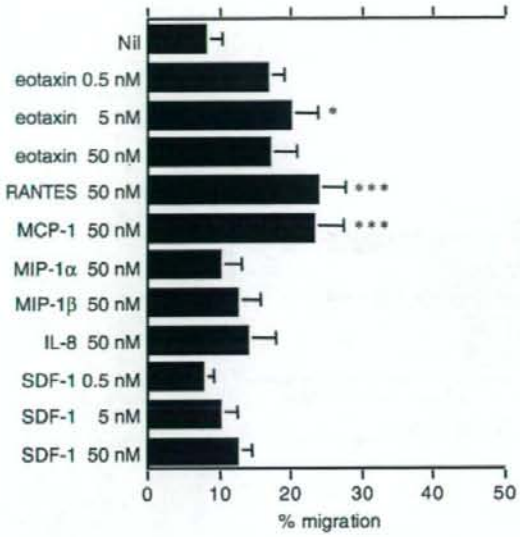

Fig. 2 Effects of various chemokines on basophil transendothelial migration (TEM).[49] HUVEC were pretreated with IL-1β (5 ng/ml) at 37°C for 4 h. TEM assay was performed in the presence of various chemokines at the indicated concentrations in the lower wells. Basophils were semi-purified by Percoll density centrifugation from peripheral blood, and immediately added to the upper wells. After 3 h of incubation at 37°C, the cells in the lower wells were collected, and the number of IgE-positive cells was determined by flow cytometry. Percentages of migrated basophils are shown, based on the total numbers of cells inoculated into the upper wells. *Bars* represent the SEM ($n = 4-7$).
$^{*}P < 0.05$, $^{**}P < 0.01$, vs. migration in the absence of chemokines.

on TEM of Percoll-separated human basophils.[49] Highly purified basophil preparations after Percoll and MACS separation gave essentially the same results. TEM was determined after 3 h of incubation using IL-1β-activated HUVEC. Among the various chemokines tested, CCR3 ligands, eotaxin/CCL11 and RANTES/CCL5 induced strong basophil TEM. Eotaxin binds specifically and exclusively to CCR3, whereas RANTES binds to both CCR1 and CCR3, although with higher affinity for CCR1. To determine the chemokine receptors responsible for RANTES-induced basophil TEM, we next performed blocking experiments using receptor-specific mAbs. As expected, eotaxin-induced TEM was almost completely inhibited by anti-CCR3 mAb. Although RANTES-induced TEM was also markedly blocked by anti-CCR3 mAb, treatment with anti-CCR1 mAb exerted virtually no effect, suggesting that CCR3 is the responsible receptor mediating the transmigratory response for both eotaxin and RANTES. This is also interesting in that it is the reverse of the relative affinities of RANTES for these two receptors, indicating that the affinity and TEM inducing activity of RANTES are not equivalent. Moreover, there was yet another chemokine receptor which induced basophil TEM: SDF-1, a specific ligand for CXCR4. Although freshly isolated basophils failed to exhibit TEM in response to SDF 1, strong TEM was observed with 24-h cultured basophils. In parallel to our previous findings regarding basophil chemotaxis,[35] these results show that both CCR3 and CXCR4 are involved in basophil TEM. We found that β2 integrin is the key adhesion molecule that mainly accounts for basophil TEM. β1 integrin is also slightly involved, since TEM of fresh basophils toward eotaxin or IL-3 was significantly suppressed by treatment of basophils with anti-CD18 mAb, and weakly suppressed by anti-CD29 mAb.[49]

The involvement of IL-3 was also assessed in that study.[49] When no chemokines were added to the lower wells, a significant increase in basophil TEM was observed when the cell suspension in the upper wells contained as low as 3 pM of IL-3. Furthermore, IL-3 showed an additive effect on eotaxin-directed TEM: the number of basophils that transmigrated toward eotaxin was significantly increased in the presence of IL-3. However, the magnitude of IL-3's effect on basophil TEM was modest compared to the strong effect of eotaxin.

TRANS-BASEMENT MEMBRANE MIGRATION

Basophil trans-basement membrane migration assay was performed using an experimental model, Matrigel (BD Biosciences, Bedford, MA, USA),[50] a gel containing basement membrane components, i.e., laminin, type IV collagen, heparan sulfate, proteoglycan and entactin.[51] Various chemokines were placed in the lower chamber. After 18 h of incubation, approximately 10 to 16% of the basophils had transmigrated spontaneously to the lower chamber. However, no apparent induction of transmigration was observed with any of the tested chemokines ("Nil" columns in Fig. 3A), showing a clear contrast to the findings for basophil TEM,[49] in which cells actively transmigrated towards certain chemokines. We next included the potent basophil-active cytokine, IL-3, at 300 pM in the upper chamber. As shown in Figure 3A ("IL-3" columns), IL-3 induced statistically significant basophil trans-basement membrane migration even in the absence of chemoattractants in the lower chambers. Moreover, among the chemokines tested, IL-8 and RANTES added to the lower chambers at 50 nM induced a significant increase in migration compared to the spontaneous migration of IL-3-treated basophils. Other chemokines, including MCP-1 and eotaxin, exhibited weak basophil attracting potency, but it did not reach statistical significance. Another well-

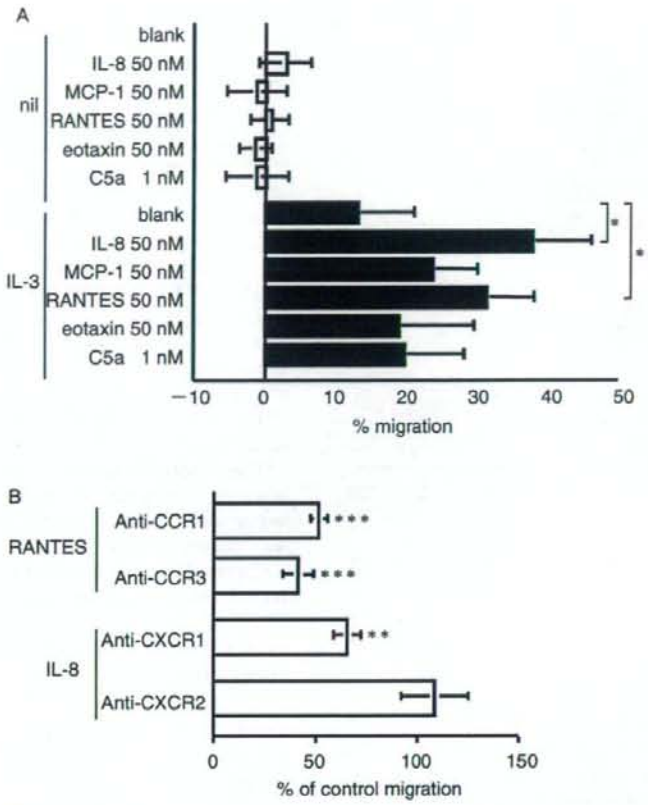

Fig. 3 Effects of chemokines on trans-basement membrane migration of human basophils.[51] (**A**) Percoll-separated basophils were suspended in medium with (*filled columns*) and without (*open columns*) IL-3 at 300 pM and subjected to the basophil trans-Matrigel migration assay. Various chemokines or complement C5a were placed in the lower chamber. After incubation for 18 h, cells that had migrated to the lower chambers were counted by flow cytometry. The percentage of migrated cells was calculated by subtracting the spontaneous migration of IL-3-untreated cells (14.0 ± 3.6% for medium only). *Bars* represent the SEM ($n = 5$). $*P < 0.05$ and $**P < 0.01$. (**B**) Basophils were preincubated with anti-CCR mAb (20 μg/ml) or isotype control mAb (mouse IgG1, 20 μg/ml), or anti-CXCR mAb (1 μg/ml) or isotype control mAb (mouse IgG2a, 1 μg/ml) at 37℃ for 60 min, and the transmigration assay was performed in the presence of RANTES or IL-8 at 50 nM in the lower chamber and IL-3 at 300 pM in the upper chamber. *Bars* represent the SEM ($n = 5$). $**P < 0.01$ and $***P < 0.001$ vs. migrated cells pretreated with control antibody. The percent migration of basophils pretreated with control antibody was 56.4 ± 10.5% (RANTES) and 35.5 ± 5.8% (IL-8).

known chemoattractant for basophils, complement C5a, failed to show significant transmigration-inducing activity. On the other hand, among the lipid mediators tested, 5-oxo-ETE and PAF at 1 μM significantly induced basophil transmigration, but only when IL-3 was included in the upper chamber (data not shown). Thus, the repertoire of attracting substances involved in this basophil transmigration system was unique compared to that in TEM or simple migration. Blocking experiments clearly demon-

strated that both CCR1 and CCR3 were involved in the RANTES-directed trans-basement membrane migration of basophils, and that CXCR1, but not CXCR2, was involved in the case of IL-8 (Fig. 3B).[51] These results showed that not only CCR3 but also two other chemokine receptors, CCR1 and CXCR1, are involved in this transmigration across Matrigel. Anti-CD18 mAb but not anti-CD29 significantly inhibited IL-3 plus RANTES-induced transmigration, indicating that β2 integrin, but not β1 integrin, plays a central role in trans-Matrigel migration of basophils.

Importantly, IL-3 was essential for chemoattractant-induced transmigration, since, in the absence of IL-3, none of the tested chemokines upregulated basophil transmigration above the control level. In addition, an elastase inhibitor that blocks certain matrix metalloproteinases (MMPs) obviously attenuated transmigration of basophils. The results of quantitative real-time PCR and immunohistochemical studies suggested that MMP-9 is synthesized, stored in the cytoplasm, expressed on the cell surface and released by basophils, and flow cytometric analysis indicated that IL-3 upregulated surface MMP-9 levels on basophils.[51] Presumably the effects of IL-3 on the expression of both β2 integrin and MMP-9 are importantly involved in the transmigration: cell-surface MMP-9 and/or MMP-9 that has been released will cooperatively help basophils expressing enhanced levels of β2 integrin cross the basement membrane.

SURVIVAL

It was already reported that IL-3 maintained the viability of purified basophils during culture.[52] In the absence of cytokines, basophils rapidly died, and survived *in vitro* for only 3 days. Addition of IL-3 resulted in marked enhancement of basophil survival, such that nearly half of the cells remained viable for 14 days, as assessed by trypan blue stain. Several years after that initial study, the anti-apoptotic potencies of cytokines were analyzed by flow cytometry.[20,53] As judged by their plateau levels, IL-5 and GM-CSF were equally effective with IL-3 in protecting basophils from apoptosis. The rank order of potency as assessed by the ED_{50} values was IL-3 > IL-5 = GM-CSF, with IL-3 being ~10-fold more potent than the others. It should be noted that even a very low concentration of IL-3 was sufficient for survival enhancement (ED_{50}: 20 fM). IFN-γ is also reported to enhance basophil survival, but IL-4 or TNF-α is not.[11] On the other hand, SCF, in concert with IL-3, was shown to prolong survival of basophils.[23]

A glucocorticoid, dexamethasone, is known to induce apoptosis in basophils. To determine whether IL-3 can overcome the apoptosis-inducing effect of dexamethasone, basophils were incubated with serially diluted IL-3 in the presence and absence of dexamethasone (100 nM).[53] The enhanced life span induced by low concentrations of IL-3 (300 fM) was clearly shortened by dexamethasone, with statistical significance. On the other hand, dexamethasone exerted no significant effect on the rate of basophil apoptosis in the presence of higher concentrations of IL-3 (3 – 300 pM). Similar results were observed for the relationships between the effects of IL-3 and basophil-apoptotic substances other than glucocorticoids.[54]

EFFECT OF A NEW CYTOKINE, IL-33 ON ADHERENCE AND ACTIVATION OF BASOPHILS

IL-33 is a recently identified cytokine that belongs to the IL-1 family.[55] This cytokine binds to the ST2 receptor (also called DER4, Fit-1 or T1), which has high homology to IL-1 receptor.[55] ST2 receptor is reported to be expressed on mast cells[56] and Th2 cells,[57] but not on Th1 cells. ST2 has been considered to mediate the biological action of its ligand, IL-33, which can cause Th2-biased allergic inflammation. Accumulating evidence suggests that IL-33 can exert significant biological effects both *in vivo* and *ex vivo*.[55,57-59] For example, IL-33 enhances production of Th2-associated cytokines by *in vitro* polarized Th2 cells. In addition, in mast cells, we recently demonstrated that IL-33 enhanced the survival of human umbilical cord blood-derived mast cells and promoted their adhesion to fibronectin as well as their production of IL-8 and IL-13.[60] IL-33 is now recognized as a potentially important cytokine that enhances Th2-balanced immune regulation, but the action of IL-33 on allergic effector cells had not been known until recently.

In our very recent study analyzing the effects of IL-33 on human basophils, we demonstrated that basophils express the transcript and protein for ST2, a receptor for IL-33. IL-33 affected several arrays of basophil functions: this cytokine upregulated CD11b expression on the cell surface of basophils, enhanced eotaxin-directed chemotaxis, induced Th2 cytokine IL-4 secretion and augmented the IgE-mediated histamine release reaction.[61] Importantly, basophil adhesion was potently enhanced by IL-33, and this action of IL-33 was stronger than that of IL-3, a well-known basophil-active cytokine. Neutralization experiments demonstrated that ST2 mediates IL-33's effects on basophils. In other studies analyzing the actions of IL-33, we found that this cytokine also activates human eosinophils.[62] However, the precise effects of IL-33 on eosinophils differ somewhat from those on basophils: IL-33 failed to enhance migration and degranulation of eosinophils but suppressed eosinophil apoptosis,[61] whereas basophil apoptosis was not affected by IL-33. The different spectra of IL-33's effects on basophils and eosinophils may in part account for the different behaviors and fates of these effector cells in the controlling mechanisms of allergic inflammation.

These findings suggest that IL-33 may be a potent and unique regulator acting not only on lymphocytes