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Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness

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ABSTRACT

Background: Although airway eosinophilia is seen as a cardinal feature of asthma, data eosinophilia are still lacking on the proportion of the asthma group exhibiting raised airway eosinophilia. This study aimed to assess the distribution of sputum eosinophil count and its relationship with methacholine bronchial hyperresponsiveness in mild to moderate steroid-naive asthmatic people.
Methods: Sputum was induced by inhalation of hypertonic saline (NaCl 4.5%) in 118 mild to moderate steroid-naive asthmatic people consecutively recruited from our outpatient clinic, and in 44 healthy people. The asthma group was selected on the basis of an forced expiratory volume in 1 s (FEV_1) of > 70% predicted, and a provocative methacholine concentration causing a fall of 20% in FEV_1 (PC_{20} methacholine; $PC_{20}M$) ≤ 16 mg/ml.
Results: In the asthma group, the median (range) of the percentage and the absolute values of sputum eosinophils were 4.8% (0-75) and 38 10^3/g (0-14 191), respectively, *vs* 0% (0-2.3) ($P < 0.001$) and 0 10^3/g (0-53) ($P < 0.001$) in healthy participants. Based on the 95% percentile for normal values calculated from our healthy group, 69% of the asthma group had significantly raised sputum eosinophil count (that is > 2%). In the asthma group, multiple regression analysis followed by a stepwise procedure revealed that sputum eosinophil count was significantly and inversely associated with $PC_{20}M$ accounting for 16% of its total variance ($P < 0.001$) while neutrophil counts positively related to $PC_{20}M$ accounting for 4% of total variance ($P < 0.05$). By contrast, no significant relationship was found between either eosinophil or neutrophil counts and the slope of forced vital capacity (FVC) *vs* FEV_1 from the methacholine challenge.
Conclusions: We conclude that two-thirds of people in the mild to moderate asthma group had increased sputum eosinophilia, which plays a limited role in determining the degree of methacholine airway hyperresponsiveness.

Key words: asthma; branchial hyperresponsiveness; eosinophils; methacholine; sputum.

Asthma is associated with a peculiar type of airway inflammation featuring Th2 cytokine overexpression and eosinophilic infiltration (1, 2). Some bronchoscopic studies have indicated that the extent of airway eosinophilic inflammation is proportional to the disease severity as reflected by the intensity of bronchial hyperresponsiveness or baseline impairment of lung calibre (3, 4). However, this issue remains controversial (5, 6).

The invasive nature of bronchoscopy has certainly been an impediment to large-scale cross-sectional studies on airway inflammation in asthma. It is reasonable to believe that the relatively small group sizes studied by bronchoscopy may account for the observed discrepancies. The newly developed noninvasive (7, 8) and reproducible (9, 10) technique of induced sputum has resolved this and has prompted studies on bronchial inflammation in asthma sufferers, on greater numbers of patients than in the past (11-13). This technique has also recently made it possible to determine sputum cell counts in a large and representative group of healthy participants (14).

This study aimed to assess the distribution of airway sputum eosinophil count in a very large group of mild to moderate steroid-naive people with asthma, and to analyze the relationship between eosinophil count and bronchial hyperresponsiveness to methacholine. Bronchial hyperresponsiveness to methacholine was not only assessed by measuring the PC_{20}forced expiratory volume in 1 s (FEV_1) but also by calculating the slope of the fall in forced vital capacity (FVC) *vs* FEV_1 from the methacholine challenge as a reflect of gas-trapping (15).

To provide a picture as close as possible to what the clinician may encounter in its daily practice, we conducted a prospective study on more than 100 consecutive asthmatic patients seen at our outpatient clinic, who were free of treatment with inhaled steroids, with baseline FEV_1 > 70% predicted. Cellular composition of sputum in the asthma group was compared with that found in 44 healthy participants without any evidence of IgE sensitization.

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MATERIAL AND METHODS

Participants

One hundred and twenty-eight mild to moderate nonsmoking asthmatic patients with $FEV_1 \geq 70\%$ predicted were consecutively recruited from the outpatient clinic at CHU Liege Sart-Tilman between July 1997 and September 1999. Asthma was diagnosed on the basis of clinical history of recurrent episodes of wheeze, breathlessness and/or cough associated with the demonstration of bronchial hyperresponsiveness to methacholine. Participants were considered to have bronchial hyperresponsiveness if a provocative concentration ofmethacholine caused a 20% fall in FEV_1 ($PC_{20}M$) of $\leq$ 16 mg/ml.

All participants recruited had a clinical history suggestive of asthma for at least 3 months before sputum induction. None used inhaled or oral steroids for at least 6 weeks prior to sputum induction. IgE-mediated asthma (16) was defined as a positive skin test (wheal $\geq$ 3 mm when compared to saline) to at least one of the most common aeroallergens from our area (house-dust mites, grass pollen, birch pollen, weed pollen, cat and dog dander, and mould mixture). Patients were excluded if symptoms suggestive of upper airway viral infection started within 4 weeks of sputum induction.

A group of 47 healthy nonsmoking participants, recruited through a local advertisement, volunteered as a control group. This group was used to define the value of "normal sputum eosinophil count". None had evidence of IgE-mediated reaction (16) or $PC_{20}M \leq 16$ mg/ml. Characteristics of all those who produced adequate sputum samples are given in Table 1.

Each subject gave written informed consent and the protocol was approved by the local ethics committee.

Methacholine challenge

Participants underwent a methacholine bronchial challenge according to a slightly modified Cockroft's method. Each subject inhaled successively by tidal breathing for 2 min fourfold increasing concentrations of methacholine chloride (from 0.06 mg/ml to a maximum of 16 mg/ml). The aerosol was generated by a jet nebuliser (Micro Mist; Hudson RCI, Temecula, CA, USA) as previously described (17).

A multifunctional electronic pocket spirometer was used to record the flow-volume curve, connected in real-time to a computer (Spirobank, MIR, Rome, Italy). Sixty seconds after each inhalation the participants made a deep inspiration to reach total lung capacity, and then immediately gave a forced maximal expiration without breath holding. This manoeuvre was repeated three times and the expiratory flow curve with the best FEV_1 value was selected by the software programme (WINSPIRO, MIR, Italy). Any forced expiration lasting less than 2 s was rejected.

Table 1. *Subject characteristics*

	Healthy subjects (*n* = 44)	Asthmatics (*n*= 118)
Age (years)	32 ±9	35 ± 13
Sex (male/female)	21/23	53/65
Skin prick positive	0	93
Height (cm)	172 ± 13	170 ± 11
FEV_1 (I)	3.92 ± 0.88	3.31 ± 0.80
FEV_1 (%)	104 ± 16	95 ± 12
FVC (I)	4.45 ± 1.02	3.95 ± 0.98
FVC (%)	98 ± 14	97 ± 12
FEV_1/FVC*	88 ± 10	81 ± 8
$PC_{20}M$ (mg/ml)	> 16	1.10 (0.03-16)
Slope FVC *vs* FEV_1	ND	0.85 ± 0.22

Results are expressed as means ± SD except $PC_{20}M$ which is expressed as geometric mean (range). * Only in 86 patients. ND, not done.

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The provocative concentration of methacholine causing a 20% fall in FEV_1 from baseline ($PC_{20}M$) was calculated by linear interpolation from the dose-response curve. After the provocative test each subject inhaled 400 µg salbutamol given by metered dose inhaler with a spacer (Volumatic, Glaxo SmithKline, Uxbridge, UK) and the sputum induction was performed 30 min later.

Sputum induction

Induction was performed using hypertonic saline (NaCl 4.5%) aerosolized by ultrasonic nebulizer (Devilbiss, PA, USA) with an output set at 1.5 ml/min for three periods of 5 min (or four periods if no sample was produced after 15 min). After each inhalation, the participants rinsed their mouths with tap-water and dried them with tissue paper to minimize contamination with saliva. Then they coughed up sputum in a plastic container placed at 4°C until processing. For safety reasons, peak expiratory flow rate was monitored after each 5 min inhalation period and the challenge was continued if it was > 250 l/min.

Sputum processing

Entire sputum was transferred into 50 ml polypropylene tubes (Becton Dickinson, Abingdon, UK), homogenized with an equal weight of dithiothreitol [DTT] (Calbiochem, La Jolla, CA, USA) 0.01 M and processed as previously described (18). Total cell count and squamous cell count were made using a hemocytometer. Samples were acceptable if the squamous cell count was <80% (10). The success rate of the sputum induction, as defined by the collection of a sample with a squamous cell count of <80%, reached 94% in healthy participants (44 out of 47) and 92% in the asthma group (118 out of 128) (Table 1). The differential cell count was performed on cytospins stained with Diff-Quick (Dade, Marburg, Germany) after counting 400 cells.

Statistical analysis

Sputum cell counts were presented as mean ± SD and median (range). Comparisons of sputum cell counts between the asthma group and healthy participants were performed by an unpaired Student's *t*-test, or by a Mann-Whitney test when distribution of the data was not parametric. The Kolomogorov-Smirnoff test was used to assessed the normality of the cell count distribution. Normal eosinophil count was determined by calculating the 95% percentile from the healthy group. Abnormally raised sputum eosinophil counts were those with values above the 95% percentile. Linear regression analysis was performed on the FVCs recorded at each step of the methacholine challenge against the corresponding FEV_1. The slope of the regression was used as an index of gas-trapping. The relationship between methacholine bronchial hyperesponsiveness and the different sputum cell counts was assessed by calculating Pearson's correlation coefficient after log transformation of both $PC_{20}M$ and absolute cell count values. As several variables were found to be associated with $PC_{20}M$, a multiple stepwise regression analysis was performed. When cell count was 0 it was arbitrarily assigned a 1-value to make log transformation possible. *P*-values < 0.05 were considered as statistically significant.

RESULTS

Sputum cell counts

Total and differential cell counts in healthy participants and the asthma group appear in Table 2. The percentage of squamous cells, macrophages, and neutrophils were normally distributed, but this was not the case for the percentages of eosinophils, lymphocytes and epithelial cells, nor the total cell counts. The asthma group had greater percentages and greater absolute values of sputum eosinophils than healthy participants ($P < 0.001$ for both). There was no difference between the groups with respect to the other cell types, nor with respect to the total cell counts.

Based on the values of sputum eosinophil count in healthy participants, we found that 69% (82/118) of the asthma group had significantly raised sputum eosinophil count (> 2% > 95% percentile of control group). The distribution of sputum eosinophil count showed that half of the asthma group had less than 4.8% sputum eosinophils, whereas those with an eosinophil percentage greater than 20% only represented 17% (21 out of 118) of the group (Fig. 1).

Relationship between sputum cell count and methacholine bronchial responsiveness

After simple linear regression $PC_{20}FEV_1$ methacholine was found to be inversely related to sputum eosinophil ($P < 0.0001$; Fig. 2A) and lymphocyte counts ($r = - 0.19$, $P < 0.05$). By contrast, the slope FVC/ FEV_1 showed no

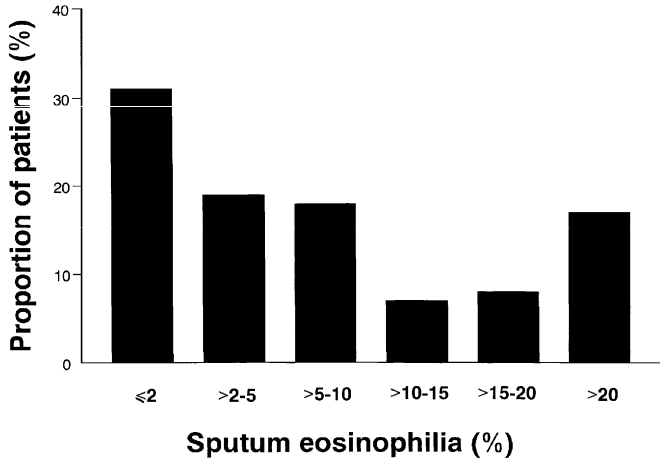
relation to sputum counts of eosinophils or other cells ($P > 0.05$; Fig. 2B).

The relationship between sputum cell counts, baseline airway calibre, and $PC_{20}FEV_1$ methacholine was further assessed by multiple regression analysis. $PC_{20}M$ was significantly influenced by sputum cell counts and baseline airway calibre with a global variance R reaching 29% ($P < 0.001$) (Table 3). The three variables significantly contributing to the global variance after the stepwise procedure were the eosinophil count, the neutrophil count and the FEV_1 % predicted, accounting for up to 16% ($P < 0.001$), 4% ($P < 0.05$) and 4% ($P < 0.05$) of the total variance, respectively. However, eosinophil count was inversely related to $PC_{20}M$, but the latter ($PC_{20}M$) positively related to both neutrophil count and FEV_1.

Table 2. *Sputum cell composition in healthy participants and mild to moderate the asthma group*

		Healthy subjects		Asthmatics	
		Mean (± SD)	Median (range)	Mean (± SD)	Median (range)
Squamous	%	13 (9.1)	13.5 (0-33)	15.8 (10.2)	14 (0-61)
Total nonsquamous	10^6/g	1.09 (0.9)	0.80 (0.17-4.42)	2.10 (4.97)	0.73 (0.16-37.8)
Macrophages	%	52.6 (17.4)	55 (9-85)	45.6 (21.2)	46.6 (0-87.8)
	10^3/g	527 (68)	382 (27-2238)	690 (1147)	325 (0-7914)
Lymphocytes	%	1.3 (1.4)	1 (0-5.2)	1.2 (1.4)	0.8 (0-7.4)
	10^3/g	15 (25)	7 (0-150)	22 (37)	10 (0-217)
Neutrophils	%	31.8 (22.8)	26.3 (0.8-87)	29.9 (23.5)	23.8 (0-86.4)
	10^3/g	415 (587)	202 (1-2890)	811 (259)	179 (0-20 390)
Eosinophils	%	0.3 (0.6)	0 (0-2.3)	10.9 (13.6)	4.8 (0-75)
	10^3/g	5(11)	0 (0-53)	361 (1515)	38 (0-14 191)
Epithelial cells	%	13 (11.5)	10 (0-55.2)	11 (10.4)	9 (0-64.5)
	10^3/g	115 (120)	68 (0-440)	185 (512)	59 (0-4909)

Figure 1. *Distribution of sputum eosinophil count expressed as a percentage of total cells in patients (n = 118) in the mild to moderate steroid-naive asthma group.*



DISCUSSION

Our study describes the distribution of sputum eosinophil counts and its relationship to methacholine bronchial responsiveness in a large population of mild to moderate steroid-naive asthma patients, consecutively recruited from our outpatient clinic. When compared to healthy nonatopic participants, 69% of the asthma group had abnormally raised sputum eosinophilia; this was significantly associated with the severity of methacholine bronchial hyperresponsiveness assessed by the $PC_{20}FEV_1$.

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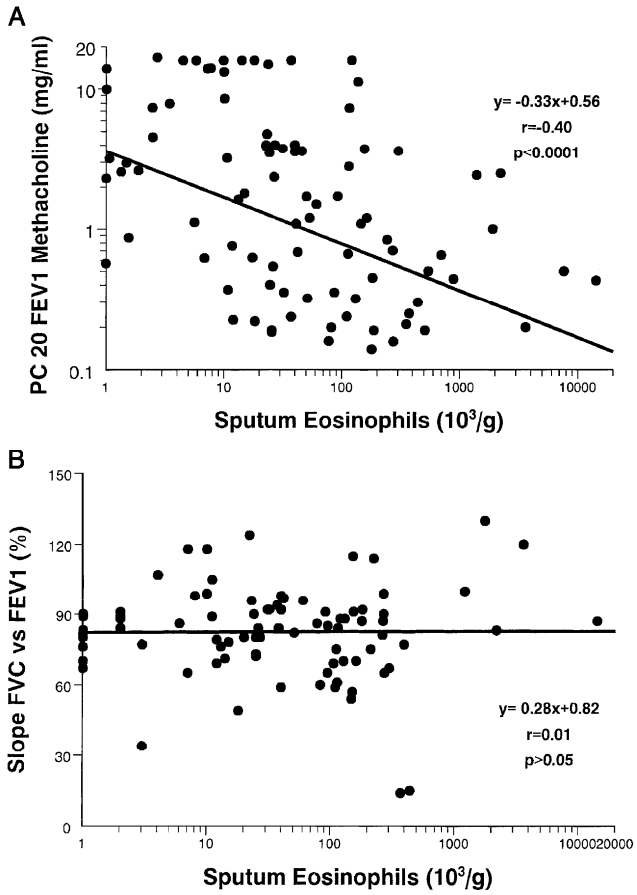
Figure 2. *Relationship between the sputum eosinophil count and PC_{20} FEV_1 methacholine (n = 118) (upper panel) and the slope FVC/FEV_1 (n = 86) (lower panel).*



Although there is general endorsement of the statement that asthma is an eosinophilic bronchitis, no data are yet published about the proportion of asthma patients with abnormally high airway eosinophilia. To our knowledge, this study is the first to look prospectively at the distribution of sputum eosinophil counts in a large population of mild to moderate steroid-naive people with asthma, to establish the proportion of who have significantly raised eosinophil counts. This obviously requires definition of normal values, so it is essential to assess airway inflammation in a sufficiently large group of healthy participants.

The normal sputum eosinophil values found in our healthy participants are very close to those recently reported by Belda et al. (14) in a group of more than 100 participants. There was a wide range of sputum eosinophil counts among the patients, but most had a percentage of sputum eosinophils significantly greater than the 95% percentile of a population of nonatopic healthy participants (> 2%), and approximately one-fifth displayed a sputum eosinophil count exceeding 20%. However, it is worth noting that approximately half of the asthma group had sputum eosinophil counts less than 5%. This may be important for screening for clinical studies that assess antiinflammatory drugs in asthma, in patients selected on the basis of sputum eosinophilia (19).

One interesting observation of this study is that about one-third of the steroid-naive asthma group are non-eosinophilic—which stems the fact that symptoms of asthma associated with methacholine bronchial hyperresponsiveness do not necessarily translate to increased sputum eosinophilia. Recent data have suggested that the efficacy of inhaled corticosteroids in attenuating methacholine bronchial hyperresponsiveness and symptom scores in asthma depends on a increased numbers of eosinophils in the airway before starting the treatment (20). Thus, those with noneosinophilic asthma certainly deserve further attention, in terms of their response to drugs such as theophylline, cromoglycate, or leukotriene receptor antagonists that are used as controller medications.

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Table 3. *Multiple regression analysis of the relationship between methacholine bronchial responsiveness and sputum cytology and baseline lung calibre in the asthma group*

	PC_{20} methacholine		
	Global variance R^2	Partial regression coefficient β (SE)	*P* value
	0.29		0.0006
Macrophages		0.08 (0.14)	0.58
Lymphocytes		- 0.10 (0.08)	0.23
Neutrophils		0.28 (0.10)	0.006
Eosinophils		- 0.20 (0.06)	0.001
Epithelial cells		- 0.03 (0.09)	0.71
FEV_1		0.02 (0.008)	0.007

$PC_{20}M$ is the dependent variable. Cell absolute counts and FEV_1 % predicted are the independent variables. SE, standard error.

The extent of sputum eosinophilia in the steroid-free asthma patients appears to contribute weakly but significantly to the severity of methacholine bronchial hyperresponsiveness. In a model including sputum cell count and baseline FEV_1 as independent variables and $PC_{20}FEV_1$ as the dependent variable, stepwise multiple regression analysis showed that sputum eosinophils accounted for 16% of the variation in $PC_{20}M$. Although weak, the contribution of eosinophils to methacholine bronchial hyperresponsiveness should not be neglected because it might underlie the fast and limited change in $PC_{20}M$ that follows treatment with inhaled steroids (20). The weak relationship between sputum eosinophil count and $PC_{20}M$ clearly points to the fact that severe bronchial hyperresponsiveness may be seen without prominent eosinophilia and that, conversely, bronchial hyperresponsiveness sometimes remains mild despite massive infiltration of the airway with eosinophils. This is in keeping with previous studies that show high sputum eosinophil counts in chronic cough without asthma (21), in rhinitis (22), or in inflammatory bowel disease (23). However, it is possible that the relationship between sputum eosinophilia and bronchial hyperresponsiveness may have been stronger if bronchial responsiveness was assessed with indirect, potentially more clinically relevant bronchocon-strictors such as adenosine (24,25) or bradykinin (26) instead of methacholine.

Surprisingly, neutrophils contributed to $PC_{20}M$ in an opposite manner to eosinophils, i.e. more neutrophils in the airway mean a higher $PC_{20}M$. This suggests that neutrophils protect against severe bronchial hyperresponsiveness in mild to moderate steroid-naive asthma even if the reasons are unclear at present. This contrasts with the supposedly active role of the cell in severe asthma, particularly in patients resistant to corticosteroids (12, 27). One interpretation is that asthma that remains mild to moderate *without* inhaled steroid therapy has very different cell mechanisms to those of asthma that is moderate to severe *with* inhaled and/or oral steroid therapy.

Baseline airway calibre is an important determinant of nonspecific bronchial hyperresponsiveness in the general population (28). However, this relationship is less clear in asthma where it is common to see patients with normal lung function and severe bronchial hyperresponsiveness. The contribution of baseline FEV_1 to the $PC_{20}M$ in our study is further weakened by the exclusion of those patients with baseline obstruction. Since neither airway eosinophilia nor FEV_1 substantially contribute to the level of $PC_{20}M$ in mild to moderate asthma, biochemical and/or structural changes in airway smooth muscle or bronchial mucosa are likely to be critical in regulating the extent of airway instability (29).

Finally, $PC_{20}FEV_1$ was significantly related to sputum eosinophil count, but this was not the case for the slope FVC/FEV_1 which appeared to be almost independent of sputum eosinophil count. This slope may reflect a propensity to develop excessive peripheral airway obstruction leading to air-trapping, thus it would suggest that sputum eosinophil count does not predict the behaviour of the small airway following inhalation of methacholine. However, this is not a surprising lack of association. Inflammation may still contribute to small airway dysfunction, as sputum samples are mainly from the proximal airway (30), the abnormal behaviour of which is more likely to effect FEV_1 than FVC.

We conclude that most mild to moderate steroid-naive asthma patients have a significantly raised sputum eosinophilia, which plays a limited role in methacholine bronchial hyperresponsiveness.

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