Sputum Induction in Corticosteroid-Dependant Asthmatics: Risks and Airway Cellular Profile

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SUMMARY Sputum induction with nebulized hypertonic saline is increasingly being used to evaluate airway inflammation. We investigated the procedure-associated risk in 16 asthmatics that were still symptomatic despite on high doses of regular corticosteroid (CS) therapy (7 on daily inhaled CS $\geq$ 800 μg budesonide or equivalent; 9 on additional daily oral CS) and their sputum cellular profile. For comparison, 12 mild stable asthmatics and 10 normal healthy subjects were included. All subjects inhaled 3%, 4% and 5% hypertonic saline sequentially via ultrasonic nebulizer as a means to induce sputum. Maximal percentage fall of Forced Expiratory Volume on One Second (FEV_1) during sputum induction was significantly greater in CS-dependent asthmatics (median % [IQR]: 16.0 [11.0-32.3]) than in mild asthmatics (5.3 [4.2-10.8], $p = 0.002$] and in normal subjects (4.6 [3.4-6.4]), $p = 0.0001$). The maximal percentage FEV_1 fall was inversely correlated with baseline FEV_1 ($R_s = -0.69$; $p < 0.0001$). Compared to mild asthmatics, induced sputum from CS-dependant asthmatics had proportionately fewer eosinophils (2.2 [0.8-7.0] versus 23.3% [10.7-46.3], $p = 0.003$) and greater neutrophils (64.2 [43.9-81.2] versus 28.7 [19.0-42.6], $p = 0.009$). Sputum neutrophils showed a significant inverse correlation to FEV_1 ($R_s = -0.51$, $p = 0.01$). We concluded that sputum induction using nebulized hypertonic saline should be performed with caution in CS-dependant asthmatics. The airway cellular profile observed suggests that the immunopathology underlying CS-dependant asthmatics may be different or a consequence of CS therapy.

The modern method of sputum induction is increasingly being used to evaluate airway inflammation in airway diseases.[1-3] With prior treatment with inhaled short-acting β_2-agonist, nebulized hypertonic saline to induce sputum is generally shown to be safe and well tolerated in normal subjects and most asthmatic patients. Sputum induction has been attempted without serious hazards in adult asthmatic subjects recovering from severe exacerbations[4] and in chronic stable asthmatics of greater severity.[5-7] This relatively non-invasive approach to study airway inflammation is an invaluable alternative to bronchoscopy. This is particularly so in corticosteroid (CS)-dependant asthmatics whose easy susceptibility to bronchospasm precludes the use of bronchoscopy to investigate airway inflammation.

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To date, the pathophysiology of airway inflammation in these asthmatics remains poorly understood. While eosinophils are widely regarded as the central effector cells in asthma pathogenesis,[8-10] neutrophils had been shown to dominate in the airways of CS-dependent asthmatics[7,11] compared to asthmatic patients of milder disease, suggesting that eosinophils may not be solely responsible in asthma pathogenesis.

In order to further explore the potential of sputum induction as a means to investigate airway inflammation, we studied the safety of sputum induction and the cellular profile of the induced sputum in symptomatic CS-dependant asthmatic patients, compared to those in mild stable asthmatic patients and in normal healthy subjects.

PATIENTS AND METHODS

Subjects

A group of asthmatic patients, being followed up in hospital chest clinics, was recruited according to eligibility as per study protocol. Normal healthy subjects were recruited by research advertisement. The diagnosis of asthma was defined according to international guidelines.[12] We defined 'CS-dependency' as dependency on inhaled budesonide $\geq$ 800 μg or equivalent daily with or without additional oral CS to provide best possible symptom control and 'symptomatic' as having ongoing asthmatic symptoms sufficient to interfere with daily living.[13] These were individuals managed as $\geq$ Step Four in the British Thoracic Society asthma guidelines[14] with concomitant regular controller therapy such as theophylline, salmeterol or ipratropium bromide. Mild stable asthmatics were defined as having infrequent symptoms (i.e. $\leq$ once weekly) that were easily relieved or prevented with rescue short-acting β_2-agonist, and methacholine PC_{20} of $\leq$ 8 mg/ml. Normal healthy subjects were defined as having no respiratory or other chronic medical conditions, non-smokers of at least 6 months duration and if previously smoked, had done so in less than 5 pack years. In addition, all normal subjects must demonstrate negative skin prick reactivity to cat, house dust mite or grass pollen, and methacholine PC_{20} of $\geq$ 16 mg/ml. The study protocol was approved by local hospital ethics committees and informed written consent was obtained from all subjects.

Study design

All eligible subjects attended at least two visits: one screening and another where sputum induction was performed. During the screening visit, subjects' clinico-demographic details and lung functions were recorded. Normal subjects and mild asthmatics also had skin prick allergen test and methacholine inhalation challenge. Asthmatic patients were required to be clinically stable with unaltered regular asthma therapy for at least two weeks before the second visit. Where indicated, subjects were seen more than once in order to establish optimal treatment. Any subjects who had an exacerbation within three weeks would require that their second visit be postponed till a later date.

Sputum induction

We followed the method originally described by Pin and colleagues.[15] Thirty minutes to one hour after treatment with salbutamol 200 μg via a meter dose inhaler (MDI) device (for mild asthmatics) or nebulized salbutamol 2.5 mg (for CS-dependant asthmatics), Forced Expiratory Volume in One Second (FEV_1) was recorded and this was taken as the baseline value for calculating the percentage FEV_1 change following hypertonic saline inhalation. Subjects then inhaled sequentially 3%, 4% and 5% hypertonic saline via an ultrasonic nebulizer, DeVilbiss Ultra-Neb 2000 (DeVilbiss Co, Heston, Middlesex, UK) in all subjects except oral CS-dependent asthmatics who were nebulized via Medix Sonic 2000 (Medix Ltd, Harlow, UK). This had occurred due to the availability of different ultrasonic nebulizers at the two different recruiting hospitals.

Each concentration of hypertonic saline was inhaled for 7 minutes, thereafter subjects rinsed their mouth and blew their nose (to minimize contamination from saliva or nose) before expectorating into a sterile container. FEV_1 was measured prior to each 7-minutes period and the procedure was terminated if FEV_1 fell below 20%. If the fall was between 10% and 20%, induction would be performed using the

same concentration of hypertonic saline as before (i.e. not to progress to higher concentration). A final FEV_1 was recorded and rescue salbutamol via nebulizer was given when clinically indicated.

Parameters for evaluating safety

The maximal percentage FEV_1 fall from baseline during the sputum induction, presence of hospital admission or prolonged observation more than one hour resulting from the sputum induction, were used as endpoints for evaluating safety.

Sputum processing and examination

The sputum was selected from saliva and processed within 2 hours according to a method described by Pizzichini and colleagues.[1] Briefly, sputum was homogenized by adding four volumes of freshly made 0.1% dithiothreitol (DTT) (Sputolysin, Calbiochem Ltd, Nottingham, UK) which was then added equal volume of Dulbecco phosphate-buffered saline (D-PBS) (Sigma, Poole, UK). The cell suspension was filtered through a 48 µm nylon gauze (BBSH Thompson, Scarborough, Ontario, Canada) and the filtrate centrifuged at 1,185 x g for 4 minutes at room temperature. The supernatant was aspirated and stored at -70°C for future assay while the cell pellet was resuspended with D-PBS, adjusted to 0.5 x 10^6 cells/ml, to be placed into cups of Shandon III cytocentrifuge (Shandon, Inc. Pittsburgh, PA, USA) and cytoslides made. After air dried, cytoslides were stained with Wright's Giemsa for differential cell count on at least 400 non-squamous cells. These cytoslides were counted by two independent observers. Cell counts were only accepted as valid if the differences of cell counts between the two observers were less than 5%. The average between the two counts was taken as the final count.

Data analysis

Descriptive statistics were used to summarize clinical characteristics of the study populations. Maximal percentage FEV_1 fall from sputum induction and the sputum cellular profile were expressed as median and interquartile range (IQR) and differences between groups were first analysed using Kruskal-Wallis test and then by Mann-Whitney U test if any significance were found. Correlations between variables were examined by Spearman rank correlation coefficient (R_s). In order to reduce the possibility of correlations occurring by chance, only those with $R_s > 0.50$ and significant at ≤ 0.01 level were considered relevant. All other tests considered a p value of ≤ 0.05 as statistically significant. All data were analysed using the statistical package GraphPad Prism® version 2.01 for Window 95 and NT.

RESULTS

Sixteen CS-dependant asthmatics (7 on high dose inhaled CS alone and 9 on additional oral CS), 12 mild asthmatics and 10 normal subjects were recruited. The clinico-demographic characteristics of these subjects are described in Table 1. Generally, CS-dependant asthmatics were older, had asthma longer and demonstrated significantly lower FEV_1 than mild stable asthmatics. Between the two groups of CS-dependant asthmatics, those requiring additional oral CS showed a trend towards older age, longer disease duration and lower FEV_1.

The maximal percentage FEV_1 fall following nebulized hypertonic saline was significantly greater in CS-dependent asthmatics than in mild asthmatics or normal subjects (on inhaled CS: median [IQR]: 16.0 [12.3-22.0]; on oral CS: 16.0 [11.0-21.0]; mild asthmatics, 5.3 [4.2-10.8]; normal subjects: 4.6 [3.4-6.4]) (Fig. 1). Between those asthmatics dependent on inhaled CS and oral CS, the FEV_1 fall was comparable. Similarly, there was no significant difference in FEV_1 fall between mild asthmatics and normal subjects. The inverse correlation between maximal percentage FEV_1 fall induced by the procedure and baseline clinic FEV_1 in all asthmatics was highly significant ($R_s = -0.69$; $p < 0.0001$) (Fig. 2).

Two inhaled CS-dependant asthmatics, 4 oral CS-dependant asthmatics and 1 mild asthmatics had FEV_1 fall $\geq 20\%$. However, among them, only 3 oral CS-dependant and 1 inhaled CS-dependant asthmatics had a clinically significant event with marked wheezing and chest tightness. Their bronchospasm were easily reversed with nebulized β_2-agonist within 30 minutes and did not required prolonged observation or hospitalisation. Three of these

Table 1 Clinico-demographic characteristics of asthmatic and normal subjects

Characteristics	CS-dependant asthmatics			Mild stable asthmatics	Healthy subjects
	Whole group	On high dose inhaled CS	On additional oral CS		
N	16	7	9	12	10
Age (years)	48** (30-60)	42 (30-55)	51 (42-60)	25 (23-28)	26 (23-30)
Male, N	11	6	5	9	6
FEV$_1$ % predicted normal	46.3** (27.4-69.4)	48.4 (27.4-69.4)	43.9 (31.2-56.6)	93.6* (86.0-101.1)	107.1 (98.8-115.4)
FEV$_1$ liters/minute	1.45** (0.9-2.5)	1.82 (1.2-2.5)	1.33 (0.9-1.8)	3.95 (3.4-4.5)	4.18 (3.5-4.9)
Disease duration (years)	31** (9-44)	21 (9-34)	33 (23-44)	16 (13-20)	-
Daily CS requirement	-	1,150 (800- 1,600) μg[†]	18.4 (9- 40) mg[‡]	none	-
Other controller medications					
Theophylline	13	5	8	none	-
Salmeterol	16	7	9	none	-
Ipratropium bromide	13	4	9	none	-
Immunosuppressants[¶]	3	0	3	none	-

Values are mean (range) unless otherwise specified
CS= corticosteroids; FEV$_1$= Forced Expiratory Volume in One Second;
[¶] Intended as corticosteroid-sparing agents. All three patients previously had cyclosporin A
[†] inhaled budesonide or equivalent; [‡] prednisolone
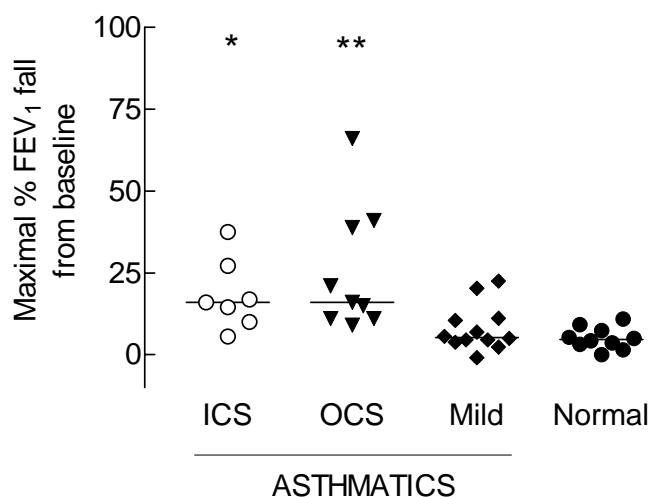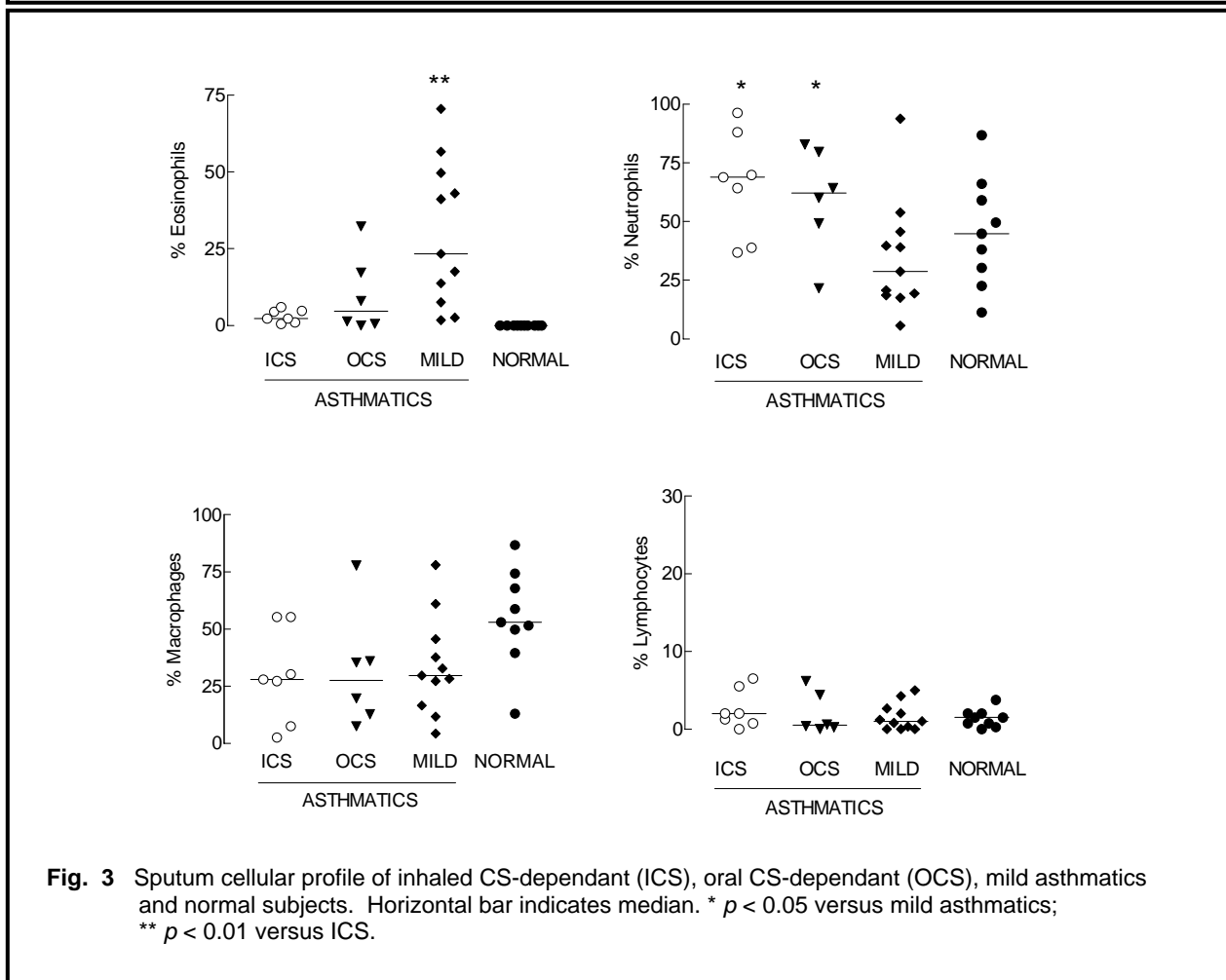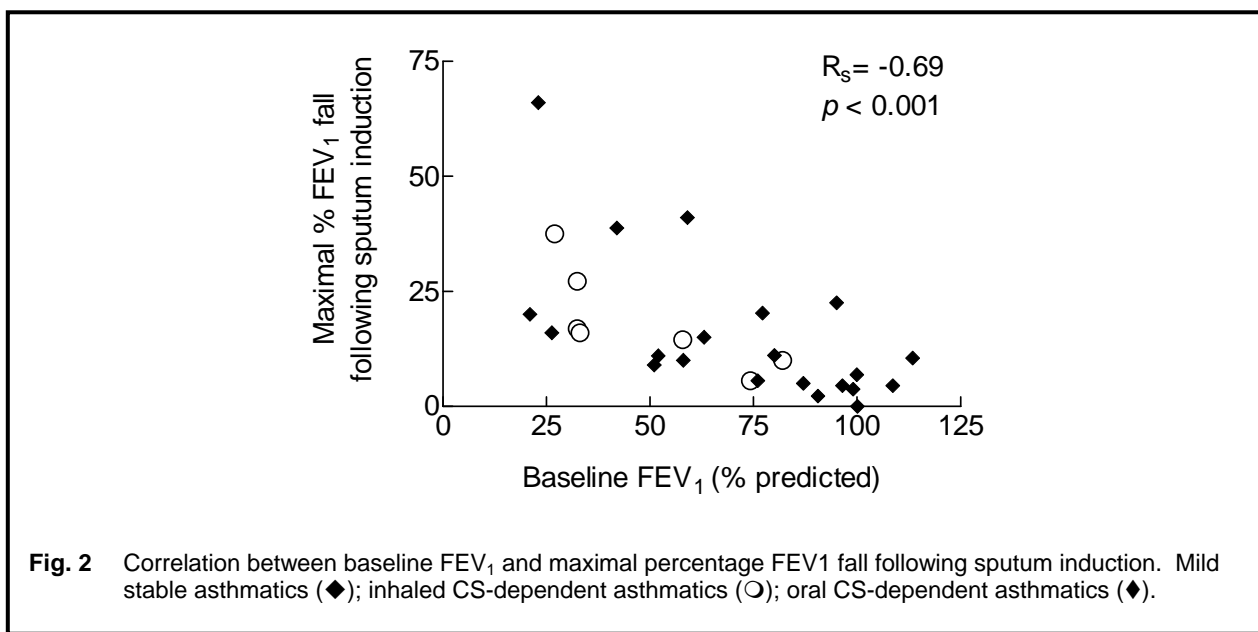*$p < 0.05$ versus normal subjects; **$p < 0.01$ versus mild asthmatics or normal subjects

Fig. 1 Maximal percentage FEV$_1$ fall from baseline following sputum induction. ICS = inhaled CS-dependent asthmatics; OCS = oral CS-dependent asthmatics; Mild = mild asthmatics; Normal = normal subjects. Horizontal bars represent median. ** $p < 0.01$ OCS versus mild and normal and ICS versus normal. *$p < 0.05$ ICS versus mild asthmatics.

Fig. 2 Correlation between baseline FEV_1 and maximal percentage FEV1 fall following sputum induction. Mild stable asthmatics (◆); inhaled CS-dependent asthmatics (○); oral CS-dependent asthmatics (◆).

Fig. 3 Sputum cellular profile of inhaled CS-dependant (ICS), oral CS-dependant (OCS), mild asthmatics and normal subjects. Horizontal bar indicates median. * $p < 0.05$ versus mild asthmatics; ** $p < 0.01$ versus ICS.

asthmatics had FEV_1 of < 1 liter. Of the 38 subjects studied, 1 normal subject, 1 mild asthmatic and 3 oral CS-dependant asthmatics could not yield sufficient sputum for processing.

In mild asthmatics, percentage sputum eosinophil was higher than in inhaled CS-dependent asthmatics (median [IQR] %: 23.3 [10.7-46.3] versus 2.3 [1.6-4.6], p = 0.006) and in oral CS-dependent asthmatics (versus 4.7 [1.0-12.6], p = 0.05). Percentage sputum neutrophil in CS-dependent asthmatics (on inhaled CS, 68.9 [51.5-78.9]; on oral CS, 62.1 [54.6-71.9]) was significantly higher than those in mild asthmatics (28.7 [19.0-42.6], p = 0.03 and 0.04, respectively), but comparable to those in healthy subjects (44.7 [30.3-49.5]). Percentage sputum macrophages and lymphocytes were comparable between these groups (Fig. 3).

Percentage sputum eosinophil did not significantly correlate to baseline FEV_1 in either CS-dependent (R_s = -0.26, p = 0.39), non-CS-requiring (R_s = -0.17, p = 0.61) or when all asthmatics were studied together (R_s = 0.38, p = 0.06) (Fig. 4, top). Similarly, there was no significant correlation between percentage sputum neutrophils and baseline FEV_1 in either CS-dependent (R_s = -0.19, p = 0.53) or non-CS-requiring (R_s = -0.15, p = 0.65) asthmatics alone. When all asthmatics were studied together, the inverse correlation between percentage sputum neutrophils and FEV_1 was statistically significant (R_s = -0.51, p = 0.01) (Fig. 4, bottom). In other sputum cell types, there was no significant correlation between percentage counts with baseline FEV_1 (data not shown).

DISCUSSIONS

We have shown that sputum induction using nebulized hypertonic saline can induce significant bronchoconstriction in symptomatic CS-dependant asthmatics, when compared to mild stable asthmatics and normal healthy subjects. The maximal percentage FEV_1 fall correlated inversely with baseline lung function of these asthmatics. Significant bronchocontriction still occurred despite pre-treatment with short-acting β_2-agonist. However, they were easily reversed with nebulized short-acting β_2-agonist and there were no subjects that required prolonged observation or hospitalization. Induction of sufficient
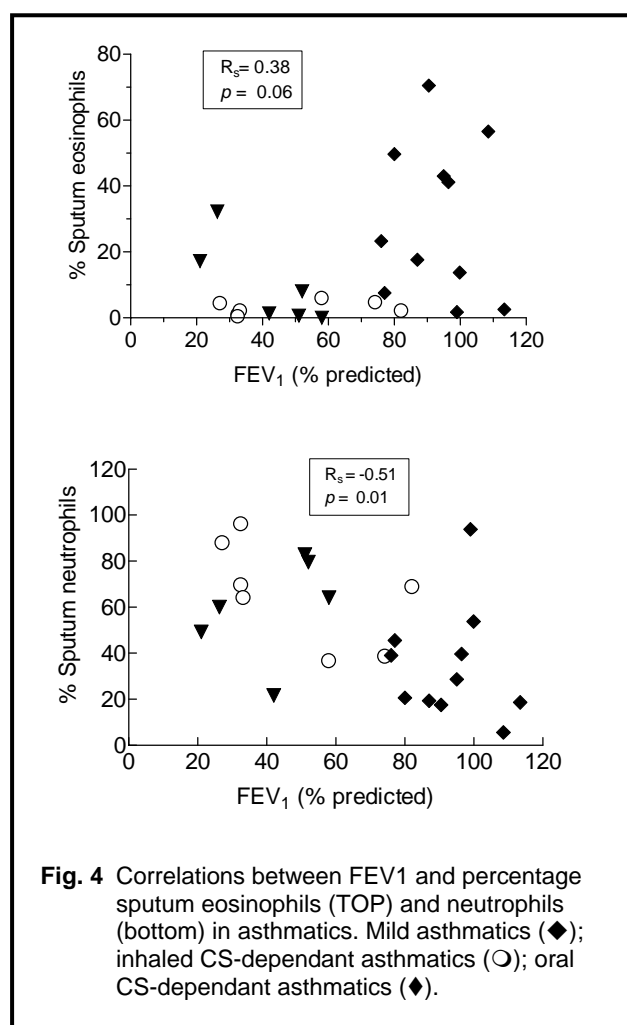

Fig. 4 Correlations between FEV1 and percentage sputum eosinophils (TOP) and neutrophils (bottom) in asthmatics. Mild asthmatics (◆); inhaled CS-dependant asthmatics (○); oral CS-dependant asthmatics (◆).

sputum for analysis using this method was generally successful in CS-dependant patients.

We also showed that the proportion of sputum eosinophils and neutrophils in induced sputum differed appreciably between CS-dependant and mild stable asthmatics. Eosinophils were significantly lower, while neutrophils, significantly higher in CS-dependant subjects. The proportion of sputum neutrophils in CS-dependant asthmatics were however comparable to those from healthy subjects and furthermore, there was considerable overlap between all study populations, suggesting that these were highly variable between subjects. While there was no significant correlation between sputum eosinophils and FEV_1, a significant inverse correlation could be demonstrated between percentage sputum neutrophils and FEV_1 when all asthmatics were studied together.

Over the last few years, the modern method of sputum induction and processing as an approach to study airway inflammation has gained wide acceptance. Many studies have now shown that the induced sputum obtained from this approach are repeatable, reliable[1-3] and responsive to change.[4,16] The obvious advantage in the use of induced sputum is that it ameliorates the risk associated with bronchoscopy when studying subjects with poor or unstable lung function.

Two studies have specifically addressed the safety issue of performing sputum induction using nebulized hypertonic saline in asthmatic patients. In 64 asthmatics of varying severity and dependency of CS therapy, de la Fuente et al.[5] showed that severe asthmatics had a significantly greater FEV_1 fall following this procedure than mild asthmatics. However, the FEV_1 fall in their subjects did not exceed 20%. Furthermore, they could not show any significant correlation between FEV_1 fall and baseline FEV_1. In another study by Grootendorst et al.[6] of 20 adolescent asthmatics with moderate-to-severe severity dependent on high dose inhaled CS, sputum induction with hypertonic saline brought about an increase of about 9% FEV_1 predicted, suggesting that there was very little risk associated with the procedure in their asthmatic patients.

Our findings add to the bulk of existing data on safety of inducing sputum with nebulized hypertonic saline in that for symptomatic asthmatics despite being treated with high doses of inhaled CS with or without additional oral CS, sputum induction with nebulized hypertonic saline represents a definite risk. In these patients, the resulted FEV_1 fall can exceed 20% and clinically significant wheezing can occur. Furthermore, baseline FEV_1 can predict a risk of significant bronchoconstriction. One likely explanation for the study of de la Fuente et al.[5] not being able to show significant FEV_1 falls is that they had intentionally excluded asthmatics with baseline FEV_1 < 1 liter in their study population. This is relevant because three of our four asthmatics who developed clinically significant wheeze had baseline FEV_1 < 1 liter.

Currently there is a move towards using isotonic saline, instead of hypertonic saline to induce sputum in patients with poor or unstable lung func-tion. This is intuitively appropriate since it has been well recognized that inhalation of hypertonic saline can cause bronchoconstriction in normal and asthmatic subjects.[17,18] Sputum cellular profile in asthmatics has been shown to be slightly altered whether they were induced using hypertonic or isotonic saline.[20] Furthermore, isotonic saline has been shown to safely induced sputum in children[20] and adults[4] during asthma exacerbation.

Our findings on sputum cellular profile support the view that in some asthmatics chronically dependent on CS, neutrophils may dominate in the airways.[11,7] Wenzel et al.[11] showed that in the bronchial mucosa of a group of symptomatic CS-dependant asthmatics not dissimilar to ours, the number of neutrophils in both bronchial biopsies and bronchoalveolar lavage (BAL) was significantly higher than in moderate asthmatics, not on CS treatment and in normal subjects. Similarly, Jatakanon et al.[7] showed that in induced sputum, neutrophil numbers were significantly higher in severe CS-dependant asthmatics than in mild asthmatics or in normal subjects. This view that CS-dependant asthmatics have more airway neutrophilia is however still being debated as there are also other studies showing that sputum eosinophils were increased in severe CS-dependant asthmatics.[21,22] More recently, studies have suggested asthmatic patients with neutrophilic airway inflammation may represent a subgroup of asthmatics that is less responsive to the effect of corticosteroids.[23,24] Another possible perspective that is worth considering is whether chronic CS use in asthma is responsible for this alternation of airway 'phenotype'. It has been recognized that while CS could increase eosinophil apoptosis,[25,26] it could paradoxically delay neutrophil apoptosis[26] and therefore lead to a dominance of airway neutrophils. Whether such neutrophils play any pathogenic role under such circumstances is unclear and warrants further research.

ACKNOWLEDGMENTS

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