Eur Respir J 1998; 11: 529–533
DOI: 10.1183/09031936.98.11030529
Printed in UK - all rights reserved

European Respiratory Journal
ISSN 0903 - 1936

PERSPECTIVE

Methods of examining induced sputum: do differences matter?

J.C. Kips, R.A. Peleman, R.A. Pauwels

Methods of examining induced sputum: do differences matter? J.C. Kips, R.A. Peleman, R.A. Pauwels. ©ERS Journals Ltd 1998.

ABSTRACT: Analysis of induced sputum has been proposed as a direct, relatively noninvasive method for the evaluation of airway inflammation in diseases such as asthma or chronic obstructive pulmonary disease (COPD). An important question in the validation of this technique concerns the potential influence of differences in the methods of examining sputum.

Up to the present time, two basic techniques for processing sputum have been described. The first approach consists of selecting all viscid portions from the expectorated sample, whereas the second approach processes the whole expectorate, containing sputum plus saliva. Both processing techniques have been shown to provide valid and reliable data on the composition of the cellular and soluble fraction of induced sputum.

From the data currently available, it would therefore appear that the usefulness of induced sputum as a method for assessing airway inflammation is not influenced by differences in the methods currently used for examining sputum.

Eur Respir J 1998; 11: 529–533.

Dept of Respiratory Diseases, University Hospital Ghent, Belgium

Correspondence: J.C. Kips
Dept of Respiratory Diseases
University Hospital Ghent
De Pintelaan 185
B-9000 Ghent
Belgium
Fax: 32 92402341

Keywords: Asthma
chronic obstructive pulmonary disease
sputum

Received: July 28 1997
Accepted after revision November 5 1997

Analysis of induced sputum has been adopted over the past few years as a relatively noninvasive method for the evaluation of airway inflammation in diseases such as asthma and chronic obstructive pulmonary disease (COPD). So far, no "gold standard" for the induction and processing of sputum has been agreed upon, and differences in methodology exist between various groups.

An important question, therefore, is whether these differences in methodology influence the validity and reliability of induced sputum as a marker of airway inflammation.

Sputum induction

When sputum cannot be obtained spontaneously, it can be induced by inhalation of an aerosol of hypertonic saline. Two slightly different approaches for induction have been used: the first consists of inhaling the same (3–4.5%) or increasing (3, 4 and 5%) concentrations of aerosolized hypertonic saline over fixed time periods [1–3], whereas the second consists of inhaling the same concentration of hypertonic saline (4.5%) over increasing time periods [4]. Choice of technique does not seem to influence the differential cell count in sputum. It has been suggested that, irrespective of the technique used, changes could occur in the cellular and biochemical composition of sequential samples collected throughout the induction procedure [5, 6]. Although these preliminary data have not all been confirmed [7], it would therefore seem advisable to standardize the duration of sputum induction.

PIZZICHINI *et al.* [8] compared spontaneously produced sputum to sputum samples induced by inhaling increasing concentrations of hypertonic saline. Both samples were collected on the same day. This study revealed that spontaneously produced sputum contains less viable cells, produces cytospins of poorer quality and shows a higher degree of squamous cell contamination. However, the differential cell count correlated quite well with the induced sample (*e.g.* intraclass correlation coefficient of 0.83 for eosinophil counts). The correlation decreased once cell viability was less than 50% in the spontaneously produced sample. Soluble mediators, such as eosinophil cationic protein (ECP) or fibrinogen, were slightly higher in spontaneously produced sputum.

Irrespective of the induction technique used, the challenge procedure should be performed in a standardized way that includes the necessary safety procedures, as hypertonic saline can cause severe airway constriction in asthmatic subjects. Airway calibre should, therefore, be monitored throughout the induction period. In addition, unless sputum induction and assessment of bronchial responsiveness to hypertonic saline are performed simultaneously, subjects should be pretreated with inhaled short-acting β_2-agonists. It has been shown that obtaining sputum from the same asthmatic subjects with or without pretreatment with salbutamol, does not influence the cellular composition. In contrast, the mean fall in forced expiratory volume in one second (FEV_1) after placebo was 20.7% as opposed to 8% after salbutamol pretreatment [9]. Another element which can increase safety, especially when moving from mild to more severe asthma, is to challenge with isotonic, as opposed to hypertonic saline [10]. Again, it has been reported that this does not alter the cellular distribution of the sputum sample [9, 11]. The only disadvantage of isotonic saline is that the success rate of inducing a sufficient sample might be lower, especially in mild asthma.

It also has been shown that delivering the aerosol with an ultrasonic nebulizer, as opposed to a jet nebulizer, yields a higher success rate, and that the choice of ultrasonic nebulizer does not influence the differential cell count [9]. This difference in success rate seems to be predominantly related to differences in particle size. Finally, using a mouthpiece and noseclip, as opposed to a face mask, for sputum induction has no effect on the sputum composition [12].

Sputum processing

Once a sputum sample has been obtained, it should be processed as soon as possible. Basically, two techniques for processing have been described. The first approach, which effectively minimizes contamination with saliva, consists of selecting all viscid or more dense portions from the expectorated sample [1, 13]. The second approach processes the whole expectorate, comprising sputum plus saliva [2]. An adaptation of this method consists of trying to collect sputum and saliva separately, so as to reduce the salivary contamination of induced sputum [14, 15]. Whatever technique is used, the sample is liquified by adding 0.1% dithiothreitol (DTT), which has been shown to be more effective in dispersing cells than the addition of saline [16].

The resulting mixture can then be used for all purposes, including assessment of cell viability by trypan blue exclusion, total cell counts in a haemocytometer or Courter counter and differential cell counts on stained cytospins. Both processing methods also allow for additional techniques to be performed on the cell fraction of sputum, including polymerase chain reaction (PCR), immunocytochemistry or flow cytometry [17–19]. Moreover, the supernatant of sputum, processed in either way, can be used for measurement of a variety of soluble mediators, including eosinophil-derived proteins, tryptase, myeloperoxidase, deoxyribonucleic acid (DNA), albumin, fibrinogen, nitric oxide (NO) derivatives and cytokines, such as interleukin (IL)-5, IL-8 or tumour necrosis factor-α (TNF-α) [2, 3, 10, 14, 20–26] (table 1). However, it has to be borne in mind that DTT can affect the expression of cellular markers [27], or measurements of soluble mediators in sputum. This needs to be evaluated for each assay, as this effect varies for different mediators. For example, treatment of sputum with DTT as opposed to saline has been shown to increase ECP levels, but not to affect IL-5 or IL-8 concentrations in the sputum supernatant [16].

Table 1. – Review of some soluble markers in supernatant of sputum from patients with asthma or chronic obstructive pulmonary disease (COPD)

Soluble markers	[Ref.]
Cytokines	
Tumour necrosis factor-α (TNF-α)	[14]
Interleukin-5 (IL-5)	[10, 24]
Interleukin-8 (IL-8)	[14]
Granulocyte/macrophage colony-stimulating factor (GM-CSF)	[24]
Eosinophil-derived mediators	
Eosinophil cationic protein (ECP)	[2, 3, 21]
Eosinophil-derived neurotoxin (EDN)	
Eosinophil peroxidase (EDP)	
Neutrophil-derived mediators	
Myeloperoxidase (MPO)	[22, 23]
Human neutrophil lipocalin (HNL)	[22, 23]
Mast cell-derived mediators	
Histamine	[2, 25]
Tryptase	[2, 25]
Others	
Fibrinogen	[2, 21]
Albumin	[2, 21]
Mucin-like glycoprotein	[20]
Deoxyribonucleic acid (DNA)	[20]
Nitric oxide (NO) derivatives	[26]
Intercellular adhesion molecule-1 (ICAM-1)	[25]

Validity and reliability of induced sputum

Important issues that need to be addressed when evaluating a new technique, such as induced sputum, as a potential outcome measure, are the reliability and validity of the technique. Elements in the assessment of reliability include the interobserver consistency and the repeatability of analyses on samples obtained on different days from clinically stable patients. Elements in the assessment of the validity of induced sputum include: its potential to distinguish between normal subjects and patients with different diseases; its conformity to other measurements of airway inflammation; and its responsiveness to intervention. Both processing techniques have been shown to provide valid and reliable data.

Firstly, the cell fraction and the fluid phase of induced sputum clearly differ between healthy subjects, asthmatics and smokers with chronic bronchitis or COPD [1, 2, 14, 28–30], irrespective of the processing technique used [13, 15, 31]. Overall, compared to healthy subjects, sputum from asthmatics contains increased numbers of eosinophils, even when collected from patients with mild stable disease. In contrast, sputum from smokers with COPD contains mainly neutrophils. The increase in sputum neutrophil counts is less pronounced in smokers with chronic bronchitis [14, 23].

A few studies have compared both processing techniques directly on samples obtained from the same patient, with either asthma or COPD. When doing so, it becomes apparent that although squamous cell contamination is higher in the whole sample approach and the quality of the cytospins somewhat less, the main information re-mains unaltered [15, 31–33]. PELEMAN *et al.* [31] induced sputum in patients with COPD. Each sample was divided into two. Half was processed together with the associated saliva, whereas from the second half viscid portions were selected. Although the quality of the cytospins was better using the second approach, the outcome remained unaltered, namely a clear increase in the percentage of neutrophils compared to normal volunteers (table 2). In a group of 11 asthmatics, GERSHMAN *et al.* [15] induced sputum on two occasions, separated by 72 h. Either the whole sample was processed, or sputum was collected separately from saliva. The second approach reduced squamous cell contamination by 28%, but the differential eosinophil counts were very similar, showing an increase in both samples. By minimizing salivary contamination and thus dilution, the concentration of soluble mediators was also seen to be higher using the second approach. This illustrates an im-portant point, namely that whatever

Table 2. – Total and differential cell counts in induced sputum from patients with chronic obstructive pulmonary disease (COPD), processed after selection of "plugs" or by homogenization (n=9)

	Selection	Homogenization
Total cells $\times 10^3$ cells·μg^{-1}	15.6±6.2	5.9±1.9
Macrophages %	28±4	30±5
Neutrophils %	69±4	67±6
Eosinophils %	0.7±0.3	0.3±0.1
Lymphocytes %	0.6±0.3	0.8±0.2
Bronchial epithelial cells %	0.5±0.3	2.3±1.6

Values are expressed as mean±SEM. Percentages are calculated on the nonsquamous cell population. No significant differences were seen, as calculated by Wilcoxon signed rank test [31].

method is being used it must be used consistently. Even though both methods generate similar results, they are not interchangeable.

The concentrations of various soluble mediators in sputum supernatant are also clearly different between normal subjects, asthmatics and COPD patients. Several groups, using different processing techniques, have reported increased levels of ECP in sputum from asthmatics when compared to healthy controls [2, 3, 21], whereas in sputum from COPD patients an increase in myeloperoxidase and IL-8 has also been found [14, 22, 23].

Furthermore, these differential cell counts and measurement of soluble mediators in samples obtained on different days from clinically stable patients prove reproducible, illustrating the reliability of the technique [1, 3, 4, 21, 34]. Again, this has been confirmed for both processing techniques. PIZZICHINI *et al*. [21] induced sputum on two occasions separated by 6 days in 19 stable asthmatics, 10 smokers with chronic bronchitis and 10 healthy subjects. Sputum was processed by selecting portions from the sample. Intraclass correlation coefficients for differential macrophage, neutrophil and eosinophil counts ranged 0.71–0.94, indicating a high reproducibility. Measurements of soluble mediators were found to be equally reproducible. Using the whole sample approach in 21 asthmatic subjects. IN'T VEEN *et al.* [3] analysed sputum samples collected on two occasions separated by at least 48 h. Again, the results were reproducible, albeit that the intraclass correlation coefficients obtained for differential cell counts were somewhat lower than in the previous study. The reproducibility of ECP measurements was similar.

One element in the evaluation of the criterion validity of induced sputum includes assessment of the conformity to other measurements of airway inflammation. This issue has been addressed by comparing induced sputum to bronchial wash, bronchoalveolar lavage (BAL) and bronchial biopsies. It has to be realized that these techniques might sample different locations within the bronchial tree, and that it is, therefore, difficult to establish with certainty what the "gold standard" is, to which sputum has to be compared. Comparative studies indicate that sputum contains more neutrophils but fewer macrophages and lymphocytes than BAL fluid [30, 35–37]. However, the cellular distribution of sputum, and especially the relative eosinophil numbers, correlates quite well with eosinophil numbers in bronchial washes or BAL. This applies not only for asthma but also for chronic bronchitis, especially during exacerbations, when increased numbers of eosinophils are found in sputum, BAL and biopsies [38]. Importantly, similar results were obtained irrespective of the technique used for processing sputum [30, 35, 37]. From the limited amount of data currently available, it would appear that the correlation with eosinophil numbers in bronchial biopsies is less clear [30, 36]. This issue remains to be further investigated.

Finally, an important element in the content validity of induced sputum is the responsiveness to intervention or the capacity of the technique to reflect changes in the degree of airway inflammation. The composition of sputum has indeed been shown to respond to factors known to affect the degree of airway inflammation. Both processing techniques show an increase in the percentage of eosinophils in sputum from atopic asthmatics following an allergen inhalation challenge [39–43]. Conversely, treatment with systemic or inhaled steroids reduces sputum eosinophil numbers and ECP levels [10, 44–47]. Again, this response to treatment can be observed irrespective of the processing technique used.

In conclusion, the data currently available seem to underline the robustness of induced sputum as a method for assessing airway inflammation in diseases such as asthma and chronic obstructive pulmonary disease. It would appear that the reliability and validity of the data derived from induced sputum are not influenced by differences in the methods currently used for inducing and processing the sample. It has to be kept in mind that, although the various methods provide similar results, they are not interchangeable. Therefore, whatever method has been chosen, it must be used consistently.

References

1. Pin I, Gibson PG, Kolendowicz R, *et al.* Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47: 25–29.
2. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147: 1126–1131.
3. In't Veen JCCM, de Gouw HWFM, Smits HH, *et al.* Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur Respir J* 1996; 9: 2441–2447.
4. Iredale MJ, Wanklyn AR, Phillips IP, Krausz T, Ind PW. Noninvasive assessment of bronchial inflammation in asthma: no correlation between eosinophilia of induced sputum and bronchial responsiveness to inhaled hypertonic saline. *Clin Exp Allergy* 1994; 24: 940–945.
5. Gershman NH, Liu H, Liu J, Wong HH, Fahy JV. Fractional analysis of sequential sputum samples during sputum induction. *Am J Respir Crit Care Med* 1997; 155: A819.
6. Holz O, Jörres RA, Koschyk S, Speckin P, Welker L, Magnussen H. Changes of cellular and biochemical sputum composition during sputum induction in healthy and asthmatic subjects. *Am J Respir Crit Care Med* 1996; 153: A289.
7. Richter K, Jörres RA, Mucke M, Magnussen H. Sequentially induced sputum in patients with asthma or chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1997; 155: A821.
8. Pizzichini MMM, Popov TA, Efthimiadis A, *et al.* Spon-

taneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996; 154: 866–869.
9. Popov TA, Pizzichini MMM, Pizzichini E, *et al.* Some technical factors influencing the induction of sputum for cell analysis. *Eur Respir J* 1995; 8: 559–565.
10. Pizzichini MMM, Pizzichini E, Clelland L, *et al.* Sputum in severe exacerbations of asthma. *Am J Respir Crit Care Med* 1997; 155: 1501–1508.
11. Bacci E, Cianchetti S, Paggiaro PL, *et al.* Comparison between hypertonic and isotonic saline-induced sputum in the evaluation of airway inflammation in subjects with moderate asthma. *Clin Exp Allergy* 1996; 26: 1395–1400.
12. Wong HH, Gershman NH, Liu JT, Liu H, Fahy JV. A comparison between induced sputum samples collected using a mask *vs* a mouthpiece. *Am J Respir Crit Care Med* 1997; 155: A819.
13. Pizzichini E, Pizzichini MMM, Efthimiades A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effect of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996; 9: 1174–1180.
14. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996; 153: 530–534.
15. Gershman NH, Wong HH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. *Eur Respir J* 1996; 9: 2448–2453.
16. Efthimiades A, Pizzichini MMM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol *vs* phosphate-buffered saline. *Eur Respir J* 1997; 10: 1336–1340.
17. Gelder CM, Thomas PS, Yates DH, Adcock IM, Morrison JFJ, Barnes PJ. Cytokine expression in normal, atopic and asthmatic subjects using the combination of sputum induction and the polymerase chain reaction. *Thorax* 1995; 50: 1033–1037.
18. Hoshi H, Ohno I, Honma M, *et al.* IL-5, IL-8 and GM-CSF immunostaining of sputum cells in bronchial asthma and chronic bronchitis. *Clin Exp Allergy* 1995; 25: 720–728.
19. Kidney JC, Wong AG, Efthimiades A, *et al.* Elevated B-cells in sputum of asthmatics: clear correlation with eosinophils. *Am J Respir Crit Care Med* 1996; 153: 540–544.
20. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA. Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147: 1132–1137.
21. Pizzichini E, Pizzichini MMM, Efthimiades A, *et al.* Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid phase measurements. *Am J Respir Crit Care Med* 1996; 154: 308–317.
22. Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma and normal subjects. *Am J Respir Crit Care Med* 1997; 155: 449–453.
23. Kips JC, Van De Velde V, Bullock GR, Peleman RA, Pauwels RA. Composition of sputum in smokers with or without airflow limitation. *Eur Respir J* 1996; 9: 118s.
24. Keatings VM, O'Connor BJ, Wright LG, Hutson DP, Corrigan CJ, Barnes PJ. Late response to allergen is associated with increased concentrations of tumor necrosis factor-alpha and IL-5 in induced sputum. *J Allergy Clin Immunol* 1997; 99: 693–698.
25. Louis R, 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: 466–472.
26. Kanazawa H, Shoji S, Yamada M, *et al.* Increased levels of nitric oxide derivatives in induced sputum in patients with asthma. *J Allergy Clin Immunol* 1997; 99: 624–629.
27. Popov T, Gottschalk R, Kolendowicz R, Dolovich J, Powers P, Hargreave FE. The evaluation of a cell dispersion method of sputum examination. *Clin Exp Allergy* 1994; 24: 778–783.
28. Chodosh S. Examination of sputum cells. *N Engl J Med* 1970; 282: 854–858.
29. Gibson PG, Girgis Gabardo A, Morris MM, *et al.* Cellular characterisation of sputum from patients with asthma and chronic bronchitis. *Thorax* 1989; 44: 693–699.
30. Maestrelli P, Saetta M, Di Stefano A, *et al.* Comparison of leucocyte counts in sputum, bronchial biopsies and bronchoalveolar lavage. *Am J Respir Crit Care Med* 1995; 152; 1926–1931.
31. Peleman RA, Rytilä PH, Kips JC, Joos GF, Pauwels RA. Sputum analysis in chronic obstructive pulmonary disease (COPD): comparison of two methods. *Am J Respir Crit Care Med* 1995; 151: A486.
32. Taylor DA, Kanabar V, Cook RM, O'Connor BJ. Selection of sputum plugs from induced sputum of asthmatic patients does not improve cellular analysis. *Am J Respir Crit Care Med* 1997; 155: A819.
33. Antonio S, Beghe B, Bianchi A, Neri M, Ind PW. Induced sputum: a comparison between selected portion and entire sputum. *Am J Respir Crit Care Med* 1997; 155: A822.
34. Spanavello A, Sharara AM, Migliori GB, *et al.* Reproducibility of induced sputum in asthmatics and normals. *Am J Respir Crit Care Med* 1996; 153: A289.
35. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 1997; 52: 372–374.
36. 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: 769–779.
37. Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995; 152: 53–58.
38. Saetta M, Di Stefano A, Maestrelli P, *et al.* Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med* 1994; 150: 1646–1652.
39. Pin I, Freitag AP, O'Byrne PM, *et al.* Changes in the cellular profile of induced sputum after allergen induced asthmatic responses 1–3. *Am Rev Respir Dis* 1992; 145: 1265–1269.
40. Fahy JV, Liu J, Wong H, Boushey HA. Analysis of cellular and biochemical constituents of induced sputum after allergen challenge: a method for studying allergic airway inflammation. *J Allergy Clin Immunol* 1994; 93: 1031–1039.
41. Gauvreau GM, Watson RM, Jordana M, O'Byrne PM. Effect of inhaled budesonide on allergen-induced airway responses and airway inflammation. *Am J Respir Crit*

Care Med 1996; 154: 1267–1271.
42. Wong BJ, Dolovich J, Ramsdale EH, *et al.* Formoterol compared with beclomethasone and placebo on allergen induced asthmatic responses. *Am Rev Respir Dis* 1992; 146: 1156–1160.
43. Pizzichini MMM, Kidney JC, Wong BJO, *et al.* Effect of salmeterol compared with beclomethasone on allergen induced asthmatic and inflammatory responses. *Eur Respir J* 1996; 9: 449–455.
44. Claman DM, Boushey HA, Liu J, Wong H, Fahy JV. Analysis of induced sputum to examine the effects of prednisone on airway inflammation in asthmatic subjects. *J Allergy Clin Immunol* 1994; 94: 861–869.
45. Fahy JV, Liu J, Ward T, Cunha H, Boushey HA. Effect of inhaled beclomethasone dipropionate (BDP) on clinical outcomes and markers of inflammation in induced sputum (IS) in asthmatic subjects. *Am J Respir Crit Care Med* 1995; 151: A41.
46. Gibson PG, Hargreave FE, Girgis-Gabardo A, Morris M, Denburg JA, Dolovich J. Chronic cough with eosinophilic bronchitis and examination for variable airflow obstruction and response to corticosteroids. *Clin Exp Al-lergy* 1995, 25: 127–132.
47. Kips JC, Inman MD, O'Byrne PM, Pauwels RA. Induced sputum reflects changes in asthma severity following modulation of the dose of inhaled steroids. *Am J Respir Crit Care Med* 1996; 153: A293.