Ⅱ. 研究成果の刊行に関する一覧表

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Ⅲ. 研究成果の刊行物・別冊

Clin Exp All Rev 2004; 4:9–14

Allergic rhinitis in children: environmental factors

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Summary
Increasing numbers of patients with allergic rhinitis are being noted on a global scale. Over 90% of Japanese patients with perennial allergic rhinitis show allergic reaction to the mite antigen and major pollen allergens such as Japanese cedar and Japanese cypress, which are carried long distances (> 100 km) by wind and hence can produce substantial harmful effects even in metropolitan areas. This situation is distinct from that in the West, where the most common anemophilous allergen, ragweed, travels much shorter distances of up to only several hundred metres. Environmental factors such as increased antigen, air pollution, diet, intestinal microflora, decreased incidence of infections, smoking, breastfeeding and vaccination may play important roles in the development and manifestation of allergic rhinitis in genetically predisposed subjects. In particular, in newborn infants, who carry the Th2 predominant state, environmental factors may greatly affect the development of balanced production of Th1 and Th2 cytokines. However, the contribution of any environmental factor to the postnatal development of allergic rhinitis has not been sufficiently determined. A better understanding of the processes involved may lead directly to better treatment or cure of allergic rhinitis.

Keywords air pollution, allergen, allergic rhinitis, dietary lifestyle, environmental factors, hygiene theory, intestinal microflora, parasite, tuberculin reaction, viral infection

Introduction

In recent years, many countries have experienced the problem of increasing prevalence of nasal allergy, with > 90% of patients with perennial allergic rhinitis showing allergic reaction to mite antigens [1]. The major pollinosis allergens in Japan are tree pollens such as sun tree (Japanese cypress) and evergreen tree (Japanese cedar), which can spread > 100 km from source and hence affect people living in metropolitan areas. This situation is distinct from that in the West, where the most common allergen is ragweed, which travels shorter distances of up to several hundred metres. Japanese cypress is widely distributed in Japan, predominantly to the west of the Kanto (Tokyo surrounding) region. Cedar pollen and cypress pollen share a common antigen and > 70% of Japanese patients with cedar pollen allergy also develop cypress pollinosis [1].

Although a well-controlled epidemiological study has yet to be conducted in Japan, questionnaire-based data suggest that cedar/cypress pollen allergy has a prevalence of 10–20/100 people nationwide. This figure varies considerably among the regions of the country, being lower than average in Hokkaido and Okinawa to the far north and far south, respectively [2, 3].

The age distribution curve of nasal allergy patients exhibits a bimodal pattern with two peaks: at ages 20s–40s affecting predominantly females and at approximately 10 years where males are more affected. It is generally regarded that the onset of perennial nasal allergy due to mite antigens begins in children and that of pollinosis is seen mainly in adult females. Recently, however, the age of onset of pollinosis appears to be decreasing.

A recent report by the Japan Ministry of Health, Welfare and Labor [4] has shown an increasing trend in the annual prevalence rate of allergic rhinitis among infants and children (Fig. 1). Furthermore, scratch tests of Japanese schoolchildren with nasal allergy indicate that besides mite antigens, children are also sensitized at high rates with many other allergens including Japanese cedar, cypress, orchard grass and ragweed pollen [3].

About 7% of patients with Japanese cedar pollinosis show spontaneous resolution of allergic reaction within a 10-year period after the onset of symptoms; elderly patients with initial symptoms after age 50 years are more likely to undergo spontaneous regression, whereas younger patients may not [5].

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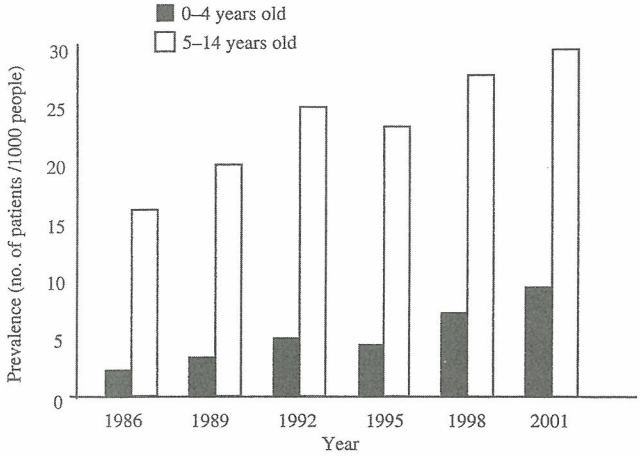

Fig. 1. Prevalence of allergic rhinitis (number of patients/1000 people) in Japanese children. Data from the Japan Ministry of Health, Welfare and Labor [4].

Onset of allergic rhinitis

As is seen with many other diseases, both genetic and environmental factors appear to be involved in the onset of allergic rhinitis. It is well known that family history is relevant to the onset of nasal allergy. For example, among identical twins concurrent onset is seen at high rates [6]. Furthermore, recent progress in genetic analysis has revealed the presence of genes regulating reactivity to leukotrienes [7] as well as cytokine-producing ability, both of which are involved in allergic responses. However, environmental factors appear to play a major aetiological role as even among identical twins the influence of genetic factors is calculated at $< 70\%$, and nasal allergy prevalence rates are known to increase among immigrant populations moving from developing to developed countries. At least one report has stated that environmental factors play the most important role in the recent increase of allergic diseases [8].

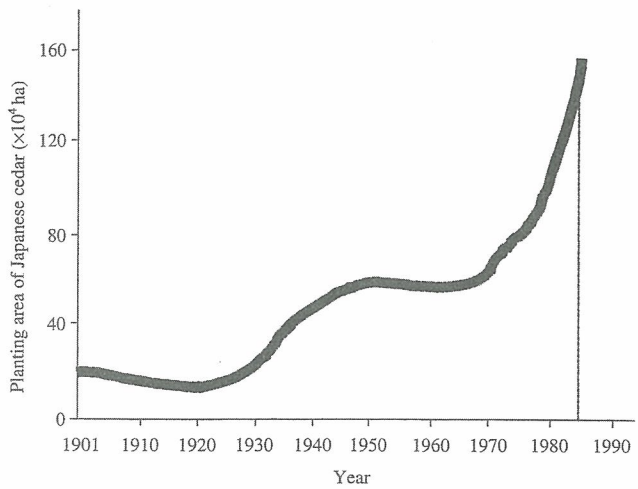

Fig. 2. Planting area of Japanese cedar aged > 30 years.

Table 1. Time-course of number of mites in house dust in Japan

Year	No. of mites (per g house dust)
1964	600
1976	1100
1983	1250

Environmental factors

Increase of allergens

Levels of environmental allergens are rising. In Japan, the amount of airborne Japanese cedar pollens has increased dramatically; after World War II, an extensive afforestation programme with Japanese cedar was initiated because of the fast growth rate of this species and its value as a building material. Figure 2 shows the planting area of Japanese cedar aged > 30 years, which produces the most pollen [9]. In addition, mites have also increased vastly in number in Japan (Table 1) due to the extensive use of concrete for houses, which provides high humidity and favourable temperatures for propagation [10].

Air pollution

Air pollution due to ozone, nitrogen oxides and sulphur oxides has been pointed out as contributing to morbidity due to allergic rhinitis [11–14]. Although not implicated directly in the development or enhancement of allergic sensitization, these environmental factors have been shown to aggravate allergic symptoms. More recently, discharge of diesel exhaust particles (DEP) from automobiles, rather than industrial soot and smoke, has attracted particular attention following reports that DEP challenge can lead to overproduction of IgE and increased permeability of the nasal mucosa membrane [15, 16]. Binding of DEP to cedar pollen grains during their long-distance travel has also been observed and may contribute to the promotion of cedar-specific IgE production.

Change of dietary lifestyle and intestinal microflora

Recently, consumption of fish rich in n-3 polyunsaturated fatty acid has been decreasing in Japan, whereas that of meat containing high amounts of n-6 polyunsaturated fatty acid is increasing [17]. Some studies have suggested that administration of n-3 polyunsaturated fatty acid improves symptoms in Japanese children with asthma [18]. However, the benefits of this treatment in allergic rhinitis have not been elucidated.

Lifestyle changes in diet tending towards high protein and fat intake also greatly affect the intestinal microflora, which in turn has significant effects on the body's immune reactions. For example, in germfree mice lacking intestinal microflora, IgE production was accelerated, whereas on normalization of intestinal bacterial flora IgE production was restored [19]. Another interesting study of the human

intestinal microflora reports that in Estonia, where the allergy prevalence rate is low, the human intestinal microflora mainly consists of eubacteria and enterococci [20]. However, in Sweden where high allergy prevalence is observed, the microflora comprises mainly *Bacteroides* and *Clostridium* species whereas 35 years ago both the microflora and prevalence were similar to those currently seen in Estonia [20]. This suggests that increases in allergy prevalence in Sweden may reflect changes in gut microflora.

Infectious diseases

Infectious diseases may also play a role in the aetiology of allergic rhinitis. Th2 cytokines such as interleukin (IL)-4 and IL-5 are involved in the stimulation of IgE production as well as migration and activation of eosinophil leucocytes and mast cells. On the other hand, the Th1 cytokines interferon (IFN)-γ and IL-2 assist in the activation of monocytes and cellular immunity. Many allergic patients exhibit dysregulated production of Th1 and Th2 cytokines, exhibiting predominant Th2 cytokines imbalance. Tuberculosis is a typical infectious disease that induces Th1 cytokine production; among representative diseases capable of activating the Th2 system, parasitic infections such as helminth infection are examples. Relating to this, there have been a number of reports suggesting that modern decreases in helminthiasis may have had a direct causative role in the rise of allergic diseases [21–23].

Parasite infection

To clarify the relationship between intestinal parasite infection and allergic rhinitis, we examined high-school students in Ecuador where parasitic infections are common. Clinical history, nasal inspection, eosinophilia in nasal discharge, skin scratch testing and measurement of antihouse dust (HD)-specific IgE and total IgE levels in serum were recorded. Parasitological evaluation included examination of stool samples and quantification of specific anti-ascaris IgE levels in serum. Figure 3 shows the incidence of nasal allergy and detection rate of parasite infection. Students with helminths in stool samples demonstrated significantly lower incidence rates of HD allergic rhinitis than those with protozoan infections. Although this would seem to indicate that helminthic infection may protect against the development of allergic rhinitis and that protozoan infection may promote this allergy, there was a significant correlation between anti-HD IgE and anti-ascaris IgE ($r = 0.615$, $P < 0.01$) (Fig. 4). Antihelminthiasis IgE is thought to participate in elimination of helminths. Allergic patients had Th1/Th2 cytokines imbalance resulting in Th2 predominance; hence, when infected, patients with allergic rhinitis produced substantially higher levels of antihelminth IgE compared with those without rhinitis. This IgE production may persist for some period to prevent chronic infection or reinfection. The rate of positive skin scratch tests for HD in students with serum anti-HD

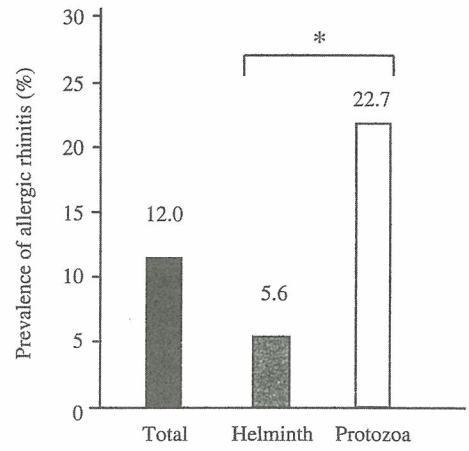

Fig. 3. Prevalence of allergic rhinitis in children with parasitic infection. Children exhibiting parasites in stool demonstrate significantly lower incidence rates of house dust allergic rhinitis than those with protozoan infections. $^*P < 0.05$.

IgE antibody showed no significant difference when comparing skin test results stratified by total IgE levels (data not shown), suggesting that even with high concentration of total serum IgE, the allergen-specific antibody is able to bind to its specific Fc receptor on mast cells, indicating a nonsaturated state. In our study, evidence for helminthic infection affecting the development of HD allergic rhinitis was inconclusive.

Tuberculin reaction and BCG vaccination

Shirakawa et al. [24] studied the relationship between tuberculin reactions and allergic reactions by comparing the same cohort of Japanese junior high-school students at ages 6 and 12 years. Students who had negative response to tuberculin test at both 6 and 12 years of age showed significantly high prevalence rates of nasal allergy and asthma as well as high IgE values in blood as compared to those who were positive. This leads to the possibility that the recent decrease in tuberculosis infection may have

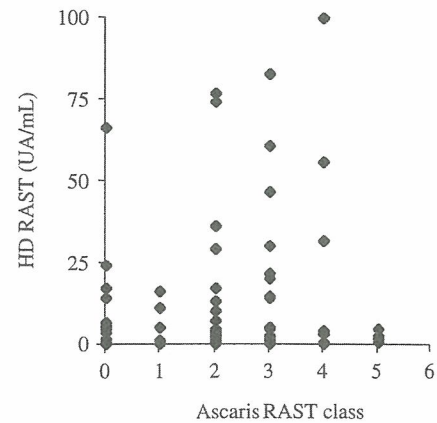

Fig. 4. Relationship between antihouse dust IgE and anti-ascaris IgE. A significant correlation ($r = 0.615$, $P < 0.01$) between anti-HD IgE RAST and anti-ascaris IgE scores is seen.

caused the high prevalence of allergic disease. However, the relationship between the tuberculin reaction and prevalence of allergic rhinitis remains controversial [25–28]. BCG vaccine is known to strongly induce Th1 cytokines and some studies have suggested that administration of BCG vaccine might reduce IgE levels of patients, including those with allergic rhinitis [29, 30]. However, it is not clear whether BCG vaccine-induced strong Th1 response in patients in whom Th2-dominant immune deviation has been established is able to improve their allergy symptoms.

Viral infection

The nasal cavity, the most frontal part of respiratory tract, is often the target of viral infection, although no relationship between such infections and allergic rhinitis has been defined [31, 32]. The common cold is the most widespread viral infection, usually induced by rhinovirus, parainfluenza virus, adenovirus and respiratory syncytial virus (RSV) [33]. We examined histamine sensitivity, defined as the lowest intranasally administered histamine concentration needed to induce sneezing attack in nonatopic patients with the common cold. During the early 3–4 days of common cold, increased nasal sensitivity to histamine was observed; however, this hypersensitivity was transient and returned to baseline values several days later. Histamine concentration of nasal wash in both the acute phase and covalescent phase of these patients showed no significant difference. Thus common cold induces transient nasal hypersensitivity that is not correlated with histamine concentration.

The mechanism of hypersensitivity to viral infection includes impairment and shedding of epithelial cells and concomitant exposure of sensory nerve endings [34]. In addition, viral infection may directly affect the nasal mucosal immunity. After RSV inoculation of isolated human nasal epithelial cells *in vitro*, both enhanced production of IL-6 and GM-CSF and increased ICAM-1 mRNA expression were observed [35]. Furthermore, tonsillar cells adherent to RSV-infected nasal epithelial cells produced more IL-4 than nonadherent cells (Fig. 5). However, no significant difference was observed for IFN-γ production in these tonsillar cells.

In another study, BALB/c mice were infected intranasally with RSV then treated with ovalbumin (OVA) by nebulizer, and the antibody response to OVA determined [36]. IgG anti-OVA antibody was significantly higher in RSV-infected than control mice, and furthermore IgE anti-OVA antibody, although in low concentrations, was detected only in the infected animals (Fig. 6). Thus RSV infection may augment the antibody responses including IgE to inhaled antigens. To examine further the influence of RSV infection in OVA-sensitized mice, B6 mice were sensitized with OVA administered intraperitoneally followed by intranasal infection with RSV (manuscript in preparation). Three days later, nasal sensitivity to OVA and to histamine was examined. Nasal rubbing attacks in response to both OVA and histamine challenge were

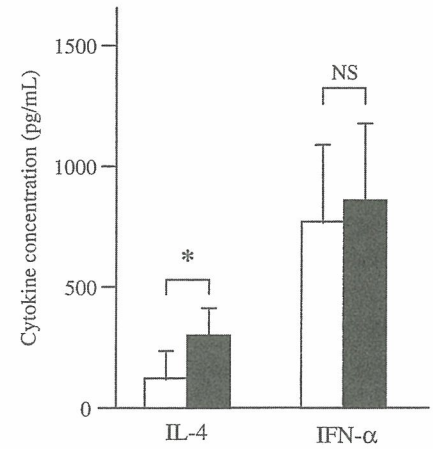

Fig. 5. Cytokine levels in tonsillar lymphocyte culture not bound (□) and bound (■) to RSV-infected nasal epithelial cells. Tonsillar cells adherent to RSV-infected nasal epithelial cells produced more IL-4 but not IFN-γ compared with nonadherent cells. $^*P < 0.05$. Reprinted with kind permission of Blackwell Publishing from Matsuzaki et al. [35].

enhanced dramatically in RSV-infected mice compared with noninfected mice (Fig. 7). The eosinophil number in the nasal mucosa was also highly increased in RSV-infected animals. Thus acute viral infections enhance the responses to subsequent OVA sensitization as well as strongly enhancing the sensitivity of prior-sensitized mice.

The hygiene theory

The hygiene theory propounds that decreases in infections acquired during early childhood may be responsible for the increasing prevalence of allergic diseases in the population [37]. In support of the theory, the frequency of several allergic diseases including allergic rhinitis has been shown to be inversely associated not only with childhood infections [38, 39] but also with the number of siblings [40, 41]. The presence of older siblings naturally exposes children to more infections during early childhood and protects

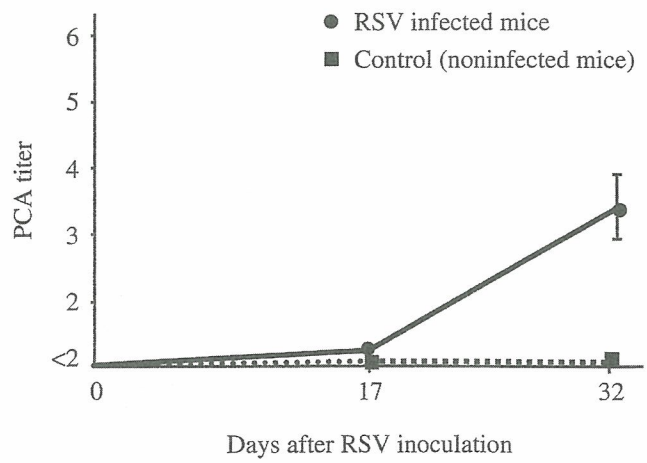

Fig. 6. Detection of IgE anti-OVA antibody in BALB/c mice intranasally infected or sham-infected with RSV then treated with OVA. Reprinted with kind permission of the Society for Experimental Biology and Medicine from Freihorst et al. [36].

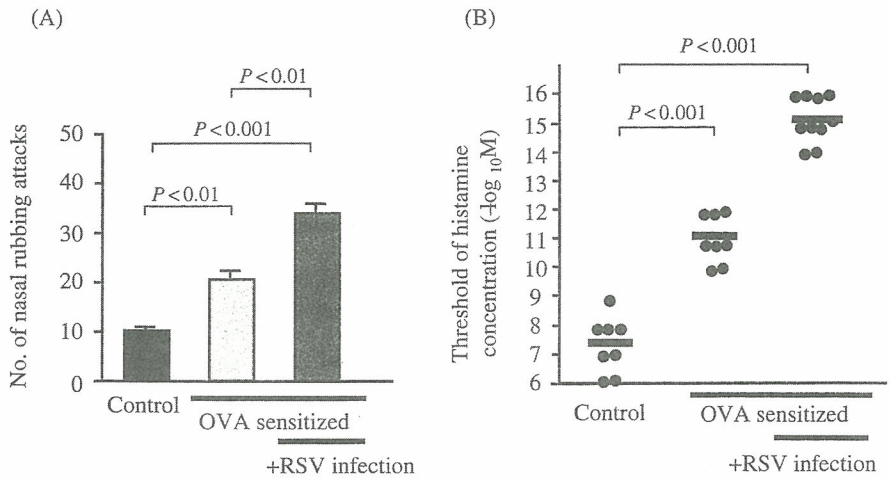

Fig. 7. Nasal rubbing attacks on provocation test with OVA (A) and sensitivity against nasally administered histamine (B) in B6 mice. Control: non-OVA sensitized, non-RSV infected mice; OVA sensitized: OVA sensitized but not RSV infected mice. OVA sensitized + RSV infection: OVA sensitized followed by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

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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.

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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

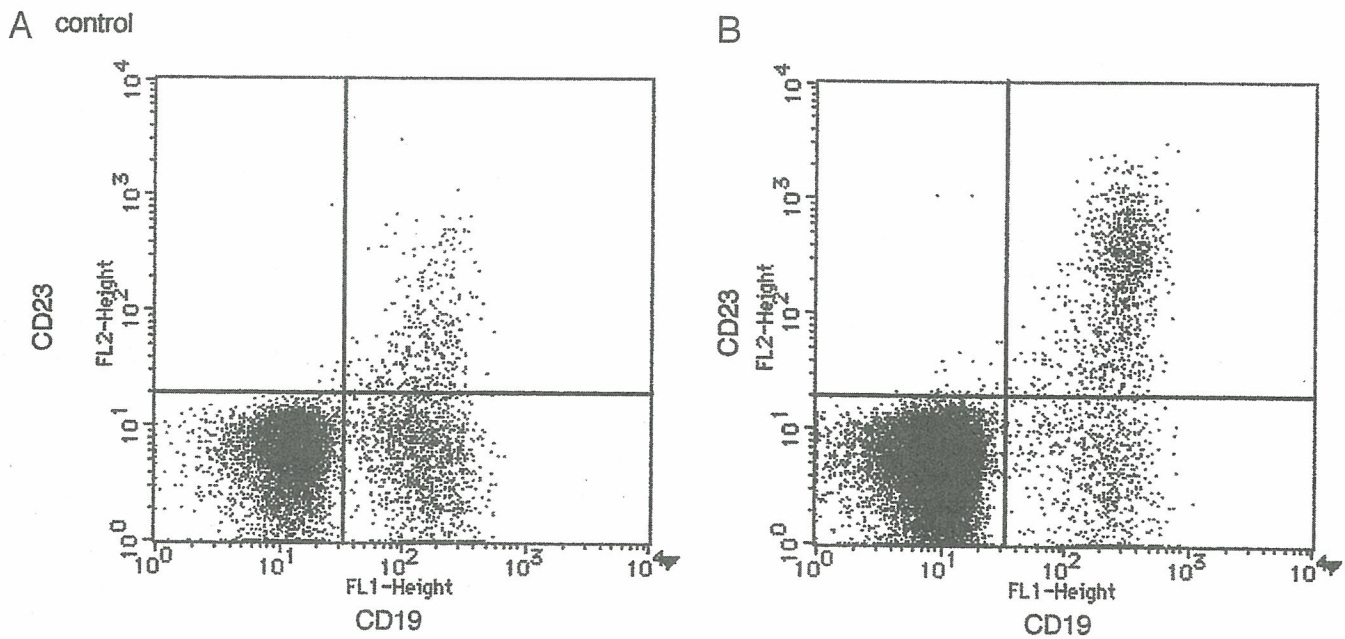

Figure 1. Representative dot plots for fluorescein isothiocyanate–conjugated anti-CD19 (FL1) and R-phycoerythrin–conjugated anti-CD23 (FL2) antibodies for the control group (A) and the allergic rhinitis (AR) group (B). The proportion of CD23-bearing B cells was measured by flow cytometry as CD19 and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

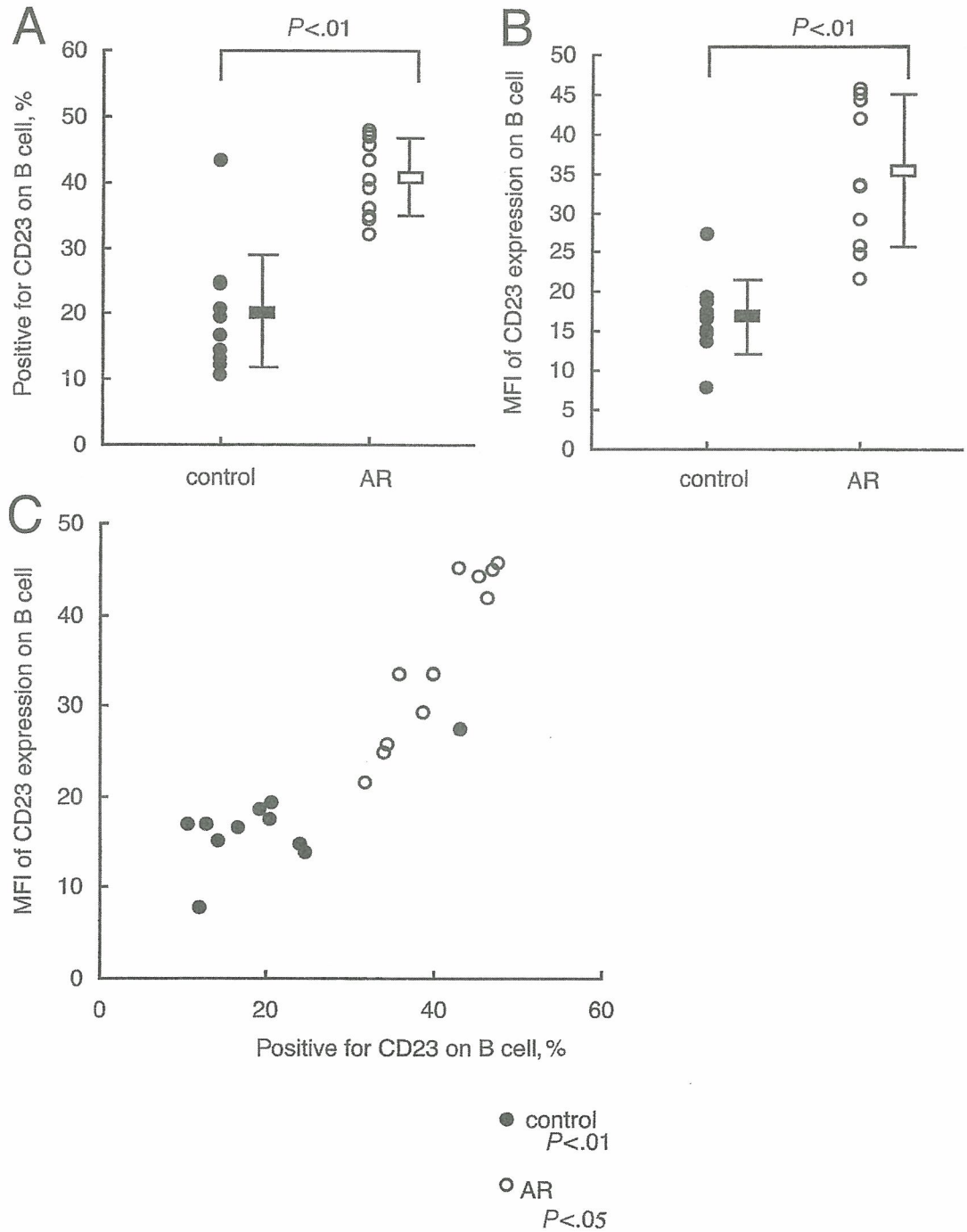

Figure 2. CD23 expression on B cells. A, Percent positive values for CD23 on B cells from patients with allergic rhinitis (AR) were significantly higher than for the control group. B, A similar tendency was seen in the mean fluorescence intensity (MFI) of CD23 expression on B cells, which was significantly 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.

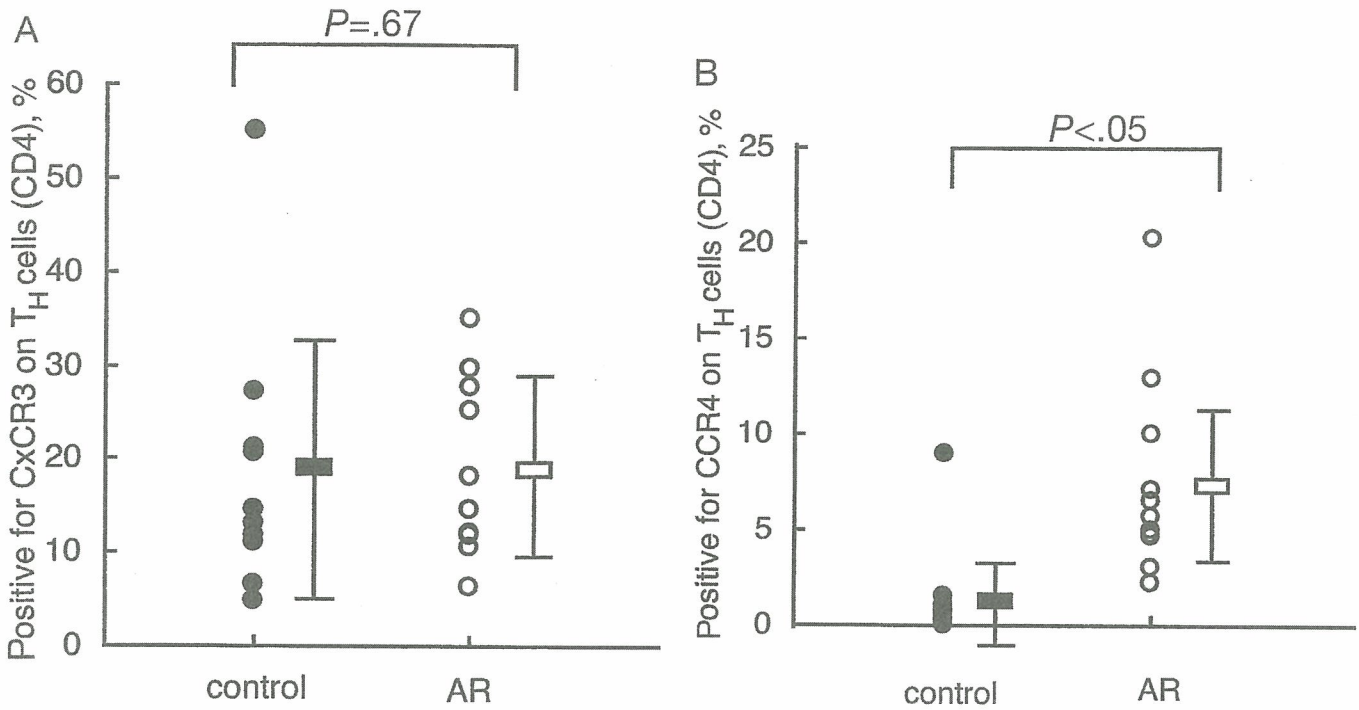

Figure 3. Percent positive values for chemokine receptor expression on mucosal CD4 T cells. A, Data for the CXCR3 subtype, assumed to be T_H1 cells. B, Data for the CCR4 subtype, assumed to be T_H2 cells. There is no significant difference in percent positive values for CXCR3 on CD4 cells from patients 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

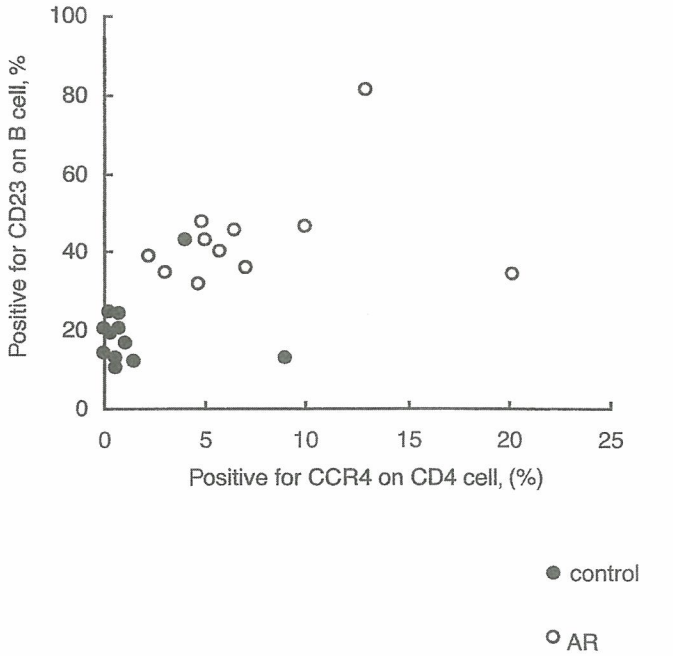

Figure 4. Correlation plot for percent positive values for CD23 on mucosal B cells and percent positive values for CCR4 on mucosal CD4 cells. There is no significant correlation between these data in the control group (P = .25) or the allergic rhinitis (AR) group (P = .17).

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]

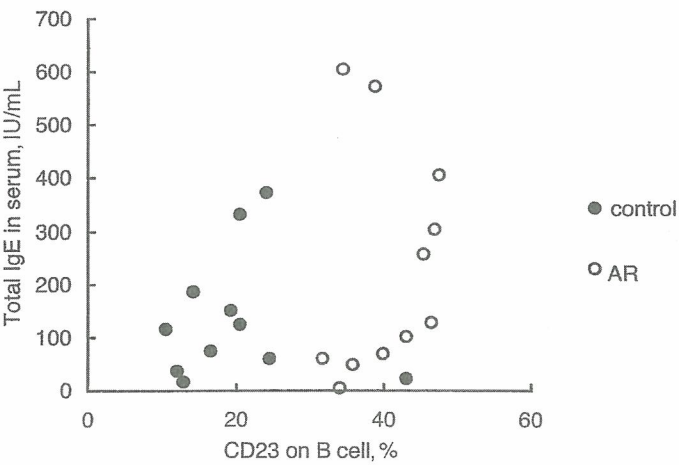

Figure 5. Correlation plot for percent positive values for CD23 on mucosal B cells and the total IgE level. There is no significant correlation between these data in the control group (P = .21) or the allergic rhinitis (AR) group (P = .66).

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

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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

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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

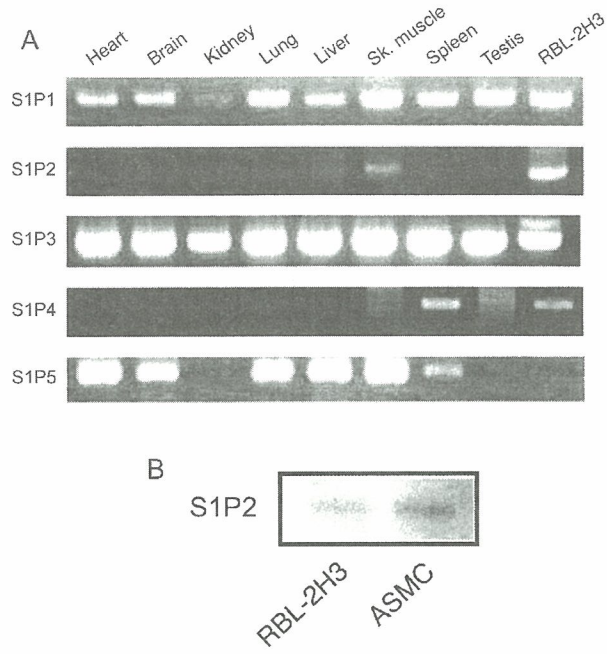

Fig. 1. **Expression of S1Ps in RBL-2H3 cells.** (A) Detection of expression of mRNA for S1Ps in RBL-2H3 cells, in comparison with in various rat tissues, was performed by RT-PCR. cDNA preparations from RBL-2H3 cells and various rat tissues were amplified for $S1P_{1-5}$. The products were resolved on 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.

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.

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

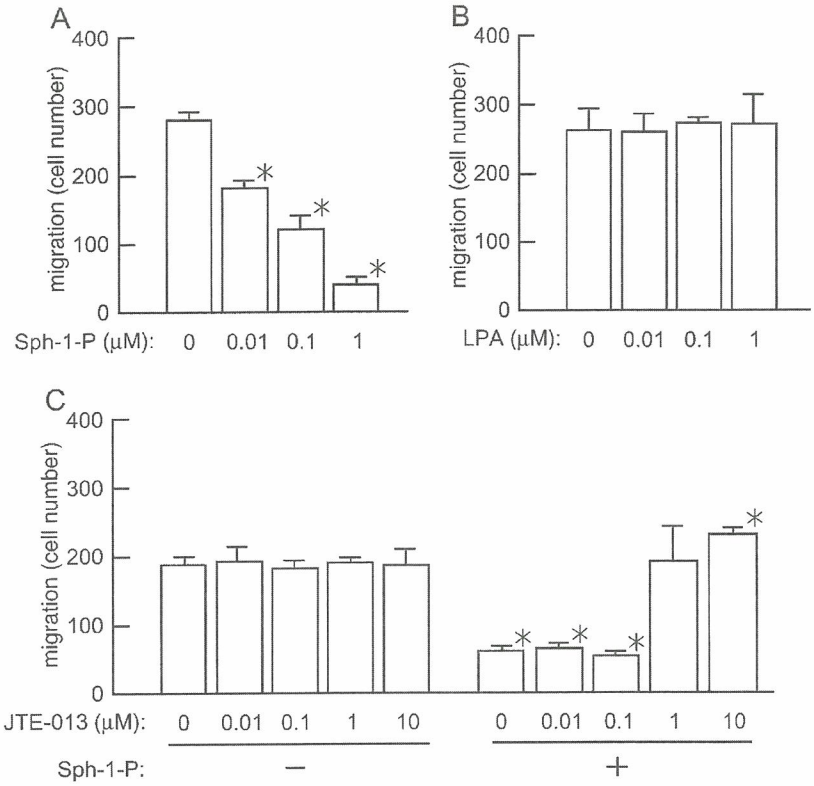

Fig. 2. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a modified Boyden's chamber assay.** (A and B) RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of Sph-1-P (A) or LPA (B) were placed. (C) RBL-2H3 cells 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 $LPA_{1\text{-}3}$ 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