

AN INVESTIGATION INTO THE VALUE OF MOLECULAR
TECHNIQUES IN HUMAN LYMPHOID MALIGNANCIES

BY

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Dedication

This thesis is dedicated to my wife Sughran, and our children Zariief, Samir and Hafisah, whose love, support and encouragement helped me to complete this work.

List of Abbreviations.

A	-	Nucleotide Adenosine
AILD/AIL	-	Angioimmunoblastic lymphadenopathy
AIDS	-	Acquired Immune Deficiency Syndrome
ALL	-	Acute Lymphoblastic Lymphoma
ATL	-	Adult T cell Lymphomas
APES	-	Aminopropyltriethoxysilane
B-cell	-	B lymphocytes
BM	-	Bone marrow
BNLI	-	British National Lymphoma Investigation Group
cDNA	-	Complementary DNA
C	-	Nucleotide cytosine/constant region
CB/CC	-	Centroblastic/Centrocytic
CsCl	-	Caesium chloride
CLL	-	Chronic Lymphatic Leukaemia
CD	-	Cluster differentiation antigen designation
CNS	-	Central Nervous System
CMV	-	Cytomegalovirus
CT	-	Computerised Tomography
D	-	Immunoglobulin/T cell receptor gene Diversity Region
DNA	-	Deoxyribonucleic acid
dCTP	-	Deoxycytidine triphosphate
DEPC	-	Diethylprocarbonate
DTT	-	Dithiothrietol
EDTA	-	Ethylenediamine triacetic acid
ESR	-	Erythrocyte sedimentation rate

FACS	-	Fluorescent activated cell sorter
G	-	Nucleotide Guanine
H	-	Immunoglobulin heavy chain
HCl	-	Hydrochloric acid
HD	-	Hodgkin's disease
HIV	-	Human immunodeficiency virus
HLA	-	Histocompatible Leucocyte Antigens
HTLV-1	-	Human T-cell Leukaemia/Lymphoma virus
IAA	-	Isoamyl alcohol
ICD	-	International Classification of Diseases
Ig	-	Immunoglobulins
LB	-	Luria-Bertani medium
LCA	-	Leucocyte common antigen
MF	-	Mycosis Fungoides
MHC	-	Major Histocompatible Complex
MRD	-	Minimal Residual Disease
mRNA	-	Messenger RNA
NaCl	-	Sodium chloride
NaOH	-	Sodium Hydroxide
NHL	-	Non-Hodgkin's Lymphoma
NK	-	Natural Killer cells
OLB	-	Oligonucleotide labelling buffer
PK	-	Proteinase-K
PEG	-	Polyethylene glycol
PCR	-	Polymerase Chain Reaction
PBS	-	Phosphate Buffered saline

RS	-	Reed Sternberg cell
RNA	-	Ribonucleic Acid
SS	-	Sezary Syndrome
SCC	-	Standard saline citrate buffer
SDS	-	Sodium Sodium dodecyl sulphate
T	-	Nucleotide thymidine
tdt	-	Terminal deoxynucleotide transferase
T-cell	-	T lymphocyte
TAE	-	Tris acetate/EDTA
TBS	-	Tris buffered saline
TCA	-	Trichloroacetic acid
TRC	-	T-cell receptor
TE	-	Tris/EDTA
UP	-	Ultra pure water
UV	-	Ultra violet
V	-	Variable region
VAB	-	Veronal acetate buffer
WHO	-	World Health Organisation

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Summary

The value of molecular techniques in providing diagnostic clarification in human lymphoid neoplasms (malignant lymphomas), was investigated in comparison to conventional methods.

Gene probe analysis (Southern blotting) and immunophenotyping were compared on biopsied tissues and peripheral blood of patients suspected of lymphoma. In the vast majority of B-cell lymphoma cases, gene rearrangement studies did not provide any additional clinically relevant information, but merely confirmed what was apparent from less costly and time consuming methods. Cases in which gene studies were required were uncommon, but included the heterogeneous and ill-defined T-cell lymphoproliferative disorders. As there is a lack of a specific phenotypic marker of malignancy in these T-cell neoplasms, gene probing provided the only definitive investigation of demonstrating clonal restrictions, and was found to be successful even on peripheral blood in lymphopenic patients.

The technique of in-situ hybridization was used to differentiate monoclonal from polyclonal B-cell neoplasms, and was applied to routine bone marrow trephines to permit identification of light chain mRNA in suspected myeloma patients. In situ hybridization was found to be rapid, sensitive and specific for demonstration of light chain mRNA expression in plasma

cell malignancies. The hybridization signal was optimised by an acid fixation and decalcification of the trephine, and by varying Proteinase K concentration. Cell morphology was well preserved and the resolution of this technique was high, with few problems of non-specific stain uptake seen in immunocytochemical conventional methods. In situ hybridization can be modified to detect any target cellular nucleic acid sequence, after synthesis of an appropriate probe.

Finally, the novel and powerful amplification technique of polymerase chain reaction (PCR) was investigated in B-cell lymphoma cases. Consistent and reproducible amplification was critically dependent on many variables including the assay conditions, the quality of Taq polymerase enzyme and the hybridization times and temperatures. However, false negative and of more concern false positive reactions still occurred. The exquisite sensitivity of PCR may not always be an advantage in clinical situations, and amplification of trace amounts of contaminants may become problematical. To be successful, PCR requires scrupulous technique and rigorous controls.

PCR was shown to be potentially highly sensitive, and specific at distinguishing clonal from polyclonal B-cells and in detecting occult or minimal residual disease.

CHAPTER 1. SETTING THE SCENE.

1.1. INTRODUCTION.

Malignant lymphomas are an important group of neoplastic disorders, of world-wide prevalence and affecting all age groups.

The lymphomas are all potentially curable, unlike the vast majority of other cancers. Hence the aim of research has been to categorize and classify these tumours accurately, with a view to identifying aetiological factors, ascribing pathogenesis and then proposing specific curative treatments for individual malignancies. In attempting to understand events in oncogenesis and the cell biology of normal and neoplastic clones, blood lymphocytes, whether reactive or malignant are eminently amenable for research and most laboratories have investigated procedures whereby all lymph nodes are sent fresh and unfixed for examination. As a result, this past decade has seen major advances in lymphoma research, both in terms of improved diagnosis and better prospects of cure for patients.

In many cases, diagnosis is readily apparent on the basis of well established clinical and morphological criteria. The mainstay of diagnosis still rests on

microscopic evaluation of routine processed sections of surgical biopsies by an experienced pathologist, aided by special stains and immunocytochemistry.

However, diagnostic difficulties are common, and in recent years immunophenotyping on cryostat sections of unfixed biopsies has been widely used as an additional procedure. The characterisation of lymphoid malignancies by immunophenotyping methods is based firstly upon the demonstration of a monoclonal origin of tumour cells and secondly the expression of surface and cytoplasmic antigens characteristic of their lineage and differentiation stage.

Immunophenotyping too, has been shown to have limitations in lymphoma diagnosis. For example, the lack of a specific marker for T-cell monoclonality, and the failure to detect B-cell clonal populations, when these tumours fail to alter the overall immunoglobulin kappa/lambda ratios remains problematical. With the advent of DNA recombinant techniques, demonstration of monoclonality within a lymphoid population became possible, by detection of rearrangement of several genes which contribute to the cells' antigen-specific receptors. Recently, developments in molecular techniques have had a tremendous impact and bearing in the diagnosis of malignant lymphomas.

1.2 The aims of this project were:-

1. To assess the value of molecular techniques in providing diagnostic clarification in human lymphoid malignancies (malignant lymphomas).

2. To evaluate the relative merits (including sensitivity and specificity) of molecular techniques in comparison to conventional histological, immunohistological and immunophenotyping methodologies.

3. To assess the feasibility of employing molecular methods as part of a routine diagnostic work-up in suspected lymphoma cases.

1.3 THE MALIGNANT LYMPHOMAS.

The malignant lymphomas comprise a heterogeneous group of neoplastic lymphoproliferative disorders.

Classically, Hodgkin's disease (HD) is separated from the other non-Hodgkin's lymphomas (NHL), on characteristic histological features, including the presence of multinucleated Reed-Sternberg cells.

1.3.1 EPIDEMIOLOGY.

The lymphoid malignancies occur throughout the world, but in relation to other cancers are uncommon.

However, prevalence studies and data collection have been hampered firstly by a lack of clear definitions of the biological boundaries of each disease group, and secondly by the variations in nomenclature over the years. The majority of international statistics are based on the ninth edition of the International Classification of Diseases (ICD) criteria (WHO 1977). This incorporated the Rye classification for HD, but divided the NHL into reticulum cell sarcoma & lymphosarcoma and other lymphomas (including malignant histiocytosis). In the UK the BNLI system (Bennett et al., 1974) has been followed by some workers. Despite introduction of lymphoma panels in the UK in an attempt at achieving consensus, a small but significant group of cases in HD and NHL could not be assigned. Cases misclassified by individual pathologists were also

apparent (Bird et al., 1984). Recently, an immunocytochemical study has shown 80% of "undifferentiated cancer" cases to be of lymphoid origin.

In England & Wales cancer incidence data have been derived from national cancer registration schemes. These data are of limited value in the case of malignant lymphomas, as the disease categories are based upon ICD criteria, diagnostic accuracy is poor (Bird et al., 1984) and reliability of registry data is suspect (Barnes et al., 1986). Bearing these reservations in mind, general epidemiological information on the lymphoid malignancies can still be obtained from this data base.

The prevalence rates for HD per 100,000 population are 3.1 (male) and 2.0 (female) for all age groups (OPCS 1978-85). MacMahon (1966) was first to note the bimodal age distribution. In developing countries, the age-specific incidence is increased in childhood, whereas in affluent countries this peak occurs later in young adults (25-34 years) and is dependent on the level of economic development of a country (Correa & O'Connor, 1971). In addition HD shows an increased incidence in higher socio-economic groups. This is

consistent with the hypothesis that HD develops as a rare consequence of a common childhood infection, occurring later in this group (Gutensohn & Shapiro, 1982). This social class difference in HD prevalence disappears in the over 55 year age group (Gutensohn, 1982).

The prevalence rates for NHL vary between 7-10/100,000 population for both sexes. The rates are much less for developing countries, where NHL tends to be rare in childhood and young adults. Exceptions are Burkitt's lymphoma in West Africa (Williams & Bamgboye, 1983), and both HD and NHL in the Middle East (Omar et al., 1985). Lymphomas remain uncommon in South & South-East Asia (O'Connor, 1984).

Certain racial groups have an increased incidence of malignant lymphomas, for example, the Japanese, American Blacks, and Jews.

There is a slight male preponderance in all lymphomas. For NHL the ratios are 1.1-1.3:1 for any disease type or age group. A wider scatter is seen for HD (1.1-2.7:1) particularly marked in middle age and childhood cases, and lymphocyte predominant subtype.

There has been an apparent increase in HD incidence in the over 65 year age group, probably related to more comprehensive investigations performed now on the elderly. An increase in NHL in the over 30 year age group is related to a real increase in follicular lymphoma cases.

1.3.2. AETIOLOGY AND PATHOGENESIS.

No single aetiological agent has been identified, and the process of human lymphoid malignancy is considered complex and multifactorial. Three separate factors are considered important, namely genetic susceptibility, infectious agents and disturbance of the immune system.

HODGKIN'S DISEASE

The genetic link is suggested by male preponderance, increased incidence in Jews and cases of familial aggregation. There is some evidence that a HLA linked gene is associated. An infectious agent is suggested by the bimodal age peaks of HD, evidence that HD may be two different diseases with the first peak caused by an infectious agent. Viruses have been implicated, as EBV capsid antigen have been found in greater proportion in HD cases than a control population (Evans & Gutensohn, 1984). The viral link however, may not be easily

corroborated, as opportunistic organisms such as viruses tend to colonize immunosuppressed patients for whatever reason (malignancy, drugs etc.). The evidence for altered immunity comes from higher incidence of atopic dermatitis and eczema in HD cases (Winkleman & Rajka, 1982). Certain occupations involving wood dust (Greene et al., 1978) may be causally linked.

NON-HODGKIN'S LYMPHOMA

The genetic link is suggested by male preponderance, increased incidence in Jews and familial aggregation. No HLA linked susceptibility gene has been identified. Further evidence is suggested by predisposing disease states which have a genetic basis, such as Ataxia telangiectasia and combined immune deficiency syndrome (Frizzera et al., 1980).

The link with viruses is stronger than in HD. EBV is causally linked to Burkitt's lymphoma (Geser et al., 1982), human T-cell leukaemia/lymphoma virus type I (HTLV-I) with Adult T-cell lymphoma (Wong-Staal & Gallo, 1985) endemic in Japan and the Caribbean (Blattner et al., 1983). HTLV-I positive lymphoma cases have also been found in UK immigrants from the Caribbean (Greaves et al., 1984). The human immunodeficiency virus (HIV) is associated with aggressive lymphomas. Aberrant immunity may be a

common factor in rheumatoid arthritis, coeliac disease and hypogammaglobulinaemia; and the development of lymphomas, usually immunoblastic or high grade (Kinlen et al., 1985). Renal transplant recipients have a 60-fold increased incidence of lymphomas (Kinlen et al., 1979), as do patients on immunosuppressive drugs (Kinlen, 1985).

Evidence of a radiation link comes from atomic bomb survivors (Kato & Schull, 1982) and studies on a cohort of ankylosing spondylitics who had received radiation treatment (Smith & Doll, 1982).

Patients with immune deficiency have a much increased susceptibility to develop lymphoid malignancies. This underlines the role of immune surveillance by the lymphoid organs in the prevention of lymphoproliferative disorders. The immune deficiency may be inherited, (Ataxia-telangiectasia, Wiskott-Aldrich syndrome, severe combined immune deficiency disease) or acquired secondary to immunosuppressive therapy for organ transplantation, malignant or non-malignant (e.g. Rheumatoid arthritis) disease. Patients with Acquired Immune deficiency syndrome (AIDS) too have an increased risk of developing malignant disease (Kaposi's sarcoma and NHL).

1.3.3. THE CLASSIFICATION OF HUMAN LYMPHOID NEOPLASIA.

The classification of human lymphoid malignancy is highly complex. Efforts have been made to relate the malignant cell to normal stages of ontogeny. With modern techniques of immunophenotyping and immunogenetics, major advances have been possible in understanding the biology of malignant change.

THE NON-HODGKIN'S LYMPHOMAS.

Substantial progress has been made in the classification of the NHL with the majority ascribed a B or T-cell lineage. The most modern classifications are Lukes & Collins (1975, 1977), Kiel (Gerard-Marchant, 1974; Stansfield et al, 1988) and the Working formulation (Rosenberg et al., 1982). However, none of these appear fully adequate, need to be continually revised. Each has a group of miscellaneous lymphomas, which require further categorisation.

The value of detailed histological subtyping is variable. For the lymphoblastic lymphomas (B & T-cell), accurate identification is important since this influences outcome and response to treatment. It is also worthwhile to type distinct clinicopathological entities for example mycosis fungoides and hairy cell leukaemia. Other lymphomas are important for

epidemiological reasons, such as Burkitt's lymphoma and adult T-cell lymphoma/leukaemia. But for the majority of lymphomas precise histological typing may be of limited clinical value at present.

The Rappaport classification (1966) has been widely used for many years and has been shown to have clinical applicability (Table 1.1). Its major disadvantage is the use of the term "histiocytic" for large cell lymphomas of lymphoid origin, as only a small number are truly histiocytic. Furthermore the terms 'poorly' and 'well differentiated' to describe lymphocytes and concepts of growth pattern (nodular/diffuse) are unfortunate and may be misleading.

To overcome these difficulties newer classifications were proposed, in keeping with contemporary concepts in cell biology. The Kiel classification has been favoured as it is up to date and divides tumours into therapeutically important low grade and high grade groups. The malignant cells in high grade tumours are analogous to blast cells, whereas in low grade lymphomas, the malignant cell is related to effector cells of the lymphoid system (i.e. lymphocytes, plasma cells and lymphoplasmacytoid cells) and cells of intermediate maturity. Recently, attempts have been made for a consensus in the classification of lymphomas

Table 1.1: Rappaport Classification of Non-Hodgkin's Lymphomas

	{	Lymphocytic well differentiated	}
	{		}
	{	Lymphocytic poorly differentiated	}
	{		}
Nodular	{		}Diffuse
	{		}
	{	Mixed (Lymphocytic-histiocytic)	}
	{	Histiocytic	}
	{	Undifferentiated)	}

(Lennert, 1983; Rosenberg, 1982), with the Working Formulation gaining some acceptance (Table 1.2). For this project a modified and recently updated Kiel classification (Stansfield et al, 1988) has been used (Table 1.3) as the basis of lymphoma classification.

Table 1.2: An outline of the Working formulation classification.

Low Grade

ML	:	Small lymphocytic
ML	:	Follicular small cell
ML	:	Follicular mixed cell

Intermediate Grade

ML	:	Follicular large cell
ML	:	Diffuse small cell
ML	:	Diffuse mixed cell
ML	:	Diffuse large cell

High Grade

ML	:	Large cell immunoblastic
ML	:	Lymphoblastic
ML	:	Small cell Burkitt's

Miscellaneous

Composite
 Mycosis fungoides
 Histiocytic
 Extramedullary plasmacytoma
 Unclassifiable
 Other

Table 1.3 Updated Kiel Classification

<u>B Cell</u>	<u>T Cell</u>
Low Grade	Low Grade
Lymphocytic	Lymphocytic
Hairy Cell Leukaemia	Small cerebriform cell
Lymphoblastic/cytoid	Lymphoepithelioid (Lennerts')
Plasmacytic	AILD
CB/CC - Follicular ± diffuse	T-zone
- diffuse	Pleomorphic, small cell
High Grade	High Grade
Centroblastic	Pleomorphic, medium
Immunoblastic	and large cell
Large cell anaplastic	Immunoblastic
Burkitt's lymphoma	Large cell
	anaplastic
Lymphoblastic	Lymphoblastic
Rare Types	Rare types

LOW GRADE B-CELL LYMPHOMAS

a). LYMPHOCYTIC.

This is equivalent to Chronic Lymphatic Leukaemia, with choice of term depending on distribution of disease. The tumour consists of small round lymphocytes, morphologically identical to mature lymphocytes, which diffusely and non-destructively infiltrate nodes leaving the capsule intact. Poorly defined proliferation centres (pseudofollicular zones) may occur containing a small number of blast cells surrounded by prolymphocytes, but no centrocytes (cf. follicular lymphomas). In about 3% of cases, transformation to a large cell lymphoma occurs so called Richter's syndrome (Armitage et al., 1978).

b). HAIRY CELL LEUKAEMIA.

This is a distinct clinicopathological entity affecting predominantly middle aged males (Burke et al., 1974; Catovsky et al., 1974). The cells have a monotonous appearance, with small round or oval nuclei and abundant clear cytoplasm and commonly infiltrate bone marrow, liver and spleen. The cytoplasmic hairy projections can be visualised by Roman^{ow}~~sky~~'s stain on peripheral blood or by electron microscopy. Hairy cells exhibit tartrate resistant acid phosphatase activity. Marrow infiltration is associated with

reticulin deposition, and hence a dry marrow aspirate is common. The B-cell nature has been confirmed by surface markers (Worman et al., 1983; Melo et al., 1984) and clonal rearrangement of the immunoglobulin gene (Korsmeyer et al., 1983).

c). LYMPHOPLASMACYTOID.

Tumours associated with autoimmune and immune deficiency syndromes are of this type (Stansfeld, 1985). These lymphomas are characterised by diffuse infiltration by lymphocytes, plasma cells and lymphoplasmacytoid cells, associated with mast cells and macrophages, and by the presence of cytoplasmic immunoglobulin. Three subtypes are described. A lymphoplasmacytic variant with predominantly plasma cells and a paraprotein, usually IgM. Lymphomas associated with Waldenstroms macroglobulinaemia are of this type, and may be associated with hyperviscosity.

A second lymphoplasmacytoid type is described with reduced plasma cells and paraprotein. This variant is usually associated with peripheral blood involvement, and hence this type is difficult to differentiate from lymphocytic lymphoma. The third (polymorphic) type consists of a pleomorphic proliferation with blast cells, multinucleated giant cells including Reed-Sternberg like cells and macrophages.

Although not lymphomas, plasma cell malignancies whether diffuse or focal are related B-cell neoplastic lymphoproliferative disorders. Their primary impact is on bone marrow rather than lymph nodes.

d). CENTROBLASTIC/CENTROCYTIC.

These tumours are usually associated with a follicular growth pattern, and consist of centroblasts and smaller centrocytes in varying proportions. There may also be zones of diffuse infiltration and sclerosis. Reactive plasma cells are frequently present but tingible body macrophages are usually absent. Dendritic reticulum cells and reactive T-helper cells are present within the neoplastic follicles. Monotypic surface IgM (or IgM + IgD) has been demonstrated on the malignant cells (Swerdlow et al., 1985), but other phenotypes have also been observed.

HIGH GRADE B-CELL LYMPHOMAS.

a). CENTROBLASTIC.

These B-cell tumours are analogous to transformation of germinal centre blast cells. They may arise de novo, or more commonly as secondary transformation from CB/CC lymphoma. This latter category has been subdivided into monomorphic blasts or polymorphic type (Stansfeld,

1985) and recently reclassified (Hui, Feller & Lennert, 1988). The polymorphic variant shows differentiate towards large centrocytes, immunoblasts with or without plasmacytoid differentiation.

b). B-LYMPHOBLASTIC.

These tumours affect children and young adults, and include endemic and sporadic Burkitt's lymphoma (BL). Initially described as a African sarcoma of jaw (Burkitt, 1958), BL was later classified as a malignant lymphoma (O'Connor & Davis, 1960) and also occurs in non-Africans (O'Connor et al., 1965; Dorfman, 1965). A close aetiological relationship was established with the Epstein-Barr virus (Epstein & Achong, 1964; 1979), with possibly malaria as a cofactor (Burkitt, 1969). BL consists of diffuse monotonous cohesive cells with round uniform nuclei and scanty cytoplasm. Mitosis are frequent, and tingible body macrophages are present giving the classical starry sky appearance. The non-Burkitt lymphoblastic lymphoma is more pleomorphic, with prominent nucleoli and has monotypic surface IgM (Flandrin, 1975).

Some cases of non-B non-T cell lymphoblastic lymphoma probably represent tissue phase of acute lymphoblastic leukaemia.

c). B-IMMUNOBLASTIC.

These cells are larger and have more cytoplasm than centroblasts. These tumours are the commonest high grade lymphoma in middle and old age. They may arise de novo or secondary to CB/CC, lymphocytic or lymphocytoplasmod lymphomas. Autoimmune or immune deficiency may precede onset. These tumours are rapidly dividing and have prominent nucleoli.

d). ANAPLASTIC LARGE CELL (Ki-1+) LYMPHOMA.

Ki-1 is a monoclonal antibody reactive against Reed-Sternberg cells of Hodgkin's disease, but also weakly reactive against other cells. It shows strong staining in 1-8% of all non-Hodgkin's lymphomas. These positive lymphomas are of large cell type with pleomorphic or round vesicular nuclei, associated with giant cells and paracortical involvement. Of the 45 cases studied by Stein et al., (1985) none reacted against macrophage markers. 26 and 7 cases were positive for T-cell and B-cell antigen markers respectively and 9 cases reacted with both sets of markers. The remaining 3 cases lacked any reaction to either. Stein proposed that these tumours are of B and T cell origin having undergone a process of activation.

LOW GRADE T-CELL LYMPHOMAS

The scheme proposed by Suchi et al., (1987) has been incorporated with the updated Kiel classification (Stansfield et al, 1988) for the T-cell lymphomas.

a). LYMPHOCYTIC.

These are much rarer than their B-cell counterpart, and are similar apart from lacking proliferation centres. These tumours are analogous to chronic lymphatic and prolymphatic leukaemias.

b). SMALL, CEREBRIFORM CELL.

Mycosis fungoides (MF) is primarily and predominantly a cutaneous neoplasm, and is characterised by localisation to the epidermis (in contrast, secondary lymphomas localise to the dermis and subcutaneous tissue). The infiltrate consists of Lutzner cells (Lutzner & Jordan, 1968) which are small lymphoid cells with convoluted nuclei, and a small number of larger cells with irregular or multilobate nuclei (Mycosis cell). The neoplastic cells are CD4 positive and form a close association with the skin HLA-DR+ dendritic cells. Transformation into a T-cell immunoblastic lymphoma may occur (Slater, 1984).

Sezary syndrome (SS) consists of chronic leukaemia and erythroderma, with neoplastic cells in the skin and blood. The skin lesions and the neoplastic cell are identical to MF. Therefore SS is a variant of MF, as circulating neoplastic cells can also be detected in MF.

Lennert's lymphoma (Lennert, 1975) is characterised by a diffuse infiltrate of small lymphocytes with irregular angular nuclei, a smaller number of blasts some multinucleate and Reed-Sternberg like, and numerous epitheloid histiocytes many forming small granulomas. The invasion begins in the paracortex but eventually involves the whole node. Differentiation from Hodgkin's disease may be difficult, particularly if also associated with an infiltration of eosinophils, neutrophils and plasma cells.

c). ANGIO-IMMUNOBLASTIC LYMPHADENOPATHY (AILD).

In the past, AILD has been considered as an abnormal immune reaction, but was subsequently proposed as a peripheral T-cell lymphoma (Shimoyama et al., 1979; Watanbe et al., 1980). AILD remains controversial, although clonal TCR gene rearrangements have been found in at least some cases.

The infiltrate is by small/intermediate lymphoid cells with irregular nuclei intermingled with larger atypical lymphoid cells, some resembling T-immunoblasts. The follicles are indistinct or absent and germinal centres are seldom found. There are a large number of high endothelial venules, epitheloid cell clusters and thickened basement membranes.

d). T-ZONE LYMPHOMA.

This tumour described by Lennert (1978), includes previous cases of peripheral T-cell lymphoma (Waldron, 1977) and some T-immunoblastic sarcoma of Lukes (1979). It is a mixed infiltrate of small/medium sized lymphocytes and blast cells in varying proportions. Multinucleated tumour giant cells (Reed-Sternberg like) may be seen. In the early phase, the tumour is confined to the paracortical area, but subsequently the nodal architecture will become effaced. Additional features include eosinophils, epitheloid histiocytes, endothelial venules and fibrosis. Transformation to a T-cell immunoblastic lymphoma may occur.

e). PLEOMORPHIC SMALL-CELL LYMPHOMA.

This consists of a monotonous small cell infiltrate with irregular nuclei and scanty cytoplasm, and spares

the B-cell follicular areas. The neoplastic cell has a T-helper phenotype (CD3+ CD4+). Both HTLV-I positive and negative cases have been described. Transformation to a large cell type heralds poor prognosis.

HIGH GRADE T-CELL LYMPHOMAS.

a). PLEOMORPHIC MEDIUM/LARGE-CELL LYMPHOMA.

The Adult T-cell lymphomas (ATL) associated with HTLV-I virus are mostly of this type. It is a diffuse variable cell sized tumour, with atypical nuclear configuration. Extensive pleomorphism is present in the HTLV-I positive cases.

ATL is the commonest peripheral T-cell neoplasm in Japan, particularly endemic in Kyushu the southern most island (Kikuchi et al., 1979). An identical disease was subsequently described in blacks from the Caribbean basin (Catovsky et al., 1982). The HTLV-I virus was isolated from these tumours (Poiesz et al., 1980). A non-endemic form is occasionally encountered in Europe and elsewhere.

It presents commonly with lymphadenopathy, leukaemia, skin involvement and hypercalcaemia. The node is infiltrated by variable sized lymphoid cells in the paracortex, with follicles initially preserved. The small lymphoid cells have a irregular convoluted nucleus, whereas the larger blasts can have a regular or irregular nucleus. Multinucleated Reed-Sternberg like cells are common. The neoplastic cells have been identified as CD4 positive post-thymic type, lacking

the enzyme tdt. The associated hypercalcaemia results from increased osteoclastic activity secondary to tumour released osteoclastic activating factor.

b). T-CELL IMMUNOBLASTIC.

This is analogous to B-cell immunoblastic lymphoma, but is much less common. It is a rapidly dividing tumour associated with multinucleated Reed-Sternberg like tumour cells, macrophages, eosinophils (more marked than B cases) and plasma cells (related to T-helper activity). T-cell immunoblastic lymphoma may arise de novo or from T-zone lymphoma and mycosis fungoides.

c). T-CELL LYMPHOBLASTIC.

This has a similar incidence as the non-endemic B-cell lymphoblastic lymphoma, and similarly affects predominantly children. It is often associated with a thymic mass, lymph node and marrow infiltration. Leukaemia frequently supervenes and CNS involvement is not uncommon. The neoplastic cell has characteristically a convoluted nucleus, but this is not a consistent feature (Nathwani et al., 1976). The cells are of variable size with frequent mitosis and are of cortical thymocyte phenotype.

d). ANAPLASTIC LARGE CELL (Ki-1+) LYMPHOMAS.

As described above under high grade B-cell lymphomas.

RARE TYPES /UNCLASSIFIED LYMPHOMAS.

HISTIOCYTIC NEOPLASMS.

The histiocytic malignancies are divided into malignant histiocytosis and true histiocytic lymphoma, although distinction between the two may be difficult.

Stansfeld (1985) believes these disorders are distinct, whereas Van der Valk and colleagues (Van der Valk et al., 1984) regard these conditions as extreme ends of a spectrum of related disorders.

Malignant histiocytosis affects all age groups, presenting with fever, wasting, generalised lymphadenopathy, hepato-splenomegaly, anaemia, purpura and leukopenia. Phagocytosis is non-specific though important diagnostic feature. The proliferation is of morphologically atypical histiocytes.

True histiocytic lymphoma has markedly decreased in frequency with better B and T cell markers, and reclassification. It presents as a localised tumour of pleomorphic blast cells with prominent nucleoli.

Multinucleated Reed-Sternberg like giant cells are present, as well as increased neutrophils, eosinophils and plasma cells.

HODGKIN'S DISEASE.

HD is an odd malignancy in which the putative neoplastic cells (mononuclear Hodgkin cell; multinucleated Reed-Sternberg cell) only form a minority of the tumour, the rest comprising lymphocytes, histiocytes, plasma cells, neutrophils, eosinophils and fibroblasts. The Reed-Sternberg (RS) cells are required for diagnosis, but are not pathognomonic of HD, occurring in the NHL, virus infections (EBV, herpes, rubella) and drug sensitivity (phenytoin). The RS cells have a eosinophilic cytoplasm with perinuclear halo and can be bi- or multi-nucleated.

Unlike the NHL, the classification of HD has been widely agreed, based on Lukes & Butler (1966) and modified by the Rye conference (Lukes et al., 1966). Originally 6 categories were recognized but reduced to 4. Recently, further modifications have been proposed. The nodular form of lymphocyte-predominant disease may be a separate entity (Timens et al., 1986), with a B-

cell origin of the RS cell (Stein et al., 1986). But B-cell rich Hodgkin's may occur in other categories (Angel et al., 1987), for instance clonal immunoglobulin gene rearrangement has been shown in 6/7 cases of nodular sclerosing HD (Weiss et al., 1986), but this remains controversial.

Two separate prognostically significant categories have been proposed for nodular sclerosis subtype (MacLennan et al., 1985), in which the grade 1 of lymphocytic/lympho-histiocytic or mixed cellularity subtype is associated with a lower relapse rate and better survival, than the grade 2 variety which is characterised by mixed cellularity with lymphocyte depletion or numerous anaplastic Hodgkin cells.

The mixed cellularity subtype has more RS cells than lymphocyte-predominant, but fewer than lymphocyte deplete HD. Reactive cells are present with lymphocytes. Foci of necrosis and neutrophils have been related to the presence of B symptoms (Colby et al., 1981).

1.3.4. CLINICAL FEATURES.

The clinical manifestation of malignant lymphomas are protean, hence differentiation from non-malignant conditions is important. As a significant proportion of lymphoma patients are potentially curable, each suspected case therefore requires a careful assessment of histological subtype and extent of disease, so that appropriate treatment is not delayed.

HODGKIN'S DISEASE.

In the vast majority of cases (70-90%), HD presents with superficial lymphadenopathy, usually affecting a single contiguous group of nodes. The cervical chain is most commonly involved (60-80%), followed by axillary (6-20%) and inguinal regions (6-12%). The mediastinal lymph nodes are involved in only 10% of cases at presentation, but become involved in upto 60% of patients during the course of the disease (Kaplan et al., 1973; Moran & Ultmann 1974), and are associated with poor prognosis (Mauch et al., 1978; Lee et al., 1980; Velentjas et al., 1981; Hoppe et al., 1982).

The retroperitoneal nodes may be involved in about 30% of patients (Ultmann et al., 1973), and are strongly associated with B symptoms (anorexia, weight loss of more than 10% body weight, fever & pruritis). Systemic

disturbance in HD implies poor prognosis (Tubiana et al., 1971; Vaughan-Hudson et al., 1985). Classical Pel-Ebstein fever is rare. Alcohol induced pain at sites of involvement is suggestive of HD, but uncommon (1-10%), and occurs in other conditions such as sarcoidosis.

Involvement of Waldeyer's ring (nasopharynx & tonsil) is unusual in HD. The spinal cord is another uncommon site, though important to recognize in view of the risk of paraplegia. The localisation is usually the epidural space of the lower dorsal or upper lumbar spine. The presentation is with nerve root pain, paraesthesiae, weakness and stiffness of legs, and disturbance of micturition. Intracerebral lesions are much less common.

Bone marrow involvement implies significant tumour burden and widespread dissemination (Rosenberg 1971), reflected by anaemia, thrombocytopenia and leucopenia, particularly lymphopenia (MacLennan et al., 1981). The raised serum alkaline phosphatase too reflects bone disease.

Skin manifestations are common in HD, but are non-specific.

NON-HODGKIN'S LYMPHOMAS.

The rate of growth is highly variable in the non-Hodgkin's lymphomas, but the tumours have a propensity for local invasion and tissue destruction.

Two thirds of patients present with painless lymphadenopathy (cervical > inguinal > axillary > multiple sites), while the remainder present with systemic symptoms or extra-nodal disease. Systemic symptoms (fever, weight loss) are associated with diffuse lymphomas and advanced disease (Patchefsky et al., 1974). About 20% of NHL present with symptoms of extra-nodal disease, usually large cell type involving Waldeyer's ring, gut, bone, skin, salivary glands, thyroid, testes and breast (Rudders et al., 1978; Reddy et al., 1980).

The spleen is enlarged in 30-40% of patients at presentation (Moran et al., 1975), and may be massive leading to pancytopenia and hypersplenism. The spleen is always involved in liver involvement, which occurs in 20-50% of patients at presentation and more commonly associated with low grade lymphomas. Bone marrow involvement occurs in 1/3 to 1/2 of patients, low grade more often than high grade lymphomas (Rosenburg 1975).

At presentation 5-16% of patients have gastrointestinal involvement (Jones et al., 1973), frequently stomach (50%) and small intestine (25%) in adults. The ratios are reversed in children.

Central Nervous System (CNS) lymphoma presentation is uncommon, but ultimately 10% of patients have CNS disease (Litam et al., 1979), manifesting as spinal cord compression or leptomeningeal spread with cranial nerve palsies or raised intra-cranial pressure. Other uncommon manifestations of lymphoma include renal obstruction by retro-peritoneal nodes, skin infiltration, and pulmonary involvement including pleural effusion.

With the NHL, various subtypes are associated with particular modes of presentation. The low grade lymphomas (predominantly follicular) have a slow indolent course, but the majority presenting late with bone marrow and blood invasion (60-85%). A paraprotein is found in 20% of cases. Retro-peritoneal involvement occurs in 90% of cases. Hypersplenism, liver and CNS involvement is not uncommon.

The intermediate grade lymphomas are more aggressive and have more frequent parenchymal involvement (liver, lungs, BM). The peripheral T-cell lymphomas are

associated with this group, presenting with generalised lymphadenopathy, anorexia, weight loss and pleuro-pulmonary involvement.

The large cell diffuse types frequently present with local disease, and CNS lymphoma is significant (10%). The high grade immunoblastic lymphomas are associated with aggressive clinical behaviour and poor prognosis. Immunoblastic lymphoma may be preceded by immune or lymphoproliferative disease. Anaemia and other manifestations of advanced disease are common.

The high grade lymphoblastic lymphomas are associated with anterior mediastinal mass (50%) and blood dissemination leading to marrow and CNS invasion. Burkitt's lymphoma is also an aggressive neoplasm, with explosive growth potential, invading marrow, CNS, gut, urinary and respiratory tracts, and associated with metabolic disturbance.

1.4. PROBLEMS ENCOUNTERED IN CLINICAL DIAGNOSIS OF MALIGNANT LYMPHOMAS.

Diagnosis begins at the bedside, and the clinician needs to consider many non-malignant possibilities for a patient presenting with lymphadenopathy.

Age, presenting features and clinical examination initially may suggest lymphoma as the most likely diagnosis in a patient. Ultimately, however every suspected lymphoma case requires the establishment of a tissue diagnosis.

Non-malignant lymphadenopathy with or without splenomegaly is frequent in childhood and infancy, related to infectious agents. Acute localised infections due to streptococci and staphylococci (acute pyogenic lymphadenitis) are easily differentiated, but chronic persistent infections may pose diagnostic difficulties. Tuberculous lymphadenitis is now much less common, usually presents as a nodal mass in the vicinity of the tonsillar lymph node, and may be associated with sinus formation and calcification on X-ray.

Viral infections (infectious mononucleosis, CMV) and Toxoplasmosis present with pyrexia, generalised lymphadenopathy and atypical lymphocytes in the

peripheral blood. A blood film and a Paul-Bunnell test may save the patient undergoing invasive procedures. Cat-scratch fever may present with persistent lymphadenopathy, usually epitrochlear with or without adjacent axilla. Contact history may be lacking, but the glands tend to be hot tender and somewhat indurated.

A drug or chemical cause of lymphadenopathy may be due to phenytoin exposure (Hydantoin pseudolymphoma), and presents with fever, skin rash and marked lymphadenopathy and occasionally splenomegaly. It is important to establish history of drug exposure. The nodes show marked histiocytic hyperplasia with or without focal necrosis. Progression to true lymphoma has been documented (Hyman & Sommers, 1966; Gams et al., 1968).

Idiopathic causes of lymphadenopathy include sarcoidosis, but other associated features (iritis, parotid enlargement, erythema nodosum with hilar lymphadenopathy, hypergammaglobulinaemia and hypercalcaemia) should suggest the correct diagnosis, but may only be confirmed on histological evaluation. Other important differential diagnoses are lymphadenopathy associated with autoimmune disorders, for rheumatoid arthritis, and systemic lupus

erythematosis.

AILD is associated with marked immunological disturbance (Frizzera et al., 1975; Lukes & Tindle, 1975), and its relationship to malignant lymphoma still remains controversial. Many cases respond to steroid therapy for a time, but progression to lymphoma has been established in some cases with T-cell lineage (O'Connor et al., 1986).

Angiofollicular lymph node hyperplasia first described by Castleman (1956) is another rare condition to consider. Histologically two distinct variants are recognized, a hyaline vascular form and a plasma cell type (Keller et al, 1972), the latter associated with a variety of conditions such as rheumatoid arthritis, autoimmune diseases and lymph nodes draining carcinomas. It is reputed to be a benign disorder but aetiology and pathogenesis remains obscure (West, 1989).

Finally, secondary neoplastic infiltration should always be considered, particularly if hard fixed tender nodes are found.

1.4.1. INITIAL INVESTIGATIONS IN LYMPHOMA DIAGNOSIS.

Laboratory Investigations may provide useful corroborating evidence for a diagnosis of malignant lymphoma. Conversely, they may help to exclude such a diagnosis.

A reactive blood picture is commonly seen in Hodgkin's disease, with neutrophilia and eosinophilia. Anaemia implies poor prognosis and advanced disease (Tubiana et al., 1971), as does lymphopenia (MacLennan et al., 1981) reflecting compromised bone marrow function due to significant tumour burden and marrow infiltration (Rosenberg 1971). Bone marrow examination is useful in HD, revealing reactive changes in many cases, and only occasionally infiltration by disease which can be difficult to recognize.

The Erythrocyte Sedimentation Rate (ESR) is a laboratory indicator of B symptoms and systemic involvement in HD, and has been identified as a consistent predictor of outcome (Haybittle et al., 1985) as does plasma viscosity (Akhtar et al., 1991) which has come to replace the ESR in many laboratories.

Besides recognising reactive changes in NHL which are more common than in HD, scrutiny of the blood film has yielded circulating lymphoma cells in a significant number (8-20%) of cases (Come et al., 1980; McKenna et al., 1975). The presence of circulating lymphoma cells is commoner with the low grade lymphomas (Jaffe, 1983) and generally reflects marrow infiltration (Dick et al., 1974; Garrett et al., 1979). Low grade lymphomas are considered to be a systemic disease (Jaffe, 1983) and peripheral blood lymphoma is of less import than with the high grade lymphomas. The examination of the bone marrow is a vital staging investigation in lymphomas, and corroborates the histopathological diagnosis based on biopsied tissue.

Other investigations reveal less diagnostic information. The blood urea and uric acid may be raised as a reflection of tumour burden. Liver function tests may be deranged and may reflect infiltration. Serum protein electrophoresis may be useful, revealing a paraprotein associated with occult low grade lymphomas. Investigations of haemolytic anaemias too may detect an underlying malignant lymphoma.

Radiological visualisation of tumour bulk and extent

has made a major contribution to diagnosis and staging of suspected cases obviating the need for staging laparotomies in the vast majority. Computerised tomography (CT) will reveal macroscopic disease in the mediastinal and retroperitoneal nodes, but may still need to be combined with lymphangiography for infiltration of normal sized lymph nodes.

Contrast media radiology (pyelography, barium meal/enema) may yield further information, although direct examination with biopsy facility (laryngoscopy, bronchoscopy, colonoscopy, gastroscopy) if possible is preferred.

1.5. CONVENTIONAL TECHNIQUES IN LYMPHOMA DIAGNOSIS.

In every suspected case of malignant lymphoma, it is mandatory to establish a tissue diagnosis. This is usually based upon microscopic evaluation of a biopsied lymph node by a histopathologist.

1.5.1. TECHNICAL DIFFICULTIES.

The establishment of a histological diagnosis may be complicated by several avoidable difficulties.

The histopathologist's interpretation of a tissue is based upon the clinical information provided. If this is inaccurate or inadequate, the morphological features may be misinterpreted.

A lack of care in choosing the biopsy site may result in delayed or missed diagnosis. The inguinal and upper cervical (tonsillar) lymph nodes are to be avoided, frequently being sites of chronic non-specific inflammatory changes. The nodes in the posterior triangle of the neck, supraclavicular fossae and epitrochlear regions are satisfactory and easily accessible for excision by local anaesthesia. The axillary nodes are less accessible and require a general anaesthetic for excision. The largest involved node should be selected, with intact capsule. The surgeon should avoid traction, and crushing by forceps

as this produces artefacts. All too often lymph node biopsy is delegated to an inexperienced member of the surgical team. Open biopsies are always preferred to needle biopsies. The tissue is then rapidly transported to the laboratory for fixation.

In cases where no superficial node is present, involved marrow or tru-cut liver biopsy may be submitted. In cases where only the mediastinal nodes are involved, a scalene node biopsy may be diagnostic, or else tissue obtained by thoracotomy. Tissue obtained by mediastinoscopy is often badly crushed. In some cases, diagnosis may await development of a superficial node.

A part of the node should be fixed without delay, and the remainder frozen using familiar procedures and fixatives. The fresh node is bisected, as intact capsule will not allow complete penetration of fixative. Autolysis will occur in unfixed areas of a node or if there has been a delay in fixation, making interpretation impossible.

The histological techniques should be of a high standard. Improperly cut and stained sections are a common cause of histological misinterpretation. Cutting sections with a freezing microtome may result in partial disintegration and collapse of necrotic areas such as caseating tuberculous lesions. Cryostat

microtomy is satisfactory, but paraffin wax embedded tissue is preferred.

1.5.2. MORPHOLOGICAL DIAGNOSIS.

The majority of lymphomas can be diagnosed and classified on morphological grounds alone, but the observer error is significant. The commonest mistake is to suggest a diagnosis on inadequate histological grounds. This was particularly the case before the widespread use of immunocytochemistry as the following study illustrates. In a study of 600 cases of lymphadenopathy with an initial diagnosis of Hodgkin's disease (Symmers, 1968), confirmation was only possible in 317. In the remaining 283 cases, 69 were diagnosed with other malignant lymphomas; 34 reticulum cell sarcoma; 29 infiltrated with metastatic carcinoma; 192 showing inflammatory or other non-neoplastic conditions and 89 with chronic non-specific lymphadenitis.

The accurate diagnosis and classification of malignant lymphomas has been beset by variations in nomenclature over the years. Each classification is inadequate, requires continual revision and contains a large number of miscellaneous lymphomas. To improve diagnostic accuracy and consistency, lymphoma panels were set up in the United Kingdom. This has resulted in consensus diagnoses and identified cases misdiagnosed or misclassified (Bird et al., 1984). Despite the introduction of these panels a small, but significant,

number of suspected lymphoma cases remain undiagnosed. Recent evidence from cell marker work has suggested that 80% of "undifferentiated carcinoma" cases were in reality of lymphoid origin (Gatter et al., 1985).

1.5.3. IMMUNO-HISTOCHEMICAL MARKERS AND TECHNIQUES.

In most cases, lymphoma diagnosis can be clearly made on the basis of histological appearance, particularly when supplemented by immuno-histochemical staining applied to fixed tissue (Stein et al., 1984; Lauder, 1988). With the advent of antibodies to leucocyte common antigen (Warnke et al., 1983) and cytokeratin (Makin et al., 1984), it became possible to differentiate malignant lymphoma from carcinoma, especially anaplastic carcinoma in the vast majority of cases (Lauder et al., 1984). However, antibodies to leucocyte common antigen (LCA) are heterogenous, recognize different epitopes and therefore show differences in pattern of reactivity. LCA may be destroyed by fixation (e.g. formalin), and frozen sections are preferred (some antibodies reactive with fixed tissue are available). LCA is expressed in the vast majority of lymphomas, but not all (Pizzolo et al, 1986). In addition some lymphoid tumours are positive for epithelial membrane antigen (Delsol et al, 1984). Therefore a panel of lymphoid and epithelial markers is

recommended (Lauder et al., 1984).

Diagnostic difficulties are common, however and in recent years immunophenotyping on cryostat sections of unfixed biopsies has been widely used as an additional procedure (Aisenberg, 1981), since many T and B cell differentiation antigens are destroyed or poorly preserved in fixed paraffin processed tissue (Curran & Gregory, 1980). Gradually monoclonal antibodies are being developed which recognize epitopes that are resistant to routine fixation and processing and encouraging results have been published (West et al., 1986).

B-CELL LYMPHOMAS.

In the case of B-cell malignancies, demonstration of clonality by detection of monotypic immunoglobulin synthesis and light chain restriction (Warnke & Levy, 1978) usually requires the use of such unfixed tissue. Anamolous cytoplasmic Ig staining in paraffin sections is well recognized (Isaacson & Wright, 1979; Isaacson et al., 1980), and is due to a large number of reactive B-cells associated with the tumour population. In addition, polytypic Ig is taken up from tissue fluids, by dead and damaged cells as well as macrophages (normal, neoplastic, Reed-Sternberg and other multinucleated forms). The staining pattern (granular,

Golgi zone with perinuclear space) and positive staining for Ig J-chains may help to distinguish.

With cryostat sections, non-specific staining for surface Ig (polytypic) may be present in the interstitial tissues of neoplastic follicles. Also some large cell lymphomas synthesize little Ig or none (Gregg et al., 1984), and diagnosis relies on pan-B markers. Another very rare complication is the presence of biclonal tumours (Sarasombath et al., 1977; Sklar et al., 1984).

Clonal analysis of blood has also been based upon immunoglobulin light chain analysis and restriction (Sobol et al., 1985), and a high degree of sensitivity has been reported with flow cytometric methodology (Johnson et al., 1985).

With lymphocytic lymphoma, cytoplasmic or surface IgM kappa or lambda is easily demonstrated using frozen tissue sections. In addition, 40% of cases react with a T-cell marker CD5. Lymphoplasmacytoid lymphoma too is characterised by monotypic cytoplasmic or surface immunoglobulin.

The follicle centre cell (follicular, diffuse or combined) lymphomas are characterised by monotypic surface Ig and HLA class II antigens by frozen sections. In 2/3 of cases, cytoplasmic Ig with J chain can be demonstrated on paraffin sections using very sensitive techniques.

Surface Ig on frozen sections, and cytoplasmic Ig on paraffin sections can also be demonstrated in immunoblastic lymphomas.

In the case of centrocytic and mantle zone lymphomas, monotypic Ig marks with IgD/IgM. Additional markers include C3B receptor and CD5.

T-CELL LYMPHOMAS.

A large number of monoclonal antibodies to T-cell antigens have become available, but results are unsatisfactory on fixed tissue. With T-cell lymphomas, no single immunological phenotype is restricted to a single morphological entity. A helper (CD4): suppressor (CD8) ratio (unlike kappa:lambda) does not differentiate neoplastic from reactive states.

Tumours are initially divided into tdt (terminal deoxynucleotidyl transferase) positive/negative cases,

and thymic/pre-thymic types. Tumours are then characterised further e.g. CD4 positive.

HISTIOCYTIC LYMPHOMAS.

Markers useful in histiocytic lymphomas are intracytoplasmic alpha-1-anti-trypsin (Isaacson et al., 1981) on paraffin sections, and macrophage-specific monoclonal antibody (Radzun et al., 1984) on cryostat sections. But alpha-1-anti-trypsin is found in quite a few T-cell lymphomas as well. Antibodies against polytypic Ig can also be shown.

HODGKIN'S DISEASE.

Immunohistological studies have been disappointing in the search for the nature or origin of Hodgkin's disease (Lee, 1989).

A panel of markers has emerged for the Reed-Sternberg associated antigens and includes the granulocyte associated CD15 (Stein et al, 1982; Hsu & Jaffe, 1984), epithelial membrane antigen CD30 and LN-1 a B-cell restricted antigen all preserved in fixed paraffin sections (Chittal et al, 1988).

The Reed-Sternberg cells will also demonstrate polytypic Ig and alpha-1-anti-trypsin activity (Payne et al., 1982). Ki-1+ expression has been found in

lymphocyte predominant HD (Stein et al., 1981), in addition to large cell NHL.

1.5.4. IMMUNOPHENOTYPING IN LYMPHOMA DIAGNOSIS.

In many case of lymphoma, immunophenotyping merely offers confirmation of a diagnosis which is clear on the basis of well established clinical and morphological criteria. But in other cases, immunophenotyping is crucial to diagnosis, prognosis and treatment.

The characterisation of lymphoid malignancies by immunological techniques is based upon the principle that the neoplastic cell is of monoclonal origin and expresses surface and cytoplasmic components characteristic of its lineage and ontogeny. The demonstration of monoclonality is usually apparent in B-cell lymphomas by immunoglobulin light chain (kappa & lambda) restriction. Unfortunately there is no convenient equivalent marker available for the T-cell malignancies.

The neoplastic phenotype is related to a particular stage within normal lymphoid differentiation pathway, the implication being that the neoplastic clone has arisen from a normal counterpart. But anomalous expression of cell lineage markers (or lineage infidelity) is known to occur and may prove to be useful diagnostic features of the neoplastic population

a). IMMUNOPHENOTYPING OF CIRCULATING LYMPHOMA CELLS.

Circulating lymphoma cells may be detected in the blood of patients with non-Hodgkin's lymphoma in the absence of a lymphocytosis or any other abnormality of the lymphocyte count (Galton & MacLennan, 1982). Therefore immunophenotyping of peripheral blood is indicated in all cases suspected with a diagnosis of lymphoma, and not just those with an unexplained lymphocytosis.

Various methods are available for surface marker analysis, but the cases in this study had blood analysed by immunofluorescent staining and flow cytometry using a Becton Dickinson FACS Analyser. The direct antiglobulin rosette (DAGR) method (Ling, Bishop & Jefferis, 1977) is used for determination of surface immunoglobulin on B-cells.

A routine panel of monoclonal antibodies (Table 1.4) is used for all lymphocyte subset analyses to characterise lymphocytes as B T or natural killer (NK) cells, and using the DAGR method to determine the presence of a surface immunoglobulin positive (sIg+) B-cell population. This is essentially based on the ratio of kappa+ : lambda+ cells being outside the normal range of 0.8 - 3.3 (mean 1.6) which suggests the presence of a monoclonal B-cell population.

Further characterisation of a monoclonal population is obtained by additional phenotyping using the panel of cell surface markers (Table 1.4). Characteristic patterns or profiles of marker expression can then be defined for a monoclonal population.

B-CELL LYMPHOMAS.

The most consistent feature is expression of sIg+ either at a low level (B-CLL) or high density expression as with prolymphocytic and hairy cell leukaemia. B-CLL expresses CD5 and CD24, whereas prolymphocytic leukaemia expresses all B-cell markers (usually strongly) with the exception of CD5. CD5 and CD24 tend to be absent or weak in hairy cell leukaemia, whereas CD22, FMC7 and CD25 (IL-2 receptor) tend to be strongly expressed or distinctive.

B-cell surface differentiation antigens and sIg+ tend to be absent in the plasmacytoid malignancies, but the cells can be phenotyped with staining for cytoplasmic immunoglobulin. Blood involvement in lymphoplasmacytoid lymphoma is well recognized (Cader et al, 1983) and can be detected by surface and cytoplasmic Ig expression. FMC7 tends to be strongly expressed.

TABLE 1.4
COMMONLY USED MONOCLONAL ANTIBODIES IN LYMPHOMA DIAGNOSIS

ANTIGEN	CELL EXPRESSION	ANTIBODY USED
CD45 (L.C.A) CD45R	All leukocytes B cells, T subsets	F10-89-4 ¹ F8-11-13 ¹
CD19 CD20 CD22 CD24 - Ig heavy chain ($\mu, \zeta, \alpha, \gamma$) Ig light chain (K, λ)	Pan-B Pan-B B-Subset Pan-B B-Subset B B	Leu-12 ² Leu-16 ² Leu14 ² Dako B ³ OKB2 ⁴ FMC7 ⁵ Anti-human Ig (light heavy chains) ²
CD1 CD2 CD3 CD4 CD8 CD5 CD7	Thymocyte Pan-T, NK Mature T T helper/inducer T suppressor/cytotoxic Pan-T, B (CLL) Pan-T, immature Blasts	OKT6 ⁴ Dako T 11 ³ Dako T3 ³ , Leu4 ² Dako T4 ³ , Leu3 ² Dako T8 ³ , Leu2 ² Dako T1 ¹ RFT2 ⁶
CD16 ?CD11 (c3b-R) HLA-DR	NK, granulocytes DRC, B, M B, M, activated T	Leu11b ² Anti-c3b receptor ³ OK-DR ⁴

Source of antibodies

- 1) Professor J. Fabre
- 2) Becton-Dickenson
- 3) Dako-Patts
- 4) Ortho Diagnostics
- 5) Sera Lab
- 6) Royal Free Hospital

Abbreviations

- LCA - leucocyte common antigen
B, T - B, T lymphocyte
NK - natural killer cells
M - macrophages
DRC - dendritic reticulum cells

Immunophenotype profiles in other B-cell lymphomas may show typical expression (sIg+, CD20) or show considerable variability of expression even in histologically uniform categories of tumours.

T-CELL LYMPHOID MALIGNANCIES.

The immunodiagnosis of T-cell disorders is much more problematical in the absence of a marker of clonality comparable to the B-cell light chain type. In addition T-cell malignancies tend to be more heterogenous even in apparently single disease states or categories.

Most cutaneous T-cell lymphomas including mycosis fungoides and Sezary syndrome are CD4+ CD8-, with non-expression of one or more pan-T marker. This phenotype with normal expression of pan-T markers is seen in cases of T-CLL, and T-prolymphocytic leukaemia. The latter can sometimes express other phenotypes (CD4+ CD8+; CD4- CD8+).

The adult T-cell leukaemia/lymphoma associated with HTLV-1 infection usually expresses CD4+ CD8- CD7- with normal expression of pan-T antigens. These neoplastic cells tend to be consistently positive for CD25 (IL-2 receptor).

A diagnosis of T-cell leukaemia or lymphoma can only be suspected with a characteristic phenotype even with anomalous or non-expression of certain T-cell markers. Its nowhere more apparent to consider the patients clinical condition, histological results and other investigations in conjunction with the immunophenotyping data before deciding on a diagnosis.

b). IMMUNOPHENOTYPING OF LYMPHOID TISSUE BIOPSIES.

Despite the fact that there is a rapid expansion of monoclonal antibodies developed for use in fixed paraffin wax-embedded biopsy material, and encouraging results published (West et al, 1986), the immunodiagnosis of lymphoma cases is best done on fresh-frozen tissue. This does then require the rapid transportation of biopsy material to the laboratory for appropriate processing.

The choice of a particular immunostaining method is open to personal preference, but the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique of Cordell et al, (1984) was used on the majority of the cases in this study.

The CD45 marker has been found to be a highly effective

and consistent marker at identifying tumours of lymphoid (or histiocytic) origin, as the initial aim in lymphoma diagnosis is to diagnose or exclude lymphoid malignancies from the rest.

B-CELL LYMPHOMAS.

The majority of NHL are of B-cell origin and the most useful markers for B-cell lineage include CD45R CD19 CD20 and CD22. However, CD19 may not be expressed on some high grade centroblastic tumours, and CD45R is not entirely B-cell specific being expressed on some normal T-cells. CD45R+ T-cell lymphomas occur but are thought to be rare (Norton & Isaacson, 1989). If a panel of B-cell markers is used (Table 1.4), then a B-cell lymphoma is unlikely to be misdiagnosed.

Expression of CD24 is more variable, tending to stain about half of the low grade B-cell cases, some of the high grade lymphomas with the B-lymphoblastic cases usually being positive.

In most B-cell lymphomas surface and cytoplasmic immunoglobulin can be demonstrated with evidence of light chain (kappa or lambda) restriction. Misinterpretation may occur with background staining particularly with some high grade cases.

T-CELL LYMPHOMAS.

The heterogeneity of T-cell malignancies has already been discussed. Using the routine lymphoma marker panel (Table 1.4) attempt is made to divide the T-lymphoblastic lymphomas from the peripheral T-cell types (Suchi et al, 1987), the former expressing an immature thymocyte phenotype (CD1+ , pan-T markers). The results tend to be heterogenous in all other cases.

Widespread T-cell marker staining may be due to small lymphocytes adjacent to or surrounding the neoplastic cells, and therefore it is obviously important to identify the neoplastic cells from the reactive population on immunostained tissue sections.

HODGKIN'S DISEASE.

Immunostaining of sections reveals the majority of background cells to be CD4+ and CD8+ lymphocytes in varying proportions. If the CD4+ population is predominant differential diagnosis from a T-cell lymphoma may be difficult. The markers for Reed-Sternberg cell associated antigens tend to work best on paraffin rather than frozen sections.

1.6. THE BASIS OF A MOLECULAR APPROACH TO LYMPHOMA DIAGNOSIS

Malignant lymphoma is a disease of unregulated proliferation, usually monoclonal, affecting B and T lymphocytes and their precursors. The molecular approach has been based on understanding the organization and expression of the Immunoglobulin (Ig) and the T-cell receptor antigen (TCR) genes, and demonstrating clonal rearrangement of these genes. This forms the basis of Southern blotting (Southern, 1975).

1.6.1. THE BASIS OF SOUTHERN BLOTTING.

The detection of monoclonality within a lymphoid population depends upon commitment of stem cells to B and T cell differentiation, associated with rearrangement of several genes specific for that differentiation. Therefore during lymphoid maturation, a series of somatic recombinations occur, which change the relative positions of base sequences that are substrates for the DNA-cutting action of restriction endonucleases. This endonuclease digestion results on electrophoresis, blotting and hybridisation with cDNA probes fragments of heterogenous size, producing new bands at different positions from those obtained with germ-line DNA.

IMMUNOGLOBULIN STRUCTURE AND EXPRESSION.

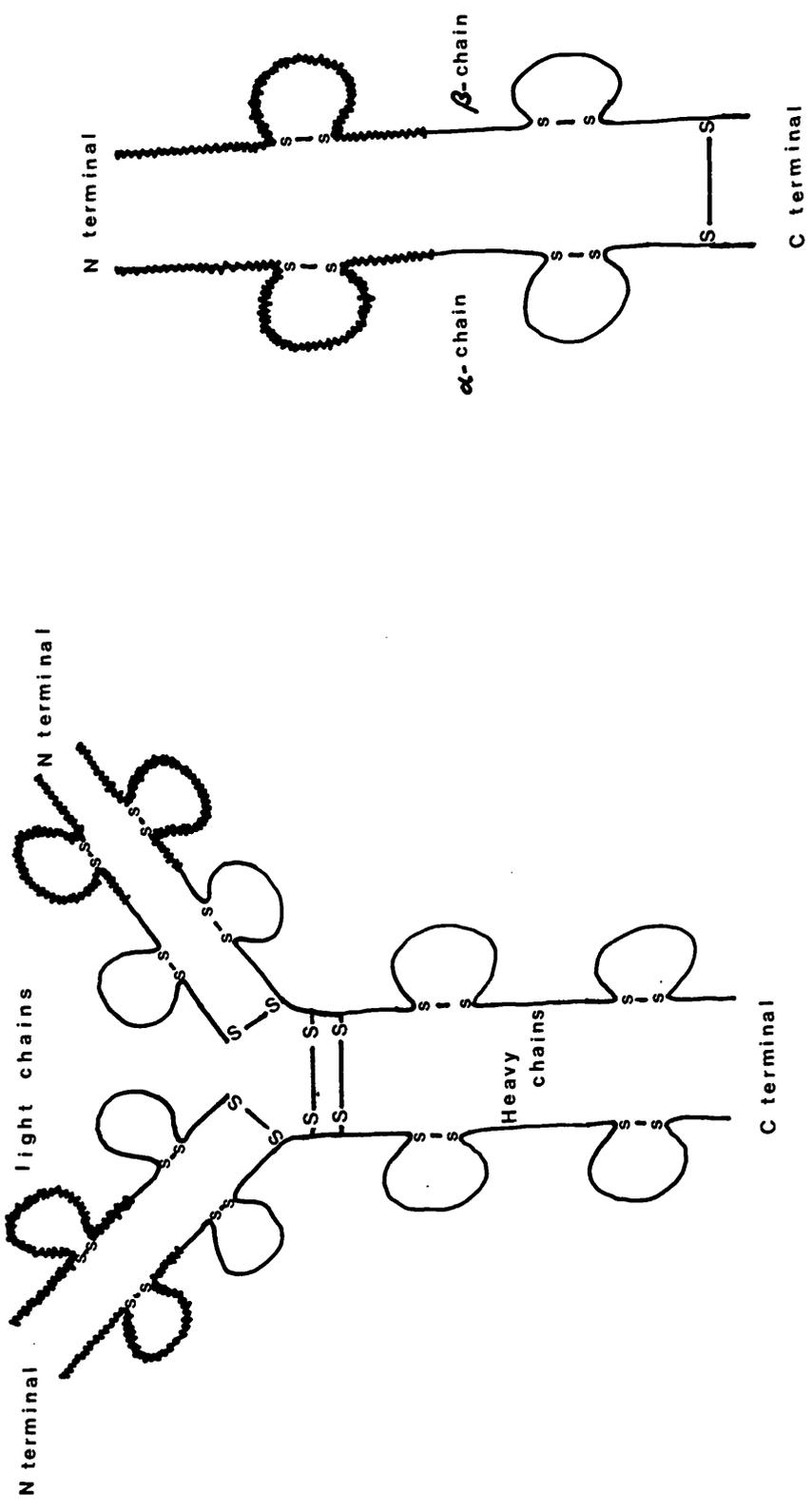
Cells of the B-lymphocyte lineage are characterised by synthesis and expression of Ig. The primary function of Ig is to bind specifically to a diverse array of presented antigens.

Ig molecule has a four-chain polypeptide structure (two light and heavy chains) linked together by non-covalent interactions and covalent disulphide bonds (Davies & Metzger, 1983) with variable, joining and constant regions (Figure 1.1). This basic structure is seen in several other protein molecules involved in the immune response, including TCR (Hood et al., 1985; Hunkapiller & Hood., 1986). Ig classes and subclasses are examples of isotypic variation involving the constant region of the heavy chain, encoded by closely linked genes on chromosome 14. Allotypes are variants inherited as alternatives, due to changes affecting the constant region genes.

CHROMOSOMAL LOCALISATION OF IMMUNOGLOBULIN GENES.

The genes encoding for the heavy chains are found on the long arm of chromosome 14 (14q32), while the sites for kappa and lambda light chains are chromosomes 2 (2p13) and 22 (22q11) respectively (Erikson et al., 1981; Kirsch et al 1982; Malcolm et al., 1982).

Figure 1.1 The structure⁶⁰ of the immunoglobulin molecule and similarities with the T cell receptor antigen can be seen.



Immunoglobulin

T-Cell receptor

ORGANIZATION AND EXPRESSION OF IMMUNOGLOBULIN GENES.

The active immunoglobulin genes are thought to be formed by a series of DNA rearrangements that bring together separate gene segments (variable V; joining J; diversity D; constant C) during the development of the B-cell lineage (Figure 1.2).

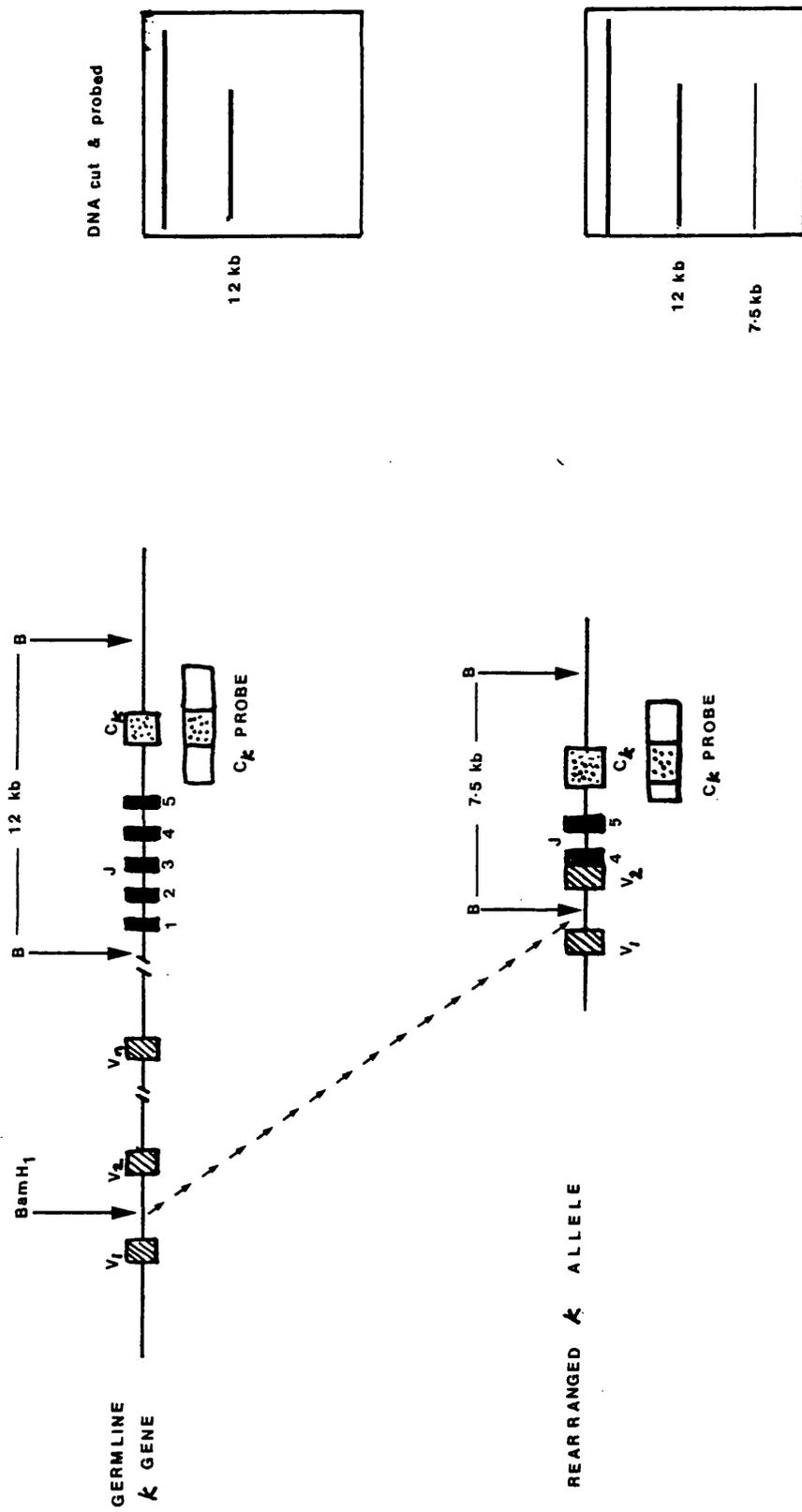
Each segment which could be integrated is flanked by short conserved sequences, which act as signals for integration - so called 12/23 base pair joining rule (Max et al., 1979 Sakano et al., 1979).

Integration events apparently occur in a specific order, with heavy chain genes before light chain genes (Maki et al., 1980; Korsmeyer et al., 1981).

There is thought to be an additional degree of ordering within the process of heavy-chain gene rearrangement, in that D to JH joining appears to precede V to D-JH joining (Yaoita et al., 1983). Regulation of gene integration occurs by allelic exclusion (expression by a single B-lymphocyte of only one of the two possible alleles at each Ig gene locus), and by isotypic exclusion namely expression of only kappa or lambda light chains, but not both. The result is that each B-cell produces Ig with only a single kind of VH region and a single kind of VL region.

Figure 1.2 Somatic rearrangement of immunoglobulin kappa gene, a process which occurs only in B-lymphoid cells.

The basis of southern blotting is explained by illustrating restriction enzyme sites for BamH, one of which is lost during rearrangement. Therefore in the germline configuration, a specific probe to the c_k region will demonstrate a 12kb fragment after DNA restriction and electrophoretic separation on an agarose gel. The B cells undergoing rearrangement will demonstrate a smaller 7.5kb fragment on the gel, so called rearranged band. The intensity of this band is proportional to the size of the monoclonal B cell population, in comparison to the reactive, polyclonal B lymphocytes.



GENOMIC CONFIGURATION OF IMMUNOGLOBULIN

k - GENE

Furthermore, an ordered hierarchy of light chain gene integration seems to occur during B-cell development, with kappa gene rearrangement preceding lambda (Hieter et al., 1981; Korsmeyer et al., 1981) and lambda gene integration only occurs after non-functional rearrangement of both kappa alleles.

The generation of V region diversity is thought to be achieved by V-J gene segment (light chain), and V-D-J gene segment (heavy chain) combinations, variation in the sequence produced at the joining sites and by somatic mutation.

The Ig gene promoter gene, as well as enhancer are thought to contain transcription control elements with tissue specificity for developing B-cells. During the development of the B-lymphocyte, the VH region is first expressed with the C μ region resulting in production of IgM. Subsequently, a switch to other Ig classes can occur containing the same VH region and light chain, but a different CH region. This heavy-chain class switch is thought to take place by DNA rearrangement to different CH gene, with deletion of all the intervening CH genes (Honjo & Kataoka, 1978).

B-CELL DIFFERENTIATION. (Figure 1.3)

The first stage of B-cell differentiation apparently occurs in the haemopoietic tissues, is antigen independent and involves the differentiation of pluripotent haemopoietic stem cells into virgin B-cells. These cells synthesize and express Ig as a surface receptor for antigen.

The second stage is considered to be initiated following an encounter with antigen, resulting in expansion of antigen-specific clones, and production of plasma cells which synthesize and secrete large amounts of antibody together with generation of long lived memory cells.

B-CELL LYMPHOPROLIFERATIONS AND NORMAL B-CELL DIFFERENTIATION.

Malignant B-cells are considered stereotypes of discrete normal maturation stages frozen by the oncogenic event (Salmon & Seligmann, 1974).

Mature B-cells emerging from the bone marrow are thought to have the same phenotype (sIgM+ sIgD+ CD19+ 20+ 21+ 22+ 24+) as B-cells in nodal primary nodules and mantle cells in secondary follicles, whereas the phenotype of germinal centre centroblasts and centrocytes (sIgM+ HLA-DR+ CD10+ 19+ 20+ sIgD- CD24-)

may result from lymphocyte activation in an appropriate microenvironment (Stein et al., 1982). A second smaller B-cell population (CD5+) appear early in ontogeny, but are subsequently confined to peripheral lymphoid organs (Campana et al., 1985).

Centroblastic/centrocytic lymphomas closely imitate the reactive germinal centre, with a follicular growth pattern and cells expressing CD10+ IgD- phenotype. Malignant expansions of CD5+ B-cells results in B-CLL and Centrocytic lymphoma (Gobbi et al., 1983).

The possible relationships between lymphoproliferations and B-cell differentiation is shown in Figure 1.3.

Fig 1.3

B lymphocyte differentiation and the relationship to the various types of B cell neoplasia.

DR	:	HLA-DR
Tdt	:	terminal deoxynucleotidyl transferase
VDJ & DJ	:	variable, diversity and joining region genes
Ig	:	Immunoglobulin
μ	:	Ig heavy chain
CD	:	cluster designation
c	:	complement receptor
ALL	:	acute lymphoblastic leukaemia
CLL	:	chronic lymphocytic leukaemia
PLL	:	prolymphocytic leukaemia
WM	:	Waldenstrom's macroglobulinaemia
NHL	:	non-Hodgkin's lymphoma
HCL	:	hairy cell leukaemia

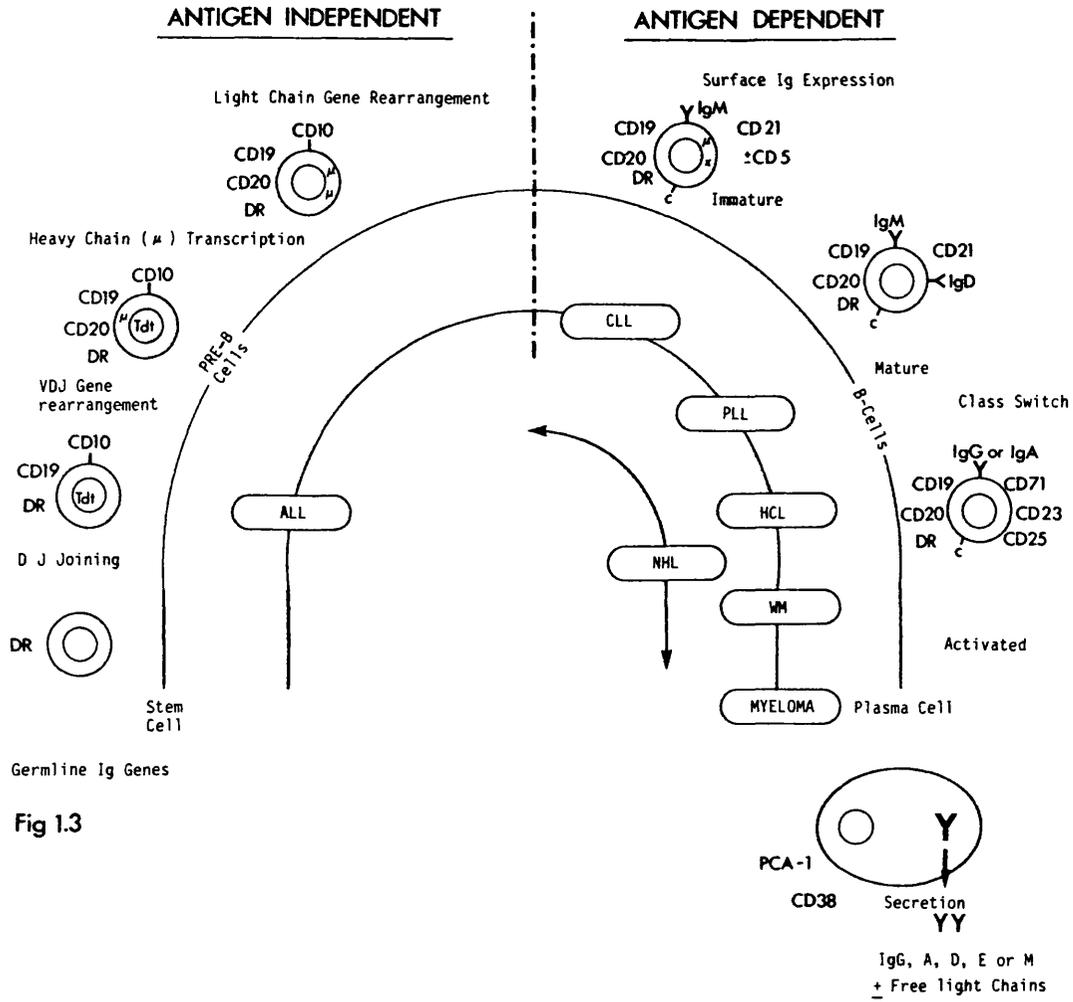


Fig 1.3

THE T-CELL RECEPTOR POLYPEPTIDES.

T-lymphocytes, unlike B-cells predominantly recognize antigen when it is associated with membrane-bound products of the major histocompatibility complex (MHC). The specific recognition molecules on T-cells is the membrane-bound T-cell antigen receptor (TCR), analogous to the surface bound Ig molecule on B-cells.

There are considered to be four distinct T-cell antigen receptor polypeptides ($\alpha, \beta, \gamma, \delta$), which form two different heterodimers ($\alpha:\beta$ and $\gamma:\delta$) that are very similar to Ig ("the immunoglobulin superfamily"). The TCR $\gamma:\delta$ heterodimer is associated with the CD3 polypeptide (Saito et al., 1984) and is present on a small percentage (1-10%) of peripheral T-cells (Brenner et al., 1986), but on the majority of dendritic T-cells in the skin (Stingl et al., 1987; Kuziel et al., 1987). The function of the $\gamma:\delta$ heterodimer is unknown, whereas TCR $\alpha:\beta$ provides specificity for all cytotoxic and helper cells.

TCR GENE ORGANIZATION AND EXPRESSION.

Like immunoglobulin, the TCR genes are divided into specific V, D (β & δ) and J segments, which are fused together to form a complete V-coding domain adjacent to a particular C region. The process of recombining gene elements is considered to be similar to the Ig gene.

TCR genes are apparently first rearranged and expressed in the thymus during the earliest stages of T-cell differentiation, in the order β , γ , δ , α . The γ : δ heterodimer precedes α : β expression, and it has been suggested (Pardoll et al., 1987) that γ : δ rearrangement may be attempted first (like Ig kappa gene) and in the event of failure, cells proceed to α : β expression.

CHROMOSOMAL LOCALISATION OF THE TCR GENES.

Chromosome 7 (7p12-7p21) is the site for TCR beta chain gene and is closely associated with the site (7p15) for the gamma chain gene. The alpha chain gene maps to chromosome 14 (14q11-14q12) (Caccia et al., 1985). The delta gene is thought to be closely associated with the CD3 antigen and maps to chromosome 11 (11q23-11qter) (Van den Elsen et al., 1985).

T-CELL DIFFERENTIATION.

T-cell precursors are thought to have an intrathymic maturation stage after leaving the bone marrow, where clonal restriction precedes release into the peripheral circulation. Therefore any peripheral T-lymphocyte subset will have an equivalent thymocyte subpopulation (Pizzolo & Chilosi, 1984). The T-cell lineage specific markers all react with a few T-cell associated antigens (CD1-8), although other non-specific markers will also be useful for categorisation of T-lymphocyte subpopulations if used in conjunction with the lineage specific markers.

T-CELL NEOPLASMS.

Rearrangement of the TCR beta-chain defines cells committed to the T-cell lineage, as well determining clonality (Isaacson et al., 1985; O'Connor et al., 1985; Waldmann et al., 1985). T-cell malignancies are classified into pre-thymic/thymic and peripheral (post-thymic) lymphomas (Suchi et al., 1987).

T-ALL and T-lymphoblastic lymphoma are ontogenically related, distinction is largely based on differential clinical presentation. The lymphocytes are usually TdT positive with CD1, CD2, CD7 phenotype. The CD1 marker and TdT positivity differentiate from the peripheral T-cell lymphomas.

The peripheral T-cell lymphomas are a heterogeneous group showing great variability in clinical presentation. All are derived from immunocompetent post-thymic T lymphocytes. They are CD1 negative, TdT negative, and have rearranged TCR genes. With T-cell lymphomas, no single immunological phenotype is restricted to a single morphological entity. A helper (CD4) : suppressor (CD8) ratio (unlike kappa:lambda) does not differentiate neoplastic from reactive states.

1.6.2. IN SITU HYBRIDIZATION

In situ hybridization allows localisation of specific nucleic acid sequences in tissue sections, and a number of protocols have been published recently (Burns et al, 1985; Beckman et al, 1985; Unger et al, 1986; Pringle et al, 1987).

This technique became available for wider clinical laboratory usage due to the development of non-radioactive nuclei acid probes and detection systems. The most successful and useful of these methods is based on incorporation of biotin ~~is~~ into the probe and subsequently detected by enzyme conjugated avidin (Leary et al 1983). These biotinylated probes can be used on cryostat (fresh tissue) or routine formalin-fixed paraffin wax-embedded sections.

The hybridization procedure is specific, because recombinant DNA techniques have enabled the synthesis of pure and highly specific probes. These probes are either biotinylated or labelled with ^{35}S and hybridization (or annealing) detected using an alkaline phosphatase/fast red naphthol capture method and by autoradiographic emulsion respectively.

1.6.3. THE POLYMERASE CHAIN REACTION.

PCR is an in vitro method for the primer directed enzymatic amplification of specific DNA sequences (Saiki et al, 1985). Two oligonucleotides (primers) hybridize to opposite strands of DNA that flank the segment to be amplified. The orientation of the primers is in opposite directions, such that DNA synthesis is specific proceeding across the regions between the primer hybridization sites, using thermostable DNA polymerase (Saiki et al, 1988; Erlich et al, 1988).

DNA synthesis is initiated with the enzyme Taq polymerase (a heat stable DNA polymerase from a thermophilic bacteria, *Thermophilus aquaticus*), and is based on a series of repeated cycles of heat denaturation (to render the DNA single stranded), annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase. Each cycle essentially doubles the amount of DNA synthesized, resulting in an exponential increase of the specific target sequence.

The repeated cycles of denaturation, annealing and primer extension are achieved by rapid alterations in temperature. Denaturation is usually performed at 95°C

for 1-2 minutes, with primer annealing at 45-55°C and primer extension at 70-75°C. Specificity of annealing is determined by the length of the oligonucleotide, its GC nucleotide content and salt concentration in the amplification buffer. The PCR procedure is now automated using commercially available "thermal cyclers".

PCR is a versatile procedure, permitting successful amplification with poor quality DNA or DNA from historical paraffin-embedded biopsy specimen material (Lai-Goldmann, 1988).

The amplified DNA can be directly visualised by staining with ethidium bromide, following electrophoretic separation. This allows determination of the size of fragment generated and of the specificity of amplification. Confirmation of specific amplification is accomplished by hybridization of radiolabelled, allele specific oligonucleotide probes, after transference of DNA onto nylon filters (Southern blotting).

1.7. THE OBJECTIVES OF THIS PROJECT

- 1) Assess the value of gene probe analysis in providing diagnostic clarification when applied to tissues of patients suspected with lymphoid malignancy. In addition, compare the relative sensitivity of detecting circulating lymphoma cells by immunophenotyping and gene probe analysis.

- 2) Assess the value of in situ hybridization in differentiating between monoclonal and polyclonal lymphoid neoplasms.

- 3) To investigate and develop further the novel technique of polymerase chain reaction in lymphoma diagnosis, specifically at detecting occult or minimal residual disease (MRD).

CHAPTER 2 : SOUTHERN BLOTTING.

Patients were studied with a clinical and histological diagnosis of non-Hodgkin's lymphoma, in whom both tissue biopsies and blood samples were obtained prior to treatment. In each case, biopsies were submitted to conventional histological examination, immunophenotyping by immuno-enzyme staining on cryostat sections of unfixed tissue, and DNA extraction for gene studies. Mononuclear cells were separated from blood for immunofluorescent analysis of lymphocyte subpopulations and gene rearrangement studies.

Clinical and staging information was obtained from case notes.

2.1. METHODS.

2.1.1. PROCESSING OF BIOPSIES.

Surgical biopsies were transported immediately, unfixed to the laboratory. Representative portions were cut for fixation in 10% formol saline and paraffin embedded for routine histology, and snap frozen (in liquid nitrogen) for both immuno-enzyme staining on cryostat sections and DNA extraction for gene studies.

Routine histological examination was done on paraffin sections stained with haematoxylin and eosin, and reticulin stains. Additional cytochemical and immunohistochemical stains were used to further delineate ambiguous cases.

2.1.2. TISSUE IMMUNOPHENOTYPING.

Fresh tissue was snap frozen in liquid nitrogen, after mounting onto cork and embedding medium. 6 μ m. cryostat sections were cut, mounted onto glass slides, air dried (30 minutes at room temperature), and fixed in dry acetone for 20 minutes at room temperature.

The technique of APAAP (Cordell et al, 1984) was used. Normal rabbit serum was used as a blocking agent, to prevent non-specific binding. Two sequential layers of monoclonal antibodies were used, beginning with mouse anti-human antigen in Tris buffered saline (TBS) for 30 minutes, intervening washes in TBS followed by rabbit anti-mouse immunoglobulin in TBS for 30 minutes and washes in TBS, after which APAAP was used. For colour reaction a substrate buffer containing fast red, naphthol and levamisole was used. The sections were counterstained with haematoxylin, washed in tap water, and dehydrated in ethanol, followed by mounting in aqueous medium for microscopic evaluation.

2.1.3. LYMPHOCYTE IMMUNOPHENOTYPING.

Mononuclear cells were separated from heparinized blood samples by centrifugation on a metrizoate/hypaque gradient, SG 1.077 (lymphocyte separating medium; Flow laboratories).

A direct immunofluorescence method was used for detecting Ig heavy and light chains on surface of lymphoid cells. The mononuclear cells (approx. 3×10^6) were resuspended in 2 ml. of acetate-buffered saline, and incubated at 37°C for 15 minutes, followed by washing in culture medium (RPMI-1640) and incubated in 2 ml. of culture medium (60 minutes at 37°C) to remove non-specifically bound cytophilic antibodies. After resuspension in fresh culture medium, and addition of 200 μ l. of FITC- conjugated anti-immunoglobulin in 0.1 M PBS, the cells were incubated at 4°C for 30 minutes, followed by washes in culture medium and resuspension in one drop of PBS/glycerol (v/v) for mounting onto a glass slide.

An indirect immunofluorescence method was used for detection of membrane antigens recognized by various monoclonal antibodies. The cells were resuspended in a PBS based buffer (PBS 100 ml., sodium azide 0.2 g., bovine serum albumin 0.2 g., pooled human AB serum 2

ml.), followed by incubation with monoclonal antibody at 4°C for 30 minutes. To the supernatant was added 35 µl. of FITC-conjugated goat anti-mouse Ig and incubated for further 30 minutes, followed by mounting in PBS/glycerol as above.

A panel of monoclonal antibodies was used (Table 1.4) and mouse immunoglobulin was included as negative control.

B-cell lymphomas were diagnosed, when tumour cells expressed monotypic κ or λ light chains and pan-B antigens. T-cell lymphomas were based on expression of pan-T or T-cell subset antigens.

2.1.4. DNA EXTRACTION.

DNA was prepared from samples of tissue (fresh frozen or fixed and paraffin embedded) and peripheral blood lymphocytes by proteinase K digestion using standard procedures (Pettersen & Sambrook, 1973).

2.1.4.1. DNA EXTRACTION FROM FRESH FROZEN TISSUE.

The tissue was homogenised, digested with a proteolytic enzyme and DNA retrieved using phenol/chloroform extractions and ethanol precipitation.

a). HOMOGENIZATION OF TISSUE.

A piece of tissue 2.5 x 2.5 x 2.5 mm (50-100 mg.) was selected and allowed to warm up slightly. The tissue was placed in a corex tube containing 2 ml. of 0.05 M Tris/HCl buffer at pH 8.0, and homogenised at low speed for 20 seconds.

b). PROTEIN DIGESTION.

The enzyme proteinase kinase (PK) was pre-prepared at a concentration of 20 mg/ml. in 0.05 M Tris/HCl pH 8.0. 20 μ l. of PK was mixed with 200 μ l. of 10% SDS, and added to the homogenised tissue. This mixture was incubated in a polypropylene tube at 37°C until digestion was complete (3 hours to overnight) and the solution became viscous.

c). PHENOL/CHLOROFORM EXTRACTIONS.

If the solution was very viscous, it was pre-diluted with further 0.05 M Tris/HCl buffer (approx. 3 mls.). To the polypropylene tubes was added 3 mls. of phenol/chloroform/isoamyl alcohol (IAA). Mixing was ensured by gentle inversions. The mixture was separated by centrifugation at 3000 G for 10 minutes. The upper (DNA) layer was removed by gentle agitation, using a pasteur pipette avoiding uptake of the precipitated protein at the phenol interface. This procedure was repeated 2-4 times, until a clear DNA layer was obtained.

Any trace of phenol solution was removed by extracting through an equal volume of chloroform/IAA a number of times.

d). DNA PRECIPITATION.

To the extracted DNA was added 5 M sodium chloride solution, to achieve a final concentration of 0.1 M, in a corex tube. To this was added 2-3 times the total volume of cold (-20°C) filtered absolute ethanol. On mixing a fluffy DNA precipitate was seen, which is then retrieved into an eppendorf tube using a pipette tip. The DNA was then rinsed in cold (-20°C) 80% ethanol, to remove excess salt.

e). DNA RESUSPENSION AND PURIFICATION.

After evaporation of the ethanol in a vacuum dessicator, the DNA precipitate was resuspended in a small volume (upto 1 ml.) of 1 x TE buffer. This was left until DNA went into solution, the process could be speeded up by incubation at a high temperature (e.g. 10 minutes at 65°C or one hour at 37°C).

Further purification of DNA could be accomplished by dialysis. The DNA solution was carefully loaded in dialysis tubing secured at each end by plastic clips. The filled dialysis tubing was placed in a large volume beaker containing 1 x TE solution. Several changes of TE buffer were required over the period of exchange (3 hours minimum, at 4°C). The dialysate was continually mixed, with magnetic stirrers.

On completion of dialysis, the DNA solution was replaced into eppendorf tubes, ready for DNA estimation. The methodologies described are based on standard protocols (Maniatis et al, 1984).

2.1.4.2. DNA EXTRACTION FROM CELL SUSPENSIONS.

The mononuclear (lymphocyte) population was separated from peripheral blood and stored in ethanol at 4°C, prior to DNA extraction.

The cell suspensions were centrifuged at 400 G for five minutes, to pellet the cells and the ethanol discarded. The cells were resuspended in 2 mls. of 0.05 M Tris/HCl pH 8.0, and 200 μ l. of 10% SDS and 20 μ l. of PK (20 mg/ml.) added. After mixing and incubation at 37°C for a variable period (minimum one hour), the DNA was extracted with phenol as described above.

2.1.4.3. DNA FROM FIXED AND PARAFFIN EMBEDDED TISSUE.

This was carried out according to the method described by Warford et al (1988), which consists of a five stage process of tissue rehydration, digestion, purification, DNA precipitation and resuspension.

Approximately 100 mg of tissue was pre-prepared into a corex tube, by cutting an appropriate number of 5-15 μ m sections from the paraffin blocks.

a). TISSUE HYDRATION.

The tissue was dewaxed by incubation in 10 mls. of xylene for 10 minutes, followed by centrifugation at 3000 g for 10 minutes and the xylene discarded. This process was repeated twice more.

The tissue was rehydrated by two 10 minute periods each in 99% ethanol, 95% ethanol and sterile ultra-pure (UP)

water in order stated. After each wash the ethanol or water was discarded following centrifugation at 3000 G for 10 minutes, to pellet the tissues.

b). PROTEIN DIGESTION.

The tissue was transferred to a polypropylene tube containing 9 mls. of 0.05 M Tris/HCl pH 8.0. To this was added 1 ml. of 10% SDS and 100 μ l. of 20 mg/ml. PK concentration. This mixture was then incubated at 37°C with gentle agitation. A fresh volume of PK was added after every 24 hours, until digestion was complete with no tissue fragments remaining, and the solution became viscous. This process of digestion could be accelerated by starting with thinner tissue sections.

c). PURIFICATION.

The DNA was purified by extracting sequentially with 7.5 mls. of phenol/chloroform/IAA (thrice) and 5 mls. chloroform/IAA (thrice). The phenol and chloroform extracts were centrifuged at 2000 G for 30 and 15 minutes respectively. The samples were back extracted by adding 5 mls. of 1 x TE to the first phenol interface after removal of the clear aqueous DNA layer followed by centrifugation as for primary extracts. This solution was then passed over the remaining interfaces of the other steps. The primary and back extracts of DNA were pooled.

d). DNA PRECIPITATION AND RESUSPENSION.

The DNA was precipitated with sodium chloride and ethanol as described above. The DNA was resuspended and purified by dialysis as described above.

2.1.5. DNA ESTIMATION.

The concentration of the extracted DNA was determined by spectrophotometry. To 20 μ l. of resuspended DNA solution was added 980 μ l. of 1 x TE in glass cuvettes. The absorbance (A) of the solution at 260 nm wavelength was measured, and the concentration of DNA was calculated as follows. In addition, readings were taken at 280 nm and 230 nm for RNA and carbohydrate content of the sample.

$$\text{Concentration DNA } (\mu\text{g}/\mu\text{l.}) = \frac{A \times 50 \times 50}{1000}$$

RNA and carbohydrate estimate by ratio of readings at 260/280 and 230/260 respectively. For DNA samples the RNA ratio should approximate 1.8 (pure RNA is 2.0). The remaining DNA was stored at 4°C.

2.1.6. ENZYME RESTRICTION OF DNA.

DNA was digested (3m/ng) with the restriction endonucleases BamH-1 or EcoR-1 (Pharmacia), in some

cases additionally with Bgl-II or Hind-III (Pharmacia) and size fractionated on 0.6% agarose gels. The DNA fragments were then transferred onto nylon filters as described by Southern (1975).

a). DNA RESTRICTION

To 20 µg of genomic DNA (10 µl.) in a 1.5 ml. eppendorf, was added 10 µl. of x10 restriction buffer, 50 units of restriction enzyme and sterile distilled water to make the total volume up to 100 µl. The DNA was restricted by incubation at 37°C overnight, with another 50 units of enzyme being added after the first hour.

b). ETHANOL PRECIPITATION

To the restricted DNA sample was added 10 µl. of 2.5 M sodium acetate and 330 µl. of cold (-20°C) absolute ethanol. After mixing, the solution was placed at -70°C for 10 minutes, followed by centrifugation at 12000 G in a microfuge at 4°C for 10 minutes. The supernatant was poured off and the DNA pellet dried in a vacuum dessicator for 15-30 minutes, before being resuspended in a small volume (16 µl.) of 1 x TE.

c). CONFIRMATION OF RESTRICTION

A standard 0.6% agarose ("mini") gel was prepared using high melt temperature agarose in Tris-acetate/EDTA

(TAE). To 2 μ l. of the restricted DNA sample was added 3 μ l. distilled water and 1 μ l. of gel loading buffer (bromophenol blue). The solution was heated in a water bath at 65°C for 10 minutes, prior to loading on to the gel. In addition, molecular weight standards were loaded. The gel was immersed in TAE in an electrophoresis tank, and a potential difference of 50 volts applied for 2-3 hrs. The gel was then stained for 45 minutes in 0.2 μ g/ml. ethidium bromide in TAE, and DNA restriction confirmed by visualisation of DNA on an ultra-violet (UV) transilluminator.

2.1.7. SOUTHERN BLOTTING

The DNA fragments were transferred out of the gel onto nylon filters as described by Southern (1975). Filters were then hybridized with appropriate radiolabelled probes.

2.1.7.1. PREPARATION OF DNA FOR SOUTHERN BLOTTING

After restriction of genomic DNA with a suitable restriction enzyme, and confirmation of restriction by means of a mini-gel, the next step is to prepare a full size standard ("maxi") gel using 0.6% high melt agarose in TAE. The remaining DNA with loading buffer was placed into the wells, and electrophoresis carried out overnight, at a potential difference of 20V. Following staining in 0.2 μ g/ml ethidium bromide in TAE, the DNA

was visualised and the gel photographed on an UV transilluminator.

2.1.7.2. DEPURINATION AND DENATURATION

To allow transference to proceed, the gel was subjected to a process of depurination and denaturation. The unused areas of the gel were cut out and discarded, and the remaining gel depurinated by immersion in a staining dish containing 0.25 M HCl. The dish was placed on a shaker at room temperature for 7 minutes, after which the gel was rinsed in UP water and the DNA denatured by immersion in a solution of 1.5 M NaCl and also 0.5 M NaOH on a shaker at room temperature for one hour. A change of solution was performed after 30 minutes. The gel was then rinsed in UP water again, and neutralized by immersion in 1.5 M NaCl in Tris/HCl pH 8.0 on a shaker at room temperature for one hour, followed by a final rinse in UP water.

2.1.7.3. DNA TRANSFERENCE ON TO A NYLON MEMBRANE

This was carried out using a modified version of the technique described by Southern (1975). A large staining dish was filled with 20 x SSC and a perspex sheet laid on top. After soaking 4 thicknesses of Whatman 3MM paper in the dish, the paper was wrapped around a perspex sheet and air bubbles removed using a glass rod, ensuring that the ends of the paper remained

immersed in the SSC solution. An adherent piece of plastic film (Saran wrap) was then placed over the soaking Whatman paper and a window cut out in the plastic film for the gel. The gel was placed inverted (DNA uppermost). Onto the gel was placed a nylon filter (Hybond N, Amersham), cut to the specifications of the gel. Four pieces of sized 3MM paper were placed on top, followed by a pile of absorbent towels, a further perspex sheet and a 1 kg weight. The transfer of DNA was allowed to proceed, but was greatly facilitated by frequent towel changes. A minimum of 2 hours was required, with the towels being changed every 5 minutes for the first 30 minutes and every 15 minutes thereafter.

The filter was removed, inverted and orientation marked. It was then placed between two sheets of 3MM paper and baked at 80°C for 5 minutes. The filter was wrapped in a plastic film, and the DNA side placed on a UV transilluminator for 30 seconds to cross-link the DNA. The filter was stored between 2 pieces of 3MM paper until required for hybridization.

To check transference of DNA onto filter, the gel was stained with ethidium bromide and the DNA visualised on an UV transilluminator.

2.1.7.4. PROBE PREPARATION

This entails labelling template DNA with alpha-³²P-dCTP and purification by gel filtration on a spun column.

The probe for the human heavy-chain locus consisted of a 2.5 kb. fragment endonucleases Ecor-1/Hind-III genomic JH insert (C76R51A). Rearrangements of the TCR beta chain locus were detected with 0.8kb Bgl-II C β 1 insert from the cDNA clone Jurkat 2. The structure of these probes is fully described elsewhere (Flanagan and Rabbitts, 1982; Yoshikai et al., 1984).

a). RADIO-LABELLING TEMPLATE DNA

DNA was radiolabelled using the random primer method (Feinberg and Vogelstein, 1983).

Prior to the labelling reaction, 10ng of DNA in 10 μ l of distilled water is denatured (heated in a hot-block to 100°C for 5 minutes, then immediately cooled on ice). To the template DNA in an eppendorf are added the following reagents in the stated order.

1. Sterile UP water to a final volume of 50 μ l.
2. 2 μ l. of OLB
3. 0.6 μ l. of 20 mg/ml bovine serum albumin (Sigma A-7906).

4. 1 μ l. of alpha-³²P=dCTP (Amersham PB 10205, 3000-4000 Ci/mM; 10 μ Ci/ μ l).
5. 0.6 μ l. lyphozyme large fragment E.Coli DNA polymerase I (Klenow) (BRL 8012 LA).

The reaction is allowed to proceed at room temperature for a minimum of 5 hours, and then terminated by the addition of 85 μ l. of stop solution.

b). PURIFICATION OF RADIO-LABELLED PROBES

The probe was purified by removing unincorporated nucleotides by gel filtration using spun columns (Maniatis et al., 1984). The bottom of a 1 ml. syringe was plugged with polyallomer wool and the syringe filled with Sephadex G50 (5g/100ml). A packed volume of 1 ml. was obtained by repeated additions of Sephadex and centrifugation at 4,000 G for 5 minutes. Finally, the column was equilibrated with 1 x TE/0.1% SDS by additions of 100 μ l. volumes and centrifugation at 4,000 G for 5 minutes, until a similar volume was retrieved from the column after each spin.

After measuring the initial activity of the probe solution, it was added to the prepared column and centrifuged at 4,000 G for 5 minutes. The purified probe solution was collected into an eppendorf tube.

c). MEASURING ACTIVITY OF THE PURIFIED PROBE.

The measurements were made using a Oncor radioactive probe counter. The activities of the initial probe solution and the purified probe solution were measured, and the approximate incorporation of the nucleotides estimated by calculating the total activity of the purified probe as a percentage of the activity of the probe solution before gel filtration.

The weight of probe DNA was estimated by assuming initial amount of each of the four nucleotides in the solution to be 4 nM and the molecular weight of DNA to be 330, as follows:

$$\text{Weight of probe (ng)} = \% \text{ incorporation} \times 16 \times 330$$

The usual incorporation of nucleotides was approximately 60% and the probes were used immediately.

2.1.7.5. HYBRIDIZATION OF SOUTHERN FILTERS

The final concentrations of the hybridization solution were 5 x SSC, 1 x PE, 10% PEG and 400 µg.ml. denatured salmon sperm DNA. The filter was prehybridized by immersion in the solution in a perspex chamber for 30 minutes at 65°C. After denaturation of the probe DNA, 0.2ng/ml. was added to the hybridization solution in the chamber (specific activity of the probe 10⁹

dpm/ μ g). The filter was allowed to hybridize overnight at 65°C.

2.1.7.6. STRINGENCY WASHINGS.

The filter was extensively washed at the appropriate stringency. For JH and TCR beta probes, four 10 minute washes of 2 x SSC and 0.1% SDS at 65°C were followed by four 10 minute washes of 0.2 x SSC and 0.1% SDS at 65°C. It was then dried at room temperature on a sheet of 3MM paper and wrapped in a plastic film ("Saran wrap").

2.1.7.7. AUTORADIOGRAPHY.

The filter was placed in an x-ray cassette and exposed to Kodak XAR OMAT film using a single intensifying screen at -70°C. The exposure time varied from 12 hours to 240 hours to allow detection of low copy sequences. The film was then developed to reveal an autoradiographic image of dark bands of probe hybridization to DNA in filter.

2.1.7.8. DEHYBRIDIZATION

The filter was dehybridized to allow re-use with another probe (e.g. JH after TCR beta and vice versa). Filters could be re-probed and dehybridized up to 6 times before any deterioration occurred.

The filter was dehybridized by washing in 0.4M NaOH for 30 minutes at 45°C, followed by a solution of 0.1 x SSC, 0.1% (w/v) SDS and 0.2M Tris/HCl pH 7.5 for 30 minutes at 45°C. The filter is dried with blotting paper, and dehybridization checked by autoradiography.

An alternative method was used for nitrocellulose-based filters. A solution of 0.1% SDS was boiled and poured onto the filter in an hybridization chamber and allowed to cool to room temperature.

2.1.8. PLASMID PREPARATION

The probe for the human heavy-chain locus consisted of a 2.5 kb. EcoR1/Hind-III genomic JH insert in bacteriophage M13 (C76R51A).

The probe for the TCR beta chain locus consisted of 0.8 kb. Bgl-II C β 1 insert from the cDNA clone Jurkat 2.

In order to produce sufficient template DNA for probing, it was necessary to grow bacteria containing the plasmid in large numbers. The method described below is based on Hanrahan, and is modified for JH insert.

2.1.8.1. PREPARATION OF JM 101 COMPETENT CELLS.

a) A frozen stock of JM 101 culture was streaked on to freshly made minimal agar plates. Minimal agar media plates were prepared, after mixing 100 mls. of minimal agar (2.5% in water) with 25 mls. 5 x A salts, 125 μ l. of 20% magnesium chloride, 1.25 mls. of 20% glucose and 125 μ l of filter sterilised thiamine (1mg./ml/) 5 x A salts consisted of 5.25% (w/v) potassium monohydrate sulphate, 2.25% (w/v) of potassium dihydrate sulphate, 0.5% (w/v) ammonium sulphate and 0.25% (w/v) of sodium citrate hydrate. 5 x A salts were autoclave prior to use.

- b) Using a single colony from agar plates, an overnight culture was set up into 10 mls. of Psi-b. (Psi-b consists, of 5g/l of bacto yeast extract, 20 g/l of bacto tryptone, 5g/l of magnesium sulphate and pH adjusted to 7.6 with potassium hydroxide. All media components must be Difco. Psi-a is Psi-b with 14 g/l of bacto agar).
- c) 100 μ l. of the overnight culture was used to inoculate a fresh 10 ml. sample of Psi-b. Growth was allowed to proceed at 37°C for 2 hours, until O.D. at 550 nm. was 0.3.
- d) A 5 ml. subculture was inoculated into 100mls. of pre-warmed (37°C) Psi-b and grown for a further 2 hours at 37° until the O.D. at 550 nm reached 0.48.
- e) The growth was split between 4 x 50 mls. conical centrifuge tubes, chilled on ice for 10 minutes and centrifuged at 4,000 G for 5 minutes at 4°C.
- f) The supernatant was decanted and each pellet resuspended in 10 mls. TfbI, which is a filtered sterilised solution of 30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride and 15% (v/v) glycerol. The pH is adjusted to 5.8 with 0.2 M acetic acid.

- g) The cells are chilled on ice for 5 minutes, and then centrifuged at 5,000 G for 5 minutes at 4°C.
- h) Each pellet was resuspended into 2 mls. filtered sterilised TfBII and pooled. (TfBII is 10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v/v) glycerol and pH adjusted to 6.5 with potassium hydroxide).
- i) The cells were left chilled on ice, and then aliquoted into prechilled eppendorfs (200 µl. per tube). The cells were then snap frozen in liquid nitrogen and could be stored indefinitely at -20°C.

2.1.8.2. TRANSFECTION OF JM 101 CELLS.

As above, minimal agar plates were prepared, frozen stock of JM 101 culture inoculated and grown overnight. A single colony was inoculated into 10 mls. Psi-b, incubated at 37°C until O.D. at 550 nm. reached 0.48 to produce an exponentially growing cells.

2.1.8.3. TRANSFORMATION

Competent JM 101 cells were thawed at room temperature, and left on ice for 10 minutes. 10 ng of JH phage DNA was added to 200 μ l. cells, and left on ice for 30 minutes. The cells were heat shocked at 42°C for 90 seconds and brought back to ice for 1-2 minutes. 4 volume of Psi-b was added at RT, and then incubated with gentle agitation at 37°C for 1 hour.

A "lawn" of exponentially growing JM 101 cells were prepared. 2-3 mls. of exponentially growing cells were added to culture plate, and excess drained. The plate was dried upside down at 37°C.

After addition of sloppy agar, 50-100 μ l. of phage transformed cells were added to the plate, cultured overnight at 37°C. Plaques (lysis sites) appear.

2.1.8.4. PREPARATION OF VECTOR DNA.

Using a pasteur pipette, a plaque is transferred into 1 ml. of Luria-Bertani (LB) medium, and incubated in a Gallenkamp laboratory shaker (300 rpm) at 37°C for 6-8 hours. A 10 ml. culture of *Escherichia coli* JM 101 was grown exponentially to O.D. at 550 nm of 0.48 and added to the 1 ml. bacteriophage preparation. This was then added to 400 ml. of 1 x LB in a 21 Ehrlenmeyer flask, and incubated in a shaker (300 rpm) at 37°C for 4 hours.

The following cultures were divided between six 300 ml. centrifuge tubes and the cells harvested by centrifugation at 11,000 G at 4°C for 20 minutes.

2.1.8.5 ALKALINE LYSIS OF CELLS

A modified Birnboim & Doly method was used (Maniatis et al., 1984) to lyse the resulting bacterial pellets. The covalently closed circular plasmids are unaffected by exposure to mild alkali, which breaks hydrogen bonds in DNA.

The plasmids regain normal configuration at neutral pH, while the *Escherichia coli* remains in a denatured state. Each pellet was resuspended in 5 mls. of lysis solution containing 5 mg./ml. lysosyme (Sigma L-6876), 50 mM glucose, 25 mM Tris chloride, (pH 8.0) and 10 mM

ethylenediamine tetracetic acid (EDTA). The suspensions were transferred to 50 ml. centrifuge tubes and allowed to stand for 5 minutes at room temperature.

To each tube was added 10 ml. of a freshly prepared solution of 0.2 M NaOH and 1% SDS wt/vol, mixed gently by inversion and then allowed to stand on ice for 10 minutes. 7.5 ml. of ice-cold 5 M potassium acetate pH 4.8 (30 g glacial acetic acid to 60 ml. of 5 M potassium acetate) was added to each tube, thoroughly mixed and allowed to stand for 10 minutes, followed by centrifugation of the suspensions at 12,000 G at 4°C for 20 minutes, to pellet the cellular DNA and bacterial debris.

The supernatant was equally divided into two 300 ml. centrifuge tubes, and 3 volumes of cold (-20°C) ethanol were added to each tube. After thorough mixing, the tubes were left at -20°C for 20 minutes, and then centrifuges at 12,000 G at 4°C for 15 minutes. The supernatant was discarded and each pellet resuspended in 10 mls. of 1 x Tris/EDTA (TE). The suspensions were transferred to two 50 mls. centrifuge tubes and 5 mls. of cold (4°C) 7.5 M ammonium acetate added to each. After thorough mixing, the suspensions were centrifuged at 12,000 G at 4°C for 15 minutes.

The pellets were discarded and the supernatant divided between four 50 ml. centrifuge tubes. Three volumes of cold ethanol (-20°C) were added to each tube and mixed thoroughly and then left at -20°C for 20 minutes, followed by centrifugation at 12,000 G at 4°C for 15 minutes. The supernatant was decanted and the pellets dried as much as possible by inversion. Each pellet was resuspended in 2 mls. of 1 x TE to give a total pooled volume of 8 mls.

2.1.8.6. PURIFICATION OF PLASMID DNA.

This was based upon the differential binding properties of the covalently closed circular DNA and the linear DNA in a caesium chloride (CsCl)-ethidium bromide density gradient, according to the method described by Maniatis et al. (1984).

The volume of DNA solution was measured accurately, after adding 1/30th of the volume of 3M sodium acetate (pH 5.2) to lower the pH and prevent CsCl precipitation.

For every 1 ml. of DNA solution was added exactly 1 g of solid CsCl and mixed gently until all the salt had dissolved. The volume was remeasured, and for every 1 ml. of solution was added 80 µl of a 10 mg./ml.

solution of ethidium bromide in water. The final density of the DNA solution was 1.55 mg./ml. and the concentration of the ethidium bromide approximately 600 µg./ml.

Adjustments to density were made using the following formula:

$$\text{CsCl volume to adjust (ml.)} = (1.55 - \text{density}/1.55) \times \text{volume in mls.}$$

Low density purple protein aggregates were formed, as a result of ethidium bromide complexing with bacterial proteins. The DNA solution (and protein aggregates) were transferred to a Sorvall 11.5 ml. polyallomer tube suitable for centrifugation in a Kontron type 65.13 titanium fixed angle rotor. The remainder of the tube was filled with paraffin oil and balanced to two decimal places with a balance tube. The tubes were then sealed and centrifuged at 90,000 G at 20° for 36 hours.

2.1.8.7. ISOLATION OF PLASMID DNA.

Following centrifugation, two bands of DNA were visualised under ordinary or u.v. light. The lower band consisted of closed covalent circular plasmid DNA, whereas the upper band was linear bacterial and nicked

circular plasmid DNA. The tube was clamped to a retort stand and a needle used to pierce the top of the tube and allow air in. The lower band of DNA was then collected using a syringe with a 21G hypodermic needle inserted with the flange uppermost just inferior to the band. The DNA solution of about 1 ml. was collected and placed into an eppendorf tube.

2.1.8.8. EXTRACTION OF DNA.

The ethidium bromide was removed from the solution by extraction with isopropanol/TE saturated with CsCl. After adding an equal volume to the DNA solution in an eppendorf tube, mixing thoroughly by inversions, the sample was centrifuged at 12,000 G in a microfuge at room temperature for 2 minutes. The upper isopropanol layer containing the ethidium bromide was discarded by pipetting. This process was repeated until the lower aqueous DNA phase was colourless.

Further purification of DNA was by dialysis and phenol/chloroform/IAA extractions as described above. The concentration of the purified plasmid DNA was determined by spectrophotometry (vide supra).

2.1.9. PREPARATION OF THE PURIFIED INSERT DNA
(FRAGMENT PREP.)

The JH DNA sequence to be used as the probe template was separated from the vector DNA by enzyme restriction and agarose gel electrophoresis (Maniatis et al., 1984).

2.1.9.1. ENZYME RESTRICTION.

The JH template is a 2.5 kb EcoR-I and Hind-III fragment in bacteriophage M13 (C76R51A) (Flanagan JD, Rabbits TH. 1982). Two consecutive restrictions were accomplished by using the enzyme requiring low salt buffer conditions first, and adjusting the salt concentration for the second enzyme. Alternatively, two separate restrictions with EcoR1 and Hind III were performed followed by ethanol precipitation and resuspension of DNA after each restriction.

To 20 μ g. of DNA was added 10 μ l. of x 10 restriction buffer, 50 units of enzyme and sterile distilled water to make the total volume up to 100 μ l. The DNA was restricted by incubation at 37°C overnight or at least 2-3 hours, with another 50 unit of enzyme being added after the first hour. This was followed by ethanol precipitation, resuspension in 1 x TE (vide supra) and restriction with second set of enzyme.

To the restricted DNA sample was added 10 μ l of 2.5 M sodium acetate and 330 μ l of cold (-20°C) for 10 minutes, followed by centrifugation at 12,000 G in a microfuge at 4°C for 10 minutes. The supernatant was poured off and the DNA pellet dried in a vacuum dessicator for 15-30 minutes before being resuspended in 16 μ l of 1 x TE.

2.1.9.2. AGAROSE ELECTROPHORESIS.

A 1.5% agarose gel was prepared using low melt temperature (LMT) agarose ("Nusieve", Bio-Rad 162-0017). 3 μ l of gel loading buffer was added to the DNA sample and the solution heated in a water bath at 65°C for 10 minutes, to denature the DNA. The gel was immersed in Tris-acetate/EDTA (TAE) in an electrophoresis and the sample loaded onto the gel along with molecular standards. Electrophoresis was carried out for 2-3 hours at a potential difference of 50 volts. The gel was then stained for 45 minutes in 0.2 $\mu\text{g/ml}$. ethidium bromide in TAE and the DNA visualised on a u.v. transilluminator.

Following electrophoresis, the separated insert and vector bands of DNA were cut out, ensuring as clean separation from gel as possible, and placed into pre-weighed eppendorf tubes. The weight of the eppendorf containing the DNA was determined, and for every 1 g.

of gel was added 3 mls. of sterile distilled water and the mixture boiled for 7 minutes and then stored at 4°C.

2.1.9.3. ION EXCHANGE CHROMATOGRAPHY.

For maximum purity of JH insert DNA, further purification was performed by ion exchange chromatography on a "NACS PREPAC" mini-column (BRL 1525NP).

The agarose gel containing the DNA was melted at 75°C, and the volume estimated. Four volumes of 0.25 M NaCl in 1 x TE was then added to the solution and mixed well, followed by incubation, at 70°C for 10 minutes, and then 45°C for 10 minutes. The column was hydrated by washing three times with 2 M NaCl in TE and then equilibrated by adding 3 ml. of 0.2M NaCl in TE and the DNA sample loaded onto it. The bound nucleic acid was washed with 5 ml of 0.2 M NaCl in TE at 42°C to remove the agarose gel and any other associated impurities. The DNA sample was then eluted with 300 µl of 2 M NaCl in TE.

The DNA was then ethanol precipitated and resuspended in 1 x TE (vide supra). The DNA sample was loaded onto a LMT agarose gel and the insert fragment prepared as before. This produced a highly specific probe, by

removing vector sequence contamination which may have homology to the human genome.

The resultant DNA sample was divided into aliquots containing 5 μ g. (for in situ hybridization probes) and 10 ng. (for Southern blot probes) and stored at -20°C .

2.2 RESULTS OF GENE PROBE ANALYSIS AND IMMUNOPHENOTYPING

The histopathological categorisation of B-cell NHL was based upon a modified Kiel classification (Gerard-Marchant et al, 1974; Stansfield et al, 1988).

Peripheral T-cell lymphomas were classified according to criteria proposed by Suchi et al (1987).

2.2.1. THE LOW GRADE B-CELL LYMPHOMAS (TABLE 2.1)

Of the 13 cases, 9 were classified as follicular centroblastic/centrocytic (CB/CC) lymphomas, and the remainder comprised lymphocytic (LYM) lymphomas of spleen (2), mantle-zone (MTZ) lymphoma of spleen (1) and lymphoplasmacytoid (LPC) lymphoma (1).

The presence of a monoclonal population of B-cells on tissue was demonstrated by finding Ig rearrangement in all cases, and monotypic surface Ig was expressed in all these samples except one (insufficient tissue).

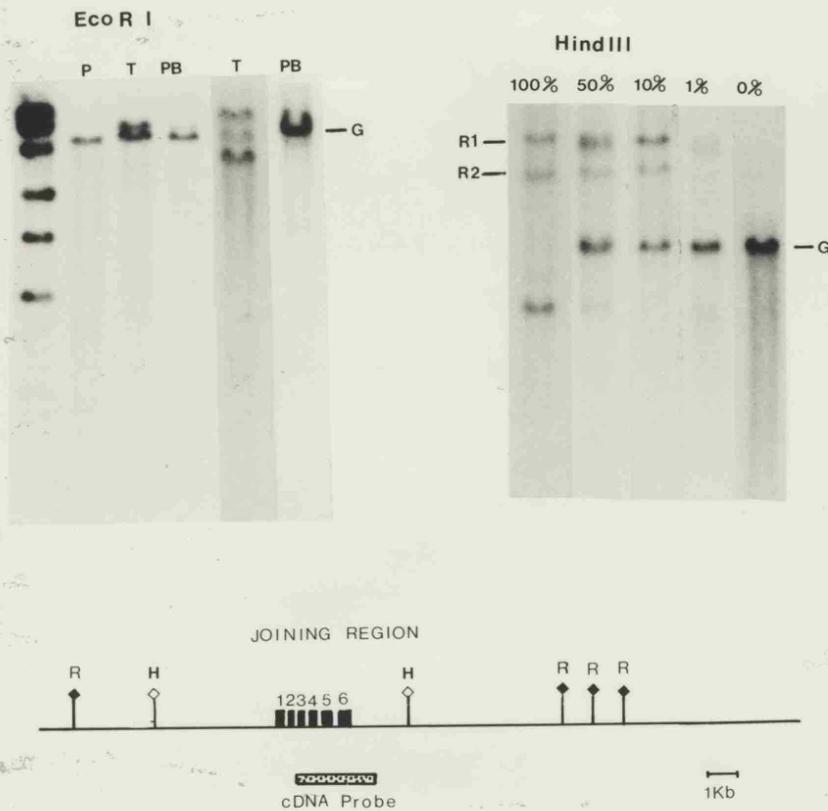
With two restriction endonucleases, the JH probe revealed 2 (6 cases), 3 (5 cases) or 4 (1 case) additional bands to the germline. Only germline bands were found with the TCR-beta probe.

A circulating monoclonal was detected in 6/13 cases by immunophenotyping, and all these cases had detectable

Table 2.1 Results on low grade B-cell lymphomas.

Case	Tissue	Histology +	Ig Phenotype	Genotype JH	lymphocyte count ($\times 10^9/L$)	% B cells	K: $\frac{\lambda}{\mu}$	Blood			Marrow involvement
								Monoclonone SIg	Genotype JH		
1	LN	CB/CC-F	IgGK	CR ₃ ^a	3.53	13.5	7:5	-	CR ₃	-	
2	LN	CB/CC-F	IgGK	CR ₃	1.16	<1.0	<1:<1	-	germline	+	
3	LN	CB/CC-F	IgMD	CR ₂	2.50	8.3	<1:12:0	IgMD	CR ₂	+	
4	LN	CB/CC-F	no tissue	CR ₃	1.0	3.3	<1:<1	-	germline	+	
5	LN	CB/CC-F	IgGK	CR	3.68	35.9	47:<1	IgGK	CR ₃	+	
6	LN	CB/CC-F	IgMD	CR ₂	1.08	3.2	2:3	-	germline	-	
7	LN	CB/CC-F	IgM	CR ₃	3.75	16.1	<1:6:0	IgMD	CR ₃	+	
8	LN	CB/CC-F	IgMDK	CR ₂	2.16	2.7	1:1	-	germline	-	
9	LN	CB/CC-F	IgMD	CR ₃	2.10	25.9	<1:5:0	IgMDGA	CR ₃	+	
10	Spleen	CB/CC-F	IgMDK	CR ₃	4.01	75.9	87:<1	IgMDK	CR ₃	+	
11	Spleen	CB/CC-F	IgMDK	CR ₄	26.2	88	78:<1	IgMDK	CR ₄	+	
12	Spleen	CB/CC-F	IgM	CR ₂	0.52	21.5	1:1	-	germline	-	
13	LN	CB/CC-F	IgMgK	CR	2.37	2.4	4:2	-	germline	+	

Plate 2.1



Example of JH rearrangement with tissue (T) and peripheral blood (PB) from two suspected B cell cases using restriction enzyme EcoR1 on the left hand side. The first two columns result from molecular weight standards and placenta respectively. Rearranged bands are most clearly seen in the tissue samples.

The sensitivity of gene probe analysis is explored (right side autoradiogram) by sequential dilution of tumour cells. Detection of rearranged bands is possible only above 1% sensitivity.

bone marrow involvement by conventional haematological methods. Gene probe analysis detected a further case, which lacked marrow involvement. Three other cases had marrow infiltration, but no detectable circulating clone. Plate 2.1 reveals an autoradiogram on two B-cell lymphoma cases, and the demonstration of the sensitivity of gene probe analysis.

2.2.2. THE HIGH GRADE B-CELL LYMPHOMAS

(TABLE 2.2)

Of the 15 high grade cases, 9 were centroblastic (CB) lymphoma and included a case with pre-existing features of follicular centroblastic/centrocytic lymphoma (case 19), with secondary transformation occurring in a submandibular node. The remainder of the high grade cases comprised lymphoblastic (LB) lymphoma (4), and one case each of immunoblastic (IMM) and pleomorphic large cell (PLC) lymphomas.

Monotypic surface Ig was expressed in all the tissue samples, and confirmed by clonal Ig rearrangement with the JH probe. A biconal population (case 23) was readily detected by both methods.

A circulating monoclonal was only detected in 2/15 cases by immunophenotyping, with another case demonstrated by gene probe analysis. In contrast to the low grade

cases, this did not necessarily reflect detectable marrow involvement.

Table 2.2 Results on high grade B-cell lymphoma cases

Case	Tissue	Histology +	Ig phenotype	genotype JH	lymphocyte count ($\times 10^9/L$)	Blood				
						% B cells	K: Λ	Monoclonal SIg	Genotype JH	Marrow involvement
14	Skin lump	CB	IgMK	CR ₂	2.23	1.0	<1:<1	-	germline	-
15	Caecum	CB	IgG	CR ₂	2.48	34.0	3:2	-	germline	-
16	Skin lump	CB	IgGK	CR ₂	1.22	5.4	5:2	-	germline	-
17	LN	CB	IgMD	CR ₂	0.83	5.9	5:4	-	germline	-
18	LN	CB	IgMD	CR ₂	0.66	31.5	2:37	IgMD	CR ₂	-
19	LN	CB	IgG K	CR ₂	1.8	15.5	9:6	-	germline	-
20	LN	CB	IgG	CR ₂	1.13	1.3	1:1	-	germline	+
21	LN	CB	IgGK	CR ₂	0.48	4.6	4:3	-	germline	+
22	Stomach	CB	IgMK	CR ₂	1.57	6.0	1:1.5	-	germline	-
23	LN	LB	IgMOK IgMO	CR ₂ (2 clones)	2.15	20.6	13:11	-	CR ₂ -1 clone	-
24	LN	LB	IgMK	CR ₂	80.0	90.0	7:<1	IgMK	CR ₂	+
25	LN	LB	IgGK	CR ₂	1.72	10.5	1:1	-	germline	-
26	LN	LB	IgMD	CR ₂	1.32	26.9	5:4	-	germline	-
27	LN	MM	IgG	CR ₂	0.54	2.8	2:1	-	germline	-
28	LN	PLC	IgGK	CR ₂	0.81	0.8	1:1	-	germline	-

2.2.3 THE T-CELL LYMPHOMA CASES

(TABLE 2.3)

Of the 15 T-cell cases, one was a T-lymphoblastic (T-LB) lymphoma arising in the mediastinum. Thirteen of the remaining 14 cases were peripheral T-cell lymphomas. On histological grounds 8 were low grade and 7 high grade (Suchi et al, 1987). The low grade lymphomas comprised a case of mycosis fungoides (MF), the node showing dermatopathic features in addition to lymphoma involvement; 3 cases of angioimmunoblastic lymphadenopathy (AIL) like lymphoma; 3 cases of T-zone (T-ZN) lymphoma and a case of pleomorphic small cell (PSC) T-lymphoma. The high grade cases were pleomorphic medium/large (PML) cell (2), immunoblastic (IMM) T-cell (3) and large cell (LC-T) T-lymphoma (1).

A case was suspected to be a T-cell lymphoma on initial histological criteria (vascular proliferation, morphology of infiltrate), was phenotyped as a B-cell neoplasm with a florid reactive T-cell proliferation.

Immunophenotyping on tissue samples confirmed T-cell lymphomas in only 4 cases (all high grade). In the remainder the data was either inconclusive, or could be interpreted to offer an alternative diagnosis. Details are given in Table 2.4. Clonal TCR rearrangements were detected in 11/15 cases, and included one case with JH

Table 2.3 Results on T-cell lymphoma cases

Case	Tissue	Histology	Phenotype	Genotype	lymphocyte count ($\times 10^9/L$)	Blood		Clinical Stage	
						% T cells	Phenotype		
29	LN	MF	Inconclusive	CR ₂	1.76	40.7	T cell clone	CR ₂	IIIB
30	LN	AIL	AIL	CR ₂	1.33	23.2	Lymphopenia	CR ₂	IIIB
31	LN	AIL	AIL	germline	0.19	67.2	Lymphopenia	germline	IIIB
32	LN	AIL	?reactive ?AIL	germline	0.83	66.9	Lymphopenia	germline	IIIB
33	LN	T-ZN	?T-lymphoma	CR ₂	2.08	86.6	?CO4 + clone	CR ₂	IIIB
34	LN	T-ZN	?T-NHL	CR ₂	0.88	69.8	Lymphopenia	CR ₂	IA
35	LN	T-ZN	?reactive	CR ₂	2.53	75.3	NAD	CR ₂	IIIB
36	mesenteric LN	PSC	Inconclusive	CR ₂	1.56	38.3	Lymphopenia	not done	IIIB
37	mediastinal LN	T-LB	T.LB	CR ₂	1.38	70.1	Lymphopenia	CR ₂	IIIB
38	LN	PML	?reactive ?AIL	germline	0.63	50.8	Lymphopenia	germline	IIIB
39	LN	PML	B-NHL	germline*	6.20	70.5	High T + NK	germline	IIIA
40	LN	IMM	T-IMM	CR ₄	0.94	35.7	T cell clone	CR ₄	IIIB
41	LN	IMM	large cell NHL(T)	CR ₂	1.33	74.6	Low CD4	CR ₂	IA
42	LN	IMM	large cell NHL(T)	CR ₂	0.20	54.6	Lymphopenia	CR ₂	IVB
43	LN	T-LC	?HD ?T-NHL	CR ₂	0.38	54.7	Lymphopenia	not done	IIIB

* clonal rearrangement shown with JH probe

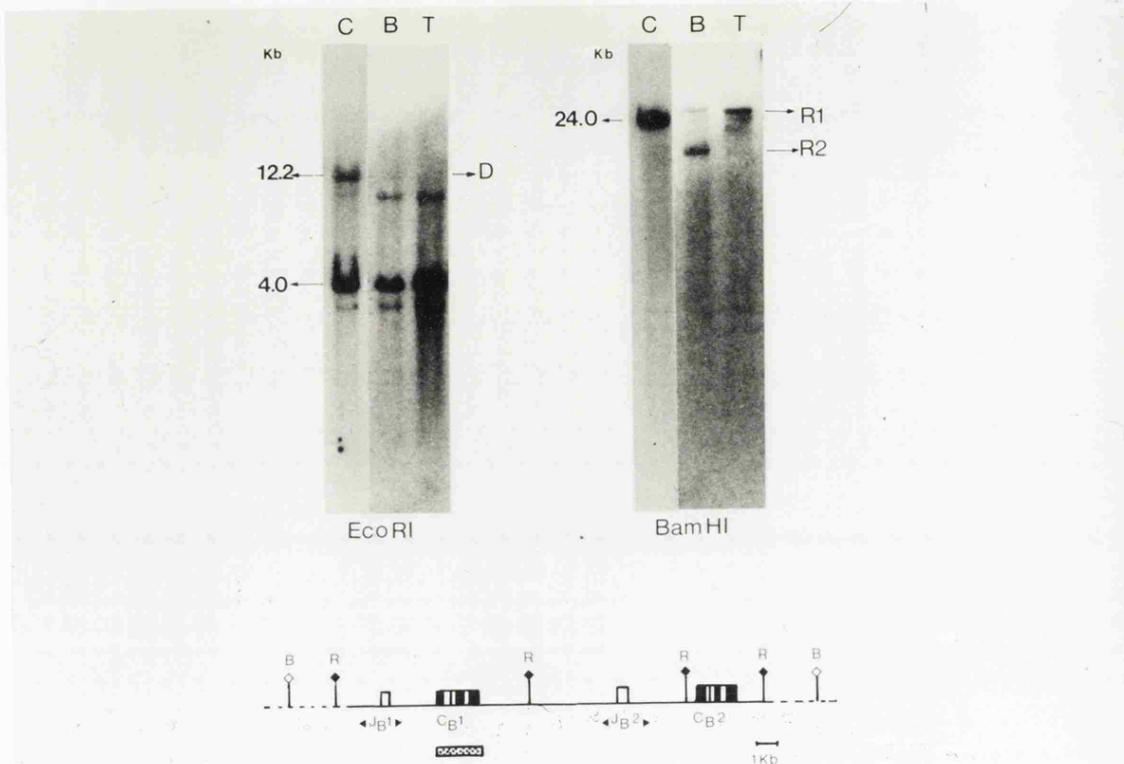
TABLE 2.4 DETAILED IMMUNOPHENOTYPING DATA ON T-CELL CASES

CASE	TISSUE	BLOOD	HISTOLOGY
29	Inconclusive	T-cell clone CD3 - 4 + 8 +	MF
30	ALL Mixed CD3 + 4+ /3+ 8+	T-lymphopenia 'Null' population 40%	ALL
31	ALL Mixed CD3 + 4+ /3+ 8+	T, B, NK lymphopenia	ALL
32	? Reactive ?ALL CD3+4+ predominant	T-lymphopenia (CD +)	ALL
33	?T-cell NHL CD3 + 4+	PCD4: CD8 (11:1) - CD4 + clone suspected	T-ZN
34	?T-cell NHL CD3 + 4+	T, B lymphopenia	T-ZN
35	Reactive? dermatopathic CD3+ 4+ predominant	No abnormality detected	T-ZN
36	Inconclusive	T lymphopenia CD4+ 8+	PSC
37	T-lymphoblastic CD1 + 7+ 5+ 2+ 3+ 4+ 8+	T lymphopenia (mixed)	T-LB
38	?Reactive ?ALL CD3 + 4+ predominant	Lymphopenia (CD4+, B)	PML
39	B-cell NHL CD45R +19+ 20+ IgA +	High T + NK	PML
40	T-immunoblastic CD1 - 7- 5- 3- CD2 + 4+ 8+ DR +	T-clone CD3 - 4+ 8+	IMM
41	Large cell (T) NHL CD1- 7- 5- 4- CD2 + 3+ 8+	Low CD4+	IMM
42	Large cell (T) NHL CD3 + 4+ DR +	Lymphopenia (T, B, NK)	IMM
43	? Hodgkins, ?T-NHL CD3+ 4+ DR +	Lymphopenia (T, NK)	T-LC

but not C-kappa rearrangement (case 35). A B-cell case was excluded by both immunophenotyping and gene probe analysis. Plate 2.2 shows an example of rearrangement seen in the blood and tissue from a T-cell lymphoma case.

A characteristic phenotype suggestive of lymphoma in blood was found in only 3 cases, whereas gene probe analysis detected evidence of monoclonones in 10 cases. The majority (10/15) of suspected T-cell cases were lymphopenic ($0.19-1.38 \times 10^9/l$) at presentation.

Plate 2.2.



An autoradiogram of a T cell lymphoma case with blood (B) and tissue (T) restricted with two restriction endonucleases EcoRI and BamHI as with control tissue (C). The 12.2 kb germline band is deleted in this case (EcoRI) and two rearranged bands R1 and R2 are seen (BamHI). Interestingly, the latter rearranged band is stronger in blood, suggesting heterogeneity of tumour cells in nodal tissue selected.

CHAPTER 2.3: DISCUSSION

Immunophenotyping and more recently gene analysis have had major impacts on lymphoma diagnosis, by demonstrating clonality and ascribing cell lineage (Aisenberg, 1981; Arnold et al, 1983; Bertness et al, 1985; Cleary et al, 1984; O'Connor et al, 1985; Picker et al, 1987). In addition, both methods have been successfully employed in the detection of circulating lymphoma cells (Johnson et al, 1985; Hu et al, 1985; Brada et al, 1987; Smith et al 1984).

This study set out to compare gene probe analysis with conventional immunophenotyping in non-Hodgkin's lymphoma cases, in providing diagnostic clarification and establishing differential sensitivity of circulating lymphoma cell detection.

INTERPRETATION OF SOUTHERN BLOTS

Electrophoresis, blotting and hybridization with cDNA probes revealed characteristic germ line bands. DNA fragments of various sizes result from endonuclease digestion. In DNA from polyclonal populations, fragments of diverse size produce a 'smudge', whereas in monoclonal B or T populations, new bands appear at positions different from those obtained with germ line (placental) DNA. Absence of bands may also indicate

monoclonality due to deletion of a gene sequence at one allele. At least 2-5% of monoclonal cells are required in a population to produce visible and detectable rearranged bands. However, interpretation difficulties are frequent and may be due to technical problems, cross-reactivity between cDNA probe and genomic fragments, or genetic polymorphism. The latter is overcome by using two or more endonucleases in the generation of DNA fragments. Non-specific hybridization can be altered by the stringency of the procedure. It is obviously important that the probe should be of high purity and free of contaminating fragments or sequences.

43 histologically diagnosed cases of non-Hodgkin's lymphoma were entered, and the results analysed separately by cell lineage and histological grade. Overall DNA analysis by Southern blots to study rearrangements of the Ig and TCR genes demonstrated monoclonal populations and assigned B or T cell lineage in 38 cases, confirming the clonal nature of human lymphomas derived from B or T lymphocytes.

Immunophenotyping confirmed diagnosis in only 31 cases, highlighting the lack of clonal markers for T-cells,

able to differentiate neoplastic from reactive hyperplasias.

The value of Ig light chain restriction as a marker of clonality (Aisenberg, 1981; Picker et al 1987) is confirmed by the results of the B-cell lymphoma cases. Except for insufficient tissue in one sample, all cases were phenotyped and assigned B-cell lineage. No additional information was made available by gene probe analysis.

About half (6/13) of the low grade B-cell cases had circulating clones and detectable bone marrow involvement, a well established relationship (McKenna et al 1975; Come et al, 1980). The finding of circulating lymphoma cells in low grade B-cell lymphoma is not surprising, as Jaffe (1983) has called these "benign" tumours of the lymphoid system, retaining some of the recirculation properties of normal lymphocytes. Experience reveals the rarity of eradicating these tumours, and many cases in apparent remission have been shown to have clonally rearranged Ig genes (Hu et al, 1985). Smith et al (1984) found circulating clones in 80% of patients with a normal bone marrow, using light chain restriction and cytofluorimetry. Subsequent studies (Hu et al, 1985; Brada et al, 1987) including

this one have failed to achieve such rates. Patient selection may have been a factor in their results.

Gene probe analysis detected one further clone in blood, which confirms the suspicion that immunophenotyping is limited, when malignant cells are intermixed with residual polytypic cells. Circulating lymphoma cells and bone marrow involvement in the high grade cases were much less frequent. These are considered poor prognostic indicators (Fisher et al 1981). However, the relevance of circulating clone detection, particularly in the low grade lymphomas is doubtful.

A diagnosis of T-cell lymphoma was considered in 15 cases (Table 2.3). Gene probe analysis detected monoclonal T-cell populations in the majority of cases. Three cases of AIL-like lymphoma showed a predominance of CD3+ 4+ and CD3+ 8+ T-subsets, with one case showing rearrangement of the TCR gene, which is in agreement with evidence favouring a T-cell origin of AIL (O'Connor et al, 1986; Weiss et al, 1986) and subsequent development of lymphoma.

The four phenotypically categorised T-cell cases were all high grade (T-lymphoblastic, large cell and immunoblastic) lymphomas, and the diagnosis was

suggested by non-expression of one or several pan-T antigens (Weiss et al, 1985). The most commonly non-expressed antigens in the large cell or immunoblastic lymphomas were CD1, 5 and 7. CD7 non-expression as a T-cell clonal marker of lymphoma has been described (Picker et al, 1987; Weiss et al, 1985). However, non-expression or anomalous expression of T-cell antigens cannot be relied upon as a marker of monoclonality in suspected T-cell neoplasms, unless corroborated by gene probe analysis.

A striking finding in the T-cell cases was the degree of lymphopenia ($0.19-1.33 \times 10^9/l$) in the majority (10/15) of cases. Despite this, gene probing detected monoclonones in 9 out of 13 pre-treatment bloods submitted. The blood samples, however were concentrated by centrifugation to achieve at least 2×10^6 lymphocytes (mononuclear cells), prior to DNA extraction.

Blood phenotype analysis demonstrated two T-cell clones (CD3+ 4+ 8+) and suspected another (CD4+) because of high CD4+ and low CD8+ subsets. A further case (case 29) had 40% "null" cell population, but phenotype data was insufficient to confirm the presence of a T-cell clone. Non-specific abnormalities were found in the other cases. Unlike B-cell malignancies, T-cell

proliferations are heterogenous and poorly categorised, and subject to a highly variable clinical course. Therefore, the establishments of monoclonal proliferations is clinically relevant.

It is generally accepted that Ig rearrangements (in addition to TCR gene) occur in T-cell tumours (Pelicci et al, 1985; Williams et al, 1987), which may cast doubt on lineage specificity of clonal DNA rearrangements. Case 35 in this study had rearrangements of both Ig and TCR genes, but was considered to be a T-cell malignancy on the basis of a germline configuration with c-Kappa probe. Furthermore, precursor T-cells may have germline DNA of TCR beta chain with rearranged T-cell gamma chain genes (Matsuoka et al, 1987) or rearrangement of both may be absent in a subset of peripheral T-cell lymphomas (Weiss et al, 1988).

Nevertheless, gene probe analysis remains a powerful diagnostic tool, particularly in cases of lymphoma of indeterminate cell lineage by immunological phenotype (Asou et al, 1987), and in assigning T-cell lineage to a heterogenous group of conditions, such as Lennert's lymphoma (O'Connor et al, 1986b), angioimmunoblastic lymphadenopathy (O'Connor et al, 1986; Weiss et al 1986), malignant histiocytosis of the intestine

(Isaacson et al, 1985) and mycosis fungoides and dermatopathic lymphadenopathy (Weiss et al, 1985).

In summary, gene probing should only be used in B-cell lymphomas, when histological and immunophenotype studies are inconclusive. However, in the case of T-cell lymphoproliferative disorders, until a specific phenotypic marker of malignancy becomes available, gene probing remains the only definitive investigation of clonal analysis. The demonstration of monoclonality on blood should be attempted despite lymphopenia, in view of the large number with circulating clones detected by gene probing.

CHAPTER 3. IN SITU HYBRIDIZATION.

This new technique was applied to routine bone marrow trephines, to permit identification of light chain mRNA with κ and λ oligonucleotide probes. The aims of the study were firstly to determine the extent to which light chain mRNA could be detected after the process of fixation and decalcification, and secondly to determine light chain (clonal) restriction in equivocal cases of myeloma.

3.1 METHODS.

3.1.1. PRETREATMENT OF GLASSWARE AND SOLUTIONS

Diethylprocarbonate (DEPC) treated and RNase-free solutions were used throughout, as mRNAs are readily degraded by RNases. DEPC was added to the solution (or water) to be treated to a concentration of 0.1% (v/v), thoroughly mixed, left overnight and autoclaved the following day.

The slides and coverslips were cleaned by successive 30 minute washes in 1% lypsol, running tap water and 95% ethanol. After drying, the slides were immersed in 2% aminopropyltriethoxysilane (APES) in acetone for 5 seconds, followed by rinses in acetone (twice) and

ultra pure water (twice). This facilitated adhesion and prevented detachment of the sections during the hybridization procedures. Each of the coverslips was dipped into dimethyldichlorosilane, dried and rinse in ultra pure water.

3.1.2. PREPARATION OF TISSUES.

An outline of in situ hybridization methodology is given in Table 3.1.

For lymph node and tonsil blocks, sections were cut at a thickness of 4 μm ., and mounted on APES coated slides and dried overnight at 37°C.

Bone marrow trephines were fixed in 2% (v/v) formal acetic acid overnight, and decalcified using 5% trichloroacetic acid (TCA) and processed for routine paraffin embedding. Additionally, as an alternative method, trephines were also fixed in 10% formal saline and decalcified with neutral ethylenediaminetriacetic acid (EDTA) pH 7.0. Five micron tissue sections were cut, and mounted on APES coated slides.

Sections were dewaxed by two 5 minute incubations of xylene and hydrated in a graded alcohol series (99% and 95%) into DEPC treated ultra pure water, to prevent degradation by nucleases.

Table 3.1
In situ hybridization methodology

No.	Stage	Methodology
1	Section adhesion	Aminopropyltriethoxysilane coated slides
2	Proteolysis	Proteinase K (0.1-100µg/ml)
3	Probe Preparation	Biotinylation
4	Hybridization conditions:	Final Concentration at (37°C)
	Formamide	50% (v/v)
	Sodium chloride	600 mM
	Sodium pyrophosphate	0.1% (w/v)
	Polyvinyl pyrrolidone	0.2% (w/v)
	Ficoll	5mM
	EDTA	10% (w/v)
	Polyethylene glycol	50mM
	Tris-HCl pH 7.5	
5	Post hybridization washes	2 x SSC/50% Formamide (v/v)
	Standard saline citrate buffer (SSC) and formamide	370
	Tris buffered saline (TBS)	
6	Detection	Streptavidin/biotinylated alkaline phosphatase
7	Visualisation	Fast Red/naphthol/levamisole

3.1.3. PROTEOLYSIS AND POST-FIXATION.

The sections were pre-treated with proteinase K (Boehringer, 161519) at various concentrations (0.1-100 µg/ml.) to reveal the mRNA sequences. The proteinase K (PK) digested protein crosslinks formed during fixation. The concentration of the enzyme required varied with the length of fixation time and site of RNA.

After dewaxing, the slides were incubated in 0.2 N HCl/DEPC at room temperature for 20 minutes, followed by a 10 minute incubation in 2 x SSC/DEPC (standard saline citrate buffer) at 70°C and rinsed twice in DEPC water. The slides were then washed in 0.05 M Tris (pH 7.65)/DEPC prior to addition of 100 µl of PK onto the sections and incubation at 37°C for one hour in a humid chamber.

The slides were then given a 30 second wash in 0.2% glycine/phosphate buffered saline (PBS)/DEPC followed by a rinse in PBS/DEPC and fixation in 0.4% paraformaldehyde/0.1 M PBS/DEPC for 20 minutes at 4°C. After a wash in DEPC water, the slides were transferred to a humid chamber for hybridization.

3.1.4. HYBRIDIZATION.

The hybridization solution was made up to a final concentration of 50% (v/v) formamide, 600 mM NaCl, 1 x PE, 10% (w/v) polyethylene glycol, 150 µg/ml. of denatured salmon sperm DNA and 2 ng./ml. of probe solution.

The sections were first prehybridized in hybridization solution without the probe. 200 µl. of solution was added to each section and incubated at 37°C for 1 hour in a humid chamber. The prehybridization solution was drained away and 50 µl. of hybridization solution containing the probe applied to each section and coverslipped. To control sections was added 50 µl. of solution without the probe. The slides were incubated in a humid chamber at 37°C for 14-16 hours.

3.1.5. PROBE PREPARATION FOR IN SITU HYBRIDIZATION BIOTINYLATED PROBES.

Cocktails of up to 15 single stranded oligonucleotide probes (25-30 mers) were prepared using a DNA synthesizer model 380B (Applied Biosystems, USA) from published sequence data of the lambda and kappa gene conserved regions. For both genes, the coding sequences for the constant region were chosen and part of the 3' non-coding sequence which showed no obvious homologies with other mRNAs.

The oligonucleotides were synthesized using phosphoramidite chemistry, and each oligonucleotide was given an aminoethylphosphate linker at the 5' hydroxyl end enabling biotinylation with biotin-11-deoxyuridyl triphosphate (Pringle et al., 1989).

a) LABELLING REACTION TO 3' END.

The labelling reaction uses terminal deoxynucleotide transferase (tdt) to add homo-polymer tail to the 3' termini of DNA, based on the method of Deng & Wu (1981;1983). The reaction was set up as follows:-

1. DNA (up to 10 µg.) x ul.
2. 10mM cobalt chloride 8.0 (Final concentration 1mM)
3. dUTP-allylamine 10.0 (0.4 mM)
4. 1.2 M sodium 9.2 (140 mM) cocodylate
5. 1M Tris. pH 8.0 2.4 (30 mM)
6. 2mM DTT 4.0 (0.1 mM)
7. tdt 4.0
8. DEPC water up to 35.2 80 µl.

To monitor the probe during purification, 9 µl. of the reaction was withdrawn to which was added 1 µl. of dilute (1:10) ³²P labelled dCTP. Both reactions were allowed to proceed at 37°C for at least 2 hours (2 hours - overnight), followed by termination with 1 µl. of 10% SDS, 2 µl. of 0.25 M EDTA and 1 x TE to a final

column of 100 μ l. The probes were then purified by gel filtration (vide supra) and activity determined.

b) PURIFICATION

The probe was then purified to remove protein introduced from the dUTP allylamine. This required two phenol extractions followed by purification repeated by gel filtration to remove the remaining phenol.

c) CHEMICAL LABELLING OF 5' END WITH CABS REACTION.

This reaction can follow 3' labelling or started de novo with 10 μ g. DNA. 10 μ l. of 1 M sodium borate (pH 8.5) is added to the DNA followed by CABS in a ratio of 1 μ g. for every 1 μ g of DNA. DEPC water is added to make up to a convenient volume (100 μ l.) and the reaction allowed to proceed (2 hours - overnight). The probe was then purified twice by gel filtration and stored at -20°C in 1 μ g. aliquots for future use.

3.1.5.1. RADIO-LABELLED PROBES FOR IN SITU

HYBRIDIZATION

^{35}S labelled DNA probes were prepared as follows:-

1. 100 ng. DNA - denatured +/- restricted with appropriate enzyme.
2. 10 μ l. OLB (dCTP instead of dATP).
3. 1 μ l. bovine serum albumin (20 mg./ml.)

4. 4 μ l. (50 μ Ci) of a 20 mM solution of 35 S-dATP-alpha-S.

5. 1 μ l. Klenow.

6. Sterile pure water up to final volume of 50 μ l.

The reaction was allowed to proceed overnight, and terminated by 50 μ l. solution containing 20 mM NaCl, 20 mM Tris/HCl pH 7.5, 2 mM EDTA, 0.25% (w/v) SDS and 1 μ l. dCTP. The probe activity was determined after gel filtration (vide supra) and used at a concentration of 0.2ng./ml.

3.1.6. POST HYBRIDIZATION WASHES.

After careful removal of the coverslips, the slides were washed in 2 x SSC/50% (v/v) formamide pre-warmed to 37°C, followed by Tris-buffered saline (50 mM Tris/HCl, 0.15 M NaCl, 2 mM magnesium chloride, 0.1% (w/v) bovine serum albumin, pH 7.6), and rinsed in 0.1% Triton X-100 in PBS for blocking non-specific binding of detection complex.

3.1.7. DETECTION SYSTEMS.

3.1.7.1. DETECTION OF THE BIOTINYLATED PROBE.

A four stage application of streptavidin 1:10,000 (Dakopatts K 391A) and biotinylated alkaline phosphatases 1:1,000 (Dakopatts K 391B) was used as described fully by Pringle et al (1989). All reagent solutions were diluted in TBS and the slides incubated

in a humid chamber, sequentially with streptavidin and biotinylated alkaline phosphatase for 30 minutes each followed by a 5 minute wash with TBS after each change.

Following detection, the slides were washed in veronal acetate buffer (VAB) one litre of which contained 3.886 g. of trihydrate sodium acetate, 5.886 g. of sodium barbitone, 5.84g of NaCl and 10.16 g. of magnesium chloride. The pH of the VAB solution was adjusted to 9 with 0.1 N HCl. The solution was stored at 4°C.

The alkaline phosphatase was visualised using a fast red, naphthol capture method. Fast red developing solution was prepared by the addition of 50 mg. of Fast red TR salt (Sigma F-1500), 25 mg of Levamisole (Sigma L-9756) and 50 mg. Naphthol AS BI phosphate sodium salt (Sigma N-2250) prediluted in dimethyl formamide, to 100 ml. of VAB. The slides were incubated in the solution for 1 hour, followed by washing in ultra pure water.

The slides were then counterstained with Mayer's haematoxylin (20 seconds) and Scott's tap water substitute (30 seconds) with ultra pure water rinses in between. Finally, the slides were mounted with Apathy's aqueous mounting medium.

3.1.7.2. DETECTION OF ^{35}S LABELLED PROBES.

Following post hybridization washes, the slides were rinsed in ultra pure water, dehydrated by immersion in 95% alcohol and air dried.

a) PREPARATION OF AUTORADIOGRAPHIC EMULSION.

Two hundred and forty μl . of glycerol was placed in a dipping jar and the volume made up to 12 ml. with ultra pure water. The solution was placed in a waterbath at 43°C and the emulsion (Ilford K5) transferred into a measuring cylinder with plastic forceps, placed in the 43°C waterbath and stirred slowly until molten. 12 ml. of emulsion as added to the glycerol and water in the dipping jar, and allowed to stand at 43°C to disperse any bubbles.

b) APPLICATION OF EMULSION ONTO SLIDES.

The slides were dipped into the emulsion, allowed to drain momentarily and then placed onto a pre-cooled metal plate for 10 minutes. The slides were transferred onto the bench for 1 hour, after which they were placed in a black plastic exposure box and left at room temperature for 4 hours to overnight.

c) DEVELOPING SLIDES.

The slides were transferred onto a staining rack and developed by immersion in Kodak D19 developer for 10 minutes followed by 3 washes in ultra pure water. The slides were then fixed by two 10 minute washes in 30% sodium thiosulphate and washed under running tap water for 20 minutes. Finally the slides were stained with haematoxylin and eosin, dehydrated (95%, 99% alcohol and xylol) and mounted in DPX.

3.1.8. TEST STRIPS.

Test strips were used in order to assess the sensitivity of the detection system for biotinylated probes, and preparation was based on a method described by Leary et al (1983). Test strips were put through the four stage detection system in parallel with the slides for each in situ hybridization experiment, using biotinylated probes.

A diluent was first prepared consisting of 9 parts 20 x SSC and 1 part denatured salmon sperm DNA. A series of probe dilutions were made to give DNA concentrations of 1 ng./ μ l; 100 pg./ μ l; 50 pg./ μ l; 10 pg./ μ l; 1pg./ μ l; 0.1 pg./ μ l and one tube with diluent only. The dilutions were boiled for 10 minutes and 1 μ l. volume of each spotted onto 8 x 1cm. strips of nitrocellulose and allowed to dry before being baked at 80°C between

sheets of 3MM paper for 2 hours. The test strips could be stored indefinitely.

The test strips were rehydrated with blocking solution (to block non-specific protein binding sites on the strips) at room temperature for 1 minute followed by incubation in fresh blocking solution at 42°C for 20 minutes. A litre of blocking solution contained 6.056 g. of 0.05 M Tris, 8.766g. of 0.15 M NaCl, 38g. of bovine serum albumin (Sigma A7906) and 500 µl. of Tween 20 (Sigma P1379), made up with ultra pure water, and the pH adjusted to 7.65 with HCl.

The strips were then blotted between two sheets of Whatman 3MM paper and dried at 80°C for 20 minutes. Finally they were rehydrated by incubation in blocking solution at room temperature for 10 minutes. The probe was then detected using the 4 stage detection technique, except that each reagent was diluted in blocking solution and the strips washed in blocking solution for 5 minutes between each stage. The strips were then developed as per slides.

3.2. RESULTS OF SITU HYBRIDIZATION EXPERIMENTS ON SUSPECTED MYELOMA CASES.

Plasma cells in the marrow of suspected myeloma cases ranged from 4 to 99% based on bone marrow aspirates, trephines and immunocytochemistry (Tables 3.2 and 3.3).

3.2.1. PRESERVATION OF LIGHT CHAIN MRNA IN PLASMA CELLS

After acid fixation and decalcification, mRNA was poorly preserved with weaker signals than EDTA decalcified tissues which produced consistent and reproducible mRNA signals. Cell morphology was also better preserved with EDTA.

Proteinase K (PK) concentration affected the strength of mRNA signals. At lower concentrations (0.1-1.0 µg/ml). the sensitivity of the signal was reduced but the pattern of staining was retained. At higher concentrations (10-100 mg/ml), there was a significant decrease in hybridization signals, deterioration in preservation of morphology due to digestion of tissue sections, and non-specific signals in other cells (e.g. neutrophils).

Table 3.2 Results of EDTA decalcified trephines in suspected myeloma cases.

Case	Bone Marrow	% Plasm a Cells	Paraprotein (g/l)	B ₂ P *	Immuno- suppression	Cytoplasmic mRNA signals in plasma cells	Final Diagnosis
1	Diffuse plasmacytosis	99	IgG K (125)	+	+	Exclusively K	Definite myeloma
2	Diffuse plasmacytosis	67	-	+	+	Almost exclusively λ	Non-secretory myeloma
3	Diffuse plasmacytosis	58	IgG λ (34.5)	+	+	Almost exclusively λ	Definite myeloma
4	Lymphoplasmacytoid + plasma cells	20	IgG λ (61.7)	+	+	Predominantly λ	Definite myeloma
5	Non-diagnostic	8	IgA λ (16.8)	-	-	Predominantly λ	Definite myeloma
6	Non-diagnostic	6	--	+	-	Predominantly K	Ideopathic B ₂ P*
7	Non-diagnostic	5	IgG λ (19.8)	-	+ border line	Both K and λ	Monoclonal gammopathy
8	Non-diagnostic	4	IgG λ (11.7)	-	-	Predominantly λ	Equivocal myeloma

* Bence-Jones Proteiuria

Table 3.3 Results of EDTA decalcified trephines in suspected myeloma cases.

Case	Bone Marrow	% Plasma Cells	Paraprotein (g/l)	BJP *	Immuno-suppression	Cytoplasmic mRNA signals in plasma cells	Final Diagnosis
9	Diffuse plasmacytosis	70	IgG λ (110)	+	+	Exclusively λ	Definite myeloma
10	Diffuse plasmacytosis	30	IgG λ (7.3)	+	+	Predominantly λ	Definite myeloma
11	Lymphoplasmacytoid + plasma cells	22	IgM K (61.6)	+	+	Predominantly K	Definite myeloma
12	Focal plasmacytoma	19	IgG K (22.1)	-	+	Predominantly K	Definite myeloma
13	Non-diagnostic	6	IgA K (8.4)			Both K and λ	Benign monoclonal gammopathy
14	Focal lymphocytosis	4	IgM K (3.8)	-	-	Predominantly K	Nodular non-Hodgkin's lymphoma.

The optimal PK concentration for tonsil was found to be 5 µg/ml (Plate 3.1). The optimal conditions for trephines were EDTA decalcification and PK concentrations between 5 and 10 µg/ml (Plates 3.2 and 3.3).

3.2.2. PLASMA CELLS IN MYELOMA CASES

(Plates 3.2-3.4)

Strong cytoplasmic signals were present in plasma cells, either with kappa or lambda probes, comparable to tonsillar controls. The signals were of uniform high intensity overlying areas corresponding to endoplasmic reticulum. In many plasma cells the Golgi zones were noted to have been spared (Plate 3.3) in contrast to immunocytochemistry, indicating differential sites of mRNA and protein localisation. Clonal populations of plasma cells tended to form into aggregates, in contrast to a more diffuse pattern in reactive states, and were easily delineated by light chain restriction. Plasma cells in non-myeloma trephines showed hybridization signals with both kappa or lambda probes with no overall predominance.

3.2.3. SPECIFICITY OF OBSERVED HYBRIDIZATION SIGNAL.

Specificity of hybridization signals were confirmed by the control results. In all cases, control sections incubated with hybridization solution lacking probe,

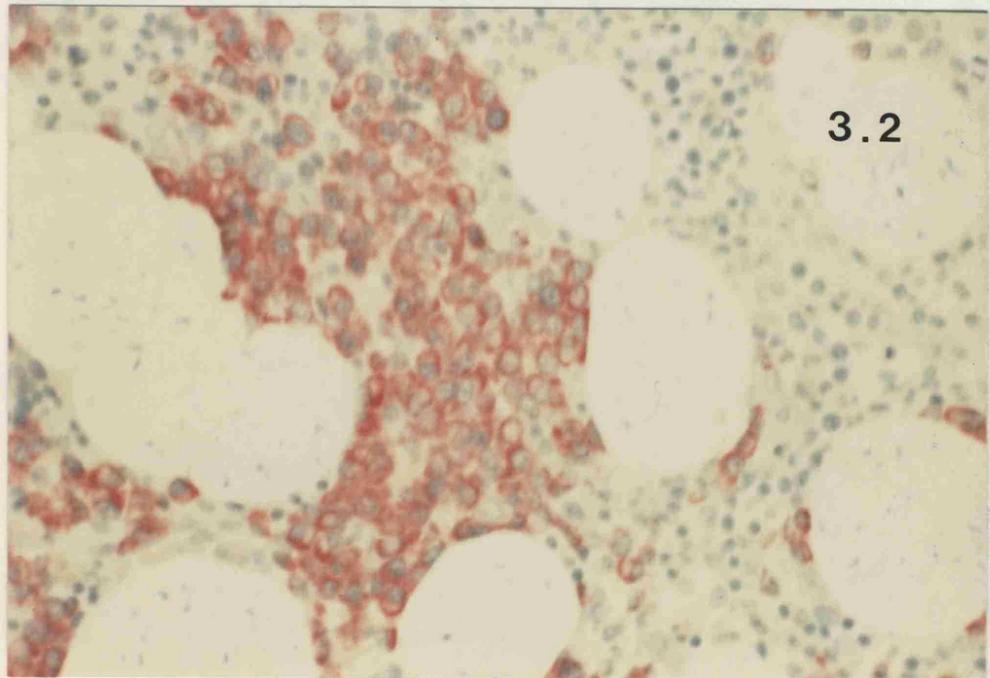
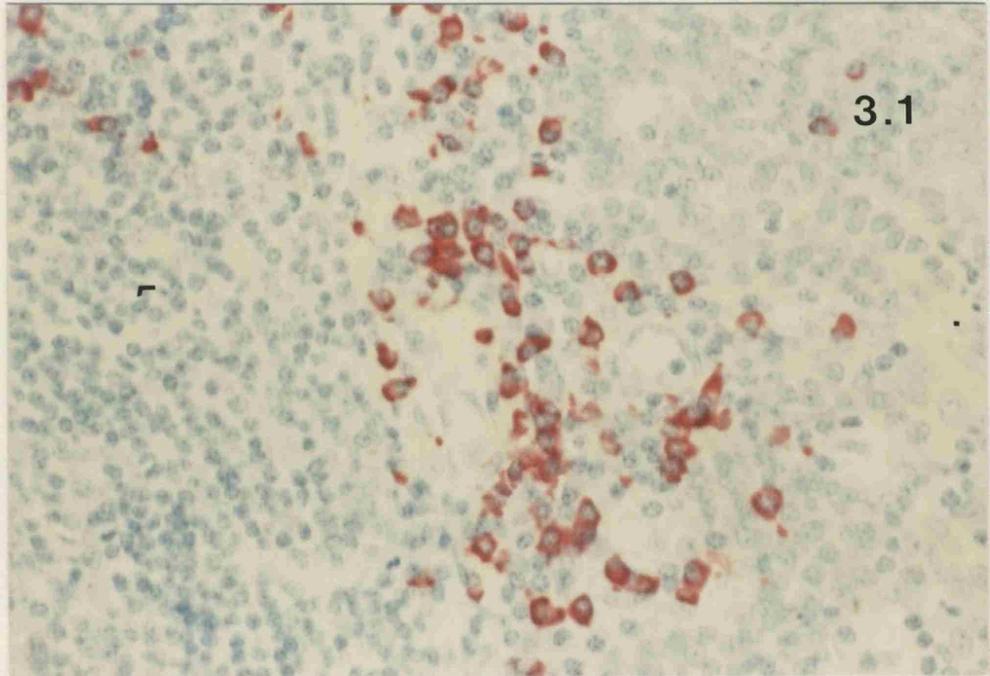
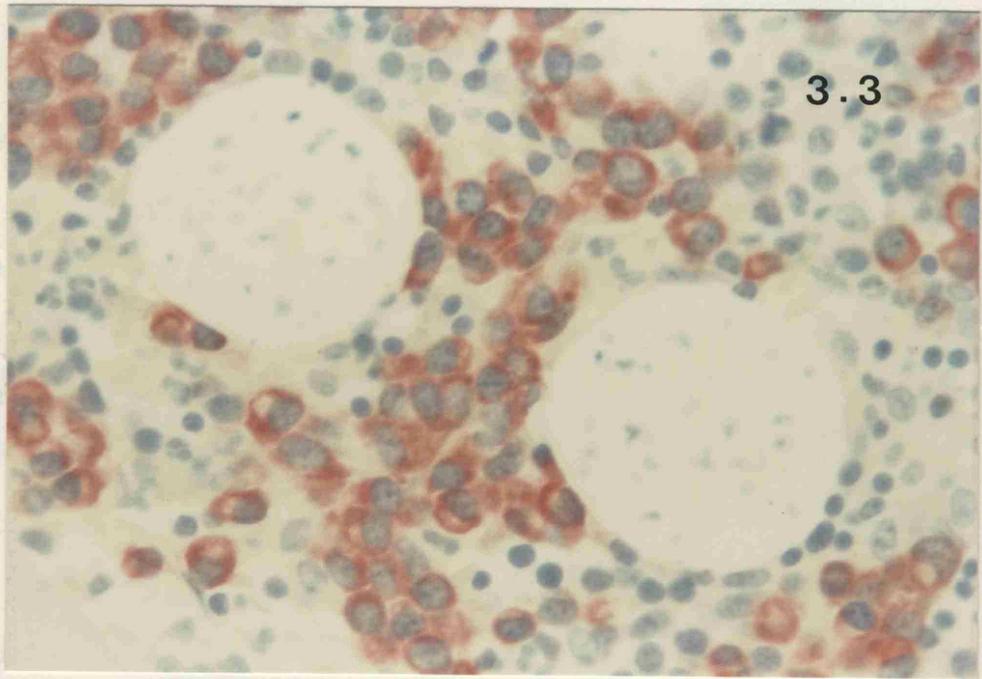
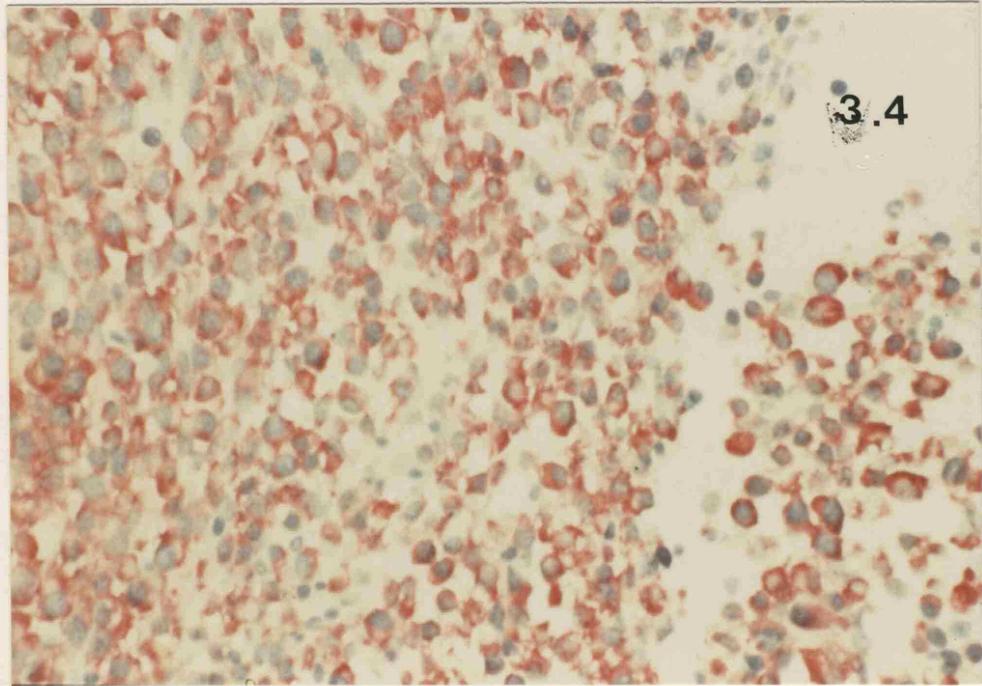


Plate 3.1-3.4 Light chain mRNA in plasma cells.

- 3.1 In situ hybridization to kappa mRNA in normal tonsil PK 5 μ g/ml.
- 3.2 In situ hybridization to kappa mRNA in bone marrow plasma cells in a case of IgG kappa myeloma (low power) EDTA trephine/PK 10 μ g/ml.



3.3



3.4

Plate 3.1-3.4 Light chain mRNA in plasma cells.

3.3 Higher power of case in figure 2, showing strong cytoplasmic signals and good preservation of morphology.

3.4 Case of IgG lambda myeloma showing sheet of plasma cells, with strong cytoplasmic signals.

showed complete absence of signal in plasma cells. Furthermore, hybridization signals were completely eliminated in sections pretreated with RNase A, confirming that the positive signals were due to specific polyadenylated RNA (mRNA) sequences.

The specificity of the observed hybridization signal for cellular, kappa or lambda mRNA sequences in myeloma cases was subsequently confirmed by serum immunoglobulin and urinary light chain analyses. Results also correlated with immunocytochemistry.

3.3 DISCUSSION.

In situ hybridization has proved an invaluable tool in the localisation of specific nucleic acid sequences in tissue sections. This technique is now suitable for clinical laboratory usage, due to the development of non-radioactive nucleic acid probes and detection systems. The most successful and useful of the current methods is when biotin is incorporated into the probe and subsequently detected by enzyme conjugated avidin (Leary et al, 1983). A number of protocols have been published using biotinylated DNA probes on cryostat or routine formalin fixed paraffin sections (Burns et al, 1985; Beckman et al, 1985; Unger et al, 1986; Pringle et al, 1987).

An oligonucleotide probe of polydeoxythymidine has been used to detect polyadenylated mRNA in paraffin sections of lymphoid tissue and the results have indicated good preservation of mRNA in routine fixed tissues (West et al, 1988; Pringle et al, 1989a). A method for the detection of immunoglobulin light chain mRNA in formalin fixed paraffin sections has recently been developed (Ruprai et al, 1988; Close et al, 1989; Pringle et al, 1989b).

In this study, this methodology was extended to routine bone marrow trephines. Acid fixation and decalcification resulted in degradation of RNA, therefore an alternative method of decalcification (EDTA) was examined and found to preserve RNA and improve detection. The sensitivity and specificity was also affected by proteinase K concentration, too much resulting in detection of endogenous biotin in cells, due to over digestion.

Under optimal conditions, in situ hybridization provided a direct molecular demonstration of a myeloma clone, in terms of light chain mRNA expression. It provided unequivocal evidence of a clonal proliferation, even in cases where plasma cells were only marginally raised. The current in situ methodology detected light chain mRNA in plasma cells, and the intensity of signal may provide an indication of relative mRNA copy number (Lawrence and Singer, 1985).

In situ hybridization has several distinct advantages over other methods. The hybridization procedure is specific, because recombinant DNA techniques have enabled the synthesis of pure and highly specific probes. The biotinylated probes can be prepared quickly and stored for long periods due to their

chemical stability. Many cases and slides can be processed with different probes at the same time. In situ hybridization can be applied to both fixed and fresh tissue, and the resolution of this technique is high, enabling precise nuclear or cytoplasmic location in cells. Cell morphology is well preserved for identification and histological location. Finally, the procedure is safe (non-radiolabelled probes) and simple to perform, in any immunocytochemical laboratory.

In situ hybridization has particular advantages in myeloma and lymphoplasmacytoid malignancies. Being free of problems associated with detection of extracellular target and its uptake by other cells, it is easier to interpret than immunohistochemistry for immunoglobulin. Additionally, this technique will type plasma cell neoplasma (undetected by routine immunocytochemistry) in post-transcriptional block or non-secretory myeloma, and is a more direct method than flow cytometric analysis of cytoplasmic immunoglobulin (Barlogie et al, 1984).

Furthermore, equivocal or indolent cases of myeloma, such as smouldering multiple myeloma (Kyle and Greipp, 1980), light chain only secretion (Kyle and Greipp, 1982) and biconal paraproteinaemia of undetermined significance (Kyle et al, 1981) can be more fully

characterised. Also poorly defined bone marrow infiltrates of lymphoplasmacytoid cells can be full typed.

In summary, in situ hybridization using biotinylated probes on routine bone marrow trephines is useful for the study of myeloma and related disorders, particularly in equivocal and non-secretory cases. The sensitivity and specificity of this technique will allow an objective assessment of disease response following cytoreductive therapy and bone marrow transplantation using standard trephine biopsies. It is a rapid (within 48 hrs), sensitive and specific method for demonstration of light chain mRNA expression in plasma cell malignancies, and should be useful for both routine and research purposes. However, it has a limited role in the vast majority of lymphoid malignancies where immunoglobulin synthesis and expression is uncertain and uncommon.

CHAPTER 4. POLYMERASE CHAIN REACTION.

The polymerase chain reaction (PCR) is a rapid method for in vitro amplification of specific nucleic acid sequences (Erlich et al 1988; Saiki et al, 1988). Basically synthetic oligonucleotides which flank the sequence of interest are used as DNA primers for the enzymatic replication of the defined DNA sequences. Repeated cycles of heat denaturation, primer annealing and enzymatic sequence extension (amplification), lead to the exponential accumulation of amplified product. The technique is therefore able to produce large amounts of product from small (DNA) samples. However, the sensitivity and specificity of PCR is dependent upon a number of variables, which require standardisation for each set of primers and source of template DNA.

4.1 PCR METHODOLOGY

4.1.1 STANDARD PCR PROCEDURE

The method used was based on published protocols (Saiki et al, 1985; Erhlich et al 1988; Saiki et al 1988). The reaction was performed using the heat stable enzyme, *Thermus aquaticus* (Taq) DNA polymerase (Perkin-Elmer Cetus and other sources), and by following the instructions supplied with the enzyme. All reagents

were prepared in sterile distilled water, and autoclaved tubes and pipette tips were used throughout.

DNA Samples

The DNA was extracted from fresh frozen tissue, fixed and paraffin embedded material or cell suspensions using standard methods (Pettersen & Sambrook, 1973). In addition, control DNA was extracted from tonsil and placental tissue. The amplification procedure was coupled with Southern blotting (Mullis & Faloona, 1987; Saiki et al 1985), or by direct inspection of the amplified DNA segments after electrophoretic separation and staining with ethidium bromide.

Standard Reaction mixture

The reactions were carried out in a total volume of 100 μ l, in sterile 1.5ml microcentrifuge (eppendorf) tubes. To each DNA sample was added an appropriate amount of amplification buffer and nucleotides to give a final concentration of 67mM Tris/HCl (pH8.8); 17mM ammonium sulphate; 3mM MgCl₂, 10mM β -merceptoethanol, 5 μ m EDTA, 22 μ m each nucleotide, 170 μ g/ml BSA and 1.0 μ m of each oligonucleotide primer. Two and a half units of Taq polymerase per tube were also included, and reactions were overlaid with 100 μ l of light mineral oil (Sigma 400-5) to prevent evaporation. Negative control reactions were performed with each batch of

experiments, replacing sample DNA with distilled water.

Amplification

The polymerase chain reaction was performed using an automatic temperature cycler (Cambio IHB, Cambridge). The cycling procedure was begun with a denaturation step, followed by annealing and primer extension. At the end of the 25-30 cycles, the final denaturation step was omitted and the extension step prolonged by 7 minutes. Following termination, the reactions were allowed to cool down to room temperature, and stored at -20°C .

Gel Analysis

The amplified DNA was visualised as bands under U.V. illumination after ethidium bromide staining, and the expected sizes confirmed with a known molecular weight length standard run concurrently on the gel. Final confirmation required DNA transference onto nylon filters (Southern blotting) and probing with a specific radiolabelled oligonucleotide.

Cetus Reaction Buffer

DNA amplification was also performed using the Cetus buffer provided, in conjunction with their Taq polymerase. The final concentrations in the reaction mixture were 10mM Tris/HCl (pH8.8), 50mM KCl, 1.5mM

MgCl₂, 0.01% (w/v) gelatin, 1.25mM each nucleotide and 0.5µm of each oligonucleotide primer.

4.1.2. ESTABLISHING OPTIMUM PCR CONDITIONS

a) TEMPLATE DNA.

Amplification was dependent on the source of template DNA used. It was easier to demonstrate amplification on chromosomal or plasmid DNA than human genomic DNA. Sample handling was also of relevance. DNA extracted from fresh frozen tissue or blood provided better template DNA for amplification in comparison to DNA extracted from fixed and paraffin embedded tissue. PCR was demonstrated on fixed tissue independent of the length of time of fixation, as long as high molecular DNA could be extracted.

b) CHOICE OF PRIMERS.

PCR was highly dependent upon the choice of primers, their purity and length. The length of sequence to be amplified was also important. Generally complementary primers over 20 bases long and guanine and cytosine (GC) rich were highly specific. To improve purity, the solution with the primers was ethanol precipitated and resuspend in 1 x TE prior to use. Selection of primer sequences from genomic DNA was a critical step for amplification to be successful. Primer concentrations became critical for amplifications over 30 cycles,

requiring 1 nmol of each primer rather than only 100 pmols.

c). PRIMING TEMPERATURE.

Initially PCR was performed manually using three pre-set water baths, and transferring the tubes by hand after set times. However, there was no indication that the solution had reached the required temperature for the allotted time. Amplification, not surprisingly, was unpredictable. More reproducible amplification was produced using an automatic temperature cycling machine (Cambio intelligent heating block), but the machines calibration had to be checked using a thermocouple and a mercury thermometer. Even with the automated temperature cycling matching, discrepant temperature were noted in the peripheral wells. The probability of reaching required temperature was increased by ensuring close (wax) contact between the sides of the well and the microcentrifuge tube.

The priming temperature was also dependent upon the length of primers. For oligonucleotides of 25bp (25-mers) or greater, a higher temperature of 65°C was used, whereas for shorter primers, a lower temperature of 55°C or 45°C was used. In addition, the higher the annealing temperature, the more specific the amplification.

The denaturation step temperature was fairly standard at 94-95°C. The primer extension step temperature was also found to be optimal around 70°C. The time for primer extension was increased for longer sequence amplifications and at the latter part of the cycles.

d). TAQ POLYMERASE ENZYME.

The quality and source of the enzyme was consistently found to be a critical factor. Enzymes were purchased from many sources, and different batches and there was much variability. Taq polymerase from Perkin-Elmer-Cetus generally produced more reproducible amplifications than alternative (usually cheaper) sources.

e). AMPLIFICATION BUFFER.

The pH of the buffer was another critical factor for successful amplification. It was found that an alkaline pH 8.1 to 8.9 facilitated amplification.

The magnesium concentration was also found to be critical. A low concentration produced better results. By a series of titration experiments, a final concentration of 1-3mM in the buffer was found to be optimal.

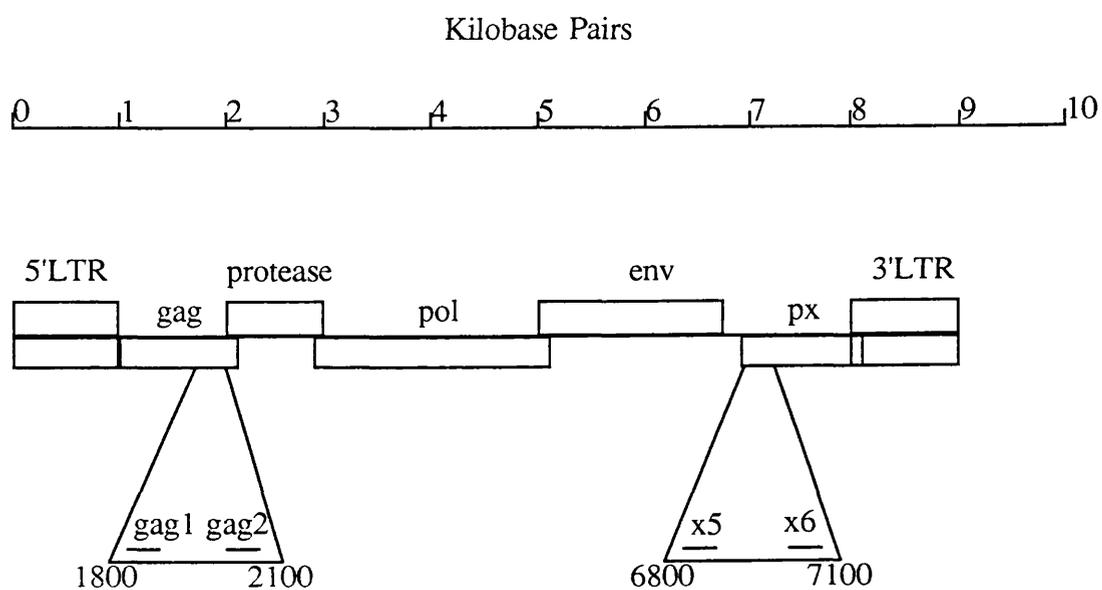
4.1.3. TEST AMPLIFICATION ON HTLV-1 TEMPLATE DNA.

The efficiency and sensitivity of PCR amplification was initially established using HTLV-I viral genome, cloned in the SST-I site of plasmid SP64 (Courtesy of the Institute of Cancer Research, London). For HTLV-I detection, primers were selected to specifically amplify intervening conserved regions among viral isolates. (Figure 4.1 for schematic diagram of HTLV-I).

To determine the sensitivity of detection, SST-I restricted cloned DNA containing the HTLV-I genomic sequences were serially diluted from 1µg to 2pg, and amplified with the HTLV-I pol and gag gene-specific primers, and analysed by Southern blotting. Amplified DNA was visualised as bands on agarose gel, after electrophoretic separation and staining with ethidium bromide. The length of the amplified fragments were compared with values predicted by sequence data. Specificity of amplification was confirmed by Southern blotting and hybridization to a specific radiolabelled oligonucleotide probe.

The details of the primers, and amplified fragments are shown in Table 4.1.

Figure 4.1 Schematic diagram of HTLV-I and sites of primers pairs used for PCR amplification.



The two primers gag1 and gag2 are homologous to nucleotides 1818 to 1840 and 2031 to 2053 respectively of the HTLV-I gag gene. The two primers x5 and x6 are homologous to nucleotides 6867 to 6890 and 7067 to 7089 respectively, of the pX region of HTLV-I virus.

Table 4.1

PCR on HTLV-I DNA using primers from the GAG and pX region of HTLV-I. Details of the sequence and amplified products are shown.

	GAG region	pX region
Primer 1	5'-GTC AGA CCT GGA CCC CCA AAG AC (1818-1840 bp; gag1)	5' CCT TCT CAG CCC CTT GTC TCC ACT (6867-6890 bp; x5)
Primer 2	CAG AGC CAG AGG AAG ATG CCC TC-3' (2031-2053 bp; gag2)	CGC TGC CGA TCA CGA TGC CTT TC-3' (7067-7089 bp; x 6)
Amplified Products	236bp	223bp
PCR temperature	denaturation 95°C x 1 minute annealing 55°C x 5 minutes primer extension 70° x 2 minutes	
Gel	4% low melt temperature ("Nuseive") electrophoresis 35V potential difference for 16 hours in TAE	
Visualisation	Gel stained in 0.2µg/ml ethidium bromide for 30 minutes, visualised under U.V. light	

4.1.4 PCR WITH β -GLOBIN PRIMERS

Using the standard PCR procedures described above, 1 μ g of DNA (placental, tonsil and unselected human genomic DNA), both unrestricted and restricted with EcoR-I and BamH-I was amplified, with commercially available β -globin gene primers. The primers were used crude and in a purified form by ethanol precipitation. The amplified product was predicted to be approximately 300bp long, and was visualised under U.V. light on a low melt temperature gel ("Nuseive") after staining with ethidium bromide (0.2 μ g.ml for 30 minutes. PCR with β -globin primers was used to establish the efficiency and sensitivity of the amplification procedure.

4.1.5. PCR WITH IMMUNOGLOBULIN KAPPA LIGHT CHAIN PRIMERS

The diagram in Figure 4.2 shows some of the structural properties of the human immunoglobulin protein. The mature of a pre-B cell to an immunoglobulin-bearing B cell requires an effective recombination of a light chain variable (VL) and joining (JL) gene segment (Hozami & Tonegawa, 1976). The kappa gene rearrangement precedes lambda (Hieter et al, 1981; Korsmeyer et al, 1981) and lambda gene integration only occurs after non-functional rearrangement of both kappa alleles. Hence demonstration of Ig kappa chain

Figure 4.2

Immunoglobulin is a four-chain structure made up of two heavy (H) chains and two (L) chains. Each H chain and each L chain consists of a variable (VH, VL) and constant part (CL, CH).

Amino acid sequences in the N-terminal half of the L and H chains (VL, VH) are highly variable (left side). Within the V region, a few amino acids are conserved, showing little or no variability (light areas), contrasting with hypervariable or complementary determining regions (CDR). Amino acids in the CDR are involved in the formation of the antigen-binding site.

The genes responsible for the biosynthesis of the VL chains (right side of figure) consist of an effect^{ive} recombination of a light chain variable (VL), a joining (JL) with a constant (CL) gene segment. Similarly for the VH chain, with the addition of a diversity (D) gene segment.

Figure 4.3

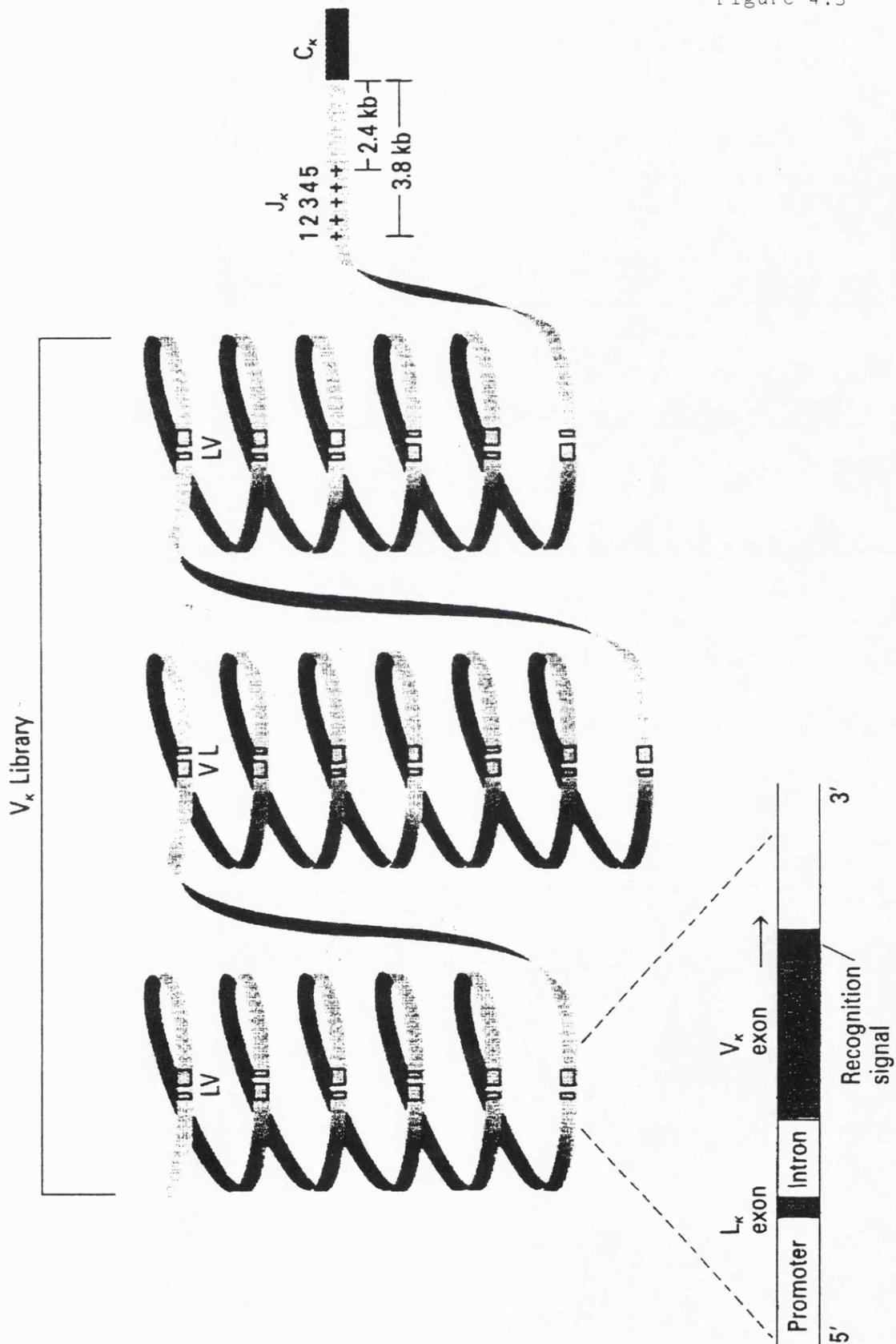


Figure 4.3

Organisation of the immunoglobulin kappa gene representing several hundred V_k , five J_k , and a C_k gene. Each V_k gene is preceded by a promoter and a leader (L_k) sequence. The leader sequence itself is not encoded by a continuous stretch of DNA, but is split by an intron.

rearrangement by PCR would be a sensitive marker of B cell clonality and lineage specificity.

The arrangement of the immunoglobulin kappa gene (Figure 4.3) consists of several hundred variable (V_k), five joining (J_k) and a constant (C_k) gene. To demonstrate V_k and J_k recombination by PCR, one would need to constrict primers to the conserved regions of each of the V_k genes and amplify with a primer judiciously placed at the 3' end of the J_{k5} gene (Figure 4.4). On rearrangement, these primers would then be sufficiently close to amplify the intervening segment. The size of the amplified fragment would depend upon which J_k gene was recombined with the V_k gene (Figure 4.5). Therefore in a polyclonal B cell population all five J_k genes would be rearranged and five separate amplified products would be seen on a gel, whereas in a monoclonal B-cell population, a single product would be predominant (Figure 4.6).

Synthesis of kappa light chain primers

A schematic representation of the sites of various primers synthesized, and the theoretical sizes of the amplified products using various pairs of primers is shown in Figures 4.7 (a and b). The V region primer was homologous to a conserved region of the V kappa gene but required a degeneracy of 64 for successful

hybridization (Figure 4.8). The sequences of the various other primers is tabulated (Table 4.2). The upper strand primers (V_{κ} , JK-1) were used in various combinations with the lower strand primers (J_{κ} , C_{κ} etc). These oligonucleotide primers were prepared using a DNA synthesizer model 380B (Applied Biosystems, USA) from published sequence data of the immunoglobulin kappa chain (Courtesy of University of Leicester).

Figure 4.4

KAPPA GENE AMPLIFICATION

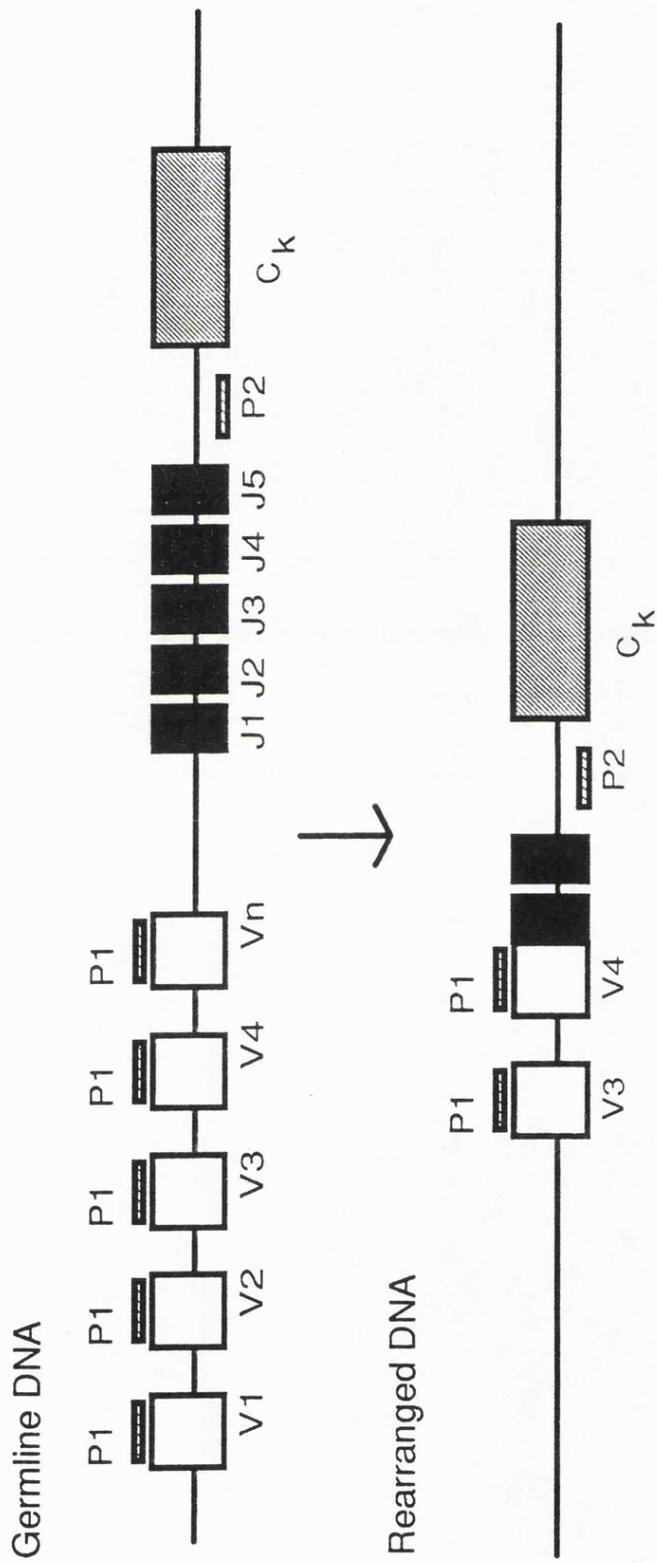


Figure 4.4

Organisation of the germline immunoglobulin kappa gene and on rearrangement. PCR amplification would be possible by selecting judicious hybridization sites in the V_k region (P1) and demonstration from the J5 gene (P2). These primers would only amplify when in close proximity, that is after V-J recombination had taken place in the rearranged DNA.

Kappa light chain gene amplification

Germline



J1 Rearranged



J2 Rearranged



J3 Rearranged



J4 Rearranged



J5 Rearranged



Figure 4.5

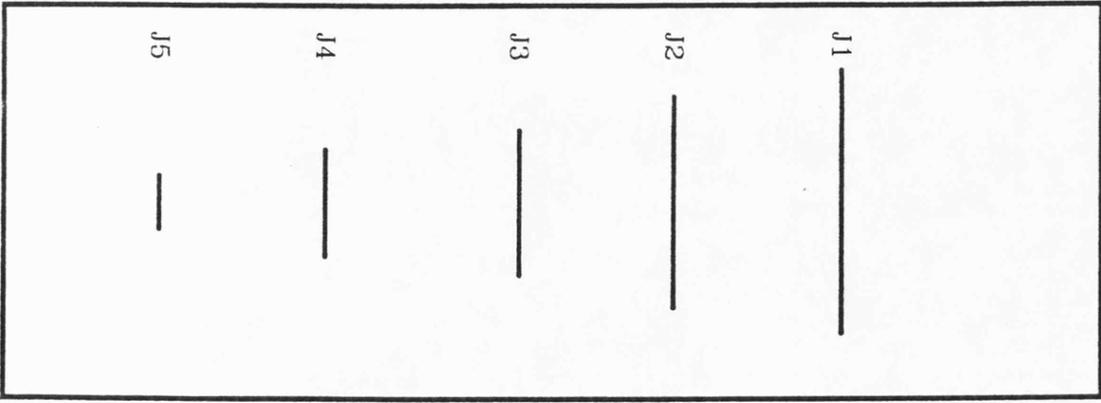


Figure 4.5

PCR amplification and product size using the kappa light chain gene, depends upon which joining (J1-J5) gene is rearranged. J1-J5 recombination are represented (left side) and related schematically to amplified PCR products visualised on a gel after electrophoresis (right side) in decreasing order of size. All five products would be seen in the DNA from polyclonal B cells.

Figure 4.6

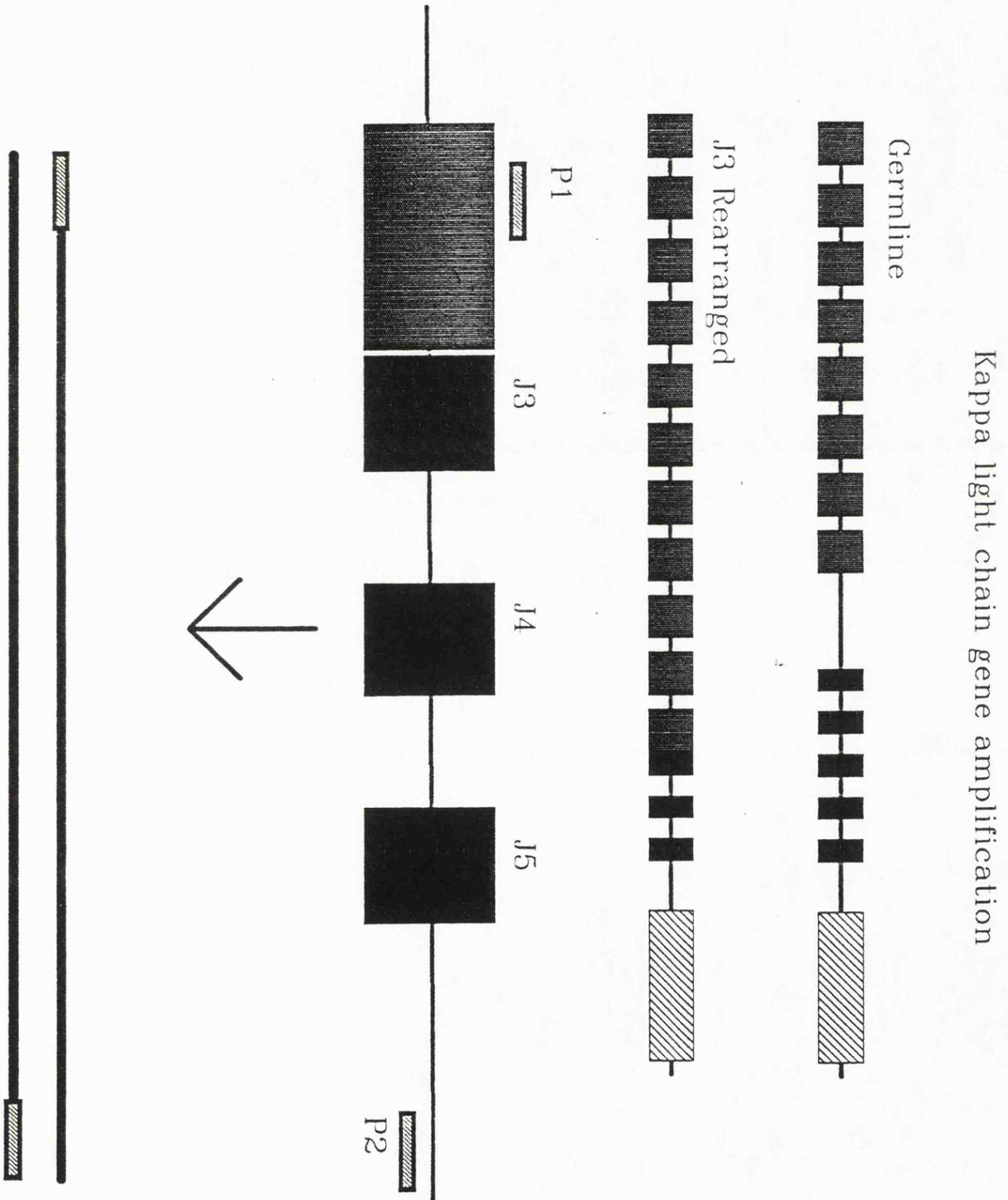


Figure 4.6

Schematic representation of kappa light chain gene amplification using PCR and DNA from a monoclonal B cell population. The product size can be related to the particular joining-variable gene recombination.

Figure 4.7

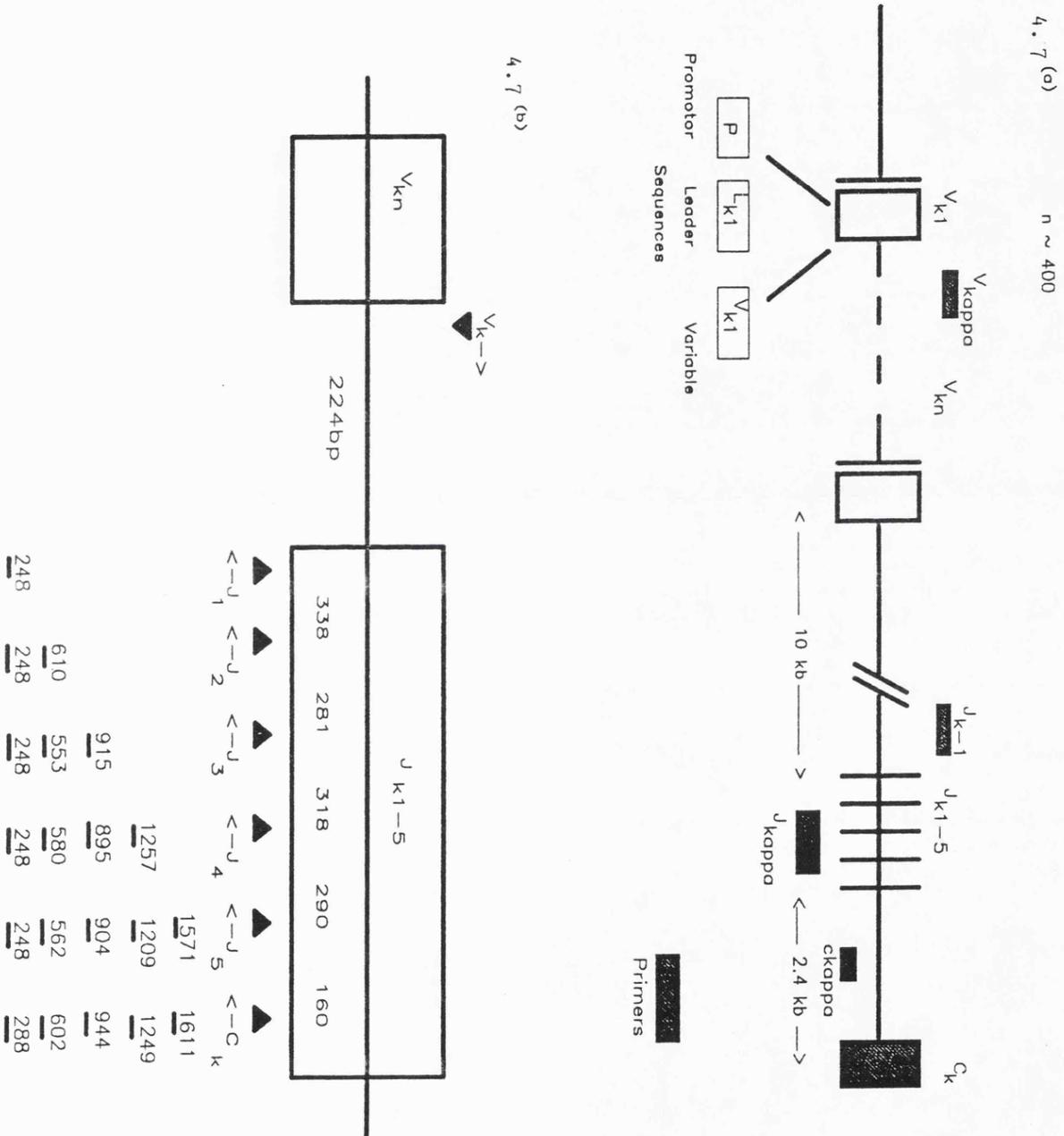


Figure 4.7

(a) Schematic representation of the organisation of the immunoglobulin kappa gene and sites of primers synthesized.

V_k - Kappa variable region genes
J_k - Kappa joining region genes
C_k - Kappa constant region gene
kb - Kilobases.

Figure 4.7

(b) Approximate sizes of amplified DNA is shown using the v_{kappa} (upper strand and J_{kappa} and C_{kappa} primers

Figure 4.8

V REGION PRIMER

5' TTA/G A/G^C/A^T/c TGG TAT/c CAG CAG AAA/gCC 3'
LEU ASP ASN TRP TYR GLN GLN LYS PRO

Figure 4.8

The V kappa region primer 24 bases long with a degeneracy of 64.

Table 4.2

Various immunoglobulin kappa gene primers to the joining and constant genes. Letters and numbers of primers and their site of hybridization are as shown previous (Figure 4.7).

Name	Sequence lower strand primers	Name	Sequence Upper strand primers
Jkappa (24 bp)	ACG TTT GAT CTC CAc/g c/TTT GGT CCC -3'	JK-1 (25bp)	5'- TCT GTT CAG CAA GAC AAT GGA GAG C
CKappa (24 bp)	GCC ATC GGA CCG AAA TTT CGG ACA -3'		
CK-1 (25 bp)	GGT CCT TCC GAA ATG CAA GGG TTC C-3'		
CK-2 (25 bp)	ACT TTG TGT CAT TCA GTT TCA GAG C-3'		
J-Kappa 1-4 (21 bp)	ACC AAa/g/c TG GAG ATC AAA CGT-3'	JKappa 1-4 (20 bp)	5'-ACG TTT GAT a/cTC CAc/g CTT GG
J Kappa 5 (21 bp)	ACA CGA CTG GAG ATT AAA CTG-3'	J Kappa 5 (20 bp)	5'- AGG TTT AAT CTC CAG TCG TG-

4.2. RESULTS OF AMPLIFICATION EXPERIMENTS

The initial PCR experiments determined the efficiency, sensitivity and reproducibility of amplification using HTLV-I viral genome, cloned in the SST-I site of plasmid SP64 (Courtesy of Institute of Cancer Research, London). The primers were judiciously selected to specifically amplify conserved regions among viral isolates. Similar experiments were performed with β -globin primers and human DNA.

Latter experiments concerned demonstration of clonality in B-cell lymphoproliferative conditions, using primers directed at the immunoglobulin kappa light chain.

The chances of successful amplification was significantly improved by switching from a manual method (3 water-baths at set temperatures) to using an automatic temperature cycling machine (Cambio IHB, Cambridge).

4.2.1. PRELIMINARY PCR EXPERIMENTS

PCR amplification of viral HTLV-I DNA was readily obtained using primers directed against the gag and pX gene regions (Plate 4.1). An amplified band at 230bp was consistent with predictions based on HTLV-I sequence data (Figure 4.1). The sensitivity of PCR amplification was tested by serial dilutions of

template (HTLV-I) DNA from 1 μ g to only 1pg (Plate 4.2).

Occasionally additional bands and other inconsistencies of amplification were investigated by varying reaction mixture (Plate 4.3). Optimal conditions for retroviral amplification were found to be an alkaline pH (8.8) and a low magnesium concentration (3mM) in the reaction mixture.

ØX174

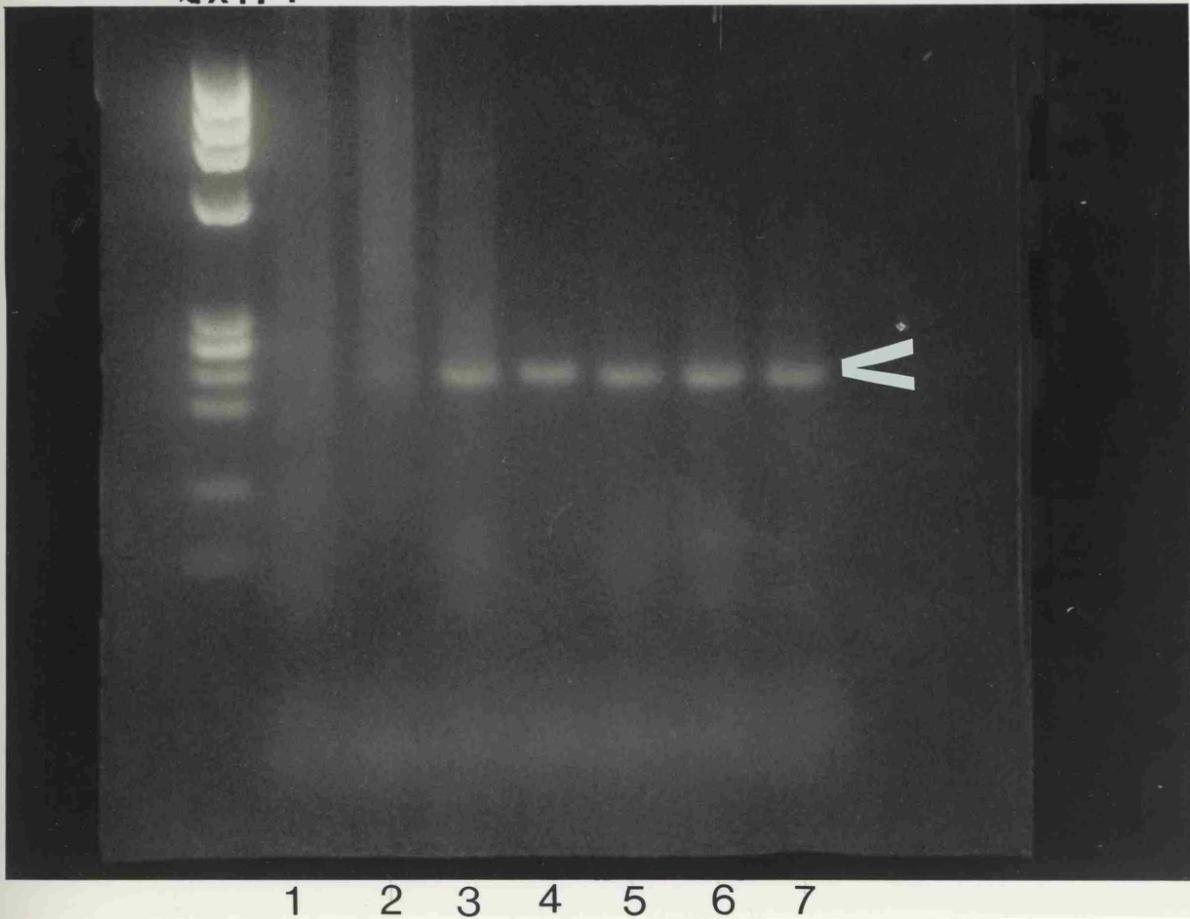


Plate 4.1

Plate 4.1

Results of HTLV-I detection with pX gene-specific primers. 1 μ g of viral DNA was amplified with 1.0 μ M of each pX primer. 10% of the reaction was placed into the wells of a 4% low melt temperature gel and electrophoresised (3 hours at 50V). The gel was stained with ethidium bromide and visualised under U.V. illumination.

Molecular standard used is 0X174/Hae III fragment showing bands at the following bp sizes:- 1353, 1078, 872, 603, 310, 281, 234, 194, 118 and 72. Lanes 1 and 2 contained human placental and tonsillar DNA. Lanes 3 - 7 all contained 1 μ g of HTLV-I template DNA. Note amplified band at 230 bp (arrowed).

Plate 4.2

Sensitivity of PCR amplification and detection of
HIV-1 RNA in plasma of patients with AIDS

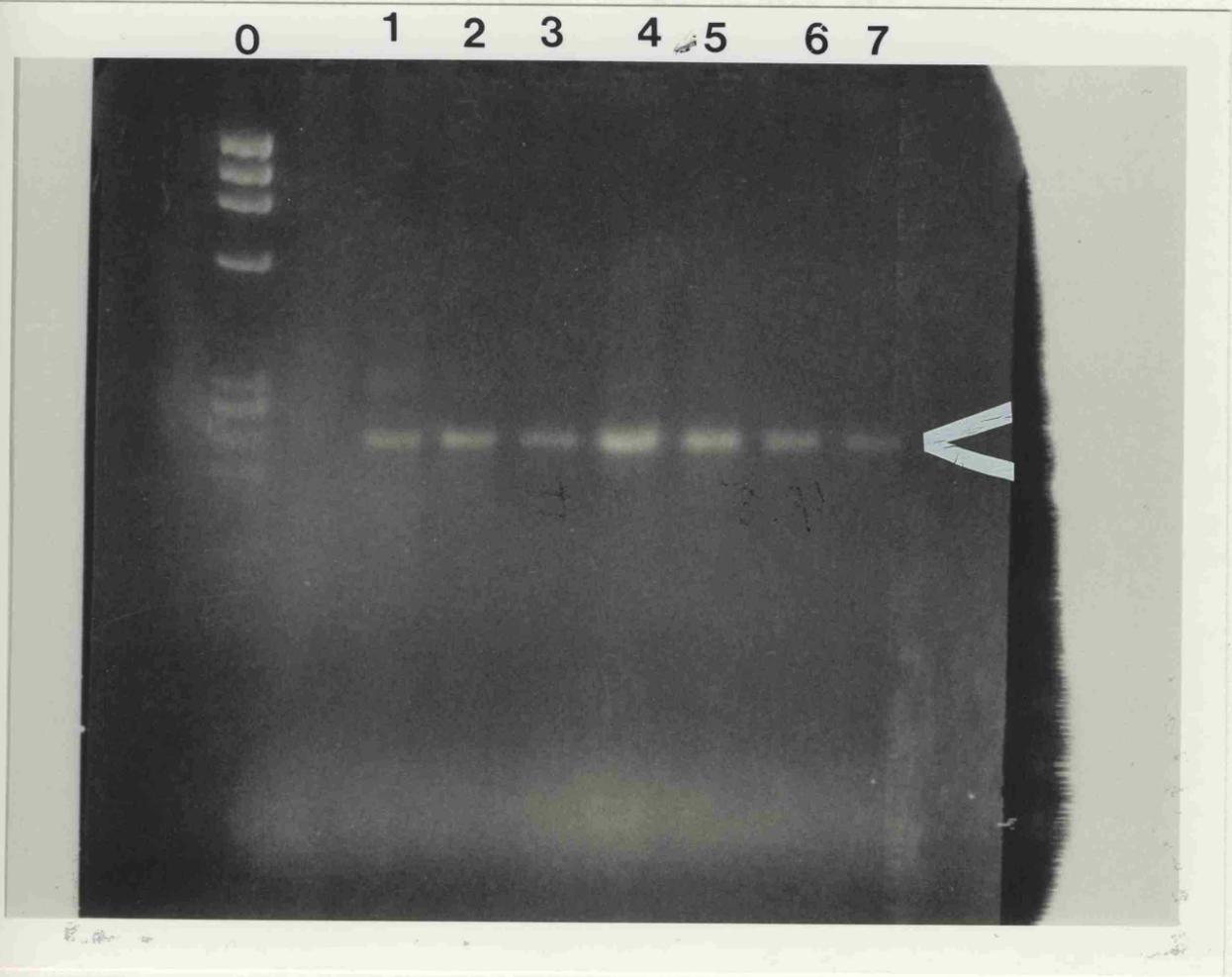


Plate 4.2

Plate 4.2

Sensitivity of PCR amplification and detection of various serial dilutions of HTLV-I template DNA.

Lane 0 molecular standard OX174/Hae III
 followed by negative control (placental DNA)

Lane 1 1ng viral DNA

Lane 2 10ng viral DNA

Lane 3 10pg viral DNA

Lane 4 1 μ g viral DNA

Lane 5 100ng viral DNA

Lane 6 100pg viral DNA

Lane 7 1pg viral DNA

Note a distinct band at 230bp is still visible at only 1pg of HTLV-I DNA.

False positive PCR amplification of HIV-1 RNA using
pol (pol) and gag gene specific primers. The of
sample RNA was amplified with 1-µg of each primer.

0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9

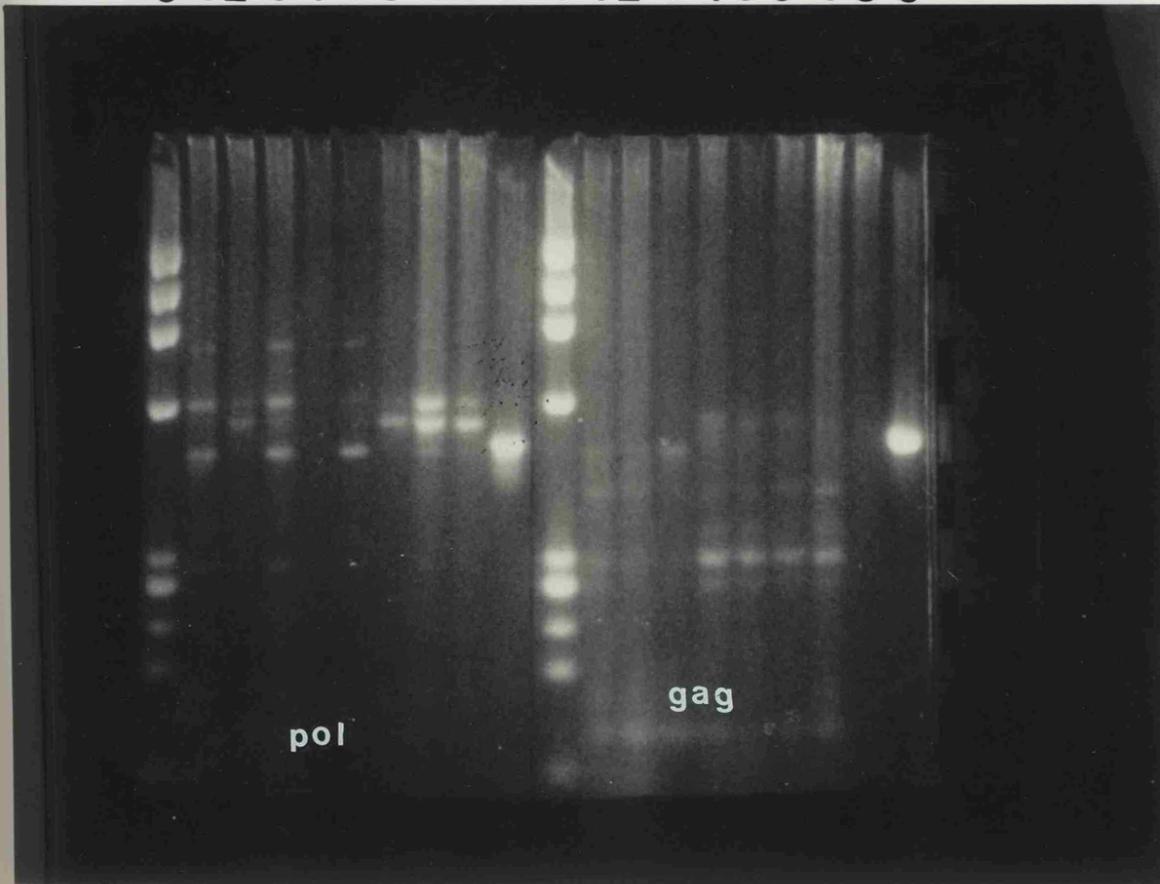


Plate 4.3

Plate 4.3

False positive PCR amplification of HTLV-I DNA, using pX (pol) and gag gene specific primers. 1 μ g of template DNA was amplified with 1.0 μ M of each primer.

Lane 0	Molecular standard OX174/Hae III
Lane 1	pH of reaction 7.5 and magnesium concentration 7mM
Lane 2	pH of reaction 8.0 and magnesium concentration 7mM
Lane 3	pH of reaction 9.5 and magnesium concentration 7mM
Lane 4	pH of reaction 8.8 and magnesium concentration 9mM
Lane 5	pH of reaction 8.8 and magnesium concentration 8mM
Lane 6	pH of reaction 8.8 and magnesium concentration 7mM
Lane 7	pH of reaction 8.8 and magnesium concentration 5mM
Lane 8	pH of reaction 8.8 and magnesium concentration 4mM
Lane 9	pH of reaction 8.8 and magnesium concentration 3mM

Note: Assay conditions in the reaction are critical for amplification. Multiple bands indicate false positive PCR amplification

These assay conditions were also found to be appropriate for amplification of human genomic DNA using β -globin primers (British Biotechnology, Oxford). Amplification was improved by non-specific restriction of DNA, before PCR procedure (Plate 4.4). Another critical factor for these experiments was the quality of (batch) variability of the enzyme Taq polymerase.

PCR amplification of human DNA from lymphoma cases was attempted, using primers directed at the Bcl-2 oncogene and the Ig heavy chain joining region (JH) gene. Amplifications were performed both manually and with automated methods, using DNA from both follicular and high grade lymphoma cases. Non consistent or reproducible amplification was produced, which subsequently led us to test reaction mixture conditions and enzyme variability (vide supra), and optimize PCR on viral DNA first.

4.2.2. AMPLIFICATION OF HUMAN DNA USING IMMUNOGLOBULIN KAPPA VARIABLE AND JOINING GENE SPECIFIC PRIMERS

The aim of this study was to develop a novel and highly sensitive and specific method of clonal (B-cell) detection. The polymerase chain reaction was used to demonstrate rearrangements of the Ig kappa variable and

joining genes in B-cell lymphomas and related disorders. DNA extracted from human tonsil and placental tissue was used as controls.

As with the Bcl-2 experiments, PCR amplification was initially inconsistent or non-existent. The method was modified to exclude the presence of a potent inhibitor of Taq polymerase, which copurifies with human genomic DNA (Franchis et al 1988). However, in our experiments no improvement of greater efficiency of amplification was observed by gel filtration (DNA enzyme restricted, boiled for 5 minutes and centrifuged through a 1ml sephadex G50 column, pre-equilibrated in 1mM Tris pH 8.0, 0.1mM EDTA).

Eventually successful amplification was produced by using high quality Taq polymerase (Cetus) and vigilance on technical detail.

Amplification of *Amphibian* DNA with *Hpa* III

X = molecular standard (2.74 Kbp III)

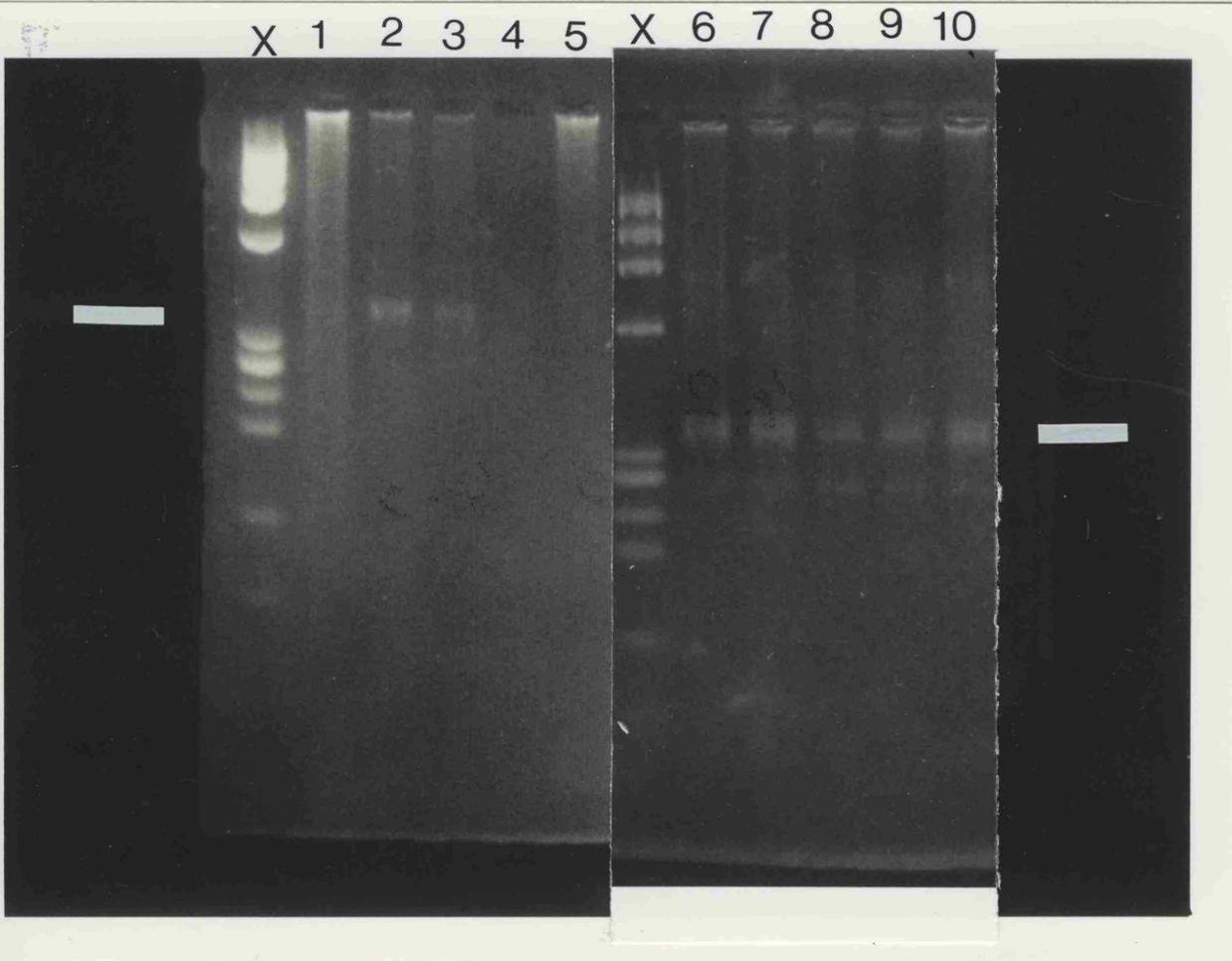


Plate 4.4

Plate 4.4

Amplification of human DNA with β -globin primers

X - molecular standard)X174/Hae III

Lanes 1-5 unrestricted DNA (1 μ g) with 1 μ M of each primer. Note inconsistent amplification.

Lanes 6-10 DNA restricted with EcoR, prior to PCR amplification. 1 μ g of restricted DNA amplified with 1mM of each primer, electrophoresed on 4% LMT gel and stained with ethidium bromide. U.V. illuminations of gel shows a band between 300 and 400 bp (marker)

With reactive tissues (tonsil, placenta) and polyclonal B-cell disorders, five amplified fragments of specific molecular sizes had been predicted (Figure 4.5) based on recruitment of each of the five joining regions to a variable gene. In practice, multiple bands were obtained, making interpretation difficult (Plate 4.5). Possibly four λ amplified products could be deduced, with the largest fragment (2kb) missing, implying selective enhancement of the shorter fragments. Results were similar with various permutations of light chain primers.

Similar band patterns were seen with DNA from B-cell lymphoma cases (presumably monoclonal or oligoclonal). Definitive confirmation of monoclonality was subsequently possible by Southern blotting and hybridization to a specific radiolabelled oligonucleotide (J_{κ} or $J_{\kappa 1}$ - $J_{\kappa 5}$ primers) probe. Classically the blot showed 4 bands in a polyclonal B-cell population and one band in a rearranged B-cell tumour (Plate 4.6). Alternatively, the results were less clear-cut, producing a smudge in polyclonal states and a band in the lymphoma case (Plate 4.7).

Scrupulous attention to technical detail resulted in some improvement in amplification success. For example, by ensuring primer concentration excess, and

replenishing Taq polymerase mid-way through the cycles resulted in more reproducible PCR amplification. In addition, increasing the initial denaturation step to ten minutes at 100°C improved results.

Overall, however PCR amplification using immunoglobulin kappa light chain primers remained inconsistent and difficult to interpret, always requiring hybridization to a radiolabelled probe for clarification. Some cases were clearly successful, while other demonstrated both cases of false positive and negative amplifications, although these were reduced by scrupulous efforts at technical detail.

Plate 4.5

PCR amplification of polyoma P-cell populations,
using immunoglobulin heavy chain primers, showing
multiple bands and making interpretation difficult.

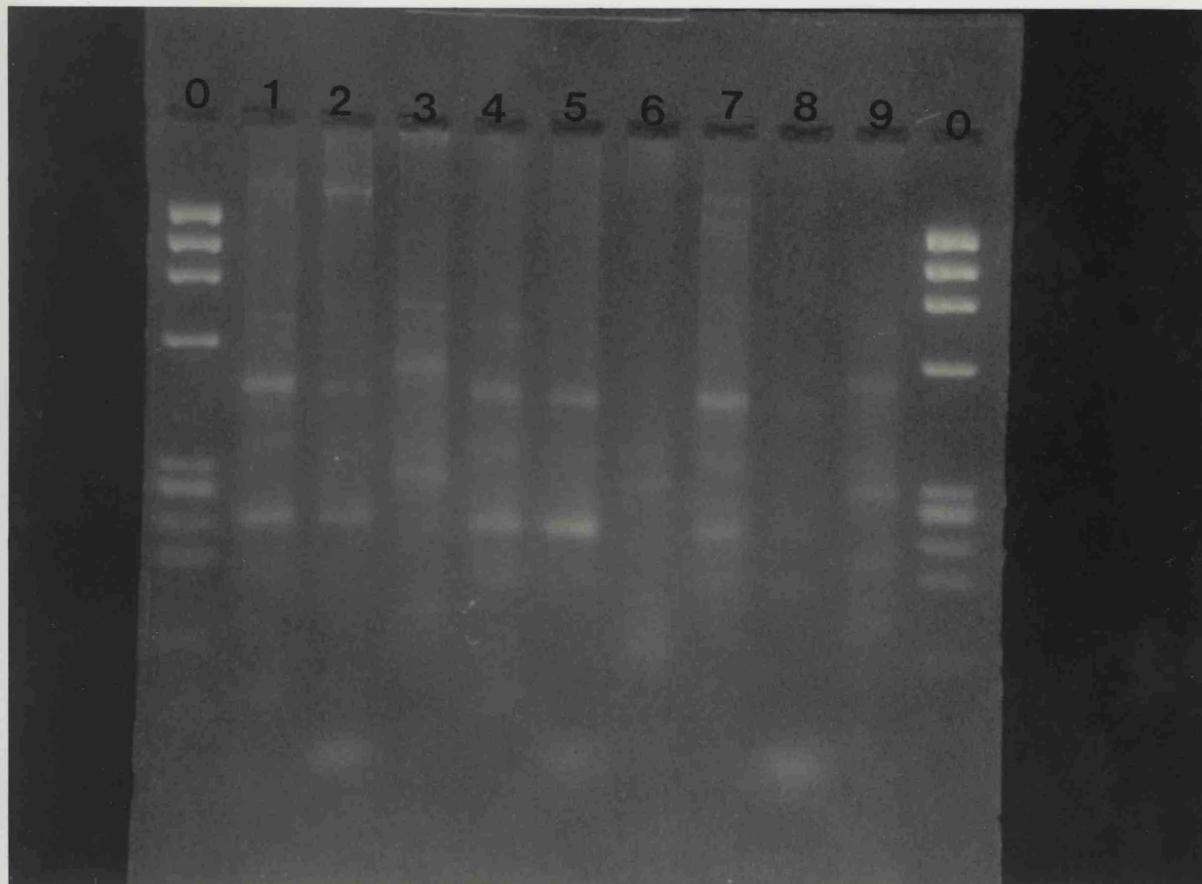


Plate 4.5

Ck1 and V_H (Lane 1)
V_H and Ck2 (Lane 2)
Ck1 and V_H (Lane 3)

On low melt temperature gel (4%) stained with ethidium
bromide and visualised under U.V. illumination.

Plate 4.5

PCR amplification of polyclonal B-cell populations, using immunoglobulin kappa light chain primers, showing multiple bands and making interpretation difficult. Certain bands occur consistently, suggesting successful amplification with at least some pairs of primers.

Lane 0 - molecular standard 0X174/Hae III

Lanes 1-3 Tonsillar DNA with various primers

J_{k1} and C_{k1} (Lane 1)

J_{k1} and C_{k2} (Lane 2)

C_{k1} and V_k (Lane 3)

Lanes 4-6 Tonsillar DNA (different source) with various primers

J_{k1} and C_{k1} (Lane 4)

J_{k1} and C_{k2} (Lane 5)

C_{k1} and V_k (Lane 6)

Lanes 7-9 Placental DNA with various primers

J^{k1} and C_{k1} (Lane 7)

J_{k1} and C_{k2} (Lane 8)

C_{k1} and V_k (Lane 9)

On low melt temperature gel (4%) stained with ethidium bromide and visualised under U.V. illumination.

Southern blot
immunoglobulin
hybridized
probe. In
and B-cell
endonuclease
polyclonal
as shown p
tissue. T
rearrange
J4.

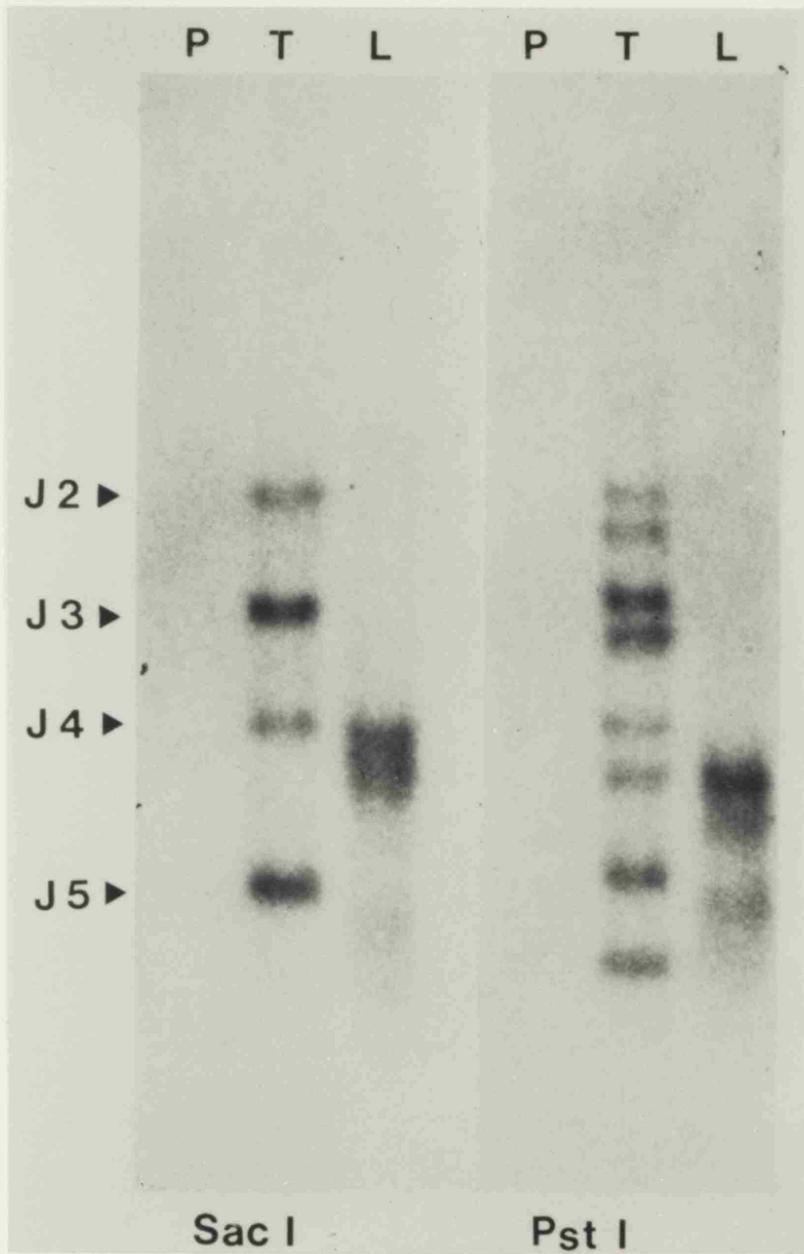


Plate 4.6

Plate 4.6

Southern blot confirmation of PCR amplification using immunoglobulin kappa light chain primers, required hybridization to a ^{32}P radiolabelled oligonucleotide probe. In this case, DNA from placenta (P), tonsil (T) and B-cell lymphoma (L) case is pre-digested with endonucleases Sac-I and Pst-I. Note confirmation of polyclonal B cell in Tonsil (2kb J1 was not amplified as shown previously), but not rearranged in placental tissue. The lymphoma case clearly shows a monoclonal rearrangement involving the immunoglobulin kappa gene J4.

Plate 4.7

Autoreadiogram after hybridization with a 32P labelled

probe in DNA

immunoglobulin

less clear-cut

two bands, in

banding however

single banding



Plate 4.7

Plate 4.7

Autoradiogram after hybridization with a ^{32}P labelled probe in DNA subjected to PCR amplification with immunoglobulin kappa light chain primers. There is less clear-cut demonstration of polyclonal B-cells from two tonsils (A and B) producing a smudge and not clear bands. However, a B-cell lymphoma case (C) shows a single band implying monoclonality.

4.3 DISCUSSION.

Comparison of gene probe analysis with immunophenotyping, reveals that for the vast majority of B-cell lymphoproliferative conditions, little additional discriminative or prognostic information was obtained with Southern Blotting. In addition, there were technical problems with Southern blot analysis of antigen receptor genes, including the limited sensitivity of the procedure (about 1-5% tumour to normal cells), the time necessary to obtain interpretable results and the reliance on radioactivity to detect rearrangements. Sensitivity is a particularly important issue in monitoring residual disease after therapy.

For this purpose we have recently adapted the polymerase chain reaction (Saiki et al, 1988; Erlich et al, 1988) technique, to provide a specific and sensitive method for detection of B-cell clones in reactive and neoplastic states. A set of gene-specific primers were synthesized to hybridize to immunoglobulin kappa variable and joining genes at selected locations, and the PCR was used to amplify the intervening segments. Amplified DNA of different kappa gene clonal rearrangements was visualised directly by ethidium bromide staining following electrophoretic separation.

Different clonal rearrangements were identified by size characteristics, compared to theoretical DNA segment amplification spanning the variable and the five joining genes and confirmed by probing with radiolabelled joining region specific oligonucleotide sequences. This method was applied to fixed paraffin-embedded archival tissue, in addition to fresh histological samples and cell suspensions.

As with any developmental work there were teething problems. Our initial experiments were designed to find the optimum PCR conditions, and identify courses for sub-optimal amplifications. The polymerase chain reaction is a powerful technique and requires a scrupulous emphasis on detail to exclude false positive and negative reactions. Initially our efforts were hampered by a lack of assurance on the quality of the enzyme Taq polymerase from several sources, and by batch to batch variability within each source.

Besides quality and reliability of Taq polymerase, other factors were also identified in producing false negative reactions. Optimal conditions for enzyme activity were dependent upon the reaction buffer and mixture, and these were established by a series of experiments. Generally an alkaline pH of 8.8 and a low

magnesium concentration of 3mM were found to be suitable.

Initially, the concentration of the primers was not considered to be critical on theoretical grounds of assumed excess. Subsequently, it was shown that the consistency and reproducibility of amplification was improved by increasing the concentration of each primer from 100 pmols to 1 μ M in the reaction.

The hybridization times and temperatures were based upon studies described in the literature and our own practical experience. It was found that amplification could be improved by having an initial prolonged denaturation step of ten minutes at 100°C, and by omitting the final denaturation step and extending the extension step for a further seven minutes. Of course, this assumes the differential temperatures machine is properly programmed, and the reaction mixture reaches the stated temperature for a the required time. In practice the machine was calibrated using a thermocouple and a mercury thermometer prior to experimentation, and commonly the outer wells were found to be wanting.

Other causes of false negativity were less easy to control. Ultimately primer annealing and amplification

is dependent upon the synthesis of pure and highly specific primers of complementary sequence. Selection of priming sites therefore is important, and effort is made to select specific sites that show no cross-hybridization to other parts of the genome, and also sequences rich in guanine/cytosine (GC-rich) which favour hydrogen bonding. However, one needs to account for genomic variability and introduce degeneracy where this is known. Self-hybridization of primers may be another reason for negative results.

In our study, the possibility of a potent inhibitor of Taq polymerase which copurified with human genomic DNA (de Franchis et al, 1988) was investigated but not substantiated.

Of more concern, are false positive reactions (Lo et al, 1988) and are usually the result of contamination particularly plasmid DNA. The advantage of the technique is also its disadvantage: the sensitivity of the method results in detection of trace amounts of contaminants (Marx, 1988). Therefore great care was taken in the preparation of template DNA before amplification. As far as possible the PCR machine and reagents were physically isolated from other molecular biology experiments, especially plasmid preparations. Buffers, primer solutions and reagents were aliquoted

out into small volumes, and each aliquot used once only and discarded to prevent cross contamination. Finally, internal positive and negative controls were used in each experiment to assess the likelihood of contamination, and verified by lack of hybridization to a specific radiolabelled probe.

Other causes of false negative reactions may be due to sequence similarities (i.e. wrong selection of priming sites) or artefact of hybridization. In general scrupulous efforts at technical detail and laboratory procedure would obviate many of the above difficulties.

The efficiency and sensitivity of PCR amplification was tested with HTLV-I viral genome and primers, human DNA and β -globin primers and DNA of lymphoid origin and Bcl-2/JH primers. Kwok et al (1988) have successfully applied the DNA amplification procedure (PCR) to facilitate detection of HTVL genomic sequences, using portion of the pol gene and x region as targets for primers. We used similar experiments to standardise and optimise PCR amplifications, as well as β -globin primers in human DNA experiments. There was a distinct lack of amplification using Bcl-2/JH primers in DNA from malignant lymphoma, despite documented evidence to the contrary (Stetler-Stevenson et al, 1988). Once the source of the failure was determined to be a poor batch

of the enzyme Taq polymerase, the Bcl-2 experiments were not repeated through lack of primers. In any case, the aim of this study was to develop novel and specific primers applicable to all B-cell malignancies, and work was started on judiciously selecting primer sites on the immunoglobulin kappa light chain, variable and joining gene.

Based upon rearrangement of each of the five joining region genes with the immunoglobulin kappa variable gene, five different fragment sizes of amplification were predicted in polyclonal or reactive B cells. However, in the event only four bands were produced, with the shortest fragment band being the most consistent. This implied selective enhancement and amplification of the shorter fragments. Despite selection of different priming sites, five fragments were not amplified. Therefore in practice, the distance between priming sites is important and shorter amplifications are preferentially produced, even if extension times were lengthened.

With monoclonal B cells, amplification produced several fragments on a gel after electrophoresis and staining, but the monoclonal rearrangement was clearly delineated by size density from the other (fainter) bands on subsequent probing. Different priming sites produced

consistent results, although reproducibility was not guaranteed.

Clearly this method requires further refinements and developments to make it more specific and reproducible. This study has shown that potentially this technique is a highly sensitive, specific and rapid way of distinguishing clonal from polyclonal B cells. This procedure requires 1 mg or less of DNA and is more rapid than the conventional Southern blotting. Other workers have recently developed a similar technique using the immunoglobulin heavy chain locus (Brisco et al., 1990) to differentiate clonal from polyclonal B cell populations, and clearly have had more reproducible and consistent results. The immunoglobulin kappa light chain, in retrospect is a less amenable gene to PCR amplification, than its sister the heavy chain gene.

CHAPTER 5: AN OVERVIEW AND DISCUSSION

The lymphomas are an important and heterogeneous group of neoplastic disorders originating from the immune system. Major progress has been made in the curability and long term remission rates due to advances in immunology, cytogenetics and molecular biology which have permitted increasingly refined clinical/pathologic correlations and definitions of specific subsets of lymphomas.

The major determinants of clinical patterns of disease and of progression are the cell type of origin (state of differentiation) and the pattern of growth within involved lymph nodes (follicular or diffuse). However, whereas in the last half century, only 2 classifications of Hodgkin's disease have been proposed, there has been a multiplicity (20 or more) of classifications of NHL resulting in widespread confusion (*vide supra*). Rarely did these classifications fulfil the criteria of reproducibility, clinical relevance or gain common usage and terminology to allow comparison of results of clinical trials or aid communication between individuals practising the same discipline world-wide.

Recently, however attempts have been made for a consensus in the classification of lymphomas (Lennert, 1983, Rosenberg, 1982), with the working formulation gaining favour in the United States, and the updated Kiel classification (Stansfield et al 1988) in Europe. Even these newer classifications will require further updating

as new clinicopathological entities become established from the application of advances in immunology, cytogenetics and molecular biology.

The use of modern immunohistochemical markers and techniques on involved tissues have been instrumental in diagnosis, but difficulties are well recognised and have already been discussed (Sections 1.5.1-1.5.3).

Immunophenotyping (Section 1.5.4) has had a major impact in the diagnosis of the lymphoproliferative disorders, and have led to important insights into lymphocyte differentiation and cellular origin of lymphoid malignancies. But here too diagnostic difficulties occur, particularly with the T cell neoplastic disorders.

The advent of DNA analysis and application of molecular biology techniques in the diagnosis and classification of lymphoproliferative disorders has shown great promise, exemplified by immunoglobulin and T cell receptor gene probe analysis of lymphoid neoplasms. This study set out to assess the value of these novel techniques in routine diagnostic lymphoma work-up.

Gene rearrangement analysis (Southern blotting) provided diagnostic classification in B and T cell lymphomas (Chapter 2, Sections 2.2, 2.3). However, this technique is neither simple or rapid, and a result usually takes about a week or 10 days to appear. Interpretation of a result as indicating monoclonality is based on the

identification of a band or bands appearing at positions disparate from control germ-line DNA. Faint or indistinct bands may require hybridization of a radio-labelled probe and autoradiography onto x-ray film up to ten days. The technique's sensitivity is such that rearranged bands are often detectable if at least 2-5% of the cells in the tissue providing the DNA are monoclonal.

Difficulties of interpretation are frequent, due to technical artifacts, genetic polymorphism within the B and T receptor gene regions, and impurities in the probe left from contaminating fragments of DNA from the vector in which it has been biosynthesised. Misleading results may also occur in cases showing paraneoplastic gene rearrangements and lineage infidelity (Section 2.3).

Nevertheless, gene probe analysis was shown to be useful in T cell neoplasms, which lack a specific phenotypic marker. In the vast majority of B cell cases, gene rearrangement analysis provides no additional information over immunophenotyping. Gene probe analysis cannot be recommended as a routine procedure in the majority of lymphomas, and should be reserved for the uncommon T cell cases and the rare B cell lymphomas which are not categorised by immunological techniques.

In situ hybridization provides direct molecular demonstration of specific nucleic acid sequences in tissue sections, with anatomical preservation of cellular detail

(Chapter 3). In this study, this methodology was extended to routine bone marrow trephines, to determine localisation and detection of immunoglobulin light chain mRNA in formalin-fixed paraffin sections based on published protocols (Burns et al, 1985; Beckman et al 1985; Unger et al, 1986; Pringle et al 1987).

The results on suspected myeloma cases under optimal experimental conditions were specific and sensitive (Section 3.2). In situ hybridization has particular advantages in myeloma and lymphoplasmacytoid malignancies, and these have been enumerated already (Section 3.3).

This study has shown the usefulness of in situ hybridization using non-radioactive biotinylated probes in routine laboratory usage. It is a rapid, sensitive and specific method. However, it has a limited role in the majority of lymphoid malignancies, as immunoglobulin synthesis and expression is uncommon. But in situ hybridization has great potential for future applications in the lymphoproliferative disorders.

In situ hybridization can be modified to detect any specific nucleic acid sequence, as recombinant DNA techniques have enabled the synthesis of pure and highly specific probes. It can be applied to both fixed and fresh tissues, and the resolution of this technique is

high, enabling precise nuclear or cytoplasmic location in cells.

Future prospects include "double labelling" allowing precise identification of cell types based on surface determinants, and additional detection of specific nucleic acid sequences. Future target sequences for oligonucleotide probes may include interleukins, interferons and other cytokines. In situ hybridization is likely to find increasing applicability in the field of lymphoid disorders.

The polymerase chain reaction (PCR) is an in vitro method for the primer directed enzymatic amplification of specific DNA sequences (Saki et al, 1985, 1988), and has rapidly become an invaluable technique for a wide variety of clinical disorders.

PCR has been used to detect neoplastic haematological disorders with well-defined, characteristic gene rearrangements, such as chronic myeloid leukaemia (Lee et al, 1988) and follicular lymphomas (Lee et al, 1987). However, many disorders including many B cell lymphomas lack such clear, consistent genetic markers. The B cell lymphoma represent progressive expansion of a clone of neoplastic lymphocytes, and all cells in the clone contain the same unique immunoglobulin (Ig) gene rearrangement. The rearranged gene thus provides a potential marker for the neoplastic clone.

CONCLUSION

The PCR once established and optimised for a set of primers is clearly the most sensitive and specific method for demonstrating clonality in human lymphoid malignancies. It surpasses gene rearrangement studies based on Southern blotting, by its speed (unnecessary to probe in many cases when specific fragment visible on a gel; Southern blot commonly takes 7-14 days for full analysis), sensitivity, (PCR can amplify picogrammes of DNA) and specificity (due to preparation of highly specific primers).

PCR can also amplify impure, fragmented DNA found in archival paraffin wax embedded tissues. DNA may be extracted from these tissues by standard methods, or DNA may be amplified directly from a previously dewaxed section (Shibata et al, 1988), obviating the need for lengthy purification procedures. Therefore PCR has the great advantage of being able to amplify poor quality DNA samples, obtained by relatively simple extraction methods.

The PCR has a great potential for the genetic analysis of tissues, particularly in relation to lymphoid malignancies. These include detection of minimal residual disease (MRD) in cases of the t(14;18) (9³²;9²¹) chromosomal translocation (Lee et al, 1987; Stetler-Stevenson et al, 1988), detection of clonality in

suspected lymphoproliferative disorders, identification of point mutations in cellular proto-oncogenes (Haliassos et al, 1989), and amplification of viral sequences present in lymphoid malignancies (Kwok et al 1988).

PCR amplification using the Bcl2/JH sequence has been successfully applied in the majority of follicular and some high grade lymphomas (Lee et al 1987; Stetler-Stevenson et al 1988). Therefore PCR in lymphoma cases with the t(14;18) chromosomal translocation is both informative and [?]/. As demonstrated in this study, good quality Taq polymerase and standardised conditions are essential for successful amplification.

The detection of viral infections in lymphoproliferative disorders are important, and PCR can be exquisitely sensitive in this area. HTLV-I is aetiologically related to adult T cell lymphoma/leukaemia, and detection is relatively simple using PCR. Using PCR amplification, other viral agents may be related to other lymphoid malignancies in the future.

A major aim of this study was the determination of MRD using PCR. As treatment becomes more thorough in eradicating lymphoid tumours, a sensitive marker of MRD is required. PCR fulfils the criteria of sensitivity, but specificity depends upon judicious selection of primary sites, and gene sequence selected for amplification. Designing specific and non-cross reacting

primers to hybridize onto suitable DNA sequences in the human genome is of critical importance.

In this study, the immunoglobulin kappa light chain was selected as being suitable for amplification in B cell malignancies, for reasons already alluded to above. Specific priming sites were selected in the variable and joining gene regions. This study demonstrated that PCR amplification across these sites was possible, although difficult. More consistent and reproducible amplification has been demonstrated using the Ig heavy gene (McCarthy et al, 1989). This study therefore demonstrates that the selection of a gene and gene sequence is also critical for successful amplification. Clearly, further investigations of the applicability of the immunoglobulin PCR technique are required, and its role in the diagnosis and detection of MRD in a variety of B cell neoplasms needs further clarification. PCR remains a powerful, investigative diagnostic tool, but at present has more of a research role, than routine clinical usage in lymphoma work-up.

PCR is a powerful technique but this advantage is also its disadvantage, the sensitivity of the method results in detection of trace amounts of contaminants (Marx, 1988) and false positive reactions (Lo et al, 1988). False negative reactions also occur, due to genomic variability and self-hybridization of primers.

Establishing PCR technique was beset with difficulties as

there are many variables to consider, which have been already discussed (Section 4.3). With meticulous attention to detail, PCR can be a powerful tool. Results suggest establishing PCR is easier with viral sequences, than complex and highly variable sequences in the human genome.

This study therefore suggests that for diagnostic lymphoma work, PCR remains a research tool despite its premature introduction into many routine laboratories. With further developments, no doubt PCR will become established as a routine diagnostic tool in lymphoid neoplasms.

Finally, the question of the place of molecular techniques in lymphoid neoplasms, given the excellent conventional methods which are now available routinely in many laboratories. Many laboratories have developed great expertise in lymphoma diagnosis and have been able to overcome the technical difficulties discussed already (Section 1.5.1). With the establishment of the standardization of immuno-histochemical markers and techniques, the vast majority of lymphomas can now be diagnosed accurately and rapidly by an expert histopathologist. Consensus in diagnosis has been facilitated by establishment of local and regional lymphoma panels, and improved classification of malignant lymphomas based more on clinicopathological entities (Rosenberg, 1982; Stansfield et al, 1988).

The immunological methods described in Section 1.5.4. are applicable and often diagnostic in lymphoma diagnosis. But in many cases, immunophenotyping merely confirms a diagnosis which is clear on the basis of well established clinical and morphological criteria, and these relatively expensive and labour-intensive methods are not necessary. However, in other area, immunophenotyping may be crucial to diagnosis, prognosis and treatment.

Molecular techniques, like immunological methods should be applied selectively, when the results are needed to clarify diagnosis, and not merely to confirm what is apparent from simpler and less expensive clinical and laboratory assessment. The mere availability of a technique should not be sufficient justification for its use.

Gene rearrangement analysis, (Southern Blotting) is now an established technique, and as shown above is useful in suspected T cell neoplasms and rarely in some B cell cases where diagnostic clarification is required. In the former, immunological markers are unsatisfactory and reactive states may be difficult to differentiate from neoplasms by conventional techniques. It must be stressed however that gene analysis is expensive, time consuming and labour intensive, and is not a technique whereby clinicians can await the results before beginning

treatment. The management will be dictated by the clinical situation and other preliminary investigations.

With low grade B cell tumours, rarely would one need to resort to molecular techniques for clinical management. There is little point in demonstrating occult or minimal residual disease, as it is well known that these tumours are rarely curable. Using highly sensitive techniques such as PCR appears inappropriate until further therapeutic options become available, as the management of these tumours is unaffected by the result of these investigations at present.

With high grade B cell lymphomas, detection of occult or minimal residual disease is more relevant particularly after cytoreductive therapy (including bone marrow transplantation). These tumours are all potentially curable, and therefore the application of highly sensitive methods at detection of residual lymphoma is appropriate.

The polymerase chain reaction would then be invaluable. The exquisite sensitivity of this technique has been demonstrated by detection of mitochondrial DNA from single hairs (Higuchi et al, 1988), and in particular by detection of one lymphoma cell in 10^6 normal cells (Cresenzi et al, 1988), which would reveal the subclinical presence of malignant cells in patients thought to be in complete remission.

The modification of this technique as described with the immunoglobulin variable and joining gene primers, would provide a rapid PCR method for distinguishing clonal from polyclonal B cell populations and would be applicable to blood, biopsy tissue and serous fluids from B cell lymphoma patients.

The fact that such complex events in the human genome can be mapped using primers to judiciously selected hybridization sites, establishes the extreme versatility and potential of PCR in human lymphoid malignancies. With suitable primers, PCR can be modified to study T cell malignancies or in fact any rearrangements in health and disease. The extreme sensitivity of PCR is however, also its disadvantage and scrupulous efforts have to be made to exclude false positive reactions. PCR remains a research tool at present and therefore has limited though important applications in lymphoma diagnosis.

In situ hybridization is an exquisite technique for localisation of specific nucleic acid sequences with morphological preservation of cells and tissues. Therefore a signal may be equated and localised to a particular cell type. This technique has been useful in mature lymphoid malignancies particularly multiple myeloma and can be applied to fresh and fixed tissues, including bone marrow trephines. This technique will continue to be useful for DNA or RNA localisation in

various cells and tissues, but remains a predominantly research tool, as other less expensive conventional techniques are available, and a certain amount of expertise is required.

This all suggest^s that molecular techniques are not going to be available in every district general hospital. For a centre to gain sufficient expertise and experience, it requires a fair amount of referrals to use these techniques. Therefore in my opinion, molecular techniques have an important though small place in diagnostic lymphoma work and should be organised regionally at University Departments, which also have a commitment to on-going research into these techniques. These regional centre would provide a diagnostic service for referring clinicians and hospitals.

Advances in the treatment of malignant lymphomas, including bone marrow transplantation and the use of lympholytic monoclonal antibodies (Hale et al, 1988) have provided a major impetus at more precise diagnostic methods and the detection of occult or minimal residual disease (MRD). In addition, clinicians are now more aware of the long term complications of chemotherapy, particularly second malignancies (Canellos et al, 1975) and therefore treatment is used judiciously based on the cellular type of malignancy and the risk of subsequent relapse. Molecular techniques therefore would have an important role at precise and sensitive localisation of

lymphoid neoplasms, and the detection of MRD after bone marrow transplantation or treatment with lympholytic monoclonal antibodies. Already, successful bone marrow engraftment has been investigated by methods which are able to distinguish between donor and host cell populations (Hutchinson et al, 1989), and found to be sensitive determinants of engraftment, leukaemic relapse and detection of chimaeric states. Future prospects in this field appear promising.

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Appendix

Abstracts and publications resulting from this project.

1. The demonstration of circulating lymphoma cells by gene rearrangement studies in patients with T cell lymphoma. J. Pathol 1989; 157: 162A.
2. The evaluation of gene rearrangement studies as a diagnostic laboratory aid in non-Hodgkin's lymphoma. J. Pathol 1989; 157: 162A.
3. Investigation comparing gene probe analysis and immunophenotyping on blood and biopsied tissues of patients with non-Hodgkin's lymphoma. Brit J. Haemat 1991 (s); 77: 69.
4. In situ hybridization detection of light chain mRNA in routine bone marrow trephines from patients with suspected myeloma. Brit J Haemat 1989; 73: 296-301.
5. Demonstration of light chain mRNA in routine bone marrow trephines using in situ hybridization, in patients with suspected myeloma. Brit J Haemat 1989(S); 71: 27.
6. The clinical relevance of plasma viscosity in Hodgkin's disease. Clin Lab Haemat 1991; 13: 1-8.