

Sputum processing: a new method to improve cytospin quality

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Summary

Background Sputum examination is being increasingly used as a non-invasive method for studying airway inflammation. However, the application of sputum still presents some methodological problems and the results of sputum analysis may be substantially flawed by salivary contamination, cell and mucus debris. In addition, much work is needed to deepen the possibility of extensive application of cell and molecular biology techniques to sputum analysis.

Objective In an attempt to improve the technique of sputum processing, we investigated the effect of: (i) 20 and 11 μ m filtration in addition to 40 μ m on salivary contamination; (ii) Percoll density gradient centrifugation on sputum slides quality; (iii) a culture medium (Minimum Essential Medium containing HEPES 22 mM, pH 7.4: MEM) as washing and suspension solution compared to PBS on cell viability.

Methods Induced sputum samples were obtained in 37 asthmatics. 21 samples were processed as selected sputum and 16 samples as entire expectorates. After dithiotreitol (DTT) homogenization, each specimen was aliquoted in two parts of equal volume. One portion was processed with the usual method, the other using a modified method: cell pellet was suspended in sterile MEM, filtered through 40, 20 and 11 μ m net filters and separated from the residual debris by Percoll gradient centrifugation.

Results As compared to the current sputum processing this method resulted in: (i) no selective bronchial cellular loss; (ii) a significant decrease of salivary contamination, particularly in entire expectorates in which squamous cells were reduced from 47 (36) to 15.5% (20) as median values and interquartile range; (iii) a higher proportion of good quality cytopins; (iv) maintenance of cell viability over the time (88% vs. 81% in MEM and PBS, respectively) 1 h after sample collection.

Conclusion In the present study we demonstrated that the proposed method is feasible and makes it possible to overcome most of the technical limits met with the commonly used method, pointing to a potential extension of induced sputum application for more sophisticated techniques.

Keywords: sputum processing, airway inflammation

Submitted 15 June 2001; revised 22 October 2001; accepted 15 November 2001

Introduction

The definition of asthma as a chronic inflammatory disease [1] has brought research to focus on the characteristics of inflammatory response. An area of particular interest is the research into the cell and molecular biology of asthma. Cells involved in the chronic airway inflammation of asthma have been obtained from lung tissues and bronchoalveolar lavage (BAL). Application of polymerase chain reaction (PCR), *in situ* hybridization (IHS), immunocytochemistry (ICC), and flow cytometry (FC) are now of common use. Recently, the less-invasive method of obtaining airway cells by induced sputum has become widespread in the assessment of airway inflammation [2,3]. Much research has been devoted to characterizing sputum

cytological and biochemical (ECP, tryptase, MPO, etc.) profile in asthma [2–7]. Current data indicate that induced sputum represents a valid and responsive method for sampling airways safely, reproducibly and serially in the clinical investigation of chronic airway inflammation in asthma [2–8]. However, the application of induced sputum still presents some methodological problems, such as the most appropriate technique of induction and processing of sputum [9]. In addition, much work is needed to deepen the possibility of the extensive application of cell and molecular biology techniques to sputum analysis. Once an adequate number of bronchial cells has been obtained by means of induced sputum, the results of sputum analysis may be substantially flawed by salivary contamination, cell and mucus debris [10–13]. The excessive presence of squamous cells, mucus and cell debris may be responsible for the poor quality of cytopins and represent a limit for the application of additional techniques on the sputum cell fraction, including PCR, IHS, ICC and FC [10–16]. Two basically different techniques of sputum processing are currently in use. The analysis of sputum samples is performed either (i) on whole

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expectorate (the patient being instructed to discard saliva before spitting sputum) [17] or (ii) on selected sputum (all the viscid portions of the sample) [5]. Selected sputum processing has the advantage of improving the quality of cytospin slides by considerably limiting salivary contamination [3,11,13]. However, some methodological issues deserve mention. First, the collection of all the more viscid portions of the sputum, instead of a few plugs free from salivary contamination as in earlier works [4,18] may result in an increased contamination of the sputum cell preparations with cell debris, mucus and fibres [12]. Second, salivary contamination may be relevant not only in homogeneous samples, but also in those specimens in which viscid portions are evident and selected out. In fact portions separated from saliva by macroscopic examination may display a non-negligible percentage of squamous cells in differential cell counts [12]. Third, the physical properties of some samples make it difficult to separate sputum from saliva even if a stereoscopic or inverted microscope is used. As a result salivary contamination, residual mucus and debris in whole sputum and, in a minority of cases in selected sputum, may (i) determine the exclusion of some samples from the analysis; (ii) make reliable cell identification difficult [12,19] and (iii) hamper the adequacy of sputum specimens for FC, ICC, ISH and PCR [12,14–16,20].

Finally, as good cell viability is needed to obtain reliable cell counts and apply other techniques (PCR, ICC, ISH and FC) [13–16,19,21] it is crucial that any modification in the method does not cause a significant decrease in cell viability.

Thus, in an attempt to improve the technique of sputum processing, we have investigated the effect of (i) additional filtration (20 and 11 µm nylon net filters) on salivary contamination; (ii) Percoll density gradient centrifugation on debris both in whole and in selected sputum and (iii) a culture medium on cell viability as washing and suspension solution compared to PBS.

Materials and methods

Subjects

Thirty-seven asthmatics with mild to moderate chronic asthma (15 males, 22 females; mean age 51.7 years) participated in the study (Table 1).

Asthma was characterized by episodes of dyspnea with wheezing; variable airflow limitation with reversible obstruction ($\geq 20\%$ increase in FEV₁ after inhalation of 200 µg of

salbutamol) and positive response to inhalation challenge with methacholine (PC₂₀FEV₁ ≤ 8 mg/mL).

Study design

In- and outpatients were screened by questionnaire, spirometry, methacholine inhalation test and allergy skin prick tests. All of the sputum from asthmatic patients was collected as induced sputum. The sputum specimens were divided into two portions of equal volume after DTT-treatment and were analysed using two different methods. Method 1: suspension with PBS and 40 µm filtration; Method 2: suspension with Minimum Essential Medium containing HEPES 22 mM, pH 7.4 (MEM), 40, 20 and 11 µm filtration and Percoll density gradient centrifugation.

Cell counts were performed blind to sputum or subject characteristics.

Clinical methods

Subjects' characteristics were documented by questionnaire. Baseline pulmonary function testing was performed by measuring static and dynamic lung volumes.

Methacholine inhalation tests were carried out according to the method described by Sterk and coworkers [22]. Allergy tests, including a battery of common aeroallergen extracts, were performed with the skin prick method.

The study protocol was approved by the local Ethics Committee.

Induction and analysis of sputum

The induction of sputum was performed according to Ronchi et al. [7]. Ten minutes after salbutamol inhalation (200 µg), hypertonic saline was nebulized with an ultrasonic nebulizer (Fisoneb) and was inhaled for 5-min periods up to 20 min. The concentration of saline was increased at intervals of 10 min from 3% to 4%. FEV₁ was measured every 5 min during inhalation of hypertonic saline solution. Subjects were asked to collect saliva in a separate bowl and every 5 min to try to cough sputum into a Petri dish.

Sputum processing

Sputum samples were processed soon after collection to prevent the sputum liquid phase absorption by the mucus and possible consequent increase in salivary cell contamination.

Selected sputum

Method 1 All the viscid portions were selected from sputum. The weight in mg of selected portions was determined and a multiple volume in µL of dithiotreitol (Sputolysin, Calbiochem, San Diego, CA, USA) equal to nine times the weight was added (DTT final concentration 0.09%, 5.85 mM). The samples were shortly mixed by vortex and softly shaken at 37 °C for 20 min. After incubation the mixture was aliquoted in two parts of equal volume. After centrifugation at 400 × *g* for 5 min and supernatant discard, one of the two portions was processed as previously described [3–5]. Briefly, the cell pellet was resuspended in PBS and filtered through a 40 µm nylon net filter (Millipore, Bedford, MA, USA). Total cell count was determined using a Burkner chamber. Cell viability was assessed using acridine orange-ethidium bromide (Sigma, Bedford, MA, USA) – which stains viable cells green and non-viable

Table 1 Characteristics of subjects

Subjects, (n)	37
Age (average age in years)	51.7 (17.5)
Sex (males/females)	15/22
Atopy*	24
FEV ₁ (% pv)	76 (24)
FEV ₁ /VC (%)	63 (22.3)
Pc20FEV ₁ †	0.74 (0.08–7.6)

Values are mean and $\pm$ SD or †geometric mean and range. *Defined as one or more positive allergy skin prick tests. FEV₁ = forced expiratory volume in 1 s; VC = vital capacity; Pc20FEV₁ = provocative concentration of methacholine causing a 20% fall in FEV₁.

orange differentially. Cells were air-dried and stained with May – Grünwald-Giemsa stain.

Method 2 The other aliquot was processed with a modified method. In method 2 the cell pellet was suspended in sterile MEM (Sigma, St Louis, MO, USA) and centrifuged at $400 \times g$ for 5 min. The supernatant was discarded and the cell pellet was resuspended in MEM. Cell suspensions were filtered through a 40, 20 and subsequently 11 μm nylon net filter (Millipore). A 20 μm filtration was performed with the aim of reducing the debris before final filtration. After filtration the cells were separated from the residual debris using discontinuous Percoll gradient centrifugation. Isosmotic Percoll was prepared by mixing nine parts of stock Percoll (Pharmacia, Uppsala, Sweden) with one part of 10-fold concentrated MEM. Working Percoll of 1.02 density was obtained by further addition of MEM. Cell suspension was gently stratified on top of Percoll 1.02 using a pasteur pipette. After centrifugation at $400 \times g$ for 7 min the cells were found to be at the bottom of the tube, while the debris was at the top of the Percoll. Finally the cell pellet was recovered using a pipette, and washed with MEM. Three sputum slides were prepared for differential count by cytocentrifugation after 40 and 11 μm filtration and density gradient centrifugation. Cells were air-dried and stained with May – Grünwald-Giemsa stain.

Total cell count was determined using a Burkert chamber after 40 and 11 μm filtration and Percoll; cell viability was assessed using acridine orange-ethidium bromide (Sigma, Bedford, MA, USA) immediately after the first suspension in PBS (method 1) or MEM (method 2), 1 and 2 h later. Cell differentials were determined by counting 200 non-squamous cells on each sputum slide.

Entire sputum

Sixteen homogeneous sputum samples were examined as entire samples.

The volume of sputum samples was determined and an equal volume of DTT (0.1%) was added. We then used the procedure described previously.

Sputum slide quality

According to Pizzichini et al. [11] a score of the quality of cell preparation on the slide was determined. Moreover we scored the degree of salivary contamination on the basis of the proportion of squamous cells on the slides. The score varied from a minimum of 4 to a maximum of 12 and was based on: debris (nil = 3, moderate = 2, excessive = 1); cell outline (preserved = 3, isolated cell damage = 2, most cells damaged = 1); degree of salivary contamination (0–10% = 3; 10–40% = 2; > 40% = 1); and overall impression (good = 3, acceptable = 2, bad = 1). A total score ranging from 4 to 6 indicated poor quality, 7–9 acceptable quality and 10–12 good quality of cell preparation on the slide.

Data analysis

Data with normal distribution were reported as arithmetic mean and standard deviation, while data with non-normal distribution were expressed as median and interquartile range. Differences in cell viability between MEM and PBS treated aliquots were assessed by multiple analysis of variance for repeated measurements. The comparison between samples

and single-time cell viability was assessed by Wilcoxon's signed rank test. Mann–Whitney *U*-test and χ^2 were used to assess the differences between groups. The repeatability of measurements was examined by the intraclass correlation coefficient (ICC) [23] and graphically reported as proposed by Bland and Altman [24].

Results

Stepwise filtration and density gradient centrifugation determined a mean total cellular loss of 28%. The median value of total cell count was initially (after the 40 μm mesh filtration) 2.4×10^6 (ranging from 0.28 to 60×10^6), whereas after Percoll centrifugation (last step of method 2) it was 1.9×10^6 (ranging from 0.19 to 46×10^6). Conversely no selective bronchial cellular loss was observed. In fact, the differential cell count was not different between method 1 and 2: the median values and IQ were 1 (5.7) and 1.5% (4.6) for eosinophils, 41.8 (45.2) and 49.1% (42.1) for neutrophils, 40.3 (44) and 43.1% (43.9) for macrophages, 0 (0.9) and 0% (0.5) for lymphocytes, respectively.

The overall repeatability of cell counts between samples was high for eosinophils, neutrophils and macrophages (Fig. 1). The ICC was 0.99 for eosinophils, 0.98 for macrophages and neutrophils, and 0.81 for lymphocytes.

Salivary cell contamination

In the slides obtained from selected sputum the median values of squamous cell contamination were 10% (14) with method 1 vs. 1% (6) ($P < 0.001$) with method 2, while in the entire sputum median salivary contamination was reduced from 47% (36) to 15.5% (20) using method 2 ($P < 0.001$) (Fig. 2).

As shown in Fig. 2, the 16 samples examined as entire sputum displayed a percentage of squamous cells ranging from 25 to 86%, while after stepwise filtration salivary contamination ranged from 2 to 48% ($P < 0.001$). Adequate sputum cytopspins (salivary contamination $\leq 40\%$) were obtained in seven out of 16 samples with method 1, while 13 cytopspins out of 16 were adequate using method 2 ($P < 0.05$). In a preliminary study, performed in 20 samples processed as entire sputum, we found a significant decrease in salivary contamination both after 20 and 11 μm filtration, with a mean reduction of 74% and 86%, respectively ($P < 0.0001$).

Percoll density gradient centrifugation

Percoll density gradient centrifugation resulted in better quality cytopspins both in selected ($P < 0.01$) and in entire sputum ($P < 0.001$), because of the reduction of residual fibres and debris (Fig. 3). The median quality score was 8 with method 1, and 10 with method 2 ($P < 0.0001$). The elimination of the majority of residual fibres and debris associated with the reduction of squamous cells due to filtration, not only resulted in a higher proportion of cytopspins of good quality slides ($P < 0.03$), but also in a higher proportion of countable cytopspins (Table 2). As a result of stepwise filtration and Percoll centrifugation 11% of cytopspins were defined as poor, compared with the 35% of method 1 (Table 2).

Cell viability

Due to the increased time needed to perform method 2 (about 30 min more than method 1) we evaluated the protective effect

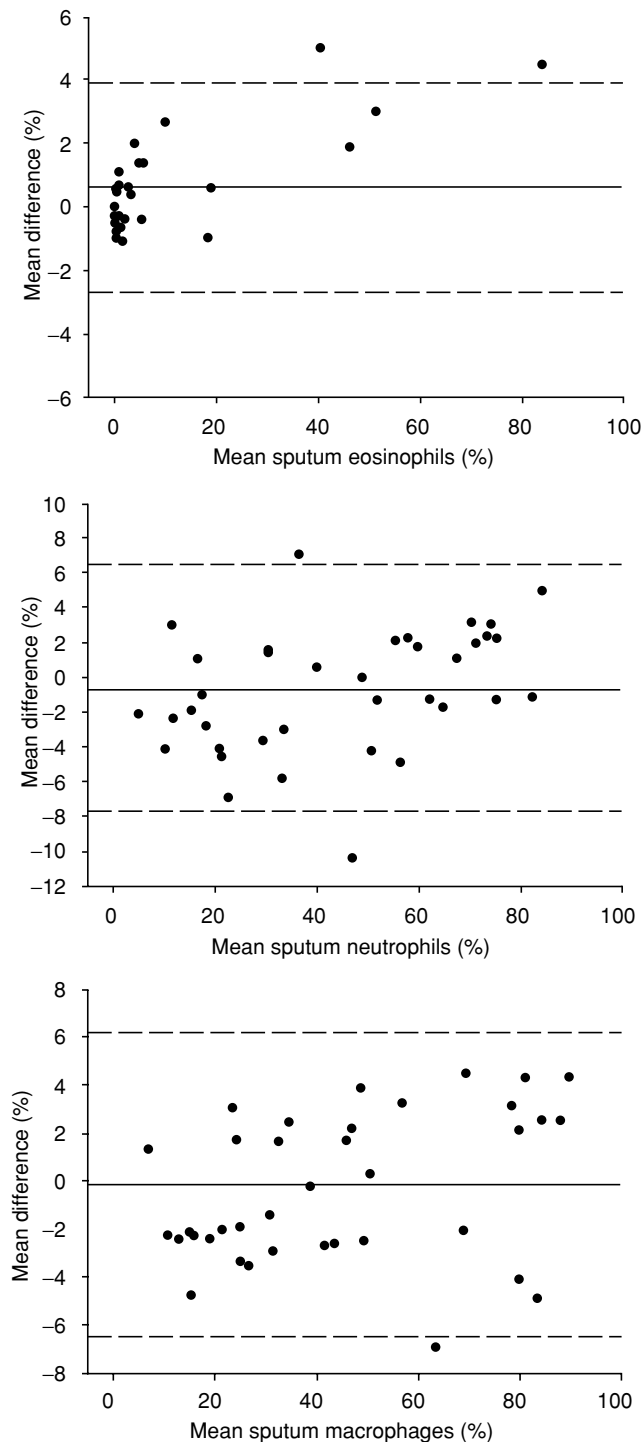


Fig. 1. Bland-Altman plots showing mean of cell differential counts against their mean difference. Continuous lines represent the mean difference in cell counts between the two methods and the dashed lines represent ± 2 SD. ICC: intraclass correlation coefficient.

of MEM over the time by suspending an aliquot of the cell pellet in PBS and MEM before the filtration steps. Cell viability was assessed three times: immediately after the first suspension in PBS or MEM (time 0); 1 and 2 hours later (time 1 and time 2, respectively). A time-related decrease of viability was observed in both aliquots, but at time 1 and time 2 cell viability was

Table 2 Cytospin slides quality

	Good No. of slides (% of total samples)	Medium No. of slides (% of total samples)	Poor No. of slides (% of total samples)
Method 1	10 (27%)	14 (38%)	13 (35%)
Method 2	18 (48%)	15 (41%)	4 (11%)

Method 1: suspension with PBS and 40 μ m filtration; Method 2: suspension with MEM, 40 μ m, 20 μ m and 11 μ m filtration and Percoll centrifugation. χ^2 : $P < 0.03$.

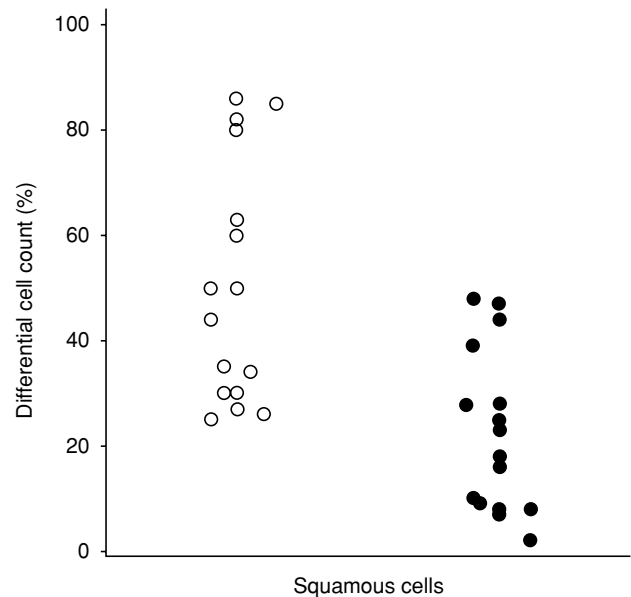


Fig. 2. Squamous cell percentages from two aliquots obtained from the same DTT-treated sputum specimen processed as entire sputum. Open circles: method 1 (suspension with PBS and 40 μ m filtration); closed circles: method 2 (suspension with MEM, 40 μ m, 20 μ m and 11 μ m filtration and Percoll centrifugation).

significantly higher in MEM-suspended samples than in PBS-suspended samples (88 vs. 81% and 84 vs. 73% as median values, respectively, $P < 0.001$). MEM was also able to preserve cell viability throughout sputum processing in spite of the longer time needed for method 2.

The median values of cell viability evaluated in a subset of randomly selected samples (10 out of 31) were 90.7% after 40 μ m filtration and 88% after 11 μ m filtration ($P = \text{ns}$)

Discussion

In this study we compared a usual method of sputum processing [3,5] with the modified method described above, by dividing every sputum sample into two aliquots after DTT homogenization. Compared with the standard technique, the aliquots processed with the modified method resulted in a higher slide quality score and an increase in adequate cytopins. A significant decrease in the percentage of squamous cells was observed particularly in those specimens treated as entire sputum as a consequence of their physical properties.

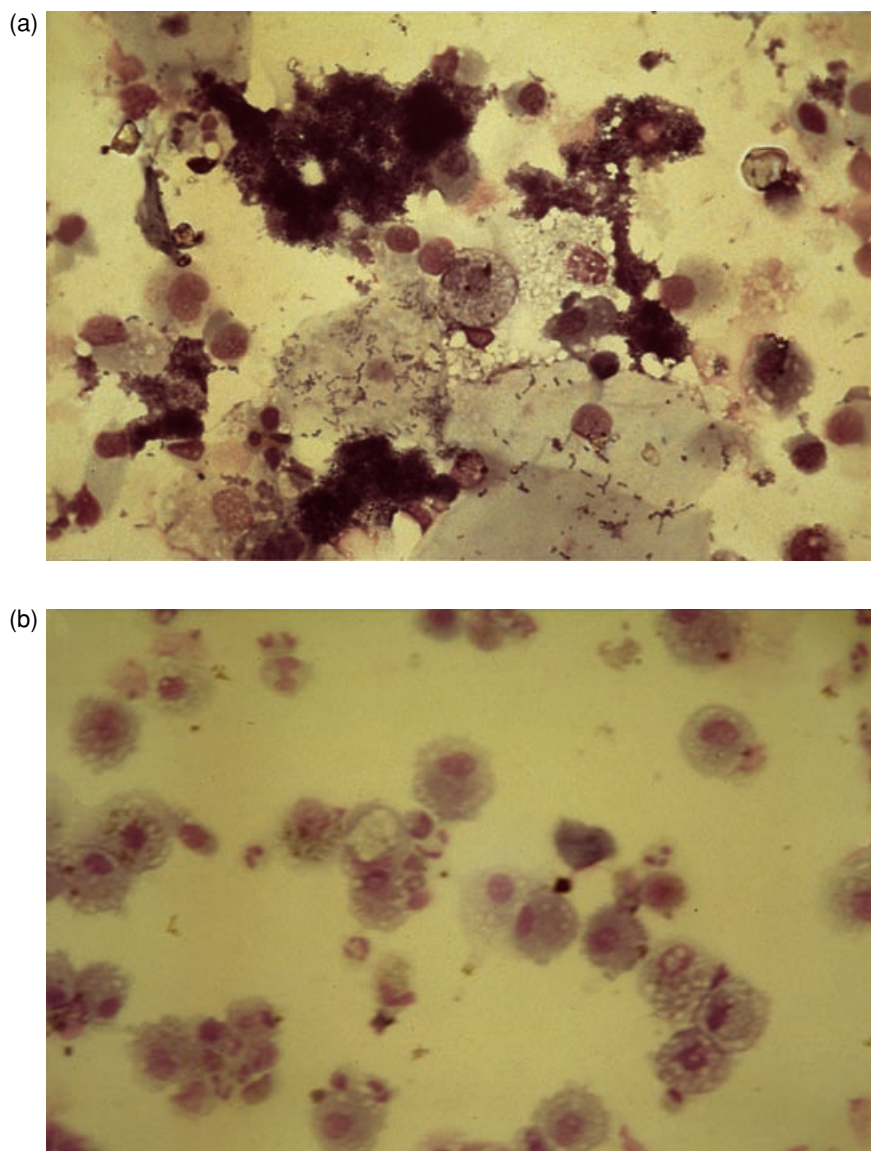


Fig. 3. Cytospins obtained from the same sputum sample, illustrating (a) a poor quality cytospin (Method 1) and (b) a high quality one (Method 2).

Stepwise filtration

One of the criteria to define the suitability of cytospin slides for differential cell counts is the amount of salivary contamination. In fact the large squamous cells occupy most of the areas of the cytospin slides tending to overlie the leucocytes, thus preventing a reliable cell count and interpretation [12,13,19,20]. This problem may be solved in the majority of cases by means of the analysis of sputum-selected portions [11]. Different limits have been stated to define an adequate selected sputum with respect to salivary contamination, ranging from 20 to 50% [6,12,20]. Even though the selected sputum method was able to effectively minimize salivary contamination, recent studies showed that a percentage of samples, ranging from 13% [5] to 17% [12] displayed a not negligible salivary contamination. In addition the selected sputum method may imply the exclusion of some liquid, homogeneous specimens or specimens in which the selection of plugs is difficult due to physical properties. In an

attempt to minimize salivary contamination we performed two additional filtrations with respect to the standard procedure of sputum processing. Stepwise filtration, without affecting differential cell counts, was able to significantly decrease the percentage of squamous cells on slides, allowing a more accurate reading and an increase in adequate sputum cell preparations. This result is particularly important in the entire sputum method in which the salivary contamination, even after separate collection of saliva and the rinsing of the mouth and throat with water before sputum collection, has a wide range with the upper limit close to 70% [25].

Percoll density gradient centrifugation

Selected sputum processing has the advantage of improving the quality of cytospin slides [11] basically as a result of reduced salivary contamination. However, an accurate reading of cell preparations on cytospins may also be hampered by the presence of debris and mucus [10,12]. Centrifugation in Percoll is

commonly used for purification of various cell types as well as cell organelles [26]. The density of the centrifugation medium can be adjusted by different concentrations of Percoll, permitting separation by density of cells from debris and mucus. In a previous work Ukkonen et al. [27] described the preparation of nasopharyngeal secretions and of a small number of sputum samples by one-step centrifugation through Percoll. However, this study showed only preliminary data on sputum and it was not aimed at investigating the effects of the Percoll centrifugation on sputum cell preparations. In the present study we applied Percoll density centrifugation both to selected and entire sputum to investigate the feasibility of the method. We were able to demonstrate that the Percoll method is feasible and effective in separating the cells from debris, residual fibres and mucus. Thus, when compared with the standard procedure, the Percoll method resulted in a higher quality slide score and in an increased number of countable cytospin slides, in particular when entire sputum was under examination. Moreover as the presence of mucus and cell debris is a limiting factor in the application of ICC, ISH, PCR and FC [12,16] the Percoll centrifugation method may be successfully employed in preparation of sputum samples for these techniques.

Cell viability

Previous studies reported a wide range of values for cell viability. In selected DTT-treated sputum induced in asthmatic patients, cell viability ranged from 66 (11) to 79.5% (2.1) as median values [28,29]. In general a lower cell viability was found in spontaneous sputum both in asthma and COPD. In the study by Pizzichini et al. [29] the median value of cell viability was 47% in spontaneous sputum in comparison with 77% in induced sputum obtained from the same subjects. We found a good cell viability and it was crucial that the modifications of the method did not cause a significant decrease in cell viability. We demonstrated that cell viability was well maintained by the use of a culture medium as washing and suspension solution. MEM is a culture medium well-suited for the growth and the maintenance of a broad spectrum of mammalian cells. Therefore its constituents can provide an optimal environment for bronchial cell preservation. Moreover the presence of HEPES 22 mM in the medium can stabilize the pH of cell suspension with respect to fluctuations resulting from changes in cellular metabolism and environment. In this way cell suspensions are adjusted to a pH of 7.3–7.4, which contributes to the maintenance of a good cell viability. Due to its characteristics MEM was used instead of PBS as an optimal medium for cell preservation. Our results showed that: (i) cell viability was higher in the aliquots suspended with MEM as compared to those maintained in PBS both 1 and 2 h after sputum collection and (ii) MEM was able to maintain cell viability throughout sputum processing, even though a more laborious procedure is needed for this method. This result may be relevant not only for achieving an accurate cell identification on slides, but also for allowing the use of sputum as a source of bronchial inflammatory cells for more complex and time-consuming techniques than differential cell count [13–16,19]. In fact the maintenance of an acceptable cell viability is the prerequisite for introducing any modification to the standard technique of sputum processing.

With regard to shortcomings, this method is more time-consuming than the usual one, and it may determine some total cellular loss. Because of this, it can be particularly useful

for samples with relevant contamination by squamous cells and debris and when sputum cells are used for more sophisticated techniques.

In conclusion, the parallel treatment of sputum specimens both by the standard technique and by the proposed method, indicates that the majority of causes associated with a poor quality slide, i.e. high squamous cell contamination, presence of debris and residual mucus, may be solved by our method. Stepwise filtration and Percoll centrifugation not only makes it possible to overcome most of the technical limits arising in the commonly used method, but make the application of sputum analysis to more sophisticated techniques easier.

Acknowledgements

Supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica of Italy and from the University of Florence.

References

- 1 NHLBI/WHO. Workshop Report 1995: Global strategy for asthma management and prevention. Bethesda, MD: National Institutes of Health, National Heart, Lung, and Blood Institute, Publication no. 95-3659.
- 2 Hargreave FE, Pizzichini MMM, Pizzichini E. Assessment of airway inflammation. In: Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ, eds. *Asthma*. Philadelphia: Lippincott-Raven Publishers 1997, 1433–50.
- 3 Pavord ID, Pizzichini MMM, Pizzichini Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax* 1997; 52:498–501.
- 4 Pin I, Gibson PG, Kolendowicz R et al. Use of sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47:25–9.
- 5 Pizzichini E, Pizzichini MMM, Efthimiadis A et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Care Medical* 1996; 154:308–17.
- 6 Spanevello A, Migliori GB, Sharara A et al. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997; 27:1138–44.
- 7 Ronchi MC, Piragino C, Rosi E et al. Do sputum eosinophils and ECP relate to the severity of asthma? *Eur Respir J* 1997; 10:1809–13.
- 8 Tarodo de la Fuente P, Romagnoli M, Godard P, Bousquet J, Chanez P. Safety of inducing sputum in patients with asthma of varying severity. *Am J Respir Crit Care Med* 1998; 157:1127–30.
- 9 Djukanovic R. Induced sputum – A tool with great potential but not without problems. *J Allergy Clin Immunol* 2000; 105:1071–3.
- 10 Fahy J. A safe simple, standardized method should be used for sputum induction for research purposes. *Clin Exp Allergy* 1998; 28:1047–9.
- 11 Pizzichini E, Pizzichini MMM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996; 9:1174–80.
- 12 Maestrelli P, Calcagni PG, Saetta M et al. Integrin upregulation on sputum neutrophils in smokers with chronic airway obstruction. *Am J Respir Crit Care Med* 1996; 154:1296–300.
- 13 Efthimiadis A, Pizzichini MMM, Pizzichini E, Kolendowicz Weston S, Dolovich J, Hargreave FE. The influence of cell viability and squamous epithelial cell contamination on the reliability of sputum differential cell counts. *Am J Respir Crit Care Med* 1995; 151:A384.

- 14 Hansel TT, Braunstein JB, Walker C et al. Sputum eosinophils from asthmatics express ICAM-1 and HLA-DR. *Clin Exp Immunol* 1991; 86:271–7.
- 15 Kidney JC, Wong AG, Efthimiadis A et al. Elevated B-cells in sputum of asthmatics: close correlation with eosinophils. *Am J Respir Crit Care Med* 1996; 153:540–4.
- 16 Riley RS, Mahin EJ, Ross W, Perfetto S, Posekany K. Techniques of cellular analysis. In: Riley, RS, Mahin, EJ, Ross, W, eds. *Clinical Applications of Flow Cytometry*. New York: Igaku-Shoin Medical Publisher, Inc. 1993, 195–250.
- 17 Gershmann NH, Wong NH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. *Eur Respir J* 1996; 9:2448–53.
- 18 Gibson PJ, Girgis-Gabardo A, Morris MM et al. Cellular characteristics of sputum from patients with asthma and chronic bronchitis. *Thorax* 1989; 44:693–9.
- 19 Ward R, Woltmann G, Wardlaw AJ, Pavord ID. Between observer repeatability of sputum differential cell counts. Influence of cell viability and squamous cell contamination. *Clin Exp Allergy* 1999; 29:248–52.
- 20 Renaud L, Shute J, Biagi S et al. Cell infiltration, ICAM-1 expression, and eosinophil chemotactic activity in asthmatic sputum. *Am J Respir Crit Care Med* 1997; 155:466–72.
- 21 Woltmann G, Ward RJ, Symon FA, Rew DA, Pavord ID, Wardlaw AJ. Objective quantitative analysis of eosinophils and bronchial epithelial cells in induced sputum by laser scanning cytometry. *Thorax* 1999; 54:124–30.
- 22 Sterk PJ, Fabbri LM, Quanjer PH et al. Airway Responsiveness. *Eur Respir J* 1993; 6 (Suppl. 16):53–83.
- 23 Chinn S. Repeatability and method comparison. *Thorax* 1991; 46:454–6.
- 24 Bland JM and Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 8:307–10.
- 25 Grootendorst DC, Sont JK, Willems LNA et al. Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allergy* 1997; 27:769–79.
- 26 Perfort HT, Laurent C, Laas TL, Kågedal L. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). *Anal Biochem* 1978; 88:271–82.
- 27 Ukkonen P and Julkunen I. Preparation of nasopharyngeal secretions for immunofluorescence by one-step centrifugation through Percoll. *J Virol Meth* 1987; 15:291–301.
- 28 Efthimiadis A, Pizzichini MMM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiotreitol vs phosphate-buffered saline. *Eur Respir J* 1997; 10:1336–40.
- 29 Pizzichini MMM, Popov TA, Pizzichini E et al. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996; 154:866–9.