Techniques in pulmonary cytopathology

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Introduction
The foundation of pulmonary cytopathology is generally attributed to Dudgeon and Barrett (1934), although physicians have inspected sputum for evidence of lung disease since the earliest days of medicine. There are few fields of cytopathology in which modern developments in fibreoptics and imaging techniques have contributed so much, not only by expanding the variety of specimens which can be obtained, but also by increasing the quantity and quality of the cellular material recovered. The chief diagnostic application remains the investigation of patients with suspected carcinoma of the lung, but through the technique of bronchoalveolar lavage, pulmonary cytopathology also contributes to the management of benign disease particularly in the immunosuppressed.

Sampling methods and cytopreparatory techniques
The different methods of obtaining cellular material for cytological examination are shown in table 1. In addition, supplementary techniques such as fine needle aspiration of lymph nodes and examination of pleural fluid can be used in staging.

Sputum
Examination of sputum is non-invasive and does not inconvenience patients or clinicians. It can provide definitive evidence of malignancy in many cases but no localisation of the lesion. It is, however, labour intensive as a laboratory investigation both with regard to preparation and microscopy and a percentage of the specimens processed will turn out to be unsatisfactory.

Specimens of early morning "deep cough" sputum should be submitted on three consecutive days. Sputum produced during physiotherapy to the chest is also suitable. Sputum obtained after bronchoscopy may yield valuable diagnostic information as it is usually highly cellular but must be labelled as such; otherwise the hyperexfoliation of epithelial cells may lead to interpretative difficulties. In patients that cannot expectorate spontaneously induced sputum can be produced by inhalation of the vapours of a warmed (37°C) mixture of 15% sodium chloride and 20% propylene glycol for 20 minutes. Sputum samples should be collected into clean, dry containers with screw-on lids and transported to the laboratory in biohazard bags. Prior fixation with 70% ethyl alcohol is not recommended as it produces a rubbery specimen which is almost impossible to smear. Preservation of cells in unfixed sputum remains reasonable for 24 hours if the container is refrigerated.

Saliva and upper respiratory tract mucus are seldom of diagnostic value, but the distinction between these and lower respiratory tract material cannot be reliably made without microscopy and all specimens must therefore be processed. Using a safety cabinet, the sputum is emptied into a Petri dish and inspected. Any thick streaks, bloodstained areas, or tissue fragments should be selectively included in the sampling which is carried out with disposable plastic forceps. A small blob is placed on a slide, squashed down with a second slide, and the two drawn apart and immediately placed into 95% ethyl alcohol for fixation. A total of four slides is generally sufficient. Staining is best achieved by the traditional Papanicolaou method which gives excellent clear morphological detail and permits inspection of the special tinctorial properties of squamous cells, an inherent part of this technique. The other traditional cytopathological stain, May-Grünwald-Giemsa (MGG), is not suitable for sputum due to the mucoid background which obscures the cells. Much less cellular detail, especially in thickly spread areas, can be seen with haematoxylin and eosin. If special stains,
Adenocarcinoma: sputum. A large three dimensional group of malignant cells (Papanicolaou).

such as Ziehl Neelsen are required, additional slides can be prepared from the original specimen which should be retained until microscopy is complete.

Several more complicated preparatory techniques exist, but none conveys any real advantage over the standard simple method as they are time consuming, result in loss of morphological detail, or disrupt the pattern of cell streaks and groups which are helpful in forming a diagnosis. The most widely used of these is the Saccomano technique in which pooled sputum is collected into a mixture of 50% ethyl alcohol and 2% polyethylene glycol (Carbowax) and the specimen emulsified in a blender in the laboratory. Apart from the inherent risk from aerosol spread of infection, the diagnostic sensitivity is inferior to the examination of fresh sputum as described above. Formalin fixation of centrifuged cell blocks which are then processed histologically is a laborious procedure and from personal experience produces a specimen which is difficult to interpret.

BRONCHOSCOPIC SPECIMENS FOR DIAGNOSIS OF NEOPLASIA

Bronchial secretions and washings

Bronchial secretions can be aspirated through a flexible catheter inserted down the bronchoscope. They generally yield only degenerate debris, mucus, and inflammatory cells and are of little diagnostic value. Bronchial washings are obtained by instilling 3–5 ml of a balanced salt solution down the bronchoscope and then aspirating the fluid. The lavage can be aimed at an abnormal area or directed along the bronchus to sample from outside the area of visual inspection. The fluid is centrifuged and about six slides prepared from the cell button. These are immediately fixed in 95% ethyl alcohol. Four are stained by the Papanicolaou method and the remainder retained in case additional material is required.

Bronchial brushings

Brushings are taken directly from the suspected tumour and can contain many well preserved cells. If the surface of the lesion appears necrotic two brushings should be obtained as the first may yield only debris. Brushing is best carried out before bronchial biopsy as the latter may cause bleeding which can interfere with the fixation of the cells harvested by the brush. Immediately the brush is removed from the bronchoscope it should be firmly rolled on to a series of glass slides, each slide being placed in a transport container of 95% ethyl alcohol as it is prepared. Great care must be taken to ensure that the slides are not allowed to dry before alcohol fixation. Sufficient material is usually available for four slides. Additional cells can be obtained by agitating the brush in 5 ml of isotonic saline and centrifuging the fluid in the laboratory.

Transbronchial fine needle aspiration

Transbronchial fine needle aspiration is used less frequently than other cytological sampling techniques and is time consuming to perform. It is useful for investigating external bronchial compression or submucosal lesions, however, especially in the upper lobes where biopsy forceps can be difficult to manipulate. Hilar, subcarinal, and low right paratracheal lymph nodes are particularly suitable for this type of investigation. Transbronchial fine needle aspiration may sometimes provide diagnostic evidence of malignancy when both brushing and biopsy fail.

The Wang disposable needle (Millrose Company, Ohio) is the device most often used for transbronchial fine needle aspiration. The apparatus consists of a 120 cm long
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Figure 3 Squamous cell carcinoma: bronchial brushings. Well differentiated keratinised malignant cells (Papanicolaou).

One to two slides should be rapidly placed in 95% ethyl alcohol for fixation and subsequent Papanicolaou staining. The remaining couple can be air dried and then stained with MGG. The latter technique is very suitable for fine needle aspirates as contamination with bronchial mucus and debris is much less than with brushings and washings.

PERCUTANEOUS FINE NEEDLE ASPIRATION
Of all anatomic regions, the lung is probably the site in which the advent of percutaneous fine needle aspiration (PCFNA) has had the greatest impact on patient management. Apart from the occasional instance of infectious disease it is almost exclusively used for the diagnosis of malignant disease. Sensitivity and specificity are high and PCFNA avoids open lung biopsy as a means of diagnosis. The method is not without its complications, however, chief among which is the risk of pneumothorax. Tumours seeding in the needle track is extremely rare.

Accurate localisation of the lesion is necessary. Skin markers and plain x-ray pictures are insufficient. Fluoroscopic control, preferably with biplane imaging, computed tomography, or for very superficial lesions, ultrasound scans, are required. Aspiration is easiest if the patient is lying down and a vertical track is chosen for the needle. The centres of very large tumours are frequently necrotic and better preserved diagnostic material is commonly aspirated from the periphery. Local anaesthetic is injected down to the pleura and the needle is then inserted and advanced under fluoroscopic or other type of imaging control. A 20 to 25 gauge needle is used. Special needles such as the Rotex screw needle are available for sampling hard lesions, such as chondromatous hamartoma. A fresh sterile needle is required for each percutaneous pass if aspiration is repeated. With the needle in position a 20 ml or 50 ml syringe is attached and suction is applied. Aspiration of pus or blood mixed with solid particles is indicative of satisfactory aspiration. The needle and syringe are then withdrawn.

PCFNA is carried out by the radiologist in the Department of Diagnostic Imaging but it is advisable for a cytotechnologist (or pathologist if it is considered that viewing the image during aspiration will assist in diagnosis) to be present to receive the needle and syringe to ensure optimal handling of the aspirated material. The equipment listed in table 2 should be laid out ready for use.

Unless a large volume of aspirate is obtained the needle is detached from the syringe and a small quantity of air drawn in. The needle is then reattached and a drop of contents “blown out” on to a slide and thinly spread with the end of a second slide as if making a blood smear. The procedure is repeated until the aspirate is fully processed. Half the slides should be placed in the ethyl alcohol as soon as they are prepared and the remainder rapidly air dried (preferably with a hairdryer) and placed in the rack. The needle

Figure 4 Large cell carcinoma: bronchial brushing. Malignant cells showing no discernible differentiation (Papanicolaou).
Table 2  Equipment for slide preparation after percutaneous fine needle aspiration

- Supply of frosted end glass slides
- Pencil to label slides
- Tray for spreading out slides
- Containers of 95% ethyl alcohol for wet fixation of slides
- Hair dryer to dry slides
- Rack for air dried slides
- Watch glass
- Paper tissues
- Disposable plastic forceps
- Disposable centrifuge tube containing 5 ml physiological saline, Hank's solution, or cell culture medium to rinse out needle and syringe
- Pot of 10% buffered formalin for tissue fragments
- Tube of medium if microbiological culture is required
- Reagents for Diff-Quik stain (Merz-Dade) as in table 3
- Request forms

and syringe are then washed out in saline or other transport medium to recover any remaining material by subsequent centrifugation. If the aspirate is very copious and contains much blood the whole contents of the syringe can be expelled into a watch glass and the excess fluid absorbed with paper tissue and smeared then prepared from the residual material. Any obvious tissue fragments are placed in the buffered formalin. If microscope facilities are available a slide can be stained by the Diff-Quik method (table 3) and examined in the Department of Diagnostic Imaging to ensure that adequate material has been aspirated before the patient returns to the ward.

In the laboratory the prepared slides are stained with Papanicolaou (alcohol fixed) or MGG (air dried), retaining several for special stains if these should be required. Additional useful material can often be obtained from the centrifuged transport medium used to rinse the needle and syringe. If there are tissue fragments these are embedded and sectioned as routine for small histological biopsy specimens.

**Diagnosis of neoplasias**

**INVESTIGATIVE PROTOCOL**

Some controversy exists as to the relative roles that sputum, endoscopic sampling (cytopathology and biopsy), and PCFNA should have in the investigation of patients with suspected malignant disease. Protocols vary from insistence on a minimum of three sputum examinations plus at least one bronchoscopy before resorting to PCFNA, to a recommendation that for reasons of speed, accuracy, and cost effectiveness PCFNA should be the primary approach in the diagnostic work-up. An intermediate position based on the clinical findings in each individual patient including the size and location of the lung mass and assessment of the relative risk of the various procedures seem most practical and the best use of resources. The various investigations are complementary, and while PCFNA is the single most sensitive technique less invasive methods are frequently fully effective.

**ASSESSMENT OF SPECIMEN ADEQUACY**

With bronchial brushings and fine needle aspirates (TBFNAs and PCFNA), this is straightforward and depends on the presence of satisfactory numbers of viable cells. For a specimen of sputum to be considered adequate, alveolar macrophages must be present. The identification of ciliated columnar cells is impractical.

**Table 3  Diff-Quik staining for rapid evaluation of fine needle aspirates**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Make smear</td>
</tr>
<tr>
<td>2</td>
<td>Rapidly air dry with hair dryer</td>
</tr>
<tr>
<td>3</td>
<td>Pot 1* 15 dips</td>
</tr>
<tr>
<td>4</td>
<td>Pot 2* 15 dips</td>
</tr>
<tr>
<td>5</td>
<td>Pot 3* 23 dips</td>
</tr>
<tr>
<td>6</td>
<td>Rinse with distilled water</td>
</tr>
<tr>
<td>7</td>
<td>Air-dry as in No 2 above</td>
</tr>
<tr>
<td>8</td>
<td>Dip in xylene</td>
</tr>
<tr>
<td>9</td>
<td>Coverslip using DPX resin mountant</td>
</tr>
</tbody>
</table>

*Commercial fixation and staining reagents supplied by Merz-Dade.

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**Figure 5**  Hamartoma: percutaneous fine needle aspirate. Mesenchymal material and cartilage cells (Papanicolaou).

**Figure 6**  Small cell anaplastic carcinoma: percutaneous fine needle aspirate. Two groups of oat cells (MGG).
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Figure 7 Large cell carcinoma: percutaneous fine needle aspirate. Large poorly differentiated malignant cells (MGG).

not sufficient evidence of lower respiratory tract sampling as these may be derived from the upper airway.

REPORTING
All available material should be examined as diagnostic cells (figs 1–7) may be irregularly distributed throughout the prepared slides. Particular attention is needed at the edges and tails of smears, especially of sputum, as these are the areas where malignant cells are most likely to be identified. Guidance on the interpretation of cytomorphology is available elsewhere. In addition to a description and conclusion, all reports must include a record of the number of slides examined, in case of future review.

Bronchoalveolar lavage
Bronchoalveolar lavage (BAL) is mainly used to investigate pulmonary infiltrates in immunosuppressed patients (figs 8 and 9) and to determine disease activity and response to treatment in cases of interstitial lung disease. It may be helpful in suspected asbestosis (fig 10) or in a few uncommon disorders such as lipoid pneumonia and alveolar proteinosis, and can also be used to retrieve cells from diffuse or localised, suspected neoplasia not amenable to diagnosis by brushing or biopsy.

LAVAGE TECHNIQUE
BAL is usually performed via the transnasal route and a 4–9 mm fiberoptic bronchoscope. Fluid is instilled by gravity or under gentle pressure with a syringe and then reaspirated by syringe or mechanical suction. A total of 100–300 mls isotonic saline in 50 ml aliquots is usually obtained, although in severely ill immunosuppressed patients smaller volumes can be used. The lavage is performed at the site of maximum radiological opacity or the middle lobe in cases of diffuse disease. About 40–60% of the volume of fluid instilled is recovered. The first aliquot generally only samples bronchial contents and alveolar material may not be obtained until subsequent specimens. A sample of fluid is reserved for the Department of Microbiology and the remainder for cytopathology and allied studies.

LABORATORY TECHNIQUES
Cell preservation is poor in saline and fluid for cytopathology should be processed within 30 minutes of BAL. Cytorepreparatory methods vary according to the clinical conditions under investigation.

Pulmonary infiltrates in immunosuppressed patients
Aliquot volumes are often small and all material should be processed.
Centrifuge fluid at 3500 rpm for 5 minutes.
Prepare slides from deposit and stain with Papanicolaou, MGG, Gram, ZN, and Grocott. With the exception of the Papanicolaou stain which requires alcohol fixation, all the other stains are performed on air-dried slides. Additional techniques—for example, fluorescent or immunocytochemical detection of Pneumocystis carinii, cytomegalovirus, or herpes simplex virus—are also possible. The morphology of the cells present and any background debris should be considered in addition to examining the

Figure 8 P carinii infection: bronchoalveolar lavage. Two characteristic alveolar casts (Papanicolaou).
Table 4 Diffuse pulmonary infiltrates in immunosuppressed patients: diagnoses possible from BAL fluid

<table>
<thead>
<tr>
<th>Opportunistic infection</th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Parasites</th>
<th>Viruses</th>
<th>Malignant infiltration</th>
<th>Lipoproteinosis</th>
<th>Pneumonitis, non-specific</th>
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<tr>
<td>-</td>
<td>Gram positive bacteria</td>
<td>Candida sp</td>
<td>Strongyloides stercoralis</td>
<td>Cytomegalovirus</td>
<td>Leukaemia</td>
<td>(Sepsis, drugs, irradiation, oxygen treatment, graft versus host disease)</td>
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<td>-</td>
<td>Mycobacterium tuberculosis (M avium, M intracellulare)</td>
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<td>-</td>
<td></td>
<td>Zygomycetes</td>
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<td>Non-specific viral inclusions (measles, adenovirus)</td>
<td>Lymphangitic carcinomatosis</td>
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<tr>
<td>-</td>
<td></td>
<td>Cryptococcus neoformans</td>
<td></td>
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<td>Kaposi’s sarcoma</td>
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<tr>
<td>-</td>
<td></td>
<td>Histoplasma capsulatum</td>
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<td>-</td>
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specimen for evidence of specific infection. The multifactorial nature of disease in the immunosuppressed patient cannot be over emphasised, and several of the conditions shown in table 4 may coexist. The morphological changes produced by pneumonitis, especially those caused by drugs or radiation, are severe and care must be taken not to confuse these with malignant infiltration.

Diagnosis of specific benign lung disease

The application of cytopathology is very limited but some special stains may be helpful provided interpretation is closely integrated with clinical findings. For example, Oil red 0 (lipoid pneumonia), Perl's (haemosiderin-laden macrophages, ferruginous bodies), diastase-periodic acid Schiff (alveolar proteinosis). If electron microscopy is required (alveolar proteinosis, histiocytosis X) 25 ml of 5% glutaraldehyde in cacodylate buffer is added to 25 ml BAL fluid as an electron microscopy fixative. Langerhans' cells in BAL react with anti-CD1, but small numbers may be found in conditions other than histiocytosis X.

Interstitial lung disease

The role of BAL is to determine disease activity and response to treatment. There is considerable variation in the results obtained which weakens the case for definitive measurement by BAL despite the initial enthusiasm of some centres.11,12 Total cell counts have little value.11 A suitable method for carrying out differential cell counts is that described by Turner-Warwick and Haslam.11

Separate the cells from the fluid by slow centrifugation and resuspend the cells in buffered tissue culture medium to 2 × 10⁶ cells/ml.

Use 100 aliquots of this suspension to prepare a set of slides by cytocentrifugation (Shandon Cytospin 450 rpm for 4 minutes). Stain with MGG and differentially count 300 to 500 cells by random point counting with × 40 objective.

Cells present include alveolar macrophages, lymphocytes, neutrophils, eosinophils and mast cells. Subsets of lymphocytes can be identified by appropriate immunocyto-
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chemical studies. Epithelial cells should be very few. Excessive numbers of ciliated columnar cells or abundant mucuspurulent debris are indicative of predominantly bronchial rather than alveolar material and cell counts will be unreliable. Differential cell counts should not be carried out in isolation and the cytomorphology of the cell population should be examined on Papanicolaou as well as MGG stained slides and by additional special stains if necessary.

It is difficult to give guidance on results of differential cell counts due to the variability of published figures, each centre establishing its own range. Details of these can be found in tabulated form in Stanley et al. Overall, idiopathic pulmonary fibrosis is associated with alveolar neutrophilia, prognosis is adversely affected by the presence of eosinophilia, and corticosteroid treatment is most likely to benefit patients with lavage lymphocytosis. Increased helper T lymphocytes are associated with sarcoidosis and increased T suppressor cells with hypersensitivity pneumonitis.