Markers of airway inflammation and airway hyperresponsiveness in patients with well-controlled asthma

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ABSTRACT: In steroid-naïve asthmatics, airway hyperresponsiveness correlates with noninvasive markers of airway inflammation. Whether this is also true in steroid-treated asthmatics, is unknown.

In 31 stable asthmatics (mean age 45.4 yrs, range 22–69; 17 females) taking a median dose of 1,000 μg inhaled corticosteroids (ICS) per day (range 100–3,600 $μg·day^{-1}$), airway responsiveness to the "direct" agent histamine and to the "indirect" agent mannitol, lung function (forced expiratory volume in one second ($FEV1$), forced vital capacity (FVC), peak expiratory flow (PEF)), exhaled nitric oxide (eNO), and number of inflammatory cells in induced sputum as a percentage of total cell count were measured.

Of the 31 subjects, 16 were hyperresponsive to mannitol and 11 to histamine. The dose-response ratio (DRR: % fall in $FEV1$/cumulative dose) to both challenge tests was correlated (r=0.59, p=0.0004). However, DRR for histamine and DRR for mannitol were not related to basic lung function, eNO, per cent sputum eosinophils and ICS dose. In addition, NO was not related to basic lung function and per cent sputum eosinophils.

In clinically well-controlled asthmatics taking inhaled corticosteroids, there is no relationship between markers of airway inflammation (such as exhaled nitric oxide and sputum eosinophils) and airway responsiveness to either direct (histamine) or indirect (mannitol) challenge. Airway hyperresponsiveness in clinically well-controlled asthmatics appears to be independent of eosinophilic airway inflammation.
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The clinical management of asthmatic patients depends on monitoring lung function and symptoms [1, 2]. However, airway inflammation can be present in asthmatic patients who are clinically well-controlled [3], suggesting that these measurements may not be sensitive enough to reflect the extent of airway inflammation.

Airway inflammation is a characteristic feature of asthma, and treatment with inhaled corticosteroids (ICS) is commonly prescribed as first-line therapy in mild, as well as moderate and severe asthma [4]. Airway hyperresponsiveness (AHR), assessed by bronchial challenge, is also a characteristic feature of asthma and seems to be related to airway inflammation [5, 6]. This relationship can be shown in steroid-naïve adults with "direct" challenges such as methacholine [5], as well as in children with "indirect" challenges such as hypertonic saline [6]. Chronic treatment with ICS reduces responsiveness to both hypertonic saline and histamine [7], although the time course to achieve this differs.

Airway inflammation can also be measured indirectly by counting the numbers of inflammatory cells in sputum [8]. Sputum collected from patients with an exacerbation of their asthma contains a very high number of eosinophils [9], but this number is reduced following ICS treatment [10], suggesting sputum eosinophil numbers may also be useful in monitoring asthma severity.

Airway inflammation may also be reflected by the levels of exhaled nitric oxide (eNO). Exhaled NO is increased during asthma exacerbations [11], and reduced in subjects taking ICS [11, 12]. These data suggest that eNO may be used as a marker of airway inflammation to monitor asthma.

This study examined whether markers of airway inflammation, as measured by inflammatory cells in induced sputum and eNO, were related to airway responsiveness, as measured by sensitivity to histamine (a pharmacological agent) and mannitol (an osmotic agent) in patients who were stable and well-controlled upon treatment with steroids.

Methods

Subjects

Fifty subjects were recruited. Data are reported for the 31 subjects from whom a sputum sample was

obtained. These 31 subjects (17 females), who were using ICS to control their asthma symptoms, met the American Thoracic Society criteria for asthma [13], had a history of wheezing and chest tightness, and were previously diagnosed by a physician as having asthma. These subjects were recruited from the Asthma Clinic of the Royal Prince Alfred Hospital, Sydney, Australia. Information on atopic status was available for 27 subjects, all of whom were atopic. Nine subjects were exsmokers. The mean age of the subjects was 45.4 (range: 22–69) yrs, the mean duration of their asthma was 24.9 (range: 2.5–60) yrs and the mean duration of their ICS use was 6.7 (range: 1–18) yrs. Nine subjects were using fluticasone, 16 budesonide and six beclomethasone, at a mean daily dose of 1,284 μg ICS·day^{-1} (95% confidence interval (CI): 1,029–1,539). Four subjects were using long-acting β-agonists (LABA) and all used short-acting β-agonists when needed. All subjects were clinically stable. In the 4 weeks before the study, they had asthma symptoms no more than twice a week, did not wake up at night because of asthma and had no respiratory tract infection. They had no changes in their dose of ICS in the last 4 weeks and the mean changes in dose of ICS were <1,000 μg daily in the last 3 months. Exclusion criteria were current smoking and the use of oral steroids within the previous 6 months.

The study was approved by the Central Sydney Area Health Service Ethics Committee. The trial was carried out under the Clinical Trial Notification Scheme of the Therapeutics Goods Administration of Australia (CTN No. 1997/373). All subjects signed a consent form prior to commencement of the study.

Study design

This was a cross-sectional study. Subjects were asked to come for two study visits within 2 weeks (7–14 days between visits). They were asked to refrain from taking short-acting β-agonists for 6 h, LABA for 24 h and antihistamines for 3 days before each study day. No ICS were taken on the day of the study. On arrival, the clinical diagnosis of asthma was confirmed by a staff physician by examination and history. On the first visit, an "indirect" challenge with mannitol was performed and on the second day a "direct" challenge with histamine. Exhaled NO and spirometry were measured before the challenge tests on both days, and sputum was collected during and/or after the mannitol challenge. Subjects were also asked to record in a diary card, their asthma symptoms, bronchodilator use and peak expiratory flow (PEF) twice daily before inhaling their asthma medication for the 4 weeks of the study.

Lung function measurements

Spirometry was performed using a MicroLoop II Spirometer (Micro Medical Ltd, Kent, UK). Forced expiratory volume in one second (FEV1) was used as an index of airway calibre. Forced expiratory manoeuvres were repeated until two readings of FEV1 within 100 mL were obtained, the largest of which was used in analyses. Values for FEV1 and forced vital capacity (FVC) were recorded as a percentage of the predicted values of KNUDSON et al. [14].

Bronchial responsiveness

Histamine challenge. A bronchial provocation test (BPT) with histamine was administered to all subjects using the rapid method [15]. Histamine diphosphate (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) was administered using DeVilbiss No. 45 hand-held nebulizers (DeVilbiss Health Care Inc., Somerset, PA, USA), in doubling doses 0.03–3.9 μmol. The test was stopped if FEV1 fell by $\geqslant$20%. Salbutamol aerosol was administered to aid recovery when necessary. The dose of histamine which provoked a 20% fall in FEV1 (PD20) was estimated by interpolation. The dose-response ratio (DRR) was calculated for all subjects as the percentage fall in FEV1 at the last dose, divided by the total dose administered [16, 17]. AHR was defined as PD20 $\leqslant$3.9 μmol histamine or a DRR >8.1.

Mannitol capsule challenge. A BPT with a dry powder of mannitol was administered to all subjects using the protocol previously described by ANDERSON et al. [18]. In brief, a noseclip was applied and subjects then performed the challenge with doses consisting of 0 (empty capsule acting as a placebo), 5, 10, 20, 40, 80, 160, 160 and 160 mg of mannitol *via* a Halermatic^{TM} (Rhône Poulenc Rorer, Collegeville, PA, USA). The 80 mg and 160 mg were given in multiple doses of 40 mg capsules. At least two FEV1 manoeuvres were performed 60 s after each dose and the highest FEV1 was used in the calculation. The FEV1 value measured after the 0 mg capsule was taken as the prechallenge FEV1 and used to calculate the percentage decrease in FEV1 in response to the mannitol challenge. If the subject had a >10% fall in FEV1 in response to a single dose, the same dose was repeated for reasons of safety. The challenge ceased when a 15% fall in FEV1 was documented or a cumulative dose of 635 mg had been administered. Salbutamol aerosol was administered to aid recovery when necessary. DRR was calculated for all subjects. The provocative dose of mannitol causing a 15% fall in FEV1 (PD15) was calculated by linear interpolation of the relationship between the percentage fall in FEV1 and the cumulative dose of mannitol required to provoke this fall. AHR was defined as a PD15 $\leqslant$635 mg of mannitol equivalent to a DRR >0.023% fall in FEV1·mg^{-1} of mannitol delivered by inhalation.

Nitric oxide measurement

Mixed expired NO was measured using a modification of the method of MASSARO et al. [11, 19]. The measurement was performed with the subject standing, without wearing a noseclip. The patient took a deep breath and exhaled for >5–15 s to residual

volume into an NO impermeable polyethylene bag (Scholle Industries Pty Ltd, Elizabeth West, Australia). The exhaled flow, measured by a rotameter (Dwyer Flowmeter Model VFASS-25, AMBIT Instruments Pty Ltd, Parramatta, Australia), was 10 L·min^{-1} at a mouth pressure >20 cmH_2O. The exhaled gas from a single breath was analysed within an hour, using a chemiluminescent analyser (Model 42C, Thermo Environmental Instruments, Franklin, MA, USA), which has a lower limit of detection of 1 part per billion (ppb). Ambient NO in the laboratory was measured at the time of testing.

Sputum collection

Sputum collection was carried out in conjunction with the mannitol challenge. If subjects had to cough during the mannitol challenge, they were asked to spit whatever was produced into a sterile container. At the end of the mannitol challenge, subjects were asked to cough and spit whatever was produced. All subjects rinsed their mouths with water at each collection point to remove any food particles and reduce salivary contamination. All specimens were retained for later examination under the microscope, even if there were no obvious sputum plugs.

Sputum preparation and differential cell count

Sputum was processed as described by Pizzichini et al. [20]. Briefly, sputum plugs were picked up and four-times the volume of diluted Sputolysin (0.1%) (Sputolysin Reagent, Calbiochem, Corp., San Diego, CA, USA) was added. The samples were placed in a shaking water bath (37°C) for 30 min and then filtered through 50 µm nylon gauze. The slides were assessed for quality before they were counted, and slides with >20% squamous cells were rejected. A total cell count was performed and cyto-centrifuge slides were prepared (Shandon Cytospin II, Sewickery, PA, USA). The inflammatory cells were expressed as a percentage of the total inflammatory cell count (400 cells) on slides fixed with methanol and stained with May-Grunwald Giemsa.

Peak flow home monitoring

Subjects were asked to perform PEF measurements while standing, twice a day, before inhaling their medication, for 4 weeks. The subjects blew three times into the peak flow meter (mini-Wright, Clement Clarke International Ltd, Essex, UK) and recorded the best of three values. The lowest PEF reading for the last of 4 weeks was calculated as a percentage of the best peak flow value achieved during the 4-week period [21, 22].

Symptoms score

Subjects were asked to fill in a diary card and to tick the level of their asthma symptoms. Morning and evening symptom scores were combined to produce a score of 0–8 (0 being symptom free and 8 having maximum symptoms).

Statistical analysis

Analysis of PD15 mannitol, PD20 histamine, DRR values of both challenge tests, eNO, eosinophils and neutrophils were carried out on log transformed data. Summary values for DRR, eNO, eosinophils and neutrophils are geometric means, with their 95% CIs. Summary values for all other parameters are arithmetic means and 95% CIs. The agreement between histamine and mannitol for classifying subjects with AHR was determined by Chi-squared analysis. Relationships were determined using the Pearson correlation for normally distributed variables, and nonparametric tests (Spearman's rho-tests) for all other variables. Significance was accepted at the 5% level.

Results

Of the original 50 subjects with well-controlled asthma recruited for this study, 31 were able to produce sputum giving a success rate of 62%. The baseline characteristics of subjects who could and could not produce sputum did not differ significantly (table 1). The values for spirometry were in the normal predicted range. There was no significant difference in FEV1 % predicted, or in the level of eNO between the two study days. However, there was a small but significant difference in FVC % pred (86.8 (95% CI: 81.4–92.2) versus 84 (95% CI: 78.8–89.2); p=0.03) and PEF % pred values (84.6 (78.8–90.3) versus 80 (74.7–85.1); p=0.006).

Of these 31 well-controlled asthmatics, 11 were hyperresponsive to histamine (PD20 histamine) and 16 to mannitol (PD15 mannitol). The DRR for histamine was significantly related to the DRR for mannitol (r=0.59, p=0.0004) (fig. 1). There was considerable overlap in airway responsiveness in subjects with and without sputum. There was significant agreement between the two challenge tests with respect to the classification of responders (PD20 histamine, PD15 mannitol) and nonresponders both for the group as a whole (Chi-squared: 11.66, p=0.0006) and for the subset of subjects with sputum (Chi-squared: 4.09, p=0.04). There were significant correlations between DRR measurements both for the group as a whole (r=0.63, p<0.0001) and for the group with sputum (r=0.61, p=0.00031). However, there was no significant relationship between FEV1 % pred and the DRR histamine or the DRR mannitol (table 2).

There was a weak correlation between sputum neutrophils and DRR for mannitol (r=0.36, p=0.046). With this exception, there was no relationship demonstrated between either the inflammatory cells collected in the sputum or eNO and airway responsiveness to either histamine or mannitol (table 2). Figure 2 shows the relationship between DRR for mannitol and sputum eosinophils. There were 15

Table 1. – Baseline characteristics of subjects from whom sputum was obtained for analysis of inflammatory cell numbers and of subjects who were unable to produce sputum

	Subjects with sputum	Subjects without sputum	p-value
Subjects n	31	19	
Sex M:F	15:16	10:9	
Age yrs	45.7 (40.6–50.8)	42.0 (35.1–48.7)	0.38
Atopic n (%)	26 (100)	13 (81)	
	(no data n=4)	(no data n=3)	
Daily ICS dose (BDP equivalent)	1402 (1092–1711)	1397 (1045–1749)	0.99
Symptom score	3.5 (3.2–4.2)	3.1 (2.9–4.2)	0.32
Baseline FEV1 % pred	82.9 (75.9–89.8)	90.1 (81.7–98.5)	0.19
Baseline FVC % pred	86.8 (81.4–92.2)	95 (88.5–98.6)	0.06
Baseline PEF % pred	84.6 (78.8–90.3)	88.9 (79.4–98.4)	0.40
PEF lowest % best	85.5 (92.9–88)	86 (82.5–89.5)	0.81
Exhaled NO ppb	19.5 (15.9–23.9)	15.8 (13.1–19.1)	0.17
DRR mannitol % fall $FEV_1 \cdot mg^{-1}$	0.063 (0.044–0.090)	0.061 (0.038–0.101)	0.94
AHR to mannitol n	16	8	0.87
DRR histamine % fall $FEV_1 \cdot \mu mol$; +3	8.68 (5.56–13.56)	7.35 (5.03–10.74)	0.60
AHR to histamine n	11	4	0.83
Max dose mannitol mg	341 (244–478)	321 (205–503)	0.83
Mannitol max % fall	14.8 (12.4–17.1)	13.8 (10.7–16.9)	0.62
Eosinophils %	2.01 (1.1–3.7)		
Neutrophils %	15.2 (10.6–21.7)		
Macrophages %	70.3 (56.6–74.6)		
Lymphocytes %	0.9 (0.6–3.3)		

Data are presented as geometric mean (95% confidence interval (CI)) for dose-response ratio (DRR), exhaled nitric oxide (eNO), eosinophils and neutrophils; and arithmetic mean (95% CI) for all other parameters unless otherwise stated. M: male; F: female; ICS: inhaled corticosteroids; BDP: beclomethasome diproprionate; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; PEF: peak expiratory flow; AHR: airway hyperresponsiveness; ppb: parts per billion. DRR is taken as the % fall in FEV_1 at the last dose per cumulative dose (for histamine, +3 is added).

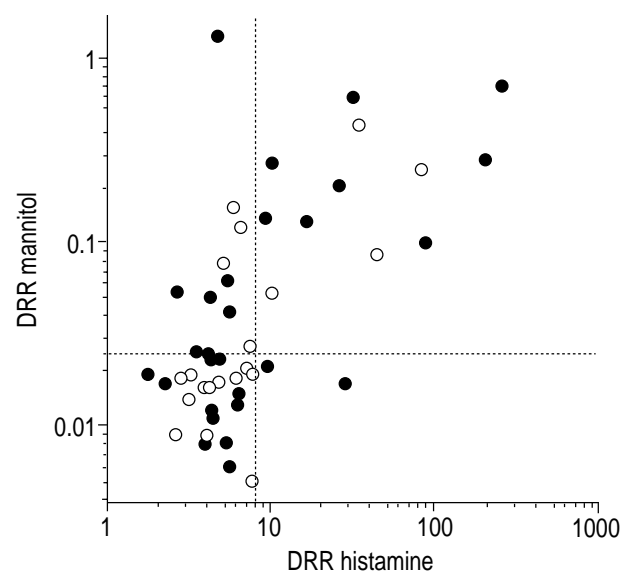subjects with sputum eosinophils >2.5% of the total cells, but these subjects did not differ from those with <2.5% eosinophils in either histamine DRR (p=0.36) or mannitol DRR (p=0.21). Mast cells were detectable in the sputum of only four subjects; two of these were hyperresponsive to histamine and three were hyperresponsive to mannitol. Exhaled NO was not significantly correlated with sputum eosinophils (r=0.23, p=0.21) or neutrophils (r=-0.03, p=0.75).

Fig. 1. – The relationship between dose-response ratio (DRR) for histamine, as per cent fall in forced expiratory volume in one second (FEV_1) per µmol histamine administered, plus 3, and DRR for mannitol, as per cent fall FEV_1 per mg mannitol administered for 50 subjects originally recruited into the study. ●: values for the subjects from whom sputum was obtained; ○: subjects who were unable to produce sputum. The dotted vertical and horizontal lines show the cutpoints for defining airway hyperresponsiveness to histamine and mannitol.

Table 2. – Relationship between responsiveness to histamine and mannitol as expressed by the dose-response ratio (DRR) and lung function variables, inhaled corticosteroid (ICS) dose, the concentration of exhaled nitric oxide (eNO) and the percentage of cells in sputum

	DRR mannitol		DRR histamine	
	r	p-value	r	p-value
FEV1 % pred	-0.292	0.11	-0.213	0.26
FVC % pred	-0.230	0.22	-0.032	0.88
PEF % pred	-0.083	0.137	-0.108	0.57
ICS dose µg	0.124	0.51	0.162	0.39
Exhaled NO ppb	0.243	0.19	0.157	0.41
Sputum eosinophils %	0.23	0.22	-0.24	0.24
Sputum neutrophils %	0.36	0.046	0.3	0.15
Sputum lymphocytes %	-0.24	0.32	0.10	0.30
Sputum macrophages %	-0.239	0.20	-0.1	0.31
Sputum mast cells %	-0.02	0.90	-0.02	0.90

r: Pearson correlation coefficient; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; PEF: peak expiratory flow; ppb: parts per billion. DRR is taken as the maximal % fall in FEV_1 per cumulative dose (for histamine, +3 is added).
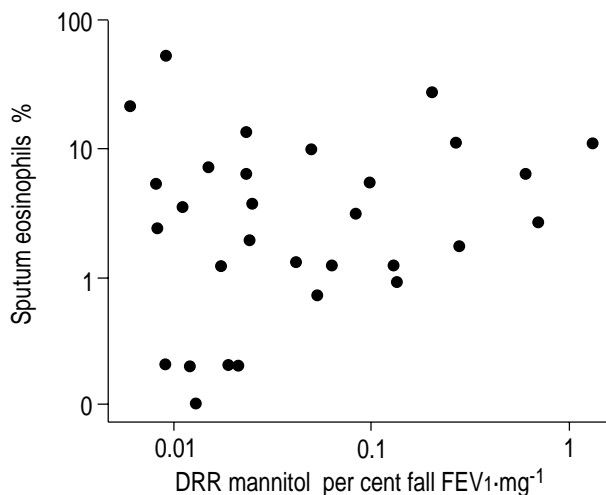
Fig. 2.–The relationship between the dose-response ratio (DRR) for mannitol, as per cent fall in forced expiratory volume in one second (FEV1) per mg mannitol administered, and sputum eosinophils, as a percentage of the total inflammatory cells counted. The correlation was not statistically significant using either parametric (Pearson's r=0.25, p=0.20) or nonparametric (r$_s$=0.14, p=0.48) analyses.

Discussion

This study has shown that in steroid-treated stable asthmatics, there is no relationship between indirect markers of airway inflammation, such as eNO or sputum eosinophils, and spirometry or airway responsiveness. Significant relationships were found only between the airway responsiveness to histamine and to mannitol and the sputum neutrophils and airway responsiveness to mannitol.

Sputum was successfully collected from 31 of a possible 50 subjects. The failure to collect sputum successfully from all subjects may have related to their well-controlled asthma. Mannitol is acting as a hyperosmolar stimulus to the airways in the same manner as hypertonic saline in terms of AHR [23]. While no formal comparison has been made between collecting sputum with mannitol and hypertonic saline, no differences have been noted between normal and hypertonic saline, suggesting that increasing the osmolarity itself does not affect the cell number [24]. While the authors may have been even more successful using a wet aerosol challenge, a success rate of 60%, which is similar to that reported by others [8], was obtained.

The present study appears to be the first one looking for a relationship between indirect markers of airway inflammation in well-controlled asthmatics who had been using ICS for several years. In this population, no clinically significant relationship could be found between markers of airway inflammation such as eNO or sputum eosinophils, and lung function values or airway responsiveness. In nonasthmatic people, sputum eosinophils can be up to 2.5% of the total cell count, especially when atopic subjects are included [3, 25]. A mean value of 2.0% eosinophils was found in this population of stable asthmatics, which is within the normal range. Treatment with ICS

decreases the percentage of sputum eosinophils and reduces the release of cytokines such as interleukin-5 and granulocyte-macrophage colony-stimulating factor [26]. CRIMI et al. [27] also found no significant correlation between the degree of airway responsiveness to the direct BPT methacholine and the numbers of inflammatory cells in sputum, bronchoalveolar lavage or bronchial biopsy.

However, a weak, but significant, correlation between sputum neutrophils and DRR for mannitol was found. These findings are supported by WARK et al. [28], who found that sputum eosinophils are highest in asthmatics not using ICS, and neutrophils are higher in subjects using ICS. There is also some evidence that isolated sputum neutrophilia does not respond to treatment with corticosteroids [29].

Airway inflammation may also be reflected by the levels of eNO. Exhaled NO is increased in steroid-naïve asthmatics and reduced in those after taking ICS [12]. The mean eNO levels in the present study's population was in the high normal range, based on a population study in the authors' region [19]. The eNO level found in the present study is similar to that found in the study of JATAKANON et al. [30], in which eNO was reduced with their highest dose of budesonide (1,600 μg) from 40.9 ppb to 18.3 ppb.

In steroid-naïve asthmatics, there is a significant relationship between eNO and provocative concentration causing a 20% fall in FEV1 (PC20) methacholine [5], PC20 histamine [31] or sputum levels of eosinophils [5]. Steroids decrease the level of eNO, probably by inhibiting the inducible NO synthase [12]. AHR also improves with ICS treatment by an effect on different infiltrative and resident cells [7, 32]. Looking at all these data, it could be expected that a relationship between these markers of airway inflammation would persist even under ICS treatment. However, VAN RENSEN et al. [33] found no relationship between changes in PC20 methacholine, sputum eosinophils and eNO after 4 weeks of ICS treatment, although all these measurements showed a significant improvement. Furthermore, LIM et al. [34] did not find a significant relationship between eNO and mucosal eosinophils (mucosal biopsy) in ICS-treated, as well as ICS-naïve asthmatic patients. It is possible that differences in the dose and duration of ICS treatment may have differential effects on inflammation, eNO and responsiveness, and thus reduce the likelihood of finding a relationship between them.

In the present study, there was a good relationship between responses to the two challenge tests, although 16 subjects were hyperresponsive to mannitol and only 11 to histamine. Cut-off points for defining AHR are widely used and established [35]. However, these cut-off points are sometimes arbitrary and therefore, may not reflect any real biological difference between subjects thus defined as normal or abnormal. Furthermore, the level of measurement error (usually at least ±one doubling dose) would mean that subjects with mild hyperresponsiveness could be classified wrongly to either the positive or negative AHR group [15, 36]. For this reason, it is better to use a continuous variable, such as DRR, to explore the relationship between the two tests. Using

this index of responsiveness, a significant relationship was demonstrated between sensitivity to histamine and mannitol. The present findings are supported by other studies, in which significant relationships between histamine and hyperosmolar saline were found for patients taking ICS [7].

It is thought that as a "direct agent", histamine acts at specific receptors on bronchial smooth muscle to cause contraction. By contrast, mannitol is an osmotic agent and acts "indirectly" to release mediators from inflammatory cells in the airways [7, 37]. These mediators, which appear to include histamine and leukotrienes [38, 39] then act on smooth muscle to cause contraction. The finding of a relationship between responsiveness to histamine and mannitol could simply be that histamine is common to both challenges. The fact that more subjects were responsive to mannitol than histamine could be due to leukotrienes being released in response to mannitol, thus providing a potent stimulus to airway narrowing. There was no relationship between airway responsiveness and baseline spirometry, suggesting that in these subjects, airway calibre explains none or only a small proportion of the variation in the response to either histamine or mannitol. Similarly, airway inflammation, as measured by eNO or sputum eosinophils, may explain only a small amount of the variation in AHR. It could be argued that eNO and sputum eosinophils may more closely reflect cells in the lumen and the superficial epithelium, but not the submucosa. Hence, the element of AHR which might result from airway wall thickening or structural changes beneath the epithelium [40] may relate poorly with measurements from the airway lumen such as eNO and sputum eosinophils, which are more sensitive to the effects of ICS. However, it is possible that the small sample size in the present study reduced the power of the study to detect significant relationships between sputum inflammatory cells and airway responsiveness.

In conclusion, in subjects with well-controlled asthma, taking inhaled steroids, there was no relationship between airway inflammation, measured either by sputum eosinophils or exhaled nitric oxide, and airway responsiveness measured by either histamine or mannitol. The relationship between responsiveness to mannitol and sputum neutrophils, while weak, was significant in this small number of subjects. This finding warrants further investigation because more subjects were hyperresponsive to inhaled mannitol than they were to histamine, and neutrophils may be important in determining this in patients taking steroids.

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