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Influence of viral infection on the development of nasal hypersensitivity

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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

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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).

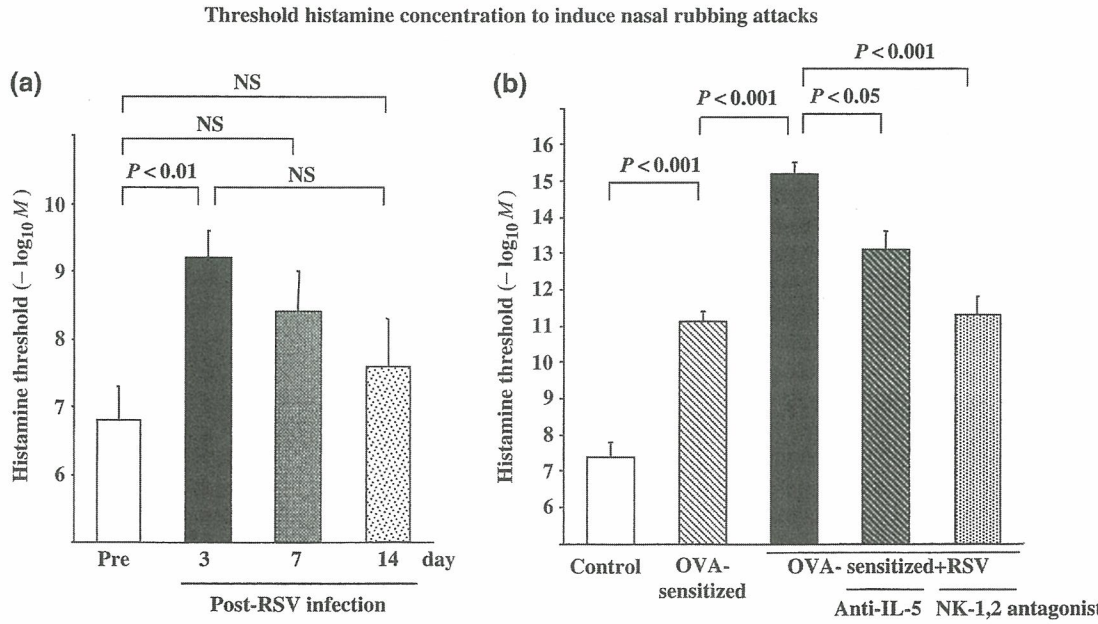

Fig. 1. Threshold histamine concentration needed to induce nasal rubbing attacks in respiratory syncytial virus (RSV)-infected non-sensitized mice (a) and in ovalbumin (OVA)-sensitized mice (b). After RSV inoculation, the threshold decreased transiently and reached its lowest on day 4. Although the threshold 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.

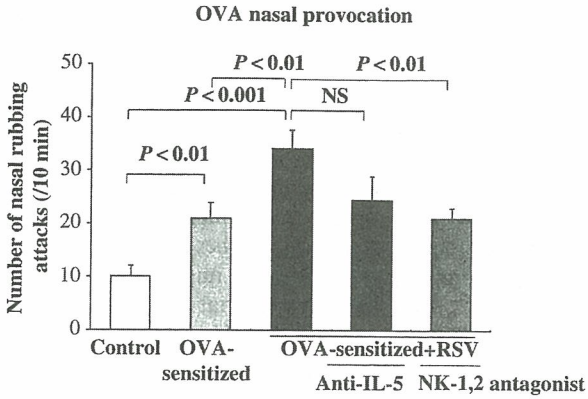

Fig. 2. The number of nasal rubbing attacks in ovalbumin (OVA)-sensitized mice following OVA provocation. Respiratory syncytial virus (RSV) infection in OVA-sensitized mice induced a dramatic enhancement of number of attacks. The anti-IL-5 treatment reduced the enhancement in some degree and the topical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

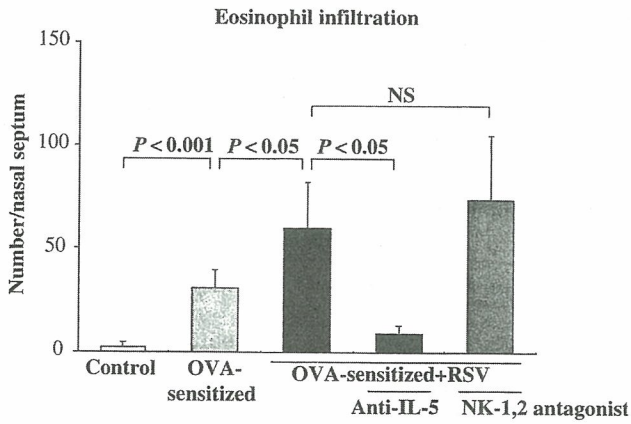

Fig. 3. The number of eosinophils in the nasal mucosa. Respiratory syncytial virus (RSV) infection markedly increased the eosinophil infiltration in ovalbumin (OVA)-sensitized mice. The treatment with anti-IL-5 antibodies reduced the number significantly but not with neurokinin (NK)-1/NK-2 receptor antagonists.

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

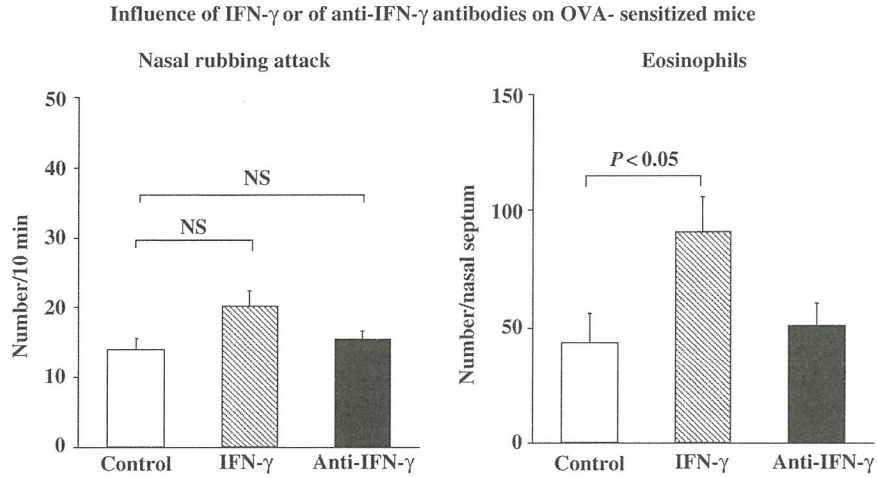

Fig. 4. Influence of IFN-γ and anti-IFN-γ antibodies on ovalbumin (OVA)-sensitized mice. The nasal administration of IFN-γ increased the number of eosinophils, but did not affect the nasal symptoms. Anti-IFN-γ treatment had no effect on either nasal symptoms or eosinophil numbers.

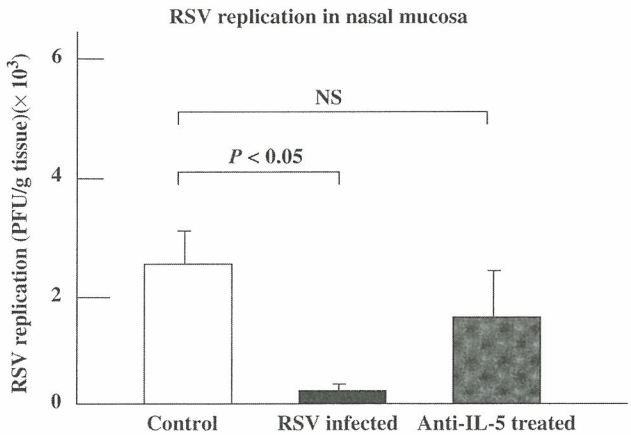

Fig. 5. Respiratory syncytial virus (RSV) replication in the nasal mucosa on day 4 after RSV inoculation. Replication was reduced in ovalbumin (OVA)-sensitized mice, but this reduction was abolished in anti-IL-5-treated OVA-sensitized mice. Non-OVA-sensitized mice were used as controls.

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.

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Role of T cells in allergic rhinitis

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Summary

A characteristic feature of allergic rhinitis (AR) is the production of allergen-specific IgE antibodies. It is widely accepted that the induction of antigen-specific type 2 $CD4^+$ helper cells (Th2) is a key event in this disease. Allergen-specific T cell–B cell interaction is indispensable for the induction of human IgE synthesis. Th2 cells also cause influx and activation of eosinophils, mast cells and basophils. Th2 cytokine-inducing cells play a major role in AR; this is known as the 'Th2 paradigm'. In this review, we examine the Th1/Th2 cell dysregulation of AR and discuss new treatment approaches to improve this dysregulation.

Keywords acquired immunity, allergic rhinitis, innate immunity, T cells, Th1 cell, Th2 cell

Allergic rhinitis and T cells

Increasing numbers of patients with allergic rhinitis (AR) are being seen on a global scale [1]. Although over 90% of patients with perennial AR exhibit allergic reactions to mite antigen, the major allergens in Japan are Japanese cedar and Japanese cypress pollens [1]. These pollens can spread more than 100 km from the tree and are, therefore, able to affect distant metropolitan areas. Japan's situation is hence distinct from that in European countries, where the most common pollinosis allergen (ragweed) can only disperse over much shorter distances of up to several hundred metres.

Symptoms of AR arise as a result of induction of allergen-specific IgE antibodies [2]. The progression of AR may be divided into immediate- and late-phase disease. Sneezing, runny nose and nasal obstruction are observed during the immediate-phase response, in which specific IgE antibody-bearing mast cells are thought to play a central role. These mast cells release various chemical mediators such as histamine, leukotrienes and prostaglandins that are causative of nasal symptoms. In contrast, the late-phase response manifests 6–8 h after allergen exposure and has a different mechanism that is characterized by recruitment and activation of eosinophils, basophils and activated T cells at the sites of allergen exposure.

It is widely accepted that induction of antigen-specific type 2 $CD4^+$ helper cells (Th2) is a key event in many allergic diseases including AR. Allergen-specific T cell–B cell interaction is indispensable for the induction of human IgE synthesis. IL-4 and other Th2 cytokines are believed to affect T cell–B cell interactions and induction of IgE synthesis from B cells [3, 4]. These Th2 cells also cause influx and activation of eosinophils, mast cells and basophils, a phenomenon known as the 'Th2 paradigm'. Although Th2 cells may have a causal role in AR, some aspects of this involvement are unclear. For example, production of allergen-mediated IFN-γ in peripheral blood mononuclear cells from AR patients is comparable with that in cells obtained from non-allergic control subjects. IFN-γ, secreted by Th1 cells, inhibits Th2 cells. Moreover, the reversible Th1 ← → Th2 hypothesis has not been reliably demonstrated *in vivo* even in successful specific immunotherapy. This suggests that Th1/Th2 dysregulation is not the cause of nasal allergy [5, 6]. Th1/Th2 cellular dysregulation in AR is yet to be clarified.

We investigated the T cell subpopulation in nasal mucosa samples from patients with AR vs. control subjects. Tissue-infiltrating lymphocytes from nasal mucosa were stained with FITC-conjugated CD4 combined with RPE-conjugated anti-CXCR3 or CCR4 monoclonal antibodies (used as Th1 and Th2 markers, respectively) and subjected to flow cytometric analysis. The percentage of mucosal $CD4^+$ cells positive for CCR4 was higher in patients than in non-allergic controls, while there was no significant difference between the percentage of mucosal $CD4^+$ cells positive for CXCR3 between the two groups (Fig. 1), suggesting that the level of Th1 in patients with AR is similar to that in non-allergic subjects. We further examined the Th1/Th2 profile in peripheral blood $CD4^+$ T cells from allergic and non-allergic subjects using the same approach [7]. As shown in Fig. 2, there was no significant difference in the number of Th1/Th2 cells in peripheral blood $CD4^+$ T cell populations between the two groups, suggesting that Th1/Th2 cellular dysregulation is not evident in peripheral blood.

Recently, we attempted direct detection of specific Th1/Th2 cell numbers in AR by major histocompatibility complex (MHC) class II epitope-mediated enzyme-linked immunospot (ELISPOT) method, and found very low population numbers of allergen-specific IL-4-producing Th cells (which represented approximately 1 spot/10^4–10^5 $CD4^+$ T cells). Interest-

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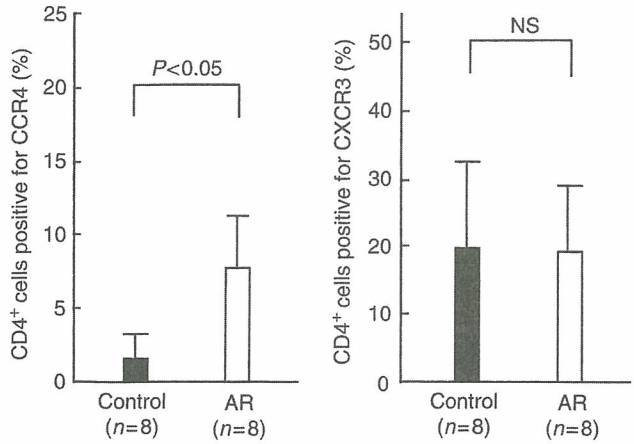

Fig. 1. T cell subpopulations in nasal mucosa of patients with allergic rhinitis and controls. RPE-conjugated anti-CXCR3 and -CCR4 monoclonal antibodies were used as T-helper type 1 (Th1) and Th2 markers, respectively.

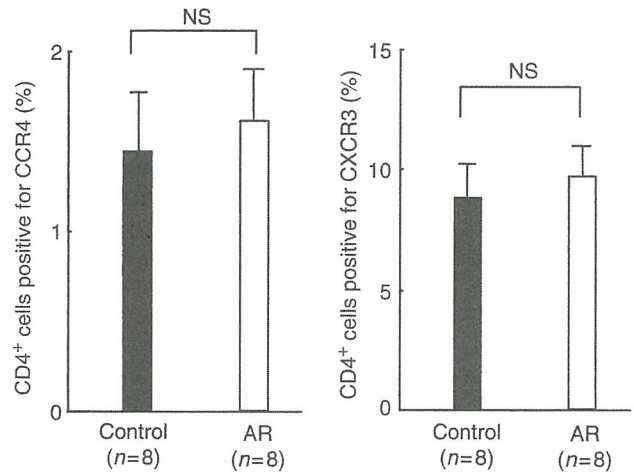

Fig. 2. T cell subpopulations in peripheral blood of patients with allergic rhinitis and controls. RPE-conjugated anti-CXCR3 and -CCR4 monoclonal antibodies were used as T-helper type 1 (Th1) and Th2 markers, respectively.

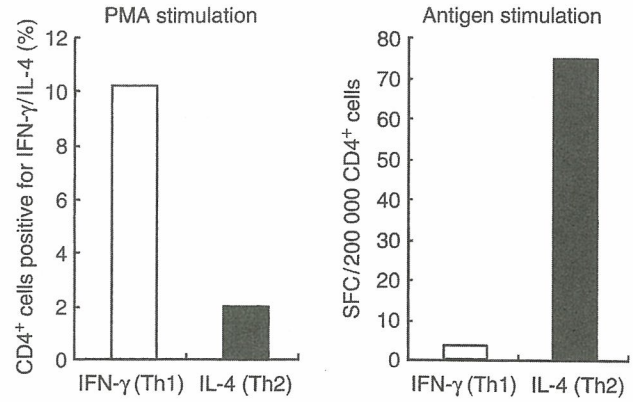

Fig. 3. T cell subpopulations in peripheral blood of a patient with allergic rhinitis. Stimulation by phorbol 12-myristate 13-acetate (PMA) was used to determine the percentage of cells positive for IFN-γ or IL-4. The number of cells secreting IFN-γ and IL-4 were measured by enzyme-linked immunospot. SFC, spot-forming cells.

ingly, specific Th2 cells were observed more frequently than specific Th1, even though non-specific total Th1 cell numbers were higher than those of Th2. Taken together, Th1/Th2 dysregulation is thought to be fundamental to the pathogenesis of AR, although very small numbers of T cells are allergen specific (Fig. 3). Therefore, it seems that levels of antigen specific Th2 cells are increased in AR; antigen-specific Th1/Th2 dysregulation induces antigen-specific IgE synthesis and activation of mast cells and chemokines, which leads to establishment of allergic inflammation.

T-helper type 2 cellular differentiation

Allergens entering the respiratory tract mucosa are captured by antigen-presenting cells (APC), which present them to $CD4^+$ Th cells. APC, which are MHC class II positive, play important roles in antigen-specific Th2 development. Various kinds of cells including monocytes/macrophages, B cells, Langerhans cells and dendritic cells (DC) have APC ability. B cells are one of the major APC in AR [8, 9], but significant numbers of Langerhans cells and DC exist in nasal mucosa and increase dramatically after allergen exposure [10, 11]. These APC including DC are thought to migrate to regional secondary lymphoid organs where they present processed antigen to Th cells in a groove of the MHC class II molecule. It is generally believed that the most important conditions of antigenic presentation are the presence of IL-4 and absence of IL-12 at the presentation site and implication of certain co-receptors determining T cell differentiation to Th2 cells. The exact source of this required IL-4 is not known, but mast cells, basophils, γδ T cells and natural killer T (NKT) cells are thought to be contributors to the Th2 response. NKT cells constitute a novel lymphoid lineage distinct from T, B and NK cells in the immune system. NKT cells are characterized by coexpression of NK1.1 NK receptor and a single invariant antigen receptor encoded by V14-J28 segments in mouse and invariant V24-J15 T cell receptor (TCR) in human. When activated, NKT cells rapidly produce large quantities of IL-4 and IFN-γ, which seem to influence subsequent adaptive immune responses and polarization of conventional-TCR αβ T cells. Recently, it has been shown that activated NKT cells in NKT-deficient mice exert a potent inhibitory effect on Th2 cell differentiation and subsequent IgE production by generating a large amount of IFN-γ. NKT cells play a crucial role in regulating the development of Th2-biased respiratory immunity against nominal exogenous antigens [12, 13].

Co-stimulatory factors can also influence Th1/Th2 differentiation, mainly by modulating contact-dependent factors, among which the extent of TCR ligation and signals delivered by OX4OL–OX40 and B7–CD28 interactions appear most important. Okano et al. have shown that B7 molecules on peripheral circulating T and B cells are up-regulated in AR; moreover, in nasal specimens, B7 molecules and their counter receptor CD28/CD152 are increased in AR patients following nasal provocation with allergen [14, 15].

Recent studies in mice have suggested the presence of several types of regulatory T cells including transforming growth factor β-inducing Th3 cells and IL-10-producing

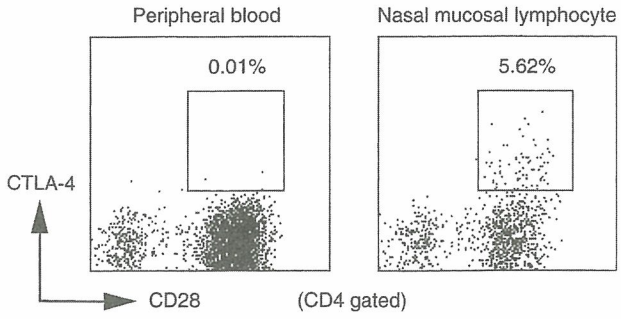

Fig. 4. Analysis of T cells in peripheral blood and mucosal lymphocyte from a patient with allergic rhinitis. CD4-gated cells are shown. The percentage of cells double positive for CD28 and CTLA-4 were 0.01% in peripheral blood and 5.62% in nasal mucosal lymphocyte, respectively.

T regulatory cells. Sakaguchi et al. [16] identified $CD4^+CD25^+$ cells that prevent $CD4^+$ T cell-mediated organ-specific autoimmune diseases. This suppression might be mediated by cell–cell interactions through CTLA-4. Our phenotype analyses of nasal T cells have shown that $CD4^+CD25^+$ $CTLA\text{-}4^+$ cells are present in mucosa of patients with perennial rhinitis but not in mucosa and peripheral circulation of non-AR patients (Fig. 4). Although, the role of $CD4^+CD25^+$ cells in allergic disease remains unclear, Iwamoto's group has reported that these T cells modulate Th1/Th2 cell balance towards Th2 cells and thus up-regulate Th2 cell-mediated allergic inflammation in the airway [17].

New treatments to improve T-helper type 2 dysregulation

To improve Th2 dysregulation, administration of Th1 cytokines or of anti-Th2 antibodies was considered to be a possible useful approach; initial experiments in animals produced some favourable results. However, clinical trials thus far have been disappointing. Administration of anti-IL-5 antibodies to patients with mild asthma significantly reduced eosinophilic infiltration in sputum but did not improve bronchial hyper-reactivity. IL-12 administration to patients with asthma also resulted in limited improvement of symptoms, and intramuscular administration of IFN-γ to patients with pollinosis did not improve symptoms or reduce IgE levels in the serum. These poor results may be because of difficulties with administering sufficient doses of Th1 cytokines and anti-Th2 antibodies to patients. Furthermore, it seems that the differences between the pathogenetic mechanisms of experimentally prepared animal models of hypersensitivity and of human allergy are too great for the successful clinical application of these treatments.

The innate immune system is the host's first line of defence and the early or initial responder to various environmental insults as diverse as Gram-positive and Gram-negative bacteria, viruses and fungi. Immune competent cells such as macrophages, DC, neutrophils and endothelial cells recognize pathogen-associated molecular patterns on the surfaces of pathogens. Toll-like receptors (TLR) are a family of pattern-recognition receptors. A wide diversity of microbial components trigger DC to produce IL-12 via TLR recognition. TLR react to components of bacterial cell wall lipopolysaccharide/lipotechoic acid and bacterial DNA such as CpG motif. IL-12 is a key cytokine influencing Th1/Th2 balance. Environmental factors may influence differentiation of allergen-specific T cells into Th2 phenotype. Shirakawa et al. [18] have demonstrated an inverse association between tuberculin responses and atopic disorders. Indeed, neonatal bacille Calmette Guérin vaccination has been shown to be associated with a lower prevalence of current asthma in a cohort study [19], and Shimada et al. [20] have demonstrated that enteric lactic acid bacteria can influence AR. In light of these results, immunotherapy with specific antigen administration and vaccine therapy using bacterial products have been attempted as ways to improve antigen-specific Th1/Th2 dysregulation [21]. Phase I/IIa studies of T cell peptide therapy against Japanese cedar pollinosis are now underway in Japan. The clinical CpG vaccine study for ragweed pollinosis in USA has shown encouraging results, and similar trials are being prepared to be conducted in Japanese patients.

Conclusions

The ratio of Th1/$CD4^+$ T cells is significantly higher in nasal mucosa of patients with AR than those without. Total numbers of Th1 and Th2 cells in peripheral blood are normal in AR patients. However, levels of antigen-specific Th2 cells are dramatically increased in allergic patients, with concomitant production of antigen-specific IgE leading to the establishment of allergic inflammation. To improve Th1/Th2 dysregulation, various new treatment approaches are under investigation, although on the whole, their effectiveness has yet to be seen.

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Bcl6 regulates Th2 type cytokine productions by mast cells activated by FcεRI/IgE cross-linking

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Abstract

Bcl6-deficient ($Bcl6^{-/-}$) mice displayed Th2 type inflammation, which caused by abnormality of non-lymphoid cells. However, initiators for the Th2 type inflammation were not clear. In order to elucidate the initiators, we investigated property and function of mast cells derived from $Bcl6^{-/-}$ mice. Mast cells were developed from bone marrow cells cultured with IL-3 (BMMCs). Although the development of BMMCs from $Bcl6^{-/-}$ mice was similar to that from wild-type mice, proliferation of $Bcl6^{-/-}$ BMMCs stimulated with IL-3 was slightly lower than that of wild-type BMMCs. When these BMMCs were stimulated by FcεRI/IgE cross-linking, $Bcl6^{-/-}$ BMMCs produced Th2 cytokines more than wild-type BMMCs did. Thus, $Bcl6^{-/-}$ mast cells are one of the initiators for Th2 type inflammation in $Bcl6^{-/-}$ mice, and Bcl6 may be a molecular target for Th2 type allergic diseases.

Keywords: Bcl6; Mast cells; Th2 type cytokines; FcεRI

1. Introduction

The human proto-oncogene *Bcl6* has been identified from chromosomal breakpoints involving 3q27 in diffuse large B-cell lymphomas (Kerchaert et al., 1993; Ye et al., 1993; Miki et al., 1994). The *Bcl6* gene encodes a 92- to 98 kDa nuclear phosphoprotein that contains the BTB/POZ domain in the NH_2-terminal region and *Krüppel*-type zinc finger motifs in the COOH-terminal region. Since the NH_2-terminal half of Bcl6 can bind to silencing mediator of retinoid and thyroid receptor protein (SMRT) and recruit the SMRT/histone deacetylase complex to silencer regions of target genes to repress expression of these genes, *Bcl6* can function as a sequence specific transcriptional repressor. To observe physiological functions of *Bcl6*, this gene was disrupted in the mouse germ line. Bcl6-deficient ($Bcl6^{-/-}$) mice showed growth retardation and abnormal immune responses. They completely lack germinal center formation (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997) and affinity maturation of antibodies specific for immunized antigens (Toyama et al., 2002).

$Bcl6^{-/-}$ mice also displayed inflammatory responses in multiple organs, especially in heart and lungs, characterized by infiltration of eosinophils and IgE bearing B lymphocytes (Th2 type inflammation), and frequently die at an early adult age (Dent et al., 1997; Ye et al., 1997; Yoshida et al., 1999). Immunization of $Bcl6^{-/-}$ mice with a protein antigen in adjuvant accelerated induction of the Th2 type inflammation (Ye et al., 1997). These data suggested that the Th2 type inflammation was originated by antigen-activated cells, especially

Abbreviations: BMMCs, bone marrow derived mast cells; $Bcl6^{-/-}$, Bcl6-deficient; SMRT, silencing mediator of retinoid and thyroid receptor protein; WT, wild-type; DNP, dinitrophenyl; HAS, human serum albumin; PI, propidium iodide

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T cells. Indeed, we have recently reported that the *IL-5* gene is one of the molecular targets of Bcl6 in T cells (Arima et al., 2002). Furthermore, recent analysis of the mechanism for the Th2 type inflammation using $Bcl6^{-/-}$ embryonic stem cell-derived chimeric mice indicated that the functional dominance of Th2 cells in $Bcl6^{-/-}$ mice is due to non-lymphoid cells including macrophages, dendritic cells, and mast cells (Toney et al., 2000). However, initiators for the Th2 type inflammation were not clear.

Mast cells are distributed throughout the vascularized tissues and play a key role in allergic reactions by releasing proinflammatory mediators such as histamine, leukotrienes and prostaglandin by antigen stimulation though a FcεRI/IgE complex. Mast cells also play a central role in innate immunity (Supajatura et al., 2002) or induce autoantibody-mediated vasculitis (Malaviya et al., 1996) by TNFα production. Furthermore, mast cells effect on immune competent cells, such as survival of eosinophils, maturation of dendritic cells, and activation of B and T cells by production of cytokines (Levi-Schaffer et al., 1998; Skokos et al., 2001; Skokos et al., 2003). Mast cells stimulated by FcεRI/IgE cross-linking produce the large amount of IL-4, which is essential for the induction of Th2 development (Schmitz et al., 1994; Fallon et al., 2002), and chemokines such as MCP-1, MIP1α, RANTES and Eotaxin, which work on Th2 development and eosinophil recruitment (Nakajima et al., 2002). These results suggest that mast cells seem to be an initiator of the Th2 type inflammation in $Bcl6^{-/-}$ mice. Therefore, we investigated property and function of $Bcl6^{-/-}$ mast cells derived from bone marrow cells cultured with IL-3 (BMMCs). When BMMCs were stimulated by FcεRI/IgE cross-linking, Th2 type cytokine productions by the $Bcl6^{-/-}$ BMMCs were larger than those by the wild-type (WT) BMMCs. We discuss a role for Bcl6 in function of mast cells.

2. Materials and methods

2.1. Mice

Bcl6-deficient ($Bcl6^{-/-}$) (Yoshida et al., 1999), (C57BL/6 × DBA2) F1(Japan SLC), C57BL/6 mice (Japan SLC) were housed in microisolate cages under pathogen free conditions. All experiments were performed according to the guidelines of Graduate School of Medicine, Chiba University (Chiba, Japan).

2.2. Culture of BMMCs

Femoral bone marrow cells of 8–12-week-old $Bcl6^{-/-}$ and WT mice were isolated and cultured in tissue culture plates as described previously (Suzuki et al., 2000). These bone marrow cells were cultured in RPMI 1640 medium containing 10% FCS and 5–10% of murine IL-3 conditioned medium (x63-IL3; kindly provided from Dr. H. Karasuyama, Tokyo Medical and Dental University) at 37 °C. Non-adherent cells in the culture were harvested every week and continuously cultured in the new plates with fresh medium.

2.3. Flow cytometry

For detection of c-Kit and FcεRI on BMMCs, BMMCs were first incubated with un-conjugated anti-CD32/16 monoclonal antibody (2.4G2; BD PharMingen, San Diego, CA) at 4 °C for 10 min, then with IgE anti-dinitrophenyl (DNP) monoclonal antibody (SPE-7; Sigma, St Louis, MO) for 50 min, followed by the incubation with biotin conjugated anti-mouse IgE antibody (BD PharMingen) for 10 min. These cells were further incubated with streptavidin-conjugated allophycocyanin (BD PharMingen) and phycoerythrin conjugated anti-c-Kit monoclonal antibody (BD PharMingen) for 10 min. Flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA).

2.4. Cell survival assay

BMMCs were washed 3 times with PBS and cultured in a 96-well-plate (1.5×10^6 cells/ml) with RPMI 1640 medium without IL-3 at 37 °C for 8 days. Viability of these cultured cells was assessed by trypan blue exclusion method, and the number of viable cells was counted on day 0, 1, 3, 5 and 8 after stimulation. For analysis of apoptosis, BMMCs were incubated with FITC-labeled annexin V and propidium iodide (PI) (Bender Medsystems, Vienna, Austria) for 15 min according to the manufacturer's instructions. Analysis of apoptosis was performed using a FACSCalibur.

2.5. Proliferation assay

BMMCs were washed 3 times with PBS and cultured in a 96-well-plate (1.0×10^6 cells/ml) with RPMI 1640 medium containing 0–10% of IL-3 at 37 °C for 36 h. These cells were incubated with 1 μCi [^{3}H]thymidine (Amersham International, Aylesbury, UK) during the last 12 h. These cells were harvested onto glass fiber filters, and [^{3}H]thymidine was counted with a liquid scintillation counter.

2.6. Stimulation of BMMCs by FcεRI/IgE cross-linking

BMMCs (1×10^6 cells/ml) were incubated with IgE anti-DNP antibody (1 μg/ml) overnight at 37 °C and washed twice with RPMI medium (Malaviya and Uckun, 2002). These cells were then challenged with various concentrations of DNP–human serum albumin (DNP–HSA; Sigma) for 30 min.

2.7. β-hexosaminidase release assay

BMMCs were stimulated by FcεRI/IgE cross-linking in Tyrode buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA, pH 7.4). The amount of β-hexosaminidase in culture supernatants and cell lysates of the activated BMMCs

was measured by hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 2 h at 37 °C. The reaction was stopped by addition of 0.2 M glycine and the optical density at 405 nm of the reaction mixture was measured by a densitometry. The percentage of specific β-hexosaminidase release was calculated as follows: the percentage released = 100 × (activity in supernatants/activity in supernatants and lysates) as previously described (Watanabe et al., 1999).

2.8. RT-PCR for cytokine mRNAs

Total RNA was isolated from BMMCs using TRIzol Reagent (GIBCO-BRL, Burlington, Canada) according to the manufacturer's instructions. The cDNA was synthesized from total mRNA (1 μg) with oligo(dT)$_{12-18}$ as a primer and Superscript II RNase H reverse transcriptase (Life Technologies, Rockville, MD). Levels of cytokine mRNA were assessed by semi quantitative RT-PCR. The 20 cycles of PCR for β-actin and 30 cycles of PCR for cytokines were carried out using the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s and polymerization at 72 °C for 60 s. PCR primers for the cDNA amplification were as follows: *IL-4* primers, 5′-ACTGACGGCACAGAGCTATTGATG-3′ and 5′-GGACTCATTCATGGTGCAGCTTATC-3′; *IL-5* primers, 5′-AGGATGCTTCTGCACTTGAGTGTTC-3′ and 5′-CCCTTGCATTTGCACAGTTTTG-3′; *IL-6* primers, 5′-GTTCTCTGGGAAATCGTGGA-3′ and 5′-TGTACTCCAGGTAGCTATGG-3′; *IL-13* primers, 5′-CAGTCCTGGCTCTTGCTTGC-3′ and 5′-AAGTGGGCTACTTCGATTTTGG-3′; *TNFα* primers, 5′-TCTCATCAGTTCTATGGCCC-3′ and 5′-GGGAGTAGACAAGGTACAAC-3′; *β-actin* primers, 5′-GTTTGAGACCTTCAACACC-3′ and 5′-GTGGCCATCTCCTGCTCGAAGTC-3′.

2.9. ELISA

Concentration of IL-6 (OptEIA, San Diego, CA) and IL-13 (R&D Systems, Minneapolis, MN) in culture supernatants was measured by ELISA according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analysis was made using unpaired *t*-test. *P*-values of <0.05 were considered to be significant.

3. Results

3.1. Development of BMMCs from Bcl6$^{-/-}$ bone marrow cells stimulated with IL-3

Bcl6$^{-/-}$ mouse peritoneal mast cells were stained with antibodies to FcεRI and to c-Kit, and analyzed on a FACS.

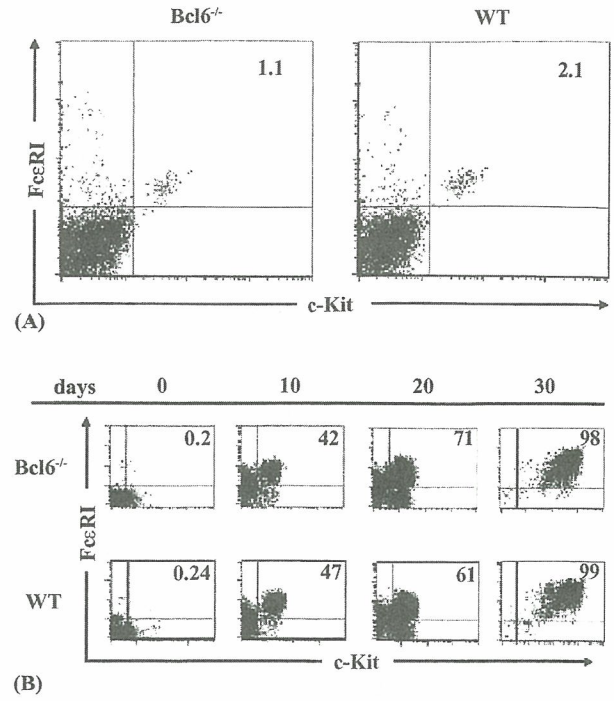


Fig. 1. Development of Bcl6$^{-/-}$ BMMCs. (A) Mast cells in peritoneal cavity of Bcl6$^{-/-}$ and WT mice were assessed by FACS analysis. The numbers in the corners indicate the percentages of mast cells in total peritoneal cells. (B) Bone marrow cells of Bcl6$^{-/-}$ and WT mice were cultured with IL-3. Development of mast cells was assessed by FACS analysis. The numbers in the corners indicate the percentages of mast cells in cultured cells.

Although the percentage of Bcl6$^{-/-}$ peritoneal mast cells was less than that of WT ones because of the larger cell infiltrations in the peritoneal cavity of Bcl6$^{-/-}$ mice, mast cells with normal surface phenotypes were observed in Bcl6$^{-/-}$ mice as well as WT littermates (Fig. 1A). In order to examine development of mast cells from bone marrow cells, Bcl6$^{-/-}$ bone marrow cells were cultured with IL-3 for 30 days. Mast cells developed in the culture were stained with anti-c-Kit and anti-FcεRI antibodies and analyzed on a FACS every 10 days. Mast cells, with more than 98% of purity, were developed in the Bcl6$^{-/-}$ bone marrow cell cultures as well as in the WT ones within 30 days (Fig. 1B). The level of FcεRI and c-Kit on Bcl6$^{-/-}$ BMMCs was similar to that on WT BMMCs, and percentages of Bcl6$^{-/-}$ BMMCs (c-Kit^{+}, FcεRI^{+}) in the cultures from day 10 to day 30 were similar to those of WT BMMCs. Thus, the deficiency of Bcl6 in bone marrow cells may not affect the development of mast cells.

3.2. Functional property of Bcl6$^{-/-}$ BMMCs

Since IL-3 is known to be an important mast cell growth factor, proliferation of Bcl6$^{-/-}$ BMMCs to IL-3 stimulation was examined by thymidine uptake. Proliferation of Bcl6$^{-/-}$ BMMCs stimulated with IL-3 was slightly lower than that of WT BMMCs (Fig. 2).

Apoptosis is induced in BMMCs by IL-3 starvation (Suzuki et al., 2000), and almost all of Bcl6$^{-/-}$ and WT BMMCs died within 8 days after IL-3 starvation (Fig. 3A).

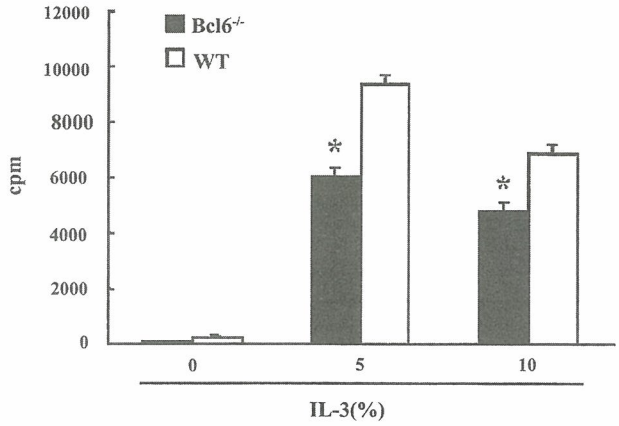


Fig. 2. Proliferation of Bcl6$^{-/-}$ BMMCs. Bcl6$^{-/-}$ (closed bar) and WT (open bar) BMMCs were cultured in the presence of various doses of IL-3 for 36 h. Data represents the mean ± S.D. of triplicate cultures. $^{*}P < 0.0001$. Results are representative of three independent experiments.

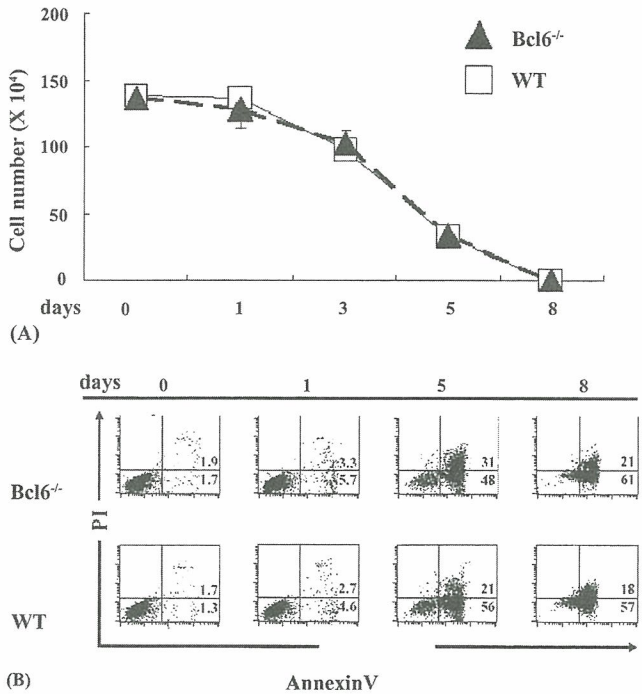


Fig. 3. Apoptosis of Bcl6$^{-/-}$ BMMCs by IL-3 starvation. (A) Live cell numbers of Bcl6$^{-/-}$ (closed triangle) and WT (open square) BMMCs were measured by die exclusion method after IL-3 starvation. (B) Apoptosis of Bcl6$^{-/-}$ and WT BMMCs after IL-3 starvation was detected by staining with annexin V and PI. The numbers in the corners indicate the percentages of cells in each quadrant. Results are representative of three independent experiments.

Thus, apoptosis of Bcl6$^{-/-}$ BMMCs after IL-3 starvation was examined by annexin V and PI staining. Percentages of apoptotic cells in Bcl6$^{-/-}$ BMMCs were similar to those in WT BMMCs until day 8 after starvation (Fig. 3B).

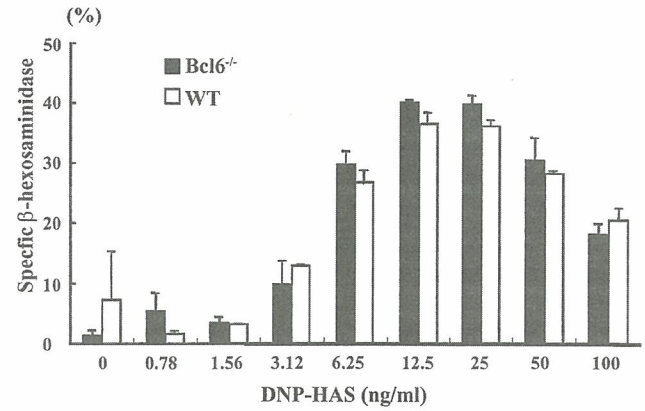


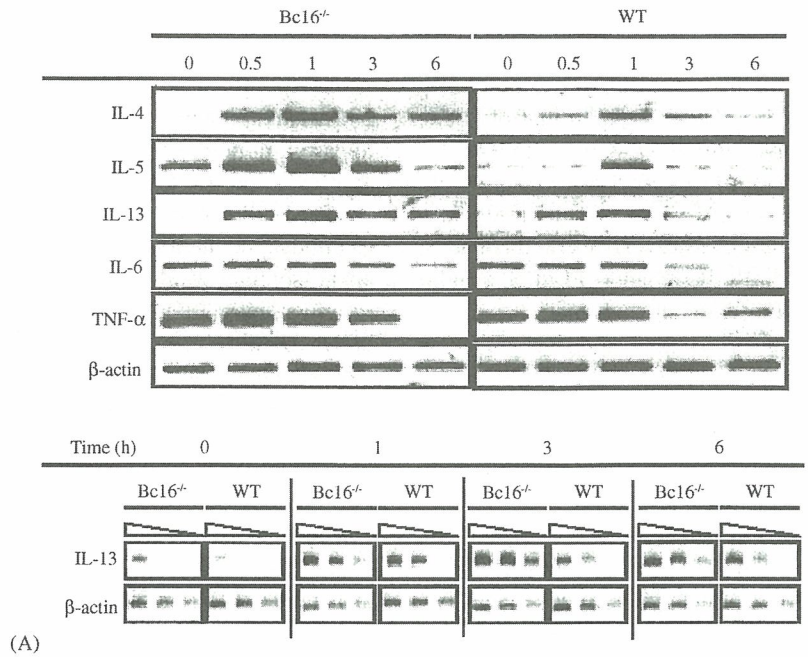
Fig. 4. Degranulation of Bcl6$^{-/-}$ BMMCs. Degranulation of BMMCs stimulated by FcεRI/IgE cross-linking was assessed by the β-hexosaminidase release assay. After Bcl6$^{-/-}$ (closed bar) and WT (open bar) BMMCs were stimulated by FcεRI/IgE cross-linking with various doses of DNP–HAS, the release of β-hexosaminidase in the culture supernatants was measured. Data represents the mean ± S.D. of triplicate cultures. Results are representative of three independent experiments.

FcεRI/IgE cross-linking is one of the major stimulations of mast cells, induces degranulation and chemical mediators release from mast cells. Since mouse mast cells release β-hexosaminidase as the result of FcεRI/IgE cross-linking (Watanabe et al., 1999; Suzuki et al., 2000), a release of β-hexosaminidase by Bcl6$^{-/-}$ BMMCs after FcεRI/IgE cross-linking was examined by the β-hexosaminidase release assay. As shown in Fig. 4, Bcl6$^{-/-}$ BMMCs released the similar amount of β-hexosaminidase as WT BMMCs did after FcεRI/IgE cross-linking with any amounts of DNP-HSA examined. Thus, degranulation of mast cells after FcεRI/IgE cross-linking is Bcl6-independent.

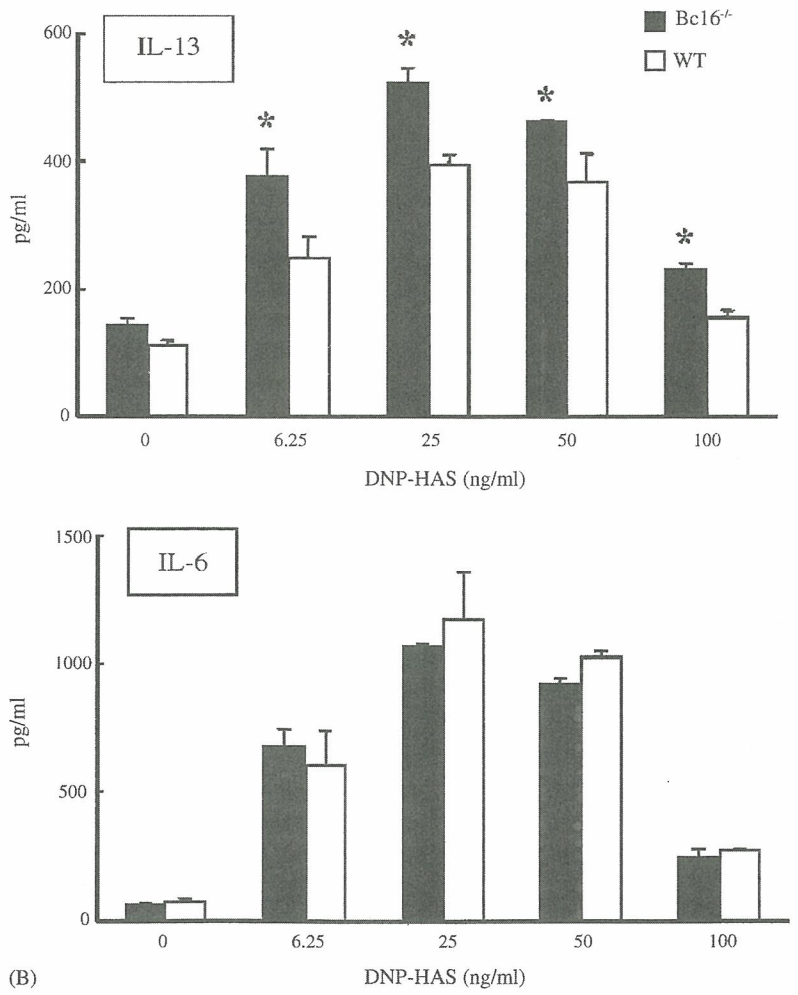
3.3. *Th2 cytokine productions of Bcl6$^{-/-}$ BMMCs stimulated by FcεRI/IgE cross-linking*

Since Th2 cytokine mRNA expression is augmented in Bcl6$^{-/-}$ T cells (Dent et al., 1997), we examined Th2 cytokine mRNA expression in Bcl6$^{-/-}$ BMMCs stimulated by FcεRI/IgE cross-linking by RT-PCR. Expression of *IL-4* and *IL-5* mRNA was clearly augmented in Bcl6$^{-/-}$ BMMCs after stimulation (Fig. 5A). Since expression of *IL-13* mRNA in Bcl6$^{-/-}$ BMMCs seemed to be more than that in WT BMMCs 3 and 6 h after stimulation, the amount of *IL-13* mRNA in those activated BMMCs was carefully examined by semi-quantitative RT-PCR. The amount of *IL-13* mRNA in Bcl6$^{-/-}$ BMMCs was 3- to 10-fold more than that in WT BMMCs 3 and 6 h after stimulation. However, there was no significant difference of *IL-6* and *TNFα* mRNA expression between Bcl6$^{-/-}$ BMMCs and WT BMMCs after stimulation.

Fig. 5. Production of Th2 cytokines by Bcl6$^{-/-}$ BMMCs. BMMCs were stimulated by FcεRI/IgE cross-linking with DNP–HAS (25 ng/ml). (A) Expression of Th2 cytokines in these BMMCs was assessed by RT-PCR (upper panel). The amount of *IL-13* mRNA was measured in cDNA with 3-hold dilutions (lower panel). (B) Production of IL-13 and IL-6 in the culture supernatants 24 h after stimulation was assessed by ELISA. Data represents the mean ± S.D. of triplicate cultures. Results are representative of three independent experiments. $^{*}P < 0.01$.


Bc16-/-
WT
0 0.5 1 3 6 0 0.5 1 3 6
IL-4
IL-5
IL-13
IL-6
TNF-α
β-actin
Time (h) 0 1 3 6
Bc16-/- WT Bc16-/- WT Bc16-/- WT Bc16-/- WT
IL-13
β-actin

(A)


IL-13
Bc16-/-
WT
pg/ml
600
400
200
0
0 6.25 25 50 100
DNP-HAS (ng/ml)
IL-6
pg/ml
1500
1000
500
0
0 6.25 25 50 100
DNP-HAS (ng/ml)

(B)

Next, we examined cytokine productions in culture supernatants of Bcl6$^{-/-}$ BMMCs stimulated by FcεRI/IgE cross-linking by ELISA. The amount of IL-13 in the culture supernatants of Bcl6$^{-/-}$ BMMCs was more than that of WT BMMCs after FcεRI/IgE cross-linking with any amounts of DNP-HSA examined (Fig. 5B). The amount of IL-6 in the culture supernatants of Bcl6$^{-/-}$ BMMCs was similar to that of WT BMMCs after stimulation. However, we could not detect IL-4 and IL-5 in the culture supernatants of Bcl6$^{-/-}$ and WT BMMCs after stimulation (data not shown).

4. Discussion

Bcl6$^{-/-}$ mice showed abnormal Th2 type inflammation (Dent et al., 1997) and the inflammation is initiated by non-lymphoid cells (Toney et al., 2000). In order to elucidate an initiator of the Th2 type inflammation, we examined functional property of mast cells from Bcl6$^{-/-}$ mice as an initiator. There was no significant difference between Bcl6$^{-/-}$ BMMCs and WT BMMCs in expression of surface FcεR1 and c-Kit, in apoptosis induced by deprivation of IL-3, and in degranulation (release of β-hexosaminidase) induced by FcεRI/IgE cross-linking. However, Bcl6$^{-/-}$ BMMCs stimulated by FcεRI/IgE cross-linking expressed more amounts of Th2 cytokine mRNAs such as *IL-4*, *IL-5* and *IL-13* than WT BMMCs did. The increased expression of *IL-13* mRNA in Bcl6$^{-/-}$ BMMCs after stimulation was reflected to the amount of IL-13 protein in culture supernatants of the Bcl6$^{-/-}$ BMMCs. However, we could not detect IL-4 and IL-5 protein in the culture supernatants of Bcl6$^{-/-}$ and WT BMMCs after stimulation. These cytokines may be difficult to detect in supernatants of the BMMC culture by ELISA, because their immunoreactivity may be changed by the heparin produced by BMMCs or these cytokines may be degraded by protease chymase produced by BMMCs as previously reported (Okayama et al., 1995).

Mast cells have been focused on as a source of IL-4 in allergic diseases, and IL-4 is the most potent factor that induces Th2 development (Fallon et al., 2002). However, cell types that produce the initial IL-4 for the induction of Th2 development are controversial. It has been speculated that IL-4 from non-Th cells, such as mast cells and basophiles, may be required for Th2 development. For example, mast cells rapidly release large amounts of Th2 cytokines including IL-4 by FcεRI/IgE cross-linking (Kalesnikoff et al., 2001) or with lipopolysaccharide stimulation (Supajatura et al., 2002), and 90% of IL-4 producing cells in the mucosa of patients with allergic rhinitis are mast cells (Bradding et al., 1992). Since Th2 cytokines, not only IL-4 but also IL-13, are known to be important for Th2 development (McKenzie et al., 1998; Chiaramonte et al., 1999), the mast cells are one of the initiators for Th2 dominancy in Bcl6$^{-/-}$ mice.

Mast cells do not mature in the bone marrow, circulate as committed progenitors and complete their maturation, and are activated in the peripheral tissues. Thus, mast cells are considered to play an important role in peripheral tissues. Mast cells activated by various stimuli including FcεRI/IgE cross-linking and lipopolysaccharide (Kalesnikoff et al., 2001; Nakajima et al., 2002; Supajatura et al., 2002) rapidly release a number of cytokines and chemokines, and recruit and activate other immune competent cells (Levi-Schaffer et al., 1998; Skokos et al., 2001; Skokos et al., 2003). Chemical mediators produced by activated mast cells are also essential for allergic reactions. For example, histamine is an essential mediator for type I allergy and anaphylaxis (Dombrowicz et al., 1993; Naclerio, 1999; White, 1999), leukotrienes are essential for vascular permeability and bronchial reconstruction (Kanaoka et al., 2001; Maekawa et al., 2002), prostaglandin works on microvascular and also bronchial reconstruction (Diaz et al., 2002). Since the number of intraepithelial mast cells increased in Bcl6$^{-/-}$ mice (Toney et al., 2000) and the production of Th2 cytokines was augmented in activated Bcl6$^{-/-}$ mast cells, Bcl6$^{-/-}$ mast cells may also be a major effector for Th2 type inflammation in Bcl6$^{-/-}$ mice.

In summary, Bcl6 regulates Th2 type cytokine productions by mast cells stimulated by FcεRI/IgE cross-linking. Thus, Bcl6$^{-/-}$ mast cells are one of the initiators for Th2 type inflammation in Bcl6$^{-/-}$ mice, and Bcl6 may be a molecular target for Th2 type allergic diseases.

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ORIGINAL ARTICLE

Comparative study of two Japanese rhinoconjunctivitis quality-of-life questionnaires

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Abstract

Conclusion. Two questionnaires were used to assess quality of life (QOL) in allergic rhinitis: the Japanese translation of the Rhino-conjunctivitis Quality of Life Questionnaire (RQLQJ) and an original Japanese QOL questionnaire (JRQLQ). Either questionnaire may be used to assess QOL depending on differences in target domains. *Objectives.* Although pollinosis is a common disease which has a major impact on patient QOL, no internationally standardized questionnaire has been available in Japan until now. The aim of this study was to compare two currently available QOL questionnaires for allergic rhinitis in Japan—the RQLQJ and JRQLQ—in terms of their appropriateness for clinical use and their psychometric properties. *Material and methods.* A multicenter, inter-group, cross-sectional study was conducted in 187 adult symptomatic patients with Japanese cedar pollinosis in 2003. Patient scores on the two questionnaires were compared in terms of both overall and comparable domains. We also examined the acceptability, construct and reliability of both questionnaires. *Results.* The questionnaires were highly correlated in terms of both overall and comparable domain scores. In addition, both questionnaires had equal and satisfactory psychometric validity, demonstrating that they are both useful tools for assessing QOL in rhinitis. However, when compared with each other, the JRQLQ focuses mainly on activities of daily life and is simpler, while the RQLQJ focuses mainly on rhinitis-related health and is more responsive.

Keywords: *Quality of life, questionnaires, rhinoconjunctivitis*

Introduction

The evaluation of health-related quality of life (QOL) has been recognized as essential to a comprehensive and holistic evaluation of diseases and treatments. Although not life-threatening, the negative impact of allergic rhinoconjunctivitis (AR) on patients' QOL is generally acknowledged.

QOL is usually assessed using generic or disease-specific self-administered questionnaires. Although various AR-specific questionnaires have been available internationally [1], until recently there have been no useful questionnaires of high quality in Japan. Of the available AR-specific questionnaires, the Rhino-conjunctivitis Quality of Life Questionnaire (RQLQ) devised by Juniper [2] is most commonly used internationally. However, the RQLQ, which was originally developed in English, must be culturally and linguistically adapted for Japanese patients following an internationally recognized methodology. Currently, the RQLQ has been translated and validated for use in Japan (RQLQJ) [3,4]. In addition, we have also developed the JRQLQ, a de novo Japanese QOL questionnaire for AR. The JRQLQ has been shown to have satisfactory acceptability, construct, concurrent and clinical validities and reliability [5]. Moreover, both the RQLQJ and JRQLQ seem to be equally valuable for assessing QOL in rhinitis patients. However, they

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differ in terms of their constructions, scale assessments and principal domains. The purpose of this study was to elucidate the relationships between the two questionnaires.

Material and methods

Subjects and administration of questionnaires

A multicenter, inter-group, comparative, cross-sectional study was conducted in 187 adult symptomatic patients with Japanese cedar pollinosis (JCP) during the 2003 pollen season (February to April) at 10 institutions in Japan. We tested two self-administered questionnaires (the RQLQJ and JRQLQ) simultaneously in the same patients.

Prior to the study, written informed consent for QOL evaluation was obtained from the patients and they were assured that their privacy would be protected. The decision regarding which questionnaire was tested first was randomly determined based on clinical record numbers. Patients were then asked to complete the questionnaires sequentially and without any assistance from physicians or nurses, with the exception of noting missing data, when necessary. The local Institutional Review Boards at Nippon Medical School approved the study from the ethical point of view.

Scoring

The questionnaire domains were scored according to the developer's recommendations. The domain scores were calculated as the mean of the total item scores. Each item score ranged from zero to six for the RQLQJ and from zero to four for the JRQLQ, with lower scores reflecting better health-related QOL. Overall scores were found by summing the means of all the items. A total symptom score was calculated for each questionnaire as the total of three nasal plus two eye symptom scores in the RQLQJ and as the total of four nasal plus two eye symptom scores in the JRQLQ. The items and domains for both questionnaires are presented in Table II.

Data analysis

The correlations of the mean scores between each of the domains in both questionnaires were investigated and compared with each other using Spearman's test. However, because the questionnaires differed in terms of their content, the correlations of the mean scores were also examined between matched comparable domains and items in both questionnaires.

The following JRQLQ and RQLQJ domains were matched and compared: nasal/ocular symptoms in the JRQLQ and nose and eye symptoms in the RQLQJ; usual daily activity in the JRQLQ and activity limitation in the RQLQJ; sleep problems; emotional function.

It is important to note that the activity limitation domain in the RQLQJ consists of 3 items selected by individual patients from 30 example items. These example items are included in similar domains in the JRQLQ, such as usual daily activity, outdoor activity and social functioning.

Psychometric evaluation

Validation studies of both questionnaires were conducted previously [3–5]. However, there has never been a direct comparison of the psychometric properties of the two questionnaires. Therefore, the validity of both questionnaires was confirmed and compared in this study by examining the following psychometric properties: acceptability; clinical validity; factor analysis; item–domain correlations (convergent and discriminate validity); and internal consistency reliability (reliability validity) [6–8]. Acceptability was defined as the percentage of missing data for individual items. To determine clinical validity, the correlation between nose and eye symptom levels and individual QOL domain scores was assessed using Spearman's test. The symptom levels were scored as mentioned above. Correlation coefficients were considered to be satisfactory when they were ≥ 0.4. To determine construct validity, factor and multitrait analyses were used. In multitrait analysis, convergent validity was tested in relation to discriminate validity. An item is expected to have stronger relationships with its own factor and weaker relationships with others. Correlations between each item and its own domain should be ≥ 0.4 [8]. Moreover, each item should have a higher correlation with its own domain than with any other domain. For factor analysis, factor loading with Varimax rotation, the communalities and the number of factors required have to be estimated from the data, usually from the sample correlation matrix. To test reliability, the internal consistency reliability was assessed using Cronbach's alpha [9]. Floor and ceiling effects (the percentages of subjects with the lowest and highest possible scores, respectively) were also calculated [9].

$p < 0.05$ was considered to significant. Other statistics are described in the relevant sections below. The software used were Excel STAT 2002 and Stat View version 4.

Table I. Patient demographics.

Sex (M:F)	49.7%:50.3%
Age (years); mean±SD	40.6±12.7
Type of AR (seasonal:seasonal+perennial)	86.6%:13.4%
Symptom score[a] (≥12: <12)	64.2%:35.8%

[a]Total nose/eye symptom score on the JRQLQ.

Results

The patient demographics and their total symptom scores (the ratio of scores of ≥12 to <12) are presented in Table I.

The mean domain score distributions for the JQRQL and RQLQJ were roughly equivalent. The numbers of items in each domain and the mean total domain scores are presented in Tables II and III.

The percentage of missing data for individual items was negligible for both the JRQLQ and RQLQJ (<1%), with the exception of the RQLQJ activity limitation domain (7.6% of total subjects), indicating good acceptability.

The correlations between domain scores for the two questionnaires were satisfactory ($r > 0.4$), with higher correlations being found for similar domains, as follows: activity limitation and usual daily activity; outdoor activity domains; sleep problem domains; non-nose/eye symptoms and general health; emotional domains (Table IV). Better correlations were obtained when the domains were adjusted for matching, as described above (Table V). Almost all correlation coefficients between domains of the two questionnaires were >0.6. These results indicate

Table II. Domain components and items in the JRQLQ and the RQLQJ.

JRQLQ	RQLQJ
1. Usual daily activities	1. Activities limitation
Reduced productivity at work/school/home	These items selected from 30 suggested, including usual daily activity, outdoor activity and social functioning
Poor mental concentration	2. Practical problems
Reduced thinking power	Inconvenience of carrying tissues paper or handkerchief
Impaired reading of book/, newspapers	Need to rub nose/eyes
Poor memory	Need to blow nose repeatedly
2. Outdoor activities	3. Sleep problem
Limitation of outdoor life	Difficulty getting to sleep
Limitation of going out	Waking up during night
3. Social functioning	Lack of a good night's sleep
Reluctance of visiting friends/ relatives	4. Non-nose/eye symptoms
Reduced contact with friends or others by telephone or in person	Tiredness
Being uneasy with people around you	Fatigue
4. Sleep problems	Thirst
Impaired sleeping	Reduced productivity
5. General health problems	Poor concentration
Tiredness	Worn out
Fatigue	Headache
6. Emotional function	5. Emotional function
Frustrated	Frustrated
Irritable	Irritable
Depressed	Impatient or restless
Unhappy	Embarrassed
7. Nasal-ocular symptoms[a]	6. Nose symptoms
Stuffy nose	Stuffy nose
Runny nose	Runny nose
Sneezing	Sneezing
Itchy eyes	Postnasal drip
Watery eyes	7. Eye symptoms
	Itchy eyes
	Watery eyes
	Sore eyes
	Swollen eyes

[a]Not included in the overall score in the JRQLQ.