COMMISSIONED PAPER

Clinical Cytology of Companion Animals: Part I Introduction

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SUMMARY

In veterinary medicine, interest in the technique of cytological examination of smears obtained by fine needle aspiration biopsy (FNAB) is a rather recent phenomenon. One might assume from this that it is a recently developed method, but nothing could be farther from the truth. As early as 1851, the Swiss pathologist H. Lebert described the method of diagnostic aspiration in his book "Traité des Maladies cancéreuses et des Affections curables confondues avec le Cancer: ("*.. and an explorative aspiration can remove all doubt. If it is a purulent or serous cyst, a stream of fluid can come out of the needle; when cancerous tissue is punctured, the needle will be surrounded by more solid structures and is less moveable. If some material is obtained it is a thin substance which when examined macroscopically, and if necessary microscopically, appears to be cancer.*") A few years earlier (1838) the Berlin physiologist Johannes Müller first described cytological criteria for the differentiation of benign and malignant tumours. Now, one and a half centuries later, this technique has also become popular in veterinary medicine.

Advantages and disadvantages of FNAB

Cytological examination of smears obtained by FNAB has several disadvantages relative to examination of histological biopsies, but there are also various advantages. When deciding whether to obtain a surgical biopsy for histological examination or an aspiration biopsy, these should be considered carefully.

The positive predictive value of cytology is higher than the negative predictive value. In other words, the absence of malignant cells in a cytological preparation is less reliable than the presence of cancer cells. The aspiration needle can unfortunately be inserted beside the tumour rather than in it, or in a necrotic or inflamed portion of the tumour, or the tumour can consist of cells that are not easily aspirated. All of these can lead to a false tumour-negative result. Another limitation of FNAB is the lack of opportunity to study the histological structure of the lesions.

The attractiveness of the FNAB rests on its advantages. It is a method that is easy to learn. It is inexpensive and quick, and the results can be known within half an hour. FNAB can also be performed without anesthesia, which makes it possible during clinical examinations. The smears do not have to be processed immediately but can be left until later. With FNAB one can obtain multiple aspirates from a lesion, which is advantageous in the follow-up of patients during the course of treatment. The risk of metastases being caused by FNAB is so small as to be negligible.

Indications for performing FNAB

On the basis of cytological examination of biopsies obtained by FNAB, it is usually possible to differentiate between inflammatory tissue and tumour, between acute and chronic inflammation, and between benign and malignant tumours. The aetiologic agents of different infectious causes of inflammation are recognisable (bacteria, fungi, and parasites such as Toxoplasma and Leishmania), and also many tumours can be classified. Cutaneous and subcutaneous masses that cannot be diagnosed by physical examination provide an excellent indication for aspiration biopsy. With FNAB, most skin tumours can not only be distinguished but also classified (e.g., mast cell tumour, histiocytoma, squamous cell carcinoma, basal cell carcinoma, and melanoma). In contrast, in the case of mesenchymal

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proliferation, it is often difficult to determine whether the process is tumourous or reactive. Chronic inflammatory tissue, the presence of malignancy and the type of tumour (fibroma/fibrosarcoma, haemanigioma/haemangiosarcoma) is often uncertain. In many cases one can come no further than the diagnosis of 'mesenchymal proliferation'. Mammary tumours also, especially for the inexperienced cytologist, can present problems as stimulated mammary tissue often contains atypical cells. In contrast, various lymph node abnormalities (including metastases) are easily diagnosed. For someone experienced in biopsy techniques, there are still more indications for FNAB: prostatice changes, interstitial changes in the lungs, clinical suspicion of abnormalities in the liver or spleen. For these indications there is, however, a greater risk of complications. Percutaneous lung biopsies in particular should not be attempted unless one is capable of handling the eventual complications.

Technique of FNAB

The material needed for performing FNAB is extremely simple: a few glass microscope slides with a matt surface at one end, a 10 ml disposable syringe, and a **thin** (22 gauge) needle (Fig. 1).
It is important to use microscope slides that have a matt surface at one end so that they may be labeled using a lead pencil. Particularly when multiple biopsies are taken, a mix up of the slides can be very unfortunate (for both the clinician and the patient). The risk of mixing slides will be even greater if you don't do the microscopic examination yourself but send the preparations to someone else. The best way to label the slides is with a lead pencil, because in contrast to ink, this will not be dissolved in the alcohol used during staining.

Fig. 1 Minimal material needed for performing FNAB: a few object glasses with a matt surface at one end, a 10 ml disposable syringe and a thin (22 gauge) needle.

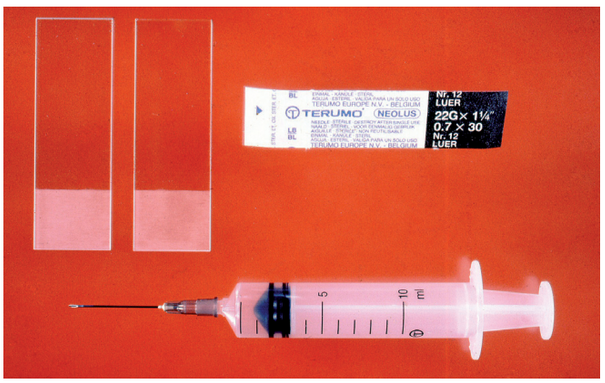


Fig. 2 Special FNA biopsy instrument (Cybio®) developed for veterinary medicine.

Syringe holders are recommended for use in aspiration biopsy in human patients. Usually a 20-ml syringe must be used, which in our experience is much less convenient. The distance from the patient is too great which, in the case of restless veterinary patients, can be a disadvantage. A small syringe has the disadvantage that its plunger must be pulled back farther to obtain sufficient vacuum. A 10-ml syringe seems to be a good compromise. Recently, in veterinary medicine a special biopsy instrument (Cybio®)[a] has been developed, which is easy to use and gives good results (Fig. 2). Some advise the use of only a fine-needle, the fine-needle capillary sampling technique, which is easier to manoeuver and may create less blood contamination. Especially in haematopoietic and epithelial tissues this may be an effective method, in mesenchymal tissues, however, the yield is often less than with FNAB.
It is a mistake to think that thicker needles can obtain better aspirates. With thick needles large clumps of tissue are aspirated. These are difficult to streak out, are easily damaged, and are difficult to stain and examine. In addition, blood is more easily aspirated with a thick needle. Experience has shown that 22-gauge needles give the best results. Only if no material is obtained with these needles is trying with a slightly larger needle worthwhile.
Anaesthesia is seldom necessary for performing FNAB. Although aspiration from the extremities and the head is occasionally painful, even in these locations it is seldom necessary to use any form of anaesthesia. The patient must be adequately restrained by the owner or assistant(s). After the hair has been clipped and the skin disinfected, the mass to be aspirated is fixed with one hand and, with the syringe held in the other hand, the needle is introduced. A vacuum of 1-2 ml is usually sufficient to aspirate material. The needle, without being withdrawn from the mass, is moved in various directions in order to obtain as representative a biopsy as possible. Often little or no material is visible in the cone of the needle. If blood suddenly enters, it is better to take a new syringe and needle and start again, as too much blood greatly hinders the evaluation of the preparation. Before the needle is completely withdrawn from the tissue, the vacuum must be released by allowing the plunger to return to a resting position otherwise the aspirated material will be drawn into the syringe and will be very difficult to retrieve. Then the needle is loosened, some air is sucked into the syringe, the needle is again placed firmly on the syringe, and the contents of the needle are expelled onto a glass microscope slide.
Various methods have been described for streaking out the biopsied material. A second object glass can be laid on top of the first and, with a turning motion, the two glasses can be pulled apart. Spreading the material with a needle or a scalpel blade is also used. Often, however, many cells are destroyed by these methods. This can be avoided if one uses the method which is also applied for streaking out a drop of blood. For this purpose a second slide, held at an angle of 45° to the first, is backed up against the material and then streaked out with a flowing movement so that the material along the back edge of the slide is taken along. For mucous material one should streak out more slowly than for more watery material.
It is advisable to take multiple aspirates from each tumour. If the tumour is large, take an aspirate from both the centre and from the edge. This increases the chances of obtaining a

representative sample. At the edge of the tumour, the certainty of obtaining cells is smaller, but a correctly obtained aspirate is then more representative because a rapidly growing tumour becomes necrotic near the centre. This also applies to lymph nodes invaded by tumour. In generalized lymphadenopathy it is reasonable to biopsy multiple nodes, since the lymph node is sometimes so necrotic that a good interpretation of the biopsy is not possible. In addition, it appears that the mandibular lymph node is less suitable for cytological examination because it is usually reactive, as a result of minor inflammatory processes within the oral cavity. Because of this, the true cause of the lymphadenopathy can be masked.

Exfoliative Cytology

Apart from FNAB of lumps or organ tissues, cytological smears of superficial lesions can also be obtained by one of the following methods.

i Impression smears

Impression smears may be obtained from skin lesions such as ulcers, exudative dermatitis and from surgically excised material. Impression smears from skin lesions can be made by pressing the slide firmly against the lesion. One option is to freshen the lesion by scraping the surface with a scalpel blade. Impression smears from surgically excised material can be best made by first cutting a fresh surface, removing the surface blood with a gauze, and then pushing the slide against the lesion. It is always best to make several impression smears. If the imprints are poorly cellular, another technique can be used to get cells from the sample. With a sterile scalpel, the surface of the tissue is scraped until some material is on the blade surface. The blade material is then spread onto a slide.

ii Swab method

Lesions such as those in the ear and nose, but also (muco) cutaneous lesions, can be sampled with a cotton swab. The material should then be smeared immediately onto a glass slide, before the material dries.

iii Direct smears

From superficial lesions or skin lesions exhibiting vesicules, pustules, etc., cytological smears may easily be obtained by scraping the lesion with a scalpel blade and then smearing the material onto a slide. However, in case of vesicules or pustules, it is better to open the lesion with a sterile scalpel blade or hypodermic needle, collecting material from within the lesion, or by making imprint smears from underneath the roof of the vesicule or pustule.

Handling of the smear

After the smear of the biopsy has been made, the method of staining determines how the preparation must be processed. For Papanicolaou staining, the smear has to be fixed immediately (by means of a fixative spray or by placing the slide into alcohol). For the so-called Romanowsky stains (Giemsa, May-Grünwald Giemsa, Hemacolor®, DifQuick®), good fixation by drying in air is necessary before staining (Fig. 3). The air-dried preparations can then be kept for a long time before staining. For Romanowsky

Table 1

Staining method	No. of dishes	Staining time
Papanicolaou	24	35 min
May-Grünwald Giemsa	3	22 min
Hemacolor® (Merck)	3	0.5 min

Table 2

Papanicolaou method			
1. alcohol 70%	20 sec	13. alcohol 80%	20 sec
2. alcohol 50%	20 sec	14. alcohol 96%	20 sec
3. distilled water	20 sec	15. Orange G	3 min
4. Harris hematoxylin	6 min	16. alcohol 96%	20 sec
5. distilled water	20 sec	17. alcohol 96%	20 sec
6. 0.25% HCl	3 dips	18. EA 50	5 min
7. tap water	20 sec	19. alcohol 96%	20 sec
8. lithium carbonate	1 min	20. alcohol 96%	20 sec
9. tap water	20 sec	21. alcohol 96%	20 sec
10. distilled water	20 sec	22. alcohol 100%	20 sec
11. alcohol 50%	20 sec	23. xylol	5 dips
12. alcohol 70%	20 sec	24. xylol	15 min

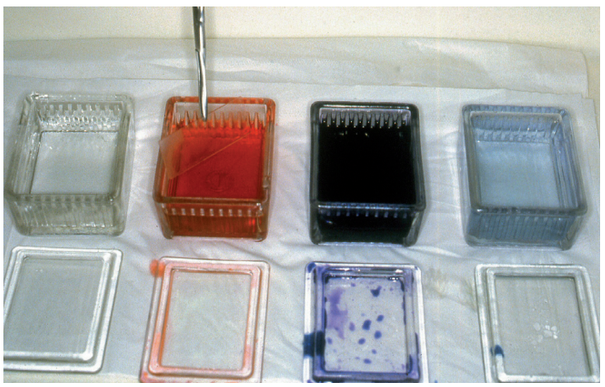


Fig. 3 Hemacolor quick stain: alcohol fixation, red stain, blue stain and buffer.

staining, fixing with spray, alcohol, heating, or formalin is *not allowed*. In addition, the smear must not be covered with glass cover-slip!
As shown below, various staining techniques are possible (Table 1). The Papanicolaou stain (Table 2) is very laborious and time-consuming. Its advantage is that it provides good detail of nuclear structure. The degree of keratinisation of cells is also brought out well. In contrast, there is often little or no staining of cytoplasmic granules. Both according to the literature and in our own experience, the Romanowsky stains (Tables 3 and 4) are more suitable for veterinary applications. These stains are suitable for demonstrating cytoplasmic inclusions and microorganisms. In addition to these general stains, various special stains are possible. An example of these is the rubeanic acid stain for demonstrating copper (e.g., in liver cells). For these stains various handbooks should be consulted.

Basic Principles for the Interpretation of Cytological Preparations

An experienced cytologist examining cytological preparations can sometimes make a diagnosis in a couple of seconds. With his experience he makes use of pattern recognition. With difficult preparations, however, a more systematic approach will lead to results sooner. This is certainly true for the inexperienced cytologist. In this paper a guideline is given for examining cytological preparations obtained by aspiration biopsy or exfoliative cytology. It is not possible in just one paper to give a complete overview of all cytological diagnoses. Those wishing to become better qualified in cytology after reading this text can pursue the subject in the references that are provided.
This paper is only concerned with the examination of cytological preparations. It should be realized, however, that for good diagnosis, the quality of the preparation is of vital importance. "In cytology the specimen is everything: Garbage in - Garbage out." (V. Perman, 1984). Good biopsy technique, good fixation of the smears, and a good staining technique are essential. In addition, a proper use of a good microscope is of course of the essence!
Apart from the possibility of poor biopsy quality, cytology has another limiting factor as mentioned earlier: a negative (e.g., tumour-negative) report is less reliable than a positive report. Severe inflammation in a tumour, necrosis of tissue within a tumour, or inadequate biopsies can result in a "false negative" report. One should always take this limitation into account. As with all diagnostic techniques, it is also advisable to consider the probability of the cytological diagnosis in terms of the probability of the clinical diagnosis, and not to see it as an independent certainty.
The examination of a preparation goes through three phases. The first is determining the tissue of origin, the second is differentiating between inflammation, hyperplasia/benign tumour and malignant tumour, and the third is classifying the inflammatory process or the tumour.

Hemacolor®	
Solution 1:	5x 1 sec
Solution 2:	3x 1 sec
Solution 3:	6x 1 sec
Wash off with buffer solution pH 7.2	

Table 4

Tissue of origin

Because the cytological characteristics of hyperplasia, inflammation, and malignancy can differ in different tissues, it is important to first determine the most frequently occurring type of cell in the cytological preparation. The following three tissues can be distinguished.

I. Haematopoietic tissue
These are cells originating from blood, bone marrow, lymph glands, spleen, and thymus. Mast cells, histiocytes, and macrophages also belong to this category. Cells of haematpoietic origin are individual cells, mostly round in shape, and have a distinct cytoplasmic membrane (Fig. 4). The smears are usually rich in cells. Preparations that have not been streaked out can incorrectly suggest a tissue organization, but this will be seen to disappear along the edges of the smear. The cell sizes can vary from small to medium. Usually they contain little cytoplasm.

Table 3

May-Grünwald Giemsa method	
Solution 1: methyl alcohol 90%	5 min
Solution 2: May-Grünwald stain 1 part, phosphate buffer pH 6.98 1 part	4 min
Solution 3: Giemsa stain 1 part, phosphate buffer pH 6.98 9 parts	15 min

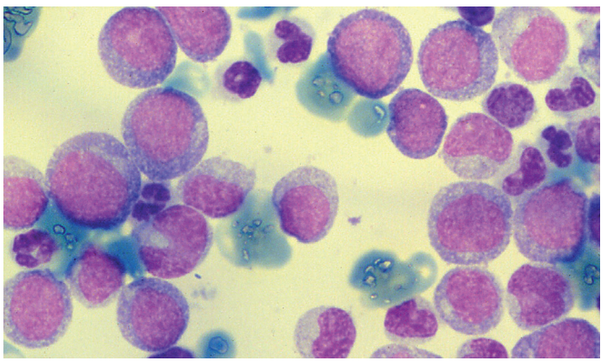


Fig. 4 Haematopoietic tissue: many individual round cells with a few erythrocytes in the background (bone marrow of dog with myeloid leukemia).

II. Epithelial glandular tissue

Cells of this type of tissue have the tendency to be connected to each other. They can form two- or three-dimensional clusters, but also monolayers (Fig. 5), acini (Fig. 6) or duct structures. Sometimes there is a stromal component present. Individual cells may also be present. The cellularity is usually slightly less than in preparations with haematopoietic cells. Benign epithelial cells also have round nuclei. The cytoplasm is often abundant and the cell borders are distinct. Sometimes, for example in endocrine tumours, the cytoplasm is stripped from the nucleus when the smear is streaked out and there are mostly naked nuclei. The cell size may vary from small (basal cells) to very large (squamous epithelial cells).

Fig. 5 Monolayer of epithelial cells (dog with epitheliosis of mammary gland).

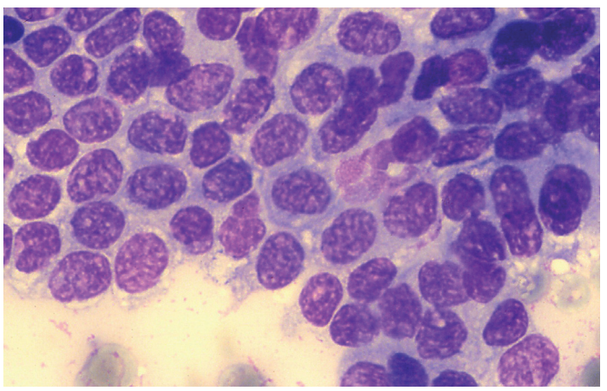
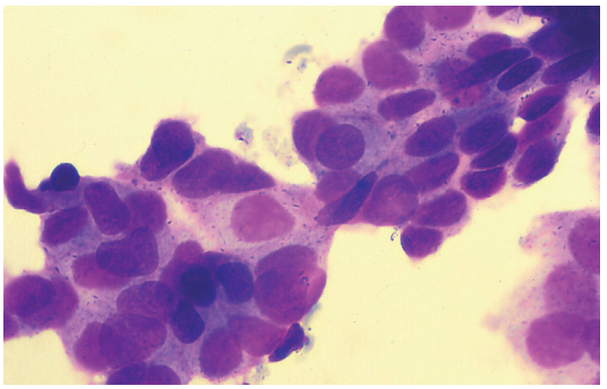


Fig. 6 Glandular epithelial tissue (dog with complex adenoma of the mammary gland).



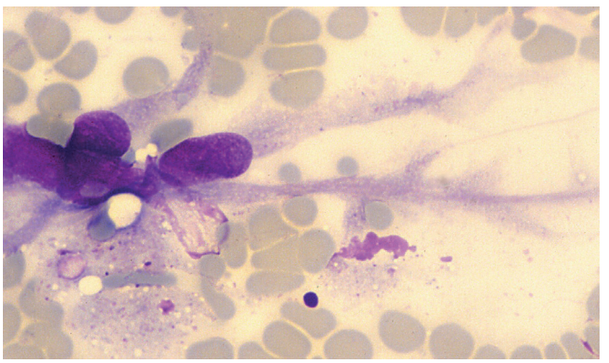


Fig. 7 Two fibroblasts in a mesenchymal proliferation (dog with a fibroma on the foreleg).

III. Mesenchymal tissue

Within this type are included the cells arising from connective tissue, muscle, cartilage, and bone. Because of the strong attachment between the cells it is difficulty to obtain material. Smears of this type of tissue are thus also characterized by a low cellularity and the cells are mostly separate from each other, although groups of cells can also be found. The amount of cytoplasm can vary markedly and the cell borders are often rather vague. The shape of the nucleus can differ but is often oval (Fig. 7). The cells can have characteristic morphologic forms, of which the spindle-shaped cell is the best known. Synovial cells, osteoblasts, and chondroblasts can have an egg-shaped outline, in which the nucleus is eccentric and sometimes lies halfway outside the outline of the cytoplasm. Fat cells can be very large and can be recognised even at a low magnification. These cells are generally connected together as a tissue, have distinct cell margins, and have an oval nucleus that lies in the middle of the light, transparent cytoplasm.

Benign pathologic processes

Inflammation

If primarily inflammatory cells are encountered in a preparation, the process may be an acute or subacute inflammation or a more chronic and sometimes granulomatous inflammation. But it may also be a secondary inflammation in a neoplastic process. It is therefore important, even in the presence of inflammatory cells, to continue looking for other types of cells. It is then sometimes difficult to differentiate between dysplastic, reactive, and neoplastic cells.

Inflammatory processes can be differentiated on the basis of their duration (acute versus chronic), type (purulent, pyogranulomatous, granulomatous, and eosinophilic), and aetiology (bacterial, parasitic, fungal, allergic, foreign-body reaction, etc.).

In a **purulent inflammation** the majority of the cells are neutrophilic polymorphonuclear leukocytes. They can account for than 85% of the cells present in an acute inflammation

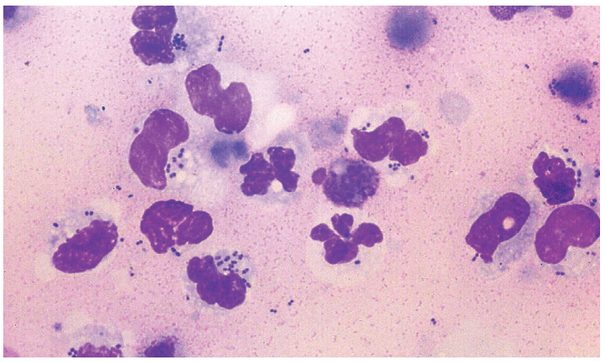


Fig. 8 Acute septic inflammation with several neutrophils with intracellular bacteria and karyolysis.

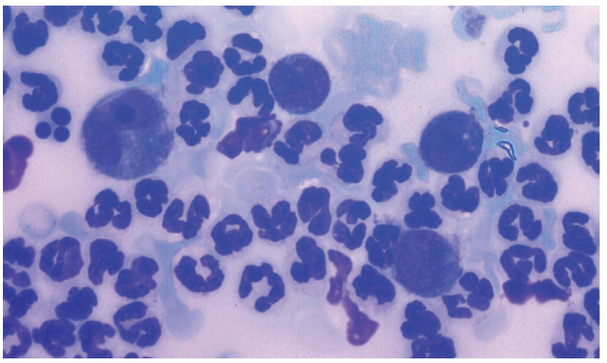


Fig. 9 Aseptic exudative inflammation. Several non-toxic neutrophils, pycnosis (on the left), karyorrhexis (in the middle), and a few macrophages.

and decrease to 30-50% in a more chronic inflammation. In a purulent inflammation there can also be macrophages. Macrophages can be present in the inflammatory process within a couple of hours and thus are no indication of chronicity. This is in contrast to the presence of plasma cells and lymphocytes. These cells only arrive at the site of the inflammation after 1-2 weeks. The inflammatory process can further be characterized by the presence or absence of degenerative changes in the inflammatory cells. Karyolysis is characterized by swelling of the nucleus, less intense staining, and then disintegration of the nucleus. This is especially seen in a severe inflammation with the release of many toxic materials and proliferation of bacteria (Fig. 8). If these characteristics are seen, a special effort must be made to look for infectious agents. In contrast, pyknosis and karyorrhexis are characteristics of a slower cell death (Fig. 9). Pyknosis causes the nucleus to shrink markedly and to lose its chromatin pattern. The nucleus becomes a uniformly darkly stained mass. If this pyknotic nucleus disintegrates, the process is called karyorrhexis.

In **granulomatous inflammation** there are fewer polymorphonuclear cells than in purulent inflammation. In contrast, there are more macrophages and epithelioid cells. The macrophages usually show fewer signs of phagocytosis than in a purulent inflammation. There can also be multinucleated giant cells (Fig. 10). If the infection becomes chronic, lymphocytes and plasma cells are encountered again. This type of inflammation should lead the clinician to consider infection by fungi, infection by actinomyces or similar bacteria, and reactions to a foreign body.

Eosinophilic inflammation contains large numbers of eosinophilic granulocytes. This type of inflammation occurs in the eosinophilic granuloma in the cat, the lick granuloma in the dog, and also in parasitic infections and allergic reactions. Eosinophilic granulocytic infiltrates are also a characteristic finding in many mast cell tumours.

Tissue regeneration

Many morphological changes related to an increased metabolic status of the cell may be present e.g. basophilic cytoplasm (high RNA- and protein content), prominent or multiple nucleoli, and increased numbers of mitoses. Differentiating this from malignancy may be difficult. Usually a normal maturation of the cells can be found.

Scar tissue may be accompanied by young connective tissue cells and blood capillaries. Some inflammatory cells may also be found.

Fig. 10 Pyogranulomatous inflammation due to fungal infection. A large multinucleated giant cell can be seen surrounded by neutrophils, some macrophages and lymphocytes.

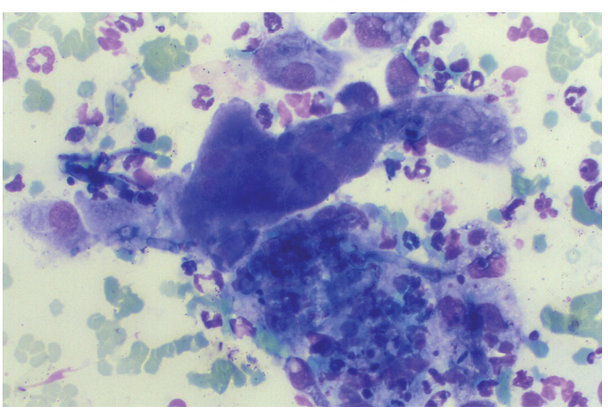
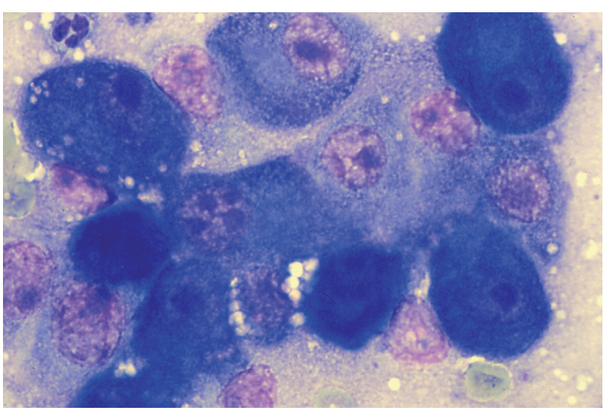


Fig. 11 Liver cell carcinoma in a dog. Note the change in basophylia, the anisocytosis and change of nuclear/cytoplasm ratio.



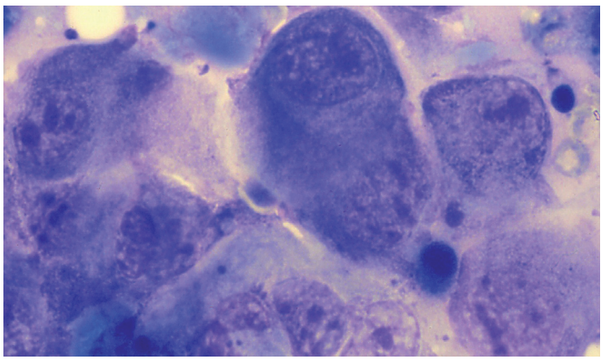


Fig. 12 Liver cell carcinoma in a dog. The nuclear malignancy criteria are especially evident: anisokaryosis, prominent and multiple nucleoli, some of them are angular and there is anisonucleoliosis. In addition irregular nuclear membranes can be seen.

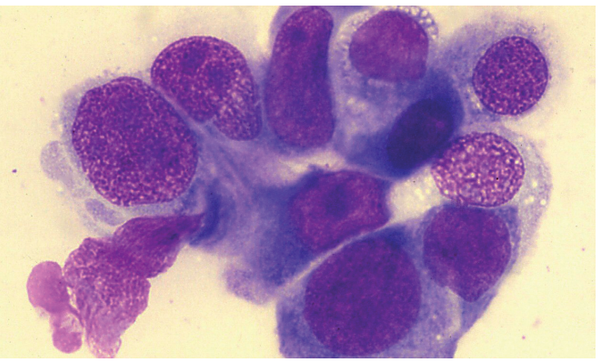


Fig. 13: Mammary gland carcinoma in a dog. Apart from change in basophilia and some microcytoplasmic vacuoles, there is anisokaryosis, coarse chromatin pattern, abnormal nuclear forms, multiple nucleoli with anisonucleoliosis.

Neoplastic processes

In order to differentiate between malignant tumours and benign tumours or hyperplasia, both general criteria and cell-related (cytoplasmic and nuclear) criteria can be used. In the following paragraphs the different criteria of malignancy will be discussed. It must be realized that no single criterion is in itself indicative of malignancy. The nuclear criteria weigh the most heavily. In addition, the criteria are not identical for different types of cells and locations. The malignancy criteria are most often used for epithelial tumours. In mesenchymal tumours they are less predictive, and the haematopoietic tumours are usually diagnosed on different criteria.

With regard to the general criteria, the appearance of the entire population of cells should be examined. The absence of inflammatory cells, the presence of a uniform cell population (i.e., one cell type) of pleomorphic cells (i.e., with variable shapes) are indications of malignancy. A high cellularity and abnormal cellular relationship (e.g., cluster forming) can also occur with malignancy. *Cluster formation* means three-dimensional cell growth without organized cell relationships. In contrast, monolayers and the formation of acini and ducts indicate better differentiation of the cell population. The occurrence of a type of cell where it does not usually occur makes the lesion immediately suspicious. A good example of this is the presence of epithelial cells in a lymph node.

The cytoplasmic criteria of malignancy include strong basophilia of the cytoplasm and even marked variability in basophilia of immediately adjacent celss. This basophilia is related to the amount of RNA and protein synthesis in the cytoplasm and is thus a measure of the cell activity. Reactive cells can, however, also be strongly basophilic. Anisocytosis (marked variation in cell size) and macrocytosis (abnormally large cells) are more often seen in malignant than in benign tumours (Fig. 11). Other cytoplasmic criteria of malignancy are the occurrence of abnormal cytoplasmic inclusions (especially cannibalism of other cells), atypical vacuolization, a high nuclear/cytoplasmic (N/C)

Table 5 Summary of cytoplasmic and nuclear characteristics of malignancy

Cytoplasmic	**Nuclear**
Anisocytosis	Anisokaryosis
Macrocytosis	Macrokaryosis
Basophilia	Multiple nuclei
Variable basophilia	Abnormal nuclear shapes
Abnormal cytoplasmic inclusions	Irreg. thickened nuclear membrane
Atypical vacuolization	Irreg. chromatin pattern
High N/C ratio	Hyperchromasia/anisochromasia
Variable N/C ratio	High mitotic index
	Abnormal mitoses
	Multiple nucleoli
	Large nucleoli
	Abnormally shaped nucleoli
	Anisonucleoliosis

ratio (relatively little cytoplasm) and a marked variation in the N/C ratio.

The most important criteria of malignancy are, however, the nuclear criteria. Giant nuclei, anisokaryosis (difference in nuclear size), multiple nuclei per cell (especially if they also differ in size and are not 2n), abnormal nuclear shapes and marked variation in shapes, irregular and thickened nuclear membranes (especially easily seen in Papanicolaou staining), and an irregular, coarse chromatin pattern are all nuclear characteristics that point to malignancy (Fig. 12 and Fig. 13). An increased mitotic index and especially abnormal mitotic forms are other nuclear criteria of malignancy. The malignancy criteria of the nucleoli are multiple nucleoli, abnormal large nucleoli, anisonucleosis and abnormally shaped nucleoli. Some authors have proposed that the presence of 3-4 of the above nuclear criteria of malignancy should be considered indicative of malignancy, as this number of many malignancy criteria should not occur in hyperplasia. In practice, however, it can still be difficult to differentiate between a malignant and a hyperplastic reaction, especially in mesenchymal proliferations.

An overview of the most important of the above-mentioned criteria of malignancy is given in Table 5.

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