

ORIGINAL ARTICLE

Allergen inhalation challenge in smoking compared with non-smoking asthmatic subjects

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Summary

Background Smoking asthmatics experience more severe symptoms, require more rescue medication and have more asthma-related hospitalizations than non-smoking asthmatics. However, studies in mice suggest that mainstream cigarette smoke may reduce airway inflammation and may attenuate airway hyperresponsiveness. A comparison of allergen-induced airway inflammatory responses of smoking and non-smoking atopic asthmatics has not been examined previously.

Objectives To determine whether allergen-induced airway responses and inflammatory profiles are attenuated in smoking when compared with non-smoking mild allergic asthmatic subjects.

Methods Allergen inhalation challenges were performed in 13 smoking and 19 non-smoking mild allergic asthmatic subjects. The forced expired volume in 1 s (FEV₁) was measured up to 7 h after allergen inhalation. Methacholine airway responsiveness was measured before and at 24 h after allergen and sputum was induced before and at 7 and 24 h after allergen.

Results Both the smoking and non-smoking groups developed similar allergen-induced falls in FEV₁ during the early and late asthmatic responses and similar increases in allergen-induced airway eosinophils. The mean maximum fall in FEV₁ during the late response was $16.3 \pm 4.3\%$ in non-smokers and $12.9 \pm 7.2\%$ in smokers. The smoking asthmatics, however, did not develop allergen-induced methacholine airway hyperresponsiveness, whereas the non-smoking controls developed a 1.18 doubling dose shift in methacholine PC₂₀ ($P < 0.05$). **Conclusions and Clinical Relevance** Mild allergic asthmatic subjects, who were current smokers with a mean 6-year pack history, develop allergen-induced eosinophilic airway inflammation and late responses, similar in magnitude to non-smoking asthmatics, but do not develop methacholine airway hyperresponsiveness associated with the allergen-induced airway eosinophilia.

Keywords airway hyperresponsiveness, allergic asthma, cigarette smoking, eosinophils, methacholine, neutrophils

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Introduction

Smoking rates in the asthmatic population are similar to rates in the general population, with 17–35% of asthmatics being current smokers, and 22–43% of the asthmatic population ex-smokers in the United States and the United Kingdom [1, 2]. Compared with asthmatics who have never smoked, asthmatic smokers have more severe symptoms [2], lower health status [3], and require rescue medication far more often than their non-smoking counterparts [3]. In addition, smoking asthmatics are admitted

to hospitals with asthma-related exacerbations at a greater rate than non-smoking asthmatics [2]. The severity of asthma symptoms and frequency of asthma exacerbations exhibit a dose-response effect in terms of cigarettes smoked, with heavy smokers suffering more than moderate or light smokers [4].

Studies in mice have suggested that second-hand cigarette smoke enhances allergen-induced airway inflammation, and facilitates allergic sensitization [5, 6]. In addition, one study using mainstream cigarette smoke provided similar information [7]; however, other studies

using mainstream cigarette smoke have provided contradictory evidence. For example, mainstream cigarette smoke has been described to attenuate ovalbumin-induced bronchoconstriction and airway hyperresponsiveness, to reduce eosinophil numbers in bronchoalveolar lavage (BAL) fluid in sensitized mice [8] and also to increase production of interleukin (IL)-13 [9], IL-10 [9], IL-5 [10] and IgE [10], but reduces allergen-induced eosinophilic inflammation [9, 10]. Most recently, Thatcher *et al.* [11] suggested that the attenuation of allergic inflammation is dose dependent, with high-dose mainstream smoke suppressing inflammation and low-dose mainstream smoke having no effect. This suppression occurred through the inhibition of T cell activity, causing reductions in BAL and tissue eosinophils, inhibition of T helper type 2 (Th2) cytokines IL-5 and IL-4 and a reduction in goblet cell metaplasia [11].

Despite the research performed in mouse models of allergic asthma, the effect that cigarette smoke has on allergen-induced airway responses in allergic asthmatics has not yet been studied. The purpose of this study was to compare and contrast allergen-induced airway responses and airway inflammation in non-smoking allergic asthmatic subjects to allergen-induced responses in smoking allergic asthmatic subjects. We hypothesized that, on the basis of the evidence from the effects of mainstream smoking in sensitized mice, current cigarette smoking would attenuate allergen-induced airway eosinophilia and the associated late asthmatic response and methacholine airway hyperresponsiveness.

Methods

Study design

Allergen inhalation challenge was performed to examine the magnitude of allergen-induced early and late asthmatic responses, allergen-induced methacholine airway hyperresponsiveness and increases in sputum eosinophils and neutrophils in smoking and non-smoking mild asthmatics. At screening, methacholine inhalation challenge and skin prick testing (SPT) were performed, followed by sputum induction. In addition, a urine sample was obtained to measure urinary cotinine. Subjects returned the following day for allergen inhalation challenge. At 7 h after challenge, subjects underwent sputum induction and blood collection. Finally, at 24 h after challenge, subjects underwent a methacholine challenge and sputum induction.

Subjects

Two groups of subjects were enrolled into the study. All subjects had a history of mild allergic asthma, were not on regular treatment for asthma and had an FEV₁ $\geq$ 70% of predicted normal, and a methacholine PC₂₀ value at baseline $\leq$ 32 mg/mL. The determination of atopy was based

on one or more positive skin responses to 19 common aeroallergens. One group ($n = 19$) was lifetime non-smokers and the second group ($n = 13$), was current cigarette smokers by history. Subjects in both groups had known allergic asthma, but had not previously experienced an allergen inhalation challenge. All subjects gave written informed consent, and this study was approved by the Hamilton Health Science Research Ethics Board.

Short-acting inhaled β_2 -agonists were not allowed within 8 h, caffeine containing products within 12 h and alcohol within 48 h of each study visit. No restrictions were placed on the smoker's smoking pattern. Long-acting inhaled β_2 -agonists were not allowed within 2 weeks of enrolment, while inhaled or intranasal corticosteroids, anti-leukotrienes, anti-histamines, cromoglycate or other anti-allergic therapy could not be taken within 6 weeks of enrolment. The only asthma medication allowed during the study was short-acting inhaled β_2 -agonists.

Skin prick tests

SPTs were conducted on the inner forearm. A total of 19 allergens, commercially prepared by Omega Inc. (Montreal, Quebec, Canada), were tested. Both positive (histamine) and negative (buffer and glycerol) controls were also used. After 10 min, the length and width of the weal of any response was measured. A weal greater than 2 $\times$ 2 mm was considered to be positive. The allergen chosen for inhalation challenge was the extract yielding the largest measured weal. On a second skin test, the selected allergen was serially diluted and applied to the forearm in the same manner as the original SPT. The lowest concentration of allergen that yielded a 2 $\times$ 2 mm weal was used to calculate the starting dose for inhalation of allergen, as described previously [12].

Methacholine challenge

Methacholine challenges were performed according to methods described previously [13]. Briefly, subjects first inhaled saline, followed by increasing concentrations of methacholine for 2-minute durations. The starting dose of methacholine was 0.03 mg/mL, and FEV₁ was measured at 30, 90, 180 and 300 s following inhalation. Subsequent doses were doubled until at least a 20% fall in FEV₁ was obtained when compared with the lowest FEV₁ value post-saline. The results were expressed as the provocative concentration of methacholine causing a 20% fall in FEV₁ (methacholine PC₂₀). The allergen-induced measurement of methacholine PC₂₀ was not made in one smoking subject and in two non-smoking subjects.

Allergen inhalation challenges

Allergen inhalation was performed as described previously [14]. The concentration of allergen required to

achieve a 20% decrease in FEV₁ was predicted using the methacholine PC₂₀ and the titration of allergen determined from the SPT [12]. The early bronchoconstrictor response (EAR) was recorded as the greatest fall in FEV₁ between 0 and 3 h, while the late bronchoconstrictor response (LAR) was recorded as the greatest drop in FEV₁ between 3 and 7 h after allergen inhalation. An EAR was defined as a fall in FEV₁ $\geq$ 20%, while an LAR was defined as a fall in FEV₁ $\geq$ 15%.

Sputum induction and processing

Sputum was induced before, 7 and 24 h following challenge using 7 min inhalations of 3%, 4% and 5% hypertonic saline as described previously [15]. Sputum was separated from saliva, treated with 0.1% dithiothreitol and processed as described elsewhere [15]. The total number of sputum cells and the percentage of eosinophils and neutrophils were measured.

Urinary cotinine

Urinary cotinine levels were measured using the COT One-Step Cotinine Test (California Biotest, Oceanside, CA, USA), which is a lateral flow chromatographic immunoassay that detects cotinine in urine samples at a cut-off concentration of 200 ng/mL. Three drops of urine (approximately 100 μ L) were transferred to the specimen well of the test device. After 5 min, the results were determined by the presence or absence of a coloured line.

Statistical analysis

All summary statistics are expressed as the mean $\pm$ 0.95 confidence intervals, with the exception of methacholine PC₂₀, which was expressed as geometric mean and range. All PC₂₀ values were log base 2 transformed before analysis. Statistical analysis was performed using SPSS version 8.0. The effect of challenge and smoking were determined using a repeated measures analysis of variance (ANOVA). The study was sufficiently powered to find at least a 30% attenuation of the allergen-induced airway eosinophilia between the groups, if such a difference existed [16]. Significance was assumed at $P < 0.05$.

Results

Subject characteristics

Thirty-two atopic, mild asthmatics, aged between 18 and 60 years were enrolled (Table 1). There were 19 non-smoking subjects and 13 smoking subjects. There were no differences between groups in baseline FEV₁ measurements, methacholine PC₂₀ and baseline eosinophil percentages (Table 1). The non-smokers had no history of

cigarette smoking and tested negative for the presence of urinary cotinine, while the smokers had an average smoking history of 6.0 pack years, and all tested positive for urinary cotinine.

Allergen-induced airway responses

In the non-smoking subjects, the mean maximum fall in FEV₁ between 0 and 3 h after inhaled allergen was $33.9 \pm 3.5\%$, while in the smoking subjects it was $31.8 \pm 4.5\%$ (Fig. 1). In the non-smoking subjects, the mean maximum fall in FEV between 3 and 7 h after inhaled allergen was $16.3 \pm 4.2\%$, while in the smoking subjects it was $12.9 \pm 7.2\%$ (Fig. 1). There were no significant differences between the groups in the magnitude of either the EAR or LAR.

Allergen-induced methacholine airway hyperresponsiveness

The geometric mean methacholine PC₂₀ values were similar between the two groups at baseline (Table 1). The non-smoker subjects exhibited significantly greater allergen-induced methacholine airway hyperresponsiveness than did the smokers. The non-smokers had an allergen-induced 1.18 ± 0.53 doubling dose concentration change in the methacholine PC₂₀, while in the smokers there was an allergen-induced 0.16 ± 0.56 doubling dose change ($P < 0.05$) (Fig. 2).

Allergen-induced changes in sputum inflammatory cells

Allergen challenge increased the total cell counts in sputum in both non-smoking and smoking subjects. The cell numbers were $4.0 \times 10^6 \pm 1.7 \times 10^6$ /g pre-allergen, $4.4 \times 10^6 \pm 1.4 \times 10^6$ /g at 7 h and $8.3 \times 10^6 \pm 3.7 \times 10^6$ /g at 24 h in the non-smokers, and $1.8 \times 10^6 \pm 0.5 \times 10^6$ /g pre-allergen, $2.6 \times 10^6 \pm 1.2 \times 10^6$ /g at 7 h and $2.9 \times 10^6 \pm 2.0 \times 10^6$ /g at 24 h in the smokers. Both groups had a significant increase in percent sputum eosinophils at 7 and 24 h after allergen. Sputum eosinophils in non-smokers increased from $3.2 \pm 3.0\%$ at baseline to $24.2 \pm 10.6\%$ at 7 h and $18.2 \pm 9.5\%$ at 24 h post-allergen ($P < 0.05$), while sputum eosinophils in smokers increased from $7.6 \pm 8.7\%$ at baseline to $23.9 \pm 15.7\%$ at 7 h and $29.0 \pm 16.3\%$ at 24 h post-allergen ($P < 0.05$). There was no significant difference in allergen-induced sputum eosinophils between the two groups (Fig. 3). Smoking subjects had a significantly higher baseline percentage of sputum neutrophils being $56.7 \pm 13.6\%$, when compared with the non-smoking subjects, which was $40.7 \pm 13.8\%$ ($P < 0.05$) (Fig. 4). Sputum neutrophils increased slightly, but not significantly, in the non-smokers at 7 h after allergen to $49.9 \pm 14.1\%$ and at 24 h to $44.4 \pm 15.2\%$, while neutrophil percentages did not change at 7 or 24 h in the

Table 1. Subject characteristics

	Gender	Age	Predicted FEV ₁ (%)	Methacholine PC ₂₀ (mg/mL)	Allergen extract inhaled	Cumulative allergen cose (BAU)	Cigarette pack years
Non-smokers							
NS01	M	22	74	0.3	Cat	18	0
NS02	F	50	96	1.0	HDM	114	0
NS03	M	20	107	10.0	HDM	457	0
NS04	F	19	95	0.1	Cat	79	0
NS05	M	21	110	1.8	Cat	152	0
NS06	M	18	76	9.5	Ragweed	611	0
NS07	M	57	79	12.3	HDM	3778	0
NS08	M	19	93	2.9	Ragweed	305	0
NS09	F	21	86	12.0	HDM	427	0
NS10	F	20	82	0.6	Cat	71	0
NS11	M	21	75	0.2	HDM	118	0
NS12	F	19	100	3.1	Cat	630	0
NS13	F	52	96	0.2	Cat	157	0
NS14	F	18	92	9.8	HDM	3778	0
NS15	M	33	81	3.6	HDM	213	0
NS16	F	19	78	5.7	HDM	213	0
NS17	F	20	91	4.7	Cat	1259	0
NS18	M	19	93	19.4	Grass	5038	0
NS19	M	58	89	19.6	Cat	315	0
Mean	10M/9F	27.7	89.1	2.54*		658.7	0.0
Confidence 95%		6.5	4.7	Range 0.1–19.6		511.1	0.0
Smokers							
S01	M	20	101	1.4	Ragweed	36	1.2
S02	M	27	82	11.7	Ragweed	30	0.8
S03	M	26	105	8.0	Cat	71	5.0
S04	F	21	100	2.6	Ragweed	66	0.8
S05	M	57	93	32.0	Ragweed	488	18.0
S06	F	49	80	5.0	HDM	1889	35.0
S07	M	24	110	32.0	Ragweed	569	1.2
S08	F	27	92	4.3	Ragweed	1219	9.8
S09	M	21	96	6.5	Ragweed	61	0.5
S10	M	24	84	1.5	Cat	2.5	1.1
S11	M	20	84	3.7	Ragweed	18	1.6
S12	M	23	83	3.3	Cat	305	2.5
S13	M	20	75	1.1	Cat	4	1.0
Mean	10M/3F	27.6	91.2	4.90*		366.0	6.0
Confidence 95%		6.3	5.8	Range 1.1–32		313.1	5.5

*Geometric means.

HDM, house dust mite; FEV₁, forced expired volume in 1 s.

smokers, being $56.3 \pm 17.4\%$, and $44.4 \pm 15.8\%$, respectively (Fig. 4).

Discussion

This study has demonstrated that both non-smoking and smoking allergic asthmatic subjects had significant and similar allergen-induced early and LAR and similar increases in sputum eosinophils at 7 and 24 h after allergen challenge. Sputum neutrophil counts were higher at baseline in the smokers. However, despite the similarity in the allergen-induced eosinophilic inflammatory response, the smoking asthmatics had significantly less allergen-in-

duced methacholine airway hyperresponsiveness when compared with the non-smoking group.

Smoking asthmatics have more severe asthmatic symptoms and more frequent hospitalizations due to asthma exacerbations than non-smoking asthmatics [1, 2]. In addition, inhaled corticosteroids (ICS), which are an effective treatment for the majority of asthmatics, are less effective in smoking asthmatics [1, 17]. These studies have suggested that the combination of cigarette smoking and asthma may promote more severe airway inflammation than asthma alone. This suggestion is supported by some of the mouse studies, which have evaluated the interaction between allergen-induced airway eosinophilia and

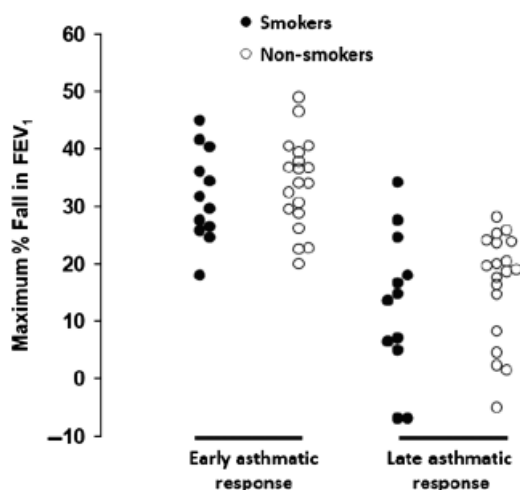


Fig. 1. The maximum % fall in forced expired volume in 1 s (FEV_1) in smoking and non-smoking asthmatic subjects during early (0–3 h) and late (3–7 h) bronchoconstrictor responses to allergen challenge. Both groups had a significant fall in FEV_1 during the early and late responses, but there were no significant differences between the groups.

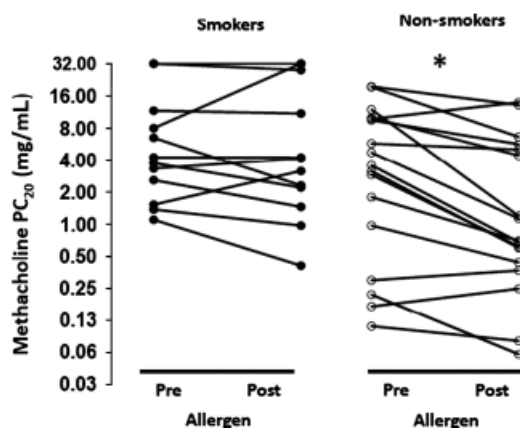


Fig. 2. The methacholine PC_{20} values measured before and 24 h after allergen challenge. Non-smoking asthmatics had a significant decline in the methacholine PC_{20} post allergen challenge, while this did not occur in the smoking asthmatics. The differences between the groups was statistically significant (* $P < 0.05$).

second-hand cigarette exposure [5–7]. Other studies, using mainstream smoke exposure, have provided opposite results [8, 9, 11]. Until now, there have been no studies of this interaction in asthmatic subjects after allergen challenge.

The subjects were selected to be mild atopic asthmatic subjects, who had not previously undergone allergen inhalation challenge. The smoking subjects were matched for atopy, baseline FEV_1 and methacholine airway responsiveness, and these baseline subject characteristics were similar in the two study groups. Furthermore, the mean allergen-induced EAR was similar between the groups; thus, the only important difference between the two

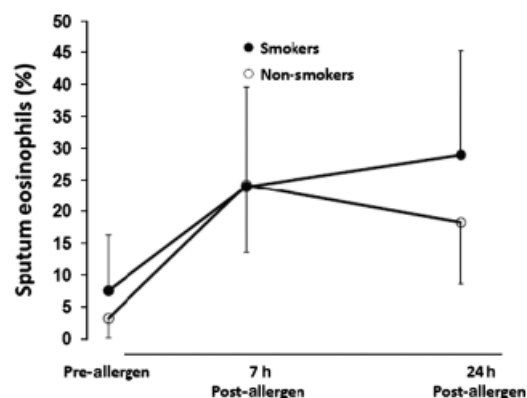


Fig. 3. The percentage of sputum eosinophils (95% confidence intervals) in smokers and non-smokers before and after allergen challenge. There was a significant increase in eosinophil percentages at 7 and 24 h compared with pre-allergen ($P < 0.05$), but no difference between the groups of smoking and non-smoking asthmatics.

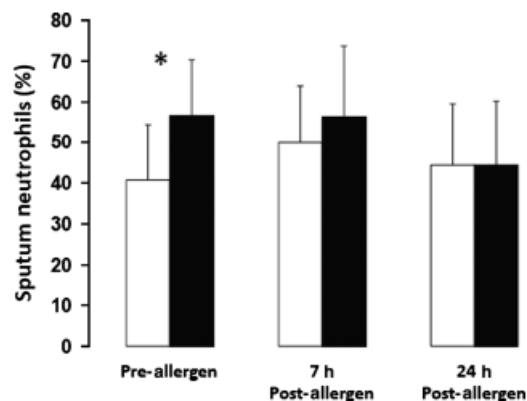


Fig. 4. The percentage of sputum neutrophils (95% confidence intervals) in smokers (closed bars) and non-smokers (open bars) before and after allergen challenge. Smokers had a significantly higher number of neutrophils at baseline when compared with non-smokers (* $P < 0.05$).

groups was whether individuals smoked or not. Although all smokers were current smokers, their pack year history of smoking was relatively short, and this was necessary in an effort to avoid the confounding of a difference in baseline FEV_1 between the groups. A variety of allergens were selected to induce airway responses in these subjects. The smokers had a higher number of subjects who were challenged with ragweed allergen (eight out of 13), when compared with the non-smokers (two out of 19), where cat allergen was the most common allergen used. In all previous studies conducted in our laboratory, the choice of allergen inhaled during the challenge, *per se*, has not been an important determinant of whether a subject will develop dual responses, airway inflammation and airway hyperresponsiveness. In the present study, this was confirmed as the mean allergen-induced LAR, and the degree of eosinophilic airway inflammation was similar in the two groups. In addition, the mean inhaled cumulative

allergen dose was lower in the smokers when compared with the non-smokers. The dose of inhaled allergen needed to induce an EAR in an individual subject depends on the degree of sensitization to the allergen and the baseline methacholine PC₂₀ [12].

Despite the fact that the magnitude of the allergen-induced change in the EAR and airway eosinophils, and its time course, was similar in the two groups, the smokers did not develop a significant allergen-induced change in methacholine PC₂₀. This was an unexpected finding, whose clinical implications are unclear. As mentioned above, the mean inhaled cumulative allergen dose was lower in the smokers; however, we have not previously identified a relationship between the cumulative allergen dose in groups of subjects and the magnitude of the allergen-induced change in methacholine PC₂₀, once the magnitude of the LAR was similar (as occurred in the present study). Hence, we do not believe that this explains the lack of allergen-induced change in the methacholine PC₂₀. Allergen-induced airway hyperresponsiveness generally lasts for between 3 and 7 days after allergen inhalation [18], as is believed to be part of the reason that allergen inhalation causes a persistence of symptoms for days after the acute responses have resolved. This study suggests that this prolonged effect of allergen exposure would be less likely to occur in allergic smoking asthmatics. This question would need to be addressed in another study, where symptoms and rescue β_2 -agonist use are formally measured for several days after allergen inhalation, in smoking and non-smoking allergic asthmatic subjects.

Carbon monoxide (CO) is one compound that may play a role in modulating airway hyperresponsiveness. Mice who inhaled low doses of CO had an attenuation of methacholine airway hyperresponsiveness both in the presence and absence of airway inflammation [19]. This effect was mediated by CO-induced increases of intracellular cGMP and relaxation of airway smooth muscle. Thus, it is possible that CO contributed to the absence of allergen-induced airway hyperresponsiveness observed in the smoking group. Unfortunately, exhaled CO was not measured as a part of this study.

We had originally hypothesized that the smoking group would have an attenuated allergen-induced inflammatory response, based on the murine studies of the effects of mainstream cigarette smoke on allergen-induced airway eosinophilia. Two previous studies [8, 9] had examined the effects of mainstream smoke and had reported a significant decrease in the number of allergen-induced airway eosinophils. A more recent study [11] examined the effects of a high dose and low dose (20% of the high dose) of cigarette smoke. The high dose of cigarette smoke had a suppressive effect on inflammation through the reduction in eosinophils and Th2-type cytokines, IL-4 and IL-5; however, the low dose of cigarette smoke showed no difference in eosinophil numbers between smoke exposed

allergic mice and naïve allergic mice, suggesting that observed differences of cigarette smoke on allergic airway inflammation are dose dependent. The smokers in our study had a low number of mean pack years. Thus, it is possible that if our population of smokers were all heavy smokers, our results may have been different.

Nicotine acts on many different receptors, and has a multiplicity of effects when inhaled. Nicotine is known to down-regulate the adaptive immune response to allergens [20]; however, the lack of difference between the smoking and non-smoking groups in relation to the allergen-induced inflammatory responses makes it unlikely this effect of nicotine was responsible for the attenuation of allergen-induced airway hyperresponsiveness. The smoking subjects had a significantly higher number of sputum neutrophils at baseline than did the non-smokers. An allergen-induced change in sputum neutrophils is often seen in non-smoking mild asthmatics [21], but the lack of change in the smoking asthmatics was an unexpected finding.

The upper or lower airway responses to allergen inhalation have been demonstrated to be enhanced by simultaneous exposure to other pro-inflammatory stimuli, such as ozone [22, 23] and diesel exhaust particles [24] in human subjects. It is therefore somewhat surprising that cigarette smoke does not induce a similar enhanced response to inhaled allergens. This may relate to the many different compounds in cigarette smoke, many of which are pro-inflammatory, but other having anti-inflammatory effects [20].

This study has a number of limitations. These include the recruitment of current smokers, but with a relatively short pack year history of smoking. This was performed in an attempt to match the two groups as closely as possible and to minimize the differences in baseline lung function between them. It is possible that smokers with a heavier daily habit, or a longer pack year history may have provided different results. In addition, the levels of carboxyhaemoglobin were not measured in the smoking subjects, so it is not possible to estimate the exposure to CO at the time of the study. Moreover, the design of the study required the smoking asthmatics to be compared with a control group, which did not have the same allergen extracts or magnitude of airway responses to inhaled allergens during the LAR. A more robust design, although ethically unacceptable, would be to study the non-smoking subjects before and during a period of cigarette smoking. However, it may be possible to study subjects when smoking and then after a period of intensive effort to quit.

In conclusion, this study has demonstrated that smoking mild allergic asthmatic subjects develop allergen-induced eosinophilic airway inflammation and LAR, which is similar in magnitude to non-smoking subjects; however, the smoking subjects do not develop methacholine airway hyperresponsiveness associated with the

allergen-induced airway eosinophilia. This suggests that smoking asthmatic subjects would not develop prolonged allergen-induced asthma symptoms, which persist for several days after allergen inhalation, as can be experienced by non-smoking allergic asthmatics.

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was completed by Z Meghji and G Gauvreau. The protocol was developed by Z Meghji, G Gauvreau and PM O'Byrne. The paper was written by Z Meghji, PM O'Byrne and G Gauvreau. All authors reviewed and approved the manuscript before submission.

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