J Clin Pathol 1987;**40**:1042–1054

The role of immunocytochemistry in diagnostic pathology

D Y MASON, K C GATTER

From the Nuffield Department of Pathology, John Radcliffe Hospital, Oxford

SUMMARY This review suggests that immunocytochemistry in diagnostic pathology can be performed using relatively small panels of antibodies and that it should be reserved for situations in which, for one reason or another, the pathologist cannot exert his or her conventional diagnostic skills. Examples include the diagnosis of tumours the true nature of which is uncertain because of anaplasia or poor morphological preservation; the demonstration of small numbers of cells which are otherwise too rare to be recognised in conventionally stained preparations; and the immunophenotyping of non-Hodgkin's lymphomas. Recently progress has been made in the context of non-Hodgkin's lymphomas by the development of monoclonal antibodies that detect T and B cell associated markers in paraffin wax sections. Most of these reagents, however, recognise either lineage *associated* (but not lineage *specific*) variants of the leucocyte common antigen CD45, or antigens that are poorly characterised. A recent promising development has therefore been the demonstration that polyclonal antisera raised against the CD3/T3 T cell specific marker (purified by affinity chromatography) are suitable for staining T cells in paraffin sections. This approach will hopefully enable antibodies to be produced which react with other well defined white cell associated markers in routine biopsy material.

This issue of the *Journal of Clinical Pathology* celebrates the foundation 25 years ago of the Royal College of Pathologists. Immunocytochemistry as a major technique in diagnostic pathology cannot claim to be of comparable antiquity: although immunocytochemical methods were used diagnostically in a number of laboratories in the 1960s for purposes such as the demonstration of autoantibodies, they did not become of practical relevance to the general histopathologist until the early 1970s. The present review traces the development since that time of immunocytochemistry as a diagnostic procedure in pathology, and considers how these methods may become of even wider scope in the future.

Early studies

Pathologists first became aware of the extent to which immunocytochemistry might be applied in diagnostic histopathology in 1974 after the publication by Taylor and Burns in the *Journal of Clinical Pathology*, which reported that immunoperoxidase labelling could show the presence of plasma cell immunoglobulin in paraffin embedded tissues.[1] In retrospect, this may seem a minor technical discovery, but at the time it was widely accepted that routinely processed paraffin embedded tissue was quite unsuitable for immunocytochemical analysis. For the first time histopathologists were offered the exciting possibility of being able not only to widen the scope of their diagnostic procedures, but also to study retrospectively tissue which had been stored for many years.

The feasibility of detecting immunoglobulin in routinely processed paraffin embedded tissue was soon confirmed by other laboratories,[2,3] although the hope that it would provide a simple and reliable means of immunophenotyping lymphomas in the diagnostic histopathology laboratory proved premature. Indeed, in retrospect, it is unfortunate that immunoglobulins, and in particular κ and λ light chains, were the first antigens to be detected in routinely processed tissue, as they are present in such high amounts in the circulation that cellular staining for immunoglobulin is as likely to represent diffusion artefacts as endogenous synthesis.[4–6]

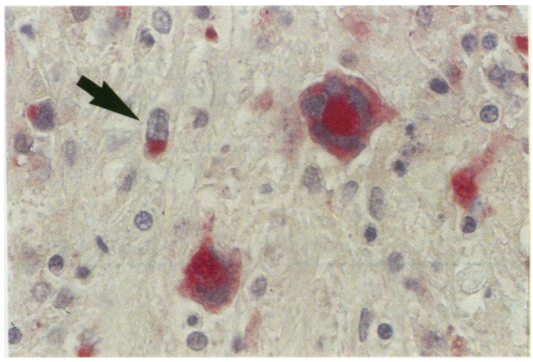

Fig 1 *Paraffin wax embedded tissue from one of Thomas Hodgkin's original cases stained for the CD15 antigen (hapten X). Note strong staining of two Reed-Sternberg cells and a mononuclear Hodgkin's cell (arrowed). (APAAP staining).*

One of the great attractions of staining paraffin embedded tissue is that it allows retrospective immunocytochemical studies to be performed. It soon became apparent that many antigenic molecules (such as hormones) can be shown even when tissue has been stored for many years.[7] Such "archeological" immunocytochemistry has recently been taken to what one assumes must be near its limit by the report of Poston and Sidhu (presented at the meeting of the Pathological Society, Oxford, 1987) that Reed-Sternberg cells in some of the tissues fixed by Thomas Hodgkin in the first half of the nineteenth century can be stained for hapten X (CD15) (fig 1).

Subsequent developments

Several major advances in immunocytochemical techniques and their applications in diagnostic pathology that have occurred since the late 1960s deserve mention. It was shown that proteolytic enzymes could strikingly enhance the intensity of staining of routinely processed paraffin embedded tissue, and this came as a revelation to many pathologists struggling to interpret weak and capricious staining reactions.[8-10] The mechanism by which proteolytic enzymes unmask antigens in this type of material remains unexplained, but many pathologists gratefully accept the element of "cookery" without question, as they are already accustomed to basing most of their diagnoses on the staining of tissue sections by a brew in which the major component (haematoxylin) is an extract of logwood chips.

Although proteolytic treatment of tissue sections opened new vistas in the immunocytochemical staining of tissue sections, the inevitable denaturation of antigens that are present in small amounts, particularly in cell surface membranes, remained a problem. Much was made in publications at that time of the ability of immunoperoxidase staining to show monotypic cytoplasmic immunoglobulin in routinely embedded paraffin sections of lymphomas. Yet mention of labelling for surface immunoglobulin was conspicuously absent, despite the fact that for several years this had been central to the immunologist's working definition of a B cell. We may now be closer to a resolution of the problem of the loss of surface membrane antigens in routine paraffin sections. In the 1970s, however, no convincing description of surface Ig labelling in paraffin sections had been published, and the report by Levy, Warnke, and others of how this obstacle could be overcome by staining cryostat sections of human tissue by immunofluorescence techniques was a major advance.[11,12]

Subsequently, the staining of cryostat sections became more "user friendly" with the introduction of immunoenzymatic procedures for staining antigens in paraffin sections.[13,14] Fortuitously this optimisation of techniques for staining human cell surface membrane antigens in tissue sections coincided with the description by Kohler and Milstein of their technique for producing monoclonal antibodies. In one leap the list of human cell surface antigens was expanded from a small handful—for example, HLA-DR, Ig—to a potentially endless list.

The introduction of monoclonal antibodies thus gave a timely boost to the use of immunocytochemistry in diagnostic pathology just when the limited range of antigens detectable in paraffin sections was beginning to dampen some pathologists' enthusiasm for the technique. The very success of monoclonal antibodies soon brought problems, however: the number of different antibody specificities is now large and many pathologists (and even professional immunologists) can find this a bewildering field to grasp. Fortunately, a series of international workshops devoted to monoclonal antibodies directed against antigens present on human leucocytes (table 1) has helped to clarify matters, at least for white cell associated molecules.[15-17] These workshops have focused on the importance of characterising monoclonal antibodies in terms of the molecules which they recognise; this in turn has led to the classification of antileucocyte antibodies according to the "CD" system (table 2). At the most recent workshop, held in 1986, 19 new CD antigens were identified, bringing the total to 45. Although this may seem a daunting number, it should be realised that it represents a distillation of data obtained in the three workshops using well over 1000 different monoclonal antibodies gathered from many different laboratories.

A comparable process of categorisation and

Table 1 *CD system for classifying human leucocyte antigens*

Three International Workshops on Human Leucocyte Differentiation Antigens have been held. Each of these meetings was based on monoclonal antibodies submitted by many different participating laboratories around the world. Aliquots were then sent out by the organisers to laboratories wishing to test the antibodies for reactivity.

Workshop	*Held at*	*Date*	*No of antibodies*	*CD antigens defined*
First	Paris	1982	207	15 (CD1–CDw15)
Second	Boston	1984	343	11 (CD16–CD26)
Third	Oxford	1986	917	19 (CD27–CD45)

Testing with antibodies is performed by three principal techniques:
(1) Immunofluorescent cell surface labelling (usually by FACS analysis);
(2) Immunoprecipitation (usually of surface labelled cells);
(3) Immunohistology on tissue sections.

The "CD" system for classifying antigens and the monoclonal antibodies which recognise them was established at the first workshop. In order for an antigen to be defined in the CD classification system it is usually necessary for it to have been recognised by at least two different workshop antibodies and for the molecular weight of the antigen to have been determined. There are exceptions to both of these rules: if only one antibody to an antigen is submitted to the workshop, but one or more antibodies of the same specificity have been described in the literature, a CD group may be established. Furthermore, certain antigens, such as glycolipids, cannot be analysed by conventional immunoprecipitation techniques and molecular weight data are hence often unobtainable.

The abbreviation "CD" stands for cluster of differentiation. It was the intention of the organisers of the first workshop that a CD number should be assigned to a group of antibodies defining an antigen (rather than to the antigen itself). Many laboratories, however, ignore this convention and use a CD designation to refer to the antigen (for example, CD15 is used synomonously with hapten X, CD2 with T11 antigen, etc).

classification of monoclonal antibodies against other cellular antigens (such as epithelial markers) has yet to be achieved. This is principally because of the difficulty inherent in performing biochemical and serological studies on antigens which are present in cells in solid tissue compared with circulating cells. Encouraging progress, however, is being made in the classification of cytokeratin molecules and of the monoclonal antibodies produced against some of them.[18]

Practical applications of immunocytochemistry in diagnostic pathology

The fact that immunocytochemistry of human tissues is now a rich and complex field (in terms both of the different techniques available and the large number of different molecular entities detectable in human tissues) has tended to cause a polarisation among pathologists. There are the "monoclonal antibody revolutionaries" and an opposing group of "contras". This polarisation is not, as far as we know, described in the literature but is nevertheless to be found in pathology departments throughout the world. Fig 2 encapsulates the arguments from these camps in two imaginary monologues.

Although these entrenched positions are extreme examples they will be familiar to many readers. The question therefore arises as to what middle position should be taken by pathologists who wish neither to ignore the value of monoclonal antibodies in diagnostic pathology, nor to accept unreservedly the more extravagant claims made on their behalf. The very large number of antibodies against human tissues which are now available makes this an urgent problem, given the almost limitless time and funds that can be spent on immunocytochemical studies. If this paper has a single message it is that although immunocytochemistry is a powerful and by now essential technique which pathologists ignore at their peril, it must be used judiciously and in clearly defined circumstances.

The median way may be found by keeping in mind that immunocytochemistry is most valuable in pathology when the normal means by which the pathologist exerts his or her diagnostic skills are thwarted for some reason. At this point immunocytochemistry comes into its own and can play an invaluable part in achieving a final diagnosis. Conversely, the clearer a diagnosis is after conventional morphological examination, the less likely it is that immunocytochemistry will have a role. Indeed, immunocytochemical investigations of samples in which the diagnosis is not in dispute are positively to be discouraged; at best they may confirm what is already known and at worst they are at odds with the diagnosis, causing ridicule and loss of face.

Guided by this rule one can identify the specific instances in which immunocytochemistry has a practical part to play. These are summarised in table 3.

DIAGNOSIS OF THE TUMOUR OF UNCERTAIN ORIGIN

One of the great successes in the application of immunocytochemistry to diagnostic pathology lies in the resolution of problems relating to the diagnosis of tumours of uncertain origin.[19 20] Such diagnostic

difficulty sometimes arises because cellular morphology, although well preserved, is uninformative. In other cases the tissue sample shows such severe distortion, either through physical damage—for example, crushing during biopsy—or from poor fixation—that normal morphological criteria can no longer be applied with confidence (fig 3). In both these instances immunocytochemical labelling is often very informative. A simple flow chart which summarises the patterns that enable different tumour cell types to be distinguished is given in fig 4. The proportion of problem cases which can be diagnosed in this way is encouragingly high (approaching 90%), and the clinical implications of making an erroneous diagnosis (in terms of inappropriate treatment) are such that most diagnostic pathology laboratories now make regular use of this application of immunocytochemistry.

SHOWING SMALL NUMBERS OF CELLS

The human visual system possesses a remarkable ability to detect atypical or aberrant cells against a heterogenous background of normal cells. This skill, the visual equivalent of the princess detecting the pea under the mattress, can nevertheless be stretched beyond its limits when abnormal cells are present only in very small numbers. In a study reported from this laboratory[21] it was shown that immunoenzymatic staining of axillary lymph nodes with antiepithelial antibodies can show micrometastases from carcinoma of the breast which escape detection on routine morphological examination. We have used a similar approach for the detection of carcinoma cells in smears of serous effusions and in bone marrow aspirates.[22 23] These studies have clearly shown that antibody based techniques can yield positive results when morphological examination is equivocal or negative, and they represent a further important area albeit of less importance than the diagnosis of tumours of uncertain type, in which immunocytochemical techniques are of value.

IMMUNOPHENOTYPING OF NEOPLASMS

The classification of tumours according to the antigens which they express can, by definition, be made only with the help of antibodies. At first sight there seems to be no contest between the conventional morphologist and the immunocytochemist, but this leaves unanswered the question of when it is necessary to perform immunophenotyping and how extensive this analysis should be.

If one considers non-Hodgkin's lymphomas it is clear that the temptation to use antibodies for their immunocytochemical analysis is strong, given the many different white cell associated antigens that can now be identified (table 2). Some studies, however, have suggested that whether a lymphoma is of T or B cell origin is of relatively little importance in terms of clinical outcome. A major distinction among large cell lymphomas of B cell type (between those that express surface immunoglobulin and those which are immunoglobulin negative) was initially thought to correlate with diagnosis. This was later shown, however, to be of no importance when the influence of other features, such as clinical staging, was accounted for.[24]

There is even sometimes an argument *against* immunophenotyping lymphoid neoplasms (or at least against issuing an unvarnished report of the results) because of the way in which such information is occasionally misinterpreted. A report that a lymphoblastic lymphoma/leukaemia carries B cell antigens occasionally causes clinicians undue alarm: they are aware that acute lymphoblastic leukaemia (ALL) of B cell type (L3 in the FAB classification) has a poor prognosis, but are ignorant of the fact that B cell associated antigens are also present on most cases of common ALL, neoplasms which usually respond much better to treatment.

Many laboratories concerned with the diagnosis of lymphoma would therefore probably now recommend limited phenotypic analysis (fig 5) and judicious consideration of how the information obtained should be incorporated into the final report.

The future

NEW ANTIBODIES

Some pathologists may be frustrated by the fact that monoclonal antibody technology has not yet yielded a range of markers which enable a computer to distinguish between neoplastic and normal tissue. Before turning in pique to in situ DNA hybridisation (which threatens to become the oldest new technique in pathology), however, they should reflect that the number of antigenic molecules currently detectable in human tissues probably represents only a minor fraction of the total number. The fact that each of the three workshops on human leucocyte differentiation antigens (table 1) identified new white cell associated molecules suggests that this field is far from exhausted. Consequently, the process of identifying new human cellular antigens, principally via the production of new monoclonal antibodies, will continue for a considerable period. Pathologists will play a major part in analysing the distribution of these molecules in normal and diseased tissues and in deciding on their practical value in clinical diagnosis.

The types of new antigenic marker that could be identified in this way include molecules specific for tumours which are currently difficult to recognise immunocytochemically, for example, Ewing's tumour and melanoma. It is also to be hoped that some of the

Table 2 *Summary of leucocyte associated antigens defined by CD nomenclature system*

Antigen	*Other title(s)*	*Molecular weight (kD)*	*Main cellular distribution*	*Comments*
CD1a		49	Cortical thymocytes, Langerhans' cells	Similar to histocompatibility antigen (linked to β_2 microglobulin)
CD1b	T6	45		
CD1c		43		
CD2	T11	46–50	T cells	Receptor for sheep red blood cells and LFA-3 involved in T cell activation
CD3	T3	19–29	T cells	Associated with T cell receptor
CD4	T4	55	Helper/inducer T cells, macrophages	AIDS virus (HIV) receptor
CD5	T1	67	T cells	
CD6	T12	85, 130	T cells	
CD7		40	T cells	Receptor for IgM
CD8	T8	32–33	Cytotoxic/suppressor T cells, splenic sinusoidal cells	
CD9		24	Granulocytes, platelets	Molecule has protein kinase activity anti-CD9 antibodies cause platelet aggregation
CD10	CALLA	100	Stem cells, renal epithelium, etc.	Widely used as a marker for "common" acute lymphoblastic leukaemia
CD11a	LFA-1	180	Many white cells	Involved in T cell cytoxicity (see CD18)
CD11b	Mac-1, CR3	170	Granulocytes, monocytes	C3bi receptor (see CD18)
CD11c	p150, 95	150	Macrophages, granulocytes	Molecule possesses C3bi receptor activity (see CD18)
CD12		No data	Myeloid cells	Little data available
CD13		180	Granulocytes, monocytes, osteoclasts, bile canaliculi, connective tissue	
CD14		55	Monocytes, Kupffer cells, granulocytes, dendritic reticulum cells	
CD15	Hapten X	50–180	Granulocytes, epithelium Reed-Sternberg cells	May be of value in the diagnosis of Hodgkin's disease
CD16	FcRIII	50–60	Granulocytes, some macrophages	Low affinity receptor for IgG
CD17	Lactoceramide	No data	Granulocytes	
CD18		95	Many white cells (see CD11a, b and c)	β chain of LFA/Mac-1 family. Homologous to integrin (fibronectin receptor)
CD19		95	B cells	
CD20		35	B cells	
CD21	CR2	140	B cells, dendritic reticulum cells	Receptor for C3d and EBV
CD22		135	B cells	Intracytoplasmic in early B cells
CD23		45	Some B cells, some dendritic reticulum cells	Low affinity receptor for IgE
CD24		42	Some B cells, granulocytes, ?endothelium	Prototype antibody: BA-1
CD25	Tac	55	Activated cells, macrophages	Interleukin-2 receptor
CDw26		120, 200	Activated T cells	
CD27		55 (reduced) 120 (unreduced)	T cells, plasma cells	
CD28		44	T cell subset	
CDw29		120–130	T cell subset, many non-leucocytes	Antibody defines subset of CD4 positive T cells which augment antibody production
CD30	Ki-1	130	Activated lymphoid cells, Reed Sternberg cells	Antigen also found on some large cell lymphomas
CD31		130–140	Granulocytes, monocytes, platelets, endothelium	? equivalent to platelet glycoprotein IIa
CDw32	FcRII	40	Granulocytes, B cells, monocytes, platelets, endothelium	Receptor for IgG
CD33		67	Early myeloid progenitors	
CD34		115	Some myeloid cells, endothelium	
CD35	CR1	220 or 250	Dendritic reticulum cells, B cells, red blood cells, granulocytes, glomeruli	C3b receptor
CD36		85	Monocytes, platelets	Gp IV (thrombospondin receptor)
CD37		40–45	B cells, also weakly on macrophages, T cells	
CD38	T10	45	Germinal centre cells, plasma cells, T cells	
CD39		80	B cells, macrophages, endothelium, other cells	
CDw40		50	B cells, interdigitating reticulum cells, carcinoma cells, other cells	? Receptor for growth factor
CDw41	GpIIb/IIIa	140 + 95	Megakaryocytes, platelets	Absent or reduced in Glanzmann's thrombasthenia
CDw42	GpIb	150	Megakaryocytes, platelets	Absent or reduced in Bernard Soulier syndrome
CD43		95	White cells, red blood cells, brain	
CDw44		65–85	White cells, brain	
CD45	LCA	180–220	Most white cells	
CD45R	LCA	Variable	Mainly B or T cells	See text

molecules being detected by molecular biological analysis (such as oncogene products and associated cellular constituents) may prove amenable to immunocytochemical analysis, although the subtle nature of the abnormalities which trigger cell proliferation (such as single amino acid phosphorylation) may make this a difficult field to exploit immunocytochemically.

Commercial sources of antibodies

Becton-Dickinson	*Coulter*	*Dakopatts*	*Ortho*
Anti-Leu-6	T6	DAKO-T6	OKT6
Anti-Leu-5	T11	DAKO-T11	OKT11
Anti-Leu-4	T3	DAKO-T3	OKT3
Anti-Leu-3	T4A	DAKO-T4	OKT4
Anti-Leu-1	T1B	DAKO-T1	OKT1
		DAKO-T12	OKT12
Anti-Leu-9		DAKO-T2	
Anti-Leu-2	T8A	DAKO-T8	OKT8 and 5
Anti-CALLA	J5	DAKO-CALLA	
		DAKO-CD11a	
Anti-CR3	Mo1	DAKO-C3bi-R	OKM1
Anti-Leu-M5		DAKO-p150, 95	
	My7		
Anti-Leu-M3	Mo2, My4		
Anti-Leu-M1		DAKO-M1	
Anti-Leu-11			
		DAKO-CD18	
Anti-Leu-12	B4	DAKO-CD19	
Anti-Leu-16	B1		
Anti-CR2	B2		
Anti-Leu-14		DAKO-CD22	
		DAKO-CD23	
Anti-interleukin-R	IL-2R1	DAKO-IL2-R	
	4B4		
		DAKO-RSC1	
	My9		
Anti-CR1		DAKO-C3bR	
		DAKO-CD37	
Anti-Leu-17			
		DAKO-GpIIb/IIIa	
		DAKO-GpIb	
Anti-leucocyte (HLe-1)		DAKO-LC	
Anti-Leu-18		DAKO-4KB5	

IMPROVEMENT IN REAGENTS

A major impetus to the production of monoclonal antibodies against human tissue antigens has come from the eagerness of immunologists to obtain new reagents for the identification of cellular components of the immune system. Many monoclonal antibodies produced by immunologists with this practical aim in view have proved valuable to pathologists. As immunohistology has usually not been the primary reason for producing such antibodies, however, the pathologist has come to depend on being able to pick up suitable "crumbs from the rich man's table." Few laboratories have set out deliberately to produce monoclonal antibodies which react with routinely processed paraffin embedded material, despite the fact that the availability of such reagents would greatly widen the scope of immunocytochemistry in diagnostic histopathology.

Currently available monoclonal antibodies suitable for use on paraffin sections

ANTIBODIES AGAINST T AND B CELL MARKERS

A series of monoclonal antibodies against T and B cells (designated MT1, MT2, MB1, MB2 and MB3) have been produced by Poppema's group[25] and they have been used in a number of laboratories for the differential typing of lymphomas.[26-28] These reagents were not submitted to any of the international workshops on leucocyte differentiation antigens. They are therefore not well characterised in terms of target antigens or their relation to other antibodies, and the only published information of this sort is to be found in a paper from the laboratory of origin.[25] Table 4 summarises the data on the staining reactions of these antibodies and the results of Western blotting analysis of the molecules which they recognise.

Interestingly, three of these antibodies (MT1, MT2, and MB1) recognise molecules with molecular weights of about 200 kD. The leucocyte common antigen (also known as T200 and now designated CD45) comprises at least four different variant polypeptide chains (arising from a single gene via alternative RNA splicing), with molecular weights of 220, 205, 190 and 180 kD. The Third International Workshop contained, in addition to 34 "conventional"

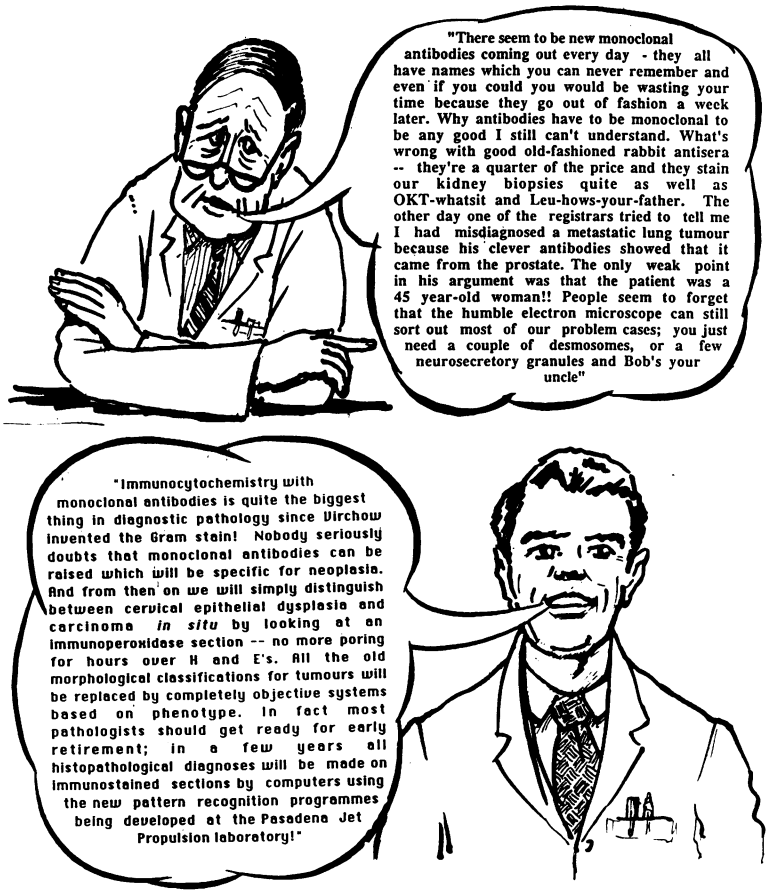

Fig 2 *The introduction of monoclonal antibodies into diagnostic pathology has been resisted by some and eagerly welcomed by others. Note that the supporter of monoclonal antibodies is quite as misguided in his opinions as is the opponent to their use. Any resemblance to living pathologists is entirely coincidental (but not improbable).*

Table 3 *Practical uses of immunocytochemistry in diagnostic pathology*

Problem	*Cause of diagnostic difficulty*	*Comment*
Unclassifiable tumour	Morphology uninformative, biopsy specimen too small, crushed, or poorly fixed	An appropriate panel of monoclonal antibodies (see fig 5) is of great assistance, especially those functioning in routinely processed material
Distinction of benign from malignant	As above	Immunocytochemistry is more restricted here, but it can be very useful in the case of lymphoma (monoclonality, loss, or acquisition of antigens, etc) or in other tumours by identifying cells inappropriate to a particular site such as epithelial cells in lymph nodes or bone marrow, glandular (Paget) cells in epidermis, etc
Identification of metastases	Too few cells to recognise by conventional examination	Appropriate monoclonal antibodies are very useful in identifying small numbers of metastatic cells in lymph nodes or bone marrow which cannot be seen by other means
Phenotyping lymphoid tumours	No reliable morphological indicators of immunophenotype	Lymphoid tumours can be extensively catagorised in frozen sections using antibodies against the different CD antigens (table 2). Of particular interest to diagnostic histopathologists are the small number of recently available monoclonal antibodies which recognise lymphoid antigens reasonably reliably in paraffin embedded sections

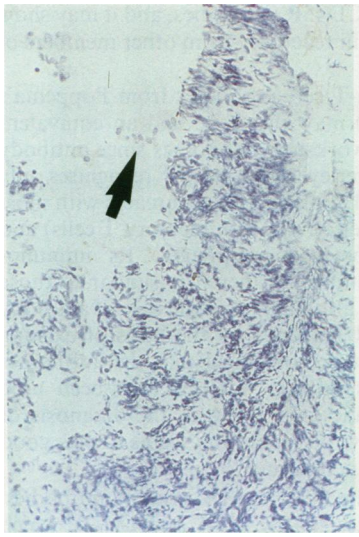

Fig 3a

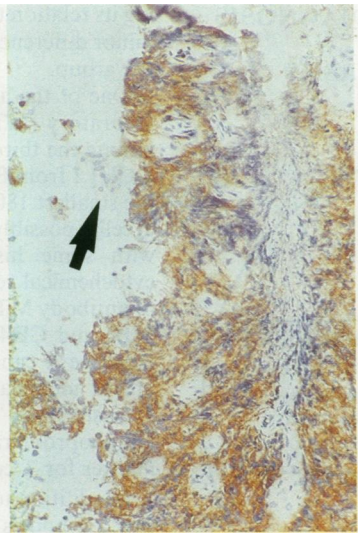

Fig 3b

Fig 3 *Demonstration of the ability of antigens to survive morphological damage to tissue. (a) This bronchial biopsy of a lung tumour (originally classified as a small cell carcinoma) was severely crushed during removal, as seen in this haematoxylin stained section. Note a few epithelial cells (arrowed) which have survived better than the underlying tumour. (b) Immunoperoxidase staining for leucocyte common antigen is clearly positive despite the extensive crush artefact, indicating that the tumour is a pulmonary lymphoma. Note the unstained epithelial cells (arrowed).*

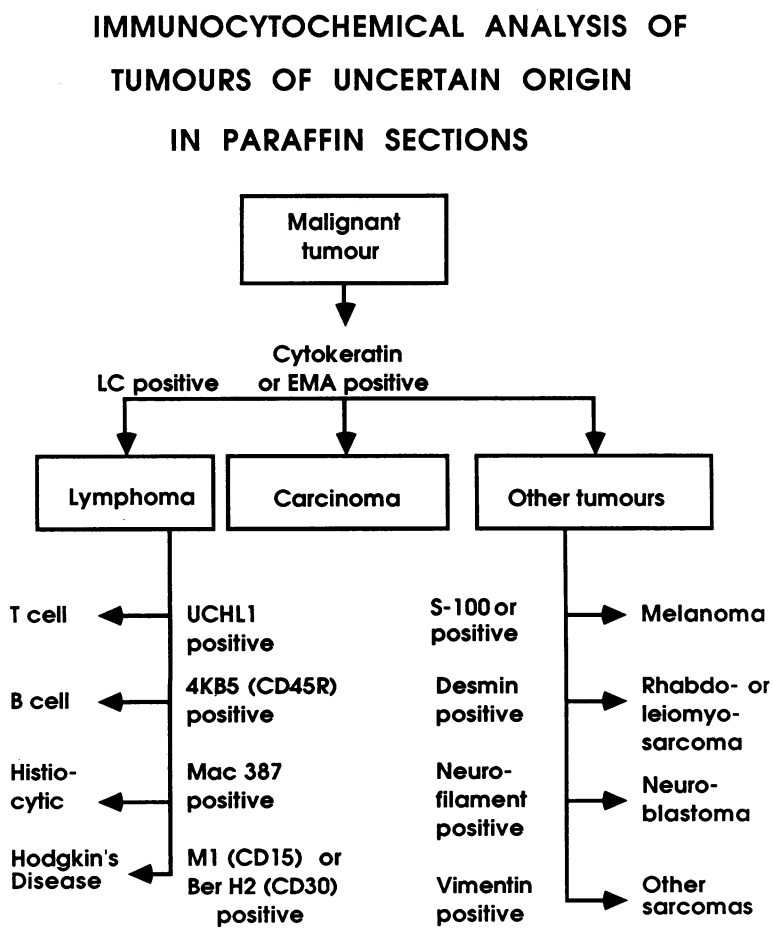

Fig 4 *Flow chart illustrating the way in which the cellular origin of a tumour may be shown by immunocytochemical staining when its nature is not apparent on routine histological examination.*

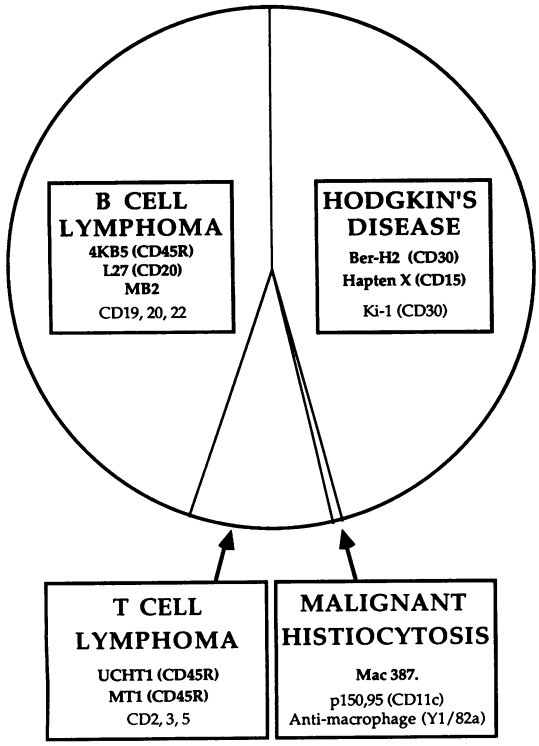

Fig 5 *Antibodies of value in distinguishing between different types of lymphoid neoplasia. Antibodies which are suitable for use on paraffin sections are shown in bold type, while those that will only stain cryostat sections are in normal type. The size of the pie segments represents the incidence in the United Kingdom of these different forms of lymphoid neoplasia.*

antileucocyte common antibodies (CD45), most of which react with all four polypeptide chains, a total of 20 antibodies which selectively recognised only some of these.[29] Almost all of them recognised the larger two polypeptide chains (of 220 and 205 kD), and they were designated CD45R (for "restricted"). They react with all B cells and with a subset of T cells and thus seem to be comparable with antibody MB1 (table 4). This is borne out by the fact that the immunocytochemical reactivity of MB1 is similar to that of the antibodies in this group. It should be pointed out, however, that, given the fact that antibody MB1 was not included in any of the international workshops, it is not possible to make a definitive statement concerning its relation to CD45R antibodies, and it may show minor differences in reactivity from other members of the group.

One of the anti-T cell antibodies from Poppema's laboratory (MT1) may also have had an equivalent among the third workshop antibodies since antibody UCHL1 from Beverley's laboratory[30] recognises only the smallest 180 kD polypeptide and reacts with most T cells (possibly selectively with memory T cells) and with some histiocytes/macrophages. Its immunocytochemical reactions are therefore similar to those of antibody MT1, although, as in the case of antibody MB1 and CD45R reagents, on the basis of existing evidence it cannot be concluded that the antibodies are of identical specificity. UCHL1 has been used extensively by Isaacson's group in the diagnosis of lymphoproliferative disorders and seems to be a good marker for T cell neoplasms.[31]

In addition to the MT and MB series of antibodies, two antibodies (designated LN-1 and LN-2), which react with B cell antigens in paraffin sections, have been reported by Epstein *et al.*[32] Their reactions (and those of a third reagent LN-3 which recognises HLA-DR) on lymphoid neoplasms were documented in a paper from the same laboratory (table 5).[33]

Table 6 provides a comparative summary of the monoclonal antibodies discussed above and attempts to relate them to each other in terms of probable specificity. Fig 6 shows an example of the use of antibodies against T cell and B cell associated variants of the leucocyte common antigen for immunophenotyping a paraffin embedded lymphoma biopsy specimen.

ANTI-HLA-DR ANTIBODIES

Epenetos *et al* reported that antibody TAL-1B5, raised against purified HLA-DR α chain, will detect HLA-DR antigen in paraffin embedded tissue.[34] Antibody LN-3, reported by Marder *et al* (table 5), and also an antibody (CR3/43) from our own laboratory, seem to behave in the same way. The broad distribution of HLA-DR limits their use as markers of neoplastic white cells to some extent; although the fact that B cell neoplasms are almost always HLA-DR positive, and T cell lymphomas only occasionally so, may be of value in the context of lymphoma phenotyping.

ANTI-HAPTEN X (CD15) ANTIBODIES

Many laboratories have raised monoclonal antibodies against this oligosaccharide grouping (which seems to be particularly immunogenic in the mouse).[15-17] Initially thought of principally as a marker for granulocytes, the antigen was subsequently shown to be present in Reed-Sternberg and Hodgkin's cells.[35,36] It appears, however, not to be

Table 4 *Summary of the MT, MB, and LN series of antibodies (from Poppema et al 1987)*

Antibody	*Molecular weight of antigen (kD)*	*Staining patterns*
MT1	190, 110, 100	*Normal tissue:* thymocytes, T lymphocytes, monocytes, macrophages (Kupffer and Langerhans' cells) *Neoplasms:* T cell and some B cell lymphomas (strongest on B lymphoblastic)
MT2	200, 190	*Normal tissue:* medullary thymocytes and T lymphocytes, mantle and marginal zone B cells, not germinal centres *Neoplasms:* T cell neoplasms unstained; all B cell tumours stained apart from B lymphoblastic
MB1	200, 110, 100	*Normal tissue:* B lymphocytes and about 50% of T cells *Neoplasms:* all B cell lymphomas stained with the exception of plasmacytomas. Several peripheral T cell lymphomas positive but not T lymphoblastic
MB2	28	*Normal tissue:* detects cytoplasmic antigen in all B cells (except for plasma cells) and reacts weakly with endothelium and epithelium *Neoplasms:* detects cytoplasmic antigen in all B cell neoplasms with the exception of plasmacytomas
MB3	31	*Normal tissue:* detects cytoplasmic antigen in B cells, monocytes, reticulum cells, macrophages and thymic "reticuloepithelium" (similar to HLA-DR but not membrane associated) *Neoplasms:* all B cell neoplasms (with the exception of plasmacytomas) and some T cell neoplasms (peripheral); all histiocytic neoplasms and also Reed-Sternberg cells
LN-1	45–85 (8 bands)	*Normal tissue:* germinal centre cells
LN-2	31 (same as MB3)	*Normal tissue:* identical to MB3
LN-3		*Normal tissue:* classical HLA-DR pattern

Table 5 *Summary of data on the LN series of antibodies (from Epstein et al 1984 and Marder et al 1985)*

LN-1	No molecular weight data but destroyed by neuraminidase	*Normal tissue:* membrane and cytoplasm of germinal cells, occasional weak staining of mantle zone B cells; cytoplasm of red cells and their precursors; a variety of different epithelial cell types *Neoplasms:* 98% of B cell neoplasms positive (cell membrane and cytoplasm). "90% unequivocal" follicular lymphomas strongest, small lymphocytic negative; five cell lymphomas negative; occasional non-lymphoid tumours positive
LN-2	35 kD	*Normal tissue:* germinal centre and mantle zone B cells (nuclear membrane and, more weakly, cytoplasm). T zone interdigitating histiocytes and thymic medullary dendritic cells *Neoplasms:* B cell neoplasms positive (94%)—four T cell lymphomas negative, scattered positive cells in one. Occasional non-lymphoid tumours positive
LN-3	HLA-DR	*Normal tissue:* reaction resembles HLA-DR pattern *Neoplasms:* reaction resembles HLA-DR pattern

Note that "only B5 fixed biopsy specimens were used ... because formalin fixed tissues were often not reactive or at best gave variable results. ... Although some reactivity was occasionally found in the formalin fixed tissues it did not approach the B5-fixed material. Most cases of formalin fixation totally blocked the ability of monoclonal antibodies to bind to their respective antigens."

Table 6 *Comparisons of reactions of MT, LN, and other monoclonal antibodies*

Poppema et al	*Epstein et al*	*Other*	*Comments*
T cell markers:			
MT1	—	UCHL1	T cell restricted form of leucocyte common antigen, good marker of T cell lymphomas
MT2	—	??	? T cell restricted form of leucocyte common antigen, but T cell lymphomas negative, and B cell NHL positive
B cell markers:			
MB1	—	4KB5	B cell restricted form of leucocyte common antigen, good marker of B cell lymphomas
MB2	—	—	28 kD cytoplasmic antigen, Poppema *et al* report that it is a good marker of B cell lymphoma
MB3	LN–2	??	Novel 30–35 kD antigen: localised to the nucleus, according to Epstein *et al*, present on B cell lymphomas but also on some T cell neoplasms; Poppema *et al* suggest that MB3 and LN–2 are identical in specificity
—	LN–1	??	No molecular weight data from originating laboratory (Epstein *et al* 1984); Poppema *et al* report multiple bands on Western blotting
—	LN–3	TAL–1B5	These antibodies recognise HLA-DR antigen and hence react with many non-B cells

Note: This table indicates that antibody UCHL1 is equivalent in specificity to MT1 and that CD45R antibodies such as 4KB5 are equivalent to MB1. These are inferences drawn from published data and remain to be proved formally.

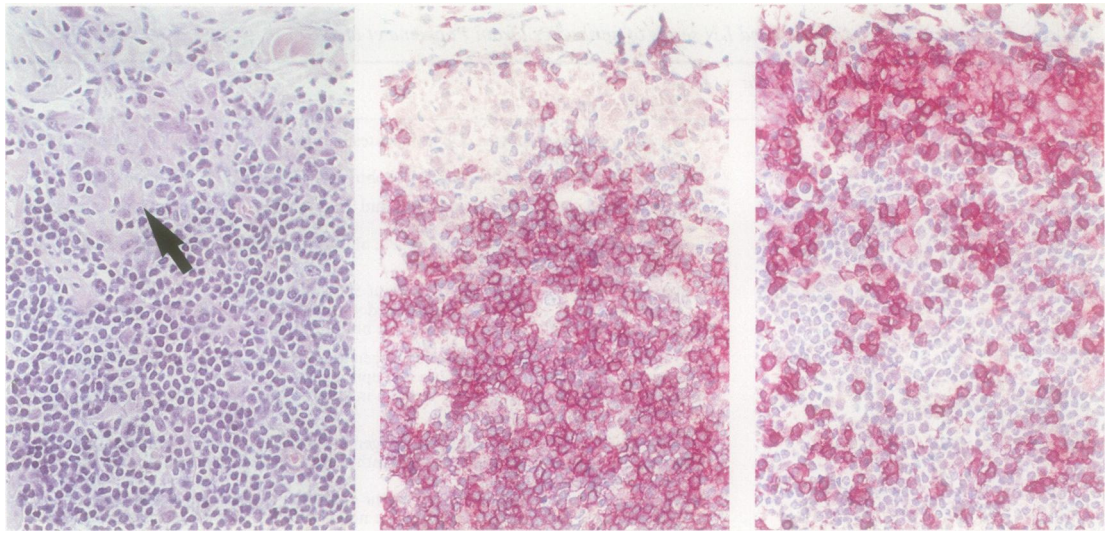

Fig 6a Fig 6b Fig 6c

Fig 6 *Phenotyping of a paraffin embedded cutaneous lymphoma using monoclonal antibodies against variants of the leucocyte common antigen. (a) A homogeneous infiltrate of tumour cells is seen in the conventionally stained section. Note clustered histiocytic cells in upper part of figure (arrowed). (b) Staining of an adjacent section with a monoclonal antibody (4KB5) against the B cell restricted form of leucocyte common antigen (CD45R) shows strong staining of many neoplastic cells. Note that the histiocytes are unstained. (c) Staining of an adjacent section with an antibody (UCHL1) against the T cell associated variant of leucocyte common antigen shows numerous unlabelled neoplastic cells, associated with scattered positive cells, presumably reactive infiltrating T cells. Note the characteristic weak staining of histiocytes by this antibody. (APAAP immunoalkaline phosphatase technique.)*

specific for this cell type among lymphomas since several authors have now reported labelling of non-Hodgkin's lymphomas by anti-CD15 antibodies.[37-39] Furthermore, many epithelial cells are stained by these antibodies,[40,41] constituting a potential trap for the unwary: metastatic carcinoma cells can occasionally be confused with Reed-Sternberg cells.

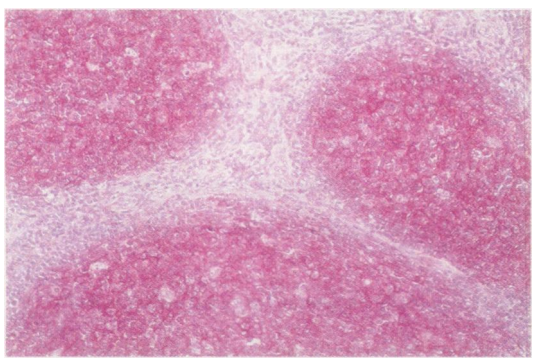

Fig 7 ***The CD20 B cell antigen (B1 antigen) can be detected in paraffin embedded tissue using monoclonal antibody L27. These figures show a follicular lymphoma stained for this antigen. Note the absence of staining in interfollicular areas. (APAAP immunoalkaline phosphatase technique.)***

Conclusions

From the preceding discussion it is apparent that there is still great scope for the production of monoclonal antibodies which will work reliably on paraffin embedded tissue sections. Most of the antibodies discussed above that are suitable for use in this way suffer from one or more of the following shortcomings: (1) they are directed against molecules which are poorly defined or even uncharacterised in terms of molecular weight; (2) the molecules they detect are only *associated* with T or B cells and are not truly *specific* for one of these cell lineages; (3) they are produced by a unique hybridoma and are hence often not widely distributed for comparative studies.

One step towards overcoming these limitations will come from directing attention to those antigens defined by the CD system that are essentially specific for T or B cells. Antigens CD1–CD8 and CD27 constitute well characterised and relatively specific markers for T cells or their subpopulations, while for B cells CD19–CD23 and CD37 would be appropriate for further study in this context. New and existing

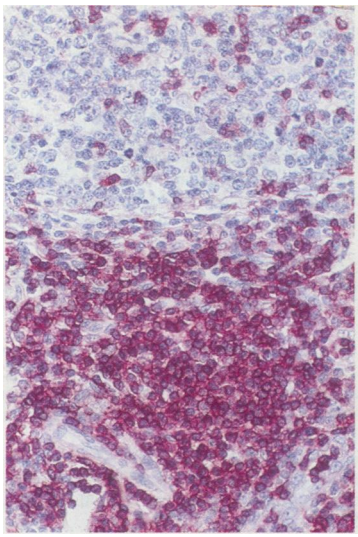

Fig 8a

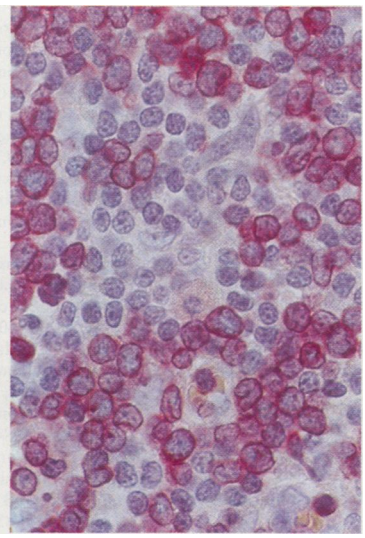

Fig 8b

Fig 8 *T cells can be detected in routine paraffin sections of human tissue using a polyclonal antiserum which detects a denaturation-resistant epitope on the CD3 (T3) molecule. (a) Paraffin embedded tonsil showing strong staining of T cells, contrasting with unstained B cells in an adjacent germinal centre. (b) Staining of a lymphoblastic lymphoma with the same antiserum showing strong neoplastic cell labelling. (APAAP immunoalkaline phosphatase technique.)*

antibodies of these specificities should therefore be screened on paraffin embedded tissue with the hope of finding reagents which detect denaturation-resistant epitopes.

In taking this approach we have recently reported that an antibody (L27) against the CD20 B cell associated molecule gives good immunocytochemical labelling of B cells in paraffin embedded tissue (fig 7) and is suitable for detecting most B cell neoplasms.[42] It is evident, however, that antileucocyte antibodies raised by conventional means (by immunising and screening against suspensions of living or lightly fixed white cells) are very rarely capable of detecting resistant epitopes, and the anti-CD20 antibody L27 represents the exception rather than the rule.

A promising field of endeavour may therefore lie in the raising of monoclonal antibodies against purified CD antigens. Many white cell antigens can now be isolated relatively easily using appropriate monoclonal antibody affinity columns, and it is even possible that the very process of purification will cause denaturation of the molecules and hence the induction of epitopes present only in paraffin embedded tissue. Preliminary immunocytochemical studies in collaboration with Dr M Crumpton's laboratory suggest the feasibility of this approach (fig 8) as a polyclonal antiserum against purified T3 (CD3) antigen (prepared by Dr G Krissansen) gives excellent staining of normal and neoplastic T cells in paraffin embedded tissue (Mason DY, Krissansen GW, Davey FR, *et al*, unpublished observations). The fact that this work was performed using a polyclonal antiserum, however, clearly limits its wider application, and it will be of great interest to see whether, in the next few years, the strategy of immunisation with purified CD3 antigen will lead to the production of monoclonal reagents which duplicate the reactivity of this antiserum. Hopefully the same approach may also yield monoclonal antibodies capable of detecting other well characterised T and B cell associated molecules in paraffin sections.

Paraffin wax embedded tissue from one of Thomas Hodgkin's original cases was kindly provided by Dr R Poston.

References

1 Taylor CR, Burns J. Immunohistological detection of intracellular immunoglobulin containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase labelled antibody. *J Clin Pathol* 1974;**27**:14–20.

2 Garvin AJ, Spicer SS, Parmley RT, Munster AM. Immunohistochemical demonstration of IgG in Reed-Sternberg and other cells in Hodgkin's disease. *J Exp Med* 1974;**139**:1077–83.

3 Davey FR, Halliday D, Marucci AA, Gottlieb AJ. Detection of intracellular and cell surface immunoglobulin in non-Hodgkin's lymphoma. *Hum Pathol* 1978;**9**:285–94.

4 Mason DY, Biberfeld P. Technical aspects of lymphoma Immunohistology. *J Histochem Cytochem* 1980;**28**:731–45.

5 Mason DY, Bell JI, Christensson B, Biberfeld P. An immunohistological study of human lymphoma. *Clin Exp Immunol* 1980;**40**:235–48.

6 Isaacson P, Wright DH. Anomalous staining patterns in immunohistologic studies of malignant lymphoma. *J Histochem Cytochem* 1979;**27**:1197–9.

7 Celio MR. Immunohistochemistry on Bouin's fixed fetal tissue stored for thirty years in ethanol. *Histochemistry* 1979;**61**: 347–50.

8 Mepham BL, Frater W, Mitchell BS. The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. *Histochem J* 1979;**11**:345–57.

9 Huang SN, Minassian H, More JD. Application of immuno-

fluorescent staining on paraffin sections improved by trypsin digestion. *Lab Invest* 1976;**35**:383–90.
10 Reading M. A digestion technique for the reduction of background staining in the immunoperoxidase method. *J Clin Pathol* 1977;**30**:88–90.
11 Levy R, Warnke R, Dorfman RF, Haimovich J. The monoclonality of human B cell lymphomas. *J Exp Med* 1977;**145**: 1014–28.
12 Warnke R, Levy R. Immunopathology of follicular lymphomas. *N Engl J Med* 1978;**298**:481–6.
13 Hoffman-Fezer G, Rodt H, Eulitz M, Thierfelder S. Immunohistochemical identification of T- and B-lymphocytes delineated by the unlabelled antibody enzyme method. I. Anatomical distribution of theta-positive and Ig-positive cells in lymphoid organs of mice. *J Immunol Methods* 1976;**13**:261–70.
14 Stein H, Bonk A, Tolksdorf G, Lennert K, Rodt H, Gerdes J. Immunohistologic analysis of the organisation of normal lymphoid tissue and non-Hodgkin's lymphomas. *J Histochem Cytochem* 1980;**28**:746–60.
15 Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF. *Leucocyte typing I.* Berlin: Springer-Verlag, 1984.
16 Haynes BF, Nadler LM, Bernstein ID. *Leucocyte typing II.* New York: Springer-Verlag, 1986:109–19.
17 McMichael AJ. *Leucocyte typing III.* Oxford: Oxford University Press, 1987.
18 Moll R, Franke WW, Schiller DL, Geiger B, Krepier R. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumours and cultured cells. *Cell* 1982;**31**:11–24.
19 Gatter KC, Alcock A, Heryet A, Mason DY. Clinical importance of analysing malignant tumours of uncertain origin with immunohistological techniques. *Lancet* 1985;**i**:1302–5.
20 Poston RN, Sidhu YS. Diagnosing tumours on routine surgical sections by immunohistochemistry: use of cytokeratin, common leucocyte and other markers. *J Clin Pathol* 1986;**39**:514–23.
21 Wells CA, Heryet A, Brochier J, Gatter KC, Mason DY. The immunocytochemical detection of axillary micrometastases in breast cancer. *Br J Cancer* 1984;**50**:193–7.
22 Ghosh AK, Mason DY, Spriggs AI. Immunocytochemical staining with monoclonal antibodies in cytologically "negative" serous effusions from patients with malignant disease. *J Clin Pathol* 1983;**36**:1150–3.
23 Ghosh AK, Erber WN, Hatton CSR, *et al.* Detection of metastatic tumour cells in routine bone marrow smears by immunoalkaline phosphatase labelling with monoclonal antibodies. *Br J Haematol* 1985;**61**:21–30.
24 Horning SJ, Weiss LM, Crabtree GS, Warnke RA. Clinical and phenotypic diversity of T cell lymphoma. *Blood* 1986;**67**: 1578–82.
25 Poppema S, Hollema H, Visser L, Vos H. Monoclonal antibodies (MT1, MT2, MB1, MB2, MB3) reactive with lymphocytes subsets in paraffin embedded tissue sections. *Am J Pathol* (in press).
26 Hall PA, d'Ardenne AJ, Butler MG, Habeshaw JR, Stansfeld AG. New marker of B lymphocytes, MB2: comparison with other lymphocyte subset markers active in conventionally processed tissue sections. *J Clin Pathol* 1987;**40**:151–6.
27 Norton AJ, Isaacson PG. An immunocytochemical study of T-cell lymphomas using monoclonal and polyclonal antibodies effective in routinely fixed wax embedded tissues. *Histopathology* 1986;**10**:1243–60.
28 West KP, Warford A, Fray L, Allen M. The demonstration of B-cell, T-cell and myeloid antigens in paraffin sections. *J Pathol* 1986;**150**:89–101.
29 Hale G, Stafford C, Lovat O, Prospero T, Waldmann H. Epitope mapping of the human leucocyte common antigen. In: *Leucocyte typing III.* Oxford: Oxford University Press, 1987.
30 Smith SH, Brown MH, Rowe D, Callard RE, Beverley PCL. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. *Immunology* 1986;**58**: 63–70.
31 Norton AJ, Ramsay AD, Smith SH, Beverley PCL, Isaacson PG. Monoclonal antibody (UCHL1) that recognises normal neoplastic T cells in routinely fixed tissues. *J Clin Pathol* 1986;**39**:399–405.
32 Epstein AL, Marder RJ, Winter JN, Fox RI. Two new monoclonal antibodies (LN-1, LN-2) reactive with B5 formalin-fixed, paraffin-embedded tissues with follicular center and mantle zone human B lymphocytes and derived tumours. *J Immunol* 1984;**133**:1028–36.
33 Marder RJ, Variakojis D, Silver J, Epstein AL. Immunohistochemical analysis of human lymphomas with monoclonal antibodies to B cell and Ia antigens reactive in paraffin sections. *Lab Invest* 1985;**52**:497–504.
34 Epenetos AA, Bobrow LG, Adams TE, Collins CM, Isaacson PG, Bodmer WF. A monoclonal antibody that detects HLA-DR region antigen in routinely fixed, wax embedded sections of normal and neoplastic lymphoid tissues. *J Clin Pathol* 1985;**38**:12–7.
35 Stein H, Uchanska-Ziegler B, Gerdes J, Ziegler A, Wernet P. Hodgkin and Reed-Sternberg cells contain antigens specific to late cells of granulopoiesis. *Int J Cancer* 1982;**29**:283–98.
36 Hsu S, Jaffe ES. Leu M1 and peanut agglutinin stain the neoplastic cells of Hodgkin's disease. *Am J Clin Pathol* 1984;**82**:29–32.
37 Wieczorek R, Burke JS, Knowles DM. Leu-M1 antigen expression in T-cell neoplasia. *Am J Pathol* 1985;**121**:374–80.
38 Dorfman RF, Gatter KC, Pulford KAF, Mason DY. An evaluation of the utility of anti-granulocyte and anti-leucocyte monoclonal antibodies in the diagnosis of Hodgkin's disease. *Am J Pathol* 1986;**123**:508–19.
39 Swerdlow SH, Wright SA. The spectrum of Leu-M1 staining in lymphoid and hematopoietic proliferations. *Am J Clin Pathol* 1986;**85**:283–8.
40 Sheibani K, Battofora H. Leu-M1 positivity not specific for Hodgkin's disease. *Am J Clin Pathol* 1984;**84**:682–3.
41 Pinkus GS, Said JW. Leu-M1 Immunoreactivity in non-hematopoietic neoplasms and myeloproliferative disorders. An immunoperoxidase study of paraffin sections. *Am J Clin Pathol* 1986;**85**:278–82.
42 Davey FR, Gatter KC, Ralfkiaer E, Pulford KAF, Krissansen GW, Mason DY. Immunophenotyping of non-Hodgkin's lymphoma using a panel of antibodies on paraffin embedded tissues. *Am J Pathol* (in press).

Requests for reprints to: Dr DY Mason, Department of Haematology, John Radcliffe Hospital, Oxford OX3 9DU, England.