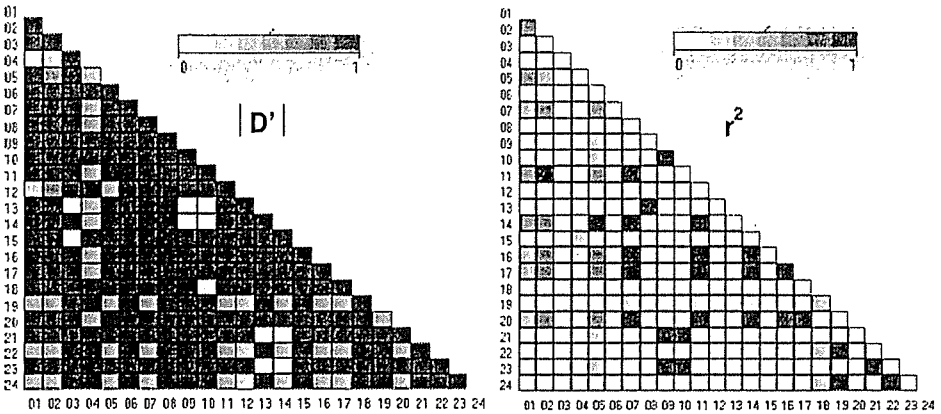

Fig. 2 Pair-wise linkage disequilibrium (*LD*) was measured by $|D'|$ and r^2 among the all of the SNPs identified in 24 sequenced samples. The blocks are *shaded* corresponding to the values obtained from the LD analysis program, SNP Alyze

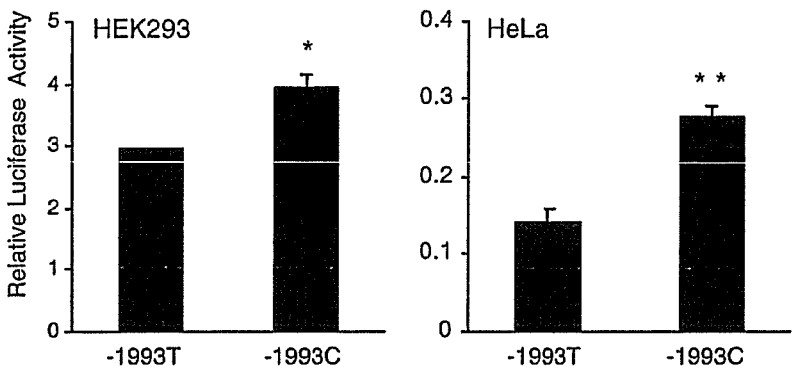

Fig. 3 Effect of the −1993T → C SNP on the transcription activity of the human *TBX21* promoter. HEK293 cells or HeLa cells were transiently cotransfected with pGL/−1993T or pGL/−1993C and pRL-TK vector. The relative luciferase activity of the *TBX21* reporter constructs is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for three independent experiments. $*P < 0.001$; $**P < 0.005$, as determined by the Student's *t* test

most common, followed by −1993C-390G and −1993C-390A. The two major haplotypes −1993T-390A and −1993C-390G were named haplotypes 1 and 2, respectively. The overall distribution of two loci haplotypes was not different between cases and controls (3 d.f.; $P = 0.10$ for AIA, and $P = 0.09$ for AS/NP), although the frequencies of two major haplotypes, haplotypes 1 and 2, were significantly different in a χ^2 test (1 d.f.; $P = 0.014$ for AIA, and $P = 0.016$ for AS/NP) (Table 4).

Transcriptional effect of TBX21 −1993T → C polymorphism

In functional assays, since the 390A → G polymorphism is a synonymous substitution, which is less likely to be directly associated with disease in general, we focused on the promoter SNP at position −1993, viz., −1993T → C. To understand the role of the T/C polymorphism at −1993 in the transcriptional regulation of the human *TBX21* gene, we performed transient expression of the −1993T and −1993C luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, in HEK293 and HeLa cells. Luciferase activity in cell extracts was analyzed after 24 h of transfection and was standardized against the internal control (*Renilla* activity). The results of this experiment showed that the −1993C construct had significantly higher luciferase reporter activity compared with the wild-type −1993T construct (33%–98% increase; $P < 0.005$). These results suggest that the −1993C allele may be associated with the increased transcriptional activity of the *TBX21* gene in human lungs.

EMSA analysis

To examine whether −1993T → C affected interaction of a nuclear factor(s) with the *TBX21* sequence around −1993, we then performed EMSAs. We prepared 2000/−1986 double-stranded oligonucleotide probes containing either the T or the C allele at −1993 bp. HEK293 nuclear extract contained nuclear proteins binding specifically to this region of the *TBX21* promoter, resulting in the formation of one major and one minor complex. Competition with 100-fold to 200-fold excess of unlabeled −1993T of −1993C probes resulted in complete inhibition of complex formation. The single band corresponding to the −1993C allele was significantly more intense than that corresponding to the −1993T allele (21% increase; $P = 0.02$ by Student's *t* test), suggesting the two different alleles had different affinities for a

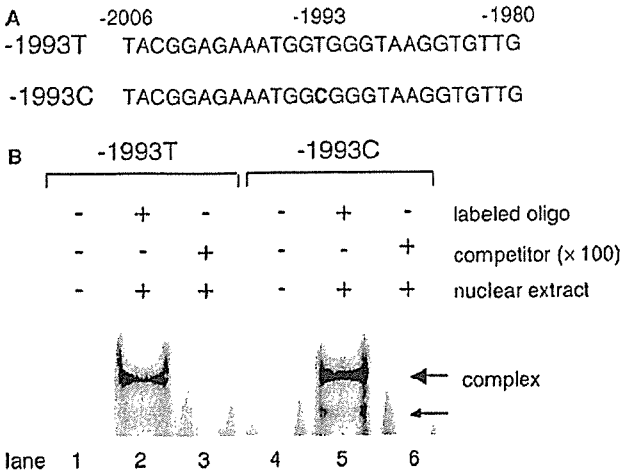

Fig. 4 EMSAs with nuclear extracts prepared from HEK293 cells. Extracts were incubated with DIG-labeled 27-bp double-stranded oligonucleotides corresponding to the −1993T or −1993C alleles of *TBX21*. Competition studies were performed by preincubating with a 100-fold excess of the unlabeled −1993T or −1993C double-stranded competitor oligonucleotides. a Oligonucleotide sequences containing T or C at −1993 bp (*bold*) and that were used as a probe or a competitor are shown. **b** Unknown nuclear protein of HEK293 nuclear extracts formed a much stronger complex with the −1993C oligonucleotide compared with the −1993T oligonucleotide (compare *lane 2* vs. *lane 5*; $P = 0.02$ by Student's *t* test). Binding complex was specifically competed by excess of unlabeled −1993T or C oligonucleotide (*lanes 3, 6*). Band intensity was quantified by using the LAS-3000 camera system and image analysis software Multi Gauge Version 2.0 (Fuji Photo Film). A representative result of three independent experiments is shown

particular nuclear factor. The same trend was also observed in HeLa cells (data not shown). Computer analysis of sequences covering −1993 bp, by using NSITE, available at http://www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter, indicated that the −1993T → C SNP is situated on a putative binding site for the E2F-1 transcription factor. To identify whether these putative consensus sites were involved in the transcriptional regulation of the *TBX21* gene, we performed a gel shift assay in the presence of specific anti-E2F-1 antibody (C-20; Santa Cruz Biotechnology, Calif., USA). However, preincubation with anti-E2F-1 antibody did not result in a supershift of the DNA-protein complexes (data not shown), suggesting that this protein (family) was not present in the complex binding to this region under these conditions. Together, these data indicate that the −1993T → C SNP in the human *TBX21* gene increases the affinity of an unknown nuclear protein to the binding site around −1993, leading to increased transcriptional activity and a higher expression of the T-bet protein.

Discussion

In the adaptive immune system, $CD4^+$ Th cells differentiate into at least two classes of effector cells, Th1 and Th2, in response to different pathogen-derived antigens. Th1 cells mediate cellular immunity and provide protection against intracellular pathogens and viruses, whereas Th2 cells produce IL-4, IL-5, and IL-13 and eradicate helminthes and other extracellular parasites (Mosmann and Coffman 1989). T-bet, a T box expressed in T cells, has recently been described as a master transcriptional regulator specific to IFN-γ-expressing lineages and is sufficient to induce IFN-γ and IL-12 receptor β2 expression, even under Th2-polarizing conditions (Afkarian et al. 2002; Szabo et al. 2000). Recent experiments have found that, without any allergic sensitization or challenge, the bronchi in mice lacking the T-bet gene, *tbx21*, are infiltrated with eosinophils and lymphocytes and exhibit signs of the airway remodeling and AHR to methacholine that are typical of allergic asthma (Finotto et al. 2002).

In order to examine whether polymorphisms in the candidate gene *TBX21* are related to the risk of human asthma phenotypes, we have characterized sites of

Table 3 Allele frequencies of *TBX21* SNPs in Japanese patients from different asthma groups and controls. Values are the number (%) of successfully genotyped chromosomes

Allele	Healthy controls ($n=640$)	Child patients with asthma ($n=361$)	P^a	Adult patients with					
				Atopic asthma ($n=313$)	P^a	Non-atopic asthma ($n=88$)	P^a	AIA ($n=72$)	P^a
−1993T → C									
T	1149 (89.8)	624 (89.1)		565 (90.3)		161 (93.6)		118 (81.9)	
C	131 (10.2)	76 (10.9)	0.67	61 (9.7)	0.74	11 (6.4)	0.11	26 (18.1)	0.004[b]
99C → G									
C	1127 (88.5)	617 (88.6)		509 (85.1)		143 (88.3)		116 (85.3)	
G	147 (11.5)	79 (11.4)	0.9	89 (14.9)	0.04	19 (11.7)	0.94	20. (14.7)	0.28
1298T → C									
T	1073 (83.8)	603 (83.5)		541 (87.0)		149 (84.7)		125 (86.8)	
C	207 (16.2)	119 (16.5)	0.86	81 (13.0)	0.07	27 (15.3)	0.78	19 (13.2)	0.35
7725G → A									
G	1062 (83.4)	577 (79.9)		502 (82.6)		139 (81.8)		122 (84.7)	
A	212 (16.6)	145 (20.1)	0.05	106 (17.4)	0.67	31 (18.2)	0.6	22 (15.3)	0.68

[a] *P*-value for the comparison with controls
[b] *P*-value statistically significant after Bonferroni correction (corrected $P = 0.016$)

Table 4 Genotype, allele, and haplotype frequencies in Japanese AIA, AS/NP cases, and controls for the *TBX21* SNPs at—1993 and 390

Locus	Haplotype number		Controls ($n=640$)	AIA ($n=72$)	Uncorrected P	Odds ratio (95% CI)	AS/NP ($n=42$)	Uncorrected P	Odds ratio (95%)
−1993T → C		Genotype TT	519 (81.1)	48 (66.7)		1.0	27 (64.3)		1.0
		Genotype TC+CC	121 (18.9)	24 (33.3)	0.004	2.15 (1.26–3.64)	15 (35.7)	0.008	2.38(1.23–4.62)
		Allele T	1149 (89.8)	118 (81.9)		1.0	68 (81.0)		1.0
		Allele C	131 (10.2)	26 (18.1)	0.004	1.93 (1.22–3.06)	16 (19.0)	0.012	2.06(1.16–3.66)
390A → G		Genotpye AA	533 (83.3)	50 (69.4)		1.0	29 (69.0)		1.0
		Genotype AG+GG	107 (16.7)	22 (30.6)	0.004	2.19 (1.27–3.77)	13 (31.0)	0.019	2.23(1.12–4.44)
		Allele A	1165 (91.0)	120 (83.3)		1.0	70 (83.3)		1.0
		Allele G	116 (9.0)	24 (16.7)	0.004	2.01 (1.25–3.24)	14 (16.7)	0.021	2.01 (1.10–3.68)
[−1993]-[390]	1	T-A	1148 (89.7)	120 (83.3)			68 (80.9)		
	2	C-G	114 (8.9)	22 (15.3)	0.014[a]		14 (16.7)	0.016[a]	
	3	C-A	17 (1.3)	2 (1.4)			2 (2.4)		
	4	T-G	1 (0.1)	0 (0.0)	0.10[b]		0 (0.0)	0.09[b]	

[a] P-value for the comparison of the frequencies of haplotype 1 and 2
[b] P-value for the overall distribution of two loci haplotypes

genetic variation in selected genomic regions of *TBX21*. Among 24 SNPs identified (five are novel), four polymorphic sites were selected for further analysis. All SNPs fulfilled Hardy–Weinberg expectations in both asthmatic and non-asthmatic subjects, and our study showed a significant association between AIA and a SNP in the regulatory region −1993T → C of the human *TBX21* gene ($P_c=0.016$); this was found to be in strong LD with a synonymous coding SNP, 390A → G, located in exon 1 ($D'=0.92$; $r^2=0.85$). Consistent with recent data (Chung et al. 2003; Ylikoski et al. 2004), these four *TBX21* SNPs lack association with any other asthma phenotype in Japanese subjects.

The percent of the −1993C or 390G allele was much higher in AIA patients than normal controls. In an attempt to extend and support these findings, we further genotyped the −1993T → C and 390A → G SNPs in independent adult AS/NP patients and also found a significant association between these SNPs and AS/NP for the allele and genotype frequencies ($P=0.008$). Furthermore, our data indicated that the single base substitution corresponding to the −1993 *TBX21* polymorphic site produced differences in the transcriptional activity of the *TBX21* gene. Unexpectedly, the *TBX21*/−1993C reporter construct was transcriptionally more active than the wild-type −1993T construct in HEK293 and HeLa cells. In addition, EMSA analysis demonstrated that the −1993T → C substitution increased the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position.

AIA refers to the development of bronchoconstriction following the ingestion of aspirin and other NSAIDs. This clinically distinct syndrome is characterized by aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis, commonly called the "aspirin triad". AIA affects 5%–20% (about 10%) of adult asthmatics with a higher prevalence in women and is infrequently found in asthmatic children (Babu and Salvi 2000; Szczeklik and Stevenson 1999). Chronic persistent inflammation is the hallmark of patients with AIA. Recently, the importance of arachidonic acid metabolites in the pathogenesis of AIA has become apparent. The cyclo-oxygenase (COX) theory is widely accepted: AIA attacks are triggered by the specific inhibition of COX in the respiratory tract, which is followed by a reduction of prostaglandin E2 (a brake on leukotriene synthesis) and an overproduction of cysteinyl leukotrienes. Thus, cysteinyl leukotrienes have been recognized as the key mediators of AIA, but the precise molecular mechanism involved in AIA remains unclear.

Surprisingly, our results have shown a significant increase in the −1993C allele, the putative higher expression of T-bet, among patients with AIA or AS/NP, compared with controls in our Japanese cohort. An inappropriate or excess Th2-biased immune response to environmental antigens has generally been considered to play a crucial role in the development of asthma. Whereas Th2 cells promote asthmatic inflammation, Th1 cells, which secret IFN-γ, have been proposed to protect against asthma by dampening the Th2 response. However, the evidence from many studies of asthma in human and animal models conflicts with this interpretation (Busse and Lemanske 2001; Salvi et al. 2001). For example, IFN-γ production is elevated in the serum of patients with asthma (Corrigan and Kay 1990), in supernatants of bronchoalveolar lavage (BAL) cells (Cembrzynska-Nowak et al. 1993), in T cells themselves in BAL (Krug et al. 1996), and in whole blood culture (Magnan et al. 2000). By using an adaptive transfer system in mice, previous reports have shown that antigen-specific Th1 cells cause considerable airway inflammation instead of attenuating Th2-mediated lung disease (Hansen et al. 1999; Li et al. 1998; Randolph et al. 1999). IFN-γ has been demonstrated to activate eosinophils in vitro, not only with an increased expression of Fcγ receptors, CD69, HLA-DR, and intercellular adhesion

molecule-1, but also with increased viability (Busse and Lemanske 2001; Krug et al. 1996). Furthermore, therapy with IL-12, a Th1-inducing cytokine, fails to reduce AHR or the late asthmatic reaction (Bryan et al. 2000). These and other recent data (Ford et al. 2001; Sugimoto et al. 2004) suggest that IFN-γ contributes to the augmentation of allergic lung inflammation partly through the activation of eosinophils, highlighting the importance of both Th1 and Th2 cytokines in the development of asthma. Thus, the classification of allergic inflammation in asthma as a Th2-mediated disease is too simplistic (Busse and Lemanske 2001), and, as pointed out by recent work (Sugimoto et al. 2004), we propose that asthma may be classified roughly into at least two subgroups, Th2-type asthma and Th1/Th2 mixed-type asthma, including AIA.

Previous reports have suggested that Th1 cells can actually cooperate with Th2 cells in vivo and enhance Th2, eosinophil, and neutrophil recruitment by increasing the expression of TNF-α, chemokines, and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) in the lungs (Randolph et al. 1999; Takaoka et al. 2001). Actually, aspirin sensitivity and AS/NP often coexist with severe asthma, and the airways of AIA patients with NP show signs of persistent inflammation with marked eosinophilia and enhanced VCAM-1 expression (Hamilos et al. 1996). The continuous airway inflammation in AIA could result from a non-IgE-mediated reaction to specific endogenous or exogenous antigens such as a virus (Szczeklik 1988; Szczeklik and Stevenson 1999). A latent or chronic viral infection has been shown to alter the expression of many cellular genes, including several constituents of the arachidonic acid pathway (Zhu et al. 1998), and virally infected cells are more prone to drug and drug-metabolite-related toxicity (Levy 1997; Nakagawa et al. 2001). Moreover, an antiviral drug, acyclovir, is reported to inhibit analgesic-induced bronchoconstriction and decrease the urinary levels of leukotrieneE4 in patients with AIA (Yoshida et al. 1998). Based upon these observations of AIA, virus-specific Th1 cells responding to a respiratory tract infection could alter the local lung environment sufficiently to increase Th2 and eosinophil recruitment, leading to strong Th2 responses to inhaled antigens induced by IL-4, 5, 13, and other mediators. We postulate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter causes a functional difference in T-bet expression, resulting in increased T-bet production and (viral-induced) an excessive Th1 inflammatory reaction in the lungs.

Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. Although we find no associations of *TBX21* SNPs with other asthma groups except for AIA, our data also indicate that the presence of the $-1993C$ allele increases the risk of AS/NP, regardless of aspirin sensitivity. Thus, in asthma phenotypes, the *TBX21* SNPs are probably not strictly associated with aspirin sensitivity itself. NP is a chronic inflammatory disease of the paranasal sinus mucosa, leading to the protrusion of edematous polyps into the nasal cavities (Mygind 1990). NP is commonly found in association with non-atopic asthma and aspirin sensitivity, and this association of NP with asthma might reflect the shared pathophysiology of these disorders of the upper and lower airways, respectively. Furthermore, previous studies have shown that NP-infiltrating T cells expressed a mixed Th1/Th2 pattern of cytokines (Hamilos et al. 1995; Sanchez-Segura et al. 1998). Together, our present data suggested that, in a variety of asthma-related conditions, the amplification of either side of the Th1/Th2 pathway, or both, could be adverse to the host. Churg-Strauss syndrome (CSS), also known as allergic granulomatosis and angiitis, is another asthma-related disorder characterized by systemic small vessel vasculitis. Indeed, analysis of the cytokine profile of T cell lines from patients with CSS has shown both type-1 cytokine and type-2 cytokine responses (Kiene et al. 2001). Of note, clinical signs of autoimmunity such as vasculitis have been observed in some patients with AIA (Szczeklik et al. 1995, 1997).

The human *TBX21* gene is located on chromosome 17q21.32, which has previously been linked with asthma and skin tests (Dizier et al. 2000). Moreover, the region on mouse chromosome 11, a region that has been linked to AHR, is syntenically homologous to human chromosome 17q12-q22 (Zhang et al. 1999). *TBX21* is likely to be a novel candidate gene in this region, in addition to other candidate genes such as eotaxin (*CCL11*). However, our data cannot exclude the possibility that $-1993T \rightarrow C$ is in LD with another polymorphism in *TBX21* or a neighboring gene. Further studies in larger or other populations will be required to confirm the effect of the *TBX21* polymorphism. To date, several candidate genes of the enzymes in the arachidonic pathway, such as *LTC4S* and *ALOX5*, have been proposed to increase the susceptibility to AIA (Choi et al. 2004; Kawagishi et al. 2002; Sanak et al. 1997); indeed many other genes, in addition to *TBX21*, probably contribute to the pathogenesis of AIA. Genetic epidemiology on larger numbers of AIA patients is an important future requirement in order to clarify the importance of our findings.

In conclusion, we have identified 24 SNPs (five novel) in the *TBX21* gene, and our studies demonstrate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter is likely to be associated with an increased risk for AIA in Japanese. This is the first report demonstrating a relationship between the *TBX21* SNPs and clinical features of human asthma. Furthermore, we have shown that the $-1993T \rightarrow C$ polymorphism affects the transcriptional activity of the gene and may contribute to an increase in T-bet expression. In certain asthma subgroups, such as AIA and AS/NP, this promoter SNP may cause inappropriate Th1 responses in the airway, leading to severe airway inflammation, in combination with antigen-specific Th2 responses. Our present data shed light on an important area of further study regarding the precise phenotype classification of asthma by using genotypes and also focus on the Th1 response in the pathogenesis of AIA.

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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

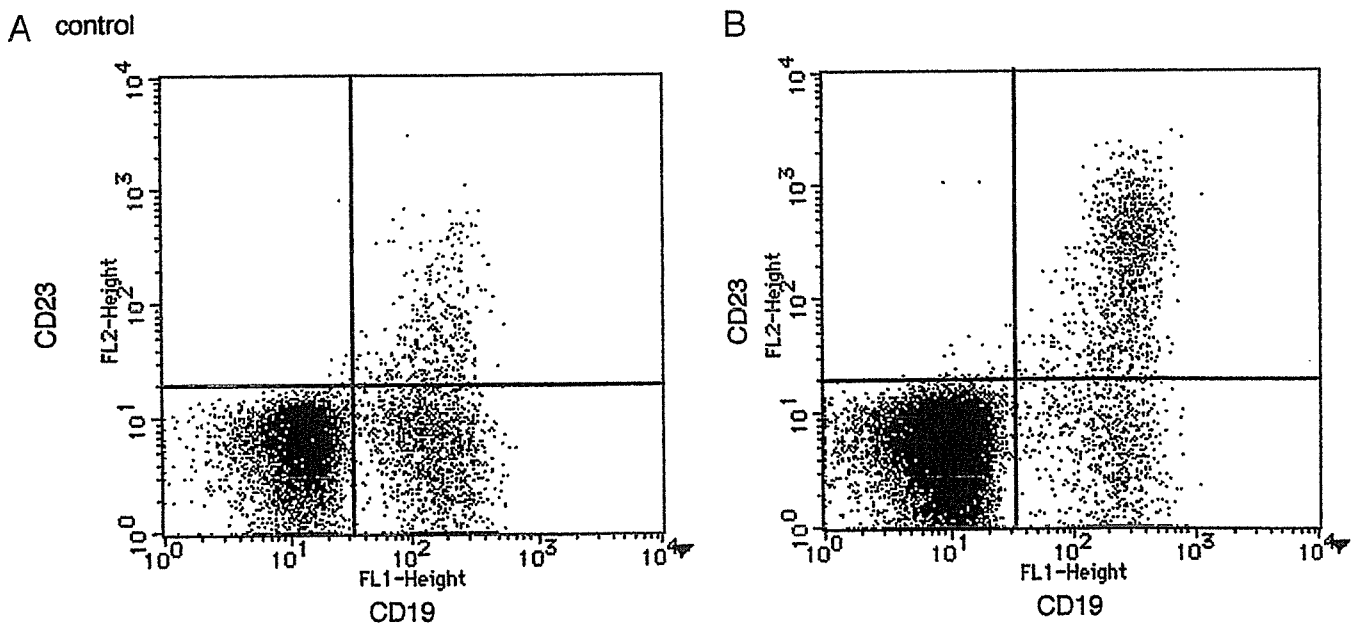

Figure 1. Representative dot plots for fluorescein isothiocyanate–conjugated anti-CD19 (FL1) and R-phycoerythrin–conjugated anti-CD23 (FL2) antibodies for the control group (A) and the allergic rhinitis (AR) group (B). The proportion of CD23-bearing B cells was measured by flow cytometry as CD19 and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

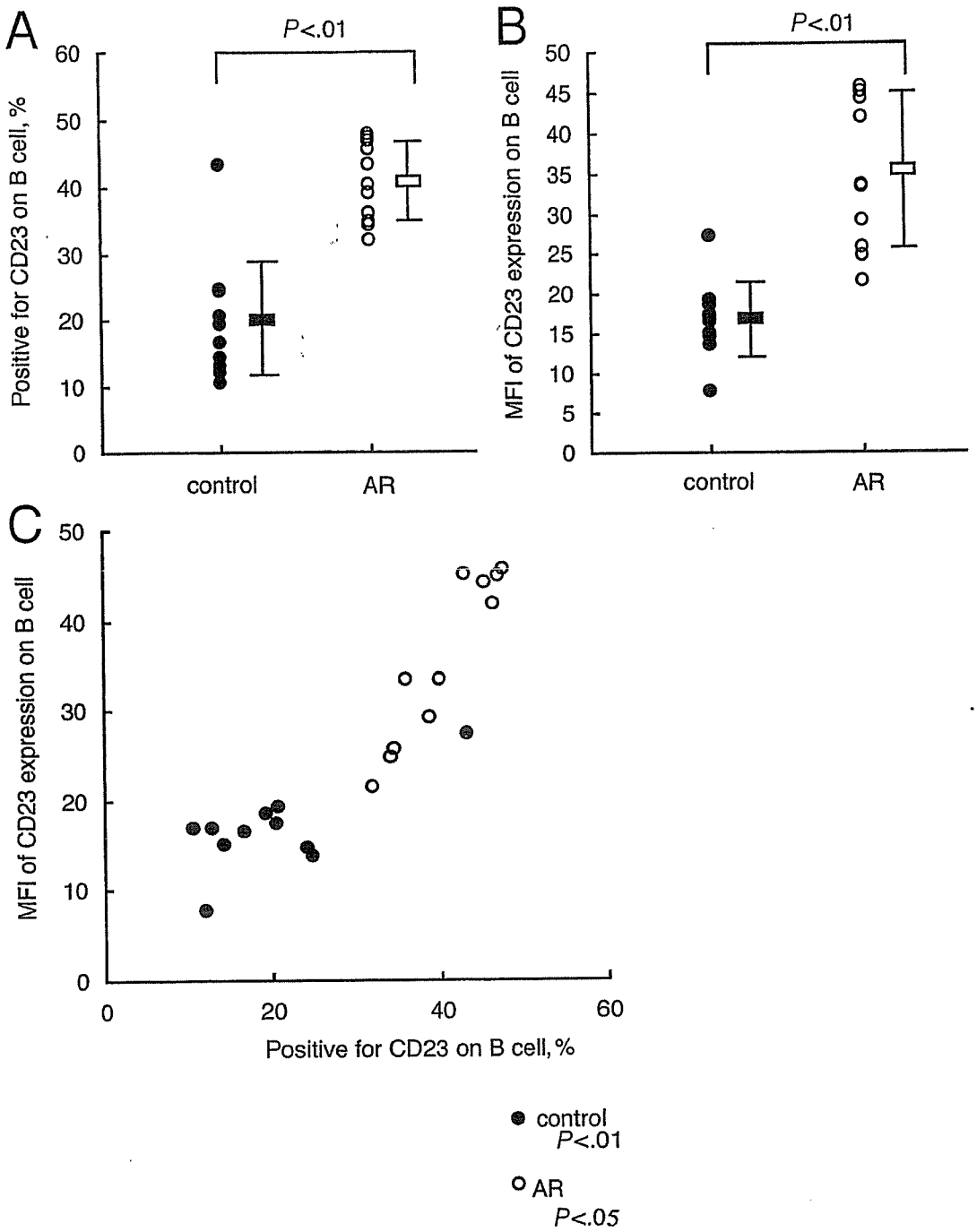

Figure 2. CD23 expression on B cells. A, Percent positive values for CD23 on B cells from patients with allergic rhinitis (AR) were significantly higher than for the control group. B, A similar tendency was seen in the mean fluorescence intensity (MFI) of CD23 expression on B cells, which was significantly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

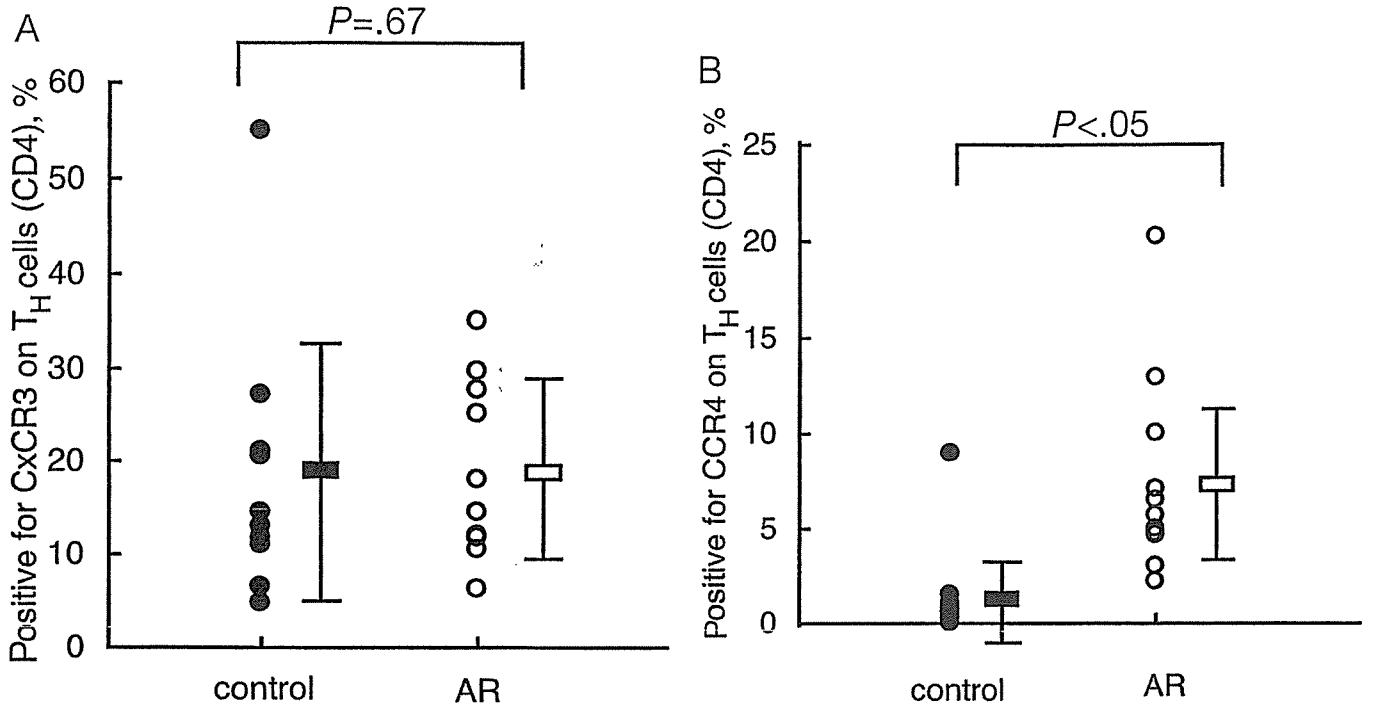

Figure 3. Percent positive values for chemokine receptor expression on mucosal CD4 T cells. A, Data for the CXCR3 subtype, assumed to be T_H1 cells. B, Data for the CCR4 subtype, assumed to be T_H2 cells. There is no significant difference in percent positive values for CXCR3 on CD4 cells from patients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

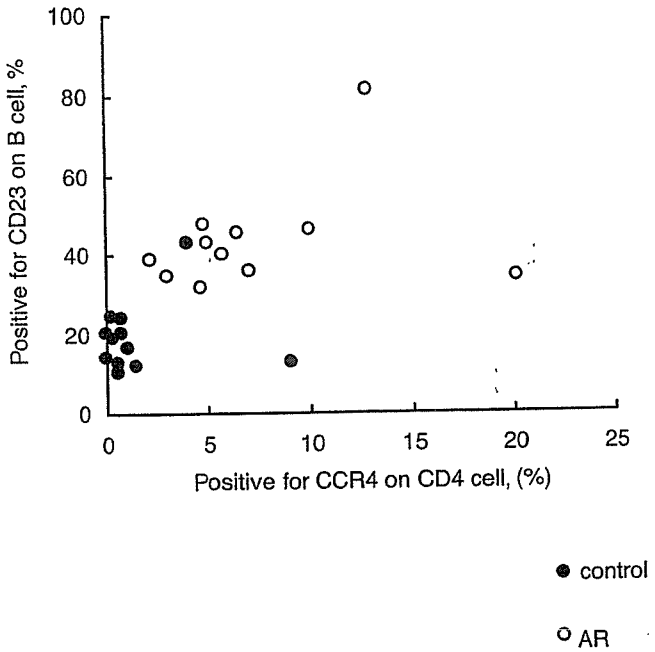

Figure 4. Correlation plot for percent positive values for CD23 on mucosal B cells and percent positive values for CCR4 on mucosal CD4 cells. There is no significant correlation between these data in the control group (P = .25) or the allergic rhinitis (AR) group (P = .17).

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]

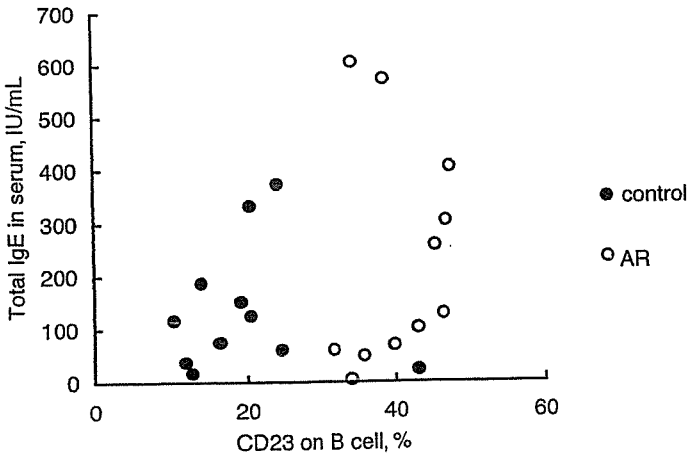

Figure 5. Correlation plot for percent positive values for CD23 on mucosal B cells and the total IgE level. There is no significant correlation between these data in the control group (P = .21) or the allergic rhinitis (AR) group (P = .66).

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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Influence of viral infection on the development of nasal hypersensitivity

Y. Okamoto*, Z. Matsuzaki†, T. Matsuoka†, S. Endo†, H. Yamamoto*, H. Chazono*, S. Horiguchi* and T. Hanazawa*

*Department of Otolaryngology, Graduate School of Medicine, Chiba University, Chiba, Japan and †Department of Otolaryngology, Yamanashi Medical University, Yamanashi, Japan

Summary

Background The underlying relationship between viral infections and allergic diseases of the upper respiratory tract has not been well clarified.

Methods In order to clarify the relationship between viral infection and nasal hypersensitivity, mice were sensitized with ovalbumin (OVA) and then infected intranasally with respiratory syncytial virus (RSV), after which their nasal sensitivity to histamine or antigen was examined.

Results Non-sensitized mice showed transient mild nasal hypersensitivity following nasal administration of histamine after intranasal RSV inoculation. In mice sensitized with OVA, RSV infection significantly exaggerated their nasal hypersensitivity to histamine and OVA. Treatment of these mice with a neurokinin (NK)-1/NK-2 receptor antagonist, but not with anti-IL-5 antibodies, reduced their hypersensitivity. The infiltration of nasal mucosa with eosinophils was temporarily associated with accelerated rate of RSV elimination in these animals.

Conclusion RSV infection induced transient nasal hypersensitivity. Several mechanisms, including impairment of nasal epithelial cells are thought to mediate this effect. In allergen-sensitized mice, RSV inoculation strongly enhanced nasal hypersensitivity.

Keywords histamine, nasal hypersensitivity, RSV

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Introduction

Recent epidemiological evidence has suggested that acute respiratory viral infections exacerbate the symptoms of pre-existing reactive airway diseases and is the most important trigger of acute asthmatic attacks [1–4]. Viruses, rather than bacteria, cause most acute respiratory tract infections, and asthma attacks in children are often preceded by viral infection [5–7].

The nasal cavity is often the first target of invading viruses, because it is the point of entry into the respiratory tract. The common cold is the most widespread viral infectious condition and is usually caused by viruses such as rhinoviruses, parainfluenza viruses, influenza viruses, adenoviruses and respiratory syncytial virus (RSV) [8, 9]. However, the relationship between viral infections and allergic diseases in the upper respiratory tract has not been well defined. The results from studies that have examined the influence of atopy on the development of the symptom after viral infections are controversial [10–13]. Bardin et al. [11] observed more severe cold symptoms in atopic subjects than in non-atopic subjects after experimental rhinovirus infection. However, in another study, augmented nasal allergic inflammation induced by antigen provocation before viral inoculation did not result in a worsening of cold symptoms [12]. The effects of the common cold on nasal hypersensitivity or allergic rhinitis have not been clearly established.

Nasal responses to viral infection are thought to differ depending on the viral species. Although rhinoviruses causes little damage to epithelial cells in the respiratory tract, RSV induces marked cytopathic effects [13]. RSV is an RNA virus infection which usually results in common cold symptoms, although progression to lower respiratory tract symptoms, the most common being bronchiolitis, frequently occurs in infants. RSV causes about 60% of the bronchitis cases in children [14, 15]. In prospective studies, as many as 75–90% of infants with a clinical diagnosis of bronchiolitis subsequently developed recurrent episodes of wheezing suggestive of childhood asthma and experienced airway histamine or methacholine hypersensitivity which persisted for several years [16–22].

In the present study, we have shown that RSV infection contributes to the exacerbation of nasal hypersensitivity in an allergic rhinitis mouse model.

Materials and Methods

Animals

Eight-week-old male C57BL/6 mice (Nippon Clea, Shizuoka, Japan) that were raised on ovalbumin (OVA)-free chow were

Correspondence: Yoshitaka Okamoto, Department of Otolaryngology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail: yoka-chiba@k4.dion.ne.jp

used in this study. Hartley strain guinea-pigs (Nippon Clea) were also used to measure passive cutaneous anaphylaxis (PCA). The use of these laboratory animals was approved by the local Animal Ethics Committee (Yamanashi Medical University) and the experiments were conducted in conformity with the guidelines of the committee.

Experimental infection with respiratory syncytial virus

The long strain of RSV (prototype RSV group A strain) was grown in HEp-2 cells in minimal essential medium (MEM) supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics. RSV was partially purified by polyethylene glycol precipitation, followed by centrifugation in a 35–65% discontinuous sucrose gradient, as described elsewhere [23]. RSV (1×10^6 plaque-forming units (PFU)) in a volume of 20 μL was administered intranasally to mice. Uninfected Hep-2 cells were processed similarly and used as controls.

Virus assay

Lungs and nasal tissues were collected and homogenized in MEM containing 2% FCS and were stored at −70 °C until they were assayed. RSV was assayed by the plaque method using HEp-2 cells in 24-well microplates. The overlay for the plaque assay consisted of MEM supplemented with 2% FCS, antibiotics and 1% methylcellulose. Plates were incubated for 7 days at 37 °C. After the methylcellulose was removed, the plaques were fixed with 10% formaldehyde and stained with 0.1% crystal violet.

Evaluation of sensitivity to histamine in nasal mucosa

One microlitre of various concentrations of histamine, diluted in phosphate-buffered saline (PBS), was administered into each nostril of the experimental mice. The number of nasal rubbing attacks that occurred during the ensuing 10 min was then counted.

Experimental protocol for sensitization with ovalbumin

Mice were immunized with 10 μg OVA (grade V, Sigma Chemical Co., St Louis, MO, USA) intraperitoneally with alum once a week for 4 weeks. Heat-killed bordetella pertussis (1×10^8 bacterial units) was used as an adjuvant in the first immunization. Five days after the last immunization, the mice were either inoculated with RSV or sham-infected with sonicated non-RSV-infected HEp-2 cells. Two micrograms OVA in 2 μL PBS was administered intranasally for 5 consecutive days after the inoculation. Sensitized mice were divided into the following experimental groups and treated as follows. Group 1 consisted of 30 mice treated with a neutralizing IL-5 antibody or a neurokinin (NK)-1/NK-2 antagonist. A rat neutralizing monoclonal antibody (mAb) directed against mouse IL-5 (PharMingen, San Diego, CA, USA) and a control isotype mouse IgGI mAb (PharMingen) were used. Antibodies were injected intraperitoneally twice a week at a dose of 0.1 mg for 1 week before RSV inoculation, and were administered intranasally for 5 consecutive days after inoculation. Group 2 consisted of 10 OVA-sensitized mice who received 0.04 μg of the NK-1/NK-2 antagonist [24] FK224 (Fujisawa Co Ltd, Osaka, Japan) intranasally for 5 consecutive days after RSV inoculation. On the day following the last nasal administration of OVA, the nasal rubbing attacks were counted for 10 min. The sensitivity of the mice to histamine was examined 24 h later in a similar manner.

Treatment of ovalbumin-sensitized mice with a neutralizing anti-interferon-γ monoclonal antibody or with interferon-γ

OVA-sensitized mice received 0.1 mg of anti-IFN-γ neutralizing mAb (PharMingen) or control mAb intraperitoneally twice a week and then intranasally for 5 consecutive days before nasal provocation with OVA. Other OVA-sensitized mice were administered 1 μg of IFN-γ (PharMingen) intranasally for 5 consecutive days before provocation with OVA.

Detection of ovalbumin-specific immunoglobulin E antibody

OVA-specific IgE antibodies were detected by PCA [25]. Briefly, 100 μL of undiluted and twofold diluted serum samples were injected intradermally into the dorsal skin of shaved guinea-pigs. Three days later, the animals were challenged intravenously with 1 mg OVA together with 1% Evans blue. A blue lesion of a diameter greater than 5 mm, as determined 30 min after the challenge, was considered to be positive. PCA titres were expressed as the reciprocal of the highest dilution giving a positive reaction.

Histological examination

On the 4th day after RSV inoculation the mice were killed by CO_2 overdose. The heads of the mice were detached along the line between the upper and lower jaws, and they were then fixed in formalin and decalcified. The section of the nasal cavity anterior to the eyeball was examined and processed for paraffin sectioning. Tissue sections were stained with PAS and the number of infiltrating eosinophils in the whole nasal septum mucosa of each section was determined.

Fluorescence-activated cell sorting analysis

Nasal mucosal tissue from the above mice was cut into small pieces, which were then teased gently through a nylon mesh using frost glass slides. The disrupted mucosa was then suspended in RPMI-1640 containing 10% FCS, penicillin (100 units/mL) and streptomycin (100 μg/mL). After washing twice with medium, $CD3^+$ T cells were purified in 0.2 mL of RPMI-1640 using magnetic beads (Dynal, Great Neck, NY, USA). Following purification, the medium was supplemented with 10% FCS. 10^6 nasal $CD3^+$ T cells collected from seven RSV-infected OVA-sensitized mice or from non-infected OVA-sensitized mice were stained with fluorescein-conjugated anti-CD4 antibody (PharMingen) and fixed overnight with 4% paraformaldehyde (Sigma Chemical Co). The fixed cells were permeabilized by incubation in PBS with 1% bovine serum albumin and 2% saponin (Sigma Chemical Co) for 10 min. A phycoerythrin-conjugated anti-IFN-γ antibody (PharMingen) or an anti-IL-5 antibody (PharMingen), diluted to 20 μg/mL in PBS, was then added. After a 30 min incubation, the cells were washed with PBS and were

analyzed using a FACScan (Becton Dickinson, Fullerton, CA, USA).

Statistical analysis

Comparisons between groups were evaluated using Student's *t* test and Wilcoxon's test.

Results

Viral replication and nasal histamine sensitivity

After nasal inoculation with 10^6 PFU of RSV, mild replication of RSV in the respiratory tract was observed with peak levels occurring in the lung on day 4 and the levels then declined until day 7 as shown previously [26]. RSV was recovered from the nasal mucosa for 12 days after inoculation.

Non-specific stimulation of the nasal mucosa of mice also resulted in nasal rubbings. The number of nasal rubbing attacks observed in 20 normal mice following nasal installation of 2 μL PBS was 9.4 ± 2.9 (mean ± SD). Thus, the lowest histamine concentration administered intranasally in a volume of 2 μL that was needed to induce more than 20 nasal rubbing attacks was defined as the threshold level of nasal histamine hypersensitivity. After RSV inoculation, the threshold decreased and reached its lowest on day 4. It returned to normal by day 14 (Fig. 1(a)).

Influence of respiratory syncytial virus infection on ovalbumin-sensitized mice

The threshold of nasal hypersensitivity to histamine decreased in OVA-sensitized mice and RSV infection in OVA-sensitized mice induced a dramatic enhancement of nasal sensitivity to histamine (Fig. 1(b)). The threshold of nasal hypersensitivity to histamine observed in RSV-infected mice increased gradually after the last nasal administration of OVA and 14 days later, it was the same as that of non-infected mice (data not shown). Fluorescence-activated cell sorting analysis of nasal mucosal T lymphocytes in the RSV-infected OVA-sensitized mice not only revealed an increased expression of IFN-γ, but also of IL-5 (Table 1). Anti-IL-5 treatment of RSV-infected OVA-sensitized mice using neutralizing antibodies reduced the histamine sensitivity in some degree ($P<0.05$) and the treatment with an NK-1/NK-2 antagonist resulted in a marked reduction ($P<0.001$) of the sensitivity (Fig. 1(b)).

After OVA nasal provocation the frequency of nasal rubbing attacks dramatically increased in RSV-infected OVA-sensitized mice, compared with non-infected sensitized mice (Fig. 2). However, anti-IL-5 treatment of RSV-infected OVA-sensitized mice did not significantly improve nasal symptoms after OVA administration. On the other hand, an NK-1/NK-2 antagonist resulted in a significant improvement (Fig. 2).

The number of eosinophils in the nasal mucosa was markedly increased in RSV-infected OVA-sensitized mice compared with those in non-infected OVA-sensitized mice (Fig. 3). The PCA titre, on the other hand, was not significantly different between the two groups (mean ± SD; 21.1 ± 21.0 in infected sensitized mice, 16.6 ± 11.4 in non-infected sensitized mice). Anti-IL-5 treatment of RSV-infected OVA-sensitized mice significantly reduced the number of infiltrated eosinophils, however, the treatment with an NK-1/NK-2 antagonist had no effect on eosinophil infiltration.

The nasal administration of IFN-γ to OVA-sensitized mice increased the number of nasal eosinophils, but had no effect on nasal symptoms (Fig. 4). Treatment with anti-IFN-γ neutralizing antibodies did not affect nasal symptoms or eosinophil infiltration (Fig. 4).

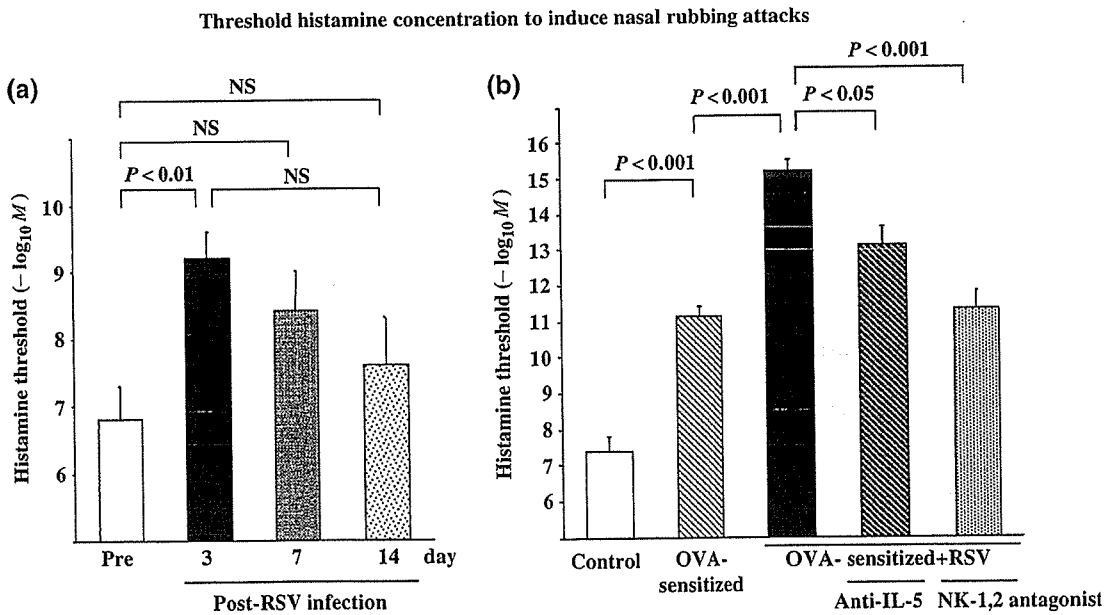

Fig. 1. Threshold histamine concentration needed to induce nasal rubbing attacks in respiratory syncytial virus (RSV)-infected non-sensitized mice (a) and in ovalbumin (OVA)-sensitized mice (b). After RSV inoculation, the threshold decreased transiently and reached its lowest on day 4. Although the threshold decreased in OVA-sensitized mice, RSV infection in OVA-sensitized mice induced a dramatic reduction of the threshold. The treatment with neurokinin (NK)-1/NK-2 receptor antagonist but not with anti-IL-5 neutralizing antibodies improved the reduction. Non-OVA-sensitized mice were used as controls.

Table 1. IL-5 and IFN-γ expression of nasal mucosal T lymphocytes from OVA-sensitized mice*

	RSV-infected mice (%)	Sham-infected mice (%)
IL-5	11.9	6.2
IFN-γ	17.4	11.4

OVA, ovalbumin; RSV, respiratory syncytial virus.
*Mean of two groups and each group consisted of T lymphocytes collected from nasal mucosa of seven mice.

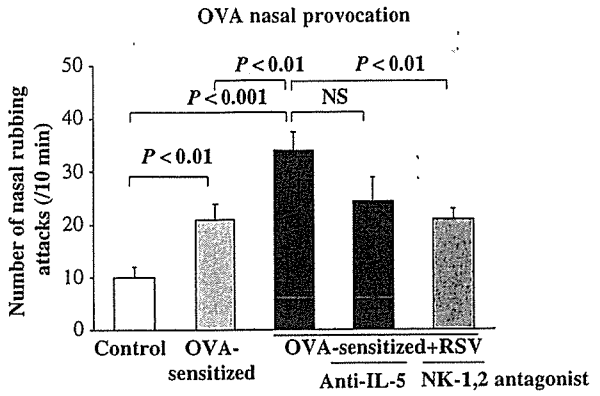

Fig. 2. The number of nasal rubbing attacks in ovalbumin (OVA)-sensitized mice following OVA provocation. Respiratory syncytial virus (RSV) infection in OVA-sensitized mice induced a dramatic enhancement of number of attacks. The anti-IL-5 treatment reduced the enhancement in some degree and the topical administration of the neurokinin (NK)-1/NK-2 receptor antagonist did more.

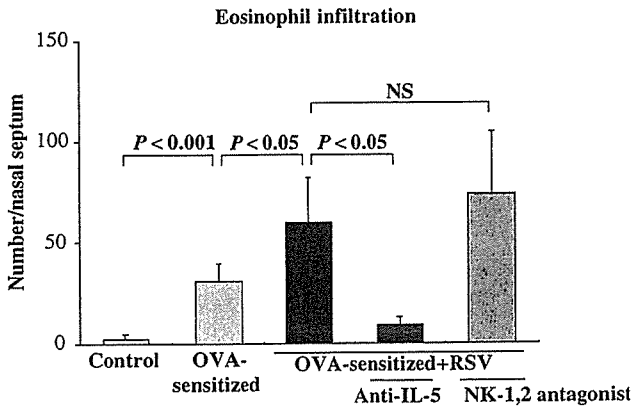

Fig. 3. The number of eosinophils in the nasal mucosa. Respiratory syncytial virus (RSV) infection markedly increased the eosinophil infiltration in ovalbumin (OVA)-sensitized mice. The treatment with anti-IL-5 antibodies reduced the number significantly but not with neurokinin (NK)-1/NK-2 receptor antagonists.

RSV replication on day 4 was significantly reduced in OVA-sensitized mice. However, the use of anti-IL-5 did not exhibit any influence on viral replication and no reduction in viral shedding was observed in anti-IL-5-treated OVA-sensitized mice (Fig. 5).

Discussion

The above studies were designed to examine the mechanism of nasal hypersensitivity observed during viral infections. A murine RSV infection model was used in which the quantitative analysis of nasal rubbing attacks was evaluated as a measure of nasal hypersensitivity. Sneezes in mice are not clearly distinguishable as in humans and are difficult to quantify precisely. The evaluation of nasal obstruction is also difficult, because mice cannot survive by breathing orally. BALB/c mice are known to be sensitive to allergic reactions [27], particularly in the lower respiratory tract, although their nasal reactivity to histamine and other antigens is quite low (data not shown). While C57BL/6 mice are known to mount a Th1 dominant immune response [28], IgE production is inducible in these animals if the correct adjuvant, such as alum, is used, and nasal hypersensitivity can be observed after the topical administration of histamine or antigens. In light of the above and because RSV replication in the nose of BALB/c mice is tolerated well by these animals, we chose to use C57BL/6 mice in our study.

The observations summarized in this report suggest that experimentally induced infection with RSV results in significant enhancement of nasal sensitivity to OVA and histamine in previously sensitized animals. OVA-sensitized animals also exhibited increased expression of IL-5 and IFN-γ and pronounced accumulation of eosinophils in the nasal mucosa after RSV infection.

The mechanisms underlying the development of hypersensitivity states after viral infections such as RSV have not been clinically defined. It is possible that viral infection-associated mucosal damage; recruitment of mast cells, eosinophils and other cellular mediators of hypersensitivity; and activation of cholenergic, adrenergic or non-adrenergic non-cholinergic neurogenic mechanisms may play an important role in the development of mucosal hypersensitivity states [29–31].

In the present studies, pre-treatment with anti-IL-5 resulted in significant decrease in the accumulation of eosinophils. However, such treatment did not influence the degree of viral induced hypersensitivity. In fact, anti-IL-5 treatment was associated with decreased viral elimination in the nasal cavity, and as a result eosinophils may be associated with accelerated RSV elimination. It has been shown that eosinophil cationic protein and eosinophil-derived neurotoxin may act as rebonuclease-dependent antiviral agents [32]. In the present studies, it is interesting to note that use of IFN-γ was associated with increasing eosinophil counts but did not influence nasal hypersensitivity reactions. Thus, although eosinophils may play an important role in viral induced allergic inflammation [33, 34], eosinophils did not seem to contribute to nasal hypersensitivity to OVA in the current experimental setting. IFN-γ is a classical Th1 cytokine that has been shown to reduce allergic reactions when administered during sensitization [35]. However, treatment of OVA-sensitized animals with anti-IFN-γ neutralizing antibody did not decrease nasal sensitivity to OVA during RSV infection.

The observation of particular interest in the current studies is the significant reduction of nasal hypersensitivity detected after the use of NK-1/NK-2 antagonists, although such treatment did not influence eosinophil counts. Recently, it has been shown that infection with RSV frequently is associated with activation of NK receptor sites [36–38]. Tachykinin family of neuropeptides such as substance P have been shown to exhibit strong blinding affinity for NK receptors especially NK-1. Such receptor-neuropeptide interactions are associated

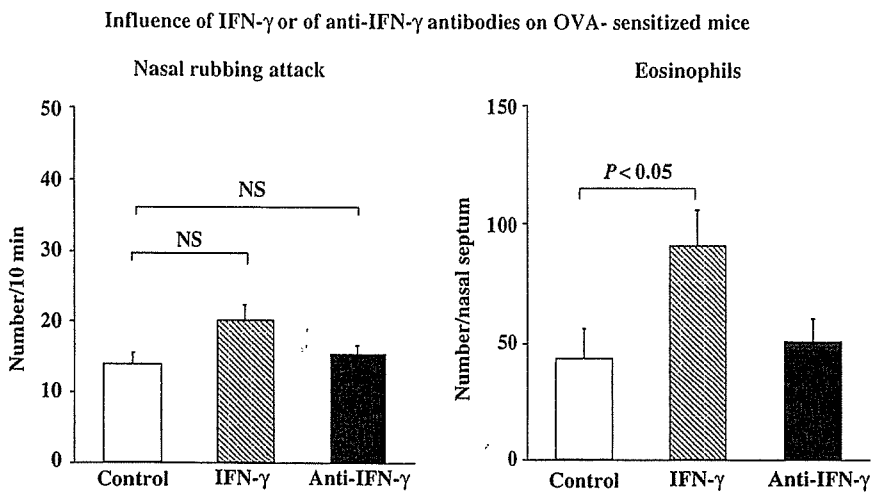

Fig. 4. Influence of IFN-γ and anti-IFN-γ antibodies on ovalbumin (OVA)-sensitized mice. The nasal administration of IFN-γ increased the number of eosinophils, but did not affect the nasal symptoms. Anti-IFN-γ treatment had no effect on either nasal symptoms or eosinophil numbers.

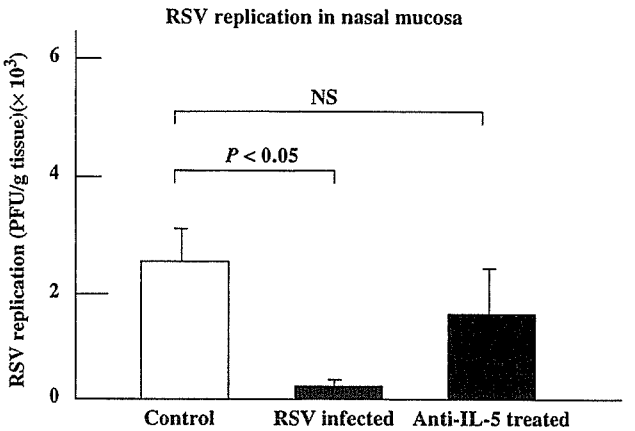

Fig. 5. Respiratory syncytial virus (RSV) replication in the nasal mucosa on day 4 after RSV inoculation. Replication was reduced in ovalbumin (OVA)-sensitized mice, but this reduction was abolished in anti-IL-5-treated OVA-sensitized mice. Non-OVA-sensitized mice were used as controls.

with a wide variety of biologic inflammatory effects, including changes in vascular permeability, mucous secretion, leucocyte chemotaxis and bronchoconstriction [39–41]. It is thus suggested that RSV-associated increase in allergic nasal hypersensitivity to OVA and possibly to other allergens may in part be related to activation of neuropeptide receptors during acute viral infection of the nasal mucosa.

It is possible that increased eosinophil recruitment is mediated by chemokines induced by IFN-γ. Recently induction of eotaxin 3 and IP-10 by IFN-γ in mucosal cell cultures has been demonstrated after experimental RSV infection in *in vivo* settings [42–44]. Based on these reports and the present studies, it is proposed that a possible relationship exists between IFN-γ and induction, recruitment and/or activation of eosinophils in allergic sensitization in the nasal mucosa during viral infections.

Acknowledgements

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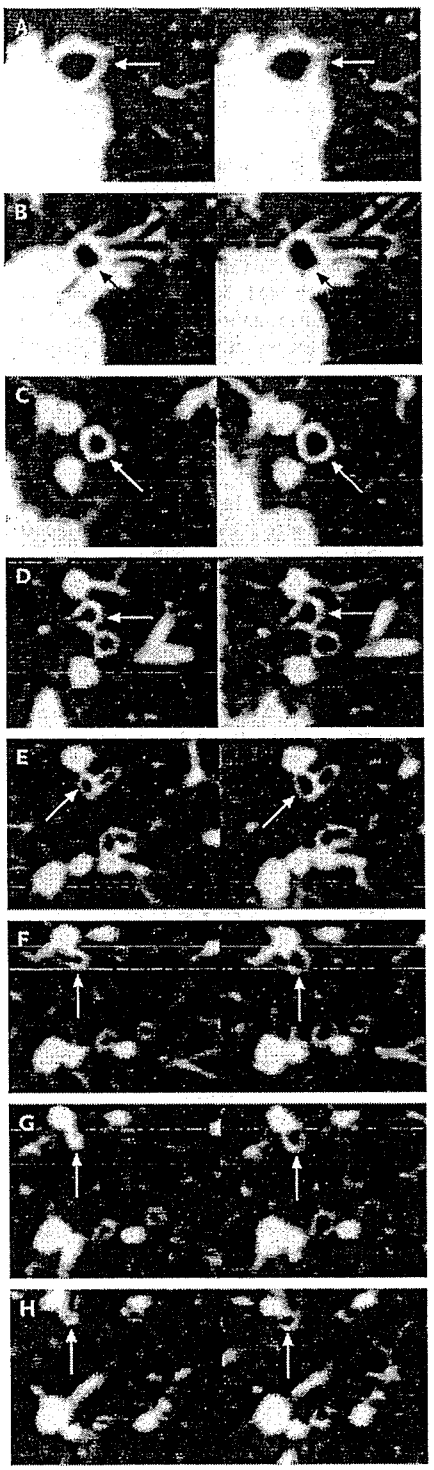
IMAGES IN CLINICAL MEDICINE

Airway Dilatation after Inhalation of a Beta-Agonist



A 37-YEAR-OLD WOMAN WITH A 25-YEAR HISTORY OF ASTHMA that had been managed with inhaled budesonide and albuterol (salbutamol) as needed underwent high-resolution multislice helical computed tomographic scanning. Thirty minutes after the inhalation of albuterol, a cross-sectional multiplanar reconstruction of the right upper lobe, obtained at maximal inspiration, revealed an increase in the airway caliber. Each panel of images from these two videos consists of the view before the inhalation on the left and the corresponding view after the inhalation on the right. Panel A shows the segmental, or third-generation, bronchus (arrows); Panel B the fourth-generation bronchus at the branching point (arrows); and Panel C more of the periphery of the fourth-generation bronchus, with full wall visualization (arrows). Panels D, E, F, and G show bronchial divisions from the fifth to the eighth generation (arrows); the same bronchial divisions are shown in the video clip. Panel H shows preinhalation airway occlusions (left-hand image), which opened up after the inhalation (right-hand image). The forced expiratory volume in one second, initially 1.27 liters (49.8 percent of the predicted value), increased by 0.49 liter after the inhalation of albuterol. It is important to note that airway resistance is an inverse function of the airway radius to the fourth to fifth power.

Gen Tamura, M.D.

Tohoku University Hospital
Sendai 980-8574, Japan

Yuji Suda, M.D.

Sendai City Medical Center
Sendai 983-0824, Japan

Murine Plasmacytoid Dendritic Cells Produce IFN-γ upon IL-4 Stimulation[1]

Akira Suto, Hiroshi Nakajima,[2] Naoki Tokumasa, Hiroaki Takatori, Shin-ichiro Kagami, Kotaro Suzuki, and Itsuo Iwamoto

IL-4 plays a key role in inducing IL-4 production in $CD4^+$ T cells, functioning as an important determinant for Th2 cell differentiation. We show here that IL-4 induces IFN-γ production in $B220^+$ plasmacytoid dendritic cells (PDCs). By searching for cell populations that produce IFN-γ upon IL-4 stimulation, we found that PDCs were a major IFN-γ-producing cell upon IL-4 stimulation in wild-type and Rag-$2^{-/-}$ splenocytes. Isolated PDCs, but not $CD11b^+$ DCs or $CD8^+$ DCs, produced IFN-γ upon IL-4 stimulation. In vivo, the depletion of PDCs by anti-Ly6G/C Ab prevented IFN-γ production induced by IL-4 administration. We also found that IL-4 induced IFN-γ production, but not IL-12 or IFN-α production, in PDCs and also strongly enhanced CpG oligodeoxynucleotide-induced IFN-γ production, but not CpG oligodeoxynucleotide-induced IL-12 or IFN-α production. However, IL-4 did not induce IFN-γ production in Stat$6^{-/-}$ PDCs. Moreover, IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism, and only the Stat4-expressing PDCs produced IFN-γ. Furthermore, IL-4 did not induce IFN-γ production in Stat$4^{-/-}$ PDCs. These results indicate that PDCs preferentially produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. *The Journal of Immunology,* **2005, 175: 5681–5689.**

Cytokine environment is critical for the differentiation and commitment of immune cells. For example, IL-4, a representative Th2 cytokine, induces further Th2 cell differentiation, whereas a Th1 cytokine IFN-γ in coordination with IL-12 induces Th1 cell differentiation (1–3). Although these positive feedback mechanisms are essential for the profound differentiation of Th cells, the immune system also has a number of intrinsic and extrinsic machinery to antagonize the excessive differentiation of immune cells (4, 5).

Dendritic cells (DCs)[3] are a migratory group of bone marrow-derived leukocytes with at least three subtypes in mouse spleen: $CD8^+$ DCs, $CD11b^+$ DCs, and $B220^+$ DCs ("plasmacytoid DCs") (PDCs) (6, 7). Although $CD8^+$ DCs and $CD11b^+$ DCs express high levels of MHC class II molecules and costimulatory molecules such as CD80 and induce T cell proliferation, PDCs express MHC class II molecules at very low levels, do not express CD80, and fail to stimulate T cell proliferation (8, 9). These findings suggest that PDCs are immature DCs with a weak ability as APCs. On the other hand, PDCs localize in the T cell zone of lymphoid tissues and produce a large amount of type I IFNs upon bacterial or viral infection (10–12). Therefore, it is suggested that PDCs play a key role in innate immune responses.

Recently, a number of experiments have suggested that innate immune responses contribute significant polarizing influences on Th differentiation (13). The global view is that TLR activation of APCs such as DCs induces cytokine production that favors Th1-type immune responses and prevents the development of deleterious Th2 responses (13). On the other hand, a recent study has shown that PDCs inhibit Th2 responses even in the absence of TLR signaling (14). However, the role of PDCs in Th differentiation is still largely unknown.

In this study, by searching for cell populations that produce IFN-γ upon IL-4 stimulation, we found that PDCs were a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induced IFN-γ production in PDCs by a Stat6-dependent mechanism. We also found that IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism and that only the Stat4-expressing PDCs produced IFN-γ. Furthermore, we found that Stat4-deficient PDCs did not produce IFN-γ upon IL-4 stimulation. Our results highlight a unique function of IL-4-induced IFN-γ production in PDCs in the immune regulation of cytokine networks.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories. Stat6-deficient (Stat$6^{-/-}$) mice (15) and Rag-$2^{-/-}$ mice were backcrossed for more than eight generations onto BALB/c mice. Stat$4^{-/-}$ mice on a BALB/c background were purchased from The Jackson Laboratory. OVA-specific DO11.10 TCR transgenic (DO11.10^+) mice (16) were backcrossed over 10 generations onto BALB/c mice. All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were performed according to the guidelines of Chiba University.

Reagents

Mouse IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 were purchased from PeproTech. Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN) 1668 (TCCATGACGTTCCTGATGCT) was purchased from Hokkaido System Science.

Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan

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[2] Address correspondence and reprint requests to Dr. Hiroshi Nakajima, Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba City, Chiba 260-8670, Japan. E-mail address: nakajimh@faculty.chiba-u.jp

[3] Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; ODN, oligodeoxynucleotide; WT, wild type.

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