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Making the Most of Your Microscopy Mini Series

Session Two: Fluid Analysis On Your Own: Rapid In House Screening

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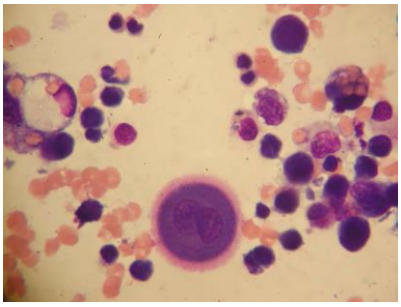

CYTOLOGY & FLUID ANALYSIS

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Introduction

This is a huge area that cannot be completely covered in one day, but sampling considerations, fundamental principles, inflammation and neoplasia, with certain fluids will be discussed.

Cytology is very useful as it is minimally invasive and quick, and with good sampling and accurate interpretation, is often diagnostic. Further more invasive, expensive and potentially risky investigations are therefore not always required and therapy can be initiated immediately. Alternatively it can reduce the number of differential diagnoses and therefore help guide further investigations. It is commonly used for superficial or palpable masses (*e.g.* skin) and is good for fluid preparations. Internal structures can be sampled under ultrasound guidance. It is rarely associated with complications but these should always be remembered where appropriate, such as possible metastatic 'seeding', bleeding or peritonitis (following aspiration of an abscess).

Whenever cytology is contemplated, the size and apparent nature of the mass should be taken into account. Also consider what diagnostic question you are asking and how this could be answered best, not only in terms of sampling technique but also the possible answers cytology can or cannot provide in that scenario.

For example, a small needle in a large mass will sample only very small areas of the mass, so useful or diagnostic material can be missed. Therefore aspirates from various locations within the mass are recommended. If large enough, ultrasound guidance can be used to target areas. **Both the centre and periphery of a (large) mass should be sampled**. Various techniques can be used to obtain cells, and these are best used, sometimes in combination, when the mass or situation dictate rather than having one technique which will not provide good material in all situations.

Sampling Techniques

Fine needle aspiration

The mass is first isolated and 'fixed' using one hand and then one of the techniques described below is used to harvest cellular material.

1. **Needle only** - initially it is recommended that the needle is used alone. A 21-23 gauge needle is preferred. It is held between the thumb and index finger, quickly introduced and moved to and fro in the mass without exiting the skin or mass itself but sampling all the areas of the mass. Spinning/rotating the needle on its axis helps to harvest tissue cells. The needle is then withdrawn. An air-filled 2.5 ml syringe is attached and the material briskly expelled onto one end of a clean labelled slide. Diagnostic material often appears as slightly 'creamy' to blood tinged fluid. Grossly the material can appear fatty if a lipoma or adipose tissue is sampled. This material is then prepared (spread) as outlined later. This whole sampling process is ideally repeated with a fresh needle. This technique minimises both cell damage and blood contamination. If no material is obtained, a suction method can then be used.

2. **Suction –** this is identical to that described above, but when the needle is in the mass, suction is applied by withdrawing the plunger on a (2.5-5ml) syringe attached before or after needle insertion. It is much easier (and better) to have two people doing this (one holding the mass and one aspirating), as otherwise the needle can move out of the mass when suction is applied. The suction can either be:

   intermittent – move the needle, apply suction and release. Redirect the needle and reapply suction, repeating several times.

   continuous – suction is applied and maintained whilst the needle is moved to and fro within the mass.

Suction must be released before removing the needle from the mass or the material is lost into the syringe itself.

The syringe is removed and filled with air to expel the material as outlined above. Suction methods increase the risk of both cell damage and blood contamination. This is especially true of continuous suction, which can also increase the risk of other tissues around the mass being sampled as the needle is moved. This method though is often essential for firmer masses (potentially requiring even 20 ml syringes) especially if they are derived (mainly) from soft tissue (fibrous) components.

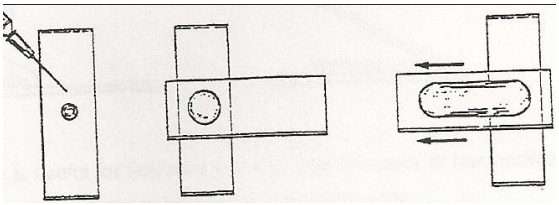
'Preparation of the Harvest'

Once the harvest / material has been obtained, it must be smeared gently to create a monolayer, otherwise all or most of the material cannot be examined for cytology.

There are several ways of dispersing/spreading it to allow staining and examination. Crucially the cells must form a monolayer and not become damaged. Strengths and weaknesses of each method vary, and very much depend upon the skill of the operator. As with blood films, only practise and adaptation or planning allow a consistently good smear to be made.

- **blood film technique** – this is identical to that for a blood film and is very good for more liquid, blood contaminated preparations, especially if the tissue cells are known to be fragile. The advancement speed should generally be slower though. For example lymph node aspirates can be prepared like this.

- **line concentration –** this is also the same as the blood film technique above, but when the material has been advanced about 2/3-3/4, the spreader slide is stopped and lifted off. This leaves a line of concentrated cells at one end. It is very useful for fluid and lavage/wash preparations, especially when they have low cellularity (do not appear grossly turbid/cloudy). Urine sediment cytology can also be prepared in this fashion.



Slide A

- **Squash –** If you have steady hands, this can be freestyle. However if not, it is advisable to hold (with thumb and index finger at either end) and rest the slide that has the material (A) on a flat bench before starting. Another labelled slide (B) is held at right angles with one end (that nearest your hand) over the droplet of material and lowered onto the material. As slide B touches, the material will naturally spread out. Without exerting any downward pressure, slide B is then smoothly and briskly pulled across over slide A. The majority of material is transferred to slide B, but it is worth using slide A too. Some people perform this with a flourish but this will only damage the cells. Others do it too slowly, allowing the slides to actually 'stick' to each other which also damages the cells.

Downward pressure is only used when actual tissue fragments are seen grossly, when gentle pressure is used to squash the fragments and create a thin layer of cells. This is also useful for solid biopsy fragments. It typically provides the best smear of all the techniques but is more prone to cell damage due to excessive pressure or speed. Only practise improves the smear quality unfortunately.

- **Star –** here the aspirate needle is used to drag the material into radiating streaks from the central droplet of material. This is minimally traumatic but unfortunately rarely spreads the cells well, so many areas are too thick for examination. It is not generally recommended, but can be good for (centrifuged/concentrated) fluid preparations that are moderately cellular or grossly turbid in appearance.

Internal organs and structures

If internal structures - ideally under ultrasound guidance - are being sampled the principles outlined above are the same, but the skin should be aseptically prepared. If a vascular organ such as the liver or spleen is being sampled, some argue that coagulation (haemostasis) screening should be performed. However, detectable or clinically significant bleeding following aspirates is (very) rarely seen, and as the spleen has a muscular capsule in many species, this often 'closes' over the point of entry.

If scanning shows diffuse architectural changes, then a simple needle method with or without suction is fine. However, if there is a focal lesion, using a stylet and needle is ideal, as the stylet is only removed once the mass has been targeted, preventing other extraneous tissue being sampled.

Once smeared, the material is allowed to air dry naturally for 10-20 mins before staining, the length of time in part depending on the thickness of the smear. This process can be quickened by applying gentle heat to the back of the slide using a hairdryer set at 'medium' at a distance of about 12 inches. If done too soon, there will be too much water in the cells when the 'fixative' is applied, creating artefacts, easily seen in red cells as punched out, refractile and central holes.

Staining

Stains are broadly and simplistically split into water versus alcohol based stains. The majority of 'in house' stains and commonly used veterinary cytological stains are Romanowsky water based, and this discussion will be confined to these.

'Diff Quik':

In house staining invariably involves three separate liquids into which the slide is 'dunked' a variable number of times ('Diff Quik'). There are a large number of these stains available.

They should be replaced (not just topped up) and the containers (*e.g.* Coplin jars) cleaned on a regular basis, as the staining material becomes depleted with use and stain precipitates form with time, as well as possibly growing fungal and bacterial colonies. All these factors will prevent or hamper examination of the cytological harvest. The first clear liquid is a fixative, the second an orange dye and the third a blue dye. Typically the manufacturer's guidelines should be followed for the timing and number of 'dunks' required. However, it is worth checking that the colour scheme produced is acceptable. For example, eosinophil granules should be orange to pink, neutrophil cytoplasm clear and their nuclei dark purple. Often people use too little blue dye or too much orange dye. As a rough rule, after fixing in the first liquid, dunk in the orange dye until an even orange covering is produced on the slide, and then similarly with the blue dye.

Incompletely or improperly stained slides severely hamper detailed examination of cells. The amount of time in each will vary depending in part on the amount of material – the more material, especially if (too) thick, the longer required in each liquid.

Diff Quik stains are great, especially when time is of the essence such as during intra-operative procedures. However it does have limitations, beyond that of the variable staining mentioned. Relative to other stains such as May Grunwald, Wrights and Giemsa, it shows less fine detail of the cells, especially nuclear detail and chromatin patterns. It can also fail to show granular substances, such as mast cell granules well. This last 'flaw' whilst often quoted rarely prevents identification of a mast cell (tumour) although it does reduce the possibility of suggestive mast cell grading. Conversely, Diff Quik stains can enhance the identification of certain inclusions, such as Distemper viral inclusions in red and white cells.

May Grunwald Giemsa (MGG):

These are often used in combination, the M-G used at stronger concentration and providing the 'bulk of the stain', whilst the Giemsa is more dilute, 'cleaning and finishing' the cells' staining. Stain times vary from about 15 minutes to 45 depending on the detail and amount of material in question. It is often considered the 'best' stain but takes the longest.

Modified/buffered Wrights:

This was principally designed for haematology and blood films where it remains the standard but is equally applicable to tissue preps and cytology, especially as it is quicker, also often being combined with Giemsa. The staining characteristics are similar to MGG but less 'colourful'.

Rinsing:

Many people finish by rinsing with tap water. This can be fine, especially if gently applied to the back of the slide and not directly to the cells. However, it is better to use distilled or specific buffered water as this is gentler to the cells and creates little artefactual change. Tap water can wash out the dyes and alter the cell shape artificially. Buffered water is easily made by adding buffer tablets to distilled water. Once rinsed, the slide is allowed to air-dry. As with initial smearing, this can be quickened by judicious use of a hairdryer.

Most in house microscopes have a dry x40 objective lens. These are designed to be used with coverslipped slides to maximise fine detail. Whilst this lens can always be skipped to use the x 100 oil immersion, this can slow examination of the entire slide. Coverslips can be either glued using material such as “Surefix” or “Sub X” mounting medium but these take time. ‘Oil immersion’ oil can be used instead, but care must be taken when moving the stage platform on the microscope as vigorous movements will shear the coverslip off the slide. The actual oil immersion lens (x 100) can still be used on such coverslipped slides.

N.B.

The majority of in house labs and commercial external labs use water based stains (such as ‘Diff Quik’ and Wrights) for cytology *etc.* It is therefore vital that any slides or fluid taken are not exposed to formalin liquid or even the fumes from biopsy material. If being sent in the post, **they should be sent separately packaged**. Formalin is designed for wet fixing cells and alcohol stains such as Papanicolauo. Otherwise it introduces cell artefacts and prevents interpretation using water based stains. A few labs do use formalin stabilised fluids, but this should be checked first with the lab. The main limitation of such stains is the loss of cytoplasmic detail and infectious agents, especially bacteria, but with the benefit of enhanced nuclear detail.

FUNDAMENTAL PRINCIPLES

Cytology uses only a handful of cells to try and diagnose a huge range of diseases and conditions. It therefore requires:

1. a detailed knowledge of normal anatomy and cell appearance - what should we be seeing if this was normal healthy tissue?
2. a detailed knowledge of disease processes – is this sample inflammatory, proliferative or both, and if proliferative, is it benign or malignant?
3. confidence that the sample examined is representative – have we got enough cells to diagnose a disease?
4. good quality samples – is there too much artefact or contamination for a diagnosis?
5. does the diagnosis ‘fit’ with the clinical history, signalment *etc*?

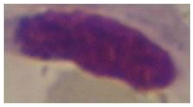
1. What is normal?

Specific cells and organs are too numerous to describe in a single day. However, all cells and tissues have common features that should always be examined.

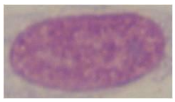
Nucleus

This stains purple with most commonly used stains. It is composed of DNA in the form of heterochromatin (dark inactive DNA) and euchromatin (light active DNA). The varying combination of these two gives the nuclei of cells characteristic textures or appearances (*e.g.* dotted/stippled, smudged or reticular/ropey). Cells in many tissues are 'resting' and so mainly have inactive (heterochromatin) nuclei which appear smooth and dark purple. As the nucleus becomes active and the cell produces (more) substances, whiter areas (euchromatin) are seen. Proliferative tissue cells therefore have more 'open' chromatin, with white and dark areas. This activity can be great enough to show as a focal 'hyperactive' point – the nucleolus. This is the area where DNA is actually being read and translated to produce proteins etc. Very active cells have large nucleoli or multiple nucleoli. Nucleolar appearance varies from an indistinct clear 'circle' to discrete dark patches within the nucleus.

The nucleus is surrounded by a nuclear membrane that is usually invisible and smooth. This membrane is often oval to circular, but can have characteristic shapes.



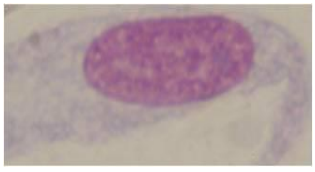
Fibrocyte nucleus

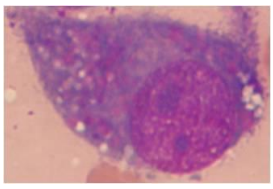


Fibroblast nucleus

Cytoplasm

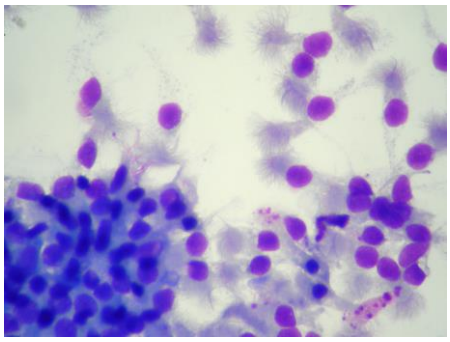
This is surrounded by an invisible membrane. However it forms certain shapes that are very useful and whether this shape is discrete and very clear, or poorly demarcated is also crucial. Inside very variable features can be seen. Conversely this time (compared to the nucleus), resting or non proliferative cells have pale smooth cytoplasm. Active cells have darker blue cytoplasm that in some cells can show focal 'intensity' in the form of a pale zone near or adjacent to the nucleus (the Golgi zone). If the cell is productive, product can be seen, as distinct granules (blue, pink, purple etc), indistinct smudges, as a diffuse hue, or in the form of clear white vacuoles. If the cell is phagocytic, infectious agents like bacteria and debris *etc.* may be seen inside.





Populations

No tissue has only type of cell in it. Therefore the combination and arrangement of cell types can be very important, not only in identifying the tissue/organ, but also whether any of the populations are absent or increased. Certain populations, such as inflammatory cells can be the disease process. Certain tissue types have cellular arrangements that are characteristic.



Palisading columnar ciliated epithelial cells in a BAL.

Background

It should never be underestimated how useful this can be. The appearance of the background is often affected by contamination but can be very useful. Proteinaceous material typically appears variably blue (basophilic) and smooth, whilst glycoproteins/carbohydrates are pinker (eosinophilic). For example, joint fluid is lightly pink and stippled, whereas lymph nodes are lightly basophilic. Chylous effusions have a very light blue background that has numerous irregular vacuoles or clear white spaces. This background will also alter cellular dispersal and therefore their appearance, potentially preventing examination of cytoplasmic detail especially, but also allowing mis-interpretation of a cell's origins.

All of these features will be present in some form in every single cell sample examined. The key is that normal tissue has similar features to proliferative tissue, whether it is benign or malignant.

There is (probably) no single cell feature that tells you a cell is malignant or not. In other words, malignant features are features that can be seen in 'normal' tissue, but not at that time, to that 'degree' or with such variation.

Normal tissue though is uniform, with cells appearing very similar and regular. Benign proliferation can be almost identical this, but may appear more crowded or 'active'. Malignant tissue can have many similar features, but crucially there will be more variation from cell to cell, in size, shape and features because the proliferation is now uncontrolled and excessive. Unfortunately though, some malignancies (such as leukaemia or lymphoma) are the direct opposite. Here the neoplastic process is very uniform (clonal) and similar from cell to cell (monomorphic).

2. Inflammatory or Proliferative

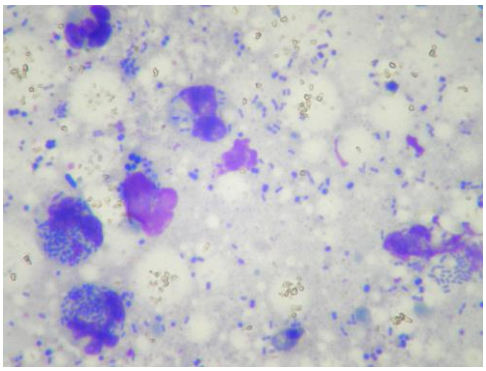
One of the main reasons for cytology is to identify whether a lesion/mass/process is inflammatory, infectious or proliferative (neoplastic). Whenever inflammation or infection is present within tissue, it will cause the surrounding tissue to react and proliferate. As outlined above, physiological reaction to inflammation (hyperplasia and dysplasia) can be very similar to neoplasia (inappropriate or excessive proliferation). When inflammation is present diagnosing neoplasia can be very difficult by cytology alone. Being able to diagnose neoplasia will in part depend on the relative proportions of inflammatory cells to tissue cells. If mostly inflammatory cells are seen and the sample is representative, the lesion is diagnosed as inflammatory. Within this there may be (very) atypical (tissue) cells, but only a few are seen. However, if many atypical cells are seen or they dominate the sample, even if inflammation is present to cause tissue reaction and proliferation, neoplasia can be diagnosed, and if the cells are very variable (pleomorphic), the proliferation is malignant. This distinction or diagnosis cannot be achieved and very much depends on how representative the sample is thought to be.

Inflammation is commonly broken into three categories based on the dominant or mixture of cells present. This classification can help identify the cause of the inflammation, but can be non specific. Inflammatory cells include: neutrophils, macrophages, eosinophils, lymphocytes, mast cells and plasma cells. Basophils are rarely seen/identified.

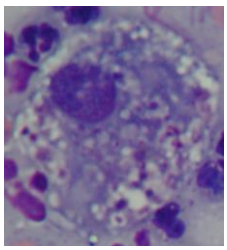
Whenever inflammation is seen, searching for infectious agents is necessary, especially in neutrophils and macrophages, but also within the background potentially. How long do you search? Until you get bored! Alternatively at least 5 mins is a practical guide.

INFLAMMATION

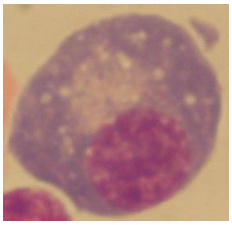
Acute or suppurative – this is dominated by neutrophils, with only low numbers of other mixed inflammatory cells. The neutrophils should be checked for infectious agents, such as bacteria, within their cytoplasm. Bacteria may not be seen, especially if antibiotics have recently been administered. So called ‘degenerate’ changes though can provide support for an infectious process. These changes involve the nucleus that becomes pale and indistinct, swelling and losing its lobulated appearance and nuclear membrane, its shape therefore being much more irregular. They are not the same as ‘toxic’ changes seen in the peripheral blood.



Pyogranulomatous or chronic active – this is when the inflammatory population is very mixed, with macrophages being prominent and about 30-50% of cells seen. Other inflammatory cells can also be present in low numbers. It is non specific, but more commonly seen in foreign body reactions or inflammation/infection that has been present for a while and may even be resolving naturally.



Macrophage



Plasma cell

Granulomatous – this is dominated by mononuclear cells, especially macrophages, along with lymphocytes and plasma cells. Other inflammatory cells are also present, especially neutrophils, but these often appear mature and 'non degenerate'. Macrophages are VERY variable in appearance.
Their key features include:
an eccentric variably shaped nucleus, low nuclear:cytoplasmic ratio, reticular chromatin and abundant very irregular cytoplasm that can contain debris, cells and miscellaneous material.

With time and especially foreign body lesions, macrophages can become giant and multinucleate. They can also form loosely cohesive sheets with more blue and smooth cytoplasm, similar in appearance to epithelial cells. These are termed epithelioid macrophages. Granulomatous inflammation can be characteristic of certain infections such as Leishmania and Mycobacteria and also very chronic foreign body reactions (such as around a retained swab).

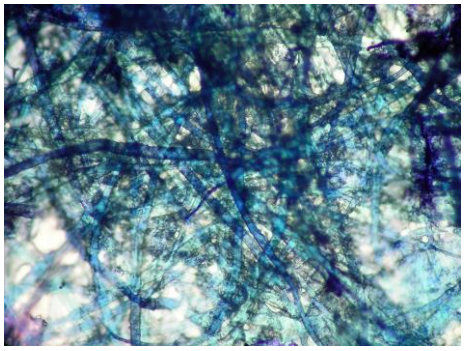
Eosinophilic – this can be dominated by eosinophils, with low numbers of mixed inflammatory cells, including mast cells. However, if significant numbers (arbitrarily >10%) are present, they are significant and suggest underlying parasitic or allergic diseases most

commonly. In cats, focal eosinophilic granulomas can be seen. Rarely, especially in this country, it can be fungal.

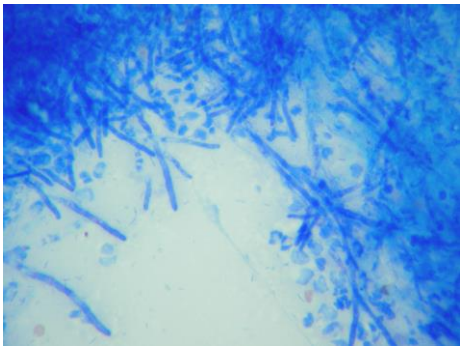
Infectious Agent or Contamination?

Contamination is an unfortunate inherent consequence of cytological preparations that must be minimised (by good sampling, smearing and staining) but appreciated to avoid inaccurate interpretations. As a rule of thumb, to ensure what you are seeing is a genuine 'agent', the following are useful but by no means absolute (see noted exceptions elsewhere in the text):

- ensure a similar appearance can (at least) be duplicated in the smear.
- Phagocytosis – genuine agents creating or forming part of the inflammatory process will be INSIDE the inflammatory cells (same plane of focus as the nucleus).
- Exclude contaminant granular material such as starch, stain dyes and ultrasound gel. Such material is typically more variable, (semi-)refractile, and extracellular.
- Infection is most commonly a single agent and so monomorphic – lots of bacterial shapes and sizes especially if extracellular may be contamination.
- Do the inflammatory response/populations and cell features (such as 'degenerate' changes) correlate with the agent in question? Bacterial infection is typically neutrophilic whilst fungal infection is typically pyogranulomatous to granulomatous. *N.B.* This is variable with the animal's individual response and in part relies on knowing the clinical timescale too.



Paper fibres



Fungal hyphae

3. Representative and therefore diagnostic?

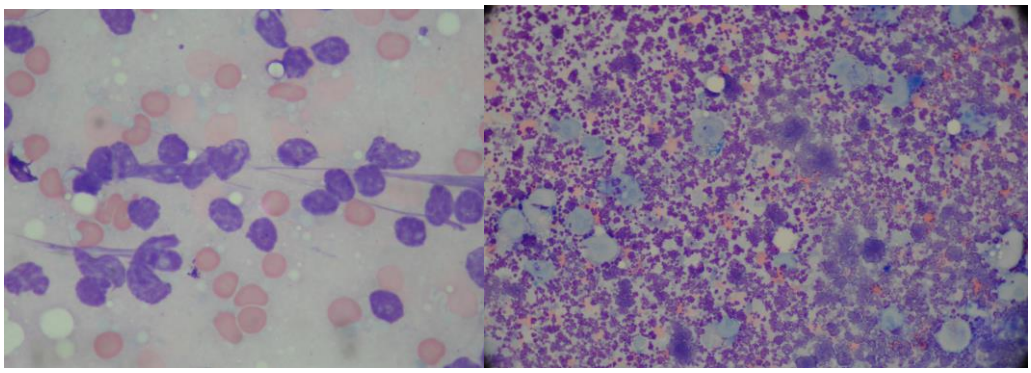
Representation and therefore confidence in a diagnosis is very difficult to assess. It certainly in part reflects experience, but the sampling itself is very influential. The more cells we have, the more likely we are to make the correct diagnosis. Certain clinicians produce very good samples. The history and description of the lesion is very important here. A large mass that has only one smear made from an aspirate is unlikely to be entirely represented in that preparation. Conversely small masses can allow the aspirating needle to exit the mass and aspirate adjacent tissue.

This combination of abnormal and normal tissue can make a diagnosis difficult if not impossible. Whilst seemingly paradoxical, the history and exact location can form the crux as to whether the interpretation is inconclusive versus diagnostic because they provide the context for interpreting the cells and their behaviour that otherwise is provided by the surrounding 'normal' tissue in histology and larger biopsies.

For example: with a history of blunt trauma to an area, proliferating endothelial (mesenchymal) cells is expected and appropriate within a resolving haematoma. Cytologically these can look (very) 'malignant' and even dominate the harvest, but are only hyperplastic. Without this historical/clinical knowledge, their behaviour can be interpreted as 'ectopic' or inappropriate and therefore neoplastic and possibly malignant. Other features such as organising haemorrhage can provide distinguishing criteria but can be variably present or appreciated.

4. Confusing or misleading artefact?

One of the commonest pitfalls of cytology is sample artefact. This is often in the form of damage to the cells. Nuclei may remain intact, but without the surrounding cytoplasm, they are difficult if not impossible to interpret. Even the nuclei can be damaged, with only streaks of material remaining. **Optimal sampling and preservation cannot be overemphasised.** Contamination can be in the form of adjacent tissues as already mentioned, which can also hamper other diagnostic tests. For example, a tracheal lavage can be contaminated with oropharyngeal material. If inflammation is seen, it is often not possible to say whether the inflammation is in the trachea or pharyngeal area. Similarly, this material often contains commensal bacteria, and so culture results can be difficult to interpret. Ultrasound guided aspirates can have too much gel which obscures the cells, or the stain may have too much precipitate which again obscures the underlying cells.



5. Appropriate clinical context?

Whenever you make a cytological diagnosis, it should be made within the context of the history, signalment *etc.* This not only gives more confidence in the representation as already outlined, but is also an external quality control check. However, it is also very easy to use this information to over diagnose, providing a diagnosis when actually there are too few cells or other differential diagnoses for the same cell populations. This factor again relies on self confidence, experience, confidence in the sampling technique/clinician etc and also overall knowledge of the 'possibilities.'

Body Cavity Fluids

Body cavities are lined by a single layer of mesothelial cells that cover the inner body wall, reflecting back as a continuous sheet over the viscera and mediastinum. Normally there is a very small amount of fluid formed as an ultrafiltrate of blood , the volume based upon the balance of production (due to blood hydrostatic and oncotic pressure) through the capillaries (wall permeability) to be resorbed and 'lost' (into the lymphatics). Alterations in any or all of these three broad 'areas' results in accumulation of fluid due to:

- increased permeability with vascular damage, dysplasia or inflammation
- decreased blood oncotic pressure due to loss of protein, primarily albumin.
- Increased blood hydrostatic pressure due to fluid overload, congestion etc.
- Reduced lymphatic drainage due to inflammation, congestion or obstruction.

Normally there is too little fluid for sampling, but when a disease process alters one or more of the above factors, the resulting fluid accumulation can be sampled, the composition providing information about the underlying disease process.

The collection of cavity effusions is covered elsewhere in more detail and will not be discussed further.

In most situations the fluid should be placed in an EDTA tube and a (sterile) plain tube. **Fresh smears of material should always be made if possible, especially if cytocentrifugation is available**. The EDTA tube is used for quantified analysis and cytology. The plain tube is for (bacterial) culture. Certain biochemical testing may require an additional plain tube, heparinised tube or possibly fluoride tube.

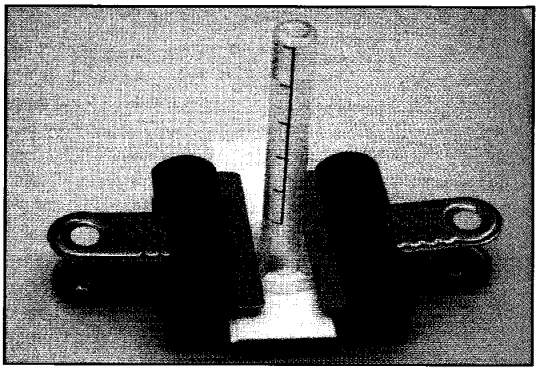
Although not specifically designed for fluid preparations, it is still recommended to fill the tube(s) accurately, especially if quantifying cell counts, protein levels or SG values. If the tube is variably filled, the EDTA (or other anti-coagulant) will have very variable effects on cell shapes and dilutional effects on fluid components such as cells and protein. Many fluids need processing and cells concentrating before they can be examined. In most commercial labs, this is automatically and easily done using a cytospin centrifuge.

However, similar preparations can be made in house by one of two techniques – sedimentation or slow centrifugation.

I**f the liquid is grossly turbid or floccular, direct (blood film or line concentration) preparations should be made. Floccules are gently removed and "squash" smeared** (see p 4).

Sedimentation:

This is simple and least traumatic to the cells in the fluid but is time consuming (as it relies on gravity). The fluid is introduced into a container that sandwiches some filter paper onto a slide. A circular hole is made in the filter paper equal in diameter to the container. In this way, the fluid component wicks away, leaving the cells to air-dry onto the central 'hole' area. A typical example is shown below, with bulldog clips holding the components together.



Centrifugation:

This is no different to urine sediment preparation, but does require a variable speed centrifuge. The speed required is similar (about 1500 rpm or 450g) for 5 minutes. Slower speeds can be better but require slightly longer times. Exact speeds and times may need adjusting to your individual centrifuge. Once centrifuged, the supernatant is removed, the sediment resuspended and then smeared onto a slide with either a blood film or line concentration method. If hypocellular fluids are being analysed, such as CSF, it can be difficult to find any cells if these methods are used. Therefore a drop of fluid can be placed on a labelled slide and then:

- gently dried using warm air from a hairdryer.
- dispersed using a needle or cocktail stick in a known pattern (see star prep earlier) and allowed to air-dry or dried using a hair dryer.

In this way, the area where the cells will be is already known. If the whole process is standardised in terms of volumes *etc.*, quantified analysis of cells can also be done. Crucially the cells must still be 'forced to spread out' if a cytospin centrifuge is not available, otherwise they will all appear as circular 'blue blobs'. **Remove as much liquid from a drop placed on a slide and then smear as outlined above or on p4-5.**

Quantitative Assessment

This only applies to fluids formed *in vivo*, iatrogenic lavages and washes not being analysed in this fashion as collection methods and numerical indices are not properly standardised or evaluated for these fluids.

Quantified analysis primarily looks at enumeration of red and nucleated cell numbers, with protein evaluation, either total protein (via refractometry or 'dipsticks') or both albumin and globulin via wet/dry chemistry, both performed on the supernatant after sedimentation/centrifugation. Other biochemical parameters, such as lipids, pH and lactate or glucose can be measured but are performed less routinely and their use/clinical interpretation is controversial, excluding confirmation of a chylous effusion via lipid ratios.

Cell counts:

This can be performed as for blood with any effusion that appears grossly cellular or turbid. However care must be taken for clotted or fibrinocellular material as this can block the sampling probe etc in the analyser. Most automated analysers cannot be used for very low cellular fluid such as CSF.

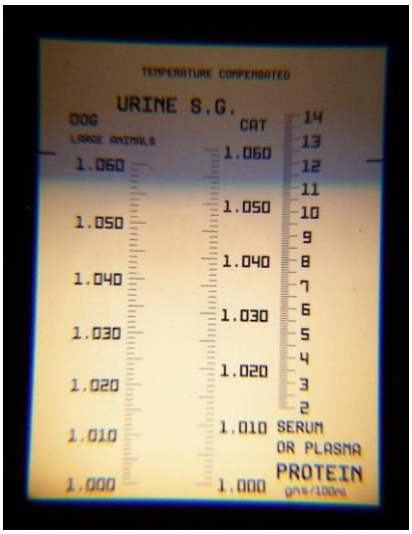
Manual counts can be performed on any fluid, but if grossly cellular/turbid, the neat fluid will have to be diluted (with physiological saline or buffer solution) before placing it in a counting chamber (*e.g* improved Neubauer haemocytometer). The exact dilution does not matter (and may vary) as long it is known and the cells individually visible and countable in the chamber used.

When using automated and manual techniques, remember it is a nucleated cell count (NCC) so does not distinguish inflammatory (white) cells from tissue cells such as mesothelial cells. Also, cohesive arrangements, such as in neoplastic effusions, will be counted as 'one cell' by automated methods.

Total Protein (TP):

REFRACTOMETRY – TP is easily measured via a refractometer as with urine SG, but using a separate scale, the value obtained being multiplied by 10 as levels are typically expressed in g/l (scales typically read in g/dl).

Whilst this is easy and reliable, interference from haemolysis, lipids in chyle, etc can occur, and differentiation into the three main categories (transudate, modified transudate and exudate) can be misleading especially at the thresholds such as 20-25 g/l, TP via this method potentially placing a transudate wrongly into the modified transudate or even exudate category. The cell counts though provide confirmatory information for categorisation. However this potential inaccuracy means some advocate more accurate assessment via a biochemical analyser.



BIOCHEMISTRY - This is the most accurate method, and allows differentiation of albumin and globulin fractions. This can be useful in identifying specific effusions, especially FIP, where characteristically low albumin:globulin (<0.4 being highly specific, <0.9 being highly sensitive) ratios, as well as high TP values (> 35 g/l) can be seen. However, many dry chemistry analysers (such as those typically seen in-house) underestimate albumin levels and can therefore be misleading. This differentiation though does not appear to be useful (or at least well assessed) in discriminating the effusive cause in most situations.

DIPSTICKS - This can be used for measuring the TP, but they are designed to detect and 'operate' at very low (micro) protein levels and therefore, typically above 20 g/l provide no further differentiation and even misclassify effusions. They are also designed to primarily detect albumin over and above other proteins such as globulins, therefore underestimating the latter. True transudates may be appropriately assessed, but their use may be best confined to CSF, where their semi-quantitative assessment is a useful guide.

Lipids:

Grossly these have a characteristic white opalescent appearance, seen in so called chyle or chylous effusions. Pseudochylous effusions are often mentioned but typically appear cloudy rather than opaque or genuinely white, the gross appearance instead being due to cellular debris than its lipid content. Biochemical assessment looks at the cholesterol (Chol) to triglyceride (Trig) ratio, not only within the fluid itself but also ideally relative to the serum/plasma at the same time. A high Trig level (> 3 mmol/l in the fluid is probably diagnostic of chyle, as is a Chol:Trig effusion ratio <1, but otherwise:

{Trig} effusion > {trig} serum & {Chol} effusion < {Chol} serum = chyle

Conversely **pseudochyle** (primarily created by chronicity and cellular degeneration) has a relatively higher cholesterol than triglyceride content.

Glucose:

Some advocate the measurement of glucose in cavity effusions, especially pleural and peritoneal, to diagnose a bacterial septic aetiology. This can be solely done on the fluid but is best compared to a simultaneous serum/plasma level. Limited studies have shown that a difference between the two of > 1mmol/l seems very sensitive and specific for sepsis in both dogs and cats, although slightly less sensitive in cats. These measurements were all done within 15 minutes of taking the sample but can be done on (all?) dry in house chemistry analysers (whole blood glucometers are not recommended due to the glucose levels expected and different mthodology). Delayed analysis will negate assessing this, especially as the study was limited and did not correlate the glucose with nucleated cell counts, neoplastic effusions and timings. In fact neoplastic exudates, especially lymphomatous are now recognised as false positives (unpublished data / in press). It may not provide any further information than that provided by standard cell counts and cytology etc. but may be an option if these are unavailable. Potentially fluoride or serum gel tubes could enhance sample stability but this has not been assessed. Other parameters used in this fashion, primarily pH and lactate levels, have similar time constraints and/or specific special sampling requirements.

Once these figures (NCC and TP) are obtained, the fluid can generally be classified into three broad categories. This classification seeks to identify the pathological source of the effusion but is by no means absolute and definitive. The cytological examination (see later) can help here, but again may not provide a definitive diagnosis though often reducing the differentials. The table outlined below is provided as a guide, but again is not absolute as there are no standardised and accepted threshold figures, and certain fluids are not placed in these three groups (see list later). Various factors will affect the appearance of these fluids, not only in terms of *in vitro* sample ageing, but also repeated iatrogenic drainage effects and the *in vivo* chronicity of the effusion itself, all factors that are unfortunately too often overlooked or are poorly understood / appreciated.

Effusion Type	Appearance	NCC / µl	TP (g/l)
Transudate	Clear serous	<1500	<15 (or <25)
Modified Transudate	Clear pale yellow	1000-7000	25-50
Exudate	Turbid, floccular	>7000	> 30

Transudates

Hypoalbuminaemia is the most common cause, due to:

- excessive protein loss - protein losing nephropathy, protein losing enteropathy with/without lymphangiectasia (rarely burns/skin loss)
- reduced production - hepatic dysfunction (rarely malnutrition)

Quoted thresholds for a sole loss of oncotic pressure causing the transudation is <15 g/l. Exactly where and how this figure is derived from appears unclear, and many 'low protein' disease processes also involve other changes that increase the hydrostatic pressure or reduce lymphatic drainage, so transudates will be seen with albumin levels between 15 - 25 g/l. In these cases, concurrent diseases should always be considered, as well as a 'transitional state' (see p29). Concurrent pathology includes portal hypertension, thromboembolus, diaphragmatic herniation with lymphatic obstruction.

When identified, initially assessing protein levels with liver enzymes and bile acids are recommended, followed by imaging and urine analysis depending on results.

Modified Transudates

These are the possibly the most non specific, arising due to a variety of causers, primarily involving increased hydrostatic pressure and reduced drainage. Cellularity can be very similar to a transudate, but crucially the TP is higher. They can arise in either the pleural, peritoneal or both cavities. They can appear serosanguinous rather than pale yellow, often due to iatrogenic blood contamination rather than genuine *in vivo* haemorrhage. Aetiologies include:

- congestive heart failure (CHF) (pleural, esp.cats, and/or peritoneal)
- liver disease (peritoneal)
- neoplasia (pleural and/or peritoneal)
- organ torsion or inflammation (pleural or peritoneal)
- trauma and diaphragmatic rupture (pleural and/or peritoneal)

When identified, initially screen for CHF (imaging, ECG and biomarkers), liver disease (enzymes and bile acids) and protein status. If clear, image internal organs and then aspirate or biopsy, possibly at ex lap if imaging is not available.

Exudates

As these arise due to infectious and inflammatory conditions, the resulting cytokine 'storm' alters the vascular permeability, so these typically have the highest protein values and cellularity, as 'larger holes' develop in the vasculature overwhelming lymphatic drainage and/or obstructing it. Conversely here, the NCC is crucial for differentiation from a modified transudate, as the TP levels can overlap.

- **Septic –** these arise due to penetrating trauma, including foreign bodies, leakage or rupture of an infected tissue/organ (especially GI) and uncommonly bacteraemia. Crucially neutrophils dominate and they are 'degenerate', with or without infectious agents, primarily bacteria in this country. These bacteria are seen both free AND phagocytosed in the neutrophils.
- **Non septic –** these arise due to exfoliating neoplasia or underlying sterile inflammatory diseases. In the former, atypical neoplastic cells dominate (see later) whereas the latter are primarily mature, possibly hypersegmented or pyknotic, neutrophils. No infectious agents are seen (phagocytosed inside the cells). Non neoplastic causes include FIP, pancreatitis and ruptured gall bladder or urinary bladder (chemical irritants).

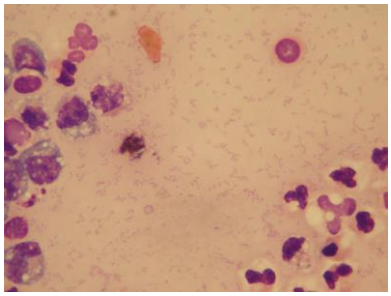
Further diagnostic tests that can be done based on clinical suspicion or findings focus on relative levels between the fluid and plasma/serum, as with glucose discussed earlier.

- **Pancreatitis** – assess amylase and lipase levels (higher levels in the fluid), but fluid volume can be very small.

- **Bile peritonitis** – measure total bilirubin (fluid levels higher but will equilibrate in time). Bilirubin (crystals) can be seen in the background with microscopy.

- **Uroperitoneum** – measure creatinine, (urea, sodium, chloride and potassium). Crucially the timescale must be borne in mind as some analytes rapidly equilibrate/change. Classically the urea and creatinine are higher in the fluid, with hyperkalemia, hyponatraemia and hypochloraemia. However, the urea can equilibrate in hours, the creatinine taking longer, in the order of days, the electrolye levels being variable dependent upon the timescale and other factors altering their homeostasis. Urine crystals, such as oxalate and struvite, may be seen in the background with microscopy.

- **FIP** – measure the fluid albumin and globulin levels to give the A:G ratio (see TP discussion earlier p25). The background here is typically lightly eosinophilic and floccular. The cell count is often lower than typical exudates, often being similar to modified transudates, or even transudates initially. However, the protein level will be high (>35 g/l)., the cells mainly being mature neutrophils and macrophages.



'Transitional fluids'

The pathological aetiology and resulting effusion type will not always correlate, and this is often due to the timescale involved. All effusions will start as a transudate but will then progress at varying speeds through to their 'true' (diagnostic) state. CHF if detected early can be associated with a transudate. Septic and especially non septic aetiologies may present as modified transudates if seen peracutely, or even a transudate (*e.g.* initial urine leakage in uroperitoneum before chemical irritation ensues).

Haemorrhagic

Grossly these appear red, the PCV being variable but on a par with the peripheral blood. The total protein is much more variable, and conclusively saying the effusion is haemorrhagic can be very difficult. Iatrogenic sampling of the spleen or a vessel within the abdominal wall etc can easily mimic this. Some crucial differences are:

- Iatrogenic contamination will often appear to 'swirl' as blood is drawn into another type of effusion, whereas true haemorrhage will be uniformly red from the start of sampling/drainage.
- In genuine haemorrhage, depending upon the intra-cavity volume, signs of haemodynamic compensation and possibly hypovolaemic shock will be evident.
- The supernatant (examine when checking the PCV) is often haemolysed with genuine haemorrhage.
- If examined immediately, microscopy will show platelets and fibrinocellular aggregates.
- Genuine haemorrhage is often not peracute/acute, so microscopy shows evidence for organisation and red cell breakdown – erythrophagia, haemosiderin, and haematoidin (see macrophage discussion p 33 later).

Cytological examination should also be performed on the buffy coat layer after microhaematocrit centrifugation as this reduces the distortion and density of red cells to facilitate identifying neoplastic cells.

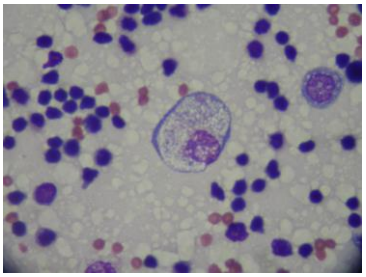
If supported or suspected, primary differentials are a bleeding tumour or lesion, coagulopathy and trauma. Imaging, history and coagulation time screening should differentiate these.

Neoplastic

These typically appear as an exudate, but can be a modified transudate and maybe associated with intra-cavity haemorrhage if the tumour is fragile, involves or invades the vasculature. Rarely they are a transudate when the neoplasm(s) cause organ dysfunction and hypoproteinaemia. **The lack of neoplastic cells in any effusion does not exclude neoplasia as the effusive cause.**

Chylous

These arise due to obstruction or damage to the draining lymphatics, most commonly in the chest but also within the peritoneum. They arise non specifically, aetiologies in many ways overlapping that of modified transudates and include idiopathic (Afghans over-represented?). They are identified by lipid analysis (see p 25-26). Cytologically they are usually dominated by small mature lymphocytes, but with chronicity the cell populations can become more mixed and potentially dominated by neutrophils and other mononuclear cells (macrophages and mesothelial). Typically the background is lightly basophilic with irregular clear white vacuoles.



Whilst rare, differentiation from pseudochyle may be recommended – see lipid discussion (p 25) earlier. Refridgeration to identify chylomicrons as they form a white layer at the top of the tube is a very insensitive way of identifying chyle. Centrifugation in a microhaematocrit tube commonly shows a white layer at the top of the tube.

In many ways its identification is more about possible surgical intervention and treatment than altering the differential aetiology list, investigations being essentially the same as for modified transudates. In cats, CHF is often quoted to be the most common cause, but different studies have found a different prevalence.

CYTOLOGICAL EXAMINATION OF FLUIDS

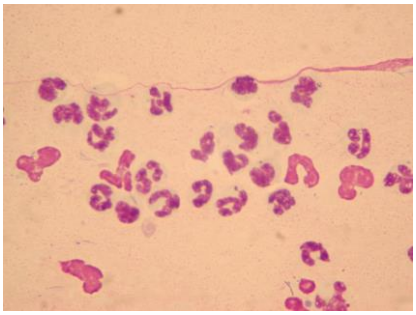
This should always be performed on any fluid to maximise its diagnostic potential but is typically most rewarding on fluids with relatively high cell counts. Discussion will focus on identifying the cells and assessing their 'behaviour', as the individual cell types, with certain exceptions, can be found in any of the different types of effusions. Please note:

- many cells, especially leukocytes (neutrophils and macrophages), originate from the blood and so will present a morphological spectrum *e.g.* 'macrophages': will initially be monocytes, then start phagocytosing, immune regulating etc – macrophages, may form multinucleate giant cells in foreign body reactions or epithelioid macrophages in longstanding inflammation.
- Fluid changes occur as the cells swell and lyse (both *in vivo* and *in vitro)*. This is seen initially in the cytoplasm and then in the nucleus, resulting in most cells becoming 'round', even epithelial carcinoma cells.

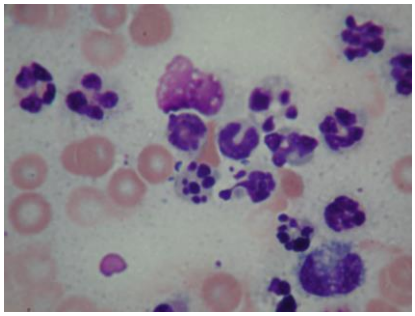
NEUTROPHILS:

Classically these have a segmented (3-5) dark purple nucleus that has internal white lines surrounded by off white cytoplasm. Check for:

- hypersegmentation (> 5 'lobes'/segments) and pyknosis (dark circular nuclear 'blobs' with no chromatin pattern) are features that suggest sterility and *in vivo* ageing. **NB Iatrogenic treatment, antibiotics and steroids especially, will mimic this.**
- Nuclear shape and colour – 'degeneration' and infection cause 'nuclear membrane damage' so it becomes paler and more pink-purple, smudged with poorly defined edges. **NB.** Hydropic changes that occur as the neutrophils swell in the fluid can mimic this (especially if the fluid preps are not fresh) but the nucleus is usually still dark purple, with crisp edges, the intersegmental strands often appearing very fine and stretched (also seen when cells are damaged during centrifuging).
- Bacteria – crucially phagocytosed inside the cytoplasm, but also in the background. If only seen in the background, these may only be sample contaminants. If mixed bacteria are seen, GI or foreign body sources are initial differentials. **NB.** Phagocytosis of contaminant bacteria will occur *in vitro* after the sample is taken.



Pyothorax

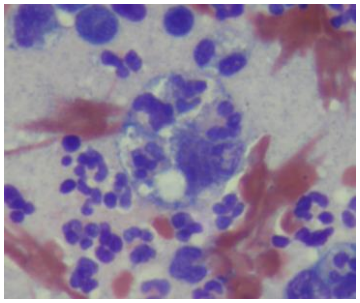


Non septic

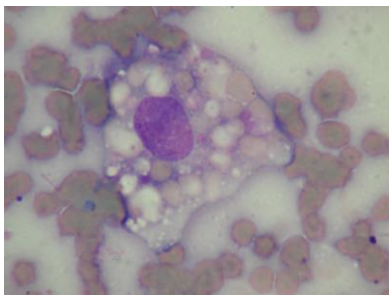
MACROPHAGES:

These are one of the most variable cells and so their identification and discrimination from other cells, especially mesothelial, can be very difficult especially in long standing effusions or those taken after repeated drainage. For identifying features please see inflammatory discussion (p14 - 15) earlier. Check for:

- Leukophagia – presence of phagocytosed white cells, especially neutrophils is more commonly seen with sterile inflammatory diseases as the inflammation starts/attempts to resolve. **NB.** When iatrogenic therapy starts, this will also be seen in septic exudates etc.

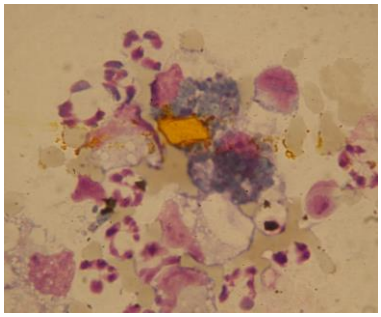


Leukophagia



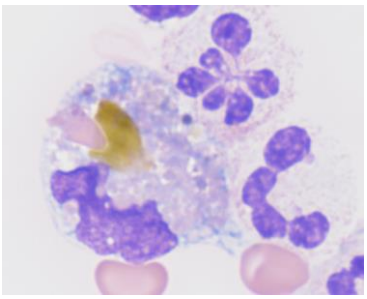
Erythrophagia

- Erythrophagia – intact red cells in the cytoplasm supports previous, recent or ongoing haemorrhage and is not expected with iatrogenic blood contamination. **NB.** *In vitro* phagocytosis also occurs, so preps that are not fresh must be interpreted cautiously and alongside the presence of haemosiderin and haematoidin.
- Haemosiderin – irregular blue-black to brown-black material in the cytoplasm supports red cell breakdown and *in vivo* haemorrhage.



Haematoidin and haemosiderin

- Haematoidin – yellow rhomboid crystals also indicate red cell breakdown, believed to result from anaerobic breakdown of red cells and abnormal bilirubin crystallisation.
- Bilirubin – yellow-green amorphous to fine needle-like crystals in their cytoplasm and also often in the background supports bile peritonitis.



- Other phagocytosed material – such as fungal spores, methylcellulose.

LYMPHOCYTES:

These are typically similar to those in the blood, being small (1-1.5 x RBC) with a very high N:C ratio, minimal basophilic cytoplasm on one side of the nucleus and dark purple chromatin with white lines. Rarely larger examples (blasts/blastoid/reactive) can be seen that are similar to those in lymphoma, but they should be rare and form part of a 'reactive continuum', often with plasma cells then too. If they form more than 40-50% of the lymphoid population, lymphoma is indicated.

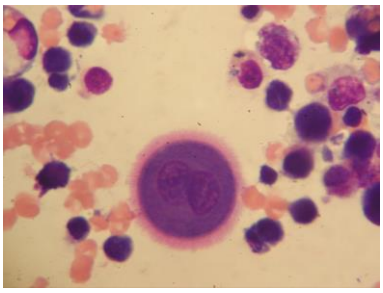
LEUKOCYTES:

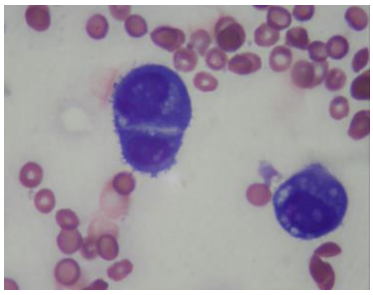
Other white cells such as eosinophils and mast cells are seen rarely in many effusions, especially if inflammatory. If they dominate or are prominent, consideration would include underlying hypersensitivity, parasitic, eosinophilic or neoplastic diseases (especially mast cell neoplasia and lymphoma).

MESOTHELIAL:

These, like macrophages, are very variable, and when proliferating in chronic or inflammatory effusions, will often display so called 'malignant' features cytologically. They are therefore often misdiagnosed as malignant and neoplastic or confused as epithelial as they exfoliate in small groups. More characteristic features include:

- Circular shape and nucleus, with a variable to low N:C ratio
- Stippled chromatin with a prominent nucleolus.
- Deeply basophilic cytoplasm that classically has peripheral blebbing, 'ruffles' or an eosinophilic corona.
- Cells are often individual or seen in pairs, the latter having a central 'window' or fenestration (*cf.* carcinoma epithelial cells next paragraph). Larger groups appear as a 'bunch of grapes' with monomorphic cell features (*cf.* carcinoma epithelial cells next paragraph)

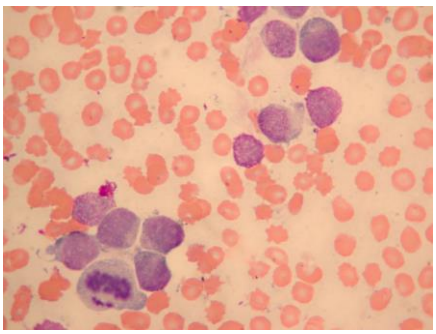




NEOPLASTIC CELLS:

This will not be comprehensive, but will highlight certain features of the more common neoplasms that exfoliate into effusions. **The most difficult discrimination is that of reactive mesothelium from mesothelioma and carcinoma, especially in the face of an inflammatory process.** Sole reliance on 'ticking off' classical malignant features will very commonly over-diagnose malignancy.

- Lymphoma – see lymphocyte discussion (on preceding page). Neoplastic lymphoid cells are typically large, with a very high N:C ratio, and irregularly smudged or clumped chromatin and variably prominent nucleoli. Unlike in leukaemia and lymph nodes, the monomorphic hallmark is often lost as the cells are fragile and variably altered by the centrifuging process.



- Carcinoma – cells show marked variation in (relative) nuclear and cell size, cell borders being crisp and well defined (*cf.* mesothelial). Seen individually and in variably sized cohesive arrangements, the latter having a 'common border' and no fenestrations.
- Mesothelioma – features are similar to those seen in reactive cells, but cell numbers are increased, there is more pleomorphism and variation in sizes etc, with more cohesive groups, these groups having an 'undulating border' similar to a bunch of grapes.

Differentiation can be very difficult and often requires a lot of experience and examination of subtle features, so if in doubt, send the slides, fresh fluid preps and the fluid itself off to an experienced cytopathologist.

Never forget to look at the background as it can be very helpful and even diagnostic in certain instances such as chyle and FIP. Other features are things like plant material and protozoa in GI rupture or iatrogenic sampling.

Some Text Book References

BSAVA Manual of Canine and Feline Clinical Pathology 3rd Ed. Editors J. Ristic and E. Villiers.

Diagnostic Cytology and Haematology of the Dog and Cat. 5th Ed. Editors Cowell, Tyler and Meinkoth. Mosby

Atlas of Canine and Feline Cytology, 2nd Ed. Editors Rose Raskin and Denny Meyer. WB Saunders