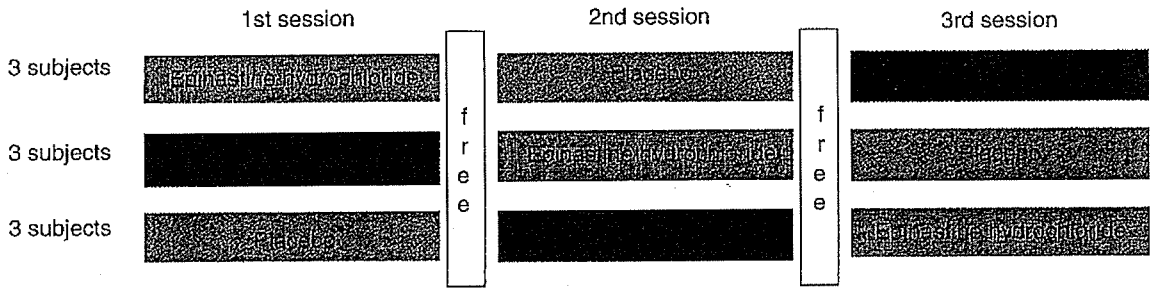

Fig. 1 Study design

Table 1 Nasal finding score

	Score			
Nasal Findings	3	2	1	0
Inferior nasal turbinate mucosal swelling	Middle turbinate not seen	Intermediate between (3) and (1)	To centre of middle turbinate	None
Nasal discharge	Filled	Intermediate between (3) and (1)	Small amount adhered to the skin	None

gen provocation tests,[7] and QOL surveys.[8] The antigen nasal provocation test is a test in which the antigen is applied directly to the nasal mucosa to elicit nasal symptoms from such a stimulation.[9] Since this is a quantitative clinical study, where a fixed amount of antigen is applied, it is possible to reproducibly elicit the allergy reactions and allows the evaluation of drugs for their treatment effect of nasal symptoms.

In this study, we examined patients with Japanese cedar pollinosis using Japanese cedar antigen discs to conduct the nasal provocation test and evaluated two second-generation antihistamines, epinastine hydrochloride tablets and fexofenadine hydrochloride tablets, for efficacy compared with placebo in a crossover clinical study.

METHODS

SUBJECTS

This study was conducted between August 14 and October 2, 2004. The subjects were male and female volunteers with Japanese cedar pollinosis who were 20 years old or older. The inclusion criteria required that the subjects have a CAP-RAST score for Japanese cedar of 2 or greater, show a positive nasal provocation reaction to the Japanese cedar antigen disc (more than two symptoms by the nasal provocation test from among nasal itching, sneezing, rhinorrhea and nasal congestion), and provide written consent to participate in this study.[10]

The following subjects were excluded.

* Subjects with a history of hypersensitivity to the components of the study drugs

* Subjects who were unable to stop smoking on the days of the clinical study

* Subjects who had used steroids within one month of the start day of the clinical study

* Subjects who within one week of the start day of the clinical study used drugs that may affect the results of the clinical study (antihistamines, antiallergic drugs, vasoconstrictors)

* Subjects undergoing desensitization therapy

* Subjects with nasal diseases that affect the assessment of the nasal provocation reaction, such as acute/chronic rhinitis, nasal polyps, hypertrophic rhinitis, deviated septum or sinusitis

* Subjects who were reactive to multiple antigens including pollens other than Japanese cedar (ragweed, mugwort), and had worsening of nasal symptoms when the nasal provocation test was conducted during the season of dispersion of the pollen

STUDY DRUGS, STUDY DESIGN

Study drugs were epinastine hydrochloride 20 mg tablets (epinastine), fexofenadine hydrochloride 60 mg tablets (fexofenadine), and placebo indistinguishable from epinastine (provided by Nippon Boehringer Ingelheim Co., Ltd., Hyogo, Japan with fees paid).

The clinical study consisted of four visits. Visit 1 consisted of screening tests, and visits 2, 3 and 4 involved nasal provocation tests using Japanese cedar antigen discs.

In the study design, the three study drugs were administered to subjects as a single dose on the days of the three nasal provocation tests in an open 3-way crossover method in the order assigned by the randomization (Fig. 1). A study drug administrator who was neither the physician nor the clinical study collaborator conducted the randomization, and the study

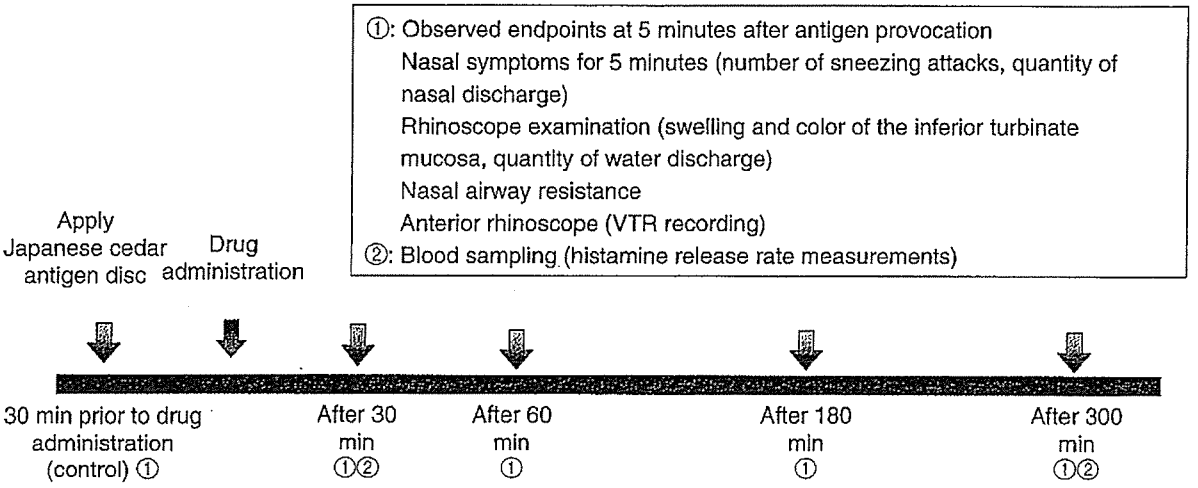

Fig. 2 Observed endpoints

Table 2 Characteristics of subjects

Sex	male	5
	female	4
Age (years)	mean ± S.E.	23.56 ± 0.57
Age at onset (years)	mean ± S.E.	16.67 ± 1.16
Disease type	sneezing, rhinorrhea	8
	nasal airway closure	1
CAP-RAST (score)	house dust	2.22 ± 0.82
	mites	2.33 ± 0.89
	Japanese cedar	3.22 ± 0.31
	mugwort	0.44 ± 0.38
	ragweed	0.56 ± 0.43
Co-existing illesses	no	1
	yes	8
	chronic allergic rhinitis	5
	seasonal allergic conjunctivitis	2
	allergic conjunctivitis	2
Allergy prior history	no	9
	yes	0
Prior therapy (desensitization, surgery)	no	9
	yes	0

drug administrator performed the drug administration in a way that could not be identified by the physician nor the clinical study collaborator.

OBSERVED ENDPOINTS

Efficacy

During the 5 minutes after provocation, the nasal symptoms were observed, including the number of sneezing attacks and quantity of nasal discharge (weight of tissues used).

At 5 minutes after provocation, a rhinoscope examination was conducted to examine and rate the swelling and color of the mucosa of the inferior nasal turbinates and nasal discharge. The rating was conducted according to the nasal finding classification of The Practical Guideline for Management of Allergic Rhinitis.[4] The extent of the inferior nasal turbinate mucosal swelling and nasal discharge were scored (3 points, 2 points, 1 point, none; Table 1), and the changes in the nasal finding score over time were determined for each drug.[10]

In addition, nasal airway resistance measurements,

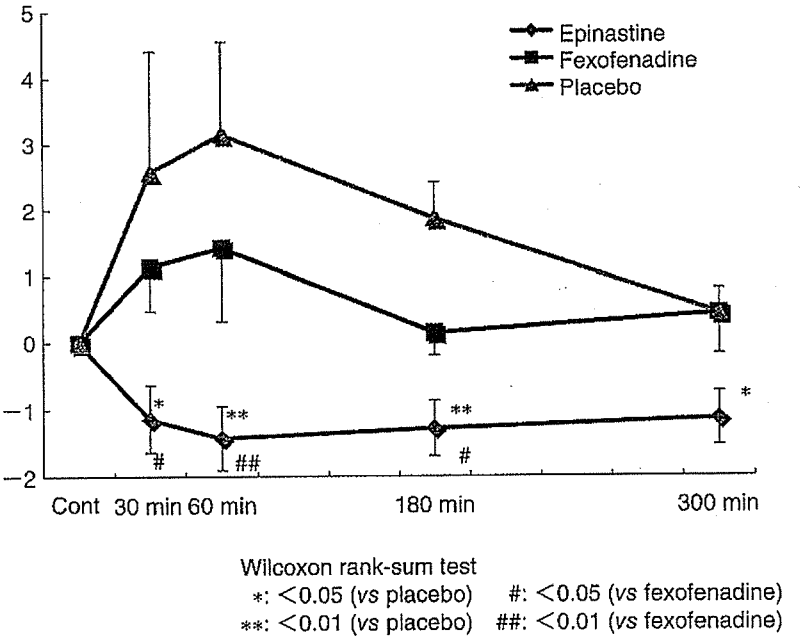

Fig. 3 Change in the number of sneezing attacks

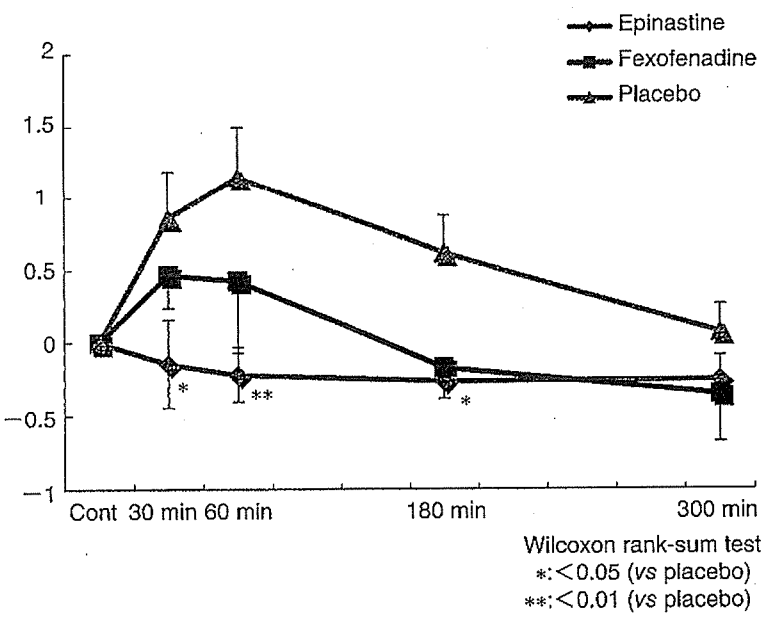

Fig. 4 Change in the quantity of nasal discharge (weight of tissues used)

VTR recording by anterior rhinoscopy for a one-minute period immediately after provocation and for a one-minute period beginning at 5 minutes after provocation, and histamine release rate (HRT Shionogi, Osaka, Japan) after first nasal provocation (30 minutes before drug administration) and at the end of the last nasal provocation (300 minutes after drug administration) were also observed.

Safety

At each visit, the physician conducted an examination at 30 minutes prior to drug administration before the nasal provocation and at 300 minutes after drug administration after the completion of the nasal provocation.

Patient-reported Evaluation

After completion of all clinical studies, subjects were

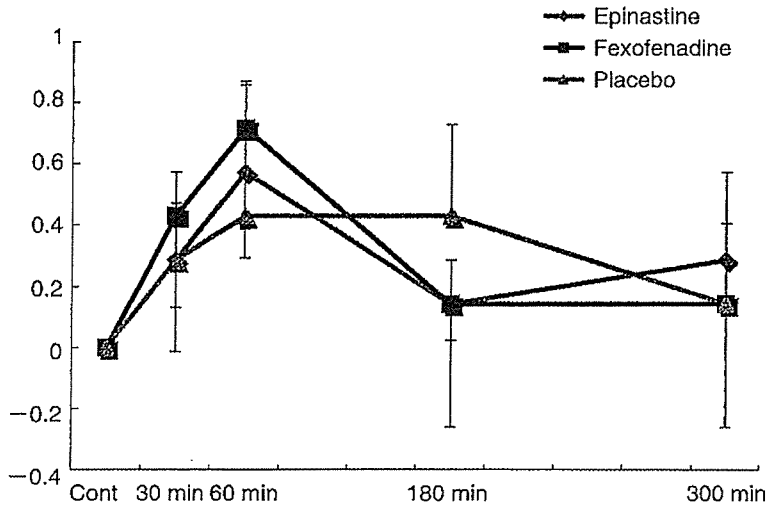

Fig. 5 Change in the inferior nasal turbinate mucosal swelling (nasal finding score)

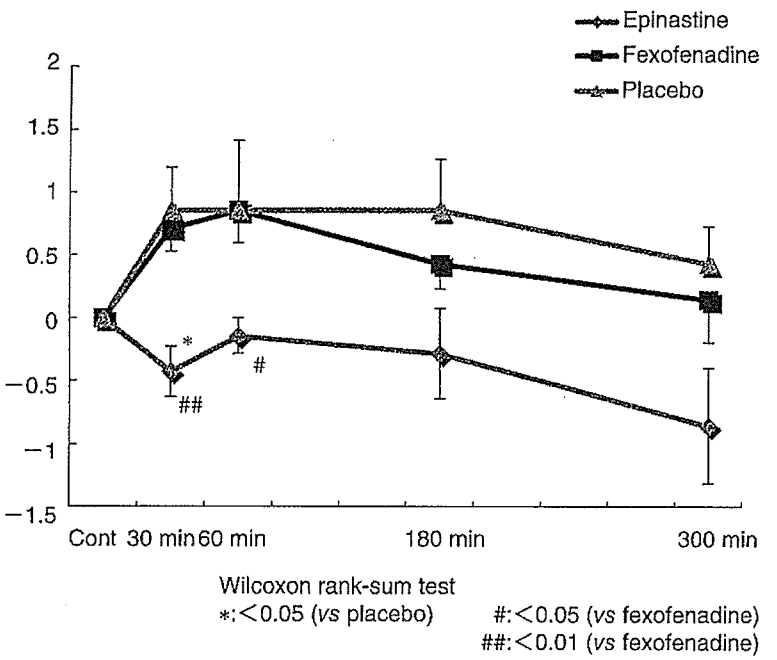

Fig. 6 Change in the nasal discharge (nasal finding score)

surveyed using questionnaires for their impressions on the efficacy of the study drugs to determine patients' opinions.

STUDY METHODS

The nasal provocation was done using Japanese cedar antigen discs containing 50 ng of Cry j1 (kindly provided by National Hospital Organization Sagamihara National Hospital, Kanagawa, Japan), and were applied to the anterior portion of both inferior nasal turbinates for 5 minutes. The procedures for each observation day are indicated below.

Day of Screening Tests (Visit 1)

Subjects were given an explanation regarding their participation in this clinical study and provided written consent.

The subjects underwent a physical examination, laboratory tests (hematology, blood chemistry, urinalysis) and the nasal provocation test. The subject's background, past medical history and co-existing illnesses, and concomitant drugs were surveyed to con-

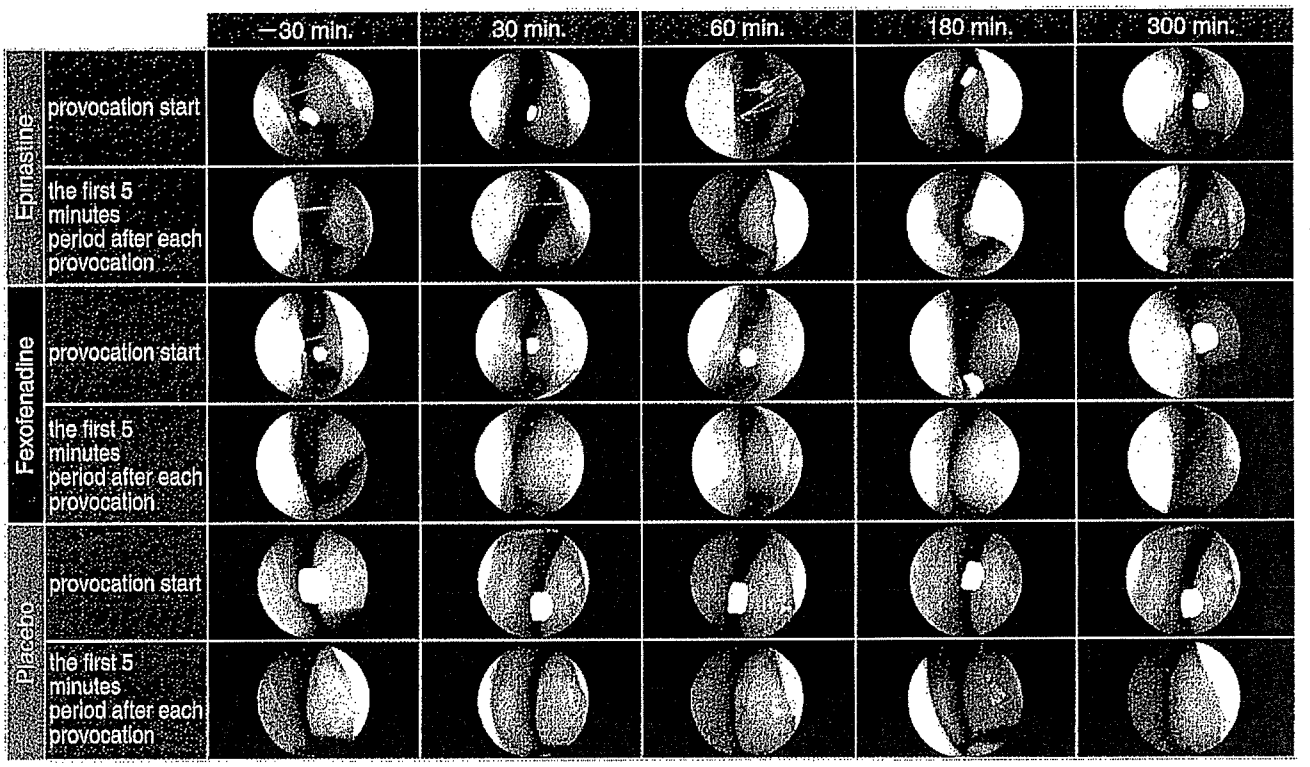

Fig. 7 The appearance of nasal mucosa by rhinoscope

firm inclusion criteria and exclusion criteria.

Days of Nasal Provocation Tests (Visits 2, 3, 4; Fig. 2)

The study drugs were administered at the three visits, and at each visit, a study drug was given as a single dose. There was a drug-free period of 14 days between visits.

The first nasal provocation was conducted at 30 minutes prior to any study drug administration, after which the study drug was administered. Then, subsequent nasal provocation was conducted at 30 minutes, 60 minutes, 180 minutes, and 300 minutes after drug administration (a total of five times). At each time point, the respective observations were conducted.

Statistical Analysis

The results were evaluated as the rate of change in the value after antigen provocation prior to drug administration compared with those after drug administration, and the value was expressed as a mean ± standard error. Comparison between study drugs was performed using the Wilcoxon rank-sum test.

This clinical study was conducted at the Tokyo Clinical Research Organization for Medicine Clinic (ToCROM). Prior to conducting the study, the study was reviewed by the Independent Ethics Committee of the Osaka Pharmacology Research Clinic and was approved by the clinic director.

RESULTS

There were nine subjects (5 men, 4 women), mean age 23.56 ± 0.57 years. The details of subjects' demographics are in Table 2. None of the subjects experienced safety problems during the study. One subject did not return for visits 3 and 4, while one subject did not return for visit 3. Thus, 7 subjects received all study drugs.

EFFICACY

Nasal Symptoms

Changes in the number of sneezing attacks and changes in the quantity of nasal discharge (weight of tissues used) are shown in Figures 3, 4. The number of sneezing attacks was maintained below baseline for up to 300 minutes after administration of epinastine. On the other hand, with fexofenadine and with placebo, the number of sneezing attacks increased during the 60 minutes after study drug administration, followed by a decrease. However, the number of attacks did not drop below baseline. Epinastine showed a significant difference against placebo from 30 minutes to 300 minutes after drug administration and against fexofenadine from 30 minutes to 180 minutes after drug administration.

The changes in the quantity of nasal discharge showed a trend that was similar to that of the number of sneezing attacks. Epinastine showed values below

Table 3 Subject impressions

	effective		neither effective nor ineffective		ineffective
Epinastine	3	42.86%	2	28.57%	2
Fexofenadine	2	28.57%	4	57.14%	1
Placebo	0	0.00%	3	42.86%	4

baseline continuously to 300 minutes after administration. The changes in the quantity of nasal discharge increased for 30 minutes after administration of fexofenadine and for 60 minutes after the administration of placebo, followed by a decrease. Epinastine showed significant differences in the changes in the quantity of nasal discharge compared with placebo from 30 minutes to 180 minutes after administration.

Nasal Findings

Figures 5, 6 show the changes over time in the swelling of the inferior nasal turbinate mucosa and nasal discharge expressed as the nasal finding score. The nasal finding score for swelling increased for 60 minutes after drug administration for all three study drugs. There were no significant differences among the study drugs.

The nasal finding score for the nasal discharge increased for 60 minutes after the administration of fexofenadine and placebo and was higher than baseline to 300 minutes after administration, while at no time point did epinastine show values above baseline. Epinastine showed significant differences compared with placebo at 30 minutes after administration and with fexofenadine at 30 minutes and 60 minutes after administration.

Nasal Airway Resistance

There were no significant differences among the study drugs.

VTR Recording By Anterior Rhinoscopy

Figure 7 shows examples of intranasal images at each measurement time point before and after nasal provocation.

The swelling of the inferior nasal turbinate mucosa was inhibited from 30 minutes after administration of epinastine and from 180 minutes after administration of fexofenadine.

Histamine Release Rates

The mean±standard error at 30 minutes before and 300 minutes after administration were 53.24 ± 15.67% and 50.16 ± 9.24% for epinastine, 43.00 ± 11.01% and 40.17 ± 10.25% for fexofenadine, and 49.56 ± 14.24% and 35.44 ± 11.06% for placebo, respectively, showing decreases with all three study drugs. There were no significant differences among the study drugs.

SAFETY

None of the subjects experienced any adverse events.

PATIENT-REPORTED EVALUATION

Table 3 shows the subjects' impressions concerning the study drugs after the completion of the clinical study. 3 of 7 subjects given epinastine (42.86%), 2 of 7 subjects given fexofenadine (28.57%) and 0 of 7 subjects given placebo reported that the "study drug was effective."

DISCUSSION

The nasal provocation test exposes a fixed amount of the antigen directly to the nasal mucosa and determines the changes in the extent of nasal allergy symptoms. Thus, it is a simple method for objectively evaluating the efficacy and duration of effect of antiallergic agents.[7,9,11] Usui *et al.* have studied the efficacy of Ketotifen oral agent and nasal agent by the nasal provocation test using house dust antigens.[12] Konno and Yoshida *et al.* have also repeated antigen nasal provocation using the Japanese cedar antigen and have reported that even during non-dispersion seasons, nasal symptoms seen in the field can be reproduced.[13,14] When there is no pollen dispersion, the specific IgE in the nasal mucosa is decreased, but when antigen in an amount sufficient to elicit the development of allergic symptoms is applied, then the disease becomes apparent. In recent years, many studies have also been conducted to observe nasal allergy symptoms and conduct drug efficacy evaluation in pollen (antigen) exposure chambers,[6] but from the standpoint of being able to expose all subjects to a fixed amount of antigen, the antigen provocation test is superior for evaluation of nasal symptoms. On the other hand, in terms of being able to reproduce symptoms of pollinosis during a pollen-dispersion season, exposure tests in pollen exposure chambers are superior to the antigen provocation test. This is because exposure chambers are closer to the exposure to pollen in the field and can elicit symptoms that do not arise in antigen provocation tests, such as symptoms in the eye and throat. However, studies like the present clinical study involving a small number of subjects, particularly studies that include detailed assessments such as rhinoscope examination, require exposure to a fixed amount of antigen. Thus, we adopted the antigen provocation test. In addition, repeated provocation reaction was used to reproduce repeated antigen exposure similar to the repeated ex-

posure in the field occurring during a pollen-dispersion season. In a simple one-time antigen exposure, the pollen is in a non-dispersion state and the allergy reaction that occurs is the pure immediate phase reaction followed by the late phase reaction.[7] However, in the actual clinical setting, since there is repeated exposure to large quantities of dispersed pollen, it may be important to determine the efficacy of drugs under conditions with immediate phase and late phase occurring simultaneously.

It was thought that repeated antigen provocation reactions can lead to better reproduction of the actual pollinosis symptoms, and that increasing the number of exposures may lead to an increase in reactivity. However, the reaction including nasal mucosal swelling after placebo administration increased to a certain point after antigen provocation but showed a trend towards a decrease beyond one hour, and by 5 hours the reactivity was at the level prior to drug administration with the first provocation. This may be because the current clinical study was started in a pollen-free state. Specific IgE in the nasal mucosa begins to increase at the time of the year when the pollen dispersion is starting,[15] and it has been shown in clinical studies in Japanese cedar pollinosis in Japan that at that time of the year, the hypersensitivity increases gradually.[13,14] For this reason, in this clinical study, the hypersensitivity becomes apparent for a short period of time, and thereafter, possibly because of the low level of specific IgE, the decreased reactivity of mast cells results in decreased production and release of histamine and decreased nasal discharge after four or five antigen provocation reactions.

The study drugs used here, epinastine hydrochloride and fexofenadine hydrochloride, are popular second-generation antihistamines in Japan. Fexofenadine hydrochloride in the pollen-dispersion season showed clinical efficacy starting around day 2 of administration, and improved QOL early in treatment.[8] There are no similar data for epinastine hydrochloride, but a rapid response has been reported in a clinical study involving the skin.[16] In this study, a comparison of these two agents and placebo was conducted under conditions close to pollen exposure in the field using repeated antigen provocations. The results indicated that epinastine hydrochloride showed the features characteristic of second-generation antihistamines, in which the number of sneezing attacks and quantity of nasal discharge decreased after antigen provocation at 30 minutes after administration. The single dose administration inhibited these symptoms for at least 3 hours, while the number of sneezing attacks was inhibited for 5 hours. Fexofenadine hydrochloride also showed a trend towards inhibition of these symptoms, but the effect was not significantly different from placebo. There are actual efficacy data for fexofenadine hydrochloride with respect to QOL in pollinosis,[8] and it is expected that the differences would become significant with a larger sample size, but no significant difference was observed in this study, possibly because the dose in this clinical study of once a day differs from the usual dosage used in clinical practice. The anti-histamine effect was not caused by only a single dose, but also by one-day dosage. If we add the second tablets of fexofenadine hydrochloride, the result of 300 minutes would be changed. Further work in the future is needed to determine whether significant differences can be seen using identical experimental methods in a larger number of subjects. On the other hand, it was evident that epinastine hydrochloride showed significant differences, and thus its rapid efficacy and usefulness need to be confirmed.

The observation that neither of the drugs showed significant differences compared with placebo with respect to efficacy measures such as the inferior nasal turbinate mucosal swelling score and nasal airway resistance may be explained by the fact that this study involved a single dose administration. The histamine release assay results may also be explained by this observation.

Based on the results of this clinical study, the efficacy of epinastine hydrochloride in the early phase of pollinosis treatment was demonstrated by the observation that a single dose administration led to the suppression of the nasal mucosal reaction elicited by repeated provocation. One may consider these data to be one line of evidence for pollinosis treatment in the early phase of pollen dispersion or when there is rapid increase in the quantity of pollen dispersed.

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分担研究報告書

各種自動花粉測定器の性能についての検討，舌下免疫療法の有効性の検討，ならびにスギ花粉症発症へのインフルエンザウイルス感染の影響についての検討

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研究要旨

正確、詳細な花粉飛散情報を得るために自動花粉測定器の役割が期待されるが、現在の自動測定器には空中浮遊粒子（SPM）との識別能力に大きな問題がある。KH3000，神栄センサーの問題点を明らかにすると共に神栄センセーの改善を目的に、花粉粒子識別率、土粒子の誤認率から新たな補正式を作り、本式を用いて花粉飛散の検討を行ったところ、ダーラム法との相関が著しく向上し、かつ黄砂の影響も軽減した。一方、2 年間舌下免疫療法を実施している患者の有効性を臨床スコアから評価したが、有意差は明らかではなかった。また、本年度スギ花粉症を初めて発症した患者の背景因子としてインフルエンザウイルス感染についても検討を行ったが、罹患の影響は明らかではなかった。

A. 研究目的

飛散花粉曝露の回避は花粉症患者の基本的治療であり、その重要性では言うまでもない。正確、詳細な花粉飛散情報の提供は有効な曝露の回避に不可欠であり、さらに患者のセルフケアーに役立つことも期待される。近年、一定量の大気を吸引しながら、粒径，形状，偏光度，あるいは花粉の自動蛍光などを利用した花粉を識別して測定するいくつかの自動花粉測定器が開発されている。しかし、昨年の検討で、これらの測定器はいずれも花粉以外の空中浮遊微粒子（SPM）をカウントしてノイズが大きいこと、時に花粉飛散数が少ない時にはダーラム法による測定値と相関が低いことが明らかであった。そこで、本年度は検討を進め、改善法についても検討を行った。

また、2 年間舌下免疫療法を行っている患者の有効性、さらに本年度スギ花粉症を発症した患者の背景因子としてインフルエンザウイルス感染についても検討を行った。

B. 研究方法

1）千葉大学医学部屋上に神栄センサーならびに KH3000 を設置して、ダーラム法による飛散カウントとの比較、さらに降雪の影響、黄砂の影響について検討を行った。また、花粉非飛散時の神栄センサーが SPM を花粉粒子と識別してしまう誤認率を計算し、さらに花粉曝露室内に置いたときの花粉粒子識別率から、花粉識別判別式により花粉と識別された粒子数を計算し、新たな補正式を算出し、本式を用いた花粉飛散の検討を行った。

2）舌下免疫療法の二重盲検試験（大久保班）は平成 16 年から行った患者のうち実薬投与群についてさらに維持量での投与を 1 年間継続して行い、本年の症状について検討を行った。

3）本年千葉県南房総市（旧 丸山町）で丸山小学校 5 年生全員、丸山中学校 1，2 年生全員を対象に、抗体検査、アンケート調査から本年スギ花粉症を発症した生徒のこの 1 年間のインフルエンザウイルス罹患について調査を行った。

（倫理面への配慮）

舌下減感作療法については、千葉大学大学院医学研究院倫理委員会の許可を得て内容について十分な説明を行い、文書による同意を得て行った。また、学童に対するスギ花粉症調査、インフルエンザウイルス感染の罹患についてのアンケート調査は、保護者の文書同意を得て行った。

C. 研究結果

1）自動花粉測定器 KH3000 は降雪、黄砂により影響を受け、カウント数は著しく増加した。一方、神栄センサーは、雪は花粉と誤認しなかったが、黄砂には影響を受けていた。SPM の影響を改善するための補正式，花粉粒子数＝2.1 ×（花粉識別判別式による花粉と識別された粒子数）－0.073 ×（神栄センサーの測定粒子数）を作り、本年殿 2 月～4 月のダーラム法との日毎の測定数との相関を検討した

ところ、補正式により相関係数は0.54から0.91に上昇した。また、黄砂の影響も補正式により著しく改善することが可能であった。

2) 舌下免疫療法2シーズン目の鼻症状スコアを検討すると、全体として低値であった。対照として参考値であるが、本年飛散数期に別の舌下免疫療法でのプラセボ群と比較して低値を呈していたが、有意差は明らかではなかった。

3) 本年度、初めてスギ花粉症を発症した生徒は6名いたがこの1年のインフルエンザウィルス罹患は不明であった。

D. 考察

自動花粉測定器はSPMとの識別に依然として大きな問題が残っている。今回のように、各地域毎のSPMの花粉誤認率を飛散シーズン前に前もって測定し、本検討で用いたような補正式を作成し使用することで精度の向上がはかれる可能性がある。

舌下免疫療法の有効性については、本年のスギ花粉飛散が著しく低値であったことも加わり、また本オープン試験で症例数も少なく有意差は明らかではなかった。スギ花粉症発症へのインフルエンザウイルスの影響についても明らかではなかったが、今後さらに検討対象数を増やして検討する必要がある。

E. 結論

自動花粉測定器はSPMとの識別に依然大きな問題がある。この改善の一つとして地域毎の機種によるSPM誤認率を計算して補正式を作ることで可能性が期待される。スギ花粉症に対する舌下免疫療法の有効性については、多人数が参加した長期の二重盲検試験が必要である。

F. 健康危険情報

なし

G. 研究発表

1論文発表

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2学会発表

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

花粉自動測定器の特徴

	大 和 (KH3000)	NTT	興 和 (KP1000)	神 栄	NTT-神栄
花粉粒子識別手段	・粒径の識別 ・形状(長・短径)の識別	・粒径の識別	・粒径の識別 ・蛍光による識別	・粒径の識別 ・偏光度による識別	・粒径の識別 ・偏光度による識別
砂塵除去手　段	・砂抜き容器により10μ以下の粒子除去	・2段階の分別手段で10μ以下、50μ以上の粒子を分別	・バーチャルインパクターを使用して微粒子を除去	・100μ以上の大粒子フィルターの使用	・100μ以上の大粒子フィルターの使用
吸気吸入量	4.1ℓ／分	30ℓ／分	4ℓ／分	0.9ℓ／分	2.2ℓ／分

問題点：・ノイズが大きい ― 微粒子をカウントしている

・特に花粉飛散が少ない時にダーラム法との相関が低い

(相関係数0.3～0.5)

手法

X ＝ 花粉粒子数
Y ＝ 土粒子数
Z ＝ 測定器が捕捉した全粒子数
α ＝ 花粉粒子識別率
β ＝ 土粒子を花粉粒子と識別する誤認率
γ ＝ 花粉識別判別式により花粉と識別された粒子数

↓ とすると

$Z = X + Y$
$\alpha * (Z - Y) + \beta * Y = \gamma$

が成立する

α ＝ 0.507 （実験値）
β ＝ 0.034 （2006年1月観測値の平均値）

より

$$X = 2.1 * \gamma - 0.073 * Z$$

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

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

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

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

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

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

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

Ann Allergy Asthma Immunol. 2005;94:286–291.

INTRODUCTION

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

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

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MATERIALS AND METHODS

Patients

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

Tissue Samples

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

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

Antibodies

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

Flow Cytometric Analysis

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

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

Statistical Analysis

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

RESULTS

Patients

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

Dot Plots for CD19 FITC and CD23 RPE

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

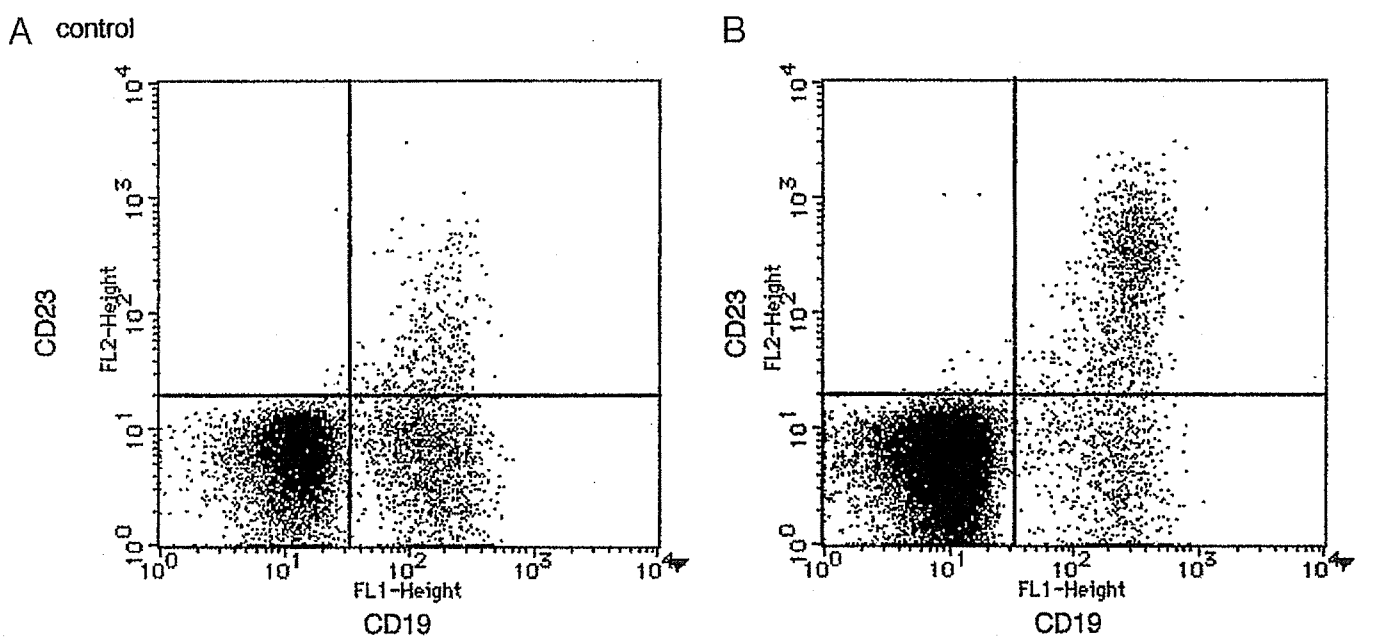

Figure 1. Representative dot plots for fluorescein isothiocyanate–conjugated anti-CD19 (FL1) and R-phycoerythrin–conjugated anti-CD23 (FL2) antibodies for the control group (A) and the allergic rhinitis (AR) group (B). The proportion of CD23-bearing B cells was measured by flow cytometry as CD19 and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

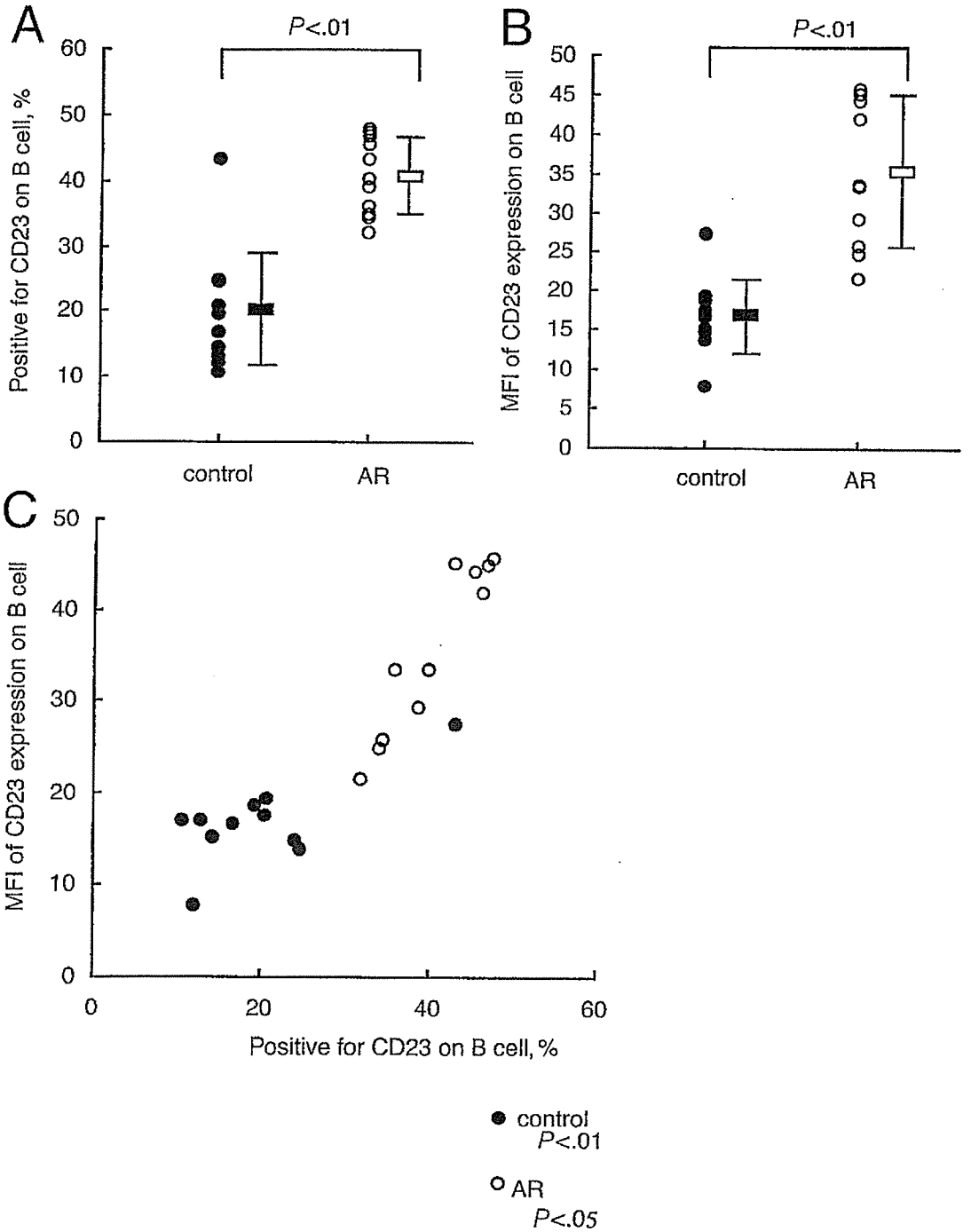

Figure 2. CD23 expression on B cells. A, Percent positive values for CD23 on B cells from patients with allergic rhinitis (AR) were significantly higher than for the control group. B, A similar tendency was seen in the mean fluorescence intensity (MFI) of CD23 expression on B cells, which was significantly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

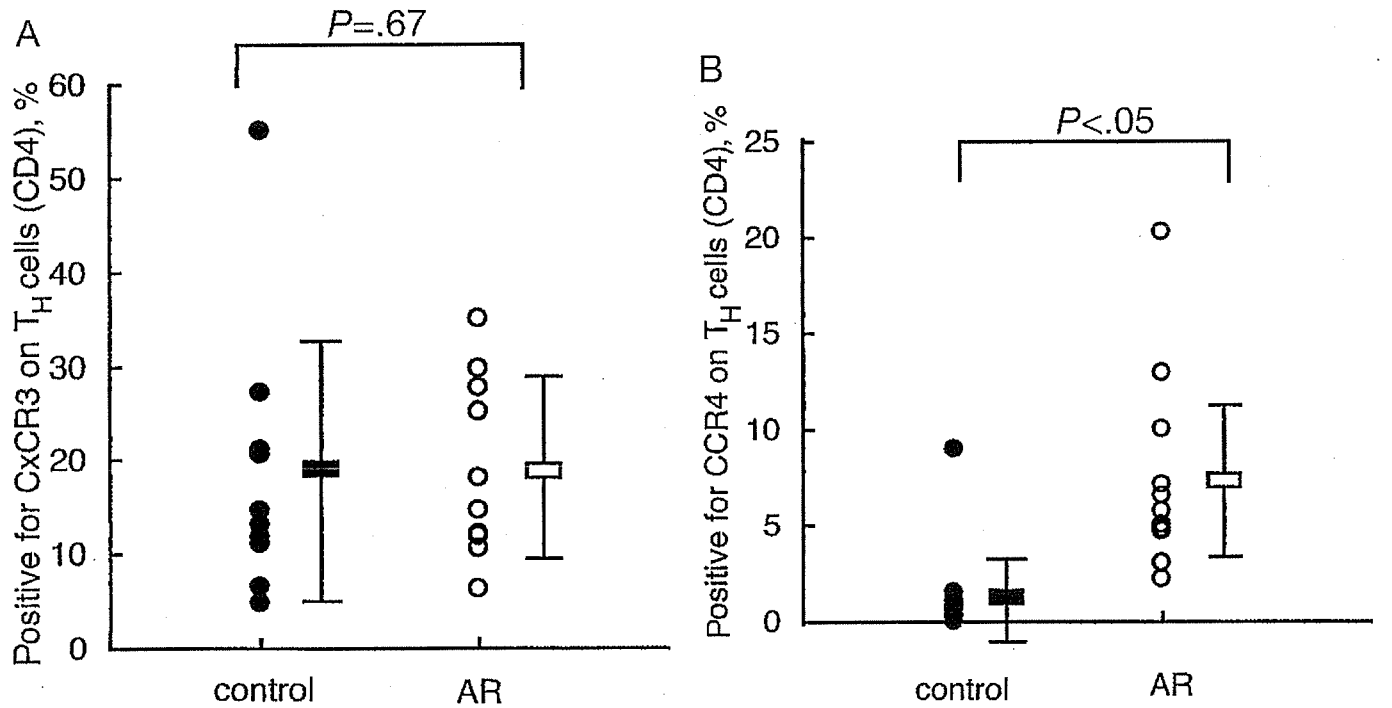

Figure 3. Percent positive values for chemokine receptor expression on mucosal CD4 T cells. A, Data for the CXCR3 subtype, assumed to be T_H1 cells. B, Data for the CCR4 subtype, assumed to be T_H2 cells. There is no significant difference in percent positive values for CXCR3 on CD4 cells from patients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

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

Expression of CD23 on Mucosal B Cells

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

Expression of Chemokine Receptors on Mucosal CD4 Cells

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

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

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

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

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

DISCUSSION

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

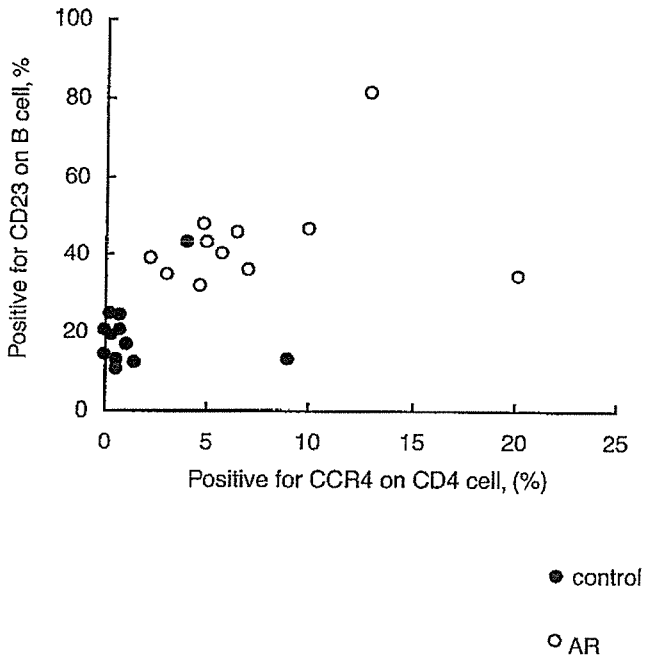

Figure 4. Correlation plot for percent positive values for CD23 on mucosal B cells and percent positive values for CCR4 on mucosal CD4 cells. There is no significant correlation between these data in the control group (P = .25) or the allergic rhinitis (AR) group (P = .17).

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

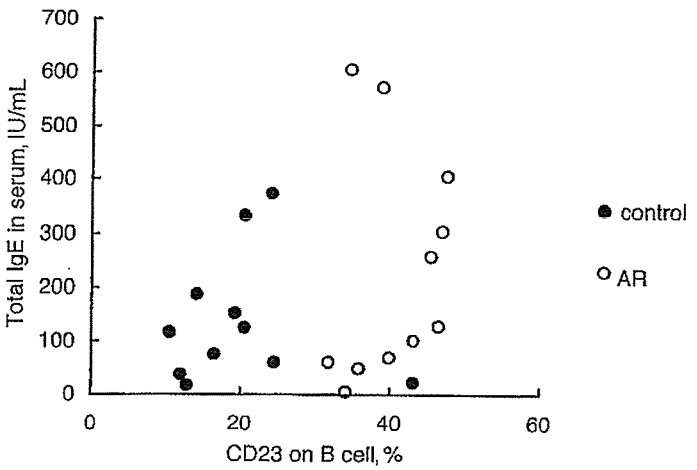

Figure 5. Correlation plot for percent positive values for CD23 on mucosal B cells and the total IgE level. There is no significant correlation between these data in the control group (P = .21) or the allergic rhinitis (AR) group (P = .66).

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

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

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

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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).

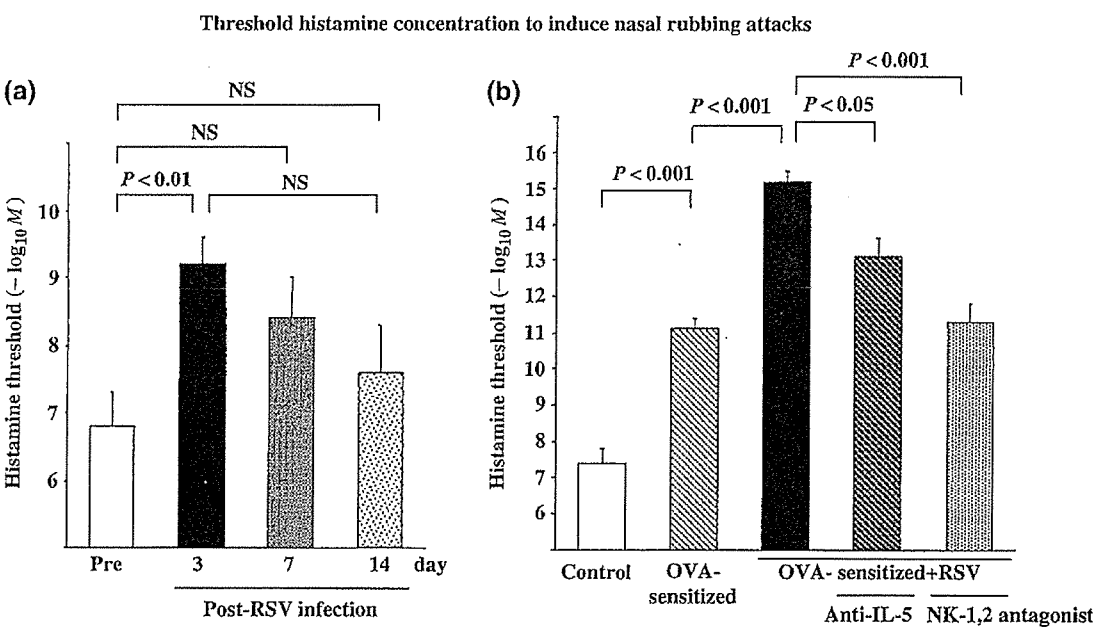

Fig. 1. Threshold histamine concentration needed to induce nasal rubbing attacks in respiratory syncytial virus (RSV)-infected non-sensitized mice (a) and in ovalbumin (OVA)-sensitized mice (b). After RSV inoculation, the threshold decreased transiently and reached its lowest on day 4. Although the threshold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