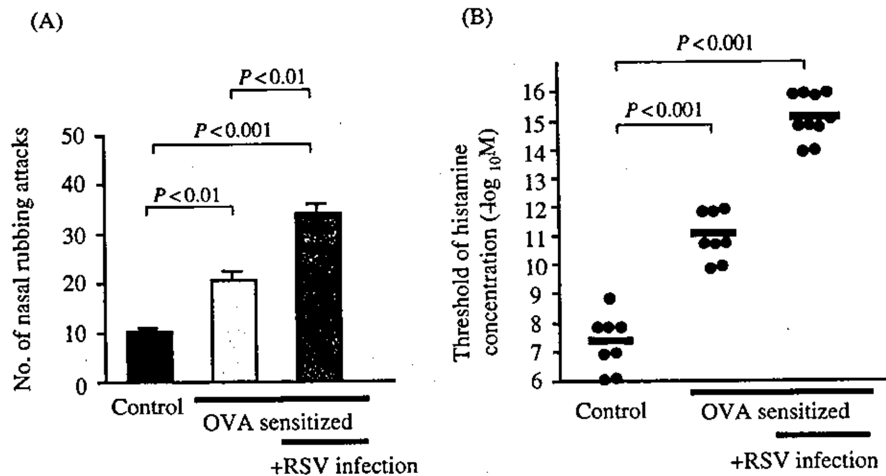

Fig. 7. Nasal rubbing attacks on provocation test with OVA (A) and sensitivity against nasally administered histamine (B) in B6 mice. Control: non-OVA sensitized, non-RSV infected mice; OVA sensitized: OVA sensitized but not RSV infected mice. OVA sensitized + RSV infection: OVA sensitized followed by RSV infected mice.

against the subsequent development of allergic diseases. Children who attend daycare are also known to have more frequent infections than those who remain at home. In a Japanese study [42], the presence of more older siblings at home had a protective effect against the development of allergic rhinitis (Table 2). However, studies examining the relationship between lower infection rates and the development of allergic rhinitis have produced either conflicting results or failed to establish any association [43]. Viral infection may induce Th2 cytokines and airway hypersensitivity and promote IgE synthesis; G glycoprotein of RSV is known to be a strong inducer of Th2 cytokines [44]. The nasal immune response to viral infection may depend on the type of virus as well as the age and immune condition of the patient [45, 46].

The role of bacterial infection in allergic rhinitis is also not clear. Although transient nasal hypersensitivity has been observed during the acute phase of common cold, the threshold for sneezing was rather higher if the patients had purulent rhinorrhoea of secondary bacterial infection [47]. However, no theory has been put forward to explain this effect.

Conclusions

To maintain pregnancy, the womb has evolved a Th2-dominant environment [48]. Following delivery, the Th2-dominant state prevails in the newborn infant into early life, when it is speculated that environmental factors such as viral infections, air pollution and diet greatly influence the development of well-balanced Th1/Th2 milieu [49].

In Japan, 50–80% of university students are estimated to be sensitized to Japanese cedar pollen [3]. Nowadays, people who are thus sensitized and carrying IgE antibody can be seen everywhere. Forecasts of cedar pollen scattering and predictions of the number of patients with Japanese cedar pollinosis are common. However, while the patient numbers keep rising, no solution has been given to the underlying problems and no end to the epidemic is in sight. The situation must be handled as a social matter. To fully understand nasal allergy, it is necessary for physicians to conduct accurate, large-scale epidemiological investigations to clarify the causes of the increased prevalence of allergic rhinitis. Subsequently, society as a whole will have to face and tackle the grave health problems presented by this epidemic.

Table 2. Difference in prevalence of allergic rhinitis in Japanese siblings

Child	Allergic rhinitis (%)
Oldest	8.2
Second	6.3
Third	4.9
Fourth	3.1

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Expression of membrane-bound CD23 in nasal mucosal B cells from patients with perennial allergic rhinitis

Shigetoshi Horiguchi, MD, PhD; Yoshitaka Okamoto, MD, PhD; Hideaki Chazono, MD; Daiju Sakurai, MD; and Kouichi Kobayashi, MD

Background: CD23 is the low-affinity receptor for IgE on B cells and is thought to play an important role in regulation of IgE production.

Objective: To measure the expression of membrane-bound CD23 in nasal B cells and examine its correlation with CD4 subtypes or serum IgE levels in patients with perennial allergic rhinitis.

Method: We used flow cytometric analysis with double, direct immunofluorescence staining of the mucosal-infiltrating lymphocytes to examine the expression of CD23 in nasal mucosal B cells of patients with perennial allergic rhinitis. The expression of CD23 in nasal B cells of patients with nonatopic rhinosinusitis served as a control.

Result: The ratio of $CD23^+$ B cells to total B cells in patients with perennial allergic rhinitis was significantly higher than in nonatopic controls, whereas that of B cells to total lymphocytes was unchanged. The ratio of $CCR4^+$ CD4 cells to total CD4 cells in allergic patients was significantly higher than in nonatopic controls, whereas the ratio of $CXCR3^+$ CD4 cells to total CD4 cells was unchanged. There was no significant correlation between the percentages of $CD23^+$ B cells and $CCR4^+$ CD4 cells. In addition, the percentage of $CD23^+$ B cells did not correlate with the total IgE level or with the specific IgE level.

Conclusions: Our results indicate that nasal mucosal CD23-bearing B cells, as well as T_H2 cells, increase in patients with perennial allergic rhinitis. However, the expression of CD23 did not directly correlate with the number of T_H2 cells in the nasal mucosa.

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INTRODUCTION

Allergic rhinitis (AR) occurs through fundamental mechanisms that involve induction of allergen-specific IgE antibodies. Allergen-specific T-cell–B-cell interactions are indispensable for the induction of human IgE synthesis, and it has recently been reported that interleukin 4 (IL-4) and other cytokines released from CD4 helper cells (T_H2 cells) affect T-cell–B-cell interactions and play a role in the induction of IgE synthesis in B cells.[1,2]

Human CD23 exists in 2 isoforms (CD23a and CD23b), which differ only in 6 or 7 amino acids at the N terminus. CD23 has the potential to associate with HLA-DR at the surface of B cells and in doing so may help to stabilize T-cell–B-cell interactions, which in turn contribute to T-cell activation.[3] The membrane-bound CD23 on B cells is thought to enhance IgE-dependent antigen presentation to T cells and also to influence IgE synthesis in the B cells. However, CD23 expression on B cells in the nasal mucosa and its possible correlation with relevant T_H2 cells in patients with allergic diseases have yet to be clarified. In the present study, we measured the expression of membrane-bound CD23 in nasal B cells and examined its correlation with CD4 subtypes or serum IgE levels in patients with perennial allergic rhinitis.

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan.
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MATERIALS AND METHODS

Patients

Japanese patients with serious perennial AR due to *Dermatophagoides pteronyssinus* were enrolled in this study. The diagnosis of AR was made based on the criteria of Okuda et al,[4] including a positive CAP radioallergosorbent test result (greater than class 2; SRL, Tokyo, Japan) against *D pteronyssinus*. None of the patients received immunotherapy or immunosuppressive drugs (including steroids) during the study. Japanese patients with nonatopic rhinosinusitis were enrolled as controls. Informed consent for participation in the study was obtained from each participant.

Tissue Samples

Inferior turbinate mucosa or paranasal mucosa was obtained by endonasal sinus surgery. After the mucosa was cut into small pieces (approximately 2 mm), tissue-infiltrating lymphocytes were collected with a cell strainer (Falcon, Discovery Labware, BD Biosciences, Bedford, MA), using the Ficoll-Hypaque separation technique (lymphocyte separation solution, Nacalai Tesque Inc, Tokyo, Japan). The tissue-infiltrating lymphocytes were washed twice with phosphate-buffered saline (PBS) and resuspended in a freezing solution

(Cell Banker, Nihon Zenyaku, Fukushima, Japan). The cells were stored at −80°C until examination.

Antibodies
Anti-human CD4, CD19, CD23, and CXCR3 monoclonal antibodies were purchased from Dako Corporation (Tokyo, Japan). Anti-human CCR4 monoclonal antibody was obtained from Genzyme (Boston, MA).

Flow Cytometric Analysis
The frozen cells were rapidly thawed and diluted 10 times with PBS that contained 1% bovine serum albumin (BSA). After 2 washes with PBS in 1% BSA, the cells were stained with an fluorescein isothiocyanate (FITC)–conjugated monoclonal anti-CD19 antibody combined with a R-phycoerythrin (RPE)–conjugated anti-CD23 antibody or with a FITC- or RPE-conjugated negative control antibody, according to the manufacturer's protocol. The cells were also stained with an FITC-conjugated anti-CD4 antibody combined with RPE-conjugated anti-CXCR3 or anti-CCR4 antibodies.

Cells were subjected to flow cytometric analysis using a flow cytometer (FACScan, Becton, Dickinson and Company, Franklin Lakes, NJ). A lymphocyte gate was set based on the pattern of forward and side scatter. A minimum of 5×10^4 cells in the gate was analyzed on the same day. B lymphocytes were identified as $CD19^+$ lymphocytes, and T_H cells were identified as $CD4^+$ lymphocytes. Cell viability was demonstrated by negative staining with 7-aminoactinomycin D (Sigma-Aldrich, St Louis, MO), which showed that at least 98% of the cells were viable.

Statistical Analysis
Statistical analysis was performed using a Wilcoxon rank sum test or a Wilcoxon signed rank test for paired and unpaired data. Statistical analysis was also performed using a Spearman rank correlation test for correlation between the data. $P < .05$ was considered statistically significant. Data are presented as mean ± SD.

RESULTS

Patients
Eleven Japanese patients (mean ± SD age, 41.1 ± 18.7 years; age range, 23–69 years; 5 men and 6 women) with serious perennial AR due to *Dermatophagoides pteronyssinus* were enrolled in the study as study patients. Eleven Japanese patients (mean ± SD age, 50.4 ± 14.3 years; age range, 24–71 years old; 7 men and 4 women) with nonatopic rhinosinusitis were enrolled as controls.

Dot Plots for CD19 FITC and CD23 RPE
Typical dot plots for CD19 FITC and CD23 RPE staining are shown for the control group and the AR group in Figure 1. Only $CD19^+$ cells expressed CD23 on mucosal lymphocytes, and CD23 expression on B cells from AR mucosa was higher than that of controls. The dot plot pattern of CD23 expression on nasal B cells suggested that this was not an all or nothing effect for a given cell but rather that B cells expressed various levels of CD23. Therefore, we measured the percent positive and mean fluorescence intensity (MFI) of CD23 on B cells, where the percent positive value indicates the relative amount

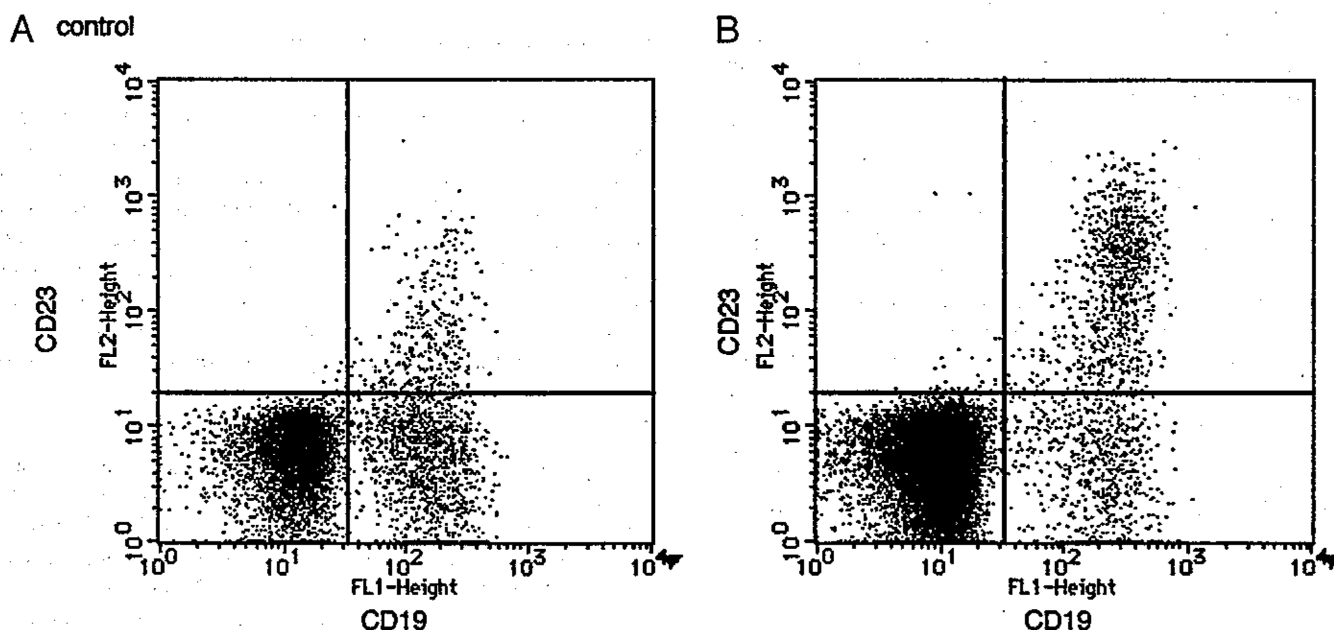

Figure 1. Representative dot plots for fluorescein isothiocyanate–conjugated anti-CD19 (FL1) and R-phycoerythrin–conjugated anti-CD23 (FL2) antibodies for the control group (A) and the allergic rhinitis (AR) group (B). The proportion of CD23-bearing B cells was measured by flow cytometry as CD19 and CD23 double-positive plots.

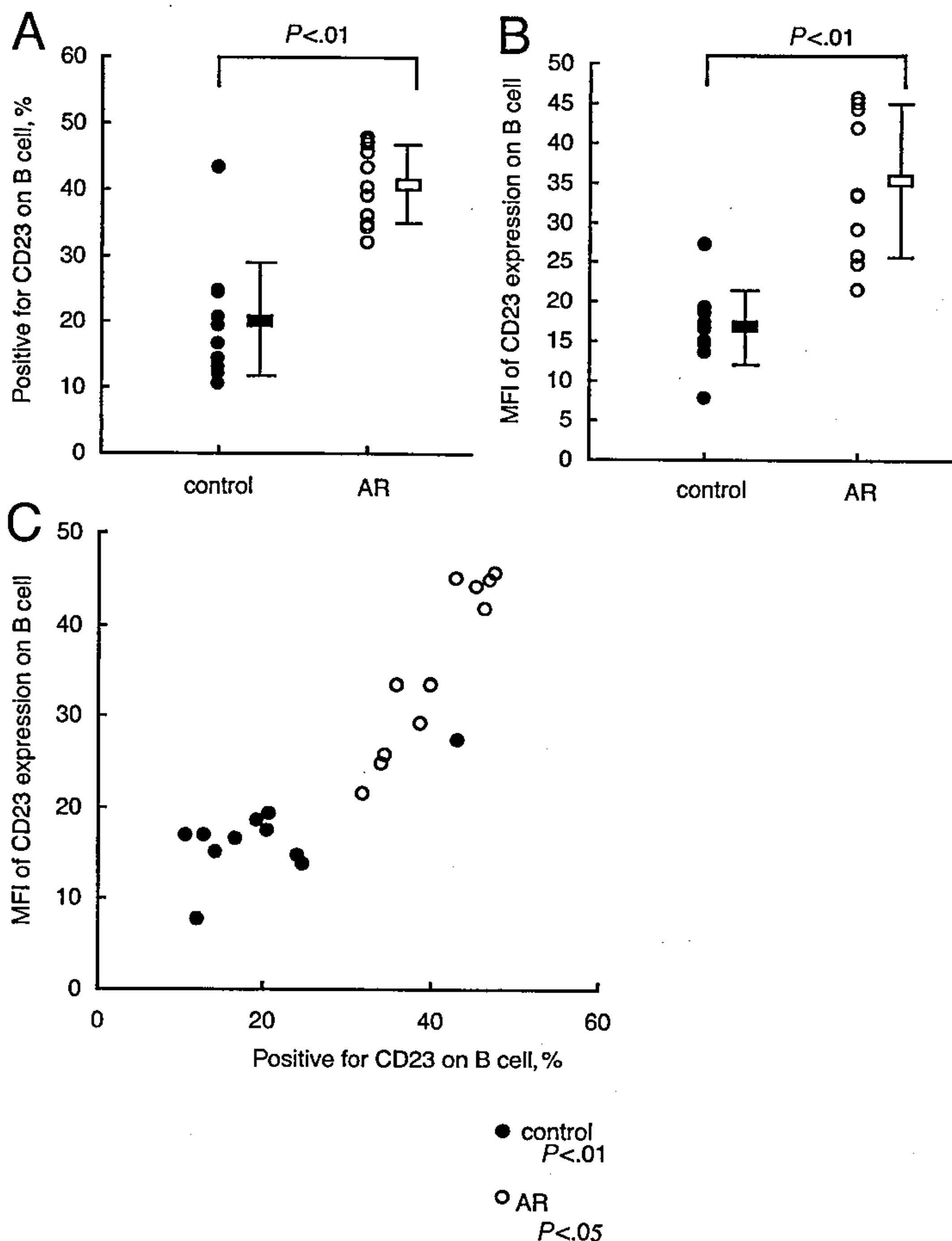

Figure 2. CD23 expression on B cells. A, Percent positive values for CD23 on B cells from patients with allergic rhinitis (AR) were significantly higher than for the control group. B, A similar tendency was seen in the mean fluorescence intensity (MFI) of CD23 expression on B cells, which was significantly increased for patients with AR compared with controls. C, Correlation plot between percent positive values for CD23 on B cells and MFI of CD23 expression on B cells. The percent positive values for CD23 on B cells was significantly correlated with the MFI of CD23 expression on B cells for the control group and the AR group.

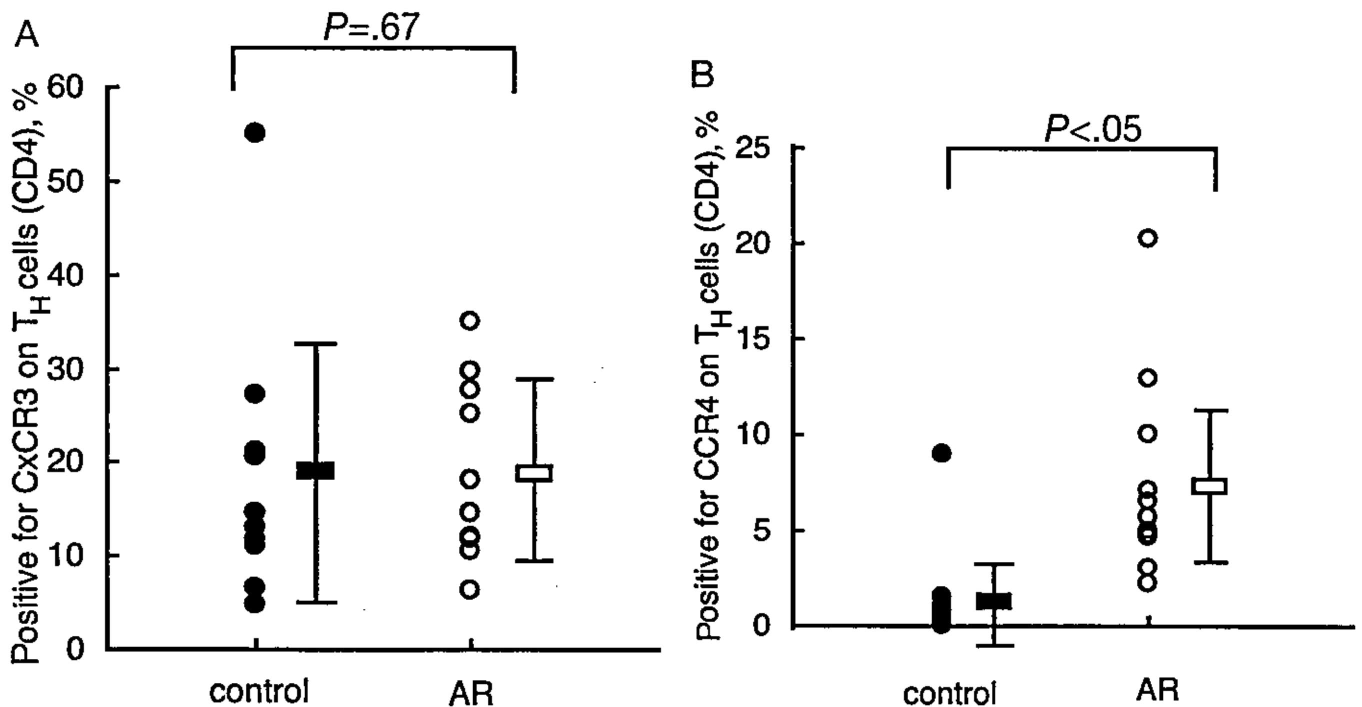

Figure 3. Percent positive values for chemokine receptor expression on mucosal CD4 T cells. A, Data for the CXCR3 subtype, assumed to be T_H1 cells. B, Data for the CCR4 subtype, assumed to be T_H2 cells. There is no significant difference in percent positive values for CXCR3 on CD4 cells from patients with allergic rhinitis (AR) and controls (A), whereas the percent positive values for CCR4 on mucosal CD4 cells from patients with AR were significantly higher than in controls.

of $CD23^+$ B cells to total B cells, and the MFI indicates the mean level of CD23 expression per B cell.

Expression of CD23 on Mucosal B Cells

The percent positive value for CD23 on mucosal B cells in the AR group (43.9% ± 5.8%) was significantly higher than in the control group (19.9% ± 9.0%, $P < .001$) (Fig 2A), whereas that of B cells to total lymphocytes was unchanged (data not shown). The MFI of CD23 on mucosal B cells in the AR group (40.76 ± 20.62) was also significantly higher than in the control group (16.9 ± 4.68, $P = .004$) (Fig 2B). The percent positive value and the MFI for CD23 were significantly correlated, with the correlation coefficients for control subjects and AR patients being 0.71 ($P = .03$) and 0.99 ($P = .008$), respectively (Fig 2C).

Expression of Chemokine Receptors on Mucosal CD4 Cells

CXCR3 and CCR4 were used as T_H1 and T_H2 markers, respectively.[5] There was no difference between the percent positive value for CXCR3 on mucosal CD4 cells in the AR group (16.3% ± 8.1%) and in the control group (18.8% ± 13.8%, $P = .67$) (Fig 3A), whereas the percent positive value for CCR4 on mucosal CD4 cells in the AR group (7.5% ± 5.7%) was significantly higher than in the control group (1.3% ± 2.5%, $P = .02$) (Fig 3B).

Correlation Between Percent Positive Values for CD23 on B Cells and CCR4 on CD4 Cells

No significant correlation was observed between the percent positive values for CD23 on mucosal B cells and CCR4 on CD4 cells from the same mucosa. The correlation coefficients for control subjects and AR patients were 0.21 ($P = .25$) and 0.26 ($P = .17$), respectively (Fig 4); hence, there was no significant correlation in either group.

Correlation Between the Percent Positive Value for CD23 on B Cells and the Serum IgE Level

The correlation between the percent positive values for CD23 on mucosal B cells and total serum IgE levels is shown in Figure 5. No significant correlation was observed between these values or between the percent positive values for CD23 on B cells and specific IgE levels (data not shown).

DISCUSSION

The role of CD23 in IgE synthesis is still controversial and remains to be elucidated. The binding of the antigen-IgE complex to CD23-bearing B cells has been shown to augment IgE-mediated responses.[6] In addition, CD23 is the enhancement of IgE-dependent antigen presentation to T cells.[3,7,8] In clinical studies, the cell surface expression in peripheral blood B lymphocytes has shown increased CD23 expression

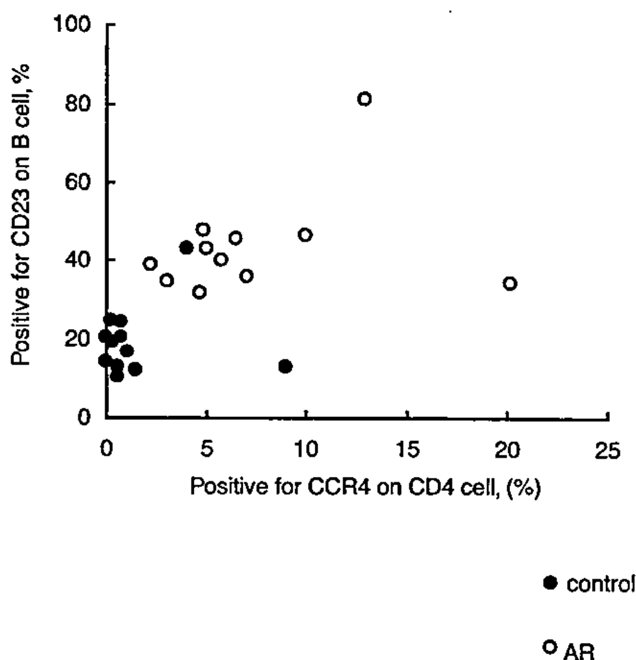

Figure 4. Correlation plot for percent positive values for CD23 on mucosal B cells and percent positive values for CCR4 on mucosal CD4 cells. There is no significant correlation between these data in the control group ($P = .25$) or the allergic rhinitis (AR) group ($P = .17$).

in allergic children and adults, including patients with AR compared with nonallergic controls,[9,10] and has further shown that CD23 expression decreased after successful hyposensitization.[11,12] Furthermore, since IgE levels in serum were evaluated as an atopy marker, significant correlations were reported between the levels of the soluble form of CD23 and the levels of IgE in serum in patients with atopy.[13]

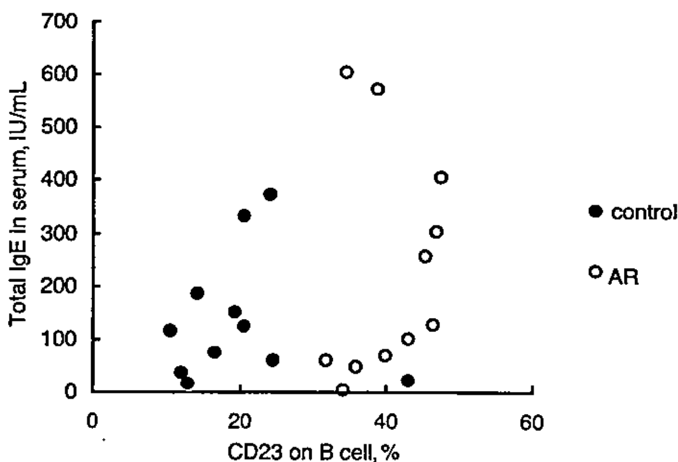

Figure 5. Correlation plot for percent positive values for CD23 on mucosal B cells and the total IgE level. There is no significant correlation between these data in the control group ($P = .21$) or the allergic rhinitis (AR) group ($P = .66$).

In the present study, we examined the expression of CD23 on mucosal B cells and found higher percentages of CD23-bearing B cells in patients with perennial AR compared with those in nonallergic patients. In addition, we investigated T_H1 and T_H2 cells in the nasal mucosa by staining for expression of CXCR3 and CCR4 chemokine receptors, respectively. The results showed that the T_H2/CD4 ratio in patients with perennial AR was indeed higher than in nonallergic controls, whereas the T_H1/CD4 ratio was unchanged. However, no significant correlation was found between the T_H2/CD4 ratio and the CD23/B-cell ratio. Furthermore, no significant correlation was found between the CD23/B-cell ratio in the nasal mucosa and the total IgE level or specific IgE level in serum (data not shown).

The T-cell–B-cell interaction must play an important roll in allergic inflammation. IL-4 and IL-13 are known to promote the switching of B cells from IgM to IgE production and expression of CD23,[14] whereas interferon-γ, IL-10, and IL-12 inhibit this effect.[15–17] Other than T_H2 cells, various kinds of cells in the nasal mucosa, such as mast cells, basophils, and CD8 cells, have been shown to produce IL-4 and IL-13.[18,19] The lack of a significant correlation between the ratio of T_H2/CD4 T cells with CD23/B cells may suggest that the total amount of IL-4 and/or IL-13 produced from not only T_H2 cells but other cells influences CD23 expression in nasal mucosal B cells. In this study, T_H2 cells were shown to make up approximately 7.15% of the infiltrating CD4 T cells in the nasal mucosa of patients with AR. However, only a small portion of these T_H2 cells could recognize the house dust mite allergen. An enzyme-linked immunosorbent spot-forming cell assay study has shown a low frequency population of allergen-specific IL-4– or IL-13–producing T_H cells, which represented approximately 1 spot per 10,000 to 100,000 peripheral CD4 T cells.[20,21] T_H1/T_H2 cytokine dysregulation is thought to be a fundamental pathogenesis of AR, but only a few T and B cells are allergen specific. The major source of IL-4 and IL-13 production in the effector phase in the nasal mucosa of patients with AR may be mast cells or basophils and not T_H2 cells.[22] The role of T_H2 cytokines from mast cells remains to be clarified, but a recent study showed that T_H2 cytokines from mast cells are induced by antigen stimulation[23] and influence not only the differentiation of naive T cells toward T_H2 cells[24] but also B-cell activation.[25] In addition, the lack of correlation between the number of nasal B cells and the serum IgE level observed in this study may suggest that the nasal mucosa could synthesize IgE independently from peripheral blood.[26]

Overall, the results of this study suggest that enhanced expression of CD23 on nasal mucosal B cells occurs in patients with AR. However, further analysis is required regarding the significance of CD23 in nasal mucosa at the site of the allergic reaction.

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Requests for reprints should be addressed to:
Shigetoshi Horiguchi, MD, PhD
Department of Otolaryngology
Head and Neck Surgery
Graduate School of Medicine.
Chiba University
1-8-1 Inohana
Chuo-ku, Chiba 260-8670
Japan
E-mail: horiguti@faculty.chiba-u.jp

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Sphingosine 1-Phosphate Inhibits Migration of RBL-2H3 Cells *via* $S1P_2$: Cross-Talk between Platelets and Mast Cells

Eiko Yokoo[1], Yutaka Yatomi[*,2], Toshiro Takafuta[3], Makoto Osada[3], Yoshitaka Okamoto[†,1] and Yukio Ozaki[3]

Departments of [1]Otolaryngology and [3]Laboratory Medicine, University of Yamanashi Faculty of Medicine, 1110 Tamaho, Nakakoma, Yamanashi 409-3898; and [2]Department of Laboratory Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655

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To analyze the involvement in allergic reactions of platelets and sphingosine 1-phosphate (Sph-1-P), a lysophospholipid mediator released from activated platelets, the effects of Sph-1-P and a supernatant prepared from activated platelets on mast cell line RBL-2H3 were examined. Sph-1-P strongly inhibited the migration of both non-stimulated and fibronectin-stimulated RBL-2H3 cells, which was reversed by JTE-013, a specific antagonist of G protein-coupled Sph-1-P receptor $S1P_2$; $S1P_2$ was confirmed to be expressed in these cells. A similar anti-motility effect of Sph-1-P was observed in a phagokinetic assay. Consistent with these results, treatment of RBL-2H3 cells with Sph-1-P resulted in a rounded cell morphology, which was blocked by JTE-013. Under the present conditions, Sph-1-P failed to induce intracellular Ca^{2+} mobilization or histamine degranulation, responses postulated to be elicited by intracellular Sph-1-P. Importantly, the Sph-1-P effect, *i.e.*, the regulation of RBL-2H3 cell motility, was mimicked by the supernatant (both with and without boiling) prepared from activated platelets, and this effect of the supernatant was also blocked by JTE-013. Our results suggest that the motility of mast cells can be regulated by Sph-1-P and also platelets (which release Sph-1-P), *via* cell surface receptor $S1P_2$ (not through intracellular Sph-1-P actions, postulated previously in the same cells).

Key words: allergy, lysophospholipid, platelets, RBL-2H3 cells, S1P/Edg receptor, sphingosine 1-phosphate.

Abbreviations: FcεRI, high affinity IgE receptor; LPA, lysophosphatidic acid; Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate.

Mast cells play a central role in triggering IgE-mediated allergic reactions. Cross-linking of allergen-specific IgE bound to the high affinity IgE receptor (FcεRI) expressed on the surface of mast cells, upon challenge with polyvalent allergens, results in the release of several chemical mediators (*1, 2*). This leads to the manifestation of allergic symptoms in atopic diseases such as bronchial asthma, atopic dermatitis, and allergic rhinitis (*1, 2*). However, like most other biological reactions, allergy should be considered as an integrated group of multicellular events; interactions between mast cells and various cell types existing at sites of allergic inflammation should be involved.

It is now established that blood platelets are involved in a variety of biological reactions other than thrombosis and hemostasis, and allergic reaction is no exception (*3–6*). For example, the involvement of platelets in bronchial asthma has been postulated. Platelets are reportedly released from megakaryocytes in the capillary bed of the lungs (*7*), and found in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced responses (*8, 9*). Furthermore, platelet-specific proteins platelet factor 4 and β-thromboglobulin, and RANTES (which is abundant in platelets) have been reported to be released into the circulation and bronchoalveolar lavage fluid during provoked or spontaneous asthmatic attacks *in vivo* (*3, 5, 10, 11*), while agonist-mediated activation of platelets *in vitro* has been shown to be augmented in asthmatics (*12*). Finally, functional expression of FcεRI, as well as the low-affinity IgE receptor (CD23), has been reported in platelets (*13*). Platelet involvement in allergic reactions has been ascribed to the release, upon stimulation, of a number of bioactive substances such as thromboxane A_2, serotonin, histamine, platelet-derived growth factor, IgE, and chemokines such as RANTES and platelet factor 4 (*3–6, 10–12, 14, 15*). Importantly, platelets of atopic individuals differ in their granular contents and in the amounts of biologically active mediators released, compared with platelets of non-atopic subjects (*15*). Analysis of the chemical mediators released from platelets may lead to a new therapeutic strategy for controling allergic diseases. In fact, the role of thromboxane A_2 in the pathogenesis of allergy, especially asthma, is now well established, and the therapeutic usefulness of thromboxane A_2 synthase inhibitors and receptor antagonists is widely known (*16, 17*).

Sphingosine 1-phosphate (Sph-1-P) was recently added to the list of bioactive lipids released from activated

*To whom correspondence should be addressed. Tel: +813-5800-8730, Fax: +81-3-5689-0495, E-mail: yatomiy-lab@h.u-tokyo.ac.jp
†Present address: Department of Otolaryngology, Graduate School of Medicine, Chiba University.

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platelets (*18, 19*). This phosphorylated sphingoid base induces a variety of biological responses in diverse cell types, mainly through interaction with the cell surface receptors S1Ps (*20–22*). Although its role in allergic diseases is not established completely, it was recently shown that Sph-1-P is increased in bronchoalveolar lavage fluid collected from asthmatic subjects (*23*), and the involvement of this bioactive lipid in asthma has been suggested (*24, 25*). Since blood platelets store abundant Sph-1-P (*18, 26*) and release it upon activation (*18, 19*), it is important to examine the effects of this bioactive lipid on mast cell functions from the viewpoint of platelet-mast cell interactions; mast cells exist abundantly along blood vessels. In this study, we examined the effect of Sph-1-P on rat basophilic leukemia cell line RBL-2H3, a tumor mast cell line used frequently as an experimental model of mucosal mast cells (*27*). We also analyzed the relative involvement of this bioactive lipid in platelet-mast cell interactions with the use of a supernatant prepared from activated platelets and a specific S1P antagonist.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA, USA); fibronectin (from bovine plasma, 0.1% solution), lysophosphatidic acid (LPA), and tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma, St. Louis, MO, USA); thrombin (Mochida Pharmaceuticals, Tokyo); fura2-AM (Dojin Chemicals, Kumamoto). Convulxin was a gift from Prof. Takashi Morita (Meiji Pharmaceutical University, Tokyo).

The pyrazolopyridine derivative JTE-013 was a gift from the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation, Osaka. JTE-013 is a specific $S1P_2$ antagonist; see "Pyrazolopyridine compounds and use thereof as drugs. PCT (WO) Patent: Publication number, WO 01/98301; Publication date, December 27, 2001". It has been confirmed that JTE-013 inhibits the specific binding of radio-labeled Sph-1-P to the cell membranes of Chinese hamster ovary cells stably transfected not only with human $S1P_2$ but also rat $S1P_2$ (*28, 29*).

Cell Culture—The rat basophilic leukemia RBL-2H3 (JCRB0023) cells (*27*) were obtained from HSRRB, Japan Health Science Foundation (Osaka), and grown in Eagle's minimal essential medium (Sigma) containing 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C under an atmosphere of 5% CO_2 and 95% room air. The RBL-2H3 cells were harvested by treatment with 0.25% trypsin plus 0.02% EDTA for 3 min at 37°C.

Washed platelets were prepared from healthy donors as described previously (*19*). Washed platelet suspensions (cell density, 5×10^8/ml) were stimulated with 50 ng/ml of convulxin, a potent platelet stimulant (*30*), for 15 min at 37°C. Then, supernatants were obtained by centrifugation. It was confirmed that convulxin, by itself, failed to affect the response of RBL-2H3 cells in the present study.

RT-PCR Analysis—Total RNA was prepared from RBL-2H3 cells with Trizol reagent (Gibco BRL, Life Technologies, Rockville, MD, USA), and the isolation of polyA$^+$ RNA was performed with a polyA$^+$ RNA purification kit (Takara Biomedicals, Shiga), according to the manufacturer's instructions. The isolated mRNA was reverse transcribed using a SuperScript™ Preamplification System (Gibco BRL, Life Technologies, Rockville, MD, USA). This reverse transcribed cDNA and eight normalized, first-strand cDNA preparations from rat tissues (Rat MTC™ Panel I; Clontech Laboratories, Palo Alto, CA) were amplified in a Perkin-Elmer 9600R thermal cycler (The Perkin-Elmer Corp., Norwalk, CT, USA) using Ex Taq™ (Takara Biomedicals).

The full-length sequences of rat $S1P_1$ (NM 017301), $S1P_2$ (NM 017192), and $S1P_5$ (AF233649) were obtained from the GenBank database. Since the full-length sequences of rat $S1P_3$ and $S1P_4$ were not available in the database, we searched for rat genomic DNA sequences encoding rat $S1P_3$ and $S1P_4$ using the Trace Blast program. The oligonucleotide primer pairs designed and used for PCR amplification were as follows: $S1P_1$-1 (sense), 5′-ATGGTGTCCTCCACCAGC-3′, and $S1P_1$-2 (antisense), 5′-AGTTCCAGCCCATGATGG-3′; $S1P_2$-3 (sense), 5′-AGCAAGTTCCACTCAGCC-3′, and $S1P_2$-2 (antisense), 5′-CATAGAGGGGCAGCACAG-3′; $S1P_3$-1 (sense), 5′-ATGGCATCCACGCATGCG-3′, and $S1P_3$-3 (antisense), 5′-CATTCACTTGCAGAGGAC-3′; $S1P_3$-4 (sense), 5′-AACTTGGCTCTCTGCGAC-3′, and $S1P_3$-2 (antisense), 5′-CAGTCGGGAAAGTTCTCC-3′; $S1P_4$-4 (sense), 5′-TGGGTGTACTACTGCCTC-3′, and $S1P_4$-2 (antisense), 5′-GCGCACACACAGTTCCAG-3′; $S1P_5$-1 (sense), 5′-ATGGAGTCCGGGCTACTG-3′, and $S1P_5$-2 (antisense), 5′-TAGGCCTTGGCGTAGAGC-3′; and $S1P_5$-3 (sense), 5′-TTACCTTGTCGGACCTGC-3′, and $S1P_5$-4 (antisense), 5′-TCCCAAGCAGTTCCAGTT-3′.

When primers $S1P_1$-1 and $S1P_1$-2 were used, a 553 bp S1P1 fragment was amplified. With primers $S1P_2$-3 and $S1P_2$-2, a 376 bp fragment of $S1P_2$ was amplified. To amplify the $S1P_3$ cDNA, we performed nested PCR; the first PCR was performed using primers $S1P_3$-1 and $S1P_3$-3, and a 317 bp fragment was amplified by the second PCR using primers $S1P_3$-4 and $S1P_3$-2. With primers $S1P_4$-4 and $S1P_4$-2, a 326 bp fragment of $S1P_4$ was amplified. To amplify the $S1P_5$ cDNA, we performed nested PCR; the first PCR was performed using primers $S1P_5$-1 and $S1P_5$-2, and a 298 bp fragment was amplified by the second PCR using primers $S1P_5$-3 and $S1P_5$-4.

Immunoprecipitation and Immunoblotting—These procedures were performed basically as described previously (*28*). Cell lysates were immunoprecipitated and then immunoblotted with 2 μg/ml of anti-human and rat EDG-5 ($S1P_2$) C-terminal monoclonal antibodies (Exalpha Biologicals, Boston, MA, USA). Antibody binding was detected using peroxidase-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH, USA) and visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Migration Assay—RBL-2H3 cell migration was assessed by means of a modified Boyden's chamber assay, *i.e.*, in Transwell cell culture chambers (Costar, Cambridge, MA, USA). Polycarbonate filters with 8 μm pores, used to separate the upper and lower chambers, were coated with Vitrogen 50 (purified collagen) (Cohesion, Palo Alto, CA, USA). The coated filters were washed with a serum-free medium and dried immediately. Then RBL-2H3 cells were added to the upper compartment of the

chamber at a density of 1 × 10^5/100 µl of medium containing 0.1% bovine serum albumin and incubated for 4 h at 37°C. RBL-2H3 cells were allowed to migrate toward an indicated reagent in the lower chamber. After the reaction, the filters were fixed and stained with trypan blue. After removal of non-migrating cells by wiping with cotton swabs, cells that had migrated through the filter to the lower surface were counted manually under a microscope in five predetermined fields at a magnification of ×200. When checkerboard analysis was performed, 0, 0.01, 0.1, 1, or 10 µM Sph-1-P was added to the upper and/or lower chamber.

Phagokinetic Assay on Gold Sol-coated Plates—Random cell motility and phagocytotic activity were jointly estimated as the area of phagokinetic tracks on gold sol particle-coated plates. Briefly, 3.5 cm dishes coated with 0.2% gelatin were incubated with colloidal gold for 45 min, and then washed twice with PBS. RBL-2H3 cells (2,000 cells) were added to each dish. After 24 h at 37°C, phagokinetic tracks were visualized using dark-field illumination under a confocal microscope. The area cleared of gold particles was measured after photography by cutting out and weighing the cleared area, and the mean value for 20 cells was calculated in each experiment.

Actin Staining—For actin staining, cells were fixed with 3% paraformaldehyde in PBS for 40 min and then permeabilized with 0.2% Triton X-100 for 8 min. Actin filaments were detected by staining with 0.1 µg/ml of tetramethylrhodamine isothiocyanate-conjugated phalloidin. Actin staining was observed and photographed under a confocal microscope.

Measurement of the Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$)—$[Ca^{2+}]_i$ measurement was performed with the use of Ca^{2+}-sensitive fluorophore fura2. Confluent RBL-2H3 cells were harvested by trypsinization, and then the cells were incubated with 3 µM fura2-AM. After 30 min at 37°C, the cells were washed twice, adjusted to 2 × 10^6/ml, and then supplemented with 1 mM $CaCl_2$. Fluorescence measurements were made with an FS100 (Kowa, Tokyo). The $[Ca^{2+}]_i$ values were determined from the ratio of fura2 fluorescence intensity with 340 and 380 nm excitation.

Histamine Release Assay—The levels of histamine in the medium were measured, after its acylation, using a Histamine ELISA (ICN Biomedicals, Aurora, OH, USA). The procedures were based on the manufacturer's instructions.

Data Presentation and Statistics—The data are presented as the means ± SD (n = 3) or representative of 3 or 4 separate experiments. When indicated, the statistical significance of the difference between the two groups was determined by means of Student's t test. $P < 0.05$ was considered significant.

RESULTS

S1P Expression in RBL-2H3 Cells—Many, if not all, of the biological responses induced by Sph-1-P are mediated by its cell surface receptors, *i.e.* S1Ps (*21, 22*). Although $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), and $S1P_3$ (EDG-3) seem widely expressed, $S1P_4$ (EDG-6) and $S1P_5$ (EDG-8) each exhibit a limited expression pattern (*21, 22, 31*). The $S1P_4$ expression profile is largely confined to the tissues and cells of the hematopoietic system (*32*), while $S1P_5$ is known to be expressed in the brain (*33*). As shown in Fig. 1A, the PCR products of $S1P_1$ through $S1P_4$ were amplified from RBL-2H3 cells. However, when the S1P expression was compared with that in other rat tissues, the most obvious finding was strong $S1P_2$ expression in RBL-2H3 cells. $S1P_1$, known to be widely expressed, and $S1P_4$, mainly expressed in the hematopoietic systems, were confirmed to be expressed in RBL-2H3 cells; $S1P_5$ expression was not detected.

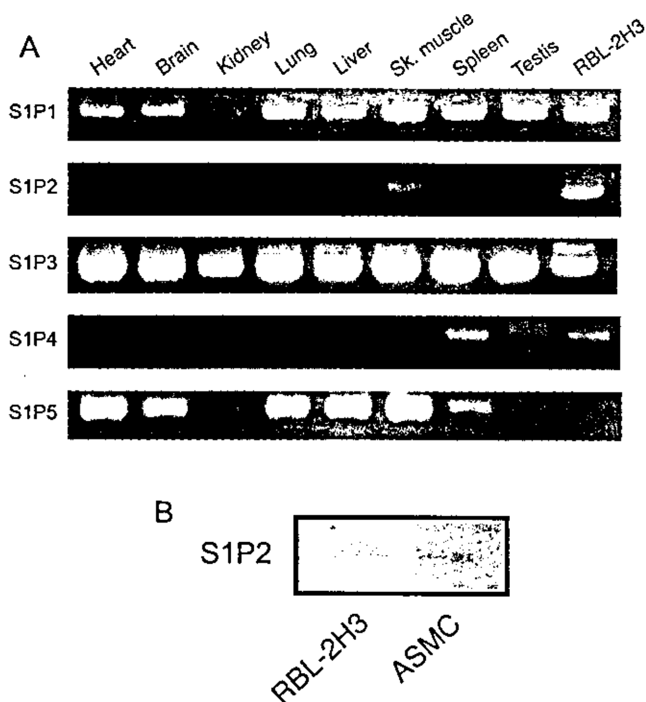


Fig. 1. **Expression of S1Ps in RBL-2H3 cells.** (A) Detection of expression of mRNA for S1Ps in RBL-2H3 cells, in comparison with in various rat tissues, was performed by RT-PCR. cDNA preparations from RBL-2H3 cells and various rat tissues were amplified for $S1P_{1-5}$. The products were resolved on 2% agarose gels. Sk. muscle, skeletal muscle. (B) Detection of $S1P_2$ protein in RBL-2H3 cells and vascular smooth muscle cells. Lysates obtained from RBL-2H3 cells and aortic smooth muscle cells (ASMC) were immunoprecipitated and then immunoblotted with anti-$S1P_2$ antibodies.

We next confirmed the protein expression of $S1P_2$ in these cells using vascular smooth muscle cells as a positive control (*21, 22*). RBL-2H3 cells were found to express $S1P_2$ protein (Fig. 1B), although precise quantitation was difficult due to the employment of immunoprecipitation.

Inhibition of RBL-2H3 Cell Migration by Sph-1-P and Its Reversal by a Specific $S1P_2$ Antagonist—One of the most unique characteristics of S1P receptors is their receptor isotype-specific, bimodal regulatory activity on cell migration (*22, 34*). While $S1P_1$ acts as a typical chemotactic receptor, $S1P_2$ acts as a chemorepellant one (*22, 34*). Since RBL-2H3 cells expressed both S1P receptors, it was considered important to examine the effect of Sph-1-P on the migration of these cells. When examined by means of the modified Boyden's chamber assay, incubation of RBL-2H3 cells in the absence of any treatment was found to lead to significant basal migration across the membrane (Fig. 2A and Table 1). Sph-1-P strongly

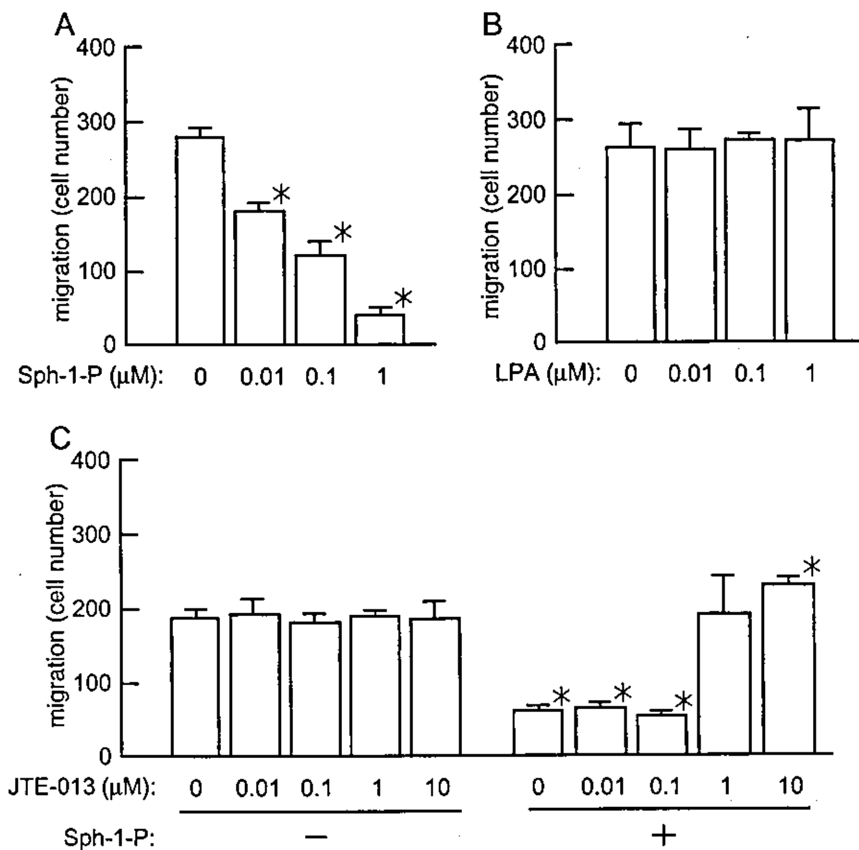


Fig. 2. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a modified Boyden's chamber assay.** (A and B) RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of Sph-1-P (A) or LPA (B) were placed. (C) RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where 100 nM Sph-1-P was present (+) or absent (–). *Statistically significant compared with the control cells (without Sph-1-P/JTE-013 treatment).

inhibited this basal RBL-2H3 cell migration in a concentration-dependent manner (Fig. 2A). To determine whether or not this migration inhibition depends on the presence of a concentration gradient of Sph-1-P between the lower and upper chambers, checkerboard experiments were conducted. Marked inhibition of migration was observed not only in the presence of a Sph-1-P concentration gradient, but also with equal concentrations of Sph-1-P below and above the membranes (Table 1). These results indicate that Sph-1-P inhibits chemokinesis (random motility) of RBL-2H3 cells, as well as chemotaxis. In contrast, LPA, which is structurally similar to Sph-1-P as a lysophospholipid and interacts with LPA_{1-3} receptors (*21*, *31*), failed to affect the migration response (Fig. 2B). Furthermore, the strong migration inhibition by Sph-1-P (observed with the use of RBL-2H3 cells) was not observed for other hematopoietic cells such as human neutrophils, lymphocytes, eosinophils, monocytic leukemia U937 cells, and myeloma-derived ARH77 cells (data not shown), indicating the uniqueness of this tumor mast cell line.

Since chemorepellant receptor $S1P_2$, strongly expressed in RBL-2H3 cells (see Fig. 1), was the most probable candidate receptor involved in the Sph-1-P inhibition of cell motility, the effect of the $S1P_2$ antagonist JTE-013 (*28*, *29*) was examined. This compound, by itself, failed to affect the basal RBL-2H3 migration (Fig. 2C). When RBL-2H3 cells were pretreated with JTE-013, the inhibition induced by Sph-1-P was reversed (Fig. 2C), indicating Sph-1-P inhibition of RBL-2H3 cell migration through $S1P_2$. It should be noted that the cell migration after treatment with Sph-1-P plus JTE-013 was even enhanced compared with the basal migration without any treatment (Fig. 2C).

Fibronectin is known to enhance RBL-2H3 cell migration (*35*), which was confirmed under the present conditions (Fig. 3A). Similar to the basal RBL-2H3 cell migration, that enhanced by fibronectin was inhibited by Sph-1-P, which was reversed by JTE-013 (Fig. 3B). Further-

Table 1. **Checkerboard analysis of RBL-2H3 cells.** Different concentrations of Sph-1-P were added to the upper and/or lower chamber, and then RBL-2H3 cells were allowed to migrate for 4 h.

[Sph-1-P, Lower chamber] (μM)	[Sph-1-P, Upper chamber] (μM)				
	0	0.01	0.1	1	10
0	206 ± 30	184 ± 13	203 ± 26	187 ± 30	182 ± 21
0.01	131 ± 8	111 ± 10	118 ± 6	111 ± 7	122 ± 15
0.1	70 ± 18	68 ± 17	55 ± 11	62 ± 10	49 ± 8
1	53 ± 17	44 ± 14	50 ± 9	40 ± 4	40 ± 6
10	33 ± 4	23 ± 3	33 ± 11	30 ± 6	27 ± 7

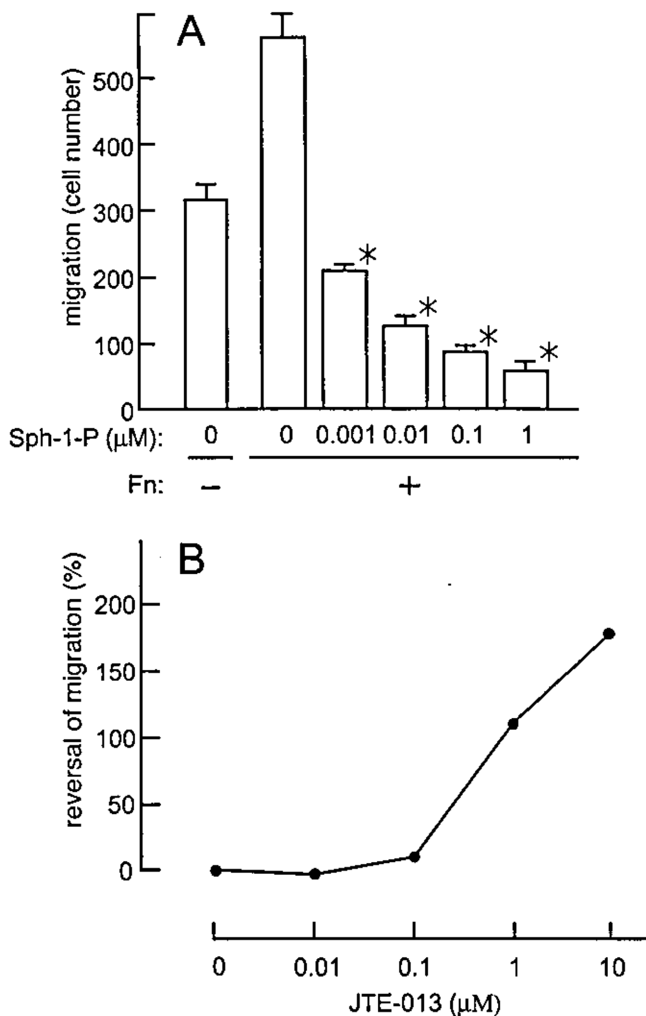
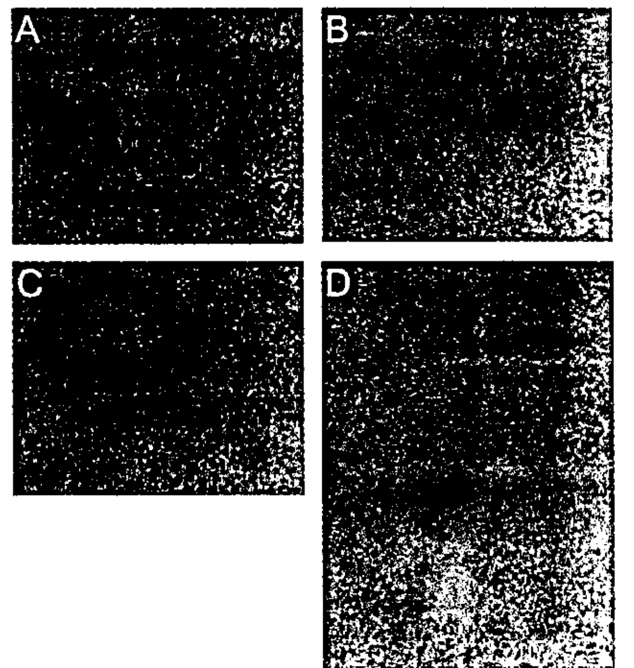


Fig. 3. **Inhibition of fibronectin-induced RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013.** (A) Various concentrations of Sph-1-P were placed in the lower chamber with (+) or without (–) 100 μg/ml of fibronectin (Fn). RBL-2H3 cells were allowed to migrate for 4 h. *Statistically significant compared with the control fibronectin-treated cells (without Sph-1-P treatment). (B) RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where 100 nM Sph-1-P was present or absent, together with 100 μg/ml of fibronectin. The reversal by JTE-013 of the Sph-1-P-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/Sph-1-P] – [migrating cells in the presence of Sph-1-P]) / ([migrating cells in the absence of JTE-013/Sph-1-P] – [migrating cells in the presence of Sph-1-P]) × 100.

more, the fibronectin-stimulated cell migration in the presence of Sph-1-P plus JTE-013 was greater than that without Sph-1-P/JTE-013 (Fig. 3B).

Inhibition of RBL-2H3 Cell Phagokinesis by Sph-1-P and Its Reversal by a Specific S1P$_2$ Antagonist—To confirm the anti-motility effect of Sph-1-P, we also performed a phagokinetic track assay. The phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P (Fig. 4). JTE-013, which by itself failed to affect the response, reversed the Sph-1-P-inhibited phagokinesis (Fig. 4). These results further strengthen the idea that Sph-1-P inhibits RBL-2H3 motility through S1P$_2$.



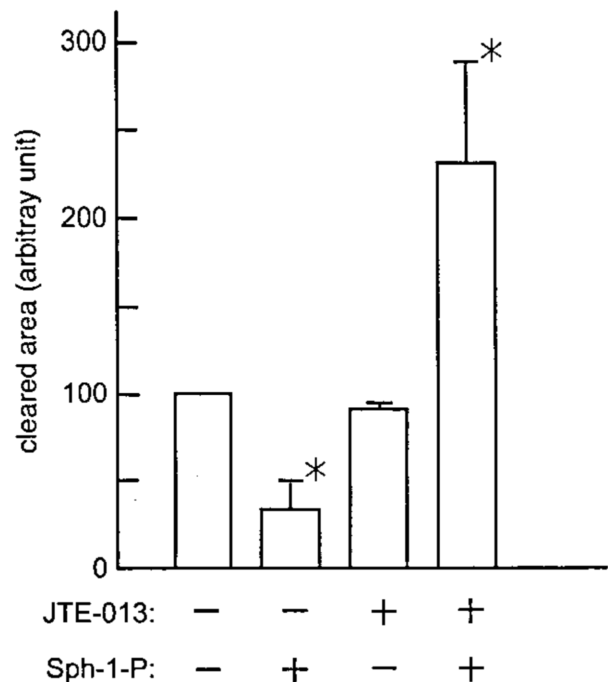


Fig. 4. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a phagokinetic assay.** (Upper panel) RBL-2H3 cells were preincubated without (A and B) or with (C and D) 10 μM JTE-013 for 10 min, and then challenged without (A and C) or with (B and D) 1 μM Sph-1-P for 24 h. Chemokinesis was evaluated with a phagokinetic assay using gold sol-coated plates. (Lower panel) RBL-2H3 cells were treated as described for the upper panel, and the area cleared of gold particles for each cell was measured. *Statistically significant compared with the control cells (without Sph-1-P/JTE-013 treatment).

Again, the response in the presence of Sph-1-P plus JTE-013 was greater than that without any treatment (Fig. 4).

Cytoskeletal Reorganization of RBL-2H3 Cells Treated with Sph-1-P—We further evaluated cytoskeletal reor-

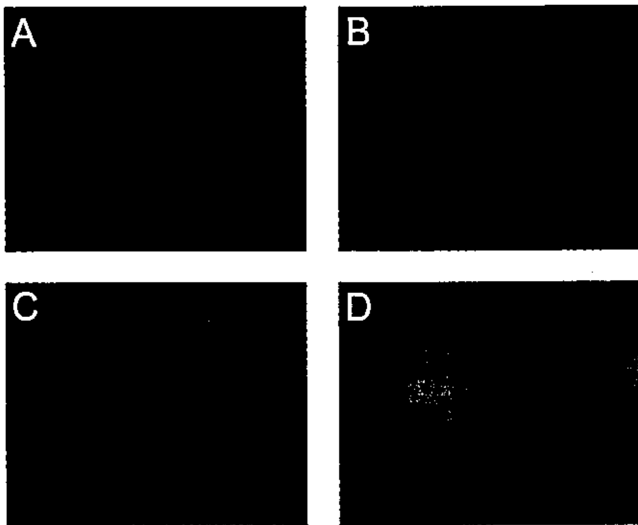


Fig. 5. **Cytoskeletal Reorganization of RBL-2H3 cells treated with JTE-013 and/or Sph-1-P.** RBL-2H3 cells were pretreated without (A and B) or with (C and D) 1 μM JTE-013 for 10 min, and then stimulated without (A and C) or with (B and D) 100 nM Sph-1-P for 30 min. Then, the cells were fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate-phalloidin for actin staining.

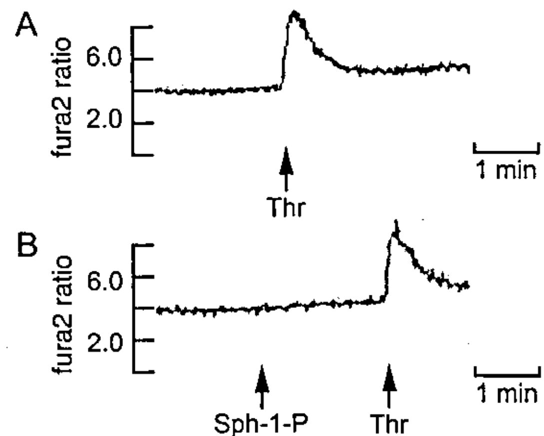


Fig. 6. **Measurement of RBL-2H3 cell $[Ca^{2+}]_i$ Fura2-loaded RBL-2H3 cells were stimulated with 1U/ml of thrombin (A) or 100 nM Sph-1-P and then 1U/ml of thrombin (B).** $[Ca^{2+}]_i$ was monitored as the ratio of fura2 fluorescence.

ganization under the conditions in which Sph-1-P exerted its anti-motility effect; RBL-2H3 cells stained to visualize F-actin are shown in Fig. 5. Low nanomolar concentrations of Sph-1-P induced stress fiber formation in RBL-2H3 cells (data not shown), possibly through Rho activation (*36*). When higher concentrations (100 nM – 1 μM) of Sph-1-P were employed, marked changes in cell morphology and increased numbers of rounded cells were observed (Fig. 5, A and B). The $S1P_2$ antagonist JTE-013, which, by itself, failed to affect the morphology of RBL-2H3 cells, blocked the Sph-1-P-induced cell shape change (Fig. 5), indicating Sph-1-P-induced cell rounding *via* $S1P_2$, as previously reported for transfected HEK293 cells (*37*). Instead, membrane ruffling-like structures were observed (Fig. 5D).

Effects of Sph-1-P on Intracellular Ca^{2+} Mobilization and Histamine Release in RBL-2H3 Cells—We next examined intracellular Ca^{2+} mobilization using Ca^{2+}-sensitive fluorophore fura2. As previously reported (*38*), thrombin caused a rapid and transient increase in $[Ca^{2+}]i$ (Fig. 5A). However, Sph-1-P, at concentrations capable of exerting the anti-motility effect, neither elicited an increase in $[Ca^{2+}]i$ nor affected the response induced by thrombin (Fig. 6B). When very high concentrations (above 10 μM) of Sph-1-P were employed, a small but significant increase was observed (data not shown), which should be independent of the effect of this bioactive lipid on RBL-2H3 cell migration shown above.

Consistent with the inability of Sph-1-P to induce intracellular Ca^{2+} mobilization (at least at nanomolar concentrations), this bioactive lysophospholipid failed to elicit histamine degranulation (data not shown), which is dependent on the Ca^{2+} signal (*39, 40*).

Imitation by a Supernatant Prepared from Activated Platelets of the Effect Triggered by Sph-1-P—We finally analyzed platelet-mast cell interactions and the relative involvement of Sph-1-P in this cell-cell communication. For this purpose, we prepared a supernatant from an activated platelet suspension. This supernatant strongly inhibited RBL-2H3 cell migration (Table 2), as did Sph-1-P. Similar results were obtained when the boiled (instead of non-boiled) supernatant was used to eliminate the effect of peptide mediators (data not shown). Accordingly, not a protein but probably a lipid component seemed to be responsible for the migration inhibition by the platelet supernatant. Although a variety of bioactive substances are released from activated platelets, ones interacting with RBL-2H3 cells or mast cells have hardly been reported. Accordingly, we postulated that the observed effect of the supernatant may be due to the presence of Sph-1-P released from platelets, based on the resemblance of the effects of Sph-1-P and the platelet supernatant. We tested this possibility by examining the effect of the $S1P_2$ antagonist JTE-013 on the response induced by the supernatant; Sph-1-P inhibits RBL-2H3 migration through $S1P_2$, as shown above. As expected, the migration inhibition of RBL-2H3 cells by both the boiled (Fig. 7A) and non-boiled (Fig. 7B) activated platelet supernatants was completely reversed by JTE-013. Furthermore, pretreatment with high concentrations of this $S1P_2$ antagonist before the addition of the supernatant even enhanced the response compared with the control without any treatment (Fig. 7).

Table 2. **Inhibition of RBL-2H3 cell migration by a supernatant prepared from activated platelets.** RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of a supernatant prepared from activated platelets were placed.

Experiment	Supernatant from activated platelets				
	0%	0.1%	1%	10%	100%
1	240	250	200	80	5
2	230	230	202	85	8
3	145			50	2
4	143			54	3

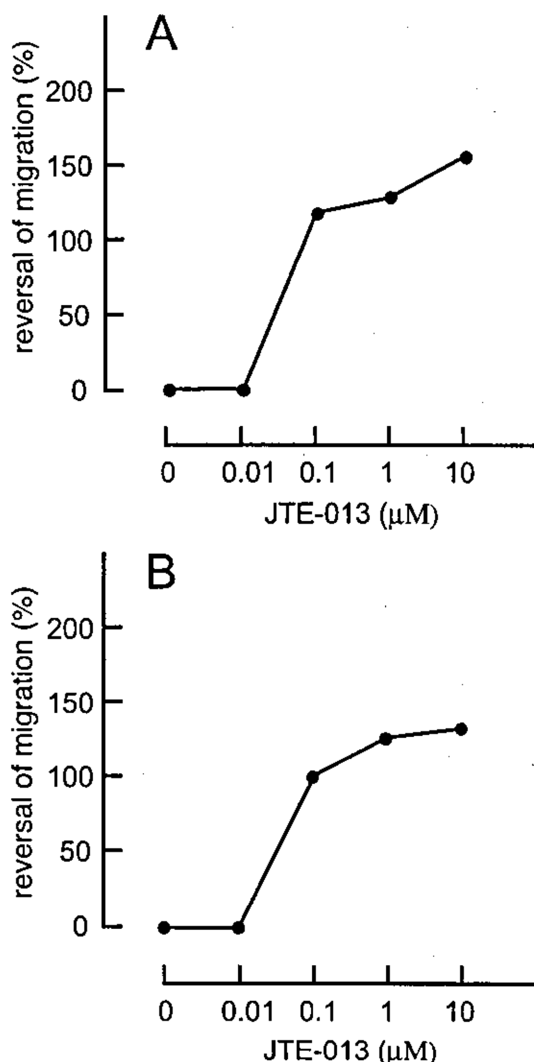


Fig. 7. **The reversal by JTE-013 of Sph-1-P-inhibited RBL-2H3 cell migration.** RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where a buffer containing 10% of the supernatant prepared from activated platelets was placed. The supernatant was boiled (B) or not boiled (A). The reversal by JTE-013 of the supernatant-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) / ([migrating cells in the absence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) × 100.

DISCUSSION

Sph-1-P Inhibition of RBL-2H3 Cell Migration—In this study, we found that Sph-1-P strongly inhibits the migration of RBL-2H3 cells through the G protein-coupled receptor $S1P_2$. This is consistent with the facts that $S1P_2$ is strongly expressed in these cells (this study), and that $S1P_2$ negatively regulates membrane ruffling and the resultant cell migration (*22*, *34*). Although $S1P_2$ has been reported to be a chemorepellant receptor (*22*, *34*), our present results show that Sph-1-P interaction with $S1P_2$ leads to inhibition of both chemokinesis (random motility) and chemotaxis, since the phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P, which was reversed by the $S1P_2$ antagonist JTE-013. Whether this difference is due to the choice of cell type remains to be solved.

It should be noted that, in cells pretreated with JTE-013 and then challenged with Sph-1-P, the migration and phagokinesis responses inhibited by Sph-1-P were not only blocked but also enhanced compared with those without Sph-1-P/JTE-013. RBL-2H3 cells express both $S1P_1$ and $S1P_2$. $S1P_1$ and $S1P_2$ exert contrasting effects on cell motility; the former stimulates membrane ruffling and migration in a Rac-dependent manner, while the latter inhibits these responses (*22*, *34*). Accordingly, when the $S1P_2$-mediated effect was blocked by its specific antagonist, the $S1P_1$-mediated effect, *i.e.*, Rac-dependent stimulation of migration, should be observed in Sph-1-P-challenged RBL-2H3 cells; Rac is involved in the migration of these cells (*41*). This is consistent with the morphological study in which a rounded cell shape (without cytosolic extension) was observed for RBL-2H3 cells incubated with Sph-1-P, while this rounded shape was abolished and membrane ruffling-like structures were observed when JTE-013 was pretreated before Sph-1-P addition.

Sph-1-P in RBL-2H3 Cells or Mast Cells: an Extracellular First Messenger or an Intracellular Second Messenger?—Sph-1-P is now considered to be a unique cell signaling molecule, functioning as both an extracellular first messenger and an intracellular second messenger (*20*). We believe that the regulation of RBL-2H3 cell motility by Sph-1-P reported in this study can be best explained by its extracellular action via the $S1P_2$ receptor since (i) $S1P_2$ is actually expressed in these cells, (ii) Sph-1-P is capable of eliciting the response at low nanomolar concentrations, corresponding to the Kd values of S1P receptors (*21*, *22*), and (iii) most importantly, the Sph-1-P-induced response was specifically blocked by an $S1P_2$ antagonist.

Our present paper is not the first to report the effect of exogenous Sph-1-P on mast cells or RBL-2H3 cells. Choi *et al.* reported that Sph-1-P increases intracellular Ca^{2+} in RBL-2H3 cells, although an extremely high concentration of Sph-1-P (such as 25 μM) was employed (*40*). It is hard to speculate that that high concentration of Sph-1-P can be attained *in vivo*; even the Sph-1-P concentration in serum, where the highest concentration of Sph-1-P could be attained given that the most important source of extracellular Sph-1-P is blood platelets, is less than 1 μM (*26*). In fact, it was postulated by Choi *et al.* that Sph-1-P, transiently formed intracellularly by sphingosine (Sph) kinase, acts as an intracellular messenger in signaling by the FcεRI antigen receptor (*40*). This Sph kinase-mediated Ca^{2+} signal, which is responsible for FcεRI-triggered mast cell degranulation, was later shown in human bone marrow-derived mast cells (*39*). Furthermore, high intracellular levels of Sph-1-P were shown to activate the mitogen-activated protein kinase pathway; again, as high as 10 μM Sph-1-P was employed in that study (*42*). Judging from the above together with our present study, it may be possible to speculate that both types of Sph-1-P action, *i.e.*, those as an intracellular messenger and an extracellular mediator, can be seen in mast cells; neither Ca^{2+} mobilization nor histamine release, possibly due to the intracellular Sph-1-P action, was observed with the

present conditions under which the anti-motility effect of extracellular Sph-1-P through $S1P_2$ was observed. This is not so surprising since a similar situation can be found in several systems (*18, 20*). For example, in human umbilical vein endothelial cells, Sph-1-P induces migration, proliferation, angiogenesis, and nitric oxide formation through cell surface receptors $S1P_1$ and $S1P_3$ (*43, 44*), while the Sph kinase pathway, through the generation of intracellular Sph-1-P, is critically involved in mediation of TNFα-induced endothelial activation (including adhesion molecule expression) (*45*).

Cross-talk between Mast Cells and Platelets—Another important finding in this study is that platelets seem to regulate mast (RBL-2H3) cell motility by releasing Sph-1-P. This is based on the facts that the Sph-1-P effect, *i.e.*, regulation of RBL-2H3 cell motility, is mimicked by a supernatant (both with and without boiling) prepared from activated platelets and that this effect of the supernatant was blocked by an $S1P_2$ antagonist. Given that 140 pmol Sph-1-P is stored in 10^8 platelets (*26*) and that 30% of it is released upon activation (*19*), the concentration of Sph-1-P in the supernatant prepared from activated platelets (5×10^8/ ml) is calculated to be 210 nM. Based on our results regarding the concentration-dependent effect of Sph-1-P (see Figs. 2A and 3A), this concentration of Sph-1-P is expected to exert an anti-motility effect on RBL-2H3 cells. At a site of inflammation, injury, or hemorrhage, platelets extravasate from the blood and adhere to the subendothelial tissue, which leads to their activation, while mast cells are residential cells adjacent to the endothelium in the connective and mucosal tissues. Therefore, activated platelets recruited to the inflamed or damaged site may modulate mast cell functions *in vivo*. The limitation of this study is the use of tumor mast cell line RBL-2H3, although this has been used frequently as an experimental model of mucosal mast cells (*27*). In this context, it should be noted that $S1P_1$ and $S1P_2$ are reportedly expressed not only in RBL-2H3 cells but also in primary bone marrow-derived mast cells (*25*). Furthermore, to strengthen our conclusion, other approaches than the usage of $S1P_2$ antagonists, *e.g.*, knockdown strategies involving siRNA, may be needed in the future.

We recently examined the effect of Sph-1-P on eosinophils. Sph-1-P acts as a chemoattractant for these cells (Yokoo, E., Yatomi, Y., Takafuta, T., and Ozaki, Y., unpublished observation), in contrast to mast cells. Accordingly, it is possible to speculate that Sph-1-P may inhibit and stimulate the locomotion of mast cells and eosinophils, respectively, thereby promoting interaction between mucosal mast cells (residing around the blood vessels) and infiltrating eosinophils. Recently, it was shown that nerve growth factor collaboratively worked with membrane lysophosphatidylserine of activated platelets to induce mast cell activation, and it was proposed that NGF released in response to inflammatory stimuli may contribute to mast cell activation in collaboration with locally activated platelets in the process of inflammation and tissue repair (*46*). We feel more attention should be paid to cross-talk between mast cells and platelets, although the significance of this cell-cell communication *in vivo* remains to be clarified in further studies.

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小児期アレルギー性鼻炎（花粉症）の長期予後

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Okubo Kimihiro
大久保公裕*
*日本医科大学耳鼻咽喉科助教授

小児アレルギー性鼻炎は増加している。疫学調査をみても両親の世代より発症が早い。また小児では通年性アレルギー性鼻炎が多いことが特徴であるが，近年花粉症も増加している。また両親がアレルギー性鼻炎であるほど小児アレルギー性鼻炎の発症が早いことが確認されている。小児では日常生活以外は成人よりQOLの低下は少ないが，これは小児があまり症状を訴えないことにも起因する。ガイドラインに沿った適切な治療で成人へのアレルギー性鼻炎の移行を重症化させないよう工夫が必要である。

QOL (quality of life)
花粉症
低年齢化
疫学
抗原回避

は じ め に

アレルギー性鼻炎はⅠ型アレルギーの典型的な疾患であり，現在では厚生労働省の定める生活習慣病としての慢性疾患でもある。治療が難しいかわりに，重症化しても

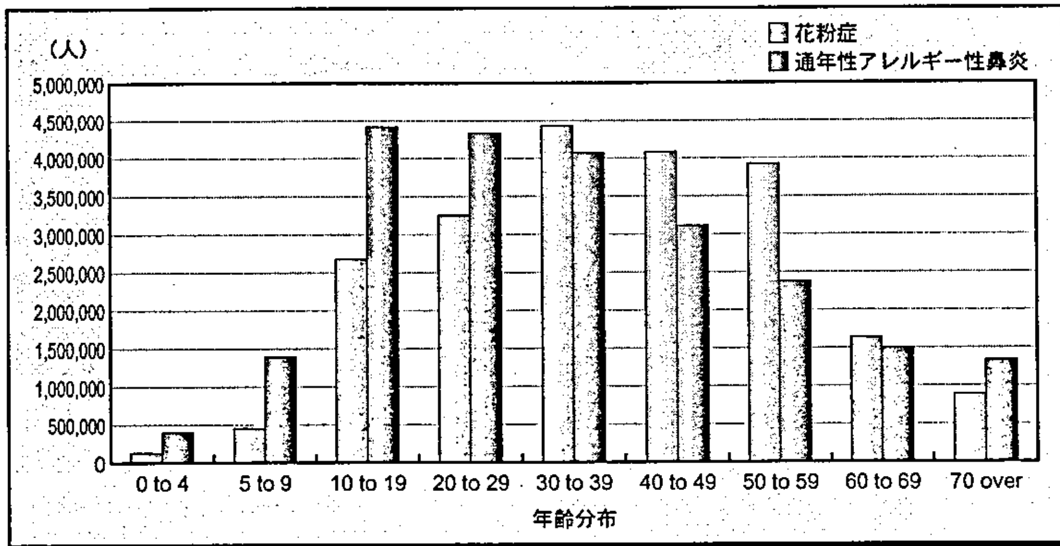

図1　年齢別アレルギー性鼻炎人口
通年性アレルギー性鼻炎は10，20，30代に有病者数のピークをもち，花粉症は30，40，50代にそのピークをもつ．
（鼻アレルギー診療ガイドライン2002年版〔ライフサイエンス〕および総務省人口推計月報より作成）

QOL（quality of life）の低下を生じるのみで死亡原因となりえない疾患がこの生活習慣病で，花粉症も含まれる[1]。実際のアレルギー性鼻炎の診断に最も重要なのは問診であり，症状を正確に把握することが必要である。しかし，小児では両親の共働きが多い現在では，問診が十分でないことも多く診断が困難になる場合がある。今回のテーマである長期予後はまずはじめの診断がはっきりしなければ予後を考えることはできない。

以前より一般的に小児では通年性アレルギー性鼻炎が多いとされていた（図1）[2]。当科で15歳以下の小児の通年性アレルギー性鼻炎と花粉症の割合を検討したところ，通年性アレルギー性鼻炎単独例が52.5%を占め，通年性アレルギー性鼻炎と花粉症の合併例も34.4%を占め，花粉症単独例も13.1%と決して少ない数字ではなかった。通年性アレルギー性鼻炎患児では，ハウスダストやダニ，ペットや昆虫など多種類の抗原に曝露されている場合が多い。小児花粉症のおもな感作抗原としてはスギ，ヒノキ科，草本類のイネ科のカモガヤ，ブタクサなどが挙げられる。現在はアレルギー疾患の既往がなかったのにスギ花粉やヒノキ科花粉症を突然発症する患児の増加傾向が目立ち，ヒノキ科花粉の飛散シーズンが終わった後もカモガヤ花粉などにより長期にわたり花粉症が継続する症例もしばしば経験する。今回，長期予後を考えるため，疫学的な調査結果，患者のインターネットによるQOLの調査[3]，そして長期の予後を左右すると考えられる治療について述べる。

Ⅰ．小児の疫学調査

花粉症発症の要因として遺伝の影響が以前から指摘されており，アレルギー家族歴がある場合は子どものアレルギー疾患発症に大きく影響し，特に母系からの影響が強いとされていた。製薬会社の会社員およびその家族を対象に行ったアレルギー性鼻炎に関するアンケート調査では，母親の発症歴が父親の発症歴に比べてより強く影響するわけではないことがわかった。対象は成人男性883人(会社員803人)，女性847人(同155人)，子ども1,285人。両親のアレルギー性鼻炎発症の有無による子どものアレルギー性鼻炎発症率を検討したところ，最も高かったのは，両親ともに発症歴がある場合で57.4%，次いで父親のみ発症がある場合で44.8%，母親のみ発症がある場合で44.1%，両親ともに発症歴がない場合は26.7%であった。両親とも発症歴がある群はいずれの群に比べても発症率が有意に高く，父親または母親のいずれかに発症がある群は両親とも発症がない群に比べて有意に高いことがわかった。しかし，父親のみ発症がある群と母親のみ発症がある群では発症率に差はなく，先行研究とは違った結果になった。

同じ調査結果から，両親とも発症歴がある場合では，いずれかの親に発症，両親とも発症歴がない場合に比べて低年齢で発症する傾向にあり，3歳でまず第1の発症ピークにあることがわかった。一方，両親とも発症歴がない場合は子どもが花粉症を発症する年齢は学童期以降となる傾向にあった(図2)。また，親の世代と子どもの世代との間で，15歳までにアレルギー性鼻炎を発症した割合と，花粉症を発症した割合を比較したところ，アレルギー性鼻炎では，親の世代では約9%であったのに対し，子どもの世代では約17%，花粉症のみで見ても，親の約5%に対して約10%であった[1]。現在の子どもの世代で発症年齢が低くなっているのは間違いない。発症が早いだけで，最終的な発症率そのものの値は変わらないという解釈も成り立つが，今後も増加する可能性も高い。低年齢の発症はアレルギー性鼻炎の自然寛解が小児ではほとんどなく，全年齢を通して5%以下と少ないことを考えると小児の発症ではアレルギー性鼻炎の罹病期間が成人での発症より長期化していることが考えられる。

Ⅱ．小児アレルギー性鼻炎のQOL

小児のアレルギー性鼻炎，特に花粉症が小児の生活に及ぼす影響を調べるためにインターネットでの調査を行った。2002年に成人で標準化された日本アレルギー性鼻炎標準QOL調査票に準拠し，インターネットでアンケートを実施した。このアンケートでは花粉症患者では日常生活，社会生活，身体，精神生活に花粉症の症状が大きく障害を及ぼすことが示された。しかし小児では成人ほどQOLは悪化しておらず，日常生活で悪化しているのみであった。どのQOLの領域でも「ややひどい」以上の率は成人より低い率であったが，特に精神生活の領域では成人と比較し，軽いことが分かった(図3)[3]。この調査では小児の受診率は55.5%で，成人の47.4%より高いが，一般的にはQOLが悪化していない小児の