IMMUNOHISTOCHEMICAL PROGNOSTIC PARAMETERS

IN BREAST CARCINOMA

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CONTENTS

ABSTRACT

The biological behaviour of breast cancer is unpredictable and present prognostic markers do not accurately indicate survival times for individual patients. Recent investigations have focused on a search for intracellular markers which might provide information unattainable by histology. This thesis examines the relationship between traditional pathological prognostic parameters and several new potential prognostic indicators, identified and quantified by immunohistochemical staining, in 115 malignant breast neoplasms.

A modified technique of identifying estrogen receptor (ER) protein in frozen sections and imprints utilizing a new commercial monoclonal anti-ER antibody is reported. Optimal preservation of the ER antigen is observed following fixation in periodate-lysine-paraformaldehyde (PLP) for 10 minutes. An improved, reproducible method of detecting ERs in formalin-fixed paraffin sections using the anti-ER antibody is described.

A recently synthesized monoclonal antibody to proliferating cells, Ki-67, is used to estimate the tumour growth fraction (GF) in all cases and an inverse relationship between GF and ER status is identified. Coexpression of cytokeratin and vimentin intermediate filaments (IFs) is documented, for the first time, in 10.4 per cent of ductal carcinomas. Acquisition of vimentin correlates strongly with a high tumour GF and the role of vimentin as a potential prognostic marker is discussed. Staining of nucleolar organizer regions (NORs) with the silver impregnation technique of

Crocker et al (1986) reveals a correlation between the NOR count and the Ki-67 count. Finally, it is recommended that all lymph nodes in cases of node-negative breast cancer be stained with anti-cytokeratins following the identification of "missed" micrometastases in 22 per cent of 55 cases studied by this technique.

Alpha-lactalbumin, pregnancy-specific ß1-glycoprotein (SP1) and prolactin, three traditional markers for breast carcinoma, are assessed and deemed non-specific and of no prognostic value. Antisera to basement membrane and myoepithelial cell antigens assist in identifying early invasive foci in intraductal carcinomas and in differentiating sclerosing adenosis from well-differentiated carcinoma. Anti-factor VIII and UEA I, employed to detect vascular invasion, provide no advantage over an assessment of haematoxylin and eosin-stained sections.

In conclusion, lymph node status and tumour GF are considered the major prognostic parameters in breast cancer. Minor prognostic markers include ER status, histological type and grade, and tumour size. Expression of vimentin IFs by breast carcinomas and NOR counts may also prove to be of prognostic value.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree of Doctor of Medicine.

WENDY A. RAYMOND

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CONTRIBUTIONS OF THIS THESIS

This dissertation makes the following contributions:

1. The relationships between traditional pathological prognostic markers of breast cancer, including lymph node status, histological grade and tumour size, and several novel potential prognostic parameters are described for a series of 115 malignant breast tumours.

2. A simplified technique of immunostaining for estrogen receptor (ER) protein in frozen sections is developed using a commercial anti-ER antibody and an avidin-biotin complex method.

3. ER staining of imprints of fresh breast tumour tissue is established as a useful technique.

4. Following an assessment of 16 fixation techniques, periodate-lysine-paraformaldehyde (PLP) for 10 minutes at room temperature is found to optimally preserve the estrophilin antigen in frozen sections and imprints.

5. An improved, readily reproducible method of detecting ERs in paraffin sections fixed in formalin for one and a half hours is described, and the percentages of ER-positive tumour cells (ER counts) are shown to correlate with the corresponding frozen section ER counts.

6.   The percentage of Ki-67-positive cells (Ki-67 count), representing the tumour growth fraction (GF), is demonstrated to correlate with tumour histological grade, but no significant relationship with lymph node status or tumour size is observed.

7.   An inverse relationship between Ki-67 and ER count is identified. A theory is proposed to explain the failure of some ER-positive tumours to respond to hormonal therapy, based on the finding of an exceptional subgroup of cases with high GFs and high ER counts.

8.   Coexpression of cytokeratin and vimentin intermediate filaments (IFs) is documented in approximately 10 per cent of breast carcinomas. Vimentin expression in a tumour of presumed epithelial origin highlights the need for caution when using immunoperoxidase stains to classify neoplasms of unknown primary site.

9.   Vimentin expression in breast carcinomas is significantly related to high tumour GF and histological anaplasia. Vimentin expression is proposed as a potential indicator of poor prognosis.

10.   A correlation between the number of silver-stained nucleolar organizer regions (AgNORs) and Ki-67 counts is described, supporting suggestions that NOR numbers may be related to proliferative activity.

11. "Missed" micrometastases are identified in 22 per cent of 55 cases of node-negative (Stage I) breast cancer studied with anti-cytokeratins, thereby "upstaging" each patient to Stage II.

12. Antisera to basement membrane components (laminin and type IV collagen) and to myoepithelial cell antigens (actin and MSA) assist in the identification of early stromal invasion and in differentiating benign epithelium from well-differentiated malignant duct structures.

13. Immunostaining with antiserum to factor VIII-related antigen and with UEA I fails to increase the detection of vascular invasion over conventional assessment using haematoxylin and eosin-stained slides.

14. Staining for alpha-lactalbumin, pregnancy-specific ß1-glycoprotein (SP1) and prolactin is found to be nonspecific and deemed of no prognostic value.

15. It reviews the extensive literature on current histological prognostic parameters.

16. Recommendations as to the major and minor prognostic markers in breast carcinoma are provided.

ACKNOWLEDGEMENTS

"It is not in the nature of things for anyone to make a

sudden, violent discovery; science goes step by step,

and everyone depends on the work of one's predecessors"

Ernest Rutherford

CHAPTER I.   INTRODUCTION

I. INTRODUCTION

Cancer of the breast is the commonest non-cutaneous cancer and the primary cause of cancer mortality in Australian women. The annual incidence is greater than 5000, with the implication that approximately one in 16 women will develop the disease at some time in their lifetime. The relatively young age of many of the victims adds to the social and emotional effects of this disease, with a sharp rise in incidence from the early third decade to 50 years of age, followed by a brief plateau, and then a further rise in incidence at a reduced rate (Giles et al, 1987). Two thousand, two hundred and fifty-eight female deaths from breast cancer were recorded in Australia in 1987, representing approximately 18.5 per cent of all female deaths due to malignant neoplasms in that year (Castles, 1987). Malignant neoplasms are the major cause of Australian female deaths in the 25 to 64 year old age group. In Australia, as in the majority of Western countries, the age standardized mortality rate from breast cancer of approximately 20 deaths per 100,000 person years has changed little over the past 30 years (McMichael & Armstrong, 1988).

The overall five year survival of breast cancer patients treated in South Australia between 1977 and 1985 was 74 per cent, similar to the survival rates of 70 per cent and 75 per cent quoted for Australia overall and the United States respectively (Giles et al, 1987). Deaths may, however, continue to occur for up to 30 or more years after diagnosis, and thus only a very small proportion of patients, if any at all, may be considered to have been cured of their disease. There is thus a need to identify markers of tumour aggression in order to define those subgroups of patients

with a good prognosis requiring minimal further treatment, and those with an anticipated unfavourable outcome for whom aggressive therapy may be beneficial.

Despite the lack of any definitive evidence as to the aetiology of breast cancer, numerous "risk factors" associated with the development of this tumour have been tabulated by epidemiologists. The principal factors established to increase breast cancer risk above the level of risk in the general population are genetic predisposition (familial history), a past history of breast cancer or benign epithelial proliferative changes, particularly atypical hyperplasia and papilloma with fibrovascular core (Dupont & Page, 1985; Sinclair, 1985), nulliparity or a late age at the first full-term pregnancy, decreasing age at menarche and increasing age at menopause, exposure to ionizing radiation, obesity in post-menopausal women and, probably, the use of estrogens (without combined progesterones) for the relief of post-menopausal symptoms. Although not an indication of prognosis, such factors do provide a crude estimate of the likelihood of developing breast cancer. Other less-well-established factors which may increase risk relate to dietary intake of saturated fat and possibly of alcohol, and the use of oral contraceptive agents in certain subgroups of women (McMichael & Armstrong, 1988).

The persistent high incidence of breast carcinoma, the high mortality and the extreme individual variability in the biological course of this disease has impelled the search over the past few decades for clinical and pathological parameters which may provide guidelines for treatment and an indication of prognosis. Clinical staging systems reflecting the anatomic extent of the disease at the time of diagnosis are widely used, two of the most popular being the International T-N-M and the

Manchester systems. These both incorporate a clinical assessment of tumour size and attachment to the underlying chest wall or overlying skin (T), the presence or absence of clinically palpable lymph nodes (N) and the presence or absence of distant metastases (M). In all systems Stage I (or A) patients have the most favourable prognosis, and those with Stage IV (or D) disease have the worst outlook (Henderson & Canellos, 1980; Beahrs, 1984). However, staging based on clinical assessment alone, even with the benefits of modern radiographic imaging techniques, has been estimated to be inaccurate in up to 40 per cent of cases when compared with the information obtained from pathological study of a surgical specimen (Beahrs, 1984).

The presence or absence of axillary nodal metastases is, to date, the single most useful pathological parameter for predicting survival. Other parameters traditionally considered helpful in the prognostication of breast carcinoma include an assessment of primary tumour size, histological type and grade, presence of vascular invasion and, more recently, hormone receptor status. Each of these will be discussed in detail in the next chapter.

Even so, traditional clinical and pathological staging methods only provide an approximate prognostic index and frequently fail to accurately predict outcome for individual patients with breast cancer. A need for more sophisticated prognostic determinants that yield information concerning the nature of the malignant process and, in particular, the metastatic potential of the tumour, has resulted in the development of new methods of assessing prognosis over the past 10 to 15 years. Techniques which measure cell proliferation and detect "tumour specific", or

potentially prognostic, intracellular markers show most promise. Such techniques and markers allow examination of the primary tumour at a cellular level. They may permit prediction of the likely behaviour of a tumour which has not yet metastasized, or in which the metastases are too small for even the most sophisticated image analysis system to recognize. Furthermore, they may predict the patient's likely response to various therapeutic modalities thereby enabling the most appropriate cancer therapy to be chosen.

The worldwide development of breast screening programmes will yield a greater proportion of small tumours for which histological assessment may be particularly unreliable. Insufficient tissue may be available for traditional biochemical hormone receptor assays. The axillary lymph node status may remain unknown for many patients with current trends towards more conservative surgery. The emphasis of pathological prognostic assessment of breast cancer is thus changing from the traditional inspection of the gross specimen and microscopic examination of haematoxylin and eosin (H & E)-stained slides, to a process which demands specialized techniques and a pathologist experienced in their interpretation.

Most of these new prognostic techniques are still in their infancy and the nature of breast cancer dictates that up to 25 years clinical follow-up is necessary to truly assess their worth (Dawson et al, 1982). However, it is impracticable to evaluate the multitude of new markers with such long-term follow-up before they are utilized in clinical practise. Assessment of novel, potential prognostic indicators, as described in this study, is therefore dependent upon identifying relationships with current parameters known to have some prognostic value in order to offset a limited

duration of follow-up.

This thesis begins by reviewing currently employed prognostic parameters and then describes several new prognostic markers, improved immunocytochemical staining techniques developed to identify these markers, and their application to 115 malignant breast neoplasms. The markers are related to historically verified prognostic indicators including histological grade and lymph node status. In conclusion, major and minor prognostic parameters are specified and recommendations for optimal prognostic assessment of surgical breast carcinoma specimens are given.

CHAPTER II. REVIEW OF CURRENT PROGNOSTIC INDICATORS

II. REVIEW OF CURRENT PROGNOSTIC INDICATORS

II.a. TUMOUR SIZE

The maximum diameter of the primary tumour, conventionally used to represent tumour size, is the only pathological prognostic feature which can be attained by macroscopic inspection of the surgical specimen. The European Group for Breast Cancer Screening (1988) recommended that tumour size be measured microscopically on a large histological section. This is no doubt more accurate than assessment of the wet tissue, but the preparation of large histological sections is seldom practicable in a routine pathology laboratory and a correction for processing shrinkage is required. Macroscopic measurements of tumour size, preferably in three dimensions, are considered satisfactory by most workers.

The true significance of primary tumour size in determining clinical outcome is disputed (Sears et al, 1982; Fisher et al, 1984; Russo et al, 1987), and critical assessment is hampered as almost all study populations include patients who have received various forms of post-operative therapy. In general, survival is better and axillary nodal metastases less likely in patients with small primary tumours (Carter et al, 1989). A tumour less than 0.5 cm in greatest dimension, termed "minimal cancer" by some authors (Fisher, 1984), is associated with the most favourable prognosis. Cancers measuring less than two cm, two to five cm, or greater than five cm in diameter may all behave differently and may thus be treated differently by the surgeon (Murad, 1975).

In 1969 Fisher et al, in a study of 2000 breast cancers, found nodal metastases in only 22 per cent of patients with tumours less than one cm in diameter, while in tumours of greater than six cm diameter, metastases were present in 63 per cent. Other workers have confirmed tumour size to correlate with lymph node status and be independently predictive of survival (Russo et al, 1987; Hutter, 1984). Life table data of 614 women treated by radical mastectomy and followed for 10 years were analysed by Fisher et al (1984) who found two cm to be the critical tumour size. Tumours of less than two cm in diameter were positively related to prolonged disease-free survival (DFS), independently of lymph node status; however, the magnitude of the difference in DFS when compared with all cancers greater than two cm in diameter was only 15 per cent. This relationship appears somewhat inconsistent in that a stepwise association between tumour size and survival is lacking. Furthermore, when the data were prepared according to nodal categories, primary tumour size correlated inversely with survival only in patients with four or more nodal metastases. When no nodes or one to three nodes contained metastatic deposits only a trend was observed. Sears et al (1982) also demonstrated that primary tumour size was not a prognostic indicator for node-negative breast cancer patients. Dawson et al (1982) compared a population of breast cancer patients surviving 25 years after initial surgical treatment with a group of patients who died within 10 years and found that amongst the "long survivors" there was a greater percentage of tumours less than two cm in diameter. However, 12 per cent of "long survivors" had tumours larger than five cm in diameter. These observations suggest that there may be at least two different breast tumour types: one in which tumours attain a large size without exhibiting regional or distant metastases, and another in

which systemic metastasis occurs before there is extensive local growth within the breast (Henderson & Canellos, 1980). A more recent study of 24740 breast cancer cases with five years of follow-up demonstrated that as tumour size increased, survival decreased, regardless of lymph node status (Carter et al, 1989). A linear relation was found between tumour diameter and the percentage of cases with positive lymph node involvement.

Thus, tumour size probably cannot be considered a prognostic parameter in isolation and its value as an independent prognostic factor in patients without nodal involvement remains controversial.

II.b. HISTOLOGICAL TYPE

Histological typing of infiltrating breast carcinoma has been shown to have prognostic significance. The majority of breast cancers are classified as infiltrating ductal carcinomas (IDCs) - not otherwise specified (NOS), comprising 60 to 75 per cent of most series (Roses et al, 1982; Hutter, 1984) and with a 10 year survival rate of between 50 and 60 per cent. This is a very heterogeneous group, the neoplastic cells being arranged as nests, cords and gland-like structures. The lack of characteristics that might assign these tumours to other groups is their only common feature (World Health Organization, 1981).

It is important to recognize certain histological types of breast carcinoma which are associated with more predictable behaviour patterns as therapeutic modifications may

be indicated. Subtypes of breast carcinoma acknowledged to have a more favourable prognosis than average include medullary carcinoma, tubular carcinoma, mucinous (colloid) carcinoma, adenoid cystic carcinoma and juvenile secretory carcinoma (Hutter, 1984; Gallager, 1984). Although invasive, these tumours all exhibit a low incidence of axillary nodal metastases and a long DFS (Gallager, 1984). If recurrences occur they tend to be local or in regional lymph nodes, distant metastases presenting only as a late complication.

Medullary carcinoma typically forms a well-circumscribed, almost spherical mass composed of poorly-differentiated epithelial cells, frequently with a high mitotic count and marked nuclear pleomorphism, associated with a prominent lymphoid infiltrate. Glandular structures and *in situ* carcinoma are notably absent, and greater than 75 per cent of the tumour exhibits a syncytial growth pattern. Only those tumours adhering to these stringent diagnostic criteria ("typical" medullary carcinomas) exhibit a favourable prognosis. Tumours fulfilling only two or three of the diagnostic criteria are designated as "atypical" medullary carcinomas. Ridolfi et al (1977) and Rapin et al (1988) described 10 year DFS rates for "typical" medullary carcinomas of 84 per cent and 92 per cent respectively, compared with rates of 74 per cent and 53 per cent for patients with "atypical" medullary carcinoma. Furthermore, "typical" medullary carcinomas showed a more favourable prognosis than ordinary IDCs, even in the presence of axillary nodal metastases. Recently, Wargotz and Silverberg (1988) reviewed the criteria for the diagnosis of medullary carcinoma and proposed that cases be classified as medullary carcinoma even in the presence of focal *in situ* carcinoma, marginal infiltration, or a sparse mononuclear infiltrate. The five year survival for this group was 94 per cent. If two or more

atypical features were present the tumours were classified as IDCs and the five year survival rate fell to 64 per cent. Wargotz and Silverberg (1988) did not believe in the subtype of "atypical" medullary carcinoma.

Tubular carcinomas are well-differentiated tumours in which a dense fibrotic stroma typically surrounds small angular tubules lined by a single layer of regular cells. They present as small localized lesions, rarely greater than one cm in diameter, and very rarely with lymph node metastases. This form of carcinoma is often observed admixed with other forms of infiltrating carcinoma but a more favourable prognosis is only associated with tumours which show at least a 75 per cent tubular component (Carstens et al, 1985). In this latter group survival at five years has been reported to reach 100 per cent (Peters et al, 1981; Deos & Norris, 1982). Peters et al (1981) noted that as the tubular proportion of the carcinoma decreased, the biological aggressiveness of the tumour increased. Based on such findings, and the observation that it is very unusual to find a pure tubular carcinoma greater than one cm in diameter (Gallager, 1984), it has been suggested that tubular carcinoma may represent the earliest form of IDC and, in time, may be capable of progressing to a more aggressive carcinoma subtype.

Mucinous (or colloid) type carcinoma, which constitutes only one to three per cent of all primary breast cancers, is characterized by abundant epithelial mucus both surrounding and within tumour cells. The 10 year survival rate for this tumour subtype was 90.4 per cent in one series when there was no invasive ductal component (Komaki et al, 1988). In general, the greater the amount of mucus production, the better the prognosis and the smaller the tumour size.

Two other subtypes of carcinoma with favourable prognoses, adenoid cystic carcinoma and juvenile secretory carcinoma, are extremely rare and have characteristic histological phenotypes. The adenoid cystic carcinoma is identical to the tumour of the same name that presents more typically in salivary glands. Secretory carcinoma is composed of cells with intracytoplasmic periodic-acid-Schiff (PAS)-positive material. The tumour cells are arranged around acinar-like spaces containing PAS-positive secretions (Gallager, 1984). A recent publication has suggested that patients with invasive cribriform carcinomas may also survive longer than those with IDCs-NOS (Dawson et al, 1986).

Two tumour subtypes have been observed to be particularly aggressive, exhibiting invariably poor prognoses. The five year survival rate of inflammatory carcinoma rarely exceeds 10 per cent (Hutter, 1984). Inflammatory carcinoma is a clinico-pathological entity characterized by a rapidly growing tumour associated with diffuse brawny induration of the breast and extensive permeation of dermal lymphatics. Carcinomas exhibiting sarcomatoid metaplasia, in the form of malignant osteoid or cartilage, tend to be of large size at first presentation and clinical history generally suggests a rapid growth rate. Five year survival rarely exceeds 35 per cent (Gallager, 1984).

Some breast tumours are easily placed into one histological category, but in many cases several different patterns are clearly identified within the one tumour. This lack of specificity hinders accurate correlation between tumour type and survival. Stenkvist et al (1983), in a study of the reliability and reproducibility of the breast

cancer classifications proposed by Ackerman, the World Health Organization (WHO) and the Armed Forces Institute of Pathology, found interobserver reproducibility varied between only 45.5 per cent (Ackerman) and 78.6 per cent (WHO) equal assessments, and intraobserver reproducibility varied between 57.0 per cent (Ackerman) and 75.7 per cent (WHO). No correlation between tumour type and five year recurrence rate was identified for any of the classification systems examined. Thus, although histological typing may sometimes help in indicating likely clinical outcome, it cannot be regarded as a reliable prognostic parameter.

One further subgroup of breast carcinoma worth considering at this point is the entity of *in situ* (or non-invasive) neoplasia. The currently accepted view that *in situ* carcinoma is a biological precursor of invasive carcinoma has been disputed, however, there is definite value in identifying this pattern as a marker of increased risk for future *in situ* and invasive carcinoma. *In situ* carcinoma is defined as a proliferation of malignant epithelial cells confined to the mammary ducts or lobules, without light microscopic evidence of invasion through the basement membrane into the surrounding stroma.

The widespread use of mammographic screening has resulted in the detection of a relatively greater number of small tumours. Many of these prove to be *in situ* ductal carcinomas, also known as intraductal carcinomas, mammographically demonstrable as minute foci of calcification. Traditionally this form of carcinoma has been treated with radical mastectomy, but with the present trends towards breast-conserving surgery and radiation therapy for invasive cancer (Fisher et al, 1985a; Recht et al, 1986) it would seem difficult to justify more aggressive treatment of non-invasive

carcinoma. If, however, *in situ* carcinoma is treated only with wide excisional biopsy it is important to be fully cognisant of the possibility of residual *in situ* or invasive carcinoma, the increased potential for subsequent neoplastic transformation of histologically normal breast ducts, and the risk of axillary metastases.

A variety of histological patterns of ductal carcinoma-*in situ* (DCIS) have been recognized and include comedo, cribriform, solid and papillary forms. Several studies have suggested the comedo type of DCIS may be more aggressive and show a higher incidence of microinvasion (Schnitt et al, 1988; Patchefsky et al, 1989), but these observations have not been universally confirmed and all types of DCIS are usually considered together in analyses of survival data. In patients with a diagnosis of pure DCIS the reported risk of synchronous occult IDC varies between 12 and 21 per cent (Carter and Smith, 1977; Rosen et al, 1979; Lagios et al, 1982). The latter authors utilized a technique of serial subgross sectioning, correlated with radiography, for examination of 53 mastectomy specimens and detected the highest rate of occult invasive tumour (21 per cent). All of these occult foci occurred in patients with DCIS extending over an area greater than 2.5 cm in diameter. The frequency of multicentric intraductal carcinoma (DCIS in other quadrants of the same breast) was 54 per cent for primary lesions greater than 2.5 cm in diameter, but only 14 per cent for smaller intraductal tumours (Lagios et al, 1982). In addition, it has been reported that 25 to 50 per cent of women with DCIS treated by biopsy alone will develop intraductal or invasive carcinoma within 10 years, a relative risk of eight to 10 times that for women who have not received a breast biopsy (Dupont & Page, 1985; College of American Pathologists, 1986; Sinclair, 1988). There is no increased risk for the contralateral breast.

Even with the most diligent pathological examination foci of invasion in DCIS may not be identified (Chapter IV.8) and the optimal management of these patients is still not known. Based on their theory that frequency of occult invasion and multicentricity are functions of tumour size, Lagios et al (1982) recommend that tylectomy (subcutaneous mastectomy) with long-term follow-up may be a reasonable alternative to total mastectomy for treatment of DCIS, in particular if the lesion is less than 2.6 cm in diameter. Fisher et al (1986) claim lumpectomy with post-operative irradiation is optimal therapy, while others advocate modified radical mastectomy (Carter & Smith, 1977). Nonetheless, whatever the management, survival rates for patients with pure DCIS remain very high (Fisher et al, 1986).

Lobular carcinoma is a distinctive infiltrating small cell carcinoma frequently associated with the formation of intracytoplasmic "mucoid globules" (signet ring cells). Lobular carcinoma-*in situ* (LCIS) is a marker of increased risk (56 to 63 per cent) of developing occult LCIS in other quadrants of the same breast (Rosen et al, 1978; Carter & Smith, 1977), while the risk of LCIS arising in the contralateral breast ranges from 26 to 35 per cent (Urbane, 1967; Haagensen, 1986). LCIS is also regarded as a risk factor for future invasive carcinoma, but it is not clear whether it is a precursor lesion *per se*. Data compiled by Anderson (1977) revealed that 20.4 per cent of 44 patients developed ipsilateral invasive carcinoma and 9.1 per cent developed subsequent contralateral carcinoma over a mean follow-up period of 15.9 years. Similar results were obtained by Rosen et al (1978) who reported that IDC was the most common invasive carcinoma to develop in such instances.

The optimal treatment of patients with LCIS is also controversial (Anderson, 1977; Rosen et al, 1978). In a large series of such patients treated with excision alone, only three per cent died of invasive carcinoma after a mean follow-up period of 14 years (Haagensen, 1986).

Thus it appears that the prognosis of patients with pure DCIS or LCIS is most favourable, approaching 100 per cent curability by mastectomy. It is not clear whether all cases of *in situ* carcinoma would eventually progress to become clinically important lesions as treatment by mastectomy in the past has prevented the study of the natural history of these tumour subtypes. It is important to recognize that biological differences exist between *in situ* lobular and ductal carcinomas and optimal therapy may therefore differ depending on the type of *in situ* neoplasia. Nevertheless, both present a definite risk for subsequent invasive carcinoma, this risk being greatest for patients with DCIS.

II.c. HISTOLOGICAL GRADE

Numerous studies have subdivided invasive ductal breast carcinomas according to histological grade or degree of differentiation and demonstrated a significant correlation with tumour recurrence and overall survival (Dawson et al, 1982; Fisher et al, 1984; Singh et al, 1988). Others, however, have not observed such an association (Roses et al, 1982). Nevertheless, an estimate of histological grade is presently a routine component of the breast carcinoma pathology report and may reflect the degree of malignancy and extent of tumour spread. Such grading

assessments may also assist in treatment planning as well-differentiated tumours are recognized to be less responsive than anaplastic lesions to radiation therapy or radiomimetic agents (Lippman et al, 1978). In general, high grade or poorly-differentiated tumours show a greater propensity to relapse than well-differentiated, or low grade, carcinomas and the closer the tumour cells approximate normal breast epithelium the less the anticipated biological aggressiveness.

The first attempt at systematic histological grading was performed by Greenough in 1925. His system was based on the degree of arrangement of cells around an "open gland lumen", the degree of uniformity of cell and nuclear size, and an assessment of nuclear hyperchromasia and mitotic activity. Most current grading systems still incorporate these three characteristics - histological pattern (with the identification of tubules), nuclear pleomorphism and mitotic activity. Only IDCs are graded, alternative tumour types carrying different individual prognoses as described in the previous section. In assigning a histological grade the estimation of nuclear grade and tubule formation is based only on the invasive component of the tumour, the intraductal element being regarded as a separate entity. The failure to adhere to these conditions of grading may account for some of the irreproducibility encountered.

The most popular grading systems are those of Black (1957), Hartveit (1971), Bloom and Richardson (1957), and a modification of the latter endorsed by the World Health Organization (1982). Bloom and Richardson's (1957) method is preferred at the IMVS and is utilized in this study. It is based on a simple numerical system in which three histological features are scored on a three point scale according to

whether each is present in slight, moderate or marked degree. The first feature is the degree of tubular differentiation, where one point is given for well-formed tubules and three points if none are present. The degree of nuclear pleomorphism (incorporating the variation in size, shape and staining of nuclei) is assessed subjectively, one point being awarded for uniform nuclei and three points for marked pleomorphism. Finally, the mitotic potential of the tumour is assessed with one point for the presence of occasional mitoses, two points for up to two mitoses per high power field and more frequent mitoses meriting three points. The points in each of the three categories are summed and histological grades are allocated arbitrarily as follows:

3, 4 or 5 points	-	Grade I (low)
6 or 7 points	-	Grade II (intermediate)
8 or 9 points	-	Grade III (high)

Bloom and Richardson (1957) described survival data which confirmed the value of their histological grading system. Survival rates in 1409 patients followed for five years were 75 per cent for patients with Grade I tumours, 47 per cent for those with Grade II tumours, and 32 per cent for patients with Grade III tumours. Three hundred and fifty-nine patients were followed for 15 years and the survival rates fell to 31 per cent, 18 per cent and 10 per cent for patients with Grade I, II and III cancers respectively. The prediction of survival was much enhanced when axillary nodal status was also taken into account. The correlation of histological grade with survival in 63 untreated patients is perhaps more pertinent as this group provides a better reflection of the natural history of breast cancer. Survival of patients with Grade I tumours was two to three times longer on average (up to 13 years) than survival of patients with Grade III tumours (up to four years). Many other workers

have utilized the Bloom and Richardson grading system and correlations with survival have been confirmed (Singh et al, 1988; Heathfield et al, 1988). Bloom and Richardson (1957) also noted that the grade of most tumours remained relatively constant over time, but alterations were occasionally observed in metastases and local recurrences, especially following radiotherapy. This could explain some inconsistencies in prediction of clinical course based solely on histological grading. When variation in grade within a tumour is observed, the assessment should be based on the most anaplastic part of the tumour.

Other commonly employed methods of histological grading are described briefly. The World Health Organization grading system (1982) is based on that of Bloom and Richardson and combines histological and cytological criteria - mitotic rate, hyperchromatic nuclei and pleomorphism. Black and Speer (1957) regarded structural differentiation (tubule formation) as unimportant and presented a four-tiered grading system based on nuclear atypia whereby the most anaplastic-appearing nuclei were designated as Grade I. Their investigations identified nuclear grade to be an independent prognostic variable. Hartveit's system (1971) was based on nuclear crowding, lobulation and diameter in relation to the total diameter of the cell, as well as an assessment of cell borders.

Fisher et al (1984) modified the schema presented by Black and proposed a grading system incorporating the presence or absence of tubules and an assessment of nuclear grade. All of the pathological material presented in the National Surgical Adjuvant Breast Project trials was classified according to this three-grade system. These studies (Fisher et al, 1984) indicated the value of histological grade in

predicting survival of patients free of axillary lymph node disease or with metastases in four or more nodes at initial diagnosis. High histological grade correlated with other parameters thought to be associated with poor prognosis including young age, lymphatic permeation and tumour necrosis. Plots of survival versus nuclear grade closely paralleled those of survival versus histological grade, but histological grade was more finely discriminatory of survival than an assessment of nuclear grade alone (Fisher et al, 1980). Finally, some pathologists rely upon "eyeballing" to gain an overall impression and simply designate the tumour as well, moderately or poorly-differentiated.

The many alternative grading systems, and the sometimes conflicting results in relation to the ability of histological grade to predict survival, attest to the fact that histological grading is not a consistent prognostic discriminant and that no one system is ideal. In particular, grading systems are subjective, arbitrary and frequently show low interobserver and intraobserver reproducibility (Stenkvist et al, 1979; Gilchrist et al, 1985). Gilchrist et al (1985) clearly demonstrated that the level of interobserver disagreement was directly proportional to the number of reviewers, there being a 46 per cent disagreement with two reviewers, 73 per cent disagreement with five and 98 per cent disagreement with eleven reviewers.

The ideal grading system must be simple to apply, rapidly performed, readily reproducible and an effective guide to prognosis. Morphometric quantitative microscopic methods, utilizing computer-assisted image analysis systems, have recently been introduced with the aim of providing objective and more accurate and reproducible prognostic data than can be provided by subjective histological grading

systems (Heathfield et al, 1988). Morphometric measurements of nuclear features (diameter, axes, perimeter and area), nuclear to cytoplasmic ratios, cellularity indices (percentage area of neoplastic cells relative to stroma) and mitotic activity indices have been used to provide a prognostic index in breast cancer. Among others, Baak et al (1982) and Tosi et al (1986) have demonstrated the superior value of morphometric parameters, in particular the mitotic activity index, the mean nuclear area and the cellularity index, over conventional histological grading and staging methods in predicting DFS. Tumours with fewer mitoses, smaller nuclear areas and a higher cellularity index are less likely to metastasize and show a longer survival. This technique appears promising, but is still in its infancy, is tedious and time consuming to perform, and lacks standardization. Furthermore, costly computer image analysers are not accessible to all pathology laboratories.

Recently fine needle aspiration cytology has gained importance in the diagnosis of breast lesions. A cyto-prognostic classification has been described by Mouriquand et al (1986) who examined 204 Papanicolaou-stained breast carcinoma aspirates or imprints and ranked these into one of three grades based on an elaborate scoring system. The seven year DFS was 95 per cent for Grade I tumours, 70 per cent for Grade II cancers and 45 per cent for the most poorly-differentiated (Grade III) lesions. This system is novel and appears promising, but has yet to be assessed by other workers.

In conclusion, histological grading systems do appear to have some prognostic value and it seems reasonable to continue the present practise of providing an indication of tumour differentiation in the surgical pathology report. Nevertheless, due to the

limitations inherent in any subjective grading system, alternatives such as morphometric analysis should be thoroughly explored to attempt to refine predictions of tumour behaviour based on histological assessment of the primary tumour.

II.d. VASCULAR INVASION

The presence of tumour emboli within vascular channels (either lymphatic or blood) is believed to influence the survival of women with breast cancer (Rosen, 1983). Patients with vascular invasion are at substantial risk of local tumour recurrence and of distant metastases. Rosen (1983) studied 38 node-negative breast cancer patients in whom lymphatic emboli had been identified in the mastectomy specimen, matched with controls who did not have evidence of vascular invasion. After five years of follow-up distant metastases were found in 43 per cent of the patients with emboli but in only four per cent of those in whom tumour emboli had not been identified. Similarly, tumour emboli had been found more frequently in women who developed recurrences than in those who remained free of disease. The adverse prognosis associated with lymphatic invasion was only independently significant in the subset of patients with T_1 tumours (TNM staging; tumour diameter less than or equal to two cm), especially those with tumours measuring between 1.1 and two cm in diameter. Furthermore, serial sectioning of the apparently negative nodes indicated that the unfavourable effects of lymphatic invasion were probably independent of occult regional nodal metastases.

Roses et al (1982) claimed lymphatic invasion to be the single most important

pathological factor in predicting disease recurrence in patients with $T_1N_0M_0$ tumours (axillary node-negative and no evidence of metastases). Recurrence occurred in 32 per cent of patients with lymphatic invasion but in only 10 per cent of patients without identifiable lymphatic emboli. Bettelheim and associates (1984a) described peritumoral vascular invasion (encompassing both lymphatic and blood vessels) in 29 per cent of a series of node-negative patients, and confirmed this parameter to be a significant predictor of recurrence. More recently Davis et al (1985), in a study of 1510 women, concluded that vascular (including lymphatic and blood vessel) invasion was a significant independent predictor of DFS and overall survival in both node-negative and node-positive patients. Weigand et al (1982) recorded blood vessel invasion, identified by the presence of either intraluminal erythrocytes, an endothelial cell lining and/or the presence of elastic tissue (around larger vessels), in 35 per cent of 175 patients. These authors also concluded that blood vessel invasion is a useful prognostic marker, independent of lymph node status.

However, dissenting opinions regarding the prognostic value of vascular invasion have been reported. Roses et al (1982) failed to find a correlation between the presence of blood vessel invasion and DFS. Fisher and associates (1984), reporting the findings of the National Surgical Adjuvant Project for Breast Cancers in patients treated by mastectomy, concluded that intramammary lymphatic invasion was not a significant independent pathological discriminant for treatment failure but did observe adverse prognostic effects of blood vessel invasion.

The reasons for these discordant results may be multiple and are likely to include sampling inadequacies and difficulties in distinguishing blood from lymphatic

capillaries. Accurate assessment of vascular invasion is also hindered by the possible occurrence of "pseudoemboli" in which artefactual tissue spaces, almost indistinguishable from true vascular spaces, contain small nests of tumour cells. This phenomenon is a result of tissue shrinkage due to fixation (Rosen, 1983). Perhaps the application of vascular invasion as a marker for prognostication is most hampered, though, by interobserver and intraobserver differences in interpretation. Even with precisely defined criteria of vascular invasion Gilchrist et al (1982) found unacceptably high levels of disagreement among three pathologists reviewing microscopic sections. All three concurred on the presence of lymphatic emboli in only 12 (34.2 per cent) of 35 cases. Agreement on the absence of blood vessel invasion was reached in 86 per cent of cases. Their data concerning lymphatic invasion has been analysed by Rosen (1983) who concluded that "the authors have been unduly pessimistic", and that if the criteria had been limited to decisions about lymphatic invasion in extratumoral tissues the level of agreement would have reached 77 per cent.

True lymphatic and blood vessel invasion is most clearly evaluated in the tissue peripheral to the margin of the invasive carcinoma (Rosen, 1983). Any neoplasm which expands and invades would be expected to destroy or extend into vascular spaces obstructing its path and thus the importance of identifying vascular invasion within the main tumour mass is unclear. Prognostic significance of lymphatic invasion has been demonstrated in those studies in which lymphatic invasion is clearly limited to emboli within peritumoral lymphatics (Rosen, 1983; Bettelheim et al, 1984a).

The majority of the aforementioned studies relied on an assessment of H & E-stained slides, with or without the aid of an elastic stain to outline blood vessels. Immunohistochemical techniques using UEA I (*Ulex europaeus* agglutinin I) and antisera to factor VIII-related antigen, type IV collagen and laminin identify endothelial cells and basement membrane respectively, and have been used to provide a more objective and reproducible means of recognizing vascular invasion (Bettelheim et al, 1984b). A detailed assessment of these techniques is discussed in Chapter IV.7.

Thus, vascular permeation appears to be a local manifestation of biological aggressiveness and pathological invasiveness of a tumour. There is evidence to support the prognostic importance of peritumoral lymphatic and blood vessel invasion in patients with or without axillary nodal metastases. Interobserver and intraobserver differences in interpretation may, however, preclude the use of this parameter as a reliable discriminant on which to base therapeutic decisions.

II.e.   LYMPH NODE METASTASES

Histological evidence of metastatic carcinoma within axillary lymph nodes is currently the most reliable indicator of a subgroup of breast carcinoma patients with a poor prognosis and the most reliable predictor of tumour recurrence (Fisher, 1986; Russo et al, 1987; Carter et al, 1989). As such, the tumour burden in axillary nodes is the major criterion influencing the selection of patients for adjuvant chemotherapy

following definitive surgical treatment.

Approximately 50 per cent of patients who present with breast cancer have histological evidence of lymph node metastases (Fisher, 1986; Carter et al, 1989). Fisher et al (1984) demonstrated that the probability of survival decreased with each additional node found to contain metastatic tumour and, in particular, that patients with one to three positive nodes exhibited significantly worse survival rates than those without nodal metastases, but better survival rates than those with four or more involved nodes. Carter et al (1989) reported similar findings based on an evaluation of data recorded by the National Cancer Institute (USA) on 24740 patients followed for five years. Fisher et al (1984) found that of women treated with mastectomy and axillary lymph node clearance the 10 year DFS was 80 per cent when no axillary node metastases were identified. This DFS fell to 53 per cent when one to three lymph nodes were involved and 29 per cent when four or more nodes contained metastatic tumour. Stratification of the latter group into categories of four to six positive, seven to 12 positive, and greater than 13 positive nodes was prognostically valuable with 10 year survival rates of 41 per cent, 31 per cent and 13 per cent respectively. However, sample sizes in many studies are too small to allow for these further subdivisions. Ten year DFS figures of 72 per cent (node-negative), 33 per cent (one to three positive nodes) and 16 per cent (four or more positive nodes) were obtained in a study of 716 patients by Valagussa et al (1978).

Study of axillary lymph node metastases in relation to clinical outcome of patients with invasive breast carcinoma has contributed to our understanding of the biology of breast cancer. The correlation between lymph node status and both the size of

the primary tumour and patient survival led to the belief that lymph nodes functioned as natural barriers or filters to prevent the dissemination of cancer cells. Breast cancer cells were originally thought to spread in a stepwise progression from the primary tumour site to regional nodes before systemic dissemination. This was the rationale behind the extensive radical mastectomy, introduced by Halsted, which became the standard treatment for breast cancer in the first half of this century (Tagnon, 1986). The value of this mutilating operation was questioned with the advent of comparative clinical trials which showed the radical mastectomy provided no survival advantage over a simple, limited, tumour resection.

Experimentation by Fisher and associates (Fisher, 1980) revealed that tumour cells may disperse through venous channels as well as lymphatic vessels. Lymphatico-venous communications are so abundant that lymph nodes could not therefore be an effective barrier to tumour cell dissemination. Malignant cells can readily pass into the vascular system via lymph nodes, the efferent lymph channels and the thoracic duct or, alternatively, through direct lymphatico-venous connections in the nodes. Furthermore, it was recently demonstrated that there was no difference in survival or recurrence after 10 years of follow-up irrespective of whether involved lymph nodes were left *in situ* or removed (Fisher et al, 1985).

Thus, positive lymph nodes are not a determinant of systemic metastases, but rather an indication of poor prognosis and the entry of cancer cells into the vascular network. Lymph node spread probably occurs at the same time as spread to more distant sites. Although lymph node removal does not alter outcome, it allows an assessment of the presence and extent of tumour dissemination and thereby provides

important prognostic information.

On the other hand, the finding of negative lymph nodes does not necessarily imply that the tumour has been removed prior to dissemination. Regional lymph nodes contain cells capable of destroying tumour cells (Fisher, 1980), or the neoplastic cells may traverse the nodes without leaving deposits. Reports of recurrent disease up to 20 years after the removal of a primary breast carcinoma from a node-negative patient support the current view that breast cancer is probably a systemic disease almost from its inception. Negative lymph nodes may also reflect the existence of tumour-host relationships which may not only prevent growth of tumour in the lymph node, but also inhibit metastases from occurring elsewhere (Fisher, 1980). Moreover, intrinsic factors in the tumour cells as well as in certain organs, particularly lymph nodes, bone marrow, lung and liver, could render these sites the most favourable for the future development of metastases.

The axilla is conventionally divided into three levels. Nodes may be found within each of these levels which are identified according to the relationship of axillary tissue to the pectoralis minor muscle. Tissue lateral (or inferior) to the lower border of pectoralis minor is designated as level I, tissue posterior to (or underlying) the muscle as level II, and tissue medial to the upper border as level III (most apical). Smith et al (1977) found a close correlation between the total number of axillary nodal metastases and the position of the involved nodes. Metastases were generally found to follow an orderly anatomic progression from level I to III.

The extent of axillary node sampling required to provide useful prognostic

information is controversial. Clinical nodal staging is grossly imprecise with up to 40 per cent false-positive or false-negative assessments (Sacre, 1986). Fisher and associates (1981) indicated that the removal of as few as three to five nodes could accurately establish the qualitative axillary nodal status (i.e. positive or negative overall) and identify whether the patient has Stage I or II disease. Most prognostic value, however, is derived from quantitation of the extent of nodal involvement which requires removal of at least 10 nodes. This is generally fulfilled by a dissection of the lower two axillary levels (levels I and II). However, recent demonstrations of "skip" metastases (tumour deposits in upper axillary levels but none in the lowest level) in up to 29 per cent of cases (Danforth et al, 1986; Sacre, 1986) argue for more extensive axillary tail dissections which identify, on average, between 15 and 20 lymph nodes (Morrow et al, 1984).

Clearing of axillary adipose tissue with Carnoy's fixative or 80 per cent alcohol renders the fat translucent and facilitates lymph node dissection. Routine use of this technique is not recommended, however, as although it significantly increases the yield of lymph nodes over formalin fixation, no additional prognostic information is gained as the extra nodes detected are generally very small and not involved with tumour (Morrow et al, 1984; Kingsley et al, 1985; Lewis & Rice, 1988). In addition, many of the smaller lymphoid aggregates identified lack sinuses and are therefore not true nodes.

Fisher et al (1984) reported extracapsular extension of nodal metastases to be an ominous finding when compared with metastatic deposits confined within the node capsule. However, the strong correlation of extracapsular extension with pathological

nodal status diminished the value of the former feature as a prognostic discriminant. Other workers have not found extracapsular extension to be of prognostic value (Dawson et al, 1982).

The size of nodal metastases may also provide useful prognostic information. In one study patients with "micrometastases" of less than two mm in diameter exhibited a more favourable outcome than those patients with deposits greater than two mm in diameter (Fisher et al, 1978). Rosen et al (1981) demonstrated that amongst patients with T_1 primary tumours, those with no axillary metastases had similar survival curves to those with single micrometastases (less than two mm in diameter) after six years follow-up, but when follow-up was extended to 12 years the patients with micrometastases fared significantly worse than the node-negative patients.

Traditionally axillary lymph node metastases are sought by examination of H & E-stained histological sections. While large tumour deposits are readily observed in such preparations, the identification of single cells or small clusters of tumour cells in the nodal subcapsular sinus can be difficult, and distinction from epithelioid histiocytes may be impossible. Between 15 and 20 per cent of patients with node-negative breast cancer die of their disease within five years and it has been suggested that many of these patients are truly Stage II at diagnosis, axillary nodal micrometastases having been "missed" on assessment of conventional H & E-stained sections. The use of monoclonal anti-cytokeratin antibodies to identify these small tumour deposits is examined in Chapter IV.1.

II.f.   HORMONE RECEPTOR STATUS

Steroid hormones act by regulating the differentiation and proliferation of cells which carry high affinity receptors. The hormone dependency of breast cancer relates to the presence of intrinsic steroid receptors in the neoplastic cells. By definition, a "hormone receptor" is a protein which exhibits specific, high affinity and saturable binding to a hormone. This then produces a biological response. Normal breast epithelial cells contain receptors for a number of steroid hormones including estrogens, progestagens, androgens and glucocorticoids, all of which play an important role in the regulation of cellular growth and activity. In normal hormone-responsive tissues there is considerable intercellular heterogeneity of receptor protein expression (Thorpe, 1988).

Nenci et al (1988) have recently proposed an elegant theory endorsing the central role of estrogens as promoters of human breast cancer. This theory is based on evidence that estrogens are responsible for the continued growth of previously transformed mammary epithelial cells. The carcinogenic process begins with induction of a heritably susceptible population of target cells. As a result of this initiation step the altered mammary epithelial cells may express excessive numbers of estrogen receptors (ERs). An essentially normal endocrine environment may then promote the development of hyperplasia due to increased sensitivity of the altered cells to an estrogenic mitogenic stimulus. Permanent expression of ERs in transformed cells will result in persistent proliferation if the favourable environment is maintained, and possible malignant transformation. Excessive exogenous estrogens or prolonged exposure to normal levels of ovarian estrogens (due to early menarche,

late menopause and/or nulliparity) may also promote abnormal proliferation of epithelial cells (Thomas, 1984). ER-rich tumours would therefore be expected to behave most aggressively in an estrogen-rich setting. However, these tumours are most susceptible to hormonal manipulation and administration of the nonsteroidal anti-estrogen agent tamoxifen citrate, which competitively binds to the ER, may block estrogen binding and inhibit further tumour growth.

The complementary DNA of the ER present in the human breast cancer cell line MCF-7 has recently been cloned and sequenced (Green et al, 1986). Remarkably, the predicted ER gene sequence shares strong homology with the *erb*-A gene of the oncogenic avian erythroblastosis virus. The v-*erb*-A gene is expressed as a cytoplasmic, non-oncogenic protein which appears to potentiate the action of the oncogenic v-*erb*-B gene, a gene partially homologous to the epidermal growth factor receptor (EGFR) gene. Thus, ER may induce cellular growth factors (such as EGFR) or may potentiate a human oncogene and precipitate the development of breast cancer.

Lipophilic steroid hormones, including estrogen and progesterone, are distributed within the cytoplasmic and nuclear compartments of the cell. The ER and progesterone receptor (PgR) were previously assumed to reside in the cell cytoplasm and thought to translocate to the nucleus upon binding of hormone. Recent studies with monoclonal antibodies have indicated, however, that all forms of ER and PgR are located in the cell nucleus. There are two types of ER. The first, "free" ER, is not bound to endogenous hormone and is easily extractable from tissue homogenates under low salt conditions. This form is measured in cytosolic ER

radioimmunoassays. The second form of receptor is bound to nuclear chromatin, occupied by endogenous estradiol ("filled") and extractable only with buffers of high ionic strength. This nuclear ER is presumably the biologically active form, directly responsible for hormone-modulated tissue responses and regulation of cell function. The demonstration of a significant correlation between cytosolic and nuclear ER levels indicates that an equilibrium is likely to exist between the two forms (Thorpe, 1988).

Based on evidence that ER values provide useful information regarding the patient's likely response to endocrine manipulation (McGuire et al, 1986; Moot et al, 1987) the ER content of all surgical breast cancer specimens is measured routinely. The overall patient improvement following endocrine treatment, either with additive hormones, anti-estrogens, or with ablative ovarian surgery, is of the order of 30 per cent. Approximately 55 to 65 per cent of patients with ER-rich cancers demonstrate tumour regression, while of those with tumours lacking ER only about 10 per cent show a response (Jensen, et al, 1975; Thorpe and Rose, 1986). Overall response to endocrine therapy can therefore be predicted with an accuracy of about 75 per cent on the basis of ER status. The PgR is an estrogen-dependent protein and its presence in normal and neoplastic epithelial cells indicates an intact estrogen response pathway (Kiang and Kollander, 1987; Thorpe, 1988). Patients with both ER-positive and PgR-positive tumours have the best chance of responding to hormonal therapy (up to 80 per cent), whereas those with tumours positive for only one receptor have a lower response rate (Benner et al, 1988).

The reason why some receptor-negative patients respond to endocrine manipulation and why a substantial proportion of receptor-positive patients fail to improve remains unknown. A study of post-menopausal patients by the Danish Breast Cancer Cooperative Group showed that only those women with high cytosolic ER levels (above 100 fmol/mg cytosol protein) responded to tamoxifen, whereas an arbitrary cut-off point of 10 fmol/mg is generally used to indicate positivity and, therefore, potential therapeutic response (Rose et al, 1985). A level of 10 fmol/mg may include too few ERs for hormonal therapy to be effective. In contrast, several other randomised controlled trials of tamoxifen therapy have identified prolonged disease-free intervals in the treated groups of patients irrespective of ER status (Nolvadex Adjuvant Trial Organization, 1985; Vogel et al, 1987). These data suggest that hormonal manipulation may also benefit receptor-negative patients.

The small group of ER-negative and PgR-negative patients who do improve with hormonal therapy may also reflect inherent inaccuracies of the biochemical assay technique used to determine the receptor status. These include sampling error, variable cell populations within the tumour, technical difficulties or false negatives secondary to high endogenous steroid levels masking the receptor site.

The relationship of ER status to the course of breast cancer and the role of ER status as a prognostic indicator are fraught with controversy, the literature presenting widely variable and conflicting views. Many authors have described increased DFS and overall survival times in women with ER-positive breast tumours (Knight et al, 1977; Allegra et al, 1979; Howell et al, 1984; McGuire et al, 1986; Shek et al, 1987; Kinne et al, 1987; Koenig, 1988; Fisher et al, 1988). McGuire et al (1986)

report that early recurrence and overall survival is significantly influenced by ER status in both node-negative and node-positive patients. Fisher et al (NSABP, 1988) studied 1157 axillary node-negative breast cancer patients who had received no adjuvant systemic therapy and found that those with ER-positive tumours had an eight per cent better DFS and a 10 per cent better overall survival at five years than those with ER-negative cancers. These differences, although slight, were significant and indicate that an ER-negative node-negative patient is twice as likely to die of her disease within five years as her ER-positive counterpart. When combined with ER, the PgR evaluation provided no independent contribution to the prediction of outcome. In a study of 448 breast cancer patients, followed at the Memorial Sloan-Kettering Cancer Center for a median of 75 months, ER levels were found to be significant predictors of DFS independent of other recognized prognostic indicators (Kinne et al, 1987). Similar findings were reported in a recent series of 207 patients with a median follow-up of 27 months (Koenig, 1988).

Howell and coworkers (1984) measured ER levels in 508 patients and found that although there was no difference in the relapse-free survival of groups with receptor-negative and receptor-positive tumours, survival from first relapse was longer in receptor-positive patients, and confined to those women who responded to hormone therapy. Shek et al (1987) reported similar findings and suggested that much of the difference in survival between ER-positive and ER-negative patients could be accounted for by better response to endocrine therapy following relapse. Those patients with ER- positive tumours who failed to respond to therapy experienced a similar survival to ER-negative patients.

The reputed prognostic worth of ER status in breast cancer has been contested, however, by a number of workers (Hilf et al, 1980; McDivitt et al, 1986; Chevallier, 1988; Gelbfish et al, 1988). Hilf and associates (1980) were unable to demonstrate any benefits of ER content analysis in predicting the course of the disease or response to therapy. ER protein data were of no value in predicting tumour recurrence or survival in a subpopulation of 275 node-negative breast cancer patients (Sears et al, 1982) or in 204 stage I and II patients studied by Gelbfish et al (1988). Bloom and Degenshein (1987) also failed to find any difference in the biological behaviour of receptor-negative and receptor-positive tumours from 110 patients who had been treated with surgery alone. These authors point out that in many studies patients had received, in addition to primary surgical treatment, some form of adjuvant therapy (the nature of which was often not specified), thereby invalidating any conclusions proclaiming prognostic value of ER status.

Most centres now routinely use a cytosol assay to measure the PgR content as well as the ER content of all mammary carcinoma specimens when sufficient tissue is available, although the relationship between PgR status and survival is also controversial. Clark and McGuire (Clark & McGuire, 1983; Clark et al, 1983) showed that PgR status was better at predicting DFS in node-positive patients than ER content, and was as important as ER content in predicting overall survival. Howell et al (1984), Gelbfish et al (1988) and Chevallier et al (1988) concurred that PgR status was valuable, but in other studies DFS and overall survival times were unrelated to the presence or absence of PgR (Allegra et al, 1979; Stewart et al, 1983; Sutton et al, 1987; Fisher et al, 1988). Brdar et al (1988) concluded that the combination of ER and PgR is a better indicator of DFS than ER or PgR alone.

Receptor values are mostly concordant (ER-positive/PgR-positive or ER-negative/PgR-negative) in keeping with the theory that PgRs are dependent on the presence of ERs (Horwitz & McGuire, 1975; Giri et al, 1988). The combination ER-negative/PgR-positive (discordant) is very uncommon and may be ascribed to methodological error since all "so-called" PgR-positive but ER-negative tumours by cytosol assay are ER-positive with immunocytochemical stains (Kiang & Kollander 1987). Seventy per cent of ER and PgR estimates were concordant in a study of 1597 women with Stage II (node-positive) breast cancer (Fisher et al, 1987). Life table analyses revealed patients with discordant assays to exhibit a DFS intermediate to the survival of patients with concordant receptor negative or positive tumours. Fisher suggested that discordant receptor status may reflect an aberration of ER metabolism and not a methodological error.

While there appears to be value in determining steroid hormone receptor content as a predicator of likely response to endocrine manipulation (ER negativity probably being a more efficient clinical predicator than ER positivity), the role of ER and PgR as prognostic parameters remains, at best, controversial. The discrepancies in the literature may reflect different laboratory methods used for assaying receptor content and a lack of standardization, limited duration of follow-up in some studies, and/or inclusion of patients who had received adjuvant therapy which could independently affect their clinical outcome. Alternatively, some other confounding variable which remains elusive may be responsible.

Receptor values have been linked to other variables and any association with clinical

outcome may merely reflect such relationships. Most information has been derived from studies of ER only. Many reports describe a highly significant association between the receptor status and the histological grade of IDCs (Santini et al, 1986; Fisher et al, 1987; Reiner et al, 1988; Thorpe, 1988), a higher proportion of ER-positive and PgR-positive neoplasms being observed among well-differentiated tumours than among the more anaplastic malignancies. Other investigators have failed to find such a relationship (Sismondi et al, 1985; Toma et al, 1987; Koenig, 1988). Stegner et al (1986) examined 60 breast cancers ultrastructurally and concluded that the formation of steroid receptor was associated with a high degree of cytoplasmic and nuclear organization, and was independent of histological tumour type. In contrast, receptor-negative tumours were rich in mitochondria and ribosomes, indicating high metabolic activity. Twelve ultrastructural variables could be defined to discriminate receptor-positive from receptor-negative tumours.

Histological type, tumour size and axillary lymph node status have also been related to tumour receptor content and contradictory data have been reported. Invasive lobular, tubular and papillary carcinomas have been found to be ER-positive more frequently than other breast cancer types, whilst the majority of medullary carcinomas were ER-negative (Sismondi et al, 1985; Reiner et al, 1988). These associations were not confirmed by Poulsen et al (1982) or Santini et al (1986).

Although most studies show no correlation between receptor status and the number of involved lymph nodes or the size of the primary tumour (Toma et al, 1987; Koenig, 1988; Reiner et al, 1988), a positive relationship with tumour size has been described by several workers (Kaplan et al, 1985; Thorpe, 1988). The presence of

elastic tissue correlated with ER content, and the lymphocytic reaction was inversely related to receptor status in several studies (Fisher et al, 1987; Reiner et al, 1988). Such discrepancies may relate not only to different methods of ER assay, but also to the variable classifications used to describe histopathological features in different laboratories. Recent work by Smyth et al (1988) established that ER and PgR levels in young (less than 50 years) patients with breast cancer are influenced by the phase of the menstrual cycle, the highest levels being observed in the early proliferative phase and the lowest in the early secretory phase, thus possibly introducing a further confounding variable.

Although ER and PgR values are generally considered to be complementary, some clinical differences are seen. Patient age is found to be associated fairly consistently with ER but not PgR content, the majority of ER-positive cancers occurring in older, or post-menopausal women (Fisher et al, 1987; Toma et al, 1987; Koenig, 1988; Brdar et al, 1988).

The steroid receptor status of breast neoplasms has been studied extensively in relation to other facets of the disease. ER-positive tumours have been linked to a greater likelihood of bony or soft tissue metastases, while visceral and brain metastases reportedly occur more often in patients with ER-negative tumours and are associated with a more malignant course (Lee, 1985). The qualitative ER status of primary and metastatic lesions is generally consistent, although the quantitative value may vary (Toi et al, 1988).

Despite the uncertainty surrounding the efficacy of steroid receptors as prognostic

parameters in breast cancer, it is now common practice to assay for both ER and PgR in breast tumour specimens whenever possible because of the evident benefits in predicting response to hormonal therapy.

Currently most available ER data are based on cytosolic radioimmunoassays utilizing tritiated steroids. Radioimmunoassays are expensive and time consuming to perform and are potentially hazardous. They require a relatively large quantity of tumour tissue (one gram), limiting their application to large biopsies. The use of homogenized tissue inevitably results in sampling errors with potential for false-negative results from dilution of the homogenate by stromal cells, non-neoplastic epithelium and/or necrotic tissue. Furthermore, the cytosolic assay is an indirect method of identifying ERs (Chapter IV.2) and the presence of endogenous hormones or other less-specific estrogen binding sites may interfere with the results. Three types of estrogen binding sites have been described. ERs (type I binding sites) are present in low concentrations in the nucleus and have the highest affinity for estrogen, achieving saturation at 10^{-9}M estradiol (Chamness et al, 1980). Type II binding sites have a lower affinity but a higher capacity for estradiol binding and are a group of different proteins located in the nucleus and cytoplasm that are saturated with estrogen at a concentration of 10^{-7}M. Type III binding sites are the most abundant, occurring in the cell membrane and cytoplasm. They have relatively low affinity for estrogen but their collective capacity is very great and at higher estrogen concentrations binding to these sites may be substantial (Chamness et al, 1980). Some authors have been prompted to question the ability of cytosolic radioimmunoassays to accurately identify a nuclear based ER and have suggested the technique may be predominantly detecting the related, less specific, cytoplasmic type

II and III binding sites (Chamness et al, 1980; Poulsen, 1981).

The recent synthesis of a monoclonal antibody specific for the   ER protein, estrophilin (Greene et al, 1984; King & Greene, 1984), now allows an accurate and direct demonstration of the true ER at a cellular level in cryostat histological sections, aspirates and imprints (King et al, 1985; Raymond & Leong, 1988). The immunocytochemical technique, commercially available as the Abbott Monoclonal ER-ICA Kit, is applicable to very small biopsies and promises to be a more accurate method of assessing tumour ER content. Whereas the ligand binding assays detect only "free" ER, the monoclonal antibody recognizes both "free" receptors and those occupied with endogenous hormone. The immunocytochemical assay has been established by a number of workers as the preferred alternative to the biochemical assay for correlating the responsiveness of mammary carcinoma to endocrine therapy (King et al, 1985; DeSombre et al, 1986; McClelland et al, 1986).

Several anti-ER monoclonal antibodies have been reported to bind to ER-bearing cells in formalin-fixed, paraffin-embedded tissues, but their reliability has yet to be confirmed (Hendler & Yuan, 1985; DeRosa et al, 1987). Other workers have modified the ER-ICA staining procedure and applied it to paraffin sections with variable success (Andersen et al, 1986; Cheng et al, 1988). Details of these immunocytochemical assays are elaborated in Chapter IV.2 and several modified techniques to highlight ERs in imprints, frozen sections and paraffin sections are described.

The PgR cytosolic radioimmunoassay is similar to that used to determine ER content and is susceptible to similar potential methodological inaccuracies. An anti-PgR antibody has only recently been released commercially (Transbio Sarl, Paris, France; Giri et al, 1988), precluding its use in this thesis. Only one other publication describes a PgR immunocytochemical assay, the authors having employed a "home-grown" monoclonal antibody, JZB39 (Pertschuk et al, 1988).

Analyses of ER and PgR in breast cancer tissue have improved our understanding of the biology of this tumour as well as provided a rationale for the selection of optimal therapy. Patients unlikely to respond to endocrine manipulation can be identified and other treatment modalities employed. However, the relationship between receptor status and prognosis remains controversial. This may be the result of non-uniform sampling of tumour tissue, variations in receptor assay methods or differences in patient selection criteria. Thus, hormone receptor status is not an ideal independent prognostic parameter.

II.g. TUMOUR PROLIFERATION

A measurement of tumour cell proliferation, representing an estimate of tumour aggressiveness, is the newest prognostic parameter to be assessed routinely in some centres, and as a research tool in many others. It is possible that this cellular characteristic may provide an explanation for the profound heterogeneity observed in the clinical course of comparably staged patients with identical morphological diagnoses.

Cell Kinetics

The growth of a tumour mass is dependent on the size of the proliferating population, or growth fraction (GF), the cycle transit times of its component cells, these together determining the number of cells produced per unit time, and the rate of loss of proliferating and non-proliferating cells. Cells within the same tumour may spend different periods of time within the various phases of the cell cycle and thus exhibit heterogeneous rates of proliferation.

The cell cycle of plant cells was first described in 1954 by Howard and Pelc (Post & Sklarew, 1985) as consisting of four discrete temporal phases, sequentially nominated the G_1 phase (pre-DNA synthesis gap), the S phase of DNA synthesis, the G_2 phase (post-DNA synthesis gap) and the M phase of mitosis (Fig.II.1). This model of cycling cells passing from one phase to the next was later confirmed by Quastler (1963) to also apply to animal and human cells. Some cell types were found to leave the cycle after a certain number of divisions and differentiate, thus losing their proliferative potential (eg neurones and myocardial cells), while others became temporarily non-proliferative, or dormant, after mitosis. The latter type were designated to be in the G_0 (resting) phase. Quiescent G_0 cells could be induced to re-enter the cycle by an appropriate stimulus. Continuously dividing cells cycled from one mitosis to the next.

The G_1 phase is the most variable period of the cell cycle and the rate of proliferation in normal cell populations is largely dependent upon the duration of this presynthetic gap. Protein and RNA synthesis are necessary for entry into the
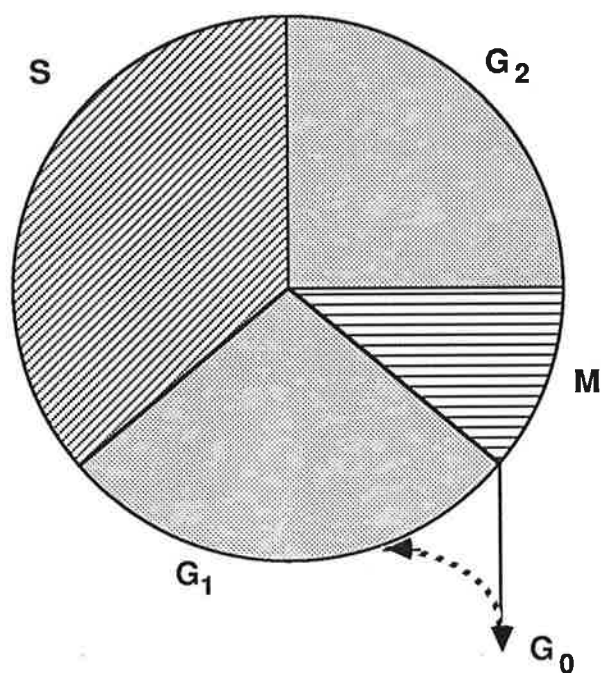
Figure II.1. Phases of the cell cycle.

S phase, the period during which DNA replicates and chromosomal proteins are laid down on newly synthesized DNA. Various studies indicate that the duration of S phase in breast cancer cells is approximately 18 hours, the interval between DNA synthesis and mitosis (G_2 phase) near four to five hours and the duration of mitosis one to two hours. The lengths of the G_o state and G_1 phase are very variable (McGuire & Dressler, 1985).

Neoplastic cellular growth involves the same essential kinetic factors as observed in normal cells (Baserga, 1981), but several important differences are noteworthy. Studies of tritiated-thymidine ($[^3H]$-thymidine) uptake have indicated that the rate of cellular proliferation is slower in many tumours than is observed in their normal counterparts. This is due to prolonged DNA synthesis times, frequent quiescence of cells for days or longer in the post-mitotic (G_o or G_1) phase with variable preparedness to enter S phase and/or cell arrest in the G_2 phase. In addition, the proportion of cells in the DNA synthesis phase is generally smaller in neoplastic populations than in populations of normal cells (Post & Sklarew, 1985). The variable periods of temporary arrest are unique to neoplastic cells and explain the phenomenon of tumour recurrence months to years after an apparent cure, a feature particularly prominent in the natural history of breast cancer.

Tumour Doubling Times

Tumour growth is thus dependent on the excess number of cells produced over the number of cells that die per unit of time, and is not simply a function of the number of mitoses or the length of the cell cycle. Early calculations of tumour doubling

times, based on these latter measurements, were gross underestimations if they failed to take account of spontaneous death of neoplastic cells and the variability of cycle transit times of tumour cell subpopulations (Baserga, 1981), but for several decades these calculations served as estimates of tumour growth rate (Kusama et al, 1972). Serial measurements of the size of palpable primary breast tumours, skin recurrences or metastatic deposits were also performed, or serial mammograms examined (Heuser et al, 1979). Alternatively, doubling times of tumours in tissue culture were measured. Breast neoplasms on average grow more slowly than many other malignancies, with doubling times cited from 30 to 300 or more days (Kusama et al, 1972; Heuser et al, 1979; Cooperman & Hermann, 1984).

It is well accepted that approximately 30 population doublings are required for a tumour to attain a diameter of one cm (10^9 cells), the smallest size at which most breast cancers may be palpable (Fisher et al, 1984). Biologically, detection even at 0.5 cm diameter (27 doublings) is considered relatively late in the lifespan of a tumour which will cause death of the host with only 10 to 20 more doublings. Tumour cells may be present up to 17 years before detection and up to 50 per cent of patients with tumours one cm in diameter at diagnosis are estimated to harbour occult metastases which may appear as recurrent disease many years later (Fisher et al, 1984). Extensive tumour necrosis or unexplained prolonged dormant phases may further slow tumour growth.

Mitotic Index, Tritiated-Thymidine Uptake and Flow Cytometry Studies

Measurement of tumour doubling time has limited practical application. The ideal

method for measuring tumour cell proliferation for use in prognostication should be accurately quantifiable, rapidly performed, capable of being automated, and have a defined relationship to known cell kinetic parameters and to clinical outcome. Traditional methods which reflect kinetic activity include determination of the mitotic index (MI; Hoffman, 1949) and measurement of the fraction of tumour cells in the DNA synthetic phase of the cell cycle, either by autoradiographic detection of [^{3}H]-thymidine uptake (Meyer & Bauer, 1975) or by DNA flow cytometry (Meyer, 1982).

The most direct measurement of cell proliferation is a count of the percentage of mitotically active neoplastic cells. This is relatively simple and can be performed on routine histological sections, but is very tedious and, due to the limited number of cells in this phase compared with the longer duration of the S phase, frequently inaccurate. The MI of most breast carcinomas is extremely low and an accurate measurement could require a count of up to 50000 cells (McGuire & Dressler, 1985). Delays in fixation of three hours have been reported to result in a reduction of the MI by up to 85 per cent (Graem & Helweg-Larsen, 1979), introducing a further source of error.

The thymidine-labelling index (TLI), which is a measure of the fraction of neoplastic cells in the S phase of the cell cycle, has been used as a correlate of tumour growth. Fresh biopsy specimens of less than one mm in thickness are incubated with [^{3}H]-thymidine, a specific DNA precursor which is incorporated into DNA, and the percentage of labelled cells estimated by subsequent autoradiography (Tubiana et al, 1981). Autoradiographic exposure for seven days is necessary for accurate TLI

measurements on human tissues. The resultant autoradiographs resemble ordinary tissue sections in which S phase cells are marked by silver grains over their nuclei. The labelling indices of breast carcinoma cells are approximately 40 times greater than the MIs and can be measured by counting 2000 nuclei, requiring approximately one hour (Tubiana et al, 1981).

DNA flow cytometry also provides a semiquantitative measure of the percentage of cells in the S phase. It is a rapid, semi-automated method of DNA analysis in which tumour cells are stained with a DNA-specific fluorochrome solution. Solid tumour tissue is disaggregated into a single cell suspension, usually by enzymatic techniques, and stained fresh, after frozen storage or after retrieval from paraffin-embedded blocks (Hedley et al, 1983; Quirke & Dyson, 1986). Alternatively the technique can be performed using fine needle aspiration specimens. The cell suspension is injected into the flow cell of the flow cytometer where stained cells are excited by a high energy light source to emit discrete fluorescent pulses. The intensities of the fluorescent pulses are analysed by computer and processed into histograms. S phase cells can be discriminated as they have more DNA, and hence more fluorescence, than G_1 cells, but less DNA and fluorescence than premitotic (G_2) or mitotic cells. Flow cytometric results may be available very rapidly as no period of photographic exposure is required and cells can be counted at rates of over 100/second electronically (Meyer, 1984). However, structural relationships are lost in the single cell suspension and the analysis must always be controlled by microscopic inspection of histological reference slides to differentiate between neoplastic and normal inflammatory cells. As expected, the S phase fraction (SPF) derived from DNA flow cytometry correlates closely with the results obtained by [3H]-thymidine labelling.

McDivitt et al (1984) demonstrated a correlation of 0.847 (p = 0.0001) between the SPF and the TLI in a population of breast cancer cells.

All of these techniques, however, are laborious, require expensive equipment, are difficult to perform in a routine diagnostic laboratory and have inherent inaccuracies. In addition, measurements of the TLI are dependent upon radioactive substrates. The labelling indices (TLI and SPF) only detect those cells engaged in DNA synthesis at a given time and give no indication of the fate of cells once they enter S phase or the proportion of cells arrested in G_o, G_1 or G_2 phases. Thus, the data must always be interpreted with circumspection.

Relationships Between Traditional Cell Kinetic Measurements and Clinical and Pathological Variables

Despite the shortcomings described above, most of the reported relationships between breast cancer cell kinetics and clinical outcome have relied upon TLIs and flow cytometric measurements of SPF. Many studies with these techniques have shown an inverse relationship between tumour proliferative activity and the relapse-free intervals and overall survival times of breast carcinoma patients (Meyer & Hixon, 1979; Gentili, 1981; Meyer et al, 1983; Tubiana et al, 1984; Silvestrini et al, 1985; Hedley et al, 1987). The TLI provides prognostic information independent of age, primary tumour size, axillary lymph node involvement and ER status, such that the higher the LI the poorer the prognosis (Meyer & Lee, 1980; Silvestrini, 1985; Meyer, 1986). Silvestrini et al (1985) measured the TLI in 258 patients without distant or axillary nodal metastases and found those patients with

high labelling indices had a DFS of only 59.6 per cent compared with 80.5 per cent for the group with low TLIs. The TLI was a predictor of survival in premenopausal as well as in postmenopausal patients, regardless of the post-relapse treatment. Similar data were obtained in a prospective study by Tubiana and associates (1981) of 128 patients of all stages with six years follow-up. They demonstrated significant inverse correlations between the TLI and both the DFS and overall survival times, such that patients with a TLI of less than 0.25 per cent had less than half of the relapses and deaths of the group with a TLI greater than 3.8 per cent. Further follow-up at 10 years confirmed DFS and total survival to be significantly longer for patients with a low TLI (Tubiana et al, 1984). These results were independent of tumour stage, primary tumour size, axillary lymph node status, the presence of an inflammatory reaction and hormonal status. However, the TLI was significantly correlated with the mitotic component of the histological grade, also an indicator of tumour proliferative activity, and when the results were adjusted for histological grade the prognostic significance of the LI was no longer apparent. This would suggest that expensive analyses of the TLI of the tumour are of no more value in assessing tumour proliferative potential than estimates of histological grade.

Meyer et al (1983), in a study of 278 breast cancer patients with no detectable distant metastases, demonstrated that the probability of relapse was dramatically higher in those patients with a TLI above the median value (of 4.55 per cent), irrespective of tumour stage, lymph node involvement, ER content and menopausal status. However, high TLIs were associated with poorly-differentiated tumours and high nuclear grade. Nodal status and the TLI were equally strong independent predictors of early relapse, whereas the predictive value of ER content was

dependent on its inverse relationship with the TLI. ER was related to probability of relapse in the below median TLI group only. A subgroup of node-negative patients with a high relapse-expectancy (50 per cent at four years) could be selected on the basis of TLI values.

The TLI also correlates with some tumour histological characteristics. Meyer et al (1986) found that poorly- differentiated tumours and those with a prominent inflammatory response or necrosis had a higher mean TLI. They confirmed, in a study of 757 invasive breast tumours, a lack of relationship between the TLI and axillary lymph node status. No relationship with vascular invasion or histological type was observed. Although an inverse correlation between tumour GF and ER status had been reported by a number of groups (Kute et al, 1981; Meyer, 1984; Silvestrini et al, 1985), Meyer et al (1986) concluded that the TLI could not be predicted strictly on the basis of receptor status. In general, GFs of cancers from younger, premenopausal patients were significantly higher than those of cancers from postmenopausal patients (Gentili et al, 1981; Meyer et al, 1986).

Similar relationships between the SPF (determined by flow cytometry) and the clinical course of breast cancer on the one hand, and various histopathological prognostic factors such as receptor status and histological grade on the other, have been identified. A low SPF predicted an increased time to relapse and overall survival, and was associated with well-differentiated and ER-positive tumours in one study by Meyer and coworkers (1980). Hedley et al (1987) found that SPFs of less than or equal to 10 per cent were related to significantly longer DFSs than SPFs of

greater than 10 per cent, in an analysis of 285 DNA histograms. High tumour GFs (greater than 10 per cent) were strongly correlated with high tumour grade, but only weakly associated with nodal, hormone receptor and menopausal status. Moran et al (1984) measured the SPF in 76 malignant breast tumours and described a range of values from less than one per cent to 37.4 per cent. The SPF was significantly higher in poorly-differentiated ductal carcinomas, ER-negative tumours, medullary carcinomas and metastatic tumour deposits. The SPF was not related to the number of axillary nodal metastases.

Flow cytometry data for 700 breast tumours identified a considerably higher SPF in tumours lacking ER and PgR when compared with tumours positive for both receptors (McGuire et al, 1986). Similar findings were reported by Feichter et al (1988) who identified a significant correlation between DNA measurements and hormone receptor status. The latter authors found no associations between SPF, tumour size and axillary nodal status.

A major problem encountered when interpreting flow cytometry and tritiated thymidine data is the lack of a standardized definition as to what constitutes a "high" or a "low" SPF or TLI. This may, in part, be due to differences in staining, in preparative procedures and in the degree of background correction. As such, considerable variations in absolute values of SPF and TLI are recorded, perhaps accounting for some of the discrepant results published by various workers (McGuire & Dressler, 1985; Feichter et al, 1988).

DNA Aneuploidy

Flow cytometric analysis offers, in addition to kinetic analysis, an estimate of total DNA content and a measurement of DNA ploidy, or chromosome content. DNA aneuploidy, defined as the presence of an abnormal DNA stem line, is identified as at least two separate G_0/G_1 peaks. The non germ-line peaks reflect the chromosome number of the abnormal cells (Meyer, 1984; Quirke & Dyson, 1986). DNA aneuploidy may be subclassified as hyperdiploid, hypodiploid, tetraploid and multiploid, and expressed as a DNA index (DI) representing the modal tumour cell DNA content divided by the modal normal cell DNA content. When the DNA content of neoplastic cells is equivalent to that of benign cells the DNA index is 1.0 (diploid), whereas a nuclear DNA index of between 1.0 and 2.0 indicates a hyperdiploid cell population (Meyer, 1984). Most tumours either have a near diploid or triploid-tetraploid mode, while a few have two distinct aneuploid populations (multiploidy). DNA content may also be measured using microdensitometry of Feulgen-stained paraffin sections, however this method is highly labour intensive and does not appear to have the resolving power of flow cytometry (Hedley et al, 1983).

DNA aneuploidy is a well-recognized feature of human solid tumours and appears to reflect aggressive tumour behaviour. Depending on the technique used DNA aneuploidy has been reported in 50 to 90 per cent of breast cancers and is generally independent of tumour stage, size and nodal status. Associations between tumour ploidy and steroid receptor status have been described, with near diploid tumours tending to be ER-positive and receptor-negative tumours more frequently aneuploid (Dressler et al, 1988). This association did not achieve statistical significance in

some studies (Friedlander et al, 1984; Quirke & Dyson, 1986), while others failed to identify any relationship between ploidy and receptor status in breast cancers (Kuo et al, 1985). Dressler et al (1988) measured the DNA content of 1184 breast tumours and detected aneuploidy in 57 per cent. Of 346 lymph node-negative patients, followed for a median of 45 months, those with aneuploid tumours had the worst prognosis and the highest risk of recurrence, regardless of the SPF. The disease-free interval was shorter in patients with aneuploid tumours compared with those with diploid tumours. In the diploid group a high SPF predicted a worse prognosis. Other workers have reported similar findings (Meyer, 1984; Moran et al, 1984; McGuire et al, 1986; Feichter et al, 1988).

Hedley et al (1987) demonstrated a correlation between aneuploidy and involvement of four or more axillary nodes, postmenopausal status, ER negativity, high tumour grade and SPFs of greater than 10 per cent. DFS and overall survival for these patients was worse than for those with diploid tumours, but DNA aneuploidy did not have independent prognostic significance when allowance was made for its correlation with the other prognostic features. Cornelisse et al (1987) also reported relationships between ploidy and current prognostic parameters, and found DNA aneuploidy to be an independent indicator of poor prognosis in the subgroup of postmenopausal patients.

No correlation between DI and the extent of nodal metastases was found by Moran et al (1984), although a high DI was associated with poorly-differentiated ductal carcinomas and receptor-negative status. DNA aneuploidy did not worsen survival of a subgroup of 135 "poor prognosis" breast cancer patients in a recent study by

Suzuki and Koike (1988). A single report examined the cellular DNA content of premalignant breast lesions and preinvasive carcinomas and suggested that aneuploidy may be of value in identifying those preinvasive lesions most likely to behave aggressively (Carpenter et al, 1987).

Thus, although some reports suggest that aneuploidy is related to receptor status and several clinicopathological prognostic parameters, there remains considerable controversy with regard to the prognostic significance of the presence and degree of various ploidy abnormalities.

Ki-67 Immunostaining

The technical problems and inherent limitations of the tritiated thymidine uptake and flow cytometric methods of assessing cell turnover have prompted the development of alternative techniques based on detecting the proportion of cells labelled by markers preferentially expressed on proliferating cells. The most promising of these markers is the monoclonal antibody named Ki-67 which is available commercially as DAKO-PC (Dakopatts, Denmark). Ki-67 recognizes a nuclear antigen expressed by cells in all phases of continuous division (G_1, S, G_2 and M phases), but absent in the G_0 (resting) phase. The antigen may be demonstrated using a simple, rapid, reproducible and specific immunocytochemical assay in frozen sections (Chapter IV.3). The percentage of tumour cells decorated by this antibody may be counted (Ki-67 count) and represents the tumour GF. The immunoreactions may also be analysed with a computerized system, such as SAMBA, which permits a multiparametric and automated analysis of coloured images (Charpin et al, 1988).

Workers utilizing Ki-67 to measure GF have reported values in accordance with those obtained by other methods of assessment (Gerdes et al, 1984a; Lellé et al, 1987; Baisch & Gerdes, 1987).

McGurrin et al (1987), Barnard et al (1987) and Lellé et al (1987) applied the Ki-67 antibody to series' of breast cancers and demonstrated correlations with MI, histological type, histological grade and patient menopausal status, confirming earlier correlations based upon the SPF of cycling cells. However, data regarding the relationships between Ki-67 counts and both ER and lymph node status are inconsistent (McGurrin et al, 1987; Barnard et al, 1987; Lellé et al, 1987, Gerdes et al, 1987).

Ki-67 counts were determined for the 115 malignant breast neoplasms in this study. Details of the staining technique and the relationships between GF and various histopathological and clinical parameters are provided in Chapter IV.3.

Novel Techniques of Assessing Tumour Growth Fraction

Various other methods of determining the tumour GF have been proposed. These are less-well-established and include immunohistochemical bromodeoxyuridine staining, detection of transferrin receptor (TfR) on proliferating cells and staining for nucleolar organizer regions (NORs). Bromodeoxyuridine is a thymidine analogue which is incorporated into DNA-synthesizing nuclei. It may be identified and quantitated in tissue sections immunohistochemically following proteolytic digestion, using monoclonal anti-bromodeoxyuridine antibody (Sugihara et al, 1986; Morstyn

et al, 1986; Veronese et al, 1989) and the results are comparable to those obtained using [^{3}H]-thymidine analysis. Although the procedure is much less cumbersome than autoradiography, bromodeoxyuridine staining still measures only those cells in the S phase of the cell cycle. Benign and malignant proliferating cells express TfR, but its utility as a marker of tumour proliferative activity is limited by its expression in non-neoplastic macrophages (Habeshaw et al, 1983).

The recently-developed silver (Ag) impregnation technique to identify NORs has been applied to breast neoplasms in two previous studies (Smith & Crocker, 1988; Giri et al, 1989). Malignant breast lesions demonstrated significantly greater numbers of AgNORs per tumour cell nucleus than benign breast epithelial cells (Smith & Crocker, 1988). A previously unreported relationship between AgNOR counts and Ki-67 counts in malignant breast neoplasms is described in Chapter IV.5.

A number of enzymes that are strictly related to DNA synthesis increase in activity during the S phase. Biochemical measurements of thymidine kinase isoenzymes may reflect cellular proliferative potential (Baserga, 1981), although this technique is not widely used.

Several other monoclonal antibodies have been reported to detect proliferating cells in cryostat or paraffin sections. These include an antibody to human DNA polymerase α (pol α); an antibody to an enzyme present in the nucleus in G_1, S and G_2 phases (Namikawa et al, 1987) and detectable only in frozen sections; C_5F_{10}, which identifies dividing cells in normal and neoplastic tissues in formalin-fixed paraffin-embedded sections (Lloyd et al, 1985) and an antibody to proliferating cell

nuclear antigen (PCNA), also known as cyclin (Mathews et al, 1984; Robbins et al, 1987). PCNA is a cell cycle-related nuclear protein that is maximally elevated in the late G_1 and S phases of proliferating cells. It has been identified in paraffin sections of various solid human malignancies and has been found to correlate with mitotic activity and tumour histological grade. All of these antibodies are recognized using an avidin-biotin complex (ABC) immunocytochemical technique, but none are commercially available as yet so that their value as potential prognostic markers remains anecdotal.

Growth Fraction and Response to Adjuvant Therapy

It has been suggested that indices of cell proliferation may provide an indication of the likely response of a patient to adjuvant therapy. Cell sensitivity to radiation or chemotherapy is well known to vary throughout the cell cycle. These agents temporarily inhibit the progression of proliferating cells at selective stages of the cycle, thus changing the proportion of cells in each phase and producing a cycling variation of the sensitivity of surviving cells (Post & Sklarew, 1985). Despite many years of experience with chemotherapy relatively little information on the effects of these agents upon human cancer cell proliferation is available, and consistent relationships between drug-induced cytokinetic alterations and improved clinical course have not been established (Post & Sklarew, 1985). In general terms, tissue damage triggers a recruitment of previously quiescent (G_o) cells into the proliferative pool so only those tumours with a large cell turnover are successfully controlled by adjuvant therapy. This is consistent with the observation that more rapidly growing tumours appear to respond better to drug therapy (Charlson & Feinstein, 1982). In

a study of patients with disseminated breast carcinoma by Sulkes et al (1979) the LI was significantly higher in 11 responders to combination chemotherapy (mean LI 15 per cent) than in 14 non-responders (mean LI 7.1 per cent)   The predictive ability of LI was independent of other pretreatment prognostic variables examined. In a controlled trial of the effect of adjuvant chemotherapy in patients with node-negative, ER-negative breast cancer the LI was the most accurate discriminator of benefit (Bonnadonna et al, 1986).

In summary, morphological evaluation of breast neoplasms, by means of histological grading and a count of mitoses, provides some insight into tumour proliferative activity but is generally not sufficiently objective or quantitative to aid in cancer prognosis.   While the proliferative rate of a tumour may not necessarily be a determinant of its metastatic capability, a high GF, as determined by the TLI or SPF, is generally believed to be an important predictor of early relapse independent of other traditional prognostic variables. Significant correlations between GF and the histological grade and hormone receptor content have been described. Proliferative indices may also be useful in predicting the likely benefit of adjuvant therapy, whereby tumours with a large GF show a significantly better response than those with a low GF (Sulkes et al, 1979).

Autoradiographic and flow cytometric techniques for measuring tumour GF are not available in many pathology laboratories. Ki-67 immunostaining offers a simple alternative which may be more widely applicable to routine surgical practice.

However, the procedure requires fresh frozen tissue and, as yet, insufficient follow-up time has elapsed to correlate Ki-67 counts directly with survival. Researchers investigating Ki-67 and other new proliferative markers are thus reliant on comparisons with parameters of some known prognostic value if material for retrospective analysis is lacking.

A high degree of correlation has been demonstrated between results obtained by autoradiography, flow cytometry, Ki-67 counts and AgNOR counts, but this does not necessarily imply that all are measuring the same process or proliferative determinant. It remains to be seen which of these markers is a true discriminator of outcome. At present, in the absence of any documented advantages, the choice of method to assess tumour GF is most likely to depend upon the ease of performance.

II.h.   MISCELLANEOUS FACTORS

A great many other pathological features have been implicated by various authors as prognostically useful in breast carcinoma (Alderson et al, 1971; Fisher et al, 1975 & 1984; Rosen et al, 1981; Roses et al, 1982). Those features which have most frequently been related to survival include the intensity and quality of the cellular infiltrate associated with the primary tumour (lymphocytic, lymphoplasmacytic, histiocytic or other), the presence of perineural invasion, stromal desmoplasia and/or elastosis, tumour necrosis, nipple involvement, the site of the primary tumour and the type of tumour border. None of these parameters were

shown to be reliable predictors of recurrence in two large studies (Roses et al, 1982; Fisher et al, 1984).

More recent work has indicated that an evaluation of the immunophenotype of lymphoid cell infiltrates in breast carcinomas may provide prognostic information, although reports of those subtypes of lymphocytes associated with poor outcome are conflicting (An et al, 1987; Underwood et al, 1987). In general, however, larger numbers of lymphocytes and macrophages were apparent in malignant compared with benign tumours, and in particular in poorly differentiated carcinomas (Zuk & Walker, 1987). Von Kleist et al (1987) found that T helper/inducer cells predominated over T suppressor/cytotoxic cells in neoplastic tissues, whereas in benign tissues the ratio of T cell types was well balanced. No medullary carcinomas were included in their series of 117 cases.

The type of immunological response in axillary lymph nodes, as a representation of host resistance to local neoplasia, has also been related to prognosis. Early work suggested that a lymphocyte predominant response (including sinus histiocytosis) was associated with a more favourable outcome (Hartveit, 1982), germinal centre predominance (or an unstimulated pattern) resulted in intermediate survival, and lymphocyte depletion in a poor outcome (Cutler et al, 1963; Tsakraklides et al, 1974). However, Fisher et al (1984) found that only a pattern of germinal centre response in regional lymph nodes adversely influenced DFS in a group of patients without nodal metastases (Stage I).

Several studies have indicated that the patient's age and menopausal status at

diagnosis are useful prognostic indicators, such that survival is significantly shorter in patients aged less than 35 years, even when other favourable prognostic factors are present (Dawson et al, 1986; Falkson et al, 1986). These results are now considered controversial and any prognostic advantage of age may relate to the differential ER status observed in different age groups (Daniell, 1988).

In one multi-institutional study it was concluded that race is an important prognostic variable, with DFS and overall survival rates significantly worse for black patients compared with white patients (Crowe et al, 1986). This prognostic disadvantage held true when survival rates were controlled for ER status, the ER-negative black subset of patients experiencing the worst prognosis.

Breast thermography has recently been reported to be a novel clinical prognostic indicator for patients with breast cancer (Isard et al, 1988). Thermographic stage was assessed in 70 patients and a scoring system indicated a shorter survival at five and 10 years follow-up for those with poor thermographic prognostic scores (asymmetry of vascular shadows, dilated hot veins, excessive focal or global heat and loss of normal breast contour). The rationale for this study is based on the observation that slow-growing cancers have minimal circulatory changes while rapidly growing lesions are accompanied by marked increases in metabolic heat production and blood flow. Further investigation in this area is warranted, although the predictive value of this parameter was not absolute and interobserver reproducibility in the interpretation of the thermal images is likely to be a problem.

One large group of markers which have gained recent attention as possible predictors

of prognosis are the proto-oncogenes. These are a family of heritable genes, normally present in the human genome, which regulate cell growth and development and were identified on the basis of their similarity to genetic sequences with known tumorigenic or transforming potential. The level of expression of several oncogenes is reported to be higher in a number of human tumours than in the corresponding normal tissues (Trent, 1985). Activation of proto-oncogenes may occur as a result of gene amplification, chromosomal rearrangement, point mutation or by the insertion of promoter DNA from another gene. The consequent oncogene protein products may be structurally altered or produced in excess and thus may play a role in malignant transformation. It has been postulated that the products of some activated cellular oncogenes may be either growth factors or their specific receptors and as such, tumours expressing several of these genes may be able to grow with autocrine stimulation (Israel & Band, 1984).

Gene amplification eventually exerts its effect through protein overexpression and therefore an estimate of gene expression may be obtained by several methods. These include a direct measure of the gene copy number (by DNA isolation and Southern blotting), extraction of RNA and Northern blotting, or measurement of the amount of protein product in a Western blot analysis or in tissue sections using immunoperoxidase techniques (Gusterson et al, 1988; Walker et al, 1989). Immunoperoxidase staining can be readily employed in routine laboratories and allows an assessment of tumour heterogeneity, however, a knowledge of the oncogene product is required and problems of loss of antigen reactivity related to fixation and paraffin embedding must be overcome (Slamon et al, 1989). In addition, oncogene mRNA may be determined by *in situ* hybridization (Walker et al, 1989).

The detection of certain abnormal or enhanced genes and/or gene products in breast cancers may identify those tumours which are particularly aggressive and likely to show early recurrence or treatment failure. However, the reported frequency and level of amplification of a number of different proto-oncogenes varies considerably between different series of patients.

One proto-oncogene which has been identified in multiple copies in up to 30 per cent of breast cancers is HER-2/*neu*, also known as c-*erb*B-2. This gene is present on chromosome 17 and encodes a transmembrane glycoprotein which is structurally similar to the EGFR. A number of workers have reported that amplification of this gene correlates with the presence of lymph node metastases and with a poor prognosis in breast cancer patients (Zhou et al, 1987; Slamon et al, 1987 & 1989; Fontaine et al, 1988; Wright et al, 1989; Tsuda et al, 1989). Others, however, found no independent relationship with tumour recurrence, survival or other recognized prognostic markers (Barnes et al, 1988; Gusterson et al, 1988; Ali et al, 1988; van de Vijver et al, 1988a). Van de Vijver et al (1988a) observed that all *in situ* ductal carcinomas showing HER-2/*neu* membrane staining had a comedo-type histological pattern and suggested that *neu* overexpression may represent an early step in the development of a distinct histological type of breast carcinoma.

Although the discrepant findings may, at least in part, be attributable to different methods of assessing gene expression (Slamon et al, 1989), there remains considerable controversy with regard to the reliability of HER-2/*neu* amplification in predicting survival.

Several other genetic alterations have been described in breast carcinoma, some of which have been associated with decreased survival. The *ras* gene family encodes for a group of closely related 21000 dalton proteins termed p21 (Walker & Wilkinson, 1988). Experimental models have strongly implicated the *ras* p21 protein in the induction of metastases (Hill et al, 1988). Point mutation of the *ras* oncogene, leading to a transforming gene, is infrequent in breast cancer, but an increased frequency of rare alleles of the gene and the allelic deletion of c-Ha-*ras*-1 have been reported (Thor et al, 1986; Theillet et al, 1986; van de Vijver et al, 1988b) and related to tumour aggressiveness in one study (Theillet et al, 1986). *Ras* p21 expression may be determined using monoclonal antibodies to p21 and an immunoperoxidase technique on paraffin sections. Two studies failed to identify any relationship between high expression of p21 protein and current prognostic parameters (Walker & Wilkinson, 1988; Czerniak et al, 1989), while others found high Ha-*ras* expression to be associated with lymph node metastases (Lundy et al, 1986; Agnantis et al, 1986; Querzoli et al, 1988). Several authors described equal staining with p21 antibodies (RAP-5 and Y13-259) in benign and malignant breast epithelium and claimed that p21 protein is a feature of certain normal cell types (Ghosh et al, 1986; Candlish et al, 1986; Walker & Wilkinson, 1988).

Amplification of c-*myc* and various *myc* rearrangements have also been reported in breast tumours but, while they may be associated with the development of breast carcinoma, there is little evidence to support a relationship to prognosis (Escot et al, 1986; Bonilla et al, 1988; Tsuda et al, 1989). Further studies with large series of patients are required to more clearly define the clinical significance of the many

genetic alterations apparent in primary breast carcinomas.

Finally, numerous articles have been published indicating the potential prognostic value of a wide variety of antibodies which reaction to varying degrees with antigens expressed by human mammary tissues. Markers implicated as prognostically significant in breast carcinoma include alpha-lactalbumin (ALA), human placental lactogen (HPL), pregnancy-specific β_1-glycoprotein (SP1), ABH isoantigens, carcino-embryonic antigen (CEA), lectins, alpha and beta HCG, NCRC II, lactoferrin, transferrin, ferritin and prolactin. Some of these will be discussed further in Chapter IV.6. In addition, a plethora of reports describe antibodies which were produced in experimental laboratories and are not commercially available (Kufe et al, 1987; Kim et al, 1988). Although their proponents may claim prognostic usefulness of these markers, the lack of widespread availability detracts from their value as experimental results cannot be verified in multicentre trials or in other laboratories. It is recognized that antibodies presently accepted as useful were all initially only research tools, but very few of the total number heralded as "new prognostic parameters" actually reach the marketplace. It is for this reason that only readily available commercial antibodies were employed in this study of prognostic markers in human breast cancer.

II.i.   CONCLUSIONS

Breast cancers can be divided into aggressive tumours exhibiting early relapse and rapid growth despite maximal therapy, and tumours which grow more slowly, only to

relapse unpredictably up to 20 years after the initial diagnosis. No single pathological parameter can reliably predict the future behaviour of a newly diagnosed breast carcinoma or indicate the most appropriate treatment modality.

The identification of axillary lymph node metastases is currently the most useful predictor of relapse in women with breast cancer, and is the basis of selection of patients for adjuvant chemotherapy in most oncology centres. Regional lymph nodes are of biological rather than mechanical significance, borne out by the fact that more aggressive regional therapy results in improved loco-regional control, but has no effect on overall survival. Knowledge of the ER status provides a good indication of the likelihood of tumour regression following hormonal manipulation, but the relationship between ER status and survival remains unclear.

Numerous other pathological features have been found to correlate with tumour virulence, the most valuable of these being histological type, histological grade, primary tumour size, and the presence of peritumoral vascular (lymphatic or blood vessel) invasion, all of which may be assessed from the routine surgical specimen. Some histological markers, however, such as vascular invasion and extranodal extension of metastases, are closely related to nodal status diminishing any independent prognostic value.

An assessment of tumour GF is also of value but requires specialized techniques. The methods most applicable to routine surgical pathology practise are discussed in detail in Chapters IV.3 & IV.5.

A combination of the pathological parameters described offers the greatest potential for predicting time to recurrence or death and assisting in treatment planning for individual patients. However, the assessment of many of these parameters is subjective, hindering accurate and reproducible results. The most useful future prognostic parameters must surely be those which may be measured objectively, thereby overcoming some of the real problems of interobserver and intraobserver irreproducibility. This study assesses a range of this latter group of readily quantifiable parameters.

CHAPTER III.   MATERIALS AND METHODS

1. MATERIALS

2. METHODS

III.1. MATERIALS

III.1. MATERIALS

Fresh breast tissue, essential for many of the techniques employed in this study, is routinely received at the IMVS from the adjacent Royal Adelaide Hospital (RAH) for frozen section diagnosis. Where possible, clinical data were obtained from case records.

Fresh tumour was received from 115 women who had undergone excision biopsy, total mastectomy or modified radical mastectomy, with or without axillary dissection, for primary breast cancer between 1st January 1987 and 31st December 1988. The patients with malignant disease were aged between 35 and 86 years, with a mean age of 64 years. The age groups were divided at 55 years to serve as a substitute for the patients' menopausal status as this was not known in all cases. Twenty-three patients were "pre-menopausal" and 84 patients "post-menopausal". In eight cases the patient's age was unknown. Clinical follow-up was necessarily limited during this two year prospective study.

For each case in the study H & E-stained sections from all of the routinely processed paraffin blocks (section III.2.c.) were reviewed. The characteristics of the primary malignant tumours (n = 115), including the corresponding axillary lymph node status, are summarized in Table III.1. There were 107 IDCs (including two metastatic deposits in axillary nodes and a primary carcinoma of the male breast), three infiltrating lobular carcinomas, a pure intraductal carcinoma, a tubular carcinoma, a mucinous carcinoma, a carcinosarcoma exhibiting osteosarcoma and

TABLE III.1.

PRIMARY TUMOUR AND LYMPH NODE CHARACTERISTICS

FOR 115 CASES

Tumour diameter in cm	**0 – 1.9**	**2 – 4.9**	**≥ 5**	**NA**
	30(26.0)[1]	74(64.3)	7(6.1)	4

Histological grade of IDCs	**I**	**II**	**III**	**NA**[2]
	29(27.6)	64(60.9)	12(11.4)	10

Positive lymph nodes	**0**	**1–3**	**≥ 4**	**NA**
	38(33.0)	31(27.0)	20(17.4)	26

ER status (cytosolic; positive ≥10 fmol/mg protein)	**Positive**	**Negative**	**NA**
	75(65.2)	35(30.4)	5

NA = Not assessed
IDC = Infiltrating ductal carcinoma

[1]Percentage of 115 cases in parentheses

[2]Includes a tubular, intraductal, mucinous and three lobular carcinomas, a carcinosarcoma, a phyllodes tumour, a male breast IDC and a metastatic IDC.

chondrosarcoma and a malignant phyllodes tumour (World Health Organization, 1982).

Primary tumour size, represented by the maximum tumour diameter measured on the gross specimen, was subdivided into three major groups following the International TNM staging system (Chapters I & IIa). Thirty (26 per cent) tumours were less than two cm in maximum diameter (T_1), 74 (64.3 per cent) ranged between two and five cm in diameter (T_2), and in only seven cases (6.1 per cent) the diameter of the primary tumour was greater than or equal to five cm (T_3). In four cases the primary tumour size was unknown.

The IDCs were graded histologically according to the criteria established by Bloom and Richardson (1957) based on the degree of nuclear anaplasia and tubular differentiation, and the number of mitotic figures. One IDC could not be graded as only the metastatic deposit was available for assessment. Male breast IDCs traditionally are not graded (Bezwoda et al, 1987) and the single example was excluded from statistical comparisons involving histological grade. Twenty-nine IDCs (27.6 per cent) were Grade I, 64 (60.9 per cent) were Grade II and 12 (11.4 per cent) were Grade III (Table III.1). It is noted that the proportion of Grade III carcinomas in this series is somewhat smaller than has been reported previously (Bloom & Richardson, 1957), but there was no selection bias and all cases were graded independently by the author and the pathologist reporting the case. In the event of disagreement the slides were reviewed and a consensus reached.

Lymph node involvement was divided into three categories: negative lymph nodes,

cases in which one to three of the nodes excised contained metastases, and those in which four or more lymph nodes contained tumour. Thirty-eight cases were lymph node-negative (33 per cent), one to three nodes were positive in 31 cases (27 per cent), and in 20 cases (17.4 per cent) four or more nodes contained metastatic tumour (Table III.1). In 26 cases axillary sampling had not been performed.

As a routine service each tumour was analysed for ER content using a dextran-coated charcoal cytosolic assay performed by Dr. David Horsfall at the Flinders Medical Centre, Adelaide, S.A. Tumours were , by convention, considered positive when estrogen binding levels were greater than or equal to 10 femtomoles (fmol)/mg of cytosolic protein (see Chapter IV.2). Seventy-five of 110 tumours (68 per cent) were ER-positive by this technique.

In addition, 12 benign fresh breast samples were obtained as mammoplasty or biopsy specimens and comprised six examples of normal breast epithelium, four fibroadenomas and two cases of fibrocystic change.

III.2. METHODS

a. **INTRODUCTION**

b. **AVIDIN-BIOTIN COMPLEX (ABC)**
   STAINING TECHNIQUE

c. **TISSUES EXAMINED**

   - **fresh frozen**

   - **paraffin-embedded**

   - **controls**

d. **ASSESSMENT OF STAINING**

e. **STATISTICAL METHODS**

III.2. METHODS

III.2.a. INTRODUCTION

Immunocytochemistry is defined as the use of immunological techniques for biochemical analysis of cells and tissues. The concept that enzymes could be used in place of fluorochromes to label antibodies prompted the expansion of this technology to the field of histopathological diagnosis. While fluorescent antibody methods were useful in some areas of pathology, for example in the study of renal disease, the poor morphological detail obtained with the limited-resolution fluorescence microscope, the requirement for fresh tissue, and the need for photography to record the results before the fluorescence faded, prevented widespread application of this technique in diagnostic pathology. By substituting the antibody label fluorescein-isothiocyanate with the enzyme horseradish peroxidase a wide range of specific cellular antigens and tumour products could be readily identified in tissue sections. Horseradish peroxidase, in the presence of hydrogen peroxide (H_2O_2), interacts with a chromogenic substrate, usually diaminobenzidine (DAB), which polymerizes to form an insoluble coloured reaction product. The product is deposited at the site of the antigen/antibody reaction and is visible by ordinary light microscopy (Lewis et al, 1983). This technique provides a permanent record and may be performed on paraffin-embedded as well as fresh tissue sections.

The simplest immunoperoxidase procedure is a one-step method in which the primary antiserum is directly labelled with peroxidase (Figure III.1), however, this
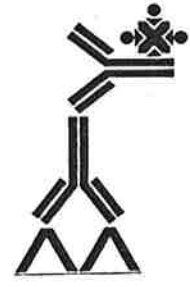
Figure III.1. Immunoperoxidase Staining Techniques

      a.   Direct Method
      b.   Indirect Method
      c.   Peroxidase-Antiperoxidase (PAP) Method
      d.   Avidin-Biotin Complex (ABC) Method

method is not particularly sensitive and is only suitable when adequate quantities of antisera are available for labelling. In the indirect ("sandwich") method the secondary antibody is peroxidase-labelled and is directed against the immunoglobulin of the species in which the first antibody is raised (Figure III.1). This method is slightly more sensitive than the direct method and utilizes readily available anti-species antibodies (Heyderman, 1979).

In 1970, Sternberger et al. introduced the peroxidase-antiperoxidase (PAP) method which employs unlabelled primary and secondary antibodies and a PAP reagent. The secondary antibody acts as a bridge between the primary antibody and the PAP complex, the latter two being raised in the same animal species (Figure III.1.). The same PAP complex may thus be utilized with many different primary antibodies. This technique is much more sensitive than the two earlier methods as an average of 2.4 molecules of peroxidase are attached to each molecule of tissue antigen and identified by chromogen staining.

The three-step technique is described in brief. After deparaffinization tissue sections are exposed to hydrogen peroxide solution which quenches endogenous peroxidase activity (predominantly present in red blood cells). The sections are then incubated with normal (non-immune) serum to suppress non-specific binding of immunoglobulin molecules to collagen. Primary antibody against the antigen of interest is then applied, followed by secondary antibody directed against immunoglobulin specific to the primary antibody species. This linking antibody is added in excess, ensuring one of the two antigen-binding sites remains free to combine with the third step reagent, the PAP complex. Following the addition of

DAB and H_2O_2 the antigen of interest is identified by brown pigment at the site of the DAB-peroxidase reaction (Lewis et al, 1983).

The PAP method, while still widely used, has been replaced in many laboratories by a technique developed by Hsu et al (1981) to amplify the antigen/antibody reaction. This newer avidin-biotin-complex (ABC) technique (Figure III.1) is based upon an unusually high affinity interaction between avidin and biotin, and represents a simplification in that the ABC reagent is universal and the need for a PAP complex especially prepared for each different animal species is eliminated. It is also much more sensitive than the PAP method and is not limited to 2.4 peroxidase-binding sites per antigen. The ABC technique, utilized in this study, is described in greater detail in the next section.

Since the advent of hybridoma technology a wide range of high affinity and specific monoclonal antibodies have been raised against various antigens including hormones, immunoglobulins, proteins, enzymes, oncofetal antigens, viruses and bacteria. Immunohistological analysis has secured an important place as an aid to diagnosis and classification of surgical biopsy material. Immunoperoxidase technology has also opened a new field for research pathologists whereby the identification and quantitation of certain tumour cell products or antigens in histological sections may be related to prognosis. Such research, however, has limited practical application if the antibodies employed are not readily available to the diagnostic pathologist. For this reason only commercially available reagents of documented specificity were examined in this project.

III.2.b AVIDIN-BIOTIN-COMPLEX (ABC) STAINING TECHNIQUE

A modified version of the ABC method of Hsu et al (1980) is employed in the immunoperoxidase laboratory at the IMVS (Leong, 1986) and was used in this study. The low molecular weight vitamin, biotin, forms an essentially irreversible union at four binding sites on the egg-white glycoprotein, avidin, of molecular weight (MW) 68000 (dissociation constant 10^{-15}; Figure III.1). A wide range of molecules, including antibodies and enzymes, can be conjugated to biotin without significant loss of biological activity. Many molecules of biotin can be coupled to the peroxidase molecule. Avidin serves as a bridge between biotin-labelled peroxidase molecules, and the biotin-peroxidase links molecules of avidin, thus forming a large three-dimensional, lattice-like complex. Avidin is added in excess and free biotin-binding sites within the latticework are attracted to sites of biotinylated secondary antibody (Figure III.1). This amplification step results in a technique estimated to be 20 to 40 times more sensitive than the PAP method (Hsu et al, 1980).

Two major problems associated with the chemical properties of avidin limit the utility of ABC immunoperoxidase systems. Avidin is highly positively-charged at neutral pH and may thus nonspecifically bind negatively-charged molecules (such as nucleic acids and phospholipids) resulting in high background labelling, and the carbohydrate component of this glycoprotein may also react with molecules such as lectins. Use of streptavidin, a related protein of MW 60000 which has been isolated from *Streptomyces avidinii*, overcomes these deficiencies. Streptavidin is effectively

identical to egg-white avidin but has an isoelectric point close to neutral pH and contains no carbohydrate moieties (Chaiet & Wolf, 1964; Shi et al, 1988). Consequently, sections stained using the streptavidin-biotin-peroxidase complex demonstrate reactivity equivalent to those employing the original ABC reagent, but with greatly reduced non-specific binding. Streptavidin was used in the ABC techniques described in the following chapter.

Appendices I and II detail the methodology employed. In brief, following fixation, sections were incubated with primary antibodies at various dilutions (Table III.2) in a humidified chamber, for one hour in the case of frozen sections and overnight for paraffin sections. The sections were then treated with appropriate biotin-labelled secondary antibodies (raised in mouse, goat, rat or rabbit), diluted with three per cent non-immune horse serum to 1:250, for 30 minutes. This was followed by incubation with ABC reagent for 60 minutes. Finally, DAB in H_2O_2 and Tris HCl buffer was applied for five to 10 minutes while controlled macroscopically and microscopically. The specific biotinylated secondary antibodies and digestion techniques are described in the relevant chapters.

All secondary biotinylated antibodies were purchased from Vector Laboratories, Burlingame, California, USA. The ABC reagent was obtained as streptavidin-biotin-horseradish peroxidase complex from Amersham International, Buckinghamshire, England, UK.

The ABC method shows increased specificity as well as sensitivity over the PAP method. An excess of biotinylated secondary antibody is not necessary in the ABC

TABLE III.2.

ANTIBODIES APPLIED TO FROZEN AND PARAFFIN SECTIONS

ANTIBODIES	SOURCE[1] (animal[2])	DILUTION	COMMENTS[3]
ER	Abbott Laboratories (rat)	1:2	Monoclonal. Requires fresh frozen tissue
Ki-67	Dakopatts (M)	1:40	Monoclonal. Requires fresh frozen tissue
CAM 5.2	Becton-Dickinson (M)	1:50	Monoclonal. Mw 39-50 kd, Digestion
AE1/AE3	Hybritech (M)	1:400	Monoclonal. Mw 40-67 kd, Digestion
Anti-EMA[4]	Sera-Lab (goat)	1:2000	Polyclonal
Vimentin	Dakopatts (M)	1:250	Monoclonal
α-lactalbumin	Nordic (R)	1:4000	Polyclonal
SP1[5]	Dakopatts (R)	1:3000	Polyclonal
Prolactin	Dakopatts (R)	1:4000	Polyclonal
Ferritin	Dakopatts (R)	1:12000	Polyclonal
Factor VIII	Dakopatts (R)	1:6000	Polyclonal, Digestion
Laminin	E-Y Laboratories (R)	1:750	Polyclonal, Digestion
Type IV collagen	Eurodiagnostics (R)	1:800	Polyclonal, Digestion
Actin	Amersham (M)	1:8000	Monoclonal IgM
MSA[6]	EnzoBiochem (M)	1:375	Monoclonal

[1] - Abbott Laboratories, Wiesbaden-Delkenheim, West Germany
  - Dakopatts, Copenhagen, Denmark
  - Becton-Dickinson, California, USA
  - Hybritech, California, USA
  - Sera-Lab, Sussex, England
  - Nordic Laboratories, Tilburg, Netherlands
  - E-Y Laboratories, San Mateo, California, USA
  - Eurodiagnostics, Apeldoorn, The Netherlands
  - Amersham, Buckinghamshire, England
  - EnzoBiochem, New York, USA

[2] Animal source of antibody production - M = mouse;   R = Rabbit

[3] All antibodies of IgG type unless otherwise stated

[4] Anti-Epithelial Membrane Antigen

[5] Pregnancy-specific β_1-glycoprotein

[6] Muscle specific actin

method compared with the relative excess of bridging antibody required to react with the PAP complex. As such, highly diluted secondary as well as highly diluted primary antibody may be used, with a resultant increase in method specificity, minimization of background staining and considerable cost-saving (Milios & Leong, 1988).

III.2.c. TISSUES EXAMINED

Fresh frozen tissue

Representative samples of each fresh breast specimen were covered in O.C.T. embedding medium (Tissue Tek, Cat. No: 4583, Miles Laboratory, Illinois, USA) and snap frozen in liquid nitrogen within 15 to 30 minutes of excision. The tissue samples chosen were as similar as possible in macroscopic appearance to the representative blocks taken for paraffin sections. H & E-stained cryostat and paraffin sections were compared to identify any histological heterogeneity which could produce inconsistent or inaccurate results. The tissue was stored for up to 12 months at -20°C.

Cases were batched for immunoperoxidase staining. Consecutive six-micron cryostat sections (Ames Cryostat II, Miles Laboratories) were thaw-mounted onto tissue adhesive-treated slides (Rentrop et al, 1986). Sections to be stained with Ki-67 (Table III.2) were air-dried overnight prior to fixation in acetone at 22°C for 10 minutes, followed by two 30-second exposures to microwave irradiation (Chapter

IV.3). Those slides for ER content analysis were immediately fixed in periodate-lysine-paraformaldehyde (PLP) at 22°C for 10 minutes (Chapter IV.2). The modified ABC technique was then employed (Appendix II; Chapter III.2.b).

Paraffin-embedded tissue

The paraffin-embedded tissue blocks had been routinely processed following dissection and sampling by the reporting pathologist. In the IMVS histopathology laboratory all breast blocks are fixed by microwave irradiation in a domestic microwave oven (700 Watts, Sharp Model No. R9570, Carousel) to 62°C in Normal Saline, and processed through absolute alcohol and chloroform before embedding in paraffin wax in an autoprocessor (Shandon Hypercenter) on an overnight (12 hour) cycle (Leong et al, 1985). The only exposure to formalin was for less than one hour during transportation to the laboratory.

Five-micron serial sections were prepared and stained with the antisera listed in Table III.2 using the modified ABC method (Appendix I). In addition, one section was stained with H & E. Work from this laboratory has previously demonstrated that fixation by microwave irradiation results in optimal preservation of most cellular antigens when compared with routine formalin fixation (Leong et al, 1988a). Paraffin sections were also impregnated with silver to identify NORs within epithelial nuclei (Chapter IV. 5).

In 32 cases, additional blocks of tumour were selected for fixation in either 10 per cent neutral-buffered formalin or Bouin's fixative for one and a half hours at 22°C,

following a report that such fixatives were optimal for preservation of estrophilin in paraffin sections (DeRosa et al, 1987). These blocks were not exposed to microwave irradiation and were used in developing a technique to identify ERs in paraffin-embedded sections (Chapter IV.2.g).

Controls

Known positive controls were included for all antisera in each immunocytochemical "run". Cytospins of MCF-7 ER-positive cultured tumour cells, provided with the Abbott ER-ICA kit, were used to verify performance of the anti-ER antibody. In addition, cryostat sections of one known ER-positive tumour from our files were stained and evaluated in terms of both intensity and extent of positivity. Sections of a highly proliferative IDC were included as standard positive controls for the Ki-67 antibody. In-built positive controls for vimentin were present in all sections examined. Specific positive control sections were included, as applicable, for antisera to the following antigens: cytokeratins - mesothelioma, alpha-lactalbumin (ALA) - lactating breast, pregnancy-specific β_1-glycoprotein (SP1) - placenta, ferritin - spleen, prolactin - prolactin-secreting pituitary adenoma, factor VIII-associated antigen and UEA I -lymph node vessels, muscle-specific actin (MSA) and actin - leiomyoma, and type IV collagen and laminin - epitheliosis of the breast. If the positive control failed to stain, or if the staining was too faint to easily differentiate positive and negative cells, the run was repeated.

Although abolition of positive staining by use of an antibody preabsorbed with the antigen under study is considered the most reliable negative control, this is often

not practical and was deemed unwarranted in this study which utilized only commercial antibodies of proven specificity. Negative control staining was obtained by substituting the primary antibody with non-immune horse serum (NHS) or with an antibody of irrelevant specificity for which the tissue should be non-reactive.

An H & E-stained frozen and/or paraffin section from each case was reviewed to exclude necrosis or other causes of false positive immunoperoxidase staining.

III.2.d. ASSESSMENT OF STAINING

Interpretation of immunoperoxidase staining requires familiarity and experience with the characteristics of a positive reaction, and an awareness of the individual nature of the antibody used. A knowledge of the tissues with which the antibody may react and of the optimal methods of antigen preservation is essential.

True positivity was recognized by distinct brown granular staining with a heterogeneous distribution within single cells and throughout the tissue. For the majority of antisera utilized in this study staining was localized in the cytoplasm of epithelial cells. The exceptions were anti-epithelial membrane antigen (EMA) which was identified as staining of cell surface membranes, and the distinctive nuclear staining produced by anti-ER and Ki-67 in cryostat sections. A quantitative assessment of the degree of positive staining was performed for each antiserum examined without knowledge of the clinical or histopathological parameters for the given case.

The percentages of Ki-67 and ER-positive cells were determined by counting 400 to 500 morphologically malignant cells, or, in the case of benign breast tissue, benign epithelial cells. Stromal cells were not included in the count. Multiple non-overlapping, random fields, covering representative areas of the tumour, were examined with a X40 objective using a cross-hatched Whipple grid divided into one hundred squares and mounted in a X10 microscopic eyepiece. Only those cells lying predominantly within the nine squares in each corner and the nine central squares (i.e. 45 squares in total) were counted (Garcia et al, 1987). The percentages of Ki-67 and ER-positive cells were termed Ki-67 and ER counts respectively. In tumours demonstrating a large intraductal component, as well as diffuse infiltration, counts were performed for each pattern.

The intensity of nuclear ER staining varied and, as this may reflect the quantity of hormone receptor within a cell, a subjective intensity grading from 0 (no staining) to + + + + (very strong staining) was assigned to each case, with a mean value being estimated for cases with variable staining intensity (Chapter IV.2).

When assessing ALA, SP1 and prolactin antisera, satisfactory results were obtained by estimating the degree of staining using a low power (X4 or X10) objective. Each case was assigned to one of four categories according to the percentage of positive cells, where 0 = no staining reaction; 1 = less than 33 per cent cells positive; 2 = 33 to 66 per cent cells positive and 3 = greater than 66 per cent cells positive. An alternative method of categorization was utilized to assess vimentin staining of epithelial cells and is detailed in Chapter IV.4.

Misinterpretation of immunoperoxidase-stained tissues may be the result of interpretative or technological errors. Melanin and haemosiderin pigments may cause difficulty, but are generally readily differentiated from the brown granules of DAB by their different texture and colour, as well as their presence on the control slide. Problematic background staining may be observed when necrotic tissue, which non-specifically absorbs antibodies, is present. Similarly, non-specific binding to connective tissues and collagen may be encountered despite pre-incubation of sections with a high concentration protein solution (eg normal/non-immune serum), especially with concentrated primary antisera. Such binding tends to be paler, more diffuse and more evenly distributed than true positive staining and, with experience, can generally be recognized. Passive absorption or active phagocytosis of antigens by histiocyte-type cells may produce the false impression that such cells are immunoreactive. Similarly, non-specific artefactual uptake of antisera by the free edges of histological sections (the so-called "edge phenomenon") may be confusing.

It is vital that tissue is appropriately fixed to ensure preservation of immunohistochemically detectable antigen and avoidance of false negative results due to "lost" antigen. The ideal fixative varies for different antigens and alternative fixation techniques utilized in this study are detailed in the appropriate sections of Chapter IV. Fresh tissue was fixed or frozen promptly to avoid drying out, another potential source of erroneous or false negative results. Similarly, care was taken to ensure sections were not left to dry during the staining process. Exposure of tissue to formaldehyde produces covalent bonding which may mask antigenic sites, hampering immunohistochemical localization of antigen. Pretreatment with various

proteolytic enzymes (most commonly trypsin) has been used to expose antigenic sites, however, this step is extremely capricious and must be tailored to the individual enzyme, tissue fixation conditions and antibody. Variable activity of protease enzymes may be encountered among different commercial suppliers. Inadequate treatment will not reveal buried intracellular targets and excessive digestion will destroy cell structure to the extent that interpretation may be impossible. The identification of the "correct" amount of digestion is made on purely empirical grounds.

Failure to block endogenous peroxidase activity with methanol H_2O_2 may lead to false positive staining, especially if large amounts of blood are present in the tissue. Some antigens are sensitive to methanol and hence it may be necessary to use an alternative enzyme, such as alkaline phosphatase, in place of peroxidase.

Finally, avidin may potentially bind to endogenous biotin, a vitamin and coenzyme found in many mammalian tissues. Such false positives are most likely in the assessment of liver and renal sections and the problem does not generally arise when staining other tissues.

With experience, misinterpretation of immunoperoxidase stains is unlikely, however, recognition of the many potential sources of error emphasizes the essential role of appropriate controls performed in tandem with the sections under investigation.

III.2.e. STATISTICAL METHODS

Spearman's rank order correlation coefficient (r) method, Mann-Whitney U tests, unpaired t-tests (t) and Chi-squared tests were employed, using the software package StatView 512+ (Brainpower Inc., Calabasas, California) on a Macintosh SE computer, to analyse the relationships between Ki-67 counts, ER counts, AgNOR counts and vimentin staining, and the association, if any, between these variables and the tumour histological grade, axillary lymph node status, primary tumour size and patient age. The differences between Ki-67 counts, vimentin staining and AgNOR counts in benign and malignant breast epithelium were also examined. P values of less than 0.05 were considered significant.

CHAPTER IV. PROGNOSTIC PARAMETERS STUDIED

1. LYMPH NODE MICROMETASTASES

2. IMMUNOHISTOCHEMICAL ESTROGEN RECEPTOR ANALYSIS

3. TUMOUR GROWTH FRACTION

4. INTERMEDIATE FILAMENT PROTEIN ANALYSIS

5. SILVER NUCLEOLAR ORGANIZER REGIONS (AgNORs)

6. OTHER TRADITIONAL PROTEIN MARKERS OF BREAST CARCINOMA

7. IMMUNOHISTOCHEMICAL ASSESSMENT OF VASCULAR INVASION

8. ASSESSMENT OF TUMOUR INVASION USING ANTIBODIES TO BASEMENT MEMBRANE ANTIGENS AND MYOEPITHELIAL CELL ANTIGENS

IV.1. LYMPH NODE MICROMETASTASES

a. INTRODUCTION

b. METHODOLOGY

c. RESULTS

d. DISCUSSION

IV.1. LYMPH NODE MICROMETASTASES

IV.1.a. INTRODUCTION

Between 15 and 20 per cent of breast cancer patients deemed to be axillary lymph node-negative on routine histopathological examination, and therefore with an anticipated favourable outcome, die of their disease within five years, and an additional 10 per cent die within the subsequent five years (Fisher et al, 1978a). This observation highlights the possible role of occult metastatic disease in contributing to the mortality of a subgroup of patients with Stage I breast cancer at presentation.

There is consensus that survival rates in breast cancer are inversely related to the number of axillary nodes containing tumour deposits (Chapter II.e). However, there is controversy concerning the prognostic role of micrometastases, or minute "occult" metastases, which in the past have been detected primarily by serially sectioning paraffin-embedded lymph nodes. Using this technique, Pickren (1961) showed that the survival rate for patients with occult metastases, identified in 22 per cent of 51 presumed node-negative cases, was similar to that for patients with no metastases. Huvos et al (1971), in a study of 227 patients with an eight year follow-up, suggested that micrometastases less than two mm in diameter in level III axillary lymph nodes influenced survival in that clinical outcome was similar to those cases with level I gross or macrometastases. However, micrometastases in level I nodes were not prognostically significant. Attiyeh et al (1977) followed 105 patients from the same

sample for 14 years and confirmed these results.

In a study of 565 cases, Fisher et al (1978b) concluded that there was no survival difference at four years between cases with micrometastases (less than two mm in diameter) and truly node-negative cases, however, patients with micrometastases experienced higher treatment failure rates. Treatment failure was defined as the appearance of local, regional or distant metastases, or death due to disease, while survival encompassed all patients living free of disease or with recurrence. Fisher and associates therefore suggested that patients with micrometastases measuring less than or equal to 1.3 mm in diameter be designated to have Stage $1\frac{1}{2}$ disease in view of their excellent prognosis. Fisher et al (1978a) also step-sectioned axillary nodes from 78 cases at five-micron intervals and found metastases in 24 per cent (19) of cases. No difference in five year survival could be found between the truly node-negative group and those who had tumour identified on resectioning.

Conflicting data were presented by Rosen et al (1981) who demonstrated a clear prognostic disadvantage of micrometastases (less than two mm in diameter) after follow-up of 147 women with T_1 tumours for 12 years, although after only six years of follow-up the survival curves of these patients were similar to those of T_1 patients with negative nodes. The diameters of the micrometastases were measured on photocopy images of lymph node sections, a technique initially described by Huvos et al (1971).

It has been considered that the detection of micrometastases is "purely academic" (Fisher et al, 1978a). While metastases greater than two mm in diameter have been

accepted as clinically significant, this dimension is only an arbitrary figure. One might argue that such lesions develop from smaller deposits, i.e. micrometastases, which are more readily able to traverse lymphatic vessels unobstructed from the primary tumour to the draining axillary lymph nodes. This study attempts to detect occult axillary node micrometastases by immunocytochemistry, thereby delineating a subgroup of patients for further study.

Only five previous reports utilize immunocytochemical techniques to detect axillary node metastases in breast cancer patients. Workers at the Institute of Cancer Research, Surrey, UK, were the first to describe a glycoprotein present on epithelial surfaces and luminal membranes in benign and neoplastic human tissues which they named epithelial membrane antigen (EMA). The antigen is recognized by antisera raised against delipidated human milk-fat-globule membrane antigens (Heyderman et al, 1979). Sloane et al (1980) utilized this polyclonal antiserum to EMA to confirm the presence of histologically diagnosed metastatic disease in the liver and lymph nodes. Anti-EMA antisera are now commercially available in various monoclonal and polyclonal forms.

Wells et al (1984) demonstrated metastatic tumour in seven of 45 (15 per cent) axillary node-negative cases of breast carcinoma by applying monoclonal antibodies to EMA and KL1 (an anti-keratin antibody raised against human epidermis and prepared in the authors' laboratory). Micrometastases were detected in four (33 per cent) of the 12 infiltrating lobular carcinomas included in the series. Metastatic deposits from lobular carcinomas might be anticipated to be even more easily overlooked in H & E sections than those from ductal carcinomas because of their

small cell size, poor cohesiveness, and resemblance to histiocytes. A more recent study examined 767 lymph nodes from 50 cases of node-negative infiltrating lobular carcinoma with antisera to EMA, HMFG-2 (Human milk-fat-globule-2), and a monoclonal antibody against 54 kd keratin. Metastatic cells were detected in 24 per cent of cases of this tumour subtype (Bussolati et al, 1986), however, EMA and HMFG-2-positive macrophages were observed in the nodes of six cases. Similar findings were described by Trojani et al (1987 a & b) who examined both infiltrating lobular carcinomas and IDCs. In addition, antisera to EMA have been used to identify breast cancer cells in up to 28 per cent of bone marrow smears when metastases had not been revealed in conventionally stained smears (Sloane et al, 1980; Redding et al, 1983).

The above studies utilized predominantly antisera to EMA, but it is now recognized that anti-EMA is a less sensitive marker than anti-cytokeratins for the detection of epithelial cells (Pinkus et al, 1986; Thomas & Battifora, 1987) and has recently been reported to also decorate plasma cells and lymphocytes (Delsol et al, 1984) as well as macrophages (Bussolati, 1986). No studies, to date, have correlated the presence of immunocytochemically detected "occult" metastases with long-term (greater than five years) clinical follow-up data.

The cytokeratins are a multigene family of polypeptides (MW 40 to 68 kd) which form part of the cytoskeleton of epithelial cells. Positive staining with cytokeratin antibodies can be found in all carcinomas, regardless of the primary tumour site or degree of differentiation of the tumour cells. Moll et al (1982) identified at least 19 different human polypeptides that may be categorized into two broad groups, those

of higher molecular weight (generally epidermal-derived) and those of lower molecular weight (predominantly internal organ-derived). Carcinomas derived from different types of epithelium express distinctive profiles of cytokeratins that are characteristic of their epithelial cells of origin. Cytokeratins displayed in metastases are identical to those in the primary tumour (Leong, 1986a).

Previous authors have relied on non-commercial antibodies to cytokeratins or less specific antisera to EMA. In this study two readily available commercial monoclonal antibodies, of proven specificity and worth (Battifora, 1988), were utilized to ensure easily reproducible data, and the benefits of such markers over standard histological procedures were evaluated (Raymond & Leong, 1989a).

IV.1.b. METHODOLOGY

The tissue blocks of all lymph nodes from 55 cases of breast carcinoma with axillary dissections (modified radical mastectomies) were retrieved from the files of the IMVS. These represented cases diagnosed in the three years following January, 1986, which had been reported as node-negative, and included some of the cases detailed in Chapter III. Assessment had been based on the light microscopic examination of a single H & E-stained section of each node by a qualified pathologist. All nodes had been fixed by microwave irradiation, then processed in alcohol and chloroform before embedding in paraffin wax, as described in Chapter III.2.c. (Leong et al, 1985).

Further sections from each node were cut, without trimming, and then stained with monoclonal antibodies to lower molecular weight cytokeratins (CAM 5.2) and to a broad spectrum of cytokeratins (AE1/AE3). Initially anti-EMA was also employed, however, after preliminary studies of six cases the use of this polyclonal antiserum was discontinued. The specificities and dilutions of the antibodies are given in Table IV.1.1. The commercial sources are detailed in Table III.2.

A modified ABC technique was performed as detailed in Chapter III.2.b and Appendix I. Prior to staining with AE1/AE3 and CAM 5.2, the sections were digested with trypsin II (Sigma T-8128, St Louis, Missouri, USA) at 0.5 mg/ml for three minutes. Following incubation with the primary antibody overnight at 4°C, biotinylated secondary antibody was applied for 30 minutes at 22°C, ABC reagent for 60 minutes at 22°C and DAB for 15 to 20 minutes. Lillie-Mayer's haematoxylin was used as a counterstain. Positive and negative controls were included as described in Chapter III.2.c. Brown granular cytoplasmic and/or membrane staining in cytologically malignant cells identified metastatic tumour. The metastases in all nodes were measured with an eyepiece micrometer and the number of cells in the plane of section of each deposit were counted. Original H & E sections of the resected lymph nodes were reviewed in conjunction with the peroxidase-stained sections.

"Occult metastasis" was defined as "tumour not previously identified on examination of the original H & E section". T-tests were employed to analyse the relationships between the presence of occult metastases and the location (quadrant), diameter, type and histological grade of the primary tumour.

TABLE IV.1.1.

ANTIBODY	SPECIFICITY	DILUTION
AE1/AE3	MW. 40-67 kd (Tseng et al, 1982; Cooper et al, 1985)	1/400
CAM 5.2	MW. 39, 43 & 50 kd (Moll et al, 1982; Makin et al, 1984)	1/50
anti-EMA*	Human milk-fat-globule membrane antigen (Heyderman et al, 1979)	1/2000

* Anti-Epithelial Membrane Antigen

IV.1.c. RESULTS

The 55 cases of mammary carcinoma comprised 49 IDCs, two pure intraductal
carcinomas, two cases of medullary carcinoma, one lobular carcinoma and one pure
tubular carcinoma. Of the 49 ductal carcinomas, 21 (43 per cent) were well-
differentiated (Grade I), 24 (49 per cent) were moderately-differentiated (Grade II)
and four (eight per cent) were anaplastic (Grade III). Between one and 27 axillary
lymph nodes were found per case (with a mean of 12.9), there being a total of 709
nodes in the 55 cases. These were examined on 439 slides, with an average of eight
slides per case.

Occult metastases were found in immunoperoxidase-stained sections of 15 nodes
from 12 cases (21.8 per cent). All cases exhibiting occult metastases were IDCs. No
more than two nodes in a single case contained tumour cells (Table IV.1.2). The
immunoperoxidase-stained tumour cells were readily detected with a X4 objective
(X10 eyepiece). Both AE1/AE3 and CAM 5.2 showed equally distinct cytoplasmic
and membranous staining. Staining for EMA was performed in six cases but was
discontinued when considerable background staining and non-specific decoration of
plasma cells was found to interfere with interpretation of the results.

The majority of the deposits were identified in the subcapsular sinus or in capsular
lymphatic spaces (Figure IV.1.1), and in one instance there was extranodal extension
of metastatic tumour (Figure IV.1.2). Three nodes also contained tumour within the

TABLE IV.1.2

CHARACTERISTICS OF 12 CASES OF DUCTAL CARCINOMA

WITH "MISSED" METASTASES

TUMOUR DIAMETER (cm)	SITE	GRADE[a]	N° OF NODES EXAMINED	N° OF NODES INVOLVED	INTRANODAL SITE	SIZE OF METASTASES (average no. of cells)
0.8	—	I	6	2	SC	40, 5
1.5	UO	II	11	1	SC	7
1.5	—	II	27	1	P	6
1.8	UO	I	8	1	P/SC*	200
2.0	SA	I	14	1	SC	6
2.0	—	I	7	1	P/SC	6
2.0	UO	II	16	2	SC	15, 5
2.5	UI	II	12	1	SC	30
3.0	UI	II	24	1	SC	50
3.3	UO	III	8	2	SC	20, 2
3.5	UO	I	1	1	P/SC	100
—	UO	ID	21	1	SC	5

a - Bloom and Richardson grade (1957)   SC - subcapsular sinus
UI - upper inner quadrant   P   - parenchymal
UO - upper outer quadrant   *   - extends outside capsule
SA - subareolar   —   - not available
ID - intraductal
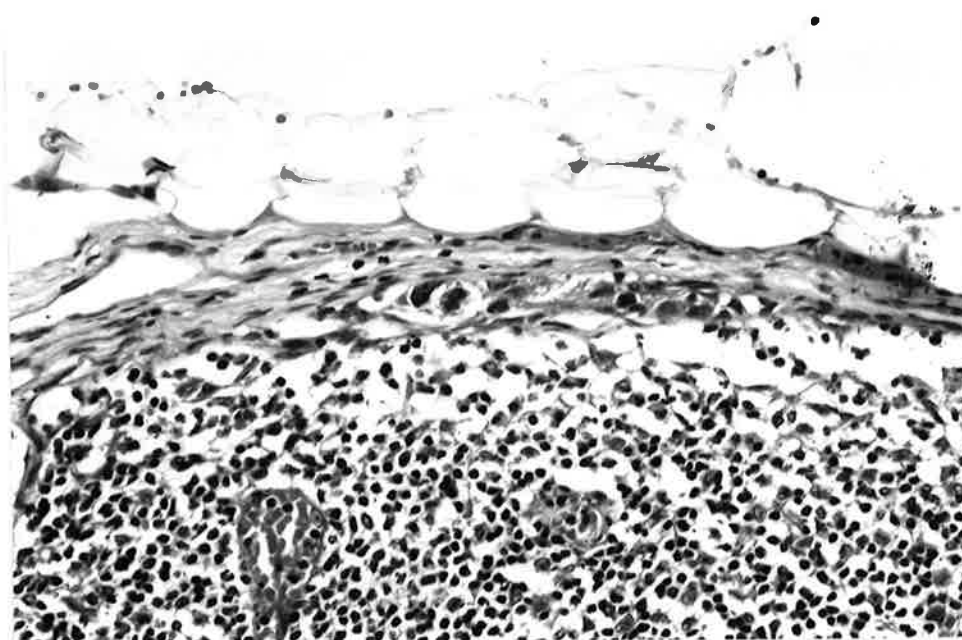
a.
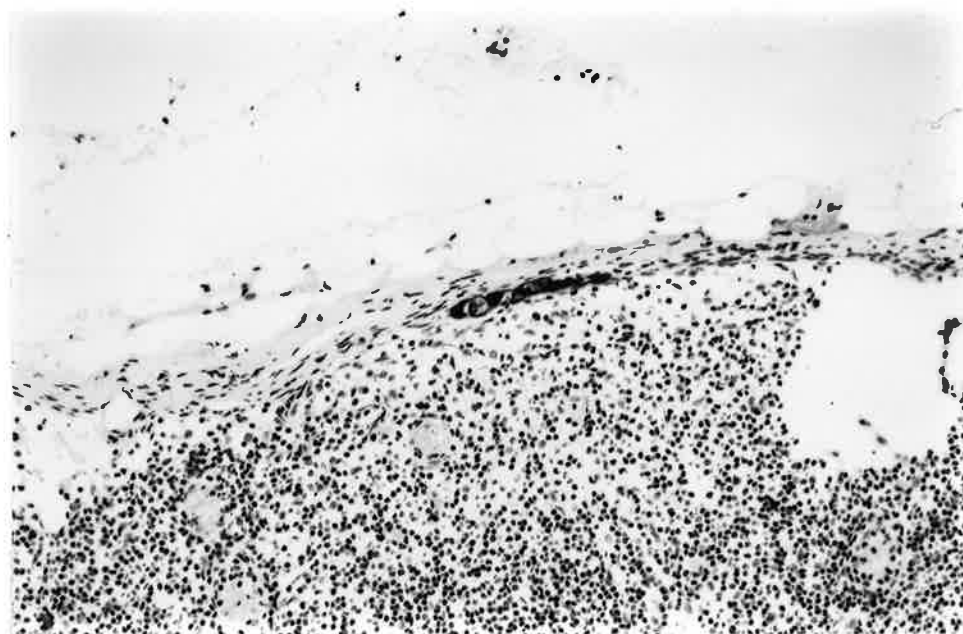
b.

Figure IV.1.1. (a) At this medium magnification (H & E, x 250) the collection of cells in the subcapsular sinus is difficult to recognize as metastatic tumour.
(b) However, the deposit is clearly identified at an even lower magnification (x125) with an immunohistochemical stain for lower molecular weight cytokeratins (CAM 5.2).
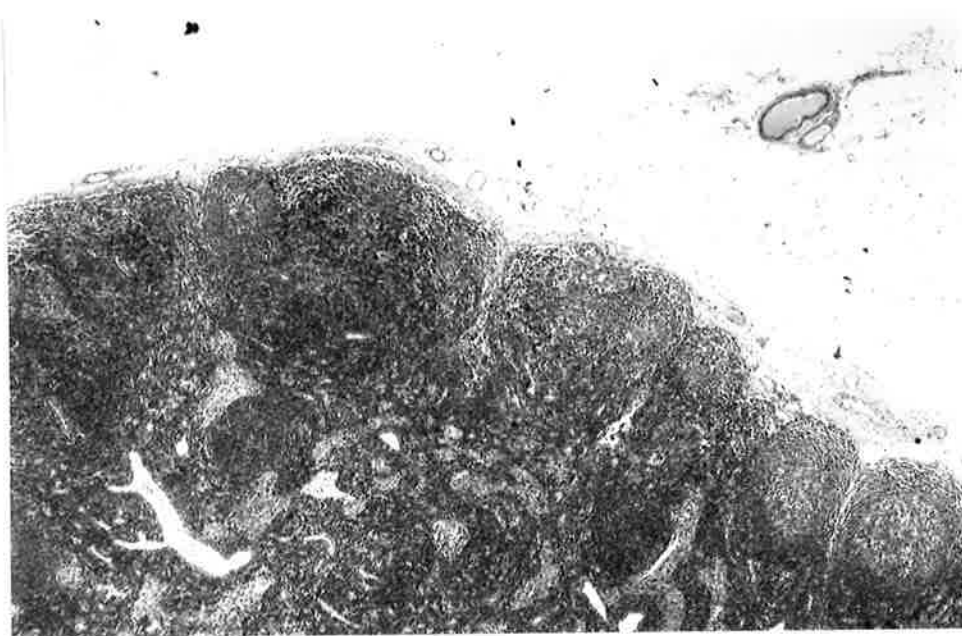
a.

b.
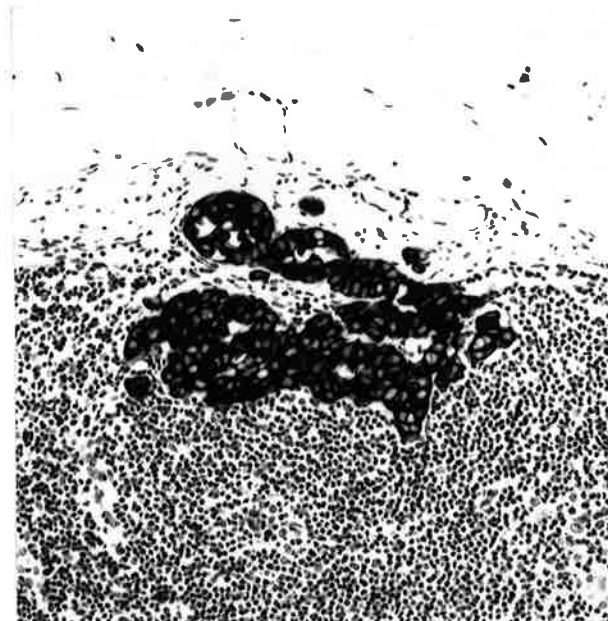
c.

Figure IV.1.2. (a) The deposit of tumour cells is barely visible in the H & E section (x 25). The adjacent section (b), stained with CAM 5.2, clearly identifies the micrometastasis, which is shown to extend beyond the capsule (c) at increased magnification (X 125).

nodal substance (Figure IV.1.3), and in one case only intraparenchymal tumour was detected. The maximum diameters of the occult metastases ranged from 0.1 to 0.6 mm and an average of 40 cells were present in each deposit (range approximately five to 200 cells).

All 12 cases were re-staged from pathological Stage I (no axillary lymph node metastases) to Stage II (axillary metastases). There were no significant differences in the number of nodes or sections studied in patients with or without occult metastases. No significant association was found between the presence of occult metastases and the location, diameter, type or histological grade of the primary tumour (t-tests), and no trend was noted.

IV.1.d. DISCUSSION

The work presented demonstrates that immunohistochemical staining with anti-cytokeratin antibodies is a sensitive method of detecting small nodal metastases (Raymond & Leong, 1989a). It highlights the fallibility of conventional microscopic assessment of axillary nodes, utilized by the majority of pathologists, in that "missed" metastatic deposits were revealed in 12 (21.8 per cent) of the 55 cases of node-negative breast carcinoma reviewed. The examination of axillary nodes naturally varies considerably in different laboratories, and in this series all sections had been screened by at least one qualified pathologist. In all of the cases the metastatic cells could be retrospectively recognized, albeit with difficulty, in the original H & E sections, indicating that the results were not attributable to sampling. However, even
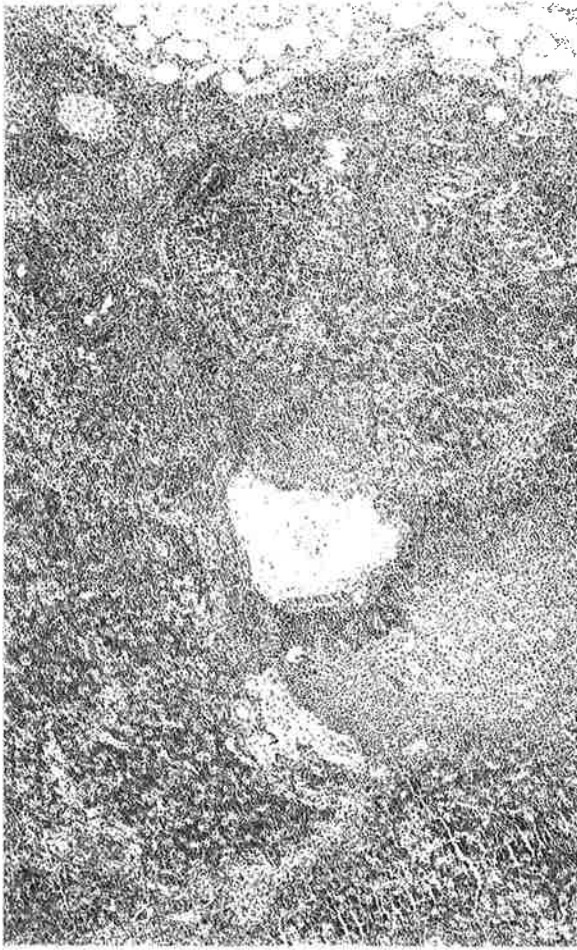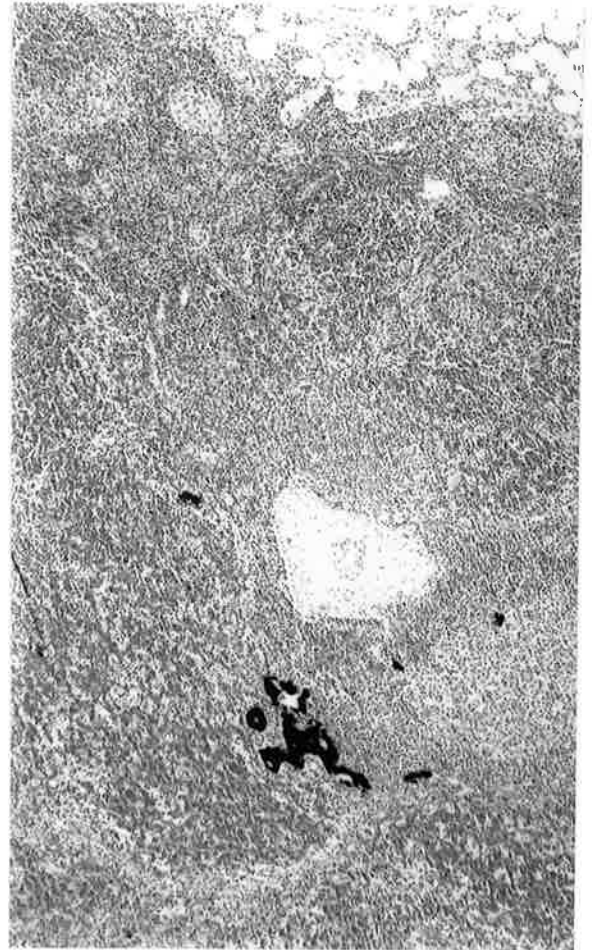
a. b.

Figure IV.1.3. The intraparenchymal deposit of neoplastic cells could easily be missed in the H & E-stained node (a), whereas it is clearly highlighted in the section stained with AE1/AE3 (b). In addition, other smaller deposits of tumour are now visible.

on challenging several senior pathologists in the department with these sections, most of the deposits were not identified despite the raised index of suspicion.

The method of serially sectioning axillary nodes, which has been used to increase the detection rate of metastatic deposits by increasing the area of the node sampled (Fisher et al, 1978a), is labour-intensive and time-consuming for both laboratory technician and pathologist. Routine histological screening of H & E sections is usually performed at low magnification (X4 or X10 objective). At this power small tumour deposits may not be differentiated from epithelioid histiocytes, blood vessels, and other elements of the lymph node. Screening at higher magnification does not appear to be a more accurate technique in that small tumour deposits may still be overlooked, and it may still be difficult to distinguish between tumour and non-tumour cells. In contrast, immunoperoxidase stains clearly highlight metastatic deposits.

Although the numbers in this study were small, there was no relationship between the presence or absence of metastases and the primary tumour grade, type, diameter or location - associations which have not been addressed in previous studies which detected micrometastases by immunocytochemical means. These characteristics of primary tumours did not correlate with the presence of occult lymph node metastases identified by serial sectioning of nodes in one study (Wilkinson et al, 1982).

Occult metastases were detected in 21.8 per cent of the present series of carcinomas, or 24.5 per cent of cases of IDC, which is comparable to the number of node-negative cases found to have micrometastases in other studies utilizing

immunocytochemical methods or employing serial lymph node sectioning. This percentage also corresponds with the proportion of patients with Stage I node-negative breast cancer in whom disseminated disease develops within five years of presentation (Fisher et al, 1978a; Wilkinson et al, 1982).

Many of the antibodies utilized in the various studies employing immunoperoxidase techniques for the detection of occult metastases (Sloane et al, 1980; Wells et al, 1984; Bussolati et al, 1986; Trojani et al, 1987 a & b) were not commercially available at the time of publication, so that the work of these authors could not be reproduced in other research laboratories or applied by diagnostic pathologists. The experience in this study is based on commercial antibodies of documented specificity and the utilization of the highly sensitive ABC staining technique. AE1/AE3 and CAM 5.2 were considered equally efficacious.

These results illustrate that experienced pathologists examining H & E sections may miss small metastatic tumour foci in axillary lymph nodes. The oversight may occur in up to one-quarter of "node-negative" cases. Immunocytochemical staining with antibodies to cytokeratins, specific markers of epithelial cells, is a simple, cheap (Raymond & Leong, 1989a) and accurate method of detecting occult metastases. Because of the possible prognostic and treatment implications of missing such deposits, routine staining of all axillary lymph nodes with AE1/AE3 or CAM 5.2 is advocated in all patients deemed to have node-negative breast cancer by conventional assessment, or in cases where cell identification is a problem.

IV.2. IMMUNOHISTOCHEMICAL ESTROGEN RECEPTOR

ANALYSIS

a. INTRODUCTION

b. DEVELOPMENT OF OPTIMAL FIXATION
   AND STAINING PROCEDURES IN
   IMPRINTS AND FROZEN SECTIONS

c. FROZEN SECTIONS

d. IMPRINTS

e. CORRELATION WITH CYTOSOLIC
   RADIOIMMUNOASSAY

f. CORRELATION WITH HISTOLOGICAL
   AND CLINICAL PARAMETERS

g. PARAFFIN SECTIONS
   - present methods
   - fixation
   - staining technique
   - correlation with frozen
     section and cytosolic assays

h. DISCUSSION

IV.2. IMMUNOHISTOCHEMICAL ESTROGEN RECEPTOR ANALYSIS

IV.2.a. INTRODUCTION

The value of determining the ER content of human breast cancers has been discussed in Chapter II.f. This chapter describes improvements and refinements of various methods of tumour ER content analysis.

Currently, the selection of breast cancer patients for hormonal therapy is largely guided by the results of cytosolic analyses of ER levels (Horsfall et al, 1986). Most of the available data that relate receptor concentrations to clinical prognostic parameters have also relied upon these radioligand binding assays which are dependent on 17ß-estradiol binding to cytosolic and/or nuclear extracts. Cytosolic ER levels for all cases in this study were measured as a routine service procedure by Dr. David Horsfall at the Flinders Medical Centre, Adelaide, using saturation analysis assays (Poulsen, 1981; Horsfall et al, 1986). A series of incubations containing the radioligand [^{3}H]-17ß-estradiol (ranging in concentration from 0.05 nM to 2.0 nM), in the presence of a 100-fold excess of unlabelled diethyl-stilboestrol, was used to estimate the degree of non-specific binding. Bound and free hormone were separated, after a 16 hour incubation, by the addition of dextran-coated charcoal. The binding data were analysed by Scatchard linear regression analysis (Poulsen, 1981; Thorpe & Rose, 1986). Tissues with greater than or equal to 10 fmol of specific estrogen binding/mg of cytosol protein are regarded by convention as receptor-positive (Horsfall et al, 1986).

There are, however, certain inherent limitations of these biochemical assay techniques. They do not measure ER already occupied by endogenous estrogen or ER in the early stages of synthesis which is incapable of binding hormone. Standardization is difficult, and charcoal has been shown to absorb between 10 and 20 per cent of ER, rendering the assay less sensitive and the estimated dissociation constant inaccurate (Poulsen, 1981). Aside from being expensive and time consuming to perform, and necessitating the use of a radiohazard, such assays also require up to one gram of tumour tissue in order to obtain concentrated cytosols, prohibiting assessment of scant biopsies or small tumours. Cytosolic assays of homogenised tissue preclude simultaneous histological confirmation that viable tumour is present in the sample, and the morphological features of the tissue, including heterogeneity of ER expression, cannot be assessed. Inadvertent assay of neighbouring non-malignant epithelium, stromal cells, and/or necrotic tissue, or insufficient numbers of cancer cells in the biopsy may lead to an erroneously low result. In one study in which control histology was performed, tumour was found to constitute less than 10 per cent of the specimen used for receptor analysis in 22 per cent of 116 cases (Steele et al, 1987).

Steroid receptors are thermolabile, unstable proteins and incorrect sample storage or transport, or suboptimal assay conditions, may destroy binding activity producing falsely low or negative values. In common with histochemical methods based on fluoresceinated ligands or anti-ligand antibodies, the validity of the cytosolic assay in detecting the true high affinity ER, which is the main determinant of breast cancer responsiveness to hormonal therapy, is doubtful. Binding of an unknown concentration of endogenous free estrogen to the receptor site prohibits binding of

the ligand and interferes with accurate estimations of the ER content. [^{3}H]-estradiol may also bind to lower affinity type II and III estrogen binding sites (Chamness et al, 1980; Chapter II.f.).

Recent studies with monoclonal antibodies have located the ER within the epithelial cell nucleus, altering the traditional assumption that the ER resided in the cytoplasm and was translocated to the nucleus following binding with estrogen (Greene et al, 1980). The validity of ER determinations based on tissue cytosol homogenates has thus been further questioned.

The development of monoclonal anti-ER antibodies allows direct identification of the actual ER protein (estrophilin) in cryosections, independent of the binding of endogenous hormone, thus circumventing many of the problems mentioned above. Anti-human ER antibodies are applied to tissue sections and the site of antibody binding visualized using PAP or ABC immunocytochemical staining methods (Greene et al, 1980 & 1984; King & Greene, 1984). Only one such antibody is available commercially. Monoclonal H222 Spγ was developed by L.S. Miller (Greene et al, 1984; McCarty et al, 1985) by immunizing Lewis rats with estrophilin isolated from MCF-7 (human breast cancer) cells and hybridizing the rat spleen cells with mouse myeloma cells. The IgG antibodies recognize a specific, stable antigenic determinant that is close to the estrogen binding site and has been shown to be specific for the ER (King & Greene, 1984; Greene et al, 1984; King et al, 1985). This antibody is obtainable in kit form as the Abbott ER-ICA Monoclonal Kit (Abbott laboratories, Wiesbaden-Delkenheim, West Germany). The immunocytochemical assay (ICA) allows a rapid and readily reproducible measurement of ER content at a cellular level and can be performed in any routine

immunoperoxidase laboratory. Direct examination of the tissue section permits an assessment of tumour mosaicism and avoids erroneously low readings due to contamination by excessive stromal elements. Only a small amount of tissue is necessary and the technique is also applicable to tumour aspirates and imprints (Flowers et al, 1986; Reiner et al, 1987; Masood & Johnson, 1987; Earl, 1987; Keshgegian et al, 1988).

Excellent correlations between the percentage of ER-positive cells in frozen sections, as determined by the ER-ICA method, and the quantitative biochemical assay scores have been reported (King et al, 1985; Pertschuk et al, 1985) and the superiority of the immunocytochemical assay over the cytosolic methods established (DeSombre et al, 1986; Bilous et al, 1987). Furthermore, the clinical value of the ER-ICA technique as a predictor of tumour responsiveness to hormone treatment has been described (DeSombre et al, 1986; McClelland et al, 1986).

Several anti-ER antibodies have been reported to bind to ERs in routinely prepared paraffin-embedded tissues (Poulsen et al, 1985; Hendler & Yuan, 1985; DeRosa et al, 1987) but these are not commercially available and their reliability has yet to be confirmed. Using modified versions of the frozen section staining technique, variable success has been reported with the H222 Spγ antibody (Shimada et al, 1985; Andersen et al, 1986; Shintaku & Said, 1987; Cheng et al, 1988).

This chapter describes the development of a simplified version of the immunocytochemical assay recommended by the ER-ICA kit manufacturers, and its application to frozen sections and imprints of fresh breast tumours. Sixteen alternative fixation procedures were examined in 20 tumours and the optimal

method determined. The extent of monoclonal anti-ER staining was related to the cytosolic radioimmunoassay results and available histological parameters in the 115 breast cancers. Finally, a simple and reproducible method utilizing pronase digestion and the commercially available anti-ER antibody was formulated to identify ER protein in formalin-fixed paraffin-embedded tissues.

IV.2.b. DEVELOPMENT OF OPTIMAL FIXATION AND STAINING PROCEDURES IN IMPRINTS AND FROZEN SECTIONS

Suitable fixatives for the preservation of antigen are of fundamental importance when performing immunohistochemical staining procedures and often the method of fixation needs to be specifically tailored to the individual antigen. Fixation arrests autolysis, stabilizes soluble and structural proteins, and protects the tissues against the deleterious effects of subsequent processing. It is essential that the type, duration and temperature of fixation be standardized in order to allow reproducible immunohistochemical staining results. The simpler the fixation technique, the greater the chance of faithful reproduction in different centres.

The method of fixation recommended by Abbott Laboratories prior to immunostaining cryostat sections with the anti-ER antibody involves sequential immersion in 10 per cent formalin (3.7 per cent formaldehyde and PBS) for 10 to 15 minutes, PBS (wash) for four to six minutes, methanol at $-10^{\circ}C$ to $-25^{\circ}C$ for three to five minutes and, finally, one to three minutes in acetone at $-10^{\circ}C$ to $-25^{\circ}C$. This sequence is very cumbersome and a working temperature of $-10^{\circ}C$ to $-25^{\circ}C$ is inconvenient. Sixteen alternative methods of fixation, all available in most surgical pathology laboratories (Table IV.2.1), were therefore applied to imprints and frozen

TABLE IV.2.1.

FIXATIVES FOR ESTROGEN RECEPTORS

IN IMPRINTS AND FROZEN SECTIONS

FIXATIVES EVALUATED	DURATION (min)	TEMPERATURE (°C)
Formalin → Methanol → Acetone*	10 → 5 → 5	-10° & 4°
Formalin → Acetone → Methanol	10 → 5 → 5	-10° & 4°
Formalin → Acetone	10 → 5	-10° & 4°
Formalin → Methanol	10 → 5	-10° & 4°
Acetone → Methanol	10 → 5	-10° & 4°
Formalin	10	22°
Acetone	10	-10° & 4°
Methanol	10	-10° & 4°
PLP	10	22°
Methacarn	10	-10° & 4°
Bouin's Medium	10	22°
70% Ethanol	10	4°
95% Ethanol	10	4°
B5	10	22°
Formol Ethanol	10	4°
Carnoy's solution	10	22°

10% formalin was used at 22°C.
PLP = Periodate-lysine-paraformaldehyde
Methacarn = Methanol (60%)-chloroform (30%)-acetic acid (10%)
Bouin's medium = Picric acid (72%)-formalin (24%)-acetic acid(4%)
B5 = Mercuric chloride (90%)-formalin (10%)
Carnoy's = Ethanol (60%)-chloroform (30%)-acetic acid (10%)
*Abbott recommendation

sections of 19 breast carcinoma specimens in order to establish a simpler procedure which adequately preserves the ER protein.

Tissue from the 19 IDCs was obtained within 15 to 30 minutes of surgical removal. Multiple imprints were prepared in each case by lightly touching freshly-cut tumour surfaces, moistened with buffered saline, on to tissue adhesive-treated slides (Rentrop et al, 1986). Each slide was immediately fixed by one of the 16 methods listed in Table IV.2.1. For eight of the methods two alternative temperatures were examined. In 12 tumours all 24 fixation methods were assessed. In the remaining cases the number of imprints prepared was limited by the size of the tumour sample, but no fewer than 10 fixatives were examined. After fixation the imprints were frozen and stored for no longer than four weeks at -20°C prior to immunostaining.

In addition, fresh tissue from these 19 cases was preserved in OCT embedding medium at -20°C for up to three months. Multiple consecutive six-micron cryostat sections were thaw-mounted on to tissue adhesive-treated slides. These were similarly fixed immediately by one of the techniques listed (Table IV.2.1) before immunostaining. Preliminary trials of fixation by microwave irradiation (Chapter III.2.c) yielded unsatisfactory ER staining in both imprints and cryostat sections and this method was discontinued.

Briefly, the sections and imprints were successively incubated with normal horse serum, monoclonal rat anti-ER (H222 Spγ, 0.1 µg/ml, ER-ICA Monoclonal Kit) at a dilution of 1:2 for 60 minutes and biotinylated secondary antibody (rabbit anti-rat IgG) for 30 minutes. This was followed by reaction with the ABC reagent for

60 minutes before development with DAB and application of a light haematoxylin counterstain (Appendix II). All steps were performed at 22^0C. Cytospins of MCF-7 ER-positive cultured tumour cells, provided with the kit as standard positive controls, served to verify assay performance in terms of both intensity and extent of staining. In addition, cryostat sections of one known ER-positive tumour from the author's files were included with each run. Negative control staining was performed as stated in Chapter III.2.c.

Epithelial cells were designated ER-positive when there was distinct brown granular nuclear staining. There was considerable variability in staining intensity and percentage positivity between different MCF-7 cytospin controls provided with the Abbott kits, while staining of known ER-positive control sections from the study sample was much more consistent for the purpose of standardizing staining runs. Minimal cytoplasmic staining for ER was observed in some tumours and occasionally in the Abbott positive control slides, the significance of which is unknown.

An ER count was derived for each of the frozen sections and imprints by counting the percentage of ER-positive nuclei in 400 to 500 morphologically malignant cells (detailed in Chapter III.2.d). Tumours with an ER count of greater than or equal to 10 per cent were arbitrarily considered to be positive, in accordance with previous authors (Pertschuk et al, 1985; Tosi et al, 1987). The intensity of ER staining was subjectively graded as this may reflect the quantity of hormone receptor within a cell. Tumours were allotted to one of five categories of staining intensity ranging from 0 (no staining) to + + + + (very intense staining). In tumours exhibiting a

range of staining intensities a mean value was estimated (McCarty et al, 1985; Flowers et al, 1986).

In four known ER-positive cases, cytospin preparations were stained with anti-ER after fixation in periodate-lysine-paraformaldehyde (PLP) or formalin followed by acetone (at -10^0C). However, this method was discontinued as artefactual nuclear crenation due to drying, and clumping of cells, caused difficulty in distinguishing positive cells from background staining.

The reagents included in the ER-ICA kit are those required to perform a standard PAP assay. The ABC method was compared to the PAP technique by running both assays in parallel using a series of Abbott control cytospins and cryostat sections, fixed by a range of alternative methods, from four IMVS cases. The ABC amplification system proved superior to the PAP procedure whether kit reagents or PAP complex and peroxidase-labelled secondary anti-rat antibodies from Dakopatts (Copenhagen) were utilized. Using the ABC method the primary antibody could be diluted to one half of the recommended strength, resulting in considerable cost saving (one kit, retailing for approximately A$950.00, provides three ml of primary antibody). Therefore, for this study only the primary antibody component of the kit was used in conjunction with the ABC method routinely employed in the IMVS laboratory (Appendix II). Other reasons for the superiority of the ABC technique were iterated in Chapter III.2.b.

This study revealed the fixation procedures of choice in both imprints and frozen sections to be PLP at room temperature (22^0C) for 10 minutes (Figures IV.2.1. &
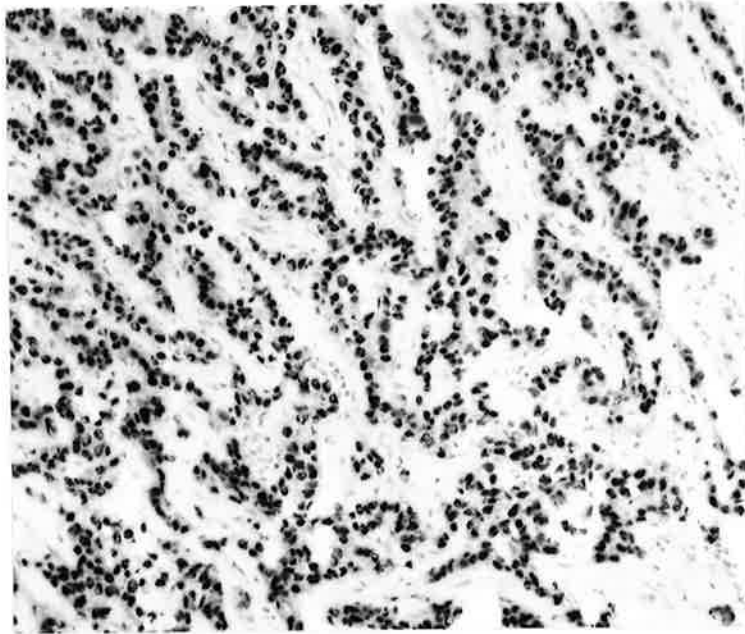
Figure IV.2.1. Frozen section of a well-differentiated IDC, fixed in PLP, with an ER count of 91 per cent. Note the strong nuclear ER staining of the tumour cells, in contrast to the stromal nuclei which are stained with haematoxylin.
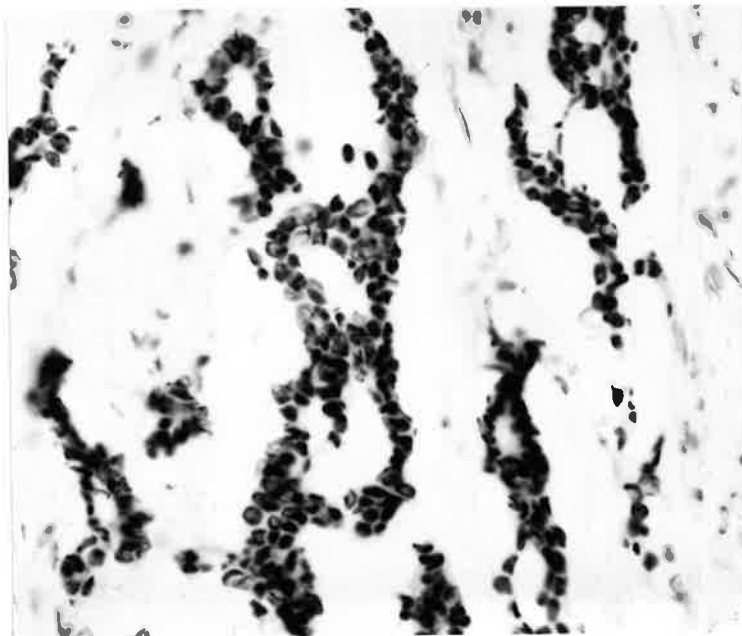
Figure IV.2.2. Frozen section of a tubular carcinoma with an ER count of 70 per cent. Fixation in PLP produces strong nuclear ER staining.

IV.2.2) or the sequence of 10 per cent formalin (22^0C) for 10 minutes followed by acetone at -10^0C for five minutes. Such preparations exhibited the best cellular detail, the greatest staining intensity and the highest percentage positivity (ER counts). Minimal background staining was observed with these two methods. Alteration in the sequence of reagents recommended by the ER-ICA kit manufacturers (i.e. formalin → methanol → acetone) to formalin → acetone → methanol had no deleterious effect on the staining intensity or morphological preservation, and exclusion of methanol from the sequence improved the staining. Fixation at -10^0C produced better results than fixation at 4^0C for this sequence.

Acetone alone at -10^0C, 10 per cent formalin alone at room temperature, the formalin (22^0C) → methanol (-10^0C) → acetone (-10^0C) sequence, and 70 per cent alcohol at 4^0C all produced adequate staining in those cases with high ER cytosol values, but were less suitable for cases with a lower proportion of ER-positive cells. Methanol alone, Methacarn, Bouin's solution, 95 per cent ethanol, B5, formol-ethanol, Carnoy's solution (Table IV.2.1) and microwave irradiation failed to preserve ER protein and were all found to be unsuitable methods of fixation. The deleterious effect of drying on the estrophilin antigen is well known (King et al, 1985; Helin et al, 1988) so the poor results obtained following fixation with microwave irradiation were not surprising. Several other authors have examined alternative fixatives (Press & Greene, 1984; King et al, 1985), however, most papers using the Abbott ER-ICA kit rely on the Abbott recommendation or a single alternative reagent such as picric acid/para-formaldehyde (Pertschuk et al, 1985).

The preservation of estrophilin antigen is thus crucial to reliable, reproducible

immunostaining results. Based on the studies described, the recommended fixatives are therefore the single reagent, PLP, at room temperature, or the sequence of 10 per cent formalin (22^0C) followed by acetone at -10^0C. These methods are simpler, more consistent, less time-consuming, and provide superior staining intensity, tumour cytomorphology and higher ER counts than the three-reagent sequence recommended by Abbott Laboratories. It is anticipated that the fixation and staining technique described would be applicable to fine needle aspirates. Ideally, however, future ER estimations will be derived from ER immunostaining of paraffin sections.

IV.2.c. FROZEN SECTIONS

For each of the 115 malignant breast neoplasms and 12 benign breast tissues examined in this study (Chapter III.1) cryostat sections were cut, immediately fixed in PLP at room temperature for 10 minutes and then stained with H222 monoclonal anti-ER antibody. Forty-five to 85 per cent of benign breast epithelial cells were ER-positive, with a mean of 66 per cent. Stromal cells were found to be consistently ER- negative. The mean ER count for the six examples of normal breast epithelium was 67 per cent. This proportion is considerably higher than the figure of seven per cent cited by Petersen et al (1987) in a study of cryosections of 18 cases of normal human breast tissue. The reason for the discrepancy in mean values is not clear but may, at least in part, reflect the greater sensitivity of this author's technique over the standard kit procedure employed by Petersen and associates. The small samples in both studies and, possibly, the older mean patient

age in this author's sample (55 years versus 30 years) may be another explanation.

Eighty-four (73 per cent) of the 115 malignant neoplasms were ER-positive, with ER counts ranging from 10 to 91 per cent and a mean ER count of 47.2 per cent (S.D. 32.5). Within each section there was considerable heterogeneity of ER expression (Figure IV.2.3). In several cases there were two distinct cell populations within the one section, with separate pools of ER-positive and ER-negative cells, as has been previously noted (King et al, 1985; Azavedo et al, 1986). The ER count in these latter cases was still taken to be the proportion of ER-positive cells in the total tumour cell population (Figure IV.2.3). In addition, the intensity of nuclear staining was assessed subjectively in 74 cases and graded as not present (0), greater than background of inbuilt controls (+), distinct (+ +), intense (+ + +) or very intense (+ + + +). For each of these cases a semiquantitative ER score, the product of the intensity value and the ER count, was calculated to produce a greater spread of values for statistical analysis (Flowers et al, 1986; Azavedo et al, 1986; DeSombre et al, 1986; Raymond & Leong, 1988 & 1989d). This value ranged from 0 to 364. There was a significant positive correlation between the ER count and the staining intensity ($r = 0.65$, $p < 0.0001$) and, as anticipated, between the ER count and the ER score ($r = 0.88$, $p < 0.00001$).

Immunostaining with an anti-ER specific antibody in histological preparations thus overcomes many of the difficulties and potential inaccuracies encountered with the cytosolic assay techniques and the earlier immunohistochemical measurements of ER based on staining for bound estrogen (Chamness et al, 1980; Pertschuk et al, 1985; DeGoeij et al, 1986).
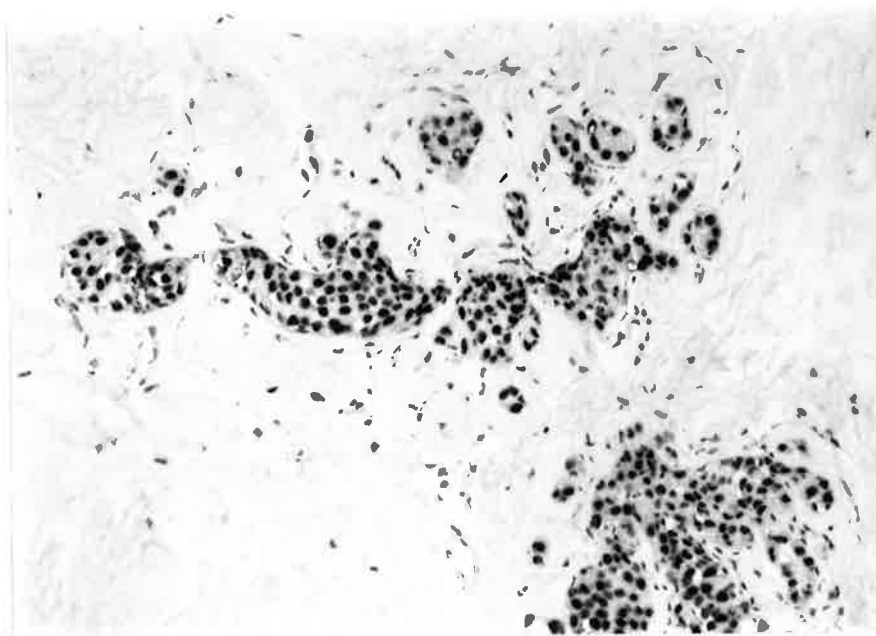
a.
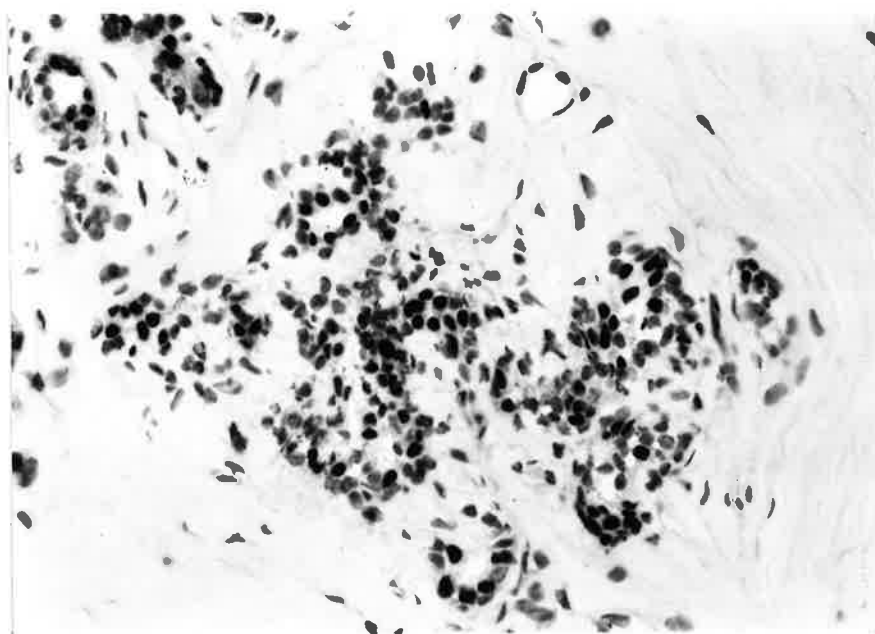
b.

Figure IV.2.3. Photomicrographs illustrating heterogeneity of ER expression in the same frozen section of an IDC (Grade II) fixed in PLP. The overall ER count was 41 per cent. In (a) greater than 75 per cent of epithelial nuclei are ER-positive, in contrast to the field shown in (b) where less than 50 per cent of nuclei stain positively. In other fields, not shown, all tumour cells were ER-negative. The cytosol ER value for this tumour was 0.

IV.2.d. IMPRINTS

ER immunostaining of cryostat sections is well-established, however, frozen section cytomorphology is often poor, sufficient fresh material may not always be available to reserve a portion for freezing, and performing a frozen section on a block of tissue prior to paraffin embedding often results in suboptimal morphology in the subsequent diagnostic histological section. This is of particular relevance as smaller tumours are now being diagnosed at a non-palpable stage with mammographic screening and detection procedures. Reliable semi-quantitative measurement of tumour ER content in cytological touch preparations (imprints), by means of an immunoperoxidase technique, combines excellent preservation of cytomorphology with the expedient use of limited tissue, such as in Trucut biopsies. After imprinting the small amount of tissue can be processed for routine histology and the imprints stained with the anti-ER antibody, either immediately or following fixation and storage at -20^{0}C to -80^{0}C.

ER immunostaining has been successfully applied to fine needle aspirates of breast carcinoma cells in numerous studies (Azavedo et al, 1986; Flowers et al, 1986; Reiner et al, 1987; Ozzello et al, 1987; Earl, 1987; Keshgegian et al, 1988), allowing information about the ER status to be available prior to surgery. A concordance of up to 87 per cent has been demonstrated between ER counts in aspirates and counts in histological biopsies of tumour tissue obtained subsequently at operation (Reiner et al, 1986). This method has also been shown to be as predictive of breast cancer response to endocrine therapy as the biochemical ER

assay (Coombes et al, 1987).

At the time of writing, however, steroid receptor determination by imprint cytology had been described in only a single report which utilized an indirect fluorescent immunocytochemical method (Masood & Johnson, 1987). Other authors had provided only anecdotal descriptions of ER staining of imprint preparations (Pertschuk et al, 1985; Ozzello et al, 1987).

Imprints in this study were prepared and stained with the H222 antibody as described in section IV.2.b. Positive staining was localized to the nuclei of malignant and benign epithelial cells (Figure IV.2.4), as observed in the frozen sections, and ER counts for the 19 cases examined ranged from 0 to 81 per cent. The ER counts for the imprints, the corresponding frozen section ER counts (range from 0 to 90 per cent) and the cytosolic ER values (fmol/mg cytosol protein) are displayed in Table IV.2.2. Statistical analysis demonstrated a significant positive correlation between the ER counts in imprints and both the ER cytosol values ($r = 0.64$, $p < 0.05$) and the frozen section ER counts ($r = 0.66$, $p < 0.05$; Figure IV.2.4), confirming the validity of the imprint technique.

Peroxidase-stained imprint preparations were ER-positive in nine cases, and in each case the corresponding frozen section was also ER-positive. Three cases exhibited ER positivity in frozen sections but no or negligible peroxidase staining was identified in the imprints, and in only one of these instances the cytosolic ER value was greater than 10 fmol/mg protein. In such situations it is possible that there was some degradation of receptor in the imprint material, despite careful attention

Figure IV.2.4. (a) Imprint from a well-differentiated ductal carcinoma fixed in formalin/acetone (-10°C) with an ER count of 81 per cent. Some ER-negative, haematoxylin-stained cells are seen (arrows).
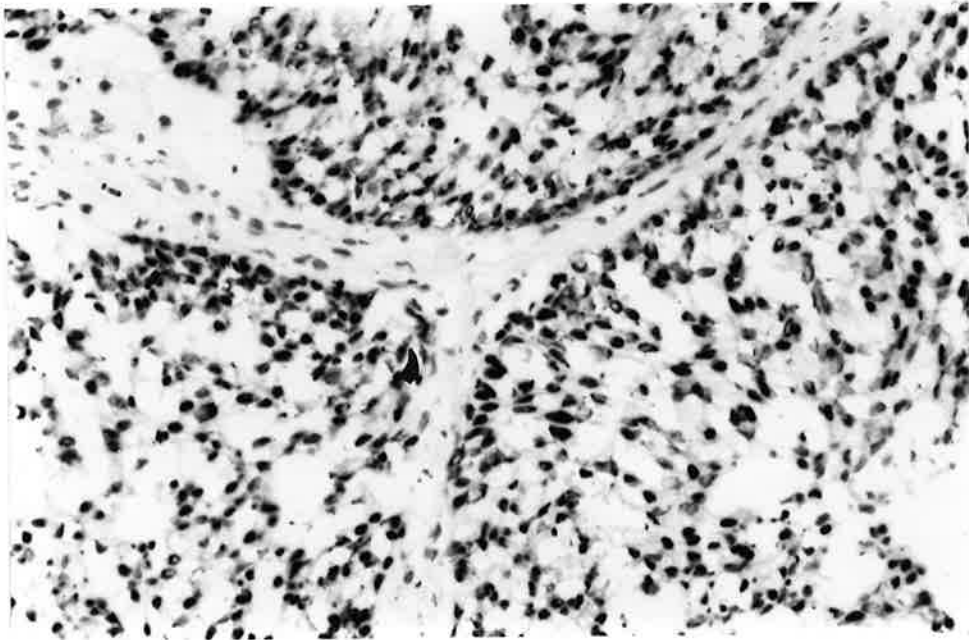

Figure IV.2.4. (b) Corresponding frozen section fixed in PLP illustrating an ER count of 90 per cent. The tumour had an ER cytosol value of 522.

TABLE IV.2.2.

ESTROGEN RECEPTOR (ER) CYTOSOL VALUES AND ER COUNTS FOR

IMPRINTS AND FROZEN SECTIONS OF 19 CASES OF BREAST CARCINOMA

ER cytosol value (fmol/mg prot)	Imprint ER Count	Frozen section		
		ER Count	Staining Intensity	ER Score
522	81	90	4	360
89	38	46.5	3	139.5
70	43	58	3	174
47	39	68	3	204
41	49	72	4	288
26	3	70	3	210
25	15	40	1	40
20	60	56	2	112
16	30	53	2	106
7	5	2	1	2
3	2	0	0	0
0	0	41	3	123
0	30	20	2	40
0	0	23	2	46
0	0	1	1	1
0	0	0	0	0
0	1	0	0	0
0	0	0.5	1	0.5
0	0	0	0	0

ER cytosol assayed by the dextran-coated charcoal method
Staining intensity: 0 = no staining; 1 = weak staining;
2 = moderate staining; 3 = strong staining;
4 = very strong staining
ER score = Staining Intensity X ER count
ER scores were determined in PLP or formalin → acetone
(-10°C) fixed tissues (see text).

to the fixation protocol and efforts to avoid drying. Tumour cell morphology was noted to be markedly distorted in one of these three cases. Alternatively, the discrepancies may reflect inadequate tumour sampling. In one other case 30 per cent of neoplastic cells in the imprint were ER-positive and the corresponding cryostat section ER count was 20 per cent, but biochemical assay indicated that the tumour was ER-negative (Chapter IV.2.e).

Although the frozen sections and imprints exhibited similar ER counts, the staining intensity in imprints was generally weaker than in the corresponding sections. Marked variability in imprint staining intensity could probably be explained by artefactual factors, such as drying, and it was considered that intensity grading of imprints was likely to be inaccurate.

Imprints were ideally prepared following application of buffered-saline drops to the freshly cut tumour surface, as drying not only resulted in poor preservation of cellular morphology, but also in loss of estrophilin antigen (Raymond & Leong, 1988). The technique described is simple and easily mastered.

Several inherent problems have, however, been identified which must be considered when examining immunostained imprint preparations, and these no doubt also apply to fine needle aspiration smears. Excess blood associated with the imprint material may interfere with accurate assessment of the peroxidase-stained cells and non-specific background staining may occasionally be seen around clumped tumour cells. In some instances, the tumour cell yield may be suboptimal and this author suggests that approximately 200 malignant cells should be evaluated to take into account

intratumoral ER variation. Furthermore, there may be intrinsic selectivity in the preparation of imprints or fine needle aspirates, for example, less cohesive tumour cells may be more readily sampled. Thus, when adequate tumour tissue is available, immunostaining of frozen sections remains the preferred option.

IV.2.e. CORRELATION WITH CYTOSOLIC RADIOIMMUNOASSAY

There was a 93 per cent agreement in positivity and negativity between the results of the steroid binding dextran-coated charcoal assays and the frozen section immunocytochemical assays in 110 patients, with a highly significant correlation ($r = 0.81$, $p < 0.00001$; Figure IV.2.5). The ER score (ER count X staining intensity) also correlated strongly with the ER cytosol values ($r = 0.82$, $p < 0.0001$, $n = 74$).

Six (5.2 per cent) cases revealed intense immunocytochemical ER staining but were classified as ER-negative by cytosolic analysis (Figure IV.2.3). In one of these cases 30 per cent of the tumour cells were ER-positive in the corresponding imprint. Two biochemically ER-positive tumours demonstrated very low ER counts (eight and 1.5 per cent) in the frozen sections. Discrepancies such as these have also been reported by other workers (Pertschuk et al, 1985; Keshgegian et al, 1988).

Those cases classified as ER-negative using the ligand-binding assay, but demonstrating ER-positive neoplastic cells in cryostat sections, may correspond to some of the 10 per cent of "ER-negative" patients (determined biochemically) that do respond to hormonal manipulation. These were considered to be "false negatives" and may reflect errors of sampling, such as excessive dilution of tumour cells by
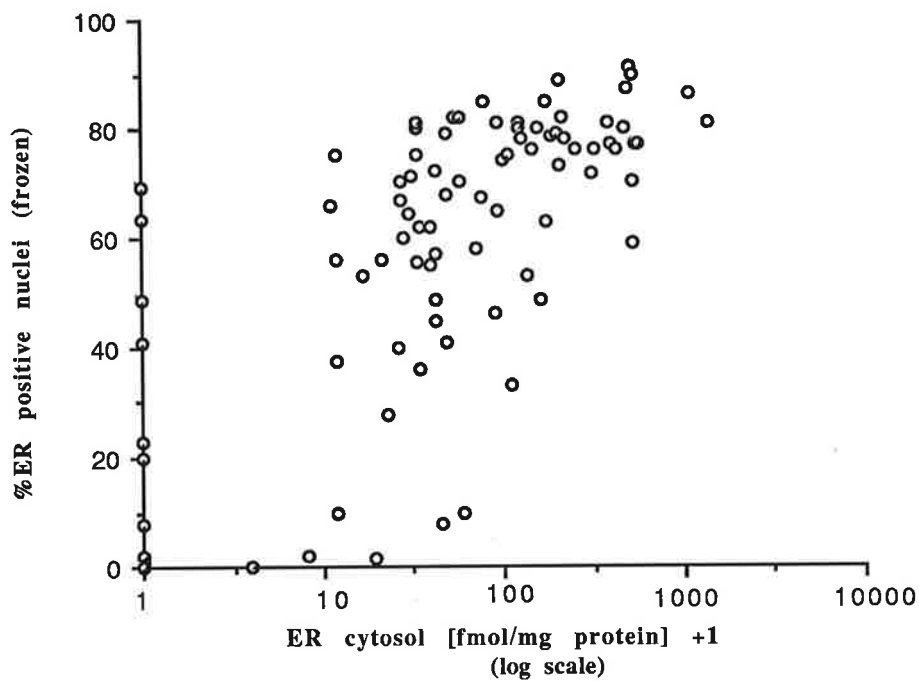
Figure IV.2.5. Relationship between the immunohistologically determined ER count and the cytosolic ER value in 110 malignant breast neoplasms ($r = 0.81$, $p < 0.00001$).

stromal elements, heterogeneity of ER content or a lack of viable tumour cells in the homogenized specimen.

In three of these discrepant cases the H & E-stained sections revealed the tumour to be associated with a marked desmoplastic stromal response, suggesting that there may have been excessive dilution of neoplastic cells by stromal cells in the tissue homogenate. Similar sampling inaccuracies may explain why several other cases had notably high ER counts and relatively low ER cytosol values. Alternatively, inaccuracies inherent in the biochemical technique such as degradation of receptor during tissue processing, or the inability to measure receptors that cannot react in a functional assay (due to binding to endogenous estrogen or altered binding sites), could be responsible.

"False positive" results from the cytosolic assay possibly reflect non-specific binding of the $[^3H]$-estradiol to type II or type III estrogen binding sites, rather than binding to the receptor itself (Chapter IV.2.a; Chamness et al, 1980).

IV.2.f. CORRELATION WITH HISTOLOGICAL AND CLINICAL PARAMETERS

A significant inverse correlation between the ER count and the histological grade of the IDCs was demonstrated ($r = -0.43$, $p < 0.001$, $n = 105$). The scattergram illustrated in Figure IV.2.6., however, reveals a wide range of ER counts for each grade. The inverse correlation was still significant, but weakened, if the histological grade was related to biochemical ER values ($r = -0.31$, $p < 0.01$, $n = 105$) or the ER score ($r = -0.32$, $p < 0.01$, $n = 64$). Histological grade was not related to ER staining intensity. An inverse relationship was apparent between the ER count and
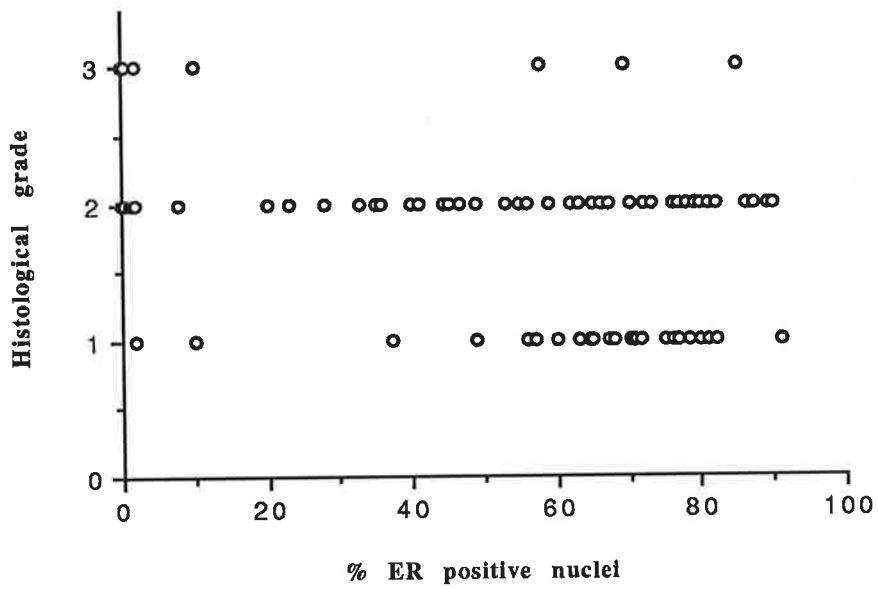
Figure IV.2.6. Relationship between the ER count (percentage of ER-positive nuclei) and the histological grade (r = -0.43, p < 0.001).

the axillary nodal status (r = -0.25, p < 0.05, n = 88), but this did not reach statistical significance if biochemical ER values were compared with the presence of axillary metastases. A wide range of ER counts was observed in both node-negative and node-positive cases (Figure IV.2.7). No relationship was identified between either the ER score or ER staining intensity and nodal status.

An inverse correlation between the ER count and the size of the primary tumour was also demonstrated (r = -0.20, p < 0.05). In addition, a significant difference in ER counts was revealed between the subgroups of premenopausal and postmenopausal patients (t = 2.5, p < 0.05, n = 108). Thus, ER-positive tumours tended to be small and well differentiated, occurred in older (postmenopausal) patients and were less frequently associated with axillary nodal metastases, confirming findings previously reported (Fisher et al, 1988; Reiner et al, 1987; Keshgegian et al, 1988).

IV.2.g PARAFFIN SECTIONS

Present Methods

Immunoperoxidase localization of ERs in paraffin sections has long been sought and a number of different procedures have recently been published, however, only a few of these employed the commercially available H222 anti-ER antibody. Shimada et al (1985) applied this antibody at high concentration (10 µg/ml) for 30 minutes at 37°C to biopsy material fixed in buffered formalin for 24 hours at 4°C and then rinsed overnight in sodium phosphate buffer. A concordance of 82 per cent was obtained with cytosolic assays, and of 86 per cent (24 of 28 cases) with frozen section
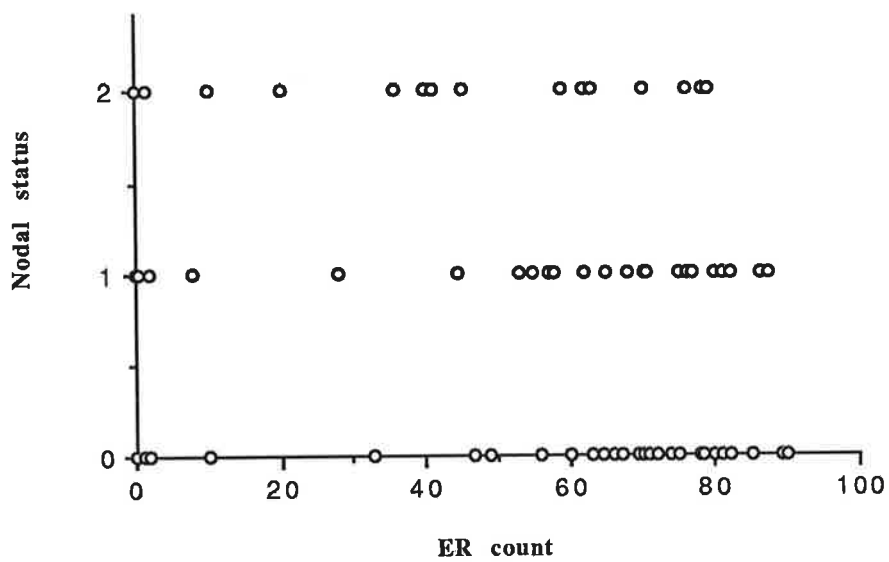
Figure IV.2.7. Relationship between ER count and axillary nodal status ($r = -0.25$, $p < 0.05$).

0 = node-negative; 1 = 1-3 nodes positive; 2 = ≥4 nodes positive.

results. However, the requirement of cold formalin fixation for 24 hours differs markedly from conventional fixation, and delays of up to 48 hours are required before embedding. Andersen et al (1986) applied full strength H222 antibody for 16 hours at 4^0C, following trypsinization, to "routinely formalin-fixed" (time unspecified) breast cancers, stored for up to five years. Endogenous peroxidase was not blocked. Agreement between DCC assay and immunocytochemically determined ER values was obtained in 30 of 35 cases, all discordant cases being attributed to cytosolic ER-positive tumours that failed to stain with the immunohistochemical method. An ABC technique was utilized in both of the above studies.

Shintaku and Said (1987) described a method of enzymatic digestion of paraffin sections with deoxyribonuclease (DNase) prior to ER immunostaining. The tissues had been fixed in formalin at room temperature for four to 12 hours and "routinely processed" before incubation with DNase I for two hours at room temperature. Primary H222 anti-ER antibody was applied overnight and the other Abbott kit immunoreagents were used to complete the reaction. It was noted that methanolic H_2O_2 was not included in the description of the methodology for this study, but this step was said not to reduce the intensity of ER staining. Good agreement was demonstrated between the results of immunoperoxidase staining of paraffin sections and cytosolic values. Furthermore, there was a 94 per cent (30 of 32 cases) concordance when ER immunostained frozen and paraffin sections were compared. In 20 cases Hiort et al (1988) digested paraffin sections with both trypsin and DNase before applying anti-ER and the reagents supplied with the ER-ICA kit. The DAB reaction product was enhanced with cobalt chloride. Their qualitative results were concordant in the 12 cases in which paraffin and frozen sections were compared.

Finally, Cheng et al (1988) compared several of the above techniques (Andersen et al, 1986; Shintaku & Said, 1987) with one using pronase enzyme (Calbiochem) pretreatment (at 30 mg/ml) and an alkaline phosphatase method. This latter method yielded the best results with 100 per cent sensitivity and 89 per cent specificity when compared with cytosolic assay ER values. The tissue blocks were "routinely formalin-fixed", but no details of fixation temperature or duration were provided. Other workers, however, have failed to demonstrate ER in formalin-fixed paraffin-embedded sections using the H222 antibody, theoretically because of loss of ER antigen expression during fixation and processing (King et al, 1985; Pertschuk et al, 1985a).

Poulsen et al (1985) identified ERs in Bouin's-fixed paraffin-embedded tissue utilizing a mixture of two monoclonal antibodies that recognize separate antigenic sites on the ER protein (D547 Spγ and D75P3γ), different to the epitope detected by H222 Spγ. These antibodies were also raised against the MCF-7 human breast cancer cell line in the research laboratory of Dr. Geoffrey Greene (Greene et al, 1980), but are not commercially available. The sections were incubated overnight at 4°C with the primary antibody mixture and a PAP technique was employed. There was no mention of digestion or of blocking endogenous peroxidase. However, ER positivity was detected in only half of the biochemically ER-positive tumours.

A recent paper by Bur et al (1987) confirmed consistent staining for ER with the monoclonal D75 antibody in formalin-fixed (between two hours and two days), routinely processed, paraffin-embedded endometrial tissues, if the sections were

pretreated with 0.1 per cent bovine pancreatic trypsin. Endogenous peroxidase was blocked and the sections were incubated with primary antibody overnight. A PAP method was preferred when compared with an ABC procedure.

Despite the above claims, "routinely" formalin-fixed tissues may not always be suitable for immunohistochemical localization of the sensitive ER protein. DeRosa et al (1987) applied the D75 antibody (at room temperature for 60 minutes) and an ABC technique to paraffin-embedded tissues fixed in Bouin's solution or buffered formalin, each at various temperatures and for varying lengths of time. The best results were obtained following fixation for one to two hours in either Bouin's solution at room temperature or in formalin at 4^0C. Prolonged fixation resulted in decreased immunoreactivity and in the appearance of nonspecific cytoplasmic and background staining. Preliminary trials using D75 antibody kindly donated by Dr. Greene, however, failed to reproduce the described ER staining in this author's formalin-fixed paraffin-embedded tissue. The reasons for this are not clear, but may, at least in part, reflect differences in the tissue processing techniques employed by the two laboratories.

Finally, there have been a number of other publications describing monoclonal antibodies which specifically stain ER-bearing cells in paraffin-embedded tissues (Hendler & Yuan, 1985; Yuan & Dawson, 1987). Many of these antibodies, however, are not freely available for use by other research workers or by diagnostic pathologists.

ER staining of formalin-fixed paraffin-embedded tissues has thus been fraught with

difficulties. The aim of this author's study was to develop a simple and readily reproducible immunocytochemical assay, using the commercial H222 antibody, to detect ER in paraffin sections prepared with minimal deviation from routine procedure.

Fixation

A major drawback of using archival tissues from a principal surgical pathology laboratory when developing new immunoperoxidase techniques is that fixation and processing of tissues is rarely uniform. This is especially a problem when a large number of cases are received from different sources, such as fresh from hospital operating theatres, or partially fixed from private local or country hospitals. Under these circumstances the time interval between excision and fixation, and the duration and temperature of fixation can vary considerably, all of which may effect the immunoreactivity of the tissue. Thus, as appropriate fixation appears to be critical when attempting to identify ERs in paraffin sections, only those cases in which fresh tissue was received from the adjoining hospital within 15 to 20 minutes of excision were examined.

Three alternative methods of fixation were compared. Two of these were chosen using the findings of DeRosa et al (1987) as a guide to the most appropriate means of preserving the estrophilin protein, although it is recognized that different anti-ER antibodies (H222 and D75) may not react in the same manner and under the same fixation conditions. In preliminary experiments with three cases, formalin

fixation times of one, two, four and eight hours at room temperature were employed. Formalin fixation at room temperature was preferred, being more convenient than fixation at 4°C. As staining was optimal after both one and two hours of fixation, one and a half hours was the duration employed for all subsequent specimens. After four hours of formalin fixation ER staining was of weaker intensity, and after eight hours or longer determination of an ER count became difficult. Fixation in Bouin's solution and by microwave irradiation was also tested.

In 30 IDCs, one lobular carcinoma and one *in situ* ductal carcinoma, separate blocks of fresh tumour were fixed by each of the following methods:

a) neutral buffered formalin (pH 7.0) for one and a half hours at room temperature

b) Bouin's solution for one and a half hours at room temperature

c) Microwave irradiation (Chapter III.2.c).

If immediate fixation was not possible fresh tissues were left refridgerated (4°C) for up to a maximum of one hour. Following fixation, all tissue blocks were processed and embedded in paraffin in an identical manner (Chapter III.2.c), without further exposure to formalin. Fresh frozen tissue had been reserved for ER immunostaining of cryosections and for cytosolic assay (as previously detailed).

Optimal and most intense staining for ER was observed in the formalin-fixed tissue (Figure IV.2.8). Staining was weaker, although still distinct, in the sections which had been fixed in Bouin's solution or by microwave irradiation. No previous study has described ER staining in microwave-fixed paraffin sections.
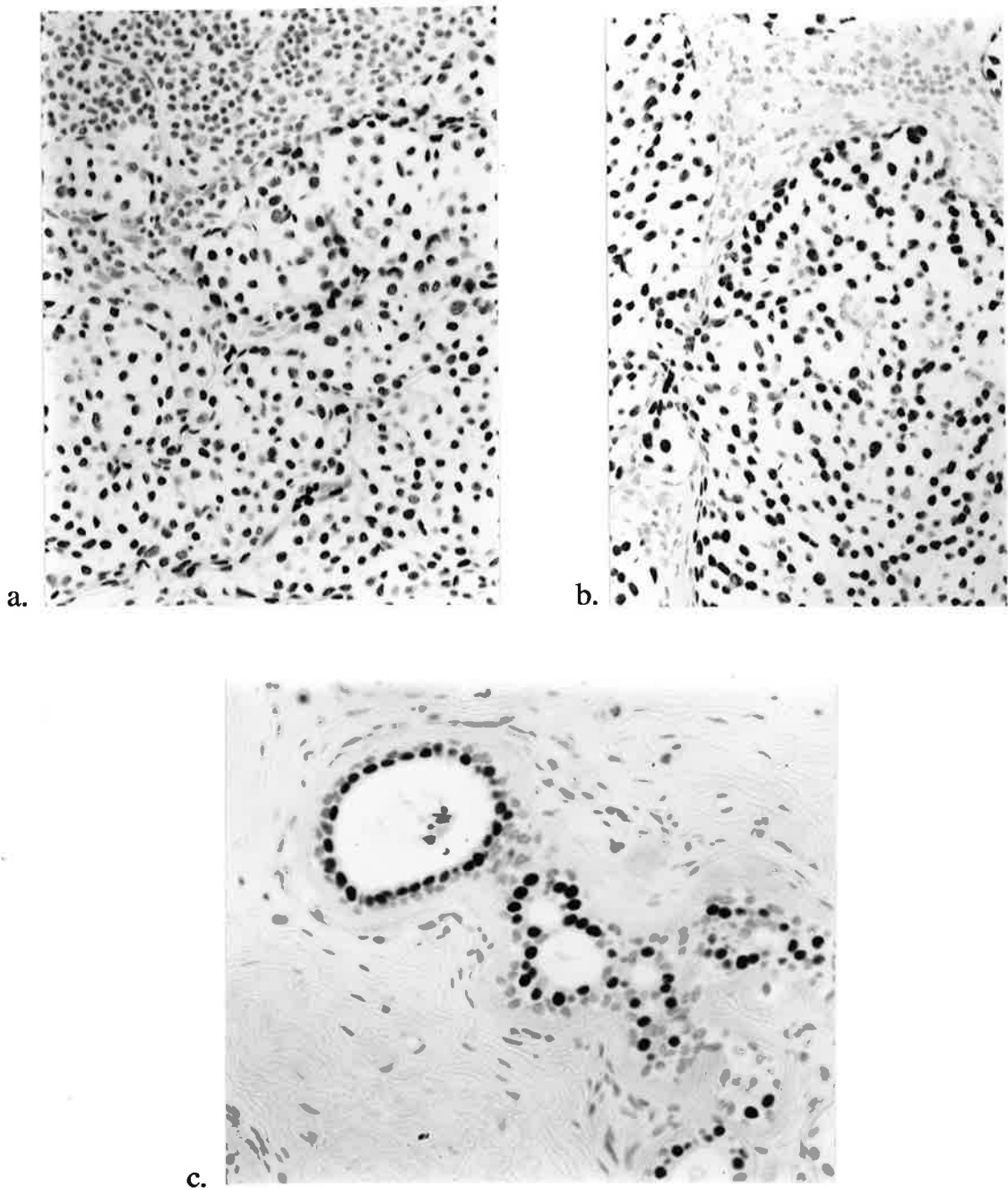
Figure IV.2.8. (a) Paraffin section of an IDC, Grade II, demonstrating ER staining of moderate intensity (++) in 89 per cent of neoplastic nuclei (Case 6). Note the contrasting ER-negative lymphocytes, stained with haematoxylin, in the upper field (formalin fixation, anti-ER stain). (b) Nickel modification of DAB enhances the contrast between ER-positive tumour cells and ER-negative lymphocytes and stromal cells in an adjacent section. Scattered ER-negative neoplastic cells are also more readily identified. (c) In this field of benign breast epithelium, from the same case, approximately 40 per cent of the epithelial nuclei are ER-positive (anti-ER, paraffin section).

Staining Technique

ER immunohistochemical staining was performed using a modified streptavidin-biotin method (Chapter III, Appendix I). Five-micron paraffin sections were cut, deparaffinized with xylene, passed through graded alcohols and rinsed successively in deionized water and PBS. Sections were then treated with pronase (Calbiochem, Cat. No. 537088, La Jolla, CA, USA), at 0.2 mg/ml for 15 minutes at 37^0C, prior to sequential application of normal horse serum and the primary antibody (H222 anti-ER, Abbott ER-ICA monoclonal kit) at a dilution of 1:2 overnight at room temperature. Biotinylated secondary antibody (rabbit anti-rat IgG) and ABC reagent were then applied at the same concentration and for the same duration as described for the cryostat sections (Chapter IV.2.b). The sections were developed with DAB in a Tris-HCl buffer for 25 to 30 minutes and a very light haematoxylin counterstain applied (Appendix I). A negative control was run parallel to each test section by replacing the primary antibody with normal horse serum. In addition, paraffin sections of a known ER-positive tumour were included with each run. It is recommended that control sections are freshly cut prior to immunostaining as it was observed fortuitously that there is an apparent decay of the ER antigen in unstained paraffin sections stored for several months. The reason for this loss of immunoreactivity is not known, but may relate to increased drying and exposure to the atmosphere.

Endogenous peroxidase was not blocked with methanolic H_2O_2 as preliminary studies revealed this step to have an adverse effect on the demonstration of the ER. The

reason for this is not apparent, but it is noted that while methanolic H_2O_2 was utilized by some workers, others have omitted or failed to mention this procedure (Shimada et al, 1985; Andersen et al, 1986; Shintaku & Said, 1987).

The enzyme pronase isolates double-stranded DNA in the nucleus and may unmask ER antigenic determinants that are usually obscured by the formalin fixation process (Cheng et al, 1988). This step may therefore increase the sensitivity of the immunocytochemical assay. During preliminary studies paraffin sections were treated with pronase at concentrations ranging from 0.1 to 30 mg/ml for one, three, six, nine, 12, 15, 18, 21 and 30 minutes, and the extent and intensity of ER staining was assessed. The anti-ER antibody was applied at full strength and at dilutions of 1:2, 1:5 and 1:10. Positive staining of receptor sites was achieved with each of the enzyme solutions, but more concentrated preparations caused partial disruption of nuclei and loss of cytological detail. Primary antibody could be applied at a dilution of up to 1:5 to reliably indicate positive or negative ER status. However, counting was most accurate if a primary antibody solution of higher concentration was used (1:2), allowing easier distinction between ER-positive cells and negative nuclei stained with haematoxylin. Nickel modification of DAB was used in a few cases (Hsu & Soban, 1982) to enhance the ER reaction product and was ideal for photography of ER-positive cells (Figures IV.2.8 & IV.2.9).

In addition, several alternative digestion methods were assessed based on the recommendations of previous workers. Attempts to reproduce the ER staining techniques described by Andersen et al (1986) and Shintaku and Said (1987), using trypsin II and DNase I respectively (both purchased from Sigma Chemical Company,
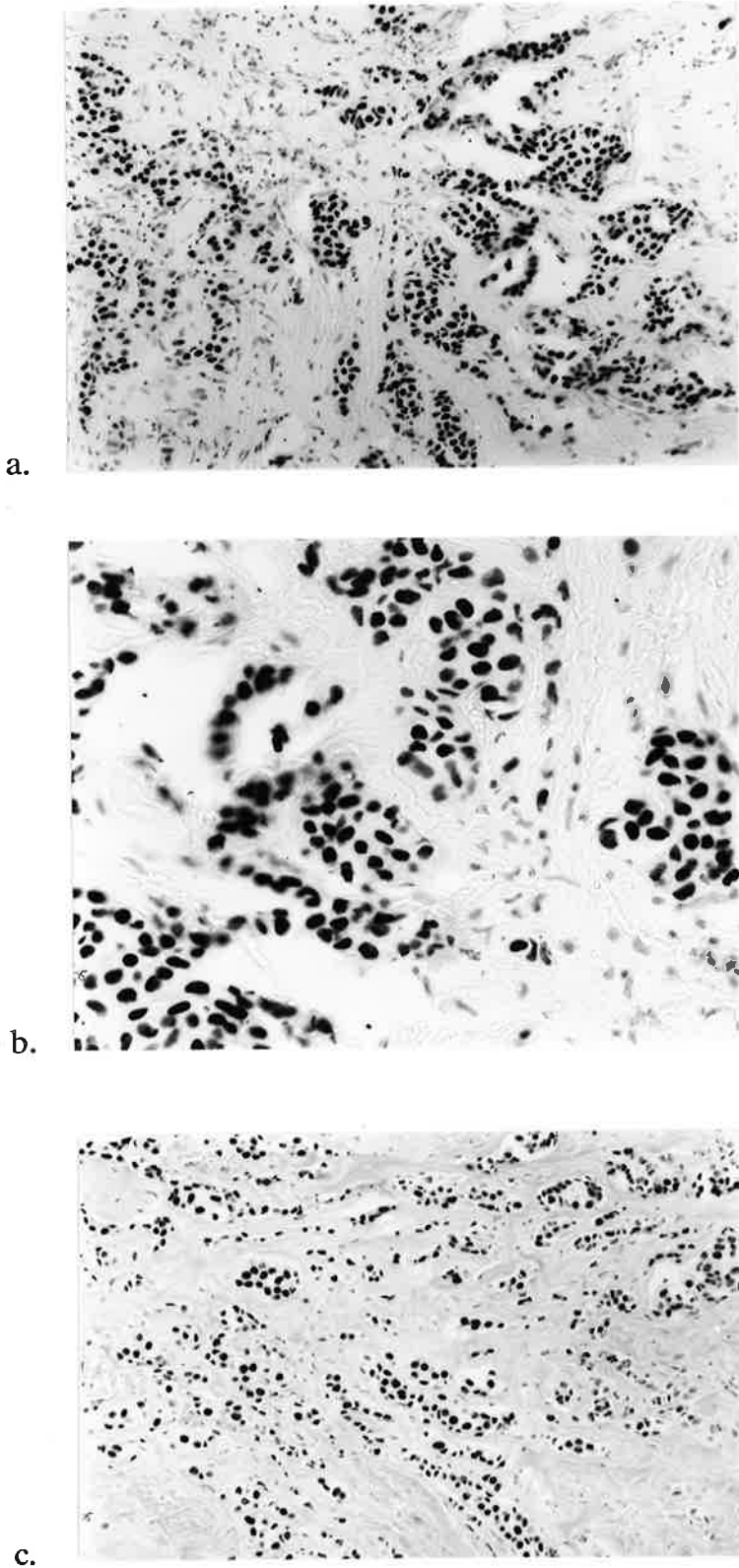
Figure IV.2.9. (a) An IDC, Grade II (Case 8), illustrating intense (+ + + +) ER staining with an ER count of 84 per cent (anti-ER, paraffin section), and demonstrated at higher magnification in (b). Nuclear staining, in the absence of cytoplasmic staining, is readily appreciated. (c) Enhancement of the reaction product by nickel modification of DAB is depicted in an adjacent section.

St Louis, MO, USA), were unsuccessful. Trypsin II was applied to formalin-fixed sections of known ER-positive cases at 0.5 mg/ml for two and for five minutes, and DNase I at 2.5 mg/ml and five mg/ml for 15, 30, 60, 90 and 120 minutes. Careful attention was paid to the published procedural details and it is not clear why these methods failed to work on this author's material. The use of "pronase E" from Sigma Chemical Company (Protease Type XXV, Cat.No. P-6911) at 0.2 mg/ml and 0.5 mg/ml for two and four minutes each was also unsatisfactory. Following digestion with these three enzymes the tissue was frequently disrupted and nuclear morphology was poor.

The most consistent and reproducible technique, therefore, was achieved with tissues fixed in neutral-buffered formalin for one and a half hours, pronase purchased from Calbiochem and a streptavidin-biotin technique.

Correlation with Frozen Section and Cytosolic Assays

Specific staining for ER was located exclusively in the nuclei of epithelial cells in all paraffin sections (Figures IV.2.8 - IV.2.10). Variations in the intensity and distribution of staining within a section were observed, as identified in ER-positive cryostat sections, and may be attributed to heterogeneity of the tumour cell population. ER-positive tumours showed staining of both *in situ* and infiltrating components when both were present in the section. Variable numbers of non-neoplastic epithelial cells were ER-positive in sections from both ER-positive and ER-negative carcinomas and served as in-built controls when present (Figure IV.2.8). Neoplastic cells in peroxidase-stained paraffin sections were much more readily
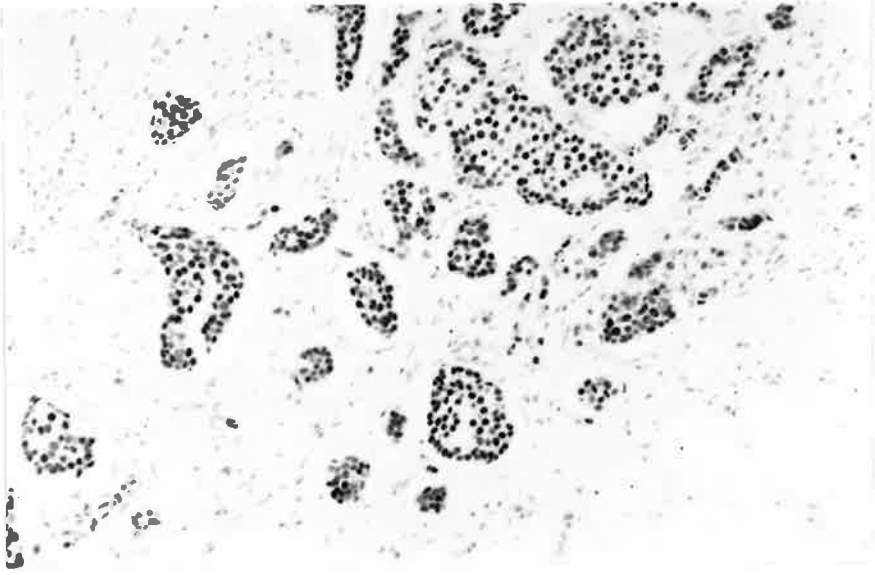
a.
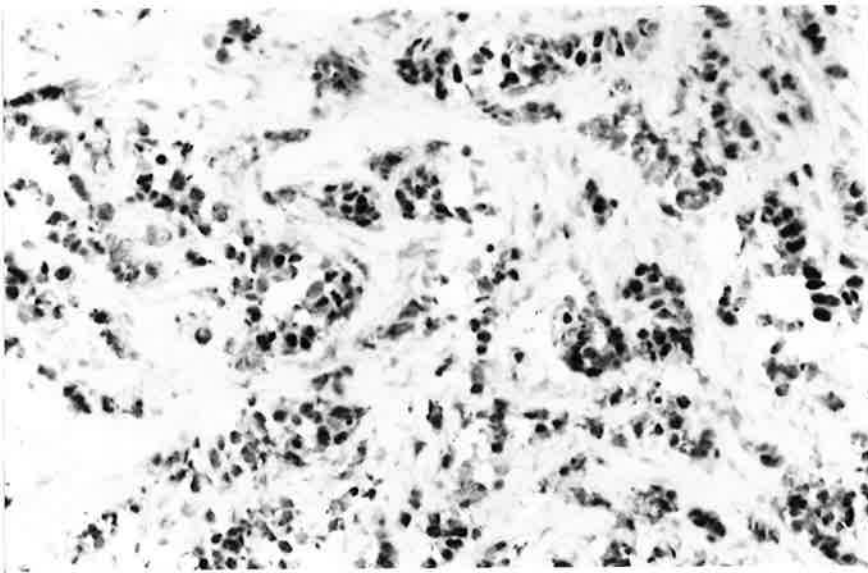
b.

Figure IV.2.10. (a) An ER count of 91 per cent is illustrated in this formalin-fixed paraffin section of a moderately-differentiated IDC (Case 2, x125), while 63 per cent of tumour cells are ER-positive in the corresponding PLP-fixed frozen section (b), depicted at higher magnification (anti-ER stain, x250). The cellular morphology and clarity of staining are clearly superior in the paraffin section (a).

differentiated from macrophages, benign epithelial cells and stromal cells than was possible in cryosections (Figure IV.2.10). Although some minor background staining was occasionally observed, there was no false positivity of epithelial nuclei in the negative controls. As expected, false positive staining was seen in cells with endogenous peroxidase activity such as erythrocytes and inflammatory cells.

The percentage of ER-positive neoplastic cells and the predominant staining intensity were assessed in a manner identical to that described for the cryostat sections. By convention, a tumour was considered "positive for ER" if greater than 10 per cent of neoplastic cells stained positive, although it is recognized that perhaps a tumour exhibiting any ER positivity should be regarded as "ER-positive".

ER counts determined by immunostaining paraffin sections, and the corresponding frozen section and cytosolic receptor values, are shown in Table IV.2.3. ER counts in the paraffin sections ranged from zero to 94 per cent and 27 of the 32 tumours were ER-positive. There was concordance between the paraffin and the frozen section-determined receptor status in 30 cases (94 per cent). The Spearman rank order correlation coefficient (r) relating ER counts in the two media was 0.76 (p <0.0001; Figure IV.2.11). In the paraffin sections of the two discordant cases there were focal areas in which up to 70 per cent of tumour cells exhibited intense ER positivity in a background of unstained or rarely stained cells (overall ER count approximately 15 per cent), while only seven to eight per cent of cells in the cryosections were ER-positive. The differences are thus most likely to be a reflection of tumour heterogeneity. Nevertheless, the finding of ER counts of greater than 10 per cent in paraffin sections of tumours deemed to be ER-negative with

TABLE IV.2.3.

IMMUNOCYTOCHEMICAL STAINING OF ERs IN

PARAFFIN AND FROZEN SECTIONS, AND CYTOSOLIC ER VALUES

Case No.	Paraffin		Frozen		Cytosolic ER Value (fmol/mg protein)
	ER Count*	Intensity#	ER Count*	Intensity#	
1	94	++++	87	++	487
2	91	+++	63	+	173
3	91	++	81	+++	121
4	90	++	89	+++	206
5	89	++++	81	+++	376
6	89	++	86	+++	1074
7	87	+++	91	++++	274
8	84	++++	66	+++	10
9	83	++	79	++	197
10	82	+++	76	++	256
11	81	+	80	++	156
12	80	+	65	++	93
13	79	++	33	+	111
14	77	+++	67	+	75
15	77	+++	81	+++	1339
16	73	++	37	+	11
17	72	+++	77	+++	391
18	72	++	82	+++	215
19	69	+	78	+++	186
20	69	++	45	++	41
21	61	+	75	++	11
22	60	+	77	+++	553
23	54	+	67	++	26
24	52	++	59	+++	516
25	49	+	49	+	42
26	15	+	8	+	0
27	15	++	7	+	45
28	0	0	0	0	0
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0	0
32	0	0	0	0	0

*ER count = Percentage of positively-stained tumour cells
#Intensity grading: 0 negative; + weak; ++ moderate;
 +++ strong; ++++ very strong (see text)
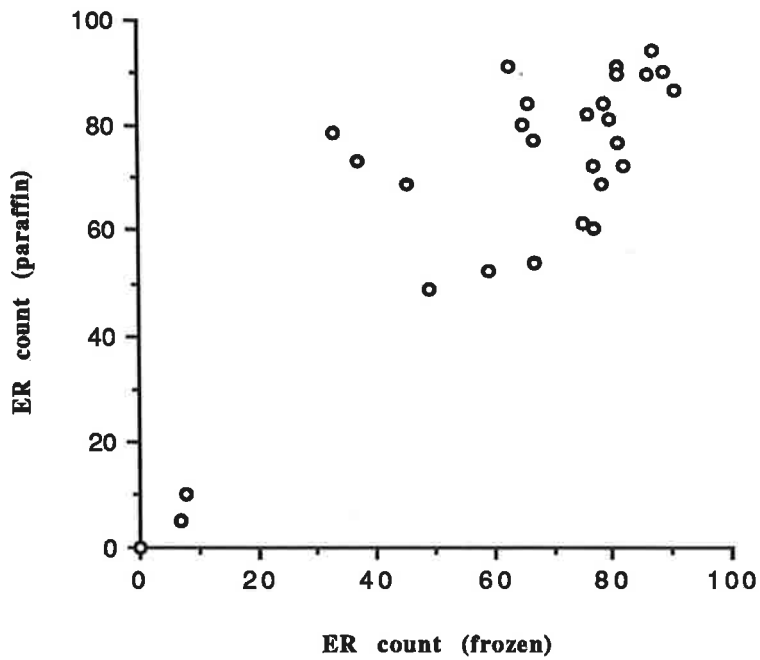
Figure IV.2.11. Relationship between paraffin section and frozen section ER counts in 32 breast carcinomas (r = 0.76, p < 0.0001).

frozen section immunostaining suggests that the paraffin technique may be more sensitive. Concordance between cryostat and paraffin section ER values is higher than most cited previously (Shimada et al, 1985; Andersen et al, 1986; Cheng et al, 1988). Shintaku and Said (1987) also reported a concordance of 94 per cent, however, the two outstanding cases in their study were ER-positive on frozen sections and negative on paraffin sections. It is possible that their method is less sensitive in failing to detect positivity in two tumours.

ER counts in paraffin sections were also compared with the ER content determined by cytosolic assay and there was concordance in 97 per cent (31) of cases (Table IV.2.3). A positive correlation was also observed ($r = 0.60$, $p < 0.001$) suggesting that the greater the percentage of positive nuclei, the higher the cytosolic value is likely to be. Using the biochemical assay as the standard, as most previous authors have done (Cheng et al, 1988), the sensitivity of this author's paraffin technique was 100 per cent. All cases ER-positive by cytosolic assay were ER-positive in paraffin sections (26 of 26). In one case (case 26) the cytosol ER value was zero and eight per cent of tumour cells were positive in the cryostat section (designated "ER-negative"), but the paraffin section demonstrated patchy areas of positive staining amounting to an overall ER count of 15 per cent. Heterogeneity of ER expression is again the most likely explanation for the discrepant values and may reflect sampling limitations inherent in the biochemical technique rather than "false positive" staining of the paraffin section. Other inaccuracies of the biochemical method have already been detailed and it thus seems more appropriate to compare the new ER immunostaining technique for paraffin sections with frozen section analyses.

Tumours which exhibited intense (+ + + or + + + +) ER staining in paraffin sections tended to have a higher percentage (greater than 70 per cent) of ER-positive carcinoma cells than weakly-stained ER-positive cases ($r = 0.79$, $p < 0.001$; Table IV.2.3). A correlation between frozen and paraffin sections was also observed in terms of the staining intensity ($r = 0.76$, $p < 0.0001$), although staining was generally stronger in frozen sections. Staining intensity and ER counts were not always proportional to the quantitative cytosolic ER values (Table IV.2.3).

There was no relationship between the ER count in paraffin sections and the histological grade of the carcinomas. This may, however, reflect the small sample size ($n = 32$) as frozen section ER counts were significantly related to tumour grade in the larger study population of 105 IDCs (Chapter IV.2.f).

In conclusion, ER-immunostaining of paraffin-embedded tissue is highly specific and, if performed on optimally fixed tissue specimens, is highly sensitive. The technique developed is simple and reliable when applied to tissues that have been embedded routinely in paraffin after fixation in formalin for one and a half hours. The advantages of paraffin section ER-immunostaining over the cytosolic assay are identical to those described in relation to ER staining of frozen sections and include direct selective assessment of ER protein, both occupied and unoccupied by endogenous estrogen. In addition, larger sections may be assessed on a paraffin section than is generally possible using cryostat sections and better cytomorphological preservation facilitates recognition of different cell types. However, as the receptor protein is very sensitive to the effects of prolonged or inappropriate fixation (Bur et al, 1987) and the duration, source and concentration of enzyme digestion, the

techniques devised by this author and others may not be as reliable when applied to inadequately fixed specimens. In such instances, for example when tissue is received from peripheral centres or malignancy was not suspected at operation, negative staining should be interpreted with caution, but when tissues are fixed immediately after excision ER staining of paraffin sections is excellent.

IV.2.h. DISCUSSION

In this chapter immunohistochemical techniques using the Abbott H222 anti-ER antibody to determine the ER content of breast tissues, prepared as imprints, cryostat sections and paraffin sections, were described. The optimal method of fixation for each medium was identified from a broad range of alternatives. The techniques are highly sensitive, simple, reproducible and readily applicable to routine diagnostic pathology, and the most appropriate method of ER determination may be chosen according to the type of specimen available.

Statistical analysis demonstrated a significant correlation between ER counts in frozen sections and imprints (n = 19). Similarly, ER counts in paraffin sections correlated with those in cryostat sections (n = 32; p < 0.0001). There was also a significant relationship between biochemically-determined ER values and receptor counts obtained by immunostaining frozen sections (n = 110), imprints (n = 19), and paraffin sections (n = 32) with the anti-ER antibody. The qualitative ER status defined by cytosolic assay and frozen section ER analysis showed a high degree of concordance with the paraffin section results.

Imprints are recognized to be suitable alternative preparations for immunohistochemical ER analysis, having greatest potential application in the study of small tumour specimens. Staining of paraffin-embedded tissues is, however, optimal and allows ER analysis of breast cancers to be performed on diagnostic tissue blocks. This technique may also potentially permit retrospective assessment of cases with a known clinical outcome.

A variable degree and intensity of ER staining was observed with the positive control section supplied with the Abbott ER-ICA Kit and it is recommended that a section from a known ER-positive breast tumour is used as an additional positive control. Prompt fixation of tissues, in particular imprints, is essential to avoid drying and to prevent loss of the estrophilin antigen.

The counting procedure employed to determine the ER count was considered accurate and reproducible, repeat counts showing no more than a five per cent variation. This counting method is somewhat tedious, but with experience it was possible to estimate the percentage of ER-positive cells on casual examination using a X10 or X20 objective. Estimates were relatively accurate when less than 10 per cent or greater than 80 per cent of neoplastic cells were receptor-positive. Errors were most likely to occur when the proportion of receptor-positive to receptor-negative cells approached a ratio of 1:1. Even so, an accurate determination of the percentage of receptor-positive cells may not therapeutically be as important as the distinction between receptor-positive and receptor-negative status (Thorpe, 1988). Decisions as to whether more than 10 per cent of the tumour cells are ER-positive can be readily made on the perusal of stained sections at low magnification (X4 or

X10 objective). Nevertheless, accurate counts were performed in this study to ensure reproducible and reliable data.

The assessment of ER staining intensity was subjective, as has been the case in most previous studies (Flowers et al, 1986; Tosi et al, 1987; Ozzello et al, 1987), and a very strong correlation between staining intensity and ER count was demonstrated for both paraffin and frozen sections. The relationships between ER status and various other histopathological parameters were not strengthened when the ER count was combined with intensity (ER score), or when intensity was analysed in place of the ER count (see also Chapter IV.3). These data suggest that assessment of intensity is not warranted as it apparently does not provide any additional information over a measure of the percentage of ER-positive cells. Similar conclusions were reached by Ozzello et al (1987) who related ER staining intensity to cytosolic assay results.

Several disadvantages of the immunocytochemical method are apparent. The tissue must be handled and processed very carefully to maintain antigenic reactivity, and results are only semi-quantitative and subjective unless complex computer-assisted image-analysis systems are employed (Bacus et al, 1988). While an assessment by such computer image analysers may be ideal, they are expensive and not accessible to all pathology laboratories.

Most of the interest in ERs relates to their value in predicting tumour response to hormonal therapy. Researchers therefore continue to seek the most accurate and reliable methods of tumour ER analysis as clinicians largely rely upon the results of

such analyses when deciding whether or not hormonal treatment is appropriate.
A large number of ER-positive cancers (up to 45 per cent), however, do not respond
to hormonal manipulation, and a small number (approximately 10 per cent) of ER-
negative cases do respond. This observation defies present explanations of the
mechanism of tumour hormone responsiveness, despite taking into account the
potential inaccuracies of the ER cytosol technique utilized in most studies published
to date. It has been suggested that in some ER-positive tumours the receptors may
be "inactive", rendering the tumour functionally ER-negative. An alternative
explanation may relate to an overriding influence of factors stimulating tumour
proliferation. Certain markers, for example, the "proliferating" cell antibody Ki-67,
may help identify an aggressive subgroup of ER-positive cancers, and it is for this
reason that the Ki-67 immunostaining technique was developed, forming the subject
of the next section.

IV.3. TUMOUR GROWTH FRACTION

a.   **INTRODUCTION**

b.   **METHODOLOGY**

c.   **RESULTS**

               1. Ki-67 staining
               2. Correlation with Histological Grade, Lymph Node Status and Clinical Parameters
               3. Correlation with ER Content

d.   **DISCUSSION**

IV.3. TUMOUR GROWTH FRACTION

IV.3.a. INTRODUCTION

Cell Kinetic Studies

Cell kinetic studies probably reflect the true proliferative activity of a given tumour and best define its potential biological aggressiveness and behaviour. Until recently, tumour doubling times (Cooperman & Hermann, 1984), mitotic cell counts (Hoffman, 1949) and procedures to detect cells in the DNA synthesis phase of the cell cycle, such as thymidine uptake studies (Meyer & Bauer, 1975; McDivitt et al, 1986), DNA analysis by flow cytometry (Meyer, 1982; McDivitt et al, 1986) and bromodeoxyuridine staining (Sugihara et al, 1986; Riccardi et al, 1988), have been the main methods of studying tumour kinetics. Using such techniques, an inverse relationship between the tumour proliferative activity and the relapse-free intervals and overall survival times of breast carcinoma patients has been demonstrated by a number of workers (Meyer & Hixon, 1979; Gentili et al, 1981; Meyer et al, 1983; Tubiana et al, 1984; Silvestrini et al, 1985). A high proliferative rate has thus been established as an important independent indicator of early relapse in breast cancer and is useful in predicting the likely benefit of chemotherapy. Tumours with a large growth fraction (GF) show a much better response to chemotherapy than those with a small GF (Sulkes et al, 1979; Charlson & Feinstein, 1982; Chapter II.g). Although these techniques have been shown to produce prognostically useful data,

their use has been largely limited to research laboratories because most are laborious and expensive to perform. For instance, the measurement of TLIs requires prolonged incubation of the excised tissue with radioactive isotopes. Furthermore, tissue relationships and morphological details are lost during the preparation of single cell suspensions for flow cytometry. Immunohistochemical analysis using the monoclonal antibody Ki-67, which stains a nuclear antigen in human proliferating cells, overcomes many of the disadvantages of the former methods.

Previous studies have reported a correlation between high rates of replication of neoplastic cells, measured by DNA flow cytometry or tritiated thymidine techniques, and paucity or absence of ER, determined by cytosolic ER evaluations (Silvestrini et al, 1979; Kute et al, 1981; Moran et al, 1984; Meyer et al, 1986; McDivitt et al, 1986; Paradiso et al, 1988). The inaccuracies inherent in each of these techniques have already been detailed (Chapters II.g & IV.2). Three studies explored the relationship between ER content and Ki-67 immunostaining in breast carcinoma (McGurrin et al, 1987; Barnard et al, 1987; Gerdes et al, 1987). McGurrin and coworkers described a complex association between Ki-67 labelling rates and biochemically determined ER levels. Although there was no obvious correlation, eight of nine tumours with Ki-67 counts over 30 per cent were ER-negative, and tumours with low and intermediate Ki-67 counts often had high ER values. Barnard et al (1987) found no association between cytosolic ER status and the Ki-67 count. An inverse correlation between Ki-67 counts and ER status determined by immunohistochemical assay has been documented only once previously, in a series of 76 breast cancers (Gerdes et al, 1987). A group of exceptional cases with positive ER status and moderate or large GFs was noted.

In this study the Ki-67 counts for 115 malignant breast neoplasms were correlated with the tumour ER content, histological grade, axillary nodal status and several clinical parameters in order to establish the reproducibility of Gerdes' work and relate tumour proliferative activity to some established histological prognostic indices.

Ki-67 Antibody

Ki-67 is a murine monoclonal antibody identified serendipitously by Johannes Gerdes and associates in Kiel, West Germany, during studies aimed at the production of antibodies to Hodgkin and Reed-Sternberg cell nuclear antigens (Gerdes et al, 1983 & 1984a). Ki-67 is specific for a nuclear epitope expressed in all but the G_0 (resting) phase of the human proliferating cell cycle. Ki-67 labelling of cells in the G_1, S, G_2 and M phases is therefore a measure of GF in tumour cell populations. The exact nature of the antigen recognized by Ki-67 is unknown. Although there are similarities between Ki-67 and monoclonal antibodies used to detect DNA polymerase-α (Namikawa et al, 1987), DNA synthesis is not essential for expression of this antigen whereas protein synthesis is vital.

There is good evidence that expression of the antigen identified by Ki-67 is closely related to cell proliferation. Firstly, Ki-67 immunoreactivity is observed in cells known to be proliferating, such as cells in the germinal centres of cortical follicles, the necks of the gastrointestinal crypts and undifferentiated spermatogonia, but is absent in cells such as hepatocytes, mature sperm cells, and neurones, known to remain in a resting state (Gerdes et al, 1983); secondly, antigen expression can be

induced in mitogen-triggered peripheral blood lymphocytes, and is lost when HL-60 cells are stimulated with phorbol esters to differentiate into mature macrophages (Gerdes et al, 1984a); and thirdly, a close relationship is observed between Ki-67 counts and the fraction of cells in the S phase of the cell cycle, as determined by tritiated-thymidine and flow cytometric studies (Gerdes et al 1984a; Schwarting et al, 1986; Baisch & Gerdes, 1987; Schwartz et al, 1989). Most recently, Kamel et al (1989) described a strong correlation between the TLI and Ki-67 counts for separately and double-labelled cryostat sections in 28 malignant breast tumours. A correlation between Ki-67 counts and uptake of bromodeoxyuridine has also been observed (Schrape et al, 1987; Sasaki et al, 1988). Ki-67 immunostaining is therefore a technically simple, reproducible, specific and rapid means of quantitating the fraction of proliferating cells in frozen sections.

A number of benign tissues and malignant tumours have been studied with the Ki-67 antibody and the percentage of Ki-67-positive cells correlated with traditional prognostic parameters. In one study, highly proliferative lung tumours, such as small cell carcinomas, exhibited a mean Ki-67 count of greater than 25 per cent, in contrast to the scores of less than 10 per cent demonstrated in carcinoid tumours of the lung (Gatter et al, 1986). A strong correlation was found between the percentage of Ki-67-positive non-Hodgkin's lymphoma cells and the Kiel classification (Gerdes et al, 1984b). High grade lymphomas exhibited Ki-67 counts of 50 to 83 per cent compared with a range of 2.5 to 25 per cent observed in low grade tumours. The mean Ki-67 count was significantly higher in prostatic carcinomas than in benign prostatic glands, whilst a significant correlation between the Ki-67 count and tumour histological grade was recorded in a study of 31 prostatic biopsies (Raymond et al,

1988).

Malignant breast tumours are reported to exhibit mean Ki-67 counts ranging from 15 to 20 per cent, compared with mean counts of between three and five per cent observed in benign breast lesions. Ki-67 counts have been related to the histological type and grade of breast tumours in several studies, higher counts correlating with higher histological grades or more anaplastic carcinomas (Gerdes et al, 1986; Lellé et al, 1987; McGurrin et al, 1987; Barnard et al, 1987; Raymond & Leong, 1989a). These studies confirm associations described in earlier papers in which only the S phase fraction of cycling cells was measured (Chapter II.g).

The Ki-67 antigen is not preserved in routinely processed, paraffin-embedded tissues, precluding retrospective analyses of tumours in which clinical outcome is known. In this study the Ki-67 count was compared to histopathological variables known to have some prognostic value.

IV.3.b. METHODOLOGY

Six-micron cryostat sections from 12 benign breast tissues and 115 malignant breast neoplasms (Chapter III.1) were thaw-mounted onto tissue-adhesive treated slides (Rentrop et al, 1986), air-dried overnight (approximately 18 hours), and then fixed in acetone at $22^{0}C$ for 10 minutes, followed by two 30-second exposures to microwave irradiation (Leong & Milios, 1986). In five cases the effect of prolonged air-drying on the intensity and extent of Ki-67 staining was assessed by storing

additional consecutive sections for up to three days prior to fixation.

Immunohistochemical staining was performed as detailed previously (Chapter III.2.b.; Appendix II). The sections were incubated with the Ki-67 antibody (Dakopatts, Copenhagen, Denmark) at a dilution of 1:40 for 60 minutes, and with biotinylated horse anti-mouse secondary antibody for 30 minutes, both at 22°C (Raymond et al, 1988; Raymond & Leong, 1989d). A highly proliferative IDC was included as a standard positive control for each run.

Cells were designated Ki-67-positive when there was granular brown staining of epithelial nuclei. The percentage of Ki-67-positive cells, termed the Ki-67 count, was determined by assessing 500 morphologically malignant cells or benign epithelial cells in multiple fields using an ocular graticule in a manner identical to that used for deriving ER counts (Chapters III.2.d. & IV.2). In carcinomas demonstrating a large intraductal component, in addition to diffuse infiltration, a Ki-67 count was performed for each pattern in order to compare the relative proliferative rates.

No additional information is likely to be gained from an assessment of Ki-67 staining intensity as any positive staining indicates the cell is part of the cycling population (Gerdes et al, 1983 & 1984a), and thus intensity values were not assigned to Ki-67-positive cells.

Consecutive cryostat sections had been stained with monoclonal anti-ER and ER counts determined (Chapter IV.2.c.).

IV.3.c. RESULTS

1. Ki-67 Staining

Ki-67 counts for the 12 cases of benign breast epithelium ranged from zero to five per cent, with a mean of 2.21 per cent (S.D. 2.62). This was significantly lower than the mean Ki-67 count of 17.4 per cent (range from two to 67 per cent, S.D. 13.18) for 112 malignant breast tumours (t = 3.97, p <0.0001; U = 77, p <0.00001). The mean Ki-67 count for the subgroup of six normal breast tissues was 1.91 per cent.

In each case distinct, granular, brown nuclear staining was observed (Figure IV.3.1). Multiple discrete intra-nuclear foci of variable staining intensity, probably representing nucleoli, were frequently apparent (Figure IV.3.2). A regional variation in the proportion of cells immunostained by Ki-67 was occasionally seen within a single malignant lesion. In some tumours, counts of up to 40 per cent could be observed in foci of intraductal carcinoma and at the infiltrating margins of the tumour, despite a mean Ki-67 count of only 15 to 20 per cent (Figure IV.3.3).

In three IDCs reliable Ki-67 counts could not be guaranteed. In one case the cryostat sections contained predominantly intraductal carcinoma with a high Ki-67 count, but there was insufficient infiltrating tumour to allow a representative estimate of the overall GF. Poor cytomorphological preservation and the presence of numerous red blood cells and lymphocytes in the frozen tissue of two cases impaired accurate assessment. Only 112 of the 115 tumours were therefore included in the
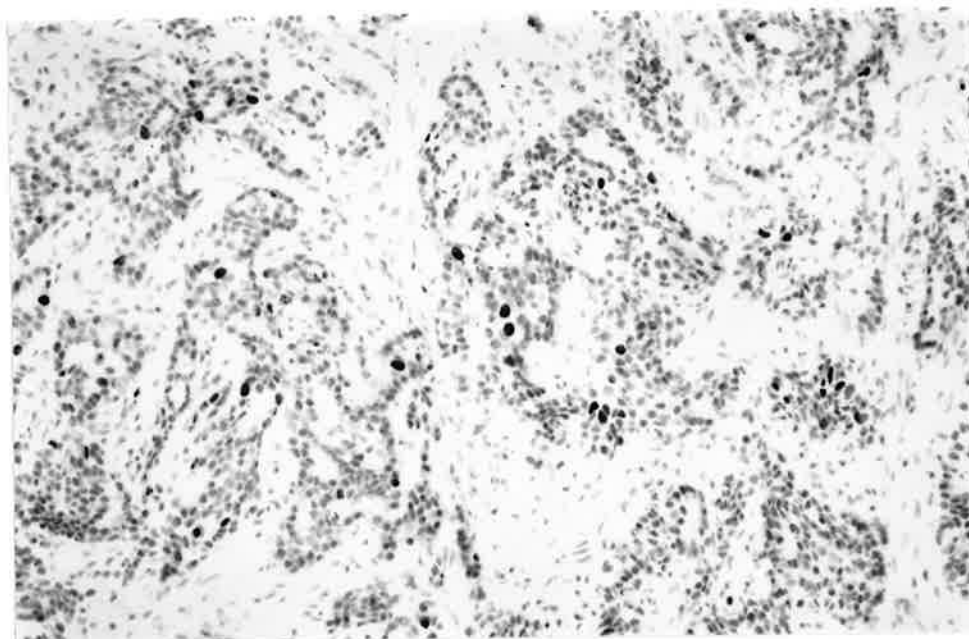
a.
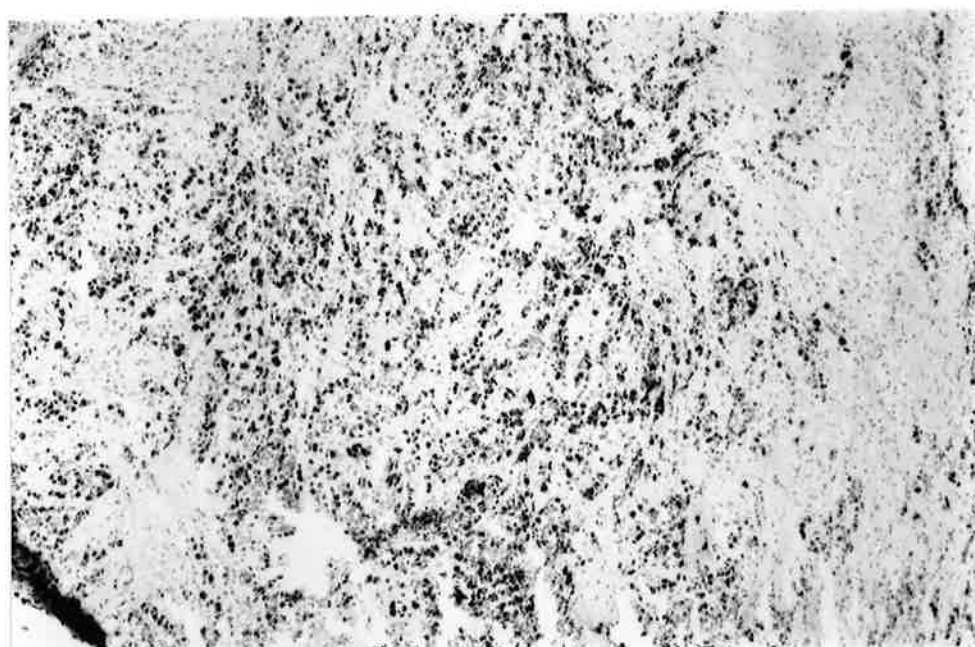
b.

Figure IV.3.1. Two IDCs illustrating contrasting GFs. (a) Ki-67 nuclear staining is identified in 15 per cent of neoplastic cells in this Grade I tumour with an ER count of 75 per cent (Ki-67 stain, haematoxylin counterstain). (b) A Grade III carcinoma with a Ki-67 count of 58 per cent and an ER count of less than 1 per cent (Ki-67 stain, haematoxylin counterstain).
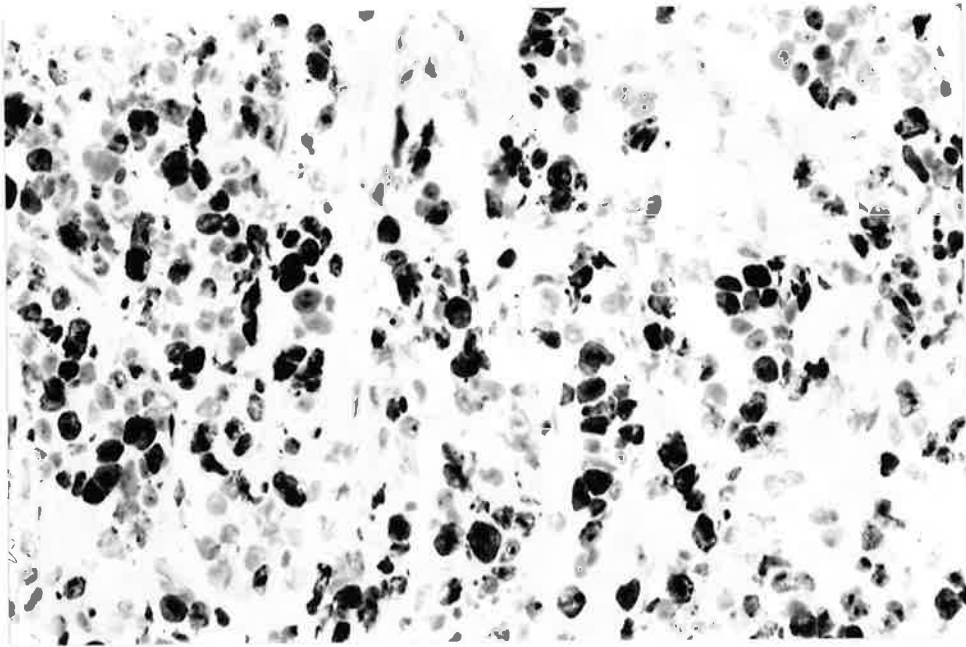
a.
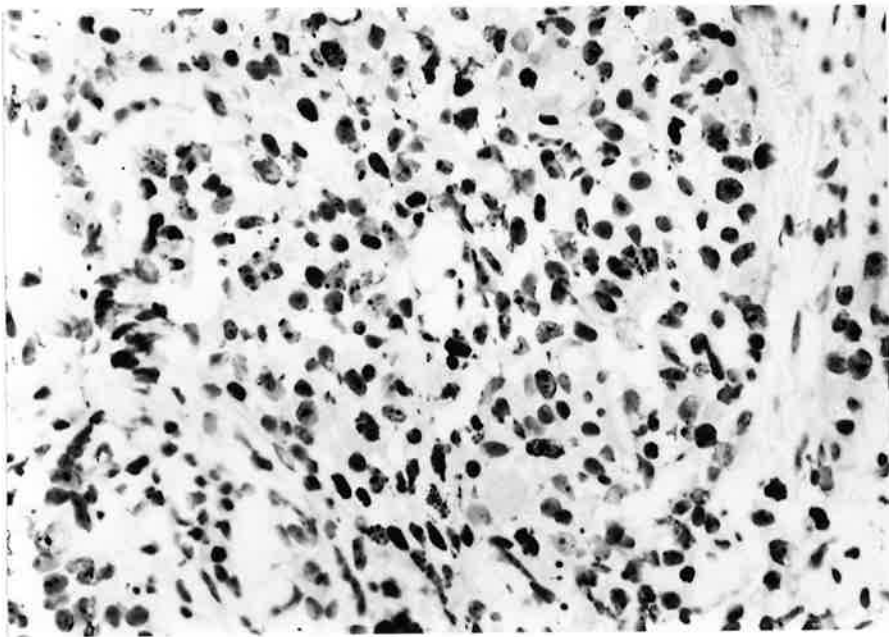
b.

Figure IV.3.2. (a) Higher magnification photomicrograph of the poorly-differentiated carcinoma illustrated in Figure IV.3.1.b, and (b) a moderately-differentiated infiltrating ductal carcinoma with a Ki-67 count of 30 per cent. In both, intranuclear foci of accentuated staining are readily identified (Ki-67 antibody, haematoxylin counterstain).
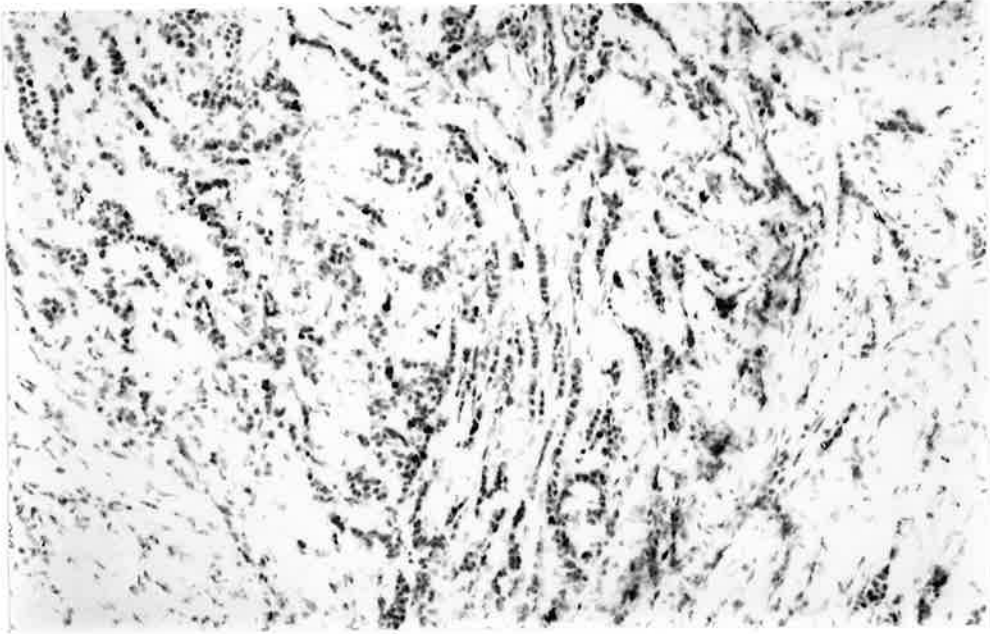
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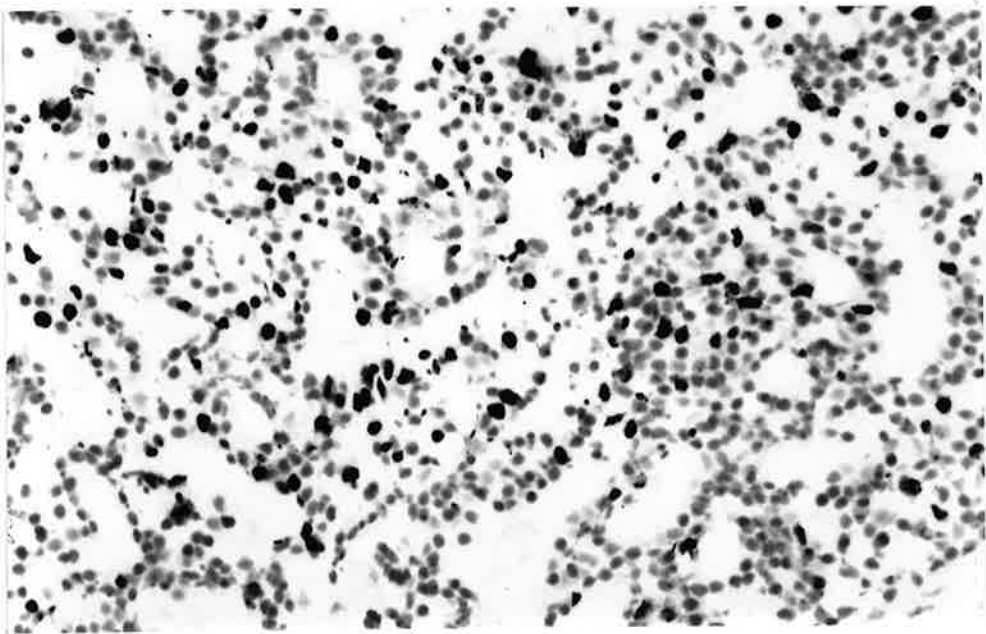
b.

Figure IV.3.3. An IDC, Grade I, demonstrating a regional variation in Ki-67 staining. In (a) approximately 10 per cent of the infiltrating tumour cells show nuclear staining, whereas in the intraductal portion of the same tumour (b), up to 40 per cent of cells stain positively. The mean Ki-67 count was 17 per cent (Ki-67 antibody, haematoxylin counterstain).

statistical analyses of Ki-67 counts.

Staining with the Ki-67 antibody was consistent and there was no loss of the Ki-67 antigen if the period of air-drying prior to fixation was extended to 48 or 72 hours. In addition, unfixed sections air-dried for up to a week were observed to still demonstrate Ki-67 positivity, although the study was not controlled for slides stored for this length of time and a comment on the degree of antigen preservation cannot be made. Weak cytoplasmic staining was noted sporadically, but never in the absence of nuclear staining, as reported by the majority of investigators utilizing the Ki-67 antibody (Gerdes et al, 1983 & 1986; Lellé et al, 1987; McGurrin et al, 1987; Barnard et al, 1987).

2. Correlation with Histological Grade, Lymph Node Status and Clinical Parameters

A strong positive correlation between the Ki-67 count and the histological grade of the IDCs (r = 0.52, p < 0.0001, n = 103) was demonstrated (Figure IV.3.4). The Ki-67 count was not significantly related to the presence of axillary metastases (either assessed as the absolute nodal status or following division into the subgroups of zero, one to three, and greater than three nodes positive), the size of the primary tumour or the patient's age.

No significant difference in Ki-67 counts was observed between the groups of premenopausal and postmenopausal patients (premenopausal population mean Ki-67 count = 22.1, n = 23; postmenopausal population mean Ki-67 count = 16.3,
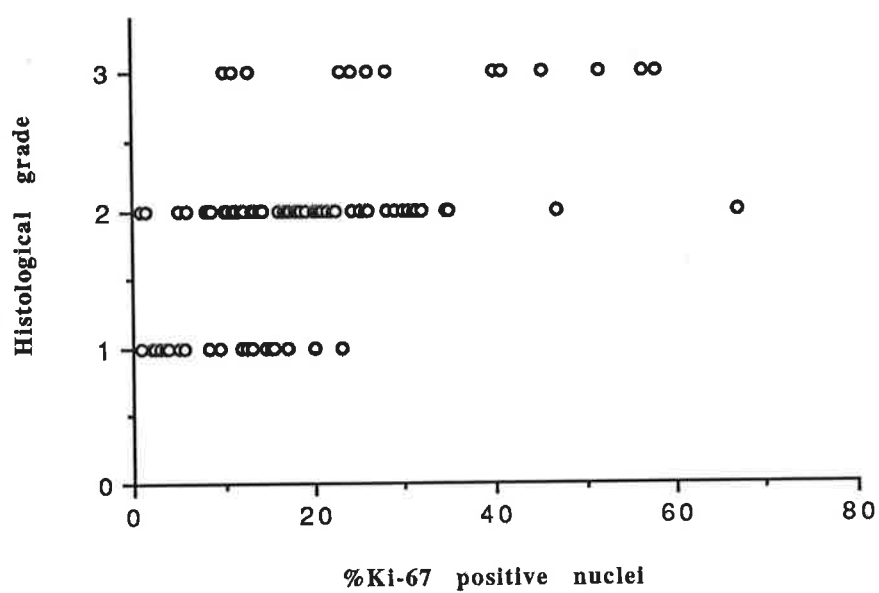
Figure IV.3.4. Relationship between the histological grade and the Ki-67 count (% Ki-67 positive nuclei) in 103 infiltrating ductal carcinomas (r = 0.52, p <0.0001).

n = 83), in agreement with data published by Barnard et al (1987). However, several studies have demonstrated GF to be higher in cancers from premenopausal women than in those from postmenopausal women (Gentili et al, 1981; McGurrin et al, 1987). These discrepant findings may be a reflection of the relatively small number of premenopausal patients compared with postmenopausal patients in this series of 115 tumours. Even so, the patient population studied in this thesis is larger than most of those previously examined.

3. Correlation with ER Content

The results of immunocytochemical assessment of ER content using the Abbott monoclonal anti-ER antibody and the ER values obtained using a standard dextran-coated charcoal cytosolic assay were detailed in the previous section (Chapter IV.2).

A significant inverse correlation (r = -0.39, p <0.001) was revealed between the tumour GF (Ki-67 count) and the immunohistologically determined ER content (ER count) in 112 malignant breast neoplasms, as depicted in Figure IV.3.5. The inverse correlation was slightly weaker, but still significant, if the biochemical ER values were compared with the Ki-67 counts (r = -0.35, p <0.001). The ER score (ER count X staining intensity) was also inversely related to the GF (r = -0.35, p <0.01, n = 74), as was intensity alone (r = -0.24, p <0.05, n = 74).

The relationship between GF and ER staining intensity has not previously been addressed. Despite the persistence of the inverse correlation between GF and ER content when taking into account ER staining intensity, the strength of the
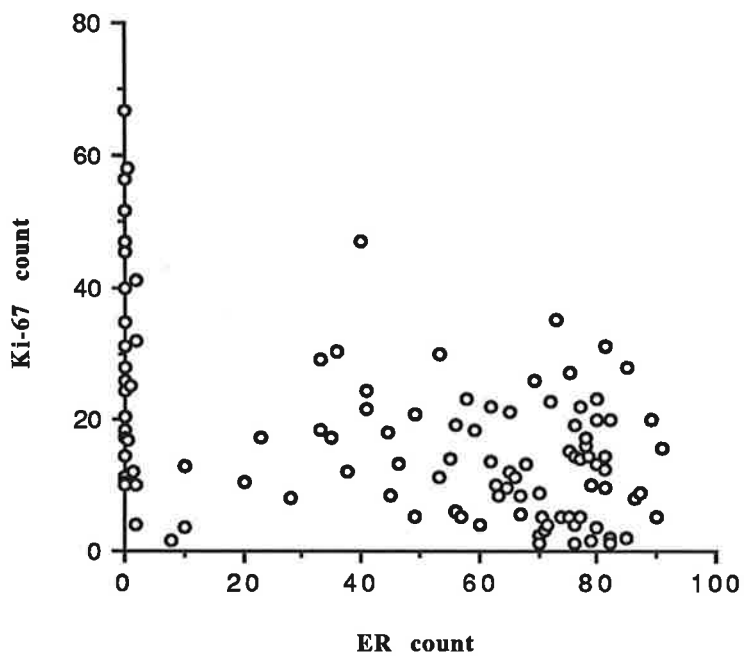
Figure IV.3.5. Relationship between the Ki-67 count and the immunohistologically-determined ER count in 112 malignant breast neoplasms (r = -0.39, p < 0.001).

correlation based on the ER count alone was greater, supporting the previous assertion that subjective assessment of ER staining intensity is probably unnecessary.

A statistical comparison of the relationship between Ki-67 and ER counts when tumours were stratified into premenopausal and postmenopausal categories was of limited value, due to the relatively small number of premenopausal patients (n = 23). Nevertheless, the inverse correlation between GF and ER content held true for each subgroup (premenopausal, n = 23, r = -0.52, p <0.05; postmenopausal, n = 83, r = -0.36, p <0.001), and no significant difference was observed between the two groups.

Three to five per cent of epithelial and stromal cells were Ki-67-positive in the malignant phyllodes tumour, with 80 per cent of the epithelial cells staining ER-positive. The male breast carcinoma and the carcinosarcoma were both ER-negative and had Ki-67 counts of 17.2 and 47 per cent respectively. There were too few lobular and intraductal carcinomas for meaningful comparisons of ER and Ki-67 counts between the different tumour subtypes.

IV.3.d. DISCUSSION

This study of 112 malignant breast neoplasms is the largest series to assess both the ER status and the Ki-67 count, representing the tumour GF. The results concur with those of Gerdes et al (1987) who examined a series of 76 carcinomas, and with studies reporting an inverse correlation between tumour GF and ER content using

non-immunocytochemical methods (Kute et al, 1981; Moran et al, 1984; Meyer et al, 1986; McDivitt et al, 1986).

A significant inverse correlation between Ki-67 and ER counts was identified. However, closer examination of the data revealed the relationship between the receptor status and the GF to be more complex. The inverse correlation was largely due to eight tumours with very high Ki-67 counts (greater than or equal to 40 per cent), of which seven were ER-negative, and a larger group of extensively ER-positive tumours with GFs of less than 10 per cent (Figure IV.3.5). Tumours with intermediate Ki-67 counts (between 10 and 40 per cent) exhibited a wide distribution of ER values, ranging from completely negative to very high counts. Thus, slowly proliferating tumours are predominantly ER-positive and very highly proliferative carcinomas are generally ER-negative, but tumours exhibiting an intermediate turnover may be either ER-positive or negative. It may be that the group of ER-positive tumours with intermediate GFs accounts for the 40 to 45 per cent of patients with ER-positive tumours who fail to respond to hormonal therapy (Lippman & Allegra, 1978; McGuire et al, 1986). These ER-positive, more rapidly proliferating tumours possibly escape hormonal control due to the presence of an aggressive, autonomous cell subpopulation. Ki-67 counts may thus be able to discriminate prognostic subgroups within the categories of ER-positive and ER-negative cancers. Insufficient follow-up time has elapsed since the Ki-67 antibody was first produced commercially to relate the presence and degree of Ki-67 positivity directly to survival, and follow-up of the patients within this author's sample is proceeding.

These data suggest that the predictive value of ER status in breast neoplasia is linked to its relationship with the tumour GF. This theory is supported by a recent study of 52 cases of advanced breast cancer in which GF was evaluated by thymidine labelling studies and ER content assessed with the cytosolic assay (Paradiso et al, 1988). An objective clinical response was observed in most patients with ER-positive indolent tumours treated with hormonal therapy, but only in a few patients with ER-positive rapidly proliferating tumours. Meyer et al (1983) found that the probability of relapse was significantly related to the TLI, independent of cytosolic ER content. ER was related to the probability of relapse in the below median TLI group only. Therefore, prior relationships described between ER content and survival (Chapter II.f) may have derived largely from the inverse correlation between ER content and the TLI (Meyer et al, 1983; Silvestrini et al, 1986). Whether ER content has any prognostic value independent of its relationship to tumour GF is uncertain and must await further investigation.

Despite a wide scatter of Ki-67 counts within each grade there was a significant relationship between the GF and the histological grade of the IDCs (Figure IV.3.4). This is in agreement with previous reports measuring GF either by thymidine labelling (Gentili et al, 1981) or Ki-67 immunoreactivity (McGurrin et al, 1987; Barnard et al, 1987; Lellé et al, 1987). Histological grading has been shown to provide some useful prognostic information (Chapter II.c) and was analysed here in an attempt to offset the limited clinical follow-up, and thus to indirectly associate Ki-67 with prognosis.

No relationship between the axillary lymph node status and tumour proliferative

activity was found, despite the fact that the presence and extent of nodal metastasis is currently the most useful predictor of breast cancer relapse (McDivitt et al, 1986; Chapter II.e). An explanation for this unexpected lack of correlation is not readily apparent, but similar results have been reported by other workers using both radioisotope labelling (Gentili et al, 1981; Meyer et al, 1986) and Ki-67 immunostaining (McGurrin et al, 1987; Barnard et al, 1987) techniques. The metastatic capability of a tumour may be determined by variables such as inherent invasiveness, the duration of the neoplasm, and/or the existence and selection of specific metastatic cell sublines that develop and spread independently of the tumour proliferative rate.

Thus, while there appears to be a negative correlation between Ki-67 and ER counts in breast carcinomas, the most appropriate use of these parameters in planning an optimal treatment régime, and in predicting survival, may result from a consideration of the GF and ER status as independent factors. A knowledge of both ER status and GF may most accurately select patients who will benefit from hormonal therapy and/or systemic cytotoxic therapy.

Tumours with high ER counts and low Ki-67 values might be treated most successfully with hormonal therapy alone, high Ki-67 counts and low ER content would suggest the need for systemic chemotherapy and/or radiotherapy, while patients possessing ER-positive tumours with an intermediate to high Ki-67 count may be recognized as a poor prognostic group who would perhaps benefit most from aggressive therapy, with or without combined endocrine manipulation.

A high GF, as a marker of tumour aggressiveness, may account for the failure of many ER-positive tumours to respond to endocrine therapy. GF may prove to be the overriding parameter in predicting outcome for patients with ER-positive tumours. Determination of the true prognostic usefulness of Ki-67 and ER values, and their role in predicting optimal treatment protocols, awaits clinical testing with long-term follow-up and assessment of survival data.

IV.4. INTERMEDIATE FILAMENT PROTEIN ANALYSIS

a. INTRODUCTION

b. METHODOLOGY

c. RESULTS

1. Coexpression of cytokeratins and vimentin in benign breast epithelium

2. Vimentin expression in breast carcinomas

3. Correlation between vimentin expression and tumour growth fraction

d. DISCUSSION

IV.4. INTERMEDIATE FILAMENT PROTEIN ANALYSIS

IV.4.a. INTRODUCTION

The expression of intermediate filaments (IFs) by neoplastic cells is one area which has received little attention in relation to tumour prognosis. The IFs are a group of filamentous protein polymers that form the principal cytoskeletal components of eukaryotic cells (Osborn & Weber, 1983). Their diameter of seven to 10 nm is "intermediate" between the diameter of the smaller microfilaments (six nm) and the larger microtubules (25 nm). Although the IFs from different cells are indistinguishable from one another at ultrastructural level, they are biochemically and immunologically distinct and can be subdivided into five major classes. The cytokeratins, previously discussed in Chapter IV.1, are expressed by all epithelial cells and represent a family of at least 19 different protein subunits with molecular weights ranging from 40 to 68 kd. Vimentin is a protein of molecular weight 52 kd and is characteristically found in cells of mesenchymal origin. Desmin is expressed by smooth, skeletal and cardiac muscle cells, glial fibrillary acidic protein (GFAP) by all types of glial cells, and neurofilaments are characteristic of neurones.

There is evidence that neoplastic cells retain the IF type characteristic of their parent tissue (Gabbiani et al, 1981; Altmannsberger et al, 1981; Osborn & Weber, 1983; Gown & Vogel, 1984 & 1985) and analysis of these proteins has proven useful in aiding histological classification of human neoplasms. With the ready availability of commercial monoclonal antibodies specific for the five different IF groups,

immunohistochemical studies of cytoskeletal IFs have become widespread in diagnostic surgical pathology.

Although individual tumours generally express the same single filament type as their progenitor cells, an increasing number of reports describe neoplasms which express additional IF classes (Leong, 1988). The expression of multiple filament types, in most instances, occurs in a predictable manner and may be helpful in tumour diagnosis.

Coexpression of vimentin, traditionally accepted to be specific for mesenchymal cells, and cytokeratins has been reported in a number of epithelial neoplasms (Azumi & Battifora, 1987). These include renal adenocarcinomas (Holthofer et al, 1983), pulmonary carcinomas (Gatter et al, 1986; Upton et al, 1986), thyroid adenocarcinomas (Miettinen et al, 1984; Buley et al, 1987), ovarian carcinomas (Miettinen et al, 1983a; Benjamin et al, 1987), endometrial adenocarcinomas (McNutt et al, 1985), salivary gland tumours (Casalitz et al, 1981), choroid plexus tumours (Doglioni et al, 1987) and prostatic adenocarcinomas (Leong et al, 1988). Despite the expression of vimentin, these tumours are still considered to retain their essential epithelial nature (Gould, 1986).

Lower molecular weight cytokeratins are present diffusely throughout the cell cytoplasm or are distributed in a subplasmalemmal or apical location in all of these epithelial tumours, but, with the exception of the pulmonary, ovarian and thyroid carcinomas, higher molecular weight cytokeratins are not identified. Vimentin expression is often regional and has been observed in endometrial (McNutt et al,

1985) and prostatic adenocarcinomas (Leong et al, 1988) as distinctive paranuclear arrays aligned in the long-axis of the tumour cells and their non-neoplastic counterparts. This finding is in keeping with the tight association observed ultrastructurally between vimentin filaments and the nucleus (Lazarides, 1980). It is possible that the IF profile of the cell directly or indirectly influences its microscopic appearance, perhaps by governing interactions between the nucleus and the cytoplasm or by supporting cytoplasmic organelles.

Expression of vimentin IFs in breast epithelium has been mentioned in only two previous papers (Leader et al, 1987; Azumi & Battifora, 1987). Hitherto, all studies utilizing anti-vimentin antibodies in the assessment of breast carcinomas have consistently reported these neoplasms as vimentin-negative (Gabbiani et al, 1981; Osborn & Weber, 1983; Gown & Vogel, 1984 & 1985).

One separate group of mammary carcinomas recently recognized to coexpress cytokeratin and vimentin IFs are the primary spindle cell carcinomas, or pseudosarcomas, of the breast (Ellis et al, 1988; Merino et al, 1988). The expression of cytokeratins by these tumours confirms their epithelial nature and justifies the use of the term "carcinoma". However, they also demonstrate spindle cell morphology, an appearance traditionally associated with vimentin-positive cells of mesenchymal origin. Spindle cell carcinomas are distinct from true carcinosarcomas in which the sarcomatous component is often morphologically differentiated into elements which do not express cytokeratins, such as bone, cartilage or muscle.

Other tumours which coexpress cytokeratins and vimentin are mesotheliomas, synovial sarcomas, epithelioid sarcomas and neoplasms demonstrating a mixture of tissue types such as carcinosarcomas, mixed Müllerian tumours, teratomas and Wilm's tumours.

Immature embryonic cells may express vimentin IFs which are lost at a later stage of cellular differentiation, possibly as the maturing cells begin to synthesize other cytoskeletal proteins (Dahl, 1981; Damjanov, 1982). The coexpression of vimentin and cytokeratins in primary epithelial tumours may be attributed to a reversion of the neoplastic cells to embryonic states, and thus be in accordance with the theory of "dedifferentiation" proposed for such malignancies (Gould, 1986). Such coexpression has been reported in epithelial cells in culture (Virtanen et al, 1981) and in metastatic carcinoma cells in serous cavities (Ramaekers et al, 1983). On the basis of their studies with mouse parietal endodermal cell cultures, Lane et al (1983) hypothesized that the acquisition of vimentin may be related to reduced cell-to-cell contact and is a property of epithelial cells that survive independently. However, the expression of vimentin is clearly modulated by poorly understood intrinsic and extrinsic cell factors.

The postulated relationship between the synthesis of vimentin IFs by epithelial cells and reversion to a more primitive form led this author to propose that the acquisition of vimentin may influence the prognosis of epithelial neoplasms. A single report of a series of lung carcinomas related vimentin expression to poorly-differentiated tumours (Upton et al, 1986); however, no studies have associated vimentin expression in human carcinomas with tumour behaviour.

A detailed study of the coexpression of cytokeratins and vimentin in a wide range of benign and malignant breast epithelium is presented in this chapter. The degree of vimentin immunoreactivity in 115 malignant breast neoplasms is related to the tumour GF, represented by the percentage of Ki-67-positive tumour cells (Chapter IV.3). Vimentin positivity is also related to histological grade, axillary nodal status, ER status, primary tumour size and patient age.

IV.4.b. METHODOLOGY

Paraffin-embedded tissues from 135 breast biopsy and mastectomy specimens received by the IMVS were examined. In addition to the 115 malignant breast neoplasms detailed in Chapter III.1, there were three examples of normal breast epithelium from mammoplasty specimens and seventeen cases of benign breast proliferations, comprising four fibroadenomas, four lactating adenomas, two intraductal papillomas, one tubular adenoma and six cases of fibrocystic change. For each malignant tumour a portion of fresh tissue had been snap-frozen in liquid nitrogen (Chapter III.2.c).

One or two representative blocks from each specimen were selected for immunocytochemical staining after reviewing all relevant H & E-stained paraffin sections. The ABC immunohistochemical staining technique has been described in detail in Chapter III (Appendix I). Briefly, five-micron paraffin sections were sequentially incubated with monoclonal anti-vimentin antibody (V9 clone, Dakopatts,

Copenhagen, Denmark; Osborn et al, 1984) at a dilution of 1:250 at 4°C overnight and biotinylated secondary antibody (horse anti-mouse IgG) for 30 minutes at 22°C. This was followed by application of the ABC reagent, development with DAB and counterstaining with haematoxylin. The monoclonal antibody CAM 5.2 (Becton-Dickinson, California, USA; Makin et al, 1984; Chapter IV.1), which recognizes lower molecular weight cytokeratins, was applied at a dilution of 1:50 to immediate consecutive paraffin sections in each case. Staining for cytokeratins followed digestion with trypsin II (Sigma Chemical Company, Missouri, USA; Catalogue No. T-8128) at 0.5 mg/ml for three minutes at 37°C.

Double antigen immunostaining was performed in four selected vimentin-positive cases to confirm the presence of vimentin and cytokeratin IFs within a single neoplastic cell. The first primary antibody (anti-vimentin) was localized by applying the ABC technique, as described above, with the chromogen DAB. Vimentin-positive cells were recognized by granular brown cytoplasmic staining. The sections were then digested with trypsin II for two minutes, following which the second primary antibody (CAM 5.2) was visualized by employing a PAP technique using cobalt chloride ($CoCl_2$)-modified DAB as the chromogen. This method was adapted from Hsu and Soban (1982). The addition of $CoCl_2$ produced a blue-black reaction product enabling cytokeratin IFs to be distinguished from vimentin IFs. No nuclear counterstain was applied to these sections.

In six of the IDCs six-micron cryostat sections were fixed in acetone at 4°C for 10 minutes followed by two 30-second exposures to microwave irradiation. The sections were then immunostained with monoclonal anti-vimentin at 1:50 dilution using the

modified ABC technique (Appendix II), and served to ensure that the methods of fixation and processing employed for the paraffin-embedded tissue were not destructive to the vimentin antigen. Twelve smears of IDCs, fixed in 10 per cent buffered formalin for one minute, were also stained with anti-vimentin antibodies.

Lesions were designated positive for vimentin when there was distinct brown granular cytoplasmic staining in epithelial cells. Each case was assigned to one of three arbitrary categories according to the percentage of vimentin-positive cells; Category 0 representing no vimentin staining, Category 1 representing less than 10 per cent of epithelial cells vimentin-positive and Category 2 representing greater than 10 per cent of cells positive. In addition, the degree of vimentin immunoreactivity was estimated to the nearest 10th percentile for each Category 2 tumour. In carcinomas with both an *in situ* and an infiltrating component the overall extent of vimentin staining was assessed.

Consecutive cryostat sections from each of the malignant neoplasms had been stained with the Ki-67 antibody (Chapter IV.3) and with the anti-ER antibody (Chapter IV.2). Known positive and negative controls were included for all antibodies (Chapter III.2.c).

The relationships between vimentin positivity and tumour histological grade, primary tumour size, patient age, Ki-67 and ER counts, and axillary nodal status were analysed statistically (Chapter III.2.e).

IV.4.c. RESULTS

1. Coexpression of Cytokeratins and Vimentin in Benign Breast Epithelium

All epithelial cells were CAM 5.2-positive. In seven (35 per cent) of the 20 examples of benign breast epithelium (Table IV.4.1) a weak to moderate intensity of vimentin staining was observed in occasional single or small groups of epithelial cells (Category 1). Vimentin-positive epithelial cells were most pronounced in areas of florid epitheliosis. The vimentin IFs were mainly localized in the basal portions of the epithelial cells and as paranuclear aggregates (Figure IV.4.1), a distribution similar to that described in benign thyroid (Miettinen et al, 1984), renal (Azumi & Battifora, 1987) and prostatic epithelium (Leong et al, 1989). There was no vimentin immunoreactivity in the epithelium of the lactating adenomas, the single tubular adenoma or the three examples of normal breast.

2. Vimentin Expression in Breast Carcinomas

Variable degrees of vimentin staining were noted in 50 (44 per cent) of the 115 malignant neoplasms (Table IV.4.2). In the majority of vimentin-expressing tumours (39) only scattered single or small clusters of malignant epithelial cells stained positively, amounting to considerably less than 10 per cent of the overall neoplastic population (Category 1). Desquamated cells within glandular lumina, notably in comedo-type intraductal components of carcinomas, often expressed vimentin and

TABLE IV.4.1

VIMENTIN STAINING OF BENIGN BREAST EPITHELIAL CELLS

	Vimentin Staining*		
	0	1	2
Benign Proliferations (n=17)			
Fibrocystic change	3	3	–
Fibroadenoma	2	2	–
Lactating adenoma	4	–	–
Tubular adenoma	1	–	–
Intraductal papilloma	–	2	–
Normal Breast Tissue (n=3)	3	–	–
TOTAL	13	7	–

*Categories of vimentin staining (see text):
0 = negative; 1 = <10% positive; 2 = >10% positive
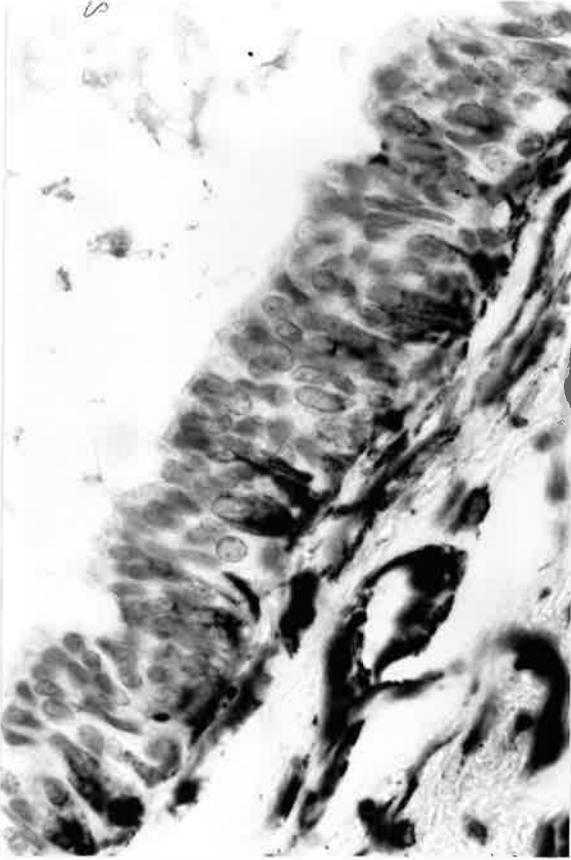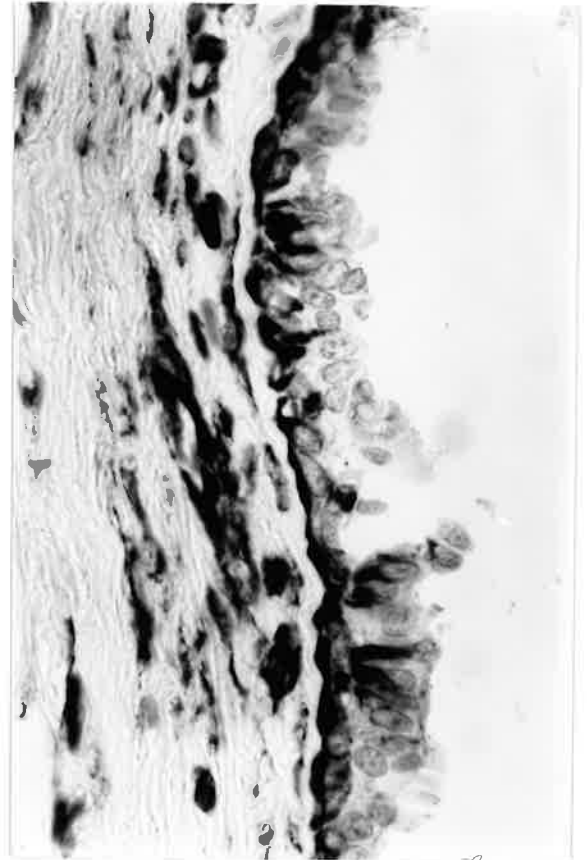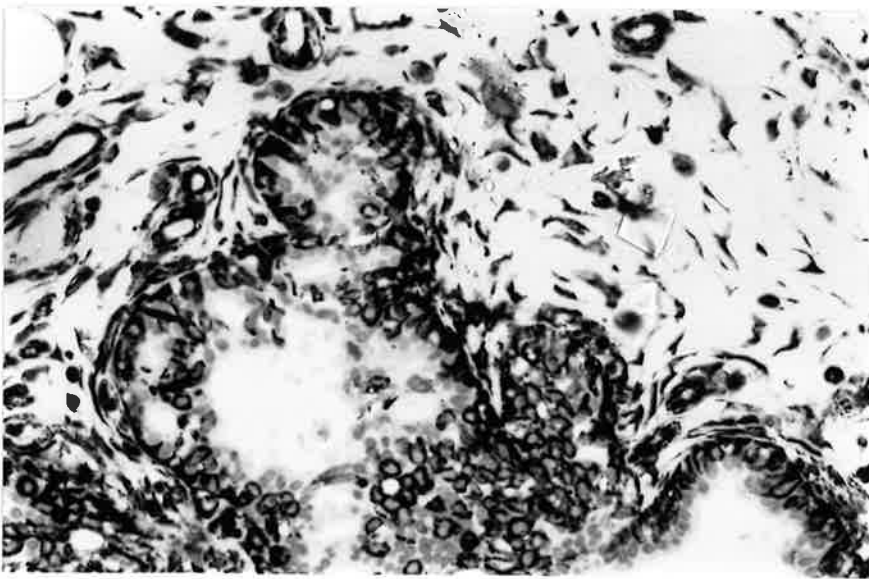
Figure IV.4.1. Three examples of epitheliosis illustrating vimentin staining in the basal portions of the epithelial cells, and as paranuclear sheaths and aggregates. Note strong positive staining of stromal cells (haematoxylin counterstain).

TABLE IV.4.2

VIMENTIN STAINING AND TUMOUR GF IN

115 MALIGNANT BREAST NEOPLASMS

	Vimentin Staining* (n = 115)			Mean Ki-67 Count** (n = 112)
	0	1	2	
Intraductal carcinoma	–	1	–	27.0
IDC Grade I	16	11	2	9.0
IDC Grade II	40	21	3	18.3
IDC Grade III	2	5	5	32.9
IDC, metastatic	1	–	–	5.0
Infiltrating lobular carcinoma	3	–	–	10.1
Tubular carcinoma	1	–	–	5.2
Mucinous carcinoma	1	–	–	10.0
Malignant phyllodes tumour	1	–	–	3.5
Male breast carcinoma	–	1	–	17.2
Carcinosarcoma	–	–	1	47.0
	65	39	11	17.4 (mean)

IDC = Infiltrating ductal carcinoma;   Bloom and Richardson (1957) histological grade

* Categories of vimentin staining (see text) :
   0 = negative;   1 = <10% positive;   2 = >10% positive

**Three cases excluded (see text)

accounted for many of the positive cells in Category 1 tumours. Vimentin staining in these cases was of weak to moderate intensity and was predominantly perinuclear in distribution.

Ten (10.4 per cent) of the 106 IDCs, comprising two histological grade I tumours, three grade II and five grade III carcinomas, and the single carcinosarcoma were assigned to Category 2 (Tables IV.4.2 & IV.4.3). With one exception (Case 1),there was diffuse and intense cytoplasmic vimentin immunostaining in greater than 50 per cent of the neoplastic population in all paraffin blocks examined of these Category 2 tumours (Figures IV.4.2-IV.4.5). Sheets of vimentin-positive neoplastic cells retained unequivocal morphological characteristics of epithelial cells, in some areas forming definite glandular structures. Case 1 was a heterogeneous carcinoma characterized by both tubular and infiltrating ductal (grade I) components. Vimentin positivity was demonstrated in 40 to 50 per cent of malignant cells in the ductal portion, but staining of the tubular component with anti-vimentin antibodies was negative. One of the grade III carcinomas represented metastatic carcinoma in an axillary lymph node (Case 6). Although blocks of the primary tumour were not available for vimentin, ER or Ki-67 immunostaining, H & E sections from the breast lesion were reviewed and confirmed a primary, grade III IDC.

There was no vimentin staining in any of the lobular, tubular or mucinous carcinomas, or in the epithelium of the malignant phyllodes tumour. In-built positive controls for vimentin were present in all sections examined and also confirmed preservation of the IF antigen in the paraffin-embedded and frozen tissue.

TABLE IV.4.3

BIOLOGICAL PARAMETERS FOR 10 CASES OF INFILTRATING DUCTAL CARCINOMA WHICH EXHIBITED EXTENSIVE VIMENTIN STAINING

Case	Ki-67 Count (%)	ER Count (%)	Grade*	Metastases** (axillary node)	Vimentin Staining (%)
1[a]	20	82	I[a]	0	40-50
2	-	56	I	0	50-60
3[b]	67	0	II	-	50-60
4[c]	47	40	II	+	70-80
5	-	8	II	-	70-80
6[d]	52	0	III	+	> 90
7	41	2	III	0	70-80
8	45	0	III	0	70-80
9	40	0	III	0	60-70
10	56	0	III	+	80-90

```
*    Tumour histological grade (Bloom & Richardson, 1957)
**   + = presence of metastases, 0 = no metastases,
     - = sampling not performed
a    Mixed tubular and Grade I ductal carcinoma; vimentin
     positivity in 40-50% of ductal carcinoma cells only
     (see text)
b    Died 18 months post operatively
c    Extensive metastatic disease one year post operatively
d    Metastatic breast carcinoma in an axillary lymph node
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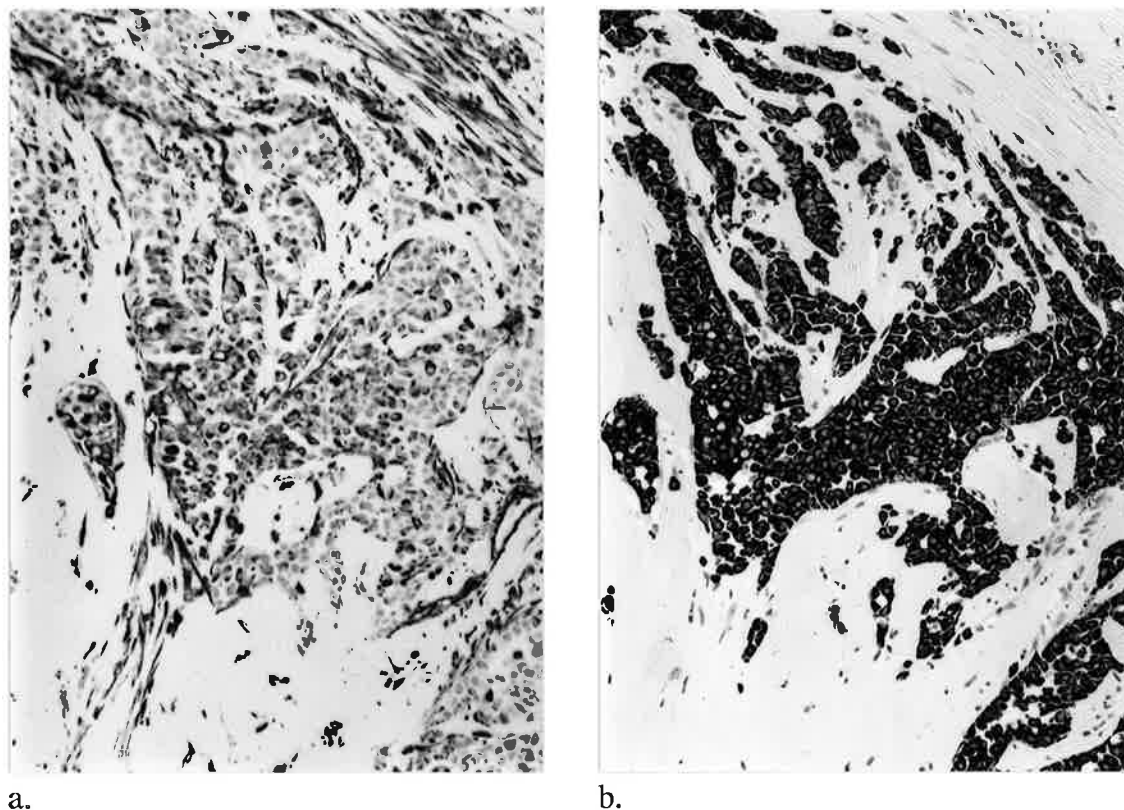
Figure IV.4.2. (a) A well-differentiated infiltrating ductal carcinoma (Case 2) demonstrating vimentin immunoreactivity in approximately 55 per cent of the tumour cells, and (b) coexpression of cytokeratins (CAM 5.2 stain) in an adjacent section (haematoxylin counterstain). Note vimentin positivity of stromal cells in the upper right field of (a).
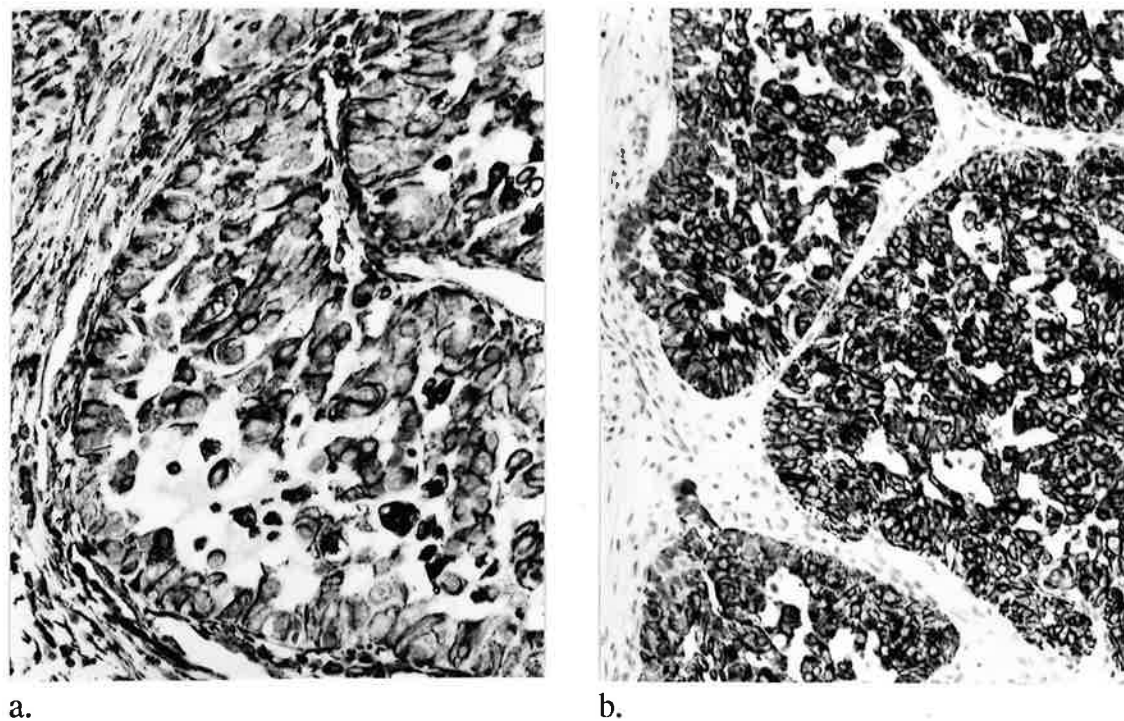
Figure IV.4.3. An intraductal portion of a poorly-differentiated adenocarcinoma (Case 8) showing extensive vimentin staining of neoplastic cells in (a), and coexpression of cytokeratins (CAM 5.2 stain) in (b).
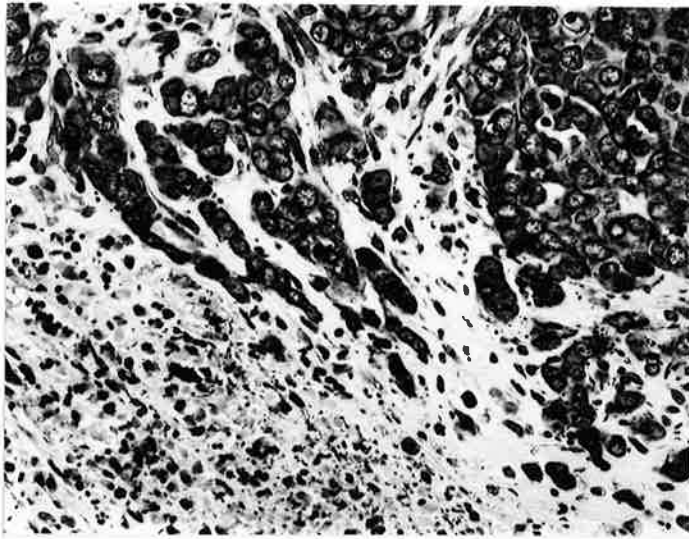
a.
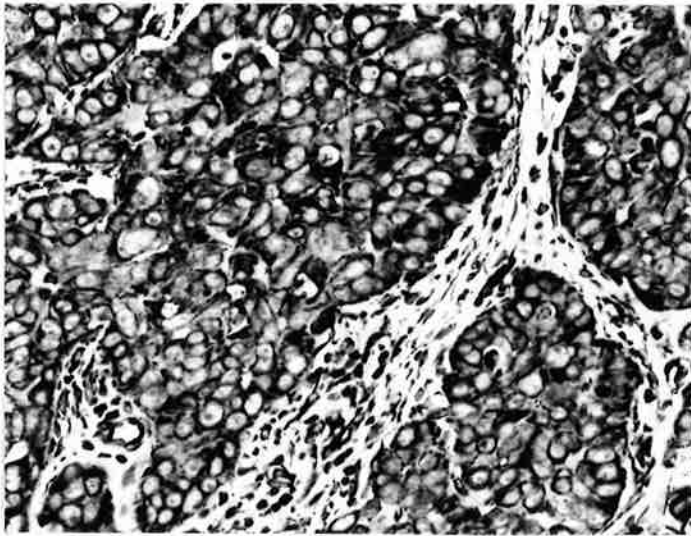
b.

Figure IV.4.4. (a & b) Two fields from a poorly-differentiated, focally necrotic (a), infiltrating ductal carcinoma (Case 10) showing cytoplasmic vimentin staining in up to 90 per cent of tumour cells.
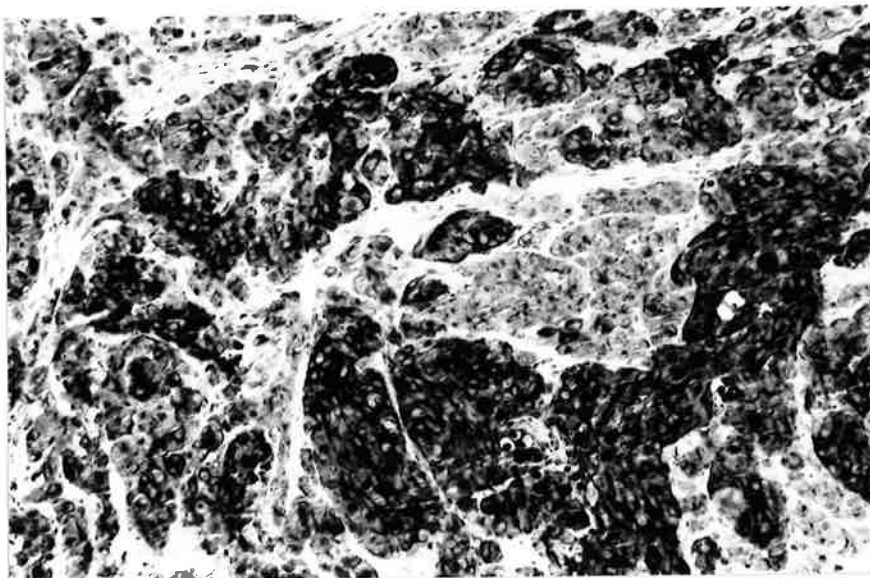
a.
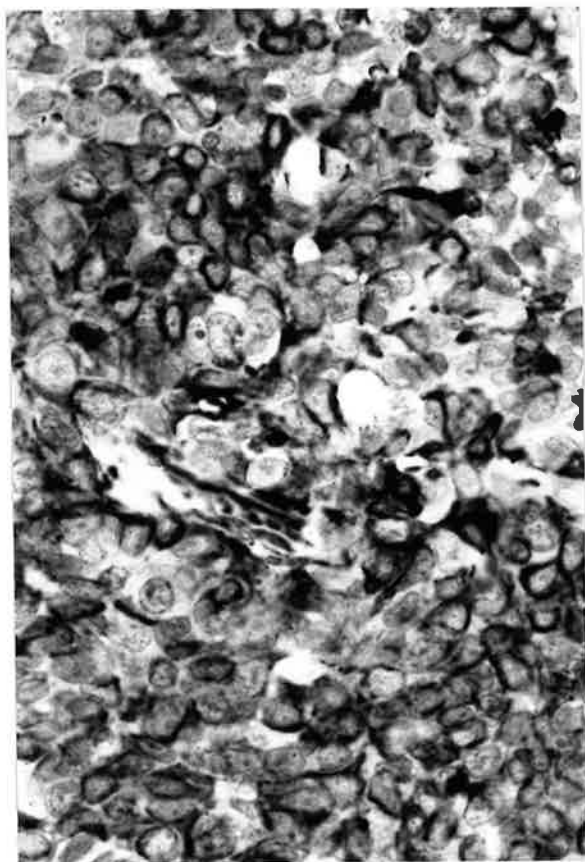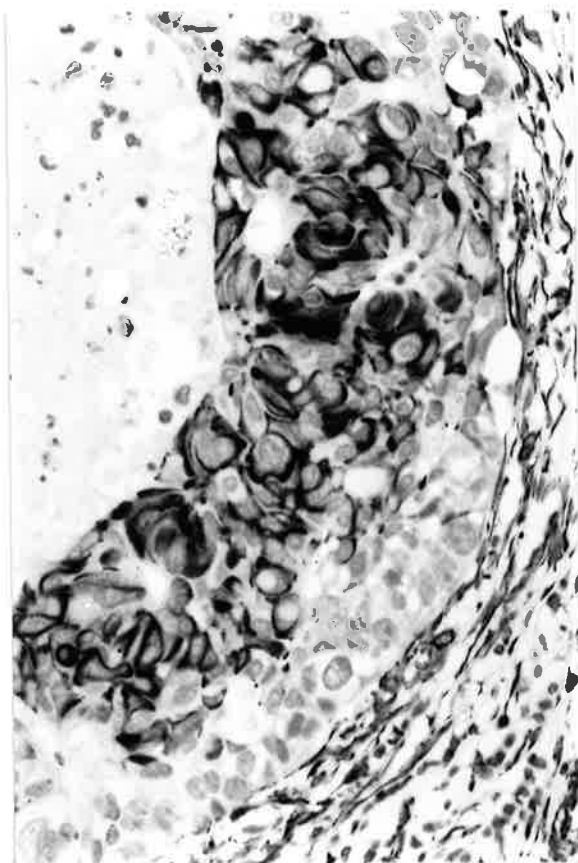
b.   c.

Figure IV.4.5. Sheets of neoplastic cells are diffusely vimentin-positive in a poorly-differentiated ductal carcinoma (a).   Perinuclear   and   circumferential subplasmalemmal accentuation of vimentin staining is well illustrated at higher magnification (b) and in an intraductal portion of the same tumour (c).

In four cases coexpression of cytokeratin and vimentin IFs was demonstrated in malignant epithelial cells using the double immunostaining technique. Although double labelling was not performed in all cases, it was felt acceptable to assume true coexpression in the remaining vimentin-positive tumours as all benign and malignant epithelial cells expressed lower molecular weight cytokeratins, and some of these same cell groups, identified in immediate consecutive paraffin sections, also expressed vimentin IFs (Figures IV.4.2 & IV.4.3).

A comparison of tumours in Categories 0 and 1 revealed no significant differences in any of the histological or immunocytochemical (Ki-67 and ER) variables examined. Thus, for statistical analyses, tumours in categories 0 and 1 (representing no or insignificant amounts of vimentin staining) were combined and termed "vimentin-negative", and were compared with Category 2 tumours (exhibiting extensive vimentin positivity), designated "vimentin-positive".

A significant difference in histological grades of IDCs was demonstrated between the two vimentin groups (t = 2.53, p < 0.05). Five of the 10 vimentin-positive ductal carcinomas were poorly differentiated (grade III), whereas only 12 of the 105 graded ductal carcinomas were classified as grade III (Table IV.4.2). Two of the 29 grade I carcinomas and three of 64 grade II carcinomas exhibited extensive vimentin positivity. Vimentin staining in well-differentiated tumours was generally less intense than that observed in high grade carcinomas (Figure IV.4.2). In many Category 1, grade I neoplasms the distribution of vimentin staining resembled the pattern seen in benign epithelium. In contrast, vimentin IFs in more anaplastic carcinomas were observed diffusely throughout the cell cytoplasm and often with distinctive

perinuclear and circumferential subplasmalemmal accentuation (Figure IV.4.5).

The vimentin-positive and vimentin-negative tumours also showed significant differences in ER counts (t = 3.37, p <0.005) and patients' ages (t = 2.41, p <0.05). If the carcinosarcoma was excluded from the statistical analyses the differences held, and vimentin-positive ductal carcinomas were predominantly ER-negative (t = 3.05, p <0.005) and more likely to occur in younger women (t = 2.98, p <0.005). No relationship was observed between vimentin positivity and primary tumour size or the presence of axillary nodal metastases (Appendix III).

Immunostaining for vimentin IFs in frozen sections and smears provided no advantage over staining of paraffin-embedded sections. Although antigen preservation is generally recognized to be superior in fresh tissues, suboptimal cytomorphology in the cryostat sections and smears prevented accurate differentiation between vimentin-positive stromal, myoepithelial, inflammatory and neoplastic cells, and thus these methods were abandoned. In the two cases in which carcinoma cells were vimentin-positive in cryostat sections, the cells were also clearly stained with vimentin in the corresponding paraffin sections. Cytological preservation in the smears was poor due to the many washes the cells were subjected to during immunoperoxidase staining. In paraffin sections cell preservation was superior; however, the abundance of vimentin-positive non-neoplastic stromal cells surrounding the neoplastic element made interpretation difficult in several cases and staining of alternative blocks was necessary.

3. Correlation Between Vimentin Expression and Tumour Growth Fraction

The Ki-67 counts ranged from two to 67 per cent in 112 malignant breast tumours (Table IV.4.2; Figure IV.4.6; Chapter IV.3.c). Three IDCs, two of which were vimentin-positive, were excluded from statistical analyses of Ki-67 counts for the reasons outlined in Chapter IV.3.c. Briefly, intraductal carcinoma, with a Ki-67 count of 47 per cent, was the predominant tumour type identified in cryostat sections of Case 5 (Table IV.4.3), and insufficient infiltrating tumour was present to allow a representative estimate of the overall GF. Accurate Ki-67 counts were not possible in Case 2 and in one vimentin-negative carcinoma as the tumour cells were poorly preserved and surrounded by numerous cells with endogenous peroxidase activity (Chapter III.2.d).

A significant difference in Ki-67 counts was identified between the vimentin-positive (Category 2) and vimentin-negative (Categories 0 and 1) groups of neoplasms ($t = 8.92$, $p < 0.0001$; Figure IV.4.6). This difference was still highly significant when only IDCs were analysed ($t = 8.38$, $p < 0.0001$). Of the eight vimentin-positive ductal carcinomas for which fresh frozen tissue was adequate, seven manifested Ki-67 counts greater than or equal to 40 per cent, well above the mean GF for this series (Table IV.4.3; Figures IV.4.7 & IV.4.8). The one exceptional case (Case 1) was the tumour composed of both tubular and well-differentiated ductal components. This carcinoma exhibited an overall Ki-67 count of 20 per cent, greater than the mean GF but less striking than in the other seven cases (Figure IV.4.9). However, it is not clear whether the frozen tissue from this composite carcinoma was representative of the vimentin-positive ductal portion or more similar to the vimentin-negative tubular
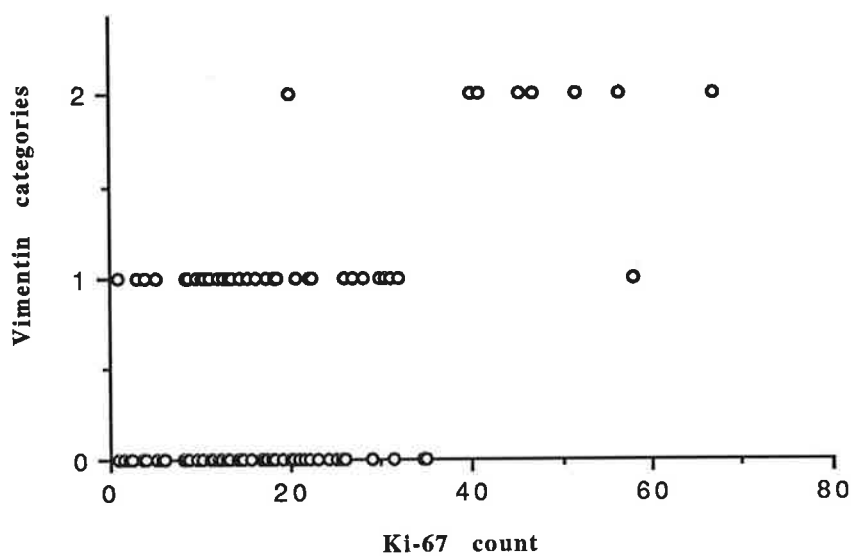
Figure IV.4.6. Relationship between Ki-67 count and vimentin expression in 112 malignant breast tumours.

Vimentin staining categories: 0 = no staining; 1 = <10% cells positive; 2 = >10% cells positive.
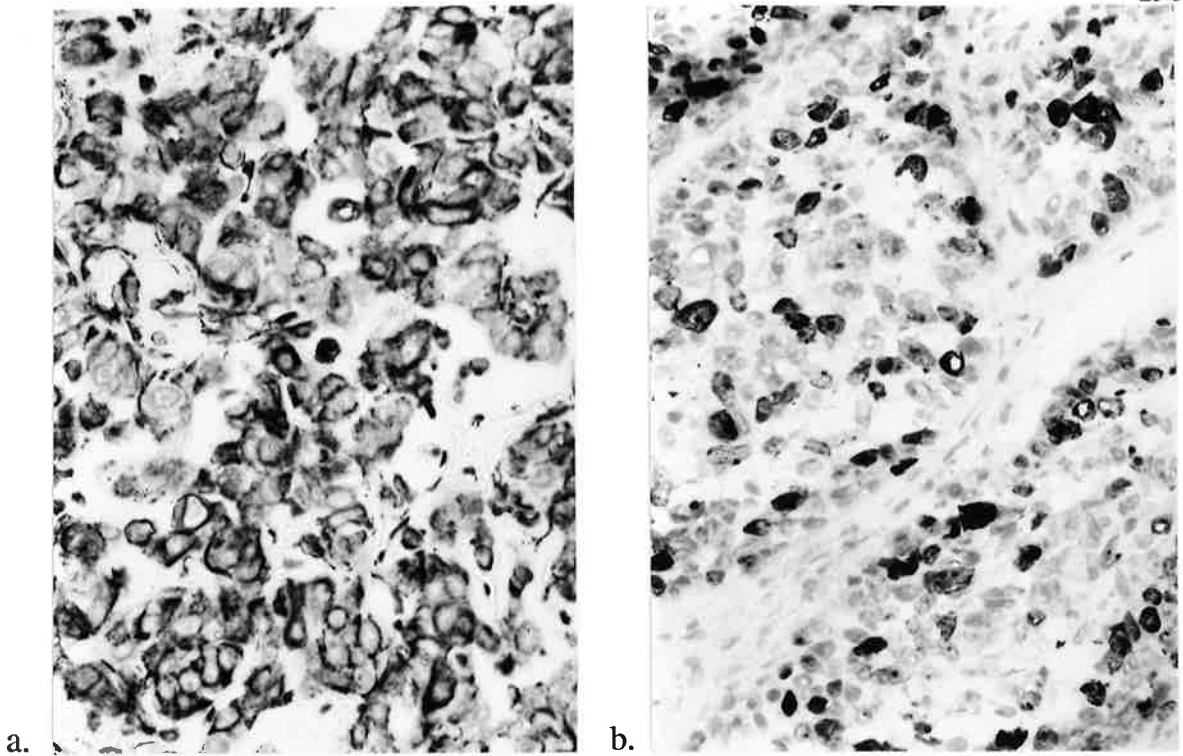
Figure IV.4.7. (a) Sheets of neoplastic cells in a poorly-differentiated ductal carcinoma (Case 8) depicting intense cytoplasmic vimentin positivity. (b) The frozen section from the same carcinoma illustrates Ki-67 nuclear staining in 45 per cent of tumour cells (haematoxylin counterstain).
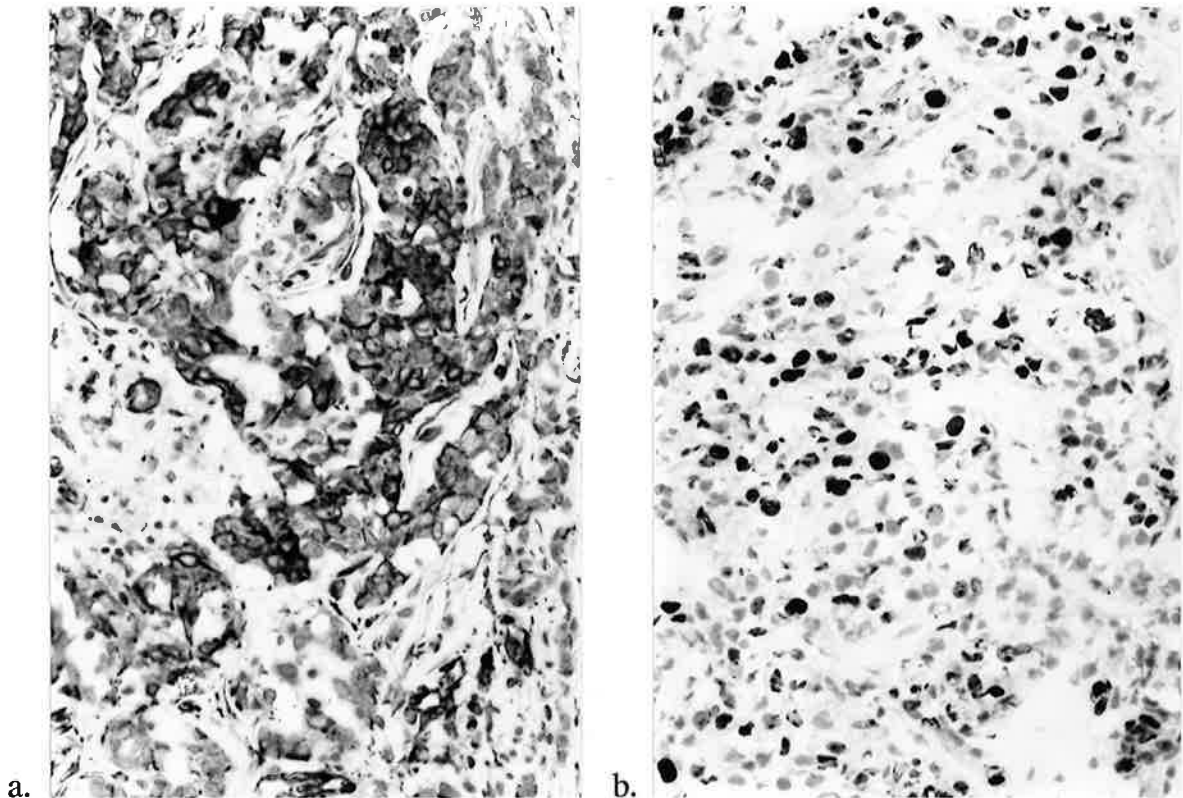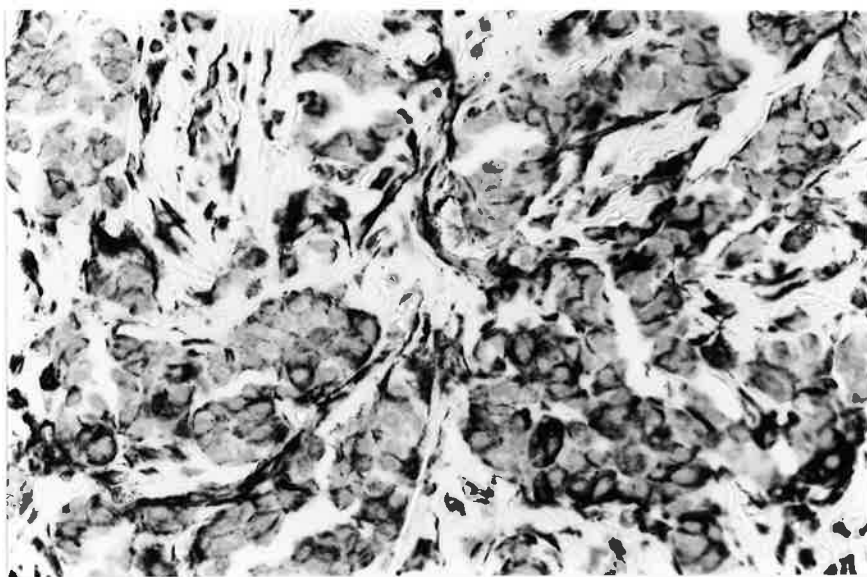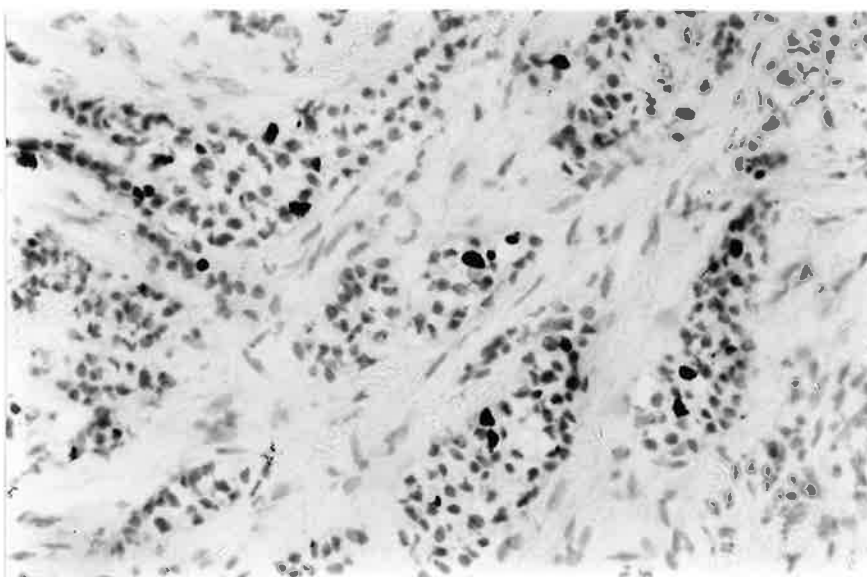

Figure IV.4.8. (a) A moderately-differentiated infiltrating ductal carcinoma (Case 4) showing vimentin staining in up to 80 per cent of tumour cells. (b) The Ki-67 count of 47 per cent is demonstrated in the corresponding frozen section (Ki-67 antibody, haematoxylin counterstain).

a.

b.

Figure IV.4.9. (a) Vimentin immunoreactivity in up to 50 per cent of neoplastic cells in the ductal component of Case 1. (b) The frozen section of Case 1 illustrates Ki-67 nuclear staining in approximately 20 per cent of tumour cells.

component. Unfortunately there was no frozen tissue reserve to allow direct staining of cryostat sections with anti-vimentin antibodies.

The carcinosarcoma had a Ki-67 count of 47 per cent and was ER-negative. One IDC with a Ki-67 count of greater than 40 per cent (58 per cent) exhibited no vimentin staining. This was a Grade III, ER-negative tumour.

IV.4.d.   DISCUSSION

This work identifies another neoplasm of epithelial origin that may express vimentin in addition to cytokeratins, thereby adding to the list of tumours which demonstrate IFs other than those of their reputed cell of origin (Raymond & Leong, 1989 c & d). Knowledge of this group of tumours may be helpful in characterizing anaplastic carcinomas and metastatic deposits of unknown primary site. Caution is required, however, when immunohistochemical analysis of IFs is used for the identification of tumours, and application of a panel of anti-IF antibodies is essential.

Variable amounts of vimentin were demonstrable in 50 (44 per cent) of the 115 primary malignant breast neoplasms studied. In 10 (10.4 per cent) of 106 cases of IDC coexpression of vimentin and cytokeratin IFs was observed in greater than 40 per cent of tumour cells (Category 2). In these cases cytoplasmic staining for vimentin was diffuse, often with distinctive perinuclear and subplasmalemmal accentuation. Desquamated tumour cells also often expressed vimentin, a finding which parallels the observations made in previous studies of carcinomas of the lung

(Upton et al, 1986) and prostate (Leong et al, 1989). Vimentin expression in breast carcinomas thus appears to occur in sheets of cells, or in scattered single or small clusters of cells, allowing simple, reproducible grouping of these tumours. Vimentin staining in benign breast epithelium was generally scant and predominantly distributed basally and as paranuclear sheaths and aggregates, with sparing of the apical portions of the cell (Category 1). The results were obtained using tissues fixed by microwave irradiation, previously demonstrated to produce optimal preservation of most cellular antigens, including vimentin, when compared with routine formalin fixation (Chapter III.2; Leong et al, 1988a).

Only two previous publications have noted vimentin staining of breast carcinomas (Leader et al, 1987; Azumi & Battifora, 1987); however, these papers did not provide details of the histological type or grade of the breast cancers, nor was the extent or distribution of staining described. In a study of 38 formalin-fixed (for 24 to 72 hours) infiltrating carcinomas of various primary sites, Leader et al (1987) found only a single vimentin-positive breast carcinoma. Azumi and Battifora (1987) observed vimentin positivity in five (12 per cent) of 43 breast carcinomas fixed in ethanol, but no staining was detected in 26 formalin-fixed tumours. Leader and coworkers utilized monoclonal anti-vimentin from Labsystems and an indirect peroxidase method, while Azumi and Battifora employed anti-vimentin from DAKO (California) and an ABC method. Other authors failed to identify vimentin in breast cancers. Gabbiani et al (1981) examined only five breast carcinomas with an immunofluorescent staining technique and polyclonal anti-vimentin antiserum. The details of fixation and staining were not provided in a review by Osborn and Weber (1983) who quoted that no vimentin staining was observed in neoplastic cells of 18

breast carcinomas. Gown and Vogel (1985) applied a non-commercial monoclonal anti-vimentin antibody to Carnoy's or methacarn-fixed paraffin sections of 23 ductal carcinomas, following pronase digestion, and reported all to be vimentin-negative. The use of microwave-fixed tissue, monoclonal anti-vimentin antibodies and the ABC amplification technique may be the most sensitive method of detecting vimentin IFs and explain the relatively high percentage of vimentin-positive carcinomas identified by this author.

While myoepithelial cells in the breast express cytokeratin and vimentin IFs, they clearly lie external to the epithelial cells of normal mammary ducts and acini, as demonstrated by immunocytochemical staining for actin (Ohtani & Sasano, 1980) and myosin (Gusterson et al, 1982), and by ultrastructural examination (Gusterson et al, 1982). Using antibodies specific to muscle actins, myoepithelial cells were demonstrated surrounding the basal layer of benign ducts and their cytoplasmic processes were not observed between ductal epithelial cells (Chapter IV.8.c). Myoepithelial cells are generally not identified amongst deposits of malignant cells (Gusterson et al, 1982; Henderson et al, 1986), but may occasionally be interposed at the stromal-epithelial junction of the infiltrating neoplasm (Gusterson et al, 1982). Thus, it is unlikely that the cells described in this chapter to express both vimentin and cytokeratin IFs are myoepithelial cells caught up in the invasive tumour. Furthermore, the coexpressing neoplastic cells exhibited unequivocal morphological features of malignant epithelial cells, some forming definite tubules and others infiltrating as sheets of polygonal-shaped cells with rounded nuclei. Such nuclear and cytoplasmic features also prohibited classification of these tumours as "spindle cell" carcinomas or as "carcinosarcomas" (which exhibit separate epithelial and

mesenchymal features).

The problems and inaccuracies inherent in the use of current prognostic markers and their limitations in predicting tumour behaviour and clinical outcome have already been outlined (Chapter II). Whilst tumour GF is recognized to be a useful independent predictor of early relapse, autoradiography and flow cytometry are not available in many pathology laboratories. Ki-67 immunostaining is a simple alternative, but fresh frozen tissue is required and, as yet, insufficient time has elapsed to meaningfully correlate Ki-67 counts with relapse-free intervals and overall survival. Other prognostic parameters are therefore being sought and the acquisition and expression of additional IF proteins by neoplastic cells may prove useful.

No significant differences in Ki-67 counts or other histological variables were observed between breast neoplasms in categories 0 and 1. Thus, breast carcinomas appeared to express insignificant amounts of vimentin (Categories 0 and 1) or were strongly vimentin-positive (Category 2). In the eight vimentin-positive cases for which fresh frozen tissue was available, the corresponding Ki-67 counts were greater than or equal to 40 per cent in seven cases, and were significantly higher than the counts determined for vimentin-negative tumours (Figure IV.4.6). The one exception was the heterogeneous carcinoma composed of tubular and well-differentiated infiltrating ductal components which demonstrated a Ki-67 count of 20 per cent (Figure IV 4.9). However, due to relatively poor histological preservation it was not possible to determine with certainty whether the frozen tissue was representative of the extensively vimentin-positive (Category 2) ductal portion, or corresponded to the tubular component in which no vimentin positivity was identified. Given the overlap

between these two tumour types, it is possible that this case represents a transition between a tubular carcinoma of indolent nature and a more aggressive ductal carcinoma. Thus, on the basis of histology alone a favourable outcome is anticipated, but the acquisition of vimentin IFs by the infiltrating ductal component suggests the pattern of behaviour may be more aggressive. The Ki-67 count of 20 per cent is higher than expected for a tubular carcinoma, but may not be a true reflection of the GF of the most aggressive (vimentin-positive) ductal component. Alternatively, this carcinoma in evolution may not yet have attained the high GF of the other vimentin-positive cases. Therefore, this case may not be an exception, but rather represent the early phase of an aggressive tumour. Follow-up of the patient is proceeding.

The mucinous, pure tubular and lobular carcinomas were all vimentin-negative, in accordance with the anticipated favourable outcomes for these tumour subtypes (Chapter II.b). While vimentin-positive carcinomas exhibited high proliferative rates, the converse did not necessarily apply. One carcinoma in the series showed a very high GF (Ki-67 count of 58 per cent) but was vimentin-negative (Figure IV.4.6). It is recognized that it may not be valid to consider the Grade III ductal carcinoma metastatic to an axillary lymph node (Case 6) in the same light as the other cases of primary ductal carcinoma, however, this case demonstrated a Ki-67 count of 52 per cent and vimentin immunoreactivity in greater than 90 per cent of tumour cells.

Despite the relationship between vimentin staining and histological grade, not all vimentin-positive carcinomas were poorly-differentiated (Grade III). Two grade II tumours, which might be expected to demonstrate more favourable prognoses, were

vimentin-positive and behaved aggressively during the short follow-up period (Table IV.4.3). A further moderately-differentiated carcinoma and two well-differentiated carcinomas were also vimentin-positive. Thus, while most vimentin-positive carcinomas are poorly-differentiated, grade I and II tumours may occasionally stain positive and it is in these cases that vimentin expression may have particular prognostic value. The patient with carcinosarcoma (Ki-67 count of 47 per cent) presented with local recurrence and multiple pulmonary metastases within a year of diagnosis.

In this series vimentin positivity was not related to the axillary nodal status (Table IV.4.3), in keeping with the lack of relationship between nodal status and tumour GF described in Chapter IV.3. Expression of vimentin may thus reflect the proliferative rate and potential aggressiveness of a carcinoma rather than its metastatic capability, while the axillary nodal status provides an indication of whether or not the tumour has metastasized.

The coexpression of cytokeratin and vimentin IFs in renal tubular epithelium, ovarian epithelium and mesothelium has been attributed to transformation from a precursor mesenchymal cell into a cytokeratin-expressing cell (Pinkerton et al, 1961; Holthofer et al, 1983; LaRocca & Rheinwald, 1984). Such an explanation is not plausible, however, for the multiple filament expression observed in breast epithelium and in many other benign and malignant epithelia.

The scant, weak intensity vimentin expression occasionally observed in benign breast epithelium is probably a variant of normal and thus it would not be unexpected to

find vimentin IFs in some breast carcinomas. The intense vimentin positivity noted in Category 2 tumours is distinctive and may be of biological significance. Acquisition of vimentin by a breast carcinoma may indicate "dedifferentiation" or regression, accounting for a more aggressive behaviour. Lane's hypothesis that vimentin expression may be related to reduced cell-to-cell contact (Lane et al, 1983) provides a further possible explanation for vimentin staining of desquamated epithelial cells.

In conclusion, this study is the first to describe a relationship between vimentin expression in breast carcinoma and high tumour GF. Vimentin expression, observed in approximately 10 per cent of IDCs, may be a new prognostic marker for IDC and may identify, independently of lymph node status, those tumours likely to exhibit aggressive behaviour and perhaps benefit from early adjuvant therapy. Two anecdotal cases of grade II carcinoma with early relapse were cited. The value of vimentin analysis also lies in its simple application to paraffin-embedded sections, allowing for retrospective studies on patients with known clinical outcome. Long-term follow-up studies are essential to establish the relative values of vimentin reactivity and Ki-67 counts as prognostic parameters in breast carcinoma.

IV.5. SILVER NUCLEOLAR ORGANIZER REGIONS (AgNORs)

a.   **INTRODUCTION**

b.   **METHODOLOGY**

c.   **RESULTS**

   1. AgNOR Staining of Breast epithelium

   - Benign
   - Malignant

   2. AgNOR Correlation with Tumour Proliferation and Other Clinicopathological Parameters

d.   **DISCUSSION**

IV.5. SILVER NUCLEOLAR ORGANIZER REGIONS (AgNORs)

IV.5.a. INTRODUCTION

Nucleolar organizer regions (NORs) are chromosomal (DNA) segments encoding for ribosomal RNA (rRNA). NORs comprise about forty transcription units each and have been located on the short arms of the human acrocentric chromosomes 13, 14, 15, 21 and 22. They are responsible for the development of the RNA-containing nucleolus or nucleoli into which they project on large loops of DNA (Fakan & Hernandez-Verdun, 1986; Underwood & Giri, 1988). For over a decade cytogeneticists used banding techniques to identify NORs in metaphase preparations when investigating chromosomal disorders such as trisomies and translocations. More recently, localization of ribosomal genes (rDNA) on chromosomes or within interphase nucleoli has been possible using *in situ* hybridization methods.

In 1975, Goodpasture and Bloom described a silver-staining (argyrophilic) technique to identify the chromosomal locations of rDNA. This technique was subsequently simplified to a one-step procedure and applied to sections of paraffin-embedded and plastic-embedded tissue (Howell & Black, 1980; Ploton et al, 1982, 1984 & 1986).

Silver-NORs (AgNORs) are argyrophilic, nucleolar, acidic, non-histone proteins associated with rDNA and are thought to play a regulatory role in decondensation and/or transcription of the rDNA, and in the maturation of the rRNA (Fakan &

Hernandez-Verdun, 1986). The silver impregnation technique produces silver binding to sulphydryl groups present on the NOR-associated proteins (Buys & Osinga, 1980). The exact biochemical nature of these proteins is still controversial, but they appear to include RNA polymerase I, C23 protein (nucleolin, 110 kd), a C-23 related protein (100 kd), and B23, a 78 kd phosphoprotein (Lischwe et al, 1979; Williams et al, 1982; Buys & Osinga, 1984; Ochs & Busch, 1984).

As NORs are loops of DNA which transcribe to rRNA, and rRNA ultimately directs ribosome and protein synthesis, it has been speculated that large numbers of NORs detected within nuclei of hyperplastic or malignant cells may reflect increased cell synthetic or metabolic activity compared with normal tissues (Crocker et al, 1988), and may partly reflect cell ploidy. The number of detectable NORs and AgNORs must depend on the level of transcriptional activity, the number of NOR-bearing chromosomes and the stage of the cell cycle (as AgNOR numbers are halved immediately following mitosis). A quantifiable increase in the AgNOR count of a cell population could occur in several situations. Firstly, if there is active cell proliferation, such as in malignancy, resulting in nucleolar dissociation and dispersion of AgNORs through the nucleus; secondly, if there is a defect of nucleolar association; thirdly, if cell ploidy increases, resulting in an increase of AgNOR-bearing chromosomes; or finally, if there is increased transcriptional activity (Underwood & Giri, 1988).

Although the novel argyrophil stain identifies NOR-associated proteins rather than the NORs themselves, it is highly specific (Ochs & Busch, 1984) and has been employed by some histopathologists to compare normal tissue, hyperplastic and

benign proliferative lesions, and malignant conditions. The technique has been extensively developed and modified by Dr. John Crocker and associates from Birmingham, England, who have demonstrated that quantitation of NORs can discriminate between high and low grade lymphomas (Crocker & Nar, 1987), and between benign and malignant cutaneous lesions (Crocker & Skilbeck, 1987; Egan & Crocker, 1988). The technique may also be of value in assessing small cell tumours, fibrous proliferations of childhood (Egan et al, 1987 & 1988) and bronchial carcinomas (Crocker et al, 1987). Smith and Crocker (1988), in a study of 46 cases, demonstrated that the numbers of AgNORs in breast carcinoma cells significantly exceeded those in the epithelial cells of benign breast lesions. The degree of overlap, however, prohibited the use of AgNOR counts as an absolute criterion for malignancy.

If the number of NORs and AgNORs truly reflects cell synthetic activity, it would be logical to expect AgNOR numbers to relate to other markers of cell proliferation. In a study of 20 non-Hodgkin's lymphomas, Crocker et al (1988) observed a correlation between the number of AgNOR sites per nucleus and the percentage of S phase cells, estimated by DNA flow cytometry. No relationship between AgNOR counts and DNA aneuploidy was found. Giri et al (1989), however, demonstrated a significant relationship between high AgNOR counts and DNA aneuploidy in a group of 46 breast cancers, and observed similar trends between high growth phase fractions, obtained by flow cytometry, and high AgNOR numbers. Finally, Hall et al (1988) described a linear relationship between the mean number of NORs in 80 cases of non-Hodgkin's lymphoma and both the percentage of Ki-67-positive cells and the histological grade of the tumours.

The present study expands on the work of Smith and Crocker (1988) in applying the argyrophil stain to a much larger series of cases, comprising 114 malignant breast tumours and 66 benign breast lesions. AgNOR counts are related to the ER status, the tumour GF and various other clinicopathological parameters.

IV.5.b. METHODOLOGY

Silver impregnation is not an immunoperoxidase stain, but the methodology was developed and the results included in this dissertation because the technique represents a further means of measuring cell proliferative activity in paraffin sections and has potential application to routine pathology practice. Representative paraffin blocks from 114 of the 115 malignant breast tumours and from 66 additional benign breast lesions, selected from the files of the IMVS, were chosen for AgNOR staining (Table IV.5.1). The only paraffin-embedded tissue available for one of the 115 malignant tumours had previously been subjected to freezing and thawing, and proved to be unsuitable for AgNOR staining (Sections IV.5.c & IV.5.d). All tissue blocks were fixed by microwave irradiation and processed as detailed in Chapter III.2.c. In 10 cases additional blocks were fixed for one and a half hours in either 10 per cent neutral-buffered formalin or in Bouin's fixative prior to routine processing in order to assess the quality of AgNOR staining under different fixation conditions.

AgNORs were demonstrated according to the method of Crocker and associates (Crocker & Nar, 1987; Crocker & Skilbeck, 1987; Leong & Gilham, 1988). The

TABLE IV.5.1

MEAN AgNOR COUNTS IN BENIGN AND MALIGNANT

BREAST EPITHELIUM

	No. of Cases	AgNOR Count
BENIGN		
Normal breast	9	1.8
Duct ectasia	5	1.6
Apocrine metaplasia	3	1.2
Papilloma	4	2.8
Fibroadenoma	15	2.3
Adenosis & epitheliosis	26	2.1
Sclerosing adenosis	1	1.7
Gynaecomastia	3	2.0
Total	66	2.05 (S.D.= 0.57)
MALIGNANT		
Infiltrating ductal carcinoma	105	5.4
Infiltrating lobular carcinoma	3	4.3
Carcinosarcoma	1	6.2
Phyllodes tumour	1	3.3
Intraductal carcinoma	1	6.2
Mucinous carcinoma	1	3.2
Tubular carcinoma	1	2.7
Male breast carcinoma	1	4.3
Total	114	5.28 (S.D.= 2.19)

colloidal silver staining solution was prepared by dissolving two per cent gelatin in one per cent aqueous formic acid, which was then mixed in a ratio of 1:2 by volume with 50 per cent aqueous silver nitrate. Five-micron paraffin sections were taken to water via xylene and graded alcohols, and covered with the silver solution for 45 minutes under darkroom conditions before washing with deionised water, dehydrating to xylene and mounting in synthetic medium. No counterstain was applied. In six IDCs air-dried whole cell imprint preparations were stained with the colloidal silver solution.

Sections of tonsil, in which lymphocytes replicating in germinal centres displayed many more AgNORs than non-replicating paracortical lymphocytes, served as positive controls (Crocker & Nar, 1987). Lymphocytes surrounding and infiltrating the tumour in test sections provided inbuilt controls.

Cryostat sections from fresh frozen tissue had been stained with the Ki-67 antibody and Ki-67 counts obtained for 112 malignant neoplasms, six examples of normal breast epithelium, four fibroadenomas and two cases of fibrocystic change, as detailed in Chapter IV.3. In each malignant tumour ER counts were also available.

Counting Procedure

NORs were visualized as distinct silver-positive intranuclear dots of varying sizes. The mean number of AgNORs per nucleus was calculated for each case following a count of between 200 and 300 morphologically malignant cells or, in the examples of benign breast tissue, benign epithelial cells. Care was taken to exclude

inflammatory and stromal cells from the count. Using a X100 oil emersion lens and a Whipple grid, multiple random fields were examined and all individually discernible and separable nuclear black dots (AgNORs) within the confines of the grid were counted. Careful focussing was necessary to visualize all AgNOR dots in the depth of the section. Where two or more dots within a nucleolus were so closely aggregated or overlapping that the precise number could not be assessed, the aggregate was counted as a single AgNOR. This method of counting has since also been found reliable by Giri et al (1989) and Crocker et al (1989). In preliminary studies of 20 cases, AgNOR counts obtained with a X60 lens were compared with values derived from using a X100 lens and oil emersion. Oil emersion increased the AgNOR count by an average of two dots per nucleus and a X100 lens was used for all subsequent cases.

Statistical Methods

The relationships between AgNOR counts and tumour histological grade, primary tumour size, patient age, axillary nodal status, and Ki-67 and ER counts were analysed using Spearman's rank correlation coefficient (r) method (Chapter III.2.e; Appendix III). Unpaired t tests (t) and Mann-Whitney U tests (U) were employed to examine the differences between AgNOR counts in benign and malignant breast epithelia.

IV.5.c. RESULTS

1. AgNOR Staining of Breast epithelium

Benign Epithelium

The AgNOR counts for the 66 cases of benign breast epithelium ranged from 0.9 to 3.6, with a mean of 2.05 (Table IV.5.1, Figure IV.5.1). There were no significant differences in AgNOR counts between the different types of benign lesions (adenosis and epitheliosis, apocrine metaplasia, sclerosing adenosis, duct ectasia, papilloma, fibroadenoma and gynaecomastia). AgNORs in these lesions and in normal breast epithelium were frequently large and central, exhibiting a circular dark periphery with a paler centre. In many cells AgNORs were not visible, while in others dots were arranged in small clusters. In addition, distinct "satellite" dots away from the clusters were observed in some cells, as noted by Smith and Crocker (1988).

Malignant Epithelium

The mean AgNOR count for all malignant neoplasms was 5.28 per nucleus (Table IV.5.1), with a range from 2.3 to 13.3 (Figures IV5.2 & IV.5.3). AgNOR counts for malignant tumours significantly exceeded those for normal breast and benign lesions ($t = 11.58$, $p < 0.0001$; $U = 142$, $p < 0.000001$). The greater number of AgNOR dots in malignant tumours was readily appreciated on casual examination of most
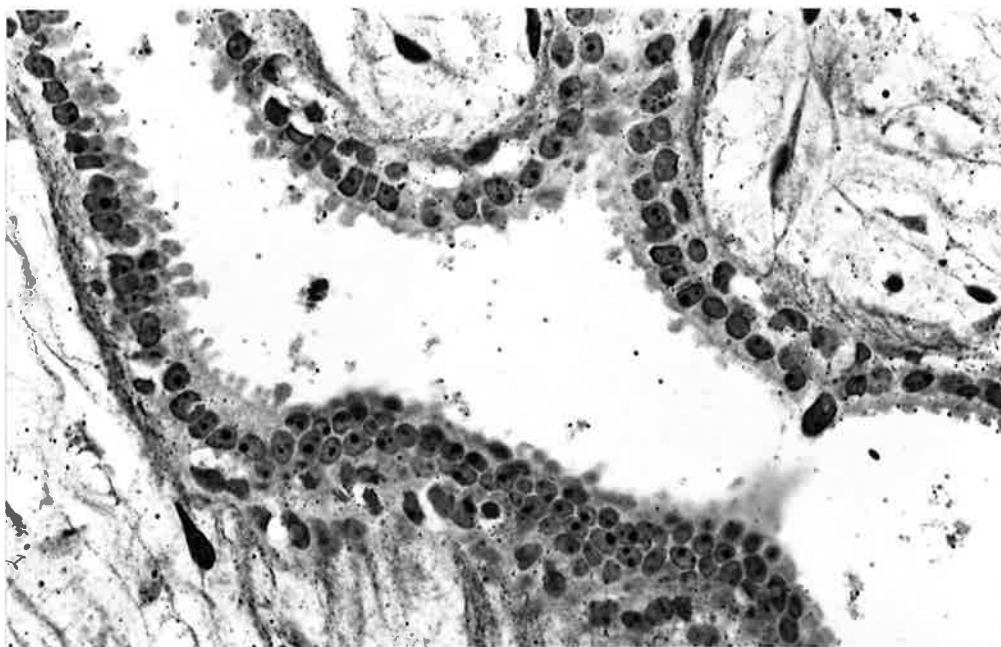
Figure IV.5.1. Epitheliosis from a case of fibrocystic change of the breast demonstrating a mean AgNOR count of 2.3. Between zero and three distinct black dots are present within the epithelial cell nuclei (no counterstain used).
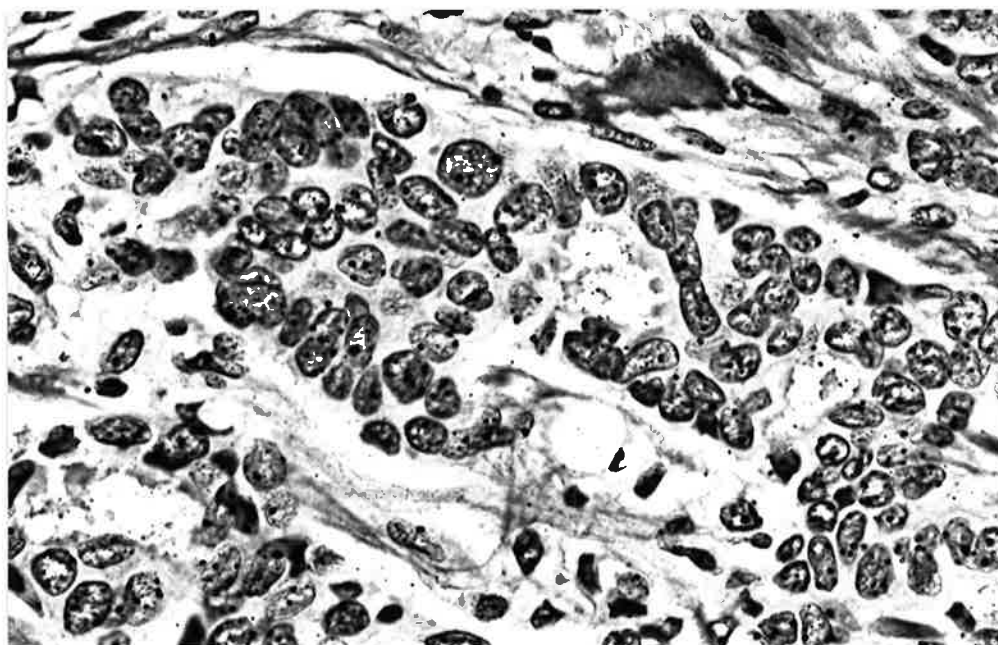
Figure IV.5.2. A moderately-differentiated infiltrating ductal carcinoma with a low AgNOR count of 3.71. The Ki-67 count for this case was 8.8 per cent.
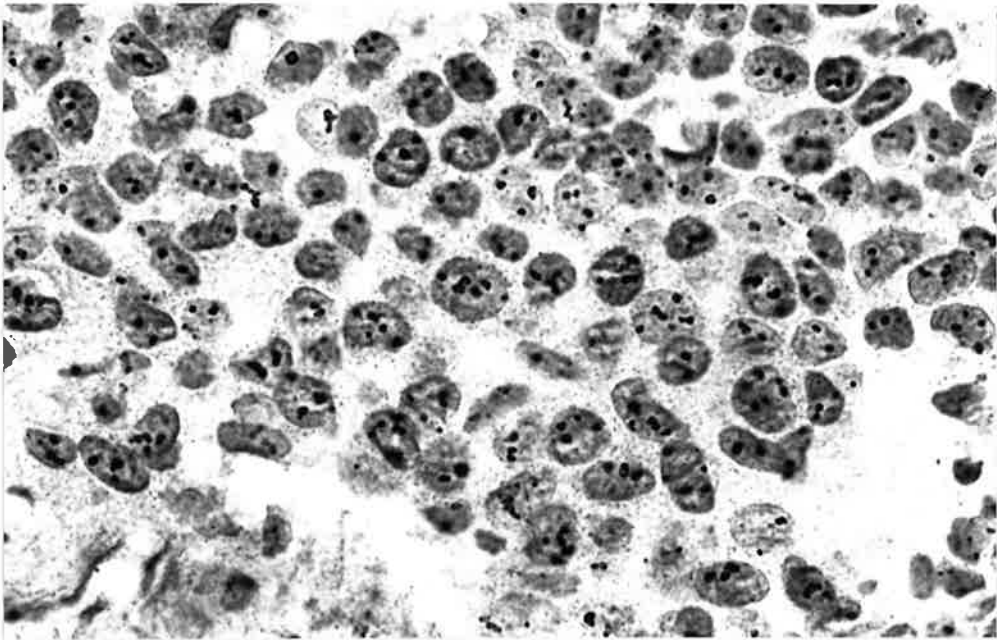
a.
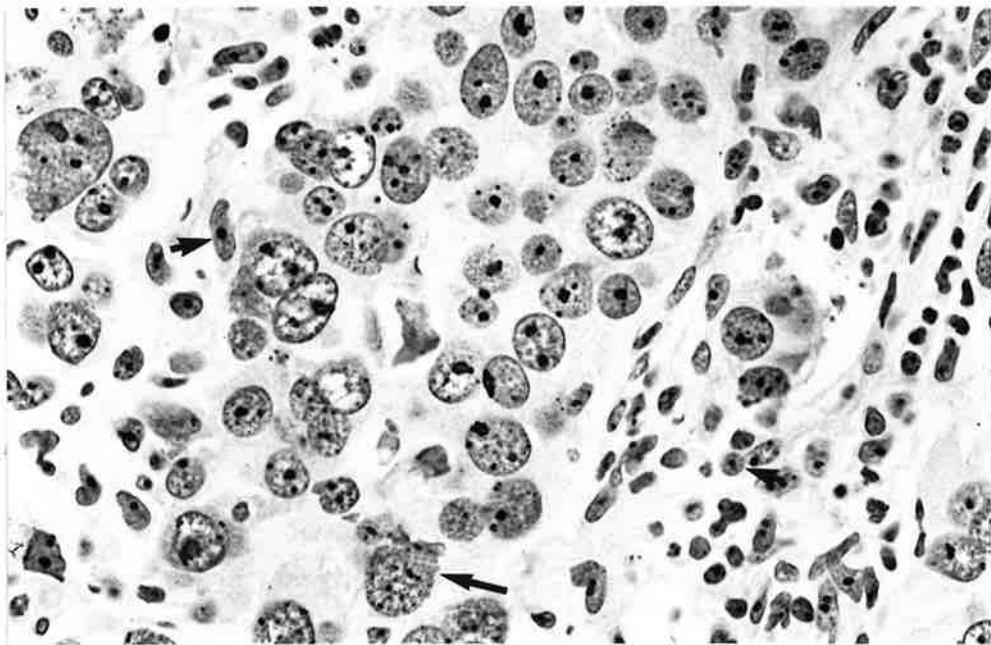
b.

Figure IV.5.3. Well-differentiated (a) and poorly-differentiated (b) infiltrating ductal carcinomas illustrating AgNOR counts of 6.36 and 5.1 respectively (a, x750; b, x500). In (b), malignant cells contain up to 10 dots per nucleus (long arrow), in contrast to background stromal and lymphoid cells which show only one to three dots per nucleus (short arrows). The Ki-67 count of the tumour depicted in (a) was 5%, while 56% of neoplastic cells were Ki-67 positive in the poorly-differentiated carcinoma (b).

sections without the need for counting, and marked intratumoral and intertumoral variation was apparent. AgNORs in malignant cells were often dispersed throughout the nucleus, and showed a greater variability in size and staining characteristics when compared with AgNORs in benign epithelial cells. Between zero and 25 dots were counted per cell, with as many as three clusters of up to eight individually discernible dots per cluster being observed. All dots, both "satellite" and those within clusters, were counted when visible as discrete entities. Repeat counts did not vary by greater than 10 per cent.

Incubation with the silver nitrate solution for 45 minutes produced readily identifiable AgNORs in all sections, although some variability in staining intensity was noted between cases. Incubation for less than 30 minutes or longer than 60 minutes, all other conditions remaining constant, produced suboptimal staining. AgNOR staining of sections from 15 paraffin blocks of tissue which had previously been frozen in liquid nitrogen for the purpose of intraoperative frozen-section evaluation either produced indistinct, smudged, pale brown intranuclear foci, impossible to accurately quantify, or no staining at all. Small "bubbles" of the order of one to two microns in diameter developed in some of the stained sections. These may represent gelatin and, although a nuisance, were readily distinguished from the AgNORs by their refractile nature and random intracytoplasmic and extracellular distribution.

Individual AgNOR dots were much more readily discerned in the imprint preparations than in the paraffin sections, and the range of mean counts increased to 6.2 to 22.6 per nucleus for the six examples (Figure IV.5.4). There were too few
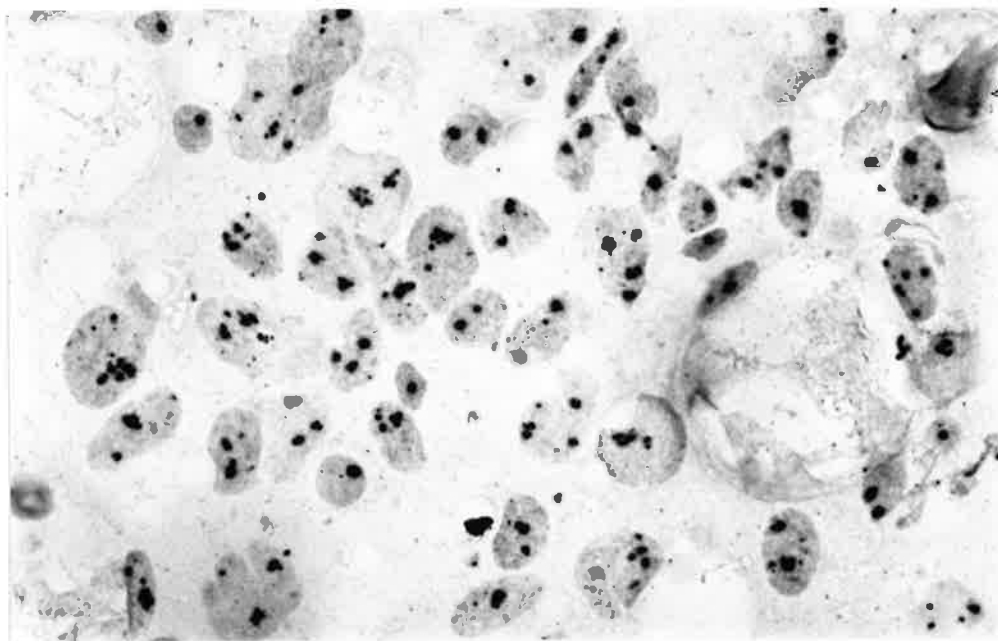
a.
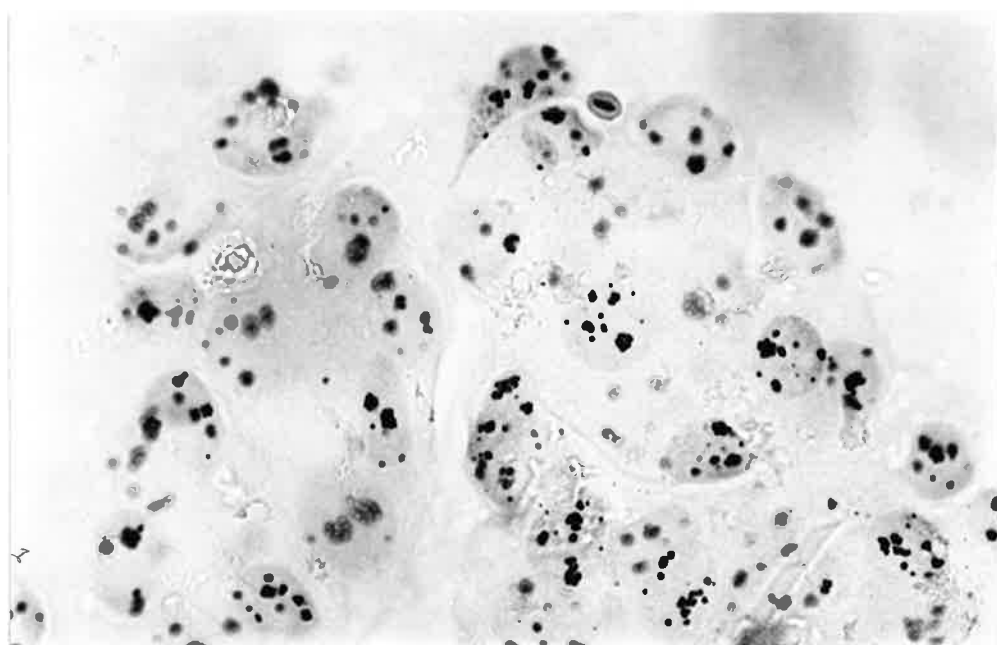
b.

Figure IV.5.4. The clarity of AgNOR staining in imprint preparations is readily apparent in these ductal carcinomas. The AgNOR count for the imprint illustrated in (a) was 12, compared with an AgNOR count of 4.9 for the paraffin section of this case. A mean count of 15 is illustrated in (b), while the corresponding paraffin section AgNOR count was only 3.5.

cases, however, to perform meaningful statistical analysis.

Case	Imprint AgNOR count	Corresponding Paraffin Section AgNOR count
1.	6.2	3.1
2.	12.0	5.1
3.	12.0	4.9
4.	15.0	3.5
5.	17.0	6.7
6.	22.6	5.2

A comparison of silver-stained paraffin sections in which portions of the same breast tumour had been fixed by one of three techniques revealed comparable AgNOR staining intensity in 10 per cent neutral-buffered formalin and microwave- irradiated tissues. AgNOR staining of Bouin's-fixed tissues was adequate, but of lesser intensity.

The Ki-67 and ER counts used for comparison with AgNOR counts were specified earlier in this chapter.

2. AgNOR Correlation with Tumour Proliferation and Other Clinicopathological Parameters

A significant correlation was identified between the mean numbers of AgNORs per nucleus and the Ki-67 count in 111 malignant breast neoplasms ($r = 0.41$, p

<0.0001), as depicted in Figures IV.5.5 & IV.5.6. In three cases Ki-67 counts were not available (Chapter IV.3) and in one case there was no tissue suitable for AgNOR staining (Chapter IV.5.b) In the 105 IDCs the AgNOR count was positively related to the tumour histological grade (r = 0.29, p <0.01), although such a correlation had not been apparent in preliminary work including only 79 ductal carcinomas (Raymond & Leong, 1989e). Nevertheless, AgNOR counts within each histological grade showed considerable overlap (Figure IV.5.7). There were too few cases of the other histological subtypes for meaningful statistical analysis, although the mucinous and tubular carcinomas were noted to exhibit relatively low AgNOR counts, in keeping with an anticipated favourable prognosis, and the count of 6.2 for the carcinosarcoma may reflect the traditionally poor prognosis associated with this tumour type. The AgNOR count correlated inversely with the ER count (r = -0.34, p <0.001; Figure IV.5.8) and with ER values obtained by cytosolic assay (r = -0.23, p <0.05).

No relationship was observed between the AgNOR count and the primary tumour size, the patient's age or the axillary nodal status. There was no difference in mean AgNOR counts when premenopausal and postmenopausal women were compared. Finally, AgNOR counts were significantly higher in the group of vimentin-positive (Category 2) ductal carcinomas than in the group of vimentin-negative (Category 0 or 1) tumours (t = 3.6, p <0.0005). The mean AgNOR count for vimentin-positive cases was 7.43, compared with a mean of 5.05 for vimentin-negative tumours (Figure IV.5.9).
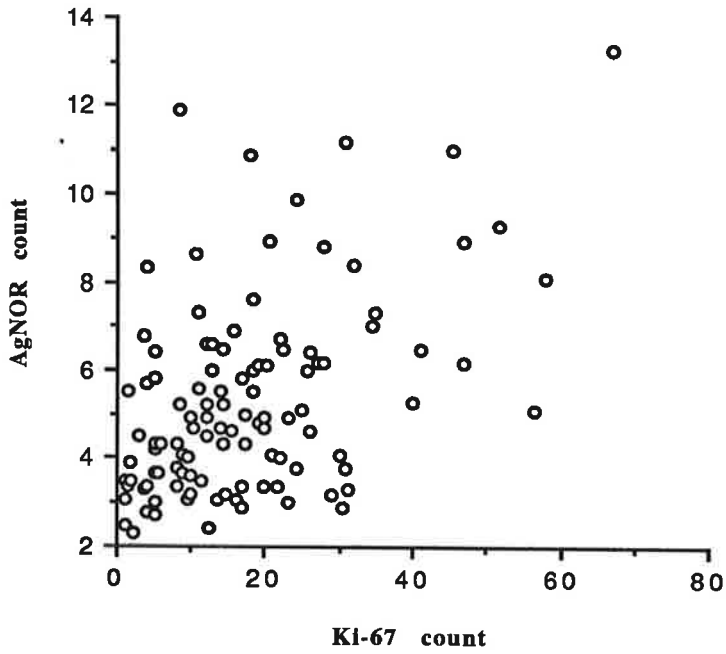
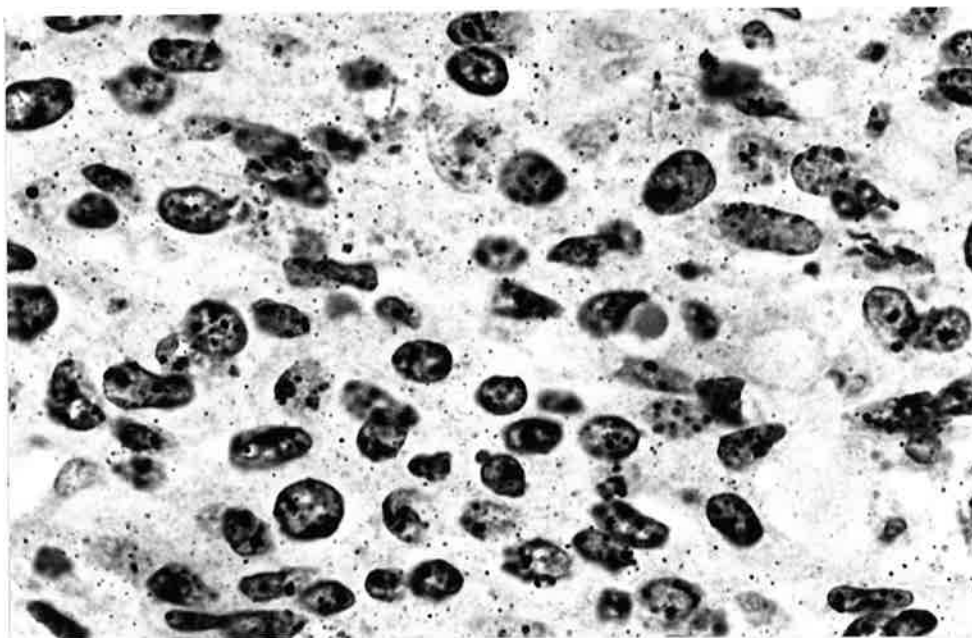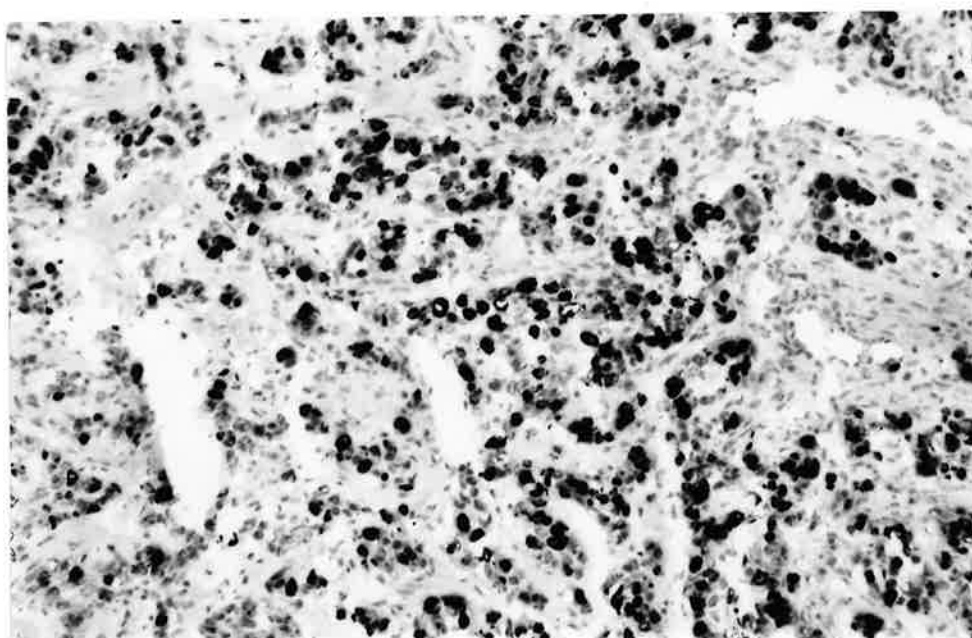**Figure IV.5.5.** Relationship between the Ki-67 count and the AgNOR count in 111 malignant breast neoplasms (r = 0.41, p <0.0001)

a.

b.

Figure IV.5.6. (a) A moderately-differentiated infiltrating ductal carcinoma demonstrating a mean AgNOR count of 13.3. Up to 18 dots are readily visible in the malignant nuclei. Note the random small extracellular spots which represent fine particles of gelatin precipitate. (b) Frozen section from the same case showing Ki-67 nuclear staining in 67% of neoplastic cells.
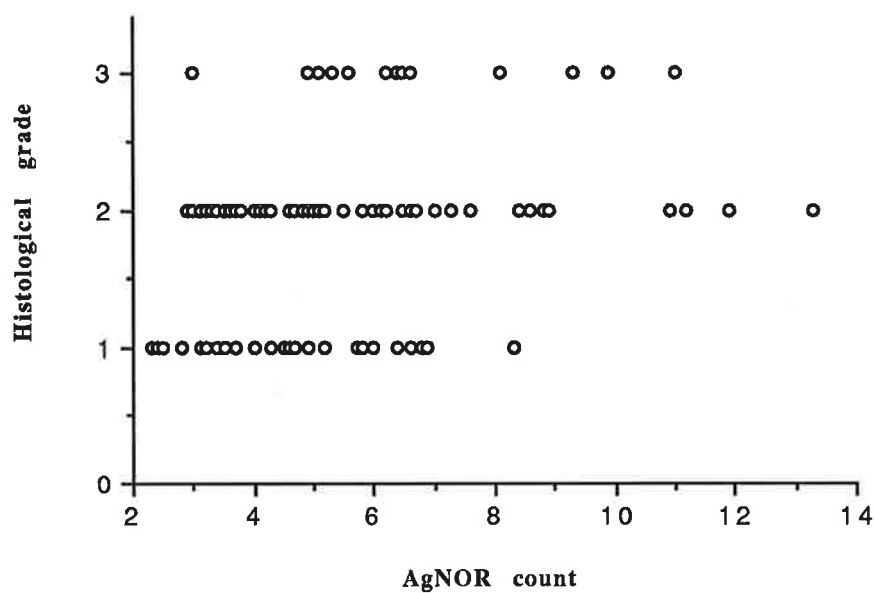
Figure IV.5.7. Relationship between the AgNOR count and the histological grade in 105 infiltrating ductal carcinomas ($r = 0.29$, $p < 0.01$)
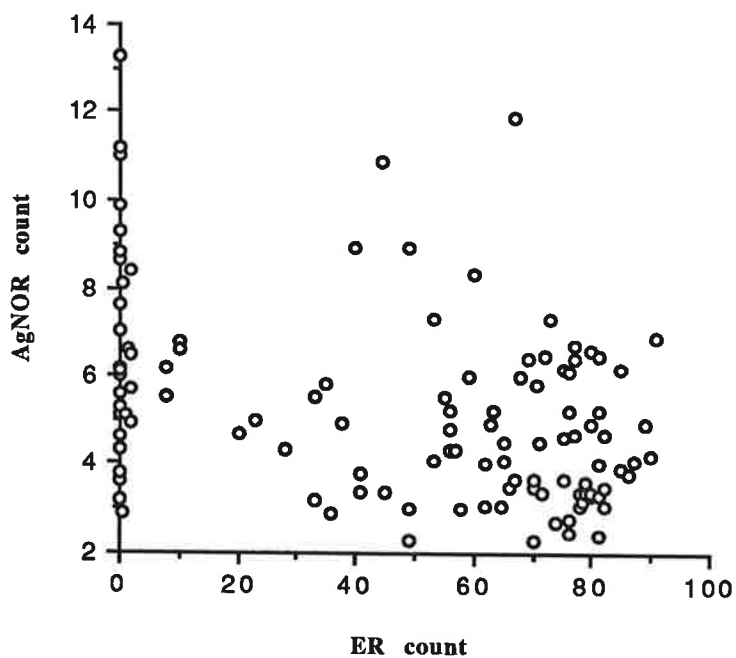
Figure IV.5.8. Inverse correlation between the AgNOR count and the immunohistochemically-determined ER count in 114 malignant breast neoplasms (r = -0.34, p <0.001)
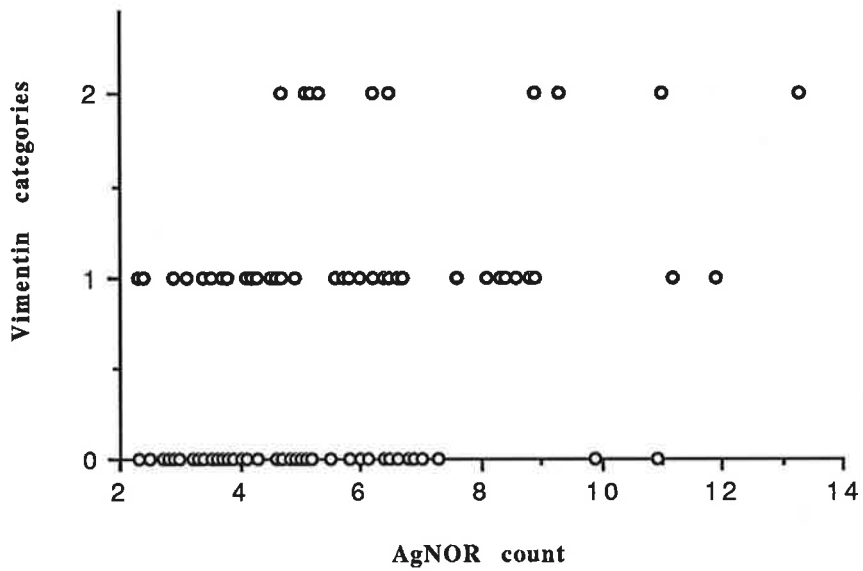
Figure IV.5.9. Relationship between the AgNOR count and vimentin expression (t = 3.6, p <0.0005)
Vimentin categories: 0 = no staining; 1 = <10% cells positive; 2 = >10% cells positive

IV.5.d.   DISCUSSION

The value of assessing tumour proliferative activity as a guide to prognosis in breast carcinoma has been discussed in Chapter II. Counting of nuclear AgNORs is a novel, simple procedure which has gained in popularity with its ready applicability to paraffin sections. A positive relationship between the tumour GF, determined by Ki-67 immunostaining, and the AgNOR count is demonstrated for the first time in a series of malignant breast neoplasms. Significantly higher AgNOR numbers were also found in malignant breast tumours (n = 114) when compared with normal breast and benign breast lesions (n = 66), confirming the results of Smith and Crocker (1988) which were based on a series of 46 cases.

The inverse correlation between AgNOR counts and ER values may be explained on the basis of the previously demonstrated inverse relationship between Ki-67 and ER counts, but adds weight to the possible prognostic value of NOR numbers. Furthermore, the correlation between AgNOR counts and the histological grade of IDCs is in keeping with current models of the behaviour of breast carcinoma. The high mean AgNOR count in vimentin-positive tumours also supports this author's theory that the latter may relate to more aggressive tumour behaviour.

The AgNOR technique, however, poses some important limitations which have not been fully discussed by the major proponents of this new marker. Despite the fact that the mean AgNOR count for malignant breast lesions was significantly higher than that observed in benign epithelium, a variable degree of overlap was observed

for lesions with two to three AgNORs per nucleus. This prohibits an absolute distinction between benign and malignant breast lesions based on AgNOR counts. Such overlap is also apparent in the data presented by Smith and Crocker (1988). High AgNOR counts (greater than four) were readily recognized on casual examination, and were only observed in malignant tumours.

The degree of dispersion of NORs was variable, and in those cases in which dots were tightly aggregated or overlapping within a cluster it was not possible to accurately or reliably quantitate the number of AgNORs because of their small size and apparent fusion. Unacceptable interobserver variation is likely if attempts to discern individual AgNORs within such a cluster are made and it is preferable to consider inseparable dots as one AgNOR. In some sections, the distinction between a neoplastic cell and a stromal cell or histiocyte was very difficult. Staining was occasionally pale (six lesions). This was improved by repeat staining in four cases, but in the other two cases extension of the staining period to 60 minutes was necessary.

Counting with a X100 oil emersion lens increased the AgNOR count by an average of two dots per nucleus compared with counting using a X60 lens. However, counting 200 cells using oil emersion is tedious and only semi-quantitative, requiring up to 15 minutes per case. Interobserver and intraobserver AgNOR counts did not vary by greater than 10 per cent (Raymond & Leong, 1989e), but inter-institutional studies may not be so reproducible. The average AgNOR count for malignant tumours in this author's study was lower than that found by Smith and Crocker (1988). This is somewhat surprising as the sections examined were two microns

thicker than the ones used by the Birmingham group. The differences most probably reflect the different methods of enumerating AgNORs. It is clear that if the AgNOR technique is to be adopted for routine use the protocol for counting must be reliable and readily reproducible.

Microwave-fixed tissue produced excellent AgNOR staining of comparable intensity to sections of the same breast carcinoma which had been fixed in 10 per cent neutral-buffered formalin (Leong & Gilham, 1988; Leong & Raymond, 1988). Hitherto, AgNOR staining in microwave-fixed tissue had not been described. Formalin fixation was superior to fixation in Bouin's solution, confirming the findings of Smith et al (1988) who examined AgNOR staining of normal tonsil fixed by a series of alternative methods. It is of interest that NORs could not be stained in paraffin sections of tissues previously subjected to freezing and thawing.

Air-dried imprints produce elegant AgNOR staining and provide a promising alternative medium for assessment of NORs as the entire cell nucleus is present, compared to only a portion of the nucleus in a five-micron section. Furthermore, individual AgNORs are more clearly stained and widely dispersed in imprints, and the higher counts obtained may allow greater prognostic discrimination amongst carcinomas, and also help distinguish between benign and malignant lesions. Similar findings were recently reported in an article describing the application of the AgNOR method to imprints of lymphoid tissue (Boldy et al, 1988).

In conclusion, the AgNOR method does not offer a reliable discrimination between benign and malignant breast epithelium, but may provide information on breast

cancer prognosis. The correlation between the mean numbers of AgNOR sites per nucleus and the Ki-67 count, representing the tumour GF, suggests that the number of AgNORs in a breast tumour may reflect the proliferative behaviour of that tumour. NOR expression may be of fundamental importance in cell activity, however, such data only provide indirect evidence about the nature of NORs and their functional significance remains uncertain. A broad scatter of Ki-67 and AgNOR counts was observed in this study. The potential application of these methods to indicate prognosis or provide a guideline for optimal therapy will require long-term clinical follow-up, in particular, to assess which parameter most accurately predicts outcome. The AgNOR method has the advantage of being applicable to conventionally fixed and processed paraffin sections.

IV.6. OTHER TRADITIONAL PROTEIN MARKERS OF BREAST CARCINOMA

a.   INTRODUCTION

b.   ALPHA-LACTALBUMIN

c.   PREGNANCY-SPECIFIC β_1-GLYCOPROTEIN (SP1)

d.   PROLACTIN

e.   DISCUSSION

IV.6. OTHER TRADITIONAL PROTEIN MARKERS OF BREAST CARCINOMA

IV.6.a. INTRODUCTION

During the past decade there has been an intense search for biological markers of breast carcinoma which exhibit tissue specificity as well as offering prognostic information. Some of these have been described in the preceding sections of this chapter, but many others have been identified which remain, at best, of controversial value.

Breast carcinomas are known to synthesize a wide variety of substances which may be detected using biochemical and immunohistochemical techniques. Immunohistochemical assays reveal the populations of tumour cells expressing these various antigens to be heterogeneously distributed in tissue sections. Some antigens, such as alpha-lactalbumin (Lee et al, 1984), lactoferrin (Charpin et al, 1985) and prolactin receptor (Bonneterre et al, 1986), are normal breast epithelial cell products. These are associated with milk production and may reflect functional differentiation of the tumour, hormone sensitivity and, possibly, a favourable prognosis. The expression of the ER-related proteins ER-D5 and P24 (Bilous et al, 1987; Smyth et al, 1987; Giri et al, 1987; Horne et al, 1988) may also indicate hormone sensitivity

of the neoplastic cells and a favourable outlook. Weak correlations between ER content in frozen sections and both ER-D5 and P24 staining have been described, but the true significance of expression of these epitopes has not yet been established.

Certain normal extramammary proteins may be anomalously produced by breast carcinoma cells and are thought to indicate "dedifferentiation" of the tumour. Such "inappropriate" antigens are generally associated with poor survival and include oncofetal antigens such as carcinoembryonic antigen (Kuhajda & Mendelsohn, 1982; Khemani et al, 1988; Fukutomi et al, 1989) and the pregnancy-associated proteins - human placental lactogen (HPL), pregnancy-specific β_1-glycoprotein (SP1) and pregnancy-associated plasma protein A (Horne et al, 1976; Kuhajda & Eggleston, 1985). *In vitro* studies with lymphocytes in culture have suggested that HPL and SP1 may have immunosuppressive properties by inhibiting the stimulation of lymphocytes induced by phytohaemagglutinin (Horne et al, 1976; Cerni et al, 1977), and it has been speculated that these ectopic substances may allow the tumour to escape recognition as "foreign" and grow unhindered by host defences.

Epidermal growth factor receptor (EGFR), a transmembrane molecule which transduces the activation and mitogenic signal of epidermal growth factor, and transferrin receptor (TfR) are two other antigens which have recently been heralded as potential indicators of poor prognosis (Tonik et al, 1986; Sainsbury et al, 1987; Wrba et al, 1988b; Horne et al, 1988). Expression of each of these receptors has been related to tumour proliferative activity and to conventional markers of poor prognosis, including histological anaplasia and ER-negative status (Chapter II.g; Tonik et al, 1986; Sainsbury et al, 1987; Delarue et al, 1988). However, Wrba et

al (1988a) failed to confirm any relationship between EGFR and tumour grade, tumour diameter, lymph node status or Ki-67 count. TfR has been identified on activated lymphocytes, histiocytes, dendritic reticulum cells and hepatocytes (Habeshaw et al, 1983), and it was concluded in one recent study that TfR is not a reliable marker for proliferating cells (Schrape et al, 1987; Chapter II.g).

In several studies paraffin sections of breast carcinomas were stained with antisera to the major iron-binding proteins - lactoferrin, ferritin and transferrin (Rossiello et al, 1984; Charpin et al, 1985). Rossiello et al (1984) detected lactoferrin only in normal breast epithelium and in benign proliferative lesions and suggested this protein could be used as a pointer to benign disease; however, lactoferrin was found in 7.5 per cent of 67 breast carcinomas studied by Charpin and coworkers (1985). Raised serum and cytosolic ferritin concentrations observed in a variety of malignant states, including breast carcinoma, have been attributed both to tumour synthesis (Weinstein et al, 1982) and to stromal reaction (Rossiello et al, 1984), and ferritin has been postulated as a possible tumour marker (Weinstein et al, 1982). Rossiello and associates (1984) observed ferritin staining mainly in the stroma and in histiocytes surrounding breast carcinoma cells, although faint ferritin positivity was observed in tumour cells of 20 of the 40 cases examined.

Paraffin sections from each of the 115 malignant breast neoplasms in this study were stained with anti-ferritin antiserum, at a dilution of 1:12000, using the ABC technique (Chapter III.2; Appendix I). Positive staining of variable intensity and extent was detected in the cytoplasm of stromal cells in 88 per cent of cases. Stromal staining was occasionally observed in the vicinity of benign epithelium, but was most intense

surrounding neoplastic cells (Figure IV.6.1a), supporting the contention that stromal cells may be responsible for raised serum ferritin levels in malignant states. However, scattered stromal histiocytes, myoepithelial cells and vascular endothelial cells also stained positively, and in four cases (3.5 per cent) there was weak ferritin staining of occasional neoplastic cells (Figure IV.6.1b), confirming the findings of Rossiello et al (1984). Due to the presence of diffuse stromal ferritin positivity in almost all cases and the scant, inconsistent staining in tumour cells, this marker is considered unlikely to be of prognostic value.

Lectins are proteins which bind to surface carbohydrate residues on epithelial cells. Those commonly used include peanut agglutinin (PNA), soy bean agglutinin (SBA), wheat germ agglutinin (WGA), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutinin I (UEA I) and Concanavalin A (Walker et al, 1985 a & b; Fenlon et al, 1987). Following reports of alterations in cell surface carbohydrates during malignant transformation (Leathem et al, 1983), lectins have been implicated as a means for differentiating benign from malignant breast epithelium and as possible prognostic indicators. Various authors have described increased binding or loss of binding by some of these lectins to breast carcinoma cells, but a consistent relationship between lectin binding and either tumour differentiation, receptor status or survival has not been identified (Walker et al, 1985 a & b; Stanley et al, 1986; Fenlon et al, 1987; Dansey et al, 1988). Most recently, HPA staining of breast tumours has been related to early relapse and shorter survival based on examination of clinical data for two series of patients, one followed for 15 years (Leathem & Brooks, 1987) and the other for five years (Fukutomi et al, 1989). The use of UEA I to outline blood vessels, and thus highlight tumour vascular invasion, will be discussed in Chapter IV.7.
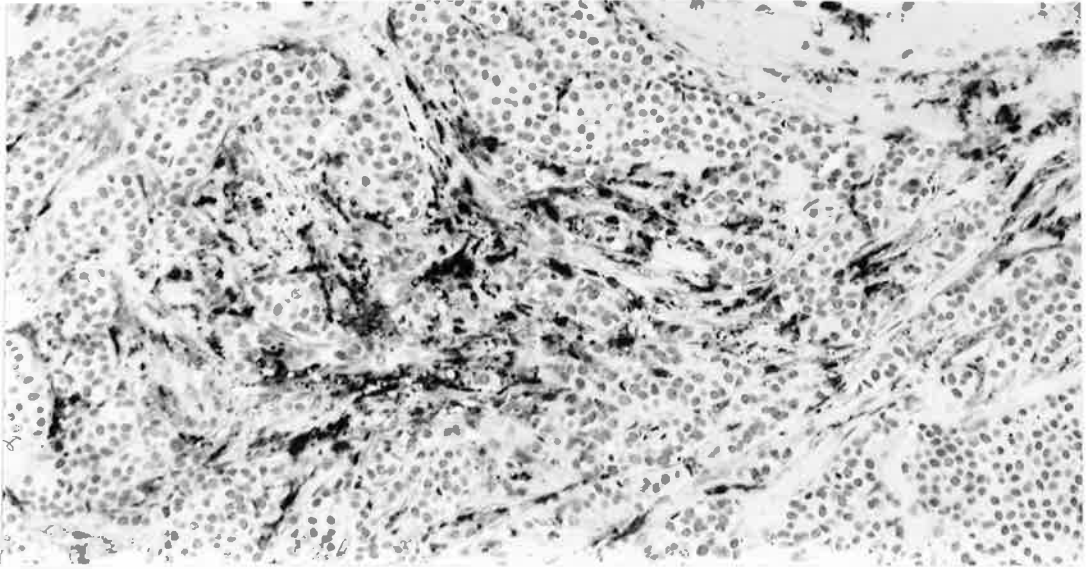
a.
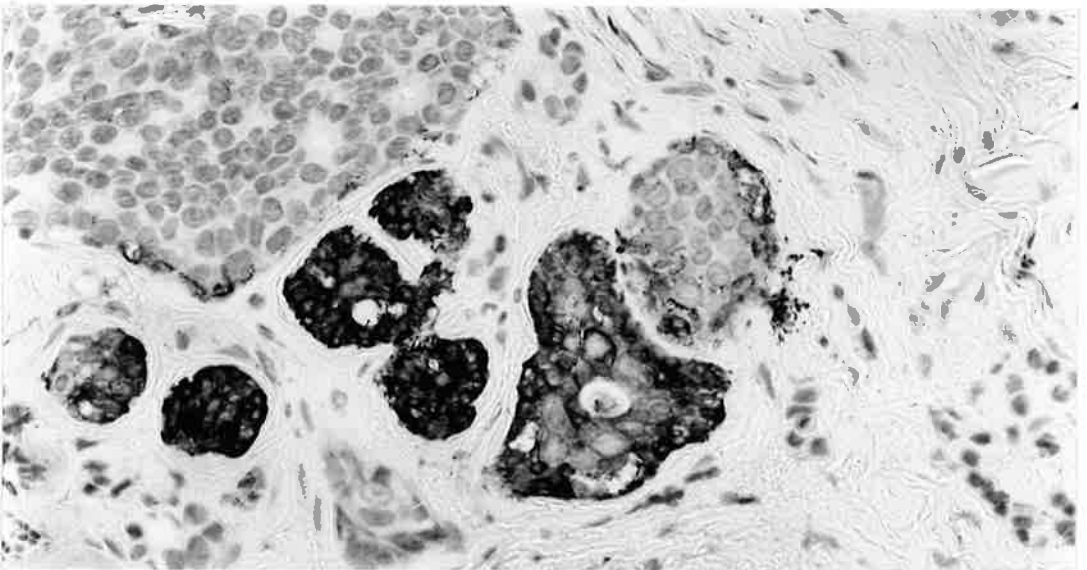
b.

Figure IV.6.1. Paraffin sections of two infiltrating ductal carcinomas stained with anti-ferritin antiserum (haematoxylin counterstain) illustrating intense stromal staining in (a), and positivity in aggregates of neoplastic cells in (b).

Human milk-fat-globule membrane (HMFG) antigens I and II (Berry et al, 1985; Parham et al, 1989), NCRC II (Ellis et al, 1985 & 1987; Wright et al, 1987; Hitchcock et al, 1989), urokinase-plasminogen activator (Duffy et al, 1988), monoclonal antibody B72.3 (Kline et al, 1989), P53 protein (Cattoretti et al, 1988), gross cystic disease fluid protein-15 (Wick et al, 1989; Mazoujian et al, 1989) and breast carcinoma-associated antigen (Yu et al, 1980) are among a host of other immunocytochemical markers, raised against human or animal mammary carcinoma cell lines, which have been heralded in the literature as either diagnostic adjuncts for breast carcinoma or as potential prognostic indicators. This multitude of markers and conflicting results leave the surgical pathologist in a quandary as to which ones are of true value. Currently none can be deemed tissue specific and none provide reliable prognostic guidance.

Several of the more popular "breast markers" - alpha-lactalbumin, SP-1 and prolactin, are evaluated in the following sections and the degree of staining related to histological grade, ER status, presence of lymph node metastases, Ki-67 and AgNOR counts, and vimentin expression.

IV.6.b. ALPHA-LACTALBUMIN

Alpha-lactalbumin (ALA), a glycoprotein of approximately 14 kd, is thought to facilitate the conversion of glucose to lactose in the production of milk (Rose & McGrath, 1975). The reported incidence of ALA expression in breast carcinomas

and their metastatic deposits, using immunoperoxidase staining techniques on paraffin-embedded tissues, ranges between zero and 76 per cent (Walker, 1979; Hall et al, 1981; Clayton et al, 1982; Lloyd et al, 1984; Lee et al, 1984 & 1985), and ALA has been widely used in the past as a mammary tissue-specific marker in the identification of metastatic carcinomas (Walker, 1979; Bahu et al, 1980; Kuhajda & Mendelsohn, 1982; Clayton et al, 1982; Lloyd et al, 1984).

In this study the value of ALA as a prognostic marker was examined. One hundred and fourteen of the 115 breast carcinomas and eight benign breast tissues were stained with ALA antiserum (Nordic Laboratories, Tilburg, Netherlands) at a dilution of 1:4000 (Chapter III.2; Table III.2). In one case the tumour was very small and no neoplastic cells were identified in the deeper levels.

ALA positivity was recognized as diffuse, granular, cytoplasmic staining of epithelial cells. The stroma was excluded from assessment. Tumours were allocated to one of four categories according to the extent of ALA staining, where Category 0 = no staining reaction, Category 1 = less than 33 per cent of cells ALA-positive, Category 2 = 33 to 66 per cent of cells positive and Category 3 = greater than 66 per cent of cells ALA-positive (Wrba et al, 1988b). Both the absolute ALA status (positive or negative overall) and the extent of staining (Categories 0 to 3) were related to the tumour histological grade, lymph node status, ER status, Ki-67 count, AgNOR count and vimentin immunoreactivity, with p values based on Spearman's correlation coefficient (r) and unpaired Student's t-tests.

ALA was localized in 44 (38.6 per cent) tumours in this series. The extent of

staining was variable and 57 per cent (25) of the ALA-positive tumours were classified as Category 1, 23 per cent (10) as Category 2 and 20 per cent (9) as Category 3 (Table IV.6.1; Figure IV.6.2). These proportions are similar to those described by Lee et al (1984). None of the three lobular carcinomas, one mucinous carcinoma, one intraductal carcinoma, one carcinosarcoma or one malignant phyllodes tumour exhibited ALA positivity. Positive staining was seen in less than a third of neoplastic cells in the tubular carcinoma. In addition, non-neoplastic epithelium, when present in the section, was focally ALA-positive as reported previously (Kuhajda & Mendelsohn, 1982; Le Doussal et al, 1984; Lloyd et al, 1984; Lee et al, 1984). One of three fibroadenomas, two cases of fibrocystic change and all examples of normal breast tissue showed patchy ALA staining. There was consistent positive staining of the control section of lactating breast (Figure IV.6.2b) and no staining in sections treated with normal horse serum in place of the primary antiserum.

Staining was diffuse throughout the cell cytoplasm (Figure IV.6.2) and varied in intensity between cases. Background staining of connective tissue, inflammatory cells (Figure IV.6.2c), blood vessels and keratinizing squamous epithelium was frequently observed and attributable to the polyclonal nature of the antiserum. The background staining was homogeneous throughout the cell, including the nucleus, and could generally be readily distinguished from true granular positivity. Two cases, however, were excluded from statistical analyses as the background reaction was too marked for accurate classification (Table IV.6.1). The presence of background staining was thought to prohibit a reliable assessment of staining intensity.
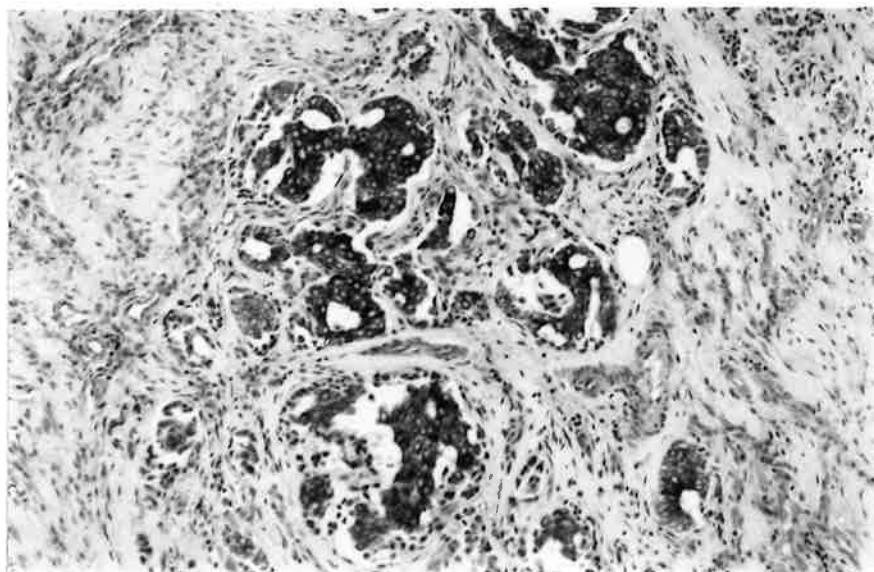
TABLE IV.6.1.

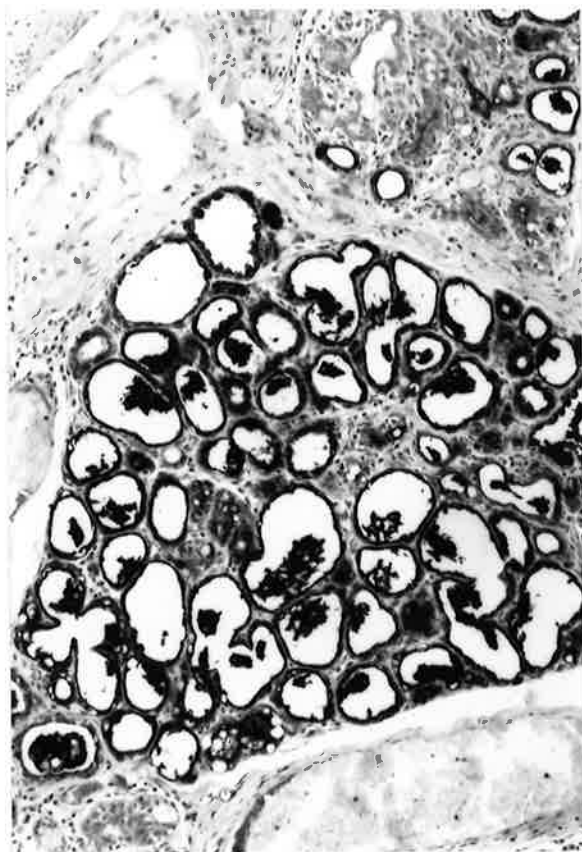STAINING OF "TRADITIONAL" BREAST CANCER

MARKERS IN 114 CASES

ANTISERUM	% POSITIVE TUMOUR CELLS*				NA
	0	1	2	3	
Alpha-lactalbumin (ALA)	68	25	10	9	2
Pregnancy-specific β_1-glycoprotein (SP1)	53	33	17	10	1
Prolactin	108	5	0	0	1

* 0 = no staining; 1 = <33% positive; 2 = ≥33% &
  ≤66% positive; 3 = >66% positive

NA = not assessed (see text)
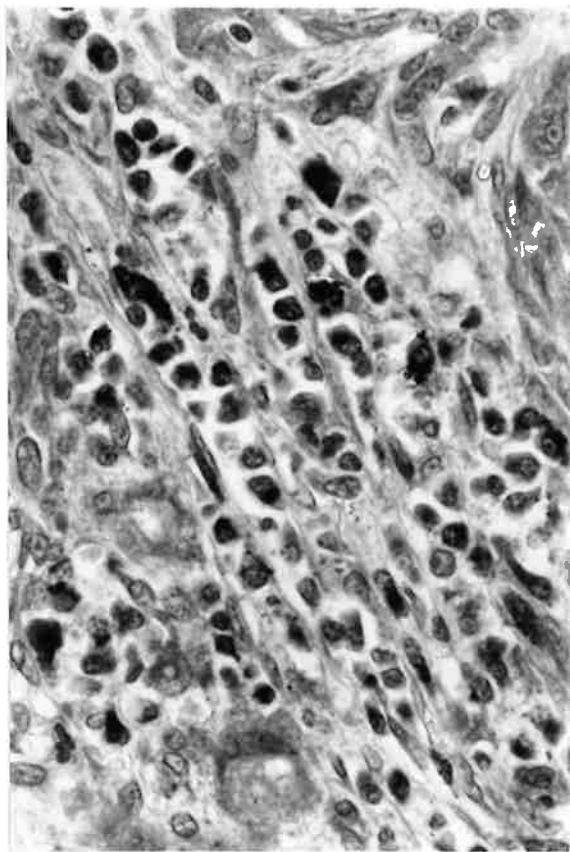
Figure IV.6.2. (a) An infiltrating ductal carcinoma illustrating ALA staining in approximately 50 per cent of neoplastic cells (haematoxylin counterstain).
(b) Strong ALA positivity in the epithelial cells and intraluminal secretions of the control section of lactating breast. (c) Background ALA staining of inflammatory cells, including plasma cells. Less than 33 per cent of neoplastic cells were ALA-positive in this case.

There was no correlation between ALA positivity, or the extent of ALA staining, and the histological grade, the presence or absence of lymph node metastases, the ER status, the AgNOR count or vimentin immunoreactivity. Fifteen (52 per cent) of 29 well-differentiated (grade I) carcinomas produced ALA, and 33 per cent (four) of 12 grade III tumours were ALA-positive. Similar findings were reported by Walker (1979), Clayton et al (1982), Lee et al (1984), Le Doussal et al (1984) and Wrba et al (1988b). Lee et al (1985), in a study of 233 breast neoplasms, concluded that ALA expression was of no value in predicting prognosis in terms of the likelihood of developing recurrent tumour, the interval to recurrence or the likelihood of harbouring metastases. The three previous reports that related ALA reactivity to ER status (Bahu et al, 1980; Le Doussal et al, 1984; Wrba et al, 1988b) also failed to find a relationship, however, two relied upon cytosolic estimations of ER content (Bahu et al, 1980; Le Doussal et al, 1984), and Bahu and coworkers (1980) utilized an indirect immunofluorescence technique to assess ALA expression.

ALA was inversely related to the Ki-67 count (r = -0.279, p < 0.01), and there was a significant difference in Ki-67 counts between the ALA-positive and ALA-negative cases (t = 3.04, p = 0.003). This suggests that ALA-positive tumours are more likely to have low Ki-67 counts (mean of 12.9 per cent), and thus are less likely to behave aggressively, than ALA-negative tumours (mean Ki-67 count of 20.7 per cent). Only three (17 per cent) of 18 tumours with Ki-67 counts greater than 30 per cent were ALA-positive compared with 43 per cent (40) of 93 tumours with Ki-67 counts less than 30 per cent. Wrba et al (1988b), in a paper published since the completion of the above work, reported no such correlation, however, their Ki-67

data were only analysed as three separate arbitrary categories (between zero and four per cent, greater than four and less than or equal to eight per cent, and greater than eight per cent) and may thus not reflect the true spectrum of Ki-67 counts. In this thesis only 26 of the 112 tumours had Ki-67 counts of less than or equal to eight per cent.

Finally, several publications have described ALA staining in non-mammary neoplasms (Lee et al, 1984; Wick et al, 1989). Although staining in salivary gland and skin appendage tumours, reported by Lee et al (1984), may be explained by the similar embryonic derivation of these glands and the mammary gland, the finding of ALA in 49 per cent of a series of 65 non-mammary carcinomas (including ovarian, colonic, pancreatic, endometrial and prostatic carcinomas) by Wick et al (1989), and of ALA-positive mesotheliomas (Lee et al, 1984), is unacceptable. It is not known whether the extramammary staining reflects the presence of cross-reacting antibodies or the elaboration of an ALA-like substance, but it is clear that ALA is not tissue specific and is of no value in discriminating between breast and non-mammary tumours.

IV.6.c. PREGNANCY-SPECIFIC β_1-GLYCOPROTEIN (SP1)

Pregnancy-specific β_1-glycoprotein (SP1) is one of the so-called pregnancy-associated proteins synthesized by human placental syncytiotrophoblast and secreted into the maternal circulation. This group of secretory glycoproteins with hormone-like activity have been detected by radioimmunoassay in the sera of healthy persons and in those

with various malignancies, including breast carcinoma (Sorensen et al, 1984; Fagnart et al, 1985; Wright et al, 1987). The serum SP1 levels in patients with breast carcinoma are generally low and are unlikely to be of use in patient monitoring (Sorensen et al, 1984; Wright et al, 1987), however, one study of 84 breast cancer patients followed for four years suggested that the serum SP1 level was proportional to poor prognosis and inversely proportional to the ER concentration (Fagnart et al, 1985).

SP1 has also been demonstrated immunohistochemically in the cytoplasm of breast carcinoma cells, with the proportion of SP1-positive tumours varying between 18 and 76 per cent (Horne et al, 1976; Walker, 1982; Sorensen et al, 1984; Kuhajda et al, 1984; Lee et al, 1985; Wright et al, 1987). Despite this, no correlation between serum levels and tissue positivity was demonstrated in one study (Sorensen et al, 1984).

The inappropriate production of placental or pregnancy-associated proteins such as SP1 suggests functional "dedifferentiation" of the tumour cells and a less favourable prognosis is anticipated. Several authors linked the presence of SP1 in breast carcinoma cells, assessed by immunohistochemical staining, with poor prognosis. Horne et al (1976) demonstrated a significantly shorter survival time for women with SP1-positive breast carcinomas compared with those with SP1-negative tumours, and Kuhajda et al (1984) found a significant association between SP1 positivity and the presence of axillary lymph node metastases in small IDCs. However, Walker (1984) found no association between the presence of SP1 in breast neoplasms and early recurrence or histological differentiation. In a retrospective series of 233 breast

carcinomas, Lee et al (1985) found no relationship between SP1 staining and the likelihood of developing recurrence, the recurrence interval, or the presence of metastases. Wright and co-workers (1987) concurred and found that SP1 staining was not associated with tumour stage and was not related to survival, except in a subgroup of patients with grade II carcinomas. Patients with SP1-negative, grade II tumours were noted to experience longer survival.

Expression of SP1 was assessed using polyclonal antiserum (Dakopatts, Copenhagen, Denmark) at a dilution of 1:3000. Tumours with any cells showing granular cytoplasmic staining were scored as "positive". Cases were divided into one of four categories (0 to 3), as detailed for the ALA-stained tumours (Chapters III & IV.6.b). Sections of human placenta served as positive controls.

In one case marked background staining prohibited accurate assessment and this case was excluded from further analysis. Sixty tumours (52.6 per cent) were scored as SP1-positive (Table IV.6.1). The heterogeneous, granular SP1 staining was localized in the cytoplasm of the tumour cells in all positive cases (Figure IV.6.3) and 33 (55 per cent) were assessed as Category 1, 17 (28 per cent) as Category 2 and 10 (17 per cent) as Category 3. Differing staining intensities were generally apparent within the same tumour. The three lobular carcinomas, one mucinous carcinoma, one tubular carcinoma, one intraductal carcinoma, one carcinosarcoma and the malignant phyllodes tumour were all SP1-negative. Staining of a few neoplastic cells in the male breast carcinoma was observed.

One of three fibroadenomas and tissue from one of the three examples of normal
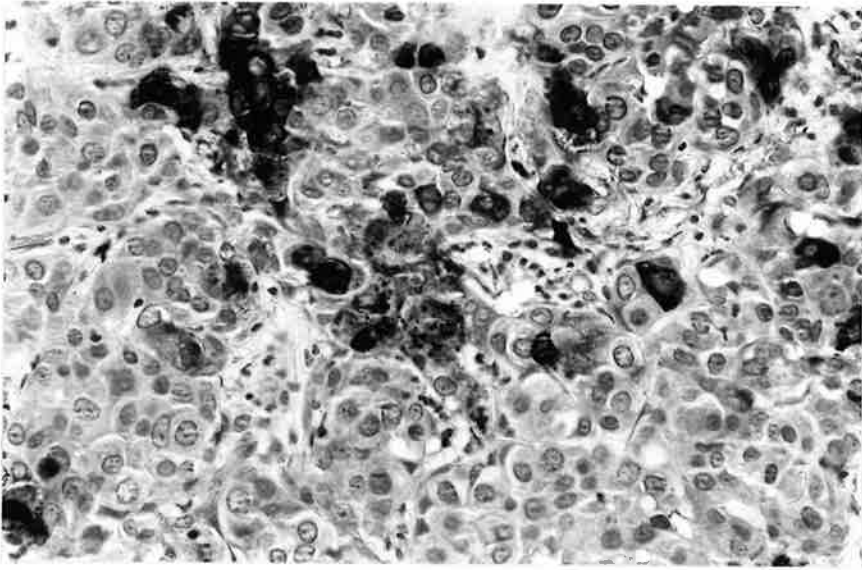
a.
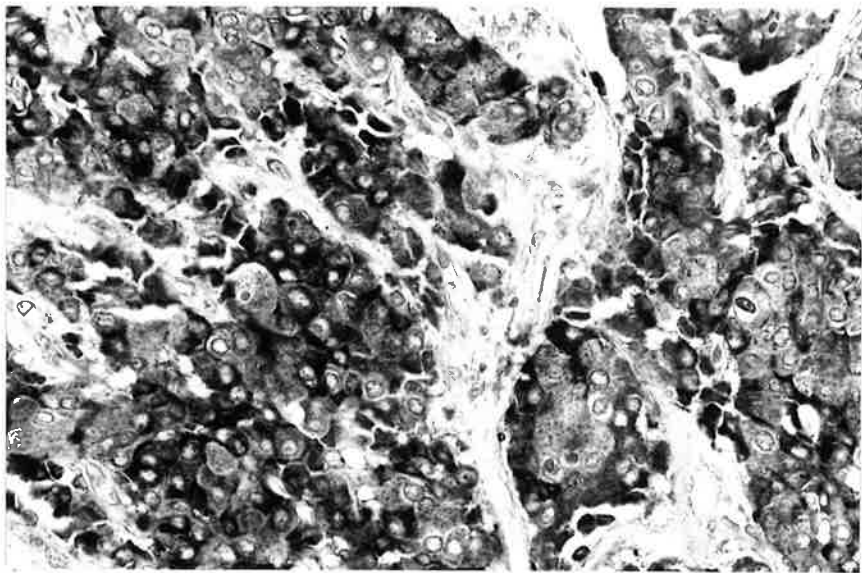
b.

Figure IV.6.3. (a) A poorly-differentiated ductal carcinoma demonstrating a heterogeneous pattern of cytoplasmic staining for SP1, in contrast to the moderately-differentiated carcinoma illustrated in (b), in which almost all neoplastic cells are SP1-positive (haematoxylin counterstain).

breast showed a focal positive reaction for SP1 in epithelial cells. Neither of the two cases of fibrocystic change showed positive staining. These findings are in accordance with those of others who described focal SP1 positivity in fibrocystic disease and in normal breast ducts and lobules (Kuhajda et al, 1984; Sorensen et al, 1984; Wright et al, 1987), although a few authors reported benign breast tissue to be consistently SP1-negative (Horne et al, 1976; Lee et al, 1985). There was generally minimal background staining, although necrotic tumour cells and occasional inflammatory cells were noted to stain positively. SP1, therefore, has no value in discriminating benign from malignant breast disease.

There was no relationship between the SP1 status (positive or negative overall) or the degree of SP1 staining and either the histological grade of the tumours, the lymph node status, the ER count, the AgNOR count or vimentin immunoreactivity. Unpaired Student's t tests revealed that SP1-negative tumours were likely to have higher Ki-67 counts (mean of 20.9 per cent) than SP1-positive tumours (mean of 14.9 per cent; $t = 2.37$, $p < 0.05$), although there was no correlation between the degree of SP1 staining and Ki-67 counts ($r = -0.11$, $p > 0.05$). Thirteen of 18 tumours with Ki-67 counts greater than 30 per cent were SP1-negative, however, the lack of correlation suggests that the data may be influenced by the relatively few cases with high Ki-67 counts.

In addition, significant correlations between both the extent of staining of SP1 and ALA ($r = 0.46$, $p < 0.0001$), and the absolute SP1 and ALA status ($t = 4.95$, $p = 0.0001$), were observed.

IV.6.d. PROLACTIN

Prolactin is a lactogenic hormone normally produced by the anterior pituitary to interact with prolactin receptors in breast tissue. The role of prolactin in the induction and growth of experimental mammary tumours is well known (Dhadly & Walker, 1983; Bonneterre et al, 1987), but the importance of this hormone in the development of human breast cancer is not established.

Dhadly and Walker (1983) applied purified human prolactin, followed by antiserum to human prolactin and a PAP technique, to cryostat sections of human breast carcinomas in order to detect prolactin receptors and found 56 per cent of 50 tumours gave a positive reaction. Extensive prolactin positivity was noted to correlate with well-differentiated tumours.

Bonneterre and coworkers (1986) measured prolactin receptor levels in tumour cytosol extracts by radioimmunoassay and described a correlation between total receptor levels and both ER and PgR status. Follow-up studies revealed prolactin receptor positivity predicted a higher RFS in node-positive and ER-positive patients (Bonneterre et al, 1987). Other investigators, however, failed to confirm this relationship (Waseda et al, 1985). Rae-Venter et al (1981) also observed a higher concentration of prolactin receptors, identified by radioimmunoassay, in moderately and well-differentiated breast carcinomas, but found no correlation between prolactin receptors and ER status. Holdaway and Friesen (1977) utilized a similar technique and identified binding sites for prolactin in only 20 per cent of breast tumours. Purnell et al (1982) used antiserum to prolactin and a PAP assay on paraffin sections

from 12 cases and revealed staining throughout the breast parenchyma, in addition to staining of breast secretory cells. Furthermore, they observed prolactin positivity in 64 per cent of 22 primary prostatic carcinomas examined.

Wilson et al (1980) studied breast tissues in culture and determined that while prolactin stimulated lactalbumin production in normal breast tissue, malignant tumours were resistant to such stimulation. They postulated that an absence or deficiency of prolactin receptors in malignant tissue may be responsible.

Thus, knowledge of the prolactin and prolactin receptor content of breast carcinomas is limited, and mostly based on radioimmunoassays of tumour homogenates. An antibody to prolactin receptor is not commercially available and immunoperoxidase staining of tissue sections is therefore dependent upon the more indirect measure of prolactin binding, utilizing antiserum to prolactin.

A preliminary series of 50 breast tumours were stained with anti-prolactin antiserum (dilution of 1:4000) purchased from Dakopatts in 1983 (Santa Barbara, CA, USA; Lot 043P) and stored at -20°C. Extensive background staining was revealed in all cases, rendering it impossible in most instances to identify any truly positive neoplastic cells. Inflammatory cells, connective tissue cells and stratified squamous epithelium stained equally, and nonspecific staining of neoplastic nuclei was observed.

Repeat staining of these 50 tumours, and an additional 63 breast carcinomas for which paraffin-embedded tissue was available, was performed with antiserum to

human prolactin purchased in 1988 from Dakopatts (Lot 047B) and applied at a dilution of 1:4000 (ABC technique; Chapter III; Appendix I). Despite reliable staining of the control sections of prolactin-secreting pituitary adenoma, scant staining of neoplastic cells was identified in only five (4.4 per cent) tumours, all cases of moderately-differentiated IDC. Benign epithelium was consistently prolactin-negative.

It is concluded that staining for prolactin is of no prognostic value, being positive in only a very small percentage of cases and bearing no relationship to any of the prognostic parameters studied. It is possible that the presence of prolactin receptor in breast carcinomas relates to ER and PgR status, and to survival (Bonneterre et al, 1986 & 1987), but the same does not appear to hold true for prolactin. Alternatively, binding of prolactin to the prolactin receptor may prohibit attachment of anti-prolactin antibodies. Batches of antisera clearly vary and may explain the discrepancy between this study and that of Dhadly and Walker (1983), who employed a non-commercial antibody.

IV.6.e. DISCUSSION

A specific marker for breast epithelial cells remains elusive. In turn, each of the proteins examined in this study - ALA, SP1 and prolactin, have been proclaimed at some time as specific breast cancer markers, but subsequent studies failed to confirm these allegations. Positive staining should not, therefore, be considered a reliable indication of breast origin when examining metastatic carcinomas of unknown primary site.

Furthermore, ALA and SP1 expression, and the presence of prolactin receptors, have been implicated by several authors as indicators of prognosis. In this series of breast carcinomas there was no relationship between ALA, SP1 or prolactin staining and the tumour histological type and grade, lymph node status, or tumour ER content, all traditional markers of prognosis. ALA and SP1 staining were also unrelated to AgNOR counts and vimentin expression.

Tumours with high GFs, represented by a high Ki-67 count, were significantly more likely to be both SP1 and ALA-negative. In addition, significant correlations between the degree of staining of SP1 and ALA, and the absolute SP1 and ALA status were observed. SP1 and ALA staining have not previously been compared. These findings are somewhat paradoxical in that SP1 expression has been considered an indicator of poor prognosis, whereas ALA production is thought to be associated with functional differentiation and longer survival. The reason for this apparent discrepancy is not clear, but may in part reflect the relatively small number of cases with high Ki-67 counts. The lack of SP1 staining in tumours with high GFs might suggest that SP1 expression is related to a better prognosis. However, the production of ectopic proteins may indicate unfavourable factors independent of the tumour proliferative activity. It is also possible that the presence of SP1 in tumour tissue represents uptake of SP1 from the circulation rather than local production of the hormone, and thus a relationship with tumour GF would not be expected. The true significance of the relationship between high Ki-67 counts and both ALA-negative and SP1-negative tumours will require clinical trials with long-term follow-up.

The SP1 antiserum was generally superior to the ALA antiserum both in terms of sensitivity of staining and degree of background immunoreactivity. In some cases, however, staining with both of these polyclonal antisera was unsatisfactory and might only be improved with the availability of monoclonal antibodies to ALA and SP1.

Differences in the immunostaining techniques and in the specificities of the various antisera utilized are most probably responsible for the wide variability in the percentages of SP1 and ALA-positive tumours reported in the literature. The figures obtained in the present study are comparable to those previously observed. Very few tumours exhibited staining with the prolactin antiserum. This situation is analogous to that of early attempts to identify ER in breast tumours using anti-estradiol. It was not until the monoclonal anti-ER antibody became available that reliable estimates of receptor content could be obtained, and perhaps a monoclonal antibody to the prolactin receptor will clarify the role of this hormone in breast cancer prognosis. In conclusion, with the currently available antisera, ALA, SP1 and prolactin should not be considered reliable prognostic markers in breast cancer.

IV.7. IMMUNOHISTOCHEMICAL ASSESSMENT OF VASCULAR INVASION

a.   INTRODUCTION

b.   FACTOR VIII

c.   *ULEX EUROPAEUS* AGGLUTININ I (UEA I)

d.   DISCUSSION

IV.7. IMMUNOHISTOCHEMICAL ASSESSMENT OF VASCULAR INVASION

IV.7.a. INTRODUCTION

Evidence supporting the prognostic value of peritumoral lymphatic and blood vessel invasion has been discussed in Chapter II.d. The reliability of routine assessment of vascular invasion in microscopic sections as an indicator of poor prognosis is probably most limited by interobserver and intraobserver differences in interpretation (Gilchrist et al, 1982). Assessment is also hindered by the well-recognized tissue shrinkage artefact that results from fixation and processing, and which may create the impression that nests of tumour cells are present within vascular spaces. These "pseudoemboli" are more likely to retain the exact contour of the surrounding stroma than true intralymphatic emboli which, although assuming the general configuration of the surrounding channel, exhibit noticeable differences in outline (Rosen, 1983). In addition, endothelial nuclei usually protrude into the vascular space whereas nuclei of stromal cells left at the margin of an artefactual space tend to be slender and resemble those of the adjacent connective tissue. In some cases, however, the distinction may still be difficult.This artefact is most frequently encountered within the tumour substance, so the evaluation of vascular invasion is most reliable when limited to mammary parenchyma peripheral to the tumour.

The differentiation of small blood vessel capillaries from lymphatic capillaries in H

& E sections is often arbitrary and depends, possibly erroneously, on the presence of luminal erythrocytes. Conventional elastic and reticulin stains are of little help in distinguishing between small lymphatics and blood vessels, neither of which have elastic tissue, or between larger vessels with elastic lamina and breast ducts (Lee et al, 1986). In this study the term vascular invasion implies invasion of both lymphatic and blood vessels.

The use of various immunohistochemical markers has thus been proposed to provide a more objective and reproducible means of recognizing vessel invasion than an assessment of H & E-stained slides, and to aid in differentiating blood vessels from lymphatic channels. The lectin UEA I (*Ulex europaeus* agglutinin I) and antisera to factor VIII-related antigen, type IV collagen and laminin have been employed with varying success.

In this study, 114 breast tumours were stained with antiserum to factor VIII-related antigen and with UEA I lectin to determine whether immunohistochemical staining is advantageous in the detection of vascular invasion by neoplastic cells. Laminin and type IV collagen are constituents of basement membranes (BMs) and envelop breast ducts and lobules as well as vascular channels (Gusterson et al, 1982; Chapter IV.8). They cannot, therefore, specifically identify vascular invasion as was suggested by Bettelheim et al (1984b). Furthermore, there are conflicting opinions regarding the localization of laminin and type IV collagen around lymphatic capillaries. Barsky and coworkers (1983), in a light microscopic study of various tissues rich in lymphatic and/or blood vessels, reported linear immunoreactivity with antisera to both laminin and type IV collagen around blood vessels, but lymphatic capillaries and artefactual

tissue spaces were uniformly negative for these BM components. Autio-Harmainen et al (1988) and Listrom and Fenoglio-Preiser (1988) contradicted these findings and illustrated laminin and type IV collagen surrounding both lymphatic and blood capillaries. Thus BM antisera do not always distinguish between the two types of vessels. The elaboration of BM material, containing laminin and type IV collagen, by invasive breast carcinomas and other tumours (Gusterson et al, 1982; Charpin et al, 1986b; Havenith et al, 1989) further restricts the use of these substances in delineating vascular channels.

Several other antibodies that are reportedly specific for endothelial cells are available and include PAL-E and BAW-200 (Lee et al, 1986). Monoclonal antibodies to ABH blood group isoantigens decorate blood vessel and lymphatic endothelium in addition to erythrocytes and breast epithelium (Lee et al, 1986; Little et al, 1986).

IV.7.b.   FACTOR VIII

Factor VIII (anti-haemophilic factor) is a plasma macromolecular protein complex involved in the intrinsic pathway of coagulation. One component, the precipitating antigen or factor VIII-related antigen, is synthesized by vascular endothelial cells and has also been demonstrated in platelets and megakaryocytes (Bloom, 1979). Several workers proposed factor VIII-related antigen may be a specific marker for vascular endothelial cells, and hence be an adjunct to identifying vascular invasion by tumour cells (Mukai et al, 1980; Sehested & Hou-Jensen, 1981). While some authors found

factor VIII-related antigen to be a reliable marker of blood vessel endothelium (Mukai et al, 1980; Bettelheim et al, 1984b; Capo et al, 1985) and failed to detect staining of neoplastic or normal lymphatics, others reported the antigen to be present occasionally in lymphatic endothelium, particularly if enzyme pretreatment was utilized (Sehested & Hou-Jensen, 1981; Lee et al, 1986). Guarda et al (1982) concluded that the presence of factor VIII-related antigen could not be used to distinguish between cells of blood vessel and lymphatic derivation, as staining for this antigen was also observed in the cytoplasm of endothelial cells lining lymphatic channels, and in the neoplastic cells in lymphangiomas.

Bettelheim et al (1984b) examined a series of breast carcinomas with immunocytochemical stains to determine whether the detection of vascular invasion would be increased. They concluded that antibodies to factor VIII-related antigen differentiated small blood vessels (factor VIII-positive) from lymphatic channels (factor VIII-negative), and that antibodies to type IV collagen were of value in delineating the BM of both blood and lymphatic channels to confirm the diagnosis of embolic tumour; but the immunocytochemical approach did not increase the detection of vascular invasion.

Differences in antisera and in the immunohistochemical techniques employed could account for the reported variable distribution of factor VIII-related antigen. Furthermore, several recent publications indicate that proteolytic digestion enhances the demonstration of factor VIII-related antigenic sites previously masked by formalin fixation (Sehested & Hou-Jensen, 1981; Miettinen, 1983; Lee et al, 1986). The fact that at least some authors have identified factor VIII staining in lymphatic

endothelium must question the specificity of this antiserum for blood vessels.

Lymphatic and blood vessel emboli were analysed separately in some of the studies in which the prognostic value of vascular invasion was assessed, whereas in others the evaluation of vascular invasion encompassed both types of vascular channels (Chapter II.d). As both appear to be equally poor prognostic parameters, it is probably not relevant to attempt to separate lymphatic from blood vessel invasion in tissue sections. Distinction of lymphatics from blood vessels is thus only really of value to distinguish malignant blood vessel neoplasms from neoplasms of lymphatic origin.

A search was made for vascular invasion in H & E-stained paraffin sections of 114 breast tumours with generous stromal edges, as would be performed during routine histopathological examination. Only tumour deposits identified within spaces lined by a definite endothelial layer were considered to be vascular emboli. These were compared with serial sections stained with antiserum to factor VIII-related antigen using an ABC technique (Chapter III.2; Appendix I). Incubation with primary polyclonal antiserum (rabbit anti-human factor VIII-related antigen; Dakopatts, Copenhagen, Denmark) at a dilution of 1:6000 overnight at 4°C followed digestion with protease type XXIV (Cat. No. P-8038; Sigma Chemical Company, St Louis, Missouri, USA) at 0.5 mg/ml for six minutes at 37°C. Blood vessels in sections of lymph nodes functioned as positive controls, and easily recognizable vessels in each of the test slides served as internal controls.

Factor VIII staining, in the form of a dense brown granular precipitate, was localized

in the cytoplasm of endothelial cells of blood vessels and capillaries (Figure IV.7.1).
Many more vessels were identified with the aid of factor VIII staining than were seen
with conventional H & E stains. Most of the extra vessels were small calibre
capillaries, particularly prominent immediately external to the BM of both benign
and malignant breast ducts. In addition, many small irregular vessels without
muscular walls, and likely to represent lymphatic channels, stained positively with the
factor VIII antiserum. In general, capillary endothelial cells showed the strongest
immunoreactivity and as the vessel calibre increased the positivity weakened, as also
observed by Mukai et al (1980). In a number of sections, medium-sized to large
blood vessels with definite muscular walls failed to show any factor VIII positivity.
Repeat staining with anti-factor VIII at a dilution of 1:2000 failed to alter these
findings and merely increased the degree of background immunoreactivity. In
addition, cytoplasmic factor VIII positivity was observed in occasional inflammatory
cells, particularly plasma cells, and in two cases there was focal positivity of
neoplastic cells (Figure IV.7.2).

Clumps of malignant cells within spaces lined by factor VIII-positive endothelial cells
were apparent in the stroma surrounding 12 (11.3 per cent) of 106 tumours. In the
remainder of the tumours there was no evidence of vascular invasion. In two of the
12 cases vascular invasion was identified as a result of increased sampling. The
vessels involved were not present on the original H & E sections and thus these two
cases had been assessed as negative for vascular invasion. In eight cases
immunoperoxidase staining with the anti-factor VIII antiserum confirmed vascular
invasion diagnosed on H & E-stained sections. In two of eight doubtful cases tumour
deposits were confirmed to be present in endothelial-lined structures, while in the
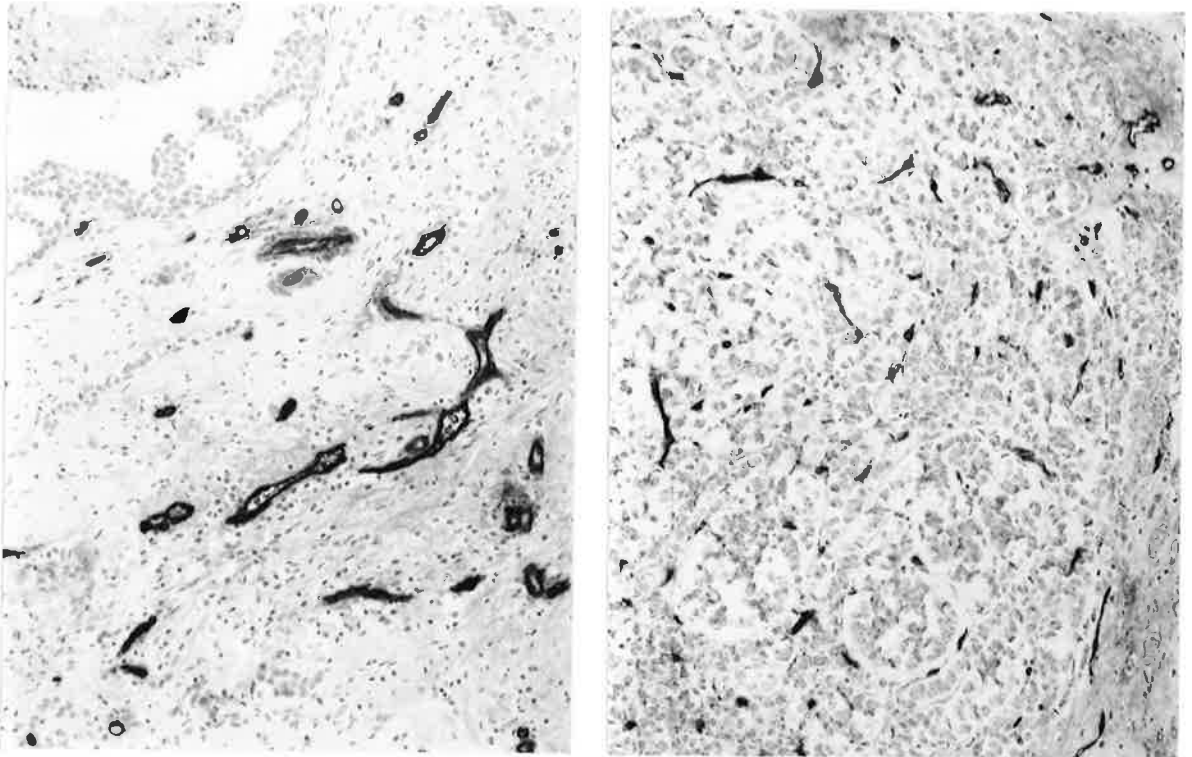
a.   b.

Figure IV.7.1. (a) Factor VIII staining highlights vascular channels in this well-differentiated infiltrating ductal carcinoma. (b) Small, irregular capillaries are readily identified amongst the sheets of neoplastic cells in a moderately-differentiated ductal carcinoma (factor VIII stain, haematoxylin counterstain).
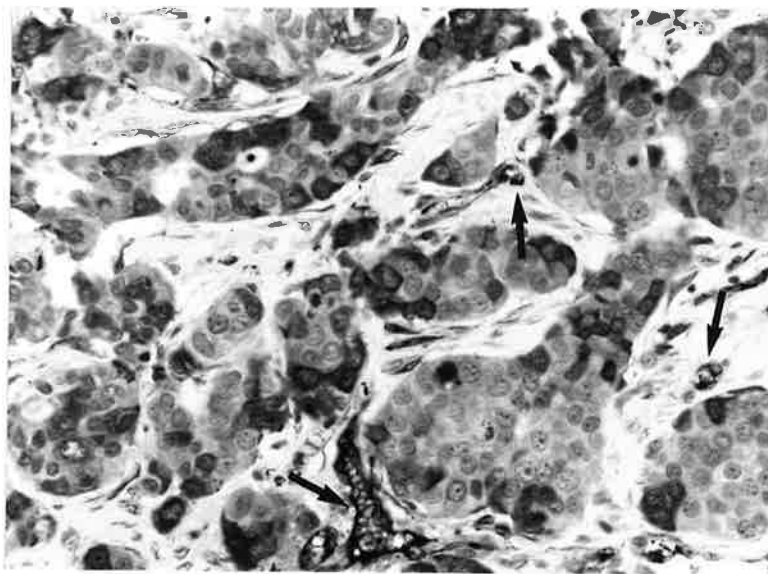

Figure IV.7.2. Scattered neoplastic cells show cytoplasmic staining with antiserum to factor VIII-related antigen in this moderately-differentiated ductal carcinoma. Note the staining of endothelial cells lining small vascular channels (arrows).

other six cases the tumour deposits were shown to be "pseudoemboli" present in artefactual stromal spaces "lined" by fibroblast nuclei mimicking endothelium. Background staining was too marked to allow accurate assessment in eight of the 114 cases.

These results confirm that the anti-factor VIII antiserum localizes factor VIII-related antigen in endothelial cells of all vascular channels, in accordance with previous studies (Bloom, 1979; Sehested & Hou-Jensen, 1981), although with marked variability of staining intensity between cases and between vessels of differing calibre. Immunostaining was of value in confirming the conventional H & E diagnosis of vascular invasion, and in excluding vascular invasion in doubtful cases. There was no increase in the incidence of vascular metastases detected over an initial examination of H & E-stained sections. Both lymphatic and blood vessels appeared to stain equally with this antiserum.

IV.7.c. *ULEX EUROPAEUS* AGGLUTININ I (UEA I)

A recently introduced endothelial cell marker is *Ulex europaeus* agglutinin I (UEA I), a lectin obtained from *Ulex europaeus* seeds (Holthofer et al, 1982). UEA I recognizes the H antigen in erythrocytes of individuals with blood group O, in addition to specifically binding α-L-fucose residues of glycoproteins along cell membranes in normal and neoplastic tissues, irrespective of the blood type of the tissue donor.

UEA I has been proclaimed a specific and more sensitive marker than factor VIII-related antigen for endothelial cells in normal tissues and various vascular tumours (Holthofer et al, 1982; Miettinen et al, 1983b; Ordonez & Batsakis, 1984; Little et al, 1986). A number of reports have described the use of UEA I in the investigation of vascular lesions and in the assessment of vascular invasion in malignant tumours. The techniques employed included application of fluorochrome-labelled (Holthofer et al, 1982; Miettinen et al, 1983b) or peroxidase-labelled (Walker, 1985) UEA I following protease digestion, and serial incubation with purified lectin, antiserum to the lectin, secondary antibody and PAP complex (Ordonez & Batsakis, 1984). Walker (1985) claimed staining with UEA I increased the number of cases identified with vascular invasion in a series of 50 breast carcinomas. In addition, she noted that UEA I failed to stain lymphatic endothelium and suggested that this difference in reactivity may be of value in distinguishing between lymphatic and blood vessels. Others (Ordonez & Batsakis, 1984; Little et al, 1986) disputed this finding and demonstrated lectin staining of endothelial cells lining both types of vascular channels.

It is noteworthy that isoantigens identical to A, B and H antigens present in erythrocytes have been detected in certain normal epithelia and some carcinomas (Ordonez & Batsakis, 1984; Little et al, 1986), posing a potential limitation of the use of UEA I in delineating vascular invasion.

For each of the 114 breast neoplasms for which sections were stained with antiserum to factor VIII-related antigen, a contiguous section was stained with UEA I using a modified immunoperoxidase technique. Slides were first treated with trypsin type III

at one mg/ml (Cat. No. T-8253; Sigma Chemical Company) and calcium chloride dihydrate at one mg/ml (pH 7.0) for 15 minutes at 37°C. Endogenous peroxidase was blocked and biotin-labelled UEA I (Cat. No. B-1065; Vector Laboratories, Burlingame, California, USA) applied at a dilution of 1:25 (80 µg/ml) at 4°C for 18 hours, followed by incubation with ABC reagent for 60 minutes at room temperature, and development with DAB (Appendix I). This technique obviates the need for applying a primary antiserum to the lectin in a three-stage immunoperoxidase method such as that used by Ordonez and Batsakis (1984). UEA I reactivity was then compared with the H & E-stained and the anti-factor VIII-immunostained sections.

The results of UEA I immunostaining were similar to those observed with the factor VIII antiserum and were in accordance with the work of others (Mukai et al, 1980; Walker, 1984). There was diffuse, granular cytoplasmic staining of all vascular endothelial cells, frequently with membrane accentuation. Many more vessels were apparent than with H & E stains alone (Figure IV.7.3). There was variation in staining intensity both between specimens and within the one section, small vascular channels generally staining much more intensely than larger arteries and veins.

Vascular invasion was confirmed in 10 cases, refuted in six, and in two cases increased sampling identified foci of vascular invasion not present on the original diagnostic sections. In nine cases considerable background staining, most probably a consequence of a large population of carbohydrate-rich cells and secretions, prohibited reliable assessment of UEA I staining. In 79 (75 per cent) of 105 tumours there was extensive cytoplasmic UEA I positivity of neoplastic cells (Figures IV.7.4
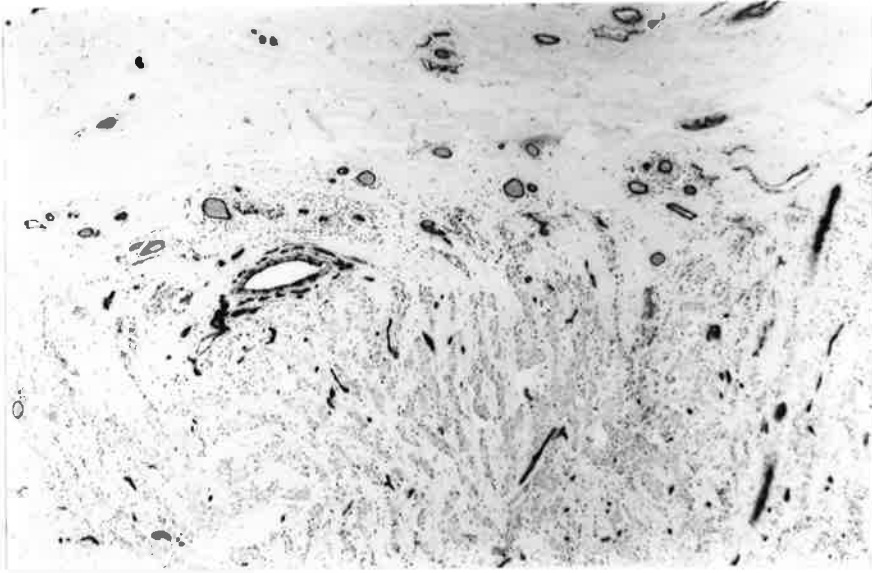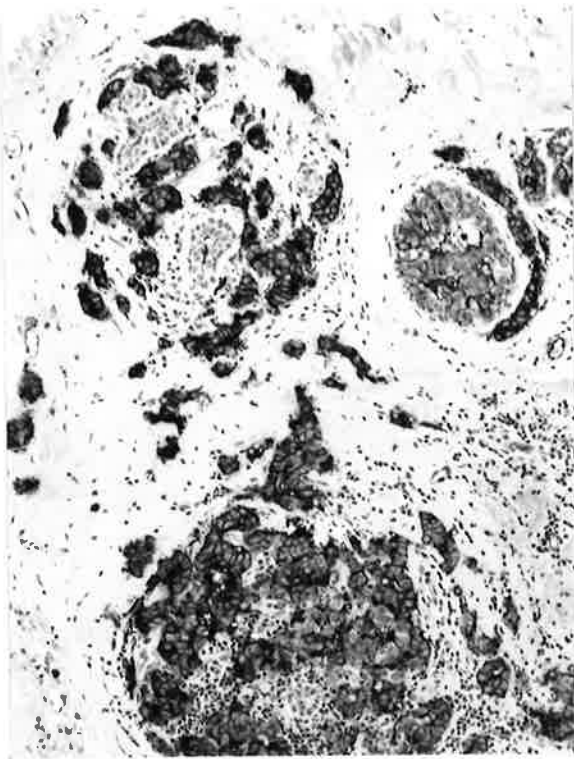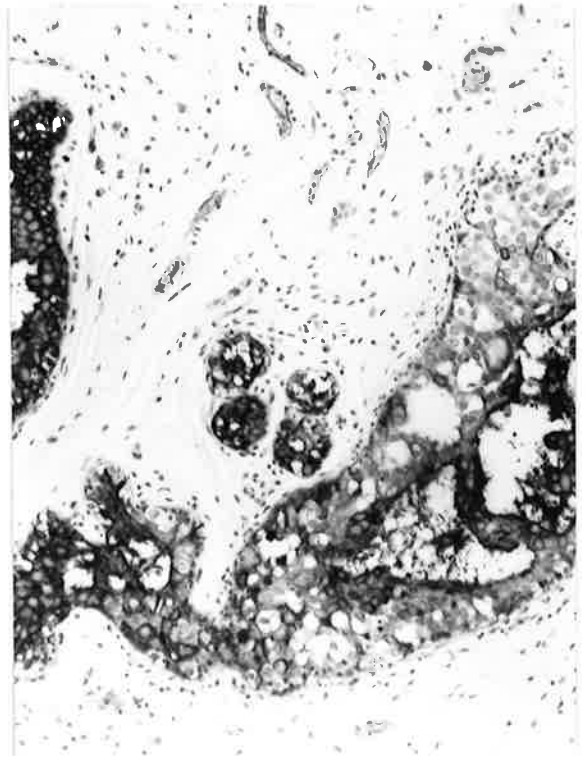
Figure IV.7.3. Staining with UEA I highlights the many small vascular channels amongst the invasive carcinoma and in the peritumoral parenchyma (haematoxylin counterstain).

a.   b.

Figure IV.7.4. (a & b) Two moderately-differentiated invasive ductal carcinomas showing diffuse cytoplasmic UEA I staining of neoplastic cells. Note the marked membrane accentuation (haematoxylin counterstain).

& IV.7.5). Intraductal carcinoma, when present in the section, was consistently UEA I-positive if the adjacent infiltrating tumour stained with the lectin. Four cases demonstrated lectin positivity of intraductal carcinoma while the infiltrating tumour was UEA I-negative. In 49 cases, benign breast epithelium was included on the section and in each example showed diffuse UEA I reactivity, regardless of whether or not the tumour cells were UEA I-positive. Lectin positivity was also observed in the epithelial cells of three fibroadenomas and four cases of fibrocystic change. Apocrine epithelium was uniformly negative. These findings suggest that UEA I reactivity may be lost as breast epithelium acquires malignant properties or undergoes metaplastic change.

In addition, myoepithelial cells surrounding both benign ducts and ducts expanded by tumour cells were frequently observed to be UEA I-positive (Figure IV.7.6). The pattern of immunoreactivity was variable, staining only some areas and occurring in both UEA I-positive and UEA I-negative tumours. As expected, inflammatory cells stained positively in some cases.

Thus, UEA I delineated vascular channels as effectively as anti-factor VIII antiserum and facilitated the positive identification of tumour emboli in several doubtful situations. UEA I staining did not, however, increase the yield of cases exhibiting vascular invasion over an assessment of conventional H & E stains. Furthermore, in a large number of cases the interpretation of vascular invasion was hindered by tumour expression of UEA I, and by UEA I positivity of myoepithelial cells (Figures IV.7.5 & IV 7.6).
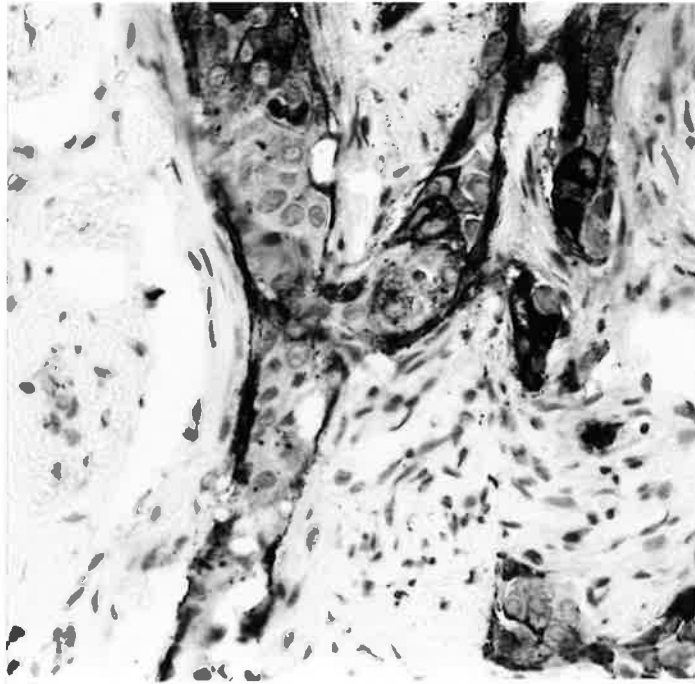
Figure IV.7.5. A moderately-differentiated ductal carcinoma stained with UEA I. Cytoplasmic staining of neoplastic cells prohibits recognition of a definite endothelial lining around this suspected intravascular deposit.
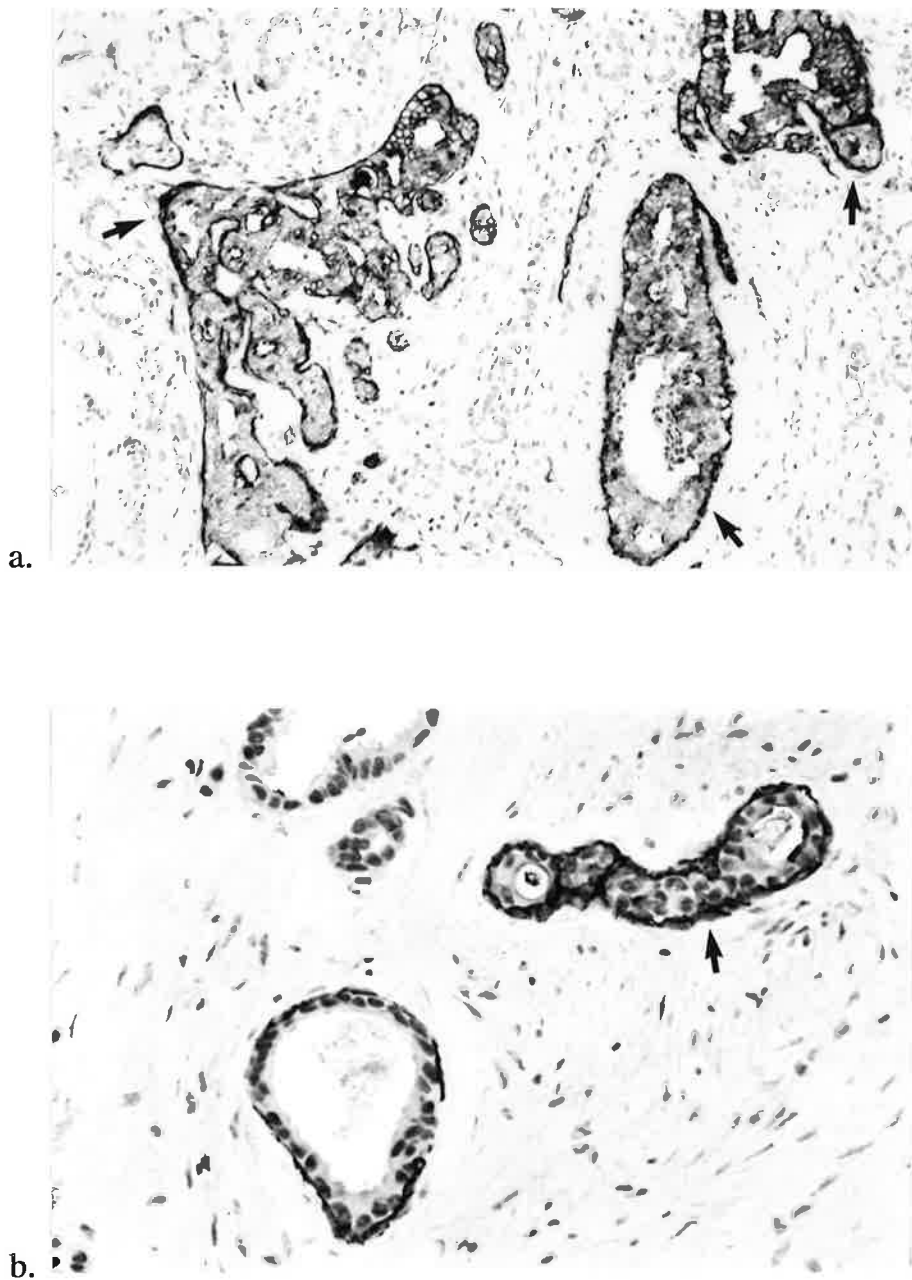
Figure IV.7.6. (a) A well-differentiated ductal carcinoma illustrating cytoplasmic UEA I staining of myoepithelial cells in a continuous rim around intraductal tumour (arrows). Note individual UEA I-positive neoplastic cells and staining of small vessels. (b) At higher magnification, UEA I positivity of myoepithelial cells is clearly identified in this tubular carcinoma (UEA I stain, haematoxylin counterstain).

IV.7.d.   DISCUSSION

Biotinylated UEA I and antiserum to factor VIII-related antigen clearly delineated both lymphatic and blood vessels, and facilitated positive identification of tumour emboli.   These immunocytochemical stains did not, however, identify vascular invasion in any examples classified as negative for invasion on conventional histological examination. This is most probably because the increased numbers of vessels identified with immunoperoxidase methods were generally of small diameter and therefore less likely to contain tumour emboli.

Immunoreactivity for factor VIII-related antigen was generally weaker than the corresponding UEA I stain, particularly in small capillaries. Miettinen et al (1983) and Ordonez and Batsakis (1984) reported similar findings in glomerular endothelium and liver sinusoidal lining cells, and suggested this probably relates to the smaller amount of antigen synthesized. Even so, anti-factor VIII antiserum appears to be more discriminatory than UEA I. In this study, UEA I occasionally decorated myoepithelial cells, rendering the stain of little value in discriminating vascular emboli from *in situ* or intraductal tumour. Furthermore, staining of carcinoma cells with UEA I, in approximately 75 per cent of cases, frequently prohibited recognition of a distinct endothelial lining around a deposit of lectin-positive cells (Figure IV.7.5).

There is considerable variability in the patterns of staining reported for both factor VIII-related antigen and UEA I. This may, at least in part, reflect the variation in

staining techniques utilized, but highlights the need for caution when interpreting staining with these markers.

In conclusion, H & E-stained sections of breast carcinomas with surrounding mammary parenchyma are adequate for the histological diagnosis of vascular invasion, and there is no place for routine staining with anti-factor VIII antiserum or UEA I. In cases deemed equivocal or inconclusive, such as when tissue shrinkage artefact hinders recognition of true vascular spaces, selected immunostaining of a serial section with anti-factor VIII may be helpful.

IV.8.   ASSESSMENT OF TUMOUR INVASION USING ANTIBODIES TO BASEMENT MEMBRANE ANTIGENS AND MYOEPITHELIAL CELL ANTIGENS

a.   **INTRODUCTION**

b.   **TYPE IV COLLAGEN AND LAMININ**

c.   **ACTIN AND MUSCLE SPECIFIC ACTIN**

d.   **DISCUSSION**

IV.8. ASSESSMENT OF TUMOUR INVASION USING ANTIBODIES TO BASEMENT MEMBRANE ANTIGENS AND MYOEPITHELIAL CELL ANTIGENS

IV.8.a. INTRODUCTION

Tumour progression is reliant upon a complex multistep process of local invasion followed by uncontrolled local growth and metastatic spread. Although the exact nature of the biochemical changes responsible for the initiation of invasion are unclear, certain histological features, including the presence of an intact basement membrane (BM) separating the epithelium from the connective tissue interstitium, have been used to recognize *in situ* carcinoma prior to the development of microinvasion.

The normal human mammary gland is composed of two layers of phenotypically distinct epithelial cells arranged around a lumen, the whole structure being encompassed by a continuous extracellular BM. The inner lining cells have a secretory function and the outside layer is formed by elongated, contractile, myoepithelial cells.

Extracellular BMs are integral structures in the organization and orientation of cells within tissues and organs. Ultrastructurally, the BM is composed of a basal lamina lucida of low electron density, adjacent to the parenchymal cells, and a basal lamina

densa of high electron density, adjacent to the connective tissue matrix. The BM forms a continuous lining along the basal aspect of all epithelial surfaces, and surrounds glandular, ductal and vascular components of tissues to separate epithelial and endothelial cells from the stroma (Christensen et al, 1989). The BM is generally extremely stable but may, in certain pathological states, undergo local dissolution. This process is likely to play a crucial role in the progression of invasive tumours (Siegal et al, 1981; Barsky et al, 1983b; Liotta, 1984). Metastasis requires a further transgression through the BM surrounding the vascular compartment.

Two major constituents of all BMs are type IV collagen and laminin. Both molecules are found exclusively in BMs and are apparently synthesized by a number of cell types including epithelial, myoepithelial and endothelial cells (Siegal et al, 1981; Barsky et al, 1983b; Liotta, 1984). Other minor components of the BM meshwork include entactin, proteoglycans and type V collagen. Type IV collagen is the structural backbone of the BM and has been localized by immunoelectron microscopy to the basal lamina densa. Laminin is a glycoprotein which is present in the lamina lucida, and is considered an important mediator for the attachment of cells to the BM.

The recent availability of antisera to BM components has stimulated increased interest in the study of the organisation of BMs associated with various benign and malignant tumours (Barsky et al, 1983b; Carter et al, 1985; Willebrand et al, 1986; Sakr et al, 1987; Autio-Harmainen et al, 1988; Havenith et al, 1989). The majority of invasive carcinomas are recognized to synthesize varying amounts of BM material, but BMs surrounding tumour nests are generally fragmented, and in many cases are

completely absent (Barsky et al, 1983b). Benign and *in situ* lesions appear to be circumscribed by intact BM.

BMs have been extensively studied in breast lesions, both immunohistochemically using antisera to type IV collagen and laminin, and ultrastructurally. Normal breast ducts and lobules, benign epithelial proliferations and *in situ* carcinomas, of ductal and lobular type, are thought to be surrounded by an intact BM. However, focal defects in continuity of the BM surrounding *in situ* carcinomas have been revealed by electron microscopy, and in zones of microinvasion the BM appears irregular, fragmented or absent in immunohistochemical studies (Siegal et al, 1981; Liotta, 1984; Charpin et al, 1986b; Sakr et al, 1988; Christensen et al, 1989). Infiltrating carcinoma and metastatic breast carcinoma cells are generally devoid of extracellular BM containing laminin and type IV collagen, but focal deposits of BM material can sometimes be identified, especially around neoplastic cells forming ducts or glandular structures (Siegal et al, 1981; Charpin et al, 1986b; Sakr et al, 1988; Christensen et al, 1989).

The loss or defective organization of the BM matrix in malignant lesions may be a result of increased breakdown by tumour-derived degradative enzymes, decreased synthesis, or decreased or abnormal assembly of the secreted BM components.

Several authors, using immunocytochemical stains and electron microscopy, have confirmed a single layer of myoepithelial cells to be located surrounding the epithelial cells of benign breast acini and ducts, and circumscribing ducts involved with *in situ* carcinoma (Bussolati et al, 1984; Tsukada et al, 1987; Eusebi et al,

1989). This layer also becomes disarrayed during invasion, and the identification of myoepithelial cells may thus also prove of value in detecting foci of microinvasion, and in distinguishing between invasive carcinoma and benign proliferative lesions, the latter showing preservation of myoepithelial differentiation.

In this study enzymatic digestion methods were developed to optimize the staining of BM with antisera to laminin and type IV collagen. In addition, antibodies to muscle actins were employed to identify myoepithelial cells, and the relative merits of the two methods of detecting early tumour invasion were compared.

IV.8.b.   TYPE IV COLLAGEN AND LAMININ

Periodic acid-Schiff (PAS) staining and silver impregnation techniques are the classical methods used to outline BMs; however, these are not specific for BM components and may stain proteins associated with or adjacent to the BM. In addition, PAS-positive mucin or glycogen may be secreted by the tumour cells (Siegal et al, 1981; Eusebi et al, 1989).

The diagnostic value of BM staining was assessed in this study using commercial antisera to type IV collagen and laminin, many previous publications having employed antisera from non-commercial sources (Siegal et al, 1981; Barsky et al, 1983b & 1984; Ekblom et al, 1984; Sakr et al, 1988). Immunohistochemical localization of these two proteins is impaired by formalin fixation and paraffin embedding, and thus the use of either frozen tissue or proteolytic enzyme

pretreatment is required (Siegal et al, 1981; Barsky et al, 1984; Finkel & Pastore, 1987; Christensen et al, 1989). When assessing features such as microinvasion, by mapping focal dissolution of the BM, paraffin sections are clearly of superior quality when compared with frozen sections. Several different digestion methods were examined in order to obtain optimal clarity of staining with each antiserum. These were digestion with pepsin (Sigma Chemical Company) at one mg/ml in 0.5M acetic acid (as recommended by Barsky et al, 1984, for formalin-fixed tissues), and with protease type XXIV at varying concentrations and for varying durations. In addition, formalin-fixed sections were compared with microwave-fixed tissue.

The presence and distribution of BM was studied in 36 sections from various breast tissues. These comprised examples of fibrocystic change (15), intraductal carcinomas (4) and invasive carcinomas (17), the latter including four tubular carcinomas, one papillary carcinoma, two lobular carcinomas and ten IDCs. In all cases, tissue blocks had been fixed by microwave irradiation (Chapter III.2), and in two examples formalin-fixed tissues were also assessed. Serial sections were stained with polyclonal rabbit antisera to human laminin and type IV collagen (Table III.2), and with H & E.

The optimal immunostaining procedures are described. Digestion with protease type XXIV (Cat. No. P-8038; Sigma Chemical Company, St Louis, Missouri, USA), at 0.5 mg/ml for 10 minutes at 37°C, was performed prior to application of anti-laminin antiserum (Cat. No. AT-2404; E-Y Laboratories, San Mateo, California, USA), at a dilution of 1:750, overnight at 4°C. Enzymic digestion with protease type XXIV (Sigma Chemical Company), at 0.5 mg/ml for one minute at 37°C, preceded

incubation with antiserum to type IV collagen (code PCO; Eurodiagnostics, Apeldoorn, The Netherlands). This was applied at a dilution of 1:800 overnight (18 hours at 4°C). A standard ABC method was employed to complete the reaction in both instances (Chapter III.2; Appendix I).

Staining of formalin-fixed tissue with type IV collagen antiserum required prolongation of the digestion time to four minutes, whereas digestion at four mg/ml for 10 minutes was necessary for optimal staining with anti-laminin antiserum. Controls, using inappropriate antisera or with omission of the primary antiserum, were uniformly negative.

The presence and extent of BM staining was assessed in each case. Similar staining patterns were observed with anti-laminin and anti-type IV collagen antisera. Staining around normal breast ducts and lobules, and encircling benign proliferations of breast epithelium (including fibroadenomatoid change, sclerosing adenosis and epitheliosis), occurred as a single, continuous, extracellular, undulating line on the basal side of the basal epithelium, corresponding to the BM region (Figures IV.8.1 & IV.8.2). The BM staining emphasized the fibrovascular core of intraductal papillomas, both staining beneath the hyperplastic epithelium and around the core vessels (Figure IV.8.1b). No BM layer lined intraductal, cytologically malignant, papillary projections or intraductal papillary carcinoma cells (Figure IV.8.3).
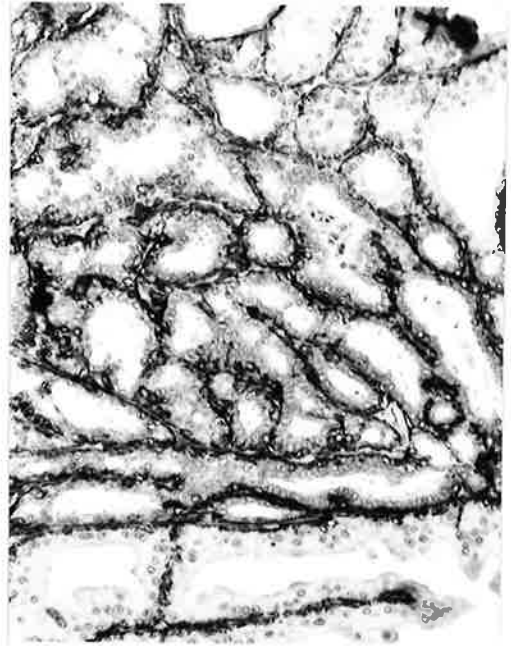
Continuous BM staining was also observed around intraductal carcinomas. Irregular thinning of the BM, with focal fragmentation and loss, was observed at sites of microinvasion (Figures IV.8.4 & IV.8.5). In two cases which had been reported as
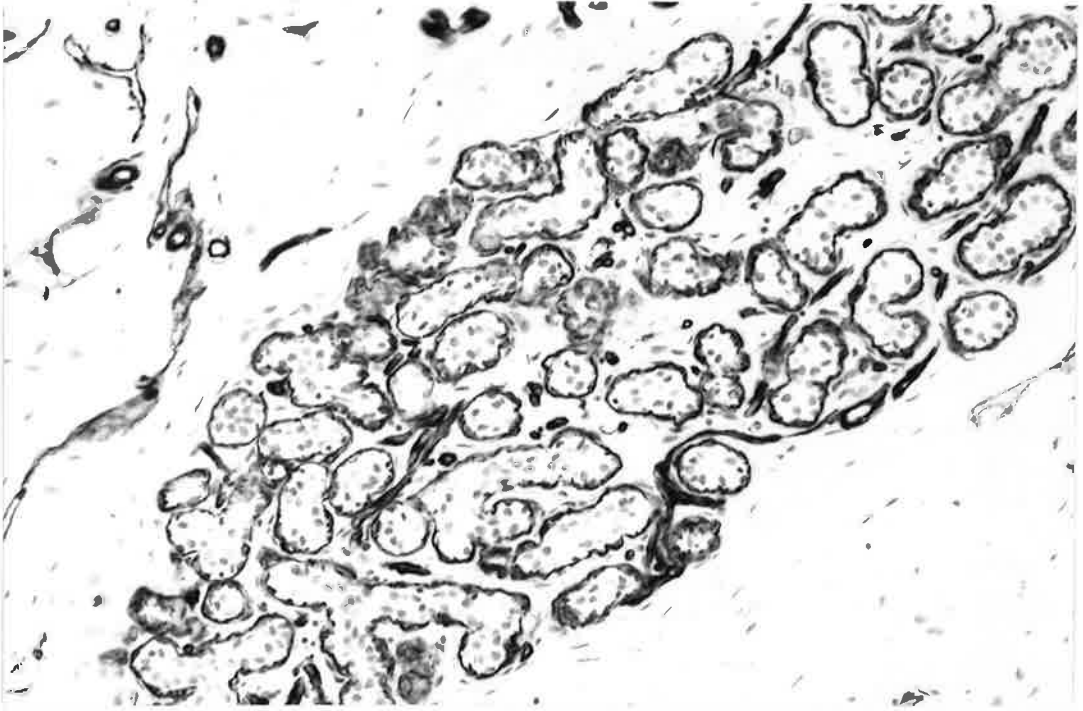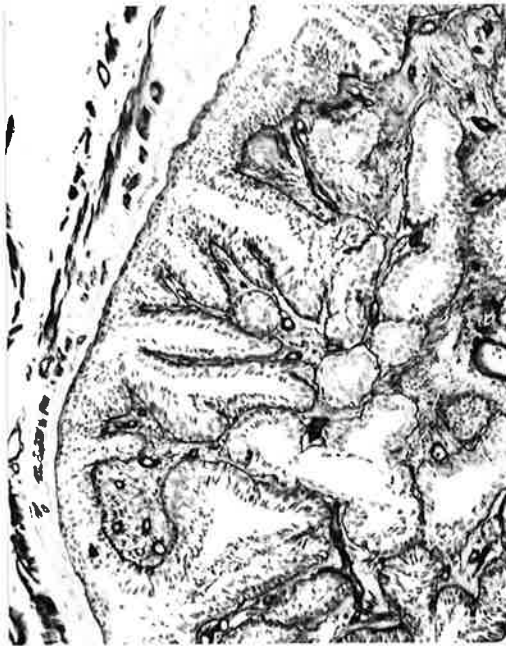
Figure IV.8.1. Staining for type IV collagen illustrates a continuous, undulating rim of BM around glands in a focus of adenosis (a), and emphasizes the fibrovascular core of an intraductal papilloma (b). Core vessels are also surrounded by intact BM (haematoxylin counterstain). (c) Staining for muscle specific actin (MSA) highlights myoepithelial cells in the intraductal papilloma (haematoxylin counterstain).
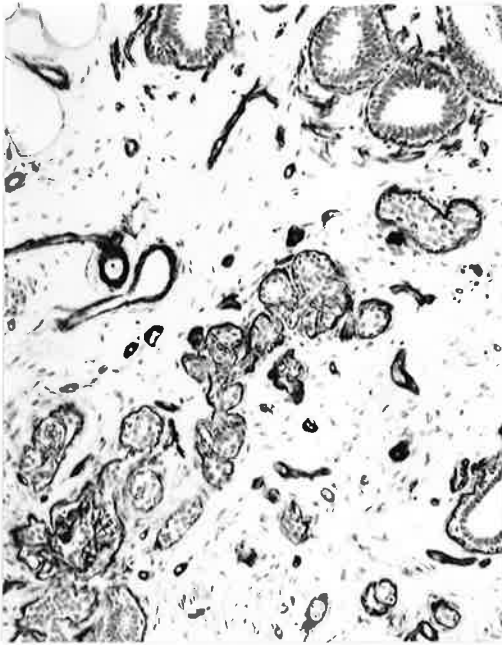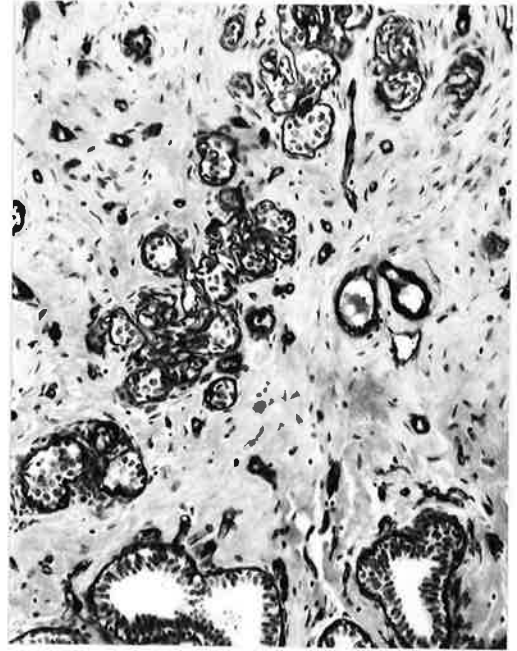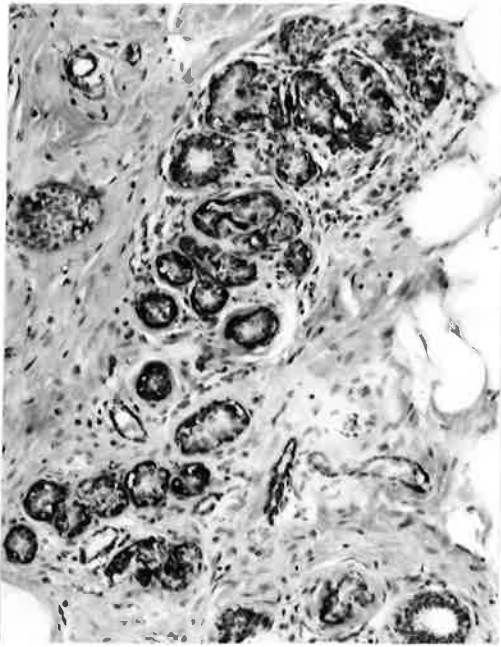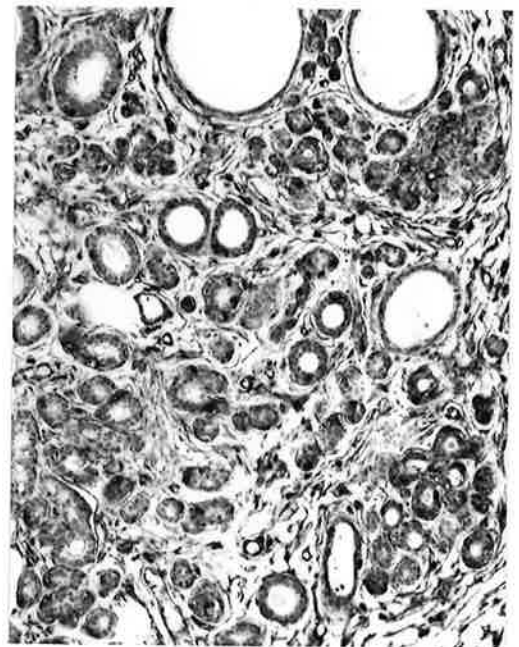
Figure IV.8.2. Sections from a case of fibrocystic change, including a focus of sclerosing adenosis, stained with anti-type IV collagen (a), anti-laminin (b), anti-MSA (c) and anti-actin (d), with haematoxylin as a counterstain. A similar pattern of continuous staining of the BM around acini, ducts and vessels is observed in (a) and (b). Cytoplasmic staining of myoepithelial cells is demonstrated in (c), but these cells are not as easily identified with the anti-actin stain (d), due to considerable background positivity.
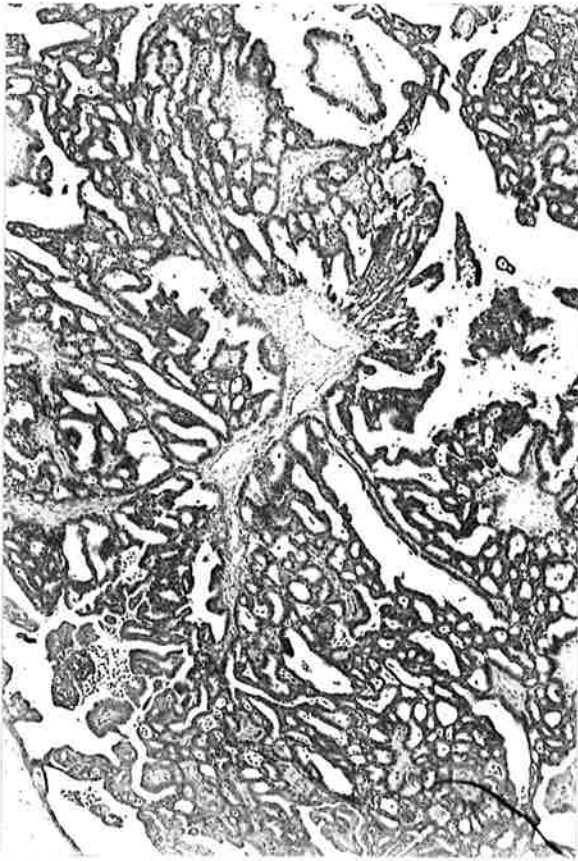
a.
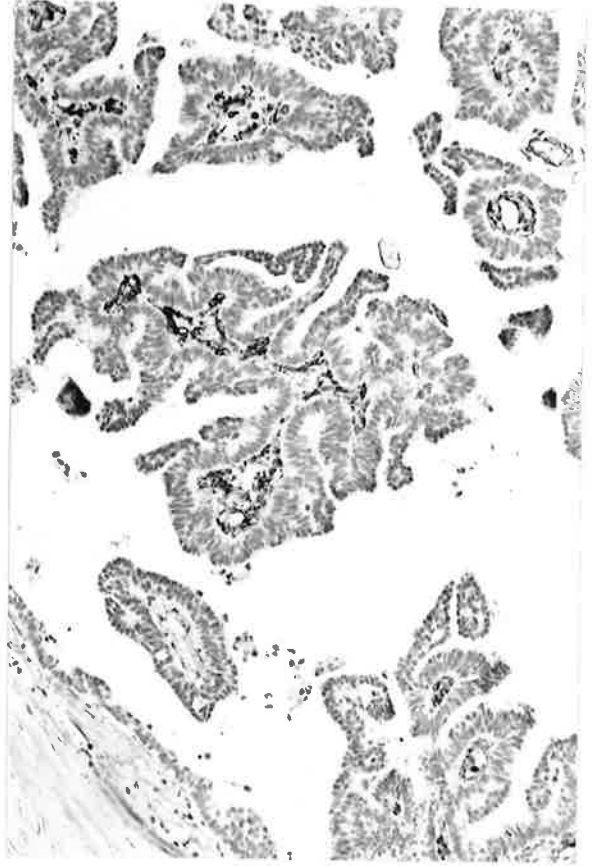
b.
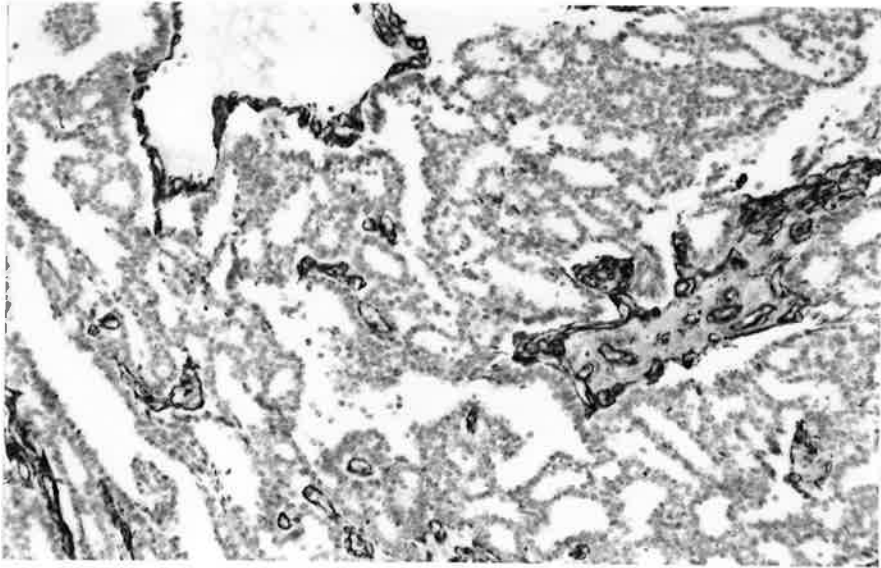
c.

**Figure IV.8.3.** Low (a) and high (b) magnification photomicrographs of a papillary carcinoma stained with anti-MSA. Myoepithelial cells are conspicuously absent, in contrast to the case of intraductal papilloma illustrated in Figure IV.8.1. Staining for type IV collagen (c) demonstrates an absence of BM material associated with the malignant cells. Note staining of pericytes in (b), and of the BM surrounding vessels in (c).
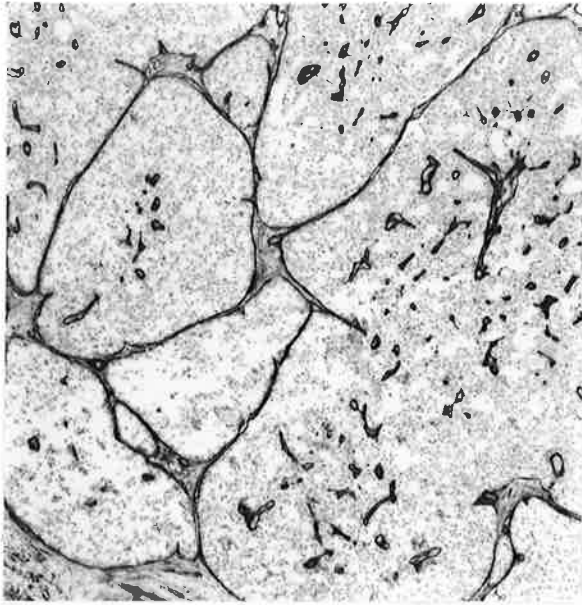
a.
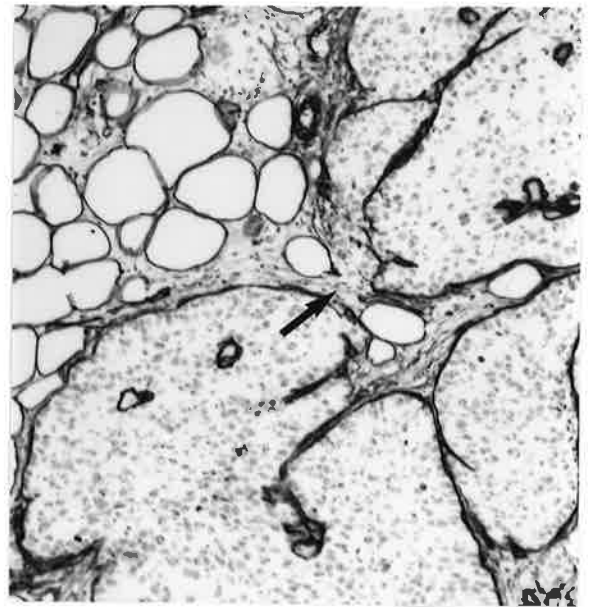
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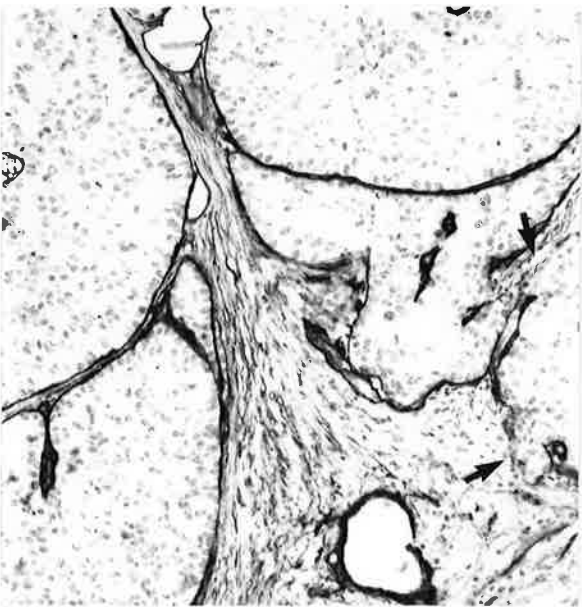
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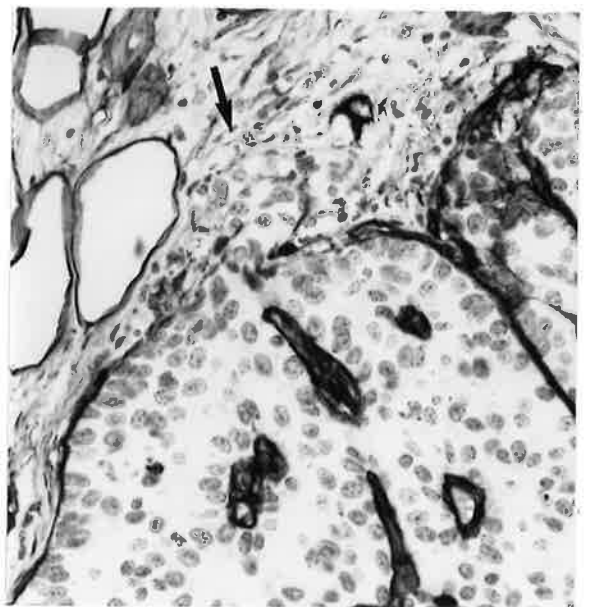
d.

Figure IV.8.4. (a) Intraductal carcinoma is clearly surrounded by continuous, linear BM in this field (type IV collagen stain, haematoxylin counterstain). However, at numerous sites (arrows) the BM is thin and fragmented (b & c), and in (d) a tongue of tumour cells can be seen breaking through the BM. This represents a focus of microinvasion which was not detected on routine H & E stains.
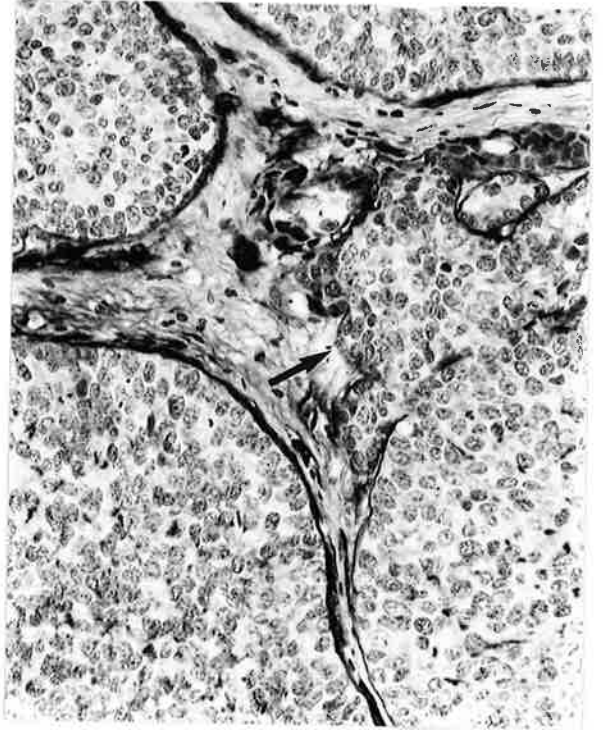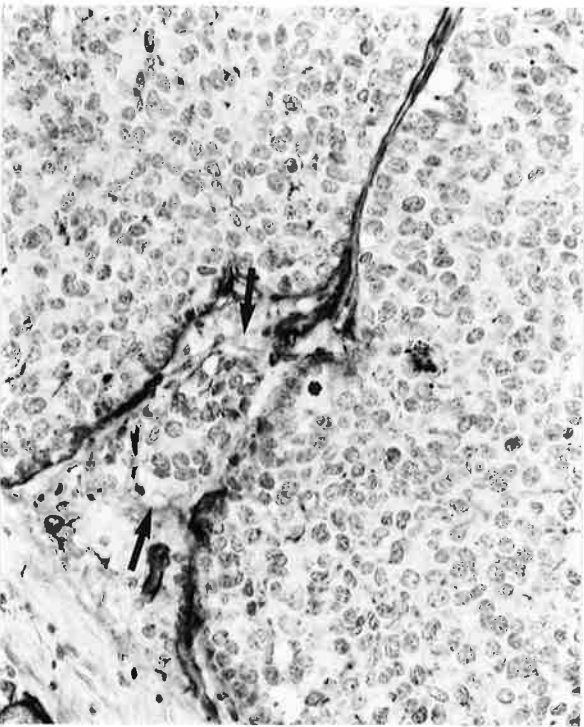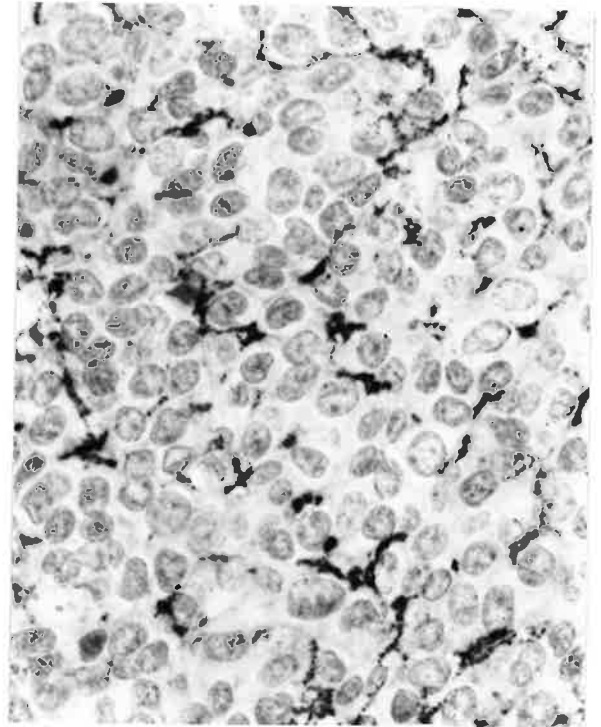
Figure IV.8.5. (a) Laminin staining of the same case of intraductal carcinoma as illustrated in Figure IV.8.4. Focal breaches of the BM at sites of microinvasion (arrows) are readily identified (b & c). In addition, deposits of BM material are intimately related to neoplastic cells (d).

pure intraductal carcinoma, invasive foci were identified by the presence of extensions of tumour through breaks in the BM (Figures IV.8.4 & IV.8.6). Invasive islands of ductal, lobular, papillary and tubular carcinomas were surrounded by variable amounts of thin BM-like structures at the tumour-stromal interface (Figures IV.8.7 - IV.8.9). The extent of BM staining varied within the same tumour and between tumours of the same grade. BM was most apparent circumscribing neoplastic cells apparently forming glandular or ductal structures, and lesser amounts were generally seen in poorly-differentiated areas. In a number of cases, linear BM material was also observed partially surrounding individual neoplastic cells within intraductal tumour deposits (Figure IV.8.5).

Blood and lymphatic vessels were surrounded by continuous BM in all tissues, and served as inbuilt positive controls. Small vessels were particularly prominent around the base of ducts, and ductal and vessel BMs often appeared to merge together. The two antisera demonstrated identical immunostaining reactions. There was minimal background staining with antiserum to type IV collagen, but a pale homogeneous background hue was observed in a number of cases stained with anti-laminin antiserum (Figures IV.8.2 & IV.8.5).

The clarity of BM staining in microwave-fixed tissues was comparable to that apparent in formalin-fixed sections, but a prolonged enzymic digestion time or a higher enzyme concentration was required for the formalin-fixed tissues.

These results support the findings of Willebrand et al (1986) and the concept that invasive carcinoma can produce a basal lamina (Gusterson et al, 1982). It has
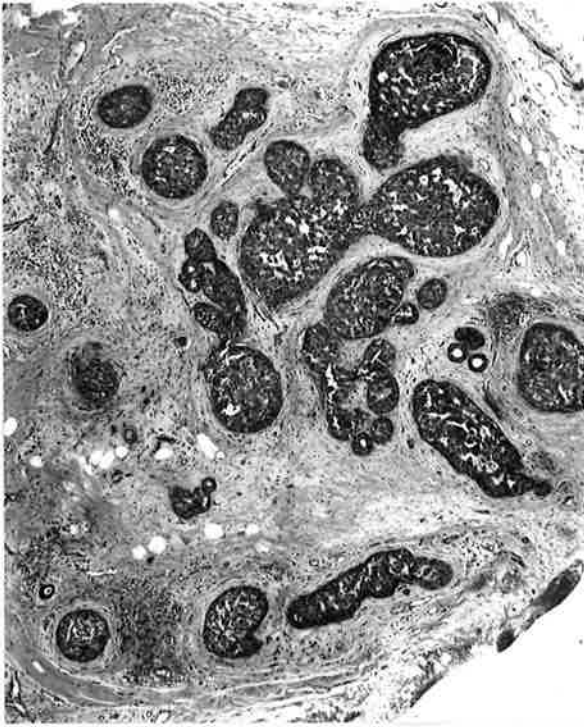
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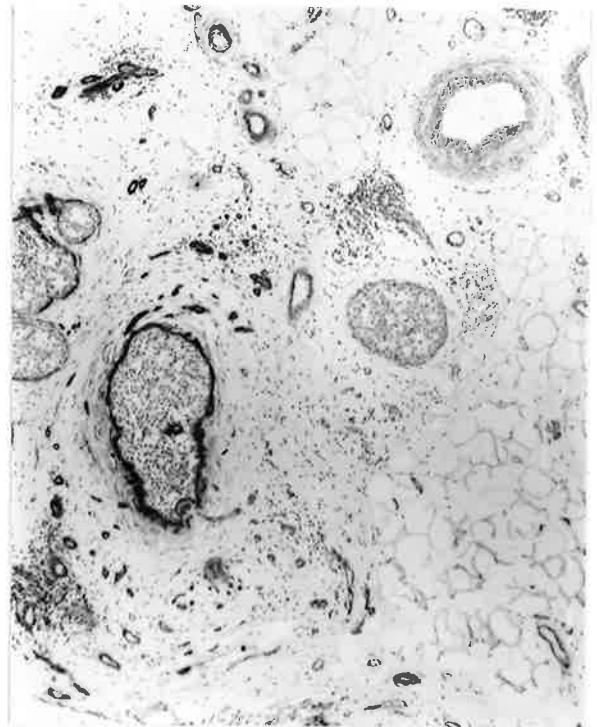
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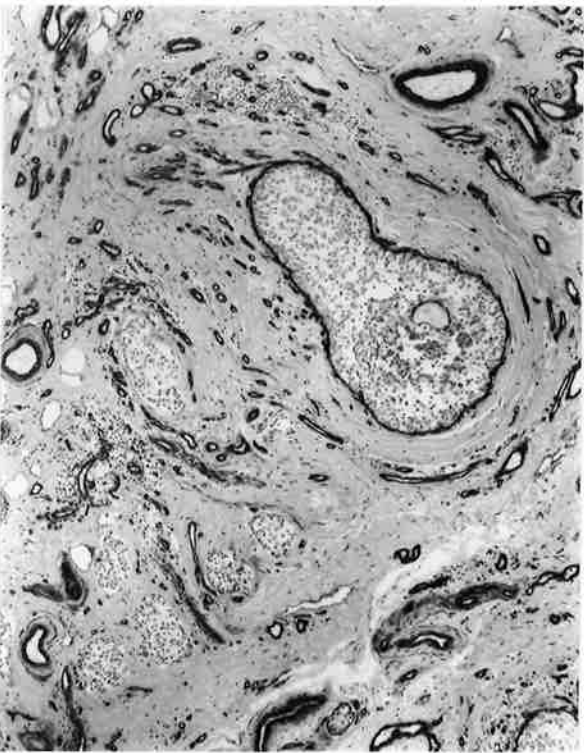
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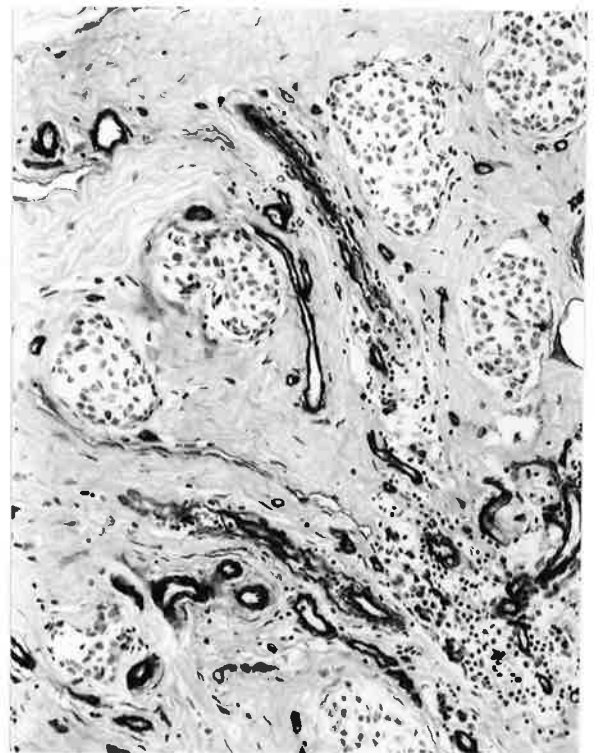
d.

Figure IV.8.6. (a) A breast carcinoma diagnosed as *in situ* ductal in type on examination of H & E-stained sections (H & E stain). Staining with anti-type IV collagen (b) and anti-laminin (c & d) reveals foci of tumour which lack a surrounding BM. These deposits are interpreted as being invasive carcinoma replicating the pattern of growth of the intraductal tumour.
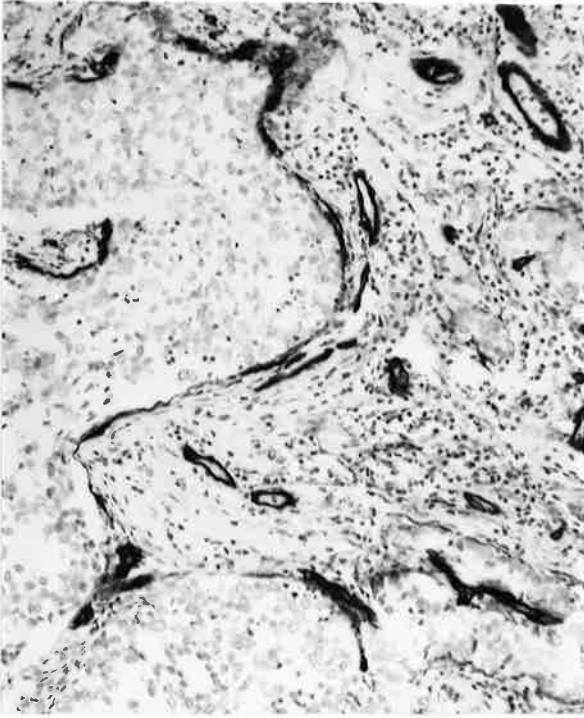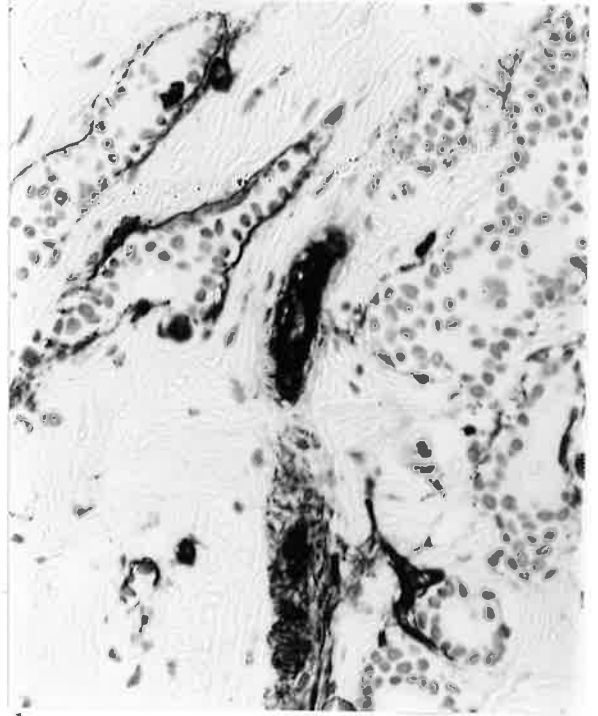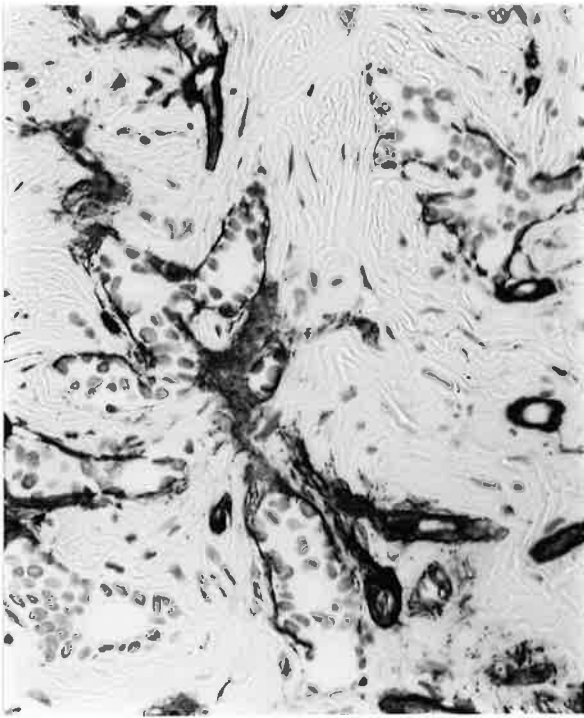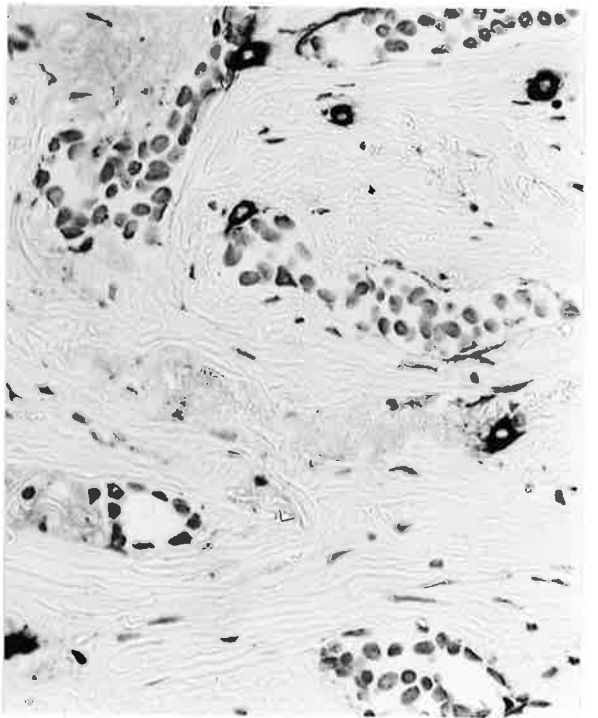
Figure IV.8.7. (a) A moderately-differentiated invasive ductal carcinoma partially circumscribed by BM. Note strong BM staining around small vessels (anti-type IV collagen stain, haematoxylin counterstain). (b,c & d) A similar pattern of BM staining is observed in this case of tubular carcinoma. BM encircles some glandular structures, while others (d) are associated with only scant or no BM material (anti-type IV collagen, haematoxylin counterstain).
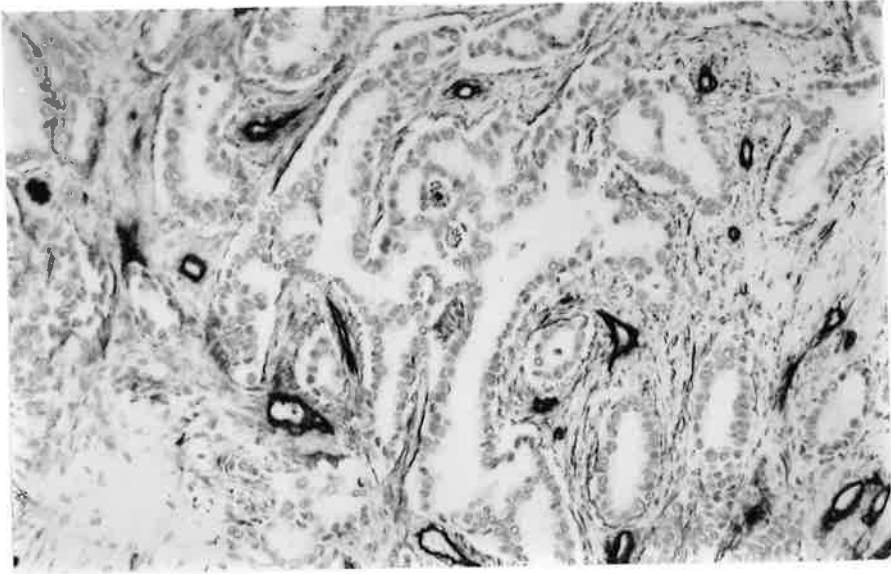
Figure IV.8.8. This tubular carcinoma is stained with anti-type IV collagen and lacks surrounding BM, in contrast to the tubular carcinoma illustrated in Figure IV.8.7 (haematoxylin counterstain). Note intense BM positivity investing small vessels.
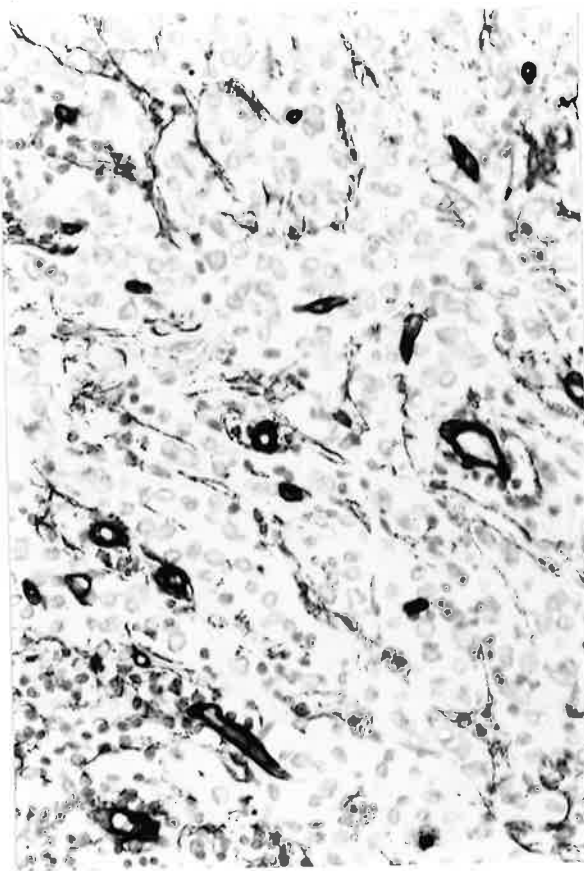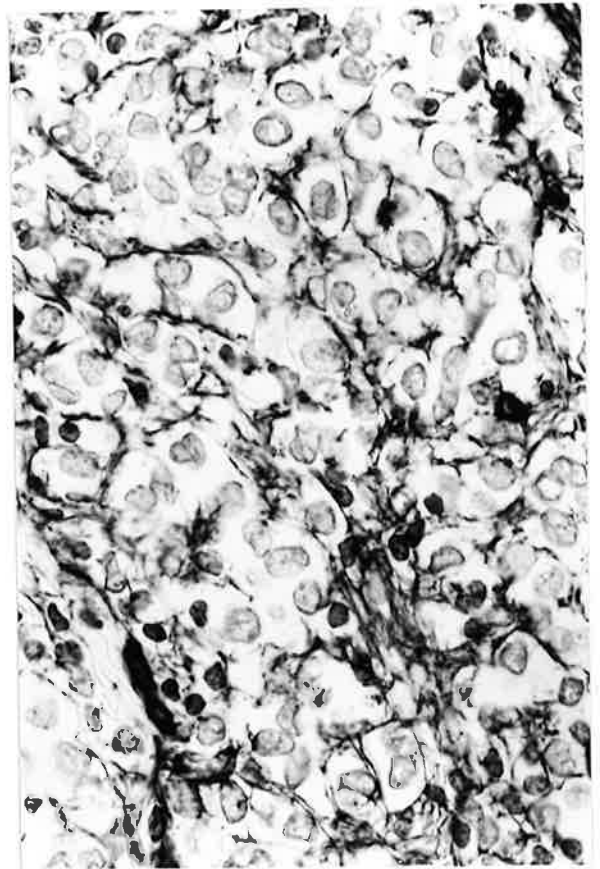
a.   b.

Figure IV.8.9. (a & b) Thin BM-like structures are intimately associated with the neoplastic cells of these two lobular carcinomas (anti-type IV collagen stain, haematoxylin counterstain).

previously been reported that intact BM is observed in areas of ductal carcinomas showing gland formation, while poorly-differentiated tumours lack an organized BM (Siegal et al, 1981; Ekblom et al, 1984; Christensen et al, 1989); however, no correlation between the extent of BM deposition and histological grade has been demonstrated (Willebrand et al, 1986; Sakr et al, 1988).

The findings in this study suggest that tubular carcinomas are similar to other well-differentiated carcinomas, as both show abnormal fragmented and focally discontinuous BM (Figure IV.8.7). This is in accordance with several other reports published since the completion of this study (Christensen et al, 1989; Charpin et al, 1989), and refutes earlier work which failed to identify BM in this rare form of breast cancer (Gould, 1975; Willebrand et al, 1986; Sakr et al, 1988; Chapter II.b). The irregular and incomplete nature of the BM surrounding glandular structures in tubular carcinomas may potentially be used as a means of distinguishing this type of carcinoma from sclerosing adenosis, in which individual tubules are invariably surrounded by continuous BM.

Intracytoplasmic staining for laminin and type IV collagen was not observed in epithelial cells in this series, concurring with reports by Charpin et al (1986b) and Sakr et al (1988). Siegal et al (1981) noted intense cytoplasmic staining of IDC cells with anti-laminin and anti-type IV collagen antisera, and occasional neoplastic cells expressed laminin in a study by Ekblom et al (1984). The reason for the latter findings are not clear, but possibly relate to cross reactivity of epitopes.

Finally, Charpin et al (1986b & 1989) described a technique for computerised quantitative analysis (Samba 200) of tissue sections stained with antisera to laminin and type IV collagen, with the aim of correlating the percentage of the cell surface immunostained with histopathological and clinical data. However, one of several drawbacks of this method is the inability to differentiate epithelial from vascular BM, potentially creating a major source of error.

Immunoperoxidase staining with antisera to specific BM components may therefore assist in differentiating *in situ* carcinoma from early invasive carcinoma, and sclerosing adenosis from tubular carcinoma.

IV.8.c. ACTIN AND MUSCLE SPECIFIC ACTIN

A number of muscle specific proteins have been described, including desmin, the IF protein typical of striated and smooth muscle cells, and actin. Actins are protein constituents of the microfilaments, cytoskeletal elements present in most cells. Six separate, closely related but biochemically and immunologically distinct, isotypes of actin have been defined, four of which (skeletal muscle alpha, smooth muscle alpha, cardiac alpha and smooth muscle gamma) are expressed exclusively in muscle cells (Tsukada et al, 1987; Miettinen, 1988).

Myoepithelial cells lie external to the epithelial cells of normal mammary, salivary and sweat gland ducts and acini, as demonstrated by immunocytochemical staining for actin (Ohtani & Sasano, 1980; Tsukada et al, 1988; Miettinen, 1988; Eusebi et

al, 1989) and myosin (Gusterson et al, 1982), and by electron microscopic studies (Gusterson et al, 1982). Myoepithelial cells coexpress cytokeratin and vimentin IFs, but staining for desmin is negative (Tsukada et al, 1987; Miettinen, 1988). Antibodies to muscle actins were therefore employed to identify myoepithelial cells in benign breast lesions, *in situ* neoplasia and infiltrating carcinomas.

Monoclonal antibodies directed against several muscle actins were applied to paraffin sections of the same 36 lesions that were studied with antisera to laminin and type IV collagen. Monoclonal anti-actin, a mouse IgM antibody, was obtained from Amersham Laboratories (Buckinghamshire, England; code N.350) and applied at a dilution of 1:8000 (Table III.2). In addition, a monoclonal IgG mouse hybridoma antibody specific for muscle actins, derived from clone HHF 35 and designated muscle specific actin (MSA), was purchased from Enzo Biochem (New York, USA; Cat. No. MA-931). This antibody has been characterized as specific for alpha actins and a muscle cell-specific fraction of gamma actins (Tsukada et al, 1987; Miettinen, 1988), and was applied at 1:375 dilution.

A standard ABC technique was employed, with incubation of the primary antibody overnight at 4°C to localize the muscle proteins (Appendix I; Chapter III.2). No digestion was required to expose the actin antigens in the microwave-fixed tissue, although Miettinen (1988) utilized pepsin to enhance staining in formalin-fixed sections. Tsukada et al (1987) claimed HHF 35 was resistant to most types of fixation, including formalin-based solutions. Both anti-actin and MSA (HHF 35) produced similar granular brown cytoplasmic staining of smooth muscle cells, although considerable background nonspecific staining was often observed with anti-

actin.

Myoepithelial cells were observed in a continuous layer bordering the periphery of ducts and acini in normal breast tissue and benign breast lesions (Figures IV.8.1, IV.8.2 & IV.8.10). Cytoplasmic processes were not observed to extend between epithelial cells (Figure IV.8.11; Chapter IV.4.d). In the four cases of intraductal carcinoma studied, the myoepithelial layer surrounding the expanded ducts was identified with anti-actin and MSA. The tumour cell population was non-reactive. These findings are in agreement with a study of multiple normal and pathological tissues by Tsukada et al (1987), and with a recently published paper examining seven breast carcinomas *in situ* (Eusebi et al, 1989). Patchy breaches in continuity of the myoepithelial layer were observed in intraductal carcinomas at foci of microinvasion, but these were not nearly as clear, or as easily identified, as the breaches in BM continuity described in Chapter IV.8.b. Infiltrating carcinomas were negative for muscle actins, but scattered myoepithelial cells were interposed at the tumour-stromal interface (Chapter IV.d), as found in immunocytochemical and ultrastructural studies by Gusterson et al (1982), Henderson et al (1986) and Eusebi et al (1989). Myoepithelial differentiation thus appears to be a rare event in breast carcinomas.

In the tumour stroma, scattered spindle-shaped actin-positive cells with dendritic-like projections were observed and interpreted to be "myofibroblasts", previously recognized in healing wounds, hyperplastic fibrous tissue responses and within the stroma of certain breast and ovarian carcinomas (Tamimi & Ahmed, 1987; Tsukada et al, 1987). In addition, cytoplasmic actin and MSA positivity was seen in the muscular layer of arteries and veins, and in pericytes surrounding the smaller blood
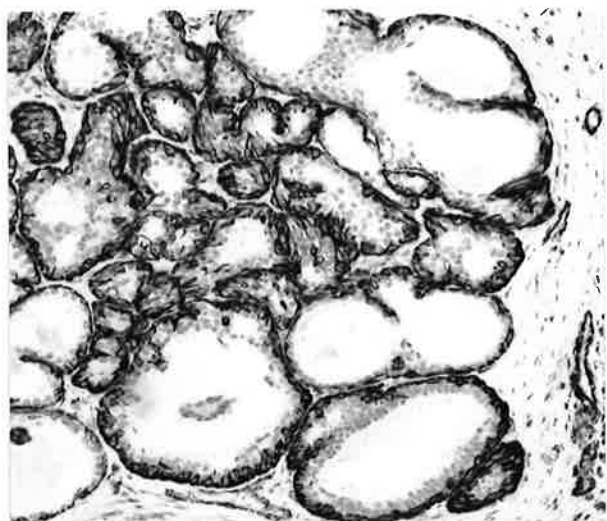
a.
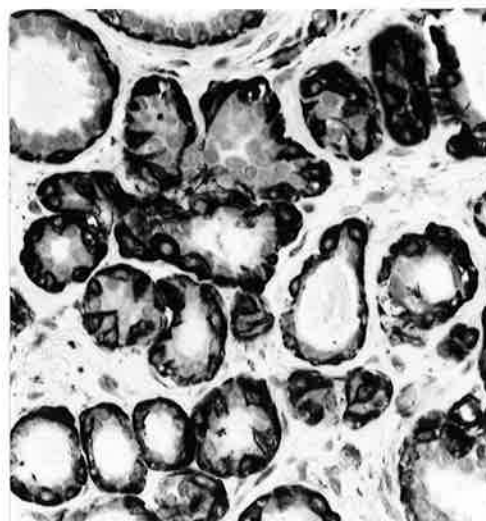
b.

c.

Figure IV.8.10. (a) Strong cytoplasmic staining with anti-MSA highlights the continuous layer of myoepithelial cells bordering the periphery of benign breast glands in a case of fibrocystic change (haematoxylin counterstain). A similar pattern is seen in a different example (b), illustrated at higher magnification. (c) Benign breast ducts demonstrating staining of myoepithelial cells with anti-vimentin (haematoxylin counterstain). Note the contrasting pattern of staining, with vimentin IFs localized as a solitary basal aggregate.
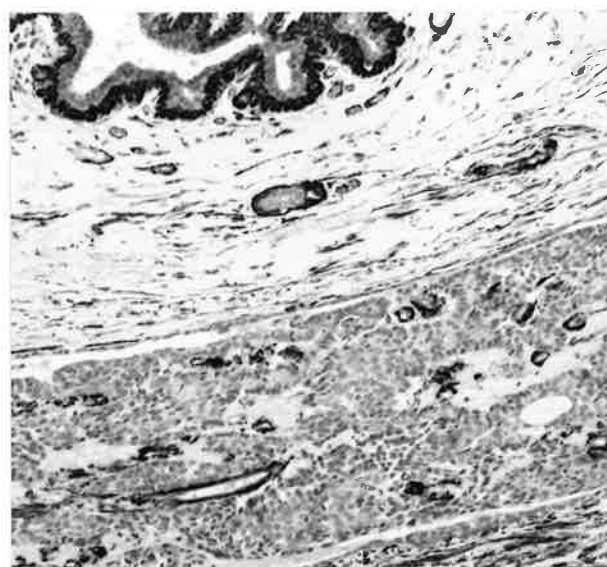
Figure IV.8.11. A moderately-differentiated infiltrating ductal carcinoma stained with anti-MSA (haematoxylin counterstain). Myoepithelial cells are prominent around a benign duct in the upper field. Positive staining is not identified amongst the sheets of tumour cells in the lower field, except in pericytes surrounding small blood vessels.

vessels (Figure IV.8.11; Tsukada et al, 1987; Miettinen, 1988).

The myoepithelial cell layer may thus be identified with antibodies to muscle actins. Observation of breaches in the continuity of the layer may be useful in evaluating microinvasion in proliferative intraductal lesions, in a manner analogous to that of assessment of BM integrity.

IV.8.d. DISCUSSION

The presence of BM, recognized by immunostaining for laminin or type IV collagen, together with MSA-positive myoepithelial cells localized close to the BM, may aid the diagnostic pathologist in differentiating benign epithelial proliferations from well-differentiated invasive carcinoma, and in identifying foci of microinvasive carcinoma. Furthermore, identification of myoepithelial cells is useful in establishing the presence of a dual cellular population, and hence the likely benign nature of a lesion. Staining for BM components, particularly type IV collagen, showed greater clarity and was subject to less background interference than was observed with the anti-actin stains.   Anti-laminin and anti-type IV collagen antisera are therefore recommended over immunostaining with antibodies to muscle actins.

The presence of myoepithelial cells and the retention of the capability of synthesizing basal lamina appear to be related, and have been viewed as evidence of differentiation in neoplastic cell populations (Gould et al, 1975). Further study of the BM will no doubt increase our understanding of the process of invasion and

metastasis. Laminin binds via specific cell surface receptors, which may be recognized using monoclonal antibodies, to normal and neoplastic cells. The degree of occupancy or the number of laminin receptors may be altered in human carcinomas (Liotta, 1984). Furthermore, whereas laminin receptors in normal epithelium may be polarized at the basal surface and occupied with laminin in the BM, receptors on invading tumour cells may be distributed over the entire surface of the cell. Experiments using animal models have shown a possible role for laminin in the interaction of tumour cells with the BM, and a means by which the laminin receptor may facilitate invasion of the extracellular matrix (Liotta, 1984). It is plausible that BM components secreted by neoplastic cells, or possibly by adjacent stromal cells, act as a scaffold for tumour growth. The observation in this study of small amounts of linear laminin and type IV collagen-positive BM material admixed with intraductal neoplastic cells supports the hypothesis that tumour cells may secrete BM.

In conclusion, staining for BM components offers a means of recognizing occult invasive carcinoma, and of distinguishing certain benign epithelial proliferations from well-differentiated invasive carcinomas. The identification of microinvasive foci in tumours which had been diagnosed on routine H & E sections as pure intraductal carcinomas may explain the occasional finding of nodal metastases in cases of "*in situ* cancer".

CHAPTER V.   CONCLUSIONS

a.   **MAJOR PROGNOSTIC PARAMETERS**

b.   **MINOR PROGNOSTIC PARAMETERS**

c.   **RECOMMENDATIONS**

CHAPTER V.   CONCLUSIONS

This thesis describes the relationships between traditional prognostic markers of breast cancer and several novel potential prognostic indicators, identified and quantified by means of immunohistochemical staining, in 115 cases of breast malignancy collected over a period of two years.   Several new techniques were developed to assist with accurate and reproducible quantification of the ER status and the tumour GF.

No infallible predictive indicator of clinical outcome has yet been determined for breast carcinoma patients, despite the immense efforts which have been directed towards this goal over the past few decades.   Neither has a specific tissue or serum marker of breast neoplasia been identified which could exclude or confirm the breast as the primary site in cases of metastatic cancer of "unknown primary".   As no single factor can predict outcome in all cases, the most accurate determinations of overall tumour burden and predictions of tumour behaviour are likely to be achieved with a prognostic index incorporating a hierarchy of the known prognostic parameters (Table V.1).

V.a. MAJOR PROGNOSTIC PARAMETERS

Histological evidence of axillary lymph node involvement remains the principal method of defining groups of breast cancer patients with a poor prognosis.   This

TABLE V.1.

PARAMETERS OF VALUE IN PREDICTING OUTCOME IN BREAST CARCINOMA

MAJOR

Axillary lymph node metastases

Tumour growth fraction (Ki-67 count)

MINOR

ER status

Histological grade

Histological type

Tumour size

POTENTIAL PROGNOSTIC MARKERS

Vimentin IF expression

AgNOR count

marker is of particular value in small laboratories where facilities for techniques such as immunocytochemical staining are lacking. "Occult" axillary lymph node micrometastases were identified in 22 per cent (12) of the 55 Stage I patients in this study using antibodies to cytokeratins to detect neoplastic cells not discernible in routine H & E-stained sections. Immunocytochemistry can therefore potentially enhance the accuracy of histological assessment of nodal status. However, with the present vogue for more conservative surgery, many patients do not receive even a partial axillary dissection and the axillary lymph node status remains unknown (18 per cent of cases in this series). Furthermore, the five year relapse rate for patients assessed as node-negative (Stage I) remains at approximately 20 per cent.

Indices of cell proliferation, or GFs, have been shown to correlate with other prognostic indicators, with DFS and overall survival, and with response to chemotherapy. Specialized techniques to measure GF, such as thymidine-uptake studies and flow cytometry, are expensive and complex to perform, but use of the monoclonal antibody Ki-67, with specificity for a nuclear antigen present only in proliferating cells, allows GFs to be readily determined in cryostat sections using immunocytochemical techniques. Immunostaining with this antibody promises to be a most important tool for prognostication.

V.b.   MINOR PROGNOSTIC PARAMETERS

Endocrine markers of breast cancer, such as ER protein, provide an indication of likely response to hormonal therapy and hence DFS, although the value of ER status

in predicting overall survival is controversial. Any relationship between positive ER status and improved survival possibly relates to the inverse correlation between ER status and tumour GF. Papers stating that 10 per cent of ER-negative tumours respond to hormonal therapy, and that 40 to 45 per cent of ER-positive cases fail to respond, are generally based upon cytosolic determinations of ER content. New immunocytochemical techniques of ER analysis highlight the degree of ER heterogeneity within breast carcinomas, overcome many of the inaccuracies of the cytosolic method, and potentially improve the predictive capability of receptor status. Nevertheless, the overriding influence of a large GF may explain why some patients with ER-positive tumours relapse quickly.

The degree of differentiation in IDCs, as assessed by histological grading, has been shown to have prognostic relevance. Problems of interobserver and intraobserver reproducibility limit the accuracy of this prognostic parameter, but patients with high grade neoplasms appear to be at a greater risk of relapse than patients with well-differentiated carcinomas.

New potential markers of proliferative activity in breast cancer are the expression of vimentin IFs, in addition to cytokeratin IFs, and the AgNOR count. The value of these parameters lies partly in their ability to relate to GF independently of lymph node status, and partly in their simple application to routinely-fixed paraffin-embedded sections, thereby allowing assessment of archival tissues.

Tumour size may also have a weak bearing on prognosis, particularly in node-negative patients, and certain tumour types exhibit a more predictable behaviour

pattern. Finally, although many authors regard vascular invasion to be of prognostic value, a number of dissenting opinions have been published and the usefulness of this marker is controversial.

V.c. RECOMMENDATIONS

Thorough routine histopathological examination of the macroscopic specimen and H & E-stained slides should always be performed, including an assessment of tumour size, tumour type, extratumoral vascular invasion and histological grade of IDCs. The number and level of lymph node metastases should be noted, including the presence of extracapsular extension.

The monoclonal antibodies Ki-67 and anti-ER should be applied to cryostat sections of breast neoplasms whenever possible, and a paraffin section of each carcinoma should be stained with monoclonal antibodies to vimentin IFs. More information can potentially be gained from a slide-based system, in which the observer can simultaneously evaluate tumour histology and either hormone receptor status or GF, than with the use of techniques such as flow cytometry, where tissue architecture and cell-to-cell relationships are lost in the tissue homogenate.

Finally, it is recommended that sections of all lymph nodes in cases of Stage I breast cancer be stained with anticytokeratins such as CAM 5.2 or AE1/AE3 to increase the rate of detection of small metastatic deposits.

With emphasis placed on the axillary lymph node status, as an indicator of metastatic capability, and on the tumour GF or Ki-67 count, to reflect proliferative potential, an assessment of each of these parameters (Table V.1) provides the best guide as to the likely clinical outcome and hence the preferred therapy option. Long-term follow-up of all patients, including an appraisal of response to treatment, is ideal to further elucidate the most significant prognostic parameters.

With the availability of future biological markers of proven prognostic value, these recommendations will no doubt need to be revised, however, this thesis offers guidelines as to the optimal way the pathologist can, at present, serve the patient with breast cancer.

APPENDICES

APPENDIX I

AVIDIN-BIOTIN PEROXIDASE TECHNIQUE
(Paraffin Sections)

1. Deparaffinize and hydrate tissue sections through xylene and graded alcohols to absolute alcohol.

2. Place sections in 0.5% hydrogen peroxide (H_2O_2) in methanol (8.3 ml of 30% H_2O_2 in 500 ml absolute methanol), for 30 minutes.

3. Wash sections in tap water, then in deionized water.

4. Place sections in PBS buffer, pH 7.4, for 10 minutes.

5. Incubate sections with appropriate 3% non-immune serum for 15-20 minutes.

6. Drain off excess serum (vacuum pump suction technique) and incubate with appropriately diluted primary antibody (in appropriate 3% non-immune serum) overnight at 4°C.

7. Wash slides in PBS buffer (pH 7.4) x 2, 7 minutes each.

8. Incubate sections with biotinylated secondary antibody (diluted to 1:250 with appropriate 3% non-immune serum)for 30 minutes at room temperature (22°C).

9. Wash in PBS buffer x 2, 7 minutes each.

10. Incubate with ABC reagent (diluted to 1:250 with 3% non-immune serum) for 60 minutes at room temperature.

11. Wash in PBS buffer x 2, 7 minutes each.

12. Freshly prepare diaminobenzidine tetrahydrochloride solution (DAB): 1 microtube of DAB (25 mg) added to 50 ml of Tris HCl buffer, pH 7.6 (0.05M), and 50 μl of 30% H_2O_2 (0.03%). Incubate sections for 5-15 minutes at room temperature with macroscopic/microscopic control of the reaction.

13. Wash sections in tap water for 2 minutes.

14. Counterstain lightly with Lillie-Mayer's haematoxylin.

15. Dehydrate, clear and mount.

APPENDIX II

AVIDIN-BIOTIN PEROXIDASE TECHNIQUE
(Frozen Sections)

Fresh tissues are embedded in O.C.T. (Miles Laboratories), snap frozen in liquid nitrogen, and then stored at -20°C. Cryostat sections are cut at 6 microns and thaw-mounted onto aminoalkylsilane-treated glass slides (Rentrop et al, 1986).

1. Fix as appropriate for individual antigens (as described in text: ER - PLP; Ki-67 - microwave irradiation followed by acetone).

2. Rinse sections in freshly prepared PBS buffer, pH 7.4, for 10 minutes.

3. Incubate sections with 3% normal horse serum for 10 minutes.

4. Drain off normal horse serum and apply primary antibody, diluted in 3% normal horse serum, for 60 minutes at room temperature.

5. Wash slides in PBS buffer x 2, 7 minutes each.

6. Incubate with appropriate secondary biotin-conjugated antibody, diluted in 3% normal horse serum (1:250), at room temperature for 30 minutes.

7. Wash in PBS buffer x 2, 7 minutes each.

8. Apply ABC reagent (diluted 1:250 with 3% normal horse serum) and incubate for 60 minutes at room temperature.

9. Wash in PBS buffer x 2, 7 minutes each.

10. Apply DAB solution (25 mg per 50 ml of Tris HCl buffer, pH 7.6, and 50 μl of 30% H_2O_2) and incubate for 5-10 minutes, controlling microscopically.

11. Rinse in PBS, pH 7.4, for 1 minute and then briefly in tap water.

12. Lightly counterstain with Lillie-Mayer's Haematoxylin.

13. Dehydrate, clear and mount.

CORRELATION MATRIX

	Ki-67	ER(%)	ER(c)	AgNOR	Nodes	Grade	Vim	Size	Age
Ki-67	1								
ER(%)	r -0.39 $p<0.001$	1							
ER(c)	r -0.35 $p<0.001$	r 0.81 $p<0.0001$	1						
AgNOR	r 0.41 $p<0.0001$	r -0.34 $p<0.001$	r -0.23 $p<0.05$	1					
Nodes	NS	r -0.25 $p<0.05$	NS	NS	1				
Grade	r 0.52 $p<0.0001$	r -0.43 $p<0.001$	r -0.31 $p<0.01$	r 0.29 $p<0.01$	r -0.24 $p<0.05$	1			
Vim	t 8.92 $p<0.0001$	t 3.37 $p<0.005$	MWU257 $p<0.002$	t 3.6 $p<0.0005$	NS	t 2.53 $p<0.05$	1		
Size	NS	r -0.20 $p<0.05$	r -0.20 $p<0.05$	NS	r 0.28 $p<0.05$	NS	NS	1	
Age	NS	r 0.24 $p<0.05$	r 0.22 $p<0.05$	NS	r -0.23 $p<0.05$	NS	t 2.4 $p<0.05$	NS	1

t   - unpaired t test
r   - Spearman rank correlation coefficient
NS - Not significant
MWU - Mann-Whitney U test

ER(%) - ER count
ER (c) - cytosolic ER value
Vim   - Vimentin

APPENDIX IV

ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex
ALA	Alpha-lactalbumin
AgNOR	Silver nucleolar organizer region
BM	Basement membrane
CK	Cytokeratin
cm	Centimetres
DAB	Diaminobenzidine
DFS	Disease-free survival
DI	DNA index
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
ER	Estrogen receptor
GF	Growth fraction
H & E	Haematoxylin and eosin
IDC	Infiltrating ductal carcinoma
IF	Intermediate filament
IMVS	Institute of Medical and Veterinary Science
kd	Kilodalton
LI	Labelling index
MI	Mitotic index
mg	Milligrams
mRNA	Messenger RNA
MW	Molecular weight
NOR	Nucleolar organizer region
NSAP	National Surgical Adjuvant Project of Breast Cancers
PAP	Peroxidase-anti-peroxidase
PBS	Phosphate-buffered saline
PgR	Progesterone receptor
PLP	Periodate-lysine-paraformaldehyde
SPF	S phase fraction
SP1	Pregnancy-specific ß1-glycoprotein
TfR	Transferrin receptor
TLI	Thymidine labelling index
μ	Micron
WHO	World Health Organization

ADDENDUM

At the completion of this thesis two recent works have come to the author's attention. Both collaborate the results presented in this dissertation and support the hypothesis that vimentin expression may be a feature of more malignant breast cancer cells.

Sommers et al (1989) cloned complementary DNAs of genes expressed in a hormone-independent breast carcinoma cell line. The same genes were not found in a hormone-dependent line. One such clone, isolated in many copies, coded for the IF protein vimentin. Vimentin was expressed in five of 11 hormone-independent mammary carcinoma cell lines tested, but was not present in five hormone-dependent cell lines. Furthermore, vimentin was expressed in several tumour lines with a more anaplastic phenotype and in a highly tumorigenic breast epithelial cell line transformed by two oncogenes. Cytokeratin expression was diminished in breast cancer cell lines which expressed vimentin.

In another paper by Cattoretti et al (1988), preferential expression of vimentin, p53 protein and EGFR was described in ER-negative and high grade breast cancers.

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