IMMUNOHISTOCHEMISTRY IN DIAGNOSTIC VETERINARY PATHOLOGY

by

Hazel M. Johnston BVMS MRCVS

Dissertation submitted for the Degree of Master of Veterinary Medicine
in the Faculty of Veterinary Medicine
University of Glasgow

Department of Veterinary Pathology
University of Glasgow
November 1997

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration

I, Hazel Johnston, declare that the work presented in this dissertation is original, and was carried out by myself or with due acknowledgement, and has not been previously presented for the award of a degree at this or any other university.

Signed:

Dated: 26/11/97

Dedication

This work is dedicated to my father
John S. Marshall BA.
1945-1997

Acknowledgements

I would like to acknowledge and thank the many colleagues who generously provided assistance during the preparation of this thesis.

Head of Pathology Section, Professor Hugh Pirie, and senior pathologists Drs. Pauline McNeil, Irene McCandlish and Sarah Toth provided advice and encouragement during my time in the Department of Pathology. Dr McNeil and Dr. David Taylor also kindly allowed me the use of their computers and printers.

Head technician Mr. Iain MacMillan and his team; Mrs. Alma Jenkins, Mrs. Lynn Stevenson and Mr. Colin Nixon, provided expert technical assistance and advice.

Mr. Richard Irvine and Mr. John Ramsay obligingly collected tissue samples and performed much of the hard work involved in Post Mortem examinations.

Mr. Alan May and Mr. Ronnie Barron took the many photographs required for this thesis and Ms. Vicki Dale and Ms. Susan Cain were kind enough to scan them onto CD for me.

Thanks are also due to my office mates, Dr. Mara Artuffo and Ms. Mary Fraser who amicably shared their computer and work space with me.

Finally, a special thank you goes to my supervisor Dr. Hal Thompson who supported this work throughout and read and corrected the manuscript. Like other aspiring pathologists before me I have learned that Hal, if you can find him, will always help you out.

Contents

CHAPTER ONE.

Putting Immunohistochemistry in Context

CHAPTER TWO

Using Immunohistochemistry in Diagnostic Veterinary Pathology

CHAPTER THREE

Investigating Cytokeratin Subclasses Using Immunohistochemistry

CHAPTER FOUR

Immunohistochemistry in tumour biology: Studies on the p53 gene product

List of Tables

List of Figures

Abbreviations

ABC	avidin biotin complex
AEC	3-amino-9-ethylcarbazole
APAAP	alkaline phosphatase anti alkaline phosphatase
APES	3-amino-propyltriethoxysilane
°C	degrees Celsius
CEA	carcinoembryonic antigen
CD	cluster of differentiation
CK	cytokeratin
cm	centimetre
CNS	central nervous system
DAB	3,3-diaminobenzidine
DHS	DNP-hapten sandwich
DNA	deoxyribonucleic acid
DNP	dinitrophenol
EBV	Epstein Barr Virus
ELISA	enzyme linked immunosorbent assay
FVIII-RAG	Factor VIII related antigen
GFAP	glial fibrillary acid protein
GUVS	Glasgow University Veterinary School
H&E	haematoxylin and eosin
HRP	horse radish peroxidase
Ig	immunoglobulin
IL	interleukin
kDA	kiloDalton

LAB	labelled avidin biotin
mg	milligramme(s)
MHC	major histocompatibility complex
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
mw	molecular weight
μm	micrometre(s)
NSE	neuron specific enolase
PAP	peroxidase antiperoxidase
PAS	periodic acid schiff
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PTAH	phosphotungstic acid haematoxylin
SV40	Simian Virus 40
TBS	tris buffered saline
TGF	transforming growth factor
UVB	ultraviolet radiation (type B)
W	watt

Summary

Immunohistochemistry is the application of antigen-antibody interactions plus a detection system to tissue sections for the purpose of identifying and localising a given substance.

Widely applied in the fields of research science and medical diagnostic pathology, immunohistochemistry is beginning to be used in diagnostic veterinary pathology and this work was performed to investigate the potential of the technique in assisting with certain histological challenges.

Chapter one provides a broad technical introduction to immunohistochemistry, highlighting the strengths and pitfalls of the technique, and concludes with a literature review which focuses particularly on the rapidly expanding veterinary literature dealing with the use of immunohistochemical methods.

Chapter two describes the use of specific antibodies to determine the cell of origin of thymic tumours in a variety of species. These commercially available antibodies were raised against human epitopes but were found to be effective on domestic animals. Immunohistochemistry was found to be invaluable in differentiating between thymic epithelial and lymphoid tumours.

In chapter three antibodies to specific subclasses of cytokeratin were applied to canine tissues and found to be effective at localising different cytokeratins in normal canine skin and in a range of epithelial tumours.

In chapter four the identification of a nuclear tumour suppressor gene protein, p53, by immunohistochemistry is described. Immunohistochemical detection of this protein indicates abnormal functional status of p53 which occupies a pivotal role in the cell's response to DNA damage. A panel of anti-human p53 antibodies were used on canine, equine and bovine tumours and very high levels of p53 protein expression were detected in equine squamous cell carcinomas.

Publications Arising From This Thesis

JOHNSTON, H.M., THOMPSON, H., PIRIE, H.M. 1996
p53 Immunohistochemistry in Domestic Animal Tumours
The European Journal of Veterinary Pathology Vol. 2 no. 3 p 135-140

JOHNSTON, H.M., THOMPSON, H., FITZPATRICK, J.L. & SHERIDAN, P. 1996
Thymic Neoplasia in Cattle
Association of Veterinary Teachers and Research Workers
Jubilee Scientific Meeting Abstracts

JOHNSTON, H.M., PIRIE, H.M., THOMPSON, H. 1995
p53 Immunohistochemistry in Domestic Animal Tumours
Proceedings of the 13th European Congress on Veterinary Pathology.

Chapter One

Putting Immunohistochemistry in Context

1.1.1) The Historical Perspective.

Microscopes have been used in the study of the minute anatomy of animals and plants for more than three hundred years. The term cell was first used in 1665 by Robert Hooke to describe the compartments he observed microscopically in a thin slice of cork. The term histology, from the Greek *histos* (tissue) and *logos* (study), was coined in 1819 by a microscopist named A.F.J.K. Mayer but the concept of different tissues existing in the body was first propounded by Marie F.X. Bichat (1771-1802), a French anatomist who introduced the word tissue, from the French *tissu* for texture or weave, to biological science. Bichat did not use a microscope in his studies but made his observations from gross dissections. By 1839, Schwann in the field of zoology and Schleiden in the field of botany had independently published their conclusions that nucleated cells were the basic unit of life and the science of modern histology, where the body was recognised to be composed of cells and tissues, began to emerge.

Considering the body to be made up of a number of basic building blocks or tissues was very important for the development of the new science of histology as recurring microscopic arrangements were recognised in successive areas of the body as they were studied. In this way, the structure and function of the individual tissue types could be considered and the study of complex structures like organs logically approached and simplified. A tissue based approach also paved the way for an understanding of the embryological development of the body.

Initially histologists examined membranes, scrapings and tissues pressed thinly using a device known as a *compressorium*, but untreated, living cells are unrewarding to examine microscopically as they are transparent and comparatively thick. Most cellular components have similar refractive indices and are difficult to identify in this way. Therefore, over the following half century this study of living (surviving) cells was superseded by the investigation of preserved dead cells as developments in fixation, processing and staining made this possible.

Although as early as 400BC the Greeks and Persians knew something of the action of mercuric salts and although the preservative effects of alcohol have been recognised for a long time it was the latter half of the nineteenth century which saw systematic investigations of fixatives undertaken. Numerous formulae were developed, many simply variations on common themes. Also around this time the production of synthetic dyes began. These investigations into the technical challenges of tissue processing were stimulated by the development of tissue sectioning and microscopic examination as carried out by the pioneers of histology.

At its beginning histology was a descriptive anatomical science but over time it has developed into a complex discipline where studies of the structure, composition and function of the tissues are linked. Modern histology is a wide science with specialised sub-divisions such as histochemistry, immunohistochemistry and autoradiography. It is, of course, also the basis of histopathology- the study of cell and tissue changes in disease.

1.1.2)Tissue Processing

Tissue processing refers to any treatment of tissues undertaken to facilitate the production of sections for microscopic examination. Processing may be considered to begin with tissue selection and appropriate trimming. The following is a brief outline of the principles and common methods of tissue processing.

Fixation

Fixation fulfils several purposes. It prevents autolysis and bacterial attack and hardens the tissue for subsequent sectioning. It stabilises structural components to preserve their *in-vivo* characteristics and can enhance staining by acting as a mordant. Importantly, fixation also protects those handling the tissues through its antiseptic properties. Stabilisation and hardening are due to crosslinking of proteins. The action of formaldehyde on proteins is reviewed by Pearse and Hopwood (1985). Reaction between formalin and proteins proceeds relatively slowly and is largely reversible by excess water whilst the reaction between proteins and glutaraldehyde is rapid and essentially irreversible. The chemistry of glutaraldehyde-protein interaction is considered by Hardy, Nicholls and Ryden (1976).

The ideal fixative is one which prevents tissue degradation whilst keeping the tissues as close to their living state as possible, renders the tissue suitable for and stable to further processing and does not allow alteration or loss of the constituent chemicals. In practical terms fixation methods are selected with reference to the types of investigation to be carried out on the tissue.

In 1960 Baker introduced a classification system which described fixatives as coagulants or non coagulants and named protocols after the worker who introduced them, disregarding minor variations. Currently fixatives may be most conveniently classified according to their chemical nature (Table 1).

Chemical Fixatives	
Aldehydes	formaldehyde paraformaldehyde glutaraldehyde
Oxidising Agents	osmium tetroxide potassium dichromate
Protein Denaturing Agents	acetic acid methyl alcohol ethyl alcohol
Undefined Mechanism	mercuric Chloride picric Acid

Table 1. List of common fixatives grouped according to chemical action.

In practice, by far the most commonly employed fixative is neutral buffered formalin prepared using 4% formaldehyde.

Dehydration

After fixation is complete the tissue blocks are dehydrated through a graded series of alcohols which remove aqueous and most lipid tissue constituents. This is necessary because the solidifying media are not, except in special circumstances, water miscible.

Clearing

After dehydration a clearing agent, a fluid miscible in both the dehydrating agent (usually alcohol) and the embedding media (usually paraffin wax) is used to remove the dehydrating agent from the tissues. Alcohol itself is not miscible with paraffin wax. Clearing agents ideally have rapid action and low capacity for tissue damage. Traditionally xylene, which can have carcinogenic effects, was the most commonly used clearing agent but now xylene substitutes of reduced toxicity are used.

Embedding

The process of embedding gives tissues physical support to allow sectioning for microscopy. Paraffin wax is a long established embedding medium and is still the most popular. It is convenient to handle, produces good staining results and is inexpensive. Alternative embedding media, including various resins, are available and are used to produce ultra-thin sections for electron microscopy or to support very hard tissues for sectioning.

Most laboratories use automated tissue processing for the bulk of their samples although very small pieces of tissue or urgently required samples may be processed by hand. The end result of either automated or manual processing is a tissue block from which sections may be taken.

Section Cutting

Sections are cut from paraffin embedded blocks using a microtome, floated out on a warm water bath and picked up on glass microscope slides. The sections are dried, usually in a 60°C oven, and are then ready to be dewaxed and stained.

Frozen Sections

In some situations, for example the handling of an urgent surgical biopsy, or for use with specialist techniques in CNS histology or enzyme histochemistry (after pre-fixing), frozen sections may be used in preference to conventionally processed ones. These sections are cut, usually unfixed, on a cryostat machine in which the tissue is cooled to a selected temperature for optimal sectioning. The sections may be briefly fixed, then stained, mounted and examined. This has the advantage of great speed but cryostat sections never possess the morphological clarity of conventionally processed sections as the tissue is unavoidably damaged by the freezing process (ice crystal artefact).

1.1.3) Histological Staining

Staining of tissues is employed to introduce contrast between different cell and tissue components. The majority of histological stains were originally used as textile dyes and almost all dyes in current use are synthetic. Haematoxylin, which is extracted from the heartwood of the tree *Haematoxylon campechianum* is an important exception.

Stains may be actively taken up by living cells in "vital" or "supravital" staining, but more usually staining is determined by tissue affinity for the dyestuff. A detailed discussion of the physico-chemical interactions which co-operate to produce tissue staining is beyond the scope of this review but processes including van der Waals, ionic, hydrogen and covalent bonding all have roles to play. In some cases the underlying mechanism of the staining is poorly defined. Pearse (1980) and Thompson (1966) provide comprehensive overviews of histochemical theory whilst Bird and Boston (1975) and Vickerstaff (1954) provide a mass of technical information on dyes from their application in the textile industry.

Several aspects of the chemical behaviour of a given stain are important. The rate of uptake of a stain by different tissue constituents can be manipulated to produce differential staining in rate controlled staining protocols. For example, Alcian Blue rapidly stains mucins and can be used to identify these substances but if the stain incubation were sufficiently prolonged, other basophilic materials, such as cell nuclei would also stain. Similarly, the rate of loss of stain can be used as a means of

differentiation. In this technique all tissue components are stained, with or without heat or a stain penetration enhancer like phenol, and then treated with a solvent which removes the stain at different rates from different structures. Luxol Fast Blue is used in this way to highlight myelin. Some dyes are particularly useful in their ability to stain different tissue components different colours. This property, known as metachromasia, allows mast cell granules, cartilage matrix and mucins to be highlighted by staining with Toluidine Blue or Methylene Blue which stain these substances reddish-purple against a blue background.

Obviously, to be demonstrated by a stain a tissue component must remain present in the section through fixation and processing. Hence the method of fixation will influence staining patterns according to the preservation of various substances. Fixatives may elute substances altogether or react with them producing derivatives which do not stain. Lipids, for example, are well preserved by fixation in osmium tetroxide but poorly retained in formalin. The reverse is true in respect of proteins.

The most useful staining procedure for routine histology and histopathology is the combination of haematoxylin and eosin. The haematoxylin stains the basophilic elements such as the cell nuclei and provides good nuclear detail. The dye produces a reddish colour which turns blue in mild alkali such as tap water in hard water areas or in Scot's tap water substitute (distilled water plus magnesium sulphate and potassium bicarbonate). Initially the sections are overstained and some of the colour removed in acid-alcohol to produce greater differentiation. Eosin, a derivative of fluorescein, is a pink/red stain particularly effective for use in combination with haematoxylin. Of particular merit is its

capacity to allow distinction between the cytoplasm of different cell types and between different connective tissue substances. This is due to differential loss of the stain through tap water washing and alcohol dehydration. The intensity of the stain may be adjusted according to the preference of the histologist or pathologist.

Histochemistry

Histochemistry is defined as that branch of histology which deals with the identification of chemical compounds in cells and tissues. Histochemical techniques are a refinement of routine histological staining.

In a diagnostic service histochemical methods are often referred to as "special stains" and are employed in the small proportion of cases where the haematoxylin and eosin section is not considered to provide sufficient diagnostic information. These stains are chosen with reference to the appearance of the standard section and are used to visualise a particular feature or answer a specific question about the tissue.

Routine histochemical stains can be used to visualise the five broad types of chemical substances found in mammalian cells. These substances, and commonly used stains for them, are listed in Table 2.

The scope and value of histochemical methods in diagnostic pathology is ably reviewed by Stoward (1990) whilst Culling (1974) provides a great deal of technical and practical information regarding histochemical staining.

Substances Identifiable by Histochemical Methods		
Substance	Group/Residue	Method
Nucleic acids	phosphate	methyl green /pyronin
	deoxyribose	Feulgen- Schiff
Proteins	amine	phloxine-tartrazine
	thiol	ferric ferricyanide
Mucosubstances	vicinal glycol	PAS
	sialic acid	Alcian Blue
	specific saccharides	lectins
Lipids	long aliphatic chains	Oil red O (all lipids)
	free fatty acids	Nile Blue Sulphate
Metals	Iron	Perl's method
	Calcium	Von Kossa

Table 2. Outline of substances commonly localised by the use of histochemical stains. (Prepared with reference to Stoward 1990.)

1.2.1) Immunohistochemistry: An introduction.

Immunohistochemistry, or immunocytochemistry as the technique is sometimes referred to, is the use of specific antigen-antibody interactions plus a detection system to identify cell or tissue constituents *in situ.*

The science of immunohistochemistry had its beginnings in 1941 when Albert H. Coons used a fluorescein labelled antibody directed against streptococcal antigens (Coons, Creech and Jones 1941). Later he also employed an indirect immunofluorescence method to trace plasma cells, again in relation to streptococcal antigen distribution. Coons' work was oriented solely towards immune responses to microbial agents and it was some time before alternative uses for immunohistochemistry were developed.

As stated above, immunohistochemistry makes use of the specific nature of antibody-antigen interaction to identify constituents of interest. Various methods exist to render the bound antibody detectable. If the antibody is to be labelled this must be accomplished without markedly compromising its ability to bind to its target antigen. Methods which employ several steps or layers avoid directly labelling the primary antibody and allow the visible end reaction to be amplified with respect to the primary antibody.

It is important to include adequate controls with immunohistochemical procedures. Tissue known to express the desired antigen should always be included to prevent technical problems producing false negatives; if the positive control fails to stain then the

test batch must be rejected and the reagents and procedures checked to identify the problem. Negative controls of each test section where the primary antibody is replaced by pre-immune serum or irrelevant antibody must similarly be included to check for spurious staining due to non-specific binding of other reagents. The test sections are then compared with their corresponding negative control to identify background staining.

1.2.2)Primary antibodies in immunohistochemistry

Primary antibodies used in immunohistochemistry may be either monoclonal (identical immunoglobulin molecules produced by a single clone of plasma cells) or polyclonal (derived from different cell clones). Licensed establishments may maintain animals for polyclonal sera or ascites production, but many commercial sources of antibody exist.

Polyclonal immune serum, produced by repeated immunisation of the animal with the required antigen, contains around 10mg/ml immunoglobulin, 1-10% of which will be the antibody of interest. The serum must be purified to remove unwanted proteins and other materials. Immunoglobulins may be precipitated using ammonium sulphate as an initial step (England and Seifter, 1990). The crude immunoglobulins isolated by this method can be purified by affinity chromatography where the antibody of interest is specifically bound and subsequently recovered. This technique is comprehensively reviewed by Ostrove (1990). Ascites fluid contains 1-10mg/ml immunoglobulin, approximately 90% of which will be the desired antibody and this is similarly purified before use.

The production of monoclonal antibodies requires tissue culture facilities for spleen fusion and hybridoma selection. Once established these cells provide an almost limitless supply of the specific antibody (Kohler and Milstein 1975, Galfre and Milstein 1981). Hybridoma culture supernatants yield 0.05-1mg/ml of immunoglobulins and the specific antibody is removed by affinity chromatography or by concentration using ammonium sulphate fractionation and dialysis.

Several characteristics are required of an antibody for it to be diagnostically useful. In particular the antibody should be of high specificity and sensitivity. These characteristics may be defined as follows:

$$\textbf{specificity} = \frac{\text{true negatives}}{\text{true negatives+false positives}} \% \qquad \textbf{sensitivity} = \frac{\text{true positives}}{\text{true positives+false negatives}} \%$$

In practice it is difficult to produce an antibody of both high specificity and sensitivity. Most markers with high specificity, e.g. >90%, are of lower sensitivity e.g. <70% and vice versa. However, carefully chosen antibodies or panels of antibodies can produce a good combination of sensitivity and specificity.

Versatility, which relates to the ability of the antibody to work successfully on a range of substrates, is another desirable quality for an antibody. Frozen sections, unfixed preparations and formalin or otherwise fixed, paraffin embedded sections are all targets for immunohistochemical investigation. In practice it has been found that antibodies known to work well on formalin fixed, paraffin embedded tissues tend to be effective on a wide range of preparations. These antibodies are also the most suitable for use in routine diagnostic procedures as formalin fixed, paraffin embedded material is convenient to work with and store (see 1.1.2).

Remaining considerations in antibody selection include cost, availability and shelf-life. The use of commercially prepared antibodies has some advantages in terms of external quality control. Although an enormous range of antibodies are available almost all of these are designed for use on human tissue (a range are also designed for mouse).

Researchers using these antibodies in other species must be especially careful to employ adequate controls to validate their results.

1.2.3) Immunofluorescence

i)Direct Immunofluorescence

This technique, originally developed by Coons in 1941, involves labelling the primary antibody with a fluorochrome (see later). Labelling the antibody directly with dye has proven to be ineffective as the result is not sufficiently visible under the microscope. The major disadvantage of this system is its lack of versatility, each individual primary antibody requires to be bound to a suitable fluorochrome, a time consuming and expensive process. Also the signal intensity tends to be poor as only one fluorescently labelled antibody molecule is bound to each antigenic site.

ii)Indirect Immunofluorescence

This technique, a progression from direct immunofluorescence, was developed in the 1950s (Coons, Leduc and Connolly 1955, Holborrow, Weir and Johnson 1957). In the first step unlabelled primary antibody is applied to the section. This is followed by a heterologous fluorochrome labelled anti-immunoglobulin secondary antibody. As the secondary antibody can be used against any primary antibody of the same species (usually mouse for monoclonals) and class (almost invariably IgG) this technique allows far greater flexibility and economy. It also offers increased sensitivity as it avoids processing of the primary antibody during labelling with its inevitable effect on activity and,

importantly, more than one labelled secondary antibody can bind to each primary antibody and amplify the fluorochrome signal.

Both immunofluorescence techniques have certain inherent drawbacks which make them unsuitable for routine use. For example, these techniques are not suitable for use on formalin fixed paraffin embedded material - the substrate of choice for routine investigations - due to the autofluorescence associated with these preparations. Also, counterstaining to reveal tissue morphology is not possible with fluorescent labelling. Another problematic feature is the impermanency of fluorescent preparations which fade and cannot be routinely mounted.

Fluorochromes

Of the many known fluorochromes only a small number are used in immunofluorescence studies. The most common is fluorescein, a green emitter with maximum fluorescence in light of 400-520 μm wavelength, which has been in use since the pioneering studies of Albert Coons. Fluorochromes are usually conjugated to antibodies via reactive isothiocyanate groups. Red emitting fluorochromes such as rhodamine and its derivatives are also quite widely used (Titus et al. 1982). More recently, phycobilliproteins, intense natural fluorochromes extracted from algae, have found applications in biological science. One major group of phycobilliproteins, the phycoerythrins, have been used successfully in immunofluorescence despite their large size (up to 2410kDa mw) which can give rise to steric hindrance of binding (Pizzolo and Chilosi 1984).

It is possible to double or multiple label sections to allow localisation of more than one antigen in the same tissue providing the fluorescent signals can be differentiated. Texas Red, a rhodamine derivative is suitable for combination with fluorescein for this purpose as their emission spectra have minimal overlap. In this technique primary antibodies which differ from each other in terms of species of origin or immunoglobulin class are applied followed by differently labelled corresponding secondary antibodies.

1.2.4) Immunoenzyme Detection Systems

Indirect immunoperoxidase method

Originally developed by Nakane and Pierce in 1966 and refined by Avrameas (1969) and Nakane and Kawaoi (1974), this enzyme labelled secondary antibody system helped to overcome the problems associated with the indirect immunofluorescence method. It is a two step method in which application of an unlabelled primary antibody is followed by a horseradish peroxidase-linked secondary antibody conjugate directed at the primary antibody species and immunoglobulin class.

This method has the advantages of simplicity, speed and wide commercial availability of reagents and it avoids problems associated with antibody bridge formation (see later). It may be applied to conventionally processed sections. Drawbacks of this technique are lack of efficiency in comparison with multi-step protocols due to lower intrinsic signal amplification, reduction in enzyme (and antibody) activity caused by their chemical coupling and potential background staining due to non-specific binding of the secondary antibody to endogenous immunoglobulins present in the tissue under examination. The last is a problem common to all methods using anti-immunoglobulin secondary antibodies.

Peroxidase-antiperoxidase (PAP) method

This technique was developed as an advance from the earlier indirect immunoperoxidase techniques (Sternberger et al. 1970). It allows the delivery of a greater number of enzyme

molecules to each antigenic site. The PAP method is a three step antibody bridge system. Unlabelled primary antibody is first applied, followed by a bridging antibody specific to the primary antibody species and immunoglobulin class. The bridging antibody will usually be IgG and must be added in excess to ensure that it binds to the primary antibody with only one of its two antigen binding sites. The free binding site must be available to react with a preformed peroxidase-antiperoxidase complex which is added in the third step. This reagent includes an antibody raised against the enzyme horseradish peroxidase (HRP) which is of the same species and class as the primary antibody. The bridging antibody therefore links the primary antibody and the PAP complex.

This technique offers very high specificity as the two binding sites of the bridge antibody are identical and produce a precise immunohistochemical correspondence between the primary antibody and the PAP complex. It is also efficient in terms of signal amplification with the primary antibody capable of binding several bridging antibodies and the PAP complex capable of delivering three HRP molecules to each bridging antibody.

However, this technique has several disadvantages. It lacks versatility, requiring PAP complexes specifically designed for each different species and immunoglobulin class of primary antibodies plus specific bridging antibodies. This naturally increases the expense of the technique. As with some of the other techniques interference from endogenous immunoglobulins can result in high background staining, especially on frozen sections. If the bridge antibody is not sufficiently in excess of the primary antibody it will tend to bind to it with both of its available binding sites leaving none available to interact with

the PAP reagent. Hence at supraoptimal primary antibody concentrations the intensity of the final staining is actually reduced. This apparently paradoxical finding, known as the Bigbee effect, is named after the researcher who determined the explanation for it (Bigbee, Kosek and Eng 1977).

Alkaline phosphatase-antialkaline phosphatase method (APAAP)

This method follows the same principles as the above PAP method except that the enzyme against which an antibody is raised is alkaline phosphatase (Cordell et al. 1984). This is less commonly used than the previous technique as background staining is often more problematic. APAAP is used in selected circumstances such as bone marrow staining where the tissue in question has endogenous peroxidase activity that is difficult to quench effectively (see 1.2.5).

Avidin-Biotin Methods

Another approach to multistep immunohistochemical labelling utilises the very high affinity between avidin, a 68kDa mw. glycoprotein found in egg white and biotin, a member of the vitamin B complex. These two molecules bind together almost irreversibly. Covalently coupling biotin to a protein (e.g. an immunoglobulin) gives that protein the ability to bind avidin which in turn may be covalently joined to ligands such as enzymes, fluorochromes or other proteins. Each avidin molecule can bind four biotins and each biotin molecule has two binding sites for avidin (Bratthauer 1994).

There are two main techniques in use in immunohistochemistry which are built around the avidin-biotin system:

i) Covalent conjugate or labelled avidin (LAB) method

In this method a primary or more usually secondary biotinylated antibody is followed by the addition of a covalent conjugate between avidin and an enzyme, fluorochrome or other marker. The "avidin" used in this method is usually streptavidin, a molecule obtained from the bacterium *Streptomyces avidinii* which has similar excellent biotin binding properties and a reduced tendency toward non-specific binding. This technique is used widely in immunohistochemistry and immunofluorescence (Elias, Margiotta and Gabore 1989, Milde et al. 1989).

ii) Avidin-biotin complex (ABC) method

In the ABC technique the application of a biotinylated primary, or again more commonly secondary, antibody is followed by the addition of a preformed complex of avidin and biotinylated enzyme which forms a lattice and stays in solution. The ratio of avidin to biotin in the complex is such that a binding site for the biotinylated secondary antibody is available (Hsu, Raine and Fanger 1981). This method is illustrated in Figure 1.

The advantages of the ABC and LAB techniques for immunohistochemistry include the reliable nature of the affinity between avidin and biotin which is not as demanding in terms of conditions for success as are some wholly immunological interactions. These two methods are also extremely sensitive due to their capacity to deposit many enzyme molecules at each antigenic site. This sensitivity allows high dilution of the primary antibody which is desirable in terms of reduced cost and also reduces background staining due to non-specific primary antibody binding. The reagents themselves are more

universal in application as biotinylated secondary antibodies are easily produced and widely available which gives these techniques further advantage over the inflexible PAP method in terms of economy.

In terms of disadvantages, steric hindrance due to the large size of the ABC complex can reduce binding capacity in some circumstances and some tissues, especially fresh or frozen tissues, have endogenous biotin or biotin-like activity and are difficult to examine by these methods.

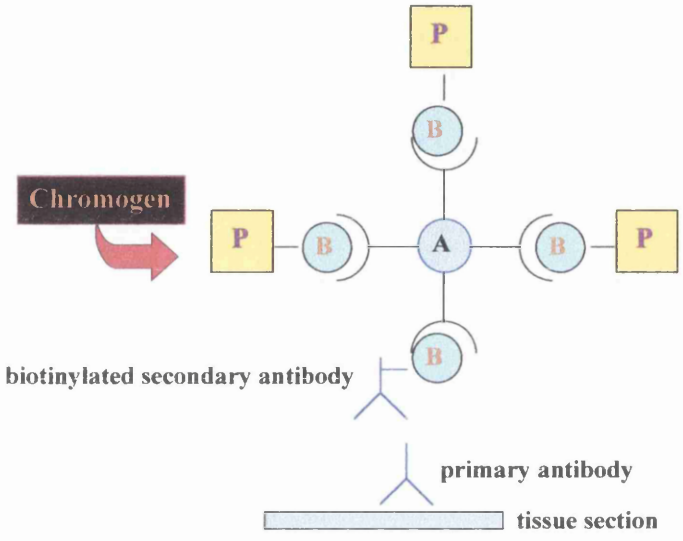

Figure 1. Schematic representation of the multiple steps in the ABC method.
A = avidin
B = biotin
P = peroxidase

1.2.5) Enzymes and Chromogens

The visible signal in immunoenzyme techniques is generated when the enzyme, acting on a substrate, catalyses a reaction in a chromogen which results in a detectable colour change. To be of use in immunohistochemistry the reaction must produce an insoluble precipitate.

The most commonly used enzyme in immunohistochemistry is peroxidase, which has an oxidative function in the presence of a source of oxygen (hydrogen peroxide, H_2O_2). Horseradish peroxidase (HRP) is most commonly used as it is easily obtained and has antigenic differences from most mammalian forms of the enzyme. For use in conjunction with this enzyme, a chromogen which changes colour and produces an insoluble pigment when oxidised is required. Many tissues have endogenous peroxidase activity with the potential to cause heavy background staining, but fortunately this activity is effectively quenched by pre-incubating the tissue sections in a hydrogen peroxide/methanol or hydrogen peroxide/ water solution (Streefkirk 1972, Burns, Hambridge and Taylor 1974). An exception to this is the myeloperoxidase activity in bone marrow for which alternative enzyme systems are preferred.

The most widely used chromogen in peroxidase systems is probably 3,3-diaminobenzidine (DAB) which produces a brown precipitate. It is favoured because it is effective in quickly adhering to the tissue at the site of its generation and its polymer is resistant to the dehydrating and clearing agents (alcohols and xylenes) allowing

conventional mounting of the slides. The colour produced is stable in long term storage. A possible disadvantage to using DAB is the similarity of its colour to some endogenous pigments such as melanin, haemosiderin and lipofuschin. It is, however, possible to modify the coloured end-product by carrying out the reaction in the presence of heavy metal ions. The presence of nickel, for example, produces a dense black pigment ideal for multiple staining or highlighting a very faint colour reaction (Shu, Ju and Fan 1988).

An alternative chromogen to DAB is 3-amino-9-ethylcarbazole (AEC) which produces a red deposit. This can produce better contrast than DAB in some circumstances. The major drawback to the use of AEC is that it is soluble in the dehydrating and mounting agents used routinely and aqueous mounting is required.

Another enzyme used in immunohistochemistry is alkaline phosphatase. This enzyme cleaves phosphates off a donor molecule which in turn acts as a mediator of colour change in a third molecule. This system produces more coloured molecules per enzyme molecule than peroxidase and is hence potentially more sensitive. Alkaline phosphatase may be preferred for examining protein or nucleic acid blots or tissue cultures but is less commonly applied to routine tissue sections as alkaline phosphatase activity is widespread in tissues. Pre-treatment with levamisole to block this activity is possible but is not as dependable as quenching peroxidase with hydrogen peroxide (Ayala et al. 1993).

A variety of chromogenic substrates are available for use with alkaline phosphatase. A combination of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) produces a permanent blue precipitate at the site of the enzyme localisation whilst

Fast Red TR/Napthol AS-MX (Sigma, Poole, Dorset, England) produces a red precipitate.

Glucose oxidase is occasionally used as the enzyme system of choice (Jasani 1992), largely in cases where background enzymatic activity is a problem, as glucose oxidase activity is not found in mammalian tissues.

1.2.6) Antigen retrieval

A vast array of antigens can be localised using immunohistochemistry but in many cases special treatment of the tissue sections is necessary to render the antigens available for binding with their specific antibody. The basic purpose of these treatments is to overcome the effects of fixation, hence they are not necessary on fresh preparations or frozen sections. As stated previously, however, formalin fixed paraffin embedded sections are in many respects the "gold standard" of histological preparations and a great deal of interest has been focused on how to maximise the use of immunohistochemistry, which was originally applied to frozen sections, in these preparations.

Formalin fixation causes cross-linking between reactive sites on the same or adjacent proteins which alters the antigenicity of the tissue, although it appears that it does not alter the secondary structure of proteins (Mason and O'Leary 1991). A minority of antigens, particularly cell surface markers, are destroyed by fixation *per se* but studies using special processing techniques have produced tissues with reactivity comparable to frozen specimens when reacted with antibodies against antigens which do not resist routine processing (Collings, Poulter and Janossy 1984). This finding suggested that fixation need not destroy antigens and stimulated further interest in the possibilities of recovering the antigenicity of routinely processed tissues. It has been found that for the majority of antigens, which are not irretrievably destroyed by conventional processing,

fairly simple treatment can unmask those which would be otherwise unavailable and enhance the reactivity of others which partially withstand fixation.

There are two basic types of antigen retrieval: enzymatic and heat-mediated. Enzymatic antigen retrieval involves the application of a solution of trypsin (usually 0.05% trypsin, 0.1% $CaCl_2$ in phosphate buffered saline {PBS}) or a similar proteolytic enzyme, such as the commercially available Pronase E (Sigma, Poole, Dorset, England), to break down the protein linkages induced by fixation. Protocols are adjusted with different incubation times and temperatures as required. There are two main methods of heat-mediated antigen retrieval; microwave oven and pressure cooker processing. In both of these methods the tissue sections are immersed in a buffered solution (usually a citrate solution) which is allowed to boil (Cattoretti et al. 1993, Shi, Key and Kalra 1991). Again the action of these treatments aims to break down the bonds formed during processing and re-expose the desired antigenic sites. The pitfalls of antigen retrieval techniques are twofold. Firstly both enzymatic and heat treatment is damaging to the tissues. This leads to poorer preservation of morphology or, at worst, destruction and loss of tissue sections. The other potential problem is alteration of antigenicity in the tissue where the pre-treatment itself generates spurious positive reactions.

It is therefore necessary to determine the least damaging protocol effective for a particular tissue and antigen and to apply meticulous controls to guard against unrecognised false positives.

1.3.1) Applications of immunohistochemistry

The applications of immunohistochemistry in research and basic science are numerous. This technique has been widely used wherever localisation of specific substances is of value. Antibodies may be raised against specific gene products, surface markers, microbial antigens, subcellular constituents, in fact almost any antigenic determinant according to requirements, making the technique extremely versatile.

Particular veterinary research fields in which immunohistochemistry is extensively utilised include endocrinology, where the technique has been applied, for example, to studies of the neuroendocrine system of the lungs (Rodrigez et al. 1992, Lopez et al. 1993), gastrointestinal endocrine function (Ceccarelli, Pedini and Gargiulo 1995, Martinez, Lopea and Sesma 1993, Calingasan et al. 1984) and pancreatic endocrine function (Taniyama et al. 1993, Furuoka et al. 1989, Atkins et al. 1988, Nakajima et al. 1988).

In the field of neuroanatomy/neurophysiology Bowker et al. (1993) used immunohistochemistry to identify sensory receptors in the equine foot, Hewicker Trautwein et al. (1994) to localise glial and neuronal cell markers in the developing bovine brain and Prieto et al. (1993) to examine the innervation of the equine ureter.

Parasitology is another discipline where the literature on work involving immunohistochemistry is very extensive. This technique allows, for example, localisation

within the parasite of antigens which provoke a serological response in the host. Specific antigens have been localised in nematodes such as *Trichinella spiralis* (Takahashi et al. 1988, Li and Bao 1994) and *Ancylostoma caninum* (Sawsngjaroen, Opdebeeck and Prociv 1995), in protozoa including *Plasmodium yoelii* (Torii et al. 1992) and *Entamoeba histiolytica* (Sherchand et al. 1994), trematodes such as *Fasciola spp.* of liver fluke (Yamasaki, Kominami and Aoki 1992) and arthropod parasites including the louse *Polyplax spinulosa* (Volf 1994) and the ixodid tick *Dermacentor variabilis* (Davis, Dotson and Oliver 1994).

This review will focus on the diagnostic and related uses of immunohistochemistry and specifically highlight those areas in the field of veterinary pathology where the use of immunohistochemistry has been reported.

Immunohistochemistry in Diagnostic Pathology

As mentioned previously in this chapter, immunohistochemical techniques have been in use since the 1940s, at first as a specialised research tool for which frozen sections were required. When, in 1974, Taylor and Burns reported the immunohistochemical detection of plasma cell immunoglobulin in paraffin embedded tissues it was realised that immunohistochemistry could be developed into a powerful tool for use in diagnostic pathology. Quickly this work was repeated in other laboratories (Garvin et al. 1974, Davey et al. 1978) and the applicability of this technique to routinely processed tissues was confirmed. As mentioned previously (1.2.6) special fixation and processing protocols

can extend the usefulness of some antibodies into paraffin embedded tissues but the range of commercially produced antibodies specifically designed for use in formalin fixed, paraffin embedded tissues, with or without antigen retrieval techniques is also increasing. These include antibodies to intermediate filaments, many leucocyte markers and tumour-associated proteins.

The examination of fixed, paraffin embedded tissues is of central importance to diagnostic histopathology. Techniques which allow the exquisitely specific and sensitive antibody-antigen interactions of immunohistochemistry to be combined with morphological interpretation of such samples probably represent the most important advance in the science of diagnostic pathology in recent years.

Another great advantage, aside from suitability for inclusion in routine diagnostic procedures, of staining paraffin embedded sections is that it allows retrospective, archival studies to be undertaken, making available a vast resource of material. Immunohistochemistry has been successfully performed on tissues stored since the last century and in at least one instance on tissues held in ethanol for thirty years (Celio 1979).

Tumour Diagnosis

Immunohistochemistry is of particular benefit to the diagnostic pathologist in certain situations. Perhaps the most important among these is the use of this technique in the diagnosis of tumours of unknown origin in cases where the morphology is not helpful or is poorly preserved. In such cases, where haematoxylin and eosin sections, together with more specific histochemical stains such as sirius red, PAS, PTAH or reticulin stains, fail

to sufficiently identify the cells a panel of carefully chosen antibodies will be applied. The choice of this panel is tailored according to the pathologist's judgement of the requirements of the particular case. It is impractical to use immunohistochemistry as a "fishing exercise" in the diagnostic situation as using a vast panel of antibodies would be prohibitively expensive and inefficient.

To determine the nature of a poorly differentiated tumour antibodies against cell lineage markers are used. The expression of these markers in a cell provides information on the neoplasm's embryological derivation. The use of these markers in tumour diagnosis relies on the fact that most tumours retain similar markers of lineage to their tissue of origin (Cooper, Schermer & Sun 1985). Lineage markers include intermediate filaments, cytoskeletal components so named because they are intermediate in size between small filaments (e.g. actin) and large filaments (e.g. microtubules), the S-100 protein, carcinoembrionic antigen (CEA), Factor VIII related antigen (FVIII-RAG) and many lymphoid subset markers divided into CD (cluster of differentiation) groups. The major classes of intermediate filament are cytokeratins, vimentin, desmin, neurofilaments and glial fibrillary acid proteins (GFAP). Lineage markers will be discussed in greater detail in chapter two.

Interpretation of the immunohistochemical results must be made in the light of the morphological appearance of the tumour as in some cases malignant transformation can alter the expression of intermediate filaments causing unexpected expression, co-expression or loss of expression (Corwin and Gown 1989).

Another problem which immunohistochemistry is well suited to help solve is the detection and identification of small numbers of cells, for example, tumour cells in smears or aspirates (Ghosh, Mason and Spriggs 1983, Ghosh et al. 1985) or micrometastases in lymph nodes (Wells et al. 1984). These studies have shown that immunohistochemical techniques can yield positive results when classical morphological or cytological examination has been negative or equivocal. This is obviously advantageous in terms of determining prognosis and appropriate treatment.

Veterinary Tumour Pathology

As in human medicine, one of the veterinary fields in which immunohistochemistry has been employed is tumour investigation. The immunophenotyping of lymphoid neoplasia is an expanding area of interest. Caniatti et al. (1996) examined fine needle aspirates of tumourous canine lymph nodes, combining morphological examination with immunocytochemical analysis of cytospin preparations which allowed immunophenotyping and classification of the neoplastic cells. In 1994 Tanimoto et al. reported applying anti-IgG, anti-PCNA, anti-EBV and anti S-100 antibodies to formalin fixed, paraffin embedded sections from cases of alimentary lymphosarcoma (lymphoma) in swine. Their findings included monoclonal immunoglobulin expression which was not associated with albumin positivity. This allowed reliable identification of these cases as B cell lymphosarcomas (lymphomas) which can be difficult to distinguish from lymphoid hyperplasia (pseudolymphoma) (Fenoglio-Prieser, Pascal and Perzin 1990).

Investigations on lymphoid cell populations in cases of Enzootic Bovine Leucosis (EBL) have demonstrated that EBL-associated tumours are neoplasms of mature B cells (Heeney and Valli 1990, Aida, Okada and Amanuma 1993). Recently, Chiba et al. (1995) used a panel of monoclonal antibodies against leucocyte differentiation molecules on lymph nodes and tumours from cases of EBL, divided the the lymph node lesions into three discernible patterns and classified the cells in the tumour masses.

Lymphosarcoma is the commonest haematopoetic neoplasm in the cat and the association of this disease with Feline Leukaemkia Virus (FeLV) is well documented (Hardy et al. 1981). However, tumourigenesis including the development of lymphosarcoma, has also been reported in association with Feline Immunodeficicency Virus (FIV) infection (Hopper et al. 1989, Alexander et al. 1989, Shelton et al. 1990). In 1996 Callanan et al. described the histopathologic and immunohistologic examination of eight cases of lymphosarcoma in domestic cats with FIV. Seven were found to be high grade B cell lymphomas of the centroblastic or immunoblastic subtypes (modified Kiel classification). Burraco et al. (1992) similarly reported the use of immunohistochemistry to define the cellular lineage of a large granular lymphoma in a FIV positive/ FeLV negative cat.

Canine plasmacytomas have been studied with antibodies against immunoglobulin (Ig) light chains. The results have confirmed their plasma cell origins by demonstating the presence of these immunoglobulin molecules in the cells and have proven the neoplastic nature of these lesions by showing the cells are monoclonal (Brunnert and Altman 1991, Kyriazidou, Brown and Lucke 1989).

Sandusky, Carlton and Wightman (1987) reported a series of sixty-five canine "round cell" skin neoplasms which they studied using the avidin-biotin-complex (ABC) method on conventionally processed tissues. Mast cells were found to react with avidin and were consequently stained positively even on negative control sections. The authors considered S-100 (amelanotic melanoma), vimentin (amelanotic melanoma and transmissible venereal tumour) and Ig light chain (cutaneous lymphoma) to be the most useful antibodies to aid differential diagnosis of these neoplasms.

A case of disseminated malignant histiocytosis in a Golden Retriever was reported by Hayden et al. (1993). The authors used histology, electron microscopy and immunohistochemistry to diagnose this case. The neoplastic cells reacted positively with antibodies to human T-cell antigen, alpha-1-antitrypsin, cathespin B and lysosyme.

Pace et al. (1994) reported the immunohistochemical examination of a series of seven cases diagnosed as feline malignant fibrous histiocytomas. Archival biopsy specimens were stained for cytokeratins, vimentin, desmin, S-100 and alpha-1-antitrypsin. Four tumours stained with all antibodies except alpha-1-antitrypsin, one for vimentin only, one for S-100 and vimentin and one was uniformly negative. The authors conclude that feline malignant fibrous histiocytomas show diverse intermediate filament expression as does the human counterpart of this tumour and that they do not appear to be histiocytic in origin. They also suggest the three discordant biopsies in the series should be reclassified.

Immunohistochemistry has also been used in the investigation of endocrine neoplasms . Heinrichs, Baumgartner and Capen (1990) demonstrated a uniform pattern of primarily

pro-opiomelanocortin (POMC) immunoreactivity in nineteen adenomas of the pars intermedia in horses with moderately strong melanocyte-stimulating hormone (MSH) immunoreactivity and weaker, more variable adrenocorticotrophic hormone (ACTH) positivity. This supports the concept of equine pituitary adenomas producing a unique clinicopathologic syndrome associated with excess production of POMC derived peptides with secondary involvement of ACTH, a different aetiopathogenesis from Cushing's Syndrome in the dog. Endocrine disease of the canine pancreas has also been a target for immunohistochemical investigation. In 1987 Hawkins et al. used the technique to examine normal and neoplastic islets in formalin fixed, paraffin embedded sections of canine pancreas. Sub-islet localisation of glucagon, insulin and somatostatin producing cells was established. No gastrin staining was detected in the normal islets. Of twenty tumours, eight had immunoreactivity for insulin, nine for glucagon, fourteen for somatostatin and one for gastrin. Three were pure insulinomas but all the others had mixed hormone production.

The immunohistochemistry of equine pulmonary granular cell tumours has been investigated by various workers. In the series reported by Kelley et al. (1995) the cytoplasmic granules in the tumour cells were stained uniformly positive with S-100 and NSE. Bouchard et al. (1995) described three cases where the tumours were positively stained with S-100 and vimentin but negative for NSE. Both authors concluded that these neoplasms are of neural crest (probably Schwann cell) origin similar to the granular cell tumours recognised in humans (Miettinen et al. 1984).

Vaan Maanen et al. (1996) reported the immunohistochemical examination of three cases of carcinoid tumour in the horse. All three were reactive for NSE and synaptophysin, confirming the neuroendocrine nature of these tumours.

Bovine pulmonary blastomas have also been examined using this technique and multiple pathways of differentiation within the tumour masses were demonstrated by Kelley et al. (1994) who used anti-vimentin, anti-actin, anti-cytokeratin and anti-NSE antibodies and found a mixed pattern of immunoreactivity in the neoplastic cells.

Tumours of neural tissue have also been investigated immunohistochemically. Ribas et al. (1989) examined a series of canine choroid plexus tumours with lineage directed markers and found evidence of epithelial but not glial differentiation. Immunohistochemical studies of unusual neural neoplasms have aided their identification and demonstrated their origins. Carrigan et al. (1996) described an equine papillary ependymoma which was positive for GFAP and weakly positive for vimentin, whilst Mattix et al. (1994) reported a ganglioneuroblastoma in a dog with mixed immunoreactivity similar to such tumours in humans and other animals.

Vascular tumours, particularly accessible cutaneous ones, have been examined by immunohistochemical techniques. Miller, Ramos and Kreeger (1992) reacted haemangiomas and haemangiosarcomas in domestic cats with antibodies to Factor VIII-related antigen confirming the vascular endothelial origin of these growths. Similarly, Johnson et al. (1996) used this method to demonstrate the orderly arrangement between

vascular endothelium and smooth muscle supporting cells in benign vascular neoplasms in young horses.

Immunohistochemistry in non-neoplastic disease

i)Viral Infections

Characterisation of neoplastic cells is not the only veterinary application of immunohistochemistry. An enormous literature exists dealing with the applications of this technique in investigating viral disease.

Haines, Clark and Dubovi (1992) screened thirty two monoclonal antibodies (mabs) to epitopes in the Bovine Viral Diarrhoea Virus (BVDV) and found only one (mab 15C5) which recognised an epitope preserved on formalin fixed, paraffin embedded tissues. This antibody was found to be effective and specific for immunohistochemical detection of BVDV in routine specimens. The same monoclonal antibody (15C5) was used by Bazler et al. in 1995 in a retrospective study of fifty cases of BVDV diagnosed by viral isolation. In the thirty-nine bovine animals in the study there was 100% concordance between immunohistochemistry and viral isolation. When testing was restricted to the intestinal tissue and/or faecal samples virus isolation detected BVDV in 65% of cases compared with immunohistochemistry which detected BVDV antigen in 100%. Immunohistochemical detection was poor in ovine and caprine cases where the virus had been isolated. Immunohistochemistry has also been used to determine antigen distribution in body tissues of specific pathogen free (SPF) calves experimentally infected with different strains of non-cytopathic BVDV (Marshall, Moxley and Kelling 1990). Once again mab 15C5 was used in this study which found different strains to have the same tissue trophism for thymus, Peyer's patches, mesenteric lymph nodes and bone marrow.

Central nervous system (CNS) lesions associated with persistent BVDV infection, Mucosal Disease and congenital malformations (attributed to in-utero infection with the virus) have been investigated immunohistochemically. In persistently infected animals and Mucosal Disease cases a widespread infection of neurons by the BVD virus was detected. In contrast, few cases with congenital CNS malformations showed viral antigen in the CNS, reflecting a competent immune response within the foetus at the time of infection (Wohrmann et al. 1992).

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) has been another target for immunohistochemical study in both natural and experimental infections. In 1996 Halbur et al. used the technique to compare antigen distribution of different American PRRSV isolates and European Lelystad Virus in experimentally infected pigs. A similar pattern of virus antigen distribution in alveolar and other macrophages, lymph nodes, tonsils and spleen was found.

The involvement of PRRSV in four pig herds with abortions was confirmed using, serology, virus isolation and immunohistochemistry It was found that immunohistochemistry could demonstrate viral antigen in tissues where virus isolation was negative (Rossow et al. 1996). The same technique was used by Larochelle, Sauvageau and Magar (1994) to demonstrate PRRSV in lung sections from field cases of porcine proliferative and necrotizing pneumonia.

Immunohistochemistry has been used to investigate many morbillivirus infections including canine distemper, rinderpest and the closely related peste de petit ruminants

(PPR). In 1991 Brown, Mariner and Olander successfully used anti rinderpest antibodies to localise PPR virus in conventionally processed respiratory tissues from experimentally infected goats. Brown and Torres (1994) used a similar technique to study the distribution of rinderpest virus in experimentally infected heifers. In these animals viral antigen was detected in lymphoid tissue and intestinal and respiratory epithelium.

Iwatsuki et al. (1995) examined lymphoid organs from dogs with natural Canine Distemper Virus (CDV) infections using anti-measles antibodies and a range of lymphoid cell markers. Histological examination showed lymphoid atrophy. The authors found that Thy-1 positive and CD4 positive T-cells were major targets for the virus during acute infection.

A survey of brain tissue from 236 wild carnivores (146 mustelids and 90 foxes) in southwestern Germany was reported by Van Moll et al. (1995). CDV antigen was detected by immunohistochemistry in 54 (37%) mustelids and in no foxes. Associated histopathological changes were found in 45% of the positive CDV cases. These results suggested a local CDV epizootic in the mustelid population.

In 1993, Alldinger, Baumgartner and Orwell conducted a detailed study of viral surface protein expression in canine distemper encephalopathies. The brains of sixteen dogs with natural infections were studied with antibodies against five major viral epitopes. The results indicated restricted CDV envelope protein synthesis in acute and more prominent synthesis in chronic distemper encephalitis.

ii)Bacterial Infections

Immunohistochemistry is also used in the investigation of non-viral infectious diseases. Recently (1996), Madrame et al. used immunohistochemistry to detect virulence-associated antigens of *Rhodococcus equi* in formalin fixed, paraffin embedded tissue from lungs of six clinically affected foals using a monoclonal antibody to the 15-17 kDa antigen of this bacterium. The authors suggest that this technique may be used as a diagnostic aid when only formalin fixed tissues are available.

As this very versatile technique can be used to localise almost any antigenic material it can also detect toxins produced by micro-organisms and be used to investigate the role of these substances in disease pathogenesis. For example, Whitely et al. (1990) used immunohistochemistry to localise *Pasteurella haemolytica* A1 derived endotoxin, leukotoxin and capsular polysaccharide in experimental cases of bovine pasteurella pneumonia. The study aimed to localise the cellular and subcellular sites of interaction of endotoxin and leukotoxin in the lungs and relate this to the location of bacteria using immunohistochemistry and immunoelectron microscopy. The authors found that endotoxin was released into the inflammatory exudate and could cross the alveolar wall and that leukotoxin appeared to be specific for leukocytes.

Immunohistochemistry can offer rapid confirmation of the diagnosis in bacterial infections where bacterial isolation is slow, expensive or difficult. One such application is the diagnosis of *Listeria monocytogenes* infection by immunohistochemistry on fixed brain tissue (Weinstock, Horten and Rowland 1995). This group used commercially

available rabbit polyclonal antiserum titrated using tissue from a sheep from which a pure culture of *L. monocytogenes* had been isolated. The authors concluded that with careful tissue selection (staining was best in microabscesses or areas of necrosis) that the technique was accurate and specific.

Haziroglu et al. (1996) used the immunoperoxidase method to investigate *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* antigens in pneumonic lungs of 2-10 month old lambs. Some discordance was found between the results of bacterial isolation and immunohistochemistry but the clear visualisation of antigen in the lesions was considered valuable in the study of respiratory disease pathogenesis. It was considered that positive isolations and negative immunohistochemistry might be due to low numbers of bacteria in the sections and the reverse effect might be due to immunohistochemical detection of antigen from non-viable bacteria.

iii)Prion-related Disease

Immunohistochemistry has also found application in the investigation of prion related diseases, for example natural scrapie in sheep. The diagnosis of scrapie classically rests on finding neuronal and neuropil vacuolation in the brains of clinically affected sheep. Problems arise when vacuolar change is absent or minimal in suspect cases or where vacuolation is observed in brains from apparently healthy sheep. Specialist techniques such as western blotting or electron microscopy may be used to investigate these cases but require specialist laboratories whereas immunohistochemistry can be used with relative ease and on fixed tissues. In 1993 Miller et al. described a protocol for immunohistochemical detection of scrapie associated prion protein (PrP^{sc}). Due to the

nature of the fixation protocol used the sections examined in this study could not also be used for histopathologic scrapie diagnosis. In 1995 Van Keulen et al. refined this technique using a slightly different fixation protocol which better preserved tissue morphology and an autoclave pre-treatment step to enhance PrP^{sc} immunoreactivity. Using antisera to ovine PrP Van Keulen and colleagues detected PrP^{sc} in the brains of fifty sheep with natural scrapie. Brains from twenty control sheep were examined and found negative. The scrapie associated protein was found both in vacuolated areas and non-vacuolated areas of the brain. The authors concluded that the immunohistochemical detection of PrP^{sc} was a useful confirmative test in scrapie diagnosis.

iv)Skin Disease

Immunohistochemistry has proven a useful technique for examination of the skin both in disease states and in the assessment of cell populations and cell activity in normal skin. One of the most common dermatopathological applications of immunohistochemistry is the investigation of immune mediated skin disease.

In an attempt to validate a more convenient method than immunofluorescence, and to improve on the previously reported PAP immunostaining on formalin fixed tissues (Suter et al. 1984) Haines, Cook and Clarke (1987) investigated the use of ABC immunohistochemistry with enzymatic antigen retrieval to detect intercellular IgG in the epithelium of formalin fixed skin biopsies from dogs with autoimmune skin disease. In their study, twenty-seven of twenty-eight cases diagnosed by histology as autoimmune skin disease (AISD) were positive by immunohistochemistry. One case of seven

diagnosed histologically as not consistent with AISD also showed intercellular IgG staining consistent with pemphigus foliaceus.

Day, Hanlon and Powell (1993) reviewed a series of cases of immune mediated skin disease in the dog and cat with special reference to immunohistochemistry using species-specific IgG and IgM antibodies. Immunohistochemical staining of sections from ten dogs with pemphigus foliaceus showed intraepithelial deposition of IgG in seven cases, this was accompanied by IgM in two cases. The location of the positive staining was associated with dermal aggregates of B-cells/plasma cells. Eight biopsies from cats with pemphigus foliaceus were examined and only two of these showed intraepithelial IgG deposition. In the dog detection of the third component of complement (C3) was also attempted, with no positive results. Smaller numbers of other disease states were similarly examined.

In 1995, Gorrell, Townsend and Ladds examined the lymphocyte populations in normal and acanthotic ovine skin using an indirect immunoperoxidase technique on frozen sections. The authors found that acanthotic skin had an eight-fold increase in the number of lymphocytes in the epidermis and at the dermoepidermal junction. They also describe changes in the distribution of T-cell subsets.

The technique has also been used to characterise the immunophentype of the lesions in canine demodicosis. Caswell et al. (1995) reported that all the lymphocytes located within the follicular walls of biopsies from affected dogs were CD3+ T-lymphocytes, compared with the mostly plasma cell perifollicular infiltrate which contained only 10% CD3+ T-

cells. The authors suggest that a cell mediated reaction which targets the follicular wall is involved in demodicosis.

This first chapter provides a technical overview of the principles and methods employed in immunohistochemistry and a review of the literature concerning the application of immunohistochemical methods in diagnostic pathology. From this it is clear that immunohistochemistry is of wide and increasing use in the investigation of animal disease.

In the following chapters immunohistochemical investigations performed by the author are described.

Chapter 2

Thymoma: A Practical Challenge for Immunohistochemistry

2.1.1) Thymoma: a practical challenge for immunohistochemistry.

Thymomas are tumours of the epithelial component of the thymus gland and are uncommon in most domestic species (Moulton and Harvey 1990, Theilen and Madewell 1987). Diagnosis of a thymoma hinges on the identification of an abnormal population of epithelial cells. These epithelial cells are, however, often obscured by lymphocytes and the use of immunohistochemistry can provide specific evidence of their lineage and allow an exact diagnosis whilst conventional techniques may only allow suspicion or tentative diagnosis in some cases.The differentiation of thymoma from the more common neoplasm of thymic lymphosarcoma presents a diagnostic challenge for the practical application of immunohistochemistry in veterinary pathology.

To assess the possibilities of the technique which, through the specificity of the antigen-antibody interactions involved, can definitively identify cell type, thymic tumour cases drawn from the Department of Pathology archive and active cases passing through the Veterinary Hospital system were examined.

To appreciate the types of thymic malignancies which may be encountered it is necessary to review the basic developmental biology of the thymus.

2.1.2) The thymus.

Embryologically the thymus is a paired organ which develops from the ectoderm of the third brachial cleft and the endoderm of the pharyngeal pouch. These tissues migrate caudally as the foetus grows (Griffith 1990). In the young calf the thymus extends from the larynx to the pericardium and rapidly increases in size during the first 6-9 months of post natal life although it reaches its greatest relative size shortly after birth (Dyce, Sack and Wensing 1987). With age active lymphoid tissue is replaced by fat and fibrous tissue but this process is variable and is influenced by many factors including stress and infectious disease. In normal animals a thymic remnant is detectable throughout life.

The thymus is a composite of lymphoid and epithelial tissues, a lobulated, encapsulated organ divisible into cortex and medulla. It is a central (primary) lymphoid organ which plays a dominant role in T lymphocyte development. The epithelial stroma is established first and attracts lymphocyte precursors. T cells originate in the bone marrow but do not differentiate there. Instead, they migrate at an early stage to the subcapsular region of the thymic cortex and in this specialised microenvironment undergo differentiation, expansion and selection (Van Ewijik, 1991, De Waal Malefit et al. 1986). Bone marrow derived macrophages also colonise the thymus along with lymphocyte precursors.

Because T lymphocytes recognise foreign antigen only in the form of peptides bound to self MHC molecules, to contribute to the immune response T cells must be able to identify the body's own MHC molecules. This is known as *self-restriction.* It is also

critically important that they do not react to self-peptides bound to self MHC molecules - they must be *self tolerant*. It is in the thymus that T cells are selected to fulfil these two requirements (Roitt 1997). Through *positive selection* cells are screened for self MHC restriction and by *negative selection* cells which react to self-peptides bound to self MHC are eliminated. Although extensive proliferation of T lymphocytes occurs in the thymus the rigorous selection they undergo means that most die there by apoptosis (programmed cell death). Only approximately 2% of the developing thymocytes (thymic lymphocytes) will leave the organ as mature T cells (Janeway and Travers 1994). Ingenious experimental work involving transgenic mice has demonstrated that positive selection of thymocytes depends on thymic cortical epithelial cell MHC expression (Cosgrove et al. 1992, Moller 1993) whilst bone marrow derived antigen presenting cells (APCs) are most important in driving negative selection (Kappler, Roehm and Marrack 1987). These APCs are also responsible for presenting antigen to mature T cells in the body.

A varied and interacting population of cells therefore exists in the thymus. Of these the thymocytes are by far the most numerous and at least in the cortical areas tend to obscure the other populations on conventional microscopic examination.

2.1.3) Tumours of the thymus.

As would be expected in any organ, tumours arising in the thymus can originate in any of the different cell types which make up that organ. In practical terms thymic tumours are likely to arise from the thymic lymphoid or epithelial cell populations. The relative prevalence and importance of these conditions in veterinary medicine is discussed below.

Thymic Lymphosarcoma

Thymic lymphosarcoma is particularly important in cattle where it is one of three major forms of Sporadic Bovine Leukosis (SBL). This condition is characterised by enlargement of the thymus by malignant lymphoid cells with variable involvement of the local nodes and bone marrow and is typically seen in animals less than three years of age, most frequently in those between six months and two years (Blood and Radostits 1989, Stober 1981). The other major manifestations of SBL are juvenile multicentric lymphosarcoma, which presents as multiple lymph node enlargement in animals less than six months old and the rarer cutaneous form where animals, usually between one and three years of age, develop plaques and nodules in their skin which regress and recur. Sporadic Bovine Leukosis is not associated with Bovine Leukaemia Virus (BLV) infection, the animals do not demonstrate antibodies to the virus and no viral DNA has been detected in the tumours (Ogawa et al. 1986).

Thymic lymphosarcoma is also an important disease in the cat. Lymphosarcoma (lymphoma) of all types is the most common tumour in this species accounting for

one third of all tumour cases (Jarrett 1990). Thymic lymphosarcoma is diagnosed over a wide age range but is more prevalent in young cats (Moulton and Harvey 1990). Presenting signs are almost always due to space occupying effects of the primary tumour as although metastasis may occur this appears to be a late event and comparatively less important in the clinical course. Thymic lymphosarcoma in the cat is strongly associated with Feline Leukaemia Virus infection, 80% of cases are FeLV positive (Jarrett 1990).

Thymic lymphosarcoma is recognised in the dog but is uncommon. Theilen and Madewell (1987) reported less than 5% of their canine lymphoma cases to be of this type.

Thymoma

Bovine thymomas are extremely rare with only a few cases in the literature reported in any detail (Altman and Streett 1968, Parker and Casey 1976, Momotani, Nakamura and Shoya 1981). In some of the older veterinary literature the term thymoma was used to denote any tumour of thymic origin which can hamper interpretation in the absence of histological description.

In contrast to the bovine situation the most common thymic tumour in goats is reported to be the thymoma. These growths are especially prevalent in dairy breeds. One American study found 25% of goats necropsied at over two years old to have clinically silent thymomas (Hadlow 1978). A series of thymoma cases have also been reported in the Angora goat (Streett et al. 1968).

In small animals there is a fairly extensive literature on thymic epithelial tumours. Thymomas are described in the dog (Robinson 1974, Bellah, Stiff and Russell 1983, Aronsohn 1985) and cat (Carpenter and Holzworth 1982, Martin et al. 1986, Gores et al. 1994) where they can be benign or malignant. Benign tumours may be asymptomatic or have space occupying effects but they are well encapsulated and do not invade locally. Malignant thymomas, however, spread by local invasion, transcoelomic seeding and through blood vessels and lymphatics with observed metastatic involvement of the lungs and liver. In human patients a number of autoimmune paraneoplastic syndromes are associated with thymoma and similar problems have been identified in the dog. Aronsohn et al. (1984) observed an autoimmune paraneoplastic syndrome in ten out of fifteen of a series of dogs with thymoma. Myasthenia gravis, hypercalcaemia and polymyositis have been identified in canine thymoma cases by several other authors (Darke, McCullagh and Geldart 1975, Harris et al. 1991, Klebanow 1992). Feline thymomas are associated with similar syndromes of myasthenia gravis (Scott-Moncrieff, Cook and Lantz 1990) and polymyositis (Carpenter and Holzworth 1982).

Classification of thymomas

Several systems of classifying primary thymic tumours have been proposed for use in the field of human medicine. These have ranged from a simple division according to whether or not the tumours were associated with myasthenia gravis (Iverson 1956) to complex systems based on embryological origin (Lewenhaupt 1948). However, most classifications are based on the microscopic appearance of the tumour with the broad categories of epithelial, lymphoid and teratomatous variously subdivided. The term thymoma is now exclusively used for epithelial tumours of the thymus and it is widely

agreed that this term should be restricted to tumours where the epithelial cells are cytologically benign (Castleman 1955, Levine and Rosai 1978, Rosai and Levine 1976) although the behaviour of these tumours may be benign or malignant. Where the cells are cytologically malignant the growths are designated thymic carcinomas (Henry 1992). The histological appearance of the thymomas reported in the veterinary literature is quite variable including clear cell variants (Mackey 1975, Gores et al. 1994) and cystic tumours (Gores et al. 1994). It is common to see a very variable cytological appearance in different fields of the same tumour. In human medicine, as the histological appearance of the cells is not a reliable reflection of biological behaviour, the appearance at thoracotomy of invasive growth or lack of it is the most important prognostic indicator (Batata et al. 1974, Walker, Mills and Fechner 1990).

Jarrett and Mackey (1974) in the World Health Organisation "International Histological Classification of Tumours of Domestic Animals" simply separate thymomas into two types; predominantly epithelial where the cells are mostly plump and elongate with abundant, faintly eosinophilic cytoplasm and predominantly lymphoid where the most abundant cells have the appearance of normal mature lymphocytes.

A fairly simple system which separates thymomas into mixed epithelial-lymphoid, predominantly epithelial, predominantly lymphoid, spindle cell and clear cell variants is probably most useful for these tumours in domestic animals in terms of histological description. No prognostic significance has been determined for these variants.

Differential diagnosis of thymic tumours

The essential criterion for diagnosis of a thymoma is the identification of a population of neoplastic epithelial cells. This can be problematic as these cells are always accompanied by lymphocytes. Even in metastases the association between neoplastic thymic epithelium and lymphocytes is maintained. It is suggested that the tumour cells provide a microenvironment in which T-lymphocytes are induced to differentiate along normal pathways much as they are in the normal thymus (Henry 1992). In many cases the accompanying lymphocytes are far more numerous than the epithelial cells and may obscure them possibly leading to misdiagnosis as thymic lymphosarcoma.

Previously, differential diagnosis between thymic lymphosarcoma and thymoma has relied on the recognition of a second population of cells, distinct from lymphocytes in conventionally stained preparations. The lymphocytes found in a thymoma will be cytologically normal in contrast with the neoplastic lymphoid cells of a thymic lymphosarcoma. It is recognised that the appearance of the epithelial cells in a thymoma may be variable and commonly recognised patterns, such as spindle-shaped cells, "rosette" patterns and whorls reminiscent of abortive Hassall's corpuscles recapitulate patterns recognised during thymic development and involution (Lattes 1962). Special histological stains have been employed to assist in the diagnosis of these tumours, for example staining for reticulin will tend to show no reticulin fibres between the cells (although this is not a constant finding) and the epithelial cells often contain PAS positive granules (Lattes 1962, Legg and Brady 1965).

Ultrastructural studies have also been carried out on these tumours. Using the electron microscope it is possible to demonstrate characteristically epithelial features; desmosomes and tonofilaments (Fukuda et al. 1992, Levine et al. 1975) in the

thymoma cells. The drawbacks to this method are obviously the expense and lack of general availability of the use of an electron microscope.

In the field of human diagnostic pathology immunohistochemistry has been used as an aid to the diagnosis of thymic tumours. Antibodies to keratin have been used to demonstrate the epithelial nature of the neoplastic cell population in thymomas (Battifora et al. 1980).

Although histologically the critical distinction which can prove most difficult may be that between a thymic lymphosarcoma and a thymoma, in the clinical situation other neoplastic processes which may present as a cranial thoracic mass must also be considered as differential diagnoses. These include ectopic thyroid or parathyroid tumours, chemodectoma or other heart base tumour and primary lung tumours.

The following work was performed to assess the potential of immunohistochemistry in veterinary diagnostic pathology with particular reference to its use as a tool for the reliable diagnosis of thymomas in domestic animals.

2.2.1) Antibodies for use in Veterinary Diagnostics

The purpose of this part of the study was to investigate the practical applications of immunohistochemistry in diagnostic veterinary pathology. To do this the array of commercially available antibodies were reviewed. A range of veterinary research antibodies available from Serotec, (Oxford, England), includes leukocyte differentiation antigens and immunoglobulins from several domestic species. However, these products are designed as research tools and are suitable in some cases for cryostat sections, flow cytometry, ELISA or immunoprecipitation but they are not effective on conventionally processed tissue. A wide range of antibodies which can be used on formalin fixed, paraffin embedded tissues are available from many sources (Dako, Glostrup, Denmark; Novocastra, Newcastle, England; Serotec, Oxford, England) but these are all designed for use on human tissue with a small number of exceptions in the form of products designed for use in experimental laboratory rodents. No data was available on the use of these antibodies in the veterinary context.

A review of the literature and the manufacturers data provided the starting point for the investigations which began on an empirical basis. The aim of the study was first of all to establish whether commercially available anti-human antibodies could be successfully applied to tissues from domestic animals and then to evaluate their possibilities in the particular field of tumour diagnosis.

Antibodies for investigation in this study were selected according to the following criteria:

i) applicability to routinely processed tissue

ii) of potential benefit in the diagnostic situation

Antigens of interest to the diagnostic pathologist

Of particular interest were the intermediate filament markers which can allow more accurate classification of morphologically undifferentiated or otherwise difficult neoplasms and the lymphoid subclass (Cluster of Differentiation, CD) markers which may be used to establish the character and clonality of lymphoid cell populations.

As described in Chapter 1 the immunohistochemical staining procedures are fairly laborious and antibodies can be expensive so it is not practical in the diagnostic situation to subject any doubtful tumour to a battery of immunohistochemical tests. In the field of medical pathology diagnostic algorithms have been designed to direct the application of immunohistochemical markers to difficult cases. The approach is to use a limited panel of the most useful antibodies and to take the microscopic appearance of the tissue in standard H&E sections as the starting point from which the pathologist uses his experience to determine what the tumour *may* be and decides which tests to employ. For example, to establish whether a poorly differentiated tumour is of epithelial origin (carcinoma) or mesenchymal origin (sarcoma) the tissues would be stained with cytokeratin and vimentin and examined. With a suitable range of antibodies the exact diagnosis may be pursued further. Figure 2 is an example of a simplified diagnostic algorithm and was prepared with reference to antibody specifities on human tissue as detailed in Jasani and Schmid (1993).

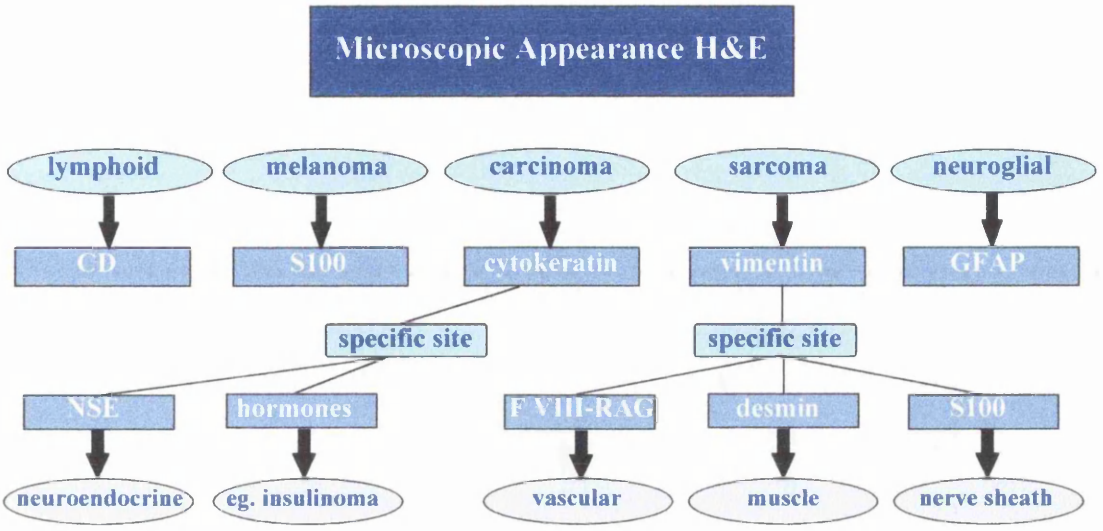

Figure 2. Diagrammatic algorithm illustrating how a panel of antibodies, including the intermediate filament markers, can be used in the identification of tumours. The tissue distribution of these markers is covered in the text to follow. See also Table 3.

2.2.2 Cytoskeletal Components

To understand the role of intermediate filaments in the cell and hence their significance in immunohistochemistry it is necessary to review intermediate filaments in the context of the cellular matrix.

Eukaryotic cells possess a complex array of filamentous proteins which constitute the cytoskeleton (Junqueira, Carneiro and Long 1986). The pattern of expression of these is tissue specific and determines the properties of each cell type. These proteins interact with both the nuclear and cytoplasmic membranes and are involved in providing mechanical support and determining cellular architecture, in cell motility, mitosis and differentiation, cytoplasmic translocation and in the coupling of cell surface receptors to cellular responses.

The major components of the cytoskeleton are: microfilaments (actin filaments)
intermediate filaments
microtubules (tubulin polymers)
(lamins - nuclear matrix proteins)

Microfilaments are present in all cells but are most abundant and well organised in muscle. These actin filaments are associated with myosin in all functions which involve cell motility, again this mechanism is best developed in skeletal muscle. Actin microfilaments form localised aggregates which are widely distributed in the cell. The filaments are labile, capable of rapid assembly (from a cytoplasmic monomer pool)

and disassembly (Toner and McCormick 1992). The assembly of actin polymers is controlled by an extensive family of actin binding proteins (Stryer 1988).

Microtubules are hollow, cylindrical structures of indeterminate length, formed from the assembly of macromolecular subunits of two similar 50 KDa protein subunits, α and β tubulin (Stryer 1988). These structures are also very labile, capable of lengthening by the addition of tubulin molecules. A state of equilibrium between assembly and disassembly may be established. Microtubule bundles form part of the mitotic spindle, are attached to individual chromosomes and control their movement during mitosis (Toner and McCormick 1992).

Intermediate filaments are mechanically strong, being polymerised from fibrous, not globular, protein subunits unlike actin filaments and microtubules. They are also chemically stable and highly insoluble (Steinert et al. 1982). Intermediate filaments are not believed to undergo rapid assembly and disassembly.

In addition, a family of nuclear proteins, the lamins, are ubiquitously present in cells. Within the lamin family are three main types of polypeptide which form part of the nuclear matrix and compose the fibrous lamina which is closely associated with the internal membrane of the nuclear envelope (Junqueira, Carneiro and Long 1986). Lamins have some sequence homology with intermediate filament proteins (Watt 1992). Lamin assembly is heavily modulated by phosphorylation and these proteins are likely to be important in chromatin organisation in interphase and nuclear envelope breakdown during cell division (Krohne and Benavente 1986).

Each of these filament types has been studied with regard to its possible pathological significance (Rungger-Brandle and Gabbiani 1983), but at present intermediate filaments appear to be of most interest and it is on these proteins that this discussion focuses.

The tumour protein expression profile usually reflects that of the cell of origin but exceptions exist (Corwin and Gown 1989). In some cases the unusual pattern of intermediate filament (co)expression in a tumour is of itself of diagnostic significance. For example, epitheloid sarcomas characteristically co-express cytokeratin and vimentin (Daimara et al. 1987).

2.2.3 Intermediate Filaments

Intermediate filaments are so named because at 7-11nm in diameter they are intermediate in size between microfilaments (5-7nm) and microtubules (22-25nm). They are long filaments formed by the polymerisation of rod-shaped coiled coil molecules. There are five major groups of cytoplasmic intermediate filament which although identical morphologically are biochemically and immunochemically distinguishable (DeLellis and Kwan 1988). Each of these intermediate filaments shows a characteristic pattern of cellular distribution which is outlined in Table 3.

Techniques utilising monoclonal antibodies and recombinant DNA have identified a multigene family of proteins that form the intermediate filaments (Franke et al. 1978, Lazarides 1982). The expression of these proteins appears to be controlled by individual genes and follows a regulated pattern of cell differentiation.

In the embryo, recognisable intermediate filaments (cytokeratins) begin to occur in the late morula/early blastocyst stage in association with the formation of nascent desmosomal structures (Jackson et al. 1980). Class switching of intermediate filament expression in embryonic cells occurs during their differentiation (Holtzer, Biehl and Holtzer 1985).

Intermediate Filament Classes and Tissue Distribution		
Intermediate Filament	Tissue	Molecular Weight (kd)
cytokeratin	**epithelial cells**	**40-68**
desmin	**muscle**	**53**
glial fibrillary acid	**glial cells**	**55**
vimentin	**mesenchyme**	**57**
neurofilament	**neurones**	**68, 160, 200**

Table 3. The distribution of intermediate filament expression in different cell types.

2.2.4 Immunohistochemistry of Intermediate Filaments

Immunohistochemistry for vimentin, desmin and glial fibrillary acid protein (GFAP) is relatively straightforward as these proteins are composed of a single type of polypeptide. Neurofilaments and cytokeratins are more complex. The former group has three major proteins and the latter at least nineteen. Immunohistochemistry of cytokeratin subclasses will be considered in greater detail in Chapter 3.

A large volume of work using recombinant DNA techniques has revealed that all the intermediate filament proteins have a central region approximately 310-350 amino acids long arranged in a repeating heptad sequence. This region has a coiled-coil structure produced by the interaction of neighbouring α-helices. This central rod domain is divided into four segments by non-helical regions (Weber and Geisler 1985). All classes of intermediate filament share two highly conserved consensus sequences at the beginning and end of the rod domain (Cadrin and Martinoli 1995). This region of the intermediate filament protein molecules is highly stable to autolysis and fixation-induced changes so in one sense is a useful target to raise antibodies against. Importantly however, because these regions are highly homologous between the different classes of intermediate filament such antibodies are prone to cross reaction. The exposed, non-helical amino terminal "head" and also non-helical carboxy terminal "tail" regions of the protein are more susceptible to the formation of cross linkages during fixation but are more specific to the intermediate filament class. These regions are highly variable in size and amino acid composition (Steinert, Steven and Roop 1985) and produce the immunological and functional specificity of the

different classes of intermediate filament. Antibodies raised against epitopes in these regions often require the use of cryostat sections to avoid changes due to fixation or, in the case of routinely processed samples, the application of enzymatic or high temperature unmasking techniques to restore antigenicity to these sites (see Chp. 1).

When undertaking immunohistochemical investigations into intermediate filament expression it is important to recognise the difference between true antibody cross reaction and intermediate filament co-expression. Some antibodies, both polyclonal and monoclonal recognise conserved epitopes and react with more than one and sometimes with all intermediate filaments. Equally some cells, for example some tumour cells, do genuinely express more than one class of intermediate filament (Daimara et al. 1987).

In doubtful cases when the tissue under investigation shows positivity to more than one intermediate filament marker, for example cytokeratin and vimentin, several strategies may be employed to ascertain what the true picture should be. First and most simply the investigator should use different antibodies to the same determinant. Using this panel approach it is possible to demonstrate whether the questionable intermediate filaments are persistently identified, which suggests genuine co-expression, or whether one particular antibody appears to be producing a spurious positive result which suggests cross reaction. Alternatively mRNA encoding intermediate filament proteins may be detected by *in-situ* hybridisation, demonstrating which proteins are being synthesised in the cell (Leube et al. 1986). Where strong staining for one intermediate filament is accompanied by weak, focal positivity for another cross reaction may be suspected, particularly if tissue pre-treatment has been

carried out, as it is recognised that proteolytic pre-treatment, whilst often necessary, can generate spurious staining results (DeLellis and Kwan 1988). It is, of course, necessary to interpret all staining with reference to appropriate positive and negative controls (as outlined in Chp. 1). With many sections, a useful internal control is provided by examining normal structures such as skin or muscle within the section and rejecting sections where these elements are inadequately or incorrectly stained.

Cell cycle dependant changes in intermediate filament structure and distribution have been documented. Some changes in intermediate filament organisation are visible ultrastructurally but rearrangements at the subunit protein level, invisible to the electron microscopist, may affect reactivity with monoclonal antibodies (Franke et al. 1983). This means that some antibodies which recognise an epitope only accessible during certain phases of the cell cycle, e.g. mitosis, may produce confusing results due to differential masking of the epitope rather than altered expression of the intermediate filament within the cell.

Another source of apparent conflict arises from the difference between antibodies for immunohistochemistry which must recognise the tertiary structure of proteins as they occur in the cell and antibodies for electrophoretic methods which require the denaturation of proteins. Antibodies are often raised against these denatured proteins and may react with epitopes unavailable in the cellular proteins (DeLellis and Kwan 1988). Although commercially produced antibodies should have been thoroughly tested for reliable performance in all the systems for which they are marketed, internal quality control is very important, especially when antibodies are being applied to non-human tissue for which little data is available.

2.2.5) Other important immunohistochemical markers.

Just as the five classes of intermediate filaments are of great importance in tumour identification so are several other groups of antigens. In particular, the lymphoid cell markers known as CD antigens which allow immunological subdivision of lymphoid neoplasms are significant.

An enormous number of lymphoid markers exist. These are mostly antibodies against determinants on human cells and are usually highly species specific. These antibodies are organised into defined groups according to the decisions of international workshops. The antibody categories are established on the basis of their behaviour in three technical systems:

1) immunofluorescent cell surface labelling by Fluorescence Activated Cell Sorter (FACS)

2) immunoprecipitation of labelled cell surface antigens followed by analysis of their molecular weights

3) immunofluorescent or immunohistochemical staining of histological sections

The tests are duplicated by independent laboratories before new antibodies are assigned to Cluster of Differentiation (CD) groups.

Many of these lymphoid antibodies are not applicable to routinely processed tissues as conventional processing destroys or alters the surface antigenic determinant which they recognise. The limited numbers of antibodies which are effective in fixed tissue are routinely used in human diagnostic pathology and include CD45 (LCA, Leukocyte

Common Antigen) a pan-leukocyte marker which identifies all cells of haematopoietic origin, CD20 a pan B-cell marker (Mason et al, 1990) CD3 a pan T-cell marker (Engelman et al 1981) and CD68 a pan macrophage marker (Falini et al 1993).

Other markers of particular diagnostic interest are CD4 (helper/inducer T-cell specific) and CD8 (suppressor/cytotoxic T-cell specific) and antibodies to these determinants which are effective in paraffin sections are now being marketed by Novocastra, Newcastle, England.

Whilst in human diagnostic pathology immunohistochemical investigation of lymphoproliferative disorders is becoming an established and invaluable adjunct to classical morphological examination in the veterinary field the use of lymphoid markers is still very much restricted to specialised research applications. Only a few of the anti-human leukocyte antigen markers are of use in domestic animal species. The results of some investigations into the application of commercially available lymphoid markers in domestic animal tissues are presented later in this chapter.

Markers such as S-100, a protein expressed by tissue of neural crest origin which is particularly useful in detecting amelanotic melanomas, *neuron specific enolase* (NSE), an isoenzyme found in primarily in neurons and neuroendocrine cells and Factor VIII related antigen (FVIII-RAG)/von Willebrand factor which is expressed on endothelial cells and megakaryocytes are all potentially useful in the diagnostic context although they are not directly relevant to the study presented in this chapter.

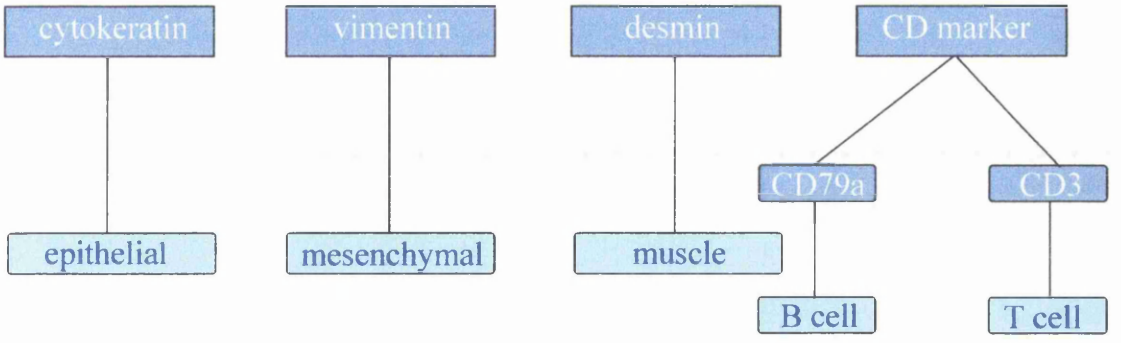

Figure 3. Simple diagramatic algorithm illustrating the selected range of immunohistochemical markers applied to the tissues in this study.

2.3.1) Materials and Methods

The archive records of the Department of Pathology were examined for possible thymoma cases and the stored microscope slides and blocks were retrieved. Where necessary fresh H&E stained sections were prepared and the tumours were examined by routine light microscopy. Tissues judged to be severely autolytic were rejected.

Although the figures in Table 4 relate to the computerised records of the hospital system (dating from 1988) from which it is possible to obtain numerical totals some older cases of thymic tumours and some from external sources were available and these were also considered.

Six canine, three feline, one bovine and one caprine putative thymomas were examined. The antibody panel selected is illustrated in Figure 3. In addition sections of normal thymus from a young pup, a kitten and a young calf were processed using the same protocol. To provide further control sections for the lymphoid markers bovine and human tonsil and canine and feline lymph node were also stained with these antibodies.

Immunohistochemical Method

Formalin fixed, paraffin embedded sections of the eleven tumours and the normal control tissues were cut at 5μm onto microscope slides coated to promote tissue adhesion (APES: 3-aminopropyltriethoxysilane). This is necessary for sections which are to undergo the fairly vigorous and prolonged processing involved in immunohistochemistry.

The following antibodies were used on the tissue sections:

CYTOKERATIN Dako, Glostrup, Denmark
MNF116, mouse anti-human monoclonal, isotype IgG1 kappa
This antibody recognises cytokeratin sub-families 5,6,8,17,19 and has a broad pattern of reactivity in epithelia. It may be used to detect normal and neoplastic cells of epithelial origin.

VIMENTIN Dako, Glostrup, Denmark
V9, mouse anti-swine monoclonal, isotype IgG1 kappa
This antibody cross reacts with human vimentin and labels cells of mesenchymal origin.

DESMIN Dako, Glostrup, Denmark
DE-R-11, mouse anti-swine monoclonal, isotype, IgG1 kappa
This antibody is prepared using antigen purified from swine stomach and cross reacts with human desmin. It labels smooth and striated muscle.

CD3 Dako, Glostrup, Denmark
rabbit anti-human polyclonal
This antibody is produced using synthetic human CD3 peptide coupled to bovine serum albumin as an immunogen. It is used as a pan-T cell marker in the detection of normal and neoplastic T cells.

CD79a Dako, Glostrup, Denmark
HM57, mouse anti-human monoclonal, isotype IgG1 kappa
This antibody is raised using synthetic human CD79a peptide as an immunogen and detects an intracytoplasmic epitope in both normal and neoplastic B cells. It is known to cross react with B cells of many mammalian species.

Within the laboratory in the Department of Pathology we have considerable experience in using the cytokeratin, vimentin and desmin antibodies listed above. These have been extensively tested in the laboratory on a wide range of species and are known to work reliably on animal tissue. The use of the lymphoid markers is a newer development hence the requirement for staining human control tissue to ensure adequate processing and use of the domestic animal lymphoid tissue where the distribution of B and T lymphocytes is known as controls.

Enzymatic pre-treatment (see Chapter 1) using 0.1% trypsin with 0.1% calcium chloride in tris-buffered saline for thirty minutes at room temperature was employed

to unmask antigenic sites before the use of the cytokeratin and desmin antibodies. In the case of the CD3 and CD79a antibodies heat mediated antigen retrieval using a pressure cooker (as described in Chp.1) was employed. For this 1600ml of citrate buffer was brought to the boil in a pressure cooker with the lid unlocked. The sections were immersed in the boiling liquid, the lid locked and the appliance allowed to pressurise (approx. 4min). The sections are then held at pressure for one minute. The vimentin antibody is effective on paraffin section without pretreatment.

Subsequent quenching of endogenous peroxidase activity (1.5% hydrogen peroxide in methanol for ten minutes) was carried out.

After this the slides were placed in a humidity chamber and the tissue sections ringed using a PAP pen (Miles Ltd, Stoke Poges, England) which produces a waxy line and creates a small well around the section into which solutions may be pipetted. At this stage all the sections were covered with PBS buffered pre-immune serum from the same species in which the secondary antibody was raised. This helps to prevent non-specific binding of the antibody to immunologically "sticky" sites and hence reduces background staining. In this case rabbit serum was used and the sections incubated for thirty minutes.

Incubation with the antibodies listed above was carried out for sixty minutes at room temperature in the humidity chamber. Following test serial dilutions the cytokeratin and desmin antibodies were used at a dilution of 1:50, the vimentin at 1:40, the CD3 at 1:200 and the CD79a at 1:500. During this time the negative control samples continue to incubate in the pre-immune serum.

Following antibody incubation the sections were rinsed in TBS and processed according to a standard avidin-biotin peroxidase detection method (Vectastain, Vector Laboratories, Peterborough, UK.). The biotinylated secondary antibody (rabbit anti-mouse IgG) was applied and the sections incubated for thirty minutes.

A further five minute rinse was followed by thirty minutes incubation with an avidin-biotin-peroxidase complex, a similar rinse and incubation with a peroxidase substrate solution.

DAB (3,3-diaminobenzidine) was used as the chromogen and the sections were counterstained with Meyer's haematoxylin. A ten minute incubation with the DAB was found to be adequate. The negative controls where the primary antibody was replaced by buffered serum were processed with the test sections.

2.4.1) Results

Archival Survey

When the archive of the Pathology Department at Glasgow University Veterinary School was reviewed for cases of thymic neoplasia thymic lymphosarcoma was by far the most common thymic neoplasm encountered. The results of the survey of the computerised database (which dates back to 1987) for canine, feline and bovine tumours are presented in Table 4. Examination of the records for caprine cases revealed only twenty cases in total and the only neoplasm identified in this group was the single thymoma described (Case 11).

The age distribution of cases of bovine thymic lymphosarcoma diagnosed in the Department of Pathology at Glasgow Veterinary School is in close agreement with published figures (see 2.1.3) and is illustrated in Figure 4 whilst the ages of the published thymoma cases (where supplied) are shown in blue in Figure 5. The purple marker in this figure represents the bovine thymoma described in this series (Case 10) which is the only such tumour recorded in the Pathology Department archives.

Figure 6 graphically displays the age distribution of feline thymic disease. Thymic lymphosarcoma has been seen at GUVS very much as a disease of young cats with the vast majority of cases being two years old or less. Nine cases of thymic lymphosarcoma included in Table 4 were from animals of unrecorded age. Of the three thymomas two were diagnosed on aged cats, the third cat was six years old.

In Figure 7 the age range of the canine thymic tumour cases is similarly illustrated. The pattern of disease in this species is quite different with the peak incidence of

thymic lymphosarcoma in mature adult dogs and a greater overlap in age at presentation for lymphosarcoma and thymoma. As can be clearly seen from Table 4 thymomas in the dog account for a far greater proportion of thymic neoplasms than is the case in the cat. One canine thymoma case was from an animal of unrecorded age.

Pathology Database					
	All Tumours	**All Thymic Tumours**	**Thymic Lymphosarcoma**	**Thymoma**	**Other thymic tumours**
Dog	**4086**	**36**	**24**	**9**	**3**
Cat	**705**	**57**	**54**	**3**	**0**
Cattle	**867**	**59**	**58**	**1**	**0**

Table 4. Numbers of thymic tumours in the computerised record of Glasgow Veterinary School. Of the canine tumour cases 0.59% were thymic lymphosarcomas, 0.22% were thymomas. The feline tumour set contained 7.66% thymic lymphosarcomas and 0.42% thymomas and the bovine tumours included 6.67% thymic lymphosarcomas and 0.11% thymomas. See also Figures 3, 4, 5 and 6.

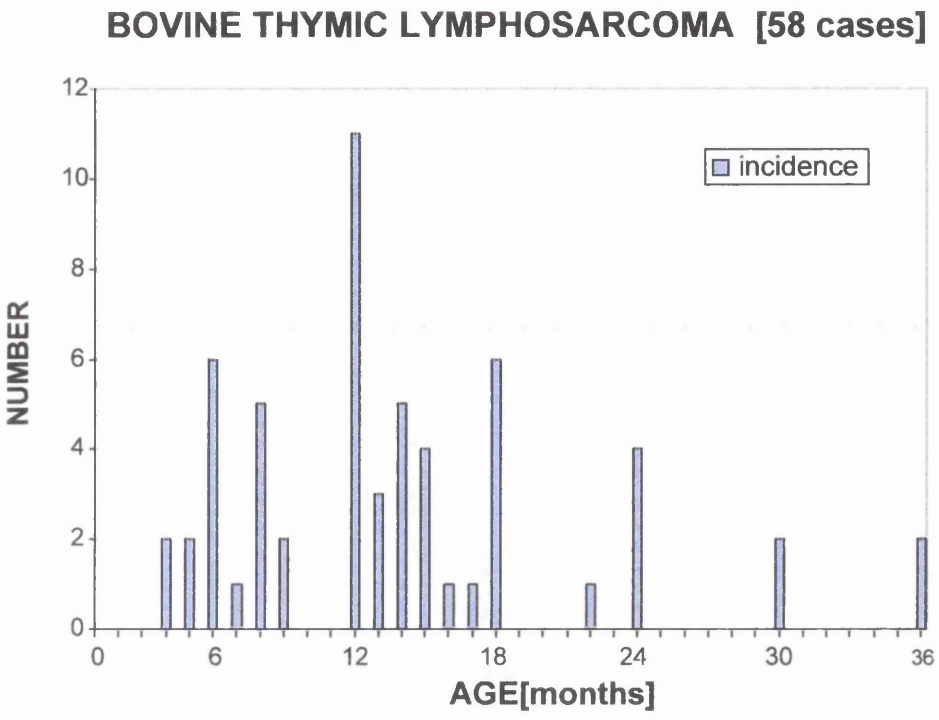

Figure 4. Age distribution of bovine thymic lymphosarcoma cases seen at Glasgow University Veterinary School.

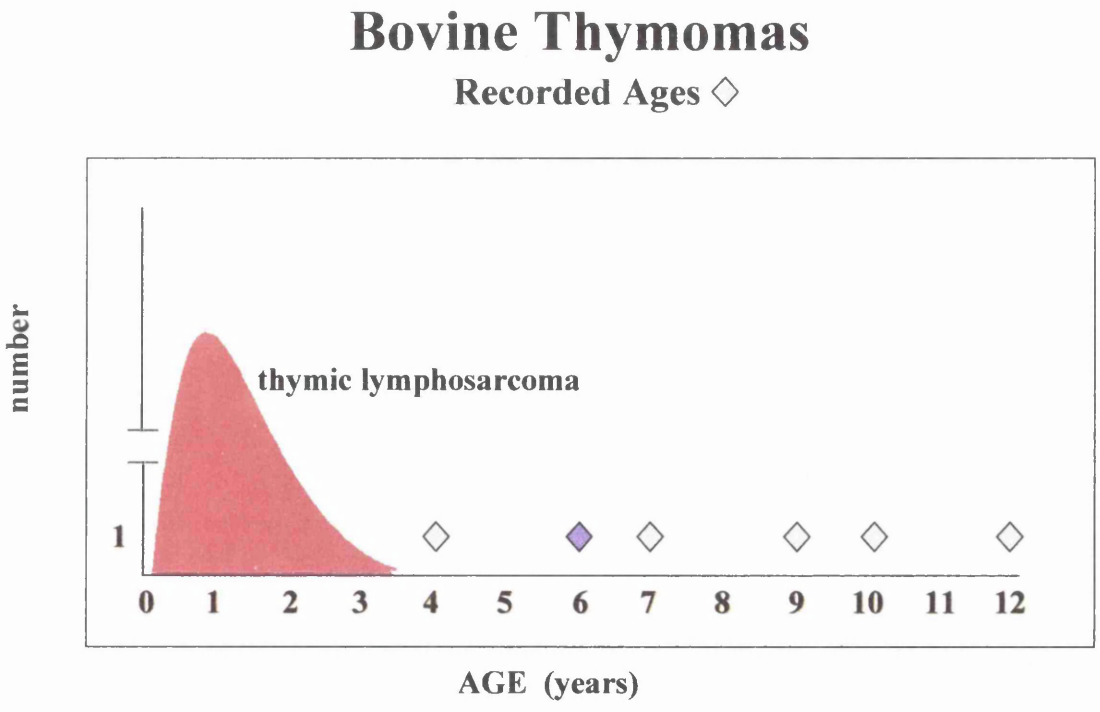

Figure 5. Ages of thymoma cases (where recorded) in comparison with age distribution of thymic lymphosarcoma cases from Pathology Department archive.

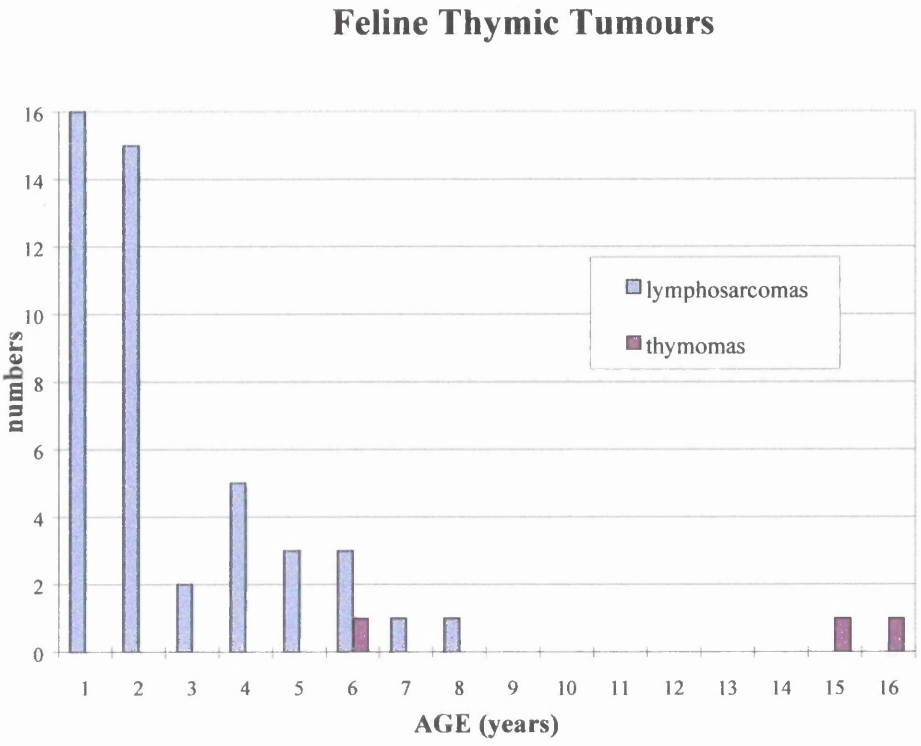

Figure 6. Graph of feline thymic tumour cases against age (where recorded).

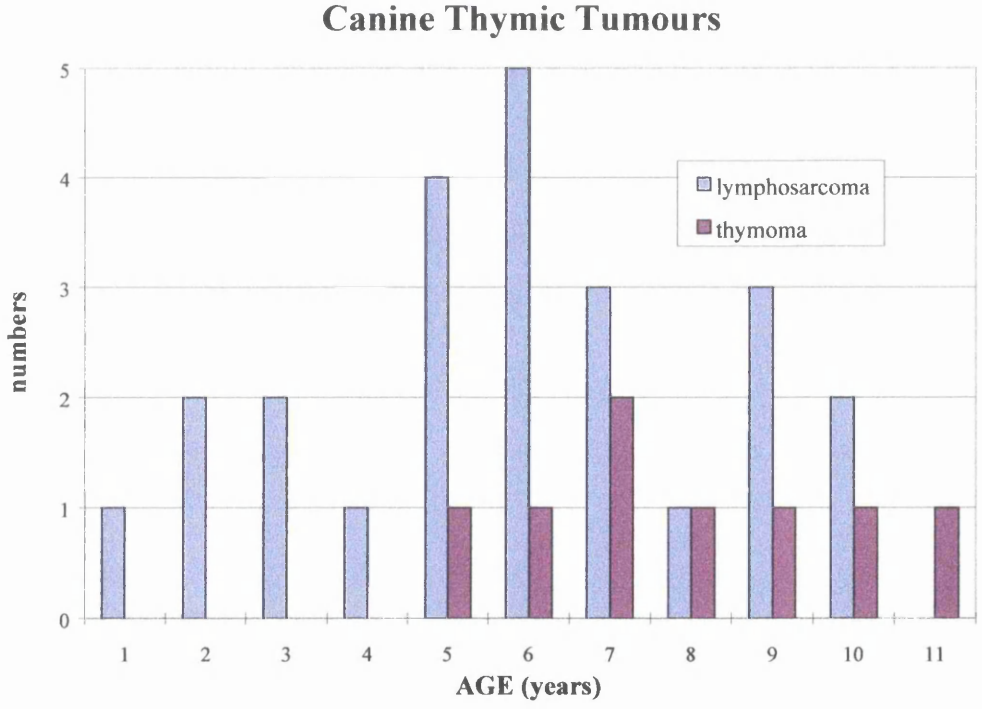

Figure 7. Graph illustrating the rather different age distribution patterns of the two types of thymic neoplasia examined in the dog.

2.4.2) Results of immunohistochemical staining and case details

Standard H&E sections from the thymus of a young dog, a kitten and a calf were prepared and examined. In each the organ was densely cellular with clear lobulation and corticomedullary distinction. The cortex appeared to be composed almost entirely of closely packed lymphoid cells and the medulla was less densely cellular with some larger epitheloid cells visible along with the thymocytes (Figure 8).

The normal thymus sections were also stained with the broad spectrum cytokeratin antibody as described in the method. This effectively highlighted the cortical epithelial cells which are otherwise obscured by the lymphoid population. In the thymic medulla the epithelial population is also stained by this technique. The DAB chromogen gives rise to a brown pigment in the positive areas and this can be seen against the blue haematoxylin background stain in Figure 9.

When stained with the CD3 antibody there was little positivity amongst the immature thymocytes of the cortex. Towards the medulla more cells stained but the results were rather pale. Small numbers of CD79a positive B cells were identified in the cortex.

In both the human and animal tonsil and lymph node controls there was strong staining of the cells of the paracortex and scattered cells of the follicular germinal centres with the CD3 antibody. Staining of the follicular mantle, some cells in the germinal centre and scattered cells throughout the paracortex was produced with the CD79a antibody.

See Tables 5 and 6 for clinical, histological and immunohistochemical case details.

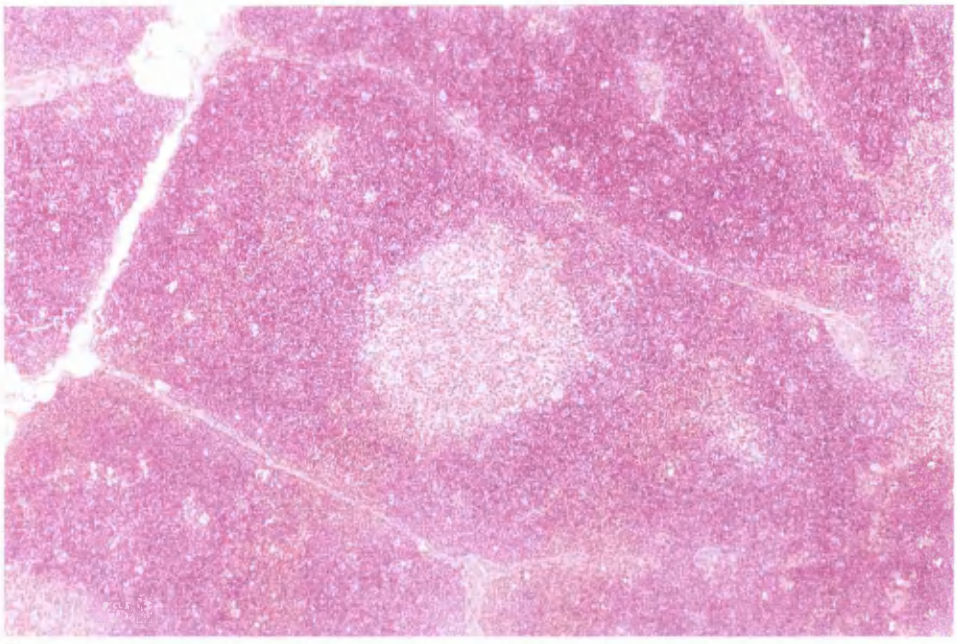

Figure 8. Normal calf thymus. Note the distinct lobulation, densely cellular cortex and paler medulla in centre of lobule. H&E (medium power)

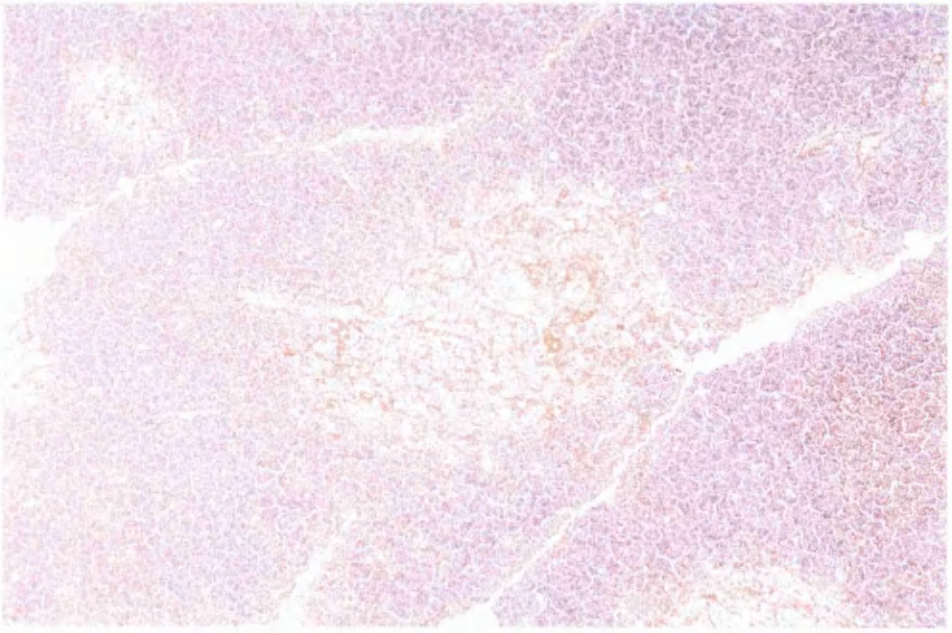

Figure 9. Normal calf thymus. The finely dispersed brown staining highlights the inconspicuous epithelial cells of the cortex whilst the medulla shows more prominent positivity for the epithelial marker. Broad-spectrum cytokeratin (medium power)

	Species	Breed	Age (y)	Sex	Histological Description	Clinical Features of Thymoma
Case 1	Canine	G. Retriever	11	FN	predominantly lymphoid	incidental finding at necropsy
Case 2	Canine	X-bred	7	M	predominantly epithelial	cough, regurgitation
Case 3	Canine	G. Retriever	10	F	mixed clear cell & epith.	regurgitation, dyspnoea,
Case 4	Canine	I. Setter	9	M	mixed lymphoepithelial	cough, dyspnoea
Case 5	Canine	Boxer	5	MN	resembled epithelial	dullness,occasional dyspnoea, V+D
Case 6	Canine	Lab. Ret.	8	M	mixed lymphoepithelial	sudden onset respiratory distress
Case 7	Feline	DSH	7	MN	predominantly lymphoid	cough, tachypnoea, hyperpnoea
Case 8	Feline	DSH	-	MN	mixed lymphoepithelial	detected at routine examination
Case 9	Feline	DSH	16	FN	mineralised, vascular	oedema, pleural effusion, CCF
Case 10	Bovine	Ayrshire	6	F	predominantly epithelial	cervical swelling, tachycardia
Case 11	Caprine	Saanen	4	F	mixed lymphoepithelial	chronic wasting

Table 5. Table listing the case details, histological types and clinical findings in the eleven putative thymomas examined in this study.
V+D - vomiting and diarrhoea
CCF - congestive cardiac failure

Case 1.

Case one was an eleven year old female neutered Golden Retriever. She was suffering from a hepatocellular tumour and had just begun chemotherapy when she died in renal failure. The thoracic mass was discovered *post mortem*. At necropsy the animal was in poor body condition and ascitic. The liver had a hepatocellular tumour in the right middle lobe and was chronically congested. The thymic mass was roughly tubular and 12x2x2cm in size. There was no significant cardiac pathology and the thymic mass was judged to be causing venous compression. The renal changes were those of acute tubular nephrosis and may have been related to chemotherapy.

Examination of the H&E section showed a vascular mass of moderate cellular density. The cells were of variable appearance, round to spindle shaped with variably sized vesicular nuclei. Intermixed with these cells was a population of smaller lymphoid cells. Sirius red staining revealed abundant stromal collagen.

Immunohistochemical examination with anti-cytokeratin antibody showed small nests of epitheloid cells to be very strongly stained (Figure 10). Many spindle cells were stained with vimentin (stromal fibroblasts). The tumour was uniformly negative for desmin and CD79a whilst very numerous CD3 positive cells were present throughout the section.

The diagnosis in this case was confirmed to be thymoma.

Case 2.

Case two was a seven year old male crossbred dog which presented with a cough and a history of regurgitation. The dog was deteriorating clinically and megaoesophagus was detected so euthanasia was performed. At necropsy a firm, lobulated red mass 6x5x5xcm in size was discovered just cranial to the heart. The oesophagus was dilated cranial to the level of this mass.

The H&E section from this tumour showed an extremely vascular tissue composed of mainly spindle shaped cells with oval to fusiform hyperchromatic nuclei forming a densely cellular network around numerous blood filled channels.

Immunohistochemical staining resulted in almost all cells staining positive with the cytokeratin marker (Figure 11). Only an occasional cell stained positive with vimentin and the section was completely negative for desmin and CD79a. Immunostaining with the CD3 marker highlighted a population of T-lymphocytes which were more difficult to identify in conventional sections.

This case was confirmed to be a thymoma.

Case 3.

Case three was a ten year old female Golden Retriever who presented with a history of regurgitation, dyspnoea, tachypnoea and exercise intolerance. A diagnosis of mediastinal neoplasia was made and the dog was euthanased. At necropsy a firm, white, nodular mass with cystic areas 18x15x12cm in size was found in the mediastinum. The left cranial lung lobe was extensively adhered to the mass and the thoracic oesophagus was flaccid and thin walled.

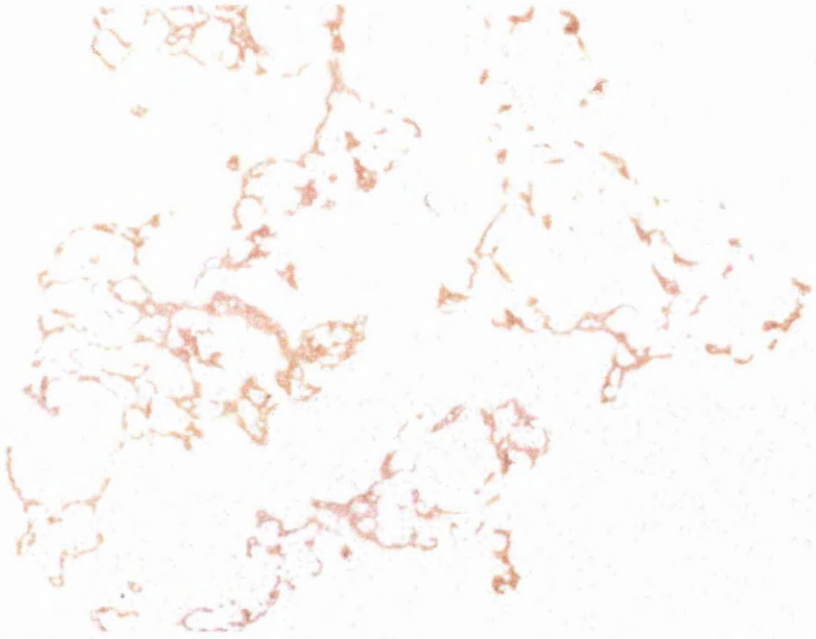

Figure 10. Canine thymoma (case 1). Note groups of strongly stained epithelial cells. Broad-spectrum cytokeratin (medium power)

Figure 11. Canine thymoma (case 2). Almost all cells in this mass are stained. Broad-spectrum cytokeratin (medium power)

Microscopic examination of H&E stained sections showed an encapsulated mass divided by connective tissue septae and containing some cystic areas. The cells were of variable appearance, there were large numbers of clear cells and streams of cells with round nuclei and densely eosinophilic cytoplasm.

Immunohistochemically, most cells in this mass stained positive with cytokeratin. Many, mostly spindle shaped cells stained positively with vimentin, particularly in the connective tissue septae. No desmin positivity was detected but small foci of CD79a positive cells with a granular staining pattern were seen. Large numbers of CD3 positive cells were present throughout the section.

Case three was also confirmed as a thymoma.

Case 4.

Case four was a nine year old male Irish Setter first presented nine months before euthanasia with a two month history of coughing and dyspnoea. Thoracic tumours were diagnosed radiographically and the dog was managed on diuretic therapy to control the associated hydrothorax. Thoracotomy was performed and a loculated cystic tumour observed in the anterior thorax and the dog euthanased. At necropsy the thoracic tumour was found to be very large, filling the anterior thorax completely and displacing the lungs dorsally and caudally. The mass was lobulated, mostly composed of firm white tissue but with cystic haemorrhagic areas.

On H&E section this densely cellular mass was shown to be encapsulated and divided by connective tissue septae. A mixed population of lymphoid and larger round cells with eosinophilic cytoplasm was observed (Figure 12).

With cytokeratin immunostaining the dominant larger cell population was strongly positive (Figure 13). Occasional vimentin positive cells were observed, no desmin or CD79a positivity was detected and very large numbers of lymphoid cells were CD3 positive.

The immunohistochemical results confirmed the diagnosis of thymoma.

Case **5.**

Case five was a five year old neutered male Boxer. He presented with a history of dullness, vomiting, diarrhoea and occasional dyspnoea over one to two months. Ultrasound examination revealed a thoracic mass and pleural effusion. The dog was euthanased. At necropsy he was found to be in good body condition and to have an anterior mediastinal mass displacing the heart and lungs. The mass was firm and white with haemorrhagic areas and surrounded the trachea and oesophagus.

Microscopic examination of H&E sections of this mass showed a moderately heavy stroma dividing sheets of round cells with large round nuclei and moderate amounts of eosinophilic cytoplasm.

These cells were uniformly negative for cytokeratin and desmin expression. Many spindle cells were vimentin positive and scattered CD79a positive cells were present in some areas. There were widely distributed CD3 positive cells.

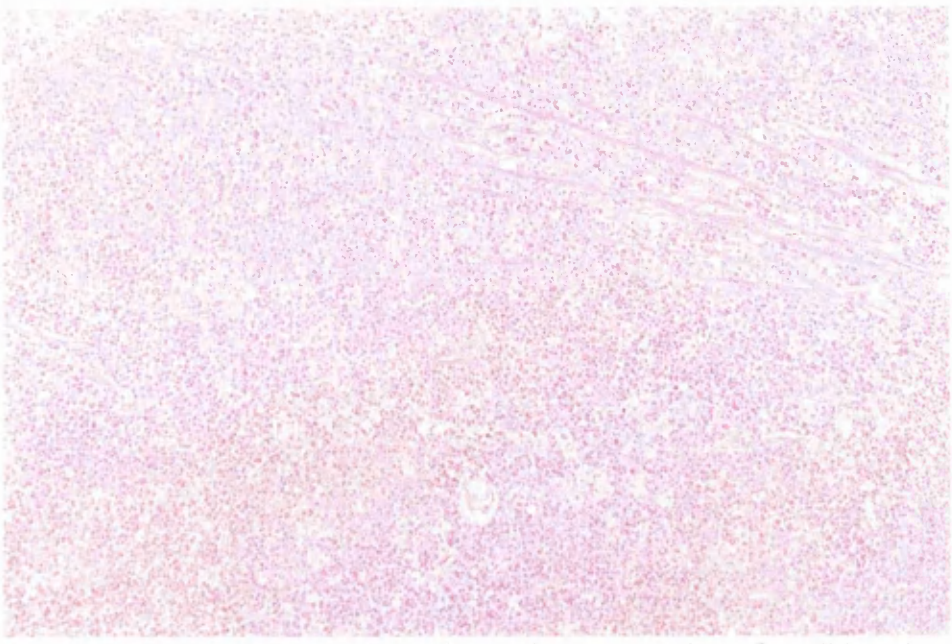

Figure12. Canine thymoma (case 4). A mixed population of small lymphocytes and larger eosinophilic cells can be seen. H&E (medium power)

Figure 13. Canine thymoma (case 4). With cytokeratin immunostaining there is widespread positivity of the larger cells. Broad-spectrum cytokeratin (medium power)

As the cells of this mass were negative for cytokeratin the original diagnosis of thymoma was found to be incorrect (see discussion).

Case 6.

Case six was an eight year old male Labrador Retriever which presented with sudden onset respiratory distress. Ultrasonic and radiographic examination showed a very large thoracic mass caudally displacing the heart. The dog was euthanased without further investigation or treatment. At necropsy the large thoracic mass was found to be firm and white with central haemorrhage and areas of necrosis. The heart was caudally displaced and the lungs partially collapsed. A small quantity of fluid was present in the pleural cavity.

On H&E section the mass was clearly composed of a mixed population of cells containing large paler staining epithelial-type cells and numerous small lymphocytes (Figure 14).

Immunohistochemical staining with cytokeratin effectively highlighted the population of larger cells, confirming their epithelial origin (Figure 15). Vimentin and desmin immunohistochemistry yielded negative results. Staining with the T cell marker CD3 produced positivity in many, but not all of the small lymphocytes (Figure 16). These negative cells were also unstained with CD79a as shown in Figure 17 (see 2.5.1).

Case six was confirmed as a thymoma.

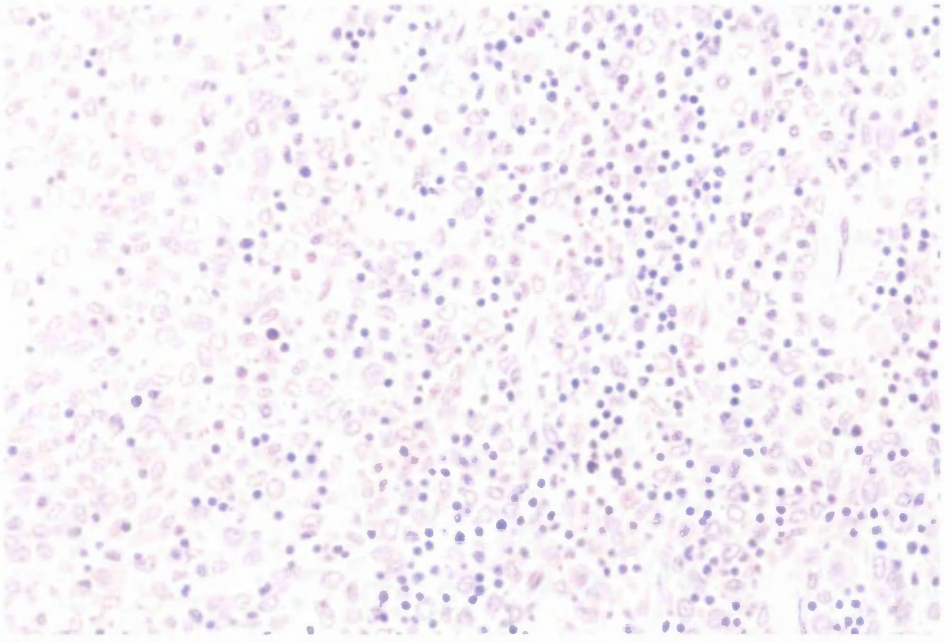

Figure 14. Canine Thymoma (Case 6). The mass is composed of a mixture epitheloid cells and smaller normal lymphocytes. H&E (high power)

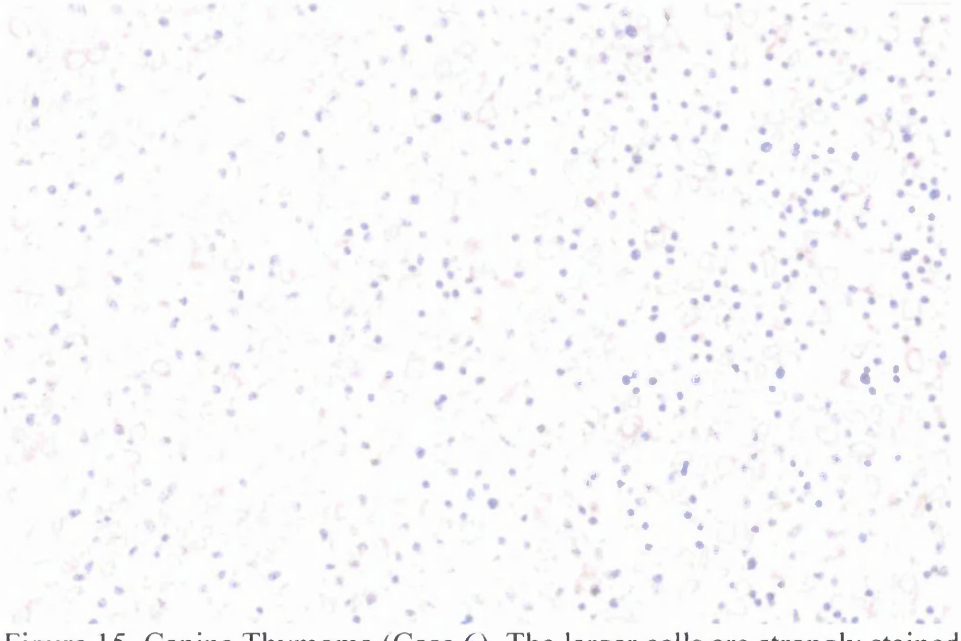

Figure 15. Canine Thymoma (Case 6). The larger cells are strongly stained whilst the lymphocytes are unstained. Broad spectrum cytokeratin (high power)

Figure 16. Canine Thymoma (Case 6). Many, but not all the lymphocytes, are positive with this T-cell marker. CD3 (high power)

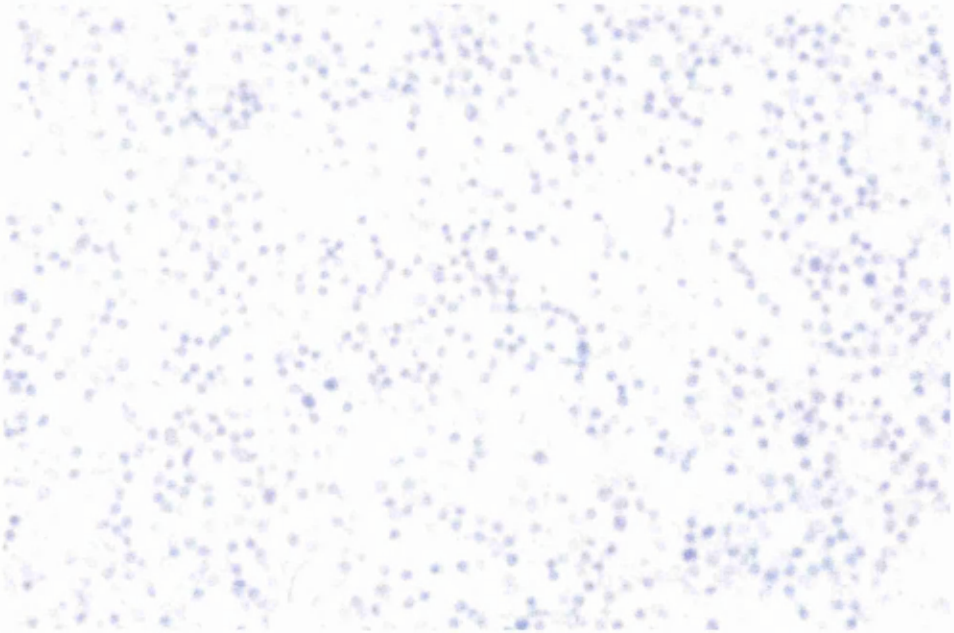

Figure 17. Canine Thymoma (Case 6). No cells are positive with the pan-B marker. CD79a (high power)

Case 7.

Case seven was a seven year old male neutered Domestic Shorthair cat which presented with a three month history of coughing, tachypnoea and hyperpnoea. Minor weight loss was also reported. Radiologically a thoracic mass was visualised. Thoracotomy was performed and the mass removed and submitted for histological examination. The mass was firm, white and lobulated measuring 7x4x4cm with a cystic centre.

On H&E section this mass was composed of admixed sheets of lymphoid and larger round cells which tended to aggregate together. Some fields were composed almost exclusively of lymphoid cells.

On immunohistochemical examination with cytokeratin there were numerous interlinked cords and clusters of strongly stained cells against a background of unstained lymphoid cells. The tissue was negative for desmin, had very few vimentin positive cells and widely distributed CD79a positive cells. These, however, were greatly outnumbered by CD3 positive cells.

This case was also confirmed to be a thymoma.

Case 8.

Case eight was a neutered male Domestic Shorthair cat of unrecorded age. When the cat was presented for routine vaccination the veterinary surgeon noticed displacement of the cardiac sounds with no thoracic fluid apparent. Radiography confirmed the

presence of a cranial thoracic mass and a needle biopsy was taken and sent to the Department of Pathology.

H&E sections of this good quality needle biopsy showed small fragments of tissue composed of admixed lymphoid and larger oval to spindle shaped cells with oval nuclei (Figure 18).

On immunohistochemical staining with cytokeratin the larger cells were strongly stained with cytokeratin and the lymphoid cells were clearly unstained (Figure 19). The sample was negative for vimentin and desmin. Foci of CD79a positivity were identified and numerous, strongly stained CD3 positive cells were present.

The biopsy diagnosis of thymoma was demonstrated to be correct for this case.

Case 9.

Case nine was a sixteen year old female neutered Domestic Shorthair cat which was on treatment for hyperthyroidism (carbimazole). The animal developed massive oedema and pleural effusion and was euthanased with a diagnosis of congestive cardiac failure. Post mortem examination revealed a very thin carcase with turbid, malodorous fluid in both the thoracic and abdominal cavities. A calcified mass was present in the mediastinum. Its origin was uncertain. Pasteurella multocida was cultured from the thoracic fluid.

This necropsy specimen had large amounts of mineralisation which had produced scoring when the sections were cut. Sheets and shreds of vascular, densely cellular tissue composed of spindle shaped cells with round to oval nuclei were observed.

This tissue was completely negative for cytokeratin. When stained with the vimentin antibody moderate numbers of cells were positive. No CD79a positivity was present but CD3 positive cells were numerous in some areas.

As no cytokeratin positivity was demonstrated in this mass the diagnosis of thymoma was rejected.

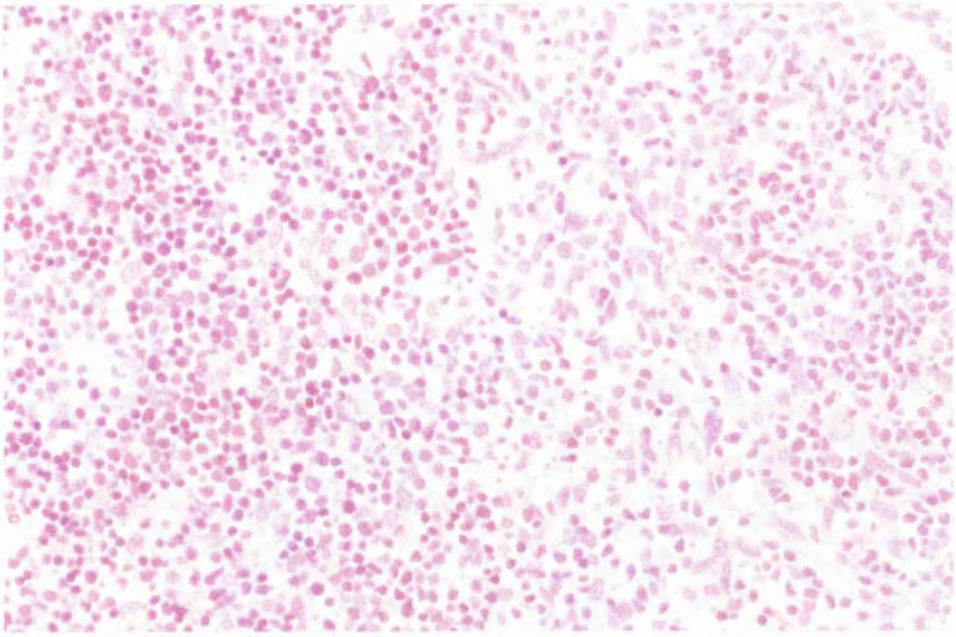

Figure 18. Feline thymoma (case 8). Note the mixture of lymphoid cells and larger, paler staining epithelial cells. H&E (medium power)

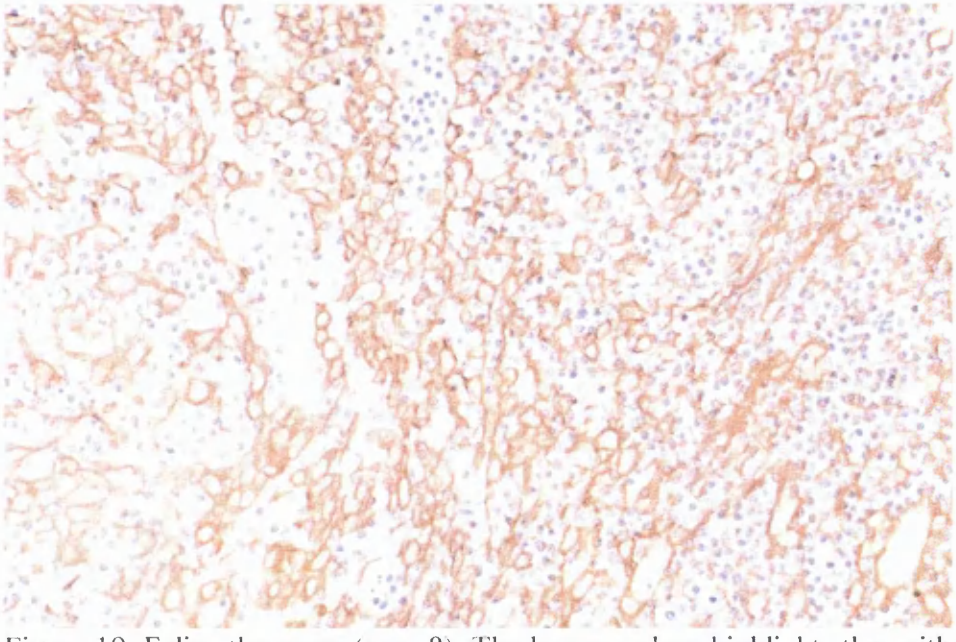

Figure 19. Feline thymoma (case 8). The brown colour highlights the epithelial cells whilst the lymphocytes are stained blue by the haematoxylin counterstain. Broad-spectrum cytokeratin (medium power)

Case 10.

Case ten involved a six year old Ayrshire cow with a six week history of neck and foreleg swelling unresponsive to treatment with antibiotics and corticosteroids which was referred with an initial diagnosis of Adder bite. The cow was non-pregnant and nearing the end of lactation. Her rectal temperature was 38.6°C, heart rate 80bpm with the cardiac sounds muffled and caudally displaced and respiratory rate 20pm with an occasional cough. There was a poorly defined, palpably irregular mass at the thoracic inlet, pre-sternal oedema and jugular distension. No sign of thoracic pain was evident on percussion. The prescapular lymph nodes were enlarged but other palpable nodes were of normal size. On arrival she had diarrhoea and later developed rumenal bloat. A tentative diagnosis of thymic neoplasia was made and the cow was euthanased due to a worsening tendency towards rumenal bloating. At necropsy the animal was found to be in reasonable body condition. Figure 20 shows her thoracic viscera plus the firm yellowish white, nodular thymic mass of 40x35x30cm in size which was compressing and caudally displacing the heart and lungs. Tumour tissue extended into the cervical and mediastinal lymph nodes which were massively enlarged and over the pleural surface of the first five ribs. The tumour mass was located in the position of the thoracic thymus and no normal remnant of this organ could be detected. There was marked oedema with lymphatic dilatation and perilymphatic fibroplasia in the presternal area. In the abdomen the rumen was found to contain a small amount of dry, firm material and to be massively distended with free gas.

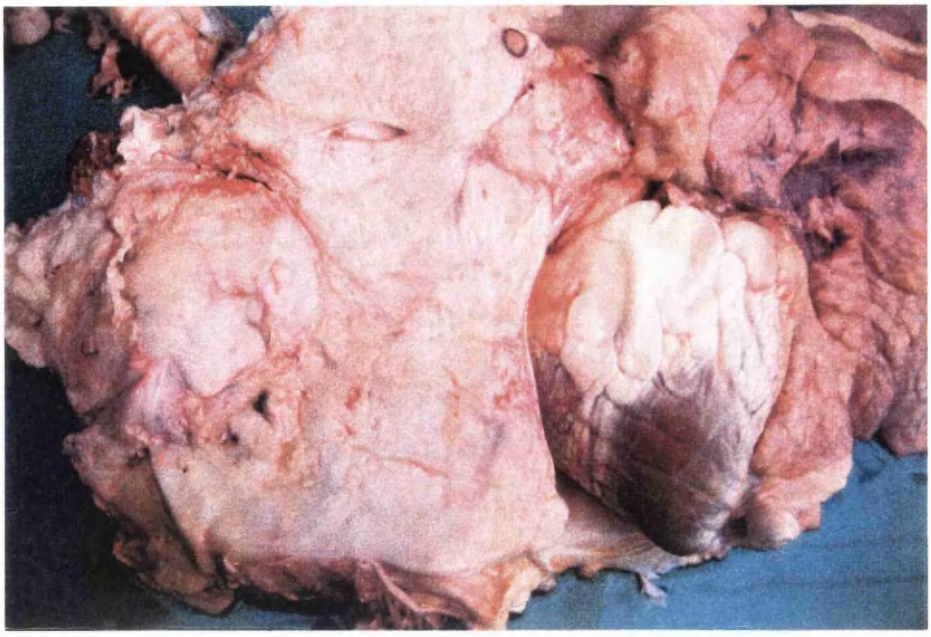

Figure 20. Thoracic viscera from case 10. Note large yellowish mass cranial to heart (cut surface shown). A small area of necrosis is present near the top edge.

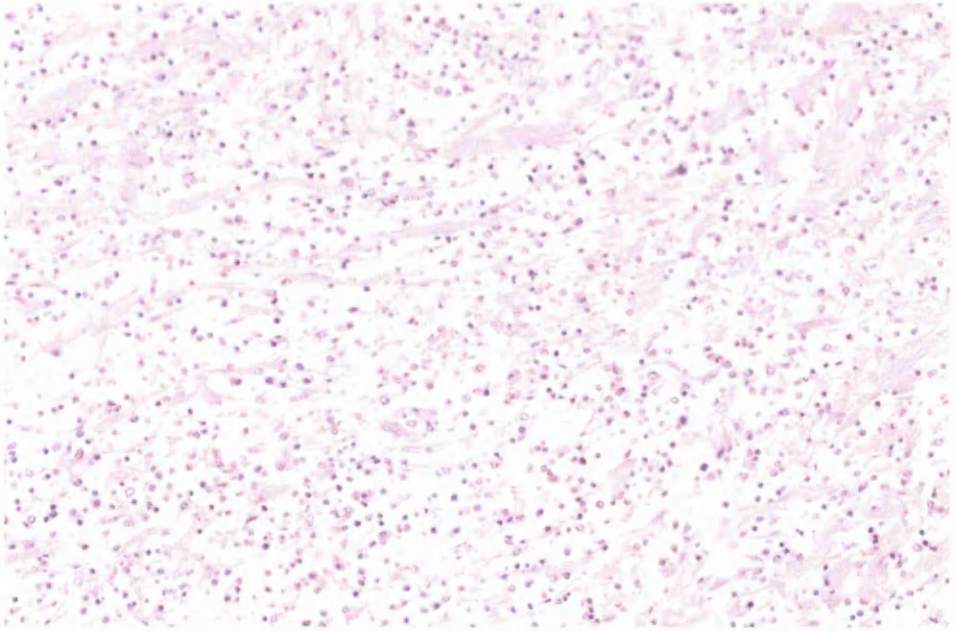

Figure 21. Bovine thymoma (case 10). The mass shows variably sized cells in a heavy connective tissue stroma. H&E (medium power)

On H&E section this mass was moderately cellular with prominent stromal collagen. There was a mixture of cell sizes, the smaller cells had round dense nuclei and the larger ones vesicular nuclei with indistinct cytoplasm (Figure 21).

When examined with anti-cytokeratin antibody the mass showed widespread positivity with a degree of background staining (Figure 22). With vimentin a population of spindle cells stained positive whilst the vast majority of cells remained unstained. Desmin staining was negative whilst a few cells were positive for CD79a. CD3 staining highlighted a population of lymphoid cells which were more numerous than expected from the conventional sections.

Immunohistochemistry confirmed the diagnosis in this case to be thymoma.

Case 11.

Case eleven was a four year old female Saanen goat which was euthanased because of chronic wasting. At necropsy the animal was observed to be in poor body condition. A firm, lobulated, creamy-yellow mass was found lying cranial to the heart. This tumour filled the thoracic inlet and is illustrated in Figure 23.

Microscopic examination of H&E sections of this mass revealed it to be densely cellular with prominent connective tissue septae. It had a mixed population of small lymphocytes and larger cells with vesicular nuclei and moderate amounts of eosinophilic cytoplasm (Figure 24).

The larger cells which were the dominant population stained strongly with the anti-cytokeratin antibody (Figure 25). Vimentin staining yielded multifocal positive

staining of fibroblasts. Desmin and CD79a staining were negative whilst large numbers of cells were CD3 positive.

Immunohistochemistry was effective in confirming the histological diagnosis of caprine thymoma.

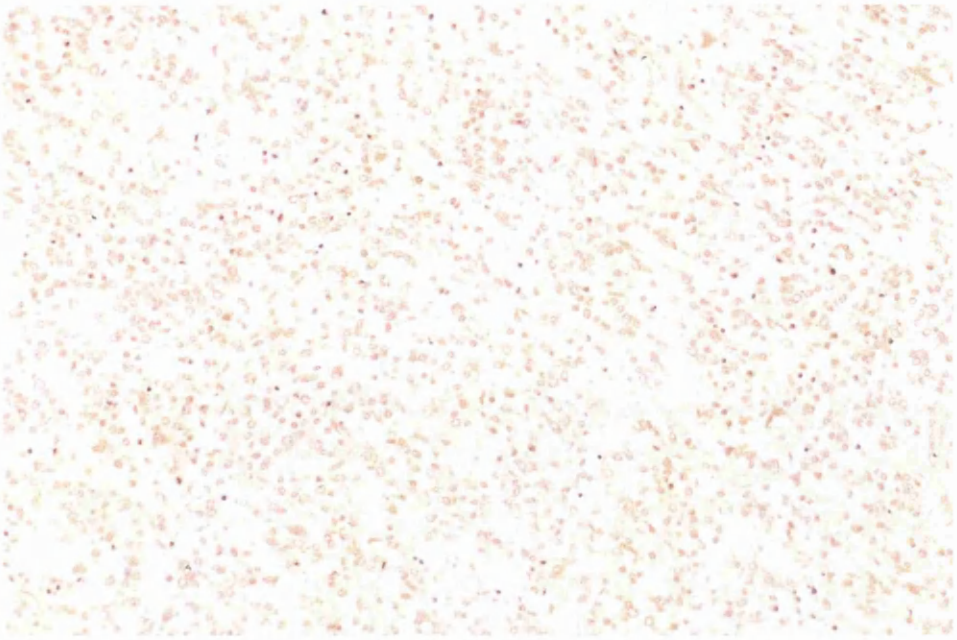

Figure 22. Bovine thymoma (case 10). Most of the cells stain with antibody to cytokeratin. Background staining is also apparent. Broad-spectrum cytokeratin (medium power)

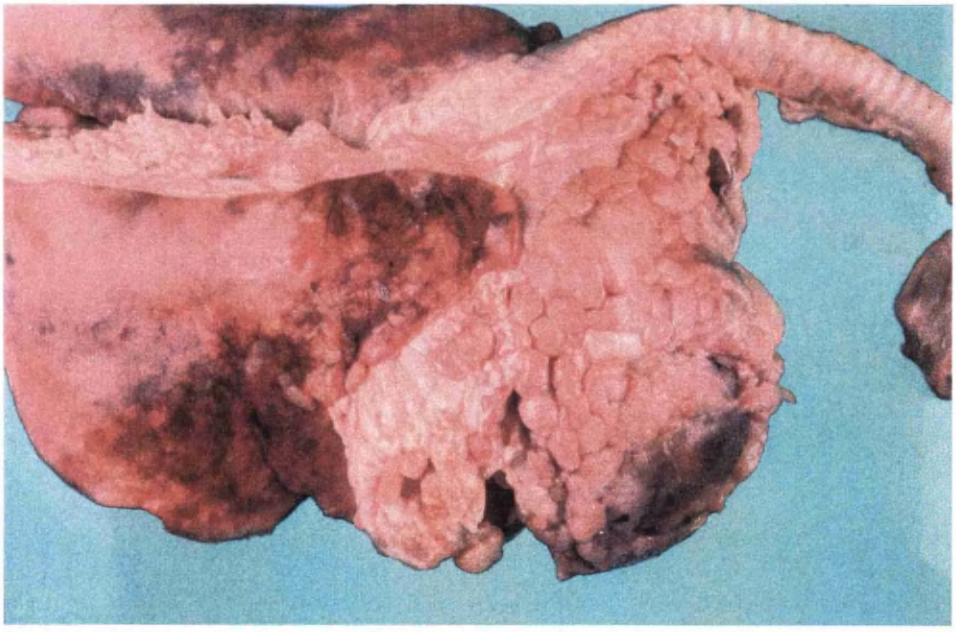

Figure 23. Thoracic viscera from case 11. Note lobulated yellowish mass over heart in anterior thorax.

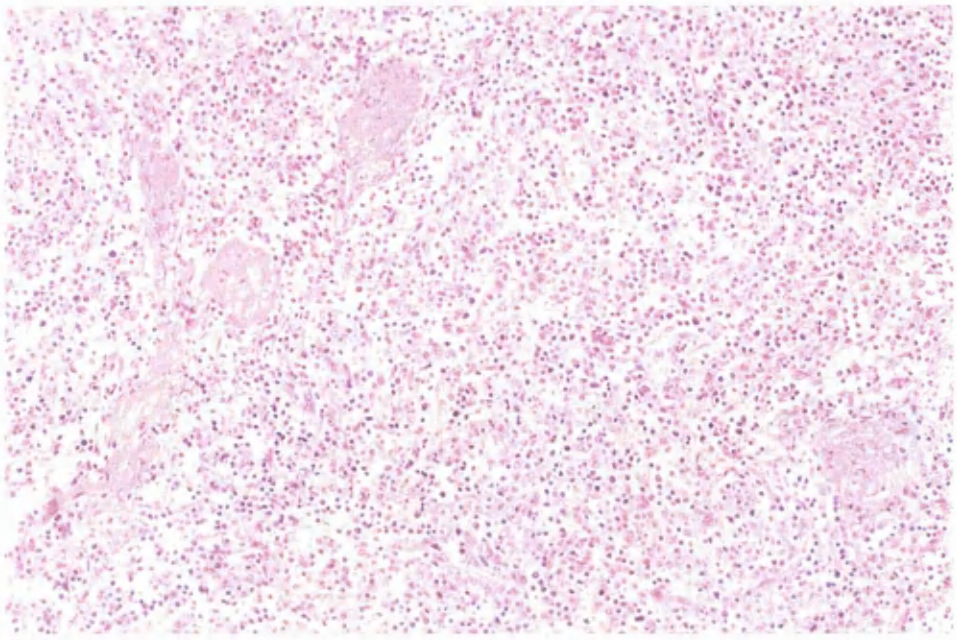

Figure 24. Caprine thymoma (case 11). A mixed population of cells are seen in a prominent connective tissue stroma. H&E (medium power)

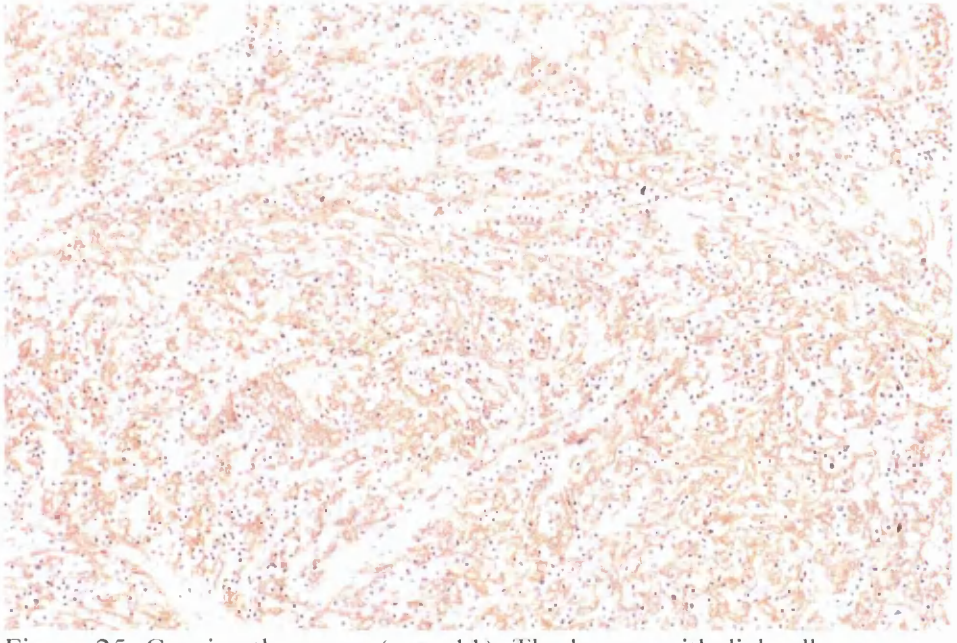

Figure 25. Caprine thymoma (case 11). The larger epithelial cells are strongly positive and the lymphocytes negative. Broad-spectrum cytokeratin (medium power)

	cytokeratin	vimentin	desmin	CD3	CD79a
Case 1	++	+	-	+++	-
Case 2	++++	(+)	-	+	-
Case 3	+++	+	-	+++	(+)
Case 4	+++	(+)	-	+++	-
Case 5	-	++	-	++	+
Case 6	+++	-	-	++	-
Case 7	++	(+)	-	+++	+
Case 8	+++	-	-	+++	+
Case 9	-	++	-	+	-
Case 10	++++	+	-	++	-
Case 11	+++	+	-	+++	-

Table 6. Tabulation of immunohistochemistry results for tumours tested.

Key: ++++ majority of cells stained
+++ large numbers of cells stained
++ moderate numbers of cells stained
+ small numbers of cells stained
(+) occasional cells stained
- no positive cells

2.5.1) Discussion

From this small survey, five out of six canine cases (cases 1-4 &6), two out of three feline cases (cases 7 and 8), the bovine and the caprine case (cases 10 and 11) were confirmed as thymomas using immunohistochemistry. In some cases the appearance of the cells by conventional staining alone was highly suggestive of this diagnosis but in others the nature of the cells was not clear nor was their distribution throughout the tumour mass. In all the positive cases the use of anti-cytokeratin antibodies was effective in highlighting the epithelial cells with an unequivocal difference between the positive and negative populations. In the negative cases there was no trace of cytokeratin positivity. In cases 5 and 8 vimentin positivity was present in moderate numbers of cells and these tumours are likely to be sarcomas.

In each case negative control samples were carefully examined to exclude the possibility of non-specific binding. This was not found to be a problem with any of these sections. There was, however, variation in intensity of cytokeratin staining between the thymomas which is likely to relate to variations in fixation time and levels of intermediate filament protein expression. The bovine tissues examined tended to produce some background staining on the test sections and the author has observed this to be a common occurrence in this species with a variety of antibodies and techniques.

As listed in Table 5, the predominant clinical findings in those cases subsequently shown to be thymomas were respiratory. Dyspnoea and coughing will have been due to space occupying effects of the thoracic tumour masses. In cases 5 and 8 where the

final diagnosis was not thymoma the clinical syndromes were also different: vomiting and diarrhoea in one case and congestive cardiac signs in the other.

The results of the CD3 immunostaining were interesting. This antibody identified populations of T cells accompanying the epithelial cells in all the thymoma cases and the lymph node and tonsil controls but produced little staining in the sections of normal thymus. In humans the CD3 molecule (also referred to as TCR or T cell receptor) is expressed during thymopoesis, although not during the very early stages of T cell maturation, as well as on mature T cells (Petrie et al. 1990). However, many "double positive" thymocytes, slightly more mature cells which express both CD4 and CD8 express only low levels of CD3 (Janeway and Travers 1994) with only those which successfully recognise self-MHC and pass the positive selection criteria going on to express high levels of CD3 (and ceasing to express one of the co-receptor molecules to become "single positive"). In case 6 it was particularly obvious that a population of lymphocytes were present which did not stain for CD3 or CD79a. It is quite possible that these cells are less mature members of the T cell series. The results on the tumours and on the peripheral lymphoid tissue control sections strongly support the assertion that this antibody is appropriate for detection of mature T lymphocytes in domestic animals. Examination of suspensions of labelled thymic cells from these species by *fluorescence activated cell sorter* (FACS) would provide a more accurate measure of their receptor status but these results may be consistent with low level expression in many cells which is not effectively demonstrated by immunohistochemistry.

The criterion for the diagnosis of thymoma is the demonstration of an abnormal population of cytologically benign epithelial cells (Castleman 1955, Rosai and Levine 1976, Levine and Rosai 1978). All the cases described as positive here fulfil this criterion. Cytological indices of malignancy; nuclear and cellular pleomorphism, increased nuclear to cytoplasmic ratio, nuclear hyperchromatism and abnormal mitoses were not present.

This was also true for the single case of bovine thymoma reported by Altman and Streett (1968) and the fifteen cases (6 canine, 1 feline, 5 bovine and 3 ovine) in the series described by Parker and Casey (1976). However, in the more recent report by Momotani, Nakamura and Shoya (1981) a single case of bovine thymoma is reported where the epithelial cells are described as resembling squamous epithelial cells with 1-3 large nuclei and conspicuous nucleoli. These cells were very variable in size, shape, nucleus to cytoplasm ratio and staining characteristics. This tumour, although a commonly quoted example of bovine thymoma, had the morphologic appearance of a carcinoma (it also had distant metastases) and would more properly be described as a thymic carcinoma under current classification (Henry 1992).

The caprine cases in Hadlow's survey (1978) were all composed of a mixture of cytologically bland epithelial cells and normal lymphocytes, as were the caprine and most other cases in this survey. From Hadlow's series, in sixteen out of seventeen thymomas normal lymphocytes were the most numerous cells.

The benign cytological appearance of thymomas is not a reliable guide to their biological behaviour. In the human literature the presence or absence of invasion through the tumour capsule at thoracotomy is considered to be the major factor

separating functionally malignant and benign thymomas (Batata et al. 1974, Walker, Mills and Fechner 1990). Aronsohn (1985) proposes a classification for canine thymomas based on the human literature. He divides thymomas into grades I-IV where grade I tumours are completely bounded by their capsule, grade II tumours show pericapsular invasion into fat and serosae, grade III tumours have invasion into neighbouring organs and/or intra thoracic metastases and grade IV tumours exhibit extra-thoracic spread. These grades of tumour are separable in prognostic terms and require different therapeutic approaches. Suitable treatment modalites are suggested.

For the cases described here the information required to grade the tumours prognostically as described by Aronsohn (1985) is not consistently available. It would seem that whilst his grading system is useful and relevant to the surgeon exploring a thoracic mass and may aid in management decisions regarding a case it cannot be readily applied to small biopsy samples. Broadly, the tumours examined in this study would fall into groups I or II in Aronsohn's classification.

What is clear from this study is that immunohistochemical examination of these tumours greatly facilitates histological classification. For example in the bovine case (case 10) it was difficult to classify the cells at all in the conventional H&E section. Most cells were then found to be epithelial through their cytokeratin positivity and CD3 staining highlighted a small but significant lymphoid component to the tumour. In some cases, particularly those where the cellular preservation was particularly good, as in Case 7, the immunohistochemical results confirmed the histological diagnosis.

The results of this study indicate that immunohistochemistry is an effective and convenient way of identifying the epithelial nature of these thymic tumours. It allowed the definitive diagnosis of what is an extremely rare tumour in the bovine in a manner not previously reported. Previous attempts to confirm the epithelial nature of such neoplasms have employed electron microscopy to demonstrate epithelial features such as desmosomes and tonofilaments ultrastructurally (Momotani, Nakamura and Shoya 1981). This technique has the disadvantages of being expensive, laborious and of limited availability. Immunohistochemistry, on the other hand, may be performed in any histology laboratory, is less expensive and more suitable for inclusion into routine diagnostic services.

In the small animal cases the capability to provide a definitive histological diagnosis between cases of thymic lymphosarcoma and thymoma has additional significance in terms of the different prognostic implications and treatment options for these two tumours.

Thymic lymphosarcoma in either a cat or dog carries a very poor prognosis. In the cat the majority of cases will be associated with Feline Leukaemia Virus infection which adds the dimension of health risk to other in-contact cats. A proportion of thymic lymphosarcomas will respond to cytotoxic chemotherapy should the owner wish to pursue such treatment options whereas these therapies are not useful in the treatment of thymoma (Bellah, Stiff and Russell 1983).

Thymomas in these species, on the other hand, are commonly, though not exclusively benign. Those without an infiltrating growth pattern may be amenable to surgical resection and there are reports of these procedures producing a cure. Gores et al.

(1994) described a series of twelve cats with thymoma which were treated with surgical resection alone. One patient died in the post operative period from persistent intrathoracic haemorrhage and one, which had an intercurrent fungal pleuritis, was euthanased. The other ten cats showed no sign of recurrence or metastases and most were followed up over a substantial period. The authors conclude that in most cats with thymomas surgical excision alone can be expected to result in either a cure or a long term disease free interval. One cat treated with surgical resection for a thymoma which subsequently recurred is described by Martin et al. (1986). Further surgery and adjuvant chemotherapy was then undertaken. In this series case 6 was successfully treated by surgical resection of the mass.

Successful surgical resection of thymomas in the dog has also been reported (Harris et al. 1991, McNeil 1980, Poffenbarger, Klausner and Caywood 1985). In some cases post operative medical therapy to control myasthenic signs was necessary (Poffenbarger, Klausner and Caywood 1984). In a case reported by Bellah, Stiff and Russell (1983) the histological diagnosis on first biopsy of a thoracic mass was thymic lymphosarcoma. When the mass failed to respond to standard chemotherapy for this disease biopsy was repeated and thymoma diagnosed. This dog was then treated by surgical excision of the mass and did well for six months at which time it died suffering from anorexia and ascites. Thoracic radiographs were normal but laparotomy showed lesions on the spleen and liver. Histopathology was not performed.

Transthoracic needle biopsy is a useful technique for the differential diagnosis of thymic disease in small animals. It is relatively non-invasive and may be simply

performed in general practice. As thymic lymphosarcoma is the most common thymic malignancy in small animals it is valuable to achieve the diagnosis in the least invasive manner as surgical resection is not indicated in the treatment of this disease. In the series reported here case 7 was diagnosed by needle biopsy. The core of tissue obtained by the practitioner was fixed and submitted for histological and immunohistochemical examination. This simple procedure was very effective in providing a high quality diagnostic sample. A needle biopsy providing a core of tissue to be processed is more informative than aspiration cytology but this method may also be used to examine thymic masses.

Rae, Jacobs and Couto (1989) compared cytological preparations and histological sections from thirteen canine and feline thymomas. Fine needle aspirate biopsy (FNAB) was found to be more useful than impression smears with seven out of nine showing recognisable features of thymoma; epithelial-like cells and cytologically normal lymphocytes. FNAB of a thymic lymphosarcoma would be expected to yield immature/atypical lymphoid cells. The pitfalls of this technique include the relatively high likelihood of producing a non-diagnostic sample by aspirating from areas which are cystic or where lymphoid cells predominate. It is possible to apply immunohistochemical techniques on cytological preparations if required. In this situation the technique is more correctly termed immunocytochemistry.

The success of this study in providing confirmation of the possibly difficult histological diagnosis of these tumours suggests that immunohistochemistry is a valuable adjunct to classical histological examination and diagnosis of thymomas in

domestic animals and that the technique may be suitable for consideration in the diagnostic situation.

Chapter 3.

Investigating Cytokeratin Subclass Expression Using Immunohistochemistry

3.1.1) Cytokeratin Immunohistochemistry.

In chapter two the use of lineage markers to determine the tissue of origin of thymic tumours in domestic animals was assessed. This chapter focuses on the investigation of cytokeratin subclass expression in normal canine skin and canine cutaneous neoplasms of epithelial origin as a progression from the previous work.

Of the five intermediate filament classes the epithelia-associated cytokeratins are the most complex. Moll et al. (1982) catalogued nineteen subsets of human epithelial cytokeratins using two-dimensional gel electrophoresis (Table 7). Other subsets also exist but these are less well defined. Cytokeratins may be divided into acidic (Type I) and neutral to basic (Type II) groups according to their isoelectric point (Table 8). Usually two to ten of these subfamilies are present in a particular epithelium.

The particular cytokeratin "finger print" of an epithelium varies according to three main factors as follows: (Cooper, Schermer and Sun 1985)

i) epithelial type: simple versus stratified
ii) differentiation type (amongst stratified) e.g. skin, cornea, oesophagus
iii) state of cellular growth: normal versus hyperproliferative

Examples of tissue cytokeratin profiles are shown in table nine. Cytokeratins are usually expressed as a pair consisting of one of each type. In these pairs the neutral/basic subunit is approximately 8kDa larger than its acid counterpart (Cooper, Schermer and Sun 1985). In general, higher molecular weight cytokeratins are present

in complex stratified epithelia such as skin or oesophagus while lower molecular weight cytokeratins are more commonly found in simple epithelia, e.g. hepatocytes or glandular acinar cells. Both high and low molecular weight cytokeratins are expressed in ductal epithelia, e.g. mammary, bronchogenic, skin adnexal and biliary and pancreatic ducts. This is also the case in transitional mucosa and mesothelium. These fairly consistent "rules" of cytokeratin expression suggest that the identification of specific types of keratin may be of diagnostic value.

Cytokeratin Subclasses						
no.	mw(kDa)	pI		no.	mw(kDa)	pI
1	68	7.8		11	56	5.3
2	65.5	7.8		12	55	4.9
3	63	7.5		13	54	5.1
4	59	7.3		14	50	5.3
5	58	7.4		15	50	4.9
6	56	7.8		16	48	5.1
7	54	6.0		17	46	5.1
8	52.5	6.1		18	45	5.7
9	64	5.4		19	40	5.2
10	56.5	5.3				

Table 7. Cytokeratin subclasses as classified according to Moll et al (1982)

Cytokeratin Subclasses	
Type I acidic	Type II neutral/basic
9	1
10	2
11	3
12	4
13	5
14	6
15	7
16	8
17	
18	
19	

Table 8. Cytokeratin subclasses grouped according to isoelectric point.

In practice there are two main applications for cytokeratin immunohistochemistry:

i) it allows an undifferentiated neoplasm to be identified as epithelial in origin

For this purpose a broad spectrum antibody reactive to an epitope conserved among the subclasses or a mixture of antibodies which identify different cytokeratins is used.

ii) it can aid histogenic subclassification of tumours (epithelial tumours) by determining whether the tumour is derived from simple, ductal or squamous epithelium. In this situation antibodies for defined cytokeratin subclasses are used.

Cytokeratin Subclass Distribution	
epidermis	1 (2) 5 10 11 14 (15)
oesophagus	4 5 (6) 13 (14,15,16, 17,19)
simple epithelium (e.g. hepatocyte)	8 18
ductal epithelium (e.g. mammary)	7 8 18 19
transitional epithelium (bladder)	(5) 7 8 (13) (18) 19

Table 9. Examples of human tissue cytokeratin profiles. (Derived from Moll et al 1982)

3.1.2) Skin Tumours of the Dog

A variety of skin neoplasms arising from the cells which compose or migrate to the skin and its associated structures are recognised in the dog (and in other domestic animal species). The classification and prognostication of these tumours composes a large part of the work of a diagnostic veterinary pathology service.

Although some of the common skin tumours of dogs are classified as "round cell" tumours, including mast cell tumours, the most common malignant skin tumour in canines (Goldschmidt and Shofer 1992) and histiocytomas - benign growths which often regress spontaneously (Taylor, Dorn and Luis 1969), a significant number of canine skin growths arise from the local epithelial structures and it is these which are examined in this chapter.

The classification of some of these tumours, for example the squamous carcinoma, is well established with little discordance between different authors. For other lesions, perhaps most notably the benign skin adnexal tumours, there are significant differences in the approach to classification between authors. Gross, Walder and Irke (1992) espouse a fairly complex system of subdivision for these tumours whilst other authorities on veterinary dermatopathology like Jaeger and Wilcox (1994) regard such efforts an exercise in semantics.

It is therefore of interest to apply the newer developments in immunohistochemical technology to these tumours to assess whether the results assist in determining a meaningful classification system based on cutaneous embryology.

In this study the immunohistochemical examination of cytokeratin subfamilies expressed in the skin and associated epithelial neoplasms of the dog is undertaken using commercially available anti-cytokeratin antibodies raised against human proteins.

Before commencing this work it was necessary to:

i) review the range of canine cutaneous epithelial tumours to be studied

ii) refer to the patterns of tissue cytokeratin expression noted in investigations of human tissue.

3.1.3) Epithelial Tumours Investigated in This Study

A classification system for neoplasms of the skin of domestic animals was published by Weiss and Frese in 1974 under the auspices of the World Health Organisation (WHO). They state that "tumours occur more frequently in the skin than any other part of the body". The following are the classifications of epithelial skin neoplasms recognised by these authors.

WHO Epithelial Skin Tumour Classification

Basal cell tumour (basal cell carcinoma)
Squamous cell carcinoma
Papilloma i)squamous cell papilloma
ii)fibropapilloma
Sebaceous gland tumour i)sebaceous adenoma
ii)sebaceous carcinoma
iii)tumour-like hyperplasia
Tumour of hepatoid (perianal) glands i)adenoma
ii)carcinoma
iii)tumour-like hyperplasia
Sweat gland tumour i)papillary syringadenoma
ii)cystadenoma of apocrine sweat glands
iii)spiradenoma
iv)mixed tumour of apocrine sweat glands
v)carcinoma of apocrine sweat glands a)papillary carcinoma
b)tubular carcinoma
c)solid carcinoma
d)signet ring cell carcinoma
Tumour of hair follicle i)trichoepithelioma
ii)necrotizing and calcifying epithelioma
Intracutaneous cornifying epithelioma
Cysts i)epidermal cyst
ii)dermoid cyst
iii)follicular cyst
iv)cyst with epithelial proliferation

Controversy exists over the classification of basal cell and appendage tumours in domestic animals. Many veterinary pathologists broadly follow the classification used

for human tumours which is similar to the above but recognises different phenotypic variants of the basal cell tumour (solid, cystic, ribbon and medusoid) and lists pilomatrixoma as a hair follicle tumour (Pulley and Stannard 1990). Some espouse a simpler classification which terms most of the histological subdivisions "basal cell tumours with follicular (or adnexal) differentiation" (Jaeger and Wilcox 1994) and some work to a complex and very comprehensive system of subclassification as suggested by Walder and Gross (1992).

Some of the major epithelial skin tumours of dogs are considered in detail in this study: Tumour types were selected for inclusion in this study on the basis of being relatively prevalent and serving as examples of different pathways of differentiation.

Squamous Cell Carcinoma of the Skin

Squamous cell carcinoma is a malignant tumour arising from the squamous epithelium of the epidermis or mucosal surfaces. This tumour affects all domestic species but is most common in the dog, cat, horse and cow. It has been reported to be the second most common *malignant* neoplasm of the skin in dogs after mast cell tumour (Walder and Gross, 1992). A large scale survey undertaken by Goldschmidt and Shofer (1992) placed squamous cell carcinoma at 5% of all epithelially-derived neoplasms in dogs, approximately 1.75% of all canine skin tumours.

In the dog the most common sites of origin include the oral cavity, the skin on the legs, trunk, scrotum, lips and the nail bed epithelium. Goldschmidt and Shofer (1992) suggest that Bloodhounds, Bassett Hounds and Standard Poodles are at increased risk

of developing squamous cell carcinomas and Madewell et al. (1982) reported that such tumours originating on the digits developed more frequently in black Labrador Retrievers and black Standard Poodles.

There is a general tendency in all species for squamous cell carcinoma to develop in unpigmented areas of skin, especially where the overlying hair coat is short or sparse. Sun exposure is a known risk factor as UVB radiation induces DNA damage in keratinocytes, and squamous carcinomas may be preceded by actinic keratosis (Hargis and Thomassen 1979, Hargis, Thomassen and Phemister 1977). In dogs with a history of sun-bathing behaviour, especially in sunny climates, the head, ventral abdomen and perineum are predilection sites for tumour development. Thermal injury of the skin may also predispose to the development of this tumour.

Squamous cell carcinomas are locally invasive but are usually slow to metastasise. An exception to this generalisation is squamous cell carcinoma of the digit, which despite its often well differentiated histological appearance tends to be highly invasive, osteolytic and more commonly metastatic. Metastasis to the local lymph nodes and lungs from tumours of the digit has been reported in up to 40% of cases in comparison with 5% for squamous carcinomas overall (Jaeger and Wilcox 1994).

Grossly, squamous carcinomas may produce papilliform growths which tend towards ulceration of the surface, or more commonly, be erosive and produce ulcers which become deeper and more severe with time (Pulley and Stannard 1990).

Histologically the tumours are composed of irregular masses, nests or cords of proliferating epidermal cells which invade the dermis and subcutis (see fig. 31). In

general the cells have large vesicular nuclei with prominent nucleoli and a moderate to high mitotic rate. Aberrant mitotic figures may be seen. The cytoplasm is eosinophilic and abundant. The growths are often fairly well differentiated with a reasonably orderly progression from cells resembling the basal layer of epithelium at the periphery of nests or masses to large, keratinising cells with eosinophilic cytoplasm and pyknotic nuclei at the centres. The amount of keratin produced depends on the degree of differentiation, in well differentiated tumours numerous keratin "pearls", composed of concentric layers of keratinising cells and keratin, are seen. Well differentiated squamous cell carcinomas also show prominent intercellular bridges, an artefactual effect produced by the stretching of desmosomes between the cells which shrink during fixation. There is a variable amount of stromal collagen production and inflammatory cells may be present.

Poorly differentiated histological variants are less common but do occur. In these growths the epithelial structures tend to be smaller and often include cords of cells with a highly invasive growth pattern. There is often less stromal collagen and the cells are hyperchromatic with amphophilic cytoplasm and no keratin pearl formation, although individual dyskeratotic cells may be seen. The mitotic rate is typically high and there is nuclear and mitotic atypia.

Papillomas

Papillomas are benign epithelial neoplasms and there are various manifestations of these skin growths. Squamous papillomas may be induced in dogs by infection by canine papilloma virus, a member of the papovaviridae family of double stranded DNA viruses. The reported incidence of such cases is between 1 and 2.5% of all canine skin

tumours (Walder and Gross 1992b). These lesions are typically exophytic and sized a few millimetres to (rarely) a few centimetres in diameter. The predilection sites for viral papillomas are the face and the oral cavity and spontaneous regression over several weeks to months is usual. Multiple oral papillomas are seen in cases of canine oral papillomatosis. This infectious condition is seen in young or immunosuppressed animals and although spontaneous regression occurs in most cases, malignant transformation to carcinoma has been recorded. Squamous papillomas with no evidence of viral involvement may also occur. These are often smaller, usually 1-5mm in diameter and occur most commonly on the face, eyelids, conjunctiva and footpads.

Histologically, typical viral papillomas are composed of finger-like projections of cytologically mature squamous epithelium. The epithelial projections are heavily keratinised and an inflammatory infiltrate is common in the stroma of the papillae. Viral cytopathic effects are manifest by koilocytosis and the formation of large keratohyalin granules. Basophilic intranuclear inclusions may be identified at the junction of keratinised and non-keratinised epithelium. Ultrastructural studies have demonstrated aggregations of viral particles in the nucleoplasm of cells in the stratum granulosum (Watrach 1969). Non-viral squamous papillomas are similar histologically but the degree of epithelial hyperplasia and hyperkeratosis is usually less as is the inflammatory infiltrate. Specific viral changes are absent (Figure 35).

Fibropapillomas are lesions where a core of excess fibrous tissue is covered by papillated squamous epithelium resembling that found in a squamous papilloma.

Basal Cell Tumour and Appendage Tumours

This group of tumours are important in the dog and cat and rare in other domestic species. The basal cell tumour is a distinct undifferentiated epithelial neoplasm of the skin. The basal cell characteristic of this tumour is not synonymous with the basal cell of the epidermis which is well differentiated and possesses intercellular bridges. It is a more primitive pluripotential cell, analogous to the primary epithelial germ cell and possesses no intercellular bridges (Pulley and Stannard 1990).

A spectrum of basal cell and appendage tumours exist from undifferentiated basal cell tumours to those which resemble fully formed but enlarged sebaceous glands or rudimentary hair follicles. Apocrine sweat glands also arise from the primary epithelial germ cells but tumours of this type do not exhibit the same spectrum of differentiation and are usually divisible into adenomas and adenocarcinomas.

Basal Cell Tumours

Basal cell tumours are composed almost entirely of undifferentiated basal cells and lack differentiation towards follicles, sebaceous structures, apocrine glands or squamous cells. Synonymous terms used for this tumour include basaloid tumour, basal cell epithelioma, basalioma and basal cell carcinoma. The aetiology of this neoplasm is unknown.

In two 1950s surveys of skin tumours of the dog basal cell tumours have been found to feature at 10% and 5% (Head, 1953 and Cotchin, 1954). A more recently published survey of biopsy specimens submitted to the University of Pennsylvania counted basal

cell tumours at 11% of epithelially derived skin neoplasms in the dog, around 3.8% of all canine skin tumours received (Goldschmidt and Shofer 1992). A survey of feline basal cell tumours found that they comprised 11-28% of feline skin tumours (Diters and Walsh 1984).

Grossly, basal cell tumours are usually solitary dermal or subcutaneous masses ranging in size from 0.5-10cm. The masses are firm, discrete and freely moveable over underlying tissues. Ulceration of the overlying epithelium is common and secondary infection may be a feature of ulcerated lesions. The skin of the head and neck is most commonly affected.

Histologically the cells of basal cell tumours have hyperchromatic oval nuclei and scant cytoplasm. Some tumours may contain spindle-shaped cells with more elongated nuclei. The cells are uniform in size and shape and are usually quite small with poorly defined cytoplasmic outlines. Melanin pigment may be present within the basal cells themselves and in scattered melanocytes within the tumour, especially in feline tumours, and the mitotic rate may be quite high. A fibrous stroma of variable extent may subdivide and surround the tumour. The tumours may be subclassified according to their histological pattern into solid, cystic, adenoid (as in Figure 38) and medusoid variants, but this approach may be unsatisfactory as the majority of tumours are composed of more than one pattern. No prognostic significance is attached to the various histological subdivisions.

Basal cell tumours in the dog carry an excellent prognosis. Local excision is curative, with adequate margins the tumours do not recur at the surgical site and metastasis has not been recorded.

Trichoepithelioma

Trichoepitheliomas are uncommon, benign skin tumours recognised in dogs and cats. These are epithelial growths which are capable of differentiating towards all three segments of the hair follicle therefore variable histological appearances are seen both between and within tumour masses. In a study by Bevier and Goldsmidt (1981), trichoepitheliomas were recorded at 1-3% of all canine *skin* tumours. In the cat trichoepitheliomas account for 1.5-4% of *skin* tumours (Theilen and Madewell 1987).

Grossly, trichoepitheliomas are solitary round to oval dermal masses usually less than two centimetres in diameter, although larger growths are possible. The skin overlying the mass is partially or completely alopecic and ulceration is common, especially in larger lesions. The dorsal trunk is a predilection site. Most cases occur in dogs aged five years or more. As a breed, the Bassett Hound is predisposed to developing multiple trichoepitheliomas (Goldschmidt and Shofer 1992).

Histologically, trichoepitheliomas are well circumscribed but unencapsulated dermal nodules composed of a mix of budding epithelial islands of basaloid (small, basophilic undifferentiated) cells which resemble primitive follicular bulbs, having scant pale cytoplasm and uniform ovoid nuclei and cystic structures of varying size which may be lined by similar cells and show extensive matrical keratinisation and some shadow cell formation (Figure 43). A hyaline basal lamina resembling the hyaline sheath of the catagen hair follicle may support the outer layer of cells The cysts may alternatively be lined by squamous epithelium resembling the follicular infundibulum or isthmus or they may have a combined appearance. The tumour may be contiguous with overlying

epithelium and the basaloid and shadow cells may be pigmented. The surrounding stroma is relatively acellular.

Rare cases of invasive trichoepithelioma where the growth extends into the subcutis and malignant variants with a similar histological appearance and evidence of lymphatic spread or node involvement are recorded.

Pilomatrixoma (pilomatricoma)

Pilomatrixomas are uncommon benign neoplasms recognised in dogs which arise from the germinal cells of the follicle matrix. Weiss and Frese (1974) quote the incidence of pilomatrixomas in dogs as 3% of all skin tumours.

Grossly, pilomatrixomas are solitary, well circumscribed masses typically two to ten centimetres in diameter which may be multinodular. Their consistency ranges from firm to possibly gritty or bony to the extent that sectioning is very difficult. The masses are usually dome-shaped or plaque like, freely moveable over deep tissues with atrophy and alopecia of the overlying epithelium. The neck, back and tail are predilection sites. The commonest age range for presentation with this tumour is five to ten years. In terms of breed disposition the Kerry Blue is greatly over-represented as are Bedlington Terriers and Schnauzers. It is suggested that in such breeds which have a continually growing haircoat there are greater numbers of active anagen hair follicles and due to this an increased susceptibility to pilomatrixomas.

Histologically the masses are well circumscribed and dermal to subcutaneous in location. The growth is composed of single or multiple cysts lined by basophilic

basaloid keratinocytes resembling the matrix of the hair bulb with their large, hyperchromatic ovoid nuclei and scant cytoplasm (Figure 47) . These cells may show fairly numerous mitoses. There may be regions of squamous epithelium formation with or without a stratum granulosum and foci of differentiation towards inner root sheath with shadow cell formation. In the centre of the tumour nodules, particularly in long-standing lesions, there is commonly extensive differentiation to shadow cells and accumulation of keratinised debris with giant cell formation which may progress to calcification or osseous metaplasia. The cysts are set in a moderately cellular stroma of collagen and mucin. Melanin pigment may be seen in tumour cells or in stromal melanophages. Rupture of the cysts leads to granuloma formation.

In their book "Skin Tumours of the Dog and Cat" (Pergamon Press, 1992) Goldsmidt and Shofer present one of the most comprehensive surveys of cutaneous neoplasia in these species using data collected from the submissions to the Pathology service at the University of Pennsylvania Veterinary School. The figures supplied in this large scale survey are expressed divided into epithelial and mesenchymal classes but when they are converted and expressed as percentages of skin tumour totals (this is also the method used in Table 10 in Results) the figures are as follows: Basal cell tumours add up to 3.9% of all canine skin neoplasms examined, squamous cell carcinomas account for 1.8%, papillomas composed less than 0.35% of this survey whilst trichoepitheliomas and pilomatrixomas numbered 4.2% and 1% respectively.

In an earlier survey, Nielsen and Cole (1960) examined 7489 skin lesions in dogs and found 153 epithelial neoplasms, excluding mammary and perianal gland tumours (2% of the surveyed material). The numbers of non-epithelial neoplasms were not recorded

so it is not possible to give figures for prevalence of individual tumour types against total skin tumour numbers. 33 basal cell tumours (21.5% of *epithelial* neoplasms), 31 squamous carcinomas (20.3%), 7 trichoepitheliomas (4.5%), 9 squamous papillomas (5.9%) and 5 calcifying epitheliomas, synonymous with pilomatrixomas (3.4%) were identified.

In a 1970 survey Brody found the skin to be the commonest site for neoplasms of the dog, representing 35.6% of 2917 neoplasms. Of these 984 skin tumours, 445, (approximately 45%) were of epithelial origin. In this survey 205 "adnexal tumours" were not separated into basal, follicular or glandular variants but it was recorded that the Kerry Blue breed was over-represented in the follicular tumour group. In agreement with the findings of Liu and Hohn (1968) the digit was found to be an important site for squamous carcinoma with these lesions being associated with increased incidence of metastases.

Bostock (1986) states that ten tumour types account for 80% of all canine cutaneous neoplasms. These are listed as mastocytoma (mast cell tumour), hepatoid adenoma, lipoma, sebaceous adenoma, fibrosarcoma, melanoma, histiocytoma, squamous cell carcinoma, haemangiopericytoma and basal cell tumour. The total frequency of canine skin tumours is reported to be approximately 450 new cases per year per 100,000 dogs.

3.2.1) Review of the Epithelial Structures of the Skin

The epidermis is a stratified squamous epithelium that forms the protective covering of the skin. In this membrane the more superficial cells are flattened while the deeper layers vary from cuboidal to columnar. Other examples of stratified squamous epithelium are found in the cornea, the mucous membrane of the oral cavity and the oesophagus. In contrast to these other epithelia the surface of the skin is dry and the superficial cells transform to provide a tough, protective, non-living layer of keratin. Associated with the epidermis are the epithelial structures of the hair follicles and adnexa.

The epidermis is renewed by a balanced process of growth (proliferation) and differentiation and is organised into layers reflecting stages in these dynamic processes. In most areas of mammalian skin the epidermis consists of four layers:

stratum basale / stratum germinativum (basal cell layer)

stratum spinosum (prickle cell layer)

stratum granulosum (granular cell layer)

stratum corneum (horny layer)

In the footpad and nasal planum skin of the dog and in the palms and soles of humans a fifth layer, the *stratum lucidum* (clear layer) exists beneath the *stratum corneum.*

In transit from the basal layer to the dead squamous layer an epidermal cell will undergo a series of morphological and biochemical changes. Various morphological and biochemical features characterise the stages of epidermal growth and differentiation.

The *stratum basale* is composed of a single layer of cells resting on the basement membrane. Within this layer there is some morphologic and functional heterogeneity as some cells function primarily in anchoring the epidermis whilst others act as proliferating stem cells (Goldsmith 1991). Cells of the *stratum basale* possess desmosomes - membrane junctions which link them in a three dimensional manner. Melanocytes (pigment producing cells) are also located at this level. Only this inner, basal cell layer of the epidermis has the capacity for DNA synthesis and mitosis (Weinstein and Van Scott 1965).

Human epidermal basal cells have in their cytoskeleton a dispersed but extensive network of cytokeratin filaments composed of equal amounts of CK5 (58kDa) and CK14 (50kDa) (Nelson and Sun 1983). These cytokeratin subfamilies are the hallmarks of a stratified epithelium.

Immediately above the basal layer is the spinous layer (*stratum spinosum*). In haired areas of canine skin this layer tends to be one to two cells in thickness. In histological sections, intercellular bridges (prickles/spines) appear to connect the cells of this layer. As in squamous cell carcinoma this distinctive appearance is a processing artefact. These cells are post-mitotic but metabolically active (Fuchs 1990).

As cells enter the spinous layer they increase steadily in size and in human studies have been shown to produce two additional cytokeratins, CK1 (67kDa) and CK10 (56.5kDa) which aggregate into thin bundles or tonofilaments (Eichner, Sun and Aebi 1986). These are produced due to changes in the transcription of specific mRNAs and their production represents a commitment to terminal differentiation. Spinous cells also

synthesise glutamine and lysine rich envelope proteins, such as involucrin, which are deposited on the inner plasma membrane of each cell (Rice and Green 1979).

The *stratum granulosum* is variable in canine haired skin and when present is one to two cells thick. In non-haired areas and at follicular infundibula this layer can be four to eight cells thick. The "granules" are insoluble keratohyalin aggregates within the cells.

As epidermal cells enter the granular layer (*stratum granulosum*) they stop synthesising cytokeratins and envelope proteins but produce filaggrin and loricrin. Filaggrin has at least two functions, it aggregates, packs and aligns keratin filaments and produces the matrix between the keratin filaments in the corneocyte and it also acts as a source of free amino acids which are important for maintaining normal hydration of the *stratum corneum* (Scott, Miller and Griffin 1995). Loricrin is a protein known to be a major component of the cornified covering of the skin (Mehrel et al. 1990).

Subsequently the cells become permeable and an influx of calcium activates the enzyme epidermal transglutaminase which catalyses the formation of bonds which cross-link the envelope proteins (Rice and Green 1979). Other, lytic enzymes are released and the metabolic activity in the cells ceases, producing squames - flattened, anuclear, eosinophilic cells made up of macrofibrils of keratin. The squames comprise the cornified layer (*stratum corneum*). This superficial layer of terminally differentiated keratinocytes is sealed together with lipids and constitutes an impermeable barrier which prevents entry of micro-organisms and loss of body fluids.

In epidermal disease states associated with hyperproliferation the expression of the differentiation-associated cytokeratins 1 and 10 is downregulated and a different set of proteins, CK6 (56kDa) and CK16 (48kDa), is induced. These are similarly located in the suprabasal epidermis (Koplan and Fuchs 1989). It is important to note that in addition to being expressed in epidermis under conditions of hyperproliferative "stress" that cytokeratins 6 and 16 together with 17 (46kDa) are normally expressed in hair follicles (Mclean and Lane 1995).

The balance between differentiation and proliferation is important for the generation of normal epidermis. In the living animal many factors interact and the functions of some of these have been elucidated using cell culture techniques.

It is known that calcium is a prerequisite for several processes in differentiation including stratification, desmosome assembly and activation of epithelial transglutaminase (Hennings et al. 1980, Watt, Mattey and Garrod 1984).

The retinoids, derived from retinoic acid (a vitamin A analogue) are an important class of morphogenic regulators in some systems. The effect of these compounds on differentiation depends on their interaction with cytoplasmic retinoic acid binding protein in the target cells (Graham 1992). Retinoids reduce the development of terminal differentiation in stratified squamous epithelium (Fuchs and Green 1981) and excess retinoids have been shown to reduce the expression of CK1, CK10, CK6, CK16, cornified envelope production and filaggrin (Fuchs 1990).

Physical factors are also important in directing normal epithelial (epidermal) development - cultures mimicking the natural situation where epidermal cells are

exposed to air and receive nutrition via diffusion show enhanced differentiation (Asselineau et al. 1986). Epithelial growth factor (EGF) stimulates keratinocyte growth as do TGFα, IL-6, IL1α and various fibroblast factors.

To commit to terminal differentiation a cell must leave the cell cycle. TGFβ inhibits DNA synthesis and cell division in basal cells, however it is expressed in greater amounts suprabasally where it may be important in maintaining growth arrest in the differentiating layers. At high levels TGFβ inhibits rather than promotes the synthesis of CK1 and CK10 and filaggrin but increases the synthesis of CK6 and CK16. The effects of TGFβ seem to differ at different stages of epidermal differentiation (Choi and Fuchs 1990).

3.3.1) Materials and Methods

The computerised database of the records of the external biopsy service operated by the Canine Infectious Disease Research Unit (CIDRU) within the Department of Pathology was reviewed for cases of the previously listed skin tumours which were selected with reference to their relative prevalence. Conventionally stained sections were examined and suitable cases selected for inclusion in the study. Normal canine skin and liver were used as control tissues.

In this study examples of each of the previously described canine skin growths; six squamous carcinomas, five papillomas, seven basal cell tumours, five trichoepitheliomas and five pilomatrixomas plus the controls were examined with four commercially available anti-human cytokeratin antibodies. In addition, a single example of each tumour type plus the control tissues was stained by the pan-cytokeratin marker routinely used in the laboratory. These antibodies were all mouse anti-human monoclonals, three purchased from Novocastra Laboratories (Newcastle-upon-Tyne, England):

antibody to cytokeratin (CK) 14 NCL-LL002

antibody to cytokeratin (CK) 16 NCL-CK16

antibody to cytokeratin (CK) 18 NCL-CK18

and one purchased from Dako (Glostrup, Denmark):

antibody to cytokeratin (CK) 10 DE-K10

The pan-cytokeratin in routine use in the laboratory is also supplied by Dako (Glostrup, Denmark):

recognises cytokeratin sub-families 5,6,8,17,(19) MNF116

Blocks of the tumours of interest were selected from the Department of Pathology archives and sections from these were cut at approximately 5μm onto coated slides (APES: 3-amino-propyltriethoxysilane) to promote adhesion. These sections were dried overnight at 37°C. For each test section a corresponding negative control section was also cut and processed in parallel.

After dewaxing and rehydrating as described in Chapter 1 the sections were subjected to a standard protocol according to the manufacturers' recommendations. A commercially available ABC detection kit (Vectastain Elite anti-mouse IgG, Vector Laboratories, Burlinghame, CA, USA.) was used.

For the CK 14, 16 and 18 antibodies this involved a high temperature antigen unmasking step. In this procedure 0.01M tri-sodium citrate buffer at pH 6.0 was brought to the boil in a pressure cooker without locking the lid. The sections were then immersed in the boiling solution and the lid locked in place. Heating was continued and after approximately four minutes the pressure indicator valve rose and the sections were incubated at this high pressure for one minute. The pressure cooker was then cooled in water and the sections were removed once the pressure had fallen. At this point the sections were placed in distilled water then in tris buffered saline (TBS).

Enzymatic pre-treatment using 0.1% trypsin with 0.1% calcium chloride in tris-buffered saline for thirty minutes at room temperature was employed to unmask the desired antigenic sites before incubation with MNF116.

In the case of the CK10 antibody neither high temperature processing nor enzymatic digestion is necessary and the sections were taken from rehydration, through rinses in tris buffered saline (TBS) to the next stage.

In all cases the next stage was quenching of endogenous peroxidase activity for which the sections were immersed in 1.5% hydrogen peroxide in methanol for ten minutes. Subsequently the sections were rinsed in distilled water for five minutes then in two changes of TBS for five minutes each.

After this the slides were placed in a humidity chamber and the tissue sections ringed using a PAP pen as described in Chapter 2. At this stage all the sections were covered with buffered pre-immune serum from the same species in which the secondary antibody was raised. In this case the serum was derived from horse. After thirty minutes incubation the test sections had the excess pre-immune serum blotted off and the primary antibody (see above) applied. Sixty minutes incubation at room temperature in the humidity chamber followed. During this time the corresponding negative controls continued to be incubated with the pre-immune serum.

After the incubation time the sections were rinsed for five minutes in TBS twice, the biotinylated secondary antibody was applied and the sections incubated for thirty minutes.

A further five minute rinse was followed by thirty minutes incubation in an avidin-biotin-peroxidase complex, a similar rinse and incubation with a peroxidase substrate solution. In this case AEC (3 amino-9 ethylcarbazole) was the substrate used. An

incubation time of fifteen to twenty minutes was found to produce good staining intensity.

After rinsing in tap water the sections were counterstained with fresh Meyer's haematoxylin with a twenty second immersion in the stain producing adequate counterstaining. Because AEC is soluble in the traditional dehydrating agents (see Chapter 1), aqueous mounting is required. For this purpose Supermount™ (Biogenex, San Ramon, CA, USA) was applied over the tissue section and allowed to dry overnight. To protect the sections they were subsequently mounted and allowed to dry before examination.

3.4.1) Results

i) Archival Survey Results

A review of the database of the external biopsy service in the Department of Pathology at Glasgow University Veterinary School over the ten year period 1986-1996 produced the following results. The five most prevalent types of cutaneous epithelial tumours were chosen for inclusion in this study. The records for cases of skin tumours of epithelial origin showed basal cell tumours to be the most common by a large margin. The second most common epithelial tumour and by far the most common malignant epithelial tumour was the squamous carcinoma. These tumours provide examples of different biological behaviour patterns and differentiation pathways. The results of the survey of the archival records are summarised in Table 10 below.

Basal cell tumour	**281**	**6.5%**
Squamous cell carcinoma	**59**	**1.4%**
Papilloma	**52**	**1.2%**
Pilomatrixoma	**100**	**2.3%**
Trichoepithelioma	**94**	**2.2%**
Sebaceous epithelioma	**89**	**2%**
Intracutaneous epithelioma	**73**	**1.7%**
Sebaceous adenoma	**85**	**1.9%**
Trichofolliculoma	**15**	**0.3%**
Hamartoma	**53**	**1.2%**
Fibroadnexal dysplasia	**4**	**<0.1%**
Papillary cystadenoma	**4**	**<0.1%**
Papillary adenoma	**2**	**<0.1%**

Table 10. Canine skin tumours of epithelial origin diagnosed in the external biopsy service. The percentages relate to the *total number of skin tumours of all types.*

ii) Cytokeratin intermediate filament expression in canine skin

After staining by the described method the sections were reviewed and the patterns of expression of each particular cytokeratin for normal canine skin, liver and each tumour under investigation were noted.

Normal canine skin

Figure 26 diagramatically illustrates the cell layers of normal canine epidermis and the cytokeratin expression of this tissue, whilst Figure 27 shows a conventionally stained section of normal canine skin.

CK14 expression was universal in the basal layers of the surface epithelium and in all layers of the hair follicle epithelium. In the thin epidermis it was sometimes difficult to discern separate layers in the immunostained sections but some suprabasal CK14 positivity, which appeared to be of a lesser intensity, was also apparent. The cells of the adnexal glands were positive and the superficial keratin crust was unstained (Figure 28).

A faint positivity to CK18 was detected in some apocrine gland cells but staining in these cells was also detected in the negative control sections so this finding was interpreted as non-specific binding.

Positive staining with CK10 antibody (Figure 29) was seen in suprabasal, but not basal, surface epithelium, in the follicular infundibula and part of the inner root sheath. The "basal" layer of the outer root sheath, the apocrine and the sebaceous gland epithelia were negative.

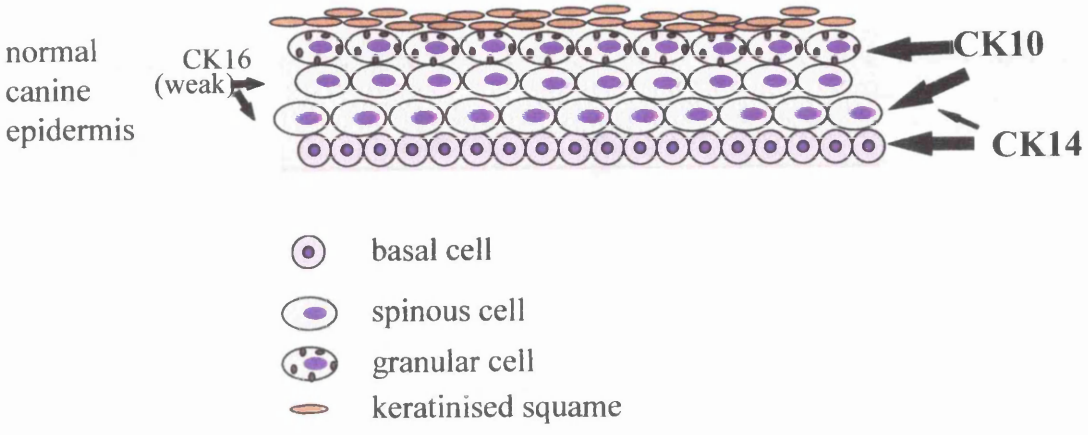

Figure 26. Diagramatic representation of canine epithelium indicating position of cytokeratin staining.

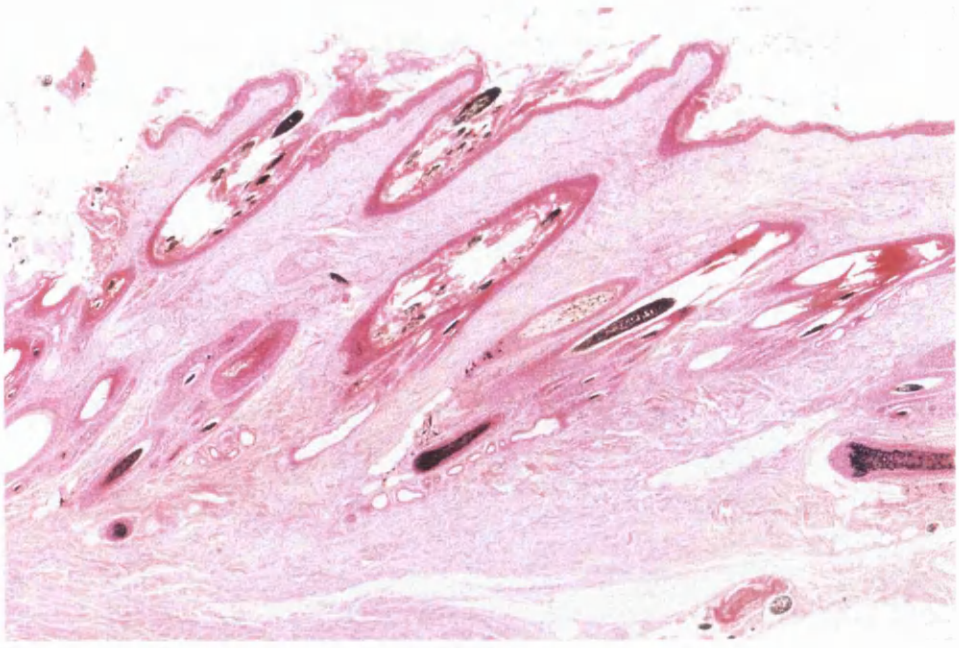

Figure 27. Normal canine skin. H&E (medium power)

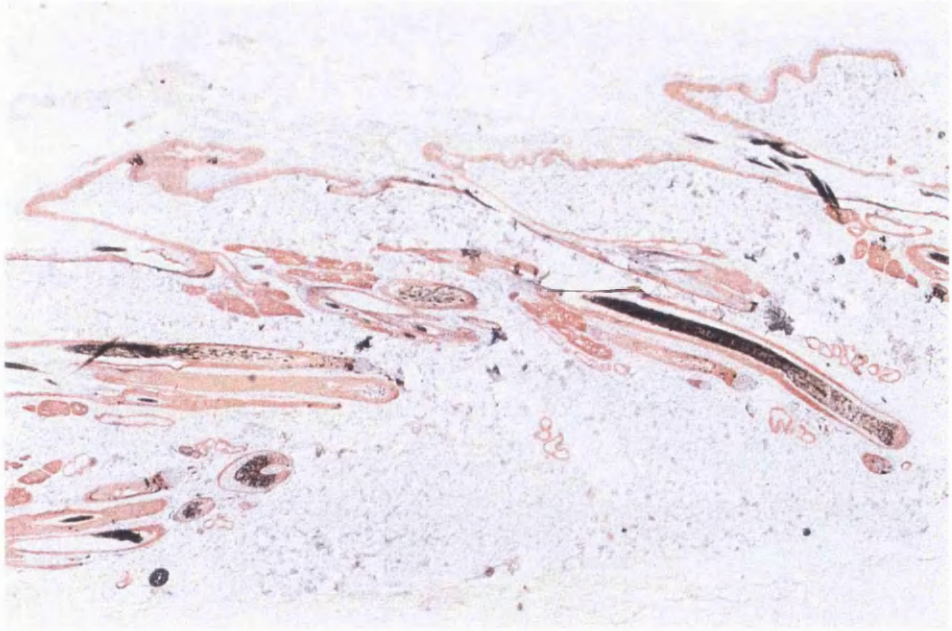

Figure 28. Normal canine skin. Note widespread staining in epidermis, especially in basal layer, in hair follicles and adnexal epithelium. CK14 (medium power)

The follicular epithelium save for the outer layer of the outer root sheath and the hair bulb itself was CK16 positive. Some cells of the surface epithelium and the sebaceous glands were faintly positive for CK16.

The pan-cytokeratin antibody produced staining in all layers of the epidermis, the walls of the hair follicles, the apocrine glands and the basal cells of the sebaceous glands. Part of the hair bulb was consistently negative (Figure 30).

Liver

A section of liver was included as an example of simple epithelium. No positivity was demonstrated for CK10, CK14 or CK16. Staining with antibody to CK18 produced faint positivity but the staining definition was not good. With the pan-cytokeratin the strongest staining was in the bile duct epithelium and there was faint staining in the hepatocytes.

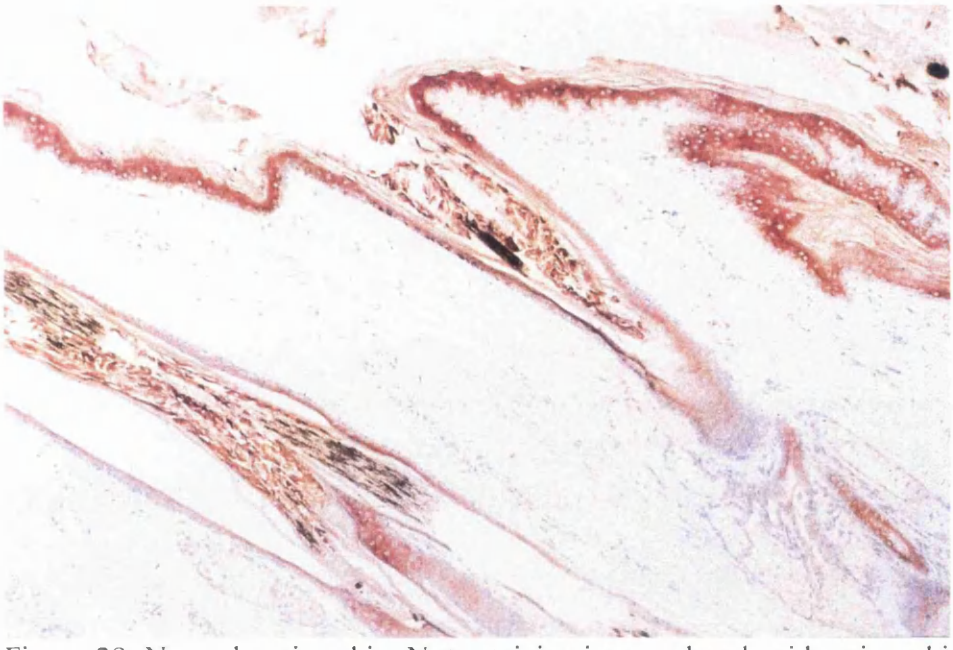

Figure 29. Normal canine skin. Note staining in suprabasal epidermis and inner root sheath. CK10 (medium power)

Figure 30. Normal canine skin. All epithelial cells except some in the hair bulb are stained. Broad-spectrum cytokeratin (medium power)

iii) Cutaneous epithelial tumours

Although in the tumours slight variations in antibody results were observed within each lesion type broadly group specific reaction patterns could be discerned. These are noted in Table 11 and described in detail in the text which follows it.

	CK10	CK14	CK16	CK18
squamous carcinoma	+ (-)	+++	++	-
papilloma	- (+)	+++	++	-
basal cell tumour	-	+++	+	-
trichoepithelioma	++ (-)	+++	++	-
pilomatrixoma	-	++ (+)	++	-

Table 11. Record of staining results for epithelial tumours.
- = no staining in any tumour case examined
(-) = no staining in some of the tumour cases examined
(+) = limited staining in some of the cases
+ = small numbers of positive cells
++ = large numbers of positive cells
+++ = majority of epithelial cells positive

Squamous carcinoma

An example of a conventionally stained canine squamous carcinoma is shown in Figure 31.

In the squamous carcinoma cases there was staining of all the epithelial cells of the tumours with CK14. This was the case for all tumours examined although staining intensity varied from section to section. This staining was cytoplasmic and granular in nature. One example of a squamous cell carcinoma stained for CK14 is shown in Figure 32.

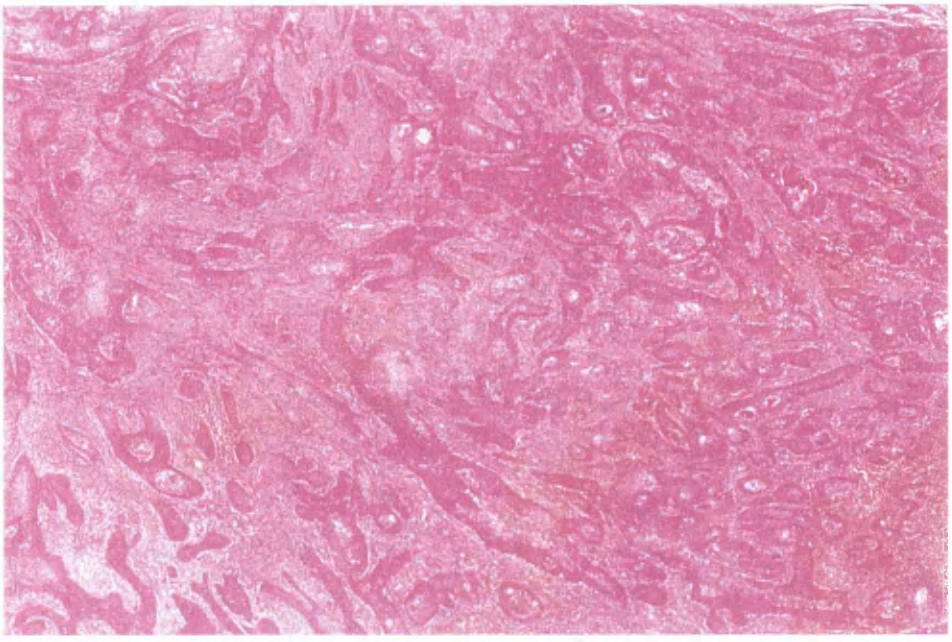

Figure 31. Canine squamous cell carcinoma. Nests and cords of malignant epithelial cells invade through the dermis. H&E (medium power)

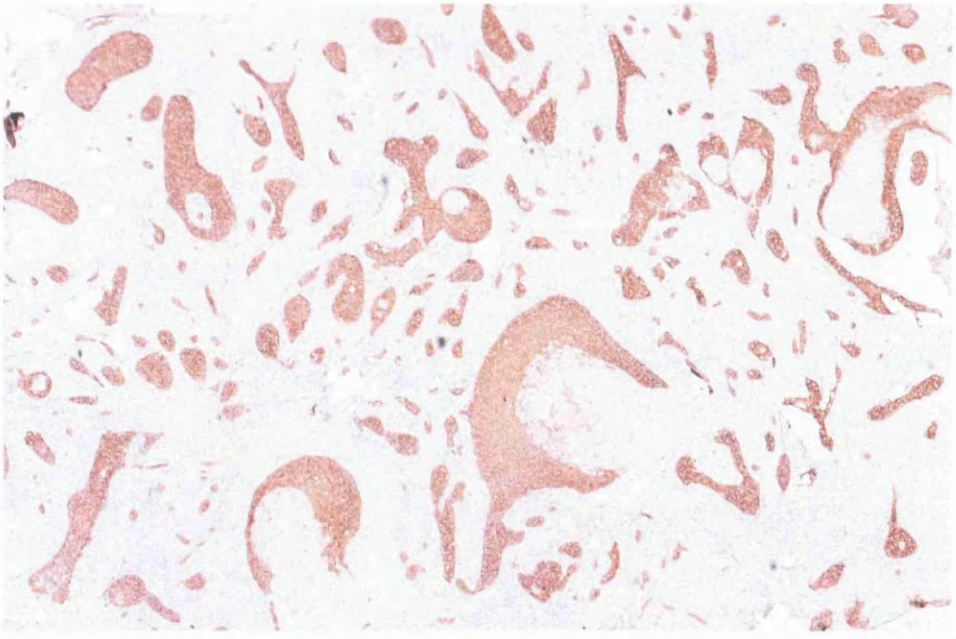

Figure 32. Canine squamous cell carcinoma. All the malignant epithelial cells are strongly stained. CK14 (medium power)

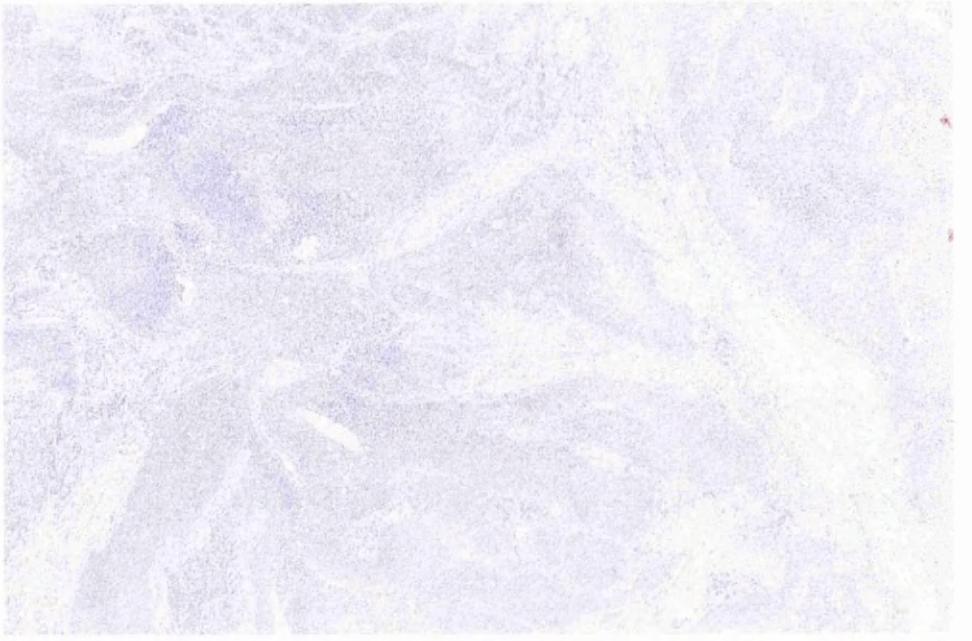

Figure 33. Canine squamous cell carcinoma. This tumour is negative for the differentiation associated CK10. CK10 (medium power)

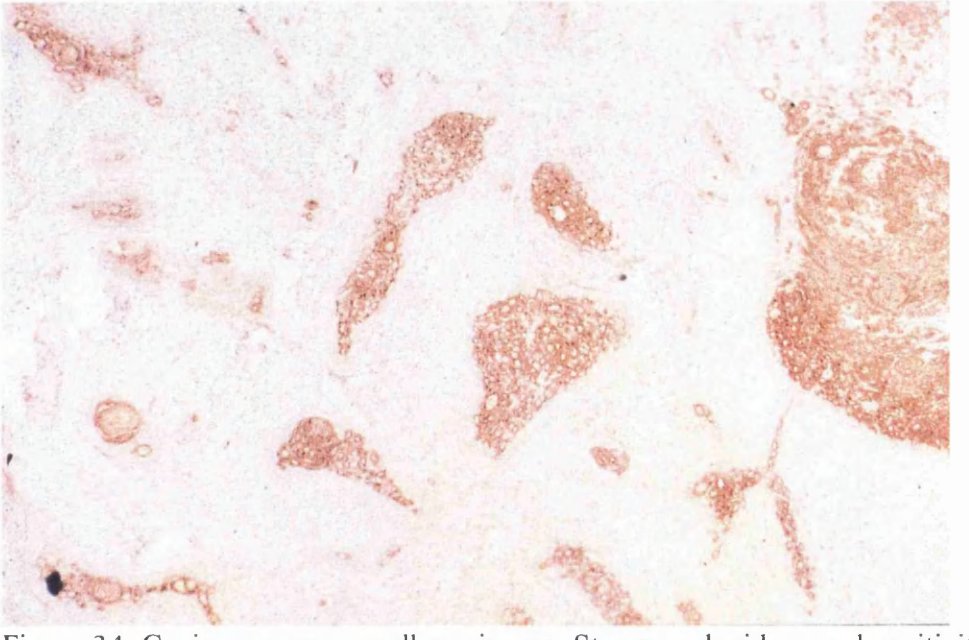

Figure 34. Canine squamous cell carcinoma. Strong and widespread positivity for CK16. CK16 (medium power)

Looking at the CK10 expression in the squamous carcinomas a slightly more variable pattern emerged. In two tumours in this study individual cells within keratin pearl formations (the most differentiated areas of the tumour) stained positive as did some spinous cells which were faintly stained. In the rest of the selected tumours no cells were positive for this marker of differentiation (Figure 33).

With the anti-CK16 antibody tumour cells with a basaloid appearance, where they appeared, were negative whereas there was widespread staining of cells resembling *stratum spinosum* in five sections. One of these is shown in Figure 34. In one case only individual cells or groups of cells were stained with rather poor intensity.

In the squamous carcinoma stained with the pan-cytokeratin the obviously epithelial "islands" of neoplastic cells were strongly positive. Some of the surrounding spindle shaped cells were weakly positive whilst some were clearly negative.

Papilloma

One example of a canine papilloma stained with haematoxylin and eosin is shown in Figure 35.

In the papillomas examined CK14 produced positive staining in all the epithelial cells. This staining varied in intensity between sections.

CK18 results were uniformly negative in this class of tumour.

A variable set of results for the anti-CK10 antibody was obtained in the papillomas. One was completely negative, two had individual positive cells and two showed widespread but weak positivity. In all cases the adjacent normal epithelial was

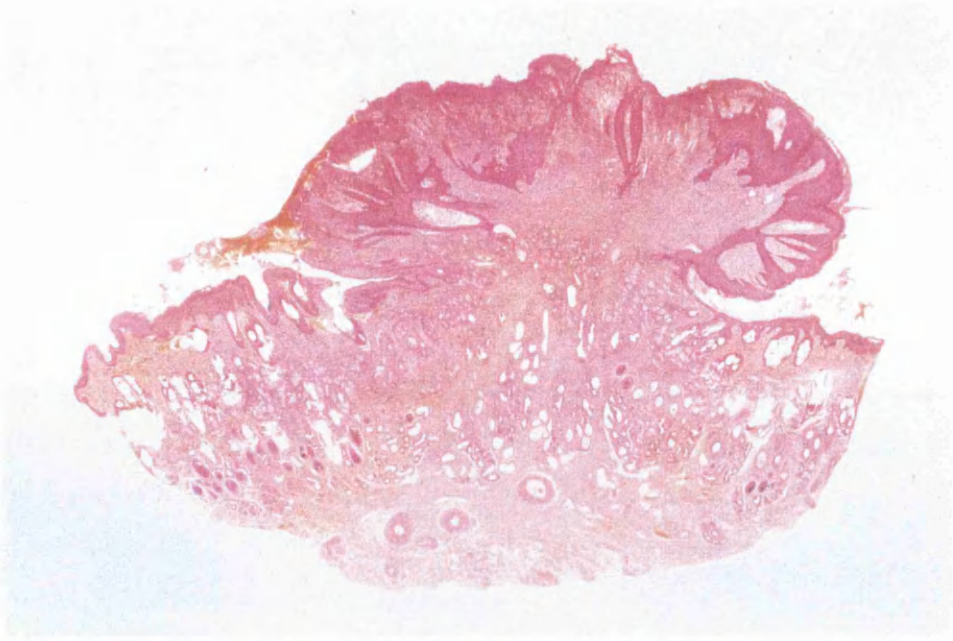

Figure 35. Canine papilloma. H&E (low power)

Figure 36. Canine papilloma. The papilloma is negative whilst the adjacent, rather hyperplastic, epithelium is positive. CK10 (medium power)

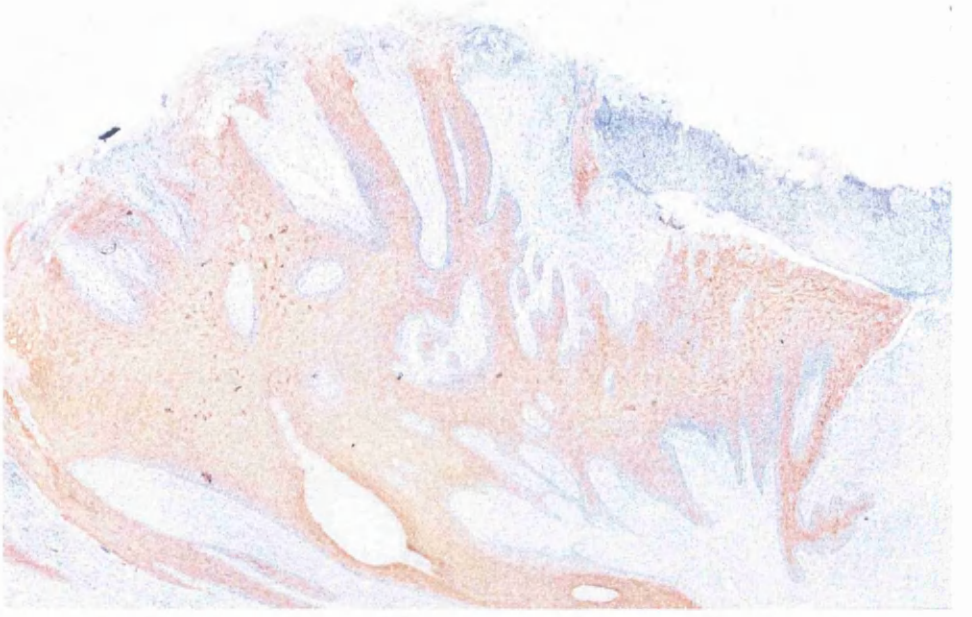

Figure 37. Canine papilloma. Widespread staining in cells with spinous morphology. CK16 (medium power)

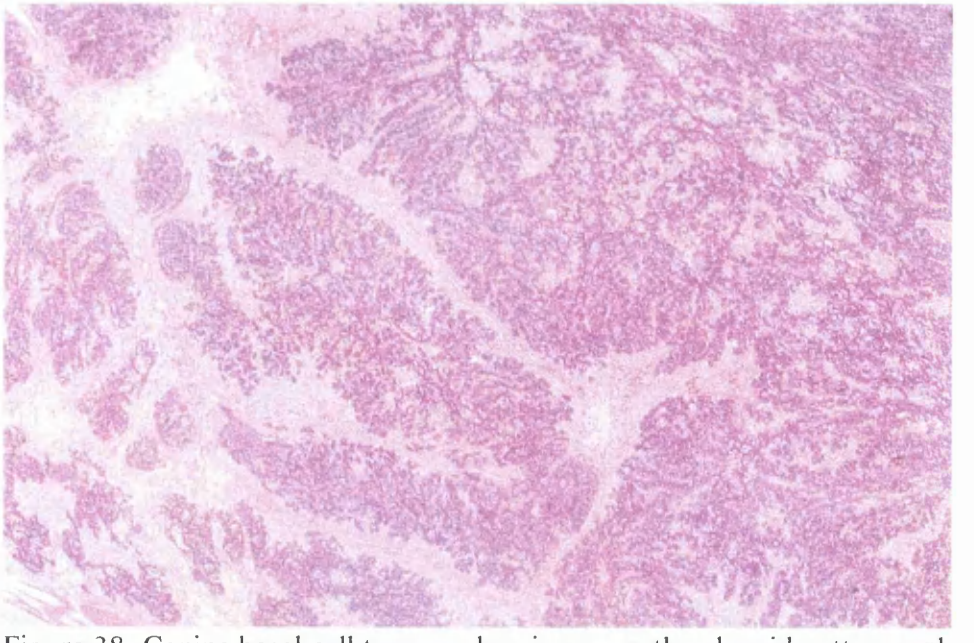

Figure 38. Canine basal cell tumour showing a mostly adenoid pattern and prominent fibrous stroma. H&E (medium power)

noticeably more stained with this antibody than was the papilloma. This is effectively illustrated in Figure 36.

The results with CK16 were largely positive in the suprabasal areas of the papillomas. Staining was restricted to cells with spinous layer morphology (Figure 37).

An inverted papilloma was stained using the pan-cytokeratin antibody and the cells resembling stratum spinosum were all strongly positive whilst the cores of the filliform growths and the parakeratotic crust were negative.

Basal cell tumour

Figure 38 shows one example of a canine basal cell tumour with a mostly adenoid pattern.

In four of the basal cell tumours a population of negative cells was present admixed with the positive ones (Figure 39) but in the others all the tumour cells were positive for CK14 (Figure 40). In no case of basal cell tumour was any positivity for CK18 detected.

Interestingly, no basal cell tumours had any positive staining when reacted with CK10 antibodies (Figure 41).

With the anti-CK16 antibody three basal cell tumours had low intensity staining in many cells and four had only small numbers of positive cells. Figure 42 shows a basal cell tumour where many cells are stained with a fairly low intensity. All the epithelial cells of the basal cell tumour tested were positive with the pan-cytokeratin marker.

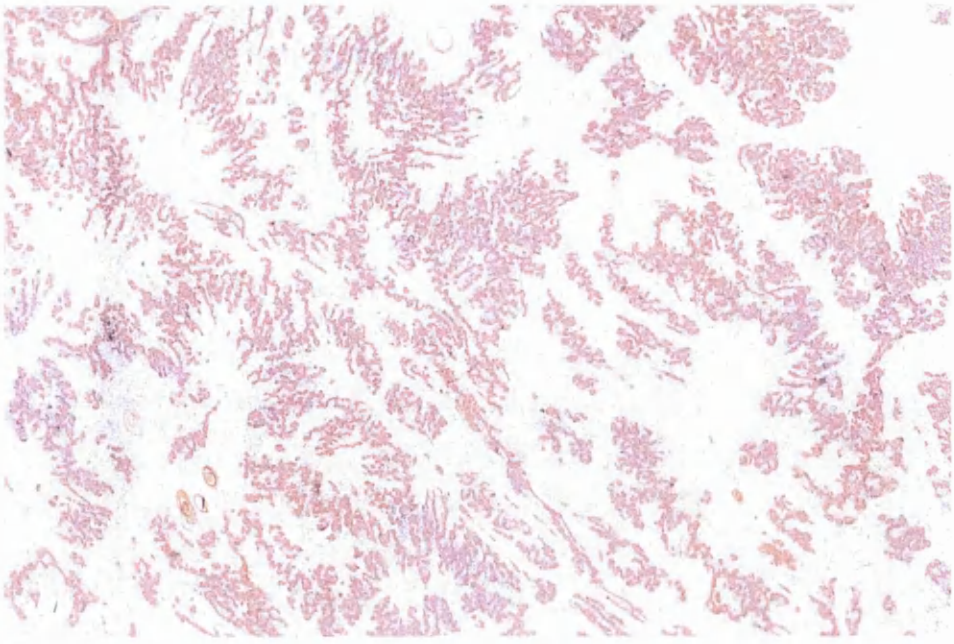

Figure 39. Canine basal cell tumour. A population of negative cells accompany the positive cells in this adenoid basal cell variant. CK14 (medium power)

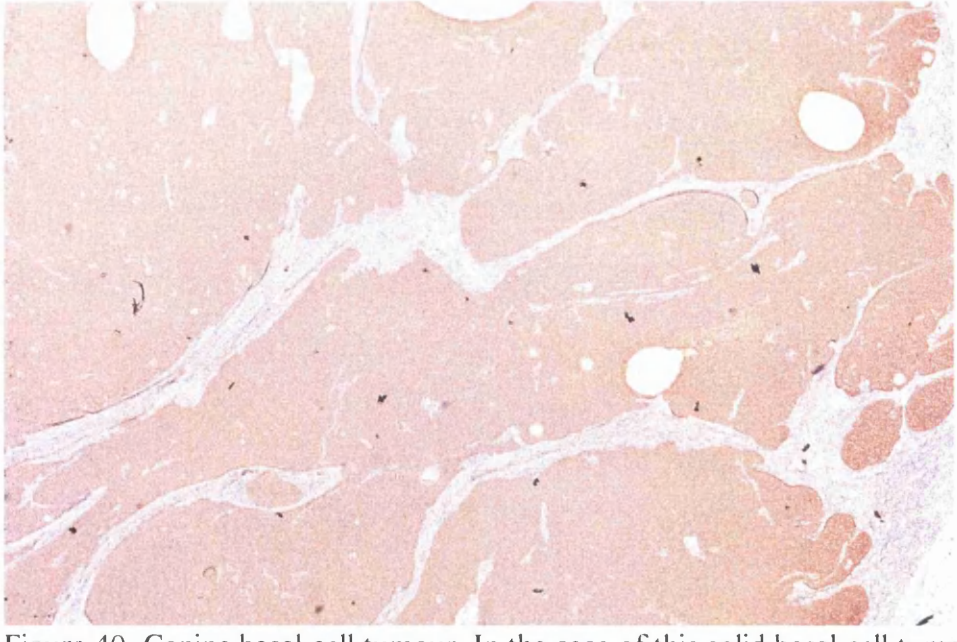

Figure 40. Canine basal cell tumour. In the case of this solid basal cell tumour all the cells are positive. CK14 (medium power)
CK14 (medium power)

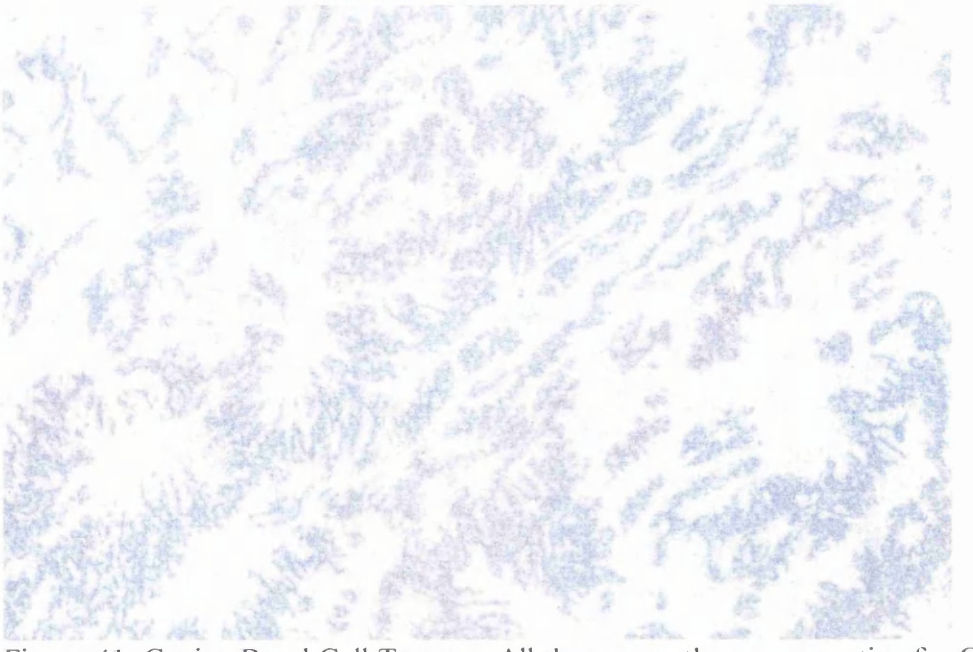

Figure 41. Canine Basal Cell Tumour. All these growths were negative for CK10. CK10 (medium power)

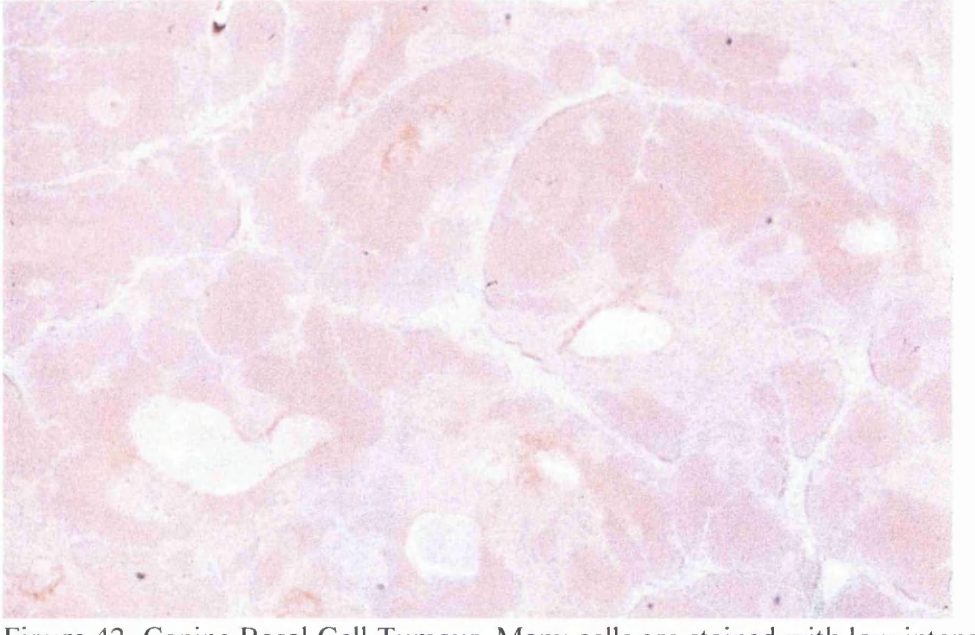

Figure 42. Canine Basal Cell Tumour. Many cells are stained with low intensity. CK16 (medium power)

Trichoepithelioma

An example of a typical canine trichoepithelioma stained with haematoxylin and eosin is shown in Figure 43.

Epithelial staining for CK14 was widespread in these growths with a consistent pattern in all sections where a few cells inside whorl formations and a few knots of cells were left unstained (Figure 44).

No positive staining was detected in trichoepithelioma sections stained with CK18.

The results obtained by staining these tumours with CK10 were rather variable. Three had occasional cell positivity in the walls of the larger keratinizing cystic elements and two were negative. One negative example is pictured in Figure 45.

With the anti-CK16 antibody there was a consistent pattern of strong focal staining in the whorls and follicular elements (Figure 46).

With the pan-cytokeratin all the epithelial structures of the trichoepithelioma examined were positively stained.

Pilomatrixoma

Figure 47 shows a conventionally stained canine pilomatrixoma.

In these tumours the epithelial lining of the cystic structures was wholly positive to CK14 in some sections and patchily positive in others (Figure 48). The staining was of variable intensity.

No epithelial structures of these tumours stained with antibody to CK18.

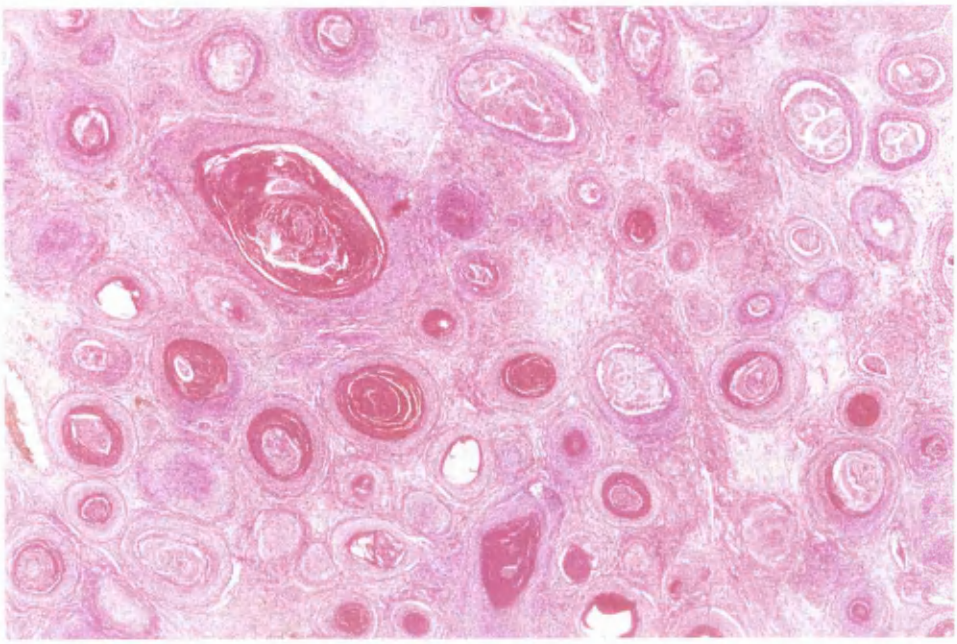

Figure 43. Canine Trichoepithelioma. H&E (low power)

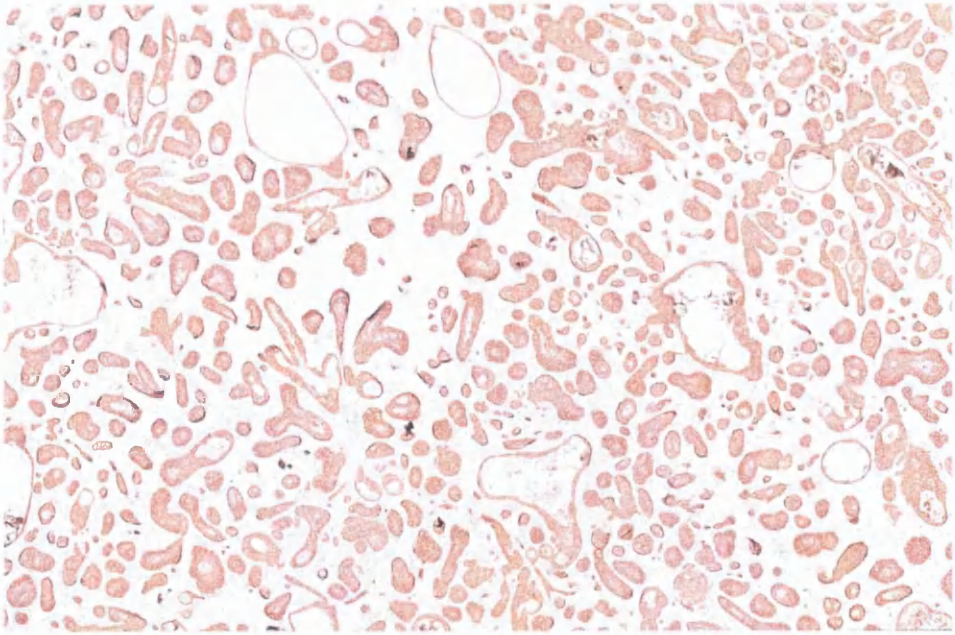

Figure 44. Canine Trichoepithelioma. Widespread positivity of all epithelial elements with only small groups of cells unstained. CK14 (low power)

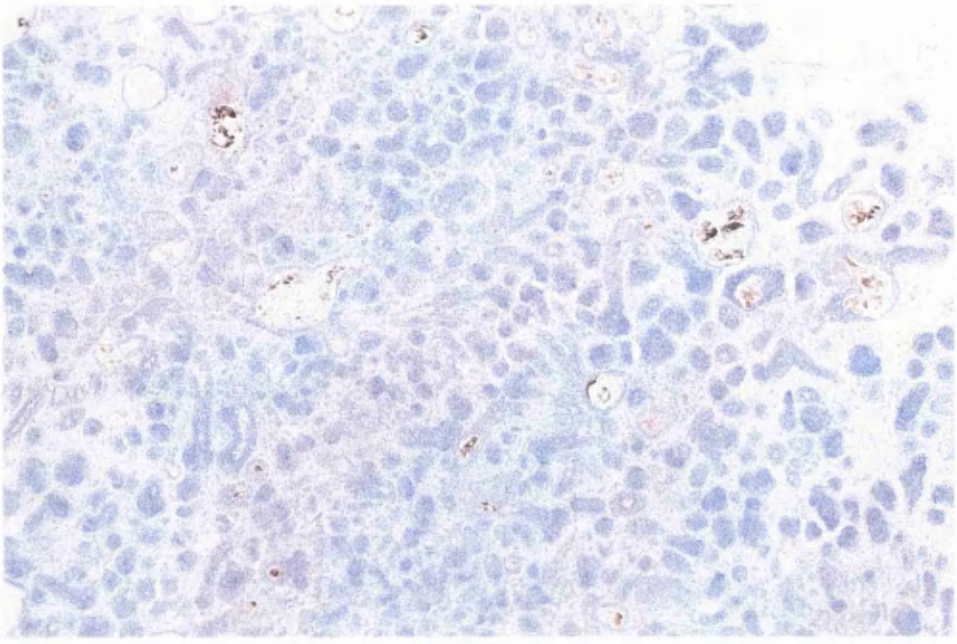

Figure 45. Canine Trichoepithelioma. No positive staining was observed in this tumour. CK10 (low power)

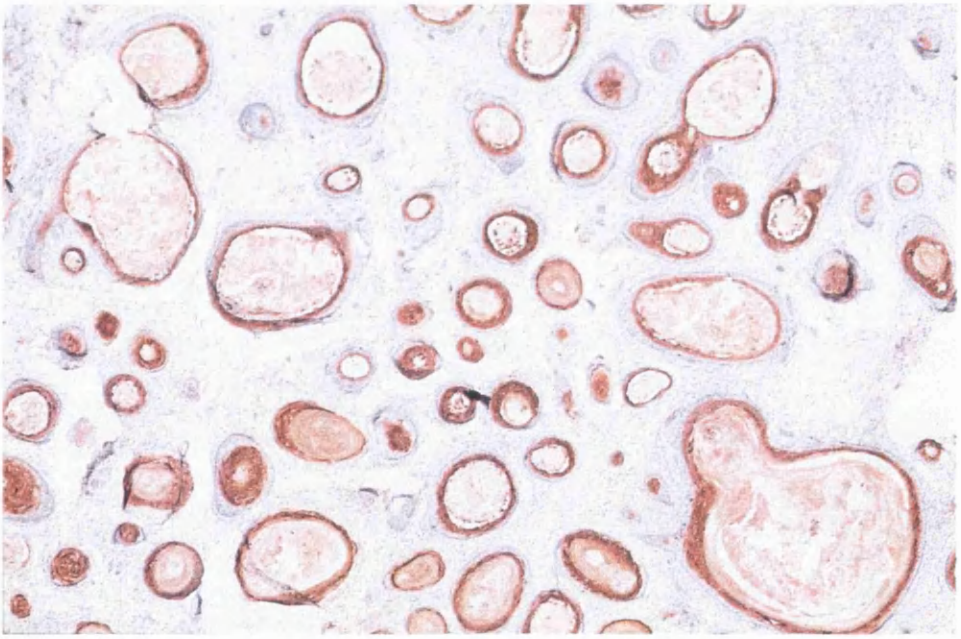

Figure 46. Canine Trichoepithelioma. All these tumours had strong focal staining in the walls of the follicular elements. CK16 (medium power)

The epithelial walls of the tumours were CK10 negative in all pilomatrixomas tested (Figure 49).

The inner (luminal) epithelial layers of the follicular/cystic structures showed positivity to CK16. Areas of squamous differentiation also tended to be CK16 positive. Focal strong positivity is illustrated in Figure 50.

With pan-cytokeratin staining many epithelial structures were wholly positive. In some of the larger cystic areas the staining was variable with negative areas.

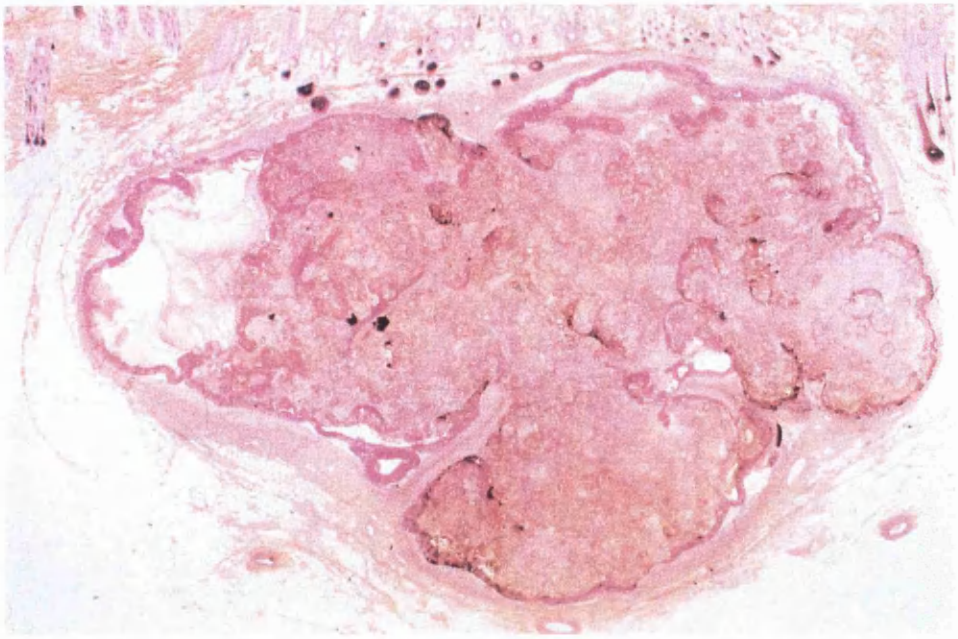

Figure 47. Canine Pilomatrixoma. Section shows a single large dermal cystic element with a well circumscribed epithelial wall. H&E (low power)

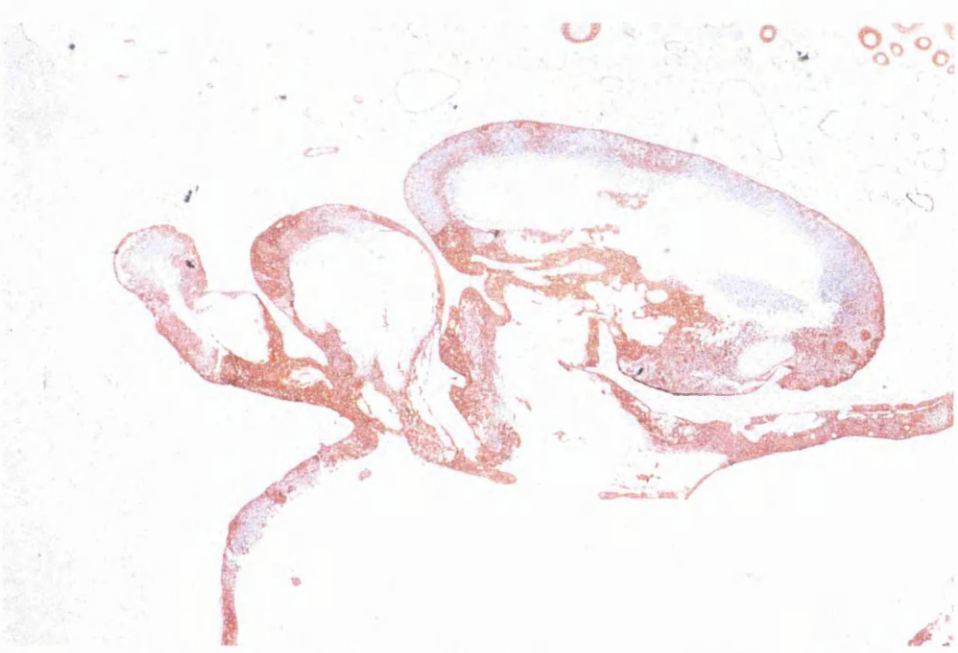

Figure 48. Canine Pilomatrixoma. Section shows part of the epithelial wall which is patchily stained. CK14 (medium power)

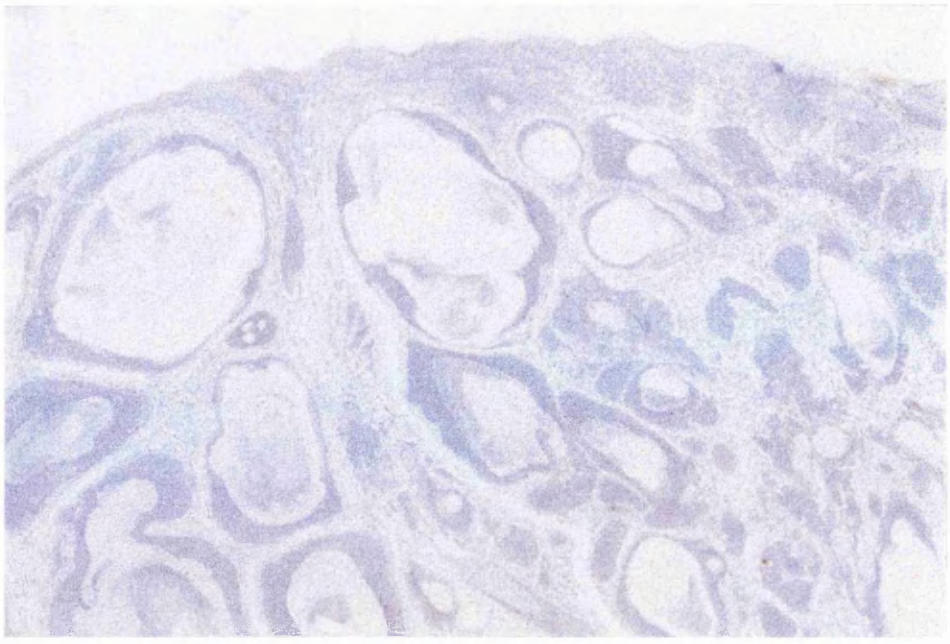

Figure 49. Canine Pilomatrixoma. The growth shown had multiple cystic elements, all unstained. No CK10 positivity was found in any pilomatrixoma tested. CK10 (medium power)

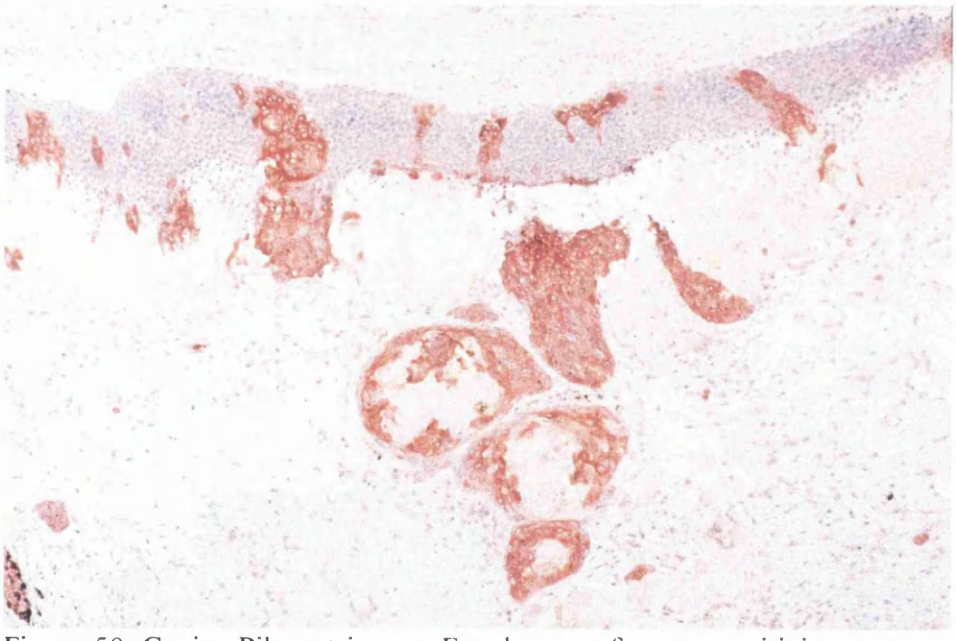

Figure 50. Canine Pilomatrixoma. Focal areas of strong positivity are seen. CK16 (medium power)

3.5.1) Discussion

The aim of this work was to investigate whether commercially available anti-human antibodies could be used to determine the cytokeratin profiles of canine skin and cutaneous epithelial neoplasms and if so to assess whether consistent profiles of protein expression existed within tumour types.

Of the tumours examined in this study the squamous carcinoma has the most serious implications for the animal as it is a malignant tumour and the papilloma is its benign counterpart. The basal cell tumour falls into a separate category of non-metastatic but locally aggressive growths and the trichoepitheliomas and pilomatrixomas are different examples of benign adnexal skin tumours. The profile of each of these was considered with reference to the staining pattern of normal canine skin.

With CK14 normal skin stained positive in the basal layer of the epidermis, all layers of the follicular epithelium and the cells of the adnexal glands. Staining of lesser intensity was also found suprabasally. This corresponds well with the expected profile as this cytokeratin is known to be expressed (along with its pair CK5) in the basal cells of stratified epithelium and to be progressively replaced in the upper layers of epidermis (Nelson and Sun 1983). In the papillomas all the epithelium is CK14 positive, presumably reflecting a failure in normal differentiation which would tend to produce down regulation of this keratin pair in suprabasal epithelium in favour of the differentiation associated CK10 (and its pair CK1). Similarly, in the squamous carcinoma CK14 was also detected in all epithelial cells.

In normal canine skin suprabasal epidermal cells, cells of the follicular infundibulum and those of the inner root sheath were CK10 positive. In the papillomas there was very little CK10 positivity with staining restricted to individual cells in the stratum spinosum or more widespread but very weak staining at this level. In the squamous carcinoma cases individual positively stained cells were detected in the keratin pearl formations where they were present (the most morphologically differentiated part of these tumours) and in a few spinous cells only. This would support the assertion that differentiation was not progressing in a normal manner in either papillomas or squamous carcinomas.

CK16 is a proliferation associated cytokeratin which together with its pair CK6 is found in proliferating stratified epithelium and in normal follicular epithelium (Eichner, Aebi and Sun 1986). In the normal canine skin tested CK16 was present in the hair follicles except in the outer root sheath and hair bulb and appeared very faintly in some epidermal cells. In both the papillomas and the squamous carcinomas suprabasal staining with this antibody was widespread in areas with spinous layer morphology.

Staining for these determinants provides a strong visual representation of the biological processes occurring in these tumours with regard to their cytokeratin expression. The expression of differentiation associated cytokeratins is very much reduced in comparison to normal skin whilst proliferation associated cytokeratins show greatly enhanced expression. The normal development of epidermis may be considered to be a balance between differentiation and proliferation producing orderly cell growth and it is apparent that the dysregulated growth which neoplasia represents is reflected in the cellular cytokeratin expression. Similarly, alterations in cytokeratin subclass expression

would be expected to accompany hyperproliferation in non-neoplastic conditions characterised by epithelial hyperplasia such as acral lick dermatitis.

Basal cell tumour and the two most common adnexal variants, the trichoepithelioma and the pilomatrixoma which were also chosen for this study may be considered under the umbrella term of benign skin adnexal tumours. These tumours have different patterns of differentiation but all are cured by excision. Whilst these tumours represent points on a continuum of differentiation ranging from wholly undifferentiated basal cell tumours to growths with purely follicular or glandular morphology it has been traditional in histopathology to group the lesions into named categories. The purpose of examining them for cytokeratin subclass expression was to see whether tumour specific patterns could be discerned.

In all three tumour types CK14 positivity was widespread although negative populations of epithelial cells were detected in all growths tested except four basal cell tumours. This marker is widespread in all stratified epithelium and its absence in the negative cells suggests they may be progenitor cells, uncommitted to stratified differentiation.

All basal cell tumours and pilomatrixomas were uniformly negative for CK10. This marker was identified in a few cells in three trichoepitheliomas and may indicate a definable, albeit small, difference in differentiation pathway in these tumours. In the normal skin CK10 was detected in the follicular infundibula and luminal root sheath but not in the outer root sheath so this finding suggests the cells of origin for the trichoepitheliomas derive from or differentiate towards the former areas and those of the pilomatrixoma do not.

The proliferation-associated CK16 was found at low levels or in small numbers of cells in the basal cell tumours and quite strongly in foci within the trichoepitheliomas. Within the pilomatrixomas specific areas also tended to stain. Expression of this cytokeratin is likely reflect rapidly growing areas in the tumours.

The opening review of this chapter drew attention to the controversy surrounding the classification of cutaneous epithelial tumours where the "lumpers" favour grouping the lesions under broad headings and the "splitters" endeavour to subdivide and categorise them. What does cytokeratin subfamily immunohistochemistry add to this debate? In the opinion of the author this work suggests that the phenotype of each tumour is the important factor.

What is meant by this is that cytokeratin expression profile is intimately linked to cellular morphology. Thus the keratin pearls in squamous cell carcinoma, which are the most morphologically differentiated, are the areas which express the differentiation-associated cytokeratin CK10. These cells are part of the same mass as their CK10 negative neighbours, yet they differ in protein expression. It is not novel to suggest that cells within a single tumour may be heterogeneous as new genetic alterations are known to occur during tumour progression, but the point here is that these same cells are morphologically, *phenotypically*, different also.

Across all the tumours tested areas of similar morphology showed similar staining patterns. For example, CK10 positivity where it appeared in the trichoepitheliomas was always seen in cells which in H&E sections were large with eosinophilic cytoplasm and vesicular nuclei resembling stratum spinosum.

Considered as a whole, the heterogeneous group of adnexal tumours seem to resist accurate placing within very precise classifications, not least because the morphology can vary markedly between different fields in the same tumour, although broad statements can usually be made about the predominant direction of differentiation. Equally, it is the opinion of the author that it is not possible to demonstrate whether any given tumour of this type actually arose from a cell of a certain location/type (i.e. outer root sheath) or whether it arose from an undifferentiated cell and "chose" to differentiate towards a particular phenotype so systems which place great weight on the cell of origin may not be entirely valid.

In summary, the work presented in this chapter demonstrates that it is possible to visualise the location of different cytokeratin subclasses in normal canine skin and epithelial cutaneous tumours using immunohistochemistry. Small differences between the tumour classes are apparent although, as would be expected, they show evidence of common differentiation pathways. A logical approach where the taxonomic diligence of the "splitters" is combined with the practical understanding that in some cases these divisions are artificial and contrived would perhaps provide the most meaningful way forward for veterinary histopathologists.

Chapter 4

Immunohistochemistry in tumour biology: Studies on the p53 gene product.

4.1.1.) Introduction to p53.

In this final chapter, moving on from the consideration of cell lineage markers as possible tools for the examination of tumours, immunohistochemical detection of accumulation of an abnormal gene product in neoplastic cells is investigated. Unlike the previously considered markers, detection of p53 protein offers no information on the derivation of a tumour but it may in time be found to aid accurate prognostic interpretation.

The p53 gene encodes a 53kd nuclear phosphoprotein which acts as a tumour suppressor. The gene product was originally discovered in the late 1970s by researchers working on rodents cells transformed by the SV40 virus and because of its association with transformation in these cells the protein was suspected to be a tumour antigen and the gene designated a proto-oncogene (Lane and Crawford 1979, Linzer and Levine 1979). Subsequently these p53 genes were discovered to be mutant forms (Hinds, Finlay and Levine 1989) and the normal (wild-type) protein product was identified and shown to be a negative regulator of the cell cycle and to suppress transformation (Finlay, Hinds and Levine 1989). p53 is involved in regulating the cell cycle but is not necessary for normal growth and development (Donehower et al. 1992). What this gene is pivotal in, it seems, is the response of the cell to "stressors"; genotoxic insults such as ionising radiation, and some chemicals or drugs (Kastan et al. 1991, Cox and Lane 1995).

In normal cells p53 is present at very low levels but if genetic damage occurs the protein accumulates and binds to target sequences of DNA and acts as a

transcriptional activator. The accumulated p53 protein binds to specific sites in the promoter of a downstream effector, the cyclin-dependant kinase (CDK) inhibitor p21 (*WAF-1, CIP-1*) and upregulates expression of this gene, which by inhibiting the action of these enzymes, mediates cell cycle arrest at G1. Cyclin-dependant kinases are necessary to phosphorylate the retinoblastoma (Rb) protein and effect cell entry into the S phase (Steinman et al. 1994). The resulting cell cycle arrest provides the cell with an opportunity to repair the damaged DNA before cell division occurs.

In addition to initiating cell cycle arrest wild-type p53 is also capable of inducing apoptosis in DNA damaged cells via a separate pathway. The mechanisms underlying the ability of p53 to induce apoptosis are still incompletely understood although they are known to be distinct from the p53 activation of the p21 cell cycle arrest pathway as deletion of the p21 gene does not affect radiation induced apoptosis (Deng et al 1995, Brugarolas et al 1995). p53 and the apoptosis-suppressing gene *bcl-2* act as effector and suppressor respectively of a common cell death pathway (Marin et al. 1994) and p53 has been demonstrated to decrease expression of *bcl-2* and enhance the expression of the related *bax* gene (Miyashita et al. 1994). It appears that the active form of *bcl-2* may be a *bcl-2/bax* heterodimer (Yin, Oltvai and Korsmeyer 1994) and that increased expression of *bax* favours the formation of *bax/bax* homodimers which antagonise the action of *bcl-2* (Oltvai, Milliman and Korsemeyer 1993). Thus if the DNA is irretrievably damaged the cell is driven into p53-mediated apoptosis, preventing propagation of the genetic error.

Mutations of p53 are the most common genetic alterations found in human cancer, appearing at high frequency in a diverse range of neoplasms (Nigro et al. 1989). The p53 gene is well conserved in evolution with regions of high homology being present in mammals, birds, fish and amphibians (Montenarh 1992). The highly homologous exons 2, 4, 5, 7 and 8 code for five clusters of amino acid sequences known as domains I-V. Of these II-V fall into exons 5-8, the area of the gene which has attracted most attention (Montenarh 1992). It is within these highly conserved and presumably functionally critical regions of the gene that the vast majority (98%) of these cancer-associated mutations occur (Hollstein et al. 1991). These mutations translate as alterations of amino acid sequence which cluster in the highly conserved central region of the protein as illustrated in Figure 51.

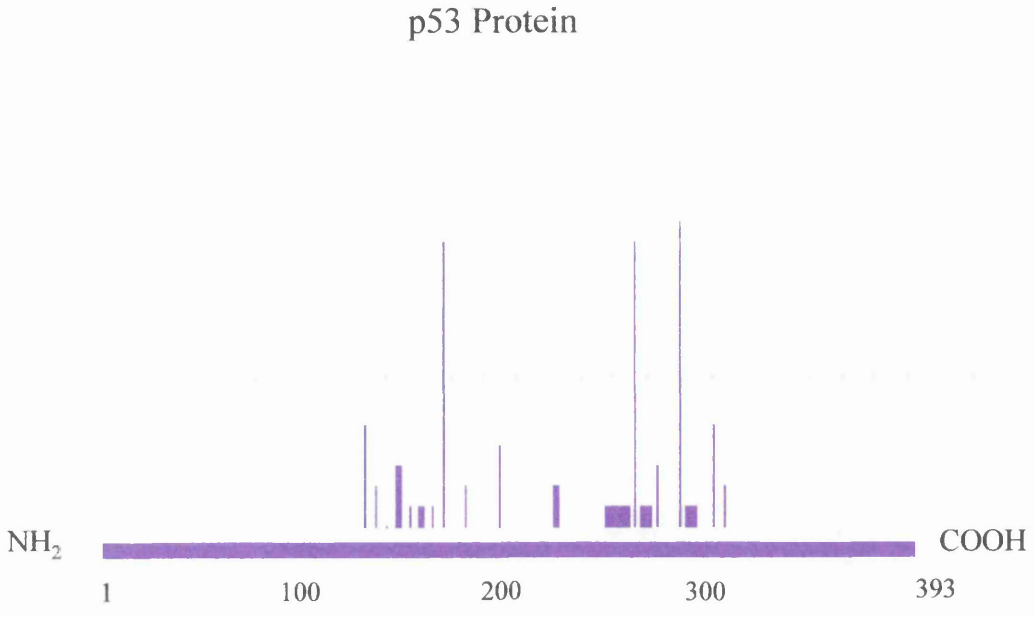

Figure 51. Frequency and distribution of amino acid changes in the p53 protein in cancer associated mutations (adapted from Levine, Momand and Finlay 1991).

Mutations of the p53 gene may have several consequences. In most human cancers in which p53 mutation has been studied; colon, breast, brain and lung tumours, (Bartek et al. 1990, Offerhaus et al. 1992, Meling et al. 1993, Iggo et al. 1990) many of the neoplasms show a "loss" of both p53 alleles usually through deletion of one and point mutation of the other. This suggests that these particular mutations are recessive to the wild type allele and only contribute to tumorigenesis when wild type activity is lost.

With mutation there can be a loss of function effect as all mutants lose the ability to bind to the specific p53 target sites (Vogelstein and Kinzler 1992) and hence cannot activate effector genes meaning that the protective guard against genetic instability which wild type p53 provides is lost. In addition, a gain of function effect which can act in the heterozygous state can occur. Some, although not all, forms of mutant p53 can act in a dominant negative fashion by complexing with and inhibiting the action of wild type p53 protein (Vogelstein and Kinzler 1992, Gannon, Greaves, Iggo and Lane 1990). Furthermore, some p53 mutants can act to enhance transformation by mechanisms which do not involve complexing with the normal protein (Levine, Momand & Finlay 1991).

4.1.2) Immunohistochemistry of p53: a possible role in diagnostic pathology

There is no doubt that in terms of the molecular biology of tumours p53 is a gene of major, possibly even central, importance. However this fact, of itself, would not contribute much towards the discipline of diagnostic histopathology. What makes p53 so interesting from a diagnostic viewpoint is that, in addition to abolishing functional activity, genetic mutation leads to the production of a stabilised mutant protein which accumulates in the cell to levels detectable by immunohistochemistry. In normal cells p53 levels are too low for this to be possible (Montenarh 1992). In certain circumstances p53 accumulation is due, not to genetic mutation, but to post-translational binding of the normal protein by cellular or viral proteins (Momand et al. 1992, Scheffner et al. 1990, Yew and Berk 1992). This also represents loss of p53 biological activity.

Therefore assessment of p53 accumulation by immunohistochemistry provides a simple method of examining the p53 functional status of these cells. In fact, in cases where the functional activity of the p53 protein is altered by post-translational changes immunohistochemistry will demonstrate the abnormality where the more sophisticated technique of gene sequencing will not.

As p53 represents such a pivotal point in cell cycle control a great deal of research has been carried out in recent years to investigate the prognostic significance of p53 dysregulation in tumours. In the field of human medicine strong associations between p53 expression and tumour progression have been found in colorectal adenocarcinoma, (Baas et al 1994, Costa et al 1995) some

types of lymphoma, (Said et al 1992, Villuendas et al 1992) breast cancer (Barnes et al 1993, Thor et al. 1992) and prostatic cancer (Visakorpi et al. 1992). As yet very little work has been published regarding p53 in the domestic species.

The aim of this study was to investigate p53 immunohistochemistry in routinely processed tissues from selected tumours of domestic animals using commercially available anti-human p53 antibodies.

4.2.1.) Materials and Methods

In this study ninety three tumours of domestic animal origin were examined. These were comprised of forty equine tumours, thirty one bovine tumours and twenty two canine tumours. In each species relatively common malignancies were chosen. In the horse squamous cell carcinoma, lymphosarcoma, sarcoid and pituitary adenoma cases were examined, the cattle samples were from squamous carcinoma and lymphosarcoma cases and the dog tumours examined were squamous carcinomas and pulmonary carcinomas.

The equine groups were selected to provide examples of different types of neoplasm for comparison.

Of the sixteen equine squamous carcinomas examined, seven were ocular (three of these confined to the third eyelid), four were penile (one is shown in Figure 52) and the remainder were single examples of preputial, palatine, vulval, perianal and gastric tumours. Squamous cell carcinoma is the most common equine neoplasm seen by this department (Figure 53) and can be locally aggressive and metastatic.

Lymphosarcoma is also quite frequently seen (Figure 54) and can sometimes be difficult to differentiate from non-neoplastic lymphoid infiltration. The equine lymphosarcoma sections in this study were from six alimentary and two splenic cases. Sarcoids are a special category of non-metastatic, locally aggressive tumours (Figure 55) and pituitary adenomas are functionally active but benign growths.

Figure 52. An example of equine penile squamous carcinoma. The tumour is very large and ulcerated.

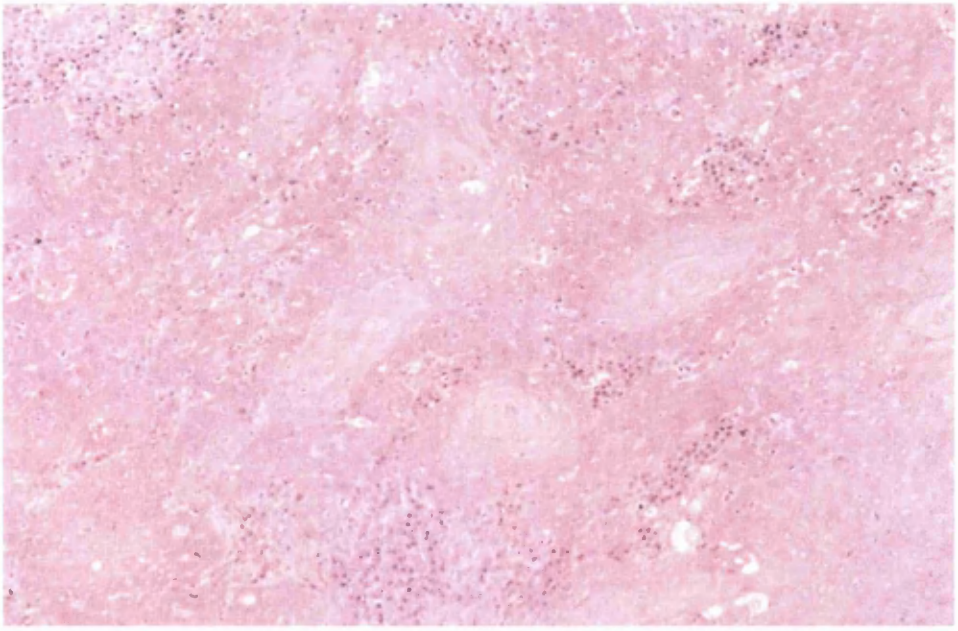

Figure 53. Equine squamous cell carcinoma. Large eosinophilic cells form nests in the dermis. Some inflammatory cells are present. H&E medium power.

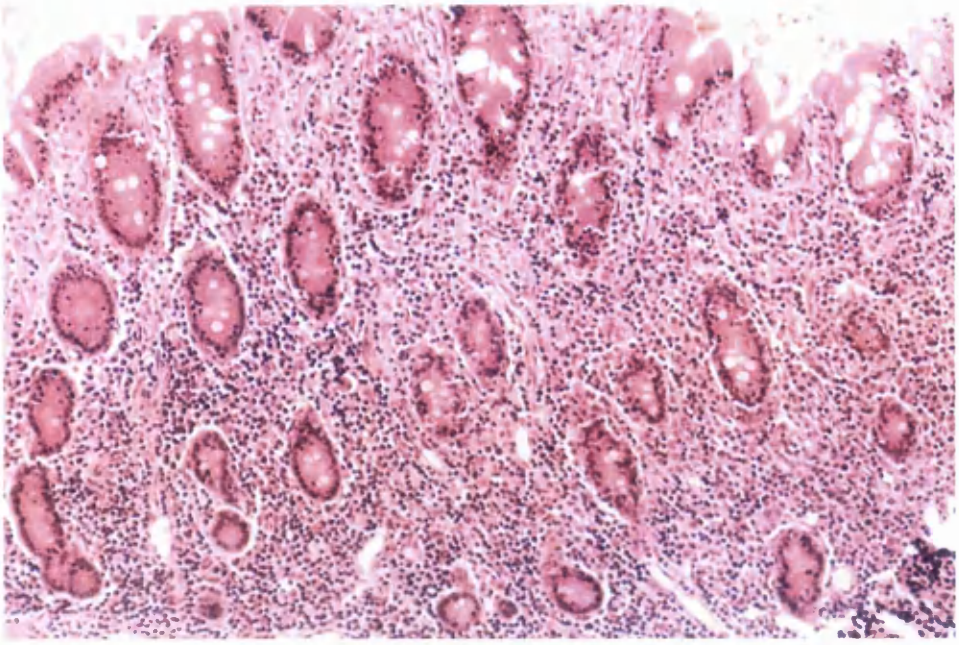

Figure 54. An example of equine alimentary lymphosarcoma. Malignant lymphocytes invade the lamina propria. H&E (medium power)

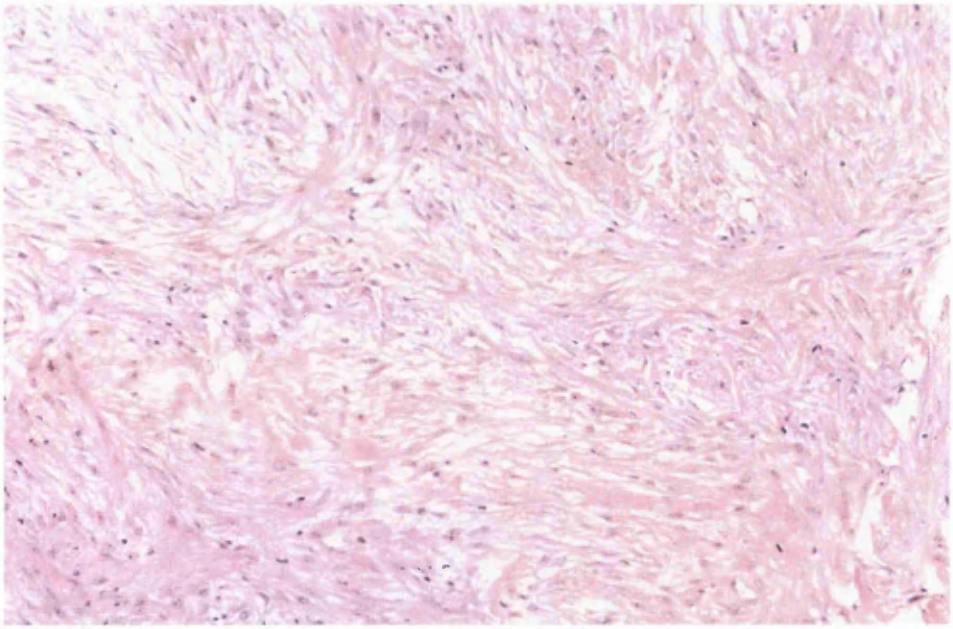

Figure 55. Equine sarcoid. The section shows interweaving bundles of spindle shaped cells. H&E (medium power)

Of the bovine carcinoma series, seven were squamous carcinomas of the upper alimentary tract: one from the pharynx, three from the oesophagus and three from the rumen. Six were uterine carcinomas. Also included were single examples of squamous carcinoma of the eyelid, the larynx, the nasopharynx and facial skin. One tumour was widely disseminated throughout the mediastinum but the primary site was not identified and two were biopsy samples where the primary site was not known.

The canine lung tumours examined were all necropsy specimens and were composed of six bronchiolar-alveolar adenocarcinomas, three papillary adenocarcinomas and two anaplastic carcinomas. The canine squamous carcinomas were biopsy specimens and three were digital, two tonsillar, two lingual, one oral (unspecified), one perianal, one preputial and one from hindlimb skin.

Immunohistochemistry was carried out on formalin fixed paraffin embedded tissues taken from the pathology department archive. Sections of 5-7μm were cut onto pre-coated slides (APES: 3-aminopropyltriethoxysilane) to promote adhesion and dried overnight at 37°C. After de-waxing the sections were immersed in citrate buffer (10mM, pH 6.0) and processed in a microwave oven at 650W for five minutes, four times in quick succession. The container was turned at the breaks in processing to minimise the effects of potential hot-spots in the microwave oven. The slides were then allowed to cool at room temperature for twenty minutes. Next, a five minute incubation with 3% hydrogen peroxide in water to block endogenous peroxidase activity was performed. This was followed by ten minutes blocking, to reduce background staining, using buffered

serum from the species in which the secondary antibody was raised. Incubation with the primary antibody for one hour at room temperature in a humidity chamber was followed by the application of a standard streptavidin-biotin peroxidase method (Vectastain Universal Quick Kit, Vector Laboratories, Burlinghame, CA, USA.) for detection. AEC (3 amino-9 ethylcarbazole) was used as a chromogen and the slides were counterstained with Meyer's haematoxylin. With AEC aqueous mounting is required so the sections were coated with Supermount™ (BioGenex, San Ramon, CA, USA.) and allowed to dry before using DPX (BDH Laboratory Supplies, Poole, England.) to mount the coverslips.

Monoclonal antibodies

In the equine and bovine tissues two monoclonal mouse anti-human p53 antibodies, DO-1 (Immunotech, Marseilles, France.) and PAb240 (Serotec, Oxford, England.) were used and in the canine a third, PAb1801 (Pharmingen, San Diego, CA, USA.) was also tried. DO-1 and PAb1801 each recognise epitopes expressed on both wild-type and mutant p53 whilst PAb240 is specific for mutant protein. DO-1 and PAb1801 were supplied pre-diluted and PAb240 was used at dilutions of 1:100 and 1:50.

Tissue sections from a known p53 positive human colonic adenocarcinoma were used as positive controls. In the negative controls which accompanied each test section the primary antibody was replaced by buffered serum. In addition, normal equine skin sections were included.

After immunostaining the sections were scored semi-quantitatively by counting the p53 positive tumour cells in each section and grouping them as follows: 0%/- , <5%/+ , 5-25%/++ , 26-50%/+++ , >50%/++++ . Each section was scored by two observers (Hazel Johnston and Hal Thompson).

4.3.1) Results

The results of the immunostaining, (Table 12.) show that extensive p53 positivity was present in the equine squamous carcinoma group with more limited positive reactions in the equine lymphosarcoma and sarcoid cases. Two bovine squamous carcinoma cases were also positive but no specific staining was identified in the the bovine lymphosarcomas, the equine pituitary tumours nor any of the canine tumours.

Of the equine squamous cell carcinomas fifteen out of sixteen tumours stained with DO-1. These tumours had specific nuclear staining and in some cases almost all the tumour cell nuclei were strongly positive (Figures 56 and 57). The same tumours stained less well with PAb240 which overall produced poorer staining intensity and failed to stain some tumours which were positive with the first antibody. The one negative result with DO-1 was from a penile squamous carcinoma. Out of eight equine lymphosarcomas, one alimentary tumour gave a positive result with both antibodies (Figure 58) and no staining was observed with the pituitary lesions. Of the ten equine sarcoids examined two had distinct staining with DO-1 which was absent with PAb240 (Figure 59). Interestingly, this staining had a perinuclear pattern (see discussion).

In the bovine series two carcinoma cases had positive nuclear staining with both antibodies. These were the eyelid lesion, in which almost all the tumour cell nuclei were stained, (Figure 60) and the pharyngeal squamous carcinoma in

Tissues	Antibodies														
	DO-1					PAb240					PAb1801				
	-	+	++	+++	++++	-	+	++	+++	++++	-	+	++	+++	++++
Equine															
Squamous carcinoma	1	4	2	4	5	6	2	2	2	4					
Lymphosarcoma	7	0	0	1	0	7	0	0	1	0					
Sarcoid	8	0	0	2	0	10	0	0	0	0					
Pituitary adenoma	6	0	0	0	0	6	0	0	0	0					
Bovine															
Carcinoma	18	1	0	0	1	18	1	0	0	1					
Thymic lymphosarcoma	11	0	0	0	0	11	0	0	0	0					
Canine															
Squamous carcinoma	11	0	0	0	0	11	0	0	0	0	11	0	0	0	0
Lung carcinoma	11	0	0	0	0	11	0	0	0	0	11	0	0	0	0

Table 12. Results of immunohistochemical staining of tumour sections using monoclonal mouse anti-human p53 antibodies. The sections were classed by percentage of tumour cells showing positive staining such that 0 / - , <5% / + , 5-25% / ++ , 26-50% / +++ , >50% / ++++ .

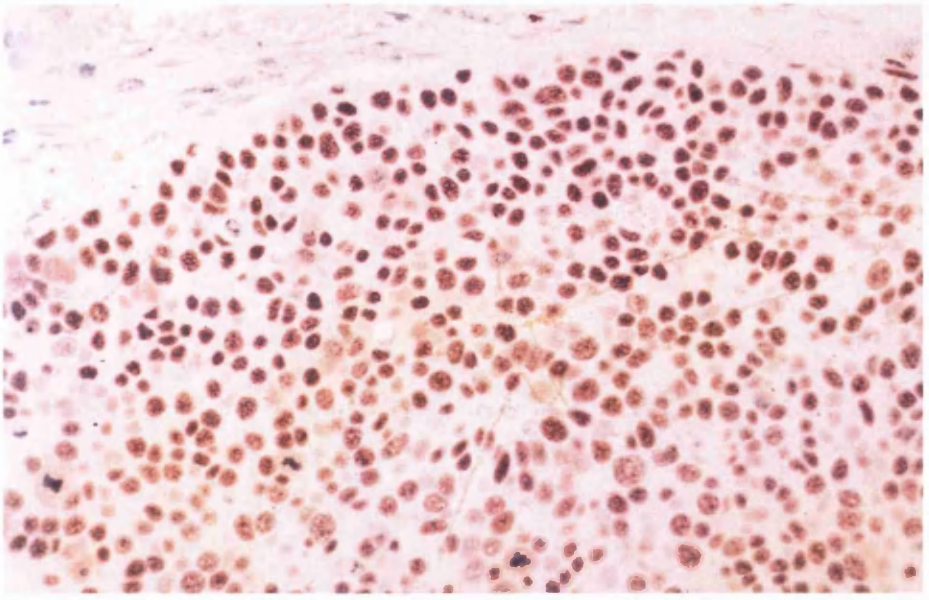

Figure 56. In this particular equine squamous cell carcinoma almost all the tumour cell nuclei are strongly positive. DO-1 (high power)

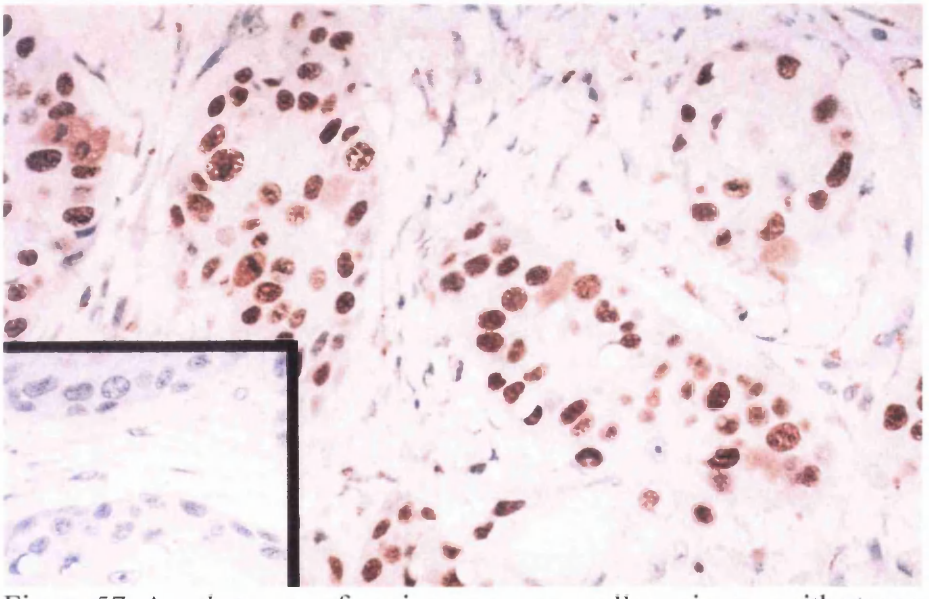

Figure 57. Another case of equine squamous cell carcinoma with strong nuclear positivity in almost all tumour cells. DO-1 (high power)
Inset: negative control showing unstained nuclei.

Figure 58. One equine alimentary lymphosarcoma was positive with DO-1 and Pab240. This sheet of cells was part of a tumour nodule. DO-1 (medium power)

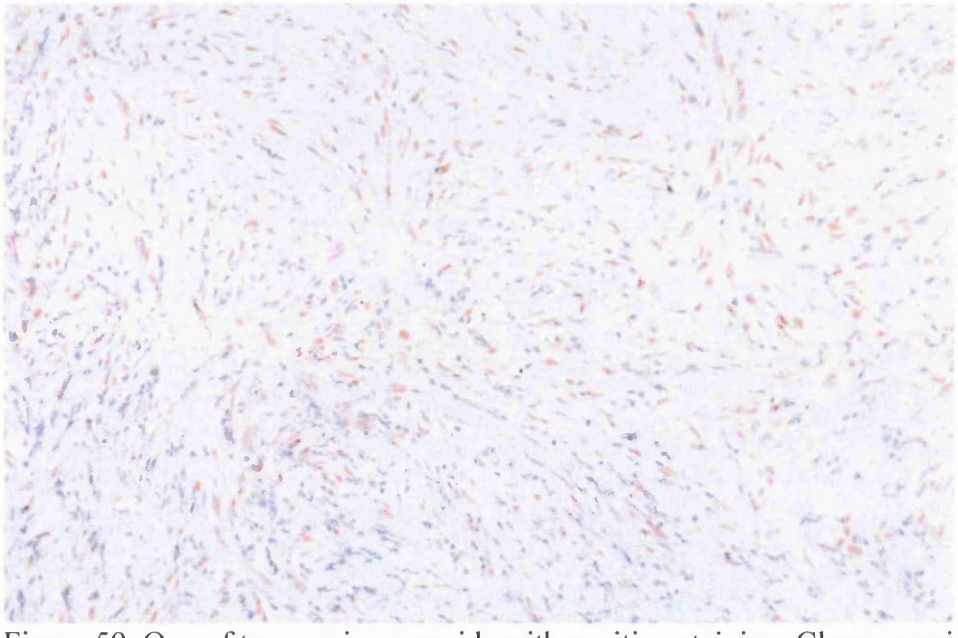

Figure 59. One of two equine sarcoids with positive staining. Close examination reveals that this staining was cytoplasmic and mostly perinuclear. DO-1 (medium power)

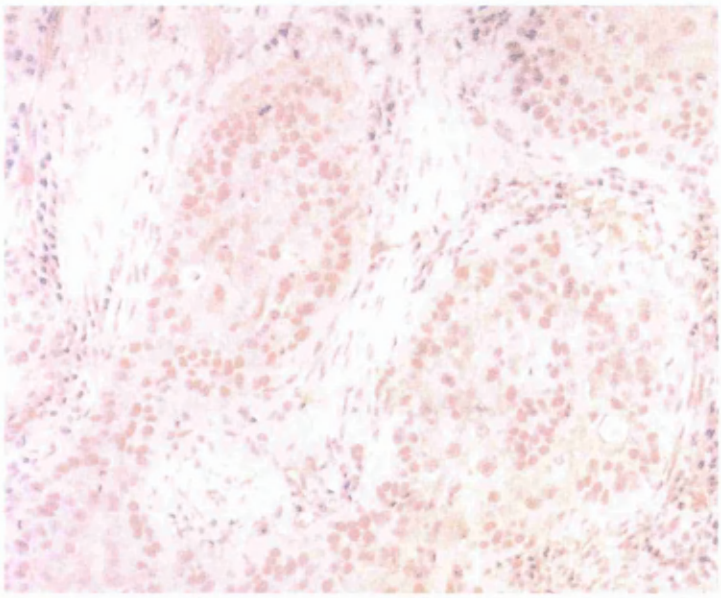

Figure 60. Bovine squamous cell carcinoma. Most of the tumour cell nuclei are positive although the staining intensity is not as great as in the equine cases. DO-1 (medium power)

which a small number of tumour nuclei were positive. Again, DO-1 produced better staining intensity. The bovine lymphosarcomas yielded no specific staining and showed a high degree of background present on both test sections and negative controls. This finding is in agreement with the observation made in chapter two that bovine tissues appear prone to high background staining despite careful blocking treatment.

No specific p53 reactivity was detected in the canine tissues examined in this study even using a third antibody, PAb1801,in addition to DO-1 and PAb240.

4.4.1.) Discussion

In this study we aimed to determine whether p53 status in selected tumours of domestic animals could be assessed using commercially available anti-human p53 antibodies. In particular we were interested in antibodies known to be effective for immunohistochemistry in formalin fixed tissue.

The technique proved most rewarding in the equine tumours where extensive expression of high levels of p53 protein were found in one particular neoplasm, the squamous carcinoma. The antibodies used also appeared to be successful in the bovine tumours, detecting p53 accumulation in two carcinoma cases. However, no p53 accumulation was detected in the canine tissues in this study.

Looking in detail at the equine results we see that fifteen out of the sixteen cases examined were positive with DO-1 and ten were positive with PAb240. In several of the tumours almost all of the neoplastic cells were stained. In all cases where staining was apparent DO-1 produced better staining intensity and the sections which were positive using DO-1 but failed to stain with PAb240 were those with a comparatively less intense stain. This suggests that the differences in detection are due to sensitivity or suitability of the antibodies with DO-1 being better for p53 detection in paraffin sections of equine (and bovine) tissues. The other possibility is that the discrepancies are due to detection, by DO-1, of stabilised wild type protein which PAb240 does not recognise. The author does not believe this to be the case as the differences consistently relate to intensity of

staining where tumours with faint but recognisable staining with DO-1 have no discernible staining with the other antibody.

Two out of ten equine sarcoids stained positive with DO-1 but not with PAb240. This staining was quite intense and the difference in result between these antibodies does raise the possibility of detection of stabilised wild-type protein. However, cytoplasmic staining is rarely associated with wild type p53. As papillomavirus has been implicated in the aetiology of the sarcoid the possibility exists that this staining may represent wild-type p53, which PAb240 would not recognise, bound to a viral oncoprotein. However, the known p53 binding protein of HPVs (Human Papilloma Virus) 16 and 18, which are involved in carcinoma of the uterine cervix in humans, is E6, which inactivates p53 by promoting its degradation, not by sequestering and stabilising it (Scheffner et al. 1990). It has been demonstrated that neoplasms may harbour both HPV transforming proteins and p53 mutations (Helland et al. 1993). Another possibility is that certain genetic factors are acting to interfere with the subcellular localisation of p53. For example, *bcl-2* and *c-myc* acting in concert have been shown to prevent nuclear localisation of p53 at critical points in the cell cycle (Ryan et al. 1994).

The results of the bovine series corresponded with the finding in the horse that DO-1 produced better staining, but neither positive bovine tumour produced as intense a stain as shown by most of the equine tumours.

The complete negativity of the canine results was perhaps surprising as our sample selection was weighted to enhance the likelihood of p53 overexpression in the test group. Thus pulmonary carcinomas were examined because mutations

of p53 are recognised in human lung cancer patients (Fontanini et al. 1994, Iggo et al. 1990). Dogs are allowed to live a full lifespan and are exposed to the same environmental conditions as their human owners which makes them interesting models for comparison with man. However, none of the canine tumours in this study showed specific reactivity with any of the antibodies used, therefore either these antibodies do not recognise canine p53 or no p53 accumulation exists in any of these tumours. The use of several antibodies which recognise different epitopes should increase the chance of detection but it remains possible that none of these are appropriate for use in the dog. It has been suggested that the overall incidence of p53 mutation is lower in the dog than in man and in one study where the highly conserved exon eight of the p53 gene was sequenced only one tumour out of fifteen was found to have a mutation (Mayr et al. 1994). It may be that these particular neoplasms in the dog do not involve p53 mutations whilst others do. Sagartz et al. (1996) used a polyclonal anti-p53 antibody (CM-1, Signet Laboratories, Dedham, MA) to investigate osteogenic tumours in dogs and found that the p53 protein was overexpressed in the majority of osteosarcomas tested. This finding appeared to correlate with aggressive biological behaviour in the tumours. In a recent study Wolf et al. (1997) used the same antibody to examine canine colorectal tumours. Their findings were contrary to data derived from studies on similar studies on humans as benign lesions were found to stain with greater frequency than malignant lesions. The authors suggest this may represent an early role for p53 alterations in these tumours and conclude that p53 is not a prognostically useful marker in this situation.

Variability in technique is a major problem in assessing the significance of immunohistochemical results. Different methods of fixation and retrieval, different concentrations and sensitivities of antibodies, incubation times and conditions and detection systems all influence results. In addition, the quantification of results is subjective.

In this study the antibodies were selected for their ability to react with formalin fixed paraffin embedded tissue to allow examination of archival materials. The range of antibodies available is ever increasing but the numbers applicable to formalin fixed tissue are still comparatively small. The manufacturers had no data regarding the use of these antibodies in the species examined here. By testing on human control tissue microwave processing was found to be necessary to unmask the relevant epitopes, even in the case of PAb240 where the data sheet stated this was not the case. This type of pre-treatment is associated with problems of the tissues floating off the slides, becoming overheated and showing increased background staining. In these experiments occasional sections proved very difficult to process due to floating but careful turning of the container during processing helped to minimise section overheating. Non-specific background staining occurred in some of the canine tissues and bovine lymphosarcomas but was not a significant problem. There is a concern that microwave processing and other pre-treatment could generate spurious positive results but if suitable control sections are used we do not believe this to be a problem, at least in terms of p53 which due to its short half life of less than twenty minutes (Reich, Oren and Levine 1983) has a normal cellular concentration below the threshold of detection for immunohistochemistry (Montenarh 1992). We found no specific

reactivity in normal tissue nor in the negative control sections and non-neoplastic cells in positive tumour sections were unstained. This strongly suggests that the processing itself does not cause staining.

Largely, immunohistochemical detection of p53 is a marker of genetic mutation but there are exceptions to this. Immunohistochemically negative cases can occur in the presence of a mutated gene if the mutation is a stop signal or a gross deletion where no p53 protein is produced at all. This situation is rare but is recognised in a small proportion of human malignancies (Wynford-Thomas 1992). Similarly, some mutations may not stabilise the protein sufficiently for immunohistochemical detection and it is not clear at present what degree of functional impairment these mutations cause, as partial stabilisation could mean only partial inactivation. Conversely, stabilised protein can be detectable in the absence of genetic mutation due to binding of the normal p53 protein by the transforming proteins of oncogenic DNA viruses such as E1b of adenovirus type 5 (Yew and Berk 1992) or cellular gene products like *mdm2* (Momand et al 1992, Wu et al. 1993) which has a regulatory effect on p53 and itself can be a target for mutation in some neoplasms.

With the caveats mentioned above, immunohistochemistry is a simple and effective way of examining p53 functional status in cells and in fact can be more informative than the more laborious and expensive process of DNA sequencing. This study suggests that p53 assessment in domestic animal tumours may in the future prove an adjunct to traditional methods of microscopic tumour diagnosis and prognostication.

References

AIDA, Y., OKADA, K. & AMANUMA, H. 1993
Phenotype and ontogeny of cells carrying a tumour-associated antigen that is expressed on bovine leukaemia virus induced lymphosarcoma.
Cancer Res. 53: 429-437

ALEXANDER, R., ROBINSON, W.F., MILLS, J.N., SHERRY, C.R., SHERARD, E., PATERSON, A.J., SHAW, S.E., CLARK, W.T. & HOLLINGSWORTH, T. 1989
Isolation of feline immunodeficiency virus from three cats with lymphoma.
Austral. Vet. Pract. 19: 93-97

ALLDINGER, S., BAUMGARTNER, W. & ORWELL, C. 1993
Restricted expression of viral surface proteins in canine distemper encephalitis.
Acta Neuropathologica 85: 635-645

ALTMAN, N.H. & STREETT, C.S. 1968
Bovine Thymoma: Case Report.
Am. J. Vet. Res. 29: 2411-2414

ARONSOHN, M.G. 1985
Canine Thymoma.
Vet. Clinics North America: Small Animal Practice 15: 755-767

ARONSOHN, M.G., SCHUNK, K.L., CARPENTER, J.L. & KING, N.W. 1984
Clinical and pathological features of thymoma in 15 dogs.
JAVMA 184: 1355-1362

ASSELINEAU, D., BERNARD, B.A., BAILLY, C., DARMAN, M. & PRUNIERAS, M. 1986
Human epidermis reconstructed in culture; is it "normal"?
J. Invest. Dermatol. 86: 181-185

ATKINS, C.E., LECOMPTE, P., CHIN, H.P., HILL, J.R., OWNBY, C.L. & BROWNFIELD, M.M. 1988
Morphologic and immunocytochemical study of young dogs with diabetes mellitus associated with pancreatic islet hypoplasia.
Am. J. Vet. Res. 49: 1577-1588

AVRAMEAS, S. 1969
Coupling enzymes to proteins with glutaraldehyde: use of conjugates for the detection of antigens and antibodies.
Immunocytochemistry 6: 43-52

AYALA, E., MARTINEZ, E., ENGHARDT, M., KIM, S. & MURRAY, R. 1993
An improved cytomegalovirus immunostaining method.
Lab. Med. 24: 39-43

BAAS, I.O., MULDER, J.W.R., OFFERHAUS, G.J.A., VOGELSTEIN, B., HAMILTON, S.R., 1994:

An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms.
J Pathol, 172, 5-12.

BAKER, J.R. 1960
Principles of Biological Microtechnique
Methuen, London.

BARNES, D.M., DUBLIN, E.A., FISHER, C.J., LEVISON, D.A., MILLIS, R.R., 1993:
Immunohistochemical detection of p53 protein in mammary carcinoma:An important new independent indicator of prognosis?
Hum Pathol, 24, 469-476.

BARTEK, J., IGGO, R., GANNON, J. & LANE D.P. 1990
Genetic and immunohistochemical analysis of mutant p53 in human breast cancer cell lines.
Oncogene 5: 893-899

BASZLER, T.V., EVERMANN, J.F., KAYLAR, P.S., BYINGTON, T.C. & DILBECK, P.M. 1995
Diagnosis of naturally occurring bovine viral diarrhoea virus infections in ruminants using monoclonal antibody based immunohistochemistry.
Vet. Pathol. 32: 609-618

BATATA. M.A., MARTINI, N., HUVOS, A.G., AGUITOR, R.I. & BEATTIE JNR, E.J. 1974
Thymomas: Clinicopathologic features, therapy and prognosis.
Cancer 34: 389-396

BATTIFORA, H., SUN, T-T., BAHU, R.M. & RAO, S. 1980
The use of antikeratin antiserum as a diagnostic tool: thymoma versus lymphoma.
Hum. Pathol. 11: 635-641

BELLAH, J.R., STIFF, M.E. & RUSSELL, R.G. 1983
Thymoma in the dog: Two case reports and review of 20 additional cases.
JAVMA 183: (3) 306-311

BEVIER, D.F. & GOLDSCHMIDT, M.H. 1981
Skin tumours in dogs I. *Epithelial tumours and tumour-like lesions.*
Comp. Cont. Edu. 3: 389-398

BIGBEE, J.W., KOSEK, J.C. & ENG, L.F. 1977
Effects of primary antiserum dilution on staining of antigen rich tissues with peroxidase-antiperoxidase techniques.
J. Histochem. Cytochem. 25: 443-447

BIRD, C.L. & BOSTON, W.S. (eds) 1975
The Theory of Colouration of Textiles
Dyers Company Publications Trust, Bradford.

BLOOD, D.C. & RADOSTITS, O.M. 1989
Veterinary Medicine. A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 7th. ed. p20
Ballliere Tindall, London.

BOSTOCK, D.E. 1986
Neoplasia of the skin and subcutaneous tissues in dogs and cats.
Br. Vet. J. 142: 1-19

BOUCHARD, P.R., FORTNA, C.H., ROWLAND, P.H. & LEWIS, R.M. 1995
An immunohistochemical study of three equine pulmonary granular cell tumours.
Vet. Pathol. 32: 730-734

BOWKER, R.M., BREWER, A.M., VEX, K.B., GUIDA, L.A., LINDER, K.E., SONEA, I.M. & STINSON A.W. 1993
Sensory receptors in the equine foot.
Am. J. Vet. Res. 54: 11 1840-1844

BRATTHAUER, G.L. 1994
In:Immunocytochemical methods and protocols. Chp 21 p.175
ed.Javois L.C.
Humana Press, New Jersey.

BRODEY, R.S. 1970
Canine and feline neoplasia.
Adv. Vet. Sci. 14; 309-354

BROWN, C.C., MARINER, J.C. & OLANDER, H.J. 1991
An immunohistochemical study of the pneumonia caused by peste de petit ruminants virus.
Vet. Pathol. 28: 166-170

BROWN, C.C. & TORRES, A. 1994
Distribution of antigen in cattle infected with rinderpest virus.
Vet. Pathol. 31: 194-200

BRUGAROLAS, J., CHANDRASEKARAN, C., GORDON, J.I., BEACH, D., JACKS, T. & HANNON, G.L. 1995
Radiation induced cell cycle arrest compromised by p21 deficiency.
Nature 377: 552-557

BRUNNERT, S.R. & ALTMAN, N.H. 1991
Identification of immunoglobulin light chains in canine extramedullary plasmacytomas by thioflavine T and immunohistochemistry.
J. Vet. Diag. Invest. 3: 245-251

BURNS, J., HAMBRIDGE, M. & TAYLOR, C.R. 1974
Intracellular immunoglobulins. A comparative study on three standard tissue processing methods using horseradish peroxidase and fluorochrome conjugates.
J. Clin. Pathol. 27: 548-557

BURRACO, P., GUGLIELMINO, R., ABATE, O., BOCCHINI, V., CORNAGLIA, E., DENICOLA, D.B., CILLI, M. & PONZIO, P. 1992
Large granular lymphoma in a FIV positive and FeLV negative cat.
Journal of Small Animal Practice. 33: 297-284

CADRIN, M. & MARTINOLI, M.G. 1995
Alterations of intermediate filaments in various histopathological conditions.
Biochem. Cell Biol. 73: 627-634

CALINGASAN, N.Y., KITAMURA, N., YAMADA. J., OOMORI, Y. & YAMASHITA, T. 1984
Immunocytochemical study of the gastroenteropancreatic endocrine cells of the sheep.
Acta Anat. 118: 171-180

CALLANAN, J.J., JONES, B.A., IRVINE, J., WILLET, B.J., MCCANDLISH, I.A.P. & JARRETT, O. 1996
Histologic classification and immunophenotype of lymphosarcomas in cats with naturally and experimentally acquired feline immunodeficiency virus infections.
Vet. Pathol. 33: 264-272

CANIATTI, M., ROCCABIANCA, P., SCANZIANI, E., PALTINIERI, S. & MOORE, P.F. 1996
Canine lymphoma: Immunocytochemical analysis of fine needle apiration biopsies.
Vet. Pathol. 33: 204-212

CARPENTER, J.L. & HOLZWORTH, J. 1982
Thymoma in 11 cats.
JAVMA 181: 248-251

CARRIGAN, M.J., HIGGINS, R.J., CARLSON, G.P. & NAYDEN, D.K. 1996
Equine papillary ependymoma.
Vet. Pathol. 33: 77-80

CASTLEMAN, B. 1955
Tumors of the thymus gland. In: Atlas of Tumor Pathology fasc. 19
Armed Forces Institute of Pathology, Washington.

CASWELL, J.L., YAGER, J.A., FERRER, L. & WEIR, J.A.M. 1995
Canine demodicosis: A re-examination of the histopathologic lesions and description of the immunophenotype of infiltrating cells.
Vet. Dermatol. 6: 1, 9-19

CATTORETTI, G., PILERI, S., PARRAVICINI, C., BECKERS, M.H.G., POGGI, S., BIFULCO, C., KEY, G., D'AMATO, L., SABATTINI, E., FEUDALE, E., REYNOLDS, F., GERDES, J. & RILKE, F. 1993
Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections.
J. Pathol. 171: 83-98

CECCARELLI, P., PEDINI, V. & GARGIULO, A.M. 1995
The endocrine cells of the gastro-enteric tract of adult fallow deer (Dama dama L.)
Anat. Histol. Embryol. 24: 171-174

CELIO, M.R. 1979
Immunohistochemistry on Bouins fixed fetal tissue stored for thirty years in ethanol.
Histochemistry 61: 347-350

CHIBA, T., HIRAGA, M., AIDA, Y., AJITO, T., ASAHINA, M., WU, D., OHSHIMA, K., DAVIS, W.C. & OKADA, K. 1995
Immunohistologic studies of subpopulations of lymphocytes in cattle with enzootic bovine leukosis.
Vet. Pathol. 32: 513-520

CHOI, Y. & FUCHS,E. 1990
TGFβ and retinoic acid: regulators of growth and modifiers of differentiation in humanepidermal cells.
Cell. Reg. 1: 791-809

COLLINGS, L.A., POULTER, L.W. & JANOSSY, G. 1984
The demonstration of cell surface antigens on T cells, B cells and accessory cells in paraffin embedded human tissues.
J. Immunol. Methods 75: 227-239

COONS, A.H., CREECH, H.J., & JONES, R.N. 1941
Immunological properties of an antibody containing a fluorescent group.
Proc. Soc. Exp. Biol. (New York) 47: 200-202

COONS, A.H., LEDUC, E.H. & CONNOLLY, J.M. 1955
Studies on antibody production I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit.
J. Exp. Med. 102: 49-60

COOPER, D., SCHERMER, A. & SUN, T-T. 1985
Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications and limitations.
Lab. Invest. 52: 3, 243-256

CORDELL, J.L., FALINI, B., ERBER, W., GATTER, K.C. & MASON, D.Y. 1984
Immunoenzymatic labelling of monoclonal antibodies using complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP)

complexes.
J. Histochem. Cytochem. 32: 219-229

CORWIN, D.J. & GOWN, A.M. 1989
Review of selected lineage-directed antibodies useful in routinely processed tissues.
Arch. Pathol. Lab. Med. 113: 645-652

COSGROVE, D., CHAN, S.H., WALTZINGER, C., BENOIST, C. & MATHIS, D. 1992
The thymic compartment responsible for positive selection of CD4+ T cells.
Intl. Immunol. 4: 707-710

COSTA, A., MARASCA, R., VALENTINIS, B., SAVARINO, M., FARANDA, A., SILVESTRINI, R., TORELLI, G., 1995:
p53 gene point mutation in relation to p53 nuclear protein accumulation in colorectal cancers.
J Pathol, 176 45-53.

COTCHIN, E. 1954
Further observations on neoplasms in dogs with particular reference to site of origin and malignancy.
Br. Vet. J. 110: 218-230

COX, L.S. & LANE , D.P. 1995
Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage.
Bioessays 17: 501-508

CULLING, C.F.A. 1974
Handbook of Histopathological and Histochemical Techniques 3rd ed.
Butterworth & Co., London

DAIMARA, Y., HASHIMOTO, H., TSUNEYASHI, M. & ENJOJI, M. 1987
Epithelial profile of epithelioid sarcoma: an immunohistochemical analysis of eight cases.
Cancer 59: 134-141

DARKE, P.G.G., McCULLAGH, K.G. & GELDART, P.H. 1975
Myasthenia gravis, thymoma and myositis in a dog.
Vet. Rec. 97: 392-394

DAVEY, F.R., HALLIDAY, D., MARUCCI, A.A. & GOTTLIEB, A.J. 1978
Detection of intracellular and cell surface immunoglobulin in non-Hodgkins lymphoma.
Hum. Pathol. 9: 285-294

DAVIS, H.H., DOTSON, E.M. & OLIVER, J.H. Jr. 1994
Localisation of insulin-like immunoreactivity in the synganglion of nymphal and

adult Dermacenter variabilis (Acari:Ixodidae).
Exp. App. Acarology 18: 111-122

DAY, M.J., HANLON, L. & POWELL, L. 1993
Immune-mediated skin disease in the dog and cat.
J. Comp. Path. 109: 395-407

DELELLIS, R.A. & KWAN, P. 1988
Technical considerations in the immunohistochemical demonstration of intermediate filaments.
Am. J. Surg. Pathol. 12 (Supp. 1) 17-23

DE WAAL MALEFIT, R., LEENE, W., ROHOLL, P.J., WORMMEESTER, J. & HOEBEN, K.A. 1986
T cell differentiation within thymic nurse cells.
Lab. Invest. 55: 25-

DITERS, R.W. & WALSH, K.M. 1984
Feline basal cell tumours: A review of 124 cases.
Vet. Pathol. 21: 51-56

DONEHOWER, L.A., HARVEY, M., SLAGLE, B.L., McARTHUR, M.J., MONTGOMERY, C.A., BUTEL, J.S., BRADLEY, A., 1992:
Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.
Nature, 356, 215-221.

DYCE, K.M., SACK, W.W. & WENSING, C.J.G. 1987
Textbook of Veterinary Anatomy Chp.25
W.B. Saunders Company, Philadelphia

EICHNER, R., SUN, T-T. & AEBI, U. 1986
The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments.
J. Cell Biol. 102: 1767-1777

ELIAS, J., MARGIOTTA, M. & GABORE, D. 1989
Sensitivity and detection efficiency of the peroxidase antiperoxidase (PAP), avidin biotin complex (ABC) and the peroxidase labelled avidin biotin (LAB) methods.
Am. J. Clin. Pathol. 92: 62.

ENGLAND, S. & SEIFTER, S. 1990
Precipitation techniques.
Methods Enzymol. 182: 285-296

ENGELMAN, E.G., WARNKE, R.A., FOX, R.I. et al 1981
Studies of a human T lymphocyte antigen recognised by a monoclonal antibody.
PNAS 78: 1791-1795

FALINI, B., FLENGHI, L., PILERI, S. et al. 1993
PG-M1: A new monoclonal antibody directed against a fixative resistant epitope on the macrophage-restricted form of the CD68 molecule.
Am. J. Pathol. 142: 1359-1372

FENOGLIO-PREISER, C.M., PASCAL, R.R. & PERZIN, K.H. 1990
Tumours of the Intestine in Atlas of Tumour Pathology 2nd series, fasc. 27
Armed Forces Institute of Pathology Washington DC

FINLAY, C.A., HINDS, P.W. & LEVINE, A.J. 1989
The p53 proto-oncogene can act as a suppressor of transformation.
Cell 57: 1083-1093

FONTANINI, G., VIGNATI, S., BIGINI, D., MERLO, G.R., RIBECCHINI, A., ANGELETTI, C.A., BASOLO, F., PINGITORE, R., BEVILACQUA, G., 1994:
Human non-small cell lung cancer: p53 protein accumulation is an early event and persists during metastatic progression.
J Pathol, 174, 23-31.

FRANKE, W.W., SCHMID, E., OSBORN, M. & WEBER, K. 1978
Different intermediate-sized filaments distinguishable by immunofluorescence microscopy.
Proc. Natl. Acad. Sci. USA 75: 5034-5038

FRANKE, W.W., SCHMID, E., WELLSTEED, J,. GRUND, C., GIG, O. & GEIGER, B. 1983
Change of cytokeratin filament organisation during the cell cycle: selective masking of an immunologic determinant in interphase pkk2 cells.
J. Cell Biol. 97: 1255-1260

FUCHS, E. 1990
Epidermal differentiation: The bare essentials.
J. Cell Biol. 111: 2807-2814

FUCHS, E. & GREEN, H. 1981
Regulation of terminal differentiation of cultured human keratinocytes and mouse epidermis.
Cell. Reg. 1: 87-97

FUKUDA, T., OHNISHI, Y., EMURA, I. & TACHIKAWA, S. 1992
Microcytic variant of thymoma: histological and immunohistochemical findings in 2 cases.
Virchow's Archive A , Pathol. Anat. 420: 185-189

FURUOKA, H., ITO, H., HAMADA, M., SUWA, T., SATOH, H. & HAKURA, C. 1989
Immunocytochemical component of endocrine cells in pancreatic islets of horses.
Jap. J. Vet. Sci. 51: 35-43

GALFRE, G. & MILSTEIN, C. 1981
Preparation of monoclonal antibodies: strategies and procedures.
Methods Enzymol. 73: 3-46

GANNON, J.V., GREAVES, R., IGGO, R. & LANE, D.P. 1990
Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form.
EMBO J. 9 55: 1595-1602

GARVIN, A.J., SPICER, S.S., PARMLEY, R.T. & MUNSTER, A.M. 1974
Immunohistochemical demonstration of immunoglobulin in Reed-Sternberg and other cells in Hodgkin's disease.
J. Exp. Med. 139: 1077-1083

GHOSH, A.K., ERBER, W.N. HATTEN, C.S.R. ET AL 1985
Detection of metastatic tumour cells in routine bone marrow smears by immunoalkaline phosphatase labelling with monoclonal antibodies.
Br. J. Haematol. 61: 21-30

GHOSH, A.K., MASON, D.Y. & SPRIGGS, A.I. 1983
Immunocytochemical staining with monoclonal antibodies in cytologically "negative" serous effusions from patients with malignant disease.
J. Clin. Pathol. 36: 1150-3

GOLDSCHMIDT, M. H. & SHOFER, F.S. 1992
In: Skin Tumours of the Dog and Cat
Pergamon Press, Oxford

GOLDSMITH, L.A. 1991
Physiology, Biochemistry and Molecular Biology of the Skin. 2nd ed.
Oxford University Press, Oxford

GORES, B.R., BERG, J., CARPENTER, J.L. & ARONSOHN, M.G. 1994
Surgical treatment of thymoma in cats: 12 cases (1987-1992).
JAVMA 204: (11) 1782-1785

GORRELL, M.D., TOWNSEND, W.L. & LADDS, P.W. 1995
The distribution of lymphocyte sub-populations in normal and acanthotic ovine skin.
Vet. Immunol. Immunopathol. 44: 151-167

GRAHAM, C.F. 1992
In: Oxford Textbook of Pathology vol. 1
eds. McGee, Isaacson & Wright
Oxford University Press, Oxford

GRIFFITH, R.C. 1990
In: Anderson's Pathology 9th ed. Vol II Chp 25
ed. Kissane, J.M.
The C.V. Mosby Company, St. Louis

GROSS, T.L., IRKE, P.J. & WALDER E.J. 1992
Veterinary Dermatopathology. A Macroscopic and Microscopic Evaluation of Canine and Feline Skin Disease.
Mosby Year Book, St. Louis

HADLOW, W.J. 1978
High prevalence of thymoma in the dairy goat.
Vet. Pathol. 15: 153-169

HAINES, D.M., CLARK, E.G. & DUBOVI, E.J. 1992
Monoclonal antibody based immunohistochemical detection of bovine viral diarrhoea virus in formalin-fixed, paraffin-embedded tissues.
Vet. Pathol. 29: 27-32

HAINES, D.M., COOK, E.M. & CLARK, E.G. 1987
Avidin-biotin-peroxidase complex immunohistochemistry to detect immunoglobulin in formalin fixed skin biopsies in canine autoimmune disease.
Can. J. Vet. Res. 104-109

HALBUR, P.G., PAUL, P.S., FREY, M.L., LANDRAF, J., EERNISSE, K., MENG, X-J., ANDREWS, J.J., LUM, M.A. & RATHJE, J.A. 1996
Comparison of the antigen distribution of two US porcine respiratory and reproductive syndrome virus isolates with that of the Lelystad virus.
Vet. Pathol. 33: 159-170

HARDY, W.D. Jr. 1981
Haematopoietic tumours of cats.
JAAHA 17: 921-940

HARDY, P.M., NICHOLLS, A.C. & RYDEN, H.N. 1976
The nature of the cross-linking of proteins with glutaraldehyde.
J. Chem. Soc. 1: 958-962

HARGIS, A.M. & THOMASSEN, R.W. 1979
Solar keratosis (solar dermatosis, senile keratosis) and solar keratosis with squamous cell carcinoma.
Am. J. Pathol. 94: 193-196

HARGIS, A.M., THOMASSEN, R.W. & PHEMISTER, R.D. 1977
Chronic dermatosis and cutaneous squamous cell carcinoma in the beagle dog.
Vet. Pathol. 14: 218-228

HARRIS, C.L., KLAUSNER, J.S., CAYWOOD, D.D. & LEININGER, J.R. 1991
Hypercalcaemia in a dog with thymoma.
JAAHA 27: 281-284

HAWKINS, K.L., SUMMERS, B.A., KUHAJDA, F.D. & SMITH, C.A. 1987
Immunohistochemistry of normal pancreatic islets and spontaneous islet cell

tumours in dogs.
Vet. Pathol. 24: 170-179

HAYDEN, D.W., WATERS, D.J., BURKE, B.A. & MANIVEL, J.C. 1993
Disseminated malignant histiocytosis in a Golden Retriever: Clinicopathologic, ultrastructural and immunohistochemical findings.
Vet. Pathol. 30: 256-264

HAZIROGLU, R., DIKER, K.S., TURKARSHAN, J. & GUBHAHER, M.Y. 1996
Detection of Mycoplasma ovipneumoniae and Pasteurella haemolytica antigens by an immunoperoxidase technique in pneumonic ovine lungs.
Vet. Pathol. 33: 74-76

HEAD, K.W. 1953
Skin diseases: Neoplastic diseases.
Vet. Rec. 65: 926-929

HEENEY, J.L. & VALLI, V.E.O. 1990
Transformed phenotype of enzootic bovine lymphoma reflects differentiation-linked leukaemogenesis.
Lab. Invest. 62: 339-346

HEINRICHS, M., BAUMGARTNER, W. & CAPEN, C.C. 1990
Immunocytochemical demonstration of proopiomelanocortin-derived peptides in pituitary adenomas of the pars intermedia in horses.
Vet. Pathol. 27: 419-425

HELLAND, A., HOLM, R., KRISTENSEN, G., KAERN, J., KARLSEN, F., TROPE, C., NESLAND, J.M., BORRESEN, A.L., 1993:
Genetic alterations of the tp53 gene, p53 protein expression and HPV infection in primary cervical carcinomas.
J Pathol, 171, 105-114.

HENNINGS, H., MICHAEL, D., CHENG, C., STEINERT, P.M., HOLBROOK, K. & YUSPA, S.H. 1980
Calcium regulation of growth and differentiation of mouse epidermal cells in culture.
Cell 29: 245-254

HENRY, K. 1992
In: Systemic Pathology Vol. 7. Thymus, Lymph Node, Spleen and Lymphatics. 3rd ed. p. 72
Churchill Livingstone, Edinburgh.

HEWICKER-TRAUTWEIN, M., KRUEGER, N., URBAN, K. & TRAUTWEIN, G. 1994
Immunohistochemical localisation of glial and neuronal cell markers in the developing bovine brain.
Anat. Histol. Embryol. 23: 154-165

HINDS, P., FINLAY, C. & LEVINE 1989
Mutation is required to activate the p53 gene for co-operation with the ras oncogene and transformation.
J. Virol. 63, 2: 739-746

HOLBORROW, E.J., WEIR, D.M. & JOHNSON, G.D. 1957
A serum factor in lupus erythematosus with affinity for tissue nuclei.
Br. Med. J. 2: 732-734

HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., HARRIS, C.C., 1991:
p53 mutations in human cancers.
Science, 253, 49-53.

HOLTZER, H., BIEHL, J. & HOLTZER, S. 1985
Induction-dependant and lineage dependant models for cell diversification are mutually exclusive.
In: Advances in Neuroblastoma Research p. 3-11
eds. Evans, E.A., D'Angio, G.J. & Seege, R.C.
Liss, New York

HOPPER, C.D., SPARKES, A.H., GRUFFYDD-JONES, T.J., CRISPIN, S.M., MUIR, P., HARBOUR, D.A. & STOKES, C.R. 1989
Clinical and laboratory findings in cats infected with feline immunodeficiency virus.
Vet. Rec. 125: 341-346

HSU, S.M., RAINE, L. & FANGER, H. 1981
Use of an avidin biotin peroxidase complex (ABC) in immunoperoxide technique: a comparison between ABC and unlabelled antibody (PAP) procedures.
J. Histochem. Cytochem. 29: 577-580

IGGO, R., GATTER, K., BARTEK, J., LANE, D., HARRIS, A.L., 1990:
Increased expression of mutant forms of p53 oncogene in primary lung cancer.
Lancet, 335, 675-679.

IVERSON, L. 1956
Thymoma; review and reclassification.
Am. J. Pathol. 32: 695-719

IWATSUKI, K., OKITA, M., OCHIKUBU, F., GEMMA, T., SHIN, Y.S., MIYASHITA, N., MIKAMI, T. & KAI, C. 1995
Immunohistochemical analysis of the lymphoid organs of dogs naturally infected with canine distemper virus.
J. Comp. Path. 113: 185-190

JACKSON, B.W., GRUND, C., SCHMID, E., BURKI, K., FRANKE, W.W. & ILLMENSE, K. 1980
Formation of cytoskeletal elements during mouse embryogenesis.
Differentiation 17: 161-179

JAEGER, J.A. & WILCOX, B.P. 1994b
In: Colour Atlas and Text of Surgical Pathology of the dog and Cat. Vol. 1 p 257-62
Wolfe Mosby Year Book Europe, London

JANEWAY, C.A. & TRAVERS, P. (1994)
Immunobiology. The Immune System in Health and Disease. p.6:24
Current Biology, London.

JARRETT, J.O. 1990
In: Feline Medicine and Therapeutics Chp. 15
eds. Chandler, E. A., Hilbery, A.D.R. & Gaskell, C.J.
Blackwell Scientific Publications, Oxford

JARRETT, W.F.H. & MACKEY, L.J. (1974)
Neoplastic diseases of the haematopoietic and lymphoid tissues.
Bull. W.H.O. 50: 1-2, 21-34

JASANI, B. & SCHMID, K.W. 1993
Immunocytochemistry in Diagnostic Pathology
Longman, Singapore.

JASANI, B., THOMAS, N.D., NAVABI, H., MILLAR, D.N., NEWMAN, G.R., GEE, J. & WILLIAMS, E.D. 1992
Dinitrophenyl (DNP) localisation system: a review of principle reagents and applications developed over the past ten years.
J. Immunol. Methods 50: 193-198

JASANI, B., WYNFORD-THOMAS, D. & WILLIAMS E.D. 1981
Use of monoclonal anti-hapten antibodies for immunolocalisation of tissue antigens.
J. Clin. Pathol. 34: 1000-1002

JOHNSON, G.C., MILLER, M.A., FLOSS, J.L. & TURK, J.R. 1996
Histologic and immunohistochemical characterisation of haemangiomas in the skin of seven young horses.
Vet. Pathol. 33: 142-149

JUNQUEIRA, L.C., CARNEIRO, J. & LONG, J.A. 1986
Basic Histology 5th ed. p53
Lange Medical Publications. Los Altos CA

Vet. Pathol. 31: 658-662

KAPPLER, J.W., ROEHM, N. & MARRACK, P. 1987
T cell tolerance by clonal elimination in the thymus.
Cell 49: 273-280

KASTAN, M.B., ONYEKWERE, O., SIDRANSKY, D., VOGELSTEIN, B. & CRAIG, R.W. 1991

Participation of p53 protein in the cellular response to DNA damage.
Cancer Res. 51: 6304-6311

KELLEY, L.C., HILL, J.E., HATNER, S. & WORTHAM, K.J. 1995
Spontaneous equine pulmonary granular cell tumour: morphologic, histochemical and immunohistochemical characterisation.
Vet. Pathol. 32: 101-106

KELLEY, L.C., PUETTE, M., LANGHEINRICH, K.A. & KING, B. 1994
Bovine pulmonary blastomas: histomorphologic description and immunohistochemistry.
KLEBANOW, E.R. 1992
Thymoma and acquired myasthenia gravis in the dog: a case report and review of 13 additional cases.
JAAHA 28: 63-69

KOHLER, G. & MILSTEIN C. 1975
Continuous culture of fused cells secreting antibody of predefined specificity.
Nature 256: 495-497

KOPLAN, R. & FUCHS, E. 1989
The use of retinoic acid to probe the relationship between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells.
J. Cell Biol. 109: 295-307

KROHNE, G. & BENAVENTE, R. 1986
The nuclear lamins. A multigene family of proteins in evolution and differentiation.
Exp. Cell Res. 162: 1-10

KYRIAZIDOU, A., BROWN, P.J. & LUCKE, V.M. 1989
An immunohistochemical study of canine extramedullary plasma cell tumours.
J. Comp. Path. 100 259-266

LANE, D.P. & CRAWFORD, L.V. 1979
T antigen is bound to a host protein in SV40 transformed cells.
Nature 278: 261-263

LAROCHELLE, R., SAUVAGEAU, R. & MAGAR, R. 1994
Immunohistochemical detection of swine influenza virus and porcine respiratory and reproductive syndrome virus in porcine proliferative and necrotizing pneumonia cases from Quebec.
Can. Vet. J. 35: 513-515

LATTES, R. 1962
Thymoma and other tumours of the thymus. An analysis of 107 cases.
Cancer 15: 6: 1224-1260

LAZARIDES, E. 1982
Intermediate Filaments: A chemically heterogenous, developmentally regulated class of proteins.
Ann. Rev. Biochem. 51: 219-250

LEGG, M.A. & BRADY, W.J. 1965
Pathology and clinical behaviour of thymomas. A survey of 51 cases.
Cancer 18: 9: 1131-1144

LEUBE, R.E., BOSCH, R., ROMANO, V., ZIMBELMANN, R., HOFLER, H. & FRANKE, W.W. 1986
Cytokeratin expression in simple epithelia III. Detection of mRNAs encoding human cytokeratin nos. 8 and 18 in normal and tumour cells by hybridization with cDNA sequences in vitro and in situ.
Differentiation 33: 69-85

LEVINE, A.J., MOMAND, J. & FINLAY, C.A. 1991
The p53 tumour suppressor gene.
Nature 351: 453-456

LEVINE, G.D. & ROSAI, J. 1978
Thymic hyperplasia and neoplasia: areview of current concepts.
Hum. Pathol. 9: 495-515

LEVINE, G.D., ROSAI, J., BEARMAN, R.M. & POLLIACK, A. 1975
The fine structure of thymoma with emphasis on its differential diagnosis. A study of ten cases.
Am. J. Pathol. 81: 49-86

LEWENHAUPT, E. 1948
Tumours of the thymus in relation to thymic epithelial anlage.
Cancer 1: 547-563

LI, J.H. & BAO, H.E. 1994
Immunohistochemical localisation of antigen and histochemical analysis of Trichinella spiralis larvae.
Chinese Journal of Parasitic Disease Control 7: 113-115

LINZER, D.I.H. & LEVINE, A.J. 1979
Characterization of a 54k dalton cellular SV40 tumour antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells.
Cell 17: 43-52

LIU, S. & HOHN, R.B. 1968
Squamous cell carcinoma of the digit of the dog.
JAVMA 153: 411-424

LOPEZ, J., BARRENECHEA,M.A., BURNELL, M.A. & SESMA, P. 1993
Immunocytochemical study of the lung of domestic fowl and pigeon: endocrine

cells and nerves.
Cell Tissue Res. 273: 89-95

McLEAN, W.H.I. & LANE, E.B. 1995
Intermediate filaments in disease.
Curr. Opin. Cell Biol. 7: 118-125

McNEIL, P.H. 1980
A thymoma as a cause of oesophageal obstruction in a dog.
NZ Vet. J. 28: 143-145

MACKEY, L.J. 1975
Clear cell thymoma and thymic hyperplasia in a cat.
J. Comp. Pathol. 85: 367-370

MADARAME, H., TAKAI, S., MORISAWA, N., FUJII, M., HIDAKA, D., TSUBAKI, S. & HASEGAWA, Y. 1996
Immunohistochemical detection of virulence-associated antigens of Rhodococcus equi in pulmonary lesions of foals.
Vet. Pathol. 33: 341-343

MADEWELL, B.R., POOL, R.R., THEILEN, G.H. & BREWER, W.G. 1982
Multiple subungual squamous cell carcinomas in five dogs.
JAVMA 180: 731-734

MARIN, M.C., HSU, B., MEYN, R.E., DONEHOWER, L.A., EL-NAGGER, A.K., McDONNELL, T.J., 1994:
Evidence that p53 and bcl-2 are regulators of a common cell death pathway important for in vivo lymphomagenesis.
Oncogene, 9, 3107-3112.

MARSHALL, D.J., MOXLEY, R.A. & KELLING, C.L. 1996
Distribution of virus and viral antigen in specific pathogen free calves following inoculation with noncytopathic bovine viral diarrhoea virus.
Vet. Pathol. 33: 311-318

MARTIN, R.A., EVANS, E.W., AUGUST, J.R. & FRANKLIN, J.E. 1986
Surgical treatment of thymoma in a cat.
JAAHA 22: 347-354

MARTINEZ, A., LOPEA, J. & SESMA, P. 1993
Development of the diffuse endocrine system in the chicken proventriculus.
Cell Tissue Res. 271: 107-113

MASON, D.Y., COMANS-BITTER, M., CORDELL, J.L. et al 1990
Antibody L26 recognises an intracellular epitope on the B cell associated CD20 antigen.
Am. J. Pathol. 136: 1215-1222

MASON, J.T. & O'LEARY T.J. 1991
Effects of formalin fixation on protein secondary structure: a calorimetric and

infra red spectroscopic investigation.
J. Histochem. Cytochem. 39: 225-229

MATTIX, M.E., MATTIX, R.J., WILLIAMS, B.H., RIBAS, J.L. & WILHELMSEN, C.L. 1994
Olfactory ganglioneuroblastoma in a dog: A light, ultrastructural and immunohistochemical study.
Vet. Pathol. 31: 262-265

MAYR, B., SCHELLANDER, K., SCHLEGER, W., REIFINGER, M., 1994:
Sequence of an exon of the canine p53 gene - mutation in a papilloma.
Br Vet J, 150, 81-84.

MEHREL, T., HOHL, D., ROTHNAGEL, J.A., LONGLEY, M.A., BUNDMAN, D., CHENG, C., LICHTI, U., BISHER, M.E., STEVEN, A.C., STEINERT, P.M., YUSPA, S.H. & ROOP, D.R. 1990
Identification of a major keratinocyte envelope protein, loricrin.
Cell 61: 1103-1112

MELING, G.I., LOTHE, R.A. , BORRESEN, A.L., ET AL. 1993
The TP53 tumour suppressor gene in colorectal carcinomas. II. Relation to DNA ploidy pattern and clinicopathological variables.
Br. J. Cancer 67: 93-98

MIETTINEN, M., LEHTENEN, E., LEHTOLA, H., EHKBLOM, P., LEHTO, V. & VIRTANEN, I. 1984
Histogenesis of granular cell tumours - an immunohistochemical and ultrastructural study.
J. Pathol. 142 221-229

MILDE,P., MERKE, J., RETZ, E., HAUSSLER, M. & RAUTERBERG, E. 1989
Immunohistochemical detection of 1,25 dihydroxyvitamen D3 receptors and estrogen receptors by monoclonal antibodies: comparison of four immunoperoxidase methods.
J. Histochem. Cytochem. 37: 1609-1617

MILLER, J.M., JENNY, A.L., TAYLOR, W.D., MARSH, R.F., RUBENSTEIN, R. & RACE, R.E. 1993
Immunohistochemical detection of prion protein in sheep with scrapie.
J. Vet. Diag. Invest. 5: 309-316

MILLER, M.A., RAMOS, J.A. & KREEGER, J.M. 1992
Cutaneous vascular neoplasia in fifteen cats: Clinical, morphological and immunohistochemical studies.
Vet. Pathol. 29: 329-336

MIYASHITA, T., KRAJEWSKI, S., KRAJEWSKA, M., WANG, H.G., LIN, H.K., LIEBERMANN, D.A., HOFFMAN, B., REED, J.C., 1994:
Tumour suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro

and in vivo.
Oncogene, 9, 1799-1805.

MOLL, R., FRANKE, W.W., SCHILLER, D.L., GEIGER, B. & KREPLER, R. 1982
The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumours and cultured cells.
Cell 31: 11-24

MOLLER, G. 1993
Positive T cell selection in the thymus.
Immunol. Rev. 135: 5-242

MOMAND, J., ZAMBETTI, G.P., OLSON, D.C., GEORGE, D., LEVINE, A.J., 1992:
The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation.
Cell, 69, 1237-1245.

MOMOTAMI, E., NAKAMURA, N. & SHOYA, S. (1981)
Morphologic evidence of the histogenesis of epithelial thymoma in a cow.
Am. J. Vet.Res. 42: (1) 114-121

MONTENARH, M. 1992:
Biochemical properties of the growth suppressor/oncoprotein p53.
Oncogene, 7, 1673-1680.

MOULTON, J.E. & HARVEY, J.W. 1990
In: Tumors in Domestic Animals 3rd ed.
ed. Moulton
Berkeley, CA. Univ. California Press.

NAKAJIMA, S., KITAMURA, N., YAMADA, J., YAMASHITA, T. & WATANABE, T. 1988
Immunohistochemical study on the endocrine pancreas of cattle with special reference to coexistence of serotonin and glucagon or bovine pancreatic polypeptide.
Acta Anat. 131: 235-240

NAKANE, P.K. & KAWAOI, A. 1974
Peroxidase labelled antibody: A new method of conjugation.
J. Histochem. Cytochem. 22: 1084-1091

NAKANE, P.K. & PIERCE, G.B. 1966
Enzyme labelled antibodies: preparation and application for the localisation of antigens.
J. Histochem. Cytochem. 14: 929-931

NELSON, W. & SUN, T-T. 1983
The 50 and 58 kD keratin classes as molecular markers for stratified epithelium:

cell culture studies.
J. Cell. Biol. 97: 244-251

NIELSEN, S.W. & COLE, C.R. 1960
Cutaneous epithelial neoplasms of the dog - a report of 153 cases.
AM. J. Vet. Res. 21: 931-948

NIGRO, J.M., BAKER, S.J., PREISINGER, A.C., JESSUP, J.M., HOSTETTER, R., CLEARY, K., BIGNER, S.H., DAVIDSON, N., BAYLIN, S., DEVILEE, P., GLOVER, T., COLLINS, F.S., WESTON, A., MODALI, R., HARRIS, C.C., VOGELSTEIN, B., 1989:
Mutations in the p53 gene occur in diverse human tumour types.
Nature Lett, 342, 705-708.

OFFERHAUS, G.A., DE FEYTER, E.P., CORNELISE, C.J. ET AL 1992
The relationship of DNA aneuploidy to molecular genetic alterations in colorectal carcinoma.
Gastroenterology 102: 1612-1619

OGAWA, Y., SAGATA, N., TSUZUKU-KAWAMURA, J., ONUMA, M., IZAWA, H. & IKAWA, Y. (1986)
No involvement of bovine leukaemia virus in sporadic bovine lymphosarcoma.
Microbiol. Immunol. 30: (7) 697-701

OLTVAI, Z.N., MILLIMAN, C.L. & KORSMEYER, S.J. 1993
Bcl-2 heterodimerises in vivo with a conserved homolog, bax, that accelerates programed cell death.
Cell 74: 609-619

OSTROVE, S. 1990
Affinity chromatography: general methods.
Methods Enzymol. 182: 357-379

PACE, L.W., KREEGER, J.M., MILLER, M.A., TURK, J.R. & FISCHER, J.R. 1994
Immunohistochemical staining of feline malignant fibrous histiocytomas.
Vet. Pathol. 31: 168-172

PARKER, G.A. & CASEY, H.W. (1976)
Thymomas in domestic animals.
Vet. Pathol. 13: 353-364

PEARSE, A.G.E. 1980
Histochemistry, Theoretical and Applied 4th ed. vol. 1.
Churchill Livingstone, Edinburgh.

PETRIE, H.T., HUGO, P., SCOLLAY, R. & SHORTMAN, K. 1990
Lineage relationships and developmental kinetics of immature thymocytes. CD3, CD4 and CD8 aquisition in vivo and in vitro.
J. Exp. Med. 172: 1583-1588

PIZZOLO, G. & CHILOSI, M. 1984
Double immunostaining of lymph node sections by monoclonal antibodies using phycoerythrin labelling and haptenated reagents.
Am. J. Clin. Pathol. 82: 44-47

POFFENBARGAR, E., KLAUSNER, J.S. & CAYWOOD, D.D. 1985
Aquired myasthenia gravis in a dog with thymoma: A case report.
JAAHA 21: 119-123

PRIETO, D., HERNANDEZ, M., RIVERA, L., ORDAZ, E. & GARCIA SACRISTAN, A. 1993
Catecholaminergic innervation of the equine ureter.
Res. Vet. Sci. 54: 312-318

PULLEY, L.T. & STANNARD, A.A. 1990a
In: Tumours of Domestic Animals 3rd ed. p.59
Ed. Moulton
University of California Press, Berkeley

RAE, C.A., JACOBS, R.M. & COUTO, C.G. 1989
A comparison between the cytological and histological characteristics in thirteen canine and feline thymomas.
Can. Vet. J. 30: 497-500

REICH, N.C., OREN, M. & LEVINE, A.J. 1983
Two distinct mechanisms regulate the levels of a cellular tumour antigen, p53.
Mol. Cell Biol. 3: 2143-2150

RIBAS, J.L., MENA, H., BRAUND, K.G., SESTERHENN, I.A. & TOIVIO-KINNUCAN, M. 1989
A histologic and immunohistochemical study of choroid plexus tumours in the dog.
Vet. Pathol. 26: 55-64

RICE, R.H. & GREEN, H. 1979
Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross linking by calcium ions.
Cell 18: 681-694

ROBINSON, M. 1974
Malignant thymoma with metastases in a dog.
Vet. Pathol. 11: 172-180

RODRIGUEZ, A., PENA, L., FLORES, J.M., GONZALEZ, M. & CASTANO, M. 1992
Immunocytochemical study of the diffuse neuroendocrine cells in equine lungs.
Anat. Histol. Embryol. 21: 136-145

ROITT, I.M. 1997
Essential Immunology 9th ed. Part IV Section 10
Blackwell Science, Oxford.

ROSAI, J. & LEVINE, G.D. 1976
Tumors of the thymus. In: Atlas of Tumor Pathology 2nd series fasc. 13
Armed Forces Institute of Pathology, Washington

ROSSOW, K.D., LAUBE, K.L., GOYAL, S.M. & COLLINS, J.E. 1996
Fetal microscopic lesions in porcine respiratory and reproductive syndrome virus-induced abortion,
Vet. Pathol. 33: 95-99

RUNGGER-BRUNDLE, E. & GABBIANI, G. 1983
The role of cytoskeletal and cytocontractile elements in pathologic processes.
Am. J. Pathol. 110: 361-392

RYAN, J.J., PROCHOWNIK, E., GOTTLIEB, C.A., APEL, I.J., MERINO, R., NUNEZ, G., CLARKE, M.F., 1994:
c-myc and bcl-2 modulate p53 function by altering p53 subcellular trafficking during the cell cycle.
Proc Natl Acad Sci USA, 91, 5878-5882.

SAGARTZ, J.E., BODLEY, W.L., GAMBLIN, R.M., COUTO, C.G., TIERNEY, L.A. & CAPEN, C.C. 1996
p53 tumour suppressor protein overexpression in osteogenic tumours of dogs.
Vet. Pathol. 33: 213-221

SAID, J.W., BARERRA, R., SHINTAKU, I.P., NAKAMURA, H., KOEFFLER, H.P., 1992:
Immunohistochemical analysis of p53 expression in malignant lymphomas.
Am J Pathol, 141, 1343-1348.

SANDUSKY, G.E., CARLTON, W.W. & WIGHTMAN, K.A. 1987
Diagnostic immunohistochemistry of canine round cell tumours.
Vet. Pathol. 24: 495-499

SAWANGJAROEN, N., OPDEBEECK, J.P. & PROCIV, P. 1995
Immunohistochemical localisation of excretory/secretory antigens in adult Ancyclostoma caninum using monoclonal antibodies and infected human sera.
Parasite Immunol. 17: 29-35

SCHEFFNER, M., WERNESS, B.A., HUIBREGTSE, J.M., LEVINE, A.J., HOWLEY, P.M., 1990:
The E6 oncoprotein encoded by Human Papilloma Virus types 16 and 18 promotes the degradation of p53.
Cell, 63, 1129-1136.

SCOTT, D.W., MILLER, W.H. & GRIFFIN, C.E. 1995
Muller & Kirk's Small Animal Dermatology 5th Ed. Chp 1. p21
W.B. Saunders Company, Philadelphia

SCOTT-MONCRIEFF, J.C., COOK, J.R. & LANTZ, G.C. 1990
Aquired myasthenia gravis in a cat with thymoma.
JAVMA 196: 1291-1293

SHELTON, G.H., GRANT, C.K., COTTER,S.M., GARDNER, M.B., HARDY, W.D. & DIGIACOMO, R.F. 1990
Feline immunodeficiency virus and feline leukaemia virus and their relationship to lymphoid malignancies in cats: a retrospective study (1968-1988).
J. AIDS 3 623-630

SHERCHAND, J.B., THAMMAPALERD, N., RIGANTI, M., THORDANIJ, S., PUNPOOWONG, B., BOREHAM P.F.L. & BOREHAM, R.E. 1994
Monoclonal antibody based immunohistochemical demonstration of Entamoeba histiolytica in liver tissues of experimentally infected hamster (Mesocricetus auratus).
Interntl. J. for Parasitology 24: 909-916

SHI, S-R., KEY, M.E. & KALRA, K.L. 1991
Antigen retrieval in formalin -fixed, paraffin embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections.
J. Histochem. Cytochem. 39: 741-748

SHU, S., JU, G. & FAN, L. 1988
The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system.
Neuroscience Lett. 85: 169-171

STEINERT, P.M., STEVEN, A.C. & ROOP, D.R. 1985
The molecular biology of intermediate filaments.
Cell 42: 411-419

STEINERT. P., ZACKROFF, M., AYNARDI-WHITMAN, . & GOLDMAN, R.D. 1982
Isolation and characterisation of intermediate filaments.
Methods Cell Biol. 24: 399-

STEINMAN, R.A., HOFFMAN, B., IRO, A., GUILLOUF, C., LIEBERMANN, D.A., EL-HOUSEINI, M.E., 1994:
Induction of p21(WAF-1/CIP-1) during differentiation.
Oncogene, 9, 3389-3396.

STERNBERGER, L.A., HARDY, P.H., CUCULIS, J.J. & MEYER H.G. 1970
The unlabelled antibody enzyme method of immunocytochemistry. Preparation and properties of soluble antigen antibody complex (horseradish peroxidase-

anti horseradish peroxidase) and its use in the identification of spirochaetes.
J. Histochem. Cytochem. 18: 315-333

STOBER, M. (1981)
The clinical picture of the enzootic and sporadic forms of bovine leukosis.
Bov. Pract. 16: 119-129

STOWARD, P.J. 1990
In: Histochemistry in Pathology 2nd ed. Chp 2
eds.Filipe, M.I. and Lake, B.D.
Churchill Livingstone, Edinburgh.

STREEFKIRK, J.G. 1972
Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol.
J. Histochem. Cytochem. 20: 829-831

SUTER, M.M., PALMER, D.G., ZINDEL, S. & SHENCK, H. 1984
Pemphigus in the dog: Comparison of immunofluorescence and immunoperoxidase method to demonstrate intercellular immunoglobulins in the epidermis.
Am. J. Vet. Res. 45: 367-369

STREETT, C.S., ALTMAN, N.H., TERNER, J.Y. & BERDJIS, C.C. 1968
A series of thymomas in the Angora goat.
Edgewood Arsenal Special Publication 100-36
Dept. of the Army, Maryland

STRYER, L. 1988
Biochemistry 3rd ed. Chp.36
Freeman & Co. New York

TAKAHASHI, Y., UNO, T., MIZUNO, N., SUZUKI, H., YAGI, J. & ARAKI, T. 1988
Immunohistochemical localisation of antigenic substances in Trichinella spiralis muscle larvae.
Jap. J. Parasitology 37: 435-440

TANIMOTO, T., MINAMI, A., YANO, S. & OHTSUKI, Y. 1994
Ileal lymphoma in swine.
Vet. Pathol. 31: 629-636

TANIYAMA, H., SHIRAKAWA, T., FURUOKA, H., OSAME, S., KITAMURA, N. & MIYAZAWA, K. 1993
Spontaneous diabetes mellitus in young cattle: histologic, immunohistochemical and electron microscopic studies of the islets of Langerhans.
Vet. Pathol. 30: 46-54

TAYLOR, C.R. & BURNS, J. 1974
Immunohistochemical detection of intracellular immunoglobulin containing cells

in formalin-fixed, paraffin-embedded tissues using peroxidase labelled antibody.
J. Clin. Pathol. 27: 14-20

TAYLOR, D.O.N., DORN, C.R. & LUIS, O.H. 1969
Morphologic and biologic characteristics of the canine cutaneous histiocytoma.
Cancer Res. 29: 83-92

THEILEN, G.H. & MADEWELL, B.R. 1987
Veterinary Cancer Medicine 2nd ed.
Lea & Febinger, Philadelphia.

THOMPSON, S.W. 1966
Selected Histochemical and Histopathological Methods
Thomas, Springfield.

THOR, A.D., MOORE, D.H., EDGERTON, S.M., KAWASAKI, E.S., REIHSAUS, E., LYNCH, H.T., MARCUS, J.N., SCHWARTZ, L., CHEN, L.C., MAYALL, B.H., SMITH, H.S., 1992:
Accumulation of p53 tumour suppressor gene protein: an independent marker of prognosis in breast cancers.
J Natl Cancer Inst, 84, 11, 845-855.

TITUS, J.A., HAUGHLAND, R., SHARROW, S.O. & SEGAL, D.M. 1982
Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorimetric and fluorescence microscopic studies.
J. Immunol. Methods 50: 193-204

TONER, P.G. & McCORMICK, D.F.W. 1992
In: Oxford Textbook of Pathology vol I Chp. 1.1
eds. McGee, Isaacson & Wright
Oxford University Press, Oxford

TORII, M., YANAGI, T., TSUBOI T. & KANBARA, H. 1992
Appearance and localisation of Plasmodium yoelii circumsporozoite protein during sporogeny in Anopheles stephensi.
J. Protozoology Res. 2: 134-140

VAN EWIJIK, W. 1991
T cell differentiation is influenced by thymic microenvironments.
Ann. Rev. Immunol. 9: 591-615

VAN KEULEN, L.J.M., SCHREUDER, B.E.C., MELOEN, R.H., POELEN-VAN DEN BERG,M., MOOIJ-HARKES, G., VROMANS, M.E.W. & LANGEVELD J.P. 1995
Immunohistochemical detection and localisation of prion protein in brain tissue of sheep with natural scrapie.
Vet. Pathol. 32: 299-308

VAN MAANEN, C., KLEIN, W.R., DIK, K.J. & VAN DEN INGH, T.S.G.A.M. 1996

Three cases of carcinoid in the equine nasal cavity and maxillary sinuses: Histologic and immunohistochemical features.
Vet. Pathol. 33: 92-95

VAN MOLL, P., ALLDINGER, S., BAUMGARTNER, W. & ADAMI,M. 1995
Distemper in wild carnivores: an epidemiological, histological and immunohistochemical study.
Vet. Microbiol. 44: 193-199

VICKERSTAFF, T. 1954
The Physical Chemistry of Dyeing 2nd ed.
Oliver & Boyd, Edinburgh.

VILLUENDAS, R., PIRIS, M.A., ORRADRE, J.L., MOLLEJO, M., ALGARA, P., SANCHEZ, L., MARTINEZ, J.C., MARTINEZ, P., 1992:
p53 protein expression in lymphomas and reactive lymphoid tissue.
J Pathol, 166, 235-241.

VISAKORPI, T., KALLIONIEMI, O., HEIKKINEN, A., KOIVULA, T., ISOLA, J., 1992:
Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation.
J Natl Cancer Inst, 84, 11, 883-887.

VOGELSTEIN, B. & KINZLER, K. 1992
p53 function and dysfunction.
Cell 70: 523-526

VOLF, P. 1994
Localisation of the major immunogen and other glycoproteins of the louse Polyplax spinulosa.
Interntl. J. for Parasitology 24: 1005-1010

WALDER, E.J. & GROSS, T.L. 1992
In: Veterinary Dermatopathology; A Macroscopic and Microscopic Evaluation of Canine and Feline Skin Disease. Section 2, Part V.
Gross, T. L., Ihrke, P.J. & Walder, E.J.
Mosby Year Book, St. Louis

WALKER, A.N., MILLS, S.E. & FECHNER, R.E. 1990
Thymomas and thymic carcinomas.
Semin. Diag. Pathol. 7: 250-265

WATRACH, A.M. 1969
The ultrastructure of canine cutaneous papilloma.
Cancer Res. 29: 2079-2084

WATT, F.M. 1992
In: Oxford Textbook of Pathology vol I Chp. 1.2

eds. McGee, Isaacson & Wright
Oxford University Press, Oxford

WATT, F.M., MATTEY, D.L. & GARROD, D.R. 1984
Calcium-induced reorganisation of desmosomal components in cultured human keratinocytes.
Cell Biol. 99: 2211-2215

WEBER, K. & GEISLER, N. 1985
Intermediate filaments: structural conservation and divergence.
Ann. NY Acad. Sci. 455: 126-143

WEINSTEIN, G.D. & VAN SCOTT, E.J. 1965
Autoradiographic analysis of turnover times of normal and psoriatic epidermis.
J. Invest. Dermatol. 45: 257-265

WEINSTOCK, D., HORTEN, S.B. & ROWLAND , P.H. 1995
Rapid diagnosis of Listeria monocytogenes by immunohistochemistry in formalin-fixed brain tissue.
Vet. Pathol. 32: 193-195

WEISS, E. & FRESE, K. 1974
Tumours of the skin.
Bull. World Health Org. 50: 79-100

WELLS, C.A., HERYET, A., BROCHIER, J. ET AL 1984
The immunocytochemical detection of axillary micrometastases in breast cancer.
Br. J. Cancer 50: 193-197

WHITELY, L.O., MAHESWORAN, S.K., WEISS, D.J. & AMES, T.R. 1990
Immunohistochemical localisation of Pasteurella haemolytica A1-derived endotoxin, leukotoxin and capsular polysaccharide in experimental bovine pasteurella pneumonia.
Vet. Pathol. 27: 150-161

WOLF, J.C., GINN, P.E., HOMER, B., FOX, L.E. & KURZMAN, I.D. 1997
Immunohistochemical detection of p53 tumour suppressor gene protein in canine epithelial colorectal tumours.
Vet. Pathol. 34: 394-404

WOHRMANN, T., HEWICKER-TRAUTWEIN, M., FERNANDEZ, A., MOENNIG, V., LIESS, B. & TRAUTWEIN, G. 1992
Distribution of bovine viral diarrhoea viral antigens in the central nervous system of cattle with various congenital manifestations.
J. Vet. Med. series B, 39: 599-609

WU, X., BAYLE, H., OLSON, D., LEVINE, A.J., 1993:
The p53 -mdm2 autoregulatory feedback loop.
Genes Devt, 7, 1126-1132.

WYNFORD-THOMAS, D. 1992
p53 in tumour pathology: can we trust immunohistochemistry?
J. Pathol. 166: 329-330

YAMASAKI, H., KOMINAMI, E. & AOKI, T. 1992
Immunohistochemical localisation of a cysteine protease in adult worms of the liver fluke Fasciola spp.
Parasitology Res. 78: 574-580

YEW, P.R., BERK, A.J., 1992:
Inhibition of p53 transactivation required for transformation by adenovirus E1B 55kd protein.
Nature, 357, 82-85.

YIN, X.M., OLTVAI, Z.N., KORSMEYER, S.J., 1994:
BH-1 and BH-2 domains of bcl-2 are required for inhibition of apoptosis and heterodimerization with bax.
Nature, 369, 321-323.