FLOW CYTOMETRIC STUDIES ON BREAST CANCER

by

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CONTENTS

Chapter 2: Materials and Methods

Chapter 5: The effect of distance on the immune response within the tumour draining axillary lymph nodes

Chapter 6: DNA and oncogene expression in primary and metastatic breast cancer

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CD	Cluster of designation
CD25	Interleukin-2 receptor
CTL	Cytotoxic T lymphocyte
DI	DNA index
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
FACScan	Fluorescence activated cell scanner
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
FL 1,2,3	Fluorescence channel 1,2 or 3
FSc	Forward scatter
HLA	Human leucocyte antigen
ICAM I	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-2R	Interleukin-2 receptor
Kb	Kilo base pairs
LFA-1	Lymphocyte function-associated antigen-1
LNL	Lymph node lymphocyte
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumour virus
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PE	Phycoerythrin

PI	Propidium iodide
sIgG	Surface immunoglobulin G
SPF	S phase fraction
SSc	Side scatter
Tac	T cell activation synonym for the CD25 antigen on the β chain of the IL-2 receptor
TCR	T cell receptor
Tc	T cytotoxic cell
TGF-β	Transforming growth factor β
Th	T helper cell
TIL	Tumour infiltrating lymphocyte

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Summary

This study investigated the potential of flow cytometry for assessment of both the immunology and oncology of breast cancer and explored the possibility of correlation between the two.

The T cell receptor γ/δ expression was studied in lymphocytes found in the tumour (TILs), in the tumour draining lymph nodes and in the blood. During this study the T lymphocytes were divided into CD3, CD4 and CD8 phenotypic subsets to assess differences. Tumour Infiltrating Lymphocytes (TILs) were observed to have a greater median expression of CD3+ CD8+ γ/δ+, whereas in contrast the lymph node lymphocytes (LNL) are shown to have a higher median expression of CD3+ CD4+ γ/δ+ lymphocytes. Interestingly the majority of γ/δ+ lymphocytes were double negative (CD4- and CD8-) which may indicate a role for this subset in immunology of breast cancer. Breast tumours often have reduced MHC class I expression, which in effect means that normal cytotoxic T cell mediated tumour killing is inhibited. Consequently other non MHC mediated killing methods, possibly mediated by the double negative γ/δ T cell subset, may be important in tumour immunology.

The immune response was assessed in a tumour invaded and a tumour free lymph node removed from the same patient. The phenotype and activation of these lymphocytes was measured by flow cytometry to study the effect of metastatic tumour on the nodal immune response. The most significant difference was noted in the T lymphocyte phenotype, with the tumour invaded lymph node having an increased percentage of CD8+ cytotoxic T cells

($p<0.001$) which were replaced in the tumour free node, by CD4+ T helper cells ($p=0.008$). The trend was clearly shown as a decreased CD4/8 ratio in the tumour invaded node, increasing significantly in the tumour free node ($p<0.001$). Significant changes were also observed for activation marker expression with HLA DR levels significantly higher in the tumour invaded nodes for both CD8+ T cells ($p=0.023$) and CD4+ T cells ($p=0.036$). The interleukin-2 receptor expression however was only significantly increased in the tumour invaded nodes for CD8+ T cells ($p=0.029$) these trends possibly being indicative of the effect of tumour invasion on the immune system.

Low and high axillary nodes which had been removed from each breast cancer patient were used to assess which of the observed lymphocyte phenotype and activation differences were local and also to compare stage I and II nodal responses. The stage II patients were then further subdivided into 4 subsets on the presence or absence of metastatic tumour within these lymph nodes. Phenotypic changes were observed in the 22 stage I patients only with a significant increase in CD3+ T cell percentage in the low node ($p=0.042$), with a concomitant decrease in the high node. The opposite was observed for CD19+ B cells ($p=0.036$), with a lower percentage in the low node. This trend was reflected in the significant decrease in the CD3/CD19 ratio ($p=0.036$). No significant phenotypic trends were noted for pooled stage II patients. However when the subsets of stage II patients were analysed a significant trend for T cells was noted in the subset with low node tumour invaded and the high node tumour free with a higher CD4/8 ratio in the low invaded node, decreasing in the high node ($p=0.05$). Lymphocyte activation trends were observed in the 22 paired stage II nodes only. The low nodes showed significantly higher percentages of CD4+ HLA DR+ lymphocytes ($p=0.01$) and of interleukin-2

receptor (Tac) expression on both CD8+ T cells (p=0.05) and CD4+ T cells (p<0.001). The stage II subsets showed significant changes in activation, with the low node tumour invaded and high node tumour free showing significantly higher percentages of CD8+ HLA DR+ lymphocytes in the low node compared to the high node (p=0.05). Trends of increasing interleukin-2 receptor (Tac) expression in the low node were observed for CD4+ T cells (p=0.005). A trend of increasing expression of IgG on CD19+ B cells was observed in the subset with both low node and high node tumour free (p=0.05). The general conclusion was that the invasion of the node was reflected primarily in the HLA DR response on CD8+ (T cytotoxic) cells while distance from the tumour was reflected by the interleukin -2 receptor (tac) response on CD4+ (T helper) cells.

Two oncoprotein markers were then analysed in the primary and metastatic tumour. p53 and c-*erb* B-2 expression was measured in primary and metastatic breast cancer by dual parameter flow cytometry (oncoprotein and DNA). Interestingly almost all tumours had a detectable number of cells positive for both oncoproteins and no tumour had 100% of the cells positive. This was true even in the node where there was no possibility of "normal" epithelial cells being present in the sample. There were no significant trends for either c-*erb* B-2 and p53, although the c-*erb* B-2 expression tended to increase in stage II patients compared to stage I. In addition aneuploid tumours tended to have an increased expression of c-*erb* B-2. The level of both c-*erb* B-2 and p53 had little variation when primary tumour was compared to matched metastasis.

p53 and c-*erb* B-2 expression and tumour ploidy were then related to the nodal immune response to deduce the effect of tumour type on the immune

system. No trends were evident for c-erb B-2 expression and the nodal immune response, whereas increased p53 expression tended to be related to increasing levels of the interleukin-2 receptor (tac) on CD4+ T cells ($p=0.009$). The ploidy of the tumour was related to the level of lymphocytic activation in the lymph nodes, with generally higher levels in the nodes draining aneuploid tumours. The only significant trend observed was for increased levels of sIgG on CD19+ B cells in nodes draining aneuploid tumours ($p=0.04$).

Overall the data illustrate inter and intra patient heterogeneity of both the tumour and the immune response to it. Thus, as each patient is unique, only a limited subset of patients are likely to respond to immunotherapy.

CHAPTER 1

INTRODUCTION

1.1. Cancer

There exists in animals a delicate balance between cell death and cell renewal. As cells die they are replace by new cells generated by proliferation. Under normal circumstances the production of new cells is so regulated that the numbers of any particular type of cell remain constant. Occasionally cells arise which do not respond to normal growth control mechanisms. These cells will divide and give rise to a group of cells collectively known as cancer.

1.1.1. The history of breast cancer and treatment

Breast Cancer is an ancient disease, being first described by the Egyptians five thousand years ago (Donegan, 1988). The Romans were the first to attempt surgery as a treatment. However it was a Greek, Galen, who in the second century A.D described Breast cancer as "Like a crab, with the appearance of large veins from the sides giving the appearance of legs " thus the term cancer was coined. Galen at this time described breast cancer as being a "systemic disease" attributed to an excess of black bile in the body for which there was no cure.

Until the nineteenth century the practice of breast surgery was extremely ill advised and barbaric often resulting in death during the operation. It was only when Descartes discovered the lymphatic system and specifically the axillary lymph nodes draining the breast that Galen's black bile systemic disease theory was questioned. Le Dran offered the first alternative theory of breast cancer being a local disease which would begin in the breast, then metastasise to the nodes and from there finally enter the general circulation.

This pointed the way to early diagnosis and surgery to halt the disease. The advance in breast surgery came with Halsted and the radical mastectomy (Halsted, 1894). This novel surgery at this time was based on an anatomical understanding of the disease, which involved the *en bloc* removal of the breast, pectoralis muscle and the axillary draining lymph nodes in all patients. The radical mastectomy was performed in its entirety unchanged until the early 1960's. The acceptance of the radical mastectomy was due to the surgeons being convinced that no improvements could be made, as the limit of radical surgery had been reached. Eventually there was an irresistible stimulus for reassessment of treatment, as, although the limit of surgery had been reached, one third of all women were still succumbing to the disease. The greater understanding of the biology of cancer and the study of the metastatic process had shown that the confinement of the disease to the lymph nodes had come to an end long before surgery was attempted. Studies initiated on the effect of surgery suggested that the radical mastectomy was perhaps too radical and more conservative surgery could well be advocated in many cases. The type of surgery was altered and combined with new secondary treatments such as radiation and chemotherapy. Resultant trials have shown this to be just as effective as the radical mastectomy.

1.1.2 Treatment of breast cancer

The treatment of breast cancer is continuously evolving and throughout the U.K. variable surgical and post operative therapy regimes exist. In the Western Infirmary, Glasgow the surgery consists of two main types, lumpectomy and mastectomy.

Lumpectomy involves the removal of the tumour when the tumour is less than 3 cm in size. With mastectomy there is removal of the whole breast down to the pectoralis major muscle when tumour size is greater than 3 cm. Simultaneously in both cases the axillary tumour draining nodes are removed and are assessed for the presence of tumour (Section 1.1.6.1.).

Post-operative therapy in the Western Infirmary is of two types depending on the presence of tumour metastases in the axillary lymph nodes.

(A) No tumour present in the axillary nodes: post-operative therapy is not given
(B) Tumour present in the axillary nodes: post-operative therapy given.

Pre menopausal women receive chemotherapy in a combination of chemotherapeutic agents known as CMF (cycloheximide, methotrexate and 5-fluorouracil) over a set period after surgery (Bonadonna, 1979) .

Post menopausal women receive tamoxifen an anti-estrogen drug if the tumour has been shown to be estrogen driven or CMF due to failure of tamoxifen treatment. As well as chemotherapy the lumpectomy patients receive wide field radiation therapy to the breast. The function of these therapies is to stop rapidly dividing cancer cells by inhibiting DNA synthesis the major drawback being that other fast dividing cells such as lymphocytes are affected and this may curtail any immune response to the tumour.

There are no guide lines on breast cancer treatment and due to this fact many differences in surgery are observed between treatment centres. It is for individual surgeons to decide on what they perceive to be the optimal patient treatment. Surgical views are often opposing and generate much debate around

topics such as the requirement for removal of the axillary nodes (Fentiman and Mansel, 1991) especially if these nodes are generating an immune response against the tumour. However in contrast, the USA has specific treatment guidelines on surgery and therapies. An example of this is the widespread practice to give pre-operative chemotherapy to shrink the tumour and to reduce tumour cell shedding during surgery. Eventually in this country regimes must be rationalised since treatment goes hand in hand with a greater understanding of the biology of the disease. This hopefully will make clinicians evaluate and question treatment regimes more often than the blind acceptance found in the past.

1.1.3. Anatomy of the Breast

1.1.3.1. Cellular composition of normal breast

The size, structure and function of the human breast is determined by complex interactions of hormones and other signals, resulting in differentiation of the epithelial cells to produce an organ capable of milk secretion.

The mammary gland, or breast, is a modified sweat gland that has developed the specialised function of secreting milk instead of sweat. The gland overlies the pectoralis major muscle and is a complex arrangement of inter-connected secretory units surrounded by fat and connective tissue, and serviced by an extensive blood, lymphatic, and nervous system. The mammary gland has an epithelial component, which constitutes the essential *glandular tissue* (parenchyma), and a connective tissue component, which forms the supporting and enclosing framework, called *stroma*. Overlying the glandular

tissue is a layer of adipose tissue. The breast is highly vascularised and well provided with lymphatic drainage, leading towards the axilla.

The epithelial components are arranged as lobes, each with 15 to 20 interconnected *lactiferous ducts*, which run radially from the base of the nipple and branch repeatedly (Figure 1.1.). Each of these ducts dilate to form a

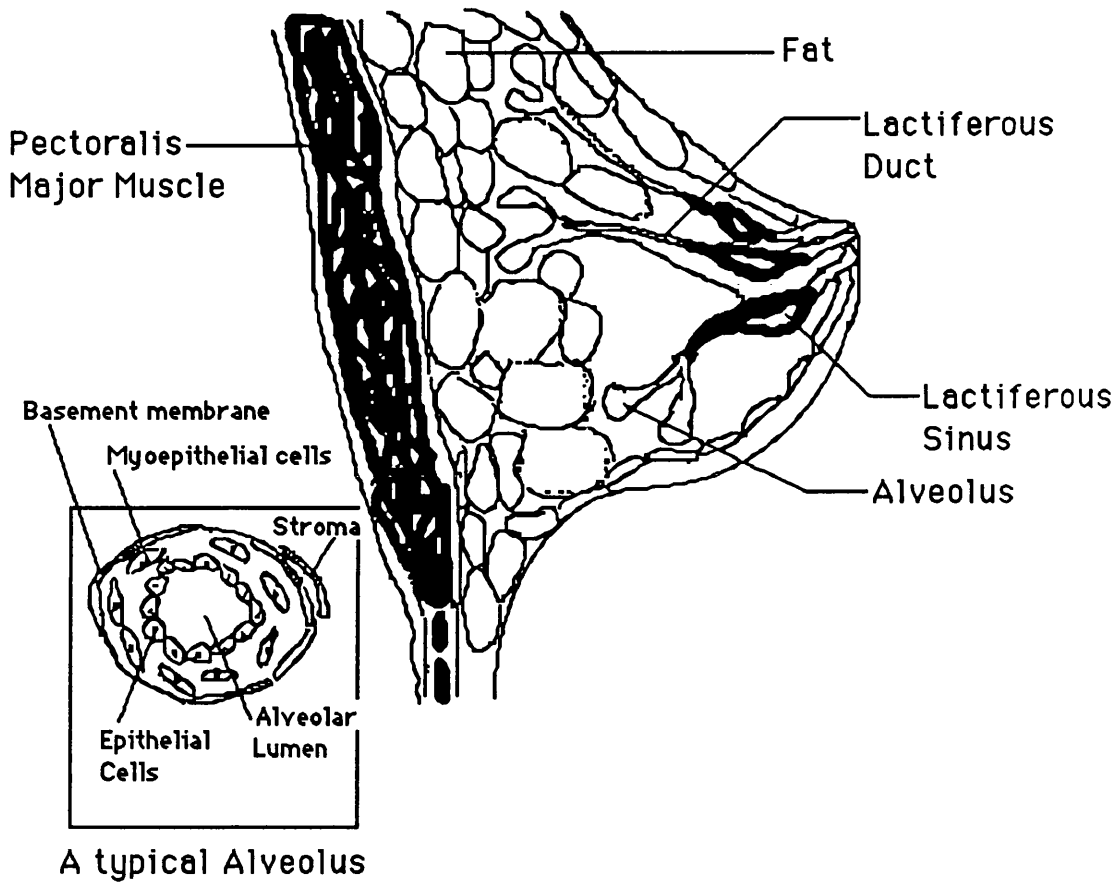


Figure 1.1 The human mammary gland and its constituent parts.

lactiferous sinus, before opening separately on the nipple. Each lobe is made up of the glandular tissue that develops from a single duct and is subdivided into lobules corresponding to the branchings of the duct system. Each functional lobe is a separate gland operating within the environment of the adjacent fat and stromal cells. The gland does not have a definite capsule, and

its connective tissue component consequently merges with the subcutaneous connective tissue. The fundamental secretory units of each lobe are the many *alveoli* of the lactiferous ducts. These secretory units are responsive to hormonal modulations and, as such, show maximum growth and regression during various hormonal changes of puberty, pregnancy, and menopause. The alveoli are surrounded by a mesh of *myoepithelial* cells which show some of the properties of epithelial and muscle cells and are involved in milk ejection. The whole structure is surrounded by the basal lamina which acts as a boundary between epithelium and stroma. The boundary is permeable to growth factors and metabolites but is normally breached only by migratory blood cells.

1.1.3.2 Lymphatic drainage of the breast

The breast has a well developed lymphatic system that drains mainly towards the axilla (Figure 1.2). The major lymphatics include a superficial group draining the skin (Rotter nodes) and a deep group draining the mammary lobules. In addition, there are the internal mammary nodes which accompany the internal mammary artery. Although the lymphatics pass in all directions from the breast, the main direction of lymphatic flow is towards the axilla with about 97% of the lymphatic drainage to the axillary nodes, with the remaining passing to the internal mammary nodes (Donegan, 1988). The axilla can be further subdivided into three positions. They include the apical, which are located farthest away, the central (mid) axillary nodes, and those nearest to the gland, the anterior axillary nodes.

The axillary lymph nodes play a very important role in breast cancer with metastatic spread of the disease frequently involving the axillary nodes.

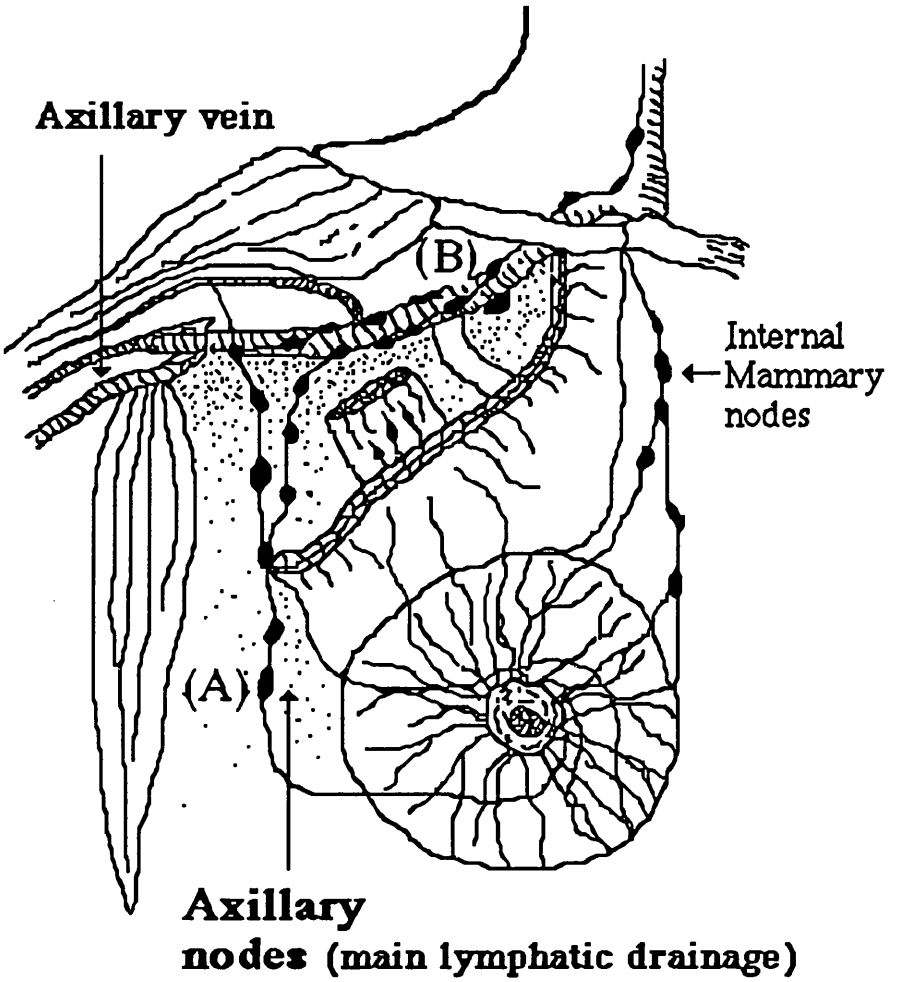


Figure 1.2 The lymphatic drainage of the breast A= low axillary nodes and B= high (apical) axillary nodes.

The status of these nodes is, therefore, an important feature in assessing the prognosis of a breast cancer patient. In effect, the surgical management of breast cancer includes not only the removal of the primary tumour but removal and examination of the axillary lymph nodes. Whether nodes are involved in a cancer and the number of nodes involved is, at present, the most important prognostic indicator (Section 1.1.6.1.). If there is metastatic tumour in the nodes it can be directly compared with the primary to deduce the changes and interactions with the immune system within these axillary nodes.

1.1.4. Biology of the breast and breast cancer

1.1.4.1. Cellular components

The main four cell types in the breast are the glandular epithelium, myoepithelial cells, the fat cells and the stroma. These are involved in the interactions producing normal breast development or cancer formation, such as direct contact with the myoepithelium, secretion of growth factors from the stroma or activation of precursors (stromal and fat cells). These interactions are an essential part of complete breast development.

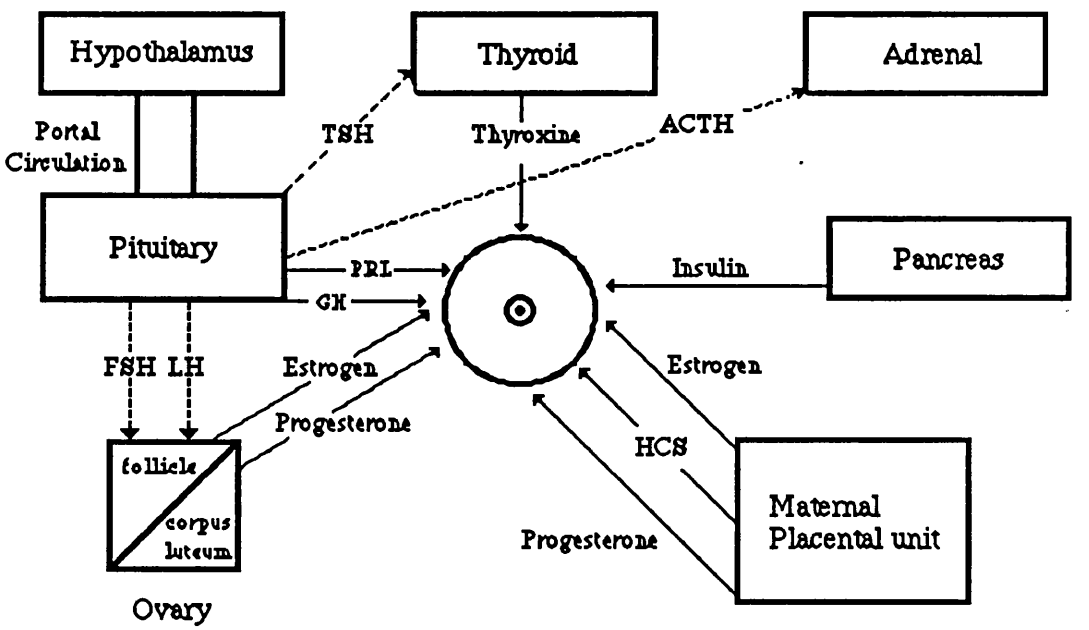


Figure 1.3 : Endocrine factors affecting breast development and function
FSH (follicle stimulating hormone); TSH (thyroid stimulating hormone); LH (luteinizing hormone); PRL (prolactin); GH (growth hormone); HCS (Human chorionic somammotropin).

1.1.4.2 Hormones

Hormones have a crucial role in the the biology of the breast and also in breast cancer (Figure 1.3). The hormones involved are of two types; (1) the polypeptides, including growth hormone and prolactin; (2) the steroids including oestrogen, progesterone and glucocorticoids, some of which are involved in both normal and abnormal breast development.

1.1.4.2.1. Polypeptide hormones

Prolactin is a polypeptide hormone released from the anterior pituitary. The release is controlled by Prolactin inhibitory factor (PIF), assumed to be dopamine or a similar molecule. Prolactin is involved in the control of growth and differentiation of the mammary gland. Mitogenic effects of prolactin have been shown on MCF-7 cells and rat mammary cells (Shafie and Brooks, 1987). A role has also been implied in oestrogen receptor regulation and the transcription of the casein gene (Guyette *et al* 1979). However the specific effect of prolactin is dependent on the differentiation state of the breast epithelium and this can be modulated by the interaction of other hormones such as insulin (Ollivier-Bousquet, 1978).

Oxytocin is produced by the hypothalamus and has the ability to interact with the myoepithelial cells located in the alveolus and the surrounding ducts. Oxytocin is involved with the phosphorylation of myosin causing contraction of the target cells for induction of milk ejection.

1.1.4.2.2. Steroid hormones

Oestrogen is a member of the steroid hormone family. In breast tissues,

the oestrogens bind to specific oestrogen receptors (ER) in the cytosol and the bound receptor then moves to the nucleus (Shayamala and Nandi, 1972). In addition to its effect on the gland, the hormones also promote prolactin secretion. Interestingly, oestrogen is believed to be produced in normal breast tissue as well as in breast cancer tissues (Edwards *et al* 1979).

Oestrogens are associated with mitogenesis in the female reproductive epithelium, especially in ductal formation (Stroudemire *et al* 1975) with progesterone and prolactin stimulating formation of lobulo-alveolae similar to that seen in pregnancy. This effect of progesterone is not seen on mammary epithelial cells in culture. The main role of progesterone is thought to be in the prevention of terminal differentiation in mammary epithelium during pregnancy (Neville and Berga, 1983).

Thus although the full role of the steroid hormones remains undefined, they appear to have a significant effect on the initiation and maintenance of differentiation. The presence of the oestrogen receptor in breast tumours is now routinely evaluated as it has been linked with patient prognosis.

Insulin has also been demonstrated to be a pre-requisite for proliferation of mammary epithelium in vitro but the precise role is as yet undefined and is thought to involve regulation of fatty acid production in lactating epithelium.

1.1.4.3. Cell-Cell interaction

The understanding of cellular interactions and adhesion are now being investigated in greater detail. Adhesion molecules such as ICAM 1, and others have an important role to play in both normal and abnormal growth of the cellular epithelium. Cell junctions in the breast are an important part of the

epithelium, giving it integrity. Gap junctions form between neighbouring cells. The small gaps which form are important for the exchange of small molecules and signals between neighbouring cell types. Tight junctions form due to the direct fusing of two different cell types together and these act as the major force in anchoring cells together (Gorby and Steinberg,1981). Changes in cell-cell interactions has been shown to be associated with the increase in metastatic potential and long term prognosis. The presence of epithelial cells and stroma may indicate an interaction between these two types of cells involving the secretion of specific growth factors. This is dependent on the structure of the basal lamina and alterations occurring in this layer have been implicated in metastatic potential (Pitelka *et al* 1984).

1.1.4.4. Growth Factors

Growth factors have an important role to play for both positive and negative regulation of breast cellular development. Two main factors implicated are Epidermal growth factor (EGF) and Transforming growth factorα (TGFα).

EGF is a growth factor of between 48 and 53 amino acids which acts on the Epidermal Growth Factor receptor (Section 1.4.3.4.). The receptor is found on most mammalian cells with the exception of differentiated white blood cells. On binding of EGF the receptor is dimerised and then immediately endocytosed. Signalling from this receptor involves the phosphorylation of the tyrosine kinase portion of the receptor leading to protein kinase C activation. High levels of EGFR protein expression has been implicated in poor prognosis for the patient (Sainsbury *et al* 1987; Nicholson *et al* 1988).

TGFα has a similar structure to that of EGF and can also bind to the EGF receptor and activate the receptor. The current speculation is that TGFα is the ligand for the EGF receptor during embryonic development and that EGF is the normal ligand produced during adult life. This would agree with the suggestion that a tumour cell has evolved into a more basic, rapidly dividing embryonic cell type with specific requirements for growth factors such as TGFα.

A third growth factor, TGF-β (Transforming growth factor β), although originally described as a positive growth stimulator, can also act as a growth inhibitor depending on the target cell type (Sporn *et al* 1987). Furthermore, although many normal epithelial cells are inhibited by TGF-β, several transformed cell lines, including breast cancer cells have been found to be resistant to its inhibitory effect (Knabble *et al* 1987). This may support the idea that escape from negative control might be an important step during carcinogenesis (Roberts *et al* 1985). Although a post receptor mechanism and not a loss of receptors is likely to be involved (Manning *et al* 1991), the mechanism of inhibition of epithelial cell growth by TGF-β is currently unclear. TGF-β is also a powerful immunosupressant and this may have an effect on any immune response including that to a tumour (Shull*et al* 1992).

1.1.5. Epidemiology and Etiology

1.1.5.1. Mortality

The frequency of mortality of breast cancer was unknown by the Greek and Roman Physicians who described it. The cases of carcinoma of the breast in the USA in 1987 were 900 males and 130, 000 females with a mortality in

the same year of 300 males and 41, 000 females. The age adjusted death was 27.1 per 100,000 women. The highest rate of mortality is found in England and Wales at 33.8 per 100,000 women and in the United States there has been no significant change in the incidence rate for the past 60 years (Donegan, 1988). However, the latest study from the USA suggests that the rate of breast cancer is increasing and now affects 1 in 9 women during their lifetime. Whereas in Japan a highly industrialised nation, the rate of breast cancer is one of the lowest in the world.

1.1.5.2. Aetiology of breast cancer

The incidence of breast cancer is high, but varies amongst different groups throughout the population. The reasons for this have been attributed to many different epidemiologic factors which have possible roles in cancer development.

1.1.5.3. Sex

The most significant high risk factor in breast cancer is the sex of the patient. More than 99% of breast cancer occurs in women. Breast cancer in men is rare and involves only 7 in a million men.

1.1.5.4. Age

The age of the patient is an important factor with nearly 85% of all breast cancers detected in women over 40. Less than 1.5% occur in women under the age of 30 (Leis, 1978).

1.1.5.5. Genetic predisposition

Female relatives of a women with breast cancer have a two-fold increase in their suceptibility, with the highest risk for their daughters. There is a difference observed between western and oriental women, but this seems to have an environmental rather than a genetic origin. Following immigration to the U.S.A, the daughters of oriental women show a rate of breast cancer equal to that of the indigenous population (Seidman, 1972).

1.1.5.6. Reproductive factors

A high incidence of cancer of the breast has long been noted in nuns and in childless women. The age of childbirth is also an important factor as women who have children before the age of 35 have a two-fold higher risk of breast cancer compared to women who have had children before the age of 20 (MacMahon *et al* 1973).

1.1.5.7. Dietary factors and Obesity

The poor diet in certain areas of the country, such as the West of Scotland will undoubtedly have a great deal of influence on the susceptibility of women to breast cancer. Associations with the dietary intake of saturated fat, cholesterol and protein have been suggested (Gray *et al* 1979). However these relationships are very tentative and often inconsistent.

Mice made artificially fat have an increased risk of breast cancer and studies, in various countries, have shown a similar, increased risk of breast cancer in postmenopausal obese women (Boyle and Leake, 1988).

1.1.5.8.Viruses

Viruses have been commonly implicated with both benign and malignant neoplasms in animals and in mice the mouse mammary tumour virus (MMTV) has been associated with breast cancer. However, while virus like particles have been discovered in human breast milk, there has been no association between this and the incidence of cancer (Spratt *et al* 1988).

1.1.6. Prognostic indices in breast cancer

Evaluation of patient prognosis is an important aspect of breast cancer as an understanding of the biology has to be eventually related to clinical treatment.

1.1.6.1 Staging of the tumour

The stage of the cancer has been shown to be the most reliable prognostic indicator in breast cancer. Evaluation by the Tumour Node, Metastases (TNM) method has long been accepted. The presence of metastatic tumour within the axillary nodes surrounding the breast is evaluated and the number and types of nodal involvement are noted and a staging is given for each patient.

Stage 1: The cancer is small and localised to the breast. No metastases are present in the tumour draining axillary nodes

Stage 2: The cancer has metastasised to the axilla but is not widely disseminated through the whole axillary tail.

Stage 3: The cancer is widely disseminated through the breast and has metastasised through the majority of the axillary nodes

Stage 4: There are widespread metastases to the bone and brain.

The 5 year survival of patients without nodal metastases varies between 67 to 85% and on nodal involvement this then drops significantly from 63 to 73% for one node involved. (Silverberg, 1975; Fisher, 1983). For patients with two or three nodes showing tumour involvement the prognosis for survival is not further decreased but, when many nodes are involved the survival rate of patients is greatly decreased.

1.1.6.2. Tumour size

The diameter of the tumour has been related to the presence of axillary node metastases and shown to be an important independent variable in long term patient survival (McGuire, 1986).

1.1.6.3. Oestrogen Receptor Status (ER)

The presence of oestrogen receptors in tumour cells is a well established predictor of clinical response to endocrine therapy. (Section 1.1.2.). Also a positive ER status has been correlated to the patient prognosis (Shek and Godolphin, 1988) and seems to have an inverse relationship to the EGFR status, with the lack of the ER leading to bad long term prognosis. ER status has been implied to be an independent prognostic indicator, and related to the degree and presence of tumour in the axilla (McCarty *et al* 1980). The degree of differentiation of the tumour measured by histological grading is related to

the presence of the ER protein (McCarty *et al* 1980) and it is likely that the biological significance may be due to this association.

1.1.6.4. Grade of the Tumour

The most common grading system for breast cancer was introduced by Bloom and Richardson (1957). The grading system looks at three features of the tumour: cytological differentiation; structural differentiation; mitotic figure frequency. To each of these figures a value of between 1 and three is given and points are then totalled. Points from 3 to 5 represent low grade (Grade 1). 6 to 7 are intermediate grade (Grade 2) and 8 to 9 represent high grade (Grade 3). Grade 1 tumours are less frequent than high grade tumours (Bloom and Richardson, 1957) and have a better prognosis.

1.1.6.5. Tumour Type

There are many types of breast cancer due to the heterogeneous nature of the tumour. The main difference is if the tumour is *in situ* (non invasive) within the ductal system or invasive and has spread beyond this area. The invasive tumours include invasive carcinoma, medullary carcinoma, invasive lobular, comedo and tubular carcinoma. The majority of non invasive tumours consist of carcinoma of ductal or lobular regions. These non invasive tumours have a much better prognosis.

1.1.6.6. Overview of prognostic indices

All of these factors are in current use for the evaluation and treatment of breast cancer patients. The use of a single parameter should not be taken as 100% reliable. Even the use of staging has drawbacks as up to 30% of all node

negative women will eventually relapse and die of the disease. Thus the absence of lymph node metastases does not ensure that primary tumour has not metastasised to other areas of the body (Redding *et al* 1983). The validity of the pathology has also been called into question as often only two contiguous slices of node are taken and the possibility of micrometastases outwith these slices is possible. Thus pathological evaluation of patients can be very prone to observer subjectivity (Gilchrist *et al* 1985). A study illustrates this point, as when the same tumour samples were sent to five different laboratories for grading, results on comparison showed 100% agreement in only 14.5% of the cases (Delides *et al* 1982). The assessment of patient pathology must be standardised more carefully as possible variation may mask important trends. However, even if better standardisation is achieved, multiple factors eventually will have to be assessed to give a better understanding of the disease due to the heterogeneity of the tumour.

1.1.7. Metastasis

Breast cancer will initially grow as carcinoma cells *in situ*, as a group of cells linked together and contained within the basement membrane. Abolition of the cell to cell contact will eventually allow individual tumour cells to invade surrounding tissues and become metastatic.

The metastasis of tumour cells is not one process but multistep interlinked steps eventually allowing the cancer to spread (Figure 1.4).

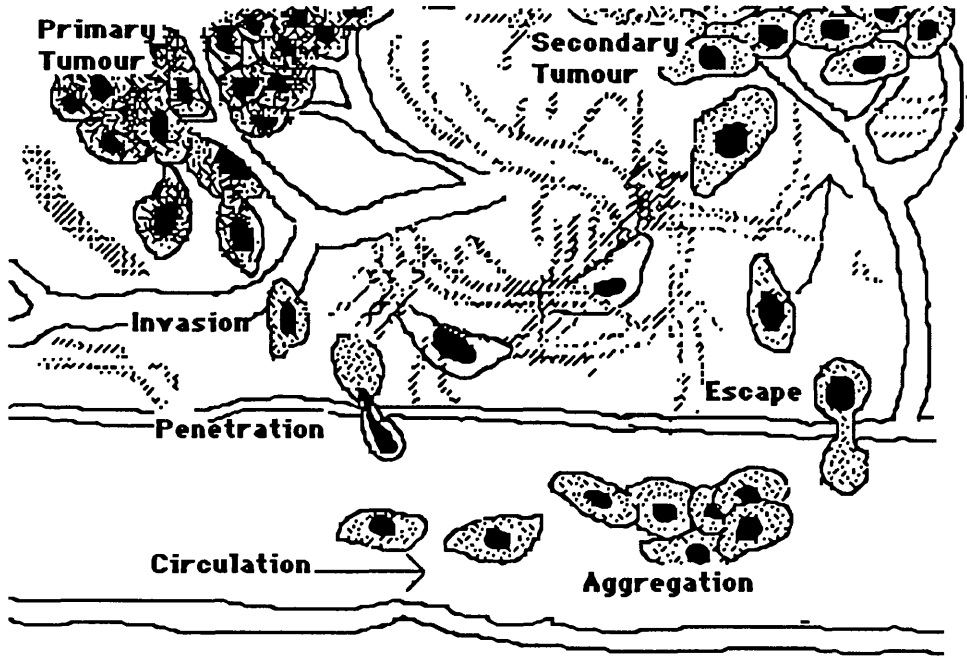


Figure 1.4 The metastatic process

There are a number of steps for metastasis to occur succesfully

(1) Release from the primary tumour accompanied by proteolytic digestion of the basement membrane and surrounding tissues

(2) Blood vessel invasion and entry into the circulation

(3) Homing to secondary sites

(4) Infiltration of the target tissues

Within primary cancer patients there is a great heterogeneity of disease with the majority of tumour localised to the breast and this type of cancer can be cured by surgery. However in some patients metastases are widespread and often incurable by surgery.

1.1.7.1. Generation of metastatic potential

Cancer is believed to be clonal, i.e derived from one transformed cell. The genetic changes initiated within the growing cancer are often unstable and, especially since cancer cells are fast growing, the DNA will be in an open conformation which is more susceptible to damage. Thus the tumour will evolve due to environmental pressures, especially those from the immune system, allowing more malignant cells to emerge from the heterogeneous population (Figure 1.5)

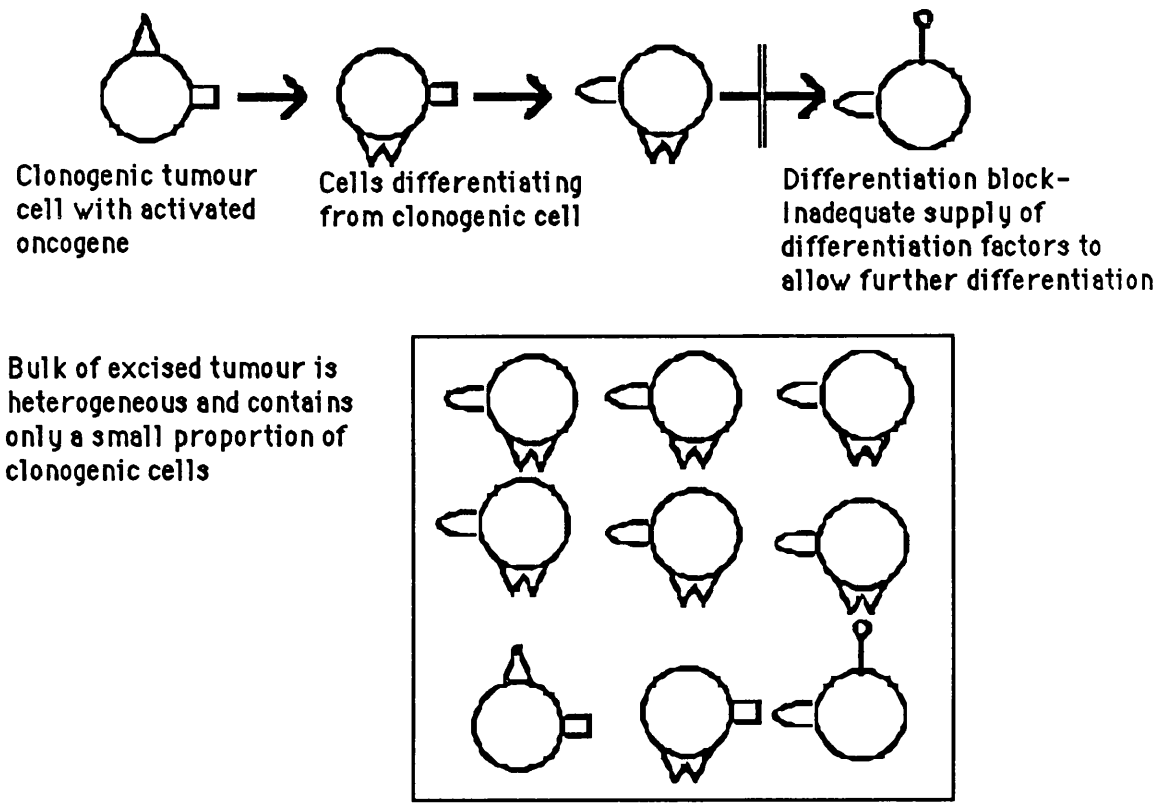


Figure 1.5 Development of tumour cell heterogeneity.

and eventually the tumour will become autonomous and escape from the host control mechanisms. Tumour heterogeneity was first noted in histology and

now has been extended to genetic, biochemical and immunologic properties. The great degree of tumour heterogeneity dampened the enthusiasm for antibody therapy as one monoclonal will in theory only recognise a small fraction of the total tumour cell population. Within this evolving tumour cell environment a subpopulation is likely to emerge and dominate. Alam *et al* (1992) investigated the change in DNA index from the primary to the metastatic tumour found within the axillary tumour draining lymph nodes. A subpopulation with a specific DNA index was shown to have metastasised to the lymph nodes, which would indicate that different subpopulations of tumour cells have differing abilities for metastasis. This has also been shown in animal models; clones derived from one tumour possess differing metastatic ability when compared to clones from other tumours (Fidler and Kripke 1977). Steeg *et al* (1988) have now isolated a gene, NM23, overexpression of which indicates good patient prognosis and low metastatic capability.

1.1.7.2. Angiogenesis

Tumours up to a certain size (usually a few millimeters) have an adequate blood supply . Greater than this size will require new capillary blood vessels and a micro circulation within the tumour. The induction of a tumour blood supply is called angiogenesis and requires a complex interplay of growth factors from the tumour and the surrounding normal tissues. The non tumourogenic cells have been reported to produce both angiogenic factors and inhibitors of angiogenesis (Rastinejad *et al* 1989). Loss of the inhibitor of angiogenesis due to either mutation or suppression can be seen in the increase in both angiogenesis and tumorigenicity. Weidner *et al* (1991) have implicated increased microvessel density surrounding the breast tumour to an increase in numbers of metastases and a decrease in patient survival. A similar relationship has also been reported for human melanomas. (Srivastava *et al* 1988).

1.1.7.3. Proteolytic enzymes and their function in metastasis

During the metastatic process the tumour cell must pass through physical barriers, including membranes and extracellular matrices, which are composed of collagen, proteoglycans and other glycoproteins. To accomplish this, proteolytic enzymes must be produced by the metastatic tumour cells. It has been shown that the production of these enzymes is one of the initial changes during neoplastic transformation (Duffy, 1987). Thus various proteases which include cathepsin D, urokinase (plasminogen activator) have been studied as indicators of metastatic potential. Recent reports have implicated their secretion in metastatic spread in breast cancer. However a major problem with this measurement is that it is often difficult to distinguish between tumour associated enzymic activities and enzymes released from contaminating normal cells.

1.1.7.4. Evasion of host immune responses

As a tumour develops and metastasises, there will be a constant interaction with the immune system taking place within the tumour in the form of tumour infiltrating lymphocytes (TIL), in the blood and also in the tumour draining axillary lymph nodes. The development of the tumour in the first place would suggest that the immune response is ineffective in controlling tumour growth. For an effective immune response the MHC class I molecule which is found normally on all nucleated cells in the body will mediate a cytotoxic T cell response against the tumour (Section 1.2.3.4). This will be in the form of a peptide in the groove of the MHC molecule that will allow the immune system to distinguish between 'self' and 'non self' proteins. The MHC class II

molecules would also be involved in the immune response (Section 1.2.3.5.), but unlike the MHC class I molecules they are expressed only on specialised antigen presenting cells such as B cells or macrophages.

In breast tumour cells both MHC class I and II molecules are presented (Whitford *et al* 1992a; Goepel *et al* 1991) though not all cells in the tumour express them. When MHC expression on primary breast cancers is compared to the situation on the corresponding metastases, the expression of MHC class I molecules is even further reduced or eliminated. The lack of MHC expression in tumours has been linked to failure to express the β-2 microglobulin component and this interferes with the associated peptide generation mechanisms. The removal of class I expression will allow the tumour cells to escape CD8+ cytotoxic T cell restricted killing and thereby allow the tumour to move through immune tissues without generating an immune response.

1.1.7.5. Expression of adhesion receptors and metastases

Cell adhesion molecules are found on the surface of all cells and bind to ligands on other cells or in the extracellular matrix. Cell adhesion is an important part of immunological recognition and re-circulation (Reviewed by Dustin and Springer, 1991). Several adhesion molecules associated with lymphocytes have now been described (Section 1.2.7.). Three of these adhesion molecules have been associated with metastases (Johnson *et al* 1989; Fawcett and Harris 1992). LFA-1(lymphocyte function antigen-1), ICAM-1 (Intracellular adhesion molecule-1) and CD44.

LFA-1 is expressed only on leucocytes (Krensky *et al* 1983), while its counter receptor ICAM-1 is expressed on a variety of cell types (Dustin *et al* 1988) including tumour cells but in widely differing amounts.

ICAM-1 is expressed at very low levels on normal tissues except endothelial cells where rather moderate levels are found. ICAM-1 is upregulated on initiation of a local immune response. Johnson *et al* (1989) indicated that a glycoprotein with the same sequence as ICAM-1 was expressed on advanced human melanomas, but absent on benign and early melanomas. The suggestion is that the expression of ICAM-1 will cause tumour cells to interact with lymphocytes causing a reduction in the adhesion of the tumour. Which would implicate the lymphocytic infiltration within the tumour as having a role in the metastatic spread of tumour.

The CD44 glycoprotein is widely expressed on a variety of different tissues and is thought to function as an adhesion molecule (reviewed by Underhill 1992, Herrlich *et al* 1993). The CD44 molecule has specifically been implicated in lymphocyte homing at the high endothelial venule (HEV) within the lymph nodes. The protein is normally present as numerous isoforms created by 'mRNA alternative splicing', a mechanism allowing cells to exclude or include specific segments of mRNA in the final transcript. It has been calculated that different splicing of the CD44 transcript could give rise to 1000 different isoforms of the protein. This may indicate a functional role rather than a structural role for the CD44 glycoprotein (Screaton *et al* 1992; Screaton *et al* 1993).

A variant of CD44 has been implicated in metastatic ability of tumour cells. Gunthert *et al* (1991) raised a monoclonal antibody (Mab) against metastatic cells and discovered a variant of CD44 which was expressed only in

metastatic rat cell lines. Hoffman *et al* (1991) showed splice variants were expressed in human cancer cell lines. Arch *et al* (1992) showed that one variant (V6) was transiently expressed on B cells, T cells and macrophages after antigenic stimulation, with anti-CD44 antibodies inhibiting lymphocytic activation. The expression of this CD44 variant has been related to metastatic capability in breast cancer (Tarin and Matsumura 1992) and colorectal carcinomas (Tanabe *et al* 1993). However this is disputed (Jackson *et al* 1993).

Metastasis is multifactorial and many factors will be necessary before the tumour spreads through the body eventually to settle and grow in tissues other than the breast. To date only a small number of the relevant contributing factors have been described and much more work will be necessary before this extremely complicated process is fully evaluated.

1.2. The Immune response

The immune system is a defense mechanism which has evolved to protect against pathogenic organisms and possibly even cancer. A great amount of diversity can be generated by the wide variety of cells, with the capability of recognising and eliminating any foreign antigen. This immune response can be divided into two areas, recognition and response. With the body discriminating between 'self' (body protein) and 'non self' (foreign protein), eventually a response is elicited against the specific antigen.

1.2.1. Lymphocytes

An immune response can be elicited by either the T cell or B cell subsets in conjunction with other lymphoid cells. The T cell will recognise mainly endogenous antigen whilst the B cell recognises cell surface or soluble antigen.

1.2.1.1. B lymphocytes

B lymphocytes mature in the bone marrow and express a unique antigen recognition molecule on the surface called an antibody, which is a membrane bound glycoprotein. After encountering cell surface or soluble antigen the cell begins to divide rapidly and differentiates into memory B cells and plasma cells to produce large quantities of soluble antibody.

1.2.1.2. T lymphocytes

T lymphocytes also arise from haematopoetic stem cell precursors in the bone marrow. T cells migrate to the thymus for maturation and during this migrationthe receptor known as the T cell receptor (TCR) is expressed. This receptor has a function similar to the antibody produced by the B cell. But the antigen recognition is mediated in conjunction with a major histocompatibility complex (MHC) molecule (Section 1.2.3.3).

1.2.2. Lymphocyte phenotypic markers

Proteins on the surface of lymphocytes can be used to define the different subsets of lymphocytes which can then be detected by monoclonal antibody. The method of monoclonal antibody (Mab) production by Kohler and Milstein (1975) led the way for specific recognition of an epitope.

Monoclonal antibodies could thus be used to type lymphocytes and other cells by recognition of specific protein expression.

Prior to Mab's, studies involving lymphocytes were crude, involving the use of sheep erythrocytes to form non antigenic rosettes for purification and estimation of the T cell population. The use of Mab's along with flow cytometry has significantly simplified lymphocyte study.

Lymphocytes express a great number of proteins on their surface and in certain cases unique proteins are found for a specific cell type. Mabs have been generated against these leucocytic phenotypic proteins. A system has been developed to reduce confusion over the many Mabs generated. This is called the CD (cluster of differentiation) system for identification of human cells of a haematopoietic lineage by Mabs. The CD designation is decided by regular international workshops. During these meetings, Mabs are compared and those with the same epitope are assigned a "cluster" and a number. At the 4th international workshop (1989), the number of defined antigens was increased to CD78.

1.2.2.1. T cell phenotypic markers

The definitive marker of the T cell is the expression of the CD3 protein (Figure 1.11.) which is expressed only on T cells in conjunction with the TCR through which antigen recognition takes place. There are two TCR the α/β (Section 1.2.4.1) and the less common γ/δ (Section 1.2.4.2) which are stably associated with the CD3 protein. The CD3 protein is a complex of five proteins associated with T cell signal transduction from the TCR. The T cell population can be further divided into two main subsets based on phenotypic markers. The helper (Th) subset which expresses the CD4 protein and the T

supressor/cytotoxic (Tc) subset which is CD8+. The CD4+ T cell recognises antigen in conjunction with the MHC class II molecule (Section 1.2.3.5.) and produces growth factors called cytokines to 'help' both the B and T cell immune responses. The CD8+ T cell recognises antigen with the MHC class I molecule (Section 1.2.3.4.) and functions, as the name suggests, to mediate cell killing.

The CD4+ T cell population can be further subdivided based on its secretion pattern of growth factors, known as cytokines, and the nature of the lymphocytic interaction. The CD4 Th cells have been subdivided into Th-1 and Th-2. The Th-1 subset 'helps' the T cell response by secreting IL-2 and INF-γ. Th-2 subset 'helps' the B cell response by secretion of growth factors and involvement in isotype switching of antibody class (Mossman and Coffman, 1989).

1.2.2.2. B cell phenotypic markers

B cells have two main functions: that of antigen presenting cells to the CD4+ T cell subset presenting the MHC class II molecule on their surface (Section 1.2.3.5) and also as mediators of the humoral antibody response against cell surface or soluble antigen (Section 1.2.1.1.). The main phenotypic markers of B cells are the CD19, CD20 and CD22 proteins.

1.2.3. Antigen processing and presentation

The recognition of antigen occurs by two main mechanisms involving the T and B cell compartments of the immune response. B cells are involved in the recognition of soluble or cell surface antigen whereas the T cells recognise

mainly endogenous processed antigen in the form of peptides presented on MHC (Section 1.2.3.3.).

1.2.3.1. Antigen presenting cells

Antigen must be in a suitable form for recognition by either B or T cells. Specialist organelles and antigen presenting cells have evolved this function. The main antigen presenting cells include macrophages, dendritic cells and B cells. These cells endocytose the antigen which is then processed within the endosome to form peptides. These peptides combine with the groove of the MHC class II molecule (Section 1.2.3.6.2) and move to the surface of the antigen presenting cell. T helper cells then recognise the peptide and the MHC molecule presented. If the peptide is foreign the T helper cell will become activated and release soluble growth factors known as cytokines which expand the response against the antigen to bring in both the T cytotoxic and B cell response.

1.2.3.2. Organs of antigen presentation

The immune system has specialised organs involved in the presentation of antigen to lymphocytes. These organs provide suitable environments for lymphocyte maturation. Important structures are the lymph nodes,which are situated throughout the lymphatic system. Their primary function is to drain lymph fluid from defined tissues and vascular spaces. They also act as a trap for antigen draining into the lymph. Within each node are specialised areas for B or T cell activation. The B cells mature in the germinal centres before entering the general circulation and the T cells mature in the paracortex. In order for lymphocytes to enter the lymph node they must adhere to and pass

between the endothelial cells lining the blood vessels. This most readily occurs in the regions called the high endothelial venules (HEV) and lymphocytes possess specialised adhesion molecules for this purpose. (Figure 1.6)

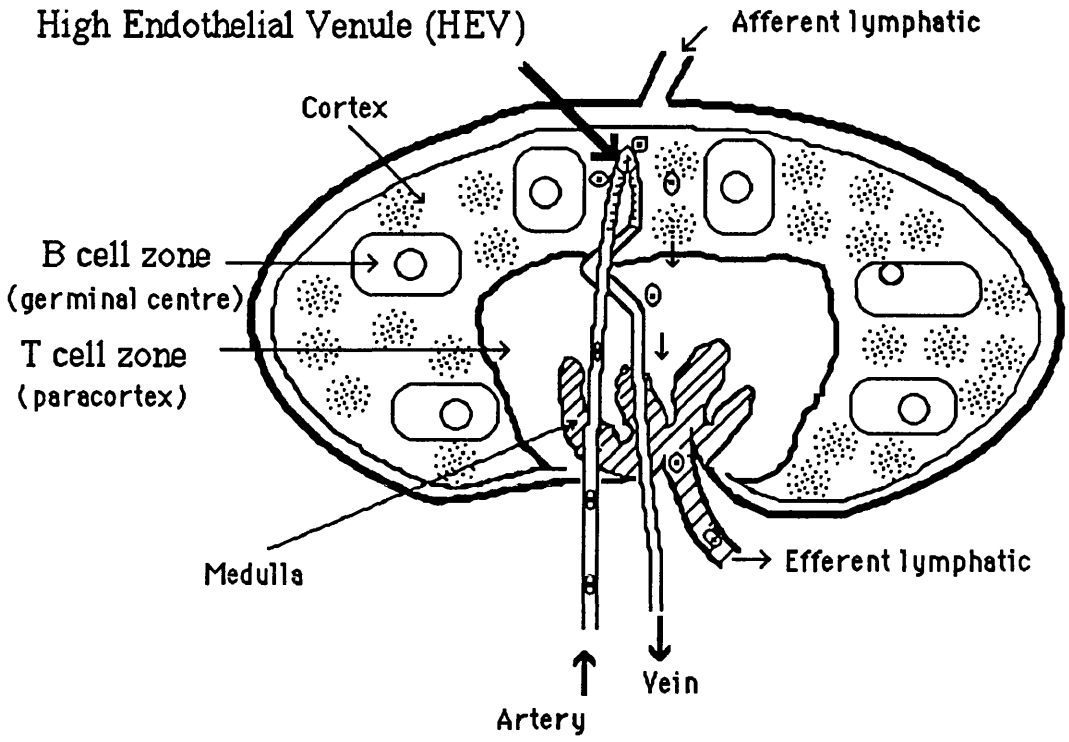


Figure 1.6. The anatomy of a lymph node

B cells are found primarily in the germinal centres, where follicular dendritic cells present the antigen. Also present are T helper cells which on B cell activation release cytokines to supplement the antigen response. Similar dendritic cells are found in the paracortex, the T cell area. Here the dendritic cells present antigen via MHC class II molecules for T helper recognition (Section 1.2.3.5).

1.2.3.3. Presentation of antigen via the MHC molecules

During the 1960s it became clear that T cell responses were restricted to allelic forms of a set of highly polymorphic glycoproteins called the Major Histo Compatibility complex (MHC). Only in the 1980's was the nature of the interaction of the T cell response with the MHC elucidated. It was found that T cells contained an α/β receptor (Section 1.2.4.) which had specificity for both the MHC and the antigen it was presenting.

T cells recognise peptides processed from antigen and presented in the groove with either MHC class I or class II (Bjorkman *et al* 1987). The MHC itself must be autologous, otherwise it will be recognised as being foreign by the cell. The processing mechanisms are different for class I and II MHC. The CD8 and CD4 proteins have a crucial role in this process in that they interact directly with the MHC (Figure 1.7). CD4 interacts with class II and CD8 interacts with the α3 domain of class I.

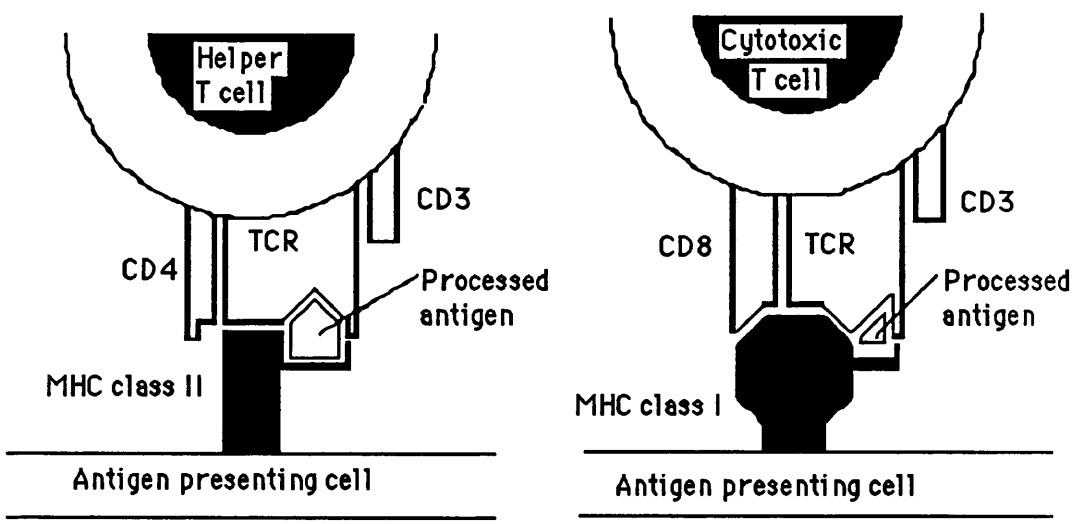


Figure 1.7 Antigen recognition by CD4+ helper and CD8+ cytotoxic T lymphocytes.

1.2.3.4. Class I MHC antigen presentation

MHC class I molecules are present on all nucleated cells (Daar *et al* 1984) and are involved in presentation of endogenous antigen such as those derived from viruses. A peptide derived from such an endogenous protein is formed and eventually associates with the MHC molecule. Evidence suggests that the peptide charging of the MHC class I molecule occurs in the endoplasmic reticulum (ER). This association will allow stabilisation of the β-2 microglobulin with the MHC molecule allowing entry into the secretory pathway. In the absence of peptide binding the class I heavy chain (1α) and the β-2 microglobulin will undergo degradation in the ER.

1.2.3.5. Class II MHC antigen presentation

The method of presentation of class I and class II antigen is similar, in that antigen is presented in the form of a peptide fragment. However the MHC class II molecule uses specialist antigen presentation cells with an entirely different method of peptide generation and presentation.

The MHC class IIα and IIβ assemble in the ER along with the invariant chain (Ii), forming a trimeric complex. The presence of Ii prevents peptide binding and it is not until the eventual loss of the Ii and the gain of a peptide that the IIα -IIβ complex transits to the cell surface for TCR recognition.

1.2.3.6. Structure of the MHC molecules

The structure of the antigen presentation molecule, the MHC, is crucial to the immune response elicited. As the MHC class I and II molecules have

similar structures, great differences occur in the groove involved in the presentation of peptide antigen.

1.2.3.6.1. Class I

The class I molecule is membrane bound consisting of a heavy chain of 46Kd and a soluble subunit of β-2 microglobulin (Figure 1.8).

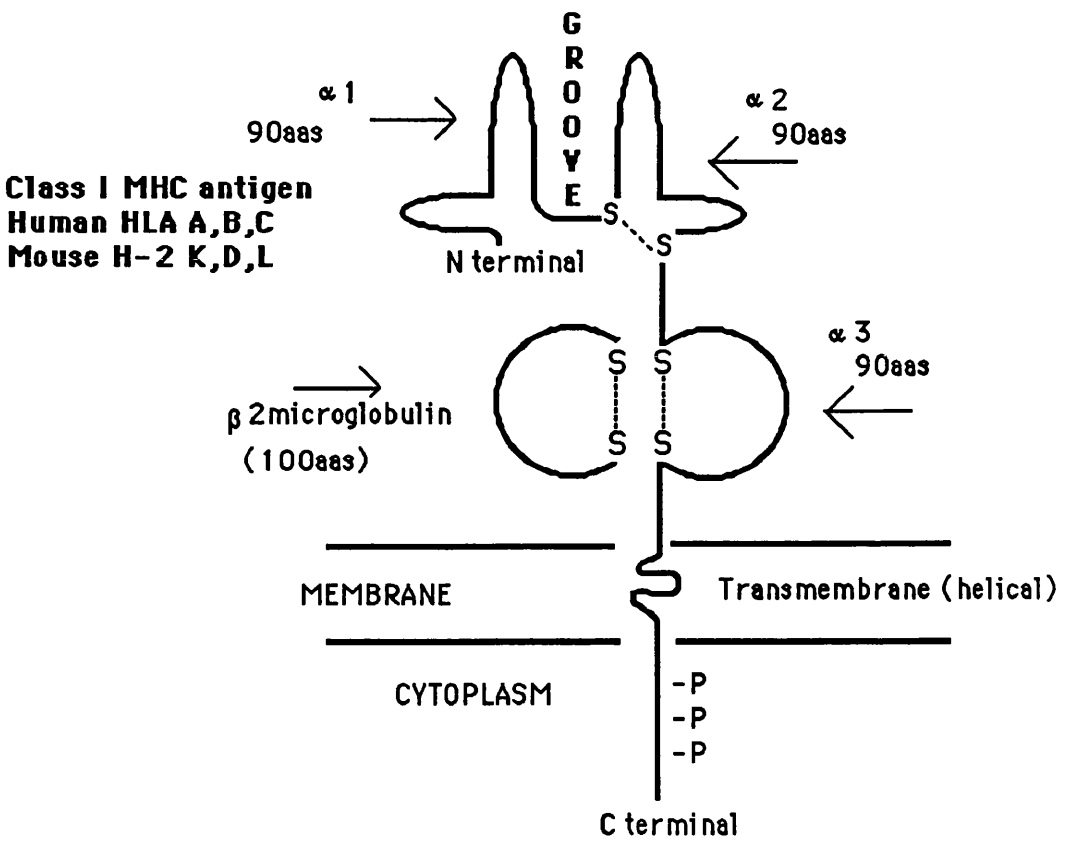


Figure 1.8.. The structure of class I MHC

The heavy chain consists of two main areas, a membrane domain and a peptide binding domain. Crystallography of the peptide binding domain has

shown that the α1α2 domain unit forms the peptide binding site, supported by a β pleated sheet making up the floor, (Bjorkman *et al* 1987) (Figure 1.9).

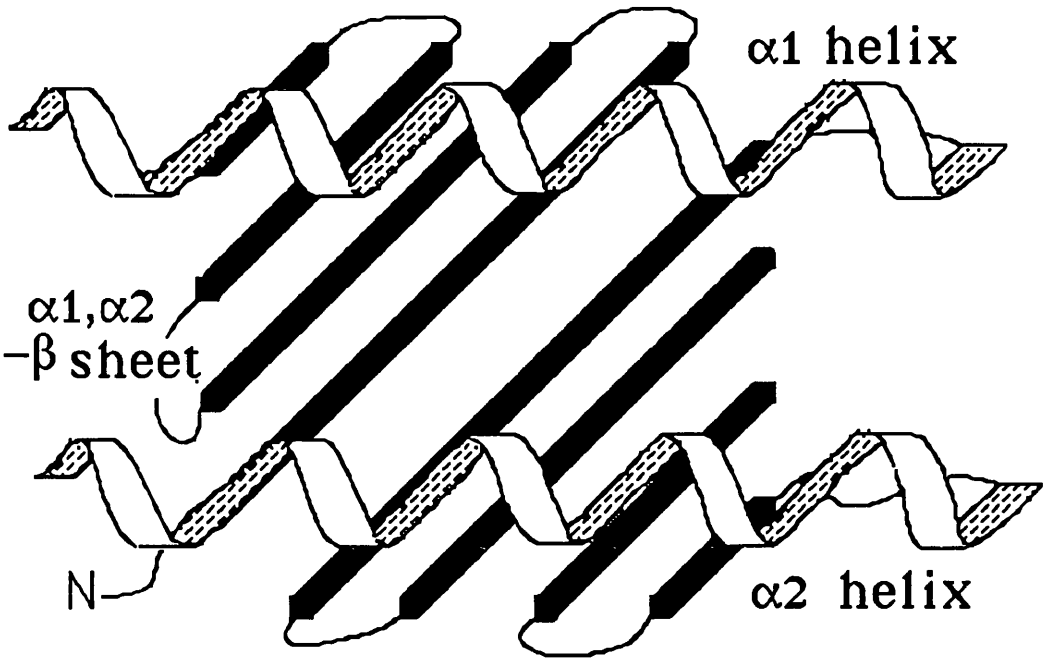


Figure 1.9.. Overhead view of the protein groove involved in the presentation of peptides by an HLA A2 molecule.

Surrounding this are found two α helices, one each from α1 and α2. The β–2 microglobulin contacts the β-floor but not the peptide binding domain. A number of pockets have been identified for peptide binding and have been given the designation A through to F.

1.2.3.6.2. Class II

Class II molecules are heterodimeric membrane proteins with each protein consisting of an α subunit and a β subunit (Figure 1.10.). Like the class 1 heavy chain, the α and β chains each consist of two regions: an extracellular hydrophilic region, a transmembrane hydrophobic region and an

intracellular hydrophilic region. The crystal structure of HLA DR1 has recently been elucidated by Brown *et al* (1993). There is similarity with the MHC class I molecule with α chains having close homology with the corresponding α1 and β-2 microglobulin of the MHC class I molecule. The peptide binding groove floor consists of 8 strands of anti parallel β pleated sheets open at both ends allowing peptides of between 12-24 amino acids to bind. The crystal structure indicates possible dimerisation of the MHC class II receptor and this may be important in stabilisation of the CD4+ TCR recognition with an antigen presenting cell.

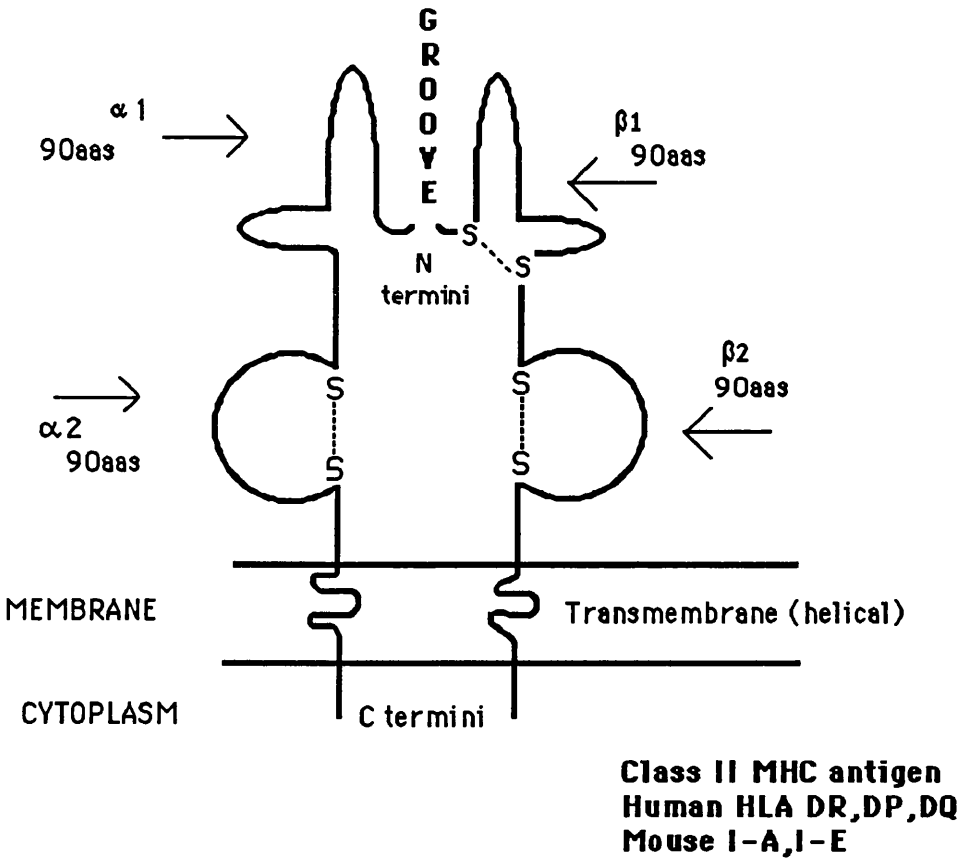


Figure 1.10. The structure of the MHC class II molecule.

The processed peptides are presented in the groove and the CD4 T cell will simultaneously recognise the MHC class II molecule and the peptide within the groove.

1.2.4 Recognition of antigen by T cells

After the discovery of the mechanism for antigen recognition of B cells utilising a specific receptor which undergoes gene rearrangement to generate diversity, it was thought that T cells would possess a receptor with a similar rearrangement for antigen recognition. Initial studies involved examining cDNA generated from T cells and, firstly, identifying genes able to somatically rearrange within the T cell population and then, secondly, identification of the protein and its structure (Hedrick *et al* 1984a). A β chain was discovered and shortly afterwards a γ and an α chain were found. The α and β were found to associate and form an immunoglobulin type receptor. Later a δ chain was found and shown to associate with the γ chain. This γ/δ protein was identified and shown to have a receptor structure similar to the α/β TCR and was also designated a TCR (the γ/δ receptor is distinct from the γ and δ subunits of the CD3 component of the T cell receptor).

1.2.4.1. α/β T cell receptor

The α/β TCR is a disulphide linked heterodimeric transmembrane protein with considerable homology with IgG. It is composed of both α and β chains, each containing two main domains, a variable amino acid sequence (V region) found at the N terminus and a constant amino acid sequence found at the carboxy terminus (C region). These domains interact with each other to form the area of recognition (Hedrick *et al* 1984b).

The β chain locus was mapped to chromosome 7 in humans (Figure 1.13.) and chromosome 6 in mice. Genomic sequences for the α locus are found on chromosome 14 in both mice and humans. The genes are formed from separate genetic elements: α from the V region and J region; β from the V the Diversity (D) regions and the J regions. The rearrangement of these genes gives a substantial potential for diversity (Geliebter *et al* 1986; Vega *et al* 1985; Seeman *et al* 1986).

The α/β TCR recognises peptides presented in conjunction with the groove of the MHC (Section 1.2.3.3.). In most cases both the α and β chain are involved in recognition of the peptide/MHC complex. The expression of CD3 is stably associated with the TCR and the expression of CD4 with the CD3 complex results in a MHC class II mediated antigen recognition. CD8 with the CD3 complex elicits a MHC class I recognition.

The association of the TCR with the CD3 complex is a crucial part of the recognition mechanism. The CD3 complex has been shown to be responsible for the transduction of signals to the nucleus and consists of five proteins (Figure 1.11.).

In humans, the Vα genomic sequence of the gene segments is estimated to contain approximately 100 members and the Vβ pool approximately 70 members which can be assembled with D, J and C regions. This leads to a very considerable potential for diversity in the TCR.

The study of autoimmunity (Zamvil 1985; Oksenberg *et al* 1990; Oksenberg *et al* 1993) and allograft rejection (Miceli and Finn, 1989) has indicated a very limited usage of the TCR genes. This restricted usage has been

implied in other disease states, such as multiple sclerosis (Oksenberg *et al* 1990; Oksenberg *et al* 1993).

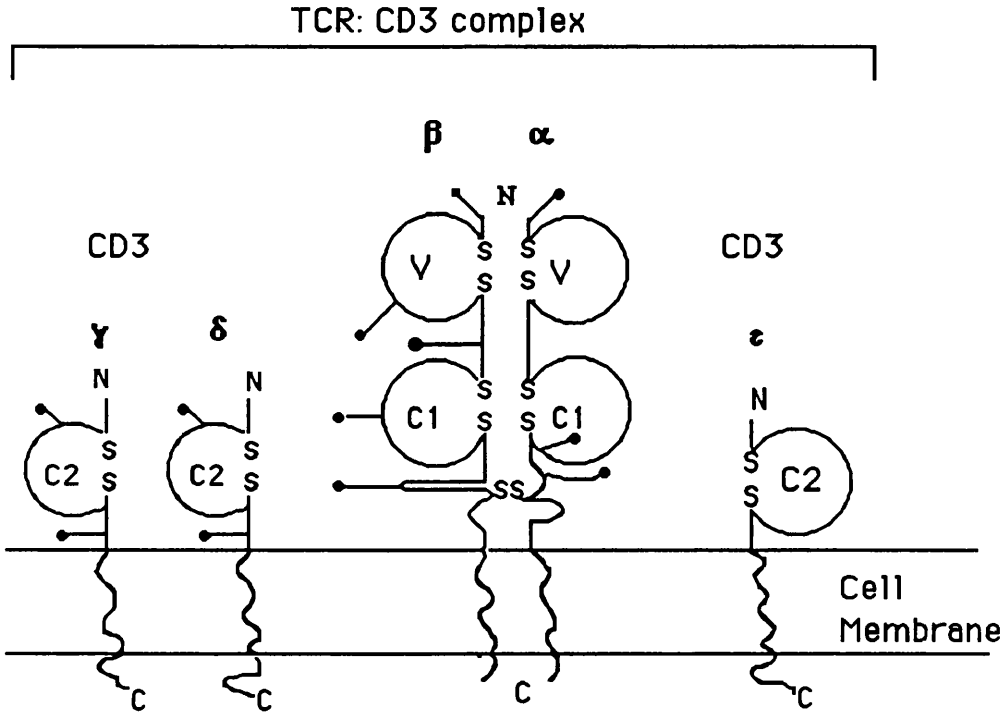


Figure 1.11. The TCR:CD3 complex of T lymphocytes.

The circles signify domains; V and C domains show homology with Id V and Ig C domains respectively. Presumed N-linked carbohydrate sites are shown by symbol ——●. A sixth member of the complex, CD ζ is not illustrated.

However, in contrast to the limited usage of the TCR found in other diseases, cancer stimulates oligoclonality on the TILs found within tumours. (Belldegrun *et al* 1989). This oligoclonality is restricted when TIL are grown in IL-2 culture leading eventually to a dominant Vβ expression (Karpati *et al* 1991).This oligoclonality for TIL is found also in eye and breast cancers (Durie *et al* 1992).

The term superantigen was first proposed by White *et al* (1989) describing novel T cell stimulation (Figure 1.12.). The superantigens described, expanded specific T cell subsets instead of the more usual polyclonal stimulation noted for mitogens such as concanavalin A. The specific superantigen stimulus binds a variable portion in the β chain of the TCR to activate a specific V subset of T cells. Two main types of superantigen are known, the bacterial toxins such as Staphylococcal

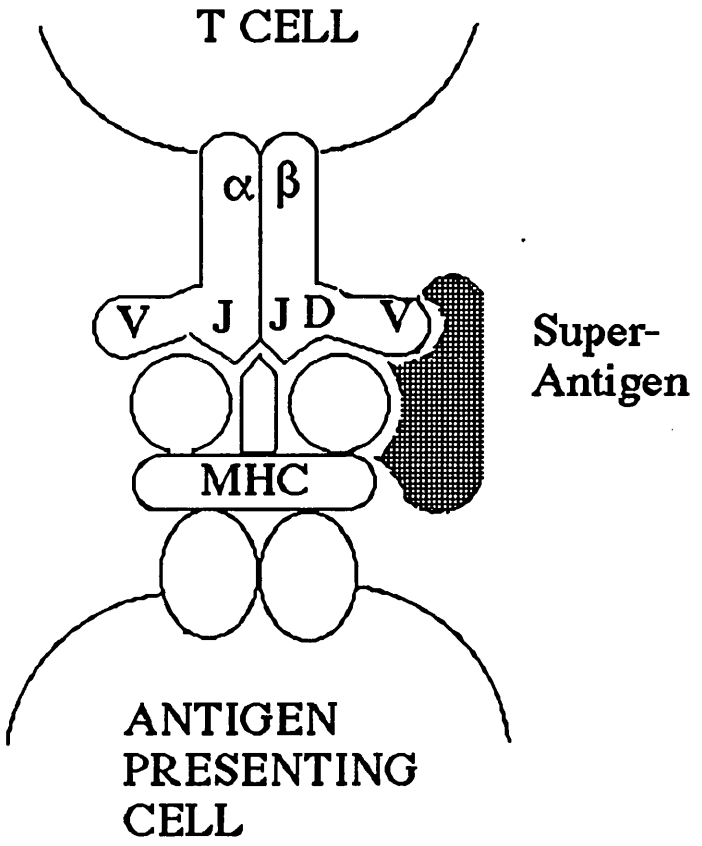


Figure 1.12. Interaction of a superantigen with the α/β TCR.

enterotoxin A (Acha-Orbea and Palmer, 1991) and the products of the mouse mammary tumour virus (MMTV).

In lymph nodes draining the tumours of pancreatic cancer patients, T cells tend to express the α/β rather than the γ/δ TCR. It has also been

postulated that the mucin polypeptide core may be acting as a superantigen, (Jerome *et al* 1991).

1.2.4.2. γ/δ T cell receptor

The second TCR to be discovered was the γ/δ. Like the α/β TCR it is a heterodimeric immunoglobulin type receptor. The γ chain locus was mapped to the short arm of chromosome 7 in humans (Figure 1.13) and on chromosome 13 in mice, while the δ locus is unusual in that it lies within the α genomic region in both species. The great diversity shown by the receptor is due to the significant junctional diversity in the γ (Jones *et al* 1986) and particularly the δ V domains. This suggests that the γ/δ TCR is as diverse as the α/β receptor (Chien *et al* 1987; Elliot *et al* 1988)

Murine γ/δ T cells can be divided into four main subsets of γ/δ T cells according to the usage of V_γ segments V5, V6, V4, and V7. Each subset localises in a specific area, which would suggest specific adhesion molecules are involved in targeting these subsets (Section 1.2.7.). Two subsets localise in the epithelia, V5 in the epidermis and V6 in the mucosa surrounding the uterus, vagina and tongue (Asarnow *et al* 1988; Itohara *et al* 1990). These γ/δ T cells originate from the thymus (Itohara *et al* 1989; Lafaille *et al* 1989) and seem to have no role in the lymph nodes. However other γ/δ T cells (V7) originate outwith the thymus and localise in the intestine (Bandeira *et al* 1991).

In humans there are two main subsets of γ/δ T cell Vδ1 and Vδ2 predominant in the post natal thymus (Casorati *et al* 1989). Most Vδ2. express CD45RO, a probable marker for memory cells, wheras the Vγ1 CD45 cells are

CD45RO negative (Hayward *et al* 1989). Unlike mice there is no dominant selection for epithelial areas of the body (Groh *et al* 1989; Bucy *et al* 1989).

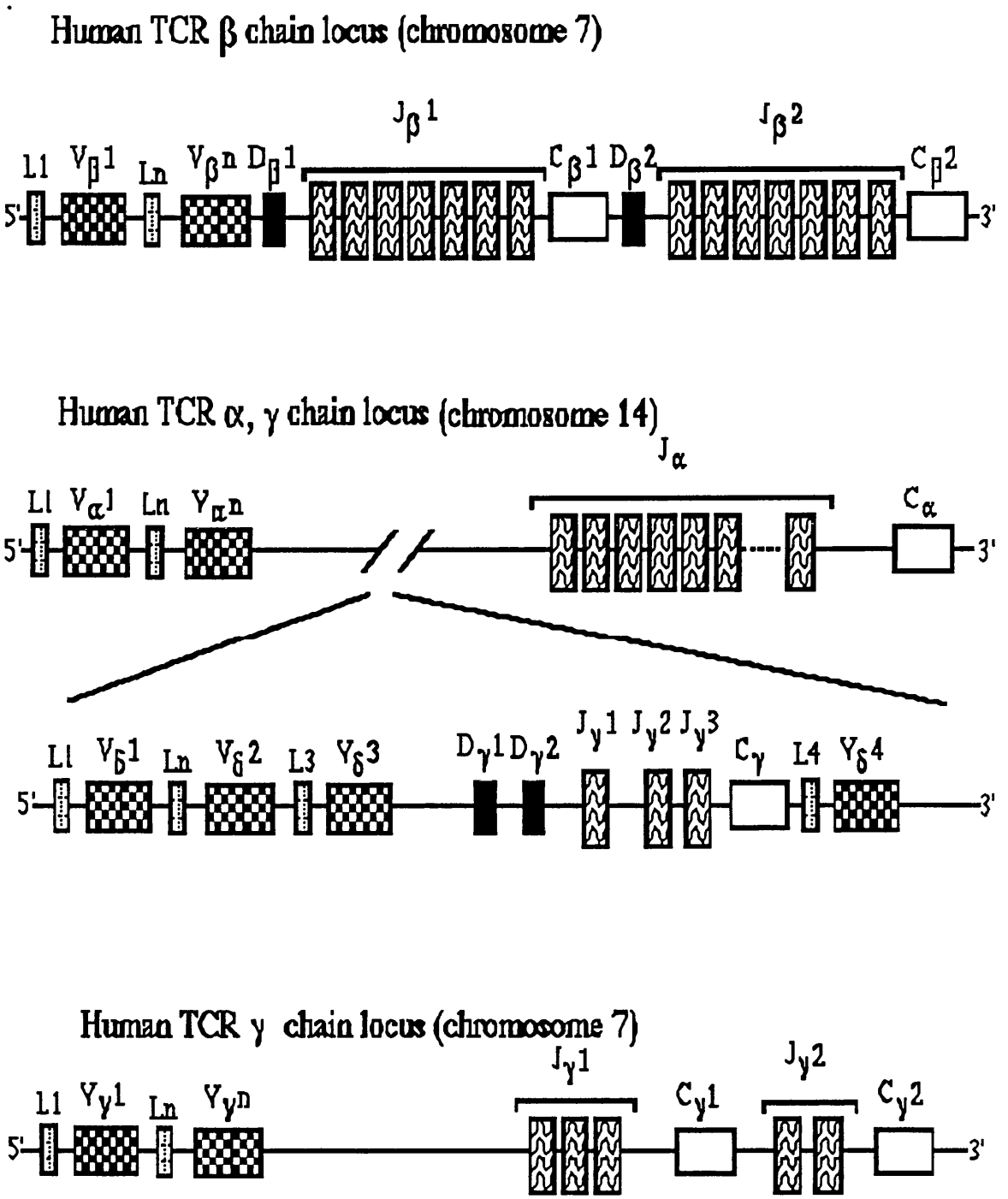


Figure 1.13. The human genomic location of the T cell receptors α/β and γ/δ

The majority of γ/δ T cells in both mice and human do not appear to recognise classical MHC molecules. The few that have classical recognition have shown specific recognition for both class I and class II antigens (Matis *et al* 1989; Rellahan 1991), with cross reactivity for different alleles (Ciccone *et al* 1989; Spits *et al* 1990).

Along with the MHC presentation, a peptide is presented in the groove for TCR γ/δ recognition (Section 1.2.3.3.). Recently it has been shown that a peptide presented on HLA DR was derived from tetanus toxins was able to be recognised by γ/δ T cells (Holoshitz *et al* 1992). However this recognition of MHC is only found in a small subset which would suggest that this type of cell does not have the same bias for MHC molecules.

Instead of recognition of MHC it is possible that these cells may recognise other antigen presenting proteins on the surface of cells. A molecule implicated in antigen recognition is CD48 (T cell target antigen), a member of the immunoglobulin superfamily (Mami-Chouaib *et al* 1990) since direct involvement of the molecule is seen in a mumber of cases in the recognition of γ/δ T cells. It seems that the γ/δ cells can recognise both MHC and non MHC molecules which would give a great variation in function as the location in different areas of the body would suggest and possible involvement of differing classes of antigen presenting cells.

γ/δ T cells have been implicated in a number of disease states such as rheumatoid arthritis and leprosy. Synovial fluid of rheumatoid arthritis patients and leprosy patients have been shown to react against heat shock proteins, with th Vδ1 and Vδ2 subsets of the γ/δ TCR expanded in the synovial fluid from rheumatoid arthritis patients (Modlin *et al* 1989; Holoshitz *et al* 1989). Elevated

levels have also been observed in coeliac disease (Raulet 1989; Spencer and Isaacson 1989).

In humans the considerable junctional diversity in Vγ9 and Vδ2 would indicate possible superantigen involvement. When superantigen stimulation of both γ/δ and α/β T cells was studied using Staphylococcal enterotoxin A (SEA) (Flesher *et al* 1991; Abe *et al* 1991). It was discovered that SEA coated cells were lysed by α/β and γ/δ T cells (Vγ9 γ/δ subset). However, unlike α/β cells, γ/δ cells do not proliferate *in vitro* in this response to SEA. The role of superantigens and their interactions with presenting molecules and the γ/δ receptor have yet to be fully elucidated.

γ/δ T cells have similar surface protein marker expression to α/β T cells and to date only the T19 protein on sheep lymphocytes is unique to γ/δ T cells. The CD3 protein has been shown to be stably associated with the γ/δ, (Goodman and Lefrancois 1988; Janeway 1988).

γ/δ T cells due to their diverse nature could have a role in the immune response to tumours, since γ/δ cells have been isolated from different human cancers. In Burkitts lymphoma γ/δ T cells have shown specific lysis of autologous tumour (Wright *et al* 1989), the interaction involving γ/δ TCR and surface IgG of the tumour. γ/δ expressing TILs have also been isolated from a variety of tumours. TILs isolated from lung tumours have shown lysis of autologous tumour. This cytotoxicity could be inhibited by an anti MHC class I antibody (Zocchi *et al* 1990), implying class I restricted antigen recognition. However no other TILs isolated have since shown cytotoxicity against autologous tumour (Nanno *et al* 1992) and to date the role of γ/δ T cells in immunosurveillance of cancer has not been proven.

The question of the role of the γ/δ T cell receptor remains as yet unanswered. The receptor has, in theory, more possible combinations than either α/β or even IgG molecules, and this could be useful in the diversity of recognition and distribution shown by γ/δ T cells. Infiltrates of γ/δ expressing cells found in many disease states have implied possible functions but to date the role of the γ/δ T cell receptor still open for debate.

1.2.5. T cell activation

The activation of the T cell results from the TCR peptide/ MHC complex interaction, transducing a signal across the plasma membrane eventually allowing resting T cells to move from G0 to G1 phase of the cell cycle.

The biochemical events of T cell activation have been studied (Abbas *et al* 1991) using a T cell line (Jurkat) which has been stimulated with mitogen (Concanavalin A). Within 15 minutes transcription of cellular oncogenes such as c-*fos* and by 30 minutes c-*myc* can be detected. The c-*fos* binds to the IL-2 gene enhancer and by 45 minutes IL-2 is secreted, with the receptor upregulated within 2 hrs. Later signals include the MHC class II molecule HLA DR 3-5 days later.

1.2.5.1. HLA DR

HLA DR like other MHC class II molecules has a heterodimeric structure of four glycoprotein domains (Brown *et al* 1988; Brown *et al* 1993). Antigens are presented to CD4+ T cells (Guillet *et al* 1986) via the groove in the structure of this molecule (1.2.3.6.2.). Expression of this molecule is restricted to certain specialised antigen presentation cells including both B cells and macrophages (Hammerling 1976). Apart from this role in antigen

presentation, HLA DR is expressed on antigen stimulated T cells 3-5 days after initial stimulation. (Table 1.1) but is absent on resting T cells (Reinhertz *et al* 1979). The exact role of this molecule is unknown, but may be part of a CD8/CD4 interaction.

1.2.5.2. IL2-R

The IL-2-R consists of three glycoprotein chains, a low affinity β chain of Mr 55,000 (Urdal *et al* 1984; Cushley and Harnett 1993) and an intermediate affinity α chain (75,000), which is involved in signal transduction.

Table 1.1

Representative molecules expressed by activated T cells

Molecule	Function	Appearance	Increase in expression
c-fos	Nuclear binding protein	15 min	<100
c-myc	proto oncogene	30 min	20
INFα	cytokine (secreted)	30 min	>100
IL-2	cytokine (secreted)	45 min	>1000
TGF-β	cytokine (secreted)	< 2 hrs	>10
IL-2R	p55 receptor	2 hrs	>50
Transferrin	receptor	14hrs	5
HLA DR	Class II MHC	3-5 days	10

Best documented is the acquisition of the receptors allowing interaction between cells of the immune system. These include the Interleukin-2 receptor (IL-2R) and the MHC class II molecule HLA DR.(Tseudo *et al* 1986; Cushley

and Harnett 1993). When α/β associate they form the high affinity IL-2 receptor. There has also been shown to be a third chain γ which has been shown to associate with the receptor (Cushley and Harnett, 1993). The binding of the ligand a cytokine growth factor (IL2) to the receptor causes progression of the cells from the G1 into the S phase of the cell cycle (Herzberg and Smith 1987). When stimulated, T cells express the low affinity chain to a greater extent (5 or 10) than the α chain and IL-2 binding to the high affinity receptor increases the expression of the β chain 10 or 20 fold. The use of the β chain (CD25 or Tac) as a measure of T cell activation is a standard test

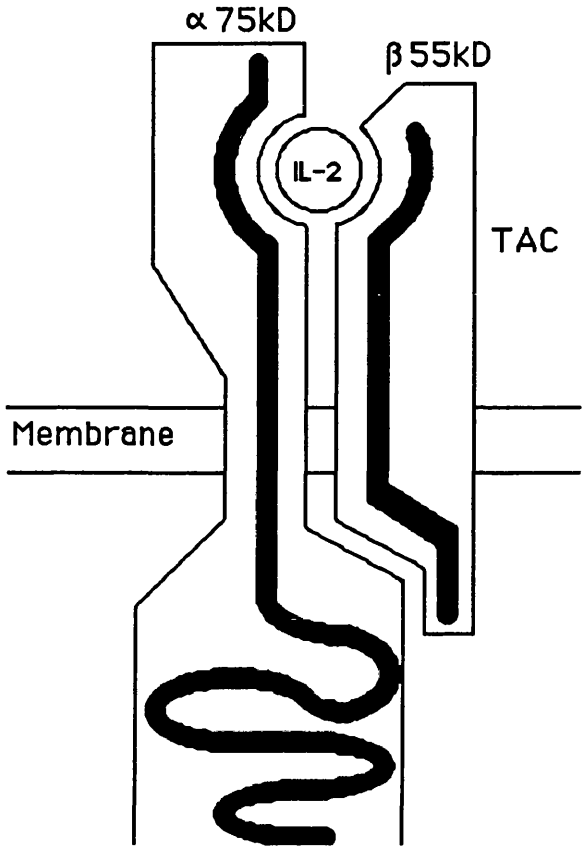


Figure 1.1.4. Structure of the high affinity Interleukin-2 receptor (IL-2R).γ chain not illustrated

for T cell activation. However IL-2 bound to the high affinity receptors undergoes internalisation leading to eventual down regulation of the surface IL-2 receptor (Duprez *et al* 1988).

1.2.6. B cell activation markers

B cells like T cells carry markers which are produced during activation. MHC II molecules are found on the surface, their function being to present antigens to CD4+ T helper cells (Section 1.2.3.5). Other markers may include the CD5 protein (Vande Velde *et al*, 1991; Hayakawa and Hardy, 1988) which is expressed on both T cells and a subset of activated B cells believed to be involved with B-T interaction and activation. B cells have had their activation defined by the presence of endogenously produced IgG on their surface. In the initial stages of a B cell response the cells express surface IgM which is polyvalent with low affinity for antigen. The isotype is then switched to the high antigen affinity IgG which marks the onset of the secondary response (Tonegawa, 1983). During antigen recognition CD4+ T helper cells secrete growth factors called cytokines to help generate the primary and secondary responses. Thus B cell maturation can be measured by the B cell marker CD19 and the presence of IgG.

1.2.7. Adhesion molecules and antigen presentation

Interaction of lymphocytes with antigen presenting cells or target cells found in a specific area of the body is an essential part of the immune recognition and activation process. Adhesion molecules play an important role.

However only a few have been identified and have been given a CD designation (Section 1.2.2.).

Adhesion molecules possess a lectin-like N terminal domain which mediates binding to a carbohydrate ligand molecule. The similarity with lectins has resulted in the the designation 'se-lectins' for this specific group. There are three families of cell adhesion molecules, integrins, the immunoglobulin super family and selectins (Springer 1990).

Selectins can be split into three subgroups

(1) E - selectins

(2) P - selectins

(3) L - selectins

L- selectin (lecam) is found on both the endothelium and the lymphocyte allowing binding during lymphocyte recirculation through peripheral lymph nodes.

P- selectin also known as CD62 has a similar function to L - selectin but allowing neutrophils and monocytes to bind to endothelial cells at sites of injury (Larsen *et al* 1989).

E- selectin (Elam 1) is predominantly found in activated and inflamed endothelium, promoting binding of neutrophils, monocytes and a lymphocyte subpopulation (Bevilacqua *et al* 1989).

In addition to CD4 and CD8 which are involved in antigen recognition and activation (Section 1.2.3.3.), there are other important molecules involved in mediating the immune response.

LFA-1 (lymphocyte function associated antigen -1) is expressed on virtually all leucocytes and has been implicated in cytotoxic T cell killing and lymphocyte adhesion to high endothelial venules (HEV) (Section 1.2.3.2.) (Springer *et al* 1987). The LFA ligand is intercellular adhesion molecule -1 (ICAM-1) (Marlin and Springer 1987) which is expressed on a variety of cell types (Dustin and Springer 1988). LFA is believed to be associated with other molecules such as CD2 and CD8, to form a stable interaction during MHC class I recognition.

Other molecules included in this group include CD28 and CD44. CD28 is a member of the immunoglobulin superfamily. The corresponding ligand is the B7 molecule. Signal transduction from this molecule has been shown to be independent of CD3 and loss of this interaction has been implied to cause T cell anergy. CD44 is expressed in a variety of cell types including both endothelial cells and leucocytes. CD44 has been shown to associate with the cytoskeleton of most cells, which may be due to a role in the adhesion of the extracellular matrix.

1.2.7.1. Lymph node homing receptors

In order to enter the various lymphoid organs, circulating lymphocytes must first adhere to the specialised endothelial cells of the HEV. This binding is very specific (Butcher,1979). Evidence for homing was found in the murine system, when an antibody designated Mel-14 was raised against the HEV and showed *in vitro* to block the lymphocyte adhesion to the HEV in lymph nodes but not in other organs. The antigen for the antibody was of a lectin structure, probably interacting with a carbohydrate determinant. (Sanders *et al* 1988).

The human homologue of MEL-14 was named Leu-8. (Camerini, 1989). Expression of this molecule is down regulated upon activation. Within tumour draining nodes of breast cancer patients expression of Leu-8 was of a higher level in the blood than the nodes. Leu-8 has yet to be proven as the human equivalent of murine MEL-14.

1.3. Tumour immunology

The immunosurveillence theory for cancer (Burnet, 1964), states that tumours are continually developing and the abnormal characteristics generated will be detected by the T cell or the B cell immune system before a tumour becomes detectable. In the late 1970's the great hope for treatment was a directed immune therapy to the antigens presented on tumour cells. This was known as the "magic bullet" treatment. To date no well defined cell surface tumour specific antigens have been characterised, although a number of internal mutated onco-proteins such as H-*ras* and p53 have been implied (Section 1.4.). Evidence for the theory is conflicting. Against a theory of a generalised immune response to cancer is the fact that patients undergoing long term immunosuppressive treatment have shown exactly the same incidence of epithelial tumours, such as breast cancer, as have normal individuals. Secondly, patients with acquired immuno deficiency syndrome (AIDS) have a higher frequency of tumours associated with DNA viruses (Penn, 1988). However, the studies involving immunosuppressed patients have been for no longer than over 10 years and long term clinical evidence by Fisher *et al* (1980) has shown long term survival of patients with the primary tumour removed and the tumour draining lymph nodes intact containing metastatic tumour. This would suggest an active immune response in these patients. The tumour itself

contains a lymphocytic infiltrate in 60% of all breast cancers, mainly of a T cell origin. This T cell response in tumours has been used as an indicator of good prognosis and TIL have been used in immunotherapy trials (Rosenberg *et al* 1988). (Section 1.3.8.2.).

1.3.1. The B cell response to cancer

If a tumour is producing cell surface or soluble antigen, then a clinically useful B cell immune response could be generated. If antibody producing cells generated from patients could be isolated they could be immortalised. This would be using two major techniques: immortalisation using the Epstein Barr virus (EBV) (Steinitz *et al* 1979) or the use of fusion techniques first developed by Kohler and Milstein,(1975). These methods would generate an immortal B cell clone to produce large quantities of antibody with an anti tumour potential. The antibodies generated have had little use in diagnosis or therapy (Campbell and Leake 1990; Campbell *et al* 1987). To date the existence of a specific and useful B cell response against breast cancer has still to be proven.

1.3.2. The T cell immune response to cancer

The T cell response is mainly restricted to internal antigens and until recently the nature of the response was unclear. Recently, however a better understanding of T cell function has expanded the understanding of T cell immune responses to cancer.

The immune response to breast cancer occurs in three areas of the body: a direct response within the tumour, the tumour draining lymph nodes and the blood.

1.3.3. Tumour Infiltrating lymphocytes (TILs)

1.3.3.1. Phenotype of the TILs

The presence of TILs has long been observed in breast and other cancers. The presence of lymphocytes within the tumour has been reported to be a good prognosis (Bloom *et al* 1970; Black *et al* 1975; Ridolphi *et al* 1977; Underwood *et al* 1976) this is disputed by a number of groups (Roses *et al*, 1982; Champion *et al*, 1972). Early studies indicated that T cells comprise the major component of the lymphocytic infiltrate (Eremin *et al* 1981; Eremin *et al* 1982). However, not until the advent of flow cytometry that it became possible to determine more accurately the phenotype and mechanism of activation of these lymphocytes. Flow cytometric studies confirmed that T cells were predominant within the tumour (Bilik *et al* 1989; Whitford *et al* 1990) and using this technique the T cell subsets could be distinguished. The data has been conflicting with groups showing a CD4+ T helper cell (von Kleist *et al* 1987; Underwood *et al* 1987; Ben Ezra *et al* 1987; Balch *et al* 1990) or a CD8+ T cytotoxic predominance (Bilik *et al* 1989; Belldegrun *et al* 1989; Whitford *et al* 1990). However, considering all tumour types the CD8+ T cells seem to be the more predominant phenotype (Itoh *et al* 1988; Rosenberg *et al* 1988; Heo *et al* 1987; Belldegrun *et al* 1988; Durie *et al* 1990).

1.3.3.2. Activation of the TILs

Most studies have concentrated on the two main lymphocyte activation markers (Section 1.2.5.2.) HLA DR and the IL-2R (Tac) (Section 1.2.5.3). These studies have shown TILs contain many HLA DR positive cells (Rowe and Beverly 1984; Lwin *et al* 1985; Ben Ezra *et al* 1987) with a few studies showing the opposite. The IL-2R was shown to be expressed in variable

amounts in the infiltrate. One study has looked extensively at TILs in breast tumours. Whitford *et al* 1992, found a predominance of HLA DR expression on T cells, with the CD4+ T cell subset showing the greater expression. Percentages of positive cells reported were consistently higher than those found in the patients peripheral blood. The Tac antigen of the IL-2 R was also expressed on more cells in comparison to the peripheral blood, with the higher expression again found on the CD4+ T cells rather than CD8+ T cells. In this study the tumour type was also compared to phenotypic and activation changes within the TIL population. The presence of HLA DR correlated with tumour grade (state of differentiation). This expression of HLA DR also correlated with MHC class I and II expression. This would indicate that antigen recognition is increased on poorly differentiated tumour (Whitford *et al* 1992a) and would agree with earlier findings that a specific tumour type such as the poorly differentiated medullary carcinoma, have a large lymphocytic infiltrate and a good prognosis. (Donegan and Spratt 1988).

Thus the presence of TILs and especially the presence of activated CD8+ T cells, indicates antigen recognition and possible tumour killing in a number of breast tumours.

1.3.4. Immune responses in the axillary tumour draining lymph nodes of breast cancer patients.

The axilla occupy an important position, as these secondary lymphoid tissues are usually the first defined area of the immune system to come into contact with tumour breakdown products or metastatic tumour. The nodes are ideally situated to interact and modulate an immune response against the tumour. The lymph nodes have defined areas for both T cell and B cell immune responses. (Section 1.2.3.2.). These nodes also have a great clinical relevance

as tumour cells with the ability to metastasise are found within them. These lymph nodes are removed routinely during surgery for the for the presence of metastatic tumour as this is the best indicator of bad prognosis for the patient (Bloom *et al* 1970). Thus the nodes have a great importance to both clinician and immunologist and as such have attracted much attention.

1.3.4.1. Phenotype of lymph node lymphocytes

Early studies using immunochemistry showed the nodes to have a greater population of B cells in contrast to the greater number of T cells within the tumour (Eremin *et al* 1976; Heidenreich *et al* 1979). The two subsets of T cells were phenotyped by Mabs in conjunction with flow cytometry and initial studies by Morton *et al* (1986) agreed with earlier findings in that B cells were predominant. The proportion of B cells increased or decreased according to the stage of the disease. The T cell population indicated that CD4+ T cells were found predominantly in stage I disease. The increase in B cell population was also noted by Mantovani *et al* (1986) and Whitford *et al* (1992b). Morton *et al* (1986) showed that distance of the lymph node had no effect on the phenotype of either T or B cells.

1.3.4.2. Activation of lymph node lymphocytes

A substantial proportion of B cells in the nodes are expressing IgG (Whitford *et al* 1992b) indicating a mature immune response against unknown tumour antigens. Whitford *et al* (1992b) compared this expression of IgG to the peripheral blood and found the expression of IgG significantly higher in the nodes. HLA DR expression was shown to be higher in the nodes than the blood (Morton *et al* 1986; Whitford *et al* 1992b), with the same trend noted for IL-2R expression. The CD4+ cells expressed higher levels of the IL-2R.

The increased proportion of CD8+ HLA DR+ T cells was related to the stage of disease.

When comparing distance from the breast tumour to activation in the axillary nodes Morton *et al* (1986) have shown that lymph nodes excised from close to the tumour were found routinely to be more activated with respect to both HLA DR and IL-2R than the apical nodes further from the tumour. This would suggest a differential immune response in the axillary nodes depending on distance from the tumour.

Overall the data suggest that the nodes are activated and responding to the tumour.

1.3.5. Comparison of immune response in cancer and non cancer patients

The comparison of cancer patients with normal controls is an important one, as even within the normal population there is wide variation due to different exposure with antigen and different HLA types.

The presence of TILs within the tumour is one of the few examples of a lymphocytic infiltrate found in a specific disease state. Blood and lymph nodes can also be obtained from cancer patients. Pattanapanyasat *et al* (1988) indicated higher levels of the activation marker HLA DR expression when compared to normal controls. To date only one study has attempted to compare both blood and nodes from cancer patients, Whitford *et al* (1992b) used nodes received from the illiac region of kidney transplant patients and nodes from patients undergoing vascular surgery. This was compared to the axillary nodes

from breast cancer patients. The ratio of CD4+ T helper cells to CD8+ T cytotoxic cells was lower in the control nodes than in the cancer patients, with the control patients showing much less variation. This large variation was also noted when activation markers were measured IL-2 receptor and MHC class II molecule HLA DR were greatly raised in the cancer patients.

This change in phenotype and activation may be due to an immune response within the patients in the absence of any other major disease. The wide range observed suggests a response in a subset of patients, whereas in the remaining the response may be impaired or undergoing active supression.

1.3.6. Metastasis and the immune response within the axillary lymph nodes

The presence of metastatic tumour within the lymph nodes is the most important indicator of bad prognosis to date. It is important to study the interaction of the metastases with the immune system to understand why the immune response is eventually defeated. The removal of the lymph nodes during surgery gives an ideal opportunity to study the tumour lymphocyte interaction. Early studies, compared the histology of normal and tumour draining lymph nodes. This indicated that the micro architecture of the tumour draining lymph nodes showed significant hyperplasia when compared to normal nodes (Hamlin, 1968; Tsakraklides *et al* 1974). Other studies indicated phenotypic changes were also occurring in the T/B cells within these nodes, related to nodal tumour spread (Eremin *et al* 1980). Later studies, utilising Mabs and flow cytometry showed little change. This disagrees with the findings of Tsakraklides *et al* (1986) showing significant differences, with a higher percentage of B cells and lower percentages of T cells in nodes with no metastatic tumour (Stage 1).

The more recent reports utilised flow cytometry and specific monoclonal antibodies. Morton *et al* (1986) showed that tumour draining nodes contain a higher proportion of helper T cells than cytotoxic T cells, the level of helper T cells dropping significantly in stage II patients being replaced by a higher level of CD8+ cytotoxic T lymphocytes. The activation state of lymphocytes within stage II patients showed an increased level of HLA DR compared to tumour free stage 1 nodes.

1.3.7. Overall view of the nodal immune response

The major alterations of both architecture, lymphocyte phenotype and activation observed within the tumour draining nodes of breast cancer patients would suggest a host mediated response against their tumour.

It will be useful to study the interactions within the nodes at a differing distance from the tumour and the change within groups of nodes with respect to the change in immune responses due to tumour cell invasion.

1.3.8. Tumour antigens

An immune response will be elicited in an immunocompetent host by any cell component that is new to the system. During cellular transformation molecules are sometimes expressed that are unique to the cell and not normally found in the host. These are referred to as tumour specific antigens and may be located either on the surface or within the tumour.

The hope in the 1980's was that the generation of monoclonal antibodies against tumour specific antigens would mean a directed 'magic bullet' treatment

for cancer. Mabs were raised against cancer cell lines, tumour or metastases. The optimism soon vanished when it became evident that although the Mabs were reactive against the tumour, they were also reactive against normal tissue, (Colcher *et al* 1981). The major problem associated with Mab generation is the use of animals which have to be hyperimmunised with pristane or another material before priming with the antigen. This procedure is of course unethical to carry out in humans.

A different approach was the immortalisation of the B cell response against cancer from the blood or the nodes of a breast cancer patient. This was even less successful as the antibodies generated were polyspecific and were of little use for either diagnosis or therapy. (Campbell *et al* 1987).

The clarification of the mechanism of the T cell immune response along with the identification of the genetic changes within tumours greatly increased the knowledge of tumour immunology. T cells were demonstrated to recognise mainly intracellular antigen presented on the surface of the target cell, in the form of a peptide, by a MHC molecule .

With the identification of genetic changes it became clearer that all major changes occurred within the tumour cell and to date no mutated protein has been discovered on the surface. Intracellular genetic changes are often unique to that type of cancer and are potentially targets for T cell recognition. In breast cancer, mutations are found in two main proteins *ras* and p53 (Section 1.4). *ras* mutations occur as a point mutation in positions 12 and 13. Even a point mutation has the ability for immune recognition. p53 mutations are the most common found in human cancer, with mutation in over 50% of all breast cancers. Other possible antigens include *bcr/abl* and *tpr/met*. These are proteins formed by the fusing together of two genes during transformation forming a

possibly antigenic protein. However the expression of these in breast cancer is still to be fully elucidated.

1.3.8.1. Evidence for T cell specific antigens

Early work by Crawford *et al* (1979) using the anti p53 antibody Pab 1801 indicated that in the sera of 10% of breast cancer patients antibodies specific for p53 were present. The presence of these antibodies is unusual as normally B cells recognise cell surface or soluble antigen, For p53 recognition the tumour cell may have broken down releasing cellular contents into the body. A recent paper has suggested that this antibody response is dependent on the presence of heat shock protein (hsp) 70 complexes in breast cancer (Davidoff *et al* 1992).

T cell responses to mutated oncogene products have been tested in murine models by transfection of the mutant gene into cells and adding them back into the animal and looking for a specific cytotoxic T cell (CTL) response. A response has been noted for *ras*, p53 and *bcr/abl* for the mutant protein but not for the normal protein. However the response is not seen in all animals and is restricted to specific HLA types.

The mechanism for antigen recognition is via a peptide presented in the groove of the MHC molecule. A new method has been developed to isolate and sequence these peptides and compare them to the actual mutant protein within the cell. This involved an HPLC linked to a mass spectrometer, to isolate a picogram of peptide from 10^8 cells. The large amounts of cells involved make it difficult to sequence peptides from fresh tumour cells. However, cell lines can supply the necessary number of cells for peptide isolation, though to date the peptides isolated remain unidentified (Eisenbach, 1993).

Van der Bruggen *et al* (1991) have defined a melanoma tumour antigen (MAGE) from the cell line M22-MEL. The antigen was isolated after an extensive screening of the cell line DNA within a cosmid vector and testing with a number of cytotoxic T cell lines derived from the melanoma patient. Eventually a 2.4kb *Bam H1* fragment was isolated and the gene was found to be a member of a family of three genes with 60-80% homology. MAGE is expressed in most melanoma lines and is not found in normal tissue except the testis.

The MAGE antigen is expressed in 25% of all cases of melanoma, and 17% in breast cancer. The expression is higher in metastases. The epitope has now been mapped and the nona-peptide isolated is found in the third exon of the MAGE 1 gene. The expression of the peptide is however restricted to HLA A1. This makes it theoretically possible for an active immunotherapy within suitable patients expressing this antigen. Thus 25% of melanomas are positive, but only 40% of these patients are HLA A1. Only 10% of patients are suitable for treatment (Boon 1993).

1.3.8.2. Immunotherapies

Traditional techniques such as surgery, radiation and chemotherapy can cure or delay cancer. However, there are a large number of people who die of the disease.

A more directed approach to cancer therapy would be the use of the immune system to target the tumour as the immune system has been implicated in spontaneous regression of the tumour in a very small number of patients.

It was not until the discovery of the important T cell growth factor interleukin -2 (IL-2) (Gillis *et al* 1978) and the introduction of methods of growing large quantities of T cells in culture that T cell immunotherapy became possible.

Initially lymphokine activated killer (LAK) cells were generated from the peripheral blood lymphocytes (PBL) removed from cancer patients and grown in IL-2. These cells possessed the capability for lysis of tumour but not normal tissue (Raynor *et al* 1985). These LAK cells were assumed to be derived from the "null" population, which make up 5% of the circulating lymphocytes. They are capable of non-MHC associated cytotoxicity. (Grimm *et al* 1985). On analysis the cells were CD3 negative, CD16 negative and are thought to be natural killer cells.

These cells were cultured in IL-2 and transferred back into the patient along with large quantities of IL-2. Limited anti-tumour effects were noted with the highest response in malignant melanomas (Rosenberg *et al* 1988).

The response rates were not as good as expected and work then concentrated on the tumour infiltrating lymphocytes (TILs) as murine models had shown a 50 to 100 fold improvement in the capability for tumour regression (Rosenberg *et al* 1986). TILs were extracted from human tumours and grown in IL-2. They were then readministered into the patients with large quantities of IL-2 (Topalian *et al* 1987). Initial data indicated complete regression in 15% of renal carcinoma and maligant melanoma patients.

Immunotherapy has its drawbacks, the administration of large quantities of IL-2 can cause major side effects such as vascular leak syndrome, weight

gain, nausea and even death in 1% of the patients. Most of the side effects are only temporary and after therapy is completed they completely disappear.

Recent TIL therapy has utilised gene therapy technology to transfer a marker neomycin gene to the removed TIL. These have been infused into patients and shown to home into the tumour. This pilot study led the way for transduction of specific genes into the TIL. Tumour necrosis factor (TNF) gene has been transferred into the TILs in the hope that these lymphocytes will home into the tumour. However the TILs do not all home into the tumour and some may reach the liver where the secretion of TNF is extremely cytotoxic to the liver.

Eventually the antigens stimulating the immune system must be identified to give a more directed immune therapy designed for each individual patient.

1.4. Genetic factors and cancer

1.4.1. The cell cycle

The disruption of cell cycle control is one of the factors which may lead to the initiation of cancer.

Two main identifiable events that characterise the cell cycle: are mitotic cell division and DNA synthesis. (Figure 1.15). Mitosis is represented by M and the period of DNA synthesis is S. G0 cells are in the quiescent, resting phase, whilst the phases G1 and G2 are gaps between M and S and S and M

respectively. The term G1 and G2 were coined due to the absence of knowledge of other identifying markers at this time (Adams, 1990).

1.4.1.1. Cell cycle control

Cells in different environments spend variable amounts of time within the phases of the cell cycle. The majority of lymphocytes are in the quiescent phase (G0) and are out of cycle, requiring a stimulus to enter the cycle. For entry into G1 a single entry point has been reported (Pardee *et al* 1974) followed by movement through the different phases of the cycle (Figure 1.15). In cancer the G1phase of the cell cycle has been reported to be highly variable (McGuire and Dressler 1985).

Thus the loss of cell cycle control in cancer is evident, however the understanding of the normal processes involved in cell cycle control is still not clear. A universal mechanism for the control of M phase entry has been proposed (Nurse 1990; Murray A.W, 1992; Pines J, 1993; Sher C.J., 1993). The protein kinase p34, first identified as the product of the cdc 2/ CDC 28 gene in yeast, is believed to function at the onset of S phase.(Mendenhall *et al* 1987) and mitosis (Pines, 1993; Sher 1993). The $p34^{cdc2}$ kinase phosphorylates key proteins which leads to the events in M phase. A second class of protein, "the cyclins" complexes with and activates $p34^{cdc2}$ to regulate M phase (Moreno *et al* 1989). Cyclin binding modifies and activates the $p34^{cdc2}$.

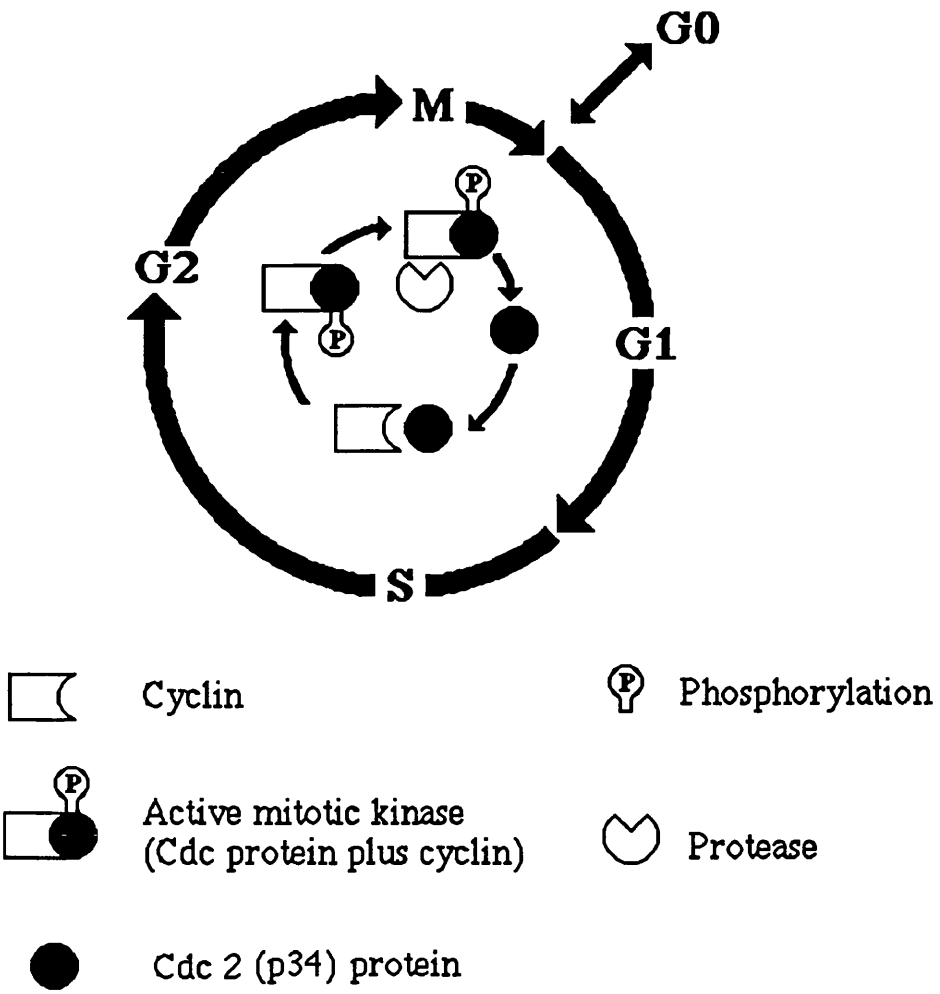


Figure 1.15. The regulation of the cell cycle by its associated proteins

S phase initiation involves the p34 kinase and G1 cyclins, which appear in G1 and are degraded in S phase. The G1 cyclins are essential at the decision to pass from G1 to S phase. This transition is known as START (Pines, 1993; Sher, 1993) and involves a class of regulatory proteins that are involved in the G1 phase of the cell cycle.

1.4.2. Measurement of DNA content

The DNA content of a normal somatic cell with 46 chromosomes is referred to as diploid (Tijo and Levan 1956). A cell with fewer or more than 46 chromosomes is described as aneuploid.

1.4.2.1. Flow cytometric DNA analysis

Flow cytometry is commonly used for the measurement of DNA content as the technique gives information on the DNA ploidy and S phase fraction (SPF), which is a measure of the proliferative rate of a growing cell population. With this method a large number of cells are analysed objectively. A DNA histogram is obtained from which ploidy and S phase can be determined. The ploidy is usually expressed in the form of DNA index which contrasts the differences from diploidy.

1.4.2.2. DNA ploidy and breast cancer

In a substantial proportion of all breast cancers the state of the DNA is aneuploid. Studies indicate between 53% and 73% of breast tumours have an aneuploid DNA content, with an average of 63% (Frierson 1988).

This change in DNA ploidy has been linked in a number of studies to lymph node involvement (Eskelinen, *et al* 1989; Toikkanen *et a* 1989; Hedley *et al* 1987). However no association has been found by others (Visscher *et al* 1990; Moran *et al* 1984; Horsfall *et al* 1986; Thorud *et al* 1986; McDivitt *et al* 1986; O'Reilly *et al* 1990). Studies involving larger numbers of patients have

only shown weak correlations to stage parameters (Hedley *et al* 1987; Ewers *et al* 1984; Dressler *et al* 1988).

DNA content correlates significantly with histopathological tumour grade (Toikkanen *et al* 1989; O'Reilly *et al* 1990; Thorud *et al* 1986; Moran *et al* 1984; Kute *et al* 1985; Kallioniemi *et al* 1987; Feichter *et al* 1988; Dowle *et al* 1987; McDivitt *et al* 1986; Visscher *et al* 1990). Generally aneuploid tumours seem to have a worse prognosis.

1.4.2.3. Multiparametric flow cytometric DNA analysis

The major problem associated with the analysis of solid tumours such as breast cancer is the presence of contaminating cells such as lymphocytes which can contribute up to 80% of the tumour mass (Whitford *et al* 1991). Histochemical analysis can easily recognise the presence of non- tumour cells, whereas single parameter DNA flow cytometric analysis cannot.

However Zarbo *et al* (1989) has described two colour multi parametric DNA analysis on intact cells, the second colour being anti-cytokeratin which stains only cells of an epithelial origin. Utilising this method Alam *et al* (1992) described the ploidy changes from primary to metastatic tumour cells showing a subpopulation which metastasised from the primary tumour to the tumour draining lymph nodes.

The use of multiparametric DNA flow cytometric analysis has been used to quantify oncogene expression in relation to the ploidy of the DNA. There are reports for p53 (Morkve and Hostmark 1991), *erb* B-2 (Kelsten *et al* 1990) and C-*myc* (Hendy-Ibbs *et al* 1987).

1.4.3. DNA oncogenes and tumour supressors

1.4.3.1. Oncogenes and proto-oncogenes

The first evidence for the cause of cellular transformation came from experiments carried out on the Rous Sarcoma virus. Later it was discovered that the presence of cancer genes called oncogenes within the viral genome was responsible for cellular transformation. In the Rous Sarcoma virus this was identified as a region called v-*src* and in later experiments homologues of v-*src* were discovered in normal cells. From these experiments other genes, similar to oncogenes, were identified and named proto-oncogenes.

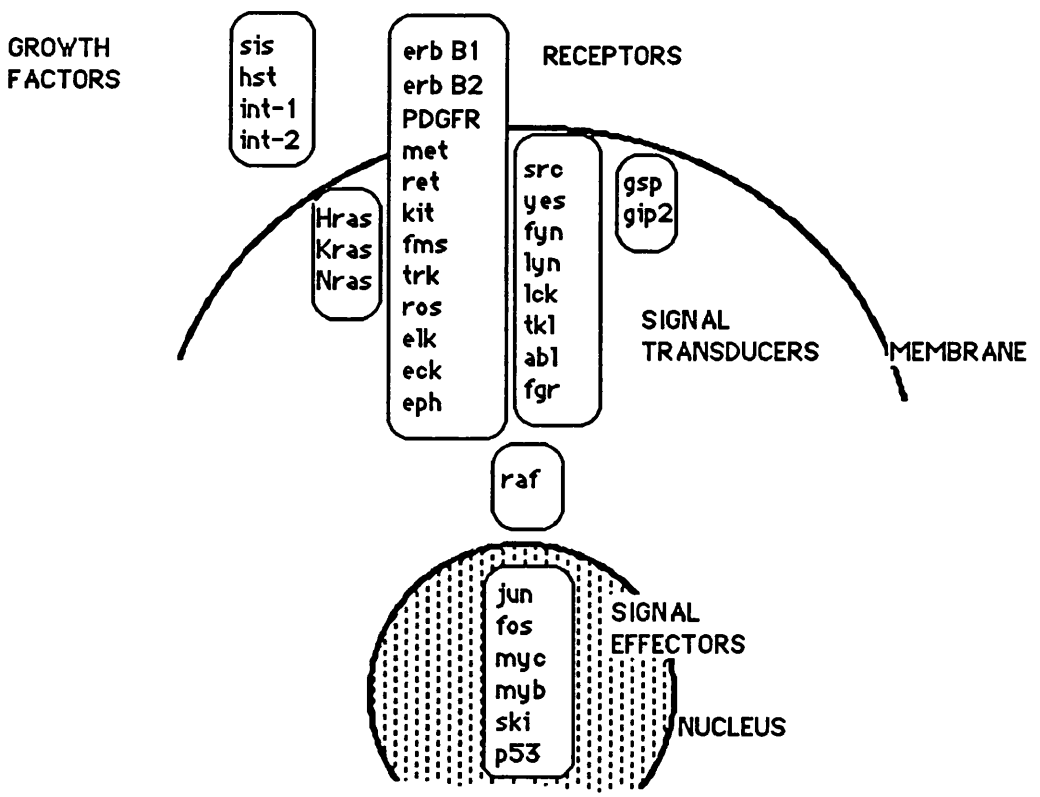


Figure 1.16. Some proto oncogenes and tumour suppressor in normal and abnormal cellular development

The cellular proto-oncogenes code for proteins which are believed to be normally expressed at low levels during precise stages of cell or embryo development but which become irreversibly and permanently expressed in tumour cells as a result of viral, chemical or radiation induced changes in the genome of the affected tissue. In some cases overexpression of the normal cellular proto-oncogene as a consequence of loss of regulatory control is sufficient to cause cell transformation. In others, the gene product itself carries a mutation and codes for a slightly altered protein. The early oncogenes (eg *src*) were isolated as a result of their ability to induce tumours in animals. Later strategies have detected new oncogenes (eg c-*met*) by random irradiation of animal DNA followed by transfection, isolation of genes which cause cellular transformation and subsequent extrapolation to the similarly hybridizing sequences within the human genome.

Most characterised oncogenes appear to encode developmental proteins, growth factors, growth factor receptors, signal transducing factors, or nuclear proteins controlling gene expression and the cell cycle. Tumour suppressor genes ("anti-oncogenes") are negative regulatory elements whose inactivation causes uncontrolled cell growth.

1.4.3.2. Oncogenes implicated in breast cancer

Oncogenes which have clearly been associated with breast cancer are shown in Table.1.2.

Table 1.2 Oncogenes implicated in breast cancer

Oncogene	Human chromosome location	Possible function of gene product	Size and location	Possible alteration in breast cancer
myc	8q24	gene expression or cell cycle control	439 amino acids nuclear	Amplification (occasional alteration)
H-*ras*	11p14	GTP binding protein	189 amino acids inner membrane	overexpression Gene deletion
erb- -B-2 (*neu*)	17q21	receptor	1260 amino acids Transmembrane	Amplification Overexpression
hst and *int* -2	11q13	Growth factors	206 and 220 amino acids secreted	Amplification

1.4.3.3 *C-myc*

The oncogene *myc* was first identified as the transforming element of avian myelocytomatosis virus-29 (Bister *et al* 1977) and subsequently the human equivalent cellular oncogenes were identified. In man, there are three related *myc* proto-oncogenes. N-*myc* is amplified and expressed in human neuroblastomas (Schwab *et al* 1983) while the L-*myc* is amplified in lung cancers (Nau *et al* 1985). C-*myc*, which is encoded by chromosome 8q24, contains three exons of which only exons 2 and 3 contain the protein coding sequence. The C-*myc* protein product is a 439 or 454 amino acid nuclear DNA binding phosphoprotein with a short half life. The precise function of this protein is unclear but it is thought to work in collaboration with other

nuclear proteins (Cole, 1991) and could be involved in the regulation of transcription of other genes important for cell growth regulation.

The deregulation of the C-*myc* proto-oncogene can take place via three different genetic mechanisms. Adjacent retroviral insertion causes activation in avian bursal lymphomas (Hayward *et al* 1981) whereas, in human and murine leukemias and lymphomas, it may be activated by chromosome translocation into actively transcribing regions encoding the immunoglobulin or T cell receptor genes (Adams *et al* 1983; reviewed by Cole, 1991). Finally, amplification of the oncogene may occur and this has been reported in several types of human tumour (reviewed by Alitalo and Schwab, 1986). It is believed that the C-*myc* oncogene may have a role in the the progression of the disease rather than in tumourigenesis.

C-*myc* amplification in breast cancer has been reported by several workers. In a study of 121 human primary breast carcinomas, Escot *et al* (1986) observed an amplification of 2 to 15-fold in 32% of the samples analysed. Tavassoli *et al* (1989) reported a similar range of amplification (3-15 fold) in a sample of 52 breast tumours and in addition, found a significant correlation between amplification of the oncogene and histological grade in these tumours. C-*myc* amplification has been correlated to early relapse and poor short term prognosis in breast cancer (Roux-Dosseto *et al* 1992; Borg *et al* 1991). Interestingly, in addition to carcinoma cells, tumour infiltrating lymphocytes (TIL) (Section 1.3.3.) also showed high levels of C-*myc* transcription. A possible reason for this high level is that C-*myc* was one of the first proto-oncogenes to be translated during T cell activation. The presence of this population expressing C-*myc* to high levels may call into question the results obtained for western blotting as TIL can make up to 80% of the tumour cell mass (Whitford *et al* 1990).

There are few reports of alterations in C-*myc* in breast cancer. Escot *et al* (1986) reported rearranged C-*myc* in only 5 samples, representing a mere 4% of the total cases studied. One patient with a particularly aggressive tumour was reported to have a deletion in exon 3 by Varley *et al* (1987b).

1.4.3.4. H-*ras*

H-*ras* is an oncogene first identified in transforming DNA from the human EJ bladder carcinoma (reviewed by Marshall, 1993) and shown to be the cellular homologue of the *ras* H gene of Harvey sarcoma virus (Der *et al* 1982; Parada *et al* 1982; Santos *et al* 1982). It is a member of a family of *ras* oncogenes which also includes K-*ras* (the proto-oncogene corresponding to Kirsten sarcoma virus) and N-*ras* (from neuroblastomas). The three functional members of the *ras* gene family are similar in that they encode a protein of Mr 21,000 in four exons (Lemione, 1990). The H-*ras* gene is situated at chromosome location 11p1.4 and the gene product contains 189 amino acids. All known *ras* proteins are GTP binding proteins with substantial homology to the normal cellular G proteins which are known to act as regulatory elements in signal transduction systems involving raf and MAP kinase (Marshall, 1993)

Ras activation is by point mutation leading to single amino acid differences from the normal cellular proto-oncogene sequence. In the human tumours studied, amino acid substitutions have been found to occur at positions 12, 13 and 61. Substitution of the normal position 12 glycine residue with any other amino acid except proline causes oncogenic activation (Seeburg *et al* 1984) and in the EJ bladder carcinoma, where the glycine is altered to valine, such a change is sufficient to cause transforming activity. A similar situation is observed for the glycine residue at position 13 although a few

substitutions like Ser 13, may not be transforming (Bos *et al* 1985). Work with rodents has indicated that chemical carcinogens can cause such point mutations.

In breast cancer there have not been many reports of *ras* gene amplification. However two recent reports have suggested *ras* amplification, in relation to nodal positivity (Saccani-Jotti *et al* 1992) and no relationship to any prognostic indices (Spandidos *et al* 1992). Three groups have reported the loss of one of the two c-H-*ras* alleles in around 25% of breast cancers (Theillet *et al* 1986; Ali *et al* 1987; Mackay *et al* 1988). These studies also found a good correlation with histological grade, with more than 74% of tumours with c-H-*ras* allelic deletion being grade 3 (Theillet *et al* 1986; Ali *et al* 1987). It has been suggested that the allelic deletion of c-H-*ras* may be indicative of the possible involvement of tumour suppressor genes located on the same chromosome. Overexpression of unmutated H-*ras* proto-oncogene as mRNA (Theillet *et al* 1986) has also been reported even in situations where one allele is missing. There have also been studies showing that the *ras* protein is overexpressed (Horan-Hand *et al* 1984; De Bortoli *et al* 1985) without gene amplification. These reports were based on early immunocytochemical studies where it is possible that the specificity of the antibodies was not as high as anticipated.

1.4.3.4. *erb* B

The *erb B* gene was originally identified in avian erythroblastosis virus as a cellular gene which had the capacity to induce avian leukemias due to integration of the virus leading to abnormal overexpression (Fung *et al* 1983). On subsequent analysis, it became apparent that the virus had led to the expression of a truncated form of a cellular proto-oncogene which was the

gene encoding the Epidermal Growth Factor Receptor (EGFR). EGFR is a transmembrane glycoprotein of Mr 170,000 and is found on many epithelial cell types, including breast cancer cells. EGFR also binds transforming growth factor α (TGF-α) resulting in a growth stimulatory effect similar to that observed with EGF. The receptor has a cysteine-rich cell external domain, which binds EGF and TGF-α, a transmembrane domain and an intracellular domain which contains a tyrosine specific protein kinase. The kinase is activated upon binding of EGF to the receptor (Reviewed by Carpenter, 1987). The truncation in v-*erb* B is such that the extracellular (N terminal) domain of the gene is lost but the transmembrane and internal domains are present. The *erb* B protein therefore, lacks the extracellular domain and is therefore not sensitive to EGF and the intracellular domain is permanently activated. It has been shown that over-expression of the normal EGF receptor gene itself is sufficient to confer an EGF dependent transformed phenotype on NIH/3T3 cells (Di Fiore *et al* 1987a).

There are reports of amplification of the normal proto-oncogene (EGF receptor) (Liberman *et al* 1985). Amplification of EGFR gene accompanied by elevated levels of mRNA and protein have been reported in only about 3% of the primary carcinomas studied (Ro *et al* 1988; Lacroix, 1989). Many groups have also obtained data showing that the expression of EGF receptor is a relevant factor in breast cancer prognosis (Harris *et al* 1989; Sainsbury *et al* 1987, Nicholson *et al* 1988). However a recent study (Reeves *et al* 1993) has shown that in many cases expression of EGFR on normal mammary ductal epithelium is higher than that found in breast cancer. This would call into question the efficacy of using EGFR overexpression in tumours as a prognostic marker.

1.4.3.5. c-*erb* B-2 (*neu*, HER-2)

The *erb* B-2 oncogene was originally identified as a result of transfection studies in which NIH-3T3 cells were transformed with DNA from chemically induced rat neuroglioblastomas (Shih *et al* 1981). Shortly after this, two groups working independently, identified *erb* B-like genes, which they called c-*erb* B-2 and HER-2 respectively (Coussens *et al* 1985; Semba *et al* 1985). In the same year, a third group found amplification of an *erb* B-like gene in a single mammary carcinoma (King *et al* 1985). All three of these are the same gene, which was shown to be the human homologue of the rat *erb* B-2 gene and found to have substantial homology (45%) with the EGF receptor, particularly in the tyrosine kinase domain (82% homology) (Schechter *et al* 1984). Cells transfected with the gene, however, showed no response to EGF.

Although closely related to, the *erb B-2* gene is distinct from the EGFR gene (Schechter *et al* 1985). It is encoded on human chromosome 17q21, as compared to band p11-p13 of chromosome 7, where the EGFR gene is located. The protein encoded by the *erb B-2* gene is 1260 amino acids long with Mr 185,000, as compared to Mr 170,000 of the EGFR protein. Like EGFR, the *erb* B-2 protein has an extracellular domain, a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular domain with tyrosine kinase activity. The ligand for the *erb B-2* protein is as yet unknown. Six possible ligands for the *erb* B-2 receptor have recently been identified only two have been characterised: a factor of Mr 30,000 (gp30) secreted by the breast cancer cell line MBA-MB-231 (Lupu *et al* 1990; Lupu *et al* 1992) and a 40Kd glycoprotein from ras transformed cells (Peles *et al* 1992). In cell lines overexpressing the *erb* B-2 protein, stimulation by the ligands was shown to initiate differentiation (Lupu *et al* 1992; Bacus *et al*

1992) and also DNA polyploidy (Bacus *et al* 1992). TGFα like activity with agonist activity for both EGF and *erb* B-2 was noted for each ligand. However the role of the *erb* B-2 ligands in normal and abnormal cellular development is still to be fully elucidated.

erb B-2 can transform cells in two ways. Over-expression of the normal cellular proto-oncogene, *erb* B-2 is sufficient to transform NIH-3T3 cells and, unlike, the erb B (EGFR) gene (Section 1.4.3.4), the transformation is not dependent on exogenous growth factors (Di Fiore *et al* 1987b). Gene amplification results in elevated levels of expression of both *erb* B-2 mRNA and protein (Venter *et al* 1987; Vijver *et al* 1988). However, this does not preclude the possibility that in some cases mutation may occur, for the *erb* B-2 oncogene product itself carries a point mutation at position 664 in the transmembrane region where valine is replaced by glutamic acid. This leads to increased tyrosine kinase activity, probably through receptor dimerisation (Weiner *et al* 1989). However a recent report suggests that the mutation at position 664 is a relatively rare event in breast cancer (Zoll *et al* 1992).

The *erb* B-2 oncogene has been extensively studied in breast cancer since the report of Slamon *et al* (1987), suggesting that its amplification correlated with poor prognosis. A large number of studies have now been published involving both *erb* B-2 gene amplification and protein overexpression in human primary breast tumours. The reported gene amplification rates range from 10 to 46%, with an average amplification of 20%. Variability between these reports probably reflects differences in patient groups, the samples used for DNA analysis, paraffin-embedded as opposed to frozen or fresh tumour, and the fact that DNA preparations from tumour samples often contain DNA from normal vascular, stromal or inflammatory cells in addition to malignant cells. Although studies in general have shown

good correlation between gene amplification and relative expression of both RNA and protein (Slamon *et al* 1989), there are also reports of a significant percentage of tumours with a single copy of the gene that overexpress the protein product (Inglehart *et al* 1990; Hanna *et al* 1990; Tandon *et al* 1989). In consequence measurement of protein expression may be a better guide to the clinical relevance of alteration (Clark and McGuire, 1991).

A characteristic feature of this onco-protein expression is that generally the *erb* B-2 protein is overexpressed in an unmutated, normal form and the potential site of mutation lies in the transmembrane domain. Immunohistochemical studies or western immunoblotting therefore, record differences in the intensity of staining. In the majority of the studies, immunocytochemical analysis with polyclonal antibodies, specific for the *erb* B-2 protein and not cross-reacting with EGFR, has been used and often groups of investigators have used the same anti-serum from the same source. A group of investigators (Lovekin *et al* 1991; Winstanley *et al* 1991; Gullick *et al* 1991; O'Reilly *et al* 1991), using one such anti-serum (21N- directed to peptides 1243-1255) reported overexpression in about 20% of breast tumours, which is similar to that reported for gene amplification. Using both polyclonal and monoclonal anti-*erb* B-2 protein antibodies, membrane or cytoplasmic staining has been found to correlate with *erb* B-2 amplification (Venter *et al* 1987; Vijver *et al* 1988, Berger *et al* 1988).

Most of the studies have also correlated *erb* B-2 amplification with prognosis in patients. In general, patients with node-positive disease have greater frequency of amplification than patients with node-negative disease. Although the original study reported by Slamon *et al* (1987) and some of the more recent studies (Tandon *et al* 1989; Borg *et al* 1991; May *et al* 1990) showed a positive association between *erb* B-2 expression and the number of

invaded lymph nodes, this has not been generally reproducible. Vijver *et al* (1988) found large cells of comedo type *in situ* carcinomas were associated with more intense staining. None of the recently published papers report any significant relationship with nodal status. But the association with histological grade has been consistently reported (Garcia *et al* 1989; Paik *et al* 1990; Wright *et al* 1989; Parkes *et al* 1990) although, investigators vary in their reports of the level of significance. A few studies also report that *erb B-2* expressing tumours are generally larger in size (Vivjer *et al* 1988; Borg *et al* 1991) and negatively associated with steroid receptor status (Borg *et al* 1991; Tandon *et al* 1989) but others have failed to confirm this (Zhou *et al* 1989; Slamon *et al* 1987).

Although the area has been widely investigated, reports regarding the correlations between *erb* B-2 amplification and clinical outcome of breast cancer patients differ considerably. Clinical and pathological characteristics of the patients included in these studies vary and in some, analysis is restricted to node positive and in others to node negative patients. Using a monoclonal antibody to stain *erb* B-2 protein, Vijver *et al* (1988) reported no significant correlation between *erb* B-2 expression and disease free survival or overall survival after adjustment for tumour size, while Thor *et al* (1989), studying over-expression of *erb* B-2 with a Mab (TA1), which interacts with the extracellular domain of the protein, reported clinical significance in certain patient subpopulations only. In general, however, a significant prognostic effect of *erb* B-2 in terms of survival and recurrence has been reported in a larger number of studies compared to the reports of those studies which show no, or only a limited, prognostic effect (Perren, 1991). In a more recent study (Clark and McGuire, 1991), *erb* B-2 oncogene amplification in breast cancer has been reported to be of marginal utility as a prognostic factor in indicating clinical outcome. Correlation with either disease-free or overall survival was

marginally significant for node positive patients only, but was not retained in multivariate analyses. In contrast, in the analysis of Gullick *et al* (1991), who combined the results of three relatively small studies with the same polyclonal ab and thus improved the total number of cases, *erb* B-2 protein over-expression was found to be significantly associated with both recurrence and survival and this has also been confirmed in a multivariate analysis. This association was observed for breast cancer patients with involved and uninvolved nodes. As more studies with higher patient number are analysed, a clearer relationship will perhaps appear. At present, however, it is unclear which antibody preparation, method of tissue fixation, immunocytochemical reagents or schemes for immunohistochemical scoring is most useful. Standardisation of these methods must take place to give reliable estimates between groups.

It is of interest that *erb* B-2 has been implicated in the initiation of breast cancer rather than progression of the disease (Allred *et al* 1992). Further evidence for this is the fact that there is little change from tumour to metastases with respect to *erb* B-2 expression. (Tommasi *et al* 1992).

1.4.3.6 *Hst* and *int* -2

Hst and *int-2* are members of the Fibroblast Growth Factor (FGF) family of genes. The *hst* gene (also known as kFGF and as int-5), now assigned the name HSTF1 for heparin-binding secretory transforming factor, was originally isolated by gene transfer experiments with DNA from human stomach cancers and Kaposi's sarcoma (Deli Bovi *et al* 1987; Taira *et al* 1987), while *int-* 2 is a proto-oncogene identified as being adjacent to one of the integration sites of the proviral DNA of mouse Mammary Tumour virus (MMTV) (Nusse and Varmus, 1982). *Hst* and *int-* 2 are closely linked and in

the human genome both are encoded on chromosome 11q13. Activation of both genes is by transcriptional deregulation giving over-expression of the normal gene product. The product of the *hst* gene is 206 amino acids long and that of the int-2 is 220 amino acids long. They have sequence homology to basic and acidic FGF but, significantly, unlike the FGF genes, they carry a signal sequence which allows them to be secreted efficiently. Although the precise function of the gene product is not known, normal expression of *int-* 2 is thought to be restricted to defined stages of embryonic development (Wilkinson *et al* 1988) and transfection with *int-* 2 constructs in cell lines has led to small but reproducible changes in cell morphology and growth rate (Vijver *et al* 1991). A receptor specific for the *int-* 2 protein has not yet been identified.

Hst and *int-* 2 have been reported to be co-amplified in stomach cancer (Yoshida *et al* 1988). In breast cancer, the degree of gene amplification is low, and is not paralleled by expression of the gene (Fantl *et al* 1989). Varley *et al* (1988) reported an amplification of *int-* 2 sequences ranging from 2- to 20-fold in 23% of the studied 40 primary breast carcinomas. While Zhou *et al* (1989) reported amplification in only 19% (9 out of 46) of the primary tumours. Other workers also report similar modest amplifications of int-2 DNA in human breast cancers (Lidereau *et al* 1988). In addition to reporting co-amplification of *int* -2 and *hst* in about 20% of primary tumours, Fantl *et al* (1989) and Roux-Dosseto *et al* (1992) observed no significant correlation with any histopathological factors. Moreover, they also concluded that amplification of *int-* 2 and *hst* genes is not accompanied by marked over-expression. Co-amplification was also observed by Ali *et al* (1989) and Theillet *et al* (1989). Theillet *et al* (1989) also reported a correlation between RNA expression and gene amplification in the case of *hst* but not of *int-* 2, while Ali *et al* (1989) found a significant association between *int-* 2 amplification and subsequent

Varley *et al* (1988) have reported that the int-2 gene is altered in several amplified tumours with loss of an *ECo* R1 site. In most of the tumours with int- 2 amplification, a whole cluster of adjacent genes, including the oncogenes *hst* and *bcl*- 1 is co-amplified (Lidereau, *et al* 1988; Yoshida *et al* 1988).

1.4.3.7. Other oncogenes implicated in breast cancer

1.4.3.7.1. c-*erb* B-3

The third member of the ERB family to be identified was a 148kDa transmembrane growth factor receptor given the designation *erb* B-3. Two groups simultaneously discovered the gene using reduced stringency hybridisation of a v- *erb* probe, due to the high levels of homology in this receptor family. c- *erb* B-3 has 40% homology to the outer portion of *erb* and *erb* B-2 and 82% homology with the tyrosine kinase receptor domains. The ligand binding region is structurally related to *erb* and *erb* B-2 and may indicate "cross talk" of ligands, as evidence has shown low ligand specificity between binding of the *erb* and *erb* B-2 ligands. However, to date, the ligand for *erb* B-3 has not yet been identified.

Only one study has investigated the prognostic significance of *erb* B-3 in breast cancer, reporting 43/195 patients (22%) over-expressing the *erb* B-3 receptor, and this correlated with the mRNA level in these tumours. The over-expression of the *erb* B-3 protein correlated with the presence of lymph node metastases but not with the overall survival of the patients. Gastric and pancreatic cancer have shown greater overexpression of c-*erb* B-3 when compared to levels in breast cancer (Lemoine *et al* 1992).

Only limited data has so far been generated for *erb* B-3 and like *erb* B-2 more detailed studies with larger patient numbers are necessary to fully evaluate the importance of the *erb* B-3 protein and its prognostic utility.

1.4.4. Tumour suppressor genes ("Anti-oncogenes")

There is evidence that normal cells are controlled by genes which regulate activity of transforming genes and oncogene products. These have been called tumour suppressor genes and deletions or mutations in these important genes may play an important role in the generation of tumours. In cancer, the suppressor protein may be mutated and over-expressed with loss of its cellular control mechanisms. Along with deletions of the gene these may play an important step in carcinogenesis.

1.4.4.1. Retinoblastoma gene (RB)

Retinoblastoma (RB) is encoded on human chromosome 13q14 and is expressed as a nuclear phosphoprotein. It has long been implicated in the generation of childhood tumours of the developing retina, collectively known as retinoblastoma. This linkage with cancer susceptibility was confirmed with the cloning of the human RB-1 gene and the demonstration that the RB genes from sufferers of retinoblastoma contain mutations for both alleles. Transfection of the wild type retinoblastoma into transformed cells containing the mutant gene showed reinstatement of growth control. The retinoblastoma phosphoprotein is also able to bind various viral transforming gene products and inactivate them (De Caprio *et al* 1990) These early experiments suggested that RB was a member of a group of negative regulators of proliferation. Transfection studies indicated growth control involvement of RB and a central

role in cell proliferation. This hypothesis was tested in a gene knockout mouse for the RB gene (Lee *et al* 1992; Jacks *et al* 1992). The knockout embryo does not survive gestation and is terminated at about 12 to 13 days. At this point millions of cell divisions will have been already made without the absolute necessity for the RB gene product. Thus the hypothesis for a central role in cell division must be questioned. The results however indicate another possible role, that of controlling cell differentiation, as the embryo ceases cell division with terminal differentiated cells at the time of abortion. At this point it could be hypothesised that RB plays a crucial role for further decisions involving cell division.

It is of interest that the heterozygous knockout mouse develops pituitary tumours whereas the humans develop retinoblastoma.

In breast cancer and other cancers there have been reports of loss of expression of the RB gene (Spandidos *et al* 1992) but no linkage to the progression of the disease.

1.4.4.2 p53 tumour suppressor gene

p53 was first detected in SV40 transformed cells (Lane and Crawford, 1979; Lizer and Levine, 1979) where the protein was found to form an oligomeric complex with the SV40 oncogene product, large T antigen. Thus initially p53 was classified as a tumour antigen. However subsequent experiments have shown transforming ability and immortalisation of primary cells (Jenkins *et al* 1984), p53 was then categorised as an oncogene. Subsequently all of the transforming clones of p53 cDNA were shown to have originated from mutant forms (Hinds *et al* 1989). Recent findings have indicated that p53 protein has properties of a tumour suppressor gene,

negatively regulating the cell cycle and requiring loss of function mutations for tumour formation, as expression of cDNA or genomic clones of wild type p53 suppress the transformation of cells in culture and the tumourigenic potential in animals (Mercer *et al* 1990).

The human p53 gene was localised to the short arm of chromosome 17 at 17p13.1 (Benchimol *et al* 1985, McBride *et al* 1986). The organisation of p53 on the genome of other species is conserved and forms 11 exons interrupted by 10 introns. The expression of the human p53 gene has been shown to be controlled by two promoters of differing strengths and disruption of these promoters or the introns within the gene can cause abrogation of p53 expression (Reisman *et al* 1988; Reisman *et al* 1990).

p53 has been shown by immunofluorescence studies to be localised primarily in the nucleus although it has also been shown have cytoplasmic localisation in non transformed cells (Dippold.*et al* 1981). Using calf thymus DNA the p53 mutant protein derived from human tumours was shown to have a higher binding affinity for single stranded(ss) DNA rather than double stranded(ds) DNA which is the opposite to what is observed with wild type p53 (Steinmeyer *et al* 1988). DNA binding characteristics of p53 were studied and when the first 40 amino acids are removed from the amino terminal end of the protein the DNA binding is not affected. However an antibody with an epitope to the carboxyl end of the p53 protein also does not inhibit DNA binding. These results would suggest that the DNA binding domain lies between the two ends of the molecule (Steinmeyer *et al* 1988). This observation was strengthened when a 33 base pair sequence was identified that specifically bound to p53 (Kern *et al* 1991). These sequences are associated with DNA replication origins and this fact along with the role in gene

expression may suggest a dual role for the p53 protein in the regulation of growth control.

As p53 binds to both ssDNA and dsDNA regulatory sequences, the obvious question arises, is p53 a transcriptional regulatory factor? P53 has been shown to enhance the activity of a 5' Long Terminal Repeat (LTR) promoter and in mice a p53 responsive element for the muscle specific creatine kinase could be activated 80 fold in the presence of wild type p53 (Luria and Horowitz 1986). In humans similar regulatory affects were seen in the control of IL-6 transcription (Santhanam *et al* 1991). The wild type p53 protein has shown the ability for repression of c-*fos* and c-*jun* (Ginsberg *et al* 1991). It was noted that the amino terminal portion of the protein is a stronger transactivator than the full length protein, indicating that other areas of the protein are important in regulation of the transactivation activity (Fields and Jang 1990).

The involvement of p53 in the cell cycle was first suggested as p53 expression was not observed in quiescent (resting) lymphocytes (Milner and McCormick 1980), but was induced on mitogen stimulation of the cells. Using quiescent lymphocytes as a model, p53 expression could be followed during cell cycle progression using fluorimetry. p53 content was shown to increase from G1 to S and also between G2 and M phase suggesting that the expression of p53 may be correlated with cell growth rather than progression through any specific phases of the cell cycle (Danova *et al* 1990). However after microinjection of p53 specific antibodies into growth stimulated quiescent cells, the entry into S phase was inhibited, indicating that p53 is an essential part of the G1 to S transition (Mercer *et al* 1984) having no effect on the S to G2M transition. Other evidence of cell cycle involvement was shown by the addition of a potent inhibitor of G1, sodium butyrate, which blocked the

synthesis of p53. These results were obtained for both mutant and wild type forms, which would suggest a role at two distinct points the G0 to G1 and the G1 to S phases of the cell cycle.

Wild type p53 has a negative effect on the cell cycle, which is in direct contrast to the positive effect of mutant p53. Mercer *et al* (1990) have shown induction of wild type p53 in a variety of cell lines caused cell cycle arrest at G0/1 preventing the cells from entering S phase. At the protein level p53 can inhibit a cellular initiation protein essential to functioning of DNA polymerase α. (Gannon *et al* 1987). Thus p53 may affect both the proteins and the genes directly involved in cellular replication mechanisms.

Early experiments, as described above, have shown that p53 had transforming capacity. This would suggest that it may have a promoter/enhancer ability in cellular transformation (Rovinski *et al* 1988). However it was later realised that the mutant form of p53 had been used in immortalisation experiments (Hinds *et al* 1989). When the same experiments were repeated with wild type p53 transfections into primary or senescent cells, it was found that these cells could not be immortalised. The wild type p53 has been shown to reduce the effect of transformation (Finlay *et al* 1989) and the development of fast growing tumours (Shaulsky *et al* 1991).

The induction of apoptosis also known as programmed cell death, is a central part of normal cellular development, which involves a selective "editing" of cells. Work on mammalian cells had shown the importance of two oncogenes *myc* and *bcl-2* in positive and negative control of this process (Evan, 1992).

It has been shown that p53 expression can be induced by DNA damage and this would suggest a role in cellular repair mechanisms (Kastan *et al* 1991). Over-expression of wild-type p53 in a number of culture systems has been shown to induce apoptosis. However other cells types have shown only growth arrest (Michalovitz, 1990). These two concepts of DNA repair and apoptosis were explored in the thymocytes of p53 gene knockout mice (Lowe *et al* 1993; Clarke *et al* 1993). Thymocytes are an ideal model as they are a well evaluated system for apoptosis and clonal selection of lymphocytes. The results indicate that thymocytes from the gene knockout mice have 20 fold greater resistance to radiation before undergoing apoptosis, than thymocytes from normal mice. When comparing heterozygous mice to homozygous, the heterozygous mice containing only one copy of the p53 gene are more resistant to radiation induced apoptosis than homozygous mice with two copies of the gene implying a gene dosage effect. Induction of apoptosis may act as a defence against mutations in the genome of these cells, selectively editing mutated cells.

The p53 gene has been examined in a wide variety of primary tumours. Mutations in and/or reduction of heterozygosity of the p53 gene locus appear to be very frequent in many human tumours, including breast cancer. p53 gene has been most extensively studied in colon carcinomas, where 75-80% show a loss of both p53 alleles. Similar reduction to homozygosity has also been reported for breast tumours (Mackay *et al* 1988). In approximately 65% of informative cases, a region (p13.1) on the short arm of chromosome 17 which includes p53, has been reported to be lost in breast cancers (Mackay *et al* 1988; Devilee *et al* 1991). It has been hypothesised that the tumour may develop through loss of heterozygosity in one allele, and subsequently the other through point mutation, which gives rise to over-expression of an altered protein. The mutated protein is unable to exert the control function of the

normal wild type protein. Like those in the H-ras oncogene, mutations in the p53 gene appear at "hot spots" on the encoding gene, with residues such as 175, 248 and 273 being particularly significant. This complex data has only been rationalised by the proposition that the mutation of p53 on one allele, confers selective growth advantage on the cell even though the other allele expresses the normal wild type gene.

Studies on the p53 gene have involved the examination of human tumours for loss of gene sequences and immunohistochemical analysis for gene expression. In assessing gene expression advantage is taken of the fact that the wild-type p53 gene product has a short half life and low intracellular concentration, whereas most mutant forms appear to have a much longer half life and a high intracellular concentration. The identification of p53 in primary tumours by antibodies is therefore, taken to indicate that the gene has been altered. In the case of breast cancer, loss of one gene locus encoding p53, or over-expression of the mutated protein have been reported. Both Bartek *et al* (1990) and Cattoretti *et al* (1988), using immunocytochemical methods found the protein to be expressed in about 50% of human breast tumours. In the study of Cattoretti *et al* (1988), the choice of antibody used was important as one antibody detected mutant proteins in more tumours than the other. In the more recent study of Varley *et al* (1991), both loss of heterozygosity and p53 protein expression were examined. 86% of breast tumours exhibited either p53 expression or loss of heterozygosity with only a smaller percentage of samples being positive for both. They also found three patients positive for p53 expression but negative for loss of heterozygosity where clear mutations in the p53 gene could be mapped by the polymerase chain reaction (PCR).

Mutations in p53 gene are the most frequently observed in human cancers. Donehower *et al* (1992) attempted to investigate the role of p53 in

mammalian development and tumourigenesis by inserting a null mutation into the gene by homologous recombination in murine embryonic stem cells. Mice homozygous for the null allele appear normal but are prone to the spontaneous development of a variety of neoplasms by 6 months of age. These observations indicate that a normal p53 gene is dispensable for embryonic development, that its absence predisposes the animal to neoplastic disease, and that an oncogenic mutant form of p53 is not obligatory for the genesis of many types of tumours.

p53 has been implicated in a variety of functions from transactivation to induction of apoptosis. However the results from the gene knockout mouse would suggest that p53 is not as essential as previously thought. The large incidence of mutations of p53 would indicate a central role in tumourigenesis and use of the gene knockout mouse model will be in the future invaluable to test hypotheses for the function of the p53 protein.

1.4.4.3. Other tumour suppressor genes

Apart from the two well defined tumour suppressor genes there exist other tumour supressor genes. These include deleted in colon cancer (DCC) and WT-1 first discovered in Wilms tumour. To date, there are no published refereed reports of these being relevant to breast cancer.

1.4.5. Aneuploidy, oncogenes and tumour suppressor genes

In breast cancer changes in the DNA ploidy are observed in approximately 70% of all tumours. A few studies have compared expression of specific oncoproteins with DNA ploidy. Of these the c-*erb* B-2 protein and the c-*myc* have been related to aneuploidy in breast cancer (Tavassoli *et al.*, 1989). With some reports relating higher c-*erb* B-2 expression in tumours with tetraploid DNA (4N) (Bacus *et al* 1990; Lee *et al* 1992). There have been no

1.5. Aim of the study

This study was designed to utilise flow cytometry to assess both immunological and oncological parameters in breast cancer and to relate these two areas together. Flow cytometry has certain advantages over other techniques in that cells are analysed objectively giving a quantitative result for fluorescence. Flow cytometry can also select for cell types of a defined size such as lymphocytes to either include or exclude them from the analysis.

The samples analysed in this study are important as the tumour can be compared to the corresponding lymph node metastases in patients. Flow cytometry can exclude the lymphocytes and thus analyse only metastatic tumour cells. The lymphocytes can then be analysed separately for immune responses due to the tumour.

The flow cytometric studies included

(i) The expression of the T cell receptor γ/δ within the lymphocyte populations of a breast cancer patient.
(ii) immune responses in tumour invaded compared with tumour free axillary nodes from the same patient.
(iii) immune responses in tumour draining lymph nodes taken from the low and high axilla
(iv) Expression of p53 and c-*erb* B-2 proteins in primary and metastatic breast cancer.
(v) Relationship of p53 and c-*erb* B-2 expression to the immune response in the tumour draining axillary nodes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Human tissue

Peripheral blood, axillary lymph nodes and tumour were obtained from patients undergoing surgical resection for breast cancer at the Western Infirmary, Glasgow , Scotland.

2.1.2. Tumour cell and lymph node lymphocyte preparations

RPMI 1640	Gibco, Paisley, Scotland.
Penicillin Streptomycin	Gibco, Paisley, Scotland.

2.1.3. Peripheral blood lymphocyte preparations

Ficoll-Hypaque	Pharmacia fine chemicals Uppsala, Sweden.
RPMI 1640	Gibco, Paisley, Scotland.

2.1.4. Cryopreservation of lymphocytes and tumour cells

DMSO	BDH chemicals Ltd, Poole, England
Foetal Calf serum	Gibco., Paisley Scotland.

2.1.5. Plasticware

Centrifuge tubes and Universals	Sterilin Ltd., Feltham, England
Falcon 2052 FACs tubes	Becton Dickinson, Cowley, England.

2.1.6. Antibodies

All antibodies were obtained from Dakopatts Ltd., High Wycombe, England.

With the following exceptions:

Leucogate	Becton Dickinson, Cowley, England.
Sheep anti-mouse IgG (Whole ab) FITC conjugated	Amersham International plc, Amersham, England.
Mouse anti-human c-*erb* B-2	Oncogene Sciences, Cambridge, England
Mouse anti-human p53 Pab1801	A kind gift from Lionel Crawford ICRF, Cambridge.
Mouse anti-human γ/δ T cell receptor	A kind gift from Dr J Borst, Netherlands

2.1.7. Other Materials

Sephadex G25 column	Pharmacia, Cambridge, England

2.1.8. Buffers

Phosphate buffered saline (PBS) pH 7.2

170mM NaCl , 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4

Sheath Fluid pH 7.2

1.3mM NaCl, 0.02mM KCl, 20mM LiCl, 15mM KH_2PO_4, 10mM Na_2HPO_4,

10mM EDTA

Carbonate Buffer pH 9.0

0.25M Na_2CO_3, 0.15M NaCl

Citrate Buffer pH3.0

0.1M Na Citrate

2.2 Methods

2.2.1. Sample preparation of tumour and lymphocytes

Samples were obtained from the Western Infirmary, Glasgow, Scotland. None of the patients had received any pre-operative chemotherapy or radiation therapy. A central representative slice of tumour was taken, with the remainder sent to pathology for routine histology and grading. The lymph nodes collected were bisected, with one half collected for analysis and the other half sent to pathology for staging. The samples were transferred aseptically to sterile containers.

2.2.2. Cell harvesting

Tumour and lymph nodes were processed within 1 hour of collection to minimise turnover of the membrane receptors. 5ml RPMI was added to the sample to remove any traces of blood with 1ml of 10^4 units/ml penicillin, 10mg/ml streptomycin subsequently added to remove any traces of infection. The samples were transferred to a 90mm sterile petri dish containing 10ml RPMI medium. The tumour tissue was teased apart and disaggregated using a scalpel and a sterile needle. The spilled cells were then harvested, washed and centrifuged at 150g for 5 minutes. They were cryopreserved if not immediately used.

2.2.3. Isolation of peripheral blood lymphocytes (PBL)

10ml of venous peripheral blood taken during surgery was layered carefully on an equal volume of Ficoll-Hypaque. The tubes were centrifuged at 1400g for 20 minutes and the lymphocyte layer was carefully removed. The

lymphocytes were transferred to a tube containing 10ml RPMI and then washed once again. The lymphocytes if not used immediately were cryopreserved.

2.2.4. Cryopreservation

The cell pellet was resuspended in 3ml freezing medium containing 10% DMSO and 90% foetal calf serum. This cell suspension was divided into 1ml aliquots and transferred into cryopreservation vials which were placed in a -70°C freezer overnight prior to storage in liquid nitrogen.

On removal from liquid nitrogen the ampoules were thawed rapidly in a 37°C water bath and transferred to a sterilin tube containing 1ml of PBS. This was centrifuged at 150g for 5 minutes and the cells were resuspended in PBS for FACScan analysis.

2.2.5. Flow Cytometry

The flow cytometer used was the Becton Dickinson FACScan (**F**luorescence **A**ctivated **C**ell **S**canner). This allows simultaneous measurement of five parameters on a single cell consisting of two physical parameters forward scatter (FSC) and side scatter (SSC) and 3 fluorescence parameters. (1) FSC is the measure of incident light scattered in the forward direction and (2) SSC measures the degree of sideways light scatter for an estimation of granularity. The two physical parameters make it possible to distinguish between different cell types such as lymphocytes and tumour cells allowing gating of populations.

Fluorochromes

Three main fluorochromes fluorescein (FITC), phycoerythrin (PE), and propidium iodide(PI) are used for fluorescence channels 1, 2 and 3 respectively.

Fluorescein (FITC) ($C_{12}H_{11}NO_5S$) is a commonly used conjugate and can be easily attached on proteins and immunoglobulins giving a stable complex. FITC is green on low density staining and yellow on high density staining. Excitation range is 400-530nm, emission maximum 530 nm (Figure 2.1).

The second fluorochrome phycoerythrin (PE) (FL 2) is a member of the phycobiliproteins, consisting of highly fluorescent orange/red macromolecules. Coupling to immunoglobulins is achieved by protein-protein crosslinking methods by direct linking to thiol groups. Excitation range is 420-580nm, max 480, 545, 565. emission maximum 580 nm (Figure 2.1).

The third dye is propidium iodide (PI) which intercalates into RNA and nuclear DNA for discrimination of live and dead cells. PI excitation max is 493 nm and emission maximum at 630nm in the red part of the spectrum (Figure 2.1).

2.2.6. General FACScan procedures

2.2.6.1. General lymphocyte staining

Cell suspensions of LNL or PBL were washed after cryopreservation to remove any traces of foetal calf serum. These cells were resuspended at a cell

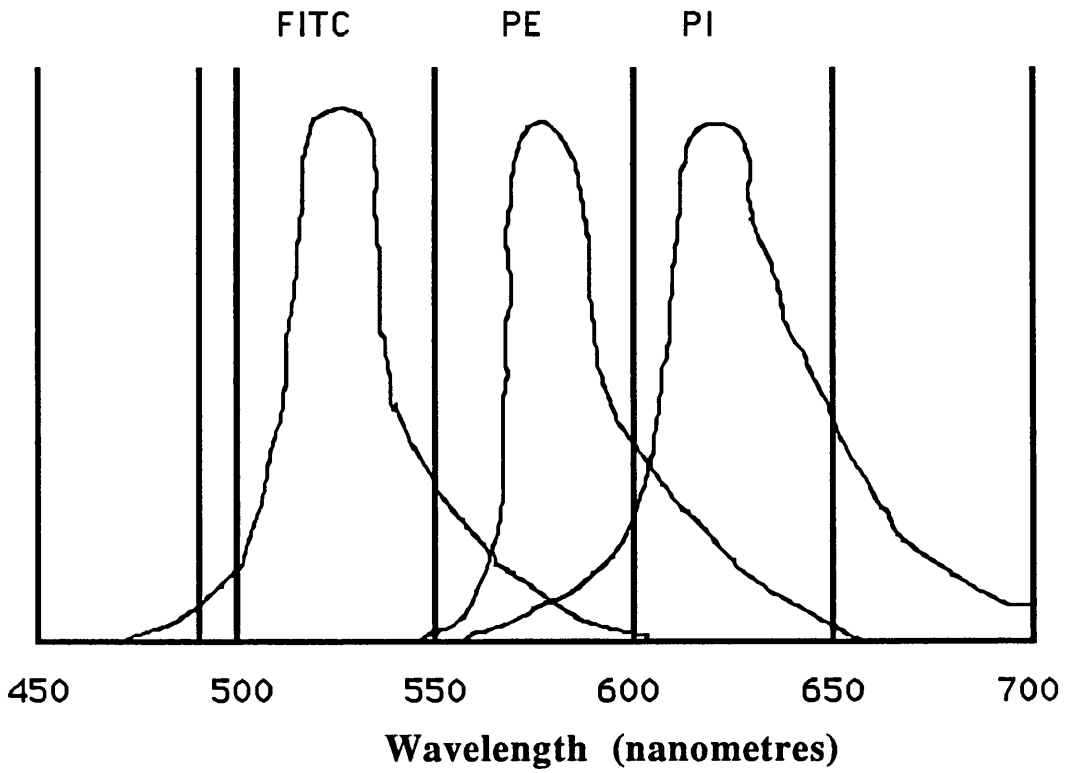


Figure 2.1 Emission characteristics of fluorescein (FITC), phycoerythrin (PE) and propidium iodide (PI).

density of 10^6/ml. Aliquots (50µl) were incubated for twenty minutes on ice with the relevant monoclonal antibody (Mab) to specific phenotypic and activation markers on the lymphocytic membrane (Table 2.1). This incubation was carried out in the dark to reduce photo-bleaching of the fluorochromes. Where the primary antibody was unconjugated the cells were washed with 1ml PBS and a suitable second antibody labelled with FITC would be added for a further 20 minute incubation. The cells were washed, centrifuged at 150g for 5 minutes and resuspended in 0.5ml PBS. Propidium iodide (2µg/ml) was added to discriminate between live and dead cells by exclusion gating (Figure 2.2).

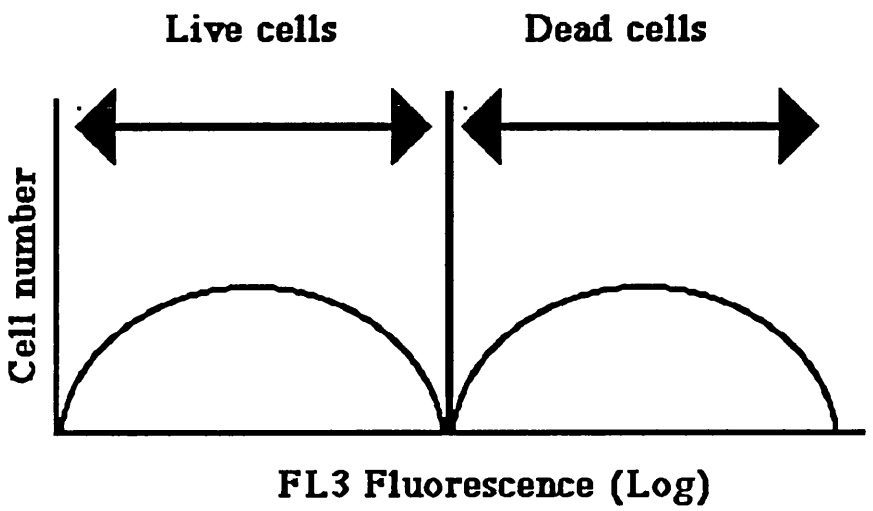


Figure 2.2 PI gating to exclude cells with breached membranes which have taken up PI and are highly FL-3 fluorescent.

2.2.6.2. Lymphocyte scatter gate.

A lymphocyte gate was set based on cell size, forward scatter (FSC) and granularity, side scatter (SSC). The validity of the gate was checked by the leucogate antibody (Figure 2.3). An irrelevant anti-mouse IgG monoclonal antibody was used to assess the non specific binding. 5000 events were collected for statistical relevance and analysed on the FACScan research program.

2.2.6.3. Live cell discrimination by propidium iodide gating

During the freezing and thawing of human tissue a proportion of cells will rupture and become non-viable due to the fragility of the cell membrane or ice crystal formation. Data from the viable cells with intact membranes are

Table 2.1. Monoclonal antibodies used for lymphocyte phenotyping and activation studies

First antibody	Second antibody	Co-expression
IgG1-FITC	IgG2a-PE	Control
Leucogate Anti-CD45-FITC	Anti-CD14-PE	Staining of leucocyte sub populations (lymphocytes, monocytes, neutrophils)
Anti-Leu 4-FITC	Anti-Leu12-PE	T-lymphocytes B-lymphocytes
Anti-Leu 3a-FITC	Anti-Leu2a-PE	CD4+ T helper cells, CD8+ T cytotoxic cells
Anti-Leu 3a-PE	Anti-HLA DR-FITC	Activated CD4+ helper T cells
Anti-Leu 3a-PE	Anti-CD25-FITC	IL-2 receptor on CD4+ helper T cells
Anti-Leu 2a-PE	Anti-HLA DR-FITC	Activated CD8+ cytotoxic T cells
Anti-Leu 2a-PE	Anti-CD25-FITC	IL-2 receptor on CD8+ cytotoxic T cells
Anti-Leu 12-PE	Rabbit $F(ab)_2$ anti-human IgG(γ chain)-FITC	Surface IgG expressing CD19+ B cells

collected since cells with ruptured membranes can bind the staining antibody non specifically. To distinguish between the populations, propidium iodide is added. The PI intercalates into DNA and this is detected on the red channel on the FACScan (FL3). Thus the red cells can be gated out of the analysis (Figure 2.2). The PI is also used for DNA staining of fixed cells (Section 2.2.6.7).

2.2.6.4. Analysis of lymphocyte staining

A quadrant was set on the G1/G2 non specific binding control to include 99.5% of cells for statistical relevance. The X co-ordinate and the Y co-ordinate of the quadrant were noted for gating. The quadrant analysis was used for T/B and H/C estimations (Figure 2.4). Analysis of lymphocyte subset activation required a dot plot gate to include only the positive staining lymphocytes (Figure 2.5a and b). A histogram was drawn and the X-coordinate from the G1/G2 (Figure 2.5c) non specific binding experiment was used as a mark to measure dual positive cells.

2.2.6.5. Lymphocyte staining method for the γ/δ T cell receptor

TILs, LNLs and PBLs were selected by FSC/SSC gating.The lymphocytes expressing the γ/δ T cell receptor (TCR) were identified with monoclonal anti-TCR-γ/δ which recognises both the disulphide and non-disulphide forms of the receptor (a gift from Dr J. Borst). For two colour FCM analysis, 1x 10^5 cells were incubated with the anti-γ/δ-TCR for 20 minutes, and washed twice before incubating with FITC-sheep anti mouse IgG for 20 minutes. After two more washes, the cells were blocked with mouse sera (diluted 1:50) for 20 minutes, and washed twice again. PE-conjugated antibodies (CD4, CD8, and CD3) were added to the cells for a 20 minute

Figure 2.3 Lymphocytic gating by forward scatter (FSC) and side scatter (SSC) parameters.

green= lymphocyte population, red=monocytes, cyan=non staining cells.
A illustrates the size parameters before forward scatter (FSC) and side scatter (SSC) before gating and **C** illustrates the lymphocyte population after gating. **B** illustrates the differential staining of the leucocyte populations before gating and **D** after lymphocyte gating.

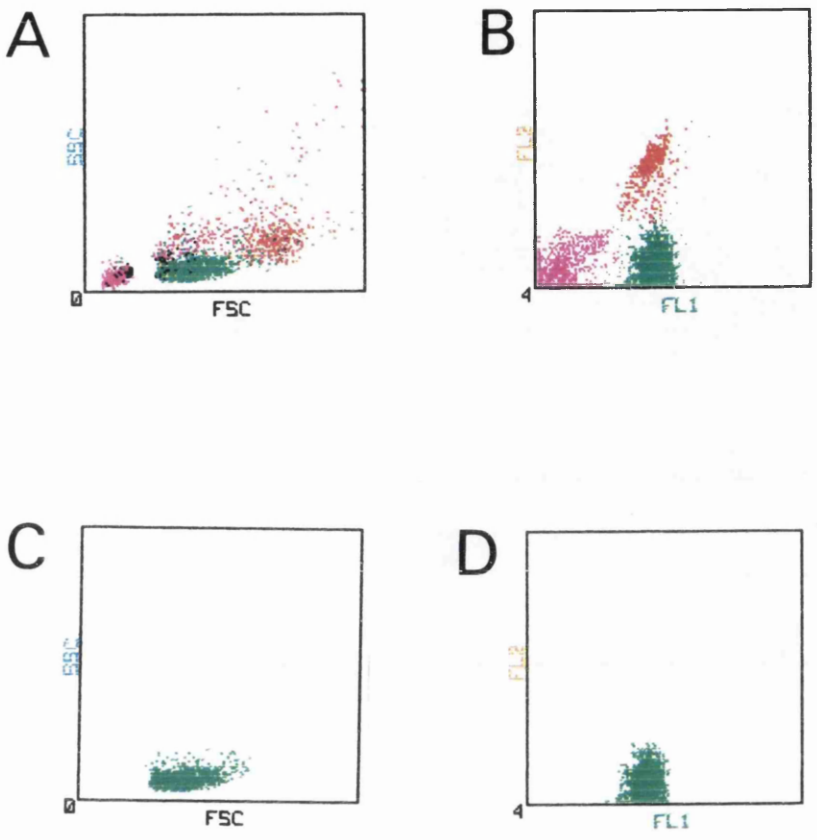

A
SSC
0
FSC
B
FL2
4
FL1
C
SSC
0
FSC
D
FL2
4
FL1

Figure 2.4 Phenotypic analysis of lymphocytes from breast cancer patients

A The G1/G2 level of non specific staining in the bottom left quadrant

B The CD3+ (T cell) staining in the bottom right quadrant and the CD19+ (B cell) staining in the top left quadrant

C The CD4+ (T helper) staining in the bottom right quadrant and the CD8+ (T cytotoxic) staining in the top left quadrant.

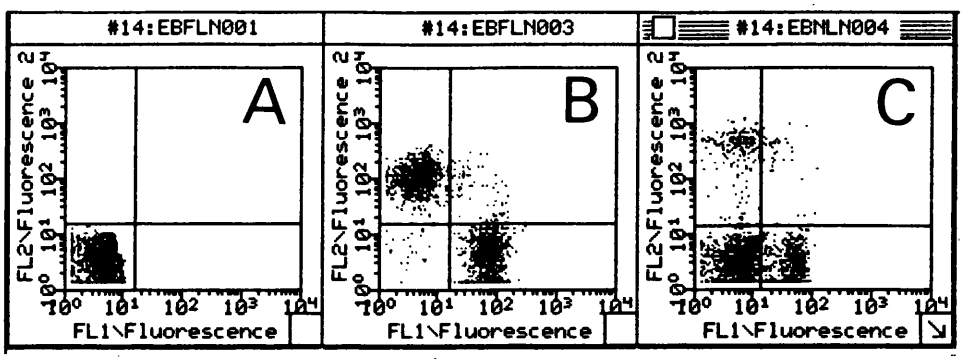

#14:EBFLN001
#14:EBFLN003
#14:EBNLN004
A
B
C
FL2\Fluorescence 2
FL1\Fluorescence
10^0 10^1 10^2 10^3 10^4

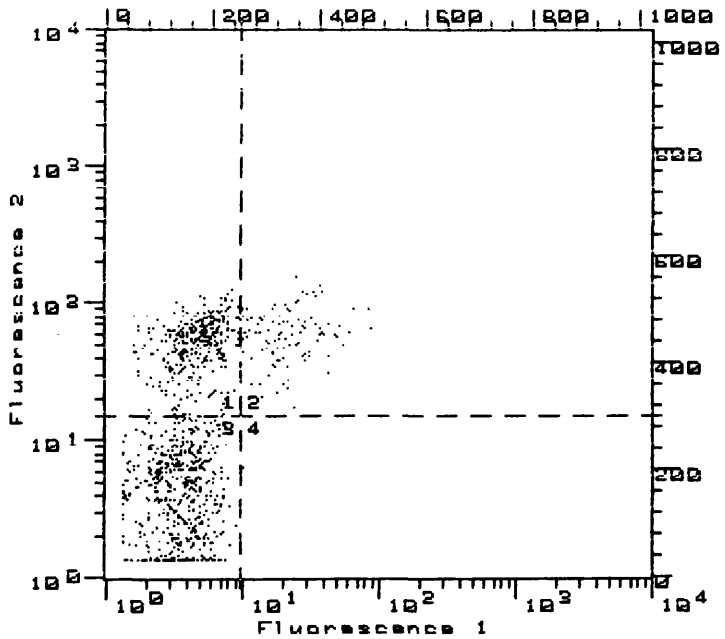
Figure 2.5 Analysis of lymphocyte activation marker expression

A Dot plot of anti-CD19 (FL-2) and anti-IgG staining (FL-1). The double stained CD19+ IgG+ show up on the upper right quadrant

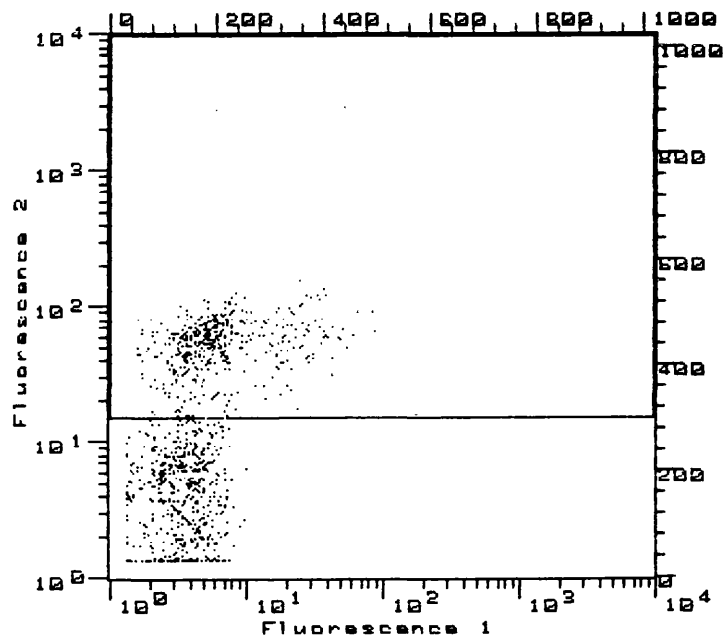
B. The proportion of cells expressing an activation marker (in this case IgG expression on CD19+ cells) is analysed by isolating the particular population on FL-2 (phenotype staining)

C. On a histogram on FL-1, the proportion of IgG + CD19+ B cells is assessed by placing a marker, the position of which is based on the control sample.

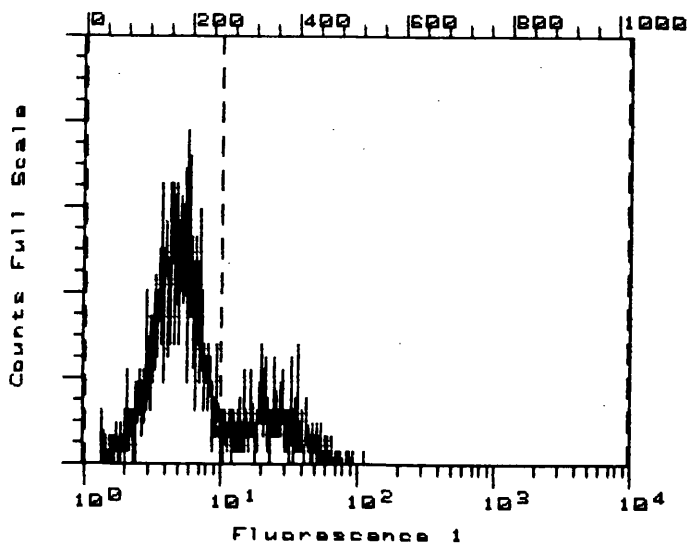
A


Fluorescence 2
Fluorescence 1
1
2
3
4

B


Fluorescence 2
Fluorescence 1

C


Counts Full Scale
Fluorescence 1

incubation before the final two washing steps. Antibodies with irrelevant specificities were used as negative controls.

2.2.6.6. Analysis of γ/δ T cell receptor expression

Percentages of γ/δ positive cells were calculated from histograms on FL1 (FITC) drawn after gating for CD3 positive cells (FL-2 channel) on an FL1 vs FL2 plot. Phenotypic analysis was performed by calculation from populations gated for CD4 and CD8 and expressing the result as a percentage of total CD3/4/8 + γ/δ + cells (Figure 2.6).

2.2.6.7. Dual Colour Flow Cytometry of oncoprotein and DNA

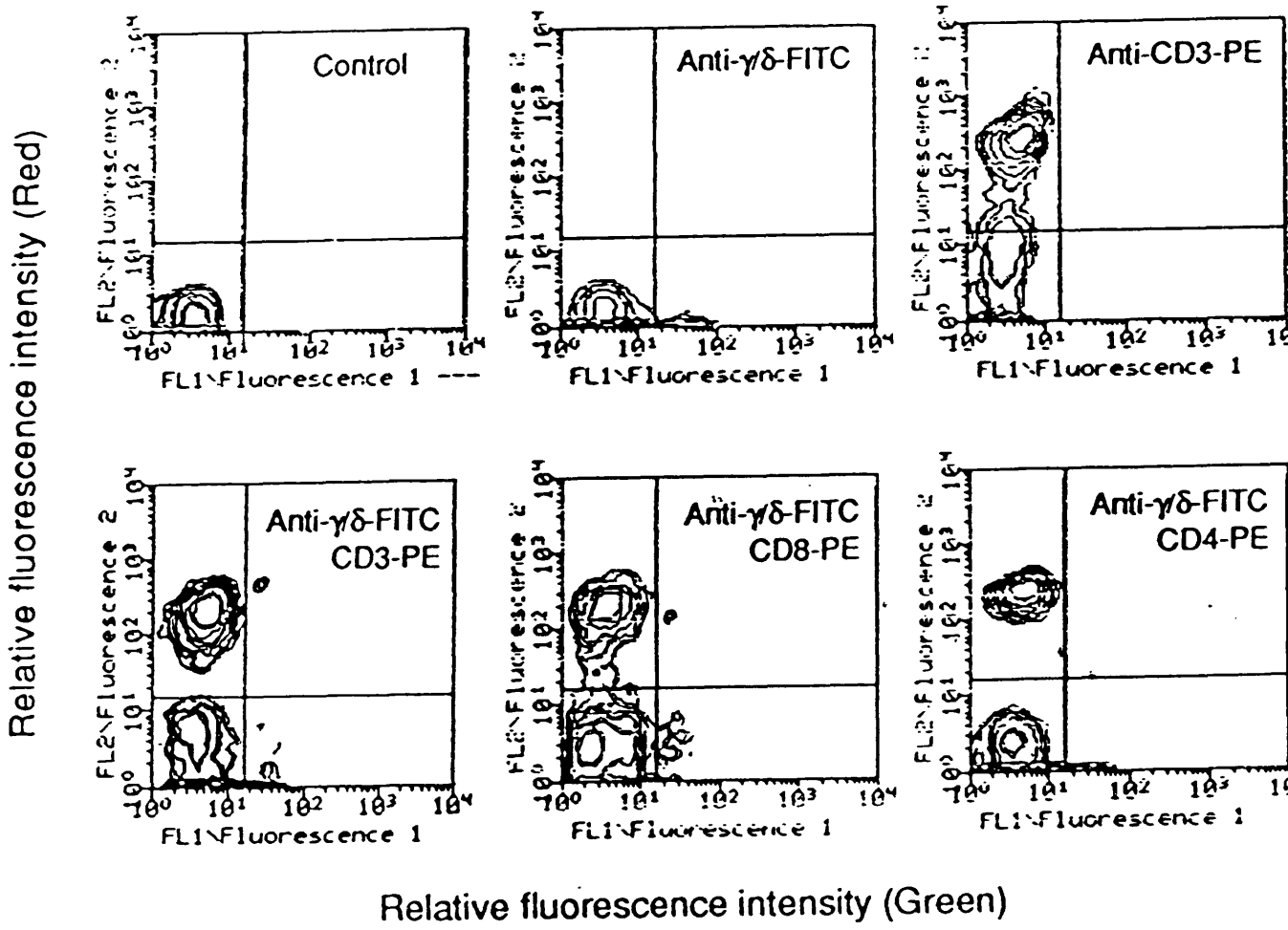
The tumour cells were washed and resuspended in 0.5ml PBS to a final concentration of 10^6/ml. 0.5ml 70% ethanol was added dropwise slowly whilst vortexing, ensuring complete mixing.The ethanol fixed cells were incubated on ice for 30 minutes. The cells were washed and resuspended in 100μl PBS. The p53-FITC, and the c-*erb* B-2 antibodies were added (10μg/ml final concentration) to the relevant tubes and incubated in the dark for 20 minutes and subsequently washed. To the c-*erb* B-2 tubes a second anti-mouse FITC labelled antibody was added. The cells were finally washed and resuspended in 500μl 20μg/ml propidium iodide to stain the DNA. The controls consisted of second FITC antibody added alone to the tumour cells and the p53 and c-*erb* B-2 staining of the PBLs.

2.2.6.8. Analysis of oncoprotein staining

The data was acquired on the Consort 30 program. The fluidics were set on the low mode to allow the nucleus of one individual cell to have the correct

Figure 2.6 Analysis of γ/δ T cell receptor expression

Two colour flow cytometry showing T cells expressing γ/δ receptors in TILs from a breast cancer patient. Lymphocytes in the cell suspension were incubated with monoclonal anti-TCR γ/δ (green fluorescence, FL-1) and or PE labelled monoclonal antibodies (orange fluorescence, FL-2) specific for the CD3 marker and the CD4 and CD8 subsets. The thresholds of positivity for green (horizontal line) and orange (vertical line) fluorescence were established by incubation with unrelated antibodies (Figure 2.4A).


Control
Anti-γ/δ-FITC
Anti-CD3-PE
Anti-γ/δ-FITC
CD3-PE
Anti-γ/δ-FITC
CD8-PE
Anti-γ/δ-FITC
CD4-PE
FL1↑Fluorescence 1
FL2↑Fluorescence 2
Relative fluorescence intensity (Red)
Relative fluorescence intensity (Green)

shape for DNA acquisition. Peripheral blood lymphocytes (PBLs) from the same patients were stained as a diploid control. For analysis of the tumour samples, the lymphocytes present in the tumour and lymph node lymphocytes were gated out using the forward scatter (FSC) and side scatter (SSC) parameters the competence of this gate being confirmed by analysis of a sample of PBLs from the same patient at the same time. The tumour cells were stained with a sheep anti- mouse IgG polyclonal antibody conjugated to FITC for assessment of non specific binding (Figure 2.7). Analysis gates were constructed on the lymphocyte samples and checked against the non specific binding to the tumour cell (Fig 2.7 B and E). The number of positive cells in the analysis gates of the control samples was usually 5% or less, this was not subtracted from the positive samples which may therefore contain up to 5% of cells showing non specific binding. For ploidy determination a simultaneous propidium iodide DNA stain was used.

2.2.7. FITC labelling of the p53 antibody

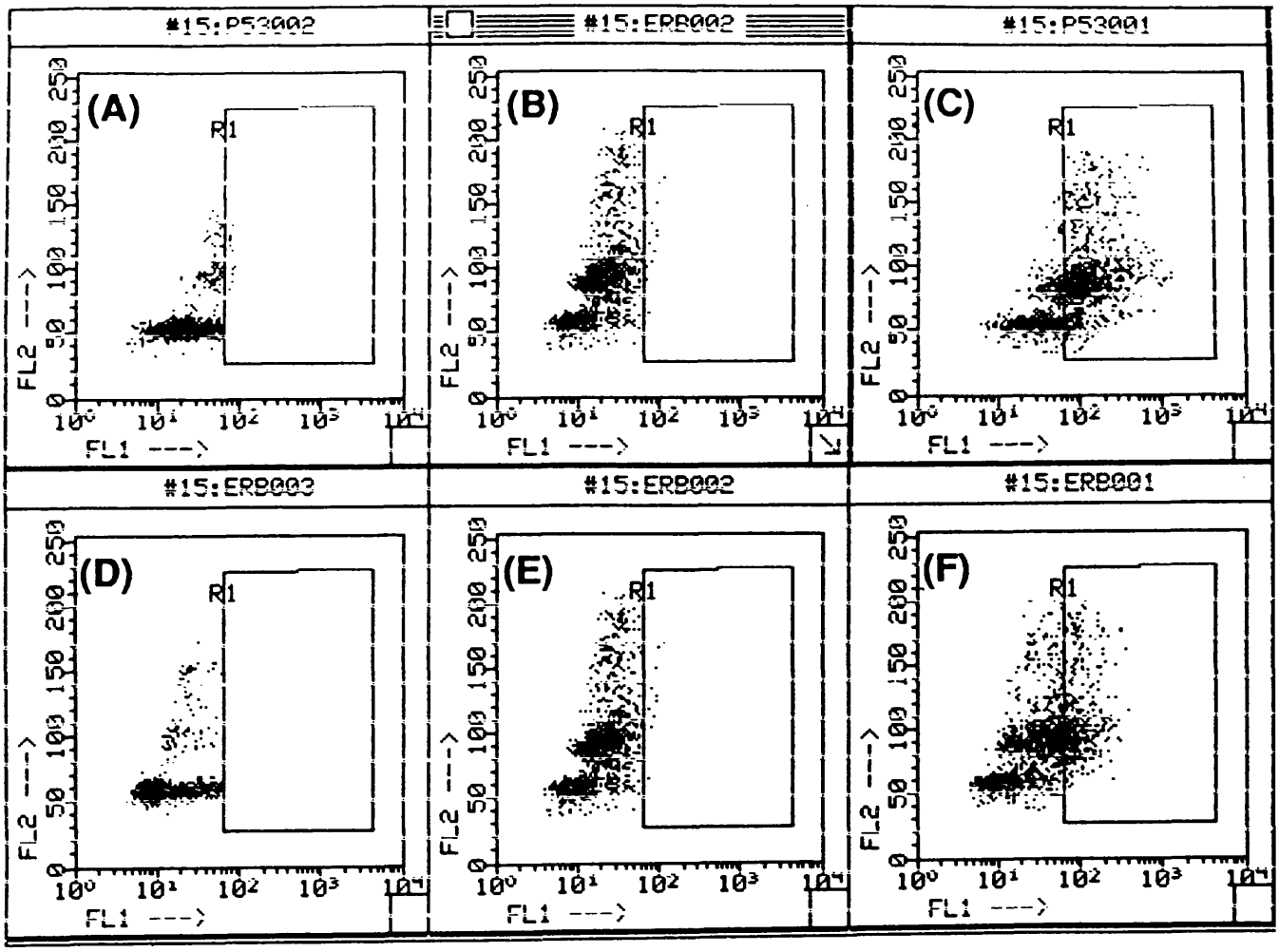
0.05mg of FITC was added to 1mg/ml antibody in a maximum volume of 0.5ml 0.25M carbonate buffer /0.15M NaCl pH 9.0. Incubation was overnight at 4^{o}C whilst shaking. Samples were eluted through a Sephadex G-25 column to separate bound from free FITC based on molecular size.

2.2.8. Fluorescence microscopy

For examination of p53 and c-*erb* B-2 staining 50µl of tumour cell suspensions (10^6/ml) were stained with 10µl (100µg/ml) of the relevant

Figure 2.7 Dual parameter flow cytometric plot of oncoprotein staining (Fluorescence 1) and DNA amplification (ploidy) (Fluorescence 2) for patient 3 showing the gating for positive cells.

A and **D** represent monoclonal antibody staining of the patient peripheral blood lymphocytes (PBLs) for p53 and c-*erb* B-2 respectively. **B** and **E** represent control staining of tumour cells with a sheep anti mouse IgG (Fluorescence 1) antibody for assessment of non specific binding.
C and **F** correspond to positive staining of tumour cells for p53 and c-*erb* B-2 respectively.


#15:P53002
(A)
R1
FL2 --->
FL1 --->
#15:ERB002
(B)
R1
FL2 --->
FL1 --->
#15:P53001
(C)
R1
FL2 --->
FL1 --->
#15:ERB003
(D)
R1
FL2 --->
FL1 --->
#15:ERB002
(E)
R1
FL2 --->
FL1 --->
#15:ERB001
(F)
R1
FL2 --->
FL1 --->
0
50
100
150
200
250
10^0
10^1
10^2
10^3
10^4

fluorescein conjugated antibody. This incubation was for 20 minutes on ice in the dark. The samples were washed in 1ml PBS and resuspended in 100µl of PBS. The samples were applied in 10µl portions to a microscope slide which was then examined in a Leitz orthoplan microscope.

2.2.9. Statistics

All statistical analyses were carried out using Statworks software for the Macintosh computer.

For all comparisons non parametric tests have been used for the comparison of different groups. Statistical significance is taken at 5%, corresponding to less than or equal to p=0.05.

The Wilcoxon signed rank test was used for the comparison of tumour free and tumour invaded lymph nodes removed from the same patient in Chapter 4.

The Mann-Whitney U test was used for the comparison of two independent groups such as the expression of p53 and c-*erb* B-2 within the diploid and aneuploid tumour subsets in Chapter 6.

The comparison of more than two independent variables was carried out using the Kruskal-Wallis test, such as for p53 and *erb* B-2 expression within the three gradings of tumour (Chapter 6).

The Spearman Rank test was used to assess the assess p53/c-*erb* B-2 and the corresponding increase or decrease in the immune response (Chapter 7).

For comparison of small numbers the students T test was used (Chapter 5).

CHAPTER 3

T CELL RECEPTOR γ/δ EXPRESSION ON LYMPHOCYTE POPULATIONS OF BREAST CANCER PATIENTS

3. T cell receptor γ/δ expression on lymphocyte populations of breast cancer patients

Relatively little is known about the function of the gamma delta subset of the T cell receptor (Section 1.2.4.2.). Recent studies have revealed that like the α/β receptor it is stably associated with the CD3 molecule and can also be associated with the CD8 and CD4 proteins on T cells. These γ/δ T cells have been shown to have significant junctional diversity within the δ gene, giving the γ/δ T cell receptor the ability to be potentially as diverse as the α/β receptor and IgG. This wide diversity is suggested to contribute to the possible functions of this receptor, which has been shown to have increased expression in coeliac disease, rheumatoid arthritis and certain bacterial infections. This has led to the suggestion of the γ/δ T cells playing a role in immunosurveillance.

In this study the quantitative distribution and phenotype of the γ/δ T cell receptor was measured within the peripheral blood lymphocytes (PBL), lymph node lymphocytes (LNL) and tumour infiltrating lymphocytes (TIL).

The methodology of lymphocyte staining and gating procedures are described in Sections 2.2.6.5. and 2.2.6.6.

Lymphocyte phenotype was defined by flow cytometry and divided into three main categories. (1)CD3+ γ/δ+, (2) CD8+ γ/δ+ as a percentage of CD3+ γ/δ+ and (3) CD4+ γ/δ+ as a percentage of CD3+ γ/δ+

The values obtained for CD3+ CD4+ γ/δ+ and CD3+ CD8+ γ/δ+ were expressed as a percentage of the total obtained for CD3+ γ/δ+, as both CD8 and CD4 glycoprotein molecules stably associate with the CD3 protein. The results are expressed in median form due to the skewed nature of the data.

3.1 Distribution of the γ/δ+ T cells in the TIL, LNL and PBL populations

The median percentages of CD3+ γ/δ+ cells in lymphocytes from each analysed source are shown in Figure 3.1 and Table 3.1. The results show TIL 5.4% (quartile range 1.6 - 7.4%), PBL 3.0% (quartile range 1.3 - 6.0%), LNL 3.6% (quartile range 3.5 - 12.5%). The greatest range of expression is found in the LNL, whereas the TIL and the PBL show much smaller ranges of γ/δ expression (Figure 3.1). With individual patients expression for TIL, PBL and LNL is illustrated in Figures 3.2a, 3.2b and 3.2c.

A small CD3- CD19- population positive for γ/δ was observed during analysis of TIL samples where the lymphocytes were not separated from non lymphocytic cells. This probably represents non specific binding of antibody to tumour cells within the TIL samples. All analyses of γ/δ TCR expression were therefore directed to the CD3+ population.

No correlation was observed with the number of γ/δ cells in the TIL population and the progression of disease as assessed by the grade of tumour and the stage of the tumour (lymph node involvement).

The overall expression of the receptor in the three tissues was similar with the exception of two patients with a higher proportion in the lymph node (33% and 25%) (Figure 3.1). Within the same patient the expression of γ/δ receptor in the TIL population did not correlate with that in peripheral blood and lymph node.

Table 3.1

Percent expression of the γ/δ T cell receptor within the CD3+, CD4+ and CD8+ T lymphocyte subsets of breast cancer patients.

Source	TCR γ/δ CD3+ as a % of CD3+ lymphocytes	CD4+ γ/δ+ as a % of CD4+ lymphocytes	CD4+ as a % of CD3+γ/δ+ lymphocytes	CD8+ γ/δ+ as a % of CD8+ lymphocytes	CD8+ as a % of CD3+γ/δ lymphocytes
TIL	5.4	10.4	4.4	14.5	45.7
PBL	3.0	12.4	3.8	11.3	15.4
LNL	3.6	9.5	18.0	12.9	0.3

Fresh lymphocytes from peripheral blood (PBL), lymph node lymphocytes (LNL) and tumour infiltrating lymphocytes (TIL) were obtained from breast cancer patients. The cells were stained with mouse monoclonal anti TCR γ/δ antibody and detected via a second anti mouse antibody conjugated to fluorescein (FITC). The CD4 and CD8 subsets were analysed using specific antibodies directly conjugated to phycoerythrin (PE). Values represent the median percentage obtained from analysis of 13 tumour and 11 peripheral blood and lymph node samples. Percentages of γ/δ+ cells were determined on gated CD3+ populations and percentages of CD4+ and CD8+ populations were calculated as a percentage of CD3+ γ/δ+ cells (Section 2.2.6.6.).

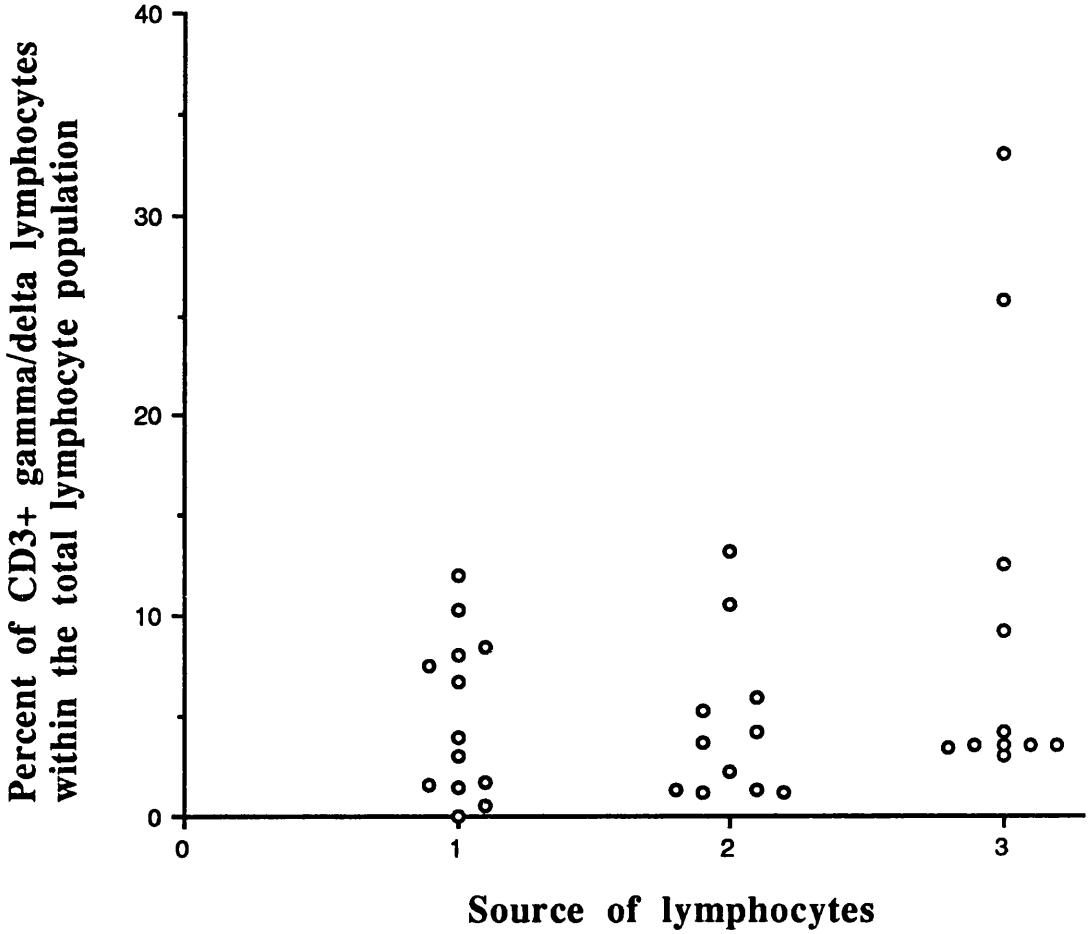


Figure 3.1 Percent of CD3+ γ/δ+ T lymphocytes within the total gated lymphocyte populations from (1) TIL (2) PBL and (3) LNL

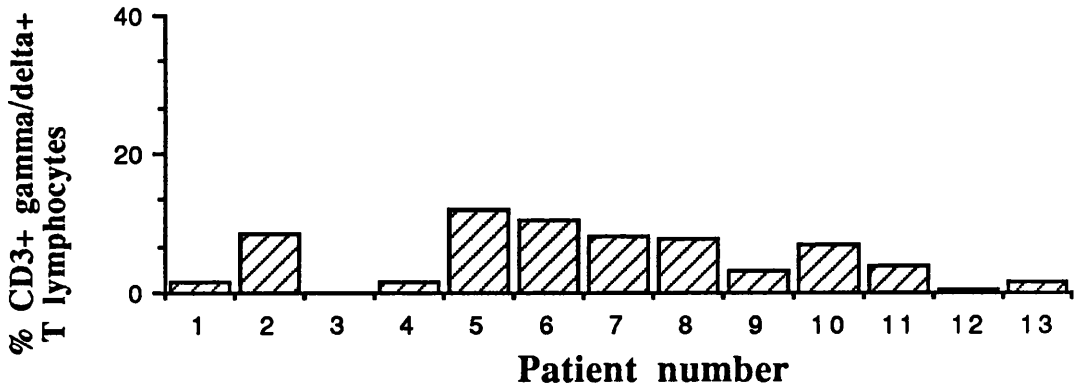


Figure 3.2a Percentage expression of CD3+ γ/δ+ T lymphocytes within CD3+ gated TIL population.of 13 breast cancer patients.

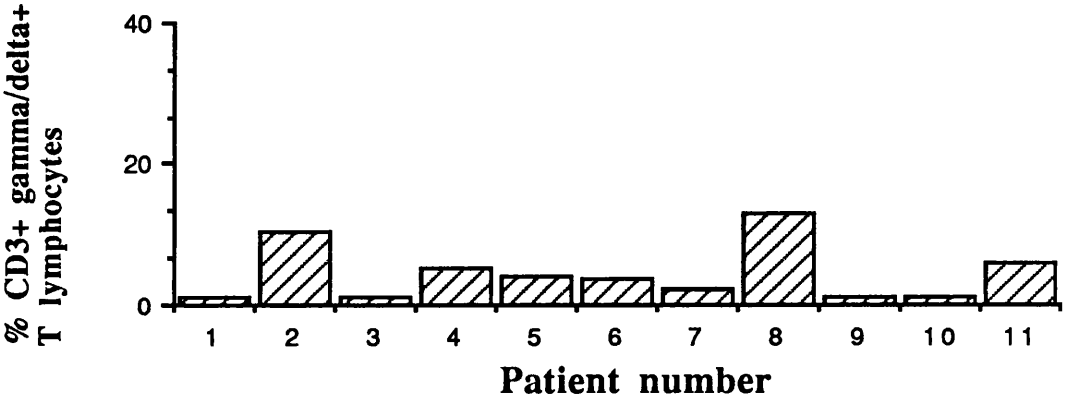


Figure 3.2b Percentage expression of CD3+ γ/δ+ T lymphocytes within the CD3+ gated PBL population of 11 breast cancer patients.

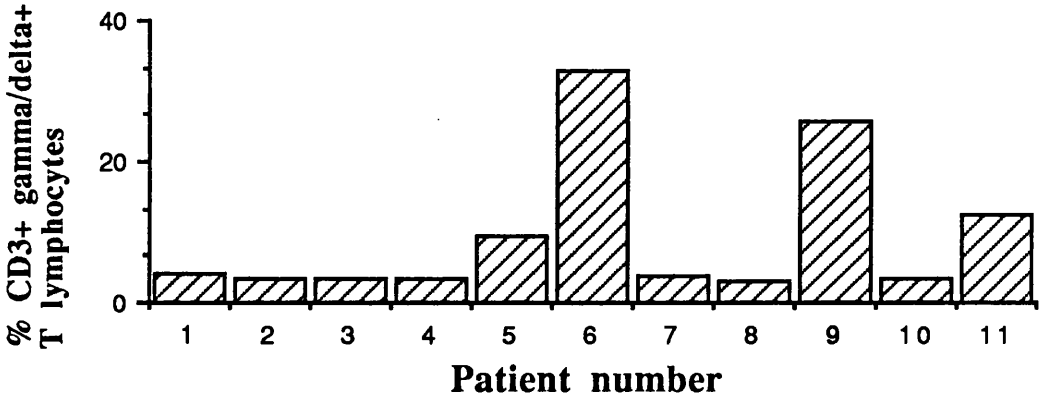


Figure 3.2c Percentage expression of CD3+ γ/δ+ T lymphocytes within the CD3+ gated LNL population of 11 breast cancer patients

3.2. Phenotypic marker expression of γ/δ T cells

The only T cell phenotype consistently expressed within the CD3+ γ/δ population of the tumour infiltrate was the CD8 marker (Table 3.1; Figure 3.3b) the median expression (45.7%) being greater than that of PBL and LNL. Patient expression in TIL, PBL and LNL is illustrated in Figure 3.4a, 3.4b and 3.4c.

In the peripheral blood within the CD3+ γ/δ+ lymphocyte population a low percentage of γ/δ+ CD8+ (median 15.4%) and γ/δ+ CD4+ (median 3.8%) expression was observed in a few cases but otherwise the majority of γ/δ cells were double negative (CD4- CD8-).

In the nodes within the CD3+ γ/δ population CD4+ γ/δ T cells were found to be predominant with a median value of 18% (Figure 3.3a). The CD4 expression on γ/δ+ cells in the lymph node was higher than CD8 expression (median 0.3%), which is the opposite to that observed in the TIL population. The majority of γ/δ+ cells in the node are double negative. Each patient expression within TIL, PBL and LNL is illustrated in Figures 3.5a, 3.5b and 3.5c.

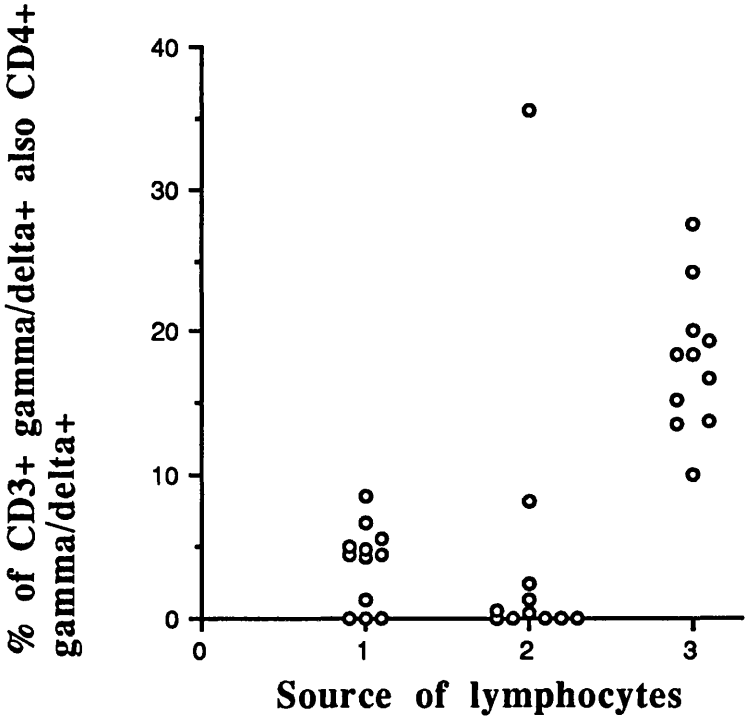


Figure 3.3a Distribution of CD3+ γ/δ+ T lymphocytes also CD4+γ/δ+ within the total gated lymphocyte populations from (1) TIL (2) PBL and (3) LNL

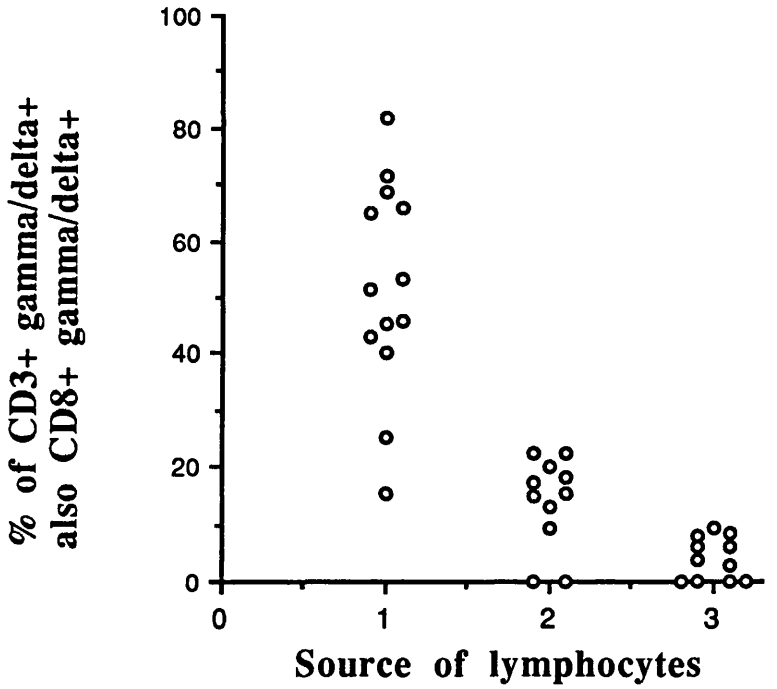


Figure 3.3b Distribution of CD3+ γ/δ+ T lymphocytes also CD8+γ/δ+ within the total gated lymphocyte populations from (1) TIL (2) PBL and (3) LNL

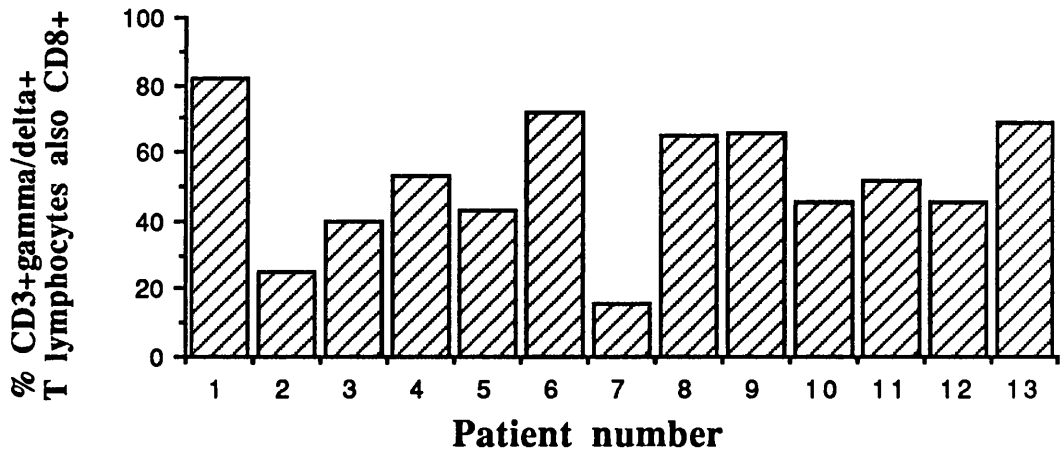


Figure 3.4a Percentage expression of CD3+ γ/δ+ T lymphocytes also CD8+ γ/δ+ within the gated TIL population of 13 breast cancer patients

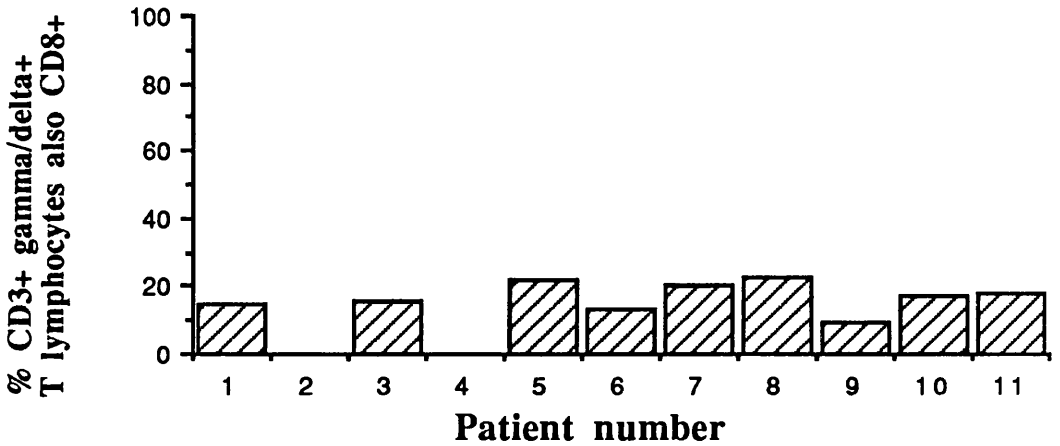


Figure 3.4b Percentage expression of CD3+ γ/δ+ T lymphocytes also CD8+ γ/δ+ within the gated PBL population of 11 breast cancer patients

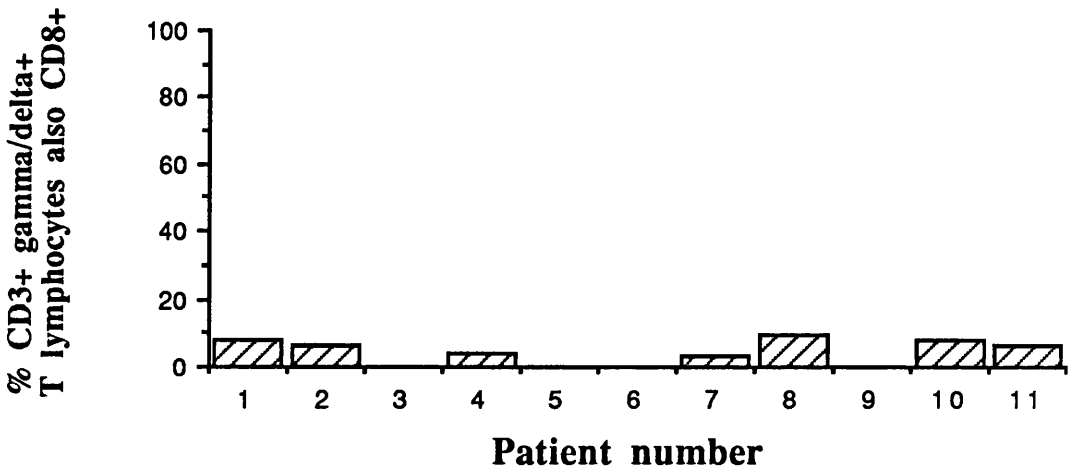


Figure 3.4c Percentage expression of CD3+ γ/δ+ T lymphocytes also CD8+ γ/δ+ within the gated LNL population of 11 breast cancer patients

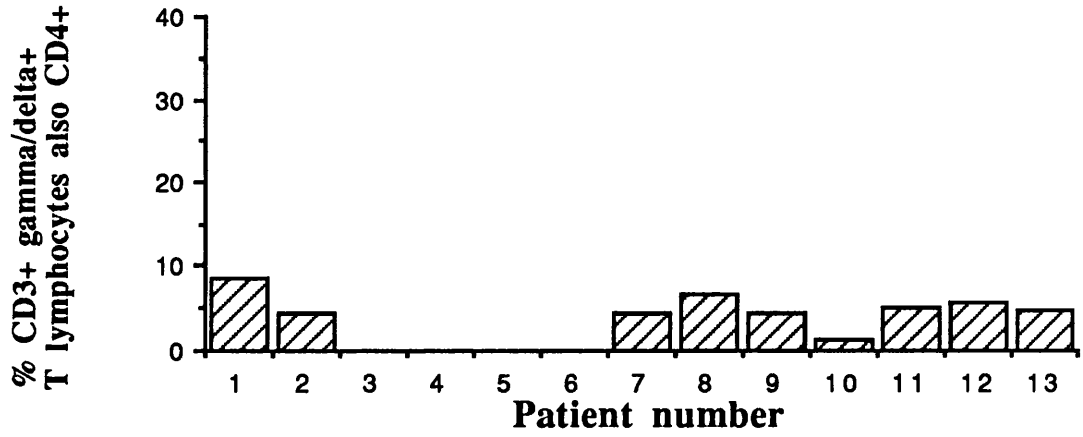


Figure 3.5a Percentage expression of CD3+ γ/δ+ T lymphocytes also CD4+ γ/δ+ within the gated TIL population of 13 breast cancer patients

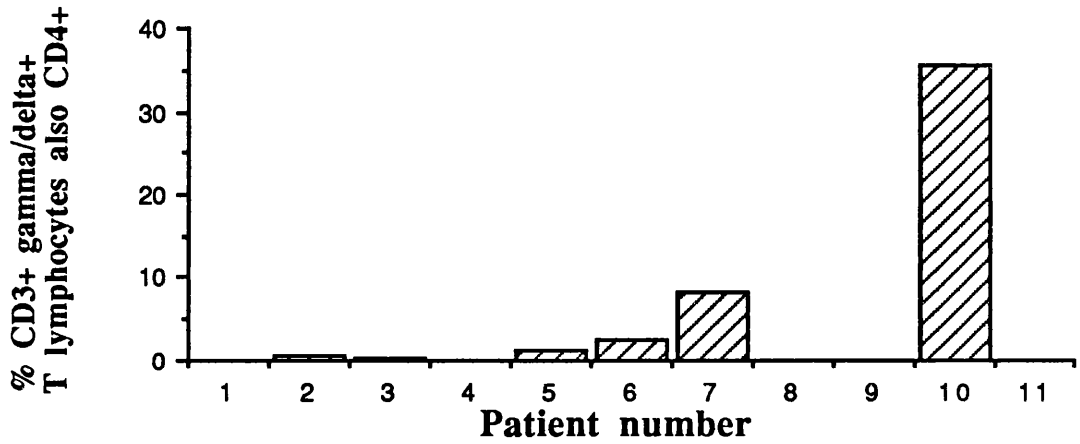


Figure 3.5b Percentage expression of CD3+ γ/δ+ T lymphocytes also CD4+ γ/δ+ within the gated PBL population of 11 breast cancer patients

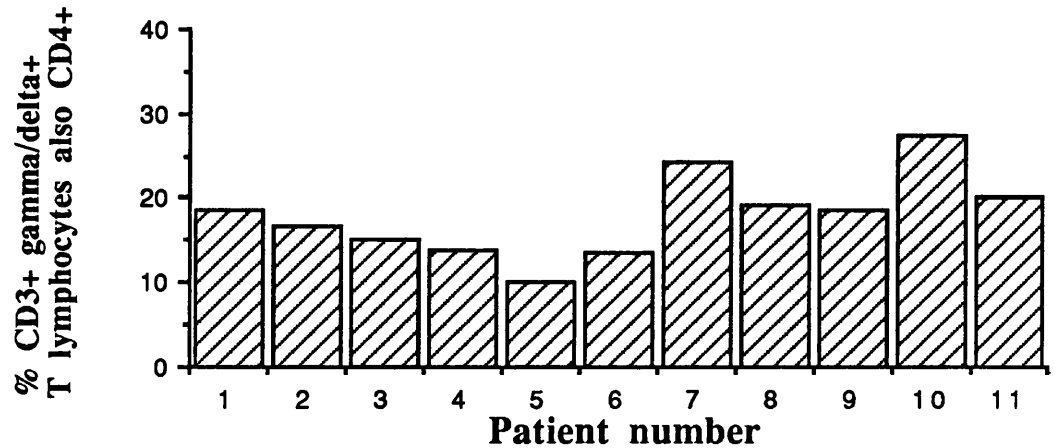


Figure 3.5c Percentage expression of CD3+ γ/δ+ T lymphocytes also CD4+ γ/δ+ within the gated LNL population of 11 breast cancer patients

3.3 Discussion

γ/δ T lymphocytes represent a small subset found in both normal patients and in some disease states. However little work has been reported on cancer and the phenotype of lymphocytes within cancer patients.

Localisation of γ/δ T cells in specific areas of the mouse such as the epithelia and mucosa has been reported depending on the Vγ usage. The exposed nature of these tissues would suggest regular direct interfacing with external antigens. However in humans this preferential homing of Vγ subsets is not seen with human γ/δ T cells possibly having a different function. Recent studies have shown that γ/δ T cells respond to heat shock proteins and also bacterial antigens which may suggest that there is a potential cytolytic role against both stressed and transformed cells. γ/δ T cells in the TIL, PBL and LNL of breast cancer patients show no relation to the disease state and tumour dissemination. The level of expression in PBL is similar to levels reported for healthy individuals (Itohara *et al* 1990), however the expression of γ/δ T cell receptor within the node and the tumour was shown to be elevated.

Within the TIL population the CD8 marker was consistently expressed on a substantial proportion of CD3+ γ/δ cells (median 45.7%). Breast cancer TILs have been reported to be predominantly CD8+ (Whitford *et al* 1991) with the infiltrate highly activated as represented by the expression of HLA DR and the IL-2R when compared with the PBL (Whitford *et al* 1991). Cytotoxicity of γ/δ TIL has been reported and in addition TIL T cell lines grown in IL-2 have been reported in certain lung tumours to have cytotoxicity for autologous tumour. However this is based on a small number of patients and so far no other isolated γ/δ T cell line from TILs has shown this specific cytotoxicity (Zocchi *et al* 1990). Other specific γ/δ T cell subsets have been shown to be

cytotoxic for a number of cancer cell lines dependent on specific Vγ usage. High expression of the γ/δ T cell receptor on cytotoxic T cells within the tumour suggests that the marker may have relevance in the possible cytolytic function of these cells.

The LNL population contained a larger proportion of CD4 cells expressing the γ/δ T cell receptor (median 18.0%).The expression within the tumour and the blood was confined to a much smaller population. The LNL which are found within the nodes of breast cancer patients have been reported to be predominantly of the CD4 phenotype (Whitford *et al* 1992) and to be highly activated compared to peripheral blood. If CD4 γ/δ T cells perform a similar function to the α/β T cells, the increase in expression of the CD4 γ/δ subset in the nodes may suggest that these may be also involved in the nodal immune response against the tumour.

The phenotypic analysis of the γ/δ T cells from the three lymphoid tissues presents one particularly interesting feature. In agreement with previous reports, the majority of the γ/δ T cells in both PBL and LNL were double negative. (CD4- CD8-). The double negative cells would represent lymphocytes not utilising MHC recognition but rather recognition mediated by another method, involving possibly undiscovered antigen presentation molecules. The large percentage of these cells may suggest that they have a crucial role within specific areas of the body.

The changes observed within the γ/δ T cell CD4+ and CD8+ subsets and more interestingly the presence of the double negative cells also reported by other groups would indicate that lymphocytes have an altered γ/δ phenotype within breast cancer patients. The further understanding of the so far ambiguous functional role of the γ/δ TCR in cancer and other disease states

may answer the question of its role in immunosurveillance and the immune response.

CHAPTER 4

THE EFFECT OF METASTATIC TUMOUR ON THE IMMUNE RESPONSE WITHIN THE TUMOUR DRAINING LYMPH NODES

4. The effect of metastatic tumour on the immune response within the tumour draining axillary lymph nodes

The immune response between two patients is extremely variable as noted in the study of Whitford *et al* (1992b), and it would be useful to reduce this interpatient variability to a minimum. As the tumour draining nodes have been shown to be eliciting an immune response against the tumour (Whitford *et al* 1992b), two lymph nodes were taken from the same patient. These lymph nodes are a site where the metastatic tumour can interact with the immune response and in consequence of this, activation and phenotype of lymphocytes within these nodes may be altered. Thus a comparison can be made within the axillary lymph nodes from the same patient, to show the changes occurring with tumour invasion in one node by comparing this with an uninvaded node, thus removing the considerable interpatient variability. Data in this chapter report such a study.

22 patients were entered in the study and from each a heavily tumour invaded lymph node and a tumour free lymph node were removed. Distance from the tumour was not assessed The methodology of staining and gating is described in Section 2.2.6.1 and Section 2.2.6.2

4.1. Phenotypic markers

4.1.1. T and B lymphocytes

Little change was noted in the mean percentage values of T and B lymphocytes between invaded and uninvaded lymph nodes (Table 4.1). The

Table 4.1.

Distribution of phenotypic markers on lymphocytes from tumour invaded and tumour free lymph nodes of 22 stage II breast cancer patients.

Phenotypic marker	STAGE II Tumour invaded	Tumour free	Statistical significance+
CD3 (% T cells)	44.8+/-3.9	43.8+/-3.9	p= 0.160
CD19 (% B cells)	42.9+/-4.5	45.8+/-3.6	p= 0.120
CD8 (% $CD8^+$ T cells)	16.7+/-2.2 (12.7+/-10.6)*	9.1+/- 0.9 (8.0+/-3.9)*	p<0.001
CD4 (% $CD4^+$ T cells)	29.8+/-2.6	36.7+/-3.3	p= 0.008
CD4/CD8 RATIO	2.6+/-0.4	4.8+/-0.6	p< 0.001

Values represent mean % +/- standard deviation of lymphocytes expressing the phenotypic marker.

* values in bracket represents median +/- standard deviation (SD). Median values and SD are cited for cases where a distribution appeared to be skewed (marked with asterisk).

+Statistical test used: Wilcoxon signed rank, when comparing tumour invaded nodes from the same patient to tumour free nodes. Statistical significance is taken at a p value equal to or < 0.05. N.S. is not significant.

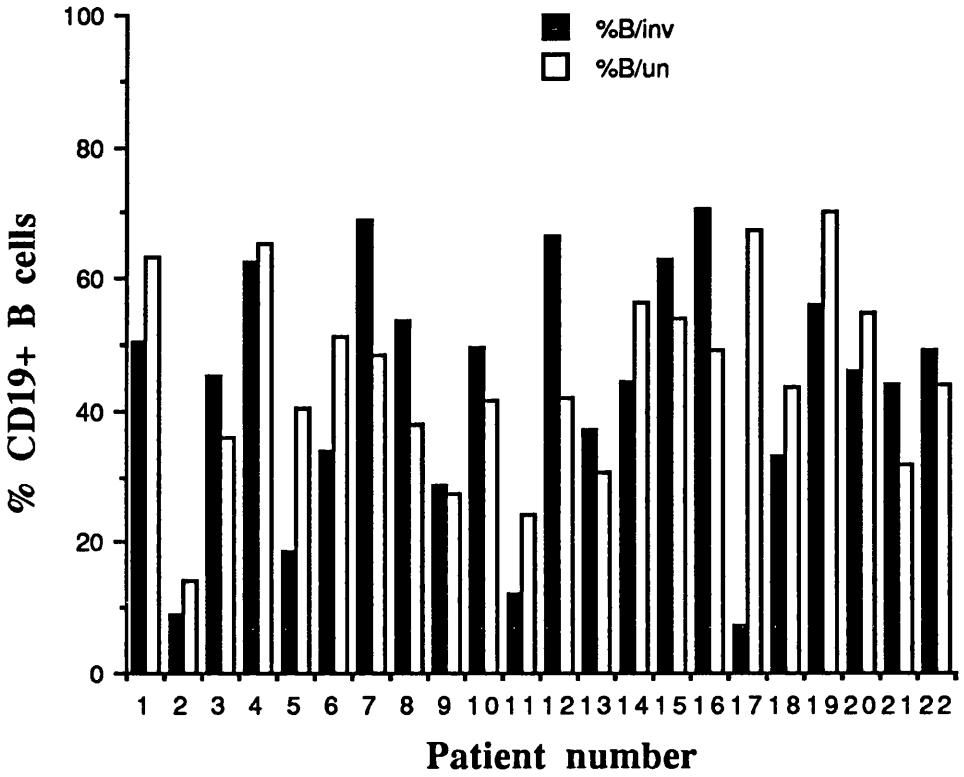


Figure 4.1 CD19+ B cell population within tumour free (un) and tumour invaded (inv) lymph nodes from the same stage II breast cancer patients.

proportion of CD19+ B cells in tumour invaded lymph nodes (42.9%) was only slightly lower than that in tumour free lymph nodes (45.8%) (Figure 4.1).

Proportions of CD3+ T cells in tumour invaded nodes (44.8%) were also very similar to tumour free nodes (43.8%) and a large variability was noted for the total numbers of T cells present (Figure 4.2).

4.1.2. T cell subsets

Although the overall proportion of T cells remains relatively constant between invaded and uninvaded nodes, analysis of the T cell subsets shows some significant changes. The mean percentage of CD8+ cytotoxic T cells in tumour invaded nodes (16.7%) was higher than the CD8+ population within the tumour free nodes (9.1%) (Table 4.1), the difference being highly significant. However the distribution of these cells was skewed (median 12.7%) with only six patients having greater than 20% CD8+ T cells (Figures 4.3 and 4.5). Within the remaining population the proportion of CD8+ T cells was confined to a much smaller range (Figures 4.3 and 4.5)

The CD4+ T helper cells on the other hand showed a decrease in the invaded nodes (29.8%) in comparison with tumour free stage II nodes (36.7%). This change was also shown to be statistically significant, the range of expression being wide in both subsets.(Figures 4.4 and 4.6)

4.1.3. CD4/CD8 ratio

The CD4/CD8 ratio agreed with the findings observed with a depressed ratio in the tumour invaded lymph nodes compared to the tumour free. Figures 4.7 and 4.8 illustrate the extensive patient variability, with some patients

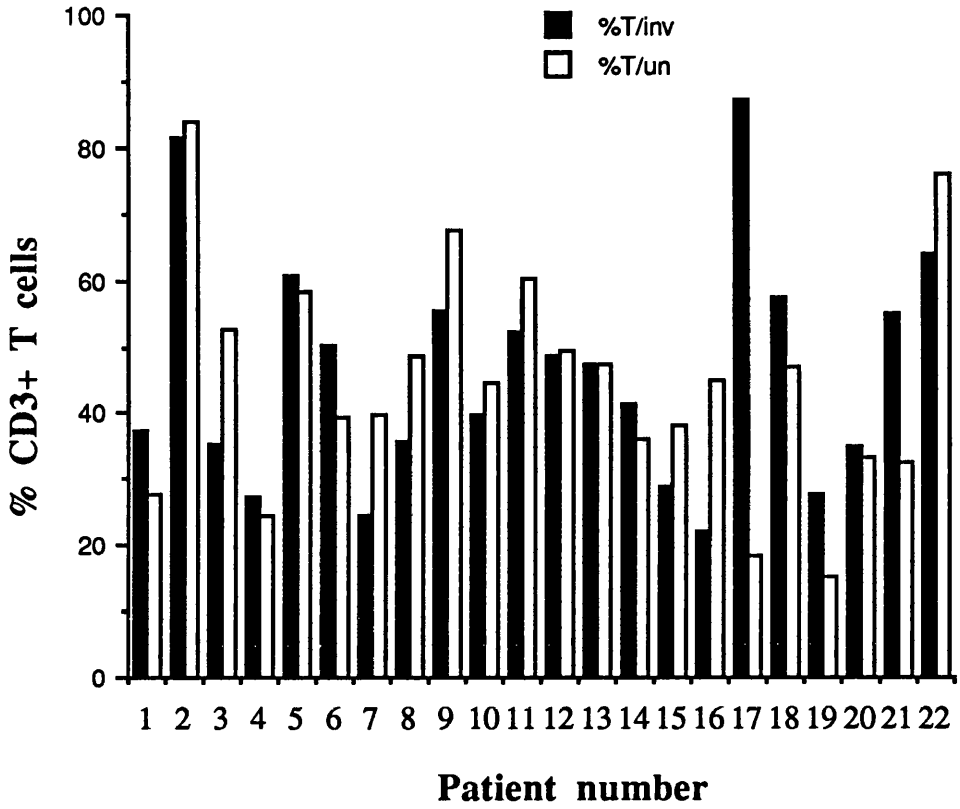


Figure 4.2 CD3+ T cell population within tumour free (un) and tumour invaded (inv) lymph nodes from the same stage II breast cancer patients.

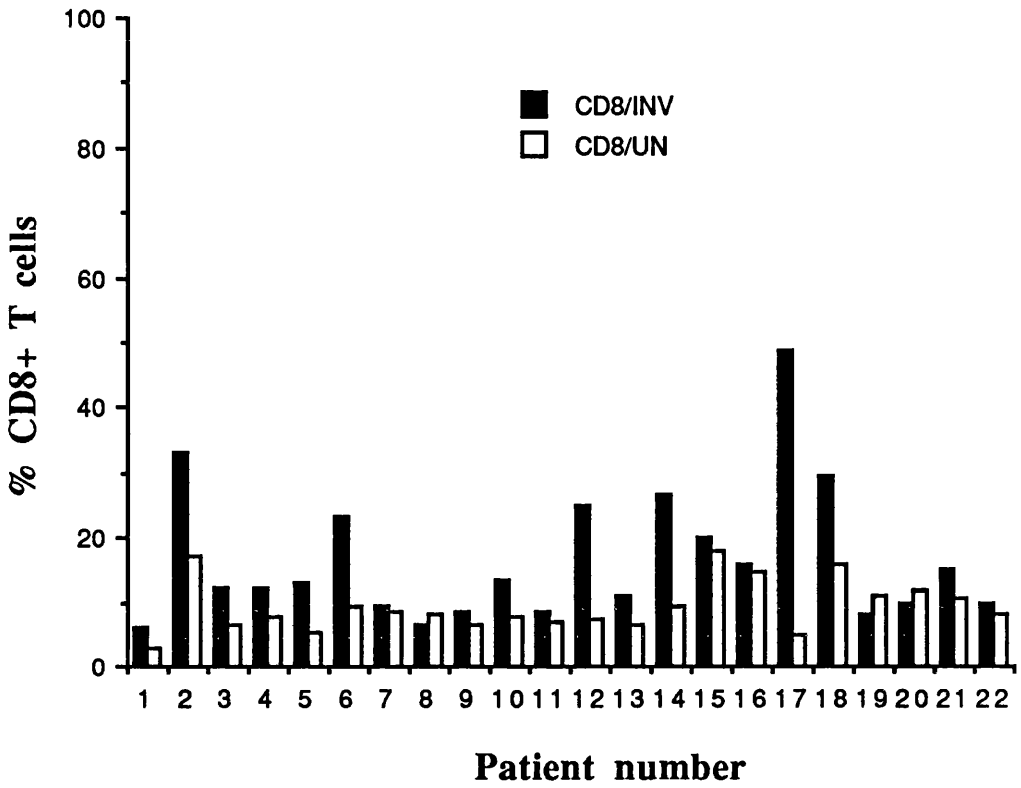


Figure 4.3 CD8+ T cell population within tumour free (UN) and tumour invaded (INV) lymph nodes from the same stage II breast cancer patients.

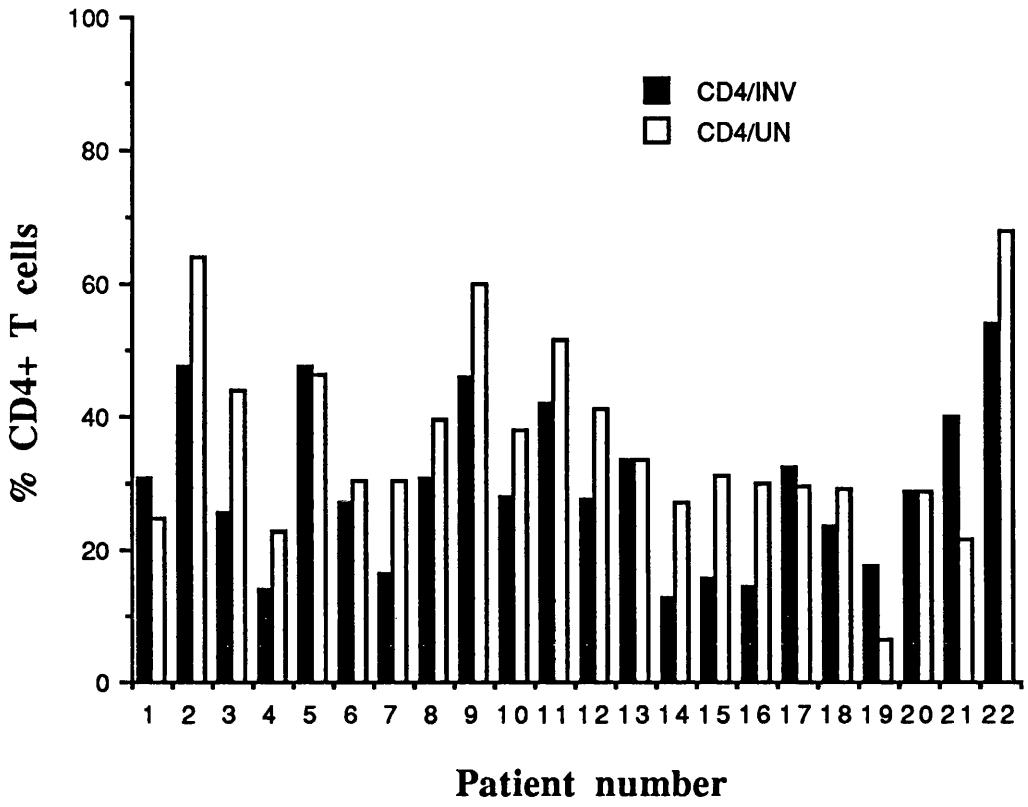


Figure 4.4 CD4+ T cell population within tumour free (UN) and tumour invaded (INV) lymph nodes from the same stage II breast cancer patients.

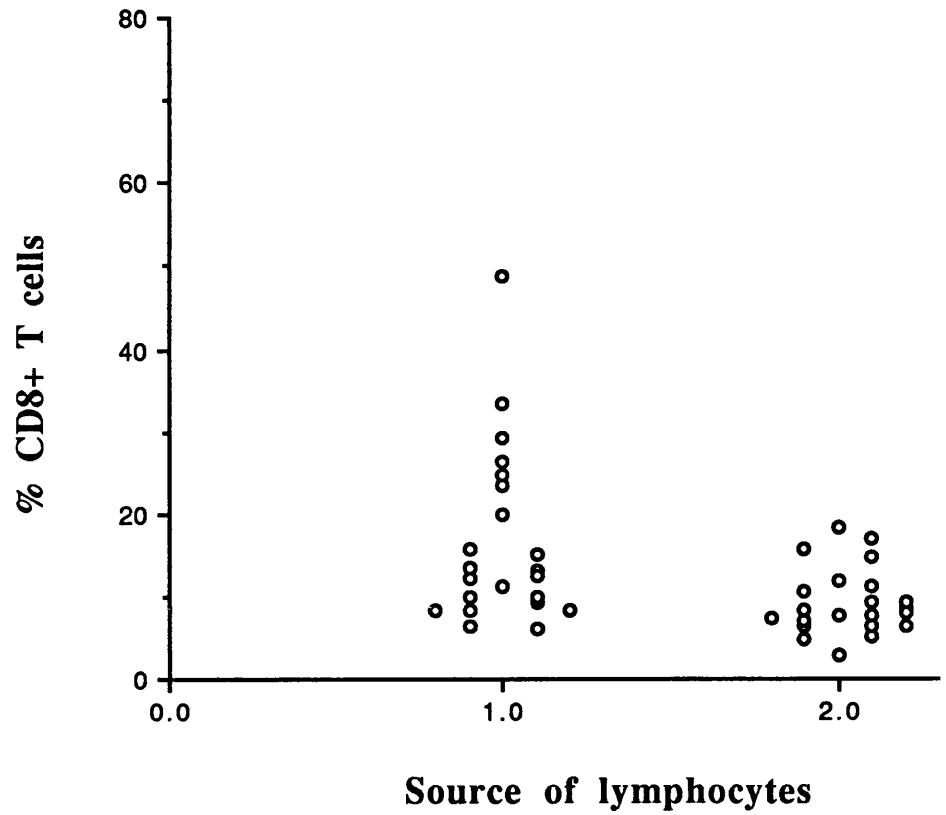


Figure 4.5 Distribution of CD8+ T lymphocytes in tumour invaded (1) and tumour free lymph nodes (2) from breast cancer patients.

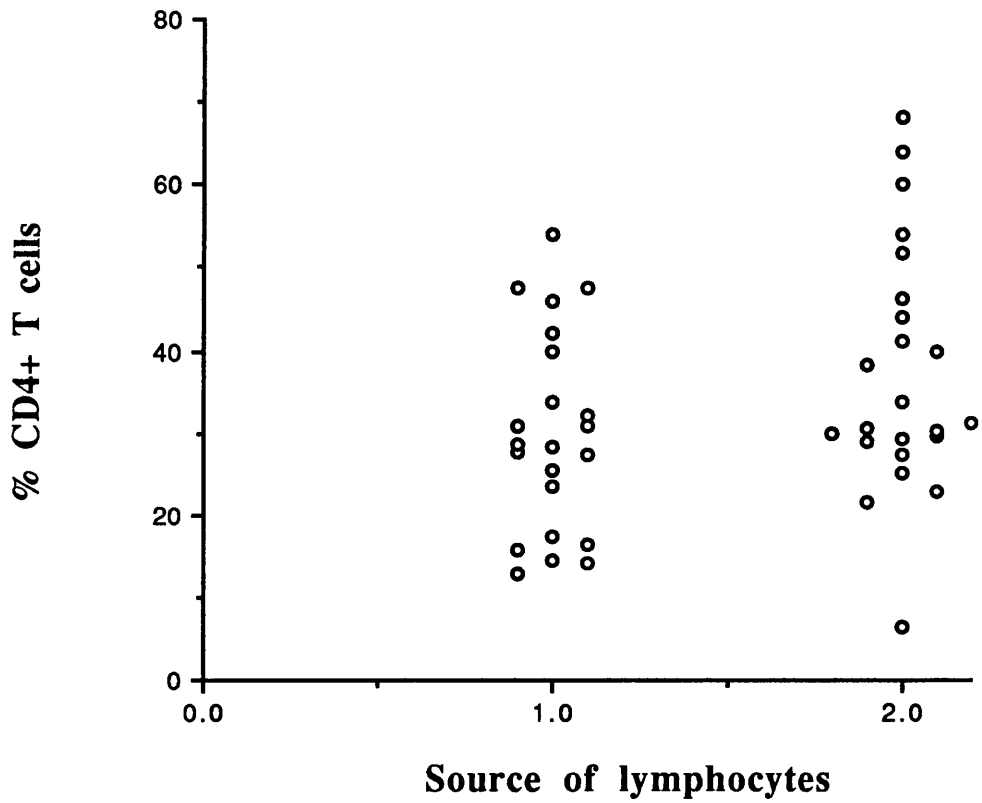


Figure 4.6 Distribution of CD4+ T lymphocytes in tumour invaded (1) and tumour free lymph nodes (2) from breast cancer patients.

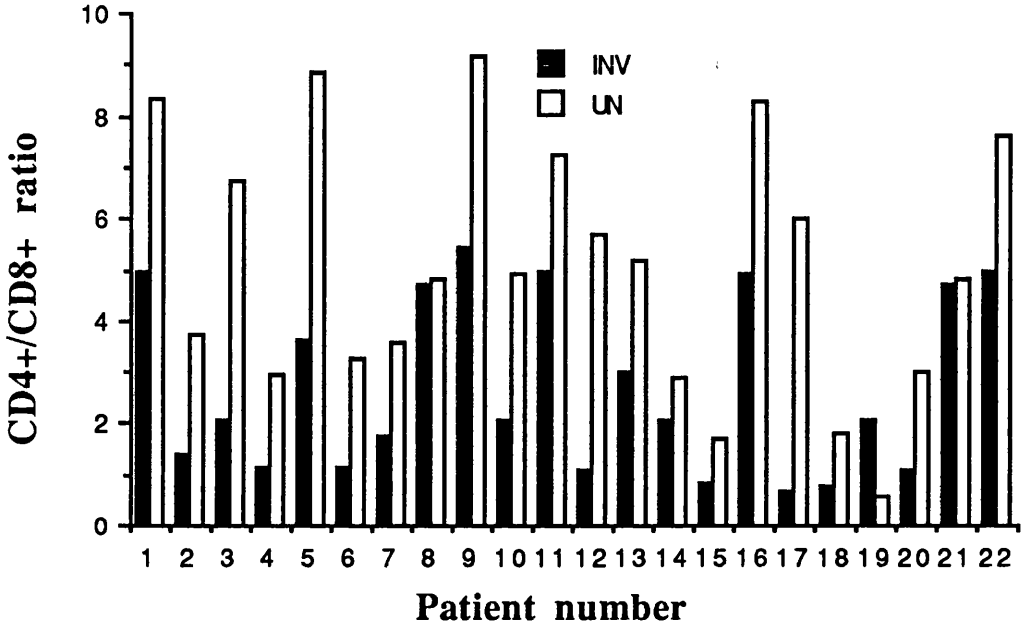
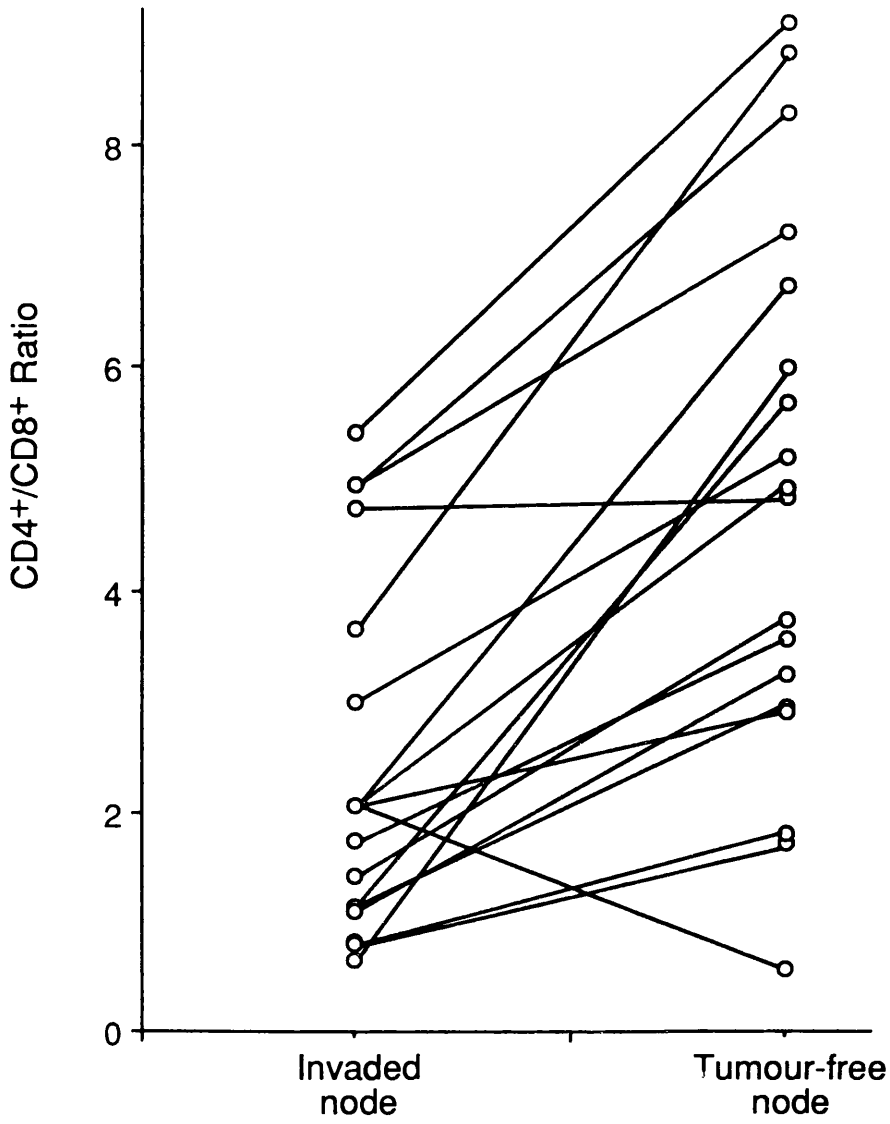


Figure 4.7 CD4/CD8 ratio within tumour free (UN) and tumour invaded lymph nodes from the same stage II breast cancer patients.

Figure 4.8 CD4/CD8 ratio of invaded and uninvaded lymph nodes showed as matched pairs for each patient.


CD4+/CD8+ Ratio
0
2
4
6
8
Invaded node
Tumour-free node

showing both a depression of the proportion of CD4+ cells and an increase in CD8+ T cells. In some patients a change was only noted within one of the T cell subsets with the depression of the CD4+T cell population occuring in the majority of cases. Figures 4.5 and 4.6 illustrate the individual changes within the CD8 and CD4 populations.

The ratios for the invaded node were within a more restricted range, between 0.8 and 5.42 whereas a much larger range was noted between uninvaded nodes from 0.4 to 9.16. The degree of change between individual patients is shown in Figures 4.7 and 4.8. Figure 4.9 illustrates the trend of a depression of the CD4/8 ratio within the invaded node.

4.2. Activation markers

The expression of the activation markers HLA DR and IL-2R (Tac antigen) on T cells and sIgG on B cells are summarised in Table 4.2. These are surface activation markers which undergo upregulation upon antigen stimulation. Lymphocytes were gated based on size characteristics as described in Section 2.2.6.2.

4.2.1. HLA DR expression

HLA DR expression overall was higher in the CD8+ T cells (Table 4.2). When invaded nodes are compared with uninvaded nodes there is a significant trend towards increased HLA DR expression on CD8+ T cells and also CD4+ T cells, within the invaded lymph nodes (Figures 4.10 and 4.11). There is a wide range of expression of HLA DR on both T cell subsets within both the invaded and uninvaded lymph nodes (Figures 4.10 and 4.11).

Table 4.2.

Distribution of activation markers on lymphocytes from tumour invaded and tumour free lymph nodes in 22 stage II breast cancer patients.

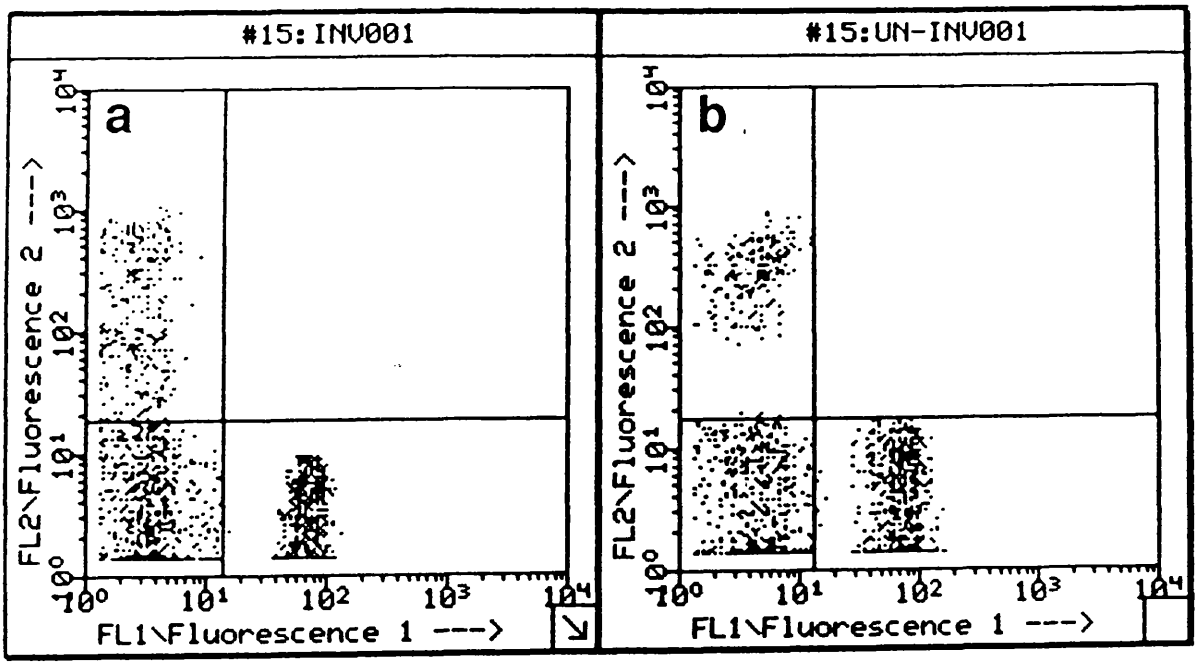
Activation Marker	STAGE II Tumour invaded	Tumour free	Statistical+ relevance
HLA DR on $CD8^+$ T cells	50.6+/-4.1	47.2+/-3.5	p=0.023
HLA DR on $CD4^+$ T cells	38.2+/-4.0	31.0+/-3.7	p=0.036
IL2R(Tac) on $CD8^+$ T cells	17.1+/-2.5 (15.7+/-12.0)*	12.9+/-2.1 (9.8+/-9.5)*	p=0.029
IL2R (Tac) on $CD4^+$ T cells	21.2+/-2.8	22.5+/-2.4	p=0.075
sIgG on B cells	37.6+/-4.6 (33.0+/-22.3)*	30.0+/-3.4 (30.9+/-15.8)*	p=0.065

Values represent mean % +/- standard deviation of lymphocytes expressing the phenotypic marker.

* values in bracket represents median +/- standard deviation (SD). Median values and SD are cited for cases where a distribution appeared to be skewed (marked with asterisk).

+ Statistical test used: Wilcoxon signed rank, when comparing tumour invaded nodes from the same patient to tumour free nodes. Statistical significance is taken at a p value equal to or < 0.05. N.S is not significant.

Figure 4.9 Representative flow cytometry diagram of (a) invaded and (b) uninvaded lymph nodes from the same patient using FITC labelled anti-CD4 (FL-2) and PE labelled anti-CD8 (FL-2).


#15:INV001
a
FL2\Fluorescence 2 --->
FL1\Fluorescence 1 --->
#15:UN-INV001
b
FL2\Fluorescence 2 --->
FL1\Fluorescence 1 --->

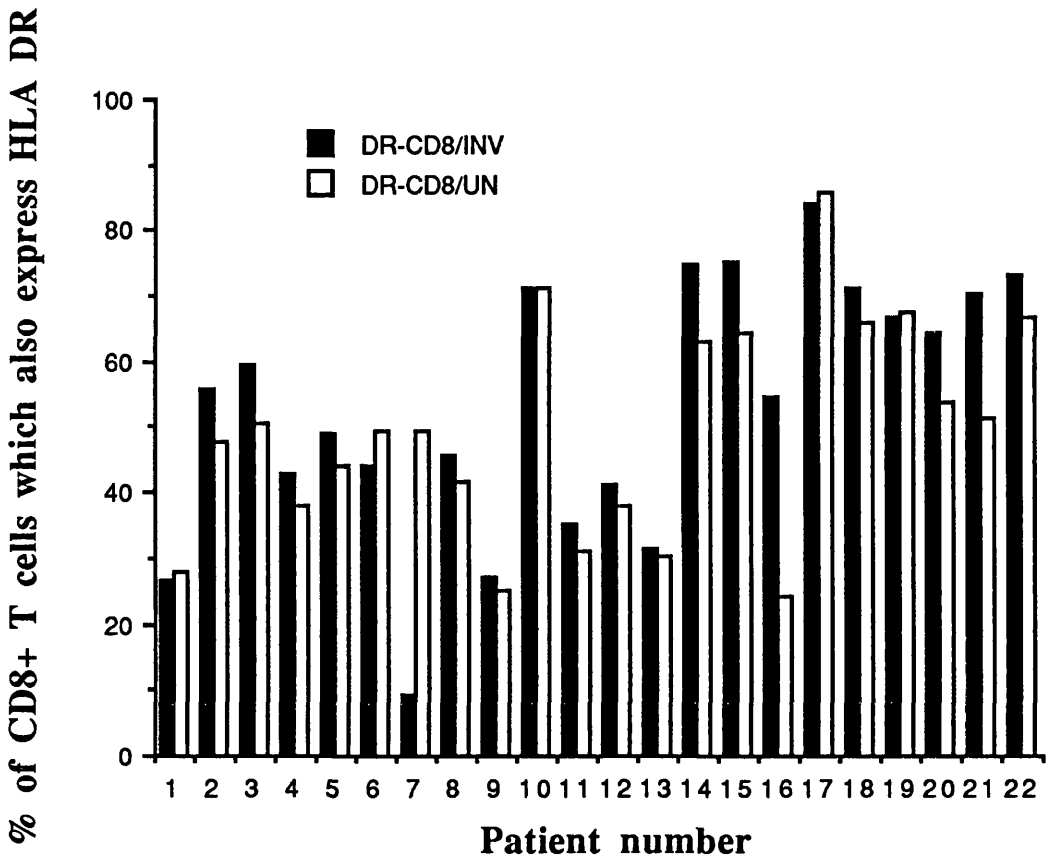


Figure 4.10 Percentage of CD8+ T cells which also expressing HLA DR in tumour free (UN) and invaded (INV) nodes from the same stage II breast cancer patients.

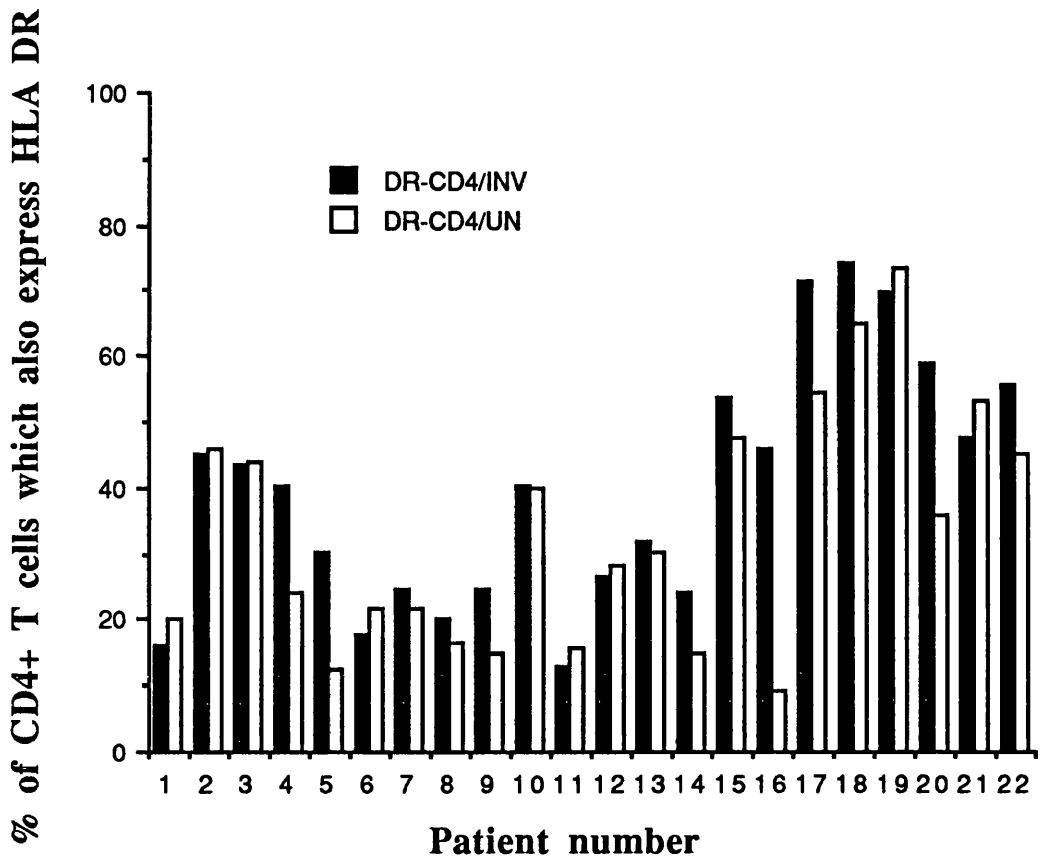


Figure 4.11 Percentage of CD4+ T cells which also expressing HLA DR in tumour free (UN) and invaded (INV) nodes from the same stage II breast cancer patients.

4.2.2. IL-2R (Tac) expression

The expression of the Tac component of the IL-2 receptor was shown to be generally higher on the CD4+ T cells than on the CD8+ T cells (Table 4.2). The mean percentage of IL-2R expressing CD8+ T cells in stage II invaded nodes (17.1%) was significantly higher when compared to the paired uninvaded lymph node (12.9%) whereas the expression of IL-2R on CD4+ T cells showed little change between invaded (21.2%) and uninvaded lymph nodes (22.5%) (Table 4.2).

The patient variability within both groups of T cells was high, with some cases of higher percentages in invaded nodes and in others the reverse. (Figure 4.12 and 4.13)

4.2.3. Surface IgG

The percentage of sIgG expressing B cells was higher in tumour invaded nodes (37.6%), compared to tumour free nodes (30.0%). This however failed to make statistical significance (Table 4.2).

The range of expression was variable and skewed. In a few patients a noticable feature was the major increase in expression in tumour invaded nodes (Figure 4.14).

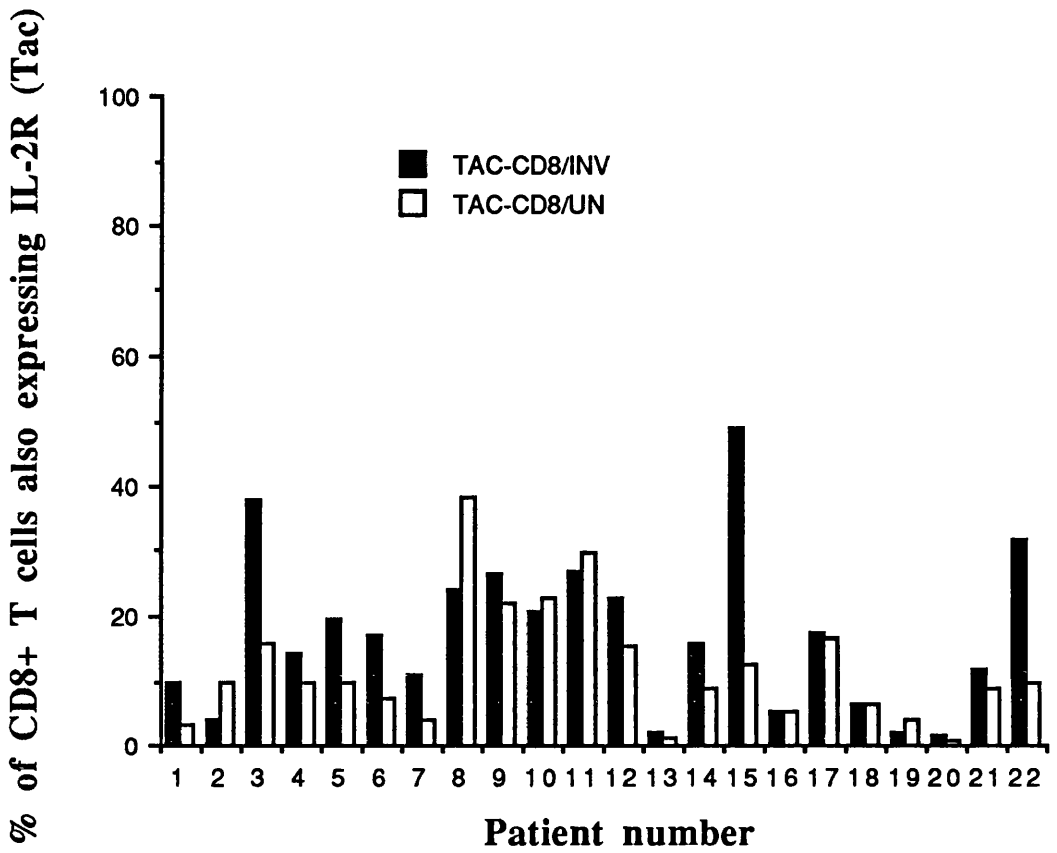


Figure 4.12 Percentage of CD8+ T cells also expressing the IL-2R(Tac) in tumour free (UN) and tumour invaded (INV) nodes from the same stage II breast cancer patients.

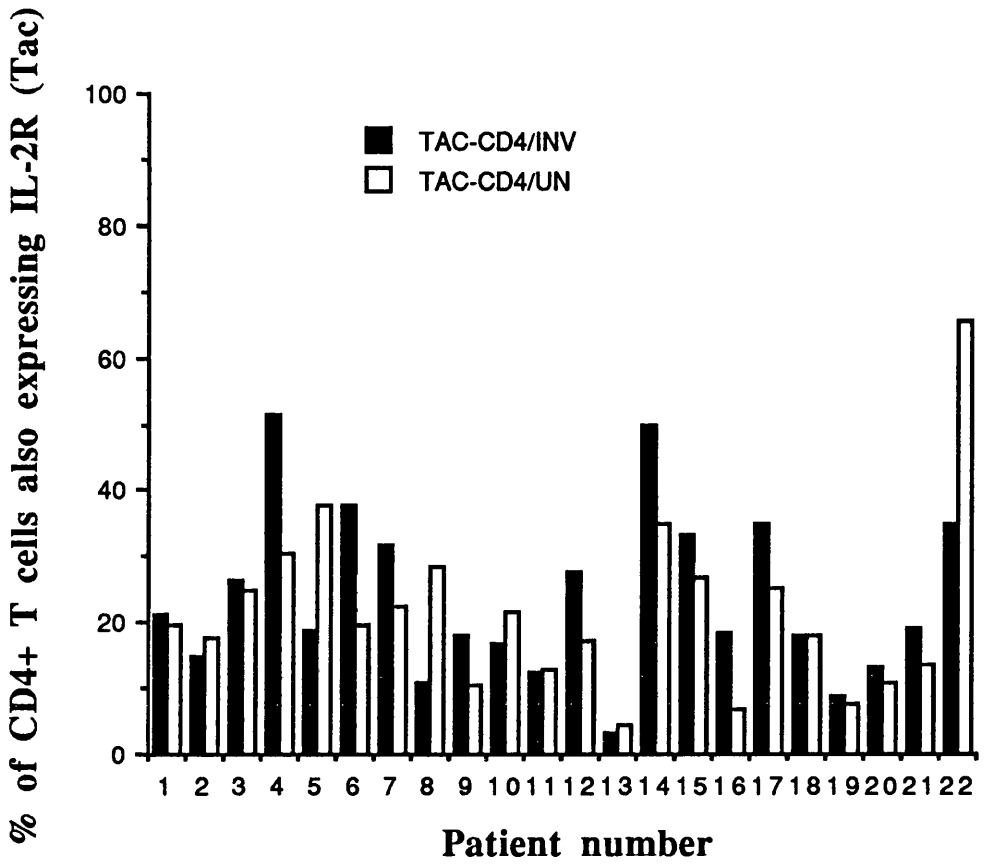


Figure 4.13 Percentage of CD4+ T cells also expressing the IL-2R(Tac) in tumour free (UN) and tumour invaded (INV) nodes from the same stage II breast cancer patients.

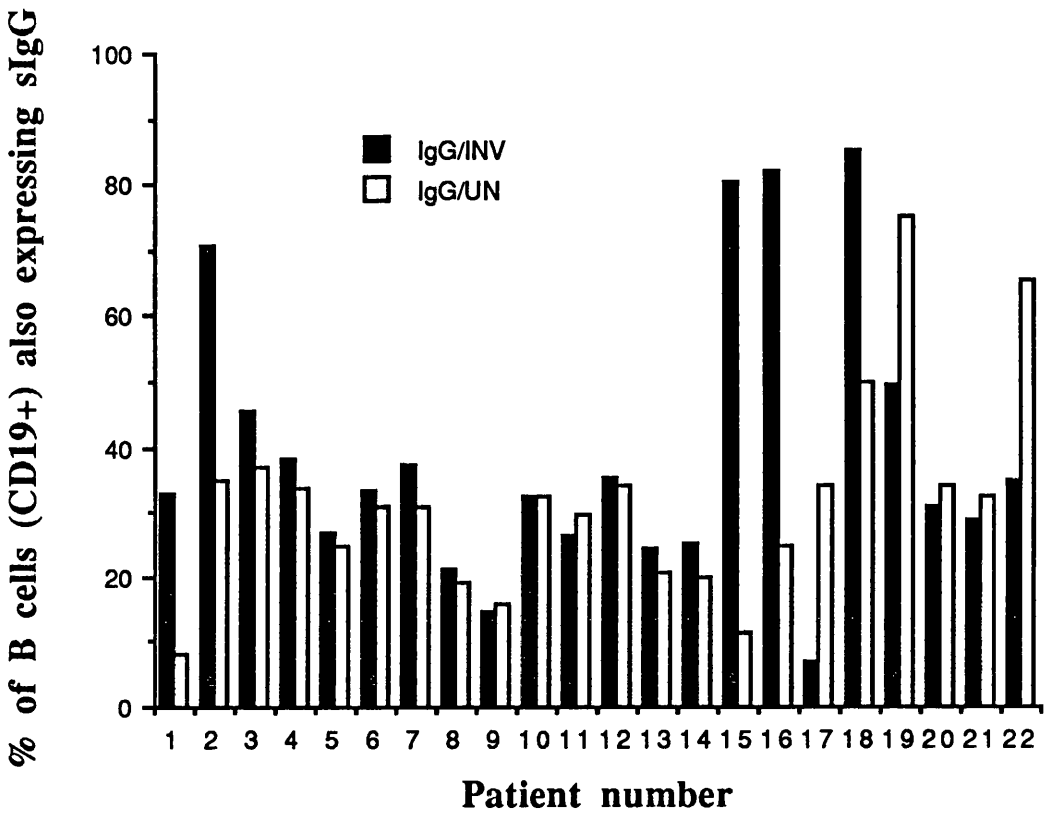


Figure 4.14 Percent of B cells (CD19+) also expressing surface IgG in tumour free (UN) and tumour invaded (INV) nodes from the same stage II breast cancer patients.

4.3. Discussion

The primary function of the axillary lymph nodes is to scavenge for antigen in the lymph fluid that drains from the breast tissues. Here they are ideally situated to encounter both metastatic tumour and tumour breakdown products possibly leading to a host mediated immune response. The metastatic tumour within these nodes may, however, have an adverse effect on the lymphocyte populations. An analysis of phenotype and activation state of nodal lymphocytes may reflect the response within the nodes and the eventual outcome of the tumour host interactions taking place.

Two previous studies have analysed lymph node lymphocytes from breast cancer patients by flow cytometry (Morton *et al* 1986; Whitford *et al* 1992). However although nodes from both stage I and stage II patients were analysed in these studies, nodes largely free of tumour were preferred and significantly invaded nodes were omitted. In this study phenotypic and activation markers on lymphocytes from matched pairs of tumour free and tumour invaded nodes taken from the same stage II patients were analysed for 22 patients. Thus inter-patient variability which is considerable (Morton *et al* 1986; Whitford *et al* 1992) is removed and only intra patient variation between the two types of nodes has been analysed.

Results obtained show that in breast cancer patients there are differences in phenotype and activation between nodes that are invaded and those that are not.

The most significant difference between tumour free and invaded nodes was the change observed within the CD4/ CD8 ratio. In invaded nodes this ratio was reduced in comparison to the tumour free nodes. The reduction in

CD4/CD8 ratio was due to an increase in the percentage of CD8+ T cells and a decrease in the CD4+ population. Within the invaded node the CD4+ T cells showed a greater decrease but also showed more variation. Similar alterations in the CD4+ and CD8+ populations have been reported within stage II breast cancer patients (Morton *et al* 1986) and melanoma patients (Farzad *et al* 1990).

The changes are most clearly illustrated in Figure 4.8. The majority of the patients show a decreased CD4/CD8 ratio within the invaded nodes but there are a small number of patients who do not show a trend.

The increased numbers of CD8+ T cells within the invaded node is of interest as tumour infiltrates mainly comprise CD8+ cells (Whitford *et al* 1991), and the increase of these cells in invaded nodes is perhaps suggestive in both cases of activated cytotoxic cells within close proximity to their target cells.

Expression of the activation marker HLA DR has been reported to be related to stage of the tumour in breast cancer patients. Morton *et al* (1986) reported higher proportions of HLA DR expressing CD4+ and CD8+ T cells in stage II patients. Within these stage II patients the change in phenotype was measured between invaded and uninvaded lymph nodes. The expression of HLA DR on CD8+ T cells was shown to be greater in invaded nodes rather than tumour free nodes with this change showing significance. The expression of the IL-2R (Tac) on CD8+ T cells showed on average an increase within the invaded nodes this also being a significant difference. The large percentage of CD8+ activated T cells within tumour invaded nodes would suggest that these lymphocytes are responding locally to the tumour, possibly in a similar way to CD8+ T cells found within a primary breast tumour. The proportion of CD4+ HLA DR cells was found to be raised in the invaded nodes with this reaching

significance. However the expression of the IL-2R (Tac) failed to reach significance.

In general the percentage of HLA DR positive T cells was higher in invaded nodes than in tumour free nodes. However the changes noted for the IL-2R must be treated with caution, as although an increased expression of the receptor indicates T cell activation, the lack of the receptor does not necessarily suggest an inactive state, as following activation the receptor undergoes down regulation (Duprez *et al* 1988).

The proportion of mature B cells expressing IgG although found to be raised in a number of patients, failed to reach statistical significance, between the two types of nodes. Thus the antibody arm of the immune response is erratic.

The results together indicate that the presence of metastatic tumour within a lymph node can cause specific alterations in both phenotype and activation status of T cells, while only affecting the B cell component in a few individuals. The differences observed in terms of phenotype can be correlated with respect to the activation status as in general it appears that a larger CD8+ T cell population and a smaller CD4+ population are present in the invaded nodes. Thus while certain T cell populations are being activated others appear not to be proliferating or are actively being suppressed by the tumour. The alterations which are observed here may have an important role to play in the metastatic process.

CHAPTER 5

INVESTIGATIONS ON THE EXTENT TO WHICH THE NODAL RESPONSE IS LOCAL

5. Investigations on the extent to which the nodal response is local

The data in Chapter 4 suggest that the majority of breast cancers elicit a measurable and statistically significant immune response in both phenotype and activation status of the lymphocytes. This Chapter investigates the extent to which such a response is local to the tumour area. The aim of this chapter was to investigate whether various therapeutic regimes such as sampling of the low nodes, removal of the entire axilla, or local radiotherapy of the axilla remove the immune response and also to indicate whether these tissues, rather than blood, should be the focus for attempts to immortalise T or B cells for subsequent immunotherapeutic strategies. The low draining axillary nodes are as close as 5cm to the tumour. The higher ones are often 15- 20cm away (Fig 1. 1). In this study, the degree of response in nodes close to the tumour as opposed to nodes distal from the tumour was analysed. The study included 44 patients, 22 stage I and 22 stage II patients. The 22 stage II patients were selected during the operation to remove the primary tumour and axilla and further subdivided thereafter by pathological grading and by the flow cytometric analysis itself into the following groups.

A. Low node invaded and high node tumour free (n=8)

These patients are similar to those in Chapter 4. The difference is that, in the operating theatre, the nodes were removed on the basis of distance from tumour rather than invasion. On average, therefore, invasion was less advanced.

B. Low node invaded and high node invaded (n=7)

C. Low node tumour free and high node tumour free (n=5)

Several nodes were obtained from these patients. The particular pair analysed contained no tumour cells by either pathological or FACS analysis. However,

other nodes, not used in the current study, were found by the pathologist to be invaded.

D. Low node tumour free and high node invaded ("skips") (n=2)

5.1 Phenotypic markers

5.1.1. Stage I paired lymph nodes (n=22)

5.1.1.1. T and B lymphocytes

The results from stage I patients are illustrated in Table 5.1. The mean level of CD3+ T cells was significantly higher in the low node (47.0%) than the high node (41.4%) (p=0.042) the individual values being shown in Figure 5.1. The mean level of CD19+ B cells show the opposite trend with the low node containing significantly lower (36.4%) than the high node (43.5%) p=0.036 the individual values being shown in Figure 5.2. This change is illustrated in the CD3/CD19 ratio in the low node (2.0) being significantly higher than the high node (1.2) p=0.036 the individual values being shown in Figure 5.3. The data are however biased by one patient with an abnormally high ratio in the low node

5.1.1.2. T cell subsets

Analysis of T cell subsets showed no significant trends within the stage I patients (Table 5.1) although the mean level of CD8+ T cells is on average higher in the low node (14.5%) than the high node (10.3%), whereas the mean level of CD4+ T cells shows no change between the low (40.8%) and high node (39.2%).

Table 5.1

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 22 stage I patients.

Phenotypic marker	Stage I Low node (n=22)	High node (n=22)	Statistical significance+
CD3 (% T cells)	47.0+/-3.5	41.4+/-3.2	p=0.042
CD19 (% B cells)	36.4+/-4.5	43.5+/-4.1	p=0.036
CD8 (CD8+ T cells)	14.5+/-3.3	10.3+/-1.4	p=0.080
CD4 (CD4+ T cells)	40.8+/-3.5	39.2+/-3.6	p=0.076
CD3/CD19 ratio	2.0+/-1.0	1.2+/-0.3	p=0.036
CD4/CD8 ratio	5.5+/-0.8	7.6+/-2.0	p=0.090

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

+Statistical test used: Wilcoxon signed rank, when comparing two lymph nodes from the same patient. Statistical significance is taken at a p value equal to or < 0.05. Only significant p values are given. N.S. is not significant.

Figure 5.1 The change in the percentage of CD3+ T cells between the low (1) and high (2) axilla of the total 22 stage I patients.

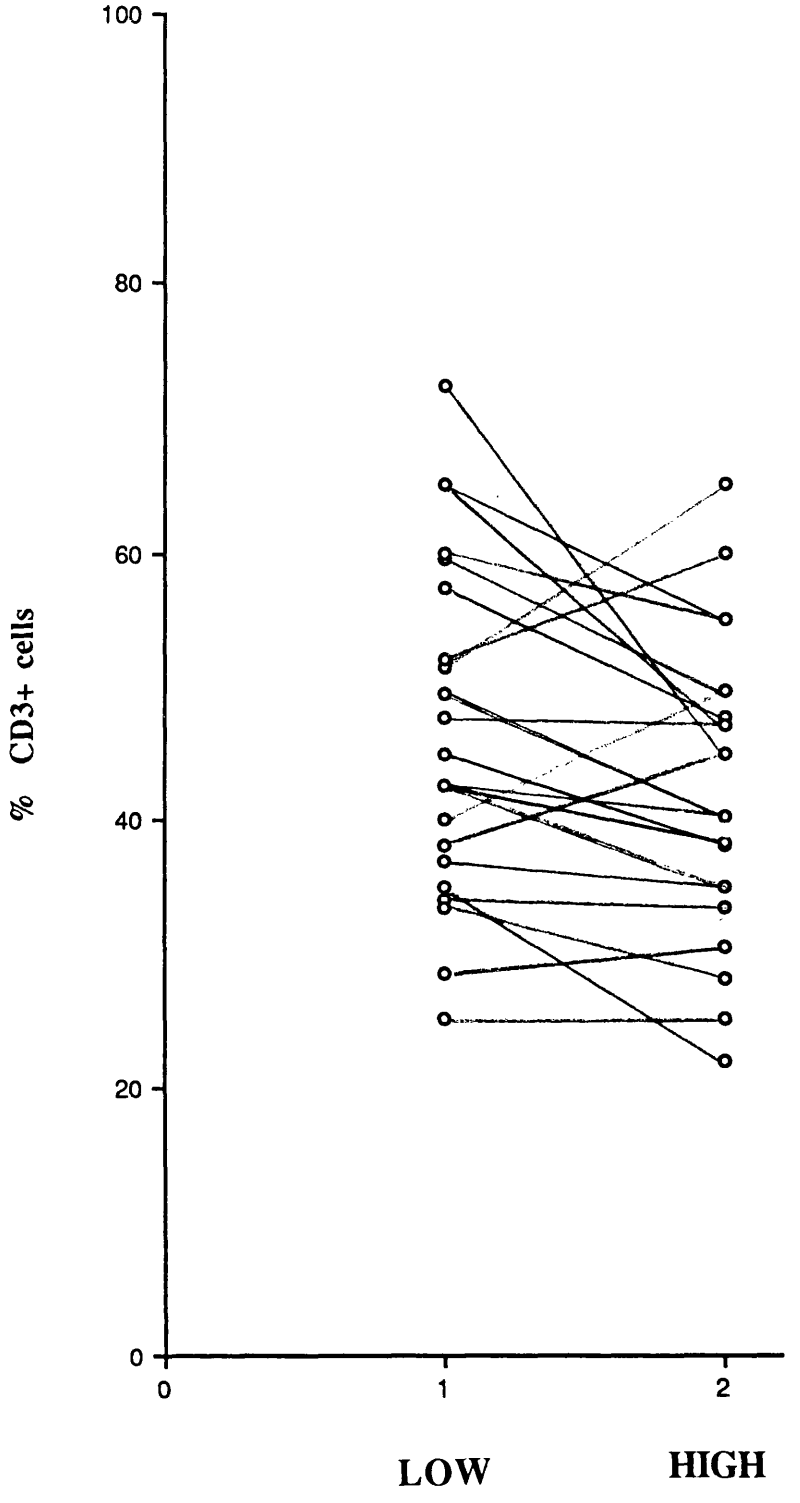

100
80
60
40
20
0
% CD3+ cells
0
1
2
LOW
HIGH
Nodal designation

Figure 5.2 The change in the percentage of CD19+ B cells between the low (1) and high (2) axilla of the total 22 stage I patients.

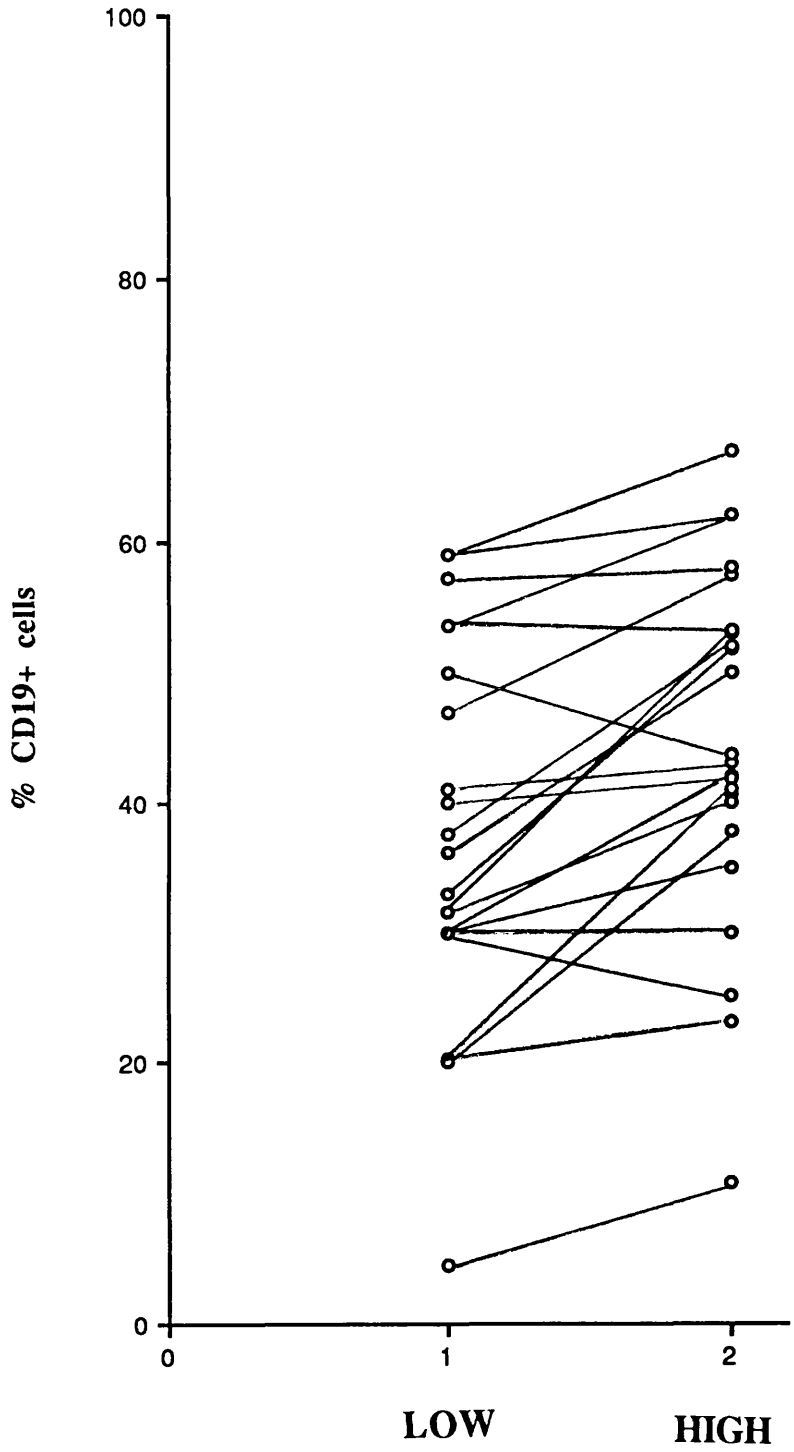
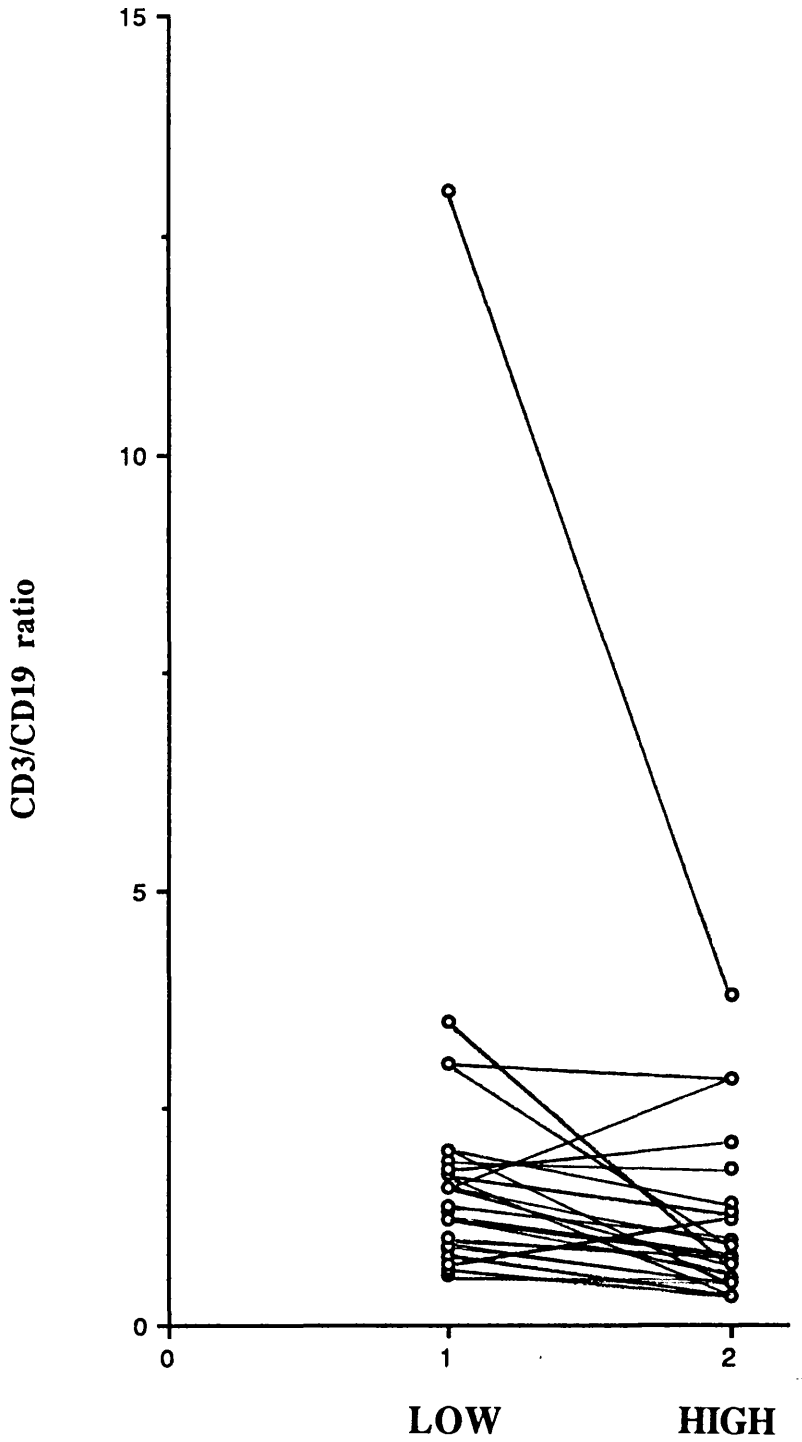

100
80
60
40
20
0
% CD19+ cells
0
1
2
LOW
HIGH
Nodal designation

Figure 5.3 The change in CD3+ T cell/ CD19+ B cell ratio in the low (1) and high (2) axillary nodes of the total 22 stage I patients.


15
10
5
0
CD3/CD19 ratio
0
1
2
LOW
HIGH
Nodal designation

5.1.2. Stage II paired lymph nodes (n=22)

5.1.2.1. T and B lymphocytes

There were no significant trends observed for changes in phenotype in stage II patients nodes (Table 5.2). However differences were noted. The mean level of CD3+ T cells was increased in the low node (47.0%) compared to the high node (43.0%). The mean level of CD19+ B cells was decreased in the low node (36.6%) to rise in the high node (43.0%). This was similar to what was observed in the stage I patients.

5.1.2.2. T cell subsets

No significant trends were observed for stage II patients (Table 5.2). However a slight increase was noted in the mean level of CD8+ T cells in the low node (13.0%) with respect to the high node (11.0%). This increase in the low node was also illustrated in the mean level of the CD4+ T cell subset within the low node (36.0%) compared to the high node (33.0%).

5.1.3. Stage II subset A: Low node tumour invaded, high node tumour free (n=8).

5.1.3.1. T and B lymphocytes

As with the total stage II patients a rise in the proportion of CD3+ T cells was observed in the low tumour invaded node (46.7%) compared to the

Table 5.2

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 22 stage II patients.

Phenotypic marker	Stage II Low node (n=22)	High node (n=22)	Statistical Significance+
CD3 (% T cells)	47.0+/-4.1	43.0+/-3.2	p=0.121
CD19 (% B cells)	36.6+/-4.0	43.0+/-3.6	p=0.113
CD8 (CD8+ T cells)	13.0+/-1.7	11.0+/-1.3	p=0.098
CD4 (CD4+ T cells)	36.0+/-2.8	33.0+/-2.3	p=0.087
CD3/CD19 ratio	2.3+/-0.7	2.3+/-1.1	p=0.097
CD4/CD8 ratio	3.2+/-0.4	3.1+/-0.3	p=0.080

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

+Statistical test used: Wilcoxon signed rank, when comparing two lymph nodes from the same patient. Statistical significance is taken at a p value equal to or < 0.05.

high tumour free node (32.5%) although this was not statistically significant (Table 5.3). Within the CD19+ B cell population there was a rise on the high tumour free node (54.8%) compared to the low tumour invaded node (40.8%). The CD3/CD19 ratio reflects this change (Table 5.3).

5.1.3.2. T cell subsets

Within the low tumour invaded node an increase (17.5%) was noted in the mean number of CD8+ T cells compared to the high node (8.8%) although this was not statistically significant (Table 5.3). An increase was observed in the mean number of CD4+ T cells in the high tumour free node (35.7%) compared to the low tumour invaded node (28.4%) this change again not being statistically relevant (Table 5.3). However when the change in ratio of CD4/CD8 was considered the trend became significant p=0.05 (Table 5.3, Figure 5.4). This was in agreement with the results of chapter 4.

5.1.4. Stage II subset B: Low node tumour invaded, high node tumour invaded (n=7).

5.1.4.1. T and B lymphocytes

There were no significant trends noted (Table 5.4) although a small decrease was noted in the mean number of CD3+ T cells in the high tumour invaded node (48.2%) compared to the low tumour invaded node (46.4%). Concurrently an increase in the mean CD19+ population was noted in the high tumour invaded node (36.8%) compared to the low tumour invaded node (28.9%). These changes are illustrated in the CD3/CD19 ratio.

Table 5.3

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 8 stage II patients with the low lymph node tumour invaded and the high node tumour free (subset A).

Phenotypic marker	Stage II Low node Tumour invaded (n=8)	Stage II High node Tumour free (n=8)	Statistical Significance+
CD3 (% T cells)	46.7+/-7.8	32.5+/-4.3	p=0.072
CD19 (% B cells)	40.8+/-6.8	54.8+/-4.9	p=0.068
CD8 (CD8+ T cells)	17.5+/-4.6	8.8+/-1.4	p=0.091
CD4 (CD4+ T cells)	28.4+/-3.7	35.7+/-3.0	p=0.67
CD3/CD19 ratio	2.5+/-1.6	3.1+/-2.4	p=0.120
CD4/CD8 ratio	2.2+/-0.4	4.4+/-0.6	p=0.05

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

Figure 5.4 The change in CD4+ T cell/ CD8+ T cell ratio in the low (1) and high (2) tumour free nodes of 8 stage II patients.

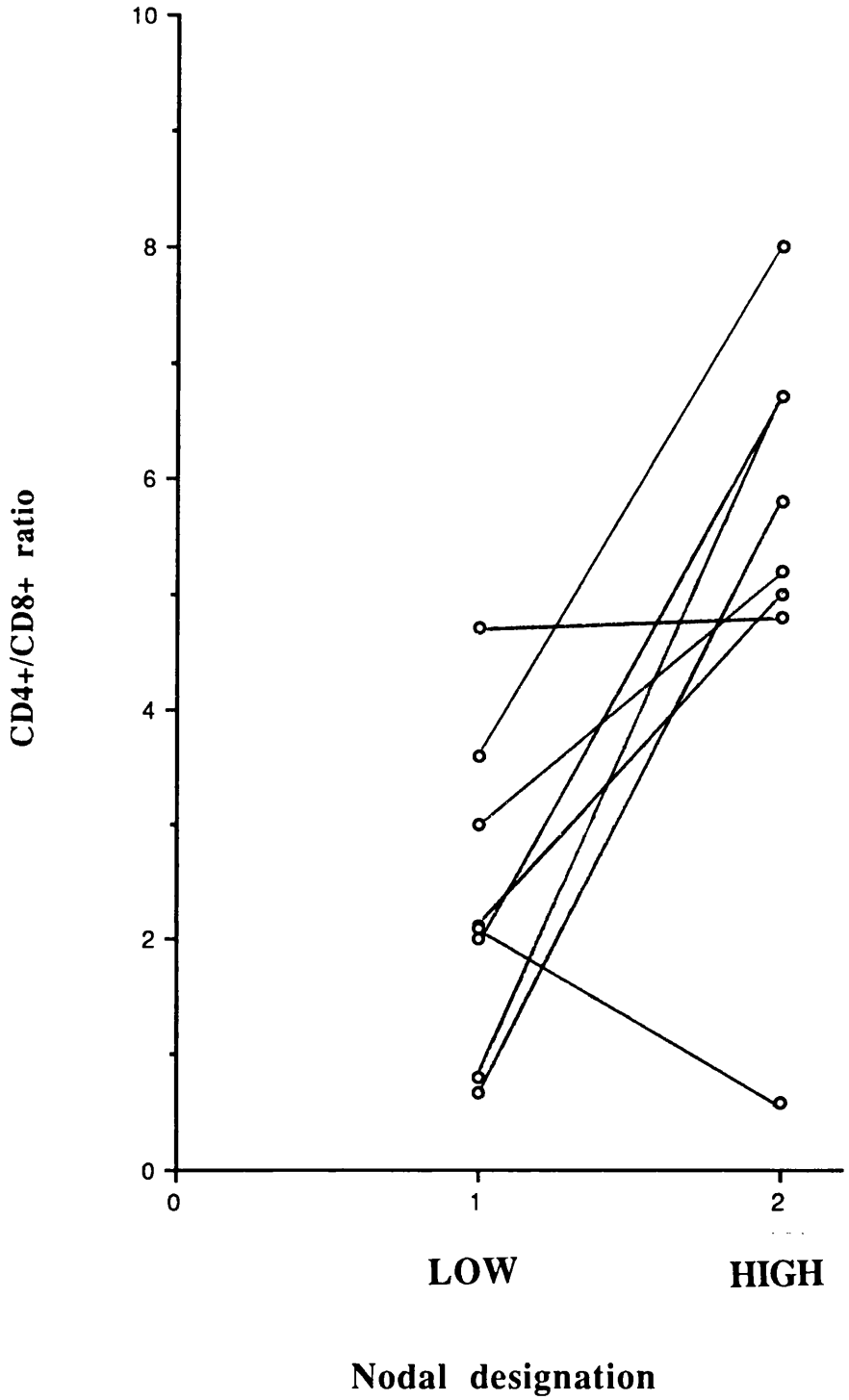

10
8
6
4
2
0
CD4+/CD8+ ratio
0
1
2
LOW
HIGH
Nodal designation

Table 5.4

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla(> 10cm from the tumour) of 7 stage II patients with the low lymph node tumour invaded and the high node tumour invaded (subset B).

Phenotypic marker	Stage II		Statistical Significance+
	Low node Tumour invaded (n=7)	High node Tumour invaded (n=7)	
CD3 (% T cells)	48.2+/-7.9	46.4+/-2.4	p=0.088
CD19 (% B cells)	28.9+/-6.4	36.8+/-1.9	p=0.078
CD8 (CD8+ T cells)	10.2+/-1.9	10.2+/-1.8	p=0.116
CD4 (CD4+ T cells)	39.4+/-5.4	38.9+/-2.3	p=0.130
CD3/CD19 ratio	2.8+/-0.8	1.2+/-0.1	p=0.089
CD4/CD8 ratio	3.4+/-0.6	3.2+/-0.6	p=0.111

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

5.1.4.2. T cell subsets

There were no significant trends within the T cell population (Table 5.4) with little change noted in the mean values of both CD8 and CD4 lymphocytes in the low and high tumour invaded nodes.

5.1.5. Stage II subset C: Low node tumour free, high node tumour free (n=5).

5.1.5.1. T and B lymphocytes

No statistically relevant trends were observed within this stage II subsets although some trends were noted (Table 5.5). As with the total stage II mean values an increase was observed in the mean CD3+ T cell population in the low tumour free node (45.0%) when compared to the high tumour free node (37.5%). An increase in the mean number of CD19+ B cells was also found in the high tumour free node (55.5%) compared to the low tumour free node (45.0%). These changes were also reflected in the CD3/CD19 ratio.

5.1.5.2. T cell subsets

There were no significant trends noted within the T cell subsets (Table 5.5). However an increase was found in the mean percentage of CD8+ T cells in the high tumour free node (18.9%) compared to the low tumour free node (12.4%). The CD4+ T cells showed the opposite trend with the numbers increasing in the low tumour free node (39.7%) compared with the high tumour free node (24.0%). This was confirmed in the CD4/CD8 ratio.

Table 5.5

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla(> 10cm from the tumour) of 5 stage II patients with the low lymph node tumour free and the high node also tumour free (subset C).

Phenotypic marker	Stage II		Statistical Significance+
	Low node Tumour free (n=5)	High node Tumour free (n=5)	
CD3 (% T cells)	45.0+/-1.8	37.5+/-14.5	p=0.056
CD19 (% B cells)	45.0+/-1.8	55.5+/-9.0	p=0.067
CD8 (CD8+ T cells)	12.4+/-1.9	18.9+/-7.4	p=0.062
CD4 (CD4+ T cells)	39.7+/-6.2	24.0+/-3.9	p=0.071
CD3/CD19 ratio	6.3+/-5.9	2.1+/-3.2	p=0.059
CD4/CD8 ratio	5.7+/-2.4	2.0+/-1.0	p=0.078

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

5.1.6. Stage II subset D: Low node tumour free, high node tumour invaded (n=2).

Due to the small sample size of n=2 no statistics were attempted on this stage II subset and any trends which were observed must be treated with caution.

5.1.6.1. T and B lymphocytes

Within the CD3+ T cell population there was a slight decrease in mean numbers from the low tumour free (57.6%) to the high tumour invaded node (54.3%) (Table 5.6). A rise in the mean number of CD19+ B cells was noted in the high node tumour invaded (23.7%) compared to the low tumour free node (20.2%) (Table 5.6).

5.1.6.2. T cell subsets

A slight rise was noted in the mean numbers of CD8+ T cells from the low tumour free node (8.6%) to the high tumour invaded node (10.5%) (Table 5.6). There was a decrease in the numbers of CD4+T cells from the low tumour free node (52.7%) to the high tumour invaded node (47.9%) with these changes being reflected in the CD4/CD8 ratio (Table 5.6).

Table 5.6

Distribution of phenotypic markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 2 stage II patients with the low lymph node tumour free and the high node tumour invaded (subset D).

Phenotypic marker	Stage II		Statistical Significance*
	Low node Tumour free (n=2)	High node Tumour invaded (n=2)	
CD3 (% T cells)	57.6+/-6.9	54.3+/-3.7	
CD19 (% B cells)	20.2+/-0.6	23.7+/-9.7	
CD8 (CD8+ T cells)	8.6+/-0.3	10.5+/-0.5	
CD4 (CD4+ T cells)	52.7+/-10.0	47.9+/-5.8	
CD3/CD19 ratio	3.2+/-0.1	2.8+/-1.3	
CD4/CD8 ratio	6.8+/-0.8	4.6+/-0.8	

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.

* Due to their being only 2 members of this stage II subset no statistics were performed.

5.2. Activation markers

5.2.1. Stage I paired lymph nodes n=22

Table 5.7 summarises the expression of the activation markers HLA DR and IL-2 receptor (Tac) on T cells and sIgG on B cells. In all cases, average levels were higher on the low node than the high node but the statistical difference of this is low

5.2.1.1. HLA DR

No significant trends in HLA DR expression were noted although differences were noted. HLA DR expression on CD8+ T cells tended to be greater in the low node (41.2%) than the high node (36.8%) (Table 5.7). A similar trend was observed for the expression of HLA DR on CD4+ T cells with a increased expression in the low node (28.8%) over the high node (23.1%) (Table 5.7).

5.2.1.2. Interleukin-2 receptor (Tac)

No significant trends were observed for the IL-2R. However some differences were noted (Table 5.7). The mean expression of the IL-2R on CD8+ T lymphocytes was higher in the low node (16.8%) compared to the high node (14.5%). The same was noted for the CD4+ T cells with the mean IL-2R expression higher in the low node (26.0%) than the high node (23.0%).

Table 5.7

Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 22 stage I patients (low lymph node tumour free and the high node tumour free).

Activation Marker	Stage I Low node (n=22)	Stage I High node (n=22)	Statistical Significance+
HLA DR on CD8+ T cells	41.2+/-4.6	36.8+/-4.1	p=0.088
HLA DR on CD4+ T cells	28.8+/-4.4	23.1+/-3.2	p=0.057
Interleukin-2 receptor (Tac) on CD8+ T cells	16.8+/-4.2	14.5+/-2.9	p=0.071
Interleukin-2 receptor (Tac) on CD4+ T cells	26.0+/-3.8	23.0+/-3.2	p=0.099
sIgG on CD19+ B cells	24.9+/-2.4	25.2+/-2.9	p=0.101

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

+Statistical test used: Wilcoxon signed rank, when comparing two lymph nodes from the same patient. Statistical significance is taken at a p value equal to or < 0.05.

5.2.1.3. Surface Immunoglobulin G (sIgG)

No significant trends were noted and no difference was observed between the CD19+ sIgG mean expression in the low (24.9%) and high nodes (25.2%) (Table 5.7).

5.2.2. Stage II paired lymph nodes n=22

Table 5.8 summarises the expression of HLA DR and IL-2R on T cells and sIgG on B cells. As with stage I, the average levels of all markers were higher on the low node nearest to the tumour

5.2.2.1. HLA DR

The mean expression of HLA DR on CD8+ T cells was found to be greater in the low node (46.0%) compared with the high node (42.9%) although this failed to reach statistical significance (Table 5.8). The mean expression of HLA DR on CD4+T cells was higher in the low nodes (31.2%) than the high node (25.0%) reaching statistical significance p=0.01 (Table 5.8, Figure 5.5). The mean expression of HLA DR, generally was found to be lower on the CD4+ T cells expressing this activation marker.

Table 5.8

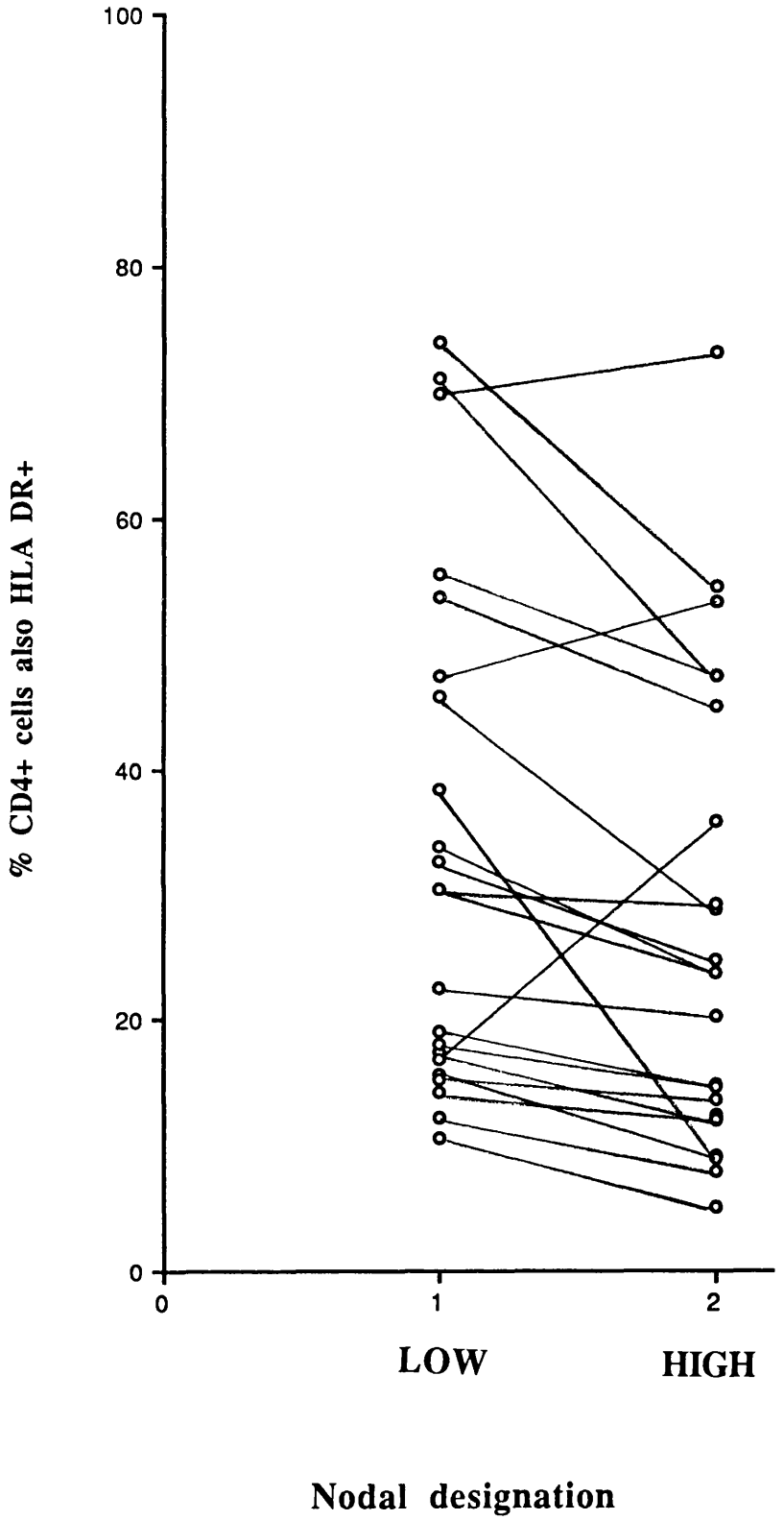
Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 22 stage II patients.

Activation Marker	Stage II Low node (n=22)	Stage II High node (n=22)	Statistical Significance+
HLA DR on CD8+ T cells	46.0+/-4.8	42.9+/-4.5	p=0.060
HLA DR on CD4+ T cells	31.2+/-0.1	25.0+/-3.9	p=0.01
Interleukin-2 receptor (Tac) on CD8+ T cells	16.0+/-3.3	12.6+/-2.3	p=0.05
Interleukin-2 receptor (Tac) on CD4+ T cells	20.7+/-2.5	16.3+/-2.2	$p < 0.001$
sIgG on CD19+ B cells	30.0+/-4.8	22.9+/-3.7	p=0.073

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

+Statistical test used: Wilcoxon signed rank, when comparing two lymph nodes from the same patient. Statistical significance is taken at a p value equal to or < 0.05.

Figure 5.5 The change in CD4+ T cell HLA DR+ population between the low (1) and high (2) nodes of the total 22 stage II patients.


100
80
60
40
20
0
% CD4+ cells also HLA DR+
0
1
2
LOW
HIGH
Nodal designation

5.2.2.2. Interleukin-2 receptor

The proportion of CD8+ T cells expressing the IL-2R was found to be higher in the low node (16.0%) than the high node (12.6%) with this trend being statistically relevant at p=0.01 (Table 5.8, Figure 5.6). A similar trend is noted with the CD4+ T cells expressing the IL-2R in the low node (20.7%) greater than the high node (16.3%) with statistical significance at $p < 0.001$ (Table 5.8, Figure 5.7). The expression of the IL-2R was found to be generally higher on the CD4+ T cells.

5.2.2.3. Surface Immunoglobulin G

The mean level of expression of IgG on CD19+ B cells was observed to be higher in the low node (30.0%) than the high node (22.9%). However this trend was not statistically relevant (Table 5.8).

5.2.3. Stage II subset A: Low node tumour invaded, high node tumour free (n=8).

In this subset, as with stage I and the pooled stage II patients the average for the low node was greater than the high for all parameters

Figure 5.6 The change in CD8+ T cell IL-2R+ population between the low (1) and high (2) nodes of the total 22 stage II patients.

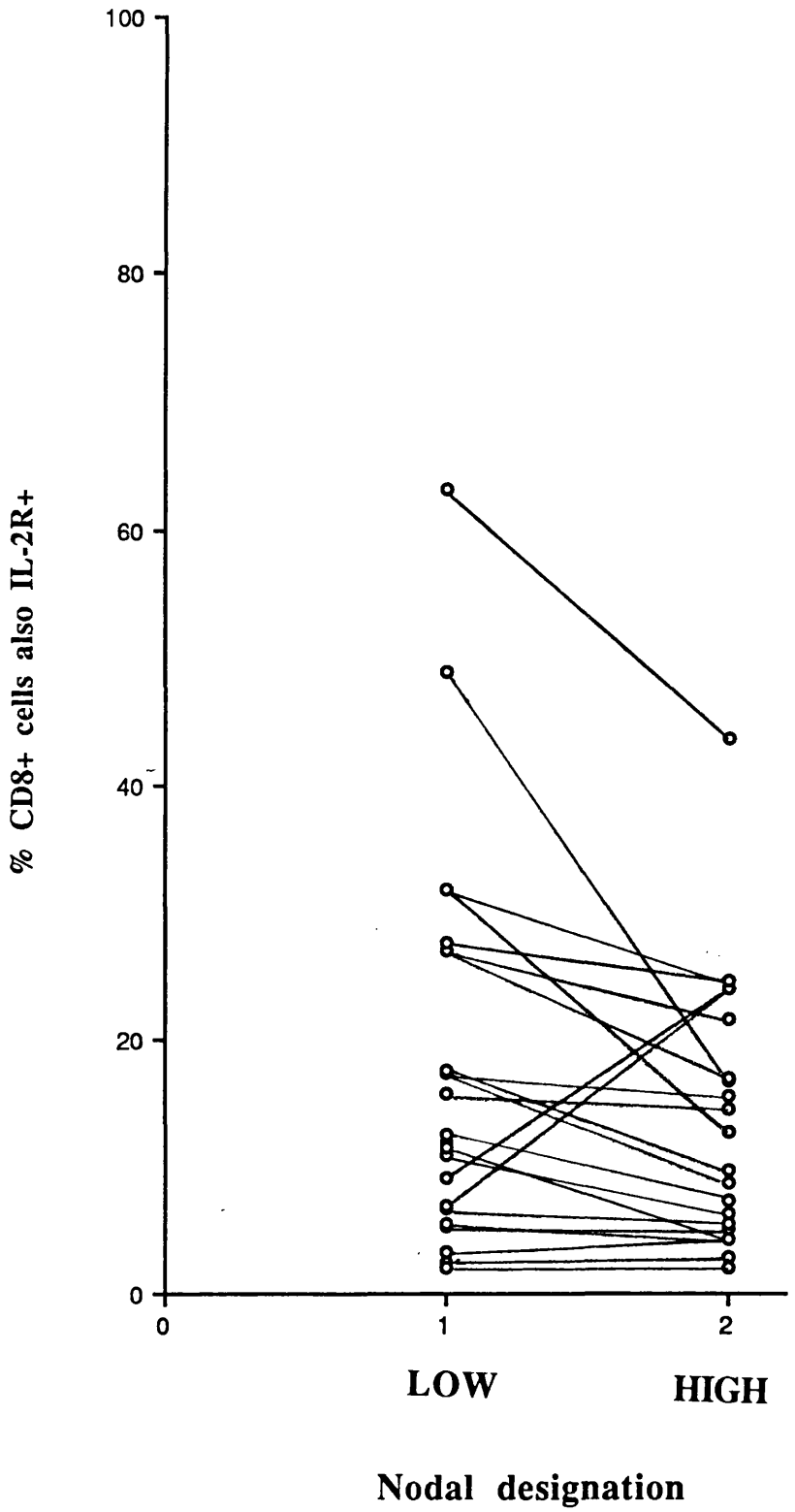
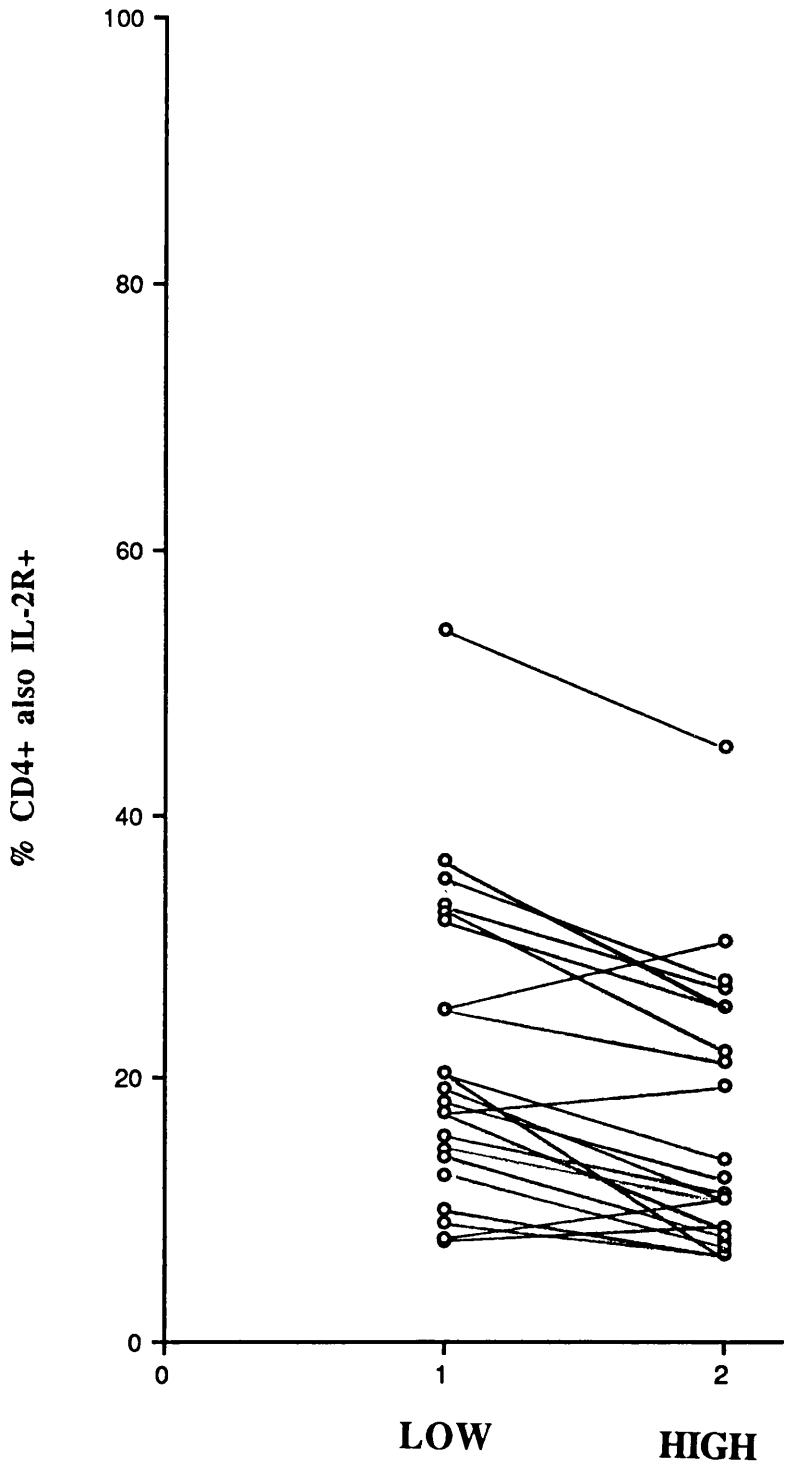

100
80
60
40
20
0
% CD8+ cells also IL-2R+
0
1
2
LOW
HIGH
Nodal designation

Figure 5.7 The change in CD4+ T cell IL-2R+ population between the low (1) and high (2) of the total 22 stage II patients.


100
80
60
40
20
0
% CD4+ also IL-2R+
0
1
2
LOW
HIGH
Nodal designation

5.2.3.1. HLA DR

The mean expression of HLA DR on CD8+ T cells in the low tumour invaded node (65.1%) was found to be significantly greater than that found in the high tumour free node (56.8%) (p=0.05) (Table 5.9, Figure 5.8). A similar trend was observed for the CD4+ T cells expressing HLA DR with a higher mean expression in the low tumour invaded node (49.0%) than the high tumour free node (40.1%), this difference did not achieve statistical significance (Table 5.9). Here the HLA DR expression was found to be generally higher on the CD8+ T cell subset. The level of HLA DR on both CD8+ and CD4+ T cell subsets in this stage II subgroup was higher than the mean values in Table 5.8.

5.2.3.2. Interleukin-2 receptor

A higher mean expression of IL-2R was observed on the CD8+ T cells in the low tumour invaded (16.9%) rather than the high tumour free node (9.2%), but this failed to reach statistical significance (Table 5.9).The mean expression of the IL-2R on the CD4+ subset was higher on the low tumour invaded node (21.6%) than the high tumour free node (14.7%) with this trend being highly significant p=0.005 (Table 5.9, Figure 5.9).

5.2.3.3. Surface Immunoglobulin G

Higher mean expression of sIgG was noted in the low tumour invaded (41.3%) rather than the high tumour free node (35.9%) the trend failing to

Table 5.9

Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 8 stage II patients. With low node tumour invaded and high node tumour free (subset A).

Activation Marker	Stage II		Statistical Significance+
	Low node Tumour invaded (n=8)	High node Tumour free (n=8)	
HLA DR on CD8+ T cells	65.1+/-4.8	56.8+/-6.7	p=0.05
HLA DR on CD4+ T cells	49.0+/-6.4	40.1+/-7.8	p=0.089
Interleukin-2 receptor (Tac) on CD8+ T cells	16.9+/-5.7	9.2+/-1.8	p=0.060
Interleukin-2 receptor (Tac) on CD4+ T cells	21.6+/-4.1	14.7+/-3.0	p=0.005
sIgG on CD19+ B cells	41.3+/-9.9	35.9+/-8.0	p=0.055

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

Figure 5.8 The change in CD8+ T cell HLA DR+ population between the low (1) tumour invaded and high (2) tumour free nodes of 8 stage II patients.

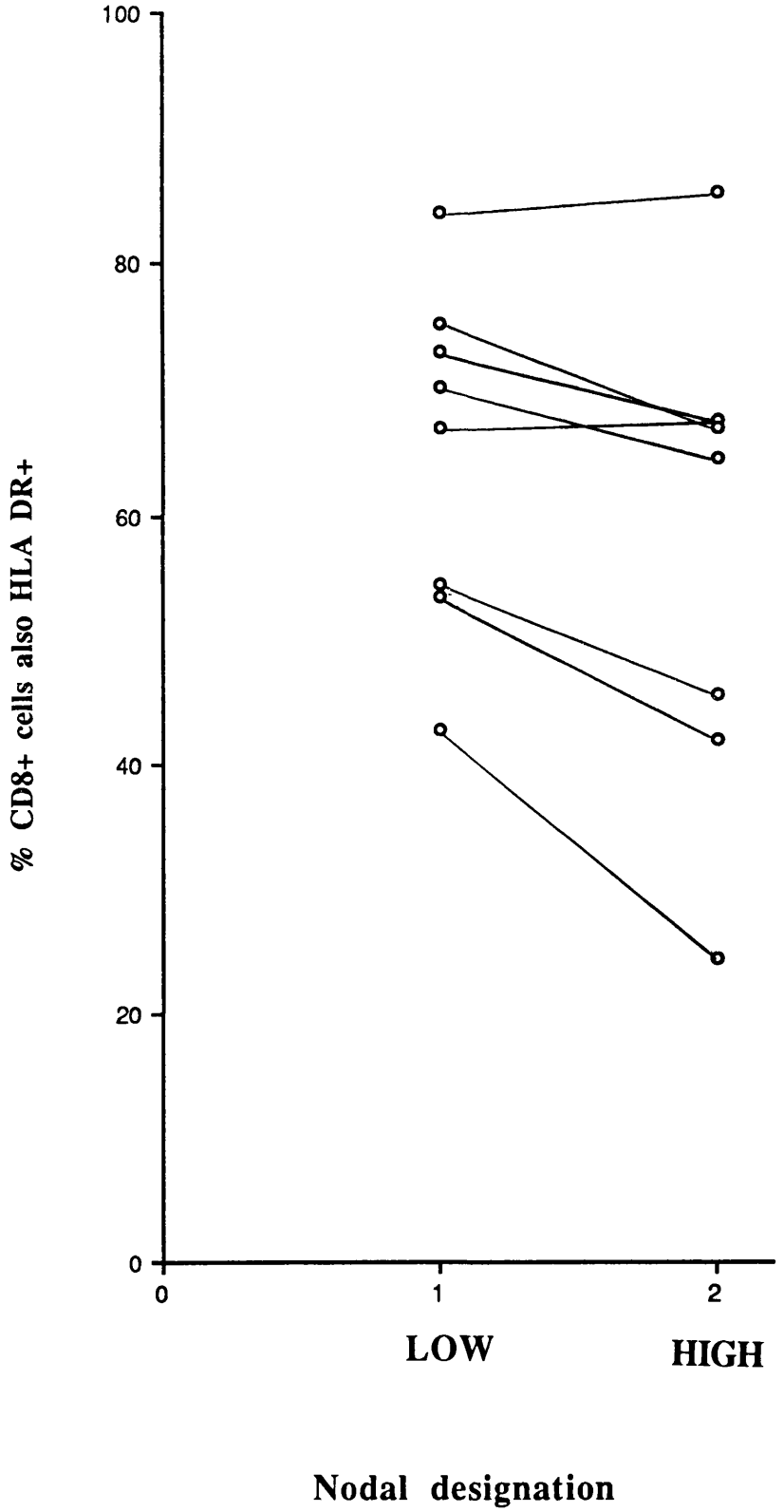
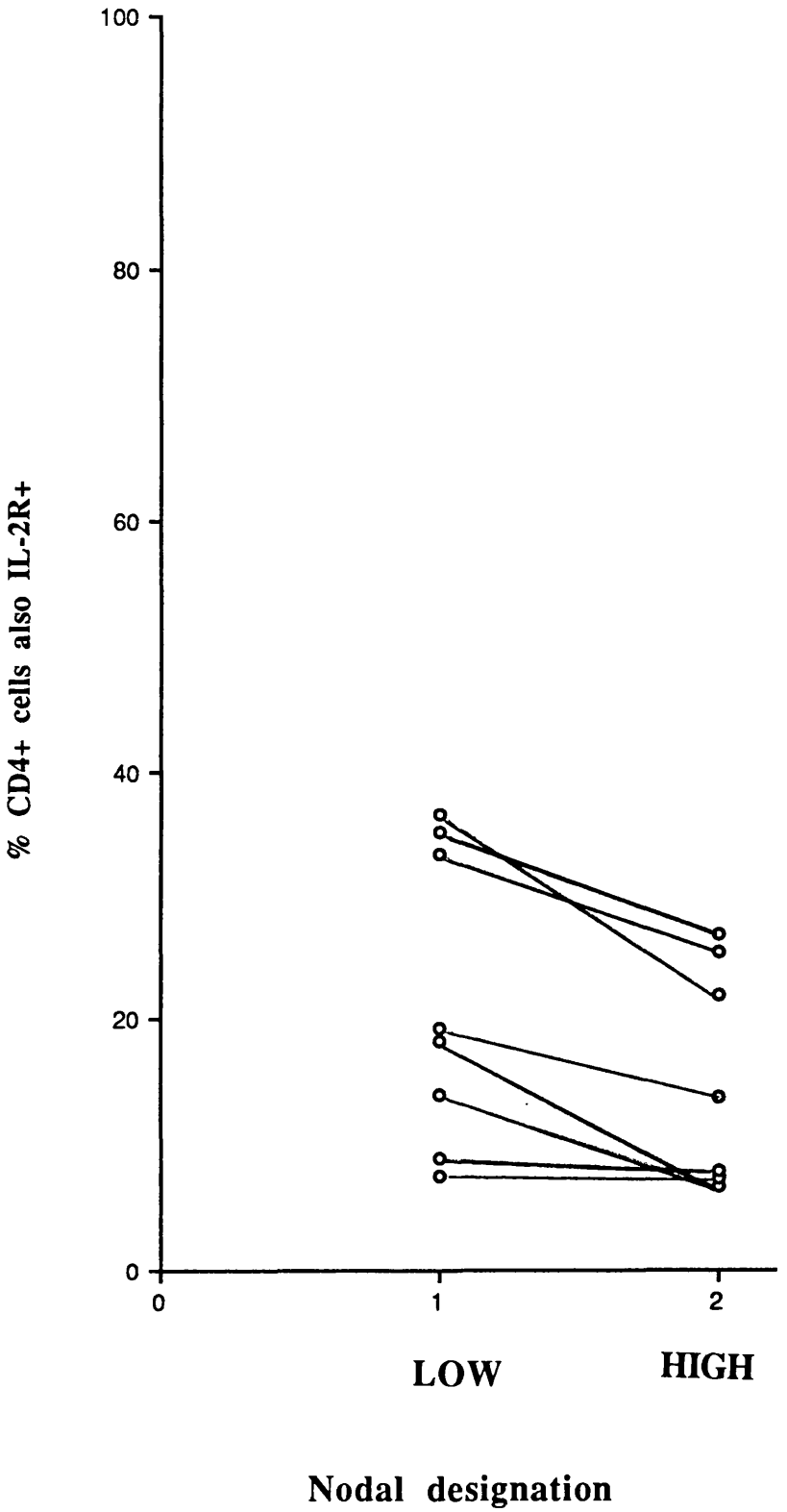

100
80
60
40
20
0
% CD8+ cells also HLA DR+
0
1
2
LOW
HIGH
Nodal designation

Figure 5.9 The change in CD4+ T cell IL-2R population between the low (1) tumour invaded and high (2) tumour free nodes of 8 stage II patients.


100
80
60
40
20
0
% CD4+ cells also IL-2R+
0
1
2
LOW
HIGH
Nodal designation

make statistical significance (Table 5.9). The level of expression in the nodes was found to be higher than the mean value obtained for stage II patients (Table 5.8).

5.2.4. Stage II subset B: Low node tumour invaded, high node tumour invaded (n=7).

The expression of activation markers is illustrated for this stage II subset in Table 5.10. No statistically significant trends were evident although some differences were noted. As with stage I patients and the pooled group of stage 2 patients, the average percentages in the low node were greater than those of the high node.

5.2.4.1. HLA DR

There was no difference in the mean expression of HLA DR on CD8+ T cells in the low tumour invaded (33.0%) and high tumour invaded nodes (32.0%). The level of expression was lower in this subset than the mean for all stage II patients (Table 5.8). The expression of HLA DR on CD4+ T cells was slightly higher in the low tumour invaded node (20.3%) than the high tumour invaded node (16.6%). The values here were lower than the mean for total stage II patients (Table 5.8).

5.2.4.2. Interleukin-2 receptor

The mean expression of the IL-2R on CD8+ T cells was higher in the low tumour invaded node (19.2%) than the high tumour invaded node (15.2%) although not statistically relevant. The same was noted for IL-2R mean

Table 5.10

Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 7 stage II patients with both nodes tumour invaded (subset B).

Activation Marker	Stage II		Statistical Significance+
	Low node Tumour invaded (n=7)	High node Tumour invaded (n=7)	
HLA DR on CD8+ T cells	33.0+/-6.6	32.0+/-4.9	p=0.110
HLA DR on CD4+ T cells	20.3+/-4.5	16.6+/-3.4	p=0.090
Interleukin-2 receptor (Tac) on CD8+ T cells	19.2+/-4.2	15.2+/-3.3	p=0.067
Interleukin-2 receptor (Tac) on CD4+ T cells	22.2+/-3.5	19.2+/-3.1	p=0.095
sIgG on CD19+ B cells	16.0+/-3.9	12.9+/-2.9	p=0.057

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

expression on CD4+ T cells in the low tumour invaded (22.2%) and high tumour invaded nodes (19.2%).

5.2.4.3. Surface Immunoglobulin G

Although not statistically relevant a higher mean expression of sIgG was noted in the low tumour invaded node (16.0%) than the high tumour invaded node (12.9%), the two values being lower than what is observed in the total mean stage II mean expression of sIgG (Table 5.8).

5.2.5. Stage II subset C: Low node tumour free, high node tumour free (n=5).

5.2.5.1. HLA DR

There is no change in mean expression of HLA DR on CD8+ T cells in the low tumour free (31.0%) and high tumour free nodes (32.0%) (Table 5.11). An increase is noted in the HLA DR CD4+ T cells in the low tumour free (31.0%) and high tumour free nodes (20.0%), but this is not statistically relevant (Table 5.11).

5.2.5.2. Interleukin-2 receptor

The mean percentage of CD8+ lymphocytes expressing the IL-2R does not significantly change between the low tumour free (8.7%) and the high tumour free nodes (10.3%) (Table 5.11) .The same is noted for the CD4+ lymphocytes in the low tumour free (11.8%) and high tumour free nodes (11.9%) expressing the IL-2R (Table 5.11). This contrasts with the greater levels of IL-2R found in the previous stage 2 subgroups.

Table 5.11

Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 5 stage II patients (subset C).

Activation Marker	Stage II Low node Tumour free (n=5)	Stage II High node Tumour free (n=5)	Statistical Significance+
HLA DR on CD8+ T cells	31.0+/-9.4	32.0+/-7.7	p=0.109
HLA DR on CD4+ T cells	31.0+/-1.4	20.0+/-7.5	p=0.058
Interleukin-2 receptor (Tac) on CD8+ T cells	8.7+/-2.9	10.3+/-3.7	p=0.103
Interleukin-2 receptor (Tac) on CD4+ T cells	11.8+/-1.4	11.9+/-3.7	p=0.146
sIgG on CD19+ B cells	33.7+/-4.7	24.5+/-5.3	p=0.05

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

+Statistical test used: Student T test as the number of paired lymph nodes has decreased due to division into stage II subsets. Statistical significance is taken at a p value equal to or < 0.05.

5.2.5.3. Surface Immunoglobulin G

The expression of sIgG was significantly higher in the low node (33.7%) than the high node (24.5%) ($p=0.05$) in this stage II subset (Table 5.11, Figure 5.10). However, with only 5 patients, even a statistically significant result is very preliminary as Figure 5.10 shows one of the five patients displaying the opposite trend.

5.2.6. Stage II subset D: Low node tumour free, high node tumour invaded (n=2).

As there were only two patients in this stage II subset, no statistics were performed and any trends which are observed must be treated with caution (Table 5.12).

5.2.6.1. HLA DR

The mean expression of HLA DR on the CD8+ T cell population was higher in the low tumour free (27.7%) than the high tumour invaded node (24.5%). This trend of greater mean HLA DR expression was found for the CD4+ T cells in the low tumour free (15.1%) and high tumour invaded nodes (10.6%). The mean expression of HLA DR on both T cell subsets (Table 5.12) was less than the average for total stage II patients (Table 5.8).

Figure 5.10 The change in sIgG expression on CD19+ B cells in the low (1) tumour free and high (2) tumour free nodes of 5 stage II nodes.

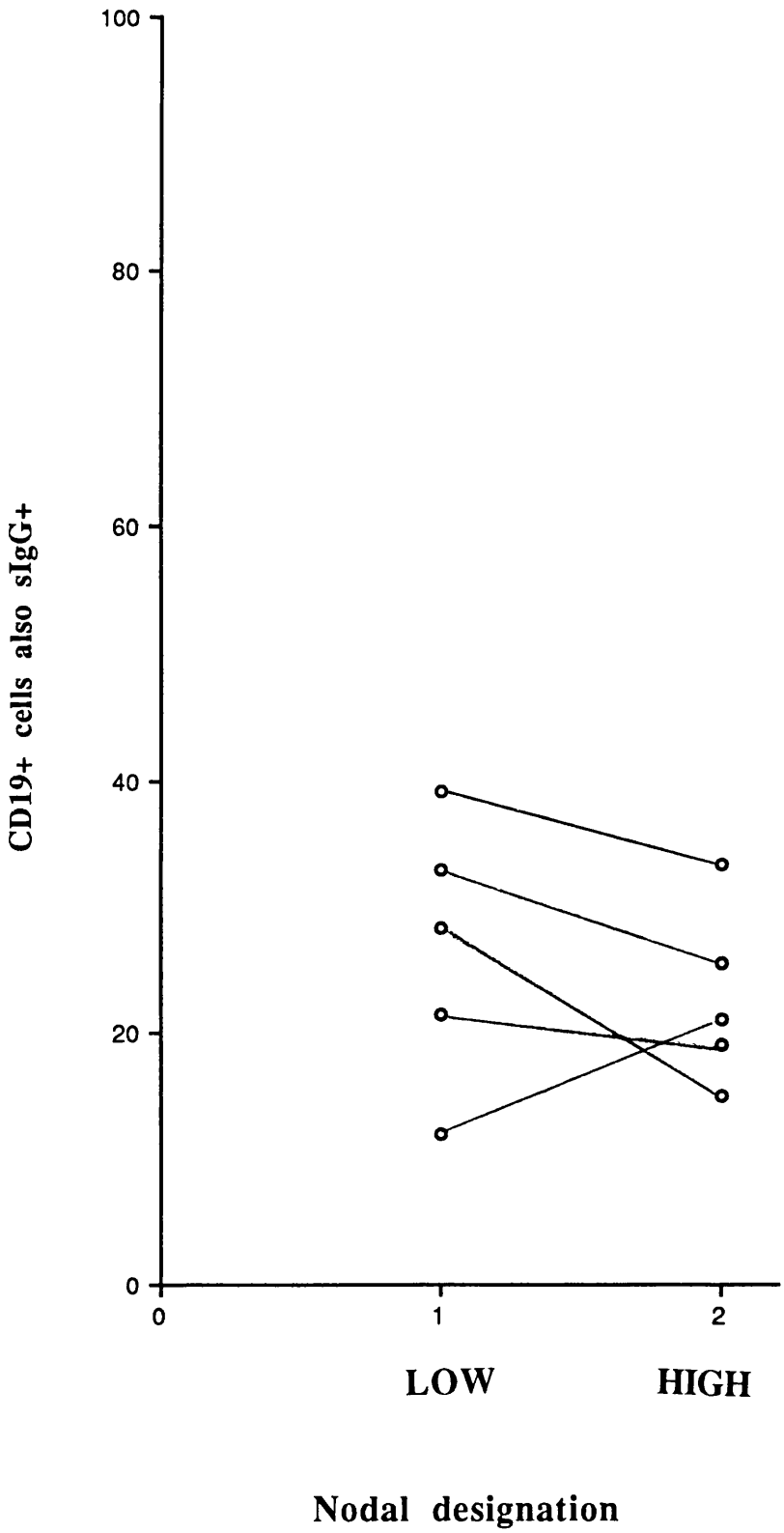

100
80
60
40
20
0
CD19+ cells also sIgG+
0
1
2
LOW
HIGH
Nodal designation

Table 5.12

Distribution of activation markers on lymphocytes from lymph nodes taken from the low axilla (5cm from the tumour) and the high axilla (> 10cm from the tumour) of 2 stage II patients with the low node tumour free and the high node tumour invaded (subset D).

Activation Marker	Stage II		Statistical Significance*
	Low node Tumour free (n=2)	High node Tumour invaded (n=2)	
HLA DR on CD8+ T cells	27.7+/-1.8	24.5+/-1.3	
HLA DR on CD4+ T cells	15.1+/-2.9	10.6+/-1.8	
Interleukin-2 receptor (Tac) on CD8+ T cells	10.0+/-0.9	7.1+/-1.6	
Interleukin-2 receptor (Tac) on CD4+ T cells	17.5+/-2.9	8.8+/-2.1	
sIgG on CD19+ B cells	12.3+/-9.4	9.5+/-6.8	

Values represent mean % +/- standard error of lymphocytes expressing the activation marker.

* Due to only 2 members of this stage II subset statistics were not performed.

5.2.6.2. Interleukin-2 receptor (Tac)

The level of mean IL-2R expression was greater for the CD8+ T cells within the low tumour free (10.0%) with decreasing levels found in the high tumour invaded node (7.1%) (Table 5.12). The same trend was observed for the IL-2R mean expression on
the CD4+ T cells in the low tumour free (17.5%) and high tumour invaded node (8.8%) (Table 5.12).

5.2.6.3. Surface Immunoglobulin G

sIgG was found to be higher in the low (12.3%) node rather than the high (9.5%) node (Table 5.12) with values less than the total stage II means (Table 5.8).

5.3. Discussion

The aim of this section was to investigate the extent to which the immune response was local. The overall conclusion is that the immune response, at least in terms of activation markers, decreases significantly in between nodes 5cm from the primary tumour and nodes 10cm from the primary tumour. Thus there is a substantive local response which will be removed by axillary sampling or clearance or destruction by local radiotherapy. In consequence, if cellular or antibody based immunotherapy is envisaged, such tissues should probably be stored in a manner which retains their suitability for this purpose immediately after the operation.

There are both significant phenotypic and activation changes occurring in the nodes of breast cancer patients in this study. Phenotype changes are observed in the overall levels of T and B cells within the nodes of stage 1 patients. With greater levels of T cells in the low node than in the high node and the opposite trend noted for B cells. The presence of greater numbers may be due to the fact that T cells are responding locally to the tumour. The T cell immune response can be observed more clearly in the trend observed for stage 2 node subset A with low node tumour invaded and high node tumour free. Here the trend was within the T cell subsets with a greater percentage of CD8+ T cytotoxic cells within the low tumour invaded node than the high tumour free. The presence of the CD8+ cells may be due to an immune response initiated due the close proximity of tumour which may be recognised by MHC class 1 restricted cytotoxicity, in agreement with the larger study in chapter 4.

However more interestingly the phenotypic trends were generally less significant than for the two activation markers HLA DR and the interleukin-2 receptor (IL-2R;Tac). The two markers of activation are both independant and

are initiated at different times after initial antigen stimulation with the IL-2R being expressed after 2 hours and HLA DR after 3-5 days (Abbas *et al* 1991; Tadatsugu and Minami, 1993). In general higher levels of HLA DR were noted in the stage 2 nodes than stage 1 agreeing with both Morton *et al* (1986) and Whitford *et al* (1992b). Trends in lymphocyte activation were only noted in stage 2 patients, suggesting that close proximity of tumour may initiate a greater immune response with HLA DR reflecting tumour invasion and the IL-2R the distance from the tumour. But within the low node the percentage of activation marker was generally greater. When the 22 stage 2 patients are divided, new trends are noted, as each group may represent a different type of tumour. A more agressive tumour may suppress the immune response and initiate few changes in the lymph nodes. Sub group B with both nodes invaded and HLA DR generally lower than the stage 2 mean may indicate this type of tumour. Whereas group A has tumour in the low node and levels of HLA DR are higher in both nodes than the mean stage 2 level, which could correspond to a local immune response throughout the nodes inhibiting the tumour from further metastasising possibly due to the type of the tumour. The presence of tumour in the nodes of breast cancer patients has been reported not to be a bad prognosis in all cases (Fisher *et al* 1980) which would agree with these findings that stage 2 patients can be subdivided with respect to their immune response in the nodes. The nodes that contain no tumour and are from an axilla with tumour present elsewhere sub group C may be interesting as they might show the first signs of tumour invasion. The activation levels of HLA DR are below the average found in stage 2 patients, however a trend is noted for increased IgG expression in these nodes above the mean stage 2 level. Possibly indicating a surface antigen or an internal mutant protein such as p53. p53 may be released due to the tumour undergoing immune attack or necrosis. However a B cell response may not be useful if the protein is internal such as p53.

In conclusion the percentage of cells bearing the activation markers HLA DR and Il-2R was observed to be higher in the low nodes rather than the high nodes which would indicate a greater immune response occurring within these nodes. This was noted for stage 1 and the pooled stage 2 patients but significance was only achieved for stage 2 patients with HLA DR mainly associated with tumour invasion and the IL-2R with distance from the tumour. These differences were seen within the stage II nodes when they were further subdivided. The main differences seemed to be from the subset with low node tumour invaded and high node tumour free. The trends of activation could signify that there are immune responses in both stage 1 and stage 2 tumours. However the presence of overall greater levels of HLA DR in stage 2 nodes would suggest that these patients are stimulating the immune system possibly due to the presence of greater levels of antigenic determinants on these tumours of stage 2 subsets.

CHAPTER 6

DNA AND ONCOGENE EXPRESSION IN PRIMARY AND METASTATIC BREAST CANCER

6. DNA and oncogene expression in primary and metastatic breast cancer

There is increasing evidence that breast cancer has a multi-factorial aetiology and that a variety of prognostic indicators may be employed to assess the aggressive capacity of a primary tumour. Among a number of parameters, those which have been shown to correlate strongly with prognosis are cell cycle analysis at the DNA level (Section 1.4.2.2.), and cellular oncogene or mutant tumour suppressor gene product estimation at the protein level (Section 1.4.3.). Cell cycle analysis, usually performed by flow cytometry, makes it possible to estimate the extent to which the primary tumour has aneuploid cells with abnormal DNA content together with the percentage of cells in the S phase of the cell cycle. Among the oncogene related proteins, which have generally been assessed by immunocytochemical methods, recent interest has centred on the c-*erb* B-2 gene product over-expressed on the surface of 20-30% of primary breast cancers (Clark and MacGuire 1991; Varley *et al* 1991; Gullick *et al* 1991) and the p53 mutant tumour suppressor gene product over-expressed in 50% of breast carcinomas (Cattoretti *et al* 1988; Bartek *et al* 1990).

Primary breast tumours, like most solid tumours of epithelial origin, generally show a heterogeneous cell population. This may reflect genuine tumour cell heterogeneity where metastatic subpopulations of the primary are most relevant to tumour progression. In addition, it may also include normal epithelial cells, stromal fibroblasts, and lymphocytes infiltrating the tumour (Whitford *et al* 1990). Thus DNA cell cycle analysis and oncoprotein expression estimation may indicate that a tumour is positive for one or both parameters but it has not been possible to judge whether all those cells within the tumour which have such genetic

abnormalities are also those which carry the mutant or aberrantly expressed oncoprotein.

Flow cytometry, routinely used in lymphocyte analysis, has the advantage that up to five parameters on the same cell may be measured at the same time (Butcher *et al* 1991). In addition, information from 10,000 cells can be objectively collected and quantitated. Flow cytometry may be used to simultaneously analyse DNA and structural protein within the same cells of primary breast tumours (Zarbo *et al* 1989; Alam *et al* 1992). This has been adapted to estimate oncoprotein products on tissue culture cell lines (Kelsten *et al* 1990; Remvikos *et al* 1990) and primary tumour (Kelsten *et al 1990* ; Morkve *et al* 1991a; Morkve *et al* 1991b; Hendy Ibbs *et al* 1987; Remvikos *et al* 1990).

This study describes an analysis of the expression of both p53 and c-*erb* B-2 in primary breast carcinomas and correlates the data with their ploidy status and metastatic capacity.

63 breast carcinomas were analysed simultaneously for DNA and p53 expression. In this group of patients 61 were analysed for stage of tumour and 50 for tumour grade.

51 breast carcinomas were analysed simultaneously for DNA and c-*erb* B-2 expression. Of this group 39 patients were analysed separately for grade of the tumour and 51 patients for tumour stage.

51 tumours were directly compared for both c-*erb* B-2 and p53 expression.

The procedure for oncogene estimation is described in Section 2.2.4.6.

6.1. Expression of the p53 and c-*erb* B-2 oncoproteins

To test the efficacy of the anti-p53 antibody and the anti- c-*erb* B-2 antibody stained with fluorescein (FITC), immunofluorescence was carried out on primary tumour. Both nuclear and cytoplasmic staining was noted for the p53 tumour suppressor protein and membrane staining was found for the c-*erb* B-2 proto-oncogene receptor.

Figure 6.1 illustrates c-*erb* B-2 and p53 flow cytometry

6.1.1. Cell lines

Three cell lines were analysed for p53 and c-*erb* B-2 expression (Table 6.1), two breast cancer lines MCF-7 and ZR-75 with known p53 and c-*erb* B - 2 expression. The other cell line HL-60 is of a neutrophil origin, with a null p53 mutation. p53 positive cells were shown to be greatest in the ZR-75 cell line (90%), with the MCF-7 cell line expressing slightly less p53 at 75%. The control HL-60 was shown to have only 1% p53 expression.

The c-*erb* B-2 expression was highest in the ZR-75 cell line at 40%, the MCF-7 having less expression at 20%. The HL-60 cell line expressed a very small proportion of c-*erb* B-2 at 5%.

The experiment for the cell lines was done in duplicate to test reproducibility, there was less than 5% variation in the result achieved. In addition, when ethanol fixed cells were compared to non ethanol fixed for c-*erb* B-2 staining, less than 5% variation was again observed in the results.

Figure 6.1 Flow cytometric analysis of the expression of the p53 and c-*erb* B-2 oncoproteins in primary breast cancer.

Diagram **A**: The control stained with an irrelevant antibody.

Diagram **B**: The p53 oncoprotein positive tumour cells.

Diagram **C**: The c-*erb* B-2 oncoprotein positive tumour cells.

Blue cells = Diploid tumour cells

Red cells = Aneuploid tumour cells

Green cells = Positive for the oncoprotein

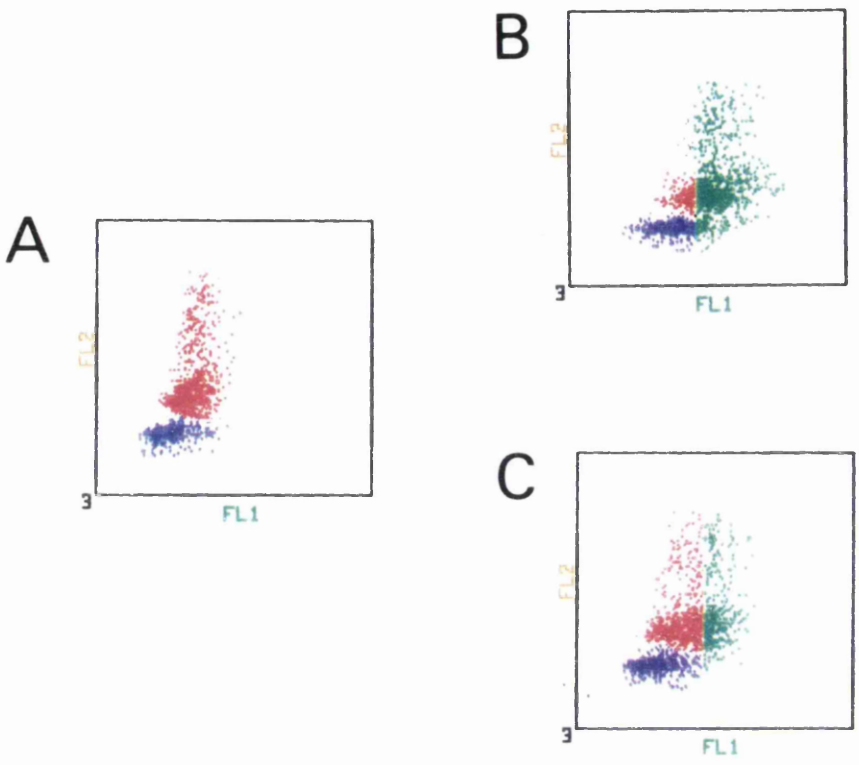

A
FL2
3
FL1
B
FL2
3
FL1
C
FL2
3
FL1

Table 6.1.
Percent positive cells for p53 and c-*erb* B-2 in cell lines

Cell line	Percent p53 positive cells	Percent c-*erb* B-2 positive cells
ZR 75	90	40
MCF-7	75	20
HL-60	1	5

The result is given as cells positive for p53 and c-*erb* B-2 within the total cells analysed.

Table 6.2.
Mean percent cells positive for p53 or c-*erb* B-2 in primary breast tumours using the data from Figure 6.4

Mean Percent positive cells	
p53 (n=63)	c-*erb* B-2 (n=51)
29	30

The values in the table are given as mean cells positive for the p53 or c-*erb* B-2 proteins

6.1.2. Tumour

The percentage of cells positive for p53 ranged from 5% to 84% (Figure 6.2) in the 63 tumours analysed, with an average positivity of 29% (Table 6.2).

The percentage of cells positive for c-*erb* B-2 ranged from 1% to 89% (Figure 6.3) in the 51 tumours analysed, with an average of 30% (Table 6.2).

6.2. Co-expression of c-*erb* B-2 and p53.

There was no strong correlation between the expression of p53 and that of c-*erb* B-2 (Figure 6.4). The patient with the highest expression of both proteins was patient 61 with 89% cells expressing c-*erb* B-2 and 60% cells positive for p53. Other patients had the opposite trend such as patient 10 with high p53 expression and low c-*erb* B-2 expression. Overall the expression of the two proteins was very variable (Table 6.2) and no correlation was found using the Mann-Whitney test.

6.3. DNA ploidy and oncoprotein expression

The simultaneous measurement of both DNA and oncoprotein gives the advantage of observing expression derived from specific DNA populations such as the aneuploid population. Figure 6.1 illustrates a tumour expression of both c-*erb* B-2 and p53 with ploidy. In the total group of tumours analysed 47/63 were aneuploid (75%) and 16/63 were diploid (25%) (Table 6.3).

Figure 6.2 p53 positive cells in 63 primary breast cancers

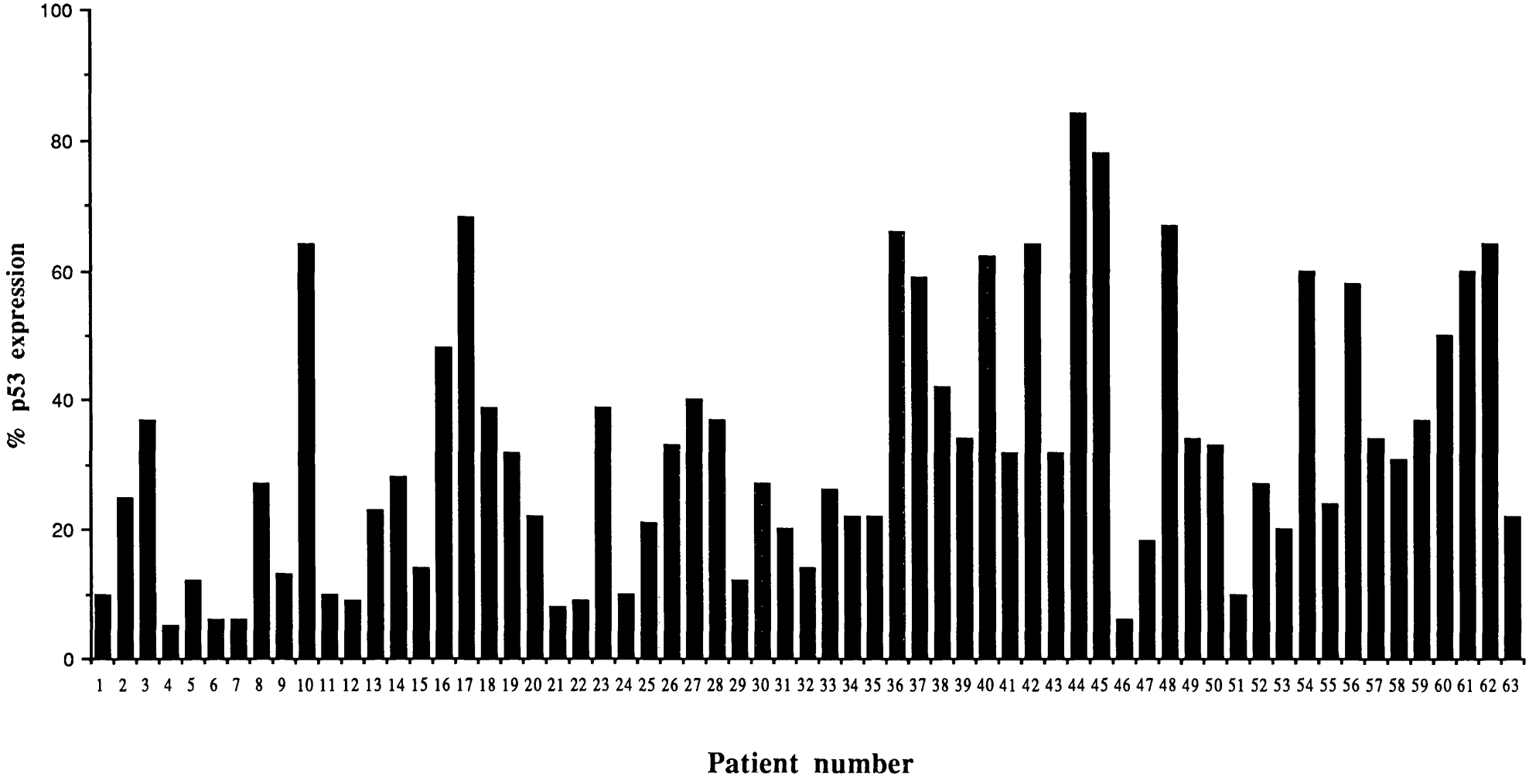

100
80
60
40
20
0
% p53 expression
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63
Patient number

Figure 6.3 c-*erb* B-2 positive cells in 51 primary breast cancers (patient numbers 1-36 and 49-63)

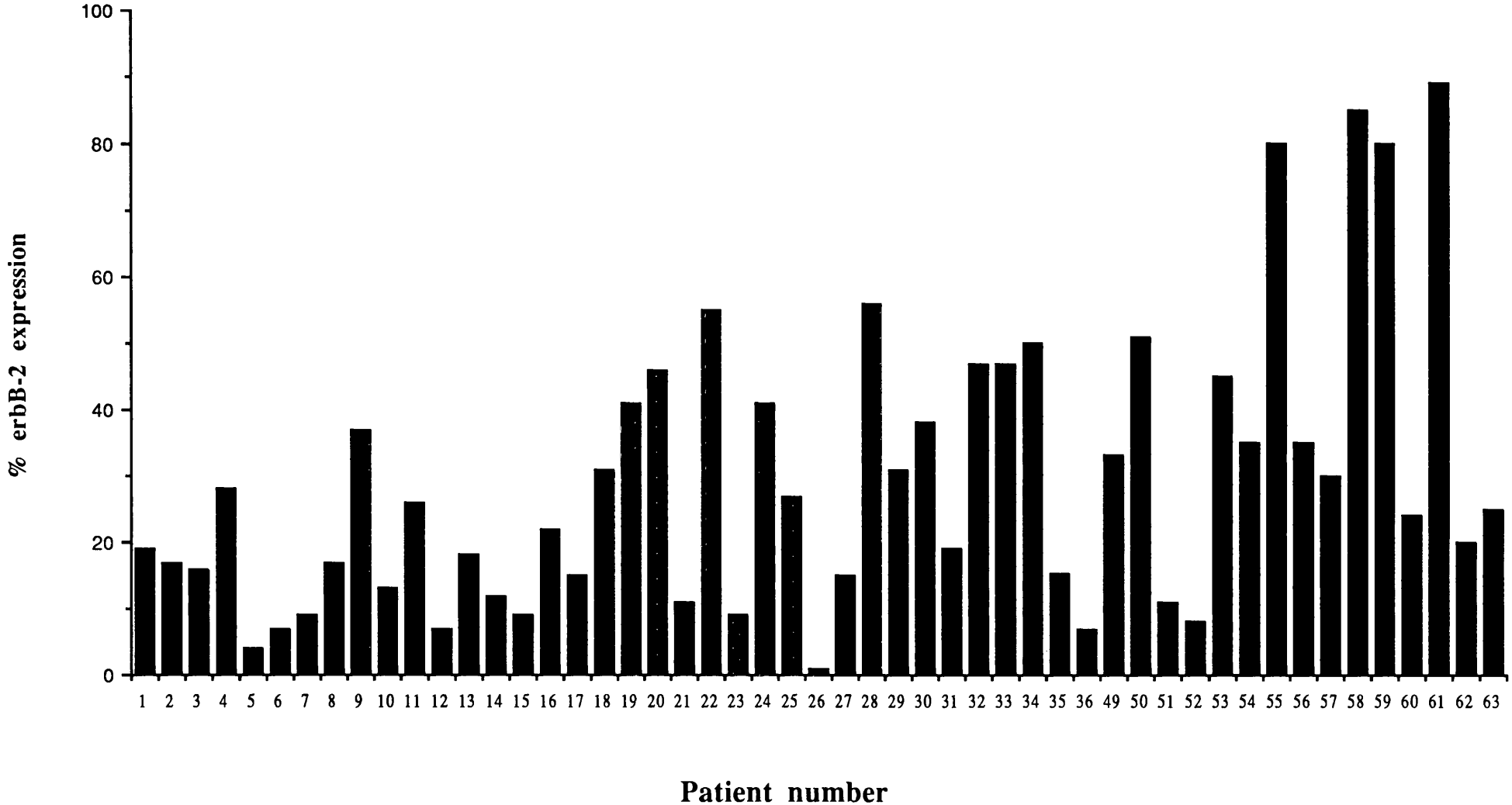

% erbB-2 expression
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63
Patient number

Figure 6.4 p53 and c-*erb* B-2 positive cells in 51 primary breast cancers (patient number 1-36 and 49-63)

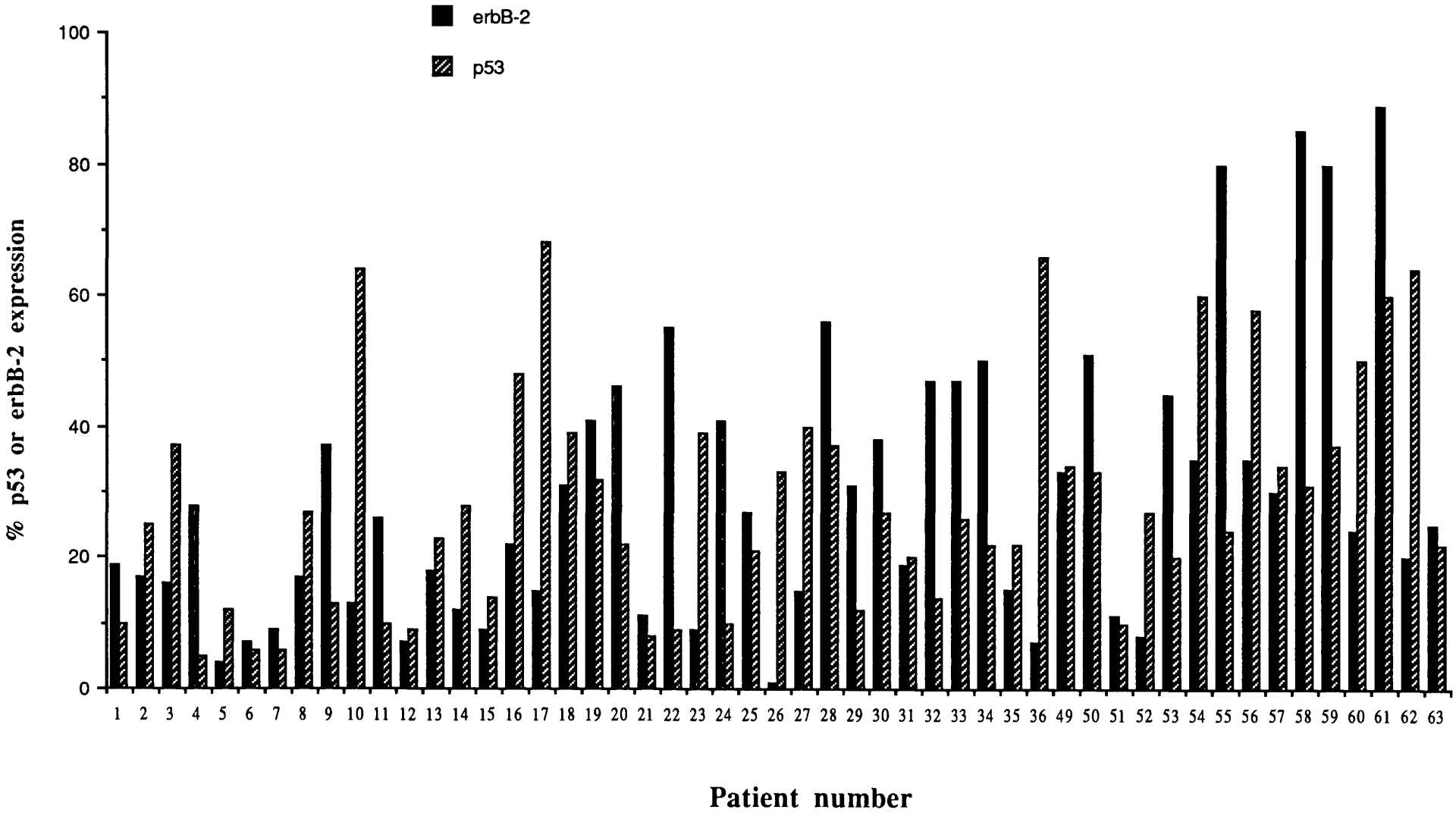

erbB-2
p53
100
80
60
40
20
0
% p53 or erbB-2 expression
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63
Patient number

Table 6.3.

Mean Percentage of cells positive for p53 in comparison to tumour DNA ploidy

	Tumour cell ploidy		Statistical significance
	Diploid (n=16)	Aneuploid (n=47)	
mean % cells p53 positive	28	35	p=0.157

The values in the table are given as mean cells positive for the p53 protein in the diploid and aneuploid tumours

For comparison of two independent groups the Mann Whitney test was used. With significance being less than or equal to p= 0.05

6.3.1. p53 and tumour DNA ploidy

The 16 diploid tumours analysed for p53 expression had a range of between 6% to 64% (Figure 6.5) with an average p53 positivity of 28% (Table 6.3). The 47 aneuploid tumours analysed had a greater range of 5% to 84% (Figure 6.5) and also a higher average expression. However the change although tending to be higher in the aneuploid tumours was not statistically relevant.

6.3.2. c-*erb* B-2 and tumour DNA ploidy

The 14 diploid tumours analysed for c-*erb* B-2 expression had a more restricted range of 1% to 48% (Figure 6.6) and a lower average expression of 15% (Table 6.4) than the diploid tumours analysed for p53 expression. The 37 aneuploid tumours had a much larger range of expression 5% to 84% (Figure 6.6) with an average expression level of 35% (Table 6.4). The increase in expression in the aneuploid population was found to be not statistically relevant.

6.4. Oncoprotein expression in relation to the stage of the tumour

The oncoprotein expression was divided into stage 1 (no nodal metastases in the tumour draining lymph node) and stage 2 (with nodal metastases).

Table 6.4.

Mean percentage of cells positive for c-*erb* B-2 in relation to tumour DNA ploidy

	Tumour cell ploidy		Statistical
	Diploid (n=14)	Aneuploid (n=37)	significance
mean % cells c-*erb* B-2 positive	15	35	p=0.123

The values in the table are given as mean cells positive for the c-*erb* B-2 protein in the diploid and aneuploid tumours

For comparison of two independent groups the Mann Whitney test was used. With significance being less than or equal to p= 0.05

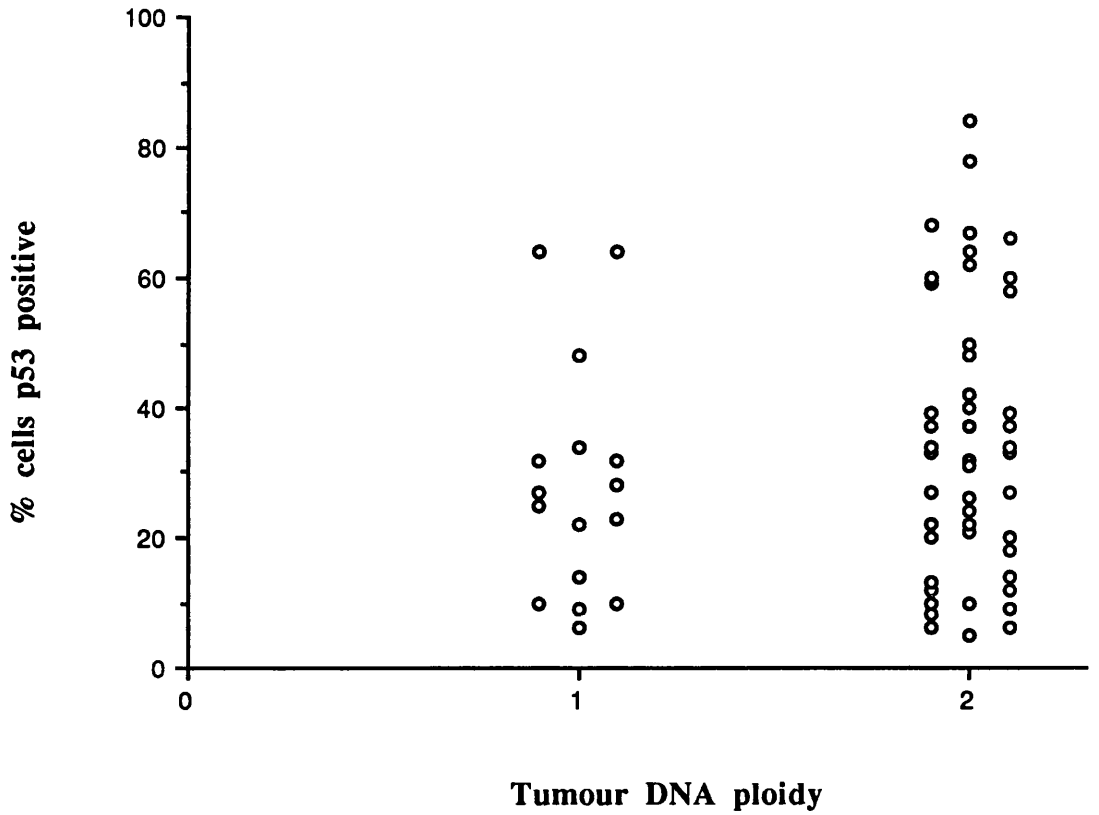


Figure 6.5 Distribution of p53 positive cells within diploid (1) and aneuploid (2) tumours

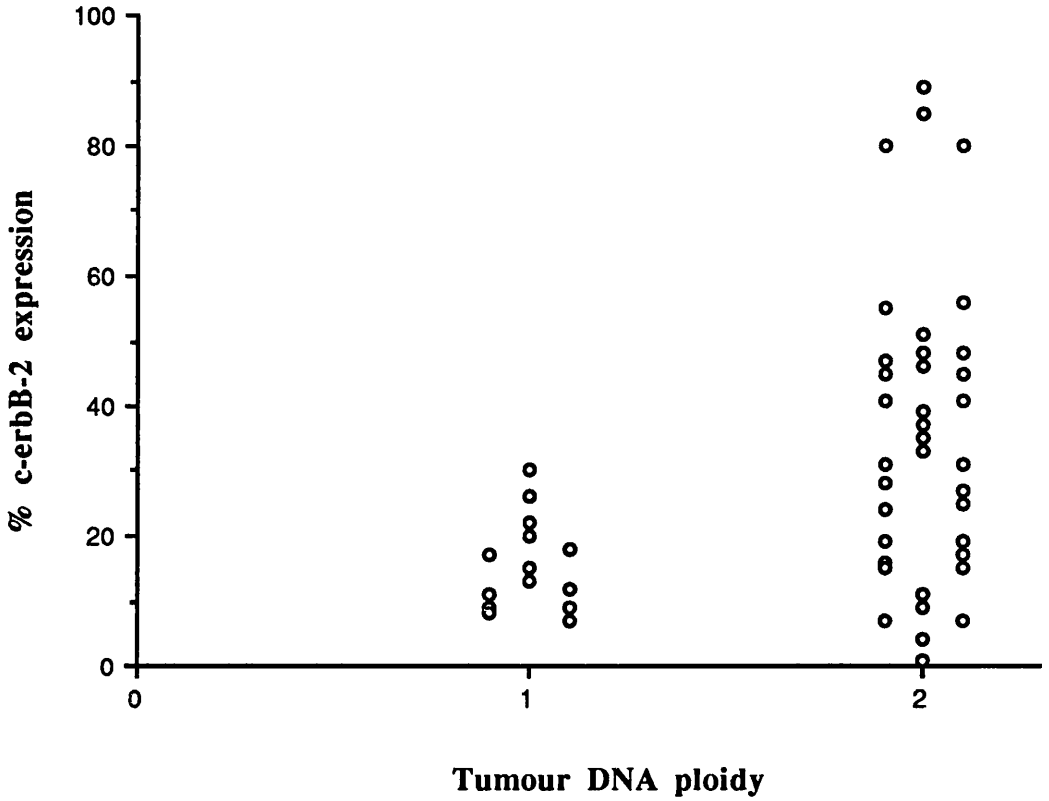


Figure 6.6 Distribution of c-*erb* B-2 positive cells within diploid (1) and aneuploid (2) tumours

6.4.1. p53 expression and stage of the tumour

p53 expression was divided into 26 stage 1 patients and 35 stage 2 patients. The range of expression was greater in the stage 1 patients 6% to 84% than the stage 2 patients range 2% to 64% (Figure 6.7). The average values reflected the range with stage 1 patients at 39% and stage 2 patients at 28% (Table 6.5). These changes do not show any statistical significance.

6.4.2. c-*erb* B-2 expression and stage of the tumour

The expression of c-*erb* B-2 was divided into stage 1 and stage 2 categories. The stage 1 tumours expressed on average lower levels of the c-*erb* B-2 protein of 22% than the stage 2 patients 36% (Table 6.6). The range of expression within the stage 1 patients 1% to 48% showed a much tighter range than that found in stage 2 patients 7% to 89% (Figure 6.8.). However this was not statistically significant.

6.5. Expression of oncoprotein in relation to the grade of tumour differentiation.

The grade of the tumour is a measure of the differentiation within the tumour from grade 1 with little differentiation to grade 3 with extensive differentiation. The p53 and c-*erb* B-2 expression was divided into the three degrees of tumour differentiation.

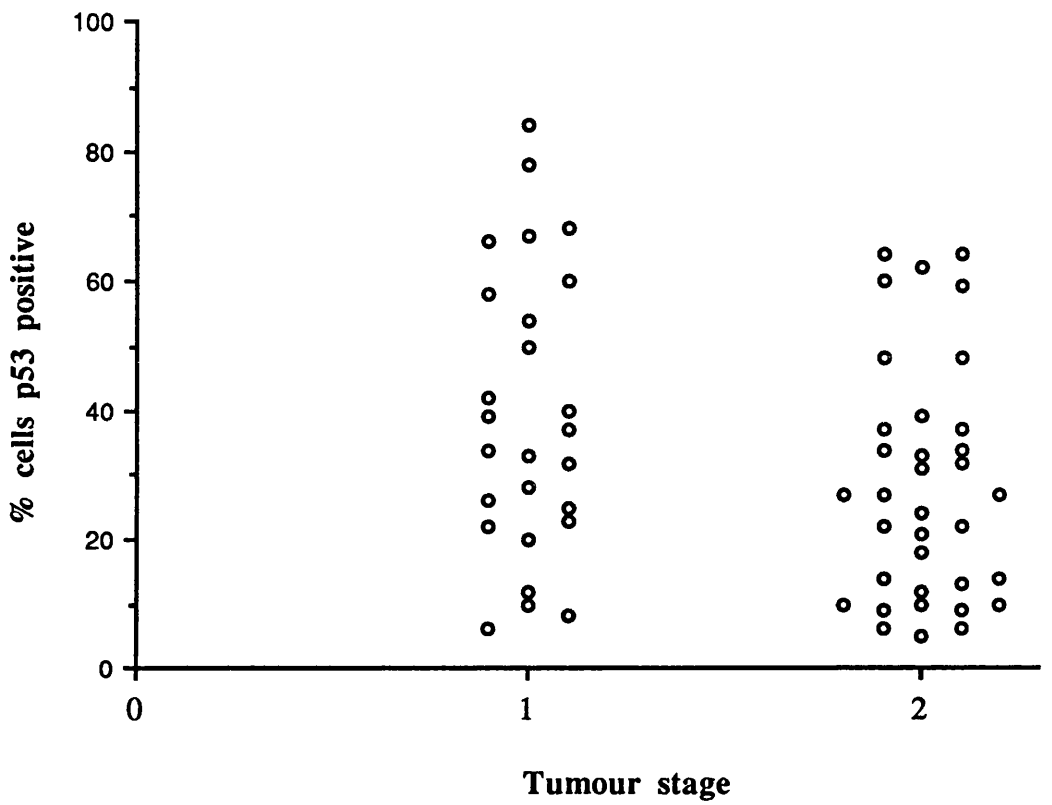


Figure 6.7 Distribution of p53 positive cells within patients with tumour free nodes (stage 1)(1) and tumour invaded nodes (stage 2)(2).

Table 6.5.

Mean percentage of cells positive for p53 in relation to the stage of the tumour (lymph node status).

	Histopathological staging of tumour draining lymph nodes		Statistical significance
	Stage 1 (n=26)	Stage 2 (n=35)	
mean % cells p53 positive	39	28	p=0.187

The values in the table are given as mean cells positive for the p53 protein in stage 1 and stage 2 tumours

For comparison of two independent groups the Mann Whitney test was used. With significance being less than or equal to p= 0.05

Table 6.6.

Mean percentage of cells positive for c-*erb* B-2 in relation to the histopathological staging of the tumour

	Histopathological staging of tumour draining lymph nodes		Statistical significance
	Stage 1 (n=21)	Stage 2 (n=30)	
mean % cells c-*erb* B-2 positive	22	36	p=0.143

The values in the table are given as mean cells positive for the c-*erb* B-2 protein in stage 1 and stage 2 tumours

For comparison of two independent groups the Mann Whitney test was used. With significance being less than or equal to p= 0.05

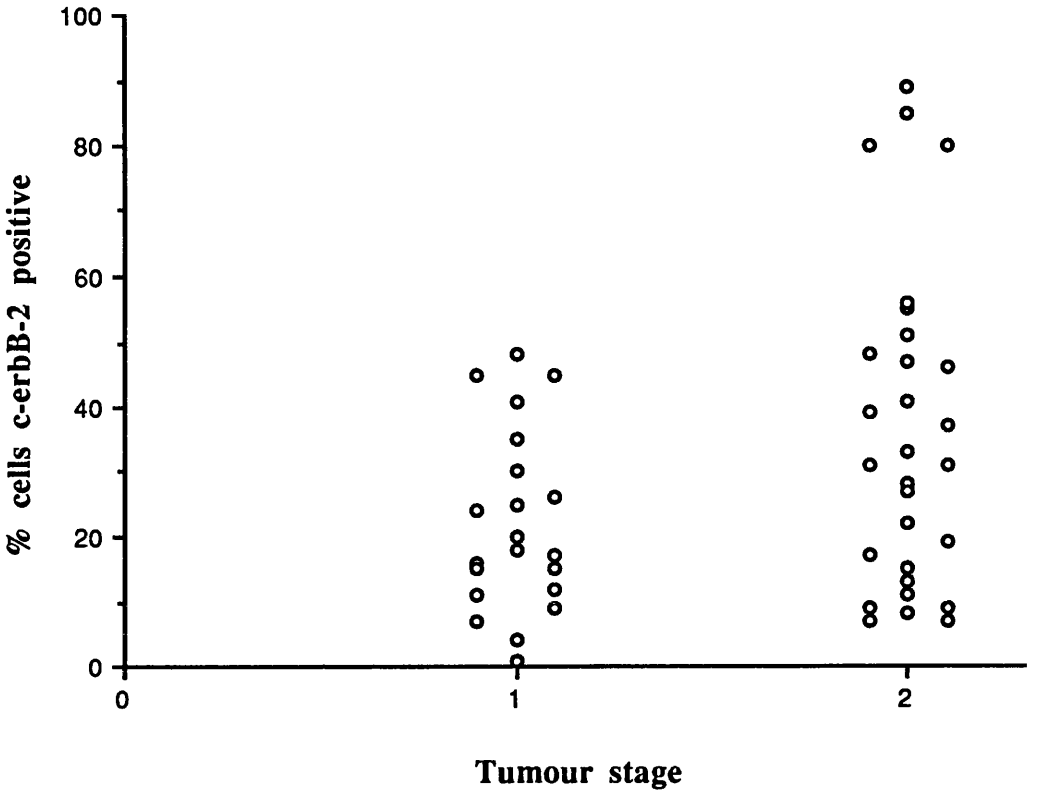


Figure 6.8 Distribution of c-*erb* B-2 positive cells within patients with tumour free nodes (stage 1)(1) and tumour invaded nodes (stage 2)(2).

6.5.1. p53 expression and tumour grade

A trend of increasing p53 expression was noted in the change from a grade 1 tumour with average expression of 29% to a grade 2 tumour of 31% and then finally to grade 3 tumours of 46% (Table 6.7). However due to the small numbers of both grade 1 and 3 tumours this trend failed to be of any significance as a result of the wide range of expression (Figure 6.9.).

6.5.2. c-*erb* B-2 expression and tumour grade

When c-*erb* B-2 was compared with grade a trend of increasing expression with increasing grade was observed with grade 1: 24%, grade 2: 25% and grade 3: 34% (Table 6.8). However this trend was not significant (Figure 6.10.).

6.6. Oncogene expression in the primary tumour and paired nodal metastases

In some of the cases the expression of p53 or c-*erb* B-2 was compared to the paired metastatic tumour within the tumour draining lymph nodes, to deduce if there are changes in expression on the oncoprotein during the metastatic process.

6.6.1. p53 expression and metastases

8 tumours were compared with their matched heavily invaded lymph nodes containing metastatic tumour (Figure 6.11a). In general the expression was similar in the metastases with in three cases little change in expression, three other cases an increase in p53 expression and in the two final cases a

Table 6.7.

Mean percentage of cells positive for p53 in relation to histological grade of tumour differentiation

	Histological grade of tumour differentiation			Statistical significance		
	Grade I (n=8)	Grade II (n=29)	Grade III (n=13)	I vs II	I vs III	II vs III
Mean % cells p53 positive	29	31	46	p=0.197	p=0.097	p=0.086

The values in the table are given as mean cells positive for the p53 protein in the three different grades of tumour differentiation.

For comparison of two independent groups the Kruskal-Wallis test was used as three independent parameters were compared. With significance being less than or equal to p= 0.05

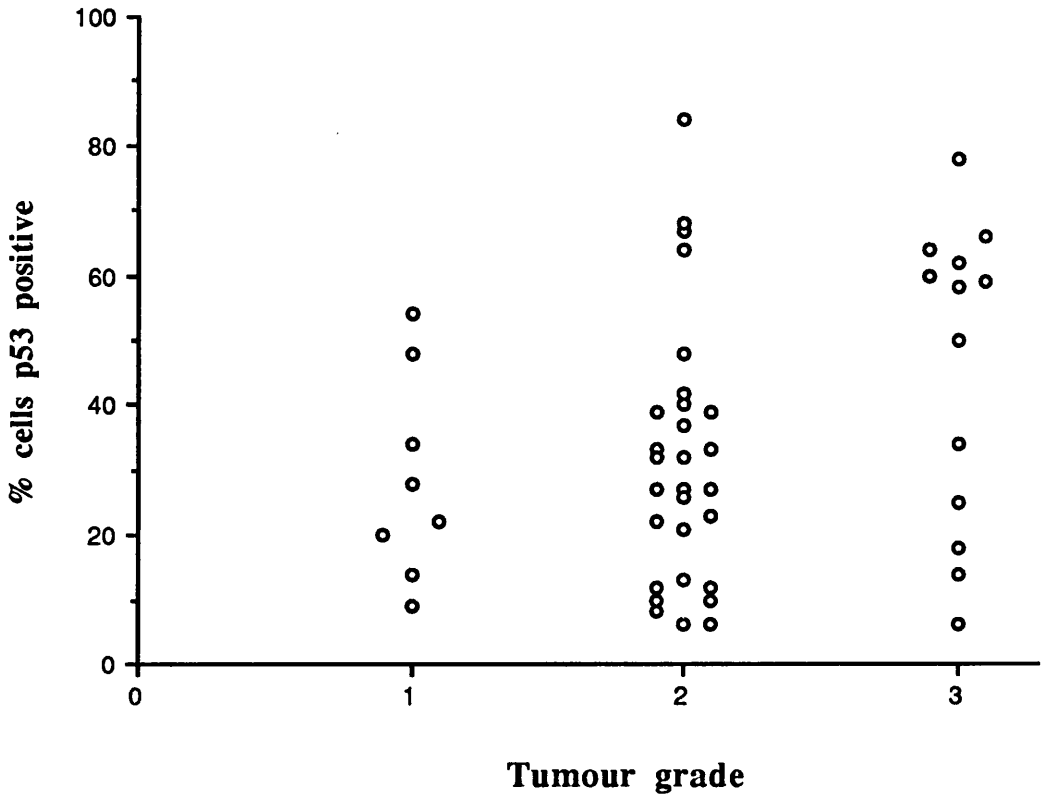


Figure 6.9 Distribution of p53 positive cells within the three different grades of tumour differentiation.

Table 6.8

Mean percentage of cells positive for c-*erb* B-2 in relation to histological grade

	Histological grade of tumour differentiation			Statistical significance		
	Grade I (n=8)	Grade II (n=24)	Grade III (n=7)	I vs II	I vs III	II vs III
Mean % cells p53 positive	24	25	34	p=0.203	p=0.116	p=0.092

The values in the table are given as mean cells positive for the c-*erb* B-2 protein in the three different grades of tumour differentiation.

For comparison of two independent groups the Kruskal-Wallis test was used as three independent parameters were compared. With significance being less than or equal to p= 0.05

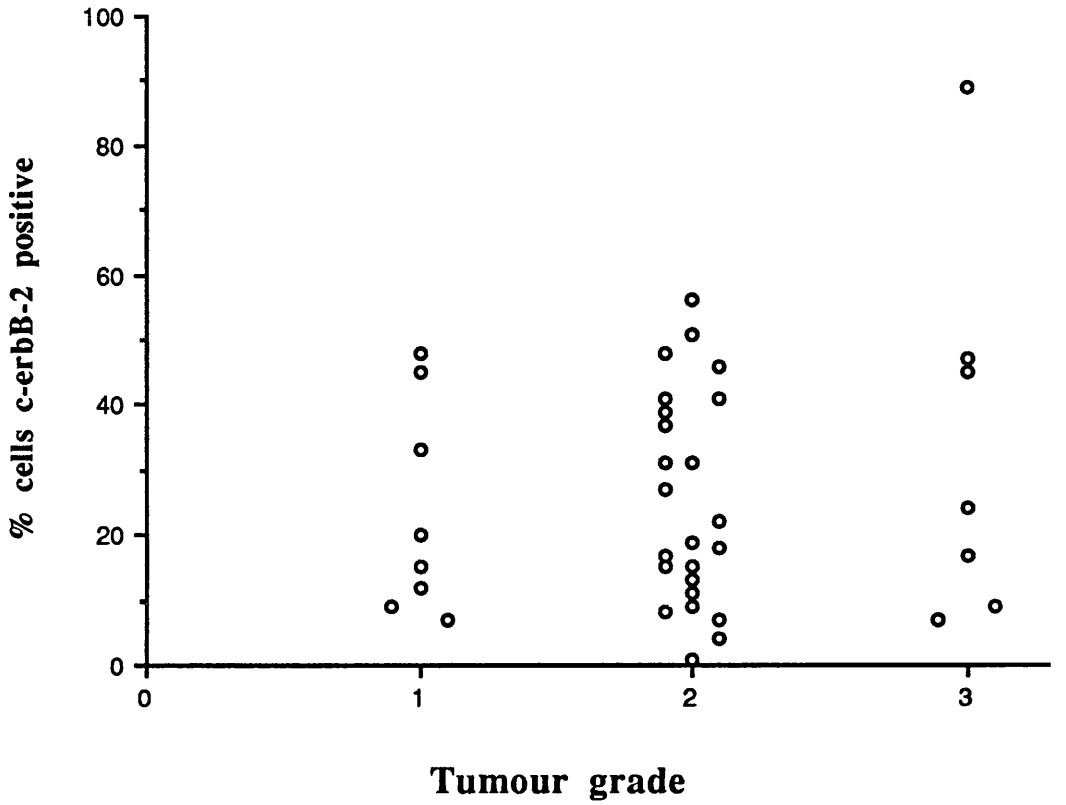


Figure 6.10 Distribution of c-*erb* B-2 positive cells within the three different grades of tumour differention.

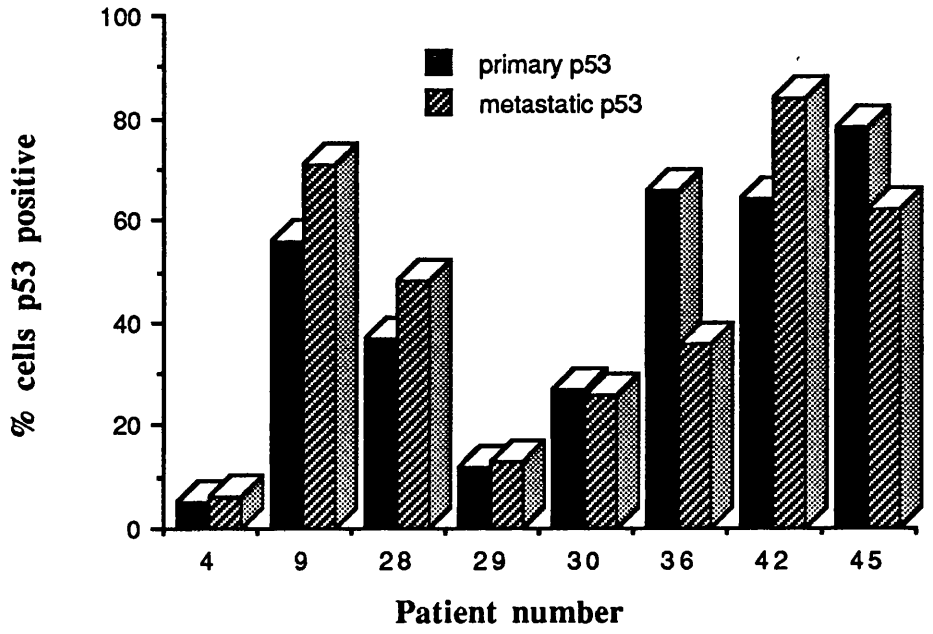


Figure 6.11a Comparison of p53 positive cells in paired primary and metastatic tumour.

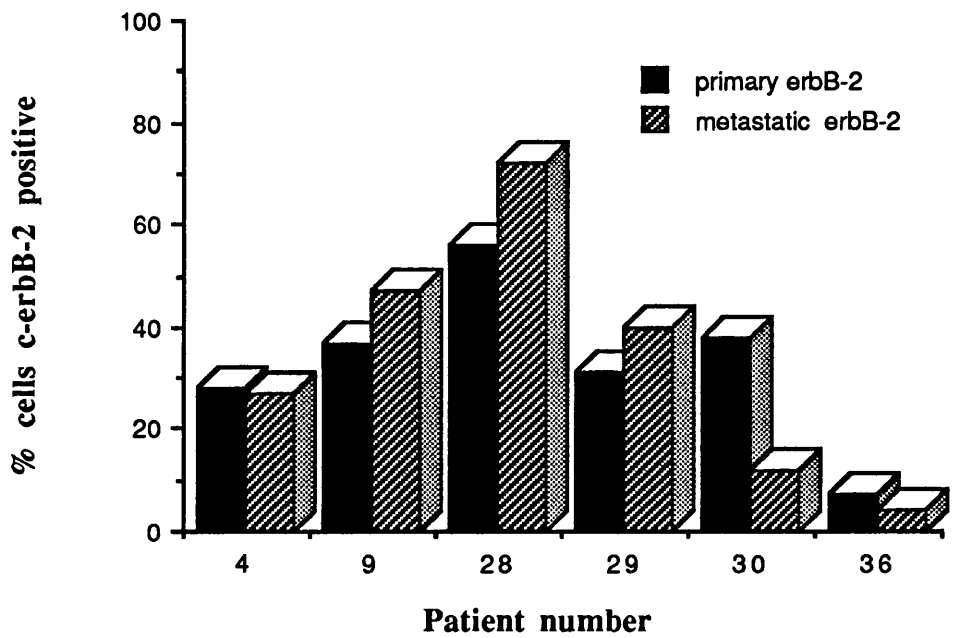


Figure 6.11b Comparison of c-*erb* B-2 positive cells in paired primary and metastatic tumour.

decrease in the p53 expression. There was no change in the average expression of p53 within the tumour and metastases (Table 6.9). The metastases show no significant change in expression, thus are similar and reflect the expression in the primary tumour.

6.6.2. c-*erb* B-2 expression and metastases

6 patients with tumour and matched lymph node metastases were analysed for c-*erb* B-2 expression (Figure 6.11b). Little change was noted in one patient, an increase in c-*erb* B-2 expression was observed in three patients and in the two remaining patients a decrease in the c-*erb* B-2 expression was noted. The metastases show no significant change in expression, thus are similar and reflect the expression in the primary tumour (Table 6.9).

Table 6.9.

Mean percentage positive cells for p53 and c-*erb* B-2 in paired primary and metastatic tumour

Tissue type	Mean percent cells c-*erb* B-2 positive (n=6)	Mean percent cells p53 positive (n=8)
Tumour	43	33
Metastases	43	34

The values in the table are given as mean cells positive for the c-*erb* B-2 protein in the three different grades of tumour differentiation.

6.7. Discussion

This study was initiated to determine whether, within a heterogeneous primary tumour genetic changes such as aneuploidy correlate with the expression of proto-oncogene (c-*erb* B-2) or tumour suppressor gene (p53) products in the same cell population within the tumour. The degree of expression of the two proteins was independently assessed in comparison to both metastatic capacity and tumour differentiation.

Flow cytometry of proteins such as p53 and c-*erb* B-2 has certain advantages over traditional techniques such as immunocytochemistry as flow cytometry examines objectively a large number of cells in a short period of time. This removes the subjective nature often associated with immunocytochemistry. The only disadvantage is that the architecture of the tumour is lost during processing of the tumour and this is compensated for by the use of scatter and marker gating. The use of multiparameter flow cytometry described here shows that more than one marker can be examined on one cell. This has traditionally been used for lymphocytes but little work has been carried out on tumour cells. Using this technique the amount of oncoprotein staining can be evaluated simultaneously with DNA profile within the primary and metastatic tumour thus providing a greater understanding of what is occurring within a heterogeneous tumour population.

The efficacy of the staining procedure was evaluated by immunofluorescence staining of the primary tumour using the anti- c-*erb* B-2 and anti- p53 antibody. Membrane staining was noted for the c-*erb* B-2 proto-oncogene receptor which was expected and little background fluorescence could be noted. The p53 staining was both cytoplasmic and nuclear which has

also been reported (Section 1.4.4.2.). The HL-60 had negligible expression of p53 which corresponded to the null p53 mutation in its genome (Table 6.1).

At the genetic level amplification of genes in cells expressing c-*erb* B-2 and p53 has been measured and no correlation was found. Analysis of the amplification of the specific oncogene may have limited relevance since ploidy analysis shows that the greater part of the entire genome is amplified in the majority of breast cancer patients, this being due to aneuploidy of the cell. The aneuploidy of the tumour seems to be related to the expression of especially the c-*erb* B-2 protein (Figure 6.6) rather than the p53 protein (Figure 6.5) with an example of the expression within aneuploid populations shown in Figure 6.1. Reports have linked expression of c-*erb* B-2 to aneuploidy (Section 1.4.5.) but the methods utilised have been immunocytochemistry and a separate DNA ploidy analysis. Here the two are simultaneous on one cell showing a direct relationship. There have been reports on tetraploid tumours expressing higher amounts of c-*erb* B-2 but in this study this was found not to be the case.

An interesting aspect of this study is the fact that even on cell lines there is never 100% expression or 0% expression of either the p53 or c-*erb* B-2 protein (Figure 6.2 and Figure 6.3) and this disputes the data obtained from immunochemistry. The theory of the tumour being clonal in origin would be questioned by this data. However the experience in the study of leukaemia suggests that all clonal tumours have the capacity to generate terminally differentiated progeny which carry a different complement of cellular markers (Greaves 1982). Thus, while the non staining cells may be normal epithelia or stroma, it is also possible that they form part of the tumour cell lineage, but at another window of differentiation. This being supported by the fact that similar expression is often observed in the metastases where stromal or normal epithelial cells are not present (Table 6.9).

The expression of the oncoproteins were compared directly with the presence or absence (stage 1 or stage 2) of tumour cells within the tumour draining lymph nodes. The p53 gave no significant trends whereas higher levels of expression of the c-*erb* B-2 tended to be greater in stage 2 patients. This agrees with results published for p53 and c-*erb* B-2 (Section 1.3.).

The degree of tumour differentiation was compared to the expression of both p53 and c-*erb* B-2 (Table 6.7 and Table 6.8). There were no statistically relevant trends noted. However the grade 3 tumours tended to express more of both p53 and c-*erb* B-2.

Analysis of the expression of oncoproteins such as p53 and c-*erb* B-2 within the metastatic tumour population of the lymph node has to date been attempted but this is difficult due to the presence of a large quantity of lymphocytes. The use of a scatter gate described in Section 2.2.6.2. provides a means to eliminate the lymphocytes due to their defined size. The lymph nodes contain pure metastatic tumour with no other stromal or epithelial contaminating cells. In consequence of this fact the nodal population assessed is almost entirely epithelial tumour cells. The percentages of both p53 and c-*erb* B-2 in the nodal metastases is variable (Figure 6.11a and Figure 6.11b) with some expression at higher levels than those found in the parent primary tumour. Overall,the metastases expressed both the proteins to a high level with all the metastases consisting of aneuploid tumour cells with a similar expression to the parent primary tumour. The result would suggest that the expression of p53 and c-*erb* B-2 could be involved primarily with the initiation of the tumour rather than progression. For the progression of the tumour multifactorial events would be necessary for successful metastatic capability.

From the results it seems that c-*erb* B-2 has trends associated with both stage (presence of metastases in the lymph nodes) and ploidy of the tumour. This suggests that c-*erb* B-2 may play an important role in the possible generation of metastatic capability of the tumour. In contrast the p53 has no trends, with only a weak tendency for increasing expression in aneuploid tumours which may indicate a possible role in early tumour generation.

CHAPTER 7

THE EFFECT OF TUMOUR TYPE ON THE IMMUNE RESPONSE IN THE TUMOUR DRAINING AXILLARY NODES

7. The effect of tumour type on the immune response in the tumour draining axillary nodes.

An immune response to breast cancer has been implicated within the primary tumour in the form of tumour infiltrating lymphocytes, in the tumour draining axillary lymph nodes and to a much lesser extent the blood. The changes observed within these areas occur in both phenotype and activation of the lymphocytes. The antigen or antigens which are stimulating the immune system in breast cancer patients are still as yet unknown. The study of the oncology of the disease provides some possible candidates. The p53 nuclear protein is found in a mutated form in over 50% of all breast cancers. As it is an internal protein a T cell response would normally be elicited. However if the protein is released from the cell then a B cell immune response would occur. Other possible antigens would include *ras* with point mutations at position 12 and 13. Other proteins such as c-*erb* B-2 over-expressed in 20-30% of all breast cancers are normal and should in theory have no effect on the immune system.

In this study the p53 protein was measured along with the c-*erb* B-2 protein in primary tumour in 22 patients (11 stage I and 11 stage II), simultaneously with the immune response within the tumour draining nodes of these patients to examine the effect of these two proteins on the nodal immune response. The ploidy of the tumour was also compared to the nodal immune response in these 22 patients.

These patients were 22 of the patients analysed in Chapters 5 and 6

The gating and staining of lymph node lymphocytes is illustrated in Section 2.2.6.1. and 2.2.6.2. along with the estimation of p53 and c-*erb* B-2 expression in primary tumour and also determination of DNA ploidy (Section 2.2.6.7 and 2.2.6.8).

7.1. Comparison of p53 and c-*erb* B-2 expression to the immune response in the low and high axillary nodes

7.1.1. Phenotypic markers

No significant trends were noted for p53 and c-*erb* B-2 positive cells in the primary tumour in relation to phenotypic change in the low and high nodes for both stage I (Table 7.1) and stage II patients (Table 7.2).

7.1.2. Activation markers

No significant trends were found for p53 and c-*erb* B-2 positive cells within the primary tumour in relation to lymphocyte activation in stage I patients (Table 7.1). The only trend noted was p53 expression in relation to increasing interleukin-2 receptor (IL-2R) on low lymph nodes of stage II patients ($p=0.009$) (Table 7.2 Figure 7.1).

7.2. Ploidy and the immune response in the axillary tumour draining nodes.

The low and the high node percentages for phenotype and activation were averaged and compared to the ploidy of the tumour (Table 7.3).

Table 7.1

Effect of p53 and c-*erb* B-2 expression in primary tumour on the immune response in the low and high axilla of stage I patients.

Lymphocyte Phenotype/ Activation	Statistical significance+ Tumour cells p53 positive	Tumour cells c-*erb* B-2 positive
CD3 (%T cells)	p=0.134	p=0.129
CD19 (B cells)	p=0.110	p=0.125
CD8 (CD8+ T cells)	p=0.089	p=0.145
CD4 (CD4+ T cells)	p=0.153	p=0.139
CD3/CD19 ratio	p=0.122	p=0.145
CD4/CD8 ratio	p=0.167	p=0.117
HLA DR on CD8+ T cells	p=0.187	p=0.156
HLA DR on CD4+ T cells	p=0.109	p=0.173
Interleukin-2 receptor (Tac) on CD8+ T cells	p=0.169	p=0.176
Interleukin-2 receptor (Tac) on CD4+ T cells	p=0.097	p=0.184
sIgG on CD19+ B cells	p=0.141	p=0.158

+ Statistical significance was assessed by the Spearman rank correlation with significance being less than or equal to p=0.05

Table 7.2

Effect of p53 and c-*erb* B-2 expression in primary tumour on the immune response in the low and high axilla of stage II patients.

Lymphocyte Phenotype/ Activation	Statistical significance+ Tumour cells p53	Tumour cells c-*erb* B-2
CD3 (%T cells)	p=0.127	p=0.23
CD19 (B cells)	p=0.162	p=0.142
CD8 (CD8+ T cells)	p=0.128	p=0.153
CD4 (CD4+ T cells)	p=0.145	p=0.142
CD3/CD19 ratio	p=0.162	p=0.176
CD4/CD8 ratio	p=0.137	p=0.124
HLA DR on CD8+ T cells	p=0.098	p=0.155
HLA DR on CD4+ T cells	p=0.093	p=0.135
Interleukin-2 receptor (Tac) on CD8+ T cells	p=0.068	p=0.168
Interleukin-2 receptor (Tac) on CD4+ T cells	p=0.009*	p=0.126
sIgG on CD19+ B cells	p=0.098	p=0.132

* Statistical significance to the low node

+ Statistical significance was assessed by the Spearman rank correlation with significance being less than or equal to p=0.05

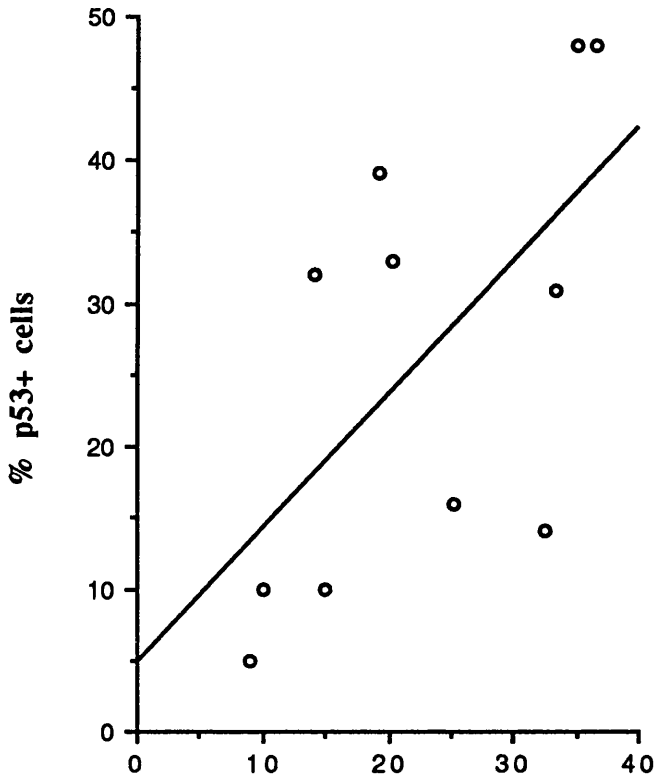


Figure 7.1 The expression of the interleukin-2 receptor (IL-2R) on stage II low node in relation to p53 expression in the primary tumour.

Table 7.3

Distribution of phenotypic and activation markers in lymphocytes from lymph nodes of breast cancer patients with diploid and aneuploid tumours.

Phenotypic/ Activation markers	Diploid (n=7)	Aneuploid (n=15)	Statistical Significance+
CD3/CD19 ratio	1.0	2.4	p=0.152
CD4/CD8 ratio	3.5	3.6	p=0.110
HLA DR on CD8+ T cells	44.5	57.0	p=0.139
HLA DR on CD4+ T cells	28.7	36.7	p=0.122
Interleukin-2 receptor (Tac) on CD8+ T cells	9.3	16.2	p=0.134
Interleukin-2 receptor(Tac) on CD4+ T cells	18.5	22.1	p=0.158
sIgG on CD19+ B cells	19.9	32.6	p=0.04

+ Statistical significance was assessed by the Mann-Whitney U test, with significance being less than or equal to p=0.05

7.2.1. Phenotypic markers

There was no difference in terms of phenotype of T or B cells with the ratio of CD3/CD19 and CD4/CD8 being similar in both aneuploid and diploid tumours.

7.2.2. Activation markers

Differences were observed in the activation states of both T and B lymphocytes compared to ploidy (Table 7.3). However, although not statistically significant HLA DR was expressed on a higher proportion of CD8+ T cells in the aneuploid tumours (57.0%) than the diploid tumours (44.5%). This was also observed for the HLA DR expressing CD4+ T cells in the aneuploid (36.7%) and diploid (28.7%) tumours. No significant trends were observed for interleukin-2 receptor (IL-2R) expressing T cells. The IL-2R+ CD8+ T cells show increased expression within the aneuploid tumours (16.2%) than diploid tumours (9.3%). Again this trend was also observed for the IL-2R expressing CD4+ T cells in the aneuploid (22.1%) compared to diploid tumours (18.5%).

The only significant difference observed for activation of lymphocytes was observed in the sIgG expression in the aneuploid tumours (32.6%) compared to diploid tumours (19.9%) with a significance of $p=0.04$.

The trend in B cell activation and the higher levels of activation in aneuploid tumours compared to diploid may indicate more antigenic determinants within aneuploid tumours.

7.3. Discussion

In this chapter the immune response in the lymph node lymphocytes was compared to some of the possible causative factors. p53 is to date the most commonly mutated protein in breast cancer with 50% tumours showing mutation of this protein. As p53 is an internal protein found mainly in the nucleus a T cell response should be initiated. This would involve the proteolysis of the protein to peptides which then associate with the MHC class I protein in the endoplasmic reticulum eventually to be presented on the surface of the tumour cell to the cytotoxic T cell (CD8) arm of the immune system. For p53 expression only one trend is observed that of the IL-2R on CD4+ T cells in the low node of stage II patients (Figure 7.1), suggesting p53 is stimulating the nodal CD4+ T helper cells. Since CD4+ T cells process largely exogenous antigen, they may use free p53 released from dying tumour cells.

In Chapter 5 the trend of increasing IL-2R in the low node was the most statistically relevant one noted. Thus the change in this subset may be an important indicator of an immune response in stage II patients with high p53 expression. A B cell response to p53 has been shown as the presence of anti-p53 IgG in sera of 10% of patients (Crawford *et al* 1979). This would be due to the tumour cell releasing its contents allowing the mutant p53 protein to be seen by the B cell arm of the immune system. This response would possibly be clinically ineffective due to the internal location of the mutant p53 as antibodies generally cannot enter live cells. Apart from this, other activation markers failed to reach significance and this may be possibly due to the loss of MHC expression in the tumour thus making a response in these patients more difficult. Also the mutation in the p53 protein may not allow presentation of

peptides derived on the MHC groove as only certain types of peptides can be presented by each MHC molecule to elicit an immune response.

No response to c-*erb* B-2 was noted for either T or B cells. This protein is normal and over-expressed in breast cancers. Thus no response may be initiated. If a functionally useful response was formed it would be seen in the form of IgG as c-*erb* B-2 is a receptor found on the surface of tumour cells.

Alterations in activation were noted in the lymph nodes in relation to ploidy. No changes were observed in the phenotype of lymphocytes when nodal lymphocytes from diploid and aneuploid patients were compared. The only significant trend was associated with the expression of sIgG on B cells which was higher in the aneuploid patients. Although no significant trends for T cell activation were observed HLA DR and IL-2R expression were higher in all cases in the aneuploid tumours. The data would possibly suggest that aneuploid tumours may express more antigens than diploid tumours. Chapter 6 indicated weak but not significant trends for both p53 and c-*erb* B-2 and aneuploidy. However the presence of a mutant protein does not necessarily mean an immune response as the full mechanism for antigen processing and presentation must be intact to initiate this response.

CHAPTER 8

GENERAL DISCUSSION

8. General Discussion

During this study a number of aspects of breast cancer oncology and immunology have been investigated by flow cytometry. For this to be accomplished samples of patient tumour, blood and tumour draining lymph nodes were obtained for investigation. The expression of the γ/δ T cell receptor was investigated in the blood, nodes and tumour infiltrating lymphocytes of breast cancer patients (TILs) (Chapter 3). The axillary tumour draining lymph nodes were investigated for the effect of metastatic tumour on their immunological function (Chapter 4) and the two factors of distance and presence or absence of tumour were also investigated as possible modulating factors (Chapter 5). The expression of two oncoproteins p53 and c-*erb* B-2 were estimated in the tumour and related to tumour type and stage of the tumour (Chapter 6). Finally the expression of c-*erb* B-2 and p53 were interrelated to the changes occurring in the lymphocytes within the tumour draining axillary lymph nodes (Chapter 7).

8.1. Flow cytometry

Flow cytometry has been used throughout this study. It has certain advantages over other techniques such as the more commonly used immunocytochemistry. The machine is run by computer which allows objective collection of data. The number of cells analysed can be as high as 50,000 for each sample in a relatively short space of time. Another advantage is that dead cells can be excluded from the analysis, as often these cells will bind the antibody non specifically and removal of these cells from the analysis will increase accuracy. Also it is possible to select for populations of defined size on the basis of FSC (forward scatter) and SSC (side scatter) such as

lymphocytes. An exclusion gate for lymphocytes can also be set as tumour infiltrating lymphocytes (TILs) which can make up 80% of the tumour cell bulk (Whitford *et al* 1992a). Different parameters can be studied on the same cell such as phenotype and activation of lymphocytes or oncoprotein expression in relation to DNA ploidy. The sensitivity of the technique with respect to immunochemistry became particularly evident in this study where several times flow cytometry detected tumour cells in nodes where conventional pathology did not. This was also shown for the detection of p53 and c-*erb* B-2 positive cells in the tumour, when a certain percentage was positive in almost every case. Some of these weak positives may be clinically relevant yet score negative in immunochemistry. However useful flow cytometry can be, there are certain drawbacks such as the sample to be analysed has to be completely disaggregated. This disrupts normal inter-cellular architecture and unlike histochemistry it is not possible to observe the relationship between staining cells and the rest of the tissue.

8.2. The immune response to breast cancer

Tumour immunology is a complex and fast moving field and one which becomes more complex as further advances are made in both immunology and oncology. Primary tumours are heterogeneous in cell population, with the sequence of genetic events leading to tumour development and metastasis probably differing between patients. On top of this, the patients' own immune response to any oncoprotein will depend on their genetic makeup, especially the HLA antigens. Thus one patient may respond to mutant p53 protein while another may not. This heterogeneity of both tumour and response capability means that, ideally, a study such as this should have used more parameters and

many more patients. Yet even if the cost and man hours had been available, modern clinical diagnostic techniques have resulted in smaller tumours due to earlier treatment. In addition the sampling of the tumour takes place at a fixed point during the progression of the disease and this will obviously vary among patients. More importantly, the full complement of oncogenes and potential immune defence mechanisms are probably not yet known. To give relevant examples it is exceedingly difficult to find "new" oncogenes and "gene knockout" mice lacking major components of the immune system which do not have accelerated rates of tumour development.

The γ/δ T cell receptor has not yet been found a well defined role in immunology and tumour immunology. The presence of the γ/δ T cell receptor varied between tissues of the immune system of the breast cancer patients (Chapter 3). Within the Tumour Infiltrating Lymphocytes (TILs) the median value was highest for the CD3+ CD8+ γ/δ subset. The presence of this (the γ/δ T cell receptor) on the CD8+ cytotoxic T cells may indicate a possible cytolytic function, as within the TIL population of breast cancer patients the majority of lymphocytes are CD8+. The lymph node lymphocytes showed a trend of increasing CD3+ CD4+ γ/δ+ lymphocytes compared to the TILs and the peripheral blood lymphocytes (PBL). This corresponds to the study by Whitford *et al* (1992b) suggesting that the CD4+ T cells make up the majority of lymph node lymphocytes. These CD3+ CD4+ γ/δ+ cells may play a role in the regulation of the immune response in these nodes. More interestingly, the double negative cells (CD3-, CD4-) make up the majority of the γ/δ subset indicating that these cells may function without the need for MHC molecules and thus, since tumours often lack MHC expression, these cells may have an important role to play.

The axillary nodes occupy an important position in breast cancer, as they are a potential site of an immune response against the cancer and also usually the first site of secondary metastatic tumour. Thus it is important to determine the role of these nodes in generating an immune response against the tumour and whether metastases affect this in any way. Earlier flow cytometric studies have indicated host mediated immune responses occur in some patients,.with both phenotypic and activation changes in the lymphocytes observed in the nodes (Whitford *et al* 1992b; Morton *et al* 1986). These studies involved both stage I and stage II patients, in general, using only tumour free nodes. The present study analysed the difference between a tumour invaded and a tumour free node taken from the same patient to compare intra patient variability in respect to the possible host tumour interactions taking place.

The presence of metastatic tumour altered both phenotype and activation of the lymphocytes. The CD8+ T cell population was found to be raised in the tumour invaded node. This reflects the observation in breast cancer TILs as here the CD8+ population make up the majority of lymphocytes. Thus in the tumour and the tumour invaded lymph node the close proximity of tumour may cause proliferation of these lymphocytes. Along with the increase in CD8+ T cells the proportion of CD4+ T helper cells decreases in tumour invaded nodes. This may indicate that although certain populations are proliferating others are possibly being actively suppressed. For example most breast cancer cells secrete TGFβ, a powerful immunosuppressant.

The indication of the changes occurring in the tumour invaded lymph nodes compared to the tumour free raised the question of proximity. How close did the tumour need to be to elicit an immune response? Distance from tumour was taken into account with a low node and a high node removed from the breast cancer patients for both stage I and stage II patients (Chapter 5).

There were significant phenotypic changes for T and B lymphocytes but only in stage I nodes. However the most significant trends were for lymphocytic activation and stage II nodes. The trend of increasing activation in a low node occurred in all cases being very significant for expression of IL-2R on CD4+ T helper cells. The presence of increased levels of the IL-2R on CD4+ T cells could indicate that these cells may be mediating a local response against the tumour in the stage II nodes where tumour has metastasised. This result must be treated with caution as down regulation of this receptor occurs soon after binding of the ligand. Thus lack of the receptor does not necessarily mean an inactive state. Fisher *et al* (1980) reported that a subset of stage II patients, survived even with suspected nodal metastases. This would suggest that the stage II patients could be divided into 4 further subgroups depending on the nodal tumour invasion in the low or high nodes. The subgroup having low node tumour invaded and high node tumour free showed some trends for both phenotype and activation. The phenotypic change was with the T cell subsets, showing a decreased CD4/8 ratio in the low node with respect to the high node. This was due to the presence of increased amounts of CD8+ T cytotoxic lymphocytes in the tumour invaded node possibly involved in an effective local response against the tumour. The same trend was observed in the study carried out on tumour invaded and tumour free nodes in Chapter 4. This subgroup also showed activation trends for both IL-2R and HLA DR with the low node having significantly higher expression. The level of HLA DR, IL-2R and IgG on B cells was also shown to be higher than the mean stage II expression possibly indicating a greater immune response against the tumour in this stage II subgroup.

This notable difference within the nodal immune response for both phenotype and activation may reflect differences in host immunity or it may reflect different tumour types. The measurement of the immune response in

relation to the tumour type may provide clues of the origin of this response. For a response to occur an antigenic protein must be presented to the immune system. The B cell arm may only be effective if the antigenic protein is external or soluble (Chapter 1). Although a B cell response to internal antigens may be generated, it is difficult to envisage any functional utility. The T cell arm main requirement is for the antigen to be presented either after internal synthesis and assembly on MHC class I antigens (CD8+ cytotoxic T cells) or after internal degredation and presentation on MHC class II antigens (CD4+ T helper cells). To date, no mutant external antigen has been recognised in breast cancer. However, the first well characterised internal antigen has been identified by van Der Bruggen *et al* (1991). This was identified from melanoma and called MAGE. The function of this protein is as yet unknown, but it is expressed on 40% of all melanomas and on a smaller proportion of breast cancers. The most commonly mutated protein in breast cancer is p53 with mutations reported in 50% of all tumours (Bartek *et al* 1990). p53 being expressed within the cell therefore, could initiate an effective T cell response. In contrast to this another common protein in breast cancer c-*erb* B-2 is a receptor protein overexpressed in 20-30% of all breast cancers (Clark and McGuire, 1991). This is "self" but overexpressed and in theory unlikely to elicit a B cell response.

The p53 (mutant) and c-*erb* B-2 (normal) proteins were measured by flow cytometry to assess their importance in both oncology and immunology of breast cancer. The p53 protein was not associated with any tumour variable. The c-*erb* B-2 protein although not significantly associated with any variable had weak trends to increased expression in aneuploid and stage II tumours possibly indicating tumour aggressiveness. The paired metastases compared to the primary showed little change in expression thus possibly indicating a role in tumour generation rather than progression for both p53 and c-*erb* B-2.

The values obtained for percent c-*erb* B-2 and p53 positive cells along with tumour ploidy were compared to the immune response in the corresponding tumour draining lymph nodes. No trends for c-*erb* B-2 were noted for either lymphocyte phenotype or activation suggesting that c-*erb* B-2 was not stimulating a B or T cell immune response as the protein is normal and overexpressed. The p53 protein was also compared to the nodal response and only one trend was observed, that of increasing p53 expression with increasing IL-2R expression on CD4+ T helper cells. This trend indicated a possible link between p53 and the immune system. Aneuploid tumours were associated with an increase in IL-2R, HLA DR and sIgG expression with the sIgG showing significance ($p=0.04$). It appears that there may be some antigenic determinants in aneuploid tumours that are absent in diploid tumours. This would suggest an antigenic protein(s) expressed in some aneuploid tumours may be mediating an immune response. This protein may not be p53 as it is not associated with aneuploidy thus some unknown proteins may be stimulating the immune system.

If the antigenic proteins can be determined then the hope lies for directed immunotherapy. The only defined antigen so far is MAGE (van Der Bruggen *et al* 1991) expressed on 40% of melanomas and 25% of breast cancers. It would be possible soon after surgery to test for this antigen. If it was present a suitable vaccination strategy could be prepared. However the MAGE antigen is only presented to the immune system in the 25% of melanoma patients who carry HLA A1. Thus with 40% total expression of MAGE and only 25% suitable, this gives only 10% of melanoma patients the possibility for vaccination. Even then an intact immune system would be be necessary to elicit an effective immune response. Tumours have been shown to have reduced expression of the MHC class I molecule necessary for a CD8+ cytotoxic T cell

response (Whitford *et al* 1992a). Thus even in this 10% subset steps may be needed to bolster the immune system.

8.3. Surgery and treatment of breast cancer

The treatment of breast cancer involves the removal of the primary tumour and the axillary lymph nodes in an attempt to limit secondary metastatic spread of tumour. As knowledge has increased, the type of surgery has changed from the Halsted radical mastectomy which favoured *en bloc* removal of the breast and the surrounding area. Today more conservative surgery is advocated with a simple mastectomy or even a lumpectomy followed by chemotherapy or radiation therapy. There still remains some controversy over breast cancer treatment with each surgeon often performing different procedures. Most surgical debate lies in the treatment of the axillary tumour draining lymph nodes. The surgeon has a number of options; either sample the low axilla for tumour, leave the axilla intact or remove the entire axillary nodes. However removal of the entire axilla is a skilled operation and in many cases only surgeons in large centres attempt it. Other surgeons argue that to leave the axilla intact will not compromise the patients survival. This is based on the Fisher trial (Fisher *et al* 1980) which showed that survival was not adversely affected in some patients in whom no axillary clearance was performed, but who statistically may be presumed to have had lymph node metastases. Thus lack of axillary clearance does not affect the survival in some breast cancer patients. However this has been disputed by others (Fentiman and Mansel, 1991), who point out that this trial was performed before the successful use of systemic adjuvant chemotherapy had been established and as such the findings are not valid. Recent trials have reinforced this, indicating that adjuvant chemo-endocrine therapy can significantly reduce both recurrence and death in breast

cancer patients (Early Breast Cancer Trialists Collaborative Group, 1992). However if any breast cancer patient is immunologically responding to the tumour, removal of the axillary lymph nodes may be detrimental.

In conclusion, a fuller understanding of the immune response and its target of of tumour specific antigens is necessary before treatments can be designed to utilise this response.

CHAPTER 9

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