UNIVERSITY OF SOUTHAMPTON

AN EVALUATION OF THE MONOCLONAL ANTIBODIES HMFG1 AND HMFG2 IN BREAST CANCER

THESIS

Submitted in accordance with the requirements of

Southampton University

for the degree of

Doctor of Philosophy

by

NICOLA BERRY

APRIL 1986

Departments of Pathology and Surgery
Faculty of Medicine
University of Southampton

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. D. B. Jones and Professor I. Taylor, for their advice and support during the last three years, particularly whilst I was writing up. Invaluable help was given by Dr. R. Marshall and Dr. N. Kirkham, who spent many hours looking at the sections with me and explaining to me the mysteries of breast tissue pathology. Many thanks, also, to the technical staff of the Southampton University Pathology Department, especially Mr. B. Mepham, Mrs. K. Britten and Mrs. K. Higginson, for their friendly interest and help in the technical aspects of the project. Finally, thanks to my friends and family for their undaunted encouragement throughout my years as a Ph.D student.

This research was funded by the Southampton University Departments of Surgery and Pathology.

To Mum and Dad

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

PATHOLOGY / SURGERY

Doctor of Philosophy

AN EVALUATION OF THE MONOCLONAL ANTIBODIES HMFG1 AND HMFG2 IN BREAST CANCER

by Nicola Berry

HMFG1 and HMFG2 are monoclonal antibodies raised against the human milk fat globule. The immunocytochemical application of these antibodies in the diagnosis and determination of prognosis in breast cancer was investigated. Immunocytochemical techniques were established for the optimal demonstration of the HMFG1 and HMFG2 antigens in cytological and histological specimens of primary and secondary breast carcinoma.

The immunohistochemical staining patterns of HMFG1 and HMFG2 in histologically normal, benign and malignant breast tissue were determined. In normal and benign tissue the staining was extracellular. Staining of malignant breast tumours was variable, both in terms of the relative intensity of stain, either extracellularly or intracellularly, and in the extent of intracellular staining.

A classification system was devised to represent these two variables and used to grade the staining patterns in tumour biopsies from both a retrospective series of 37 patients and a prospective series of 115 patients with breast carcinoma. The staining patterns classified in this way were indicative of the state of differentiation of the tissue but not of relapse free survival.

The most accurate prognostic indicator in breast cancer is the lymph node status. In 50 cases, a comparison was made of the detection of breast carcinoma metastases in the axillary lymph nodes by routine histological diagnosis and by immunohistochemical staining with a panel of monoclonal antibodies, which included HMFG1 and HMFG2. Immunohistochemical staining increased the detection of metastases by 17%.

Immunocytochemical staining with HMFG1 and HMFG2 failed to distinguish between needle aspirates of benign and malignant breast tumours.

The evaluation of the monoclonal antibodies HMFG1 and HMFG2 in breast cancer has been discussed with respect to the biochemical nature and cellular synthesis of the antigens.

ABBREVIATIONS

3A9E	3 Amino 9-ethylcarbazole
anti-EMA	antiserum directed against epithelial membrane antigen
anti-HME	antiserum directed against human mammary epithelial antigen
cytoprep.	cytocentrifuge preparation
DAB	3´ 3 Diaminobenzidine tetrahydrochloride
EMA	epithelial membrane antigen
ER	oestrogen receptor
GaM IgG	goat anti-mouse IgG
H&E	Haematoxylin and Eosin
HME	human mammary epithelial antigen
HMFG	human milk fat globule
HPO	horseradish peroxidase
IAP	immunoalkaline phosphatase
IP	immunoperoxidase
MAAPAP	mouse anti-alkaline phosphatase / alkaline phosphatase conjugate
MFG	milk fat globule
NHS	normal human serum
TBS	Tris buffered saline

CHAPTER 1

INTRODUCTION

> "It is better to give no treatment in cases of hidden (breast) cancer; treatment causes speedy death, but to omit treatment is to prolong life."

This quote was the opinion of Hippocrates around the year 400 B.C. Since then there have been many changes in the approach to breast cancer resulting from both an increase in understanding of the nature of cancer and an improvement in surgical procedures and the tools available for therapy.

1.1. Breast Cancer.

1.1.1. A Change in the Concept of Cancer Pathology.

The treatment of breast cancer varied and was not based on any concept of the pathology of the disease until the turn of this century. Then Halsted (1907), based treatment on a hypothesis of "centrifugal spread" in which the cancer spreads in continuity with the primary tumour. Extensive removal of all of the tissue around the primary tumour would hopefully eradicate all of the spreading cancer. Accordingly Halsted (1907), recommended a radical mastectomy for the cure of breast cancer. This procedure was widely adopted and was supported by an increase in survival in the early 1900's.

In the 1940's and 50's experiments on animal models (Schabel, 1977), and observations of patient survival after radical mastectomy (Fisher et al., 1968), showed that cancer did not spread in continuity with the primary tumour but by dissemination of the tumour cells into either the blood or lymphatic circulation. Removal of the primary tumour and surrounding tissues would eliminate the source of the

disease but not the metastatic cells. The originally observed improvement in survival after introduction of the radical mastectomy was retrospectively considered due to an increased awareness of breast cancer both in the medical world and the general public so that more patients were treated in earlier stages of the disease (Stehlin et al., 1979). Public awareness also resulted in a greater emphasis on the psychological aspects of breast cancer and the demand for less extensive surgery. These factors, together with the development of alternative methods of treatment such as radiotherapy, chemotherapy and endocrine therapy (Henderson & Canellos, 1980), contributed to a radical change in the management of breast cancer.

1.1.2. Detection and Diagnosis of Breast Cancer.

Experiments of tumour kinetics have shown that breast cancer has a long preclinical phase so that by the time a tumour is noticed by the patient it is at an advanced stage in it´s growth (Spratt, 1977). If it is assumed that there is a phase in the growth of a neoplasm before metastasis occurs, then the earlier a tumour is detected, the greater the chance that cells have not disseminated. Removal of a primary tumour that had not metastasised should cure the patient.

In order to detect breast tumours at an earlier stage of their life-history, programmes have been established to educate women in breast self-examination (Hinton, 1984). Also, several screening programmes using clinical examination and mammography have been initiated in America, Europe and Scandinavia (Forrest & Roberts, 1980). In these studies there was detection of more tumours at an earlier stage of disease in the screened than in the control population. Results of the HIP study in America have shown that, after a nine year follow up, screening by mammography and clinical examination reduces the mortality rate in women over 50 years of age (Shapiro, 1977). A longer follow up period is needed to confirm these results. It is possible that mammography and clinical examination is not sufficiently sensitive to detect tumours at a size when they have not metastasised. This and the counteracting carcinogenic effects of

irradiation in mammography has prompted investigation of other methods of screening. These include methods such as magnetic resonance imaging; sonography; computed tomography; transillumination; immunodetection and minimal dose digital radiography (Kopans, 1984). So far there is no evidence to suggest that these alternative methods would improve the results obtained by clinical examination and mammography.

After detection of a breast lesion, the tumour is determined as benign or malignant by three criteria, which are used to varying degrees of magnitude depending on the clinicians involved. These are the clinical assessment, radiography, such as mammography and ultrasonography and aspiration cytology (Smallwood et al., 1984). After a definitive diagnosis the patient can be treated accordingly. In cases where the diagnosis is equivocal the tumour must be excised before futher decisions can be taken. This is time-consuming, uneconomical and subjects the patients to considerable psychological stress. Any improvement in the reliability of diagnosis would be appreciated by both the patients and medical staff.

1.1.3. Treatment of Breast Cancer.

After a breast tumour has been diagnosed as malignant it can be treated in a variety of ways. The primary tumour is surgically removed and the extent of removal of the surrounding tissue depends on the surgeon involved. Less extensive surgery can be followed by radiotherapy to eradicate locally disseminated cells (Stehlin et al., 1979). Treatment of metastatic carcinoma is more complex. The most popular forms of treatment presently employed, chemotherapy and endocrine therapy, do not achieve complete remission but an increase in survival time (Henderson & Canellos, 1980).

As various forms of therapy are available, it is important to treat each patient appropriately to achieve the maximum response. The choice of treatment depends partly on the expectation of survival (Henderson & Canellos, 1980), defined by prognostic indices which are

described in detail in Chapter 4 (4.1.). Follow up of patients with breast cancer who have refused any form of treatment shows that the course of breast cancer is variable. Whilst the majority of patients develop secondary tumours and die within 2-3 years, some patients survive 10 years or more (Bloom et al., 1962). Different cancers have widely varying metastatic capabilities. The object of prognostic indices, therefore, is to define the metastatic potential of the cells in each neoplasm. A combination of variables which determine whether the cells of the primary tumour are capable of metastasis with those which assess how aggressive the cells are is likely to be most accurate in predicting prognosis. A prognostic index combining such variables has been described but is not entirely accurate (Haybittle et al., 1982). Development of more accurate methods of assessing the metastatic capability of the tumours would be valuable in the treatment of breast cancer.

Within this century changes in the approach to the management of breast cancer have improved the life expectancy. Despite this, mortality rates have not changed in the last 30 years (Studies on Medical and Population Subjects, 1981) and breast carcinoma remains the most commonly occuring cancer in women (Mould, 1983). Treatment of breast cancer might be obviated by determining its cause but, despite both experimental and epidemiological studies providing evidence both for viral and for hormonal involvement, the aetiology of breast cancer is not yet clear (Miller, 1980). The search continues, therefore, for new methods or improvements of existing methods in detection, diagnosis and treatment to solve the problem of breast cancer.

1.2 Monoclonal Antibodies

1.2.1. Tumour-Specific and Tumour-Associated Antigens.

The possibility that tumours might be identified and treated via tumour specific antigens has stimulated much research since the early 1900's. The first evidence for tumour specific antigens was presented

by Gross (1943), who demonstrated that mice immunised against a chemically induced sarcoma from syngeneic animals would not accept tumour transplants from these animals. Further evidence was provided by Prehn & Main (1957), showing that a mouse immunised against a chemically induced fibro-sarcoma from a syngeneic animal would reject a tumour graft but accept a skin graft from that animal. With this evidence for tumour immunology in mice, the search for human tumour-specific or tumour-associated antigens began.

Antibodies are necessary tools for establishing the specificity and possible usefulness of an antigen isolated from a tumour. The presence and distribution of the antigen in cell lines and in tissues can be determined by immunocytochemistry.The possibility of antigen mediated targeting of drugs, toxins and isotopes can be tested using the antibodies.

1.2.2. The Production of Polyclonal and Monoclonal Antibodies.

Immunisation of an animal with any complex antigen, including purified protein, induces a B cell response resulting in many clones of B cells each producing antibody directed to a different epitope or determinant in the molecule. In 1975, Kohler & Milstein developed a technique which has made possible the production of monoclonal antibodies (FIG 1.1). In this technique stimulated B cells from the spleen of a mouse immunised with the appropriate antigen are fused with a myeloma cell line in vitro. The myeloma cells used are deficient in either hypoxanthine guanine phosphoribosyl transferase (HPRT), or thymidine kinase (TK), which are enzymes required for the salvage pathway of nucleotide synthesis for DNA. Hybrid cells can be selected by their growth in HAT medium. This contains aminopterin, which blocks the major pathway of nucleotide synthesis, and hypoxanthine and thymidine, which are substrates used in the salvage pathway of nucleotide synthesis. Only cells able to synthesise HPRT and TK can grow in this medium (FIG 1.2). In this system hybrid cells with the growth characteristics of myeloma cells and the salvage pathway enzymes contributed by the spleen cells survive. The hybrid

FIG 1.1

Production of Monoclonal Antibodies

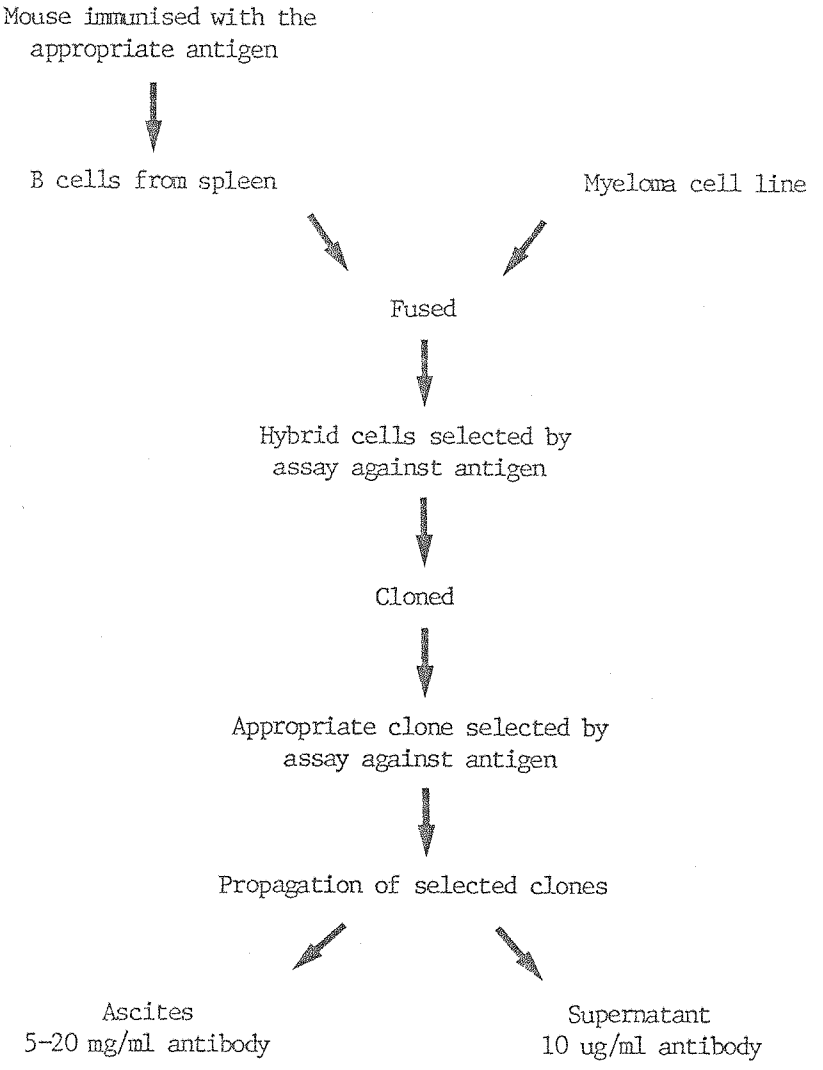
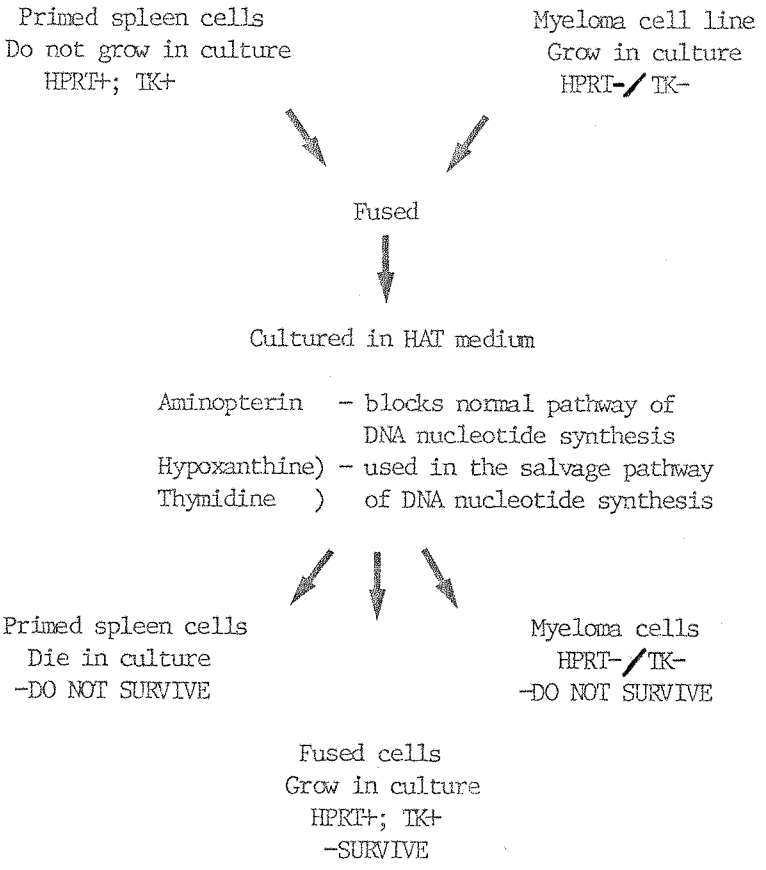


FIG 1.2

Selection of Fused Cells by Growth in HAT Medium



HPRT - Hypoxanthine guanine phosphoribosyl transferase
TK - Thymidine kinase
These are enzymes required for the salvage pathway of DNA nucleotide synthesis.

cells are cloned and the required clone is selected by assay against the antigen using techniques such as radioimmunoassay or ELISA. The selected clone can be propagated either in vitro or as an ascites. Monoclonal antibodies are thus of uniform specificity and are chemically homogenous.

Both polyclonal and monoclonal antibodies have been raised against various antigens in cancer, each having certain advantages. Polyclonal antibodies have a wider range of activity detecting all variations of a complex antigen whilst monoclonal antibodies are monospecific and discriminate between variations of a complex antigen.

1.2.3. The Search for Tumour-Specific and Tumour-Associated Antibodies.

The production of polyclonal and monoclonal antibodies provided new tools for use in the detection, diagnosis and management of cancer. This has prompted many avenues of research which have resulted in a vast array of tumour-specific and tumour-associated antibodies. Some antibodies have been raised against antigens associated with the abnormal characteristics of malignant cells and are clinically applicable in a variety of cancers. Malignant cells often divide and grow more rapidly than normal cells and may express larger amounts of antigens associated with proliferation. Examples of these are the transferrin receptor, the Ki67 antigen and, in the case of epithelial cells, epidermal growth factor receptor (Trowbridge & Domingo, 1981 Gerdes et al. 1984 Gusterson et al. 1984). The distribution of cytoskeletal proteins in normal and malignant cells is different due to the altered cellular adhesivity and activity in malignancy. Antibodies have been raised against such cytoskeletal proteins (Rungger-Brandle & Gabbiani, 1983). Other antibodies have been sought in specific types of cancer.

In breast cancer one approach to the production of tumour-specific and tumour-associated antibodies was to use malignant cells as the

immunogen and then screen against breast carcinoma cell lines. Antibodies have been raised against malignant cells or membrane enriched fractions from metastatic breast carcinoma (Ellis et al., 1984; Nuti et al., 1982) and cells from breast cancer cell lines (Yuan et al., 1982; Mariani-Costantini et al., 1984; Papsidero et al., 1983; Thompson et al., 1983; Soule et al., 1983). All of these antibodies recognise antigens present in normal or benign as well as in malignant breast tissue (Table 1.1). They are not tumour-specific but are defined as tumour-associated antigens because their expression in malignant tissue is different from that in normal or benign tissue. Tumour-associated antibodies raised against malignant cells from organs other than breast, such as the larynx and colon, have been used to demonstrate that the antigens are also present in breast tissue (Holley et al., 1983; Holmgren et al., 1984).

Schlom et al. (1980), approached the problem differently. Their research was based on the finding that the serum of women with breast cancer contains antibodies directed against primary cultures of breast cancer cells. They fused lymphocytes from the axillary nodes of patients undergoing mastectomy for primary breast cancer with a myeloma cell line. One resulting monoclonal antibody, MBE6, identified a tumour-associated antigen which was present on cells from normal, benign and malignant breast tissue (Teramoto et al., 1982), (Table 1.1).

Another source of tumour-specific and tumour-associated antigens is the oncodevelopmental proteins. These are normally present in foetal or placental tissues but are expressed in malignant tissues because of activation or derepression of the genes involved in their synthesis (Mc Laughlin et al., 1982). The most well known pregnancy associated protein is the carcino-embryonic antigen (CEA), originally detected in adenocarcinoma of the colon and normally present in the digestive organs of foetuses between 2 and 6 months gestation (Gold & Freedman, 1965). CEA has been demonstrated in normal, benign and malignant breast tissue using polyclonal antisera (Wahren et al., 1978) (Table 1.1). It is, however, a complex molecule consisting of

TABLE 1.1 Tumour-Specific and Tumour-Associated Antibodies in Breast Carcinoma

Antibody	Reference	Antigen	Immunohistochemical Detection of Antigen in Normal Breast	Benign Breast	Malignant Breast
1. RAISED AGAINST MALIGNANT CELLS eg:-					(%)
NCRC-11	Ellis et al., 1984	Metastatic breast carcinoma cells	+	+	50/50 (100)
B72.3	Nuti et al., 1982	Metastatic breast carcinoma cell membranes	-	+	19/41 (46)
H59	Yuan et al., 1982	Breast carcinoma cell line: ZR-75-1	nt	+	38/71 (53)
MBr1	Mariani-Costantini et al., 1984	Breast carcinoma cell line: MCF7	+	+	80/127 (63)
F36/22	Papsidero et al., 1983	Breast carcinoma cell line: MCF7	+	+	17/22 (77)
24.17.2	Thompson et al., 1983	Breast carcinoma cell line: MCF7	+	nt	5/5 (100)
10-3D2	Soule et al., 1983	Breast carcinoma cell line: BT-20	-	nt	+
CA-1	Holley et al., 1983	Cultured laryngeal carcinoma cell membranes	nt	+	18/19 (95)
CA-50	Holmgren et al., 1984	Colo-rectal carcinoma cell line	nt	nt	+
2. USING HUMAN LYMPHOCYTES AS SOURCE OF ANTIBODY:					
MBE6	Teramoto et al., 1982	Lymphocytes from axillary lymph nodes of patient with primary breast carcinoma fused with myeloma cells	-	+	54/67 (81)
3. ONCODEVELOPMENTAL PROTEINS					
	Wahren et al., 1978	Carcinoembryonic Antigen	+	+	21/50 (42)
	Walker, 1978	HCG (a sub-unit)	-	-	12/53 (23)
	Wachner et al., 1984	HCG (b sub-unit)	-	+	16/129 (12)
	Sorensen et al., 1984	SP1	nt	+	6/34 (18)
	Gugliotta et al., 1981	Placental lactogen	nt	nt	few
	McDicken et al., 1983	Placental alkaline phophatase	-	-	5/7 (71)

CONTINUED OVERPAGE

TABLE 1.1 CONTINUED

Antibody	Reference	Antigen	Immunohistochemical Detection of Antigen in Normal Breast	Benign Breast	Malignant breast
4. ECTOPIC HORMONES AND ASSOCIATED PROTEINS					(%)
	King & Green, 1984	Estrophilin	nt	nt	+
	Ciocca et al., 1983	24KD oestrogen regulated protein	+	+	18/70 (26)
	Spring-Mills et al., 1984	Insulin	-	-	7/9 (78)
	"	Somatostatin	-	+	2/9 (22)
	Cohle et al., 1979	ACIH	nt	nt	1/1 (100)
	Seppala & Wahlstrom, 1980	LH	nt	-	7/11 (64)
5. ONCOGENE PRODUCTS					
	Horan Hand et al., 1984	Human ras oncogene product, p21	-	+	27/30 (90)
6. ANTIBODIES RAISED AGAINST VIRAL ANTIGENS					
	Mesa Tejada et al., 1978	52KD protein immunologically related to MMTV	-	-	51/131 (39)
D2	Crawford et al., 1981	53KD protein associated with SV40	-	nt	+
6. PRODUCTS OF NORMAL CELLS					
	Klein et al., 1979	TF antigen	+	+	+
	Bussolati et al., 1975	Casein	+	+	+
	Walker et al., 1979	a Lactalbumin	+	+	51/100 (51)
	Purnell et al., 1982	Prolactin	+	+	9/24 (37)
		HMFG - See table 1.3			

many antigenic epitopes of which only some are present in breast carcinoma (Grunert et al. 1983). Monoclonal antibodies raised against one antigenic epitope in CEA might show a more restricted specificity for malignant breast tissue. Other oncodevelopmental antigens expressed in breast carcinoma are the placental proteins, including the and B sub-unit of human chorionic gonadotrophin (HCG), pregnancy specific Bl glycoprotein (SPl), placental lactogen (PL) and placental alkaline phosphatase (Walker, 1978, Wachner et al. 1984 Horne et al. 1976 Sorensen et al. 1984 McDicken et al., 1983). Antibodies raised against these antigens have been used to determine their expression in breast tissue (Walker, 1978, Wachner et al. 1984 Sorensen et al, 1984 McDicken et al, 1983 Gugliotta et al. 1981). The results from these studies are contradictory, probably because of the different reagents and detection methods used. The overall impression gained is that the placental proteins are expressed in none or very few of the benign breast samples and heterogenously in a low percentage of breast carcinoma samples (Table 1.1).

Activation or derepression of genes in malignant cells also results in the inappropriate production of hormones, one of which is oestrogen. Antibodies have been raised to the oestrogen receptor and other proteins associated with the action of oestrogen (Greene et al. 1984 Ciocca et al. 1983). Antibodies have also been used to detect the presence of immunoreactive insulin, somatostatin, adenocorticotrophic hormone (ACTH) and luteinising hormone (LH) in primary breast cancer (Spring-Mills et al., 1984 Cohle et al., 1979, Seppala & Wahlstrom, 1980) (Table 1.1).

Oncogenes are involved in the formation or growth of malignant tumours. Oncogene products may be tumour-associated or tumour-specific antigens. Recently much effort has been put into raising antibodies against oncogene products. Horan Hand et al. (1984), have raised a series of monoclonal antibodies (RAPl - RAP5) against a human ras oncogene product, p21. Horan Hand et al. (1984), immunohistochemically demonstrated these antigens in benign and

malignant breast tissue (Table 1.1).
Viruses also are possibly involved in the aetiology of breast cancer. Antibodies have been raised against a 52KD glycoprotein immunologically related to the mouse mammary tumour virus (MMTV) and against a 52KD protein associated with Simian Virus 40 (SV40) (Mesa tejada et al. 1978 Crawford et al. 1981). Both of these antigens are apparently tumour-specific, expressed in malignant but not normal or benign breast cells and tissue (Table 1.1).

A range of tumour-associated antigens have been raised against products of normal cells. Because of genetic variability (Nowell, 1976), these are expressed differently in malignant and normal or benign breast tissue. Springer et al. (1976), used antibodies to demonstrate the presence of the Thompson-Friedenrich, or TF antigen, in primary breast cancer. As this is a precursor of the blood group MN antigens it was thought that the TF antigen would not be expressed in normal cicumstances. Klein et al. (1979), however, demonstrated its' presence in normal and benign breast tissue (Table 1.1).

In breast cancer specifically the products of differentiated lactating cells have been used as immunogens for clinically applicable antibodies. These include casein and prolactin, which are present in malignant as well as in normal and benign breast tissue (Bussolati et al, 1975 Purnell et al. 1982) (Table 1.1). The tumour associated antibodies used in this study fall into the last category for they are raised against the human milk fat globule membrane.

1.2.4 The Human Milk Fat Globule Membrane

The milk fat globule (MFG) is a glycoprotein present in the membrane of differentiated breast cells. In lactation the milk fat is secreted from the apical region of the mammary epithelial cell into the mammary ducts by pinocytosis (Wooding, 1971). In the milk the fat, surrounded by the membranous envelope, is called the MFG. This,

because of it's high lipid content, has a low density and floats above the other constituents of the milk to form the cream fraction. To obtain an almost pure membrane fraction, the cream fraction is churned to separate the fat (butter) from the cell membrane which is spun and harvested. This membrane preparation is treated by solvents and lyophilised to obtain the MFG antigen (Ceriani et al., 1977).

1.2.5. Antibodies Raised Against MFG.

1.2.5.1. Polyclonal Antisera Raised Against MFG.

Initially polyclonal antisera were raised against the delipidated human MFG (HMFG). Ceriani et al. (1977), raised a polyclonal antiserum against the HMFG called anti-Human Mammary Epithelial (HME), antiserum. The HME antigen was located by indirect immunofluorescence on viable cells derived both from normal and malignant breast cell lines and from mechanically disaggregated benign and malignant breast cell tumours (Ceriani et al., 1977; 1980). The antigen was not observed on cells from other normal and malignant cell lines such as kidney, lung, colon, melanoma and lymphoma or from a cell line derived from the myoepithelial elements of a breast carcinoma (HS578 Bst). The HME antigen was demonstrated by immunofluorescence on the breast epithelial cell surface. The antigen expression was different for each breast tumour cell line both in staining intensity and in the percentage of cells staining. Quantitation of HME antigen levels by flow cytofluorimetry and radioimmunoassay showed that they are present in higher quantities on normal and benign epithelial breast cells than on breast carcinoma epithelial cells (Ceriani et al., 1980). Separation of the HME antiserum on affinity chromatography columns showed that the antiserum is directed against three major components in the HMFG (Ceriani et al., 1980).

Another polyclonal antiserum raised against HMFG in the same way is the anti-Epithelial Membrane Antigen (EMA) antiserum (Heyderman et al., 1979). The specificity of this antiserum was determined by

identification of EMA in formalin fixed, paraffin embedded tissue using the indirect immunoperoxidase staining technique (Sloane & Ormerod, 1981). EMA was present in normal resting, lactating, benign and malignant breast tissue. In lactating and benign breast tissue and in well differentiated breast carcinoma staining was concentrated on the luminal border of the acini of breast ducts and lobules. In poorly differentiated breast carcinoma the staining was strongest in the cytoplasm of the epithelial cells, around the periphery of the cells and in intracytoplasmic luminae. All other normal epithelial tissues, except for squamous epithelium and the proximal convoluted tubule of the kidney, stained positively. Where acini were present, staining was observed on the luminal border of the epithelial cells. The same staining pattern was observed, but with greater intensity, in non-neoplastic diseases of epithelial tissue. Normal mesothelial tissues, pleural ovarian and peritoneal, showed weak inconsistent staining of the surface membrane.

When a range of neoplastic tissues was examined, the majority of tumours stained positively with anti-EMA, including squamous carcinoma. Epithelial carcinomas which did not stain included basal cell carcinoma, malignant melanoma, hepatocellular carcinoma, adrenal cortical carcinoma and carcinoid tumours of the small intestine, thymus and lung. Of the non-epithelial neoplasms examined, 2 synovial sarcomas and a malignant mesothelioma stained positively. These results showed that EMA has a widespread but specific distribution in both normal and malignant epithelial tissues.

The difference in specificity between anti-HME and anti-EMA is interesting since they were both raised against the HMFG. The antisera could be recognising different antigenic epitopes within the HMFG molecule. Alternatively the precise specificity of antisera as determined by ELISA differs from that determined by immunohistochemical staining on tissue sections. Thompson et al. (1983), found that immunohistochemical staining was the best technique for demonstrating the tissue specificity and distribution of an antigen. It is possible that the specificity of anti-HME, as

determined by ELISA differed from that for anti-EMA established by immunohistochemical staining.

In the purification of EMA from human milk, Ormerod et al. (1983), observed that it consisted of a range of molecules with varying molecular weights. They concluded that the antigenic determinants of EMA were carried on a heterogeneous set of molecules, maybe representing the products of a much larger component of the membrane. EMA is composed of more than 50% carbohydrate, with galactose and N-acetyl glucosamine as the predominant sugars. Shimizu & Yamuchi (1982), isolated a large molecular weight protein (>300K) from the HMFG composed of more than 50% carbohydrate and with many properties similar to EMA. The 2 proteins may be analogous.

To obtain a more restricted antibody specificity, Imam & Tokes (1981), raised a polyclonal antiserum against a 70KD glycoprotein, the Secretory Epithelial Membrane Antigen (SEMA), which was purified from the HMFG. The immunohistochemical staining patterns of this antigen in normal, benign and malignant breast tissue are similar to those of EMA. The expression of SEMA in non mammary tissues has not been reported. The introduction of hybridoma technology made possible the production of monoclonal antibodies against different antigenic epitopes within the HMFG. These might have a different tissue specificity from the polyclonal antisera.

1.2.4.2. Monoclonal Antibodies Against HMFG.

The monoclonal antibodies used in this study are HMFG1 and HMFG2 (originally called 1.10.F3 and 3.14.A3 respectively), (Taylor-Papadimitriou et al., 1981), and E29 (Gatter et al., 1984). There are no publications about the nature or tissue specificity of E29. HMFG1 and HMFG2 were formed from the fusion of female BALB/c mice immunised against delipidated HMFG with the myeloma cell line P3/NS1/1-Ag4-1 (Taylor Papadimitriou et al., 1981). The precise method of production of the 2 antibodies differed slightly. For HMFG1, the mouse was immunised by 2 injections of delipidated HMFG

membranes. After fusion of the immunised spleen cells with the myeloma cell line, the hybrid cells were assayed for production of antibody against cultured milk epithelial cells. They were screened against Bristol 8 cells, a lymphoblastoid cell line, and Hum F, a human mammary fibroblast cell line. For HMFG2, the mouse was immunised by 1 injection of delipidated HMFG membranes followed by a booster injection of epithelial cells cultured from milk. After hybridisation the cells were tested for production of antibody to HMFG attached to plates. They were screened against the Bristol 8 and Hum F cell lines.

When titred against 6 ng of HMFG, HMFG1 showed a greater activity than HMFG2. Both HMFG1 and HMFG2 were shown, from ouchterlony gel reactions, to be IgG isotype. The specificity of HMFG1 and HMFG2 was first tested against established cell lines and short term cultures. Both reacted with epithelial cells cultured from human milk and with 7/8 breast cancer cell lines. The antibodies were not reactive against the breast carcinoma cell line Hs578T. Both antibodies gave a negative reaction with a large range of fibroblast lines, including those derived from the mammary gland and negative or slight reactions with 10 lymphoblastoid lines derived from B cells. They were also predominantly negative with epithelial cell lines except a pharyngeal carcinoma cell line and HeLa cells.

The specificity of the antisera was then tested on a range of formalin fixed, paraffin embedded blocks of normal and tumour tissue by the indirect immunoperoxidase staining technique (Arklie et al., 1981). As with anti-EMA, both HMFG1 and HMFG2 stained normal, lactating, benign and malignant breast tissue. Staining was present in 3/5 specimens of normal breast on the luminal cell surface and in secretions of 10-20% of the alveoli and ducts. In lactating breast the staining was more intense and uniform. Similar staining was observed in papillomas and fibroadenomas representative of benign breast disease. Both antibodies stained a range of histological types of breast carcinoma and metastases of breast carcinoma in lymph nodes. It was originally reported that only HMFG2 was present in

metastatic cells in the axillary lymph nodes. Since then the HMFG1 antigen has also been demonstrated in these metastatic deposits. Neither antibody stained sarcomas and HMFG2, but not HMFG1, stained mucoid tumours. Immunostaining with HMFG1 and HMFG2 in malignant breast tissue was generally intracellular but variable in both the pattern and intensity of staining.

The staining of non-mammary normal and malignant tissue showed a similar distribution to that of anti-EMA but was more restricted (Table 1.2). Positive staining was observed on the luminal surface of epithelial cells in exocrine glands, such that both antibodies stained sebaceous glands and salivary glands and HMFG2 stained sweat glands. There was staining on the luminal surface of other normal tissues with a secretory function: the liver, pancreas, lung and uterus. HMFG2 also stained the ductule efferentes of the epididymis. Also, as with anti-EMA, the luminal surface of cells lining the collecting ducts in the kidney stained positively. Tissues which did not stain with either HMFG1 or HMFG2 included the stomach, small intestine, large intestine, appendix, thymus, thyroid, skin, testis, fallopian tube, bladder and gall bladder. From the limited number of carcinomata studied, positive staining reactions were observed with adenocarcinoma of the lung, ovary and uterus but not of the intestinal tract, cervix, nasopharynx and liver.

Since the beginning of this study 2 more series of monoclonal antibodies have been raised against the HMFG. Foster et al. (1982a; 1982b), raised 4 monoclonal antibodies against the HMFG, called M3, M8, M18 and M24 (the M series). Hilkens et al. (1984), reported the production of a series of monoclonal antibodies directed against 9 epitopes within the HMFG (MAM series). These epitopes were named MAM1, MAM2, MAM3a, b, c, MAM5, MAM6a, b, c. The tissue specificity of these antibodies is similar to that of anti-EMA, HMFG1 and HMFG2. They recognise antigens present in a range of normal and malignant tissue, generally, but not exclusively, of epithelial origin. A comparison of the specificity of these antibodies with anti-EMA, HMFG1 and HMFG2 in a selection of normal and malignant tissues has

TABLE 1.2 The Immunohistochemical Specificity of Antibodies Raised Against HMFG in Normal and Malignant Tissue.

	ANTI-EMA	HMFG1	HMFG2	M3	M8	M18	M24	MAM1	MAM2	MAM3 a b c	MAM5	MAM6 a b c
NORMAL TISSUE												
Oesoghagus	+	nt	nt	+	−	−	+/−	−	−	− − −	−	+ + +
Stomach	+	−	−	+	+	+	+	−	−	+ + +	−	+ nt+
Small intestine	+	−	−	+	−	−	+	−	−	+ + +	−	− − −
Large intestine	+	−	−	+	+	+	+	−	−	+ + +	−	− − −
Liver	+	+	+	+	+	+	+	−	−	+ + +	−	− − −
Pancreas	+	+	+	+	+	−	+	−	−	+ + +	−	+ + +
Kidney	+	+	+	+	+	+	+	−	−	− + −	−	+ + +
Bladder	+	−	−	nt	nt	nt	nt	−	−	+ + +	−	+ + +
Uterus	+	+	+	+	+	+	−	−	−	− − −	−	+ + +
Ovary	+	nt	nt	+	−	−	−	−	−	− − −	−	+ nt+
Testis	−	−	−	+	+	+	+	−	−	− − −	−	− − −
Sebaceous Gland	+	+	+	+	+	+	−	−	−	− + +	−	+ + +
Salivary Gland	+	+	+	nt	nt	nt	nt	−	−	+ + +	−	+ + +
Sweat Gland	+	+	−	+	+	−	+	−	−	+ + +	−	+ − +
Thyroid	+	−	−	+	+	+	−	−	−	− − −	−	+ + +
Lung	+	+	+	−	+	+	−	−	−	+ + +	−	+ nt+
Skin	−	−	−	+	−	+	−	−	−	− − −	−	− − −
Spleen and Lymph Nodes	−	nt	nt	nt	nt	nt	nt	−	−	− − −	−	− − −
Activated Mesothelial Cells	+	nt	nt	nt	nt	nt	nt	−	−	− − +	−	+ + +
MALIGNANT TISSUE												
Carcinoma of G.I. Tract	+	−	−	nt	nt	nt	nt	−	−	+ + +	−	+ nt+
Carcinoma of Ovary	+	+	+	nt	nt	nt	nt	−	−	+ + +	−	+ nt+
Carcinoma of Bladder	+	nt	nt	nt	nt	nt	nt	−	−	+ ntnt	−	+ nt−
Carcinoma of Lung	+	+	+	nt	nt	nt	nt	−	−	+ + +	−	+ nt+
Sarcoma	+	−	−	nt	nt	nt	nt	−	−	− − −	−	− − −

Sources of information: Sloane & Ormerod, 1981 Arklie et al., 1981 Foster et al., 1982a 1982b, Hilkens et al., 1984.

been made in Table 1.2. Differences in results found in a comparison of immunohistochemical staining between several studies may be due to the various methods of tissue processing and staining used. Differences in tissue specificity of the monoclonal antibodies were observed, however, both between and within the studies. The monoclonal antibodies showed a more restricted tissue specificity than the polyclonal anti-EMA. The exception to this was that antibodies in the M series detected antigens in the testis and in skin which were not recognised by anti-EMA. The tissue specificity of each monoclonal antibody was different. The antigenic epitopes within the HMFG, as determined by monoclonal antibodies, are therefore expressed mainly in epithelial cells but each with a unique specificity in non-mammary tissues. The polyclonal anti-EMA contains antibodies recognising most but not all of these antigens.

A comparison has also been made of the staining of these antibodies in mammary tissue (Table 1.3). The general staining patterns observed are extracellular in normal and benign tissue and heterogenous with both extracellular and intracellular staining in malignant tissue. Within this framework there are differences, however, between the presence and distribution of the various antigenic epitopes in normal, benign and malignant breast tissue (Table 1.3).

The most exciting prospect of the clinical application of monoclonal antibodies is in immunodetection or immunotherapy. There are many uses for tumour-specific and tumour-associated antibodies in cancer research (Neville & Gusterson, 1985). One of these is in pathology, where the immunocytochemical detection and localisation of antigens is a valuable aid to the diagnosis of the nature and extent of malignancy.

1.3 Immunocytochemistry.

The principle of immunocytochemistry is the specific detection of antigens in cells or tissue by antibodies which are labelled so that they can be visualised by microscopy. There are a variety of labels

TABLE 1.3 A Comparison of the Immunohistochemical Staining Characteristics of Antibodies Against HMFG in Normal, Benign and Malignant Breast Tissue. ( ) = number of tumours staining

Reference	Antibody	Immunohistochemical Staining Characteristics Observed in Breast Tissue Histologically Diagnosed as Normal	Benign	Malignant
	A (General HMFG)	Extracellular in the apical surface of cells lining and in secretions within lumena of ducts and tubules	Similar to normal tissue but greater in extent and intensity	Heterogenous, with both extracellular and intracellular staining
Sloane & Ormerod, 1981	Anti- EMA (Polyclonal)	As for A but with some intracellular staining	As for A but with some intracellular staining	As for A.
Arklie et al., 1981	HMFG1	As for A	As for A	As for A (32/32)
	HMFG2	As for A	As for A	As for A (29/32)
Foster et al., 1982 a, b	M3	As for A but also stains interepithelial membranes and myoepithelial cells.	None tested (nt)	As for A with intracellular staining predominant (26/33)
	M8	As for A	nt	As for A with extracellular staining predominant (31/33)
	M18	As for A	nt	As for A (4/33)
	M24	As for A	nt	No extracellular staining observed (19/33)
Hilkens et al., 1984	MAM 1	As for A	No staining	No staining
	MAM 2	As for A in lactating breast only	No staining	No staining
	MAM 3a; b; c	As for A	As for A	As for A (36/71; 11/18; 4/18)
	MAM 5	As for A in lactating breast only	No staining	No staining
	MAM 6a; b; c	As for A	"	As for A with extracellular staining predominant (49/50; nt; 4/18)

and immunocytochemical methods so that the technique is flexible and can be appropriately applied on each occasion.

1.3.1. Variety of Labels Used in Immunocytochemistry.

Coons et al. (1941; 1942), were the first to report the immunocytochemical detection of antigens in cells and tissues. They used antibodies labelled with fluorescent molecules. The most widely used fluorescent labels are fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) which fluoresce green and red respectively in ultraviolet light (Riggs et al., 1958). The fluorescence of these labels is readily distinguished from the blue autofluorescence of nucleic acids, tyrosine and tryptophane in cells and tissues. Because they fluoresce in contrasting colours, FITC and TRITC labelled antibodies can be used to detect 2 different antigens in a cell or tissue preparation (Sternberger, 1979a). There are several disadvantages in immunofluorescence such as the temporary nature of fluorescent staining; the requirement of an ultra-violet microscope to observe the staining and the restriction of the technique to frozen or to specially treated tissues (Petts & Roitt, 1971).

These disadvantages are overcome when enzymes are used to label the antibodies. The enzymes take part in a chemical reaction resulting in a coloured reaction product which is visible by light microscopy. There are a few enzymes which act in conditions suitable for immunocytochemistry but the 2 enzymes mostly used are horse-radish peroxidase (HPO) and alkaline phosphatase (Nakane & Pierce, 1967; Sternberger, 1979b).

Each enzyme can react with a variety of substrates resulting in different coloured reaction products. Peroxidase, for example, reacts with 3´3´ Diaminobenzidine tetrahydrochloride (DAB) to give a brown reaction product; 3 amino 9-ethylcarbazole (3A9E) to give a red reaction product; and p-phenylenediamine and pyrocatechol (Hanker-Yates reagent) to give a blue-black reaction product (Graham

& Karnovsky, 1966; Graham et al., 1965; Hanker et al., 1977). Using chromogens which result in reaction products of contrasting colours, antibodies labelled with different enzymes can be used to detect 2 antigens simultaneously (Mason & Sammons, 1978).

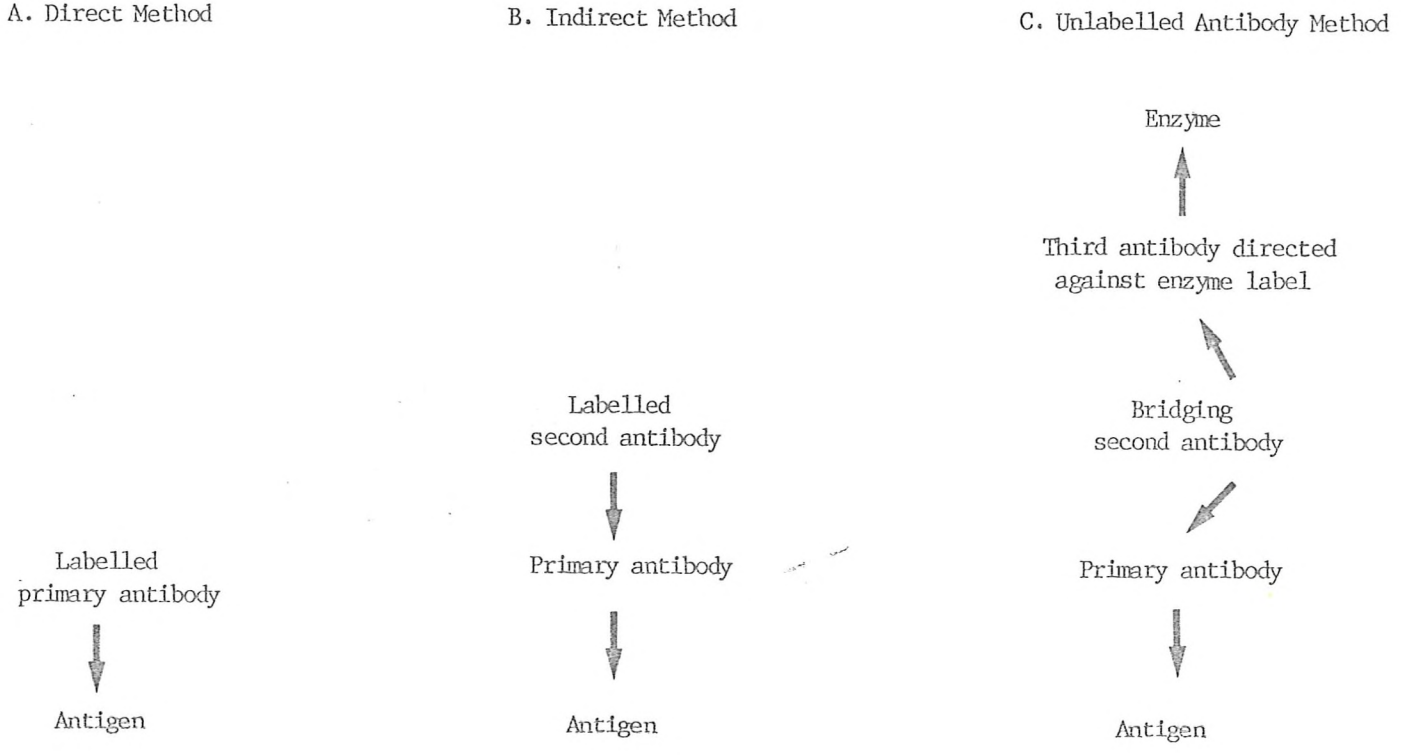
A disadvantage of enzyme immunocytochemistry is that the substrate will also react chemically with endogenous enzyme in the cells and tissue. This effect can be minimised by using an appropriate enzyme for the tissue used. For example, in formalin fixed, paraffin wax embedded tissue where endogenous peroxidase is a problem, alkaline phosphatase can be used as a label as this enzyme is mostly destroyed by tissue fixation and processing. Alternatively the endogenous enzyme can be inhibited prior to staining or developed with a chromogen which results in a contrasting colour to that of the immunocytochemical staining (Sternberger, 1979b).

The reaction product of DAB and Hanker-Yates reagent can be made electron dense by treatment with osmium tetroxide or nickel chloride for use in ultrastructural studies. Other suitable labels for electron microscopy are ferritin and colloidal gold (Singer & Schick, 1961; Faulk & Taylor, 1971).

1.3.2. Various Methods Used in Immunocytochemistry.

The flexibility achieved with a variety of labels is further enhanced by a choice of several immunocytochemical methods. The simplest immunocytochemical procedure is the 'direct' method (FIG 1.3A) where the antigen is located by labelled antibodies. The disadvantage of this method is that all of the antibodies of different specificities must be labelled. This is overcome by using an 'indirect' method (FIG 1.3B) where the primary antibody is used to locate the antigen and then acts as an antigen for a second labelled antibody. The second antibody is directed against immunoglobulins from a particular species of animal and can be used to detect all of the primary antibodies raised in that species (Weller & Coons, 1954; Mellors et al., 1955). The indirect method also increases the sensitivity of the

FIG 1.3 Variety of Immunocytochemical Methods



immunocytochemical technique because more than one labelled second antibody may bind to one primary antibody. The sensitivity can be further increased by the use of strept-avidin and biotin. In this method a second biotin labelled antibody is followed by strept-avidin. This is multivalent and binds both to a biotin molecule conjugated to the second antibody and to several other peroxidase conjugated biotin molecules. Large numbers of enzyme molecules are thereby bound to each antigen (Hsu et al., 1981).

In all procedures where an antibody is labelled, background staining will result from the attachment of the antibody to molecules other than the specific antigen. ´Immunologic´ background staining occurs most frequently when labelled polyclonal antisera are used. These contain antibodies with a range of specificities. Some are directed against the specific antigen, others will immunologically bind to different molecules in the cells and tissue. This type of background staining is reduced if the polyclonal antiserum is purified or if monoclonal antibodies are used. In these cases immunologic background staining will occur only when the antibodies directed against the specific antigen cross react with other molecules in the cells and tissue.

´Methodologic´ background staining results from the non-specific adsorption of proteins to cell and tissue components by electrostatic and hydrophobic bonds (Mayersbach, 1967; Sternberger, 1979b). This can be reduced by titration so that there is sufficient antibody only for the specific binding sites (Pearse, 1980a). Also non-immune sera can be used to inhibit this type of background staining. Non-immune sera from the same species of animal as the antibody competes with the antibody for the non-specific binding sites. Non-immune serum from the same species of animal as the cell or tissue preparation competes with the non-specific binding sites for the antibody (Sternberger, 1979b).

In order to eliminate the problems of background staining associated with labelled antibodies, the unlabelled antibody method was

developed (FIG 1.3C), (Sternberger, 1969; Cordell et al., 1984). In this method the primary antibody is used to locate the antigen. A bridging second antibody is then added in excess so that one reactive site binds to the primary antibody and the other binds to a third antibody. The primary and third antibody are raised in the same species of animal. The third antibody is directed against the enzyme label and these can be added sequentially or as complexes (Burns, 1975). None of the antibodies used are labelled and so non-specific attachment of the antibodies to molecules other than the specific antigen does not result in background staining.

As with the indirect method, inclusion of another layer of antibody increases the sensitivity of the technique. If greater sensitivity is required more antibody-enzyme complexes can be bound to the antigen via bridging antibodies. The sensitivity is particularly increased when a complex of HPO and anti-peroxidase antibodies (PAP) is used. These reagents form a chemical complex consisting of 2 antibodies and 3 molecules of HPO (Sternberger et al., 1970). The unlabelled antibody technique is at least 20 times more sensitive when PAP is used than when the antibody and enzyme are applied sequentially (Burns, 1975).

It is possible to use controls to check that any staining observed is a result of specific interactions between the primary antibody and the antigen. Specificity controls, where the primary antibody is replaced with antibodies of a different specificity but raised in the same species, confirm that the primary antibodies are solely binding immunologically to the specific antigen. This is also shown by a control where the primary antibody is preabsorbed with specific antigen. These controls can be disgarded once the specificity of the primary antibody has been established. Controls where the primary antibodies or other layers of the immunocytochemical methods were replaced by TBS or by non-immune serum establish whether background staining or the presence of endogenous enzyme is contributing to the staining observed. These, and controls which are known to stain positively or negatively with a particular antibody, can be included

in each experiment. An internal control is present in a series of tissue sections whereby areas of the section act as positive and negative controls.

The variety of immunocytochemical methods and labels outlined above can be appropriately applied depending on the amount of antigen within a tissue and the nature of the tissue.

1.4. Aims of This Study.

The introduction has shown that the HMFG1 and HMFG2 antigenic determinants are present on normal and benign breast epithelial cells as well as breast carcinoma cells (1.2.4.2.). They cannot, therefore, be used in the detection or treatment of breast carcinoma. There may, however, be other clinical applications for these antigens in breast cancer. As the HMFG1 and HMFG2 antigens are specific for epithelial cells, their most obvious use is in the detection and diagnosis of metastatic breast carcinoma in non-epithelial tissues. The presence of the antigens intracellularly in malignant tissue may help in the distinction between benign and undifferentiated malignant breast tumours in breast needle aspirates. Finally, the heterogenous immunohistochemical staining patterns observed in malignant tissue may be related to the nature of the tumour and used as an indication of prognosis. The aims of this study were, therefore, determined as:

i) To characterise the immunocytochemical staining patterns of the monoclonal antibodies HMFG1 and HMFG2 in tissue sections and in cytological preparations of normal, benign and malignant breast.

ii) To investigate the use in prognosis of immunocytochemical detection of HMFG1 and HMFG2 in primary and secondary breast carcinoma.

iii) To determine the use of immunocytochemical staining of HMFG1 and HMFG2 in the diagnosis of breast needle aspirates.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. Breast Material.

2.1.1. Tissue Used in Technique Control and Development.

2.1.1.1. Tissue used in experiment 3.2.2.

Five sections, approximately 10 x 10 x 2 mm in size, were cut from fresh biopsy material from 4 women undergoing surgery for breast disease in 1982. One section from each case was fixed in Carnoy Solution (2.2.1.3.), one in Bouin's Solution (2.2.1.2.), one in neutral buffered formalin (NBF) (2.2.1.1.), one in formol sublimate (2.2.1.4.) and one in formol-acetic acid (2.2.1.5.). All 5 sections of tissue were embedded in paraffin wax (2.2.1.8.). From the routine sections stained with Haematoxylin & Eosin (H&E), 2 cases were diagnosed as benign, 1 fibroadenoma and 1 cystic mastopathy, and 2 were infiltrating ductal carcinoma.

2.1.1.2. Tissue used in experiment 3.2.3.

One section, approximately 10 x 10 x 2 mm in size, was cut from fresh biopsy material from each of 10 women undergoing surgery for breast disease in 1982. The sections were rapidly frozen in liquid nitrogen and stored in nitrogen vapour. The remainder of the biopsied material was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). From the routine H&E stained sections, 3 of these cases were histologically benign, 1 diagnosed as cystic mastopathy, 1 as fibroadenoma and 1 as intraductal papilloma. Seven were histologically diagnosed as carcinoma and comprised infiltrating ductal carcinoma (6) and infiltrating mixed ductal & lobular carcinoma (1).

2.1.1.3. Tissue used in experiment 3.2.4.

Normal breast tissue removed at post-mortem and breast biopsies from 2 women undergoing surgery for breast disease in 1982 were fixed in NBF (2.2.1.1.), and embedded in paraffin wax (2.2.1.8.). From the routine H&E stained sections, one biopsy was benign and was diagnosed as cystic mastopathy. The other biopsy was malignant and diagnosed as infiltrating ductal carcinoma.

2.1.1.4. Tissue used in experiment 3.2.5.

Biopsy material from 20 women whose breast was surgically removed in 1982 was studied. The tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). Histological diagnosis described the cases as infiltrating ductal carcinoma (16), infiltrating lobular carcinoma (1), medullary carcinoma (1), mixed lobular & ductal carcinoma (1) and mixed medullary & lobular carcinoma (1).

2.1.2. Breast Tissue Used in the Investigation Relating Immunohistochemical Staining Patterns of HMFG1 and HMFG2 to Prognosis.

Biopsy material from 189 women undergoing surgery for breast disease was studied. All of the material was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). From the routine H&E stained sections, 35 of the cases were diagnosed histologically as benign and 154 as carcinoma. In 3 cases a bilateral mastectomy was performed so that 157 malignant biopsies were available for staining. Histologically normal tissue surrounding the lesion was present in 29 cases of breast carcinoma. Patients with breast cancer were of two chronological groups. Thirty seven were treated in 1974/5 and their relapse and survival data until 1982 was documented. A prospective series of 117 cases were treated in 1982/4. Two of these patients were lost to follow-up. The relapse and survival data of the remaining 115 patients was recorded for between 11 and 40 months with a mean follow-up of 24.2 months. Three patients died from causes

other than breast cancer. All of the patients in the retrospective and prospective study received additional therapy only after relapse. The oestrogen receptor (ER) status of 103 specimens was determined by Tenovus Laboratories (Cardiff) using the dextran charcoal method (Cooke et al, 1979). The lymph node status was known for 148 specimens and the tumour size for all of the specimens. The Bloom's Grade for each specimen was determined independently from the routine H&E stained sections.

The benign cases comprised the following: fibroadenoma (9), cystic mastopathy (14), duct ectasia (2), intraduct papilloma (3), adenosing sclerosis (2), epitheliosis (2), gynecomastia (1) and cystosarcoma phyllodes (2). The carcinomata were of the following types: infiltrating ductal (111), infiltrating lobular (12), mixed ductal & lobular (4), medullary (4), infiltrating comedocarcinoma (5), infiltrating cribriform (5), mucoid (3), tubular (4), mixed medullary & lobular (1), carcinoid (2) and squamous metaplasia of carcinoma (1).

2.1.3. Breast Tissue Used in the Comparison of Detection of Breast Carcinoma Metastases in the Axillary Lymph Nodes by Routine Histological Diagnosis and by Immunohistochemical Staining.

2.1.3.1. Tissue used in the pilot study.

The primary tumour and lymph nodes from 13 patients who had a Patey mastectomy carried out by surgeons from the Southampton University Surgical Unit between the years 1973 and 1982 were studied. The primary tumours from these patients were histologically diagnosed as infiltrating ductal carcinoma (10) and medullary carcinoma (3). All of the patients were histologically diagnosed as lymph node negative and yet relapsed within 6 years. The tumour size was known for all of these patients. All of the tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.).

2.1.3.2. Tissue used in the main study.

The primary breast tissue and axillary lymph nodes from 50 patients who had undergone a Patey mastectomy carried out by surgeons from the Southampton University Surgical Unit in 1983-4 were available for study. The primary tumours from these patients were histologically diagnosed as: infiltrating ductal carcinoma (38), infiltrating lobular carcinoma (7), infiltrating carcinoma with a cribriform pattern (2), mixed infiltrating ductal and lobular carcinoma (1), tubular carcinoma (1) and squamous metaplasia of carcinoma (1). The lymph node status and tumour size were recorded for all of these patients. The ER status and Bloom's grade were determined for each tumour as in 2.1.2. All of the tissue was fixed in NBF (2.2.1.2.) and embedded in paraffin wax (2.2.1.8.).

2.1.4. Tissue Used in the Investigation of Immunocytochemical Staining in Breast Needle Aspirates.

2.1.4.1. Cytocentrifuge preparations of T47D cells.

The technique controls for the study of immunocytochemical staining of aspirated cells from breast needle biopsies with HMFG1 and HMGF2 were carried out on a breast carcinoma cell line, T47D. This stains immunocytochemically with HMFG1 and HMFG2 (Taylor-Papadimitriou et al., 1981). T47D cells grow on the surface of plastic culture flasks in RPMI (1640) with L-glutamine (GIBCO) containing 10% foetal calf serum. They are grown at 37°C and 5% CO_2.

For cell preparations, the cells were removed from the culture flask surface by agitation with medium from a pipette to form a cell suspension. This was diluted to a concentration of 1×10^5 cells/ml and then cytocentrifuged at 50g for 5 minutes onto several microscope slides.

2.1.4.2. Aspirated cells and breast tissue used in experiments 5.2.1.4., 5.2.1.5. and 5.2.2.

Aspirated cells (2.3.1.) and the corresponding NBF fixed (2.2.1.1.), paraffin wax embedded (2.2.1.8.) breast tissue from 65 women who had diseased breast tissue surgically removed in 1982/3 were studied. The histological diagnosis of each case was made from the routine H&E stained section. Twenty two of the cases were benign, comprising fibroadenoma (12), cystic mastopathy (9) and papilloma, (1). Forty three of the cases were carcinoma, comprising infiltrating ductal (35), lobular (4), tubular (1), mixed ductal & lobular (2) and mixture of adenocarcinomatous & squamous features (1).

In 10 benign cases and 20 malignant cases, both direct (2.3.2.) and indirect (2.3.3.) aspirates were available for comparison in 5.2.1.4.

Material from 2 of the fibroadenoma and 2 of the infiltrating ductal carcinoma was used in experiment 5.2.1.5.

2.1.4.3. Aspirated material used in experiment 5.2.1.6.

Cells were aspirated from a suspicious breast lump in 50 women attending a breast clinic in Southampton. In each case, after smears were made from the aspirated cells for routine diagnosis, a needle wash (2.3.4.) was made. Not all of the lesions were subsequently removed and so the histological diagnosis of the complete series is not known.

2.2. Methods.

2.2.1. Fixation Methods.

2.2.1.1. 10% Neutral buffered formalin (NBF), (Bancroft, 1967).

The tissue was incubated in NBF for 12-24 hours at room temperature.

NBF:

Formaldehyde (40%)	10 ml
Distilled water	90 ml
Sodium dihydrogen phosphate (anhydrous)	350 mg
Disodium hydrogen phosphate (anhydrous)	650 mg

2.2.1.2. Bouin´s Solution (Bancroft, 1967).

The tissue was incubated in Bouin´s Solution for 6-12 hours at room temperature.

Bouin´s Solution:

Picric acid (saturated aqueous)	75 ml
Formaldehyde (40%)	25 ml
Glacial acetic acid	5 ml

2.2.1.3. Carnoy Solution (Bancroft, 1967).

The tissue was incubated in Carnoy Solution for 6-8 hours at room temperature.

Carnoy Solution:

Ethyl alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

2.2.1.4. Formol sublimate (Bancroft, 1967).

The tissue was incubated in formol sublimate for 12-24 hours at room temperature.

Formol sublimate:

Mercuric chloride (saturated aqueous)	90 ml
Formaldehyde (40%)	10 ml

2.2.1.5. Formol-acetic acid (Curran & Gregory, 1980).

The tissue was incubated in formol-acetic acid for 12-24 hours at room temperature.

Formol-acetic acid:

Glacial acetic acid	2.5 ml
Formaldehyde (10%)	97.5 ml

2.2.1.6. Frozen tissue (Judd & Britten, 1982).

Fresh tissue was rapidly frozen in liquid nitrogen and stored in nitrogen vapour. Six μm sections of the tissue were cut on a Slee rotary cryostat and stored over silica gel at -20^{o}C. Just before staining, the sections were brought to room temperature and fixed in dehydrated acetone at room temperature for 20 minutes.

2.2.1.7. Fixing and permeabilising cell preparations.

Cell preparations of either T47D cells or needle aspirated cells were stored in dehydrated acetone at -20^{o}C for up to 2 weeks. Just before staining the cell preparations were removed from the acetone, brought to room temperature and air-dried.

2.2.1.8. Embedding in paraffin wax.

Fixed tissue was taken through alcohols and chloroform to paraffin wax on an automatic processor (Tissue Tek II, Ames Company Ltd.) and embedded in paraffin wax.

2.2.2. Preparation of the Fixed Tissue for Staining.

2.2.2.1. Preparation of sections from tissue blocks

Paraffin sections were cut on a Leitz rotary microtome, floated out on water at 45-50^{o} and mounted on microscope slides. They were dried by placing the slides section downwards on a ridged hotplate at 56^{o}C for 1 hour. The sections were stored overnight in an incubator at 37^{o}C.

2.2.2.2. Dewaxing and rehydrating tissue embedded in paraffin wax.

Sections of tissue embedded in paraffin wax were dewaxed by two successive immersions in xylene for 10 minutes each. They were rehydrated by immersing them successively twice in absolute alcohol and once in 70% alcohol for 30 seconds each. The mercury pigment was removed from tissue fixed in formol sublimate prior to staining. This was done by treating the sections with iodine and thiosulphate. The sections were incubated at room temperature in 0.5% iodine for 5 minutes and then washed with tap water for 1 minute. The sections were then incubated at room temperature in 2.5% sodium thiosulphate in distilled water for 1 minute and washed in tap water.

2.2.2.3. Inhibition of endogenous peroxidase (Adapted from Streefkerk, 1972).

Engogenous peroxidase was inhibited by incubating the tissue sections in freshly prepared 0.5% H_2O_2 in methanol for 10 minutes at room temperature.

2.2.2.4. Trypsin digestion of tissue sections (Mepham et al., 1979).

To standardise the trypsin activity, the tissue sections were incubated in distilled water at 37^{o}C for 10 minutes. Then the sections were transferred to a solution of 0.1% trypsin in 0.1%

calcium chloride (adjusted to pH 7.8 with 0.1M NaOH) at 37°C for 10 minutes. The sections were washed in tap water for 5 minutes. (The optimum incubation time should be determined for each new batch of trypsin used)

2.2.3. Staining Techniques.

2.2.3.1. Indirect immunoperoxidase (IP) technique in tissue sections and cell preparations (Adapted from Burns, 1975).

All of the incubations and washes were carried out at room temperature on racks in humid chambers. In all of the washes the paraffin sections were washed for 10 minutes and the frozen sections and cell preparations for 2 minutes. The washes were made with Tris buffered saline (TBS) at pH 7.6 (2.2.5.1.) and then drained of excess fluid. All of the antibodies were diluted to the predetermined titre in TBS at pH 7.6. The sections were washed twice and 100 µl of primary antibody was applied to each tissue section and incubated for 30 minutes. After 3 washes, the sections were incubated with 100 µl of peroxidase-conjugated second antibody for 30 minutes. After 3 washes, the sections were incubated with 200µl of DAB (2.2.5.2.) for 10 minutes. The sections were washed briefly in TBS at pH 7.6, then for 5 minutes in running tap water and counterstained in Haematoxylin (2.2.3.3.). Then the sections were successively immersed in 70% alcohol, absolute alcohol (x2) and xylene (x2) for 2 minutes each and mounted in DPX.

2.2.3.2. Unlabelled immunoalkaline phosphatase (IAP) technique in tissue sections (adapted from Cordell et al., 1984).

All of the incubations and washes were carried out at room temperature on racks in humid chambers. In all of the washes the paraffin sections were washed for 10 minutes and the frozen sections were washed for 2 minutes. The washes were made with TBS at pH 7.6 (2.2.5.1.) and then drained to remove all excess fluid. All of the antibodies were diluted to the predetermined titre in TBS pH 7.6. The

sections were washed twice and 100 µl of primary antibody was applied to each tissue section and incubated for 30 minutes. After 3 washes the sections were incubated with 100 µl of a second bridging antibody. A further 3 washes were followed by incubation of the sections with 100 µl of mouse anti-alkaline phosphatase/alkaline phosphatase conjugate (MAAPAP) (10 mg alkaline phosphatase : 1000 µl mouse anti-alkaline phosphatase). The sections were washed x3. For double- and triple-bridge enhancing, the sequence of incubating the sections with the second bridging antibody and with MAAPAP were repeated once and twice respectively, only with 10 minute incubation times. 200 µl of New Fuschin substrate (2.2.5.4.) was filtered onto each section and incubated for 30 minutes. After a brief wash in TBS and a 5 minute wash in running tap water, the sections were counterstained in Mayer´s Haematoxylin (2.2.3.4.), and mounted in glycerin jelly.

2.2.3.3 Haematoxylin & Eosin (Culling, 1974a)

The sections were incubated in Harris´ Haematoxylin for 2-3 minutes and washed in running tap water for 5 minutes. They were differentiated by immersion in acid alcohol for 5 seconds and washed in running tap water for 10 minutes to ´blue´ them. Then they were incubated in Eosin for 5-10 minutes and washed in running tap water for 10 minutes. The sections were then immersed successively in 70% alcohol, absolute alcohol (x2) and xylene (x2) for 2 minutes each and mounted in DPX.

For Haematoxylin staining the incubation in Eosin was omitted.

2.2.3.4. Mayers Haematoxylin (Culling, 1974a).

The sections were incubated in Mayers Haematoxylin for 2-3 minutes, washed in running tap water for 10 minutes to ´blue´ them and dehydrated and mounted as in 2.2.3.3..

2.2.3.5. Methyl Green (Culling, 1974a).

Methyl Green was filtered onto each section for 45 seconds. Then the sections were washed in tap water for 5 minutes and mounted in glycerin jelly.

2.2.4. Standard Staining Procedures.

2.2.4.1. Standard procedure adopted for the investigation of the immunohistochemical staining patterns with HMFG1 and HMFG2 in breast tissue.

Fresh breast tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). Four μm thick sections were cut from each block (2.2.2.1.). The sections were dewaxed and rehydrated (2.2.2.2.), endogenous peroxidase was inhibited (2.2.2.3.) and the sections were digested in trypsin (2.2.2.4.) for 10 minutes. The indirect IP technique was carried out (2.2.3.1.) using HMFG1 and HMFG2 (gift from J. Taylor-Papadimitriou, ICRF, London but also available from Seward Laboratories) at a dilution of 1:3 as the primary antibody. The second antibody was peroxidase conjugated swine anti-mouse IgG (Dako) at a dilution of 1:50 containing Normal human serum (NHS) at a dilution of 1:50.

2.2.4.2. Standard procedure adopted for the comparison of detection of breast carcinoma metastases by immunohistochemical staining with HMFG1 and HMFG2 and by routine histological diagnosis.

Fresh lymph nodes were fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). Four μm thick sections were cut from each node (2.2.2.1.). The sections were dewaxed and rehydrated (2.2.2.2.) and digested in trypsin (2.2.2.4.) for 30 minutes. A double bridge IAP technique was carried out (2.2.3.2.), using HMFG1 and HMFG2 (gift from J. Taylor Papadimitriou, ICRF, London but also available from Seward Laboratories), both at a dilution of 1:2, E29 (gift from D. Mason, Oxford), at a dilution of 1:10 and CAM 5.2 (gift from

P.Isaacson, UCL, London) at a dilution of 1:8 as the primary antibody. The second bridging antibody was goat anti-mouse IgG (Miles-Yeda) used at a dilution of 1:200 with NHS at 1:100. MAAPAP (gift from D. Mason, Oxford but, also available from Dako) was used at a dilution of 1:8

2.2.4.3. Standard procedure adopted for the investigations of immunocytochemical staining with HMFG1 and HMFG2 in breast cell preparations.

The breast cell preparations were fixed and permeabilised for 24 hrs-2 weeks in dehydrated acetone at -20°C (2.2.1.7.). They were stained in the indirect IP technique (2.2.3.1.), using HMFG1 and HMFG2 (gift from J.Taylor-Papadimitriou, ICRF, London, but also available from Seward Laboratories), at a dilution of 1:2 as the primary antibody. The second antibody was peroxidase-conjugated swine anti-mouse IgG (DAKO), used at a dilution of 1:50, containing NHS at a dilution of 1:50.

2.2.5. Substrates and Reagents Used in the Immunocytochemical Procedures.

2.2.5.1. TRIS Buffered Saline pH 7.6 (TBS)

Sodium Chloride	80	gm
TRIS (TRIS hydroxymethyl methylamine)	6.05	gm
N Hydrochloric Acid	38	ml
Distilled water to	10,000	ml

The pH was adjusted to 7.6 if necessary.

2.2.5.2. 3, 3´ Diaminobenzidine tetrahydrochloride (DAB) (Graham & Karnovsky, 1975).

Five mgm DAB (Sigma), was added to 10 cm^3 TRIS/HCl buffer, pH 7.6*. Immediately before use 100 µl of freshly prepared 1% H_2O_2 was added.

*TRIS/HCL Buffer, pH 7.6:-

0.2 M TRIS (24.228 g/l)	12	ml
0.1 M HCl	19	ml
Distilled water	19	ml

2.2.5.3. 3 Amino 9 Ethylcarbazole (3A9E), (Graham et al., 1965)

5mgm 3A9E (Sigma) and 3cm^3 dimethyl sulphoxide were added to 25cm^3 of 0.2M acetate buffer (pH 5.0)*. Immediately before use 0.1 cm^3 of freshly prepared 0.5% H_2O_2 was added.

*0.2M acetate buffer (pH 5.0):-

0.2M acetic acid	30	ml
0.2M sodium acetate	70	ml

2.2.5.4. New Fuchsin (Cordell et al., 1984)

5 mg sodium nitrite was dissolved in 125 µl distilled water and added to 50 µl New Fuschin (Raymond A. Lamb) in 2M HCl. This was added to a freshly prepared solution of 6.75 cm^3 TRIS/HCl buffer (pH 8.7)* in 18.75 cm^3 distilled water. Finally, a freshly prepared solution of 12.5 mg sodium naphthol ASBI phosphate (Sigma) in 150 µl dimethyl formamide was added.

* TRIS/HCl buffer (pH 8.7):

0.2M TRIS	12.5	ml
0.1 M HCl	5	ml
distilled water	32.5	ml

2.2.5.5. Fast Blue.

7.5 mg of Fast Blue (Sigma) was dissolved in 10 ml of freshly prepared TRIS/HCl buffer at pH 9.0.* 3 mg of Naphthol AS phosphate (Sigma) was added.

* TRSI/HCl buffer (pH 9.0):

0.2M TRIS	12.5 ml
0.1M HCl	2.5 ml
distilled water	35.0 ml

2.3. Preparation Of Cells From Breast Needle Aspirates For Immunocytochemical Staining.

2.3.1. Aspiration of Cells from Breast Tumours.

Cells were aspirated from fresh breast biopsies immediately after surgical removal using a method identical to that used to aspirate cells from palpable breast lumps in the Southampton Breast Clinic. The plunger of a 10 ml syringe was slightly withdrawn and the needle (21g bore) was inserted into the breast tumour. The plunger was pulled out to exert a suction pressure and the needle was moved back and forth in the tumour to remove as many cells as possible. The plunger was released to its previously slightly withdrawn position and the needle removed from the tissue. The plunger was completely released to expel all the aspirated cells from the needle.

2.3.2. The Direct Aspirate.

Two drops of aspirated cells were expressed onto each of several microscope slides and smeared.

2.3.3. The Indirect Aspirate.

Aspirated cells were expressed into either 0.5 ml of sterile RPMI medium with L-Glutamine (GIBCO), or Husains mucolytic medium * in a

microtest tube. The tube was centrifuged at 50g for 10 minutes to pellet the cells, the supernatant was pipetted off and the cells were resuspended in 0.5 ml of fresh medium. This was cytocentrifuged at 50g for 5 minutes onto several microscope slides.

*Dr. Husain of Charing Cross Hospital, London, supplied the details of Husain's Mucolytic Medium:

Hanks balanced salt solution	445 ml
Absolute alcohol	50 ml
Glacial acetic acid	5 ml

2.3.4. Needle Wash.

Once the aspirated cells had been expressed onto microscope slides to make smears for routine diagnosis (2.3.2.), 0.5 ml sterile RPMI medium, was sucked up into the needle used for the aspiration. The medium, containing aspirated cells remaining in the needle, was expressed into a microtest tube which was treated as for the indirect aspirate (2.3.3.).

CHAPTER 3

TECHNIQUE CONTROL AND DEVELOPMENT

3.1. Introduction.

In any immunohistochemical study it is important to investigate the effects of tissue processing and the staining procedure on the results. The limits of accuracy of the study can then be determined.

3.1.1. Tissue Fixation.

In all histochemical studies the tissue must be treated so as to preserve the tissue morphology and, in the case of immunohistochemical staining, the nature and the distribution of the antigen. The morphological and biochemical characteristics of fresh tissue can be preserved in a variety of ways (Pearse, 1980 b,c). The tissue may be initially frozen in liquid nitrogen and the immunohistochemical staining techniques performed either directly on cryostat sections or on chemically fixed cryostat sections. Judd & Britten (1982), used a variety of chemical fixatives in a range of different conditions. They found that the optimum tissue preservation combined with demonstration of immunoglobulins occurred when the cryostat sections were fixed in acetone for 20 minutes at room temperature.

Tissue may also be preserved by chemical fixation immediately after surgery. The structure of a tissue depends mainly on the configuration of its constituent proteins such as those present in the cell membrane, the extracellular glycoproteins and the globular proteins in the cytoplasm and extracellular fluid. Chemical fixatives maintain the tissue structure either by precipitation of the proteins or by the formation of cross links between certain components of the protein (Pearse, 1980c). There are several types of both

precipitating and cross-linking fixatives, each with different properties and consequently different actions on the tissue components. The choice of fixative depends on the antigens being studied and the nature of the tissue used (Bosman et al., 1977; Brandtzaeg & Rognum, 1983). It is important to establish the best fixative for both the maintenance of the cell and tissue structure and a true representation of the antigen distribution. In this study the effects of five fixatives on the immunohistochemical localisation of HMFG1 and HMFG2 in breast tissue were investigated.

Firstly, 10% neutral buffered formalin (NBF) (2.2.1.1.), the fixative used routinely for surgical specimens in the Southampton Hospitals. Formaldehyde forms methylene cross bridges between protein end groups, namely, amino, imino, imido, peptide, guanidyl, hydroxyl, carboxyl, SH, and aromatic rings (Pearse, 1980c). The buffered saline present maintains a suitably high pH (Fox et al., 1985). Formol-acetic acid (2.2.1.5.) contains formaldehyde, but differs from NBF in two ways. Acetic acid preserves intracellular membranes and consequently cytoplasmic antigens. It also lowers the pH of the solution, which increases the formaldehyde activity (Curran & Gregory, 1980). Formol sublimate (2.2.1.4.) combines the properties of formaldehyde and mercuric chloride. Mercuric chloride is a powerful precipitant, but has the disadvantage of causing tissue shrinkage and penetrating the tissue slowly. This is balanced by formaldehyde activity (Brandtzaeg, 1982). Bouin's Solution is composed of formaldehyde, acetic acid and picric acid (2.2.1.2.). The picric acid precipitates proteins, combining with them to form picrates, and also produces intermolecular salt links. This fixative penetrates tissue rapidly and causes little shrinkage (Pearse, 1980c). The fifth fixative, Carnoy Solution (2.2.1.3), penetrates the tissue rapidly, coagulates proteins and nucleic acids and extracts lipids. Carbohydrate components are also well preserved (Kiernan, 1981).

The 5 fixatives chosen display a variety of properties, including protein precipitation and cross-linking, each with a different effect

on the tissue and antigen. A suitable fixative for use in this study should be determined from this selection.

The formation of cross-links during formalin fixation can prevent the antibodies from reaching the antigenic sites, thereby 'masking' the antigens (Kuhlmann et al., 1974). An indication that the antigen is masked by cross-linking of the surrounding proteins, rather than by irreversible antigen denaturation, is that the antigenic reactivity in a tissue treated with a cross linking fixative can be restored by proteolytic digestion (Curran & Gregory, 1977). It has been suggested (Mepham et al., 1979), that enzyme digestion allows contact between the antibody and the corresponding antigenic determinant by breaking peptide bonds.

The degree of antigenic masking depends on the number of cross-links formed during fixation. When formaldehyde is present in solution an equilibrium is formed between formaldehyde and methylene glycol. In a formaldehyde based fixative, variables such as the pH and temperature affect the equilibrium, regulating the amount of formaldehyde available for fixation and, therefore, the number of cross-links formed (Fox et al., 1985). Such variables can be controlled so that the fixation process is standardised. The number of cross-bridges formed during fixation also depends on the concentration of proteins surrounding an antigen (Brandtzeag, 1982). This variable cannot be controlled so that uneven antigen masking may occur. The effect of fixation and, if necessary, a suitable enzyme digestion time for each antigen in a particular tissue must be determined so as to optimally expose the antigen without digesting away either the antigen or the tissue. There are a choice of enzymes for proteolytic digestion but trypsin the most easily regulated (Mepham, 1982b). The effect of trypsin digestion on the immunohistochemical staining of HMFG1 and HMFG2 in breast tissue fixed in the five solutions detailed previously has been determined.

There are a variety of methods and labels which can be used in an immunocytochemical study (1.3). The appropriate staining procedure

depends on the antibodies and tissues used. The effect on staining of the different stages in the staining procedure such as the inhibition of endogenous enzyme (1.3.1), titres of the antibodies and use of non-immune serum (1.3.2) should be established. The optimum staining method for the localisation of each antigen in a particular tissue can be determined and a standard staining procedure adopted to ensure comparability within a study.

3.1.2. Aims of the Investigation.

1. To determine the effects of various fixatives and the procedures involved in an appropriate immunohistochemical staining technique on the staining patterns observed with HMFG1 and HMFG2 in breast tissue.

2. To establish a standard procedure of tissue preparation and immunohistochemical staining to ensure comparability of staining patterns in a series of breast lesions.

3.2. Experiments.

3.2.1. Determination of Suitable Titres of HMFG1 and HMFG2 and the Optimum Trypsin Digestion Time for the Control Experiments.

Four μm thick sections were cut (2.2.2.1.) from a block of NBF fixed (2.2.1.1.), paraffin wax embedded (2.2.1.8.), malignant breast tissue histologically diagnosed as infiltrating ductal carcinoma. After dewaxing and rehydration (2.2.2.2.), the sections were either left undigested or trypsin digested (2.2.2.4.) for 10, 20 or 30 minutes. Then they were stained in the indirect IP technique (2.2.3.1.), using HMFG1 and HMFG2 neat, or at a dilution of 1/10 or 1/100 as the primary antibody. Peroxidase-conjugated swine anti-mouse IgG was used as the second antibody. This antiserum had been titrated in the Southampton University Pathology Laboratories and was used at 1/50. Controls were included where TBS was used to replace the primary antibody (TBS controls).

HMFG1 and HMFG2 gave similar results in two separate titrations and are treated together in the results (Table 3.1). Faint staining of the connective tissue was observed in all of the sections, including the TBS controls. This was reduced by trypsin digestion for 10 or 20 minutes. When the antibodies were used neat, intracellular staining, often particularly intense in focal areas, was observed in many of the epithelial cells. This staining was not observed when the primary antibody was diluted to 1/10 or 1/100.

The strength and intensity of staining in the undigested tissue section was increased by 10 minutes trypsin digestion. After 20 minutes trypsin digestion, the intensity and extent of staining had decreased significantly and there was loss of clarity in the tissue morphology. After 30 minutes trypsin digestion, the tissue section had been digested away leaving faintly brown fragments of tissue for observation.

3.2.2. A Comparison of the Immunohistochemical Staining Patterns Observed With HMFG1 and HMFG2 in Breast Tissue Fixed in Five Different Ways.

Tissue from 4 patients (2.1.1.1.) was fixed in Carnoy Solution (2.2.1.3.), Bouin's Solution (2.2.1.2.), formol-acetic acid (2.2.1.5.), formol sublimate (2.2.1.4.) and NBF (2.2.1.1.). Two consecutive 4 μm thick sections for each of HMFG1 and HMFG2 and a TBS control were cut from each block (2.2.2.1.) and dewaxed and rehydrated (2.2.2.2.). One of each pair of sections was digested in trypsin (2.2.2.4.) for 10 minutes and then all of the sections were stained with neat HMFG1 or HMFG2 in the indirect IP technique (2.2.3.1).

Similar staining patterns were observed for both HMFG1 and HMFG2 in all of the differently treated sections for each case and will be presented together (Table 3.2) (FIGS 3.1; 3.2). Staining in the 2 benign cases was observed mostly extracellularly in the ducts and tubules (FIG 3.1). In malignant tissue the staining throughout the

TABLE 3.1

Determination of Suitable Titres of HMFG1 and HMFG2 and the Optimum Trypsin Digestion for the Control Experiments.

Trypsin Digestion Times	Dilutions of HMFG1 and HMFG2		
	Neat	1 : 10	1 : 100
0′	++/XXX	0/XXX	0/XXX
10′	+++/XXX	0/XXX	0/XXX
20′	+/XXX	0/XX	0/XX
30′	0/X	0/X	0/X

This table shows the results from two titrations of HMFG1 and HMFG2 carried out on sections from a block of infiltrating ductal breast carcinoma fixed in NBF and embedded in paraffin wax. The sections were digested in trypsin from a range of 0-30′ and stained with varying dilutions of HMFG1 and HMFG2 in an indirect immunoperoxidase technique.

0 - No staining
+ - Weak staining intensity
++ - Strong staining intensity
+++ - Very strong staining intensity
X - Complete digestion of tissue
XX - Overdigestion of tissue
XXX - Tissue morphology maintained

TABLE 3.2

A Comparison of the Immunohistochemical Staining Patterns Observed With HMFG1 and HMFG2 in Breast Tissue Fixed in Five Different Ways.

Fixative	Undigested Tissue		Trypsin Digested Tissue	
	Staining Characteristics	Cell and Tissue Morphology	Staining Characteristics	Cell and Tissue Morphology
Carnoy's Solution	+++/—	XX	+++/—	X
Bouin's Solution	+++/—	X	+++/-	X
Formol Sublimate	++/—	XX	+++/—	XX
Formol Acetic Acid	++/—	XXX	+++/-	XXX
NBF	+/—	XXX	++/-	XXX

This table compares the immunohistochemical staining of two benign breast lesions and two breast carcinomata fixed in five different ways and embedded in paraffin wax. Both undigested sections and sections digested in trypsin for 10' were stained with neat HMFG1 and HMFG2 in an indirect immunoperoxidase technique.

+ weak; ++ strong; +++ very strong staining intensity
- none; — weak; —— strong background staining
X poor; XX good; XXX very good cell and tissue morphology

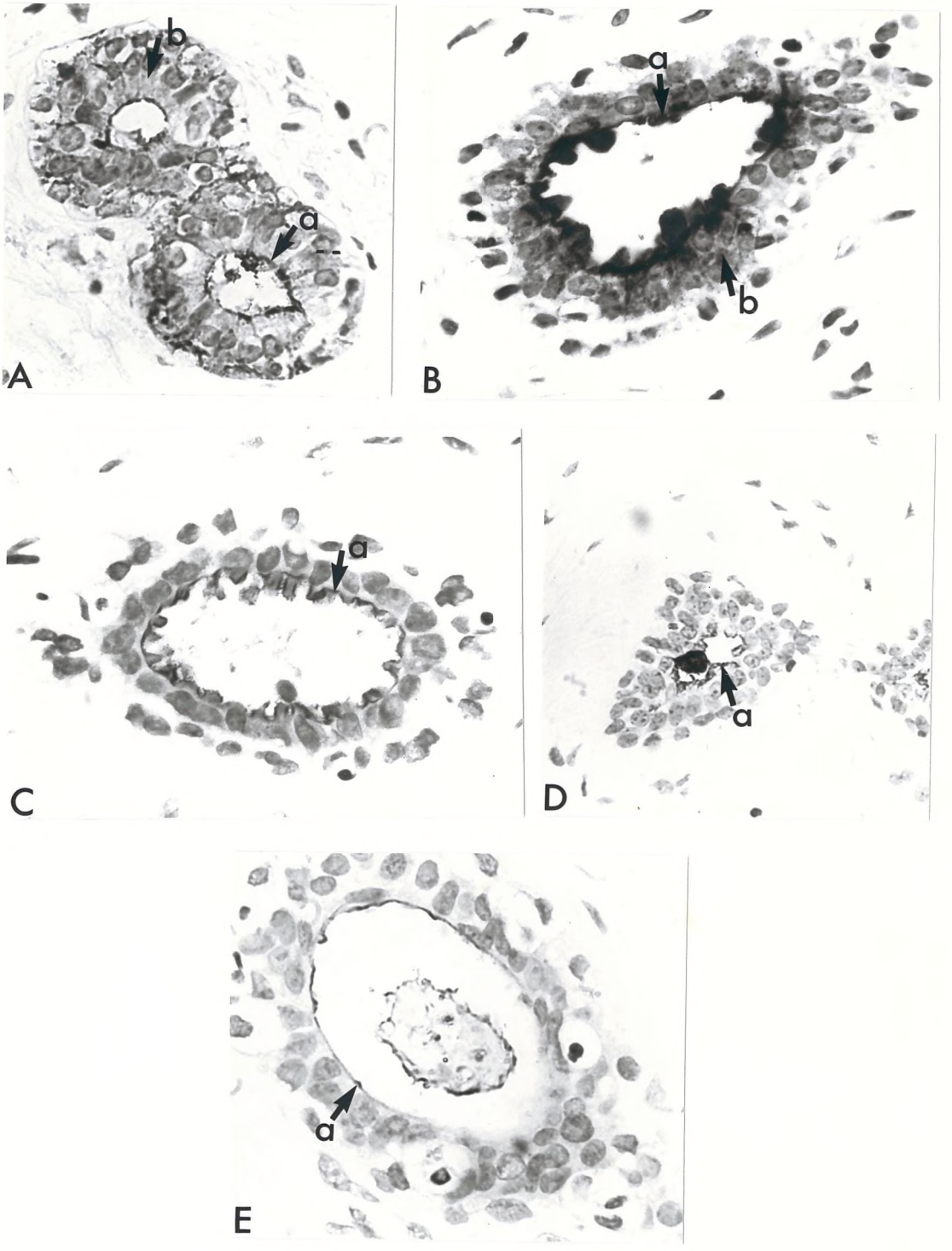


FIG 3.1

One case of histologically benign breast disease (fibroadenoma) fixed in either Carnoy Solution (A), Bouin's Solution (B), formol-acetic acid (C), formol sublimate (D), or NBF (E) and stained with HMFG1 in an indirect IP technique (3.2.2.). Magnification x450

Tissue fixed in all five fixatives shows the same extracellular staining pattern (a), although intracellular staining (b), was observed in tissues fixed in Carnoy Solution and Bouin's Solution. Tissue preservation in tissue fixed in these two solutions was inferior to that fixed in formol-acetic acid, formol sublimate and NBF.

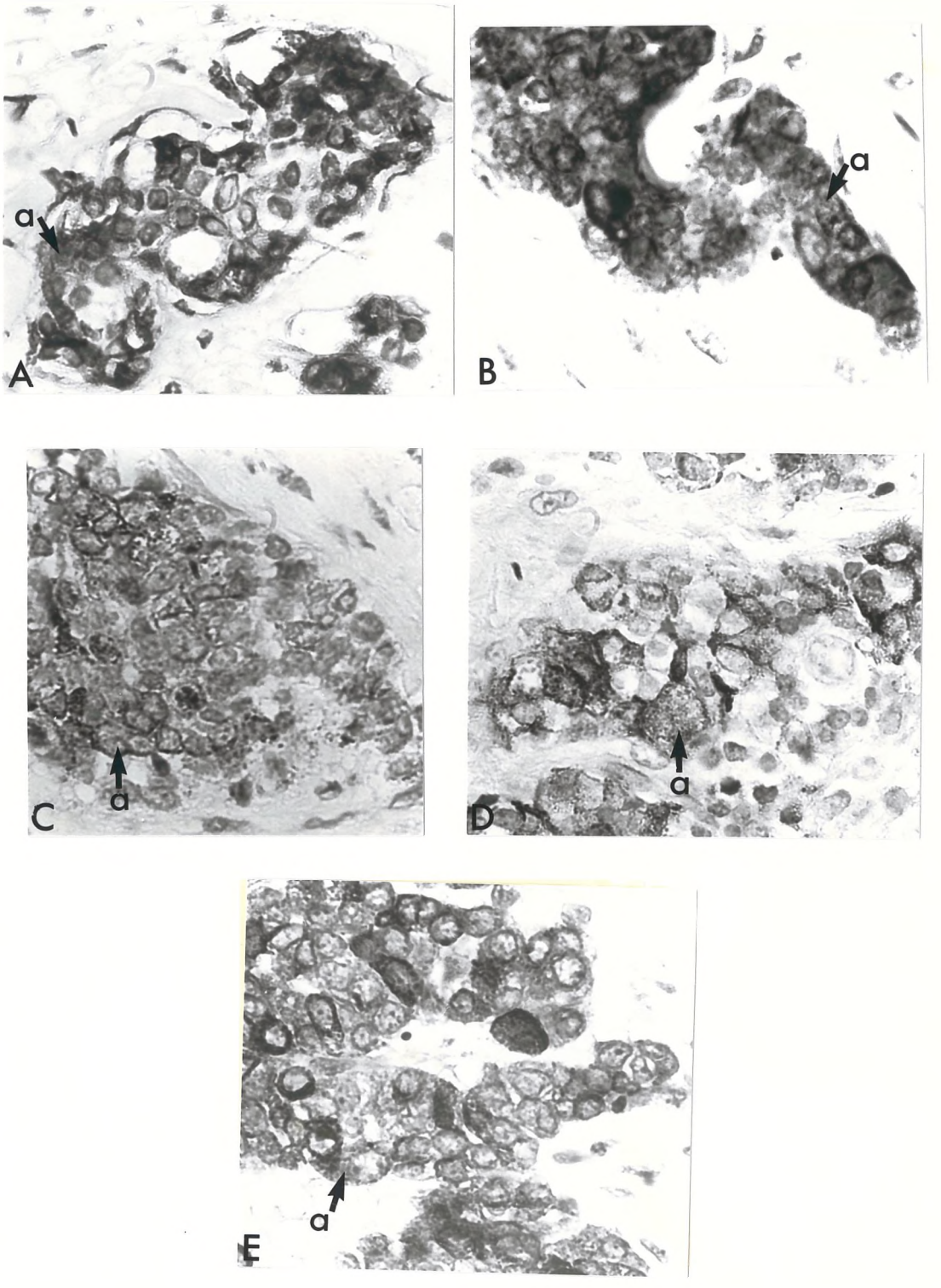


FIG 3.2

One case of infiltrating ductal carcinoma fixed in either Carnoy Solution (A), Bouin's Solution (B), formol-acetic acid (C), formol sublimate (D) or NBF (E) and stained with HMFG2 in an indirect IP technique (3.2.2.). Magnification x450.
Tissue fixed in all five fixatives shows the same intracellular staining pattern (a), but tissue fixed in Carnoy Solution and Bouin's Solution is less well preserved than that fixed in formol-acetic acid, formol sublimate and NBF. The staining pattern in the former two is consequently less distinguishable.

sections was variable but extensive intracellular staining was present (FIG 3.2).

The greatest intensity of staining of both malignant and benign tissue was observed in tissue fixed in Carnoy Solution and Bouin's Solution (FIGS 3.1; 3.2). Faint intracellular staining of the epithelial cells lining the ducts and tubules in benign tissue was observed only when these two fixatives were used. Staining in tissue fixed in formol sublimate, formol-acetic acid and NBF was less intense. Background staining of the connective tissue was present in all sections, as shown by staining in the TBS controls, but was particularly strong in the tissue fixed in formol sublimate (FIG 3.3).

The cell and tissue morphology was not well maintained in Bouins and Carnoys fixed tissue. Consequently the finer detail of staining, particularly in malignant breast tissue, was not discernible (FIGS 3.1; 3.2). The antigen distribution could be easily discerned in breast tissue fixed in formol sublimate, formal-acetic acid and NBF. In all of the Bouin's fixed tissue, 2 cases of Carnoys fixed tissue and in 1 case of formol sublimate fixed tissue the sections were over digested by trypsin (FIG 3.4). In all other cases trypsin digestion increased the intensity of staining, whilst decreasing the background staining. Cell morphology and the fine detail of staining was particularly clear in tissues fixed in formol-acetic acid or NBF and which had been digested in trypsin.

3.2.3 . A Comparison of the Staining Patterns Observed in Fixed and Unfixed Frozen Tissue With NBF Fixed, Paraffin Wax Embedded Tissue, Trypsin Digested and Undigested. Also, the Effect of Inhibiting Endogenous Peroxidase on Staining.

In 10 cases (2.1.1.2.), both NBF fixed (2.2.1.1.), paraffin wax embedded (2.2.1.8.) tissue and fresh tissue, rapidly frozen in liquid nitrogen, were available. Three consecutive sections were cut from each NBF fixed tissue block for each of HMFG1, HMFG2 and a TBS

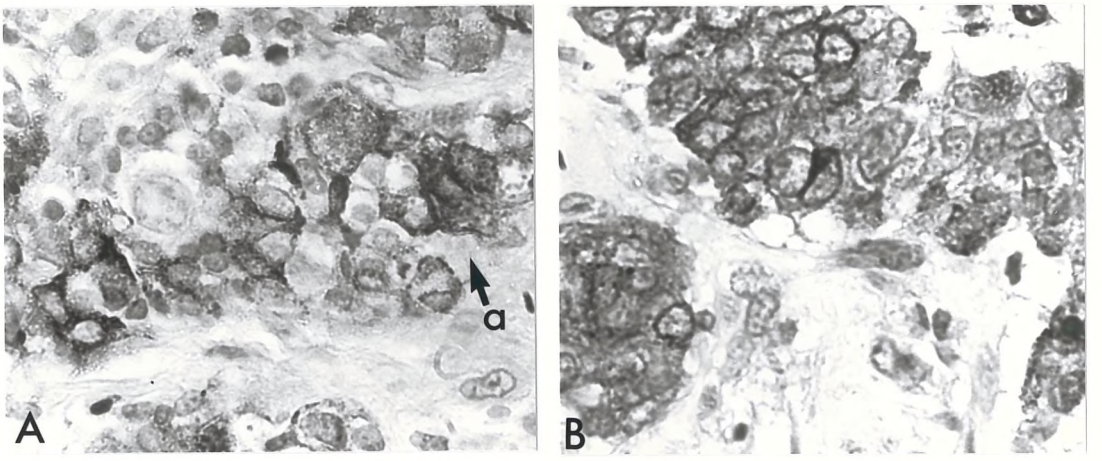


FIG 3.3

One case of infiltrating ductal carcinoma fixed in formol sublimate and stained with HMFG1 in an indirect IP technique (3.2.2.). Magnification x450.
Background staining (a), present in undigested tissue (A) is reduced in trypsin digested tissue (B).

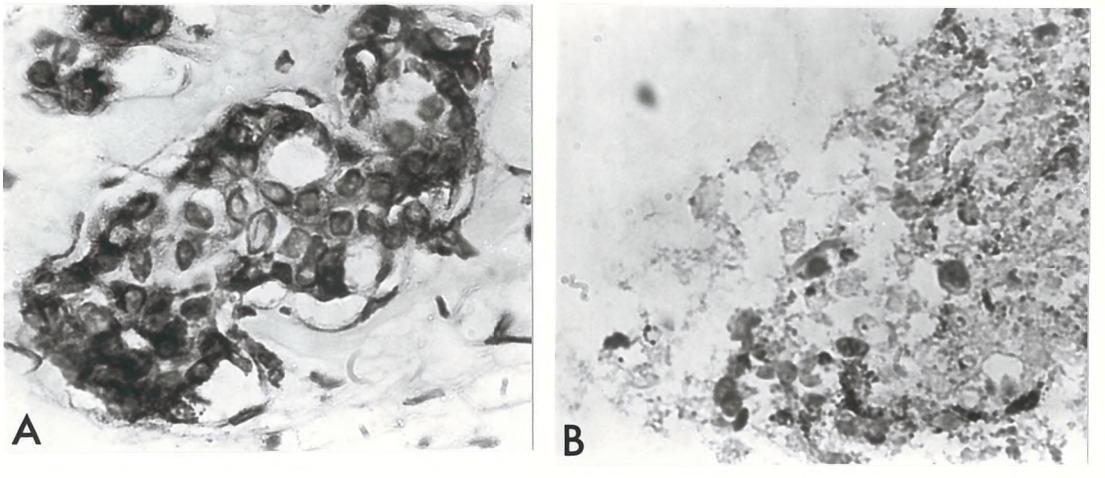


FIG 3.4

One case of infiltrating ductal carcinoma fixed in Carnoy Solution and stained with HMFG2 in an indirect IP technique (3.2.2.). Magnification x450.
Undigested tissue (A) is stained but the tissue is digested away after treatment with trypsin (B).

control (2.2.2.1.). After dewaxing and rehydration (2.2.2.2.), two were digested with trypsin (2.2.2.4.), in one of which endogenous peroxidase was inhibited (2.2.2.3.). The third section was left untreated. Two consecutive sections were cut for each of HMFG1, HMFG2 and a TBS control from each block of frozen tissue. One of these was fixed in dehydrated acetone at room temperature for 20 minutes (2.2.1.6.), the other was left unfixed. All of the sections were then stained with HMFG1 or HMFG2 in the indirect IP technique (2.2.3.1.).

The results obtained with HMFG1 and HMFG2 were similar and will be treated together (Table 3.3) (FIGS 3.6; 3.7). In all of the preparations the basic staining patterns observed were similar, that is, extracellular in the ducts and tubules of benign tissue (FIG 3.6) and variable, but with some intracellular staining in malignant tissue (FIG 3.7). Background staining was observed in the connective tissue of both the TBS controls and the test sections and was reduced by trypsin digestion in the NBF fixed tissue. The cell and tissue morphology in unfixed and fixed frozen tissue was less well preserved than in NBF fixed tissue. The distribution of stain was most easily distinguished, therefore, in NBF fixed tissue. In unfixed frozen tissue 4/10 sections were entirely destroyed and in the remaining 6 sections the cell morphology was unclear. It was more difficult, for example, to discern the presence of focal intracellular staining in frozen than in NBF fixed material. Fixation of the frozen tissue improved the preservation of cell and tissue morphology.

In 2 cases of infiltrating ductal carcinoma extensive intracellular staining was present in the frozen sections, whilst in the NBF fixed sections it was extracellular between the cells and in tubules. Pathological examination of these cases showed that the histology of the NBF fixed section of the tumour was more structurally differentiated than in the frozen sections. The histology of the 8 remaining cases was comparable. In 3 cases staining present in the frozen sections was not present in the undigested NBF fixed tissue but was present in a similar pattern in the trypsin digested NBF fixed tissue. In all other cases similar staining patterns were

TABLE 3.3

Determination of the Effect of Fixation and Preparation for Staining on the immunohistochemical Staining Patterns of HMFG1 and HMFG2 in Breast Tissue.

Tissue Treatment	Cell and Tissue Morphology	Distinction of Staining Pattern	Staining Characteristics
Frozen, Unfixed	X	*	+++/——
Frozen, Acetone Fixed	XX	**	+++/——
NBF Fixed, Paraffin Wax Embedded, Undigested	XXX	***	++/——
NBF Fixed, Paraffin Wax Embedded, Trypsin Digested	XXX	***	+++/—
NBF Fixed, Paraffin Wax Embedded, Trypsin Digested, Endogenous Peroxidase Inhibited	XXX	***	+++/—

In this experiment variously treated tissue sections (as detailed in the table) from 10 cases of benign and malignant breast tumours were stained with HMFG1 and HMFG2, neat, in an indirect immunoperoxidase technique.

X poor; XX good; XXX excellent preservation of tissue
* poor; ** good; *** very good distinction of staining pattern
+ weak; ++ strong; +++ very strong staining intensity
- none; — weak; —— strong background staining

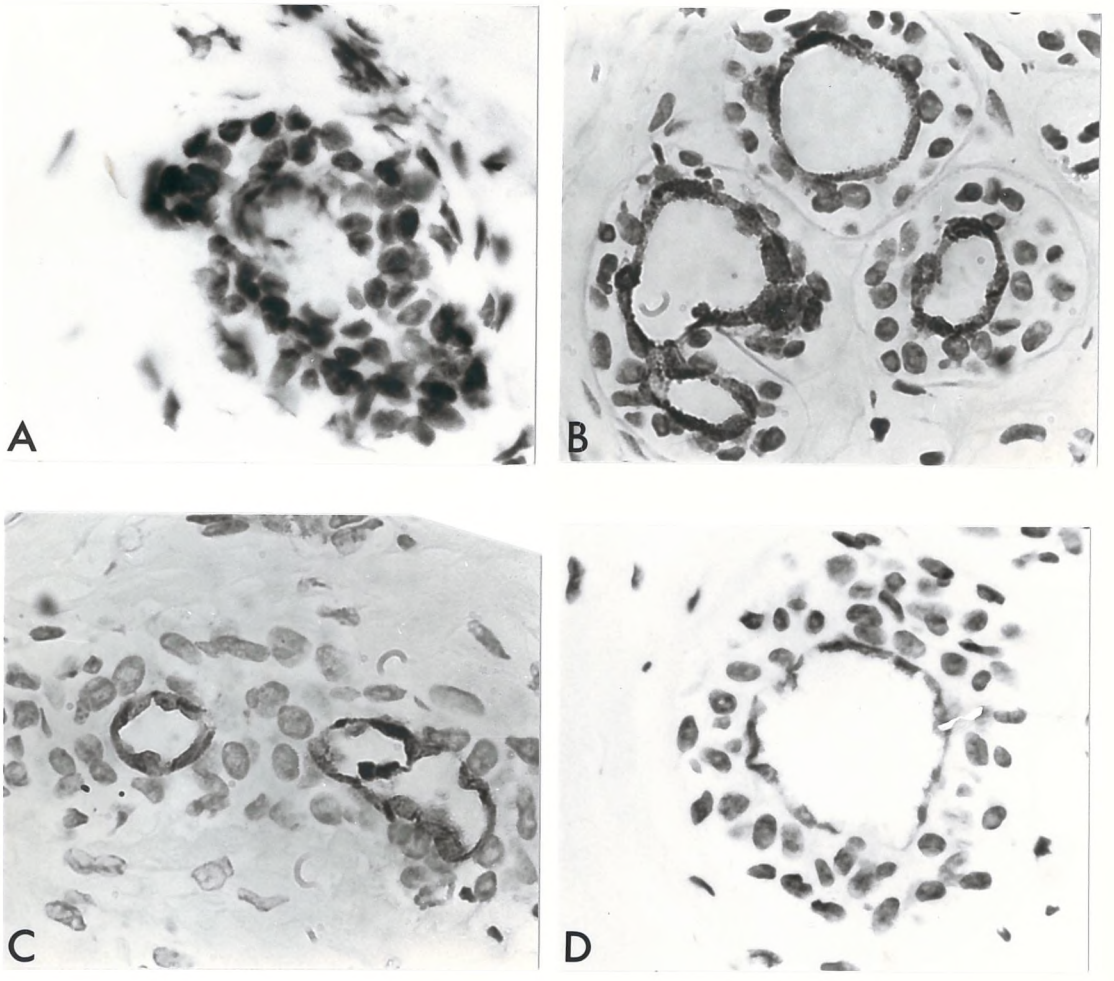


FIG 3.5

One case of histologically benign breast tissue (fibroadenoma) stained with HMFG1 in the indirect IP technique (3.2.3.). Magnification x 450.
The preservation of unfixed frozen (A) and fixed frozen (B) tissue is inferior and the staining pattern more intense and less distinguishable than in NBF fixed, paraffin wax embedded tissue (C). Trypsin digestion of NBF fixed, paraffin wax embedded tissue (D) restores the staining intensity, whilst maintaining distinguishable staining patterns

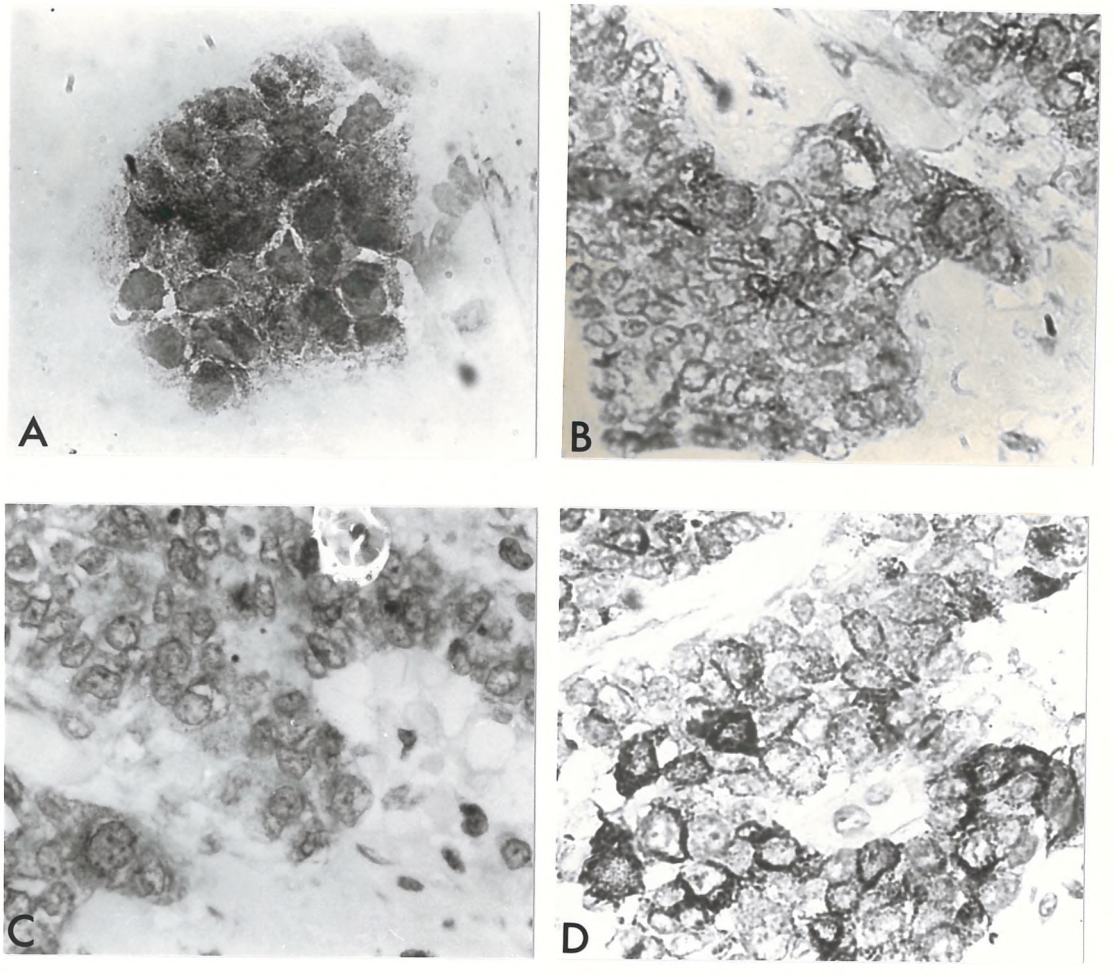


FIG 3.6

One case of histologically malignant breast tissue (Infiltrating ductal carcinoma) stained with HMFG1 in the indirect IP technique (3.2.3.). Magnification x450. The preservation of unfixed frozen (A) and fixed frozen (B) tissue is inferior and the staining pattern more intense and less distinguishable than in NBF fixed, paraffin wax embedded tissue (C). Trypsin digestion of NBF fixed, paraffin wax embedded tissue restores the staining intensity, whilst maintaining distinguishable staining patterns.

observed in frozen and NBF fixed tissue, although the staining in undigested NBF fixed tissue was generally weaker and less extensive than that observed in the other sections. Inhibition of endogenous peroxidase with H_2O_2 in methanol did not affect either the intensity or distribution of the stain.

3.2.4. Determination of the Optimum Titres of HMFG1 and HMFG2 as the Primary Antibody and Peroxidase-Conjugated Swine Anti-Mouse IgG, With NHS, as the Second Antibody Used in the Indirect IP Technique.

One block of histologically normal tissue, one block of histologically benign tissue and one block of anaplastic carcinoma tissue were sectioned for titration (2.2.2.1.). All of the tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). Four μm thick sections were cut from each tissue block (2.2.2.1.) and dewaxed and rehydrated (2.2.2.2.) before inhibition of endogenous peroxidase (2.2.2.3.) and trypsin digestion for 10 minutes (2.2.2.4.). The sections were stained in the indirect IP technique (2.2.3.1.) using various dilutions of HMFG1 or HMFG2 as the primary antibody and peroxidase-conjugated swine anti-mouse antiserum as the second antibody. A further variable was introduced by the addition of NHS at various dilutions to the second antibody to reduce methodologic background staining. To ensure comparability of staining, all of the sections were treated simultaneously. Specificity controls were included using anti-human thyroglobulin monoclonal antibodies at the current working dilution in the Southampton University Pathology Laboratories to replace HMFG1 and HMFG2. TBS controls were also included.

A preliminary titration (3.2.1.), showed that no specific staining was observed when the antibodies HMFG1 and HMFG2 were diluted to 1:10. They were, therefore, used neat and at dilutions of 1:2, 1:4 and 1:8. Peroxidase-conjugated swine anti-mouse IgG and NHS were already in use in the Southampton University Pathology Laboratories, both at dilutions of 1:50. They were therefore titrated at dilutions of 1:25, 1:50 and 1:75. The results from the 3 different cases for

both HMFG1 and HMFG2 were similar and are shown in Table 3.4.

Strong staining of the breast epithelial cells was observed when HMFG1 and HMFG2 were used neat and remained strong at a dilution of 1:2, decreasing slightly at a dilution of 1:4 and becoming very weak at a dilution of 1:8. At all dilutions of the primary antibody the staining intensity was similar when the swine anti-mouse IgG was used at dilutions of 1:25 and 1:50 but decreased at a dilution of 1:75. The NHS eliminated staining of the connective tissue in the TBS and the specificity controls at a dilution of 1:50. At this dilution staining of the connective tissue in the test sections was also eliminated.

3.2.5. Reproducibility of the Indirect IP Staining Technique.

Twenty cases of malignant breast tissue (2.1.1.4.) were used to determine the reproducibility of the standard procedure of tissue processing and staining adopted in this study (2.2.4.1). Six consecutive 4 μm thick sections were cut (2.2.2.1.) from each block for each of HMFG1, HMFG2 and the TBS control. Two of each set of sections were stained on three separate occasions. The staining of each of the sections was compared by two independent observers. Comparison of the staining was complicated by the heterogeneity of staining within a tissue section. Care was taken to compare the same region of the sections.

No staining was seen in any of the TBS controls. The staining intensity was the same in each pair of sections stained simultaneously but was different for all the sections stained on separate occasions. This was more obvious in some sections than others. The staining intensity increased uniformly throughout the section so that the staining pattern in all six sections from a case was the same. In 5 cases an increase in staining intensity resulted in weak staining in some cells where previously no stain had been observed.

TABLE 3.4

Determination of the Optimum Titres of HMFG1, HMFG2, Peroxidase-Conjugated Swine Anti-Mouse IgG and NHS for Use in the Indirect Immunoperoxidase Technique in This Study.

Dilutions of 2nd Antibody (Px conj SwaM IgG)	Dilutions of NHS	Dilutions of Primary Antibody (HMFG1 and HMFG2)			
		Neat	1 : 2	1 : 4	1 : 8
1 : 25	1 : 25	++++/0	++++/0	+++/0	+/0
	1 : 50	++++/0	++++/0	+++/0	+/0
	1 : 75	++++/-	++++/-	+++/0	+/-
1 : 50	1 : 25	++++/0	++++/0	+++/0	+/-
	1 : 50	++++/0	++++/0	+++/0	+/-
	1 : 75	++++/0	++++/0	+++/-	+/-
1 : 75	1 : 25	+++/0	+++/0	++/0	0/0
	1 : 50	+++/0	+++/0	++/0	0/0
	1 : 75	+++/0	+++/0	++/0	0/0

This table shows the results from titrations on sections of NBF fixed, paraffin wax embedded normal, benign and malignant breast tissue. After trypsin digestion and inhibition of endogenous peroxidase the sections were stained in an indirect immunoperoxidase technique with varying dilutions of HMFG1 and HMFG2 as the primary antibody and of Px conj SwaM IgG with NHS as the second antibody. The optimum titre is underlined.

0 none; + weak; ++ fairly strong; +++ strong; ++++ very strong staining intensity
0 none; - weak background staining

3.3. Discussion.

3.3.1. Preparation of the Tissue for Staining.

The effect of a fixative on the localisation of a particular antigen by immunohistochemistry depends on the mode of action of the fixative, the nature of the antigen and the location of the antigen in a tissue (3.1.1.). In this study a range of precipitating and cross-linking fixatives was selected for a comparison of their effect on the immunohistochemical demonstration of the HMFG1 and HMFG2 antigens in breast tissue (3.1.1.).

The immunohistochemical staining patterns of HMFG1 and HMFG2 in breast tissue were not affected by the fixative, but the extent and intensity of the staining was altered. In breast tissue fixed by precipitation i.e. Carnoys fixed and Bouin's fixed tissue, the staining intensity was strong but the preservation of cell and tissue morphology was poor and resolution of the staining was lost. In NBF and formol-acetic acid fixed tissue, the maintenance of morphology was good but the cross-linking had masked the antigens and staining was weaker. In formol sublimate fixed tissue the preservation of the tissue morphology was good and the staining was strong but there was considerable background staining. This reduced the clarity of the staining pattern. The extensive background staining in formol sublimate fixed tissues has been reported in other tissues (Mepham, 1982a). This is a result of the mercury conferring a negative charge on proteins in the tissue, thereby increasing the non-specific electrostatic attraction between the proteins and the antibodies (Mayersbach, 1967).

After 10 minutes' trypsin digestion, the staining in NBF, formol-acetic acid and formol sublimate fixed tissue was stronger, whilst background staining was reduced. Treatment with trypsin reduces background staining by digesting the surface of the tissue section, including the proteins which non-specifically bind the antibodies. Fixation by Bouin's and Carnoy Solutions did not

withstand trypsin digestion, for all of the Bouin's fixed tissue and 2/4 cases of the Carnoys fixed tissue were overdigested. One case of formol sublimate fixed tissue was also overdigested. The combination of strong staining, good tissue preservation and negligible background staining in NBF and formol-acetic acid fixed tissue which had been trypsin digested resulted in clarity of the staining pattern. All breast biopsies in Southampton General Hospital are routinely fixed in NBF. The advantages of using routinely processed material are two-fold. Firstly, the material is readily available for the study. Secondly, if there was a suitable application of immunohistochemical staining in pathology, it could be incorporated into the routine histological study of the tissue. NBF was, therefore, chosen as the standard fixative in the study.

Two other studies have involved an immunohistochemical investigation of HMFG1 and HMFG2. In these studies "formalin" (Arklie et al., 1981) and "buffered formalin" (Wilkinson et al., 1984) were used as fixatives. In some cases of Wilkinsons' study the tissue was post-fixed in mercuric chloride. Direct comparisons with these studies were, therefore, tentatively made. In an immunohistochemical investigation of EMA in breast tissue, Sloane & Ormerod (1981), state that the "best preservation and localisation of the antigen" was obtained using 10% formol saline. These workers also tested formol alcohol, formol sublimate and glutaraldehyde and found them "inferior". Anti-EMA is raised against HMFG (Heyderman et al., 1979) and so these results are compatible with those noted for HMFG1 and HMFG2.

Rasmussen et al. (1984), investigated the immunohistochemical detection of the MAM antigenic epitopes of the HMFG (1.2.4.2) in variously fixed malignant breast tissue. They found that the immunohistochemical staining was less intense in formalin fixed tissue than in that fixed by Lillie's acid-alcohol formalin solution. The latter fixative contains formaldehyde, glacial acetic acid and alcohol (Lillie & Fulmer, 1965) and so would have properties similar to both formol-acetic acid and Carnoy solution. These results

correspond with those in this study where the intensity of staining is greater in tissue fixed in Carnoy solution than that fixed in NBF. The maintenance of cell and tissue morphology by the formalin and by Lillie's acid-alcohol formalin solution were not compared. Rasmussen et al. (1984), also found that enzyme digestion destroyed any antigen reactivity observed in formalin fixed sections. The enzyme used, however, was pronase which is more active and consequently less easily controlled than trypsin (Mepham et al., 1979). It is possible that antigenicity was destroyed because of overdigestion by pronase.

NBF fixation, followed by trypsin digestion, resulted in apparently good antigen localisation but the staining might still be artefactual. The number of cross-links formed during fixation varies according to the concentration of proteins in the tissue surrounding the antigen. This unequal masking effect might not be rectified by uniform trypsin digestion (3.1.1). To test the accuracy of antigen localisation, the immunohistochemical staining of HMFG1 and HMFG2 in frozen breast tissue, fixed and unfixed, and in NBF fixed tissue, undigested and trypsin digested, were compared. Tissue morphology was poorly preserved in unfixed frozen tissue such that 4/10 sections were damaged. In the remaining 6 sections the staining patterns were not clear. Preservation of morphology was improved in fixed frozen tissue but was superior in NBF fixed paraffin wax embedded tissue.

Similar staining patterns were observed with both HMFG1 and HMFG2 in all of the differently treated tissue sections, showing that fixation and trypsin digestion of breast tissue does not affect the immunohistochemical staining of these antibodies. Staining in unfixed frozen tissue is more diffuse than in fixed frozen tissue, presumably due to the diffusion of unfixed antigen. In those cases where histology was comparable, staining in the NBF fixed tissue was weaker and less extensive than in the frozen tissue. This was because of antigen masking by the cross-links formed during fixation. After trypsin digestion, the staining in NBF fixed tissue was similar in intensity and distribution to that in fixed frozen tissue.

Comparable results were obtained by Rasmussen et al. (1984), who investigated the effects of fixation on the immunohistochemical staining patterns of the MAM epitopes of HMFG in malignant breast tissue. Unfixed frozen tissue was poorly preserved but showed the same staining patterns as acetone fixed frozen tissue. Staining patterns were also the same in fixed frozen and formalin fixed paraffin wax embedded malignant tissue. The staining intensity, however, was reduced in the latter. This was not restored by pronase digestion for the reasons given above.

It is impossible to know precisely the effects of fixation and processing on antigen expression because it is not possible to compare with the in vivo situation. From the results of this study, fixation in NBF and digestion by trypsin gives the most likely distribution of antigen in breast tissue. To avoid misleading results due to variation in methods, a standard procedure of tissue fixation and trypsin digestion was adopted throughout this study (2.2.4.1.).

3.3.2. Optimisation of the Staining Procedure for Accurate Antigen Localisation.

The use of an indirect immunohistochemical method (1.3.2) in the initial control experiments proved sufficiently sensitive, after trypsin digestion, to demonstrate the HMFG1 and HMFG2 antigens in NBF fixed, paraffin wax embedded breast tissue. As the components of malignant breast tissue do not exhibit much peroxidase activity, a peroxidase label was chosen, with DAB as the substrate. The reaction product of this enzyme-substrate system is brown which contrasts with the blue Haematoxylin counterstain. It is insoluble in alcohols and xylene and so can be mounted in DPX which is a superior mountant both in the clarity and permanence of the preparation (Sternberger, 1979b).

Monocytes and erythrocytes, containing myeloperoxidase and pseudoperoxidase respectively, are occasionally present in malignant

breast tissue. As these enzymes react with DAB, endogenous peroxidase was inhibited. Mepham (1982b), found that the most effective method of inhibiting endogenous peroxidase was 0.5% H_2O_2 in methanol as described by Streefkerk (1972). As there was no observable effect on the immunohistochemical demonstration of HMFG1 and HMFG2 in NBF fixed, paraffin wax embedded breast tissue using this method it was included in the standard staining procedure adopted in this study.

Inaccurate observations can result from the presence of background staining. Methodologic background staining of connective tissue in the TBS controls was reduced by trypsin digestion. This is because the trypsin digests the surface of the tissue section, removing the proteins which non-specifically attach to the antibodies (1.3.2.). Trypsin digestion also reduces background staining because exposure of the antigenic sites allows the use of a more dilute primary antibody. There is, therefore, no excess antibody to bind non-specifically to the tissue (Sternberger, 1979b). Methodologic background staining was further reduced by titration of all the antibodies used and incorporation of non-immune serum (NHS) into the second antibody (1.3.2.). The final result was that there was negligible background staining observed in the tissue sections (3.2.4.).

3.3.3. Reproducibility and Standardisation of the Staining Technique.

Once the optimum conditions for localisation of HMFG1 and HMFG2 in breast tissue by an indirect IP technique were determined, they were combined in a standard procedure used throughout the study (2.2.4.1.). For each new batch of antibody used, the original blocks of tissue used in the titrations (2.1.1.3.) were stained using this procedure to check that the staining throughout the study was comparable.

The reproducibility of immunohistochemical staining using the standard procedure was checked by comparing the staining in consecutive sections from each of 20 blocks of malignant tissue.

Pairs of sections stained simultaneously gave similar results. The distribution of stain in the cells and tissues of the sections stained on separate occasions was reproducible but the staining intensity was different. In 5 of the 20 cases an increased intensity of stain revealed weak intracellular staining in cells where there was no staining in the corresponding sections. It is not known precisely what affects staining intensity, but since the final reaction is a chemical one, conditions, such as temperature, may be involved. This limitation was recognised and little importance was attached to the staining intensity. If reliability of the staining intensity was important, sections of a control block could be included each time the sections were stained and the procedure repeated until the same staining intensity was achieved in the control. An internal control was present in each series of sections stained, where some parts of the section acted as a negative control and positive staining was always observed in at least one of the sections (1.3.2.).

3.3.4. Comments on Technique Control and Development.

There are many varieties of histological methods and the technique control and development could have been pursued further. It would have been interesting to include more fixatives for comparison of their effect on immunohistochemical staining with HMFG1 and HMFG2. Baker's formol calcium, for example, is recognised as an efficient fixative of phospholipids and, thus, of cell membranes (Pearse, 1980c). Including it in the fixation experiments may have revealed interesting locations of HMFG1 and HMFG2. It would also have been valuable to combine experiments such that a comparison of staining of HMFG1 and HMFG2 in both unfixed and fixed frozen tissue could have been compared with differently fixed tissue. Interesting information would have been obtained if the comparisons of staining in differently fixed tissue had been made at varying dilutions of HMFG1 and HMFG2 as in the fixative comparisons of Curran & Gregory (1980). Such experiments would reveal the extent of cross-linking caused by the 3 formaldehyde based fixatives and whether the background

staining observed in formol sublimate tissue was reduced at a lower titre of antiserum. There was a limitation in the control experiments, however, for the intensity was not reproducible each time the staining procedure was performed. Therefore, all of the differently treated sections in an experiment had to be stained simultaneously so that the results were comparable. The numbers of sections stained in each experiment, to be adequately stained, had to be below 75.

Despite these drawbacks, the control experiments allowed a standard method of tissue processing and staining to be established and the limits of confidence of the study determined.

CHAPTER 4

AN INVESTIGATION OF THE PROGNOSTIC VALUE OF IMMUNOHISTOCHEMICAL STAINING WITH HMFG1 AND HMFG2 IN BREAST CANCER.

4.1. Introduction.

4.1.1. The Importance of Prediction of a Patient's Survival.

Changes in the approach to the management of primary breast cancer over the years were discussed in Chapter 1. With the development of a variety of treatments it has become necessary to establish which treatment is appropriate for each patient (1.1.3.). Also, in the investigation of new and more effective treatments it is important to compare groups of patients with similar expectations of survival (Schabel, 1977). There has been considerable research into factors which might be indicative of a patient's prognosis. The results from these studies often conflict, due to inadequate numbers of patients and length of follow up or to variability in sampling techniques such as pathological examination and biochemical quantification. This investigation was based on studies involving a large number of patients, with a long follow up where the results were comparable.

The conclusions of these studies are represented by Haybittle et al. (1982), who carried out a multiple regression analysis of the relapse free survival (RFS), over 1-6 years, of 387 patients with a variety of factors. These included: age, menopausal status, tumour size, lymph node status, histological grade, cellular reaction,sinus histiocytosis and oestrogen receptor status. All of these factors had some effect on RFS, but the 4 factors which significantly affected it were lymph node status, tumour size, tumour grade and oestrogen receptor status.

4.1.2. Lymph Node Status.

The presence or absence of metastases from the primary breast cancer to the axillary lymph nodes, the lymph node status, as detected by histological examination, is the single most accurate prognostic indicator (Valagussa et al., 1978; Haybittle et al., 1982; Fisher et al., 1983). Both the presence of metastases and the number of nodes containing metastases is indicative of RFS. Valagussa et al. (1978), related the lymph node status to the relapse rate in 716 women over a 10 year period. They found that relapse occurred in 27.9% of node negative patients compared with 66.5% in patients with 1-3 nodes involved and 83.6% in patients with >3 nodes involved. Fisher et al. (1983), confirmed these results in relating disease free survival to the lymph node status in 505 patients. After 5 years follow up, 84% of patients with no node involvement were free of disease, compared with 60.1% when 1-3 nodes were involved, 41.9% when 4-6 nodes were involved, 27.7% when 7-12 nodes were involved and 16.4% when >13 nodes were involved.

The presence of metastatic deposits in the lymph nodes is an indication of the metastatic potential of the cells, that is, whether the cells are capable of entry into the circulation and invasion of target organs to establish secondary deposits. The extent of metastasis in the axillary lymph nodes is a monitor of the extent of metastasis in other target organs (Poste & Fidler, 1980; Fisher, 1980).

Lymph node status is not entirely accurate in predicting RFS. Valagussa et al. (1978), found that 28% of women with no histologically detected metastases in the axillary lymph nodes at the time of surgery relapsed after 10 years. Several factors could contribute towards this inaccuracy. Firstly, metastatic cells may have entered the circulation and invaded tissue other than the axillary lymph nodes. Breast carcinoma cells have been histologically and cytologically detected in the internal mammary nodes (Valagussa et al., 1978), in the intramammary lymphatic vessels (Nime et al.,

1977), and in the mammary blood vessels (Friedell et al., 1965) of patients whose axillary nodes were diagnosed as not containing metastatic deposits. The presence of malignant cells in all of these locations was related to poor survival.

Secondly, metastases in the axillary lymph nodes may not have been present in the sections examined or may not have been histologically detected. Several studies have shown that serial sectioning of nodes from patients revealed occult metastases in 17-33% of patients originally diagnosed as node negative (Saphir & Amromin, 1948; Pickren, 1961; Fisher et al., 1978b; Wilkinson et al., 1981). In the latter three studies the survival of those patients whose nodes contained occult metastases was no worse than in those whose nodes contained no metastases. These results may be misleading for Fisher divided 19 cases containing occult metastases into 10 cases where the malignant cells were lying "free within the peripheral or capsular lymphatic spaces" and 9 cases where the cells were present in the lymphatic tissue and spaces. All together, the 19 cases had the same survival rate as 59 cases not containing occult metastases. When analysed separately, however, none of the patients in the first sub-group had relapsed within 5 years whilst in the second sub group 5/9 patients had relapsed. The latter relapse rate (55%) was significantly higher than the relapse rate of patients with no metastases (22%). This indicates that cells in the sub-capsular sinus have a different metastatic potential to those which have invaded the lymph tissue. The other studies mentioned did not distinguish between the two types of occult metastases.

Valagussa et al. (1978), also found that 17% of women with more than 3 nodes invaded by metastases were free of disease after a 10 year follow-up. This may have resulted from malignant cells invading the lymph nodes but being unable to establish a secondary deposit or overcome the patients immunologic defence system (Fisher 1980). The effect on survival of macrometastases ($>$ 2 mm in diameter), and micrometastases ($<$ 2 mm in diameter), has been investigated (Huvos et al., 1971; Attiyeh et al., 1977; Fisher et al., 1978a; Rosen et al.,

1981). In all of these studies the survival of patients with micrometastases was intermediate between that of patients with no metastases and those with macrometastases. As with all metastases in the axillary lymph nodes, the survival of patients with micrometastases was worse in patients where the primary tumour was more aggressive as indicated by it's size and histological nature (Rosen et al., 1981). The length of follow up is important. Rosen et al. (1980), found that the survival of patients with micrometastases was significantly better than that of patients with macrometastases after 3 years but declined rapidly to be similar to patients with macrometastases after 10 years.

These results suggest that the cells of different micrometastases do not have the same metastatic potential. Cells of some micrometastases establish large secondary deposits. Cells of other micrometastases are either destroyed by the patient's defence system or are unable to carry out the processes required to establish large secondary deposits. As with occult metastases, this may be indicated by the position of micrometastases in the tissue. The metastatic potential of cells in different locations might be different. No study has detailed the effect of the position in the tissue of micrometastases on survival.

It is possible that by further distinction of the size and location of metastases in the axillary lymph nodes, the lymph node status may become a more accurate prognostic indicator but presently it must be supported by other variables.

4.1.3. Tumour Size.

The size of a tumour at surgery, as measured by the maximum diameter of the tumour, is indicative of a patient's prognosis (Goldenberg, 1961; Treves & Holleb, 1958). There is a difference in survival between women with tumours <2 cm, 2-4/5 cm, and >4/5 cm in diameter (Cutler & Myers, 1967; Elston et al., 1982; International Union Against Cancer, 1960). Fisher et al. (1969), investigated the

combined effects of tumour size and lymph node involvement on prognosis and showed that relapse rates are related to the size of the tumour only in women with evidence of lymph node involvement. This was confirmed by Valagussa et al., (1978). These results suggest that the tumour size is not an indication of the length of time the tumour has existed and, consequently, whether cells are likely to have disseminated. It is, instead, an indication of how aggressive the primary tumour was and the secondaries are likely to be (Fisher et al., 1969).

4.1.4. Differentiation Grade.

Various aspects of the histology of the primary breast cancer can be related to prognosis (Haybittle et al., 1982). Patey & Scharff (1928), devised a grading system of the epithelial elements of the tumour in which 3 histological features are assessed:-
i) The degree of structural differentiation as shown by the tubular arrangement of the cells. 1 point is awarded for a high degree of tubule formation, and 2 or 3 points are awarded as the tumour becomes progressively less structurally differentiated.
ii) Nuclear pleomorphism. 1-3 points are awarded as the nuclei become more pleomorphic.
iii) Frequency of hyperchromatic and mitotic figures. 1 point is awarded when there are no mitoses observed and the nuclei stain normally to 3 points when there are 4-5 mitoses per field of view at a magnification x100 and the nuclei are hyperchromatic.

A combination of the 3 assessments makes a total of between 3 and 9 points. The lowest degree of malignancy is represented by 3-5 points and is Grade 1, whilst 6-7 points represents an intermediate degree of malignancy and is Grade 2. The highest degree of malignancy is represented by 8-9 points and is Grade 3.

Bloom & Richardson (1957), adopted this grading system and related it to the RFS of 1409 patients at 5, 10 and 15 years follow-up. A significantly different RFS was observed in patients graded

histologically as 1, 2 or 3. Patients with Grade 1 tumours had the best survival and Grade 3 tumours the worst. This grading system has become known as the Bloom's Grade.

Another grading system devised by Black et al. (1956), is based on the tumour-host relationship and takes into account:-
i) Nuclear structure and pleomorphism.
ii) The presence of sinus histiocytosis in the lymph nodes.
iii) The extent of lymphoid infiltration into the primary tumour.
This grading system is not as widely used as Bloom's Grade. When the two grading systems were compared for their effectiveness as a prognostic indicator, Bloom's Grade was found to be more accurate (Parl & Dupont, 1982). This is because Bloom's Grade includes an assessment of the tubule formation, which is the most determinitive histological factor in prognosis.

When Bloom & Richardson (1957), related Bloom's Grade to RFS, they found that patients with Grade 2 and 3 tumours relapsed most frequently in the first 5 years and then with less frequency from 5 to 15 years, whilst patients with Grade 1 tumours relapsed with constant frequency over the 15 years. This suggests that histological grading, as with tumour size, is a measure of the aggressiveness of the breast cancer secondaries. It is also a measure of the metastatic potential of the cells in the primary tumour for both Bloom & Richardson (1957) and Haagensen (1933), noted that at 5 and 10 year follow-up, lymph node negative patients with Bloom's Grade 2 or 3 tumours had a significantly worse RFS than lymph node positive patients with Bloom's Grade 1 tumours.

As with other prognostic indicators Bloom's Grade is not entirely accurate in it's predictions. One reason for this is the variation of histology within a tumour. Bloom (1950), found that sections cut from different parts of the tumour have a comparable Bloom's Grade. Haagensen (1933), however, using the same grading system, showed that in 11% of 164 sections the histology was sufficiently heterogenous as to create difficulties in grading. This problem can be overcome to

some extent by taking into account the full histological picture of the tumours and then classifying the tumours according to either the most predominant or the most malignant features. To obtain a complete histological picture of large tumours, 2-3 sections should be examined. Another source of inaccuracy is inter-observer variation. This occurs because the division between grades is an arbitrary point on a continuing increase in malignant potential. Extremes of either well differentiated or undifferentiated tumours are easily identifiable but classification of intermediate tumours is more difficult. Accuracy of Bloom's Grade assessment increases with experience (Marshall, personal communication).

Another reason why Bloom's Grade is inaccurate as a prognostic indicator is that the secondary deposits of tumour may be more or less aggressive than the primary tumour. The histological grade of metastases in the lymph nodes is identical to the primary tumour in 70-80% of cases (Bloom & Richardson, 1957; Haagensen, 1933). Thus, in 20-30% of cases, the secondary has a different histological grade than the primary and this may affect survival.

Because of the inherent problems, Bloom & Richardson (1957), propose this grading system as a guide to prognosis rather than a mathematically accurate indicator. Nevertheless, it is a valuable tool in indicating prognosis.

4.1.5. Oestrogen Receptor Status.

A high proportion of human mammary carcinomas contain specific receptors for oestrogen (ER) which are not detected in normal breast tissue or in the majority of benign lesions (Feherty et al., 1971; Terenius et al., 1974). There have been a large number of studies on the presence or absence of ER (ER status) in breast cancer, with varying results. This is due to both the number of methods used, with different sensitivities for the detection of ER, and tumour heterogeneity. In one study the ER status in several samples of tumour was compared (Leclercq et al., 1975). In 1/16 cases, 2 samples

of a tumour were positive and the third sample was negative. Also the value of ER varied greatly in all of the positive samples.

Despite the variation in results it is generally considered that the ER status is useful both as an indicator of the response of the patient to hormone therapy and as a prognostic indicator. Hawkins et al. (1980), collated the results of studies relating the ER status to different types of endocrine therapy in 1180 patients treated in several centres throughout the world. They found that 54% of patients with ER positive tumours responded to various modalities of endocrine therapy compared with 6.8% of patients with receptor negative tumours. The reason that ER status is not totally accurate in it's indication of response to therapy may be partially due to variation in the methods used to quantify ER as described above. It is also suggested that there is some interaction of ER with other receptors, such as progesterone receptors, which may affect the response (Hawkins et al., 1980).

There has been controversy over the use of ER status as a prognostic indicator. Howat et al. (1983), found that after 34 months there was no significant difference in the RFS of 157 patients with ER positive or ER negative tumours. The majority of investigators, however, found that survival was significantly better in patients whose primary tumour was ER+ than in those whose primary tumour was ER- (Knight et al., 1977; Rich et al., 1978; Cooke et al., 1979). Bishop et al. (1979), found that the ER status affected survival in this way only in post-menopausal women.

Why the ER status should predict prognosis has been a subject of debate. It has been suggested that as ER are found on normal, differentiated tissues, ER status is a measure of the differentiation of the tissue (Rich et al., 1978). Studies associating ER status with the histological grade of the tumour have shown varying results due to variability of both the methods used to assay ER and inter-observer variation in histological grading (Poulsen et al., 1982). It is generally agreed that, although the relationship is not

statistically significant, Bloom's Grade 3 tumours are predominantly ER negative and Bloom's Grade 1 tumours are predominantly ER positive (Terenius et al., 1974; Cooke, 1982; Howat et al, 1983). Cooke (1982), related the value of ER content in primary breast tumours to the tumour labelling index (TLI), a measure of cell growth and division. He found that there is an inverse relationship between the ER value and the TLI, such that ER negative tumours had a very high TLI whilst ER positive tumours had a lower one. Similar results were reported by Silvestrini et al. (1979). ER status and Bloom's Grade may be linked because both are partially an assessment of cell growth and division in the primary tumour.

A combination of ER status with lymph node status improves the prognostic accuracy of both factors independently (Cooke, 1982). This is particularly marked when the tumour has already metastasised, as determined by lymph node status (Knight et al., 1977; Maynard et al., 1978a). ER status is useful as a predictor of prognosis because it indicates the rate of cell growth and, therefore, the aggressiveness of the primary tumour and the secondaries.

4.1.6. Prognostic Indices Combining Independent Prognostic Factors.

The lymph node status, tumour size, histological grade and oestrogen receptor status are all of some significance in predicting prognosis. A combination of these factors might provide a more accurate prognostic index. Several studies have investigated the prognostic accuracy of classifications based on the TNM system where the determination of prognosis by tumour size (T), lymph node status (N) and the presence of distal metastases (M) are combined (Zippin, 1966; Cutler & Myers, 1967). Each of these classifications gives a more accurate prediction of survival than either lymph node status or tumour size alone.

A combination of ER status with either lymph node status or histological grade also gives a more accurate prognostic index (Cooke, 1982; Maynard et al., 1978b). Combining lymph node status,

tumour size and histological grade improved the accuracy of prediction of prognosis (Thorensen et al., 1982). Haybittle et al. (1982), combined all four factors in a prognostic index which selected a group of patients with a very high risk of relapse and another group with a very low risk of relapse. Two thirds of the patients, however, were in the intermediate group between these two extremes. Combining these factors, therefore, increased the prognostic accuracy but left room for improvement and the search for new prognostic indicators continues.

4.1.7. The Use of Immunohistochemical Staining as a Prognostic Indicator.

The use of immunohistochemical staining in primary and secondary breast cancer as an indication of patient survival has been investigated in this study. In the primary tumour, immunohistochemical staining was used to assess the cell and tissue distribution of the antigens. Malignant cells differ from normal cells in their nuclear activity and consequently in their cellular functions (Cairns, 1978). These changes are associated with altered composition or distribution of the cell constituents and variations in such alterations might be an indication of the metastatic potential of the cells. Immunohistochemical staining can be used to investigate the variation of the presence and distribution of antigens.

The tissue specificity of the monoclonal antibodies HMFG1 and HMFG2 and their distribution in normal, benign and malignant tissue has been described in detail previously (1.3.2.). The antigens are present extracellularly in the ducts and tubules in normal and benign tissue and distributed heterogeneously, both extracellularly and intracellularly, in malignant tissue. In this study the various immunohistochemical staining patterns of HMFG1 and HMFG2 in primary breast cancer have been related to prognosis.

Another application of immunohistochemical staining is in secondary

deposits of breast carcinoma in the axillary lymph nodes. Valagussa et al. (1978), showed that 28% of women with no histologically detected metastases relapse within 5 years of surgery. One reason for this may be that metastases present in the lymph nodes are not detected by routine histological examination. Immunohistochemical staining of antigens present in breast carcinoma cells, but not cells of the lympho-reticular system, might reveal previously undetected metastases in the axillary lymph nodes. The original characterisation of HMFG1, HMFG2 and E29 antigens showed that they are specifically found on cells of epithelial origin and not cells of lympho-reticular origin (1.2.5.2.). More recently, Delsol et al, (1984) have shown that HMFG antigens are present on plasma cells present in the axillary lymph nodes. Although plasma cells are morphologically distinguishable from breast carcinoma cells, the staining by the monoclonal antibodies against HMFG was confirmed by using a monoclonal antibody against a cytokeratin antigen.

Cytokeratins are intermediate filaments found specifically in epithelial cells (Moll et al., 1982). CAM 5.2 is a monoclonal antibody which recognises a low molecular weight intracellular cytokeratin present in secretory epithelia (Makin et al., 1984). It was included in this study to confirm that staining by HMFG1, HMFG2 and E29 was specifically of breast carcinoma cells. The application of immunohistochemical staining techniques as tools of prognosis in primary and secondary breast cancer was investigated in this study.

4.1.8. Aims of the Investigation.

4.1.8.A - In the Primary Tumour:-

1. To characterise the immunohistochemical staining patterns of HMFG1 and HMFG2 in normal, benign and malignant breast tissue.
2. To classify the immunohistochemical staining patterns of HMFG1 and HMFG2 in malignant breast tissue.
3. To relate the classified staining patterns to RFS and to existing prognostic indicators, namely, the lymph node status, tumour size,

Bloom's Grade and ER status.

4.1.8.B - In the Secondary Tumour:-

1. To determine the tissue preparation and the staining conditions for the optimum detection of breast carcinoma metastases in axillary lymph nodes.

2. To compare the detection of metastases from primary breast carcinoma to the axillary lymph nodes by routine histological examination and by immunohistological staining.

4A. An Investigation of the Prognostic Value of Immunohistochemical Staining With HMFG1 and HMFG2 in Primary Breast Cancer.

4A.2. Experiments.

4A.2.1. Characterisation of the Immunohistochemical Staining Patterns of HMFG1 and HMFG2 in Normal, Benign and Malignant Breast Tissue.

Immunohistochemical staining was carried out on 29 blocks of histologically normal breast tissue, 35 blocks of abnormal but histologically benign, tissue and 157 blocks of breast carcinoma (2.1.2.). All of the tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). Several 4 μm thick sections were cut from each block (2.2.2.1.) and stained with HMFG1 and HMFG2 in the standard IP procedure determined for this study (2.2.4.1.). A TBS control was included for each block of tissue. The sections were examined by 2 observers and the staining patterns in normal, benign and malignant tissue characterised (Table 4A.1).

The staining of both HMFG1 and HMFG2 in histologically normal tissue was extracellular. It was located both on the luminal surface of the epithelial cells and in secreted material within the lumina of the ducts and tubules (FIG 4A.1). The extent of staining was variable between cases and within a tissue block, both between lobules and between the acini in a lobule. There was no staining in some lobules and different percentages of acini staining in other lobules. When a ductule was associated with the acini in a lobule, a similarity in the extent of staining was present in both structures (FIG 4A.2). In all of the 29 cases, staining of HMFG1 was more intense than that of HMFG2.

Staining of the benign tissue was also extracellular but generally of stronger staining intensity and more extensive than in normal tissue. As in the normal tissue, staining was variable between cases and,

TABLE 4A.1

Summary of the Immunohistochemical Staining Patterns of HMFG1 and HMFG2 in Breast Tissue.

Tissue Histology		Description of Immunohistochemical Staining With HMFG1 and HMFG2
Normal	Extracellular	- Staining on the luminal surface of epithelial cells and secretions within the ducts and tubules. No intracellular staining.
	Variable	- Between patients and between lobules and acini within a tissue block.
Benign	Extracellular & Variable	- As above, but with weak intracellular staining in the epithelial cells lining ducts and acini which contained much secretion in 3/37 cases.
Malignant	7/157	- Extracellular, as normal above.
	10/157	- No staining with either HMFG1 or HMFG2
	140/157	- Intracellular with marked heterogeneity of both the extent of intracellular staining and the relative intensity of staining extracellularly and intracellularly.

These staining patterns were observed in breast tissue fixed in NBF, embedded in paraffin wax and stained in the standard indirect immunoperoxidase technique established for this study.

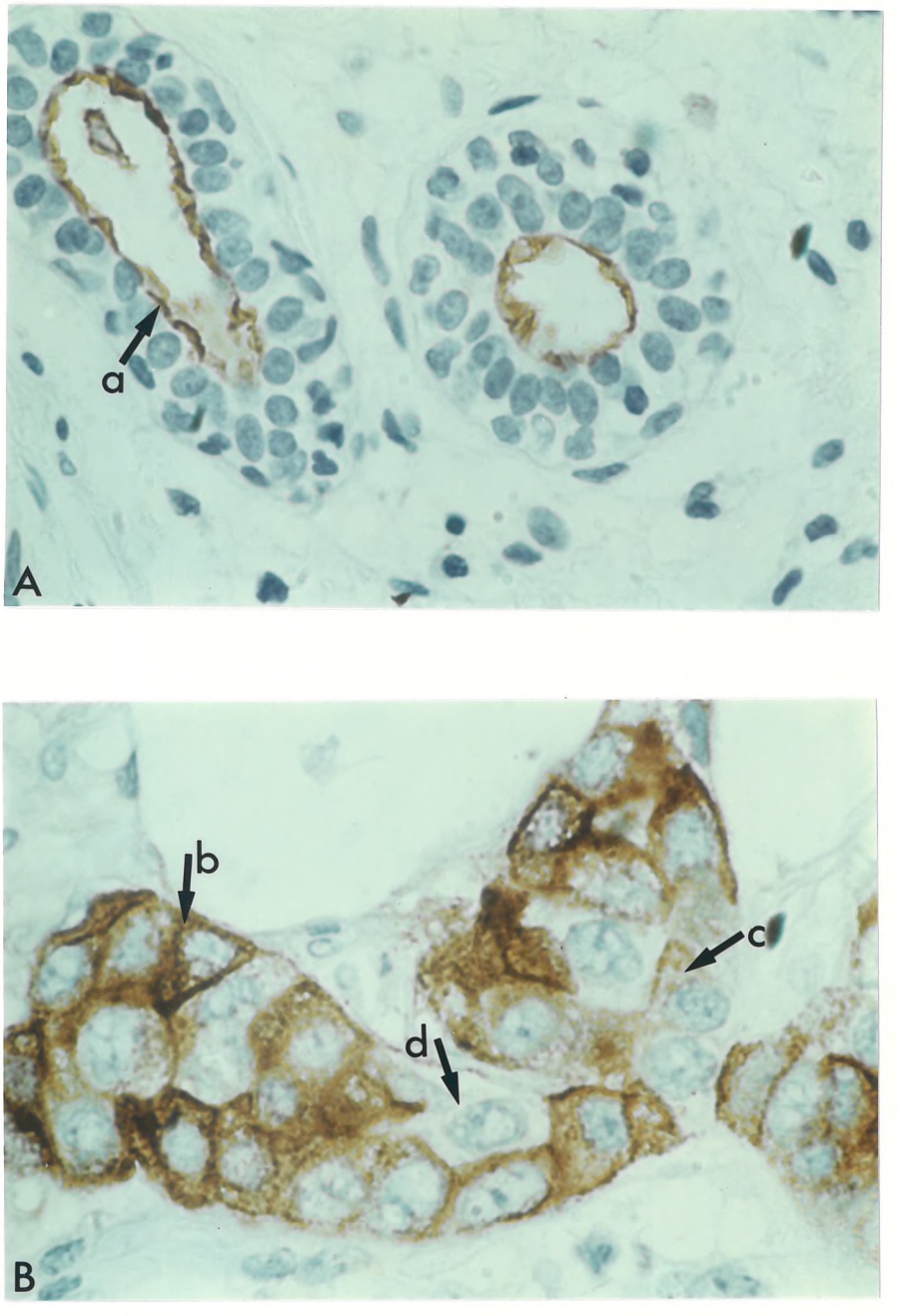


FIG 4A.1

Staining of normal (A) and malignant (infiltrating ductal carcinoma), (B) breast tissue with HMFG1 in the indirect IP technique (4A.2.1). Magnification x 660.
Staining is extracellular (a) in normal tissue. In malignant tissue staining is intracellular but heterogenous - some cells stain strongly (b), some weakly (c) and some not at all (d).

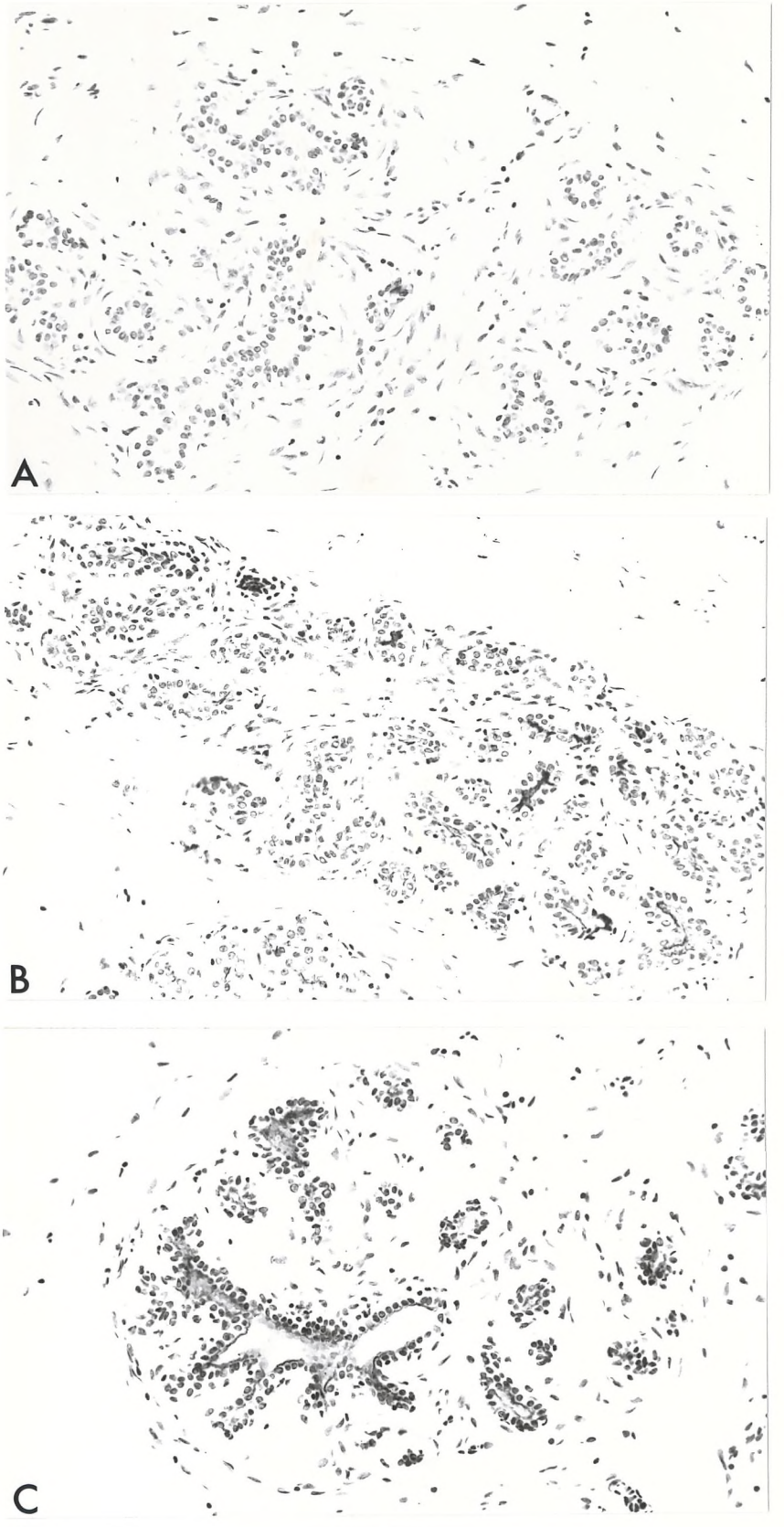


FIG 4A.2

Lobules from one section of normal breast tissue stained with HMFG1 in the indirect IP technique (4A.2.1). Magnification x225.
There is variability in staining, with no staining in lobule A, staining of some acini in lobule B and staining of all the acini in lobule C. The extent of staining is similar in the acini and the ductule in each lobule.

within a lesion, between different lobules. The particular staining characteristics of the different histological types of benign lesion are shown in Table 4A.2. Intracellular staining was observed in the epithelial cells of the ducts and acini in 2 cases of fibroadenoma and 1 case of intraduct papilloma (FIG 4A.3). Also, intracellular staining was present in the myoepithelial cells in 1 case of fibroadenoma and 1 case of cystic mastopathy. In all of these cases the staining was present only in cells of the ducts and tubules which contained particularly large amounts of secretion. The connective tissue surrounding these structures was also stained.

Apocrine metaplasia was present in 9 cases of cystic mastopathy and 1 case of fibroadenoma. The apocrine cells in these lesions were stained in a diffuse intracellular pattern (FIG 4A.4). Two cases were histologically diagnosed as phyllodes tumours. No staining was observed with either HMFG1 or HMFG2 in one of these tumours and in the other tumour extracellular staining was observed only in the section stained by HMFG1. There were 2 cases of adenosing sclerosis, 1 from a man and 1 from a woman. Extracellular staining with HMFG1 and HMFG2 was observed in both blocks of tissue and there was no difference in the staining characteristics. The staining intensity of HMFG1 was stronger than HMFG2 in all of the lesions except 1 case of cystic mastopathy and 1 case of fibroadenoma.

Staining of malignant tissue varied from case to case and within a section, between different areas (FIG 4A.5) and between adjacent cells (FIG 4A.1). HMFG1 and HMFG2 generally gave the same staining patterns although the relative intensities of the 2 antibodies varied. In 10 cases neither antibody stained the tissue. In 7 cases where there was extensive tubule formation, the staining pattern was similar to that seen in normal and benign tissue. All of the remaining 140 cases showed intracellular staining to some extent with one or both of the antibodies. In some cases the staining intensity was strongest extracellularly, either in tubules, or, where there were no tubules, in intercellular spaces and intracytoplasmic vacuoles. In other cases the staining intensity was strongest

TABLE 4A.2

The Immunohistochemical Staining Patterns of HMFG1 and HMFG2 in Benign Breast Tissue.

Tissue Histology	No. of Cases	Presence of Extracellular Staining	Presence of Intracellular Staining	Staining of Myoepithelial Cells	Staining of Apocrine Cells	Intensity of Staining HMFG1 > HMFG2	Intensity of Staining HMFG2 > HMFG1
Fibroadenoma	9	9	2	1	1	8	1
Cystic Mastopathy	14	14	0	1	9	13	1
Duct Ectasia	2	2	0	0	0	2	0
Intraduct Papilloma	3	3	1	0	0	3	0
Sclerosing Adenosis	2	2	0	0	0	2	0
Epitheliosis	2	2	0	0	0	2	0
Gynecomastia	1	1	0	0	0	1	0
Phyllodes Tumour	2	1 (HMFG1 only)	0	0	0	-	-

These staining patterns were observed in benign breast tissue fixed in NBF, embedded in paraffin wax and stained in the standard indirect immunoperoxidase technique established for this study.

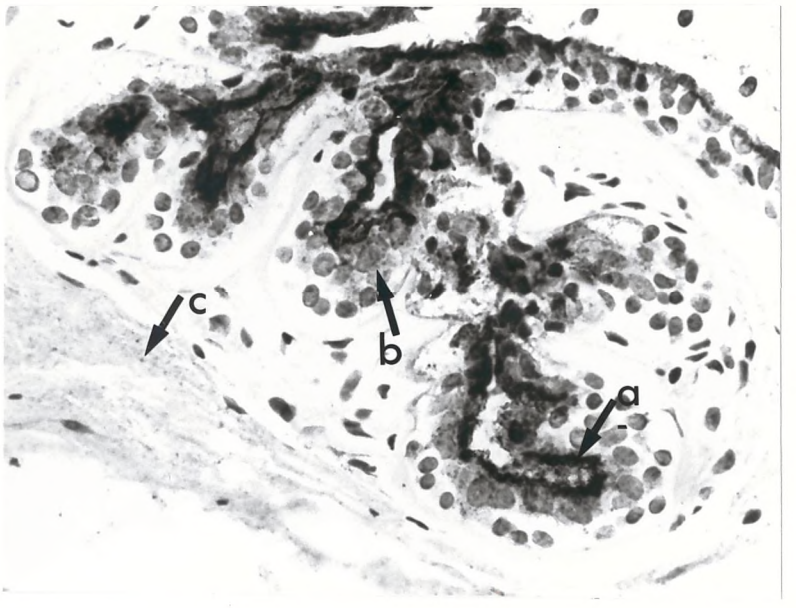


FIG 4A.3

Ductules of a section of benign breast tissue (fibroadenoma) stained with HMFG1 in the indirect IP technique (4A.2.1). Magnification x 450.
Particularly intense extracellular staining is present in the lumina (a). There is also intracellular staining in the epithelial cells (b) and staining of the connective tissue (c).

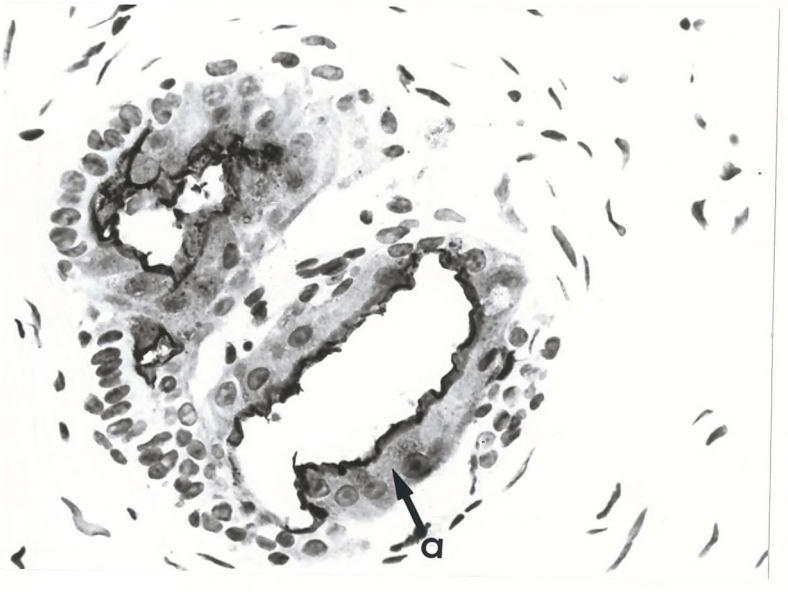


FIG 4A.4

Ductules of a section of benign breast tissue with apocrine metaplasia, stained with HMFG1 in the indirect IP technique (4A.2.1). Magnification x 450.
Diffuse intracellular staining is present in the apocrine cells (a).

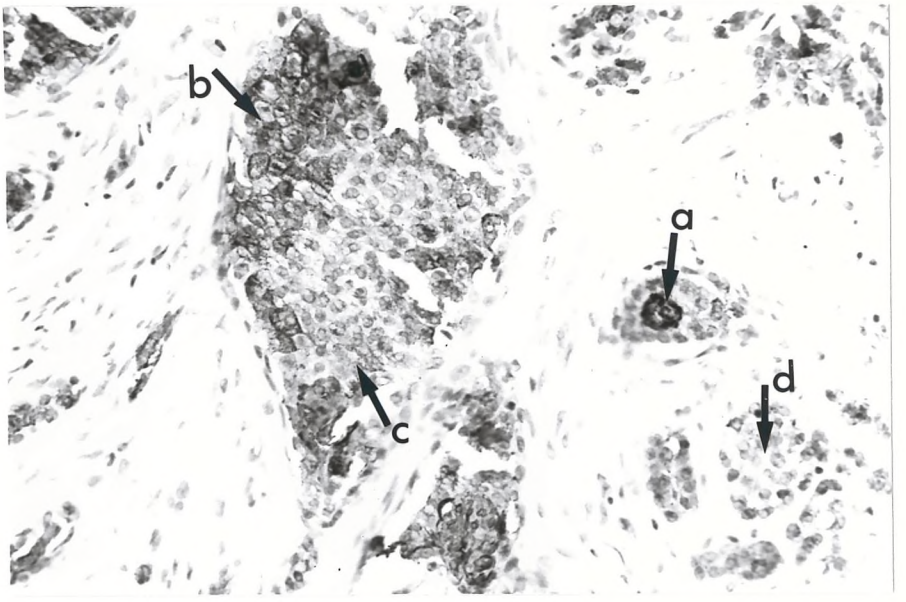


FIG 4A.5

A section of infiltrating ductal breast carcinoma stained with HMFG1 in the indirect IP technique (4A.2.1). Magnification x 225.
Staining is variable, showing extracellular staining (a), intense (b) and weak (c) intracellular staining and no staining (d).

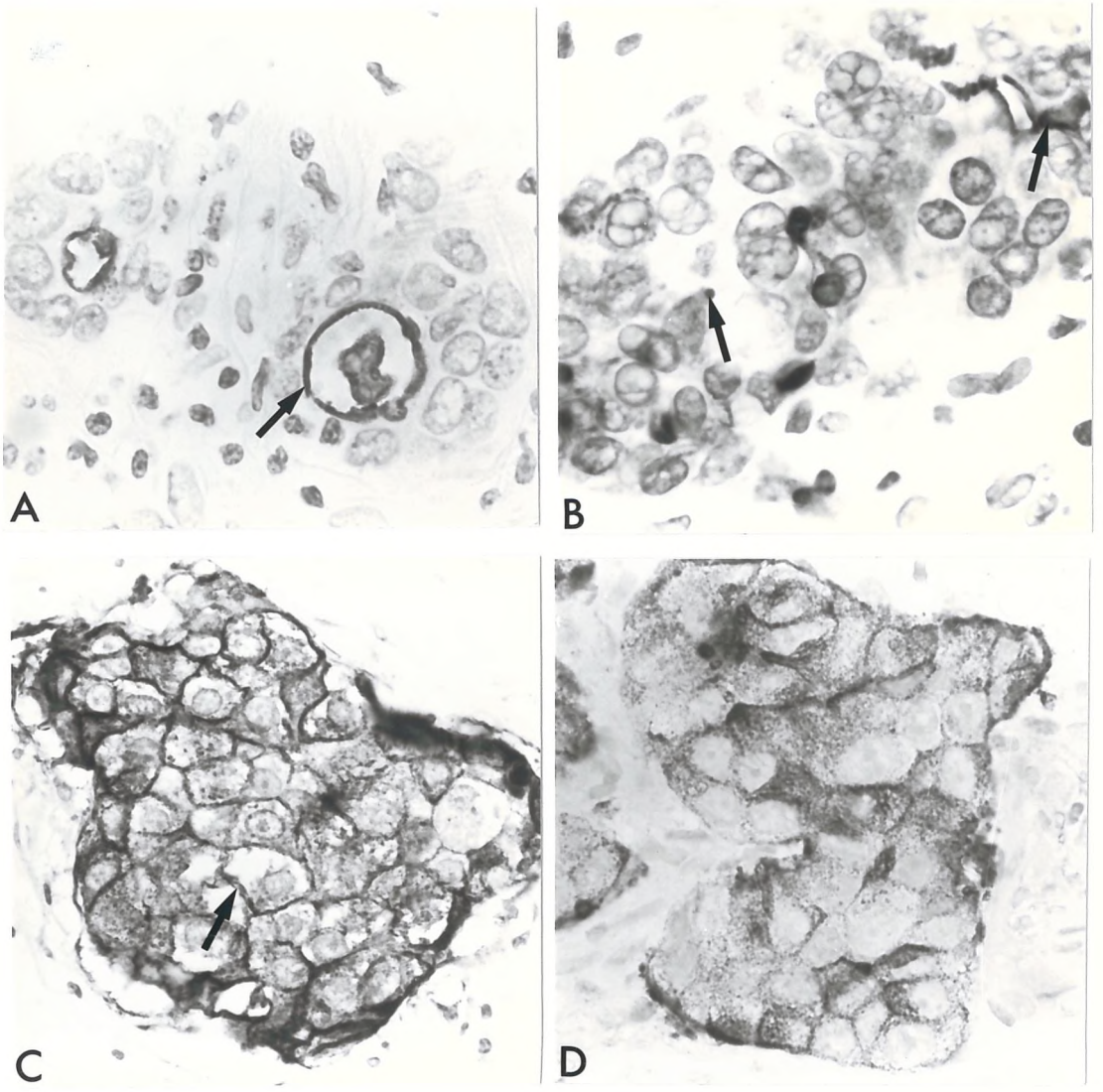


FIG 4A.6

Staining of four cases of infiltrating ductal breast carcinoma with HMFG2 in the indirect IP technique (4A.2.1.). Magnification x660.
A variety of staining patterns are observed. Extracellular staining is observed in the ducts and tubules of structurally differentiated tissue (A) or in intercellular spaces and in intracytoplasmic vacuoles (B). Intracellular staining is observed, either located towards the periphery of the cell (C), or diffuse in the cytoplasm (D).

intracellularly and was distributed either towards the periphery of the cell or diffusely in the cytoplasm. When diffuse intracellular staining was particularly intense it appeared as granular staining. (FIG 4A 6).

Intraduct tumour cells stained more homogenously than infiltrating tumour cells but still showed marked variation. Particularly strong staining was observed in the strands of tumour cells typical of lobular carcinoma and no staining was present in the carcinoid tumours or in the metaplastic tumour. There was no other general association of the staining pattern with the histological type of tumour.

A comparison was made of the staining in 2 tumours from each of 5 patients. The tumours were excised from the same breast in 2 cases and both showed similar staining patterns. In 2/3 cases where the tumours were excised from each breast the staining patterns were different.

4A.2.2. Comparison of the Staining of Monoclonal Antibodies Against Different Epitopes Within HMFG.

4A.2.2.1. A Comparison of the Immunohistochemical Staining Patterns of Three Monoclonal Antibodies Raised Against the HMFG.

E29 is another monoclonal antibody raised against HMFG (Gatter et al., 1984). It was titrated on normal, benign and malignant tissue as in 3.2.4 and the optimum titre was determined at 1/30.

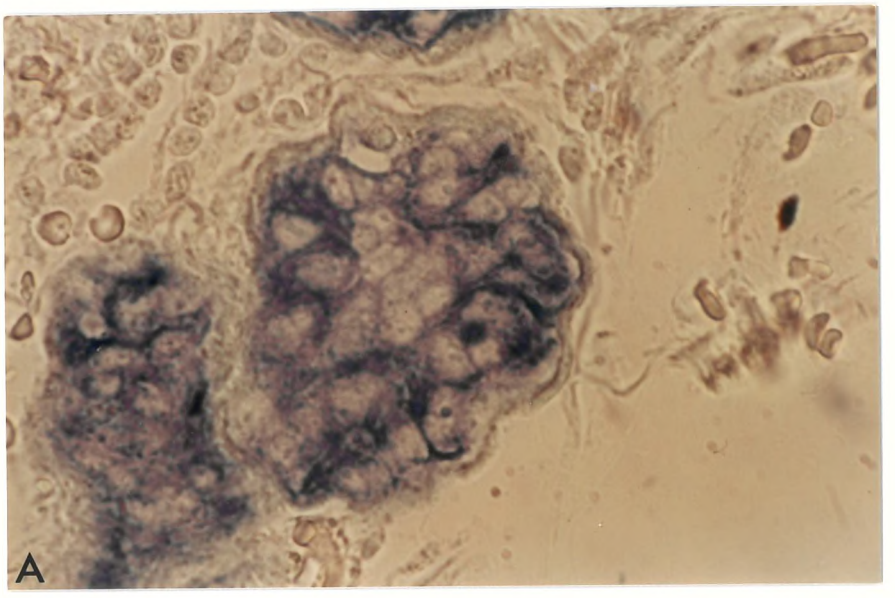
All of the benign tissue and 100 of the malignant tissue blocks used in 4A.2.1 were stained with E29 as well as with HMFG1 and HMFG2 in the standard IP technique as in 4A.2.1.. Similar staining patterns to those previously described for HMFG1 and HMFG2 (4A.2.1) were observed in tissue stained with E29. Staining in the benign lesions was extracellular and the staining intensity of E29 was intermediate between that of HMFG1 and HMFG2. Staining in malignant tissue was heterogenous and corresponding tissue sections stained with HMFG1,

HMFG2 and E29 showed similar staining patterns although the relative intensity of staining of the 3 antibodies varied.

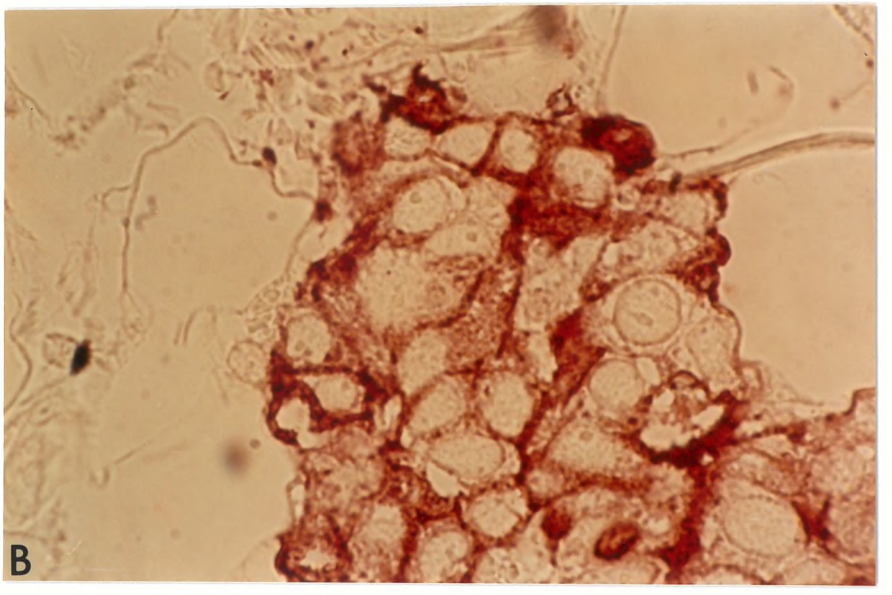
4A.2.2.2. A Comparison of the Staining of HMFG1 and HMFG2 in the Same Tissue Sections.

Ten of the malignant tissue blocks used in 4A.2.1. were selected for a comparison of the staining of HMFG1 and HMFG2 in the same tissue section. The tissue selected consisted of 8 blocks of infiltrating ductal carcinoma, ranging from poorly to well differentiated, and 2 blocks of lobular carcinoma. A series of 4μm thick sections were cut from each block (2.2.2.1.), dewaxed and rehydrated (2.2.2.2.). Endogenous peroxidase was inhibited (2.2.2.3.) and the tissues were digested in trypsin for 10 minutes (2.2.2.4.).

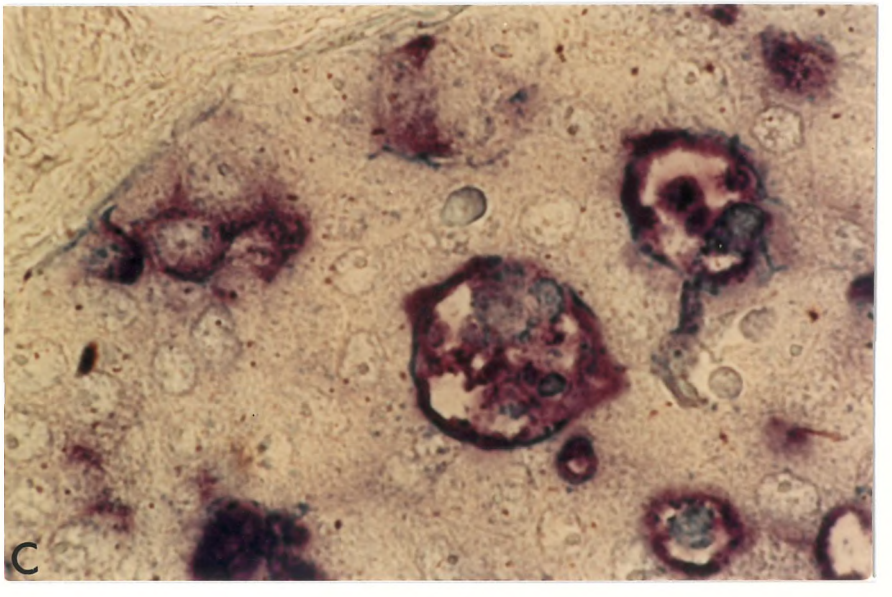
The sections were then stained with both HMFG1 and HMFG2 in a double staining immunohistochemical method modified from that used by Mason & Sammons (1978). The principle of this method is that an indirect immunohistochemical technique (2.2.3.1.), was carried out twice on each tissue section. The tissue sections were sequentially incubated with the first monoclonal antibody and then an anti-mouse antibody. This was followed by the second monoclonal antibody and then another anti-mouse antibody. The presence of the two antigens was distinguished by using two different anti-mouse antibodies, peroxidase (P) labelled and alkaline phosphatase (AP) labelled. The P was then reacted with 3A9E (2.2.5.3.) for 15 minutes to give a red coloured product (FIG 4A.7). The AP was reacted with Fast Blue (2.2.5.5.) for 15 minutes resulting in a blue coloured product (FIG 4A.7). A purple colour was the result of a close association of the P labelled and the AP labelled antibodies (FIG 4A.7). Methyl Green was used as a counterstain (2.2.3.5.). The sections were washed and mounted in glycerin jelly. HMFG1 AND HMFG2 (gift from J. Taylor-Papadimitriou), were used at a dilution of 1:3. The P labelled and the AP labelled second antibodies (Dako), were used at a dilution of 1:50, with NHS at 1:50.



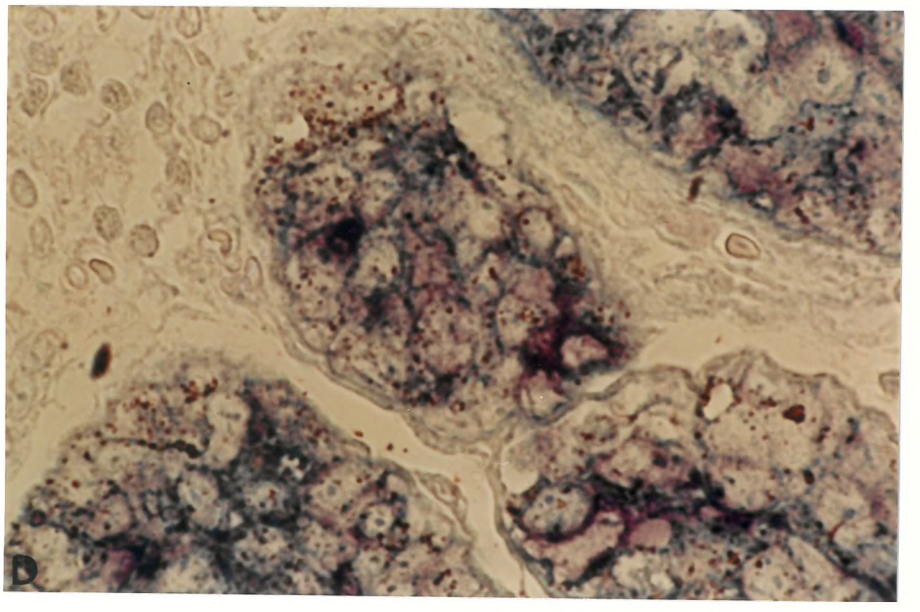
A



B



C



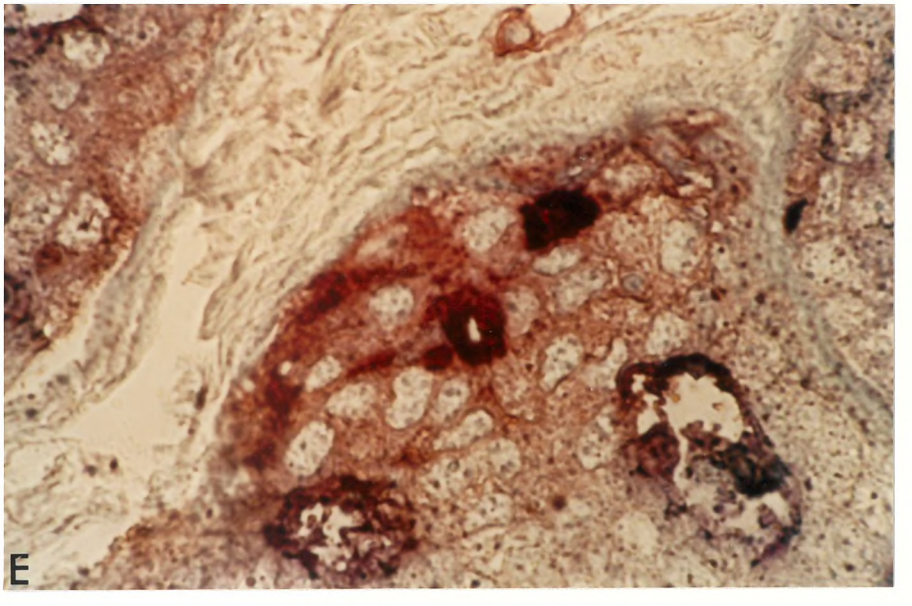


FIG 4A.7

A section of infiltrating ductal carcinoma stained with both HMFG1 and HMFG2 in a double staining IP technique (4A.2.2.2.). Magnification x660.
HMFG1 is identified by the blue colour (A) and HMFG2 is identified by the red colour (B). A close association of HMFG1 and HMFG2 results in a purple colour (C, D). Extracellularly, HMFG1 and HMFG2 were always closely associated (C). Intracellularly, they were found both closely associated (D) and alone (E).

A series of sections from each block were stained as follows:-

A. HMFG1 with P labelled 2nd antibody.

B. HMFG1 with AP labelled 2nd antibody.

C. HMFG2 with P labelled 2nd antibody.

D. HMFG2 with AP labelled 2nd antibody.

E. HMFG1 with P labelled 2nd antibody, then HMFG2 with AP labelled 2nd antibody.

F. HMFG2 with P labelled 2nd antibody, then HMFG1 with AP labelled 2nd antibody.

G. HMFG1 with AP labelled 2nd antibody, then HMFG2 with P labelled 2nd antibody.

H. HMFG2 with AP labelled 2nd antibody, then HMFG1 with P labelled 2nd antibody.

I. TBS control with P labelled 2nd antibody.

J. TBS control with AP labelled 2nd antibody.

A comparison of the sections E and F with G and H showed that the AP reaction product with Fast Blue was decreased in intensity if it was developed before the P with 3A9E (G, H). The preferred method of double staining was to develop the P with 3A9E first (E, F). The TBS controls showed that there was no methodologic background staining.

Staining of the sections where HMFG1 was applied first (E) and where HMFG2 was applied first (F) were identical, showing that neither antibody interferes with staining by the other. Staining of both the HMFG1 and HMFG2 antigens in well differentiated tumours was extracellular, resulting in purple stain (FIG 4A.7). In some areas of less well-differentiated tumours both antibodies stained the same cells intracellularly shown by intracellular purple stain (FIG 4A.7). In other areas of these tumours the cells stained intracellularly with only one antibody (FIG 4A.7). This was not an artifact as it correlated with the staining in the tissue sections where only 1 antibody was used (A, B, C & D). Both HMFG1 and HMFG2 stained the majority of the cells in the lobular carcinomas.

4A.2.3 Classification of the Immunohistochemical Staining Patterns

Observed With HMFG1 and HMFG2 in Malignant Breast Tissue.

The staining patterns of malignant tissue were graded according to whether the staining pattern was most extensive extracellularly or intracellularly and according to the extent of intracellular staining. In a previous experiment (3.2.5.), consecutive sections from 20 cases were stained on separate occasions. In 5 cases differences in staining intensity between the sections resulted in weak staining of some cells where previously no staining had been observed. Because of this and because of the heterogeneity of staining, it was necessary to take an overall view of staining in the tissue section. Also, to further standardise the classification, only the staining pattern in the infiltrating areas of the tumour was graded. For this reason the 5 cases of intraduct carcinoma were excluded. Each of the tissue sections were graded as described below (Table 4A.3).

An overall assessment of the relative intensity of staining observed in the infiltrating regions of the tumour was made at a magnification of x40 and 1-4 points were awarded. A representative area of the section was then assessed at a magnification of x400 and the presence of intracellular staining in 100 randomly observed cells noted and awarded 1-4 points. The 2 scores obtained for each tissue section were then added such that a tissue with a completely extracellular staining pattern scored 2 points, 1 for the strong extracellular staining in tubules and 1 because 0-25% of the cells stained intracellularly. A tissue section with strong intracellular staining would score 8 points, 4 because of the strong intracellular staining diffusely distributed in the cells and 4 because 76-100% of the cells stained intracellularly. The cases were then divided into 3 grades: those not stained at all were grade C, those scoring 2-5 points were grade A and those scoring 6-8 points were grade B.

To check the reproducibility of grading, 100 sections of breast carcinoma stained with HMFG2 were coded and then graded on 2 separate occasions. When the 2 assessments of the tissue sections were

TABLE 4A.3

Classification of the Immunohistochemical Staining Patterns of HMFG1 and HMFG2 in Malignant Breast Tissue.

Staining Pattern	Points Scored
1. Relative intensity of staining strongest:	
Extracellularly - in tubules	1
Extracellularly - intercellularly and in intracytoplasmic vacuoles	2
Intracellularly - localised towards periphery of cell	3
Intracellularly - diffusely distributed in the cytoplasm	4
2. % of Cells with intracellular staining:	
0 - 25%	1
26 - 50%	2
51 - 75%	3
76 - 100%	4

compared, the grade was the same in 84% of the cases. The remaining 16% were particularly difficult to grade because of the variability of staining throughout the section.

After exclusion of the cases of intraduct carcinoma, the immunohistochemical staining patterns of HMFG1 and HMFG2 in the remaining 152 specimens were graded according to the classification described above. The results of the grading are shown in Table 4A.4.

The immunohistochemical staining patterns of both HMFG1 and HMFG2 showed the same relative intensity of staining in 121 specimens and the same extent of intracellular staining in 93 specimens. A comparison of the staining grades in the sections stained with HMFG1 and HMFG2 showed that there was little difference in the number of cases in either the final staining grade or in the component parts of the grade, that is, relative intensity of staining and extent of intracellular staining. A low percentage of tissues did not stain (11.2% and 7.9% for HMFG1 and HMFG2 respectively). The staining patterns were more frequently graded A than B. The predominant staining pattern was, therefore, a combination of strong extracellular staining with less than 50% of the cells staining intracellularly. Both an intracellular staining pattern around the periphery of the cell and intracellular staining in 50-75% of the cells was less frequently seen than the other staining patterns. The staining grade B was, therefore, a result of tissues staining either in a diffuse intracellular stain or where 76-100% of the cells stained intracellularly.

4A.2.4. Correlation of the Staining Grades with RFS and Other Prognostic Indicators.

The staining grades of the tissue sections assessed in 4A.2.3. were correlated with the lymph node status, tumour size, oestrogen receptor status, and Bloom's Grade using a Chi-squared test. Survival curves of the different staining grades of both the retrospective series from 1974/5 and the prospective series from 1982/4 were

TABLE 4A.4

Classification of the Immunohistochemical Staining Patterns of HMFG1 and HMFG2 in 152 Primary Breast Cancer Biopsies.

		Number of Biopsies Stained With: HMFG1	HMFG2
Points Awarded for Relative Intensity of Staining	1	37	36
	2	45	49
	3	13	14
	4	40	41
Points Awarded for Extent of Intracellular Staining	1	37	39
	2	38	38
	3	12	19
	4	48	44
No Staining		17	12
Staining Grade	A	76	85
	B	59	55
	0	17	12

All of the tissue was fixed in NBF, embedded in paraffin wax and stained with HMFG1 and HMFG2 in the standard IP technique used in this study.

plotted and analysed using the SPSS "SURVIVAL" sub-programme (Nie et al.,1981). Pairwise comparison of the curves was performed using the Lee Desu statistic (Lee & Desu, 1972).

4A.2.4.1 Relation of the Staining Grades to the Presence of Nodal Metastases.

There were 54/148 node positive cases and 94 node negative cases. There was no significant association between the staining grade A, B or C and the presence or absence of nodal metastases for either HMFG1 or HMFG2 (χ^2= 3.05;χ^2= 2.44) (FIG 4A.8; Table 4A.5).

4A.2.4.2. Relation of the Staining Grades to the Size of the Primary Tumour.

Of 152 specimens of primary tumour, 31 were categorised as <2cm in diameter, 96 between 2-5 cm and 25 >5 cm. The association of the staining grades of HMFG1 and HMFG2 with tumour size is shown in Table 4A.6 and FIG 4A.9. A high proportion of tumours <2 cm in diameter were staining grade A. This was reflected in the high χ^2 values (6.37 and 10.34 for HMFG1 and HMFG2 respectively). The correlation was not significant.

4A.2.4.3. Relation of the Staining Grades to Oestrogen Receptor Status.

59 of 103 cases from 1982-4 were oestrogen receptor positive and 44 were oestrogen receptor negative. There was no significant association between the staining grade A, B or C and the oestrogen receptor status of the tumours. (Chi-squared test.) (Table 4A.7; FIG 4A.10) The lack of correlation was present in sections stained with either HMFG1 or HMFG2. (χ^2= 5.10; χ^2= 1.70).

4A.2.4.4. Relation of the Staining Grades to Bloom's Grade.

Of the 152 carcinomas, 23 were Bloom's grade 1. The staining pattern

TABLE 4A.5

Relation of the Staining Grades of HMFG1 and HMFG2 In Primary Breast Cancer to the Presence of Metastases in the Axillary Lymph Nodes.

Staining Grade	Number (%) of Biopsies Stained With: HMFG1 Lymph Node Status LN+	HMFG1 Lymph Node Status LN-	HMFG2 Lymph Node Status LN+	HMFG2 Lymph Node Status LN-
A	22 (14.9)	52 (35.1)	27 (18.2)	56 (37.8)
B	24 (16.2)	33 (22.3)	24 (16.2)	30 (20.3)
C	8 (5.4)	9 (6.1)	3 (2.0)	8 (5.4)

LN+ - Metastases present in the axillary lymph nodes
LN- - Metastases not present in the axillary lymph nodes

148 biopsies of primary breast carcinoma were stained with HMFG1 and HMFG2 in the standard indirect IP technique established for this study. The staining patterns were graded according to the classification described in 4A.2.3

FIG 4A.8

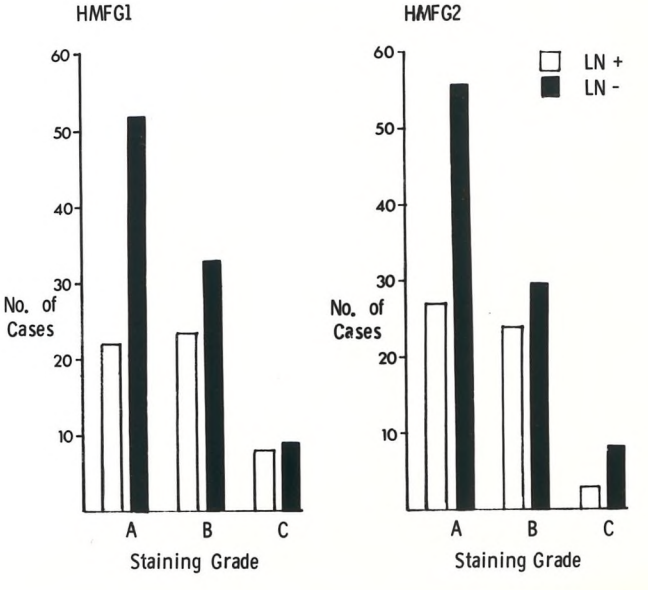


TABLE 4A.6

Relation of Staining Grades of HMFG1 and HMFG2 in Primary Breast Cancer to the Size of the Primary Tumour

Staining Grade	Number (%) of Biopsies Stained With: HMFG1 Size of the Primary Tumour			HMFG2 Size of the Primary Tumour		
	<2 cm	2-5 cm	>5 cm	<2 cm	2-5 cm	>5cm
A	18 (11.8)	44 (28.9)	12 (7.9)	21 (13.8)	49 (32.2)	14 (9.2)
B	9 (5.9)	45 (29.9)	8 (5.3)	8 (5.3)	44 (28.9)	7 (4.6)
C	4 (2.6)	7 (4.6)	5 (3.2)	4 (2.6)	3 (2.0)	4 (2.6)

152 biopsies of breast carcinoma were stained with HMFG1 and HMFG2 in the standard indirect IP technique established for this study. The staining patterns were graded according to the classification described in 4A.2.3

FIG 4A.9

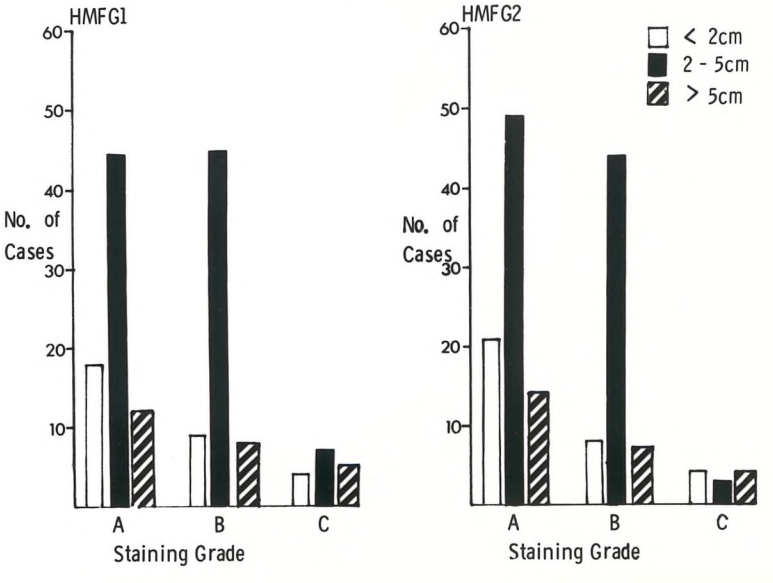


TABLE 4A.7

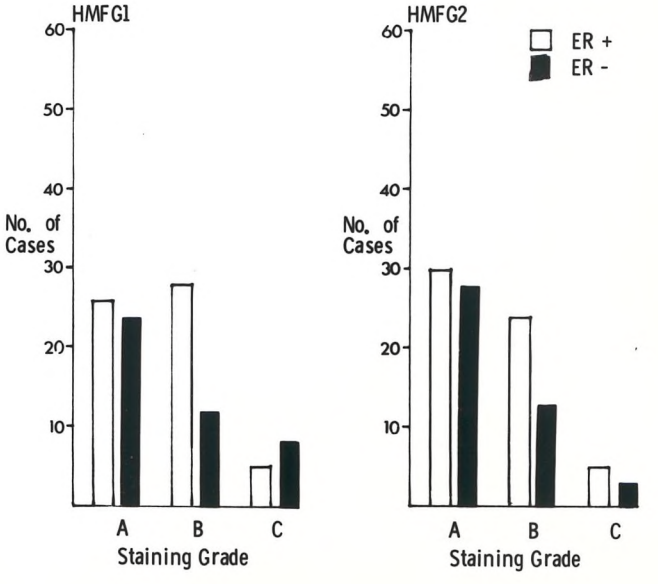
Relation of the Staining Grades of HMFG1 and HMFG2 in Primary Breast Cancer to the Oestrogen Receptor Status

Staining Grades	Number (%) of Biopsies Stained With: HMFG1 Oestrogen Receptor Status		HMFG2 Oestrogen Receptor Status	
	ER+	ER-	ER+	ER-
A	26 (25.2)	24 (23.3)	30 (29.1)	28 (27.2)
B	28 (27.2)	12 (11.6)	24 (23.3)	13 (12.6)
C	5 (4.8)	8 (7.8)	5 (4.8)	3 (2.9)

ER+ = Oestrogen receptor positive
ER- = Ostrogen receptor negative

103 biopsies of breast carcinoma were stained with HMFG1 and HMFG2 in the standard indirect IP procedure established for this study. They were graded according to the classification system described in 4A.2.3

FIG 4A.10



was Grade A with HMFG1 and HMFG2 in 18 and 19 cases respectively. 106 cases were Bloom's grade 2 and 23 were Bloom's grade 3. (Table 4A.8; FIG 4A.11) There was no association between the staining grades A, B or C with Bloom's grade 2 or 3. The association of staining grade A with Bloom's Grade 1 tumours was statistically significant for HMFG1 (χ^2= 13.85). The association was not statistically different for HMFG2 but there was some correlation as shown by the high value of χ^2 (8.76).

4A.2.4.5. Life Table Analysis Comparing the Staining Grades.

A) Survival curves of 37 patients from 1974-5 with staining grade A, B and C are illustrated in FIG 4A.12. There were some differences between the curves, particularly with HMFG2, whereby patients with a staining grade C had a high RFS and RFS was higher in patients with staining grade B than with staining grade A. Statistical analysis of the 3 curves showed that there was no significant difference in the survival of each staining group in sections stained with either HMFG1 or HMFG2.(p = 0.29; p = 0.12).

B) Survival curves of 112 patients from 1982/4 with staining grade A, B and C are illustrated in FIG A.13. There was no statistically significant difference between the curves of each staining group in sections stained with either HMFG1 or HMFG2 (p = 0.58; p = 0.63).

4A.3. Discussion.

4A.3.1. The Staining Characteristics of HMFG1 and HMFG2 in Normal, Benign and Malignant Breast Tissue.

Immunohistochemical staining of HMFG1 and HMFG2 in normal, benign and malignant breast tissue could generally be described as heterogenous. Staining in normal tissue was extracellular on the luminal surface of epithelial cells and in secretions in the ducts and tubules. It was variable in the presence and extent of staining between patients, between lobules in a biopsy and between acini within a lobule.

Staining of benign tissue was, likewise, extracellular and heterogenous between cases and within a section. In both normal and benign tissue the heterogenous staining was related to distinct units of ducts and lobules. A duct and the acini in the same lobule stained to the same extent with HMFG1 and HMFG2. This was also observed by Foster et al. (1982b), using other monoclonal antibodies raised against the HMFG, M3, M8, M18 and M24.

Intracellular staining was observed in the epithelial cells or myoepithelial cells of ducts and tubules containing much secretion in 5 cases of benign breast disease. In all of these cases staining was also present in the connective tissue surrounding the ducts and tubules, so the intracellular staining was attributed to leakage of the antigen from the lumina to other parts of the tissue. The cells of apocrine metaplasia were stained in a diffuse intracellular pattern. The change in cell morphology in metaplasia, therefore, resulted in a change in antigen distribution.

In malignant tissue staining was heterogenous both in the extent of intracellular staining and in the relative intensity of staining. This heterogeneity was observed both between tumours and, within tumours, between different areas of a tumour and between adjacent cells. In some cases extracellular staining was observed both in tubules and in the intercellular spaces. Staining of the intracytoplasmic vacuoles was also considered extracellular, as electron microscopy has shown that the surface of the inner membrane of the vacuole has features typical of the exterior surface of the cell membrane (Battifora, 1975). Also, intracytoplasmic vacuoles were most frequently observed when the staining pattern was extracellular in the intercellular spaces. In other cases, staining was stronger intracellularly, distributed either towards the periphery of the cell or diffusely throughout the cytoplasm. When intracellular staining was particularly intense it was expressed as granular staining. There was no staining with either HMFG1 or HMFG2 in 6.6% of the tumours and a further 4.6% and 1.3% did not stain with HMFG1 and HMFG2 respectively.

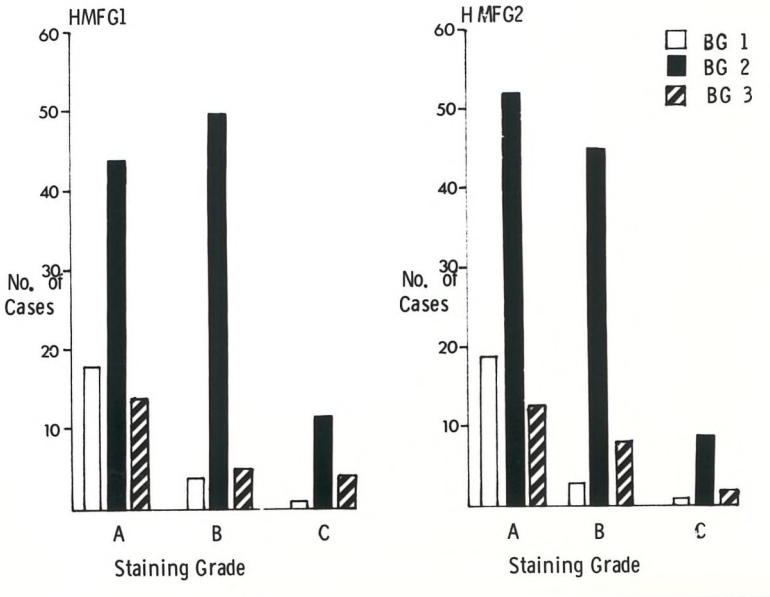
TABLE 4A.8

Relationship of the Staining Grades of HMFG1 and HMFG2 in Primary Breast Carcinoma to Bloom's Grade

Staining Grade	No. (%) of biopsies stained with: HMFG1 Bloom's Grade 1	2	3	HMFG2 Bloom's Grade 1	2	3
A	18 (11.8)	44 (28.9)	14 (9.2)	19 (12.5)	52 (34.2)	13 (8.5)
B	4 (2.6)	50 (32.9)	5 (3.2)	3 (2.0)	45 (29.6)	8 (5.3)
C	1 (0.7)	12 (7.9)	4 (2.6)	1 (0.7)	9 (5.9)	2 (1.3)

152 biopsies of breast carcinoma were stained with HMFG1 and HMFG2 in the standard indirect IP procedure established for this study. They were graded according to the classification system described in 4A.2.3

FIG 4A.11



The immunohistochemical staining patterns of HMFG1 and HMFG2 in breast tissue have been described previously (Arklie et al., 1981). Results in Chapter 3 have shown that differences in tissue preparation and staining technique affect the staining characteristics of HMFG1 and HMFG2. Caution should, be exercised, therefore, when comparing immunohistochemical staining characteristics between studies. Nevertheless, the immunohistochemical staining characteristics described by Arklie et al. are similar to those in this study. Similar staining patterns have also been noted with anti-EMA, except that weak intracellular staining was present in normal and benign tissue (Sloane & Ormerod, 1981). This may have been the result of antibodies present in the polyclonal antiserum directed against antigens other than HMFG.

Immunohistochemical staining of another monoclonal antibody raised against HMFG, E29 (Gatter et al., 1984), was compared with that of HMFG1 and HMFG2 and showed similar staining characteristics.

Immunohistochemical staining of 2 other series of monoclonal antibodies raised against HMFG in breast tissue has been characterised (Foster et al., 1982a, b; Hilkens et al., 1984). These antibodies showed differences in the presence and extent of staining in normal tissue and in the presence of staining and the predominance of the particular staining pattern in malignant tissue (Table 1.3). When present, however, the staining could also be associated with the staining of HMFG1 and HMFG2.

4A.3.2. Grading the Staining Characteristics of HMFG1 and HMFG2 in Breast Cancer.

The staining grades were designed to reflect the immunohistochemical staining patterns observed, whilst being in a form which could be related to indices of prognosis and to survival. The staining pattern in normal, benign and structurally differentiated tumours (those with extensive tubule formation) was extracellular, and became more

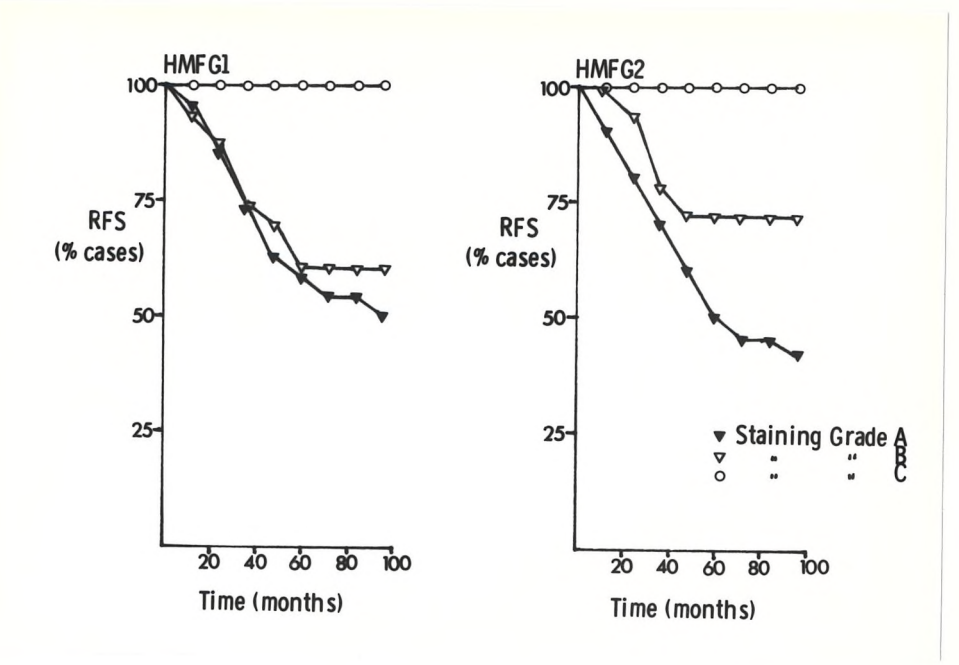


FIG 4A.12

Life table analysis comparing the staining grades A, B and C of HMFG1 and HMFG2 with RFS in 37 cases of breast carcinoma (4A.2.4A).

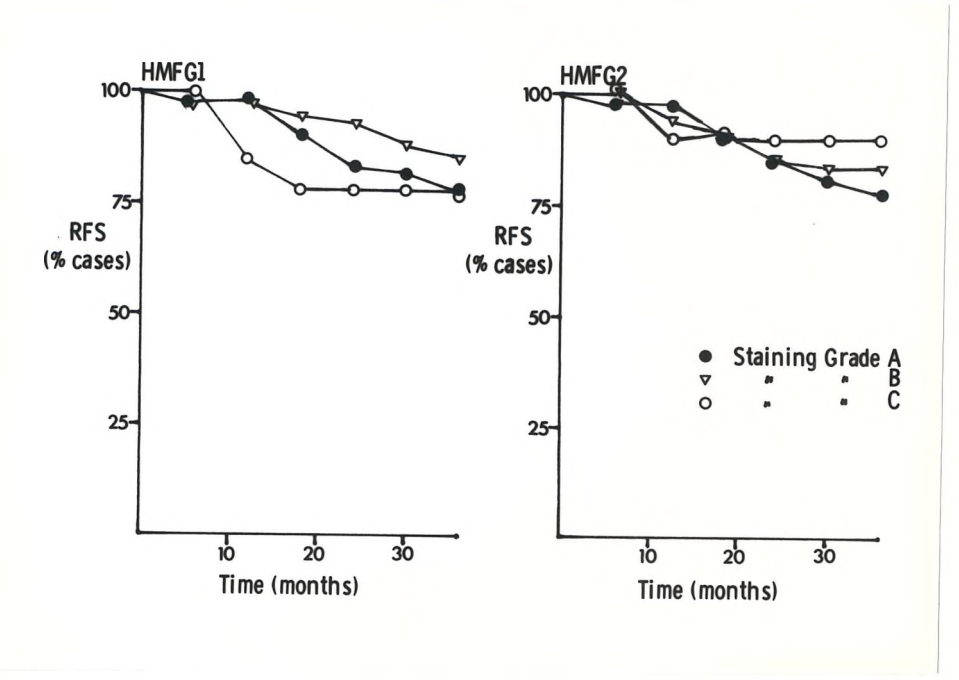


FIG 4A.13

Life table analysis comparing the staining grades A, B and C of HMFG1 and HMFG2 with RFS in 112 cases of breast carcinoma (4A.2.4B).

intracellular as structural differentiation was lost. The grading system, therefore, took into account the relative intensity of extracellular and intracellular staining and also the number of cells with intracellular staining. The 20 control sections where 2 consecutive sections were stained on separate occasions showed the same staining pattern on both sections but the overall intensity of stain throughout the section varied. Little emphasis, therefore, was placed on the overall intensity of the stain.

Heterogeneity was observed between different areas of a tissue section and was a limitation of the grading system. An overall view of the staining pattern at a low magnification was included in the staining classification to counteract this. Despite this precaution, a group of cells with a particular staining pattern might influence the survival of the patient and yet not be included in the grading if the predominant staining pattern was different. The heterogeneity also made difficult the grading in many cases, but when the sections were graded on 2 separate occasions 84% reproducibility was obtained.

Other methods of grading immunohistochemical staining patterns in breast tissue have been devised. Wilkinson et al. (1984), graded the immunohistochemical staining patterns of HMFG1 and HMFG2 in primary breast cancer according to the uniformity of the presence or absence of staining in a section, the number of cells staining and the intensity of the stain. This assessment was made for each of 3 staining patterns, extracellular, cytoplasmic and on the membrane-associated. Essentially, this staining classification assessed the extent of each particular staining pattern in the tissue. Rasmussen et al. (1985), stained malignant tissue with 3 monoclonal antibodies raised against the HMFG. For each antibody the tissue was divided into 2 groups on the basis of positive or negative staining. Positive staining was determined firstly at >10% of the tumour cells staining and then at >50% of the tumour cells staining. This classification system measures the extent of staining regardless of the staining distribution. Neither of the 2 authors mentioned above commented on the reproducibility of their staining

classification. All of the classification systems described above were used to grade a series of malignant breast tumours and then related to indices of prognosis and to RFS.

4A.3.3. Relation of the Staining Grades of HMFG1 and HMFG2 in Primary Breast Carcinoma to Indices of Prognosis and to Survival.

The staining grades of HMFG1 and HMFG2 were first related to indices of prognosis. There was an association of the staining grade A, essentially an extracellular staining pattern (4A.2.3) in Bloom's Grade 1 tumours. This association was statistically significant for HMFG1 and, although not significant, was reflected in the high values for HMFG2. Bloom's grade 2 and 3 tumours were not associated with any particular staining pattern. High χ^2 values were also obtained for the correlation of staining grade 1 with tumours less than 2 cm in diameter, although the association was not statistically significant. There was no relation of the staining grades of either HMFG1 or HMFG2 to nodal status or to oestrogen receptor status.

Wilkinson et al. (1984), also found that with HMFG1 there was a statistically significant association of the staining grades which reflected extensive extracellular staining with structurally well-differentiated tumours. There was no correlation of the staining grades with other indices of prognosis. Rasmussen et al. (1985), graded the staining patterns of 3 monoclonal antibodies raised against different epitopes in the HMFG, Mam3a, Mam3b and Mam3c. Presence of the Mam3b antigenic epitope in >10% of the cells was significantly related to a well differentiated histological grade. There was also a significant correlation of >10% of cells staining with Mam3a and Mam3b antigenic epitopes with ER+ tumours.

These results show that the staining patterns of monoclonal antibodies against HMFG and against membrane proteins are indicative of the state of differentiation of the tumour. In all of the studies there was a significant relationship of either extracellular staining or extensive staining with histologically well-differentiated

tumours. There was no association of a particular staining grade with less well-differentiated tumours. The associations of staining with small tumours and with ER+ tumours may have been because such tumours are well-differentiated.

The staining grades of HMFG1 and HMFG2 were related to the survival of 37 patients who had a mastectomy in 1974-5 and had been followed up until 1982. A comparison of the survival curves of the 3 staining grades showed that with both HMFG1 and HMFG2, absence of staining in the tumour was associated with a good survival. Also patients with a tumour with staining grade A had a better survival than those with a tumour graded B. These differences were not statistically significant and so none of the staining grades identified a distinct group of patients with a good or a bad survival.

A prospective study was initiated to determine if the associations of the staining grades with survival observed in the retrospective study became statistically significant with a higher number of patients. The grades with HMFG1 and HMFG2 were related to the survival of 115 patients from 1982-4. The average follow up time for these patients was 24.2 months. The difference in survival of patients with a staining grades A, B and C which was observed in the retrospective study was not maintained. The survival of these patients will be documented over the next few years to determine whether a longer follow up reveals associations of the staining grades with RFS.

A limitation of the staining classification was the heterogeneity of staining patterns. One staining pattern might influence patient survival but not be detected because it is not the predominant staining pattern.

Differing results were obtained when other classification systems of the staining characteristics were related to survival. Wilkinson et al. (1984), found that the staining grades were not related to survival when HMFG2 was used but identified a group of 13 patients whose tumours did not stain with HMFG1 and who had a particularly

poor prognosis. These results are different from those in this study, for in the retrospective study there were 3/37 patients whose tumours did not stain with HMFG1. All of these survived for 8 years with no recurrences. In the prospective study there were 14 patients whose tumours did not stain with HMFG1, 3 of which recurred 7, 9 and 11 months after mastectomy. The 11 remaining patients had an aversge follow up time of 29 months. Similarly Taylor-Papadimitriou et al. (personal communication), found that there was no association of the absence of staining with a poor survival in a large retrospective series of patients.

Wilkinson et al. (1984), also showed a significant association of extensive extracellular staining with a good prognosis. Extensive extracellular staining was also correlated with a low histological grade. This relationship was not absolute and several Bloom's Grade 2 tumours showed extensive extracellular staining. Statistical analysis was not carried out to determine whether the presence of extracellular staining was a more accurate prognostic indicator than histological grading. There were 17 LN+ patients whose tumours showed extensive extracellular staining and who did not experience early recurrence, as expected. These patients were followed up for an average of 36 months. As the presence of extracellular staining is an indication of the well-differentiated state of the tumour it is possible that the metastases are less aggressive and take a longer time to establish secondary tumours (4.1.4). It will be interesting to follow the survival of these patients over a longer time-span.

The results of Wilkinson et al. (1984), cannot be directly compared with those in this study because the method of grading the tissue was different. Staining grade A represented those tissues where extracellular staining was predominant and where there was little intracellular staining. There was no association of this staining grade with a good prognosis. Similarly a separate analysis of the section of the classification system which measured whether staining was strongest extracellularly or intracellularly showed that extracellular staining was not related to a good prognosis.

Rasmussen et al. (1985), associated the extent of staining of Mam3a, Mam3b and Mam3c in breast tissue with survival and found that there was no significant association.

There are other antibodies directed against breast tumour-associated antigens where the pattern of immunohistochenical staining could be of prognostic value.(1.2.). The only antibody which has been reported as useful in this area is NCRC11 (Ellis et al., 1984). NCRC11 antibodies were raised against metastatic breast carcinoma cells. The antigen expression in breast tissue is very similar to that of antigenic epitopes in the HMFG and the antibodies cross-react with antibodies raised against the M8 antigen in the HMFG (Ellis et al., 1984). The immunohistochemical staining of antibodies against NCRC11 in primary breast carcinoma was graded according to the percentage of positively staining cells. No account was taken of the localisation of the antigen in the cells or of the tumour heterogeneity. No staining was observed in the tumours of 7 patients. There was no association of the lack of antigen expression with survival. There was, however, an association of the percentage of positively stained cells with survival. A high percentage of positively staining cells predicted a good survival and the converse was true. This grading system has been included in the prognostic index devised at Nottingham and research continues to confirm these results.

In this study, it is surprising that, although not statistically significant, patients with a staining grade B had a better survival than patients with a staining grade A in the retrospective series and prospective series for both HMFG1 and HMFG2. Staining grade A includes those patients with extracellular staining and one would expect this group to have a better survival. Staining grade B was composed mainly of patients with extensive intracellular staining (4A.2.3). It is possible that this classification was not only an assessment of the staining pattern but also of the homogeneity of staining.

A heterogenous tumour is composed of many genetically different cells which may increase the chances of a cell overcoming the hosts' resistance to metastasis and establishing a secondary tumour. The heterogeneity of a particular breast carcinoma can be observed in the immunohistochemical localisation of HMFG antigens. Wilkinson et al. (1984), considered the uniformity of a staining pattern in their classification system, but this was combined with the number of cells staining and the intensity of the stain. An attempt was made in this study to grade the tumours according to the extent of heterogeneity as determined by the staining of HMFG1 and HMFG2 but no method could be devised that was reproducible. This was because particular staining patterns are not localised in clearly defined areas but gradually change throughout the tissue. An assessment of antigen and, therefore, cellular heterogeneity could be made more accurately using a microdensitometer, which does not rely on subjective impressions.

In conclusion, presently there is no convicing evidence that the immunohistochemical staining patterns of monoclonal antibodies raised aginst the HMFG antigen in primary breast cancer define groups of patients with a particular prognosis.

4B An Investigation of the Prognostic Value of Immunohistochemical Staining With HMFG1 and HMFG2 in Secondary Breast Cancer.

4B.2 Experiments.

4B.2.1. Determination of the Staining Procedure for the Optimum Demonstration of HMFG1, HMFG2 and E29 in Breast Carcinoma Metastases in the Axillary Lymph Nodes.

4B.2.1.1.
Determination of the Optimum Titre of Monoclonal Antibodies HMFG1, HMFG2 and E29 for IAP staining in Breast Carcinoma Metastases in the Axillary Lymph Nodes.

A titration of the monoclonal antibodies HMFG1, HMFG2 and E29 was carried out on 2 nodes from each of 2 patients with primary infiltrating ductal carcinoma. The tissue was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.). In both patients macrometastases (> 2mm diameter) were present in one of the nodes and micrometastases (< 2mm diameter) were present in the other. A series of 4μm thick sections were cut from each node (2.2.2.1.), dewaxed and rehydrated (2.2.2.2.). They were stained with either HMFG1, HMFG2 or E29 at varying dilutions in the IAP technique with double-bridge enhancement (2.2.3.2.). HMFG1 and HMFG2 are used at a dilution 1:2 in primary breast carcinoma (3.2.4.) and so were used neat and at double dilutions to 1:32. E29 is used at a dilution of 1:30 in primary breast carcinoma (4A.2.2.1.) and so was used neat and at double dilutions to 1:512.

The second antibody, goat anti-mouse IgG (GaMIgG), and the third antibody, mouse anti-alkaline phophatase/alkaline phosphatase conjugate (MAAPAP), were titrated in checkerboard fashion by the University Pathology Department at Southampton General Hospital. GaMIgG was used at a dilution of 1:200 with NHS at 1:100 and MAAPAP

was used at a dilution of 1:8. TBS controls were included. The staining of both macro- and micrometastases with HMFG1, HMFG2 and E29 was not sufficiently strong for the purposes of detection of metastases. No staining was observed in the TBS controls and there was no background staining or staining of cells other than breast carcinoma cells. All surgically removed lymph nodes are routinely fixed in NBF and embedded in paraffin wax, so it was necessary to alter the staining procedure used so as to obtain staining intensities sufficiently strong for the detection of breast carcinoma cells.

4B.2.1.2. Experiment to Determine the Effect of Fixation, Trypsin Digestion and the Degree of Staining Enhancement on the Immunohistochemical Localisation of HMFG1, HMFG2 and E29 Antigens in Breast Carcinoma Metastases in Axillary Lymph Nodes.

This experiment was carried out on a palpable lymph node from a patient who had a Patey mastectomy for a primary infiltrating ductal breast carcinoma in Southampton in 1983. Immediately after surgery the lymph node was divided and half was snap frozen in liquid nitrogen and subsequently stored in nitrogen vapour. The other half was fixed in NBF (2.2.1.1.) and embedded in paraffin wax (2.2.1.8.), (A). Another NBF fixed, paraffin wax embedded lymph node containing macrometastases (4B.2.1.1.) was also used (B).

Three sections, 6 μm thick were cut from the frozen node for each antibody and fixed in dehydrated acetone for 20 minutes (2.2.1.6.). Similarly, 8 sections, 4μm thick were cut from each of the NBF fixed, paraffin wax embedded nodes (2.2.2.1.), and were dewaxed and rehydrated (2.2.2.2.). Of each set of 8 sections, 2 were left undigested and 2 were digested in trypsin for 10, 20 and 30 minutes. All sections were then stained with HMFG1, HMFG2 or E29, undiluted, in the IAP technique (2.2.3.2.). The second antibody was GaMIgG, used at 1:200 with NHS at 1:100, and the third antibody was MAAPAP, used at 1:8. One of each pair of sections was stained with double-bridge enhancement and the other was stained with triple-bridge enhancement. TBS controls were included for each experimental condition.

The results are summarised in Table 4B.1. No staining was observed in the TBS controls, in connective tissue or in any other cell type than breast carcinoma cells. For each monoclonal antibody, in both frozen and NBF fixed, paraffin wax embedded tissue, the staining intensity was slightly stronger when triple-bridge, rather than double-bridge enhancement was used.

The staining in frozen tissue was stronger than in the corresponding undigested NBF fixed, paraffin wax embedded tissue. When the latter was digested in trypsin, the staining intensity increased so that after either 20 or 30 minutes trypsin digestion, depending on the antigen being localised, it was as strong as the staining in the frozen tissue. The tissue and cell morphology was still preserved after 30 minutes trypsin digestion.

A comparison of two NBF fixed, paraffin wax embedded nodes showed that staining with each of the monoclonal antibodies varied. HMFG1 was more intense in B than in A, whilst HMFG2 was more intense in A than in B. Trypsin digestion so greatly increased the staining intensity that after 30 minutes trypsin digestion the differences in staining intensity between the different tissues and between the monoclonal antibodies were less marked. The staining patterns in the metastases from different primary tumours were also different. In A the staining of all 3 antibodies was patchy and located particularly in the intercellular spaces, whilst staining in B was uniform and intracellular. HMFG1, HMFG2 and E29 gave the same staining pattern in each case but differed in the extent of intracellular staining. Trypsin digestion did not alter the staining pattern.

It was determined that trypsin digestion of NBF fixed, paraffin wax embedded lymph nodes for 30 minutes followed by double-bridge IAP staining with a panel of monoclonal antibodies, HMFG1, HMFG2 and E29 results in sufficiently strong staining for the detection of breast carcinoma cells. This was established as the standard procedure throughout this study (FIG 4B.1).

TABLE 4B.1

A Comparison of the Effect of Fixation, Trypsin Digestion and Double-Bridge or Triple-Bridge Enhanced IAP Technique on the Detection of HMFG Antigens in Breast Carcinoma Metastases in the Axillary Lymph Nodes.

Trypsin Digestion Times	Frozen Tissue		NBF Fixed, Paraffin Wax Embedded Tissue			
			A		B	
	x2	x3	x2	x3	x2	x3
A. HMFG1						
0′	++	+++	0	+	+	++
10	-	-	+/++	++	++	+++
20	-	-	++	++	+++	+++
30′	-	-	++	+++	+++	+++
B. HMFG2						
0′	++	+++	+/-	++	+/-	+
10′	-	-	+	+++	+	+/++
20′	-	-	++	+++	++	++
30′	-	-	+++	+++	++	+++
C. E29						
0′	+	++	0	+/++	+/-	+
10′	-	-	+/-	++	+	+/++
20′	-	-	+	++	+	++
30′	-	-	+	++	++	++

A comparison was made of the detection of HMFG1, HMFG2 and E29 antigens using IAP staining with either double (x2) or triple (x3) bridge enhancement in frozen and NBF fixed, paraffin wax embedded lymph node tissue. Two blocks of NBF fixed, paraffin wax embedded tissue were used, one corresponding to the frozen tissue (A) and another containing macrometastases (B). These were either undigested or digested in trypsin for 10, 20 or 30 minutes.

0 No staining
+ Weak; ++ strong; +++ very strong staining intensity

4B.2.1.3. Determination of the Optimum Titre of HMFG1, HMFG2 and E29 for Immunohistochemical Staining of Breast Carcinoma Metastases in the Axillary Lymph Nodes Using the Standard Staining Technique Established for This Study.

A titration of HMFG1, HMFG2 and E29 was carried out as in 4B.2.1.1, except that the standard IAP staining procedure established for this study was used (2.2.4.2.). No staining was observed in the TBS controls, in connective tissue or any other cell than breast carcinoma cells. The optimum titres were determined as the greatest dilution of antibody before staining intensity was decreased. The titres were established as:-

HMFG1 - 1:2

HMFG2 - 1:2

E29/68 - 1:10.

4B.2.1.4. Determination of the Staining Characteristics and Optimum Titre of CAM5.2 in Breast Carcinoma Metastases in the Axillary Lymph Nodes.

The monoclonal antibody CAM5.2 was included in this study after the standard IAP staining procedure had been established. This experiment was carried out to determine whether CAM5.2 could be incorporated into the study with no alteration to the staining procedure. A series of 4 μm thick sections were cut from the 4 lymph nodes used in 4B.2.1.1 and from the corresponding primary tumour. They were stained with CAM5.2, neat and at double dilutions to 1:512, in the standard IAP staining procedure established for this study (2.2.4.2.). TBS controls were included.

No staining was observed in the TBS controls, in connective tissue or in any other cells than breast carcinoma cells. In the primary tumour staining was intracellular, diffusely distributed in the cytoplasm of the neoplastic epithelial cells. There was heterogeneity in the staining intensity in adjacent cells but not between different areas

of the tumour or between primary tumours from different patients. Intracellular staining was also observed in the histologically normal breast epithelial cells surrounding the tumour.

Staining of the metastatic cells in the axillary lymph nodes was intracellular, distributed diffusely in the cytoplasm and heterogenous in staining intensity, as in the primary tumour. There was no difference in the staining pattern between different areas of the metastasis or between metastases from different primary tumours. No staining was observed in any other components of the lymph nodes than the metastatic cells. These results showed that CAM5.2 could be incorporated into this study with no alteration to the standard staining procedure. The optimum titre was determined as the greatest dilution before staining intensity decreased, which was 1:8.

4B.2.2. Pilot Study: To Determine if IAP Staining With HMFG1, HMFG2 and E29 Reveals Breast Carcinoma Metastases in the Axillary Lymph Nodes of Patients Diagnosed as Node Negative but with a Poor RFS.

Lymph nodes from a retrospective series of 13 patients with primary breast carcinoma were histologically diagnosed as node negative and yet the patients had a short RFS. A total of 58 lymph nodes was available. The number of nodes excised from each patient ranged from 1-12 with a mean number of 4 and a mode number of 1. The clinical details of the patients are described in (2.1.3.1.).

Four consecutive 4 μm thick sections were cut from each block of tissue. The primary tumour was stained with HMFG1, HMFG2 and E29 in the standard IP staining procedure (2.2.4.1.). The lymph nodes were stained with the same antibodies in the standard IAP staining procedure (2.2.4.2.). TBS controls were included. The lymph node sections were all examined by a scientist so that metastases were detected by the presence of staining and not by the morphological characteristics of the malignant cells. Subsequently the IAP stained sections, the routinely diagnosed H&E stained sections and the IP stained primary tumour were examined together by the scientist and a

pathologist.

Cells stained with HMFG1, HMFG2 and E29 were present in one lymph node of each of 2 patients and cells staining only with HMFG2 were present in one lymph node of a third patient. The staining in the primary tumour of the former 2 patients was extensively intracellular with all 3 monoclonal antibodies. A similar staining pattern was observed in the metastases. In the latter case the primary tumour was stained only with HMFG2 extracellularly in the intercellular spaces. A similar pattern was observed in the metastasis but there was more extensive intracellular staining. The pathologist confirmed that, morphologically, the stained cells were breast carcinoma.

The stained metastases were present in the routinely diagnosed H&E stained section in 2 of the patients but not in the third. In the latter case the metastasis had been revealed by further sectioning through the node. This was the metastasis stained only by HMFG2. The H&E stained sections of the other 2 nodes were circulated amongst the pathologists in the Southampton University Department of Pathology for routine diagnosis. In no instance were the metastases detected.

One of the 2 metastases detected by IAP staining was composed of several clumps of 10-30 cells situated in the sub-capsular sinus. The other was a larger metastasis of many cells located within the lymph tissue towards the periphery. The primary tumour of the first patient was a medullary carcinoma between 2-5cm in diameter and of the second patient was an infiltrating ductal carcinoma <2 cm in diameter.

4B.2.3. A Comparison of the Detection of Breast Carcinoma Metastases in the Axillary Lymph Nodes by Routine Histological Diagnosis and by Immunohistochemical Staining.

The primary breast tumour and axillary lymph nodes from 50 patients were used in this study (2.1.3.2.). Four μm thick sections were cut from each block (level A), stained with H&E (2.2.3.3) and routinely diagnosed by members of the Southampton University Department of

Pathology. Several 4 μm thick sections were cut from each of the lymph nodes (level B). Four from each block were stained with HMFG1, HMFG2, E29 and CAM5.2 in the standard IAP staining procedure (2.2.4.2.). One section was used as a TBS control and one was stained with H&E (2.2.3.3.) and diagnosed by a pathologist. Similarly, sections of the primary tumour were stained with HMFG1, HMFG2, E29 and CAM5.2 in the standard IP staining procedure (2.2.4.1.). One section was included as a TBS control.

A total of 371 lymph nodes were studied. The number of lymph nodes excised for each patient ranged from 1-20 with a mean value of 7 and a mode value of 7. The routine histological examination diagnosed 19 cases as lymph node positive. Metastases were present in 1, 2 or 3 nodes in 14 cases and in 3 nodes in 5 cases.

The IAP stained sections were examined by a scientist so that metastases were detected by staining and not by cell morphology. The IAP stained sections, the H&E stained sections from both level A and level B, and the IP stained primary tumour were examined together by the scientist and the pathologist to confirm all observations.

Comparison of the IAP stained sections with the H&E stained sections from level A and level B distinguished between metastases which were revealed by further sectioning through the tissue and those which were revealed by immunohistochemical staining. After this distinction was made, the detection of metastases by immunohistochemical staining was compared with the detection of metastases by routine diagnosis of H&E stained sections at level A. It was expected that diagnosis of H&E stained sections at level B would be biased.

A comparison of the presence of metastases detected by IAP staining in the H&E stained sections at level A and at level B showed that metastases were present at only 1 level in 4 nodes. The 4 cases where a metastasis was present at either level A or level B but not both levels are shown in Table 4B.2. This table shows that in 2 cases the patient would be diagnosed as lymph node positive at one level of

TABLE 4B.2

Details of the Cases Where Sectioning Through the Axillary Lymph Nodes at Two Levels Revealed More Breast Carcinoma Metastases Than When Sectioned at One Level.

Case Number	No. of Nodes Containing Metastases at Level A	No. of Nodes Containing Metastases at Level B
E253	0	1
E229	8	7
E230	1	0
E181	2	1

These results are from a comparison of the detection of breast carcinoma metastases in H&E stained sections from two levels of each of 371 axillary lymph nodes (4B.2.3.).

sectioning and as lymph node negative at the other level. In the other 2 cases metastases were detected in more lymph nodes at one level of sectioning than at the other. Metastases were present in 23 cases and 71 nodes at level A and in 23 cases and in 69 nodes at level B. Sectioning at 2 levels, both A and B, revealed metastases in 24 cases and 72 nodes. These results were taken into account in the comparison of detection of metastases by immunohistochemical staining and by routine diagnosis.

Routine diagnosis detected metastases in 65 nodes whilst IAP staining detected metastases in 79 nodes, an increase of 17.7%. The characteristics of the metastases detected by IAP staining alone are shown in Table 4B.3. Metastases were detected by IAP staining in the lymph nodes of 4 patients routinely diagnosed as node negative. This resulted in a 17.4% increase of metastasis detection from 19 cases to 23 cases. In 2 of these patients the metastases were small clumps of 2-4 cells situated in the sub-capsular sinus. In both of these cases the metastasis was present only in the section stained by HMFG2. The pathologist confirmed that, morphologically, the cells were breast carcinoma. In 1 patient the metastasis consisted of small clumps of cells and single cells situated within the lymph tissue at the periphery (FIG 4B.2) and although the primary tumour stained with all four monoclonal antibodies, the metastases stained only with HMFG2 and CAM5.2. In the fourth case clumps of approximately 50 metastatic cells were observed in 2 lymph nodes and were not originally identified because the morphology of the cells was similar to that of the surrounding histiocytes (FIG 4B.2). In this case the metastatic cells were stained with all of the panel of monoclonal antibodies. The remainder of the metastases were detected in 4 patients routinely diagnosed as lymph node positive. All of these metastases were situated within the lymphoid cells and all except 1 were deposits of more than 100 cells. All of the metastases detected by immunohistochemical staining were micrometastases (<2mm in diameter).

The clinical details of the patients in whom an improvement in the detection of metastases was made are shown in Table 4B.4. In all of

TABLE 4B.3

The Characteristics of the Metastases from Primary Breast Cancer to the Axillary Lymph Nodes Detected by IAP Staining with HMFG1, HMFG2, E29 and CAM 5.2 but Not by Routine Examination.

Patient	Size and Position of Metastasis	IAP Staining by: HMFG1	HMFG2	E29	CAM5.2
E169	A clump of 4 cells in the sub-capsular sinus	/	+	/	/
E176	A clump of several cells and single cells in the periphery of the lymph tissue	+	-	-	+
E178	Several clumps of approximately 50 cells situated in the cortex of 2 lymph nodes	+	+	+	+
E219	A clump of 3 cells in the sub-capsular sinus	/	+	/	/
E220	A clump of > 100 cells; A clump of 3-4 cells both in the periphery of the lymph node	+	+	+	+
E222	Clumps of > 100 cells in the periphery of the lymph node	+	+	+	+
E237	A clump of > 100 cells; Several small clumps and isolated cells within the lymph tissue	+	+	+	+
E258	A clump of > 100 cells and several single cells situated within the lymph tissue	+	+	+	+

+ metastasis stained
- metastasis not stained
/ metastasis not present in section

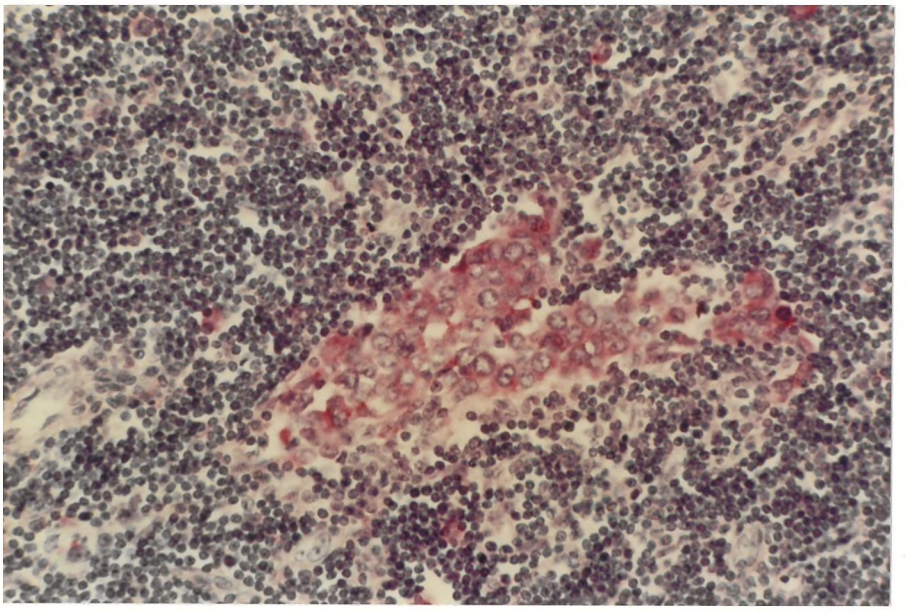
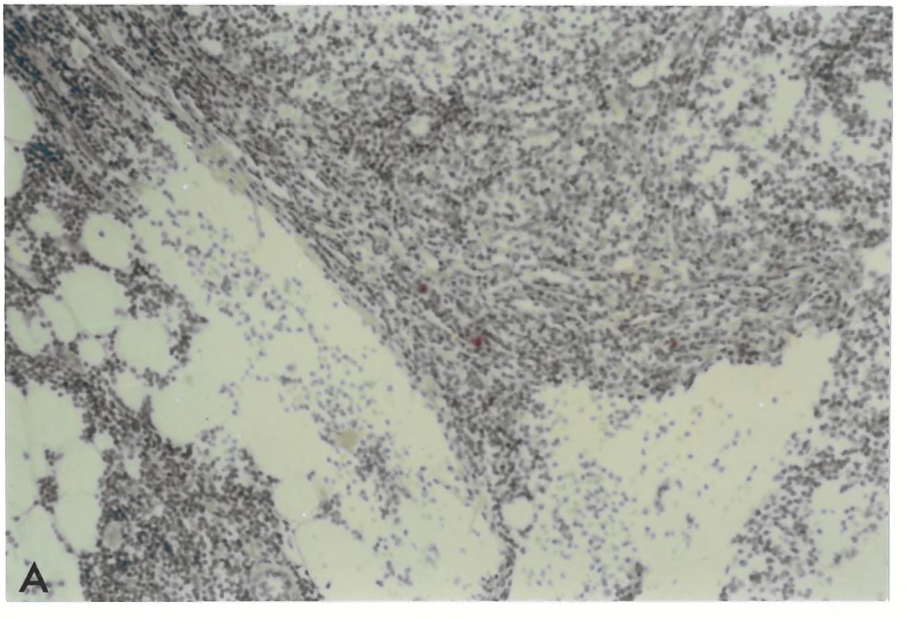
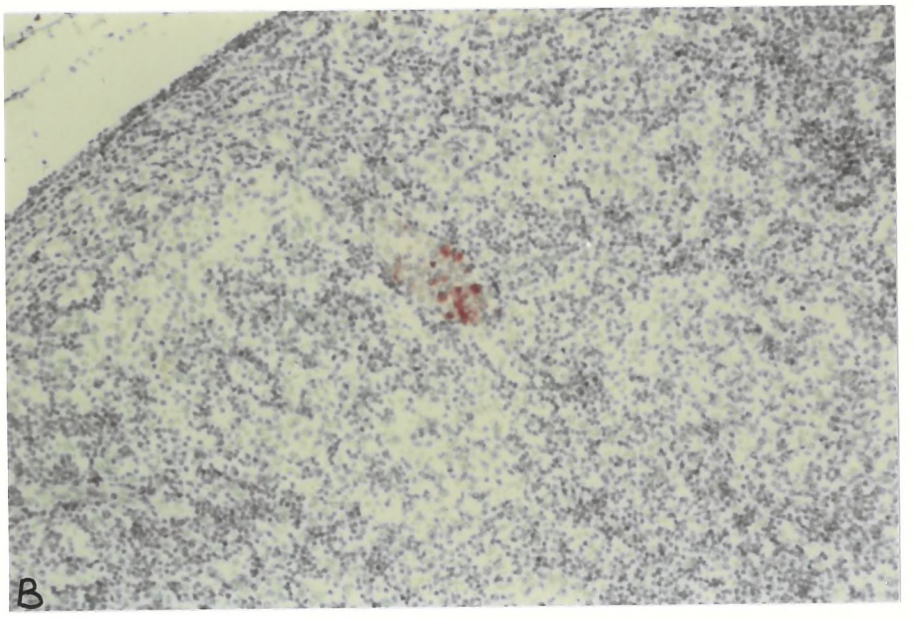


FIG 4B.1

A metastasis of primary breast cancer to the axillary lymph node stained with HMFG2 in a double-bridge IAP technique (4B.2.1.2.). Magnification x 350.





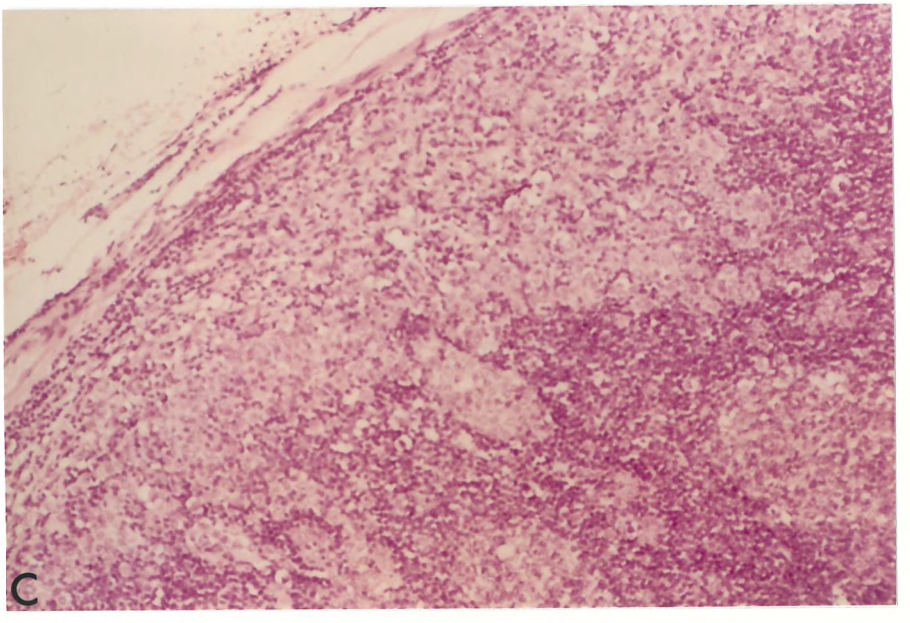


FIG 4B.2

IAP staining with a panel of monoclonal antibodies directed against HMFG and against cytokeratins detects both single (A), and large clumps of (B), metastatic breast carcinoma cells in the axillary lymph nodes. These were not detected by routine histological examination of the H+E stained section (C) (4B.2.3). Magnification x150.

TABLE 4B.4

The Clinical Details of Patients in Whom Metastases Were Detected by Immunohistochemical Staining but Not by Routine Diagnosis

Case Number	No. of +ve Nodes Detected by Routine Diagnosis	No. of +ve Nodes Detected by Routine Diagnosis	Tumour Size (cm)	ER Status	Bloom's Grade	Histological Type of Tumour
E222	1	2	2-5	+	2	Infiltrating ductal Ca
E220	3	5	2-5	+	2	Infiltrating ductal Ca
E219	0	1	2-5	-	2	Infiltrating ductal Ca
E237	7	12	2-5	+	2	Infiltrating lobular Ca
E176	0	1	2-5	+	1	Infiltrating ductal Ca
E178	0	2	2-5	+	2	Mixed ductal & lobular Ca
E169	0	1	2-5	+	2	Infiltrating ductal Ca
E258	9	10	2-5	+	2	Infiltrating lobular Ca

the cases the primary tumour was 2-5 cm in diameter, and in all but 1 case the ER status was positive. In 7 cases the Bloom's Grade was 2 and in the eighth case it was 1. An increase in the detection of metastases was made in 5/38 (13.1%) cases histologically diagnosed as infiltrating ductal carcinoma, 2/7 (28.6%) cases of lobular carcinoma and 1/1 case of mixed ductal and lobular carcinoma.

Two further cases were routinely diagnosed as node positive but with some doubt as to whether the cells might be sinus histiocytes. In both of these cases the metastases stained with all 4 monoclonal antibodies which confirmed that they were carcinoma cells. The primary tumour from both of these cases was histologically diagnosed as infiltrating ductal carcinoma and showed no unusual histological features.

An examination was made of the IAP staining with the 4 antibodies in the lymph nodes of the 23 node positive patients. Histologically the metastases showed the same variability observed in primary carcinoma both between tumours and between different areas of a tumour. The histology of the secondary tumour was representative of that in the primary tumour although variability resulted in some differences. All of the metastases from a single primary tumour also showed variable histology, part of which corresponded with that in other metastases and in the primary tumour.

Double-bridge enhanced IAP staining did not give the same cytological detail of antigen distribution as IP staining. Despite this, similar staining patterns to those observed in primary breast carcinoma (4A.2.1.) could be identified in the metastases, although staining was more extensive intracellularly. Where the metastasis had formed tubules, staining intensity with the monoclonal antibodies against HMFG was strongest extracellularly on the luminal surface of the tumour cells and in luminal secretions. In less differentiated tissue the staining intensity was strongest either extracellularly in the intercellular spaces, or intracellularly around the periphery or diffusely in the cell. There was an area of diffuse intracellular staining in all except one metastasis. Staining of intracytoplasmic

lumina was rarely observed. The staining intensity of the 3 monoclonal antibodies raised against HMFG differed slightly in each metastasis. Granular intracellular staining of plasma cells was occasionally observed with these antibodies. Staining with CAM5.2 was also heterogenous but always gave a diffuse intracellular staining pattern as in the primary tumours. CAM5.2 did not stain plasma cells.

A comparison was made of the IAP staining in the corresponding primary and secondary tumours. Both the primary and the secondary tumour stained with all 4 monoclonal antibodies in 17 of the 23 node positive cases. In 2 cases (mentioned previously) metastases were present only in 1 section and were stained only with HMFG2. The secondary tumour was stained with HMFG1 and CAM5.2 in 1 case and with HMFG2 and CAM5.2 in 2 cases where the primary was stained with all of the antibodies. The staining pattern of the antibodies against HMFG in the primary tumour of these cases was negligible, with traces of extracellular stain in the intercellular spaces, and no staining. In the remaining node positive case the primary tumour stained only with CAM5.2 whilst the metastases stained with all 4 monoclonal antibodies in a diffuse intracellular staining pattern.

Different procedures were used to stain the primary and the secondary tumours, so staining intensity in the primary and secondary tumours was not compared and comparisons in the staining pattern were made tentatively. The staining pattern of antibodies against HMFG in the secondary tumour reflected that in the corresponding primary tumour but staining of the metastasis was always more extensive intracellularly than in the primary tumour. The staining of different metastases from the same primary tumour was also similar. The staining pattern of the metastases did not resemble that of the primary in 4 cases, (including the case where the metastases stained but not the primary). IAP staining of the metastases with CAM5.2 was always similar to that in the primary tumour, diffusely intracellular with slight heterogeneity in the staining intensity between different areas of the tumour.

Immunohistochemical staining made clear the pattern of metastatic infiltration. In most cases the metastases infiltrated from the sub-capsular sinus and medullary sinus into the lymph tissue. In some cases the tumour was established on the periphery of the lymph tissue near the sinuses. In others the metastatic cells appeared to infiltrate the lymph node singly and establish a larger secondary deposit nearer the centre of the node. Occasionally metastatic cells were observed both in a large metastatic deposit and dispersed singly throughout the node in such areas as the germinal centre. These cells stained with the monoclonal antibodies against both HMFG and CAM5.2. Metastases from both infiltrating ductal and lobular carcinoma consisted of clumps of cells but the arrangement of the cells within the clump differed. In metastases from infiltrating ductal carcinoma the cells were either randomly arranged or were structurally differentiated into tubules. Cells in the metastases of lobular carcinoma were arranged in columns.

4B.3 Discussion.

4B.3.1. Immunohistochemical Staining of Metastases from Primary Breast Cancer to the Axillary Lymph Nodes.1.

The aim of this study was to compare the detection of metastases from primary breast cancer to the axillary lymph nodes by immunohistochemistry and by routine diagnosis. An appropriate immunohistochemical method for this purpose is the unlabelled bridge IAP method described by Cordell et al. (1984). These workers suggest that there are 3 advantages to using this method over the indirect IP method. Firstly, using a bridge method emphasises the detection of antigens so that they are clearly visible by light microscopy. The cytological distribution of the antigen was not so clear as when the IP method was used (4B.2.3) but such precise information was not required for this study. Secondly, the bright red colour of the reaction product of alkaline phosphatase with Naphthol ASBI contrasts with the blue nuclear H&E counterstain more than the brown colour of the peroxidase-DAB reaction. Thirdly, unlike breast tissue, lymph

tissue contains eosinophils which exhibit peroxidase activity. Although endogenous peroxidase activity can be blocked, any possible confusion of results is eliminated if the IAP technique is used. Tissue fixation and processing destroy the alkaline phosphatase enzyme. This was proven by absence of staining in the TBS controls (4B.2.1.2). An IAP technique, either double-bridge or indirect has been successfully used in the immunohistochemical detection of metastatic breast carcinoma cells in the bone marrow (Dearnaley et al., 1981; Redding et al., 1983).

It was important that the monoclonal antibodies used in this study were specific for antigens present in breast carcinoma cells but not cells of the lympho-reticular system. Also that the antigens were preserved in NBF fixed, paraffin wax embedded tissue, as that was the only source of lymph nodes available. It was originally reported that the monoclonal antibodies raised against HMFG, HMFG1, HMFG2 and E29, recognise antigenic epitopes present only in epithelial cells (Arklie et al., 1981; Taylor-Papadimitriou et al., 1981; Gatter et al., 1984). After this study was begun, work was published showing that the HMFG2 and E29 antigenic epitopes were present on plasma cells in a variety of lymph nodes, including 46 of 53 axillary lymph node samples (Delsol et al., 1984). In all of these lymph nodes less than 50% of the plasma cells were stained and in many samples only occasional positive plasma cells were detected. Retrospective observation of the sections after this work was published showed that granular intracellular staining was occasionally observed in the plasma cells.

To eliminate any possibility of confusion of plasma cells with metastatic breast carcinoma cells, a monoclonal antibody against an antigen other than HMFG was included in the study. Cytokeratins are intermediate filaments present only in epithelial cells (Moll et al., 1982). The immunohistochemical detection of cytokeratin in cells in a lymph node indicates that the cells are of epithelial and not lymphoid origin. CAM5.2 is a monoclonal antibody directed against a low molecular weight cytokeratin present in normal and malignant breast epithelial cells (Makin et al., 1984). It is also preserved in

formalin fixed paraffin wax embedded material and was, therefore, suitable for this study.

The preliminary experiments to determine a standard IAP staining procedure were completed before the inclusion of CAM5.2. The same lymph nodes used in the preliminary experiments were stained with CAM5.2 in the standard IAP staining procedure. Both the primary breast carcinoma and the metastatic cells stained intracellularly and diffusely with CAM5.2 at an appropriate titre. This is the same staining distribution as that described for CAM5.2 by Makin et al. (1984). These results allowed CAM5.2 to be included in the study with no alterations to the standard IAP staining technique. As well as staining all of the lymph nodes with monoclonal antibodies against HMFG and CAM5.2 antigens, a further check that the cells were metastatic breast carcinoma was pathological confirmation of their morphology.

In the initial experiment (4B.2.1.2.) only weak staining was observed in the breast carcinoma metastases when the lymph nodes were stained with varying dilutions of HMFG1, HMFG2 and E29 in the IAP technique. A comparison was made of the staining in frozen tissue and in NBF fixed, paraffin wax embedded tissue from the same lymph node (4B.2.1.2.). Staining intensity was strong in the frozen tissue but negligible in the NBF fixed, paraffin wax embedded tissue, suggesting that immunohistochemical detection of HMFG antigens was reduced by the process of fixation and embedding in paraffin wax. Staining intensity was increased slightly by trypsin digestion for 10 minutes, but, depending on the antigenic epitope and the lymph node stained, was as strong as the staining intensity in the frozen sections after 20 or 30 minutes trypsin digestion.

The cell and tissue morphology was not affected by trypsin digestion for 20 or 30 minutes. These results differ from the immunohistochemical staining of primary breast tissue where 10 minutes trypsin digestion was sufficient to reveal HMFG antigen in NBF fixed, paraffin wax embedded tissue and the tissue was overdigested after 20

minutes trypsin digestion (3.2.1.). The effect of tissue fixation on immunohistochemical staining is discussed in Chapter 3 (3.1.1). The number of cross-links formed by an aldehyde based fixative, such as NBF, depends on the number of reactive sites available. This varies in different tissues (Brandtzaeg & Rognum, 1983). Immunohistochemical detection of an antigen can be prevented by these cross-links but is restored by digesting the tissue in trypsin which etches the surface of the section (Curran & Gregory, 1977). The more cross-links formed by the fixative, the greater is the trypsin digestion time required to reveal the antigen. Results from this study suggest that NBF forms more cross-links in lymph nodes than in breast tissue and consequently requires a longer trypsin digestion time to reveal the HMFG antigens. This result is consistent with those in the Southampton University Pathology Department where immunohistochemical demonstration of many antigens in NBF fixed, paraffin wax embedded lymph nodes requires a longer trypsin digestion time than other tissues.

A difference in staining intensity between the three monoclonal antibodies against HMFG in any one metastasis was observed in undigested tissue sections. These differences were reduced by trypsin digestion. This is because the exposure of larger amounts of antigen by trypsin digestion results in maximum staining intensity so that differences between the antigens cannot be detected. This was an advantage in this study, for strong staining intensity with all of the monoclonal antibodies gave the maximum opportunity for detection of metastases.

There was a marginal difference in staining intensity between the sections stained with double-bridge and triple-bridge enhancement. After 30 minutes' trypsin digestion this difference was negligible. As strong staining intensity is achieved by the double-bridge enhancement and this is more economical in time and reagents, it was the method used in this study.

The standard staining procedure was therefore established as 30 minutes' trypsin digestion of the NBF fixed, paraffin wax embedded

lymph nodes, followed by a double-bridge enhanced IAP technique. The procedure is described in full in (2.2.4.2.). With each new batch of antibody or reagent the standard staining procedure was carried out on the tissue used in the control experiments (4B.2.1.2), and any suitable modification made to maintain comparability of staining.

4B.3.2 Immunohistochemical Staining of Metastases from Primary Breast Cancer to the Axillary Lymph Nodes.2.

Similar staining patterns to those in the primary tumours were identified in the secondary tumours. Staining of the 3 monoclonal antibodies against HMFG was similar in each lymph node. Extracellular staining was present either on the luminal surface of cells forming tubules or in the intercellular spaces. Intracellular staining was strongest either towards the periphery of the cells or diffusely in the cytoplasm. Differences in staining intensity between extracellular and intracellular staining were not as great in the lymph nodes as in the primary tumour and there was more extensive intracellular staining. This is because the staining of antigens in the lymph nodes was enhanced in the IAP technique as compared with the IP staining of the primary tumour. Staining of both the primary tumour and the secondary tumour with CAM5.2 was diffusely intracellular in the metastatic cells.

Wells et al. (1984), stained breast carcinoma metastases in the axillary lymph nodes with HMFG2, E29 and a monoclonal antibody directed against a cytokeratin, KL1, in an indirect IAP technique. They found that each of the 3 monoclonal antibodies gave approximately equal intensity and distribution of staining. These results are not consistent with those in this study. Differences in staining procedure, such as the antibody titre and use of trypsin may account for some of the inconsistency in results but it is surprising that there was no difference between the staining distribution of the HMFG and KL1 antigens.

The immunohistochemical staining pattern of a metastasis usually

reflected that in the corresponding primary tumour and other metastases from the same primary. Some differences were observed because of the heterogeneity between different areas of a tumour. Two of the 23 node positive patients contained metastases in only the section stained with HMFG2. Similar staining patterns were observed in the primary and secondary tumour in 17 of the 21 remaining cases. In 3 cases staining in the primary tumour with the antibodies against HMFG was negligible with traces of stain in the intercellular spaces. The secondary tumour was stained with only HMFG1 and CAM5.2 in 1 of these cases and with only HMFG2 and CAM5.2 in 2 cases. The staining pattern in all 3 of these metastases was predominantly extracellular. The primary tumour of one patient stained only with CAM5.2 whilst the metastases stained intracellularly with all 4 monoclonal antibodies. In 3 other cases the distribution of the HMFG antigens was different in the primary and secondary tumour. These results are consistent with those from a study by Foster et al. (1982b), where breast carcinoma metastases in the axillary lymph nodes were immunohistochemically stained with M8, M18, M19 and M24. The primary and the secondary tumour "generally showed the same staining patterns". MBr1 is a monoclonal antibody raised against membranes of the breast carcinoma cell line MCF7. This showed an 85% concordance of staining of the primary tumour and metastases to the axillary lymph nodes (Mariani Costantini et al., 1984). CAM5.2 stained both the primary tumour and the secondary tumours in a similar diffuse intracellular staining pattern.

As the secondary breast tumours did not always stain with all of the monoclonal antibodies and the presence or absence of stain in the secondary tumours could not be reliably predicted by staining in the primary tumour, it was considered necessary to use a panel of monoclonal antibodies to ensure detection of all the metastases. It could be argued that as CAM5.2 stained all of the breast carcinoma metastases in a strong intracellular stain, it could be used to detect metastases without the support of other antibodies. Metastasis detection, however, is safer if staining by one antibody is confirmed by staining of at least 1 other antibody. The inclusion of E29 was

superfluous in this study and in future studies the panel of monoclonal antibidies used would be HMFG1, HMFG2 and CAM5.2.

As staining in the established secondary tumours was heterogenous, it was impossible to determine whether the original cells which metastasised were stained in a particular staining pattern or in a variety of staining patterns. No particular staining pattern could, therefore, be associated with a cell type which metastasised. Metastases composed of between 3-20 cells were immunohistologically detected in 4 cases. Two of these were situated in the sub-capsular sinus and were stained in a diffuse intracellular pattern. Two were present within the lymph tissue, 1 was stained in a diffuse intracellular pattern and the other was structurally differentiated into a tubule and stained extracellularly. The primary tumour of this last metastasis was also structurally well-differentiated.

There are currently 2 opposing hypotheses of the process of metastasis. One is that the heterogeneity of cells is a representation of phenotypically variant sub-clones, each with a different potential for metastasis (Poste & Fidler, 1980). The other is that it is the properties of the tumour as a whole which determine whether metastasis occurs (Alexander, 1984). It is not known whether the metastases composed of a few cells would all establish substantial secondary deposits. Despite this, the micrometastases detected in this study, including those which had invaded the lymph node tissue rather than remained in the sub-capsular sinus, showed different staining patterns to one another. This suggests that cells with different staining characteristics, and therefore from phenotypically variant sub-populations of tumour cells are able to metastasise and supports the latter hypothesis of metastasis.

4B.3.3. A Comparison of the Detection of Metastases by Immunohistochemical Staining with the Monoclonal Antibodies HMFG1, HMFG2, E29 and CAM5.2.

In the pilot study metastases were immunohistochemically detected in

the lymph nodes of 2/13 (15.4%) patients who were histologically diagnosed as node negative and yet who relapsed within 6 years. This showed that immunohistochemical staining with a panel of monoclonal antibodies improves the detection of breast carcinoma metastases in the axillary lymph nodes. The clinical details or histological type of tumour in these 2 patients gave no indication as to whether there might be a group of patients whose metastases were particularly difficult to identify.

It is surprising that only 15.4% of these patients with a poor survival showed evidence of metastasis in the axillary lymph nodes. The reason for this could be that metastatic cells had spread along routes other than the lymph circulation (4.1.2). Also, the average number of lymph nodes available for examination was 4, which may be too few for adequate representation of the lymph node status.

The main study was carried out on 371 lymph nodes from 50 patients with primary breast carcinoma. Sections of the lymph nodes were immunohistochemically stained with a panel of monoclonal antibodies HMFG1, HMFG2, E29 and CAM5.2 in the standard IAP staining procedure established in this study. Immunohistochemical detection of metastases improved the number of positive nodes from 65 by routine diagnosis to 79, an increases of 17.7%, and the number of positive cases from 19 by routine diagnosis to 23, a 17.3% increase. The metastases detected by immunohistochemical staining alone were micrometastases ranging from 2-3 cells to more than 100. Two metastases consisting of 2-3 cells were present in the sub-capsular sinus and the remaining 12 metastases were present in the lymph tissue.

Two of the metastases detected were present only in the section stained with HMFG2. The pathologist confirmed that morphologically these cells were breast carcinoma. All of the other metastases stained with CAM5.2 and at least one of the antibodies raised against HMFG, confirming that they were metastatic breast carcinoma. Granular intracellular staining with the antibodies raised against HMFG was observed in a small percentage of plasma cells. Plasma cells did not

stain with CAM5.2 and were morphologically distinct from breast carcinoma cells.

There have been 2 other studies where a comparison has been made between the detection of breast carcinoma metastases in the axillary lymph nodes by routine diagnosis and by immunohistochemical detection. Sloane et al. (1980), stained lymph node biopsies of 31 patients with anti-EMA in an indirect IP method. All of the histologically detected metastases stained with anti-EMA but no staining was observed in the 23 cases (173 nodes) histologically diagnosed as negative. These workers do not state how strong the staining intensity in the metastases was. If the staining was weak, small metastases may have remained undetected, especially as the staining reaction product was brown and did not contrast well with the H&E counterstain.

Wells et al. (1984), stained the axillary nodes of 57 cases of breast carcinoma with HMFG2, E29 and a monoclonal antibody directed against a cytokeratin, KL1, in a double bridge IAP technique. Metastases not detected by routine diagnosis were immunohistochemically detected in 12/57 (20%) of the cases. As in this study, metastases were present either as single cells or as small clusters of cells either in the sub-capsular and medullary sinuses or within the lymphoid cells. Occasionally the metastases were widely disseminated through the lymph tissue.

In both this study and that of Wells et al., more metastases were detected when the tissue was sectioned at 2 levels than when it was sectioned at 1 level. Wells et al. did not distinguish between metastases detected by further sectioning through the tissue and those detected by immunohistochemical staining. In this study, those metastases revealed by further sectioning through the block were identified before the comparison of metastasis detection between routine diagnosis and immunohistochemical staining.

An improvement in the detection of metastases was made in 3/8 (37.5%) cases where the primary tumour contained a lobular component and in

5/38 (13.1%) cases where the primary tumour was an infiltrating ductal carcinoma. Although the tumour cells of metastases from lobular carcinomata were in clumps, the arrangement of the cells in the clump was in columns, rather than randomly or into tubules as in the metastases from a ductal carcinoma. This cell arrangement probably made difficult the identification of metastases. Similar results were found by Wells et al. (1984), where metastases were immunohistochemically detected in a higher proportion of cases where the the primary tumour was a lobular tumour than when it was a ductal tumour. In one case where a metastasis was detected by immunohistochemical staining, the morphology of the cells was similar to sinus histiocytes and may have been regarded as such in the routine diagnosis. Immunohistochemical staining helped in the detection of metastases whose histology or whose small size made difficult their identification by histological diagnosis.

The effect of metastatic invasion of the axillary lymph nodes on the survival of patients with breast carcinoma has been discussed in the introduction to this chapter (4.1.2). The presence of metastases in the axillary nodes is an indication of poor RFS (Valagussa et al., 1978; Fisher et al., 1983). The RFS is worse when >3 nodes are involved. If a treatment protocol was based on the presence and extent of metastases in the lymph nodes, patient management would have been altered in 5/50 cases in this study.

It is not appropriate, however, to base patient management on the presence of immunohistochemically detected metastases in the lymph nodes, for the effect of such metastases on survival is not accounted for in the original studies. All of the metastases detected by immunohistochemical staining were micrometastases and in several cases were not identified by routine diagnosis because of their particularly small size. The effect of micrometastases on survival was discussed in the introduction (4.2.1) and the site of the metastasic deposit is an important factor. Metastatic cells in the sub-capsular sinus have a different effect on survival than those in the lymph tissue (Fisher et al., 1978b). A study of the effect on survival of such metastases is

beyond the scope of this project as it would involve staining very large numbers of lymph nodes in order to obtain sufficient data for statistical analysis.

Before clinical applications are considered, it is also necessary to establish that small clumps of cells detected by immunohistochemical staining are metastatic breast carcinoma cells and not circulating normal breast cells or sinus histiocytes containing circulating epithelial antigens. Material was not available to determine whether the clumps of cells detected in the axillary nodes from breast carcinoma patients are also present in the nodes from patients with benign breast disease. Hendler & House (1985), have used a panel of monoclonal antibodies, H59, H71 and H72, to stain, by radioimmunoassay, the axillary lymph nodes of women either with breast cancer or who underwent mastectomy because of a high risk of breast cancer or because of hyperplastic fibrocystic changes. These antibodies are raised against the breast carcinoma cell line ZR-75-1 and are directed against cell surface glycoproteins. The H59 and H72, but not the H71 antigenic determinants are secreted. There was binding of the H59 and H72 antibodies to sinus histiocytes and perivascular cells within the sinuses in the axillary nodes of women both with and without breast carcinoma. They suggested that this staining was a result of secreted antigens phagocytosed by the sinus histiocytes. This staining was not observed in this study, probably because of the different techniques used to detect the antigens. This binding was distinguished from the more intense staining of all three antibodies to clumps of cells and to isolated cells found in the axillary nodes only from patients with breast cancer.

Immunohistochemical staining helped both in the detection of metastases and, in 2 cases, in the determination of whether clumps of cells were sinus histiocytes or metastatic breast carcinoma. Also the contrast between breast carcinoma cells and lymph cells shown by IAP staining improved the efficiency as well as the accuracy of diagnosis. Immunohistochemical staining may also improve the accuracy of the lymph node status as a prognostic indicator.

CHAPTER 5

AN INVESTIGATION TO DETERMINE WHETHER IMMUNOCYTOCHEMICAL DETECTION OF HMFG1 AND HMFG2 ANTIGENS CAN IMPROVE THE DIAGNOSTIC ACCURACY IN NEEDLE ASPIRATES OF BREAST TUMOURS.

5.1. Introduction.

A suspicious breast lump is presently diagnosed as benign or malignant by using three facilities, the clinical examination, the radiographic evidence (e.g. xeromammography, ultrasonography) and the cytological diagnosis of needle aspirates (Meirion Thomas et al. 1978). When combined, these three facilities can give a diagnostic accuracy of 95% (Meirion Thomas et al., 1978). Cytological diagnosis of needle aspirates from palpable tumours is the most accurate. (Meirion Thomas et al., 1978; Coleman et al., 1975; Smallwood et al., 1984). Needle aspiration is not considered a useful technique in diffuse, non-palpable lesions because it is not possible to aspirate accurately from the diseased area of the breast (Gardecki et al., 1980). It is possible, however, to aspirate from a mammographically detected lesion using ultrasonography as a guide.

To prepare the needle aspirate for diagnosis, it is smeared onto microscope slides. Then it is either air-dried and stained with either May-Grunwald Giemsa or Diff-quik (Harleco), or fixed in alcohol and stained using the Papanicolaou technique (Franzen & Zajicek, 1968). The diagnosis of these preparations is based upon three criteria (Franzen & Zajicek, 1968):-

a) Cell spread. Cells which are tightly adhesive and remain in clumps are more likely to be benign than if the cells are isolated.

b) Cell and nuclear morphology. Cells with large nuclei, showing the nuclear abnormalities of malignancy, such as an irregular nuclear margin or pronounced chromatin are indicative of a malignant lesion.

c) Component cells. The presence of bipolar cells, which are the

myoepithelial cells, suggests a benign lesion.

When evaluable smears are obtained, the diagnosis of needle aspirates is around 90% accurate (Table 5.1). The frequency of technically unsatisfactory aspirates ranges from 5% to 25% (Table 5.1). Technically unsatisfactory aspirates are usually due to the presence of too few, or no cells. This type of aspirate is more frequently aspirated from benign than from malignant breast lesions because greater cellular adhesivity in the former prevents the cells from being sucked up the needle (Gardecki et al. 1980). When the number of technically unsatisfactory aspirates is taken into account, the diagnostic accuracy is decreased to about 70% (Table 5.1).

When patient management and treatment is based wholly or partially on any diagnosis it is necessary to take into account the possibility of false positive and false negative results. In aspiration cytology, incorrect diagnoses can result when there are a few cells present with the distinctive characteristics of a particular condition but not sufficient for a definitive diagnosis. Also, cell characteristics can be misleading. For example, in well-differentiated carcinoma or small cell carcinoma the cells could be confused with those of a benign lesion (Zajdela et al., 1975). The possibility of inappropriate mastectomy as a result of inaccurate needle aspirate diagnosis is kept to a minimum by dividing the diagnoses of malignancy into two groups, "probably carcinoma" and "definitely carcinoma". Similarly, diagnosis of benign lesions can be divided into "probably benign" and "definitely benign". The patient can then be treated appropriately. The number of cases in the groups where the diagnosis is not definite is between 3-10% (Table 5.1).

In the Southampton Breast Clinic, the results of the clinical examination, mammography and ultrasonography, and needle aspiration cytology are combined (Smallwood et al., 1984). "Definitely malignant" patients undergo a mastectomy, whilst "probably malignant" patients have either a tru-cut or, rarely, a frozen section performed. All of the benign diagnoses have an excision biopsy and if the histological examination of the tumour shows that it is

TABLE 5.1

A Comparison of Investigations of the Accuracy of Cytological Diagnosis of Needle Aspirates from Palpable Breast Lesions.

Reference	Number of Lesions	Technically Unsatisfactory Aspirates	Overall Correct Diagnosis of Evaluable Aspirates	Number of Cases Diagnosed as 'Possibly' Benign or Malignant	False +ves	False -ves
Franzen & Zajicek, 1968	1,686	-	75.8%	8.4%	1	94
Furnival et al., 1975	237	24.8% (59/237)	95.5%	1%	2	5
Zajdela et al., 1975	2,772	5.5% (155/2772) Benign 6.4% carcinoma 5.1%	93.3%	3.7%	3	63
Meirion-Thomas et al., 1978	196	18.9% (37/196)	95%	8.7%	-	-
Gardecki et al., 1980	211	26.5% (56/211)	81%	6%	-	-
Smallwood et al., 1984	224	-	98.6%	Equivocal and unsatisfactory not separated.	-	2

malignant, the patient returns to surgery for appropriate treatment. In Southampton there have been no false positive diagnoses in aspiration cytology. Incorrect diagnoses have, however, been documented. False positive results are diagnosed at a frequency of less than 1% (Table 5.1).

Immunocytochemical staining of needle aspirates for an antigen which distinguishes normal and benign breast cells from malignant breast cells, either by it's presence or by it's cellular distribution, might be a valuable tool in cytodiagnosis. It is possible that fewer cells would be required for an accurate diagnosis based on the presence and distribution of antigen than are required for classical cytological diagnosis. The number of technically inadequate cases may then be reduced. Also the distinction between benign and malignant tumours would be clearer in those cases previously diagnosed as "possibly malignant" and "possibly benign". This would facilitate patient treatment, as frozen sections or 2-stage surgery could be eliminated or reduced in number. This would reduce both the psychological burden of uncertainty on the patient and the time and money spent in surgery.

Unfortunately, such a hypothetical situation cannot be realised presently, as, to date, there is no antibody which distinguishes completely between benign and malignant cells. Immunocytochemistry with breast carcinoma-associated antigens, in combination with cytological diagnosis, however, might improve the diagnostic accuracy of needle aspirations. This method has been used to distinguish between malignant cells and benign mesothelium in pleural and peritoneal effusions (Table 5.6).

HMFG1 and HMFG2 antigens are possible candidates for the distinction of benign and malignant breast cells in needle aspirates. Immunoperoxidase staining of normal, benign and malignant breast tissue sections shows that HMFG1 and HMFG2 antigens can be found extracellularly in the ducts and tubules of normal, benign and well-differentiated malignant breast tissue. Intracellular staining

was rarely observed in normal and benign breast tissue, whilst 95% of breast carcinomata stained intracellularly to some extent (4.2.1.). It might be possible to distinguish between aspirates from benign and malignant breast lesions on the basis of intracellular staining with HMFG1 and HMFG2. As intracellular staining is not entirely confined to malignant breast lesions, the diagnosis of aspirates by immunostaining should be combined with routine cytological diagnosis.

5.1.2. Aims of this Study.

1. To establish a procedure which would result in distinct and accurate immunostaining of aspirated breast tumour cells with HMFG1 and HMFG2, and which could be combined with routine cytological diagnosis. This involved:-
a) Observation of the effects of permeabilising and processing aspirated breast tumour cells on the staining of HMFG1 and HMFG2 antigens.
b) Establishment of the optimum technique for demonstration of HMFG1 and HMFG2 by immunocytochemical staining.
c) Combination of immunocytochemical staining of the aspirate with routine cytological diagnosis.

2. To investigate the use of the monoclonal antibodies HMFG1 and HMFG2 in improving the distinction between benign and malignant breast tumour aspirates using the established staining procedure.

5.2. Experiments.

5.2.1. Technique Control and Development for Cell Aspirations.

5.2.1.1. Titration of the Monoclonal Antibodies HMFG1 and HMFG2 in Breast Cell Preparations.

A series of cytocentrifuge preparations (cytopreps) of T47D cells (2.1.4.1.) were permeabilised and fixed for 20 minutes (2.2.1.7.). The cytopreps were stained in an indirect IP technique (2.2.3.1.). HMFG1

and HMFG2 were used as the primary antibody and peroxidase-conjugated swine anti-mouse serum was used as the second antibody. The antibodies were used at various dilutions and NHS was added to the second antibody at various dilutions as in 3.2.4..

Both HMFG1 and HMFG2 gave similar results. No background staining was observed and no staining was observed in the TBS controls (Table 5.2). The optimum titres were determined as :-
HMFG1 & HMFG2 - 1/2
Peroxidase-conjugated swine anti-mouse serum - 1/50

Although no staining was observed in the TBS controls, NHS at a dilution of 1/50 was added to the second antibody to prevent background staining of material present in breast tumour aspirates but not present in cytopreps of T47D cells. Using the antibodies at these dilutions resulted in strong staining of the T47D cells.

5.2.1.2. The Effect of the Inhibition of Endogenous Peroxidase With H_2O_2 in Methanol on the Immunocytochemical Staining of HMFG1 and HMFG2.

Six cytopreps of T47D cells (2.1.3.1.), were permeabilised and fixed for 20 minutes (2.2.1.7.). Three of the cytopreps were treated with a 0.5% solution of H_2O_2 in methanol for 10 minutes to inhibit endogenous peroxidase activity (2.2.2.3.). Both the untreated and the treated cytopreps were stained in the indirect IP technique (2.2.3.1), using HMFG1 and HMFG2 as the primary antibody at 1/2 and peroxidase-conjugated swine anti-mouse antiserum as the secondary antibody at 1/50, with NHS at 1/50. TBS controls were included.

No staining was observed in the TBS controls. In the untreated cytopreps HMFG1 and HMFG2 stained the T47D cells variably. Strong diffuse staining was present in some cells and weaker focal staining was present in others. In the cytopreps where endogenous peroxidase activity was inhibited, the cells were stained more weakly than in the untreated cytopreps. For the remainder of this study endogenous

TABLE 5.2

Determination of the Optimum Titres of HMFG1 and HMFG2 as the Primary Antibody, Peroxidase-Conjugated Swine Anti-Mouse IgG as the Second Antibody and NHS in the Indirect IP Technique on Cytopreps of T47D Cells.

Dilutions of 2nd Antibody (Px conj SwaM IgG)	Dilutions of NHS	Dilutions of Primary Antibody (HMFG1 and HMFG2)			
		Neat	1 : 2	1 : 4	1 : 8
1 : 25	1 : 25	++++	++++	++	0
	1 : 50	++++	++++	++	0
	1 : 75	++++	++++	++	0
1 : 50	1 : 25	++++	++++	++	0
	1 : 50	++++	++++	++	0
	1 : 75	++++	++++	++	0
1 : 75	1 : 25	+++	+++	+	0
	1 : 50	+++	+++	+	0
	1 : 75	+++	+++	+	0

0 none; + weak; ++ fairly strong; +++ strong; ++++ very strong staining intensity

This table shows the results of a titration on permeabilised and fixed cytopreps of T47D cells. Varying dilutions of HMFG1 and HMFG2 were used as the primary antibody, and of Px conj SwaM IgG with NHS as the second antibody in an indirect IP technique (5.2.1.1.).

There was no background staining or staining of the TBS controls.

peroxidase was not inhibited in preparations of breast tumour aspirates.

5.2.1.3. The Effect of Acetone Treatment on Immunocytochemical Staining of HMFG1 and HMFG2.

Cytopreps of T47D cells (2.1.4.1.), were left unfixed or permeabilised and fixed in dehdrated acetone at -20° (2.2.1.7), for either 20 minutes, 1 week or 2 weeks before staining with HMFG1 and HMFG2 in the indirect IP technique (2.2.3.1.). The antibodies were used in the same dilutions as in 5.2.1.2.. TBS controls were included.

The results for HMFG1 and HMFG2 were similar. No staining was observed in the TBS controls. Most of the cells in the unfixed cytopreps were either lost or lysed in the staining procedure. Both intact and lysed cells stained with HMFG1 and HMFG2 but the staining was weak. The cytopreps stained after 20 minutes, 1 week or 2 weeks immersion in acetone showed the same distribution of stain, although the intensity of stain in the cytopreps fixed for 20 minutes was weaker than those fixed for a longer time. From these results it was established that it was necessary to fix and permeabilise cells prior to immunocytochemical staining, and that the HMFG1 and HMFG2 antigens were not destroyed by storage in dehydrated acetone at -20^{0} C for up to 2 weeks.

5.2.1.4. A Comparison of the Direct and Indirect Preparation of Breast Tumour Aspirates for Use in the Diagnosis of Malignancy by Immunocytochemistry.

Immunocytochemical staining of direct (2.3.2.), and indirect (2.3.3.), breast tumour aspirates and of the corresponding NBF fixed (2.2.1.1.), paraffin wax embedded (2.2.1.8.) primary tumour with HMFG1 and HMFG2 was compared in 30 cases (2.1.4.2.). The aspirates were prepared and stained in the indirect IP assay determined for the breast cell aspirate study (2.2.4.3.). TBS control sections were included. The breast tissue was stained in the standard indirect IP

technique determined for this study (2.2.4.1.). TBS control sections were included.

It was difficult to relate the staining of disaggregated cells to the staining of cells composed into structures in a tissue. Also, aspirated cells represent all areas of a lesion, whilst a tissue section represents only part of it. As the immunohistochemical staining of HMFG1 and HMFG2 is heterogeneous , there are likely to be discrepancies when comparing the immunocytochemical staining of a tissue section with that in needle aspirates.

It was more difficult in direct aspirates than in indirect aspirates to relate the staining to that observed in the tissue section. In direct aspirates the cells were smeared over the slide which made difficult the application of the immunocytochemical reagents. This resulted in artefactual variability of staining (FIG 5.1). Also, smeared cells made observation an inefficient task. In indirect aspirates the cells were confined to a well defined area of 6mm by cytocentrifugation, which made efficient both the application of reagents and observation of the preparation.

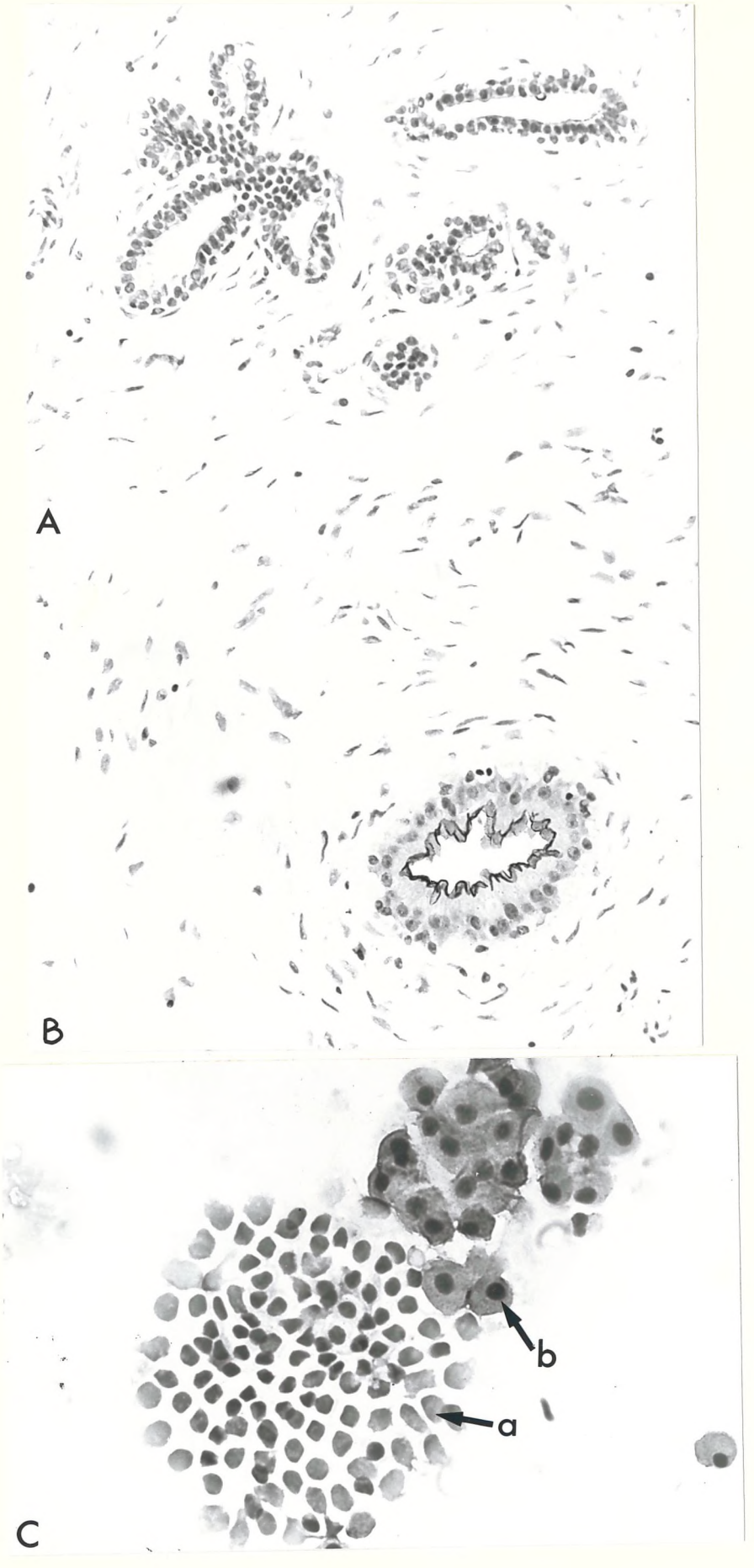
Another problem with direct aspirates was that of background staining which was confused with the staining of the aspirated cells (FIG 5.1). In 6 of the direct aspirates, background staining was observed in the TBS controls as well as in the test preparations. This was methodologic background staining where the second antibody was binding to components in the cytopreps other than the primary antibody. In 22 of the direct aspirates, staining was observed in the test slides only. This was immunologic background staining where the primary antibody was recognising the HMFG antigens. In 8 cases immunologic background staining obscured staining of the cells (FIG 5.1). Immunologic background staining was more intense when the immunohistochemical staining of the tissue section showed extracellular staining of secreted HMFG in the lumina of ducts and tubules. In indirect aspirates methodologic background staining was not observed in any of the cases and immunologic background staining

TABLE 5.3

A Comparison of the Extent of Background Staining Resulting From Immunohistochemical Staining with HMFG1 and HMFG2 of Direct and Indirect Preparations of Needle Aspirates from Palpable breast Lesions.

	Number of Cases with Background Staining in:	
	Direct Preparation	Indirect Preparation
Staining as a result of endogenous peroxidase	0/30	0/30
Methodologic Background Staining	6/30	0/30
Immunologic Background Staining	22/30	2/30

This table compares the background staining observed in direct and indirect preparations of needle aspirates from 30 palpable breast lesions. The preparations were permeabilised, fixed and stained in an indirect IP technique (5.2.1.4.).


A
B
C
b
a

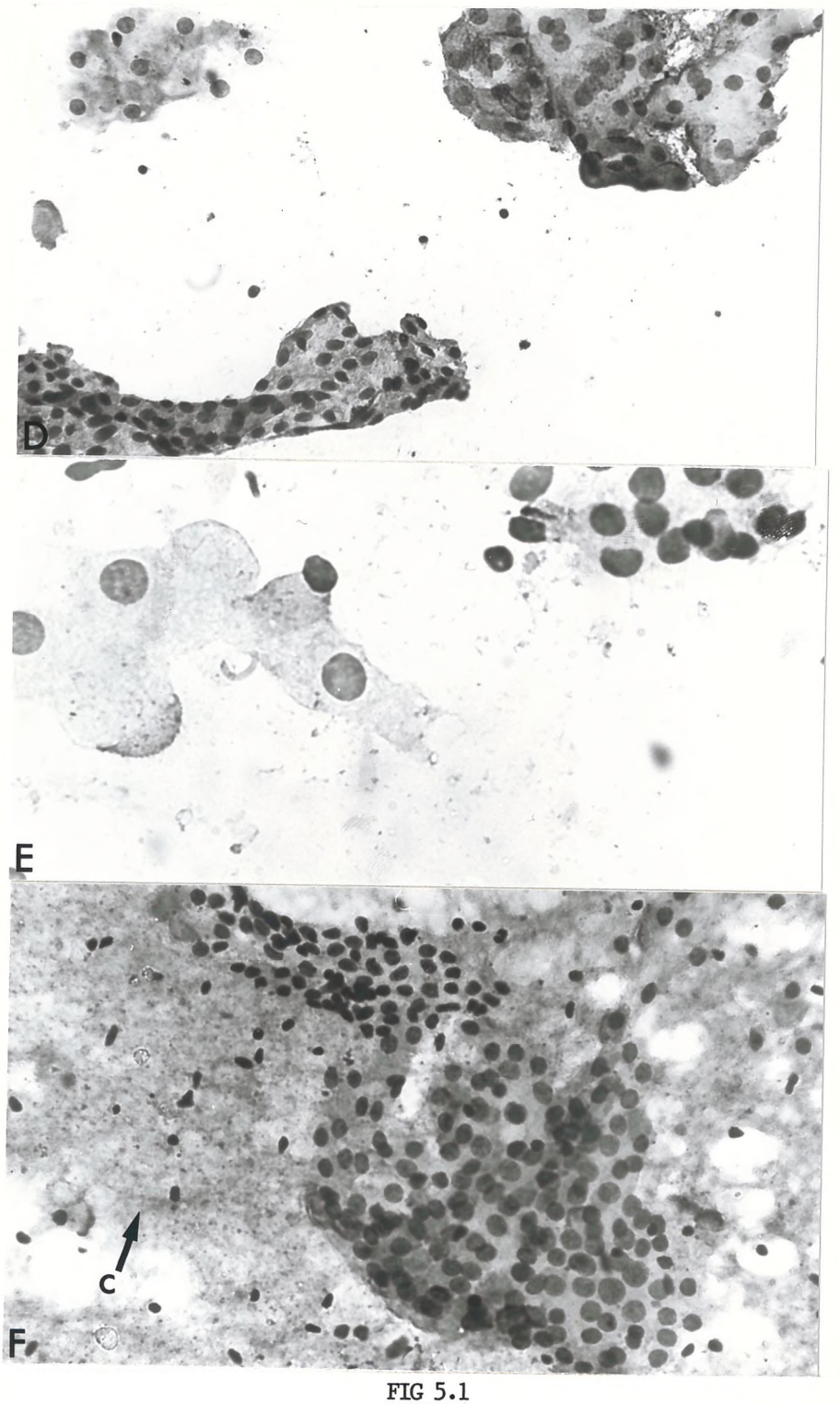


FIG 5.1

Staining of NBF fixed tissue (A, B), an indirect aspirate (C) and a direct aspirate (D, E, F) from the same case of benign breast disease (fibrocystic disease) with HMFG1 in an indirect IP technique (5.2.1.4.). The proliferating ducts and tubules show none, or very weak extracellular staining (A) whilst staining is stronger in tubules with apocrine metaplasia (B).
The staining pattern in the tissue is well represented by the staining in the indirect aspirate (C) where the epithelial cells (a) are not stained and the apocrine cells (b) are diffusely stained. Staining in the direct aspirates is variable (D, E, F), often masked by background staining (c). Also both the epithelial and apocrine cells are stained and staining is not representative of that in the tissue section.
Magnification of tissue x375; Magnification of aspirates x660.

was observed in 2 cases. In neither direct nor indirect aspirates was background staining due to endogenous peroxidase observed.

Despite the problems, there was a similarity between the staining in the aspirated cells and in the tissue section (FIG 5.1). In 2 histologically benign cases and in 2 carcinoma cases there was no staining was present in the indirect aspirate, direct aspirate or tissue section. In the remaining 26 cases heterogeneous staining was observed in the aspirates, both in the number of cells staining and in the distribution of stain. The various types of staining distribution included the following: diffuse staining, granular staining, intense staining around the periphery of the cell and diffuse but with an intense focal area of staining in the cell. Extracellular staining observed in the ducts, tubules and intercellular spaces in tissue sections was not observed as such in the aspirates. In some aspirates extracellular staining was represented by an intense rim of staining around the periphery of the cell. In others, it could not be distinguished from intracellular staining. Extracellular staining was also represented in direct aspirates by immunologic background staining. Intense staining of a focal area within the cell was observed in the needle aspirates, although not to the same extent as in the tissue sections.

The preparation of direct aspirates gave minimal disturbance to the aspirated cells, except that smearing spread out loosely adhering cells. There was little cell damage. The preparation of indirect aspirates occasionally separated cell aggregates and the cells were flattened onto the microscope slide during cytocentrifugation. There was cell damage and, in the washing, cell loss.

The conclusion of a comparison of the immunocytochemical staining of direct and indirect aspirates with HMFG1 and HMFG2 was that cell morphology was better maintained in direct aspirates, but the immunocytochemical staining was distinct and more representative of the immunohistochemical staining pattern in indirect aspirates.

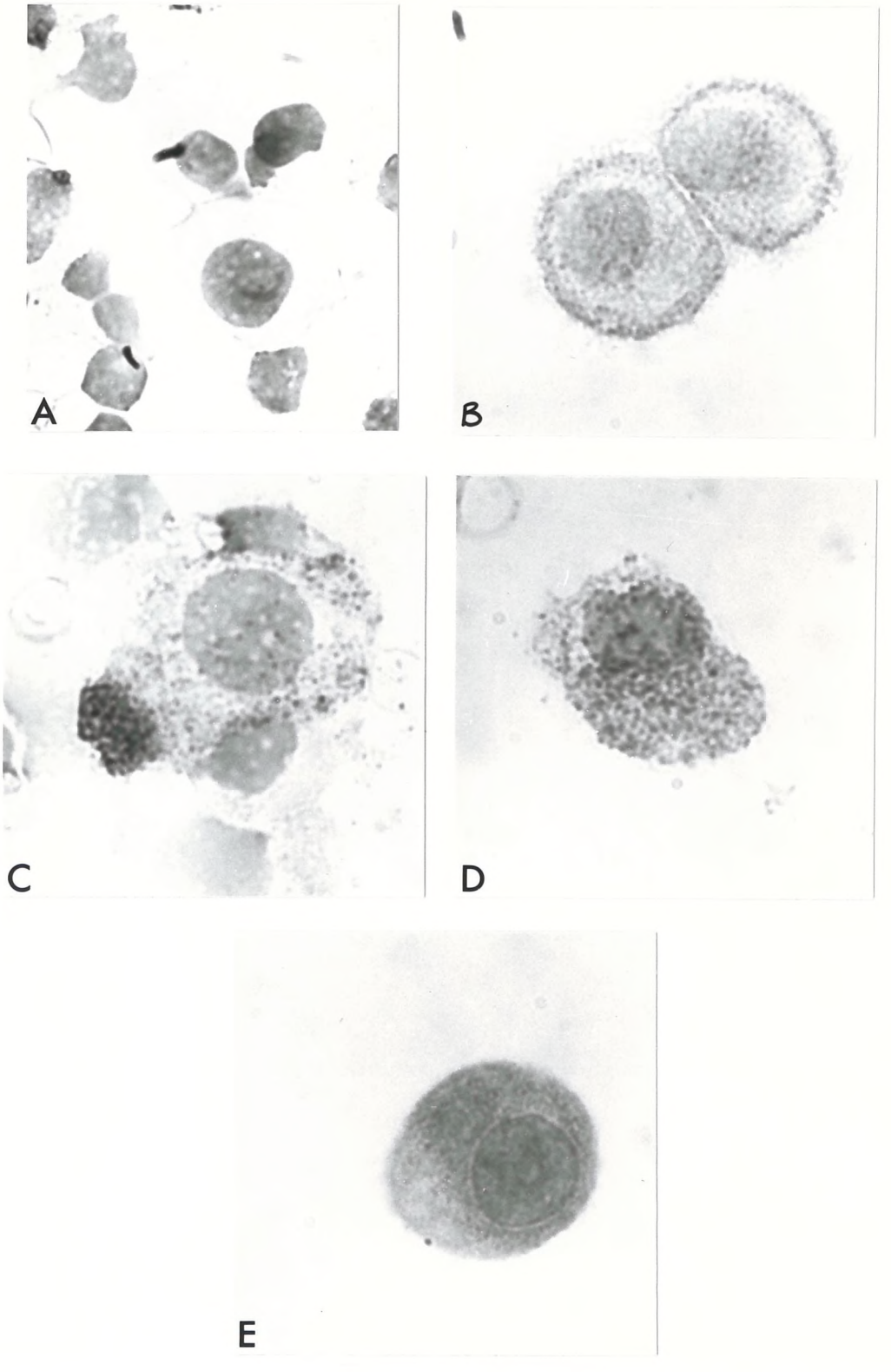


FIG 5.2

Indirect preparations of aspirated cells from 5 cases of infiltrating ductal breast carcinoma stained with HMFG2 in an indirect immunoperoxidase technique (5.2.1.4.). Variable staining patterns are observed: no staining (A), an intense rim of staining around the periphery of the cell, indicative of extracellular staining (B), an intense focal area of staining in the cell (C), granular staining (D) and diffuse staining (E). Magnification x945.

TABLE 5.4

A Comparison of the Immunocytochemical Staining with HMFG1 and HMFG2 of Direct and Indirect Preparations of Needle Aspirates from 30 Palpable Breast Lesions.

	Direct Aspirate	Indirect Aspirate
Clarity of Staining Pattern	1. Cells spread over microscope slide, resulting in inaccurate application of staining reagents and inefficient observation	1. Cells confined to a circle 6mm in diameter. Therefore both the staining procedure observation was accurate and efficient
	2. Extensive background staining	2. Little background staining
Maintenance of Cell Structure and Morphology	1. Cell aggregates and cell morphology retained	1. Cell aggregates and cell morphology often not retained
	2. Occasional cells damaged	2. Several cells Damaged
Representation of the Staining Pattern in the Tissue	Staining of cells is confused by artefactual variability of staining and presence of background staining	The staining patterns in the aspirates and the corresponding tissue section were similar

5.2.1.5. A Comparison of the Immunohistochemical Staining of HMFG1 and HMFG2 Washed in RPMI 1640 or Husains Mucolytic Medium.

In an attempt to further improve the immunocytochemical staining observed in indirect aspirates, aspirated tumour cells from 4 patients (2.1.4.2.) were expressed into 0.5 ml of each of RPMI 1640 and Husains Mucolytic Medium (2.3.3.) The resulting preparations were permeabilised and fixed for 24 hours (2.2.1.7.) and stained in the indirect IP technique determined for this study (2.2.4.3.). In all 4 cases, the cells suspended in Husain's Mucolytic Medium had lysed and staining was observed on the lysed contents of the cells (FIG 5.3). In comparison, the cells washed in RPMI 1640 medium were still intact and the staining of individual cells was accurately observed.

5.2.1.6. Evaluation of Combining Immunocytochemical Staining in Indirect Aspirates with the Routine Diagnosis from Direct Aspirates.

In 49 cases (2.1.4.3.), both direct and indirect aspirates were prepared from the same breast tumour needle aspirate by first preparing direct aspirates (2.3.2.) and then preparing an indirect aspirate from the needle wash (2.3.4.). Generally, 4 direct aspirates were routinely prepared for cytological diagnosis. Two of these were air-dried and stained with Diff-quik (Harleco) and 2 were fixed in absolute alcohol and stained using the Papanicolaou technique (Culling, 1974). These preparations were routinely diagnosed cytologically. Two indirect aspirates were prepared from the needle wash of each case. One of these was stained with HMFG2 in the standard IP technique determined for this study (2.2.4.3.) and the other served as a TBS control. These preparations were observed "blind" and then compared with the cytological diagnosis.

In 17 of the direct aspirates there were insufficient cells present for an accurate cytological diagnosis. Nine of the corresponding indirect aspirates also had insufficient cells present for an immunocytochemical diagnosis. Of the remaining 8, in 7 there were between 10-20 cells and in 1 there were many cells. In these 8

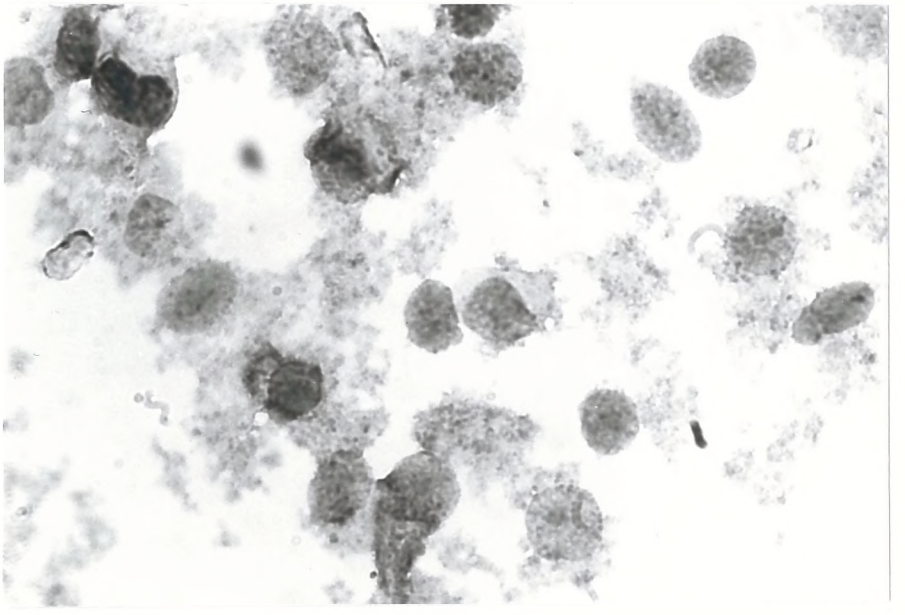


FIG 5.3

Cells from an infiltrating ductal breast carcinoma expressed into Husain's Mucolytic Medium in the indirect preparation of aspirates and stained with HMFG1 in the indirect immunoperoxidase technique (5.2.1.5.). The cells are lysed. Magnification x 945.

cytopreps there were sufficient cells to observe the immunocytochemical staining characteristics. In the remaining 32 aspirates, both direct and indirect aspirates were adequate for a diagnosis. In 23 of the indirect aspirates background staining due to endogenous peroxidase in erythrocytes was observed.

5.2.2. Determination of Whether the Immunocytochemical Staining Patterns with HMFG1 and HMFG2 Can Differentiate Between Benign and Malignant Breast Aspirations.

Indirect aspirates (2.3.3.) were prepared from 65 breast tumours (2.1.4.2.), fixed and permeabilised for 24 hours-1 week (2.2.1.7.) and stained in the standard indirect IP technique determined for this study (2.2.4.3.). The corresponding breast lesions were routinely fixed in NBF, (2.2.1.1.), embedded in paraffin wax (2.2.1.8.) and 4 μm thick sections (2.2.2.1.) were routinely stained with H&E for histological diagnosis. As stated previously (5.2.1.4.), the immunocytochemical staining patterns observed in the needle aspirates were heterogenous. For classification, if there was no staining at all, the aspirate was described as "no staining". If staining was present, the predominant distribution of stain was noted as one of the following: diffuse, granular, an intense rim of stain around the periphery of the cell or diffuse with an intense focal area of stain. The aspirates were classified and the results were compared with the routine histological diagnosis of the corresponding H&E stained section (Table 5.5).

Focal staining was observed only in malignant breast aspirates. Apart from this there was no association of any of the staining classifications with benign or malignant tumours or with any of the histological types of tumour.

TABLE 5.5

A Comparison of the Immunocytochemical Staining Patterns of HMFG1 and HMFG2 with the Histological Diagnosis in Breast Tumour Needle Aspirates.

Histological Diagnosis	Immunocytochemical Staining Pattern				
	No Staining	Diffuse	Granular	Rim Around Periphery of Cell	Focal
MALIGNANT (Carcinoma)	4	8	13	15	3
comprising:					
Infiltrating ductal	2	7	11	13	2
Infiltrating lobular	–	–	1	2	1
Mixed ductal and lobular	–	1	1	–	–
Tubular	1	–	–	–	–
Adenocarcinoma with squamous features	1	–	–	–	–
BENIGN	2	7	5	8	–
comprising:					
Fibroadenoma	2	4	2	4	–
Cystic Mastopathy	–	3	3	3	–
Papilloma	–	–	–	1	–

Indirect aspirates were made from needle aspirates of 65 palpable breast lesions, fixed and permeabilised, and stained with HMFG1 and HMFG2 in an indirect IP technique (5.2.2.).

5.3. Discussion.

5.3.1. Establishment of the Standard Processing of Aspirates for Observation of Immunocytochemical Staining.

The immunocytochemical technique to be used in this study was first standardised so as to establish comparability within the study. The immunocytochemical stain essentially relies on chemical parameters, which should be controlled (To et al. 1981). The titre of both HMFG1 and HMFG2 in the indirect IP technique was determined at 1/2. The titre of the second antibody was determined at 1/50 with NHS at 1/50. To et al, (1982), noted a difference in the intensity of staining of benign mesothelial cells and malignant cells in pleural and peritoneal effusions with anti-EMA and increased the dilution of the antibody to enhance this difference. There was no such difference in intensity between the immunostaining of benign and malignant breast cells with HMFG1 or HMFG2. As the basis of distinction of malignant from benign cells with HMFG1 or HMFG2 was the presence of intracellular staining, it was necessary to permeabilise the aspirated cells as well as fix them. This was achieved by immersing the prepared aspirates in dehydrated acetone at $-20^{0}C$.

Without fixation or permeabilising;, cytocentrifuged T47D cells were either lost or lysed in the IP technique and staining was weak. After 20 minutes' immersion in the acetone, the cells were adequately fixed but the staining was weaker than the cells fixed for a longer time. This could be a result of the variation in staining intensity each time the cells are stained (3.2.5.) or because the cells were not sufficiently permeabilised for all of the intracellular antigens to be stained. As all of the aspirates used in this study were immersed in acetone for at least 24 hours, no further experimentation was carried out to ascertain which was the case. To et al., (1981), found that storage of smears of pleural effusions in ethanol for more than 2 weeks destroyed the antigen to which anti- EMA is directed. In this study the maximum storage time for the aspirates was 2 weeks.

Inhibition of endogenous peroxidase with 0.5% H_2O_2 in methanol reduced the expression of the HMFG antigens in T47D cells. Endogenous peroxidase activity was, therefore, not inhibited in this study. Holley et al (1983), investigated the immunocytochemical detection of malignant cells in breast needle aspirates with the Cal antibody. They increased the concentration of H_2O_2 in methanol to ensure complete blockage of endogenous peroxidase in erythrocytes. This apparently had no effect on the expression of the Cal antigen.

Background staining due to the endogenous peroxidase activity in erythrocytes, macrophages and granulocytes was not observed in either the indirect or the direct breast tumour aspirates. This is because the cells were aspirated directly from the excised lesion at surgery to obtain sufficient cells for experimentation. Erythrocytes are normally present in routinely prepared breast tumour aspirates and were present in 23/49 indirect aspirates prepared from the needle wash of a routine aspiration. The endogenous peroxidase activity due to erythrocytes was not a problem because their morphology is sufficiently distinct from that of malignant cells to prevent confusion. If endogenous peroxidase activity were to be a difficulty in subsequent experiments, the IP technique could be substituted by the IAP technique (Cordell et al., 1984). This has been done in studies of various antigens in pleural fluids (Ghosh et al, 1983b). The IAP technique was not originally adopted in this study because of the unavailability of the necessary reagents. Alternatively the endogenous peroxidase could be reacted with a different substrate, such as 3A9E (Graham et al., 1965), prior to staining. The red colour of the 3A9E would contrast with the brown colour of DAB. To et al. (1981) removed the erythrocytes, the source of the background staining, from pleural effusions by ficoll-triosil separation. There were too few cells in the aspirates to remove the erythrocytes in this way.

5.3.2. A Comparison of the Immunocytochemical Staining Observed in Indirect and Direct Needle Aspirates.

Aspirated breast tumour cells are more accurately stained and efficiently observed in an indirect preparation where all of the cells are confined to a circle 6mm in diameter. In the direct preparation they are smeared over the slide. Abele et al. (1985), reported alternative methods of smearing aspirated cells resulting in improved quality of preparations. Employment of these methods might improve the quality of the direct aspirates.

Background staining is a problem in the direct aspirates. In 6/30 cases, methodologic background staining was observed, where the second antibody was adhering to components of the aspirate other than the primary antibody. In 22/30 cases there was immunologic background staining of extracellular HMFG. This was particularly intense when there was extensive extracellular staining in the tissue section and was a result of the release of both extracellular HMFG by the break up of tissue structure and intracellular HMFG from the lysis of cells. The background staining made difficult the distinction of the various staining patterns and in 8 cases obscured the cellular staining. During the preparation of the indirect aspirate the cells were washed. This removed most of the extracellular fluid and cell debris that was causing the background staining. Consequently, no methodologic background staining was observed in the indirect aspirates and in 2/30 cases there was immunologic background staining.

Immunocytochemical staining in indirect aspirates, direct aspirates and the corresponding tissue section was not easily compared. This is because a staining pattern related to the structure of a tissue section is appears different when related to individual cells removed from that tissue. Also, the staining of the HMFG1 and HMFG2 antigens in both benign and malignant breast sections is heterogeneous (4A.2.1). During aspiration of cells from a palpable breast lesion, the needle is moved about within the lesion so that cells from the

whole lesion are represented (Smallwood et al., 1984). In comparison a tissue section represents only one area of the lesion.

Despite these limitations, there was a similarity in the staining of the tissue section and the aspirated cells, particularly in the indirect aspirate. It was not possible, however, to distinguish between extracellular and intracellular staining in the aspirates. In a tissue section the cells are sectioned and extracellular and intracellular staining can be distinguished. In aspirates the whole cell stained and extracellular and intracellular staining is confused. The only indication of extracellular staining was an intense rim of staining around the periphery of the cell. This was observed in some of the aspirates from lesions where extracellular staining was present in the tissue section. The rim of staining was also observed with immunocytochemical staining of anti-EMA on both benign mesothelial and malignant cells in pleural effusions (To et al., 1981). Extracellular staining was otherwise observed as diffuse or granular and could not be distinguished from intracellular staining. Extracellular and intracellular staining were distinguished by Holley et al. (1983) but they did not give precise details of the staining observed to indicate how this was done.

Other staining patterns observed included no staining, diffuse or granular staining and diffuse staining wih an intense focal area of stain. Focal staining was not observed as frequently in needle aspirates as in tissue sections. This is because the presence of extracellular staining obscured the focal intracellular stain. Only particularly intense focal staining was observed.

Indirect aspirates are the choice of preparation for the observation of immunocytochemical staining. In these preparations the staining is accurately and efficiently observed and most representative of staining in the tissue section. A similar method to the indirect aspirate was used by Holley et al. (1983), who expressed the aspirated cells into 0.5 ml of heparinised saline before cytocentrifugation. These preparations were then stained

immunocytochemically with the Cal antibody.

It would be appropriate to combine the immunocytochemical staining of indirect aspirates with routine cytodiagnosis to improve the accuracy of diagnosis of needle aspirates.

5.3.3 Combination of Immunocytochemical Staining of Indirect Aspirates with Routine Cytology in the Diagnosis of Breast Tumour Needle Aspirates.

Routine diagnosis of breast tumour aspirates depends upon 3 criteria, the cell spread, cell and nuclear morphology and the component cells (5.1). The cytocentrifugation involved in the processing of the indirect aspirates destroys cell morphology and patterns of cell aggregates. There is also some loss of cells, including the bipolar cells, in the washing. Furnival et al. (1975), suspended a needle wash of the cells from a breast tumour aspirate in saline and passed the suspension through a Millipore filter. They found that this method gave an excellent preparation of the aspirate but that the characteristic patterns of cell aggregates were lost. Direct aspirates are better preparations for cytological diagnosis, therefore, but indirect aspirates are preferred for diagnosis of immunocytochemical staining. It was necessary to establish a way of obtaining both an indirect and a direct aspirate from the same breast tumour aspirate.

A comparison was made of routinely prepared direct aspirates with immunocytochemically stained indirect aspirates prepared from the needle wash in 49 cases. Seventeen of the 49 direct aspirates were technically unsatisfactory for diagnosis by morphological criteria. There were no cells present in 9, 10-20 cells present in 7 and >100 cells present in 1 Of the corresponding indirect aspirates. In the remaining 32 cases both indirect and direct aspirates were adequate for diagnosis. As a suitable antibody for the distinction of benign and malignant breast cells has not yet been discovered, it is not possible to compare the number of cells required for a definite

diagnosis by cytology and by immunocytochemistry.

It is possible that the combination of diagnosis from a routinely prepared smear and from an immunocytochemically stained indirect aspirate would decrease the percentage of technically unsatisfactory aspirates and increase the possibility of diagnosis. Even without immunocytochemical staining the two preparations might be complementary. Furnival et al. (1975), prepared conventional smears of breast tumour aspirates and then prepared an indirect aspirate from the needle wash. Both preparations were routinely stained with Giemsa and Papanicolaou staining and diagnosed cytologically. They found that both the smear and the needle wash were technically unsatisfactory in 30 cases. In 11 cases a diagnosis could be made from the smears whilst the needle washes were technically unsatisfactory and the reverse situation occurred in 25 cases. Cytological observation of both a direct and an indirect aspirate can, therefore, increase the diagnostic accuracy of needle aspirates.

5.3.4. The Value of Monoclonal Antibodies Against HMFG Antigens in Discriminating Between Benign and Malignant Cells in Indirect Preparations of Breast Tumour Needle Aspirates.

The immunocytochemical staining of indirect preparations of needle aspirates with HMFG1 and HMFG2 was correlated with the routine histological diagnosis from the corresponding NBF fixed, paraffin wax embedded H&E stained sections in 65 cases. Depending on the predominant staining pattern the aspirates were noted as one of the following: diffuse, granular, with an intensely stained around the periphery of the cells, with an intense focal area of stain or as no staining. Cells with an intense focal area of staining were observed only in 3 aspirates, which were all carcinoma. Apart from this, the types of staining did not distinguish either between benign and malignant cells, or between the histological type of tumour. The lack of distinction between intracellular and extracellular staining in needle aspirates meant that the malignant cells could not be identified by intracellular staining. It was concluded that

immunocytochemical staining of indirect needle aspirates wih HMFG1 and HMFG2 was of no practical use in helping the cytologist to distinguish between benign and malignant lesions.

Holley et al. (1983), have investigated the use in diagnosis of immunocytochemical staining of breast needle aspirates with the Ca-1 antibody. Ca-1 is an antibody raised against membrane glycoproteins of a human laryngeal carcinoma cell culture (Ashall et al. 1982). In an immunohistochemical investigation of frozen and NBF fixed, paraffin wax embedded tissue, Ca-1 stained 22 breast carcinomas but none of 3 fibroadenomas or sections of normal breast tissue (McGee et al., 1982). In the study of breast needle aspirates, however, staining was observed in both the needle aspirate and frozen tissue section of 6/8 cases diagnosed histologically as benign. As noted with HMFG1 and HMFG2, there was considerable variation in the staining of aspirates, both in the number of cells staining and in the distribution of stain. The aspirates were classified according to extracellular or intracellular staining, which could apparently be distinguished, and also according to the presence or absence of stain. Neither classification distinguished malignant from benign tumours.

Several workers have investigated the diagnostic usefulness of various antigens in pleural and peritoneal effusions where there is difficulty in distinguishing malignant cells (carcinoma, lymphoma and mesothelioma) from reactive mesothelial cells in 15-40% of effusions (To et al. 1981; Woods et al., 1982). Antisera against a variety of antigens have been investigated, to varying degrees of success (Table 5.6). Both polyclonal and monoclonal antibodies raised against the HMFG (To et al., 1981; Epenetos et al., 1982; Ghosh et al, 1983a) showed heterogenous staining of the malignant cells in pleural and peritoneal effusions. Strong staining, in these studies, was confined to malignant cells which was useful in diagnosis. O'Brien et al (1979) found that anti-EMA, in combination with other antisera, showed no distinction between benign and malignant cells in 10% formalin fixed, paraffin wax embedded cell blocks of pleural and

TABLE 5.6 A Comparison of Studies Investigating the Use in Diagnosis of the Immunocytochemical Staining of Pleural, Peritoneal and Pericardial Effusions.

Reference	Antibodies Used	Material Used	Staining Observations	Detection in Cytologically +ve Cases	Detection in Suspicious or Cytologically -ve cases	Staining of Benign Effusions (False +ves)
To et al., 1982	Polyclonal anti-EMA	246 pleural & peritoneal effusions	Strong staining observed only in malignant cells	63/116 (54%)	8/48 (17%)	3/127 (2%)
Woods et al., 1982	Monoclonal Ca-1	50 pericardial, peritoneal & pleural effusions	Staining observed only in malignant cells	23/27 (85%)	-	2/25 (8%)
Mariani-Constantini et al., 1982	Monoclonal MBr1	9 pleural & peritoneal effusions	Staining discriminates between reactive mesothelial and breast carcinoma cells	9/9 (100%)	-	-
Smith et al., 1980	Monoclonal abs anti- ABH blood group iso-antigens	Pleural & peritoneal effusions	Normal and atypical cells mesothelial cells stain, malignant cells do not stain	-	-	-
Epenetos et al., 1982	A combination of monoclonal abs HMFG2 & AUA-1	65 pleural & peritoneal effusions	Heterogeneity of staining of malignant cells. Reactive mesothelial cells do not stain	26/26 (100%) carcinoma, 0/8 lymphoma	4/31 (13%)	0
Ghosh et al., 1983	A combination of monoclonal abs HMFG2, Ca-1 & CEA	41 pleural effusions cytologically diagnosed as benign	The combination of abs enabled distinction between malignant cells and reactive mesothelial cells	-	12/41 (29%)	-

peritoneal effusions. To et al. (1981) attributed this difference in results to the difference in processing and staining of the effusion.

Immunocytochemical staining of pleural, peritoneal and pericardial effusions with a monoclonal antibody raised against Cal antigen (Woods et al., 1982; Ghosh et al, 1983a) was heterogeneous but was confined to malignant cells. Similarly in another preliminary study (Mariani-Constantini et al., 1982), investigated immunocytochemical staining with the monoclonal antibody MBrl, which was raised against the membrane fraction of the MCF-7 cell line. They found that MBrl distinguished between mesothelial cells and breast carcinoma cells in 9 cases.

An alternative approach has been to use antibodies which recognise antigens on normal cells and reactive mesothelial cells but not malignant cells, such as the blood group iso-antigens (Smith et al. 1980). Similalarly Singh et al. (1979), used antibodies directed against mesothelial cells but not epithelial cells. In preliminary studies both of these appeared to be of some diagnostic use. In order to overcome the problem of heterogeneous staining in malignant cells, combinations of antibodies were used (Epenetos et al. 1982; Ghosh et al, 1983a; 1983b) (Table 5.6). In this way all of the malignant cells were identifed with either one or other of the antibodies. Also, the different staining characteristics of reactive mesothelial and malignant cells with each of the panel of antibodies eliminated the possibility of false positives.

Some success has, therefore, been achieved in distinguishing between benign and malignant pleural and peritoneal effusions using immunocytochemical staining. The success has been due to the differential staining characteristics of reactive mesothelial cells and malignant epithelial cells. There is no such difference in staining between benign and malignant epithelial cells with HMFG1 and HMFG2, which, therefore, cannot be used to distinguish between benign and malignant needle aspirates of breast tumours.

CHAPTER 6

DISCUSSION

The purpose of this study was to investigate the use of immunocytochemical staining with the monoclonal antibodies HMFG1 and HMFG2 in the prognosis and diagnosis of breast cancer. To accomplish this, it was necessary to establish the immunohistochemical staining characteristics of HMFG1 and HMFG2 in breast tissue.

6.1 The Immunohistochemical Staining Characteristics of HMFG1 and HMFG2 in Breast Tissue.

The HMFG1 and HMFG2 antigens were accurately expressed in NBF fixed, paraffin wax embedded breast tissue which was digested in trypsin before staining (3.3.1). An indirect immunohistochemical procedure, using a peroxidase label and DAB as the substrate, was the most suitable method for the determination of the antigen distribution in breast tissue (3.3.2). A standard procedure (2.2.4.1.) was followed to establish the characteristic immunohistochemical staining patterns in normal, benign and malignant breast tissue (4A.2.1).

HMFG1 and HMFG2 were similarly distributed in corresponding sections of normal, benign and malignant breast tissue. In normal and benign tissue the antigens were present extracellularly on the luminal surface of epithelial cells and in secreted material within the lumina of ducts and tubules. The presence and intensity of staining was heterogenous between cases, between lobules within a tissue section and between the acini within a lobule. Chang & Taylor-Papadimitriou (1983), stained live and fixed cultures of normal breast epithelial cells (Hum E), with HMFG1 and HMFG2 in an indirect immunofluorescence technique. They found that there was a difference in the expression of the two HMFG antigens. The HMFG1 antigen was predominantly extracellular, whilst the HMFG2 antigen was

cell-associated. Also, a Hum E cell culture was fluorescently labelled with HMFG1 and passed through a fluorescent activated cell sorter. The HMFG1 negative cells had a significantly higher growth rate than the HMFG1 positive cells and, during growth, gave rise to both HMFG1 positive and negative cells. These results suggested that the HMFG1 antigen was present extracellularly or membrane-associated at higher levels in well-differentiated cells.

The expression of HMFG1 and HMFG2 antigens in malignant breast tissue is most appropriately described as heterogenous. In the primary tumour they may be present extracellularly, either in a similar pattern to that observed in normal and benign tissue or intercellularly and in intracytoplasmic vacuoles. Alternatively, they may be present intracellularly, distributed either towards the periphery of the cell or diffusely in the cytoplasm. These staining patterns were variably expressed between tumours, between different areas within a tumour and between adjacent cells. The same staining patterns were also heterogenously expressed in metastatic breast carcinoma in the axillary lymph nodes (4B.2.3). The development of a standard IP staining procedure in Chapter 3 showed that the heterogeneity was not an artifact caused by fixation and staining but was a true representation of antigen expression.

The relative intensity of staining of the HMFG1 and HMFG2 antigens varied and neither was expressed in a particular pattern more frequently than the other (4A.2.3). Although they were similarly expressed in normal, benign and malignant tissue, HMFG1 and HMFG2 are directed against different antigenic epitopes. This was shown by double staining of tissue sections with both HMFG1 and HMFG2 where neither antibody interfered with staining by the other (4A.2.2.2).

The immunohistochemical staining patterns of HMFG1 and HMFG2 in 2 tumours from each of 5 patients was compared. In 2 cases, the staining patterns differed which showed that heterogeneity is not an expression of the individuality of the patient (4A.2.1). In structurally differentiated malignant tissue, where there was

extensive formation of tubules, the staining pattern was similar to that in normal and benign tissue (4A.2.1). This was confirmed by the association of staining grade A, the representative grade for extracellular staining, with Bloom's Grade 1 (4A.2.4.4). The expression of HMFG1 and HMFG2 in breast tissue is, therefore, related to the state of differentiation of the cells.

This is confirmed by the results from experiments on the biochemical nature of the HMFG1 and HMFG2 antigens. Burchell et al. (1983), prepared Western blots from components of the HMFG separated on gels. Enzyme-linked immunosorbent assay (ELISA), with HMFG1 and HMFG2 were performed on these blots. Both antibodies recognised determinants present in high molecular weight components. Kinetic experiments were carried out on the binding of HMFG1 and HMFG2 to cultures of Hum E cells and cells of a breast cancer cell line (T47D). Also, ELISA assays were performed on Western blots of gel separated Hum E and T47D cell lysates. These experiments demonstrated that the HMFG1 determinant is present in molecules of large molecular weight predominantly found on the cell surface of Hum E cells. HMFG2 has different affinities for molecules with a range of molecular weights, which are present in both Hum E cells and T47D cells. Lectin blocking experiments showed that both antibodies recognise oligosaccharide sequences containing galactose, N-acetyl glucosamine and N-acetyl galactosamine. The HMFG2 antigen may contain, or be adjacent to, a non-terminal sialic acid residue. Burchell et al. proposed that the HMFG1 determinant is present in large glycoprotein molecules with complex carbohydrate side-chains. The HMFG2 determinant is more commonly found on simpler carbohydrate side-chains where glycosylation may be incomplete and the sialic acid residues more exposed.

The HMFG contains several glycoproteins (Murray et al., 1979). Both the HMFG1 and HMFG2 antigenic determinants are present on a large molecular weight glycoprotein characterised by Shimizu & Yamauchi (1982), (Burchell et al, 1983). This has been called the Epithelial Membrane Antigen because it contains the antigenic determinants to

which the polyclonal anti-EMA is directed (Ormerod et al., 1983).

Glycoproteins are synthesised in the cell by the enzymatic addition of monosaccharide residues, first to an amino acid residue in a polypeptide chain, and then to carbohydrate sequences as the oligosaccharide chain is completed. The enzymes involved in glycosylation are collectively known as the glycosyl transferases (Hughes, 1983). The expression of the HMFG1 and HMFG2 antigens has been likened to that of the blood group antigens, where the addition of monosaccharides in a glycoprotein results in the expression of different antigenic determinants (Springer et al., 1976; Hounsell & Feizi, 1982). The HMFG2 determinant is present at an early stage of glycosylation of the oligosaccharide whilst the HMFG1 determinant is present in the terminal stages (Taylor-Papadimitriou, personal communication).

Genetic variability in malignant tumours results in cellular heterogeneity which may be expressed in a variety of ways (Nowell, 1976; Woodruff, 1983). One expression of heterogeneity is in the presence and quantity of enzymes, including the glycosyl transferases (Bernacki & Kim, 1977). It is likely that the cellular site of a glycoprotein is determined by the pattern of glycosylation. Altered glycosylation will result in the inappropriate cellular distribution of glycoproteins (Hughes, 1983).

The immunohistochemical staining patterns of HMFG1 and HMFG2 in breast tissue can be explained in terms of glycosylation. In normal, benign and well-differentiated malignant breast tissue the glycoprotein which bears the HMFG1 and HMFG2 antigenic determinants is expressed at the cell surface. Immunhistochemical staining of the HMFG2 antigen is less intense than that of HMFG1 because it is masked by the terminal monosaccharides. Heterogeneity in the presence of staining may be a result of inactivity of the glycosyl transferases in some breast cells. In lactating breast the HMFG1 and HMFG2 antigens are present uniformly on the luminal surface of epithelial cells and in secretions in the lumina of ducts and tubules (Arklie et al., 1981).

In less well-differentiated breast tissue the pattern of glycosylation is altered because of variability in the presence and quantity of glycosyl transferases. This results in differences in the cellular location of the glycoprotein so that the immunohistochemical staining patterns of HMFG1 and HMFG2 are heterogenous. Intracellular staining of HMFG1 and HMFG2 is observed in the apocrine cells of benign metaplasia (4A.2.1) and is presumably a result of altered glycosylation. Glycoproteins are transported from the site of polypeptide synthesis to the cell surface in vesicles (Wooding, 1971). Changes in the pattern of glycosylation may result in the localisation of the glycoproteins intracellularly in vesicles. This may explain why intense intracellular immunohistochemical staining of HMFG1 and HMFG2 appears granular (4A.2.1). The altered glycosylation pattern changes the configuration of the oligosaccharide side-chains and the HMFG2 antigen is more exposed. The relative intensity of staining of HMFG1 and HMFG2 is, therefore, variable.

No staining was observed in 11.2% and 7.9% of breast carcinoma tissues stained with HMFG1 and HMFG2 respectively (4A.2.3). In these cases the antigens were either not expressed or masked by changes in the configuration of the oligosaccharide. Gooi et al. (1983), showed that the M18 antigenic determinant which is present on a glycoprotein molecule is revealed by desialylation of apparently non-reactive tumours.

The immunohistochemical staining of another antigen in the HMFG, E29, was investigated in this study (4A.2.2.1). The staining patterns of E29 in normal, benign and malignant breast tissue were similar to those of HMFG1 and HMFG2. The intensity of staining of E29 was intermediate between that of HMFG1 and HMFG2. The antigenic determinant is presumably located in the same glycoprotein where it is masked more than HMFG1 and less than HMFG2.

The polyclonal anti-EMA, and other monoclonal antibodies raised

against the HMFG also show similar staining patterns to the HMFG1 and HMFG2 in normal, benign and malignant breast tissue (1.2.4.2). Some of these are particularly similar in tissue specificity and distribution in breast tissue, and competitively inhibit one another in cell binding assays. There are 2 groups of such antibodies, each of which recognise antigens on the same glycoprotein. One group consists of M8, 115-D8 (MAM6), F36.22, HMFG1 and HMFG2 (Ormerod et al., 1983; Hilkens et al., 1984; Thompson et al., 1983). The other consists of 67D11 (MAM6) and M24 (Hilkens et al., 1984). Similarly, monoclonal antibodies raised against malignant cells recognise antigens on the same glycoproteins as these 2 groups of antibodies. NCRC11 is associated with the first group and B72.3 with the second (Ellis et al., 1984; Hilkens et al., 1984).

The majority of monoclonal antibodies raised against antigens on the epithelial cell surface are directed against epitopes in glycoproteins and are heterogenously expressed in breast cancer. This reactivity is not restricted to antibodies in breast cancer but encompasses antibodies directed against most epithelial specific antigens (Edwards, 1985).

6.2 The Clinical Applications of Immunohistochemical Staining with Monoclonal Antibodies Directed Against HMFG1 and HMFG2 in the Prognosis and Diagnosis of Breast Cancer.

The clinical applications of tumour-associated antibodies depend on the antigen distribution in normal, benign and malignant tissue. The previous section has shown that the majority of antibodies associated with the epithelial cell surface are heterogenously expressed in malignant tissue.

The immunohistochemical staining patterns of HMFG1 and HMFG2 in primary breast carcinoma were classified and related to survival. The classification system was designed to reflect the immunohistochemical staining patterns, whilst being in a form which could be related to survival (4A.3.2). Breast carcinoma sections stained with HMFG1 and

HMFG2 were graded according to the relative intensity of staining, and to the extent of intracellular staining (4A.2.3). Inaccuracies introduced by the heterogeneous staining were overcome by grading the most predominant staining pattern in the tissue section. The classification system would not, therefore, detect the association with survival of a staining pattern which was not predominant in the tissue section. Despite the precautions taken, the grading was not reproducible in 16% of the cases because of the staining variability. The heterogeneity of immunohistochemical staining may, itself, be indicative of survival (4A.3.3). It was not possible, in this study, to classify the heterogeneity of staining reproducibly.

There was no correlation of the staining grades with survival of either a retrospective or prospective series of patients (4A.3.3). Of all the monoclonal antibodies raised against the HMFG and other epithelial surface antigens in primary breast cancer, only immunohistochemical staining of NCRC11 is presently used in the determination of prognosis (4A.3.3).

One application in prognosis of the immunohistochemical staining of the HMFG1 and HMFG2 antigens was in the detection of metastatic breast carcinoma cells in the axillary lymph nodes (4B.3.1). A panel of monoclonal antibodies was used for two reasons. Firstly, staining of cells in the lymph node with more than one antibody confirmed that they were metastatic breast carcinoma cells. Secondly, if a cell did not express one of the antigens, it would still be detected by it's expression of another antigen. The panel consisted of 3 antibodies raised against the HMFG, HMFG1, HMFG2 and E29, and one against a simple cytokeratin, CAM5.2. A double-bridge IAP staining procedure was used to identify cells which expressed these antigens. Heterogeneity of antigen expression, particularly of the HMFG antigens, was apparent in the metastases. A few metastases did not express all of the antigens. Despite this, all of the metastases were stained with at least one antibody (4B.2.3).

A comparison was made of the detection of breast carcinoma metastases

by routine histological diagnosis and by immunohistochemical staining. Immunohistochemical staining increased the detection of metastases of both the number of positive nodes and the number of positive cases by 17%. The metastases detected ranged from small clumps of 2-3 cells to large clumps of >100 cells. It is important to determine if the prediction of survival is improved if immunohistochemically detected metastases are incorporated into the determination of lymph node status.

Finally, the application of the immunocytochemical staining of HMFG1 and HMFG2 antigens in the diagnosis of breast tumour aspirates was investigated. A technique was developed so that the diagnosis of breast tumour aspirates by immunohistochemical staining could be combined with the routine cytological diagnosis (5.3.3). When HMFG1 and HMFG2 were investigated in this procedure, there was no distinction between benign and malignant cells (5.3.4). One antigen which might be suitable for use in this procedure is the MAM-4 epitope in HMFG which is expressed in benign but not in malignant breast tissue (Hilkens et al., 1984).

There have been several clinical applications of the monoclonal antibodies HMFG1 and HMFG2 in breast cancer. They have been used in pathology to distinguish between carcinoma, lymphoma, mesothelioma and reactive mesothelium (Gatter et al., 1983; Marshall et al., 1984). HMFG2 has been used to detect metastatic carcinoma cells in pleural effusions (Epenetos et al., 1982) and to target malignant ovarian, breast and gastro-intestinal tumours in vivo (Epenetos et al., 1982). They have been detected in the serum of patients with breast carcinoma, which may have clinical applications (Burchell et al., 1984). To add to this list, the results from this study show that HMFG1 and HMFG2 can be used as part of a panel of antibodies used in the immunohistochemical detection of breast carcinoma metastases in the axillary lymph nodes. This may improve the accuracy of the prediction of prognosis by lymph node status.

Such applications have not dramatically altered the survival rate of

patients from breast cancer but in such a widespread and psychologically disturbing disease each minor improvement is to be welcomed.

REFERENCES

ABELE, J. S., MILLER, T. R., KING, E. B. & LOWHAGEN, T. 1985. Smearing techiques for the concentration of particles from fine needle aspiration biopsy. Diagnostic Cytopathology, 1, 59-65.

ALEXANDER, P. 1984. The biology of metastases. Cancer Topics, 4, 116-117.

ARKLIE, J., TAYLOR-PAPADIMITRIOU, J., BODMER, W., EGAN, M. & MILLIS, R. 1981. Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. International Journal Of Cancer, 28, 23-29.

ASHALL, F., BRAMWELL, M. E. & HARRIS, H. 1982. A new marker for human cancer cells. I. The Ca antigen and the Ca-1 antibody. The Lancet, ii, 1-6.

ATTIYEH, F. F., JENSEN, M., HUVOS, A. G. & FRACCHIA, A. 1977. Axillary micrometastasis and macrometastasis in carcinoma of the breast. Surgery, Gynecology & Obstetrics, 144, 839-842.

BANCROFT, J. D. 1967. Fixation. In : An Introduction To Histochemical Technique. Butterworths & Co. (London) 51-61.

BATTIFORA, H. 1975. Intracytoplasmic lumina in breast cancer. A helpful histopathologic feature. Archives of Pathology, 99, 614-617.

BERNACKI, R. J. & KIM, U. 1977. Concomitant elevations in serum sialyltransferase activity and sialic acid content in rats with metastasising mammary tumours. Science, 195, 577-580.

BISHOP, H. M BLAMEY, R. W., ELSTON, C. W., HAYBITTLE, J.L., NICHOLSON, R.I. & GRIFFITHS, K. 1979. Relationship of oestrogen-receptor status to survival in breast cancer. The Lancet, ii, 283-284.

BLACK, M. M., SPEER, F. D. & OPLER, S. R. 1956. Structural representation of tumor-host relationships in mammary carcinoma, biologic and prognostic significance. American Journal Of Clinical Pathology, 26, 250-265.

BLOOM H. J. G. 1950. Prognosis in carcinoma of the breast. British Journal of Cancer, 4, 259-347.

BLOOM, H. J. G. & RICHARDSON, W. W. 1957. Histological grading and prognosis in breast cancer. British Journal Of Cancer, 11, 359-377.

BLOOM H. J. RICHARDSON, W. W. & HARRIES, E. J. 1962. Comparison of untreated and treated cases according to histological grade of malignancy. British Medical Journal (ii), 213-221.

BOSMAN, F. T., LINDEMAN, J. KUIPER, G., VAN DER WAL, A. & KREUNIG, J. 1977. The influence of fixation on immunoperoxidase staining of plasma cells in paraffin sections of intestinal biopsy specimens. Histochemistry, 53, 57-62.

BRANDTZAEG, P. 1982. Tissue preparation methods for immunocytochemistry. In Techniques In Immunocytochemistry Volume1. (ed. G. R. Bullock & P. Petrusz) Academic Press (London) 1-76.

BRANDTZAEG, P. & ROGNUM, T.O. 1983. Evaluation of tissue preparation methods and paired immunofluorescence staining for immunocytochemistry of lymphomas. Histochemical Journal, 15, 655-689.

BURCHELL, J. DURBIN, H. & TAYLOR-PAPADIMITRIOU, J. 1983. Complexity of expression of antigenic determinants recognised by monoclonal antibodies HMFG1 and HMFG2 in normal and malignant human mammary epithelial cells. The Journal Of Immunology, 131, 508-513.

BURCHELL, J., WANG, D. & TAYLOR-PAPADIMITRIOU, J. 1984. Detection of the tumour-associated antigens recognised by the monoclonal antibodies HMFG1 and HMFG2 in serum from patients with breast cancer. International Journal of Cancer, 34, 763-768.

BURNS, J. 1975. Background staining and sensitivity of the unlabelled antibody-enzyme (PAP) method. Comparison with the peroxidase labelled antibody sandwich method using formalin fixed paraffin embedded material. Histochemistry, 43, 291-294.

BUSSOLATI, G., PICH, A. & ALFANI, V. 1975. Immunofluorescence detection of casein in human mammary dysplastic and neoplastic tissues. Virchows Archiv A. Path. Anat. & Histol., 365, 15-21.

CAIRNS,J., 1978. Experimental Cancer Research (2): The Cancer Cell. In : Cancer - Science and Society. W. H. Freeman & Co. (San Francisco) 119-142.

CERIANI, R. L. 1980. Mammary epithelial cell identification by means of cell surface antigens. In : Cell Biology Of Breast Cancer (ed. C. M. McGrath, M. J. Brennan & M. A. Rich) Academic Press (New York)

CERIANI, R. L., THOMPSON, K. E., PETERSON, J. A. & ABRAHAM, S. 1977. Cell surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule. Proceedings of the National Academy of Science, 74, 582-586.

CHANG, S. E. & TAYLOR-PAPADIMITRIOU, J. 1983. Modulation of phenotype in cultures of human milk epithelial cells and its relation to the expression of a membrane antigen. Cell Differentiation, 12, 143-154.

CIOCCA, D. R. ADAMS, D. J. EDWARDS, D. P. BJERCKE, R. J. & MCGUIRE, W. L. 1983 Distribution of an estrogen-induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. Cancer Research, 43, 1204-1210.

COHLE, S. D., TSCHEN, J. A., SMITH, F. E., LANE, M. & MCGAVRAN M. M. 1979. ACTH-secreting carcinoma of the breast. Cancer, 43, 2370-2376.

COLEMAN, D., DESAI, S., DUDLEY, H., HOLLOWELL, S. & HULBERT, M. 1975. Needle aspiration of palpable breast lesions : a new application of the membrane filter technique and its results. Clinical Oncology, 1, 27-32.

COOKE, T. 1982. The clinical application of oestrogen receptor analysis in early cancer of the breast. Annals Of The Royal College Of Surgeons Of England, 64, 165-170.

COOKE, T., GEORGE, D., SHIELDS, R., MAYNARD, P. & GRIFFITHS, K. 1979. Oestrogen receptors and prognosis in early breast cancer. The Lancet, i, 995-997.

COONS, A. H., CREECH, H. J. & JONES, R. N. 1941. Immunological properties of an antibody containing a fluorescent group. Proceedings of the Society for Experimental Biology and Medicine (N.Y.), 47, 200-202.

COONS, A. H., CREECH, H. J., JONES, R. N. & BERLINER, E. 1942. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. Journal of Immunology, 45, 159-170.

CORDELL, J. L., FALINI, B., ERBER, W. N., GHOSH, A. K., ABDULAZIZ, Z., MACDONALD, S., PULFORD, K. A. F., STEIN, H. & MASON, D. Y. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). The Journal of Histochemistry and Cytochemistry, 32, 219-229.

CRAWFORD, L. V., PIM, D. C., GURNEY, E. G., GOODFELLOW, P. & TAYLOR-PAPADIMITRIOU, J. 1981. Detection of a common feature in several human tumor cell lines - a 53,000-dalton protein. Proceedings of the National Academy of Sciences, 78, 41-45.

CULLING, C. F. A. 1974a. Routine stains. In : Handbook Of Histopathological and Histochemical Practices 3rd edition. Butterworth (London) 211-220.

CULLING, C. F. A. 1974. Exfoliative cytology and chromosome techniques. In : Handbook of Histopathological and Histochemical Techniques 3rd edition. Butterworth (London) 489-505.

CURRAN, R. C. & GREGORY, J. 1977. The unmasking of antigens in paraffin sections of tissue by trypsin. Experientia, 33, 1400-1401.

CURRAN, R. C. & GREGORY, J. 1980. Effects of fixation and processing on immunohistochemical demonstration of immunoglobulin in paraffin sections of tonsil and bone marrow. Journal of Clinical Pathology, 33, 1047-1057.

CUTLER, S. J. & MYERS, M. H. 1967. Clinical classification of extent of disease in cancer of the breast. Journal of the National Cancer Institute, 39, 193-207.

DEARNALEY, D. P., SLOANE, J. P., ORMEROD, M. G., STEELE, K., COOMBES, R. C. CLINK, H. MCD., POWLES, T. J., FORD, H. T., GAZET, J-C. & NEVILLE, A. M. 1981. Increased detection of mammary carcinoma cells in marrow smears using antisera to epithelial membrane antigen. British Journal of Cancer, 44, 85-90.

DELSOL, G., GATTER, K. C., STEIN, H., ERBER, W. N., PULFORD, K. A. F., ZINNE, K. & MASON, D. Y. 1984. Human lymphoid cells express epithelial membrane antigen. Implications for diagnosis of human neoplasms. The Lancet, ii, 1124-1129.

EDWARDS, P. A. W. 1985. Heterogenous expression of cell-surface antigens in normal epithelia and their tumours, revealed by monoclonal antibodies. British Journal of Cancer, 51, 149-160.

ELLIS, I. O., HINTON, C. P., MACNAY, J., ELSTON, C. W., ROBINS, A., OWAINATI, A. A. R. S., BLAMEY, R. W., BALDWIN, R. W. & FERRY, B. 1985. Immunocytochemical staining of breast carcinoma with the monoclonal antibody NCRC11: a new prognostic indicator.

ELLIS, I. O., ROBINS, R. A., ELSTON, C. W., BLAMEY, R. W., FERRY, B. & BALDWIN, R. W. 1984. A monoclonal antibody, NCRC11, raised to human breast carcinoma. I. Production and immunohistological characterisation. Histopathology, 8, 501-516.

ELSTON, C. W., GRESHAM, G. A., RAO, G. S., ZEBRO, T., HAYBITTLE, J. L., HOUGHTON, J. & KEARNEY, G. 1982. The Cancer Research Campaign (King's/Cambridge) trial for early breast cancer: Clinico-pathological aspects. British Journal of Cancer, 45, 655-669.

EPENETOS, A. A., BRITTON, K. E., MATHER, S., SHEPERD, J., GRANOWSKA, M., TAYLO-PAPADIMITRIOU, J., NIMMON, C. C., DURBIN, H., HAWKINS, L. R., MALPAS, J. S. & BODMER, W. F. 1982.Targeting of iodine-123-labelled tumour-associated monoclonal antibodies to ovarian, breast and gastrointestinal tumours. The Lancet, ii, 999-1004.

EPENETOS, A. A., CANTI, G., TAYLOR-PAPADIMITRIOU, J., CURLING, M. & BODMER, W. F. 1982. Use of two epithelium-specific monoclonal antibodies for diagnosis of malignancy in serous effusions. The Lancet, ii, 1004-1006.

FAULK, W. P. & TAYLOR, G. H. 1971. An immunocolloid method for the electron microscope. Immunochemistry, 8, 1081-1083.

FEHERTY, P., FARRER-BROWN, G. & KELLIE, A. E. 1971. Oestradiol receptors in carcinoma and benign disease of the breast: An in vitro assay. British Journal of Cancer, 25, 697-710.

FISHER, B. 1980. Laboratory and clinical research in breast cancer- A personal adventure: The David A. Karnofsky memorial lecture. Cancer Research, 40, 3863-3874.

FISHER, B., BAUER, M., WICKERHAM, D. L., REDMOND, C. K. & FISHER, E. R. 1983. Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. Cancer, 52, 1551-1557.

FISHER, B., RAVDIN, R. G., AUSMAN, R. K., SLACK, N. H., MOORE, G. E. & NOER, R. J. 1968. Surgical adjuvant chemotherapy in cancer of the breast. Annals of Surgery, 168, 337-356.

FISHER, B., SLACK, N. H., BROSS, I. D. J. & Co-operating Investigators. 1969. Cancer of the breast: Size of neoplasm and prognosis. Cancer, 24, 1071-1080.

FISHER, E. R., PALEKAR, A., ROCKETTE, H., REDMOND, C. & FISHER, B. 1978a. Pathologic findings from the National Surgical Adjuvant Breast Project (Protocol No. 4), v. Significance of axillary nodal micro- and macrometastases. Cancer, 42, 2032-2038.

FISHER, E. R., SWAMIDOSS, S., LEE, C. H., ROCKETTE, H., REDMOND, C. & FISHER, B., 1978b. Detection and significance of occult axillary node metastases in patients with invasive breast cancer. Cancer, 42, 2025-2031.

FOSTER, C. S. DINSDALE, E. A. EDWARDS, P. A. W. & NEVILLE, A. M. 1982b. Monoclonal antibodies to the human mammary gland. II. Distribution of determinants in breast carcinomas. Virchows Archiv A. Pathol. Anat. & Histopath. 394, 295-305.

FOSTER, C. S. EDWARDS, P. A. W. DINSDALE, E. A. & NEVILLE, A. M. 1982a. Monoclonal antibodies to the human mammary gland. I. Distribution of determinants in non-neoplastic mammary and extra mammary tissues. Virchows Archiv A.Pathol. Anat. & Histopath., 394, 279-293

FORREST, A. P. & ROBERTS, M. M. 1980. Screening for breast cancer. British Journal of Hospital Medicine 23, 8-21.

FOX, C. H., JOHNSON, F. B. WHITING, J. & ROLLER, P. P. 1985. Formaldehyde fixation. The Journal of Histochemistry and Cytochemistry, 33, 845-853.

FRANZEN, S. & ZAJICEK, J. 1968. Aspiration biopsy in diagnosis of palpable lesions of the breast- critical review of 3479 biopsies. Acta Radiologica (Therapy Physics Biology), 7, 241-262.

FRIEDELL, G.H. BETTS, A. & SOMMERS, S. C. 1965. The prognostic value of blood vessel invasion and lymphocytic infiltrates in breast carcinoma. Cancer, 18, 164-165.

FURNIVAL, C. M., HUGHES, H. E. HOCKING, M. A., REID, M. M. W. & BLUMGART, L. H. 1975. Aspiration cytology in breast cancer, its relevance to diagnosis. The Lancet, ii, 446-448.

GATTER, K. C., ABDULAZIZ Z., BEVERLEY, P., CORVALAN, J. R. F., FORD, C., LANE, E. B., MOTA, M., NASH, J. R. G., PULFORD, K., STEIN, H., TAYLOR-PAPADIMITRIOU, J. WOODHOUSE, C. & MASON, D. Y. 1982. Use of monoclonal antibodies for the histopathological diagnosis of human malignancy. Journal of Clinical Pathology, 35, 1253-1267.

GATTER, K. C., ALCOCK, C., HERYET, A., PULFORD, K. A., HEYDERMAN, E., TAYLOR-PAPADIMITRIOU, J. STEIN, H. & MASON, D. Y. 1984. The differential diagnosis of routinely processed anaplastic tumours using monoclonal antibodies. American Journal of Clinical Pathology, 82, 33-43.

GARDECKI, T. I. M., HOGBI, B. M., MELCHER, D. H. & SMITH, R. S. 1980 Aspiration cytology in the pre-operative management of breast cancer. The Lancet, ii, 790-792.

GERDES, J. LEMKE, H., BAISCH, H. WACKER, H., SCHWAB, U. & STEIN, H. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki67. The Journal of Immunology, 133, 1710-1715

GHOSH, A. K. MASON, D. Y. & SPRIGGS, A. I. 1983a. Immunocytochemical staining with monoclonal antibodies in cytologically "negative" serous effisions from patients with malignant disease. Journal Of Clinical Pathology, 36, 1150-1153.

GHOSH, A. K. SPRIGGS, A. I. TAYLOR-PAPADIMITRIOU, J. & MASON, D. Y. 1983b. Immunocytochemical staining of cells in pleural and peritoneal effusions with a panel of monoclonal antibodies. Journal of Clinical Pathology 36, 1154-1164.

GOOI, H. C., UEMURA, K. I., EDWARDS, P. A. W., FOSTER, C. S., PICKERING, N. & FEIZI, T. 1983. Two mouse hybridoma antibodies against human milk fat globules recognise the I (MA) antigenic determinant B-D Gal p-(1-4)- B-D-Glc p N Ac-(1-6). Carbohydrate Research, 120, 293-302.

GOLD, P. & FREEDMAN, S. O. 1965. Specific carcino-embryonic antigens of the human digestive system. Journal of Experimental Medicine, 122, 465--481.

GOLDENBERG, I. S., BAILER, J. C., HAYES, M. A. & LOWRY, R. 1961. Female breast cancer: A re-evaluation. Annals of Surgery, 154, 397-404.

GRAHAM, R.C. & KARNOVSKY, M. J. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. Journal of Histochemistry and Cytochemistry, 14, 291-302.

GRAHAM, R. C., LUNDHOLM, U. & KARNOVSKY, M. J. 1965. Cytochemical demonstration of peroxidase activity with 3-amino-9 ethylcarbazole. Journal of Histochemistry and Cytochemistry, 13, 150-152.

GREENE, G. L., SOBEL, N. B., KING, W. J. & JENSEN, E. V. 1984. Immunochemical studies of estrogen receptors. Journal of Steroid Biochemistry, 20, 51-56.

GROSS, L. 1943. Intradermal immunisation of C3H mice against sarcoma that originated in animal of same line. Cancer Research, 3, 326-333.

GRUNERT, F., LUCKENBACH, G. A., HADERLIE, B., SCHWARZ, K. & VON KLEIST, S. 1983. Comparison of colon-, lung-, and breast-derived carcinoembronic antigen and cross-reacting antigens by monoclonal antibodies and fingerprint analysis. Annals of the New York Academy of Sciences, 417, 75-85.

GUGLIOTTA, P., BOTTA, G. & BUSSOLATI, G. 1981. Immunocytochemical detection of tumour markers in bone metastases from carcinoma of the breast. Histochemical Journal, 13, 953-959.

GUSTERSON, B., COWLEY, G., SMITH, J. A. & OZANNE, B. 1984. Cellular localisation of human epidermal growth factor receptor. Cell Biology International Reports, 8, 649-658.

HAAGENSEN, C. D. 1933. Bases for histologic grading of carcinoma of the breast. American Journal of Cancer, 19, 285-327

HALSTED, W. S. 1907. The results of radical operations for the cure of carcinoma of the breast. Annals of Surgery, 46, 1-19.

HANKER, J. S., YATES, P. E., METZ, C. B. & RUSTIONI, A. 1977. A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochemical Journal, 9, 789-792.

HAWKINS, R. A., ROBERTS, M. M. & FORREST, A. P. M. 1980. Oestrogen receptors and breast cancer: Current status. British Journal Of Surgery, 67, 153-169.

HAYBITTLE, J. L., BLAMEY, R. W., ELSTON, C. W., JOHNSON, J., DOYLE, P. J., CAMPBELL, F. C., NICHOLSON, R. I. & GRIFFITHS, K., 1982. A prognostic index in primary breast cancer. British Journal of Cancer, 45, 361-366.

HENDERSON, I. C. & CANELLOS, G. P. 1980. Cancer of the breast: the past decade. New England Journal of Medicine, 302, 17-30 & 78-90

HEYDERMAN, E., STEELE, K. & ORMEROD, M. G. 1979. A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissues. Journal of Clinical Pathology, 32, 35-39.

HILKENS, J., BUIJS, F., HILGERS, J., HAGEMAN, P. H., CALAFAT, J., SONNENBERG, A. & VAN DER VALK, M. 1984. Monoclonal antibodies against human milk fat globule membranes detecting differentiation antigens of the mammary gland and its tumours. International Journal of Cancer, 34, 197-206.

HINTON, C.P. 1984. Results of a programme of education and breast self-examination. In : Breast Cancer. (Ed. R. W. Blamey) Update Publications (London) 10-14.

HOLLEY, M. P., CLOUGH, D. G. F., DUGUID, H. L. D. & CUSCHIERI, A. 1983. Evaluation of the Ca-1 antibody in the detection of malignant cells in fine needle aspirates from breast lumps. Clinical Oncology, 9, 325-330.

HOLMGREN, J., LINHOLM, L., PERSSON, B., LAGERGARD, T., NILSSON, O., SVENNERHOLM, L., RUDENSTAM, C. M., UNSGAARD, B., YNGVASON, F., PETTERSSON, S. & KILLANDER, A. F. 1984. Detection by monoclonal antibody of carbohydrate antigen CA50 in serum of patients with carcinoma. British Medical Journal, 288, 1479-1482.

HORAN HAND, P., NUTI, M., COLCHER, D. & SCHLOM, J. 1983. Definition of antigenic heterogeneity and modulation among human mammary carcinoma cell populations using monoclonal antibodies to tumour-associated antigens. Cancer Research, 43, 728-735.

HORAN HAND, P., THOR, A., WUNDERLICH, D., MURARO, R., CARUSO, A. & SCHLOM, J. 1984. Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas. Proceedings of the National Academy of Science, 81, 5227-5231.

HORNE, C. H. W., REID, I. N. & MILNE, G. D. 1976. Prognostic significance of inappropriate production of pregnancy proteins by breast cancers. The Lancet, ii, 279-282.

HOUNSELL, E. F. & FEIZI, T. 1982. Gastrointestinal mucins. Structures and antigenicities of their carbohydrate chains in health and disease. Medical Biology, 227-236.

HOWAT, J. M. T., BARNES, D. M., HARRIS, M. & SWINDELL, R. 1983. The association of cytosol oestrogen and progesterone receptors with histological features of breast cancer and early recurrence of disease. British Journal Of Cancer, 47, 629-640.

HSU, S. M., RAINE, L. & FANGER, M. 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques. Journal of Histochemistry and Cytochemistry, 29, 577-580.

HUGHES, R. C. 1983. In : Glycoproteins (Outline Studies in Biology) Chapman & Hall, London.

HUVOS, A. G., HUTTER, R. V. P. & BERG, J. W. 1971. Significance of axillary macrometastases and micrometastases in mammary cancer. Annals of Surgery, 173, 44-46.

IMAM, A. & TOKES, Z. A. 1981. Immunoperoxidase localisation of a glycoprotein on plasma membrane of secretory epithelium from human breast. The Journal of Histochemistry and Biochemistry, 29, 581-584.

INTERNATIONAL UNION AGAINST CANCER, 1960. Clinical stage classification and presentation of results. Malignant tumours of the breast, 1960-1964. Committee on Clinical Stage Classification and Applied Statistics, International Union Against Cancer.

JUDD, M. A. & BRITTEN, K. J. M. 1982. Tissue preparation for the demonstration of surface antigens by immunoperoxidase techniques. Histochemical Journal, 14, 747-753.

KIERNAN, J. A. 1981. Fixation. In : Histological And Histochemical Methods: Theory & Practice. Pergamon Press Ltd. (Oxford) 8-24.

KING, W. J. & GREENE, G. L. 1984. Monoclonal antibodies localise oestrogen receptor in the nuclei of target cells. Nature, 307, 745-747.

KLEIN, P. J., NEWMAN, R. A., MULLER, P., UHLENBRUCK, G., CITOLER, P., SCHAEFER, H. E., LENNARTZ, K. J. & FISCHER, R. 1979. The presence and significance of the Thomsen-Friedenrich antigen in mammary gland: II Its topochemistry in normal, hyperplastic and carcinoma tissue of the breast. Journal of Cancer Research and Clinical Oncology, 93, 205-214.

KNIGHT, W. A. III, LIVINGSTON, R. B., GREGORY, E. J. & MC GUIRE, W. L. 1977. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. Cancer Research, 37, 4669-4671.

KOPANS, D. B. 1984. "Early" breast cancer detection using techniques other than mammography. American Journal of Roentgenology, 143, 465-468.

KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256, 495-497.

KUHLMAN, W. D., AVRAMEAS, S. & TERNYNCK, T. 1974. A comparitive study for ultrastructural localisation of intracellular immunoglobulins using peroxidase conjugates. Journal of Immunological Methods, 5, 33-48.

LECLERCQ, G., HEUSON, J. C., DEBOEL, M. C. & MATTHEIEM, W. H. 1975. Oestrogen receptors in breast cancer: A changing concept. British Medical Journal, i, 185-189.

LE DOUSSAL, V., ZANGERLE, P. F., COLLETTE, J., SPYRATOS, F., HACENE, K., BRIERE, M., GEST, J. & FRANCHIMONT, P. 1984. Immunohistochemical detection of alphalactalbumen in breast lesions. European Journal of Cancer and Clinical Oncology, 20, 1069-1078.

LEE, E. & DESU, M. 1972. A computer program for estimating survival functions for the life table. Computer programs in biomedicine, 2, 315-321.

LILLIE, R. D. & FULLMER, H. M. 1965. Fixation. In : Histopathologic Technic and Practical Histochemistry. Mc Graw-Hill Book Company.
(New York) 25-68.

MAKIN, C. A., BOBROW, L. G., & BODMER, W. F. 1984. Monoclonal antibody to cytokeratin for use in routine histopathology. Journal of Clinical Pathology, 37, 975-983.

MARIANI-CONSTANTINI, R., BARBANTI, P., COLNAGHI, M. I., MENARD, S., CLEMENTE, C. & RILKE, F. 1984. Reactivity of a monoclonal antibody with tissues and tumours from the human breast. American Journal of Pathology, 115, 47-56.

MARIANI-CONSTANTINI, R., MENARD, S., CLEMENT, C., TAGLIABUE, E., COLNAGHI, M. I. & RILKE, F. 1982. Immunocytochemical identification of breast carcinoma cells in effusions using a monoclonal antibody. (Abstract) Journal of Clinical Pathology, 35, 1037.

MARSHALL, R. J., HERBERT, A., BRAYE, S. G. & JONES, D. B. 1984. Use of antibodies to CEA and HMFG to distinguish carcinoma mesothelioma and reactive mesothelium. Journal of Clinicla Pathology, 37, 1215-1221.

MASON, D. Y. & SAMMONS, R. 1978. Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. Journal of Clinical Pathology, 31, 454-460.

MAYERSBACH, H. V. 1967. Principles and limitations of immunohistochemical methods. Journal of the Royal Microscopical Society, 87, 295-308.

MAYNARD, P. V., BLAMEY, R. W., ELSTON, C. W., HAYBITTLE, J. L. & GRIFFITHS, K. 1978a. Estrogen receptor assay in primary breast cancer and early recurrence of the disease. Cancer Research, 38, 4292-4295.

MAYNARD , P. V., DAVIES, C. J., BLAMEY, R. W., ELSTON, C. W., JOHNSON, J. & GRIFFITHS, K. 1978b. Relationship between oestrogen receptor content and histological grade in human primary breast tumours. British Journal of Cancer, 38, 745-748.

MC DICKEN, I. W., STAMP, G. H., MC LAUGHLIN, P. J. & JOHNSON, P. M. 1983. Expression of human placental-type alkaline phosphatase in primary breast cancer. International Journal of Cancer, 32, 205-209.

MC GEE, J. O'D., WOODS, J. C., ASHALL, F., BRAMWELL, M. E. & HARRIS, H. 1982. A new marker for human cancer cells. 2. Immunohistochemical detection of the Ca antigen in human tissues with the Ca-1 antibody. The Lancet, ii, 7-10.

MC LAUGHLIN, P. J., CHENG, M. H., SLADE, M. B. & JOHNSON, P. M. 1982. Expression on cultured human tumour cells of placental trophoblast membrane antigens and placental alkaline phosphatase defined by monoclonal antibodies. International Journal of Cancer, 30, 21-26.

MEIRION-THOMAS, J., FITZHARRIS, B. M., REDDING, W. H., WILLIAMS, J. E., TROTT, P. A., POWLES, T. J., FORD, H. T. & GAZET, J-C. 1978. Clinical examination, xeromammography and fine-needle aspiration cytology in diagnosis of breast tumours. British Medical Journal, ii, 1139-1141.

MELLORS, R. C., SIEGEL, M. & PRESSMAN, D. 1955. Histochemical demonstration of antibody localisation in tissues, with special reference to the antigenic components of kidney and lung. Laboratory Investigation, 4, 69-89.

MEPHAM, B. L. 1980. A study of the peroxidase-antiperoxidase technique for the demonstration of intra-cellular immunoglobulin in paraffin section. (Thesis, Southampton University)

MEPHAM, B. L. 1982. Influence of fixatives on the immunoreactivity of paraffin sections. Histochemical Journal, 14, 731-737.

MEPHAM, B. L., FRATER, W. & MITCHELL, B. S. 1979. The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. Histochemical Journal, 11, 345-357.

MESA-TEJADA, R., KEYDAR, I., RAMANARAYANAN, M., OHNO, T., FENOGLIO, C. & SPIEGELMAN, S. 1978. Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. Proceedings of the National Academy of Sciences, 75, 1529-1533.

MILLER, A. B. 1980. Breast cancer etiologic influences. In : Breast Cancer: New Concepts in Etiology and Control. (ed. M. J. Brennan, C. M. McGrath & M. A. Rich. Academic Press (London) 17-27.

MOLL, R., FRANKE, W. W., SCHILLER, D. L., GEIGER, B. & KREPLER, R. 1982. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell, 31, 11-24.

MOULD, R. F. 1983. Cancer Statistics. Adam Hilger Ltd. (Bristol).

MUNRO NEVILLE, A. & GUSTERSON, B. A. 1985. Monoclonal antibodies and human tumours: Pathological and clinical aspects. European Journal of Cancer and Clinical Oncology, 21, 355-369.

MURRAY, L. R., POWELL, K. M., SASAKI, M., EIGEL, W. N. & KEENAN, T. W. 1979. Comparison of lectin receptor and membrane coat-associated glycoproteins of milk lipid globule membranes. Comparitive Biochemistry and Physioligy, 63B, 137-145.

NAKANE, P. K. & PIERCE, G. B. 1967. Enzyme labelled antibodies for the light and electron microscopic localisation of tissue antigens. Journal of Cell Biology, 33, 307-318.

NIE, N. H., HILL, C. H., STYEINBRENNER, K. & BENT, D. H. 1981. In : SPSS Updates McGraw Hill (New York) 218-267.

NIME, F. A., ROSEN, P. P., THALER, H., ASHIKARY, R. & URBAN, J. A. 1977. Prognostic significance of tumour emboli in intramammary lymphatics in patients with mammary carcinoma. American Journal of Surgery and Pathology, 1, 25-30.

NOWELL, P. C., 1976. The clonal evolution of tumour cell populations. Science, 194, 23-28.

NUTI, M., TERAMOTO, Y. A., MARIANI-CONSTANTINI, R., HORAN HAND, P., COLCHER, D. & SCHLOM, J. 1982. A monoclonal antibody (B72.3) defines patterns of distribution of a novel tumour-associated antigen in human mammary carcinoma cell populations. International Journal of Cancer, 29, 539-545.

O'BRIEN, M. J., KIRKHAM, S. E., BURKE, B. & 6 Others. 1980. CEA, ZGM, and EMA localisation in cells of pleural and peritoneal effusion: a preliminary study. Investigations in Cell Pathology, 3, 251-258.

ORMEROD, M. G., STEELE, K., WESTWOOD, J. H. & MAZZINI, M. N. 1983. Epithelial membrane antigen: Partial purification, assay and properties. British Journal of Cancer, 48, 533-541.

PAPSIDERO, L. D., CROGHAN, G. A., O'CONNELL, M. J., VALANZUELA, L. A., NEMOTO, T. & MING CHU, T. 1983. Monoclonal antibodies (F36/22 and M7/105) to human breast carcinoma. Cancer Research, 43, 1741-1747.

PARL, F. & DUPONT, W. 1982. A retrospective cohort study of risk factors in breast cancer patients. Cancer, 50, 2410-2416.

PATEY, D. M. & SCARFF, R. W. 1928. Position of histology in prognosis of carcinoma of breast. The Lancet, i, 801-804.

PEARSE, A. G. E. 1980a. Immunocytochemistry. In : Histochemistry Theoretical & Applied Volume 1 : Preparative and Optical Technology. Chuchill Livingstone (New York) 159-252.

PEARSE, A. G. E. 1980b. Cryotomy and ultracryotomy; Freeze-drying of biological tissues; Freeze-substitution of tissues and sections. In : Histochemistry Theoretical & Applied Volume 1 : Preparative and Optical Technology. Chuchill Livingstone (New York) 15-96

PEARSE, A. G. E. 1980c. The chemistry and practice of fixation. Histochemistry Theoretical & Applied. Volume 1 : Preparative and Optical Technology. Chuchill Livingstone (New York) 97-158.

PETTS, V. & ROITT, I. M. 1971. Peroxidase conjugates for demonstration of tissue antibodies: Evaluation of the technique. Clinical and Experimental Immunology, 9, 407-418.

PICKREN, J. W. 1961. Significance of occult metastases. A study of breast cancer. Cancer, 14, 1267-1271.

POSTE, G. & FIDLER, I. J. 1980. The pathogenesis of cancer metastasis. Nature, 283, 139-146.

POULSEN, H. S., OZZELLO, L. & ANDERSON, J. 1982. Oestrogen receptors in human breast cancer- Problems of correlation with histopathological features. Virchows Archiv A. Pathol, Anat & Histopathol., 397, 103-108.

PREHN, R. T. & MAIN, J. M. 1957. Immunity to methylcholanthrene-induced sarcomas. Journal of the National Cancer Institute, 18, 769-778.

PURNELL, D. M., HILLMAN, E. A., HEATFIELD, B. M. & TRUMP, B. F. 1982. Immunoreactive prolactin in epithelial cells of normal and cancerous human breast and prostate detected by the unlabelled antibody peroxidase-antiperoxidase method. Cancer Research, 42, 2317-2324.

RASMUSSEN, B. B., HILGERS, J. & HILKENS, J. 1984. The influence of formalin and paraffin embedding on the immunohistochemical reaction of monoclonal antibodies applied to female breast tissue. Acta Pathologica, Microbiologica et Immunologica Scandinavica. Section A., 92, 167-175.

RASMUSSEN, B. B., PEDERSEN, B. V., THORPE, S. H., HILKENS, J., HILGERS, J. & ROSE, C. 1985. Prognostic value of surface antigens in primary human breast carcinomas, detected by monoclonal antibodies. Cancer Research, 45, 1424-1427.

REDDING, W. H., COOMBES, R. C., MONAGHAN, P., CLINK, H. M., IMRIE, S. F., DEARNALEY, D. P., ORMEROD, M. G., SLOANE, J. P., GAZET, J. C., POWLES, T. J. & NEVILLE, A. M. 1983. Detection of micrometastases in patients with primary breast cancer. The Lancet, ii, 1271-1273.

RIGGS, J. L., SEIWALD, R. J., BURCKHALTER, J. H., DOWNS, C. H. & METCALF, T. G. 1958. Isothiocyanate compounds as fluorescent labelling agents for immune serum. American Journal of Pathology, 34, 1081-1097.

RICH, M. A., FURMANSKI, P. & BROOKS, S. C. 1978. Prognostic Value of Estrogen Receptor determinations in patients with breast cancer. Cancer Research, 38, 4296-4298.

ROSEN, P. P., SAIGO, P. E., BRAUN, D. W., WEATHERS, E., FRACCHIA, A. A. & KINNE, D. W. 1981. Axillary micro- and macrometastases in breast cancer. Annals of Surgery, 194, 585-591.

RUNGGER-BRANDLE, E. & GABBIANI, G. 1983. The role of cytoskeletal and cytocontractile elements in pathologic processes. American Journal of Pathology, 110, 361-392.

SAPHIR, O. & AMROMIN, G. D. 1948. Obscure axillary lymph node metastasis in carcinoma of the breast. Cancer, 1, 238-241.

SCHABEL, F. M. Jr, 1977. Rationale for adjuvant chemotherapy. Cancer, 39, 2875-2882.

SCHLOM, J., WUNDERLICH, D. & TERAMOTO, Y. A. 1980. Generation of human monoclonal antibodies reactive with human mammry carcinoma cells. Proceedings of the National Academy of Science, 77, 6841-6845.

SEPPALA, M. & WAHLSTROM, T. 1980. Identification of luteinizing hormone-releasing factor and alpha sub-unit of glycoprotein hormones in ductal carcinoma of the mammary gland. International Journal of Cancer, 26, 267-268.

SHAPIRO, S. 1977. Evidence on screening for breast cancer from a randomised trial. Cancer, 39, 2772-2782.

SHIMIZU, M. & YAMAUCHI, K. 1982. Isolation and characterisation of mucin-like glycoprotein in human milk fat globule membrane. Journal of Biochemistry, 91, 515-524.

SILVESTRINI, R. DAIDONE, M. G. & DIFRONZO, G. 1979. Relationship between proliferative activity and estrogen receptors in breast cancer. Cancer, 44, 665-670.

SINGER, S. J. & SCHICK, A. F. 1961. The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. Journal of Biophysical and Biochemical Cytology, 9, 519-537.

SINGH, G., WHITESIDE, T. L. & DEKKER, A. Immunodiagnosis of mesothelioma. Use of antimesothelial cell serum in an indirect immunofluorescence assay. Cancer, 43, 2288-2296.

SLOANE, J. P. & ORMEROD, M. G. 1981. Distribution of epithelial membrane antigen in normal and neoplastic tissues and its value in diagnostic tumour pathology. Cancer, 47, 1786-1795.

SLOANE, J. P., ORMEROD, M. G., IMRIE, S. F. & COOMBES, R. C. 1980. The use of antisera to epithelial membrane antigen in detecting micrometastases in histological sections. British Journal of Cancer, 42, 392-398.

SMALLWOOD, J., KHONG, Y., BOYD, A., GUYER, P., HERBERT, A., COOKE, T. & TAYLOR, I. 1984. Assessment of a scoring scheme for the pre-operative diagnosis of breast lumps. Annals of The Royal College of Surgeons of England, 66, 267-269.

SMITH, N. J., DZIURA, B. R. & GONDOS, B. 1980. Use of blood group iso-antigens in distinguishing benign and malignant cells in effusions. (Abstract). Acta Cytologica, 24, 66-67.

SORENSEN, S., ANDERSON, J. & NORGAARD, T. 1984. Pregnancy-specific B1 glycoprotein (SP1) in serum and tissue from patients with benign and malignant breast tumours. British Journal of Cancer, 49, 663-667.

SOULE, H. R., LINDER, E. & EDGINGTON, T. S. 1983. Membrane 126-KD phosphoglycoprotein associated with human carcinomas identified by a hybridoma antibody to mammary carcinoma cells. Proceedings of the National Academy of Science, 80, 1332-1336.

SPRATT, J. S. 1977. Growth kinetics in mammary cancer. In : New Aspects of Breast Cancer, Volume 3. Secondary Spread in Breast Cancer. (Ed. Stoll, B. A.) William Heinemann Medical Books Ltd. (London).

SPRINGER, G. F., DESAI, P. R. & SCANLON, E. F. 1976. Blood group precursors as human breast carcinoma-associated antigens and "naturally" occurring human cytotoxins against them. Cancer, 37, 169-176.

SPRING-MILLS, E. J., STEARNS, S. B., NUMANN, P. J. & SMITH, P. H. 1984. Immunocytochemical localisation of insulin- and somatostatin- like material in human breast tumours. Life Sciences, 35, 185-190.

STEHLIN, J. S., GUTIERREZ, A. E. & GREEFF, P. J. 1979. Treatment of carcinoma of the breast. Surgery, Gynecology and Obstetrics, 149, 911-922.

STERNBERGER, L. A. 1969. Some new developments in immunocytochemistry. Mikroscopie, 25, 346-361.

STERNBERGER, L. A. 1979a. Immunofluorescence. In : Immunocytochemistry 2nd edition. (Ed. S. Cohen & R. T. McCluskey) John Wiley & Sons (New York) 24-58.

STERNBERGER, L. A. 1979b. Enzyme-labeled antibodies. In : Immunocytochemistry 2nd edition. (Ed. S. Cohen & R. T. McCluskey) John Wiley & Sons (New York) 82-103.

STERNBERGER, L. A., HARDY, P. H. Jr, CUCULIS, J. J. & MEYER, H. G. 1970. The unlabelled antibody enzyme method of immunohistochemistry. Journal of Histochemistry and Cytochemistry, 18, 315-333.

STREEFKERK, J. G. 1972. J. Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. The Journal of Histochemistry and Cytochemistry, 20, 829-831.

STUDIES ON MEDICAL AND POPULATION SUBJECTS. NO. 43. 1981. Cancer statistics, incidence, survival and mortality in England and Wales. HMSO. (London)

TAYLOR-PAPADIMITRIOU, J., PETERSON, J. A., ARKLIE, J., BURCHELL, J., CERIANI, R. L. & BODMER, W. F. 1981. Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: Production and reaction with cells in culture. International Journal of Cancer, 28, 17-21.

TERAMOTO, Y. A., MARIANI, R., WUNDERLICH, D & SCHLOM, J. The immunohistochemical reactivity of a human monoclonal antibody with tissue sections of human mammary tumours. Cancer, 50, 241-249.

TERENIUS, L., JOHANSSON, H., RIMSTEN, A. & THOREN, L. 1974. Malignant and benign human mammary disease: Estrogen binding in relation to clinical data. Cancer, 33, 1364-1368.

THORENSEN, S. 1982. Histological grading and clinical stage at presentation in breast carcinoma. British Journal Of Cancer, 46, 457-458.

THOMPSON, C. H., JONES, S. L., WHITEHEAD, R. H. & MCKENZIE, I. F. 1983. A human breast tissue-associated antigen detected by a monoclonal antibody. Journal of the National Cancer Institute, 70, 409-419.

TO, A., COLEMAN, D. V., DEARNALEY, D. P., ORMEROD, M. G., STEELE, K. & NEVILLE, A. M. 1981. Use of antisera to epithelial membrane antigen for the cytodiagnosis of malignancy in serous effusions. Journal of Clinical Pathology, 34, 1326-1332.

TO, A., DEARNALEY, D. P., ORMEROD, M. G., CANTI, G. & COLEMAN, D. V. 1982. Epithelial membrane antigen - Its use in the cytodiagnosis of malignancy in serous effusions. American Journal of Clinical Pathology, 78, 214-219.

TREVES, N. & HOLLEB, A. I. 1958. A report of 549 cases of breast cancer in women 35 years of age or younger. Surgery, Gynecology & Obstetrics, 107, 271-283.

TROWBRIDGE, I. S. & DOMINGO, D. L. 1981. Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumour cells. Nature, 294, 171-173.

VALAGUSSA, P., BONNADONNA, G. & VERONESI, U., 1978. Patterns of relapse and survival in operable breast cancer with positive and negative axillary lymph nodes. Tumori, 64, 241-258.

WACHNER, R., WITTEKIND, C. & VON KLEIST, S. 1984. Immunological localisation of B HCG in breast carcinomas. European Journal of Cancer and Clinical Oncology, 20, 679-684.

WAHREN, B., LIDBRINK, E., WALLGREN, A., ENEROTH, P. & ZAJICEK, J. 1978. Carcinoembryonic antigen and other tumour markers in tissue and serum or plasma of patients with primary mammary carcinoma. Cancer, 42, 1870-1878.

WALKER, R. A. 1978. Significance of a sub-unit HCG demonstrated in breast carcinomas by the immunoperoxidase technique. Journal of Clinical Pathology, 31, 245-249.

WALKER, R. A. 1979. The demonstration of a lactalbumen in human breast carcinomas. Journal of Pathology, 129, 37-41.

WELLER, T. H. & COONS, A. H. 1954. Fluorescent antibody studies with agents of Varicella and Herpes zoster propagated in vitro. Proceedings of the Society for Experimental Biology and Medicine, 86, 789-794.

WELLS, C. A., HERYET, A., BROCHIER, J., GATTER, K. C. & MASON, D. Y. 1984. The immunocytochemical detection of axillary micrometastases in breast cancer. British Journal of Cancer, 50, 193-197.

WILKINSON, E. J., HAUSE, L. L., KUZMA, J. F., ROTHWELL, D. J., DONEGAN, W. L., CLOWRY, L. J. & 7 Others, 1981. Occult axillary lymph node metastasis in patients with invasive breast carcinoma. Laboratory Investigation, 44, 83A.

WILKINSON, M. J. S., HOWELL, A., HARRIS, M., TAYLOR-PAPADIMITRIOU, J., SWINDELL, R. & SELLWOOD, R. A. 1984. The prognostic significance of antigens expressed by human mammary tumour cells. International Journal of Cancer, 33, 299-304.

WOODING, F. B. P. 1971.The mechanism of secretion of the milk fat globule. Journal of Cell Science, 9, 805-821.

WOODRUFF, M. F. A. 1983. Cellular heterogeneity in tumours. British Journal of Cancer, 47, 589-594.

WOODS, J. C., SPRIGGS, A. I., HARRIS, H. & MC GEE, J. O'D. 1982. A new marker for human cancer cells. 3. Immunocytochemical detection of malignant cells in serous fluids with the Ca-1 antibody. The Lancet, ii, 512-514.

YUAN, D., HENDLER, F. J. & VITETTA, E. S. 1982. Characterisation of a monoclonal antibody reactive with a subset of human breast tumours. Journal of the National Cancer Institute, 68, 719-728.

ZAJDELA, A., GHOSSEIN, N. A., PILLERON, J.P. & ENNUYER, A. 1975. The value of aspiration cytology in the diagnosis of breast cancer : experience at the Fondation Curie. Cancer, 35, 499-506.

ZIPPIN, C. 1966. Comparison of the international and American systems for the staging of breast cancer. Journal of the National Cancer Institute, 36, 53-62.

WORK PUBLISHED AND PRESENTED IN CONNECTION WITH THIS THESIS

Br. J. Cancer (1985), **51**, 179–186

The prognostic value of the monoclonal antibodies HMFG1 and HMFG2 in breast cancer

N. Berry[1], D.B. Jones[1], J. Smallwood[2], I. Taylor[2], N. Kirkham[1] & J. Taylor-Papadimitriou[3]

[1]*Departments of Pathology* & [2]*Surgery, Faculty of Medicine, University of Southampton, SO9 4XY;* [3]*Imperial Cancer Research Fund, Lincolns Inn Fields, London WC2A 3PX, UK.*

Summary The monoclonal antibodies HMFG1 and HMFG2 identify antigens of the milk fat globule membrane which are also found on breast epithelial cells. Immunohistochemical staining was performed using both antibodies on formalin fixed, paraffin embedded sections of 93 breast carcinoma, 36 histologically benign lesions and 29 histologically normal breast tissue blocks. In both normal and benign breast disease the staining was largely extracellular whilst in malignant tissue the staining was variable and often intracellular. Nine carcinomas did not stain with either antibody. The staining patterns of malignant tissues were graded and no correlation was found between the grades and survival or indices of prognosis, (the oestrogen receptor status, Bloom's grade and the presence or absence of metastases to the axillary nodes.)

This study indicates that with the present methods available for grading staining patterns, although of diagnostic value, these monoclonal antibodies are unlikely to assist in determining either the degree of tumour differentiation or prognosis in breast carcinoma.

The clinical application of antibodies in the diagnosis and staging of human cancer has been widely investigated. In breast cancer, oligoclonal antisera to Epithelial Membrane Antigen (EMA) (Heyderman *et al.*, 1979) and anti human mammary epithelial antigens (HME Ags) (Ceriani *et al.*, 1977) have been raised against the delipidated milk fat globule membrane (MFGM), and have been used in the early detection and diagnosis of cancer. (Sloane & Ormerod, 1981; Sloane *et al.*, 1980, To *et al.*, 1982; Dearnaley *et al.*, 1981; Ceriani *et al.*, 1982).

A more widespread application could be achieved by using monoclonal antibodies with a greater specificity for one antigenic epitope. Recently, the monoclonal antibodies HMFG1 and HMFG2 have been raised against the delipidated MFGM (Taylor-Papadimitriou *et al.*, 1981). These react against different antigenic epitopes present on the same molecule to which EMA antibodies are directed (Ormerod *et al.*, 1983). The immunohistochemical staining patterns of both oligoclonal antibodies and monoclonal antibodies to MFGM are extracellular in the ducts and tubules of normal and benign breast tissue and markedly heterogenous in malignant breast tissue (Sloane & Ormerod, 1981; Arklie *et al.*, 1981).

Correspondence: N. Berry.
Received 29 June 1984; and in revised form 5 November 1984.

In the present study the heterogenous immunohistochemical staining patterns of HMFG1 and HMFG2 in malignant breast tissue have been characterised and graded. The grades have been related to relapse-free survival and to prognostic indicators presently in use; namely the nodal status (Valagussa *et al.*, 1978), Bloom's grade (Bloom & Richardson, 1957) and oestrogen receptor status (Cooke *et al.*, 1979).

Materials and methods

Biopsy material from 130 women undergoing surgery for breast disease was studied: From the routine sections stained with haematoxylin and eosin (H & E), 36 of the cases were diagnosed histologically as benign and 93 as carcinoma. Of the carcinoma cases, histologically normal tissue surrounding the lesion was present in 29. Patients with breast cancer were of two chronological groups. Thirty-seven were treated in 1974 and 1975 and their subsequent survival until 1982 was known. Fifty-six cases were treated in 1982 and 1983 and their oestrogen receptor status was determined by Tenovus Laboratories (Cardiff) using the dextran charcoal method (Cooke *et al.*, 1979). The presence of absence of metastatic spread of the primary tumour to the axillary lymph node was known for 89 of the patients with carcinoma. The Bloom's grade for each case was determined

independently from the routine H & E stained sections.

The benign cases comprised the following: fibroadenosis (16), fibroadenoma (11), lipoma (2), papilloma (3), gynaecomastia (2), cyst (1), and duct ectasia (1). The carcinomas were of the following types: infiltrating ductal (67), lobular (5), mixed ductal and lobular (3), medullary (3), infiltrating comedocarcinoma (4), infiltrating cribriform (3), mucoid (3), tunular (2), mixed medullary and lobular (1) and carcinoid (2).

In each case the tissue was fixed in 10% neutral buffered formol saline for ~24–48 h, taken through alcohols and chloroform to paraffin wax on an automatic processor, and embedded in paraffin wax.

Staining of sections

Sections (4 μm) were cut from each block of tissue. Endogenous peroxidase activity was inhibited using 0.5% H_2O_2 in methanol for 10 min. The sections were digested using 0.1% trypsin in 0.1% calcium chloride solution at 37°C for 10 min (Mepham *et al.*, 1979). In 10 cases control sections were not digested in trypsin. The sections were stained using an indirect immunoperoxidase assay (Burns, 1978). The sections were incubated at room temperature for 30 min with HMFG1 or HMFG2 (gift from J. Taylor Papadimitriou) as the primary antibody and, after extensive washing, incubated for a further 30 min with peroxidase conjugated rabbit anti mouse IgG (DAKO) as the second antibody. A brown reaction product was developed using 3,3 diaminobenzidine tetrahydrochloride (DAB) (Sigma Biochemicals).

For each sample negative controls using Tris Buffered Saline (TBS) pH 7.6 in place of the primary antibody were also included. In 20 cases 2 consecutive sections of the tissue were stained with HMFG1 and HMFG2 on separate occasions to determine the reproducibility of the staining patterns.

Grading of staining patterns in malignant tissue

The staining patterns observed in malignant tissue were graded according to the details in Table I. An overall assessment of the relative intensity of staining observed in the infiltrating regions of the tumour was made at a magnification of ×40, taking into account the heterogeneity of staining. A representative area of the section was then assessed at a magnification of ×400 and the presence of intracellular staining in 100 randomly observed cells noted.

The 2 scores obtained for each tissue section were then added such that a tissue with a completely extracellular staining pattern scored 2 points, 1 for the strong extracellular staining in tubules and 1 because 0–25% of the cells stained intracellularly. A tissue section with strong intracellular staining would score 8 points, 4 because of the strong intracellular staining diffusely distributed in the cells and 4 because 76–100% of the cells stained intracellularly.

The cases were then divided into 3 grades: those not stained at all being grade 0, those scoring 2–5 points grade A and those scoring 6–8 points grade B. To check the reproductibility of grading, all of the 93 carcinoma cases were coded and then graded on 2 separate occasions.

Statistics

The staining grades from the tissue sections were correlated with Bloom's grade, Oestrogen receptor status and involvement of axillary lymph nodes using a Chi-squared test. Survival curves of the different staining grades were plotted and analysed using the SPSS "SURVIVAL" sub-programme (Nie *et al.*, 1981). Pairwise comparison of the curves was

Table I Scoring system for immunoperoxidase staining

Staining observed	*Points scored*
1. Relative intensity of staining strongest:	
Extracellularly – in tubules	1
Extracellularly – intercellularly and in intracytoplasmic vacuoles	2
Intracellularly – localised towards periphery of cell	3
Intracellularly – diffusely distributed in the cytoplasm	4
2. Extent of intracellular staining:	
0–25%	1
26–50%	2
51–75%	3
76–100%	4

performed using the Lee Desu statistic (Lee & Desu, 1972).

Results

Controls

A comparison of the immunohistochemical staining pattern in undigested and trypsin digested tissue sections showed that trypsin digestion gave greater staining intensity and reduced background staining without affecting the distribution of the stain.

Of the 20 cases where 2 consecutive sections were stained on separate occasions, the staining pattern was reproducible, although the overall intensity of staining in the sections often differed.

Normal and benign tissue

The staining pattern of histologically normal and benign tissue with both HMFG1 and HMFG2 was located on the luminal surface of cells lining and secreted material within the ducts and tubules. (Figure 1 & Figure 2). Staining of benign tissue was generally greater than in normal tissue and varied in extent and intensity both between histological types of cases and between cases. No intracellular staining was observed except in some benign cases where weak intracellular staining was occasionally observed in the apical region of cells lining ducts and tubules which contained secretion.

Malignant tissue

Staining of malignant tissue varied from case to case and within different areas of the same section. HMFG1 and HMFG2 generally gave the same staining patterns although the relative intensities of the 2 antibodies varied (Table II). In 9 cases neither antibody stained the tissue. In 5 cases where there was extensive tubule formation, the staining pattern was similar to that seen in normal and benign tissue. All of the remaining 79 cases showed intracellular staining to some extent with one or both of the antibodies. In some cases the staining was strongest extracellularly, either in tubules, (Figure 3) or, where there were no tubules, in intercellular spaces and intracytoplasmic vacuoles (Figure 4). In other cases the staining was strongest intracellularly, and was distributed either towards the periphery of the cell (Figure 5) or diffusely in the cytoplasm (Figure 6). Particularly strong staining was observed in the strands of tumour cells typical of lobular carcinoma and no staining was present in the carcinoid tumours. There was no other general association of the staining pattern with the histological type of tumour.

Reproducibility of the staining grades

Eighty four percent of the sections were given the same staining grade on 2 separate occasions. The remaining 16% had been particularly difficult to

Figure 1 Immunohistochemical staining of histologically normal breast tissue with HMFG2. Extracellular staining is apparent in the lumen of tubules. (Indirect immunoperoxidase, ×400).

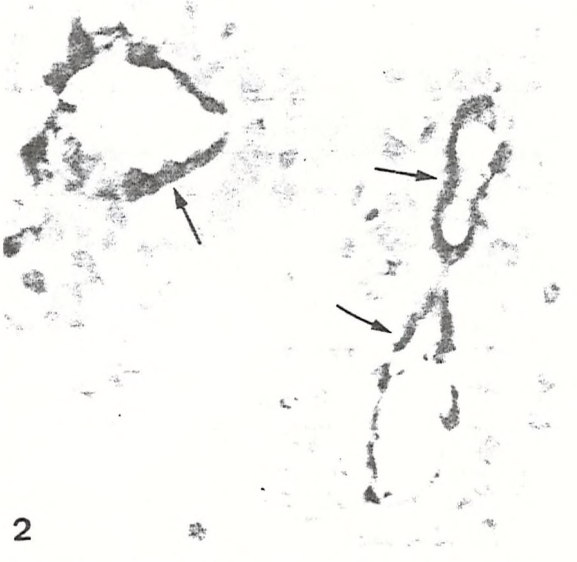
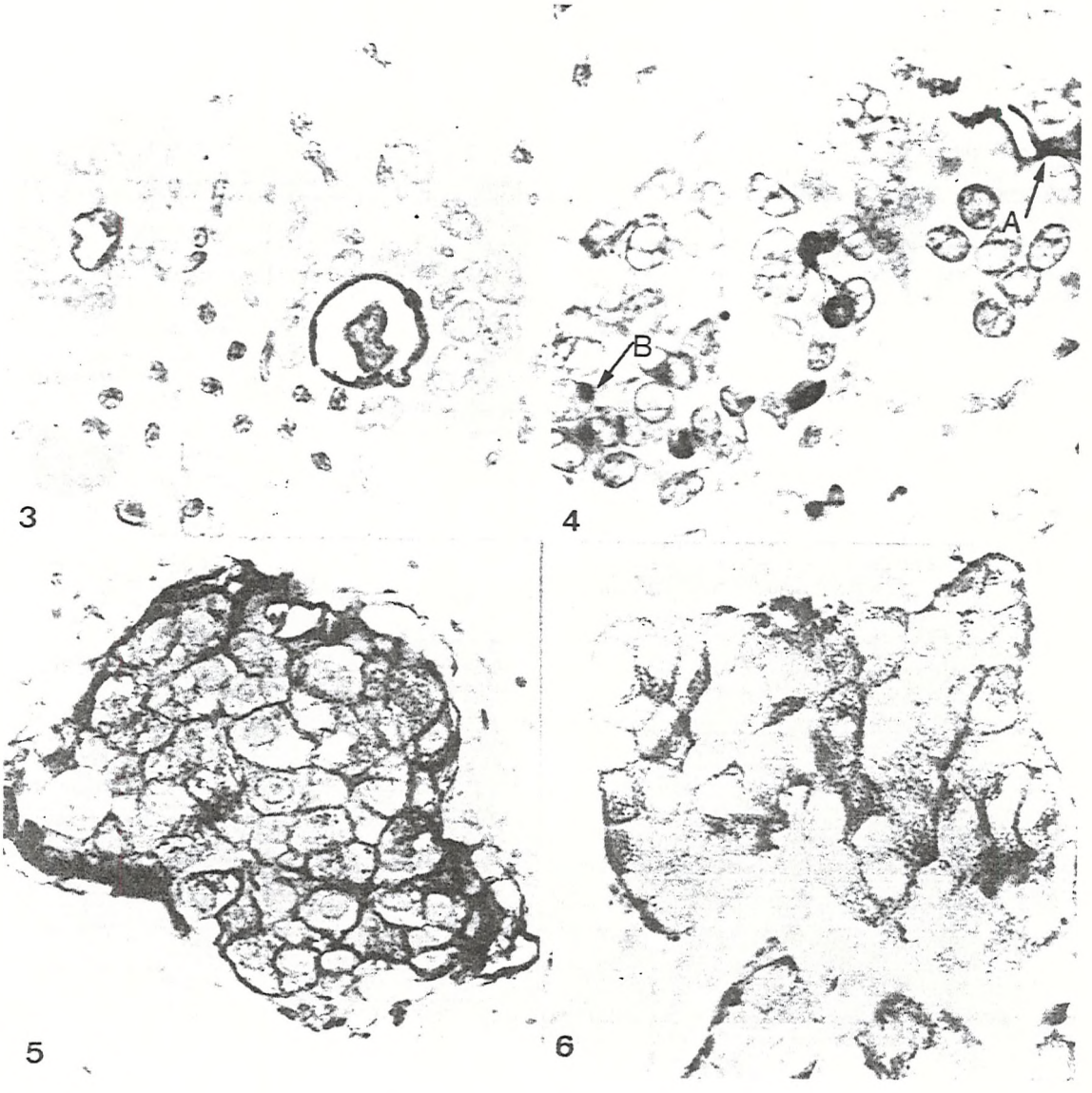


Figure 2 Immunohistochemical staining in a histologically benign breast lesion, (fibroadenoma), with HMFG2. The pattern of extracellular staining is similar to normal tissue but shows greater intensity. (Indirect immunoperoxidase, ×400).



Figures 3–6 (3) Well differentiated infiltrating ductal carcinoma. Strong extracellular staining of HMFG2 is present on the luminal surface of cells lining tubules. (Indirect immunoperoxidase, ×400). (4) Infiltrating ductal carcinoma. Immunoperoxidase staining shows HMFG2 antigen extracellularly, both between the cells (A) and in intracytoplasmic vacuoles (B). (Indirect immunoperoxidase, ×400). (5) Infiltrating ductal carcinoma. Illustrates granular cytoplasmic staining of HMFG2 antigen which is more pronounced at the cell membrane. (Indirect immunoperoxidase, ×400). (6) Infiltrating ductal carcinoma. Diffuse intracellular staining of HMFG2 antigen is present. (Indirect immunoperoxidase, ×400).

Table II Table of immunohistochemical staining patterns of HMFG1 and HMFG2 in breast cancer

Tissue histology	*Description of immunohistochemical staining with HMFG1 and HMFG2*
Normal	Extracellular – staining on the luminal surface of cells lining and secretions within the ducts and tubules. No intracellular staining.
Benign	Extracellular – As above, but occasionally with weak intracellular staining in the apical region of cells lining ducts and tubules which contained secretion.
Malignant	5/93 Extracellular – As normal above 9/93 No staining with either HMFG1 or HMFG2 79/93 Intracellular – marked heterogeneity of both the extent of intracellular staining and the relative intensity of staining from stronger extracellularly to stronger intracellularly.

grade because of the variability of staining throughout the section.

Relation of the staining grades to bloom's grade

Twelve of the 93 carcinomas were Bloom's grade 1. In 9 of these the staining pattern was grade A with both HMFG1 and HMFG2. One of the 12 cases did not stain with either antibody and in 1 the staining pattern was Grade A with HMFG2 only. Fifteen cases were Bloom's grade 3 and 66 were Bloom's grade 2 (Table III). No significant association was found between the staining grades A, B or 0 and Bloom's grade 2 or 3 (Chi-squared test). The lack of correlation was obtained in sections stained with either HMFG1 or HMFG2. ($P=7.23$; $P=6.77$).

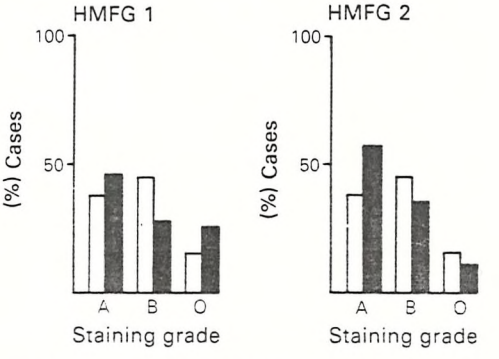


Figure 7 Relation of the staining grades to oestrogen receptor status. (□) = E.R. +; (■) = E.R. –.

Table III Relationship of the staining grades obtained with HMFG1 and HMFG2 to Bloom's Grade in 93 breast carcinomas

	Bloom's Grade					
	HMFG1			*HMFG2*		
Staining grade	*1*	*2*	*3*	*1*	*2*	*3*
A	9	26	7	10	30	6
B	2	30	4	1	28	7
O	1	10	4	1	8	2

Relation of the staining grades to oestrogen receptor status

Twenty-four of the 56 cases from 1982–3 were oestrogen receptor positive and 32 were oestrogen receptor negative. There was no significant association between the staining grade A, B or 0 and the oestrogen receptor status of the tumours (Chi-squared test) (Figure 7). The lack of correlation was present in sections stained with either HMFG1 or HMFG2. ($P=1.93$, $P=2.04$).

Relation of the staining grades to the presence of nodal metastases

Thirty-five of the 89 cases were reported as having metastases in the axillary nodes and 55 had no nodal metastases. There is no significant association between the staining grade A, B or 0 and the presence or absence of nodal metastases (Chi-squared test) (Figure 8). The correlation was insignificant in sections stained with either HMFG1 or HMFG2 ($P=3.09$; $P=0.92$).

Life table analysis comparing the staining grades

Survival curves of patients from 1974–5 with staining grade A, B and 0 are illustrated in Figure 9. Although there appeared to be some differences between the curves, particularly with HMFG2, statistical analysis of the 3 curves showed that there was no significant difference in the survival of each

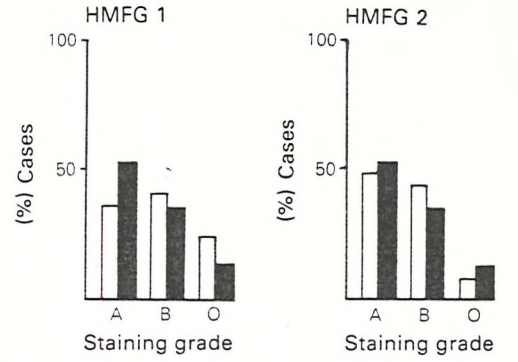


Figure 8 Relation of staining grades to the presence of nodal metastases. (□) = Node +; (■) = Node −.

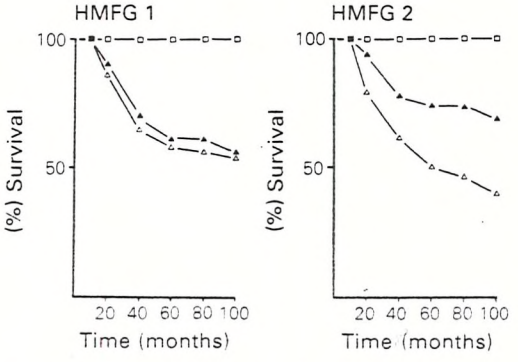


Figure 9 Life table analysis comparing the staining grades. (□) = Grade O; (△) = Grade A; (▲) = Grade B.

staining group in sections stained with either HMFG1 or HMFG2 ($P=0.79$; $P=0.18$).

Discussion

The immunohistochemical staining patterns of the monoclonal antibodies HMFG1 and HMFG2 in breast tissue have been described previously (Arklie *et al.*, 1981). The patterns observed in this study are similar, that is, extracellular in the ducts and tubules of normal and benign tissue and variable in malignant tissue.

The heterogeneity of staining in malignant breast tissue was apparent both in the extent of intracellular staining and in the relative intensity of staining. In some cases extracellular staining was observed both in tubules and between cells. Staining of the intracytoplasmic vacuoles was also considered extracellular, as electron microscopy has shown that the surface of the inner membrane of the vacuole has features typical of the exterior surface of the cell membranes (Battifora, 1975). In other cases staining was stronger intracellularly distributed either towards the periphery of the cell or diffusely throughout the cytoplasm. Nine percent of the tumours did not stain with either HMFG1 or HMFG2 and a further 2% did not stain with one of the antibodies. Wilkinson *et al.* (1984) have noted a similar percentage of negatively staining tumours.

Caution should be exerted when comparing immunohistochemical staining characteristics between studies, since differences in tissue preparation and staining technique may be of importance (Brandtzaeg & Rognum, 1982).

The change in the immunohistochemical staining pattern from extracellular in normal and benign breast tissue to mixed with varying amounts of intracellular positivity in malignant breast tissue has been observed using other antibodies (Sloane & Ormerod, 1981; Foster *et al.*, 1982) and lectins (Franklin, 1983).

The staining grades were designed to reflect the immunohistochemical staining patterns observed, whilst being in a form which could be related to indices of prognosis and to survival. Since the staining pattern in normal, benign and structurally differentiated tumours (those with extensive tubule formation) was extracellular, and became more intracellular as structural differentiation was lost, the grading system took into account the relative intensity of extracellular and intracellular staining and also the number of cells with intracellular staining. The 20 control sections where 2 consecutive sections were stained on separate occasions showed the same staining pattern on both sections but the overall intensity of stain throughout the section varied. Little emphasis, therefore, was placed on the overall intensity of the stain. Heterogeneity was observed between different areas of a tissue section and was a limitation of the grading system, for one area of tissue with a less predominant staining pattern, and therefore not graded, might influence the survival of the patient unnoticed. This heterogeneity also made grading difficult in many cases, but when the sections were graded on 2 separate occasions 84% reproducibility was obtained. This method of grading the stains contrasts with that used in another study relating the immunohistochemical staining of HMFG1 to survival and to indices of prognosis (Wilkinson *et al.*, 1984), where the grading was based on the uniformity, extent and the overall intensity of the stain.

There was no significant correlation when the staining grades were related to indices of prognosis and relapse-free survival. A significant correlation might have been expected when relating the staining grades to Bloom's grade since tumours with

extensive tubule formation had an extracellular staining pattern. Three measures of differentiation contribute to Bloom's Grade of which tubule formation is one. The other 2 factors might combine to put a tumour with much tubule formation into Bloom's grade 2. Since extracellular staining was also recognised in intercellular spaces and intracytoplasmic vacuoles as well as in tubules, significant correlation with Bloom's grade is unlikely.

There was no association of the staining grades with oestrogen receptor status. In addition no correlation of the staining grades with involvement of axillary lymph nodes was observed. There was, however, a trend, in that a Grade A staining pattern with both HMFG1 and HMFG2 was more frequently seen in patients without lymph node involvement.

For HMFG1 and HMFG2 to be useful in determining the prognosis of breast cancer patients, the different staining patterns observed should be either closely correlated to existing prognostic indicators or be clearly related to survival, even in a relatively small series of cases. Relating the relapse-free survival of 34 patients to the staining grade, although there appeared to be some difference between the survival curves of the 3 staining grades, statistical comparison of the curves showed that they were not significantly different when either HMFG1 or HMFG2 was used. The antibodies are therefore of little use in routine diagnosis. Similar results were obtained by Wilkinson *et al.* (1984) where there was no significant association of the staining patterns observed to indices of prognosis such as Bloom's grade, the presence of metastases in the axillary nodes and the oestrogen receptor status. However, these authors identified a group of patients whose tumours did not stain with HMFG1 and who had a particularly poor prognosis. High levels of extracellular staining were considered indicators of a good prognosis. Although the immunoperoxidase staining technique was different in some respects to the one used in this study, a comparison of the staining patterns observed using the 2 techniques in 10 cases showed agreement. The different results must therefore be either due to the different method of grading used or, in the case of those patients with negatively stained tumours, the small number of cases.

The results of this study show that with present methods available for grading staining patterns the immunohistochemical staining patterns of HMFG1 and HMFG2 in malignant breast tissue do not help in determining the overall prognosis in an indivudual patient.

We acknowledge the work of the technical staff in the Pathology Dept., Faculty of Medicine, University of Southampton in preparing the illustrations.

References

ARKLIE, J., TAYLOR-PAPADIMITRIOU, J., BODMER, W., EGAN, M. & MILLIS, R. (1981). Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. *Int. J. Cancer*, **28**, 23.

BATTIFORA, H. (1975). Intracytoplasmic lumina in breast carcinoma. *Arch. Pathol.*, **99**, 614.

BLOOM, H.J.G. & RICHARDSON, W.W. (1957). Histological grading and prognosis in breast cancer. *Br. J. Cancer*, **11**, 359.

BRANDTZAEG, P. & ROGNUM, T.O. (1982). Evaluation of tissue preparation methods and paired immunofluorescence staining for immunocytochemistry of lymphomas. *Histochem. J.*, **15**, 655.

BURNS, J. (1978). Immunohistochemical methods and their application in the routine laboratory. In *Recent Advances In Histopathology*. p. 337. (Eds. Anthony & Woolf) Churchill & Livingstone.

CERIANI, R.L., THOMPSON, K.E., PETERSON, J.A. & ABRAHAM, S. (1977). Surface differentiation antigens on human mammary epithelial cells carried on the human milk fat globule. *Proc. Natl. Acad. Sci.*, **74**, 582.

CERIANI, R.L., SASAKI, M., SUSSMAN, H., WARA, W.M. & BLANK, E.W. (1982). Circulating human mammary epithelial antigens in breast cancer. *Proc. Natl Acad. Sci.*, **79**, 5420.

COOKE, T., GEORGE, D., SHIELDS, R., MAYNARD, P. & GRIFFITHS, K. (1979). Oestrogen receptors and prognosis in early breast cancer. *Lancet*, **i**, 995.

DEARNALEY, D.P., SLOANE, J.P., ORMEROD, M.G. & 6 others. (1981). Increased detection of mammary carcinoma cells in marrow smears using antisera to epithelial membrane antigen. *Br. J. Cancer*, **44**, 85.

FOSTER, C.S., DINSDALE, E.A., EDWARDS, P.A.W. & NEVILLE, A.M. (1982). Monoclonal antibodies to the human mammary gland II. *Virchows Arch. (Pathol. Anat.)*, **394**, 295.

FRANKLIN, W.A. (1983). Tissue binding of lectins in disorders of the breast. *Cancer*, **51**, 295.

HEYDERMAN, E., STEELE, K. & ORMEROD, M.G. (1979). A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissues. *J. Clin. Pathol.*, **32**, 35.

LEE, E., & DESU, M. (1972). A computer program for estimating survival functions for the life-table. *Comput Progr Biomed.*, **2**, 315.

MEPHAM, B.L., FRATER, W. & MITCHELL, B.S. (1979). The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. *Histochem. J.*, **11**, 345.

NIE, N.H., HILL, C.H., JENKINS, J.C., STEINBRENNER, K. & BENT, D.H. (1981). *SPSS Updates 7–9*, McGraw Hill, New York.

ORMEROD, M.G., STEELE, K., EDWARDS, P.A.W. & TAYLOR-PAPADIMITRIOU, J. (1983). Monoclonal antibodies which react with epithelial membrane. (In press).

SLOANE, J.P., ORMEROD, M.G., IMRIE, S.F. & COOMBES, R.C. (1980). The use of antisera to epithelial membrane antigen in detecting micrometastases in histological sections. *Br. J. Cancer*, **42**, 392.

SLOANE, J.P. & ORMEROD, M.G. (1981). Distribution of epithelial membrane antigen in normal and neoplastic tissues and it's value in diagnostic tumour pathology. *Cancer*, **47**, 1786.

TAYLOR-PAPADIMITRIOU, J., PETERSON, J.A., ARKLIE, J., BURCHELL, J., CERIANI, R.L. & BODMER, W.F. (1981). Monoclonal antibodies to epithelium specific components of the milk fat globule membrane: production and reaction with cells in culture. *Int. J. Cancer*, **28**, 17.

TO, A., DEARNALEY, D.P., ORMEROD, M.G., CANTI, G. & COLEMAN, D.V. (1982). Epithelial membrane antigen. Its use in the cytodiagnosis of malignancy in serous effusions. *Am. J. Clin. Pathol.*, **78**, 214.

VALAGUSSA, P., BONADONNA, G. & VERONESI, U. (1978). Patterns of relapse and survival in operable breast cancer with positive and negative axillary nodes. *Tumori*, **64**, 241.

WILKINSON, M.J.S., HOWELL, A., HARRIS, M., TAYLOR-PAPADIMITRIOU, J., SWINDELL, R. & SELLWOOD, R.A. (1984). The prognostic significance of antigens expressed by human mammary tumour cells. *Int. J. Cancer*, **33**, 299.

PRESENTATIONS:

British Association for Cancer Research Annual Conference York, 1983.
"Immunohistochemical Localisation of Milk Fat Globule Antigens In Routine Breast Biopsy Material". N. Berry, D.B Jones, N. Kirkham, J. Smallwood, I. Taylor & J. Taylor-Papadimitriou.

The National Institutes of Health Breast Cancer Task Force Symposium entitled "New Methods for Early Detection and Diagnosis of Breast Cancer", Washington D.C., 1982
"Needle Biopsy and Monoclonal Antibodies". N. Berry.

Surgical Research Society Conference, Dublin, 1985.
"Increased Detection of Axillary Lymph Node Metastases from Primary Breast Cancer by Immunohistochemical Staining with Monoclonal Antibodies". N. Berry, D.B. Jones, R. Marshall, J. Smallwood & I. Taylor.