

DIAGNOSTIC IMMUNOHISTOCHEMISTRY: CURRENT APPLICATIONS AND FUTURE DIRECTIONS

SABARIAH RAHMAN. MBBS, MPath and ANTHONY S-Y LEONG, MBBS. MD. FRCPA. FRCPath, FCAP

Division of Tissue Pathology, Institute of Medical & Veterinary Science, Adelaide, *South* Australia.

Note: Dr Sabariah Rahman is a visiting Pathologist from the Department of Pathology, Universiti Sains Malaysia, Kubang Kerlan, Kelantan, Malaysia.

Summary

In this review, the applications of enzyme immunohistochemistry in diagnostic pathology are discussed. Antibodies for diagnostic use are grouped into eight categories, viz, antibodies for tumour typing, for lymphocyte antigens, to hormones, peptides, amines and hormone receptors, to oncofetal antigens, to oncogenes and their products, to cell proliferation antigens, to infectious agents and for the study of glomerular diseases. The practical applications of these antibodies are reviewed with particular emphasis on the use of antibody panels for the identification of anaplastic round cell tumour in adults and children and of spindle cell neoplasms. Future directions for immunohistochemical techniques include their interphase with molecular biology and their applications in immunoelectron microscopy, in in-situ hybridisation procedures, in automation of routine staining and in image analyses.

Keywords: Diagnostic immunohistochemistry, antibodies, oncogenes, lymphocyte typing, automation, in-situ hybridisation.

INTRODUCTION

The past two decades have witnessed several important developments in medical technology which are relevant to the diagnostic surgical pathologist. These include the applications of electron microscopy to tissue diagnosis, electron probe microanalysis, flow cytometry, morphometry, image analysis, molecular biology, chromosomal analysis, hybridoma technology and enzyme immunohistochemistry. While all of these techniques have made significant contributions to the practice of surgical pathology, the development of enzyme immunohistochemistry has allowed the surgical pathologist an immediate extension of his morphologic skills through the ability to stain for cell protein products in the same histological sections which he or she routinely examines. Refinements in the technique have resulted in enhanced sensitivity and reproducibility of results, and, more importantly, the development of hybridoma technology has allowed the production of an infinitely wider range of antibodies, extending further the capabilities and applications of the enzyme immunohistochemical stain. The ability to stain for the cellular protein product which is a reflection of the genomic composition of the cell, now provides pathologists with a means of identifying the immunophenotype of the cell and is a powerful tool to aid the recognition of many pathological lesions,

particularly neoplasms which have a similar morphological appearance. Immunohistochemistry has also contributed significantly to a greater understanding of the pathogenesis of inflammatory and non-neoplastic conditions, having many direct and indirect influences on patient management.

This report serves to review the applications of enzyme immunohistochemistry in diagnostic pathology and discusses the interphase between this new technology and other advances which have application in diagnostic pathology, including electron microscopy, molecular biology and cell imaging.

PRIMARY ANTIBODIES

Immunohistochemistry started with the fluorochrome labelling of primary antibodies to detect antigens in fresh frozen tissue sections. Immunoenzyme techniques are based on the attachment of an enzyme to a specific antibody. The enzymatic activity induces a visible colour change in the substrate and chromogen at the sites of localisation of the antibody-enzyme complex within the tissue. Horseradish peroxidase is the prototype of the immunoenzyme method and in the presence of an appropriate substrate such as hydrogen peroxide and a chromogen, such as diaminobenzidine, a brown reaction product is produced at the site of antibody-antigen

reaction. The development of the immunoenzyme staining procedure dispensed with the need for fresh frozen tissue and allowed the pathologist to work with permanent paraffin sections which produced superior morphologic preservation. The subsequent development of bridge techniques such as the peroxidase anti-peroxidase system¹ and later, the avidin-biotin peroxidase complex procedure,² resulted in amplification and enhanced sensitivity so that the applications of immunolabelling expanded tremendously. With greater consideration being given to fixation techniques and the processing of tissues, immunoenzyme labelling has established itself as a routine and sometimes indispensable adjunct to morphological examination.

The development of hybridoma technology³ has made it possible for the production of monoclonal antibodies which have the property of being extraordinarily specific against certain epitopes or antigenic determinants. It is now possible to produce antibodies which recognise fixation-resistant epitopes and concurrent developments in molecular biology has enabled peptide sequencing of antigens and the production of "designer antibodies" to a large range of specific antigens. While this is an advantage in diagnostic pathology, the plethora of antibodies on the market can make it equally confusing and there is an important requirement for laboratories to be fully familiar with the characteristics of the antibodies and reagents which they employ.

Antibodies for use in diagnostic pathology can be categorised as follows and their characteristics are discussed.

1. Antibodies for tumour typing
2. Antibodies to lymphocyte antigens
3. Antibodies to hormones, peptides, amines and hormone receptors
4. Antibodies to oncofetal antigens
5. Antibodies to oncogene and oncogene products
6. Antibodies to cell proliferation antigens
7. Antibodies to infectious agents
8. Antibodies for the study of glomerular diseases

ANTIBODIES FOR TUMOUR TYPING

Initial enthusiasm and attempts at producing an antibody to a cancer-associated antigen⁴ have abated.⁶ Indeed, there are few antibodies which are tissue specific and most antibodies used in tumour typing, at best, detect only tissue-associated antigens.

Intermediate filaments

Intermediate filaments are filamentous protein polymers of 7–11 nm diameter which form the cytoskeleton of eukaryotic cells. These filaments are identical at ultrastructural level but show distinct biochemical and immunological characteristics which allow them to be divided into five major classes. Cytokeratins are expressed in epithelial cells, vimentin in mesenchymal cells, are expressed in epithelial cells, vimentin in mesenchymal cells, desmin in myogenic cells, glial fibrillary acetic protein in astrocytes, and neurofilaments in neurons and certain other cells of the peripheral neuroendocrine system.^{7,8,9} The restricted expression of these filamentous proteins is conserved in both benign and malignant neoplastic proliferations so that their identification is a useful means of classifying tumours, particularly those with anaplastic morphology.

Cytokeratins

The cytokeratins are a multigene-coded group of at least 19 different cytokeratin proteins which are present in virtually every true epithelial cell.^{7,8,9,10} While the terms "cytokeratins" and "prekeratins" have been used in the past to refer to the keratins of simple epithelia which traditionally are not considered to show keratinisation, there is no convincing evidence that a distinct biochemical form of keratin exists in such epithelia. Therefore, the term "keratins" is now synonymous with "prekeratins" and "cytokeratins". Moll et al classified the cytokeratins of human epithelia into 19 groups according to their molecular weight;¹ Keratin 1 corresponding to the heaviest (67 Kd) and Keratin 19 to the lightest (40 Kd). Cytokeratins may be divided into two subfamilies based on their molecular weight, their immunoreactivity with monoclonal antibodies of restrictive specificity, and their isoelectric points.² Antibodies to cytokeratins are powerful tools in the identification of epithelial neoplasms.

Vimentin

Vimentin is a 57 Kd intermediate filament protein which is characteristically expressed by normal and neoplastic cells of mesenchymal origin, including endothelial cells, fibroblasts, macrophages, lymphocytes, chondrocytes and vascular smooth muscle cells.^{9,10,13} The expression of vimentin as the sole form of intermediate filament is indicative of mesenchymal differentiation, however,

expression of this protein is also seen in many non-sarcomas such as spindle cell carcinomas, anaplastic carcinomas of the breast, prostate and lung, so that in tumour typing, analysis of vimentin expression should be employed in conjunction with the examination for other intermediate filament expression.^{4,15}

Desmin

Desmin is a 55 year Kd intermediate filament protein which is coexpressed with vimentin in sarcomas manifesting muscle differentiation, i.e., rhabdomyosarcomas and leiomyosarcomas. Its expression is highly specific and "pathognomonic" of muscle differentiation.^{8,9,10,13}

Neurofilaments

The neurofilaments are protein triplets composed of three major subunits of 68, 150 and 200 Kd. They are found in neurons and their processes. Neurofilaments have been found in the differentiated ganglion cells of the central and peripheral nervous system neoplasms, in tumours of mixed cell origin such as gangliogliomas, ganglioneuroblastomas, neuroblastomas, medulloblastomas, differentiated retinoblastomas and the neuroelements of teratomas. In addition, these intermediate filaments have also been found in tumours of the peripheral neuroendocrine system, including tumours of the adrenal medulla, pancreatic islet cell tumours, carcinoid tumours, Merkel cell carcinomas, pulmonary oat-cell carcinomas and parathyroid adenomas.

Glial fibrillary acidic protein (GFAP)

GFAP is a 49 Kd protein expressed by astrocytes but may also be found in non-astrocytic central nervous system tumours such as ependymomas, subependymomas, oligodendrogliomas, choroid plexus papillomas and the stromal cells of haemangioblastomas. GFAP positivity may also be found in non-myelinating Schwann cells, cells of the myenteric plexus, folliculostellate cells of the pituitary, cells lining Rathke's pouch, epithelial cells of the eye lens, Kupffer cells, cartilage cells of the epiglottis and in some tumour cells in Schwannomas, neurofibromas, paragangliomas, salivary gland tumours and neoplasms of Mullerian origin.^{7,8,9,10,13}

TISSUE ASSOCIATED ANTIGENS

Antibodies to tissue associated antigens are particularly useful as they allow the specific identification of anaplastic tumours especially when they occur as metastases from unknown

primary sites. Unfortunately, very few antigens are truly tissue-specific and, at best, are only tissue-associated. This is important to remember as the demonstration of such antigens may not specifically indicate the source of the tumour and the less common exceptions should also be considered. For example, while prostatic acid phosphatase is most commonly expressed by tumours of prostatic origin, the antigen may also, less commonly be found in islet cell tumours, transitional cell carcinomas of the bladder, renal cell carcinomas and carcinoid tumours. Other tissue associated antigens include prostate specific antigen which is expressed by prostatic cells and their tumours,^{6,17} factor VIII-associated protein which is expressed by endothelial cells of blood vessels and their tumours, such as angiosarcoma and Kaposi's sarcoma,^{18,19} HMB45, a melanoma specific antigen which is expressed by epidermal naevus cells and malignant melanomas,²⁰ muscle specific actin which is expressed by cells and tumours showing myogenic differentiation including myoepithelial cells;¹ chromogranin which is expressed by neuroendocrine cells^{2,23} and placental alkaline phosphatase which is expressed by germ cells and their tumours.^{24,25}

ANTIBODIES FOR LYMPHOCYTE TYPING

A great deal has been learned about the maturation sequence of normal lymphoid cells through the study of neoplastic lymphocytes.²⁶ Immunotyping of lymphomas has passed through several phases. Initially, phenotyping was limited to the use of polyclonal antibodies reactive against lymphocyte antigens such as immunoglobulin, alpha-1-antitrypsin and lysozyme. The detection of light chain restriction identified a monoclonal population of lymphocytes which was equated to a neoplastic proliferation.^{26,27} During this period, with the limited repertoire of antibodies, many lymphomas were recorded as being of "null" phenotype and T cell lymphomas were largely unrecognized. On the basis of staining for alpha-1-antitrypsin and lysozyme, some large cell lymphomas of T cell lineage were incorrectly designated malignant proliferations of histiocytes or true histiocytic lymphomas.²⁸ The second phase of development followed the introduction of hybridoma technology which led to a proliferation of lymphocyte antibodies many of which were highly specific. This resulted in an often bewildering array of new monoclonal antibodies, some of which were

directed to the same antigen, but were produced by different laboratories, derived from different clones and assigned different names. It took the subsequent International Workshops on Leucocyte Differentiation to solve the problem of nomenclature with the introduction of the CD (cluster designation) classification system.²⁹ Laboratories were invited to submit monoclonal antibodies which were then distributed and tested biochemically, immunologically, and histologically by participants. Antibodies which recognised the same or a very similar antigen were assigned to the same CD group. With the development of a wide range of monoclonal differentiation antibodies, detailed immunophenotyping of lymphomas became possible, however, this was largely limited to cryostat section immunohistochemistry or flow fluorocytometry as many epitopes recognised at the phase of development were not fixation-resistant. The mid-1980's witnessed the third phase of development in which the products of hybridoma fusions also resulted in antibodies which recognised fixation-resistant epitopes. However, most of the initial antibodies produced have not been lineage specific and are variants of CD45 or the leucocyte common antigen (e.g. LCA, MB1, MT2, UCHL1, 4KB5).^{30,31} Lymphocyte typing is currently at a phase where major advances in molecular biology have enabled peptide sequencing of many of the CD antigens. Synthetic peptide sequences can now be made, enabling the production of highly specific monoclonal and polyclonal antibodies against formalin-resistant epitopes. Examples of these are the polyclonal CD3 antibody to T lymphocytes, the beta-F1 antibody to T lymphocytes, and OPD4, an antibody to T helper cells. Details of the applications of antibodies to differentiation antigens for the typing of lymphomas are beyond the scope of this paper and readers are referred to some excellent reviews.^{26,27,30,31}

ANTIBODIES TO HORMONES, PEPTIDES, AMINES AND HORMONE RECEPTORS

Some hormones are tissue specific. For example, thyroglobulin is largely expressed only by cells of the thyroid and their tumours. However other hormones such as calcitonin are not only expressed by the parafollicular C-cells of the thyroid and their tumours, but also by other endocrine tumours such as Merkel cell carcinoma³² and oat cell carcinomas of the lung. The analysis of various

hormones, peptides and amines not only indicates endocrine differentiation in the tumour but also allows the correlation of clinical symptoms with tumour secretory product. The analysis of oestrogen and progesterone receptor proteins has also been used as a prognostic markers as well as for the prediction of tumour response to hormonal therapy such as in carcinoma of the breast.^{33,34}

ANTIBODIES TO ONCOFETAL ANTIGENS

The detection of oncofetal antigens such as carcinoembryonic antigen, alpha-fetoprotein, and placental alkaline phosphatase are of diagnostic relevance in the identification of certain tumours which acquire these oncofetal proteins during neoplastic transformation. For example, the demonstration of alpha-fetoprotein is useful in the differentiation of hepatocellular carcinoma from other neoplasms of the liver and from non-neoplastic nodular hyperplasias.³⁵ Carcinoembryonic antigen is expressed by 50–60% of adenocarcinomas and can be used to distinguish these tumours from mesothelioma which does not express this oncofetal antigen.³⁶

ANTIBODIES TO ONCOGENES AND ONCOGENE PRODUCTS

Monoclonal antibodies in diagnostic pathology have largely been used to detect the gene products or proteins of cells. While this is a satisfactory method of studying cell function and cancer biology, the detection of gene-specific sequences, using DNA or mRNA probes would provide even more information relating to the cell genome. Recently, immunoenzyme techniques have been applied to the labelling of nucleic acids using chromogen-linked DNA and mRNA for the immunolocalisation of gene specific sequences by in-situ hybridisation. The combination of this technique and monoclonal antibody procedures allows the simultaneous study of tumour cell populations for genes of interest, as well as their protein products. Some of these include the c-myc transcripts and their gene product p62 in colorectal cancer and the ras gene family and its protein p21 in benign and malignant breast,^{37,38} prostate³⁹ and colonic diseases.⁴⁰ There is some information to indicate that the expression of these oncogenes and their products can be correlated with other prognostic variables, thereby serving as markers of tumour progression. Monoclonal antibodies RAP-5 and Y13-259 have been used to detect c-Ha-ras P21 expression in human gastric

adenocarcinomas and benign gastric **tissue**.⁴¹ The ability to immunostain for HER-2 oncogene products is also of prognostic relevance in breast **carcinoma**.⁴²

ANTIBODIES TO CELL PROLIFERATION ANTIGENS

There is strong evidence to indicate that the growth fraction of a tumour such as breast carcinoma, provides important prognostic information. Until recently, tumour growth fraction was measured by tritiated thymidine uptake **which** is a cumbersome and time-consuming procedure. The advent of flow cytometry provided another accurate method of obtaining information regarding **S-phase** fraction although it carries the inherent weakness of measuring a homogenised preparation of mixed cells. Recently, the development of two antibodies which define proteins expressed in restricted portions of the cell cycle allows the ability to immunostain for cells in their cycling phase, with the added advantage of simultaneous tissue identification.

Ki-67 is a monoclonal antibody which defines an as-yet unknown nuclear protein expressed in cells of the non-G₀ portion of the cell cycle."³ Ki-67 indices are higher than S-phase indices as the antibody detects cells in the entire cycling phase. A strong correlation has been shown between S-phase indices and Ki-67 **counts** in different **types** of tumours and it is anticipated that the antibody would have wide application in the study of tumour biology and prognostication. Unfortunately, the Ki-67 antibody is only immunoreactive in frozen **sections**.^{34,44,45}

PCNA or cyclin is a 36kD protein whose expression peaks in S-phase and it is possible to identify proliferating tumour cells with this antibody in **paraffin-embedded sections**.⁴⁶ BrdU is a thymidine analogue that can be administered to viable tumour cells either through intra-operative injection or incubation following tumour removal. The analogue is incorporated during S-phase and can be detected using highly specific monoclonal antibodies to BrdU. The technique, however, is impractical and not widely used.

ANTIBODIES TO INFECTIOUS AGENTS

Standard culture **techniques** to identify an infectious process may require from 48 hours to several weeks to isolate the bacteria, virus or parasite. The production of monoclonal antibodies against a variety of microbial agents have, therefore, produced a major impact on the diagnosis as well as research of infectious diseases. Immunologic assays provide a rapid

method for the detection of small quantities of antigens associated with micro-organisms. Monoclonal antibodies have been raised against bacteria including *B. abortus*, *E. coli*, *H. influenzae*, *L. pneumophila*, *M. tuberculosis*, *M. Leprae*, *N. gonorrhoeae*, *N. meningitidis*, *P. aeruginosa*, *T. palladium* and the staphylococcus species. In addition, monoclonal antibodies have been raised against viruses and protozoa and helminths. Among these are cytomegalovirus, Dengue virus, E-B virus, hepatitis B virus, herpes simplex virus, measles virus, polio virus, mumps virus, reovirus, rubella virus, human papilloma virus, plasmodium species, toxoplasma gondii and *trypanosoma*.⁴⁷

ANTIBODIES FOR THE STUDY OF GLOMERULAR DISEASES

The contributions of immunostaining in the diagnosis and understanding of renal diseases are well established. While many laboratories still employ a direct or indirect **immunofluorescence** technique for the study of glomerular diseases, the ability to employ immunoenzyme techniques allows the simultaneous visualisation of a paraffin section which has the advantage of better morphology, permanence of section, convenience of examination and storage of tissues, as well as obviating the necessity for a **second** core of tissue for immunofluorescence studies. Paraffin section immunostaining probably suffers from the inability to distinguish a granular pattern from an even, diffuse pattern of immunoglobulin deposition, which is sometimes important in the diagnosis of glomerular diseases. More recently, our ability to immunostain **resin-embedded** renal sections extends the scope of **morphologic** examination further and takes us closer to the goal of using the same core of tissue for all modalities of investigation, namely, morphologic, immunologic and ultrastructural examination."⁸

APPLICATIONS

The development and refinements in enzyme immunohistochemistry together with hybridoma technology have significantly expanded the vistas of morphologic diagnoses. Through the examination of differentiation markers in lymphoid malignancies, much has been learned about the maturation sequence of normal lymphocytes. The identification of infective organisms has been made much more rapid with immunostaining employing specific antibodies and the ability to identify various hormones, **peptides** and amines has

allowed a greater understanding of the differentiation potentials of neuroendocrine tumours and has had major influence on current classifications of peripheral neuroepithelial neoplasms. To the surgical pathologist, an important area of contribution of immunohistochemistry lies in the diagnosis and classification of poorly differentiated neoplasms. While it is not possible to provide a detailed discussion of the many applications of immunohistochemistry in tumour diagnosis, this review will indicate some principles of application, particularly in the identification of poorly differentiated or anaplastic tumours.

While the large majority of neoplasms can be diagnosed by morphological examination alone, a significant proportion remain "undifferentiated" or "poorly differentiated malignant neoplasms" in H & E stained sections. In the past, ultrastructural examination, cell culture studies, chromosomal analysis, and more recently, molecular biology studies have been employed to provide additional information. While useful, these ancillary procedures are time-consuming and expensive, and, perhaps with the exception of electron microscopy, these techniques often represent excursions into areas which may not be familiar to the morphologist. With increasing sophistication or cancer treatment regimes, some malignant neoplasms, including germ cell tumours, Hodgkin's disease, acute lymphoblastic leukaemia and lymphomas, osteogenic sarcoma, rhabdomyosarcoma and Wilm's tumour have proven to be potentially curable. Other tumours may have a high rate of remission and many are amenable to significant palliative treatment. The function of the pathologist in the accurate identification of such tumours is critical, not only in their broad categorisation, such as distinguishing between carcinoma, lymphoma and melanoma but also in their further subclassification. The latter function is often based on subtle and frequently subjective features which are interpreted to indicate a particular line of differentiation of the tumour. The pathologist is also often required to predict the site of origin of metastatic tumours through the examination of their morphological characteristics.

On morphological grounds, poorly differentiated or undifferentiated malignant neoplasms can be largely separated into a group of round cell tumours and a group of spindle cell tumours. Morphological examination is still the primary means of assigning anaplastic neoplasms into either of these two broad categories. When considered in conjunction with the clinical features,

particularly the age of the patient and the site of the tumour, a list of differential diagnoses can be made. Through the application of an appropriate panel of antibodies, in accordance with this list, it is often possible to further accurately identify the tumour. It is emphasised that antibodies should be applied not only to positively characterise the tumour in question but also to exclude the other entities considered in differential diagnoses. They should, therefore, be used as

TABLE 1
PHENOTYPING OF ANAPLASTIC ROUND
CELL TUMOURS IN ADULTS

	<u>CK</u>	<u>VIM</u>	<u>LCA</u>	<u>S100</u>	<u>HMB45</u>
Carcinoma	+	±	-	±	-
Melanoma	--	t	-	t	+
Lymphoma	-	t	+	-	-

CKF = cytokeratin; **VIM**= vimentin;
LCA = leucocyte common antigen;
S100 = S100 protein; **HMB45** = a melanoma-specific antigen.

a panel and not solely for the identification of the presence or absence of a single antigenic constituent. Negative stains also make a contribution by exclusion and the interpretation of immunostains should not be made in isolation of the clinicopathological features of the individual case.

Anaplastic round cell tumours in adults

In the adult, the main entities considered in the differential diagnosis of anaplastic round cell tumours would be anaplastic carcinoma, malignant melanoma and malignant lymphoma, their distinction having important therapeutic implications. These three lesions can, in a large number of instances, be differentiated by means of the panel of antibodies shown in Table 1.^{49,50}

Among this group of tumours, cytokeratins are exclusively seen in carcinomas. It is important to appreciate that cytokeratins of lower molecular weights are found in simple epithelia and is the first to appear during embryonic life, whereas, the higher molecular weight cytokeratins are associated with stratified epithelia. The antibody CAM 5.2 (Becton Dickinson), for example, detects lower molecular weight cytokeratins of 39 and 43kD, whereas, the polyclonal antibody to callus keratin (Dako) detects cytokeratins of 56 and 64kD. The polyclonal antibody to keratin (Dako) is a useful broad spectrum

anti-keratin which detects cytokeratins of 48, 50, 52, 56, 58 and 60kD. While vimentin is expressed by both melanoma and lymphoma, it may infrequently be coexpressed with cytokeratins in epithelial neoplasms. Carcinomas which express vimentin in a consistent and predictable manner include those from the breast, lung, ovary, kidney, prostate, thyroid, endometrium, salivary gland and liver.^{4,15} Carcinoma cells growing in serous cavities may also express vimentin.¹ LCA is specific to lymphomas.^{5,2} S100 protein is strongly expressed by melanoma but may uncommonly be seen in some carcinomas such as breast, genitourinary tract, pancreas, salivary glands, sweat glands and lungs.^{5,3, 5,4}

HMB45 is largely melanoma specific^{2,0} although it has uncommonly been seen in breast carcinomas.⁴

Anaplastic round cell tumours in childhood

In the child, the differential diagnoses of anaplastic round cell tumours is different from that in the adult and includes Ewing's sarcoma, peripheral neuroectodermal tumours, neuroblastoma, lymphoma/leukaemia, and rhabdomyosarcoma. While Wilm's tumour may appear similar morphologically, it is readily recognised from its site of occurrence. The panel of antibodies suitable for the differentiation of these entities and their staining characteristics are shown in Table 2.^{5,5}

TABLE 2
PHENOTYPING OF ROUND CELL TUMOURS IN CHILDHOOD

	LCA	VIM	CK	DES	MSA	NF	S100	NSE	Leu7
Lymphoma/leukaemia	+	+	-	-	-	-	-	*	-
Rhabdomyosa	-	+	-	+	+	-	-		±
Ewing's sarcoma	-	+	±	-	-	-	-	±	±
Neuroblastoma	-	+	-	-	-	+	±	+	+
PNET	-	+	±	-	-	±	-	+	±

LCA = leucocyte common antigen; VIM = vimentin; CK = cytokeratins; DES = desmin; MSA = muscle specific actin; NF = neurofilaments; S100 = S100 protein; NSE = neuron specific enolase; Leu7 = CD 57

The diagnosis of lymphomas/leukaemias in childhood is no different to that in the adult in that the tumour cells invariably express LCA and vimentin, while staining is negative for the other antigens. Having established the tumour to be of haemato-lymphoid derivation, it may be necessary to further characterise the lesion by lineage typing employing some of the monoclonal antibodies which are immunoreactive in paraffin-embedded sections. Vimentin expression is seen in all the tumours considered in differential diagnosis although Ewing's sarcoma and PNETs may occasionally show cytokeratin. Desmin and muscle specific actin are specific to cells displaying myogenous differentiation. Myoglobin would separate striated muscle from smooth muscle but this antigen is only expressed by well differentiated striated muscle cells so that it is often of little value in identifying poorly differentiated rhabdomyosarcoma.^{5,4} In any case, the distinction of skeletal muscle tumours from smooth muscle tumours can often be made on morphologic grounds. Neurofilaments are the hallmark of neuroblastomas and may

occasionally be found in PNETs. S100 staining is occasionally seen in neuroblastomas which show sustentacular cells or Schwann cell differentiation. Neuron specific enolase is characteristic, but not specific for neurons and axons and for endocrine and neuroendocrine cells. While this antigen is consistently expressed by neuroblastoma and PNETs, it may also be seen in rhabdomyosarcomas and its expression in Ewing's sarcoma is taken to indicate possible neuronal differentiation by these tumours.⁶ Leu7 (HNK-1) or CD 57 was originally described as an antigen found on natural killer cells in the blood. It was fortuitously found to be also expressed in selected non-haemopoietic cells such as neuroendocrine cells and cells of the peripheral nerve sheath.^{7,7} It is, therefore, expressed by neuroblastomas and PNETs and very occasionally is found in rhabdomyosarcomas and Ewing's sarcomas.

Spindle cell tumours

The differential diagnoses of spindle cell tumours include spindle cell carcinoma, smooth muscle tumours, malignant fibrous

hsitiocytoma (**MFH**), haemangiopericytoma, Kaposi's sarcoma, angiosarcoma, peripheral nerve sheath tumours (PNST), synovial sarcoma and epithelioid sarcoma. While this list is comprehensive, in actual practice, the list of differential diagnoses can be narrowed to no more than three or four entities depending on the clinicopathologic setting and the morphologic appearances. A panel of antibodies which may be used to differentiate these spindle cell tumours is provided in Table 3.^{5,8} Spindle cell carcinomas will express cytokeratin and will also coexpress vimentin,⁵ however, the other tissue-associated markers will not be found in these tumours. Smooth muscle tumours have, on rare occasions, been demonstrated to also express cytokeratin^{5,9} and synovial sarcomas and epithelioid sarcomas show the phenotypic pattern of carcinosarcomas in that they express both cytokeratins and vimentin. In synovial sarcomas, both the glandular as well as spindle cell component may express cytokeratin. Smooth muscle tumours are identified by the expression of vimentin, desmin and muscle specific **actin**. Uncommonly, there may be faint staining for **S100** protein. **MFH** displays no characteristic phenotype although the multinucleated and round cells often stain strongly positive for alpha-1-antitrypsin and **alpha-1-antichymotrypsin**, and less frequently for lysozyme. The expression of these markers was initially thought to indicate their histiocytic lineage but it is now well accepted that these enzymes may be expressed in varying degrees by other neoplasms of both mesenchymal as well as epithelial origin. The occasional expression of muscle specific **actin** in MFH denotes myofibroblastic differentiation in the tumour cells. Haemangiopericytoma is characterised by the absence of any of the markers in the panel except for vimentin. Kaposi's sarcoma and angiosarcoma are mesenchymal tumours which express factor VIII related protein, a marker of vascular endothelial cells. In addition, they show **positivity** for Ulex europeus agglutinin I, which is a more sensitive marker than factor VIII related protein but is also expressed by lymphatic endothelial cells. PNST is a mesenchymal tumour showing vimentin **expressin** as well as the nerve sheath markers **S100** protein and **Leu7**. In addition, they also stain for myelin basic protein, and, for a more recently **defined** antigen, nerve growth factor receptor which is a marker characteristic of nerve sheath cells, especially of the epineurium. Lastly, synovial sarcoma and epithelioid sarcoma are characterised by the coexpression of cytokeratin and vimentin.^{60,61}

Type IV collagen and **laminin** are proteins found in the basement **membrane** and are useful markers of basement membrane in the immunohistochemical typing of **tumours**.^{62,63} Among mesenchymal cells and their corresponding tumours, basement membrane expression is largely restricted to repertoire of tissue-associated markers currently available. Immunohistochemistry was not contributory in 11.8% and the major reason for this was the suboptimal preservation of tissue antigens, especially in material received from other laboratories for consultation. Among the round cell tumours, immunohistochemical typing provided a definitive diagnosis in 70% of cases compared to the 92% obtained for spindle cell tumours.

FUTURE DIRECTIONS AND CONCLUSIONS

The adaptation of enzyme-linked techniques to in-situ hybridisation procedures allows the detection of genomic sequences in the cell. Chromogen-linked DNA and **mRNA** probes are viable alternatives to radioactive labelling and allow for better morphological localisation and **visualisation**.⁶⁴ Non-radioactive methods are also more convenient, faster and less expensive. Currently, **radio-nucleotide** labelling appears to be more sensitive in some situations but with improvements in the sensitivity of commercial DNA and **mRNA** probes, the convenience endothelial cells, nerve sheath cells, smooth muscle cells and their tumours. Tumours negative for basement membrane production include synovial sarcomas and **MFH**.⁶³

In a recent analysis of the use of immunohistochemistry in our laboratories for the diagnosis of tumours, we found that among 557 consecutive tumours, the procedure allowed a definitive diagnosis in 75.4% of cases.⁸ Immunostaining provided contributory but nondiagnostic information in 12.8%. Most of these tumours were confirmed to be metastatic carcinoma, but their primary source remained undetermined because of the limited of chromogen linked techniques and their other advantages will be an important extension to the technique armamentarium in surgical pathology. The combined ability for immunolocalisation of gene-specific sequences and the ability to detect their protein products **are powerful** tools in the study of oncogenesis, especially as the "oncogene story" unfolds with emerging information from seemingly different disciplines in biomedical research. The relationship between oncogenes and growth factors, plasma membrane receptors, and transduction signalling is perhaps the best known example of this phenomenon.

TABLE 3
PHENOTYPING OF SPINDLE CELL TUMOURS

	<u>CK</u>	<u>VIM</u>	<u>DES</u>	<u>MSA</u>	<u>F8/UEA</u>	<u>S100</u>	<u>LEU7</u>
Spindle carcinoma	+	±	-	-	-	-	-
Smooth muscle	±	±	+	+	-	±	-
MFH	-	±	-	±	-	-	-
Hemangiopericytoma	-	±	-	-	-	-	-
Kaposi's sarcoma	-	+	-	-	+	-	-
Angiosarcoma	-	+	-	-	+	-	-
PNST	-	+	-	-	-	+	+
Synovial sarcoma	+	+	-	-	-	-	-
Epithelioid sarcoma	+	+	-	-	-	-	-

CK = cytokeratin; VIM = vimentin; DES = desmin; MSA = muscle specific actin;
F8 = Factor VIII related protein; UEA = ulex europaeus agglutinin I; S100 = S100 protein;
LEU7 = HNK-1 (CD57); MFH = malignant fibrous histiocytoma;
PNST = peripheral nerve sheath tumour.

The relationship between human papilloma virus and the pathogenesis of cervical condylomas, dysplasias and invasive carcinomas has emerged from in-situ hybridisation studies and the viruses have been detected by immunohistochemical techniques using monoclonal antibodies as well as chromogen linked DNA probes.⁶⁵⁻⁶⁷ The measurement of c-myc transcripts and their gene product p62 in colorectal cancer and the ras gene family and its protein p21 in benign and malignant mammary,^{37,38} prostatic³⁹ and colonic diseases⁴¹ have suggested that these parameters can be correlated with other prognostic variables, thereby serving as markers of tumour progression.

The differences in immunoreactivity using various monoclonal antibodies and DNA or mRNA probes can be semi-quantitated by densitometric methods. The development of sophisticated hardware and software for computer-based systems provides the interface between image analysis and immunohistochemistry. While the **microspectrophotometric** quantitation of DNA content for the determination of DNA ploidy is an established technique with prognostic implications in tumours of the bladder, prostate, ovary and breast, the quantitation of immunofluorescence and immunohistochemical staining is a more recent and exciting application of image analysis. For example, with the ability to immunostain for oestrogen and progesterone receptors and HER-2 oncoprotein in breast cancer and multidrug resistance proteins in a variety of neoplasms, it has been suggested

that not only is the presence of these markers prognostically important, but the quantity of the marker or the pattern of distribution among cells is also significant. Image analysis allows the quantitation of immunostaining for these various markers and would enhance the power of immunologic and molecular technologies.

Monoclonal antibodies have also breathed new life into older techniques. **Immunoelectron** microscopy has proven to be a valuable adjunct for the precise immunolocalisation of antigens at ultrastructural level and improvements in the technology offer continued hope for sharper definition and improved resolution. Immunoelectron microscopy offers the ability to localise specific immunoreactants to cells which may not have sufficient antigen for an identifiable reaction product to be visualised by light microscopic immunohistochemistry and thus can be useful in characterising poorly differentiated neoplasms.

With the increasing use of immunohistochemical procedures in routine diagnostic laboratories, it would appear that there is a role for automated routine **immunohistochemical** staining. The repeated cycles of buffer wash, reagent application, and buffer wash, seem ideally suited to automation, however, a significant scope for individual slide flexibility has to be maintained in such equipment so as to provide for **reaction** with several primary antibodies, low anti-sera **volumes** to match manual costs, as well as a rigorous quality assurance system to ensure that the sections

are treated only with correct reagents. Two such systems have been reported^{68,69} one of which is commercially available.⁶⁸

Undoubtedly, the applications of immunohistochemistry will continue to expand and the ever increasing range of monoclonal antibodies will continue to be spurred by a parallel rise in the biotechnology industry. Research reports of a new antibody whose nomenclature is that of a seemingly random sequence of letters and numbers, many be tomorrow's commercially available monoclonal antibody to a diagnostically important antigen. While some antibodies are of tremendous diagnostic relevance, the rapid proliferation of reagents has prompted the call for a consideration of reagent standardisation and other problems related to quality control.⁷⁰ Diagnostic immunohistochemistry is here to stay and will continue to expand the diagnostic ability of the surgical pathologist, freeing the pathologist from dependence on purely morphologic criteria in the identification and characterisation of neoplastic tissues and other lesions and allowing the identification of the cell or infectious agent by their specific polypeptide products and constituents, or nucleic acid content.

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