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Induced sputum to assess airway inflammation: a study of reproducibility

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Summary

Background Infiltration of the airways mucosa with activated inflammatory cells appears to be a major factor in the pathogenesis of asthma and other airway diseases. Examination of sputum provides a direct method to investigate airway inflammation non-invasively.
Objectives The aim of the present study was to evaluate the reproducibility of cell counts on cytospins and fluid phase (eosinophil cationic protein, ECP) measurements in a selected portion of induced sputum. We aimed to confirm the validity of the tecnique by comparing measurements between stable asthmatics, allergic rhinithis and healthy subjects.
Methods Sputum was induced with hypertonic saline (4.5%) twice within one week in 53 stable asthmatics, 16 subjects with seasonal rhinitis (out of the pollen season), and 19 healthy subjects. Reproducibility was examined within sample (two different plugs of the same sample) between sample (two specimens of induced sputum obtained within one week) and between examiners on stable subjects taking into account sample size, number of examinations per patients and Confidence Interval (CI) of the estimates.
Results We have found that the method is highly reproducible within sample and between examiners for all types of cells and fluid phase measurements of ECP. It is reproducible between sample for eosinophils, macrophages, neutrophils and ECP, but not for lymphocytes and weakly for epithelial cells. Sputum from asthmatics, in comparison with the sputum of healthy subjects and subjects with rhinitis had higher eosinophils (asthmatics: $12.2\% \pm 12.9$, rhinitis: 0.4 ± 0.8, normals: 0.4 ± 0.7 (%) and ECP (asthmatics: 827 ± 491 μg/L, rhinitis: 127 ± 82 normals: 157 ± 203). No significant differences were found between healthy subjects and subjects with rhinitis. Eosinophil counts were inversely correlated with FEV_1 ($r = -0.37$) expressed as percentage of predicted, but not significantly correlated with PC_{20} methacholine ($r = -0.28$) or blood eosinophils ($r = 0.26$).
Conclusions The importance of this study is the confirmation, within important statistical guidelines for a study of reproducibility, that the methods examined are reproducible and valid.

Keywords: asthma, rhinitis, sputum induced, reproducibility

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Introduction

Infiltration of the airways mucosa with activated inflammatory cells appears to be a major factor in the pathogenesis of asthma and other airway diseases. Examination of sputum

provides a direct method to investigate airway inflammation non-invasively [1]. When sputum cannot be produced spontaneously it is successfully induced by inhalation of an aereosol of hypertonic saline [2–4].

Two techniques for processing sputum have been described, selection of a portion of expectorated sputum from coexistent saliva [5] and processing of the whole sample [3]. The first technique presents a crucial point considering that different selected portions of the same sample could be representative of different parts of the respiratory tract. It could be interesting to evaluate if different plug of the same sample are similar in the cell counts.

Limited studies of cell [1,2] and fluid phase determinations [6] of selected portions of sputum have demonstrated reproducibility of the methods. However, only one study has evaluated the reproducibility within a sample of spontaneous sputum using smears [1]. To our knowledge no study has analysed the reproducibility within sample examining cells in induced sputum by cytospin.

Moreover, in reproducibility studies the roles played by the number of subjects examined, the number of measurements per subjects, and the level of uncertainity of the estimate (Confidence Intervals, CI) is critical [7].

The aim of the present study was to evaluate the reproducibility of cell counts on cytospins and fluid phase (eosinophil cationic protein, ECP) measurements in a selected portion of induced sputum. Reproducibility was examined within sample (two different plugs of the same sample) between sample (two specimens of induced sputum obtained within one week) and between examiners on stable subjects taking into account sample size, number of examinations per patients and CI of the estimates. We aimed to confirm the validity of the technique by comparing measurements between stable asthmatics, allergic rhinithis and healthy subjects.

Methods

Subjects

We studied 53 subjects with bronchial asthma, 16 subjects with seasonal rhinitis (out of the pollen season), and 19 healthy subjects. (Table 1). Asthma was defined as a clinical history of intermittent wheeze, cough, chest tightness, or dyspnoea, and documented reversible airflow limitation with an improvement in $FEV_1 \geq 20\%$ after salbutamol (200 μg) when FEV_1 was $\leq 70\%$ predicted [8]or methacholine airway responsiveness ($PC_{20} \leq 8$ mg/mL) when FEV_1 was $\geq 70\%$. All subjects were stable as demonstrated by the low daily variability (< 15%) of peak flow measurements during the study (2 weeks) [9]. Medications were unchanged during the study and for at least 15 days before, except for short-acting bronchodilator taken as required. Severity of asthma was assessed using the Aas score [10]. Healthy subjects were nonsmokers and had no history of asthma or other respiratory symptoms, had $FEV_1 > 80\%$ predicted, and methacholine airway responsiveness $PC_{20} > 16$ mg/mL. Subjects with seasonal rhinitis had a history of rhinitis and or conjuntivitis during the grass pollen season. They showed a positive skin-prick test to grass pollen extracts. On enrolment subjects had no symptoms of nasal itch, sneezing, nasal obstruction, or rhinorrhoea; they had $FEV_1 > 80\%$ predicted and methacholine airway responsiveness $PC_{20} > 16$ mg/mL. They had never had symptoms of asthma or other respiratory diseases.

Table 1. Characteristics of subjects

	Asthmatics	Healthy subjects	Rhinitis
Subjects, no	53	19	16
Age, years	40 ± 16	37 ± 12	35 ± 14
Sex males/females	24/29	9/10	11/5
Smoking (ex)	1 (6)	0 (3)	0 (3)
Atopy°	28	0	16
FEV_1 % pred	91 ± 21	110 ± 14	115 ± 14
FVC % pred	104 ± 15	112 ± 14	113 ± 17
Eosinophil serum/L × 10^9	0.42 ± 0.33	0.15 ± 0.08	0.16 ± 0.11
PC_{20}, mg/mL*	1,8	> 16	> 16
Inhaled steroid therapy	17	–	–

Data are expressed as mean and standard deviation.
* Methacholine PC_{20} geometric mean.
° Atopy was defined as one or more positive allergy skin prick tests; rhinitis have positive skin-prick tests for pollen grass.
Inhaled steroids included bechlomethasone dipropionate, budesonide or fluticasone.

The study was approved by the ethics commitee of Fondazione Maugeri and Royal Postgraduate Medical School, and all subjects gave written informed consent.

Study design

Subjects attended the laboratory on 3 days within a 2 week period, at the same time of day. On the first visit subject characteristics were documented by questionnaire, skin-prick tests to a range of antigens (dog hair, cat fur, grasses, *Cladosporium herbarum, Alternaria alternata, Dermatophagoides pteronyssinus, Dermatophagoides farinae, Aspergillus fumigatus, Parietaria*) and spirometry (Vitalograph, Buckinghamshire, UK) was performed. If spirometry showed a $FEV_1 \geq 70\%$ a methacholine inhalation test was carried out by the method described by Dixon and

Ind [11] and the results were expressed as the PC20 in non-cumulative units; if $FEV_1 < 70\%$, reversibility of airway obstruction was performed using 200 μg inhaled salbutamol. Subjects were instructed to record peak flow measurements as the best of three blows in the morning and in the evening before drug administration for 2 weeks and report values in a diary.

On the second and third visits the diary was checked, sputum was induced after inhaled salbutamol. On the second day a selected portion of sputum was analysed (occasion 1) while on the third day two selected plug of the same sample were processed separately (occasion 2), coded and analysed to determine the reproducibility within sample. On the second day a blood sample was obtained to measure differential leucocyte formula.

Sputum induction

Inhalation procedure

FEV_1 and FVC were measured before and 10 min after salbutamol inhalation (two puffs; 200 μg) and then subjects inhaled hypertonic (4.5%) saline nebulized for increasing time periods, 1,2,4,8,16 min, FEV_1 was repeated 1 min after each inhalation period [4]. Saline solutions were nebulized by an ultrasonic nebulizer (DeVilbiss 65, DeVilbiss Corporation, Somerset, PA, USA).

Sputum processing

The collected sputum samples were examined within 2 h. Selected portions of the sputum sample originating from the lower respiratory tract, were chosen using an inverted microscope and weighed. Dithiothreitol (DTT, Sputolysin, Calbiochem Corp, San Diego, CA, USA), freshly prepared in a dilution of one in 10 with distilled water, was added in a volume (in μL) equal to two times the weight of the sputum portion (in mg). Selected sputum was placed in a shaking water bath at 37 °C for 20 min to ensure complete homogenization. It was further diluted with phosphate buffered saline in a volume equal to the sputum plus DTT. The suspension was filtered through gauze to remove mucus and was centrifuged at 1000 *g* for 5 min. The supernatant was aspirated and frozen at −70 °C for later ECP (μg/L) analysis by radioimmunoassay (RIA; Kaby Pharmacia Diagnostic AB, Uppsala, Sweden). The cell pellet was resuspended in a volume of PBS equal to that the sputum plus DTTand PBS as above. Total cell count (TCC) and viability (Trypan blue exclusion method) were determined using a Burkers chamber haemocytometer. The cell suspension was placed in a Shandon 3 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA) and cytospins were prepared at 450 rpm for 6 min. Cytospin slides were fixed by methanol and were stained by May Grunwald Giemsa for an overall differential cell count on 500 nucleated non-squamous cells by two examiners. Only samples with cell viability >50% and squamous cell contamination <20% have been considered adequate [12,13]. All sputum counts and measurements were performed blind to the clinical details.

Statistical analysis

Descriptive statistics were used to summarize clinical and demographic characteristics of the subjects. Grouped data were reported as the arithmetic mean and standard deviation. PC_{20} data were log transformed and reported as geometric mean. The repeatibility of measurements was examined by the reliability coefficient (intraclass correlation coefficient = R) as the proportion of the variance in the measures due to the true variance between subjects [14] and graphically reported as proposed by Bland and Altman [15]. The repeatability of cell counts between samples was assessed as the percentage of cells of the sample on occasion 1 with respect to the the mean value of percentage cell count of the two plugs (occasion 2) of the same sample normalized for weight. The comparison between groups was assessed by unpaired *t*-test. The correlation between variables was examined by linear regression. A value of $P < 0.05$ was considered statistically significant.

Sample size, number of examination per subjects and Confidence Intervals of the estimate

From our preliminary data suggesting high values of R, we decided to test H_0: $R < 0.80$ vs H_1: $R > 0.80$ at a α significance value of 0.05 and with 80% (1-β) power [7]. The required number of measurements (n) for a given number of subjects (k) increases very rapidly as k declines, and there is a tendency towards a 'threshold' level of k beyond which any increases in k, with n held constant, brings very little return. We calculated for at $R = 0.80$, with $n = 2$, the minimum sample size required is 50, according to the equations relating k, n, α and 1-β [7]. Limits of Confidence Interval (CI) were calculated as suggested by Donner and Fleiss [7,14].

Results

Sputum induction was performed in all subjects (no: 88) on two occasions without any significant adverse effects times. On 176 attempts 156 adequate samples (88.6%), were obtained. Only four subjects failed on both occasions. Considering the failure of 20 attempts (nine on the first occasion) the calculation of the reproducibility within sample was performed on 77 subjects, between sample and between observers on 72 and 79 subjects, respectively. Salivary contamination indicated by the proportion of

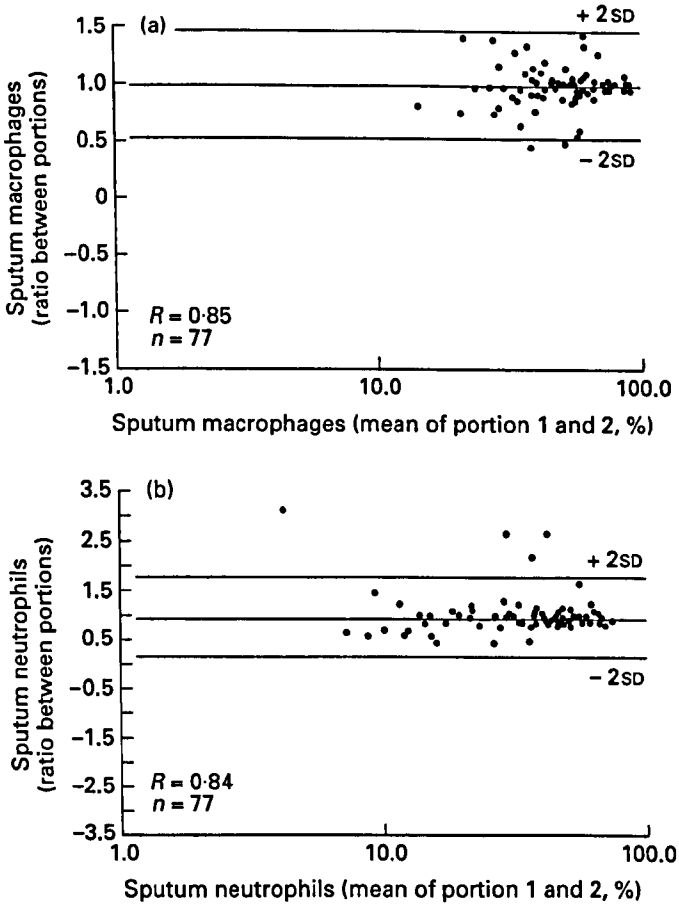

Fig. 1. Bland and Altman plot for sputum macrophages (%) (a) and sputum neutrophils (%) (b) as mean value of two portions (portion 1 and portion 2) of the same sample compared with the ratio of the first (portion 1) and second (portion 2) count. R is the global intraclass correlation coefficient. SD is standard deviation. *n* is the number of measures plotted included asthmatics, rhinitics and healthy subjects. All 77 subjects are included but multiple points are not visible.

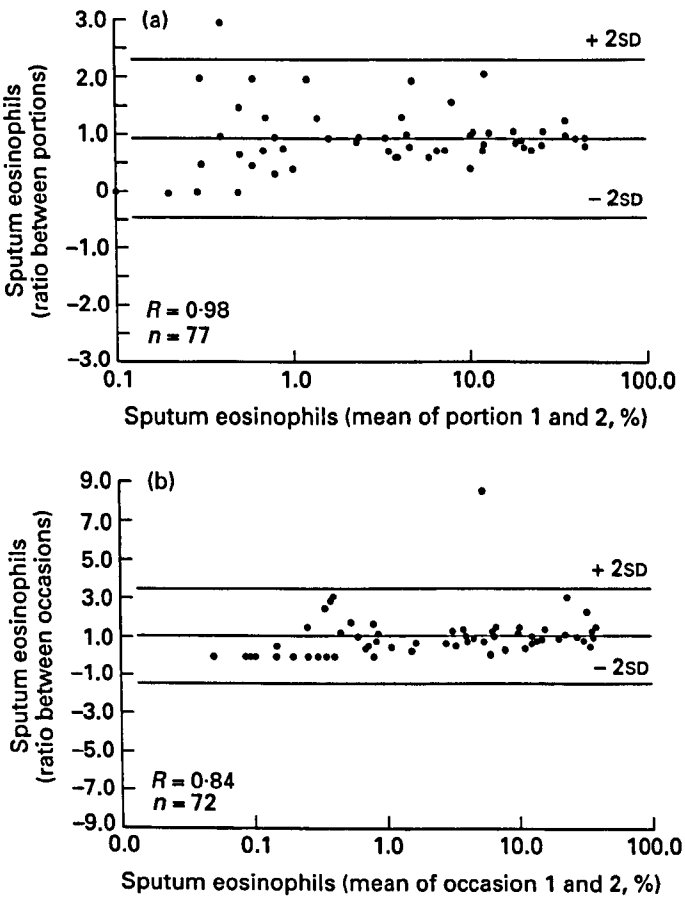

Fig. 2. Bland and Altman plot for sputum eosinophils (%). (a): mean value of two portions (portion 1 and portion 2) of the same sample compared with the ratio of the first (portion 1) and second (portion 2) count. (b): mean value of two different days (occasion 1 and occasion 2) compared with the ratio of the first (occasion 1) and the second (occasion 2) count. R is the global intraclass correlation coefficient. SD is standard deviation. *n* is the number of measures plotted included asthmatics, rhinitics and healthy subjects.. All 77 subjects (a) and 72 (b) are included but multiple points are not visible.

squamous cells was 4.8% ± 3.2 in asthmatics, 6.2 ± 4.0 in subjects with rhinitis, 6.6 ± 4.5 in normals. No correlation between the ratio of the two counts by the two observers for any cell type in adequate samples and squamous cells (%) contamination was found.

Repeatability

The overall repeatability (R) of cell counts whithin sample (two plugs of the same sample) was high for macrophages [0.86] and neutrophils [0.85](Fig. 1), eosinophils [0.98] (Fig. 2), lymphocytes [0.77], epithelial cells [0.83], total cells [0.92] and ECP [0.92]. The repeatability between samples (on two different days) was high for macrophages [0.76], neutrophils [0.75], eosinophils [0.84] (Fig. 2) and ECP [0.70], low for lymphocytes [0.39], epithelial cells [0.56] and total cells [0.44]. The repeatability between examiners (two examiners for the same slide) was high for all cells. The coefficients of repeatability are shown in Table 2.

In asthmatics the repeatability of cell counts within sample was high for macrophages [0.81], neutrophils [0.82], eosinophils [0.97], lymphocytes [0.79], epithelial cells [0.84], total cells [0.89] and ECP [0.91]. The repeatability between samples was high for macrophages [0.75], neutrophils [0.78], eosinophils [0,81], and ECP [0,70], low for lymphocytes [0.45], epithelial cells [0.53] and total cells [0.28]. The repeatability between examiners was high for all cells.

Table 2. Repeatibility of differential, total cell counts and ECP

	R/Bt (CI)	R/W (CI)	R/Ex (CI)
Total cell count	0.44 (0.27)	0.92 (0.88)	–
Macrophages	0.76 (0.66)	0.86 (0.80)	0.97 (0.96)
Neutrophils	0.75 (0.65)	0.85 (0.79)	0.98 (0.96)
Eosinophils	0.84 (0.77)	0.98 (0.97)	0.99 (0.98)
Lymphocytes	0.39 (0.21)	0.77 (0.68)	0.88 (0.82)
Epithelial cells	0.56 (0.41)	0.83 (0.76)	0.95 (0.93)
ECP	0.70 (0.53)	0.92 (0.88)	–

Data are expressed as interclass correlation coefficient (R) and the lower bound of confidence interval (CI) in parenthesis.
Bt, between two samples on two different days within 1 week; W, two plugs of the same samples; Ex, between two examiners.

Measurements in asthmatics, healthy subjects and subjects with rhinitis

Sputum from asthmatics, in comparison with the sputum of healthy subjects and subjects with rhinitis had higher eosinophils (asthmatics: 12.2% ± 12.9, rhinitis: 0.4 ± 0.8, normals: 0.4 ± 0.7) and ECP (asthmatics: 827 ± 491 μg/L; rhinitis: 127 ± 82 normals: 157 ± 203). There was no difference in total cell count between asthmatics and other two groups (asthmatics: 7.1 ± 8 × 10^5/mL; rhinitis: 8.2 ± 6; normals: 7.1 ± 3). No significant differences were found between healthy subjects and subjects with rhinitis. The values of cells (%) and standard deviations are shown in Fig. 3. There were no difference in viability (%) between asthmatics [71 ± 8], rhinitis [69 ± 6] and healthy subjects [73 ± 8].

Correlation between sputum cells and ECP or clinical measurements in asthmatics

There was a significant correlation between the percentage of sputum eosinophils and the concentrations of ECP ($r = 0.61$; $P = 0.005$). Eosinophil counts were positively correlated with Aas score ($r = 0.40$; $P = 0.003$) and inversely correlated with FEV_1 ($r = -0.37$; $P = 0.009$) expressed as percentage of predicted, but not significantly correlated with PC_{20} methacholine ($r = -0.28$). The correlation between percentage of eosinophils in sputum and number of blood eosinophils ($r = 0.26$) did not achieve statistical significance.

Discussion

In this study of reproducibility we examined the intraclass correlation coefficient R within sample, between sample, and between examiners on stable subjects taking into account sample size, number of examinations per patient and CI of the estimates. We have also confirmed the validity of the method comparing measurements among stable asthmatics, allergic rhinitis and healthy subjects.

We have found that the method is highly reproducible within sample and between examiners for all types of cells and fluid phase measurements of ECP. It is reproducible between sample for eosinophils, macrophages, neutrophils and ECP, but not for lymphocytes and weakly for epithelial cells.

Comparison of sputum measurements in asthmatics, healthy and subjects with seasonal rhinitis showed significant differences. Asthmatics had higher proportions of eosinophils and fluid phase levels of ECP compared to other groups. Healthy subjects did not differ significantly from subjects with seasonal rhinitis without hyperresponsiveness.

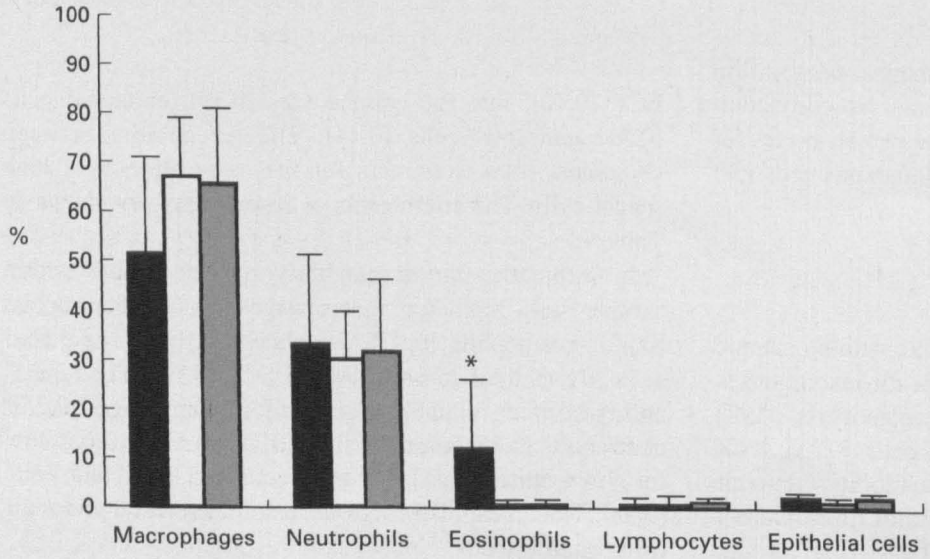

Fig. 3. Values (%) for cells and standard deviation in asthmatics (■), healthy subjects (□) and rhinitis (▨). * $P < 0.01$; eosinophils (%): asthmatics vs healthy subjects and rhinitis.

In asthmatics the proportion of eosinophils significantly correlated directly with ECP, score of severity and inversely with FEV_1% of predicted but not with methacholine PC_{20}.

This is the first study of repeatibility of sputum cell counts on cytospin that has measured the reproducibility with an adequate sample size justified statistically [7]. Moreover, it is the first large study that analyses repeatability within sample, between sample and between examiners in the same group of subjects.

Repeatability

Statistical calculations indicate that a sample of at least 50 subjects is required and 53 stable asthmatics were enrolled in the study. We have included data from two other groups of subjects (rhinitis and healthy) to evaluate also the validity of the method in assessing airway inflammation. We found good reproducibility for eosinophils, neutrophils, macrophages and ECP in line with the results of others [1,2,6]. Pizzichini *et al.* recently demonstrated excellent reproducibility, between specimens on 2 days within one week for macrophages, neutrophils, eosinophils, methacromatic cells and fluid-phase measurements [6]. We have also found reproducibility for epithelial cells moderate and low for lymphocytes. This last finding fits with the results of others [1,2,6]. They suggested that the low reproducibility of lymphocytes was probably due to the difficulty in the accurate recognition of this cell type. However, our good reproducibility for lymphocytes between examiners in the same subjects suggests that low numbers of cell in the specimen rather than the difficulty in the recognition is the main factor. In a large number of cases we did not count any lymphocytes in one sample while there were a low number of lymphocytes in the other specimen resulting in poor reproducibility in that subjects. Reproducibility of epithelial cell percentages has not previously been reported in cytospins but was also almost perfect in a study of sputum smears [1]. This was confirmed within sample but, as for lymphocytes between sample, repeatibility was moderate because of the relativity low numbers and the characteristic dumping which can occur leading to a group being counted in one slide but not on another one.

Reproducibility within sample has been reported in only one previous study with the selection of two plugs from the same spontaneous expectorate [1]. Plugs were analysed using the smears technique in 25 subjects included asthmatics, bronchitics and normals. The authors showed good reproducibility for macrophages, neutrophils, eosinophils, epithelial cells. We can confirm these data in a larger sample of induced sputum using cytospin. The practical implication of these data is that different plugs from the same sample are similar and representative of airway inflammation.

In this study we have also confirmed excellent reproducibility between examiners as already demonstrated by others [4,17]. This further confirms the accurate recognition of all cell types using the method of selection of portions of sputum, in particular taking in account samples with low squamous cells contamination. Induced sputum is a relatively new method for evaluating airway inflammation *in vivo*. Its strength lies in its lack of invasive nature and the ability to study large numbers of patients safely and repeatedly. These data suggest with adequate care the technique is reproducibile. It is important to note that a statistically valid number of subjects has been studied and the reproducibility is confirmed in a large number of stable asthmatics without including other subject groups.

Measurements in asthmatics, healthy subjects and subjects with rhinitis

In asthmatics the increase in proportion of eosinophils was similar to that reported in other studies using smear [1–4] whole sample of expectorate [3]and selected portions of sputum [6]. A single study has recently reported an increased proportions of neutrophils in stable asthmatics in comparison to healthy subjects [6]. We did not find this difference in line with previous report [1–4]. This is the first study to report data of induced sputum from pollen allergic rhinitis subjects studied out of the pollen season in the absence of airway hyperresponsiveness. Sputum cell composition of this group of subjects did not differ from healthy subjects, confirming the lack of airway inflammation as showed in a preliminary study by Foresi *et al.* [18].

In asthmatics, the increase in ECP confirms the results of earlier studies [3,6,19].

To exclude the possible interference of DTT with ECP assay, a standard curve using DTT was constructed and compared with the curve using normal saline. With DTT the standard curve retained the shape of the standard curve without DTT as demonstrated prevoiusly [20].

Correlation between sputum cells and ECP or clinical measurements in asthmatics

The validity of sputum examination was also evaluated by correlations between sputum eosinophils (which differentiated asthmatics from other groups) and asthma severity, FEV_1 and airway responsiveness. In line with previous study [6] our data showed a significant correlation between percentage of eosinophils and severity of asthma or FEV_1. In asthmatics we did not find any significant correlation between percentage of eosinophils and airway responsiveness to methacholine. In a previous study Pizzichini *et al.* [6] reported a significant inverse correlation between proportion of eosinophils and methacholine PC_{20} FEV_1

including asthmatics, healthy subjects and smokers but the significance disappeared when in the correlation only asthmatics were included. Also Iredale *et al.* [4] showed no correlation between eosinophilia of induced sputum and bronchial responsiveness to inhaled hypertonic saline. This is in line with recent morphological and functional studies have shown that airway hyperresponsiveness may be sustained by airway wall remodelling [21] and inability to dilate constricted airways [22]. Therefore, a close relationship between hyperresponsiveness and numbers of inflammatory cells in the airways should not be expected in a cross-sectional study.

Conclusions

The importance of this study is the confirmation, within important statistical guidelines for a study of reproducibility, that the methods examined are reproducible and valid. Taking in account previous studies [23–25] and considering sample size and the number of observations, induced sputum can be used to follow airway inflammation non-invasively in subjects with airway disease.

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