STUDY OF HUMAN TUMOUR DRAINING LYMPH NODE HOMING RECEPTORS IN BREAST CANCER PATIENTS

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Degree of

DOCTOR OF PHILOSOPHY

by

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To my family

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List of Abbreviations

APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of designation
CD25	Interleukin-2 receptor (Tac)
CS	Chondroitin sulphate
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
EBV	Epstein-Barr virus
ECM	Extra cellular matrix
EDTA	Ethylene diamine tetra-acetic acetate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACScan	Fluorescence activated cell scanner
FCM	Flow cytometry

FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL 1,2,3	Fluorescence channel 1, 2 or 3
FLN	Far lymph node
FSC	Forward scatter
$G_{0,1,2}$	Gap phase 0, 1, 2 of the DNA cell cycle
GAG	Glycosaminoglycan
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
GTP	Guanosine triphosphate
HAT	Hypoxanthine, aminopterin and thymidine
HEBF	High endothelial binding factor
HEC	High endothelial cell
HEV	High endothelial venule
HLA	Human leucocyte antigen
HS	Heparan sulphate
HT	Hypoxanthine and thymidine
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-2	Interleukin-2

IL-2R	Interleukin-2 receptor
IP_3	Inositol trisphosphate
Kb	Kilo base pairs
kDa	Kilo dalton
LAK	Lymphocyte-activated killer
LAM	Leukocyte adhesion molecule
LCAM	Liver cell adhesion molecule
LECAM	Leukocyte-endothelial cell adhesion molecule
LEC-CAM	Lectin-EGF-complement-cell-adhesion-molecule
LFA-1	Lymphocyte function associated antigen-1
LNL	Lymph node lymphocyte
LPAM	Lymphocyte-Peyer's patch adhesion molecule
LuLN	Lung lymph node
M	Mitotic phase of the DNA cell cycle
Mab	Monoclonal antibody
Mac-1	Macrophage-1
MadCAM	Mucosal addressin cell adhesion molecule
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node

Mls	Minor lymphocyte stimulating
M6P	Mannose-6-phosphate
NCAM	Neural cell adhesion molecule
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NLN	Near lymph node
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PEG	Polyethylene glycol
PG	Proteoglycan
PI	Propidium iodide
PIP_2	Phosphatidylinositol bisphosphate
PIPES	Piperazine-N,N'-bis-2-ethane sulfonic acid
PKC	Protein kinase C
PLC	Phospholipase C
PLN	Peripheral lymph node
PP	Peyer's patch

PTK	Protein tyrosine kinase
RPMI	Roswell Park Memorial Institute
S	Synthetic phase of the DNA cell cycle
SDS	Sodium dodecyl sulphate
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
sIgG	Surface immunoglobulin G
SPF	S phase fraction
SSC	Side scatter
Tac	T cell activation synonym for the CD25 antigen on the α chain of the IL 2 receptor
TCR	T cell receptor
Tc	Cytotoxic T cell
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	Helper T cell
TIL	Tumour infiltrating lymphocyte
TGR	Trans-Golgi reticulum
TGF	Tumour growth factor
TNF	Tumour necrosis factor
VA	Vascular addressin

VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

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Summary

The cell surface molecule LEU-8 has been reported to identify the human homologue of the MEL-14 lymph node homing receptor by sequence comparison of the two antigens (Camerini *et al.*, 1989). However, it has never been directly tested for its ability to specifically detect lymphocytes which home to human lymph nodes. This study was designed to generate monoclonal antibodies against the nodal lymphocytes in order to identify marker unique to the node, and to compare this with the homing function suggested for the LEU-8 marker. At the same time, LEU-8 was tested for a homing function in humans by comparison of its relative expression in lymph node and peripheral blood of breast cancer patients using two-colour flow cytometry.

This work also set out to assess local immunological responses in the nodes and peripheral blood, and to analyse the expression in the nodes and its relationship to the expression pattern of the human lymphocyte homing receptor CD44.

The antigens of human nodal lymphocytes identified by three monoclonal antibodies were not unique to the node, and no specific homing molecule was identified despite a logical strategy. The monoclonal antibodies produced were to B cell antigens that were unlikely to be MHC Class I or immunoglobulin. B cells were not quantitatively dominant in the human lymph node but were highly immunodominant.

The data showed that CD3+ or CD19+ cells did not express LEU-8 preferentially in the human lymph node, and indeed the latter showed a preference for location in the blood. LEU-8 was irrelevant to T lymphocyte homing and a negative indicator of B cell homing. Taken together, there was

no functional evidence from this study that the LEU-8 cDNA homologous to mouse MEL-14 antigen does indeed encode a human lymph node homing receptor.

In the local lymph nodes of breast cancer patients, the activation marker IL-2 receptor (Tac) was present on a higher proportion on CD4+ than CD8+ cells in two axillary lymph nodes (near and far from their draining tissue) and peripheral blood. In contrast, the percentage of CD8+ T cells bearing the activation marker HLA DR was higher than the percentage of HLA DR expressing CD4+ T cells in all cases.

CD44 was preferentially located in the human lymph node, but did not show significance difference between lymph node and peripheral blood such that was difficult to deduce homing. This study suggest that CD44 might be a homing receptor in humans, but more data is required for statistical validity. More interestingly both CD44 and LEU-8 were present on primary and metastatic tumour cells, and may therefore have prognostic and diagnostic potential in primary and metastatic breast carcinomas.

CHAPTER 1

INTRODUCTION

1.1 The Human Immune Response

1.1.1 T and B Cells

The function of the immune response in humans is to employ specific cells and molecules to protect the host from foreign substances and itself. This response involves multiple cellular interactions mediated by soluble growth and differentiation factors. The immune specificity is determined by T and B lymphocytes. Extracellular bacteria are susceptible to attack by antibody produced from B cells in conjunction with complement or phagocytes, while intracellular bacteria or viruses require an intact T cell system to ensure effective eradiction.

1.1.1.1 B Cells (CD19)

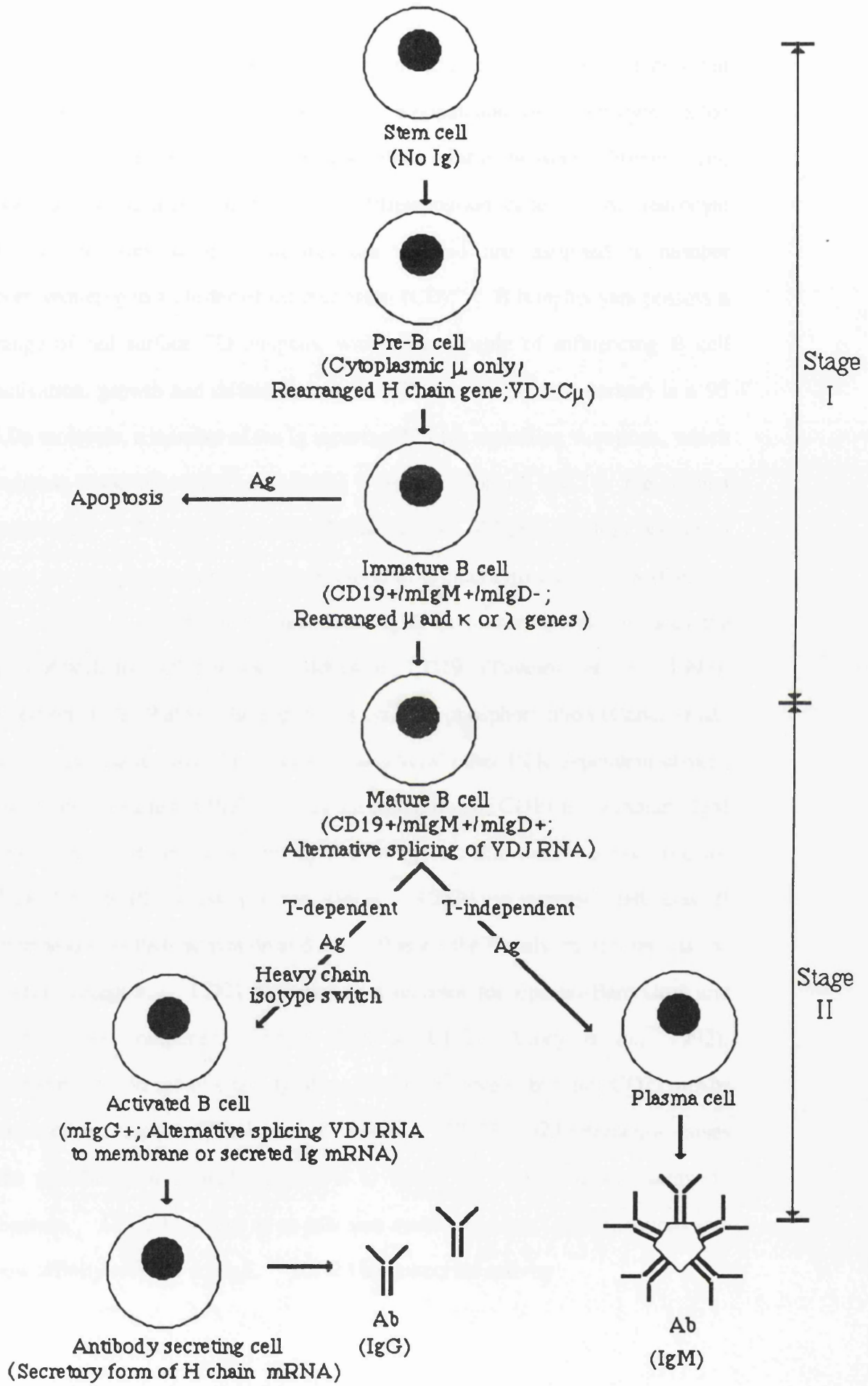
B lymphocytes are involved in the humoral immune response, which is the main defence mechanism against extracellular microbes. A substantial repertoire of antibodies or immunoglobulins in the immune system is provided by B lymphocytes. This specificity and diversity of the immune response is based on clonal selection hypothesis (Burnet, 1959). The diversity of antigen-binding V regions of immunoglobulin (Ig) molecules for an antigen is made up by combination of domains in the variable (V) regions of the heavy (H) and light (L). The development of B cells into mature antibody secreting cells can be divided into two stages, antigen independent and dependent steps (Figure 1.1). All B lymphocytes are derived from a stem cell in the bone marrow that does not produce immunoglobulin, and also gives rise to the erythroid and myeloid lineages of blood cells and to T lymphocytes (Smith *et al.*, 1991). B lymphocytes are continuously generated throughout life because of the self-renewing capacity of stem cells. Antigen-sensitive B cells occur in the circulation and secondary organs, such as spleen and lymph nodes.

In pre-B lymphocytes, the Ig gene product containing μ heavy chain composed of variable and constant (C) regions is synthesised in the cytoplasm. The immature B cell expresses a complete heavy and light chain, either κ or λ, and then produces assembled IgM molecules on the cell surface. After having acquired a complete Ig, B cells migrate out of bone marrow and continue to mature in the peripheral circulation and lymphoid tissues. Stimulation by antigen through the surface Ig molecule activates the B lymphocytes to proliferate and mature. Mature B cells co-express μ and δ heavy chains in association with the original κ or λ light chain and express both monomer IgM and IgD. These mature B cells can respond to T-independent antigens by proliferation and maturation to IgM-secreting cells without Ig class switching or hypermutation of the Ig V gene regions. Alternatively, they can respond to T-dependent antigens, again by proliferation and maturation, first to IgM secretion, then to surface expression, and later to secretion of other classes of Ig. T cell-dependent responses induce not only class switching but also hypermutation in the V region segments of Ig H and L chain genes. This reaction occurs in secondary lymphoid organs in germinal centers (Rolink and Melchers, 1991).

The activated B cells differentiate into antibody secreting cells, which begin to express Ig heavy chain classes (e.g. γ, α, or ε) other than μ and δ, undergoing heavy chain class (isotype) swiching (Tonegawa, 1983), and memory cells, which may not secrete antibody but have potential to participate in the secondary antibody response by secondary stimulation from the same antigen.

Figure 1.1 B cell development

Stage I : Ag-independent, Stage II : Ag-dependent


Stem cell
(No Ig)
Pre-B cell
(Cytoplasmic μ only;
Rearranged H chain gene;VDJ-Cμ)
Ag
Apoptosis
Immature B cell
(CD19+/mIgM+/mIgD- ;
Rearranged μ and κ or λ genes)
Mature B cell
(CD19+/mIgM+/mIgD+;
Alternative splicing of VDJ RNA)
T-dependent
T-independent
Ag
Ag
Heavy chain
isotype switch
Activated B cell
(mIgG+; Alternative splicing VDJ RNA
to membrane or secreted Ig mRNA)
Plasma cell
Antibody secreting cell
(Secretory form of H chain mRNA)
Ab
(IgG)
Ab
(IgM)
Stage
I
Stage
II

Surface Ig on B cells can couple to independent pathways of G-protein regulated PLC- and PTK-mediated PLC-activation and/or G-protein-dependent PTK-mediated PLC activation (Harnett and Rigley, 1992; Cushley and Harnett, 1993) (Figure 1.2). PLC mediates hydrolysis of phosphatidylinositol bisphosphate(PIP_2) to generate the intracellular second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Bijsterbosch *et al.*, 1986). The activation of PTK leads to the tyrosyl-phosphorylation of target proteins (Campbell and Sefton, 1990). The identity of the PTK(s) is likely to be one or more of the *src*-related PTKs, *blk*, *lyn*, and *fyn* recently shown to be associated with the mIg receptors (Yamanishi *et al.*, 1991; Burkhardt *et al.*, 1991). The antigen receptors are coupled to various PLC isoforms via G-proteins and/or PTKs depending on their state of activation or differentiation.

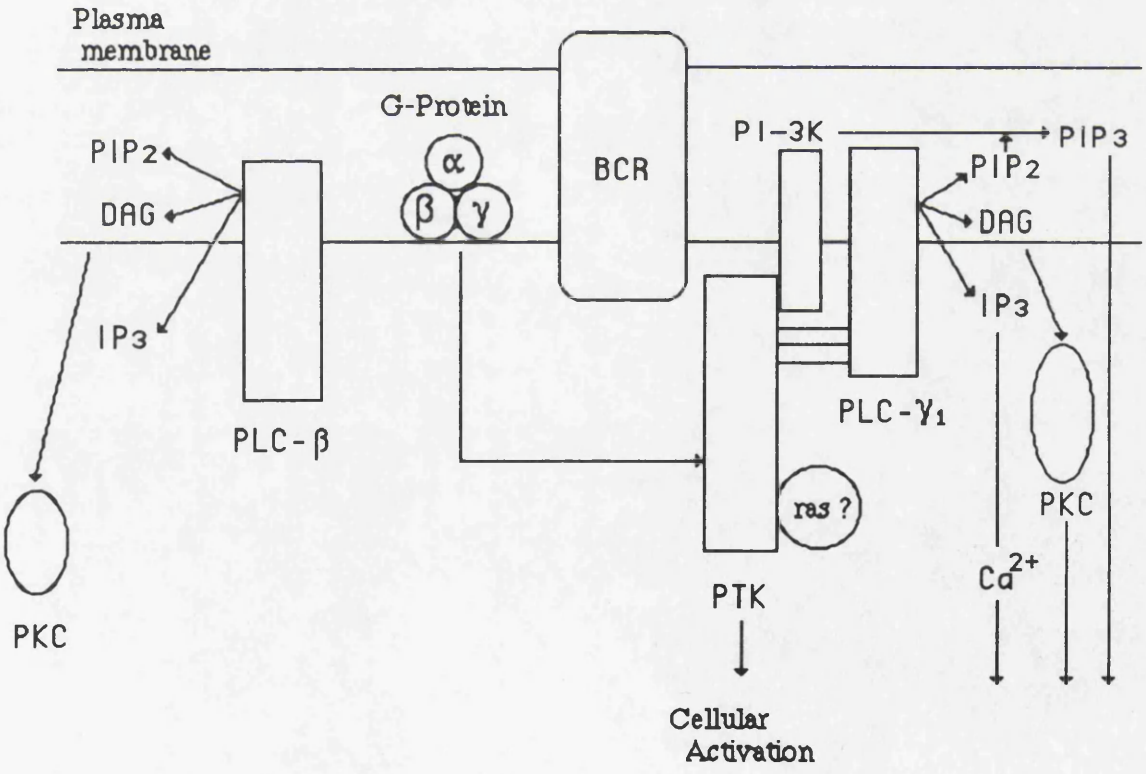


Figure 1.2 Signalling pathways of antigen receptor coupling of interactive G-protein/PTK mediation of B cell signalling

1.1.1.2 T Cells (CD3)

T cells are involved in cellular immunity. They recognise and respond to cell surface-associated but not soluble antigens, and cause the lysis of infected cells. T lymphocytes are derived from a common lymphoid-committed stem cell (CD34), which migrates from the bone marrow to colonize the thymus. Within the thymic microenvironment, T cells differentiate to become mature, functional cells which are responsive to antigen in the context of major histocompatibility complex (MHC) proteins (Section 1.1.1.5). The specificity for antigen is determined by the T-cell receptor (TCR) expressed on the surface of T lymphocytes.

1.1.1.2.1 CD4 and CD8

The majority of T lymphocytes can be divided phenotypically into two subsets, CD4 and CD8 which are encoded by genes which are cloned and characterised. One consists of cells, helper (Th), expressing the CD4 surface marker and the other consists of cytotoxic cells (Tc), expressing CD8 on their cell surface (Figure 1.3). CD4 is a monomer of 55-67 kDa with a highly conserved cytoplasmic domain (Maddon *et al.*, 1985). CD8 is a 32 kDa structure that forms homomultimers or heterodimers with CD1 or MHC class I (Snow *et al.*, 1985; Blue *et al.*, 1988). This phenotypic distinction has generally been correlated with a fundamental functional difference, namely that CD4+ T lymphocytes apparently perform "helper" functions and exhibit an antigen response restricted to MHC class II molecules, whereas CD8+ T cells are cytotoxic to antigen-bearing target cells and usually are MHC class I-restricted (Glasebrook *et al.*, 1983). The CD4 and CD8 antigens are glycoproteins expressed on reciprocal subsets of T cells within the peripheral blood compartment and have also been implicated in the regulation of T-cell

growth (Reinherz *et al.*, 1983). Both antigens are members of the immunoglobulin gene superfamily (Williams and Barclay, 1988). CD4 and CD8 function to increase the avidity of the T cell for antigen by binding to MHC class II and I antigens, respectively. This has led to the suggestion that CD4 and CD8 may generate intracellular signals (Section 1.1.1.2.3) either independently and/or in conjunction with the TCR/CD3 complex. CD4+ helper T lymphocytes appear to perform most functions via the action of secreted lymphokines (Prystowsky *et al.*, 1982). These factors modify the activity of the T cells that produce them in an autocrine fashion, as well as modulating responses of other cells in a paracrine fashion. CD8+ cytotoxic T lymphocytes also secrete some lymphokines in response to antigenic stimulation, but the range is generally comparatively restricted. CD4 and CD8 have influence in the selection of TCR repertoire in the thymus (Von Boehmer *et al.*, 1989). The T-cell receptor complex (TCR/CD3) is therefore clearly linked to the specific recognition of MHC and antigenic determinants and the subsequent activation of T cells. Interactions between TCR/CD3 and these T-cell antigens control the expansion of certain T-cell subsets with specific immune functions such as help, cytotoxicity and suppression.

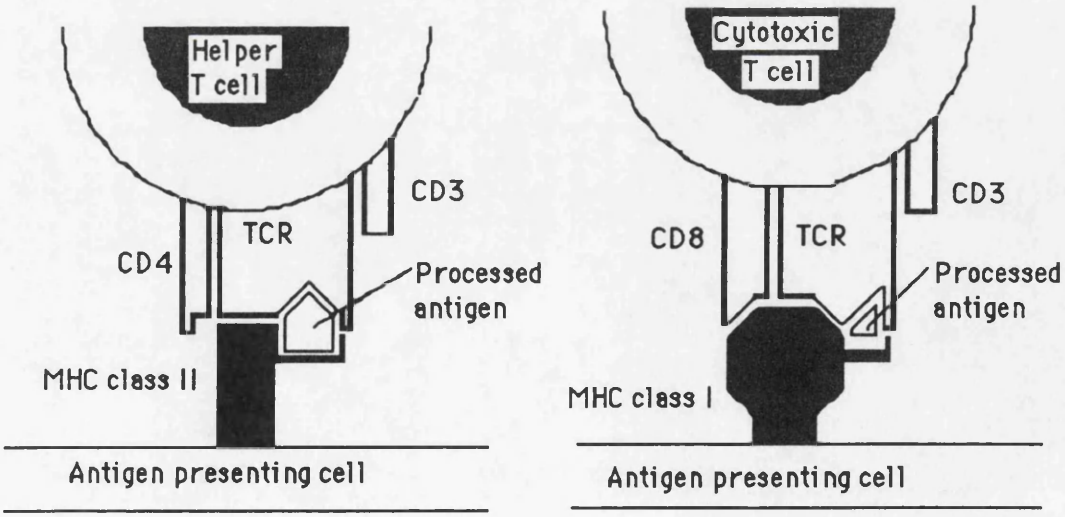


Figure 1.3 Function of the CD4 and CD8 molecules.

1.1.1.2.2 Regulation of T cell activation

The growth of many cells is regulated by intracellular molecules and integral membrane receptors which possess protein-tyrosine activity (Hunter and Cooper, 1985). T cell activation is a series of intracellular events, beginning with second messenger generation and ending with the development of effector functions and T cell proliferation. Current models of lymphocyte activation involve the relative roles of guanine nucleotide binding proteins (G-proteins) and *src*-related protein tyrosine kinases (PTKs) in antigen receptor-mediated signalling (Harnett and Rigley, 1992) followed by the release of intracellular Ca^{2+} and the activation of protein kinase C (Weiss *et al.*, 1986). Protein kinases have different kinds and each type may be responsible for phosphorylating different transcriptional regulatory proteins. Protein-tyrosine kinases are known to a effect on regulation of cell growth. At present the model of T cell activation is that, following ligation of the T cell antigen receptor, *src* - related PTKs - mediate regulation of PLC activation (Figure 1.4). The T cell receptor (TCR) is coupled to phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two potent intracellular second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Nishizuchi, 1988). IP3 can diffuse into the cytosol contributing to the release of Ca^{2+}, mostly from the endoplasmic reticulum. DAG, on the other hand, can bind and activate protein-kinase C (PKC), thereby initiating the phosphorylation of numerous substrates at serine and threonine residues. TCR can also couple to *ras* activation (Downward *et al.*, 1992) and the CD4/CD8 molecules which form a signalling complex, not only with the PTK *lck* (Section 1.1.1.2.3) but also with a novel 32 kDa GTP-binding protein (Telfer *et al.*, 1991). G protein regulation of intrinsic PTK growth factor is associated with T cells that are differentially coupled to inositol phosphate generation, depending on their state of activation or maturation.

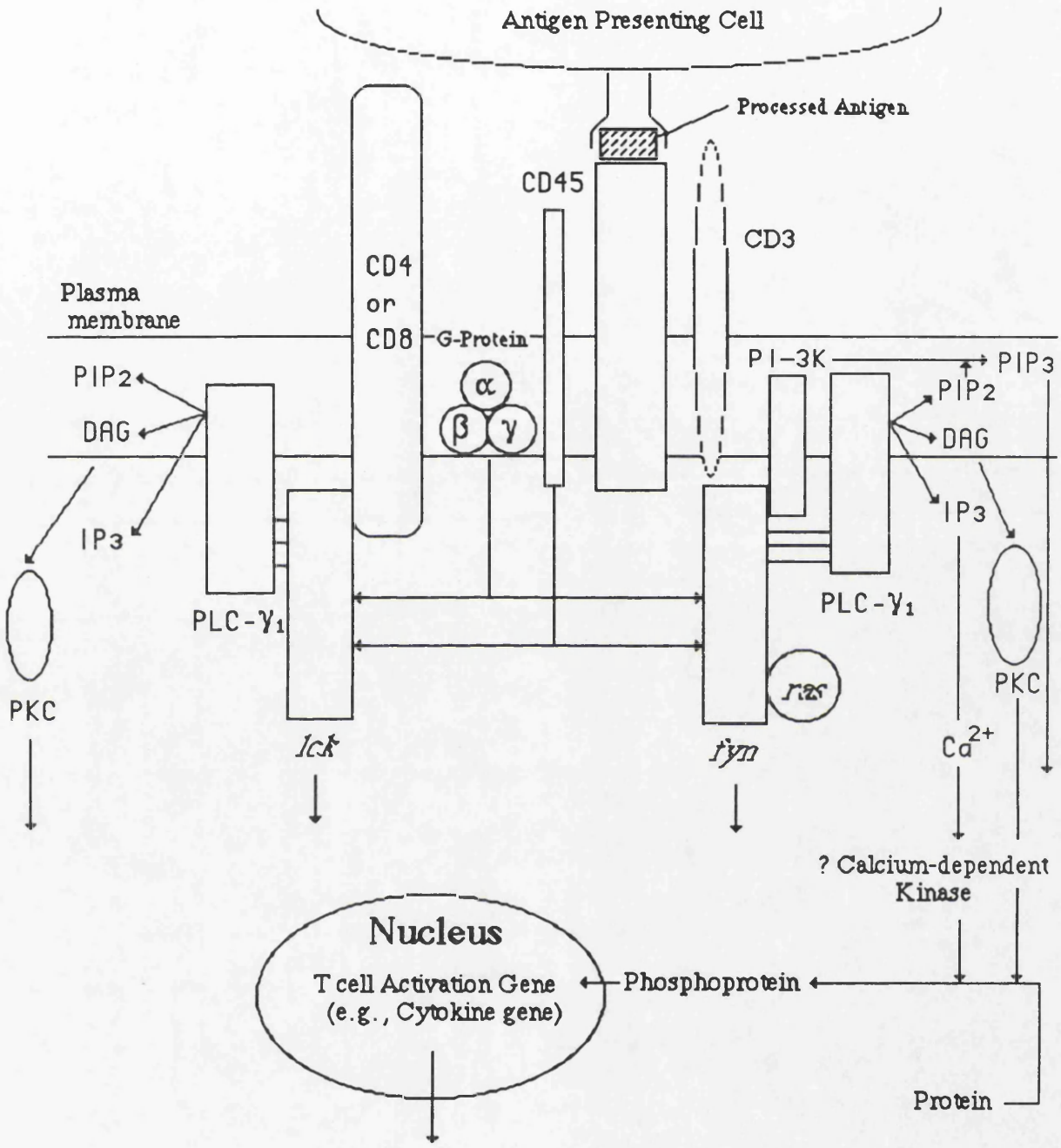


Figure 1.4 Model of G-protein/PTK mediated coupling of antigen receptors on T cells

1.1.1.2.3 The function of CD4/CD8 in human T cell activation

The primary pathway of clonal expansion occurs by means of antigen recognition by the T cell receptor (TCR)/CD3 complex (Section 1.1.1.2.4). Several accessory interactions involving CD4:MHC class II, CD8:MHC class I and LFA-1:ICAM-1 (Section 1.3) synergize with the TCR/CD3 complex in this response to foreign antigen. The CD4 and CD8 antigens abrogate or potentiate activation depending on their proximity to the TCR/CD3 complex. The effects of proximity might be also correlated with the tyrosine phosphorylation of the CD3 ζ chain (Samelson *et al.*, 1985). Cell adhesion and conjugate formation are important consequences of these binding events. These accessory interactions may also trigger intracellular signals that modify antigen-induced proliferation via the TCR/CD3 complex. Significantly, several structures (CD4, CD8 and CD45) define subsets of T cells with distinct functional programmes. CD4, CD8 and CD45 in conjunction with the TCR/CD3 complex appears to play a major role in regulating the activation and effector functions of distinct T-cell subsets. The leukocyte common antigen, CD45 has a variable ectodomain (Thomas 1989) and a constant cytoplasmic domain consisting of two tyrosine-specific phosphatase domains in tandem (Charbonneau *et al.*, 1988; Tonks *et al.*, 1989). CD45 has tyrosine-specific phosphatase activity (Tonks *et al.*, 1989) which plays a role in the process of signal transduction in the T-cell activation appearing to involve tyrosine kinases. Different CD45 isoforms associate differentially with the T cell receptor and with the coreceptors CD4 and CD8. CD45 is a tyrosine-specific phosphatase that occurs in various distinct isoforms on T cells with different functions or activation requirements. On naive T cells expressing the ABC isoforms, CD4, CD45, and the TCR all migrate independently on the T-cell surface (Dianzani *et al.*, 1992). On memory T cells expressing the null isoform of CD45, CD4, TCR and CD45 RO are all associated on the T-cell surface (Volarevic *et al.*, 1990). Finally, on Th2 cloned lines, an undefined low

molecular weight CD45 isoform binds to CD4 but this complex does not bind the TCR. T-cell activation occurs optimally when the TCR, CD4, and CD45 are brought together on the T-cell surface. This may reflect the interplay between the TCR and CD4 associated tyrosine kinases and the tyrosine phosphatase activity found on the cytoplasmic domains of CD45. CD4 and CD8 function is to associate with the T cell-specific protein-tyrosine kinase $p56^{lck}$. Engagement of the CD4/CD8 receptor by MHC antigens in conjunction with the TCR/CD3 complex would be expected to stimulate $p56^{lck}$ activity (Figure 1.5). The lck binds to a distinct sequence in the cytoplasmic tail of CD4 and CD8 (Shaw *et al.*, 1990; Turner *et al.*, 1990). The lck molecule contains a conserved carboxyl terminus, and the tyrosine at amino acid residue 505 is a regulatory tyrosine. The lck function is probably regulated through the phosphorylation and dephosphorylation of this regulatory Tyr^{505}. CD4 leads to increased levels of phosphorylation lck as well as increased kinase activity, while dephosphorylation of tyrosine 505 activates kinase activity in lck (Luo and Sefton, 1990). The coreceptor CD4 and CD8 participate in transmembrane signal transduction that correlate with tyrosine phosphorylation of the TCR ζ chain via $p56^{lck}$. The importance of this event in T-cell activation is not yet known. The CD4/CD8:$p56^{lck}$ complex may constitute the underlying molecular basis of the CD4 and CD8 function and their ability to synergize with the TCR/CD3 complex. Potentially important targets of the CD4- and CD8-associated $p56^{lck}$ are the CD3 subunits of the TCR/CD3 complex. $p56^{lck}$ could provide the molecular basis for an interaction between CD4/CD8 and the TCR/CD3 complex in the recognition of foreign antigen. The CD3 γ, δ and ε chains are also phosphorylated at tyrosine residues by the CD4/CD8:$p56^{lck}$ complex. The net result of CD3 phosphorylation may be to alter the inter-molecular associations within the TCR/CD3 complex leading to a cascade of events linked to DNA replication. Alternatively, CD3 could act to regulate the activity of the kinase and/or alter its substrate specificity. Surface

expression of CD4 antigen does not prevent the association of $p56^{lck}$ with the CD8 antigen, and vice versa.

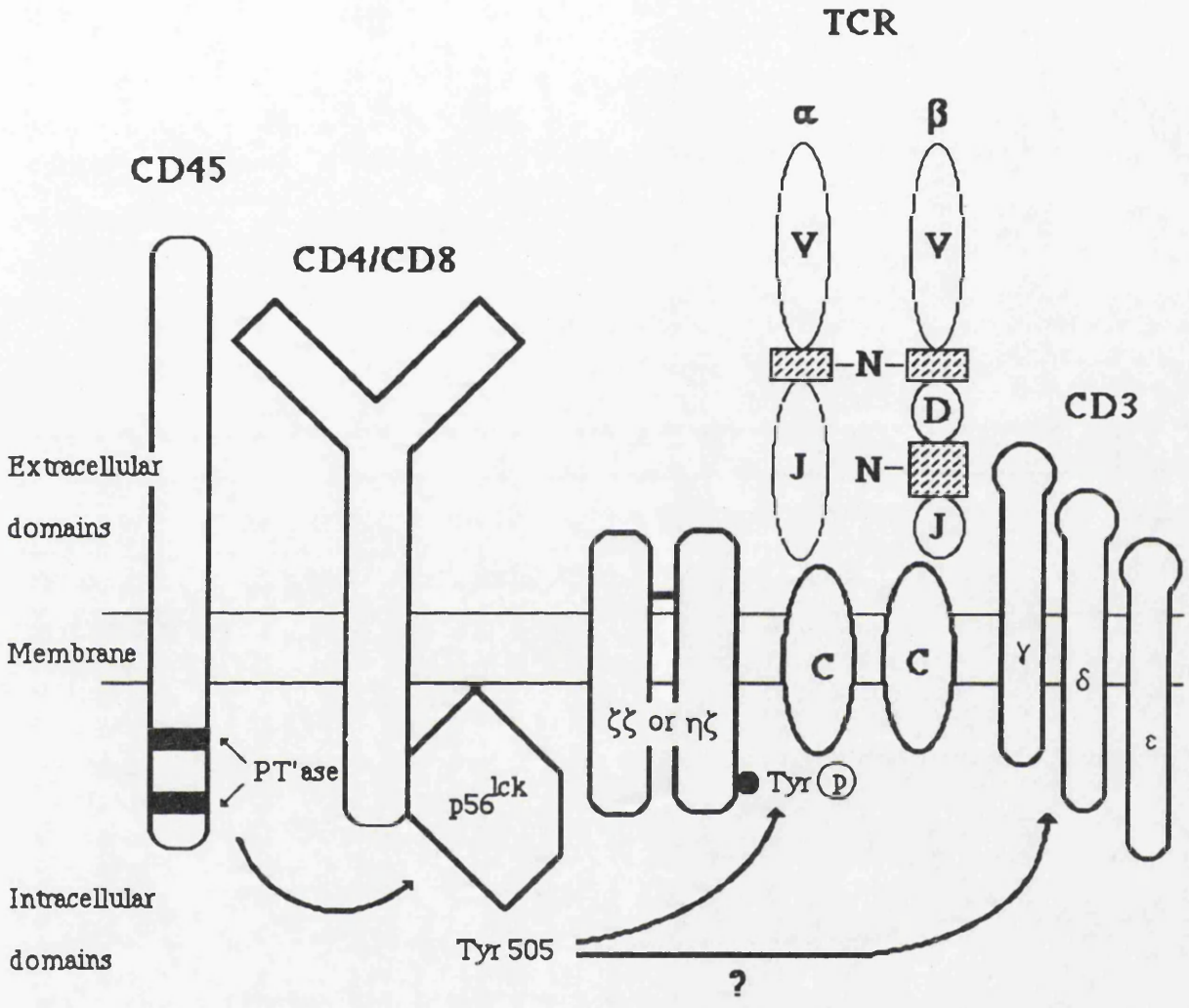


Figure 1.5 CD4/CD8:$p56^{lck}$ complex and its intracellular association with CD45 and the TCR/CD3 receptor complex in T-cell activation. T cell receptor polypeptides showing the contribution of variable(V), diversity(D), joining(J) and constant(C) gene segments. N represents areas of N region addition.

The association of $p56^{lck}$ with both antigens when expressed on the same cell is consistent with the notion that both receptors could utilize the kinase in signals driving the differentiation of $CD3^{\pm}CD4^{+}$ $CD8^{+}$ thymocytes to a more mature $CD3^{+}CD4^{+}CD8^{-}$ or $CD3^{+}CD4^{-}CD8^{+}$ phenotype.

1.1.1.2.4 The T cell receptor and the CD3 complex

Antigen-induced stimulation and subsequent events are directly linked to an interaction with the TCR/CD3 receptor complex. Subsequent biochemical analysis showed that the human antigen receptor is comprised of two subunits at 49 (a) and 42 kDa (b) that are disulphide linked to form a heterodimer of 80-90 kDa (Acuto *et al.*, 1983), and specifically bind antagonistic peptides presented by MHC molecules (Section 1.1.1.5) and a complex termed "CD3". Genetic analysis further revealed a close structural relationship between immunoglobulin and the T-cell receptor with each subunit being encoded by germline V, D, J segments which undergo rearrangement during T-cell differentiation (Hendrick *et al.*, 1984; Yanagi *et al.*, 1984). Transfection of cells with defined α and β chain cDNAs has revealed that the receptor can recognize both antigen and MHC specificities (Yague *et al.*, 1985). Other T-cell receptors termed γ/δ and $\gamma\gamma$ have been defined biochemically (Brenner *et al.*, 1987; Bank *et al.*, 1987; Borst *et al.*, 1987), and genetically (Saito *et al.*, 1984; Chien *et al.*, 1987). The various types of antigen receptors (α/β, γ/δ and $\gamma\gamma$) form multimeric complexes with the various chains of the CD3 complex. The same assortment of CD3 chains are associated with the α/β and γ/δ heterodimers which are closely related in regions proximal to the plasma membrane and in their cytoplasmic tails (Krissansen *et al.*, 1986).

The human T cell antigen receptor

The T cell receptor genes are split in the germline DNA into a number of gene segments comprising V, D, J, and C segments. These gene segments recombine in a developing T cell to produce a contiguous V(D)J exon. The α chain gene is located on chromosome 14 at 14q11 (Caccia *et al.*, 1985; Collins *et al.*, 1985) and the δ TCR locus lies between Vα and Jα. The TCR β chain gene complex spans around 600 kb on chromosome 7 (Lai *et al.*, 1988) and incorporates some 57 Vβ gene segments (Robinson, 1991). The γ-chain locus gene is localized to the short arm of chromosome 7 (Murre *et al.*, 1986). 8 of 14 Vγ segments are functional, located upstream of two sets of Jδ segments, each with a constant region complex. Cγ1 is composed of three exons: the first encodes the extracellular domain, the second codes for a connecting region, and the final exon encodes the transmembrane domain and cytoplasmic tail. The human δ chain gene complex lies within the chain complex, between the Vα and Jα segments (Hata *et al.*, 1987; Chien *et al.*, 1987). The γ gene is rearranged and expressed at an early stage in T cell development and forms either disulphide-linked or non-disulphide-linked dimers with the so-called δ protein. The four distinct T-cell antigen receptor polypeptides (α, β, γ, δ) form two heterodimers (α/β and γ/δ). The α/β heterodimer plays the part of recognition of antigenic peptides combining the MHC molecules, while the γ/δ TCR for antigen is expressed in only 1-2% of T lymphocytes in systemic lymphoid organs or blood of men. This minor fraction mainly consists of cells that carry the CD3 antigen, termed $CD3^+CD4^-$ $CD8^-$ or double-negative. Alam *et al.*, (1992) reported that the majority of cells in both PBL and LNL were "double negative" and in TILs, the CD8 marker was consistently present on a substantial proportion of $CD3^+$ γ/δ^+ cells in breast cancer patients. In humans there is no evidence of γ/δ T cells preferentially homing or localizating in epithelia (Itohara *et al.*, 1990).

The CD3 molecule

The CD3 molecule was initially identified as a cell surface antigen present on mature human thymocytes and peripheral T cells by T-cell specific monoclonal antibodies (Reinherz *et al.*, 1979). CD3 is actually a complex of 5 distinct polypeptides termed γ, δ, ε, ζ and η identified in noncovalent association with the TCR α/β. Molecular weights of these proteins range in size between 16,000 and Mr 28,000. Each of γ, δ and ε chains shows a high degree of sequence similarity to the others and the genes encoding these three subunits are clustered in a small region of human chromosome 11 (Clevers *et al.*, 1988; Gold *et al.*, 1987; Tunnacliffe *et al.*, 1987). The γ and δ subunits contain 1 to 3 extracellular N-glycosylated sites, while ε, ζ and η are non-glycosylated. In contrast to α/β chains, the mass of the polypeptide chains for each of these subunits is distributed between extracellular and cytoplasmic domains. The predominant form of ζ is as a homodimer and 10% of ζ is found as a heterodimer with a 22K protein, termed η (Orloff *et al.*, 1989). ζ chain has a 9 amino acid extracellular domain and a larger cytoplasmic domain of 112-113 amino acids. ζ chain plays a critical role in the signalling function of the TCR (Section 1.1.1.2.3.). When T cells are activated by antigen the ζ chain is phosphorylated on tyrosine residues. The cytoplasmic region of ζ chain has 7 tyrosine residues in human that are substrates for tyrosine protein kinases and also posses a ATP binding sequence (Weissman *et al.*, 1988).

1.1.1.3 T and B Cell Interaction

Early experiments by Mitchison (1971) demonstrated that T dependent antigens are involved in the recognition mechanism between B and T cells. Inside the macrophages and B cells, the bacterial toxins and other foreign antigens in the blood are degraded, and their processed peptides are then

transported to the surface by class II MHC molecules. Once TCR and the CD4 coreceptors of a pre-helper T cell are bound by the same MHC molecules on a presenting cell (Section 1.1.1.4.5.), the T cell starts to divide and produces active helper T cells. The dynamic interaction of multiple cell surface receptor-coreceptor pairs is involved in both signal transduction and adhesion for T-cell activation.

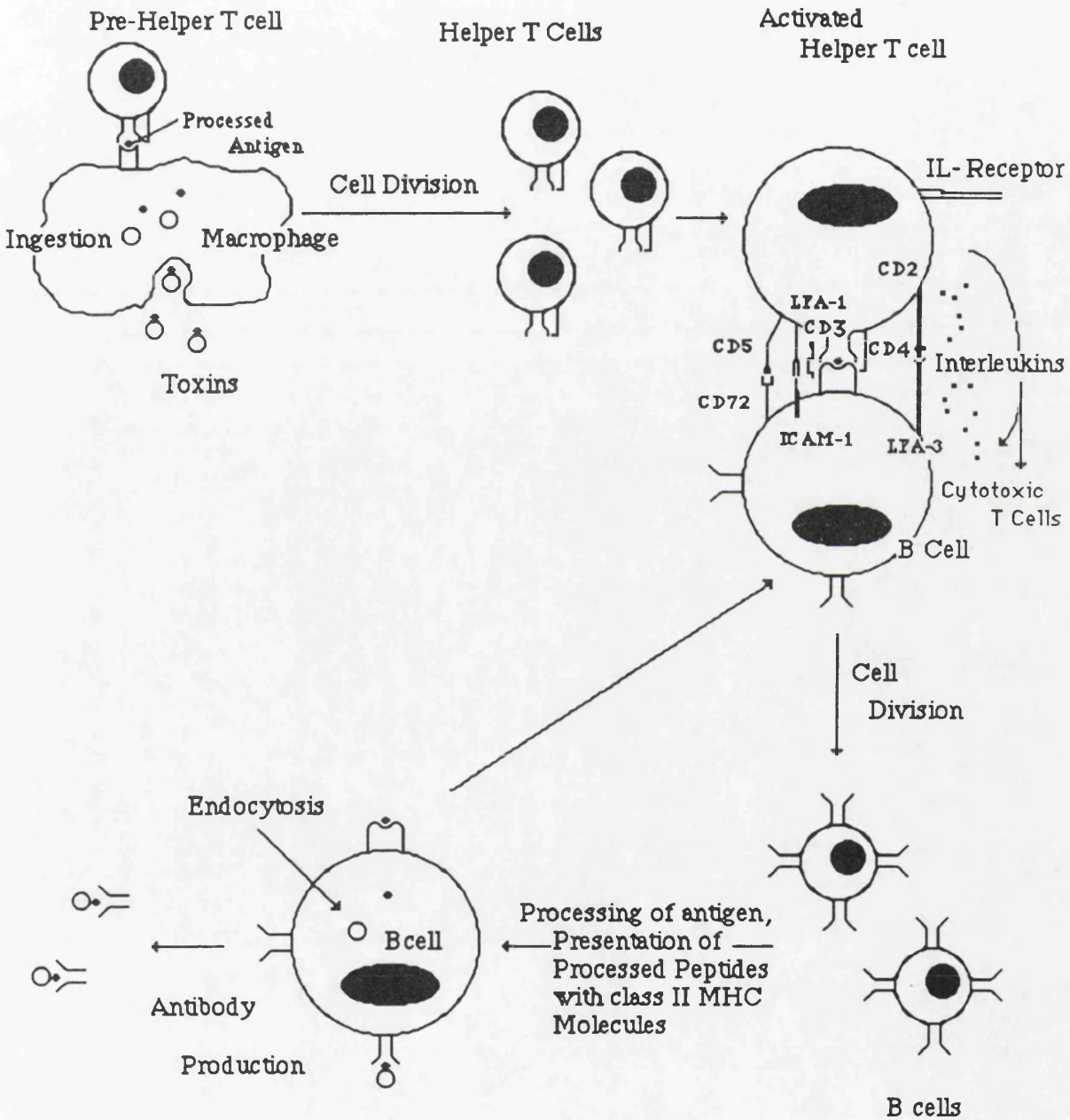


Figure 1.6 The response of helper T cells to antigens presented by B cells.

These accessory molecules specifically bind other molecules (ligand) present on the other cell surfaces, which result in increase the strength of adhesion between a T cell and B cell. And they are nonpolymorphic and invariant, which implies that have no capacity to specifically recognize many different and variable ligands. The active helper T cells produce a variety of lymphokines including interleukin(IL)-2,3,4,5,6, and 10, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor α (TNF-α), and interferon-gamma (IFN-γ). T cell-B cell interactions control lymphokine gene expression by regulating both the rate of transcription and mRNA stability (Fraser *et al.*, 1993) that further stimulate the B cells to divide and to secrete large amounts of their specific antibodies, which circulate freely in the blood, bind to the toxin and neutralise it (Boehmer and Kisielow, 1991) (Figure 1.6). The B lymphocyte in its resting state is little more than a nucleus surrounded by a thin enclosure of cytoplasm. Once a B cell meets a matching antigen, it develops an extended body containing polyribosomes, which make antibodies, and an elaborate channel system for exporting these antibodies (Nossal, 1993).

1.1.1.4 Major Histocompatibility (MHC) Antigens

The MHC class I and II molecules are highly polymorphic membrane glycoproteins. The alpha-beta receptors of T lymphocytes recognise antigen in association with the polymorphic products of the class I and II loci of the major histocompatibility complex. The molecular structure responsible for antigen presentation was revealed by resolution of the crystal analysis of the human class I molecule, HLA-A2 (Bjorkman *et al.*, 1987). The specificity of TCR recognition depends on both polymorphic residues in the antigen-binding groove and the primary sequence of the bound antigen-derived peptide.

The class I MHC α-chain is encoded on human chromosome 6 (HLA-A,B,C); that of the mouse are on chromosome 17 (H-2K,D,L) (Klein, 1986), and β2m is encoded by chromosomes 15 and 2, respectively. Invariant chain linked to the class II MHC molecule processing is encoded by human chromosome 5 and mouse chromosome 18. The human class II molecules are HLA-DR DQ, DP and the mouse are I-A and I-E. Each species has dozens of MHC loci, some of which contain pseudogenes. Klein (1979) distinguished two classes of MHC genes, class I and II, a distinction that well anticipated the different physiological function of the respective gene products.

1.1.1.4.1 Function of MHC

The function of MHC molecules is to collect peptide fragments of intact proteins inside the cell and transport them to the cell surface, where they can be surveyed by the T cells. The interaction between T-cell receptor(TCR), MHC molecule and the peptide presented by the MHC molecule is central to the cellular immune system of self/nonself discrimination. That is, MHC molecules function as the targets for immunologic rejection of transplants. Recognition of the appropriate peptide-MHC complex by the antigen specific receptor of T lymphocytes leads to cell proliferation and a cascade of cellular immune responses. The optimal peptide length for binding is between 8 and 12 amino acids with the central 5~7 residues contributing the majority of the specific contacts.

1.1.1.4.2 The structure of Class I MHC

MHC class I molecules consist of a polymorphic integral membrane glycoprotein heavy chain of about 46 kDa and the light chain, noncovalently associated

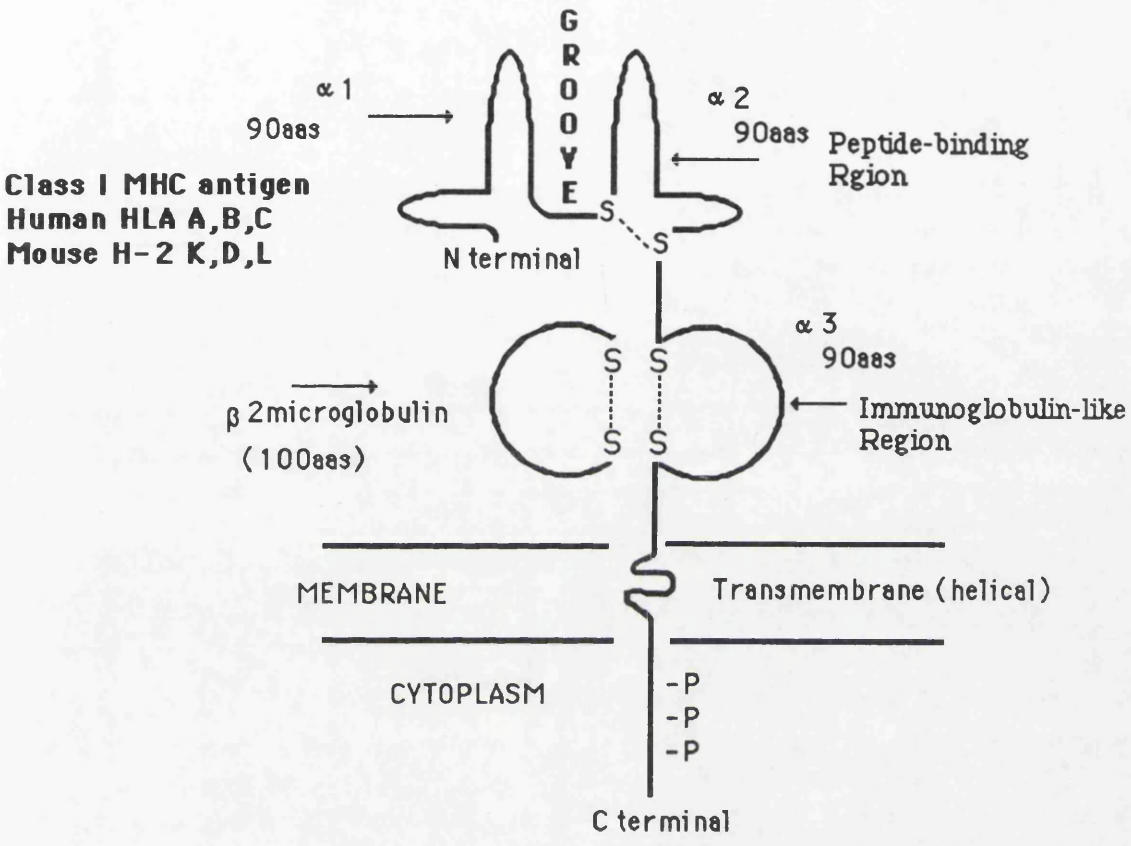


Figure 1.7 The structure of the class I MHC molecule and peptide receptor

with a 12 kDa soluble subunit, β2-microglobulin (β2m) (Bjorkman and Parham, 1990) (Figure 1.7). The heavy chain consists of three extracellular domains - the membrane distal, peptide binding region formed by the intimate

association of the α1 and α2 domains, and the membrane proximal, CD8-binding portion termed α3 (Germain and Margulies, 1993) linked with a cytoplasmic tail at the carboxyl terminus. Class I MHC heavy chains possess separate exons that each encode a distinct domain of the protein. β2m is a single, compact immunoglobulin-like domain. β2m makes contact not only with the immunoglobulin-like α3 domain, but also with the β sheet floor of the α1α2 peptide binding region. The α1α2 domain unit forms a peptide binding groove, as revealed by X-ray crystallography by Bjorkman and colleagues in 1987. The groove is bordered by two α-helices; the floor is a β-pleated sheet containing eight strands. Both amino and carboxyl termini of the peptide are tightly bound via H-bonds by conserved residues (Rammensee *et al.*, 1993). The peptide backbone can bend more or less, to accommodate a different number of amino acids between the fixed MHC sites binding peptide amino and carboxyl termini as recently visualised by X-ray crystallography (Guo *et al.*, 1992) depending on for many class I molecules, such as HLA-A68, HLA-B27 and HLA-A2.

1.1.1.4.3 The structure of Class II MHC

Class II molecules are heterodimers composed of a 33-35 kDa α-chain and a 25-29 kDa β chain. Class II HLA is polymorphic in the human population and different types selectively bind different sets of peptides (Chicz *et al.*, 1993) that may be responsible for variations in the immune response of different individuals to different antigens (Todd *et al.*, 1988). The Class II human leukocyte antigens(HLA) are cell surface αβ heterodimeric (Mr ~60,000) integral membrane proteins forming a groove that is similar to that of the class I HLA molecule (Brown *et al.*, 1988). The α2-and β2-domains have predicted homology to immunoglobulin domains. A structural

difference between class I and II is that in class II the ends of the groove appear to be open to allow overhanging of both amino acid and carboxyl termini of bound peptides. The length of 12-20 amino acid peptides is predicted for clsss II MHC molecule based on primary sequence (Brown *et al.*, 1988). Each dimer consists of one α and one β chain in noncovalent association (Figure 1.8).

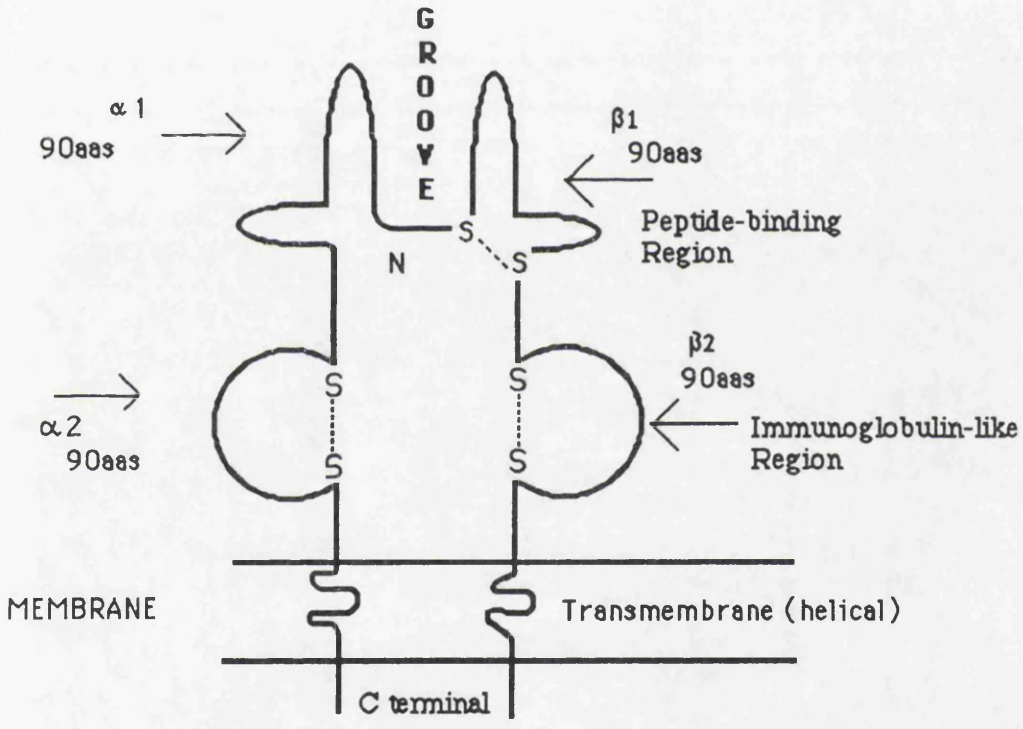


Figure 1.8 The structure of the class II MHC molecule and peptide receptor

The intron-exon organization of genes encoding class II α and β chains corresponds to functional domains in the protein molecule as with class I (Germain and Margulies, 1993). Recent X-ray crystallography work has shown that a parallel dimer of the class II HLA-DR1 αβ heterodimers suggests the capacity of class II HLA dimerization which could lead to an increased affinity for the CD4 coreceptor and to initiate the cytoplasmic signalling events in T-cell activation (Brown *et al.*, 1993).

1.1.1.4.4 Class I MHC antigen presentation

Cytosolic and nuclear proteins are degraded by large ATP-dependent proteosomes, and the resulting peptides are then translocated to the lumen of the endoplasmic reticulum (ER) membrane by a heterodimeric peptide transporter consisting of two multimembrane spanning proteins, TAP1 and TAP2. The peptides may be further trimmed in the lumen of the ER to proper class I binding peptides of restricted length (8~11 amino acids) (Falk *et al.*, 1991). At the same time MHC class I heavy (H) chain and the light chain, β2m, are synthesized in the ER and assemble, with the help of accessory molecules like p88 (88 kDa ER protein) (Degen *et al.*, 1992) that may participate in the correct folding of class I molecules, to a stable class I MHC heterotrimer (H chain, β2m and peptide) . The resulting class I heterotrimer is then transported through the Golgi apparatus and the trans-Golgi reticulum (TGR) to the cell surface after release from the ER (Neefjes and Ploegh, 1988) (Figure 1.9). The rate of intracellular transport and of assembly of class I molecules shows allele - specific differences (Degen and Williams, 1991).

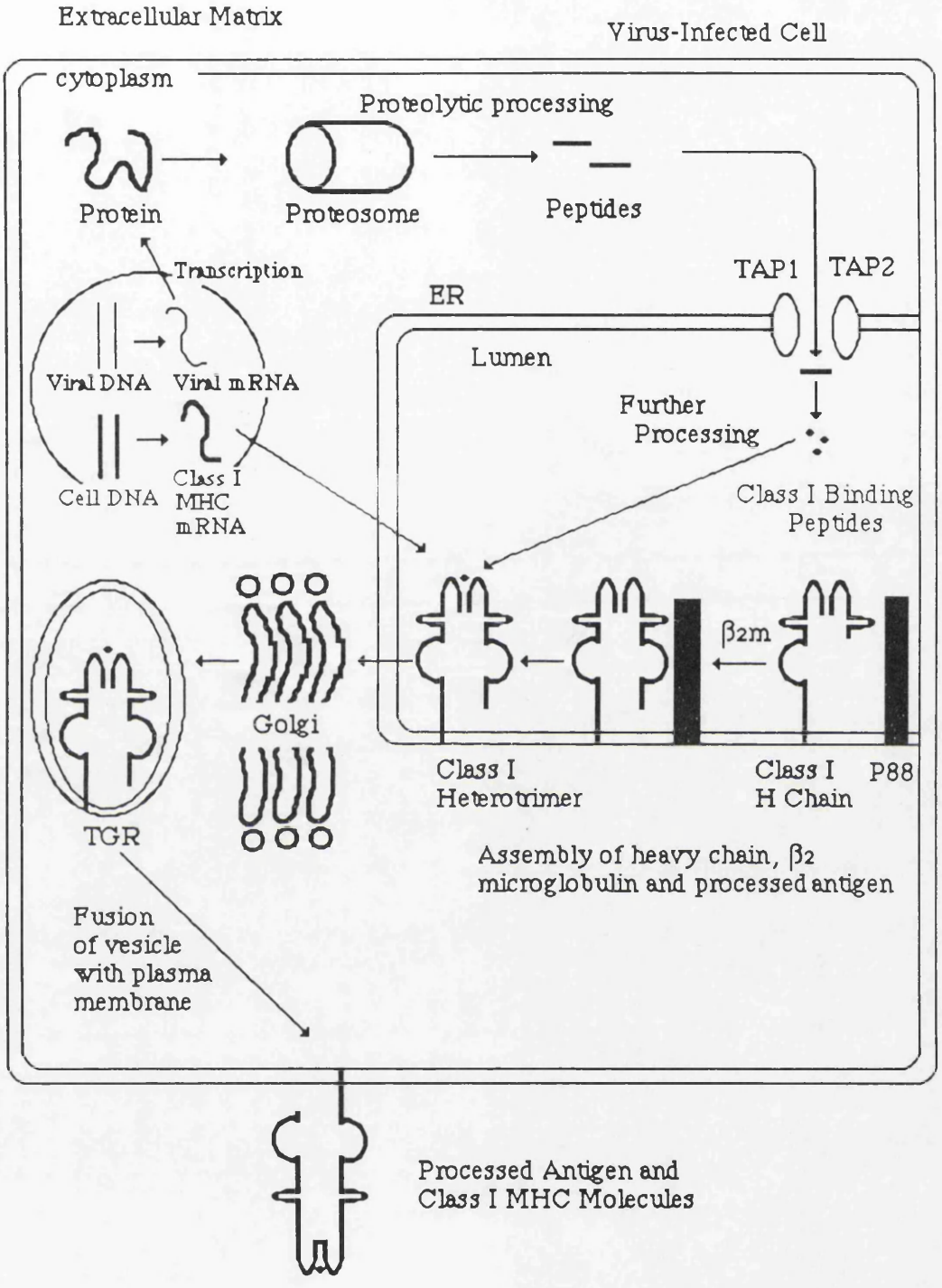


Figure 1.9 Schematic pathway of antigen processing and intracellular transport of the class I MHC molecules

1.1.1.4.5 Class II MHC Antigen Presentation

Class II MHC molecules generally present peptides derived from extracellular or surface proteins, in contrast to the antigen presentation of intracellular synthesised proteins by class I MHC molecules. These synthesized MHC class II molecules in the ER bind to an invariant chain which induces efficient transport of class II molecules from the ER (Anderson and Miller. 1992) (Figure 1.10). Invariant chain is a glycoprotein (31~43 kDa) composed of an extracellular carboxyl-terminus, a single transmembrane domain (~26 amino acids), and an amino-terminal cytoplasmic tail. The class II presentable peptides are generated by enzymes, cathepsin D (Van Noort *et al.*, 1991) as well as cathepsin E (Bennett *et al.*, 1992) which unfold antigens reducing disulfide bonds. Truncation of the cytoplasmic tail of the invariant chain by proteases (Blum and Cresswell, 1988) results in transport of the class II αβ-dimer associated with the truncated invariant chain to the cell surface (Roche *et al.*, 1992). The site of generation of presentable antigen for class II molecules is the lysosome (Harding *et al.*, 1991), so class II molecules can bind peptide in lysosomal structures (Peters *et al.*, 1991). MHC class II molecules are transported after release of invariant chain from the endosomal pathway to the cell surface. Soluble protein internalized by binding to surface Ig or to a soluble antibody-antigen complex to Fc receptors result in more efficient presentation of this antigen to CD4+ T cells (Lanzavecchia, 1988). The antibody can influence the degradation of bound antigen by protecting certain protease-sensitive sites (Davidson and Watts, 1989).

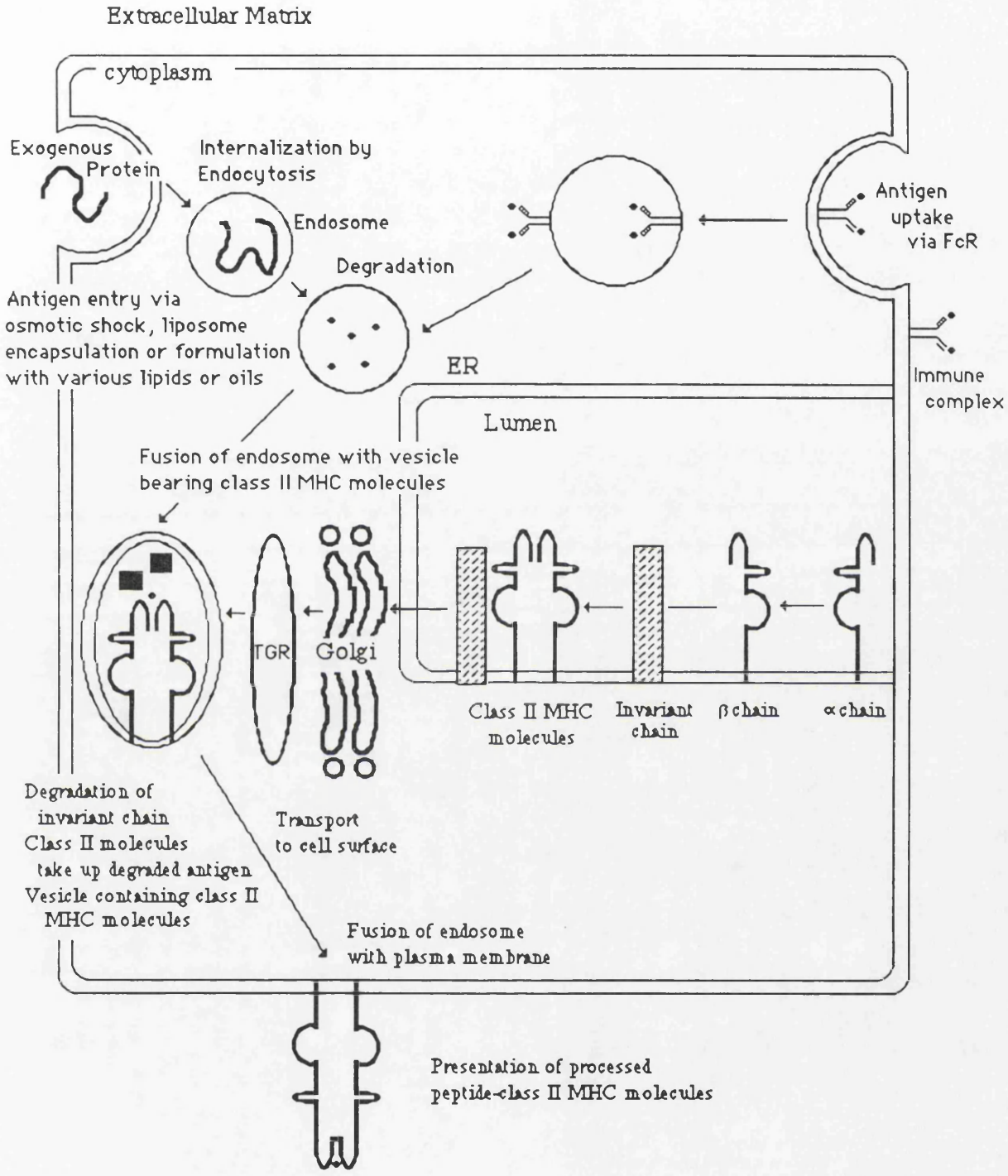


Figure 1.10 Schematic pathway of assembly and intracellular transport of MHC class II molecules

1.1.2 LYMPHOKINES

1.1.2.1 The Function of Lymphokines in Lymphocytes

Cytokines, as protein hormones, play a vital role in response to viral infection (as interferons), inflammation and immunity (as monokines and lymphokines) and hemopoiesis (as colony stimulating factors, CSFs) (Arai *et al.*, 1990). Cytokines produced by macrophage and endothelial cells are involved in the establishment of innate (or natural) immunity, whereas cytokines produced by activated T cells are the responsible for acquired (or specific) immunity. Cytokines are produced during the effector phases of innate and acquired immunity and function as server to mediate and regulate immune and inflammatory responses. Lymphokines are soluble protein mediators produced by activated T lymphocytes which participate in the regulation of activation and immune response of lymphocytes. These proteins are involved in lymphocyte activation, growth and differentiation as regulator, and in nonspecific inflammatory cells as activator by elicitating response to specific antigen recognition.

1.1.2.2 Interleukin-2

Interleukin-2 (IL-2), one of the first lymphokines to be identified originally called T cell growth factor (TCGF), has had a considerable impact on basic and clinical immunology (Rosenberg *et al.*, 1985). IL-2, produced by CD4+ T cells only upon activation by antigens, functions as an autocrine growth factor, and also acts on both CD4+ and CD8+ cells as paracrine growth factor. IL-2 plays a main role in the clonal expansion of activated T cells by interacting with the IL-2 receptor (Smith, 1988). Secreted IL-2 is a 14~17 kDa glycoprotein (monomer) encoded by a single gene on chromosome 4 in humans (Abbas *et al.*, 1991). The clonal proliferation of matured , resting T

cells is initiated via a process of signal transduction, in which the specific interaction of the antigen/MHC molecule with the TCR complex triggers the expression of IL-2 and its homologous receptor (IL-2R) (Taniguchi and Minami, 1993). The interaction of IL-2 with IL-2R leads to signal transduction pathways resulting in cell proliferation (Waldmann, 1989).

1.1.2.3 The Biological Effects of IL-2

IL-2 mediates growth and differentiation of human B cells (Waldmann *et al.*, 1984) as both a growth factor and a stimulus for antibody synthesis. IL-2 generates lymphokine activated killer cells (Grimm *et al.*, 1982) by stimulation of growth of NK cells, enhancement of their cytolytic function, and proliferation and maturation of oligodendroglial cells (Benvenisto and Merrill, 1986). IL-2 also stimulates T cell growth and synthesis of other T cell-derived cytokines such as IFN-γ and lymphotoxin. In addition, IL-2 can act in negative regulation of cell growth, programming T cells for apoptosis following TCR stimulation (Lenardo, 1991). So IL-2 is involved in delivery of various signals to a wide range of cell types through the interaction with IL-2 receptor (Minami *et al.*, 1993).

Cancer Immunotherapy using IL-2

IL-2 administration has become an clinical trial of adoptive immunotherapy of cancer. The incubation of lymphocytes in IL-2 results in the generation of activated lymphoid cells capable of lysing autologous or allogenic tumour cells (Lotze *et al.*, 1981 ; Chang and Rosenberg, 1989), but not normal cells. These lymphocyte-activated killing (LAK) cells can cause the regression of cancer in tumour-bearing host by tumour-specific killer cells

after selective expansion in vitro by culture in IL-2 (Rosenberg, 1989), but severe side effects are observed. The combination of both are more effective than either alone. Interferon- or tumour necrosis factor can upregulate class I MHC antigen expression on tumours (Houghton *et al.*, 1984), and they may exert synergistic in vivo antitumour effects for successful IL-2 therapy (Brouckaert *et al.*, 1986). Tumour-infiltrating lymphocytes (TILs) appear to be more specific and potent for individual tumours than LAK cells (Rosenberg, 1990 ; Yamaue *et al.*, 1990) and can traffic to the tumour site. Metastasis can be correlated with decreased IL-2 production in vivo, and reduced IL-2 production is more frequent in patients with a low CD4/CD8 ratio (Lissoni *et al.*, 1990).

1.1.2.4 IL-2 Receptors

The specific cell surface IL-2 receptor (IL-2R) that binds IL-2 is a true cytokine receptor complex, possessing three distinct polypeptides, IL-2Rα, IL-2Rβ and IL-2Rγ chains (Minami *et al.*, 1993) (Figure 1.11). The human IL-2Rα is a 55 kDa membrane glycoprotein (p55) capable of binding IL-2, originally described as Tac antigen or CD25 (Robb and Greene, 1983) on activated T cells. IL-2Rα is a mature protein 251 amino acids (aa) with a signal peptide of 21 aa in length and also contributes to the high-affinity receptor (Robb, 1986). Another component is the 75 kDa IL-2Rβ subunit (Takeshita, *et al.*, 1989) consisting of 286 amino acids (Hatakeyama *et al.*, 1989). The cytoplasmic region of IL-2Rβ can be divided into three subregions, serine-rich, acidic and proline-rich region, based on their amino acid compositions. IL-2Rβ binds IL-2 with intermediate affinity (Kd=1nM) and IL-2Rα with low affinity (Kd=10nM), but the noncovalent complex of the two chains gives rise to the high-affinity receptor complex. IL-2Rγ (p64) is involved in the formation of the functional intermediate- and high affinity IL-

2Rs and is required for the receptor-mediated internalisation of IL-2 (Takeshita *et al.*, 1992). Noncovalently associated IL-2Rα/β/γ heterotrimer forms a high-affinity receptor for IL-2.

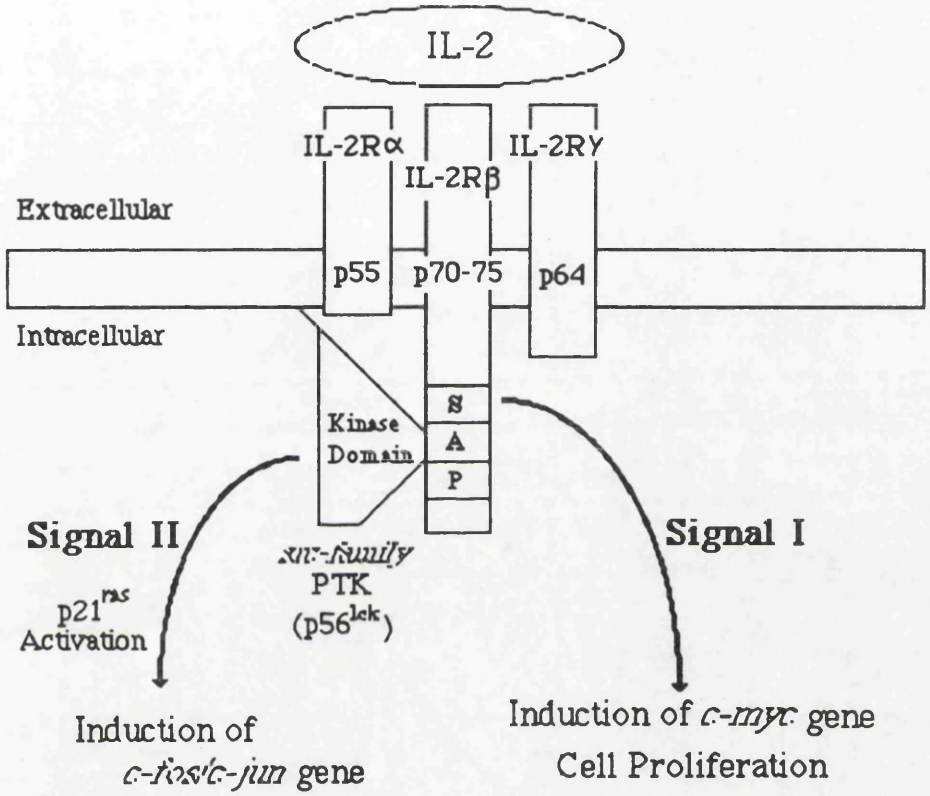


Figure 1.11 A schematic diagram of the high-affinity IL-2 receptors and two distinct signalling pathways

1.1.2.5 Signal Transduction in the IL-2 Receptors

The acidic-rich region of IL-2Rβ plays a critical role in mediating growth signal transduction via the T cell-specific tyrosine kinase $p56^{lck}$, a member of *src*-family PTKs, which is associated with the CD4/CD8 membrane proteins (Turner *et al.*, 1990), following IL-2 stimulation (Hatekayama *et al.*,

1991) that results in the increased tyrosine phosphorylation of cellular substrates. This pathway leads to the induction of the nuclear proto-oncogenes, *c-fos* and *c-jun* via the activation of p21, a membrane of the signal transducing GTP-binding proteins (Satoh *et al.*, 1991) in T lymphocytes (Cleveland *et al.*, 1987) and B lymphocytes (Kelly *et al.*, 1983). Another pathway is that the serine-rich region of IL-2Rβ leads to *c-myc* gene induction by an as yet unknown mechanism (Shibuya *et al.*, 1992) which functions upstream of certain cell cycle genes (Figure 1.11).

1.1.3 SUPERANTIGENS

Two groups of superantigens have been described so far : first, endogenous murine products (self-superantigens) that include the minor lymphocyte stimulating (Mls) determinants, and second, bacterial products (foreign superantigens) such as Staphylococcal enterotoxin (SE) (Herman *et al.*, 1990). Self- and foreign superantigens show some differences in their ability to stimulate T cells. CD4+ and CD8+ T cells respond to Mls determinants (Janeway *et al.*, 1980), and proliferate in response to the SEs (Fleischer and Schrezenmeier, 1988). Staphylococcal enterotoxin A (SEA), one of the most potent T-cell mitogens known ranges in size from 20 to 30 kDa, and interacts with class II MHC on antigen presenting cells and a region on the surface of one of the β-pleated sheets of the V_β domains of the TCR on T cells (Gascoigne and Ames, 1991; Pontzer *et al.*, 1992) (Figure 1.12). Recent experiments show that the Mls superantigen and SEA bind to at least one common region on class II MHC antigens (Torres *et al.*, 1993). Superantigen expression results in T cell proliferation and, during early ontogeny, T-cell deletion (Choi *et al.*, 1991). Superantigens indiscriminately activate any T cells whose receptors include selected V_β segments. Bound T cells secrete IL-2, resulting in abnormally high levels of the substance, and as a result,

massive numbers of T cells. They thereby activate millions of helper T cells, which may subsequently die, making them unavailable to combat later infections. This immune depression may help counteract autoimmunity (Johnson *et al.*, 1992).

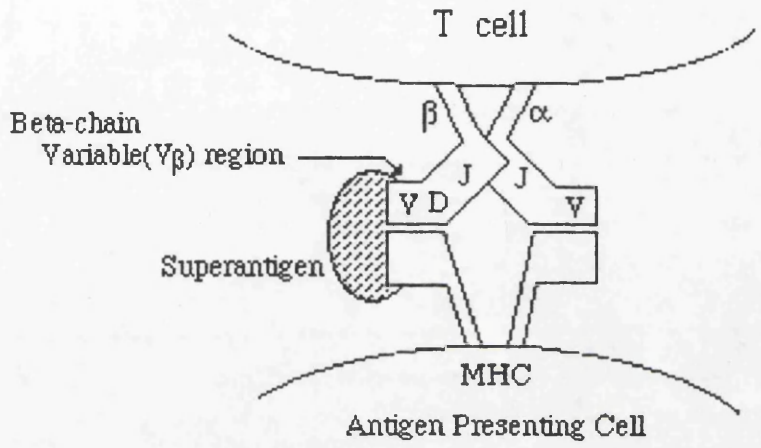


Figure 1. 12 Schematic diagram of the trimolecular complex

(superantigen/TCR/class II MHC)

1.2 Lymph Nodes

Most human immunological research is performed on blood which is readily accessible and can be sampled without surgical intervention. However, most of the important immunological activity occurs in specialised areas of the body such as the spleen, the thymus and the regional lymph nodes. The regional lymph nodes "drain" lymphatic fluid from areas of the body (the head, the chest, the gut etc.) and monitor it for the presence of foreign antigens.

The regional lymph nodes organise the local immune response in particular areas of the body. They do this either by recruiting newly emerged cells with the appropriate binding capacity to act within their area or by allowing the expansion of clones of cells with the appropriate binding capacity already within their area.

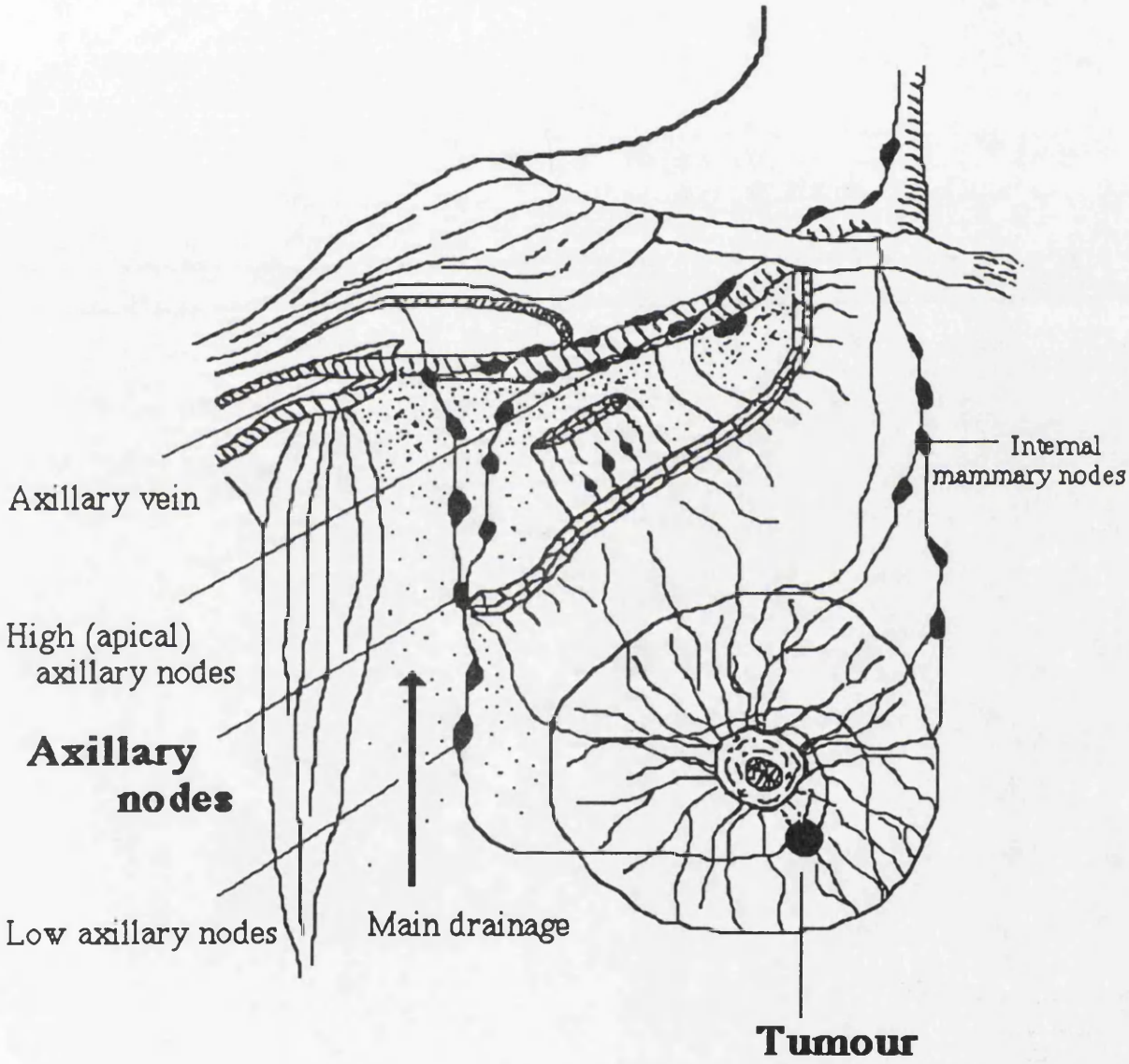


Figure 1. 13 Diagram of illustrating of lymphatic drainage of the breast cancer patient

Lymph nodes are distributed throughout the body, but particularly large and numerous lymph nodes are found in the armpits (axillary lymph nodes), groin (inguinal lymph nodes), in front of the abdominal aorta (coeliac lymph nodes), in the neck (cervical lymph nodes) and in the mesentery (mesenteric lymph nodes) (Klein, 1990). The axillary lymph nodes are located under the arm, variable in accessible number between individuals and only available as fresh tissue in cancer patients or kidney donors. They drain the chest/breast lymphatic fluid but not the lungs or more deeply situated tissues (Figure 1. 13). The axillary lymph node is a major contributing tissue in the organisation of regional immune responses in the body attracting lymphocytes bearing lymph node homing receptors (Section 1.4.2). Responses in such nodes can be precisely localised with one node showing strong immune involvement while a neighbouring node is quiescent. Similarly, in tumour patients, one or more nodes may be invaded by tumour cells while neighbouring nodes remain tumour free. The status of these nodes is important features in assessing the prognosis of a breast cancer patient. Therefore these tissues will be suitable samples for analysis of local immune response by lymphocytes in breast cancer patients.

1.2.1 Structure of Lymph Nodes

Lymph nodes are small nodular aggregates of lymphoid tissue, the filtering stations through which lymph percolates on its way to the blood stream. Each lymph node is enveloped by a fibrous thin capsule which sends many projections by numerous afferent lymphatics, which empty the lymph into a subcapsular sinus (Figure 1. 14).

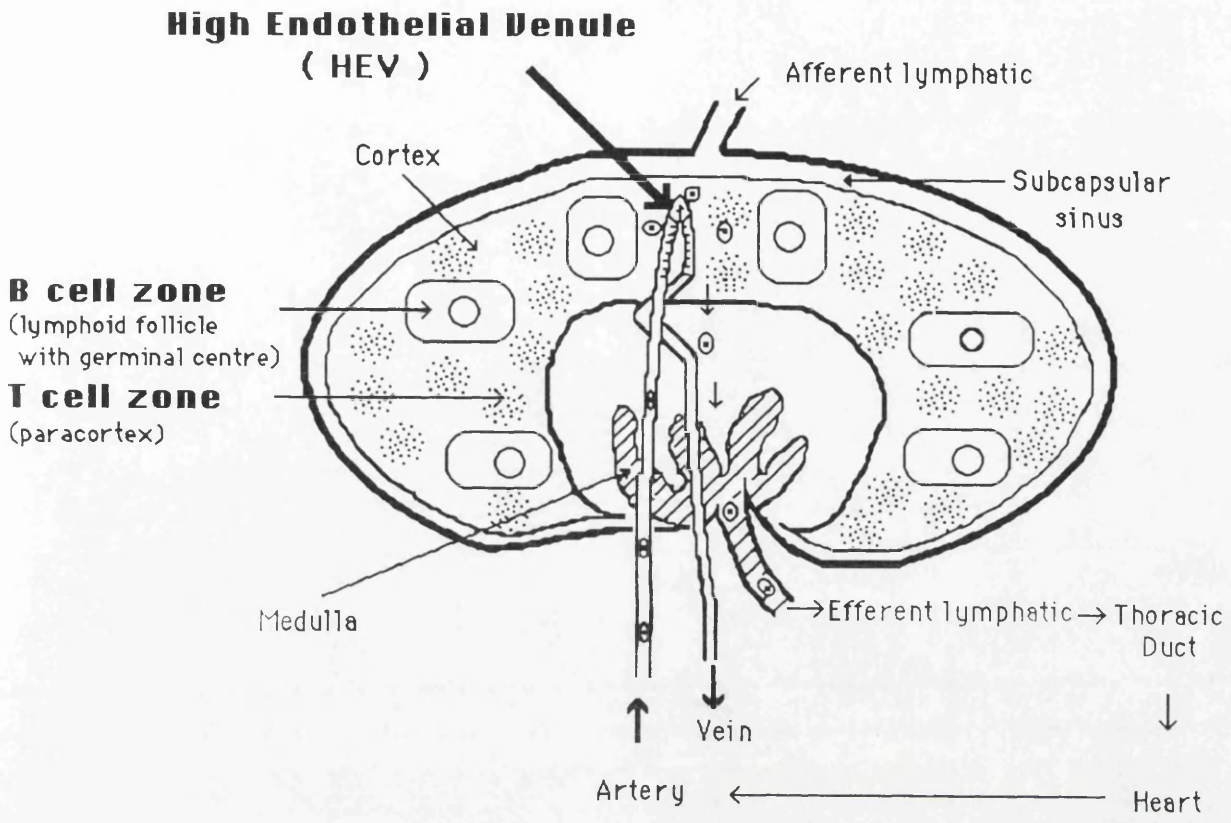


Figure 1. 14 The structure of human axillary lymph node and lymphocyte recirculation via the high endothelial venule

1.2.2 Lymphocyte subsets in Lymph Tissue

Lymph nodes have a defined structure. T lymphocytes, mainly CD4+ helper cells, are largely located in the interfollicular areas of the cortex and paracortical areas of the medulla (Figure 1. 14). The role of these cells may be to help the proliferation and differentiation of the antigen - stimulated B lymphocytes on the germinal centres of the follicles. The lymphoid follicle with its germinal centre is a site of intense cell proliferation and cell death. B -

cell follicles are found in secondary lymphoid organs throughout the body, and follicular B cells can take up antigen from follicular dendritic cells (FDCs) and process this into a form that will activate CD4+ T cells. Antigen-driven B - cell proliferation, somatic, positive and negative selection (Weiss *et al.*, 1990), and memory and plasma cell development appear to take place within the follicle (Liu *et al.*, 1992). Whitford *et al.* (1992) observed that the axillary lymph nodes are not only the major source of B cells but also the site of a large proportion of mature B cells with surface IgG in breast cancer patients, and moreover the proportion of HLA DR expressing T cells in the nodes are higher than those in the peripheral blood. This study also reported that CD4+ T cells expressing the CD25 IL-2 receptor are found to be in greater numbers than CD8+ T cells. Entry of lymphocytes into the node is reported to be initiated by a cell - cell adhesion mechanism operating between lymphocytes and High Endothelial Venules (HEV) within the nodes that then direct the cellular traffic.

1.2.3 Structure of HEV

HEV are found in all secondary lymphoid organs including lymph nodes, Peyer's patches, tonsils, adenoids, appendix, small aggregates associated with the mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts. But HEV are absent in the primary lymphoid organs (bone marrow and thymus) and are normally not present in nonlymphoid organs (Yednock and Rosen, 1989). HEV have distinctive morphological, ultrastructural, and biochemical characteristics that underlie their specialised function in lymphocyte adhesion and extravasation. High endothelial cells (HEC) have domed surfaces that protrude into the HEV lumen, and are cuboidal to columnar in height (10~12 μm) and are linked by discontinuous macular junctions at their apical and basal aspects. The HEV develop from flat endothelial venules in response to cytokines, such as γ interferon, that are

secreted by antigen-stimulated T cells. HEV express tissue-specific determinants or address signal for lymphocyte recognition. Carbohydrates may serve as essential recognition determinants of the HEV ligands. A further unusual property of HEC is their ability to synthesise and secrete a sulphated glycolipid, apparently in association with a nonsulphated glycoprotein (Andrews *et al.*, 1983). Specific HEV ligands appear to direct selectively the homing of particular lymphocytes to lymphoid tissues at distinct anatomical sites, "vascular addressins (VA)".

1.2.4 Vascular Addressins

Vascular addressins are expressed in an organ- or tissue-selective manner by a cellular or extracellular element of wider distribution, and have functionally related counterparts expressed by the same cellular or extracellular element in other tissue sites. They also mark address of such cellular or extracellular components for the purpose of directing cell-cell interactions or cellular positioning in the body. Streeter *et al.* (1988) defined a 58-66 kDa endothelial cell protein antigen expressed in mucosa-associated lymphoid organs, such as Peyer's patches, using Mab MECA-367, whereas MECA-79 monoclonal antibody recognises a glycoprotein of 92 kDa in peripheral lymph node (PLN) (Streeter *et al.*, 1988a) in the mouse and human. This mucosal vascular addressin, glycoprotein is a tissue-specific endothelial cell - adhesion molecule for lymphocytes and regulate lymphocyte traffic into mucosal tissues by mediating attachment of blood - borne cells to endothelium (Nakache *et al.*, 1989). Other researchers designated the endothelial - leukocyte adhesion molecule-1 (ELAM-1) recognised by Mab H18/7 and H4/18, which functions as an induced endothelial cell-surface molecule in IL-1, TNF, lymphotoxin and bacterial endotoxin that can support the adhesion of blood polymorphonuclear leukocytes (PMN) (Bevilacqua *et al.*, 1987). Recent works have shown that

several independent HEV ligands for the homing receptor (L-selectin) exist, some of which are tightly associated with the endothelial cell surface, such as the ~90 kDa sulphated ligands (Imai *et al.*, 1991), or the PLN addressins (Streeter *et al.*, 1988; 1988a), and the ~50 kDa - ligand (Brustein *et al.*, 1992), that are secreted.

1.3 Monoclonal Antibody Detection of Lymphocyte Populations in Human Axillary Lymph Nodes

Dual - colour parameter flow cytometric analysis of cell surface markers shows the different antigen densities on the membranes of lymphocytes in normal and cancer patients. The lymphocyte subpopulations of melanoma tumour - draining lymph nodes (Farzad *et al.*, 1990) and of early breast cancer patients in regional lymph node and peripheral blood (Morton *et al.*, 1986) have been analysed according to (a) whether or not the nodes contained metastatic tumour, (b) their distance from the primary tumour, and (c) pathological staging. There was a significant increase in CD8+ T cells associated with Stage II (with nodal metastases) in lymph node lymphocyte (LNL) populations, with corresponding decrease in CD4+ T cells. The CD4/CD8 ratio in Stage II was less than half that of Stage I (without metastasis). Lymph nodes closest to the tumour in Stage I patients contained a smaller percentage of CD19+ B cells. In Stage II, tumour - free nodes nearest to the tumour showed an increase in CD19+ B cells. There was also no significant difference between Stage I and II in peripheral blood T- or B-cells. These alterations in local regional immunocompetent cell subsets may be essential elements in the metastasis of tumour to the regional nodes and in the progression of the cancer.

1.4 Lymphocyte Homing and Adhesion Mechanisms

1.4.1 Lymphocyte Recirculation and Homing

The overall process by which the body's repertoire of immunocompetent lymphocytes is continuously cycled in and out of lymphoid organs is called "lymphocyte recirculation" (Figure 1.14). Whereas lymphocytes are generally thought to reside in the circulatory system, their protective function is more frequently required in the tissue. In consequence blood - borne lymphocytes move from the blood through the tissues and local secondary lymphoid organs such as the nodes or Peyer's patches (PP) in the gut, and then back to the blood via the efferent lymphatic ducts and the thoracic duct. On the other hand, the spleen is not supported by lymphatic vessels, and so migrating cells that extravasate in the spleen migrate directly back into the blood (i.e. intravasate). A single lymphocyte encountering a mass of infected cells can have little effect, but in the local node it can undergo clonal expansion to obtain reinforcements of the same specificity. In contrast, those lymphocytes which have not encountered antigen can pass through the node back to the blood vascular system, whereon they can enter the same or another lymphoid organ. This path of recirculation takes about 24 hours (Rosen, 1989). Thus a lymphocyte which has encountered antigen must have the ability to express receptors which enable it to home on the node rather pass through it. That is, the selective migration of lymphocyte populations to particular anatomical sites is referred as "homing". Homing mechanisms play a role in the maintain of specialised microenvironments, such as distinct complement of lymphocyte subsets and localisation in particular tissues, and are critical for the dispersal and targeting of naive and memory lymphocyte populations that are required for effective immune inspection. The physiology of lymphocyte homing/recirculation is largely determined by the differential regulation of lymphocyte adhesion and deadhesion.

1.4.2 Homing Receptors

A number of homing receptors (Figure 1.15) have been identified by laboratories, as evidenced by the ability of specific antibodies to block lymphocyte - high endothelial cell binding in PLN or gut-associated organs (Table 1.1).

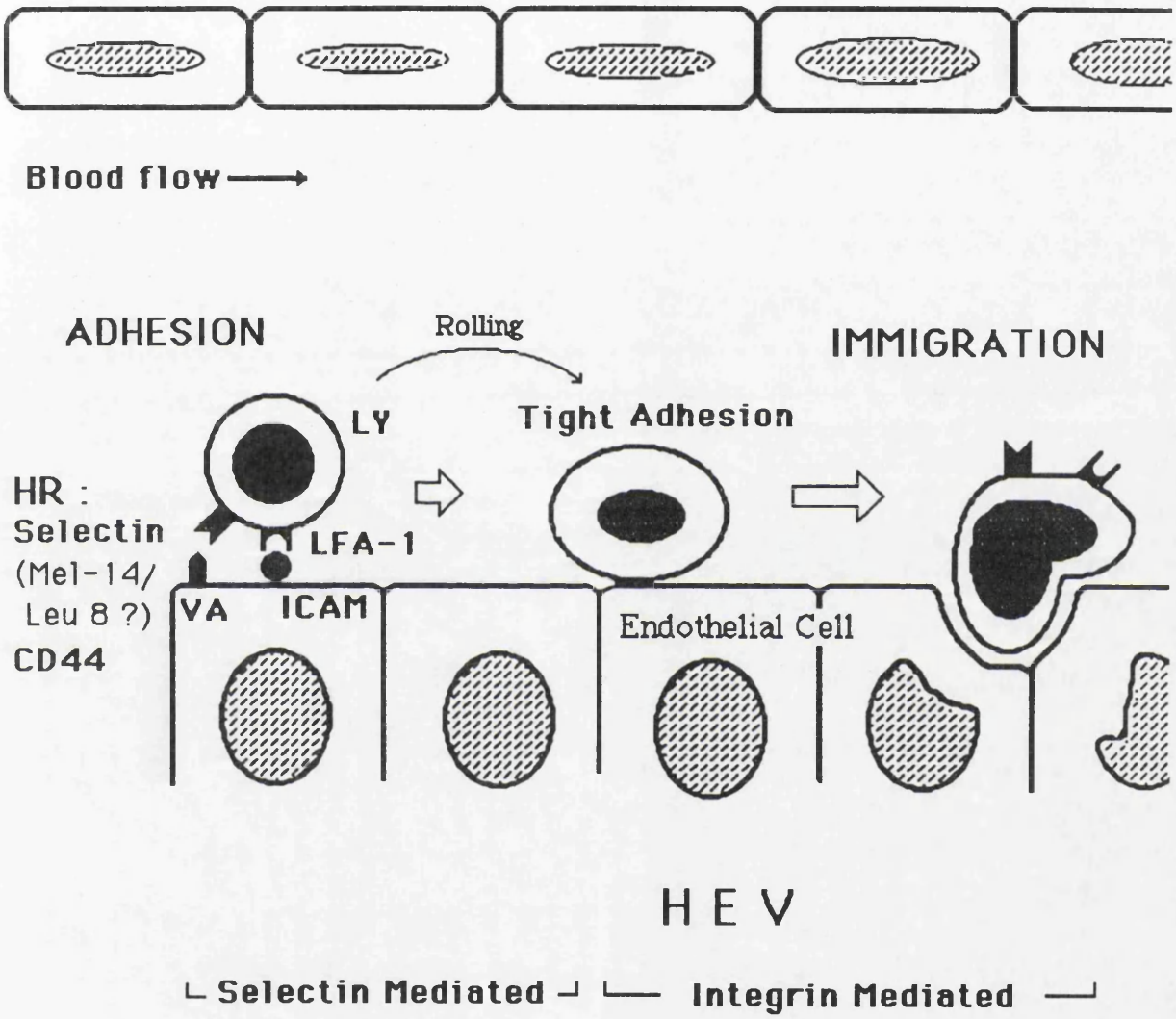


Figure 1.15 Diagram of involvement of homing receptors in adhesion between lymphocyte and HEV, and cell migration

Table 1.1 Summary of some immunologically relevant cell surface molecules proposed to be involved in lymphocyte-HEV interactions based on antibody inhibition studies.

Cell Surface Molecule	Other Names	M.W. (kDa)	Species	Family	Antibodies	Associated Molecules	Ref
Mel-14	DREG 56, LAM-1, LECAM-1 LEC-CAM-1, Leu-8(?), TQ1 L - Selectin	80 - 95	Mouse	L - Selectin	Mel-14	MECA-79 Carbohydrate ($sLewis^x$ Determinant), E - Selectin	1
Leu-8	DREG 56, LAM-1, LECAM-1 LEC-CAM-1, Mel-14(?), TQ1 L - Selectin		Human	L - Selectin	Leu-8		2
CD44	pgp-1, Ly-24, $gp90^{Hermes}$	85 - 95	Human	Proteoglycan	Hermes-1,2,3 Polyclonal anti-$gp90^{Hermes}$	Hyaluronate, Collagen, Fibronectin, MIP-1β Chemokin	3
$HEBF_{PP}$		80	Rat		1B.2		4
$HEBF_{LN}$		40, 63, 135	Rat		A.11		5
CT - 4		30	Guinea Pig		CT 4		6

References:

1. Gallatin *et al.*, 1983; Foxall *et al.*, 1992; Picker *et al.*, 1991
2. Carmerini *et al.*, 1989; Bowen *et al.*, 1989; Tedder *et al.*, 1990
3. Jalkanen *et al.*, 1987; Toyama-Sorimachi *et al.*, 1993
4. Chin *et al.*, 1986
5. Rasmussen *et al.*, 1985
6. Kraal *et al.*, 1986

Abbreviations:

LEC-CAM : Lectin-EGF-Complement-Cell-Adhesion-Molecule
LAM : Leukocyte Adhesion Molecule
LECAM : Leukocyte-Endothelial Cell Adhesion Molecule
HEBF : High Endothelial Binding Factor

1.4.2.1 MEL-14

MEL-14 is an murine monoclonal antibody which was first described as defining a cell surface molecule which was specific to lymphocytes which have the capacity to home to the nodes in the mouse (Gallatin *et al.*, 1983). This MEL-14 antigen was isolated and sequenced independently (Siegelman *et al.*, 1989; Lasky et al., 1989). MEL-14 inhibits human lymphocyte attachment to PLN HEV and has no effect on human lymphocyte attachment to mouse PP HEV or to HEV in the human appendix. Hermes-1 (Section 1.4.2.3) and MEL-14 both precipitate a 90 kDa protein from the surface of human lymphocytes. The approximately 120 amino-terminal amino acids resemble the carbohydrate binding domain of a variety of Ca^{2+}-dependent lectins (Drickamer, 1988), expressed at calcium concentrations of 10^{-4} M or greater. This cell surface glycoprotein ($gp90^{MEL}$), binding to phosphomannosyl residues (Yednock *et al.*, 1987), directly bridges lymphocytes to the HEV in peripheral lymph node (PLN), which shows specific sugars and polysaccharides can block the biological activity of the isolated receptor (Geoffroy and Rosen, 1989). Adjacent to the lectin domain is an epidermal growth factor - like domain of 33 amino acids, and closest to the membrane are two identical repeats of 62 amino acids (Figure 1. 16). These sequence repeats resemble a motif found in a number of proteins, including the complement regulatory proteins that bind C3 and C4, and the IL-2R. MEL-14 antigen has ten potential N-glycosylation sites, six of which are in the complement - binding homology repeats. There are no clusters of serines and threonines, which is consistent with the lack of O - glycosylation (Reviewed by Coombe and Rider, 1989). Data from cloning of these antigens show that the structure of CD44 (Section 1.4.2.3) and the MEL-14 antigen is different. Siegelman *et al.* (1990) suggest that lymphocyte cell surface marker Ly-22 epitope (Toda *et al.*, 1983) is an allelic determinant on the mouse lymph node homing receptor resulting from a single amino acid interchange within the EGF

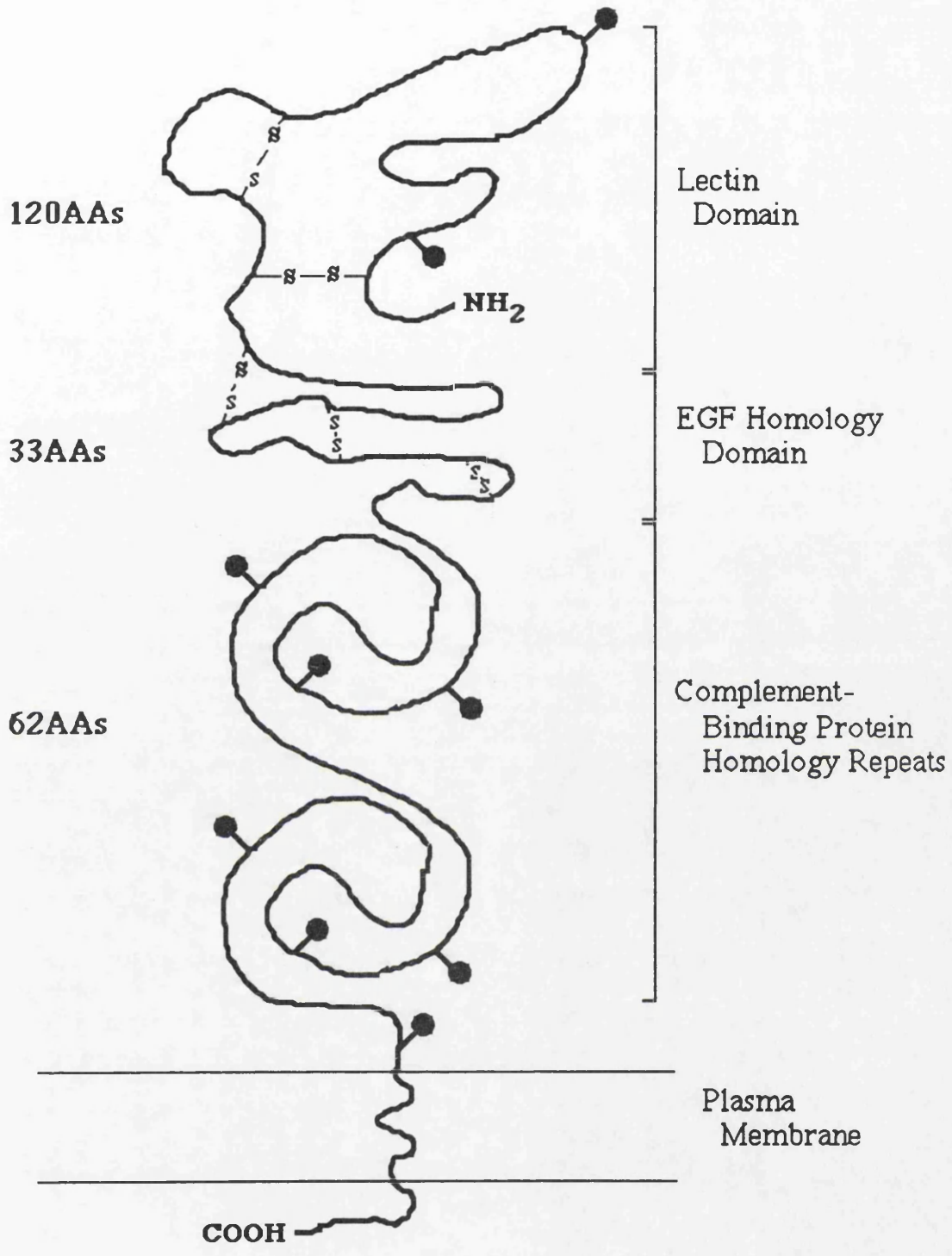


Figure 1.16 Diagram of structure of MEL-14 antigen.
—● ; Putative N-glycosylation site. Disulphide bonding of cysteine may also occur in the complement-binding protein homology repeat domains

domain, and this localisation of the Ly-22 have a role of participation of the EGF domain in the adhesion of lymphocyte to HEV. MEL-14 antigen, endothelial leukocyte adhesion molecule-1 (ELAM-1) and granule membrane protein (GMP-140), molecular weight 140 kDa, share a common organisation of homology units by the data from recent cloning. MEL-14 antigen adhesion molecule may be expressed on HEV-like vessels at sites of *Bordetella pertussis* vaccine (BPV) - induced chronic inflammation (Dawson *et al.*, 1992).

1.4.2.2 LEU-8

The LEU-8 molecule (L-selectin, LECAM-1) is distributed on many kinds of leukocytes and acts as a marker of different lymphocyte subsets. CD4+ LEU-8$^-$ T cells show high helper activity (Kanof *et al.*, 1987) and MHC class II-restricted cytolytic activity (Takada *et al.*, 1989), whereas CD4+ LEU-8$^+$ T cells show both a suppressor - inducer activity (Damle *et al.*, 1984). These data show that LEU-8$^+$ T cells in the CD4+ T cell lineage may have either suppressor or helper function. In addition, LEU-8$^+$ B cells have a higher response to *Staphylococcus aureus* Cowan-I (SAC) activation than LEU-8$^-$ B cells (Kansas *et al.*, 1985). Thus the interaction of LEU-8 with its ligand has the effect on signal transduction during T and B cell activation and differentiation (Murakawa *et al.*, 1991). Murakawa *et al.* (1992) suggest that LEU-8 participates in the activation of T cells via its ability to association with the TCR/CD3 complex.

This cell surface molecule recognised by the monoclonal antibody LEU-8 (as well as TQ1, LAM-1, DREG Mab) (Gallatin *et al.*, 1983; Lasky *et al.*, 1989; Siegelman *et al.*, 1989; Kishimoto *et al.*, 1990) has been reported to identify the human homologue of the MEL-14 lymph node homing receptor by sequence comparison of the two antigens (Camerini *et al.*, 1989; Tedder *et al.*,

1990; Bowen *et al.*, 1989), but it has never been directly tested for its ability to specifically detect lymphocytes which home to human lymph nodes. This study set out to test whether it had an obvious homing function in humans using two - colour flow cytometry (Section 2.2.3.4.4) to measure the LEU-8$^+$ populations on human axillary lymph nodes and peripheral blood.

1.4.2.3 CD 44

In humans, the CD44 (as well as Hermes, pgp-1, HUTCH-1, ECM-III, In (Lu)-related p80) cell surface antigen is involved in lymphocyte binding to HEV, lymphohemopoiesis (Miyake *et al.*, 1990), tumour metastasis (Section 1.4.3) and cell activation (Haynes *et al.*, 1989; Coombe and Rider, 1989). Unlike the MEL-14 antigen, CD44 is a ubiquitous glycoprotein molecule found in different organs and tissues (Dalchau *et al.*, 1980), and existing in different isoforms, that derive from the differential splicing of a single CD44 gene transcript, diversified molecular weight and numerous functional roles.

The CD44 molecule was originally described by Dalchau *et al.* (1980) as a human molecule defined by monoclonal antibody F10-44-2. F10-44-2 antigen, encoded in the short arm of human chromosome 11 (Goodfellow *et al.*, 1982), is expressed during T-cell maturation and widely distributed on T cells, granulocytes, brain cells and on cortical thymocytes (Flanagan *et al.*, 1989). Other groups had described a polymorphic major surface glycoprotein of 80 kDa, expressed on T-cell precursors from mouse 3T3 cells, named phagocytic glycoprotein-1 (pgp-1), which was encoded in murine chromosome 2 (Hughes and August, 1981; Hughes *et al.*, 1983; Jacobson *et al.*, 1984). This pgp-1 antigen was subsequently named Ly 24 and has at least two alleles, such as Ly-24.1 and Ly-24.2 according to various mouse strains (Lynch and Ceredig, 1989). Also in 1982, Carter described a 90 kDa surface molecule of human

fibroblasts, called extracellular matrix receptor type III (ECM-III), that was associated with the cytoskeleton, and bound both types I and VI collagen and fibronectin (Carter and Wayner, 1988). Haynes *et al.* (1983) identified an 80 kDa molecule (p80) on erythrocytes and T cells, regulated by the rare autosomal dominant Lutheran inhibitor (In(Lu)) gene (Haynes *et al.*, 1983). This antigen is required during intrathymic T-cell maturation and involved in cell adhesion and T-cell activation. In 1986, Jalkanen *et al.* described the Hermes series of antibodies (Hermes 1, 2 and 3) mediating lymphocyte - endothelial cell binding. Hermes-1 reacts with lymphoma cell lines that bind to either PLN or PP HEV, and Hermes-3 blocks the binding of lymphocytes to HEV in either mouse PP or human appendix, but not to mouse or human PLN HEV. Hermes-1 and Hermes-3 have different HEV-binding specificities. Gallatin *et al.* described a p90 molecule called HUTCH-1 involved in lymphocyte-endothelial cell binding (Gallatin *et al.*, 1989) and upregulated in T-cell activation. At the Third International Workshop on Leukocyte Differentiation Antigens, this molecule was named CD44 in humans.

The CD44 molecule is an acidic, sulphated protein that is synthesised with the addition of chondroitin sulphate side chains yielding a higher molecular weight form of 180~200 kDa, that contains O-linked and N-linked oligosaccharide side chains, multiple phosphoserine residues and several intrachain disulphide bonds (Jalkanen *et al.*, 1988) (Figure 1. 17). A subset of lymphocyte CD44 molecules is modified by covalent linkage of chondroitin sulphate (Jalkanen *et al.*, 1988). Chondroitin sulphate is involved in endothelial cell recognition of lymphocytes. The chondroitin sulphate form of invariant chain can function as an accessory molecule at the cell surface, facilitating T cell interaction with APCs through interaction with CD44 (Naujokas *et al.*, 1993). The binding site on fibronectin is the COOH-terminal heparin binding domain (Jalkanen and Jalkanen, 1992). CD44 has significant homology to functional domains in the cartilage link protein and the

cartilage proteoglycan monomer for hyaluronate receptor. Link proteins can also bind to native collagen (Chandrasekhar *et al.*, 1983). Thus, CD44 linked with cartilage protein in the amino - terminal sequence by differential glycosylation or expression of structurally similar CD44 variants (Section 1.4.3) represents a polymorphic family of cell adhesion molecules for diverse cell - cell and cell - matrix interactions. Comparison of cloning and sequencing with MEL-14 gene shows that CD44 is structurally unrelated to MEL-14 (Siegelman *et al.*, 1989). The cytoplasmic tail domain has a variable length of three residues (Goldstein *et al.*, 1989) or 72 amino acids (Stamenkovic *et al.*, 1989) by spliced exons encoding the carboxyl - terminal sequences. Alteration in the length of the cytoplasmic tail may modulate intracellular signalling, as the cytoplasmic tail has potential sites for the action of both protein kinase A and C (Camp *et al.*, 1991). The larger cytoplasmic tail is involved in the interaction of CD44 with cytoskeletal elements (Kalomiris and Bourguigon, 1989) which has functional implications in cell adhesion and migration. The effective organ specific component of lymphocyte - HEV interaction is mediated by synergy of multiple adhesion molecules, such as VLA-4, CD44 and other molecules (Holzmann *et al.*, 1989). That is, CD44 regulates the rapid appearance of Ca^{2+}-, Mg^{2+}-independent, LFA-1/ICAM-1, CD2/LFA-3, and VLA-4/VCAM-1-independent adhesion in lymphocyte - endothelial cell interaction (Toyama-Sorimachi *et al.*, 1993). Soluble circulating CD44 molecules potentially regulate lymphocyte homing and other types of CD44 - mediated cellular interactions. Expression of CD44 is dependent on the stage of T-cell differentiation, and upregulation of CD44 expression on T cells is an early event during T-cell activation via the CD3 - TCR complex (Willeford *et al.*, 1989). The ligated CD44 molecule initiates an activation cascade for a variety of T cell - mediated functions that may use protein tyrosine kinase - dependent signal transduction pathways at least in part similar to those of the CD3/TCR complex (Galandrini *et al.*, 1993), and can

direct a transmembrane signal for natural killer (NK) cell activation (Tan *et al.*, 1993).

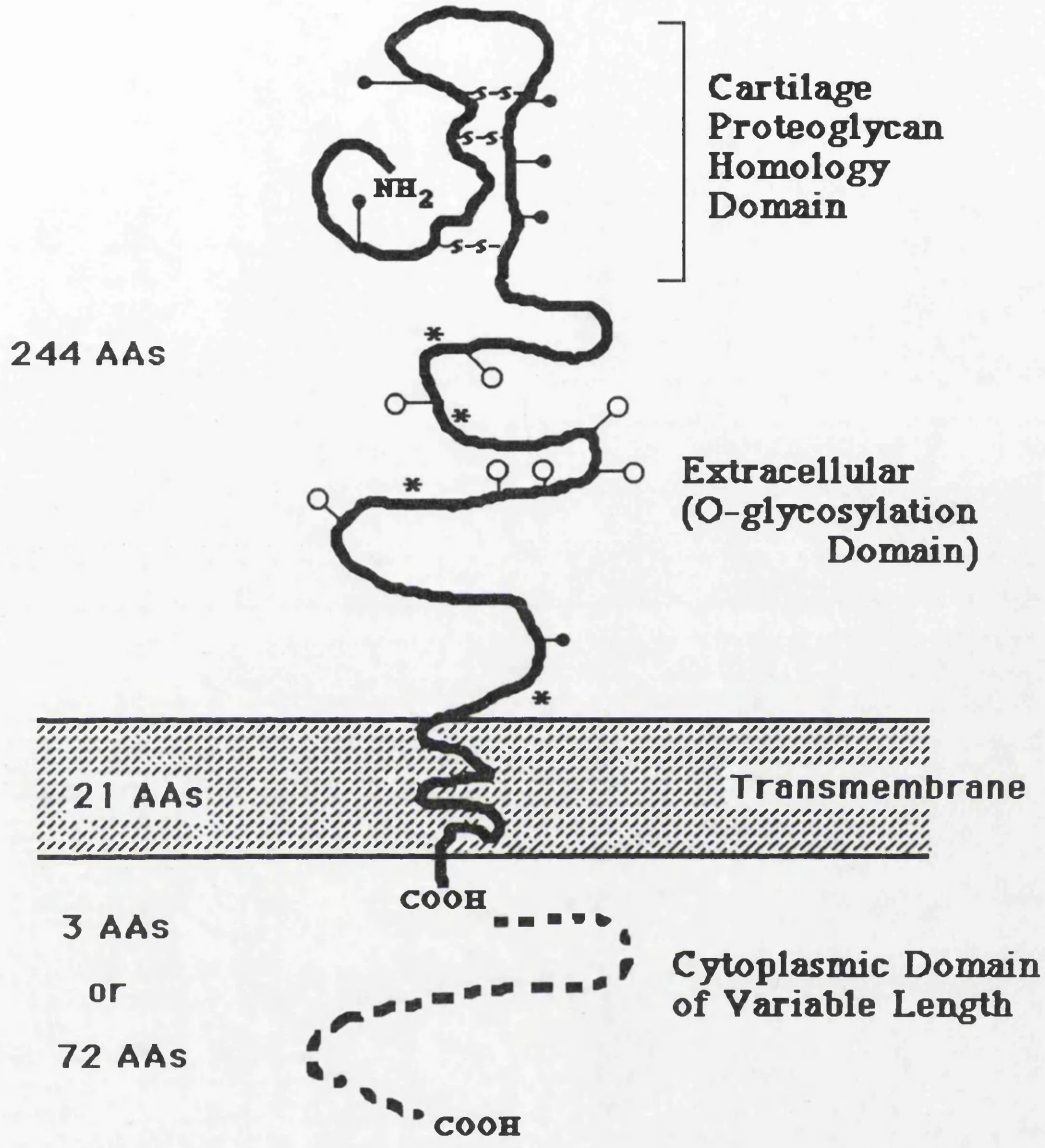


Figure 1.17 Schematic presentation of the CD44 molecule.
-• represents potential N-linked glycosylation (-CHO-),
-O represents chondroitin sulphate glycosaminoglycan chain in proteoglycan variant (*).

1.4.3 CD 44 and Metastasis

Activated lymphocytes and metastasising tumour cells share many properties, including invasive behaviour, migration involving reversible adhesive contacts, accumulation and expansion in draining lymphoid tissue, release into the circulation and extravasation (Reviewed by Herrlich *et al.*, 1993). Metastatic spread involves interaction between tumour cells and extracellular matrix (ECM) as well as between tumour cells and endothelial cells. Thus, metastasising tumour cells mimic the trafficking of lymphocytes. Tumour cells carry various members of the integrin family that are involved in the metastatic spread. After lymphocytes contact antigen, they transiently express a variant of CD44 (CD44v) which is involved in B and T cell activation in vivo (Arch *et al.*, 1992). The CD44 protein is normally present as numerous isoforms, considerable size heterogeneity producing functional changes, created by mRNA alternative splicing (Jackson *et al.*, 1992). This glycoprotein CD44v shares exon V6 (metastasis) sequences with a variant causally involved in metastasis formation. The 85 kDa form, presenting as smallest splice product involved in lymphocyte homing, is found on the surface of many cancer cells irrespective of their metastatic abilities (Pals *et al.*, 1989). The mode of action of CD44 V6 variants may resemble that of the 85 kDa isoform in that both mediate interaction with the ECM and possibly other cells and promote growth probably indirectly. Activated lymphocytes and metastasising tumour cells bind a specific ligand in the lymph nodes through CD44 V6 variants (Figure 1.18). In rat model systems, antibodies to the common variant portion of these variants block the settlement of the tumour cells in the draining lymph nodes and block consequently any further spread (Gunthert *et al.*, 1991). On the other hand, Seiter *et al.* (1993) reported that a monoclonal antibody binding within this variant part of CD44 interferes with

are in a process of activation probably express CD44v. Activated human T lymphocytes upon stimulation by mitogen or antigen and aggressive human non-Hodgkin's lymphomas (NHL) upregulate expression of a homologue of the rat metastasis - associated variant of CD44 (Koopman *et al.*, 1993). A significant proportion of aggressive NHLs overexpress CD44 variants that carry the metastasis - associated exon V6. Moreover, overexpression of the metastasis - specific CD44 variants in nonmetastasising tumour cell lines led to lymphogenic metastatic spread. Splice variants containing V6 are also expressed on several normal human epithelia and on carcinoma lines from lung, breast and colon.

1.4.4 Lymphocyte Homing to Regional Tissues

High endothelial cells express organ-specific HEV ligands for lymphocyte attachment and that lymphocytes express complementary organ-specific homing receptors (Figure 1.19). The specific interaction between lymphocytes and flat-walled vessels in a particular organ is similar to that of the HEV within the associated lymphoid organs.

Peripheral Lymph Node and Peyer's patches

PLN and PP HEV express distinguishable HEV - ligands for lymphocyte homing. Some lymphoma lines bind exclusively to PP HEV, others to PLN HEV, and still others bind well to HEV in both organs (Butcher *et al.*, 1980). Mesenteric lymph node (MLN) blasts bind to HEV in section of gut - associated Peyer's patches better than to HEV in PLN.

Breast

The specificity of lymphocyte homing to endothelial - binding in the lactating breast is similar to the small intestine has been supported by studies with the monoclonal antibody MECA-367 (Streeter *et al.*, 1988).

Lung

Lung lymph node (LuLN) blasts localised poorly within the intestine, instead, these cells entered lung - associated nodes better than did PLN and mesenteric lymph nodes (MLN) blasts (McDermott and Bienenstock, 1979). While MLN and LuLN blasts are retained equally well within the spleen or liver, MLN blasts enter the intestine five times better than LuLN blasts, and LuLN blasts enter lung twice as well as MLN blasts. The lymphocyte population in the lung is composed of 15% B-cells and 62% T-cells (almost all of which are of the CD4 subset) (Yednock and Rosen, 1989).

Naive and Memory/Effector Lymphocytes

Naive lymphocyte populations (CD45RA+) (not yet stimulated by antigen) express homing receptors (L-selectins), which mediate the selectin interaction for lymph node homing, and can bind promiscuously to several types of HEV. Naive B cells are IgM+, IgD+ and most exist in efferent lymph and thoracic duct as the naive type. Memory/effector cell precursors (CD45 RO+) express limited sets of homing receptors and increased levels of LFA-1 CD2 and VLA-4, all of which promote adhesion to endothelial cells, and have migratory or homing specificities restricted to the anatomic site of the original antigen stimulation.

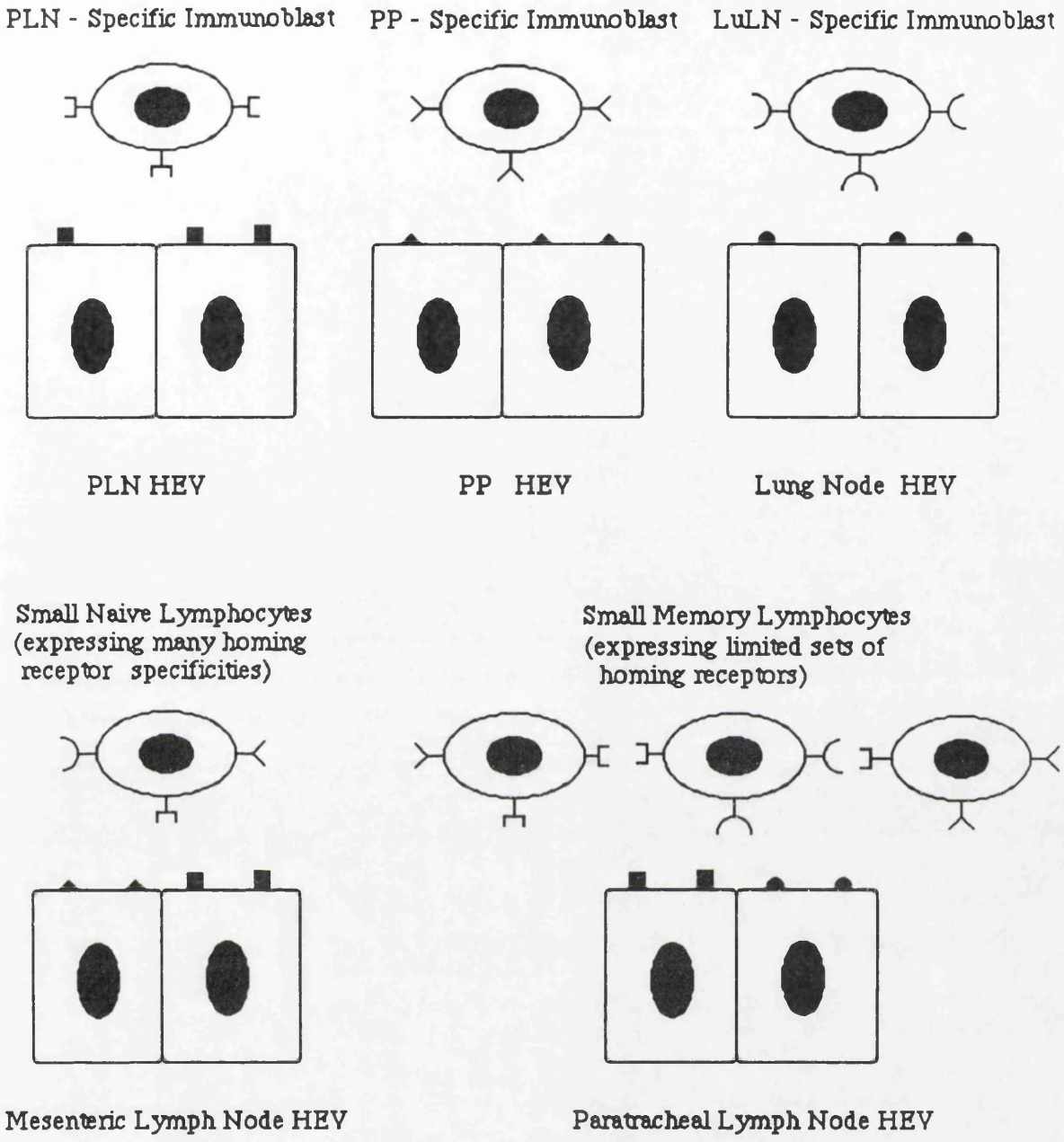


Figure 1.19 Diagram of organ-specific lymphocyte attachment to HEV of different lymphoid tissues.

When B and T cells are compared, they show different migratory behaviour through various lymphoid and non - lymphoid tissues. Whereas B cells bind preferentially (twofold to threefold) to PP HEV and T cells bind better (1.5 fold) to PLN HEV, both cell types bind equally well to HEV in the bronchus tissue. Moreover, within a certain class of lymphocyte, the state of

differentiation or activation affects the distribution and migration of such lymphocytes. The CD4 and CD8 T cell subsets also show slight binding differences to PLN HEV (Kraal *et al.*, 1983). Each of these subpopulations express both PLN- and PP-specific homing receptors, but one receptor type is expressed to a relatively higher degree on the cell surface.

1.4.5 Adhesion

1.4.5.1 Involvement of carbohydrates as recognition determinants in cell adhesion

Carbohydrates are ideal candidate recognition determinants or informational molecules because of their enormous potential for structural diversity (Rademacher *et al.*, 1988). Carbohydrates can carry much more information per unit weight than either nucleic acid or proteins because of their greater potential for structural diversity. All cell - surface carbohydrates serve as targets for receptors involving lectins, antibodies, toxins, enzymes, hormones, cancer cells, viruses and bacteria or have some other informational role. Carbohydrate - directed receptors are also important in lymphocyte attachment to HEV. The recognition determinants of the HEV ligands contain carbohydrates as essential elements. The lymphocyte attachment activity of PLN HEV is abrogated by treatment with a variety of sialidases (Rosen *et al.*, 1989). Imai *et al.* (1991) identified an endothelial ligand for PLN homing receptor as 50 and 90-kDa sulphated, fucosylated and sialylated glycoprotein (Sgp50, Sgp90; sulphated glycoprotein), indicating that sialylation of the component is a required component of the recognition determinant. As mannose-6-phosphate (M6P) inhibits lymphocyte attachment to PLN HEV, apparently by competing with the PLN homing receptor, it was relevant to determine whether M6P moieties are essential for the activity of PLN HEV

Table 1.2 Families of adhesion molecules and their counter-receptors involved in leukocyte binding to endothelium. LFA; Lymphocyte Function associated Antigen, ICAM; Intracellular Adhesion Molecule, VCAM; Vascular Cell Adhesion Molecule, NCAM; Neural Cell Adhesion Molecule, PECAM; Platelet Endothelial Cell Adhesion Molecule, Mac-1; Macrophage-1, VLA; Very Late Antigen, LPAM; Lymphocyte - Peyer's patch Adhesion Molecule, LCAM; Liver Cell Adhesion Molecule, MadCAM; Mucosal Addressin Cell Adhesion Molecule

Adhesion Molecule	Counter - Receptor	Implicated in :	Main Tissue Distribution
Ig Superfamily			
CD2	LFA-3		T cells
LFA-3 (CD58)	CD2		
ICAM-1 (CD54)	LFA-1, Mac-1	Secondary Adhesion	Widespread
ICAM-2/ICAM-3	LFA-1	Secondary Adhesion	Mostly endothelial cells/ All Leukocytes
VCAM-1	VLA-4	Homing to inflamed tissues	
NCAM (CD56)	NCAM, Heparan Sulphate		All tissues including nerve cells and NK cells
PECAM-1 (CD31)	?		Platelets, Leukocytes Endothelial cells
Integrin Family			
LFA-1 (CD11a/CD18) ($\alpha L \beta 2$)	ICAM-1, ICAM-2		Leukocytes
Mac-1 (11b/CD18) ($\alpha MB2$)	ICAM-1, C3b		NK cells, Monocytes, Granulocytes
p150,95 (CD11c/CD18) ($\alpha X \beta 2$)	?		Mostly granulocytes, Monocytes, NK cells Lymphocyte Subsets
VLA-1 (CD49a/CD29)	Laminin, Collagen		Widespread
VLA-2 (CD49b/CD29)	Laminin, Collagen		Widespread
VLA-3 (CD49c/CD29)	Laminin, Collagen, Fibronectin		Widespread
VLA-4 (CD49d/CD29) ($\alpha 4 \beta 1$)	Fibronectin, VCAM-1, $\alpha 4 \beta 7$		Widespread Peyer's patch Homing
VLA-5 (CD49e/CD29)	Fibronectin		Widespread
VLA-6 (CD49f/CD29)	Laminin		Widespread
LPAM-2 (CD49d/CD-)	?		Widespread
GpIIb/III9 (CD41/CD61)	Fibrinogen		
CD51/CD29	Fibronectin		
CD51/CD61	Vitronectin,		
MadCAM-1	Fibrinogen, Thrombospondin $\alpha 4 \beta 7$		Gut Homing
$\alpha 6 \beta 1$	?		Thymus Homing
Selectin Family			
E- Selectin	$sLewis^x$ antigen		Leukocyte Rolling
L- Selectin	Vascular Addressins		Lymphocyte Homing, Leukocyte Rolling
P- Selectin (CD62)	p150 $sLewis^x$ (CD15)		Leukocyte Rolling
Cadherin Family			
Uvomorulin	Homophylic		
LCAM	Homophylic		
E- Cadherin	Homophylic		
N- Cadherin	Homophylic		
P- Cadherin	Homophylic		
Unclassified			
CD44	Hyaluronic acid	Many Functions	Ubiquitous
Sialylated CD15	E- Selectin		

and the Ig superfamily includes the antigen - specific receptors of T and B lymphocytes (Springer, 1990).

Selectins

The selectins are integral membrane proteins characterised by an amino - terminal lectin - binding domain, epidermal growth factor (EGF) - like repeated domain and further repeated elements based on complement binding proteins known as short consensus repeats (SCRS). The selectins, namely L-, E- and P- selectin, are involved in the phase of 'tethering' and 'rolling' of leukocytes to endothelium under conditions of flow, and direct their entry into inflamed tissues. P- (previously celled PADGEM or GMP 140), E- (previously named ELAM-1) and L- selectin (involved in the lymphocyte homing receptor; Section 1.4.2.2) all function as Ca^{2+} - dependent lectins recognising sialylated, fucosylated and sulphated (not E- selectin) glycoconjugates, particularly the sialylated Lewis x antigen (sLe^{x}) (Hogg and Landis, 1993). IL-2 induces L-selectin down-regulation, whereas IL-6 and particularly TGF-1 promote L-selectin up-regulation. Local microenvironments within particular secondary lymphoid tissues influence homing receptor expression at the time of CD45 RA/RO conversion, and thereby contribute to the formation of $CD45^{low}/RO^{high}$ memory/effector T cell populations with tissue - selective homing behaviour (Picker *et al.*, 1993). A single ligand for P- selectin is a homodimer of 120 kDa subunits conjugated with O-linked carbohydrate (Moore *et al.*, 1992). The effect of leukocyte binding to P- selectin allows a tumbling movement of the leukocyte over the endothelial surface before it is completely arrested by a

second interaction between the LFA-1 on its surface and ICAM-1 on the endothelial surface (Lawrence and Springer, 1991; Fawcett and Harris, 1992). E- selectin is an inducible endothelial cell surface molecule that mediates the adhesion of neutrophils to activate endothelium (Bevilacqua *et al.*, 1989) and the adhesion of a subpopulation of resting $CD4^+$ memory T cells to activated endothelium (Shimizu *et al.*, 1991). Both E- and S- selectin bind to specific sialylated carbohydrates.

Integrins

The integrins, heterodimeric proteins consisting of α and β polypeptide chains of approximately 1,100 and 750 amino acids that are noncovalently associated and mediate the firm addition and endothelial transmigration phases of leukocyte entry into inflamed tissue. Both integrin subunits are required for ligand binding which is dependent on the presence of magnesium and calcium. The α subunit has divalent cation binding sites and β subunit is linked between the ligand and the cytoskeleton. The $\beta 1$ subunit interacts with a cytoskeletal protein, and the $\beta 1$ and $\beta 3$ subunits have sites for tyrosine phosphorylation. Integrin binding capabilities vary with cell type, indicating that at least some of the transition stages to full activation status can be stable. Lymphocytes bind the mucosal vascular addressin MadCAM-1, an Ig superfamily adhesion molecule that has a complex multidomain receptor, via the $\alpha 4\beta 7$ integrin. The interaction of $\alpha 4\beta 7$ with vascular MadCAM-1 may play a critical role in regulating lymphocyte homing to mucosal sites in Peyer's patches (Berlin *et al.*, 1993; Briskin *et al.*, 1993). Very late antigens (VLA)-4, so called because they appear late in the course of leukocyte activation, bind both an extracellular matrix component, fibronectin, and a cellular ligand on endothelial cells, vascular cell adhesion molecule (VCAM), which appears late after cytokine stimulation of endothelium. LFA-1, consisting of a 180 kDa α

chain and a 95 kDa β chain, predominates over VLA-4 during the interaction of leukocytes with their targets. LFA-1 can function in parallel with the MEL-14 antigen to strength the adhesion of lymphocytes to PLN HEV, and presumably with different homing receptors for interaction with other HEV. The adhesive contribution of LFA-1 can vary depending upon the strength of the homing receptor interaction. Both the LFA-1 and VLA-4 integrins play major roles in T-cell adhesion to activated endothelium by binding their respective cell surface ligands, ICAM-1 and ICAM-2 (for LFA-1) and VCAM-1 (for VLA-4). Fresh T cells and monocytes use LFA-1 with the assistance of VLA-4 to bind stimulated endothelium, whereas neutrophils, which lack VLA-4, depend on LFA-1 with the assistance of CD3/Mac-1. A counter - receptor for LFA-1, ICAM-1 is expressed on a wide variety of cells and its induction in inflammation is an important means of regulating LFA-1/ICAM-1 interactions. ICAM-1 and ICAM-2 are much more similar to one another, and thus represent a subfamily specialised to interact with LFA-1. Ligand or antibody binding to integrins stimulates the tyrosine phosphorylation of a cytoplasmic kinase called $pp125^{FAK}$. Thus integrins and other classes of adhesion receptors, may signal by mechanisms similar to the T-cell antigen receptor, which itself has no intrinsic enzyme activity, but interacts with proteins in a larger functional complex (Gumbiner and Yamada. 1992).

Immunoglobulin superfamily

The Ig superfamily consists of molecules that share the basic structure of immunoglobulin, composed of 90-100 amino acids arranged in a sandwich of two sheets of anti-parallel β-sheets, which is usually stabilised by a disulphide bond at its centre (Springer, 1990). The polypeptide backbone is arranged as domain structures, and each domain is built from multiple β-pleated

sheets of amino acid residues. This superfamily has intradomain disulphide bridges between cysteine residues, and a transmembrane hydrophobic region and a variable - length cytoplasmic domain. ICAM-3, homologous to ICAM-1 and ICAM-2, is expressed on lymphocytes, monocytes, neutrophils, endothelium and epithelium. ICAM-3 has a key role in the initiation of immune proliferative events. ICAM-1 can serve as a pathogenic adhesion molecule for the infected erythrocyte stage, which spares the physiological interaction with LFA-1. The extent of glycosylation on ICAM-1 may regulate adhesion to LFA-1 or Mac-1 in vivo (Diamond *et al.*, 1991). Two binding sites of VCAM-1 are involved in the firm adhesion of leukocytes to endothelium. CD31 functions as a homotypic adhesion receptor on endothelium and activates the adhesion mechanisms of naive CD8+ T cells (Tanaka *et al.*, 1992), since CD31 can potently induce integrin-mediated adhesion. Expression of CD31 is regulated by phosphorylation of serine and tyrosine after T-cell activation. CD59 acts as a second ligand for murine and human CD2. CD58 and CD59 function synergistically to activate T-cells via CD2.

Cadherins

Cadherins are single - chain integral membrane proteins that are homotypic adhesion molecules, and cadherin binding is calcium dependent. Cadherins interact with specific populations of cytoskeletal elements. The function of cadherins is not only adhesive but serves another role in organising the polarity of epithelial cell layers (McNeill *et al.*, 1990).

1.4.5.3 Cytokines and cell adhesion

When a tissue is infected, it defensively secretes proteins called cytokines, such as IL-1 and tumour necrosis factor (TNF). Cytokines produced within the tissue are important inducers of leukocyte subset - specific adhesion. Cytokines are emerging as excellent candidates for physiological adhesion triggers (Rot, 1992). They are released at sites of inflammation, act at short distances and induce inflammatory infiltrates of leukocytes in vivo. Proteoglycans (PGs) are one of the ECM components that participate in the molecular events that regulate cell adhesion, migration and proliferation, responsible for immobilisation of pro - adhesive cytokines. Proteoglycans are proteins modified by the addition of glycosaminoglycan (GAG) which are long linear polysaccharide chains made up of disaccharide subunits. This form enhances the formation of multiple cytokine binding sites to provide enormous diversity and flexibility to cytokine - cell interactions, thereby yielding a mechanism for the control of tissue - specific trafficking (homing) of circulating lymphocytes. Cell surface heparan sulphate (HS) PGs are necessary for the formation of stable local adhesion sites on fibronectin - coated substrates (Wight *et al.*, 1992). ECM proteoglycans bind cytokines/growth factors (Nathan and Sporn, 1991), and then proteoglycans mediate the initial uptake and presentation of the cytokine to receptors on the same cell (Rapraeger *et al.*, 1991). Finally, cytokines bound to proteoglycans on one cell can be presented to passing leukocytes that have come into contact with the endothelium after the initial tethering step of the adhesion cascade.

IL-8 can trigger granulocyte addition to endothelial ligands via β2 integrins (Huber *et al.*, 1991) and the macrophage inflammatory protein (MIP-1β) can trigger adhesion of T-cell subsets to the vascular cell adhesion molecule (VCAM-1) via β1 integrins. MIP-1β is localised at the high endothelial venules of reactive lymph nodes which are the site of lymphocyte migration into

lymph nodes. MIP-1β and other cytokines with glycosaminoglycan - binding sites bind to and can be presented by endothelial proteoglycans to trigger adhesion selectively not only of lymphocyte subsets, but also of other cell types (Tanaka *et al.*, 1993). Transmembrane growth factors also promote cell - cell adhesion and signalling that leads to proliferation and differentiation of cells. The endothelial receptor, vascular addressin, is inducible by severe pro - inflammatory cytokines, including TNF-α and IL-1β. Degradation of L-selectin is induced by granulocyte-macrophage-colony-stimulating factor (GM-CSF) and TNF-α (Nathan and Sporn, 1991). Resting lymphocytes do not express ICAM-1, which is rapidly induced after treatment with IL-1, TNF-α, and to a lesser extent with IFN-γ (Springer, 1990).

1.4.5.4 Cell adhesion and migration

Lymphocytes not only adhere strongly to inflamed endothelium and play a critical role in the inflammatory response (Osborn, 1990), but they also interact in a precisely regulated fashion with normal endothelium and thereby migrate into lymphoid and nonlymphoid tissue (Stoolman, 1989; Shimizu *et al.*, 1992). Adhesion sites are continually disrupted and reformed as cells change shape and move. Chondroitin sulphate (CS) PGs and hyaluronan destabilise adhesion sites promote cell motility, while the PGs that contribute to the formation of tight cellular adhesion sites inhibit cell migration (Wight *et al.*, 1992). Cells of different lineages (e.g. T cells vs. neutrophils vs. platelets) and subsets within each lineage, such as CD4+ and CD8+ T cells, differ in their interactions with endothelial cells, which results in distinct patterns of migration. Native and memory T cells have different trafficking patterns. Memory T cells preferentially migrate into both normal and inflamed nonlymphoid tissue. Naive T cells account for most of the torrent of cells

entering lymph nodes. Memory CD4+ T cells express higher levels of several adhesion molecules than do naive CD4+ T cells. The enhanced expression of LFA-1, VLA-4, VLA-5 and VLA-6 on memory cells is associated with their increased capacity to bind to the relevant ligands ICAM-1, VCAM-1, fibronectin and laminin. Fibrinogen binding to a variety of vascular cell receptors mediates a specific pathway of cell to cell adhesion by bridging together leukocytes and endothelial cells (Languino *et al.*, 1993). Inflammation not only influences the influx of memory cells into nonlymphoid tissue, but also dramatically increases lymphocyte (presumably naive T cells) entry into lymph nodes. Lymphocyte adhesion and migration to endothelial cells clearly involves multiple adhesion molecules - ligand interactions, and follow sequences of events (Figure 1.20). Stimulation of PKA and PKC induces lymphocyte binding to endothelial cells via different adhesion molecules (Turunen *et al.*, 1993). In step 1, inflammatory cytokines induce endothelial adhesion molecules and rolling of leukocytes. L- selectin and E-selectin mediate adhesion of resting T cells to endothelium, and initiate the rapid reaction rate of the carbohydrate - lectin interactions. The cytoplasmic domain of L- selectin regulates leukocyte adhesion to endothelium independent of ligand recognition, by controlling cytoskeletal interactions and/or receptor avidity (Kansas *et al.*, 1993). Step 2 is a triggering of lymphocytes by chemokines or molecules such as CD31 that activates integrins. Weak binding of VLA-4 to VCAM-1 on the endothelium provides a feedback loop to augment integrin adhesion. A rapid receptor - mediated activation event involving G protein signalling can trigger stable lymphocyte attachment to HEV in vivo, and may play a critical role in regulating lymphocyte homing (Bargatze and Butcher, 1993). In step 3, conformational changes of integrins play a major role as the main adhesive force that results in strong adhesion. The predominant pathways are thought to be VLA-4 - VCAM-1, LFA-1 - ICAM-1, and LFA-1 - ICAM-2. Lymphocyte sticking can occur within 1~3 sec after initiation of

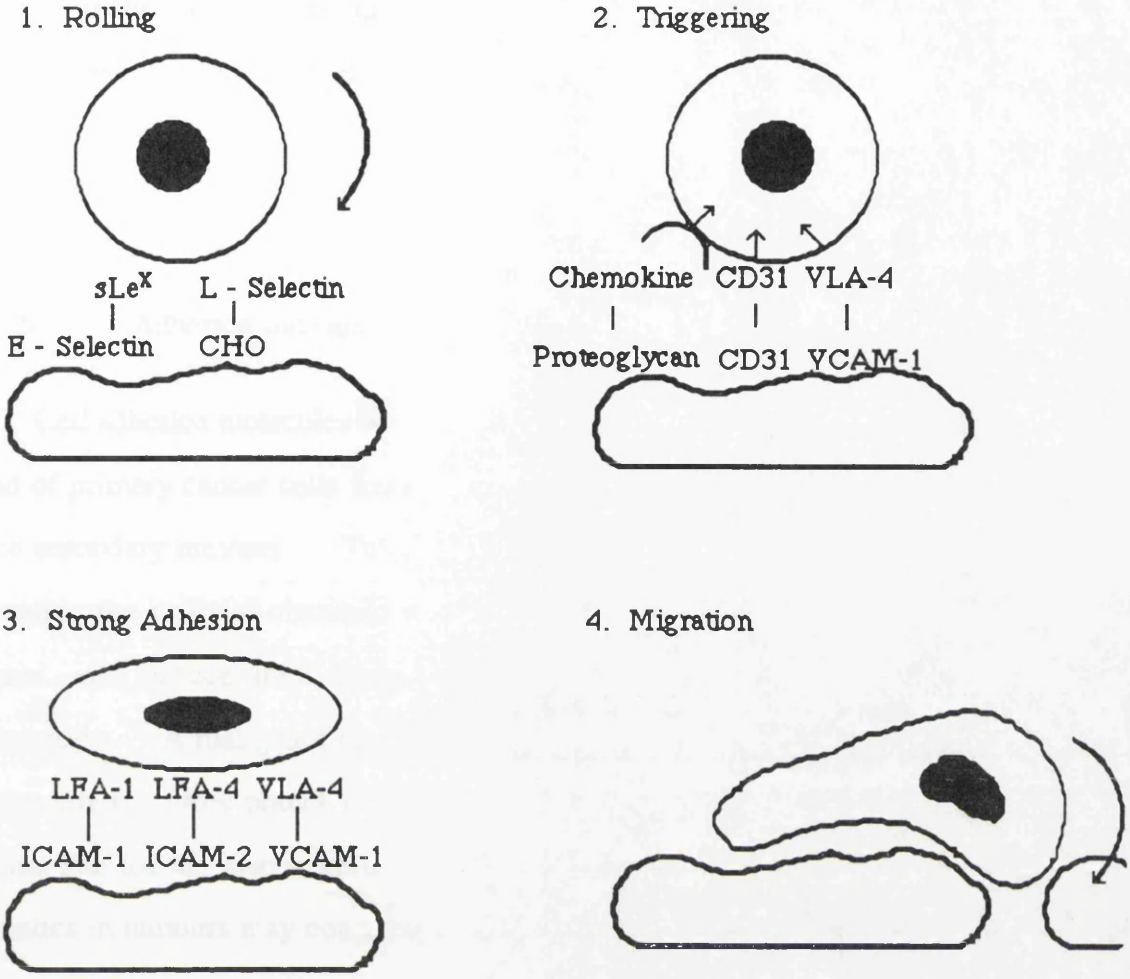


Figure 1.20 The sequence of events involved in the binding and transendothelial migration of lymphocytes

rolling. Finally, the lymphocyte migrates through the endothelium into the surrounding tissue. This requires a reduction in adhesion, followed by orderly migration. Degradative enzymes, produced by either the lymphocyte or high endothelial cell (HEC), may also be required for lymphocyte penetration of the HEV basement membrane. The majority of HEC in contact with lymphocytes have their Golgi complexes oriented toward the lymphocyte, whereas HEC not in contact have their Golgi's oriented toward the luminal surface. The fact that lymphocytes exhibit directed migration from the lumen

to the parenchyma of the lymphoid organ suggests the possibility that a haptotactic or chemotactic gradient directs the migrating cells (Czinn and Lamm, 1986).

1.4.5.5 Adhesion and cancer metastasis

Cell adhesion molecules play a role in diseases, such as metastasis - the spread of primary cancer cells from the main tumour throughout the body to induce secondary tumours. Tumour cells exit from the primary tumour by penetrating the walls of channels in the vascular and lymphatic circulatory systems, and induce their own supply of new blood vessels, called angiogenesis. A malignant tumour has a poorly defined border, called the invasion front. Any branch of the vascular tree within a tumour mass is a potential site for the discharge of cells into the circulation, and the lack of lymphatics in tumours may contribute to the build up of hydrostatic pressure within the tumour mass (Liotta, 1992). Local growth factors, hormones produced by the host and autostimulatory growth factors produced by the tumour cells themselves stimulate that proliferation. After the adhesion of the tumour cell to the basement membrane, destructive enzymes, such as metalloproteinases, are secreted by the tumour cell to cleave or unravel basement membrane molecules, such as collagens, in the extracellular matrix making up the structural backbone of the basement membrane barrier. Subsequently, the tumour cell's pseudopodia (amoeboid false feet) protrude into the zone of lysis, followed by migration of the entire tumour cell. TIMP (tissue inhibitor of metalloproteinase) (Liotta *et al.*, 1991) produced in normal tissues, such as cartilage and bone, as well as tumour cells and nm23 (nonmetastatic 23) protein (Leone *et al.*, 1991) suppress the invasion and metastasis.

The array of carbohydrates on cancer cells is strikingly different from that on normal ones, and cancer cells have unusual carbohydrates on their surface, which may account for many of their invasive properties.

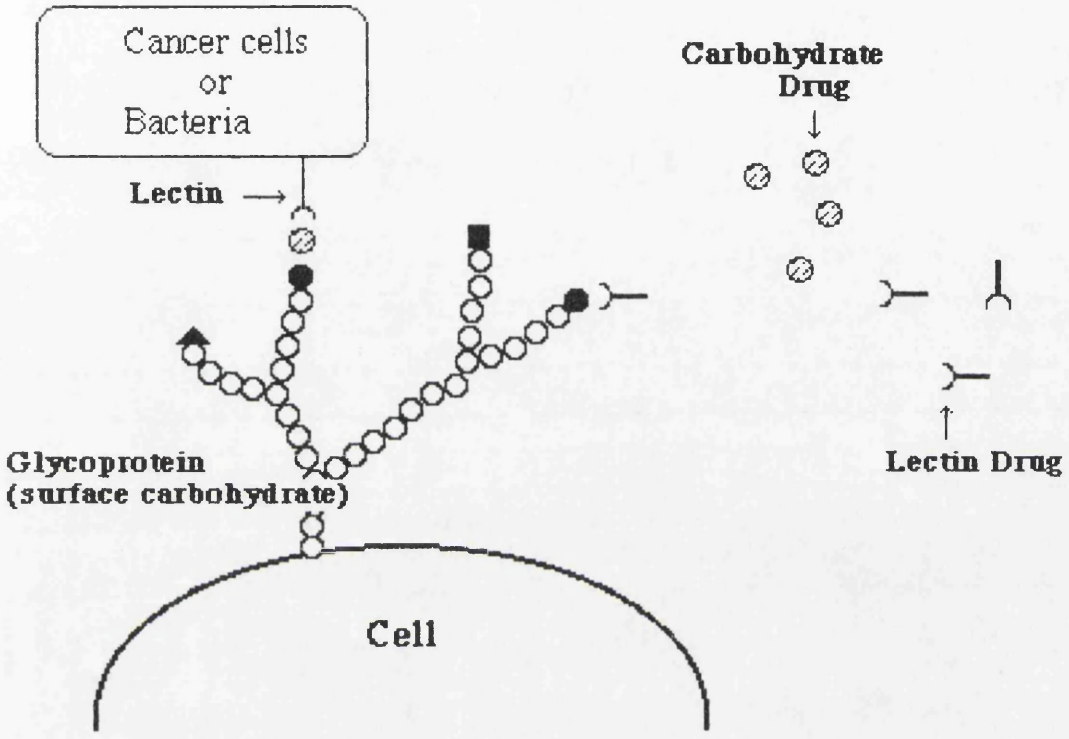


Figure 1.21 Diagram of antiadhesive therapy

Tumour metastasis and invasion are mediated not only by tumour cell lectins but also by carbohydrates on the surfaces of cells (Sharon and Lis, 1989). The carbohydrate recognised by E-selectin is expressed on cells from diverse tumours, and at least one type of human cancer cells binds specifically to E-selectin expressed on activated endothelium. Some malignant cells recruit the adhesion molecules that are part of the body's defences to promote their own

metastasis. A number of researchers are attempting to develop antiadhesive drugs containing similar carbohydrates could prevent the attachment by binding to the lectins, that is, interference of the adhesiveness of abnormal cells, as antimetastatic therapy (Sharon and Lis, 1993) (Figure 1.21).

1.5 Monoclonal Antibody Technology

Monoclonal antibody technology, developed by Kohler and Milstein in 1975, has been applied clinically to the immunoassays, diagnosis and therapy of human disease including cancer, immunocytopathology, definition of cell surface molecules (Knapp *et al.*, 1989) and modulation of immune responses. Recent advances in linkage of toxins and isotopes to monoclonal antibodies and in genetic engineering of antibodies (Morrison *et al.*, 1988) has led to reduced immunogenicity, and has improved effector function. Monoclonal antibodies are specific for single antigens, can be produced in large quantities from ascites fluid or by tissue culture production techniques with high degrees of purity, and can be efficiently coupled to isotopes, drugs and toxins. An alternative approach for the delivery of cytotoxic agents to cancer cells involves the use of monoclonal antibodies as carriers for enzymes to tumour cell surfaces (Bagshawe, 1989). The enzymes are chosen for their ability to convert drug precursors injected into active antineoplastic drugs. The active cytotoxic agents formed can then penetrate nearby tumour cells and cause the death of these cells. A number of prodrugs (drugs in an inactive form that can be transformed at the tumour into active anticancer drugs by antibody - enzyme conjugates) have been developed (Reviewed by Waldmann, 1991a). To be effective, antibodies must not cross - react with antigens on normal tissues or at least the antigen density should be less than on tumours, and the antigen - antibody complex should not dissociate from the cell membrane. For the antibody to react with tumour cells, the tumour must be vascular.

Humanised monoclonal antibodies (Winter and Harris, 1993), combination therapy (monoclonal antibody and cytokine or chemotherapeutic agent), and bifunctional monoclonal antibodies have been reported to overcome the technological barriers to successful antibody - dependent immunotherapy. Combination therapy with other biological response modifiers, such as a combination of IL-2 and LAK cells incubated with an appropriate monoclonal antibody in vitro, might increase the antitumour effect of monoclonal antibodies. The anti - Tac monoclonal antibody that blocks the binding of IL-2 to IL-2Rα can be useful treatment of patients with human T-cell leukaemia/lymphoma virus-I (HTLV-I) (Waldmann, 1991). However, such therapeutic strategies remain in early stages and the clinical evidence is on small numbers of patients.

1.6 Aims of Study

1. There is no published report on any antibody generated against the lymphocytes localised in human lymph nodes. This study was designed to generate antibodies against these cells in order to determine whether they contained a marker reactive with lymph nodes.

2. Flow cytometric analysis was used to determine whether the LEU-8+ antigen, assumed to be the human lymph node homing receptor, has functional homology with the MEL-14 antigen by examining LEU-8 expression in matched samples of human nodes and peripheral blood.

3. Several analytical parameters of immunological responses in the nodes and peripheral blood were assessed by studying both phenotypic markers and activation status of lymphocytes by flow cytometry.

4. The expression of the human lymphocyte homing receptor CD44 in the nodes and the metastasis pattern in both primary tumours and tumour - free/ - invaded nodes in breast cancer patients was analysed by flow cytometry.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All routine chemicals used in this study except where indicated were supplied by Sigma Chemical Company or BDH Chemicals, both of Poole, Dorset, England and were of the highest grade available. Plasticware was from Sterilin Ltd., Feltham, England.

2.1.1 Cell Culture Reagents

RPMI-1640	Gibco Ltd., Paisley, Scotland
Penicillin/Streptomycin	Gibco Ltd., Paisley, Scotland
Foetal Calf Serum (FCS)	Imperial Laboratories, Andover, Hants
Cyprofloxacin	Bayer, Germany

FCS was inactivated at 56 ^{o}C for 30 minutes and all these solutions were stored at 4 ^{o}C.

2.1.2 Animals

Mice	Male and female 8~12 weeks old BALB/c

Mice were supplied from the Biochemistry Department colony in University of Glasgow.

2.1.3 Human Tissue

Peripheral Blood and Lymph Nodes	Female patients with breast carcinoma between 40~90 years old (Courtesy of Professor W.D. George, Department of Surgery, Western Infirmary, Glasgow)

Patients were not previously treated with chemotherapy and lymph nodes were all assessed as tumour free by pathology and flow cytometry.

2.1.4 Peripheral Blood and Lymph Node Lymphocyte Preparation

RPMI-1640	Gibco Ltd., Paisley, Scotland
Penicillin/Streptomycin (P/S)	Gibco Ltd., Paisley, Scotland
Dimethyl Sulphoxide (DMSO)	BDH Chemicals Ltd., Poole
Ficoll - Hypaque	Pharmacia Fine Chemicals, Uppsala, Sweden
Foetal Calf Serum(FCS)	Imperial Laboratories, Andover, Hants

2.1.5 Lymphocyte Population Separation Materials

Petri Dishes	Sterilin Ltd., Feltham, England
Sheep Anti-Human IgG (H/L chain specific)	Scottish Antibody Production Unit, Lanarkshire, Scotland
Bovine Serum Albumin (BSA)	Sigma Chemical Company

2.1.6 Buffers

Phosphate Buffered Saline(PBS), pH 7.4

170 mM NaCl

3.4 mM KCl

10 mM Na_2HPO_4

1.8 mM HK_2PO_4

Sheath Fluid, pH 7.2 (Flow Cytometry Fluid)

1.3 mM NaCl

0.02 mM KCl

20 mM LiCl

15 mM HK_2PO_4

10 mM Na_2HPO_4

10 mM EDTA

Electrophoresis Running Buffer

125 mM Tris-HCl , pH 8.3

660 mM Glycine

1% (w/v) SDS

Western Blot Transfer Buffer

25 mM Tris-HCl , pH 8.6

0.16M Glycine

20% (w/v) Methanol

0.02% SDS

Citrate Buffer, pH 7.6

250 mM Sucrose

40 mM Trisodium Citrate $2H_2O$

5% (v/v) DMSO

2.1.7 Enzyme-Linked Immunosorbent Assay (ELISA) Reagents

PBS-Tween 20	BDH Chemicals Ltd., Poole
Orthophenylene Diamine (Substrate for HRP)	Sigma Chemical Company
Anti-Mouse Ig G (γ specific)	Sigma Chemical Company conjugated HRP

2.1.8 Cell Fusion Materials

Polyethylene Glycol (M.W. 1500)	Boehringer Mannheim, Germany
Hypoxanthine/Thymidine (HT)	Sigma Chemical Company
Aminopterin (A)	Sigma Chemical Company
Tissue Culture Plates	Cambridge, U.S.A.

HAT Medium

HT 1 ml (1/100 to give a final concentration of 0.1mM Hypoxanthine; 0.016mM Thymidine)

A 0.4 ml (1/100 to give a final concentration of 0.4 μM)

20% RPMI-1640 (20% FCS; 79% RPMI-1640; 1% P/S) 100 ml

2.1.9 Polyacrylamide Gel Electrophoresis Reagents

Acrylamide	BDH Chemicals Ltd., Poole
N,N'-methylene bisacrylamide	BDH Chemicals Ltd., Poole
N,N,N',N'-tetramethylethylenediamine (TEMED)	BDH Chemicals Ltd., Poole
Ammonium persulphate	BDH Chemicals Ltd., Poole
Sodium dodecyl sulphate	BDH Chemicals Ltd., Poole
Amberlite MB-2 (ion exchange resine)	Sigma Chemical Company

Low molecular weight marker proteins	Sigma Chemical Company
Coomassie brilliant blue R stain	Sigma Chemical Company
Silver nitrate	Johnson Mattey Chemicals Limited, England

2.1.9.1 Stock solution

Solution A	45% (w/v) Acrylamide
	1.2% (w/v) N,N'-methylene bis acrylamide

The solution was deionised with amberlite monobead resin and filtered.

Solution B	1.5M Tris-HCl, pH 8.8
	0.13% (v/v) TEMED
Solution C	0.65M Tris-HCl, pH 6.8
Solution D	12% (w/v) SDS
Solution E	Ammonium persulphate
Loading Buffer (x5)	65mM Tris-HCl, pH 6.8
; Stored at -20 ^{o}C	2% (w/v) SDS
	10% (v/v) Glycerol
	5% (v/v) 2-Mercaptoethanol
	0.001% (w/v) Bromophenol blue

2.1.9.2 Gel preparation

Separating Gel Preparation

12.5% separating gel was prepared from stock solution as follows:

Solution A	16.6ml
Solution B	15ml
Solution D	0.5 ml
Solution E	0.5 ml
TEMED	60µl
Deionised water	27.4ml

Stacking Gel Preparation

Solution A	2.64ml
Solution C	2.4ml
Solution D	0.2ml
Solution E	0.2ml
TEMED	24µl
Deionised water	8.67ml

2.1.9.3 Molecular weight markers

10µl low molecular weight markers proteins mixed with an equal volume of loading buffer were runned on the gel.

Protein	M.W.(subunit)	Source
Phosphorylase b	94,000	Rabbit muscle
Albumin	67,000	Bovine serum
Ovalbumin	43,000	Egg white
Carbonic anhydrase	30,000	Bovine erythrocyte
Trypsin inhibitor	20,100	Soybean
α-lactalbumin	14,400	Bovine milk

2.1.9.4 Staining solutions

Coomassie Blue Staining

Coomassie brilliant blue R stain 1g

Methanol 250mg

Acetic acid 100ml

Coomassie blue stain was dissolved in methanol acetic acid and then, made up to 1 litre with deionised water.

Silver Staining

Solution A	0.8g Silver nitrate in 4ml deionised water
Solution B	0.36% (w/v) NaOH 21ml
	14.8M Ammonia 1.4 ml
Solution C	

Solution A was added dropwise with vigorous stirring to solution B and then made up to 100ml with deionised water.

Developing Reagent	1% (w/v) Citric acid 2.5ml
	38% (v/v) Formaldehyde 250µl

The two solutions were mixed and then made up to 500ml with deionised water.

Destain

Methanol	400ml
10% Glacial acetic acid	100ml
Deionised water	500ml

2.1.10 Monoclonal Antibody Purification

50% (w/v) $(NH_4)_2S0_4$

Protein A Sepharose CL-4B Pharmacia, Uppsala, Sweden

Dialysis Tubing Medicell International Ltd., London

2.1.11 Fluorochrome Attachment to Immunoglobulin

0.25M Sodium Carbonate, pH9.0

0.1M Sodium Chloride

Fluorescein isothiocyanate(FITC) Sigma Chemical Company

Sephadex G-25M Column Pharmacia, Uppsala, Sweden

2.1.12 The Lowry Protein Assay

2% (w/v) Na/K Tartrate

1% (w/v) $CuSO_4$ $5H_2O$

0.1M NaOH / 2% (w/v) Na_2CO_3

2.1.13 Cell Protein Preparation

0.15M NaCl

Homogenisation Buffer (10mM PBS, pH7.4;1mM EDTA;0.5M Sucrose)

Piperazine-N,N'-bis-2-ethane sulfonic acid (PIPES)

2.1.14 Western Blot

Anti-human IgG Protein A-Peroxidase conjugate	Sigma Chemical Company
Rabbit anti-mouse IgG (whole molecule)	Sigma Chemical Company
4-Iodophenol	Aldrich Chemical Co. Ltd., Germany
Luminol : 5-amino-2,3-dihydro-1,4-phthalazinedione	Sigma Chemical Company

2.1.14.1 Photographic materials

X-Omat-S X-Ray Film	Kodak Ltd., London
Developer	Kodak Ltd., London
Fixer	Kodak Ltd., London

2.1.14.2 Chemiluminescence substrate

10mg 4-Iodophenol was dissolved in 1ml DMSO and added to a solution of 40mg Luminol dissolved in 5 ml 0.1M Tris-HCl, pH8.5. This solution was made up to 100ml with 150mM NaCl and then mixed with 32µl H_2O_2 immediately prior to use. This substrate was stored at 4 ^{o}C in the dark.

2.1.14.3 Stain solution for proteins

Stain

0.1% (w/v) Naphthol Blue-Black (Amido black) in 10%.(v/v) acetic acid, 50% (v/v) Methanol.

Destain

50% (v/v) Methanol

10% (v/v) Acetic acid

2.1.15 Solutions for Flow Cytometric DNA Analysis

Stock solution, pH7.6	3.4mM Trisodium citrate $2H_2O$
	0.1% (v/v) Nonidet P40
	1.5 mM Spermine Tetrahydrochloride
	0.5mM Tris
Solution A, pH 7.6	15mg Trypsin (Sigma Chemical Company) dissolved in 500ml stock solution.
Solution B, pH7.6	250mg Trypsin inhibitor (Sigma Chemical Company) and 50mg Ribonuclease A (Sigma Chemical Chemical Company) dissolved in 500ml stock solution.

Solution C, pH7.6	208mg Propidium Iodide (Sigma Chemical Company) and 580mg Tetrahydrochloride (Sigma Chemical Company) dissolved in 500ml stock solution.

Solution C was protected against light with tinfoil during preparation. All these solutions were stored at -80 ^{o}C in 5ml aliquots.

2.1.16 Antibodies for Flow Cytometry

All antibodies were obtained from Dako A/S (Table 4.1) with the following exceptions.

Sheep anti-mouse IgG(whole molecule) FITC conjugated	Amersham International, plc, Bucks
Rabbit F(ab)$_2$ anti-human IgG(γ chain) FITC conjugated	Amersham International, plc, Bucks
Anti-human CD44 FITC conjugated	Sigma Chemical Company
Anti-Leu 8 FITC conjugated	Becton Dickinson, Cowley, Oxford

2.1.16.1 Miscellaneous

FACScan tubes were supplied by Becton Dickinson, Cowley, Oxford.

2.1.17 Cell Lines

2.1.17.1 Human B cell lines

Raji was established in 1963 from Burkitt's lymphoma in an 11 old Negro male. Growth is in the form of single cells without attachment and as macroscopically visible clumps containing many hundreds of cells. This cell line produces interferon when stimulated by Newcastle disease virus (Epstein *et al.*, 1986). Raji has no surface immunoglobulin but MHC Class I and MHC Class II.

Daudi was established from a 16 year-old male Negro with Burkitt lymphoma. The cells have surface IgM very strongly (Klein E. *et al.*, 1968) and MHC Class II but no MHC Class I.

Ramos was derived from an American Burkitt's lymphoma which does not possess the EBV(Epstein-Barr Virus) genome. Ramos has surface IgM, MHC Class I and MHC Class II.

2.1.17.2 Human T cell line

Jurkat E6.1 was derived from Jurkat FHCRC. It is an interleukin-2 producing cell line, human leukaemic T cell lymphoblast, derived by incubating the cells at 41 ^{o}C for 48 hours followed by a limiting Jurkat E6.1 dilution cloning over macrophages.

2.2 Methods

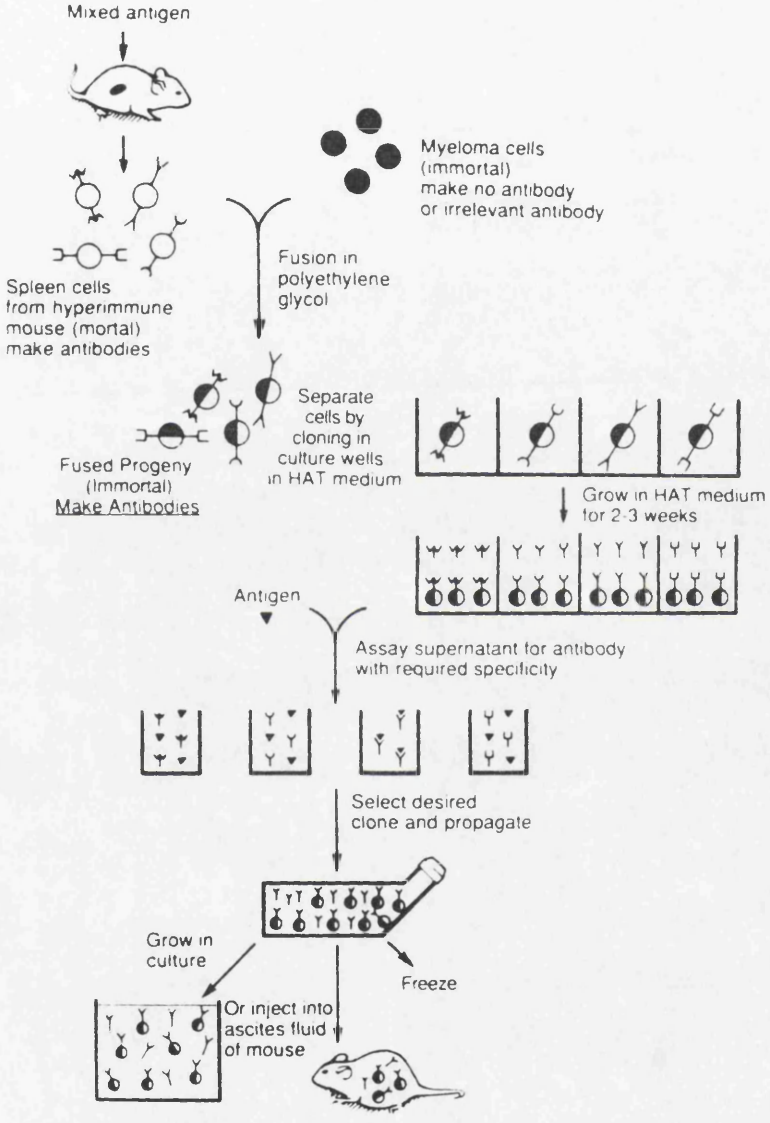
2.2.1 Monoclonal Antibody Production

Monoclonal antibodies (Mabs) reactive with lymph node lymphocytes were produced by methods described previously (Campbell,1991). Figure 2.1 outlines the steps in the production of monoclonal antibodies made in this study. Balb/c mice were immunised with a highly enriched population of nodal cells, and once a good humoral response appeared in the immunised animal, screening of serum by FACScan was used to indicate when fusion was appropriate. Animals were boosted intravenously with 10^5 cells 4 days before fusion. Spleen cells from the immunised mice were then mixed with the myeloma SP2/0 cells, and fused in 50% polyethylene glycol (M.W. 1500). Cells from positive wells were grown in complete medium (89% RPMI-1640; 10% FCS; 1% P/S) and then single-cell cloned. The resultant monoclonal antibodies were screened on FACScan or ELISA and tested as for Leu-8 (Chapter 4) in some cases with a direct label. Three clones were produced in this study, all using separate immunisation schedules. One clone was from a mouse immunised with whole lymph node lymphocytes, and two clones from mice with immunised with panned nodal T cells, were selected either by FACScan or by ELISA.

2.2.1.1 Antigen preparation

In general, fresh intact cells were used since these are excellent antigens, particularly for generating monoclonal antibodies to membrane proteins. Injection was of 10^5 to 10^6 cells. In this study the first antigen tested was unfractionated lymph node lymphocytes. Lymph node cells were collected by the method described in Section 2.2.2.3. When this response was to be dominated by B cells, two further fusions were under taken with enriched

Figure 2.1 The general procedure of monoclonal antibody production


Mixed antigen
Myeloma cells
(immortal)
make no antibody
or irrelevant antibody
Fusion in
polyethylene
glycol
Spleen cells
from hyperimmune
mouse (mortal)
make antibodies
Separate
cells by
cloning in
culture wells
in HAT medium
Fused Progeny
(Immortal)
Make Antibodies
Grow in HAT medium
for 2-3 weeks
Antigen
Assay supernatant for antibody
with required specificity
Select desired
clone and propagate
Grow in
culture
Freeze
Or inject into
ascites fluid
of mouse

populations of lymph node T cells. Preparation of T lymphocytes (panning) was carried out under sterile conditions at 4 ^{o}C. Firstly, two sterile 10cm petri dishes were coated with 10ml sterile filtered 130mM NaCl and 0.05M Tris-HCl, pH 9.0 containing 0.5ml anti-human IgG (Sheep, H/L chain specific). The petri dishes were incubated for 1 hour at 37 ^{o}C and then overnight at 4 ^{o}C. The petri dishes were then washed with 5% BSA and then with PBS five times. 5ml lymphocyte suspension adjusted to a cell density of 10^7 cells/ml was added to the plate and incubated for 1 hour at 4 ^{o}C, gently rotating the plate after 30 minutes to allow cells to coat more evenly. The plate was tilted and unbound cells were repanned on another plate to remove any residual bound cells. Finally, after the repeated panning step the supernatant containing the unbound cells was collected and centrifuged at 150g to obtain enriched T cells.

2.2.1.2 Immunisation

Primary immunisation was with 10^6 cells in saline, intraperitoneally four weeks before fusion. This was repeated after 2 weeks to stimulate isotype switching and the production of high-affinity antigen induced memory cells. After serum test (Section 2.2.1.3), final intravenous boost was with 10^5 cells 4 days before the fusion to active the memory B cells in the spleen.

2.2.1.3 Serum collection and FACScan analysis

The sera from tail bleeds was obtained 10 days after secondary immunisation and used to check the presence of required antibodies. The serum as first antibody was diluted in 1 in 100 with PBS and analysed by FACScan. The procedures were followed as described in Section 2.2.3.3.1.

When the serum titre was positive with an IgG specific probe with respect to control serum the mice are rested for a short period of time. The second antibody used in this work was anti-mouse IgG (Sheep, γ specific) conjugated to FITC (Amersham).

2.2.1.4 Cell fusion

Once a good immune response had developed in mice, preparations for fusion were made. All operations were performed in the sterile hood. The myeloma (SP 2/0) cells used in this study were at least 35% in S-phase of cell cycle on the morning of the fusion. 2×10^7 myeloma cells were harvested in sterile universal containers and washed twice with centrifugation at 150g for 5 minutes and then resuspended in RPMI-1640. The spleen tissue was isolated from the immunised mice and put into a 6 cm petri dish containing 5ml RPMI-1640. The spleen cells were teased out from the capsule using 21G needles to break clumps. The spleen cell suspension was drawn into a 10ml syringe through a 21G needle, pipetted out again and this operation was then repeated twice with a 25G needle. The spleen cells were washed twice in RPMI-1640. The final cell density of spleen cells for fusion was adjusted to 10^8 cells. Separate aliquots of spleen and myeloma cells were removed for controls. The remainder were then mixed together and centrifuged at 150g for 5 minutes. The cell pellet was tapped to loosen it and 2ml of PEG (M.W. 1500) solution was added evenly over a period of 30 seconds shaking the cells by gently flicking of the tube. The PEG and cell suspensions were dispersed over the next 30 seconds with pipetting up and down and then allowed to stand for 30 seconds. 5ml of RPMI-1640 was then added dropwise evenly to this cell suspension over a period of 90 seconds shaking very gently to dilute out the PEG slowly and then another 5ml of RPMI-1640 was added immediately. The cell suspension stood for 5 minutes and was then centrifuged at 150g for 5

minutes. The fused cells were resuspended in HAT medium (Section 2.1.8) and then 200 µl of this cell suspension was aliquotted into each well of a 96 well plate with the exception of control wells on each plate. The plate were incubated in a 5% CO_2 humid incubator at 37 ^{o}C.

2.2.1.5 Cell culture and selection of clones

Between 4 and 5 days after fusion, 0.1ml of old medium was gently removed and replaced with fresh medium. If the myeloma cells were not dead they were fed with fresh medium containing HAT. The clones appeared clearly 7 days after fusion. When clones were $<10^2$ cells in size, 0.1ml of supernatant was removed for assay and replaced with fresh medium without HAT. In this point the spleen cells in control wells were important for assay to give an indication of background antibody secretion from unfused spleen cells. The screening assay was carried out according to Section 2.2.1.6. The positive wells of plates were selected and immediately cloned on 96 well mouse spleen feeder plates. The cloning by limiting dilution was performed according to the method of Goding (1980). A very small number (10^3 cells) were required for cloning. The relevant positive clones from the fusion plates were aspirated by holding the pipette tip vertically over them and removing them into 10ml of complete medium. Next, 1 in 2 doubling dilutions were carried out as follows:

(a) 2ml from the cell suspension solution above were added to 18ml of fresh medium and then plated out at 100 µl/well into rows B and C. This was approximately 1 cell/well.

(b) This was repeated as in (a) above to give 0.1 cell/well.

(c) This was repeated as in (a) above to give 0.01 cell/well.

The clones were visualised after 1 week and assayed again. The clones secreting desired antibody were selected and recloned using the same strategy. Finally, clones positive at the highest dilution was collected and expanded in tissue culture flasks containing 10% complete medium. This colony was grown fully in tissue culture flasks. Some cells were resuspended in freezing medium (90% FCS; 10% DMSO), and frozen in liquid nitrogen and other cells were propagated by injection into mice for ascites fluid production.

2.2.1.6 Screening

The selection of positive clones from cell fusion (Section 2.2.1.5) was performed using both ELISA and FACScan analysis. FACScan can detect all surface reactive Mabs but is time consuming with >1000 primary clones. ELISA readily adapts to mass screening but the cell membranes are inevitably breached such that intracellular antigens may dominate the fusion selection.

2.2.1.6.1 ELISA assay

Nodal T cells were stored bound to ELISA plates containing 0.02% (w/v) sodium azide (to avoid bacterial contamination) and blocking buffer both of which were washed off immediately before use. Freshly prepared ELISA plates were coated with 100µl/well of a T cell suspension of 10^5 cells/ml in RPMI-1640, and fixed with 0.25% glutaraldehyde for 1 hour at room temperature. The plates were washed with PBS and then incubated with 100µl of blocking reagent (5% BSA/PBS) for a minimum of 1 hour at room temperature, followed by washing in PBS. 200µl of serum or cell culture supernatant was added to the second column of the ELISA plate using a multipipette with sterile tips and then diluted serially using 5% BSA/PBS as

diluent. The first column was left with only PBS added as a negative control without first antibody and the assay plates were covered with clingfilm and incubated for 1 hour at 37 °C before washing twice with PBS containing 0.05% Tween 20. 100µl of 1 in 1000 dilution of second antibody (Section 2.1.7) in PBS/0.05% BSA/0.01% Tween 20 was added to all wells, incubated for 1 hour at 37 °C, and plates were then washed in PBS/0.05% Tween 20. 100µl of substrate solution (0.04 mg/ml orthophenylenediamine in 0.05M Na citrate; 0.15M Na phosphate; 0.01% H_2O_2 pH 6.0) was added to each well and incubated for 15 minutes in the dark at room temperature. The reaction was stopped by the addition of 50µl 4N H_2SO_4 and wells were scanned at 492nm in a multiscan spectrophotometer.

2.2.1.6.2 FACScan analysis

FACScan screening was only performed on wells where there was a clearly visible clone. The stored frozen lymph node lymphocytes in freezing medium (90% FCS; 10% DMSO) at -140 °C were washed in PBS twice at 150g for 5 minutes, and 25µl of culture supernatant from wells of culture plates was used as first antibody. The second antibody was FITC labelled anti-mouse IgG (γ chain specific) and the method was carried out according to section 2.2.3.3.1.

2.2.1.7 Monoclonal antibody purification

Ammonium sulphate precipitation is one of the most commonly used methods for subfractionating proteins from solution. In general, antibodies can be separated by affinity chromatography on insolubilised protein A according to their heavy chain constant region. This affinity procedure can be

used to isolate the majority of IgG directly from ascites fluid or culture supernatant. The purification technique was followed in the method of Johnstone and Thorpe, 1987. The 10ml of 100% saturated (>770g/l) ammonium sulphate solution at pH 7.0 was added very gradually to 10ml tissue culture supernatant or ascites fluid (Section 2.2.1.9) with vigorous stirring slowly for 1 hour at 4 °C. The solution was centrifuged at 500g for 20 minutes at 4 °C and then the pellet was dialysed overnight in small dialysis tubing against three changes of PBS at 4 °C. On the next day a small amount of filtered sample solution was layered onto the protein A coupled to Sepharose CL-4B column (Section 2.1.10) and washed with 5ml 0.1M sodium phosphate buffer, pH 8.0. 0.5ml fractions were collected and their absorbance was monitored at 280nm. The column was eluted with 5ml 0.1M sodium citrate buffer, pH 2.5 and 0.5ml fractions were collected directly into tubes containing 60µl 2.0M Tris-HCl, pH 8.5, to neutralise the solution. The absorbance was measured at 280nm and the antibody was stored for immunoassay.

2.2.1.8 Fluorochrome attachment to immunoglobulin

This method was performed with technique of Johnstone and Thorpe, 1987. The immunoglobulin prepared in section 2.2.1.7 was dialysed at 4 °C overnight in 0.25M carbonate buffer to give a protein concentration of 10-20 mg/ml. 0.05 mg of FITC per mg of protein was added and mixed at 4 °C overnight with shaking. The mixture was applied to a Sephadex column (Section 2.1.11) and the fractions were eluted with PBS. The fluorescence/protein ratio was calculated by measuring the absorbance of the conjugate at 280nm and 495nm. Three fractions were used for most purposes.

$$\text{molar ratio} = \frac{2.87 \times A_{495}}{A_{280} - 0.35 \times A_{495}}$$

2.2.1.8.1 FACScan analysis

Monoclonal antibodies conjugated to FITC (Section 2.2.1.8) were produced as two types: as IgG monoclonal antibody conjugated FITC from culture supernatant and ascites fluid and identified using FACScan. 10µl of each monoclonal antibody produced was added to antigen and then stained with B cell marker CD19 conjugated PE (10µl) or T cell marker CD3 conjugated PE (10µl). The method in detail was followed in according to section 2.2.3.3.1.

2.2.1.9 Ascites fluid

Expansion of hybridoma cells *in vivo* was carried out in ascites fluid in mice. The mice were primed by the injection of 0.5ml Pristane(2,6,10,14-tetramethyl pentadecane) which depresses the normal immune system of the mouse so that internally injected cells are able to grow without rejection (Freund and Blair, 1982), intraperitoneally using a 21G needle. After 10 days of priming, 0.5ml of washed hybridoma cells (10^6 cells) in active logarithmic growth were injected into the peritoneal cavity of mice using a 21G needle. 7-14 days post injection the mice showed abdominal growth and were killed and the ascites fluid containing hybridoma and antibody was extracted into a heparinised tube by inserting and draining with a 21G needle. The ascites fluid was gently layered over 10ml of Ficoll-Hypaque solution and centrifuged

for 20 minutes at 500g. The supernatant containing monoclonal antibodies was collected gently and stored at 4 °C for immunoassay. The hybridoma cell layer at the interface was obtained by carefully pipetting, washed in RPMI-1640 twice at 150g for 5 minutes and then resuspended in 24 well cell culture plates containing complete medium.

2.2.2 Preparation and Storage of Lymphocytes

2.2.2.1 Patients

Axillary lymph nodes not invaded with tumour and peripheral blood were obtained from breast cancer patients whose age ranged from 40 to 90 from the Department of Surgery, Western Infirmary in Glasgow, U.K. Patients were not previously treated with chemotherapy and samples of these nodes were sent to the Department of Pathology for routine histological examination. Histopathological reports confirmed that the nodes were not invaded.

2.2.2.2 Preparation of lymphocytes from human peripheral blood

Peripheral blood was obtained from the same patients on the day of surgery. 10ml of blood was stored in potassium-EDTA anti-coagulant until ready for processing, usually within 1 hour of removal from the patients. Blood was gently layered over on equal volume of Ficoll-Hypaque and centrifuged for 20 minutes at 500g. The lymphocyte layer was obtained by carefully pipetting from the interface and added to a clean tube. The plasma, Ficoll solution and red blood cells were discarded. The cells were washed in RPMI-1640 medium twice at with centrifugation 150g for 5 minutes. The pellet was resuspended in freezing medium consisting of 90% heat-inactivated foetal calf serum and 10% DMSO to achieve a final concentration of 1×10^6

cells/ml. This cell suspension was transferred in 1 ml aliquots to freezing vials. They were placed in a - 70 °C freezer overnight and then finally stored in a liquid nitrogen freezer for later analysis. On removal from liquid nitrogen, cells were thawed quickly in a 37 °C water bath to avoid damage by ice crystal formation within the cells and immediately transferred to a universal container containing filtered PBS pH 7.4. These cells were washed twice with centrifugation at 150g for 5 minutes and resuspended at the required cell concentration of 2 x 10^7 cells/ml in filtered PBS for FACScan analysis.

2.2.2.3 Preparation of lymphocytes from human lymph nodes

Lymph nodes were also obtained from patients on the day of surgery and processed immediately in order to avoid alteration in membrane receptor expression. Half of each node transferred into a 60 mm sterile petri dish containing RPMI-1640 tissue culture media were teased out using a sterile needle and scalpel. The lymphocytes were then harvested, washed by centrifugation at 150g for 5 minutes, counted with a Haemocytometer and adjusted to 1 x 10^6 cells/ml in RPMI-1640 medium. They were then resuspended in a freezing mixture and finally stored in liquid nitrogen until analysis.

2.2.3 Flow Cytometry

PBLs, LNLs, and other cells used in this study were analysed by flow cytometry. Flow cytometry was developed as a technique for quantifying the visual picture obtained by microscopic examination of cells stained with specific antibodies. It operates by projecting a single cell stream through a focused laser beam one at a time. The light scattered from the cells is detected

by a photomultiplier, recorded and displayed as a two or three-dimensional graph on a computer monitor. The main applications of flow cytometry are for analysis of the expression of specific cell surface molecules and quantitation of cellular DNA using determination of cell cycle phases. The flow cytometer used was a Becton-Dickinson designed FACScan (Fluorescence Activated Cell Scanner).

FACScan consists of a bench-top cytometer which is a laser driven and the computer system and is capable of detecting and analysing cells using 5 optical detectors. The 5 parameters include 2 physical parameters, consisting of forward scatter (FSC) and side scatter (SSC), and 3 different fluorescence (red, red/orange and green) components. The FSC parameter detects the amount of incident light scattered in the forward direction and measures the cell volume. The amount of incident light scattered at 90 degrees is detected by SSC parameter which gives the information of the granularity of the cell. The specific size and granularity of cells are identified by these parameters, and it is possible to distinguish a particular cell population from other cell types present.

2.2.3.1 Mechanical system

(a) Optical system

The laser used in FACScan optical system is ionic, an air cooled 15 mV Argon laser set at 488nm. The output beam is transformed into an elliptical shape through the refracting beam expander and focus on a stream of cells in the flow chamber. The physical dimension of flow chamber limits the numerical aperture of the collection lens. The forward collection lens gathers scattered light from about 1 to 2 degrees off the laser-beam axis. This angle minimises

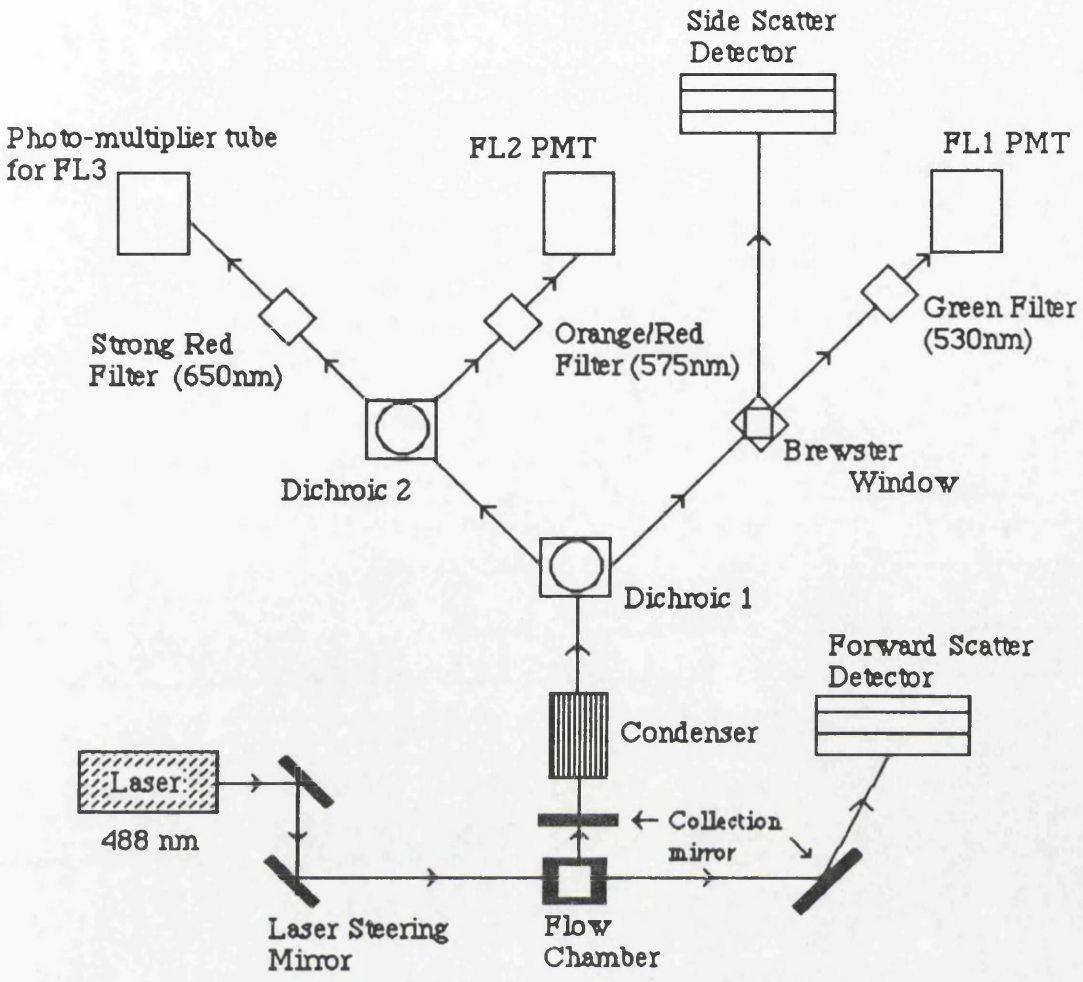


Figure 2.2 Diagram illustrating the generalised optical system of the flow cytometer

the effect of refractive index changes and maximises the dependence on particle size. The different wavelength are separated by the use of dichromic mirrors and adsorption filters. The long wavelength incident at the dichromic mirror (dichromic 1) passes on towards the green fluorescence detector and through a

530nm filter with a 30nm half-peak bandpass, optimised for FITC detection (FL 1). The transmitted path scatter is further split from the green fluorescence by a Brewster-angle beam splitter which takes advantage of the polarised nature of 90 degrees scatter and the mostly unpolarised state of fluorescence. The side scatter channel does not require a filter. A 45 degree dichromic mirror (dichromic 2) splits off the orange/red fluorescence which is optimised for the detection of PE (FL 2) through a 575nm filter with a 42nm bandwidth from the red fluorescence. The red fluorescence channel (FL 3) has a emission filter which transmits long wavelength light above 650nm (Figure 2.2).

(b) Fluidics system

The function of the fluidics system to deliver a laminar, single file flow of cells one by one to a specific point in space by the illuminating beam. Flow chambers can transport particles to the detection point with an accuracy of better than ±1 micron. The sheath fluid line of two fluid lines feeding the flow chamber flows isotonic sheath fluid continuously and is controlled by regulated air pressure. The sample is driven into the capillary tube, the sample line, by a differential pressure. The sample flow rate is controlled by a pressure regulation acting on the sample chamber. The high flow rate (60µl/min) is suitable for immune monitoring, whereas the low flow rate (12µl/min) allows higher resolution in applications such as DNA cell cycle analysis.

2.2.3.2 Fluorochrome dyes

Fluorescence occurs when a molecule excited by light returns to the ground state by emitting light of a longer wavelength. The different wavelengths between exciting and emitted light can be separated from one another using optical filters. The ability to detect fluorescence simultaneously from two or three compounds at different wavelengths enables several parameters to be measured. Fluorescein-5-isothiocyanate (FITC; $C_{12}H_{11}NO_5S$) dye is used to covalently label antibodies giving bright distinctive fluorescence (FL 1) absorbing at 488nm and emitting a green fluorescence signal at 525nm. R-phycoerythrin (PE) is from the phycobiliprotein family of highly fluorescent macromolecules (M.W. 240,000).

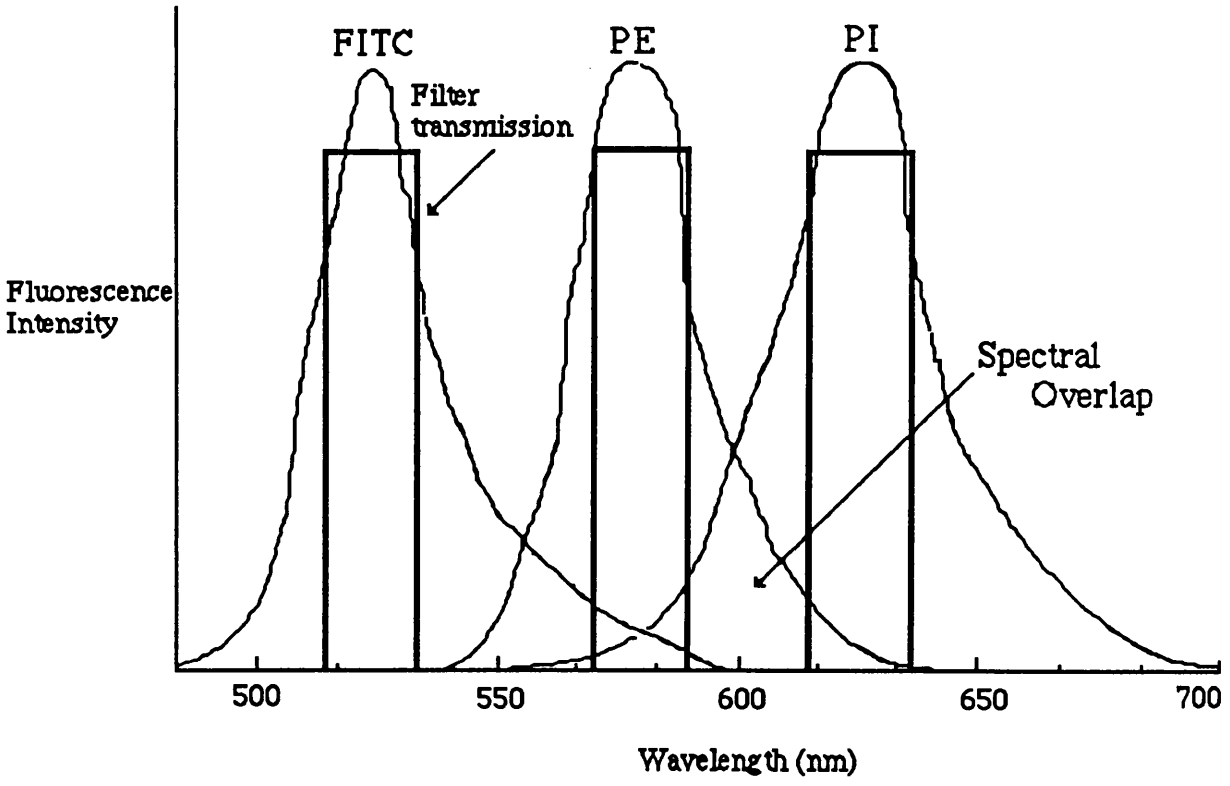


Figure 2.3 Diagram of filter transmission for the separation of fluorescence

PE is required as the label of second chromic and can be excited at 488nm but emits at a wavelength of 575nm with a strong orange/red coloured signal. It can be easily distinguished from the green fluorescence of fluorescein and detected by FL2 channel. For a third dye, propidium iodide (PI) is used to perform live and dead cell discriminations by intercalation to double-stranded nucleic acid. This dye absorbs at 488nm and emits light at greater than 650nm detected in the red part of spectrum channel (FL3). The shorter wavelengths between 500nm and 650nm pass on towards the FITC fluorescence detector and through a 530nm filter. Wavelengths greater than 650nm are reflected toward the PE fluorescence detector and through a filter centred at 578nm. The PI emission covers the range from 550nm to over 700nm and through a 640nm filter. Emission filters in each of the detector channels are selected to minimise spectral overlap (Figure 2.3).

2.2.3.3 Preparation of samples

2.2.3.3.1 General

The cells were washed twice in PBS and resuspended at a final cell density of approximately 10^7 cells/ml. 50 µl of this cell suspension was incubated in the flow cytometry tubes (Falcon 2052) with the appropriate monoclonal antibodies (Table 4.1) to specific phenotypic and activation markers on the lymphocyte membrane for 20 minutes on ice. All samples were maintained in the dark to prevent bleaching of the fluorochrome. In the case of double staining, after the initial 20 minutes incubation the cells were washed in 1 ml of PBS and centrifuged at 150g for 5 minutes, the supernatant was gently removed, and the cells were incubated in the next antibody for 20 minutes on ice. After the final incubation step the cells were washed and resuspended in 0.3 ml of PBS. Propidium iodide (10µg/ml) was then added

to each tube to discriminate live cells from dead cell populations from the analysis. Data on 5000 live cells were then acquired on FACScan.

2.2.3.3.2 Trypsin-detergent method

Preparation of nuclei for flow cytometric DNA analysis was performed according to the method of Vindelov *et al.* (1983) (Section 2.1.15). Nuclei were prepared from SP2/0 myeloma cells prior to cell fusion. Firstly, 900µl of solution A was added to 200µl of the cell suspension in citrate buffer and the contents were mixed by inverting the tube gently. After incubation for 10 minutes at room temperature, during which the tubes were inverted five times, 750µl of solution B was added. This was followed by a further 10 minutes incubation at room temperature and again the solutions were mixed by inversion of the tube. Finally, the cells were stained with 750µl of ice-cooled solution C and the tubes were incubated at 4 ^{o}C in the dark until analysed between 15 minutes and 60 minutes later.

2.2.3.3.3 Ethanol fixation method

This work was performed by the cell suspension fixation method of Zarbo *et al.* (1989). The washed cells were resuspended in PBS and then distributed into 100µl each to sample tubes. To this cell suspension, 100µl of cold 70% ethanol was added dropwise slowly with vortexing After incubation for 30 minutes on ice, the cells were washed in 1 ml PBS for 5 minutes with centrifugation at 150g and stained with monoclonal antibodies. The tubes were again incubated for 30 minutes on ice and centrifuged for 5 minutes at 150g. Finally, the cells were resuspended in 200µl PBS and 10µl of PI was added to each these while creating access for nuclear DNA dye

penetration. This method is particularly useful in studies to screen the intracellular and cell surface molecules simultaneously with cell cycle analysis.

2.2.3.4 FACScan analysis

2.2.3.4.1 DNA cell cycle analysis

The quantitative analysis of cell cycle distribution was done by FACScan flow cytometer using 10,000 nuclei per sample.

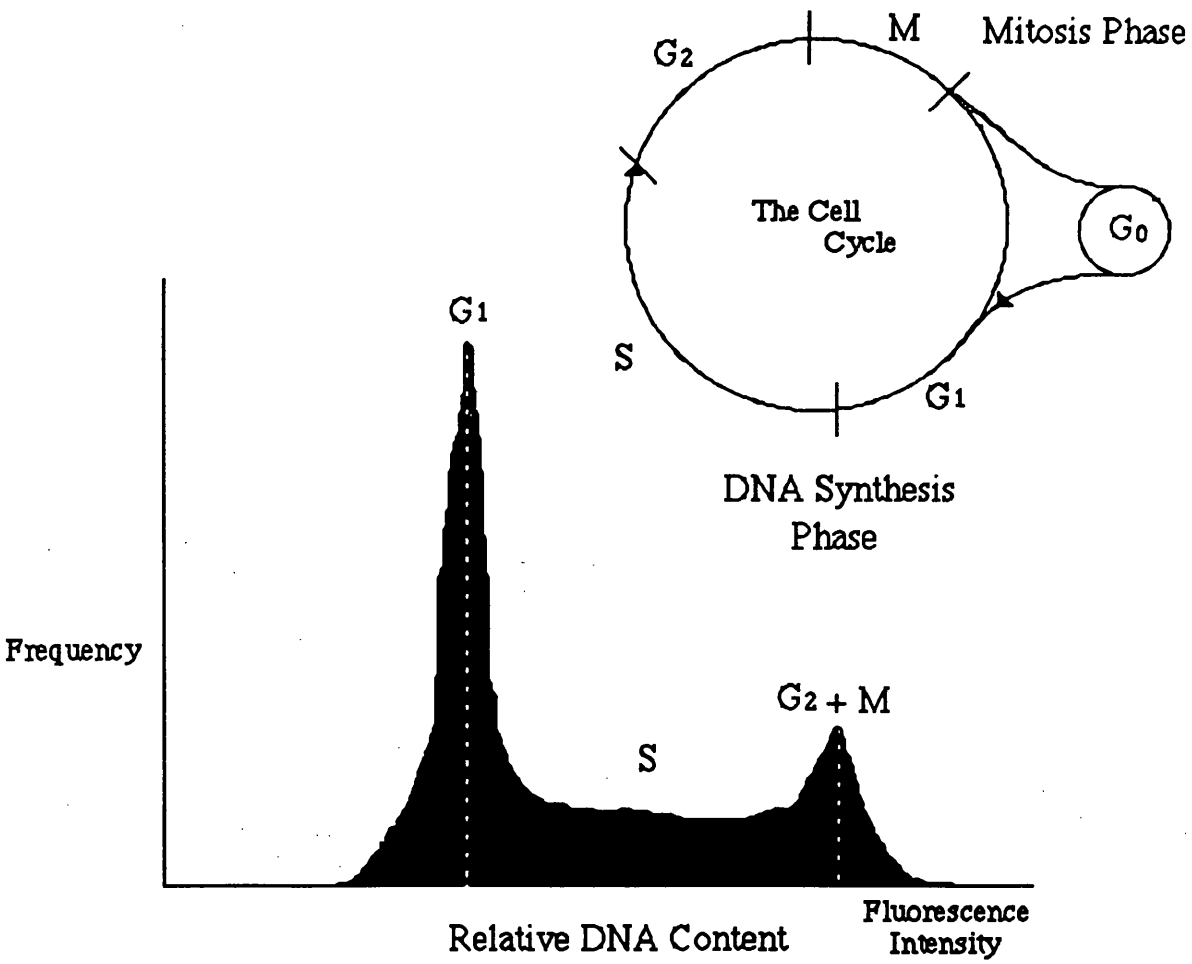


Figure 2.4 Diagram illustrating flow cytometric analysis of the cell cycle together with a DNA histogram

Analysis of such data gives information on both the nuclear content (DNA ploidy), which is frequently abnormal in breast tissue, and the S phase fraction (SPF), which is the measure of the proliferation rate of a growing cell population. Cells in G2/M phase of the cell cycle have double the DNA content, while cells in S-Phase will have a DNA content lying between Go/G1 and G2/M phase (Figure 2.4). After the nuclei were stained (Vindelov *et al.*,1983), the data was collected and analysed using available DNA cell cycle analysis software. The DNA content measured produces a histogram of the number of cells, and this will reflect the cell cycle.

2.2.3.4.2 Discrimination of live and dead cells

Data from cell samples acquired on FACScan were collected by removing dead cells by using a live selection gate.

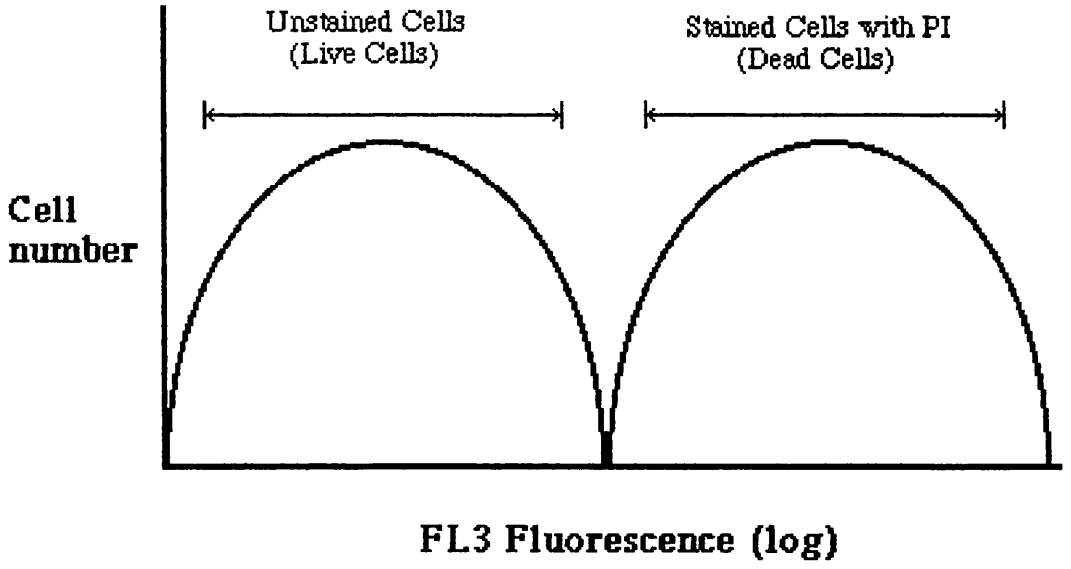


Figure 2.5 Diagram of collection gate to exclude dead cells stained with propidium iodide (PI) from live cell population

This method makes it possible to collect data from live cells with intact membranes and exclude data from dead cells with breached membranes using propidium iodide (PI), a DNA stain. PI intercalates with DNA and RNA but cannot to do so in the case of cells with intact membranes. Thus dead cells, nucleated and non-viable cellular debris, can be preferentially selected and excluded on a FL3 channel (Figure 2.5).

2.2.3.4.3 Discrimination of individual cell populations

The specific size and granularity of cells can be identified by using a Forward and Side scatter plot and these properties can be used to differentiate between lymphocytes, monocytes and neutrophils. It is possible to gate any unwanted cells positioning in the lower left hand portion of the Scatter plot staining with fluorescently labelled antibodies, and such cells therefore can be identified as distinct cell populations on FL1 versus FL2 plot. Also drawing a gate around the lymphocyte population, it is possible to collect data confined only on lymphocytes. Becton-Dickinson software package called "paint-a-gate" gives a information about the identification of cell populations in one parameter and then identify the same cells in another parameter. In this study, all analyses of lymphocytes were performed with both the PI gate and the lymphocyte scatter gate. The proportion of cells positive for the phenotypic or activation markers were analysed using four quadrant analysis of FACScan software. The positive cells were identified on each quadrant according to the fluorescence channel and relative percentage of these cells measured on the computer.

2.2.3.4.4 Dual-parameter colour analysis

Dual immunofluorescence techniques have been developed to analyse the degree of expression of multiple markers on the surface of cells, enabling the fluorescence intensity of multiple monoclonal antibodies (Table 4.1) to be measured simultaneously on each cell. Basically, the two fluorescence dyes used were fluorescein isothiocyanate (FITC) and phycoerythrin (PE) to perform two-colour analysis. The samples were incubated with a primary antibody (10µl), washed, incubated again with second antibody (10µl) and analysed. The positive cells for a given activation marker (FITC labelled) were measured on the FL1 channel and another cell population for a PE labelled antibody were measured on the FL2 channel. Finally, the distinct double-stained cell population was displayed on quadrant 2.

2.2.4 Preparation of Cell Membrane Proteins

This procedure was prepared by the method of Earp *et al.*,1984. Human B cell lines were centrifuged at 150g for 5 minutes and then washed twice with 10ml of 0.15M NaCl. The cells were gently resuspended in 2.5ml homogenisation buffer (10mM PBS, pH 7.4; 1mM EDTA) and broken by sonication on ice. 2.5ml of homogenisation buffer (10mM PBS, pH 7.4; 1mM EDTA; 0.5M sucrose) was added to the broken cell solution and centrifuged at 150g for 5 minutes to remove nuclei and unbroken cells. The supernatant was transferred and centrifuged for 60 minutes at 105,000g in a Ti 70.1 Beckman rotor. Finally, the pellet was resuspended in 20 mM PIPES, pH 7.0.

2.2.5 Estimation of Purification of Cell Proteins

To ensure the method used for preparation of cell membrane proteins, the homogenised cells prepared as described in section 2.2.4 were assayed according to the Lowry protein assay (Lowry *et al.*,1951). Firstly, 400µl of the Lowry concentrate (Section 2.1.12) was added to a 400µl of sample and incubated at room temperature for 20 minutes. After addition of 0.2N Folin reagent, this solution was mixed with vortexing immediately and then incubated for an additional 30 minutes at room temperature. The samples were monitored within 10 minutes of each other on an LBK spectrophotometer at 500nm absorbance using glass cuvettes. Finally, the protein concentration was estimated on the standard curve.

2.2.6 Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Samples in loading buffer were heated in a boiling water bath for 2 minutes and loaded into individual wells in the stacking gel. Electrophoresis was performed at a constant current of 45mA for 4 hours or 10mA for 18 hours at room temperature until the dye front reached the bottom of the gel.

2.2.6.1 Coomassie blue staining

After electrophoresis gel was stained in coomassie blue stain solution (Section 2.1.9.4) for 1 hour and then destained until protein bands were revealed. The gel was dried onto Whatman 3mm filter paper in a vacuum drier at 80 °C.

2.2.6.2 Silver staining

This is a method for highly sensitive staining of a gel developed by Wray *et al.* (1981). The SDS-PAGE gel was fixed in 40% ethanol, 5% glacial acetic acid and 55% double deionised water overnight to remove SDS. It was then stained in staining solution (Section 2.1.9.4) for 20 minutes with gentle shaking and then washed with deionised water with three changes. The gel was developed using developing reagent and protein bands were appeared within 20 minutes. The reaction was terminated by rinsing the gel in 50% methanol.

2.2.7 Western Blot

This technique was performed by the method of Batteiger *et al.* (1982) with modifications. After electrophoresis the gel was removed from the gel apparatus and placed on Whatman 3mm filter paper keeping it wet with transfer buffer (Section 2.1.6) at all stages, avoiding air bubbles. The nitrocellulose paper was covered over with a second piece of filter paper and then encased between the gause squares within the plastic cassette soaked in the same buffer. This assembly was fitted into the transfer tank, in such a way that the nitrocellulose paper was nearest to the positive electrode. Electro-transfer was achieved at 350mAmps for 4 hours at room temperature and then the nitrocellulose paper was blocked in the blocking buffer (5% BSA in PBS, pH 7.4) for 1 hour at room temperature. Washing was carried out with 3 x 100ml of buffer PBS, pH 7.4 for over 30 minutes, and nitrocellulose strip was incubated with culture supernatant of monoclonal antibody for 1 hour on shaker at room temperature. After further washing, the nitrocellulose paper was incubated with the second antibody (Section 2.1.14), rabbit anti-mouse Ig G (whole molecule) which was diluted in 1/500 with 5% BSA/PBS, pH 7.4 for 1

hour at room temperature and then washed in the PBS, pH 7.4. Anti-human Ig G protein A conjugated peroxidase diluted in 1/500 with 5% BSA/0.01% Tween 20/PBS, pH 7.4 was involved in final incubation of second antibody for 2 hours at room temperature and then finally washed in the PBS buffer. This nitrocellulose paper was developed in chemiluminescence substrate (Section 2.1.14.2) for 5 minutes and then covered with clingfilm before exposure to X-ray film (Section 2.1.14.1).

Staining for protein

The nitrocellulose paper which was blocked with 0.1% Tween 20 in the blocking step could be stained with Amido black (Section 2.1.14.3) to identify the antigenic bands after developing. Staining was achieved in a 2 minutes incubation and destained in 50% methanol, 10% acetic acid in water.

2.2.7.1 Autoradiography

After exposure to X-ray film which was sandwiched between nitrocellulose paper and a Dupont "Cronex" intensifier screen in a light proof cassette, autoradiographic images were visualised by processing of films in a Kodak X-OMAT processor.

2.2.8 Statistical Analysis

Statistical analysis was performed by using the Apple Mac Statworks software, and the data was analysed using the Mann-Whitney U test. A p value ≤ 0.05 was considered significant. The results were expressed as mean $\pm$ standard deviation.

CHAPTER 3

THE PRODUCTION OF MONOCLONAL ANTIBODIES REACTIVE WITH CELL SURFACE ANTIGENS PRESENT ON HUMAN LYMPH NODE LYMPHOCYTES

3. The production of monoclonal antibodies reactive with cell surface antigens present on human lymph node lymphocytes

3.1 Aim and summary of study

Previous Mabs identifying putative nodal "homing" markers were generated from established tissue culture lines and, in the case of LEU-8, by cDNA homology analysis from human to mouse. No other study has set out to specifically identify markers unique to the human lymph node as its first aim. This chapter describes an approach designed to do this. In order to identify marker unique to the node, monoclonal antibodies reactive with nodal T cells were generated. Immunisation and screening by FACScan showed that the nodal B cells were highly immunodominant and a clone reactive with these was isolated and characterised on B and T cell lines. Screening with ELISA on "panned" T cells was also then employed and used to isolate monoclonal antibodies which were expanded and characterised. Three resultant monoclonal antibodies were identified by dual-parameter flow cytometric analysis on lymph node lymphocytes, and were compared for reaction with peripheral blood lymphocytes.

3.2 Results

3.2.1 Cell fusion and screening

The procedure for immunisation, fusion, selection of hybrid cells and screening of culture were by methods described previously in Section 2.2.1.

Sera of Balb/c mice immunised with a highly enriched population of nodal cells were tested by using FACScan or ELISA assay for the appropriate

fusions (Section 2.2.1.3 and 2.2.1.6.1). Data from serum from a mouse immunised with whole lymph node lymphocytes and two sera from mice immunised with panned nodal T lymphocytes are shown in Figures 3.1, 3.4, 3.7a and 3.7b respectively. The mouse immune sera examined all reacted with a proportion of nodal lymphocytes. In the case of Figures 3.4 and 3.7b, all cells were positive in the node; implying that the serum contained antibodies reactive with both T and B cells in the node. The second antibody alone sometimes reacted with cells (almost certainly B cells) and sometimes didn't. This may have been because some second antibody supplies were less specific so that the anti-mouse IgG antibody reacted with human IgG on human B cell surfaces.

Myeloma (SP 2/0) cells were at least over 35% in the S-phase of cell cycle (Figures 3.2 and 3.5) on the fusion days.

After fusion, the three clones were cultured according to Section 2.2.1.5, and the relevant positive clones from the fusion plates were screened (Section 2.2.1.6) and selected by FACScan or ELISA assay. SP 2/0 cells and spleen cells were used as negative controls, and serum titre was used as positive control. Tissue culture supernatant was used as the source of the monoclonal antibodies for FACScan and ELISA assay analysis. The results are illustrated in Figures 3.3, 3.6 and 3.8. Figure 3.3 showed that more cells were positive in the subcloning stage because there were more B cells in the node sample used. Figure 3.9 shows the cells of the mouse Balb/c hybridoma.

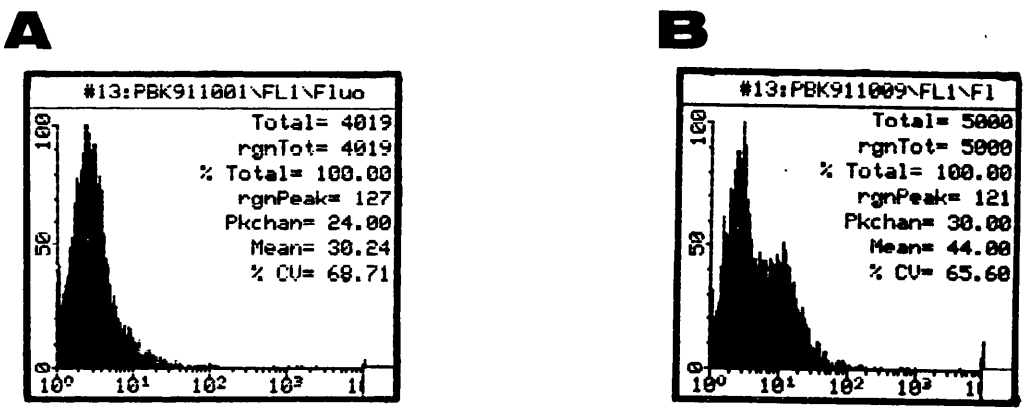
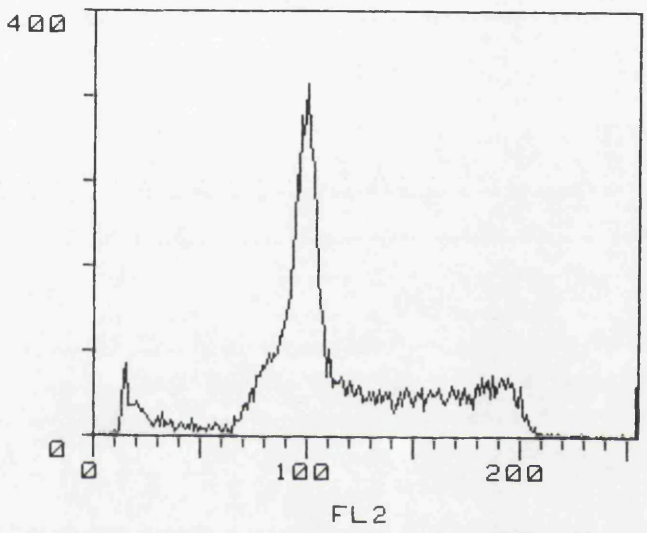


Figure 3.1 FACScan analysis of serum test from a mouse immunised with whole lymph node lymphocytes for the first approach to monoclonal antibody production. **A** represents the histogram of control (2nd antibody only) and **B** shows the histogram of positive serum on nodal lymphocytes.



Cell Cycle Statistics

Phase	Percent
G1	54
S	38
G2+M	7

Figure 3.2 Analysis of the cell cycle of SP 2/0 cells used for the fusion which produced the first monoclonal antibody.

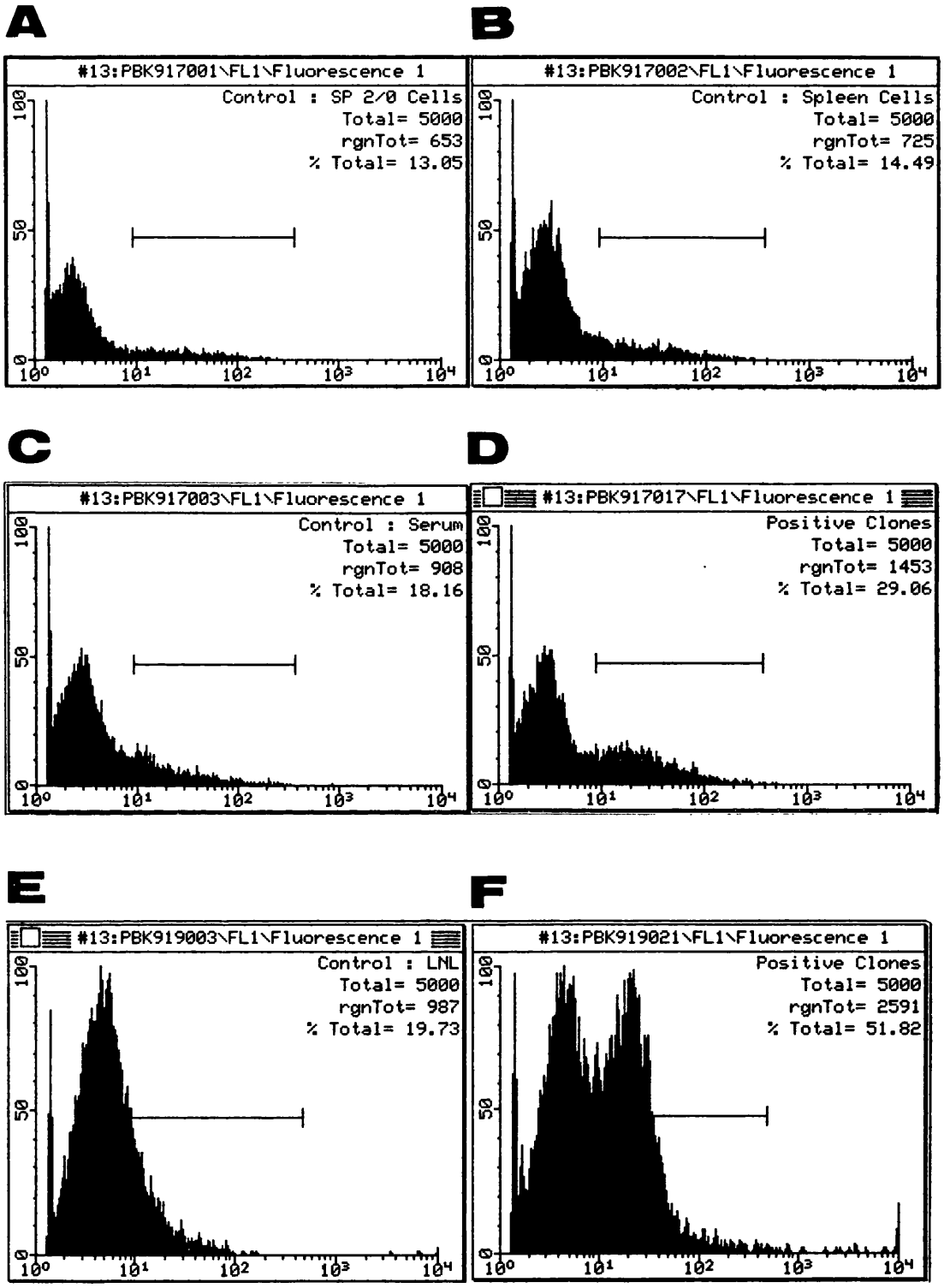


Figure 3.3 Selection of positive clones from first fusion by FACScan analysis. **A** shows SP 2/0 cell supernatant, **B** shows spleen cell supernatant, **C** shows the serum control, and **D** shows the histogram of the selected clone. **E** and **F** show the selected clone after selection and subcloning (**F**) compared to the control of 2nd antibody only (**E**).

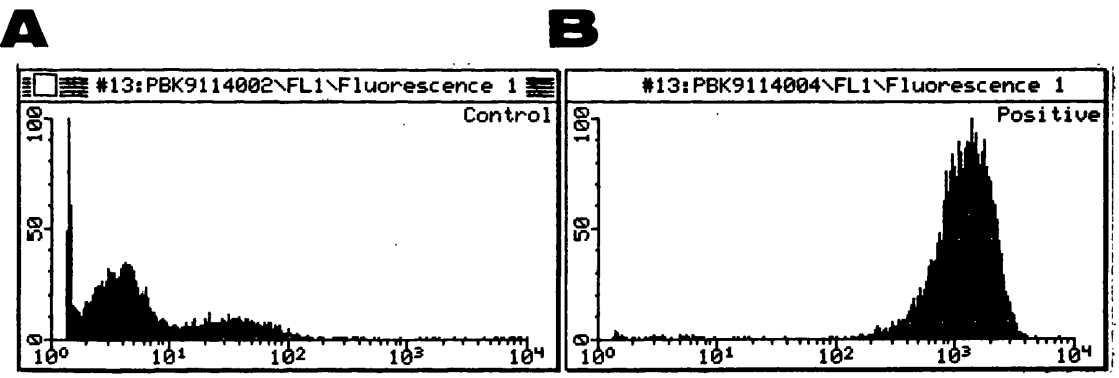


Figure 3.4 FACScan analysis of serum test from a mouse immunised with panned nodal T cells for the second approach to monoclonal antibody production. **A** represents the histogram of control (2nd antibody only) and **B** shows the histogram of positive serum on nodal lymphocytes.

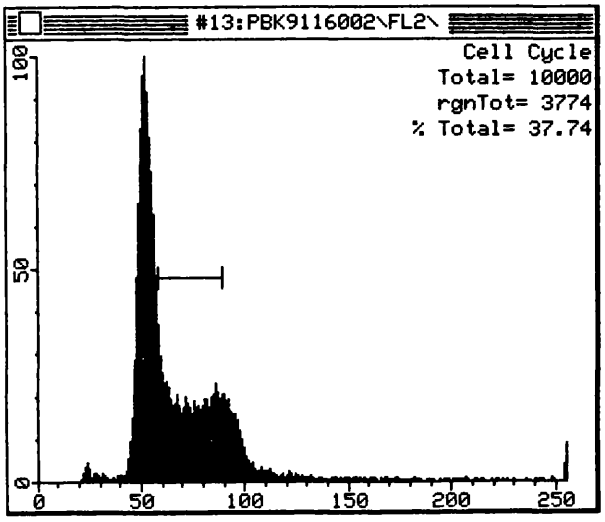


Figure 3.5 Analysis of the cell cycle of SP 2/0 cells used for the fusion which produced the second monoclonal antibody. S-Phase: 37.7%

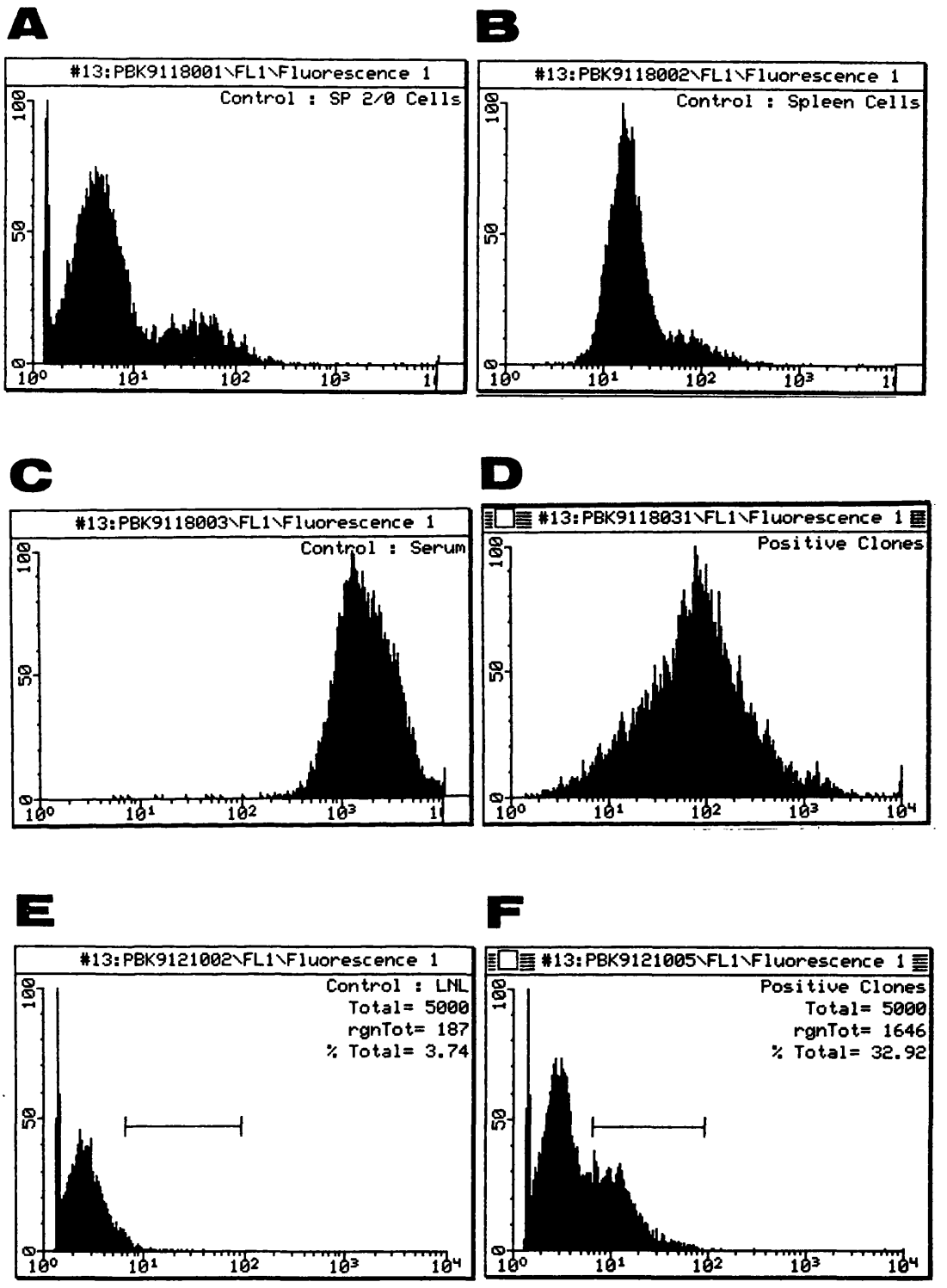


Figure 3.6 Selection of positive clones from second fusion by FACScan analysis. **A** shows SP 2/0 cell supernatant, **B** shows spleen cell supernatant, **C** shows the serum control, and **D** shows the histogram of the positive clone in screening. **E** represents the histogram of control (2nd antibody only), and **F** shows the histogram of the positive clone in recloning.

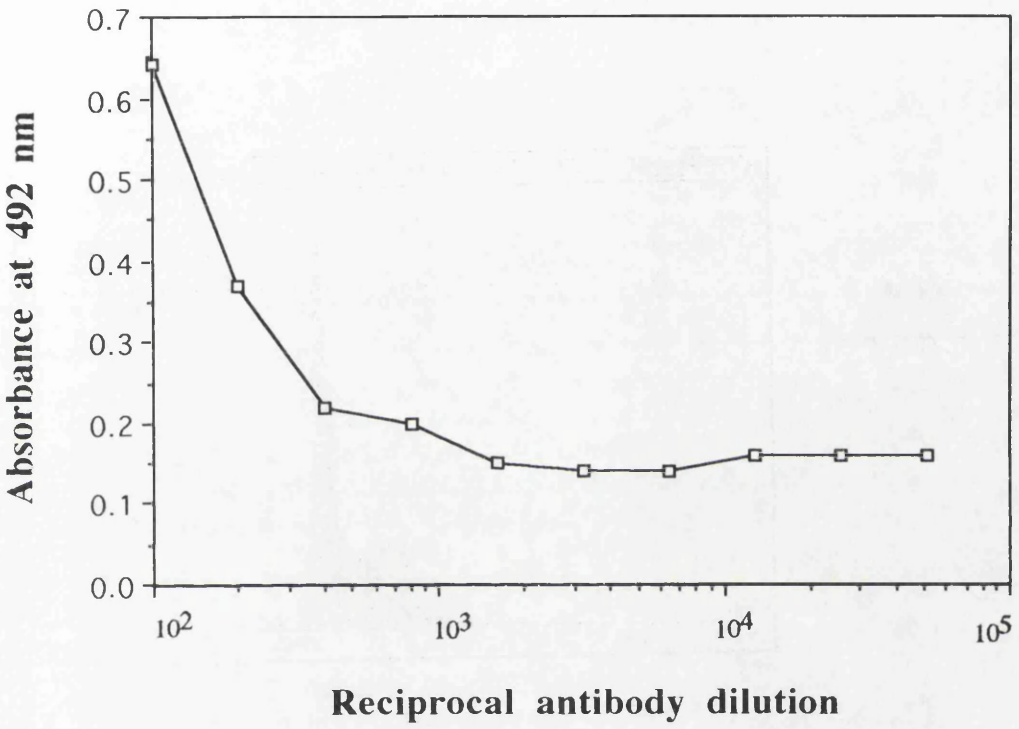


Figure 3.7a Serum test by ELISA assay of plate bound lymph node lymphocytes for third monoclonal antibody production strategy.

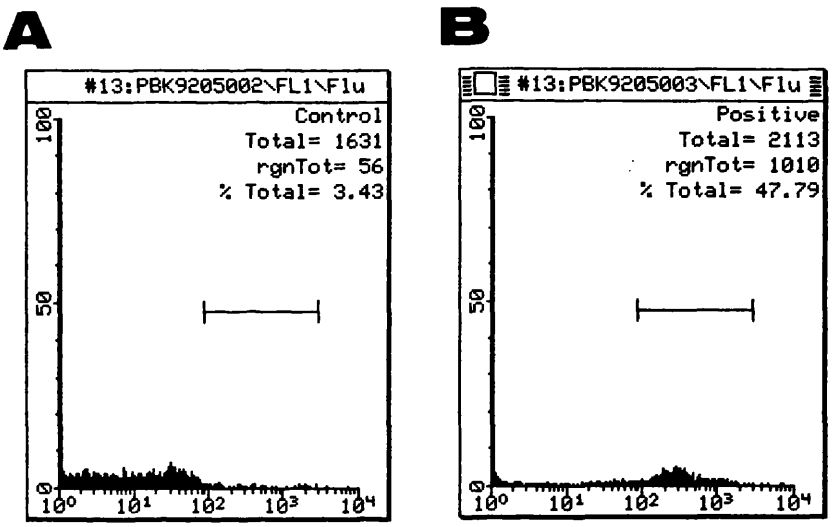


Figure 3.7b FACScan analysis of serum test from a mouse immunised with panned nodal T cells for the third approach to monoclonal antibody production. **A** represents the histogram of control (2nd antibody only) and **B** shows the histogram of positive serum on nodal lymphocytes.

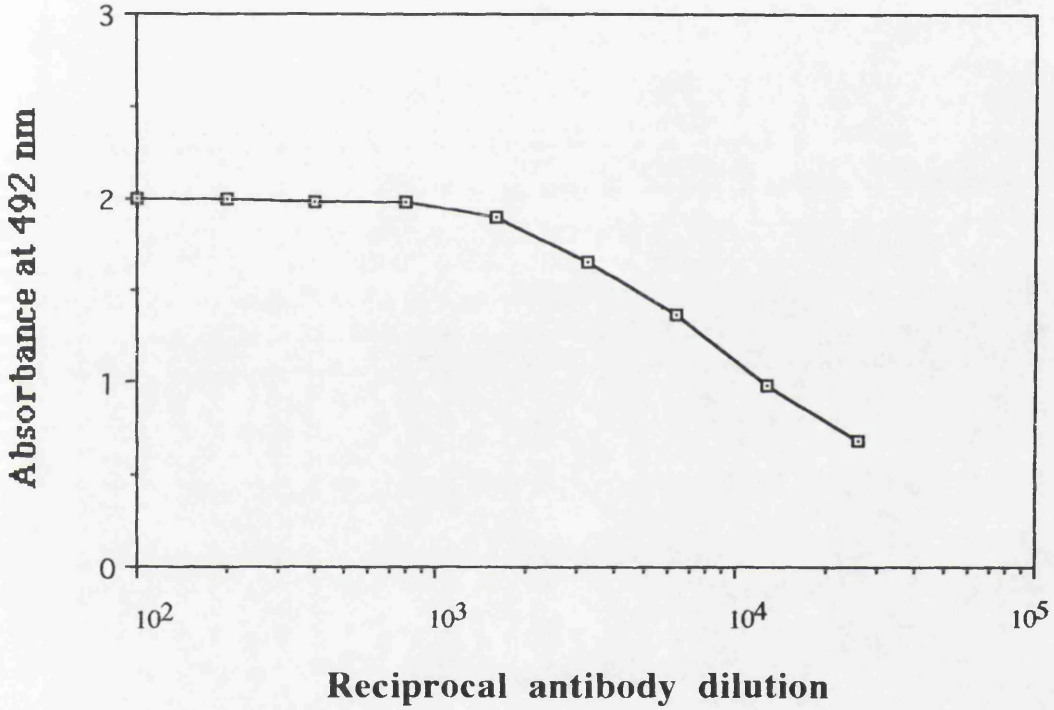


Figure 3.8 Selection of positive clone from third fusion by ELISA assay

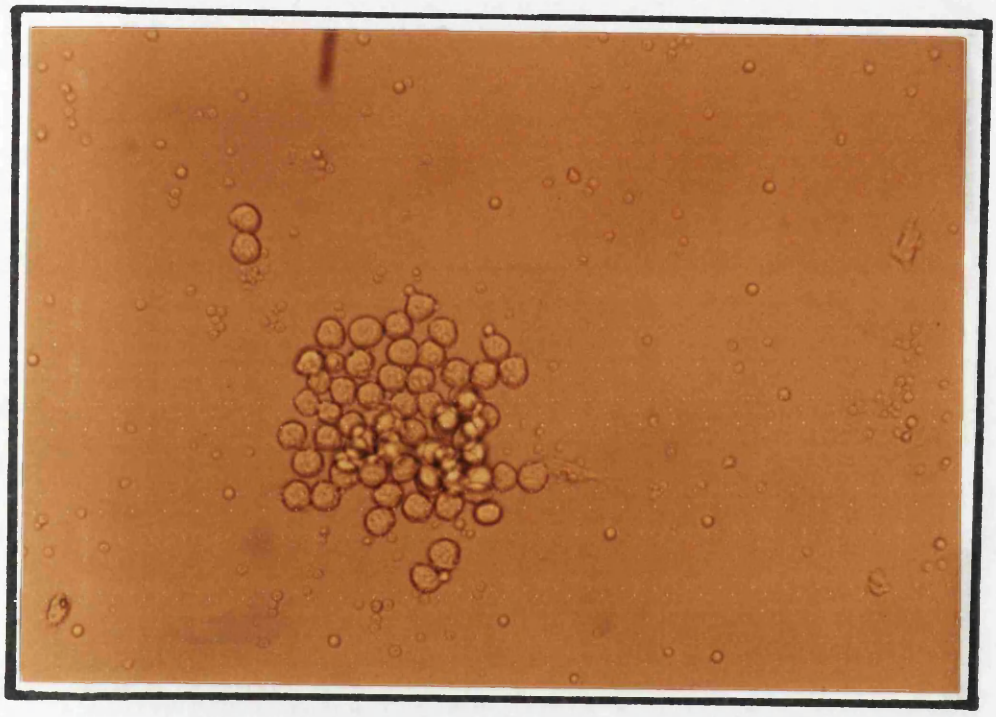


Figure 3.9 Cells of the first Balb/c mouse hybridoma one week after fusion. Magnification x 300

3.2.2 Characterisation

Purified IgG Mab labelled with FITC and IgG Mab from tissue culture supernatant and ascites fluid as described previously in Section 2.2.1.7 and 2.2.1.8 were prepared and tested by FACScan analysis of antigen binding activity. As depicted in Figures 3.10a, 3.10b and 3.10c, the monoclonal antibodies reacted with a subset of lymph node lymphocytes. In particular, Figure 3.10c shows Mab 3 binding to an antigen more strongly after ethanol permeabilisation of lymph node lymphocytes, indicating that it may detect an intracellular antigen.

3.2.2.1 Human B cell lines

Three human B cell lines (Raji, Daudi and Ramos) (Section 2.1.17.1) were used to investigate the phenotype specificity of Mabs 1, 2, and 3. Figures 3.11a, 3.11b and 3.11c showed that all three reacted with human B cell lines.

In addition, ELISA assay revealed that Mab 3 also bound to the human B cell lines. Figure 3.12 shows the ELISA profile of Mab 3 in tissue culture supernatant reacting Raji or Daudi cells.

The epitope recognised by the Mab was analysed on human B cell lines. A panel of different cell surface antigens were separated by a preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver stain as described earlier in Section 2.1.9 (Figure 3.13). Western blotting (Figure 3.14) demonstrated that Mab 2 recognised an epitope in molecular weight region of the 95 kDa antigen (CD19) of human B cell lines. Mab 1 and 3, however, failed to detect the antigen in Western blotting due to high backgrounds.

3.2.2.2 Reactivity with human T cell line

To investigate the reaction of the Mabs with T cell antigens, the three produced Mabs were analysed by using FACScan. Figure 3.15 shows that these Mabs did not bind to the human Jurkat T cell line (Section 2.1.17.2).

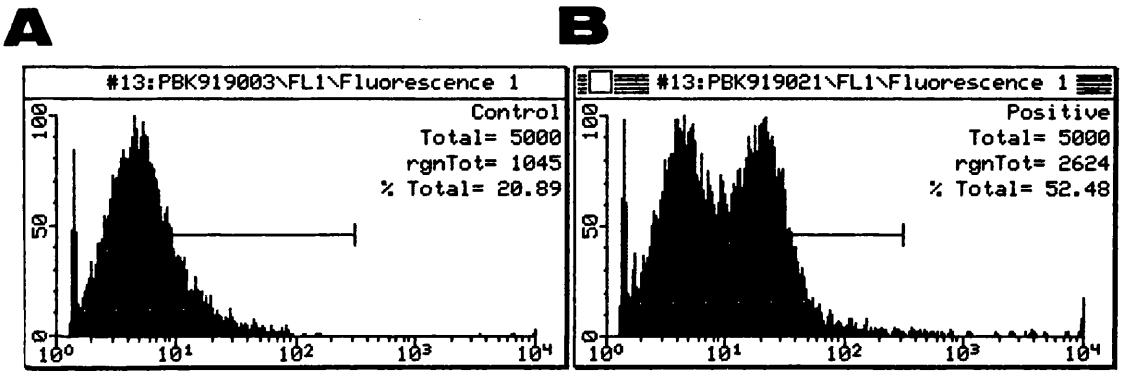


Figure 3.10a Characterisation of Mab 1 against lymph node lymphocytes by FACScan analysis. **A** represents the histogram of the control (2nd antibody only) and **B** shows the histogram of the positive clone.

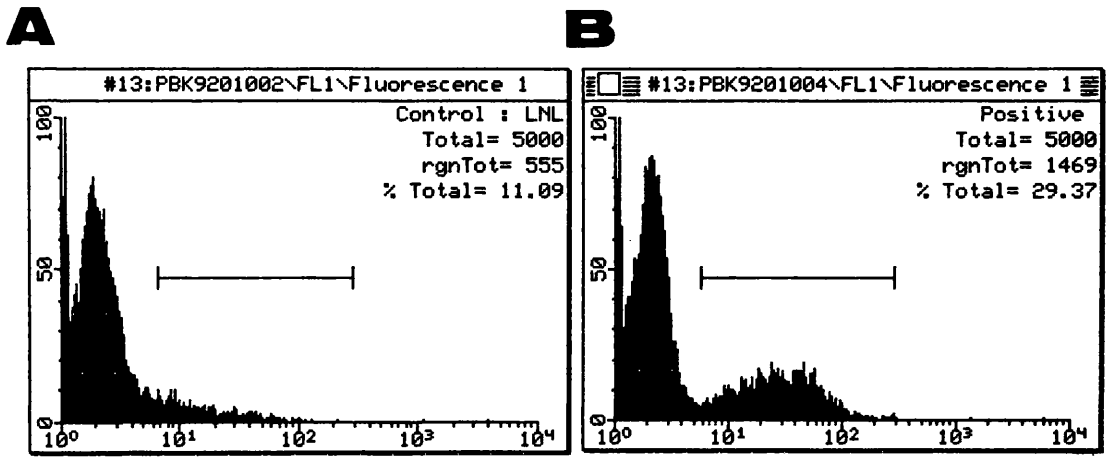


Figure 3.10b Characterisation of Mab 2 against lymph node lymphocytes by FACScan analysis. **A** represents the histogram of the control (2nd antibody only) and **B** shows the histogram of the positive clone.

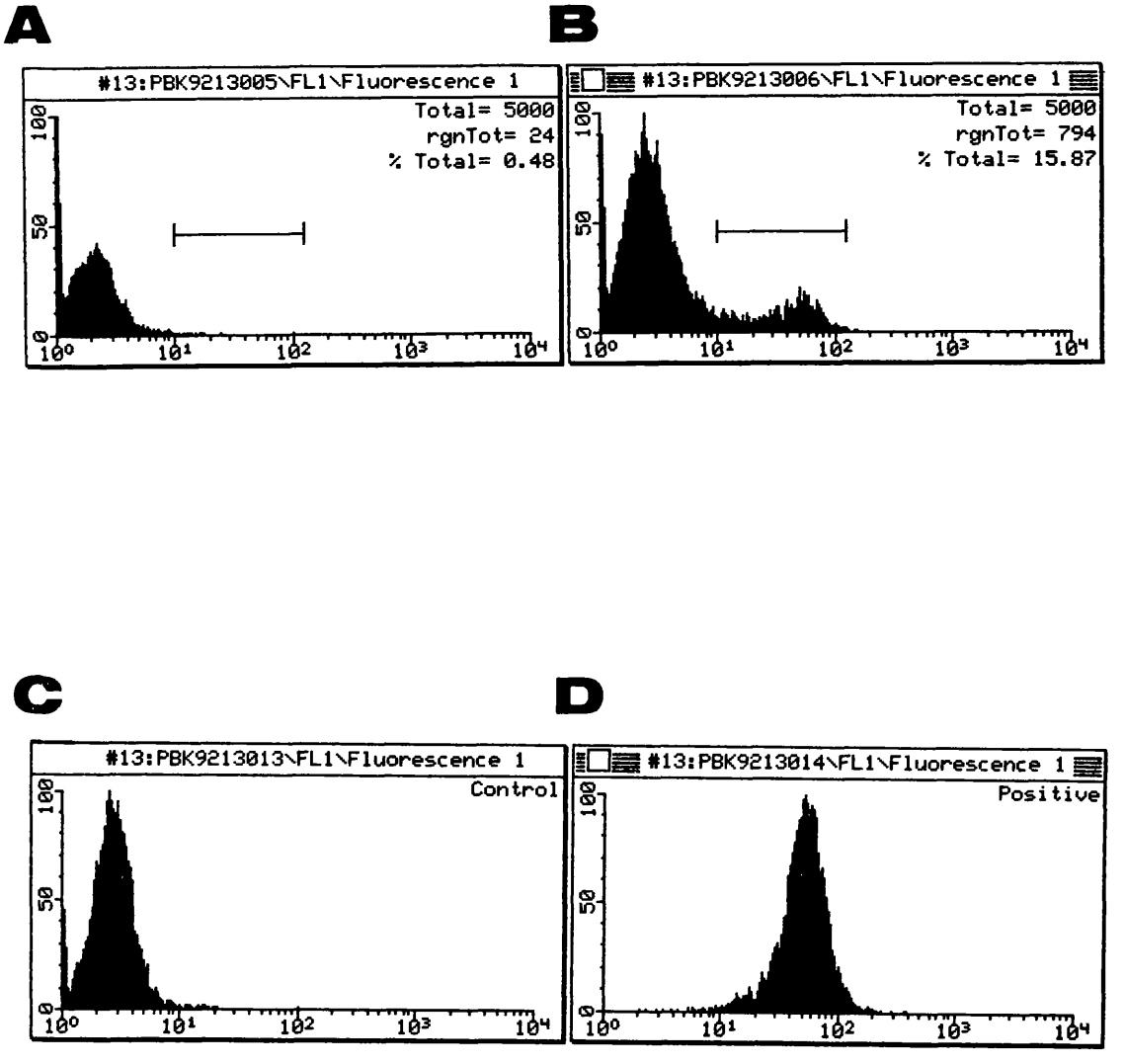


Figure 3.10c Characterisation of Mab 3 against lymph node lymphocytes by FACScan analysis. **A** and **C** represent the histograms of the control. **B** and **D** show the histograms of the positive clone. In **C** and **D** the cells have been permeabilised with ethanol to allow access to intracellular antigens.

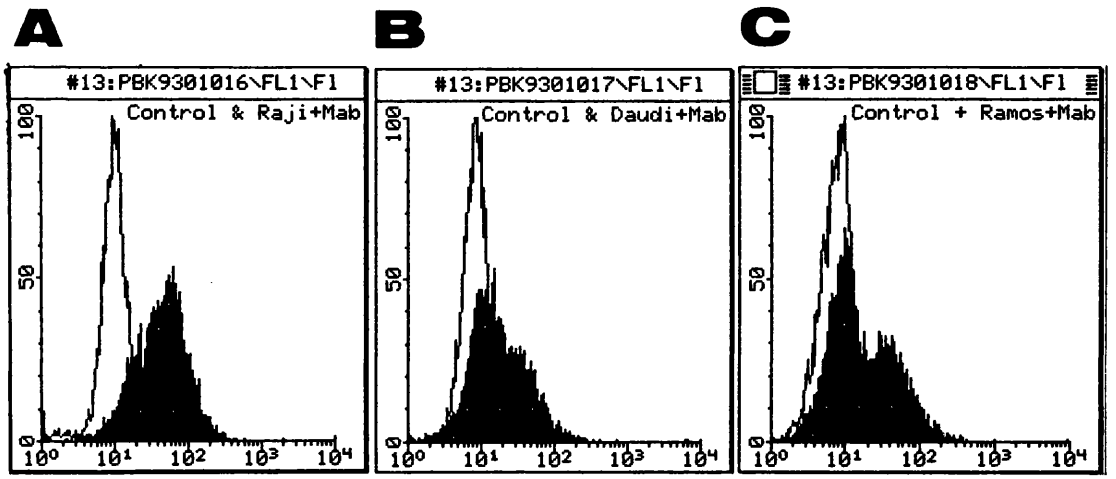


Figure 3.11a Characterisation of Mab 1 on human B cell lines against control of 2nd antibody only. **(A)** Raji cells **(B)** Daudi cells **(C)** Ramos cells

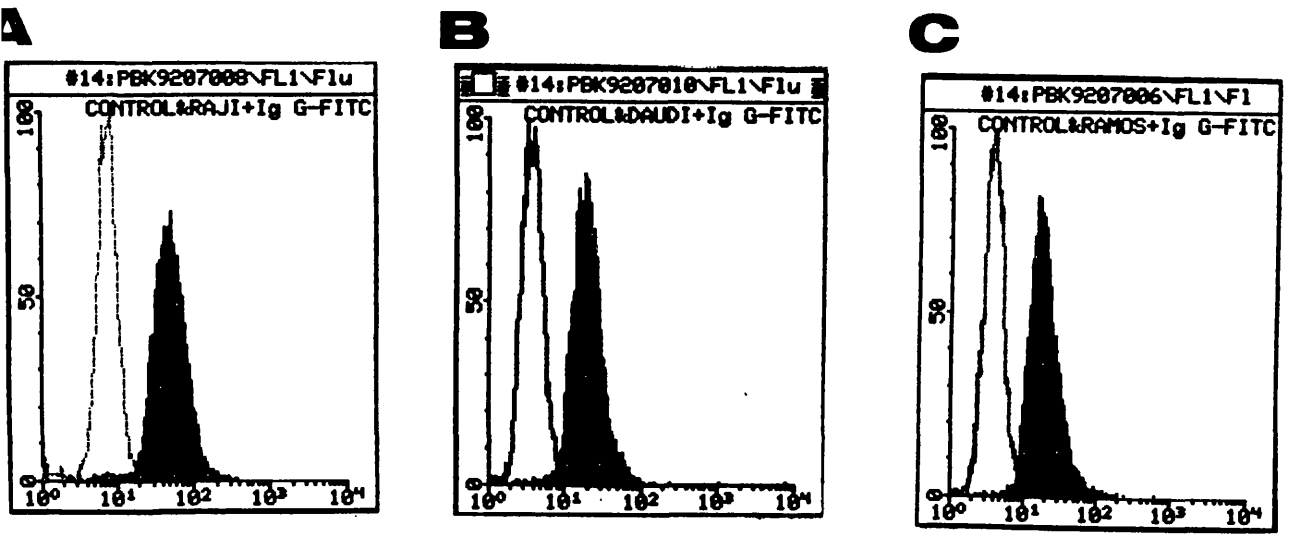


Figure 3.11b Characterisation of Mab 2 (ascites) directly FITC labelled on human B cell lines against control of 2nd antibody only. **(A)** Raji cells **(B)** Daudi cells **(C)** Ramos cells

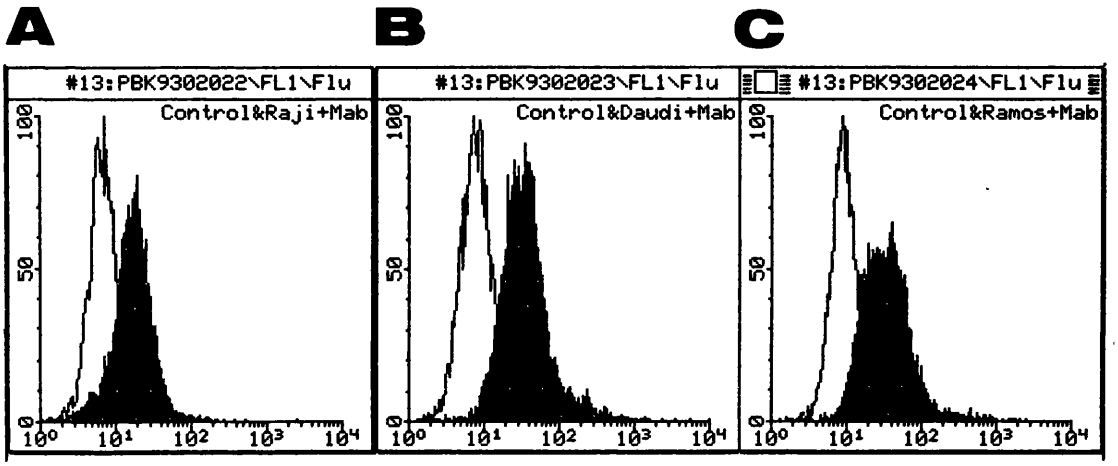


Figure 3.11c Characterisation of Mab 3 (ascites) on human B cell lines against control of 2nd antibody only. The cells have been permeabilised with ethanol to allow access to intracellular antigens. **(A)** Raji cells **(B)** Daudi cells **(C)** Ramos cells

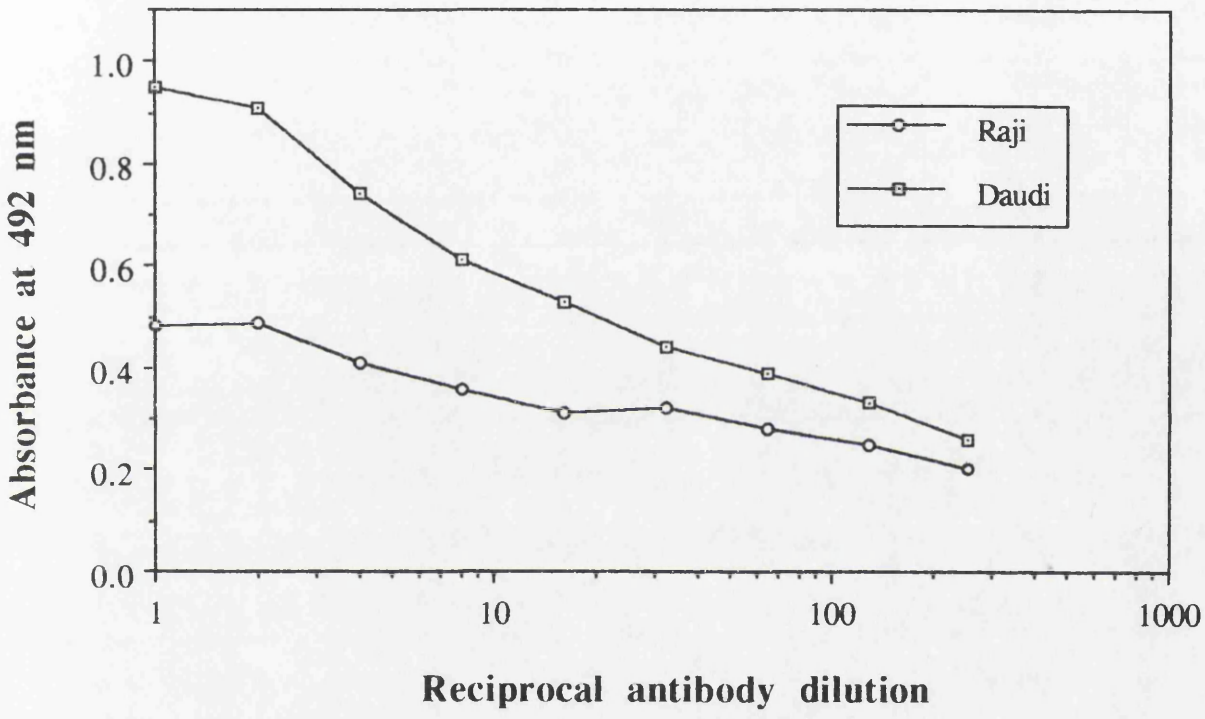


Figure 3.12 Characterisation of Mab 3 on human B cell lines by ELISA assay.

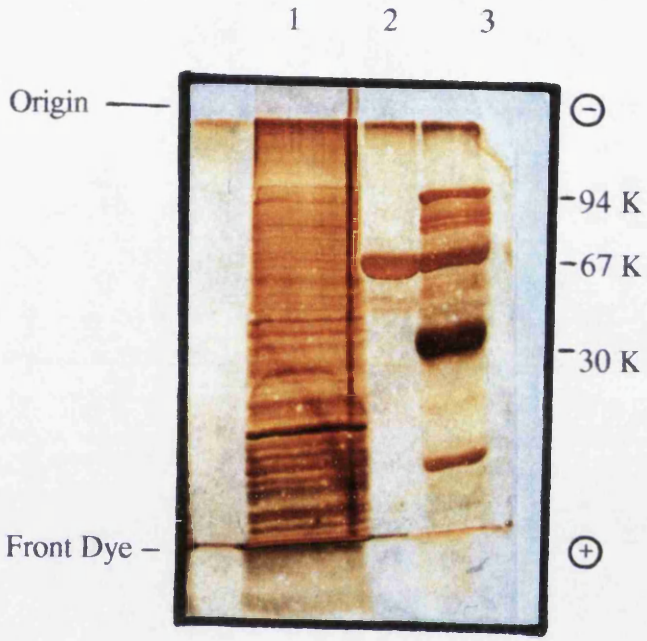
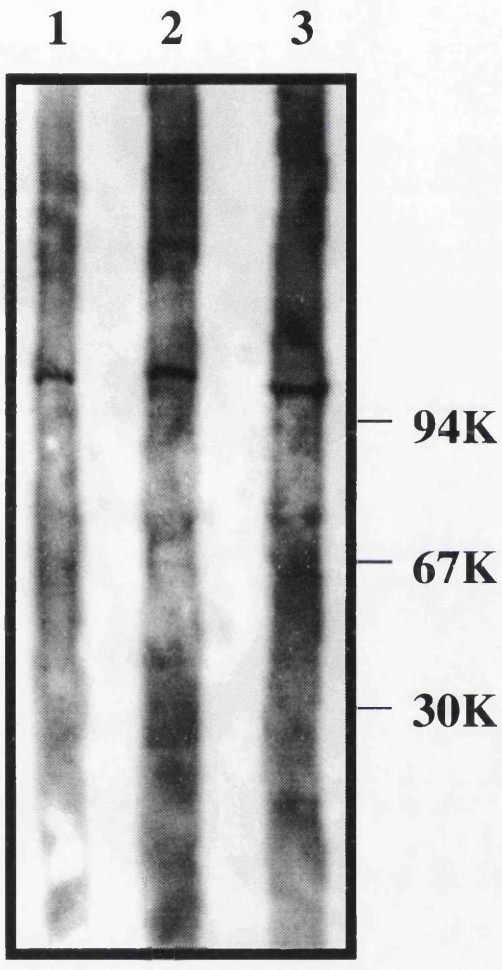


Figure 3.13 SDS-PAGE gel of human B cell line, Raji stained by silver stain. Lanes : 1, Raji cell membrane proteins; 2, BSA; 3, Markers

Figure 3.14 Western blotting of monoclonal antibody 2 on cell membrane of human B cell lines.



Lane 1: Ramos cell membrane
2: Daudi cell membrane
3: Raji cell membrane

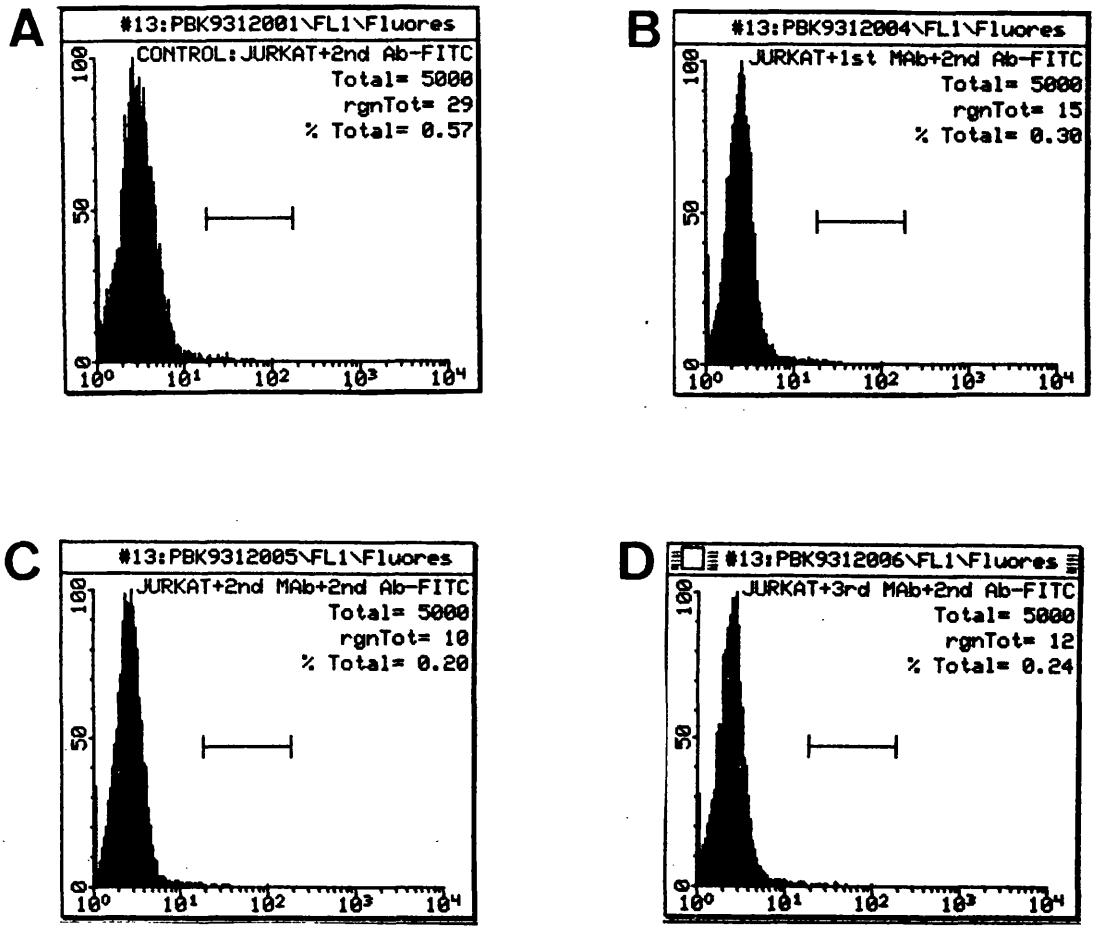


Figure 3.15 Characterisation of monoclonal antibodies on the Jurkat human T cell line. **(A)** FITC labelled 2nd antibody alone **(B)** Mab 1 **(C)** Mab 2 **(D)** Mab 3

3.2.3 Dual parameter analysis

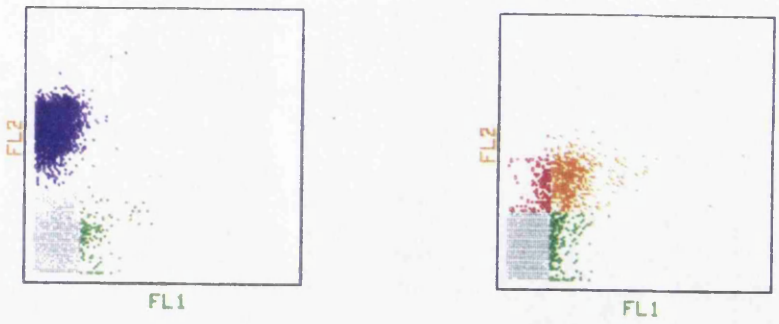
In order to identify whether the nodal cells identified by the Mabs were B or T cells, dual-parameter flow cytometric analysis was performed. All cells were stained with Mabs specific for cell surface markers (CD3 and CD19), washed, incubated with the appropriate Mab and detected in a FACScan flow cytometer. Figure 3.16 confirmed that all three previously produced Mabs identify a B-cell epitope in the lymph node.

3.2.4 Reaction to PBL

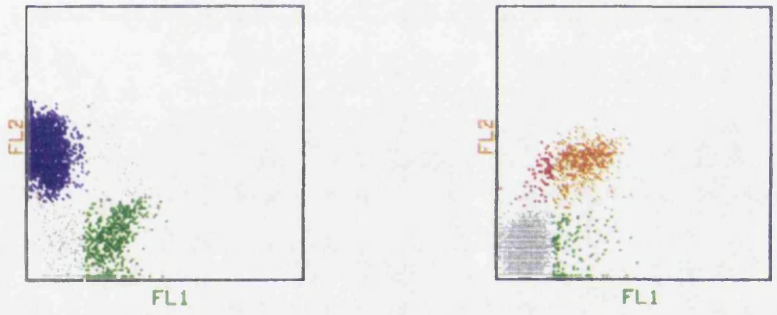
Two of the Mabs reacted with peripheral blood lymphocytes as well as nodal lymphocytes, but the reactivity of Mab1 was not tested in this work. Figures 3.17 and 3.18 indicate that the epitope is located in not only LNL but also PBL. This result is compared with LEU-8 expression on PBL described in Section 4.2.3.

Figure 3.16 Dual parameter analysis of all three monoclonal antibodies on lymph node lymphocytes. **A** left shows anti CD3-PE plus Mab 1-FITC and no dual stained cells. **A** right shows anti CD19-PE plus Mab 1-FITC with most cells double stained. **B** left shows anti CD3-PE plus Mab 2-FITC and no dual stained cells. **B** right shows anti CD19-PE plus Mab 2-FITC with most cells double stained. **C** left shows anti CD3-PE plus Mab 3-FITC and no dual stained cells. **C** right shows anti CD19-PE plus Mab 3-FITC with most cells double stained.

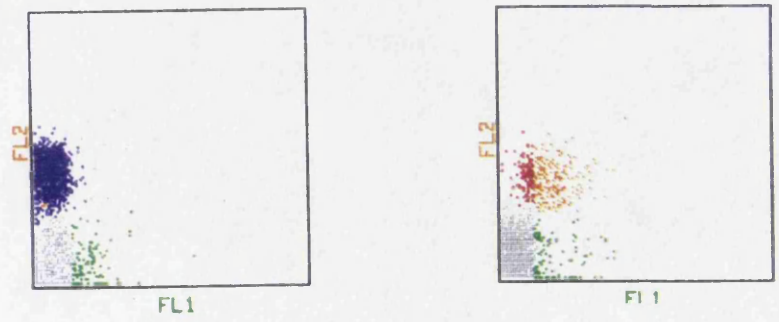
A



B



C



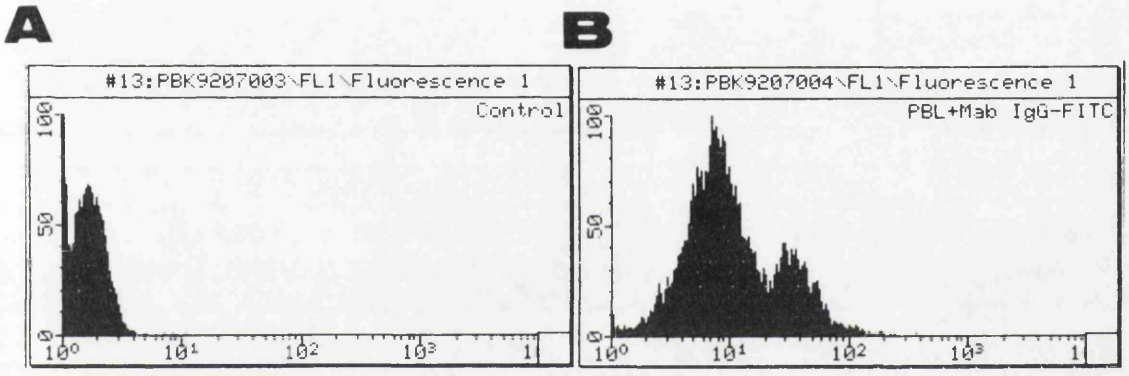


Figure 3.17 Mab 2 (ascites) on PBLs **(A)** Control (2nd antibody alone) **(B)** Mab 2 and 2nd antibody

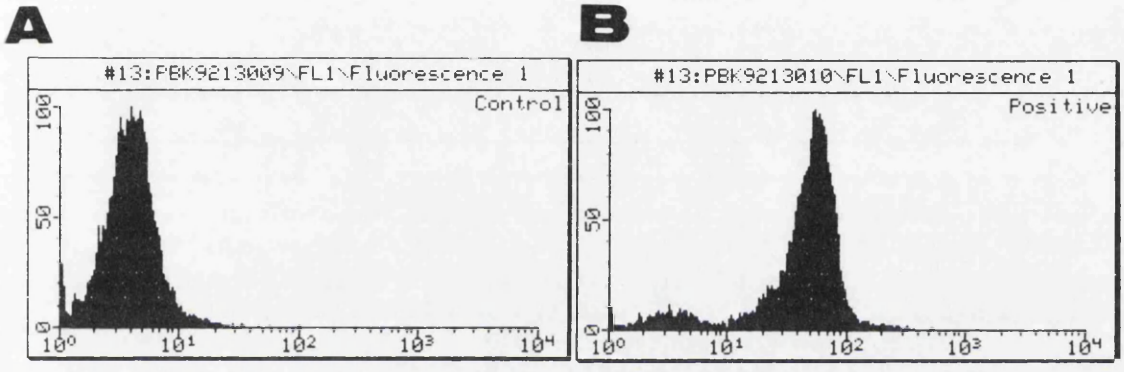


Figure 3.18 Mab 3 on PBLs after ethanol fixation (A) Control (2nd antibody alone) (B) Mab 3 and 2nd antibody

3.3 Discussion

A putative nodal homing marker, LEU-8, identified by cDNA homology analysis between human and mouse, has been suggested to participate in the process of leukocyte extravasation into lymphoid organs. This study was designed to identify markers unique to the human lymph node, and to compare these with the homing function suggested for the LEU-8 marker (Chapter 4).

Three monoclonal antibodies reacting with nodal cells were screened, selected and characterised by flow cytometric analysis and ELISA assay. In this study the only way of getting fresh human lymph nodes was during an operation for cancer. Post mortem nodes were too necrotic because of the time consuming legal formalities involved. The fact that the same Mab didn't show the same pattern each time was because a different node was from a different patient, and because there were only a few cells in each node and each node had a different proportion of B and T cells. The resultant monoclonal antibodies reacted with PBL as well as LNL, and bound to B cell lines and nodal B cells not the Jurkat T leukaemic cell line and nodal T cells. This was surprising as mice were immunised with panned nodal T cells contaminated with < 3% B cells. Thus B cells must be very immunodominant. The fact that all three monoclonal antibodies were to B cells further suggests that within the B cells there are particularly immunodominant antigens. In Western blotting analysis Mab 2 recognised an epitope in the molecular weight region of the CD19 molecule (95 kDa). It is not surprising that Mabs raised against membrane proteins may sometimes fail to detect them on immunoblot. Many membrane spanning proteins cross the membrane several times presenting a non-contiguous array of conformational epitopes to an external antibody. This will be lost on SDS-PAGE. These results indicate that the antigens identified were not to the node, but were distributed through out the peripheral

blood and nodal lymphocytes. This evidence will be compared with LEU-8 expression on PBL and LNL, and discussed in Chapter 4.

In summary, the monoclonal antibodies produced to date are to B cell antigens which are unlikely to be MHC Class I or immunoglobulin. In addition, B cells, where not quantitatively dominant in the human lymph node, are highly immunodominant. Despite a logical strategy, no specific homing molecule was identified. It was therefore of interest to find if other strategies had been more successful.

CHAPTER 4

FLOW CYTOMETRIC ANALYSIS OF LEU-8 EXPRESSION ON LYMPH NODE AND PERIPHERAL BLOOD LYMPHOCYTES OF BREAST CANCER PATIENTS

4. Flow cytometric analysis of LEU-8 expression on lymph node and peripheral blood lymphocytes of breast cancer patients

4.1 Aim of study

The LEU-8 cell surface molecule was previously reported as a human homologue of the murine MEL-14 lymph node homing receptor (Section 1.4.2.2). However, this homing function has not yet been proven in humans. If LEU-8 is the lymph node homing receptor there may be expected to be more LEU-8+ cells in the nodes than in the blood. This study measured the percentage of all T (CD3) and B (CD19) cells in the blood that are also LEU-8+ and compared this with the percentage of all CD3+ or CD19+ cells in the nodes that are also LEU-8+ by using dual-colour flow cytometric analysis.

Tumour draining lymph nodes in breast cancer patients represent an important site where malignant cells may be recognised by the host immune system, eliciting an immune response against the tumour (Whitford *et al.*, 1992). The immune response between neighbouring lymph nodes of breast cancer patients is variable, and activation of lymphocytes within tumour draining lymph nodes may be altered. This work therefore also set out to define the contribution of two cell surface antigens, the IL-2 receptor and HLA-DR, in the communication between two lymph nodes or blood and nodes in the local immune response.

4.2 Results

The methodology of lymphocyte staining and gating procedure is described in Section 2.2.3.3.1. Sample aliquots were incubated for twenty minutes on ice with the relevant monoclonal antibodies (Table 4.1) to specific phenotypic and activation markers on the lymphocyte membrane. The validity of the gate was checked by the leucogate antibody (Figure 4.1a). 5000 events were collected for statistical relevance and analysed on the FACScan research program. A quadrant was set on the IgG1-FITC/IgG2a-PE non-specific binding control to include 99.8% of cells for statistical relevance (Figure 4.1b). Analysis of lymphocyte subset activation required a dot plot gate to include only the positive staining lymphocytes. A histogram was drawn and the X co-ordinate of the quadrant from the IgG1-FITC/IgG2a-PE non-specific binding experiment was used as a marker to measure dual positive cells (Figures 4.8 and 4.10).

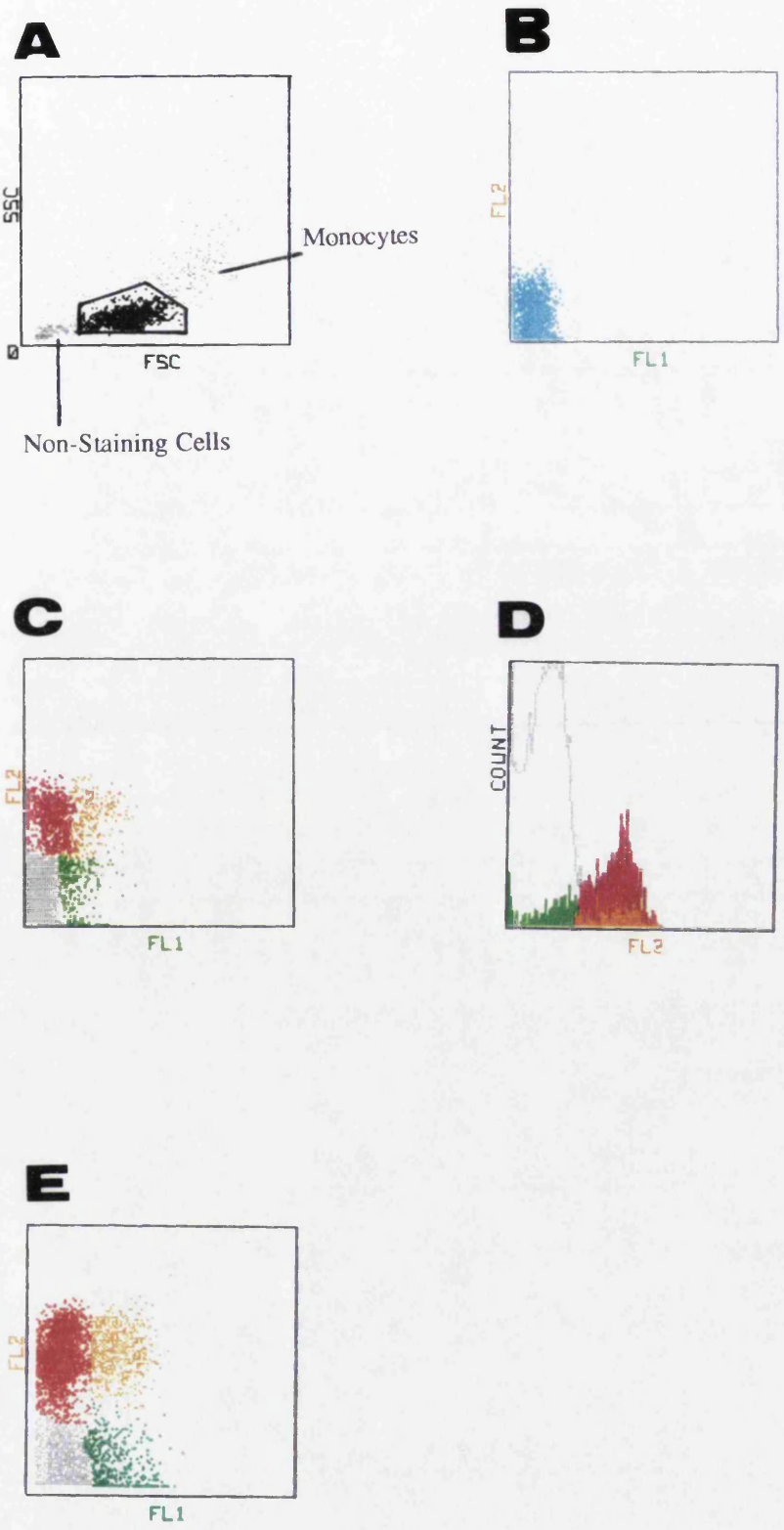
In this study the relative quantitative expression of LEU-8 on CD3/19 cells and activation markers (CD25 or HLA-DR) on lymphocyte subsets was measured on the peripheral blood lymphocytes (PBL) and two types of lymph nodes, with one close to the breast cancer tissue (NLN) and one distant (FLN).

4.2.1 LEU-8+ expression on T lymphocyte populations of breast cancer patients

T lymphocytes expressing the LEU-8 molecule were identified with monoclonal anti-LEU 8-FITC (FL1) and PE labelled monoclonal antibodies (FL2) specific for the CD3 cell marker, and percentages were calculated on FL1 vs FL2 plot (Figures 4.1c, d and e).

The values obtained for CD3+ LEU-8+ were expressed as a percentage of total CD3+ cells. The mean percentages of CD3+ LEU-8+ cells in lymphocytes from each analysed source are shown in Table 4.2 : PBL 18.1% (range 2.0 - 63.0%), NLN 17.5% (range 0.4 - 85.4%), FLN 13.8% (range 0.3 - 50.9%). The highest average expression is found in the PBL, whereas the NLN shows similar expression pattern to that of PBL and FLN shows a smaller value. No statistical significance was observed between the three lymphocyte sources (Table 4.4). Within individual patients expression of LEU-8+ CD3+ for PBL, NLN and FLN is illustrated in Figure 4.2. The overall expression of LEU-8 in the three tissues was very similar with the exception of the one among 25 patients with a higher proportion in the near lymph node (Figure 4.3). Individual patients vary greatly (Figure 4.4).

Figure 4.1 Double stained colour dot plots of LEU-8+ expression on T (CD3) cells of a single patient. (A) Control scatter plot. The selected area represents the gated lymphocyte populations selected by forward scatter (FSC) and side scatter (SSC) parameters. (B) The IgG1-FITC/IgG2a-PE level of non-specific staining in control scatter plot. (C) The Fluorescence 1 (FITC) vs Fluorescence 2 (PE) in lymph node lymphocytes. The green area shows the FITC labelled LEU-8+ cells. The red area represents the PE labelled CD3+ T cells. The yellow populations represents cells expressing both LEU-8+ and CD3+. (D) The yellow area represents the LEU-8+ population on T cells in the gated histogram. (E) The Fluorescence 1 (FITC) vs Fluorescence 2 (PE) in peripheral blood lymphocytes. The yellow area represents the LEU-8+ CD3+ cells.


A
SSC
FSC
Monocytes
Non-Staining Cells
B
FL2
FL1
C
FL2
FL1
D
COUNT
FL2
E
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FL1

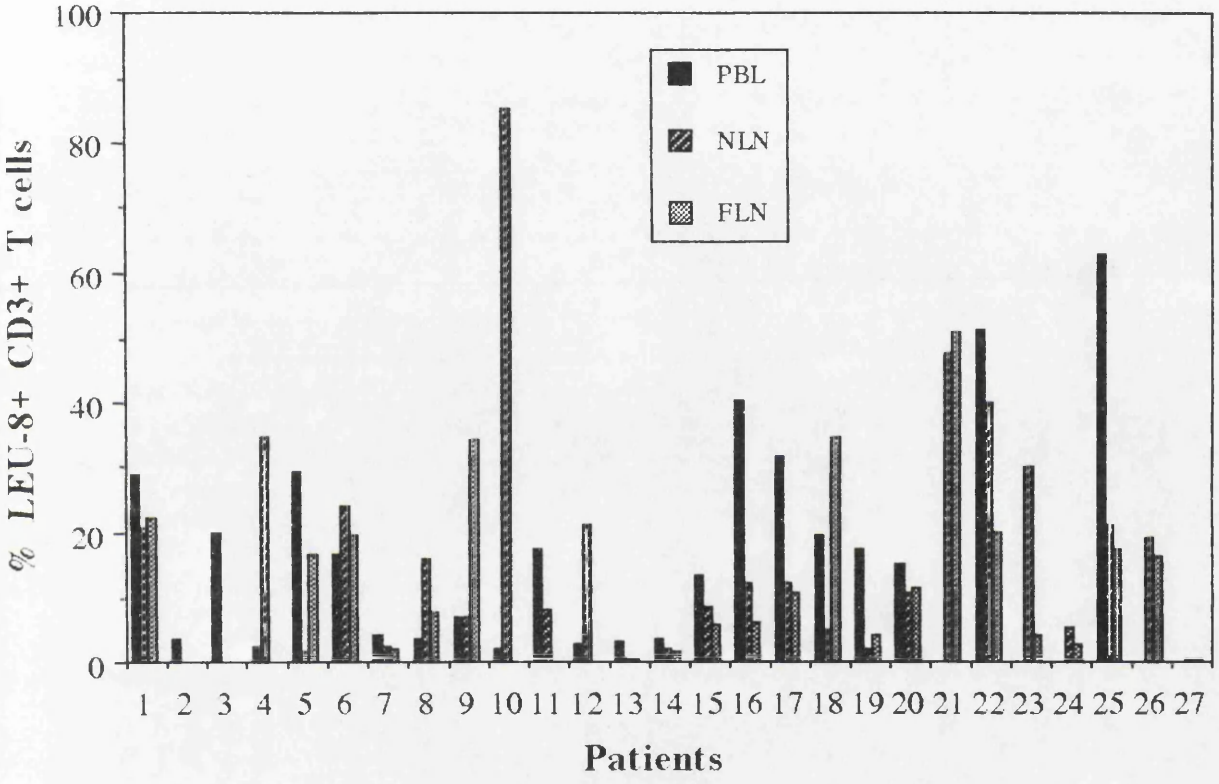


Figure 4.2 Percentage of CD3+ expression within the total gated lymphocyte populations of breast cancer patients

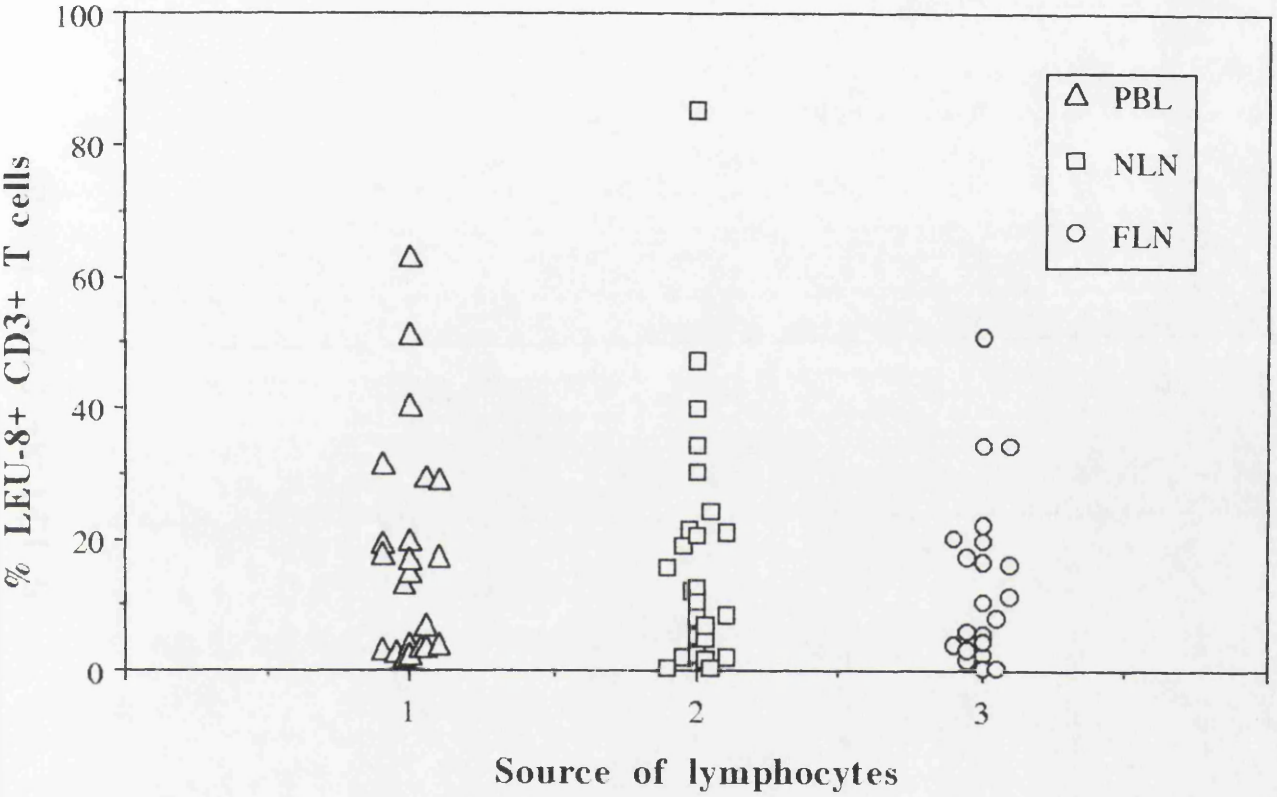


Figure 4.3 Distribution of LEU-8+ on CD3 lymphocyte populations from PBL, NLN and FLN of breast cancer patients

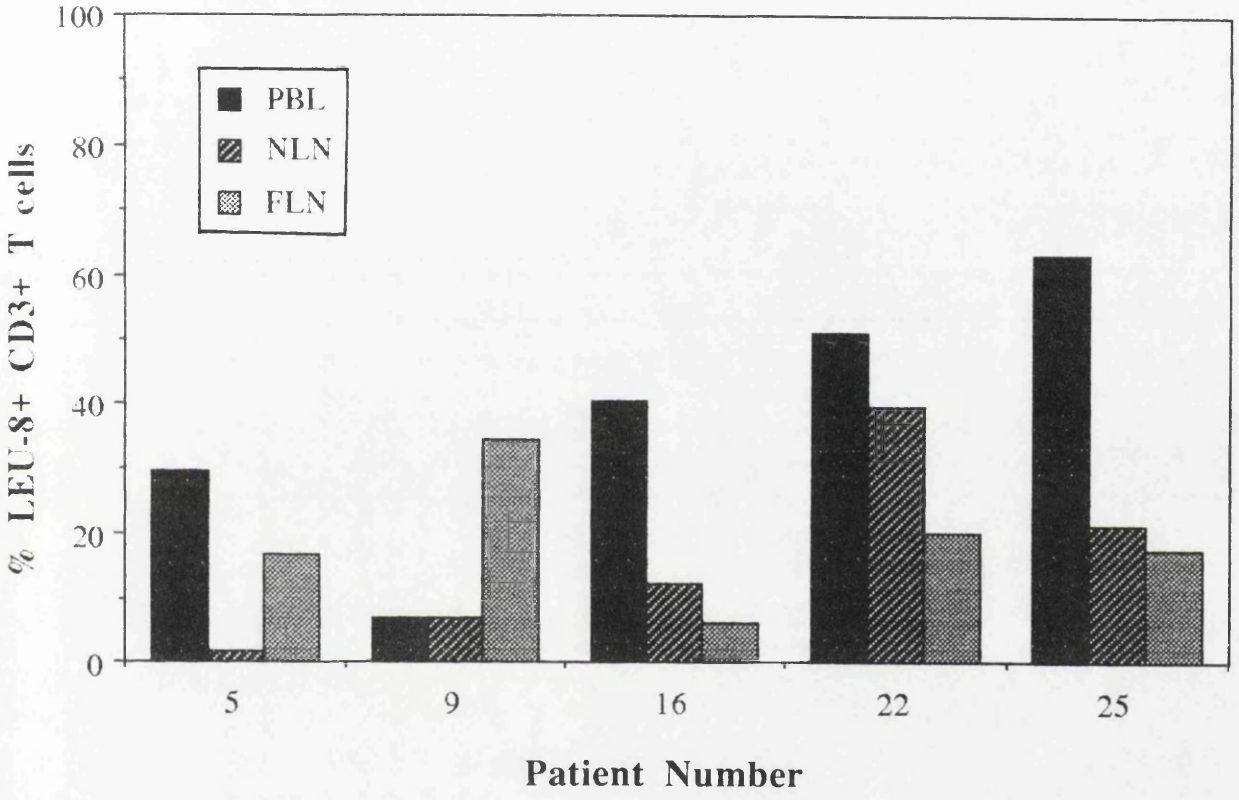


Figure 4.4 Patient variability of LEU-8+ CD3+ expression between the three lymphocyte sources in breast cancer patients

Table 4.1 Monoclonal antibodies used for flow cytometric analysis of phenotypic and activation markers of human lymphocytes

First antibody	Second antibody	Function
IgG1-FITC	IgG2a-PE	Control
Leucogate		
Anti-CD45-FITC	Anti-CD14-PE	Control : Differential staining of leucocyte subpopulations. (lymphocytes, monocytes and neutrophils)
Anti-CD3-FITC/PE		T lymphocytes
Anti-CD19-FITC/PE		B lymphocytes
Anti-Leu 3a-PE	Anti-CD25-FITC	IL-2 receptor on CD4+ helper T cells
Anti-Leu 2a-PE	Anti-CD25-FITC	IL-2 receptor on CD8+ cytotoxic T cells
Anti-Leu 3a-PE	Anti-HLA DR-FITC	Activated CD4+ helper T cells
Anti-Leu 2a-PE	Anti-HLA DR-FITC	Activated CD8+ cytotoxic T cells
Anti-Leu 8-FITC		Cell surface molecule

Table 4.2 Comparison of the average percentage of LEU-8+ CD3+ expression on blood and lymph nodes

LEU-8 on CD3	PBL (n=22)	NLN (n=25)	FLN (n=21)
Minimum	2.0	0.4	0.3
Median	15.9	12.1	10.6
Maximum	63.0	85.4	50.9
Mean	18.1	17.5	13.8
Standard Error	3.6	3.8	2.9
Standard Deviation	16.9	19.1	13.3

4.2.2 LEU-8+ expression on B lymphocyte populations of breast cancer patients

B lymphocytes expressing the LEU-8 molecule were identified with FITC labelled monoclonal anti-LEU 8 (FL1) and PE labelled monoclonal anti-CD 19 (FL2), and percentages were calculated on FL1 vs FL2 plot.

The expression of LEU-8+ CD19+ B cells showed a similar trend to that of LEU-8+ CD3+ with the mean expression on PBL (38.5%, range 2.3-58.4%) being greater than that of NLN (6.8%, range 0.1-17.0%) and FLN (10.5%, range 0.5-33.0%) (Table 4.3). The size of the LEU-8+ CD19+ population expressed as a percentage of total lymphocytes was not significantly different in all tissues (Table 4.4). Individual patient expression within PBL, NLN and FLN is illustrated in Figure 4.5. On average there is more LEU-8+ expression on B cells in the blood than in both nodes (Figure 4.6). Individual patients vary greatly (Figure 4.7).

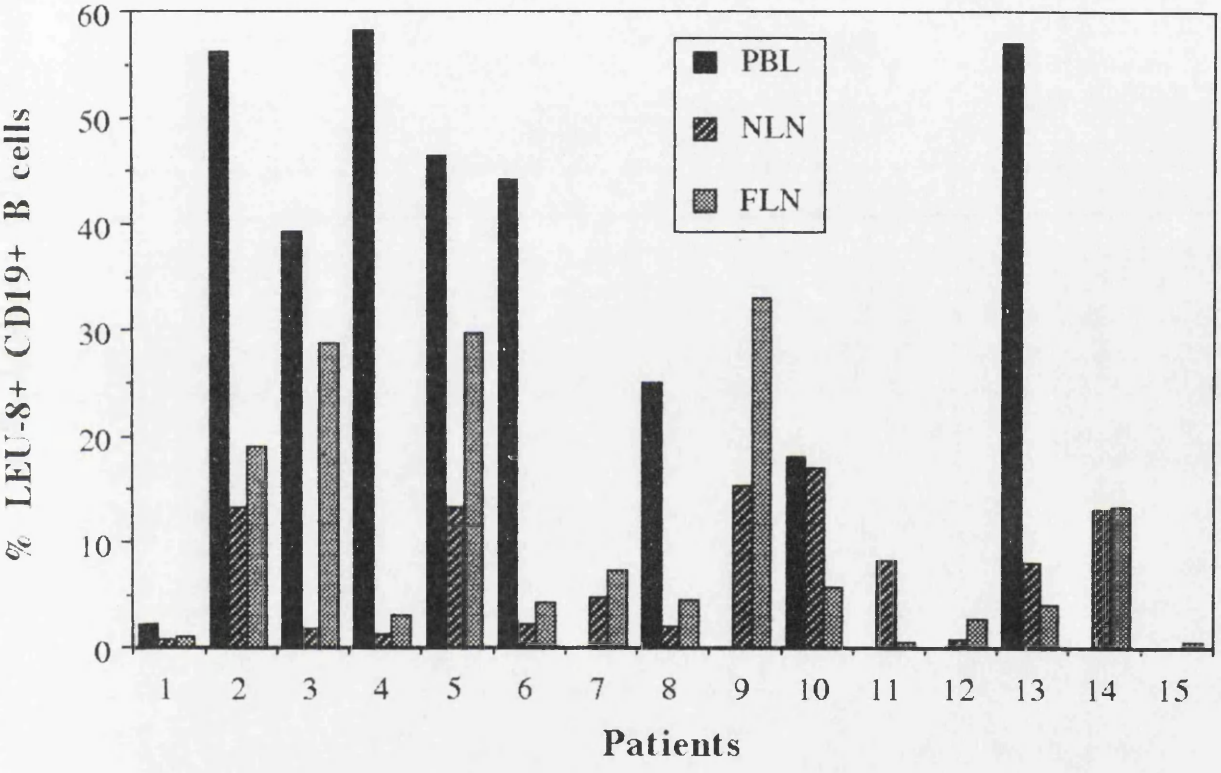


Figure 4.5 Percentage of LEU-8+ CD19+ expression within the total gated lymphocyte populations of breast cancer patients

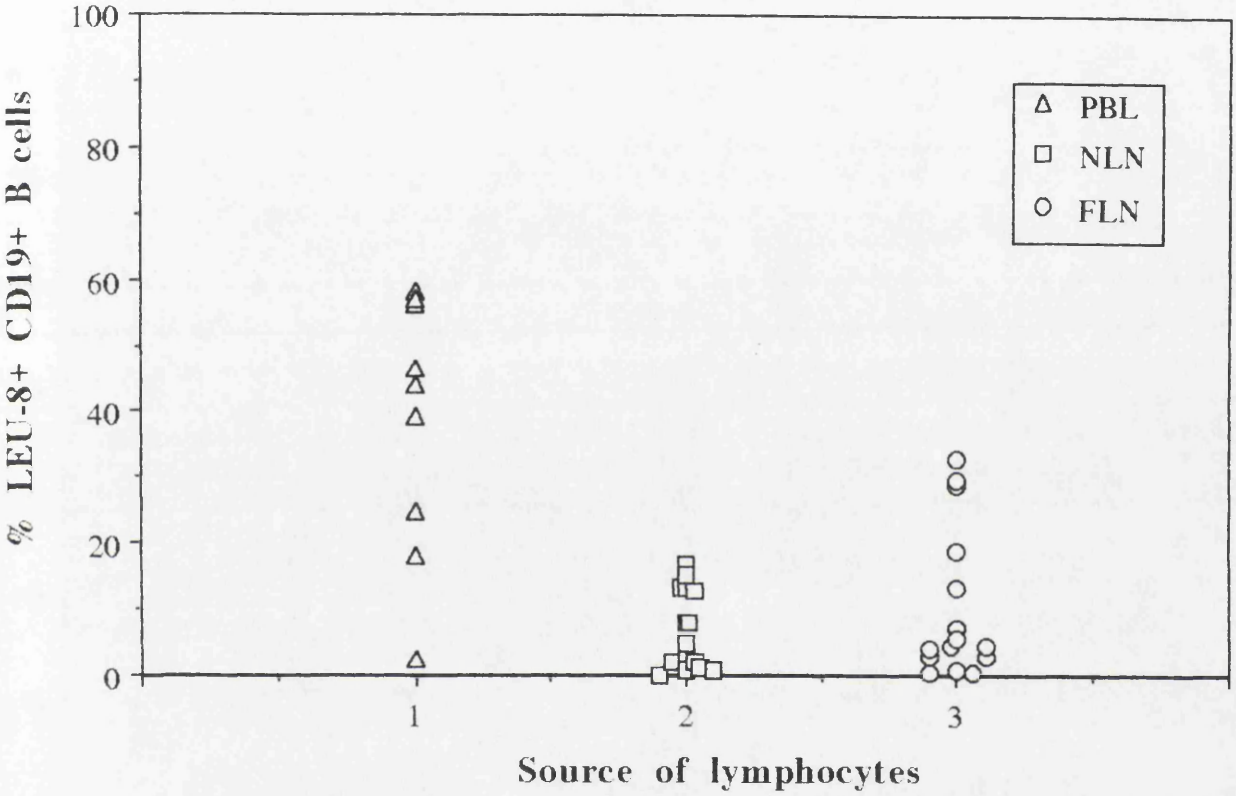


Figure 4.6 Distribution of LEU-8+ on CD19 lymphocyte populations from PBL, NLN and FLN of breast cancer patients

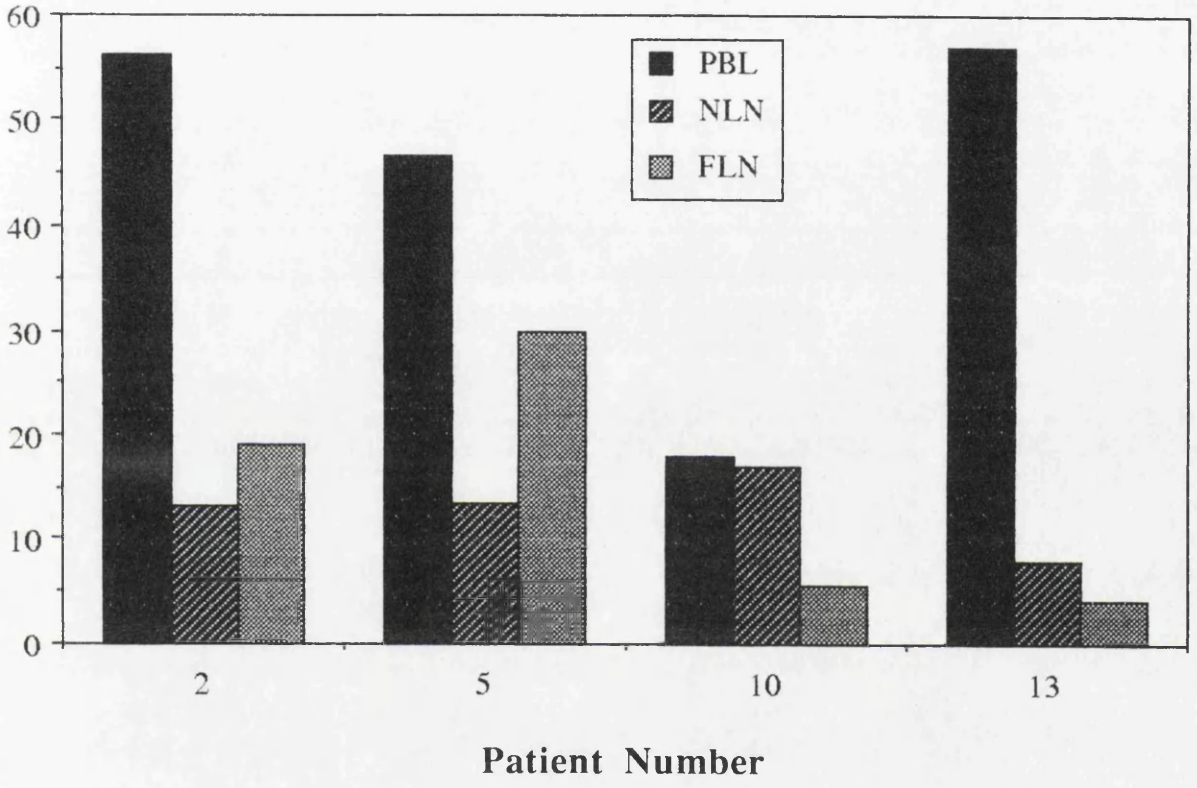


Figure 4.7 Patient variability of LEU-8+ CD19+ expression between the three lymphocyte sources in breast cancer patients

Table 4.3 Comparison of the average percentage of LEU-8+ CD19+ expression on blood and lymph nodes

LEU-8 on CD19	PBL (n=9)	NLN (n=15)	FLN (n=15)
Minimum	2.3	0.1	0.5
Median	44.2	4.7	4.4
Maximum	58.4	17.0	33.0
Mean	38.5	6.8	10.5
Standard Error	6.5	1.6	3.0
Standard Deviation	19.6	6.1	11.5

Table 4.4 Comparison of LEU-8 expression on lymphocytes from peripheral blood, axillary near lymph node and far lymph node of breast cancer patients

Parameter	PBL	NLN	FLN	Statistical significance
	(n=22)	(n=25)	(n=21)	
				a. p= N.S.
LEU-8+ CD3+	18.1 ± 16.9	17.5 ± 19.1	13.8 ± 13.3	b. p= N.S.
				c. p= N.S.
	(n=9)	(n=15)	(n=15)	
				a. p= N.S.
LEU-8+ CD19+	38.5 ± 19.6	6.8 ± 6.1	10.5 ± 11.5	b. p= N.S.
				c. p= N.S.

Values represent % mean ± standard deviation of lymphocytes expressing the LEU-8 cell surface molecule.

(a) is the comparison of PBL and NLN; **(b)** that of PBL and FLN; **(c)** that of NLN and FLN

Statistical test used : Mann-Whitney U test as the number of unpaired samples was performed. Statistical significance is taken as a $p \leq 0.05$. (N.S. : Not significant)

4.2.3 Comparison of LEU-8 expression with that of the antigen identified by monoclonal antibodies described in Chapter 3

The antigens on the node identified by the "in house" generated monoclonal antibodies were not specific to the node and were distributed throught out the peripheral blood and nodal lymphocytes (Chapter 3). Similarly, the LEU-8, cell surface antigen previously thought to be involved in homing in humans, was expressed in the peripheral blood as well as the lymph node of breast cancer patients. These data indicate that the antigen is not unique to the human lymph node, and LEU-8 is, from these studies, irrelevant to T cell homing and a negative indicator of B cell homing.

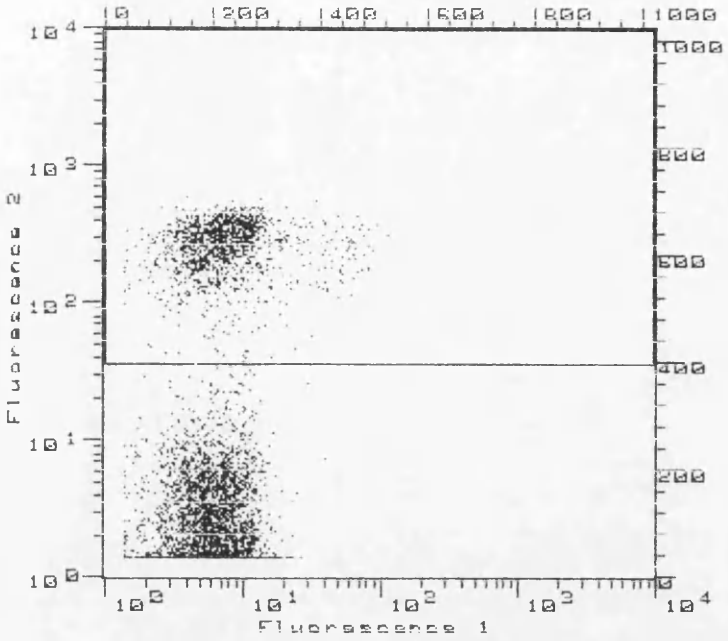
4.2.4 Analysis of activation markers

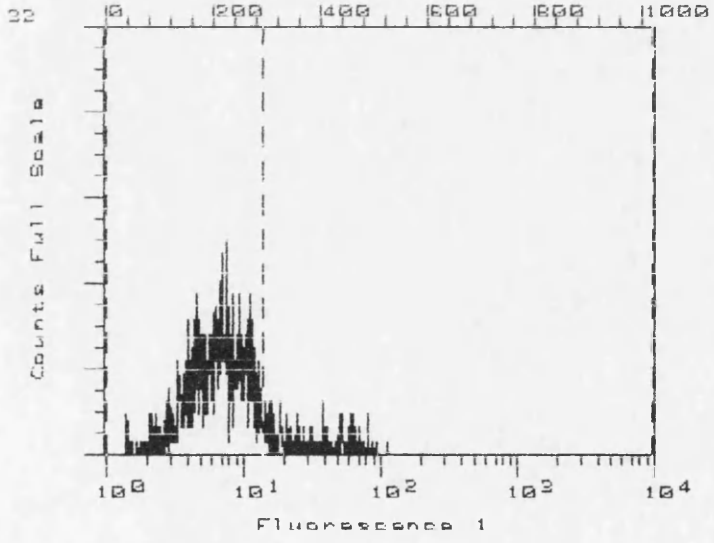
The expression of the activation markers IL-2R (CD25; Tac antigen) and HLA DR, both of which undergo upregulation upon antigen stimulation, on three lymphocyte populations is summarised in Table 4.5 and 4.6. Lymphocytes were gated based on size characteristics.

4.2.4.1 IL-2R (Tac) expression

The expression of the Tac component of the IL-2 receptor was shown to be higher on the CD4+ T cells than on the CD8+ T cells in each tissue (Figure 4.9). The mean percentage of IL-2R expressing CD4+ T cells in NLN (23.6%) was higher than in FLN (19.1%), whereas the expression of IL-2R on CD8+ T cells showed little change between three lymphocyte populations.

Figure 4.8 A dot plot of dual immunofluorescence of T cells bearing Tac (CD25) analysed using quadrant statistics. The upper figure shows the analysis gate used to isolate the population expressing the activation marker based on the FL-2 channel. The lower figure shows the number of cells from the upper 2 quadrants bearing the activation marker based on non-specific FITC binding in a control sample containing irrelevant antibody.


Fluorescence 2
Fluorescence 1


Counts Full Scale
Fluorescence 1

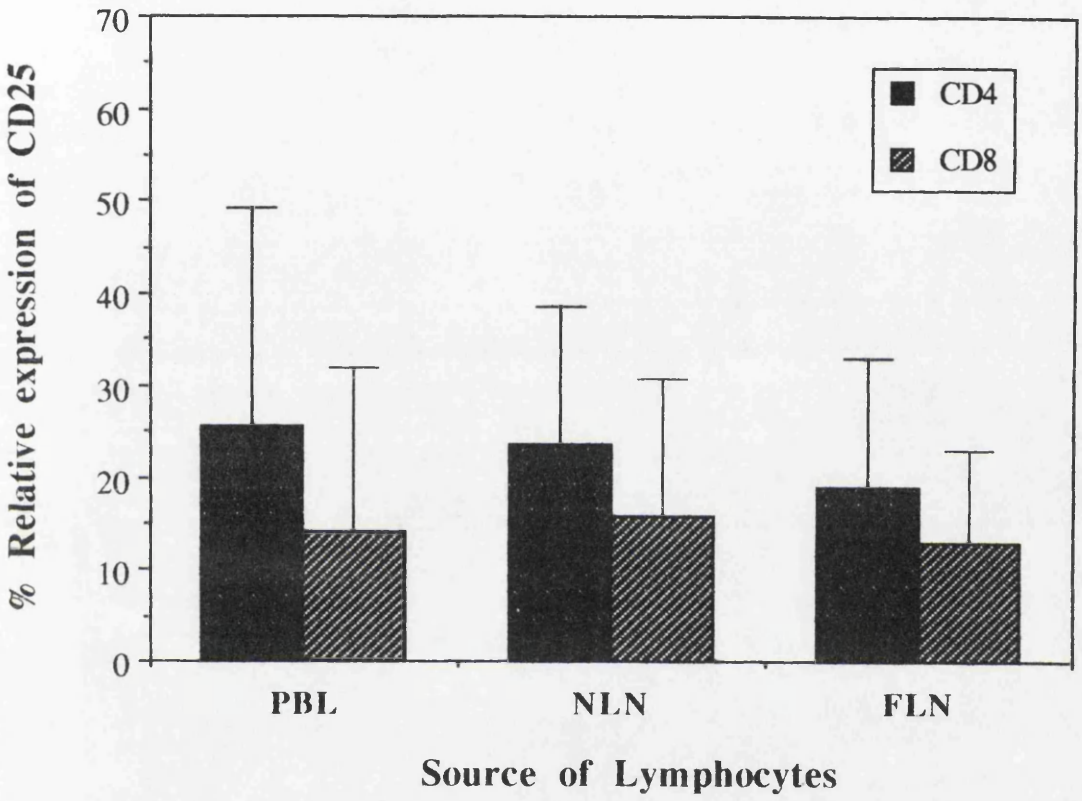


Figure 4.9 Comparison of the Tac (CD25) expression on CD4+ and CD8+ T cells in the peripheral blood, near lymph node and far lymph node lymphocytes of breast cancer patients

Table 4.5 Comparison of CD25 expression on lymphocytes from peripheral blood. axillary near lymph node and far lymph node of breast cancer patients

Parameter	PBL	NLN	FLN	Statistical significance
	(n=16)	(n=25)	(n=25)	
				a. p= N.S.
CD4+ CD25+	25.6 ± 23.7	23.6 ± 14.9	19.1 ± 13.9	b. p= N.S.
				c. p= N.S.
	(n=17)	(n=23)	(n=22)	
				a. p= N.S.
CD8+ CD25+	14.0 ± 18.0	15.9 ± 14.8	13.0 ± 10.0	b. p= N.S.
				c. p= N.S.

Values represent % mean ± standard deviation of lymphocytes expressing activation cell marker

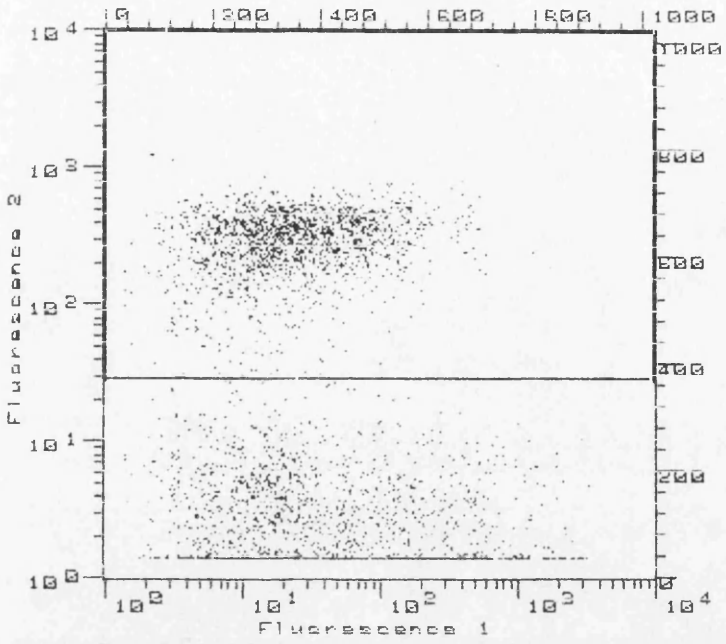
(a) is the comparison of PBL and NLN; **(b)** that of PBL and FLN; **(c)** that of NLN and FLN

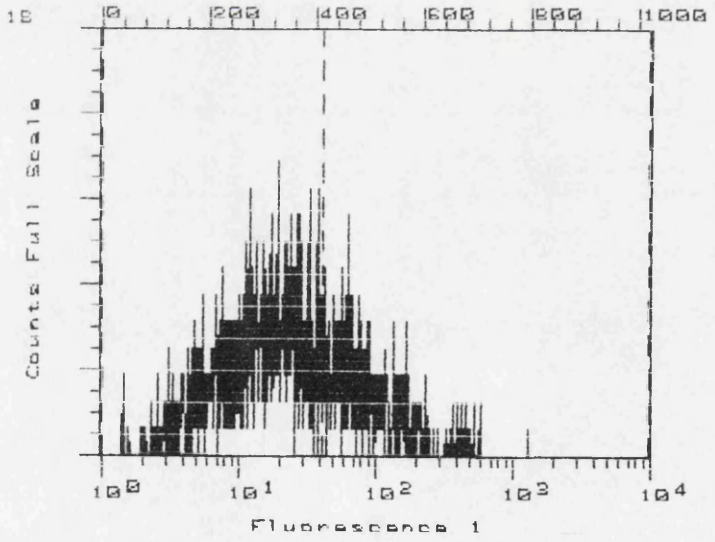
Statistical test used : Mann-Whitney U test as the number of unpaired samples was performed. Statistical significance is taken as a $p \leq 0.05$. (N.S. : Not significant)

4.2.4.2 HLA DR expression

As can be seen in Figure 4.11, unlike the CD25 surface antigen the HLA DR surface marker was found to be on many more CD8+ T cells from the three tissues than CD4+ T lymphocytes. The mean expression of HLA DR on CD8+ T cells was found to be greater in the NLN (46.8%) compared with the PBL (29.1%) or the FLN (44.0%).

Figure 4.10 A dot plot of dual immunofluorescence of T cells bearing HLA DR analysed using quadrant statistics. The upper figure shows the analysis gate used to isolate the population expressing the activation marker based on the FL-2 channel. The lower figure shows the number of cells from the upper 2 quadrants bearing the activation marker based on non-specific FITC binding in a control sample containing irrelevant antibody.


Fluorescence 2
Fluorescence 1


Counts Full Scale
Fluorescence 1

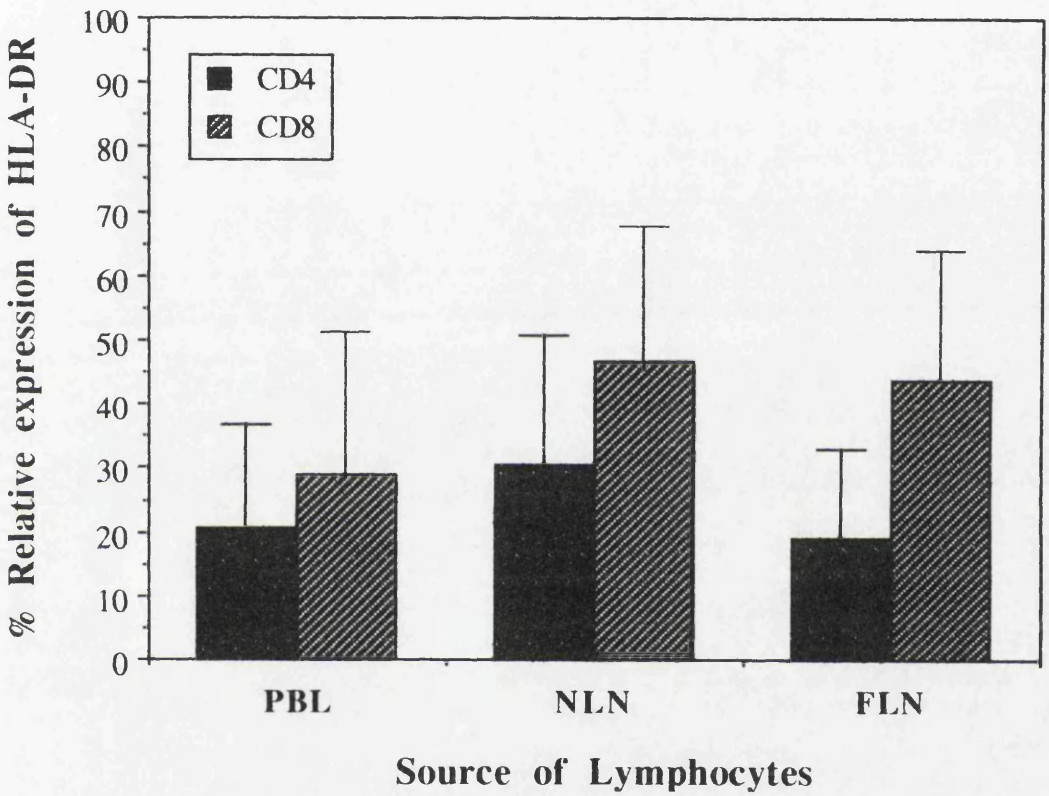


Figure 4.11 Comparison of HLA DR expression on CD4+ and CD8+ T cells from the three lymphocytes sources in breast cancer patients

Table 4.6 Comparison of the activation status of lymphocytes from peripheral blood, axillary near lymph node and far lymph node of breast cancer patients

Parameter	PBL	NLN	FLN	Statistical significance
	(n=16)	(n=25)	(n=25)	
				a. p= N.S.
CD4+ HLA-DR	20.5 ± 16.3	30.4 ± 20.5	19.1 ± 13.9	b. p= N.S.
				c. p= N.S.
	(n=17)	(n=24)	(n=24)	
				a. p= N.S.
CD8+ HLA-DR	29.1 ± 22.0	46.8 ± 21.1	44.0 ± 20.2	b. p= N.S.
				c. p= N.S.

Values represent % mean ± standard deviation of lymphocytes expressing activation cell marker

(a) is the comparison of PBL and NLN; **(b)** that of PBL and FLN; **(c)** that of NLN and FLN

Statistical test used : Mann-Whitney U test as the number of unpaired samples was performed. Statistical significance is taken as a $p \leq 0.05$. (N.S. : Not significant)

4.3 Discussion

Lymphocytes are engaged in constant trafficking from the blood into secondary lymphoid tissues, by binding to high endothelial venules (HEV) to give maximal efficiency of response. A human peripheral lymph node homing receptor fully equivalent in function and expression to the mouse MEL-14 antigen has not yet been described and tested because of obvious ethical problems. Clearly, the identification of a human peripheral node-specific homing receptor is an important goal, both for understanding the physiology of human lymphocyte traffic and for its potential clinical diagnostic and therapeutic value. The LEU-8 antigen is a human homologue of the murine MEL-14 molecule, which has been suggested to be to have a homing function, but has not to date been tested in humans (Section 1.4.2.2). This study was designed to assess the homing function of the LEU-8 molecule by comparison of its relative expression in lymph node and peripheral blood of breast cancer patients using two-colour flow cytometric analysis. The present data supports the view that LEU-8+ CD3+ or CD19+ cells show no signs of accumulating in the node, and indeed the latter show a preference for location in the blood. It is clear that lymphocytes do not express LEU-8 preferentially in the node, and individual lymph nodes differ greatly. Thus these experiments showed no evidence that LEU-8 is a homing receptor. It remains however possible that the LEU-8 receptor may be downregulated when the lymphocytes enter the node. Andersson *et al.* (1994) have claimed that a number of cell adhesion molecules, including VLA-4, LFA-1 and ICAM-1 are up-regulated on CD8+ cells, whereas the lymph node homing receptor MEL-14 is down-regulated (Kishimoto *et al.*, 1990; Stibenz and Buhrer, 1994) during systemic virus infection. In this context the human lymph node homing receptor CD44 (Chapter 5) is up-regulated (Lynch *et al.*, 1989) to operate successively in controlling extravasation, and has its special role in the interaction between lymphocyte and endothelium. It should be noted that, in this study, the CD44

receptor was expressed in a higher percentage of cells in lymph nodes than in peripheral blood (Chapter 5). This is of interest because decreased expression of MEL-14 is linked to up-regulation of CD44. Thus, there was no functional evidence from this study that the LEU-8 cDNA homologous to mouse MEL-14 antigen does indeed encode a human lymph node homing receptor.

The immune response within human breast cancer patients is highly variable and may be influenced not only by the immunological status of the patient but also by the oncogene complement of the tumour. Breast cancer patients show a substantial locoregional immune response within the axillary lymph nodes. These lymph nodes in breast cancer patients represent an important site, where malignant cells may be recognised by the host-mediated immune system draining the breast primarily. Alam *et al.* (1993) reported that the percentage of CD8+ T cells expressing HLA DR and IL-2 receptors (Tac) was significantly higher in invaded nodes and, while CD4+ T cells expressing HLA DR were also in a higher proportion in invaded nodes, the difference in the proportion of Tac expressing CD4+ T cells failed to reach significance within the same stage II patients. These results suggest that the presence of metastatic tumour cells in a lymph node has an effect on the specific alterations of the lymphocyte subpopulations. In consequence, the general activation state of the lymphocytes in the nodes may be altered. As such, the comparison is likely to be most relevant between two axillary lymph nodes or between peripheral blood and lymph nodes. In this study the activation marker IL-2 receptor (Tac) was present on a higher proportion on CD4+ than CD8+ lymphocytes from all three sources. In contrast, the percentage of CD8+ T cells bearing HLA DR was higher than that of CD4+ T cells in all cases. This activation marker was also found to be expressed on higher numbers of CD4+ and CD8+ T cells in near lymph nodes than in peripheral

blood from matched pairs of the same stage I patients. It is not known whether this reflects any functional difference in the expression of the markers on these T cell subsets. IL-2 receptor and HLA DR as activation markers, however, should be interpreted with caution. An increased expression of the activation markers indicate an activated state, but these may subsequently undergo down-regulation following binding of the relevant ligand.

CHAPTER 5

CD44 AND LEU-8 EXPRESSION ON LYMPHOCYTES AND TUMOUR CELLS FROM BREAST CANCER PATIENTS

5. CD44 and LEU-8 expression on lymphocytes and tumour cells from breast cancer patients

5.1 Aim of study

The human CD44 cell surface glycoprotein is a polymorphic molecule found on a wide variety of different tissues. CD44, which has been implicated in lymphocyte homing, tumour metastasis, T cell activation and extracellular matrix attachment, consists of a large number of related isoforms that derive from the differential splicing of a single CD44 gene transcript. Different CD44 isoforms may be present in the serum of cancer patients and serum CD44 concentrations may be an indicator of tumour metastasis in patients with malignant diseases (Guo *et al.*, 1994). This study was designed to investigate whether CD44 has a homing function in breast cancer patients. If CD44 is the lymph node homing receptor there may be more CD44+ cells in the nodes than in the blood of breast cancer patients. The percentage of all lymphocytes in the blood expressing CD44+ was measured by flow cytometric analysis, and was compared with the percentage of all lymphocytes in the nodes that are also CD44+. This work additionally asked whether tumour cells carry CD44 or LEU 8 and, if so, whether the expression on primary tumour was lower or higher than on metastatic tumour in the node.

5.2 Results

5.2.1 CD 44+ expression on lymphocyte populations of breast cancer patients

The lymphocytes expressing the CD44 molecule were identified with monoclonal anti-CD44-FITC (FL1), and percentages were calculated on the FL1 vs FL2 plot. The results obtained for CD44+ expression on lymphocytes are illustrated in Figure 5.1. There were no significant trends observed between three lymphocyte sources of breast cancer patients (Table 5.1), but differences were noted. While the mean percentage of CD44+ cells in NLN was higher (84.5%; range 36.3 - 99.0%) than that of CD44+ in PBL (71.7%; range 0.1 - 98.9%), the mean percentage of CD44+ cells in FLN (86.1%; range 45.2 - 94.0%) showed a similar pattern to that of CD44+ on NLN (Table 5.2). The overall percentage of CD44+ cells in the three tissues is shown in Figure 5.2. Although the expression of CD44 on PBL had a similiar average to other two groups, the distribution was broader. In 8/10 patients virtually all lymphocytes in the node were CD44+ whereas this is only the case for 9/16 PBL samples. Individual patients showed a large variability, with some cases of higher percentages on PBL and others with the reverse (Figure 5.3).

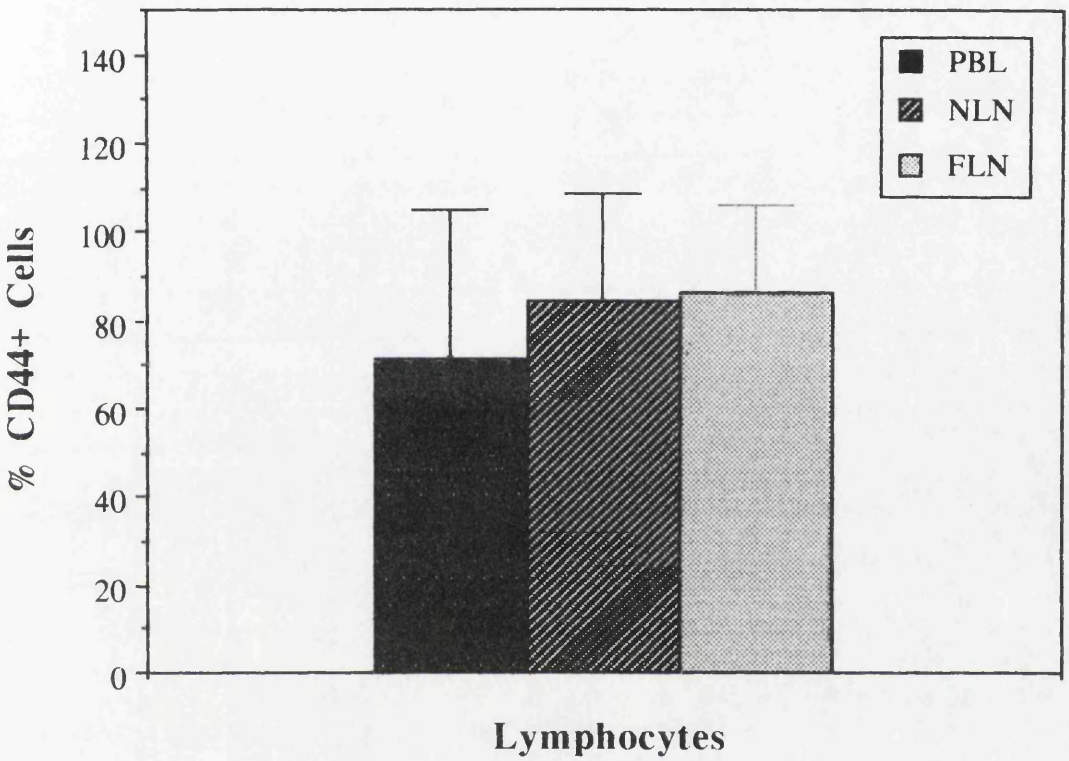


Figure 5.1 Percentage of CD44+ expression within the total gated lymphocyte populations of breast cancer patients

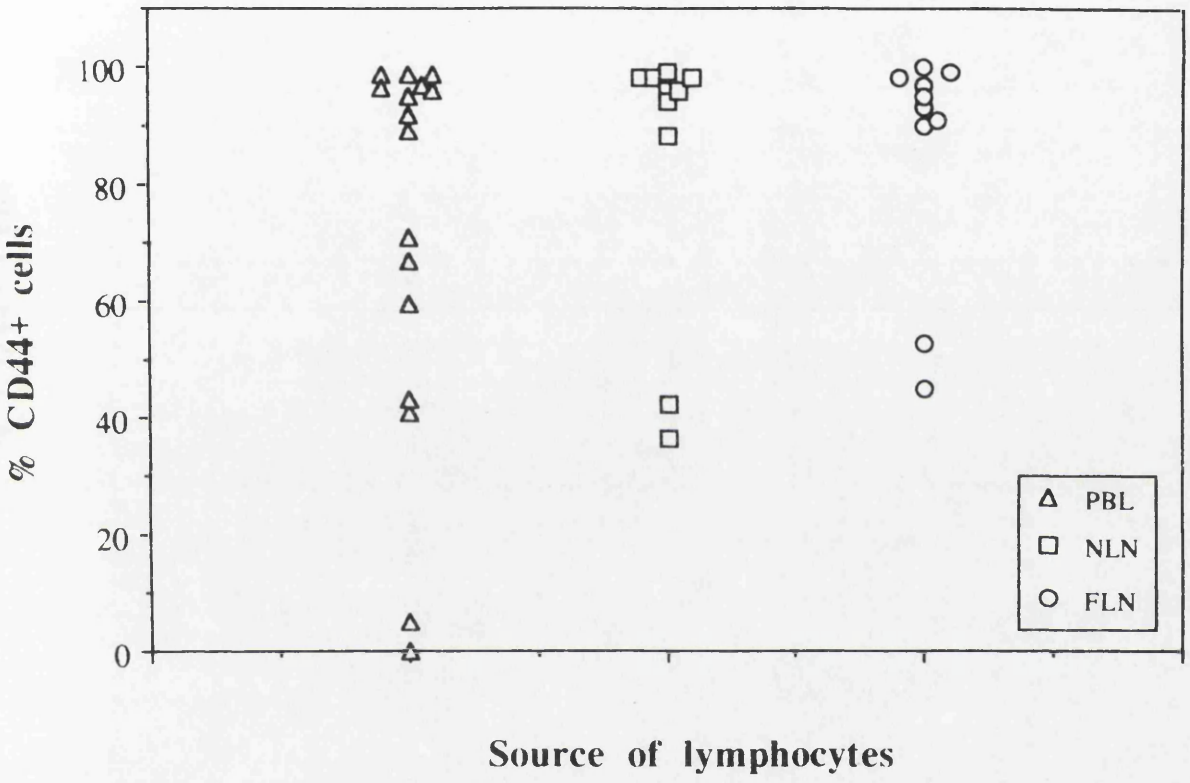


Figure 5.2 Distribution of CD44+ cells in peripheral blood, and near and far lymph node

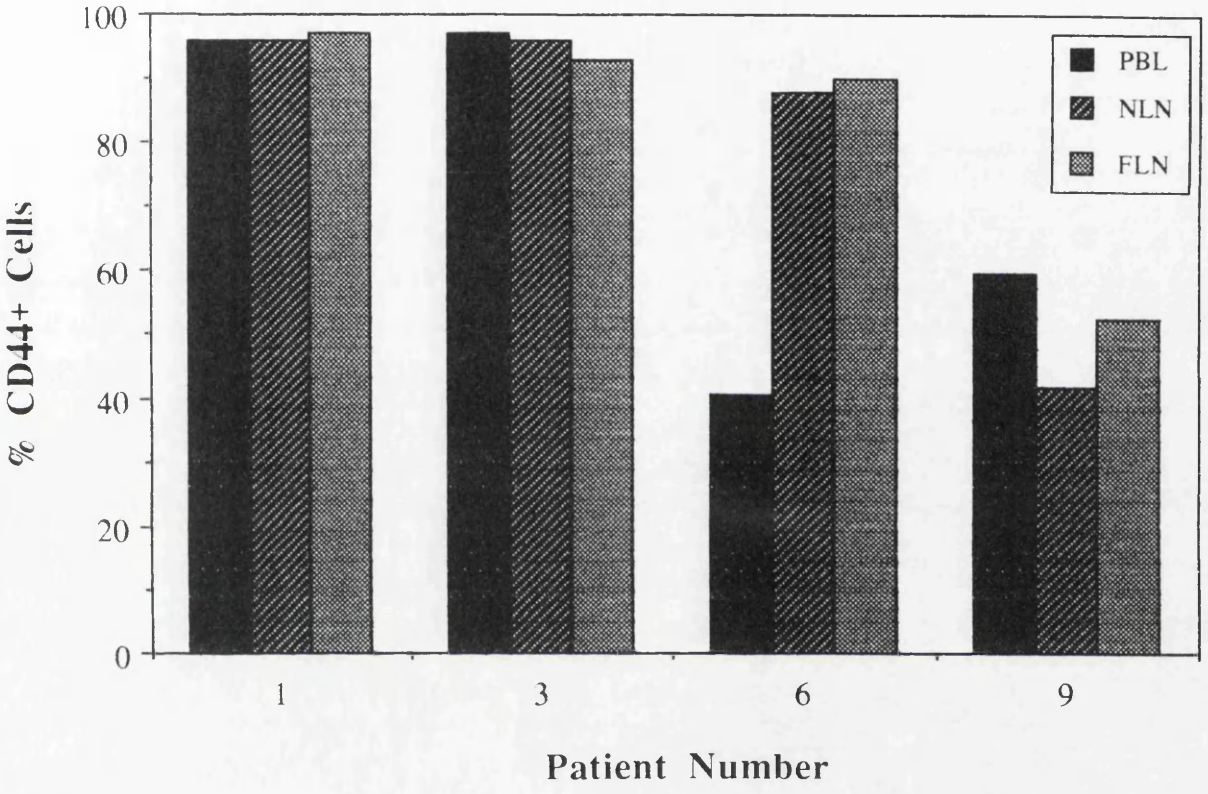


Figure 5.3 Patient variability of CD44+ expression between the three lymphocyte sources in breast cancer patients

Table 5.1 Comparison of % of CD44+ lymphocytes in peripheral blood, axillary near lymph node and far lymph node of breast cancer patients

CD44	PBL (n=16)	NLN (n=10)	FLN (n=10)	Statistical significance
				a. p= N.S.
	71.7 ± 33.4	84.5 ± 24.1	86.1 ± 20.0	b. p= N.S.
				c. p= N.S.

Values represent % mean ± standard deviation of lymphocytes expressing CD44 cell surface molecule.

(a) is the comparison of PBL and NLN; (b) that of PBL and FLN; (c) that of NLN and FLN

Statistical test used : Mann-Whitney U test as the number of unpaired samples was performed. Statistical significance is taken as a $p \leq 0.05$. (N.S. : Not significant)

Table 5.2 Comparison of the average percentage of CD44+ expressing cells within the three lymphocyte populations of breast cancer patients

CD44	PBL (n=16)	NLN (n=10)	FLN (n=10)
Minimum	0.1	36.3	45.2
Median	90.3	95.9	94.0
Maximum	98.9	99.0	94.0
Mean	71.7	84.5	86.0
Standard Error	8.4	7.6	6.3
Standard Deviation	33.4	24.1	19.9

5.2.2 Comparison of CD44+ and LEU-8+ expressing cells in PBL of breast cancer patients

The mean percentage of CD44+ cells (72.1 ± 30.3%) was almost two-fold higher than the mean percentage of LEU-8+ cells (41.3 ± 9.2%) in matched patient samples. Only 2/12 patients showed lower CD44 expression than LEU-8 expression. Frequencies of cells expressing these two antigens in peripheral blood are shown in Figure 5.4.

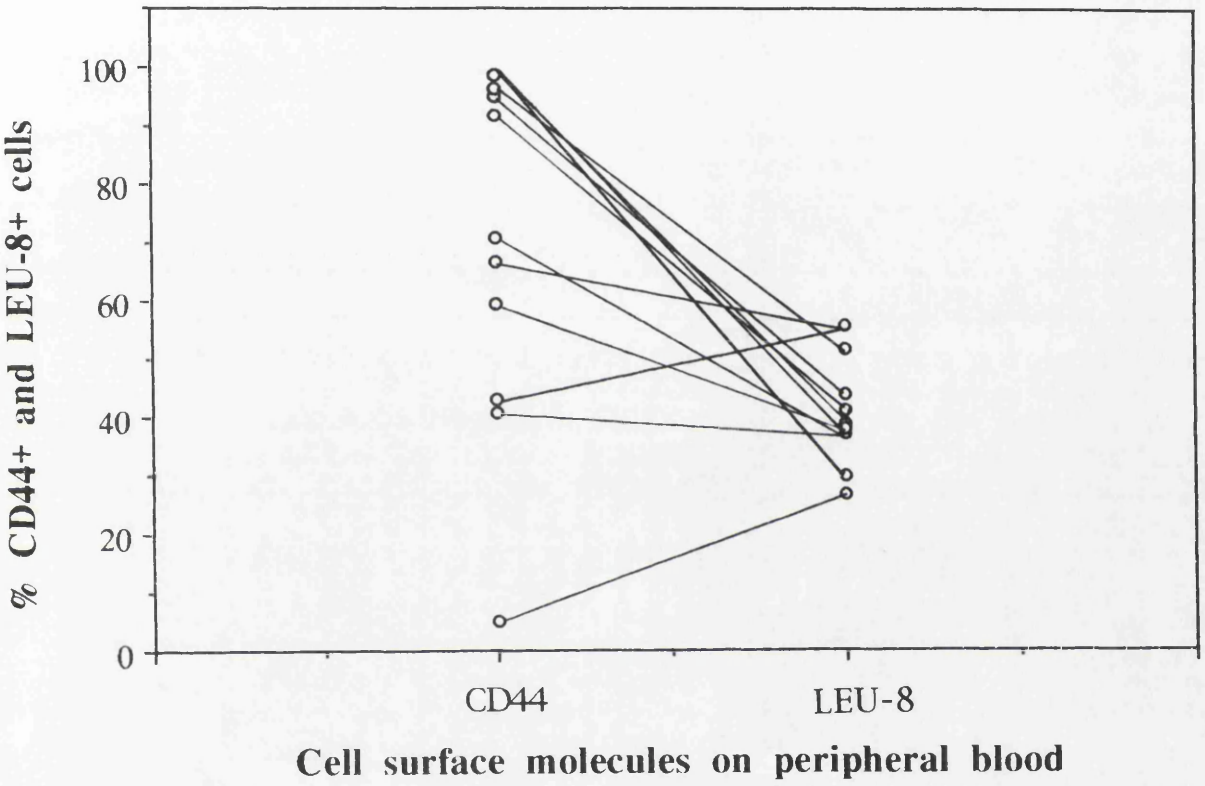


Figure 5.4 Comparison of percentage CD44+ and LEU-8+ expressing cells in peripheral blood lymphocytes of 12 samples from breast cancer patients

5.2.3 Metastasis pattern of CD44 and LEU-8 on tumour cells from breast cancer patients

Expression of CD44 and LEU-8 on the primary tumour and metastatic tumour invaded lymph nodes of breast cancer patients was determined utilising flow cytometric analysis. The low invaded draining axillary nodes are as close as 5cm to the tumour, and the high tumour free ones are often 15-20cm away (Figure 1.13). Figure 5.5 shows a scatter plot which excluded the lymphocytes from the analysis. There was a mean percentage of 56.1 ± 27.6% CD44+ in primary tumour cells and 58.0 ± 19.5% in metastatic tumour cells. This difference was not statistically significant (p=0.89) (Figure 5.6). LEU-8+ was also higher in metastatic tumour cells, being 41.3 ± 25.2% compared to the primary tumour value of 33.9 ± 15.2%. This change was also not statistically relevant (p=0.44) (Figure 5.7).

5.2.4 Expression of CD44 and LEU-8 within regional tumour draining lymph nodes

Expression of CD44 in the lymphocytes of tumour invaded or uninvaded lymph nodes was compared with that of LEU-8. The overall expression of CD44 and LEU-8 in the tumour invaded lymph nodes is illustrated in Figure 5.8. The mean expression of CD44 in the 11 tumour invaded lymph nodes was found to be greater (64.7 ± 35.8%) than that of LEU-8 (19.2 ± 12.9%) reaching statistical significance p=0.005. The mean percentage of CD44 expression was higher in the 5 uninvaded lymph nodes (35.4 ± 22.0%) than that of LEU-8 (13.4 ± 7.4%) although this failed to reach statistical significance (p=0.139) (Figure 5.9). The mean percentage of expression of CD44 in the 5 invaded lymph nodes (35.5 ± 26.7%) was similiar to the uninvaded lymph nodes (35.4 ± 22.0%), however this trend was not

statistically relevant (p=0.993) (Figure 5.10). Higher mean expression of LEU-8 was noted in the 5 invaded lymph nodes (20.9 ± 4.6%) than in the uninvaded lymph nodes (13.4 ± 7.4%) the trend failing to make statistical significance (p=0.179) (Figure 5.11).

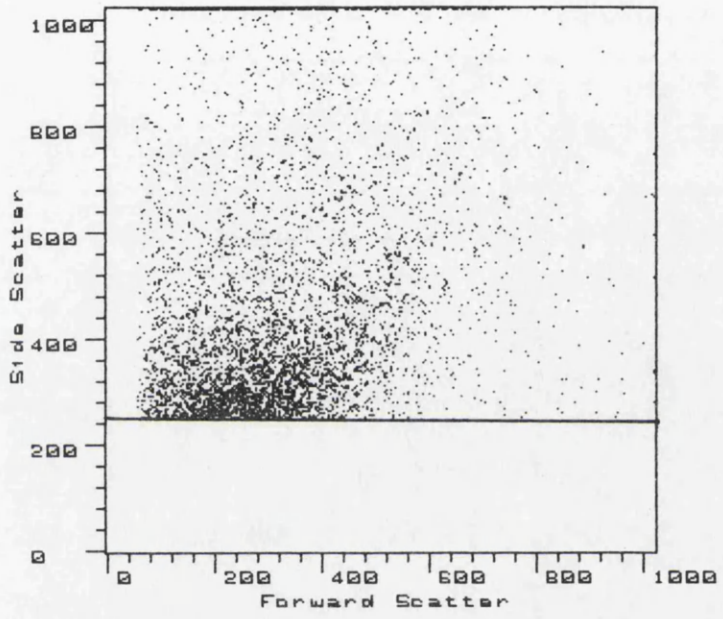


Figure 5.5 Scatter plot of tumour gate. The selected area represents the gated tumour populations by forward scatter (FSC) and side scatter (SSC) parameters. Lymphocytes fall in the lower, excluded part of the plot.

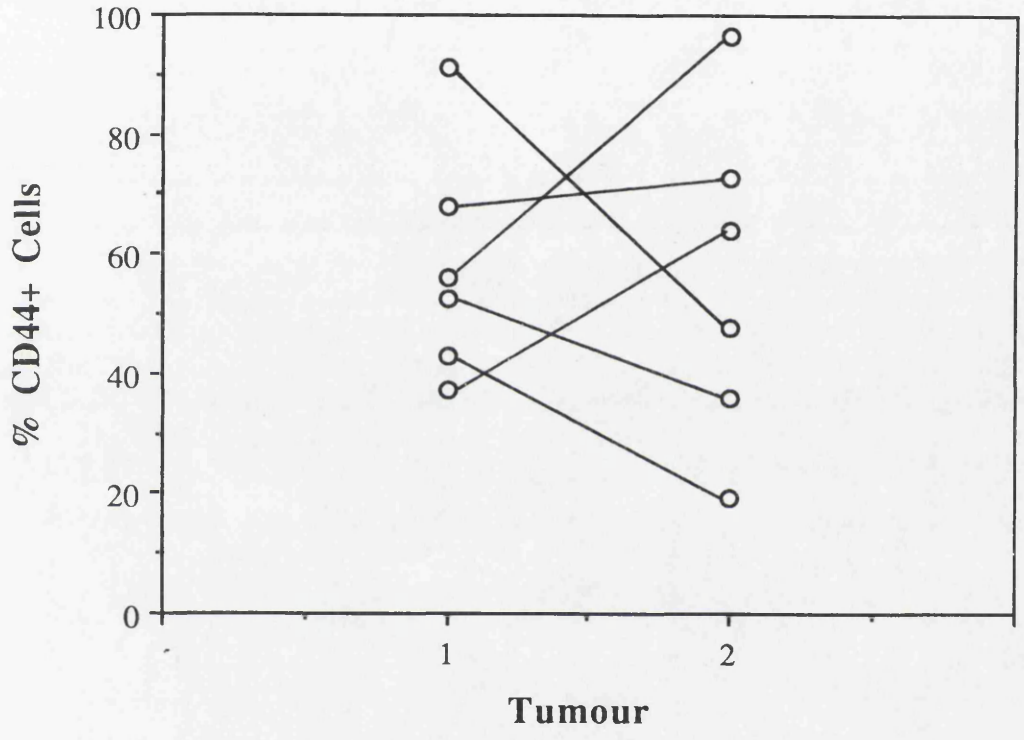


Figure 5.6 The comparison of % CD44+ cells between (1) metastasis in node and (2) primary tumour of matched samples from 6 breast cancer patients

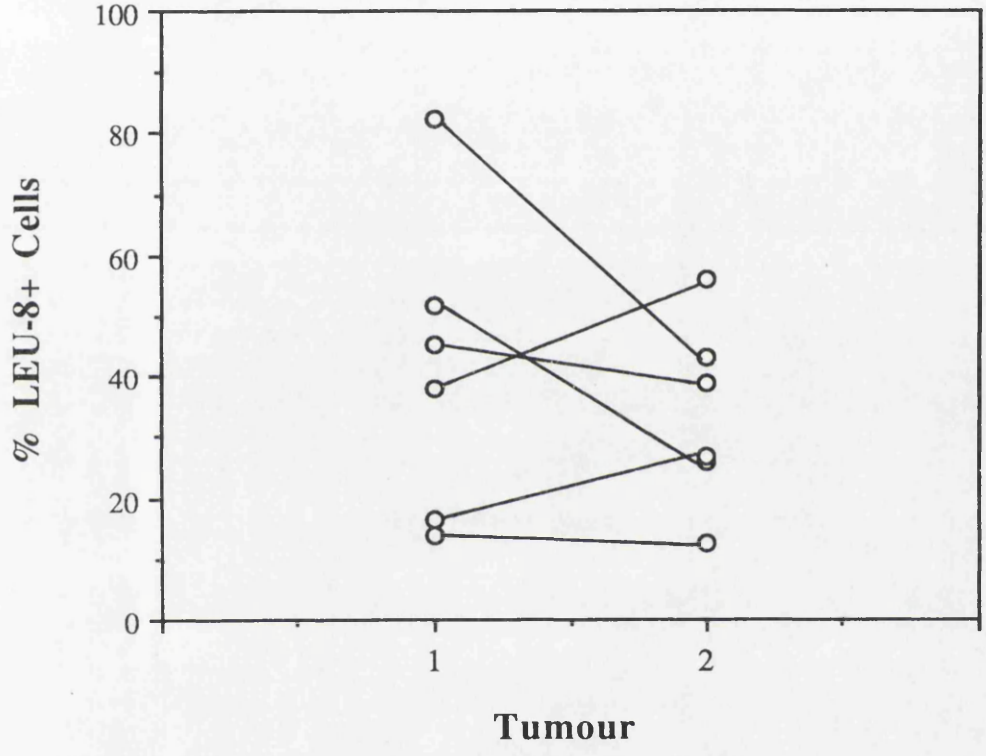


Figure 5.7 The comparison of % LEU-8+ cells between (1) metastasis in node and (2) primary tumour of matched samples from 6 breast cancer patients

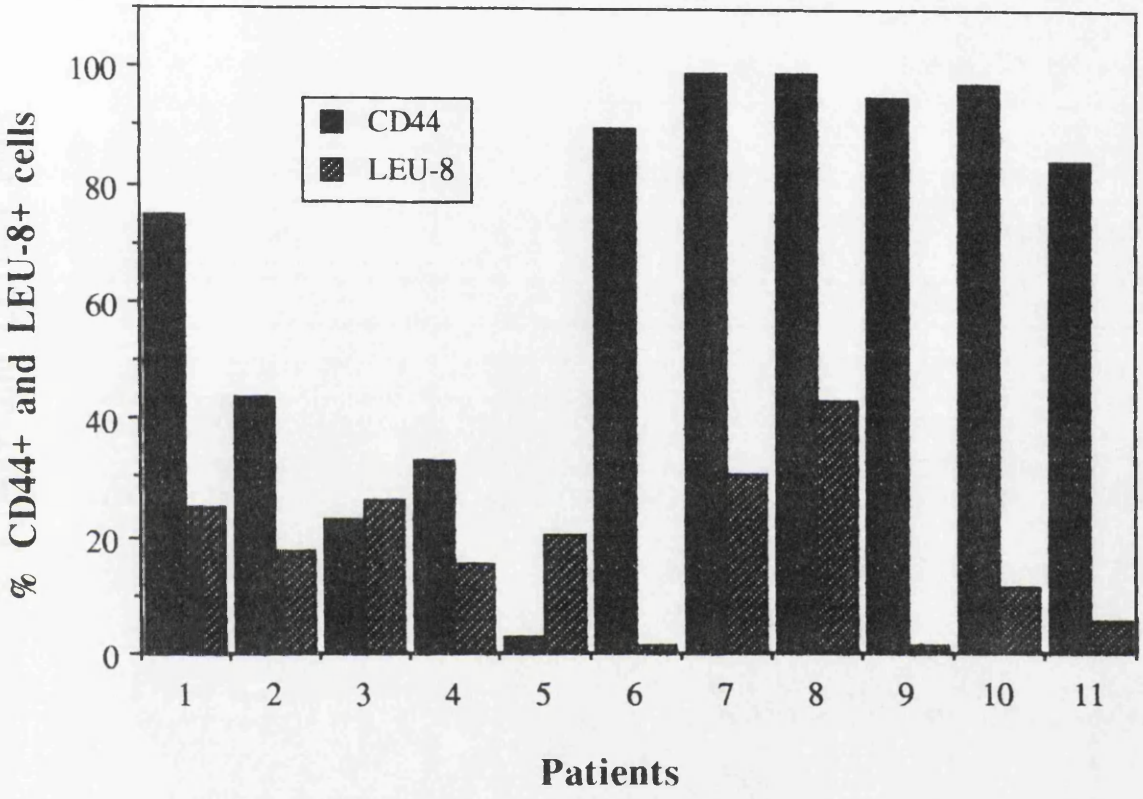


Figure 5.8 Percentage of CD44+ and LEU-8+ expression in lymphocytes from tumour invaded lymph nodes shown as matched pairs for the same breast cancer patients

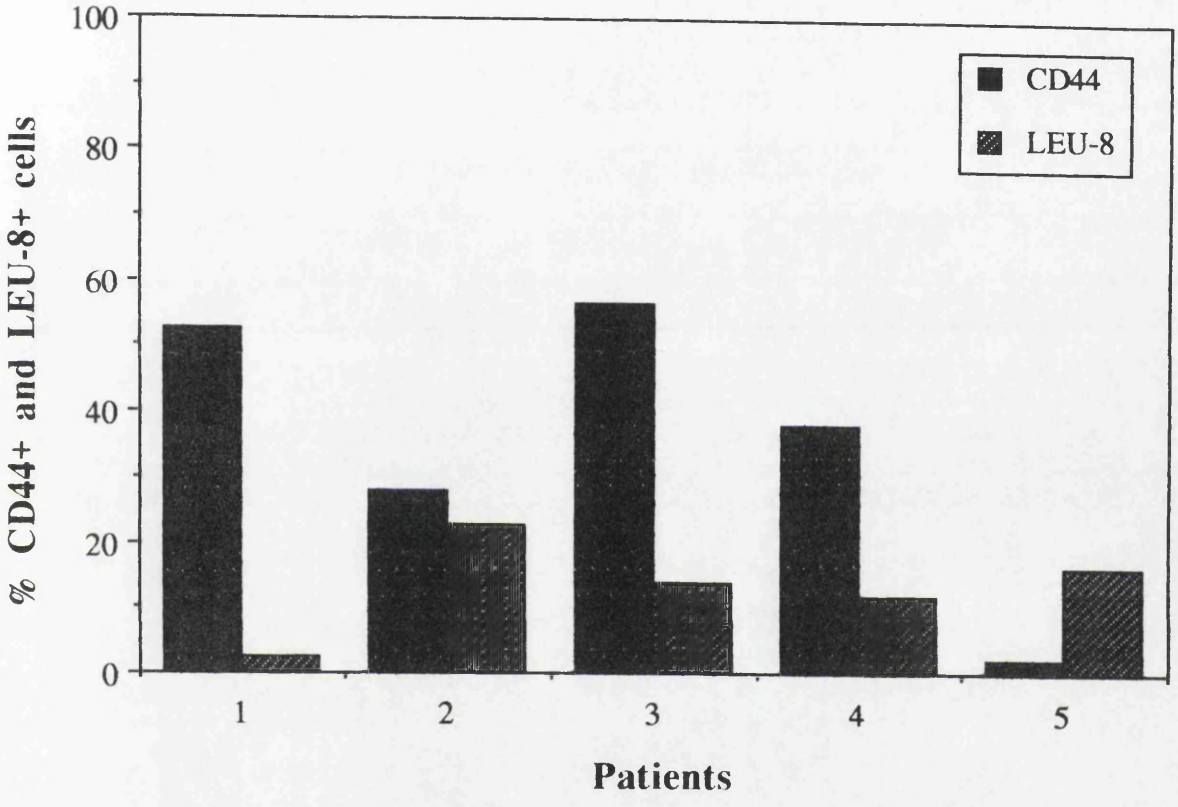


Figure 5.9 Percentage of CD44+ and LEU-8+ expression in lymphocytes from tumour free (uninvaded) lymph nodes shown as matched pairs for the same breast cancer patients

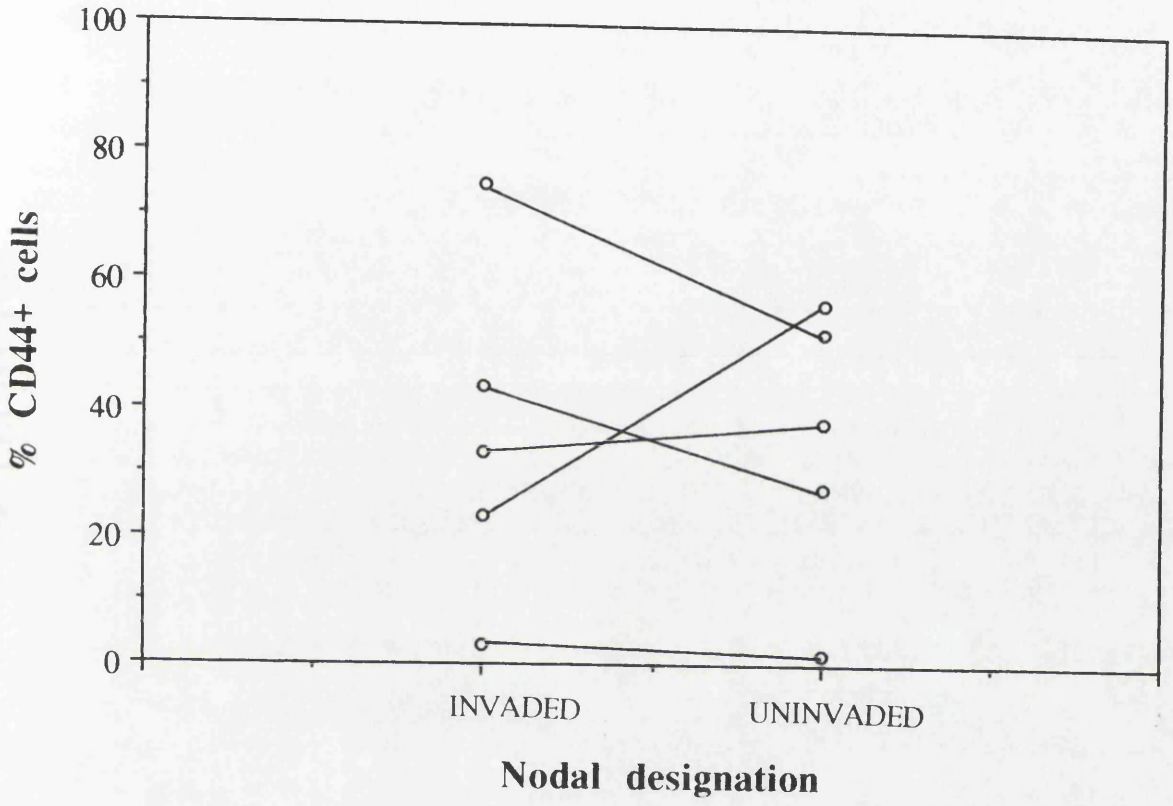


Figure 5.10 The percentage change of CD44+ expression between lymphocytes of the tumour invaded and free (uninvaded) lymph nodes of matched pairs for the same breast cancer patients

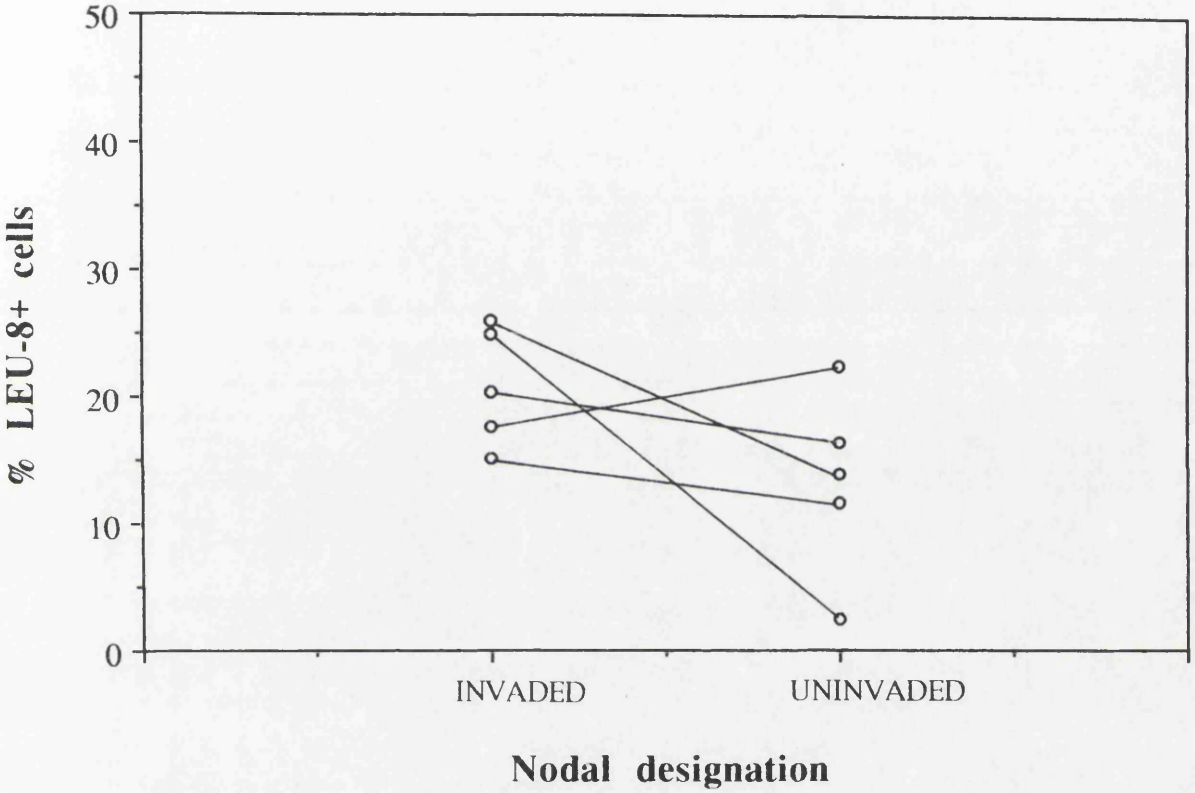


Figure 5.11 The percentage change of LEU-8+ expression between lymphocytes of the tumour invaded and free (uninvaded) lymph nodes of matched pairs for the same breast cancer patients

5.3 Discussion

Axillary lymph nodes in breast cancer patients are ideally situated to encounter tumour antigen and mediate immune responses to tumours. At the same time, metastases/tumour invaded nodes influence the lymphocytes within such nodes and determine the tumour-host interaction. This section was designed to study two main hypotheses, (a) the potential homing function of CD44 and (b) the potential involvement of cell surface glycoproteins, CD44 and LEU-8, in cancer metastasis. Expression of CD44 and LEU-8 on lymphocytes from breast cancer patients was defined by flow cytometric analysis using monoclonal antibodies to cell surface markers. Furthermore, two groups of nodes, tumour-free and tumour invaded lymph nodes, from the same patients were compared.

In general, CD44+ cells were preferentially located in the node. However the percentage of CD44+ cells was so high on most human lymphocytes, and there was so little difference between lymph node and peripheral blood that it was difficult to deduce evidence of homing . On the other hand, an interesting feature in this study was that there were more cells expressing CD44 than LEU-8 in the peripheral blood. This suggests that CD44 might be a homing receptor in humans, but more data is required before statistical analysis may have validity

An analysis of CD44 and LEU-8 expression on lymphocyte populations from local lymph nodes and tumours showed correlation with metastatic cells, and may indicate important elements in the metastatic process. Although the mean percentage of CD44 expressing cells was higher than the mean of percentage of LEU-8 expressing cells in primary tumour and axillary nodal metastases, CD44 and LEU-8 were not significantly different in the tissues of patients studied. The observations showed that tumour cells in both primary and metastatic tumours express CD44. More interestingly they also express

substantial amounts of LEU-8 which is generally described as a lymphocyte marker. This result provides evidence that CD44 and LEU-8 cell surface molecules may be involved in tumour metastasis. Jackson *et al.* (1993) observed that many tumour types do not express CD44 and that CD44 may therefore not be a tumour-specific marker for malignant disease in all cases. Taken together, the results of this study suggest that cell surface adhesion molecule CD44 may be involved in the lymphatic spread of tumour cells, possibly by mimicking circulating lymphocytes, and measurement of the CD44 and LEU-8 may have prognostic and diagnostic potential in primary and metastatic breast carcinomas. However the patient number is too small for any firm conclusion to be drawn.

CHAPTER 6

GENERAL DISCUSSION

6. General Discussion

This study was initiated to investigate whether the putative human lymph node homing receptors (LEU-8 and CD44) have an obvious homing function in humans by comparison of their relative expression in lymph nodes and peripheral blood of breast cancer patients using dual-colour flow cytometry. For the work described in this thesis tumours, blood and tumour draining lymph nodes of breast cancer patients were obtained from Department of Surgery, Western Infirmary, Glasgow. The experimental procedure in Chapter 3 was designed to generate antibodies against human lymph node cells in order to determine whether they contained a marker reactive with lymph nodes. At the same time, these antigens were compared with the homing function suggested for the LEU-8 marker (Chapter 4). Chapter 5 analysed whether CD44 has a homing function and investigated the metastasis pattern in both primary tumours and tumour-un/invaded nodes of breast cancer patients. Additionally, it asked whether tumour cells carrying CD44 and LEU-8 show lower or higher expression on primary or metastatic tumours. This work also defined the contribution of both phenotypic markers (CD4 and CD8) and activation markers (IL-2 receptor and HLA-DR) in the communication between two lymph nodes (near and far from their draining tissue) or blood and nodes in the local immune response (Chapter 4).

6.1 Flow cytometry

The flow cytometer used throughout this research was a FACScan (Becton Dickinson, Ltd., U.K.). This instrument performs DNA analysis (Chapter 3), preferential screening of individual cell populations (Chapter 3), analysis of phenotypic and activation status (Chapter 4) and has many clinical research applications (Chapter 4 and 5). Flow cytometry is an excellent tool

for analysing the expression of cell surface antigens of tumour cells and/or lymphocytes with the ability of discriminating live from dead cells that will increase accuracy of data analysis. Cells bearing the specific markers were simultaneously identified in one sample using combinations of two different monoclonal antibodies conjugated to different fluorochromes (FITC/PE). The number of cells analysed can be as high as 50,000 for each sample in a relatively short space of time. Within the five-parameters (three fluorescence channels, forward scatter; FSC and side scatter; SSC), it is possible to select for defined populations, and gate out tumour cells from the associated lymphocyte population. However, both selection and gating of a particular cell population from an irrelevant one depend on personal judgement. This can lead to an element of inaccuracy in the quantitative analysis of the data in every case. Tumour cells and lymphocytes were tested (5000 cells/run) for CD44/LEU-8 expression by flow cytometry. The appropriate negative controls establishing background fluorescence and non-specific staining by the primary and secondary antibodies were employed. Means and standard deviations of LEU-8/CD44 surface fluorescence values were calculated.

6.2 Production of monoclonal antibodies to LNLs

The methodology of MAb generation has developed with rapid progress from polyclonal animal antibodies, rodent monoclonal antibodies and now humanised antibodies during over the past 20 years. For the generation of good monoclonal antibodies, the SP2/0 myeloma cell line carried as tumour from the Balb/c mouse was fused in PEG with the spleen cells from a hyper immune mouse.

There has been no previous study on any antibody generated against the lymphocytes from human lymph nodes for identification of unique markers.

Three clones were produced in this study, all using separate immunisation schedules, of which one clone was from a mouse immunised with whole lymph node lymphocytes and two clones from mice immunised with panned nodal T cells contaminated with <3% B cells. The positive clones selected were analysed either by ELISA or by FACScan. The MAbs did not show the same reactivity pattern each time because a different node was from a different patient, and because there were only a few cells in each node and each node had a different proportion of B and T cells. Three resultant MAbs reacted with lymph node as well as peripheral blood lymphocytes, and this result indicated that the antigens defined were distributed through out the peripheral blood and lymph node lymphocytes. In Western blot analysis, however, MAb 1 and 3 failed to detect antigen, while MAb 2 recognised an epitope in the molecular weight region suggested as CD19 B cell marker (95 KDa). Dual-colour flow cytometry analysis also showed that the B cells must be very highly immunodominant in the human lymph node despite immunisation with >97% T cells. All MAbs also bound to B cell lines and nodal B cells not T cell lines and nodal T cells. Taken together, the epitope recognised by the MAbs was on human B cells, and is unlikely to be MHC I or immunoglobulin. Finally, the antigens identified were not specific to the node, and this result suggests that LEU-8 molecule has no homing function in human lymph nodes (Chapter 4).

6.3 Analysis of established lymph node homing receptor antigens

Over the past decade, the interaction between leukocytes and endothelium has been suggested to be of extreme importance in the communication of the elements of the immune system because of the discovery of various adhesion molecules expressed during early formation and differentiation of the elements in the bone marrow, the thymus or at other

peripheral sites. In addition, these adhesion molecules are thought to play an important role in antigen-driven activation of lymphocytes, and in the guiding and retaining of the cells on the extracellular matrix components within lymphoid organs and connective tissue. As a result of the multitude of adhesion molecules involved in the adhesion and extravasation of leukocytes to endothelium, many therapeutic approaches are based on the reaction of MAbs against adhesion molecules or structural parts of the ligands. Lymphocyte interactions with high endothelial venules (HEV) also involve very similar sequential rolling, activation, and activation-dependent sticking events. In final step (diapedesis), the recruitment of leukocyte from the blood into sites of inflammation or into lymphoid tissues requires the presence of specific chemoattractant gradients and appropriate substrates for leukocyte migration.

In considering this model, L-selectin functions as a dominant lymphocyte homing receptor in lymphocyte trafficking to distinct sites, whose HEV express high levels of the peripheral node addressin/ligand for the L-selectin. In addition, CD44 may also be involved in lymphocyte homing, immune function, and tumour metastasis. In certain conditions, it may be desirable to inhibit particular lymphocyte or leukocyte homing interactions as selectively as possible, and they represent an attractive target for suppression of inflammation in chronic inflammatory diseases and other immune-mediated pathologies. Clearly, the important goal of this research of lymphocyte trafficking is both for understanding of physiology of immune mechanism and for its potential clinical diagnostic and therapeutic value; of course, in relatively acute clinical syndromes, the selectivity of the therapy may be less important than its overall effectiveness.

In this research, a major problem in the analysis of LEU-8 or CD44 expression was a variability of samples between patients. The reasons for variability are mainly physiological and technical. The only way of getting

fresh human lymph nodes, tumours and blood was during an operation for cancer. Thus the cells were obtained at one single time point. Post mortem samples were too necrotic because of the time consuming legal formalities involved. The physiological factors in each patient, for example, the difference in age, or whether pre-menopausal may affect the cellular composition. Infection with opportunistic pathogens which activate the immune system also can affect the expression of a number of phenotypic and activation markers. Additionally, genetic variation can involve the protein synthesis mechanism of cell surface markers. On the other hand, expression may be lost during the handling of samples in the laboratory, in the steps of thawing the frozen cell suspension after long storage in liquid nitrogen, and in subsequent washing. Although the samples used in this research did not have the optimum conditions for analysis, the relative comparison of LEU-8 or CD44 expression on samples gave an indication of the conclusions which might be obtained using a larger sample.

The Mann-Whitney test used for the comparison of the difference in the medians of the two independent groups did not show statistical validity, because the numbers of samples in each group was small (Chapter 5).

6.3.1 LEU-8

A few papers have reported that the LEU-8 marker is human homologue of the Mel-14, L-Selectin, as previously mentioned (Section 1.4.2.2). The putative human nodal "homing" marker LEU-8 by cDNA homology analysis from human to mouse has not been proven in humans because of obvious ethical problems. This study was designed to assess the homing function of LEU-8 marker using measurement of the relative expression of LEU-8+ populations on human axillary lymph node and peripheral blood of breast

cancer patients by dual-colour flow cytometry. However, there was no indication that CD3+ or CD19+ cells express LEU-8 preferentially in the node, and individual lymph nodes differ greatly. Moreover, no specific nodal antigen for homing was identified (Chapter 3). Thus, this work showed that, although LEU-8 cDNA has some homology to Mel-14 DNA, the encoded proteins have different biological functions. More interestingly, the LEU-8 cell surface molecule may be involved in tumour metastasis (Chapter 5).

6.3.2 CD 44

The integral membrane glycoprotein CD44 is expressed on a variety of cell types including peripheral blood leukocytes (B and T lymphocytes, monocytes and granulocytes) and red cells. It is also weakly expressed on platelets, and in soluble form in plasma. CD44 has long been known to be involved in the interaction of lymphocytes and endothelium via interaction with extracellular matrix components such as hyaluronic acid. The CD44 molecule mediates a variety of functions: leukocyte-endothelial cell binding, lymphocyte homing, extracellular matrix binding, enhancement of T cell activation and adhesion to monocytes (Section 1.4.2.3). The finding of splice variants also showed that these molecules are associated with aberrant migration and metastasis (Section 1.4.3). Although quantitative and qualitative changes in CD44 can be used to discriminate primary tumours from corresponding normal tissue in a number of system, there is no consensus as to the role of CD44 in the progression of tumours.

In this experiment, monoclonal anti-human CD44 (mouse IgG1 isotype) derived from the clone A3D8 recognising an epitope on both standard and variant forms of CD44, was used to define its homing function and metastasis pattern. This study suggestes that CD44 marker might be a homing receptor

in humans although the validity of the statistical analysis was limited by small sample number. The CD44 molecule was also expressed in the primary tumour and the axillary metastatic lymph node.

6.4 Local immunological response in human lymph nodes

In breast cancer patients, tumour draining lymph nodes represent an important site where malignant cells may be recognised by eliciting a host immune response to the tumour. The axillary lymph node metastasis of breast cancer patients is currently the important prognostic clinical indicator. The lymphocyte subpopulations in regional lymph node and peripheral blood of early breast cancer patients were analysed according to their distance from the primary tumour. In this study, the comparison was likely to be most relevant between two axillary lymph nodes or between peripheral blood and lymph nodes from the same patient. While the IL-2 receptor was present on a higher proportion on CD4+ than CD8+ cells, the percentage of CD8+ cells bearing HLA-DR was higher than that of CD4+ cells in all three sources. This activation marker was also found to be expressed on higher numbers of CD4+ and CD8+ T cells in near lymph nodes than in peripheral blood, and in near than far lymph node. This general activation of the lymph node is reflected in the specific alterations of the lymphocyte subpopulations. The alteration in local immunocompetent cell subsets may represent essential elements in the activated state of regional lymph node and in the progression of the cancer.

6.5 Conclusions

In the human lymph node, B lymphocytes were highly immunodominant, not quantitatively dominant. This research showed that the suggested LEU-8 marker has no homing function in humans, and CD44 might be a human lymph node homing receptor. Both CD44 and LEU-8 may have prognostic and diagnostic potential in primary and metastatic breast carcinomas. The local regional lymph nodes of breast cancer patients show a specific alteration of lymphocyte subpopulations and activation status. Finally, it may be noted that there were trends of variability in the results obtained from this study.

In further work, human nodal antigen should be identified using, possibly, a protein sequencing technique, and more samples are required for any firm conclusion to be drawn with greater statistical validity.

CHAPTER 7

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