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Application of Immunocytochemistry to Sputum Cells to Investigate Molecular Mechanisms of Airway Inflammation

Kittipong Maneechotesuwan and Adisak Wongkajornsilp
Faculty of Medicine Siriraj Hospital,
Mahidol University
Thailand

1. Introduction

The definition of *sputum* is not equivalent to *mucus*, but sometime clinicians use both interchangeably. The definition includes the pathological secretion dispelled by cilia and expectorated with coughing. It maintains airway hydration and traps particulates, bacteria, and viruses.

The contents of sputum comprise damaged ciliated epithelium and inflammatory cells. Airway mucus possesses antioxidant, antiprotease, and antimicrobial activities. Its volume is increased during chronic airway inflammation. The sputum composition can be altered by the underlying disease and its severity. The expansion of sputum neutrophil is variably in severe asthma or in chronic obstructive pulmonary disease (COPD).

Cough and ciliary clearance greatly depend on the viscosity of the secretion to the ciliary surface (Voynow & Rubin 2009). Surface tension and surfactant interactions can overpower surface forces. Tenacity or adhesivity is the greatest determinant for the efficiency of cough to eliminate secretion (Voynow & Rubin 2009).

Sputum analysis is a non-invasive approach to dissect underlying pathophysiology of inflammatory airway diseases. The cellular and biochemical constituent of sputum correlates well with both bronchial wash (BW) and bronchoalveolar lavage (BAL), but to a lesser degree with bronchial biopsies (Fahy, et al. 1995, Pizzichini, et al. 1998, Maestrelli, et al. 1995). These observations imply the differences in the luminal and mucosal phase of airway inflammation. The fraction of neutrophils decreases from central (20-30%) to peripheral airways (<2%) while the opposite is true for macrophages (Rankin, et al. 1992). Based on this observation, sputum represents the more proximal airway, whereas BW and BAL represent the more peripheral airways. Successive sputum collection after a single sputum induction exhibited a stepwise neutrophil decrement and macrophage increment (Holz, et al. 1998a, Richter, et al. 1999) reflecting the sampling of more distal airway from later collection. The standardization of sputum induction is required to reduce inter- and intra-subject variability.

2. Sputum induction

The sputum induction technique allows the noninvasive collection of the airway content and provides an opportunity to identify biomarkers of airway inflammation in several conditions (e.g., asthma and COPD). It is superior to spontaneous sputum expectoration in the higher quantity of collected secretion from the lower airways. Sputum induction requires a high degree of cooperation from the patient. The procedure should be performed in a quiet environment and conducted by an experienced technician under the supervision of an experienced physician.

Ultrasonic neubulizers are recommended for sputum induction since other nebulizers do not provide the sufficient output of saline aerosol. The output of nebulizers should be accurately tested. A spirometry provides real-time assessment of the baseline airway caliber and promptly alerts for the excessive bronchoconstriction during the saline induction (Paggiaro, et al. 2002). A spirometer is superior to a peak flow meter since it provides greater sensitivity to measure the decline in force expiratory volume in one second (FEV_1) that alludes to saline-induced bronchoconstriction. Oxygen saturation should be monitored if there is any suspicion of resting hypoxemia. Oxygen supplement should be in reach for COPD patients with hypoxemia exacerbation (Paggiaro et al. 2002).

Sterile saline solution should be freshly prepared. Rescuing medications, bronchodilator (inhaled or nebulized salbutamol or other β_2-agonists) (Paggiaro et al. 2002) and other resuscitation medicines must be nearby. Sputum induction should be performed under aseptic environment. Hypertonic saline can induce bronchoconstriction in asthmatics (Smith & Anderson 1989) with unknown mechanism, possibly through the activation of mast cells (Gravelyn, et al. 1988) or sensory nerve endings (Makker & Holgate 1993).

The pretreatment with a short-acting β_2-agonists is recommended as the standard protocol to prevent excessive bronchoconstriction (Pin, et al. 1992, Wong & Fahy 1997, Jatakanon, et al. 1998) that could pose asthmatics at risk of an exacerbation. Excessive bronchoconstriction might bring about the premature termination of the induction resulting in an inadequate sputum collection. Salbutamol (200-400 μg or 2-4 puffs from a metered dose inhaler) can be used for pretreatment. Pretreatment with higher doses of salbutamol cannot provide additional advantage for the prevention of hypertonic saline-induced bronchoconstriction (Wong & Fahy 1997, Cianchetti, et al. 1999, de la Fuente, et al. 1998, Peleman, et al. 1999), but may induce more severe subsequent bronchoconstriction. Therefore, a single dose of salbutamol 200 μg is recommended both before and after the measurement of FEV_1 for 10 min. Salbutamol pretreatment does not interfere the inflammatory cell percentage in induced sputum (Cianchetti et al. 1999, Popov, et al. 1995). Regarding the effects on soluble mediators in sputum supernatant, salbutamol has no effect on eosinophil cationic protein (ECP) levels, but tends to reduce histamine concentrations (Cianchetti et al. 1999). There has been no study for the effect of salbutamol on the levels of other soluble mediators (i.e., cytokines, albumin, and neutrophil elastase) or the expression of cell activation markers as detected by immunocytochemistry. Also, the data on the comparison between different bronchodilators (β_2-agonists and anticholinergic drugs) are not available.

Monitoring pulmonary function during sputum induction is indispensable for safety precaution. However, no standardized monitoring protocol for pulmonary function has been recommended. Most studies measure FEV_1 every 5-10 min, with additional

measurement if any symptom develops (Iredale, et al. 1994, Bacci, et al. 1998, Pin et al. 1992, Jatakanon et al. 1998, Wong & Fahy 1997, Maestrelli, et al. 1994). Since poor perception of dyspnea can exist while bronchospasm can occur early, the measurement of pulmonary function within the first minute of nebulization should be performed to identify supersensitive subjects. FEV_1 should be periodically monitored with an interval of ≤ 5 min during aerosol inhalation. A single measurement is appropriate if the change in FEV_1 is < 10% of the postbronchodilator FEV_1 value.

2.1 Selecting saline concentrations

The concentrations of saline solution for sputum induction varied from 0.9% to 7% in different studies. Some investigators raised the concentration in a stepwise manner (3%, 4% and 5%) during sputum induction. Saline concentration and nebulizer output might influence the safety, tolerability and success rate of the induction as well as the cellular and biochemical constituents. The 3% hypertonic saline achieved the same success rate as 3-5% given sequentially. Hypertonic saline solutions are more effective than isotonic saline for sputum induction. The latter should be reserved for patients at high risk of bronchoconstriction. There is a consensus to advocate sputum induction with 4.5% sodium chloride solution that is commercially available.

Hypertonic or isotonic saline did not elicit any significant alteration in cellular ratio or their quantity in the sputum. However, the concentration-effect relationship between different saline concentrations and the levels of most soluble mediators in the sputum supernatants remain unknown. There is no difference in the levels of ECP and histamine in the sputum supernatant induced by isotonic or hypertonic saline (Bacci, et al. 1999). Sputum supernatant osmolarity fluctuates between 70-360 mOsm as a result of the natively high variation in sputum concentrations of sodium, chloride and magnesium from individual subjects.

2.2 Selecting nebulizer

The selection of nebulizer based on type and output is important for the attainment of sputum induction. The ultrasonic nebulizer offers higher success rates than does jet nebulizer (Popov et al. 1995). The exact volume of inhaled saline solution that might be required to induce an adequate sputum sample is still unclear. The optimal duration of inhalation and the optimal output are also unclear. Longer duration might yield samples from more distal airways. Other factors include the size of aerosols and their deposited locations. The deposition at different locations might yield different sputum composition and success rates. The common practice employing ultrasonic nebulizer with an output of ~ 1 mL/min can achieve a satisfactory quantity.

2.3 Duration of nebulization

Duration of nebulization can influence the cellular components of the resulting sputum. At the early phase (0-4 min), neutrophils, eosinophils and mucin are major components in the sputum. At the later phase (16-20 min), lymphocytes, macrophages and surfactant are increasingly noticeable (Holz et al. 1998a, Gershman, et al. 1999). This pattern suggests that central airways are sampled at the early phase, whereas peripheral airways and alveoli are

sampled at the later phase. The early-phase sputum may be discarded to avoid the saliva contamination. Subsequent samples may be more suitable for the analysis. Although the maximal duration of induction has not been properly studied, it depends on the conciliation between the success rate and the tolerability/safety. Shorter inhalation times (e.g. 15-20 min) have similar success rates and practicability to longer inhalation times (30 min). It is critical to keep the duration of inhalation constant between inductions in the same subject to obtain comparable results. Common practice employs a cumulative duration of nebulization for 15-20 min. Other influencing factors might include the respiratory frequency during nebulization and the patterns of inhalation (i.e., slow deep inhalation or tidal breathing) during the challenge.

2.4 Variations of expectoration

Some investigators recommend that subjects clean their oral cavity with gargle, dry with napkin, spit for saliva, and finally cough for sputum. Others argue that mouth rinsing and drying may increase oropharyngeal inflammation. Some authors encourage the use of nose clips. Sputum induction protocols are different with respect to the schedule of sputum collection. Subjects may be asked to stop inhalation at regular intervals to cough up sputum (e.g., every 5 min), or to stop only when they feel the urge to cough. Some protocols require subjects to spit saliva into one container before coughing sputum into the other. Spitting saliva before coughing sputum decreases the percentage of squamous cells in sputum by 30% and increases ECP in the supernatant by 80% (Gershman, et al. 1996). The production of a good sputum sample relies heavily on the characteristics of the individual subject rather than the technical factors.

2.5 Repeating sputum induction

Repeated sputum induction can heighten airway inflammation resulting in an iatrogenic change in cellular components. Repeating sputum induction at 8-24 h after the initial induction can increase neutrophil recruitment in the second sputum sample (Nightingale, et al. 1998, Holz, et al. 1998b). An interval of 48 h between two inductions gave comparable cell counts in normal subjects (Purokivi, et al. 2000). It is currently recommended that subsequent induction should be conducted at least 2 days apart.

2.6 Sputum induction protocols

2.6.1 Classical procedure

1. The detailed information should be provided to the patient prior to the procedure.
2. All equipments including the ultrasonic nebulizer should be checked for safety and calibrated for the output of ~ 1 mL/min.
3. The baseline FEV_1 is measured prior to the bronchodilator inhalation.
4. The bronchodilator (200 μg salbutamol) is inhaled before the commencement of sputum induction.
5. The FEV_1 is measured after the bronchodilator inhalation for 10 min.
6. Either a fixed concentration (3 or 4.5%) or increasing gradient (3, 4 and 5%) of sterile saline is nebulized as an inducer. Each nebulization lasts 5 min follows by a brief expectoration. The total duration will last ≤ 20 min. Alternatively, the continuous 20-

min nebulization may be interrupted at 1, 4, 5, 10, 15, and 20 min for expectoration. The subjects will be allowed to spit outside the schedule whenever they develop the urge to cough.

7. The FEV_1 at each interruption will be measured. The induction will be terminated if there is ≥ 20% fall in FEV_1 from the postbronchodilator value or the symptoms develop.

2.6.2 Customized procedure for high-risk subjects

1. The detailed information should be provided to the patient prior to the procedure.
2. All equipments including the ultrasonic nebulizer should be checked for safety and calibrated for the output of ~ 1 mL/min.
3. The baseline FEV_1 is measured prior to the bronchodilator inhalation.
4. The bronchodilator (200 µg salbutamol) is inhaled before the commencement of sputum induction.
5. The FEV_1 is measured after the bronchodilator inhalation for 10 min.
6. The 0.9% sterile saline solution will be nebulized with interruptions at 30 sec, 1, and 5 min for FEV_1 measurement as a safety precaution. If this fails to induce sputum, the 3% saline concentration will be nebulized with interruptions at 30 sec, 1, and 2 min for FEV_1 measurement. If this also fails to induce sputum, the 4.5% saline concentration will be nebulized with interruptions at 30 sec, 1, 2, 4 and 8 min for FEV_1 measurement. The induction will be terminated if there is ≥ 20% fall in FEV_1 from the postbronchodilator value or the symptoms develop.
7. If the subjects cannot produce spontaneous cough, they will be driven to cough and spit after 4 and 8 min.

2.7 General consideration

1. The protocol should be strictly enforced, especially the inhalation timing.
2. The induction should not be repeated within 48 h after the first induction.
3. The safety procedure must be ensured and readily accessible.

2.8 Validation of sputum

The outcome of downstream application relies on the quality of sputum. The procurement of secretions from the lower respiratory tract (sputum) induced by the inhalation of aerosol from hypertonic saline usually is contaminated with saliva. The assessment of sputum quality relies on the quantitation of contaminated squamous epithelial cells.

The collection method has been optimized to improve the induced sputum quality by the selection of viscous portion of the specimen that should minimize the contamination with squamous epithelial cells (Pizzichini, et al. 1996b). This selective collection could obtain at least two-thirds of the viable nonsquamous cells. After DTT treatment, the cellular contents of the selectively collected sample would be dispersed and were ready for cytospin examination. This selected portion usually held a similar proportion of neutrophils, eosinophils and lymphocytes. However, it contained much higher concentrations of the fluid-phase ECP than did the whole expectoration. The unaltered cellular proportion suggested that the selection for viscous portion did not alter the indices of airway inflammation. Normally, saliva contains mainly squamous epithelial cells (99%) but very

low levels of ECP (2–90 ng/mL). The selected viscous portion was generally contaminated with very little squamous cells (1.2%), whereas the remaining clear portion contained a large portion of squamous cells (70%) (Pizzichini et al. 1996b). The lessening squamous cell content unmasked the inflammatory cells, resulting in better quality after the cytospins. When the squamous contamination was less than 20%, the accuracy of differential cell counts was better, as indicated by high inter and intraobserver reproducibility. The processing of cytospin is also quicker, especially when 1,000 or so cells were counted for more accurate identification of metachromatic cells and lymphocytes, the minority cellular contents. Also, the small proportion of squamous cells provides a homogeneous population of cells for examination by flow cytometry (Kidney, et al. 1996).

Alternatively, the quality of induced sputum specimen can be evaluated by the presence of high proportion of viable nonsquamous cells. Cell viability is an important requirement for accurate cell identification. With greater than 50% viability, the reproducibility of cell counts is better. Higher viability also provided an advantage for immunological staining to determine subpopulations and activation markers (Hansel, et al. 1991, Kidney et al. 1996, Vatrella, et al. 2010).

In summary, the physical selection of viscous portion from the mixed expectorate has several advantages over the whole specimen. It is almost free of squamous cells and is therefore essentially undiluted. Cells are in better condition and the concentrations of eosinophilic cationic protein are higher. If the expectorate is not processed within 2 h, a large error might be introduced. The serous portion, which is rich in squamous cells, could be homogeneously mixed with the otherwise desirable viscous portion. The reproducibility of cell counts is threatened if squamous cell contamination represents > 20% of all recovered cells.

3. The conventional practice for cytologic study

The sputum should be processed as soon as possible or within 2 h to ensure optimal cell counting and staining (Pizzichini et al. 1996b, Fahy, et al. 1993). The dithiothreitol (DTT) is used to split the disulfide bonds in mucin to release the cells (Cleland 1964). Cells anchoring to the mucus tend to get dark stain that hinders accurate identification. DTT provides more effective cellular liberation from the mucus than does phosphate buffered saline (PBS) and has no effect on cell counts.

The duration and temperature of mixing can vary between 10-30 min and 4-37°C respectively. This range of DTT exposure time at room temperature has no effect on the differential cell count (Popov, et al. 1994). The mixing of sputum with DTT can be performed with either a shaking water bath at 37°C followed by periodic aspirations, or a tube rocker at 22°C (Popov et al. 1994, Fahy et al. 1993, Spanevello, et al. 1998). The use of a plastic transfer pipette for aspiration and expulsion of sputum is not recommended since it decreases cell yield due to incomplete mixing (Popov et al. 1994, Hansel et al. 1991).

A sample filtration is strongly recommended for removing residual mucus and debris to improve slide quality. A single filtration through a 48-µm nylon mesh results in a slight reduction in the total cell count but the differential cell count remains unchanged (Efthimiadis, et al. 1996, Efthimiadis, et al. 2000). However, little is known about the effect of repeated filtrations on differential cell count.

Since the total cell count could be lessened after centrifugation (Parameswaran, et al. 2000, Rerecich, et al. 1999), it is therefore recommended that total cell count be performed prior to centrifugation to exactly obtain the original cell count. The currently automated machine is not reliable for determining total cell count and differential cell count, and is still not recommended.

Centrifugation is generally used to separate sputum cells from the fluid phase. The centrifugation force should be set between 300 - 1500 × g for 5-10 min to obtain adequate separation of the cells and the supernatant (Fahy et al. 1993, Louis, et al. 1999, Pizzichini, et al. 1996a). The storage temperatures for cells and supernatants are -20°C and -70°C respectively (Pizzichini et al. 1996a).

The optimum cell density for cytospins is 40-60 × 10^3 cells / slide that provide a more accurate estimate for cell distribution than does the smearing technique (Pizzichini et al. 1996a, Popov et al. 1994). The centrifugation force of 22 × g for 6 min is generally employed (Pizzichini et al. 1996a, Popov et al. 1994). Although this speed is below the limits of minimal cell distortion, there is a risk of losing lymphocytes at low speeds due to the dispersion to the supernatant (Fleury-Feith, et al. 1987, Mordelet-Dambrine, et al. 1984). This should be taken into account in the investigations of sputum lymphocytes.

The differential cell counts can be accomplished using either Wright's or Giemsa stain (Efthimiadis, et al. 2002). The buffers must be titrated to the optimal pH (7.1-7.2) (Efthimiadis et al. 2002) to allow accurate characterization of cells based on their optimal staining while maintaining original morphology. The differential cell count requires a minimum of 400 nonsquamous cells (Efthimiadis et al. 2002). The report should contain the relative numbers of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells expressed as a percentage of total nonsquamous cells. The percentage of squamous cells should always be reported separately.

4. Immunocytochemistry procedures

The sputum suspension is centrifuged at 300 × g for 10 min, resuspended in PBS or Hank's balance saline solution (HBSS). The cytospins are prepared on L-polylysine-coated slides to ensure minimal cell loss during multiple washing steps. The attached cells are air-dried for 10 min and fixed appropriately. After fixation, cytospins should be wrapped in foil and stored at -20°C pending for staining.

The method of fixation is critical and needs to be optimized for any particular antigen to obtain the best quality of immunostaining. The selected fixation method should allow the proper preservation of antigens / cellular morphology and the penetration of antibodies into the entire cells. The fixation regimens may include either 2 or 4% paraformaldehyde, formalin, acetone/methanol (60/40) or periodate-lysine-paraformaldehyde (PLP). The latter is a fixative for surface glycoprotein staining but may also be used for cytokine staining (McLean & Nakane 1974). For instance, PLP fixation provided better morphology of cryostat sections but poorer immunostaining than conventional acetone immersion. However, a brief acetone fixation followed by PLP fixation offered excellent morphology preservation and good quality of immunostaining (Hall, et al. 1987). PLP was proposed for the fixation of multiple membrane antigens in skin biopsies (Pieri, et al. 2002). The benefit of PLP fixation is in the preservation of cellular ultrastructures (i.e., immunoglobulins) for

immunofluorescence or immunoperoxidase staining of paraffin-embedded specimens (Rantala, et al. 1985). For marker staining of induced sputum cells, PLP-sucrose provided the best results with the highest percentage of $CD3^+$ cells and a better staining quality than did the paraformaldehyde and acetone-methanol-fixed cells that provided the worst staining of CD68 (St-Laurent, et al. 2006). Simultaneous fixation and permeabilization using Ortho PermeaFix for flow cytometry are required for the best identification of intracellular antigens (i.e., eosinophil cationic protein, eosinophilic peroxidase, neutrophil myeloperoxidase) in cytocentrifuged cells (Metso, et al. 2002). Organic solvents as fixatives are not suitable while the crosslinking fixatives (e.g., paraformaldehyde) alone could not provide a complete penetration of antibodies into the cell interior (Metso et al. 2002).

To detect intracellular antigens, sputum cells must be permeabilized after the fixation with paraformaldehyde and glutaraldehyde. Permeabilization allows the antibody to gain an access to intracellular or intraorganellar antigens. Two common permeabilizing agents are organic solvents (i.e., methanol and acetone) and detergents.

The organic solvents work through dissolving lipids from cell membrane, thereby disrupting the membrane and allowing the influx of the antibodies. The ability of the organic solvents to coagulate proteins provides an additional advantage of cell fixation. The shortcoming of organic solvents is the removal of lipidic antigens or lipid associated antigens from cells.

One of the most commonly used detergents is saponin, a plant glycoside. Saponin permeabilizes cells through the removal of cholesterol, thereby puncturing holes over the membrane (Seeman, et al. 1973). Saponin can form micelle with antibody and cholesterol that facilitates the entry through the punctured holes. However, saponin cannot effectively permeabilize mitochondrial membranes and the nuclear envelope due to their low composition of cholesterol (Goldenthal, et al. 1985a). Therefore, saponin is suitable for immunostaining of intracellular membrane antigens localized over lysosomal membrane, plasma membrane, endocytic vesicles and endoplasmic reticulum (Goldenthal, et al. 1985b). However, Triton X-100 and NP-40 interfere the staining at these sites (Goldenthal et al. 1985b).

Other commonly used detergents are the non-ionic detergents such as Triton X-100 and Tween 20 (Maneechotesuwan, et al. 2010, Maneechotesuwan, et al. 2008). They carry uncharged, hydrophilic head groups of polyoxyethylene moieties. Antigens localized in mitochondria and the nucleus required Triton X-100 for their detection (Goldenthal et al. 1985b). Their shortcoming is their non-selective nature that could produce a false negative during immunostaining through the removal of proteins along with the lipids. A combination of different permeabilizing agents may be customized for each antigen (Goldenthal et al. 1985a).

Immunocytochemical staining can be performed with different varieties, including avidin/biotin complex, peroxidase/antiperoxidase and alkaline phosphatase / antialkaline phosphatase techniques. The use of immunoenzymatic techniques eliminates the need for expensive fluorescent microscopy. The alkaline phosphatase / antialkaline phosphatase method is preferable. The staining with the monoclonal antibodies on fixed slides should be titrated for appropriate concentrations and can be incubated overnight at 4°C. The secondary antibodies are then applied and the antibody/antigen complex is visualized

using the alkaline-phosphatase-linked substrate, with either fast red or fast blue counterstains. Negative controls must always be included to exclude the potential false positive staining. The peroxidase staining methods are not recommended for sputum.

5. Modifications of immunocytochemistry for sputum specimen

Homogenization with low-concentration DTT (0.5 mM) would liberate the otherwise anchored cells from the surrounding mucus that facilitate the exposure of the cells to the staining antibodies (Tockman, et al. 1995). However, the use of DTT to disperse cells may hamper cellular functions (e.g., the release of elastase and myeloperoxidase (MPO) from neutrophils (van Overveld, et al. 2005)) or hinder antigenic epitopes for immunocytochemical staining. Some investigators recommended the use of paraformaldehyde to fix the sputum cells prior to the treatment with low concentration of DTT.

The method employing avidin-biotin complex (ABC) is suitable for the immunocytochemistry of sputum cells (Maneechotesuwan et al. 2008, Maneechotesuwan et al. 2010). The biotin / avidin system possesses several advantages to sputum immunocytochemistry. 1) The binding affinity of avidin to biotin is higher than that of any antibody directing against its epitope. 2) The binding of avidin to biotin is almost irreversible. 3) The multiple binding sites on each molecule (four binding sites for biotin on each avidin; two binding sites for avidin on each biotin) provide the formation for macromolecular complexes between avidin and biotinylated enzymes. The ABC-alkaline phosphatase (AP) lattice complex consists of several biotinylated alkaline phosphatase molecules cross-linked by avidin. The two biotin molecules can be joined via an avidin molecule that eventually forms a complex of avidin and biotinylated enzyme or biotinylated secondary antibody. The stoichiometry of the forming complex will contain an available biotin binding site on ABC for the binding of the biotinylated secondary antibody. The formation of the complex is developed by gradual mixing avidin and biotinylated alkaline phosphatase with predefined ratios prior to use. The ABC complex can be stable until 24 h after the formation. This technique provides multiple enzymes attaching to the antigenic site, thereby enhancing the detection sensitivity. The sensitivity provided by this method is generally higher than that obtained with the conventional peroxidase-anti-peroxidase (PAP) technique. However the size of the ABC complex can be inappropriately high that it sterically interferes the overall binding, resulting in decreasing the resolution.

The ABC method can also be modified to incorporate different enzymes that provide different chromogenic properties (Bratthauer 2010). These enzymes include alkaline phosphatase (AP). The high sensitivity of the ABC-AP system permits the detection of the small amount of antigen using higher dilution of a primary antibody. Therefore, the ABCAP system is recommended for staining situation in which high sensitivity is a prerequisite such as sputum immunocytochemistry. The advantages of these techniques lie in the availability of suitable secondary agents. The shortcoming of this system is the presence of endogenous alkaline phosphatase, which is more ubiquitous than endogenous peroxidase and is tougher to remove. However, the alkaline phosphatase can generate more color producing molecules per enzyme molecule than can peroxidase, resulting in higher sensitivity. Endogenous alkaline phosphatase can be partially blocked by incubation with 3 mM levamisole for 15 min with some remaining residual activity.

Labeling of 2 epitopes can employ the simultaneous peroxidase and AP methods. An ABC assay system can be applied to the detection of more than one antibody on an individual specimen. In double labeling, experiments having two completely different assay systems would minimize the cross-over reactivity. The first antigen can be detected with the standard ABC procedure, while the second antigen can be detected using the PAP system. The two techniques provide minimal cross-over reactivity, especially if alkaline phosphatase is employed along with the peroxidase enzyme (Gillitzer, et al. 1990).

Various sugar moieties interfere with the binding of streptavidin or avidin to biotin. The most effective inhibitory sugar is mannose, followed by other saccharides. The inhibitory action probably involves the interactions of the sugars with reactive residues at the binding sites (Houen & Hansen 1997).

6. Practical considerations

1. Commercial PBS or TBS are recommended to maximize the reproducibility. The buffer should be prepared according to the manufacturer's recommendations. The commercial buffer usually makes up 5 L of solution.
2. The primary antibody should be diluted with TBS or PBS to an optimal concentration. These should be empirically titrated on a known positive specimen. Working antibody concentrations usually lie between 10-20 μg/mL. Depending on the individual reagent, this concentration could vary considerably. The initial dilution can start at 1:10 with subsequent serial 1:10 dilutions, resulting in 10, 100, 1000, and 10,000-fold dilutions of the original antibody. Optimal staining can be obtained from this wide range of dilutions. A higher resolution for the optimal staining can be obtained through serial 1:2 dilutions of the formerly obtained wide dilution. The antibodies can be aliquoted and stored in concentrated form at -70 to –80°C indefinitely. The antibodies should be thawed once and used immediately. Refreezing antibodies should be avoided. However, the manufacturer recommends that the ABC reagent components should be stored at 4°C. The antibody can be thawed and diluted to a concentrated stock solution from which more diluted working solutions can be prepared. These stock solutions can be kept at 4-8°C for a week.
3. An antibody in either polyclonal or monoclonal format can be applied for sputum staining with distinct advantages / disadvantages. The polyclonal antibody generally provides strong signal with reasonably good specificity, but can generate some background noise. The signal strength of a monoclonal antibody depends on its qualities and affinity but its specificity for antigen binding is far better than that of the polyclonal format. The preference for a monoclonal antibody over a polyclonal antibody largely depends on the availability of a qualified antibody with matching application. The chosen antibody has to be specific with no cross reaction to other cell components. The antibody should contain a high affinity to the antigen be produced in high titer. The monoclonal antibody generally creates minimal background noise. Its high affinity can withstand multiple processing steps of staining and washing with minimal loss of the attaching antibody.
4. The incubation with 10% xenogeneic serum will mask all non-specific binding sites on the specimen. The 10-min incubation with the 10% xenogeneic serum can prevent the non-specific antibody binding. The proportion of the serum and the incubation time can be optimized to generate acceptable signal.

5. The choice of blocking serum depends on the originating species of the secondary antibody. A universal type of blocking serum can be used if the secondary antibody has multiple host species to avoid the cross-reactivity. The pooled or universal secondary antibody can be used regardless of the originating species of the primary antibody. Proteins from other sources (e.g., milk, or casein-based solutions) can be employed as blocking agents, but they may not produce better results.
6. The specimens should be kept hydrated throughout the staining procedure. The inadvertent drying can generate nonspecific antibody binding. A chamber rack can be used to prevent the flowing of antibodies away with gravity.
7. All reagents and slides should sit at room temperature before the staining. The antibodies should be fully dissolved or reconstituted. The staining results can even be better if the antibody solutions are prepared the day before and left at 4°C overnight. The incubation time and temperature can be raised to optimize the reactivity. However, this might increase the background noise too. The reaction may be slightly improved without cumulative background by the incubation with primary antibody overnight at 4°C (Clements & Beitz 1985).
8. Vigorous rinsing is recommended to lessen the background noise. However, direct splashing to the specimen surface with the wash stream may dislodge antibodies with low affinity. The stream should be rapidly running from one end of the slide, crossing the slide surface to the other end. The washing step is the most critical factor to lessen the background noise and could be extended if the background noise is heavily concerned. The inclusion of non-ionic detergent (i.e., 0.25% Triton X-100 or 0.1% Tween 20) may also lessen the background (Laitinen, et al. 1983, Maneechotesuwan et al. 2008). However, these detergents may interfere the charge interactions of antibody-antigen binding, resulting in decreasing reactivity. These detergents should be applied to poly-L-lysine-coated or charged slides to improve the hydrophobicity of these slides. The reaction can be strengthened through extending incubation times (60 min for primary and 45 min for the secondary or ABC incubation), raising chromogen concentration to prevail over the dampening effect of the detergents. The chromogen concentration may be raised from 2-5 times. If background noise is highly concerned, a preabsorption of the slide with blocking agents may be required. The antibody may be diluted in buffer containing 2% bovine serum albumin, or secondary species serum to avoid the reaction from contaminating non-specific antibodies against a serum-based constituent.
9. The formation of ABC complex takes at least 30 min to be stable. Only after the ABC complex is stably formed can the secondary antibody be added. The complex can be kept in the refrigerator for at least 72 h.
10. The counterstain should not be stronger than the principal reaction; otherwise the staining sites can be masked by darker counterstain.
11. Many substrates for alkaline phosphatase provide permanent straining with different color. A combination of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) yield permanent blue precipitates at the site of alkaline phosphatase, while the Fast Red TR/Naphthol AS-MX yields a permanent red precipitates.
12. The staining sensitivity can be increased through the use of multiple chromogens and multiple enzymes targeting numerous antigens. More substrate precipitation or greater color resolution improves the sensitivity.

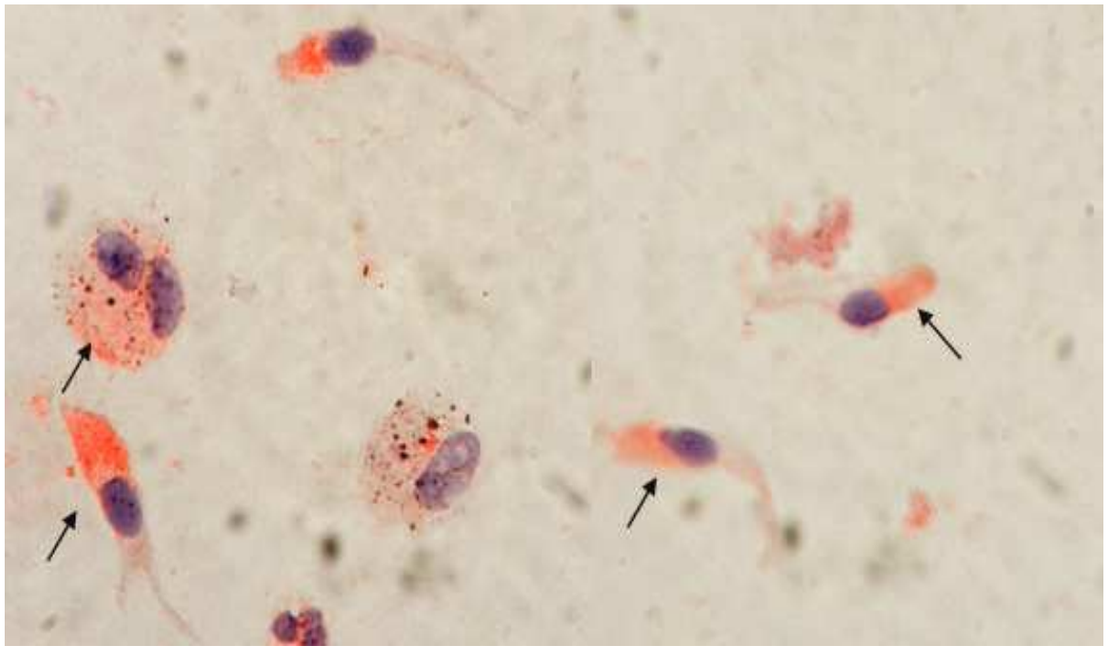

Fig. 1. The sputum cells were immunocytochemically stained for indoleamine 2, 3 dioxygenase with VECTOR® ABC-AP KIT.

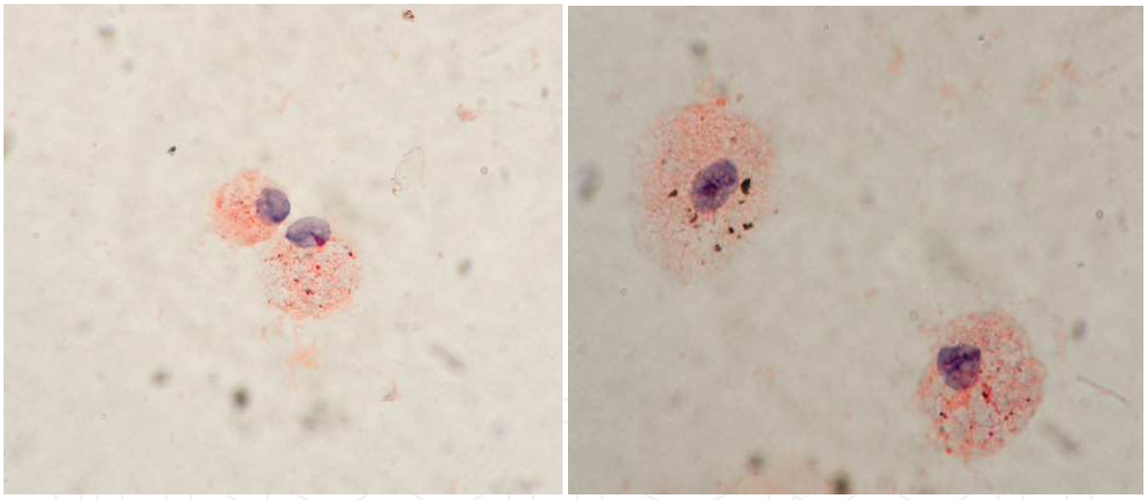

Fig. 2. The sputum cells were immunocytochemically stained for interleukin-10 with VECTOR® ABC-AP KIT.

6.1 Prototypic procedure for immunocytochemical staining of a sputum specimen (VECTASTAIN® ABC-AP KIT)

1. The cytospin slide will be removed from the freezer (-20°C) and left at room temperature for 30-60 min.
2. The cellular spot on the slides will be encircled with the wax pen.
3. The slide will be washed once with PBS for 5 min.
4. The washed slide will be placed horizontally in a humidified chamber.

5. The sputum cells will be fixed with 2% paraformaldehyde (PFA) for 5 min.
6. The slide will be completely washed once with PBS to remove residual PFA for 5 min.
7. The cell membrane will be permeabilized with 0.5% NP-40 diluted in PBS for 10 min.
8. The slide will be washed once with PBS for 5 min.
9. Nonspecific binding will be blocked with the blocking serum (normal serum) from the Vector Kit (3 drops of stock to 10 mL PBS with 0.05% tween-20) for 30 min.
10. The slide will be incubated with the primary antibody pre-diluted in PBS with 0.05% tween-20 (1:100) for 1 h.
11. The slide will be washed thrice with PBS containing 0.05% tween-20 for 5 min.
12. Incubate the slide with the biotinylated secondary antibody pre-diluted in PBS with 0.05% tween-20 (add 1 drop of stock to 10 mL PBS with 0.05% tween-20) for 45 min.
13. The VECTASTAIN® ABC-AP Reagent will be prepared by adding exactly 2 drops of Reagent A to 10 mL PBS with 0.05% tween-20, followed by adding 2 drops of Reagent B. The mixture will be immediately mixed and left at room temperature for at least 30 min before the next step.
14. The slide will be washed thrice with PBS containing 0.05% tween-20 for 5 min each.
15. The slide will be incubated with VECTASTAIN® ABC-AP Reagent for 30 min.
16. The slide will be washed once with PBS without tween-20 for 5 min.
17. The Vector® Red substrate working solution will be prepared immediately before use in test tube by adding 2 drops of Reagent 1 to 5 mL 100 mM Tris-HCl (pH 8.2-8.5) followed by a thoroughly mixing. The mixture will be subsequently reconstituted with 2 drops of Reagent 2 and 2 drops of Reagent 3 sequentially with a thoroughly mixing after each reagent addition.

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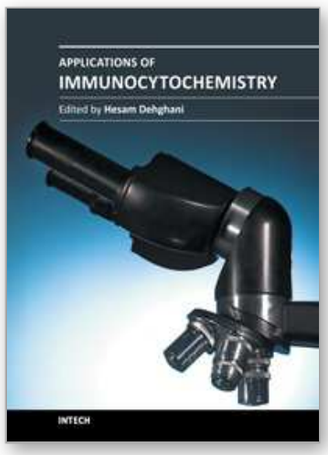

Applications of Immunocytochemistry
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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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