

Monitoring of seasonal variability in bronchial hyper-responsiveness and sputum cell counts in non-asthmatic subjects with rhinitis and effect of specific immunotherapy

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Summary

Bronchial hyper-responsiveness (BHR) is documented in a proportion of non-asthmatic individuals with allergic rhinitis (NAAR) and reflects inflammatory events in the lower airways. Natural exposure to allergens is known to modulate BHR and the level of airway inflammation in asthma, but less consistently in NAAR. Specific immunotherapy (SIT) attenuates symptoms possibly by reducing BHR and airway inflammation. The influence of natural exposure to *Parietaria* pollen on BHR and sputum cell counts of NAAR was investigated and the effect of *Parietaria* SIT examined. Thirty NAAR, monosensitized to *Parietaria judaica*, participated in a randomized, double-blind, placebo-controlled, parallel group study of the effects of a *Parietaria* pollen vaccine on symptoms/medication score, BHR to inhaled methacholine and adenosine 5'-monophosphate (AMP), and cell counts in the sputum collected out of and during the pollen seasons for 36 months. Seasonal variation in BHR to inhaled methacholine and AMP and changes in sputum cell counts were documented. Changes were consistent for AMP, but not methacholine, and invariably associated with modifications in sputum eosinophils and epithelial cells. The clinical efficacy of *Parietaria* SIT was associated with a decline in the seasonal deterioration of BHR to AMP, whereas no significant effect was observed on BHR to methacholine or sputum cell differentials. Between-groups comparison of the seasonal changes in PC₁₅ methacholine values and sputum cell differentials calculated as the AUC were not statistically significant, whereas a significant difference in PC₁₅ AMP was demonstrated throughout the study ($P = 0.029$), the median (inter-quartile range) AUC values being 2478.5 (1153.3–3600.0) and 1545.5 (755.3–1797.9) for the SIT- and placebo-treated group, respectively. Bronchial airways of NAAR exhibit features of active inflammation that deteriorate during natural allergen exposure, particularly with regard to BHR to AMP. The clinical efficacy of *Parietaria* SIT was exclusively associated with attenuation in seasonal worsening of PC₁₅ AMP, suggesting that AMP may be useful in monitoring changes in allergic inflammation of the airways.

Key words adenosine challenge, bronchial hyper-responsiveness, induced sputum, *Parietaria* allergy, specific immunotherapy, seasonal allergic rhinitis

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Introduction

Allergic rhinitis is a common IgE-mediated inflammatory condition of the upper airways, which is often associated with several co-morbidities that include allergic conjunctivitis, sinus disease, otitis media, and asthma [1]. Asthma and rhinitis share a common genetic background [2], and are characterized by a similar inflammatory process [3]. Allergic rhinitis may also predispose for the development of asthma and primarily in those with evidence of bronchial hyper-responsiveness (BHR) [4–7]. The occurrence of non-specific BHR in a proportion of non-asthmatic individuals with allergic rhinitis appears to reflect sub-clinical inflammatory

processes in the lower airways [8, 9]. We have recently demonstrated that such patients, at the peak of their pollen season, have increased eosinophilic counts and high levels of eosinophilic cationic protein (ECP) in the sputum [10].

Little is known about the critical factors that determine and influence non-specific BHR and inflammatory changes within the lower airways in subjects with allergic rhinitis, but it is likely that, as for bronchial asthma, eosinophil cell products, allergen exposure, and the resulting shedding of the bronchial epithelium may have an important pathogenetic role. Natural exposure to aeroallergens is likely to modulate the degree of non-specific BHR and the level of airway inflammation. Although the literature has repeatedly shown seasonal changes in non-specific BHR and airway inflammation in allergic asthma during the pollen season, the data appear to be less consistent in subjects with allergic rhinitis [11–13]. The studies that failed to identify a seasonal pattern in the changes

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of non-specific BHR were either too small or had other design limitations. It is also possible that enhancement of BHR during natural allergen exposure is more strongly associated with specific characteristics of some inhalant allergen type compared to others. In contrast to most natural environmental allergens, *Parietaria* pollen has strong allergenic properties, which often reaches exceptionally high peak levels during season [14]. *Parietaria* pollen, with its long persistence in the atmosphere (*Parietaria* pollen season in Sicily ranges from February to October), appears to be the major cause of respiratory allergy in the Mediterranean area and particularly in Eastern Sicily, where the study was carried out. In addition, *Parietaria* pollen is more important than *Olea* and *Graminaceae* in causing BHR and increase in blood eosinophilia during pollen season in subjects with allergic rhinitis [15].

Allergen-specific immunotherapy (SIT) is a widely used form of therapy for allergic conditions. Although the evidence of its effectiveness in asthma remains controversial [16], its efficacy in reducing the severity of symptoms related to allergic rhino-conjunctivitis is well established [17, 18]. In patients with seasonal allergic rhinitis, grass pollen immunotherapy results in long-term clinical benefit for at least 3 years after discontinuation of treatment [19] and appears to be effective in preventing progression to asthma [7]. The reasons for these beneficial effects are not known, but one explanation rests in the potential of SIT to attenuate BHR and airway inflammation.

The aim of the present study was to document the influence of variation in natural exposure to *Parietaria* pollen on non-specific BHR to methacholine and adenosine 5'-monophosphate (AMP), and cell counts in the sputum of non-asthmatic subjects with rhinitis over a 36-month study period. We have recently shown that the bronchial epithelial cell count in the sputum of allergic asthmatics is sensitive to topical corticosteroids, indicating that it is a useful surrogate marker of epithelial damage [20]. Epithelial cells in the sputum have never been investigated in non-asthmatic patients with allergic rhinitis, and their shedding may be in relation to the level of non-specific BHR; therefore, it was sensible to evaluate bronchial epithelial cell counts in their sputum as well. To exclude interference of exposure to other aeroallergens, we only selected patients with an isolated allergy to *Parietaria* pollen. This restriction of the inclusion criteria is critical to the whole investigation because it provides a clean immuno-inflammatory model to verify study objectives. In addition, the effect of treatment with *Parietaria* SIT on the study outcome measures was evaluated in a randomized, placebo-controlled manner throughout the study.

Methods

Patients characteristics

Thirty non-smoking patients (15 females and 15 males; mean age: 33 years, age range: 20–54 years) with a documented history of seasonal allergic rhinitis were recruited from the allergy clinic of our institution (Istituto Malattie Apparato Respiratorio, University of Catania, Sicily) (Table 1). These patients were selected on the basis of positive skin prick tests

Table 1. Clinical characteristics of participants at inclusion

	<i>Parietaria</i> SIT	Placebo	P-value
No. of subjects included	15	15	
Sex (male : female ratio)	10 : 5	5 : 10	
Age (years)	32 (21–54)	34 (20–53)	NS
Duration of rhinitis (years)	7.8 (4–14)	8.2 (4–16)	NS
Mean FEV ₁ (% predicted $\pm$ SEM)	92.5 ($\pm$ 3.2)	96.0 ($\pm$ 4.1)	NS
PC ₁₅ FEV ₁ Methacholine (mg/mL)	8.74 (0.17–16.00)	7.47 (0.26–16.00)	NS
PC ₁₅ FEV ₁ AMP (mg/mL)	442.3 (52.6–800.0)	460.5 (87.2–800.0)	NS
% Eosinophils in sputum	3.0 (0.5–6.5)	4.5 (1.5–5.0)	NS
% Epithelial cells in sputum	3.0 (1.5–4.0)	2.5 (1.0–3.5)	NS

Age is expressed as mean (range). Percentage of eosinophils and epithelial cells in sputum is expressed as median (inter-quartile range). PC₁₅FEV₁ methacholine and PC₁₅FEV₁ AMP values are expressed as geometric mean (range). AMP – adenosine 5'-monophosphate.

(SPTs) (weal > 3 mm) to *Parietaria judaica* and negative tests to a panel of 21 common aeroallergens (including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass, trees, dog hair, cat, *Olea europea*, *Aspergillus fumigatus*). Exclusion criteria were a past or present history of asthma, previous asthma symptoms or asthma medication intake, and/or abnormal spirometric values. Asthma was defined as recurrence of at least two of the following three symptoms: breathlessness, cough, and wheeze. None of the patients studied had ever received specific immunotherapy. The local Ethics Committee approved the study, and written informed consent was obtained from each patient.

Study design

The study was performed out of and during the pollen season when the subjects were symptomatic. It took the form of a randomized double-blind, placebo-controlled, parallel group study investigating the effects of SIT with high-dose standardized *P. judaica* extract on cell counts in the sputum, airway responsiveness to inhaled methacholine and AMP, and symptoms/medication scores in rhinitic patients allergic to *Parietaria*.

Upon entry of patients into the study (December 1997 – out of *Parietaria* pollen season), a detailed history was taken and physical examination, spirometry, and bronchial challenges with methacholine and AMP were carried out (Fig. 1). Sputum induction was performed at least 1 week later.

Patients were randomized to receive either active treatment, consisting of increasing doses of a standardized extract of *P. judaica* (Alutard SQ, ALK-Abellò, Milan, Italy) given subcutaneously, followed by monthly maintenance treatment, or placebo (0.01 mg/mL of histamine acid phosphate in allergen diluent – phosphate-buffered saline (PBS)). A maintenance dose of 80 000 standard quality units (SQ-U) was achieved in all participants. Each 0.8-mL maintenance injection of 80 000 SQ-U was equivalent to 8000 biological units and contained 4.8 µg of the major allergen Par J 1.

From March to July 1998 and 1999 (throughout two consecutive *Parietaria* pollen seasons), participants recorded daily symptoms and rescue medication on diary cards. Bronchial challenges with methacholine and AMP, and

	OUT of season Dec-97	IN season June-98	OUT of season Dec-98	IN season June-99	OUT of season Dec-99
Case history	+				
Physical examination	+				
SPT	+				
Spirometry + BPT	+	+	+	+	+
Sputum induction	+	+	+	+	+
Diary cards					
SIT/Placebo					

Fig. 1. Design of the study. At inclusion (December 1997 – out of *Parietaria* pollen season), case history was taken, physical examination completed, SPT to a panel of 21 common aeroallergens performed, and bronchoprovocation tests (BPT) with inhaled methacholine and AMP carried out. Challenges were performed at least within 5 days of each other and always in the same order (methacholine challenge first). Sputum induction was performed at least 1 week later. Patients were randomized into treatment groups. The participants recorded daily symptoms and rescue medication on diary cards throughout two consecutive *Parietaria* pollen seasons. BPT and sputum induction were repeated throughout the study during the peak and outside pollen seasons.

sputum induction were repeated during the peak and outside the *Parietaria* pollen seasons in 1998 and 1999 (Fig. 1). Relief medications for symptoms of rhinitis were withheld for at least 3 days before each study visit.

Pollen counts

A Burkard 7-day volumetric spore trap (Burkard, Rickmansworth, UK) placed on the exposed roof of the hospital building at a height of approximately 20 m was used to collect pollen grains. *Parietaria* pollen were measured daily and expressed as grains per cubic metre of air. Weekly average counts were calculated for the whole duration of the study. During this study, *Parietaria* pollen count in Catania had its highest peaks in March 1998 and April 1999. The pollen counts for *Parietaria* were comparable in 1998 and 1999, and the area under the curve (AUC) showed a slightly higher (8.4%) value in the season of 1999.

Symptoms/medication scores

The patients gave an overall assessment of their rhinitis symptoms during the pollen season. Symptoms of nasal blockage, nasal itching, sneezing, rhinorrhoea, and eye streaming/itching were rated on a four-point scale (0 = no symptoms, 1 = mild, 2 = moderate; and 3 = severe) and computed daily. Respiratory symptoms (breathlessness, cough, wheeze) were also recorded if present, and scored as either 0 (absent) or 1 (present). Daily medication scores were totalled for the allowed rescue treatments: each actuation ($\times 2$ nostrils) of oxymetazoline and each inhalation (2 puffs) of salbutamol was scored 1; each 10-mg loratadine tablet was scored 2. During the course of the study, the subjects were not allowed to take nasal or oral corticosteroids. In order to maintain the blindness of the study protocol, the physicians who consulted the participants were not involved in the data analyses.

Bronchoprovocation testing with inhaled methacholine and AMP

BHR was evaluated by methacholine and AMP bronchial challenge, as described previously [21]. In brief, methacholine (Lofarma, Milan, Italy) and AMP (Sigma Chemical Co., St Louis, MO, USA) were dissolved in PBS (pH 7.4) and normal saline, respectively, to produce increasing doubling concentrations (0.125–16 mg/mL for methacholine; 12.5–800 mg/mL for AMP) and immediately used for bronchial challenge. The solutions were administered as aerosols generated from a starting volume of 3 mL in a disposable Inspiron Minineb (C.R. Bard International, Sunderland, UK) driven by compressed air at 8 L/min. The patients inhaled the aerosolized solutions in five breaths from functional residual capacity to total lung capacity via a mouthpiece. The patients were trained to reach total lung capacity in 3 s. After five breaths of diluent's solution, the patients inhaled increasing doubling concentrations of agonist. Forced expiratory volume in 1 s (FEV_1) was measured at 1 and 3 min after administration of each concentration of agonist and the better of two values recorded for analysis. The challenges were stopped when a decrease of 15% in FEV_1 had been achieved or when the maximum concentration of agonist had been inhaled. The bronchial responses to the inhaled agonists were expressed as the provocative concentration causing a 15% decline in FEV_1 (PC_{15}), which was calculated by linear interpolation from the concentration–response curve constructed on a logarithmic scale by plotting the percentage change in FEV_1 from the post-diluent value against the cumulative concentration of agonist administered.

Sputum Induction and Processing

Induction was performed according to our previously published method [10]. Briefly, the participants inhaled hypertonic saline (4.5%) aerosolized by an ultrasonic

nebulizer (UltraNeb 99, DeVilbiss, Feltham, Middlesex, UK) with output set at 3 mL/min. The subjects wore a nose clip and quietly inhaled aerosol for up to five consecutive 5-min periods until an adequate volume of sputum was collected.

Sputum plugs were transferred into 50 mL polypropylene tubes (Becton Dickinson, Abingdon, UK), weighed, and an equal weight of 0.01 M dithioerythritol (DTE; Fluka, Gillingham, Dorset, UK) solution added to solubilize the mucus. The specimens were then vortexed for 10 s, rocked for 30 min at room temperature, and again vortexed for 10 s. They were then filtered through a 70- μ m strainer (Becton Dickinson) and the collected fluid centrifuged at 400 *g* for 10 min at 4 °C. Cell pellets were resuspended in 1 mL of PBS without Ca^{2+} and Mg^{2+} and viable cells counted in a haemocytometer. Only samples in which squamous cells comprised <30% of total cells were considered satisfactory for analysis. Differential counting was carried out on cytopspins stained with May–Grunwald–Giemsa on 600 cells (excluding squamous cells). However, for epithelial cells immunochemical staining, separate cytopspins were fixed in cold methanol for 10 min and incubated with a mouse anti-human mAb, which recognizes a number of cytokeratin proteins, including CK5, CK6, CK8, CK17, CK18, and CK19 (pan-anti-CK Ab by Dako, Wycombe, UK). Binding was detected by using peroxidase-conjugated secondary antibodies and visualized by using 0.5 mg/mL diaminobenzidine in PBS containing 0.01% H_2O_2 . All the experiments included control slides lacking primary antibody, as well as an isotype-matched antibody. Slides were coded and examined by one investigator and counts expressed as a percentage of the number of total cells and as absolute counts.

Statistical analyses

All analyses were performed with the Statistical Package for Social Science (SPSS, Chicago, IL, USA) for Windows version 10.0. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

Symptoms and medication scores were summed up together for the 8 weeks corresponding to the peak pollen counts for each season (1998 and 1999) and expressed as AUC. AUC data and sputum variables were not normally distributed and expressed as medians (inter-quartile ranges).

PC_{15} values were logarithmically transformed to normalize their distribution and expressed as geometric mean (range). However, a 15% fall in FEV_1 could not be obtained in a number of participants (non-responsive subjects) at the highest concentration of agonist administered, and a conservative estimate was obtained by calculating the cumulative PC_{15} at the highest concentration administered. Because of these censored data, PC_{15} results were compared for significance using non-parametric statistical analysis.

Seasonal variability in % cells in the sputum and PC_{15} values within treatment groups was investigated by Friedman's two-way ANOVA test, as their summary measures were not normally distributed. Where Friedman's test indicated there were significant differences between time points, for the pre-planned comparisons of interest, Wilcoxon's signed rank test for paired data was performed. To assess differences in sputum variables and PC_{15} values over time between treatment groups, the AUC for each treatment period was

calculated by plotting all individual PC values over time (a total of five PC values for each patient for each treatment period) and compared using Mann–Whitney *U*-test.

Results

Effects of SIT on symptoms/medication scores

Patient groups were comparable with respect to age, duration of rhinitis, airway responsiveness to methacholine and AMP, sputum eosinophilia, and epithelial cell counts in the sputum (Table 1). The treatment was well tolerated, and no systemic reactions occurred throughout the study.

No significant difference in scores was observed between groups after 1 year of SIT; median (inter-quartile ranges) scores were 150 (88–222) and 175 (90–246) in the placebo and SIT groups, respectively. However, by the end of the study, an important difference in scores was observed between the groups; the median (inter-quartile range) score of 319 (212–430) in the placebo group was significantly higher when compared to the score of 136 (35–284) in the SIT group ($P < 0.05$).

Effects of SIT on seasonal variation in PC_{15} methacholine and PC_{15} AMP

All the patients completed their follow-up visits until completion of the study (December 1999); we obtained PC values for both methacholine and AMP on all occasions. We reported seasonal variation of BHR in this group of non-asthmatic subjects with rhinitis and an isolated sensitization to *Parietaria* pollen. Although seasonal differences in PC_{15} methacholine values were observed throughout the study in both treatment groups (Figs 2a and b), significant changes were consistently observed only for the data obtained in June 1999 for the placebo-treated group (for details of the statistical comparisons, see Table 2).

Between-groups comparisons throughout the study showed no significant difference in PC_{15} methacholine at any time point; their geometric mean (range) values were 7.47 (0.26–16) vs. 8.74 (0.17–16) mg/mL (placebo vs. SIT – Dec 1997), 5.21 (1.03–16) vs. 6.36 (0.26–16) mg/mL (placebo vs. SIT – June 1998), 7.00 (1.31–16) vs. 10.56 (1.05–16) mg/mL (placebo vs. SIT – Dec 1998), 3.86 (0.23–16) vs. 8.01 (1.02–16) mg/mL (placebo vs. SIT – June 1999), and 9.42 (2.44–16) vs. 12.09 (3.27–16) mg/mL (placebo vs. SIT – Dec 1999), (Figs 2a and b). Similarly, when seasonal changes in PC_{15} methacholine values over time were expressed as the AUC, no statistically significant difference was observed between treatment groups ($P = 0.082$), the median (inter-quartile range) AUC values being 642.8 (436.8–720.0) and 357.6 (221.9–680.2) for the SIT- and placebo-treated group respectively.

Significant seasonal differences in PC_{15} AMP values were reported throughout the study in both treatment groups (Figs 3a and b) and were consistently observed for most of the dataset with the exception of the comparison between June 1998 vs. Dec 1999 for the placebo-treated group (for details of the statistical comparisons, see Table 2).

Between-groups comparisons throughout the study showed a significant difference in PC_{15} AMP at two out of five time points, the geometric mean (range) values being 97.1 (24.1–415.7)

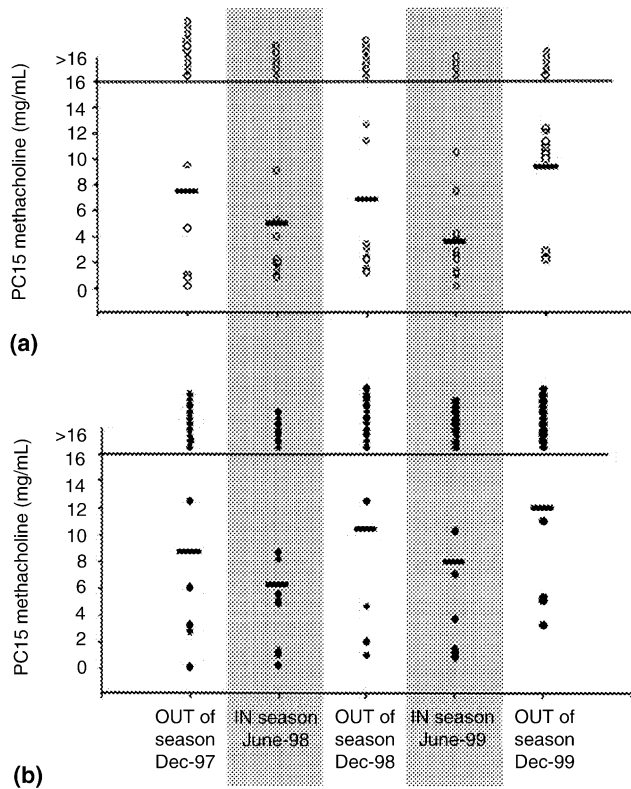


Fig. 2. Individual methacholine PC_{15} values in the placebo-treated (a – open circles) and SIT-treated (b – closed circles) group during and outside *Parietaria* pollen seasons. The geometric mean values are shown as horizontal bars. Negative methacholine responders are represented as circles above the 16 mg/mL line.

vs. 217.7 (7.2–800.0) mg/mL ($P < 0.05$; placebo vs. SIT – June 1999), and 299.5 (77.7–800.0) vs. 568.8 (211.0–800.0) mg/mL ($P < 0.01$; placebo vs. SIT – Dec 1999) (Figs 3a and b). Moreover, between-groups comparison of the seasonal changes in PC_{15} AMP values over time calculated as the AUC was also statistically significant ($P = 0.029$), the median (inter-quartile range) AUC values being 2478.5 (1153.3–3600.0) and 1545.5 (755.3–1797.9) for the SIT- and placebo-treated group, respectively.

Effects of SIT on seasonal variation in cell counts in the sputum

Sputum samples were obtained on all but three occasions (all in 1999). In addition to the change in BHR, seasonal variations were also reported for the number of eosinophil and epithelial cells in the sputum. Statistically significant seasonal differences in % eosinophils counts were observed throughout the study in both treatment groups (Figs 4a and b) and were consistently reported for each time point (for details of the statistical comparisons, see Table 2). During the peak of both *Parietaria* pollen seasons, median (inter-quartile range) % eosinophil counts in the sputum increased up to 10.0 (3.0–28.0)% and 10.5 (3.5–30.0)% respectively, during June 1998 and June 1999 in the placebo-treated group (Fig. 4a). A similar rise in sputum eosinophils was also recorded

Table 2. Statistical comparisons of the changes in PC_{15} values and % cell counts in the sputum observed throughout the study within treatment groups

Comparisons for		P-values (by Wilcoxon signed rank test)	
		Placebo group	Immunotherapy group
<i>PC₁₅ methacholine values</i>			
Dec 1997	vs. June 1998	NS	0.045
	vs. Dec 1998	NS	NS
	vs. June 1999	0.021	NS
	vs. Dec 1999	NS	NS
June 1998	vs. Dec 1998	0.049	0.017
	vs. June 1999	NS	NS
	vs. Dec 1999	NS	NS
Dec 1998	vs. June 1999	0.024	0.028
	vs. Dec 1999	NS	NS
	vs. Dec 1999	0.019	NS
<i>PC₁₅ AMP values</i>			
Dec 1997	vs. June 1998	0.009	0.005
	vs. Dec 1998	NS	NS
	vs. June 1999	0.001	0.047
	vs. Dec 1999	0.023	NS
June 1998	vs. Dec 1998	0.046	0.007
	vs. June 1999	0.012	NS
	vs. Dec 1999	NS	0.016
Dec 1998	vs. June 1999	0.001	0.028
	vs. Dec 1999	NS	NS
	vs. Dec 1999	0.003	0.026
<i>% eosinophils count</i>			
Dec 1997	vs. June 1998	0.018	0.006
	vs. Dec 1998	NS	NS
	vs. June 1999	0.002	0.018
	vs. Dec 1999	NS	NS
June 1998	vs. Dec 1998	0.004	0.003
	vs. June 1999	NS	NS
	vs. Dec 1999	0.004	0.003
Dec 1998	vs. June 1999	0.001	0.005
	vs. Dec 1999	NS	NS
	vs. Dec 1999	0.002	0.003
<i>% epithelial cells count</i>			
Dec 1997	vs. June 1998	0.016	NS
	vs. Dec 1998	NS	NS
	vs. June 1999	0.012	0.003
June 1998	vs. Dec 1998	0.001	0.018
	vs. June 1999	NS	0.018
	vs. Dec 1999	0.015	NS
Dec 1998	vs. June 1999	0.005	0.001
	vs. Dec 1999	NS	NS
	vs. Dec 1999	0.035	0.003

NS = non-significant.

during June 1998 and June 1999 in the SIT-treated group (Fig. 4b).

Between-groups comparisons throughout the study showed no significant difference in % sputum eosinophils at any time point; their median (inter-quartile range) counts were 4.5 (1.5–5.0) vs. 3.0 (0.5–6.3)% (placebo vs. SIT – Dec 1997), 10.0 (3.0–28.0) vs. 12.0 (3.5–26.0)% (placebo vs. SIT – June 1998), 1.5 (0.5–5.5) vs. 2.0 (1.0–3.2)% (placebo vs. SIT – Dec 1998), 10.5 (3.5–30.0) vs. 10.8 (1.0–49.0)% (placebo vs. SIT – June 1999), and 1.5 (0.0–6.0) vs. 2.0 (1.0–4.0)% (placebo vs. SIT –

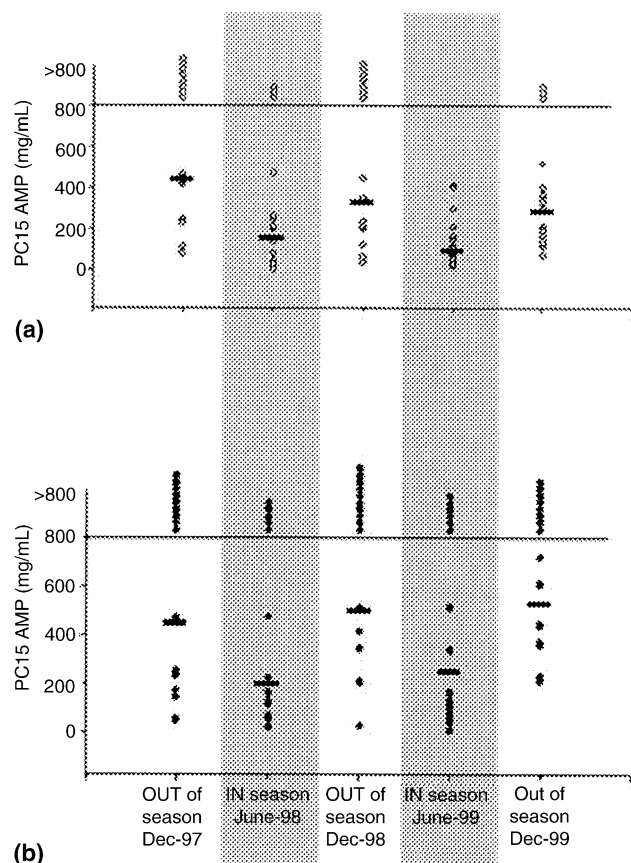


Fig. 3. Individual AMP PC₁₅ values in the placebo-treated (a – open circles) and SIT-treated (b – closed circles) group during and outside *Parietaria* pollen seasons. The geometric mean values are shown as horizontal bars. Negative AMP responders are represented as circles above the 800 mg/mL line.

Dec 1999), (Figs 4a and b). Similarly, when seasonal changes in % eosinophil counts over time were expressed as the AUC, no statistically significant differences were observed between treatment groups ($P = 0.84$), the median (inter-quartile range) AUC values being 277.0 (83.7–1351.3) and 330.8 (107.8–852.5) for the SIT- and placebo-treated group, respectively.

We also reported statistically significant seasonal differences in % epithelial cell counts throughout the study in both treatment groups (Figs 5a and b), which were consistently observed for each time point for the placebo-treated group (for details of the statistical comparisons, see Table 2). During the peak of both *Parietaria* pollen seasons, median (inter-quartile range) % epithelial cell counts in the sputum increased up to 4.5 (2.0–5.0)% and 5.0 (4.0–8.5)%, respectively, during June 1998 and June 1999 in the placebo-treated group (Fig. 5a). A similar rise in sputum epithelial cells was also recorded during June 1998 and June 1999 in the SIT-treated group (Fig. 5b).

Between-groups comparisons throughout the study showed no significant difference in % sputum epithelial cells at any time point; their median (inter-quartile range) counts were 2.5 (1.0–3.5) vs. 3.0 (1.5–4.0)% (placebo vs. SIT – Dec 1997), 4.5 (2.0–5.0) vs. 6.5 (1.0–9.0)% (placebo vs. SIT – June 1998), 2.0 (0.5–5.0) vs. 2.0 (1.0–3.5)% (placebo vs. SIT – Dec 1998), 5.0 (4.0–8.5) vs. 8.0 (3.5–14.0)% (placebo vs. SIT –

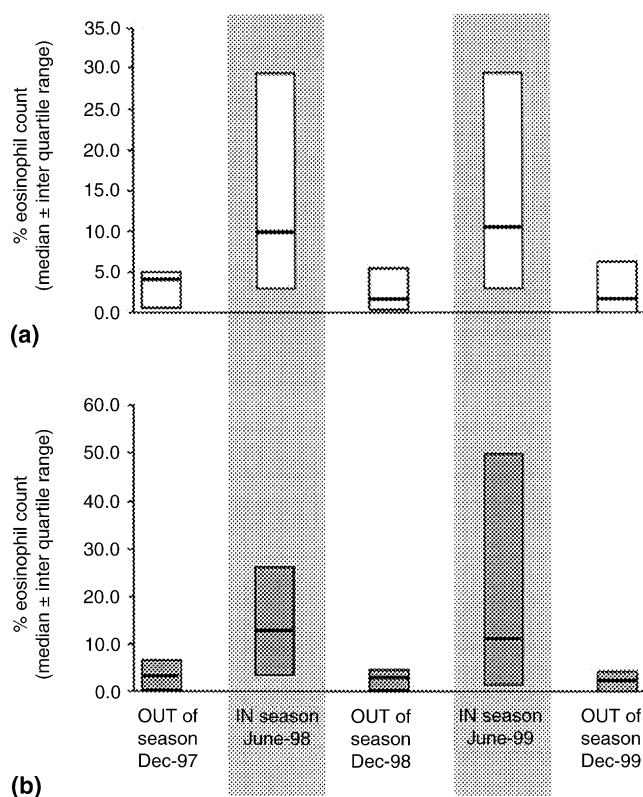


Fig. 4. Changes in median ($\pm$ inter-quartile range) % eosinophil count in the placebo-treated (a – open boxes) and SIT-treated (b – shaded boxes) group during and outside *Parietaria* pollen seasons. The medians are shown as horizontal bars.

June 1999), and 2.0 (0.5–3.5) vs. 2.5 (2.0–5.0)% (placebo vs. SIT – Dec 1999) (Figs 5a and b). Similarly, when seasonal changes in % epithelial cell counts over time were expressed as the AUC, no statistically significant differences were observed between treatment groups ($P = 0.27$), the median (inter-quartile range) AUC values being 218.0 (95.0–255.3) and 130.8 (105.2–195.5) for the SIT- and placebo-treated group, respectively.

Discussion

Prospective monitoring of seasonal changes in airway responsiveness indicates for the first time that these were consistent for AMP but not methacholine and invariably associated with modifications in sputum eosinophils and epithelial cells. It was also observed that the clinical efficacy of *Parietaria* SIT is associated with a decline in the seasonal deterioration of BHR to inhaled AMP, whereas no significant effect was observed on BHR to methacholine or eosinophil and epithelial cell counts in the sputum.

In asthma, natural exposure to aeroallergens is known to be invariably associated with non-specific BHR and airway inflammation. However, seasonal changes of BHR appear to be less consistent in subjects with allergic rhinitis alone. Gerblach et al. [11], the first to focus on seasonal variation in airway reactivity in patients with allergic rhinitis, found that sensitivity to methacholine did not change significantly in a small group of eight patients. Madonini et al. [12] performed

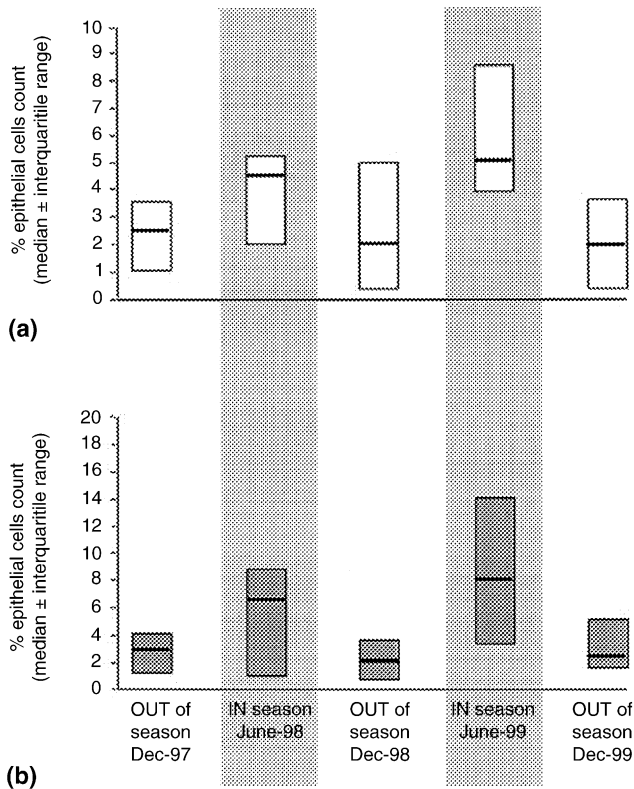


Fig. 5. Changes in median ($\pm$ interquartile range) % epithelial cell count in the placebo-treated (a – open boxes) and SIT-treated (b – shaded boxes) group during and outside *Parietaria* pollen seasons. The medians are shown as horizontal bars.

carbachol inhalation challenge in 27 subjects with allergic rhinitis and demonstrated that a positive response in three patients out of season increased significantly to 13 during the pollen season. In eight pollen allergic subjects without asthma, Boulet et al. [13] showed that the mean methacholine PC_{20} fell from 170 mg/mL prior to the pollen season to 136 mg/mL during the pollen season, returning to pre-seasonal levels after the pollen season; this difference was not statistically different. In our study, although prospective monitoring of changes in airway responsiveness to inhaled methacholine indicates that seasonal differences in PC_{15} methacholine were present throughout the study, significant changes were observed only for the data obtained in June 1999. This could be due to the fact that pollen counts for *Parietaria* were slightly higher (8.4%) in the season of 1999. In contrast, significant changes in airway responsiveness to inhaled AMP were consistently documented throughout the study, and particularly during June 1999 when all participants in the placebo group became hyper-responsive to AMP (see Fig. 3a). The reason for the superior sensitivity of AMP airway responsiveness to methacholine in detecting seasonal variation in non-specific BHR is not known, but may be explained by the seasonal increase in the number and function of airway mast cells [22], which are known to be crucially involved in the airway response to inhaled AMP through their degranulation [23, 24]. In addition, the exquisite responsiveness of AMP to changes in allergic inflammation of the airways [25] is likely to better detect inflammatory modifications associated with the onset of the pollen season.

Little is known about the critical factors that determine and influence seasonal changes in BHR in non-asthmatic subjects with allergic rhinitis. One plausible possibility is that allergen-driven BHR variations may result from IgE-mediated inflammatory changes of the lower airways. Indeed, the occurrence of BHR in a proportion of non-asthmatic individuals with allergic rhinitis appear to reflect sub-clinical inflammatory processes in the lower airways, as indicated by the increase in the sputum eosinophils of such patients at the peak of their pollen season [10]. In the present paper, we have documented important seasonal modifications in the inflammatory response of the lower airways in non-asthmatic subjects with allergic rhinitis in the course of natural exposure to *Parietaria* pollen. In addition to the change in BHR, seasonal variations for the number of eosinophils and epithelial cells in the sputum were consistently observed throughout the 36-month study period in both treatment groups.

Although airway eosinophilic inflammation is normally associated with the presence of BHR in asthma [26], very little is known about the inflammatory features in the bronchial airways of subjects with allergic rhinitis and no evidence of asthma. Bronchoalveolar lavage (BAL) and bronchial biopsy studies have shown that subjects with allergic rhinitis may exhibit inflammatory features in the lower airways with eosinophils being frequently observed [8,9]. Moreover, the occurrence of non-specific BHR in non-asthmatic individuals with allergic rhinitis, is distinctly associated with increased eosinophil counts and high levels of eosinophil cationic protein (ECP) in the sputum [10]. In line with these observations, our study proves that a significant elevation in the number of sputum eosinophils is invariably present in the course of the pollen season when changes in the level of BHR occur. It may be speculated that exposure of the bronchial epithelium to aeroallergens may promote airway eosinophilia through IgE-dependent pathways. In support of this view, Chakir et al. [27] have shown by immunohistochemistry that natural pollen exposure is associated with an increase in eosinophil numbers and IL-5 expression in the bronchial mucosa of non-asthmatic subjects with allergic rhinitis. The release of IL-5 in the bronchial mucosa of these subjects may contribute to the increase in airway eosinophilia. Allergens may also alter epithelial cell function and integrity directly through the induction of a stress response via stimulation of proteinase-activated receptors [28] or indirectly by injurious secretory products (such as major basic protein – MBP) released from activated airway eosinophils [29]. This damage results in a selective loss of columnar epithelial cells from their attachment to basal cells, which appears to be well correlated with non-specific BHR in asthma [30]. The present finding that bronchial epithelial cells count in the sputum of non-asthmatic subjects with rhinitis is invariably increased during the peak of *Parietaria* pollen season, has never been previously reported and indicates that epithelial shedding induced by the proteolytic activity of *Parietaria* pollen occurs in allergic individuals even in the absence of clinical asthma. In support of this, Piacentini et al. [31] have recently demonstrated that a period of antigen avoidance at high altitude (in the Italian Alps) significantly reduces the percentage of cytokeratin-positive epithelial cells in the sputum of children with allergic asthma.

The evidence of the present study, that SIT is beneficial in preventing the severity of symptoms related to seasonal allergy due to *Parietaria* pollen in allergic rhinitic patients, is in accordance with the results from previous randomized controlled trials [32, 33]. Although SIT is effective in controlling seasonal symptoms/medication scores, this was not associated with a decline in bronchial responsiveness to methacholine or with significant modifications in the number of eosinophils and/or epithelial cells in the sputum. The observed lack of effect of *Parietaria* SIT against methacholine airway responsiveness and sputum eosinophil counts confirms earlier findings by other investigators [34, 35], who also failed to detect a significant effect of *Parietaria* SIT on non-specific BHR, eosinophil numbers, and ECP values in patients with seasonal asthma and rhinitis. However, our findings on BHR and sputum eosinophils are somewhat at variance with previous studies on mite and grass pollen allergy [36–39]. The reasons for this are not known, but may be related to different approaches in the statistical analyses (parametric vs. non-parametric tests) or to specific characteristics of the inhalant allergen type. In contrast to mite allergens, *Parietaria* pollen has very strong allergenic properties and reaches very high peak levels during season [14]. Therefore, *Parietaria*-sensitive patients are likely to be exposed to very high allergen levels, and this high allergenic load may counterbalance the protective effect of SIT against airway responsiveness to methacholine and sputum eosinophilia. In contrast, a marker that is more sensitive to changes in allergic inflammation of the airways is likely to better detect the immunological effects of SIT. Airway responsiveness to AMP may represent such a marker, as indicated by the selective protective effect of *Parietaria* SIT against BHR to inhaled AMP.

The results of our study indicate that SIT can prevent seasonal deterioration of airway responsiveness to inhaled AMP in subjects with allergic rhinitis who do not have symptoms of asthma. Whereas all participants in the placebo group became hyper-responsive to AMP, no new cases of AMP hyper-responsiveness were observed in the SIT-treated group (rather, one patient developed hyposensitiveness to AMP – compare Figs 3a and b). The reason for the selective protective effect of *Parietaria* SIT on AMP airway responsiveness is not known. This may be explained by a reduction in the number and function of intermediate effector cells crucially involved in the airway response to inhaled AMP. AMP airway responses are known to be mediated through mast cell degranulation following the activation of specific cell-surface purinoreceptors [23, 24]. Recent studies on mechanisms of immunotherapy have demonstrated that changes in pathophysiological mechanisms responsible for mediator release from mast cells are associated with successful immunotherapy. In particular, important reductions in the number of mast cells in the nose and skin after SIT in patients who are sensitive to mites and grass pollens have been reported [40, 41].

In conclusion, we have shown that bronchial airways of non-asthmatic subjects with allergic rhinitis exhibit features of active inflammatory response characterized by BHR, recruitment of eosinophils, and epithelial cell shedding. This inflammatory process increased in association with natural allergen exposure, particularly with regard to BHR to AMP,

which was invariably associated with changes in eosinophils and epithelial cells in the sputum. It was also observed that the clinical efficacy of *Parietaria* SIT is exclusively associated with the decline in seasonal deterioration of BHR to inhaled AMP. Taken together, these findings indicate that AMP is useful in monitoring the inflammatory modifications associated with the onset of the pollen season and the progress of immunotherapy. Although the demonstration of ongoing bronchial inflammation in non-asthmatic subjects with rhinitis might point to an increased risk for development of asthma, further studies will be necessary to evaluate its full clinical and physiological consequences.

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