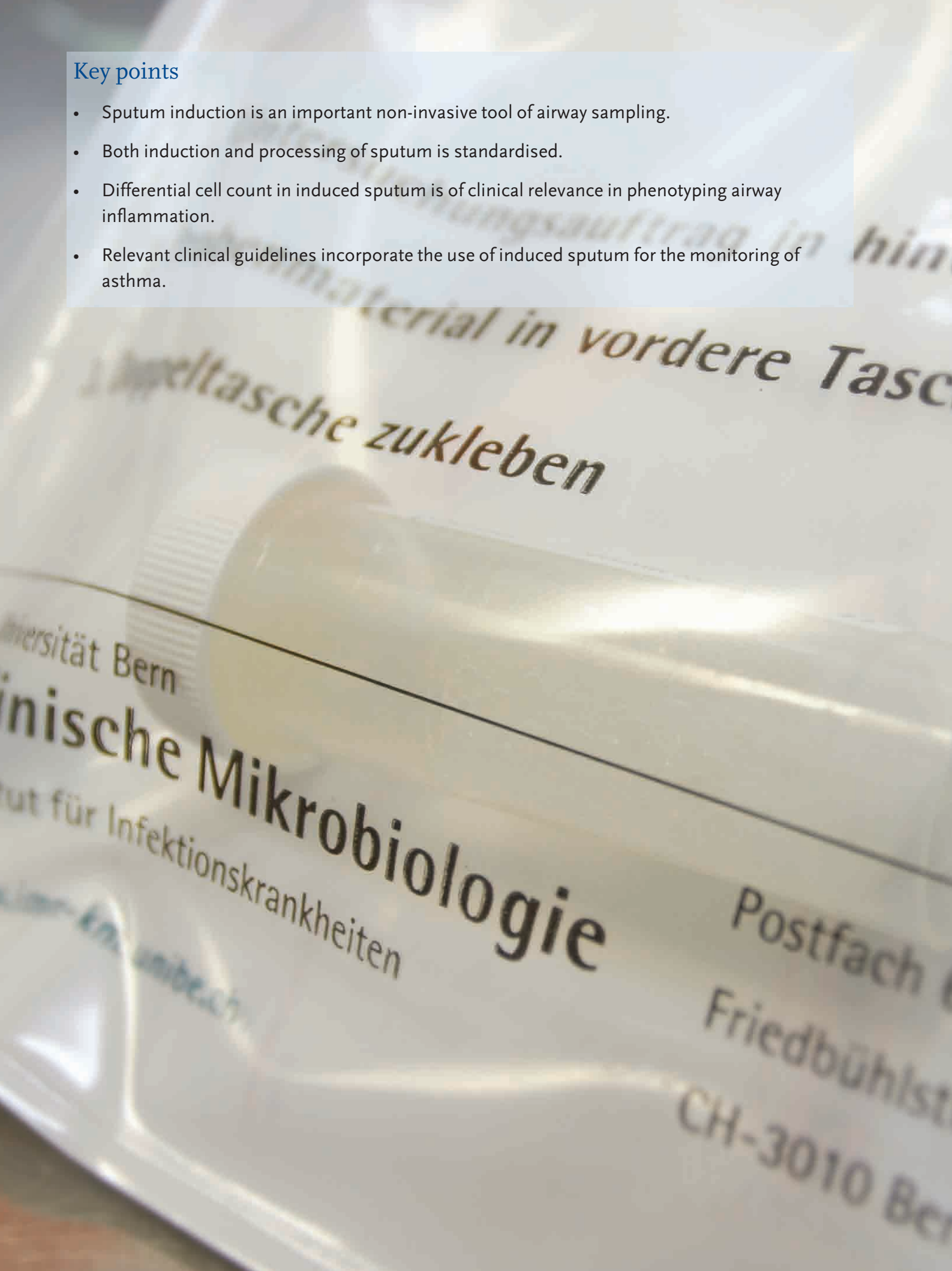
Key points

- Sputum induction is an important non-invasive tool of airway sampling.
- Both induction and processing of sputum is standardised.
- Differential cell count in induced sputum is of clinical relevance in phenotyping airway inflammation.
- Relevant clinical guidelines incorporate the use of induced sputum for the monitoring of asthma.



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Induced sputum analysis: step by step

Educational aims

- To introduce the standardised method for sputum induction
- To provide a guide for sputum processing
- To highlight important methodological aspects that may influence results
- To briefly summarise potential clinical and research use

Summary

Sputum induction is a relatively non-invasive mode of airway sampling that provides an opportunity for analysis of cellular components and infective agents, including bacteria and viruses, together with fluid-phase constituents. Both induction and processing of sputum samples are standardised and several manuals are available to help to educate professionals to perform the technique to the highest standard [36]. Using this standardised technique, the results are reproducible and comparable between different laboratories. Detailed studies on methodological issues provided enough evidence for standardisation and careful use of the methodology enabled the field to develop rapidly from a research tool to a useful clinical test. Eosinophil differential count in induced sputum is a recommended, evidence-based mode of assessing airway inflammation in asthma and its use is incorporated into the relevant guidelines.

Statement of Interest
None declared.

Introduction

Sputum analysis has been used as a diagnostic technique for centuries, and reports on sputum in different diseases, containing important aspects of sample processing, were published more than a century ago [1]. For patients without spontaneous sputum production lower airway secretions can be sampled by sputum induction. This method was first described in detail by BICKERMAN *et al.* [2], in 1958, for lung cancer diagnosis. The application of induced sputum in the assessment of airway pathology has grown rapidly; however, research groups applying the method use very different modes of induction and processing. Therefore, a task force was set up by the European Respiratory

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Society (ERS) and it published its recommendations for standardisation of sputum induction and processing in 2002 [3–8]. This set of recommendations forms the basis for the use of this sampling technique in both adults and children. Induction and processing of sputum samples in a standardised manner is a key component to provide valuable information for clinical decision making [9–14]. The current paper provides a brief overview of the recommended induction and processing methods. For further information readers are advised to use additional sources of information, such as the task force report [3–8], the ERS website for video-based educational material on sputum induction and the material from the ERS School course on Monitoring of Asthma, Chronic Obstructive Pulmonary Disease (COPD) and Other Airway Diseases (www.ersnet.org/education/courses/item/4559-monitoring-of-asthma-copd-a-other-airway-diseases.html).

How to induce sputum?

Standard procedure

Sputum induction is conducted by inhalation of nebulised sterile saline solution (isotonic or hypertonic) followed by coughing and expectoration of airway secretions. Since saline inhalation may cause bronchoconstriction, careful safety measures should be taken, including the measurement of lung function before induction, pretreatment with inhaled salbutamol and monitoring of lung function during the process. Spirometry (forced expiratory volume in 1 s (FEV_1)) is preferred over the measurement of peak expiratory flow (PEF) determination and the use of a single dose of 200 µg salbutamol is recommended for pretreatment. FEV_1 should be measured before (baseline) and 10 min after salbutamol inhalation. It is important to note that baseline FEV_1 does not have predictive value for the occurrence and severity of bronchoconstriction caused by induction. Resuscitation equipment should be available in the place where the sputum induction is undertaken and a physician should be available to supervise the procedure, which can be carried out by an experienced technician. Induction is carried out using a sterile, freshly prepared saline solution. The use of 4.5% sodium chloride solution is recommended for general use. The use of hypertonic saline results in more sample than the use of of isotonic saline; however, importantly there is no difference in cellular composition between samples induced by isotonic or hypertonic solutions [15]. For nebulisation, an ultrasound nebuliser is recommended and a there is an expert consensus suggesting that an output of $\sim$1 mL·min^{-1} is sufficient to achieve successful sampling. In general, 15–20 min is enough to provide an adequate amount of sample, during which the subject is asked to cough and expectorate at 5 min intervals. In each period, lung function is measured to detect potential bronchoconstriction and if FEV_1 decreases by more than 20% compared with post-salbutamol baseline, induction is stopped.

Alternative procedure for high-risk patients

For high-risk patients (including subjects with FEV_1/forced vital capacity $<$0.7 post-salbutamol and unstable asthmatic patients) isotonic solution (0.9%) should be used to avoid potential bronchoconstriction during induction. The concentration of saline can only be increased in a stepwise manner to 3–4.5%, if FEV_1 does not fall during inhalation. However, there is no need to increase saline concentration if an adequate amount of sample can be generated. FEV_1 (or PEF) measurements should be performed frequently (a short period of time, such as 30 s, 1 min and 5 min, after the procedure). Because saline inhalation time and the timing of expectoration may vary, because of safety or other reasons, and the inhalation time may influence sample composition, it is recommended to note the total inhalation time. The sample obtained should be kept at 4°C and processed less than 2 h after the end of induction.

If sputum induction is part of a series of non-invasive assessments of airway function and inflammation the order of the tests should be from least invasive towards more invasive.

Generally sputum induction is the last: start with exhaled breath condensate collection (that requires only tidal breathing), measurement of exhaled nitric oxide, other exhaled volatiles, lung function, bronchoprovocation test followed by sputum induction.

For monitoring airway diseases, non-invasive measurements can be repeated with different time intervals between two consecutive tests with reproducible results. The procedure of sputum induction influences the composition of airway surface fluid for a few hours. Therefore, there is a consensus suggesting that it is

advisable to leave 24 h between sputum inductions to obtain reproducible results.

With the recommended technique sputum induction is safe and in more than 90% of cases an adequate sample can be obtained successfully [5]. The sample obtained is a complex material containing large amounts of mucin, different cells, fluid-phase mediators and some saliva. To obtain reproducible results, a standardised mode of sample processing is also of great importance.

The detailed, expert consensus-based recommendations for sputum induction, with a list of relevant evidence, can be found in the ERS Task Force report [4].

Processing of induced sputum sample

The detailed expert consensus-based recommendations for induced sputum processing with a list of relevant evidence can be found in the ERS Task Force report [6].

For processing two different approaches can be followed, depending on the use of either the entire sputum or selected sputum plugs. Sputum plugs are the dense, viscid portions of samples that can be selected by using an inverted microscope with the aim of minimising saliva contamination.

Sputum processing can be conducted at room temperature for cell counts. For specific research methods (*e.g.* for fluid-phase mediator determination) handling temperature may be different according to the method applied.

First the volume and weight of the sample is recorded. The sample is then diluted with phosphate-buffered saline containing 0.1% dithiotreitol (DTT) or dithioerythritol (DTE) for 10–30 min and gently vortexed at room temperature for homogenisation. The volume of homogenisation solution is equal to the volume of sample for the entire sample method (1:1) and 4:1 for selected sputum plug samples. Filtration through a nylon mesh (48 µm) is recommended to obtain good quality slides. The cells are stained for viability assessment (mixing 10 µL tryptan blue with an equal volume of cell suspension) and cells are counted using a haemocytometer counting chamber. Cell viability is reported as %. The Task Force recommends this is done manually to obtain reliable results. This is followed by centrifugation (300–1500 × *g* for 5–10 min; usually 400 × *g* for 10 min) to separate cells and supernatant. The supernatant can be stored at

Standard procedure: step by step

1. Explain the procedure in detail to the subject (rinse mouth before procedure, saline inhalation with tidal breathing, saliva handling during inhalation; after 5 min intervals cough and try to expectorate into the sputum cup).
2. Set nebuliser (output ~1 mL·min^{-1}), fill it with sterile saline solution (usually with concentration of 4.5%).
3. Measure baseline (pre-salbutamol) FEV_1 (or PEF).
4. Premedicate the subject with inhaled salbutamol (200 µg) and repeat FEV_1 (or PEF) measurement after 10 min.
5. Start nebulisation and ask the subject to perform tidal breathing (set the clock for 15–20 min). Ask the patient to perform inhalation for 5 min intervals followed by coughing and expectoration (the clock should be stopped at each coughing episode). Encourage the subject to cough and spit at any time during the induction if he/she feels the urge to do so.
6. After each 5 min interval carry out FEV_1 (or PEF) repeat spirometry. If FEV_1 or PEF falls more than 20% from the post-salbutamol value, stop the procedure. If induction is stopped due to an adverse effect (or for any other reason), record the total induction time.

Alternative procedure for high-risk patients: step by step

1. Explain the procedure in detail to the subject.
2. Set nebuliser (output ~1 mL·min^{-1}), fill it with sterile, isotonic (0.9%) saline solution.
3. Measure baseline (pre-salbutamol) FEV_1 (or PEF)
4. Premedicate the subject with inhaled salbutamol (200 µg) and repeat FEV_1 (or PEF) measurement after 10 min.
5. Start nebulisation and ask the subject to perform inhalation with tidal breathing for 30 sec, 1 min and 5 min intervals. Encourage the subject to cough and spit at any time during the induction if he/she feels to urge to do so.
6. After each interval carry out FEV_1 (or PEF) repeat spirometry. If FEV_1 or PEF falls more than 20% from the post-salbutamol value or if symptoms develop, stop the procedure. If induction is stopped due to an adverse effect (or for any other reason), record the total induction time.
7. If isotonic saline induces sputum, continue with it. Only increase the saline concentration (first to 3% and, if it does not induce sputum, to 4.5%) if sampling is inadequate with the previous concentration. When the concentration is increased follow with 30 sec, 1 min and 2 min intervals for induction.

List of required equipment and solutions

Ultrasonic nebuliser with tubing and disposables (cups, lids)
Clock
Hypertonic (4.5%) or isotonic (0.9%) saline
Salbutamol inhaler (200–400 µg)
Spirometer (or peak flow meter) and noseclip
Glass of water for mouth rinse, cup for saliva and tissue
Petri dish for sample collection
Worksheet, calculator

-80°C for subsequent assays. The next step is cytospin preparation. The cell pellet is resuspended in balanced salt solution (or a similar buffer). Cell concentration should be adjusted to 1.0×10^6 cells·mL^{-1}. Approximately 40–65 µL of sample ($450–650 \times 10^3$ cells) should be used in each cytospin. The recommended speed for cytospin centrifugation is $10–51 \times g$ for 6 min. Wright's or Giemsa stain can be used for cytospin staining. Potentially, other methods for staining can also be applied. For differential cell counting, count 400–500 nonsquamous cells and report the percentage of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells present in the total nonsquamous cell count. The proportion of squamous cells (contamination) should be reported separately. A sputum sample is considered inadequate when the percentage of nonsquamous cells is <80%, because the reproducibility of cell counts is lower if the squamous cell proportion is >20% of the total cells (fig. 1). The normal value for sputum eosinophil differential count is <3% (fig. 2) [16].

Standard operation protocols for sputum induction and processing should contain quality control measures for each sputum laboratory to ensure reproducible results. This includes regular equipment calibration and monthly internal slide reading checks.

Differential cell count is the most well-validated marker in sputum, but there are several other methods applied to induced sputum samples. These include recently developed, cutting-edge technologies, such as molecular microbiology, viral and bacterial culture, PCR, chip technology, proteomics, lipidomics and functional assays, together with analysis of soluble mediators in fluid (sputum supernatant) including cytokines and chemokines by ELISA, fluorescent bead-based multiplex sandwich immunoassays, high performance liquid chromatography (HPLC) or microRNA [17–22]. Since the majority of cells obtained by sputum induction are viable, the sample can provide a good source of airway cells for research use, starting from cell culture they can be used in western blotting or other techniques [23–25].

Clinical application of induced sputum

Induced sputum has been used in clinical practice in a number of different ways [2, 9–11, 26]. The differential cell count of induced sputum is a widely used marker for phenotyping airway inflammation (fig. 3). Publication of several lines of evidence has demonstrated that sputum eosinophil differential cell counting provides an important means of phenotyping airway inflammation and facilitates personalised treatment choices [9–11]. In the current guidelines for asthma, sputum eosinophils are placed as an evidence-based tool for assessing airway inflammation and, therefore, predicting and assessing corticosteroid response [13–14]. The measurement has a good reproducibility and its use has been has been shown to improve asthma control. The recent guidelines for clinical end-points in asthma trials, created by the American Thoracic Society and the ERS, have also incorporated the use of induced sputum eosinophil counts as an outcome measure [13]. The updated guideline recommendations outline a role for inclusion of

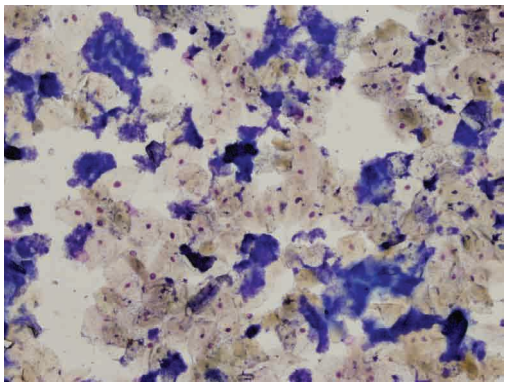

Figure 1
Inadequate induced sputum sample from a healthy subject (the proportion of squamous cells is >20%).

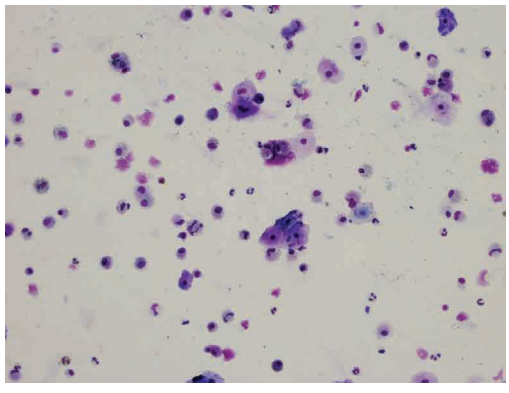

Figure 2
Induced sputum cytospin from a healthy subject (normal differential cell counts, squamous cells make up <20% of the total cell count).

Sputum processing step by step using the entire sample

1. Pre-weigh a polystyrene tube, pour in the entire sputum sample, weigh and record the weight.
2. Add an equal volume of DTT or DTE.
3. Using a pipette, disperse the fluid several times and use a vortex for further dispersion.
4. Shake for 15 min at room temperature using a rocker or water-bath at room temperature (22°C).
5. Filter the fluid and weigh the filtrate.
6. Assess cell viability and count total cell count.
7. Calculate total cell count per millilitre of entire sputum.
8. Prepare cytospin slides and stain with Giemsa or Wright's stain.
9. Count 400–500 nonsquamous cells and determine the differential cell count.

Sputum processing step by step for selected sputum plugs

1. Pour the entire sputum sample into a cell culture dish and select sputum plugs (saliva free dense areas of the sample). Pre-weigh a polystyrene tube, add the plugs to it, weigh and record the weight.
2. Add DTT or DTE with four times the sample volume.
3. From this step follow the steps described for entire sputum without modification.

assessment of sputum eosinophils, in addition to standard measures of asthma control, to guide adjustment of controller therapy in adults with moderate-to-severe asthma.

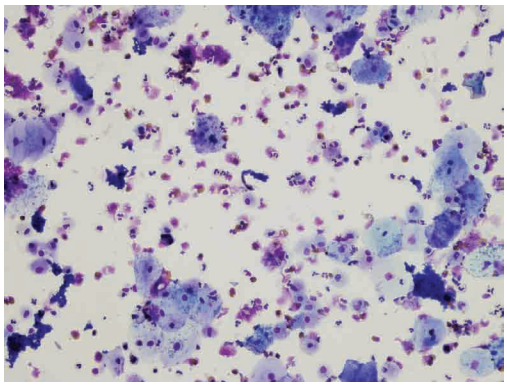

Figure 3
Induced sputum sample from an asthmatic subject.

In occupational asthma it can also be used as a diagnostic tool [27]. Similarly, in patients with COPD, the method can be used to determine steroid responsiveness based on sputum eosinophil differential count [28]. As a diagnostic tool, the method is used for diagnosing different pulmonary diseases including lung cancer, interstitial lung diseases, tuberculosis and opportunistic infections in immunocompromised hosts [1, 29–33].

Research application of induced sputum

Induced sputum samples are used in a wide range of studies. The application of cutting-edge technology provides not only new scientific understanding through application of this technique, but also new information about the source and characteristics of the sample [17–25, 34, 35].

Educational questions

1. Measurement of lung function is necessary in sputum induction:
   a. To detect the presence of reversible airway obstruction before induction
   b. To predict the likelihood of bronchoconstriction during induction
   c. To detect bronchoconstriction caused by induction
   d. To achieve deep inhalation induced bronchodilation
2. If the proportion of squamous cells is >20% in induced sputum samples:
   a. Reproducibility of differential cell count is lowered
   b. Intensive neutrophilic airway inflammation is present
   c. Airway inflammation is likely to be steroid-responsive
   d. It suggests emphysema
3. If eosinophil differential cell count in induced sputum is 15%, it suggests the following except:
   a. Ongoing eosinophilic airway inflammation
   b. Good steroid responsiveness of the airway disease
   c. An accompanying increase in neutrophil differential cell count
   d. Increased eosinophil differential cell count

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Suggested answers

1. c.
2. a.
3. c.

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