Studies on metastatic human breast cancer

A Thesis Presented for the
Degree of
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by

Syed Munir Alam

Department of Biochemistry
University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Chapter 3: The effect of metastasis on the immune function of the axillary lymph nodes

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LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
CA	Carcinoma-associated
CD	Cluster of designation
CK	Cytokeratin
Con A	Concanavalin A
DI	DNA Index
EBV	Epstein Barr virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
FCM	Flow cytometry
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL1, 2, 3	Fluorescence channel 1, 2, or 3
FSC	Forward scatter
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetyl galactosamine
GlcNAc	N-acetyl glucosamine
HMFG	Human milk fat globule
HPA	*Helix pomatia* agglutinin
ICAM-1	Intercellular adhesion molecule-1
IL-2	Interleukin 2
Kb	Kilo base pairs
LFA-1	Lymphocyte function-associated antigen-1

LNL	Lymph node lymphocytes
LNM	Lymph node metastasis
LOH	Loss of heterozygosity
Mab	Monoclonal antibody
Man	Mannose
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumour virus
MW	Molecular weight
PBL	Peripheral blood lymphocytes
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PI	Propidium iodide
Pro	Proline
cRBC	Chicken red blood cells
RFLP	Restriction fragment length polymorphism
Ser	Serine
sIgG	Surface immunoglobulin G
SPF	S-phase fraction
SSC	Side scatter
Tac	T activation, synonym for the CD25 antigen on the β chain of the IL-2 receptor
Tc	Cytotoxic T cells
TGF-β	Transforming growth factor β
Th	Helper T cells
TILs	Tumour infiltrating lymphocytes
TLI	Thymidine labelling index

UK-PA	Urokinase-plasminogen activator
WGA	Wheat germ agglutinin

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Summary

The main clinical problem in breast cancer is metastasis and the initial spread frequently involves the axillary lymph nodes. Surgical removal of the primary tumour is not always sufficient to prevent further spread of the disease and mortality is high in breast cancer. At present, lymph node metastasis (stage) remains the most important prognostic indicator but clinical staging can be inaccurate and 30% of node negative (without nodal metastasis) patients still die from their disease. The metastatic process is complex in breast cancer especially as primary tumours differ widely and while prognosis in some patients after surgery is good, in others there is early recurrence and widespread metastasis. Even within a tumour, much variation exists and it is believed that metastatic cells may represent only a subpopulation of the primary tumour. Various factors both contribute towards as well as act against the generation of such metastatic cells. Recent research has been directed towards identifying such features in breast tumours and their relationship to metastasis. The surgical practice at Western Infirmary includes axillary clearance in addition to removal of the primary tumour. It was, therefore, possible to design a study which involved analysis of both primary tumours and lymph node metastases from breast cancer patients. This study involved the measurement of different molecular parameters (including lymph node immunological responses, cell surface carbohydrate expression, DNA flow cytometric analysis, and DNA fingerprinting) in relation to metastasis to the lymph node.

The surgical removal of axillary lymph nodes along with the primary tumour still remains a matter of debate. Recent studies suggest that the lymph nodes may contribute to both a cellular and humoral immune response. There is a potential two-way interaction in lymph nodes in breast cancer patients. As

these nodes primarily drain the breast, they are ideally situated to encounter tumour cells and, as such, tumour antigen recognition leading to host-mediated immune response can occur. At the same time, however, invading tumour cells can adversely influence such a response. Alterations in both the phenotype and activation status of lymph node lymphocytes from breast cancer patients have been detected by flow cytometry with the use of fluorescent monoclonal antibodies against surface markers. In this study, in order to assess the effect of metastasis on lymph node response, lymphocytes from tumour-invaded and tumour-free lymph nodes from the same breast cancer patients have been analysed by flow cytometry for expression of phenotype and activation markers. Lymph node lymphocytes from 12 such patients (stage II) were also compared with lymphocytes from 14 patients without metastasis (stage I). High patient variability with respect to both the phenotype and activation status was observed, and there were differences in the proportion of T cell subsets and activated B cells between nodes that were invaded and those that were not. The proportion of CD8+ T cells was increased in tumour-invaded nodes compared to tumour-free nodes ($p=0.002$). In contrast, the proportion of CD4+ T cells, which was the predominant T cell subset in lymph nodes, was decreased in invaded nodes ($p=0.003$). In consequence, the CD4+/CD8+ ratio was markedly depressed in invaded nodes ($p=0.001$), while the ratio in tumour-free nodes was similar to those in stage I nodes. The proportions of both CD8+ and CD4+ T cells in tumour-free nodes were also similar to stage I nodes. Among the T cell activation markers, HLA DR, which was expressed on a higher percentage of CD8+ cells than CD4+ cells, was not significantly different between invaded and tumour-free nodes. Tac expression (IL-2 receptor), which was on a higher percentage of CD4+ T cells, was also not significantly different but was found on a higher proportion

of T cells in invaded nodes compared to tumour-free nodes. On the other hand, although the mean percentage of surface IgG-expressing B cells was slightly higher in invaded nodes when compared to both tumour-free nodes in the same patient ($p<0.05$) and nodes from stage I patients ($p<0.05$), such a difference was observed to be strong in some patients. The differences observed in the lymph nodes were not reflected in the peripheral blood lymphocytes from the same patient. These results suggest that the presence of metastatic tumour cells in a lymph node is associated with specific alterations of the phenotype and activation status of lymphocytes and may reflect the host-tumour interaction in breast cancer patients.

In previous immunohistochemical studies, lectin binding has been used to detect alterations related to malignancy in primary breast cancer. In this study, using fluorescently-labelled *Helix pomatia* (HPA) and Concanavalin A (Con A) lectins, cell surface carbohydrate expression on 32 fresh primary tumours and 12 lymph node metastases was analysed by flow cytometry. It was possible to quantitate the extent of lectin binding and the percentage of live positive cells was accurately determined from 10,000 and 5000 cells per sample for tumour and nodal metastasis respectively. While primary tumours were positive for both HPA and Con A binding (53% and 44% of analysed tumours, respectively), comparison with clinicopathological features showed that HPA, but not Con A correlated positively with lymph node metastasis ($p<0.01$). This association with nodal metastasis held for tumours of higher grade (II and III), and HPA positive tumours were also more likely to be oestrogen receptor (ER) negative ($p<0.05$). In 12 patients, where both primary tumours and lymph node metastases were analysed, a strong positive correlation was found between the percentage of cells binding HPA in lymph

nodes and those in autologous primary tumours (p=0.001). This suggests that HPA binding defines an N-acetyl galactosamine-containing cell surface marker on breast tumours which may be associated with lymph node metastasis. The presence of HPA binding tumour cells in invaded nodes further substantiates the hypothesis that HPA binding represents a phenotypic marker of metastatic potential.

It is generally assumed that only a subpopulation of tumour cells has the capacity to metastasise, and it is possible that the generation of such subpopulations occurs as a result of alterations in the DNA. A number of studies have now shown that breast cancer patients with aneuploid, high S-phase tumour DNA profiles have a poor prognosis. Aneuploidy, which represents abnormal DNA content, and high SPF (S phase fraction), which represents a high proliferation rate, can be assessed by flow cytometric (FCM) DNA analysis. Although the prognostic relevance of FCM DNA analysis has been widely studied on primary tumours, very few studies have also analysed lymph node metastases from the same patient. Fresh paired primary tumours and lymph node metastases were compared in this study. In breast cancer, the occurrence of aneuploid tumours is frequent, and 63% of 57 primary breast tumours analysed in this study were found to be aneuploid. Comparison with histopathological prognostic factors showed no significant association between DNA ploidy and tumour grade ($p<0.1$), while diploid tumours were found more likely to be ER-positive ($p<0.02$). On the other hand, aneuploid tumours were found to behave more aggressively with respect to metastasis to the nodes ($p<0.01$). However, the detection of aneuploid peaks in corresponding nodal metastasis was rare in a single-parameter DNA analysis; in only 6 cases out of 25 was an aneuploid peak, present in the primary tumour, also detected in the

node. Using an anti-cytokeratin (CK) antibody and employing a dual-parameter analysis the sensitivity of detection of aneuploid peaks in invaded nodes was increased by gating out the CK-negative lymphocytes. Moreover, it was also possible to assess whether the diploid peak in the primary tumour was also epithelial in nature. Examination of 12 primary tumours and lymph node metastases for CK+ cells revealed that tumour cells in invaded nodes can be either diploid or aneuploid, reflecting the ploidy of the primary tumour. In patients with multiploid primary tumours, only a subpopulation of the primary aneuploid cells was detected in the nodal metastases. The results suggest that aneuploidy is not a harmless feature of a cell's uncontrolled growth but is an integral feature of metastatic cells and that within a primary tumour a subpopulation may have a higher metastatic potential.

Using four different multi-locus DNA satellite probes, DNA from both primary tumours and lymph nodes from 13 patients was also analysed by DNA fingerprinting. DNA fingerprinting allows detection of individual-specific restriction fragment length polymorphisms (RFLP), which can be used to detect alterations in tumour DNA. Using the satellite probes, it is possible simultaneously to detect changes on regions widely dispersed in the human genome. The analysis in this study involved comparison of DNA samples from peripheral blood with those from primary tumours and lymph nodes in both stage I and stage II breast cancer patients. A number of instances of loss of bands and appearance of new bands was observed in both primary tumours and node metastasis, but these changes were observed in the case of both diploid and aneuploid tumours. Although DNA from only a small number of patients was analysed, the observed changes were found not to relate to either stage or tumour grade.

Finally, it was possible to determine if there was a correlation between the different parameters analysed in patients where more than one parameter was studied. DNA aneuploidy was related with both HPA staining and the lymph node response. Although the association with HPA binding was not statistically significant in the 28 patients studied, aneuploid tumours were found to be positive for HPA staining in patients with lymph node metastasis. In 15 patients where both lymph node responses and DNA ploidy were analysed, the proportion of HLA DR-expressing T cells, both CD4+ and CD8+, was higher in patients with aneuploid tumours ($p<0.05$). Although no differences were observed in Tac expression, the proportion of IgG-expressing B cells was higher in patients with aneuploid tumours ($p=0.05$). This suggests that some aneuploid tumours may express certain antigens that can elicit a lymph node response.

This study provides evidence to suggest that in breast carcinoma patients, HPA binding and aneuploidy are parameters associated with metastasis to the lymph nodes and that metastatic cells may affect the immunological response in the lymph nodes. Analyses of HPA binding and DNA aneuploidy may help to identify aggressive tumours while immunological responses in the lymph node may be used to assess host-tumour interaction. These parameters have the potential to be used both in assessing prognosis and in designing therapeutic strategies in breast cancer patients.

CHAPTER 1

Introduction

1.1 Introduction to breast cancer

1.1.1 History of breast cancer

Breast cancer is a disease which has been observed and recorded over many centuries. Although the early Egyptians did not perform any surgery, they left records of the description of the disease (Donegan, 1988). Breast cancer was also known to the Greek and Roman physicians, and its record continued through the Middle Ages and into modern times. During these times the knowledge about the cancer grew progressively. Early physicians discouraged treatment as such procedures as were available only aggravated it. There then followed a period when the cancer was believed to be a systemic disease which could not be expected to be cured. This was replaced by an optimistic view, when it was considered to be local and therefore curable. At present, one can only appreciate its complexity and the fact that yet more has to be learned about breast cancer in order to be able to understand it and devise local strategies for treatment.

1.1.2. Epidemiology and aetiology of breast cancer

Cancer of the breast affects almost exclusively females, although it can infrequently afflict the male. It ranks as one of the leading cause of cancer death in women. Although the disease occurs throughout the world, the incidence rates vary in different parts of the world. The incidence rate is reported to be increasing each year in almost all countries but there are also a few places like Bombay in India, where the rate is significantly lower. In general, breast cancer is regarded as a disease of the industrialised nations and

the current estimate is that one in every 12 women will develop breast cancer. A notable exception is Japan, a highly urban and industrialised nation but with a remarkably low death rate from breast cancer. The highest death rate has been reported for England and Wales and in the United States there has been no significant change in the death rate since 1930 (Donegan, 1988). The risk for the disease is age-related, with the majority of patients first presenting being women over the age of 40 years (Leis, 1978).

Apart from age other risk factors have also been associated with breast cancer.

Genetic predisposition. Female relatives of breast cancer patients have a two-fold risk of developing cancer and that such a risk is highest for daughters (Anderson, 1974). There is also a difference observed between Oriental and Western women, but it appears that environmental factors also have an influencing effect as Japanese immigrants to the United States display a rise toward the higher risk observed in the United States (Seidman, 1972). It is difficult to examine familial influence as a risk factor as many other synergistic factors may affect the level of risk (Cairns, 1981).

Endocrine factors. As the mammary gland is an endocrine-dependent organ, an adverse hormonal environment may contribute towards the promotion of the disease. Although the possible involvement of androgen and prolactin have been suggested, the hormone oestrogen is most important in potentiating or promoting the disease (Lilienfeld, 1963). The level of estriol (E3) and abnormality in the metabolism of oestrogen may play a role in creating an adverse environment (Lemon, 1970).

Reproductive function. Since the first observation that the celibate life led by nuns may be a contributing factor to higher incidence of breast cancer, a number of observations now reveal that full-term pregnancy at an early age substantially reduces the risk of breast cancer. Women who give birth to their first child at or after the age of 35 have a twofold higher risk of breast cancer than those who had their first child when less than 20 years of age (MacMahon *et al.*, 1973). Childlessness is in itself a risk factor but if first pregnancy is delayed beyond age 30, then the risk for breast cancer increases above that of nulliparous women. This relationship reflects the complex interplay of hormones associated with breast cancer.

Diet and obesity. Although ingested food may contain small amounts of carcinogens, none of them have been linked with breast cancer in humans. It is more the quantity and the composition of the diet that has received much attention. In this respect, an excessive fatty diet appears to be influential. Mice made artificially obese are known to develop mammary cancers faster than controls (Carroll, 1975). Since the incidence and mortality rates for breast cancer vary in different countries, researchers have compared dietary fat intake in various countries. Several such studies indicate that breast cancer rates are positively correlated with total fat intake, animal protein, and animal fat (Reviewed by Gray *et al.*, 1979). Moreover, studies carried out in various countries points to an increased risk of breast cancer in postmenopausal obese women (Boyle & Leake, 1988).

Viruses. Viruses are a very common cause of both benign and malignant neoplasms in animals and one retrovirus associated with breast cancer is the

mouse mammary tumour virus (MMTV). MMTV has proved to be an ideal model for investigating mammary tumours in animal models. Although viral particles morphologically similar to MMTV and a few other particles have often been found in human breast milk and in some human mammary cancers (Spratt *et al.*, 1988), there is no conclusive evidence to suggest the role of any virus associated with breast cancer in humans. Recent studies concerning the minor lymphocyte stimulating (Mls) antigens in mice, however, have revealed that it is the retroviral MMTV genes that encode the "superantigens" responsible for the deletion of T cells bearing receptors with specific Vβ sequences (Acha-Orbea & Palmer, 1991; Choi *et al.*, 1991; Acha-Orbea *et al.*, 1991). A significant finding is that the viral antigen is maternally transmitted in milk (Marrack *et al.*, 1991).

1.1.3. Anatomy of the Breast

1.1.3.1. Cellular composition of normal breast

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 16 to 24 interconnected *lactiferous ducts*, which run radially from the base of the nipple and branch repeatedly (Figure 1.1.1). Each of these ducts dilate to form a *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

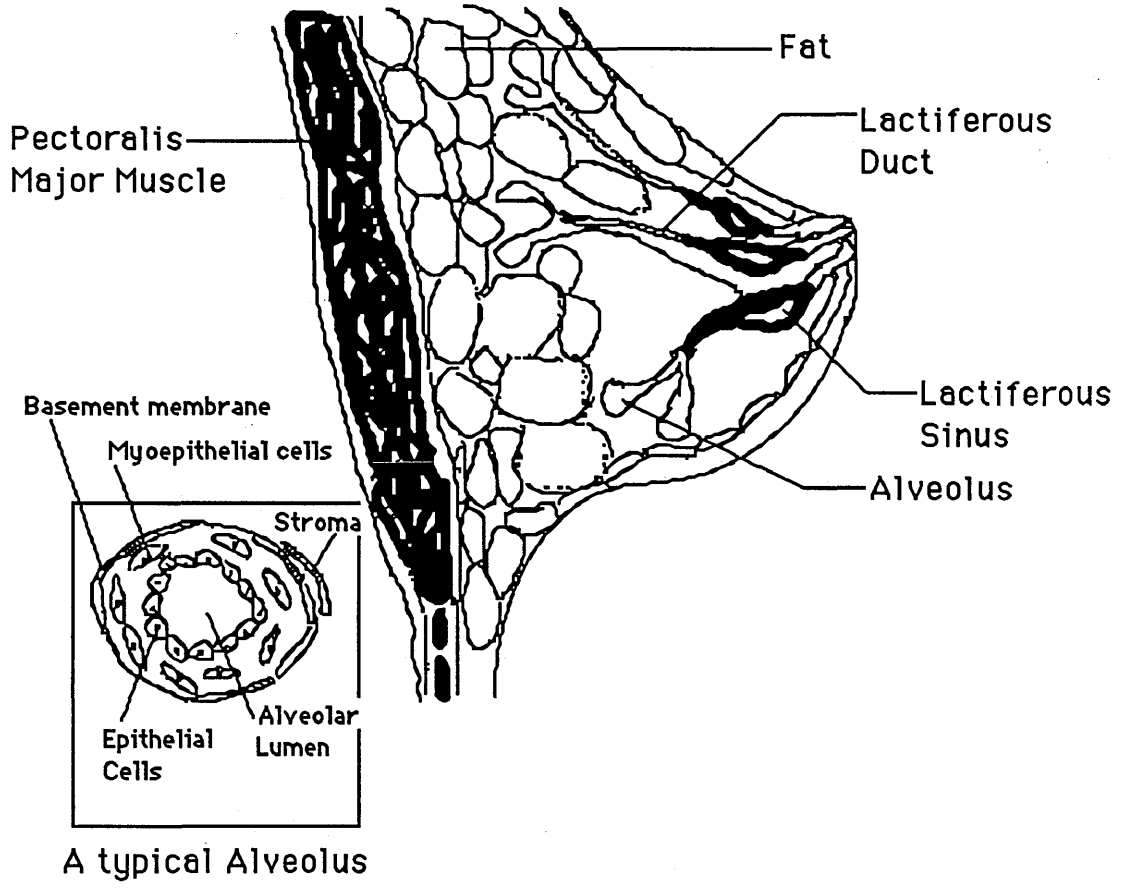


Figure 1.1.1. The human mammary gland and its constituent parts.

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* or saccular invaginations 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. These alveoli are surrounded by a mesh of

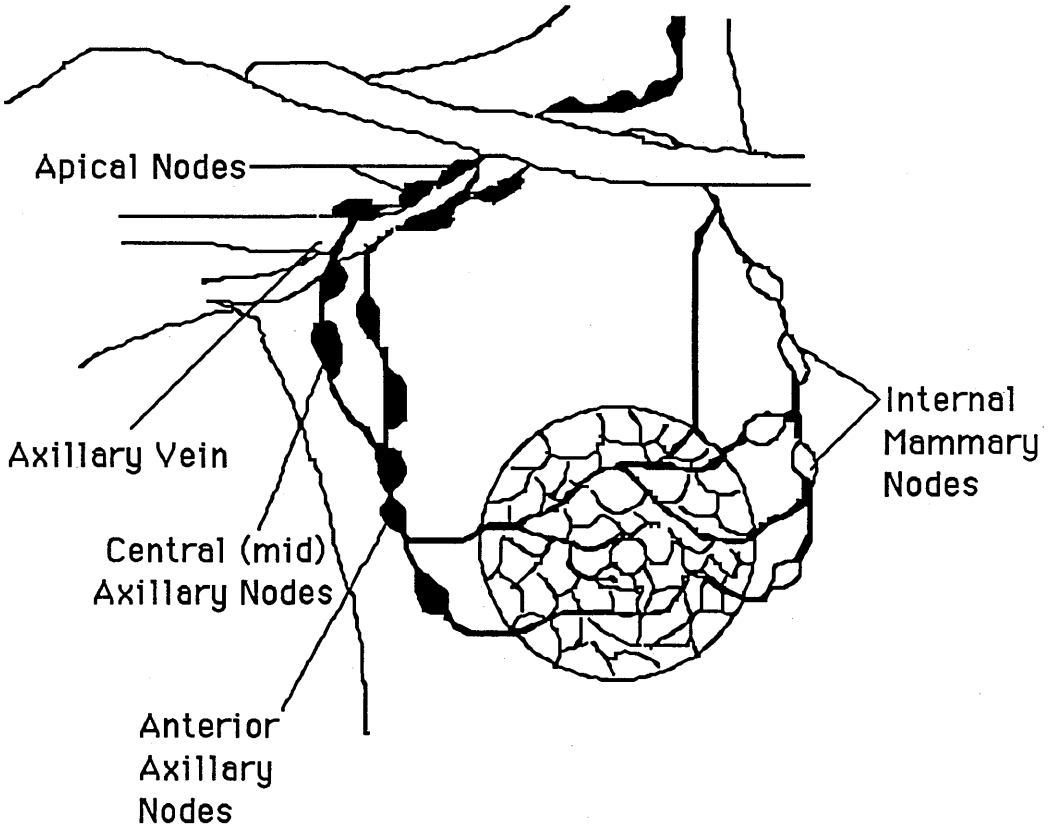


Figure 1.1.2. The lymphatic drainage of the breast.

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 1.1.2). The major lymphatics includes a superficial group draining the skin 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 direction from the breast, the main direction of lymphatic flow is towards the axilla. About 97 to 99% of the lymphatics pass laterally to the axillary nodes, with only 1 to 3% passing to the internal mammary nodes. At the axilla, the lymph nodes are arranged such that they can be grouped into three with respect to their position from the breast. 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. Metastatic spread of the disease frequently involves the axillary nodes. The status of these nodes are, therefore, important features 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 are, at present, the most important prognostic indicator of breast cancer. Secondly, these lymph nodes are also

probably involved with generating and modulating immunological responses against the tumour. The deposition of metastatic tumour cells in the nodes means that these highly malignant tumour cells can be isolated and their characteristics studied and compared with the primary tumours.

1.1.4. Biological features of breast cancer

The growth of the breast depends on hormones and growth factors and the interaction of the various cell types in the gland. The glandular epithelium interacts through direct contact with the myoepithelium, and is acted upon by growth factors, which are secreted by the stroma, and blood-borne hormones. These interactions are important both in the development of the normal breast and in sustaining the growth of a malignant tumour.

1.1.4.1. Hormones

The principal hormones involved in the growth of the mammary gland are the steroids and the lactogenic hormones. The steroids include oestrogens, progesterone, and glucocorticoids, while the polypeptide lactogenic hormones include growth hormone, placental lactogen, and prolactin. Of these hormones, oestrogen and progesterone are important in breast cancer, as they not only control and promote the growth of the normal tissue but are also involved in sustaining the growth of some breast cancers.

Oestrogens act as mitogens and are responsible for the proliferation and differentiation of mammary epithelium, especially the ductile portions of the gland. In breast tissues, the oestrogens bind to specific oestrogen receptors

(ER) in the cytosol and the bound receptor then moves to the nucleus (Shaymala & 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).

Progesterone combines with oestrogen and prolactin synergistically for lobuloalveolar development during pregnancy. An important physiological role of progesterone is believed to be the prevention of terminal differentiation of mammary epithelium during pregnancy.

The steroid receptors on breast tumour cells are now routinely monitored to assess prognosis in breast cancer patients.

1.1.4.2. Cell-cell interactions

Cell-cell adhesion plays an important role in maintaining the integrity of the mammary epithelium. Communications between the different cell types are mediated by the transmission of growth factors across cell junctions. In recent years, a number of adhesion molecules (Section 1.1.6.6) both on the surface of breast tumours and on cells interacting with them, such as lymphocytes (Section 1.2.7), have been identified. Some of these molecules appear to play an important role in the metastatic spread of the disease (Section 1.1.6.6).

1.1.4.3. Growth factors

The growth and differentiation of cells of the mammary gland is believed to be under the control of both positive and negative regulators of growth. Various such growth factors have been implicated with the growth of normal breast tissues and it is likely that in cancers there may exist an imbalance in the production of growth factors and/or a loss of responsiveness to negative growth regulators. Of the positive regulators, the role of epidermal growth factor (EGF) is well documented. EGF acts by binding to a specific cell-surface receptor (EGFR). The receptor has an external domain, which binds EGF, a transmembrane section, and a cytoplasmic portion, which contains a tyrosine-specific protein kinase (Downward *et al.*, 1984). The growth factor acts in an autocrine fashion and the cytoplasmic portion of EGFRs contain sequences closely related to the v-*erb* B oncogene (Section 1.4.2.2.4) such that once the receptors are established, tumours might be able to sustain their own growth. In breast cancers, EGF receptors have been reported to be expressed in inverse proportion to ER (Sainsbury *et al.*, 1987), although not all studies support this (Fitzpatrick *et al.*, 1984). Prognosis in breast cancer patients has been found to be significantly related to EGFR status. Both relapse-free and overall survival were significantly worse for patients with EGFR-positive tumours compared with EGFR-negative tumours (Sainsbury *et al.*, 1987; Nicholson *et al.*, 1988).

A second 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.

1.1.5. Clinical management and prognosis in breast cancer

1.1.5.1. Surgical practice

The primary tumour itself is not generally fatal to the patient and most clinical strategies are designed to minimise the possibilities of metastatic growth which causes the main damage. The initial signs of metastasis are usually to be found in the axillary nodes which drain the tumour. In most centres in the UK, the breast itself is removed entirely (mastectomy) or in part (lumpectomy) and, in addition, the axillary nodes are removed and examined for metastatic spread of the tumour. This axillary clearance is considered by surgeons to be necessary not only to remove the potential metastatic deposits but to aid decisions on patient management (Fentiman & Mansel, 1991). For example, a patient whose nodes are not invaded may be treated by local radiation therapy whereas one with invaded nodes is more likely to be given general chemotherapy. Other factors which influence clinical management include tumour size, tumour grade, and oestrogen receptor status, with receptor negative tumours having a poorer prognosis and therefore possibly requiring more aggressive therapy (Reviewed by Perren, 1991). While tumour

grade and lymph node involvement (Bloom & Richardson, 1970) are currently used as the main prognostic indicators, neither gives definitive information and much effort has been put into the search for other markers which may be used at the time of presentation to give more accurate forecasts of the outcome of the tumour.

1.1.5.2. Prognostic factors in breast cancer

As indicated above, a very difficult problem faced with breast cancer patients is in forecasting prognosis. The outcome of the disease is highly variable with some patients enjoying a recurrence-free life after having the tumour removed, while others experience rapid metastatic progression and a short survival time. Histological examination of the breast tissue not only aids in the diagnosis of breast cancer but once the cancer is detected, it also aids in the assessment of prognosis in breast cancer patients. Although a number of prognostic factors are currently in use, there is a definite need for other factors of higher prognostic value to be defined.

Stage

Stage has consistently been shown to be the most important factor influencing patient outcome in breast cancer. The commonly used staging system (TNM, Tumour Node Metastasis) employs four stage categories. In stage I, the tumour is small and limited to the breast, while in stage II, there is metastasis to axillary lymph nodes. In stage III, advanced locoregional disease is present, and in stage IV, there is widespread metastasis to distant places.

Axillary lymph node metastasis

Microscopic examination of an adequate number of axillary nodes to detect whether metastatic tumour is present, remains the single most important prognostic factor. The 5-year survival rate of patients without nodal metastasis varies from 67 to 85 percent, and drops significantly to 63 to 73 percent for patients with a single node metastasis (Silverberg, 1975; Fisher *et al.*, 1983). Although patients with one to three node metastases are considered to have a similar prognosis, overall survival and disease-free survival declines in a steady, incremental manner as the number of involved nodes increases. Generally, cancers of the breast will metastasise first to the lower (anterior , Fig. 1.1.2) axillary nodes and although patients with metastases in multiple levels are in a poor prognostic group, there is no prognostic significance of 'high level' versus 'low' or 'middle level' involvement (Barth *et al.*, 1991; Smith *et al.*, 1977).

Tumour size

The diameter of a primary breast tumour is strongly related to the likelihood of axillary node metastasis. Moreover, tumour size has also been found to be a significant independent factor associated with disease-free and overall survival (McGuire, 1986).

Tumour grade (Differentiation)

The most commonly used tumour grading system was first introduced by Bloom & Richardson (1957). This grading system assigns equal

importance to three features: structural differentiation, cytological differentiation, and mitotic figure frequency. A numerical value (1, 2, or 3) is assigned to each of these features and the sum of these defines the grade of a tumour. Points total of 3 to 5 represent grade I (low grade), 6 to 7 are grade II (intermediate grade), and 8 to 9 are grade III (high grade). Generally, grade I tumours are relatively less frequent compared to higher grade tumours (Bloom & Richardson, 1957).

Oestrogen receptor (ER)

The presence of ER in tumour cells is well established as a predictor of clinical response to endocrine therapy in breast carcinoma. In addition, there are also reports of a correlation between favourable patient outcome and positive ER status (Shek *et al.*, 1988). Breast cancer patients whose tumours lack ERs appear to be at a disadvantage in terms of both disease-free survival and overall survival. However, there are also reports of ER not being an independent prognostic factor (Parl *et al.*, 1984; Aamdal *et al.*, 1984). ER status has not been correlated with node status, but the presence of ER protein has been related to histological differentiation (McCarty *et al.*, 1980) and it is likely that the biological significance of ER is due to this association.

All of these tumour characteristics together are currently in use to provide prognostic information useful in managing patients with breast cancer. Although no single characteristic is, on its own, of value in assessing prognosis, axillary node status remains the most important one among them. Nonetheless, even this parameter, is of limited value since up to 30 percent of all women with node-negative breast cancer will eventually relapse and die from their disease (Fisher *et al.*, 1985). Thus, the absence of regional lymph

node metastases does not ensure that distant or systemic seeding of tumour cells has not occurred (Redding *et al.*, 1983), only that it is less likely. The major drawback of these factors is that they require histological assessment and as such are prone to observer subjectivity. Gilchrist *et al.* (1985) showed that pathological assessment is not a reproducible feature when the same tumour is viewed by a panel of pathologists using routine methodology. Other studies also report interobserver variation (Stenkvist *et al.*, 1983) while Delides *et al.* (1982) found agreement among six pathologists on tumour grade in only 14.5 percent of cases. In addition, these histological features are highly inter-related and often prognosis is relevant in certain patient groups. There is, therefore, a need for the development of other factors that would provide both a better understanding of the disease and help in assessing prognosis in breast cancer patients.

1.1.6. The metastatic spread of breast tumour cells

The majority of breast tumours result from transformation of ductal or lobular epithelial cells, with ductal breast cancer being dominant. After the initial transformation step and once the tumour is established, it continues to grow as carcinoma *in situ*. These are a confined group of cells still linked by cell-cell contact and contained within a basement membrane. At a later stage,

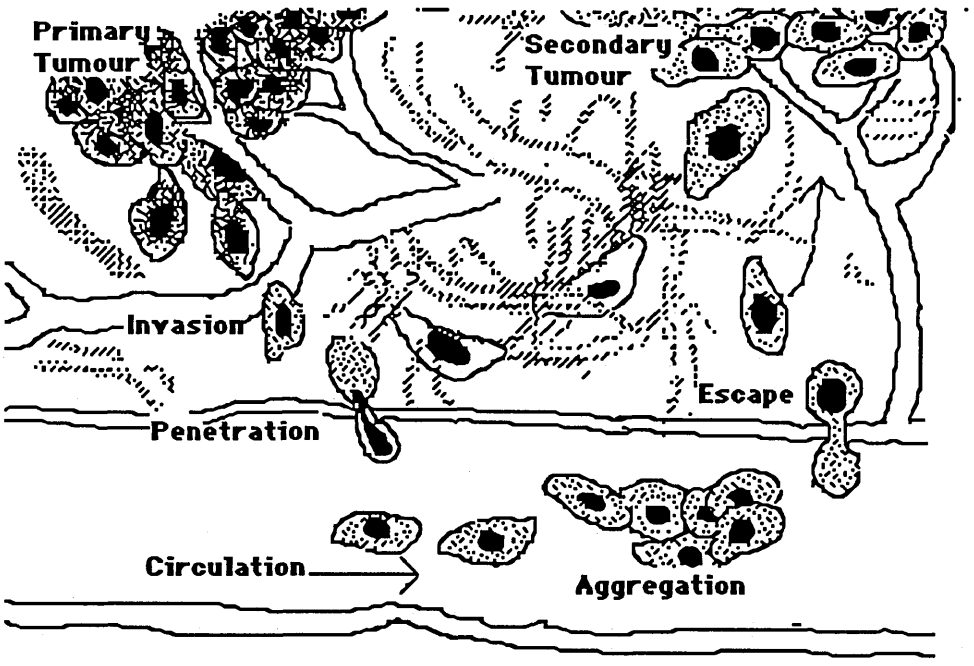


Figure 1.1.3. The metastatic process.

cell-cell contact breaks down and the tumour becomes invasive and invades the surrounding tissues. Cells from such a tumour secrete digestive enzymes and progressively the metastatic process begins.

1.1.6.1. The Metastatic process

The main clinical problem of cancer is metastasis, the migration in the blood and lymph system of tumour cells that give rise to tumours in other parts of the body. The dissemination of tumour cells is a complex and multistep process (Figure 1.1.3). This complex process can be represented in a simplified manner in the following sequences: i) release from the primary

tumour, which accompanies proteolytic degradation of basement membrane and surrounding tissues, ii) invasion into blood vessels, transport and survival of blood-borne cells, iii) homing to secondary sites and finally, iv) infiltration of the target tissues. Although these events are not clearly understood, a few characteristics of human tumours have been related to some of the events in the metastatic process.

In breast cancer, the metastatic process is even more complex. Primary tumours differ widely in nature, in some patients there is widespread metastasis while in others they are more or less contained at the primary site and thus surgically operable. Even within a tumour, much variation exists and the tumour gradually changes and finally evolves into a highly metastatic tumour. Various factors both contribute towards as well as act against the generation of such metastatic cells. Recent research has been directed towards identifying such features of breast tumours and their relationship to metastasis.

1.1.6.2. Generation of tumour heterogeneity and metastatic potential

It is generally believed that cancers develop from single transformed cells (Nicolson, 1987), whose transformation itself is initiated by a genetic event. Then as the tumour grows, multiple genetic alteration takes place and in a complex interplay with the selective pressures from the environment, one of which is likely to be host immunity, the tumour progresses from a less malignant to a higher malignant form. The underlying mechanism of the generation of the malignant phenotype is therefore alterations at the level of the gene. Gene expression, however, manifests itself in phenotypic expression

and with time a tumour acquires a heterogeneous nature, changing gradually and independently and eventually gaining autonomy from host controls, such as hormone regulation of cell growth and control of cellular differentiation. Thus, the tumour mass within a patient at surgery is a collection of cells of varying phenotype and the bulk of these cells may not contain the marker for the clonogenic cells (Figure 1.1.4). Tumour heterogeneity was first noted histologically and has now been extended to include a wide variety of genetic, biochemical, immunological, and biological properties. In breast cancer, heterogeneity in tumours has been established principally with the use of Mabs directed at antigens associated with a tumour (Reviewed by Moss *et al.*, 1988; Edwards, 1985). A given antibody reacts with only some of the tumour cells and as such the antibody defines a distinct population of cells. It was this phenomenon of tumour heterogeneity that dampened the much optimistic view concerning the use of Mabs directed against tumour-associated antigen, most of which were expressed not only by some tumour cells but also by some normal tissues (Section 1.3.1). A key question concerning tumour heterogeneity is whether any particular subpopulation of tumour cells has a higher malignant potential. In the interplay of various selective processes, it is likely that the tumour subpopulation with a growth advantage will outgrow their counterpart and if it so happens these are the cells with a higher metastatic potential, then the secondary deposits will comprise of only these cells. That tumour cells can differ in their ability to metastasise have been shown by transplanting melanoma clones in mice and it was observed that certain clones produced more metastases than others (Fidler & Kripke, 1977). This also means that only a collection of cells are highly malignant and theoretically it only amounts to finding the right antigen on these cells to be able to identify

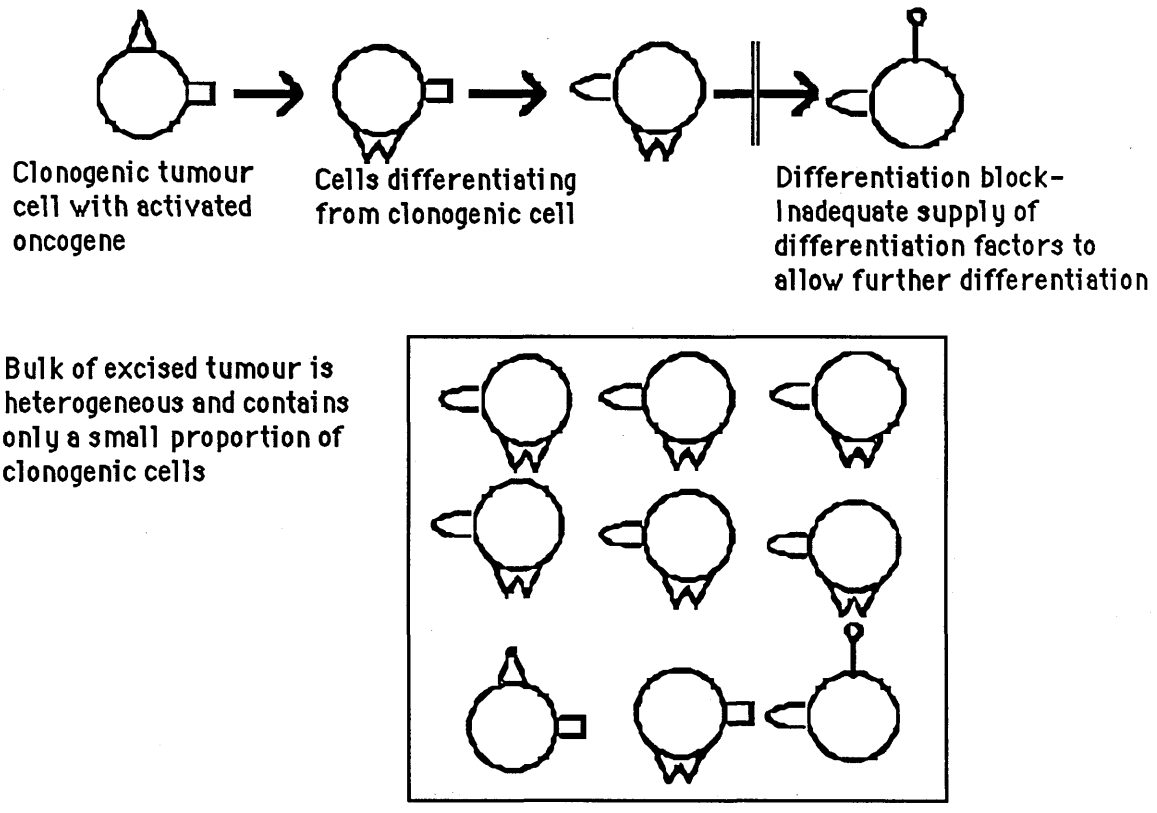


Figure 1.1.4. Development of tumour cell heterogeneity.

them. Yet, after extensive search, no defined tumour-specific antigen have been described, much less defining a specific antigen associated with malignant potential. However, recent studies have identified genetic alterations associated with breast cancer and the involvement of at least one specific gene, the nm23 gene (Section 1.4.2.4.), with metastasis have been reported. Moreover, heterogeneity in the expression of surface carbohydrates has been observed both with Mabs and the use of lectins and some of these expressions appear to be related to metastatic spread (Section 1.3.5.3).

1.1.6.3. Tumour angiogenesis and metastasis

Angiogenesis involves the formation of new capillary blood vessels and a functional microcirculation, which allows further growth of the tumour and also increases the opportunity for these cells to enter the circulation. Generally, the growth of solid tumours beyond a certain size (usually a few millimeters) depends on the induction of such a functional microcirculation from surrounding host tissue (Folkman, 1990). Angiogenesis does not depend on the release of a single diffusible factor as was once thought, but rather on a more complex interaction which probably involves the release of several angiogenic polypeptides by both tumour cells and infiltrating normal cells (Furcht, 1986). Non-tumourigenic cells have been reported to produce both angiogenic factors and an inhibitor of such factors of Mr 140,000 (Rastinejad *et al.*, 1989). Loss of this inhibitor, which normally blocks angiogenesis, due to the loss of a tumour suppressor gene was found to correlate with simultaneous expression of angiogenesis and tumourigenicity. Recently, Weidner *et al.* (1991) reported that the assessment of tumour angiogenesis in breast cancer may predict the occurrence of metastatic disease in axillary lymph nodes or at distant sites. The investigators counted the numbers of microvessels per microscopic field in samples obtained from 49 patients with invasive breast carcinoma and found a significant correlation between microvessel density and metastasis. Similar relationships between intensity of angiogenesis and probability of metastasis have also been reported in human melanomas (Srivastava *et al.*, 1988).

1.1.6.4. The role of proteolytic enzymes in metastasis

One of the initial changes occurring after neoplastic transformation is an increase in cell-released and cell-surface degradative enzymes. Indirect evidence from model tumour systems suggest that proteolytic enzymes play a role in cancer invasion and metastasis (Duffy, 1987). It is believed that cells are invasive by virtue of their ability to secrete or induce secretion of proteinases capable of degrading the molecules which compose the barriers they must cross. These barriers include basement membranes and extracellular matrices, which are composed primarily of collagen, proteoglycans, and other glycoproteins. Thus various proteases, which include the collagenases, cathepsin D, and the urokinase form of plasminogen activator, have been studied as an indicator of metastatic potential. Recent reports indicate that the enzymes cathepsin D and plasminogen activator are related to metastatic spread in breast cancer. Assay of these enzymes is, however, made difficult by the fact that often it is difficult to distinguish between tumour-associated enzymatic activities and enzymes released from contaminating normal cells (Nicolson, 1982).

Urokinase plasminogen activator (UK-PA)

UK-PA is a serine protease which converts plasminogen to the active plasmin, which can convert collagenase to the active enzyme and has a wide substrate specificity, cleaving most proteins. Therefore, PA through its activation of plasminogen can degrade many component molecules of the barrier that tumour cells must cross during metastasis. In most tissues PA

exists as two forms, the tissue type (t-PA) and the urokinase type (UK-PA), while either or both forms are variably found in tumours (Duffy *et al.*, 1988a). The significance of this has been shown by Ossowski & Reich (1983),when they reported that antibodies against UK-PA, but not against t-PA, inhibited metastases of tumour cells into chick embryos. Compared with benign tumours, UK-PA has been found to be significantly higher in malignant breast tumours (O'Grady et al., 1985) and related to both size of tumour and number of axillary nodes with metastases (Duffy et al., 1988a). In a later report (Duffy *et al.*, 1990), the latter group of investigators extended the study to reveal that cancers containing high levels of UK-PA antigen had significantly higher risk of early disease recurrence and shorter overall survival. Similar results have also been reported by Janicke *et al.* (1989). Interestingly, total plasminogen activator correlates with good prognosis (Duffy *et al.*, 1988b). Further study with larger samples are needed to confirm the importance of this protease.

Cathepsin D

Cathepsin D is an oestrogen-induced lysosomal acidic protease. A precursor glycoprotein of Mr 52,000 was first described in the culture medium of a hormone-dependent cell line and later found to be constitutively produced in ER negative cancer cells (Rochefort *et al.*, 1988). In normal cells, only a small amount of cathepsin D is secreted as the precursor. The bulk of the protein is targeted to the lysosomes, where it is proteolytically processed to an intermediate of Mr 48,000, and the mature, stable two-chain form of Mr 34,000 and 14,000 (Yonezawa *et al.*, 1988). Two distinct activities have been assigned to the secreted precursor. It has been found to have an oestrogen-regulated autocrine mitogenic effect on MCF-7 cells, and it has an acidic

proteolytic activity on various substrates, including proteoglycans and basement membranes (Rochefort *et al.*, 1987). Both these properties may relate to metastatic capability in cancer cells. In a follow-up of about 4.6 years, the cytosolic cathepsin D concentration by radioimmunoassay in 122 primary breast cancers was found to be strongly related to both metastasis-free and disease-free survival (Spyratos *et al.*, 1989). More recently, Tandon *et al* (1990) measured by western blotting the mature form of the enzyme (Mr 34,000) and found that high levels of the enzyme in node-negative patients were a significant predictor of reduced disease-free survival. Moreover, high levels of cathepsin D were found more often in tumours with an abnormal DNA content (aneuploid, Section 1.4.3.3.1) than in diploid tumours, and among aneuploid tumours, higher levels of cathepsin D identified patients at extreme risk of recurrence.

1.1.6.5. Evasion of host immune response

Tumour cells encounter the cells of the immune system while growing locally, and throughout the metastatic process. With the initiation of the metastatic process, tumour cells have to evade immune surveillance as they enter blood vessels and in the circulation until they are finally able to settle at a secondary site. Evaluation of lymphocytic cells both for their phenotype and their activation state has therefore, been done on lymphocytes present locally (tumour infiltrating lymphocytes), in blood, and in draining lymph nodes (Section 1.2.9). In breast cancer, lymph nodes are important sites both for the modulation of an immune response and as a primary site for metastatic deposit. Evaluation of lymphocytes from the axillary nodes in breast cancer is thus important in understanding the effect of metastasis on the immune response (Section 1.2.10).

1.1.6.5.1. Lack of MHC class I expression

An alternative strategy to evaluate how tumour cells evade the immune response is to look for expression on the tumour surface themselves. One particular antigen, the MHC molecules has received much attention in this context. MHC class I and II genes encode for proteins which are intimately involved in intracellular communications, and in the discrimination of self from non-self or foreign. Class II MHC antigens have a restricted expression and are generally found on specialised cells of the immune response, while MHC class I antigens are normally present on all nucleated cells in the body (Benacerraf, 1981; Linsk & Goodenow, 1986). In breast tumour cells both MHC class I and II molecules are variably expressed (Whitford *et al.*, 1992a; Goepel *et al.*, 1991) with some tumours or cells within a tumour often failing to express them. The lack of expression of MHC class I on tumour cells has an important implication. Since immunological recognition of antigens by cytotoxic T lymphocytes (Tc) is restricted to MHC class I determinants (Zinkernagel & Doherty, 1979), failure to express class I, or the expression of inappropriate or altered MHC class I components on tumour cells, is a possible mechanism whereby tumour cells may escape killing by antigen specific Tc. Similar to many other human tumours, breast tumour cells have been found to lack MHC class I molecules (Perez *et al.*, 1986; Wintzer *et al.*, 1990; Whitford *et al.*, 1992a; Goepel *et al.*, 1991). Although the study of Goepel *et al.* (1991) involved a small number of samples, both primary tumours and lymph node metastases were analysed. Expression of HLA-A2 and the β–2-microglobulin component of class I molecules were reduced or absent in the majority of primary tumours and in each of these cases their corresponding metastases showed a decreased expression.

1.1.6.5.2. Suppression of immune response

It is likely that in addition to evading immune surveillance, tumours may have a direct suppressive action on the cells of the immune system. Such immunosuppressive effect can possibly be mediated through inhibitory factors. A likely candidate is TGF-β (Section 1.1.4.3), which has been found to inhibit B and T-lymphocyte function and proliferation (Hsuan, 1989). A number of studies have now analysed lymphocytes from breast cancer patients and have reported a large variation both in terms of their phenotype and activation states (Section 1.2.9).

1.1.6.6. Expression of adhesion receptors and metastasis

The property of cell adhesion is important in any cell-cell interaction. Cells of the immune system adhere to other cells and extracellular matrix in the process of immunological recognition and recirculation (Reviewed by Dustin & Springer, 1991). Several adhesion receptors associated with lymphocytes have now been described (Section 1.2.7). Two such adhesion molecules, LFA-1 (Lymphocyte function antigen-1) and ICAM-1 (Intracellular adhesion molecules-1), both of which are specialised for use in immunological recognition, appear also to be involved with metastasis (Johnson *et al.*, 1989). LFA-1 is expressed only on leukocytes (Krensky *et al.*, 1983), while its counter-receptor ICAM-1, which binds LFA-1 directly , is expressed on diverse cell types (Dustin *et al.*, 1988) including target tumour cells. Although found to be expressed on tumour cells, ICAM-1 is generally absent from most cells in normal, nonlymphoid tissues except for a low level expression on

endothelial cells. However, local immune responses can cause a rapid increase in ICAM-1 expression on endothelial cells and induction of ICAM-1 on epithelial cells (Dustin & Springer, 1988). Recently, Johnson *et al.* (1989) reported that a surface glycoprotein of Mr 89,000 and identical in sequence to ICAM-1 was expressed on advanced human melanomas but not on benign or early melanomas. The authors suggested that since ICAM-1 is a ligand for LFA-1, ICAM-1 positive tumour cells can interact with lymphocytes within a tumour infiltrate and as such reduce homotypic adhesion between tumour cells. This enhanced adhesion of tumour cells to migratory and invasive lymphocytes may aid in dissociating cells from the primary tumour, subsequently lodging them to a secondary site, for instance the lymph nodes, and thereby help in the metastatic spread of the disease. This suggests that tumour infiltrating lymphocytes not only can cause cytotoxic killing of cells but by inducing expression of adhesion molecules can also contribute to metastatic spread of the disease.

1.1.7. Basic research on breast cancer

Research on breast cancer involves a number of diverse aspects of a cancerous cell. It has generally been studied both at the cellular and the genetic level. At the cellular level, the cell surface has been at the focus, where the aim has been to detect biochemical alteration of surface molecules on a tumour cell. While such alterations has as yet not been found to be unique for breast cancer, tumour-associated changes have been detected (Section 1.3). At the genetic level, the principal aim is to define specific genetic defects and at the same time to be able to characterise the gene products in terms of their biochemical functions. Although the identification of oncogenes and their products and of

tumour suppressor-genes associated with breast cancer underlies the main efforts in this area, it is important to point out that defects at the DNA level in breast cancer is complex and involves gross chromosomal alterations (Section 1.4).

As indicated above the obvious importance of metastatic spread in breast cancer has led to much efforts to increase understanding of the process itself. Although the metastatic process can be outlined in a simple scheme (Fig. 1.3), there is very little known about the biochemical basis of these events. Here again, in a simplistic approach, the aim is to identify both specific cell surface changes and specific events at the level of the gene which are related to the metastatic process. Associated with the spread of the disease is the interaction of the host environment with the cancer cells. One such interaction which must play a very important part is host immunity (Section 1.2). Both at the local site and during its detachment from the primary tumour cells leading to its spread to a secondary site, cancer cells have to deal with the host immune system. An understanding of the effect of cancer on the immune system is therefore, of much importance in breast cancer. Typically, the ultimate aim will be to assign a specific change to a specific defect at the DNA level and to be able to understand the role played by such a change in contributing towards tumourigenicity or metastatic potential.

1.2 Immune response to breast cancer

1.2.1. Historical background

As a consequence of elucidation of the role of the immune system in combating bacterial and viral infections, initial assumptions were that tumours also must elicit a host immune response which failed in tumour patients in the same way as it failed in those who succumbed to infections. Many experimental animal tumours appeared to be antigenic. Thus the prospect of augmenting this immune response and of vaccinating individuals against cancers appeared to be encouraging. Initially interest was focussed on the antibody response as antibodies are easy to handle and produce in large amounts, can be used between individuals of different genetic background, and were much better understood, until recently, than were T cell responses. In the 1970s and early 1980s, it was hoped that antibodies to tumour specific antigens could be used to target on the tumour cells in the manner of "magic bullets". However, more than a decade later, there is limited direct evidence to suggest that an anti-tumour immune response is generated in patients with epithelial tumours. Apart from some tumour-associated antigens no well characterised tumour-specific antigen has been identified (Section 1.3). Negative evidence also comes from post transplant patients on long term immunosuppressive therapy or those suffering from acquired immune deficiency syndrome (AIDS). These patients have no increased incidence of malignancies such as breast carcinoma and only tumours thought to be caused by DNA viruses are more frequent (Penn, 1988). Finally, as increasing numbers of proto-oncogenes are discovered it has become apparent that very few of these have a product located on the surface of the cell (Section 1.4.2.5) and therefore vulnerable to attack by the B cell arm of the immune system, and of those within the cell, the changes from the normal cell product may be

minimal, making detection by the T cell arm of the immune system difficult. In addition, the slow rate of development of such tumours may well lead to immune tolerance and their heterogeneity could give them a natural means of evading total destruction by the immune system by a process of antigenic drift analogous to that occurring in viruses. It is, therefore, possible to argue that there is little proven human response, that one would not expect a strong response, and that any weak response that is mounted can readily be circumvented by the tumour.

To counter these arguments, it is possible to suggest that the follow-up of immunosuppressed transplant patients is still relatively short compared to the aetiology of breast cancer (Campbell & Leake, 1990). Clinical evidence is also available from many sources. Fisher *et al* (1980) showed that some patients having undergone surgical removal of the primary tumour had a long survival even with residual malignancy in their lymph nodes. Others showed that stress conditions which depress the immune system, like bereavement (Bartrop *et al.*, 1977) can also cause recurrence of the disease. Finally, there is the evidence that 60% of primary breast cancers carry an infiltrate of T lymphocytes (Section 1.2.9.1.) which would appear to indicate an ongoing immune response at the time of removal. T cell recognition of antigen has become better understood in recent years and, since oncogene products are largely intracellular and only potentially visible to T cells, it is largely the T cell response that has become the focus of interest (Bodmer, 1991). Recent studies, therefore, have been directed towards the analysis of this infiltrating immune response both as an indicator of prognosis and in developing methods of immunotherapy (Rosenberg, 1988). The rationale behind such studies is the hope of augmenting the immune response in patients showing signs of mounting anti-tumour immunity and also the further understanding of the nature of this disease.

1.2.2 Assessment of the immune response in cancer patients

1.2.2.1 The B cell immune response to tumours

While murine Mabs were being used to detect tumour-associated antigens (Section 1.3), it gradually appeared that these were mouse responses to human tumours (Section 1.3). It was realised that the relevant antibodies may perhaps be derived from cancer patients themselves and these would provide a correct assessment of the human B cell response to cancer. The investigation of humoral immune response to tumour antigens generally involved the use of two phenomena. The use of EBV infection to transform human lymphocytes which have recently been exposed to tumour antigens (Steinitz *et al.*, 1979) and the cell fusion technique for Mab production (Kohler & Milstein, 1975). Theoretically, an advantage with this approach is that tumour-specific B-lymphocytes can be propagated indefinitely for the production of human Mabs and then these antibodies can be used to investigate the nature of the antigens that are stimulating the response and perhaps also in tumour diagnosis and therapy. The source of such lymphocytes for the generation of human Mabs has been either the peripheral blood or the lymph nodes from breast cancer patients. The antibodies were selected by using primary tumours, or more frequently tumour cell lines. Although a few of these antibodies from breast cancer patients were of IgG isotype (Imam & Taylor, 1989), the majority were of the IgM isotype, which reflect an early and immature immune response (Campbell *et al.*, 1987). It, therefore, turned out that the human Mabs were of little use in tumour diagnosis or therapy (Reviewed by Campbell & Leake, 1990; Campbell *et al.*, 1987). The difficulty in generating human Mabs stems from the limitations of hybridoma technology to provide a stable antibody and also from the poor selection methods used in

some early studies. As it is possible to generate irrelevant antibodies, with some degree of multispecificity for intracellular antigens, from both normal and diseased individuals (Ghosh & Campbell, 1987; Damato *et al.*, 1988), it is likely that these were selected in some of the early studies involved with human Mabs. Although a number of human Mabs from breast cancer patients have been reported (Imam & Taylor, 1989; Ronai & Solitzeanu, 1986; Gentile & Flickinger, 1972), none of them are in use in either diagnosis or therapy of breast cancer. With the apparent failure of this technique, the existence of a human B cell response to breast tumours remains to be established.

1.2.2.2 The T cell immune response

A major problem faced by early workers involved with the study of immune response in cancer patients was the assessment of T cell-mediated host immunity. Both lack of sound methodology for the study and a poor understanding of the T cell response contributed to such difficulties. In early studies, chemically and virally-induced tumours in experimental animal models were used. These studies provided evidence to indicate that host immunity can protect from inoculation of tumour (Morton *et al.*, 1969) and that such a protection could be transferred from the host to another test animal (Bard *et al.*, 1969). These experimentally induced tumours and those arising spontaneously in animals were, however, found not to resemble the human condition (Hewitt *et al.*, 1976). This then led researchers to isolate lymphocytes from cancer patients themselves in order to test their effectivity using *in vitro* tests.

1.2.2.2.1 T cell effector function: *in vitro* assays

Although the current *in vitro* assay of T cell effector function involves the chromium release assay, some of the earlier studies employed the blastogenesis assay to assess immunocompetence of lymphocytes from cancer patients. The latter assay, which involves the use of mitogens such as phytohemagglutinin (PHA) to induce uptake of tritiated thymidine, is not very reliable and studies on breast cancer reveal conflicting results (Fisher *et al.*, 1972; Humphrey *et al.*, 1981; Mandeville *et al.*, 1982).

The chromium release assay is used to determine cytotoxic capability of test lymphocytes on target cells and as such an advantage with it is that autologous tumour cells can be tested. But this requires tumour cells to be grown in culture and then allowed to take up chromium (^{51}Cr). On subsequent lysis by the test lymphocytes the labelled tumour cells release the isotope which is then assayed. There are a number of difficulties associated with this assay system. Firstly, lymphocytes from cancer patients have been found to be highly selective with respect to cells they can lyse (Topalian *et al.*, 1987; Belldegrun *et al.*, 1988; Balch *et al.*, 1990) and that too at an effector:target ratio of 40:1 (Topalian *et al.*, 1987). Although lymphocytes from most tumour infiltrates can lyse target cell lines like K562, a more acceptable feature for cytotoxicity tests is to have the autologous tumour themselves as targets. This is not always the case as reported by Balch *et al.* (1990) with TILs from breast and colon cancer and often the cytotoxicity is much greater against cell lines when compared to autologous tumour (Yamaue *et al.*, 1990). Secondly, the growth of the autologous tumour may not be an easy matter as quite often colonies of non-neoplastic cells such as fibroblasts overgrow the true epithelial tumours. Primary cells from epithelial tumours do not grow readily in culture (Section 1.4.1; Section 5.1.3) and it may be difficult to obtain a large number

of viable autologous tumour cells containing the chromium isotope. Finally, primary cultures have a high rate of background cell death, and in consequence the low signal/noise ratio may make it difficult to detect specific lymphocyte induced cytotoxicity. However, with no alternative assay to replace this method, the cytotoxicity assay is still very much in use.

1.2.3. Monoclonal antibodies against lymphocyte surface markers

One of the major developments to take place has been the discovery of the technology for the production of monoclonal antibodies by Kohler and Milstein (1975). Prior to this time, studies on lymphocytes thought to be associated with anti-tumour immune responses, took advantage of the fact that unlike B cells, human T cells form non-antigen-specific rosettes with sheep erythrocytes. Such "E-rosette"-forming cells provided a convenient method for obtaining relatively pure populations of T cells. This is reflected in some of the early studies involved in assessing immune response in cancer patients. However, with the advent of monoclonal antibody technology it became possible to characterise lymphocytes and their subgroups in terms of antibodies against markers on the cell membrane. More importantly these antibodies can be used to identify functional groups of the lymphocytes based on specific markers expressed on the cell surface.

1.2.3.1. Phenotypic markers

Lymphocytes express a large number of different molecules on their surfaces and some of these molecules are characteristic of different cell

lineages. Such molecules can be identified by monoclonal antibodies and are thus called "phenotypic markers". The CD (Cluster Designation) system for identification of human cells of haemopoietic lineage is one of the most advanced demonstrations of the extent to which Mabs can be used to chart cell phenotype and differentiation status. The system is a result of international workshops which meet regularly to group together monoclonal antibodies produced in different laboratories with the same antigen recognition characteristics. This "Cluster" of Mabs is then assigned a number according to the type of molecule identified by all the Mabs in the group. In consequence, the CD status of a molecule also defines the antigen itself. Such antigens not only define cell phenotype and lineage, but also the activation status of the cell bearing that phenotype and its possession of molecules with the ability to adhere to other cells through the LFA, ICAM and VLA family of adhesion molecules (Section 1.2.7). At the 4th International Workshop in 1989 the number of antigens defined in this way rose to a CD value of 78, with many having newly discovered subsets so that the total number of defined molecules is now close to 100 (Table 1.1) (Knapp *et al.*, 1989).

1.2.4. Cells of the immune system and their phenotypic markers

T and B lymphocytes are the major cell components of the immune system. They however have different lineage pathways and functions. In the case of the B cell, the molecules responsible for specific recognition are the different classes of immunoglobulins that can be expressed either as cell surface molecules acting as receptors, or in secreted forms. The equivalent recognition molecule on T cells is the membrane-bound T cell antigen receptor (TCR) which is specific for a combination of foreign antigen with a molecule

Table 1.1. The CD classification of some of the human leucocyte differentiation antigens

CD	Antigen	Location	Function where known
2	Mr 50,000,gp	T cells	Rosette receptor, LFA-3 receptor
3	5 protein complex	T cells	Signal transduction for TCR
4	Mr 59,000, gp	T helper	MHC class II recognition
5	Mr 67,000, gp	T, Bsubset	
8	gp dimer, Mr 32,000	T cyt/sup	MHC class I recognition
10	Mr 90,000, gp	pre-B	CALLA
11a	Mr 180,000, gp	leucocytes	alpha-chain of LFA-1
11b	Mr 155.00, gp	Mono, Gran, NK	C3b complement receptor
11c	Mr 150,000, gp	Mono, Gran, NK	
16	Mr 50-60,000, gp	Gran, NK	Fc gamma receptor III
18	Mr 96,000, gp	Leukocytes	Beta-chain for 11a,b, & c
19	Mr 95,000, gp	B cells	
20	Mr 35,000, gp	B cells	
21	Mr 140,000, gp	B subset	EBV receptor, C3d receptor
22	Mr 135,000, gp	B subset	
23	Mr 45.000, gp	Activated B	Low affinity IgE receptor
25	Mr 55,000, gp	Activated T	TAC, IL-2 receptor, beta-chain
28	Mr 44,000, gp	T subset	
29	Mr 135,000, gp	Broad	VLA beta
32 (w)	Mr 40,000, gp	Mono, Gran, NK	Fc gamma receptor
35	Mr 2220,000, gp	Gran, Mono, B	Complement receptor 1
45		Leukocytes	Leukocyte Common Antigen
45 RA	Mr 220,000, gp	T subset, B, Gran, Mono	
45 RB		T subset, B, Gran, Mono	
45 RO	Mr 180,000, gp	T subset, B, Gran, Mono	
52 (w)	Mr 21-28,000, gp	Leukocytes	Campath 1
54		Broad	ICAM-1
58	Mr 40-65,000, gp	Leukocytes, epithelial	LFA-3
71	Mr 97,000, gp	Growing cells	Transferrin receptor

CALLA= Common Acute Lymphoblastic Leukemia Antigen; gp= glycoprotein; Gran= granulocytes; ICAM= Intercellular Adhesion Molecule; LFA= Lymphocyte function Antigen; Mono= Monocytes; TCR= T cell receptor; VLA= Very Late Antigen; w= workshop.

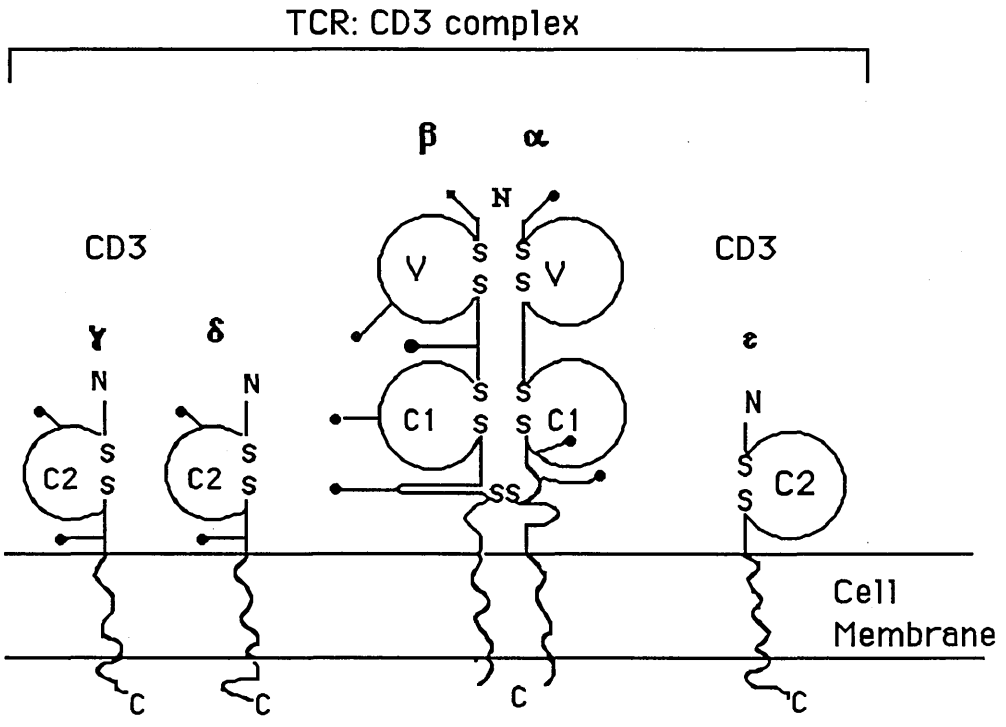


Fig. 1.2.1. The TCR:CD3 complex of T lymphocytes.
The circles signify domains; V and C domains show homology with Ig V and Ig C domains respectively. Presumed N-linked carbohydrate sites are shown by symbol —●. A fourth member of the complex, CD ξ, is not illustrated.

of the MHC (Katz *et al.*, 1973; Rosenthal *et al.*, 1973; Zinkernagel *et al.*, 1974).

1.2.4.1. T cell markers

The definitive T cell marker is the T cell receptor (TCR), which is expressed exclusively on the membrane surface of T lymphocytes. T cell recognition of antigen occurs through the TCR and forms the basis of a range of immunological phenomena including MHC-restricted cytotoxicity and T cell helper and suppressor activity (Davis and Bjorkman, 1988). The typical TCR molecule expressed on mature T cells is a disulphide-linked heterodimer consisting of two chains, α and β (Fig 1.2.1). These two chains are of similar

size; both chains are transmembrane proteins and both show considerable homology with Ig and are thus viewed as part of the "Ig superfamily" (Koning, 1991; Sprent, 1989). A minority population of T cells expresses a quite different receptor molecule composed of γ and δ chains (Janeway, 1988) rather than the α and β polypeptides. These cells are rare in the lymphoid tissues, comprising a few percent of cells in the thymus and peripheral blood but are found in large numbers in skin epidermis (Koning *et al.* 1987; Kuziel *et al.*, 1987) and gut epithelium (Goodman & Lefrancois, 1988). The proportion of $\gamma\delta$+ T cells however, have been reported to be elevated in coeliac disease (Spencer *et al.*, 1989; Trejdosiewicz *et al.*, 1989; Halstensen *et al.*, 1989) while it was variable but generally small in breast cancer patients (Alam *et al.*, 1992a). Both $\alpha\beta$ and $\gamma\delta$ T lymphocytes express their TCR in a molecular complex involving polypeptide chains of the CD3 complex (Fig 1.2.1) (Reviewed by Clevers *et al.*, 1988). In the mouse, the CD3 molecules comprise of five different polypeptide chains, γ, δ, ε, ζ, and p21, of which only the first three have been identified in man. The CD3 molecules are non-polymorphic polypeptides that are non-covalently associated with the TCR and to each other. Each chain is a transmembrane peptide and approximately one-third of the γ chain is intracytoplasmic. There are active phosphorylation sites on the intracytoplasmic portions of the γ chain and it is widely believed that the CD3 complex mediates signal transduction when T cells are activated by antigen to the TCR. Fluorescently labelled antibodies against the CD3 complex acts as a definitive tag for identifying T cells in a mixed population of lymphocytic cells.

1.2.4.2. T cell subsets

Based on definitive surface markers and associated functions, T cells can be subdivided further into two distinct non-overlapping populations: the T Helper (Th) subset which is CD4+ and the T suppressor/cytotoxic (Tc/s) subset which is CD8+ (Fig. 1.2.2). CD4+ T cells recognise antigens in association with the major histocompatibility complex (MHC) class II molecules, while the CD8+ T cells recognise antigens in association with class I MHC molecules. The CD8+ T cells are stimulated by endogenous antigens and therefore, can be expected to participate in defence against viruses, intracellular eukaryotic parasites and against tumour cells.

The ability of CD4+ T cells to secrete large amount of IL-2 and other lymphokines makes them a "helper" cell. The CD4+ Th cells control and modulate the development of immune responses and are thus involved in mediating both T-T and T-B cell interaction involving both direct cell-cell interaction and those mediated through diffusible lymphokines. CD4+ cells have been further subdivided in murine systems into Th1 and Th2 subsets (Mossman & Coffman, 1989). The Th1 subset secretes Il-2 and IFN-γ while the Th2 subset secretes Il-4 and Il-5. The rationale is that Th1 "helps" the T cell immune response while Th2 "helps" the B cell immune response and isotype switching of antibody class. In a less acceptable way, CD4+ Th cells have also been subdivided on the basis of expression of different isoforms of the common leukocyte antigen CD45, and a fibronectin receptor, CD29 (Rudd *et al.*, 1987), which belongs to the integrin class (VLA β) of adhesion molecules. Th cells which express the CD45RA isoform represents the 'virgin' or 'inducer of suppression' T cells (Tedder *et al.*, 1985), while 'memory' and 'inducer of help' T cells express high density CD29 and high density CD45RO isoform (Sanders *et al.*, 1988). According to this

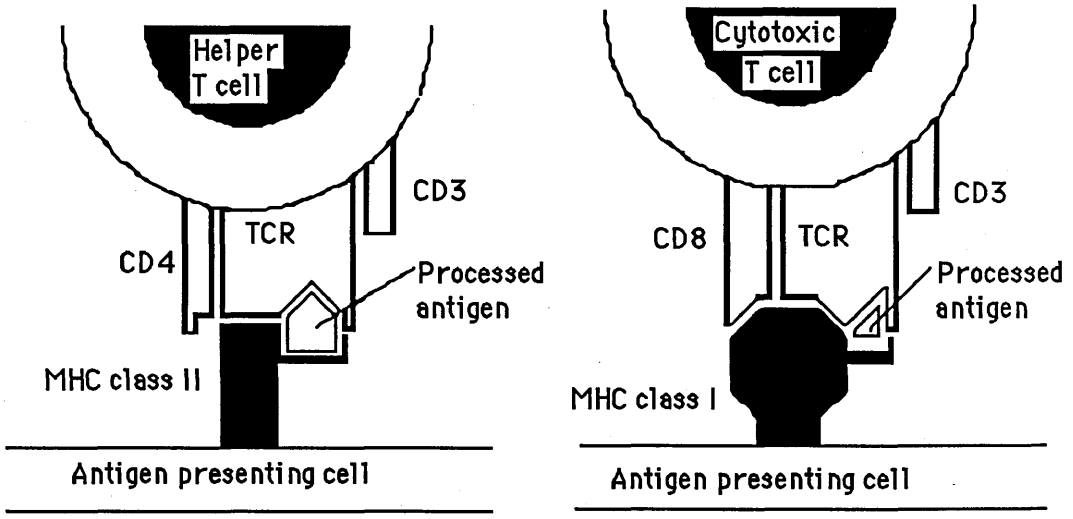


Fig. 1.2.2. Antigen recognition by CD4+ Helper and CD8+ cytotoxic T lymphocytes.

classification, CD45RA^{-}CD29^{hi} Th cells respond maximally to recall antigen and can induce optimal B cell differentiation and Ig production.

1.2.5. Activation markers and T cell activation

Presentation of an antigen on MHC class II molecules by an antigen presenting cell (APC) and subsequent recognition of this complex by Th cells via their TCR marks the activation of Th cells and the beginning of an effective immune response (Fig. 1.2.2). During the activation process the surface of T cells alters and the best documented of these alterations is the acquisition of receptors which mark the activation process and allow efficient interaction between cells of the immune system. Surface changes include the acquisition

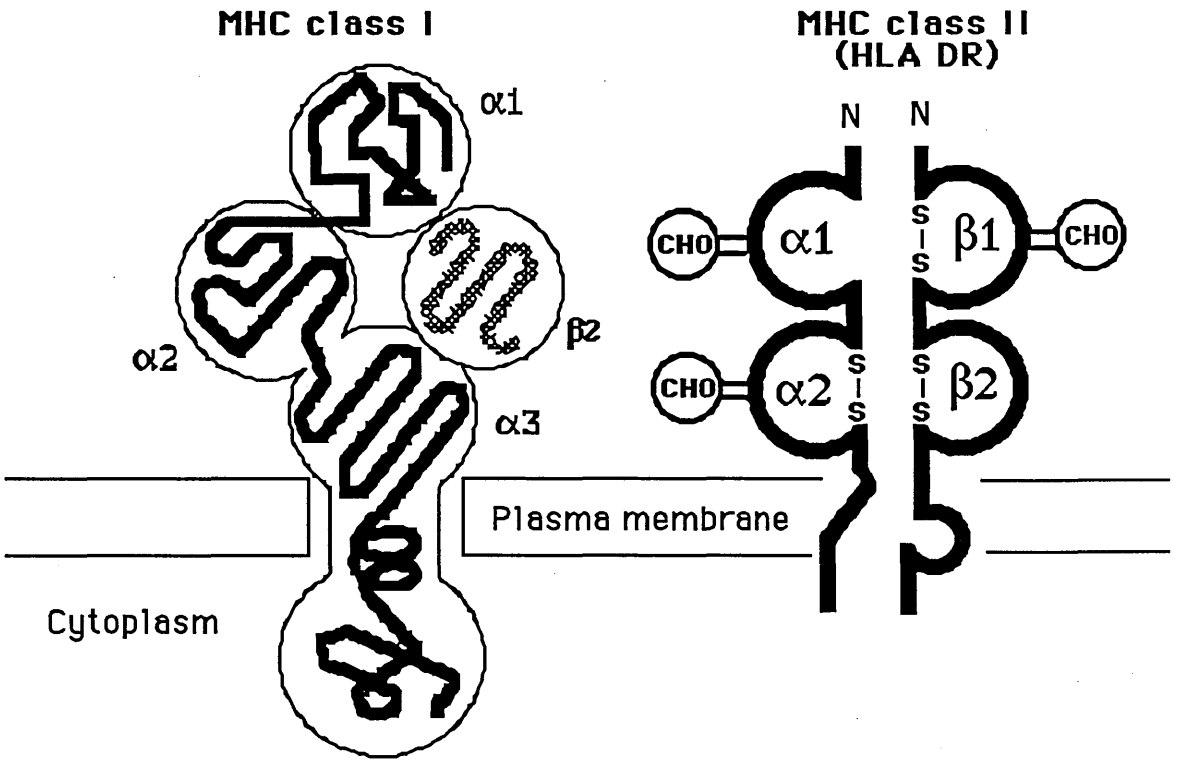


Fig. 1.2.3. Structure of MHC class I and II (HLA DR) antigens.
The three α domains of the class I complex associates with β2 microglobulin to be expressed on the cell membrane. The α and β domains of class II structure are similar in size and are both transmembranous.

of receptors for IL-2 and of HLA class II antigens. Subsequently transferrin receptors appear as iron is essential for cell division.

1.2.5.1. HLA DR

HLA DR, which is the predominant antigen of the class II MHC complex, has four domains arranged on two transmembrane polypeptide chains (Fig.1.2.3) of similar size (Brown *et al.*, 1988). Antigens are presented

by this MHC class II determinant to CD4+ T cells (Guillet *et al.*, 1986). Unlike MHC class I molecules, which are expressed on the majority of normal nucleated cells (Daar *et al.*, 1984a), the distribution of class II molecules in normal tissue is more restricted (Daar *et al.*, 1984b). The HLA DR molecule is generally expressed on the cell membrane of antigen presenting cells (APC), which include B cells and macrophages (Hammerling, 1976). Apart from its role in antigen presentation, HLA DR has also been found to be expressed on T cells following mitogenic stimulation (Ko *et al.*, 1979) and in mixed lymphocyte cultures (Pawelec *et al.*, 1982; Davey *et al.*, 1984) but absent on resting T cells (Reinherz *et al.*, 1979). Thus, the expression of HLA DR on T cells acts as a marker of T cell activation. The exact role played by this molecule when expressed on T cells is unknown.

1.2.5.2. Interleukin 2 (IL-2) receptor

The IL-2 receptor, which is expressed following T cell activation, is composed of two glycoprotein chains, a low affinity beta chain of Mr 55000 (Urdal *et al.*, 1984) and an intermediate affinity alpha chain (Mr 75000) which is involved in signal transduction (Tsudo *et al.*, 1986). Together the α–β heterodimers form the high affinity receptors for IL-2 (Fig. 1.2.4), which are effective in driving T cell growth (Reviewed by Smith, 1989). The high affinity form represents a receptor with a thousand fold higher affinity and this is brought about by the kinetic cooperation between the α and the β chain (Fig. 1.2.4). Binding of the ligand (IL-2) to the receptor mediates G1 progression to S-phase of the cell cycle (Herzberg & Smith, 1987). A characteristic feature of the IL-2 receptor expression is that on stimulation, T cells express the low affinity β chain at a density 5 to 10 fold greater than the α chain and IL-2 binding to the high affinity receptor increases the expression of the β chain 10

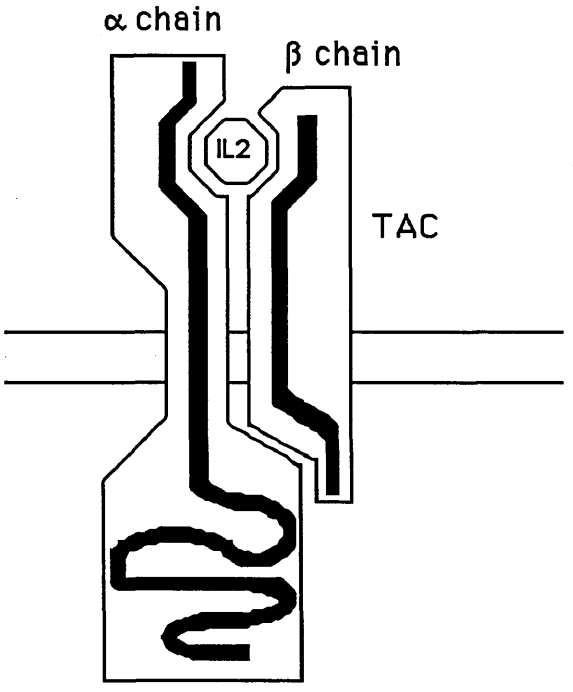


Fig. 1.2.4. Structure of the high affinity interleukin-2 receptor.

to 20 fold. The β chain (CD25), which is recognised by the use of anti-TAC monoclonal antibody, thus acts a marker of T cell activation and growth. However, IL-2 bound to high-affinity receptors also undergo internalisation and this leads to down regulation of the surface receptors (Duprez *et al.*, 1988).

1.2.6. B cells and their activation markers

The majority of B cells carry MHC class II antigens which function in antigen presentation to Th cells. CD19, CD20 and CD22 are the main markers currently used to identify human B cells. Apart from these a few others like

the complement receptors for C3b and C3d, which are associated with activation and possibly homing of the cells, and the CD5 marker, which is expressed on both T cells and a subset of B cells, is believed to be involved with T-B cell interaction (van de Velde *et al.*, 1991; Hayakawa & Hardy, 1988).

B cells are classically defined by the presence of endogenously produced immunoglobulins (Ig). Activation of a B cell involves interaction of the surface Ig with an antigen coupled with lymphokines generated by Th cells, which drives the cell through stages of growth and differentiation. Although in the initial stages of a primary humoral immune response the IgM molecule appears, this Ig is polyvalent and of comparatively low affinity. Following isotype switching and affinity maturation, the production of the IgG marks the onset of a mature secondary humoral response. B cells at this stage secrete antibodies of much higher affinity. The expression of surface IgG on CD19+ B cells can thus be utilised as an indicator of a mature humoral immune response.

1.2.7. Adhesion molecules

Implicit with the interaction of lymphocytes with antigen-presenting cells or target cells and the migratory nature of lymphocytes is the phenomenon of adhesion. In fact, multiple adhesive interactions are probably required both for homotypic and heterotypic interactions associated with the immune functions of lymphocytes. Although a number of adhesion molecules have now been described not all are well characterised and only a few have been given a CD designation. These adhesion markers were identified principally with the use of monoclonal antibodies to carbohydrate antigens on the

leukocyte surface and have been found to have lectin-like N-terminal domains with carbohydrate structures as their putative ligands (Reviewed by Springer, 1990). Now it appears that they are members of three different families of cell-adhesion molecules: the integrins, the immunoglobulin superfamily, and selectins.

Receptors involved with the interactions of circulating leucocytes with endothelial cells and secondary lymphoid tissues have been designated the 'selectins' or LECCAMS (Springer & Lasky, 1991), because of their lectin-like nature. The homing receptor selectin, also called LAM-1, LEC-CAM-1, or LECAM-1, is expressed on leukocytes and facilitates their binding to endothelium during lymphocyte recirculation through peripheral lymph nodes. The PADGEM, GMP-140 or CD62 molecule, a glycoprotein of platelets and endothelial cells, is expressed following stimulation by thrombogenic agents and allows these cell to bind neutrophils and monocytes at the site of tissue injury (Larsen *et al.*, 1989; Johnston *et al.*, 1989). While the ELAM-1 glycoprotein is synthesised by endothelial cells in response to inflammmatory agents and promotes adhesion of neutrophils, monocytes and a subpopulation of lymphocytes (Bevilacqua *et al.*, 1989a).

Apart from the CD8 and CD4 molecules, other accessory molecules involved with cell-cell adhesion during immunological recognition and interactions have now been identified (Reviewed by Dustin & Springer, 1991) Among these LFA-1 and ICAM-1 are well documented.

LFA-1 (lymphocyte function-associated antigen-1) is a member of an integrin subfamily and is found exclusively on leucocytes. On lymphocytes, LFA-1 functions as an accessory molecule in a number of lymphocyte adhesive interactions, which include T cell and natural killer cell-mediated lysis of target cells, antibody-dependent cytotoxicity and the binding of lymphocytes to

cultured endothelium (Springer *et al.*, 1987). LFA-1 is the counter-receptor of ICAM-1 and it is believed that LFA-1 works in concert with CD2, CD8, and the T cell receptor on cytotoxic cells to form a stable adhesive interaction with ICAM-1, LFA-3 and class I MHC plus antigen, respectively (Fig. 1.2.5).

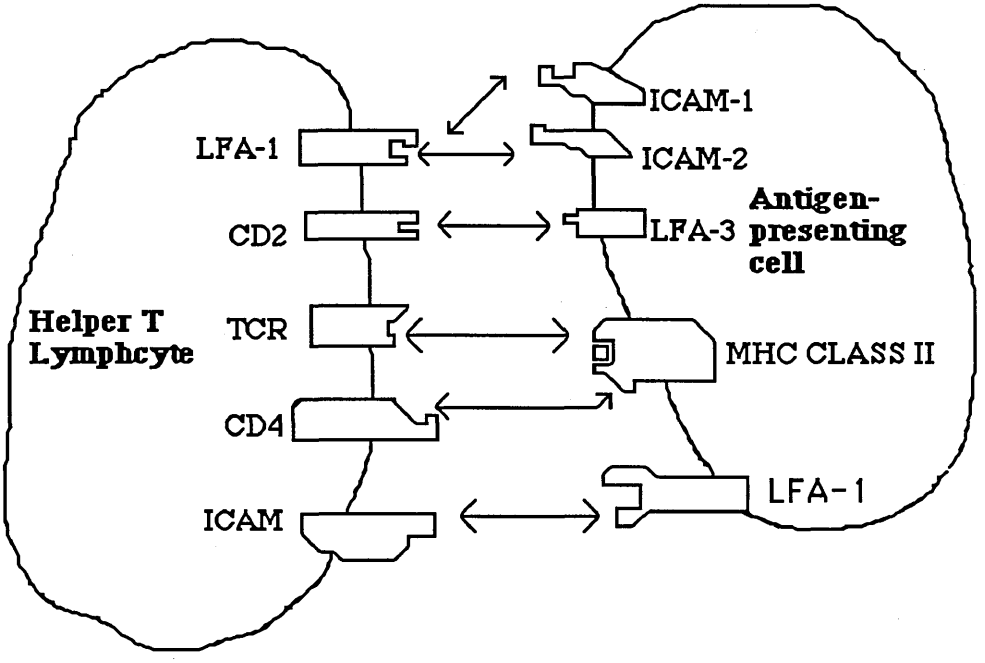


Fig. 1.2.5. Receptors in CD4+ T lymphocyte adhesion.

ICAM-1 (intercellular adhesion molecule-1), a ligand for LFA-1, (Marlin & Springer, 1987) along with LFA-3 and CD2 structurally resembles those of the immunoglobulin family. ICAM-1 is expressed on diverse cell types (Dustin *et al.*, 1988) and prominently represented on the HEV of various lymphoid organs (Dustin *et al.*, 1986). Lymphokines and monokines secreted during an immune response causes upregulation of ICAM-1 expression (Dustin & Springer, 1988).

The distribution of CD2, LFA-3, LFA-1, and ICAMs are such that CD2 and LFA-1 are specialised for use by leucocytes, while LFA-3 and ICAMs facilitate T cell interactions with any cell in the body. CD2 and LFA-3 undergo alteration by surface charge and receptor density and are involved in the regulation and modulation of T lymphocyte activation. Interaction of LFA-1 with ICAM-1 is also regulated in concert with cell activation such that LFA-1 avidity for ICAM-1 is increased by T cell activation. Although the mechanism of signalling by these molecules are not defined, these adhesion molecules play an important role in antigen recognition and lymphocyte activation.

1.2.8. Lymph node homing receptors

The ability of lymphocytes to recirculate continuously is dependent on their ability to leave the circulation and enter lymphoid organs via specialised endothelial cells, which comprise the postcapillary high endothelial venules (HEV). Binding of lymphocytes to peripheral lymph nodes and other lymphoid organs is highly specific and believed to utilise distinct recognition mechanisms (Butcher *et al.*, 1979). Evidence for such a mechanism was provided when a monoclonal antibody (MEL 14) recognising a cell surface molecule on murine lymphocytes with the capacity to "home" on the HEVs of lymph nodes was described by Gallatin *et al.* (1983). The antibody was shown to block *in vitro* lymphocyte binding to HEVs in peripheral lymph nodes but not in Peyer's patches and inhibition of *in vivo* homing to peripheral lymph nodes suggest that the antigen involved is likely to be organ specific. The antigen appears to be a mammalian lectin and probably interacts with unique carbohydrate determinant on peripheral node HEVs (Sanders *et al.*, 1988). Recently, the human leucocyte antigen, Leu-8 has been identified as

the human equivalent of Mel-14 (Camerini *et al.*, 1989). The marker appears to be rapidly down-regulated upon cell activation (Kishimoto *et al.*, 1990) and has been reported to be expressed by a proportion of human CD4+ cells with memory function ($CD45RA^{-}CD29^{hi}$) (Tedder *et al.*, 1990). The marker, variously termed LAM-1, Leu-8, Mel-14, thus remains rather elusive and laboratories working on this antigen have all now agreed that it be called LECAM-1 and classed as an adhesion molecule (Anderson *et al.*, 1991). It is likely that other accessory molecules act in concert to bring about the adhesion required for migration and homing of lymphocytes to specific lymphoid organs or sites of inflammation.

1.2.9. The immune response in breast cancer patients

In the case of breast carcinoma, the existence of an effective host response is considered to be shown by the presence of certain morphologically recognisable phenomena. One of these is the infiltration of the tumour mass by mononuclear cells (Section 1.2.7.1) and another is in the reaction of the axillary lymph nodes (Section 1.2.7.2). Histopathological examination showing micro-architectural and histologically observed lymph node responses have been found to be a favourable prognostic sign (Hamlin, 1968; Silverberg *et al.*, 1970; Hunter *et al.*, 1975; Riegrova & Jansa, 1982). Researchers have therefore, focussed on the analysis of lymphocytes, their phenotype and their functional competence, in both the tumour infiltrate and the axillary lymph nodes.

1.2.9.1. Tumour infiltrating lymphocytes (TILs)

Since the early observation of Sistrunk & MacCarty (1922) that breast cancer patients with a tumour infiltrate have an improved survival, a considerable number of studies have been carried to assess this response. The most recent of these has shown that about 60% of breast tumours have a detectable lymphocytic infiltrate and in some the infiltration may be as substantial as comprising 80% of the tumour cell suspension (Whitford *et al.*, 1990). Whether patients with a lymphocytic infiltrate in their tumours have a better prognosis is still a matter of debate. Studies have both confirmed (Bloom *et al.*, 1970; Black *et al.*, 1975; Ridolphi *et al.*, 1977; Underwood, 1974) and refuted the survival advantage (Roses *et al.*, 1982; Champion *et al.*, 1972) for breast cancer patients with TILs. The presence of tumour infiltrate was found not to be as strong a prognostic indicator as tumour grade (Bloom & Richardson, 1957). However, immunohistochemical and flow cytometric analyses have revealed interesting data on the phenotype and the activation states of the TILs.

Early studies employing rosetting technique revealed that T cells comprise the major component of the infiltrate in breast cancer patients (Eremin *et al.*, 1981; Eremin *et al.*, 1982). Later studies using Mabs and employing both histochemistry and flow cytometry have also shown that in TILs, T cells predominate and there are fewer B cells (Bilik *et al.*, 1989; Whitford *et al.*, 1990). However, analyses of subsets of T cells reveal conflicting results. In some a predominance of CD4+ helper T cells was reported (von Kleist *et al.*, 1987; Underwood *et al.*, 1987; Ben Ezra & Sheibani, 1987; Balch *et al.*, 1990) while in others, a higher proportion of CD8+ T cells was found (Bilik *et al.*, 1989; Belldegrun *et al.*, 1989; Whitford *et al.*, 1990). Reports involving other

human solid tumours, however, favour CD8+ T cells as the predominant cell component (Itoh *et al.*, 1986; Rosenberg *et al.*, 1988; Heo *et al.*, 1987; Belledegrun *et al.*, 1988; Durie *et al.*, 1990).

There have been fewer studies of the activation markers on the lymphocytes in patients with breast carcinoma. Although Whiteside *et al.* (1986) found very few HLA DR bearing cells, other studies (Rowe & Beverly, 1984; Lwin *et al.*, 1985; Ben-Ezra & Sheibani, 1987) have shown a fairly large proportions of TILs bearing this marker in breast cancer patients. This variability in reports is also seen with the expression of IL2-R (Tac), with most studies revealing few cells with this receptor and only Lwin *et al.* (1985) reporting Tac expression on an appreciable percentage (30%) of TILs. The most extensive study on TILs from breast cancer patients was carried out by Whitford *et al.* (1990). It was found that a large number of T cells express HLA DR, the marker was present on more CD8+ than CD4+ cells, and that these proportions were higher than in the peripheral blood. The Tac antigen was consistently present on greater numbers of CD4+ than CD8+ cells. The most interesting finding in this study was the strong correlation between tumour grade and HLA DR expression on the T cells. Furthermore, the presence of the activation marker correlated with both MHC class I and class II expression on the tumour cells (Whitford *et al.*, 1992a). It was suggested that, in the tumour infiltrate, the cytotoxic T cells are able to detect tumour antigen in the context of MHC antigens on poorly differentiated cells. Thus, in patients with a lymphocytic infiltrate and a poorly differentiated tumour, there is evidence of a cell-mediated immune response with a possible cytotoxic activity against the tumour. The study suggested that some form of tumour antigen is present on breast carcinoma cells, particularly in poorly differentiated tumours (Whitford, P., 1991), which is attracting immune recognition.

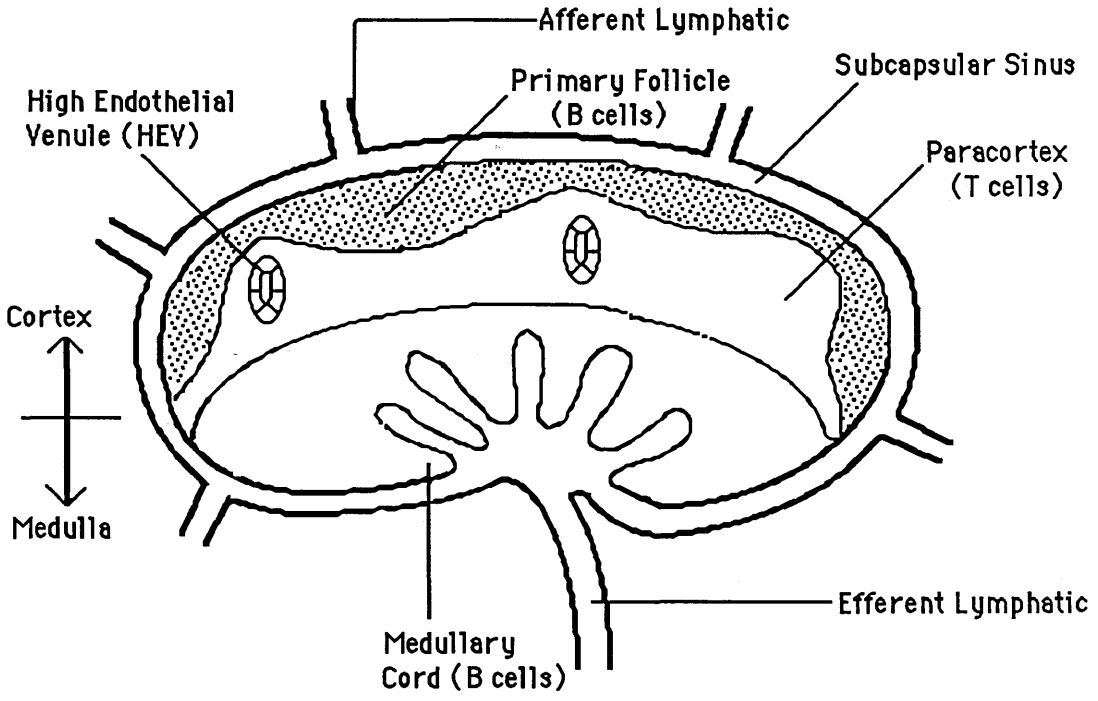


Fig. 1.2.6. Microanatomy of a lymph node.

1.2.9.2. The role of the axillary lymph nodes in breast cancer

The axillary lymph nodes draining the mammary gland occupy an important anatomical site. Lymphatics from the breast drain chiefly to nodes in the axilla, and in the female they are especially numerous and well developed (Section 1.1.3.2). As a secondary lymphoid tissue (Fig. 1.2.6), the tumour-draining lymph nodes are ideally situated to interact with tumour antigens and in modulating an immune response. Lymphocytes and antigens (if present) pass into the sinus via the afferent lymphatics from surrounding tissue spaces or adjacent nodes. The cortex contains aggregates of B cells (primary follicles) most of which have a site of active proliferation (germinal centres). The paracortex contains mainly T cells and are found in close apposition to the

antigen-presenting cells (interdigitating cells). Lymphocytes enter the node from the circulation through the specialised HEV (Section 1.2.8) in the paracortex and can re-enter the circulation through the efferent lymphatics.

In breast cancer, this lymphoid tissue is also susceptible to invasion by tumour cells as it drains the tumour tissue. If the tumour cells have the ability to metastasise, nodal invasion is the first sign of this capacity. Metastases in axillary lymph nodes are not life threatening in themselves. Rather their presence indicates the probability that carcinoma has spread beyond the local area and the greater the number of tumour positive nodes the greater that probability (Fisher *et al.*, 1983). The surgical treatment of breast cancer (Section 1.1.5.1) not only involves the excision of the primary lesion but often also removal of the axillary nodes in order to examine whether the disease has spread to the nodes. In this respect, nodal involvement still remains the best prognostic guide in breast cancer patients (Section 1.1.5.2; Bloom & Richardson, 1970). The role of the axillary lymph nodes in breast cancer has therefore, attracted much attention and investigators in this field have also been assessing the contribution of these nodes to the immune response in patients.

1.2.9.2.1. Phenotypic and activation marker studies

Early rosetting techniques comparing nodally derived lymphocytes with peripheral blood lymphocytes showed a greater B cell population in the nodes (Eremin *et al.* 1976; Heidenreich *et al.*, 1979). A more extensive phenotypic analysis was carried out using Mabs and flow cytometry by Morton *et al.* (1986), who reported an increased proportion of B cells and fewer CD4+ T cells in the lymph nodes with CD4+ T cells predominating in the Stage I patients while B cells predominated in the nodes of patients with stage II

disease. An increased B cell population, irrespective of metastatic involvement, was also observed by Mantovani *et al.* (1989) and Whitford *et al.* (1992b). The axillary lymph nodes were found not only to be the major source of B cells in breast cancer patients but also the site of a large proportion of mature B cells with surface IgG (Whitford *et al.*, 1992b) suggesting a secondary humoral immune response against the tumour. Moreover, the proportion of HLA DR-expressing T cells in the nodes were higher than those in the peripheral blood (Morton *et al.*, 1989; Whitford *et al.*,1992b). In the study of Whitford *et al.* (1992b) which covered 50 patients, CD4+ T cells expressing the CD25 IL 2-R were found to be in greater numbers than CD8+ cells and the expression of HLA DR on CD8+ cells related to the tumour stage. These studies provide evidence indicating that the axillary lymph node may have a role to play in mediating an immune response against breast cancer.

The limitation of the work of Whitford *et al.* (1992b) lies in having no indication of whether the nodes were invaded or not while the limitation of the work of Morton *et al.* (1986) lies not only in the unknown invasion status of the nodes but also the lack of normal controls and the comparatively crude development of flow cytometry at that time.

1.2.9.2.2. Cancer patients versus control subjects

Studies on the immune response in cancer patients often provide evidence to suggest that tumour antigen recognition may occur in some patients leading to the onset of a response. These studies have primarily determined the surface phenotypic and activation markers or observed major alterations in cancer patients with some changes being related to the progression of the disease. It is, however, of obvious importance to determine whether such

changes are due to the presence of cancer alone in these patients or whether similar alterations would also be observed in normal individuals. Both groups are outbred with different MHC antigens. Patients coming into the ward for surgery the next day may be mounting immune responses to a wide range of environmental antigens, for instance those encountered through contact with food or other individuals over the preceeding days. Likewise, "normal" controls come from different environments. In this context, TILs remain unique - the presence of lymphocytic infiltrate in the tumour is in itself a condition relating a response not to be seen other than in a diseased state. On the other hand, peripheral blood, although readily available from a healthy volunteer, may reflect too many of the central responses described above, and practical and ethical considerations limit the availability of truly normal locally responding nodes. This is reflected in the near absence of phenotyping studies on normal nodal lymphocytes in the literature. Although investigators go to the extent of making certain that the cancer patients selected for study are without any other ailment at the time of sampling, the only comparison of normal peripheral blood and lymph node lymphocytes with those of breast cancer patients comes from the study of Whitford *et al.* (1992b). Only a handful of other workers have previously done similar comparative studies, with Khuri *et al.* (1989) finding an increase in the CD8+ population in the nodes of breast cancer patients and, in contrast, Mantovani *et al.* (1989) finding no alteration in the phenotype of the nodal population. They, however, observed a slight increase in the number of HLA DR bearing cells in the PBL from breast cancer patients, and this is in agreement with the finding of Pattanapanyasat *et al.* (1988). In the study by Whitford *et al.* (1992b), lymph nodes taken from the iliac region of kidney transplant donors and patients undergoing vascular surgery served as control. The variations observed in terms of both the phenotypic and the activation markers in the cancer patients were much wider than the fairly narrow range observed in lymphocytes from both the peripheral

blood and the lymph nodes from the control patients. In the control nodes the CD4+/CD8+ ratio, which was lower than the average ratio in the cancer patients, fell within a fairly tight range, suggesting that the large variation observed in tumour patients reflected their disease. Much higher proportions of HLA DR- and IL-2- expressing T cells and of IgG-expressing B cells were observed in cancer patients when compared to the control subjects. In the absence of any other ailment in breast cancer patients, it could therefore be suggested that the alterations observed in the phenotype and the activation state of the lymphocytes from the axillary nodes is due to the presence of breast cancer cells and that this is a sign of host-mediated immunity against cancer. As high patient variability was observed, apparently in some breast cancer patients there may be an ongoing immune response against the cancer, while in others the response may not be generated or may even be impaired or suppressed.

1.2.10. Impairment of the immune response and metastasis

Apart from cases of advanced metastatic disease (Kansas *et al.*, 1985; Ludwig *et al.*,1985) there is little evidence of impairment of the immune system in the peripheral blood of breast cancer patients. The immunological competence of the draining lymph nodes, therefore, represents an interesting and important area of study for both those who are involved with the management of breast cancer patients and those interested in the assessment of the role of the lymphoid tissue. In this respect, an important issue is whether an impairment of the immune response occurs in patients with metastatic spread to the axillary nodes.

The very early observation, as assessed by histological examination of the lymph nodes draining mammary carcinoma has been the observed alteration observed of the micro-architecture (Hamlin, 1968; Tsakraklides *et al.*, 1974) of the nodes. As described in the more recent study of Eremin *et al.* (1980) and in agreement with some earlier studies (Black & Speer, 1958; Anatassiades & Pryce, 1966) alterations of lymphocyte subsets were described. Lymph nodes with a normal or increased T and/or B lymphocyte compartment were found to have a low incidence of nodal tumour spread (Eremin *et al.*, 1980). On the other hand, hypocellularity of the T- or B-cell dependent areas was associated with a significant increase in metastatic invasion (Eremin *et al.*, 1980). Using T cell rosetting techniques for cell surface marker study, Heidenreich *et al.* (1979) found no significant difference between tumour free and metastatic lymph nodes of breast cancer patients. However, Tsakraklides*et al.* (1975) had earlier found a significant difference between the two groups of nodes, with lymph nodes with disease metastatic to them having a higher percent of B cells and a lower percent of T cells than the nodes that did not have metastatic disease. In two more recent studies employing Mabs and flow cytometry, both the phenotypic and the activation markers were determined. In the study of Morton *et al.* (1986), regional lymph nodes from stage I patients, were found to contain a higher proportion of helper/inducer T cells which dropped significantly in stage II patients. Elevated levels of B cells, HLA DR-expressing activated Th and Tc cells and natural killer (NK) cells were found in stage II lymph nodes when compared to stage I. The second study on nodes regional to human melanoma (Farzad *et al.*, 1990) showed a significant decrease in CD4+ T cells with a corresponding increase in CD8+ cells in stage II nodes compared to those from stage I.

1.2.11. Overall view

It therefore appears that there are alterations both in terms of the phenotype and the activation state of the lymphocytes in tumour draining nodes. The progression of the disease and the growth of metastasis may have an effect on the immunocompetence of the lymphocytes in the nodes. But with very little work done on quantification of the lymphocytic population in the nodes and since the role of the node itself in breast cancer is still unclear, the precise nature of the impairment of the immune response is not yet elucidated. No studies have asked whether the presence of tumour cells within the nodes regional to breast cancer has any effect on the lymphocyte subpopulations. The only study to date (Farzad *et al.* ,1990), involved nodes regional to human melanoma from stage II patients and found that the presence of tumour within a node was associated with extreme variability in the number of CD3+ T cells, with some patients having very low values. This is the only report in which nodes invaded with tumour has been studied by flow cytometry. Although two studies involving breast cancer have been reported (Morton *et al.*, 1986; Whitford *et al.*, 1992b), these used nodes from stage I patients and uninvolved nodes from stage II patients, neither having direct contact with tumour.

There is a potential two way interaction in the node. One can examine the tumour cells to see if they are manifesting new features which may help them to escape immune detection. Conversely one can look at the lymphocytes themselves and ask how they have responded to invasion by epithelial cells, themselves foreign in a lymph node, which may or may not also bear foreign antigens. Assessment of the effect of metastasis on the immune function of the

regional nodes has obvious relevance to the surgical management of breast cancer patients.

1.3. Breast cancer-associated antigens: Alteration of surface carbohydrate structures

1.3.1. Breast cancer-associated antigens defined by monoclonal antibodies

During the early 1980s, there was much optimism about murine hybridoma-derived monoclonal antibodies (Mabs) in defining tumour-specific antigens. Several Mabs have been described that are reactive with human mammary carcinomas. These antibodies have generally been raised by two methods, using either breast tumour cell lines and extracts of breast carcinoma metastases or by using membranes from milk fat globule as immunogen. Antibodies with a preferential reaction with breast cancer tissue have a potential value in immunohistopathology as diagnostic tools and antigens that are secreted or shed may be useful in the development of serum assay and in the radio-immunodetection of metastases. It, however, became evident that although these Mabs are reactive against breast tumour cells, most of them also react with normal breast tissue, other normal tissues and other tumours (Colcher *et al.*, 1981). A particular problem with the use of antibodies to detect tumour-associated antigens is that the methodology of Mab generation relies on the immune response of animals as it is unethical to hyperimmunise a human. Any component foreign to the animal will elicit a response and as such, a highly dominant antigen will tend to mask other more tumour-specific antigen which are more likely to be of value.

An alternative was the immortalisation of human B cell response to a tumour by developing human Mabs using lymphocytes from blood or lymph nodes (Section 1.2.2.1). But this attempt was even more disappointing as the antibodies were polyspecific and of little use in diagnosis or therapy (Campbell

et al., 1987). The murine Mabs, although not being tumour-specific, have been shown, at least in some cases, to have preferential reaction with breast tissues (Reviewed by Tjandra & McKenzie, 1988). This has led to the identification of a few breast cancer-associated antigens and a general awareness of the alteration in the carbohydrate structures on the cell surface.

1.3.1.1. Mucin molecules and anti-HMFG antibodies

The dominant cell in breast cancers expresses both the epithelial keratins and the mucin molecules expressed by the lumenal or secretory epithelial cells in the normal gland (Taylor-Papadimitriou, 1987). Mucins are characterised as large glycoproteins, containing 50-60% carbohydrate in which the sugar side chains are O-linked to serines and threonines through the sugar N-acetyl-galactosamine. In an attempt to generate immunological reagents for the identification of differentiation markers specific for breast epithelial tumour cells, a large number of antibodies have been developed which react with the mucins expressed by normal and malignant mammary epithelial cells. While some of these antibodies demonstrate a relative specificity for malignant breast tumour cells, other Mabs do not differentiate between normal and cancerous tissues. The related characteristics of these antibodies indicate that the antibodies recognise an antigen of normal epithelial cellular origin, which appear to represent a family of mucin molecules. The mucin molecules are very immunogenic in mice and the antibodies generated in these studies have put into focus the mucin molecules as a tumour-associated breast antigen. Many of these antibodies were raised against the human milk fat globule (HMFG) membranes, which are surface membranes that surround milk triacylglycerol droplets secreted from mammary epithelial cells (reviewed by Patton & Keenon, 1975). Antibodies raised against the HMFG mucin also

react with components expressed by normal and malignant breast epithelial cells. Conversely, antibodies raised against carcinoma cells can react with the mucin produced by the lactating mammary gland. Although the exact nature of the epitopes recognised by these antibodies have not been determined, the components appear to be O-linked glycoproteins of Mr > 400,000 (Tjandra & McKenzie, 1988). One such mucin component found in the membrane of HMFG and also in human milk has been variously termed PAS-O (Shimizu & Yamauchi, 1982), EMA (Ormerod *et al.*, 1983), NPG (Ceriani *et al.*, 1983) and MAM 6 (Hilkens *et al.*, 1984). Two most widely used Mabs in this class are HMFG-1 and HMFG-2, raised by immunising mice with the HMFG membrane and cultured human milk cells (Taylor-Papadimitriou *et al.*, 1981). HMFG-1, which is highly reactive with the milk mucin, appears to recognise an epitope on the fully processed mucin, a characteristic of normal differentiation (Wilkinson *et al.*, 1984). In tumours, the size of the component carrying the antigenic determinant differs among tumours and with HMFG-2, the components can differ over a range from 80,000 to 400,000 (Burchell *et al.*, 1983). Griffiths *et al.* (1987) generated second-generation antibodies using the purified HMFG-2 reactive components and only reported the immunological complexity of the large components. Two of their antibodies showed cross-reaction with a smaller glycoprotein of Mr 68,000, which was found in breast cancer cell extracts. Using HMFG-1 affinity chromatography followed by treatment with hydrogen fluoride to remove all O-linked sugars, Burchell *et al.* (1987) isolated the mucin core protein of a similar size (Mr 68,000) which carried epitopes for HMFG-1 and HMFG-2. Although similar in size, the deglycosylated milk core protein is however, a different molecule from the glycoprotein detected by the second generation antibody (Griffiths *et al.*, 1987).

Mab (SM-3) was directed against the mucin core polypeptide and found to react with 47 of 50 (91%) breast cancers studied, while not staining normal tissues and HMFG-2- positive benign lesions. An unexpected finding was that both HMFG-1 and HMFG-2 which react with the milk mucin also showed a positive reaction with the extensively carbohydrate stripped mucin molecule. This suggests that the reaction of HMFG antibodies with the mucin molecule includes not only the carbohydrate portion but a part of the core protein component as well. Moreover, the SM-3 epitope appears to be masked by oligosaccharide moieties in normal cells but gets exposed, perhaps due to aberrant glycosylation, in malignant cells.

A second Mab with a relative specificity for breast cancer tissue is H23. This antibody was generated by immunisation with purified human MMTV-the retroviral-like particles released from a breast cancer cell line (T47D) (Keydar *et al.*, 1989). It is reactive with a glycoprotein of Mr 68,000 and it is not known whether this glycoprotein is coded by viral genes. In a survey of 532 breast cancer tissues, the H23-reactive antigen was detectable in 91% of the tested paraffin-embedded tissue sections and in all of 29 metastatic tissues (Keydar *et al.*, 1989).

cDNA clones coding for the core protein of the milk mucin now termed Polymorphic Epithelial Mucin (PEM) and recognised by SM-3 have recently been isolated and the amino acid sequence fully determined (Gendler *et al.*, 1988 and 1990). From such sequence data, individual-specific genetic polymorphism in the core protein PEM have been reported and the protein found to contain varying numbers of a tandem repeat element comprising of 20 amino acids. The various Mabs (DF3, HMFG-1, HMFG-2, SM-3, and H23) all recognise an epitope found within this tandem repeat but differ regarding their immunoreactivities with breast tissue (Siddiqui *et al.*, 1988; Gendler *et*

al., 1988). While HMFG-1 and HMFG-2 do not distinguish between normal and malignant breast tumours, the SM-3 and H23 Mabs are relatively tumour-specific. Recently, Wreschner *et al.* (1990) isolated and characterised cDNAs which code for the mucin protein core. Complete sequence analysis in this study reveal the complexity of the epithelial antigen with the existence of multiple forms of the antigen. It is of interest that the mucin gene is located on chromosome 1 (Taylor-Papadimitriou, 1987) in a region where alterations in breast cancer have been reported in high frequency (Section 1.4.1; Section 1.4.2.3.4).

Many more related Mabs have been generated, like the Mam 6 antibodies raised against HMFG (Hilkens *et al.*, 1984) and the NCRC antibody (Ellis *et al.*, 1984), which although raised against a breast carcinoma metastasis detects high MW glycoproteins similar to those recognised by the anti-HMFG antibodies. The exact nature of the epitopes these Mabs recognise still remains unidentified and as such the epitope which is exposed in breast cancers is yet to be characterised. These studies, however, show that glycoproteins are altered in breast cancer and some of these alterations are tumour-associated. The reactivity of these Mabs also highlights a particular common problem related to the generation of murine Mabs and used to detect tumour-specific antigens. It is clear that the mucin molecules are highly immunogenic in mice and as such it is not surprising that all these Mabs, although raised against different immunogens, react with the same high MW glycoprotein. No matter what the immunogen, be it breast tumour tissue or established cell lines, the antibodies generated turned out to be directed against the same class of molecules- the mucins. It is likely that the mouse response against the highly immunogenic mucin molecule takes precedence over other more specific tumour antigens. This perhaps explains the poor specificity of

some of the antibodies. The likelihood of these Mabs being used to diagnose or in the therapy of breast cancer, therefore, appear not to be too promising.

1.3.1.2. Tumour-associated glycoprotein 72 (TAG-72)

The TAG-72 antigen is defined by Mabs B72.3 and CC49 (Colcher *et al.*, 1981; Thor *et al.*, 1986; Muraro *et al.*, 1988). Mab B72.3 was generated using membrane-enriched extracts of a breast tumour metastasis to the liver as immunogen (Colcher, *et al.*, 1981). The antigen reactive with this antibody was shown to be a high molecular weight glycoprotein complex of approximately 220,000 to 400,000 and is termed tumour-associated glycoprotein 72 (TAG-72). Studies employing radioimmunoassay and immunohistochemistry have revealed that B72.3 reacts with approximately 50 % of breast carcinomas (Nuti *et al.*, 1982) and 85% of colon carcinomas (Stragmignoni *et al.*, 1983) tested but with no reactivity with normal human tissues. Analysis of TAG-72 carried out by Johnson *et al.* (1986) suggests that it is a mucin-like molecule.

1.3.1.3. Other breast cancer-associated glycoproteins identified by monoclonal antibodies

A number of other antibodies have also been generated using human breast carcinoma tissues or cellular fraction from breast cancer cell lines as immunogen. All of them react with breast cancer-associated glycoproteins, which remain uncharacterised. These Mabs appear not to react with the normal breast tissue although, cross reacting with a limited number of normal tissues.

One such Mab is 83D4, which was generated by immunisation with cell suspensions from a paraffin block of human breast carcinoma tissue (Pancino *et al.*, 1990). The antibody showed reactivity with breast cancer tissue in paraffin and frozen sections and with other carcinomas including ovarian and colonic carcinomas. It showed no reactivity with normal breast epithelium or HMFG membranes or skimmed milk, although reacting with a limited number of other normal tissues which include colon, stomach and endometrium. Recently, Pancino *et al.* (1991) reported the purification and characterisation of a 83D4 reactive antigen, which was expressed in breast cancer but not in normal breast. The antigen appears to be a high MW glycoprotein (apparent Mr: 300-400 to over 1000 x 10^3) and the epitope recognised by the antibody involves carbohydrate but not sialic acid. In an ELISA assay the purified antigen was found not to display reactivity for HMFG-1 antibody, but bound two other Mabs, CC49 and B72.3, which define a tumour associated glycoprotein TAG-72. Competition radioimmunoassays distinguished the 83D4 defined epitope from those recognised by the other two Mabs and it thus appears that these antibodies identifies antigens which are part of the same family of carcinoma associated glycoproteins. A few other B series antibodies have been developed and they are all directed to uncharacterised high MW glycoproteins (Schlom *et al.*, 1984).

1.3.2. Tumour-associated antigens as markers of prognosis in breast cancer patients

1.3.2.1. Serum tumour marker and prognosis

Markers in the sera of cancer patients are much sought after due to the ease of collection of blood and more importantly in the continuous monitoring

of the progress of the disease, which also has the potential for the early detection of metastases or recurrences. Most of the earlier serum markers were the traditional 'broad spectrum' tumour markers, like the oncofetal glycoproteins carcinoembryogenic antigen (CEA) and Tissue polypeptide antigen (TPA), both with very little usefulness. In later years, increasing numbers of carcinoma-associated (CA) markers were identified. Of these CA 15.3 has been relatively more widely assessed as a breast tumour marker.

CA 15.3 has been found to be more specific and appears to be more sensitive than CEA (Gion *et al.*, 1991). The antigen is defined by two different antibodies, DF3, which was raised against a membrane fraction of breast carcinoma cell line (Hilkens *et al.*, 1984), and 115 D8, raised against antigens of HMFG membranes (Kufe *et al.*, 1984). Although Gion *et al.* (1991) lately reported a significant relationships between CA 15.3 preoperative serum level and tumour size and number of positive nodes, in previously published papers serum CA15.3 levels in breast cancer patients were studied anecdotically and results are conflicting (Pons-Anicet *et al.*, 1987; Schmidt-Rhode *et al.*, 1987; Safi *et al.*, 1987). There are reports of preliminary studies involving other CA markers detected in the sera of breast cancer patients. CA 549 has been reported to be of promise in advanced disease (Bray *et al.*, 1987) and another marker termed MCA (mucin-like carcinoma-associated antigen) (Bombardieri *et al.*, 1989) has shown high sensitivity in preliminary trials being of value in assessing metastatic spread (Miserez *et al.*, 1991). While another breast CA antigen called mammary serum antigen (MSA) have been found to be more sensitive than the markers, CA 15.3 and CEA (Stacker *et al.*, 1988; Sacks *et al.*, 1987).

1.3.2.2. Anti-HMFG and related Mabs and prognosis

HMFG and related antibodies have been used more widely in assessing prognosis in breast cancer patients. But these studies have also produced conflicting results. Wilkinson *et al.* (1984) concluded that the HMFG-1 antibody could be useful as a prognostic indicator, while Berry *et al.* (1985) could find no relationship between the extent of staining with HMFG-1 and HMFG-2 and survival. Ellis *et al.* (1985, 1987) using the antibody NCRC 11 reported a clear relationship between immunoreactivity of carcinomas and the clinical course of the disease. But Angus *et al.* (1986) found no significant relationship with prognosis and NCRC 11 staining. In the more recent study of Walker (1990), the three above antibodies and four of the Mam 6 series were applied to 115 stage I and II paraffin-embedded breast tissues to assess their value as prognostic indicator. In a follow-up of 36 months, only NCRC 11 staining showed a relationship to development of recurrent disease and overall survival, but this was not an independent prognostic indicator above that provided by histological grade. It was, therefore, concluded that for short-term prognosis, none of these markers can give independent prognostic information.

Although some of these Mabs generated against surface antigens associated with breast cancer appear to be promising in clinical applications (Reviewed by Tjandra & McKenzie, 1988), they appear to be of little use in the management of cancer patients. None of these Mabs detect tumour-specific antigens and they are all directed against ill-defined epitopes. The antibodies however, reveal that alteration in the oligosaccharide moieties of surface glycoproteins occur. Increased reactivity of some of these antibodies with breast carcinomas suggest that such alteration lead to unmasking of certain

epitopes. Very little information is available concerning the exact nature of these epitopes. There is a need for the characterisation of these glycoproteins especially since the Mab-defined surface antigens appear to be high MW glycoproteins. A number of studies have examined the nature of carbohydrates associated with surface glycoproteins on tumour cells.

1.3.3. Expression and alteration of blood group antigens in human cancer

The earliest chemical evidence indicating the occurence of aberrant glycosylation in human cancer was the changes in blood groups (Reviewed by Hakomori, 1989). Changes in blood group antigens in terms of incompatible expression of A or B determinants, or the reduction of A or B determinants were observed. Related to the change of blood group antigens in human cancer, a large quantity of several fucose-containing glycolipids was found to be accumulated in various types of human adenocarcinomas, some of which were subsequently identified as the Lewis (Le) antigens (Hakomori *et al.*, 1967). The backbone structure (Galβ1-3GlcNAcβ1-3Gal) of these Le antigens represents the major carrier for the blood group ABH determinants. Le and Le related antigens represent the lacto-series antigens whose epitopes are defined by a large number of Mabs directed against various human tumours and are expressed in both glycolipids and glycoproteins. Some of these antigens like the Le^x antigen have been found to be associated with mucin-type glycoprotein or with embryogenic antigen, carcinoembryogenic antigen (CEA).

A second group represents the core structure of O-linked glycans and includes the T (Thomsen-Friedenreich), Tn and sialyl Tn antigens. These

antigens are the components of alpha sialoglycophorin (glycophorin A), which is an important constituent of the membrane of erythrocytes and is responsible for the antigenic determinants of the MN blood group system (Springer & Desai, 1985). Sialoglycoprotein bears two cryptic antigenic determinants, T and Tn, and removal of sialic acid of sialoglycoprotein uncovers the T antigen (Galβ1-3GalNAcα1- O-Ser/Thr) and this can possibly occur due to the action of bacterial neuraminidase following bacterial infection (Roxby *et al.*, 1987). Moreover, anti-T and anti-Tn antibodies have been found in all individuals and are believed to be due to a response against intestinal organisms carrying related antigens. Removal of the penultimate galactose residue reveals the Tn antigen, first discovered by Moreau and co-workers on untreated polyagglutinable RBCs in a patient with haemolytic anaemia (Springer, 1989). The expression of Tn corresponds to exposure of N-acetyl-D-galactosamine residues O-glycosidically linked to serine or threonine.

Tn expression has been reported in a few cases of malignant or premalignant haemopoietic disorders. A somatic mutation at the gene locus of two enzymes, β-1-3-D-galactosyl transferase and α-2-6-N-acetylneuraminyl transferase have been implicated with Tn expression (Springer & Desai, 1985). Mabs have been used to detect both T and Tn antigens in human cancers, including breast cancer. Using human anti-Tn antibodies, Springer *et al.* (1985, 1989) found immunoreactive Tn epitopes in greater than 90% of 69 primary breast carcinomas but absent in benign breast tumours. High density of Tn epitopes on breast, lung and urinary bladder cancers have been related to poor prognosis (Springer, 1984; Coon *et al.*, 1985; Springer *et al.*, 1985; Nishiyama *et al.*, 1987). With GalNAc as its terminal residue, the Tn antigen can be detected by the reactivity of the lectin *Helix pomatia* and other Gal NAc lectins (Section 1.3.5). This led Springer (1989) to propose that Tn might be

the biological marker associated with metastatic potential in breast cancer patients (Section 1.3.5.2).

Kurosaka *et al.* (1987) and Kjeldsen *et al.* (1988) established Mabs that were reactive with various types of human cancer. The epitope was identified as sialyl Tn (NeuAcα2 - 6GalNAcα1 - R) and found to be highly expressed in gastric, colonic, and pancreatic cancer, but less in liver and breast cancer. In contrast to Tn and sialyl Tn antigens, although the T antigen is often expressed in cases of cancer the nature of tumour-associated T antigen is still unknown. Longenecker *et al.* (1987) reported that Mabs directed against the β-disaccharide (Galβ1 - 3GalNAc) exhibits cancer-associated activity but not those reactive with the α- anomeric configuration. Another Mab against Gal-A (Galβ1 - 3GalNAcα1 - 3Gal) sequence did not react with any tumour tissue (Clausen *et al.*, 1987).

1.3.4. Glycosylation in tumour cells

The proportion of carbohydrates covalently linked to the plasma membrane of eukaryotic cells ranges from 2 and 10% of plasma membrane weight (Smets & Van Beek, 1984). Most of this carbohydrate makes up components of glycoproteins, in which the carbohydrate is linked to proteins, and glycolipids, in which the carbohydrate is linked to ceramide. The membrane glycoproteins, which contain at least 80% of all surface-located carbohydrate, act as transport systems, signal-transducing agents, antigens and receptors. As antigens and receptors, the glycoproteins play an important role in tumorigenesis. Numerous studies now reveal that the carbohydrate structures are altered in human malignant tumours.

1.3.4.1. Structure of glycoproteins

Glycoproteins are proteins to which carbohydrates are covalently bonded through glycosidic bonds. The linkage is either N-glycosidic via the amide nitrogen of an asparagine residue or O-glycosidic via the hydroxyl of serine, threonine, hydroxylysine or hydroxyproline. For the sake of convenience, glycoproteins are often differentiated from proteoglycans (eg. cartilage proteoglycan), which are a subclass of glycoproteins with distinctive features of carbohydrate structure and found in animal connective tissues (Beeley, 1985). A common structural characteristic of proteoglycans is that they contain linear polysaccharide chains which have a repeating unit containing an amino sugar derivative, which are often uronic acid or sulphate substituents. These carbohydrate chains are known as glycosaminoglycans. Glycoproteins and proteoglycans make up an overwhelming majority of extracellular proteins, which include the secreted glycoproteins, the mucins, serum, structural and the membrane glycoproteins.

1.3.4.2. O-linked glycoproteins

In comparison to N-linked glycoproteins, relatively little is known about the carbohydrates of carcinoma associated mucins or the sequence of events involved in glycosylation of the core protein. Events during addition of O-linked carbohydrates to proteins are not well established. The only common structural element in O-linked sugar chains is the GalNAc attached to the hydroxyl group of threonine or serine , which is also the first step in the biosynthesis of O-linked oligosaccharides. There is evidence for initiation of O-linked oligosaccharide synthesis occurring early (Strous, 1979; Cummings *et al.*, 1983; Jokinen *et al.*, 1985), late (Hanover *et al.*, 1982; Roth, 1984) and

continuously (Spielman *et al.*, 1987) in the biosynthetic pathways of different proteins. Following initiation, chains may be elongated, usually proceeding first with the addition of galactose to give Galβ1 - 3GalNAc.

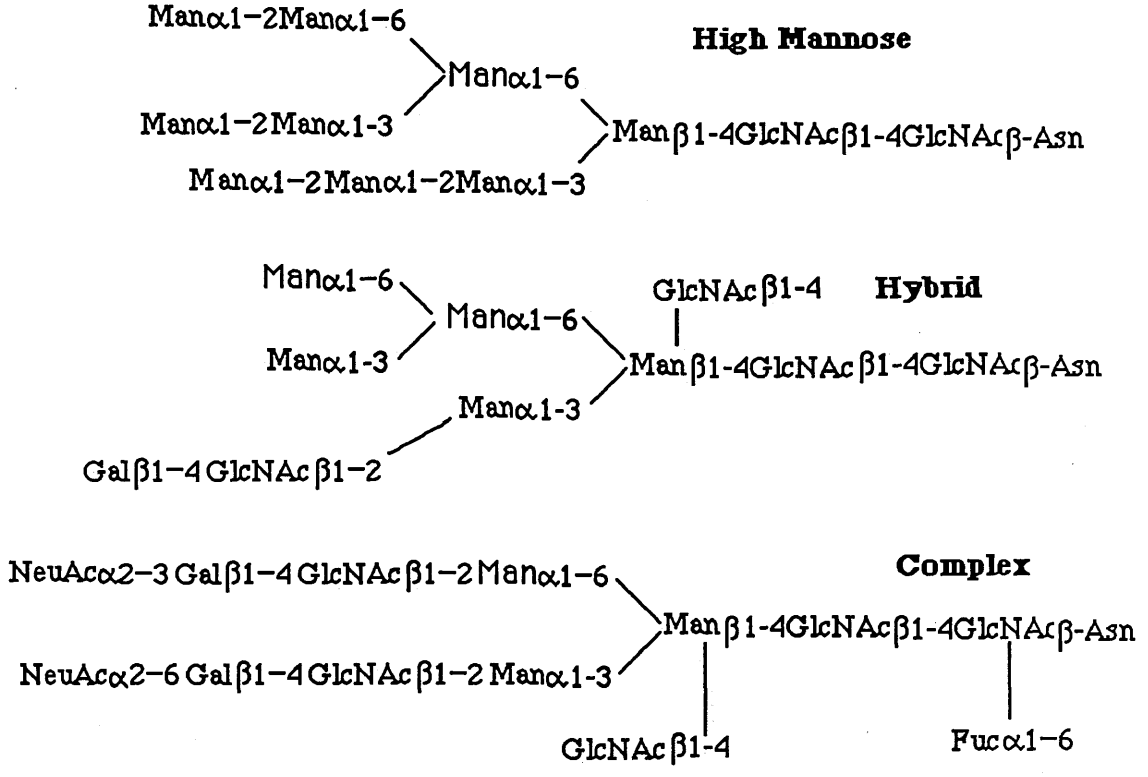


Figure 1.3.1. Structures of the major types of N-linked sugar chains of glycoproteins.

1.3.4.3. N-linked glycoproteins

Many secretory and membrane proteins are glycoproteins carrying N-linked oligosaccharides. Studies of these oligosaccharide sequences have revealed three fundamental types of structures: high mannose, hybrid and complex (Fig. 1.3.1) (Kornfeld & Kornfeld, 1985). They all share the common core structure Man α1-3(Man α1-6)Man β1-4GlcNAc β1-4GlcNAc-Asn, but differ in their outer branches. It is particularly the substitutions of

sialic acids, fucose and galactose at the termini of these oligosaccharide sequences and the variable presence of fucose linked 1-6 at the core GlcNAc which appear to be important events in malignancy (Foster, 1990). The majority of complex-type oligosaccharides in human epithelia contain between two and four outer branches and although a similar branched structures also occur in malignant tissues, some unique types of branching have recently been reported.

1.3.4.4. Biosynthesis and processing of N-linked glycoproteins

Biosynthesis of N-linked glycoproteins of the complex type proceeds via the high-mannose intermediate. Prior to glycosylation, N-linked oligosaccharide moieties are initially built on an activated lipid-carrier, dolichol phosphate. Upon phosphorylation, the dolichol moiety sequentially acquires the various monosaccharides starting with the transfer of a single N-GlcNAc residue (Parodi & Leloir, 1979). Addition of another N-GlcNAc, 9 mannose and 3 glucose residue units follow sequentially (Li *et al.*, 1978) leading to the precursor structure $Glc_3Man_9GlcNAc_2$. The complete precursor-type oligosaccharide structure is then transferred to the nascent polypeptide chain, the acceptor site of which is a specific tripeptide sequence consisting of Asp-X-Thr/Ser, X being any amino acid except Asp/Pro (Marshall, 1972).

Processing of N-linked oligosaccharides involves a complex series of trimming reactions in which glucose and mannose residues are sequentially removed (Reviewed by Kornfeld & Kornfeld, 1985). The processing which takes place in the endoplasmic reticulum (ER) involves trimming glucosidases and mannosidases which remove 3 glucose residues and 1 mannose residue. After transport of the glycoproteins into the Golgi compartment, mannosidase I

removes 3 additional Man residues. The sequential actions of β1-2 GlcNac transferase I , mannosidase II and β1-2GlcNAc transferase II produce a biantennary structure. At this point, GlcNAc transferases IV and V can initiate β1-4 and β1-6 linked antennae respectively (Gleeson & Schachter, 1983). Finally terminal sugars such as N-GlcNAc, galactose, sialic acid, and fucose are added to the branched structure which results in the conversion of the high-mannose structure to a glycan of the complex type.

The biosynthetic and processing pathways afford potential sites at which malignancy-linked alterations may occur. There are now a number of accounts of alteration of the carbohydrate structure on the surface of malignant cells.

1.3.4.5. Alteration in Asn-linked oligosaccharide structures in cancer

Cellular transformation has been shown to be associated with modification in the composition, content and distribution of cell surface oligosaccharides of glycoproteins (Skutelsky *et al.*, 1988). As the protein-bound carbohydrates are synthesised during a complex interplay of enzymes in which carbohydrate residues are sequentially added and removed and which leads to the generation of intermediate structures of varying composition, it is possible that cancer-related surface changes may be generated along this route. There is now considerable evidence that malignant transformation is accompanied by changes in the oligosaccharide structures of glycoproteins. One of the more common alterations in transformed cells is the presence of larger N-linked oligosaccharides (Smets & Van Beek, 1984; Santer & Glick, 1979; Warren *et al.*, 1978). This alteration has been widely observed in rodent

tumour cells transformed by activated H-*ras*, v-*src* oncogenes (Santer *et al.*, 1984; Dennis *et al.*, 1989) and by DNA tumour viruses (Yamashita *et al.*, 1983; Pierce & Arango, ,1986). The increased size has been attributed to increased sialylation and more recently to increased -GlcNAcβ1-6Manα1-6Manβ- branching of complex-type oligosaccharides. The increased β1-6 branching is due to an increased GlcNAc-transferase V in malignant cells (Yamashita *et al.*, 1985). In addition, increased β1-6 branching of complex-type oligosaccharides in a transfected mouse mammary cell line has been shown to be related to invasiveness and metastatic potential (Dennis *et al.*, 1987). In addition, somatic mutations and drugs which inhibit N-linked processing and reduce sialylation or branching of complex-type oligosaccharides were found to severely reduce the metastatic potential of human melanoma and B16 melanoma cells (Dennis, 1986a; 1986b; Finne *et al.*, 1982; Ishikawa *et al.*, 1988; Humphries *et al.*, 1986). These studies reveal that the oligosaccharides are required for metastasis to occur and therefore, their increased presence or alterations in primary tumours may be diagnostic of metastatic disease. The plant lectin (Section 1.3.5) leukoagglutinin (L-PHA), which has high affinity for galactose and the β1-6 linked antenna in tri- and tetra-antennary structures, has proved useful for the detection of β1-6 branched complex-type oligosaccharides in malignant cells (Dennis *et al.*, 1987). Fernandes *et al.* (1991) performed histochemical analysis with L-PHA on paraffin sections of human breast and colon tissues. The study revealed that β1-6 branched N-linked oligosaccharides are consistently increased in carcinomas of human breast and colon and that the level of L-PHA staining correlates with tumour progression in the breast and with metastatic disease in the colon. Although ductal epithelial cells in normal and benign tissue showed weak to moderate staining, strong staining was also observed in lymphoid and endothelial cells.

1.3.5. The use of lectins in studying altered glycosylation in tumour cells

1.3.5.1. Lectins

Lectins are sugar-binding proteins and glycoproteins of non-immune origin. The agglutinating property of lectins were first reported by H. Stillmark when he observed that the Ricin extract agglutinated erythrocytes and hence the name 'haemagglutinins' and 'phytohaemagglutinins' (Sharon & Lis, 1989). Years later the lectin PHA (phytohaemagglutinin) was found to act as a mitogen for lymphocytes. In addition to their agglutinating and mitogenic property, the high affinity and specificity of the lectins for certain carbohydrate residues has led to their use as probes to give information about the location, abundance and function of

TABLE 1.3.1. Lectins and their specificity

Lectin	Sugar specificity
Arachis hypogea (Peanut)	D-Galactose
Concanavalin A, *Canavalia ensiformis* (Jack bean)	D-Mannose, D-Glucose
Dolichos biflorus (Horse gram)	N-Acetyl-D-Galactosamine
Helix pomatia (snail)	N-Acetyl-D-Galactosamine
Lotus tetragonolobus I, II & III (asparagus or winged pea)	L-Fucose
Triticum vulgaris (Wheat germ)	N-Acetyl-D-glucosamine
Ulex europeaus (UEA I, gorse)	L-Fucose

glycoconjugates at the cell surface. Lectins possess certain very useful properties; they are multivalent and their interactions with oligosaccharides are specific and reversible and as they are not internalised they make a good probe for detecting carbohydrate structures on the cell surface (Beeley, 1985). Quite a number of lectins with differing specificity have been isolated from a wide range of plant and animal sources (Table 1.3.1) and are available commercially in a highly purified form.

1.3.5.2. Lectin binding in primary breast cancers

The availability of fluorescently-labelled lectins with differing specificity from various sources has made possible in recent years the use of a number of lectins in the study of carbohydrate expression on tissues from breast cancer patients. These studies have employed histochemical analysis on primary paraffin tissue or frozen sections from breast cancer patients to detect differences in carbohydrate expression. A number of such studies have now shown that such differences between normal and malignant cell can be of prognostic significance. There is however, disagreement between investigators.

The most widely studied and one of the earliest lectin to be used, peanut agglutinin (PNA) lectin, was used on human breast carcinomas as the binding sites for the lectin in rat were found to be hormone-dependent. In erythrocytes, the lectin binds to a component of the MN blood group antigen known as the T antigen (Thomsen-Friedenreich antigen), which is exposed only after desialation by neuraminidase. Binding of the lectin was also found to be a constant feature of normal human breast tissue, once sections have been treated with neuraminidase (Walker, 1984a). In breast carcinomas, positive

staining for PNA was associated with tumour differentiation (Walker, 1985), although the study by Bocker *et al.* (1984) disagrees. No significant correlation was, however, observed with hormone receptor content (Stanley *et al.*, 1986, Walker *et al.*, 1985; Dansey *et al.*, 1988).

Binding to Concanavalin A (Con A), which has specificity for D-Glc or D-Man, has been implicated in the progression of the disease in breast cancer patients (Furmanski *et al.*, 1981). The study employed a haemadsorption assay, in which human erythrocytes treated with various concentrations of Con A were incubated with the test mammary epithelial cells isolated from cultures of primary breast cancers. Con A reactivity of the tumours was classified as high or low with the Con A half-maximum value defined as the concentration of Con A at which approximately 50% of the test cells adsorbed erythrocytes. With a follow-up of about 22 months, the study showed that patients with high-reactivity tumours were at a significantly greater risk of early recurrence than those with low-reactivity tumours. There was no correlation with any of the pathological parameters. However, in an immunohistochemical analysis on paraffin-embedded tissues Dansey *et al.* (1988) reported that Con A reactivity was negative in normal breast tissue, whereas positive Con A staining of primary tissues was related to stage and disease-free survival in patients followed for 41 months. The relationship with disease-free survival was not independent but related to the stage of the disease as found in a multivariate analysis.

Both wheat germ agglutinin (WGA) and *Ulex europeaus* (UEA I) binding have also been studied to detect changes in carbohydrate expression in malignant breast tissues. Walker (1984b) reported a significant correlation between decreased WGA reactivity and the presence of lymph node metastasis. However, other studies reported either no differences in WGA binding to

breast carcinomas with, or without, axillary lymph node metastasis (Khan & Baumal, 1985) or a lack of correlation between WGA binding and the clinical outcome of the patients (Dansey *et al.*, 1988). UEA I, with specificity for L-Fucose residues showed variable binding to normal breast epithelial cells as well as malignant breast epithelium (Walker, 1984c). Fenlon *et al.* (1987) found UEA I staining not to be related to any of the prognostic factors but significant relationships were shown with disease-free interval and length of survival. A greater cell staining proportion being related to earlier local recurrence and shorter survival.

The most extensive study regarding the *Helix pomatia* lectin (HPA), which has specificity for N-Acetyl-D-Galactosamine residues, has been carried out by Brooks and Leathem (1991). In their initial pilot study, a strong correlation was observed between HPA binding to primary tumour and the presence of axillary lymph node metastases (Leathem *et al.*, 1984; Leathem *et al.*, 1985). In a histochemical analysis of paraffin-embedded sections of primary tumours from 179 breast cancer patients and with a follow-up of 20 years, HPA binding was found to be related to long-term prognosis (Leathem & Brooks, 1987). The study indicated that HPA positive tumours are more likely to have metastasised locally and to distant sites, that is patients with HPA positive tumours have shorter disease-free and overall survival times. This has been confirmed in a follow-up study involving 373 primary breast cancers. It further showed a strong association between HPA binding and presence of lymph node metastases, but no association with tumour size, histological grade or S-phase fraction (Brooks & Leathem, 1991). Although in the initial report significant differences between premenopausal and postmenopausal patients were observed, no association was observed between HPA staining and patient age in the later report. The relationship between HPA binding and lymph node

stage, local recurrence and survival has also been reported by other workers (Fenlon *et al.*, 1987; Fukutomi *et al.*, 1989).

There are also a limited number of reports of studies involving other lectins like *Lotus tetragonolobus* and *Dolichos biflorus*. But the binding of these lectins to fixed breast tissues has been found to be unsuitable, probably due to denaturation of binding sites or due to the solubility of the glycosylated substances in acqueous fixatives (Walker, 1984a) and as such of little use in retrospective studies. This highlights a particular problem with histochemical studies and the use of paraffinised tissues. Most of the above studies were done retrospectively and as such were carried out on paraffin sections of formalin-fixed, paraffin-embedded tissues. Fixation of tissues has the potential of altering the binding site if not denaturing it. In assessing the effect of fixation on lectin binding, Walker (1984a) reported that not all lectins gave consistent results and for some pretreatment with enzymes was a prerequisite, while still others were unreliable with fixed tissues when compared to frozen tissues. For lectins like soy bean agglutinin and *Ulex europeaus*, trypsinization of formalin-fixed tissue was a prerequisite and additionally soy bean agglutinin and *Dolichos biflorus*, both specific for N-GalNAc, showed binding patterns which vary between specimens and often between themselves. Similar problems was also reported for the lectin *Lotus tetragonolobus*. Moreover, histochemical analysis involves the determination of the extent of lectin binding by a simple visual assessment, often involving identification of subtle differences in staining intensity classified as moderate and strong. Such differences are often apparent only in an overall impression when multiple areas of tissues are scanned. This problem was encountered by Dansey *et al.* (1988) in assessing differences in binding patterns of PNA and WGA to normal and neoplastic breast tissues. These studies, however, provide important clues that there exist alteration on the carbohydrate structures of

breast tumour cells and although these structures are still not completely understood, recent evidences suggest that carbohydrates may play an important role as adhesion molecules and as such in the metastatic spread of the disease (Section 1.1.6.6).

1.3.5.3. Lectin-binding in primary breast cancers and their metastases

That tumour cell surface glycoconjugates play a pivotal role in the specific homing of metastases has been shown with the metastatic spread of mouse and rat melanoma cells (Fidler, 1973; Nicolson & Custead, 1982). Moreover, differences in cell surface carbohydrates have been demonstrated with animal tumours selected for high and low metastatic sublines (Altevogt *et al.*, 1983; Irimura & Nicolson, 1984; Steck & Nicolson, 1983). Studies using lectins afford a useful method for studying alteration in carbohydrate structures associated with malignancy in breast cancer. All of these reports involve retrospective analysis of primary tumours. There is therefore, a need for a prospective study using preferably fresh tissues. In the case of the lectin HPA considerable evidence indicate that HPA recognises a glycoprotein that is associated with metastasis. It would be interesting to compare carbohydrate expression on primary tumours and their metastases in the same patient. Very few studies have analysed metastatic tissues for carbohydrate expression and only in a recent report Krogerus and Anderson (1990) analysed carbohydrate expression on both primary tumours and their metastases from the same 18 breast cancer patients. In a histochemical analysis of paraffinised tissues and using a panel of seven fluorochrome-labeled lectins, the study reported that although the basic staining pattern of axillary lymph node metastases was similar that of the primary tumour, there was several quantitative and

qualitative differences. Compared with lymph node metastases, primary tumours were found to display a pattern of lectin-binding specificities that was more diversified in terms of both the extent of staining and reactivity with different lectins. In a small number of cases, in which distant metastases were also analysed, the distant metastases were found to be more restricted and displayed a generally uniform reactivity when compared to both nodes and the primary tumour. The study concluded that selected subclones with glycosylation patterns different from most of the nonmetastatic cells in the primary tumour invade lymph nodes and that further selection occurs for distant metastases. More similar studies are needed to define the surface carbohydrate structure associated with metastatic potential in breast cancer. A useful design of study would be is to analyse by flow cytometry lectin binding to fresh tissues from both primary tumour and axillary lymph node metastasis from the same patient. Flow cytometry would be advantageous in various aspects (Chapter 4, Section 4.9) as histochemical analysis is plagued with various technical difficulties.

1.4. DNA analysis of tumour cells

It is believed that during the progression of tumours, cancerous cells accumulate increasing genetic alterations that are generated by random, somatic mutational events (Nowell, 1976; Nowell, 1986). During this period of genetic instability and under host selective pressures, which are both immune and non-immune in nature, the tumour may progress to a highly malignant phenotype (Cifone & Fidler, 1981; Nicolson, 1987). This has lead researchers to assess both chromosomal abnormality, in terms of karyotype analysis, DNA content, and overexpression of oncogenes or gene loss, and the proliferation of tumour cells, in terms of its cell cycle. DNA analyses have not only revealed that solid tumours frequently have high proliferation rate, but that they also frequently have gross chromosomal abnormalities (Section 1.4.3). Detection of such abnormalities is important as they can be related to prognosis in patients and, as such, make possible characterisation of tumours with higher malignant capability.

The analysis of DNA of a tumour can be performed at various levels - by cytogenetics, molecular biology, cytophotometry and flow cytometry. Molecular biological analysis generally involves the use of oncogene probes which allows the detection of abnormalities of defined chromosomal loci (Section 1.4.2.). Additionally multi-locus recombinant DNA probes can be used to detect restriction fragment length polymorphism (RFLP), which has led to the detection of gene loss (Section 1.4.2.3.). Cytogenetic analysis and more recently DNA flow cytometric analysis allow detection of DNA abnormality on a much larger scale. Cytogenetic analysis has been used to provide invaluable information in certain malignancies and DNA flow cytometry provides information on both the DNA content and the fraction of cells in the DNA synthesis phase (SPF) of a tumour. DNA abnormality

assessments may at times be hampered by technological constraints and while each of these techniques has some advantages over the other, the growing use of flow cytometry in breast cancer is quite apparent.

1.4.1. Cytogenetic (chromosomal) analysis in breast cancer

Initially, the clues that tumours can have detectable genetic alterations came from the analysis of tumour cell chromosomes. In a chromosomal or karyotypic analysis, single cell suspension from a tumour is exposed to a mitotic inhibitor (eg. colcemid), swelled in a hypotonic solution and fixed, and then the chromosomes are spread on glass slides. Alkylating fluorochromes (eg. quinacrine mustard) and histochemical stains (eg. Giemsa) are used to produce multiple characteristic bands which can be identified on the metaphase chromosomes (Caspersson *et al.*, 1970). Recent refinements have improved resolution and have allowed enhanced identification of chromosomal rearrangements in the cells (Verma & Dosik, 1982). Abnormalities detected by chromosomal analysis include those in chromosome 1 and 11 of breast carcinoma cell lines (Cruciger *et al.*, 1976; Satya-Prakash *et al.*, 1981). Thereafter, a number of studies on both direct preparations and from primary cultures of breast tumour cells confirmed the structural involvement of chromosome 1 (Kovacs, 1981; Zhang *et al.*, 1989; Bello & Rey, 1989. There are also reports of aberrations involving chromosomes 11 (Bello & Rey, 1989; Ferti-Passantonopoulou & Panani, 1991), 3, and 6 (Bello & Rey, 1989; Ferti-Passantonopoulou & Panani, 1987). Ferti-Passantonopoulou & Panani (1991) additionally reported the consistent involvement of band 11q23-24 and of band 11p15. Further involvement of the loss of chromosomes 8, 13, and 16 have also been reported (Rodgers *et al.*, 1984; Hill *et al.*, 1987). These cytogenetic studies were performed on a small number of cells from a small patient sample

and showed multiple complex rearrangements, which include both translocations and deletions. However, no consistent cytogenetically detectable chromosome abnormality has been shown to be associated with breast cancer.

Although it is possible to identify the nature of rearrangement in terms of translocation and deletion and the chromosomal arm responsible for the aberration in leukemia and retinoblastoma, the application of such an analysis on solid tumours like breast cancer poses difficult problems (Reviewed by Sandberg *et al.*, 1988). The major problem is the scarcity of analysable chromosome spreads obtained from breast tumours. In primary cell cultures of breast tumours, only a minority of cells is mitotic and only a few of these mitoses can be analysed (Rodgers *et al.*, 1984). This makes it necessary to rely on tumour cell culture, which may not be successful in all cases or may lead to overgrowth by normal diploid cells. Moreover, the dominant cells following prolonged culture may not necessarily represent the major clone in the tumour *in vivo* and generally cultured cells have extensive chromosomal anomalies. It is, therefore, not surprising that breast cancer has not yet been associated with any unique karyotypic change. In studies where it was possible to analyse cultured well-characterised primary breast tumour cells, karyotypic changes were surprisingly reported to be rare and the cells were predominantly diploid (Wolman *et al.*, 1985; Zhang *et al.*, 1989; Smith *et al.*, 1985). In contrast, effusion metastases were found to be aneuploid (Smith *et al.*, 1985). Although the study of Smith *et al.* (1985) showed that the diploid cells retained their malignant phenotype, these cell cultures clearly represented only a subpopulation of the original tumours. The requirement for culture of tumour cells prior to analysis and the generally experienced inability to propagate aneuploid cells severely limits the applicability of the technique and

this is especially so as about 63% of human breast tumours are aneuploid (Frierson *et al.*, 1991).

1.4.2. Oncogenes or tumour suppressor genes in breast cancer: A molecular biological analysis of tumour DNA

1.4.2.1 Background

Oncogenes are derived by alterations of normal genes, proto-oncogenes, such that they are activated (Reviewed by Spandidos & Anderson, 1989). Cellular proto-oncogenes code for proteins which are important in controlling growth and differentiation of cells but which become irreversibly and permanently expressed in tumour cells. 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. Most characterised oncogenes appear to encode proteins with diverse functions, e.g. some encode growth factors, growth factor receptors, signal transducing factors, or nuclear proteins controlling the gene expression and the cell cycle (Anderson & Spandidos, 1988). Their function at the molecular level is, however, unknown.

Tumour suppressor genes ("anti-oncogenes") share the property that their expression inhibits the cancer phenotype and whose inactivation or deletion is a step in tumour formation.

1.4.2.2. Oncogenes implicated in breast cancer

1.4.2.2.1. Methodology

Many oncogenes have been implicated in breast cancer, but only a small group have been regularly reported by a wide variety of labs. In most cases, these reports have been of gene amplification or overexpression. There are various methods of obtaining evidence. Molecular biological analysis involves extraction of DNA from a piece of tumour which may well contain normal cells (including infiltrating lymphocytes) and examine the Southern blotting profiles of oncogene probes. Pathological, *in situ* hybridisation, and immunocytochemical assays are conducted on a thin tissue slice which may be unrepresentative of the tumour but which preserves the original architecture of the tumour. Such methods can therefore be used to identify individual cells within the tumour and exclude any cells in the sample which are clearly normal.

Amplification/ overexpression may be further classified:

(i) That a proto-oncogene is amplified in the tumour as evidenced by Southern (DNA probes detecting genomic DNA) blotting of restriction fragments of tumour DNA.

(ii) That a proto-oncogene is rearranged or mutated in the tumour DNA.

(iii) That a tumour contains elevated levels of proto-oncogene messenger RNA as evidenced by Northern (DNA probes detecting mRNA) blotting, or cytochemical blotting (" in situ hybridisation") of mRNA populations.

(iv) That the oncogene product is inappropriately expressed in tumours to give excess protein.

Oncogenes which have clearly been associated with breast cancer by some of these methods are shown in Table 1.4.1.

Table 1.4.1. 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 depletion
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

1.4.2.2.2. c-*myc*

The oncogene *myc* was first identified as the transforming element of avian myelocytomatosis virus-29 (Bister *et al.*, 1977) and subsequently the homologous sequences in man 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 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 has been observed by three different 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 (Adams *et al.*, 1983; reviewed by Cole, 1991). In Burkitt's lymphoma, chromosomal translocation juxtapose the *myc* gene on chromosome 8 to one of the immunoglobulin loci, either on chromosome 14, or chromosome 2, or chromosome 22. Consequently, the constant feature displayed by all Burkitt's lymphomas is that the *myc* gene on the derivative chromosome is expressed constitutively whereas that on the normal chromosome 8 is transcriptionally silent. Finally, amplification of the oncogene may occur and this has been reported in several types of human tumour (reviewed by Alitalo & Schwab, 1986).

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 a 2 to 15-fold amplification 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. Correlation between c-*myc* amplification and poor short-term prognosis was reported by Varley *et al.* (1987a), who reported levels of amplification of 2-10 fold. Mariani-Constantini *et al.* (1988) studied the transcription of the c-*myc* proto-oncogene by RNA:RNA *in situ* hybridisation in the same 18 primary breast carcinomas which were previously examined by Escot *et al.* (1986) for gene amplification. They found high levels of hybridisation in four of seven

tumours with strong amplification of the gene. But two of nine cases which had an apparently normal c-*myc* locus also showed high levels of hybridisation.

There are few reports of alterations in c-*myc* gene 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).

1.4.2.2.3. H-*ras*

H-*ras* oncogene was first identified in transforming DNA from the human EJ bladder carcinoma 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). There is a family of *ras* oncogenes which also includes K-*ras* (the proto-oncogene corresponding to Kirsten sarcoma virus) and N-*ras* (identified in neuroblastomas). These 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 11p14 and encodes a protein of 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 (Barbacid, 1987).

Ras activation is commonly by point mutation leading to single amino acid differences from the normal cellular proto-oncogene sequence. In human tumours studied, amino acid substitutions have been found to occur at specific positions, viz., 12, 13 and 61. Substitution of the normal glycine residue at

position 12 with any other amino acid except proline causes oncogenic activation and in the EJ bladder carcinoma, where the glycine is altered to valine, such a change is sufficient to cause transforming activity (Seeburg *et al.*, 1984). A similar situation is observed for the glycine residue at position 13 although a few substitution like Ser 13, may not be transforming (Bos *et al.*, 1985). The result of the point mutation is an increase in the effective binding of GTP to the protein, thus maintaining it in a more highly activated state (Barbacid, 1987; Bos, 1989).

Apart from human cervical cancer, gene amplification is considered to be an infrequent mechanism of *ras* activation in most other tumours (Yokota *et al.*, 1986). Three groups have, however, reported the loss of one of the two c-H-*ras* alleles in about 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* allele loss being grade 3 (Theillet *et al.*, 1986; Ali *et al.*, 1987). 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. The significance of these results is not clear, moreover, these reports were based on early immunocytochemical studies where it is possible that the specificity of the antibodies was not as high as anticipated.

1.4.2.2.4. *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 (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 epithelial cells. EGFR binds transforming growth factor α (TGF-α) resulting in a growth stimulatory effect similar to that observed with EGF (Reviewed by Carpenter, 1987). The receptor has a cysteine-rich extracellular 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, which is normally quiescent, but acts as a tyrosine kinase when the extracellular domain makes contact with its ligand and this leads to a signalling cascade. The truncation in v-*erb* B is such that the extracellular (N terminal) domain of the protein is lost but the transmembrane and internal domains are present. The *erb* B protein, therefore, lacks the extracellular domain and is not sensitive to EGF and the intracellular domain is permanently activated. It has, however, 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).

This is a case where the oncogene encodes only part of a normal cellular gene, which is known to be expressed in both breast cancer and normal breast epithelial cells. It is, therefore, difficult to devise antibody probes to indicate abnormal expression. There is no evidence that this gene is amplified in breast cancer. There are, however, reports of amplification of the normal proto-oncogene (EGF receptor) (Liberman *et al.*, 1985). Amplification of the 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). Several groups (Harris *et al.*, 1989; Sainsbury *et al.*, 1987, Nicholson *et al.*, 1988) have also obtained data showing that the expression of EGF receptor is a relevant factor in breast cancer prognosis (Section 1.1.4.3).

1.4.2.2.5. 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). Two groups working independently, subsequently 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 reported 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 the protein has 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 it, the *erb B-2* gene is distinct from the EGFR gene (Schechter *et al.*, 1985). It is encoded on human chromosome 17q21, whereas the EGFR gene is located at chromosome 7p11-p13. 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 gene product. Like the 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, although a candidate ligand has recently been identified (Lupu *et al.*, 1990). A factor of Mr 30,000 (gp30) secreted by cells from a human breast cancer cell line (MDA-MB-231) was found to interact directly with both EGFR and the *erb* B-2 transmembrane protein In two cell lines that overexpress *erb* B-2, tyrosine phosphorylation was induced only by gp30 but

not by EGF. Although the role of gp30 in the growth and development of normal and malignant cells remains unresolved, it is of interest that the ligand specifically inhibited the growth of cells that overexpressed *erb* B-2.

erb B-2 can transform cells in two ways. Over-expression of the normal cellular proto-oncogene, c-*erb* B-2 is sufficient to transform NIH-3T3 cells and, unlike, the *erb* B (EGFR) gene, 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; van de Vijver *et al.*, 1988). The oncogene itself may carry a point mutation; valine at position 664 in the transmembrane region being replaced by glutamic acid. This leads to increased tyrosine kinase activity, probably through receptor dimerisation (Weiner *et al.*, 1989).

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 and protein amplification in human primary breast tumours. The reported gene amplification rates range from 10 to 46%, with an overall average of 20% (Clark & McGuire, 1991). Variability in these reports probably reflects differences in patient groups, the samples used for DNA analysis, paraffin-embedded as opposed to frozen or fresh, and the fact that the tumour samples from which DNA is prepared often contain 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; Borresen *et al.*, 1990), there are also reports of a significant percentage of tumours with a single copy of the gene that overexpress the protein product (Iglehart *et al.*, 1990; Hanna *et al.*, 1990; Tandon *et al.*, 1989). As such,

measurement of protein expression may be a better guide to whether *erb* B-2 is involved in breast cancer (Clark & McGuire, 1991).

Although the potential site of mutation lies in the transmembrane domain, a feature of this onco-protein expression in breast tumours is that generally the normal *erb B-2* protein is overexpressed. Immunohistochemical analysis or Western immunoblotting, therefore, involves a record of differences in the intensity of staining. In most of the studies, immunocytochemical analyses with polyclonal antibodies, specific for the *erb B-2* protein and not cross-reacting with EGFR, have been used and often groups of investigators have used the same anti-serum from the same source. Several groups 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 *erb B-2* protein antibodies, membrane or cytoplasmic staining has been found to correlate with *erb* B-2 amplification (Venter *et al.*, 1987; van de 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. Van de Vijver *et al.* (1988) found large cells of comedo type *in situ* carcinomas were associated with more intense *erb* B-2 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 (van de Vijver *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 widely investigated, reports regarding the correlations between *erb B-2* amplification and clinical outcome of breast cancer patients differ considerably. The patients groups included in these studies are variable and in some analyses are restricted to node positive and in others to node negative patients. Using a monoclonal antibody to stain *erb* B-2 protein, Van de 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 overexpression 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 & 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 and thus improved the total number of cases, *erb B-2* protein overexpression was found to be significantly associated with both

recurrence and survival and which 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.

1.4.2.2.6. *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 and 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 (Delli Bovi *et al.*, 1987; Taira *et al.*, 1987). *int*- 2 is a proto-oncogene originally identified as being adjacent to one of the integration sites of the proviral DNA of mouse Mammary Tumour virus (MMTV) (Nusse & Varmus, 1982). *Hst* and *int*- 2 in man, are both located on chromosome 11q13 (Yoshida *et al.*, 1988). Activation of both genes is by transcriptional deregulation giving overexpression 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 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 a small but reproducible changes in cell morphology and growth rate (Van de Vijver &

Nusse, 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.* (1988) reported amplification in only 9% (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) 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 overexpression. Co-amplification was also observed by Ali *et al.* (1989) and Theillet *et al.* (1989a; 1989b). Theillet *et al.* (1989a) 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 development of metastasis or local recurrence. In addition to amplification, Varley *et al.* (1988) have reported that the *int* -2 gene is altered in several amplified tumours with loss of an EcoR1 site. In most of the tumours with *int* - 2 amplification, adjacent genes including the oncogenes hst and bcl- 1 are co-amplified (Lidereau, *et al.*, 1988; Yoshida *et al.*, 1988). As *int* -2 and *hst* genes are not expressed in normal breast tissue, it is perhaps not surprising that they are not activated by amplification in human breast tumours.

1.4.2.2.7. Other oncogenes implicated in breast cancer

There are a small number of reports of other oncogenes being amplified or expressed in breast cancer. In some cases, the oncogenes are co-amplified with the more commonly reported oncogenes. One such gene, c-*erb* A, the gene for the thyroid hormone receptor, is on chromosome 17q21 and therefore often amplified with *erb* B-2 (Tavassoli *et al.*, 1989).

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

1.4.2.3.1. Introduction

In many human tumours, including breast cancer, there is now increasing evidence that a loss of specific chromosomal regions play an important role in tumour development. Such deletions usually involve only one of the two parental chromosomes present in normal cells. These allelic losses have been interpreted as evidence that the regions affected contain anti-oncogenes or tumour-suppressor genes. The gene products of the tumour-suppressor genes are believed to normally regulate growth and differentiation in a negative fashion and thus indirectly suppress neoplastic development (Knudson, 1985). In tumours, therefore, loss of a tumour-suppressor gene leads to a loss of the natural control of cellular growth. The most extensively characterised is the Rb (retinoblastoma) gene, encoded by human chromosome 13q14. The Rb product is a phosphorylated protein which binds to DNA, but its function is not known (DeCaprio *et al.*,1989). In recent years, there has been a general awareness of the role of p53 as a tumour-suppressor gene in solid tumours, including breast cancer.

1.4.2.3.2. Methodology

It is now well established that when malignant cells are fused with diploid fibroblast of the same species, random chromosome loss with the acquisition of malignancy may occur in hybrid cells and as such it has been possible to correlate the loss of particular chromosomes with malignancy (Stanbridge, 1986). Karyotypic analysis to detect gene loss would then be required to detect the involvement of a tumour-suppressor gene. Although chromosomal losses in tumours were first detected cytogenetically, more recently, probes that detect restriction fragment length polymorphisms (RFLP) have been used to determine whether one of the two parental alleles is lost specifically in tumour DNA. In RFLP analysis, tumour and peripheral blood DNA are compared to show allele loss in the tumour, which in turn may represent the involvement of a tumour suppressor gene. All such studies relate to Southern blotting of DNA samples extracted from the tumour and, as with the proto-oncogene studies these cannot take account of normal cells such as fibroblasts or lymphocytes within the sample. Thus, allele loss is generally defined to occur when the density of various bands is reduced, rather than abolished, in comparison to the same bands from peripheral blood lymphocyte DNA from the same patient. In the case of anti-oncogenes like p53 the protein product is also identified by immunocytochemistry.

1.4.2.3.3. The p53 tumour suppressor gene

The p53 gene is located on human chromosome 17p13 and encodes a protein of 393 amino acids. Like the Rb gene, it is a nuclear protein and was first discovered by immunoprecipitation with antisera from animals bearing

SV40 induced tumours (Linzer & Levine, 1979; Lane & Crawford, 1979). Initially p53 was regarded as a nuclear oncogene and expression of p53 was shown to immortalise primary cells (Jenkins *et al.*, 1984) while mutation of p53 would enhance its transforming activity (Jenkins *et al.*, 1985). Recent findings however, indicate that p53 may actually be a tumour suppressor gene. p53 sequences have been found to be lost or inactivated in a number of malignancies and that the presence of wild-type p53 could suppress transformation of cells by other oncogenes (Finlay *et al.*, 1989). The precise role of p53 is not clear but it has been suggested to interfere with the cell cycle (Reviewed by Levine *et al.*, 1991). Introduction of the wild-type gene or cDNA into a transformed cell in culture stops cell growth (Baker *et al.*, 1990) at the G1 phase of the cell cycle (Section 1.4.3.1), possibly by regulating transcription of genes that effect the passage from late G1 to S phase.

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 more than 75% of carcinomas show a loss of a large portion of chromosome 17p (Vogelstein *et al.*, 1988; Delattre *et al.*, 1989). Similar losses have also been reported for breast tumours (Mackay *et al.*, 1988; Devilee *et al.*, 1989). In approximately 65% of informative cases, a region (p13.3) on the short arm of chromosome 17, where p53 is located, has been reported to be lost in breast cancers (MacKay *et al.*, 1988; Devilee *et al.*, 1989). It has been suggested in a model for colorectal tumorigenesis (Fearon & Vogelstein, 1990), that the tumour may develop through loss of one allele and subsequently the other through point mutation, which gives rise to overexpression 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 p53 appear at "hot

spots" on the encoding gene, with residues such as 175, 248 and 273 being particularly significant (Levine *et al.*, 1991). 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 (Fearon & Vogelstein, 1990).

Study on p53 gene generally involves the examination of human tumours for loss of 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 possible if considerable quantity is present and, therefore, taken to indicate that the gene has been altered. In the case of breast cancer, loss of p53 allele, or overexpression 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 their samples being positive for both. They also found three patients positive for p53 expression but negative for loss of heterozygosity where mutations in the p53 gene could be mapped by PCR.

1.4.2.3.4. Other possible tumour suppressor genes in breast cancer

There is now evidence that additional tumour-suppressor genes may be involved in breast cancer. Loss of heterozygosity has been reported for several loci in breast cancer and these are tabulated below (Table 1.4.2.). In some of these cases the actual gene involved has not been identified. The region on chromosome 11 is involved in about 20% of the cases studied. The deletions often involve the c-Ha-*ras* gene and always involve the region between the β-globin and parathyroid hormone loci on the short arm of chromosome 11 (Ali *et al.*, 1987).

Table 1.4.2. Loss of heterozygosity (LOH) and oncogenes and tumour suppressor genes in breast cancer.

LOH (chromosomal location)	Possible oncogenes or tumour suppressor genes in that area	References
1q	Unspecified	Chen *et al.*, 1989
1p	Unspecified	Genuardi *et al.*, 1989
3p21	c-erb A	Ali *et al.*, 1989
11p	H ras	Ali *et al.*, 1987; Mackay *et al.*, 1988.
13q	Rb	Lundberg *et al.*, 1987; Lee *et al.*, 1988; Sato *et al.*, 1990.
16q	Unspecified	Sato *et al.*, 1990.
17p13	p53	Mackay *et al.*, 1988; Cropp *et al.*, 1990; Varley *et al.*, 1991.
18q	DCC (Deleted in Colon Cancer)	Cropp *et al.*, 1990; Devilee *et al.*, 1991

In the recent study of Sato *et al.* (1990), who used a panel of 39 RFLP markers to determine allele loss in 79 primary breast tumours, at least four tumour suppressor genes were identified on chromosomes 13q, 16q, and 17p (a suppressor gene in addition to p53 was implicated). Moreover, a group of tumours with loss of both 13q and 17p showed more malignant histopathological features, while another group with loss of 17p presented with frequent lymph node metastasis. This suggest that multiple allele losses are involved in breast cancer and it is possible that the cumulative effect of these losses contribute to a more malignant phenotype.

1.4.2.4. Genes controlling metastatic behaviour

Regulation of the metastatic process, which develops late in tumour progression, has been presumed to result from the activation or repression of a number of specific genes (Nicolson, 1988). It is therefore, of obvious importance to attempt to identify specific genes which are related to the metastatic process. Ideally, identification of alterations at various stages of tumour formation is the ultimate aim of such a study. This approach was applied successfully on colorectal tumours such that it was possible to construct a model for tumour development in which a number of genetic alterations were identified (Fearon & Vogelstein, 1990). It was however, found that the progressive accumulation of these changes, rather than the order suggested in the model, was most important in the development of colorectal tumour. It has been possible to construct a model for tumorigenesis in the case of colorectal tumour, because unlike the situation in most other common human tumours, colorectal tumours of various stages of development can be obtained for study. Genetic elements involved with metastatic spread can also

be identified by comparing primary and secondary growths. Such a study involving breast cancer was carried out by Iglehart *et al.* (1990). To determine whether alterations in *erb* B-2 is an early event, the investigators examined gene amplification and expression in both the *in situ* and invasive tumour cells, in different stages of the tumour and compared primary and lymph node metastases. *erb* B-2 alterations were found to be present in all clinical stages and in both the *in situ* as well as the infiltrating component. Moreover, the gene copy number in lymph node metastasis was identical or within 2-fold of the copy number in the primary tumour. Based on these findings the investigators concluded that *erb* B-2 alterations are selected for early and may be important in the initiation of certain mammary cancers. This lends support to the report of Borg *et al.* (1991) that *erb* B-2 activity is related to increased tumour growth but not directly to metastasising ability. Although other oncogenes like *ras* have been shown in transfection studies to transform a cell line into one with metastatic capability, such oncogenes are infrequently implicated in breast cancer. One other approach in detecting novel genes which may modulate invasion and metastasis is to utilise differential screening of cDNA libraries. The objective here is to detect cDNA clones corresponding to mRNAs which differ in abundance between related cell populations, for example between cell lines of metastatic and non-metastatic behaviour. Applying such an approach on related low and high metastatic potential murine K-1735 melanoma cell lines, Steeg *et al.* (1988) identified a novel gene, nm23, whose mRNA levels were 10-fold higher in low metastatic potential lines than in high metastatic potential lines. Subsequently, lack of nm23 expression in human breast cancers was found to be associated with lymph node metastases (Bevilacqua *et al.*, 1989b). An nm23 protein of Mr 17,000 has been identified in both murine and human cells. The biochemical functions of the protein are currently unknown but its homology (75%) to the Drosophila *awd* gene (abnormal wing discs) suggests that it may participate in normal development.

Steeg & Liotta (1990), using antibodies raised against peptides corresponding to the N-terminus of the protein, showed that the situation at the protein level mirrors that occuring at the mRNA level. Recently, Leone *et al.* (1991) transfected a murine nm23 expression construct into highly metastatic murine melanoma cells and demonstrated the suppressive effect of nm23 on several aspects of the cancer process, including tumour metastasis. This identifies nm23 as a metastasis suppressor gene, the first of its kind to be detected in breast cancer. With time it is likely that more of such genes will be identified.

1.4.2.5. The extent to which known oncogene products can elicit an immune response.

Although the genetic alterations associated with breast cancer are still a subject of further research, a few specific genetic elements and their products have been implicated with the disease (eg. p53, *erb* B-2). An interesting aspect of this feature would be to relate the existing known genetic alterations with both the malignant nature of a tumour and the immune response in breast cancer patients. With respect to immunity against cancer, a mutated onco-protein is likely to induce a response. Identification of such tumour-associated antigens against which there is a likelihood of a response is important in the design of therapeutic strategies.

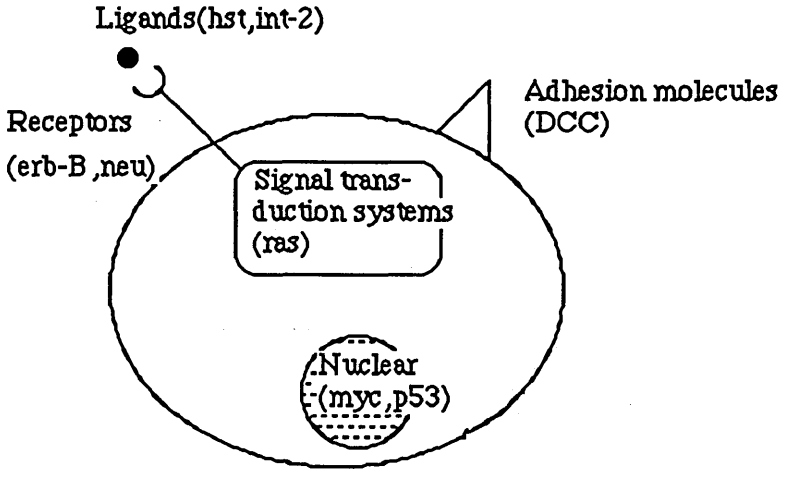


Figure 1.4.1. Oncogenes and tumour suppressor genes implicated in breast cancer.

1.4.2.5.1. Oncogene induced tumours and the immune response

Advances in the field of immunology has provided a better understanding of the nature of the immune response in human and molecular genetics has made it possible to identify specific genetic alterations in a tumour. It is now possible to rationalise the possible basis for immune response to tumours. The great majority of human tumours are of unknown origin. Any immune response may relate to the extent to which oncogene products as have been characterised are "foreign". Thus during tumour progression any change in a cellular protein, whether on the surface or inside a cell, makes it a potential target for immune recognition. Even if the coding sequence has been altered by even one amino acid (as occurs in H-*ras* and p53), there is ample classical evidence that the immune system has the capacity to detect it. In this context, it may be relevant to note that where analysis has been more detailed (Varley *et al.*, 1991), point mutations in coding regions are detected. Thus all oncogenes are potential targets for the immune response.

1.4.2.5.2. B or T cell responses

A B cell (antibody) response is only of value if the relevant antigen is expressed outside the cell or on the surface. Thus *hst* , *int*- 2, *neu* or DCC could potentially elicit such a response is they were sufficiently different from the normal gene product. The vast majority of oncogene products, including those known to carry mutations such as p53, are expressed within the cell (Figure 1.4.1.) and consequently can only elicit a T lymphocyte immune response. T lymphocytes can detect peptides derived from foreign proteins inside the cell if they are presented on the appropriate Class I (Townsend, 1989) or Class II MHC antigens (Grey *et al.*, 1989; Unanue *et al.*, 1989). No study to date has related the immune response to gene amplification, gene loss, or gene activation.

1.4.2.5.3. Variation between patients

Immune responses initiated by oncogenes or chromosome loss will also be affected by the MHC antigens borne by the tumour bearing patient. The human population is outbred and no two individuals have the same capacity to present antigenic peptides to T cells. Thus, the ability to mount a response against a particular antigen will also be determined by the ability of a patient's MHC molecules to present the antigen.

In consequence the nature of human tumours such as breast cancer together with the interpersonal variation of the immune response suggests that there will be wide patient variability. Homogeneity in tumour phenotype or immune response cannot necessarily be anticipated, in fact tumours are known to be heterogenous in their expression of antigens. An additional feature of a

tumour that is relevant to the generation of a response is that many tumours lose the expression of some or all of their MHC class I determinants (Section 1.1.6.5.1). Using monoclonal antibodies to detect class I antigens, several studies have shown the loss of class I expression in many human tumours which include colorectal cancer (Lopez-Nevot *et al.*, 1989; Momburg *et al.*, 1986; Momburg & Koch, 1989; Smith *et al.*, 1989), skin melanoma (Brocker *et al.*, 1984) and breast cancer (Perez *et al.*, 1986; Goepel *et al.*, 1991; Whitford *et al.*, 1992a). Additionally, Perez *et al.* (1986) and Whitford *et al.* (1992a) reported that both MHC class I and class II are variably expressed. Tumour cells lacking MHC class I determinants are protected from cytotoxic T-cell attack with respect to any determinant (Bodmer, 1987). An interesting study design would be to relate all these parameters with specific genetic changes in a tumour and to determine how such changes affect the immune response in a breast cancer patient.

1.4.3. Analysis of cell cycle and DNA content (Ploidy) in breast cancer.

As described in Section 1.4.1, karyotype analyses in breast cancer are limited by technical factors. Many groups have, therefore, focussed on other means of analysing the abnormalities in the DNA and the growth rate of a tumour. Various methods have now been developed to investigate the two distinct but related parameters- **cell cycle analysis**, in which the proliferation rate is determined and **ploidy analysis**, which determines the DNA content of a tumour. Most of these techniques determine either of the two parameters, while DNA flow cytometry allows the determination of both parameters.

1.4.3.1. Analysis of the cell cycle

1.4.3.1.1. Phases of the cell cycle

In any growing cell population, there is a sequence of events which ultimately leads to cell duplication. Two identifiable events that can be used to characterise the cell cycle are the mitotic cell division and DNA synthesis. As shown in Fig 1.4.2., the period of mitosis is represented by M and the period of DNA synthesis is S. Go cells are in the resting phase, while the phases G1 and G2 are gaps between the phases M and S, and S and M, respectively. G1 and G2 have been termed gaps due to the absence, until recently, of knowledge of any markers in these areas (Adams, 1990).

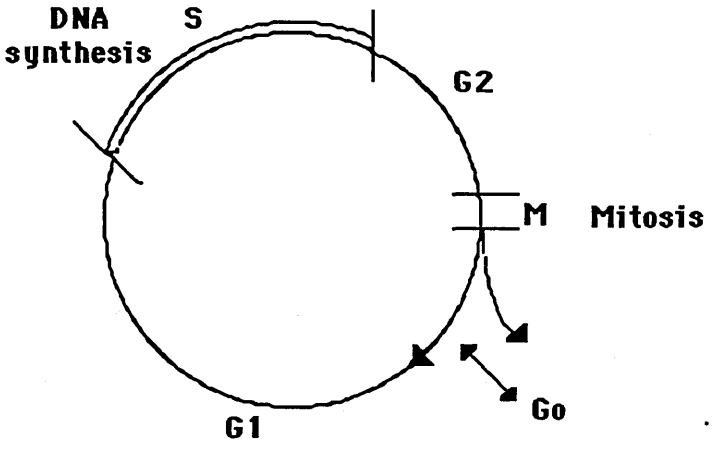


Figure 1.4.2. The phases in the cell cycle.

1.4.3.1.2. Control of the cell cycle

The period of time cells spend in various phases of the cell cycle is variable and depends on the type of tissue and culture conditions for cells in culture. For some cell populations, such as lymphocytes in the human peripheral blood or cells in sub-optimal culture conditions, the majority of cells are generally in the quiescent phase (Go) and are said to be 'out of cycle'. These cells require a stimulus (antigenic or mitogenic stimulation in the case of lymphocytes) to initiate their re-entry into the cycle. Generally a single restricting point in G1 regulates their entry (Pardee, 1974) and the cells then moves through the different phases of the cycle (Fig 1.4.1), and following mitosis enter a second round of the cycle. Data from various sources including those for breast cancer cells (McGuire & Dressler, 1985) suggest that the duration of G1 phase can be highly variable. Entry of cells into the G1 phase initiates the cell cycle, and is followed by DNA synthesis during S phase. The cells then proceed towards M phase. In the case of at least some tumours, unlike the quiescent lymphocytes, a commonly observed feature is

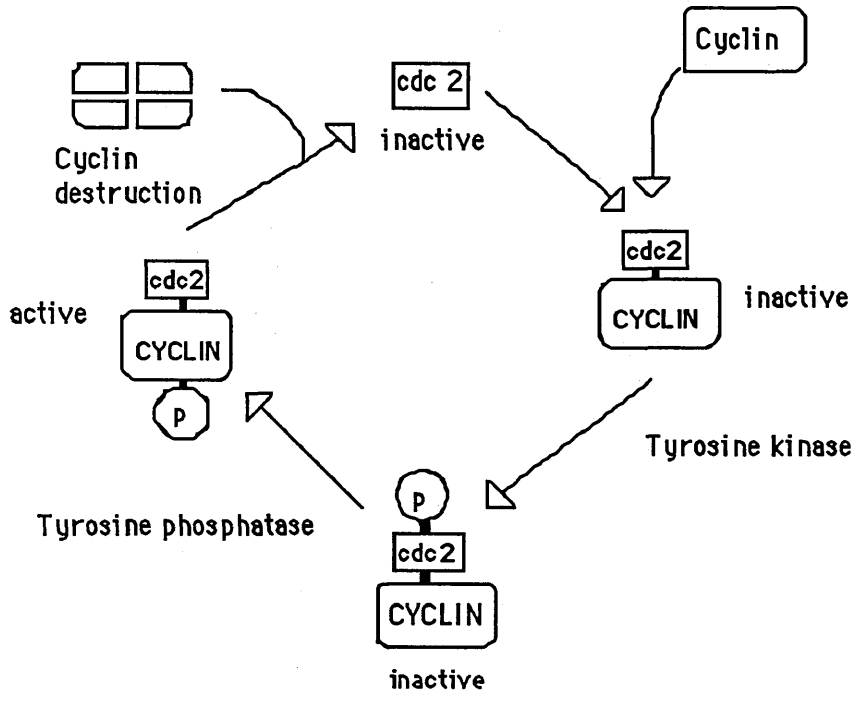


Figure 1.4.3. Cycle of the cdc2 subunit (P= protein phosphorylation).

the high proportion of cells in the proliferating phases of S and G2/M. Moreover, the cell cycle profile, i.e. the distribution of cells in the different phases, of breast cancer cells is not only different from the normal counterpart but also dramatically different from one patient to another.

Although, a loss of control of the cell cycle in cancer is evident, very little is known concerning the regulation of the cell cycle. The ordered sequence of events in the cell cycle suggest that the two crucial points of control lies at the transition between G2 and M phases and at G1, a point at which cells are commited to division. Recently, a universal mechanism common to all eukaryotic cells controlling the onset of M-phase (reviewed by Nurse, 1990) has been proposed. Central to this mechanism is the protein kinase p34 (Mr

34,000), which was first identified as the product of the *cdc* 2/ *cdc* 28 gene in yeast mutants and is believed to function both at the onset of S-phase (Mendenhall *et al.*, 1987) and at mitosis (Nurse, 1990). The $p34^{cdc2}$ kinase phosphorylates key proteins which leads to the events in M-phase. A second class of protein, the cyclins (Moreno *et al.*, 1989), forms a complex with and activates the $p34^{cdc2}$ kinase, whose high activity maintains the cell in the M-phase. The binding of the cyclin initiates covalent modification of $p34^{cdc2}$ (Fig. 1.4.3.). The phosphorylation of $p34^{cdc2}$ on tyrosine 15 keeps its kinase activity turned off, and activation of $p34^{cdc2}$ is associated with dephosphorylation at tyrosine 15 (Hunt, T. 1991). The cyclin joining is required for activation both to get the cdc2 into the correct phosphorylation state and to provide a companion subunit for the kinase. The process by which $p34^{cdc2}$ kinase is inactivated is not understood but a possible way of exit from mitosis could occur via cyclin degradation, which would disrupt the p34 cyclin complex and thereby inactivate the kinase. The timing of mitosis, as in the case of fission yeast, is determined by a regulatory gene network which includes two further putative protein kinases (*nim* 1, *wee* 1), and the *cdc* 25 gene, which encodes a phosphoprotein (Mr 80,000) (Moreno *et al.*, 1990) believed to be involved with the cell cycle timing of p34 kinase activation and thus of the mitotic onset.

The initiation of S phase also involves the p34 kinase and the G1 cyclins, which appear in G1 and are degraded as cells enter S phase. The budding yeast G1 cyclins, first discovered by their essential role at the decision point at the G1 to S transition known as Start (Reviewed by Nasmyth, 1990), represent a class of regulatory proteins that are made and involved in the G1 phase of the cell cycle. It appears that there is a positive feedback loop in which the G1 cyclins activate transcription of their genes, CLN1 and CLN2 subset, by activating, in concert with *cdc* 28, the genes SW14 and SW16,

which are required for Start (North, 1991). Recently, a candidate for a human G1 cyclin has been described. Motokura and co-workers (1991) have succeeded in isolating a cDNA of a putative oncogene, PRAD1, which appears to encode a polypeptide that is a member of the cell-cycle regulating cyclin family. Interestingly, PRAD1 has been found to be overexpressed in a subset of parathyroid tumours, in which a chromosomal rearrangement fused the PRAD1 gene to the parathyroid hormone gene. PRAD1 has also been implicated in non-parathyroid tumours, which includes breast and squamous cell carcinomas (Lammie, *et al.*, 1991).

Deregulated expression of other cyclins may also be involved in abnormal cell proliferation. Moreover, potential substrates of p34/*cdc*2 kinase include p60/c-*src*, the large T antigen of simian virus 40, and the tumour suppressor gene product p53 (Hunter, 1991). It is likely that mutations in cell cycle regulators cooperate with other mutations that activate proto-oncogenes or inactivate growth suppressor genes (Hunter & Pines, 1991) and together contribute toward the multistep process of tumourigenesis.

1.4.3.1.3. Cell cycle analysis of breast tumours

Although the regulatory mechanism of the cell cycle is gradually being unravelled, cell cycle analyses of breast tumours have been extensively studied. Several methods have been devised to quantify the distribution of cells in the various phases of the cell cycle (reviewed by Visscher *et al.*, 1990a; Quinn & Wright, 1990) and such measurements have been related to the biological behaviour of the cell. Early investigators, using crude estimates of gross doubling times based on radiographic observations or chest wall recurrences (Pearlman, 1976), observed large variations in growth rates among

individual tumours. More recent methods have greatly improved the assessment. Among the techniques that allow the assessment of only the kinetic measurements are the Thymidine Labelling Index, Bromodeoxyuridine and Ki-67.

i) Thymidine Labelling Index (TLI)

TLI, which is a measure of the percentage of tumour cells in the DNA synthetic S phase, involves incubation of unfixed viable, thin slices of tumour tissue with radioactive-labeled thymidine. The thymidine is incorporated into cells synthesising DNA and these cells are quantitated by counting radiolabeled nuclei in autoradiographs. This technique was first applied to breast cancer by Johnson & Bond (1961) who demonstrated increased TLI in fibroadenoma. Higher TLI values have been shown to be associated with larger and locally advanced primary tumours (Meyer *et al.*, 1986). However, no significant correlation between TLI and regional status was observed (Visscher *et al.*, 1990a). Two studies also compared primary tumours and corresponding metastasis (Meyer *et al.*, 1986; Tubiana *et al.*, 1981), but observed no significant differences in their TLI values. In these same studies and that of Silvestrini *et al.* (1985), histological grade was significantly correlated with TLI values, which were found to be higher in premenopausal and younger patients. With respect to outcome of the disease following mastectomy, both the study of Meyer *et al* (1986) and of Tubiana *et al* (1981) demonstrated a significant association between high TLI and early relapse. Moreover, the prognostic significance of TLI has been shown to be independent of stage (Meyer *et al.*, 1986). From the practical point of view, the use of TLI has several limitations as an indicator of tumour proliferative activity. It is a laborious and a technically difficult method. It requires careful preparation of the slices which have to be thin enough to allow maximum thymidine

permeation. This is then followed by counting of hundreds of nuclei and the fact that the uptake of thymidine is affected by incubation conditions makes comparability between observers difficult. Moreover, slices prepared from tumours may not be true representative of the larger mass as most tumours are known to be heterogenous.

ii) Bromodeoxyuridine

Being a pyrimidine analogue, bromodeoxyuridine (BrdU) is readily incorporated into cells synthesising DNA and enhances cell sensitivity to X-rays. It can thus, be used to measure S phase labelling index and has also been used widely in radiotherapy regimes for human malignant tumours. More recently, use has been made of monoclonal antibodies to BrdU (Gratzner, 1982; Morstyn *et al.*, 1983) and the proliferation of cancer cells assessed by immunocytochemical techniques or by flow cytometry. Due to instant immunofluorometric detection and being non-isotopic, the method has advantages over TLI. Moreover, by infusing BudR prior to sampling, the method can also be used for *in vivo* kinetic studies (Wilson *et al.*, 1988). Extensive assessment of cell proliferation as judged by BrdU has not been performed. Although studies on gastric carcinoma shows association between labelling index and stage of the tumours (Kikuyama *et al.* , 1988), there is very little data concerning breast cancer. Recently, Remvikos *et al.* (1991) used flow cytometry to assess simultaneously both S-phase fraction and BrdU incorporation on fine needle aspirates samples from breast cancer patients. The study found good correlation between BrdU labeling index and SPFs measured on samples taken from the same patient.

iii) Ki-67

Among the Mabs to be used to recognise antigens associated with cell proliferation, Ki-67 has been increasingly used in histopathology. The mouse antibody Ki-67 produced by Gerdes *et al* . (1983), reacts with a nuclear antigen expressed on all human proliferating cells. This antigen, recently shown to be a component of the nuclear matrix (Verheijen *et al.*, 1988; Verheijen *et al.*, 1989) is expressed preferentially during late G1, S, G2 and M phases of the cell cycle. Cells in the Go phase consistently lack the antigen (Gerdes *et al.*, 1983; Gerdes *et al.*, 1984) and thus, the Ki-67 labelling index is an indicator of the growth fraction in human neoplasms. The growth fraction is determined using immunohistological methods on frozen tissue sections or by flow cytometry on cell suspension. However, the use of the antibody in breast cancer has not been very encouraging. In the study of van Dierendonck *et al.* (1989) on the breast cancer cell line MCF-7, it was shown that compared to growth fraction estimation by BUdR, Ki-67 fractions were invariably higher. In addition, the study pointed out that Ki-67 fractions may not always be a reliable indicator of growth fractions, as under sub-optimal conditions non-proliferating cells may retain the antigen for a considerable period of time. Lelle *et al.* (1987) found increased Ki-67 positive cells in node-positive breast and Isola *et al.* (1990) revealed Ki-67 staining to be significantly higher in DNA aneuploid tumours. However, in the study of Lelle *et al.* (1987), the positivity was found to decrease with increasing numbers of positive nodes. Other workers (Barnard *et al.*, 1987; Bouzebar *et al.*, 1989) only observed a poor correlation between Ki-67 staining and other pathological parameters. Walker & Camplejohn (1988) compared Ki-67 reactivity of breast carcinomas with histological grade and found that although the presence of nuclear staining

was associated with a poorer grade, the extent of staining did not correlate with grade.

1.4.3.2. Measurement of DNA content

The terminology of 'ploidy' or the DNA content was defined in cytogenetics, with normal human somatic cells with 46 chromosomes referred to as diploid (Tijo & Levan, 1956), while a cell with fewer or more than 46 chromosomes is described as aneuploid, and a cell with a balanced gain or loss of chromosomes or with structural rearrangement but with 46 chromosomes, is pseudodiploid (Friedlander *et al.*, 1984). The term aneuploidy is reserved for chromosomal studies, and DNA aneuploidy is used for flow cytometric data.

Although the Feulgen-Schiff technique has been used in earlier studies, currently the preferred technique for the quantitative measurements of cellular DNA involves the use of fluorescent dyes which bind directly to DNA. The procedure relies on the stoichiometric binding of the dyes to DNA such that the degree of stain is directly proportional to the amount of DNA present. The DNA content of individual cells is then assessed either by static cytometry or, by flow cytometry.

1.4.3.2.1. Measurement of DNA content by static cytometry

In static cytometry, stained cells are visualised microscopically and the amount of DNA bound dye is determined directly by absorption or fluorimetric methods. Histological sections of formalin fixed tissue or fixed smear of sections from tumours can be analysed using this procedure. Lymphocytes and normal epithelial cells present in the tissue section which complicate flow

cytometric analysis, can be easily visualised and are used as a normal diploid control. Studies that have utilised this technique in assessing the DNA content in breast cancer patients showed a strong relationship between DNA content and recurrence-free survival (Fallenius *et al.*, 1988; Auer *et al.*, 1980a) and to tumour size and ER level (von Rosen *et al.*, 1989). However, data acquisition by static cytometry is relatively slow and measurement of DNA content is usually restricted to several hundred cells in any one sample. Moreover, the resolution of the technique is poor compared to those obtained from flow cytometric analysis and the technique has lost favour to the latter method.

1.4.3.3. Flow cytometric DNA analysis

In contrast to the above described techniques, flow cytometric (FCM) DNA analysis provides dual information. The technique gives information on both the nuclear content (DNA ploidy), which is frequently abnormal in breast tumours, and the S phase fraction (SPF), which is the measure of the proliferative rate of a growing cell population. A statistically meaningful number of cells, usually 10,000 and greater, can be measured in a short time and with the technique being automated, the ease of measurement being one strong point of the method. Either nuclei or intact cells from tumour tissues are stained with DNA specific dyes (such as propidium iodide) and the end result of a computer analysis produces a DNA histogram, from which both ploidy and the S phase content and the proliferative activity (S+G2M) can be determined. Ploidy is expressed in terms of DNA index, which allows comparability between observers, while the S phase can be determined manually or with the use of a computerised analysis programme. Although in FCM DNA analysis one can employ either nuclear suspensions or intact cell suspensions obtained from various sources (fresh tissue, unfixed frozen

tissue, ethanol- or formalin-fixed cells, and formalin-fixed paraffin-embedded tissue) there are clearly differences in the quality and the nature of information acquired. Retrospective analysis with clinical follow-up, and selection of a large number of cases predefined by clinical factors can be done on archival tissue kept frozen or embedded in paraffin. Although a few studies that have compared ploidy results from formalin-fixed, paraffin-embedded cells with that from fresh cells show good agreement (Frierson, 1988; Kallioniemi, 1988a; Owainati *et al.*, 1987), the quality of DNA histograms obtained from deparaffinised tissue is relatively poor. Samples from deparaffinised tissue contain a large amount of debris, which compromises the sensitivity of detection of abnormal DNA content and in a greater number of specimens, calculation of SPF is not possible (Visscher *et al.*, 1990a). Frozen tissue can be used for both prospective and retrospective studies, while with fresh tissues only a prospective study is possible. It is technically easier to extract nuclei from frozen or fresh tissue specimens and the DNA histograms produced are of a much higher quality.

A number of studies have been done to assess the potential of DNA FCM as a prognostic indicator. These studies have now shown that DNA ploidy and SPF data are of prognostic value in patients with breast cancer. Both these FCM parameters have been correlated with histopathological factors and in retrospective studies, the clinical outcome of the patients with respect to the DNA content and the SPF of their tumours have been assessed.

1.4.3.3.1. Correlation of DNA content (ploidy) with histopathological factors

A universal finding finding in all the studies that assessed the ploidy of primary tumours is the presence of a substantial proportion of aneuploid cells in a high percentage of breast cancer patients. Taking into account the 56 publications, reviewed by Frierson (1991), 63% of breast neoplasms were DNA aneuploid, the precise figure ranging between 53 and 75%. The biological role and the mechanism underlying DNA abnormality at this level is not known. Correlation with histopathological factors, however, showed that DNA ploidy has prognostic relevance.

There are conflicting reports concerning the association between DNA aneuploidy and lymph node involvement in breast cancer. DNA aneuploidy has been found to be significantly related to lymph node involvement (Eskelinen, *et al.*, 1989; Toikkanen *et al.*, 1989; Hedley *et al.*, 1984). However, others have found such association not to be statistically significant (Visscher *et al.*, 1990a; Moran *et al.*, 1984; Horsfall *et al.*, 1986; Thorud *et al.*, 1986; McDivitt *et al.*, 1986; O'Reilly *et al.*, 1990). Studies employing a larger series of patients (Hedley *et al.*, 1987; Ewers *et al.*, 1984; Dressler *et al.*, 1988) observed only a weak correlation between DNA content and staging parameters. In studies that found a statistical correlation between size and ploidy, larger tumours were noted more often to be aneuploid (Dowle, *et al.*, 1987; Ewers *et al.*, 1984; Thorud *et al.*, 1986; Visscher *et al.*, 1990a).

DNA content correlates significantly with histopathological tumour grade and in this the agreement between workers is highly consistent (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; Hedley *et al.*, 1987; McDivitt *et al.*, 1986; Visscher *et al.*, 1990a). An association between tumour ploidy and steroid receptor status has also been shown (Moran *et al.*, 1984; Dressler *et al.*, 1988; Olszewski *et al.*, 1981; Horsfall *et al.*, 1986), with aneuploid tumours tending to be receptor negative. It is perhaps relevant to point out that oestrogen receptor-negative tumours are more poorly differentiated compared to oestrogen-positive tumours (McCarty *et al.*, 1980). The relationship of ploidy to hormone status is perhaps an effect of the strong association between ploidy and tumour grade.

1.4.3.3.2. FCM analysis of SPF

In a FCM DNA histogram, S phase cells (SPF) represent the area between Go/G1 and G2/M peaks (Fig 1.4.4). Although some of the cells may be arrested in SPF (So), SPF in non-synchronised tumour populations accurately reflect cellular proliferation (Visscher *et al.*, 1990a). The relative area of the DNA histogram is calculated by making use of mathematical models.

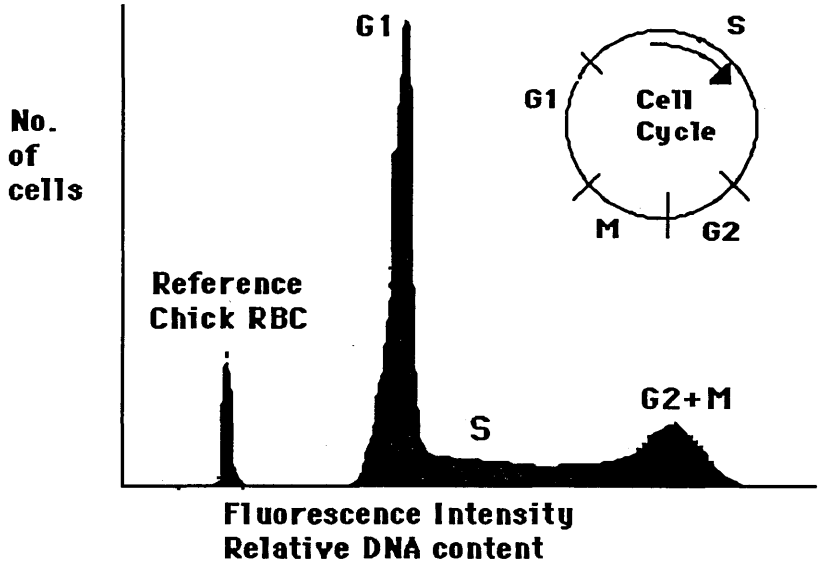


Figure 1.4.4. Flow cytometric analysis of the cell cycle.

Calculation of SPF is, however, difficult because of the lack of a theoretically-based demarcation between G1 and S phase and that between S phase and G2 phase. The calculation can be attempted manually by assigning markers to discriminate the S phase or with the use of a rectangular model described by Baisch *et al.* (1982) or more easily with the use of computerised analysis programmes like the Sum of Broadened Rectangluar (SOBR) model (Becton Dickinson). Manual calculation involves placing of markers to define the phases in the cycle (Fig 1.4.4).

Discrimination of S phase is difficult and it is more of a choice of the researcher and therefore, will give rise to inter-observer variation. Although computerised models make the calculation a very simple matter, these models are based on assumptions about the phases of the cell cycle, and at times the analysis may differ with the choice of the model. In addition, in many tumours, the presence of aneuploid populations overlapping with the diploid population or with contaminating non-tumour diploid cells, makes the precise discrimination of S phase cells impossible. A further problem associated with

SPF calculation is the presence of debris, probably representing DNA fragments, which can give rise to background and thus, a higher SPF than the true situation. Background DNA staining can be particularly prominent in nuclei extracted from paraffin-embedded tissues, while fresh tissues are certainly the best choice. Recently, it has been shown that background noise can be managed in calculating SPF by exponential background subtracting (Visscher *et al.*, 1990b) and computerised analysis programmes have also been developed which can assess SPF even in cases where there are overlapping of peaks. Ideally, a substantial improvement can be the use of an antibody specific for the tumour cells.

The difficulty is assessing SPF is evident from the reported success of various investigators in calculating SPF, the values ranging from 55 to 100% (Visscher *et al.*, 1990a). The SPF, as reported in the literature for breast tumours range from 1 to 30%, with a mean between 8% (McDivitt *et al.*, 1986) and 15.7 % (Coulson *et al.*, 1984).

1.4.4.3.3. Correlation of SPF with histopathological factors

The most significant finding concerning SPF determination by FCM is that aneuploid tumours from breast cancer patients have a higher SPF than diploid tumours. A point to make here is that in diploid cases, the true nature of the cell population has not been determined and that in some cases at least, the diploid cells or a proportion of them constitute non-cycling stromal and even lymphocytic cells, which can often make up a good percentage of the total population (Whitford *et al.*, 1990), with a low SPF. This, again, would require the use of tumour-specific markers to correctly identify the cells within the diploid population. In general, however, the large number of studies support the general consensus that aneuploidy is associated with a higher SPF.

As with ploidy, most of the studies find higher SPF to be associated with poorer histopathlogical features. Dressler *et al.* (1988) found a significantly higher mean SPF in axillary node positive diploid tumours than in node-negative diploid tumours. This difference, however, was not present among aneuploid tumours. Hedley *et al.* (1987) found only a weak association between regional nodal status and SPF. Relationship with grade was highly significant (Hedley *et al.*, 1987; McDivitt *et al.*, 1986; Feichter *et al.*, 1988; Moran *et al.*, 1984; Kallioniemi *et al.*, 1987; Olszewski *et al.*, 1981). High SPF in breast cancer is also related to oestrogen and progesterone- negative status (Hedley *et al.*, 1987; Moran *et al.*, 1984; Dressler *et al.*, 1988; Olszewski *et al.*, 1981).

1.4.3.3.4. DNA ploidy and SPF and prognosis in breast cancer

Employing retrospective and follow-up studies, several investigators have analysed the results of DNA content and SPF and compared them with the clinical outcome of breast cancer patients. Two parameters, relapse or disease-free survival (DFS) and overall survival have been assessed in follow-up studies. The period of follow-up used, however, varies between observers. Most of the studies having a follow-up from 5 to 8 years, with only Toikkanen *et al.* (1989) having a follow-up as long as 25 years. The finding from these investigations are however, relatively consistent.

In the earlier study of Kallioniemi *et al.* (1987), the overall 8-year survival rate of patients with diploid tumours was 74%, compared to 51% in aneuploid cases. The difference was statistically significant and was found to apply to both node-negative and node-positive patients. Moreover, in a

multivariate analysis, DNA ploidy emerged as a significant independent prognostic indicator. In a later study (Kallioniemi *et al.*, 1988b), the same group of workers, combined DNA index with SPF, and reported that in patients with diploid tumours and low SPF, prognosis was especially favourable. O'Reilly *et al.* (1990), employing the same follow-up period, however, found that only SPF but not ploidy was associated with the clinical course of the patients. Even this significance was lost in a multivariate analysis, when histological grade was included. The other report that failed to observe any significance of FCM studies with prognosis, was that of Dowle *et al.* (1987), who observed that the significant advantage of diploid tumours were lost on a longer-term follow-up of 84 months. Studies employing much longer follow-up times (Eskelinen *et al.*, 1989; Toikkanen *et al.*, 1989) provide evidence to indicate that FCM studies can be of importance in predicting the outcome of patients. The mean follow-up in the study of Eskelinen *et al.* was 12.4 years and DNA aneuploidy showed a significant association with decreased survival. 33 percent of patients with diploid tumours and 65% with aneuploid tumours died during the follow-up. High SPF were also associated with distant metastases or death. The follow-up period of about 25 years used by Toikkanen *et al.* (1989) is the highest so far reported. This study found that although survival rate of patients with diploid tumours was better, the ploidy significance held only in node-negative patients, while SPF were equally important for both node-negative and node-positive patients. Moreover, SPF was also found to have a prognostic value independent of other parameters.

That SPF may be a parameter of greater biological importance than ploidy have been implicated (Visscher *et al.*, 1990a) and in the study of Stal *et al.* (1989), only SPF was found to be a predictor of disease recurrence. Clark *et al.* (1989), however, found that SPF was important only in cases of diploid tumours. In an attempt to identify a sub-group with poorer prognosis within

node-negative patients, Clark *et al.* (1989) found that DFS at 5 years were significantly lower for patients with diploid tumours and with aneuploid tumours SPF were not an additional significant predictor. This is perhaps a reflection of the fact that aneuploid tumours have been found to be associated with high SPF. The study of Clark *et al.* (1989) also showed that breast cancer patients can be divided into 3 groups in terms of decreasing DFS: i) Diploid with low SPF; ii) Aneuploid tumours, irrespective of SPF; and iii) Diploid with high SPF. The DFS rates found for the three groups being 90, 74 and 70 % respectively. Although the follow-up in the Clark *et al.* (1989) study was only 5 years, it is consistent with the findings reported in studies with longer follow-up time and perhaps, summarises the relationship between ploidy and SPF and patient outcome, that is, breast cancer patients with diploid, low SPF tumours have a good prognosis. This then indicates that both aneuploidy and high SPF are associated with aggressive nature in breast cancer.

1.4.3.3.5. FCM DNA studies on primary and metastatic tissue from the same patient

The present knowledge of abnormality in tumours has been related to known prognostic indicators or, in some of the studies, to the outcome of the disease. Such studies have shown that DNA FCM data provide information pertinent to the behaviour of tumours in breast cancer patients. The precise relationship between the abnormality detected by FCM and the prognosis of patients, although reported to be significant in some studies, is not clearly understood. In breast cancer, the clinical outcome depends largely on the development of distant metastases and their biological aggressiveness and therefore, the characterisation of metastases is also relevant. Although

extensive DNA analyses of primary tumours from breast cancer patients have been undertaken, these were carried out principally to assess the potential of DNA flow cytometry as a prognostic indicator and were involved with the DNA ploidy and SPF of the primary tumours alone. Very little attention has been directed towards examining the direct relationship between metastatic potential and DNA ploidy and as such in comparing the ploidy in metastases with that in the primary tumour. In breast cancer surgical practice, the availability of invaded lymph nodes along with the primary tumour from the same patient makes them an excellent candidate for the study of aneuploid cells and their ability to invade lymph nodes. There are, however, only a few reports in breast cancer where lymph node metastases were analysed by DNA FCM, along with the primary tumour from the same patient.

In the few studies carried out involving breast cancer, a high degree of concordance between metastases and autologous primary tumours has been reported by most, but not all investigators. In the earlier study of Auer *et al.* (1980b), who used static cytophotometry and Olszewski *et al.* (1982), who used flow cytometry and studied only aneuploid tumours, the DI of primary tumours were found to be identical to those of lymph node metastases. This constancy with respect to ploidy, DI and SPF have also been reported in two later studies (Feichter *et al.*, 1989; Beerman *et al.*, 1991). However, these studies also report DNA profile differences between primary tumours and lymph node metastases in a small number of cases. Feichter *et al.* (1989) reported agreement of DI between the two tissues in 61 out of 80 cases, while SPF showed less consistency with only 62% displaying similar percentages of SPF. Additionally, the study reported that diploid tumours and their metastases revealed more consistency than aneuploid tumours. Altered kinetics have also been reported by Olszewski *et al.* (1982), who reported a significant reduction of SPF in the metastases of tumours with a positive content of

oestrogen receptors. Differences in the ploidy of primary tumours and lymph node metastases in only a small number of cases have also been observed by Beerman *et al.* (1991) and in agreement with other studies they found that lymph node metastases could be both diploid and aneuploid suggesting that diploid primary tumours can give rise to aneuploid metastatic tumours and vice versa. Although the authors in these studies concluded that ploidy is a generally stable phenomenon which is established early in the development of the primary tumour, their reports do show that in a small number of cases, compared to the primary tumour a lymph node metastasis can have a different DNA profile. The greatest extent of changes in the ploidy between primary and metastases have been reported by Hitchcock *et al.* (1989). In a study of 36 breast cancer patients, they found that 44% of metastases to the ovary and axillary lymph nodes had a ploidy different from the corresponding primary sites. Along with grade, reactivity for CEA and to NCRC-11, which detects a surface glycoprotein (Section 1.3.1.1), the report concluded that changes in ploidy in a metastasis is not unidirectional and is not related to the ploidy status of the primary. Unlike the previous observation, this suggests that changes in ploidy and other variables may occur after metastasis. In this context, an important issue is whether aneuploidy itself or a specific aneuploid subpopulation has a higher metastatic potential. As only a small number of studies have been done it is not clear whether a tumour is stable in terms of its DNA profile, and although heterogeneity in terms of ploidy of the primary tumour have been reported (Hitchcock *et al.*, 1989; Beerman *et al.*, 1991), there is no direct evidence to indicate that the development of clones with metastatic potential is related to the DNA profile of a tumour.

An important point to make here is that tumour cells analysed for DNA flow cytometry are extracted from a slice of an excised tumour mass which contains in addition to epithelial breast tumours, surrounding tissue which

comprise both normal epithelial cells and non-epithelial stromal, fibroblast and lymphocytic cells. The proportion of these cells relative to epithelial tumour cells, which are the object of study, can affect both the determination of ploidy and the measurement of SPF. Apart from the study of Feichter *et al.* (1989) which attempted to remove contaminating lymphocytes by centrifugation, in none of the other studies were the true identity of the diploid population known and, in cases where the lymph node metastases were reported to be diploid, the population observed may have been lymphocytes. That aneuploid cells in metastases can go undetected due to the presence of lymphocytes was explained by Tribukait (1987), who observed a diploid metastasis in cases where the primary tumour was aneuploid in 30% of bladder carcinomas examined. Moreover, it is also possible that aneuploid populations in tumours designated diploid may have been gone unnoticed simply because the aneuploid cells made up only a small percentage of the total cell population under study. There is therefore, a need for further analysis of both primary tumours and metastases in breast cancer by DNA flow cytometry with the help of a methodology which would allow discrimination of a tumour cell from its normal counterpart or simply from contaminating cells like fibroblasts and lymphocytes.

1.4.3.3.6. Multiparametric FCM DNA analysis

The presence of contaminating cells, both epithelial and non-epithelial normal cells, is a major problem often encountered in analysing cell suspensions from a solid tumour like breast cancer. Although in a histochemical analysis such cells can be easily recognised, it is not so in FCM DNA analysis, in which such contaminants make both the assessment of ploidy and the measurement of SPF inaccurate. In a study of ploidy or SPF of

primary tumours and their corresponding metastases, this is an issue that needs to be resolved. Ideally, a tumour specific antigen is what one would prefer in order to sort this problem, but surface expression heterogeneity and the absence of any known breast tumour-specific antigen makes this impractical. Unlike haemaopoietic tumours, breast tumour cells cannot be distinguished in terms of their intrinsic light scatter properties, which reflect cell size and granularity (Section 2.2.1.4).

Recently, Zarbo *et al.* (1989), described the use of a multiparametric DNA analysis using dual-labelled intact cells. Use was made of the epithelial tissue-specific, cytokeratin-antibody, which has been previously employed in the FCM analysis of urothelial (Feitz *et al.*, 1985; Smeets *et al.*, 1987) and colonic carcinoma (Crissman *et al.*, 1988). With this technique, specific gating on cytokeratin-positive epithelial cells was possible. Non-staining stromal, fibroblast and lymphocytic cells were therefore, excluded from the analysis and this greatly refined the detection of both aneuploidy and the calculation of SPF of primary tumours. Employing this technique in a later study of breast carcinomas, Visscher *et al.* (1990b) found that both aneuploidy and SPF was significantly related to accepted histopathologic and hormonal factors in the identification of high risk patient populations. The investigators concluded that the use of multiparametric analysis with cytokeratin gating significantly refines the biologic relevance of FCM data. Although this technique was performed mainly on primary tissues, it holds the prospect of being used to analyse primary tumours and lymph node metastases taken from the same patient. This method would allow the true identification of ploidy status of both primary and metastatic cells and therefore, a clearer understanding of the relationship between FCM data and metastatic potential.

1.4.3.3.7. Aneuploidy, oncogenes and tumour suppressor genes

It is evident that in breast cancer, there are both alterations at specific gene level as reported in terms of gene amplification or loss and genetic alterations on a much larger scale as observed in terms of tumour ploidy. Of the most widely studied oncogene *erb* B-2 alterations have been detected in only about 20% of breast tumours, whereas aneuploid data indicates that the genome of >60% of tumour cells is highly abnormal. There is obviously a requirement that the area of gene amplification or loss be related to the ploidy of the original tumour. Only in a few of the recent studies have investigators also reported ploidy status of breast tumours for which gene amplification has been studied. Studying *erb* B-2 amplification, Borg *et al.* (1991) found that aneuploid, but not diploid, tumours with gene amplification were more aggressive. In an earlier study, Tavassoli *et al.* (1989) also reported that the tumours with *erb* B-2 and *myc* amplification were aneuploid. Correlation between *erb* B-2 protein expression and DNA Index have also been reported by Baak *et al.* (1991), while Bacus *et al.* (1990) found that tumours that overexpress the *erb* B-2 protein have tetraploid DNA content. It is important to point out that in all of these studies DNA ploidy and gene amplification or protein overexpression were determined on the same tumour specimen but not on the same tumour cell. With respect to protein overexpression, a direct correlation between the two features can perhaps be best established in a dual-parameter flow cytometric analysis. Lately it has been shown that both cellular integrity and *erb* B-2 antigen reactivity were preserved in cells and tissues following fixation in ethanol and thereby, allowed a dual parameter analysis (Kelsten *et al.*, 1990).

1.4.4. DNA fingerprint analysis of tumour DNA

It appears that mutations in several genes may affect the control of growth and malignant capability of a breast tumour. Not only is there an alteration at a specific site on a chromosome but that several of such sites are affected before a highly malignant clone of tumour appears. In breast cancer, where oncogenes or tumour suppressor genes have been implicated such studies were carried out simply because the specified gene was found to be affected in another cancer type or in an experimental cell line or animal models. There are clearly more such sites involved yet unidentified. Therefore, determination of the total extent of alterations on all chromosomes rather than losses on individual chromosomes might more accurately measure the level of genetic change and perhaps would be useful for assessing prognosis. To record all the major changes in a tumour a battery of DNA probes representing different loci have to be used.

An alternative approach only recently developed is the use of DNA fingerprinting analysis for screening human tumour DNA. The DNA fingerprint technique makes use of the existence of multiple hypervariable tandem repetitive sequences (or minisatellites) that are dispersed throughout the genome and are largely polymorphic due to allelic variation in repeat copy number (Jeffreys *et al.*, 1985). Minisatellite probes consisting of the core sequences present on the majority of human chromosomes rather than on a single chromosome provide a set of genetic markers that have been found to be of value in detecting changes in tumour DNA (Thein *et al.*, 1987; Fey *et al.*, 1988). This technique was first tested on a small number of tumours of various human cancers (Thein *et al.*, 1987) and later applied on gastrointestinal (Fey *et al.*, 1988) and ovarian cancers (Boltz *et al.*, 1990, Saito *et al.*, 1991).

The investigators showed that DNA fingerprint analysis can be applied on human tumours to detect deletion or reduction in intensity of a band suggesting loss of heterozygosity, increased intensification of single bands and the appearance of novel DNA fragments. A similar study can be carried out to analyse breast tumours to detect changes during progression and metastasis. Primary and lymph node metastasis can be compared from the same breast cancer patients to detect changes which would reflect alterations associated with metastasis.

1.5. Aim of study

This study has been designed to assess different parameters in relation to metastasis to the lymph node in breast cancer patients. In order to do this, primary tumours and lymph node samples, both normal and with metastasis, were obtained from the same patient undergoing surgery for breast cancer.

The parameters studied include (i) immunological responses in the tumour-draining lymph nodes, (ii) carbohydrate expression at the tumour cell surface, and at the DNA level, (iii) the DNA content by flow cytometry and (iv) DNA fingerprinting with multi-locus satellite DNA probes.

The immunological responses of the lymph nodes were assessed by studying both phenotypic markers and activation status of lymphocytes by flow cytometry. The effect of metastasis on lymph node immunological responses was assessed by examining both tumour-free and tumour-invaded nodes taken from the same patient.

Cell surface carbohydrate expression was studied by flow cytometric analysis of lectin binding. The expression of lectin-binding marker was also assessed in metastatic cells in the lymph nodes.

FCM DNA analysis was used to assess DNA content. Using a cytokeratin-antibody, a dual-parameter flow cytometric analysis was found to be useful in assessing DNA ploidy of both primary tumours and lymph node metastases.

Four different DNA satellite probes were used in DNA fingerprinting analyses. Fingerprints from tumour DNA was compared with peripheral blood

from the same patient to determine changes in terms of band loss, appearance of new bands and changes in hybridisation intensity.

The prognostic value of these parameters were assessed by comparison with histopathological data and tested in a statistical test. Finally, in a group of patients, the different parameters have been interrelated in an attempt to assess their overall value in providing additional information.

The use of flow cytometry and fresh tissues provided a relatively non-subjective analyses of antibody- or lectin-stained intact cells.

CHAPTER 2

Materials and Methods

2.1. Materials

All chemicals used in this study were supplied by Sigma Chemical Company or BDH Chemicals, both of Poole, Dorset, England. These chemicals were of the highest grade available. All plasticware were from Sterilin Ltd., Feltham, England.

2.1.1. Tumour cell and lymphocyte preparation.

RPMI-1640 (Gibco Ltd., Paisley, Scotland)

Penicillin/Streptomycin (Gibco Ltd., Paisley, Scotland)

Dimethyl sulfoxide (DMSO) (BDH Chemicals Ltd., Poole)

FCS (Imperial Laboratories, Andover, Hants.)

Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden)

Collagenase (Worthington Biochemical Corp.)

2.1.2. Reagents for tissue culture media

RPMI-1640 (Gibco, Paisley)

Penicillin/Streptomycin (Gibco, Paisley)

FCS (Imperial Laboratories, Andover, Hants.)

2.1.3. Antibodies

Except for those listed below, all antibodies were purchased from Becton Dickinson, Cowley, Oxford and Dakkopatts A/S. These antibodies were either conjugated to FITC or PE (Table 2.1).

Sheep anti-mouse Ig-FITC conjugated (whole antibody) (Amersham International plc, Bucks.)

Rabbit $F(ab)_2$ anti-human IgG(γ chain)-FITC (Dakkopatts)

2.1.4. Lectins

All fluorescently-labelled and biotinylated lectins were obtained from Sigma Chemical Co.

Streptavidin-PE to label biotinylated lectins was from Becton Dickinson, Cowley, Oxford.

2.1.5. General Buffers

PBS (phosphate buffered saline), pH 7.4:

170 mM NaCl; 3.4 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4.

Sheath fluid, pH 7.2 (FACScan fluid):

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

2.1.6. Solutions for DNA flow cytometric analysis

These solutions were prepared as described by Vindelφv *et al.* (1983).

Citrate buffer, pH 7.6: 250 mM Sucrose, 40 mM trisodium citrate, $2H_2O$; 5 % (v/v) DMSO.

Stock solution , pH 7.6 : 3.4 mM Trisodium citrate, $2H_2O$; 0.1% (v/v) Nonidet P40; 1.5 mM Spermine tetrahydrochloride; 0.5 mM Tris.

Solution A: Trypsin (15 mg, T0134, Sigma Chemical Co.) dissolved in 500 ml stock solution, pH 7.6.

Solution B: Trypsin inhibitor (250 mg, T9253, Sigma Chemical Co.) and Ribonuclease A (50 mg, R4875, Sigma Chemical Co.) dissolved in 500 ml stock solution, pH 7.6.

Solution C: Propidium iodide (208 mg) and spermine tetrachloride (580 mg, Sigma Chemical Co.) dissolved in 500 ml stock solution, pH 7.6.

Solution C was protected against light with tinfoil and all these solutions were aliquoted and stored in plastic tubes at -70 ^{o}C.

2.1.7. Solutions and buffers used in DNA manipulations

2.1.7.1. Extraction of DNA from primary tumours, lymph nodes and PBL

Nuclei lysis buffer: 10mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, pH 8.2.

10% SDS.

Proteinase K (Boehringer Mannheim, West Germany) solution: 1 mg/ml in 1% SDS, 2 mM Na_2EDTA.

Saturated NaCl (approximately 6M)

TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM Na_2EDTA.

TE buffer, pH 7.4: 10 mM Tris-HCl, 1 mM Na_2EDTA

2.1.7.2. Plasmid DNA preparation

Solution I: 50 mM glucose, 25 mM Tris, pH 8, 10 mM EDTA, pH 7.5, 2 mg/ml lysosyme (fresh).

Solution II: 0.2 N NaOH, 1% SDS.

Solution III: 5M K-acetate, pH 4.8 (60 ml 5M K-acetate + 11.5 ml acetic acid, and the volume made to 100 ml with water).

2.1.7.3. Transformation buffers

TSB (Transformation & storage buffer): L broth (pH 6.1) containing 10% PEG, 5% DMSO and 20 mM Mg^{++} (10 mM $MgCl_2$ + 10 mM MgSO4).

50 mM $CaCl_2$,.

2.1.8. Bacterial media and reagents

Reagents for bacterial media were from Difco Laboratories, Detroit, Michigan.

L-broth (500 ml): bacto-tryptone 5g, yeast extract 2.5g, NaCl 5g, pH 7.4.

H plates (per l): 8g NaCl, 10g bacto-tryptone, 15g bacto-agar, pH 7.5

Top agar (per l): 8g NaCl, 10g bacto-tryptone, 7g bacto-agar, pH 7.5.

X-Gal (5-bromo-4-chloro-3 indolyl-β-D-galactoside) plates: 40 µl of 2% X-Gal in dimethyl formamide and 40 µl 100 mM IPTG (Isopropyl thiogalactoside) added to ampicillin-containing (100 µg/ml) agar plates.

2 x TY broth (per l): 16g tryptone, 10g yeast extract, 5g NaCl, pH 7.4.

2.1.9. Bacteria

All bacterial strains used as hosts were generously provided by Dr. M.L.M. Anderson, Dept. of Biochemistry, University of Glasgow.

E. coli strain DS941 rec F^{-}143, proA7, str31, thr1,leu6, tsx33, intl2, his4, argE3, galK2, ara14, supE44,xyl15,lacI,lac ZΔM15.

E. coli strain JM101 supE, thi-1, Δ(lac-proAB), [F', traD36, proAB, lac^{q}ZΔM15], λ^{-}.

Phage strain MV1190 Δ(lac-pro), thi, supE, Δ(srl - recA), Tn10 (tet^{R}), F',traD36, proAB.

2.1.10. Agarose gel electrophoresis

Electrophoresis grade agarose was from BRL, Paisley.

Tris-borate (TBE) buffer : 89mM Tris-borate, pH 8.3, 1mM EDTA, 0.2 μg/ml ethidium bromide.

Tris-acetate (TAE) buffer: 40mM Tris-acetate, pH 7.8, 1mM EDTA, 0.2 μg/ml ethidium bromide.

Gel Loading buffer: 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 0.25% bromophenol blue, 20% Ficoll (Type 40), 10 μg/ml ethidium bromide.

2.1.11. Purification of probe DNA

Purification of DNA fragments (representing each of the DNA probes) from acetate gels was carried out using a Geneclean II kit from BIO 101 Inc. La Jolla, California.

2.1.12. Restriction enzymes and DNA standards

All restriction enzymes were obtained from Gibco BRL (UK), Paisley, and Pharmacia LKB Biotech., Uppsala, Sweden.

Standard DNA markers λ DNA-Hind III, and
ϕ-X174 DNA-Hae III were from Gibco BRL (UK). The sizes are as follows:
Hind III/λ 23.7, 9.5, 6.6, 4.3, 2.3, 2.1, 0.6 kb
Hae III/ϕX 1.3, 1.08, 0.87, 0.6, 0.3, 0.28, 0.27, 0.23 kb.

2.1.13. Satellite probes

Plasmid DNA (100 ng in 100 μl Tris buffer) containing multi-locus minisatellite probes 33.6 (in pUC18) and 33.15 (in pUC19) were purchased from Cellmark Diagnostics, Abingdon, Oxfordshire.

Recombinant phage containing 228S (13 μg, 5 μl of 2.6 μg/μl) and 216S (2 x 5 μl from a single plaque in TE buffer) satellite DNAs in phage M13 were generously provided by Dr L. Burgoyne, The Flinders University of South Australia, Australia. These were treated as stock and were diluted 1:500 for use in transformation (Seection 2.2.4.3.5).

2.1.14. Nick Translation

DNA probes were radiolabelled using the nick translation kit (code N 5000) from Amersham plc.

2.1.15. Pre-hybridisation buffer

Both pre-hybridisation buffer and hybridisation solution were prepared according to Anderson & Young (1985).

Solution A: 50 ml deionised formamide;

25 ml 20 x SSC (SSC is 0.15M NaCl, 0.015M trisodium citrate, pH 7.0);

5 ml 100 x Denhardt's solution (100 x Denhardt's solution contains 2% Ficoll (mol. wt. 400 000), 2% polyvinyl pyrrolidone (mol. wt. 400 000) and 2% bovine serum albumin):

5 ml 1M sodium phosphate buffer, pH 6.8 (25.5 ml of 1M NaH_2PO4 and 24.5 ml of 1M Na_2HPO4);

0.5 ml 20% SDS

volume adjusted to 95.5 ml with water.

Solution B: 2 ml sonicated salmon sperm DNA at 5 mg/ml;

denatured in a boiling water bath for 5 min. and then quenched in ice.

Solution B was added to solution A and volume made to 100 ml with water.

2.1.16. Hybridisation solution

All solutions were prepared as in pre-hybridisation buffer.

Solution A: 50 ml deionised formamide;

25 ml 20 x SSC;

1 ml 100 x Denhardt's solution;

2 ml 1 M sodium phosphate buffer, pH 6.8;

1 ml 20% SDS;

10g dextran sulphate (mol. wt. 500 000)

Volume adjusted to 95.5 ml.

Solution B: 2 ml sonicated DNA (5 mg/ml)

Denatured in a boiling water bath for 5 min. and then quenched in ice. Solution B added to solution A and volume made to 100 ml with water.

2.1.17. Radiochemicals and autoradiography

[α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol in acqueous solution at 10 mCi/ml) was obtained from Amersham International plc., Amersham, Bucks.

[α-^{35}S]-ATP (600 mCi/mmol) used in sequencing was also from Amersham.

Kodak X-OMAT S film was used for autoradiography.

2.2. Methods

2.2.1. Sample preparation for immunological analysis

2.2.1.1. Patient samples

Samples of ipsilateral axillary lymph nodes and peripheral blood were obtained from 26 patients undergoing breast cancer surgery at the Western Infirmary, Glasgow. Twelve of these patients had stage II breast cancer (with nodal metastasis- Section 1.1.5.2), while the other 14 had stage I. For stage I (without metastasis) patients, specimen of one half of nodes were aseptically sampled. For the study of tumour invaded versus tumour free lymph nodes in stage II patients, one half of a lymph node invaded with tumour and one half of a node half free of tumour from the same patient were separately aseptically sampled. The other halves of these nodes were sent to Pathology for routine histological examination. Histopathological reports confirmed that the lymph nodes initially assessed by the surgeon had been correctly allocated in terms of invasion status.

The age of the patients ranged from 43 to 85 with a mean of 57. All but one patients were subsequently found by histopathological examination to have ductal infiltrating carcinoma of the breast. The one exception as found by histopathology was lobular breast carcinoma. None of these patients had received preoperative cytotoxic, endocrine or radiotherapy.

2.2.1.2 Harvesting of cells

Lymph node lymphocytes (LNL)

The aseptically obtained lymph nodes were processed immediately in order to avoid alteration in membrane receptor expression. The cells were released by teasing them apart with a scalpel and needle. The lymphocytes were isolated from the lymph node cell suspension by layering the cell

suspension over an equal volume of Ficoll Hypaque. After centrifugation at 500 g for 20 min, the lymphocytes were isolated from the interface by careful pipetting. The lymphocytes were then washed in RPMI-1640 medium, resuspended in a freezing mixture consisting of 90% heat-inactivated foetal calf serum (FCS) and 10% DMSO and finally stored in liquid nitrogen until they could be analysed. The recovery of lymphocytes from the lymph nodes was quite variable as also reported by Whitford *et al.* (1992b). The number of lymphocytes recovered from tumour invaded nodes were found to vary from greater than 10^7 to 4 x 10^6. Low recovery of lymphocytes was also found in cases of some nodes which were small but otherwise tumour free. On two occasions, no attempt was made to isolate lymphocytes from the cell suspension by Ficoll Hypaque density centrifugation as the lymph node appeared to be replaced by tumour cells to a great extent. A previous attempt to do so with a grossly tumour invaded half lymph node had resulted in the loss of the lymphocyte layer. Therefore, in order to treat samples from invaded and tumour free lymph nodes in an identical manner, the cell suspensions from both pairs of nodes were washed twice in RPMI and resuspended in freezing mixture before storing in liquid nitrogen.

Peripheral Blood Lymphocytes (PBL)

Peripheral venous blood were also obtained from each of the patient on the day of surgery. 10 ml of blood, collected in potassium EDTA tubes was gently layered over 10 ml of Ficoll Hypaque and centrifuged for 20 min at 500g. The lymphocytes were obtained by carefully pipetting the layer at the interface. The cells were washed in RPMI-1640, resuspended in freezing mixture and finally stored in liquid nitrogen.

2.2.1.3 Lymphocyte preparation for flow cytometry

Cell samples kept frozen in liquid nitrogen were quickly thawed in a water bath at 37°C, to avoid damage by ice crystal formation within the cells. The cells were washed twice in filtered PBS and resuspended at a cell density of approximately 2 x 10^7 cells per ml. 50 µl of this cell suspension was placed into flow cytometry tubes (Falcon 2052) and monoclonal antibodies (MAbs) to specific phenotypic and activation markers on the lymphocytic cell membrane were then added. The full panel of combinations of MAbs in Table 2.1 was used with each lymphocyte preparation from lymph nodes and peripheral blood. After addition of the antibodies, the content of the tubes were mixed on a vortex briefly and the tubes were incubated on ice for 20 min. The incubation was carried out in the dark to prevent bleaching of the fluorochromes and to avoid capping and internalisation of the antibody/antigen complexes, 0.02% w/v sodium azide was added and the tubes kept on ice. The cells were next washed and resuspended in filtered PBS. Propidium iodide to a final concentration of 2 µg/ml was then added to each tube to allow the identification and exclusion of dead cells from the analysis. Data on 5000 live cells were then acquired on a Becton Dickinson FACScan flow cytometer.

TABLE 2.1. Flow cytometric examination of monoclonal antibody defined phenotypic and activation markers of human lymphocytes.

Antibody 1	Antibody 2	Predominant reactivity
IgG1-FITC	IgG2a-PE	Control
Leucogate Anti-CD45-FITC	Anti-CD14-PE	Differential staining of leukocyte subpopulations (lymphocytes, monocytes, neutrophils)
Anti-Leu4-FITC	Anti-Leu12-PE	T-lymphocytes, B-lymphocytes
Anti-Leu3a-FITC	Anti-Leu2a-PE	CD4+ Helper T cells, CD8+ Suppressor/cytotoxic T cells
Anti-Leu 3a-PE	Anti-HLA DR-FITC	Activated CD4+ Helper T cells
Anti-Leu 3a-PE	Anti-CD25-FITC	IL2-receptor on CD4+ Helper T cells
Anti-Leu 2a-PE	HLA DR-FITC	Activated CD8+ cytotoxic T cells
Anti-Leu 2a-PE	Anti-CD25-FITC	IL2-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 cell

2.2.1.4 Flow cytometry of lymphocytes

All lymphocyte samples were acquired on the FACScan with two acquisition gates operative. This involves exclusion of dead cells and the use of scatter gate to collect only the lymphocyte population.

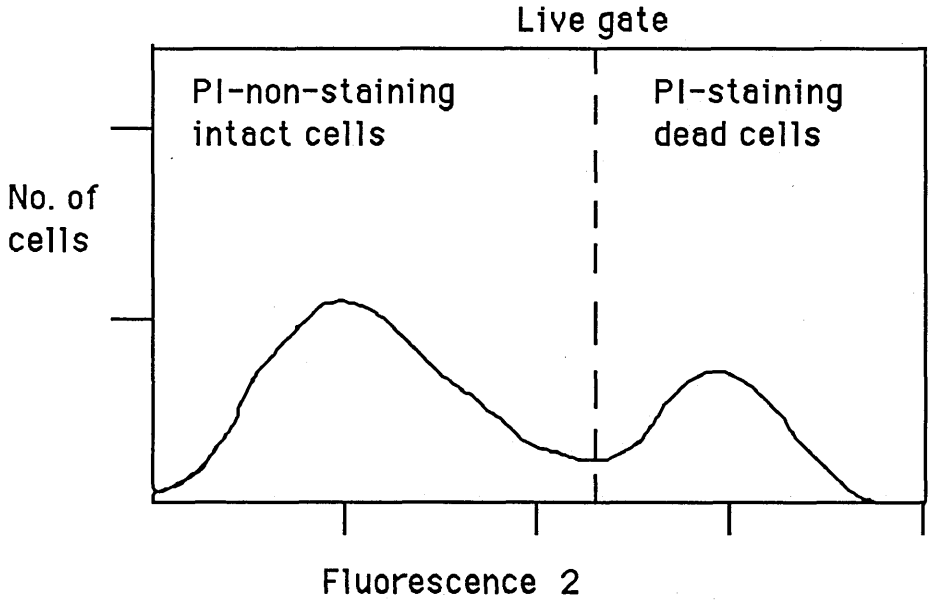


Fig. 2.1. PI-gating to exclude cells with breached membranes.

Exclusion of dead cells

An advantage with flow cytometry is that it allows exclusion of dead cells. Using PI, it is possible to segregate live cells with intact membrane from dead cells with breached membrane. PI binds to DNA but cannot to do so in the case of cells with intact membrane thus all non-staining PI cells represent live cells. These non-staining cells can be preferentially selected in flow cytometry by using a live selection gate (PI gate, Fig. 2.1). This is particularly important in studies involving antibodies to surface markers as these antibodies can bind to irrelevant intracellular antigens if the cell membrane is breached (Section 4.8).

Scatter gate to collect lymphocytes

Forward and side scatter of laser light reflect the size and granularity of of cells respectively and these properties can be used to differentiate between lymphocytes, monocytes and neutrophils (Fig. 2.2). Simultaneous with this and using the cocktail Leucogate antibodies (Table 2.1), these leucocyte subpopulations can be differentially stained with antibodies and therefore can be identified as distinct cell populations on FL1 versus FL2 plot (Fig. 2.2B). By drawing a gate around the lymphocyte population and as shown in Fig 2.2C & 2.2D, it is possible to exclude the other cell types and the collected data can be confined only on lymphocytes.

In this study, all analyses of lymphocytes were carried out with both the PI-gate and the lymphocyte scatter gate operative. The data presented here is therefore, on live lymphocytes only.

Analysis gate and assessment of positive cells

To exclude non-specific binding, all assessment of cells positive for a phenotypic or activation marker is based on markers set on the control sample, which was stained with irrelevant antibodies of matched isotype (Table 2.1). The proportion of cells positive for the phenotypic markers were analysed using four quadrant analysis. The quadrants were set on the control sample such that at least 95% of these cells fell within quadrant 3 (Fig. 2.3). The proportions of positive cells falling in each quadrants were identified on the basis of the fluorescence channel and the relative percentages of these cells measured on the computer using the FACScan software. Activation markers were measured by isolating the cell population under study on FL2 (using a PE-labelled antibody) and the percentage of these cells positive for a given

Figure 2.2. Flow cytometric analysis of lymphocytes.

A. The forward (FSC, cell size) and side (SSC, cell granularity) scatter properties of leucocytes allow them to be differentiated in a dual-parameter dot plot. This is shown here for a lysed whole blood preparation.

Green colour represents lymphocytes, blue is monocytes, red is granulocytes, and yellow represents cellualr debris.

B. The above sub-populations of blood cells stained differentially with the leucogate antibodies (Table 2.1) and identified as distinct cell populations on FL1 (FITC-staining) and FL2 (PE-staining) plot.

C. Lymphocytes gated on FSC versus SSC. In this case, the instrument has been set so as not to acquire cellular debris.

D. The efficiency of the lymphocyte gate is shown upon staining with leucogate antibody.

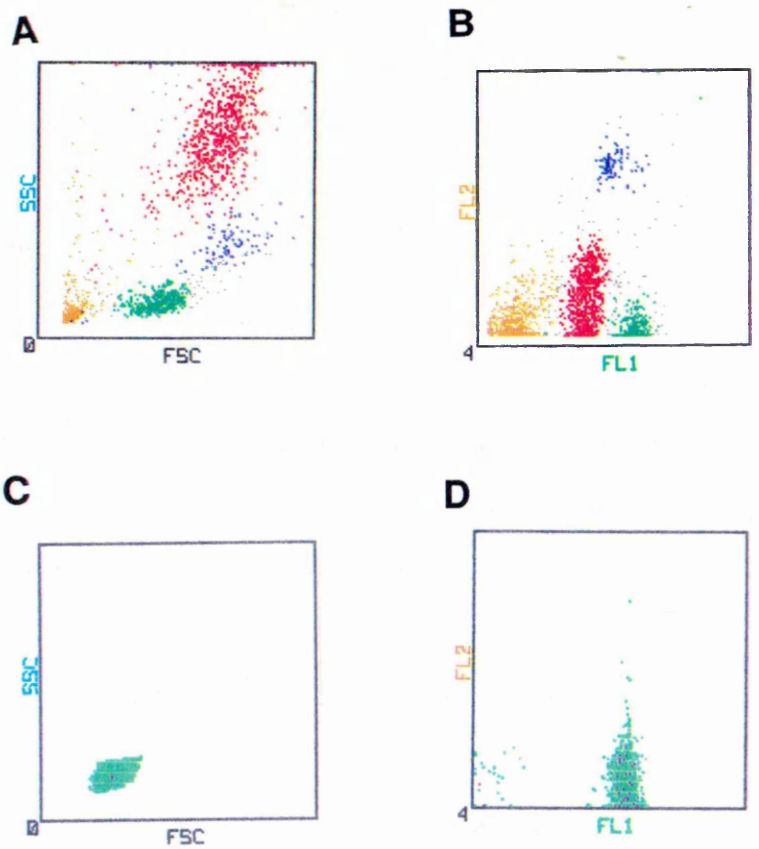

A
SSC
0
FSC
B
FL2
4
FL1
C
SSC
0
FSC
D
FL2
4
FL1

Figure 2.3. Phenotypic analysis of lymphocytes from breast cancer patients

A. The control sample. The cells have been stained with irrelevant antibodies to exclude non-specific binding. This is done by setting a four-quadrant analysis marker such that at least 95% of these cells fell within quadrant 3 (the origin). All analyses is then based on the marker set on the control sample.

B. Lymph node lymphocytes stained with anti-Leu 4-FITC and anti-Leu 12-PE to assesss the proportions of T and B lymphocytes respectively.

C. Phenotypic proportions of T cell subsets. CD8+ T cells on quadrant 1 (FITC-stained) and CD4+ T cells on quadrant 4 (PE-stained).

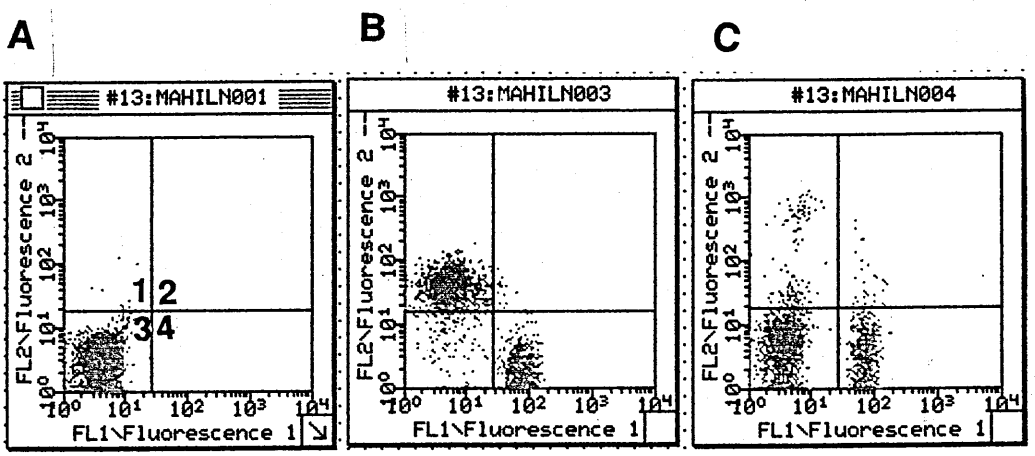

A
B
C
#13:MAHILN001
#13:MAHILN003
#13:MAHILN004
FL2\Fluorescence 2
FL1\Fluorescence 1
1
2
3
4

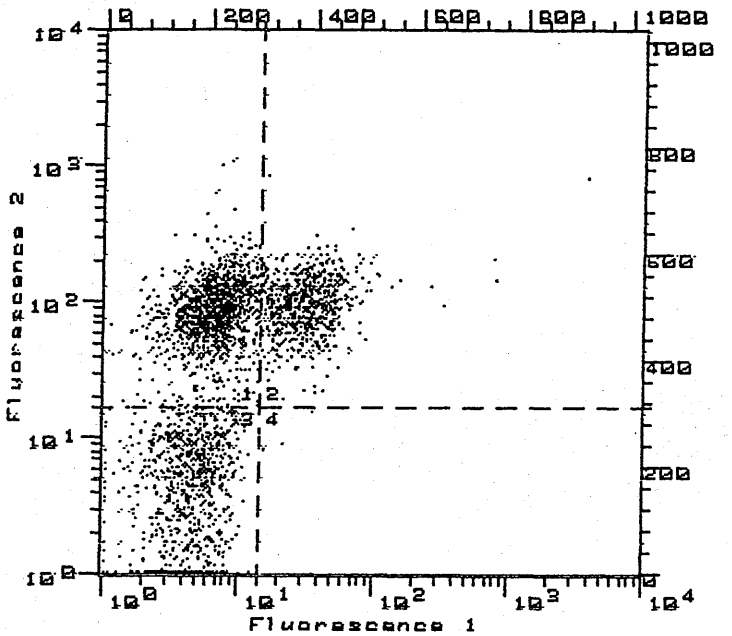
Figure 2.4. Analysis of activation marker expression.

A. Dot-plot display of anti-CD19 (FL2) and anti-IgG staining (FL1). The distinct doubly-stained IgG+ B cells show up on quadrant 2.

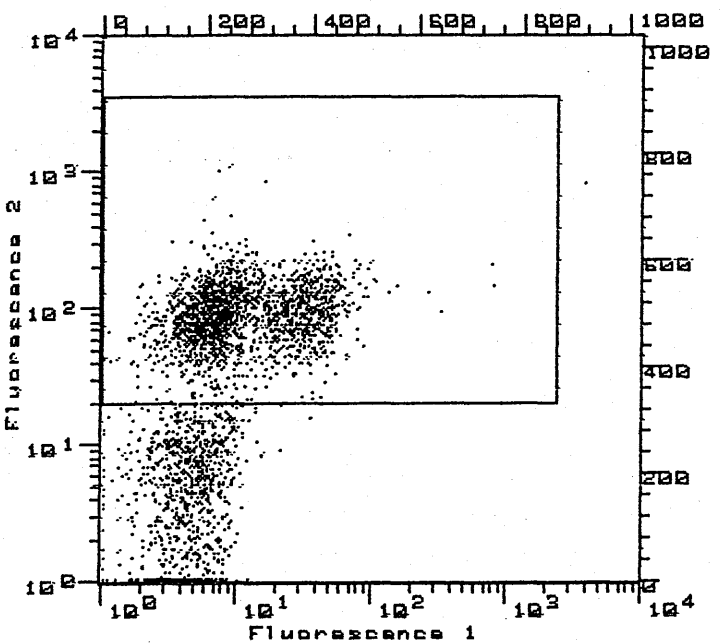
B. The proportion of cells expressing an activation marker (in this case IgG expression on CD19+ cells) is analysed by isolating the particular population on FL2 (phenotype staining).

C. On a histogram on FL1, the proportion og IgG+ B cells is assessed by placing a marker, the position of which is based on the control sample.

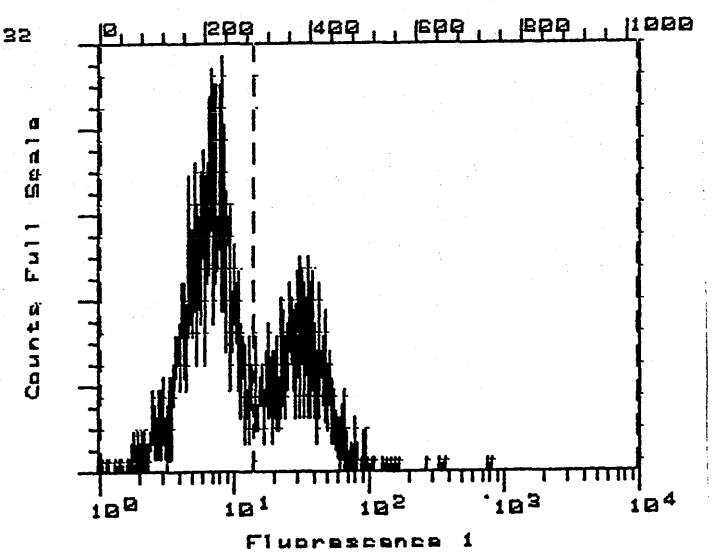
A


Fluorescence 2
Fluorescence 1

B


Fluorescence 2
Fluorescence 1

C


Counts Full Scale
Fluorescence 1

activation marker (FITC-labelled) were then measured on a histogram on FL1. This is illustrated in Fig. 2.4, which shows the measurement of IgG+ B cells.

2.2.2. Sample preparation and flow cytometric analysis of lectin binding

2.2.2.1. Patient samples

32 primary tumour samples, half lymph node samples from 12 lymph node metastases and 8 non-involved lymph nodes were obtained breast cancer patients immediately after surgery at the Western Infirmary, Glasgow. Tissue samples were sliced and the tumour and lymph node cells were spilled by extensive chopping with a scalpel blade and washed in RPMI-1640. The cells were then centrifuged for 5 minutes at 300g. Spilled cells were epithelial in morphology and marker staining with monoclonal antibody to HMFG 2. All samples were analysed for lectin binding on the day the tissues were collected, as freezing and thawing resulted in considerable cell death.

The spillage technique for cell preparation was suitable for flow cytometry as it provided a clean suspension of single cells with very little cell aggregate and debris. Both spilled cells and preparation obtained from collagenase-treated samples have been found to show similar staining for both HPA and Con A (Alam *et al.*, 1990).

Table 2.2. Ingredients for complete growth medium (50 ml).

Heat inactivated Dextran charcoal coated FCS	1.5 ml
Insulin	5 µg/ml
Hydrocortisone (Cortisol)	6×10^{-5} mM
EGF	0.1 µg/ml
Ethanolamine	0.1 mM
Phosphoethanolamine	0.1 mM
Transferrin	5 µg/ml
Prolactin	1 µg/ml
Prostaglandin E1	2.5×10^{-5} mM
Penicillin/Streptomycin	1.0 ml
Fusidic acid	5 µg/ml
RPMI-1640	44.0 ml

The ingredients are added together and then filter sterilised into the RPMI medium. FCS is inactivated at 56 °C for 30 min.

2.2.2.2. Sample preparation of normal breast cells

A sample preparation from reduction mammaplasty served as the source of normal breast tissue. Cells from this tissue sample were cultured by the method of Wolman *et al.* (1985). The tissue sample was sliced and treated with collagenase (15 mg) at 37 °C overnight. After washing in RPMI-1640 medium, the cells were seeded in two 25 cm tissue culture flasks containing the complete growth medium (Table 2.2). Three weeks later considerable growth of breast epithelial tissue was observed in one of the flasks. Cells were removed with brief trypsin treatment and assessed for MHC class I antigens to

ensure that cell surface proteins had not been damaged by this treatment. A single cell suspension from this culture was prepared by passing the cells through a 21G needle (Becton Dickinson).

2.2.2.3. Flow cytometric analysis of lectin binding

FITC-conjugated HPA and biotinylated succinyl Con A lectins were used at a concentration of 1 µg/ml and the staining was carried out as described by Alam *et al.* (1990). Both tumour and lymph node samples were disaggregated immediately after resection into single cell suspensions by mechanical mincing with scalpel blades in a petri dish containing RPMI-1640 medium. Spilled cells from primary tumours and lymph nodes were washed twice in PBS and resuspended at a cell density of approximately 2 x 10^7 cells per ml. 50 µl of this cell suspension was then incubated with 100 µl of the lectin solution on ice for 30 minutes in the dark and then washed twice in PBS. Cell suspensions treated with biotinylated Con A were further labelled with streptavidin-PE solution by adding 10 µl of streptavidin-PE solution per test and then incubated for 20 minutes. All stained cells were washed in PBS and finally resuspended in 500 µl PBS. 10 µl of PI were added to give a final concentration of 2 µg/ml before acquisition to provide live/dead discrimination.

Controls were included with each batch of tissue processed to give autofluorescence of tumour cells. 10,000 events per test were acquired for each tumour samples and 5000 events per test for each lymph node samples. All samples were collected with a live gate to exclude dead cells as described in Section 2.2.1.4. For both primary tumours and lymph node metastases, a live scatter gate on FSC scatter versus SSC scatter was used to exclude the lymphocytes and as such to restrict the analysis to tumour cells alone (Fig. 2.5). This scatter gate was essential with the cell suspension from lymph nodes as it invariably contained a high percentage of lymphocytes. Near

absence (< 5%) of lymphocytes in the acquired lymph node samples was confirmed using the leucogate antibody.

The specificity of the lectins were assessed by sugar inhibition test. The appropriate sugar (0.1M) was added to a separate tube which was otherwise treated as above.

2.2.2.4. Fluorescence microscopy

For examination of lectin binding by fluorescence microscopy, tumour cell suspensions were stained as described for flow cytometry (Section 2.2.2.3) except that the lectins were used at a concentration of 10 μg/ml. Stained cells were then applied to multispot microscopic slides, which were previously washed with ethanol and air dried. The slides were examined in a Leitz Orthoplan fluorescent microscope.

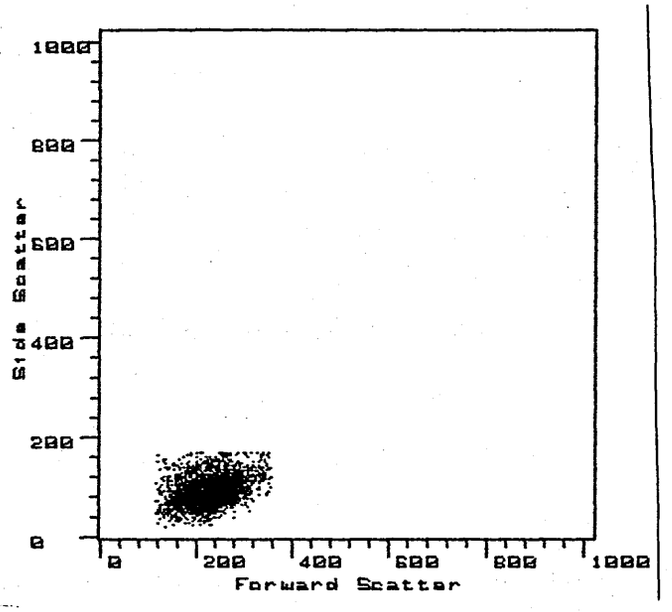
Figure 2.5. Analysis of tumour cells for lectin binding.

a) Dual parameter dot-plot display of FSC and SSC of lymph node lymphocytes.

b) Scatter plot of a cell suspension from a lymph node, showing the gate drawn to exclude lymphocytes.

All surface marker analysis of tumour cells (from both primary tumours and lymph node metastases) were carried out using this gate.

a


1000
800
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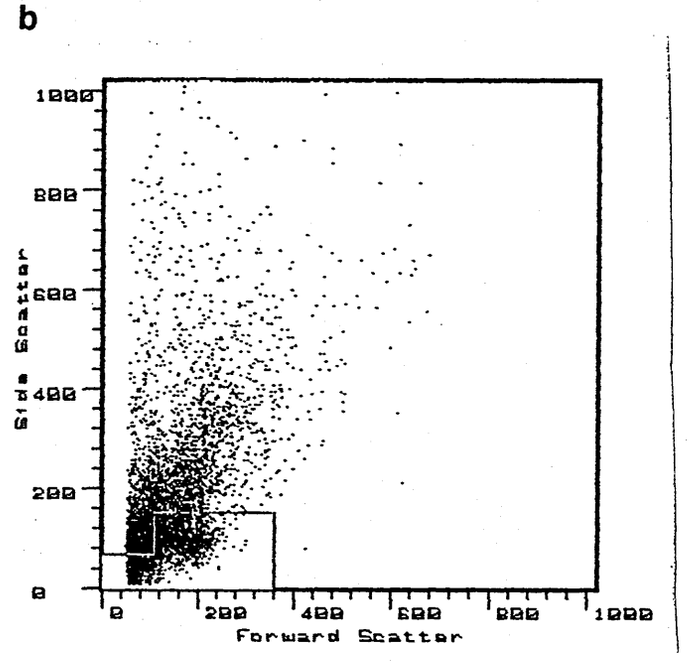
400
200
0
Side Scatter
0
200
400
600
800
1000
Forward Scatter

b


1000
800
600
400
200
0
Side Scatter
0
200
400
600
800
1000
Forward Scatter

2.2.3. Flow cytometric DNA analysis

2.2.3.1. Patient samples

Fresh, primary tumour and axillary lymph node samples were obtained from 57 consecutive patients undergoing definitive surgery for breast carcinoma in the Western Infirmary, Glasgow. 31 of these patients had metastasis in their lymph nodes while the remaining 26 lymph nodes were free of tumour. Involvement of lymph nodes was confirmed by histological examination of matching halves of nodes sent to pathology.

2.2.3.2. Sample preparation from primary tumours and lymph nodes

Cell suspensions from both primary tumours and lymph nodes were prepared by mechanical disaggregation as described in Section 2.2.2.3. The cells were washed and resuspended in citrate buffer at a cell density of 5×10^5 cells per test.

2.2.3.3. Preparation of nuclei

Nuclei for flow cytometric DNA analysis were prepared and stained according to the method of Vindelov *et al.* (1983). This involved the treatment of the cell suspension with solution A (trypsin, Section 2.1.6) and the contents were mixed by inverting the tube. The suspension was incubated for 10 minutes at room temperature, during which the tube was inverted five or six times. This was followed by a further 10 minutes incubation with solution B (trypsin inhibitor and RNAse, Section 2.1.6) and again the solutions were mixed by inversion of the tube. Finally, the cells were stained with PI by adding ice-cold solution C (Section 2.1.6) which also contained spermine

All samples were analysed twice to assess DNA ploidy. The CV (coefficient of variation) of the aneuploid peaks (Go/G1) of tumour cells ranged from 2.0 to 7.0. Any sample with an aneuploid peak showing a CV greater than 10 was not included in the study.

tetrachloride. At this stage the tubes were incubated in the dark at 0 °C until analysed 15-60 minutes later.

Chicken red blood cells (CRBC) were stained identically in a separate tube and then added to an amount no more than 10% of the total acquisition. CRBC peak served as internal standard and was set at FL2 channel 24-26. PBL were also available from each patient and these were stained and analysed separately with each sample being evaluated for DNA content.

2.2.3.4. DNA analysis by flow cytometry

10,000 nuclei were acquired per sample on a FACScan flow cytometer (Becton Dickinson) and as described in Alam *et al.* (1992b). DNA content was confirmed to be diploid if the Go/G1 peak of blood lymphocytes was superimposable with that of the tumour sample. Any additional peak was considered aneuploid if the suspected aneuploid G0/G1 peak contained at least 20% of the total sample events and the corresponding G2/M peak was also detectable. This was done to ensure that the detected peak was well above background and was clearly representative of a distinct population. The DNA index (DI), which is a measure of aneuploidy, is calculated as the ratio of the peak channel number of the aneuploid G0/G1 peak to the peak channel number of the diploid G0/G1 peak.

2.2.3.5. Measurement of SPF

Various alternatives were available for the measurement of SPF of primary tumours. Two computer-run models for SPFmeasurement, the Polynomial model and the SOBR (Sum of broadened rectangles) model were available with the DNA software. Alternatively, SPF can also be assessed by placing markers manually to define the SPF region and then the percentage of cells under the region can be calculated on the computer.

Table 2.3. Calculation of SPF using the computer-run polynomial model, sum of broadened rectangles model (SOBR) and manually by placing markers.

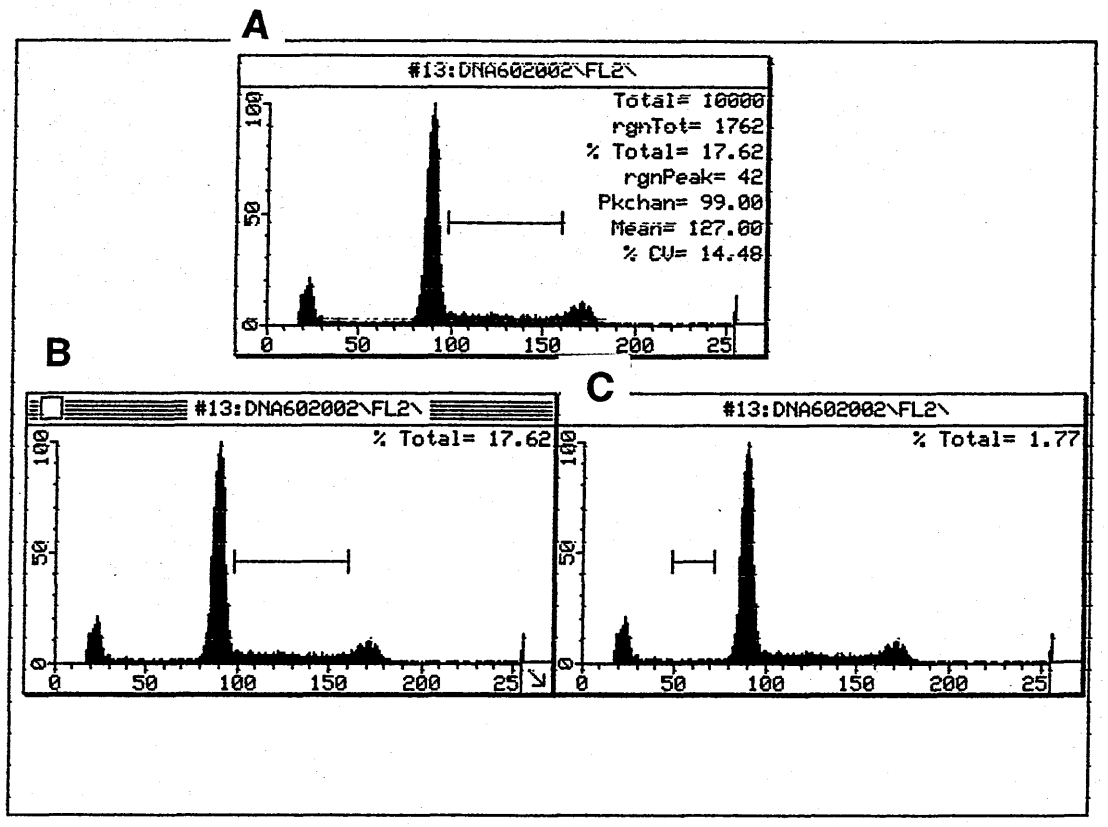
Tumour	Method	SPF
Diploid	Polynomial	7%
	SOBR	2%
	Manual	2.04%
Aneuploid	Polynomial	7%
	SOBR	6%
	Manual	3.3%

Both the models were unsuitable in cases where there was overlapping of peaks. The SOBR model offered more flexibility, while the polynomial model was highly restricted to well-defined single cell populations. The limitation of SOBR was observed in tumours with high SPF and G2/M cells. In these cases, SPF could not be calculated as the histogram did not fit the model. It was also observed that the two model at times gave different values for SPF (Table 2.3).

In the light of these difficulties, the computer-run program was not used in assessing SPF of primary tumours. The measurement of SPF was carried out by placing markers manually. SPF were measured for all diploid tumours and for a limited number of aneuploid tumours, 9 cases where there was no complication from overlapping peaks and in 4 tumours, where the diploid population was gated out using CK-antibody. The manual measurement of SPF was carried out as described by Visscher *et al.* (1990b) and is illustrated in Fig. 2.6. It shows that S-phase has been defined by assuming that both Go/G1 and G2/M are gaussian distribution. Markers are

Figure 2.6. Manual SPF determination.

Distribution limits are set with markers to define the S-phase (A). This is done by assuming that both Go/G1 and G2/M peaks are gaussian distribution. The region within the S-phase is calculated on the computer (B). Next, a separate distribution region range for background fluorescence is also set. Background events are then substracted from the total events found in the S-phase to give the true SPF (C).


A
#13:DNA602002\FL2\
Total= 10000
rgnTot= 1762
% Total= 17.62
rgnPeak= 42
Pkchan= 99.00
Mean= 127.00
% CV= 14.48
B
#13:DNA602002\FL2\
% Total= 17.62
C
#13:DNA602002\FL2\
% Total= 1.77

also set to assess background and this is substracted from the S-phase region. The background was at times found to be variable and appears to depend on the quality of the preparation. Its substraction, therefore, gives the true relative proportion of cells in the S-phase.

2.2.3.6. Dual parameter DNA flow cytometry with PI and CK staining

It was important to exclude non-epithelial cell components, which includes leukocytes, stromal fibroblasts and endothelial cells, from both primary tumour and lymph node metastases. This was achieved in a multi-parametric two-colour FCM analysis using an FITC-labelled antibody to CK (CAM 5.2-FITC). The CAM 5.2 monoclonal antibody detects CK8, CK18, and CK19, which are CK peptides consistently present in all layers of normal and malignant mammary epithelium (Moll *et al.*, 1982; Moll *et al.*, 1988; Ramaekers *et al.*, 1984). Gating for CK positivity allowed DNA analysis to be restricted to epithelial cells and this allowed both the determination of the true identity of diploid cells in primary tumours and also to determine the ploidy of small proportions of tumour cells in lymph node metastases.

Cell suspensions from both primary tumours and lymph nodes were fixed in ethanol according to the method of Zarbo *et al.* (1989). 12 paired primary tumours and lymph node samples from the same breast cancer patients were spilled as described above in Section 2.2.2.3. The washed cells were resuspended in 1 ml of RPMI-1640 plus 1 ml of FCS. 6 ml of cold 70% ethanol was then added slowly, dropwise, while vortexing, to give a final ethanol concentration of 50%. Ethanol fixation preserved the antigenicity of cytoplasmic and surface markers while at the same time creating access for nuclear DNA dye (PI) penetration (Zarbo *et al.*, 1989). Ethanol-fixed cells were then washed twice and resuspended in PBS.

The efficiency of ethanol-permeabilisation may be tested by staining of ethanol-treated cells with anti-CK antibody. These stained cells may then be counted by fluorescence microscopy.

Staining of the cells were carried out according to the method of Zarbo *et al.* (1989) and as described in Alam *et al.* (1992b) a directly labelled anti-CK-FITC antibody (Becton Dickinson) was used. Cells were first treated with anti-CK-antibody, incubated on ice and kept in the dark for 20 minutes. After two washes in PBS, 1 ml of PI (0.05 mg/ml) and 100 µl of RNAse (1 mg/ml) were added to the sample in each tube and incubated at room temperature for 30 minutes prior to analysis on FACScan. Cells stained with FITC-mouse IgG of irrelevant specifcity served as a green fluorescence negative control, while those treated with PBS was used as an autofluorescence control.

2.2.3.7. Dual-parameter analysis using a CK+ gate

Gating for CK-positive (CK+) cells and analysis of a DNA histogram is illustrated in Fig 2.7 and 2.8. CK+ cells were identified during analysis by comparing single-parameter FL1 (green) intensity to negative controls. Next a gate was drawn on FL1 (CK-FITC) versus FL2 (PI stain) to include only the cells positive for CK (Fig. 2.8). DNA profile analysis (FL2 parameter) was pereformed as described above. When primary tumours were analysed for CK+ cells, diploid peaks were always reduced and in some cases (Fig. 2.8) to a negligible level.

2.2.3.8. Sensitivity test of the dual-parameter DNA analysis.

Sensitivity tests of the dual-parameter analysis using CK was performed using a breast tumour cell line (ZR-75). ZR-75 cells are CK+ and aneuploid with a DI of 1.65. To determine the limit of detection of CK+ cells, ZR-75 cells were mixed with human PBL at following proportions: 30% of total cells, 5% of total cells, 3% of total cells, 1% of total cells, and 0% of total cells (PBL alone). Ethanol fixation, staining and analysis of CK+ cells was carried out as described above in Section 2.2.3.6. and 2.2.3.7.

Figure 2.7. Multiparametric flow cytometric DNA analysis

Ethanol-fixed intact breast tumour cells were labeled with anti-cytokeratin-FITC and then stained with PI for DNA.

A. FL1 versus FL2 dot-plot showing CK-positive (green population) tumour cells and CK-negative population (red).

B. FSC versus FL1 plot showing CK-positive (green) tumour cells and CK-negative lymphocytes (red).

C. Single-parameter DNA histogram showing CK-negative and CK-positive cell populations having diploid (red) and aneuploid (green) DNA content respectively.

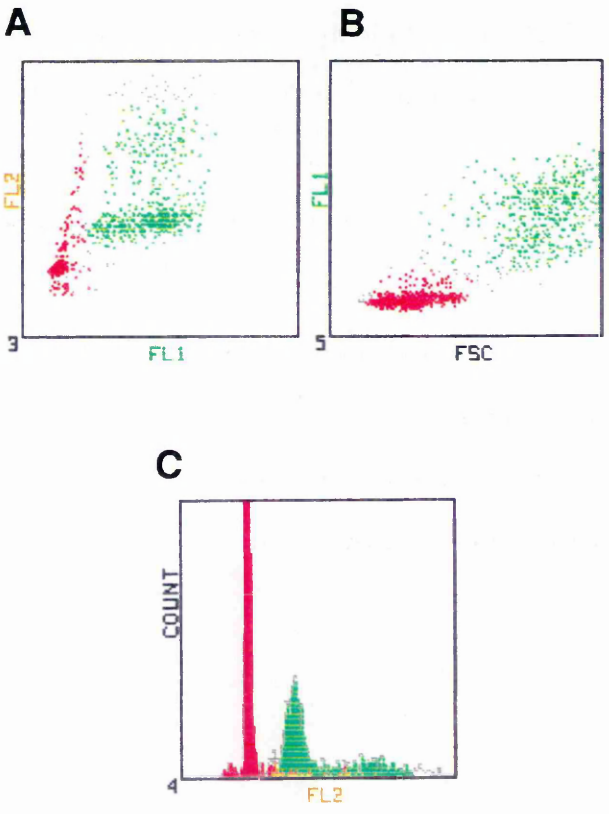

A
B
FL2
FL1
3
FL1
FL1
FSC
5
C
COUNT
4
FL2

Figure 2.8. Dual-parameter gating for CK-positive cells.

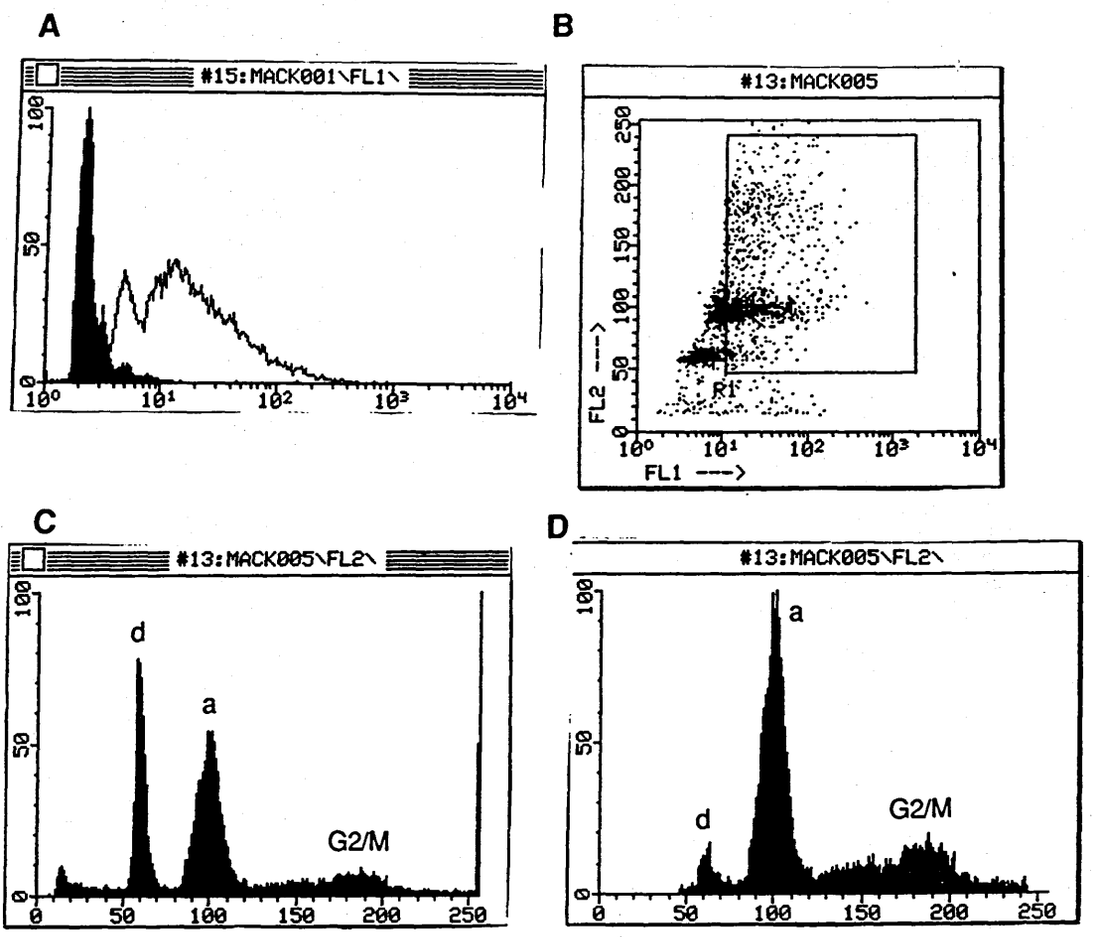
A. The reaction of the anti-CK antibody with tumour cells (unshaded area) overlaid on the reaction with lymphocytes (shaded area).

B. A flow cytometric "gate" (area within box) drawn on the plot of FL1 (green-CK+) and FL2 (red-PI) to analyse the ploidy status of CK+ cells only.

C. An ungated DNA histogram of a primary tumour showing a diploid population (d) and an aneuploid population (a= Go/G1 peak, G2/M peak).

D. A CK-gated histogram of the same tumour showing CK+ epithelial cells. The diploid peak (d), representing non-epithelial cells, has been largely gated out.

d= diploid; a= aneuploid; G2/M= G2/M peak of the aneuploid population.


A
#15:MACK001\FL1\
B
#13:MACK005
FL2 --->
FL1 --->
R1
C
#13:MACK005\FL2\
d
a
G2/M
D
#13:MACK005\FL2\
d
a
G2/M

2.2.3.9. Growth of cells from primary breast tumours

4 primary breast tumours were put into short-term culture. Tissue slices were mechanically disaggregated as described in Section 2.2.2.1 and culture of these cells were carried out as described in Section 2.2.2.2.

2.2.4. DNA fingerprinting of primary tumours and lymph node metastases

2.2.4.1. Extraction of DNA

High MW DNA from primary tumours, lymph nodes and PBL from 20 breast cancer patients was extracted according to the method of Miller *et al.* (1988). This involved twice washing spilled tumour, lymph node cells and PBL from the same patient in PBS. About 1 x 10^6 cells were resuspended in 1 ml of nuclei lysis buffer. To this 66 µl of 10% SDS were added with gentle mixing, then 167 µl proteinase K solution was added. The cell extract was incubated overnight at 37 °C with gentle shaking and then decanted into Falcon tubes and 0.33 ml of saturated NaCl was added. The contents were mixed for 15 secs by shaking the tube fairly vigorously. Next the tube was centrifuged at 800g for 6 min. The supernatant was removed carefully and the DNA was precipitated by adding exactly 2 volume of ethanol at room temperature. Strands of DNA were then spooled and transferred to an Eppendorf tube and washed in 70% ethanol. The DNA precipitate was dried briefly under vacuum and then allowed to resuspend in 200 µl TE buffer by incubating the tube at 37 °C. Finally, the DNA solution was transfered into a collodian sac (Sartorius) and dialysed overnight against TE buffer at 4 °C.

DNA extracted by this method was of high MW and gave a yield ranging from 25 to 50 µg per 1x10^6 cells. The 260/280 ratio was generally about 1.9.

2.2.4.2. Satellite DNA probes

Minisatellite probes 33.6 and 33.15 were obtained as inserts cloned into stable plasmid vectors pUC18 and pUC19 respectively and were removed by EcoRI-Hind III digestion. The inserts 33.6 and 33.15 are of sizes 0.72kb and 0.60 kb respectively (Jeffreys *et al.*, 1985). The recombinant plasmids were transformed into an appropriate host and from bulk growth of these cells the insert DNA was excised from purified plasmid DNA on gels. Finally insert DNAs were purified. This is described in Section 2.2.4.2.1.

Multi-locus satellite probes 216S and 228S were inserts obtained from recombinant M13 phage DNA. The inserts were subcloned into a more convenient plasmid vector and recovered in large amounts. The amplification and purification of these DNA satellite inserts are described in Section 2.2.4.3.

2.2.4.3. Amplification and purification of minisatellite sequences 33.6 and 33.15

2.2.4.3.1. Transformation of host *E.coli* cells

E.coli strains JM101 and DS941 were found to be suitable hosts for plasmids carying DNA probes 33.6 and 33.15 respectively in that they reproducibly yielded inserts of correct size.

Host cells were grown overnight in L-broth at 37 ^{o}C. These were then subcultured into pre-warmed L-broth, shaken at 37 ^{o}C and allowed to grow to the early log phase (2-4 x 10^{7} cells/ml).

Transformation was carried out by the method of Chung & Miller (1988). The bacterial cells were harvested by centrifugation (1000g for 10 mins at 4 ^{o}C) and resuspended in 1/10th volume of transformation and storage buffer (TSB) at 4 ^{o}C, and incubated on ice for approximately 10 mins. For

transformation, 0.1 ml aliquots of the cells were pipetted into cold sterile tubes and mixed with 100 pg of plasmid DNA. The tubes were then put on ice for 5-30 mins. Next, 0.9 ml TSB containing 20 mM glucose was added and the cells incubated at 37 ^{o}C with shaking for 60 mins. Finally, 0.1 ml of these cells were plated on ampicillin-containing (100 µg/ml) agar plates and grown overnight at 37 ^{o}C. Control plates with and without ampicillin were also set up. While there was no growth in the negative controls (nontransformed cells in the presence of ampicillin), colonies of transformants appeared for each recombinant plasmids.

2.2.4.3.2. Plasmid mini-prep

It is important to test a number of transformants to check that they contain the full length probe fragment, especially as the cloned repetitive DNA may be unstable. DNA prepared by a mini-prep method is suitable for this purpose. Three colonies were picked from both 33.6- and 33.15-plasmid containing hosts and grown in 1 ml of ampicillin-containing L broth in an Eppendorf tube with good aeration. The protocol of Birnboim & Doly (19) for a plasmid mini-prep was followed. Overnight cultures of bacterial cells were pelleted by centrifuging for 15 secs. All medium was removed and the pellet was resuspended in 100 µl solution I (Section 2.1.7.2) by vortexing and kept at room temperature for 5 mins. 200 µl of solution II (Section 2.1.7.2) was added and mixed by rocking tube sharply. After incubation on ice for 5 mins, 150 µl of precooled solution III (Section 2.1.7.2) was added and the contents of the tube mixed by gently inverting the tube. Following incubation on ice for 5 mins, the tube was spun in a microfuge for 1 min. The supernatant was carefully removed, transfered to a fresh tube and washed with 500 µl 70% ethanol. The tube was spun for 1 min and the supernatant carefully removed. The plasmid pellet was dried briefly under vacuum and resuspended in 25-30 µl TE buffer by vortexing for 5 min.

2.2.4.3.3. Restriction digestion and electrophoretic analysis of digests

2-3 µl aliquots from the mini-prep were digested with enzymes EcoRI and Hind III according to the manufacturer's instructions. The enzymes were used at 5-10 units per reaction (usually 0.5 µl/reaction) and the reaction was usually carried out in a total volume of 20 µl at 37 °C for 45 min. After which 1 µl of boiled RNAse (0.5 µg/µl) was added to each tube and incubated at 37 °C for 15 mins. Standard DNA markers were heated to 68-72 °C for 5 min and quenched on ice to disrupt the hydrogen-bonding of the lambda DNA cohesive ends. Finally, 1 µl 0.4M EDTA and 2 µl loading buffer was added to each 20 µl digest and the digested DNA fragments were separated by electrophoresis in a 1% agarose gel containing TBE buffer and ethidium bromide (0.2 µg/ml). Gel electrophoresis was carried out as described in Sambrook *et al.* (1989). Gels were run at approximately 100 volts until the bromophenol blue dye front was near the bottom of the gel. Polaroid photographs were taken under uv light with an orange filter. Both the 0.72 kb (33.6 insert) fragment and the 0.6 kb (33.15 insert) fragments were detected (Fig.2.9) and it was, therefore, adjudged that the the plasmid was stable in that particular host.

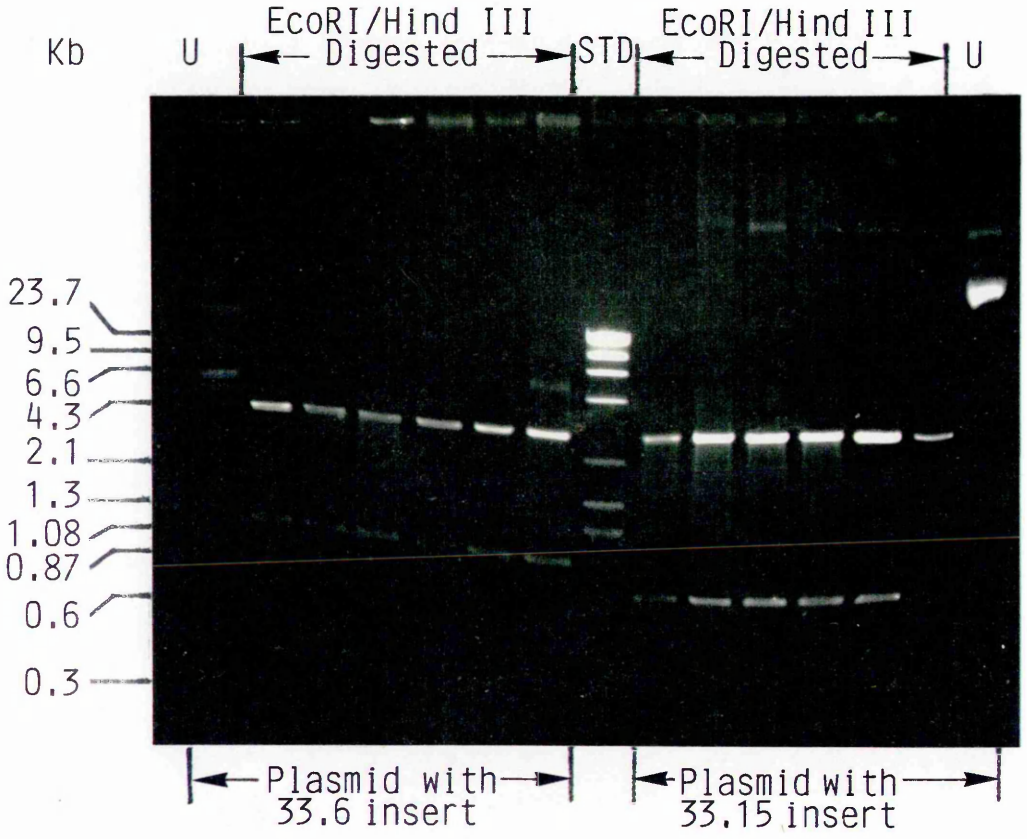
2.2.4.3.4. Bulk preparation of plasmid DNA carrying DNA inserts 33.6 and 33.15

Bulk DNA preparations were made from recombinants in suitable hosts. Colonies were picked from plates into 10 ml L broth containing ampicillin (100 µg/ml) and incubated at 37 °C with shaking overnight. These cells were subcultured by transferring the culture to 500 ml L broth plus ampicillin in 2 liter indented flasks and incubated overnight with shaking. The cells were then pelleted by centrifuging at 5000g for 5 min at 4 °C. The

Figure 2.9. Isolation of mini-satellite sequences 33.6 and 33.15.

Electrophoresis of EcoRI + Hind III digested recombinant plasmid DNA show 0.72 kb and 0.6 kb inserts representing minisatellite sequences 33.6 and 33.15 respectively. U represents undigested plasmid DNA. The first three digested lanes (from left) are plasmid DNA extracted from DS941 and the last three lanes (from left) are from JM101 strains.

ST represents standard DNA markers.


Kb
U
EcoRI/Hind III
Digested
STD
EcoRI/Hind III
Digested
U
23.7
9.5
6.6
4.3
2.1
1.3
1.08
0.87
0.6
0.3
Plasmid with
33.6 insert
Plasmid with
33.15 insert

supernantant was decanted and the buckets were wiped well to remove as much of medium as possible. The pellet was gently resuspended in 18 ml solution I (Section 2.1.7.2) and incubated at 0 ^{o}C for 30 mins. 40 ml of solution II (Section 2.1.7.2) was then added and the contents were swirled gently before incubating on ice for 5 mins. Next, 20 ml of cold solution III (Section 2.1.7.2) was added and the contents mixed gently and incubated on ice for 15-60 mins. The suspension was then centrifuged at 9000g for 5 mins at 4 ^{o}C. The supernatant was poured through gauze into a measuring cylinder to remove floating clumps. 0.6 volumes isopropanol was then added to the supernatant and left at room temperature for 15 mins. After centrifuging at 9000g for 10 mins at room temperature, the supernatant was drained well and the pellet resuspended in 5 ml of 50 mM Tris-HCl, pH 8, 10 mM EDTA.

For equilibrium centrifugation in CsCl-ethidium bromide gradients, it is necessary to adjust the refractive index of the plasmid DNA-CsCl solution. Solid CsCl and 0.5 ml of ethidium bromide (10 mg/ml) were added to the DNA solution. The refractive index (RI) of each DNA preparation was adjusted using a sodium lamp refractometer to 1.3902. The DNA solutions were then transfered to ultracentrifuge tubes and the tubes were accurately balanced by layering paraffin oil on top. The tubes were then centrifuged at 100,000g for 40 hr at 25 ^{o}C in a Beckman L7 ultracentrifuge. The ethidium bromide staining lower plasmid bands (supercoiled DNA) were visible without use of uv light, while RNA was pelleted.

The top layer was carefully pipetted out and the lower plasmid band was carefully removed. This was then extracted with n-butanol saturated with TE (10 mM Tris, pH 8, 1 mM EDTA) at least 5 times or until all traces of ethidium bromide were removed. 2 volumes of water was added and mixed well, followed by the addition of 2.2 volumes of ethanol and the contents were mixed well. The plasmid DNA solution was left overnight at -20 ^{o}C. The DNA was then pelleted by centrifuging at 14000g for 10 mins at 4 ^{o}C. The

supernatant was decanted and the pellet resuspended in 3 ml TE buffer. 1.5 ml 7.5M ammonium acetate was then added and the contents mixed well. This was followed by the addition of 10 ml of ethanol and the contents again mixed and incubated overnight at -20 ^{o}C. The supernatant was drained completely after centrifuging at 14000g for 10 mins. The tube was then rinsed in 70 % ethanol and centrifuged again at 14000g for 10 mins and decanted and drained completely. Finally, the pellet was dried briefly under vacuum and resuspended in 0.5 ml TE buffer.

2.2.4.3.5. Gene-clean purification of DNA sequences 33.6 and 33.15

In order to use the DNA satellites as probes for DNA fingerprinting, it was necessary to excise and purify each of the inserts from the vectors carrying them. Purification of the satellite inserts were carried out using the Gene-clean purification kit. 150 µg each of plasmid DNA with insert 33.6 and 33.15 were digested with EcoRI and Hind III according to the manufacture's instructions and as described in the previous section. The digested DNA fragments were then separated by electrophoresis on 1% agarose gel in TAE. The 0.72 kb fragment and the 0.60 kb fragment representing satellite DNA 33.6 and 33.5 respectively were excised from the gel with a sterile scalpel blade. The excised bands were then purified using the Geneclean II kit and following the manufacturer's instructions. The excised bands were weighed and cut into small cubes and 3 volumes of NaI stock solution was added and the tube incubated for 5 mins at 45 to 55 ^{o}C to dissolve the agarose. Then an appropriate amount of Glassmilk suspension (5 µl of Glassmilk suspension to solutions containing 5 µg DNA and an additional 1 µl for each 0.5 µg of DNA above 5 µg) was added and the contents incubated for 5 mins to allow DNA to adsorb to the glass milk. The glassmilk/DNA complex was then pelleted by centrifuging for 5 seconds. The supernatant was removed and set aside and

the pellet washed 3 times with NaCl/ethanol/water wash (New Wash). Finally, the DNA was eluted by incubating in 2 x 15 µl TE buffer for 5 min at 50 °C. The final recovery of DNA probes is shown in Table 2.4. Aliquots of purified satellite DNAs were run on a agarose gel and their sizes verified (Fig. 2.11)

2.2.4.4. Purification of satellite DNA sequences 216S and 228S

2.2.4.4.1. Tranformation of *E. coli* with phage DNA carrying 216S and 228S inserts

From the supplied recombinant M13 phage DNA, it was necessary to grow up bulk stock of the insert DNA. Preparation of competent host cells for transformation with phage DNA containing 216S and 228S inserts was carried out using the $CaCl_2$ method (Dagert & Ehrlich, 1979) as the Chung & Miller (1988) method failed to produce any transformants. Dried recombinant phage DNA containing 216S insert was provided as 2 x 5µl from a single plaque, while there were 13 µg of phage DNA carrying 228S insert (5 µl of 2.6 µg/µl). The dried probe DNAs were resuspended in 10 µl of TE buffer (stock DNA). *E. coli* strains MV1190 and JM101 were grown overnight by shaking in 2 x TY medium at 37 °C. 0.5 ml of bacterial cultures were transferred to flasks containing 50 ml 2 x TY media and allowed to grow to log phase at 37 °C. 40 ml of the cell cultures were pelleted by centrifuging at 5000g for 10 mins at 4 °C. The remaining cells were used as non-competent cells to provide background to plaque formation. The supernatant was gently decanted and the cells were resuspended gently in 1/2 volume of cold, sterile 50 mM $CaCl_2$ and kept on ice for 20 mins. The cells were spun down at 5000g at 4 °C and resuspended a second time in 1/16th volume of cold, sterile 50 mM $CaCl_2$. To 0.3 ml of these transformation competent cells were added 1 µl (from a stock diluted 500 x in TE bufer) of 216S and 228S DNA and the cells were

incubated on ice for 30 mins. The cells were then heat treated at 42 °C for 2 mins, after which 100 µl of respective non-competent bacterial cells were added to each tube. To these cells 3 ml of warm top agar was added, mixed gently and poured on H plates. Control cultures without probe DNA and those with non-competent cells were also plated. All plates were incubated overnight at 37 °C. Plaques were observed for both DNA probes and in both JM101 and MV1190 cells. With JM101 as host cells, there were fewer but well separated plaques while numerous plaques all over the plates were observed with MV1190.

2.2.4.4.2. Growth of 216S and 228S "plaques"

Plaques were picked from both JM101 and MV1190 plates using a sterile micropipette and inoculated into a bijoux containing 2 x TY medium and grown overnight at 37 °C with good aeration. Cells were pelleted and a plasmid mini-prep of both DNA probes were prepared as described in Section 2.2.4.2.1. The DNA was digested with EcoRI-Hind III and the fragments separated by agarose gel electrophoresis as described above. The fragment sizes of 210 bp and 330 bp representing DNA probes 216S and 228S respectively were observed in the plasmid DNA isolated from both transformed JM101 and MV1190. The 216S and 228S inserts were excised and purified using the gene-clean purification kit as described above in Section 2.2.4.2.2.

2.2.4.4.3. Subcloning of 216S and 228S inserts

It was desirable to subclone inserts 216S and 228S from recombinant M13 into a more suitable plasmids from which the inserts could be more easily isolated in bulk. The DNA inserts 216S and 228S isolated from M13 recombinants in JM101 and MV1190 were subcloned into vector pTZ18U. pTZ18U plasmids provided a stable vector in both strains of *E. coli*. These plasmids have a polylinker region and contain ampicillin resistance genes

which can be used in transformant selection. Screening of host transformants is made easier as the plasmids also have the lac Z gene, so that in the presence of IPTG and X-gal, non-recombinant clones will produce active β-galactosidase, thus producing blue colonies. But when DNA is inserted in the lac Z gene, no active β-galactosidase is produced, and these clones will be white.

216S (0.21 kb) and 228S (0.33 kb) fragments (10 ng DNA) were ligated to EcoRI/Hind III pTZ18U plasmids in presence of Tris HCl, $MgCl_2$. The fragments were incubated at 16 ^oC overnight in a 10 µl reaction mixture containing 2 µl buffer (2 µl of 5x) and 1 unit DNA ligase enzyme. To monitor that the enzymes and buffers were functional a control was set up; Hind III cut λ DNA was incubated with and without ligase enzyme. After incubation, ligated and unligated samples were run on a gel against markers to check if the ligation had worked.

2.2.4.4.4. Transformation with pTZ18U plasmids ligated with 216S and 228S DNA

E. coli JM101 and DS941 cells were made competent for transformation with $CaCl_2$ as described above (Section 2.2.4.5). 3 µl of 216S- and 228S- ligated plasmid DNA (10 ng/µl) were used to transform 200 µl of competent JM101 cells and the transformation was carried out by the CaCl2 method (Section 2.2.4.4.1). 100 µl of transformed cells per plate were spread on X gal/IPTG plates containing ampicillin (100 µg/µl) and the plates incubated overnight at 37 ^oC. Transformant white colonies in DS941 and JM101 for each for the two inserts were detected.

Transformant colonies were picked from all plates (both JM101 and DS941) and streaked on to ampicillin containing plates and incubated overnight at 37 ^oC. Growth of ampicillin-resistant cells were observed with all colonies picked. These cells were restreaked a second time and the stability of the

Figure 2.10. Isolation of satellite sequences 216S and 228S.

Electrophoresis of EcoRI + Hind III digested (D) recombinant plasmid DNA show 0.21 kb and 0.33 kb inserts representing minisatellite sequences 216S and 228S respectively. D represents undigested plasmid DNA. The first D lane and the last D lane (from left) are plasmid DNA extracted from DS941 and the remaining lanes represent plasmid extracted from JM101 strains.

ST represents standard DNA markers.


Kb
U D U D U STD D U D U D
23.7
9.5
6.6
4.3
2.3
2.1
1.3
1.08
0.87
0.6
0.3
Plasmid with 216S insert
Plasmid with 228S insert

transformants was checked. Mini-prep DNA was digested with EcoRI+Hind III and electrophoresed on agarose gel. The 210 bp band of probe 216S and 330 bp band of probe 228S were detected (Fig. 2.10).

2.2.4.4.5. Gene clean purifications of DNA fragments 216S and 228S

Plasmid DNA containing 216S and 228S inserts were extracted from bulk cultures of 500 ml of recombinant cells as described for probes 33.6 and 33.15. 150 µg of plasmid DNAs were digested with EcoRI+Hind III and the satellite DNA fragments were excised and purified using the geneclean kit as described above. The final recovery of the satellite DNAs is listed in Table 2.4. Aliquots of purified satellite DNA were run on a gel and the sizes verified (Fig.2.11).

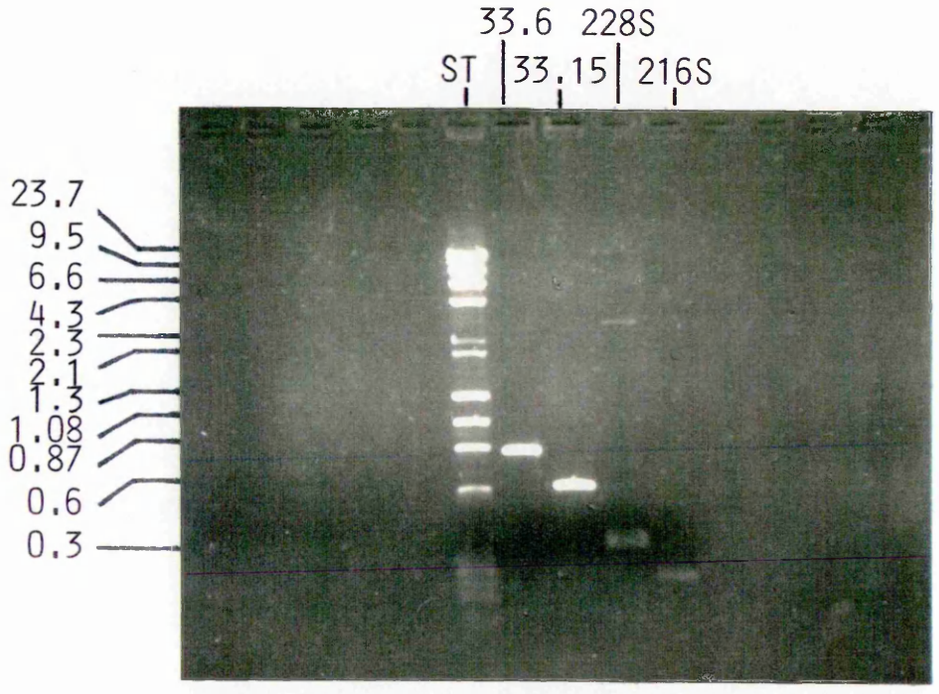
Table 2.4. Recovery of satellite DNA probes after geneclean purification.

Probe	Insert size (in base pairs)	Amount of insert recovered	recovery* (as % of expected)
33.6	720	22.5 µg	56
33.15	600	30 µg	86
216S	210	6 µg	65
228S	330	12 µg	69

*Recovery after gene-clean purification of excised satellite inserts from 150 µg of plasmid DNA.

Figure 2.11. Gene clean purified satellite DNA sequences 33.6 (0.72 kb), 33.15 (0.6 kb), 28S (0.33 kb) and 16S (0.21 kb).

ST represents standard DNA markers.


33.6
228S
ST
33.15
216S
23.7
9.5
6.6
4.3
2.3
2.1
1.3
1.08
0.87
0.6
0.3

2.2.4.5. Sequencing of satellite DNA

The purification of satellite DNAs involved a number of manipulations which then generated recombinant plasmids carrying the desired sequences. This is evident in the case of satellites 216S and 228S, which were originally inserts in M13 phage and were finally isolated as pTZ18U recombinants. Nucleotide base sequences for all the isolated DNA satellites were therefore analysed to check the conformity of the isolated sequences with those of the reported sequences.

2.2.4.5.1. Sequencing reaction

Purified plasmid DNAs carrying the desired satellite DNAs were used for sequencing. 3 µg of plasmid DNA in 9 µl of TE buffer was denatured by adding 1 µl of 2M NaOH and incubating for 15 min at 37 °C. To anneal the sequencing primer (Universal & Reverse) to the denatured template 10 pmoles (1 µl of a 10µM stock) of primer was added and briefly vortexed prior to neutralisation of the NaOH with 3M potassium acetate (3 µl). The DNA was then ethanol precipitated (75 µl) overnight at -20 °C. Following centrifugation in a microfuge for 10 min, the pellet was washed with 100 µl of 75% ethanol and finally dried under vacuum.

A commercial "Sequenase" kit (USB) was used for sequencing and the reaction carried out according to manufacturer's instructions.

Sequenase buffer 5x: 200 mM Tris HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl.

Labelling mix: 15 M dITP; 7.5 M dCTP; 7.5 M dTTP

Enzyme dilution buffer: 10 mM Tris HCl, pH 7.5; 5 mM DTT; 0.5 mg/ml BSA.

Stop solution: 95% formamide; 2 mM EDTA; 0.05% EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF.

2.2.4.5.2. Annealing mixture

Annealing reaction was carried out in a 10 µl mixture containing 7 µl DNA (denatured), 2 µl sequencing buffer and 1 µl of primer. The mixture was heated at 65 ^{o}C for 2 min and then slowly cooled to less than 35 ^{o}C. While the mixture was cooling, tubes were labelled and filled with 2.5 µl of each termination mixture. 4 termination tubes (dATP, dGTP, dCTP, dTTP) were set up for each sample to be sequenced. Labelling mix was diluted 1:4 (6 µl mix + 24 µl water) and enough of Sequenase (3 µl + 21 µl ice cold TE) was diluted 1:7 for all templates. The sets of 4 termination tubes were prewarmed in 37 ^{o}C water bath.

2.2.4.5.3. Labelling reaction

To annealed DNA mixture (10 µl) was added 1µl DTT (0.1M), 2 µl diluted labelling mix, 0.5 µl [^{35}S]-dATP and 2 µl diluted Sequenase. The contents were mixed and incubated at room temperature for 5 min.

2.2.4.5.4. Termination reaction

3.5 ml of labelling reaction was transferred to each termination tube (A,G,C,T), the contents were mixed and incubation at 37 ^{o}C was continued for another 5 min. Finally the reaction was stopped by adding 4 µl stop solution.

Scrupulously clean glass plates were taped together on three sides. A 50 ml urea/acrylamide gel was prepared. The gel was pre-run for 3 min to warm before loading samples. All samples were heated to 90 ^{o}C for 15 min and then 3 µl were immediately loaded on to the gel. Gels were run at 4 mA and 1.4 kV constant power. Two sets of gels were run for each sample: a short gel run for 3 h to allow the first dye, bromophenol blue (runs at 18 nucleotides) to reach the bottom of the gel, and a long gel was run for for 6 h which allows both dye fronts to run off the gel (dye xylene cyanol runs at 70 nucleotides). After the run was complete glass plates were prized apart and the gel washed in 10% glacial acetic acid, 10% methanol for 10 min. This treatment allows urea to diffuse out and fixes the DNA in the gel. A dampened piece of Whatman 3MM paper was then placed over it and the gel transferred on to the paper. The gel was then covered in Saran wrap and dried in a gel-dryer for 1-1.5 h. The gels were finally autoradiographed for 1-4 days at room temperature.

2.2.4.5.5. Sequence analysis

Sequences were analysed from both the short 3h and the long 6h gels and overlapping sequences from both these gels were linked where possible. All sequences were analysed and compared to those in the EMBL database. Complete sequences for satellite DNAs 216S and 228S were available from Fowler *et al.* (1988) and Fowler *et al.*, (1987) respectively. Sequences for minisatellites 33.6 and 33.15 were obtained from a computer search and then compared on the computer. For all 4 analysed satellite sequences good alignments with reported sequences were observed. Nucleotide base sequences for satellite 228S and 216S are shown in Fig. 2.12. The computer print-out of analysed sequences and its alignment with the reported sequences are given in Fig. 2.13a and 2.13b.

Figure 2.12. Sequencing of satellite DNA 228S and 216S.

The sequencing was carried out as described in Section 2.2.4.5. The figure shows the 3-hr gel.

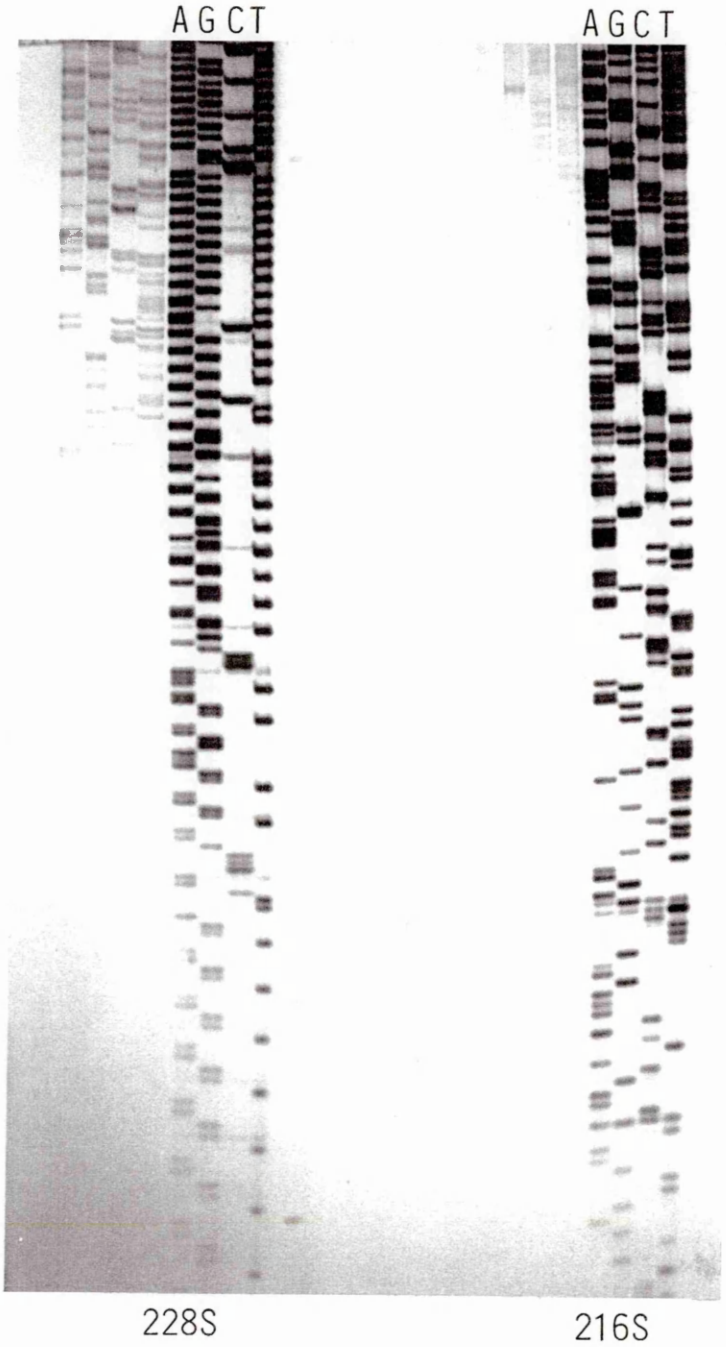

A G C T
A G C T
228S
216S

Figure 2.13a. Sequencing data on satellite DNA 228S.

The nucleotide base sequences as obtained in this study was compared with sequence data obtained from EMBL database

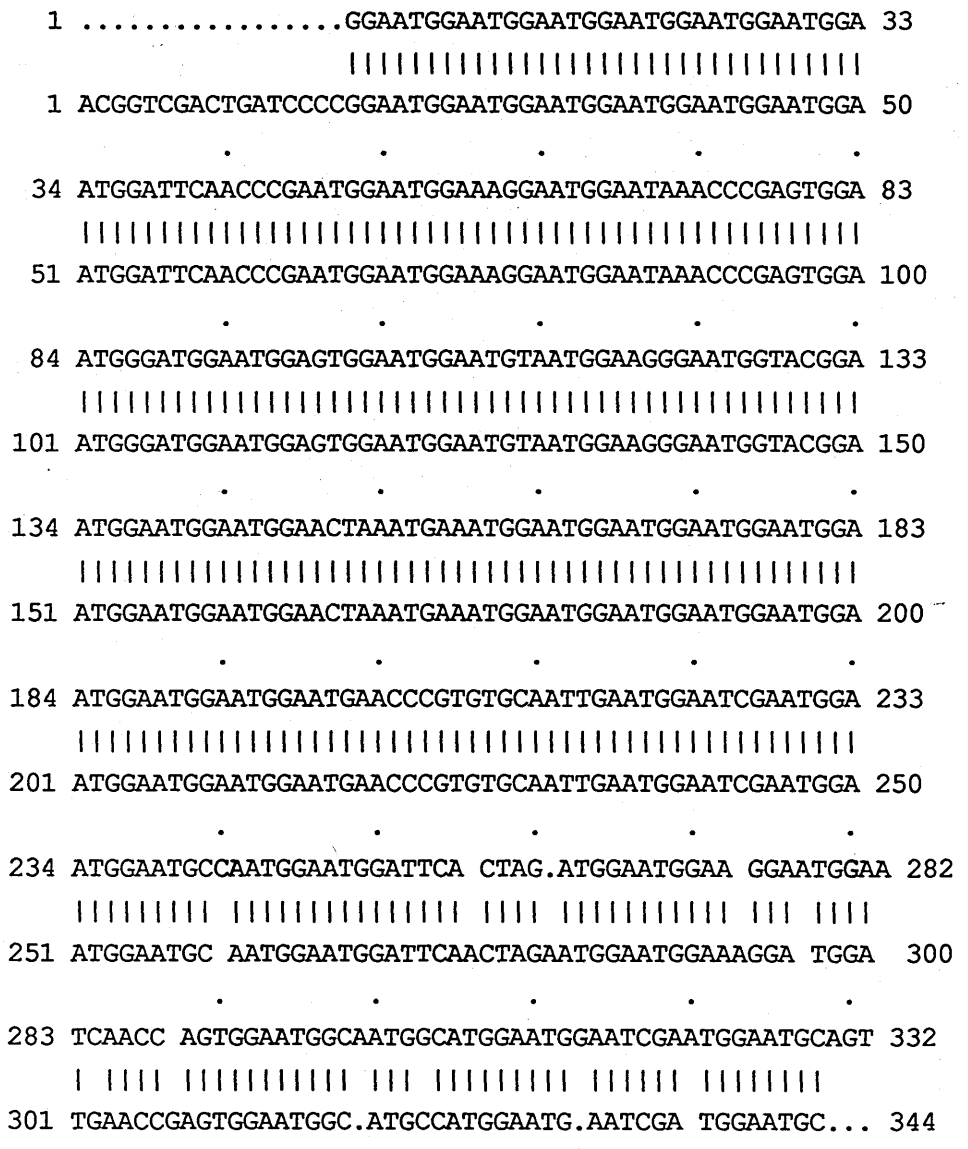
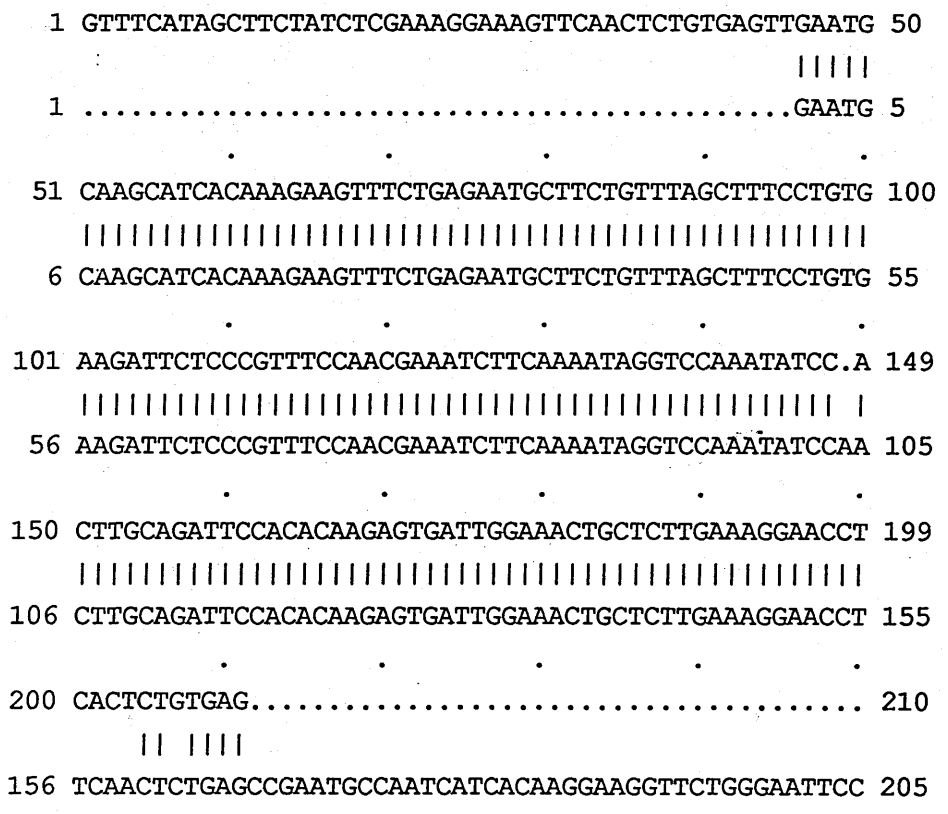

1 .....GGAATGGAATGGAATGGAATGGAATGGAATGGA 33
1 ACGGTCGACTGATCCCCGGAATGGAATGGAATGGAATGGAATGGAATGGA 50
34 ATGGATTCAACCCGAATGGAATGGAAAGGAATGGAATAAACCCGAGTGGA 83
51 ATGGATTCAACCCGAATGGAATGGAAAGGAATGGAATAAACCCGAGTGGA 100
84 ATGGGATGGAATGGAGTGGAATGGAATGTAATGGAAGGGAATGGTACGGA 133
101 ATGGGATGGAATGGAGTGGAATGGAATGTAATGGAAGGGAATGGTACGGA 150
134 ATGGAATGGAATGGAACTAAATGAAATGGAATGGAATGGAATGGAATGGA 183
151 ATGGAATGGAATGGAACTAAATGAAATGGAATGGAATGGAATGGAATGGA 200
184 ATGGAATGGAATGGAATGAACCCGTGTGCAATTGAATGGAATCGAATGGA 233
201 ATGGAATGGAATGGAATGAACCCGTGTGCAATTGAATGGAATCGAATGGA 250
234 ATGGAATGCCAATGGAATGGATTCA CTAG.ATGGAATGGAA GGAATGGAA 282
251 ATGGAATGC AATGGAATGGATTCAACTAGAATGGAATGGAAAGGA TGGA 300
283 TCAACC AGTGGAATGGCAATGGCATGGAATGGAATCGAATGGAATGCAGT 332
301 TGAACCGAGTGGAATGGC.ATGCCATGGAATG.AATCGA TGGAATGC... 344

Figure 2.13b. Sequencing data on satellite DNA 216S.

The nucleotide base sequences as obtained in this study was compared with sequence data obtained from EMBL database


1 GTTTCATAGCTTCTATCTCGAAAGGAAAGTTCAACTCTGTGAGTTGAATG 50
1 .....GAATG 5
51 CAAGCATCACAAAGAAGTTTCTGAGAATGCTTCTGTTTAGCTTTCCTGTG 100
6 CAAGCATCACAAAGAAGTTTCTGAGAATGCTTCTGTTTAGCTTTCCTGTG 55
101 AAGATTCTCCCGTTTCCAACGAAATCTTCAAAATAGGTCCAAATATCC.A 149
56 AAGATTCTCCCGTTTCCAACGAAATCTTCAAAATAGGTCCAAATATCCAA 105
150 CTTGCAGATTCCACACAAGAGTGATTGGAAACTGCTCTTGAAAGGAACCT 199
106 CTTGCAGATTCCACACAAGAGTGATTGGAAACTGCTCTTGAAAGGAACCT 155
200 CACTCTGTGAG..... 210
156 TCAACTCTGAGCCGAATGCCAATCATCACAAGGAAGGTTCTGGGAATTCC 205

2.2.4.6. Digestion of genomic DNA from primary tumours, lymph nodes and PBL

Approximately 10 μg of genomic DNA were digested with the appropriate DNA restriction endonuclease. Hinf I was used for probing with 33.6 and 33.15 satellites (Jeffreys *et al.*, 1985; Fey *et al.*, 1988; Boltz *et al.*, 1990) and Taq I for probing with 216S and 228S satellites (Boltz *et al.*, 1990). Restriction digestion was carried out under conditions recommended by the manufacturer (Pharmacia LKB).

The extent of digestion was tested on 0.4 μg aliquots from each sample by electrophoresis on a 1% agarose gel. After digestion was judged complete, 5μg DNA was electrophoresed on a 20 cm long 1% agarose gel till the bromophenol blue dye had run off the gel. DNA samples from PBL, primary tumour and lymph node were run in neighbouring lanes in the above order for each patient. Standard DNA markers were loaded on the extreme left and right lanes of the gel. All gels were photographed with a ruler with markings in centimeters placed alongside.

2.2.4.7. Southern Blotting

Southern blotting of the electrophoresed DNA was carried out using the standard capillary transfer method (Southern, 1975) and as described in Sambrook *et al.* (1989). The DNA was transferred on to Hybond-N nylon membranes (Amersham plc). Covalent attachment of DNA to the membrane was carried out by placing the membrane under uv light for 5 mins.

2.2.4.8. Radioactive labelling of DNA probes

All DNA probes and standard DNA were radioactively labelled with [α-^{32}P]-deoxycytidine triphosphate using the nick-translation kit from Amersham plc. The labelling was carried out according to the manufacturer's instruction. This involved incubation of 1-2 μg of satellite DNA with buffer, α-^{32}P-dCTP (10μCi/μl), and enzyme solution and incubated at 15 ^{o}C for 2 h. The reaction was stopped by adding 10 mM EDTA, 1% SDS and un-incorporated nucleotide was removed by gel filtration through agarose A1.5 (Bio-Rad). The specific activity of the DNA probes ranged from 2 to 4 x 10^7 c.p.m./μg.

2.2.4.9. Hybridisation with radiolabelled probes

Heat-denatured radiolabelled probes were hybridised to the Southern blots in the presence of formamide as described Anderson & Young (1985).

2.2.4.9.1. Pre-hybridisation

The filters were wetted evenly in 1% Triton X-100 and blotted gently with Whatman 3MM paper to remove excess liquid. Each filter was placed in a separate bag. 10 ml of prewarmed (37 ^{o}C) pre-hybridisation buffer (prepared as described in Section 2.1.15) was added and the bags heat-sealed. The filters were incubated overnight at 37 ^{o}C with shaking.

2.2.4.9.2. Hybridisation

Radiolabelled satellite DNA probes were mixed with radiolabelled marker DNA at a ratio of 50:1 cpm and placed in a boiling water bath for 5 min to denature the double-stranded DNA. After which the probes were quenched in ice and added to pre-warmed hybridisation buffer (prepared as described in Section 2.1.15). Pre-hybridisation buffer was removed from each bag by cutting a corner open and 8-10 ml of radiolabelled probes were added to each bag to give a probe concentration of 10^6 c.p.m./ml. Hybridisation was at 37 ^oC for 12-18 h.

2.2.4.9.3. Washing

The hybridisation buffer was removed from each bag and the filters were immersed in 200 ml of 2 x SSC, 0.1% SDS at room temperature. Filters were rinsed 4-5 times for 5 min in this solution. Next, the filters were shaken in 400 ml 0.1 x SSC, 0.1% SDS at 55 ^oC for 1h. This was repeated twice. Finally, the filters were blotted to remove excess liquid and placed in a fresh bag and exposed to Kodak X-Omat S film with intensifying screens at -80 ^oC. All filters were exposed for a short time, which was usually overnight, and for a longer exposure which varied from 4 days to a week.

2.2.4.9.4. Reprobing of filters

All filters with Hinf I-digested DNA were first hybridised with DNA probe 33.15 followed by probe 33.6. Similarly, filters with Taq I-digested DNA were first hybridised with DNA probe 228S and then with probe 216S.

Filters were stripped of the first probe by heating in 0.01 x SSC, 0.1% SDS at 85-90 °C for 1h. The filters were then blotted dry and autoradiographed to ensure that no traces of the first probe remained before the second probe was applied.

2.2.5. Statistical analysis

Non-parametric statistical tests have been used to compare different groups of patients and to assess the association between different parameters. With the exception of chi-square test, all statistical analysis have been carried out using the Statworks software. Significance is taken at the level of 5%. In most cases, p values were derived on the computer. For cases with small n values, probability Tables have been consulted and in which case p values are given as either < than or > than the given value.

When comparing tumour-free and invaded lymph nodes from the same patient, Wilcoxon signed rank test was used. This same test was used when comparing HPA binding cells in lymph node metastases and autologous primary tumours.

When comparing groups of unrelated patients, stage I versus stage II, diploid versus aneuploid, the Mann Whitney U test was used. This same test was also used when comparing HPA binding between stage I and stage II patients.

All correlations have been assessed with the Spearman correlation test. This includes correlation of both tumour-free and invaded lymph nodes with peripheral blood lymphocytes from the same patient and the correlation between HPA binding in primary tumour and lymph node metastases.

All frequency tables have been analysed by the chi-square test with Yates correction. Both the chi-square (χ^2) statistic and the p value derived from the Table are given.

2.2.6. Histopathology and ER status

Histopathological and ER content data were available as a result of routine clinical practice carried out at Western Infirmary. Tumour grade, which was assessed by the method of Bloom & Richardson (1957) and the number of lymph nodes invaded out of the total nodes examined were assessed by a pathologist at the Western Infirmary. Pathological examination was carried out on one half of the same nodes that were examined in this study.

ER content of primary tumours were determined by the ligand binding method (Leake *et al.*, 1981).

3. The effect of metastasis on the immune function of the axillary lymph nodes.

It is apparent (Section 1.2) that the immune response within human patients is highly variable and may be influenced not only by the immunological status of the patient but also by the oncogene complement of the tumour. Tumour draining lymph nodes in breast cancer patients represent a site where malignant cells may be recognised by the host immune system and additionally metastatic cells invading the nodes may adversely affect the response. In consequence, the general phenotype and activation state of the lymphocytes in the nodes may be found altered. As such, the comparison likely to be most relevant is of two axillary lymph nodes, one invaded and one tumour-free, both obtained at the same time from the same patient. However, to test the hypothesis that any breast tumour affects the nodal response at long range so that stage I patients are able to remain in that state because they have kept the tumour from invading, it is also useful to compare stage I derived nodes with both invaded and tumour-free nodes from stage II patients. This section describes pilot studies addressing such questions.

The methodology of lymphocyte staining and gating is described in Section 2.2.1.3 & 2.2.1.4.

3.1. Phenotypic markers

3.1.1. T and B lymphocytes

From the results in Table 3.1, it can be seen that the mean proportions of T and B lymphocytes in tumour-free and invaded nodes from stage II patients were similar. The proportions of CD3+ T cells in tumour invaded nodes (mean 45.7%) were only slightly lower compared to those in tumour

TABLE 3.1. Distribution of phenotypic markers on lymphocytes from tumour invaded and tumour free lymph nodes of stage II breast cancer patients and from lymph nodes in stage I breast cancer patients.

Phenotypic Marker	STAGE II Tumour Invaded (n=12)	STAGE II Tumour Free (n=12)	STAGE I (n=14)	Statistical Significance+
CD3 (% T cells)	45.7+/-4.6	49.7+/-4.8	51.5+/-3.0	a) N.S. b) N.S. c) N.S.
CD19 (% B cells)	41.6+/-6.0	41.0+/-4.35	36.4+/-3.2	a) N.S. b) N.S. c) N.S.
CD8 (% $CD8^+$ T cells)	14.4+/-2.4 (12.3+/-8.4)*	7.9+/-1.0 (7.4+/-3.3)	8.9+/-0.8 (9.5+/-3.0)	a) p= 0.002 b) 0.05>p<0.1 c) N.S.
CD4 (% $CD4^+$ T cells)	32.0+/-3.3 (29.5+/-11.5)*	41.1+/-3.8 (40.5+/-13.0)	42.1+/-3.3 (40.5+/-12.3)	a) p= 0.003 b) N.S. c) N.S.
CD4/CD8 RATIO	2.9+/-0.5 (2.05+/-1.7)	5.8+/-0.7 (5.3+/-2.2)	6.0+/-1.4 (4.8+/-5.1)*	a) p= 0.001 b) N.S. c) N.S.

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.
* values in bracket represents median +/- standard deviation (SD). Median values and SD are cited for all cases where a distribution appeared to be skewed (marked with asterisk).
+ Statistical test used: Wilcoxon signed rank test, when comparing tumour invaded and tumour free nodes from the same patient; Mann Whitney U test, when comparing independent samples of stage I and stage II patients (Methods and Materials, Section 2). Statistical significance is taken at a p value equal to or < 0.05. Only significant p values and those with p<0.1 are given. N.S. is not significant.
a) is tumour invaded versus tumour free stage II lymph nodes; b) is tumour invaded stage II versus stage I nodes; and c) is tumour free stage II versus stage I nodes.

free nodes (49.7%). While the proportions of CD19+ B cells were similar in both node types in stage II patients, there were slightly fewer CD3+ T cells and more CD19+ B cells in stage II invaded nodes in comparison to nodes from stage I patients. None of these differences were, however, statistically significant. When comparing tumour-free nodes with invaded nodes in the same stage II patient (Fig. 3.1a & 3.1b), differences were observed more with respect to B cells than T cells, then again this was in some but not all patients. There was, however, no consistent trend for T or B lymphocytes to be higher or lower in the two nodal groups (Fig. 3.1a & 3.1b).

The major differences with respect to the proportion of B and T lymphocytes were observed when stage II patients were compared with each other. Here again, patient variability was greater with respect to B cells than with T cells (Fig. 3.1a, 3.1b). A few patients were found to have very low levels of B cells (Fig. 3.1b) and in this, patient 2 was notable in having less than 10% of B cells and over 80% T cells.

3.1.2. T cell subsets

Analysis of T cell subsets showed differences between tumour-invaded and tumour-free nodes. The mean percentage of CD8+ suppressor/cytotoxic T cells in tumour invaded nodes (14.4%) was higher when compared with tumour free nodes (7.9%) of matched stage II patients (Table 3.1). This difference was statistically significant ($p=0.002$). The distribution in the invaded nodes was, however, skewed (median 12.3%) with only three patients having greater than 20% of CD8+ T cells (Fig. 3.3a). In the remaining cases,

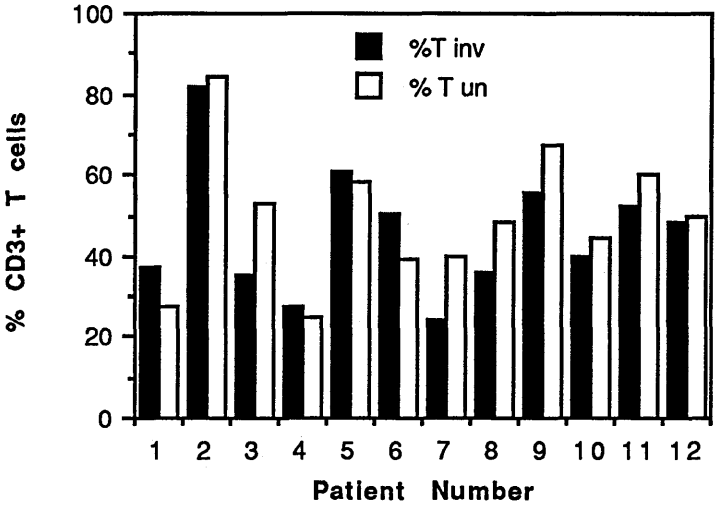


Figure 3.1a. CD3+ T cells in tumour-free (un) and invaded (inv) lymph nodes within the same stage II patients.

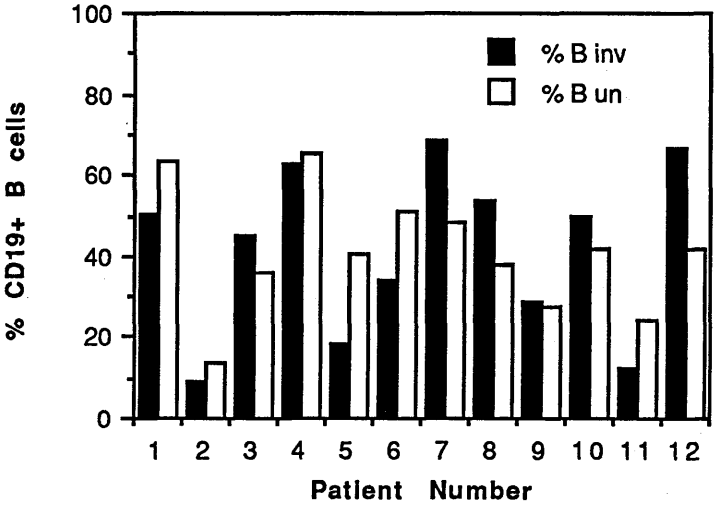


Figure 3.1b. CD19+ B cells in tumour-free (un) and invaded (inv) lymph nodes within the same stage II patients.

the differences in the proportion of CD8+ T cells were much smaller. Conversely, the CD4+ helper T cells in the stage II invaded nodes were decreased (32%) in comparison with tumour free stage II nodes (41.1%) (p=0.003). Figures 3.2a and 3.2b show the differences in CD4+ and CD8+ T cells within the same stage II patients. Although the majority of invaded nodes had higher CD8+ cells, the difference between the two groups was smaller when compared to the differences observed with CD4+ T cells (Fig. 3.2b).

The alterations in the T cell subsets within individual stage II patients however, did not reach statistical significance when invaded nodes from stage II and tumour free nodes from unrelated stage I patients were compared (Table 3.1). The mean proportions of both CD8+ and CD4+ cells in stage I patients were the same as those for tumour-free nodes in stage II patients. The distribution of CD8+ cells varied highly in the invaded nodes compared to both tumour-free nodes in the same patient and unrelated stage I patients (Fig 3.3a). Variations in the proportion of CD4+ T cells were observed in all three groups (Fig. 3.3b), but invaded nodes had lower values of this T cell subset compared to the other two groups.

3.1.3. CD4+/CD8+ Ratio

In consequence of the above differences, the CD4/CD8 ratio was significantly different in the comparison between invaded nodes from the same stage II patients (p=0.001). Although the median value of this ratio in invaded nodes (2.0) was 2 fold lower in comparison to stage I patients (4.8), this difference failed to reach statistical significance (Table 3.1).

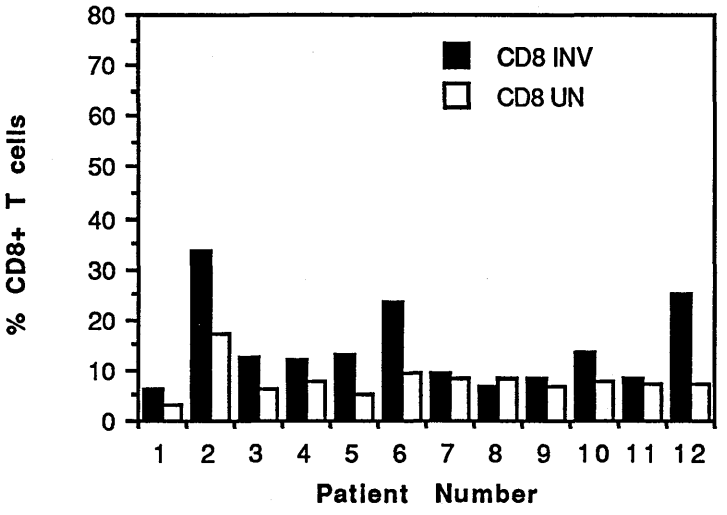


Figure 3.2a. CD8+ T cells in tumour-free (UN) and invaded (INV) lymph nodes within the same stage II patients.

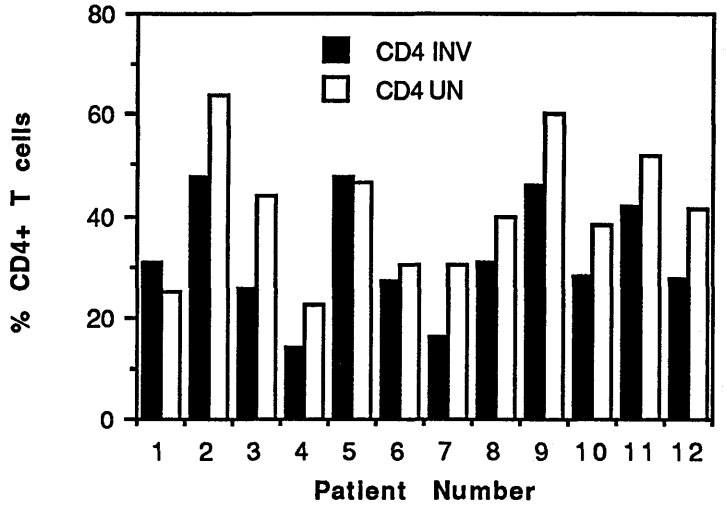


Figure 3.2b. CD4+ T cells in tumour-free (UN) and invaded (INV) lymph nodes within the same stage II patients.

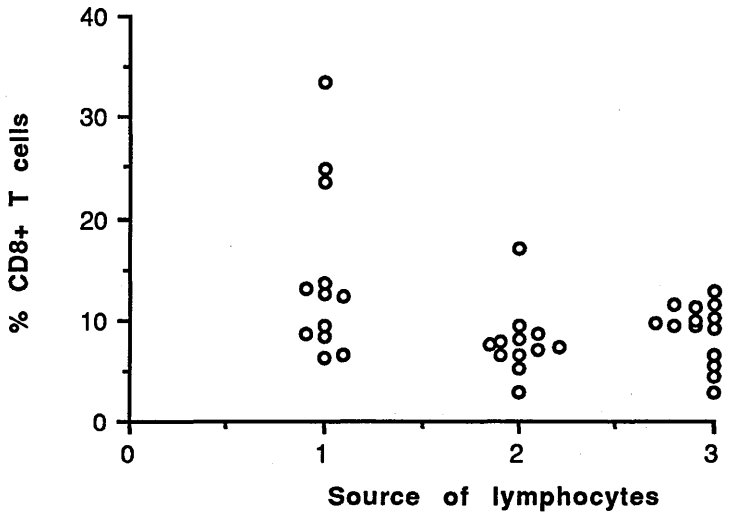


Figure 3.3a. Distribution of CD8+ T cells in tumour invaded (1), tumour free (2), and stage I (3) lymph node lymphocytes from breast cancer patients.

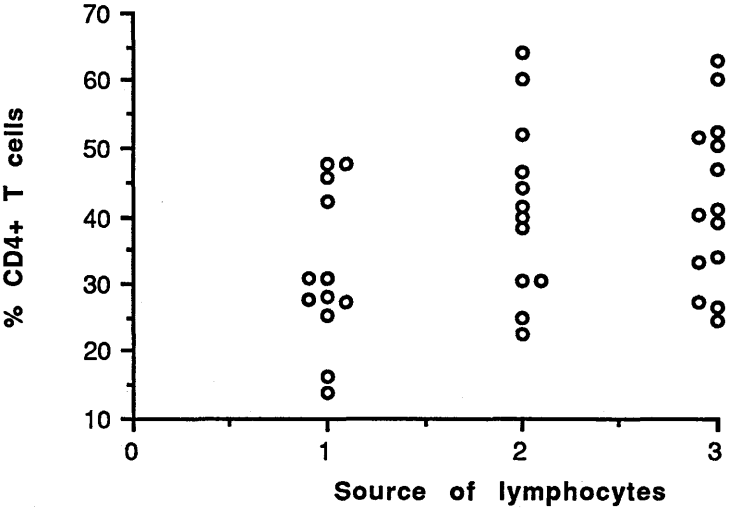


Figure 3.3b. Distribution of CD4+ T cells in tumour invaded (1), tumour free (2), and stage I (3) lymph node lymphocytes from breast cancer patients.

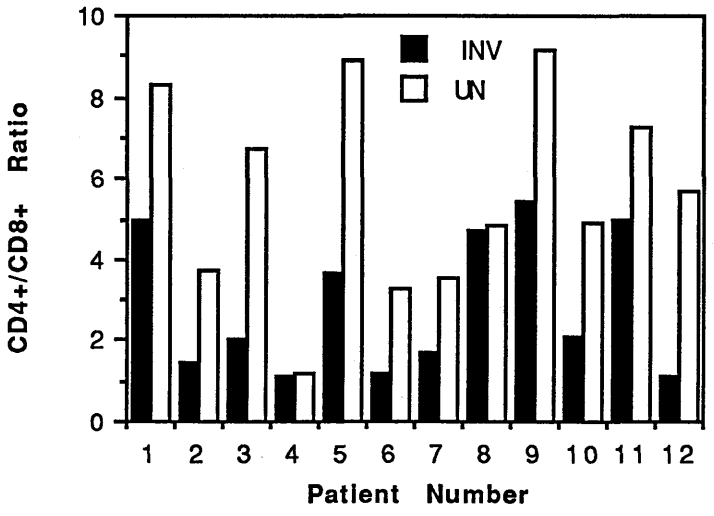


Figure 3.4a. CD4+/CD8+ ratio in tumour-free (UN) and invaded (INV) lymph nodes within the same stage II patients.

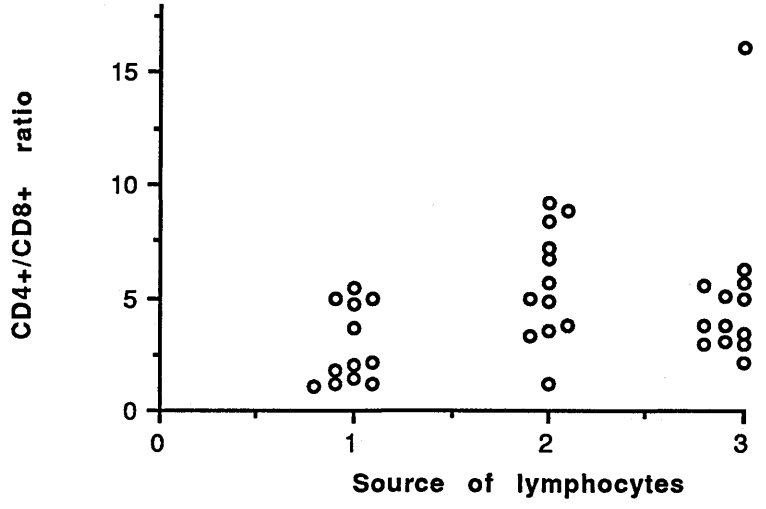


Figure 3.4b. Distribution of CD4/CD8 ratio in tumour invaded (1), tumour free (2), and stage I (3) lymph node lymphocytes from breast cancer patients.

The CD4/CD8 data are further examined in Figures 3.4a and 3.4b. Inspection of the data shows extensive patient variability. In some patients, there was both a depression of the proportion of CD4+ cells and an increase in CD8+ T cells with cases where the difference in values reached 2 fold. There were, however, also cases where the difference in the ratio was contributed by changes in only one of the T cell subsets. The depression of the CD4+ population in the tumour invaded nodes was, however, more pronounced and more consistently observed (Fig 3.2b). Figure 3.4b shows the precise distribution of the proportion of CD4+ and CD8+ T cells in invaded and tumour free nodes. The CD4/CD8 ratios for the invaded nodes were within a relatively tight range of 1.1 to 5.42 compared to the higher range of 1.15 to 9.16 for tumour free nodes.

3.2. Activation markers

Table 3.2 summarises the expression of the activation markers HLA DR and IL-2 receptor (Tac) on T cells and sIgG on B cells. With the exception of sIgG on B cells, no significant differences in terms of expression of any of these markers were observed. However, slight differences did occur in some cases.

3.2.1. HLA DR

In all cases the percentage of CD8+ cells bearing HLA DR was higher than HLA DR-expressing CD4+ cells. The proportion of HLA DR+ T cells was slightly higher in invaded nodes with a mean value of 69.9% compared to 64.8% in tumour free nodes (Table 3.2). The mean proportions of these HLA

TABLE 3.2. Distribution of activation markers on lymphocytes from tumour invaded and tumour free lymph nodes in stage II breast cancer patients and from lymph nodes in stage I breast cancer patients.

Activation Marker	STAGE II Tumour Invaded (n=12)	STAGE II Tumour Free (n=12)	STAGE I (n=14)	Statistical+ Significance
HLA DR on T cells	69.9+/-5.8	64.8+/-6.7	73.2+/-8.9	a) N.S. b) N.S. c) N.S.
HLA DR on $CD8^+$ T cells	42.4+/-4.8	42.9+/-3.6	48.1+/-6.0	a) N.S. b) N.S. c) N.S.
HLA DR on $CD4^+$ T cells	28.5+/-3.3	25.4+/-3.4	32.7+/-4.1	a) N.S. b) N.S. c) N.S.
Tac on T cells	44.9+/-3.9	37.7+/-3.5	45.3+/-6.3	a) N.S. b) N.S. c) N.S.
Tac on $CD8^+$ T cells	19.6+/-2.6 (20.2+/-9.2)	15.7+/-3.1 (12.7+/-10.8)*	16.4+/-3.2 (12.8+/-11.5)*	a) N.S. b) N.S. c) N.S.
Tac on $CD4^+$ T cells	24.0+/-3.4	21.9+/-2.2	28.8+/-3.6	a) N.S. b) N.S. c) N.S.
sIgG on B cells	34.6+/-4.1 (33.2+/-14.0)	27.6+/-2.6 (30.8+/-8.9)	25.8+/-6.6 (18.0+/-24.6)*	a) $p<0.05$ b) $0.02<p<0.05$ c) $0.05<p<0.1$

Values represent mean % +/- standard error of lymphocytes expressing the phenotypic marker.
* values in bracket represents median +/- standard deviation (SD). Median values and SD are cited for all cases where a distribution appeared to be skewed (marked with asterisk).
+ Statistical test used: Wilcoxon signed rank test, when comparing tumour invaded and tumour free nodes from the same patient; Mann Whitney U test, when comparing independent samples of stage I and stage II patients (Methods and Materials, Section 2). Statistical significance is taken at a p value equal to or < 0.05. Only significant p values and those with $p< 0.1$ are given. N.S. is not significant.
a) is tumour invaded versus tumour free stage II lymph nodes; b) is tumour invaded stage II versus stage I nodes; and c) is tumour free stage II versus stage I nodes.

DR+ cells were not significantly different between autologous nodes from stage II patients or in comparison between stage I and stage II patients. This was also true for T cell subsets, and although in some cases there was a slightly higher proportion of HLA DR+ T cells in invaded nodes when compared to tumour-free nodes, there were also cases where the reverse was true (Fig. 3.5a & 3.5b). Thus, no general trend was apparent.

3.2.2 Interleukin-2 receptor (Tac)

In all the three groups of nodes analysed, there was a higher percentage of Tac-expressing CD4+ T cells than CD8+ T cells (Table 3.2). The mean percentage of Tac+ T cells in stage II tumour invaded nodes (44.9%) was higher than in stage II tumour free nodes (35.3%) but similar to stage I nodes. Although CD8+ population from invaded nodes (median 20.2%) expressed the marker on a slightly higher proportion of cells when compared to tumour free nodes (median 12.7%), the difference was not statistically significant (Table 3.2). Patient variability was high, with some cases of higher percentages in invaded nodes and others with the reverse (Fig.3.6a & 3.6b). Moreover, in a few patients, the expression of this marker was markedly low (Fig. 3.6b).

3.2.3 Surface Immunoglobulin G (sIgG)

The percentage of sIgG-expressing B cells in tumour invaded nodes (median 33.2%) was slightly higher in comparison to tumour-free nodes (median 30.8%) and about 2 fold higher in comparison to stage I nodes (median 18%). The small difference between tumour-free and invaded nodes reached statistical significance ($p<0.05$). The distribution of stage I nodes

It is important to point out that IgG-expressing B cells as detected in this study, may also reflect the presence of cytophilic antibodies. These antibodies would bind to the Fc receptors and as such would be included in the IgG+ population. This may be tested in two ways-

i) a control may be set up in which lymphocytes may be stained with anti-kappa-FITC and anti-lambda-PE antibodies. In a dual-parameter analysis, the cytophilic antibodies would show up as double positive as opposed to single-stained B cells expressing specific IgG (either kappa or lambda).

ii) IgG+ B cells may be stripped of their surface immunoglobulin (and cytophilic antibodies) by treating with papain and then put into culture to allow regeneration of sIgG.

(mean 25.8%, median 18%) was skewed (Fig. 3.7b) and the difference between stage I and tumour-invaded stage II nodes from unrelated patients also reached statistical significance ($p< 0.05$; Table 3.2).

A notable feature of IgG-expression was that the differences between invaded and tumour-free nodes were often strong in a few patients. A representative example where the invaded node had a considerably higher proportion of sIg-bearing B cells than the tumour free nodes is shown in Fig 3.8.

3.3 Correlation with peripheral blood lymphocytes (PBL)

PBL from breast cancer patients have fewer CD4+ T cells and a higher proportion of CD8+ T cells than those from LNL (Whitford *et al.*, 1992b). A strong positive correlation with CD4+ T cells and an inverse relation with CD8+ T cells was found in lymphocytes from these sources. To determine whether alterations observed in the invaded nodes also reflect similar changes in the cell populations from PBL, lymphocytes from these sources from the same patient were compared in a correlation test.

In this study, comparison of the phenotypic markers and sIgG expression between tumour-invaded nodes and those from the PBL from the same patient (Table 3.3) revealed no significant correlation, either positive or negative. This suggests that the peripheral blood reflects neither the depression of CD4+ cells nor the elevation of the CD8+ T cells observed in tumour invaded nodes. Similarly, sIgG expression, which was found in only a small number of cells (Fig 3.8), was not reflected in B lymphocytes from peripheral blood.

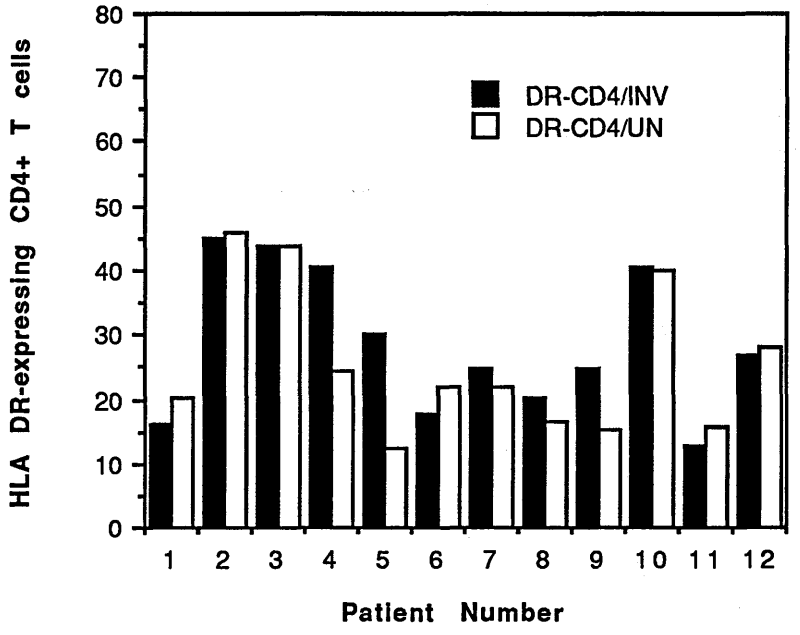


Figure 3.5a HLA DR expressing CD4+ T cells in tumour free (UN) and invaded (INV) lymph nodes

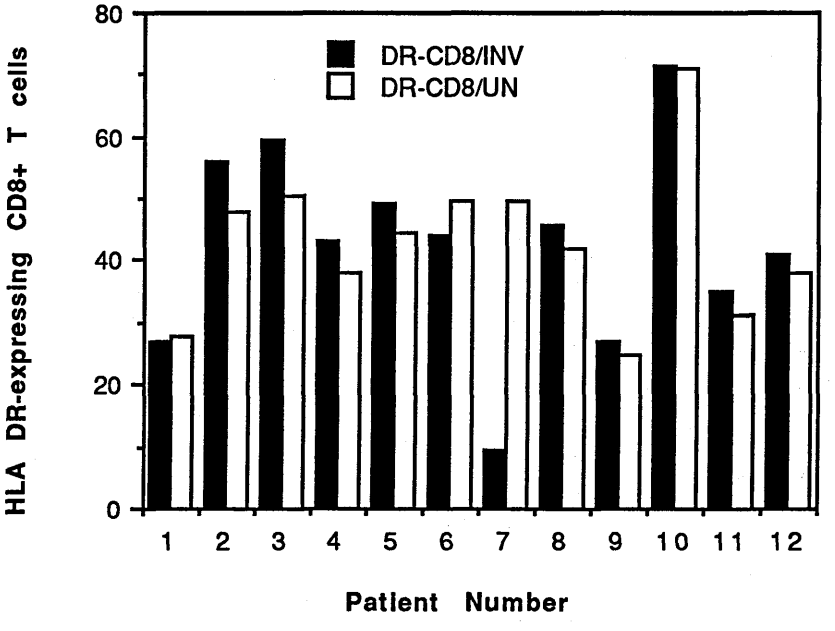


Figure 3.5b HLA DR-expressing CD8+ T cells in tumour free (UN) and invaded (INV) lymph nodes within the same stage II patients.

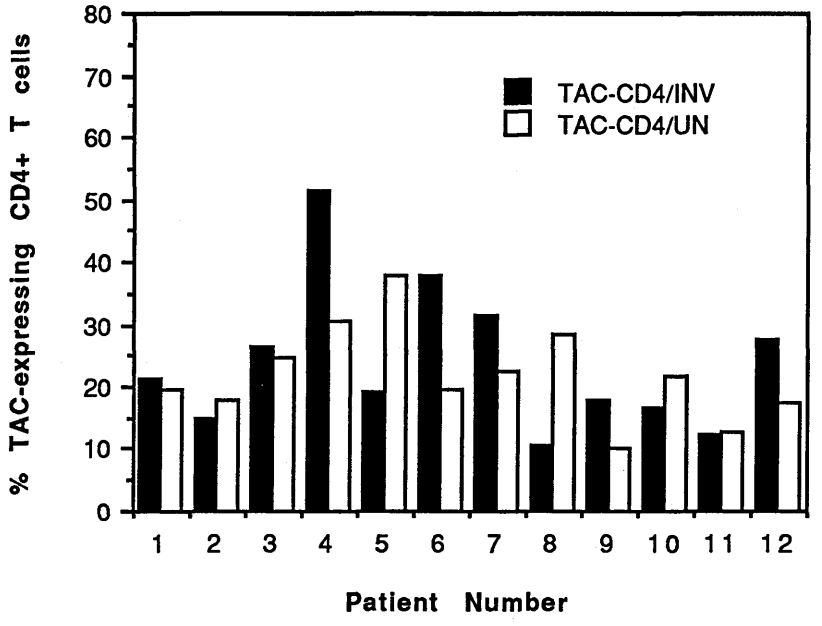


Figure 3.6a. Tac-expressing CD4+ T cells in tumour free (UN) and invaded (INV) lymph nodes within the same stage II patients.

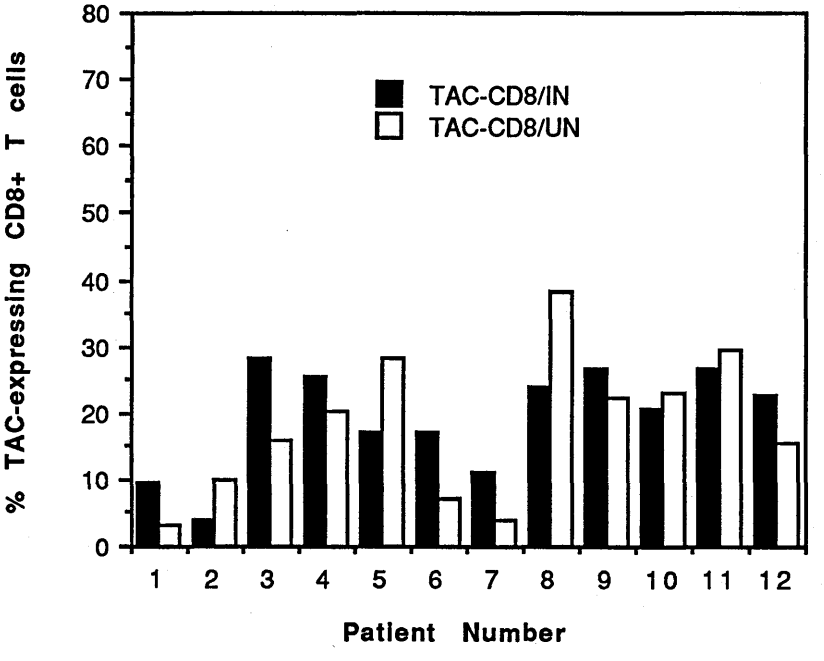


Figure 3.6b. Tac-expressing CD8+ T cells in tumour free (UN) and invaded (INV) lymph nodes within the same stage II patients.

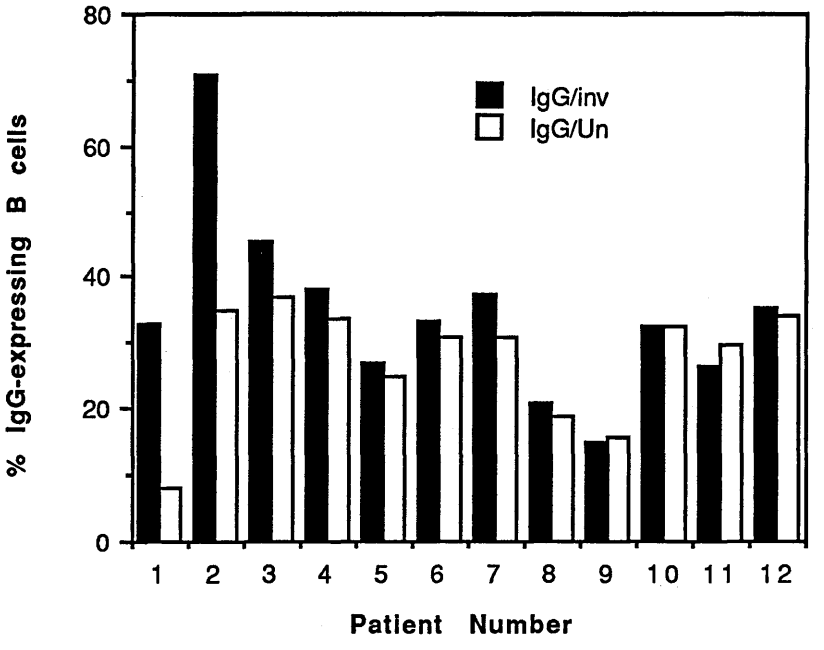


Figure 3.7a. IgG-expressing B cells in tumour free (un) and invaded (inv) lymph nodes within the same stage II patients.

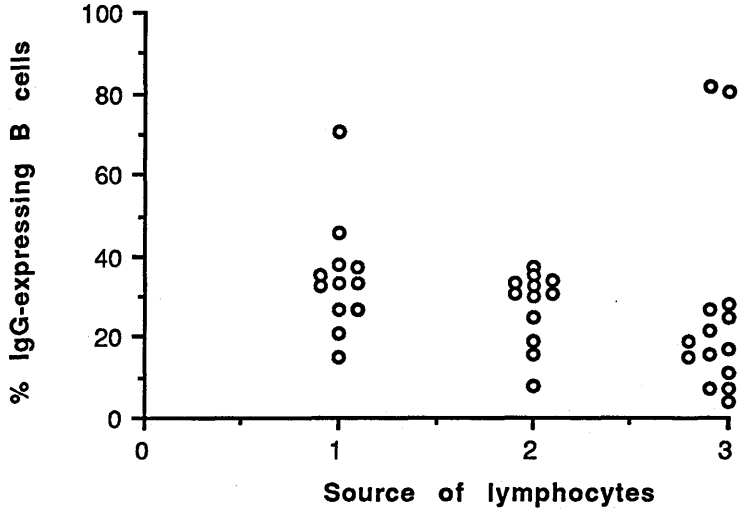


Figure 3.7b. Distribution of IgG-expressing B cells in tumour invaded (1), tumour free (2), and stage I (3) lymph node lymphocytes from breast cancer patients.

Figure 3.8. sIgG-expressing B cells (CD19+) in lymphocytes from peripheral blood (A), tumour-free (B), and invaded (C) lymph nodes from the same breast cancer patient.

Such strong differences between invaded and tumour-free nodes were observed in some stage II patients.

Anti-CD19 staining is plotted on FL2 and anti-IgG staining is on FL1.

% IgG+ CD19+ cells: PBL- 0.6; tumour-free nodes- 8.1;

tumour-invaded nodes- 33.5.

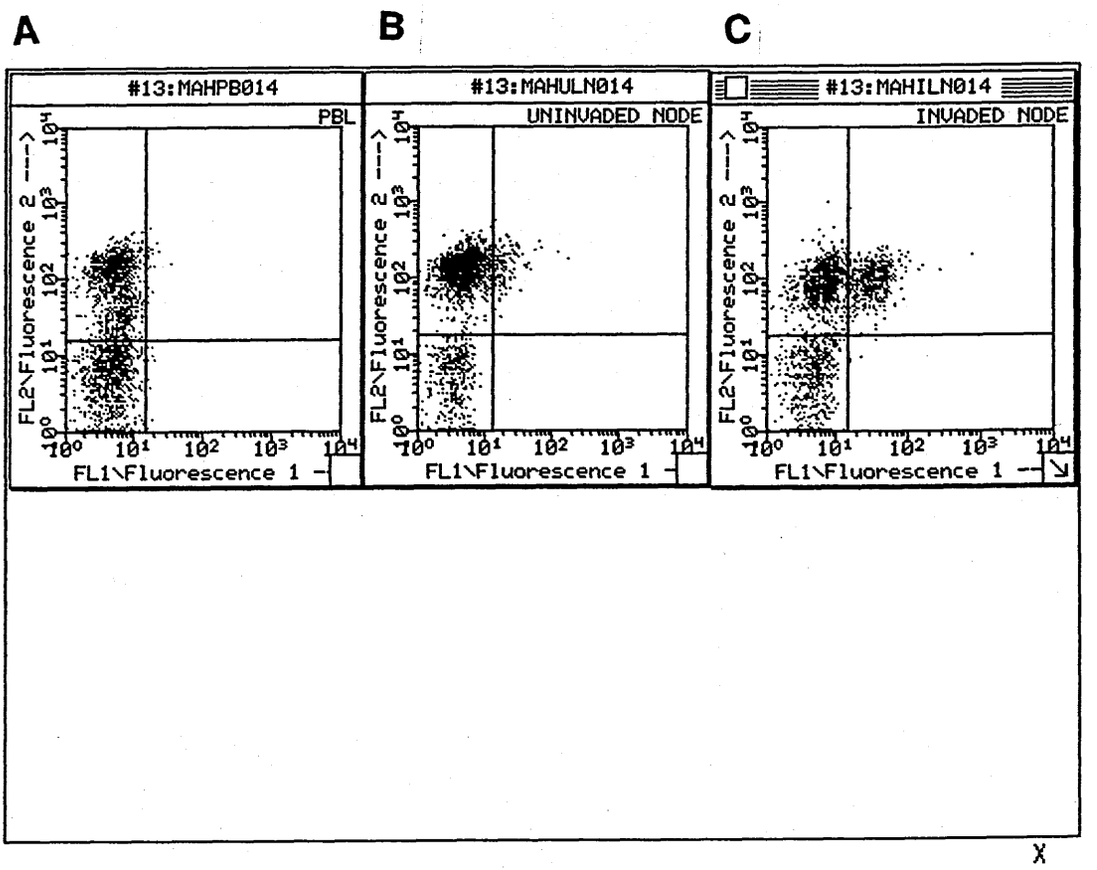

A
B
C
#13:MAHPB014
PBL
#13:MAHULN014
UNINVADED NODE
#13:MAHILN014
INVADED NODE
FL2\Fluorescence 2 --->
FL1\Fluorescence 1 --
10^0 10^1 10^2 10^3 10^4
X

TABLE 3.3. Correlation of phenotypic marker on lymphocytes from lymph node (LNL) and peripheral blood (PBL) of stage II breast cancer patients.

Phenotypic/ Activation Marker	LNL- Tumour Invaded (n=12)	LNL- Tumour Free (n=12)	PBL (n=12)	Spearman Correlation*
CD8	14.4+/-2.4	7.9+/-1.0	26.9+/-3.5	a) N.S. b) N.S.
CD4	32.0+/-3.3	41.1+/-3.8	35.6+/-3.0	a) N.S. b) N.S.
CD4/CD8 RATIO	2.9+/-0.5	5.6+/-0.7	1.6+/-0.3	a) N.S. b) N.S.
sIgG on B cells	34.6+/-4.1	27.6+/-2.6	3.95+/- 0.88	a) N.S. b) N.S.

* a) is tumour invaded nodes versus PBL; b) is tumour free nodes versus PBL.
values represent mean % +/- standard error of lymphocytic subset expressing the phenotypic marker.

3.4 Comparison of two invaded lymph nodes from the same stage II patient and two (by definition) non invaded lymph nodes from the same stage I patient.

This study was undertaken to find out whether lymph nodes operate independently within an individual. In stage I patients, a necessary control is to test two anatomically independent lymph nodes in each other. In stage II patients, it is necessary to find whether tumour invasion causes the same changes in both cases. Technically, the latter is difficult as the precise degree of tumour invasion is difficult to judge and therefore a matched pair from the same patient is not readily obtained. However, the status of the selected lymph nodes were verified by histopathology.

Comparisons of the phenotype and activation state of lymphocytes from two tumour invaded nodes within a single stage II patient and two tumour free nodes within a single stage I patient were peformed (Table 3.4). The two tumour free nodes from a stage I patient were similar in all respect with good conformity between the CD4/CD8 ratio and in the proportion of cells expressing the activation markers. The similarities between the two invaded nodes from the stage II patient were much less. The relatively lower value of CD4+ T cells and the slight elevation of CD8+ T cells in node A gave a CD4/CD8 ratio for this node similar to the mean value for the invaded nodes given in Table 3.1. But node B had a much higher CD4+ population and a CD4/CD8 ratio which was more similar to the tumour free nodes. Likewise, with respect to activation markers, node B had a higher proportion of HLA DR expressing T cells, but the two nodes showed better conformity with respect to Tac expression. Although the proportions of B cells were similar in all of these nodes, there was a higher percentage of IgG+ B cells in the invaded

TABLE 3.4. Comparison of the phenotype and activation state of lymphocytes isolated from two invaded lymph nodes removed from a stage II and two tumour free nodes from a stage I breast cancer patient.

PHENOTYPIC/ ACTIVATION MARKER	TUMOUR INVADED		TUMOUR FREE	
	NODE A %	NODE B %	NODE A %	NODE B %
CD3+	59.0	71.5	64.6	70.6
CD19+	22.5	16.8	28.8	21.5
CD8+	11.5	9.6	7.3	7.8
CD4+	46.2	62.1	55.4	62.1
CD4+/CD8+	4.0	6.4	7.5	8.0
HLA DR+CD8+	13.8	27.6	27.7	26.4
HLA DR+CD4+	8.7	19.8	14.6	14.5
TAC+CD8+	17.3	22.4	12.6	10.3
TAC+CD4+	14.6	18.7	26.2	22.5
IgG+ CD19+	15.3	15.9	9.0	7.6

nodes and in this respect both node A and B in the invaded group had almost identical proportions of these activated cells.

This data, therefore, suggest that the presence of tumour cells within a node may have variable effects, while tumour free nodes in a patient are similar with respect to the phenotypic and activation markers. It should, however, be emphasised that only a single pair of these nodes was analysed.

3.6. Discussion

Axillary lymph nodes in breast cancer patients may be an important site for mediating immune responses to tumours. As these nodes primarily drain the breast, they are ideally situated to encounter tumour cells and, as such, tumour antigen recognition leading to host-mediated immune response can occur. At the same time, however, metastatic tumour cells invading the nodes can adversely influence the lymphocytes within such nodes. An analysis of the phenotype and the activation states of lymphocytes within lymph nodes from patients with metastatic breast cancer can reflect the nature of the response and may also determine the outcome of the tumour-host interaction.

Only a few studies have analysed lymph node lymphocytes from breast cancer patients by flow cytometry (Morton *et al.*, 1986; Whitford *et al.*, 1992b). Although nodes from both stage I and stage II patients were analysed in the above studies, nodes free of tumour were preferred and grossly invaded nodes were omitted. Moreover, these studies compared nodes in groups with respect to the stage of the disease and no study has compared nodes within the same patient. In this study, phenotypic and activation markers on lymphocytes from a tumour-free and an invaded node taken from the same stage II patient (with metastasis) was analysed by flow cytometry. Furthermore, the two groups of nodes were compared to lymph node lymphocytes from patients without metastasis (stage I).

Results described in this section reveal that in some breast cancer patients, differences in the proportion of T cell subsets and activated B cells exist between nodes that are invaded and those that are not. Notable but not significant differences were also observed between invaded nodes and stage I nodes, while the phenotype and the activation states of lymphocytes from

tumour-free nodes from both stage I and stage II patients were found to be similar. The mean proportions of T and B lymphocytes were similar in both invaded and tumour-free stage II nodes, but there were more B cells in stage II patients. The lymph node is the major site of the B lymphocyte population and elevation of B cells in stage II patients was also observed by Morton *et al.* (1986). The proportion of T cells in invaded nodes although varying among patients was not significantly lower when compared to those in tumour-free nodes. Lower proportions of T cells in invaded nodes from melanoma patients has been reported by Farzad *et al.* (1990).

There were, however, notable differences in the proportion of T cell subsets and the most significant difference between tumour-free and invaded nodes was observed with the CD4+/CD8+ ratio. In invaded nodes, this ratio was reduced to less than half that of tumour-free nodes. The reduction in CD4+/CD8+ ratio was due to an increase in CD8+ T cells and a decrease in the CD4+ population. Similar alterations in the CD4 and CD8 populations have been reported for stage II breast cancer (Morton *et al.*, 1986) and melanoma (Farzad *et al.*, 1990) patients. However, in the study of melanoma patients, the significant depression of CD4+ cells was observed in both tumour-free and invaded nodes and while CD8+ cells were higher in both these nodes, only the tumour-free nodes were significantly higher than stage I nodes. In this study, the T cell subsets in stage I nodes were found to be similar to tumour-free nodes, suggesting that only the presence of tumour cells in the nodes affects the T cell subsets such that the CD4+/CD8+ ratio drops in invaded nodes. It must be pointed out that with the exception of three patients, the increase in CD8+ T cells was small and was not always accompanied by a decrease in CD4+ cells in the same patient. The decrease in the proportion of CD4+ T cells in invaded nodes was, however, more pronounced and showed more variability among patients. It is of interest to note here that lymphocytes that

infiltrate tumours (TILs) have been found to comprise predominantly of CD8+ T cells (Whitford *et al.*, 1990; Durie *et al.*, 1990) and the increase in the same phenotype in invaded nodes is perhaps suggestive, in both cases, of activated cytotoxic cells in close proximity to their target cells.

The expression of the activation marker HLA DR was similar in both tumour-free and invaded nodes and was not significantly different from stage I nodes. This is in contrary to the finding of Whitford *et al.* (1992b), who found the expression on CD8+ T cells to be related to stage, and Morton *et al.* (1986), who reported higher proportions of HLA DR-expressing CD4+ and CD8+ T cells in stage II patients. However, in agreement with all other reports, this marker was found to be expressed on higher numbers of CD8+ than CD4+ T cells. In contrast, the IL-2 receptor (Tac) was present on twice as many CD4+ than CD8+ cells in lymphocytes from all three sources. It is not known whether this reflects any functional difference in the expression of the receptor on these T cell subsets or if this is a reflection of the activated state of the respective cells. In general, the percentage of Tac-expressing T cells were slightly higher in invaded nodes when compared to tumour-free nodes, while being similar to stage I nodes. The proportions of T cells expressing Tac were, thus lower in tumour-free nodes compared to invaded nodes, but these differences were not statistically significant. IL-2 receptor as a activation marker, however, should be interpreted with caution. Although an increased expression of the receptor indicates an activated state of a T cell, lack of the receptor does not necessarily suggest an inactive state as the receptor is known to undergo down-regulation following binding of its ligand (Duprez *et al.*, 1988).

The proportion of B cells was not only higher in stage II nodes but the mean percentage of sIgG-expressing B cells in invaded nodes were higher than

stage I nodes. The difference between invaded and tumour-free nodes within the same stage II patient was also statistically significant. In the other study to assess this marker but which did not include invaded nodes, no significant difference was observed with stage (Whitford *et al.*, 1992b). However, in this study an interesting feature of IgG-expression in lymph node lymphocytes was that in some patients, compared to tumour-free nodes there was a considerably higher numbers of these activated B cells in invaded nodes. The expression of sIgG on B cells marks the onset of a mature humoral response. It is thus probable that in some of the invaded nodes, showing high percentage of IgG+ B cells, the presence of tumour cells provide adequate antigens for this secondary B cell response.

A characteristic feature observed with respect to both the phenotype and activation marker expression by lymph node lymphocytes from breast cancer patients is patient variability. This variability was more often observed in the case of invaded nodes than tumour-free nodes. This may reflect both the capability of host-immunity and the oncogenic or antigenic complement of the tumour. A parameter that could be related with host response is the differentiation state of the tumour as assessed in terms of histological grade. But as only 12 patients were studied and as there was a bias for grade 3 tumours, no relationship could be determined between any of the changes observed with the grade of the primary tumour. In addition to variation among patients it is likely that variation may also exist within a patient, such that variation is also observed among nodes of the same status in the same patient. Analysis of two invaded and two tumour-free nodes suggest that while tumour-free nodes were similar, the presence of tumour cells within a node may exert varying influences. This probably reflects differences in the degree of involvement by the tumour cells.

Taken together, these results suggest that the presence of metastatic tumour cells in a lymph node can cause specific alterations of the phenotype of the T cells and the activated state of the B cells in a proportion of the patient. While certain cell populations are being activated others appear not to be proliferating or perhaps are being actively suppressed. In effect, such alterations in the lymphocytic populations reflect the presence of metastatic cells and may be important elements in the metastatic process.

CHAPTER 4

FCM analysis of cell surface carbohydrates

4. FCM analysis of cell surface carbohydrates in breast cancer

Surface carbohydrate structures and their alterations in human breast cancer have been studied with the intention of identifying both tumour-associated changes and structures related to metastatic spread. Recent evidence suggests that in the role of adhesion molecules, surface glycoproteins may be involved in the metastatic spread of cancer (Section 1.1.6.6). In breast cancer, alterations in cell surface carbohydrate expression have been detected using lectins and in a number of studies such differences have been shown to be of prognostic significance (Section 1.3.5). Most of these studies were histochemical analysis and have been performed on paraffin sections of primary tumours.

This section presents data on a FCM analysis of lectin binding to both primary and lymph node metastases from breast cancer patients. Recognising the evident limitations of histochemical analysis and the use of paraffinised tissue, fresh samples were used and the extent of staining has been quantified in a well-defined manner. Two fluorescently-labelled lectins, Concanavalin A (Con A) and *Helix pomatia* (HPA), which were found to show detectable changes in binding to breast tumours, have been used to assess the value of lectin binding to a large number of live cells (10,000) from fresh breast cancers. Reactivity to both these lectins has been previously reported to be related to malignancy in breast cancer (Leathem & Brooks, 1987; Dansey *et al.*, 1988; Furmanski *et al.*, 1981). Although these studies analysed primary tumours, metastatic tissues have not been analysed. In this study, metastatic cells from axillary lymph node metastases have also been analysed for their binding to these lectins. Data presented here shows that HPA binds to a surface molecule present on tumour cells with higher malignant potential.

4.1. Quantification of fluorescence intensity by Flow cytometry

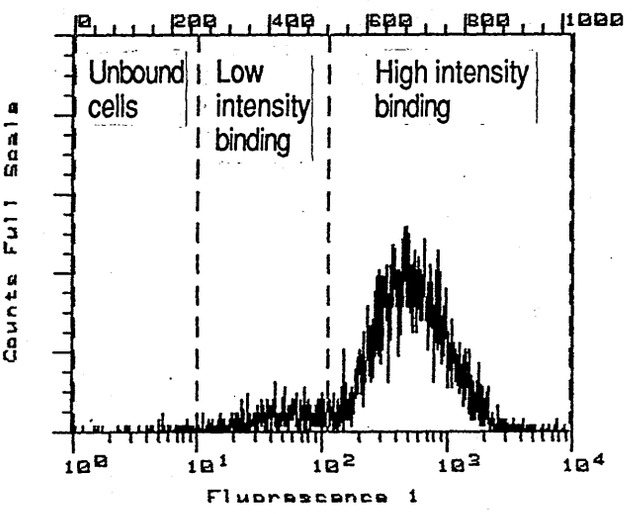
The methodology of staining and analysis of lectin binding on gated tumour cells have been described in Section 2.2.2. A gated analysis of breast tumour cells was necessary as tumour cells frequently have a substantial percentage of infiltrating lymphocytes (Whitford *et al.*, 1990). At the concentration of lectins used, lymphocytes were, however, found not to bind HPA and showed only low-intensity binding to Con A (Alam *et al.*, 1990).

In general, the binding of both HPA and Con A to both malignant and normal breast epithelial cells could be categorised into two groups - a low-intensity binding and a high-intensity binding cell population (Figure 4.1). Cell populations showing fluorescence intensity 10 times brighter than the autofluorescence were taken to be the low-intensity binding group and the high-intensity binding group showed a fluorescence intensity which was 100 times brighter. It was the percentage of cells in the high binding group that varied with each specimen analysed and the extent of which could be correlated with clinicopathological factors. Henceforth, both HPA and Con A binding refers to these populations of these cells showing high intensity binding to the lectins.

4.2. High-intensity lectin binding cells in normal breast tissue

Binding to cells from normal breast tissue (a reduction mammaplasty sample, Section 2.2.2.2) was observed for both HPA and Con A. However, the proportion of high-intensity HPA-binding cells in the normal breast epithelial tissue was markedly lower (18%) in comparison to high Con A binding cells (39%). These values were considered to assign cut-off points

Figure 4.1. Markers set to define regions of unbound cells, low intensity and high intensity binding of the tumour cells to the lectins.


0
200
400
600
800
1000
Unbound cells
Low intensity binding
High intensity binding
Counts Full Scale
10^0
10^1
10^2
10^3
10^4
Fluorescence 1

when relating lectin binding to clinicopathological features.

4.3. Con A binding to primary breast tumours

Although high-intensity Con A binding cells were detected in primary breast tumours, the proportion of these cells were quite variable with one tumour not having any of these cells and a few tumours having greater than 80% of these cells. The values ranged from 0 to 96%, with a mean of 38.5 +/- 5.5%. The mean is similar to the percentage of high-intensity Con A binding cells detected in normal breast cells (39%).

When correlating Con A binding with histopathological factors, it was necessary to assign a cut-off value to distinguish tumours with higher percentage of high-intensity Con A binding cells from those with lower proportions of these cells. The cut-off value >40% was found to be most informative in relating Con A binding and henceforth tumours with >40% of these cells will be termed Con A positive.

Of the 32 primary tumours analysed, 14 were positive for Con A binding. The relationship between Con A binding and various clinicopathological factors is shown in Table 4.1. Although a higher number of Con A positive tumours had metastasised to the nodes, the difference was not statistically significant. As with lymph node involvement, no correlation was observed between Con A binding and either tumour grade or ER status.

Table 4.1. Clinicopathological features of breast cancer patients and their relation to HPA and Con A binding.

Clinicopathological features	Total	Positive HPA	Positive Con A
All patients	32	17 (53%)	14 (44%)
Node involvement:			
invaded	16	13 (81%)	9 (56%)
non-invaded	16	4 (25%)	5 (31%)
Histological grade:			
I	4	0	1 (25%)
II	14	7 (50%)	6 (43%)
III	14	10 (71%)	7 (50%)
Oestrogen receptor:			
ER+ve (>20 fmol/mg protein)	14	6 (43%)	6 (43%)
ER -ve (<20 fmol/mg protein)	11	10 (91%)	5 (45.5%)

ER receptor (25 cases) data were not available for all patients.
HPA positive tumours have > 20% of cells showing high-intensity binding.
Con A positive tumours have > 40% of cells showing high-intensity binding.
Some of the tumours were positive for both lectins (9 cases), some were negative for both (10 cases), while a few others (13 cases) were positive for only one lectin.

4.4. HPA binding to primary breast tumours

Among the 32 primary tumours analysed, there was much variation in the proportion of cells showing high-intensity HPA binding. The values ranged from 0 to 83%, with a mean of 28.74 +/- 4.5%. This mean is higher than the proportion of high HPA cells observed for normal breast cells.

As with Con A binding, HPA positive tumours have been defined as those having >20% of high-intensity HPA binding cells. Of the various cut-off points assessed >20% offered the most informative cut-off value, especially when related to grade and node involvement.

4.5. Correlation of HPA binding with clinicopathological features

Of the total 32 primary tumours, 53% (17 cases) were designated HPA positive (Table 4.1). Correlation of positive HPA binding with clinicopathological factors revealed that there was strong correlations with lymph node involvement and ER status.

Lymph node involvement

As shown in Table 4.2, a significant positive correlation was observed between HPA binding and lymph node involvement ($p<0.01$). 81.25% (13/16) of tumours with invaded nodes were HPA positive, while only 25% (4/16) of HPA positive tumours were without nodal metastasis. Such a difference was also observed when stage I and stage II tumours were compared with respect to the presence of high-intensity HPA binding cells ($p= 0.019$). Among tumours with lymph node metastasis (stage II), the proportion of cells with high-intensity HPA binding ranged from 0.4 to 83% (Fig. 4.2), with a mean of 39.1 +/- 6.2%. Such HPA binding cells in tumours without metastasis (stage I) were also widely distributed (Fig. 4.2), ranging from 0.0 to 67%, but with a two-fold lower mean value (18.3 +/- 5.6%).

Table 4.2. Correlation of HPA binding to lymph node involvement.

Histopathology of lymph node	HPA positive (n=17)	HPA negative (n=15)	Statistical significance (Chi-square test)
Invaded	13	3	χ^2= 8.03, D.F.= 1 p<0.01
Non-invaded	4	12	

HPA postive tumours have been taken as those with >20% of cells showing high-intensity HPA binding.

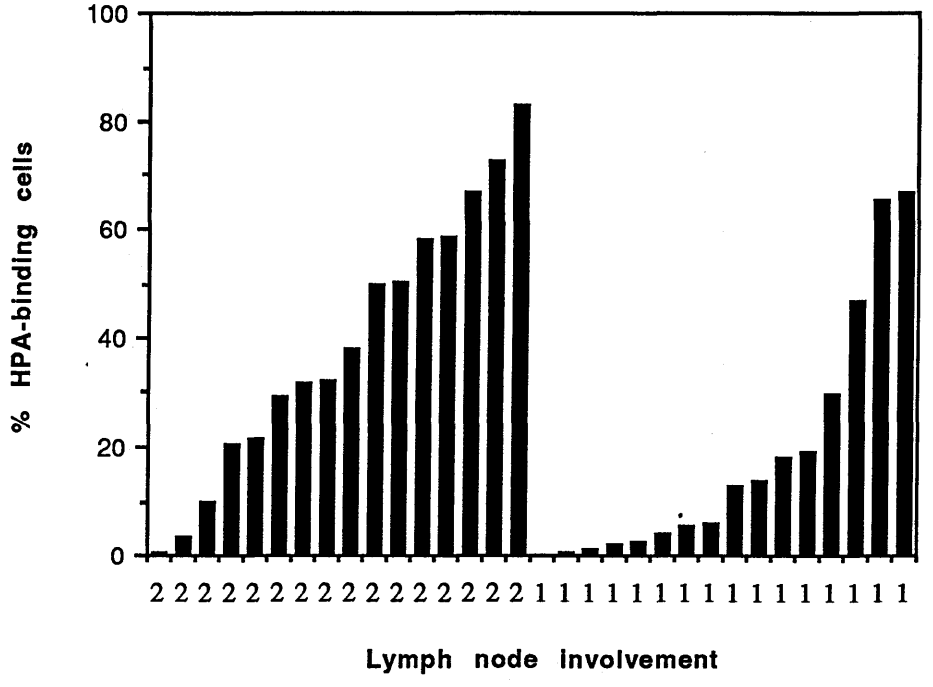


Figure 4.2. Distribution of HPA binding cells in primary tumours with metastasis (2) and without metastasis (1).

Table 4.3. Positive HPA binding related to tumour grade and lymph node involvement.

Histopathology of lymph node	Grade I (n=4)	Grade II (n=14)	Grade III (n=14)
Invaded (n=16)	0/2	3/4	10/10
Non-invaded (n=16)	0/2	4/10	0/4

Values represent number of HPA positive tumours/total.

Tumour grade

The number of HPA positive tumours and their histological grade, as assessed by the method of Bloom & Richardson (1957), is shown in Table 4.1. None of the 4 grade I tumours were positive for high HPA binding. However, there was a marked increase in the number of positive HPA tumours in the grade II and III tumours, with grade III tumours showing a higher incidence (71%) of HPA binding when compared with grade II tumours (50%). There were, therefore, marked differences between HPA binding to grade I tumours and tumours of higher grade (II and III) but these differences are statistically not significant.

The relationship between HPA binding and lymph node involvement and tumour grade is further examined in Table 4.3. Since HPA binding related significantly to lymph node involvement, it was examined to see how node involvement and tumour grade related to HPA binding when considered together. With grade II and III tumours, node involvement appear to relate

with HPA binding irrespective of grade, since with the exception of one all the grade II and III tumours with invaded nodes were positive for HPA binding. In contrast, only 4 of the grade II tumours and none of the grade III tumours without metastasis were positive for HPA binding. This relationship, however, does not hold with grade I tumours. None of the grade I tumours, whether with metastasis or not, were positive for HPA binding, suggesting that HPA binding correlates with lymph node involvement only with tumours of higher grade (II and III). The small number of grade I tumours should, however, be noted.

Table 4.4. Correlation of HPA binding to oestrogen (ER) receptor status of primary breast tumours.

	ER +ve (n= 14)	ER -ve (n= 11)	Statistical significance (Chi-square test)
HPA +ve	6	10	χ^2= 4.26, D.F.=1 $p<0.05$
HPA -ve	8	1	

HPA +ve tumours have >20% cells showing high-intensity binding. ER status cut-off point is 20 fmol/mg protein.

Oestrogen receptor (ER) status

ER data was available for 25 of these patients and this has been also been correlated with HPA binding (Table 4.4). 91% (10/11) of ER -ve tumours were found to be HPA positive while among ER +ve tumours about

43% (6/14) were HPA positive. This association between ER status and HPA binding was statistically significant ($p<0.05$).

4.6. Relationship between HPA binding and Con A binding in primary tumours: a dual-label analysis.

Figure 4.3 illustrates examples of a dual-label analysis of HPA and Con A binding to breast tumours. This was attempted to see if HPA binding cells were also positive for Con A binding. Plot f is the profile of the normal breast tissue, having a small proportion of doubly-labelled (quadrant 2, plot f, Figure 4.3) and HPA binding cells (18%), while the rest of the cells bound exclusively to Con A (quadrant 1, plot f). Though each tumour displayed a characteristic profile of HPA/Con A binding cell distribution, roughly 4 distinct features appeared when all the analysed samples were considered. 3 tumour samples showed binding patterns consistent with Plot b, where the tumours had >70% cells which bound exclusively to HPA (quadrant 4, plot b) and all of these samples were from grade III tumours with invaded node. If the 4 tumours which were positive for HPA binding are excluded, plot e appears to be representative of tumours without nodal metastasis, which included all the 4 grade I tumours as well as some grade II and III tumours. Another group with a higher intensity of Con A binding (not shown) could also be included in the plot e group. Plots c and d include tumours of grade II and III and stage I tumours which were high on HPA binding. There was, therefore, no consistent positive or negative correlation between HPA binding and Con A binding.

Figure 4.3. Dual-label analysis of HPA and Con A binding to breast tumour cells.

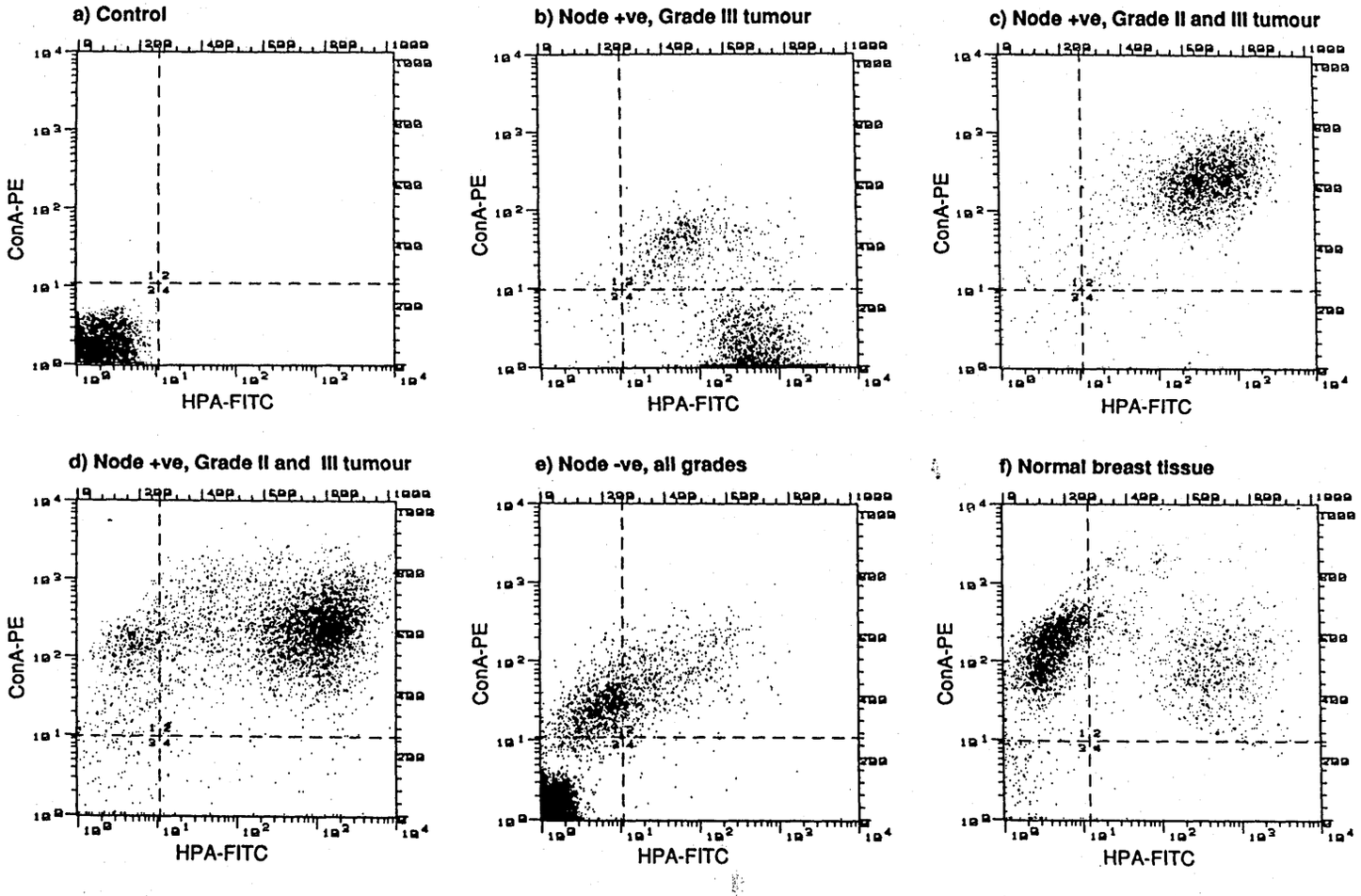
a, is a control sample;

b, is representative of grade II, node-positive tumours. 3 samples showed binding patterns consistent with this profile. >70% tumour cells showed exclusive HPA binding in all these samples.

c, and **d**, represents patterns observed with most of the higher grade (II and III) tumours with invaded nodes.

e, represents tumours without lymph node metastasis.

f, is the profile for the normal breast epithelial tissue as cultured from a reduction mammaplasty sample.


a) Control
b) Node +ve, Grade III tumour
c) Node +ve, Grade II and III tumour
d) Node +ve, Grade II and III tumour
e) Node -ve, all grades
f) Normal breast tissue
ConA-PE
HPA-FITC

4.7. HPA binding to tumour cells from invaded lymph nodes

In the same group of patients in whom primary tumours were analysed, lymph node samples were also available from all pateints without metastasis and from 12 of these patients with metastasis. The methodology for staining and gating for tumour cells in lymph nodes is decribed in Section 2.2.2.

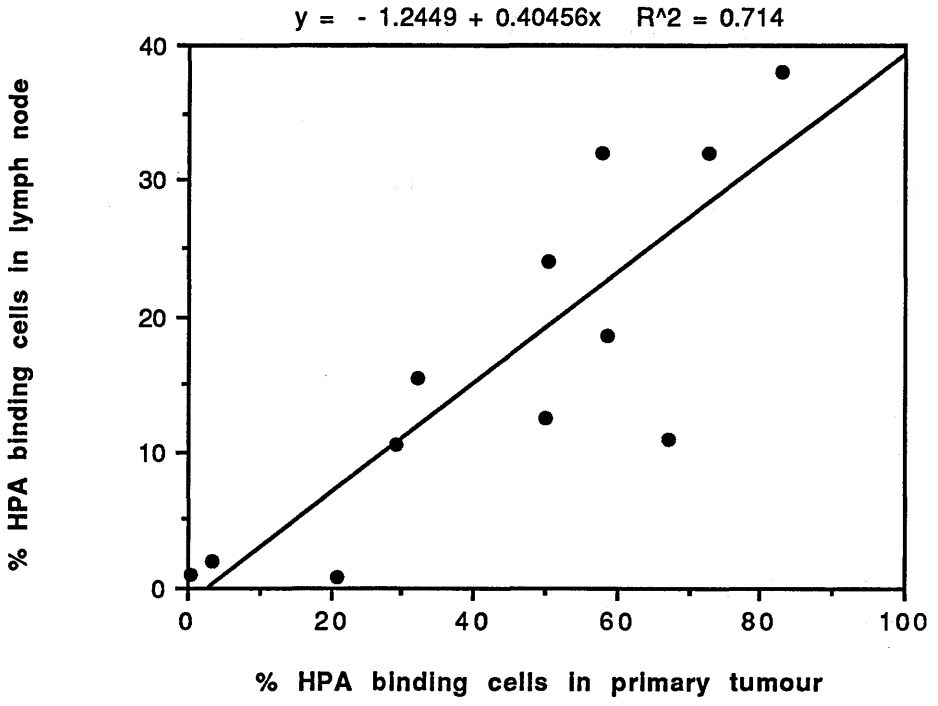


Figure 4.4. Correlation of HPA binding to tumour cells in invaded lymph nodes with autologous primary tumours.

In an attempt to see if high binding HPA cells could also be detected in invaded lymph nodes, spilled cells from all available lymph nodes were analysed for binding to HPA. For subsequent correlation with histopathology, only nodes which were invaded and for which tumour samples were also available were chosen. It is of interest to note that all non-invaded node samples were negative for HPA binding.

There were 12 invaded lymph node samples available for analysis and for each of these node samples corresponding autologous primary tumour samples were also analysed. As shown in Fig. 4.4, there was a strong positive correlation between percentage HPA binding cells in lymph nodes and those in autologous primary tumours (p= 0.001). The proportion of HPA binding cells in invaded lymph nodes were, however, less than those observed for the primary tumours. In lymph nodes, HPA binding cells ranged from 0.7 to 38.0%, with a mean of 16.5 +/- 3.7%. This mean is less than that observed for primary tumours (39.1%). It should, however, be pointed out here that about 5% of lymphocytes were collected within the tumour gate (Section 2.2.2.3) and as the number of tumour cells present in a nodal metastasis was variable only 5000 events were acquired for lymph node samples, compared to 10,000 events for primary tumours.

Table 4.5. HPA binding in tumour cells (non-lymphocytes) from invaded lymph nodes and autologous primary tumours.

% examined nodes invaded	HPA positive/total	
	Lymph node	Primary tumour
< 50	4/7	5/7
> 50	5/5	5/5

In breast cancer, both the number of invaded nodes and the total number of nodes examined vary with each patient. To further assess the relationship between HPA binding tumour cells in invaded lymph nodes with

those in primary tumours, the number of nodes invaded and the number of nodes examined were taken into consideration. All the cases with invaded nodes were grouped into having either less than 50% or greater than 50% of examined nodes invaded. The relatively lower cut-off value of 10% (compared to 20% for primary tumours) was found to be informative in defining HPA positivity in tumour cells in invaded lymph nodes. As shown in Table 4.5, all the samples (5/5) with >50% of examined nodes invaded were positive for HPA binding in cells from the lymph nodes, whereas samples with <50% of invaded nodes showed a lower incidence (4/7) of positive HPA binding.

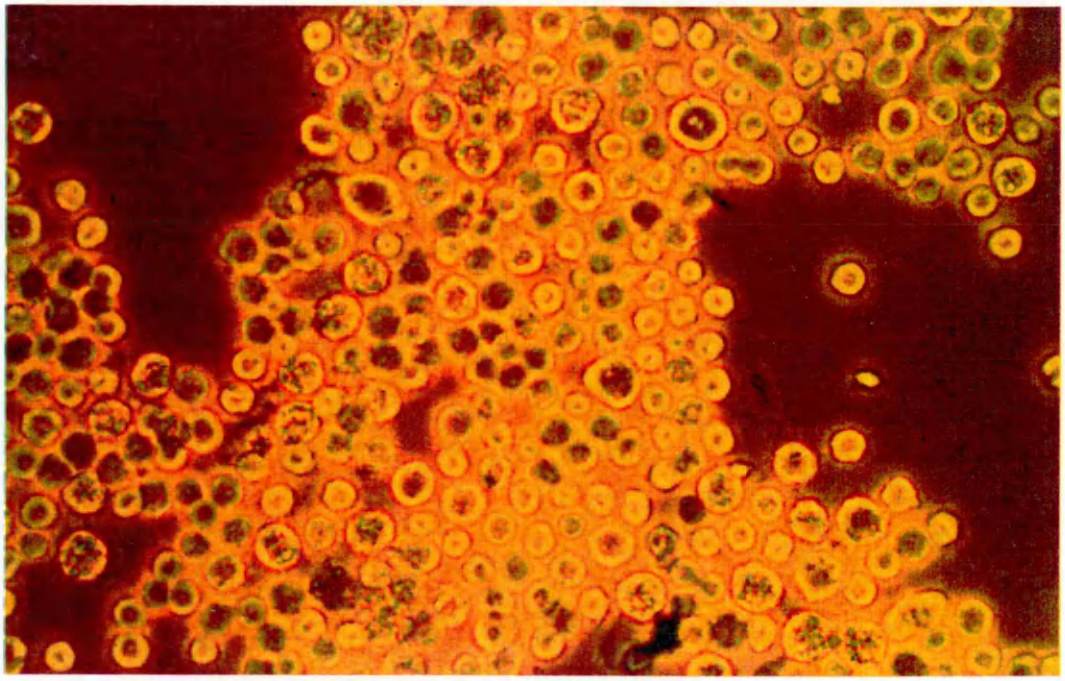
4.8. Examination of lectin binding by fluorescence microscopy

It was of interest to examine the nature of the lectin binding to the tumour cells as this information cannot be obtained from a flow cytometric (FCM) analysis. By visualising the sample directly it is possible to determine whether the staining is uniform or localised in certain areas. Fresh primary breast tumour cells were stained for examination by fluorescence microscopy as described in Section 2.2.2.4. A 10-fold higher concentration of the lectin (10 μg/ml) than that used for FCM analysis was used to visualise the staining.

Figure 4.5 shows the staining pattern of lectin-stained (FITC-conjugated) primary tumour cells under both bright field examination and fluorescent microscopy. It is apparent that only a small population of the cells stain, and that in this case the brightly staining cells are largely the ones which have poorly defined membrane structure. With this respect, FCM analysis affords an advantage as the "PI-gating" methodology allows exclusion of cells with breached membrane (Section 2.2.1.4) and therefore, the FCM analysis described above can be taken to define binding of the lectins to surface

Figure 4.5. Examination of lectin binding by fluorescence microscopy. In A, tumour cells are observed under a bright field (x 25) and in B, the same cells stained with FITC-labelled Con A are observed under a dark field (x 25).

A



B

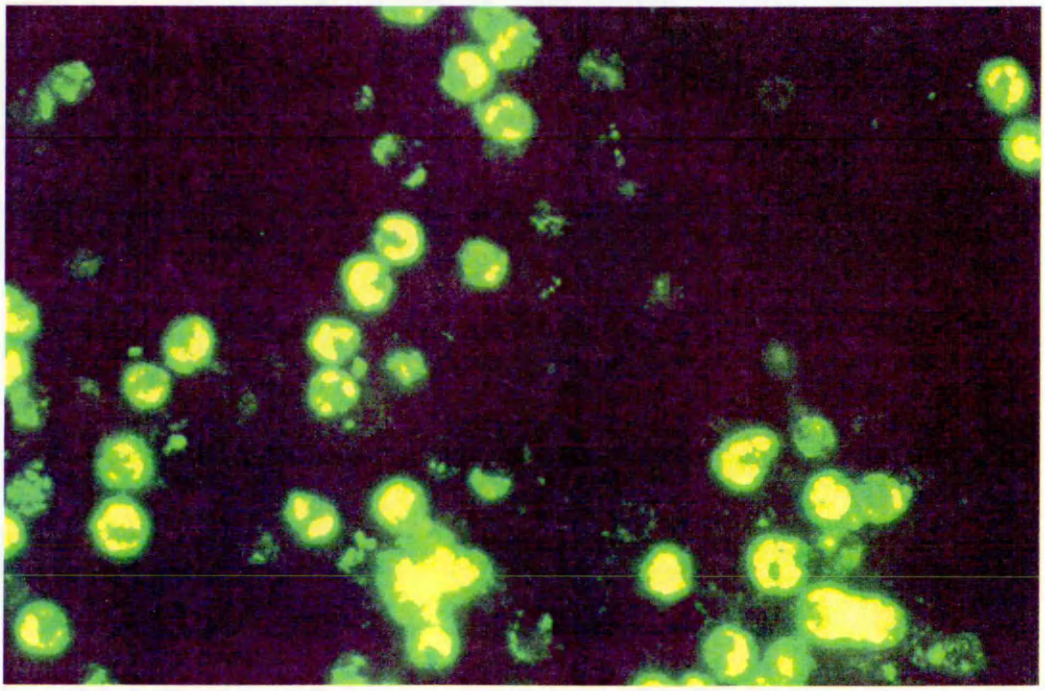
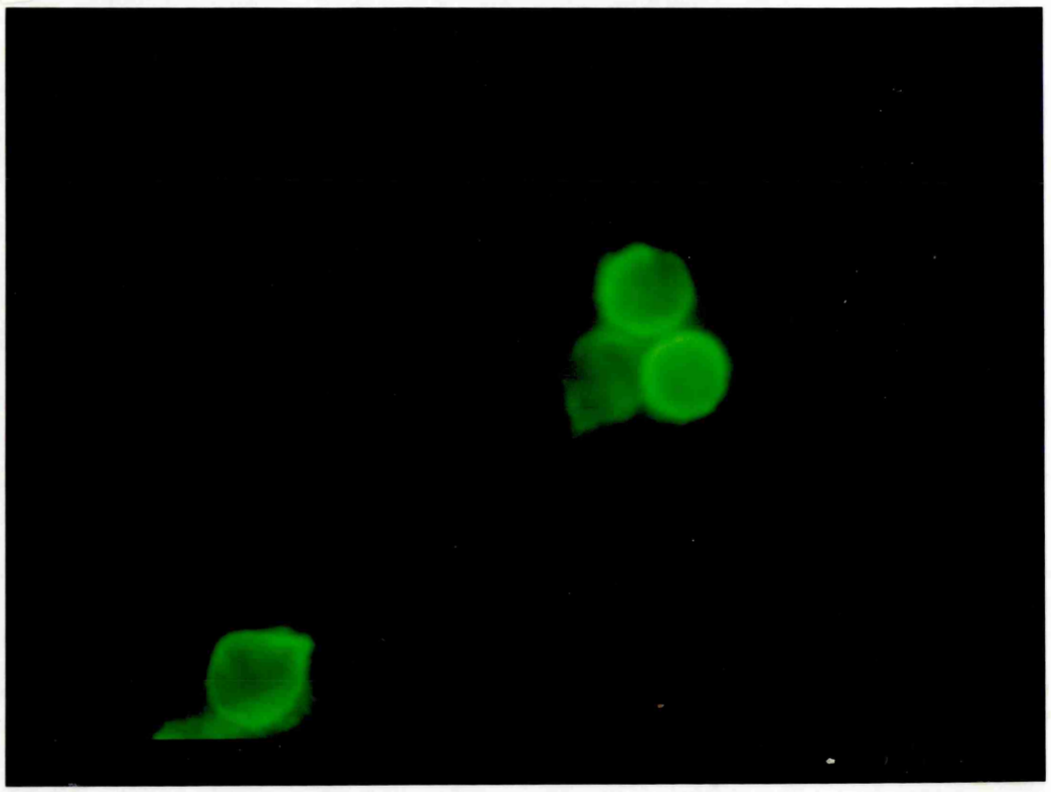


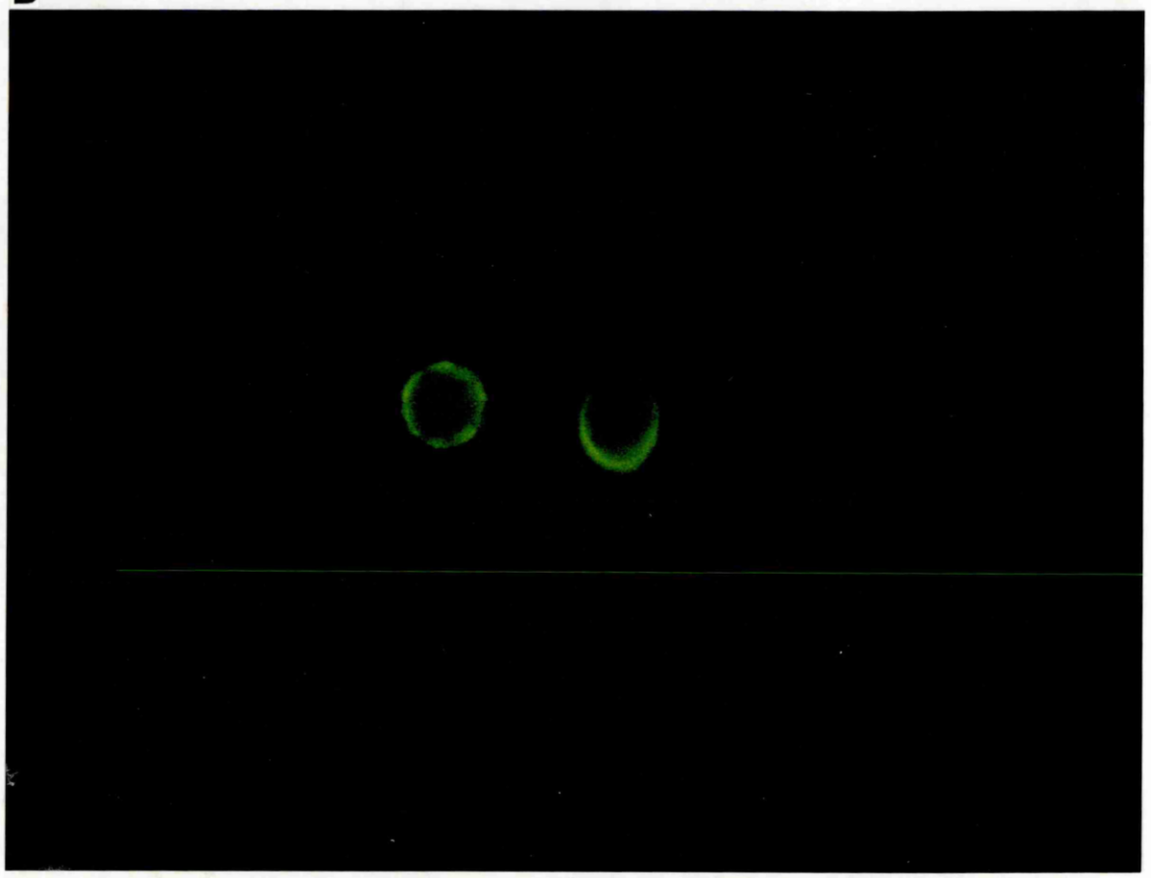
Figure 4.6. Microscopic examination (x 40) of breast tumour cells stained with A) FITC-Con A and B) FITC-HPA.

These photomicrographs show the surface staining pattern of tumour cells.

A



B



molecules on cells with intact membranes. Exclusion of dead cells is particularly important for a lectin which has a wide binding specificity and may stain intracellular sugars irrelevant to the metastatic potential of the tumour.

On the few cells which were more clearly intact, the membrane staining in the cases of HPA and Con A was highly variable, sometimes uniform and sometimes polarised to sections of the membrane (Fig. 4.6).

4.9. Discussion

From the results described in this section, it is apparent that HPA binding defines an N-acetyl galactosamine-containing cell surface marker on breast tumours which is associated with metastasis to lymph nodes. Tumour cells that express this HPA marker were also found in lymph node metastasis and the strong correlation between HPA binding in primary tumour and their corresponding lymph nodes further substantiates the conclusion that HPA binding represents a phenotypic marker of metastatic potential. The bulk of this data has been published (Alam *et al.*, 1990).

This study also demonstrates the use of flow cytometry as an effective technique in detecting differences in lectin binding in spilled cells from fresh breast cancer tissues. In all previous studies involving lectins, histochemical analysis was performed on paraffin sections of formalin-fixed tissues and the extent of lectin binding was determined by a simple visual assessment. Histochemical analysis suffers from a number of limitations and not all lectins give consistent results when fixed tissues are used (Walker, 1984a). Additionally, it is difficult to quantitate fluorescence intensity and as observed by Dansey *et al.* (1988) this can be highly subjective (Section 1.3.5.2). In this study, therefore, fresh tissues have been used and the use of flow cytometry demonstrated some distinct advantages of the technique. A large number of cells per samples have been analysed (10,000 for primary tumours, 5,000 for lymph node metastases). Moreover, the use of "PI-gating" has effectively allowed the exclusion of dead cells from the analysis. This is particularly important in assessing lectin binding as breached membranes can expose irrelevant intracellular sugars. Finally, the extent of lectin binding has been expressed in a defined quantitative fashion, thereby allowing the

percentage of positive cells to be readily determined. However, it must be pointed out that with flow cytometry, the nature of staining cannot be determined and as only free cells are studied, the normal architecture of the tissue is lost.

The data in this study show a significant correlation between HPA binding and lymph node involvement ($p<0.01$). This association appeared to be related to tumours of higher grade (Grade II and III). Although only 4 Grade I tumours were available, all these tumours were found to be negative for HPA binding, irrespective of their nodal status. There were more HPA positive tumours in the grade III group compared to grade II, but the relationship was statistically not significant. HPA positive tumours were also more likely to be ER negative ($p< 0.05$). In the recently reported follow-up study of Brooks & Leathem (1991), the strong association between HPA binding and the presence of lymph node metastasis has been confirmed. In this and other studies, HPA binding was not only related to lymph node stage, but also to local recurrence and survival (Brooks & Leathem, 1991; Fenlon *et al.*, 1987; Fukutomi *et al.*, 1989). HPA binding, however, has been found not to relate to either tumour grade (Brooks & Leathem, 1991; Fenlon *et al.*, 1987) or ER status (Fenlon *et al.*, 1987).

No correlation with any of the histopathological features was observed with Con A binding. Although Dansey *et al.* (1988) have reported no Con A binding to normal breast epithelial tissue, significant positive binding (39%) was observed in this study. It must, however, be added that normal breast tissue in this study was cultured from a reduction mammaplasty sample.

In this study, the binding of Con A and HPA were also directly compared in a simultaneous dual-label flow cytometric analysis. Although a

few characteristic features of lectin binding were observed, in general each tumour behaved individually and no consistent pattern could therefore, be recognised.

Tumour cells in invaded lymph nodes were analysed by gating out the bulk of the lymphocytes, which were invariably present in large numbers but were found not to show any high-intensity binding to HPA. Although lymph node metastases also showed abnormal HPA binding, there were fewer of these cells in the nodes when compared to the primary tumour. However, the invaded nodes reflected the binding of the lectin in the primary tumours and a strong positive correlation was observed between HPA binding in primary tumours and those in tumour cells from lymph node metastasis in the same patient ($P=0.001$). In only one recent study has lectin binding been studied in metastatic tissues from breast cancer patients (Krogerus & Anderson, 1990). Using a panel of lectins, which also included HPA, the study reported that while primary tumours were heterogeneous in their reactivity to lectins, lymph node metastases from the same patient showed a more restricted reactivity. This was suggested to indicate the presence of selected clones of primary tumour cells in the lymph node metastasis. A further implication of this is that carbohydrate structures detected by lectin binding can identify clones of tumour cells with higher metastatic potential. The presence of a percentage of cells with high-intensity binding to HPA observed in this study can then, be considered as a subpopulation of tumour cells with higher metastatic potential. In lymph nodes invaded with tumours, the presence of these HPA binding cells, therefore, provides further evidence of HPA being a surface marker of metastatic potential.

CHAPTER 5

DNA analysis of primary tumours and lymph node metastases

5.1 Flow cytometric (FCM) DNA analysis of primary tumours and lymph node metastases from breast cancer patients

A number of studies have now shown that in breast cancer patients, high SPF and abnormality in the DNA content of breast tumours correlate with poor prognosis (Section 1.4.3). These studies reveal that ploidy, which is abnormal in about 65% of breast tumours, and SPF reflect important aggressive features of a primary breast tumour. However, little is known about the true biology of aneuploid cells, especially whether these cells have a higher metastatic potential. As in the past and also described in this section, growth of aneuploid cells in culture has been difficult and as such characterisation of aneuploid breast tumour cells in terms of surface markers and oncogene expression has not been possible. The majority of FCM DNA studies have focussed on primary tumours and were carried out principally to assess the potential of DNA flow cytometry as a prognostic indicator. Very little attention has so far been directed towards comparing the ploidy in metastases with that of the primary tumour. Lymph node metastasis remain the most important indicator of the tumour's biological aggressiveness and the availability of invaded lymph nodes together with the primary tumour from the same patient makes it possible to conduct a simultaneous comparative DNA analysis of the two tissues.

This section describes data from a FCM DNA analysis of primary tumours and its correlation with histopathological factors. In addition, it also describes a comparative study between the DNA profiles of fresh primary tumours and metastatic cells from the axillary nodes of the same patients. The principal aim of the study was to investigate whether metastatic population in invaded nodes included the cells with grossly abnormal DNA. Detection of the

tumour DNA profiles in the node has generally been difficult because of the comparatively small number of metastatic tumour cells present. In this study, therefore, using an antibody against cytokeratin, a dual-parameter FCM DNA analysis was performed. This allowed study of true epithelial cells in the primary tumours while in the lymph nodes, small proportions of tumour cells which normally go undetected were analysed for their DNA ploidy.

5.1.1. Ploidy status of primary tumours and its correlation with histopathological features

The study initially involved the determination of DNA ploidy status of primary tumours from breast cancer patients. Samples from 57 primary tumours were suitable for analysis. 63% (36) of these were aneuploid and the remaining 21 cases (37%) were diploid. The majority of aneuploid tumours (31/36) were hyperdiploid (1.0<DI<1.9) with a single aneuploid peak. One tumour was classified hypodiploid (DI<1.0) and 4 were multiploid, having two aneuploid stemlines in addition to the normal diploid peak. The DNA index (DI) of the hyperdiploid tumours ranged from 1.2 to 2.95, median 1.7. In most tumours, a diploid peak was always present in addition to the aneuploid peak. However, in 2 tumours no diploid peak was detectable and the DNA histogram showed a single aneuploid population for both these cases. Representative examples of DNA profiles of primary breast tumours is shown in Fig. 5.1.

Each primary tumour appeared to be characteristically different from the other and this is reflected in the distribution of their DI shown in Fig. 5.2. In general, the distribution is bimodal with peaks in the diploid region and the hyperdiploid region of DI 1.7.

Figure 5.1. Representative examples of DNA histograms of primary breast tumours.

I. A diploid tumour;

II. Aneuploid tumour with a predominantly aneuploid population (A).

III. Aneuploid tumour with both diploid (D) and aneuploid populations (A). In this tumour the proportion of diploid cells is much greater than the aneuploid cells.

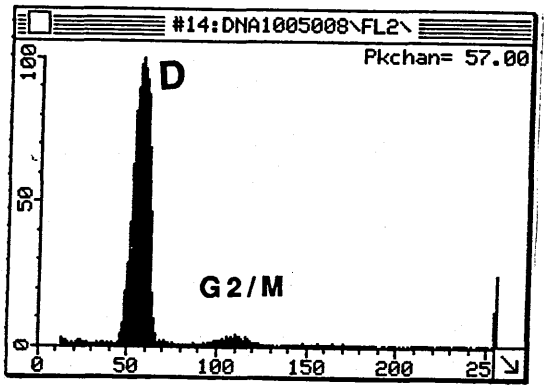
IV. Aneuploid tumour with both diploid (D) and aneuploid populations (A).

V. Multiploid tumour with two aneuploid peaks (A1 & A2). A2 peak appear as a shoulder to the main A1 peak.

VI. Multiploid tumour with more distinct aneuploid peaks, A1 and A2.

c represents chicken RBC. In aneuploid tumours, G2/M cells of the aneuploid population is marked.

I



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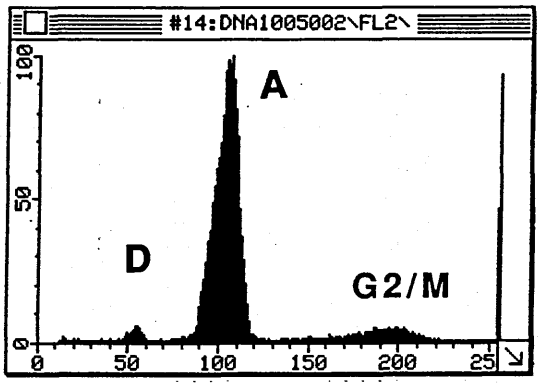

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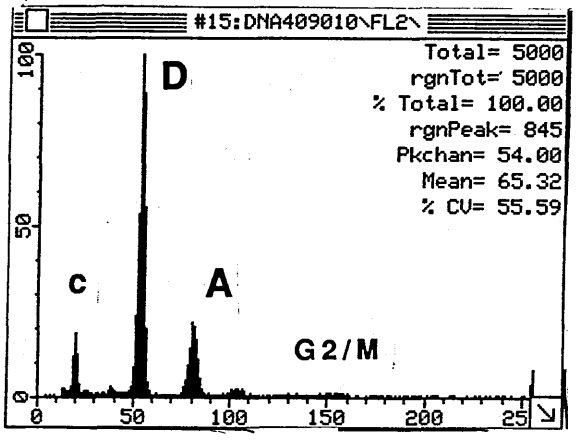
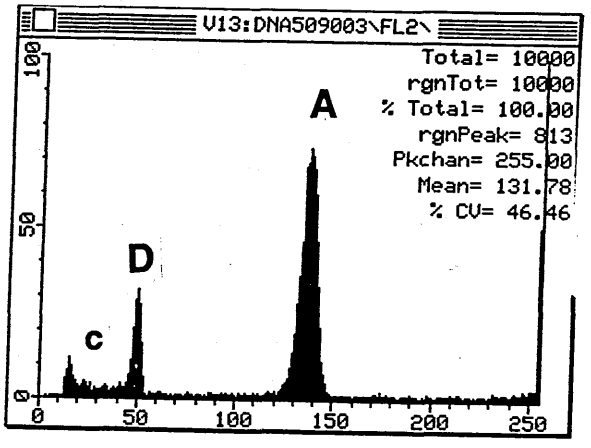
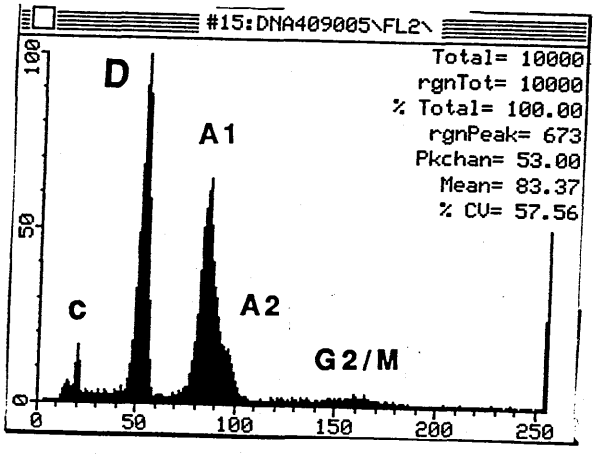


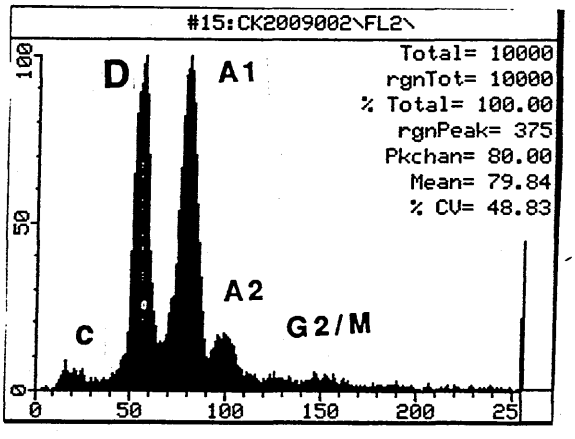
IV



V



VI



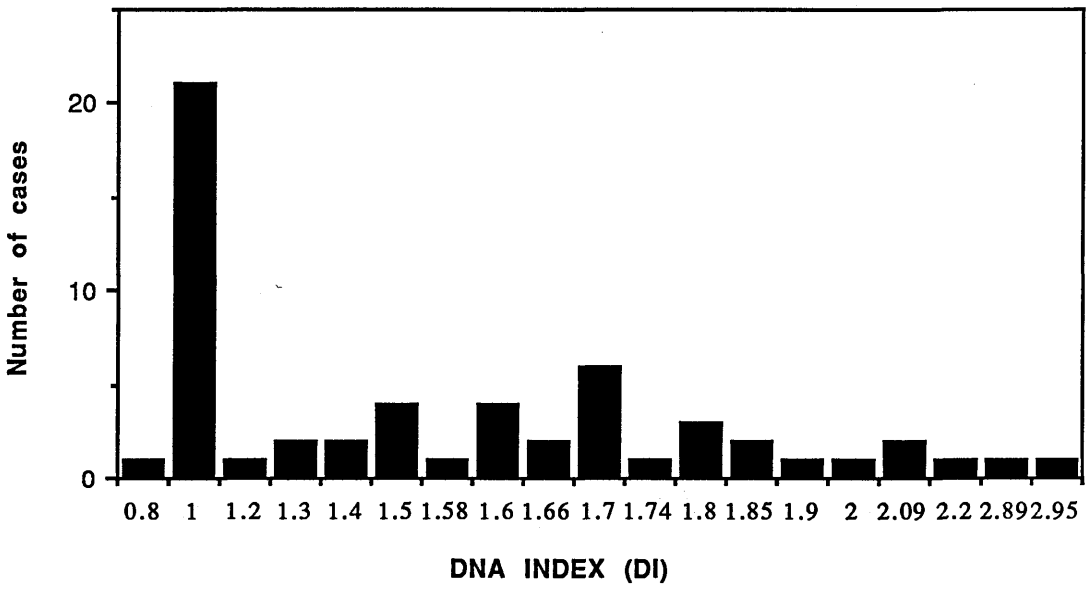


Figure 5.2. Distribution of DNA Index (DI) in all 57 cases of breast cancer analysed by DNA flow cytometry.

Lymph node involvement (Stage II)

DNA ploidy correlated significantly with lymph node involvement ($p<0.01$). In 80.6% (25/31) of the patients with invaded nodes, the primary tumours were aneuploid (Table 5.1). But it should be noted that among the patients with non-invaded nodes, a substantial percentage (11/26; 42%) of the tumours was also aneuploid (Table 5.1). At the same time, 30.5% (11/36) of patients with aneuploid tumours were without metastasis in their nodes.

Tumour grade

Although the correlation of ploidy with tumour histological grade failed to reach statistical significance ($0.5<p<0.1$), a trend indicating that poorly differentiated tumours are more likely to be aneuploid than diploid was observed (Table 5.2). As there were only 3 grade I tumours in the total

Table 5.1. DNA ploidy of primary tumours and lymph node status of 57 patients with breast cancer.

Histopathology of lymph node	FCM DNA analysis Aneuploid (n=36)	Diploid (n=21)	Statistical Significance (Chi-square test)
Invaded	25	6	$\chi^2 = 7.36$, D.F.=1
Non-invaded	11	15	$p < 0.01$

Table 5.2. DNA ploidy and histological tumour grade of 57 primary breast tumours.

Histological Grade of differentiation	FCM DNA analysis Aneuploid (n= 36)	Diploid (n = 21)	*Statistical Significance (Chi-square test)
I (Well)	1	2	$\chi^2 = 2.88$, D.F.= 1
II (Moderate)	15	13	$0.05 < p < 0.1$
III (Poor)	20	6	

*As only 3 grade I tumours were available for analysis, grade I and grade II tumours were grouped and compared with grade III in the chi-square test.

Table 5.3. DNA ploidy and Oestrogen receptor (ER) status of 44 breast cancer patients.

ER status	FCM DNA analysis		*Statistical
	Aneuploid (n= 27)	Diploid (n = 17)	Significance (Chi-square test)
ER -ve (<20 fmol/mg protein)	14	2	$\chi^2 = 5.6$, D.F.=1 $p < 0.02$
ER +ve (>20 fmol/mg protein)	13	15	

samples analysed, the differences observed are between grade II and grade III tumours.

ER status

The ER content data was available for 44 of the 57 primary tumours. The ER status was compared with the ploidy status of these patients (Table 5.3). Of the diploid tumours, 15/17 (88%) were ER positive compared with 13/27 (48%) of aneuploid tumours. There was thus a strong trend for diploid tumours to be ER positive, and this was found to be statistically significant ($p < 0.02$). However, it should be noted that among aneuploid tumours, almost equal number of tumours were ER+ve and ER-ve.

5.1.2. S phase fraction (SPF) of primary tumours

The methodology of SPF measurement is described in Methods and Materials (Section 2.2.3.5). Aneuploid tumours were analysed for their SPF only in cases (9 tumours) where there were no overlapping peaks from the diploid stemlines and in 4 cases in a gated analysis of CK+ single cell population (Section 3.3.5.1). The SPF of diploid tumours ranged from 0.65 to 12.0%, while those of aneuploid tumours ranged from 7.0% to 25%. The distribution of SPF differed significantly (P= 0.001) between diploid and aneuploid tumours, with means of 3.5+/-0.69 and 8.6+/-1.3 percent, respectively.

As SPF could be determined only for a selected number of patients with aneuploid tumours, no attempt was made to relate SPF of primary tumours with histopathological prognostic parameters.

5.1.3. DNA ploidy of primary breast tumours in short-term culture

4 primary breast tumours, of which 3 were aneuploid and one diploid, were put into short-term culture as described in Section 2. DNA ploidy of the tumours both prior to and after culture were compared. Of the 3 aneuploid primary tumours all but one culture gave rise to epithelial cells. That one culture comprised only of colonies of fibroblast cells. DNA ploidy analysis revealed that both from the diploid and the aneuploid primary tumour cells, a single population of cells with diploid DNA content grew in culture. Fig 5.3a

Figure 5.3a. FCM DNA analysis of breast tumour cells before (A) and after short-term culture (B).

From these 3 tumour samples, it was possible to grow epithelial cells as opposed to one sample which morphologically resembled fibroblasts.

While tumour from patient A was diploid, both patient K and T had aneuploid tumours. Cells that came out in culture were all diploid (B).

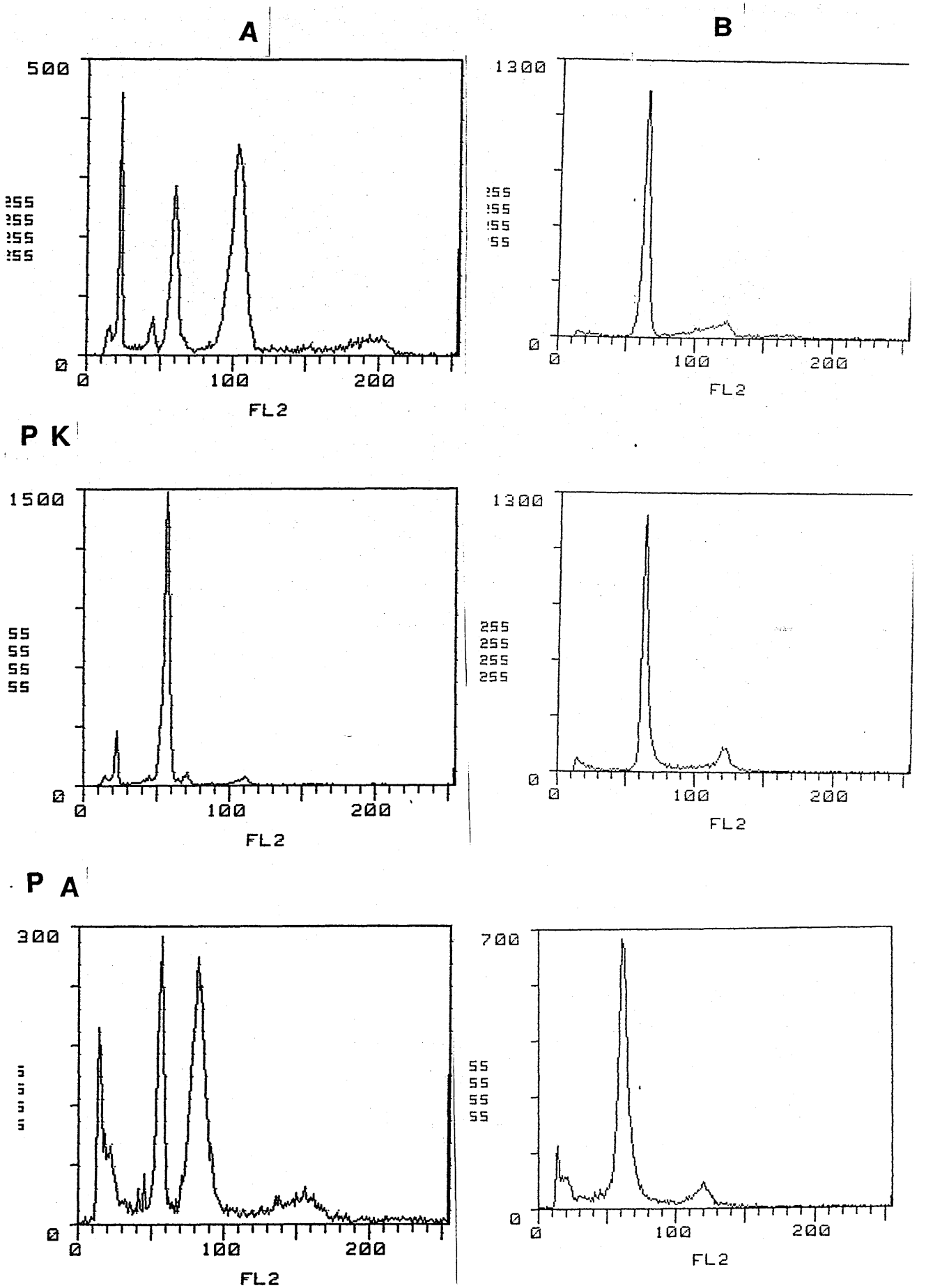
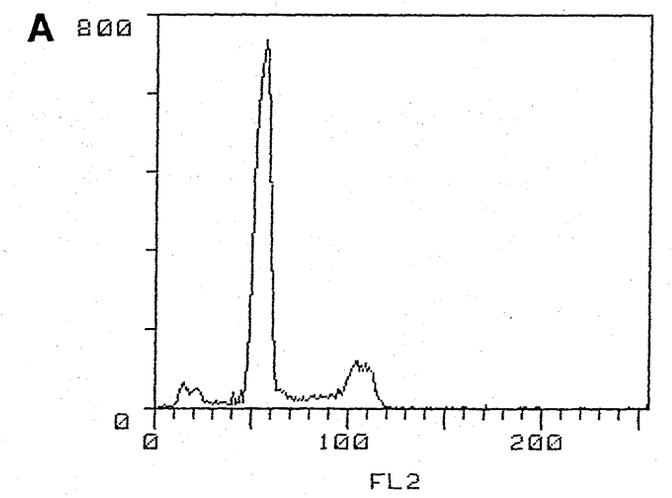
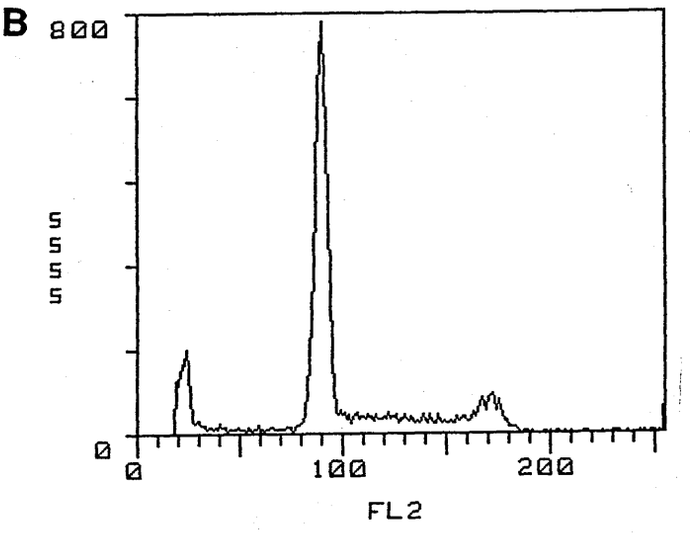

A
B
500
1300
0
100
200
FL2
P K
1500
1300
P A
300
700
P T

Figure 5.3b. FCM DNA analysis of a breast cell line (ZR-75, B) and normal breast tissue (A) from a reduction mammaplasty sample.
While cells in ZR-75 line are aneuploid, normal breast cells are diploid.


A
800
0
0
100
200
FL2


B
800
S
S
S
S
0
0
100
200
FL2

shows the DNA histograms of these samples both before and after culture. Although only a small number of primary tumours were put into culture, the results suggest that while the culture condition allows growth of diploid tumour cells, it is unable to propagate aneuploid cells. It is interesting to note that as with many established cell lines, the breast cell line ZR-75 was found to be aneuploid, while the cultured normal breast cells from the reduction mammaplasty sample were diploid (Figure 5.3b).

5.1.4. Single-parameter ploidy studies on invaded lymph node and tumour samples from the same patients

The distribution of DNA indices of all primary tumours with invaded nodes is shown in Figure 5.4. The distribution of DI over such a wide range suggests that there is considerable differences between patients with respect to their DNA ploidy but within a patient, the majority of tumour cells carry the same amount of DNA. In addition, the wide range of DI suggests that the metastatic capability is not inherent in any aneuploid cell population with a specific DI.

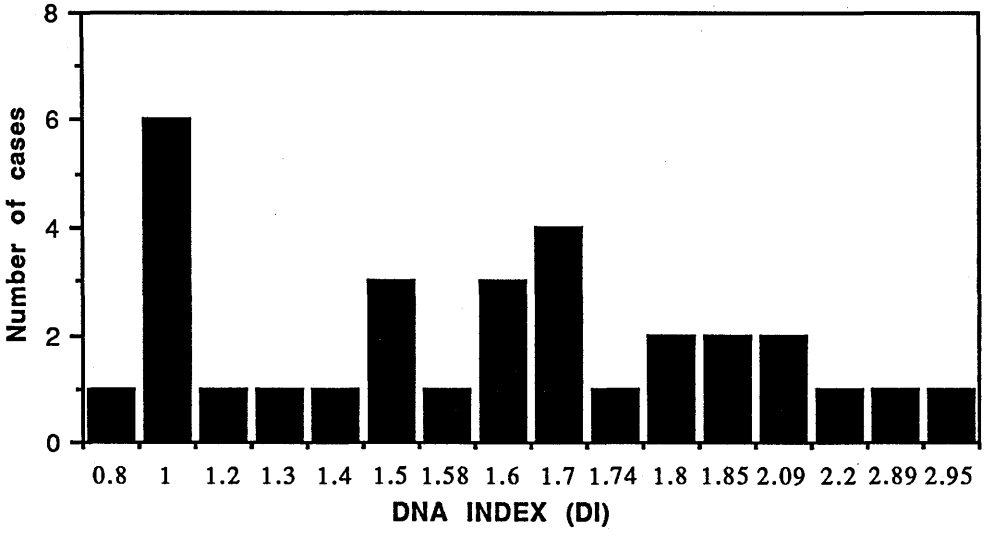


Figure 5.4. Distribution of DNA Index (DI) in 31 breast cancer patients with lymph node metastasis (Stage II).

As primary tumours with aneuploid DNA are likely to be more aggressive than those with diploid DNA, a relevant question is whether it is possible to detect metastatic tumour cells in lymph nodes and if so, then to determine the ploidy of these metastatic cells. Using a single-parameter analysis no aneuploid cells were detected in lymph nodes, whether invaded or not, from patients with diploid tumours. This suggests that at least with diploid tumours, the ploidy status of primary tumours is maintained after metastasis. This apparently did not hold for aneuploid tumours. Within the 25 invaded lymph nodes from patients with aneuploid primary tumours, an aneuploid peak was detected in only 6 cases. However, the DI of the aneuploid peak in all these lymph node metastases were identical to the DI of the primary tumours. Figure 5.5 shows two such cases. In these cases the nodes were grossly invaded by tumour cells and as such tumour cells were detectable. It can, however, be seen in Fig. 5.5 that proportion of tumour cells in a lymph node can vary considerably.

Figure 5.5. DNA content of primary tumours (A) and associated heavily tumour invaded lymph node metastases (B) from two breast cancer patients. The two examples cited here represent cases where the lymph nodes were grossly invaded and aneuploid peaks (a) were detected in a single parameter DNA analysis.
c= chicken red blood cells; d= diploid Go/G1 peak; a= aneuploid Go/G1 peak; G2/M= G2/M peak of the aneuploid population.

A. Primary tumour

B. Lymph node metastasis

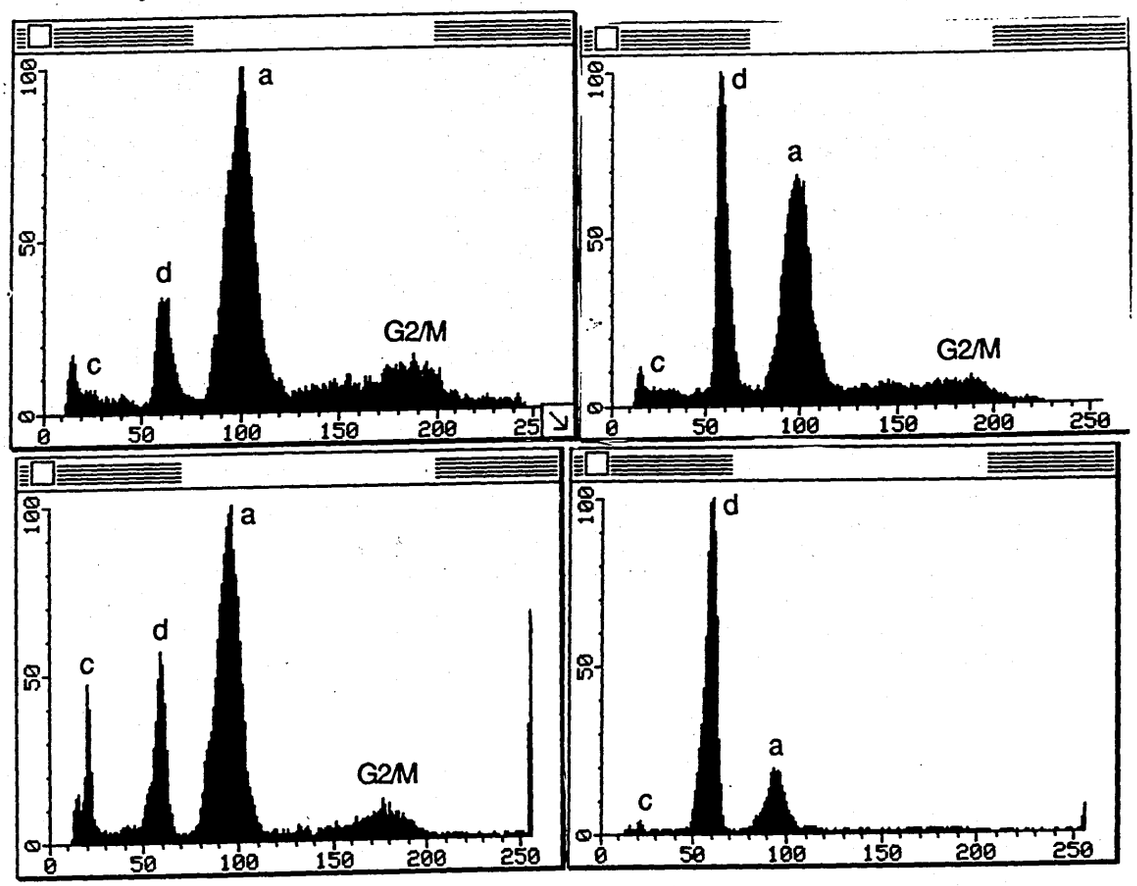


Figure 5.6. Single-parameter DNA profiles of two patients presenting with double primary tumours.

In patient A, the aneuploid population in the invaded right node has a DI of 1.75, identical to that in the primary tumour. The left tumour has a different aneuploid population (DI=1.6) and no aneuploid population can be detected in the left tumour-free node.

Patient B, with both primary tumours on the same side, had a diploid tumour (not shown) and an aneuploid tumour as shown (DI= 1.6). The aneuploid peak can be seen in the invaded lymph node.

The CV of the aneuploid Go/G1 peaks are:

Patient A, right tumour- 3.7

Patient A, right lymph node- 2.6

Patient A, left tumour- 2.8

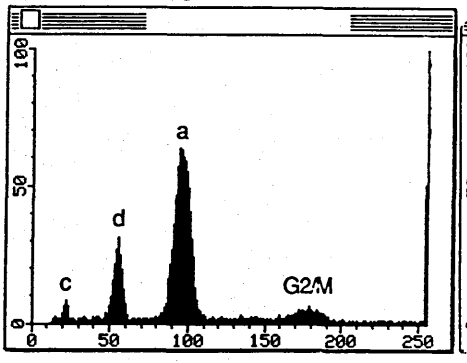
Patient A, left lymph node- 2.4

Patient B, primary tumour- 6.1

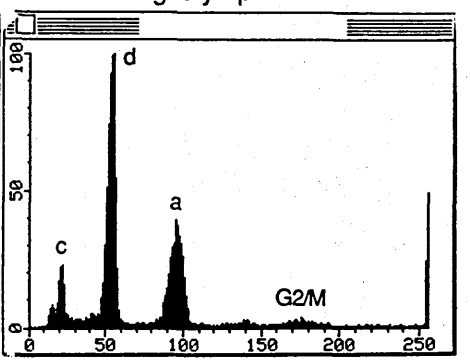
Patient B, lymph node- 5.4

Patient A:

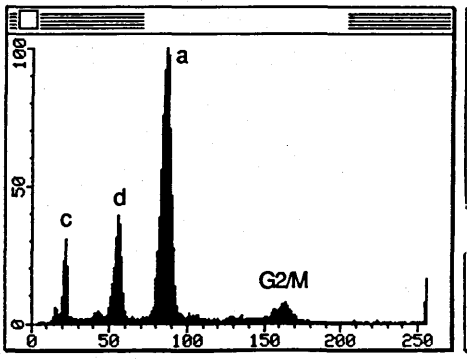
Right tumour



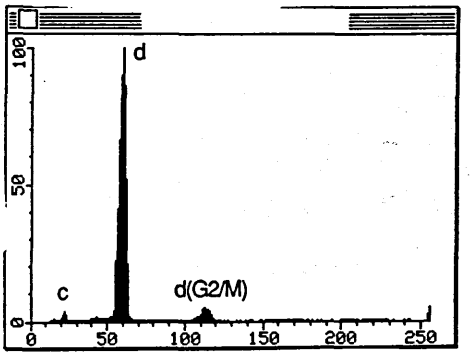
Right lymph node



Left tumour

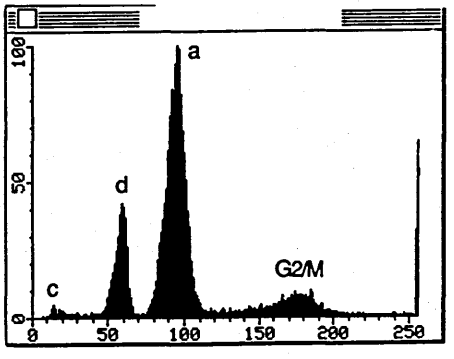


Left lymph node

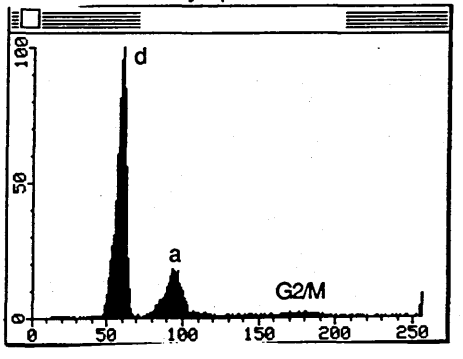


Patient B:

Primary tumour



Lymph node



5.1.5. Single-parameter DNA ploidy analysis of double primary tumours and their corresponding lymph nodes within the same patient

In the course of this study two patients presented with rare double primary tumours. In both cases, only one of these appeared to have metastasised. In the case of Patient A , who had bilateral carcinoma, both right and left tumour samples and the corresponding lymph nodes were available for DNA analysis. Histopathology revealed both tumours to be poorly differentiated (Grade III) infiltrating carcinoma. DNA analysis revealed both tumours to be aneuploid but with differing DI (Patient A, Figure 5.6). The DNA histogram of lymph node cells from the right,which was invaded, showed aneuploid cells with DI identical to the right primary. Although the left tumour was aneuploid, no aneuploid population was detected in the left lymph node, which on histopathological examination was found to be tumour free. Comparison of the DNA histograms of the two tumours reveal that it is likely that the left tumour represents a second primary and not a secondary deposit of the right primary tumour.

The second double primary, Patient B, presented with two distinct primary tumours on the same side. Tumour specimens from the two regions and lymph nodes were analysed (Patient B, Figure 5.6). Histological evaluation, which revealed carcinoma of Grade II, was possible in only the larger of the two tumours. The smaller tumour was diploid, wheras the invaded lymph node had a detectable aneuploid peak with DI identical to the larger, presumably primary tumour (Figure 5.6).

Ploidy analysis in these patients with double primaries reveal that within a patient, two tumours can have different ploidy and that they may differ

in their ability to metastasise. In the lymph node, cells from only one of these tumours were apparently present as the ploidy matched with only one of the primary tumours. In addition to the difference between being diploid or aneuploid, there also appears to be a distinct difference between aneuploid tumours in terms of their aggressive nature.

5.1.6. Dual-parameter FCM DNA analysis of primary tumours and their corresponding lymph nodes from the same patient

Although detected in a relatively small number of cases, the above results indicate that invaded lymph nodes harbour aneuploid tumour cells which appear identical to a subpopulation of cells within the corresponding primary tumour with respect to their DNA content. However, aneuploid cells were detected in only 24% (6/25) of the analysed lymph nodes with aneuploid primary tumours. As diploid populations were almost always detected in aneuploid tumours, this could reflect the presence of a substantial malignant diploid population from which terminally differentiated and essentially harmless aneuploid cells evolve. However, an alternative explanation is that it reflects the low sensitivity of detection of DNA peaks from small numbers of highly aggressive aneuploid cells, effectively swamped by the large dilutional effect caused by the inevitable presence of lymphocytes in the node preparation. To resolve this matter it was, therefore, necessary to exclude lymphocytes from lymph nodes. With respect to the primary tumour, it is not known whether the diploid cells in such tumours represent epithelial cells, in which case they could be both normal and/or tumour cells, or whether they represent non-epithelial cells from the surrounding tissues. It is highly relevant to add here that often large number of lymphocytes infiltrate breast tumours and as such are also a potential candidate for the diploid population. Thus as

for the lymph node, in the case of the primary tumour too, it is necessary to correctly establish that the diploid peak is contributed by epithelial tumour cells and not by contaminating stromal and inflammatory cells. This has been accomplished in a dual-parameter DNA FCM analysis.

5.1.7. Dual staining of tumour cells with anti-cytokeratin (CK) antibody and propidium iodide (PI)

Contaminating lymphocytes from the lymph node and non-epithelial cells from the primary tumour were excluded in a dual-parameter DNA analysis using CK staining. When primary tumours were analysed for CK and PI staining, diploid peaks were always reduced, sometimes to a negligible level (Section 2.2.3.7, Fig 2.9). In the lymph node, the sensitivity of the analysis could be exploited by restricting the analysis to invading tumour cells and thereby the assessment of whether invading cells in the lymph node are diploid and/or aneuploid was possible. Moreover, it improved detection in one lymph node metastasis, where an aneuploid peak which was undetectable was subsequently made visible by gating for CK+ cells.

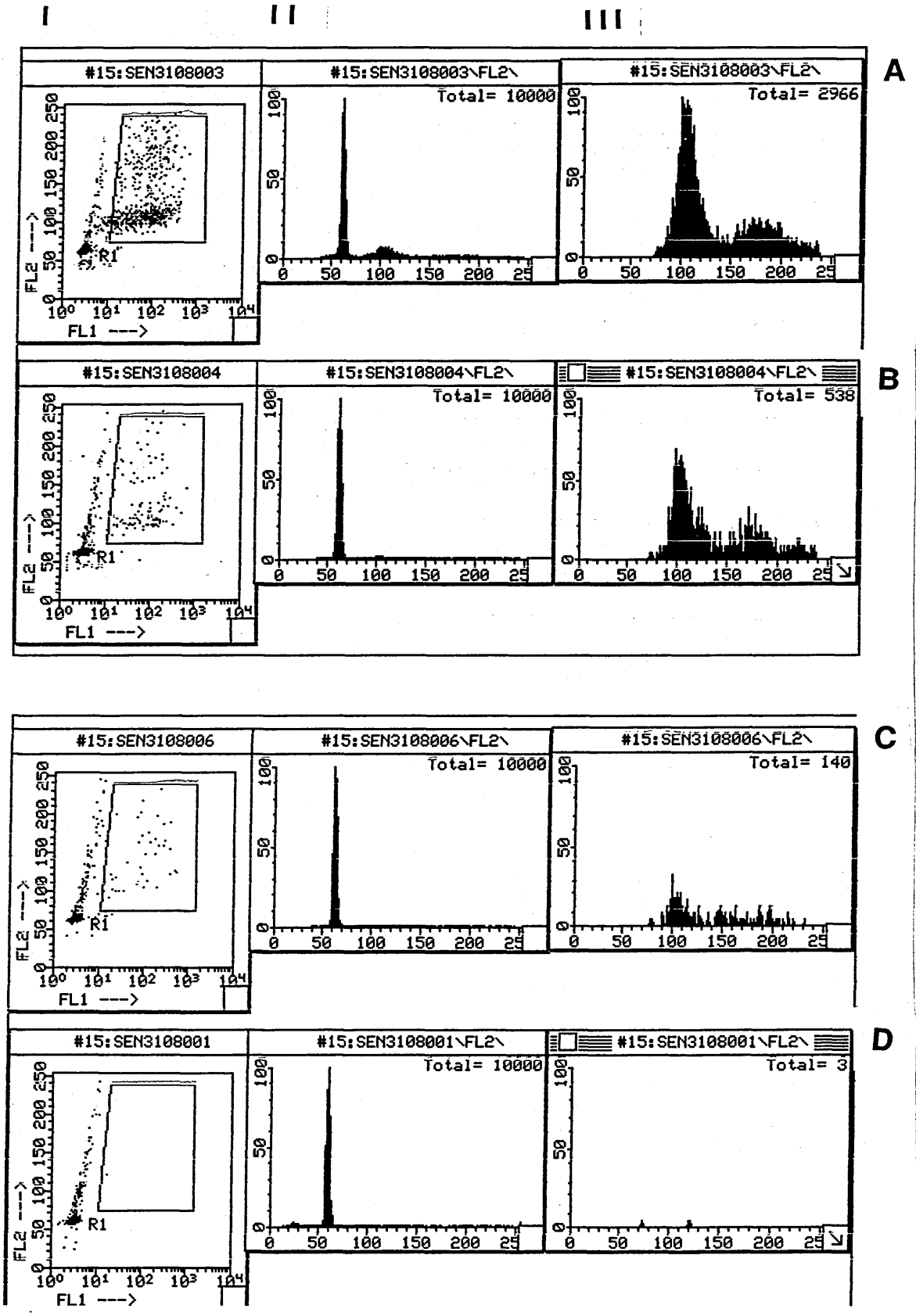
The sensitivity of CK+ gating was tested in a mixing experiment as described in Section 2.2.3.8. The sensitivity was high enough to detect as low as 5% of tumour cells in involved lymph nodes, allowing a 20 fold enrichment. As shown in Fig.5.7, the position of the aneuploid peak remained the same at all concentrations of tumour cell and was identical to that of the tumour cells alone. When 5% of tumour cells were present, both the Go/G1 and the G2/M peaks were clearly visible above the background, while below 5% (Fig 5.7, C), although the Go/G1 peak was still detectable, the G2/M peak was not.

Figure 5.7. Sensitivity test of the dual-parameter DNA analysis in detecting CK+ cells.

Breast tumour cell line (ZR-75), which are aneuploid with a DI= 1.65, were mixed in varying proportions with human blood lymphocytes.

A- 30% ZR-75 cells; B- 5% ZR-75 cells; C- 3% ZR-75 cells of total cells; and D-lymphocytes alone.

I is scatter plot with the analysis gate, II is ungated DNA histogram, and III is gated (CK+) DNA histogram.


I
II
III
A
#15:SEN3108003
#15:SEN3108003\FL2\
Total= 10000
#15:SEN3108003\FL2\
Total= 2966
R1
FL2 --->
FL1 --->
B
#15:SEN3108004
#15:SEN3108004\FL2\
Total= 10000
#15:SEN3108004\FL2\
Total= 538
C
#15:SEN3108006
#15:SEN3108006\FL2\
Total= 10000
#15:SEN3108006\FL2\
Total= 140
D
#15:SEN3108001
#15:SEN3108001\FL2\
Total= 10000
#15:SEN3108001\FL2\
Total= 3

5.1.8. Dual-parameter FCM DNA analysis of primary tumours and their corresponding lymph node metastases

Twelve paired primary tumours and lymph nodes from the same patients were selected to determine the ploidy of the CK+ primary tumour cells and their corresponding lymph nodes were analysed to detect the ploidy of the invading tumour cell population (CK+). The results are summarised in Table 5.4.

In tumour-free lymph nodes, CK+ cells were absent (Table 5.4) and the diploid cells in the ungated histogram clearly represented lymphocytes. This was true whether the primary tumour was diploid or aneuploid.

Comparison of CK+ gated primary tumours and lymph node metastases in the same patient reveal that aneuploid tumour cells differ in their metastatic capability and where a tumour was multiploid, there was evidence to suggest that there is selective metastasis of a subpopulation of aneuploid cells (Table 5.4). Three such cases are detailed in Fig. 5.8.

In patient 1, although both aneuploid and diploid cells were present in the primary tumour, only aneuploid cells were detected in the lymph node metastasis and they had a DI identical to the aneuploid cells in the primary tumour. The invaded lymph node in patient 2 revealed a single aneuploid peak with a DI identical to peak a2 of the primary tumour. In patient 3, an aneuploid peak with DI similar to the shoulder (a2 in Fig. 5.8) in the main aneuploid peak of the primary tumour was detected in the lymph node. An additional peak (a3, DI= 2.7) which was not detectable in the tumour also appeared in the lymph node histogram.

TABLE 5.4. Results from Dual-parameter DNA analysis of CK-gated paired primary tumour and lymph node from the same patient.

Patient No.	Histopathology of lymph node	Ploidy and DI of CK+ve cell population	
		Tumour	Lymph node
1	Invaded	Diploid & Aneuploid DI=1.0+1.7	Aneuploid DI=1.7
2	Invaded	Diploid & Aneuploid DI=1.0+1.45 +1.8	Aneuploid DI=1.8
3	Invaded	Diploid & Aneuploid DI=1.0+1.6+1.8	Aneuploid DI=1.85+2.7
4	Invaded	Diploid DI=1.0	Diploid DI=1.0
5	Invaded	Aneuploid DI= 1.3	Aneuploid DI= 1.3
6	Invaded	Diploid & Aneuploid DI= 1.0+ 1.4	Aneuploid DI= 1.4
7	Invaded	Diploid DI= 1.0	Diploid DI= 1.0
8	Non-invaded	Diploid DI= 1.0	None
9	Non-invaded	Aneuploid DI= 2.2	None
10	Non-invaded	Aneuploid DI=1.6	None
11	Non-invaded	Aneuploid DI=1.8	None
12	Non-invaded	Diploid DI=1.0	None

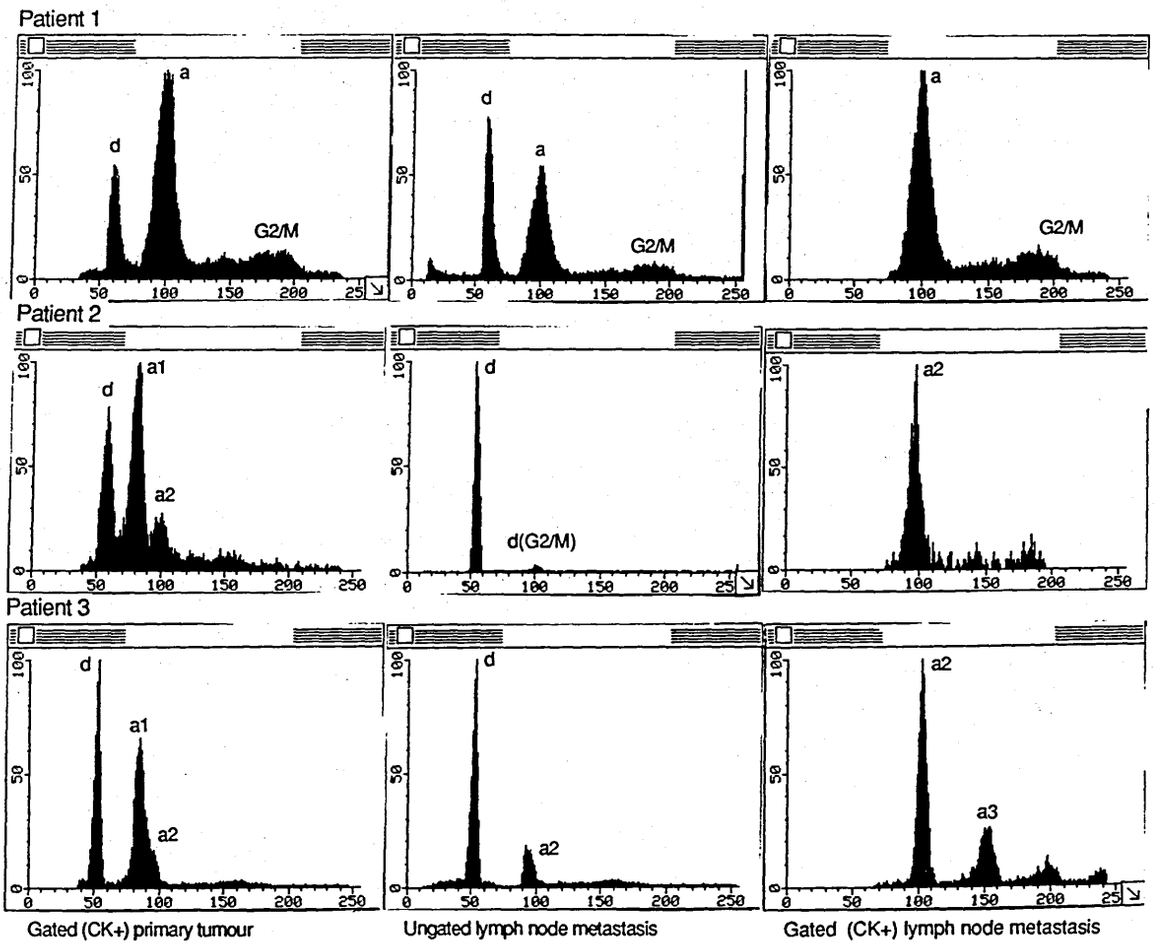
The presence of CK+ve diploid cells in both the primary tumour and lymph node metastases from Patients 4 and 7 suggests that diploid cells are as potentially metastatic as the aneuploid cells (Table 5.4).

Figure 5.8. CK+ gated and ungated DNA profiles of 3 lymph node metastases together with their primary tumours.

In patient 1, the diploid peak of the primary tumour was not represented in the lymph node following CK+ gating and the DI of the aneuploid population in the node matches the DI (1.7) of the aneuploid population in the primary tumour.

In patient 2, the aneuploid peak in the node is detected only after CK+ gating and represents the smaller of the two aneuploid peaks (a2, DI= 1.8) seen in the tumour.

In patient 3, two aneuploid peaks appear in the node after CK+ gating. Peak a2 (DI= 1.85) has a DI close to a2 peak in the primary tumour (DI= 1.8), whereas a3 (DI= 2.7) was not detected either in the ungated node or in the primary tumour.


Patient 1
d
a
G2/M
d
a
G2/M
a
G2/M
Patient 2
d
a1
a2
d
d(G2/M)
a2
Patient 3
d
a1
a2
d
a2
a2
a3
Gated (CK+) primary tumour
Ungated lymph node metastasis
Gated (CK+) lymph node metastasis

5.1.9. Discussion

This study presents data involving FCM DNA analysis of primary tumours and their corresponding lymph node metastases derived from the same breast cancer patient. Analysis of 57 primary tumours revealed that 63% of these breast tumours were aneuploid. This is in agreement with all previous reports (Frierson, 1991). Although abnormal in DNA content, these tumours appear to be characteristically different from one another and this was evident in the wide spread in the distribution of DNA indices in primary tumours whether or not the patient had metastasis. Correlation with histopathological prognostic factors revealed that aneuploid tumours are more likely to be poorly differentiated, and behave more aggressively with respect to metastasis to axillary lymph nodes. Presence of aneuploidy was found to be significantly related to lymph node metastasis. However, it is important to point out that a good percentage of patients (42%) without metastasis also had aneuploid tumours. This probably explains the conflicting reports in the literature concerning the relationship between DNA aneuploidy and lymph node involvement (Section 1.4.3.3.1). In studies where a larger number of patients were employed, the relationships observed were weak (Dressler *et al.*, 1988; Hedley *et al.*, 1984; Ewers *et al.*, 1984). Correlation with grade showed a trend suggesting that tumours of higher grade are more likely to be aneuploid. This relationship failed to reach statistical significance, presumably as there were not enough grade 1 tumours available for analysis. Almost all workers have reported a significant association between tumour ploidy and the differentiation state of the tumour (Section 1.4.3.3.1). Association between tumour ploidy and steroid receptors has also been reported by other workers (Dressler *et al.*, 1988; Moran *et al.*, 1984; Horsfall *et al.*, 1986; Olszewski *et al.*, 1981).

Assessment of SPF poses certain problems as discussed in Section 1.4.4.3.3. As the available computer models (Section 2) were not reliable for assessing SPF of aneuploid populations with high SPF, the percentage of cells in the S phase was determined by placing markers to define S-phase and assuming that both Go/G1 and G2/M peaks are a Gaussian distribution (Section 2). This made assessment of SPF possible in only cases where there were no complications from overlapping peaks and where the tumours were diploid. No correlation was, therefore, attempted between tumour SPF and histological prognostic factors. However, in agreement with other reports, aneuploid tumours were found to be significantly higher in SPF compared to diploid tumours. Recent findings identify both aneuploidy and high SPF to be related to bad prognosis in breast cancer patients (Toikkanen *et al.*, 1989; Eskelinen *et al.*, 1989; Clark *et al.*, 1989).

It is generally assumed that only a subpopulation of tumour cells have the capacity to metastasise and it is possible that the generation of such populations occur as a result of abnormalities in the DNA. However, there is no direct evidence to indicate that clonal development of cells with a higher metastatic potential is related to the DNA profile of a tumour and the biological events leading to the generation of aneuploid tumours is not clear. From the ploidy analysis of primary tumours presented in this section, it is evident that some aneuploid tumours are likely to be more aggressive than others and a tumour can have both a diploid and an aneuploid population. It is, therefore, relevant to determine the ploidy of secondary deposits in lymph nodes. With the evident difficulty in assessing SPF, this study focussed on DNA ploidy determination of primary tumours and comparing them with those of metastatic cells in the lymph nodes of the same patient. Only a few studies have compared primary tumours and metastases by DNA FCM in breast cancer patients. Although these studies report a high degree of concordance between

the ploidy of the two tissues (Feichter *et al.*, 1989; Beerman *et al.*, 1991), there are also reports of discordance in a substantial proportion of cases (Hitchcock *et al.*, 1989). All these studies employed single parameter DNA analysis and Feichter *et al.* (1989), who attempted to remove contaminating lymphocytes from the tumour cell suspension, reported that in some cases the ploidy and SPF of primary tumours can differ from metastatic cells. In this study attempts were made to determine whether the metastatic population in invaded nodes included the cells with grossly abnormal DNA. Using a single-parameter DNA analysis the detection of aneuploid cells in lymph node metastases was a rare event (only 6 cases out of 25). However, in a dual-parameter DNA analysis, which involved the use of a directly labelled antibody to cytokeratin, it was possible to detect subpopulations of aneuploid cells in the lymph node. Results described in this section suggest that these cells are capable of metastasis and indeed that within these cells, certain further sub-populations are capable of preferential metastasis.

The use of a CK antibody to differentiate epithelial cells from other cell types in both tumour and node samples confers a significant advantage. It is possible to analyse the ploidy status of the tumour cells alone and this becomes important when they form a small percentage of the cells under analysis. In the lymph node where there are extensive numbers of lymphocytes, this is of particular value. However, the primary tumour may also contain as much as 50% lymphocytes (Whitford *et al.*, 1990) in addition to stromal and other cell types and therefore this antibody will be of use in the flow cytometric analysis of tumour markers.

Although the refined technique of multi-parameter FCM DNA analysis, first described by Zarbo *et al.* (1989), has previously been used to study primary breast tumours (Visscher *et al.*, 1990b), no study has used it to

analyse primary and secondary deposits in the same breast cancer patient. Results from such a comparative analysis described in this study provides evidence to suggest that although diploid tumours can be found in lymph node metastases where the primary tumour was itself diploid, this is not the only cell population to invade lymph nodes in breast cancer patients. In multiploid tumours, double primaries and in tumours consisting of both diploid and aneuploid populations, only a subpopulation of these cells comprises the secondary deposits in lymph nodes. The DNA analysis performed in this study provided several noteworthy observations. Firstly, in 4 cases diploid peaks, although present in the primary tumour, were absent in nodes. Secondly, in cases of multiploid primary tumours, preferential appearance of a single aneuploid peak of higher DI was observed. Thirdly, there are the examples of the bilateral carcinoma patient and the patient with two tumours on the same breast. They show that even in the same patient two tumours may have different ploidy and since one of the bilateral tumours metastasised and the other did not, this raises the question of the relationship between metastatic potential and aneuploidy. These observations suggest that with respect to DNA content some of the primary tumours contain a heterogeneous population of cells with differing metastatic capability and that the subpopulation detected in the lymph node represent tumour cells of higher metastatic potential. Alternatively, the fact that only a subpopulation of tumour cells proliferate in the lymph nodes of some breast cancer patients suggests the possibility of a local inhibitory effect of the lymph node on tumour cell proliferation. Such an effect has been suggested by Olszewski *et al.* (1982), who observed a significant reduction of S-phase cells in lymph node metastases compared to those in primary tumours of the same patient. In all of these patients, the primary tumours were oestrogen receptor-positive.

Although in lymph node metastases only a subpopulation of tumour cells were detected, the observed peak had a DI identical to that in the primary tumour. The high degree of concordance between primary tumours and their metastases have led previous workers to suggest that ploidy is established early in tumour development and remains stable in the metastatic process. One invaded lymph node, in this study, was found not only to have an aneuploid peak similar to a subpopulation in the primary tumour, but also to have an additional peak not present in the primary tumour. Differences in ploidy and surface antigen expression in metastases have been reported by Hitchcock *et al.* (1989). This led them to suggest that changes in ploidy in a metastasis is not unidirectional and that changes in ploidy and other variables can occur after metastasis. In this study, at least in one nodal metastases such an event was found to occur.

Attempts to culture aneuploid tumour cells resulted in the growth of diploid cell population only. The restricted ability of current tissue culture methodology to propagate primary aneuploid tumour cells has been previously reported (Smith *et al.*, 1985; Wolman *et al.*, 1985). This, however, means that only rarely can a primary tumour or metastasis with abnormal ploidy be subjected to conventional cytogenetic analysis, internal labelling with isotope, or analysis for cell surface or enzyme markers. In effect, aneuploid cells are often assumed to be terminal cells resulting from the general disorganisation of tumour cell cycle control. Data described in this section suggest that despite their abnormal chromosome load, they are more than capable of either themselves metastasising or of constituting the bulk of metastatic deposits. The importance of this observation has recently been made more relevant by the observation that both amplification and overexpression of the *erb* B2 oncogene was observed in tumours with abnormal DNA content (Tavassoli *et al.*, 1989; Bacus *et al.*, 1990; Baak *et al.*, 1991).

5.2. DNA fingerprint analyses of primary breast tumour and lymph node metastases

As discussed in Section 1.4, mutations in several genes are probably involved in the generation of a highly malignant tumour. Most recent studies on breast cancer have focussed on detecting changes involving specific oncogenes. But it appears that there is a progression of events in the tumorigenesis pathway and it is the final accumulation of these events that triggers the metastatic state. Therefore, not only is the knowledge about specific activation of a proto-oncogene important but also the sequences of events preceding or leading to it are equally important. Moreover, there is the likelihood of the existence of more of yet unidentified oncogenes involved in this process.

In order to record these changes, therefore, a panel of DNA probes representing different loci have to be used. The recently established technique of DNA fingerprinting affords an alternative to this. Using minisatellite DNA probes, it is possible to detect simultaneously highly variable regions widely dispersed in the human genome. These repeat sequences provide individual-specific RFLP (Jeffreys *et al.*, 1985) and therefore, any localised changes in tumour DNA which also include the satellite sequences can be detected by comparison with DNA from normal tissues. The advantage of the technique is that it can simultaneously detect changes in a number of chromosomal sites. DNA fingerprinting has been applied in a few preliminary studies on gastrointestinal and ovarian cancers and has been shown to detect loss and appearance of novel bands as well as changes in hybridisation intensity (Fey *et al.*, 1988; Boltz *et al.*, 1990; Saito *et al.*, 1991).

This section describes results from such an approach on primary tumours and lymph node metastases from breast cancer patients. It is relevant to point out that as described in section 5.1, aneuploidy is a frequent event in breast cancer and in some patients the presence of aneuploid tumours is associated with metastasis. It is of much interest to determine whether aneuploidy in these patients is associated with any specific change in the tumour DNA. Changes observed in the DNA fingerprint pattern of primary tumours and lymph node metastases have, therefore, been compared with the ploidy status of the primary tumour.

Four satellite probes were used to obtain the DNA fingerprints. Minisatellites 33.6 and 33.15 detect sequences derived from approximately 30 loci dispersed throughout the human genome (Jeffreys *et al.*, 1985). The macro-satellite 228S is present on the majority of chromosomes and is predominantly centromeric in location (Boltz *et al.*, 1990), while 216S is a satellite III DNA probe which gives an invariant banding pattern (Boltz *et al.*, 1990). DNA satellites 33.6 and 33.15 were obtained as inserts in pUC18U and pUC19U recombinants respectively. These recombinant plasmids were, therefore, used to transform *E. coli* strains and the inserts were then isolated and purified from bulk growth of these cells. DNA probes 216S and 228S, which were originally in an inconvenient M13 phage DNA, were subcloned into a stable pTZ18U vector and then isolated and purified from these recombinants. The cloning and purification of the DNA satellite sequences is described in Section 2.2.4.3 and 2.2.4.4. DNA from primary tumour, lymph node and peripheral blood samples from 13 breast cancer patients was extracted as described in Section 2.2.4.1. Digestion with restriction enzymes, gel electrophoresis, southern blotting and hybridisation with radioactive probes were carried out as described in Section 2.2.4.6.

The selection of patients was performed without prior knowledge of the histopathology and although DNA FCM analysis was previously undertaken on these cases, each sample was coded such that the fingerprint analysis could be performed as a blind test. To eliminate bias, interpretation of autoradiographs was also carried out independently by a second observer. Both patient histopathology and DNA ploidy data was consulted after the complete analysis had been recorded. Changes in DNA fingerprint pattern described below are based on comparison with DNA from PBL, which can be considered as constitutional DNA, from the same patient.

5.2.1. DNA fingerprint analyses using minisatellite probe 33.6

Using minisatellite probe 33.6, in most cases only 2 distinct bands were observed. Changes were detected between primary tumour and PBL for some but not all patients. These included both loss of a band and the appearance of a band which was not detected in PBL. Changes in intensity of hybridisation was difficult to assess as in some cases DNA loading was not uniform in all lanes. DNA loading was assessed by comparing autoradiographs with photographs of ethidium bromide stained gels. In 6 cases, there were either no differences between primary tumour or lymph node when compared to PBL or in some cases the autoradiograph was uninterpretable. In the remaining 7 patients, both loss of bands and appearance of new bands were observed. The data is presented in Table 5.2.1.

The fingerprint patterns were different for each patient. A few common changes, for instance, the loss of bands 1.4 kb and 1.35 kb were observed in primary tumours. The loss of the 1.4kb band was also seen in two cases of lymph node metastasis (patient B5 and L11). Appearance of band 1.3 kb in

Table 5.2.1. DNA fingerprint analyses of primary tumours and lymph node metastases from breast cancer patients using minisatellite probe 33.6.

Patient	DNA source	Changes in DNA fingerprint		Lymph node status, Ploidy & Tumour grade
		Loss of bands	New bands	
F3	PT	1.4kb	1.3kb	without metastasis,
	NLN	identical to PBL		diploid, grade I
B5	PT	1.4kb, 1.35kb	1.5kb, 1.45kb	with metastasis,
	LNM	1.4kb, 1.35kb	1.5kb, 1.45kb	aneuploid, grade II
H6	PT	1.45kb, 1.35kb	1.4kb, 1.3kb	with metastasis,
	LNM	1.45kb	1.3kb,1.2kb	aneuploid, grade III
M9	PT	-	1.2kb	with metastasis, aneuploid, grade III
D10	PT	1.25kb	1.3kb	with metastasis,
	LNM	1.25kb	1.3kb	diploid, grade II
L11	PT	1.4kb	1.3kb, 1.2kb	with metastasis
	LNM	1.4kb	1.3kb, 1.2kb	aneuploid, grade II
N12	PT	-	1.3kb	with metastasis,
	LNM	-	1.3kb	diploid, grade II

PT=primary tumour, LNM=lymph node metastasis, NLN= normal lymph node. Differences observed are in comparison to PBL DNA from the same patient.
Band sizes were measured relative to marker DNAs.

Figure 5.2.1. Autoradiograph of DNA samples from patients B5 and H6 using minisatellite probe 33.6.

Peripheral blood (P), primary tumour (T) and lymph node metastasis (L) were digested with Hinf I and the southern blot filters were hybridised to radiolabelled probe 33.6. Both patient B5 and H6 had aneuploid tumours. The changes observed are marked with arrows. In patient B5, the 1.5kb and 1.45kb bands are present in both T and L, while the 1.4kb and 1.35 kb bands observed in P, are absent. In patient H6, lymph node metastasis is different from primary tumour. Bands of different sizes appear in T (1.4kb, 1.3kb) and L (1.3kb, 1.2kb).

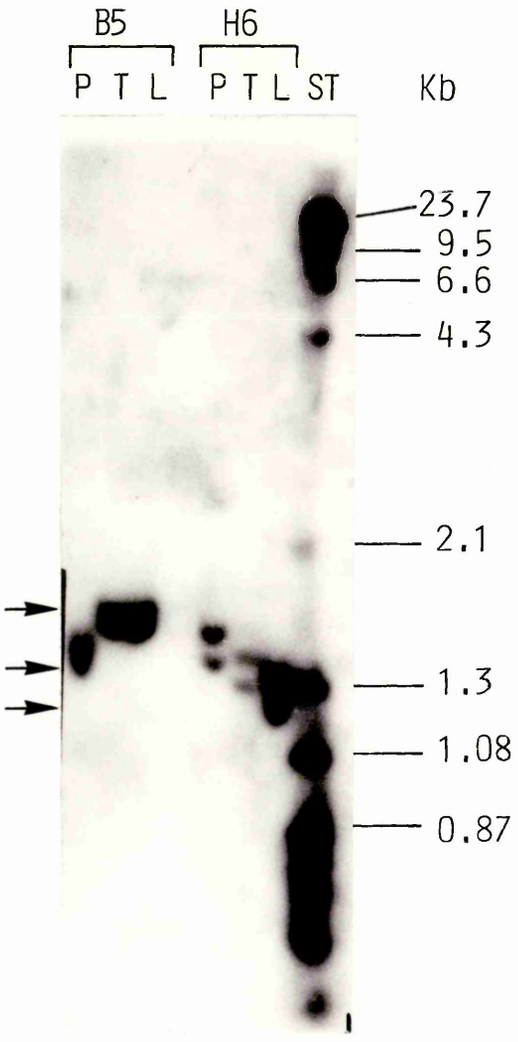
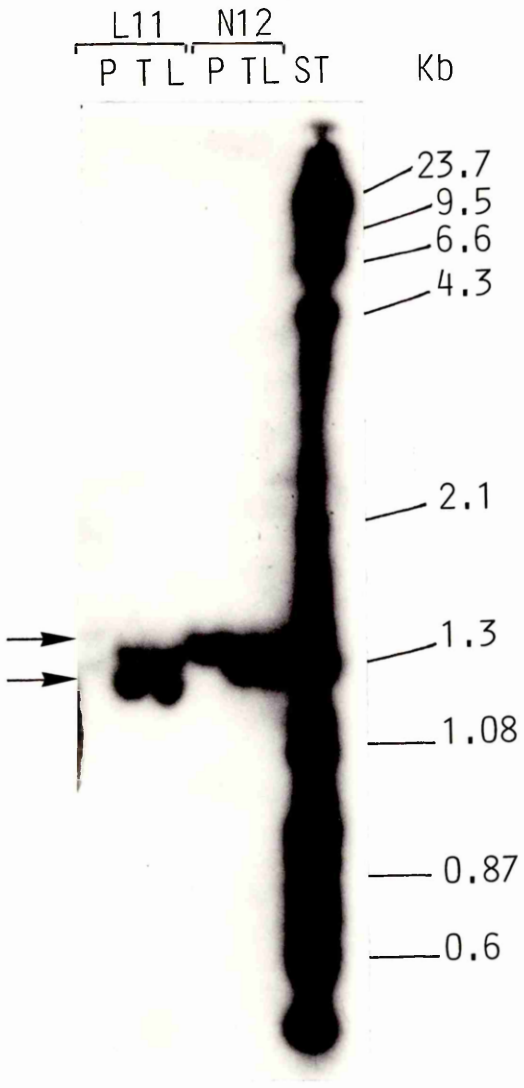

B5
H6
P T L
P T L ST
Kb
23.7
9.5
6.6
4.3
2.1
1.3
1.08
0.87

Figure 5.2.2. Autoradiograph of DNA samples from patients L11 and N12 using minisatellite probe 33.6.

Peripheral blood (P), primary tumour (T) and lymph node metastasis (L) were digested with Hinf I and the southern blot filters were hybridised to radiolabelled probe 33.6. Patient L11 had aneuploid tumour, while the tumour from patient N12 was diploid. The changes observed are marked with arrows. In both L11 and N12, the changes observed in the primary tumours are also seen in the metastases. In patient L11, bands of 1.3kb and 1.2kb appear, while 1.4kb band in P, is absent from both T and L. In patient N12, the 1.3 kb (not seen in P) bands appear in both T and L.


L11
N12
P T L P TL ST
Kb
23.7
9.5
6.6
4.3
2.1
1.3
1.08
0.87
0.6

primary tumours was also more commonly observed and in these cases a band of the same size was also detected in lymph node metastases from the same patient. In general, therefore, in cases where changes in the primary tumour were detected, similar changes were also seen in lymph node metastases (e.g., patient B5, L11 & N12, Fig. 5.2.1 & Fig. 5.2.2). In one case, however, the fingerprint from the lymph node metastasis was found to be different from the primary tumour (patient H6, Fig. 5.2.1).

The changes observed did not correlate with the ploidy status of the primary tumour as changes in the fingerprint from both diploid and aneuploid tumours were observed. Although the changes in tumours were observed in majority for stage II patients, in one stage I patient changes in tumour fingerprint was also seen. There appears to be no relationship with tumour grade either, but this is only based on a small number of patients.

5.2.2. DNA fingerprint analyses using minisatellite probe 33.15

Using probe 33.15, autoradiographs from 6 patients could be interpreted. In the remaining cases it was difficult to interpret the fingerprint pattern as the bands were hazy. In 4 of the 6 cases, changes in the fingerprint pattern in both primary tumours and lymph node metastases were observed. The data is presented in Table 5.2.2. Numerous bands ranging from 15 to 20 in number were clearly resolved in each DNA samples. In general, both loss of bands and appearance of new bands were observed. Here again, changes in hybridisation intensity could not be determined. With respect to band sizes, the changes observed with probe 33.15 were different from those seen with probe 33.6.

Table 5.2.2. DNA fingerprint analyses of primary tumours and lymph node metastases from breast cancer patients using minisatellite probe 33.15.

Patient	DNA source	Changes in DNA fingerprint		Lymph node status, Ploidy & Tumour grade
		Loss of bands	New bands	
F3	PT	-	1.9kb	without metastasis, diploid, grade I
B5	PT, LNM	identical pattern in both tissues*		with metastasis aneuploid, grade II
H6	LNM	4.3kb, 2.25kb	3.7kb, 1.7kb	with metastasis, aneuploid, grade III
D10	PT	-	8kb	with metastasis, diploid, grade II
	LNM	-	8kb	
N12	PT	-	-	with metastasis, diploid, grade II
	LNM*	5.2kb	5kb	

PT=primary tumour, LNM=lymph node metastasis. Differences observed are in comparison to PBL DNA. Band sizes were measured relative to marker DNAs.
*In these cases, the PBL DNA was uninterpretable, and only PT and LNM could be compared.

Unlike probe 33.6, in each of the primary tumours where changes were observed the band sizes differed in each patients. Thus, no common changes could be identified. The highest number of changes were observed in thelymph node metastasis (LNM) of patient H6. Compared to PBL, DNA from this node showed loss of two bands and the appearance of two new bands (Table 5.2.2; Fig 5.2.3). Although the fingerprint of primary tumour (PT) DNA could not be interpreted in this patient, it is of interest to note that with probe 33.6, the DNA from LNM was different from the PT.

As with probe 33.6, changes observed with probe 33.15 did not correlate with the ploidy status of the primary tumour. Changes observed in the primary tumours were not restricted to stage II patients as in one stage I patient changes in primary the tumour was observed. Although the final analysis was possible in only a small number of patients, the observed changes were not restricted to tumours of any particular histological grade.

5.2.3. DNA fingerprint analysis using macrosatellite probe 228S

With probe 228S, the observed bands were often diffuse and the fingerprint patterns were identical in all samples (Fig. 5.2.4). Thus no changes in either primary tumour or lymph node metastasis could be detected with this probe.

5.2.4. DNA fingerprint analysis using macrosatellite probe 216S

With probe 216S, no differences were observed in the majority of cases and all such samples gave an identical banding pattern (Fig. 5.2.5). However, in two stage II patients (H4 and H6), changes in both primary tumour and LNM were observed (Table 5.2.3). In patient H4 both loss of bands and appearance of new bands in PT and LNM were of similar sizes (Fig. 5.2.5). Likewise in patient H6, the loss of a 7.1 kb band was observed in both tissues. In this case, however, changes in hybridisation intensity could be interpreted. A 1.3kb band in the PT of patient H6 was more intense than both LNM and PBL.

Figure 5.2.3. Autoradiograph of DNA samples from patients B5 and H6 using minisatellite probe 33.15.

Peripheral blood (P), primary tumour (T) and lymph node metastasis (L) were digested with Hinf I and the southern blot filters were hybridised to radiolabelled probe 33.6. Both patient B5 and H6 had aneuploid tumours. In patient B5, the primary tumour and lymph node metastases show identical fingerprint patterns. The PBL from this patient could not be interpreted and as such it could not be determined whether there were any specific changes in these tumour cells. In patient H6, the T lane is uninterpretable but the lymph node metastasis (L) show numerous changes when compared to PBL (P). Some of the observed changes in L are marked with arrows (loss of 2.25kb band; appearance of 3.7 kb and 1.7 kb bands).

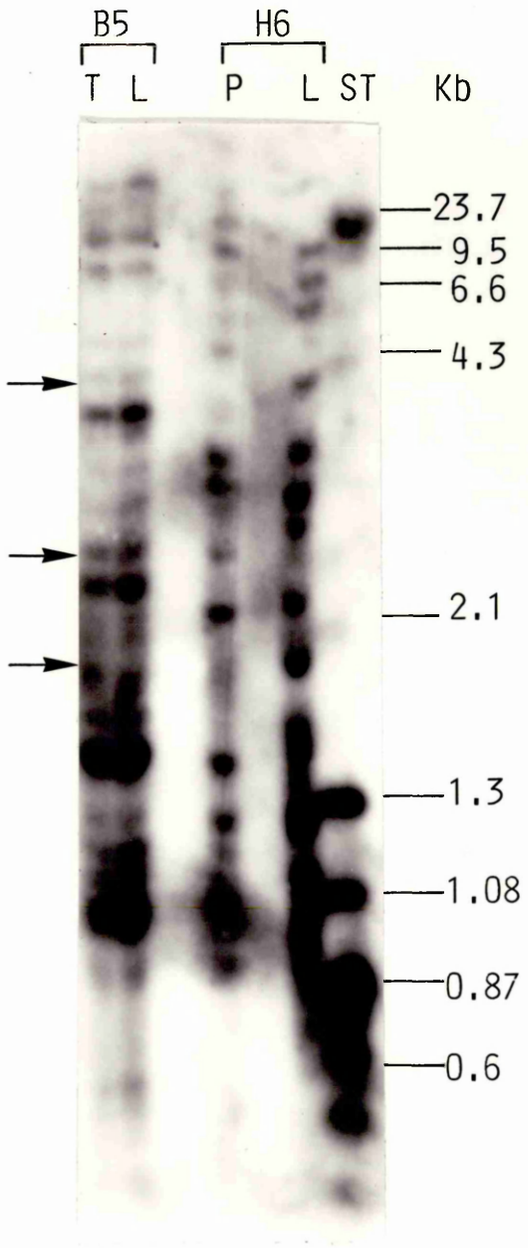

B5
H6
T L
P L ST
Kb
23.7
9.5
6.6
4.3
2.1
1.3
1.08
0.87
0.6

Figure 5.2.4. Autoradiograph of DNA samples from 4 breast cancer patients using satellite probe 228S.

Peripheral blood (P), primary tumour (T) and lymph node (L) samples were digested with Taq I and the southern blot filters were hybridised to radiolabelled probe 228S. With probe 228S no change in the fingerprint was observed.

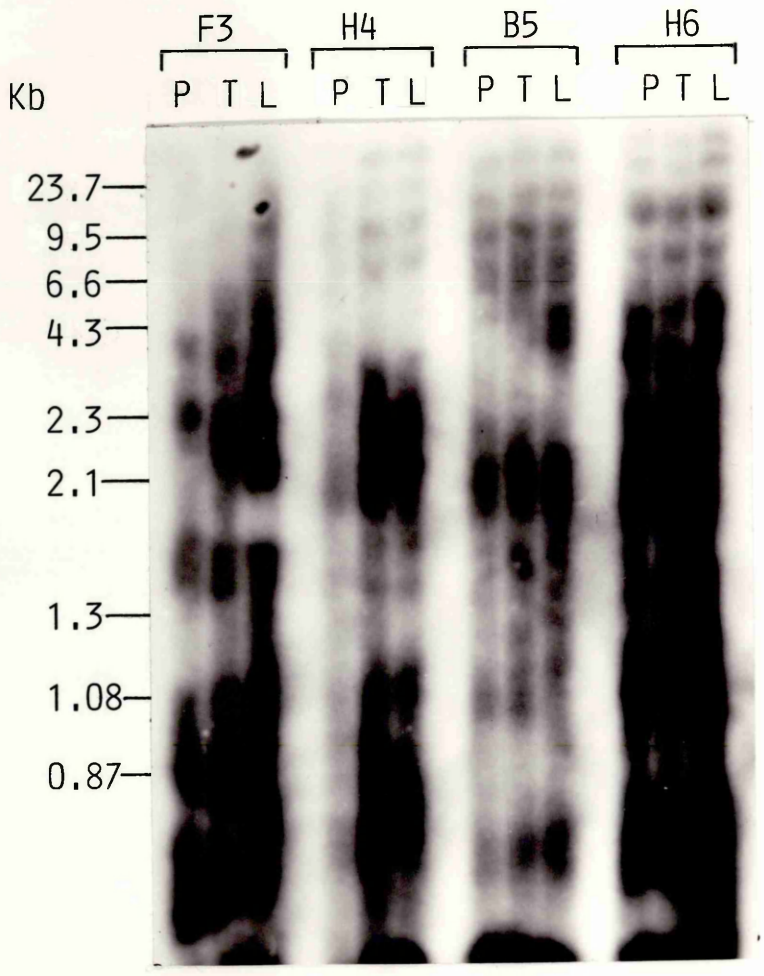

F3
H4
B5
H6
P T L P T L P T L P T L
Kb
23.7
9.5
6.6
4.3
2.3
2.1
1.3
1.08
0.87

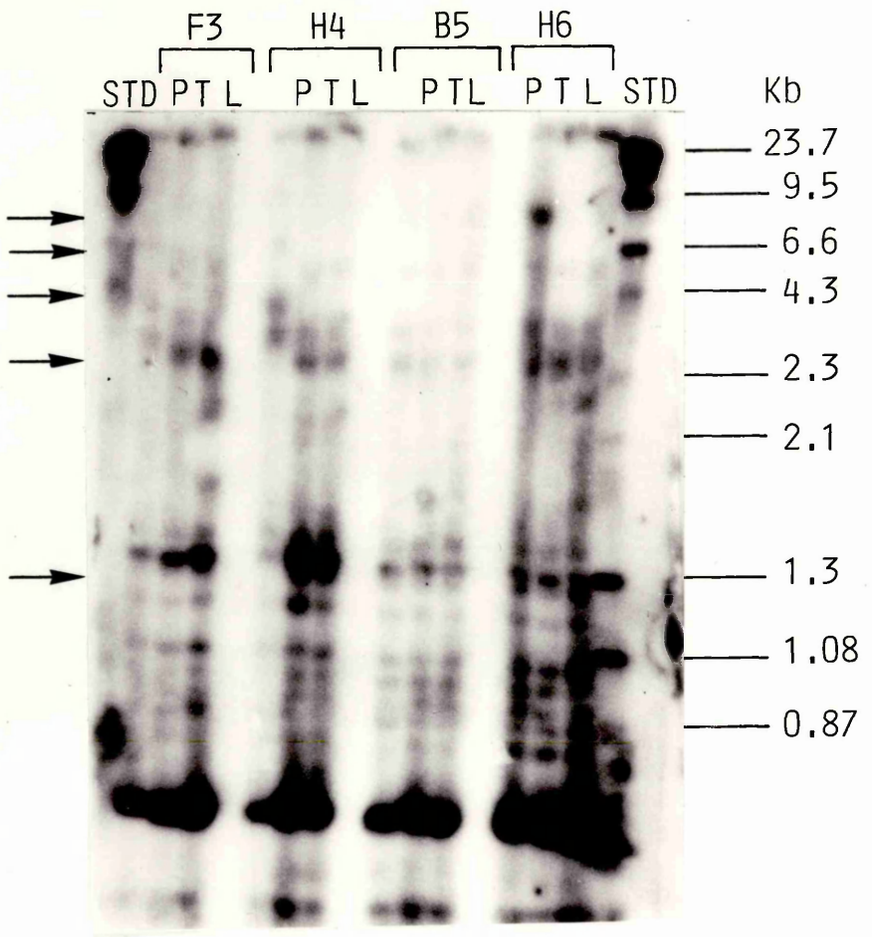
Table 5.2.3. Changes detected in DNA fingerprint of primary tumours and lymph node metastasis from 2 breast cancer patients using minisatellite probe 216S.

Patient	DNA source	Changes in DNA fingerprint			Lymph node status & Ploidy of PT
		Loss of bands	New bands	Change in intensity*	
H4	PT, LNM	5.8kb, 3.7kb	4.3kb, 2.7kb	- -	with metastasis, aneuploid, grade II
H6	PT	7.1kb	-	1.3kb (PT>LN,PBL)	with metastasis, aneuploid, grade III
	LNM	7.1kb	-	-	

PT=primary tumour, LNM=lymph node metastasis, 0=no differences observed. Only cases where differences with PBL DNA were observed are shown here. For all other cases, both PT and LNM DNA was indistinguishable from PBL DNA (Fig. 5.2.5).
*changes in hybrisation intensity is recorded as greater (>) or less (<).

Figure 5.2.5. Autoradiograph of DNA samples from 4 breast cancer patients using satellite probe 216S.

Peripheral blood (P), primary tumour (T) and lymph node (L) samples were digested with Taq I and the southern blot filters were hybridised to radiolabelled probe 216S. In the majority of cases and as shown here for patient F3 and B5 no change in the fingerprint between the DNA from the three sources was observed. Changes were observed in patient H4 and H6, both having aneuploid tumours. In the case of patient H6, the loss of a 7.1kb band was observed in both tumour and lymph node metastasis, while a 1.3kb band was more intense in the T. Similar changes in both tumour and lymph node metastasis were observed in the case of patient H4 (loss of 5.8kb & 3.7kb; new bands of sizes 4.3kb & 2.7kb).


F3
H4
B5
H6
STD P T L
P T L
P T L
P T L STD
Kb
23.7
9.5
6.6
4.3
2.3
2.1
1.3
1.08
0.87

5.2.5. Discussion

DNA fingerprint analysis provides a useful new approach in which changes in tumour DNA can be studied. The advantage of the technique is that changes at several chromosomal sites can be simultaneously detected. Moreover, it is also possible to repeatedly use several mini-satellite probes on the same filter and this increases the chance of detecting a change. In this study, DNA from both primary tumours and lymph node metastases have been compared with DNA from peripheral blood lymphocytes from the same patient. Samples from both stage I and stage II breast cancer patients have been analysed using 4 different probes. The changes observed were found not to be associated with the ploidy of the primary tumour. In both aneuploid and diploid tumours changes in DNA pattern were observed. Although only a small number of tumours were analysed, it appears that changes in fingerprint were not restricted to tumours of any histological grade.

Four different probes have been used for hybridisation and the DNA fingerprint patterns were found to depend on the satellite probe used. Most of the changes observed were with probes 33.6 and 33.15. As these two probes detect DNA fragments derived from different sets of loci (Jeffreys *et al.*, 1986), probing of the same filter with 33.15 followed with 33.6 provided additional information. Hybridisation with probe 228S produced hazy bands and in all cases no differences could be detected. Boltz *et al.* (1990), however, have reported detectable changes in ovarian tumours with 228S, although the number of changes detected were less than those obtained with probe 33.15. As also reported by Boltz *et al.* (1990), the hybridisation pattern with 216S was invariant in the majority of cases. However, in two instances in this study, changes in both the primary tumour and lymph node metastasis were observed. Changes in tumour fingerprint with probe 216S in a small number

of cases have also been reported by Boltz *et al.* (1990). As has been shown for ovarian and gastrointestinal cancers, it thus, appears that the DNA fingerprint probes 33.15 and 33.6 can be used to detect changes in breast tumours. Only 4 primary breast tumours were previously analysed by Thein *et al.* (1987) and only shifts in hybridisation intensity were observed in two of these cases. Although in one case changes in the hybridisation intensity of a band was detected with probe 216S, this was difficult to assess in this study as DNA loading was often not uniform. With probe 33.15, some 15 to 20 bands were resolvable, which is similar to the number of bands reported by other workers (Fey *et al.*, 1988), while only 2 distinct bands were observed with probe 33.6. Although the two probes were hybridised and washed under identical conditions (Section 2.2.4.9), this probably reflect differences in washing conditions used in this study as compared to other studies.

Although the changes detected in the fingerprint pattern appear not to be related with aneuploidy, differences were observed in both the primary tumours and lymph node metastases. In both primary tumours and lymph node metastases, loss of bands and the appearance of new bands were observed. The fingerprint patterns were highly patient-specific and with probe 33.15 the observed changes in a patient did not match with any of the other patients. However, with probe 33.6, band loss of 1.4kb and a new band of 1.3kb were found to be common changes. In general, where changes in primary tumours were detected, there was also changes in LNM. These changes when detected in the primary tumour were also present identically in lymph node metastases. However, for one patient (H6), tumour DNA from the two tissues were found to differ when probed with 33.6. This probably suggest that in this case further alterations may have occurred following metastasis or alternatively, it might reflect changes in the growth characteristics of subpopulations of tumour cells (Fey *et al.*, 1988), for instance dormant cells

which grow rapidly in metastases (Alexander, 1985). Differences in DNA fingerprints of primary tumours and metastasis have also been reported for gastric cancers (Fey *et al.*, 1988). It is of interest to point out here that FCM DNA analysis of lymph node metastasis has also revealed in one case the probable existence of an aneuploid population of cells which was not detected in the primary tumour (Section 5.1).

It is necessary to be cautious in assessing the implication of the changes observed with DNA fingerprinting. Firstly, although the minisatellites used as probes in this study detect sequences derived from a number of different loci, this represent only a fraction of the genome. Secondly, changes in DNA methylation have been shown to occur in colorectal cancer (Goelz *et al.*, 1985) and these changes may alter Hinf I cleavage sites. However, using Alu I and Hae III, Thein *et al.* (1987) have reported that the changes observed with Hinf I were not due to tumour-specific DNA methylation changes. Finally, the number of samples analysed was low and then this was further restricted to cases where changes could be clearly interpreted. Thus, these changes could not be tested statistically. However, in cases where changes could be detected, alterations in both primary tumour and lymph node metastases were observed. It appears this approach is likely to be fruitful when applied on a larger number of samples.

It is generally believed that loss of a band or decrease in intensity is suggestive of loss of heterozygosity. However, in DNA fingerprinting, mutation in a single base of an enzyme restriction site can generate differences in fingerprint pattern. Loss of bands were frequently observed and while no consistency was observed with probe 33.15, it appears that with probe 33.6 the loss of 1.4kb band in primary tumours, which was also absent in LNM, could represent a more common occurrence in patients with metastasis.

Increased intensity of bands is probably related to localised amplification of DNA and this may be relevant as amplification of oncogenes in breast cancer have been reported (Section 1.4). This could not be assessed in this study as in most of the cases, changes in intensity could not be correctly recorded.

The objective of this study was to detect abnormalities in tumour DNA and by comparing primary tumours and lymph node metastases to detect changes which might be related to metastatic potential. As the changes observed did not appear to relate with the tumour DNA content or with tumour grade, it is not clear whether these changes are related to the pathogenesis of the disease. The data presented here, however, show that DNA fingerprinting can be used to detect simultaneously several changes in breast tumours. A clear finding here is that there may be numerous alterations in the DNA of breast tumours. Any one of such alterations may not be important and the general belief is that the cumulative effect of these changes in specific genes may explain the aggressive behaviour of a metastatic tumour. The DNA fingerprinting approach used here complements the conventional method of using a single oncogene probe. The changes observed not only reflect the known DNA sequences, but also provide the possibilty of identifying new genes involved in breast cancer. Using this technique, it would be possible to obtain DNA hybridisation probes by isolating and cloning selected fragments obtained from breast cancer patients showing some specific alterations (for instance the loss of 1.4kb band detected in this study) in their DNA fingerprint pattern. This approach has proved fruitful in detecting chromosome 7 loss in myelodysplasia (Thein *et al.*, 1988).

CHAPTER 6

DNA content, cell surface expression, and activation states of lymph node lymphocytes

6. DNA content, cell surface expression, and activation states of lymph node lymphocytes.

It is evident from the results presented in previous sections, that both DNA aneuploidy and HPA binding are related to metastasis in breast cancer patients. HPA binding significantly correlated with lymph node metastasis (Section 4) and more than 60% of primary tumours were found to be aneuploid (Section 5.1). However, while primary tumours may have both diploid and aneuploid populations, dual-parameter analysis of lymph node metastases revealed that only a subpopulation of the primary tumour appears in these nodes. There is evidence to suggest that, with respect to DNA ploidy, breast tumours not only differ among patients, but also within the same tumour, so that only a subpopulation may possess a higher malignant capability. Since tumour cells in lymph node metastases were also found to show HPA binding, it is of much interest to determine whether there also exists a relationship between HPA binding and aneuploid DNA content. In this context, the relevant question to ask is whether tumours that are aneuploid also express HPA binding markers on their surface.

Results described in Section 3 suggest that at least in some patients, the presence of tumour cells can cause significant alterations in the lymphocyte populations from axillary lymph node metastases. Alterations both in T cell phenotype and the activation states of B cells were observed. It is therefore, also relevant to determine whether such alterations are related in any way to the DNA content of the primary tumour. Recent studies reveal that tumours that overexpress *erb* B-2 are likely to be aneuploid (Bacus *et al.*, 1990; Tavassoli *et al.*, 1989) and that such tumours are more aggressive (Borg *et al.*, 1991). Expression of oncoproteins is likely to induce a host-mediated response,

provided that the protein is recognised as foreign and in this respect, a mutated protein would be a likely candidate.

In this section, therefore, attempts have been made to relate aneuploidy with both HPA binding and phenotype and activation states of lymphocytes from lymph node metastases.

6.1. DNA aneuploidy and HPA binding

Ideally, a dual-colour analysis would have revealed whether in a breast tumour, the aneuploid cells also express HPA binding markers. However, such an attempt was unsuccessful, as HPA binding was found not to be stable after ethanol treatment, which is a prerequisite for PI staining for simultaneous DNA ploidy analysis. A dual-parameter analysis on the same tumour cells was therefore, not possible. Alternatively, a correlation of HPA staining with ploidy has been attempted on the same breast tumour samples.

In 28 tumour samples, data from both HPA binding and DNA ploidy was available. As shown in Table 6.1, among the HPA positive tumours, the majority (81%) were also aneuploid (13/16). However, the converse was not true. There were equal numbers of diploid and aneuploid tumours among HPA -ve tumours. There was no statistically significant association between HPA positivity and DNA ploidy ($p>0.1$). It, however, appears that HPA positivity may be of value in patients with aneuploid tumours. This is further examined in Table 6.2, in which stage (lymph node status) has been included to relate aneuploidy with HPA binding. The data shows that the majority of aneuploid tumours positive for HPA binding (12/14) have metastasised to the

lymph node. In contrast, the majority of aneuploid tumours negative for HPA binding (4/5) are without lymph node metastasis. This relationship, could not be examined in a statistical test. The number of stage I patients was only 5 and this reduced the frequency when patients were further put into subgroups (<5 cases). However, the data does show that aneuploid tumours with metastatic capability are also positive for HPA binding.

Table 6.1. Relating DNA ploidy and HPA binding in breast cancer patients.

	HPA +ve	HPA -ve	Statistical significance (Chi-square test)
Aneuploid	13	6	$\chi^2 = 1.8$, D.F. 1
Diploid	3	6	N.S. ($p > 0.1$)

Table 6.2. DNA aneuploidy and HPA binding and their relation with lymph node metastasis (stage).

	With metastasis (Stage II)	Without metastasis (Stage I)
Aneuploid, HPA +ve	12	1
Aneuploid, HPA -ve	2	4

6.2. Phenotype and activation states of lymph node lymphocytes in patients with aneuploid primary tumours.

The phenotype and activation state of lymph node lymphocytes and DNA ploidy of autologous primary tumours from 15 breast cancer patients were analysed. All these patients had metastasis in their axillary lymph nodes (stage II). 9 of these primary tumours were aneuploid while the remaining 6 were diploid. The data is presented in Table 6.3. There were no differences in terms of the phenotype of T cells. The CD4/CD8 ratio was more variable in patients with diploid tumours, ranging from 1.4 to 9.0, but the mean ratio was similar to those observed in patients with aneuploid tumours.

Significant diferences were observed in terms of the activation states of both T and B lymphocytes. However, Tac-expressing T cells were similar in both groups and this was true for both the CD8+ and CD4+ T cells. With respect to expression of the activation marker HLA DR on T cells, the differences observed beteween aneuploid and diploid tumours was statistically significant ($p<0.05$). This marker was expressed on a greater proportion of T cells in patients with aneuploid tumours (76.7%) than those with diploid tumours (42.7%). This difference was also maintained when expression of HLA DR was compared in T cell subsets. The proportions of HLA DR-expressing CD8+ and CD4+ T cells were lower in patients with diploid tumours (28.3 and 17.9% respectively). The mean proportions of these activated T cell subsets in patients with aneuploid tumours were 48.0 and 32.4% for CD8+ and CD4+ cells respectively, which are similar to the mean values observed for stage II lymph nodes (Table 3.2, Section 3). Similarly,

Table 6.3. Distribution of phenotypic and activation markers in lymphocytes from lymph nodes of breast cancer patients with aneuploid and diploid primary tumours.

Phenotypic/ Activation markers	Aneuploid tumours (n=9)	Diploid tumours (n=6)	Statistical significance
CD4/CD8 ratio	3.2+/-0.4 (3.3+/-1.2)	3.8+/-1.3 (2.5+/-3.1)*	N.S.
HLA DR on T cells	76.7+/-7.5	42.7+/-7.0	0.01<p<0.05
HLA DR on CD8+ T cells	48.0+/-5.0	28.3+/- 4.5	0.01<p<0.05
HLA DR on CD4+ T cells	32.4+/-4.5	17.9+/-1.8	0.01<p<0.05
Tac on T cells	39.2+/-5.6	33.9+/-2.4	N.S.
Tac on CD8+ T cells	16.8+/-3.8	19.4+/-3.4	N.S.
Tac on CD4+ T cells	22.2+/-3.1	21.2+/-3.2	N.S.
IgG on B cells	34.3+/-4.0	22.6+/-3.3	p=0.05

Values represent mean percentage +/- standard error 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: Mann Whitney U test was used in comparing patients with diploid and aneuploid tumours. Statistical significance is taken at a p value equal to or < 0.05. Only significant p values are given. N.S. is not significant.

the mean proportion of IgG-expressing B cells (34.3%) in patients with aneuploid tumours was also higher when compared to those in patients with diploid tumours (22.6%). This difference just reached statistical significance (p=0.05).

These results, therefore, suggest the possibility of the presence of some antigenic determinants in aneuploid tumours that are absent in diploid tumours. The activation states of both T and B cells suggest that the probable antigen(s) is likely to induce recognition by T cells and also to generate a B cell response.

6.3. Discussion

In this section, aneuploidy of primary tumours has been related with both HPA staining and the activation status of lymph node lymphocytes. While the association with HPA staining was not statistically significant, aneuploid tumours were found to be positive for HPA staining in patients with lymph node metastasis. There was a significant association between DNA ploidy of primary tumours and the activation state of lymphocytes from lymph node metastasis.

Both DNA ploidy and HPA staining were found independently (described in Sections 5.1 and 4 respectively) to be related to lymph node metastasis. However, a good proportion of aneuploid primary tumours was also found to be without nodal metastasis. This suggest that aneuploidy does not represent an aggressive feature in all primary breast tumours. Results in this section show that lymph node metastasis was present in the majority of aneuploid tumours showing HPA positivity. It can, therefore, be concluded that HPA positivity in aneuploid tumours define that group of patients in whom aneuploidy represents an aggressive feature. It should, however, be pointed out that after selecting for aneuploid tumours, there was only a small number (6) of HPA negative tumours available. Both HPA staining and DNA ploidy have not been included in the same study previously, while Hitchcock *et al.* (1989), who compared surface antigen (NCRC-11, CEA) expression and DNA ploidy between primary and metastatic tissues, only recorded changes between the two tissues.

Alterations in the activation status of lymphocytes in lymph node metastasis was found to be significantly associated with the ploidy of the primary tumour. While no differences were observed in the phenotype of

these lymphocytes when patients with aneuploid and diploid tumours were compared, aneuploidy was associated with both HLA DR and IgG-expression. In patients with aneuploid tumours, the percentage of cells expressing HLA DR on both CD8+ and CD4+ T cells were higher than those in patients with diploid tumours. Likewise IgG-expressing B cells were also higher in patients with aneuploid tumours. The mean values of these activated T and B cells were the same as those observed for stage II patients, suggesting that were fewer activated lymphocytes in the lymph nodes of patients with diploid tumours. Since only stage II patients were studied, it is not known whether lymph nodes in stage I patient would also show similar differences with respect to the ploidy of the primary tumour. It should be noted that in this study, no differences were observed in the activation states of lymphocytes between stage I and stage II patients, although in the study of Whitford *et al.* (1992b), which analysed a larger number of samples, and Morton *et al.* (1986) HLA DR expression was found to be higher in stage II patients.

Although a small number (15 samples) of lymph nodes were analysed, the data presented here suggest that aneuploid tumours probably express some antigenic determinant which is not present in diploid tumours. The activation states of the lymphocytes in these lymph nodes suggest that the antigen(s) probably induces the T cell populations and may also generate a B cell response. No differences were observed with Tac-expression, and it is difficult to interpret this as the IL-2 receptor can undergo internalisation and as such the loss of the receptor does not necessarily means an inactive state. Data for HLA DR expression, however, suggest that both the T cell subsets are in an activated state in patients with aneuploid tumours. In addition to the previous reports of increased HLA DR expression in stage II patients (Whitford *et al.*, 1992b; Morton *et al.*, 1986), the results described here shows that the expression of this T cell activation marker is higher in stage II patients

who have aneuploid tumours. The implication of this finding is however, not clear as no specific oncogene or tumour antigen has been associated with aneuploid DNA content. In some of the studies, where oncogene and DNA ploidy have been analysed, gene amplification and protein overexpression of c-*erb* B-2 (Baak *et al.*, 1991; Bacus *et al.*, 1990; Borg *et al.*, 1991, Tavassoli *et al.*, 1989) have been found to significantly correlate with DNA ploidy. Although in the study of Tavassoli *et al.*, a large number of aneuploid tumours also showed *myc* amplification, the correlation was not significant. It needs to be established in a dual-colour analysis, whether aneuploid cells, indeed are also overexpressing these oncogenes.

A relationship that is also relevant but not studied here is that between the activation states of lymph node lymphocytes and HPA binding. Although the affinity of the lectin is for N-acetylgalactosamine residue, the precise marker that HPA binds to in breast tumours is not known and HPA binding remains an 'undefined biological marker" (Leathem & Brooks, 1987). Springer (1989) however, has proposed that the predominant structure recognised by HPA may be the Tn antigen. It is interesting to note that Tn, which is a close precursor of the M and N blood group antigens is believed to be a cell-adhesion molecule on tumour cell membranes (Springer, 1989). It is therefore, of interest to determine whether the HPA marker is antigenic in breast cancer patients.

CHAPTER 7

General Discussion

7. General Discussion

This study has focussed on various parameters which relate to the metastatic potential of breast tumours. In breast cancer, axillary lymph node metastasis is a frequent occurrence and is currently the most important prognostic indicator (Bloom & Richardson, 1970). The surgical practice at the Western Infirmary provided both primary tumour and lymph node specimens from each patient. Each aspect of this study has, therefore, involved measurement of different molecular parameters in relation to metastasis to the lymph node. Although a number of histopathological factors are in use to assess prognosis in breast cancer patients (Section 1.1.5.2), there is clearly a need to identify other parameters which would provide additional prognostic information. In this study, parameters both at the cell surface and at the DNA level have been analysed. Tumour cell characteristics studied here included carbohydrate expression at the cell surface level (Chapter 4), and at the DNA level, the DNA content by flow cytometry and alterations at multiple sites of the tumour DNA as determined by DNA fingerprinting (Chapter 5). In addition, the effect of metastasis on the lymphocyte populations in the lymph node has been studied (Chapter 3). The axillary lymph nodes are not only a site for secondary deposits of tumour cells but being a lymphoid tissue may also be involved in modulating the immune response (Section 1.2). This is particularly important in breast cancer, as in some patients there is clinical evidence to suggest that the lymph nodes may be involved in generating a host-mediated response against the disease (Crile, 1968; Crile, 1969; Fisher *et al.*, 1980). Finally, an attempt has been made to interrelate these parameters in a group of patients (Chapter 6).

7.1. Flow cytometry

Flow cytometry (FCM) has been used both to analyse the lymphocyte populations and in studying tumours for their surface carbohydrate expression and DNA ploidy. While DNA FCM analysis provides new information, the use of this technique in the study of surface expression has certain advantages over the more commonly used technique of immunocytochemistry. Firstly, the instrument is computer-run and this makes it relatively objective. The task of assessing surface antigen expression or DNA analysis can be carried out in a very short time while the number of cells analysed can be as high as 10,000 per sample. In this respect, immunocytochemistry is relatively subjective. Moreover, only a few hundred cells can possibly be analysed and as experienced with lectin binding to breast tumour cells, often the general impression of the binding can only be assessed after a number of a fields have been scanned (Dansey *et al.*, 1988). In effect, it is a laborious and time-consuming process. In this study, using FCM it was possible to quantify the intensity of lectin binding and this allowed the percentage of positive cells to be readily determined. Secondly, dead cells could be excluded from the analysis by simply adding PI and using a live acquisition gate (Section 2.2.1.4). This operation is simple but very important as both lectins and Mabs to surface antigens can bind to irrelevant intracellular molecules. In contrast to most of the previous studies, fresh or carefully frozen single cell suspensions were used in this study and this ensured that the proportion of dead cells was relatively low. Finally, using FCM it was possible to perform a dual-parameter analysis on intact lymphocytes from lymph nodes of breast cancer patients (Chapter 3). Lymphocyte populations were selected on the basis of their FSC (forward scatter) and SSC (side scatter) and cells stained simultaneously with FITC- and PE-labelled Mabs allowed the measurement of

the relative proportions of lymphocyte subsets (Section 2.2.1.4). Dual-colour analysis also allowed the assessment of activation markers on a selected lymphocyte subset (Section 2.2.1.4). The five-parameter analysis operative in the flow cytometer was also found to be useful in analysing lectin binding to tumour cells in lymph node metastasis and in assessing DNA ploidy of metastatic cells in the nodes. Lymph nodes invariably contain a large number of lymphocytes and it was possible to exclude them during assessment of lectin binding by gating on FSC versus SSC (Section 2.2.2.3) while during DNA analysis, lymphocytes could be excluded on the basis of cytokeratin staining (Section 2.2.3.7). There are, however, a few limitations with flow cytometry. As only free cells are stained for analysis, the tumour tissue has to be completely disaggregated. This disrupts the normal architecture of the tissue and unlike histochemistry, it is not possible to observe the relationship between staining cells and the rest of the tissue.

7.2. Factors associated with metastasis in breast cancer patients

7.2.1. A study of primary tumours and lymph node metastases

The various parameters assessed in this study have been found to be associated with metastasis to the lymph nodes in breast cancer. As is apparent throughout this thesis heterogeneity in breast tumours is a common finding and it is likely that such heterogeneity may represent populations of cells with differing metastatic potential, that is, populations of metastatic and non-metastatic cells can pre-exist within the same tumour (Fidler & Kripke, 1977). It is generally believed that the progression of cancer proceeds through multiple alterations in the DNA and some of these may provide growth advantage or higher metastatic potential (Fearon & Vogelstein, 1990) to a subpopulation of

tumour cells. In this respect a relevant question is whether cells with higher malignant potential have a distinct phenotype and whether there are any specific alterations in the tumour DNA which give rise to the malignant phenotype. A number of studies, the majority of which have concentrated on the primary tumour, have revealed that certain tumour characteristics, which involve both cell surface expression (Section 1.3) and tumour DNA (Section 1.4), are associated with metastatic potential. However, in assessing metastatic potential, while it is necessary to study primary tumours, it is also worthwhile to examine tumour cells from secondary deposits. Cells from metastases may comprise of a population of cells which are more aggressive and may only represent a subpopulation of cells in the primary tumour. In breast cancer, the availability of lymph nodes along with the primary tumour makes such a comparison possible. Therefore, by comparing primary tumours and lymph node metastases from the same breast cancer patient, it is possible to determine whether tumour cells that invade the node represent any specific sub-population of the primary cells.

As demonstrated with FCM DNA analysis, assessment of primary tumours is generally representative of cells which make up the bulk of the tumour specimen and may also include contaminating normal cells (Chapter 4). In lymph node metastases, the detection of tumour cells is made difficult by the presence of lymphocytes in large number. These difficulties have been overcome in a dual-parameter flow cytometric analysis. Similarly, it was also possible to confine the measurement of lectin binding to tumour cells alone.

7.2.2. Cell surface carbohydrate expression

At the cell surface, alterations in carbohydrate structures are well documented in breast cancer (Section 1.3). This has been highlighted both with the use of lectins and with Mabs reacting with breast tumour cells. Studies with murine Mabs directed against breast tumours have failed to reveal any well-characterised tumour-specific antigen but in most cases they appear to react with high MW glycoproteins, the most dominant of which are the mucins (Section 1.3.1.1). Although restricted in reactivity to carbohydrate residues, lectins can be used to detect alterations in carbohydrate expression. In a tumour, these lectin-reactive carbohydrate residues may represent exposed sugar residues due to alteration in oligosaccharide processing or overexpression of a surface marker. In this study, HPA binding, which was initially reported to be a marker of metastatic potential (Leathem & Brooks, 1990), was found to be significantly associated with lymph node metastasis (Chapter 4; Alam *et al.*, 1990). Tumour cells that invade lymph nodes also express this marker and this correlated significantly with HPA binding in primary tumours. Thus the HPA marker may represent a metastatic phenotype. However, the particular molecule in breast tumour cells that HPA binds to is not known. A particular limitation in interpreting lectin binding being that since lectins are specific for a sugar residue, this sugar residue may be common to several glycoproteins present within the same cell. It is not, therefore, known whether the binding represents a number of markers which are all expressed together or it represents one particular marker which is being overexpressed. Although it has been proved that the combining groove of HPA reacts specifically with N-acetyl galactosamine, it is believed that the total combining site is considerably larger and complex formation involves interactions between surfaces and not sugar units *per se* and this reaction

remains ill defined (Baker *et al.*, 1983). Thus, minor variations in the composition of oligosaccharide chains, not necessarily involving the lectin-specific sugars, can also affect binding. At this stage, therefore, it may only be concluded that HPA binds to N-acetyl galactosamine-containing glycoproteins or proteoglycans that are expressed on tumour cells which have a higher metastatic potential.

7.2.3. DNA aneuploidy

A more frequently observed breast tumour characteristic is the abnormality in the DNA content. This has been widely studied and a universal finding is that more than 60% of breast tumours are aneuploid (Frierson *et al.*, 1991). In this study it was observed that although aneuploid tumours were more frequent in patients with metastasis, such tumours were also detected in patients without metastasis (Section 5.1). This was also evident in DNA fingerprinting of breast tumours, where changes in tumour DNA was observed in both tumours with and without metastasis (Section 5.2). However, it appears that among aneuploid tumours some are more aggressive than others. While most of the previous studies were on primary tumours, in this study lymph node metastases have also been analysed. Using an anti-cytokeratin antibody and employing a dual-parameter DNA analysis it was possible to exclude contaminating cells and the analysis could be restricted to epithelial cells alone. This is particularly important in lymph node metastases, which contain a large number of lymphocytes and as such contain tumour cells which may not be detected in a ploidy analysis. Using this technique it was possible to ask the question whether aneuploid cells have a higher metastatic potential in case of a primary tumour with both diploid and aneuploid cells. In general, both diploid and aneuploid tumours were detected in lymph node metastases

suggesting that both tumour cell types can be metastatic. Although aneuploid tumour cells were detected in lymph nodes in cases, where the primary tumours were also aneuploid, it was observed that with multiploid tumours, a subpopulation of aneuploid cells appeared in lymph node metastases. This, therefore, implies that at least in some primary tumours, differences in DNA content may reflect differences in metastatic potential. An alternative explanation, however, could be that there might be a local inhibitory effect of the lymph nodes on the proliferation of some tumour cells such that only a subpopulation of them survive. In any case, this suggests that in some breast tumours there are differences in tumour cell populations with respect to DNA content.

A relevant question to ask here is whether there is a role of aneuploidy in respect of gene deletion and expression. The inability of aneuploid tumour cells to grow in culture had led to a belief that aneuploidy is perhaps a harmless feature of a cell's uncontrolled terminal growth with no inherent metastatic property. In this study it was also not possible to grow aneuploid cells and as such these cells could not be studied specifically (Section 5.1.3). But this only reflects the inability of the *in vitro* culture conditions to allow growth of aneuploid cells. The exact mechanism which leads to aneuploidy in a cell is not known. It is, however, possible to speculate that aneuploidy may increase the instability of a tumour cell and thus cause an increase in the rate of gene deletion and activation. This has not been examined as until very recently, researchers studying oncogenes in breast cancer were apparently not concerned with aneuploidy and no study attempted to correlate the two features. However, in a few recent studies the amplification and overexpression of the *erb* B2 oncogene was observed in breast tumours which were also aneuploid in their DNA content (Tavassoli *et al.*, 1989; Bacus *et al.*, 1990; Baak *et al.*, 1991). This supports the view that aneuploidy may be associated with gene

overexpression and perhaps also deletion in breast tumours. In turn, some of these changes may be the precise events necessary to confer a higher metastatic potential on the tumour. Therefore, only those aneuploid tumours with the right genes activated and/or deleted will behave aggressively. To test this hypothesis, it is necessary to analyse both the relevant gene expression and DNA ploidy on the same breast tumour cells. This can be performed by employing a dual-parameter analysis similar to the one used to analyse CK+ epithelial cells and DNA ploidy.

With respect to surface expression, it appears that among aneuploid tumours HPA binding may identify a group of patients in whom aneuploidy represents an aggressive feature (Chapter 6). However, to confirm this association a much larger number of patients have to be analysed.

7.3. Metastasis and the immunological role of lymph nodes in breast cancer

The axillary lymph nodes that drain breast tumours not only represent a site for the secondary deposit of tumour cells but are also an attractive subject for the study of immune response against cancer (Section 1.2.9.2). With respect to breast cancer patients, it is most relevant to determine whether the axillary nodes are involved in mediating immunity against cancer and as such whether in some patients the absence of metastasis is the effect of such a response. The role of lymph nodes in mounting an immune response against breast cancer still remains controversial. Although not many studies have analysed lymphocytes from the nodes, some of the recent studies provide evidence to suggest that host-mediated immunity against breast cancer may occur at least in some patients (Section 1.2.9.2). HLA DR-expressing T cells

and IgG-expressing B cells were found to predominate in the nodes of breast cancer patients (Morton *et al.*, 1989, Whitford *et al.*, 1992). Although both stage I and stage II patients were studied, only tumour-free nodes were analysed and as such the effect of metastasis on the immune function of the lymph nodes could not be assessed. This study involved the analysis of tumour-free and tumour-invaded lymph nodes from the same patient and these were then compared with lymph nodes from patients without metastasis (Chapter 3). Such analyses reflect the nature of the response and may also determine the outcome of the tumour-host interaction as encountered in lymph nodes.

The response in breast cancer patients was highly patient specific and the differences observed were often seen in only a proportion of patients. The analysis carried out in this study, therefore, reflect trends observed in comparing two groups of nodes in the same patient. The presence of metastatic tumour cells in a lymph node were found to cause specific alterations of the phenotype of the T cells and the activation state of the B cells. It appears that in some patients the presence of tumour cells provide antigens for a secondary B cell response. It is of interest that the alterations in the T cell subsets in the nodes reflect those observed in tumour infiltrating lymphocytes (TILs) (Section 1.2.9.1). As in the TILs, the CD8+ T cells were found to be in higher proportion in invaded nodes of some patients. Here again, close contact of the tumour cells in the nodes with CD8+ T cells may have caused proliferation of these cells. On the other hand, however, lower percentages of CD4+ T cells were also observed in invaded nodes. This reflects the complexity of the host-tumour interaction and it appears that while certain populations are being activated others appear not to be proliferating or are perhaps being suppressed. It is perhaps the final outcome of this interaction which determines whether the host response will be able to contain the tumour.

This can only be established in a follow-up study where recurrences or survival in these patients may be used to assess the implication of a host-mediated anti-tumour response.

The variability between patients in terms of both the phenotype and the activation states of lymphocytes was highly notable. This probably reflects differences in host-immunity but on the other hand may also reflect the oncogenic or antigenic complement of the tumour. Due to the small number of patients studied it was not possible to assess if the alterations observed in lymph node lymphocytes were associated with the differentiation state (grade) of the tumours. However, it was possible to assess this with respect to the DNA content of the primary tumour. Both HLA DR expression on T cells and IgG-expression on B cells were found to be related to the ploidy of the primary tumour. It appears from this that there may be some antigenic determinants in aneuploid tumours that are absent in diploid tumours. This in turn would suggest that a putative oncoprotein, which is probably expressed in some aneuploid tumours, may be antigenic in mediating a host-mediated response. Therefore, it is necessary to determine whether any particular breast cancer-associated antigen/oncoprotein is involved in the observed response. For instance, p53 is known to carry mutations and the mutated protein reported to be expressed in 50% of breast tumours (Bartek *et al.*, 1990; Cattoretti *et al.*, 1988). The p53 protein is expressed within the cell and can, therefore, elicit a T cell response. Although there are no reports of mutation in the c-*erb* B2 gene, the other oncogene widely studied in breast cancer (Slamon *et al.*, 1989; Borresen *et al.*, 1990; Iglehart *et al.*, 1990; Clark & McGuire, 1991), the gene product carries an extracellular domain and, therefore, theoretically could elicit a B cell response

7.4. Surgical and clinical relevance

The treatment of breast cancer not only involves the removal of the cancer at the primary site but essentially it involves an attempt to arrest further spread of the disease. The once used Halsted radical mastectomy, which involved en-bloc removal of the breast and adjoining area, has been abondoned in favour of a much conservative approach. Nowadays, a simple mastectomy or even lumpectomy is performed followed by adjuvant therapy, which includes chemotherapy and loco-regional radiotherapy to the breast, axilla, or both. But there still remains much controversy in the surgical treatment of breast cancer. The controversy centres around treatment of axillary lymph nodes. Here surgeons have various options- a surgeon can choose to ignore the nodes, to sample only the lower nodes, or can make a complete axillary clearance. Nodal clearance is a skilled operation and is associated with significant potential morbidity and is therefore, only performed in large centres where the surgeons have regular experience in it. Surgeons who prefer not to perform a clearance probably have more faith in the Fisher Trial (Fisher *et al.*, 1980), which showed in a randomised trial that survival was not adversely affected in some patients in whom no axillary clearance was performed but who may be presumed to have had lymph node metastasis. Moreover, the survival rates for node positive patients randomised to receive either radical mastectomy alone or mastectomy without axillary dissection but with regional irradiation were equivalent at 10 years. The trial indicated that axillary clearance whether carried out early or only after clinical presentation does not affect survival in breast cancer patients. However, in counterarguments others point out that these trials were performed before the successful use of systemic adjuvant therapy has been established and as such the findings are not valid (Fentiman & Mansel, 1991). Accurate assessment of nodal status is believed to be important as a recently reported worldwide collaborative trial has shown

that adjuvant chemo-endocrine therapy can significantly reduce both recurrence and death in breast cancer patients (Early Breast Cancer Trialists' Collaborative Group, 1992). Although both node-positive and node-negative patients were found to benefit from a combined chemo-endocrine therapy, the improvement in 10-year survival was twice as great for patients with nodal involvement. On the other hand, an additional feature that may be relevant here is the patient's lymph node response. If any of the breast cancer patients are responding to their cancer and since the response appears to be localised to the lymph nodes, then in this case removal of these nodes would definitely compromise the patient's ability to fight the disease.

The major implication of axillary clearance is that it allows accurate assessment of nodal status and lymph node metastasis remains the most important prognostic indicator. However, it must be pointed out that staging is not always accurate and 30% of stage I patients still die from their malignant disease (Bloom *et al.*, 1970; Fisher *et al.*, 1980). There is therefore, a need for identification of other biological features of breast tumours, features which might provide additional information and help in correctly assessing prognosis in breast cancer patients. Ideally, these features should be detectable pre-operatively, for instance on fine-needle aspirates. Although a few biological features have been identified, these are still at an investigational phase. Each aspect of this study has involved measurement of different molecular parameters in relation to metastasis to the node. Although these parameters need to be established in further studies involving a larger number of patients, it appears that these parameters may be of value as prognostic factors. Ideally, it should be possible to build up a database from each patient with all available information- stage, grade, ER status, lectin binding capacity, lymph node immunological responses, aneuploidy status and oncogene content. It might then be possible to make a logical correlation between a set or subset of these

parameters and tumour metastasis in a substantial number of patients. This can perhaps be illustrated by two representative cases where some of these parameters were assessed (Table 7.1). Although both the tumours are aneuploid, the patients differ in their lymph node responses. Patient 1 appears to have a favourable response in the lymph node, more HLA DR+ T cells and and a higher percentage of IgG+ B cells. Patient 1 may, therefore, be regarded

Table 7.1. Data on lymph node response, DNA ploidy and histopathology for two breast cancer patients.

Patient 1	Parameter analysed	Patient 2
9.5	% CD8+ T cells	6.5
36.1	% CD4+ T cells	31.0
3.8	CD4+/CD8+ ratio	4.7
70.0	% HLA DR+ CD8+ cells	44.0
53.0	% HLA DR+ CD4+ cells	18.5
46.0	% sIgG+ B cells	20
Aneuploid	DNA ploidy	Aneuploid
II	Tumour grade	III
without metastasis	Nodal status	with metastasis

to have a good prognosis and this bears out in the tumour grade and the lymph node status. On the other hand, patient 2 is apparently responding poorly and the aneuploid primary tumour is more aggressive, which is reflected in tumour grade and lymph node status. Additional information may be gathered by assessing HPA binding capacity and oncogene content, and together these

would provide a more comprehensive assessment of the biological nature of the tumour and the patient's response to it. This illustration further implies that each patient may have to be treated individually as the cancer may be biologically different, at a different differentiation stage and moreover, the host-tumour interaction is likely to be patient-specific.

Technically, such an approach has two major problems-

(i) It requires a synthesis between the work of surgeons, pathologists, immunologists, biochemists, geneticists and finally a good computer database technologist to keep a datafile for each patient.

(ii) It requires more tissue than is currently available either from the primary or the node. Moreover, more representative material may be needed as primary tumours received in the clinical laboratory are heterogeneous in nature. For instance, the sample sent to pathology for grading may be different from that sent to biochemistry for ER analysis and different again from the sample sent to genetics for oncogene analysis.

If these problems can be resolved, it might then be feasible to analyse only the features relevant to the metastatic capacity of a primary breast tumour. There is clearly a lack of understanding of the true biology of breast cancer and further basic research in this field is much warranted.

CHAPTER 8

References

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