Clinical Immunology (2012) 143, 170–179

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

Sublingual administration of *Lactobacillus paracasei* KW3110 inhibits Th2-dependent allergic responses via upregulation of PD-L2 on dendritic cells

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Received 6 December 2011; accepted with revision 16 January 2012
Available online 27 January 2012

KEYWORDS
Allergy;
Dendritic cells;
Vaccination;
Sublingual administration

Abstract Lactic acid bacteria have potential in immunomodulation therapy, but their clinical efficacy and underlying mechanisms are unclear. We aimed to clarify the anti-allergic immune responses induced by intragastric and sublingual administration of heat-killed *Lactobacillus paracasei* KW3110 and *Lactobacillus acidophilus* L-92. The KW3110 strain (but not the L-92 strain) enhanced ovalbumin (OVA)-induced expression of CCR-7 and PD-L2 in murine dendritic cells (DCs), and strongly inhibited IL-5 and IL-13 production *in vitro* in co-cultures with Th2-skewed $CD4^+$ T cells from DO11.10 transgenic mice. Sublingual administration of low-dose KW3110 (but not L-92) to OVA-sensitized mice selectively suppressed serum IgE production and Th2 cytokine expression in cervical lymph nodes, and significantly improved symptoms after OVA provocation *in vivo*. KW3110 probably accelerates DC migration into the regional lymph nodes and inhibits Th2 cytokine production through enhanced CCR-7 and PD-L2 expression. Thus, sublingual KW3110 administration may be effective in reducing allergic inflammation.

Abbreviations: PBMC, Peripheral blood mononuclear cell; SLIT, Sublingual immunotherapy; DC, Dendritic cell; OVA, Ovalbumin; BM, Bone marrow; LPS, Lipopolysaccharide; CLN, Cervical lymph node; ILN, Inguinal lymph node; ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-linked immunospot; TLR, Toll-like receptor; LGG, *Lactobacillus rhamnosus* GG; FACS, Fluorescein-activated cell sorter; FITC, Fluorescein isothiocyanate; GM-CSF, Granulocyte macrophage colony-stimulating factor; TCR, T cell receptor; OD, Optical density

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doi:10.1016/j.clim.2012.01.008

1. Introduction

An increased prevalence of allergic rhinitis has been observed in many countries over the recent years [1]. A variety of medications have been used to relieve the symptoms of this condition; however, these drugs do not treat the underlying disease and have a high risk of adverse events, particularly when taken over a long period [2]. Antigen-specific immunotherapy can effectively change the natural course of allergic disease, prevent the development of other allergic diseases, and reduce new allergic sensitization [3]. However, conventional subcutaneous administration necessitates frequent visits to the physician and is associated with a risk, albeit low, of anaphylactic shock [4].

The efficacy of lactic acid bacteria in suppressing the development of allergic diseases has been demonstrated [5]. For example, when expectant mothers were treated with *Lactobacillus rhamnosus* GG (LGG), and their infants were also treated with LGG from birth to 6 months of age, there was inhibition of the development of atopic dermatitis at ages 2 and 4 years [6]. In a study of infants with milk allergy treated with LGG, placebo or other *Lactobacillus* strains, LGG significantly enhanced IFN-γ production from peripheral blood mononuclear cells (PBMCs) [6]. However, in another study, there was no evidence of significant clinical benefit or differences in cytokine production from PBMCs in infants treated with LGG compared to a placebo [7].

Xiao et al. [8] observed marked improvements in nasal symptoms and modulation of Th2-skewed immune responses after 13 weeks of administration of the probiotic strain *Bifidobacterium longum* for treatment of Japanese cedar pollinosis. There is no other evidence of benefit after 22 weeks of administration of LGG for treatment of birch pollinosis [7]. The use of different probiotics and the study methodologies, such as dose, period of administration, and study sample size may explain these contradictory results. Intestinal health and factors such as diet and antibiotic therapy can also have a significant influence, which makes it difficult to evaluate the roles of probiotics [8,9].

In this study, we examined 2 strains of heat-killed lactic acid bacteria, *Lactobacillus paracasei* KW3110 and L-92, which are widely used in Japan and are reported to produce unique and different immune responses *in vitro*. KW3110 induces higher levels of IL-12 *in vitro* than the LGG strain, making it a more effective strain [9]. KW3110 may inhibit Th2 cytokine-mediated allergic inflammation through mechanisms independent of toll-like receptors (TLRs) [10,11], since the induction of IL-12 is not abrogated in bone marrow (BM)-derived dendritic cells (DCs) in TLR2-, TLR4- and TLR9-deficient mice [12]. L-92 activates DCs in a TLR2-dependent manner [13] and may exhibit anti-allergic activity through induction of regulatory T cells *in vivo* [14]. We administered the *Lactobacillus* strains directly to the oral mucosa, rather than as probiotics, and examined the influence of the KW3110 and L-92 strains on allergic responses in antigen-sensitized mice *in vivo* and *in vitro*.

We have previously observed higher CCR-7 and PD-L2 expression in mature DCs upon stimulation with KW3110 but not with L-92, which accelerated DC migration to draining lymph nodes and increased inhibitory signals for Th2 cytokine production. Similar immunomodulation was observed in cervical lymph nodes (CLNs), which play an important role in allergic rhinitis as draining lymph nodes, after sublingual administration of KW3110 *in vivo*. This therapeutic approach may therefore be effective for allergic rhinitis.

2. Material and methods

2.1. Mice

Seven-week-old female BALB *c* mice were purchased from SLC Inc. (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. The use of these mice was approved by the Chiba University Institutional Animal Care and Use Committee and the experiments were conducted in conformity with the guidelines of the committee.

2.2. Reagents and medium

The heat-killed KW3110 [11] and L-92 [14] strains were obtained from the Central Laboratories for Frontier Technology, Kirin Holdings Co. (Kanagawa, Japan), and from the R&D Center, Calpis Co. (Yokohama, Japan) respectively. Ovalbumin (OVA) (grade 5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). We used RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, L-glutamine (2 μM), penicillin (100 $U.mL^{-1}$), streptomycin (100 $\mu g.mL^{-1}$), HEPES (10 mM), 2-mercaptoethanol (55 μM), 1% non-essential amino acids, and 1 mM sodium pyruvate (all from Gibco BRL, Grand Island, NY, USA) in the cell culture experiments. OVA was dissolved in endotoxin-free D-PBS (Wako Pure Chemical Industries, Osaka, Japan), which had an endotoxin level below the detection limit (0.05 ELISA units per milligram protein). The reagents used for stimulation were also tested for endotoxin contamination; these also had levels below the detection limit.

2.3. Maturation of DCs

DCs from murine BM were cultured in a 100-mm bacteriological petri dish with 10 $ng.mL^{-1}$ granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) [15]. The medium was replaced twice. The immature DCs were stimulated with 1 $mg.mL^{-1}$ of OVA in endotoxin-free PBS with or without 1 $\mu g.mL^{-1}$ KW3110 or L-92, and 10 $\mu g.mL^{-1}$ LPS (Lipopolysaccharide) (O111:B4, Sigma-Aldrich), and cultured for 24 h in 24-well plates at 1×10^6 cells per well. The DCs were analyzed by fluorescein-activated cell sorter (FACS) analysis of surface markers (FACSCalibur; Becton Dickinson, Sunnyvale, CA).

2.4. Phagocytosis by DCs *in vitro*

KW3110 or L-92 (1 mg of each) was suspended in 1 mL of 100 mM carbonate buffer (pH 9.5), reacted with fluorescein 5(6)-isothiocyanate (100 $\mu g.mL^{-1}$) (Sigma-Aldrich) at 37 °C for 60 min, and then washed with sterile PBS [16]. Fluorescein isothiocyanate (FITC)-labeled *Lactobacillus* (1 $\mu g.mL^{-1}$) was cultured with immature DCs (1×10^6 $cells.mL^{-1}$) for 24 h. After washing with PBS, the DCs were fixed with a cold methanol:acetone (1:1) solution for 10 min and then stained with biotin-labeled CD11c (N418; BioLegends, San Diego, CA, USA), followed by Cy5-coupled streptavidin. For

FACS analysis, incubated DCs were stained with Cy5-labeled CD11c and MHC class II Alexa555, and then fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 (in 50 mM NaCl, 5 mM EDTA, and 0.02% NaN_3; pH 7.5) for 10 min on ice. After blocking with 3% BSA in PBS for 15 min, the cells were washed thoroughly with PBS supplemented with 1% FCS and 0.1% NaN_3. Phagocytosis of DCs was examined by confocal microscopy (Olympus, Tokyo, Japan) and FACS analysis. We used the fluorescence intensity of immature DCs after incubation for 24 h with labeled *Lactobacillus* as an indicator of *Lactobacillus* uptake.

2.5. Cytokine production in the co-culture of DCs with Th2 cells

Th2-skewed $CD4^+$ T cells were prepared from OVA-specific T cell receptor (TCR) (DO11.10) transgenic mice in a BALB *c* background, expressing TCR α/ß specific for OVA peptide, presented in the context of I-Ad [17]. Immature DCs (5×10^5) prepared from BALB *c* mice using the method described above, were pre-incubated with 0.4 μM $OVA_{323-339}$ peptide (Loh15) with 1 μg mL of KW3110 or L-92 for 24 h, and were then co-cultured with Th2-skewed $CD4^+$ T cells (1×10^5 cells) for 48 h. After preincubation, some DCs were treated for 30 min with 10 $\mu g.mL^{-1}$ of a neutralizing rat monoclonal antibody directed against PD-L2 (TY25 [18], rat IgG2a; BioLegends, San Diego, CA, USA) or a control isotype rat IgG2a monoclonal antibody. The cells were washed 3 times to remove excess antibody before co-culture. The culture supernatants were collected for ELISA analysis of cytokine production.

2.6. Migration of DCs after sublingual administration

We administered 0.5 mg of FITC-labeled KW3110 with 100 μg of OVA in 0.01 mL PBS sublingually to BALB *c* mice, using a micropipette tip placed under the tongue, while holding the back of the mouse for 20 s. The oral mucosa and CLNs were obtained, and freshly frozen 6-μm thick sections were fixed in a cold methanol:acetone (1:1) solution for 10 min. These were examined after staining with biotin-labeled CD11c followed by Cy5-coupled streptavidin.

2.7. *In vivo* immunization study protocol

BALB *c* mice were sensitized intraperitoneally with 100 μg of OVA and 4 mg of alum (Pierce, Rockford, IL, USA) once a week for 3 weeks. From 1 week after the last sensitization, 500 μg of OVA in 20 μL PBS was administered intranasally for 7 consecutive days [19]. Sensitized mice (n=18 in each group) received 5 mg or 0.5 mg of KW3110 or L-92 in 0.1 mL PBS, administered through a gavage tube for 7 consecutive days at the same time every day. A second group of sensitized mice (n=16 in each group) received 0.5 mg of KW3110 or L-92 in 0.01 mL PBS by sublingual administration for 7 consecutive days, as described above. After the last nasal administration of OVA, the behavior of the mice was recorded by video camera, and sneezing and nasal rubbing events were counted for 5 min [19]. The mice were then sacrificed and the serum, CLNs, spleens, inguinal lymph nodes (ILNs), and BM were collected. Serum OVA-specific IgE Ab was analyzed by ELISA.

2.8. Treatment with a neutralizing anti-PD-L2 antibody

We investigated the preventive effect of treatment *in vivo* with a neutralizing anti-PD-L2 antibody. The OVA-sensitized mice were injected intravenously with 250 μg of anti-mouse PD-L2 mAb (TY25, rat IgG2a) or rat control IgG2 mAb 12 h after the last sensitization. We then administered 0.5 mg of KW3110 sublingually for 7 consecutive days.

2.9. Analysis of cytokine production from $CD4^+$ T cells in CLNs

Single-cell suspensions were prepared from CLNs and incubated with biotinylated anti-CD4 antibody (GK1.5; BioLegends, San Diego, CA, USA) at 4 °C for 30 min, followed by incubation with anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ cells were sorted using the MACS systems (Miltenyi Biotec) and suspended in complete RPMI 1640 medium. Cells were then cultured at a density of 1.5×10^5 cells per well in the presence of 1 $mg.mL^{-1}$ of OVA with $CD4^+$ T cell-depleted and irradiated splenic feeder cells (5×10^5 cells per well) in round-bottom 96-well microculture plates for 48 h. Cytokines in the supernatants were then analyzed by ELISA.

2.10. Detection of OVA-specific IgE-secreting cells

The number of OVA-specific IgE-secreting cells among CLN, ILN, spleen, and BM cells was evaluated in 96-well filtration plates (Multiscreen; Millipore Corp., Bedford, MA, USA) coated with 50 $\mu g.mL^{-1}$ OVA or BSA in PBS at 4 °C overnight, and then blocked with 10% FCS in RPMI. CLN cells (3×10^5 cells per well) were incubated on the plates at 37 °C under 5% CO_2. After incubation for 5 h, the plates were washed with PBS containing 0.1% Tween 20 (Sigma Chemical Co.) and then stained with alkaline phosphatase-conjugated anti-IgE antibodies (23 G3; Southern Biotechnology, Birmingham, AL, USA). Alkaline phosphatase activity was visualized using a BCIP/NBT phosphatase substrate (KPL, Gaithersburg, MD, USA). The plates were scanned and spots were counted automatically using an ELISPOT image analysis system (CTL Analyzers LLC, Cleveland, OH, USA).

2.11. ELISA analysis

Cytokine levels in the culture supernatant were measured by ELISA. Flat-bottom 96-well Nunc-Brand Immuno plates (Nalge Nunc International) were coated overnight at 4 °C with affinity-purified anti-IL-4 Ab (11B11), anti-IL-5 Ab (TRFK5) (all from Mabtech AB, Nacka, Sweden) or anti-IL-13 Ab (the ELISA Ready-SET-Go! Kit; eBioscience, San Diego, CA, USA). The wells were then washed and blocked with 1% BSA in PBS, and the culture supernatants were titered onto a treated plate and incubated overnight at 4 °C. Bound cytokines were detected using biotin-conjugated anti-IL-4 Ab (BVD6-24G2), anti-IL-5 Ab (TRFK4) (all Mabtech Ab), or anti-IL-13 Ab (eBioscience) followed by streptavidin–alkaline phosphatase

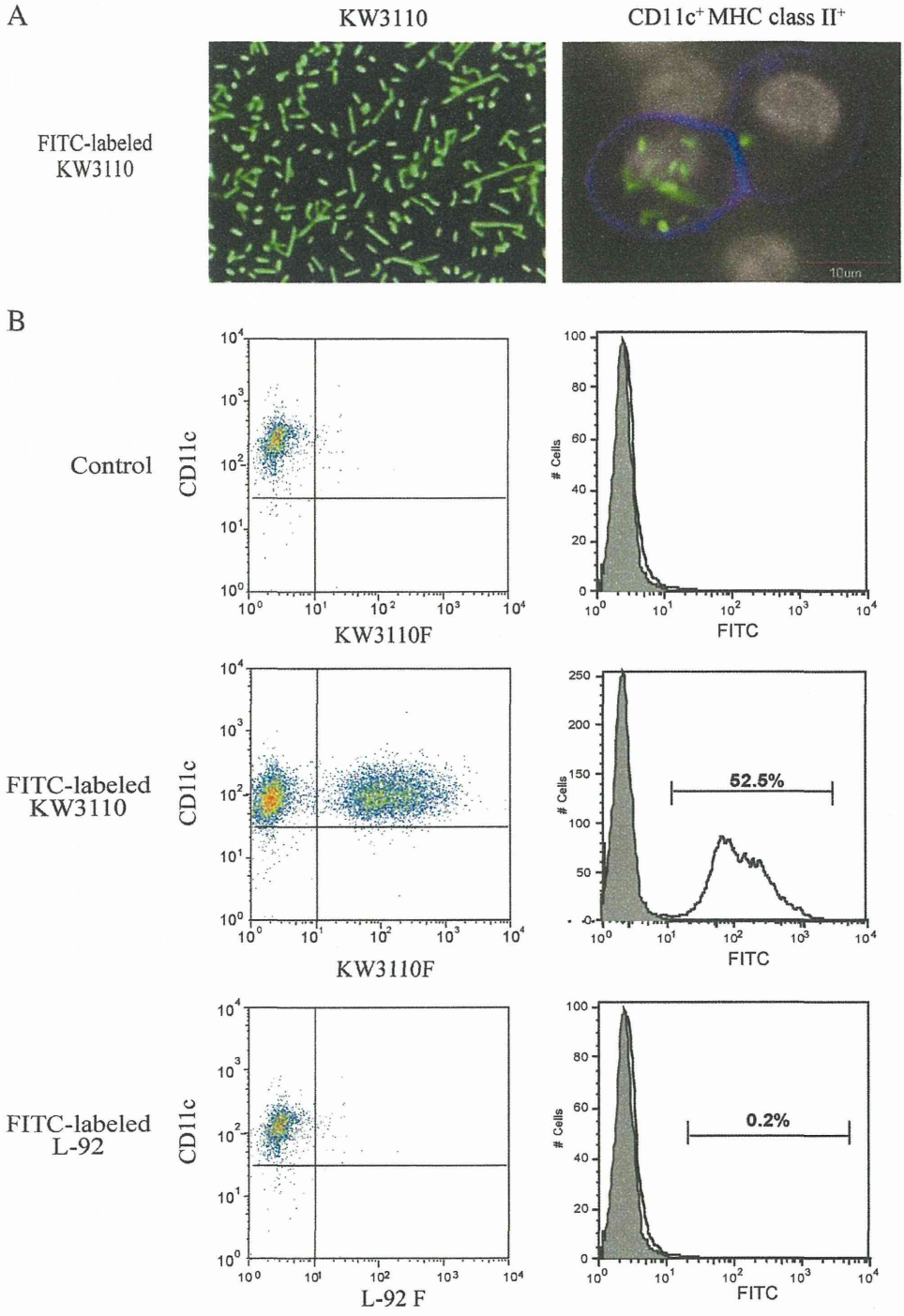

Figure 1 Phagocytosis of immature DCs analyzed by confocal microscopy (A) (green: KW3110, white: DAPI (nuclear), blue: CD11c-Cy5, red: MHC class II) and flow cytometry; (B) magnifications are 200× and 400× for the left and right images respectively. DCs phagocytosed many KW3110 bacteria in their intact form, and 52% of the cultured $CD11c^+$ DCs were found to contain FITC-labeled KW3110 after 24 h, based on FACS analysis. Only a few L-92 bacteria were phagocytosed.

(AP). The plates were developed with *p*-nitrophenyl phosphate tablets (Pierce), with the reaction terminated by the addition of 2N NaOH. Specific absorbance was measured and optical density (OD) was quantified at 410 nM using a Multiskan JX plate reader (Thermo LabSystems, Beverly, MA, USA). OVA-specific IgE was measured with a mouse anti-OVA IgE antibody assay kit (Chondrex, Redmond, WA, USA).

2.12. Flow cytometric analysis

Four-color cytometry was performed as follows. Briefly, cells were harvested from culture plates, preincubated with 10 $mg.mL^{-1}$ of unlabeled anti-CD16/32 (24G2) (BD Biosciences), and then stained on ice with a combination of Abs. We used combinations of FITC-conjugated mouse CD11c (N418)

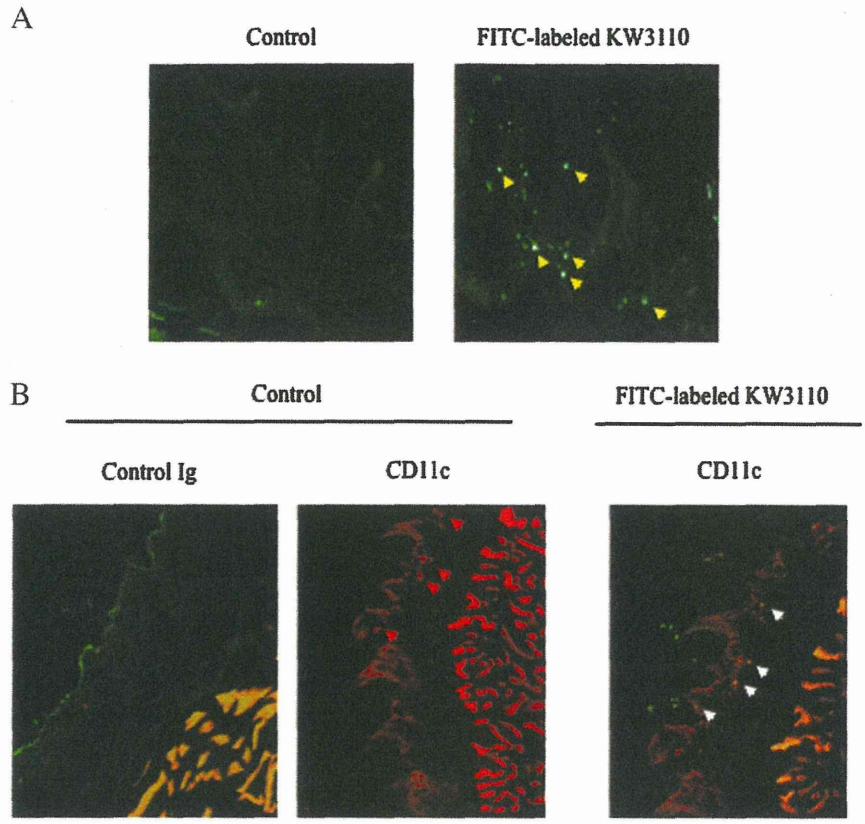

Figure 2 FITC-labeled KW3110 bacteria were detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (A, yellow arrow). Many fragmented and some intact forms of KW3110 were observed to be phagocytosed by DCs in the double staining with anti-CD11c (B, white arrow). Magnification is 100× for all images.

(eBioscience) with PE-conjugated Abs to MHC Class II IA+IE (M5/114.15), CD80 (16-10A-1), CD86 (GL-1), B7RP-1 (HK5.3), PD-L2 (122), OX40L (RM134L), 4-1BBL (TKS-1) (all eBioscience), PD-L1 (9G2), or APC-conjugated CCR-7 (4B12) (BioLegend). Data were analyzed using the Cellquest software (Becton Dickinson).

2.13. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Data are shown as mean ± SEM or SD.

3. Results

3.1. KW3110 enhanced CCR-7 and PD-L2 expression

After incubation for 24 h with LPS, L-92, or KW3110, the expression of MHC class II^{high}, CD40, CD80, CD86, B7RP-1 and PD-L1 similarly increased; however, that of CCR-7 and PD-L2 was enhanced only by KW3110 in OVA-stimulated DCs, and not by LPS or L-92 (data not shown). Administration of *Lactobacillus* without OVA stimulation induced a low level of the surface markers, and the concentration of *Lactobacillus* and LPS required for expression of the highest levels of these markers with OVA stimulation was determined to be 10 $\mu g.mL^{-1}$ in a preliminary analysis.

3.2. KW3110 was phagocytosed in the intact form by DCs

As shown in Figure 1A, the DCs phagocytosed KW3110 bacteria in the intact form, and 52% of cultured $CD11c^+$ DCs showed uptake of FITC-labeled KW3110 over 24 h based on FACS analysis (Figure 1B). In contrast, only a few L-92 bacteria were phagocytosed, and even the uptake of FITC was very low.

3.3. KW 3110 decreased cytokine production from Th2 cells

In co-culture study of DCs and Th2-skewed $CD4^+$ T cells from DO11.10 Tg mice, KW3110 decreased IL-5 and IL-13 production (from 0.210 ± 0.13 pg/mL to 0.041 ± 0.06 pg/mL for IL-5, and from 0.206 ± 0.35 pg/mL to 0.082 ± 0.19 pg/mL for IL-13, respectively); however, L-92 did not have this influence. IL-4 production was low, with no difference between various stimulations. Treatment of KW3110-treated DCs with anti-PD-L2 antibodies abrogated the inhibition of IL-5 and IL-13 production. The level of PD-L2 on DCs was the same with stimulation by OVA alone and with L-92 in OVA-stimulated DCs. In the response provoked by OVA alone, the PD-L2 antibody did not affect IL-5 or IL-13 production.

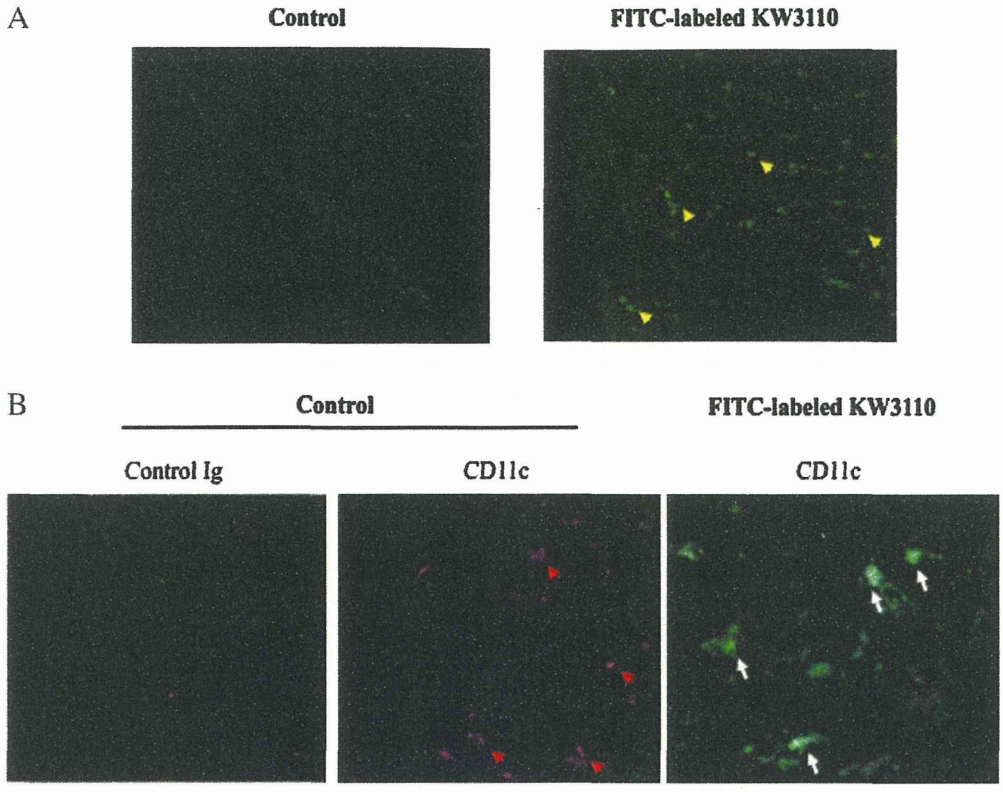

Figure 3 At 3 h after sublingual administration of FITC-labeled KW3110 stimulated with OVA, KW3110 bacteria were detected in CLNs by confocal microscopy (A, yellow arrow). CD11c-positive cells were identified by treatment with biotin-labeled anti-CD11c$^+$ (B, red arrow). Many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) bacteria were phagocytosed by DCs, as identified by double staining (B, white arrow), but were not found in the CLNs of control mice that did not receive KW3110. Magnification is 400× for all images.

3.4. Sublingually administered KW3110 migrated to CLNs

FITC-labeled KW3110 was detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (Figure 2A). The presence of CD11c-positive cells was shown by treatment with biotin-labeled anti-CD11c. Double staining with anti-CD11c showed that many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) organisms were phagocytosed by DCs (Figure 2B). Similarly fragmented and some intact forms of KW3110 were phagocytosed by DCs in CLNs 3 h after administration (Figure 3). Twelve hours later, these KW3110 were rarely detected in the oral mucosa and the CLNs.

3.5. KW3110 attenuated the nasal symptoms and serum IgE level in the OVA sensitized mice through PD-L2 expression

After OVA nasal provocation, the frequency of sneezing and nasal rubbing events decreased significantly in mice that received sublingual administration of KW3110 at 0.5 mg/day compared with those that received PBS (Table 1A). This response was dose-dependent and was stronger in mice receiving 0.5 mg.mL^{-1} KW3110 compared to those receiving a 0.05 mg.mL^{-1} dose. The response reached a plateau at a dose of 0.5 mg.mL^{-1} and there was no significant difference between mice receiving 0.5 and 5 mg.mL^{-1} KW3110 (data not shown). The frequency of sneezing and nasal rubbing events was not decreased in OVA-sensitized mice receiving sublingual L-92 or 0.5 mg of intragastric KW3110. Nasal symptoms were decreased to some extent at a ten-fold higher intragastric dose (5 mg.day^{-1}) of KW3110, but the effect was less marked than for sublingual administration at 0.5 mg/day (data not shown). Serum OVA-specific IgE was significantly reduced in mice receiving sublingual KW3110 but not in others, including those receiving intragastric KW3110 at 5 mg.day^{-1}. Treatment of OVA-sensitized mice with anti-PD-L2 mAb before KW3110 sublingual administration abrogated the effects of KW3110 (Table 1B).

3.6. Sublingually administered KW3110 decreased Th2 cytokine production and the number of IgE-secreting cells in CLNs of sensitized mice

Cytokine production from CD4$^+$ T cells in CLNs is shown in Table 2B. Of the Th2 cytokines examined, the IL-4, IL-5, and IL-13 levels were reduced in CD4$^+$ T cells from mice that received KW3110 sublingually, but not in those from mice that received intragastric KW3110. Reduction of IFN-γ or enhancement of IL-10 was not observed in CD4$^+$ T cells from any mice.

Table 1A Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		Sneezing Counts		Nasal rubbing Counts		Serum anti-OVA IgE (ng /ml)	
Nasal symptoms and serum IgE							
Gavage tube (0.5 mg)	PBS	61±15	n.s.	48±08	n.s.	425±015	n.s.
(n=18 in each group)	KW3110	51±13	n.s.	54±08	n.s.	478±029	n.s.
	L92	68±23		44±06		468±087	
Sublingual. (0.5mg)	PBS	55±06	*	48±10	*	568±054	*
(n=16 in each group)	KW3110	23±05		20±03	n.s.	278±054	
	L92	50±15	*	35±05		457±084	*
Non immunized mice (control)		10±04 **		15±11 **		n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 1B Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		IL-4 (ng /ml)		IL-5 (ng /ml)		IL-13 (ng /ml)		IFN-Ω (ng /ml)		IL-10 (ng /ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes											
Gavage tube (0.5 mg)	PBS	0.48±0.07	n.s.	2.55±0.56	n.s.	3.47±0.97	n.s.	23.08±2.98	n.s.	0.23±0.04	n.s.
(n = 18 in each group)	KW3110	0.51±0.18	n.s.	2.22±0.58	n.s.	3.89±0.18	n.s.	29.25±6.24	n.s.	0.18±0.04	n.s.
	L92	0.39±0.18		2.93±0.43		4.07±0.58		12.53±3.14		0.28±0.05	
Sublingual. (0.5 mg)	PBS	0.52±0.08	*	2.47±0.48	*	4.62±0.08	*	24.62±4.08	n.s.	0.12±0.06	n.s.
(n = 16 in each group)	KW3110	0.22±0.13		1.09±0.23		1.57±0.13		22.16±0.13	n.s.	0.15±0.03	
	L92	0.36±0.03	*	2.38±0.17	*	3.17±0.71	*	28.23±3.46		0.14 ± 0.02	n.s.
Non-immunized mice (control)		n.d.		n.d.		** n.d.		n.d.		n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable.

The number of IgE-secreting cells was higher in CLNs than in BM cells, ILNs, and the spleen from OVA-sensitized mice (Figure 4A). This number was significantly decreased in CLNs from mice that received submucosal administration of KW3110. Treatment with anti-PD-L2 antibody abrogated the reduction of Th2 cells (Tables 2A and 2B) and IgE-secreting cells (Figure 4B).

4. Discussion

DCs play a crucial role in the induction of primary T-cell-dependent immune responses [20]. Upon uptake of antigens on the mucosal surface, immature DCs in the oral mucosa are thought to migrate to the regional CLNs [21]. This suggests that the oral mucosa provides a potential route for vaccine administration and thus the interaction of oral DCs with T cells in regional lymph nodes is of interest [22,23]. In this study, we showed that *Lactobacillus* spp. administered sublingually might migrate to the CLNs and influence the maturation of DCs induced by uptake of antigen, but that the patterns differ significantly among strains.

KW3110 strongly enhanced the expression of CCR-7 and PD-L2 on DCs, compared to L-92. Sublingual administration of low doses of KW3110 in OVA-sensitized mice *in vivo* decreased IgE production and nasal symptoms induced by nasal OVA provocation, whereas the same dose of the L-92 strain or intragastric administration of KW3110 had no effect. Th2 cytokine expression and IgE synthesis in CLNs (the major area of antigen presentation and induction of effector cells in the OVA-sensitized mice) were strongly suppressed by sublingual KW3110 but not by L-92 treatment. Pre-treatment with neutralizing antibodies to PD-L2 abrogated the anti-allergic effects of sublingual KW3110. Earlier studies have shown that CCR-7 expression accelerates migration of DCs to the regional lymph nodes [24]. It is also reported that PD-L2 expression on DCs is associated with inhibition of Th2 cytokine production [18,24,25]. Matsumoto et al. showed that PD-L2 was highly expressed on pulmonary DCs and macrophages of the allergic sensitized mice, and administration of anti-PD-L2 antibody enhanced airway hyperreactivity and production of Th2 cytokines [18]. These observations suggest that DCs with high CCR-7 expression that phagocytose KW3110 migrate to the CLNs and subsequently suppress production of Th2 cytokines and IgE, mainly through expression of PD-L2.

The anti-allergic actions of L-92 observed in previous studies were brought about by induction of Treg through TLR2 [13]. However, L-92 may not stimulate DCs directly to induce CCR-7 or PD-L2, and may not effectively activate DCs in the oral submucosa when administered sublingually, due to poor

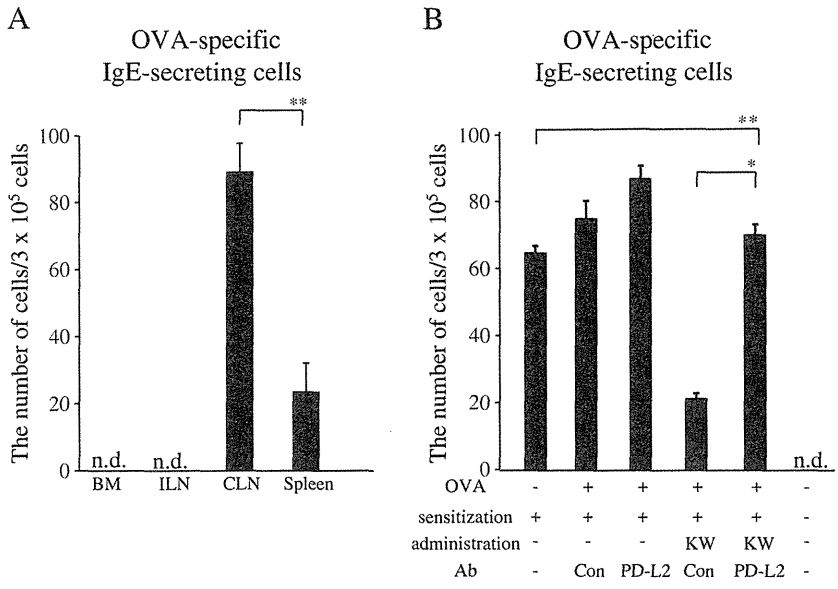

Figure 4 Inhibition of Ag-specific IgE production by KW3110. The number of OVA-specific IgE-secreting cells among cells from lymphoid organs and BM was analyzed by ELISPOT (A). Anti-PD-L2 treatment before oral submucosal administration of KW3110 blocked the reduction of IgE production in cells derived from CLNs (B).

phagocytosis. *In vitro*, DCs phagocytose a higher number of KW3110 organisms than L-92, as shown in this study. This may be because the L-92 strain is twice as large as the KW3110 strain. It is unclear if regulatory T cells were induced by sublingual administration of KW3110, because IL-10 induction or suppression of IFN-γ was not observed in the CLNs. Although the different responses among the strains of *Lactobacillus* may reflect different surface compositions and interactions with different TLRs, the roles of TLR2 or TLR9 in the immune responses induced by KW3110 are unclear [12].

KW3110 is used widely as an additive in yoghurt, beverages, and other foods, and its safety has been certified. However, in our previous study of patients with Japanese cedar pollinosis, 12 weeks intake of 50 mg day^{-1} of heat-killed KW3110, which was commenced 4 weeks before pollen dispersal, had only a limited clinical effect [26]. The KW3110 in that study was administered in capsule form; this is digested and dissolved by gastric juice or digestive enzymes, but the metabolites are unknown. In the present study, there was no benefit from intragastric administration of KW3110, but direct administration of the same dose of intact KW3110 to the oral mucosa did have an effect, with improvement in symptoms and reduced Th2 cytokine production.

5. Conclusions

Our results suggest that sublingual administration of KW3110 may reduce allergic inflammation, but the optimum dose and administration protocol require clarification. A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) for allergic rhinitis suggests that this approach is safe and may be effective as an alternative route of administration [27]. Improved efficacy of SLIT could be achieved by adjuvant therapy with sublingual administration of KW3110, since the anti-allergic effects of sublingual administration were enhanced by simultaneous stimulation with an antigen in this study. A clinical study in patients with allergic rhinitis will be performed to examine this hypothesis, and may yield further information on the potential of *Lactobacillus* therapy.

Table 2A Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Administration	Neutralizing Ab	Sneezing Counts		Nasal rubbing Counts		Serum anti-OVA IgE (ng/ml)	
Nasal symptoms and serum IgE								
–	–	–	61±9	n.s.	68±11	n.s.	651±051	n.s.
OVA	–	Control Ig	52±12	n.s.	67±07	n.s.	695±037	n.s.
OVA	–	PD-L2	64±13	n.s.	70±19	n.s.	677±017	n.s.
OVA	KW3110	Control Ig	18±05	*	24±08	*	202±058	*
OVA	KW3110	PD-L2	49±07	*	54±17	*	623±015	*
Non-immunized mice (control)			01±02	**	05±03	**	n.d.	

Values are presented as the mean±SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 2B Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Stimulation	Neutralizing Ab	IL-4 (ng/ml)		IL-5 (ng/ml)		IL-13 (ng/ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes								
–	–	–	0.41±0.13	n.s.	2.94±0.14	n.s.	4.16±0.14	n.s.
OVA	–	Control Ig	0.34±0.05	n.s.	2.83±0.58	n.s.	3.15±0.25	n.s.
OVA	–	PD-L2	0.49±0.19	n.s.	2.80±0.32	n.s.	3.49±0.36	n.s.
OVA	KW3110	Control Ig	0.14±0.04	*	0.78±0.07	*	1.08±0.09	*
OVA	KW3110	PD-L2	0.48±0.09		2.56±0.27		3.17±0.14	
Non immunized mice (control)			n.d.		n.d.		n.d.	

Values are presented as the mean±SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We are grateful to all members of Department of Otolaryngology and Immunology of Chiba University for help with the discussion. This study was partially supported by an Allergic Disease and Immunology of Health Labor Sciences Research Grant in Japan, and by a Global Center for Education and Research (COE) in Immune System Regulation and Treatment Program, MEXT, Japan. The authors have received funding support from Kirin Holdings Co. The funding sources played no other role in the research conducted.

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Comparison of nasal steroid with antihistamine in prophylactic treatment against pollinosis using an environmental challenge chamber

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ABSTRACT

Environmental challenge chambers (ECC) have been used to expose people to pollen allergens within a stable atmosphere and to examine the efficacy of treatment. Although pollinosis is one of the typical IgE-mediated type I allergic diseases, allergic inflammation is thought to contribute to the fundamental pathogenesis and prophylactic treatment may reduce exacerbations of pollinosis. The purpose of this study was to compare the efficacy of prophylactic treatment with nasal steroid (mometasone furoate nasal spray) or an antihistamine (fexofenadine) in the control of cedar pollinosis using the ECC. In a randomized, double-blind two-way crossover study, 48 patients received nasal steroid or antihistamine for 7 consecutive days (days 1–7). On day 8, patients were exposed to cedar pollen (8000 grains/m^3) in the ECC for 3 hours. Nasal symptoms induced by pollen exposure were assessed. Total nasal symptom scores (TNSSs) during the exposure in the ECC were not significantly different between the antihistamine and the nasal steroid groups. Nasal symptoms induced by pollen exposure using the ECC persisted for up to 3 days. TNSSs after pollen exposure on days 8–11 were significantly lower in the nasal steroid group compared with the antihistamine group. Prophylactic treatment with nasal steroid is more effective than antihistamine against pollinosis, particularly in the late phase. Clinical trial registration JAPIC CTI 101182 (www.clinicaltrials.jp/user/ctiMain_e.jsp).

(Allergy Asthma Proc 33:397–403, 2012; doi: 10.2500/aap.2012.33.3594)

Recently, allergen challenge testing using an environmental challenge chamber (ECC) has been widely used to examine the efficacy of various treatments.[1–6] The ECC can be used to induce symptoms in patients with allergic rhinitis and asthma under well-controlled stable conditions and is expected to offer consistent results.

However, the differences in the symptoms of pollinosis induced by pollen exposure in the ECC versus conventional provocation tests, *i.e.*, using a disk coated with pollen allergen extract or a spray of the extract, and those observed during the natural pollen season have not been well clarified. Hohlfed *et al.* reported the reproducibility of total nasal symptom scores (TNSSs) during natural seasonal exposure and found that the restricted reproducibility was caused by differences in the dispersion of pollen in the natural environment.[7] Although the levels of pollen exposure varies among individuals by season, local regional differences of the residence and the lifestyle of each subject could also significantly influence the symptoms and severity. Therefore, evaluation of the treatment should be performed carefully and a large number of subjects must be enrolled in a clinical study during the natural pollen dispersal season. However, pollen exposure investigations using the ECC have a significant advantage in this regard.

Arboreal pollen, Japanese cedar (*Cryptomeria japonica*) pollen, in particular, is the most common allergen in Japan.[8,9] Enormous amounts of Japanese cedar pollen are produced every year, with large annual variations occurring due to climate changes. Distinct from grass pollen, which can be dispersed <100 m, cedar pollen can travel >100 km, thus causing widespread pollinosis.[10] Repeated exposure to pollen induces allergic inflammation and hypersensitivity of the nasal mucosa, and early intervention against mild pollinosis, just after the start of the pollen dispersal season, may have a significant effect on the severity of symptoms when pollen dispersal reaches its peak.[11–13] During drug treatment, antihistamines, in particular, the second-generation antihistamines, are among the most widely prescribed in Japan, owing to their early onset of action and safety.[14] Although randomized controlled studies have suggested that antihistamines are less effective than nasal steroids,[15] recent *in vitro* studies have suggested that antihistamines work as inverse agonists against the H_1-receptor and that con-

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Funded by a grant from the Japan Allergy Foundation
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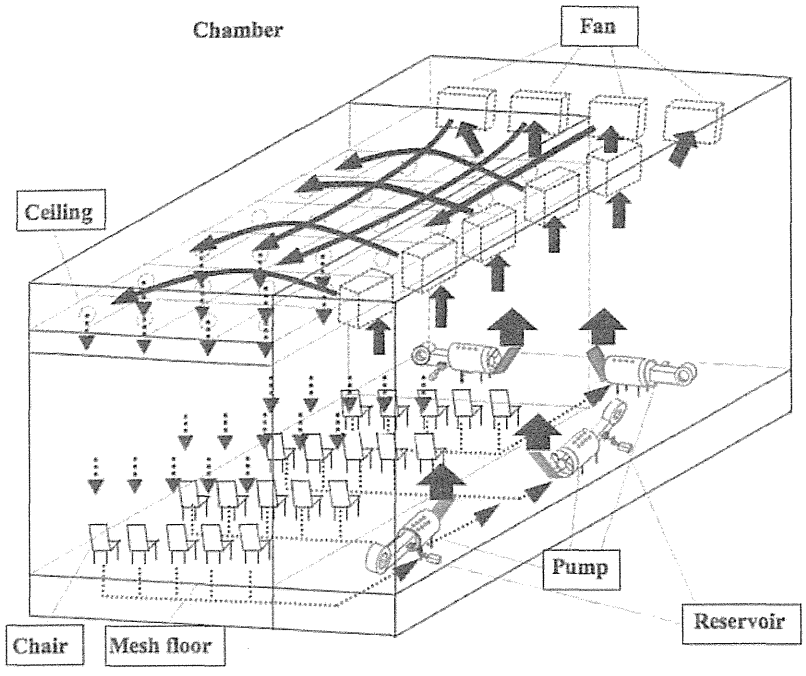

Figure 1. Chamber. The chamber can accommodate up to 50 subjects. Pollen is supplied from a reservoir using a piston pump and is discharged from the holes in the ceiling into the chamber room. The pollen is collected from underneath the mesh floor and circulated back into the chamber through the ceiling to maintain pollen dispersal throughout the chamber and avoid accumulation on the floor.

tinuous administration increases the efficacy and works effectively in prophylactic treatment.[16]

Most of the patients who are exposed to Japanese cedar pollen for only a limited number of hours in the ECC, outside of the pollen season, usually exhibit symptoms both during the pollen exposure in the chamber and after they have left the chamber, *i.e.*, a late-phase reaction. In conventional provocation studies, the symptoms are usually mild and, in particular, nasal secretion and sneezing are rarely observed in the late phase. The symptoms induced by continuous pollen exposure in the ECC more precisely reflect the symptoms observed with natural pollen exposure. Although it is difficult to evaluate the acute and late-phase reaction separately during the natural pollen season, only a few ECC studies have evaluated the late-phase symptoms. In this study, we examined the efficacy of nasal steroid and antihistamine on the symptoms of the acute-phase reaction in the ECC and of the late-phase reaction after leaving the ECC in a double-blind crossover study of patients with Japanese cedar pollinosis. We evaluated the characteristics of these drugs and determined the effectiveness of medications for relieving the symptoms during natural pollen dispersal.

METHODS

The ECC (α-Chamber)

The chamber, which was built at Chiba University in 2008, has an area and ceiling height of 71.8 m^2 and 2.6 m, respectively, and can accommodate up to 50 subjects who are under constant supervision by medical staff. The chamber unit is controlled at a constant temperature and relative humidity. Pollen is supplied from a reservoir using a piston pump and discharged from the holes in the ceiling into the chamber room. Any pollen that falls through the mesh floor into a catchment area is circulated back into the chamber to maintain pollen dispersal throughout the chamber and to avoid the accumulation of pollen on the floor (Fig. 1). The pollen level is monitored during the exposure at 56 points (on the back of each chair) within the chamber using automatic pollen counters (online supplement Fig. E1 *a*), to maintain the pollen at constant levels (500–16,000 grains/m^3).

Each patient records the frequencies of induced sneezing and rhinorrhea and subjectively assesses symptoms using mobile communication devices that allow their precise evaluation (online supplement Fig. E1 *b*). In the chamber, patients wear clean disposable clothes, including hair caps and shoe covers, to avoid transport of any pollen out of the chamber unit after the exposure (online supplement Fig. E1 *c*).

Subjects

The study population consisted of patients with Japanese cedar pollinosis who lived in Chiba City. Their ages ranged from 18 to 64 years and subjects met the following inclusion criteria: a positive allergen-specific intradermal skin test result (wheal diameter ≥10 mm) to a standardized cedar pollen extract (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) and serum cedar pollen–specific IgE level score of ≥2 by the CAP radioallergosorbent test (CAP-RAST; SRL, Tokyo, Japan). The exclusion criteria included complication of moderate/severe perennial allergic rhinitis that required treat-

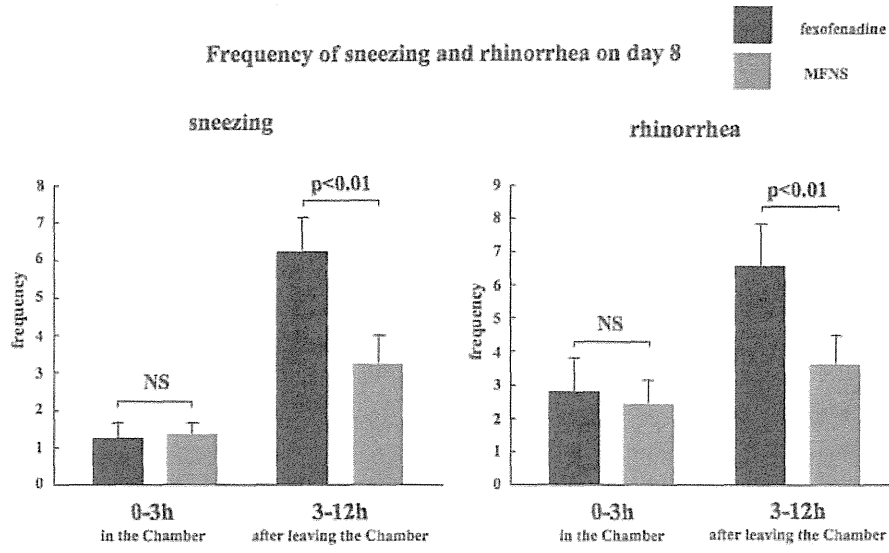

Figure 2. Frequency of sneezing and rhinorrhea on day 8. There were no significant differences in the average number of sneezing and rhinorrhea episodes during the exposure in the environmental challenge chamber (ECC) (0–3 hours) between the nasal steroid and antihistamine groups. Sneezing and rhinorrhea were observed in all subjects after leaving the chamber (3–12 hours). However, the average incidences of both symptoms were significantly reduced in the nasal steroid group. Data are expressed as the mean ± SD.

ment, a history of severe asthma, use of antiallergic drugs within 4 weeks, and a prior history of any allergen-specific immunotherapy, pregnant women, or women of childbearing potential.

The study was conducted at a university hospital in compliance with the Ethical Guidelines for Clinical Studies[17] and Good Clinical Practice[18] and the Declaration of Helsinki (2000 revision).[19] The protocol was approved by the Ethics Committee of Chiba University and Chiba University Hospital Clinical Research Center. Each of the subjects received a detailed explanation of the study and of the possible side effects and written informed consent was obtained before participation in the study.

Study Protocol

This randomized double-blind study used a two-way crossover double-dummy design to compare the efficacy of prophylactic treatment with nasal steroid (mometasone furoate nasal spray [MFNS]) and antihistamine (fexofenadine). The study was performed outside of the pollen season with enrollment and allocation by the Chiba University Hospital Clinical Research Center from July to August 2010. In 2010, the cedar pollen season was from January to April. A treatment allocation number was given to each subject. This information was closely guarded by the controller and by one member of the ethical committee who was not directly involved with the study. The study schedule is shown in online supplement Fig. E2. Before the study, subjects were interviewed regarding their medical history and underwent a CAP-RAST and an allergen-specific intradermal skin test, to measure specific serum antibodies against cedar pollen, and were preliminarily exposed to cedar pollen (8000 grains/m^3) in the chamber to confirm the elicitation of an allergic response.

Subjects were randomized to receive either treatment A (MFNS at 200 μg and placebo capsule) or treatment B (nasal placebo spray and fexofenadine at 120 mg) for 7 consecutive days (days 1–7) in a random order using a two-way crossover design, with a washout period of 14 days between drugs. The MFNS and nasal placebo spray were placed inside opaque containers and the fexofenadine and placebo tablets were placed inside opaque capsules that were identical in shape, size, color, and smell to allow the double-blind administration. On day 8, subjects were exposed to cedar pollen (8000 grains/m^3) in the chamber for 3 hours (online supplement Fig. E2).

The subjects recorded the frequencies of sneezing and rhinorrhea using mobile communication devices and medical staff checked these symptoms during the 3 hours of cedar pollen exposure in the ECC. Subjective assessment of the severity of nasal congestion was conducted just before the subject entered the chamber and then every 30 minutes during the 3 hours of cedar pollen exposure.

For examination of nasal symptoms after the pollen exposure, subjects were asked to record the symptoms using an allergy diary for 5 days (days 8–12) after leaving the chamber.

Nasal Symptom Scores in the Chamber and after Leaving the Chamber

The TNSS was the sum of the three major nasal symptoms based on both records (*i.e.*, mobile communication device and daily nasal allergy diary) according to the following criteria.

For nasal symptoms, the severity of sneezing (number of sneezes per day), rhinorrhea (number of times the subjects blew their nose per day), and nasal congestion were evaluated on a 5-point scale (0–4), based on the Clinical Guidelines for the Management of Al-

lergic Rhinitis in Japan, as follows: 0, no sensation; 1, mild; 2, moderate; 3, severe; and 4, extremely severe. Episodes of sneezing and rhinorrhea per day were rated from 0 to 4 as follows: 0, none; 1, 1–5 episodes; 2, 6–10 episodes; 3, 11–20 episodes; and 4, >21 episodes. The TNSS system is different from that used in United State rhinitis trials, which is a 0- to 3-point scale including four nasal symptoms (sneezing, rhinorrhea, nasal congestion, and itchy nose).

Primary Efficacy Variables

The primary efficacy variables were the mean TNSS for 0–12 hours on day 8. We investigated the efficacy of prophylactic treatment with nasal steroid or antihistamine on both the early and the late-phase reaction.

Secondary Efficacy Variables

Secondary efficacy variables included the mean TNSS in the chamber, the number of sneezing and rhinorrhea episodes in the chamber (0–3 hours) and after leaving the chamber (3–12 hours) on day 8, and the mean TNSS on days 9–12.

We investigated the efficacy of prophylactic treatment with nasal steroid or an antihistamine on both the early and the late-phase reaction separately.

Sample Size

Forty-four subjects were needed to detect an effect size of 0.6 TNSS with MFNS compared with fexofenadine on day 8, with a power of 80% and using a 5% two-tailed test. Therefore, we planned to recruit 48 subjects in anticipation of dropouts.

Statistics

The subjects on whom analyses were conducted represented an intention-to-treat population. Data analysis was performed with two-tailed tests at a significance level of 5%, using paired *t*-tests in SAS Version 9.1.3 (Cary, NC). Paired sample *t*-tests were used to compare symptom scores, *i.e.*, the number of sneezing and rhinorrhea episodes. The primary variables were analyzed using an ANOVA model adapted for the crossover design with the baseline score as a covariate.

RESULTS

Environmental Challenge Chamber

The pollen concentration was set to 8000 grains/m^3 because all of the subjects exhibited nasal symptoms in the preliminary study, as described previously. Furthermore, this pollen concentration has been used previously in other chamber studies with Japanese cedar pollen.[20]

After the initiation of the pollen spread, the pollen concentration in the chamber reached 8000 grains/m^3 within 15 minutes. The concentration of pollen was affected by the movement of the patients in the chamber but was maintained at constant levels within an error range of 10%.

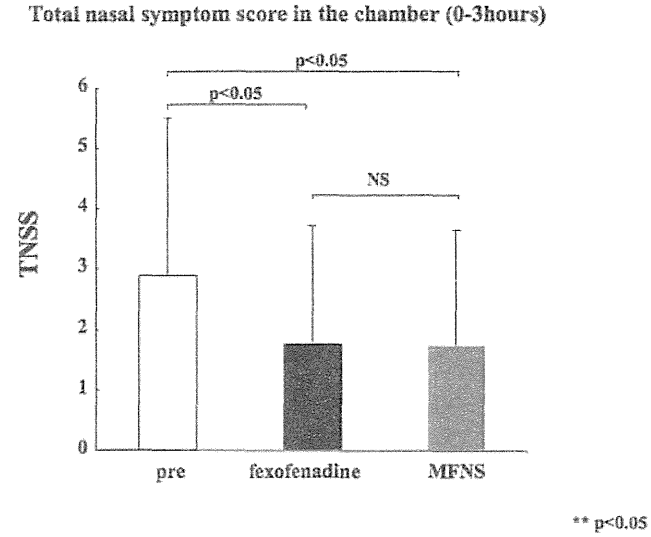

Figure 3. Total nasal symptom scores (TNSSs) in the chamber (0–3 hours). The mean TNSS after 0–3 hours in the chamber was not significantly different between the nasal steroid and antihistamine groups. However, when compared with the preliminary exposure at the beginning of the study, the mean TNSS observed in the chamber improved in both the nasal steroid and the antihistamine groups. Data are expressed as the mean ± SD.

The Subjects

Forty-eight subjects were enrolled in the study, and none dropped out or failed to record the data. The mean age of the subjects was 37.4 ± 11.8 years, and 29 (59.2%) were women. The mean cedar-specific IgE level was 23.3 ± 27.4 UA/mL (cedar pollen RAST score; class 2, 7 subjects; class 3, 24 subjects; class 4, 12 subjects; class 5, 2 subjects; class 6, 3 subjects).

All subjects underwent 3-hour exposure to cedar pollen on day 8, after receiving either treatment A (MFNS at 200 μg and placebo capsule) or treatment B (nasal placebo spray and fexofenadine at 120 mg) for 7 consecutive days (days 1–7) in a random order, using a two-way crossover design.

Number of Sneezing and Rhinorrhea Episodes and Nasal Symptom Scores during and after the Chamber Exposure

The number of sneezing and rhinorrhea episodes increased with time and all of the subjects exhibited sneezing and rhinorrhea that reached a plateau after 2 hours during the exposure in the ECC in the preliminary pollen exposure. Of the subjects, 67% exhibited nasal congestion. These symptoms continued after leaving the chamber in all subjects. The peak of sneez-

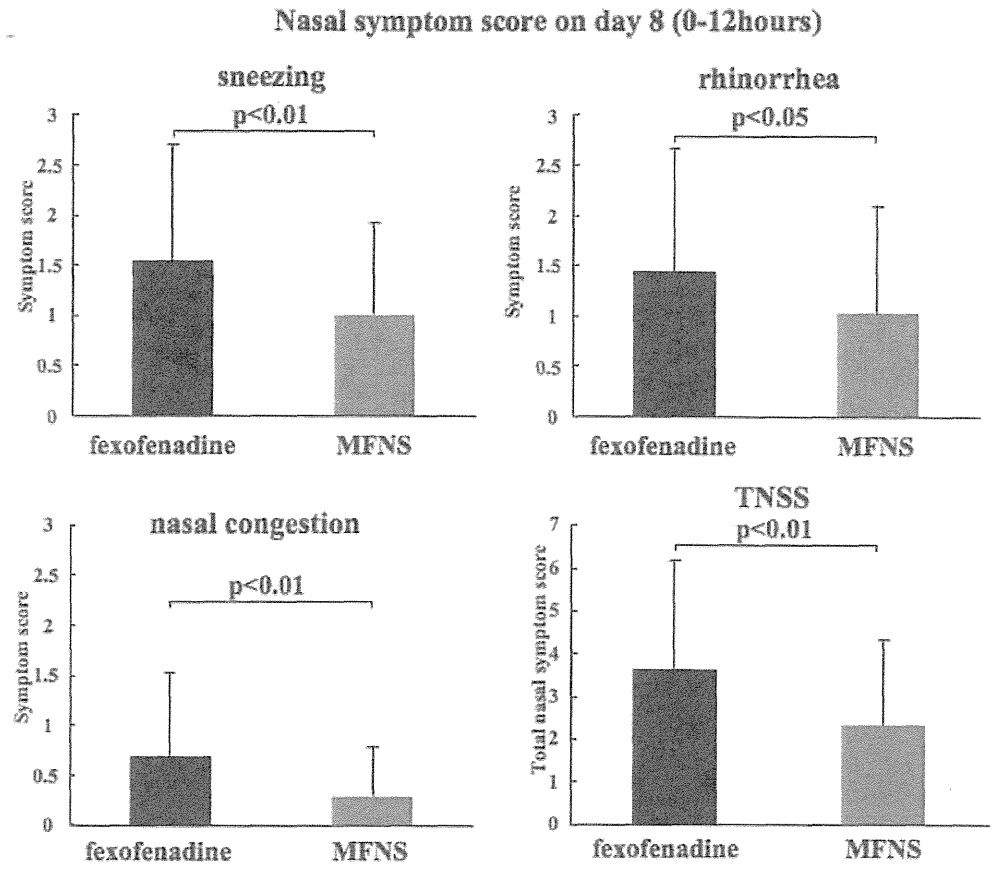

Figure 4. Nasal symptom scores on day 8 (0–12 hours). The symptom scores for sneezing, rhinorrhea, and nasal congestion in the nasal steroid group were significantly lower than those in the antihistamine group. The score of nasal congestion was also lower in the nasal steroid group. The mean total nasal symptom score (TNSS) for 0–12 hours postexposure on day 8 was decreased significantly in the nasal steroid group compared with that in the antihistamine group. Data are expressed as the mean ± SD.

ing and rhinorrhea was between 3 and 9 hours after leaving the chamber, although there was significant variation among the subjects. The nasal congestion was evaluated subjectively and also continued after leaving the chamber; however, its severity decreased gradually.

There were no significant differences between the nasal steroid and antihistamine groups in the average number of sneezing and rhinorrhea episodes (Fig. 2), the subjective severity of nasal congestion (data not shown), or TNSS (Fig. 3) during the exposure in the ECC chamber (0–3 hours). Sneezing and rhinorrhea were observed in all subjects after leaving the chamber (3–12 hours). However, the average incidences of both symptoms were significantly reduced in the nasal steroid group (Fig. 2).

On day 8 (0–12 hours), the symptom scores for sneezing, rhinorrhea, and nasal congestion in the nasal steroid group were significantly lower than those in the antihistamine group (Fig. 4). The score of nasal congestion was also lower in the nasal steroid group. The mean TNSS for the first 0–12 hours postexposure on day 8 was decreased significantly in the nasal steroid group compared with that in the antihistamine group (Fig. 4).

The lower TNSS in the nasal steroid group lasted from days 9 to 11 (Fig. 5).

Carryover or period effects were analyzed by testing the interaction terms between the intervention type and the date of intervention. No carryover effect was anticipated because of the use of a sufficient washout time between treatment periods, during which the subjects received no medication or pollen exposure (online supplement Fig. E3). The 2-week washout period was considered to be sufficient.

DISCUSSION

The aim of this study was to compare the efficacy of prophylactic treatment with MFNS or fexofenadine in the control of cedar pollinosis using the ECC. In other studies using the ECC, only nasal symptoms during the exposure have been examined[3,4] and those occurring after the subjects left the ECC have rarely been reported.[7] In this study, after leaving the pollen exposure chamber, all of the subjects exhibited nasal symptoms, including sneezing and rhinorrhea. These symptoms continued for around 3 days after a single session of pollen exposure for only 3 hours. The severity of the symptoms in the late phase did not correlate with the severity of those in the chamber. Because the patients were clothed in disposable overalls, including hair caps and shoe covers, it is not likely that they carried the pollen outside the chamber.

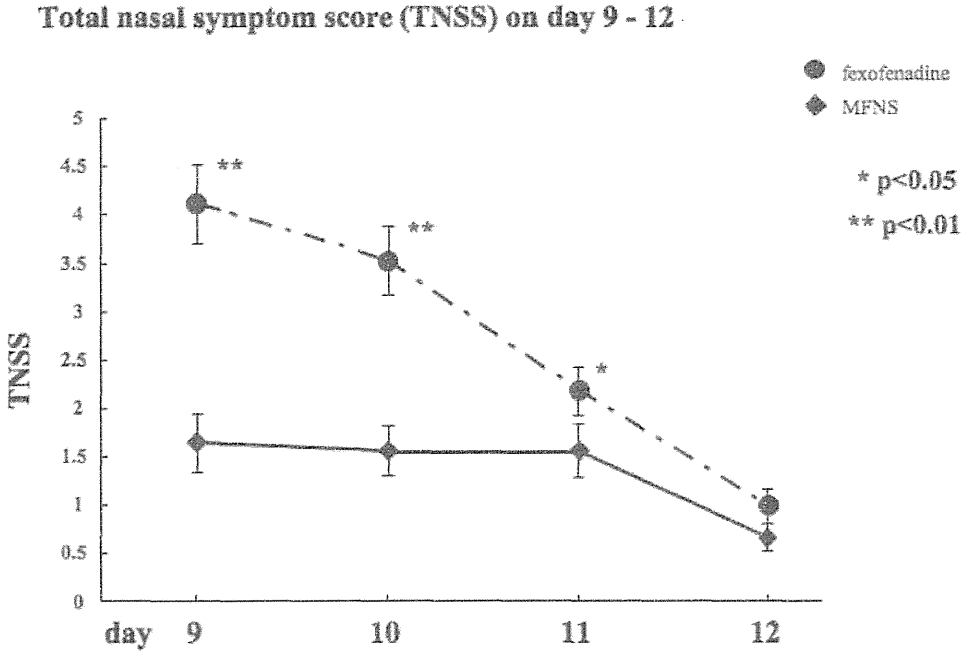

*Figure 5. Total nasal symptom scores (TNSSs) on days 9–12. TNSSs on days 9–12 in fexofenadine (●) and mometasone furoate nasal spray (MFNS; ◆) groups. The lower TNSS in the nasal steroid group lasted from days 9 to 11. Data are expressed as the mean ± SE *$p < 0.05$; **$p < 0.01$.*

The pollen exposure in the chamber might result in enhanced expression of adhesion molecules, increased infiltration of the nasal mucosa by inflammatory cells, hyperpermeability of epithelial cells, and increased neural sensory response. The nasal allergic inflammation and increased sensitivity are believed to contribute independently to the induction of symptoms, including sneezing and rhinorrhea during the late phase after an acute phase reaction to specific pollen allergens.[21,22] Examination of the inflammatory mediators in this study was difficult because the nasal washing procedure might influence the nasal symptoms and interfere with the precise evaluation of the treatment. In our previous study using eight volunteers with Japanese cedar pollinosis, increased concentrations of IL-4, IL-5, IL-13, IL-33, eotaxin, leukotrienes, and eosinophilic cationic protein, as well as the numbers of eosinophils and basophils were observed in the nasal washings collected 6 hours after pollen exposure in the chamber compared with those collected immediately before the pollen exposure. Thus, the mechanisms underlying the late-phase response need to be elucidated in further study.

No significant differences were observed in the symptoms in the exposure chamber between the antihistamine and the nasal steroid treatment groups. Compared with the symptoms observed during the preliminary exposure at the beginning of the study, the antihistamine improved the sneezing and rhinorrhea, as well as nasal congestion. However, each symptom was significantly reduced in the nasal steroid treatment group compared with that in the antihistamine group in the late phase. The symptoms in the late phase observed in the antihistamine group were not different from those observed during the preliminary exposure and the improving effect of the antihistamine treatment was not clear. The antihistamine, fexofenadine, is effective for at least 12 hours, which is shorter than MFNS. However, the symptoms observed up to 12 hours postexposure were significantly less in the nasal steroid group, after taking the drugs immediately before pollen exposure.

During the natural pollen season, pollen dispersal is known to increase with temperature and to be much greater in the daytime than at night.[14,23] However, many patients with pollinosis suffer from the symptoms at night and experience sleep problems.[24,25] It is impossible to distinguish between the early and late-phase reactions under the conditions of natural pollen dispersal. We can not ignore the direct contribution of the transport of some pollen into the house, but the late-phase reaction could play a major role in the onset of these symptoms either indoors or at night.

Repeated exposure to pollen induces allergic inflammation and hypersensitivity of the nasal mucosa, and early intervention against mild pollinosis immediately after the start of the pollen dispersal season could have a significant effect on the severity of symptoms during peak pollen dispersal.[11–13]

Histamine plays an important role in the induction of acute-phase symptoms and antihistamines have been known to exhibit rapid efficacy and improve sneezing and rhinorrhea as well as some degree of nasal congestion,[26–29] as observed in this study. However, to more effectively control the symptoms of the late-phase reaction, the inclusion of anti-inflammatory medications in the treatment is necessary. Additional studies that include immunologic parameters are

needed to elucidate the detailed mechanisms of the late-phase response to improve the control of these symptoms.

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Allergology International. 2011;60:87-92
DOI: 10.2332/allergolint.10-OA-0230

ORIGINAL ARTICLE

Upregulation of *IL17RB* during Natural Allergen Exposure in Patients with Seasonal Allergic Rhinitis

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ABSTRACT

Background: Seasonal allergic rhinitis (SAR) to Japanese cedar (*Cryptomeria japonica*; JC) is an IgE-mediated type I allergy affecting the nasal mucosa. However, the molecular mechanisms that underlie SAR are only partially understood. The aim of the study was to identify novel genes related to SAR during natural exposure to pollens, by using microarray analysis.

Methods: Subjects were 32 SAR patients and 25 controls. Total RNA was extracted from $CD4^+$ T cells isolated from peripheral blood mononuclear cells and subjected to microarray analysis with Illumina Human Ref8 BeadChip arrays. The Mann-Whitney test was performed to identify genes whose expression was altered during allergen exposure. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on samples collected from SAR patients and controls to verify the microarray results.

Results: Microarray analysis showed that the expression of 3 genes was significantly altered during allergen exposure. Among these 3 genes, the expression of interleukin 17 receptor beta (IL17RB) was confirmed to be upregulated in SAR patients compared to that of the *IL17RB* gene in healthy, non-allergic controls. The average fold change of *IL17RB* expression in the real-time RT-PCR experiment was 3.9 ($P = 0.003$).

Conclusions: The present study identified upregulation of *IL17RB* during natural allergen exposure in patients with SAR, which may further elucidate the molecular mechanisms underlying SAR.

KEY WORDS

allergen exposure, microarray, nasal mucosa, quantitative real-time RT-PCR, seasonal allergic rhinitis

INTRODUCTION

Allergic diseases such as asthma and allergic rhinitis are major causes of morbidity in developed countries, and their incidence is increasing. Seasonal allergic rhinitis (SAR) to Japanese cedar (*Cryptomeria japonica*) is an IgE-mediated type I allergy affecting the nasal mucosa. It is one of the most common allergic diseases in Japan, affecting 19.4% of the Japanese population,[1] and thus is a major public health issue. According to a national survey, the prevalence of rhinitis in Japan was 0.16 in 1992 and 0.21 in 2002.[2] We recently reported the prevalence of allergic rhinitis in an adult population of the Fukui area of Japan was 44.2% (681 of 1,540 subjects aged between 20 and 49 years), and the most common allergen in allergic rhinitis was Japanese cedar pollen (89.6%, 610 of 681 subjects with SAR).[3] SAR therefore contributes to the undermining of quality of life and decline in labor productivity.[4]

SAR is a chronic, inflammatory disease of the nasal mucosa caused by the infiltration of lymphocytes, mast cells, and eosinophils into the nasal mucosa. T-helper type 2 (Th-2) cytokines play a crucial role in orchestrating inflammatory responses. However, the molecular mechanisms that underlie SAR development are only partially understood. To understand the molecular basis of SAR, it would be helpful to examine the expression of genes in subjects with SAR during allergen exposure. Microarray techniques permit simultaneous analysis of the expression of many

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Received 19 May 2010. Accepted for publication 13 September 2010.

Table 1 Characteristics of the study population

	SAR (n = 32)	Control (n = 25)	P value
Age (year ± SD)	36.8±8.8 (25-50)	32.8±7.1 (18-47)	0.052
No. of Male/Female	16/16	12/13	0.334
Whole blood			
Neutrophil (%)	55.2 (range 39.5 to 76.0)	53.7 (range 36.3 to 76.0)	0.596
Lymphocytes (%)	33.7 (range 12 to 48.5)	37.4 (range 16.0 to 57.0)	0.154
Monotytes (%)	5.4 (range 3.3 to 8.0)	5.5 (range 3.6 to 7.3)	0.71
Eosinophils (%)	5.2 (range 0 to 18.0)	2.8 (range 0.3 to 7.3)	0.008
Basophils (%)	0.6 (range 0 to 1.5)	0.6 (range 0 to 1.8)	0.92
Total serum IgE (IU/mL)	50.1 (range 7 to 880)	28.4 (range 5 to 160)	0.049
JC-specific IgE (U_A/mL)	8.08 (range 0.79 to 86.1)	0.11 (range <0.34 to 0.56)	<0.00001

genes. Therefore, large-scale gene expression analysis by microarray may clarify which novel molecules are related to SAR.

Two types of tissues have been used for human microarray studies of allergic rhinitis. One is tissue from the nose, such as nasal polyps and nasal mucosa. Zhang *et al.*[5] performed microarray studies with nasal mucosa obtained from subjects with and without allergic rhinitis and found that several chemokine genes such as CC chemokine receptor (CCR) 2, CCR3, CCR5, CCR8, and CX3 chemokine receptor 1 were highly expressed in the nasal mucosa of subjects with allergic rhinitis compared to the expression of these genes in the mucosa of subjects without allergic rhinitis.

The other type of tissue is peripheral blood obtained from patients and controls. Larsson *et al.*[6] examined the transcriptional profiles of dendritic cells (DCs) after stimulation with grass pollen allergens and co-culture with autologous $CD4^+$ memory T cells. This study found a distinct T-cell-induced DC profile in atopic individuals, suggesting that T cells have a key instructive role in educating DCs in Th2-type responses. Benson *et al.*[7] performed microarray analysis using allergen-challenged $CD4^+$ T cells from patients with SAR and compared the expression level of $CD4^+$ T cells challenged with diluents only. This study found that tumor necrosis factor receptor superfamily member 4 (TNFRSF4), which is related to apoptosis, is significantly upregulated in allergen-challenged $CD4^+$ T cells. Allergic diseases are thought to be involved in the dysregulation of T cells, including $CD4^+$ lymphocytes. Therefore, examining changes in gene expression levels in $CD4^+$ T cells from SAR patients and healthy, non-allergic controls may improve our understanding of the molecular mechanism underlying SAR.

In the present study, we performed microarray analysis to identify changes in gene expression that reflect the status of SAR during natural allergen exposure and found that interleukin 17 receptor B (*IL17RB*) is upregulated during natural allergen exposure in SAR patients.

METHODS

SUBJECTS

Between 2003 and 2007, 1575 hospital workers and university students were invited to participate in an epidemiological survey of allergic rhinitis. All participants were of Japanese origin and were residents of Fukui Prefecture, Japan. The characteristics of the study population have been described in detail previously.[3] Total and specific IgE (produced in response to Japanese cedar, Dermatophagoides, *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans*, and *Aspergillus*) were measured using the CAP-RAST method (Pharmacia Diagnostics AB, Uppsala, Sweden).

We invited 56 of the 1575 survey subjects to participate in a gene expression analysis study and collected a 150-ml blood sample from each subject between February and April 2009, the time during which subjects were naturally exposed to Japanese cedar pollens. We also collected blood samples from the same individuals between November and December 2008, when they were not exposed to Japanese cedar pollens. Cases of SAR due to Japanese cedar polinosis (SAR group) were diagnosed on the basis of a positive history of rhinitis between February and April and high levels of Japanese cedar-specific IgE antibodies in the serum (RAST score ≥ class 2). We included only those SAR patients who were sensitized to Japanese cedar (i.e., no detectable allergen-specific IgE against dust mites, *D. glomerata*, *A. artemisiifolia*, *C. albicans* or *Aspergillus*; RAST score ≤ class 1). Subjects without allergies (control group) had to satisfy the following criteria: (1) no symptoms or history of allergic diseases, (2) no detectable, specific IgE antibodies against 6 common inhalant allergens (RAST score ≤ class 1), and (3) total serum IgE levels below the general population mean. The characteristics of subjects are listed in Table 1 and Table 2. The sample from one SAR patient (No. 19) was used only for realtime RT-PCR analysis because cRNA amplification for microarray experiment was not successful.

All participants provided written informed consent

Table 2 Treatment of SAR patients during natural pollen exposure

Sample No.	Age	Sex	Treatment
2	45	Female	Pranlukast Bepotastine besilate
3	41	Female	Epinastine hydrochloride
4	47	Female	Epinastine hydrochloride
11	27	Female	Olopatadine hydrochloride
12	30	Male	No treatment
14	25	Female	No treatment
15	28	Female	Fexofenadine hydrochloride
19	30	Male	Epinastine hydrochloride
23	30	Female	No treatment
24	47	Male	No treatment
25	27	Male	No treatment
30	50	Male	Olopatadine hydrochloride
32	49	Female	Epinastine hydrochloride
34	31	Male	No treatment
36	27	Male	No treatment
39	43	Male	Cetirizine hydrochloride
40	49	Male	No treatment
41	44	Female	No treatment
45	45	Male	Fexofenadine hydrochloride
53	45	Female	Epinastine hydrochloride
55	26	Female	Epinastine hydrochloride
56	28	Female	Epinastine hydrochloride
57	44	Male	Cetirizine hydrochloride
58	37	Male	Bepotastine besilate
59	25	Female	No treatment
60	25	Female	No treatment
65	44	Male	Epinastine hydrochloride
68	32	Male	Olopatadine hydrochloride
69	45	Female	Cetirizine hydrochloride
73	42	Female	No treatment
75	38	Male	Cetirizine hydrochloride
81	30	Male	Bepotastine besilate Olopatadine hydrochloride

to participate in the study. The study was approved by the ethical committees of the University of Tsukuba and the University of Fukui, Japan.

RNA EXTRACTION

Peripheral blood (150 ml) was taken from each subject. Peripheral blood mononuclear cells (PBMCs) were purified with Ficoll-Paque™ gradient (GE Healthcare, Piscataway, NJ, USA). $CD4^+$ T cells were isolated from PBMCs with a human CD4 Isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. RNA was extracted from PBMCs with an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

We used the Illumina BeadArray with single-color array (Illumina, San Diego, CA, USA) as a microarray platform. For the Illumina BeadArray assay, cRNA was synthesized with an Illumina® RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. In brief, 500 ng of total RNA from $CD4^+$ T cells were reverse transcribed to synthesize first- and second-strand cDNA, purified with spin columns, and then *in vitro* transcribed to synthesize biotin-labeled cRNA. A total of 750 ng biotin-labeled cRNA was hybridized to each Illumina Human Ref8 BeadChip array (Illumina) at 55℃ for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 (GE Healthcare) and then scanned with the Illumina BeadStation 500 System (Illumina). The scanned image was imported into BeadStudio software (Illumina) for analysis. Twenty-two thousand transcripts representing 8 whole-genome samples can be analyzed on a single Bead-