EVALUATION OF THE MONOCLONAL ANTIBODY SM3 - SPECTRUM OF REACTIVITY IN THE BREAST AND AT OTHER SITES, AND VALUE AS A CLINICAL IMAGING AGENT

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ABSTRACT

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SM3, a monoclonal antibody raised against the deglycosylated mucin from human skimmed milk (SM stands for stripped mucin) has been evaluated. Its reactivity has been compared with that of HMFG2, a monoclonal antibody reactive with the fully glycosylated mucin.

Immunohistochemical studies have shown that the SM3 epitope is highly tumour associated, being expressed in more than 90% of mammary carcinomas and in tumours from other sites. It is virtually undetectable, however, in breast tissue showing secretory change, and present at very low levels in normal resting breast tissue, benign breast lesions and normal tissues from elsewhere in the body.

Immunoblotting studies have shown that both in breast cancer cell lines and primary mammary carcinomas, SM3 recognises an epitope carried predominantly on low molecular weight components.

The potential use of SM3 in immunolocalisation studies has also been investigated. Radiolabelled antibody has been administered pre-operatively to breast cancer patients in an attempt to detect axillary lymph node metastases. Although the results of the imaging studies have so far been disappointing, an ideal model system for future studies of this type has been established.

The immunohistochemical and immunoblotting data are compatible with recent work showing that the SM3 epitope consists of a five amino acid sequence on the core protein of the milk mucin. It appears that in fully differentiated mammary epithelial cells highly glycosylated mucins are produced in which the SM3 epitope is masked. In malignancy,

however, incompletely glycosylated molecules are produced. These have simpler and shorter carbohydrate side chains and the SM3 epitope on the core protein is exposed. These incompletely processed mucin molecules appear to correspond to the lower molecular weight SM3 reactive bands seen in the immunoblots.

Work with SM3 has, therefore, led to further understanding of the processing of mucins in normal and malignant epithelial cells.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Bq = Becquerel

BSA = Bovine serum albumin

Ci = Curie

COU = Clinical Oncology Unit

CT = Computerised tomography

FCS = Fetal calf serum

H+E = Haematoxylin and eosin

HMFG = Human milk fat globule

HMFGM = Human milk fat globule membrane

ICRF = Imperial Cancer Research Fund

MFG = Milk fat globule

MRI = Magnetic resonance imaging

NPG = Non-penetrating glycoprotein

PAGE = Polyacrylamide gel electrophoresis

PBS = Phosphate buffered saline

PEM = Polymorphic Epithelial Mucin

SDS = Sodium dodecyl sulphate

TEMED = Tetramethyl ethylenediamine

Chapter 1

INTRODUCTION

1.1 BACKGROUND : PROGNOSTIC MARKERS IN BREAST CANCER - CURRENT STATUS AND THE NEED FOR FURTHER INFORMATION

Carcinoma of the breast is the most common malignancy in females affecting one in twelve women in the Western world. Despite extensive study of this disease little progress has been made in understanding the aetiology and factors determining outcome. It is a notoriously heterogeneous disease in which the presentation and progression of malignancy varies enormously from one patient to another. Attempts to explain this variation have shed some light on factors which affect the course of the disease but many questions remain unanswered.

Clinical features are clearly important and staging systems based on these have been devised which divide patients into prognostic groups. A summary of the recognised clinical stages and the 5-year survival within these is given in Table 1.1. Although, within each clinical stage, clear differences in prognosis are seen, there are exceptions. A number of patients who present with early (stage I) disease die within ten years of mastectomy as a result of metastatic disease at other sites and it must be assumed, that in these cases, occult malignant cells had already spread beyond the breast and regional nodes before surgery. In contrast, some patients who present with more advanced disease have a better survival than might be expected. In particular, patients with large tumours and negative nodes (after histological assessment) have a lower recurrence rate at five years than those with small tumours and histologically positive nodes (Henderson and Canellos 1980).

Table 1.1

SURVIVAL OF PATIENTS WITH BREAST CANCER RELATIVE TO CLINICAL STAGE*

	CRUDE 5-YEAR SURVIVAL (%)
Stage I Tumour < 2 cm in diameter Nodes, if present, not felt to contain metastases Without distant metastases	85
Stage II Tumour < 5 cm in diameter Nodes, if palpable, not fixed Without distant metastases	66
Stage III Tumour > 5 cm or, Tumour any size with invasion of skin or attached to chest wall Nodes in supraclavicular area Without distant metastases	41
Stage IV With distant metastases	10

*Based on data from American Joint Committee for Cancer Staging (Henderson and Canellos 1980)

In addition to clinical factors, prognosis is also known to be affected by various pathological features. The most important of these is the pathological assessment of axillary nodal metastases which yields more accurate information than does clinical evaluation. Not only is the presence or absence of involved nodes important but also, to a lesser extent, the number of involved nodes. The survival of patients with breast cancer relative to the extent of axillary node involvement (histological stage) is shown in Table 1.2. Other well recognised pathological predictors of prognosis are tumour size, tumour type and the presence of vascular invasion. Additional features, such as tumour grade, the degree of stromal elastosis and inflammatory cell reaction and reactive changes in the regional lymph nodes are of less certain value. The pathological features which are of prognostic significance are summarised in Table 1.3; the first three listed give the most valuable information.

With regard to tumour type, the single most important distinction is that between carcinoma in situ and infiltrating carcinoma, as the former by definition is still confined within the basement membrane and therefore does not have the potential to metastasise. Certain special types of infiltrating carcinoma e.g. tubular, mucoid and medullary carcinoma with lymphoid stroma are associated with a relatively favourable prognosis.

Grading of malignant tumours has been shown to be of prognostic significance at many sites in the body, and was applied to tumours of the breast as long ago as 1925 (Greenhough 1925). Most systems currently used in this country (see Table 1.4) are based on that originally described by Bloom and Richardson (1957). Grading appears to have its greatest prognostic significance in the early years after diagnosis. After ten years little difference is seen between grades II and III and after longer follow-up, differences between all grades are less significant.

Table 1.2

SURVIVAL OF PATIENTS WITH BREAST CANCER RELATIVE TO HISTOLOGICAL STAGE*

	CRUDE SURVIVAL (%)	
	5 YR	10 YR
All patients	63.5	45.9
Negative axillary lymph nodes	78.1	64.9
Positive axillary lymph nodes	46.5	24.9
1-3 positive axillary lymph nodes	62.2	37.5
4 positive axillary lymph nodes	32.0	13.4

*Based on data from National Surgical Adjuvant Breast Project (Henderson and Canellos 1980)

Table 1.3

PATHOLOGICAL FEATURES OF PROGNOSTIC SIGNIFICANCE IN BREAST CANCER

Axillary nodal status
Tumour size
Tumour type
Tumour grade
Vascular invasion
Stromal elastosis
Tumour necrosis
Stromal inflammatory response

Table 1.4

GRADING SYSTEM FOR BREAST CARCINOMAS

Adapted from the Bloom and Richardson grading method (1957)
by Elston (1987)

The following features are assessed and scored from 1-3

TUBULE FORMATION	1 - majority of tumour (>75%)
	2 - moderate amount (10-75%)
	3 - little or none (<10%)
NUCLEAR PLEOMORPHISM	1 - regular, uniform
	2 - larger with variation
	3 - marked variation
FREQUENCY OF MITOSES*	1 - 0-11
	2 - 12-22
	3 - >23

Addition of the three scores gives the overall histological grade

Grade 1 = score of 3-5
2 = score of 6-7
3 = score of 8-9

*Assessed at tumour periphery. Number/10HPF based on a Diaplan x40 objective. True mitotic figures only are scored. "Hyperchromatic figures" (apoptotic cells) are not included.

One of the major problems with grading systems is that they are subjective with considerable inter and even intraobserver variation. Recently, attempts to make assessment more objective have employed morphometric methods (Baak et al 1982).

Although some information can be gained from both clinical and pathological staging this is limited and cannot accurately predict outcome in an individual case. Current trends in the management of early breast cancer are away from radical and towards conservative treatment. As in many centres the latter does not include axillary dissection this valuable piece of prognostic information is not available. It is therefore necessary to look for other markers in breast cancer which may help both to predict prognosis and possibly to guide approaches to treatment. What are these markers and how can they be identified?

Fundamental to the understanding of the development of malignancy at any site in the body is the characterisation of properties of malignant cells and distinction of these from properties of their normal counterparts. In the breast it is therefore important to study malignant epithelial cells and the normal mammary epithelial cells from which they are derived.

The PHENOTYPE of a cell may be defined as the combination of characteristics <u>expressed</u> by a particular cell or type of cell. This is in contrast to the GENOTYPE which is the sum of the genetic material of the cell or its <u>potential</u> characteristics. Differences between the malignant phenotype and the normal phenotype may be assessed in a number of ways.

Differences in morphology have long been and remain the cornerstone of histopathological diagnosis. Such features, however, provide no information as to the functional or structural properties of cells which may be so important in malignancy. While the histochemical features of cells may provide some information as to function, with the advent of immunohistochemistry it has become possible both to study cell products, and to characterise cell surface, cytoplasmic and nuclear antigens. By such means the antigenic expression or IMMUNOPHENOTYPE of cells can be defined and differences between malignant and normal cells investigated. Immunological markers are increasingly being used both in diagnostic histopathology laboratories and research institutions. Numerous immunological markers are now available and some which are useful in the study of the breast are listed in Table 1.5.

In the context of the breast, a group of antibodies of particular interest are those directed against cell surface antigens because these may correspond to surface molecules which have important functions in cell recognition and communication (Ceriani et al 1977). Many antibodies of this type, both polyclonal and monoclonal, have been described. Recently many of the monoclonal antibodies raised against cell surface antigens have been shown to recognise different epitopes on the same high molecular weight glycoprotein molecule (mucin) found in human milk. These antibodies are tumour-associated rather than tumour-specific. Work with an antibody of this type, SM3, forms the basis of this thesis.

As an introduction to this study it is appropriate to look at both the breast in general and some of the techniques used to study breast disease. The normal structure, organisation and development of the human breast will be considered. Methods of both in vivo and in vitro study of differentiation and malignancy in the breast will be reviewed. The use of immunological markers of differentiation in the study of breast disease will be described. The technique of immunohistochemistry will be described and a brief history of its development given. Antibodies raised against the human milk fat globule membrane, a surface membrane antigen will be discussed. Finally, the background to the development of the antibody SM3 will be described and the aims of this thesis summarised.

Table 1.5

ANTIBODIES IN COMMON USE IN BREAST PATHOLOGY, IN BOTH DIAGNOSTIC WORK AND RESEARCH

I Antibodies to structural components of cells

To intermediate filaments	eg cytokeratins in epithelial cells vimentin in mesothelial cells
To contractile proteins	eg actin myosin
To basement membrane components	eg laminin collagen IV

II Antibodies to surface antigens

To components of MFGM	eg EMA HMFG1 HMFG2
To other surface antigens	eg common leucocyte antigen factor VIII related antigen

III Antibodies to cell products

To milk proteins	eg casein alpha lactalbumin
To other cell products	eg steroid hormone receptors oncogene products

1.2 ANATOMY AND DEVELOPMENT OF THE NORMAL BREAST

I ANATOMY OF THE MATURE FEMALE BREAST

The human breast is a specialised gland composed of epithelial-lined structures supported by a variable amount of fibrofatty stroma. Although the epithelium forms a minor component of the total volume of the breast, the majority of diseases, including carcinoma, arise from it and it will therefore be described in some detail.

The mammary epithelium is arranged in 10-20 segments, each consisting of a branching tubular structure that has been likened to a flowering tree (Jensen 1981) (Figures 1.1A and 1.2). Collecting ducts which open onto the surface of the nipple are analogous to the trunk, smaller ducts and ductules to branches and twigs and the lobules to flowers (Figure 1.1B). Hundreds of lobules are present in each breast. They measure from 1-8 mm in diameter and are most numerous in the upper outer quadrant and the central part of the breast. Each lobule is composed of a variable number of acini (from 10-100) sometimes called terminal ductules.

Ducts, ductules and lobules are all lined by a double layer of cells, the inner or luminal epithelial cells and the outer basal or myoepithelial cells. The intralobular stroma is loose, fibrovascular and cellular containing histiocytes, lymphocytes, plasma cells and mast cells. In contrast the interlobular stroma is denser and less cellular. Elastic tissue is present in variable amounts around the ducts but not within the lobules.

Figure 1.1. The microarchitecture of the mammary epithelium has been likened to a flowering tree (A) in which collecting ducts are analogous to the trunk, smaller ducts and ductules to branches and twigs and the lobules to flowers (B).

A

B

II THE TERMINAL DUCTAL LOBULAR UNIT (TDLU)

As described above, each lobule consists of a variable number of acini drained by a single terminal duct which has both an intralobular and extralobular component. This has been called the Terminal Ductal Lobular Unit (TDLU) (Figure 1.2). This term was coined by Wellings and Jensen in 1973 who demonstrated by very elegant subgross studies that the majority of pathological changes in the breast (including most carcinomas) arise at this site (Wellings and Jensen 1973, Wellings, Jensen and Marcum 1975, Jensen 1981). Many pathological lesions which appear to orginate in ducts actually arise in lobules which have "unfolded" to produce duct-like structures. This process is represented diagramatically in Figure 1.3.

The epithelial cells within a given TDLU are probably monoclonal in origin and are "labile" cells i.e. cells which are constantly undergoing division to replace obsolete cells (Jensen 1981). They are thus very vulnerable to hormonal or physical influences which may affect growth.

Figure 1.2. The anatomy of the breast including the Terminal Ductal Lobular Unit (TDLU). The anatomy of the breast is illustrated above (A) and the detailed structure of the TDLU shown below (B).

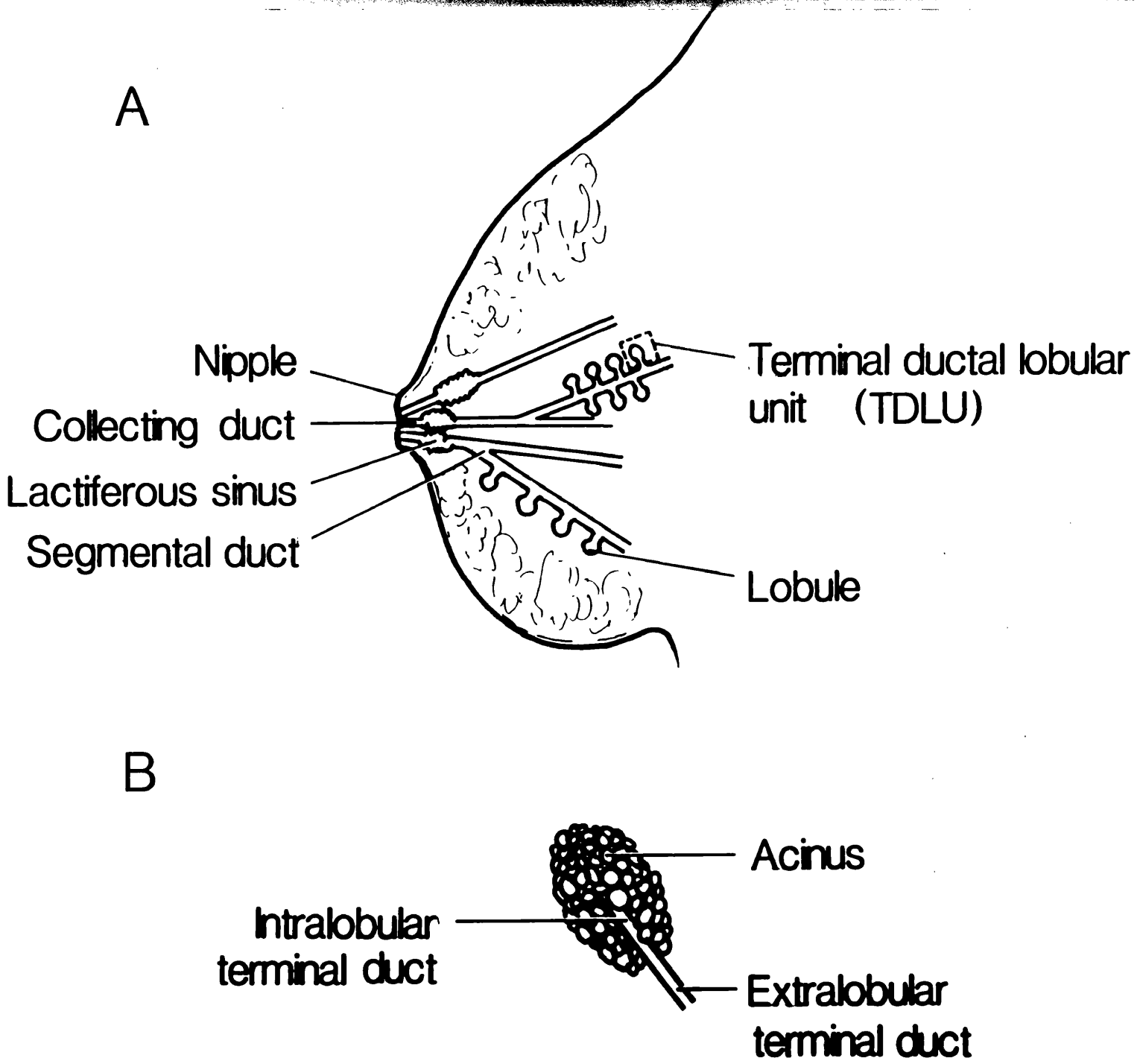
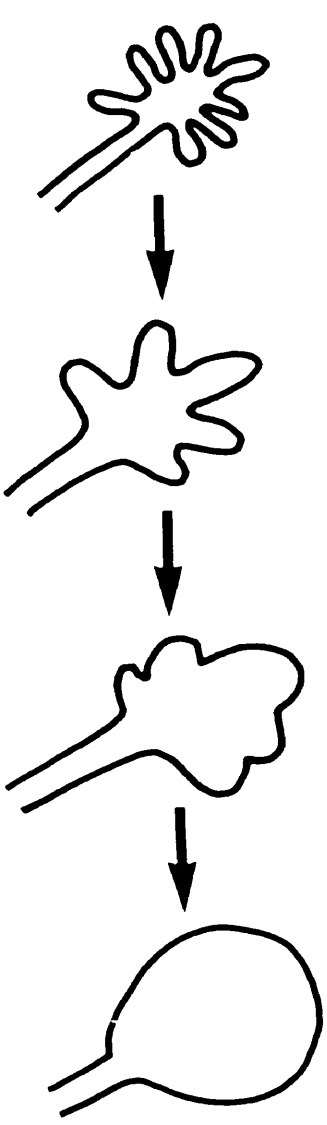
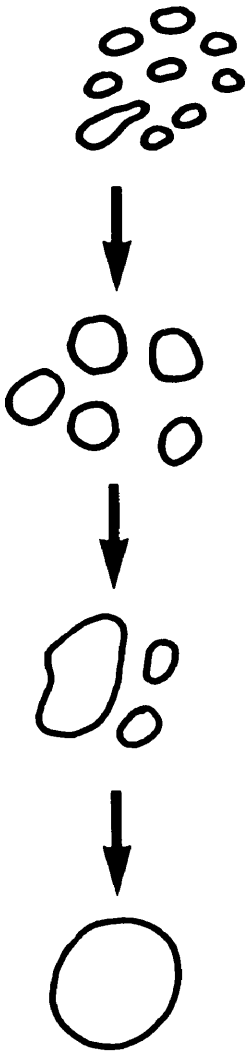

A
Nipple
Collecting duct
Lactiferous sinus
Segmental duct
Terminal ductal lobular unit (TDLU)
Lobule
B
Intralobular terminal duct
Acinus
Extralobular terminal duct

Figure 1.3. The unfolding lobule. Many pathological lesions which appear to originate in ducts actually arise in lobules which "unfold" to produce duct-like structures. This process is depicted diagramatically in two dimensions in the left hand column; the corresponding microscopic appearances are shown in the right hand column.





III DEVELOPMENT OF THE BREAST AND PHYSIOLOGICAL CHANGES

A. Embryology and development from infancy to puberty

The breast develops from the milk line or ridge which at six weeks gestation extends from the axilla to the anterior aspect of the groin. Much of this then atrophies and by nine weeks gestation the nipple appears in the pectoral region and development continues only at this site. An ectodermal bud gives rise to cords of cells which are later canalised to become primary ducts. These branch to form secondary ducts (Rehman 1978). The rudimentary breast is thus composed of primary and secondary ducts. There is some disagreement in the literature as to whether lobules are present in the pre-pubertal breast. In a recent review, Russo and Russo (1987) described personal observations of primitive lobular structures in the mammary glands of five newborn females and this is in agreement with our own observations in a small number of samples of pre-pubertal breast tissue (see Chapter 3).

The male breast remains in the rudimentary form but in the female, thelarche (breast development) starts approximately one year before menarche. At thelarche both the primary and secondary ducts grow and divide to form blindly ending buds. At the same time there is an increase in the volume of connective tissue and fat as well as the vascularity of the breast. Lobular development is generally said to begin at menarche and continues, with considerable variation from one individual to another, between the ages of 16 and 25 (Jensen 1981). Thus the fully developed breast attains the structure described above.

B. Changes with the normal menstrual cycle

In the resting female breast there is considerable variation in the appearance of the lobules. Some are better developed than others and some show minor evidence of secretory activity. Changes during the menstrual cycle have been described by a number of authors but these are of rather a subtle nature and there has been some disagreement over the exact details of these changes. Some of the disagreement may be due to the fact that morphology can vary from lobule to lobule in the same breast and it is necessary where possible to examine multiple samples from each patient and to take an average value for each feature described.

Ferguson and Anderson described a peak in mitotic activity in the second half (secretory phase) of the menstrual cycle (day 25) and a peak in apoptotic activity at day 28 (Ferguson and Anderson 1981, Anderson, Ferguson and Raab 1982). Vogel and colleagues (1981) and later Longacre and Bartow (1986) described morphological changes in both the mammary epithelium and stroma. In the proliferative phase, breast lobules were small and the intralobular stroma condensed. In the secretory phase, lobular size increased and basal cells showed vacuolation; the stroma became looser and oedematous with an increased number of lymphocytes. With the onset of menstruation the lobules contracted and there was necrosis and sloughing of the epithelum. The two groups disagreed, however, over the timing of peak mitotic activity; Vogel and colleagues found a peak early in the cycle, at days 3-7, whereas Longacre and Bartow agreed with the work of Ferguson and Anderson, finding a peak during the secretory phase of the cycle, days 20-26. A peak in mitosis at this time is in agreement with studies on thymidine labelling indices (Masters, Drife and Scarisbrick 1977, Meyer 1977) where the highest labelling has been found in the second half of the menstrual cycle.

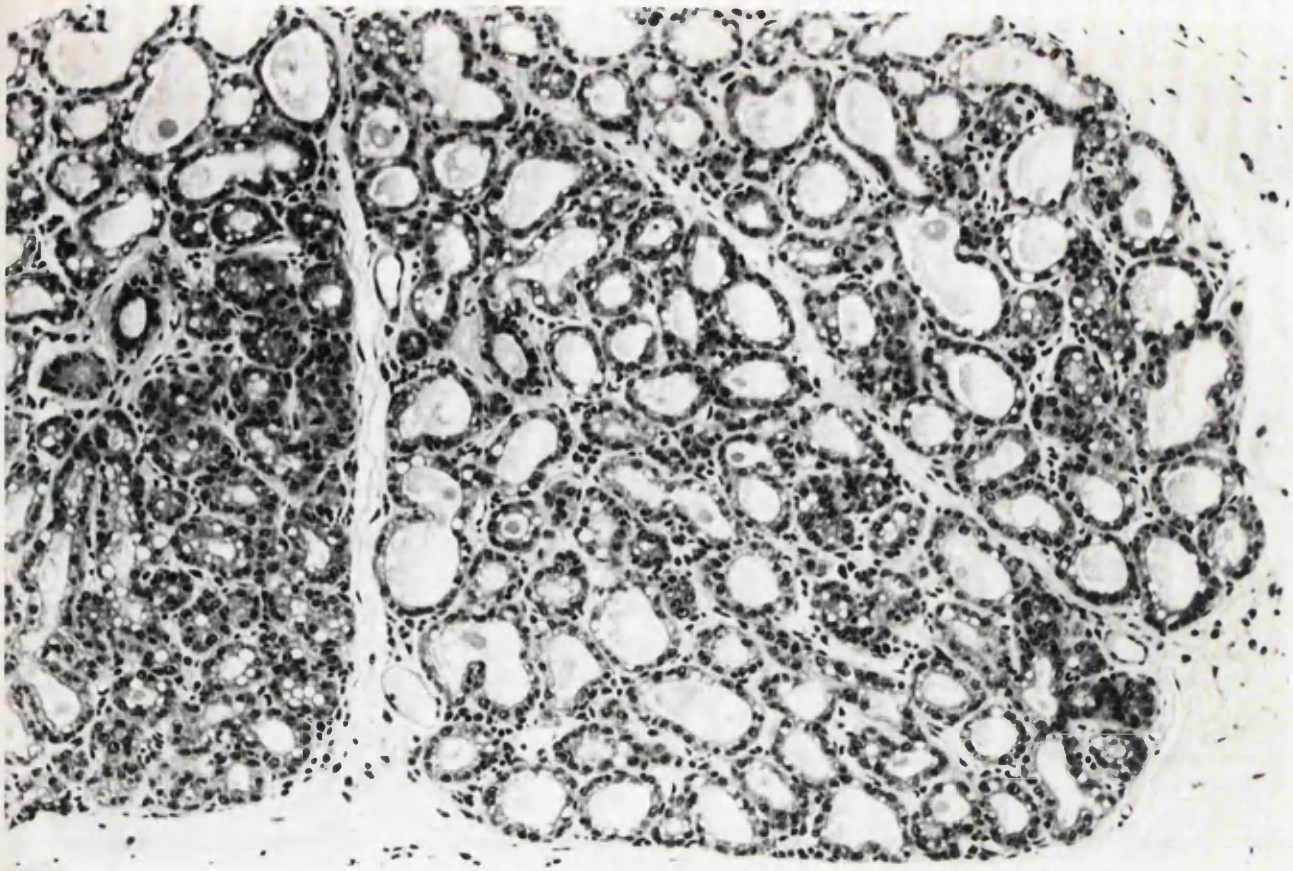
Changes have also been described at the ultrastructural level in the menstrual cycle. Fanger and Ree (1974) found that epithelial cells in the proliferative phase of the cycle had a simple structure characteristic of a relatively inactive physiological state. In the secretory phase in contrast the cells showed features suggestive of potential secretion, i.e. prominent membrane bound ribosomes and Golgi complex, numerous microvilli and accumulation of glycogen.

C. Changes in pregnancy and during lactation

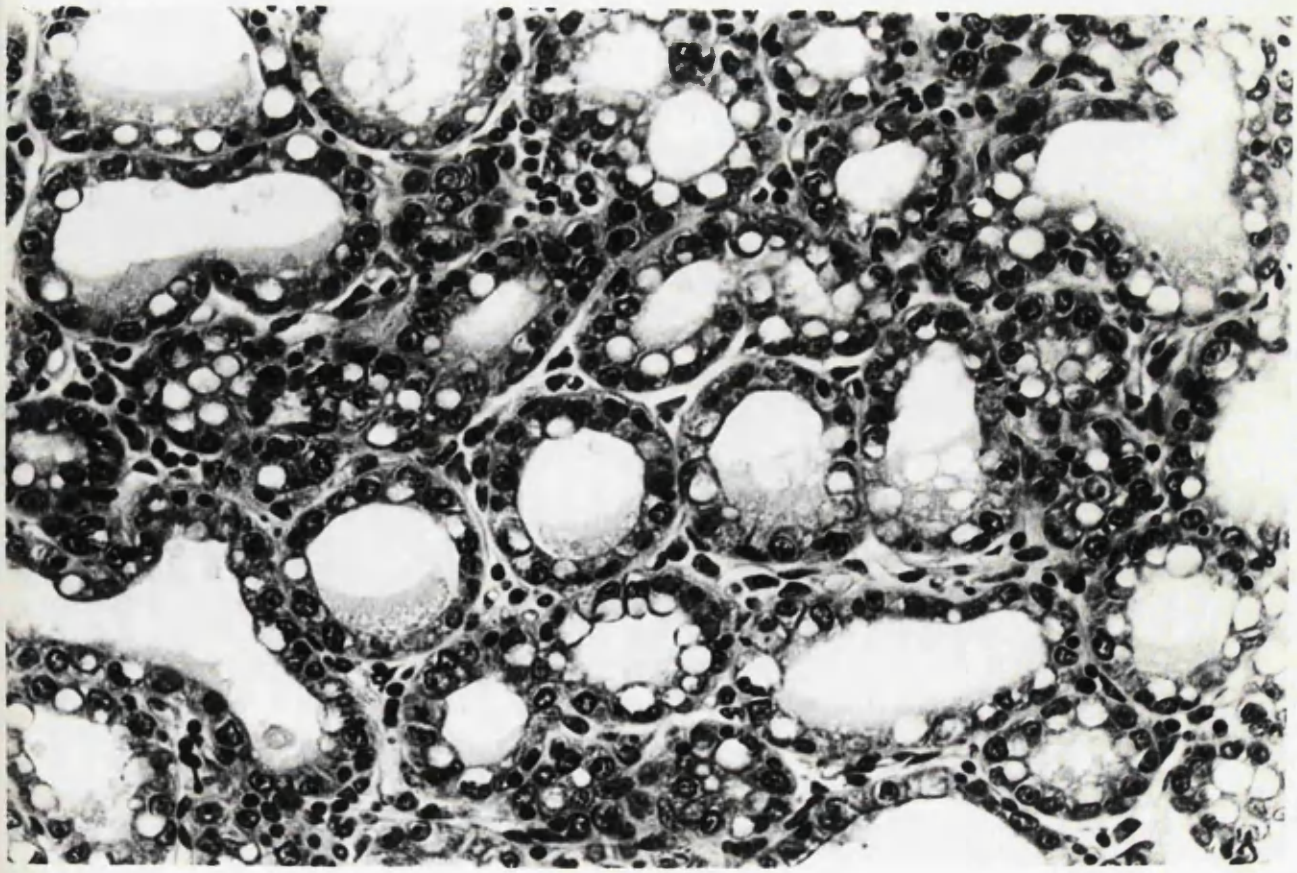
It is only during pregnancy and lactation that the breast achieves its fully differentiated state. It has been suggested that the stimulus of pregnancy, which induces the epithelial cells to undergo full functional differentiation may protect the epithelium from later abnormal growth stimuli (Jensen 1981) accounting for the protective effect from malignancy of parity. This is most marked when the first full term pregnancy is before the age of 20; indeed if the first birth is after the age of 35 years, the risk is actually increased (Willett 1989). Mammary epithelial cells showing changes consistent with pregnancy or lactation may thus be considered to be fully differentiated. Such cells, and the antigens expressed by them are therefore of interest; any differences between fully differentiated and malignant cells are of great importance in understanding the development of malignancy.

Histological changes during pregnancy and lactation are striking. There is proliferation of the glandular tissue with an increase in both the number of lobules and the number of acini within them, at the expense of both the inter and intralobular stroma (Figure 1.4A). The luminal epithelial cells show evidence of secretory activity with vacuolation of the cytoplasm which protrudes into the glandular lumina (Figure 1.4B). Myoepithelial cells become stretched and may be difficult to see in conventional H&E sections. In pregnancy, in contrast to the resting state, all lobules show secretory activity (Jensen 1981) but in lactation, the changes are not uniform and secretion is asynchronous (Sloane 1985a).
After the cessation of lactation, however, there is involution and the appearances return to those of the resting state.

Figure 1.4. Breast tissue from a pregnant woman showing secretory change. The glandular tissue has proliferated - both the number of lobules and the number of acini within them has increased at the expense of the stroma (A). The cytoplasm of the luminal epithelial cells is vacuolated and protrudes into the glandular lumina (B).
(Haematoxylin and eosin).



A



B

D. Changes with ageing

Involutional change within the resting breast starts some time before the menopause (Hutson, Cowen and Bird 1985). The amount of glandular tissue actually diminishes from the third to the sixth decade. The number of lobules is reduced as is the number of acini within them and the specialised intralobular stroma is lost. Thus breast tissue from elderly subjects usually consists of scattered ducts with few lobules. To continue the analogy mentioned earlier, the epithelium of the breast now resembles a "deciduous tree which has lost its leaves" (Jensen 1981). This process, however, is not uniform. Sometimes cyst formation may occur during the process of involution and occasionally lobules persist.

IV EPITHELIAL CELL TYPES IN THE BREAST

The epithelium of the breast is bilayered consisting of inner, or luminal, epithelial cells and outer, myoepithelial or basal, cells. These are surrounded by a continuous basement membrane.

A. Morphology

Morphological differences between the luminal and basal cells can be seen both with the light microscope and ultrastructually.

Luminal cells are cuboidal or columnar in the resting state and show secretory change with vacuolation of the cytoplasm during pregnancy and lactation. The nuclei, which are round or oval, occupy an approximately central position in the cells. The processes of luminal cells extend between the myoepithelial cells to reach the basal lamina. Ultrastructurally, the cytoplasm of the luminal cells contains numerous free ribosomes, a few scattered vesicles of rough-surfaced endoplasmic reticulum, few mitrochondria, a supranuclear Golgi complex and bundles of tonofilaments. Microvilli are seen at the apical membrane. The cells are joined at the luminal surface by watertight junctional complexes and by desmosomes elsewhere (Ahmed 1978).

Myoepithelial cells are less conspicuous than the luminal epithelial cells and show considerable variation in morphology. They are often elongated with central nuclei and inconspicuous cytoplasm. Sometimes, however, the cytoplasm is more prominent and clear but on other occasions it appears markedly eosinophilic. Ultrastructurally, myoepithelial cells contain large numbers of fine filaments which exhibit dense bodies and resemble the myofilaments of smooth muscle cells. Myofilaments are better developed in the interlobular and terminal ducts than in the acini. Myoepithelial cells are joined to the basement membrane by hemidesmosomes and to the luminal cells by desmosomes (Ahmed 1978).

In addition to luminal and basal cells described above there are a number of cells which, ultrastructurally, are difficult to categorise, either because they show characteristics of both cell types or because they lack any distinguishing feature. These have been called "indeterminate cells". They appear to represent either progenitors which can give rise to both cell types or a transition between the two (Ozzello 1971).

B. Histochemistry

Myoepithelial cells which are rich in alkaline phosphatase can be demonstrated histochemically by staining frozen material for this enzyme. Other markers of myoepithelial cells include tannic acid phosphomolybdic acid (TPA), phosphotungstic acid-haematoxylin (PTAH) and Heidenhain's iron haematoxylin (HIH) which demonstrate myofibrils (Pulley 1973, Macartney, Roxburgh and Curran 1979). Plasma membrane ATPase activity may also be used to distinguish myoepithelial cells from luminal epithelial cells (Russo and Wells 1977).

Luminal epithelial cells secrete both neutral and acidic mucins as demonstrated by the alcian blue-periodic-acid Schiff stain at pH 2.5 (Sloane 1985b).

Elastic tissue may be demonstrated around ducts but not lobules by conventional elastic stains.

Other histochemical stains may be useful in the diagnosis of malignant disease e.g. silver stains in argyrophilic breast carcinomas.

C. Immunohistochemistry

A summary of the wide range of immunohistochemical markers used in the study of breast disease has been given in Table 1.5.

Myoepithelial cells which contain the contractile proteins actin and myosin may be demonstrated with antibodies to these components (Bussolati et al 1980, Gusterson et al 1982). Although useful these antibodies are not specific for myoepithelial cells; they will also demonstrate muscle in blood vessels and stromal myofibroblasts.
Antibodies to basement membrane components such as laminin and type IV collagen, have also been described and can be used to demonstrate basement membrane around the epithelium of the normal breast and around blood vessels (Gusterson et al 1982).
Cytokeratins, the intermediate filaments of epithelial cells, can be identified immunohistochemically with a wide range of polyclonal and monoclonal antibodies. For a review of this see Taylor-Papadimitriou and Lane 1987a. Basal epithelial cells express keratins 5 and 14 (in common with basal cells from the skin) but lack keratins 8 and 18 which are characteristic of the luminal epithelial cells. A more complex pattern of expression of keratin 19 is seen and keratin 7 is expressed by both luminal and basal cells, (Bartek et al 1985a, Taylor-Papadimitriou et al 1989).
Milk proteins can be identified in lactating breast tissue with antibodies to casein (Burchell, Bartek and Taylor-Papadimitriou 1985) and alpha lactalbumin (Bailey et al 1982).
Antibodies to cell membrane antigens will be discussed extensively in section 1.4.
Steroid receptors as assayed biochemically are undetectable or present at very low levels in the normal breast. With the use of recently developed immunohistochemical techniques their presence can be demonstrated in epithelial cells. It is generally necessary to use frozen material as, although methods allowing the use of fixed tissue have been reported (Andersen, Orntoft and Poulson 1988, Cheng et al 1988), in our laboratory these do not correlate well with biochemical measurements.

Some of the above markers are useful in histopathological differential diagnosis. For example, the demonstration of myoepithelial cells may be helpful in distinguishing a benign sclerosing lesion from carcinoma. Intact basement membrane is found around the epithelium of benign breast lesions; antibodies to basement membrane components may also be useful in this context.

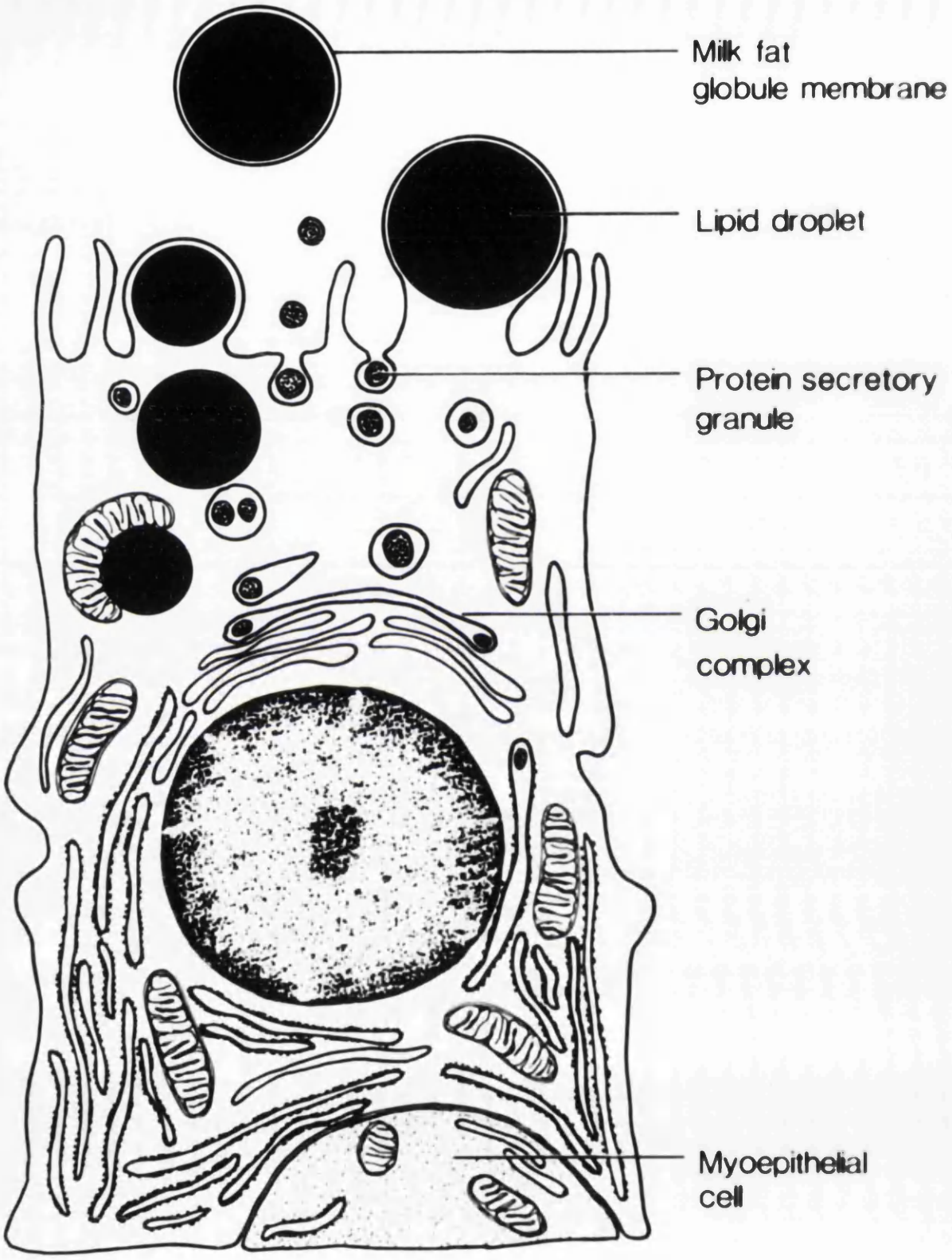
The measurement of oestrogen and progesterone receptors in primary breast carcinomas is useful in assessing prognosis and predicting response to hormone treatment (Howell et al 1984). Despite previous reports to the contrary, the milk proteins casein and alpha lactalbumin have not been demonstrated in mammary carcinomas (Bailey et al 1982, Bartkova et al 1987). Infiltrating carcinomas of both ductal and lobular types express cytokeratins characteristic of luminal epithelial cells (Gusterson et al 1982, Taylor-Papadmitriou, Lane and Chang 1983 and Bartek et al 1985a). Thus the malignant epithelial cell in vivo has a luminal phenotype with regard to keratin expression.

Other immunohistochemical markers which have been used in the study of breast disease include anti-CEA, anti-HCG and antibodies to oncogene products.

D. Secretion

The lactating mammary gland secretes two major products, protein and fat. These are formed and released by different mechanisms (Figure 1.5). The protein constituent of the milk is elaborated in the ribosomes of the luminal epithelial cells and is then found in membranous vesicles associated with the Golgi complex. These are transported to the cell surface where the membrane of the vesicles fuses with the plasma membrane and the protein is secreted into the acinar lumen. In contrast, the fatty part of the milk arises as lipid droplets free in the cytoplasmic matrix. These move to the apex of the cell and then project into the lumen covered by the cell membrane and a small amount of cytoplasm. The droplets are secreted surrounded by cytoplasm and cell membrane. This membrane is thus representative of the fully differentiated luminal mammary epithelial cell. It is known as the Milk Fat Globule Membrane (MFGM) and will be discussed in some detail below (Section 1.4).

Figure 1.5. Diagramatic representation of epithelial cells from the lactating mammary gland. Granules of protein are elaborated in the ribosomes of the luminal cells and then found in membranous vesicles associated with the Golgi complex. They are released by coalescence of the vesicles with the plasma membrane. Lipid droplets arise free in the cytoplasmic matrix and are secreted from the apex of the cell surrounded by a small amount of cytoplasm and cell membrane. This is known as the Milk Fat Globule Membrane.
(Diagram modified from Bloom and Fawcett 1967).


Milk fat
globule membrane
Lipid droplet
Protein secretory
granule
Golgi
complex
Myoepithelial
cell

1.3 METHODS AVAILABLE FOR THE STUDY OF DIFFERENTIATION AND MALIGNANCY IN THE HUMAN BREAST

I IN VIVO STUDIES

Experimental studies of carcinogenesis in human subjects are obviously unacceptable on ethical grounds. In vivo work, therefore, has focused on epidemiological studies of risk factors and on analysis of the relationship of benign to malignant breast disease; in particular on the assessment of which, if any, types of benign breast disease are associated with an increased risk of subsequent carcinoma. The epidemiological data shown in Table 1.6 suggests that hormonal influences play a major role in the aetiology of breast cancer. Furthermore it has long been recognised that the completion of a full term pregnancy at an early age protects women from breast cancer (Willett 1989). From such data it has become apparent that stimulating the mammary epithelial cells to fully differentiate i.e. to show active secretion means that these cells are less susceptible to abnormal growth stimuli later in life (Jensen 1981). As mentioned above, mammary epithelial cells showing secretory change may be considered to be fully differentiated, and it is of interest to compare features of these cells with those of abnormally differentiated malignant cells.

Of particular interest to pathologists are the numerous studies which have been performed to assess the relationship of benign breast disease to risk of subsequent carcinoma. These have produced inconsistent results. There are probably several reasons for this. Firstly, how do we define benign breast disease? Do all women who have symptoms referable to the breast have benign breast disease, or is it necessary for there to be a palpable abnormality? Should the term be used only when a biopsy shows pathological change? Can we distinguish pathological from physiological change? Secondly, even if we can agree on the definition of benign breast disease, can we agree on the terminology used for the pathological changes seen? Many different terms are used, often loosely, in the diagnosis of benign breast disease.

Table 1.6

MAJOR RISK FACTORS FOR BREAST CANCER*

Factor	High Risk	Low Risk
Demographic		
Sex	Female	Male
Age	Old	Young
Race	White	Black
Social class	Upper	Lower
Country of residence	N. America/N. Europe	Africa/Asia
Place of residence	Urban	Rural
Menstrual History		
Age at menarche	Early	Late
Age at menopause	Late	Early
Reproductive History		
Parity	Nulliparous	Parous
Age at first birth	>30 years	<20 years

*Based on data from Kalache and Vessey 1982

cont/....

Table 1.6 (continued)

MAJOR RISK FACTORS FOR BREAST CANCER*

Genetic Factors

Breast cancer in first degree relative	Relative risk x 2-4
First degree relative with premenopausal bilateral breast cancer	Relative risk > 4

Past Medical History

Previous breast cancer	Relative risk > 4
Benign breast disease	Relative risk x 2-4

Nutritional Factors

Increased risk with consumption of animal fat and obesity in postmenopausal period

Radiation Exposure

Increased risk especially when exposure at an early age

Exogenous Hormone Administration

Oral contraceptive pill - contentious issue. Recent data suggests may be slight increased risk.
Hormone replacement therapy - probably small but significant risk with long term high dose usage.

*Based on data from Kalache and Vessey 1982

In studies of mastectomy specimens where benign lesions co-existing with carcinomas have been compared with those in control specimens of either surgical or post mortem breast tissue, a higher incidence of epithelial proliferation and particularly epithelial atypia has been shown in cancer bearing than in control breasts (Foote and Stewart 1945, Ryan and Coady 1962, Black and Chabon 1969, Gallagher and Martin 1969, Wellings et al 1975).

Retrospective studies (Black et al 1972) looked at patients with benign breast disease who subsequently developed carcinoma and compared them with age matched controls with benign disease who had not developed carcinoma. An increased risk of carcinoma was found to be associated with the presence of severe epithelial hyperplasia.

It is difficult to carry out prospective studies to assess breast cancer risk as a very long follow-up period is needed. In practice, such studies are usually carried out in retrospect but the histological data is interpreted without knowledge of the clinical outcome. Recently, in carefully controlled studies, Page and Dupont performed detailed pathological examination and classification of benign breast lesions in a large number of women with a long follow-up period. Their conclusions may be summarised as follows. Firstly, the majority of women with benign breast disease are at no increased risk of subsequent carcinoma. The risk is confined to patients whose biopsies show epithelial hyperplasia. Furthermore, there is a substantially increased risk (to five times that of the normal population) only when there is epithelial hyperplasia with atypia. This, however, is an uncommon finding seen in only 4% of benign biopsies. It should be noted that the increased risk doubles (to ten times that of the normal population) if there is also a family history of breast cancer in a first degree relative. (Dupont and Page 1985, Page et al 1985).

Following these findings a consensus meeting in New York defined risk factors associated with the different categories of benign breast disease. (Consensus meeting 1986). These are summarised in Table 1.7.

Table 1.7

RELATIVE RISK FOR INVASIVE BREAST CARCINOMA BASED ON PATHOLOGICAL EXAMINATION OF BENIGN BREAST TISSUE

(Consensus statement New York, 1986)

No Increased Risk	Sclerosing adenosis Apocrine metaplasia Cystic change Duct ectasia Fibroadenoma Fibrosis Mastitis Squamous metaplasia Mild hyperplasia (>2 but <4 epithelial cells in depth)
Slightly Increased Risk (1.5 to 2 x that of comparable women who have had no breast biopsy)	Moderate or florid hyperplasia Papilloma with fibrovascular core
Moderately Increased Risk (5 x that of comparable women who have had no breast biopsy)	Atypical hyperplasia of ductal or lobular type

The role of viruses and chemical carcinogens in the aetiology of mammary cancer is well established in animals (Fisher 1975a, 1975b) but there is no such proven aetiology for human cancer.

II IN VITRO STUDIES

Due to the obvious limitations of in vivo work, much of the experimental work on human breast cancer has involved in vitro studies.

A. Tissue and cell culture: Background

Tissue culture was first described by Harrison who utilised tissue fragments from the frog (Harrison 1907). The technique provides a method of studying the behaviour of animal cells free from systemic variations occurring either during normal homeostasis or under the stress of an experiment. Since Harrison's original description, interest in the field of tissue culture has expanded enormously. Work in the field of medical science has led to growth of mammalian and in particular, human tissue. Although the study of cellular activity in tissue culture has many advantages, there are also limitations.

Perhaps the main advantage of tissue culture is control of the physicochemical environment (e.g. pH, temperature etc.) as well as the physiological conditions. Most cell lines, however, require enrichment of the culture medium with serum or other supplements. As these are prone to batch variation, the physiological conditions, although constant, cannot always be strictly defined. A further advantage is homogeneity of cell type with the most vigorous growing selectively. Experimental replicates are therefore virtually identical, in contrast to tissue samples which are always heterogeneous and where cell types vary even in replicate samples from the same tissue. Tissue culture is also economical in terms of use of reagents as cells may be exposed directly to these at low concentrations.

The disadvantages of tissue culture are firstly that it is essential that strictly aseptic growth conditions are observed and a complex environment which simulates that of plasma or interstitial fluid is needed. Therefore, although economical in use of reagents, tissue culture itself is costly in effort and materials. Secondly, some continuous cell lines are unstable due to their aneuploid chromosomal constitution. Thirdly, cells cultured in vitro will also differ from their in vivo counterparts due to loss of special cell-cell and cell-substrate interactions which exist in the three dimensional in vivo situation. Finally, although control of the environment is an advantage of tissue culture, the lack of nervous and endocrine control which exists in vivo means that cultured cells may not be truly representative of the tissue from which they are derived. The inclusion of hormones in most culture media goes some way to addressing this problem.

In summary, although there are a number of disadvantages to tissue culture, many specialised cellular functions are retained in the culture situation and provided that the limits of the experimental system are appreciated, valuable results can be obtained (Freshney 1987).

B. Tissue and cell culture: The Breast

The culture of human mammary epithelial cells is an important technique in the investigation of both normal differentiation and the development of malignancy in the breast. Under controlled conditions in vitro culture permits the study of cell growth and the factors which influence it, cell physiology, antigen expression, and drug or hormone sensitivity. Any differences between malignant epithelial cells and their normal counterparts can, potentially, be exploited in the treatment of malignancy.

(i) Culture of cells from human breast carcinomas

The culture of neoplastic cells from the human breast is difficult. Problems arise for several reasons. 1) cultures are often contaminated with fibroblasts; 2) tumour cells may initially multiply in primary cultures but then die; 3) tumour cells may not survive serial transfers.

In 1958 Lasfargues and Ozzello first described the establishment of a cell line (BT20) from a primary mammary carcinoma. The difficulty in establishing permanent cell lines from primary tumours is emphasised by the fact that BT20 is the only cell line available from this source. Subsequently, many workers have utilised cells from metastatic mammary carcinoma usually from pleural effusions (Soule et al 1973, Cailleau et al 1974, Engel et al 1978 and Keydar et al 1979). These metastatic cells have the following advantages. 1) large amounts of material are available and it may be possibile to obtain sequential samples; 2) a high percentage of metastatic tumour cells are viable; 3) there are few, if any, fibroblasts; 4) tumour cells can be separated from other cell types by differences in their speed or degree of attachment to the culture flask.

The main disadvantage is the fact that these cells have metastasised and therefore probably represent a hardy population of cells which has adapted to growing in a liquid environment. They may thus differ in some respects from the cells of the primary tumour. As mentioned above, however, due to the difficulty of establishing cell lines from primary carcinomas those derived from metastases are usually used in practice.

Immunohistochemical studies of malignant mammary epithelial cells in culture have shown that they express both cytokeratins and surface antigens characteristic of luminal rather than basal epithelial cells (Burchell 1986, Taylor-Papadimitriou et al 1987a). Thus both in vitro and in vivo (as described above), the malignant mammary epithelial cell has a luminal phenotype.

(ii) Culture of cells from normal mammary epithelium

It is difficult to culture normal epithelial cells from surgical biopsy specimens as they are often overgrown by accompanying fibroblasts. Epithelial cells free from fibroblasts can be obtained from two sources, either from human milk which does not contain fibroblasts (Buehring 1972, Taylor-Papadimitriou, Shearer and Tilly 1977) or from reduction mammoplasty specimens using a medium which selects against the growth of fibroblasts (Stampfer, Hallowes and Hackett 1980, Hallowes, Bone and Jones 1980).

a) Culture of normal epithelial cells from human milk

Human milk provides a readily available and accessible source of mammary epithelial cells, free from contaminating fibroblasts. The cells are often aggregated into grape-like clusters which contain large refractile globules. They may be separated from the milk by low speed centrifugation, and grown in Eagles medium containing serum and antibiotics (Buehring 1972, Taylor-Papadimitriou et al 1977). Growth is enhanced by the addition of a cyclic AMP elevating agent such as cholera toxin to the medium (Taylor-Papadimitriou, Purkis and Fentiman 1980) and by the use of feeder cells and enriched medium (Smith et al 1981).

As well as epithelial cells, milk and colostrum both contain large round vacuolated cells variously referred to as colostrum bodies or foam cells. There is some debate as to the exact nature of these; some believe that they may be degenerative cells, originally arising from the ductal epithelium (Papanicolau et al 1958) whereas others (Taylor-Papadimitriou et al 1977) have presented evidence showing that both functionally and ultrastructually they have the properties of macrophages. Whatever their origin, these cells have an important function, acting as feeders for the epithelial cells.

Cultured milk epithelial cells form colonies which begin as compact groups of cells with a smooth colony outline. As the colonies enlarge, however, various different growth patterns are seen (Taylor-Papadimitriou et al 1977, Stoker, Perryman and Eeeles 1982).

When examined by transmission electron microscopy, desmosomes and tonofilaments can be demonstrated in all early colonies; in some junctional complexes can be seen. Scanning electron microscopy reveals microvilli on the cell surface of early colonies (Taylor-Papadimitriou et al 1977).

Immunohistochemical studies show that cultured milk epithelial cells express the luminal cytokeratins 7, 8 and 18 as might be expected from cells which are shed into the milk. The majority (85%) also express keratin 19 and 30% of colonies keratin 14. Although they do not express the secretory markers casein and alphalactalbumin, they express mucin molecules characteristic of cells of the luminal epithelial (secretory) lineage (Taylor-Papadimitriou et al 1987a, 1987b and 1989) (see Section 1.4).

In summary, therefore, cultured cells from human milk provide a source of normal mammary epithelium. Apart from the keratin 14 expression and the lack of secretion of casein and alphalactalbumin, these cells have a phenotype identical to that seen in vivo and thus may be compared and contrasted with neoplastic cells from breast carcinomas.

b) Culture of normal epithelial cells from reduction mammoplasty specimens

A disadvantage of working with milk cells in culture is that only luminal epithelial cells are studied. In theory, both luminal and basal epithelial cells can be cultured from reduction mammoplasty specimens so that normal cell-cell interactions can be studied. In practice, however, although one starts with "organoids" (cell clusters resembling ductal and acinar structures and composed of both luminal and basal epithelial cells), depending on the subsequent growth conditions, different cell types grow out. On plastic, in milk mix (developed for growth of luminal cells from milk) only the luminal cell type proliferates whereas in complex media (Stampfer et al 1980, Hammond, Ham and Stampfer 1984) there is selective growth of cells with a basal phenotype (Taylor-Papadimitriou et al 1989). In collagen gels, the growth of organoids resembles the in vivo picture morphologically but there are some differences in antigen expression (Taylor-Papadimitriou et al 1987b); moreover, collagen gels are difficult to work with.

Although reduction mammoplasty specimens may therefore appear to have advantages over milk cells in culture, their pattern of antigen expression is clearly complex and changeable. For this reason, the in vitro studies reported in this thesis have been performed using cultured epithelial cells from human milk; as described above these show a similar pattern of antigen expression to luminal epithelial cells in vivo.

1.4 IMMUNOLOGICAL MARKERS OF DIFFERENTIATION IN THE BREAST, WITH PARTICULAR REFERENCE TO ANTI-MUCIN ANTIBODIES, THE POLYMORPHIC EPITHELIAL MUCIN AND THE DEVELOPMENT OF SM3

I INTRODUCTION

As previously mentioned, the analysis and understanding of differences between normal and malignant human mammary epithelial cells is of great importance, particularly as such differences have potential for exploitation in the management and treatment of malignancy in the breast.

The morphological features and histochemical properties of cells provide a great deal of information which should not be under estimated as it is these which are used in the course of everyday diagnostic pathology to distinguish benign from malignant conditions. Such data may, however, be greatly supplemented by analysis of the immunological profile or "immunophenotype" of cells. Cellular antigens may be present on the surface of a cell, in the cytoplasm or at both sites. Knowledge of antigen expression provides information about the behaviour and function of different cell types and enables comparison of these characteristics in benign and malignant cells. Immunological markers used in the study of the breast have been listed above (Table 1.5) and discussed in Section 1.2.

Using the techniques of immunohistochemistry and immunocytochemistry the expression of antigens in tissue sections and cells respectively can be studied. A brief discussion of these techniques now follows.

II IMMUNOHISTOCHEMISTRY

A. Definition

Immunohistochemistry or immunocytochemistry may be defined as the technique by which labelled antibodies are used as specific reagents for the localisation of tissue or cellular constituents (antigens) in situ.

B. History and development

Coons and colleagues (Coons, Creech and Jones 1941, Coons and Kaplan 1950) were the first to use a fluoroscein conjugated antibody to identify antigens in tissue sections. The main disadvantages of working with fluoroscein labelled antibodies are 1) the impermanent nature of the product 2) difficulty in interpreting which cells are labelled and 3) the morphology of labelled cells cannot be studied.

The introduction of enzymatic labelling of antibody - initially with horseradish peroxidase (Nakane and Pierce 1967, Avrameas and Uriel 1966), and later alkaline phosphatase (Mason and Sammons 1978) and glucose oxidase (Suffin et al 1979) made it possible in some cases to work with fixed paraffin embedded sections. Permanent preparations, accurate localisation of staining and good histological data could thus be obtained.

Further developments have included the use of intrinsically electron dense labels such as ferritin and colloidal gold so that the site of antigens can be identified at the ultrastructural level, and the use of radioactively labelled antibodies so that reactions may be visualised by autoradiography.

Initial immunohistochemical work was performed with polyclonal antisera but the introduction of a technique for the production of monoclonal antibodies (Kohler and Milstein 1975) has resulted in the potential for producing an unlimited supply of antibody of known specificity in a purified form. "Hybridoma technology" has expanded rapidly and monoclonal antibodies are now widely used in both diagnostic and research laboratories.

Early immunohistochemical work was performed on frozen tissue as fixation resulted in loss of antigenic activity. Many antibodies are, however, now available against antigens which are preserved after fixation or which can be revealed by digestion with enzymes such as trypsin or pepsin.

III CELL SURFACE ANTIGENS IN MAMMARY EPITHELIUM : THE HUMAN MILK FAT GLOBULE MEMBRANE

Many antibodies have been raised to cell surface antigens; those to the human milk fat globule membrane will be discussed in detail as it is work in this area which led to the development of SM3, the monoclonal antibody described in this thesis.

The essential function of the mammary gland is the production of milk. During pregnancy and lactation, the breast undergoes dramatic changes in morphology which have been described in detail above. The production and secretion of fat globules, enveloped in the apical plasma membrane of the luminal epithelial cell has also been described and illustrated in Figure 1.5. The milk fat globule membrane can easily be obtained from human milk and, as it is representative of the membrane of the normal fully differentiated mammary luminal epithelial cell, it has been widely studied and used to develop markers of differentiation. The membrane itself and the many antibodies raised against it will now be described.

A. Composition of the human milk fat globule membrane (MFGM)

The membrane consists of a glycocalyx on its external surface and a 10-50 nm dense layer on its internal cytoplasmic side (Horisberger, Rossett and Vonlanthen 1977, Freudenstein et al 1979,). High molecular weight glycoproteins containing a high percentage of carbohydrate and showing characteristics of mucin like proteins have been identified in the membrane (Shimizu and Yamauchi 1982, Shimizu et al 1986). Mucins have been a subject of considerable interest as they are found not only in normal epithelial cells but also in tumours from the breast and many other sites. They are complex molecules and are categorised by the fact that they contain a high level of carbohydrate which is

attached in O-linkage to serine and/or threonine molecules on the core protein via the linkage sugar N-acetyl galactosamine. Mucins are constituents of the mucus produced by cells lining the respiratory and gastro-intestinal tracts but are also produced by other epithelial cells such as those of the salivary gland, sweat gland, breast and ovary.

B. Antibodies to the milk fat globule membrane (MFGM)

Two methods have been described for extracting membranes from the milk fat globule. Firstly by the use of organic solvents (Ceriani et al 1977) and secondly by churning the cream fraction of the milk (Mather and Keenan 1975, Imam, Laurence and Neville 1981). Membranes extracted by either method have been used as immunogens by a number of groups and many of the antibodies thus produced react with the mucin molecules mentioned above which appear to be the most immunogenic component of the membrane.

(i) Polyclonal antibodies to the human milk fat globule membrane

Polyclonal antibodies to the human MFGM have been produced by a number of different groups (Table 1.8). In 1977, Ceriani and colleagues produced an antiserum from rabbits injected with delipidated HMFG (Ceriani et al 1977). This antibody, termed anti-human mammary epithelial cell antiserum (anti-HMEC) was said to be specific for breast epithelium. It reacts with three components of the MFGM of molecular weight 46, 70, and 150 KD. In 1979, Heyderman and colleagues, also using human MFGM, produced anti-epithelial membrane antigen (EMA) serum. This antiserum is epithelium specific rather than breast epithelium specific. It reacts mainly with a high molecular weight component (mucin) found in the human milk fat globule (Heyderman, Steele and Ormerod 1979). In 1986 Imam and colleagues developed a further polyclonal antiserum reactive with a 155 KD protein present in human MFGM. This is said to discriminate between lobular and ductal epithelial cells in the normal breast and between lobular and ductal carcinomas (Imam et al 1986).

Table 1.8

POLYCLONAL ANTIBODIES RAISED TO THE HUMAN MILK FAT GLOBULE MEMBRANE

Antibody	Antigen	Reference
Anti-human mammary epithelial cell antiserum (anti-HMEC)	3 components of MW 150 KD 70 KD 46 KD	Ceriani et al 1977
Anti-epithelial membrane antigen (anti-EMA) serum	High molecular weight component of HMFGM	Heyderman et al 1979
Antiserum to MFGM-gp 155	155 KD protein present in HMFGM	Imam et al 1986

(ii) Monoclonal antibodies to the human milk fat globule membrane

As some of the polyclonal antisera described above were found to be useful in clinical practice e.g. for imaging, with the advent of hybridoma technology several groups used the MFGM to develop monoclonal antibodies. The large number produced makes this a bewildering subject. A recent review (Burchell and Taylor-Papadimitriou 1989a) has clarified the situation by dividing the antibodies into two groups. 1) those which react with high molecular weight mucin molecules 2) those which react with smaller more discrete components. Table 1.9 lists monoclonal antibodies raised against the human milk fat globule membrane; they are subdivided as suggested in this review.

As mentioned previously the most immunogenic component of the MFGM appears to be a high molecular weight (>400 KD) mucin component (Shimizu et al 1982). The many antibodies which react with different epitopes found on this component include not only those raised against the MFGM itself but also some originally developed against tumour cells which also express the mucin (Table 1.10) (Burchell, Durbin and Taylor-Papadimitriou 1983, Ceriani et al 1983, Hilkens et al 1984, McIlhinney, Patel and Gore 1985, Price et al 1986, Abe and Kufe 1987, Griffiths et al 1987, Lan et al 1987). Some confusion has resulted from the fact that different groups have given different names to the mucin, for example EMA (Heyderman et al 1979), non-penetrating glycoprotein (NPG) (Ceriani et al 1983), MAM6 (Hilkens et al 1984), Ca antigen or epitectin (Ashall, Bramwell and Harris 1982, Bardales et al 1989) and PAS-O (Shimizu et al 1982). Burchell and Taylor-Papadimitriou proposed the use of the term "the milk mucin" (Burchell et al 1989a) but the name Polymorphic Epithelial Mucin (PEM) has also been suggested (Taylor-Papadimitriou and Gendler 1988). The reason for this terminology and the mucin itself will be discussed at this point as it was work in this field which led to the development of SM3, the subject of this thesis.

Table 1.9

MONOCLONAL ANTIBODIES RAISED TO COMPONENTS OF THE HUMAN MILK FAT GLOBULE MEMBRANE

Group 1 - React with high molecular weight mucin molecules

Antibody	Antigen	Reference
MAM 6 series	Milk mucin	Hilkens et al 1984
HMFG1	Milk mucin	Taylor-Papadimitriou et at 1981
HMGF2	Milk mucin	"
M8	Milk mucin	Foster et al 1982
M18	Milk mucin	"
BLMRL-HMFG-Mc5	Milk mucin	Ceriani et al 1983
D274*	Milk mucin	Greenwalt et al 1985

Group 2 - React with smaller, more discrete components

Antibody	Antigen	Reference
MAM-3C	Diffuse band of 40-50 KD	Hilkens et al 1984
IIID5	53 KD	Ashorn and Krohn 1985
BLMRL-HMFG-Mc3	46 KD	Ceriani et al 1983
BLMRL-HMFPG-McR2	70 KD	"

*Raised against guinea pig MFG but reacts with human

Table 1.10

MONOCLONAL ANTIBODIES WHICH CROSS REACT WITH THE POLYMORPHIC EPITHELIAL MUCIN

Antibody	Immunogen	Reference
Ca1	Extracts from H.Ep2 cells (human laryngeal carcinoma cell line)	Ashall et al 1982 Bramwell et al 1983
F36/22 M7/105	Human breast cancer cells	Papsidero et al 1983
19-9	Human colonic cancer cells	Magnani et al 1983
Du-PAN-2	Human pancreatic cancer cells	Lan et al 1985
DF3	Membrane enriched fraction of human breast cancer cells	Kufe et al 1984 Sekine, Ohno and Kufe 1985
NCRC 11	Human breast cancer cells	Price et al 1985

C. The Polymorphic Epithelial Mucin (PEM)

As described above, work with monoclonal antibodies to the MFGM focussed attention on this molecule which is produced in abundance by the lactating mammary gland.

A similar mucin is found in urine and a genetic polymorphism was first detected at the protein level using antibodies to the mucin to detect differences in molecular weight (Karlsson et al 1983, Swallow et al 1986). Similar differences in molecular weight of the mucin associated with breast cancers were then observed (Griffiths et al 1988) and later this genetic polymorphism was shown to be due to differences in the size of the core protein (Gendler et al 1987, Swallow et al 1987, Gendler et al 1988a).

As mentioned above, mucins are complex molecules and although many anti-mucin antibodies are in common use, few of the epitopes recognised by them had until recently been identified. The need to analyse mucins at the structural level prompted the cloning of the gene coding for the PEM core protein. To this end, monoclonal antibodies were produced to the deglycosylated or "stripped" mucin (Burchell et al 1987). This mucin was free of carbohydrate as shown by lectin binding experiments and the antibodies to it are therefore reactive with epitopes on the core protein. The antibodies were used to select clones from a λgt 11 expression library of cDNA made from mRNA of MCF7 cells (a breast cancer cell line) (Gendler et al 1987). Clones isolated in this way and therefore containing epitopes recognised by the core protein antibodies all contained a large domain coding for a 20 amino acid tandem repeat peptide (Gendler et al 1988b). The structure of the tandem repeat peptide is illustrated in Figure 1.6. It contains a high proportion of serine and threonine molecules and therefore multiple glycosylation sites (see arrows in Figure 1.6).

Figure 1.6. The tandem repeat peptide from the core protein of the Polymorphic Epithelial Mucin. The structure of the twenty amino acid tandem repeat peptide from the core protein of the Polymorphic Epithelial Mucin (PEM) is illustrated. The peptide contains a high proportion of serine and threonine molecules and therefore has multiple glycosylation sites (indicated by the arrows). It was identified using antibodies to the PEM core protein and therefore contains the epitopes recognised by these antibodies.


Val—Thr—Ser—Ala—Pro—Asp—Thr—Arg—Pro—Ala—Pro—Gly—Ser—Thr—Ala—Pro—Pro—Ala—His—Gly

SM3, an antibody to the PEM core protein is the subject of this thesis (SM stands for stripped mucin). It was one of the antibodies developed as a tool to clone the PEM gene. In an initial immunohistochemical study, SM3 was found to react strongly with breast carcinomas but to show no reactivity with either lactating or normal breast tissue. This apparent difference in expression of the SM3 epitope by malignant and normally differentiated mammary epithelial cells prompted the further study and detailed evaluation of the antibody described in this thesis.

A surprising finding in the course of the studies mentioned above was that the antibodies HMFG1 and HMFG2 which were raised against the intact PEM molecule showed strong reactivity with the stripped mucin (Burchell et al 1987). These antibodies differ from SM3 in that they react strongly with lactating mammary tissue as well as with carcinomas. Their epitopes, therefore, while also protein in nature, clearly differ from that recognised by SM3. In this thesis, HMFG2 reactivity has been compared with that of SM3 in order to see if any differences in epitope expression could lead to further understanding of mucin processing in malignant and normal mammary epithelial cells.

Before setting out the objectives of this thesis, previous work with HMFG1 and HMFG2 will be summarised and the development of SM3 described.

D. The monoclonal antibodies HMFG1 and HMFG2: their production, properties and clinical applications

The monoclonal antibodies HMFG1 and HMFG2 were produced in 1981 (Taylor-Papadimitriou et al 1981). The immunogens used were different for the two antibodies; HMFG1 was derived from a fusion in which a mouse had received two injections of delipidated MFGM and HMFG2 from an animal injected once with delipidated MFGM and then boosted with proliferating epithelial cells cultured from human milk. Both are monoclonal antibodies of the subclass IgG1.

Immunohistochemical staining with HMFG1 and HMFG2 in tissue sections was originally reported by Arklie and colleagues (1981). As mentioned above, strong staining of lactating breast tissue and most primary mammary carcinomas was found with both antibodies. Interestingly, staining of the mucin pools in mucoid carcinomas was either absent or very weak (Taylor-Papadimitriou et al 1986) indicating that the mucin recognised by these antibodies differs from that detected by conventional "anti-mucin" histochemical stains. In normal breast tissue staining was originally reported in less than 10% of mammary epithelium; these studies were, however, carried out on formalin fixed tissue and later work has shown that at least for HMFG2, reactivity is more extensive in methacarn fixed tissue (Burchell et al 1987). Numerous immunohistochemical studies have been carried out using HMFG1 and HMFG2; some of these are summarised in Table 1.11.

In cell culture HMFG1 and HMFG2 react with breast cancer cell lines and also with epithelial cells cultured from human milk. The antibodies differ however in their strength of reactivity with cells from these two sources. HMGF1 binds to cultured milk cells more strongly than HMFG2; the reverse is true for breast cancer cell lines (Burchell et al 1983).

The molecular weight of components carrying the HMFG1 and HMFG2 epitopes has been studied using the technique of immunoblotting. In cultured epithelial cells from human milk HMFG1 recognises only a high molecular weight component whereas HMFG2 reacts with both high and some lower molecular weight components (Burchell et al 1983). In breast cancer cell lines, high molecular weight bands are detected; the size of these varies according to the cell line and they are thought to result from the genetic polymorphism of the PEM described above. There are, however, also some lower molecular weight components seen in preparations from both breast cancer cell lines and breast cancer tissue. These are thought to represent incompletely glycosylated mucin molecules. Reactivity with these lower molecular weight components is commoner with HMFG2 than with HMFG1 (Turnbull et al 1986).

Table 1.11A

REVIEW OF PUBLISHED DATA ON IMMUNOHISTOCHEMICAL STAINING WITH HMFG1 + HMFG2

	Summary of Staining with both antibodies	Reference
BREAST TISSUE		
Carcinomas		
Primary	Majority strongly positive	Arklie et al 1981
Metastatic	Majority strongly positive	Taylor-Papadimitriou et al 1986, Epenetos et al et al 1982a
Paget's disease of nipple	Positive, usually very strong	Vanstapel et al 1984
Benign breast disease		
Papillomas	Strong positive staining HMFG2 greater than HMFG1	Arklie et al 1981
Fibroadenomas	10% epithelium stained HMFG1 greater than HMFG2	Taylor-Papadimitriou et al 1986
Cystic change	Variable, especially strong in areas of apocrine metaplasia usually HMFG2 greater than HMFG1	"
Normal breast		
Lactating	Very strong positive staining	Arklie et al 1981
Resting	Weak staining in 10-20% of epithelium	Taylor-Papadimitriou et al 1986

NB Original studies with these antibodies were carried out on formalin fixed tissue

Table 1.11B

REVIEW OF PUBLISHED DATA ON IMMUNOHISTOCHEMICAL STAINING WITH HMFG1 + HMFG2

	Summary of Staining		Reference
	HMFG1	HMFG2	
NORMAL TISSUES FROM OTHER SITES			
Liver - bile duct epithelium	+	+	Arklie et al 1981
Pancreas - acini	+	+	"
Skin - epidermis	-	-	"
			Kirkham et al 1985
sebaceous gland	+	+	Cotton 1986
			Arklie et al 1981
sweat gland	-	+	"
			Vanstapel et al 1984
			Cotton 1986
apocrine glands	+	not done	"
Salivary gland - serous demilunes	+	+	Arklie et al 1981
Kidney - collecting ducts and distal tubules	+	+	Arklie et al 1981
Lung - bronchioles	+	+	"
alveoli	-	+	"
Epididymis	-	+	"
Uterus - glandular epithelium	+	+	"
			Nouwen et al 1987
Ovary - epithelium	usually -	1/23 +	"
Fallopian tube epithelium	+	+	"
Stomach epithelium	+	+	Gatter et al 1982
Colon epithelium	+	+	"
Thyroid epithelium	+	+	"
Tonsil epithelium	+	+	"

Table 1.11C

REVIEW OF PUBLISHED DATA ON IMMUNOHISTOCHEMICAL STAINING WITH HMFG1 + HMFG2

	Summary of Staining with both antibodies	Reference
TUMOURS FROM OTHER SITES		
CARCINOMAS		
Lung - small cell carcinoma	Majority positive - staining heterogeneous	Arklie et al 1981, Moss et al 1986
squamous carcinoma	Majority positive - also heterogeneous	"
adenocarcinoma	All positive - usually strong	"
Colon - carcinoma	Majority positive	Gatter et al 1982 Epenetos 1982a
Ovary - carcinoma	Vast majority positive	Arklie et al 1981 Epenetos et al 1982a Ward, Lowe and Shepherd 1987
Endometrial adenocarcinoma	Positive	Arklie et al 1981, Gatter et al 1982
Metastatic squamous cell carcinoma of skin (in node)	"	"
Renal adenocarcinoma	"	"
Gastric adenocarcinoma	"	"
Papillary carcinoma of thyroid	"	"
NON-EPITHELIAL TUMOURS		
Lymphoma - B cell	Negative	Gatter et al 1982
T cell	Positive with HMFG2	Delsol et al 1984
Sarcoma	Very weak and focal	Zotter (personal
Myeloma	reaction in a small	" communication)
Brain tumours	number of cells	"
Reactive mesothelial cells	Negative Positive	Epenetos et al 1982b Ghosh, Spriggs and Charnock 1986

HMFG1, therefore, appears to recognise an epitope which is abundant on the normally processed mucin produced by fully differentiated epithelial cells whereas that recognised by HMFG2 is present both on this mucin and also on smaller incompletely glycosylated molecules.

Clinical applications of HMFG1 and HMFG2 have included studies of prognostic significance. Wilkinson and colleagues in an immunohistochemical study found that a high level of extra-cellular staining with HMFG1 was associated with a good prognosis in breast cancer patients whereas total absence of staining with this antibody was associated with a very poor prognosis. No such association was found with HMFG2 (Wilkinson et al 1984). Other groups, however, (Berry et al 1985, Parham, Slidders and Robertson 1988 and Parham, Coghill and Robertson 1989) failed to confirm these results. Studies of tumours from other sites have shown that HMFG2 staining is a predictor of disease progression in superficial bladder cancer (Conn et al 1988) and is inversely related to survival in small cell cancer of the lung (Allan et al 1987).
As well as immunohistochemical studies studies, immunoblotting data has shown that expression of HMFG1 and HMFG2 epitopes on high molecular weight ($>$300 KD) components in breast tumours is associated with some factors which are related to good prognosis (Turnbull et al 1986).

HMFG1 and HMFG2 have also been used in histological diagnosis, both as part of a panel to distinguish anaplastic carcinoma from lymphoma or sarcoma (Gatter et al 1982) and for the identification of small metastases in axillary lymph nodes or bone marrow. This is particularly useful in the detection of lymph node metastases from infiltrating lobular carcinoma (Bussolati et al 1986). The antibodies have also been used to identify malignant cells in serous effusions. Epenetos and colleagues (1982b) showed that HMFG2 was useful in this context particularly in combination with the antibody AUA1 although others (Ghosh et al 1986) found false positive reactions in cells from peritoneal fluid in patients without malignant disease.

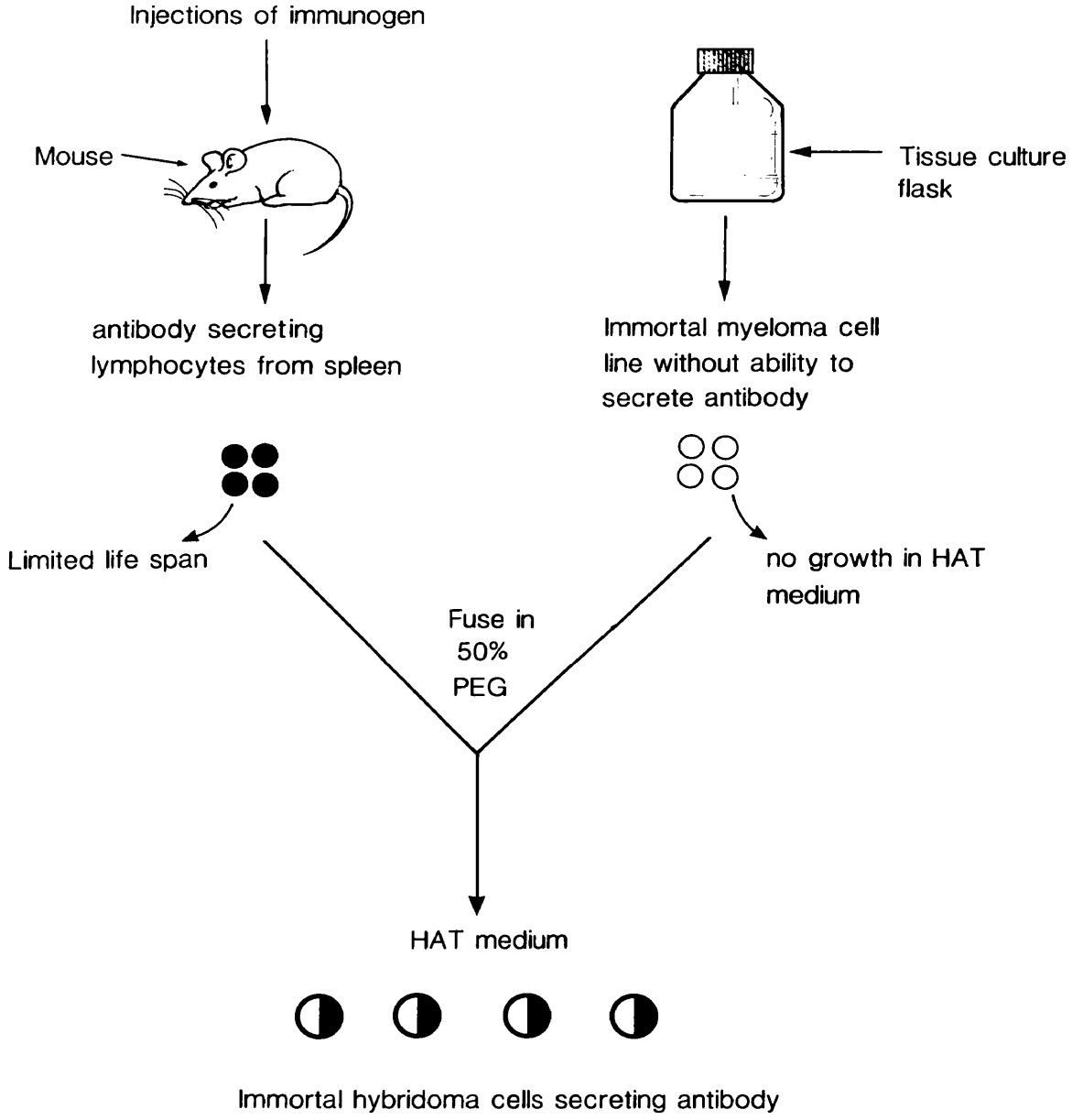
The use of HMFG1 and HMFG2 in the in vivo imaging of tumours has also been reported. Successful imaging of primary and metastatic ovarian, breast and colonic carcinomas using these antibodies labelled with radioactive isotopes of iodine has been reported (Epenetos et al 1982a, Granowska et al 1986). This subject will be discussed further in Chapter 5 where imaging studies with SM3 are described.

Other uses of HMFG1 and HMFG2 include the measurement of PEM levels in serum which may be helpful in monitoring the progress of patients with breast cancer (Burchell, Wang and Taylor-Papadimitriou 1984). Finally, in the treatment of malignancy HMFG2 has been used to administer local radiotherapy (Epenetos et al 1984).

E. The development of SM3

As described in Section 1.4IIIC, SM3 was one of several antibodies raised against the milk mucin or PEM core protein. The PEM was purified from human skimmed milk using an HMFG1 affinity column (Burchell et al 1987) and the carbohydrate stripped from the core protein by treatment with anhydrous hydrogen fluoride (Mort and Lamport 1977). Treatment with hydrogen fluoride for one hour at 4°C produced a "partially stripped preparation" whereas treatment for three hours at room temperature produced the "extensively or totally stripped mucin". This was free from carbohydrate as shown by the absence of lectin binding (peanut, wheatgerm and helix pomatia lectins) on immunoblots. Monoclonal antibodies were produced in mice by standard hybridoma technology. (Figure 1.7). In the immunisation schedule, two injections of the partially stripped mucin were followed by one injection of the extensively stripped mucin. Resulting clones were screened in a Protein-A assay against the extensively stripped, partially stripped and intact mucin each labelled with ^{125}Iodine. Hybridomas which reacted with the partially and extensively stripped but not the intact mucin were selected for further study.

Figure 1.7. The production of monoclonal antibodies by hybridoma technology. A mouse is injected with the immunogen. The animal is later sacrificed and antibody secreting lymphocytes from the spleen are fused with cells from an immortal myeloma cell line grown in culture. Hybridoma cells secreting antibody are selected out by growth in HAT medium. These are then cloned and hybridomas of the required specificity selected.



HAT = hypoxanthine, aminopterin, thymidine

PEG = polyethylene glycol

As mentioned above, one antibody, SM3 proved particularly promising as it was shown in a preliminary study to react immunohistochemically with breast carcinomas but not with lactating or normal breast tissue. It is an antibody of the IgG1 subclass and its detailed characterisation is the subject of this thesis.

1.5 OBJECTIVES OF THIS THESIS

The antibody SM3 has been evaluated and its applications as an imaging agent in breast cancer patients assessed. The work has involved three main areas of study, immunohistochemistry, immunoblotting and immunolocalisation. The materials and methods for each technique are described in Chapter 2.

I IMMUNOHISTOCHEMISTRY has been performed on a variety of tissue sections from the breast and other sites in the body, as well as on monolayers of cultured cells (Chapter 3).

II IMMUNOBLOTTING has been used to study the molecular weight of components carrying the SM3 epitope in lysates of cultured cells and cytosols prepared from primary mammary carcinomas (Chapter 4).

III A pilot IMMUNOLOCALISATION study has been undertaken to assess the potential clinical use of SM3 in the detection of metastatic disease in patients with proven primary breast cancer (Chapter 5).

Chapter 2

MATERIALS AND METHODS

The materials and methods used in the immunohistochemical, immunoblotting and immunlocalisation studies will be described separately. Details of reagents and cell lines used are given in the Appendix to this chapter.

All the work described in this thesis was carried out by the author with the exception of the following:

1. Immunohistochemistry - initial runs of staining on both fixed and frozen tissue sections were carried out by the author to establish the method and optimum fixation conditions. Subsequent staining was carried out by staff in the Clinical Oncology Unit (COU) laboratory, Guy's Hospital.

2. Immunoblotting - cytosols from fresh mammary carcinoma tissue were prepared and their protein content estimated by staff in the COU laboratory, Guy's Hospital.

3. Immunolocalisation - the protocol for this study was drawn up in collaboration with colleagues in the Departments of Clinical Oncology and Nuclear Medicine. The clinical aspects of this study were supervised initially by Dr. C. Twelves and later by Mr. I. Bennett, who also injected the antibody and collected the blood samples. Antibodies were iodinated by Dr. S. Mather at the ICRF Department of Nuclear Medicine Research at St. Bartholomew's Hospital. All imaging, both in vivo and in vitro and counting of radioactivity in resected specimens was performed in the Department of Nuclear Medicine at Guy's under the supervision of Miss S. Allen and Dr. S. Clarke. Mastectomies were performed by surgeons in the Clinical Oncology Unit at Guy's. Resected specimens were dissected initially by the author

and later by other staff in the COU laboratory. Pathological and immunohistochemical assessment was performed by the author. Data was collated in collaboration with the colleagues mentioned above and analysed in the Department of Nuclear Medicine.

4. Photography - some of the black and white photographs were taken in the Photography Department at the Imperial Cancer Research Fund (ICRF) and some of the art work was prepared by Miss D. Savva in the Medical Illustration Department, Guy's Hospital.

2.1 IMMUNOHISTOCHEMISTRY

I TISSUE SECTIONS

A. Selection of tissues

(i) Breast tissues

The breast tissue studied was obtained from surgical specimens sent for routine diagnostic purposes to the Clinical Oncology Unit (COU) Laboratory at Guy's Hospital. This laboratory handles all surgical specimens from the Guy's Hospital Breast Unit (amounting to some 1100 cases a year). There was thus a readily available supply of both frozen and fixed tissue from breast carcinomas of a range of histological types, benign breast biopsies, and normal breast tissue including cases showing secretory change (i.e. from pregnant or lactating patients). The vast majority of the cases were originally diagnosed by Dr. R. R. Millis but all histological sections were reviewed by the author. A single block containing representative tissue was then selected for immunohistochemical study. Comprehensive clinical and other prognostic data was also available on all the cases. Fetal, neonatal and pre-pubertal breast tissue was obtained at post mortem by Dr. J. Bartek and supplied as paraffin blocks of methacarn fixed tissue.

(ii) Tissue from other sites

The majority of the normal tissue examined was obtained at autopsy specifically for this study. Tissue was removed within a few hours of death and in all cases routine haematoxylin and eosin (H&E) examination of the tissue confirmed that this was histologically normal and that there was no evidence of autolysis. Tissue in which autolysis was observed was excluded. Some tissue, for example that from the gastro-intestinal tract and lung posed a particular problem as autolysis at these sites occurs rapidly. Specimens of normal lung, stomach and colon were therefore taken from surgical specimens in which a carcinoma was present elsewhere. In these circumstances the normal tissue was taken from a point as far away from the tumour

as possible. Again H&E stained sections were examined to confirm that the tissue selected was normal.

Fixed tissue from primary carcinomas (other than breast tumours) was obtained from a variety of sources. Primary lung tumours were kindly supplied by Dr. E. Sheffield of the Brompton Hospital. The majority of the tumours of colon and ovary were obtained from the Imperial Cancer Research Fund's tissue bank at the Royal College of Surgeons (most specimens orginated from either University College or St. Mark's Hospital).

As with the normal tissue, H&E stained sections from all cases were reviewed to confirm the diagnosis and to select a suitable block or blocks of tissue containing representative tumour for immunohistochemical study. In the majority of cases a single block was selected.

B. Preparation of Tissue Sections

Where possible, it is preferable to use fixed rather than frozen material in immunohistochemical studies. There are several reasons for for this. 1) tissue morphology in paraffin sections is superior to that in frozen sections and it is therefore easier to assess the distribution of staining both within the tissue and within individual cells, 2) fixed material is easier to store and handle than frozen tissue, 3) material for study can be selected retrospectively and cases referred from elsewhere can also be assessed, 4) larger pieces of tissue can be examined than is possible using frozen sections. This is a particular advantage when working with an antibody which shows a heterogeneous staining pattern. It is, however, important to ensure that no loss of antibody reactivity occurs as a result of fixation.

A preliminary study was therefore carried out to compare the results of immunohistochemical staining with SM3 on frozen sections

and sections of tissue treated with several different fixatives (see below). This comparison was performed both on breast tissue (malignant, benign and normal) and normal tissues from other sites from which frozen material was available. Staining of frozen and fixed tissue sections was carried out at the same time under the same conditions.

(i) Frozen Sections

Material for frozen sections was quickly frozen in liquid nitrogen and then stored in cryo-tubes (Nunc) in liquid nitrogen until required. Prior to sectioning, the tissue was embedded in OCT compound (Tissue Tek) on a metal chuck, frozen on to the chuck, and sections cut at 5 μm in a Bright's croysat. These were mounted on glass slides coated with poly-L-lysine, air-dried, fixed in acetone for 15 minutes at room temperature and stained immediately. In all tissues assessed results on frozen sections were similar to those obtained on paraffin embedded tissue which had been methacarn fixed (for details see Chapter 3).

(ii) Paraffin Sections

The fixatives assessed were methacarn, Carnoy's, Bouin's, glutaraldehyde and a variety of different formalin based reagents (neutral buffered formalin, formal saline, formal calcium, formal sublimate and formal alcohol). Details of these are given in the Appendix to this chapter. As will be described in detail in Chapter 3, staining in methacarn, Carnoy's and Bouin's fixed material was identical to that in frozen tissue. All the above were superior to glutaraldehyde and formalin based fixatives in terms of antigen preservation. As breast tissue in the COU laboratory is routinely fixed in methacarn as well as formalin, the former was chosen for further study. Tissue obtained from other sources was fixed in the same way, in some cases by special arrangement. Tissue was sliced, immersed in methacarn for approximately 1 hour, then taken to 70% alcohol and processed to paraffin in the usual way using an automatic tissue processor (Shandon Ltd.). Sections were cut at 3-4 μm and mounted on clean glass slides.

C. Antibodies

Details of the development and characterisation of the monoclonal antibodies SM3 and HMFG2 have been given in Chapter 1. Both were produced in the Epithelial Cell Biology Laboratory at the Imperial Cancer Research Fund under the direction of Dr. Joyce Taylor-Papadimitriou. They are mouse monoclonal antibodies of the subclass IgG1.

Hybridoma cells were grown in sterile tissue culture flasks in RPMI 1640 medium containing 10% fetal calf serum (FCS). The cells were split (1:3) three times a week, and the supernatant harvested the day after splitting.

For routine immunohistochemical staining, sodium azide was added to the culture supernatant (at a concentration of 0.02%) and the solution used neat. This contains approximately 20-30 µg/ml of HMFG2 and 10-20 µg/ml of SM3.

D. Staining method

Sections were stained in batches of approximately 30 slides at any one time. Staining with the two antibodies (SM3 and HMFG2) was always performed in parallel. Positive and negative controls (see below) were included with each batch.

Staining was performed using the conventional indirect immunoperoxidase method routinely employed in the COU laboratory. This is based on that described by Bartek and colleagues (1985b) with minor modifications. Details are as follows:

1. Dewax paraffin sections and take to absolute alcohol (2 changes).
2. Place sections in 0.5% hydrogen peroxide in methanol for 10-15 minutes when inhibition of endogenous peroxidase activity is required (see below - Section E).

3. Wash in tap water for 5-10 minutes.
4. Digest with trypsin or pronase if required*.
5. Wash twice (5 minutes each) in phosphate buffered saline (PBS).
6. Drain and wipe away excess buffer from around sections.
7. Incubate in 10% fetal calf serum (FCS) for 10 minutes to block non-specific binding of antibody.
8. Drain and wipe away excess serum.
9. Incubate with primary antibody, SM3 or HMFG2, approximately 50-100 µl of solution per slide, for 60 minutes.
10. Wash in 2 changes of PBS (10 minutes each).
11. Drain and wipe away excess PBS.
12. Incubate with second antibody (peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO P161) diluted 1:50 in PBS containing 15% FCS and 3% human serum) for 60 minutes.
13. Wash in 2 changes of PBS (10 minutes each).
14. Incubate with the chromogen diaminobenzidine (1 mg/ml in PBS containing 0.03% hydrogen peroxide) for approximately 10 minutes.
15. Rinse in PBS and then tap water.
16. Counterstain nuclei with Harris's haematoxylin (1 minute), dehydrate through graded alcohols, clear in xylene and mount in a synthetic resin, such as Depex (Gurr).

*No digestion was required for methacarn fixed tissue.

NB Stages 1-4 above were omitted when staining frozen sections. All reactions were carried out at room temperature unless otherwise stated.

The schedule described above, using diaminobenzidine as the chromogen results in a permanent brown reaction product.

E. Assessment of staining and controls

The following features were assessed -

1. Staining - present or absent?
2. If present (a) what cell types are stained?
   (b) what is the site of staining within the cells - nuclear? cytoplasmic? membranous? a combination of these?
   (c) is the staining homogeneous or heterogeneous within any one cell type?
   (d) is the staining weak or strong?
3. Is staining with HMFG2 and SM3 -
   (a) similar with both antibodies?
   (b) different, either in terms of cell type stained, site of staining within cells, intensity or distribution?

Positive Control. After preliminary assessment of SM3, a breast carcinoma which showed an intermediate level of staining with the antibody was included with each batch of slides. Reproducible staining of this case ensured consistency from one batch to another.

Negative Control. A negative control in which PBS replaced the primary antibody was included for each case.

Endogenous Peroxidase Activity. Endogenous peroxidase activity is present in a variety of cells, particularly erythrocytes and polymorphonuclear leucocytes. It can be inhibited by incubation with a solution of hydrogen peroxide in methanol (Streefkerk 1972) as described above (Section D). In the tissues examined in this study endogenous peroxidase activity did not pose a problem and so this step was not performed. Any problems which did arise were readily resolved by comparison with the negative control.

II CULTURED CELLS

A. Culture of cells

Staining patterns with SM3 and HMFG2 were studied in two human breast carcinoma cell lines, MCF7 and T47D and in epithelial cells grown from human milk. Cells were grown in sterile tissue culture flasks or plastic petri dishes, the carcinoma cells in Eagle's medium with 10% fetal calf serum (+ 10 μg/ml insulin in the case of MCF7s) and the normal epithelial cells (milk cells) in RPMI 1640 medium containing 10% fetal calf serum, 15% human serum, 10 μg/ml insulin, 5 ng/ml hydrocortisone and 50 mg/ml cholera toxin. Both were maintained at 37°C in a humidified atmosphere containing 8% carbon dioxide. The milk cells were grown in 50 mm dishes and the carcinoma cells in 75 cm^3 flasks; for the purposes of staining, these cells were split, transferred to 30 mm dishes and stained at subconfluency.

B. Staining method

To document the exact site of antibody binding, both carcinoma and milk cells were stained live. This was necessary as fixation renders the cell membrane permeable and may result in the loss of membrane associated antigens. The immunofluorescence technique was used as the peroxidase reactivity product may precipitate out making the exact location of binding difficult to determine.
Fixed cells were also stained (fixed for 5-10 minutes in methanol/acetone 1:1 by volume); both the immunoperoxidase and the immunofluorescence techniques were used as the former is a more sensitive method.

Details of the staining methods are as follows:-
(Steps 1-5 and 8 are the same for both methods. Step 6 applies only to the immunoperoxidase method).

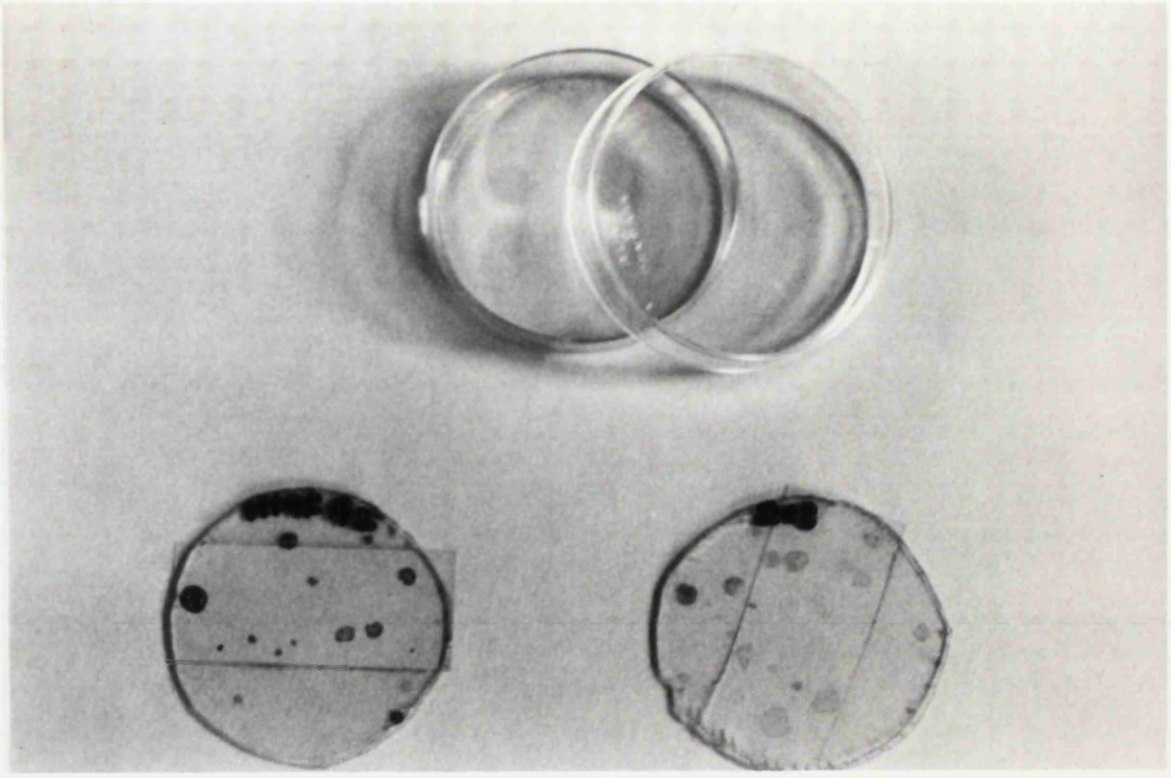
1. Wash cells twice with PBS.
2. Apply primary antibody (SM3 or HMFG2 approximately 2 ml per 30 mm dish) Incubate for 60 minutes ensuring that the cells are kept covered (ideally using a rocker). Keep lids on dishes to avoid drying.
3. Wash in 3 changes of PBS*.
4. Apply second antibody - either peroxidase conjugated or fluorescein conjugated rabbit anti-mouse (DAKO P161 or DAKO F232) each at a dilution of 1:40 in PBS containing 15% FCS and 3% human serum. Incubate for 60 minutes.
5. Wash in 3 changes of PBS*.
6. IMMUNOPEROXIDASE METHOD ONLY. Incubate with diaminobenzidine at a concentration of 1 mg/ml in PBS containing 0.03% hydrogen peroxide. Allow reaction to continue for 6 minutes. Counterstain with haematoxylin (30 seconds) then wash with warm tap water. Dry.
7. LIVE CELLS ONLY - Fix with methanol acetone (1:1 by volume) for 5 to 10 minutes.
8. BOTH METHODS. Wash with water. Cut edges from dishes leaving the base only as a perspex disc covered with cells. (Figure 2.1). Mount in Gelvatol 20:30 resin (Monsanto Massachusetts) - a water based mountant.

*Sodium azide was added to PBS for washes when staining live cells to prevent the phenomenon known as "capping". (Roitt 1988).

Immunoperoxidase staining, using diaminobenzidine as the chromogen results in a permanent brown reaction product which was visualised by conventional light microscopy as for the tissue sections.

Immunofluorescence staining using a fluorescein conjugated second antibody results in green fluorescence when viewed in ultraviolet light.

Figure 2.1. Dishes used to stain cultured cells. Cells from established breast cancer cell lines and epithelial cells from human milk were grown and stained in the plastic Petri dishes illustrated (above) either by the immunoperoxidase or the immunofluorescence method. The edges were then cut from the dishes leaving the stained cells on the base. These were mounted under glass cover slips (below).



C. Assessment of staining and controls

Staining in cultured cells was assessed in a similar way to that described for tissue sections. When staining had been performed by the immunofluorescence method, it was necessary to first visualise the cells by phase contrast microscopy to determine the number of cells in a given area and then under ultraviolet light to determine the number of cells exhibiting immunofluorescence. The following features were assessed:

1. Staining - present or absent?
2. If present (a) what cell types are stained/does the morphology of stained cells differ from that of unstained cells?
   (b) what is the site of the staining within the cells - nuclear? cytoplasmic? membranous? a combination of these?
   (c) is the staining homogeneous or heterogeneous within any cell type?
   (d) is the staining weak or strong?
3. Is staining with HMFG2 and SM3 -
   (a) similar with both antibodies?
   (b) different, (either in terms of cell type stained, site of staining within cells, intensity or distribution?)

Positive Control

In the studies on fixed cells (immunoperoxidase staining), LE61 was used as a positive control. This is an antibody to keratin 18 (Lane 1982) which reacts with both the breast carcinoma cell lines studied. In the studies on live cells (immunofluorescence), HMFG1 was used as a positive control. This antibody reacts strongly with cultured milk epithelial cells.

Negative Control

PBS was used in place of the primary antibody.

III. PHOTOMICROSCOPY

Peroxidase stained tissue sections and cultured cells were photographed on a Leitz Dialux microscope using a Wild camera system and Kodak Ektachrome 64 film.

Cells stained by the immunofluorescence method were photographed using a Zeiss photomicroscope and Ilford HP5 film.

2.2 IMMUNOBLOTTING

In order to study the mucin recognised by SM3 and HMFG2 at the molecular level, lysates were prepared from breast carcinoma cell lines and cytosols from fresh tissue from primary mammary carcinomas. The samples were then separated electrophoretically on sodium dodecyl sulphate gels. This method, described by Laemelli (1970) is a means by which proteins are separated purely according to their molecular weight.

In the technique of immunoblotting (Towbin, Staehelin and Gordon 1979) otherwise known as Western or protein blotting, separated proteins are electrophorectically transferred from a gel to nitrocellulose paper. A replica of the original gel pattern is produced on the paper and proteins can then be detected immunologically using the indirect immunoperoxidase staining technique.

Details of the method and reagents used are given below.

I SOURCE OF MATERIAL AND PREPARATION OF SAMPLES

A. Source of material

Lysates were prepared from MCF7, T47D, BT20 and ZR75-1 mammary carcinoma cell lines. The growth media for MCF7 and T47D cells have been described previously (2.1 II A). BT20 and ZR75-1 cells were grown in Eagle's medium with the addition of 15% FCS and 10 µg/ml insulin in the case of BT20 cells, and 10% FCS and 10-8M/oestradiol for ZR75-1 cells. A lysate of MRC5 cells, a fibroblast cell line served as a negative control.
The tumour cytosols utilised were those routinely prepared from the majority of primary mammary carcinomas diagnosed in the COU laboratory at Guy's Hospital.

B. Preparation of samples

(i) Cell Lines

Lysates were prepared from cells growing in 75 cm^3 tissue culture flasks. The culture medium was aspirated from the flask and the cells washed three times in PBS. Sodium dodecyl sulphate (SDS) sample buffer was then added to the cells (3 ml per flask) to lyse them. Buffer and lysed cells were transferred to glass tubes and the samples boiled for three minutes. This procedure was performed as rapidly as possible to inhibit any protease activity.

Samples were frozen until required and reboiled immediately before loading onto the gels. Either 50 or 100 µl of sample was applied to each channel on the gel.

(ii) Tumour Cytosols

Samples of tissue (approx. 500 mg) taken from primary tumours as soon as possible after surgical removal (within ten minutes), were frozen immediately in liquid nitrogen, and stored in cryo-tubes (Nunc) until required. To prepare the cytosol, frozen tissue was ground to a powder in a mikro-dismembrator (Braun). Buffer (10mM TRIS, 1.5mM EDTA, 5.0mM sodium molybdate) was added to the powder to lyse the cells and the specimen spun for ten minutes at 1000 rpm to remove nuclei, fat and cellular debris. The supernatant cytosol was stored in cryo-tubes in liquid nitrogen. Tissues and cytosol were kept frozen throughout.

The protein concentration of the cytosols was assayed using the Pierce Protein Assay Reagent (Coomassie blue G-250) in an adaptation of the well known Bradford method (Bradford 1976). Immediately prior to use, SDS sample buffer was added to the cytosols; the volume added was calculated so that the total amount of protein was approximately the same in each sample loaded onto the gel (This was approximately 100 µg in a volume of between 60 and 90 µl). Specimens were boiled immediately prior to loading onto the gel.

II SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The immunoblotting method described in this and section III was identical for all the samples described above.

Gels were prepared from a stock solution of acrylamide (30% acrylamide, 0.8% N_1N^1 methylene-bis-acrylamide, BIS(BioRad)) diluted in 0.4 M TRIS-HCl pH 8.8, 0.1% SDS, 0.06% TEMED*. The stack gel was prepared in the same buffer but at a pH of 6.8. Ammonium persulphate was added to the prepared solutions immediately before pouring the gels to give a final concentration of 0.03%.

Specimens were run either in 5% gels or 5-15% gradient gels each overlaid with a 3% stacking gel. (See Appendix to this chapter for details of reagents).

Samples were loaded in the volumes described above. Pre-stained molecular weight markers were loaded in one track of each gel. In order that results with two separate antibodies (SM3 and HMFG2) could be compared, specimens were loaded in duplicate. Prior to staining, the nitrocellulose paper could then be cut into two identical halves, each bearing the same samples and the two halves probed with a different antibody.

Electrophoresis was carried out in a running buffer of 0.025M TRIS, 0.192M glycine and 0.1% SDS. Current was applied at 50 Volts through the stack and 100 Volts through the running gel until the dye front (as indicated by bromophenol blue in the sample buffer) reached the bottom of the gel.

*TEMED = tetramethyl ethylenediamine

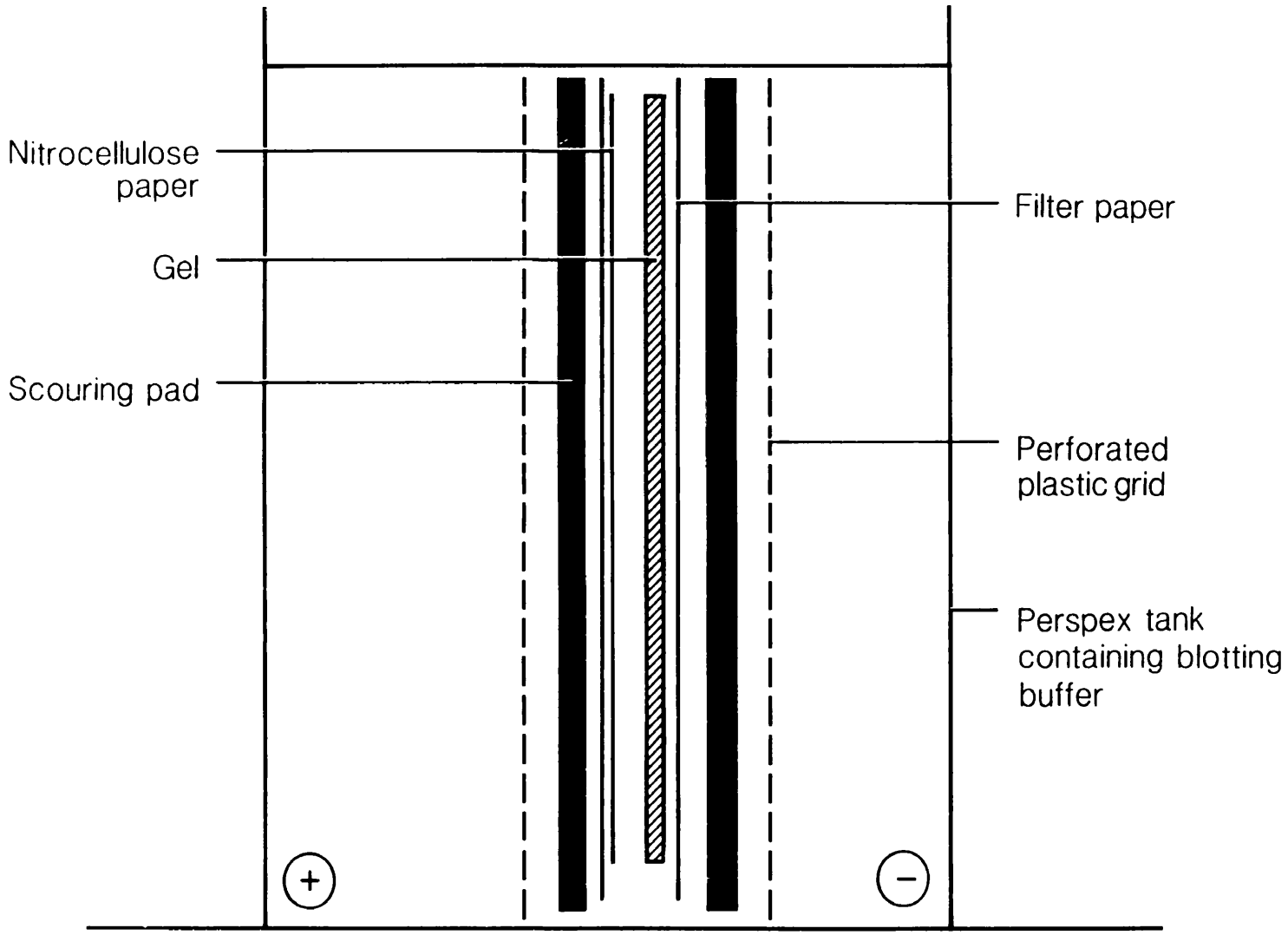
III TRANSFER TO NITROCELLULOSE PAPER, and STAINING

When the dye front reached the bottom of the gel, the current was switched off, the gel removed from the tank and applied firmly to pre-wetted nitrocellulose paper (Hybond-C extra, 0.45 μm pore size, Amersham) taking care to remove all air bubbles. Proteins were blotted from the gel to the nitrocellulose paper using the apparatus shown in Figure 2.2 (Bio-Rad Trans-Blot). The blotting buffer was 0.025M TRIS, 0.192M glycine in a solution containing 20% methanol and the transfer was carried out overnight in the cold.

The next day, the paper was soaked in a solution of 5% bovine serum albumin (BSA) in PBS, to saturate additional protein binding sites. The paper was rinsed in PBS, cut in two and each half incubated separately with either SM3 or HMFG2, each as neat tissue culture supernatant. The incubation period was two hours in a humid chamber. After extensive washing (five changes of five minutes each in PBS containing 0.05% Tween) the paper was incubated with the second antibody (peroxidase conjugated rabbit anti mouse (DAKO P161) diluted 1:50 in PBS containing 5% FCS for one hour, again in a humid chamber. Further extensive washing was then performed as described above and the paper finally incubated in the substrate solution of 4-chloro-1-napthol (a saturated solution in ethanol, diluted 1:100 in PBS and filtered) containing 0.03% hydrogen peroxide.

This reaction was allowed to continue until bands were visible on the paper (approximately 10 minutes). The reaction was terminated by washing in distilled water. The nitrocellulose paper was then dried between two pieces of filter paper.

Figure 2.2. Immunoblotting (Western blotting) apparatus. Samples are separated electrophoretically in SDS gels and then transferred from the gel to nitrocellulose paper using the apparatus illustrated. The transfer is carried out overnight in the cold.


Nitrocellulose paper
Gel
Scouring pad
Filter paper
Perforated plastic grid
Perspex tank containing blotting buffer
+
−

IV INTERPRETATION OF RESULTS

Bands appearing on the nitrocellulose paper after probing with either SM3 or HMFG2 were compared. Usually the time taken for bands to appear in the half probed with SM3 was longer than for that treated with HMFG2.

The presence and position of bands recognised by the two antibodies was noted and compared. It was not possible to make any quantitative assessment of the amount of antigen detected.

V PHOTOGRAPHY

Nitrocellulose paper was mounted on white card and photographed by the Photography Department at the ICRF.

2.3 IMMUNOLOCALISATION

A pilot immunolocalisation study in patients with primary breast cancer was undertaken. The aim was to evaluate the usefulness of radiolabelled SM3 for imaging or localising axillary lymph node metastases. The antibody was labelled with a radioactive isotope of iodine and injected by either the subcutaneous or the intravenous route. After injection, patients were scanned using a gamma camera at set intervals prior to surgery. The patients then underwent mastectomy including axillary dissection and images of the resected specimen were compared with those obtained in vivo. Individual nodes were dissected out, weighed and radioactivity counted in a gamma counter. Radioactivity per gram in the resected nodes was compared with subsequent histological findings.

I SELECTION OF PATIENTS

Informed consent was obtained from all patients entered in this study. Patients invited to participate were those with biopsy proven primary breast cancer which was to be treated with axillary clearance as part of a modified radical mastectomy. The patients were interviewed by the co-ordinator of the study (either Dr. C. Twelves or Mr. I. Bennett). The majority of patients approached were willing to participate.

II SELECTION OF RADIO-ISOTOPE AND IODINATION OF ANTIBODY

SM3 was initially labelled with ^{123}I. This isotope was chosen for several reasons:

i) It has a short half life (13 hours), ideally suited to the clinical circumstances. Patients in the COU at Guy's who are due to undergo major surgery are admitted two days before their operation. Using this antibody the patients could be injected and scanned the day before surgery and sufficient radioactivity would still remain at the time of surgery for the patient and surgical specimen to be imaged and radioactivity in the resected nodes counted.

ii) It has a photon energy of 159 KeV suitable for efficient detection and resolution.
iii) The patient receives a low radiation dose.

In later studies, SM3 was labelled with ^{125}I which has the following properties:
i) It has a longer half life than ^{123}I (59.6 days); to fit in with the clinical schedule it was necessary for patients to be injected six days before surgery, then to be discharged and readmitted the following week.
ii) It has a photon energy of 35 KeV which is too low to allow imaging.
iii) As with ^{123}I the patient receives a low radiation dose.

Iodination of the antibody was performed by Dr. S. Mather in the ICRF Nuclear Medicine Research Unit at St Bartholomew's Hospital, using the iodogen method (Fraker and Speck 1978) which is relatively simple and is said not to cause loss of antibody reactivity.

III INJECTION OF ANTIBODY

Antibody labelled with either ^{123}I or ^{125}I was injected subcutaneously into the finger web spaces or intravenously into the antecubital fossa. The different routes of injection were used to see whether injection into the regional lymphatics gave better results than injection into the blood stream as reported by others (Weinstein et al 1983, Mandeville et al 1986). The amount of antibody given was the same whether administration was by the subcutaneous or intravenous route, and was 1 mg in a total volume of 1.6 ml in the case of ^{123}I labelled SM3 and 300 mg in the same volume for ^{125}I labelled SM3. This contains 37-185 mBq (1-5 mCi) of radioactivity in the case of ^{123}I labelled antibody and 3.7 mBq (100 μCi) in the case of ^{125}I labelled antibody. For subcutaneous injections an insulin syringe was used so that the small volume could be distributed evenly between the four finger web spaces (four injections, each of 0.4 ml). Twelve

patients received subcutaneous injections and 13 patients intravenous. Injections by both routes were well tolerated by the patients who reported minimal discomfort. Injections were given into both the potentially affected side i.e. that of the primary tumour and non-affected side so that comparisons could be made between the potentially involved and the normal axilla.

Uptake of radioactive iodine by the thyroid gland was blocked by oral administration of potassium iodate and potassium perchlorate each starting the day before injection and continuing for two days after in the case of patients given ^{123}I labelled SM3 and five days after for patients receiving ^{125}I labelled SM3.

IV IN VIVO IMAGING

This was only possible when antibody labelled with ^{123}I was used. ^{125}I is unsuitable for imaging due to its low gamma energy. Scans were performed on both the day of the injection (i.e. the day before surgery) and the following day (immediately prior to surgery) according to the schedule detailed in Table 2.1. All imaging was performed using a gamma camera (Figure 2.3A) in the Department of Nuclear Medicine at Guy's Hospital.

V SURGERY AND IMAGING OF INTACT SPECIMEN

Axillary clearance was performed within 24 hours of injection in the case of ^{123}I labelled antibody and six days later in the case of ^{125}I labelled antibody. The resected specimen was collected from the operating theatre and where appropriate i.e. when the patient had been injected with ^{123}I labelled antibody, taken to the Department of Nuclear Medicine, orientated and scanned so that a comparison could be made with the images obtained in vivo.

Table 2.1

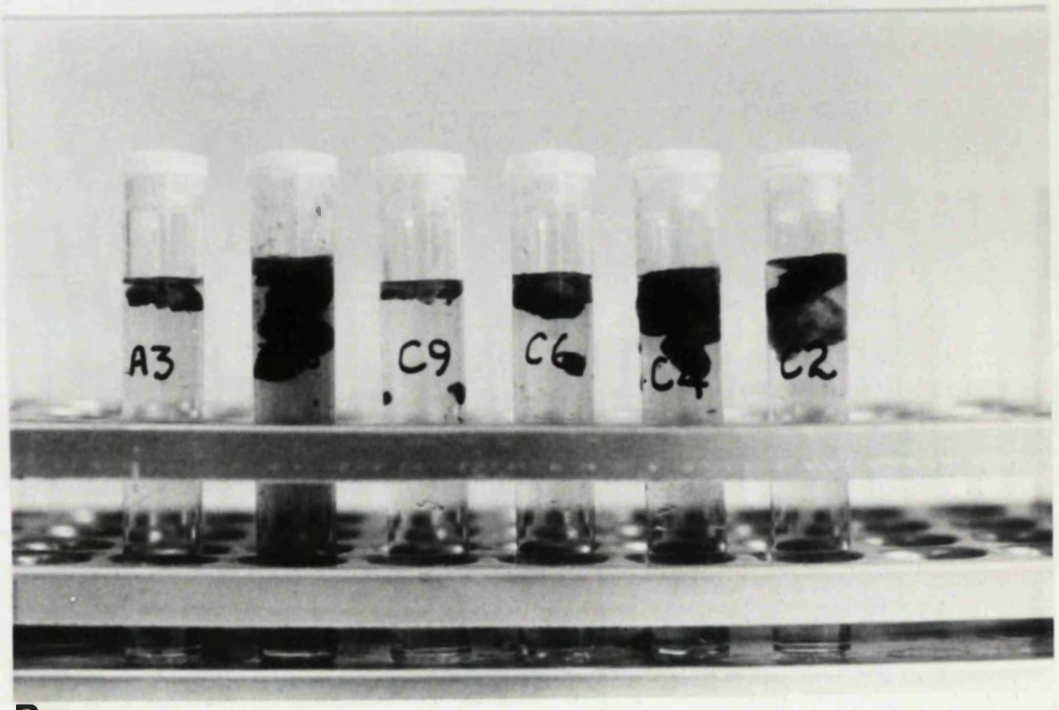
SCHEDULE FOR INJECTION OF RADIOLABELLED ANTIBODY AND IN VIVO SCANNING

(ONLY APPLICABLE TO PATIENTS GIVEN ANTIBODY LABELLED WITH ^{123}I)

Time	
Day 1	
t = 0	^{123}I labelled SM3 injected
t = 0-10 minutes	Scan of hands (dynamic)
t = 60 minutes	Scan of axillae, anterior and posterior, 15 minutes each
t = 240 minutes	Scan of axillae, anterior and posterior, 10 minutes each
Day 2	
t = 20 hours	Scan of axillae, anterior and posterior, 10 minutes each
t = 22-23 hours	Surgery - axillary clearance, with mastectomy

Figure 2.3A. Gamma camera used for imaging patients and surgical specimens during immunolocalisation studies. Patients injected with ^{123}I labelled SM3 were scanned using the gamma camera illustrated as were the mastectomy specimens from these patients. **Figure 2.3B.** Glass tubes containing fixative. Dissected axillary lymph nodes and breast tissue were weighed, placed in the labelled tubes illustrated, taken to the Department of Nuclear Medicine and counted in a multiwell gamma counter. The letters A, B and C denote axillary lymph nodes from the high, middle and low groups respectively.

A



B

VI DISSECTION OF SPECIMEN AND IN VITRO COUNTING OF NODES

Mastectomy specimens were dissected fresh using the standard method employed in the COU Laboratory. Lymph nodes are divided into the three recognised levels which are defined by sutures placed in the axilla at the time of operation. Nodes dissected in this way were grouped together and carefully labelled so that they could later be separately identified. The nodes were weighed, placed in glass tubes containing fixative (Figure 2.3B) and taken to the Department of Nuclear Medicine for counting in a multiwell gamma counter. Each sample was counted for 1000 seconds. Nodes were later processed in the usual way. An individual group of nodes counted ultimately corresponded to a single tissue block and histological section.

To assess uptake of antibody into the breast itself, tissue from the site of the primary tumour was sampled, weighed and its radioactivity counted. In some instances no tumour could be identified grossly in the mastectomy specimen but in cases where the diagnostic biopsy was either an incisional or needle core specimen a large amount of residual tumour was present. Tissue from a macroscopically normal area elsewhere in the breast was also counted.

VII HISTOLOGICAL ASSESSMENT OF NODES AND IMMUNOHISTOCHEMISTRY

The usual cutting and reporting procedure of the COU laboratory was followed. All lymph nodes are routinely examined at two separate levels i.e. sections are cut at two different depths into the paraffin wax block, the first at a superficial level and the second deeper into the tissue. This method facilitates the detection of very small metastatic deposits. Recently in the COU laboratory we have been assessing the merits of immunohistochemical staining using a combination of two anti-epithelial antibodies, CAM5.2* and HMFG2 on sections cut at the second level; in our hands this has further facilitated the detection of small metastases. The presence and extent of involvement by metastatic carcinoma in any node was carefully noted so that a comparison could be made between the histological findings and the radioactivity counted.

*Recognises lower molecular weight cytokeratins (Makin, Bobrow and Bodmer 1984).

As part of the study immunohistochemical staining with SM3 was performed on all the primary tumours from patients studied and on any nodal metastases identified. When staining the primary tumours methacarn fixed tissue was always assessed. Lymph nodes are routinely fixed only in formalin, and so only tissue fixed in this way was available for immunohistochemical staining.

To ensure that the iodination procedure had not resulted in any loss of antibody reactivity, staining was performed with both non-iodinated (as neat tissue culture supernatant) and iodinated SM3 (surplus antibody remaining after the patient had been injected). In all cases staining with iodinated SM3 was as strong as with the non-iodinated antibody. Indeed staining with the iodinated antibody was often considerably stronger, probably due to the fact that this was a purified solution and contained 1-2mg/ml antibody compared with approximately 20µg/ml in the tissue culture supernatant.

In an attempt to demonstrate that injected antibody had bound to any metastatic tumour in lymph nodes, a one stage direct immunoperoxidase staining method was used. No primary antibody was applied. Peroxidase conjugated rabbit anti-mouse immunoglobulin was added to fixed tissue sections from nodes containing metastatic carcinoma. Sections were washed, incubated with diaminobenzidine and counterstained with haematoxylin in the usual way.

VIII COLLECTION OF BLOOD AND URINE SAMPLES

In order that pharmacokinetic data could be obtained blood and urine was collected from some of the patients in the study. This was only possible from patients given ^{123}I labelled antibody as this group were in-patients at the relevant times. The schedule was as follows:
Blood samples (5 ml heparinised) were taken at t=15 minutes, t=1 hour, t=5 hours, t=20 hours and immediately post operatively.
Urine samples - the bladder was emptied prior to injection and all urine passed before surgery was collected in separate containers, the time being noted on each.

IX ANALYSIS OF DATA

Radioactivity in involved and non-involved nodes was compared, as was radioactivity in any residual breast tumour and normal breast tissue. Results, initially expressed as counts per gram of tissue were related to a standard made up from the same batch of radioisotope as was used for the injection. Data was then expressed as percentage of injected dose per gram of tissue. As the total number of patients in each group was small the data for positive and negative nodes was pooled (see Chapter 5 for details) and the mean values compared using the Student's t-test.

2.4 APPENDIX: REAGENTS AND CELL LINES

I FIXATIVES

A. Formaldehyde containing fixatives

Formal saline -	40% formaldehyde	100 ml
	tap water	900 ml
	sodium chloride	9 g
Formal calcium -	40% formaldehyde	100 ml
	tap water	800 ml
	10% calcium chloride	100 ml

Neutral buffered formaldehyde -

	40% formaldehyde	100 ml
	tap water	900 ml
	sodium dihydrogen phosphate monohydrate	4 g
	disodium hydrogen phosphate anhydrous	6.5 g

Alcholic formaldehyde -	40% formaldehyde	100 ml
	95% alcohol	900 ml

B. Alcohol containing fixatives

Carnoy's fixative -	absolute alcohol	600 ml
	chloroform	300 ml
	acetic acid	100 ml
Methacarn -	methanol	600 ml
	chloroform	300 ml
	acetic acid	100 ml

C. Picric acid containing fixatives

Bouin's fluid -	saturated aqueous picric acid	750 ml
	40% formaldehyde	250 ml
	acetic acid	50 ml

D. Mercuric chloride containing fixatives

Formal sublimate -	mercuric chloride	6 g
	sodium acetate	1.25 g
	distilled water	90 ml
	40% formaldehyde	10 ml

E. Glutaraldehyde

A 2-4% solution in phosphate buffered saline

II BUFFERS

A. Phosphate buffered saline

Sodium dihydrogen phosphate	15.3 g
Disodium hydrogen phosphate	71.6 g
Sodium chloride	90.0 g

Dissolved in 10 litres distilled water pH 7.4

B. Running buffer for sodium dodecyl sulphate polyacrylamide gel electrophoresis

0.025M Tris base	3.03 g/litre
0.192M glycine	14.42 g/litre

Adjust pH to 8.3 if necessary

C. Blotting buffer for use in Western blotting apparatus

0.025M Tris base	3.03 g/litre
0.192M glycine	14.42 g/litre

Make up in distilled water containing 20% methanol

III GELS FOR ELECTROPHORESIS

A. Running gels

	5%	5% (for gradient gels)	15% (for gradient gels)
Acrylamide (stock solution*)	5.0 ml	2.5 ml	8.0 ml
1M Tris Ph 8.8	11.2 ml	5.6 ml	5.6 ml
20% SDS	0.15 ml	0.075 ml	0.075 ml
Distilled water	13.7 ml	7.85 ml	-
Glycerol	-	0.45 g	3.0 g

Add 100 µl 10% ammonium persulphate and 20 µl TEMED** just before pouring

B. Stack gel (3%)

Acrylamide (stock solution*)	1 ml
1M Tris pH 6.8	1.25 ml
20% SDS	0.05 ml
Distilled water	7.8 ml

Add 50 µl 10% ammonium persulphate and 10 µl TEMED** just before pouring

* Stock solution of acrylamide - 30% acrylamide
0.8% BIS

** Tetramethyl ethlylenediamine

IV DETAILS OF CULTURED CELLS AND CULTURE MEDIA

Cell Line	Derived from	Medium	Reference
T47D	2° breast carcinoma (pleural effusion)	Eagle's + 10% FCS	Keydar et al 1979
MCF7	2° breast carcinoma (pleural effusion)	Eagle's + 10% FCS + 10 µg/ml insulin	Soule et al 1973
BT20	1° breast carcinoma	Eagle's + 15% FCS + 10 µg/ml insulin	Lasfargues et al 1958
ZR-75-1	2° breast carcinoma (ascitic fluid)	Eagle's + 10% FCS + 10^{-8}M oestradiol	Engel et al 1978
HumE	Primary normal breast epithelial cells from human milk	RPM1 1640 + 10% FCS + 15% human serum + 10 µg/ml insulin + 5 µg/ml hydrocortisone + 50 ng/ml cholera toxin	Taylor-Papadimitriou et al 1977

Chapter 3

IMMUNOHISTOCHEMICAL STUDIES

Details of the selection of tissues, preparation of sections and immunohistochemical staining methods have been given in Chapter 2. In most cases, both frozen and fixed, paraffin embedded tissue was examined.

3.1 PRIMARY AND METASTATIC MAMMARY CARCINOMA

Tissue from 127 cases of primary mammary carcinoma has been studied, together with a further 22 cases of metastatic mammary carcinoma. In the latter group, 19 of the deposits were in axillary lymph nodes, two in skin and one in soft tissue.

I PRIMARY MAMMARY CARCINOMAS

A variety of carcinomas of different histological types and grades was studied. Details are given in Table 3.1.

A. Comparison of staining in fixed and frozen tissue

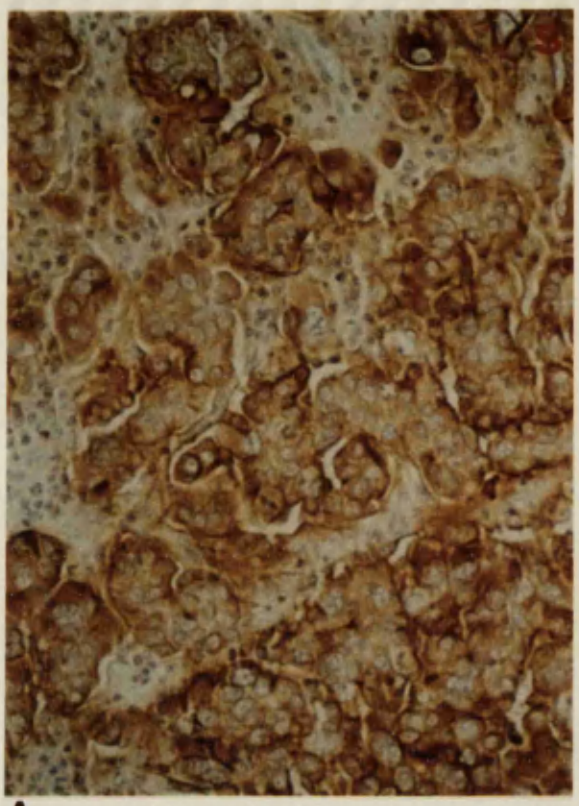
In the ten cases studied, SM3 staining in methacarn fixed material was almost identical to that in frozen tissue (Figures 3.1A and 3.1B). The only difference observed was marginally less staining in some of the frozen tissues. As described later in this chapter staining patterns with SM3 are often heterogeneous in distribution and in the small pieces of tissue used for frozen sections this heterogeneity may lead to an erroneous impression of a reduced amount of staining. Fixation in formalin, however, resulted in a marked diminution of both the intensity and distribution of staining (Figure 3.1C). The effect of digestion with trypsin was also assessed but no improvement was seen. In some cases, background staining was noted in the frozen but not in the fixed tissue sections.

Table 3.1

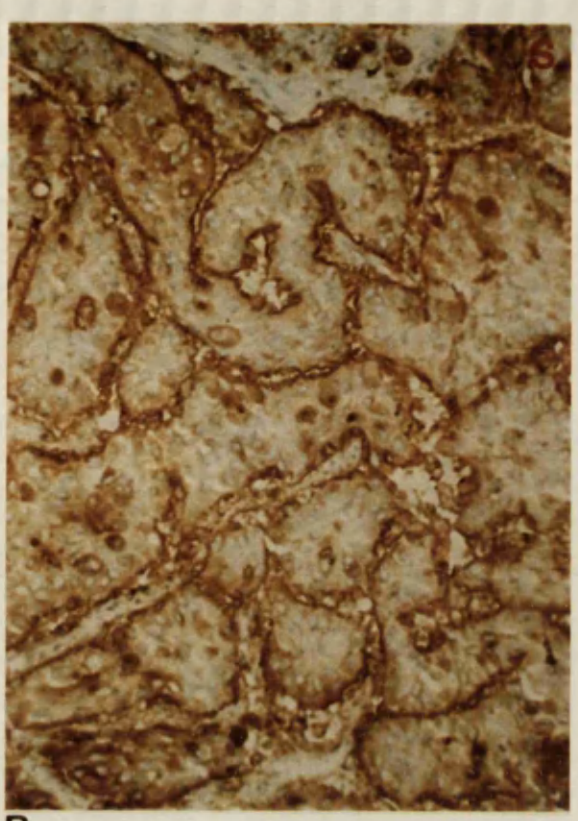
HISTOLOGICAL CLASSIFICATION OF PRIMARY MAMMARY CARCINOMAS STUDIED

Histological Type	Number of Cases
Infiltrating ductal	77
Infiltrating lobular	29
In situ ductal	5
In situ lobular	2
Special types of mammary carcinoma	
Medullary with lymphoid stroma	4
Tubular	2
Mucoid	3
Adenoid cystic	2
Spindle cell/metaplastic	1
Paget's disease of the nipple	2
Total	**127**

Figure 3.1. SM3 staining in primary mammary carcinomas: effect of different methods of fixation. SM3 (S) staining in methacarn fixed (A), frozen (B) and formalin fixed (C) tissue from a primary mammary carcinoma is illustrated. Staining in methacarn fixed material was virtually identical to that in frozen tissue but fixation in formalin resulted in a marked reduction (in this case virtual abolition) of staining.
(Immunoperoxidase).



A



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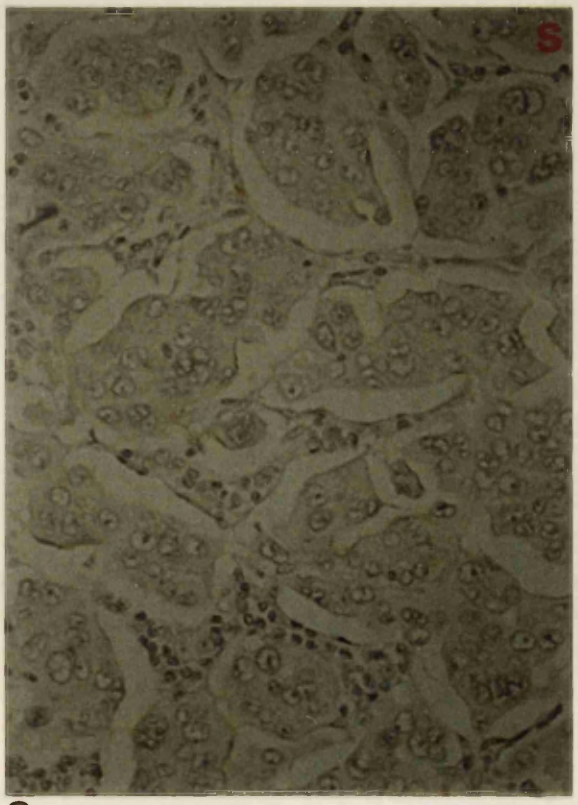


C

Staining with HMFG2 was also similar in methacarn fixed and frozen tissue. Although there was some diminution in staining in formalin fixed tissue this was much less marked than with SM3.

In a single case, tissue fixed in a number of different reagents was studied; similar staining with SM3 was seen in tissues fixed in methacarn, Carnoy's and Bouin's solution but in tissues fixed in either formalin based reagents (see Chapter 2 for details) or glutaraldehyde staining was markedly reduced.

In conclusion, therefore, in tissue from breast carcinomas, fixation in methacarn resulted in no loss of antigen expression when compared with frozen tissue and was superior to fixation in either formalin based reagents or gluteraldehyde. As mentioned in Chapter 2, all tissue received in the COU Laboratory is routinely fixed in methacarn as well as formalin; methacarn fixed tissue was therefore used for subsequent immunohistochemical studies.

B. Summary of results

A detailed assessment of the staining is given below but the major observations are summarised here. 1) of the 127 primary mammary carcinomas, 120 (94%) stained with SM3 and 126 (99%) with HMFG2; 2) staining with SM3 was generally more heterogeneous* and in some cases less intense than with HMFG2; 3) no correlation was seen between either tumour type or grade and staining with SM3.

*Heterogeneous is a term which has been used throughout this thesis to describe immunohistochemical staining patterns. The term refers to a variable distribution of staining in which cells in some areas of the tissue section were antibody positive and others failed to stain. This was often, but not always accompanied by a variation in the intensity of staining.

C. Details of staining patterns

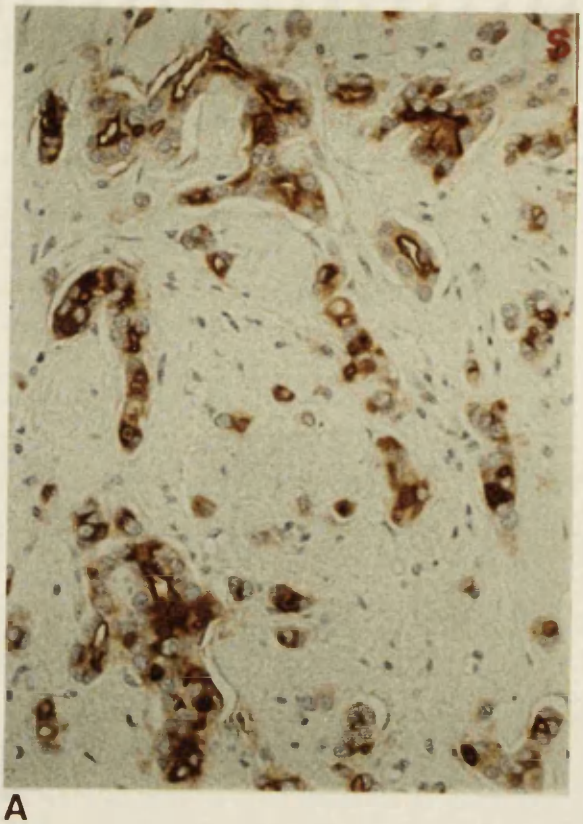
As mentioned in Chapter 2 the features assessed were 1) presence or absence of staining, 2) cell types stained, 3) site of staining within the cells, 4) homogeneity or heterogeneity of staining, 5) intensity of staining (weak or strong) and 6) comparison with HMFG2 staining.

SM3 staining was observed in 120 of the 127 primary carcinomas. In all cases staining was confined to malignant cells and was not observed in the stroma, inflammatory cells or blood vessels. Nuclear staining was never observed. Cytoplasmic staining was seen in all positive cases (Figure 3.2). In some, this was accompanied by membrane staining. The latter was most obvious at the luminal edge of cells lining tubules in tumours showing glandular differentiation (Figure 3.2A) but was also sometimes seen around individual cells (Figure 3.3A).

In many cases, heterogeneity of staining was seen (Figure 3.3). Of the 120 SM3 positive carcinomas, 78 showed a heterogeneous and 42 a homogenous pattern. The degree of heterogeneity varied considerably from case to case and within an individual case. The intensity of staining also varied in the same way from case to case and within an individual case. Sometimes all the cells in one field stained strongly whereas those in another field stained weakly or not at all. (Compare Figures 3.3A and 3.3C with 3.3B and 3.3D). In some fields the majority of cells failed to stain or stained only very weakly and one or two showed intense positivity (Figure 3.3B).

HMFG2 stained all but one of the 127 primary mammary carcinomas. The only HMFG2 negative tumour was a metaplastic carcinoma which was also SM3 negative. The six other cases which failed to stain with SM3 were HMFG2 positive. Four of these were only weakly stained with HMFG2, one was moderately stained and one stained strongly. The cell type stained and the site of staining within cells was the same as for SM3.

Figure 3.2. SM3 and HMFG2 staining in different grades of infiltrating ductal carcinoma. SM3 (S) staining in infiltrating ductal carcinomas of malignancy Grade I (A), Grade II (C) and Grade III (D) is illustrated. Staining with HMFG2 (H) in the same well differentiated (Grade I) carcinoma is also shown (B). Staining with SM3 and HMFG2 is very similar in this case, both in intensity and distribution. Staining with both antibodies is cytoplasmic (A, B, C and D) and membranous, particularly at the luminal edges of cells lining tubules (A and B). (Immunoperoxidase).



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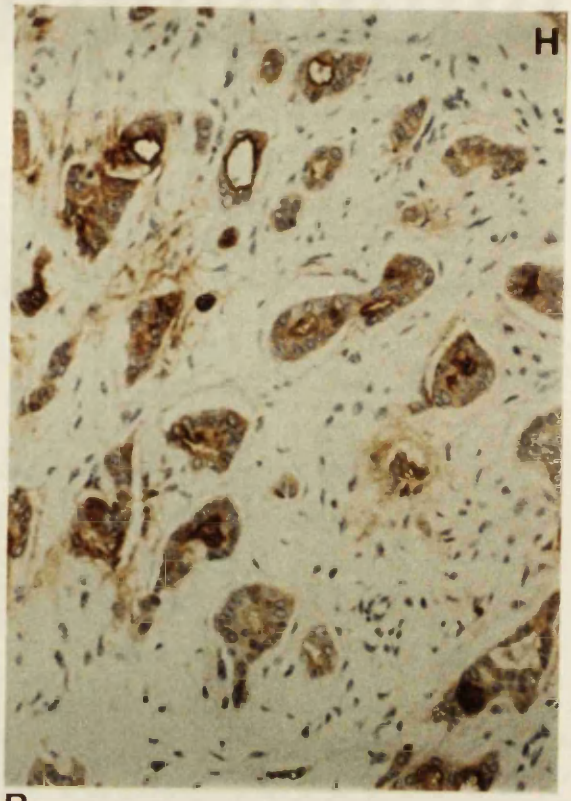

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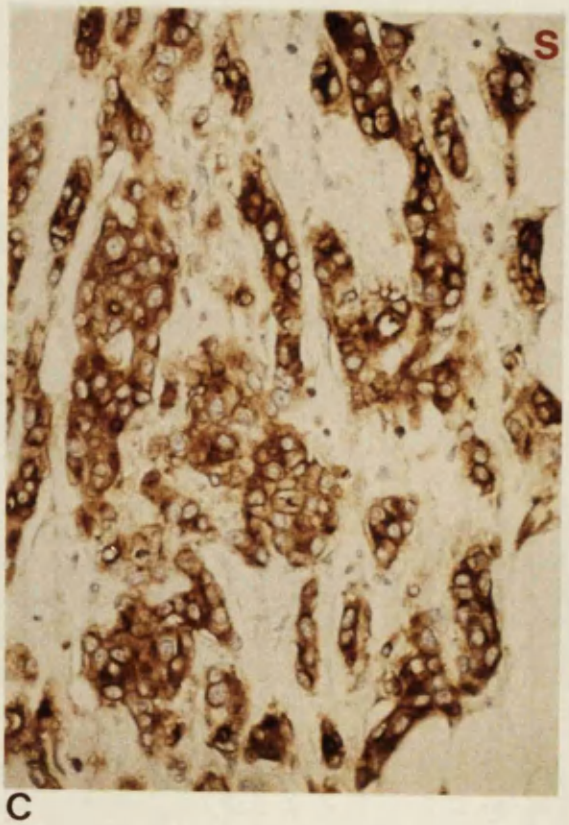

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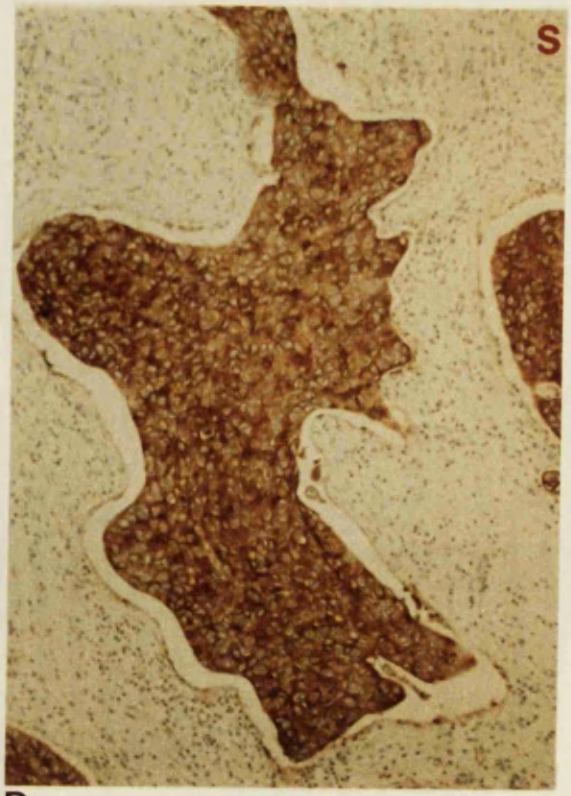
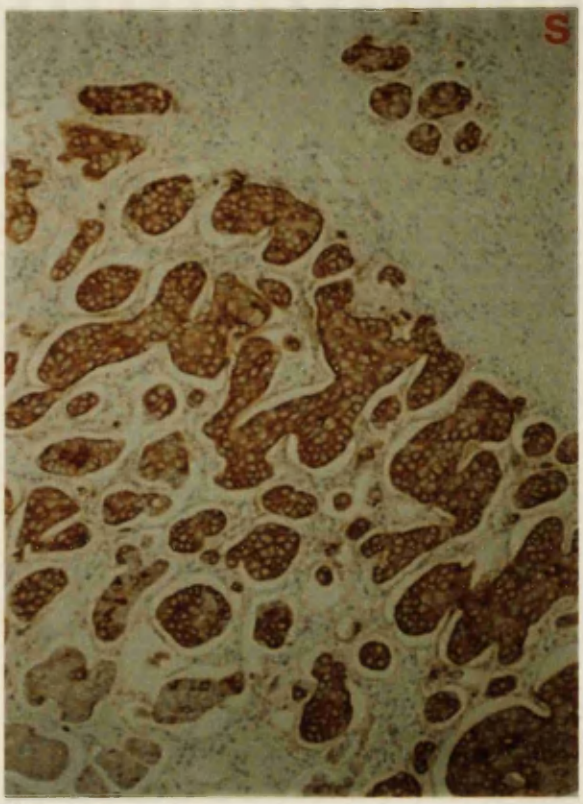


D

Figure 3.3. SM3 staining in primary mammary carcinomas demonstrating heterogeneity and variable intensity of staining.
SM3 (S) staining in two different areas of two primary mammary carcinomas is illustrated. In Figures A and B there is variation in the intensity of staining both from field to field and within an individual field. Figures C and D show heterogeneity of staining.
Note that there is membranous as well as cytoplasmic staining in the tumour illustrated in Figure A.
(Immunoperoxidase).



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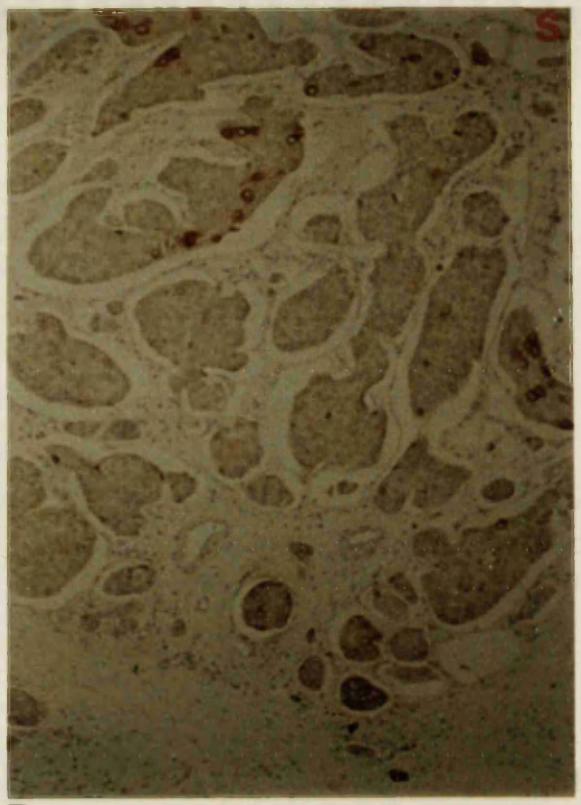

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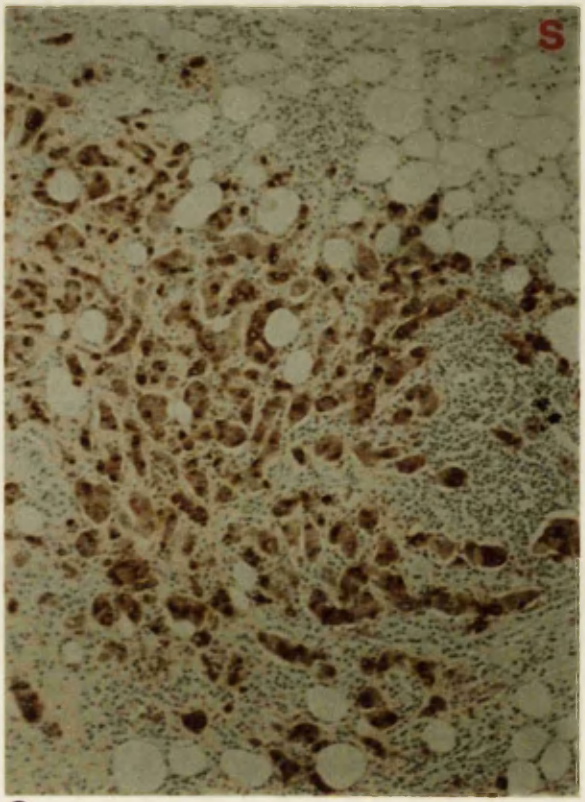

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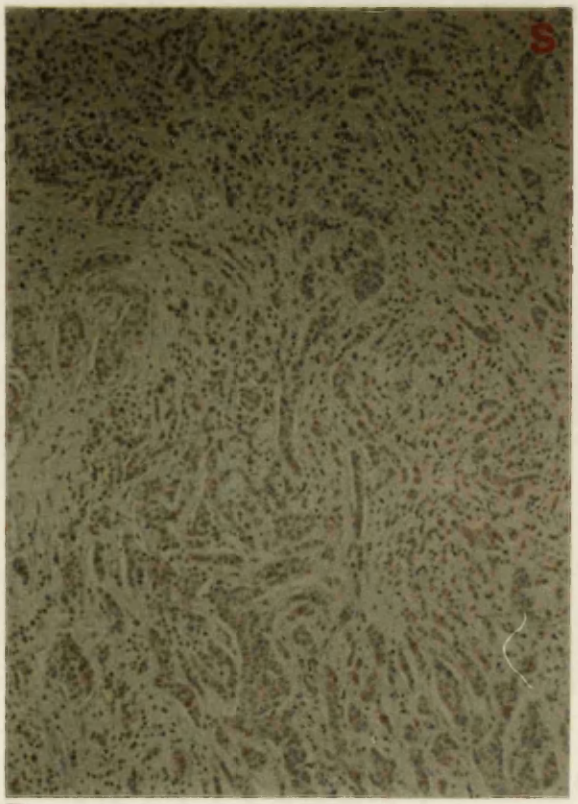


D

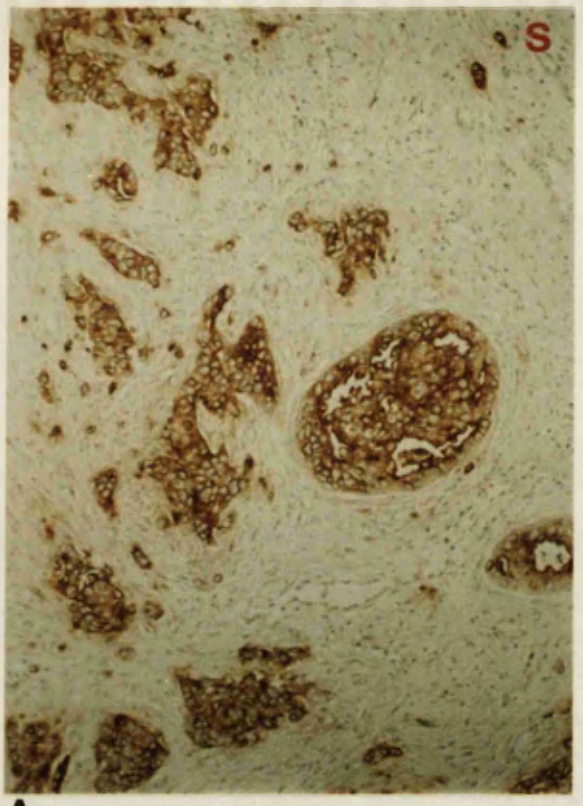
Although a moderate degree of heterogeneity was observed with HMFG2, in general this was less than with SM3. In cases showing heterogeneity the same areas of tumour stained with both antibodies. The intensity of staining was similar with both antibodies in 20 cases (Figures 3.2A and 3.2B, Figures 3.4A and 3.4B) whereas in 100 cases staining with HMFG2 was stronger than with SM3 (Figures 3.4C and 3.4D).

D. Correlation of staining with histological and other features

(i) Tumour type

Staining with SM3 was seen in all histological types of tumour except metaplastic carcinoma (Figure 3.5A). As shown in Table 3.2 cases which failed to stain with SM3 were not of any particular histological type. A similar proportion of infiltrating ductal and infiltrating lobular carcinomas stained with SM3 (75 of 77 (97.4%) and 28 of 29 (96.5%) respectively). Examples are shown in Figures 3.2 - 3.4 and Figure 3.5B. Where present, associated in situ carcinoma showed a similar staining pattern to the infiltrating tumour. The seven cases of pure in situ carcinoma were all SM3 positive (Figures 3.5C and 3.5D). The numbers of special types of mammary carcinoma are small. Both tubular carcinomas were SM3 positive (Figure 3.6A) and showed mild heterogeneity of staining; the intensity of staining varied from moderate to strong. All of the medullary carcinomas stained with SM3 but the distribution was heterogeneous and the intensity low (Figure 3.6B) except in one case of atypical medullary carcinoma which stained uniformly and strongly. Two of the three mucoid carcinomas were SM3 negative and in the one positive tumour the staining was weak and confined to only a few cells (Figure 3.6C). One of two adenoid cystic carcinomas stained with SM3. Staining was heterogeneous and of moderate intensity; the positive areas were at the apical cell membranes of cells lining glandular spaces and within secretions. Two cases of Paget's disease of the nipple showed positive staining with SM3 which was homogeneous and strong (Figure 3.6D).

Figure 3.4. Comparison of SM3 and HMFG2 staining in two primary mammary carcinomas. Figures A and B show a tumour in which staining with SM3 (S) and HMFG2 (H) is similar. Figures C and D show a tumour in which the intensity of HMFG2 staining is greater than that with SM3.
(Immunoperoxidase).



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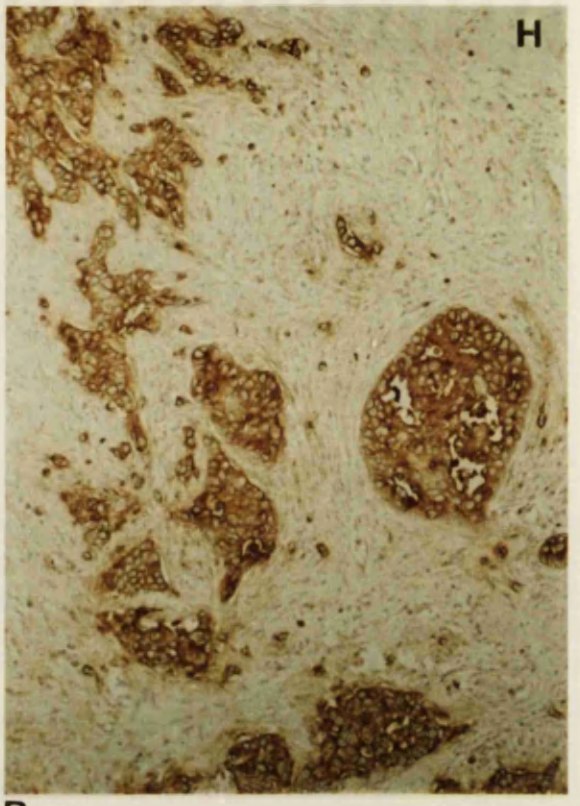

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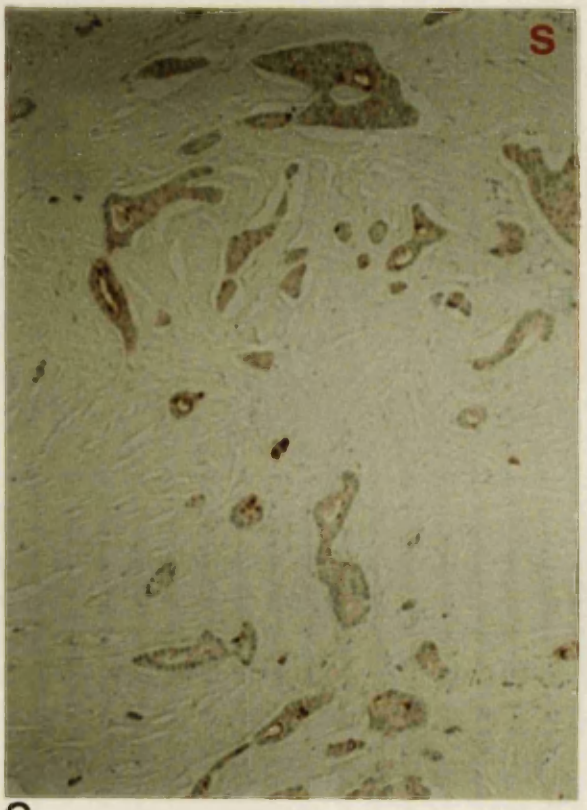

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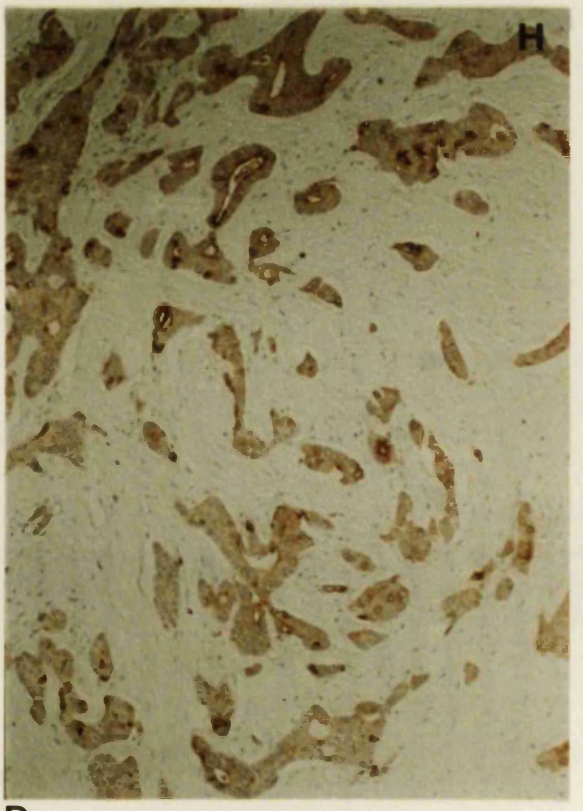
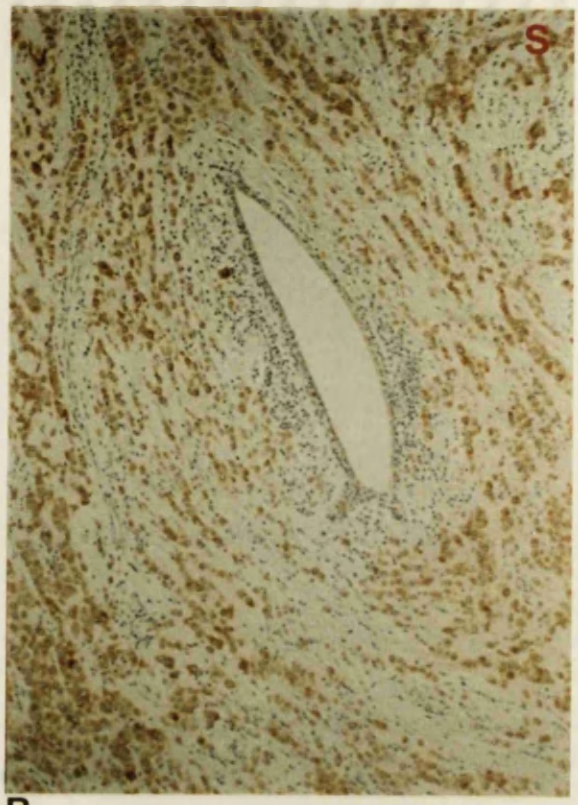


D

Figure 3.5. SM3 staining in carcinomas of different histological types. SM3 (S) staining was seen in all histological types of tumour except metaplastic carcinoma (A). This tumour also failed to stain with HMFG2. Staining in an infiltrating lobular carcinoma is illustrated in Figure B. Strong staining in carcinoma in situ of ductal type, comedo pattern is illustrated in Figure C and heterogeneous positivity in lobular carcinoma in situ in Figure D.
(Immunoperoxidase).

A



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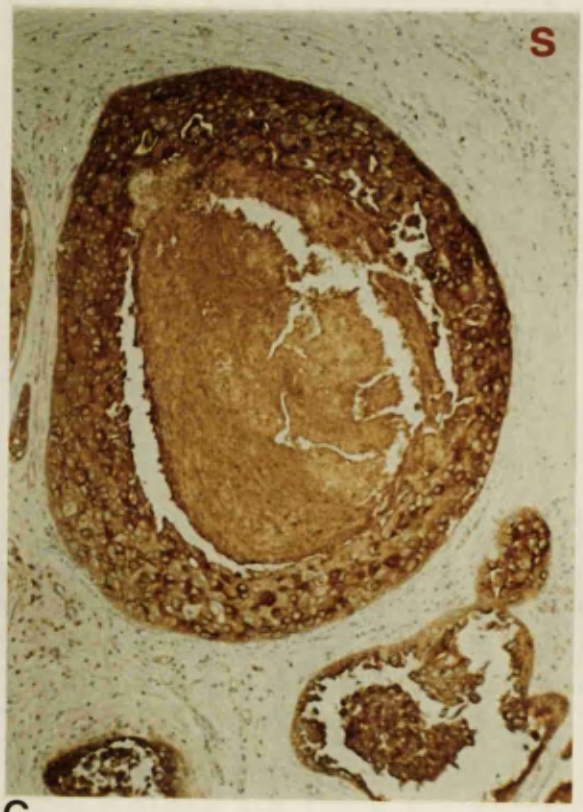
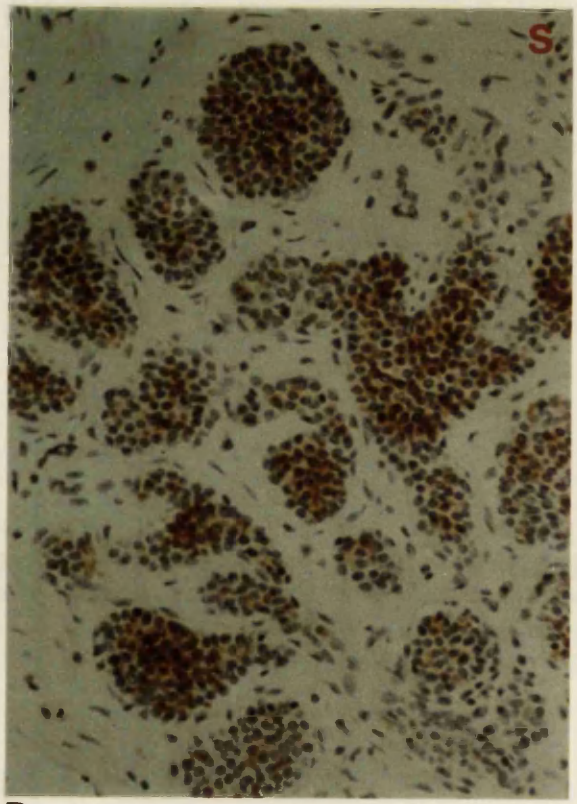
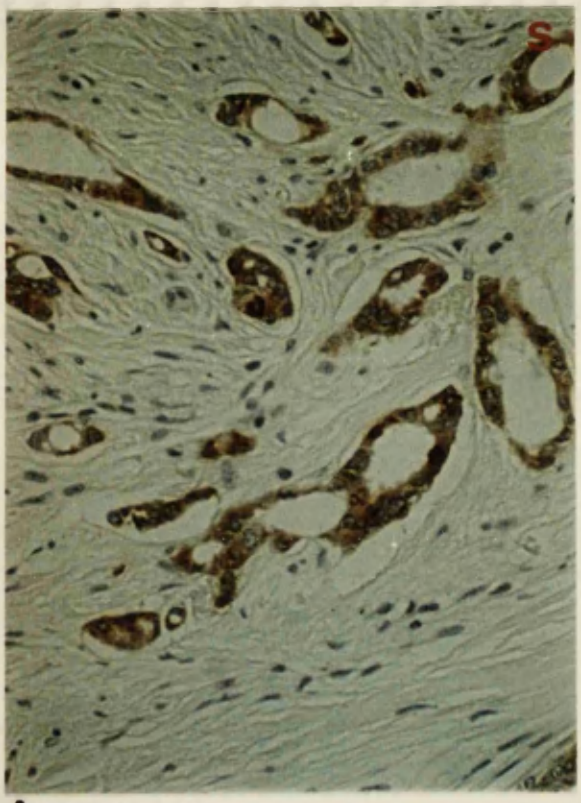


C



D

Figure 3.6. SM3 staining in carcinomas of different histological types. SM3 (S) staining is strong in this example of tubular carcinoma (A), but heterogeneous and weak in both a medullary (B), and a mucoid carcinoma (C). Note that the "mucin pools" in the latter do not stain. Staining in Paget's disease of the nipple is homogeneous and strong (D).
(Immunoperoxidase).



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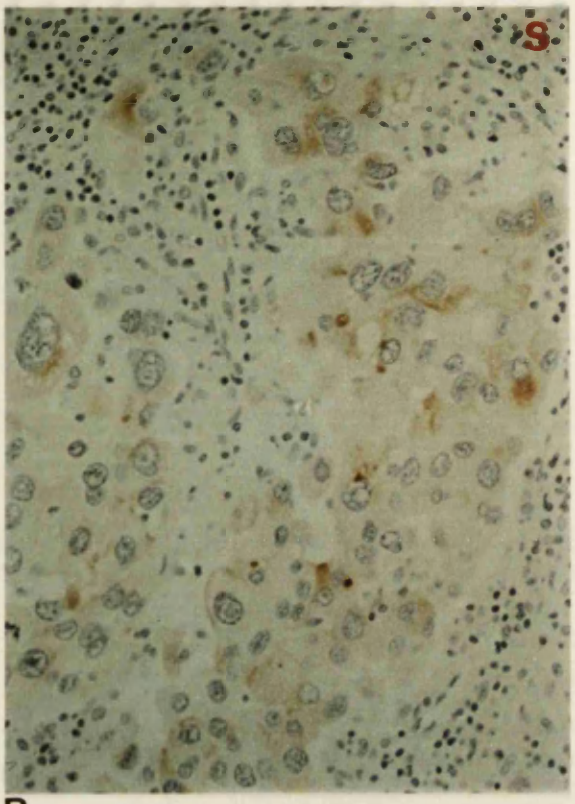

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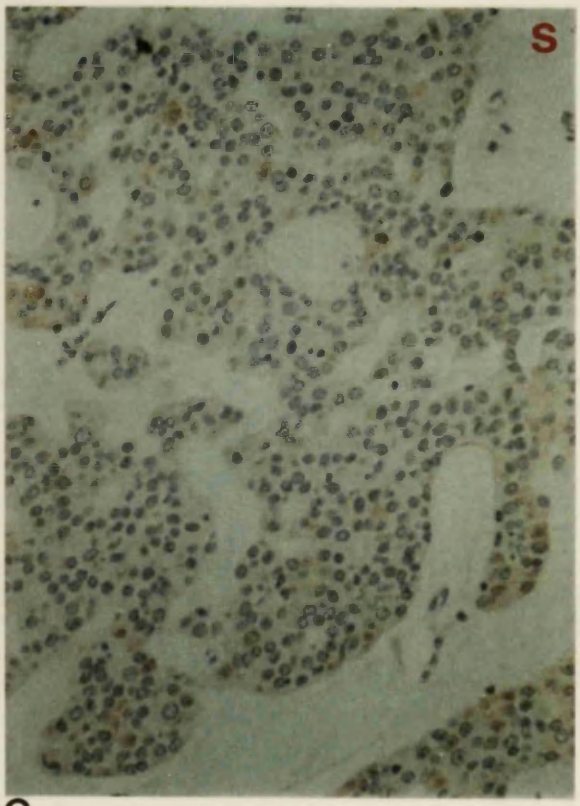

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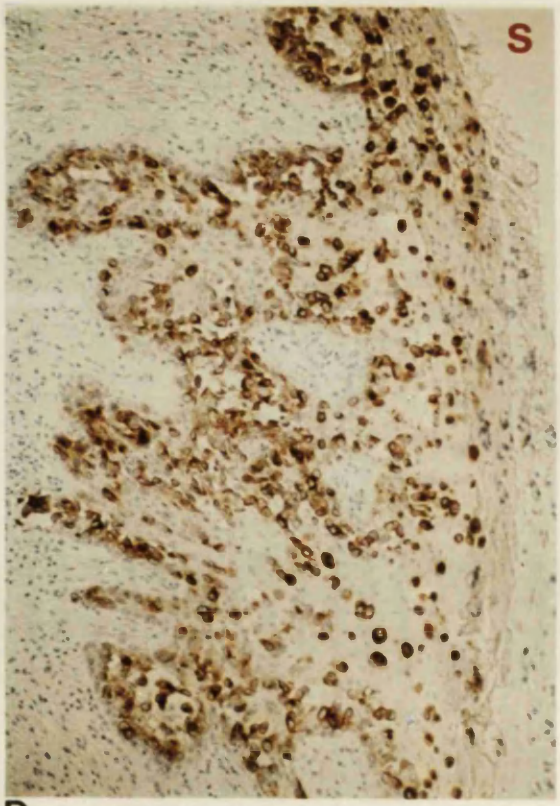


D

(ii) Tumour grade

SM3 staining was compared with histological grade (using the Bloom and Richardson system of grading). All grades of infiltrating ductal carcinoma showed SM3 positivity (Figure 3.2). There was no correlation between presence or absence of staining and tumour grade (see Tables 3.2 and 3.3).

(iii) Other features

Other features of the tumours were reviewed to see if there was any relationship between staining and either gross pathological size, oestrogen receptor (ER) status or pathological nodal status. As shown in Table 3.3, when looking for any correlation, some attempt was made to take into account both heterogeneity and intensity of staining. No significant correlation was found with any feature using the Chi-squared test. See Table 3.3 for p values for each feature.

Table 3.2

IMMUNOHISTOCHEMICAL STAINING OF PRIMARY MAMMARY CARCINOMAS WITH SM3 AND HMFG2 ACCORDING TO HISTOLOGICAL TYPE AND GRADE

Histological Type	Total No.	No. SM3 +ve	No. HMFG2 +ve
Infiltrating ductal	77	75	77
Grade I	7	7	7
Grade II	41	39	41
Grade III	29	29	29
Infiltrating lobular	29	28	29
In situ ductal	5	5	5
In situ lobular	2	2	2
Special Types of Mammary Carcinoma			
Medullary with lymphoid stroma	4	4	4
Tubular	2	2	2
Mucoid	3	1	3
Adenoid cystic	2	1	2
Spindle cell/metaplastic	1	0	0
Paget's disease of the nipple	2	2	2
Total	**127**	**120**	**126**

Table 3.3

CORRELATION OF SM3 STAINING WITH PATHOLOGICAL FEATURES AND OESTROGEN RECEPTOR (ER) STATUS

		SM3 Staining* ++ (n=29)	+ (n=54)	± (n=34)	- (n=7)	
Grade (Bloom & Richardson)	I	1	4	2	0	
	II	9	17	12	2	
	III	7	12	11	0	$p > 0.84$
Tumour size	≥ 2 cm	12	29	19	3	
	< 2 cm	8	16	8	1	$p > 0.86$
	Not known**	9	9	7	3	
ER status	Positive	18	33	19	3	
	Negative	7	15	10	2	$p > 0.93$
	Not known**	4	6	5	2	
Nodal status	Positive	12	21	16	0	
	Negative	9	15	13	4	$p > 0.16$
	Not known**	8	18	5	3	

* ++ indicates homogeneous positive staining
+ indicates heterogeneous staining but some positive areas stain strongly
± indicates heterogeneous staining but all positive areas stain weakly
- indicates complete absence of staining

** Tumour size was unknown either in cases in which an incision biopsy was performed, where a tumour was impalpable or in the cases of pure in situ carcinoma.
ER status was not measured in some small tumours and in situ carcinomas.
Nodal status was not available in patients whose primary treatment did not include axillary dissection.

II METASTATIC MAMMARY CARCINOMA

In addition to the primary tumours studied, SM3 staining was assessed in a number of metastatic mammary carcinomas and compared with that in the primary tumours.

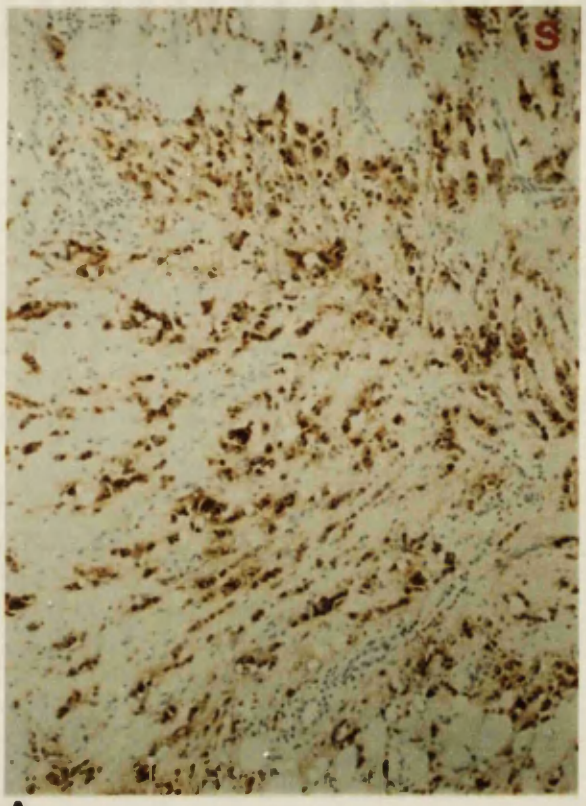
Of the 22 metastases studied (19 in axillary lymph nodes, two in skin and one in soft tissue) SM3 staining was found in all but one. The negative case was a skin metastasis. All 22 primary tumours were SM3 positive. Staining was seen only in epithelial cells, lymphoid tissue being consistently negative. The site of staining within the cell was the same as that described for primary mammary carcinomas. In the majority of the nodal metastases both the distribution and the intensity of staining was similar to that in the primary carcinoma (Figure 3.7) i.e. in cases in which there was heterogeneity of staining in the node, a similar pattern was observed in the primary tumour. There was, however, one case in which the node stained strongly and homogeneously but the primary tumour had stained weakly and heterogeneously; in this case staining in the metastasis was equivalent to that in the strongest area of the primary tumour. HMFG2 staining showed a similar relationship to that with SM3 in lymph node metastases as in primary carcinomas, i.e. was often more homogeneous and of greater intensity.

III SUMMARY OF STAINING IN MAMMARY CARCINOMAS

In both primary and metastatic mammary carcinomas there is a high level of expression of the SM3 epitope. It is detectable immunohistochemically in 94% of primary carcinomas and 95% of metastases. SM3, therefore, like HMFG2, recognises a tumour associated epitope. Differences in staining are, however, seen between HMFG2 and SM3. HMFG2 generally shows stronger and more homogeneous staining and the HMFG2 epitope is expressed by some carcinomas which fail to stain with SM3.

The differences in staining patterns between HMFG2 and SM3 indicate that the antibodies recognise different epitopes. This question will be discussed in some detail later (Section 3.7).

Figure 3.7. SM3 staining in metastatic mammary carcinoma and comparison with staining in the primary tumour. SM3 (S) staining in two metastases, one in the chest wall (A) and one in a lymph node (C) is illustrated. Both the distribution and intensity of staining in the primary breast carcinomas (B and D) is similar to that in the corresponding metastasis.
(Immunoperoxidase).



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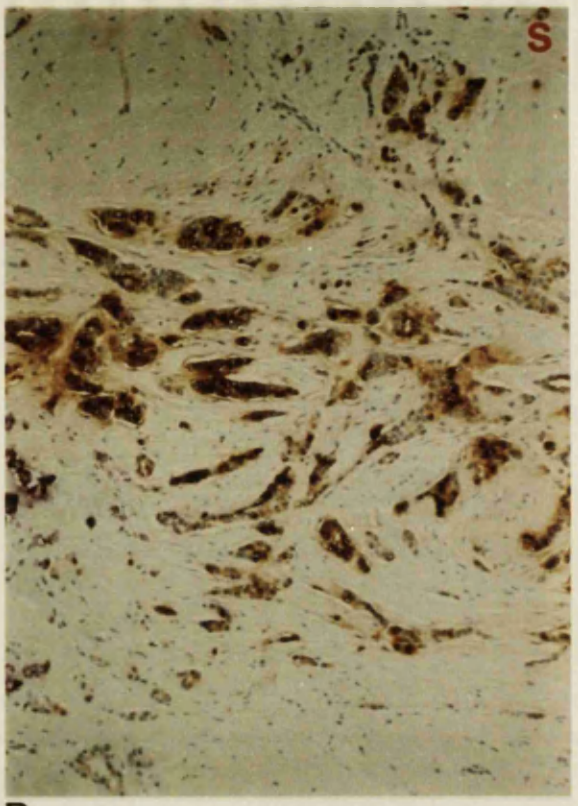

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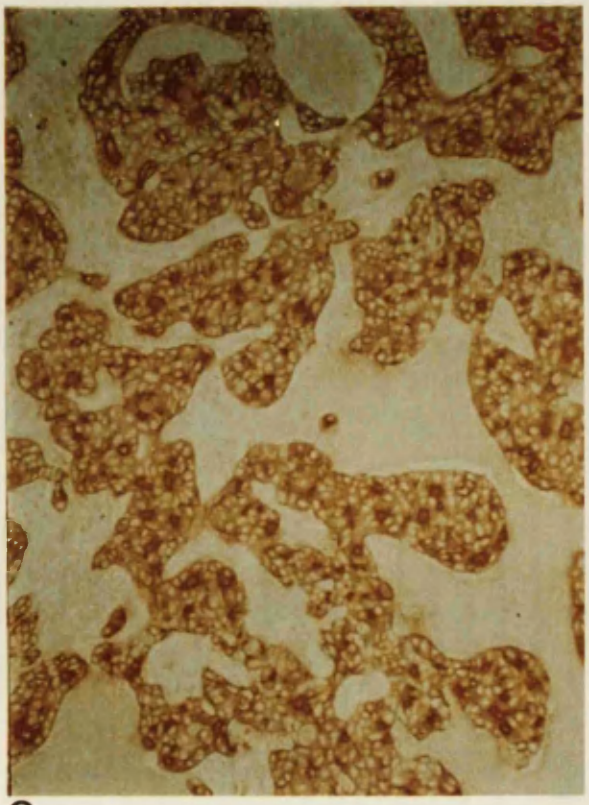

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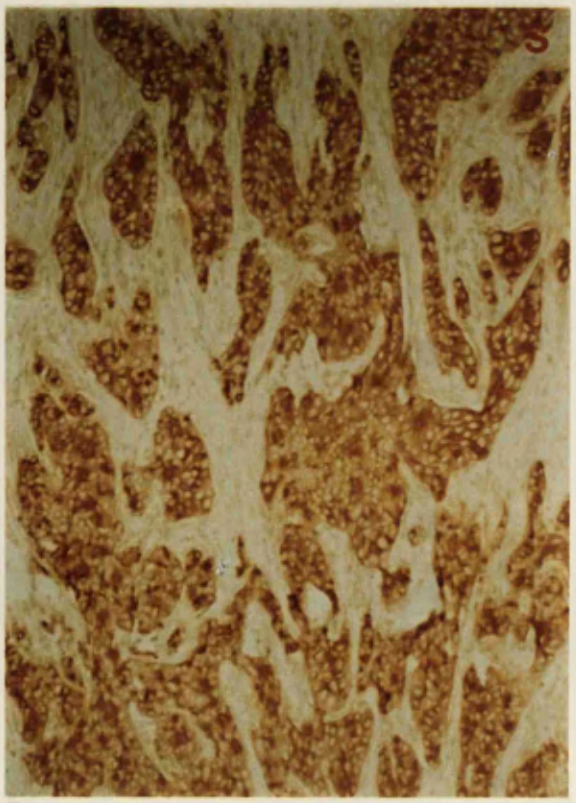


D

3.2 NORMAL BREAST TISSUE

Early immunohistochemical studies with SM3 suggested that the antibody was not only tumour associated but also more tumour specific than its predecessors HMFG1 and HMFG2. A large number of specimens of normal breast tissue were therefore screened for SM3 reactivity. These included both resting breast tissue and tissue from pregnant and lactating women, hereafter referred to as "secretory" breast tissue. A number of specimens of mammary tissue at different stages of development were also available for study. These included fetal tissue and tissue from children and adolescents.

I NORMAL RESTING BREAST

Tissue was selected from biopsy material sent for diagnostic purposes to the COU Laboratory. In some cases the normal tissue was that adjacent to a resected benign lesion such as a fibroadenoma. In others, all the tissue biopsied was histologically unremarkable or showed only minor changes such as stromal fibrosis. A representative block containing glandular tissue without excess fat was selected from each case.

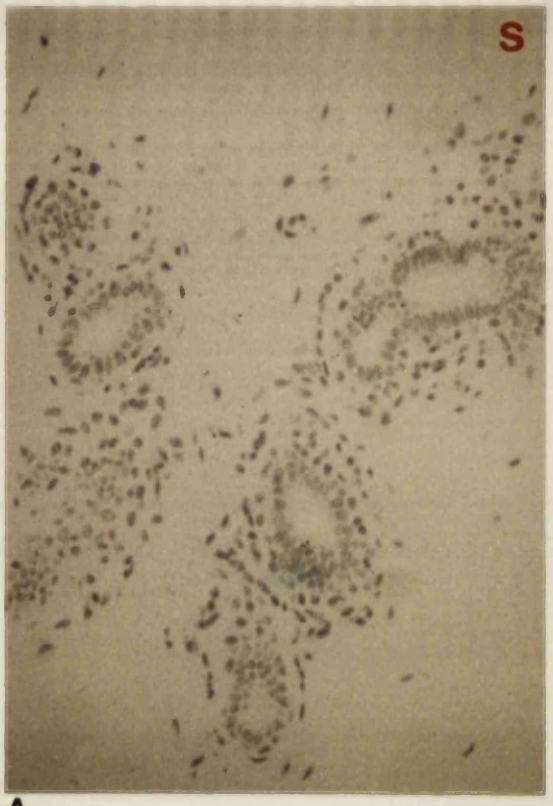
A. Comparison of staining in fixed and frozen tissue

As with the primary mammary carcinomas, staining in frozen tissue was compared with that in methacarn and formalin fixed material. SM3 staining was again similar in methacarn fixed and frozen material but markedly reduced after fixation in formalin.

B. Summary of results

Seventeen samples of normal resting breast tissue were studied. In nine of these there was no staining at all with SM3 (Figures 3.8A and 3.8C). In the eight positive cases staining was very heterogeneous and usually weak. In four cases it was confined to only very small foci, a few acini or a single duct and in the other four to between 10 and 50% of the epithelium. This contrasted with the findings with HMFG2 which stained all cases, often strongly and usually more than 50% of the epithelium (Figures 3.8B, 3.8D and 3.9B).

Figure 3.8. SM3 and HMFG2 staining in normal "resting" breast tissue. HMFG2 (H) staining in mammary ducts of different sizes is shown in Figures B and D. There is no staining with SM3 (S) in the corresponding areas (A and C) in these two cases. (Immunoperoxidase).



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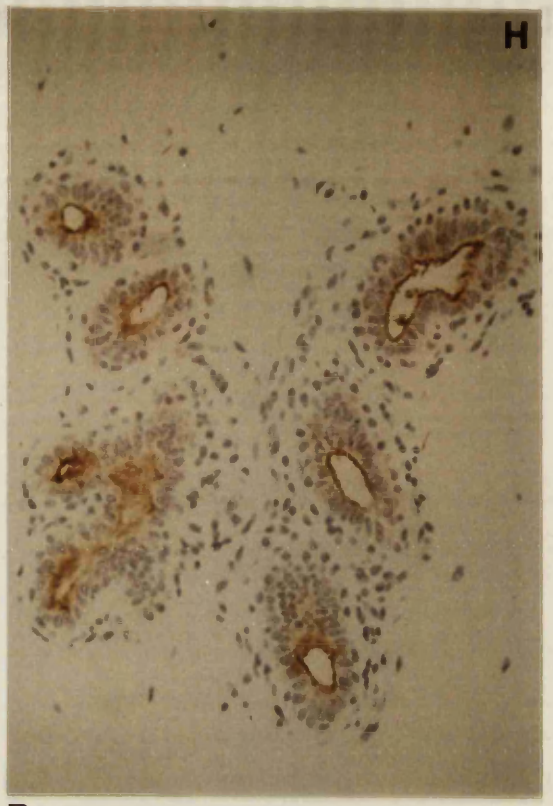

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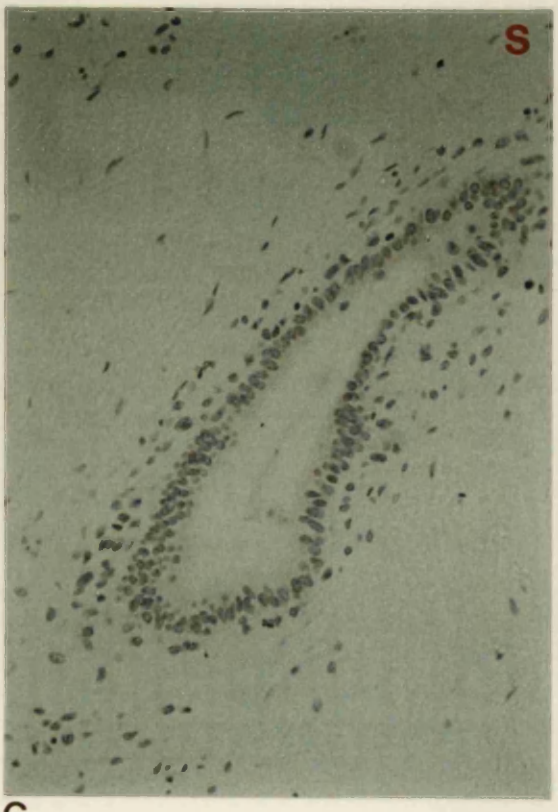

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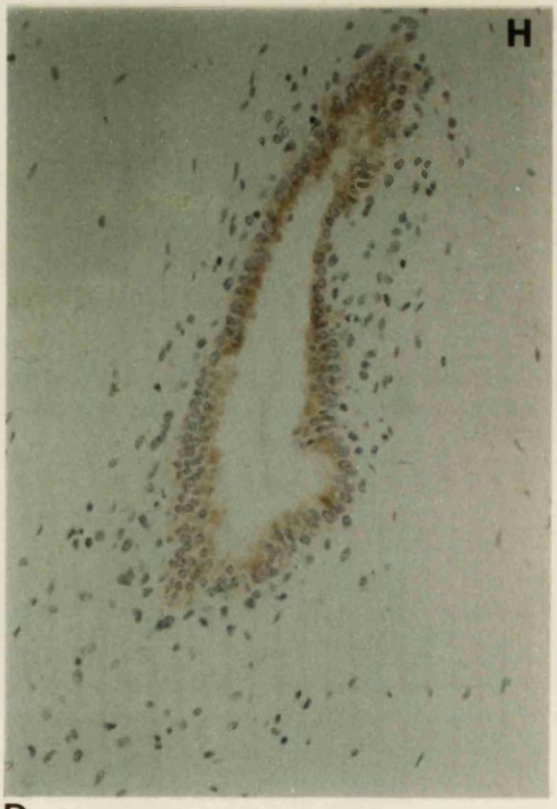


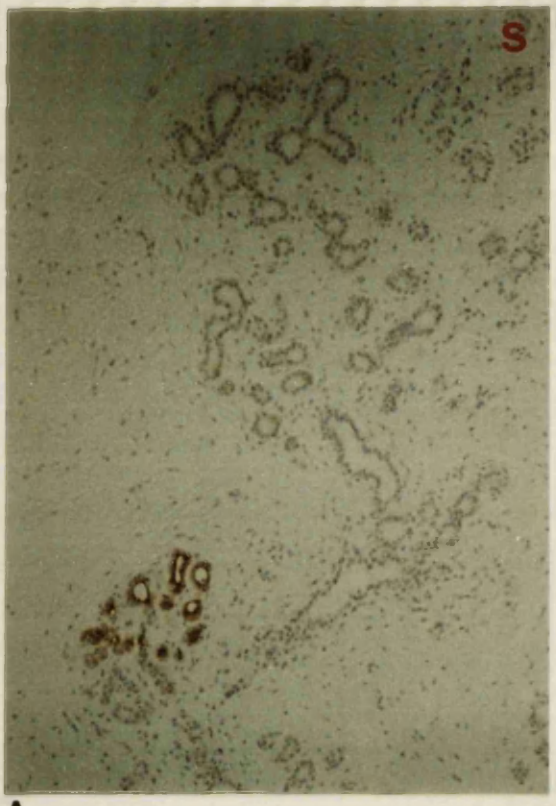
D

C. Details of staining patterns

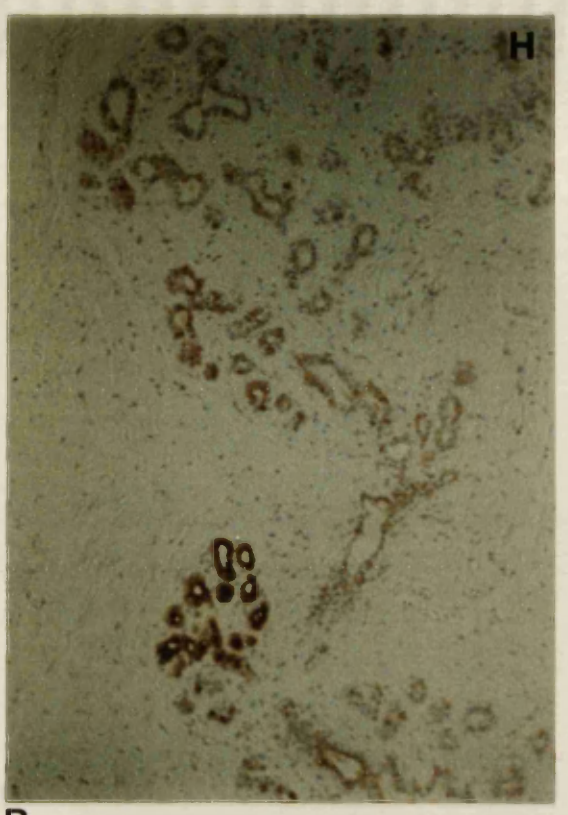
Ducts and lobules were both stained with SM3 as were intraglandular secretions when these were present. In general staining was greater in lobules than in ducts (Figure 3.9A). Positivity was confined to the luminal epithelial cells and was never observed in either myoepithelial cells or stroma (Figures 3.9C ad 3.9D). Staining was seen both in the cytoplasm and at the apical cell membrane i.e. the membrane adjacent to the glandular lumen. Membrane staining was more intense than cytoplasmic staining which was usually weak and confined to the apical region of the cell (Figure 3.9D). Sometimes membrane staining occurred without cytoplasmic staining. As mentioned above, heterogeneity was marked. This was seen from one duct to another in an individual case, although when positive, all the epithelial cells within a cross section of a duct stained. There was also heterogeneity from lobule to lobule as well as between acini within an individual lobule (Figures 3.9C and 3.9D). Again, when an acinus was positive, all the luminal epithelial cells were stained. The intensity of staining was often low.

HMFG2 staining was seen in all cases. Although there was some degree of heterogeneity, this was less than that seen with SM3. Staining was seen in between 10-70% (usually approximately 50%) of the luminal epithelial cells. The site of staining was identical to that with SM3; again membrane staining tended to be more intense than that in the cytoplasm (Figure 3.8B). In the eight SM3 positive cases HMFG2 staining was similar in two but was more homogeneous and of greater intensity in the other six. Of the nine SM3 negative cases, seven stained strongly with HMFG2 (Figure 3.8B) whereas two stained weakly (Figure 3.8D).

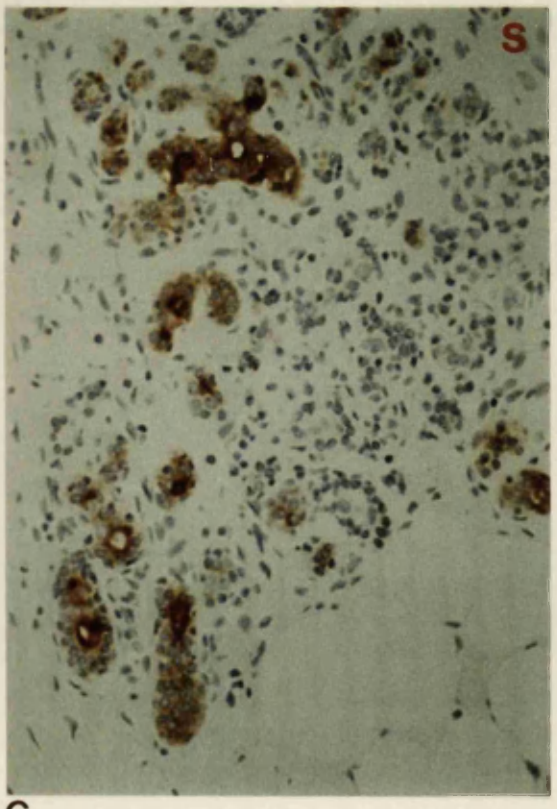
Figure 3.9. SM3 and HMFG2 staining in normal "resting" breast tissue. Heterogeneity of staining with SM3 (S) in normal resting breast tissue is illustrated. In general, staining is greater in lobules than in ducts (A). Positivity is confined to the luminal epithelial cells; the myoepithelium and stroma do not stain (C and D). Staining is strongest at the apical border of cells. HMFG2 (H) staining is both more widely distributed and of greater intensity (B). (Immunoperoxidase).



A



B



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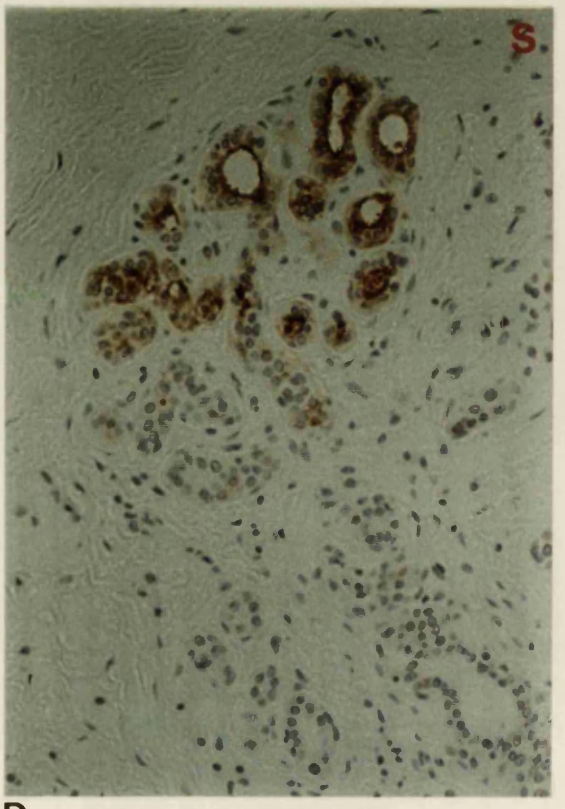


D

II NORMAL "SECRETORY" BREAST

Material from 13 pregnant or lactating women was studied. As such tissue is scarce and in great demand for research purposes in four cases the tissue was taken from a mastectomy specimen performed as part of treatment for primary mammary carcinoma. In these cases tissue was selected from a quadrant other than that containing the primary tumour and H&E examination confirmed that there was no evidence of malignancy in the tissue studied. The other nine specimens were either histologically normal, or consisted of secretory tissue adjacent to a benign lesion.

A. Comparison of staining in fixed and frozen tissue

No significant difference was observed between SM3 staining in frozen and methacarn fixed tissue but a marked loss of reactivity was seen in tissue which had been formalin fixed.

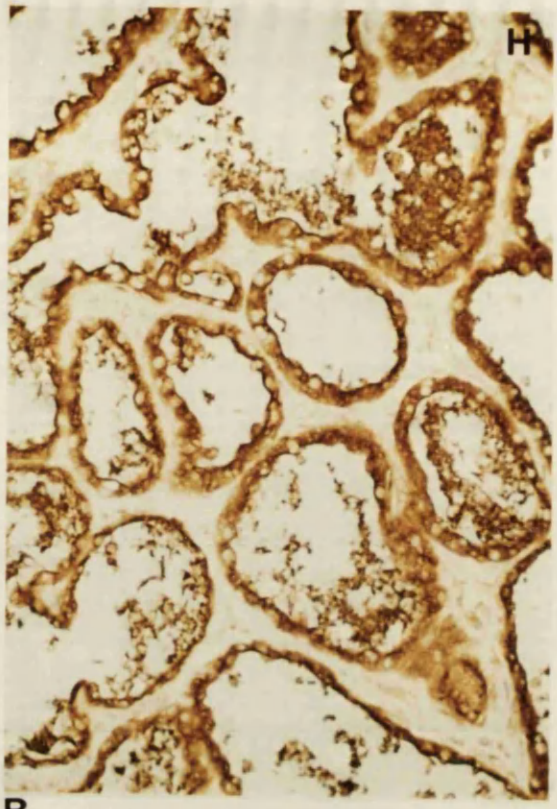
B. Details of staining patterns

In five of the 13 cases studied staining with SM3 was totally absent (Figure 3.10A) and in the other eight was focal (being confined to very small areas of the section) and of very low intensity (Figure 3.10C). No morphological differences could be identified between cells which stained positively and the vast majority which failed to stain. As the staining was so weak, the site of positivity was difficult to assess but it appeared to be within the cytoplasm of the luminal epithelial cells rather than on the cell membrane. Secretions within the acini were unstained.

These findings were in marked contrast to those with HMFG2 which showed strong and usually homogeneous staining in all 13 cases (Figures 3.10B and 3.10D). HMGF2 positivity was seen both in the luminal epithelial cells (cytoplasm and apical cell membrane) and glandular secretions.

Figure 3.10. SM3 and HMFG2 staining in normal "secretory" breast tissue. Staining with SM3 (S) is either totally absent (A) or barely detectable (C) whereas HMFG2 (H) staining is strong and homogeneous (B and D).
(Immunoperoxidase).

A



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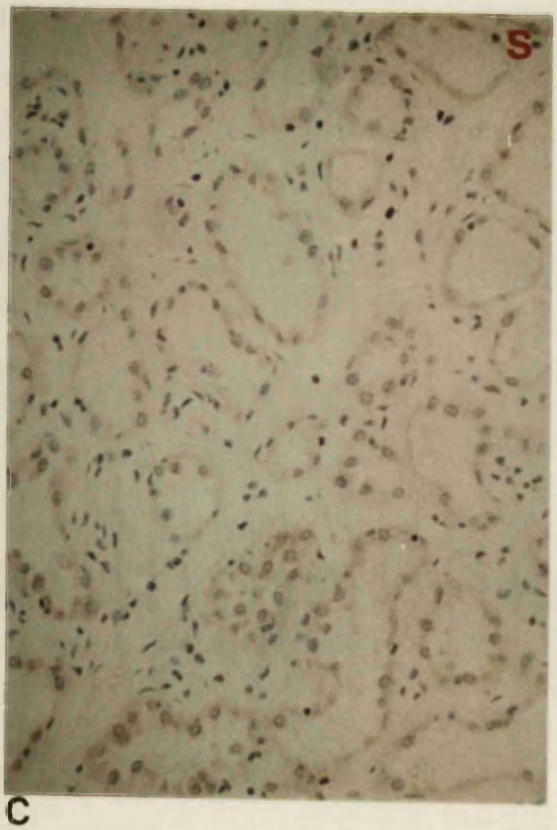

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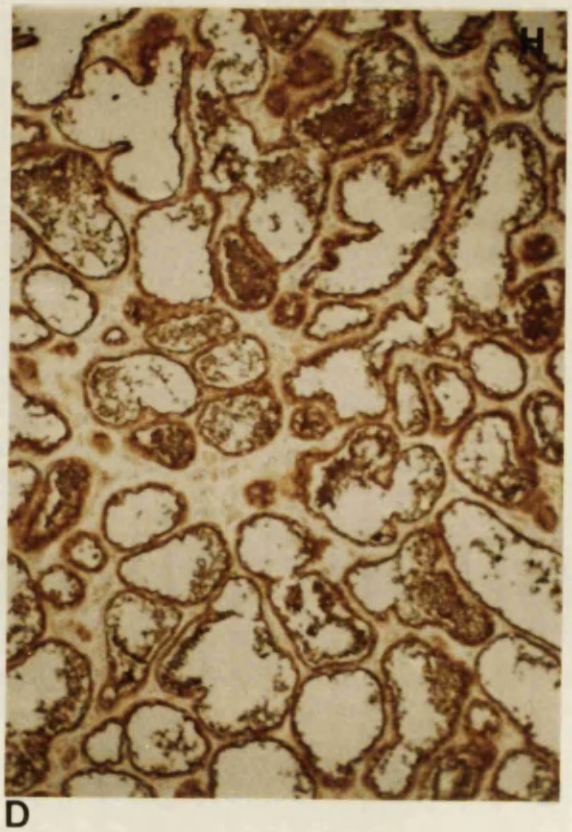


D

III BREAST TISSUE AT DIFFERENT STAGES OF DEVELOPMENT

As described in Chapter 2, tissue obtained at post mortem was fixed in methacarn. Although an attempt was made to study fetal tissues, none of the specimens of this type examined in H&E stained sections contained glandular structures. Fourteen samples of neonatal and prepubertal breat tissue were thus examined immunohistochemically. Clinical details and results of SM3 staining are given in Table 3.4. In all specimens mammary ducts were present. In addition, lobules or lobule-like structures were identified in five cases, four of which were specimens from patients less than one year in age. As mentioned in Chapter 1, the presence of lobules in the prepubertal breast is a somewhat contentious issue but our findings are in agreement with those of Russo and Russo who described the presence of primitive lobular structures in five newborn females (Russo et al 1987).

Details of staining patterns

SM3 positivity was seen in six of the 14 cases examined. The only case in which lobules were present failed to stain. Positivity was seen in the luminal epithelium of ducts and was particularly marked in areas in which the epithelial cells showed an apocrine-like appearance. Staining was both cytoplasmic and membranous. As in fully developed adult breast tissue, heterogeneity of staining was marked. In most cases only one or two ducts stained. The intensity of staining was often weak and always less than that seen with HMFG2.

Positive staining with HMFG2 was seen in all cases. Although there was some degree of heterogeneity, both in ducts and lobules, this was not as marked as that with SM3. In 11 of the 14 cases all the glandular tissue stained; in the remaining three there were some negative areas.

In summary, staining with SM3 and HMFG2 in the developing breast was similar to that in the fully developed breast.

Table 3.4

CLINICAL DETAILS AND IMMUNOHISTOCHEMICAL STAINING WITH SM3 IN SPECIMENS OF BREAST TISSUE AT VARIOUS STAGES OF DEVELOPMENT

Case No.	Sex	Age	SM3 Staining
1	F	25 days	Negative
2	F	"pubertal"	Negative
3	F	12 years	Negative
4	F	6 years	Negative
5	M	3 months	Positive
6	M	12 years	Positive
7	F	15 years	Positive
8	F	6 weeks	Positive
9	F	7 years	Negative
10	M	6 months	Negative
11	F	7 months	Negative
12	NK	NK	Negative
13	M	10 months	Positive
14	F	3 months	Positive

NK = not known

3.3 BENIGN BREAST TISSUE

Fifty two cases were selected from biopsy material to include the spectrum of changes seen in benign breast disease. The histological details are summarised in Table 3.5.

A. Comparison of staining in fixed and frozen tissue

Because of sampling difficulties, comparison was only possible in two cases of cystic disease complex and four fibroadenomas. In keeping with our previous findings, SM3 staining was very similar in frozen and methacarn fixed tissue.

B. Summary of results

The individual categories of benign breast disease will be discussed separately below but several observations were made that applied to all types of benign breast disease. SM3 staining was observed in 42 of the 52 cases studied. Positivity was confined to the luminal epithelial cells and secretions; myoepithelial cells and stroma were negative. The apical cell membrane was often more strongly stained than the cytoplasm. In all types of benign breast disease staining was particularly noted in areas of apocrine metaplasia. Staining with SM3 was always heterogeneous and often weak. HMFG2 staining in contrast was more homogeneous and usually stronger. The cell type and site of staining with HMFG2 was the same as with SM3.

C. Details of staining patterns

(i) Fibroadenomas

In five of the 15 fibroadenomas there was no staining at all with SM3 (Figure 3.11A). In the other ten, focal staining was observed. Usually between 10 and 30% of the epithelium stained positively (Figure 3.11C).

Table 3.5

HISTOLOGY OF BENIGN BREAST BIOPSIES STUDIED

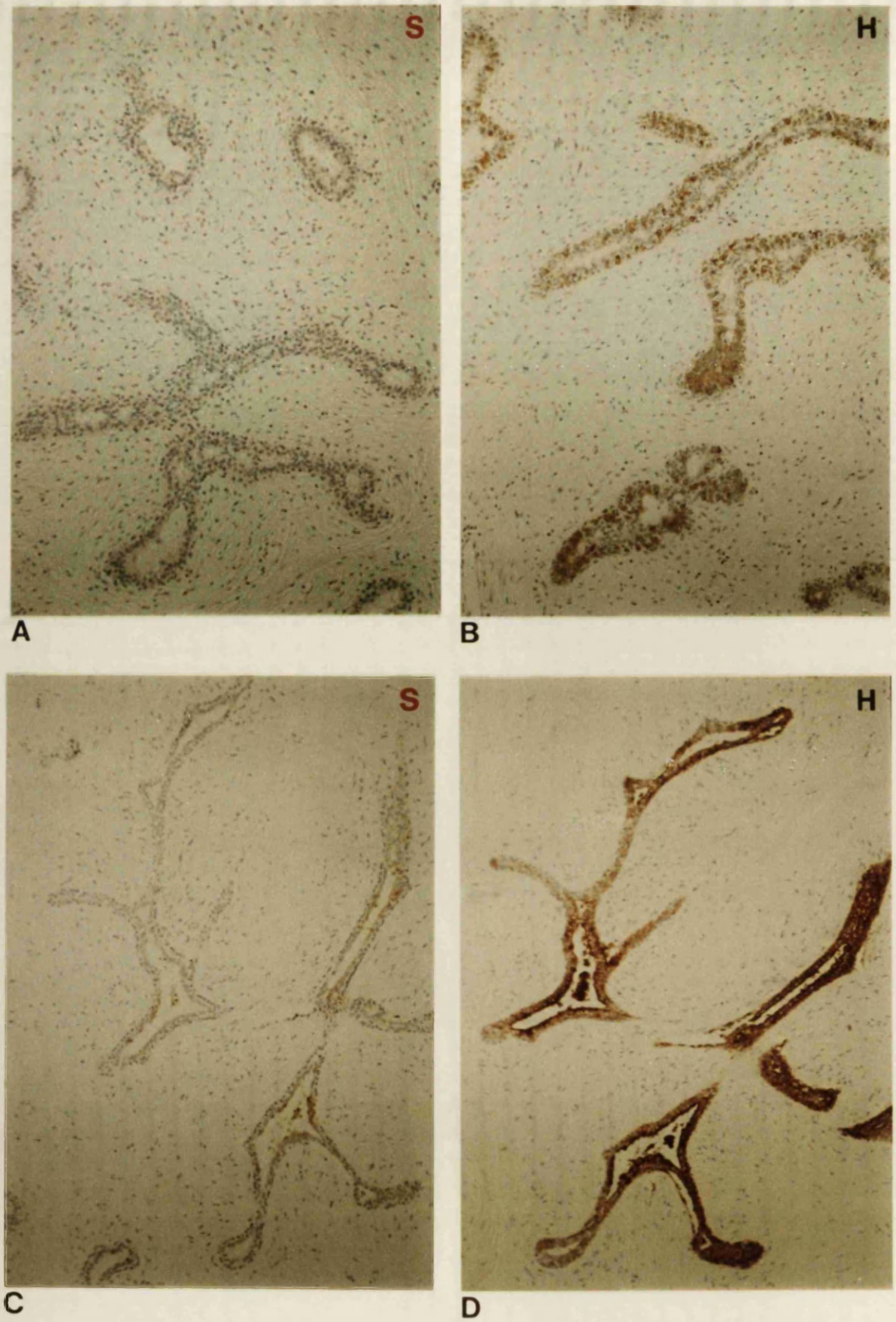
Histology	Number of Cases
Fibroadenoma	15*
Cystic disease complex	31**
Papilloma	6***
	52

* Associated features included apocrine metaplasia in one case and epithelial hyperplasia in another.

** Associated features included apocrine metaplasia in 12 cases, sclerosing adenosis in two and epithelial hyperplasia in nine.

*** Three single intraduct papillomas, one showing marked sclerosis, two multiple papillomas and one ductal adenoma. Associated features included apocrine metaplasia in two cases (one single, one multiple) and epithelial hyperplasia in one case.

Figure 3.11. SM3 and HMFG2 staining in fibroadenomas. SM3 (S) staining is either absent (A) or focal (C) (confined to between 10 and 30% of the epithelium). HMFG2 (H) stained all cases examined (B and D); staining is both more widely distributed and of greater intensity than that with SM3.
(Immunoperoxidase).


S
H
A
B
S
H
C
D

The single case in which there was associated epithelial hyperplasia failed to stain. The intensity of staining varied; in some it was weak and barely detectable but in others was moderate.

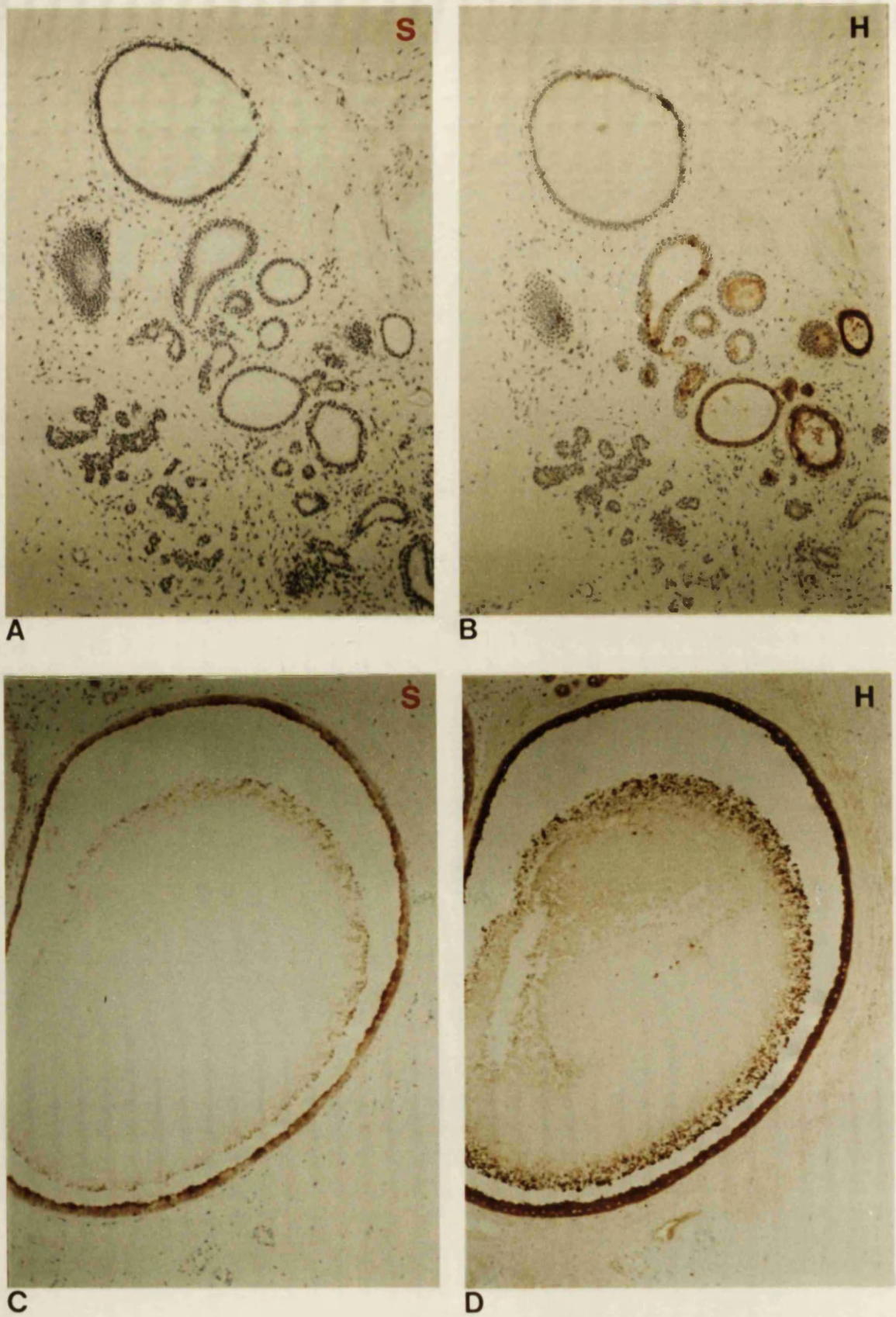
All of the fibroadenomas stained with HMFG2. Usually staining was seen in at least 50% of the epithelium; the intensity was often strong (Figures 3.11B and 3.11D). In only one case was staining focal and weak.

(ii) Cystic disease complex

Interpretation of the cases of cystic disease complex was difficult as areas showing cystic change were often intimately associated with morphologically normal breast tissue (Figure 3.12A). The combination of heterogeneity of morphology and heterogeneity of staining made interpretation difficult. In 26 of 31 specimens of this type, SM3 stained the cysts heterogeneously. The intensity of staining was also variable both from case to case and within an individual case. As mentioned previously, the strongest staining was seen in cysts lined by epithelium showing apocrine change (Figure 3.12C). In two cases there was associated sclerosing adenosis; one of these showed weak heterogeneous positivity and the other failed to stain (Figure 3.13A). In nine cases, moderate to florid epithelial hyperplasia was present. One failed to stain (Figure 3.13C) and the others stained heterogeneously. The intensity was weak in four, moderate in three and strong in one case. In two of the positive cases, staining in the areas of epithelial hyperplasia was less than in adjacent areas of apocrine change (Figure 3.13D).

All cases of cystic disease complex stained with HMFG2 (Figure 3.13B). Staining was also heterogeneous (Figure 3.12B) but less so than with SM3. The intensity of staining with HMFG2 was generally greater than that with SM3 (Figure 3.12D).

Figure 3.12. SM3 and HMFG2 staining in cystic disease complex. Cystic change was often intimately associated with morphologically normal breast tissue (A). SM3 (S) failed to stain the cysts in five cases (A). In the 26 positive cases staining was heterogeneous. Positivity was particularly marked in cysts showing apocrine metaplasia of the lining epithelium (C). All cases were HMFG2 (H) positive (B and D); HMFG2 staining was also heterogeneous but generally of higher intensity (D).
(Immunoperoxidase).


S
H
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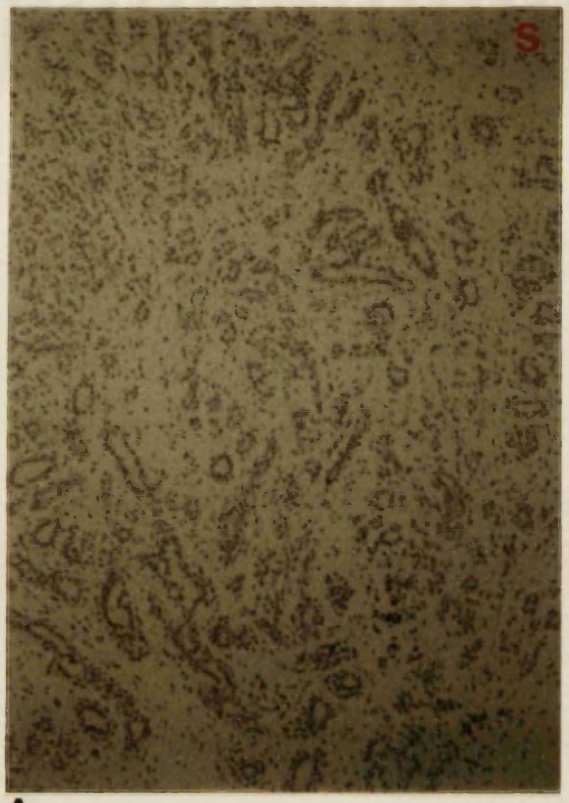
D

Figure 3.13. SM3 and HMFG2 staining in sclerosing adenosis and epithelial hyperplasia. In areas of sclerosing adenosis, SM3 (S) staining was either absent (A) or weak and heterogeneous. HMFG2 (H) staining in the same case (B) was positive.
In cases showing epithelial hyperplasia, SM3 staining was either absent (C) or when present, heterogeneous in distribution and of lower intensity than in adjacent areas of apocrine change (D).
(Immunoperoxidase).



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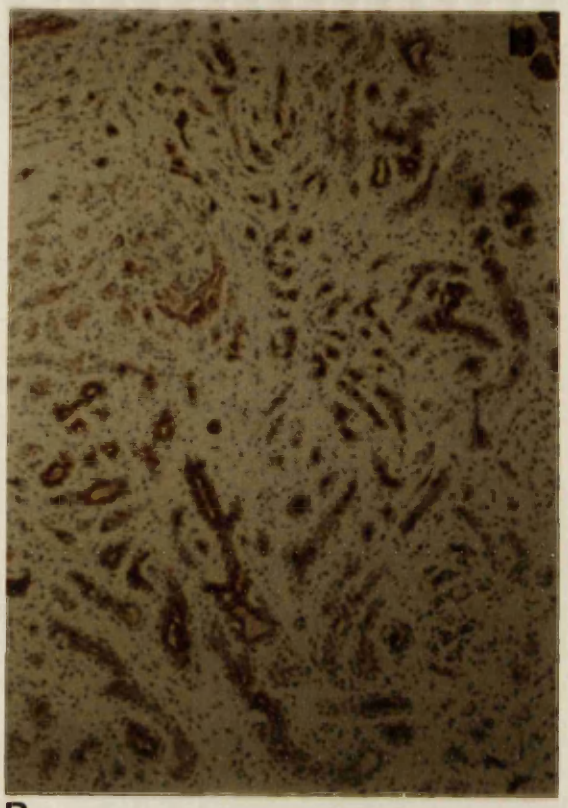

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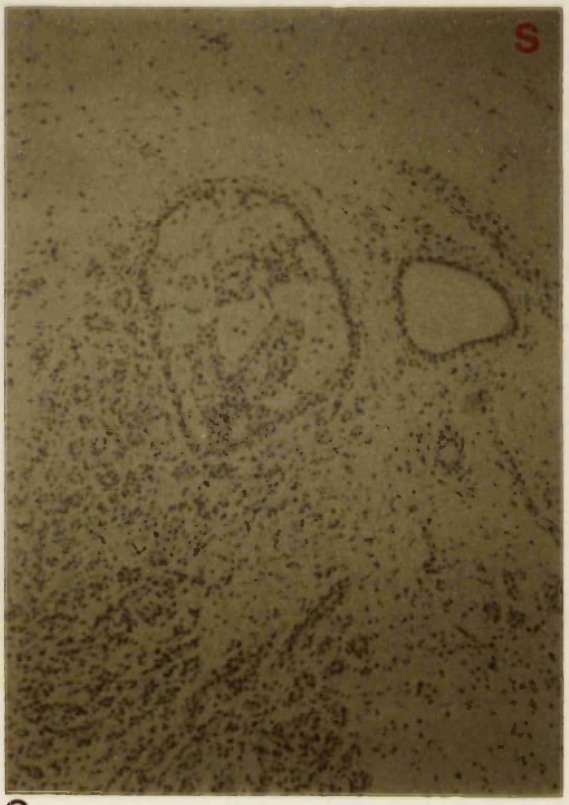

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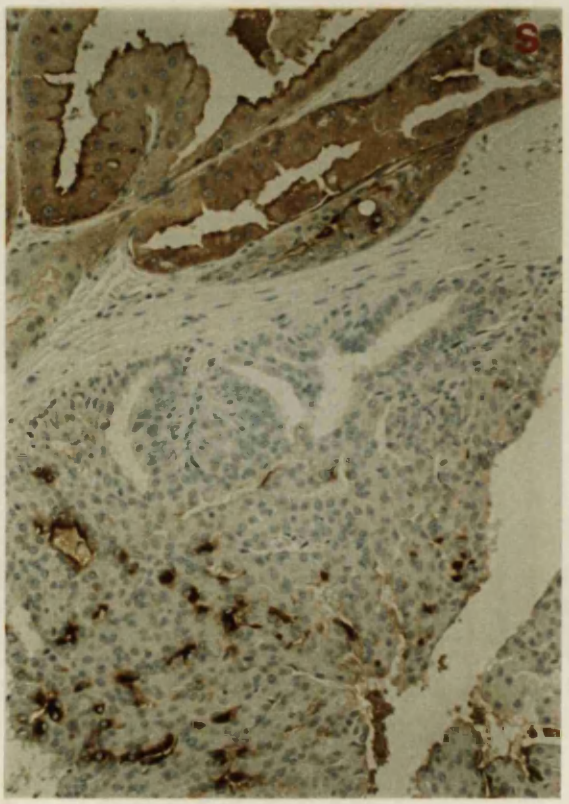


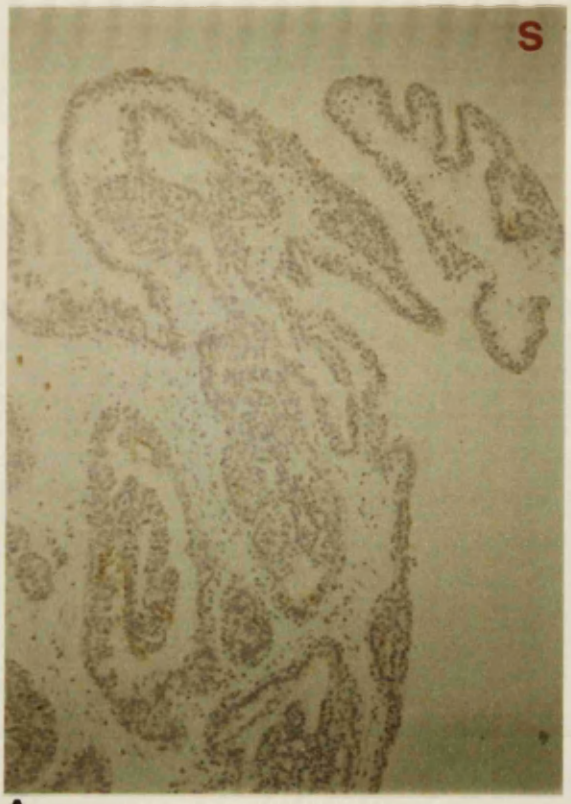
D

(iii) Papillomas

Six cases were studied and all were SM3 positive. The three single intraduct papillomas all stained heterogeneously with SM3 (Figures 3.14A, 3.14C and 3.14D) as did the one ductal adenoma. The two cases of multiple papillomas showed an even more heterogeneous staining pattern with only focal areas of positivity. Areas of epithelial hyperplasia failed to stain (Figure 3.14D). The intensity of staining was generally low; the strongest staining was seen in the ductal adenoma.

HMFG2 staining was also heterogeneous in some cases. In general the intensity of staining was greater than that with SM3 (Figure 3.14B).

Figure 3.14. SM3 and HMFG2 staining in benign papillomas. SM3 (S) staining was seen in all six benign papillomas studied. The distribution of staining was heterogeneous (A, C and D) and areas of epithelial hyperplasia failed to stain (D). HMFG2 (H) staining was also heterogeneous in some cases but the intensity was greater overall (B).
(Immunoperoxidase).



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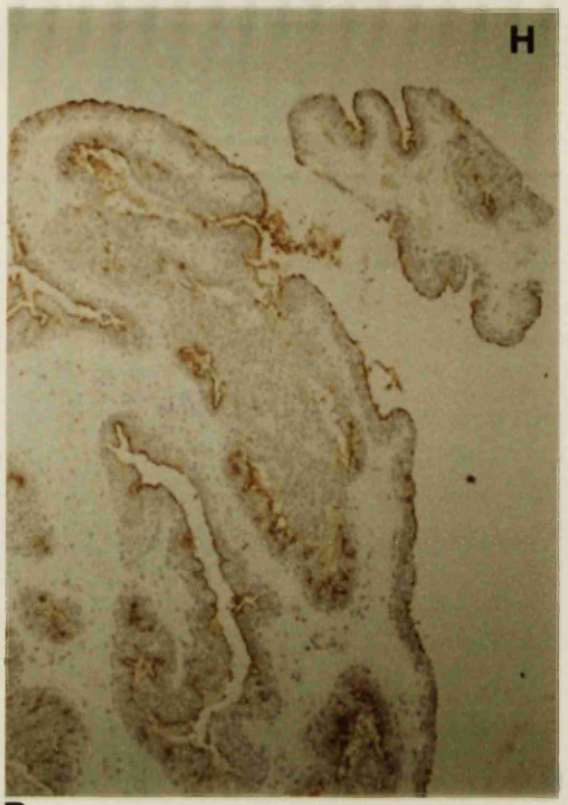

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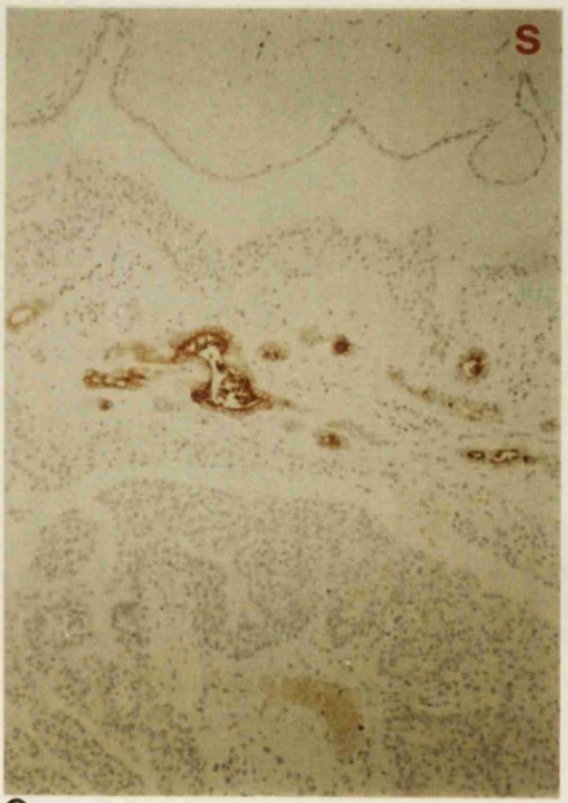

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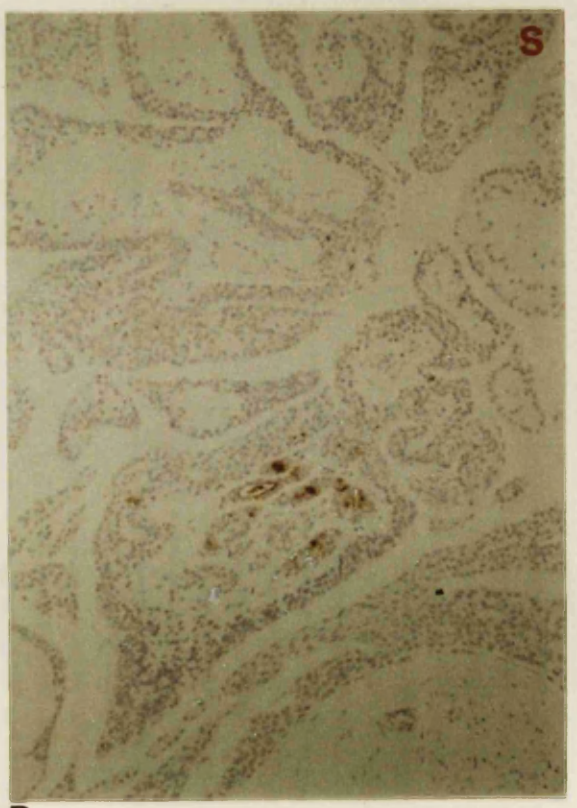


D

3.4 NORMAL TISSUES

A. Comparison of staining in fixed and frozen tissue

In some of the post mortem tissue studied a comparison was made between staining of methacarn fixed and frozen specimens. As in the breast tissue, no difference was observed between the two after taking into account the problems of heterogeneity of staining previously described.

B. Summary of results

The results are summarised in Table 3.6. A more detailed description of staining in SM3 positive tissues is given below. SM3 positivity was seen only within epithelial cells and never in tissues of either mesenchymal or lymphoid origin. Homogeneous positive staining was uncommon being seen mainly in the distal tubules and collecting ducts of the kidney and sebaceous glands of the skin. Elsewhere, staining was usually heterogeneous and often of low intensity.

C. Details of staining patterns: SM3 positive tissues

(i) Lung

Staining was seen in the epithelial cells lining the bronchioles, and in both type 1 and type 2 pneumocytes (Figure 3.15A). In the bronchiolar epithelium there was heterogeneous cytoplasmic staining of moderate intensity of both the respiratory epithelial cells and cells with granular cytoplasm thought to be either endocrine cells or Clara cells. Strong and homogeneous staining was seen in type 2 pneumocytes; that in type 1 cells was heterogeneous and of moderate intensity.

Table 3.6

IMMUNOHISTOCHEMICAL STAINING WITH SM3: NORMAL TISSUES

TISSUE (no. of cases studied)	PATTERN OF STAINING (see footnotes and text for full explanation)		
	Homogeneous Positive	Negative	Heterogeneous Positive or Homogeneous very Weak Positive
Lung (5)			
Bronchiolar epithelium			b
Alveoli - Type 1 cells			b
Type 2 cells	+		
Alveolar macrophages		-	
Pleura		-	
Colon (13)*			c
Ovary (15)		-	
Fallopian tube (2)			b
Skin (8)			
Epidermis		-	
Sebaceous glands	+		
Sweat glands (eccrine)			b
Endometrium (5)			b
Endocervix (4)			b
Ectocervix (4)		-	
Prostate (10)		-	
Testis (2)		-	
Epididymis (1)		-	
Kidney (3)			
Glomeruli		-	
Proximal tubules		-	
Distal tubles	+		
Collecting ducts	+		
Bladder (3)		-	
Thymus (2)		-	
Adrenal (2)		-	
Thyroid (3)		-	
Pancreas (3)			
Acini			b
Ducts		-	
Islets of Langerhans		-	
Liver (2)		-	
Oesophagus (2)		-	
Stomach (3)**			
Surface epithelium	+		
Glands			b
Small intestine (3)		-	
Appendix (2)		-	

cont/.....

Table 3.6 continued

Salivary gland		
Parotid (5) - ducts		a
- acini	-	
Submandibular (3) - ducts		a
- acini	-	
Sublingual (3) - ducts	-	
- acini	-	
Larynx (2)	-	

*tumour associated tissue in two cases
**tumour associated tissue in one case

a = strong foci of staining - very heterogeneous in distribution
b = weak to moderate staining - heterogeneous in distribution
c = uniform weak staining - barely detectable

(ii) Colon

Staining although very weak was a consistent finding (Figure 3.15B). It was uniformly distributed but confined to the cytoplasm of the goblet cells; the mucus within them failed to stain.

(iii) Skin

Sebaceous glands stained strongly and homogeneously (Figure 3.15C). Sweat glands (eccrine) showed a more heterogeneous staining pattern; approximately 50% were positive. The epidermis was negative.

(iv) Endometrium

Endometrial glands showed either a heterogeneous staining pattern (two cases) or no staining (one case). In the positive cases approximately 50% of the glands stained. Staining was weak and cytoplasmic.

(v) Cervix

In the endocervix, staining was similar to that in the endometrium i.e. heterogeneous positivity of the glandular epithelium of weak to moderate intensity. The squamous epithelium of the ectocervix was consistently negative.

(vi) Fallopian tube

The epithelium showed cytoplasmic staining which was heterogeneous and of low intensity.

(vii) Kidney

Positivity was seen in the distal tubules and collecting ducts of the kidney but not in the glomeruli and proximal tubules (Figure 3.15D). Staining was cytoplasmic, homogeneous, and of moderate intensity.

(viii) Pancreas

Heterogeneous staining of the pancreatic acini was seen (Figure 3.16A). Approximately 50% of acini were positive and the intensity was weak to moderate. No staining was seen in either the ducts or the endocrine pancreas.

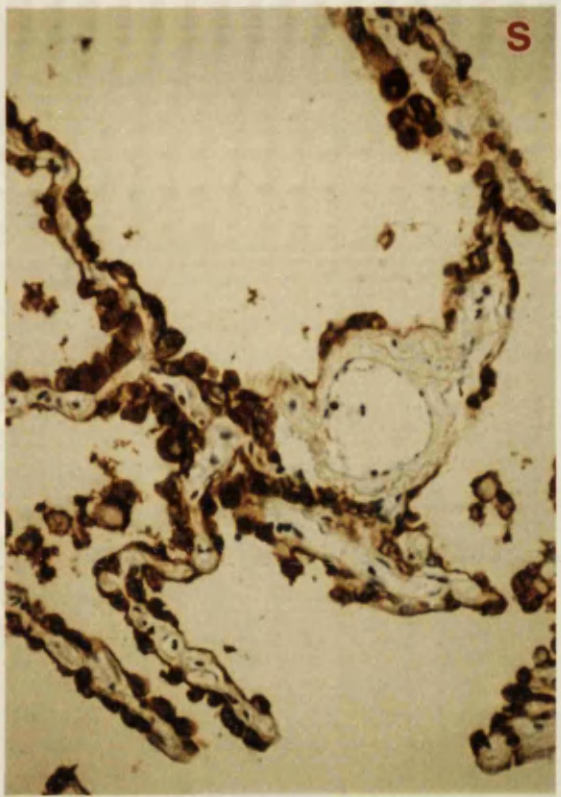
(ix) Stomach

A complex pattern of staining was seen in the gastric mucosa. Tissue from both the body and antrum was examined. In the body, the mucus secreting cells of the surface and foveolar region stained as did mucus cells lining the glands in the neck region. Within the specialised glands the parietal cells stained in the neck region but not at the bases of the glands whereas the chief cells were positive in the bases of the glands but not in the neck region (Figure 3.16B). The intensity of staining was moderate. In the antrum there was again moderate staining of the mucus secreting surface epithelium but little in the underlying glands.

(x) Salivary glands

Tissue from the three major salivary glands was examined. Some differences in staining were noted between the different types of gland. In the parotid, small and medium sized ducts showed heterogeneous moderate to strong staining but the acini were consistently negative (Figure 3.16C). In the submandibular gland, two cases showed heterogeneous positive staining of the small and medium sized ducts; in one of these, occasional acinar cells were also positive. The intensity of staining was moderate to strong. In a third case, however, there was no staining at all. Tissue from the sublingual gland failed to stain with SM3.

Figure 3.15. SM3 staining in normal tissues. SM3 (S) staining in normal lung tissue is strong (A) although heterogeneous. In normal colon (B) there is homogeneous but very weak positivity. In sebaceous glands (C) staining is strong and homogeneous. In the kidney there is moderate to strong staining of the distal tubules and collecting ducts but the glomeruli and proximal tubules are unstained (D).
(Immunoperoxidase).



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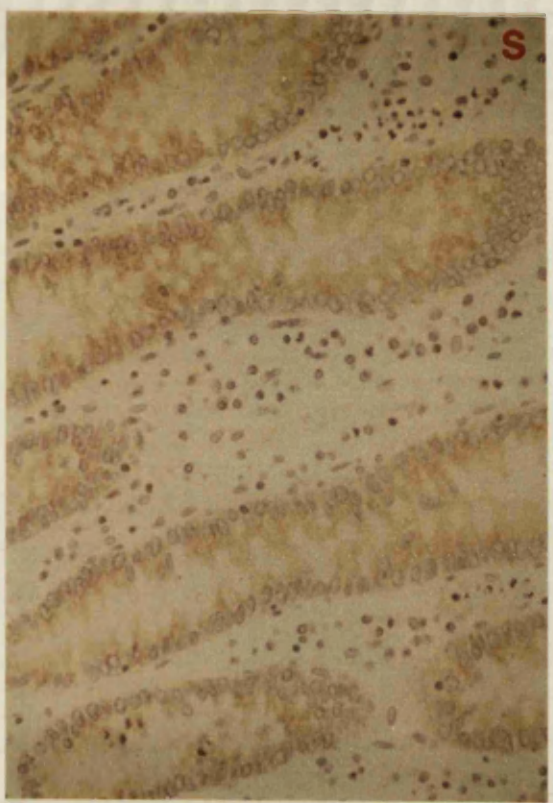

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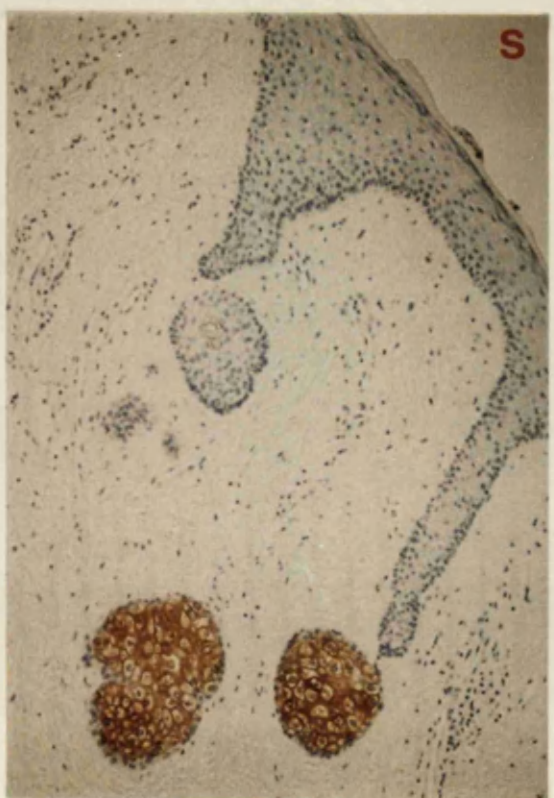

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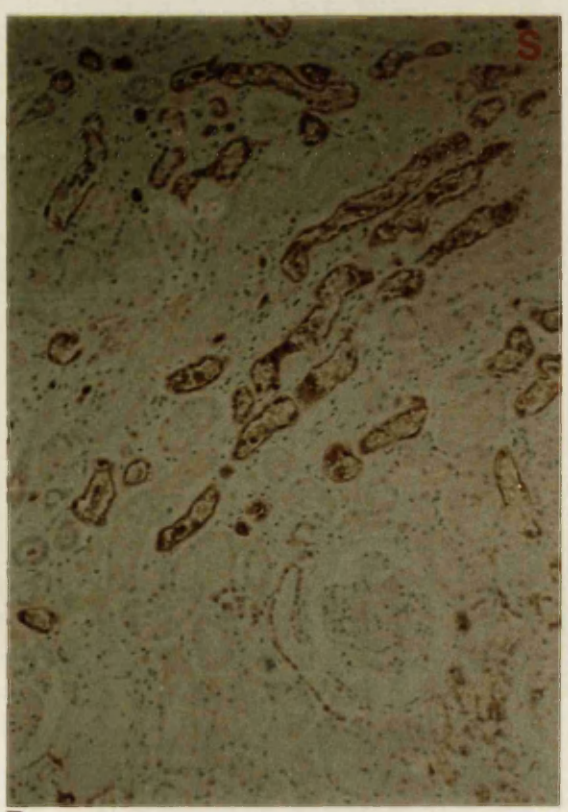
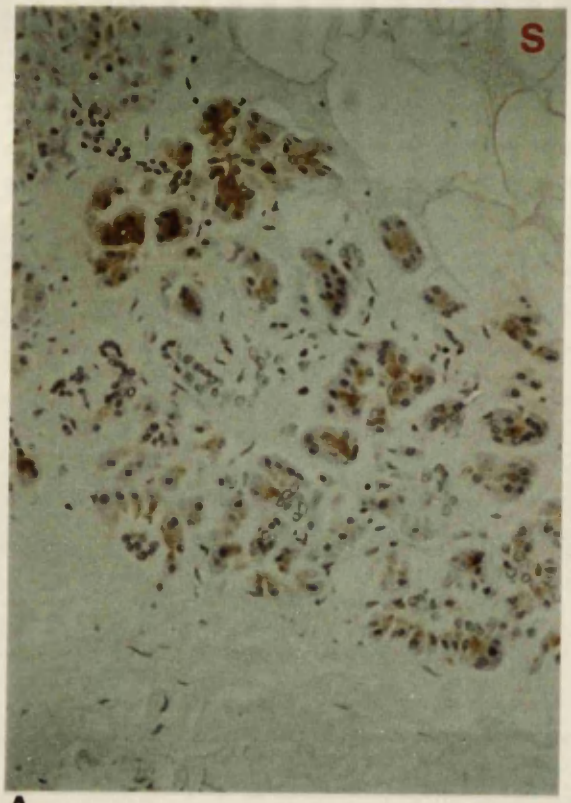


D

Figure 3.16. SM3 staining in normal tissues. Staining with SM3 (S) is heterogeneous and of weak to moderate intensity in pancreatic acini (A). Ducts and the endocrine pancreas are negative. Staining in the body of the stomach is shown in Figure B. The mucus secreting cells of the surface epithelim and those lining the necks of the glands are stained. Staining in the specialised glands is heterogeneous in distribution - see text for details. In the parotid gland staining is strong but heterogeneous and seen only in ducts (C). The surface epithelium of the ovary is SM3 negative (D).
(Immunoperoxidase).



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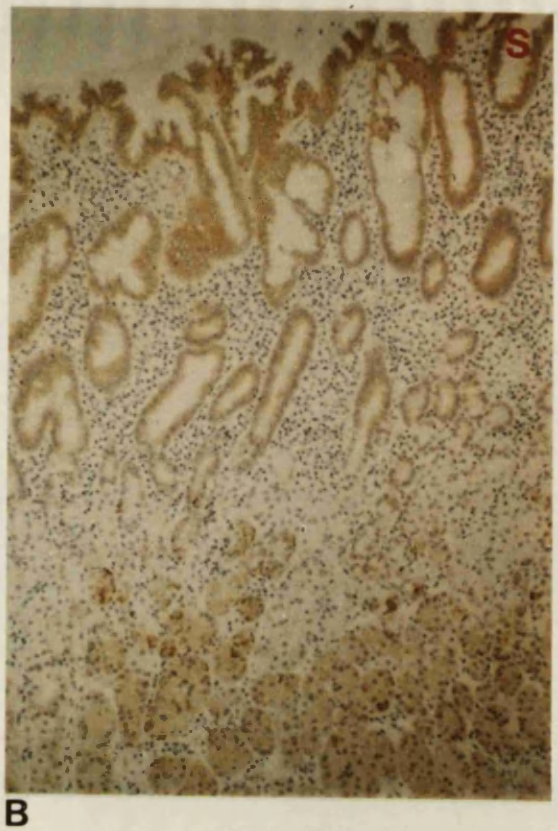

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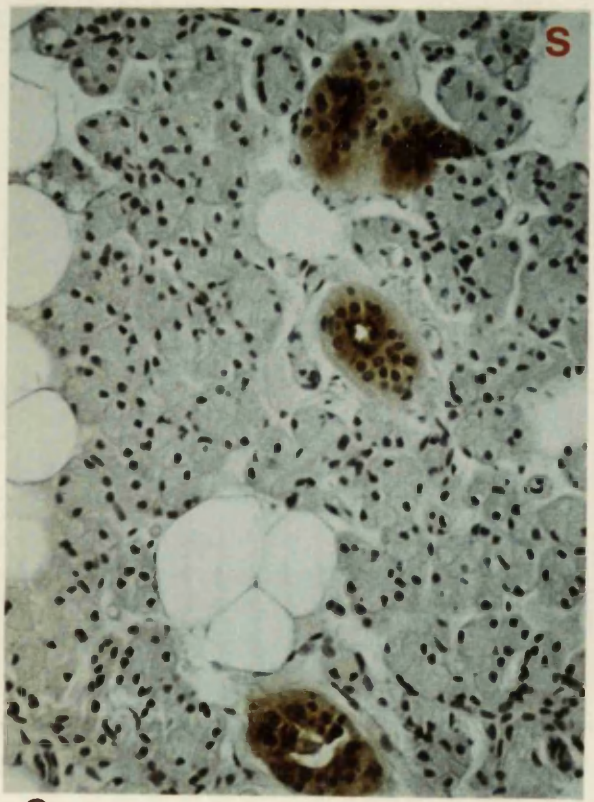

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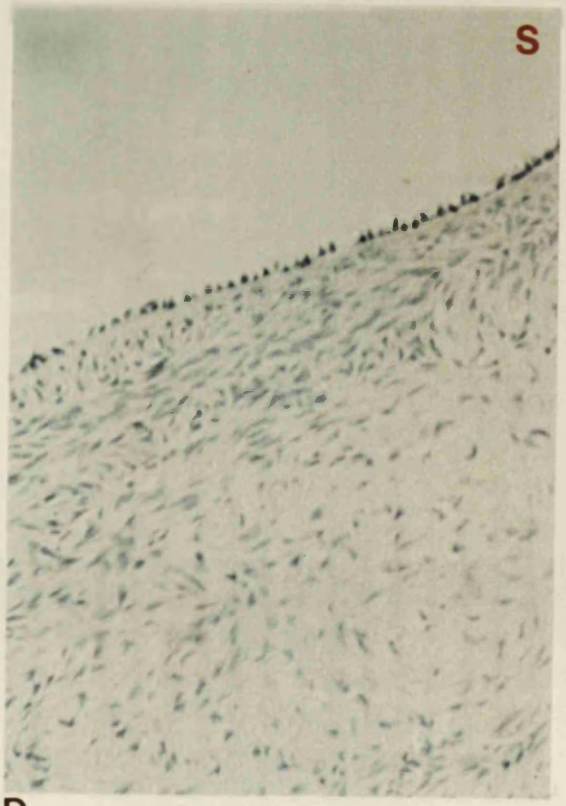


D

D. Comparison of staining with HMFG2 and SM3

In many tissues, the staining patterns with SM3 and HMFG2 were similar. There were, however, some tissues which were SM3 negative and HMFG2 positive, and others in which HMFG2 staining exceeded that with SM3 either in terms of distribution, intensity or both.

(i) Tissues showing SIMILAR staining with HMFG2 and SM3

Tissues **positive with both** antibodies - sebaceous glands of skin, lung (type 2 pneumocytes) and colon.

Tissues **negative with both** antibodies - ectocervix, vagina, vulva, placenta, testis, bladder, ureter, kidney (glomeruli and proximal tubules), adrenal, pancreas (ducts and islets of Langerhan's), small intestine and appendix.

(ii) Tissues NEGATIVE with SM3 but POSITIVE with HMFG2

Lung (alveolar macrophages), larynx, oesophagus, liver (bile duct epithelium), thymus (Hassall's corpuscles), ovary (surface epithelium) (Figure 3.16D), sublingual gland, prostate, epididymis (weak), thyroid (epithelium-weak) and skin (epidermis).

(iii) Tissues in which staining with HMFG2 exceeded that with SM3 in intensity, distribution or both

Lung (bronchiolar epithelium and type 1 pneumocytes), Fallopian tube, skin (sweat glands), endometrium, endocervix, kidney (distal tubules and collecting ducts), pancreas (acini), stomach (body and antrum) and salivary glands (parotid and submandibular).

3.5 PRIMARY CARCINOMAS FROM OTHER SITES (COLON, LUNG AND OVARY)

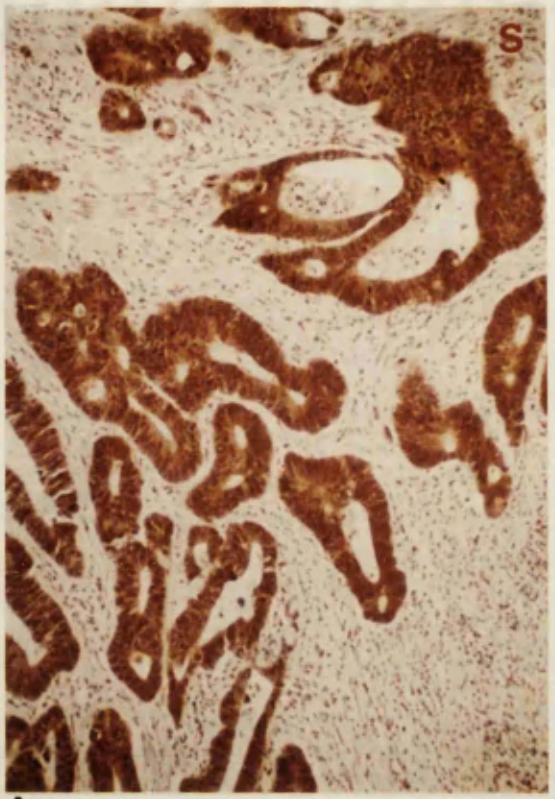
As studies on breast tissue (Sections 3.1 - 3.3) had shown a very high incidence of SM3 positivity in primary carcinomas it was decided to investigate the staining of primary carcinomas from other sites in the body. Tumours of colon, lung and ovary were chosen as they are among the commonest epithelial malignancies in adults and tissue from these sites is therefore readily available. Tissue for this part of the study was obtained from other institutions (for details see Chapter 2) and was supplied fixed in methacarn. Frozen tissue was not available. As described in Chapter 2, H&E examination was performed to confirm that representative tumour was present.

I PRIMARY COLO-RECTAL CARCINOMAS

All of the 17 tumours studied were moderately differentiated adenocarcinomas of either the colon or the rectum and all stained positively with SM3 (Figures 3.17A, 3.17B and 3.17C). Staining was seen in the tumour cells, in both the cytoplasm and on the cell membrane, the latter usually being of greater intensity. Secretions within glandular lumina of tumours also stained (Figures 3.17B and 3.17C). There was both heterogeneity of staining and variation in intensity. In eight cases staining was homogeneous and strong (Figure 3.17A). In four, all tumour cells stained but there was variation in intensity from one field to another. In the remaining five there were only small foci of positive staining which were mostly of low intensity. There were no obvious morphological differences between SM3 positive and SM3 negative areas of a tumour.

All the tumours were also HMFG2 positive. Heterogeneity was generally less marked than with SM3 but was notable in the tumours which had shown only focal SM3 positivity. The intensity of staining was generally greater with HMFG2 than with SM3; the site of staining within cells was similar (Compare Figure 3.17C with 3.17D).

Figure 3.17. SM3 and HMFG2 staining in primary colorectal carcinomas. SM3 (S) staining was homogeneous and strong in eight of 17 cases (A). Staining was cytoplasmic and membranous; secretions also stained (B). Staining with HMFG2 (H) was usually of higher intensity than that with SM3 (compare Figures C and D). (Immunoperoxidase).



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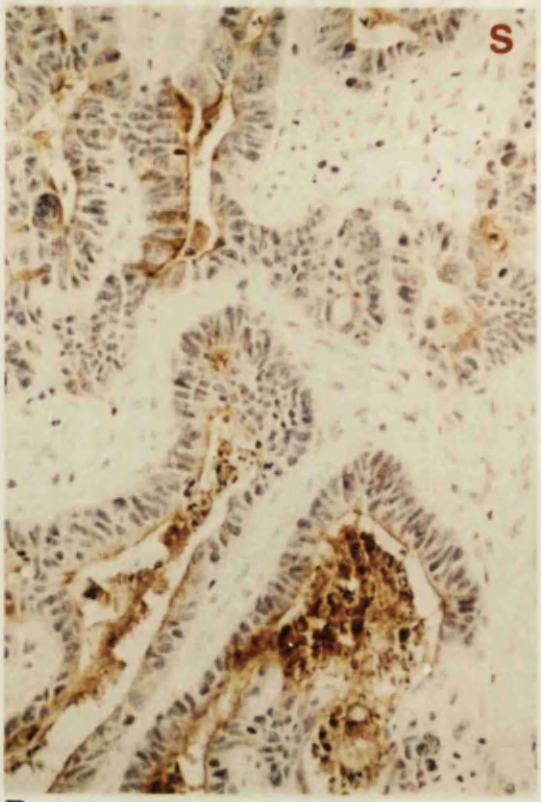

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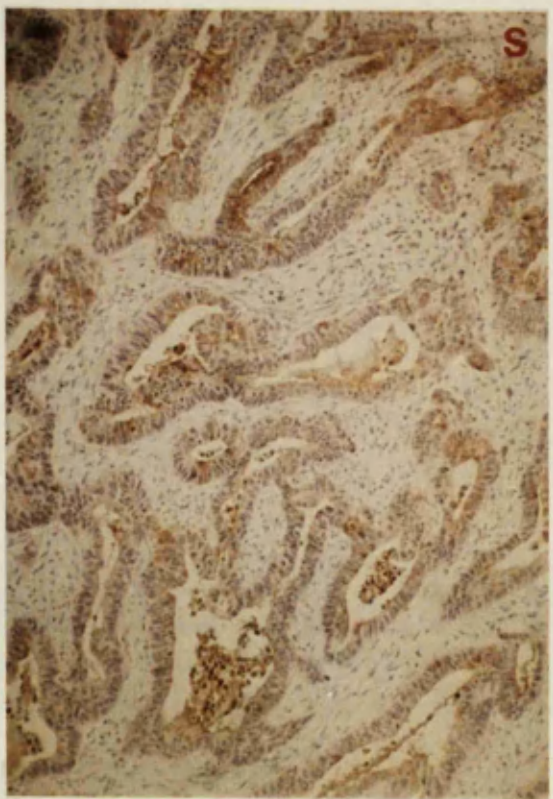

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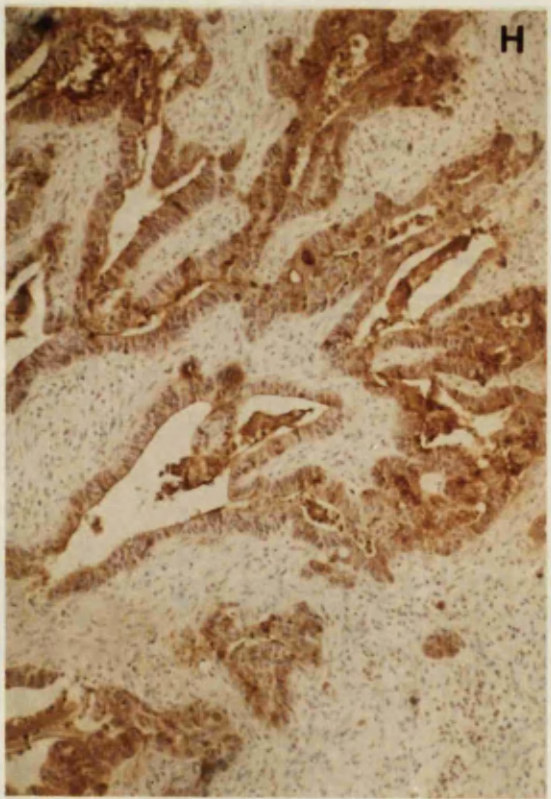


D

II PRIMARY LUNG CARCINOMAS

Fifteen tumours of different histological types were studied. The results are summarised in Table 3.7. In five there was no staining at all and in the ten positive cases the pattern and intensity of staining varied according to histological type.

A. Adenocarcinomas

Five tumours of this type were studied. All were SM3 positive. Staining was seen both in the cytoplasm of the tumour cells and at cell membranes around glandular lumina; the latter was usually stronger. Staining was homogeneous and strong in one case (Figure 3.18A) but in the other four, the intensity of staining was variable; in two cases staining was also heterogeneous.

B. Squamous carcinomas

Five tumours of this type were studied. Three were SM3 positive (Figure 3.18C). Staining was cytoplasmic. The distribution was very heterogeneous and the intensity variable.

C. Neuroendocrine and undifferentiated large cell tumours

Three neuroendocrine tumours were studied. Staining was present in only one tumour in which the cytoplasm of a few cells was weakly positive. Two undifferentiated large cell tumours were also studied. Again weak staining was present in only a few cells of one tumour.

The incidence of HMFG2 positivity (Figure 3.18B) was the same as that for SM3 in adenocarcinomas but higher in squamous, neuroendocrine and large cell tumours (See Table 3.7). In general both the distribution and intensity of staining was greater with HMFG2 (Figure 3.18D) than with SM3 but the site of staining within cells was identical.

Table 3.7

IMMUNOHISTOCHEMICAL STAINING WITH SM3 AND HMFG2: PRIMARY LUNG TUMOURS

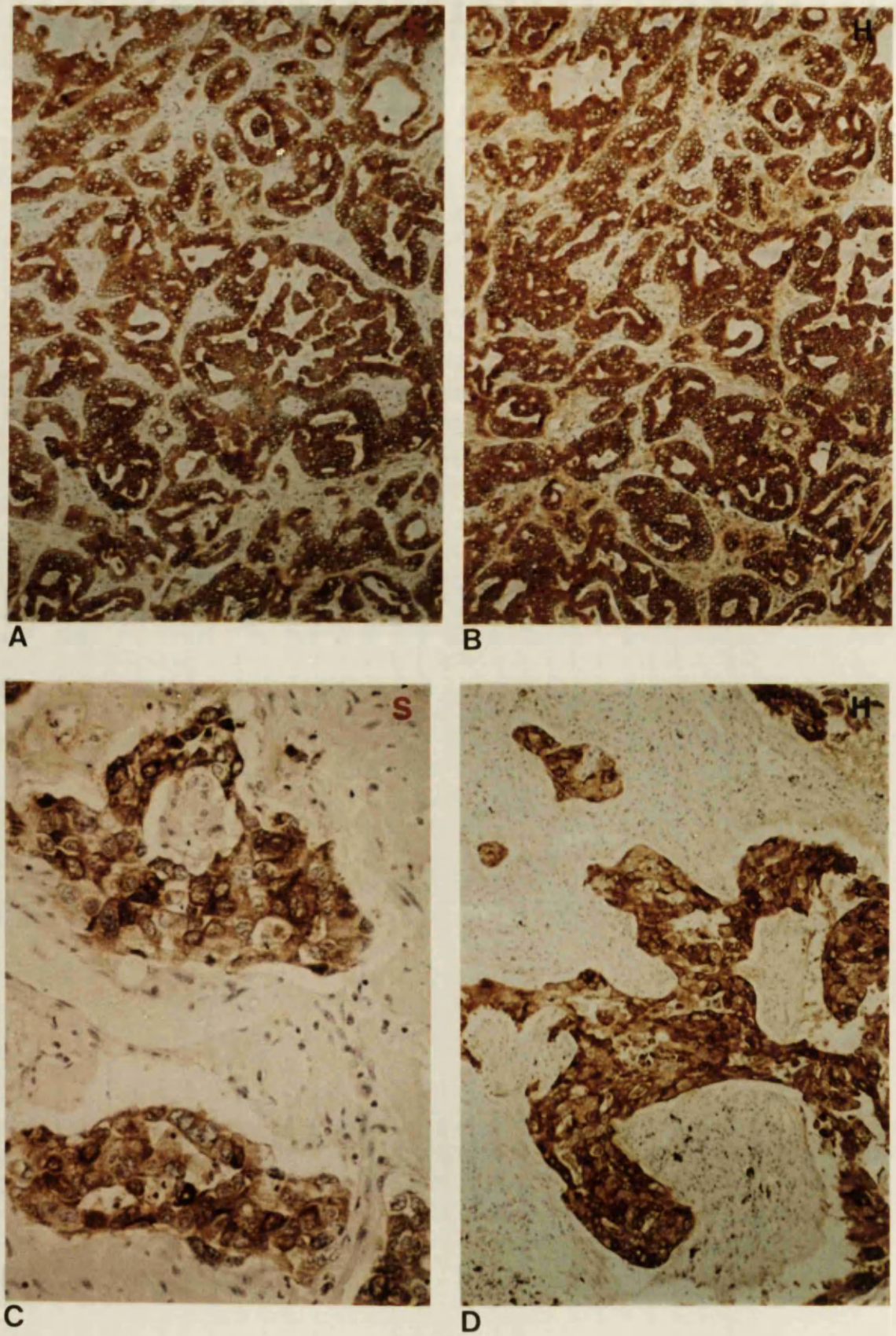
Histological Type	Total No.	No. SM3* positive	No. HMFG2 positive
Adenocarcinomas	5**	5	5
Squamous carcinomas	5***	3	4
Neuroendocrine tumours	3	1	2
Undifferentiated large cell carcinomas	2	1	2
Total	**15**	**10**	**13**

* See text for detailed description of staining pattern and intensity.

** Two well differentiated and three moderately differentiated carcinomas.

*** One well differentiated, two moderately differentiated and two poorly differentiated carcinomas.

Figure 3.18. SM3 and HMFG2 staining in primary lung carcinomas.
Staining with SM3 (S) was of highest intensity in adenocarcinomas (A). In the tumour shown, HMFG2 (H) staining is similar to that with SM3 (compare Figures A and B).
In squamous carcinomas, staining with SM3 (C) was more heterogeneous and HMFG2 positivity exceeded that with SM3 both in distribution and intensity (D).
(Immunoperoxidase).


A
B
H
C
S
D
H

III PRIMARY OVARIAN CARCINOMAS

Five adenocarcinomas of different histological types were studied. All were SM3 positive. Staining was both cytoplasmic and membranous, the latter usually being stronger (Figures 3.19A and 3.19B). The distribution and intensity of staining varied from one case to another.

A. Poorly differentiated tumours

The two tumours of this type showed strong staining; one stained homogenously and one heterogeneously.

B. Mesonephroid tumour

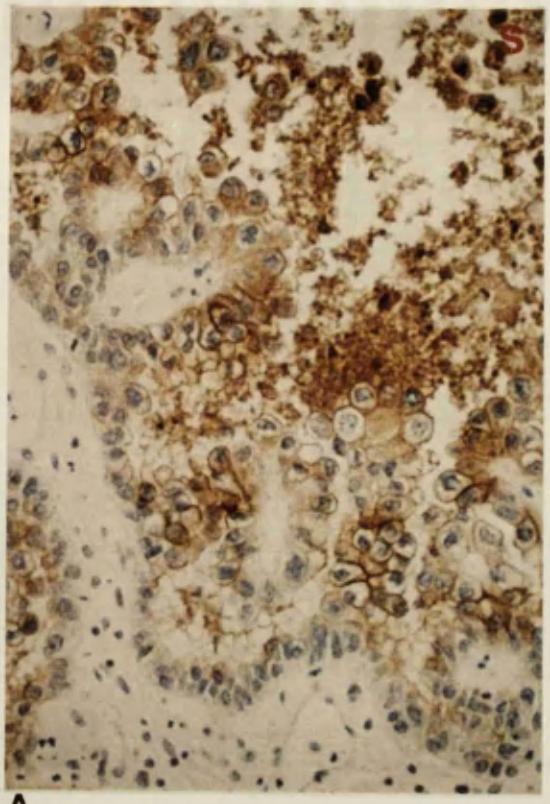
The one tumour of this type also stained moderately strongly and homogeneously (Figure 3.19A).

C. Serous and mucinous tumours

The two tumours (one serous and one mucinous) both stained heterogeneously and with variable intensity (Figure 3.19B and C). There were no apparent morphological differences between the SM3 negative and positive areas.

HMFG2 staining was similar both in distribution and intensity in the poorly differentiated and mesonephroid tumours. In both the serous and mucinous carcinomas, however, HMFG2 staining was more homogeneous and of greater overall intensity than that with SM3 (Figure 3.19D).

Figure 3.19. SM3 and HMFG2 staining in primary ovarian carcinomas. SM3 (S) staining in a mesonephroid carcinoma (A) was moderately strong and homogeneous. A more heterogeneous staining pattern with variable intensity was seen in a papillary serous adenocarcinoma (B and C). Staining with HMFG2 (H) in this case was more homogeneous and of greater intensity than that with SM3 (compare Figures C and D).
(Immunoperoxidase).



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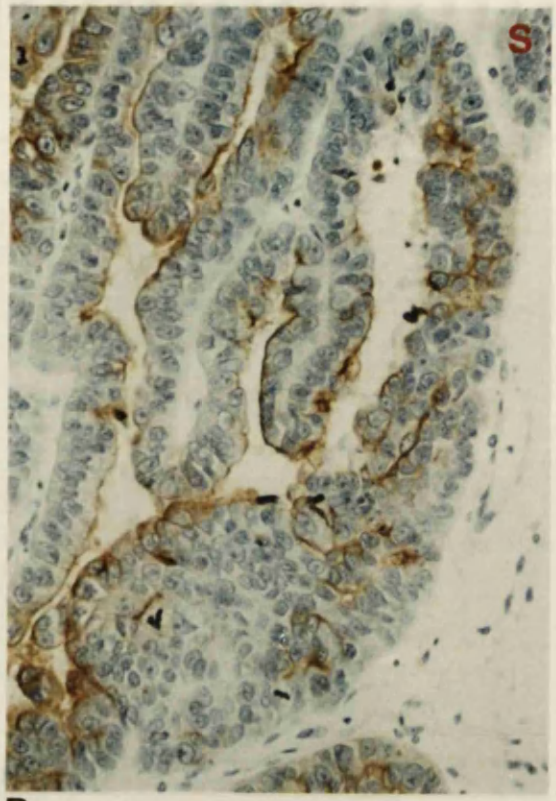

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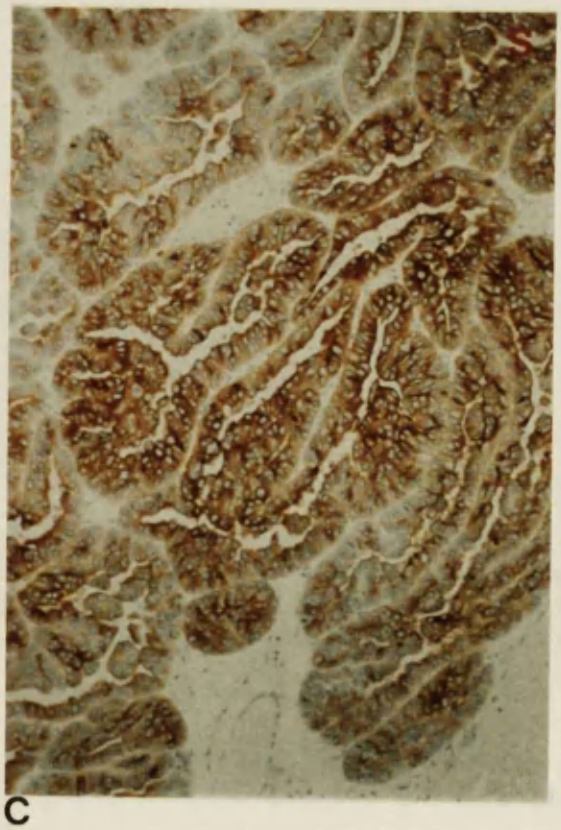

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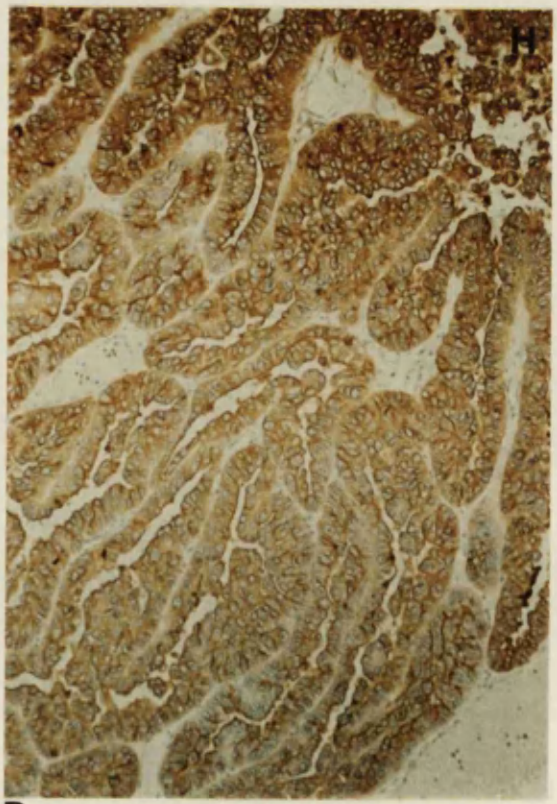


D

3.6 CULTURED CELLS: BREAST CANCER CELL LINES AND NORMAL MAMMARY EPITHELIAL CELLS

The advantages and disadvantages of studying cells in culture have been discussed in Chapter 1. Having studied the expression of the SM3 epitope as detected immunohistochemically in tissue sections, it was of interest to compare the in vivo (breast tissue sections) with the in vitro (mammary cells in culture) situation. The breast cancer cell lines MCF7 and T47D were studied as were normal mammary epithelial cells grown from samples of human milk. The culture of these cells was described in Chapters 1 and 2. It was of particular interest to determine the site of antigen expression within cells. As described in Chapter 2 (Section 2.1 II B) the process of fixation may result in the loss of membrane associated antigens. In order therefore to accurately localise the site of staining, cells were stained both live and after fixation. Both the immunofluorescence and the immunoperoxidase techniques were used.

I BREAST CANCER CELL LINES

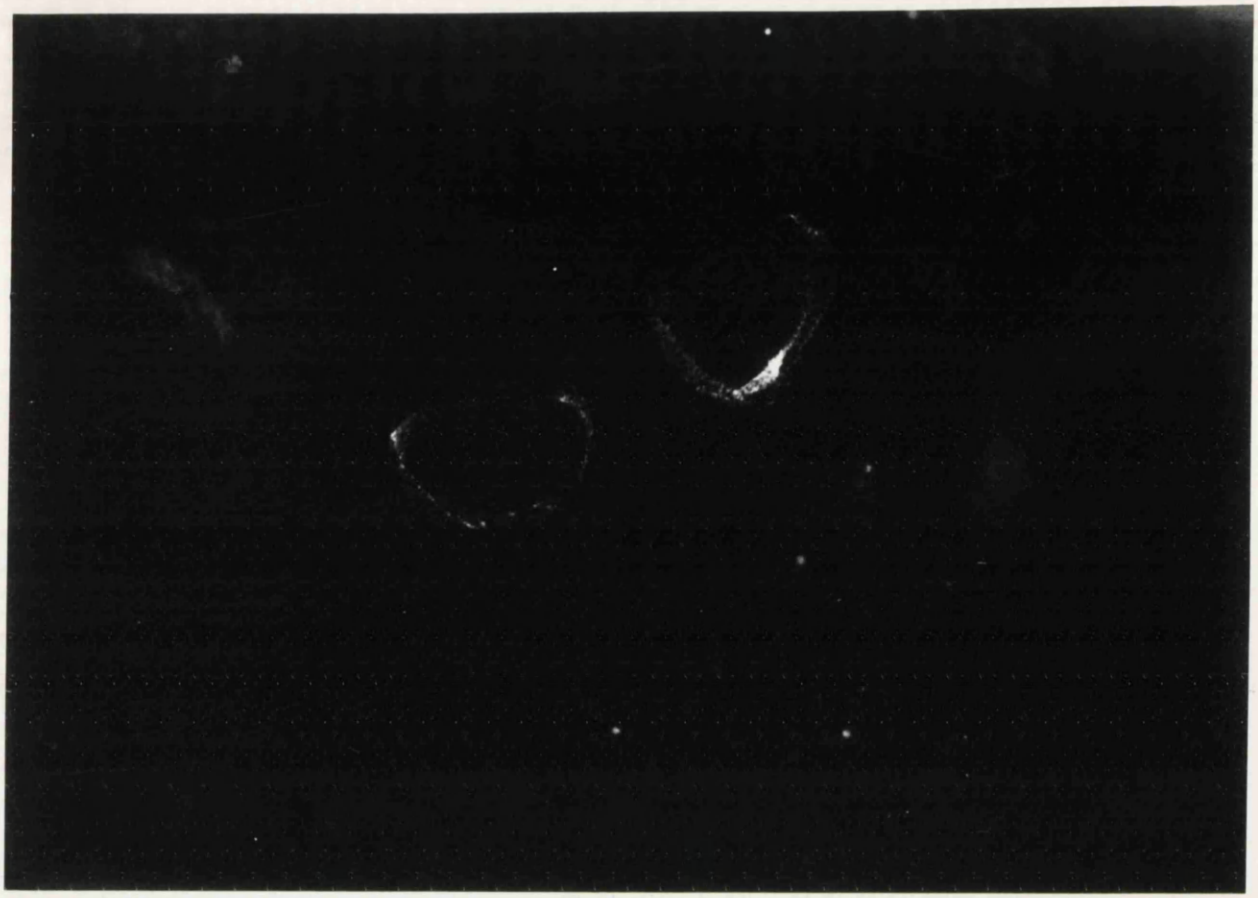
A. Live cells - staining with SM3 (immunofluorescence)

When live, only a few MCF7 cells showed membrane staining of moderate intensity; the vast majority failed to stain (Figure 3.20A). There were no morphological differences between the cells which were SM3 positive and the majority which did not stain.

B. Fixed cells - staining with SM3 (immunofluorescence and immunoperoxidase)

After fixation, using the immunofluorescence technique the majority of MCF7 cells stained; staining was cytoplasmic and of variable intensity (Figure 3.20B).
Findings were similar using the more sensitive immunoperoxidase method. Staining was cytoplasmic and generally of low intensity; only a few cells in a given field stained strongly (Figure 3.21A).

3.20. SM3 staining in cultured breast carcinoma (MCF7) cells. In live MCF7 cells (A) SM3 staining is membranous and restricted to only one or two cells. The rest of the cells in this field are unstained. After fixation, however (B), cytoplasmic positivity is seen in the majority of cells. (Immunofluorescence).



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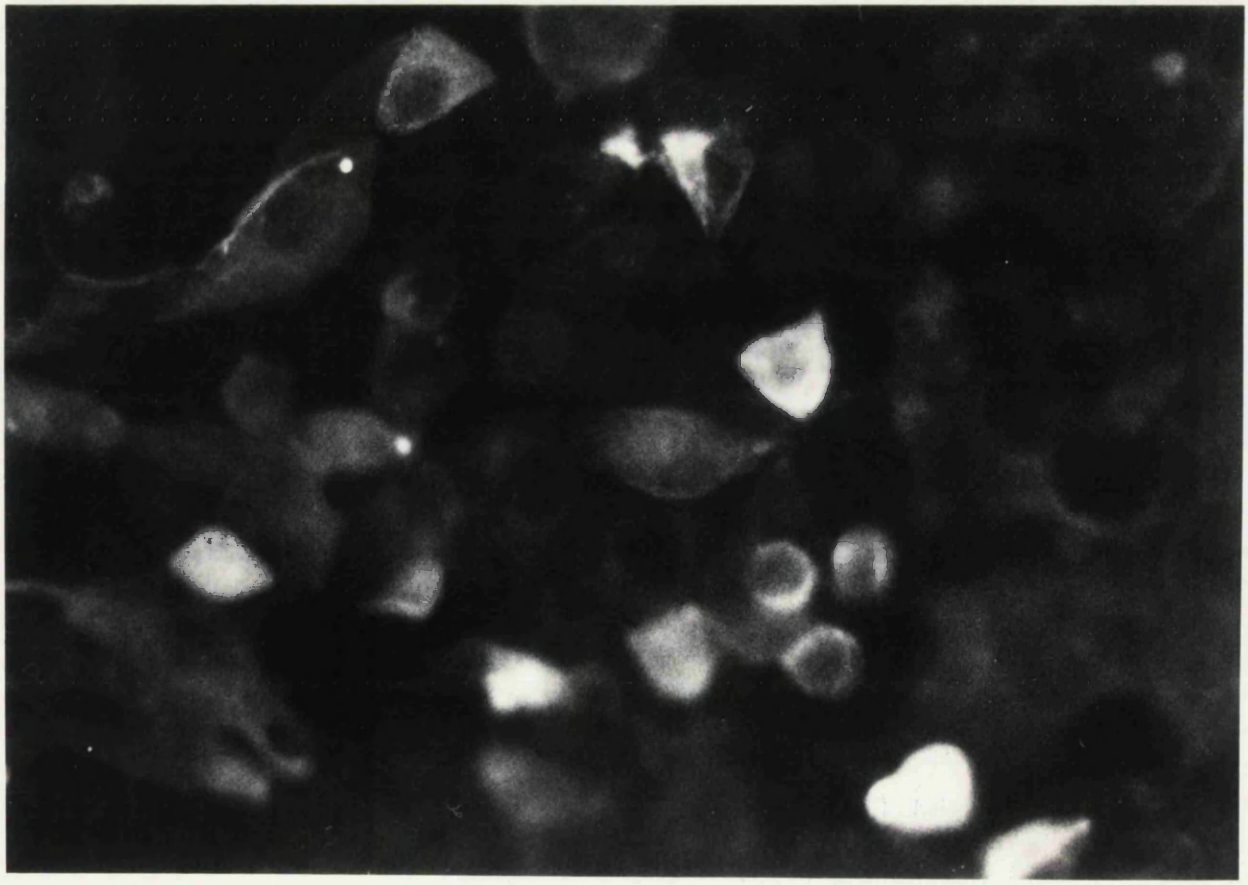


B

In T47D cells findings were similar (immunoperoxidase only).
Homogeneous cytoplasmic staining was seen in the positive control (fixed MCF7 and T47D cells stained with LE61 antibody).

C. Live and fixed cells - staining with HMFG2 (immunofluorescence and immunoperoxidase)

In live MCF7 cells, immunofluorescence staining showed a similar pattern to that with SM3, namely fluorescence on the cell membrane of only a few cells. After fixation, however, HMFG2 stained the majority of MCF7 cells using either the immunofluorescence or immunoperoxidase technique. In T47D cells findings were similar (immunoperoxidase only). The intensity of staining (in both cell lines) was greater overall with HMFG2 than SM3 (Figure 3.21B).

These results indicate that in malignant cells in vitro both SM3 and HMFG2 recognise epitopes carried on a predominantly cytoplasmic antigen.

II NORMAL MAMMARY EPITHELIAL CELLS (MILK CELLS)

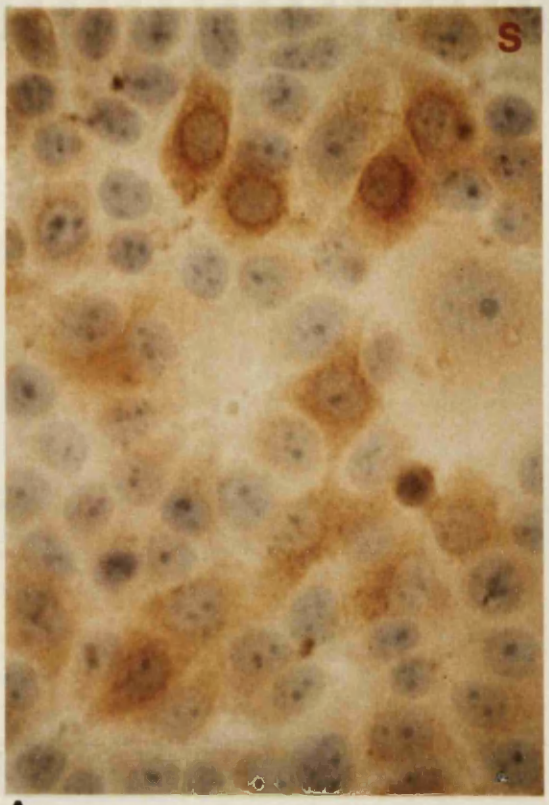
A. Live cells - staining with SM3 (immunofluorescence)

No SM3 staining was seen in live milk cells.
Heterogeneous but strong extracellular staining was seen in the positive control (live milk cells stained with HMFG1 antibody).

B. Fixed cells - staining with SM3 (immunofluorescence and immunoperoxidase)

After fixation, no SM3 positivity was seen with the immunofluorescence method, but with the more sensitive immunoperoxidase technique small foci of positivity were detected. Staining was minimal however; of several colonies on a dish, the vast majority failed to stain; only a few cells in two or three colonies showed any reactivity. Staining was cytoplasmic and varied in intensity from one cell to another (Figure 3.21C). There were no morphological differences between the cells which were SM3 positive and the majority which did not stain.

Figure 3.21. SM3 and HMFG2 staining in cultured breast carcinoma (MCF7) cells and epithelial cells from human milk. The majority of fixed MCF7 cells stain with SM3 (S). Staining is cytoplasmic and the intensity varies from cell to cell (A). Staining with HMFG2 (H) in the same cell line (B) is variable but of higher overall intensity.
In cultured epithelial cells from human milk, only occasional cells are SM3 positive and there is some variation in staining intensity from cell to cell (C). HMFG2, in contrast, stains the majority of cells; again the intensity varies (D).
(Immunoperoxidase).



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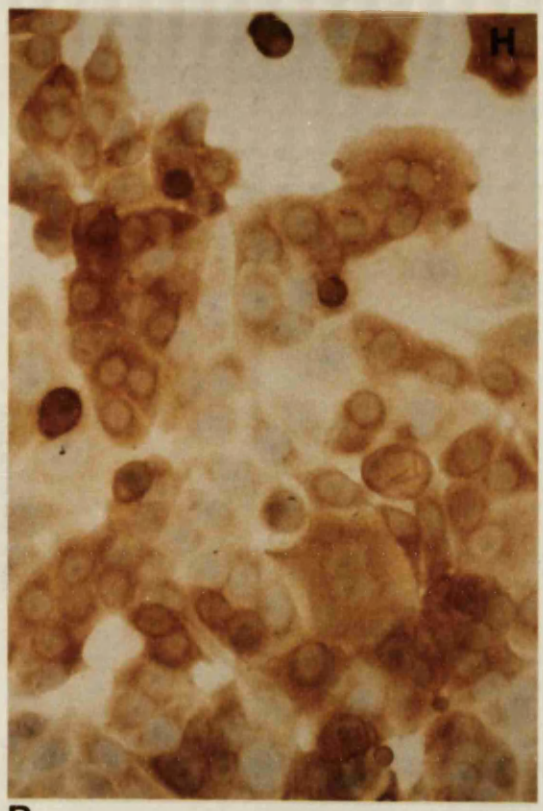
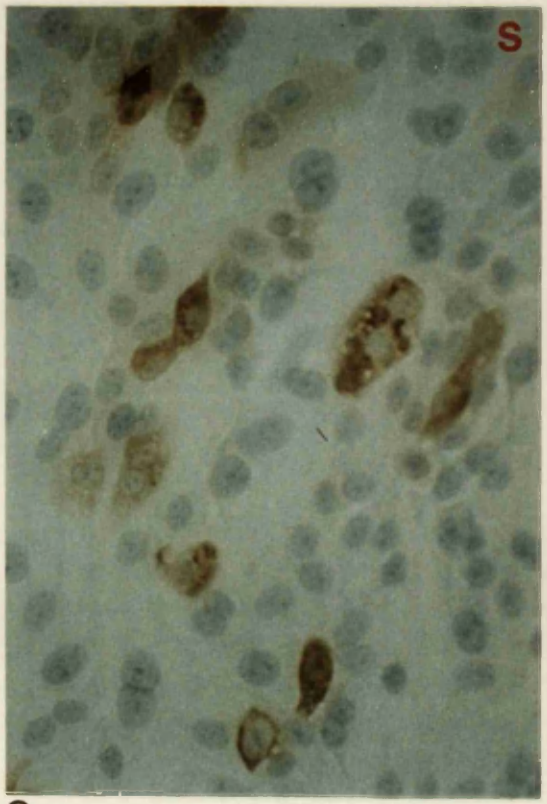
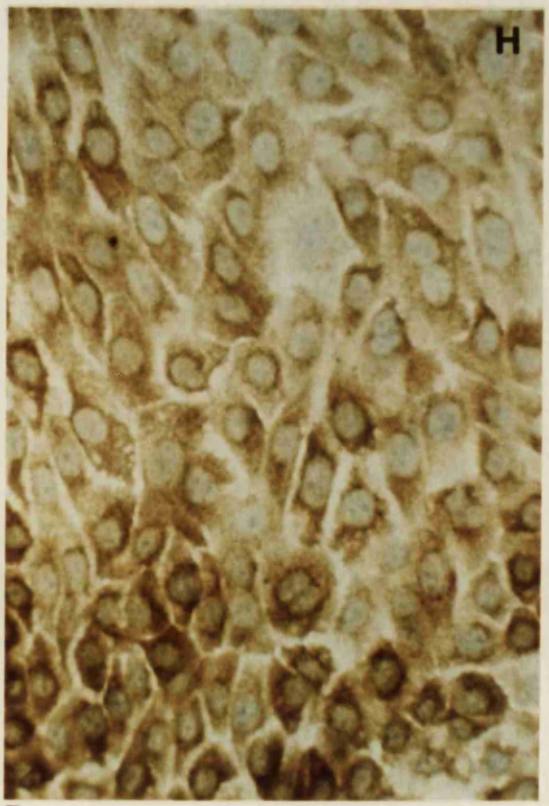


B



C



D

C. Live and fixed cells - staining with HMFG2 (immunfluorescence and immunoperoxidase)

With HMFG2, live cells showed some membrane staining. This was heterogeneous and of moderate intensity. There was more staining in fixed than in live milk cells using either the immunofluorescence or the immunoperoxidase method. In contrast to the findings with SM3, the cytoplasm of the vast majority of cells stained homogeneously. There was, however, some variation in intensity from cell to cell (Figure 3.21D).

In normal epithelial cells in vitro, therefore, the epitope recognised by SM3 is present only in occasional cells but appears to be carried on a cytoplasmic antigen. That recognised by HMFG2, is however, expressed in the majority of normal cells; although there is some staining on the cell surface the antigen is predominantly cytoplasmic.

3.7 DISCUSSION

The studies described in this chapter have shown that the SM3 epitope is highly tumour associated being expressed by the vast majority of primary mammary carcinomas and also by breast cancer cell lines. In the normal breast, however, particularly in tissue showing secretory change (fully differentiated cells),the epitope is expressed either at a very low level or not at all. The HMFG2 epitope, in contrast, whilst again highly tumour associated, is also expressed in normal and secretory breast tissue. How can this difference in expression of the SM3 epitope in malignant and normal epithelial cells be explained? How do the differences in SM3 and HMFG2 immunoreactivity arise?

As explained in Chapter 1, the gene coding for the Polymorphic Epithelial Mucin (PEM) has recently been cloned. A large domain of this gene containing both the HMFG2 and SM3 epitopes is composed of a 60 base pair tandem repeat sequence which codes for a 20 amino acid repeat peptide (Figure 1.6) (Gendler et al 1987, 1988a and 1988b). More recently, the exact structure of the peptide epitopes recognised by SM3 and HMFG2 has been elucidated. Using overlapping octamers covering the sequence of the 20 amino acid tandem repeat, it has been found that the minimum SM3 epitope consists of the five amino acids Pro-Asp-Thr-Arg-Pro. The minimum HMFG2 epitope, however, is smaller and as illustrated in Figure 3.22 consists of only three of these five amino acids Asp-Thr-Arg (Burchell et al 1989b).

The epitope mapping data allows us to suggest explanations for the immunohistochemical findings described in this chapter. As illustrated in Figure 3.23, it appears that the normally processed mucin produced by fully differentiated cells has long, branched carbohydrate side chains (Hanisch et al 1989) which mask at least part of the SM3 epitope on the core protein. The mucin produced by malignant cells, however, is abnormally glycosylated and has shorter and less branched carbohydrate side chains, so that the complete SM3 epitope can be exposed. The HMFG2 epitope, on the other hand, which is smaller and further from glycosylation sites can be exposed in the mucin produced both by fully differentiated and malignant epithelial cells.

Figure 3.22. Structure of the tandem repeat peptide from the core protein of the Polymorphic Epithelial Mucin illustrating the epitopes recognised by SM3 and HMFG2. The tandem repeat sequence of 20 amino acids is illustrated. (Arrows indicate potential glycosylation sites). The SM3 epitope consists of the five amino acids indicated (Pro-Asp-Thr-Arg-Pro). The HMFG2 epitope is smaller and consists of only the central three (Asp-Thr-Arg).

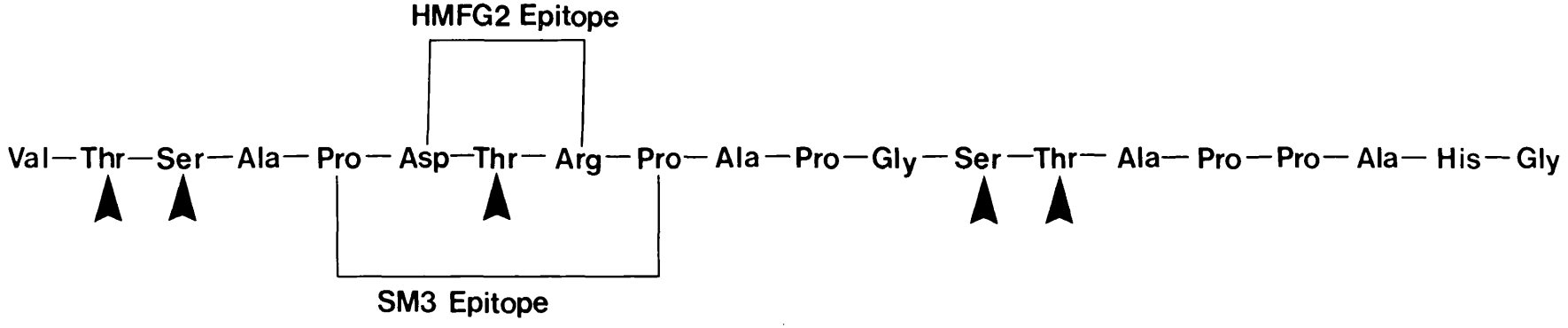

HMFG2 Epitope
Val—Thr—Ser—Ala—Pro—Asp—Thr—Arg—Pro—Ala—Pro—Gly—Ser—Thr—Ala—Pro—Pro—Ala—His—Gly
SM3 Epitope

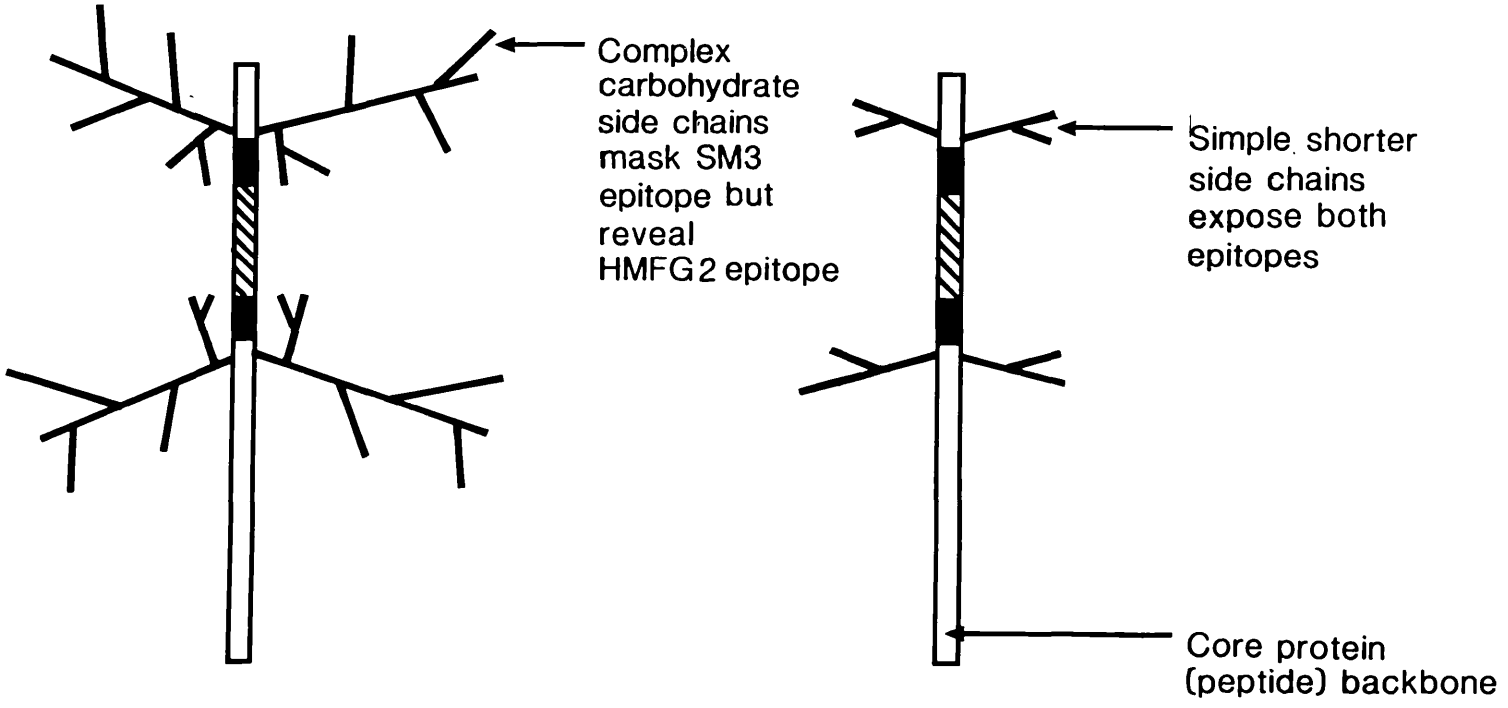
Figure 3.23. Schematic represenation of mucin processing in normally (fully) differentiated and malignant mammary epithelial cells. The structure of part of a mucin molecule is illustrated diagramatically. On the left, the mucin produced by normally (fully) differentiated epithelial cells is shown. It has complex branched carbohydrate side chains which mask part of the SM3 epitope. The smaller HMFG2 epitope is, however, exposed. The mucin produced by malignant epithelial cells is shown on the right. It is incompletely glycosylated and has shorter and less branched carbohydrate side chains; both epitopes are therefore exposed.

NORMALLY DIFFERENTIATED CELL

(FULLY GLYCOSYLATED MUCIN)

MALIGNANT CELL

(INCOMPLETELY GLYCOSYLATED MUCIN)



SM3 epitope (Pro - Asp - Thr - Arg - Pro)

HMFG2 epitope (Asp - Thr - Arg)

The concept of abnormal glycosylation of mucins in malignant cells has some support from previously published work. Firstly, as mentioned in Chapter 1, it was the suggested explanation for the variation in size of the lower molecular weight HMFG2 reactive components seen in Western blots of breast cancer cell lines and tumour cytosols (Burchell et al 1983). Secondly, in certain breast cancer cell lines, HMFG2 reactive material accumulates in the Golgi apparatus. This is the site of O-linked glycosylation and this material may represent precursor mucin molecules (Griffiths et al 1987). Thirdly, it has been shown that breast cancer patients whose tumours stain with helix pomatia lectin have a poor prognosis. As helix pomatia recognises the sugar which links carbohydrate side chains to the mucin core protein its exposure implies abnormal glycosylation (Leatham and Brooks 1987). Finally, the normal mucin isolated from human milk has been shown to contain extended and/or branched poly-N-acetylactosamine side chains (Hanisch et al 1989) whereas the oligosaccharide side chains of the mucin produced by the breast cancer cell line BT20 are short, consisting of only three or four sugars (Hull et al 1988).

The immunohistochemical data presented in this chapter also show differences in the site of staining between tissues; in the in vivo situation, cytoplasmic staining predominates in breast carcinomas and membrane staining in benign and normal breast tissue. These observations can be explained if we assume that SM3 reacts with precursor mucin molecules which, due to abnormal processing build up in the cytoplasm of malignant cells. In normal cells, in contrast, the mucin is more normally glycosylated and therefore able to be transported to the cell surface. In vitro, the level of expression of the SM3 epitope is minimal in cultured milk cells compared with breast carcinoma cell lines. Where SM3 positivity is seen, however, in both milk cells and carcinoma cells the site of staining is cytoplasmic. This difference in the site of staining in normal mammary epithelial cells in vivo and in vitro (membranous and cytoplasmic respectively) may be related to culture conditions. The reactivity of SM3 with a predominantly cytoplasmic antigen has important implications for the imaging studies which will be described in Chapter 5.

In normal resting and benign breast tissue, although SM3 staining is considerably less than in carcinomas, its presence indicates that there are foci in which abnormally glycosylated mucins are produced even in these situations. How might these arise? Work with HMFG1 has shown that antigen expression may be related to growth potential of cells (Chang and Taylor-Papadimitriou 1983). In ovarian carcinomas, SM3 expression has been reported to be related to phase of the cell cycle (van Dam P. personal communication) and it is possible that this may apply to breast tissue as well. In this context, it would be interesting to relate immunostaining with SM3 to proliferative activity. As SM3 expression is highly tumour associated, one might expect SM3 staining in benign breast disease to reflect malignant potential. In the benign biopsies so far studied, however, no such clear relationship has emerged. Eight out of nine cases of epithelial hyperplasia were SM3 positive but staining was no more intense than in other benign conditions. It would be of interest to look at a larger number of cases of epithelial hyperplasia and in particular to study examples of atypical hyperplasia. Ideas for future work with SM3 in these and other areas will be discussed further in Chapter 6.

A repeated finding in breast carcinomas was heterogeneity of staining with SM3 which was observed both from one tumour to another and within a given tumour. This may reflect differences in the degree of abnormal glycosylation between different tumours and between different cells in the same tumour. Staining with HMFG2 was generally less heterogeneous and of greater overall intensity. This probably reflects not only the fact that the HMFG2 epitope is exposed in both incompletely and more fully processed mucin molecules but also the fact that HMFG2 is intrinsically a higher affinity antibody than SM3 (Gendler et al 1988b).

In summary, therefore, differences in expression of the SM3 epitope in the breast appear to result from differences in glycosylation of mucin molecules produced by malignant and fully differentiated mammary epithelial cells.

At other sites in the body, the SM3 epitope is highly expressed in carcinomas of the ovary, colon and lung whereas in normal tissue from these sites it is either undetectable (ovary), present at only a very low level (colon) or heterogeneously expressed (lung). It therefore appears that there is either ectopic production or abnormal processing of the PEM in carcinomas from these sites. It should be emphasised, however, that mucins other than PEM are also produced by the normal ovary, colon and lung and these too may be expressed by tumours from these sites.

In the comprehensive study of normal tissues described in this chapter, the only tissues showing significant SM3 staining were sebaceous glands of the skin and proximal tubules and collecting ducts of the kidney. This positivity probably reflects a tissue specificity in processing of the PEM although an alternative explanation is a non-specific cross reactivity. The weak or sporadic reaction seen in some of the other normal tissues may be explained in the same way.

In conclusion, therefore, immunohistochemical studies with SM3 and HMFG2 have identified important differences in the immunophenotypes of malignant and normal (fully differentiated) mammary epithelial cells due to differences in mucin processing by these two cell types. Similar observations have been made in carcinomas of the colon, lung and ovary and their corresponding normal tissues.

Chapter 4

IMMUNOBLOTTING STUDIES

4.1 INTRODUCTION

Immunohistochemical studies with monoclonal antibodies such as those described in Chapter 3 provide a means of evaluating epitope expression at the cellular level. A parameter which is not assessed, however, is the molecular weight of the component or components which carry the epitope.

Many of the monoclonal antibodies reactive with mucins recognise epitopes which in carcinomas are carried on components of different molecular weights; in the case of HMFG2 these range from 80-400 KD (Burchell et al 1983, Turnbull et al 1986). The lower molecular weight components are thought to represent mucin molecules which are incompletely processed. As described in Chapter 3, SM3, like HMFG2, recognises a highly tumour associated peptide epitope; the SM3 epitope, however, is expressed at a much lower level in normal resting and secretory breast tissue than the HMFG2 epitope. It is revealed only when glycosylation of mucins is abnormal. It is therefore of interest to study and compare the molecular weights of components carrying the two epitopes.

Immunoblotting studies have been performed using both lysates prepared from breast carcinoma cell lines (to study in vitro epitope expression) and cytosols prepared from primary mammary carcinomas (to study in vivo expression). Samples were prepared, run on SDS gels, blotted onto nitrocellulose paper and then reacted with the antibodies SM3 and HMFG2 in an immuno-enzyme assay. Full details of the methods have been given in Chapter 2.

4.2 RESULTS

I BREAST CANCER CELL LINES

The cell lines studied were T47D, MCF7, ZR75-1 and BT20. As explained in Chapter 1, all are established cell lines, BT20 being derived from a primary breast tumour and the others from metastatic carcinoma cells.

Immunoblots with SM3 and HMFG2 are shown in Figure 4.1. Figure 4.1A shows results in a 5-15% gradient gel and Figure 4.1B results in a 5% gel. The epitopes recognised by both antibodies are carried on components of different molecular weights which vary from one cell line to another and range from less than 200 to approximately 350 KD. No reactivity is seen in the negative control (Figure 4.1A track C), a lysate prepared from MRC5 cells (a fibroblast cell line).

In MCF7 and T47D cells, there are differences in the components recognised by HMFG2 and SM3 (Figure 4.1A). In MCF7 cells, HMFG2 (track 3) recognises an epitope on a pair of high molecular weight components; the lower of these bands is somewhat diffuse and may represent a doublet. SM3 (track 7) shows no reaction with the high molecular weight components. Both antibodies, however, react with two pairs of lower molecular weight (around 200 KD) components. Furthermore, the lower bands recognised by both antibodies are sharper than the high molecular weight bands recognised by HMFG2 suggesting a lower carbohydrate content. Interestingly, the lowest molecular weight bands in MCF7 cells are clearly defined as a doublet suggesting that the bands recognised by both antibodies represent precursors of the higher molecular weight HMFG2 reactive bands. In T47D cells the results are more difficult to interpret due to the heavy background staining with HMFG2. HMFG2 (Figure 4.1A track 4) reacts both with material of very high molecular weight (approximately 350KD) and with some sharper bands around 200 KD. SM3 in contrast (track 8) reacts

strongly with the lower and very weakly with the high molecular weight bands. The same pattern of staining in these two cell lines is shown more clearly in Figure 4.1B. Again, the HMFG2 epitope is carried on both high and low molecular weight components (T47D cells in track 1 and MCF7 cells in track 2) whereas the SM3 epitope is carried predominantly on the low molecular weight bands (T47D cells in track 3 and MCF7 cells in track 4).

In BT20 and ZR75-1 cells, both HMFG2 (Figure 4.1A tracks 1 and 2) and SM3 (Figure 4.1A tracks 5 and 6) recognise similar epitopes on both high (greater than 200KD) and lower molecular weight components. Again, the higher molecular weight bands are diffuse indicating a high carbohydrate content.

II CYTOSOLS FROM PRIMARY MAMMARY CARCINOMAS

Figure 4.2 (a 5% gel) shows an immunoblot in which cytosols from three primary mammary carcinomas were reacted with SM3 and HMFG2 (tracks 1-6). The T47D breast cancer cell line is included for comparison (tracks 7 and 8). The tumours were all infiltrating ductal carcinomas, two were grade II and one was a grade III tumour (Bloom and Richardson grading). Immunohistochemical staining with SM3 was heterogeneous but moderately strong in all three tumours. As in the breast cancer cell lines, the immunoreactive bands differ in molecular weight from one tumour to another (compare Figure 4.2 tracks 1,3 and 5) although all reactions with tumour cytosols are much weaker than with the lysates (Figure 4.2 tracks 7 and 8). In tumour 1 (track 1) there are two bands of molecular weight just over 200 KD which react with HMFG2. These are also recognised by SM3 although the intensity of the reaction is minimal (track 2). In tumour 2 (track 3) there are two very high molecular weight bands and a further low band which all react with HMFG2; only the low band is recognised by SM3 and the reaction is very faint (track 4). Tumour 3 (track 5) contains four high molecular weight components (200 KD and above) which react weakly with HMFG2 but not with SM3 (track 6).

Figure 4.1. Immunoblotting studies: lysates from breast cancer cell lines. Figure 4.1A illustrates an immunoblot (a 5-15% gradient gel) in which lysates from four different breast cancer cell lines were reacted with HMFG2 (tracks 1-4) and SM3 (tracks 5-8). The cell lines studied were BT20 (tracks 1 and 5), ZR75-1 (tracks 2 and 6), MCF7 (tracks 3 and 7) and T47D (tracks 4 and 8). There is no reactivity in the negative control, MRC5 cells (a fibroblast cell line) (track C).
Figure 4.1B shows an immunoblot (a 5% gel) in which lysates from T47D (tracks 1 and 3) and MCF7 (tracks 2 and 4) cell lines were reacted with HMFG2 (tracks 1 and 2) and SM3 (tracks 3 and 4).

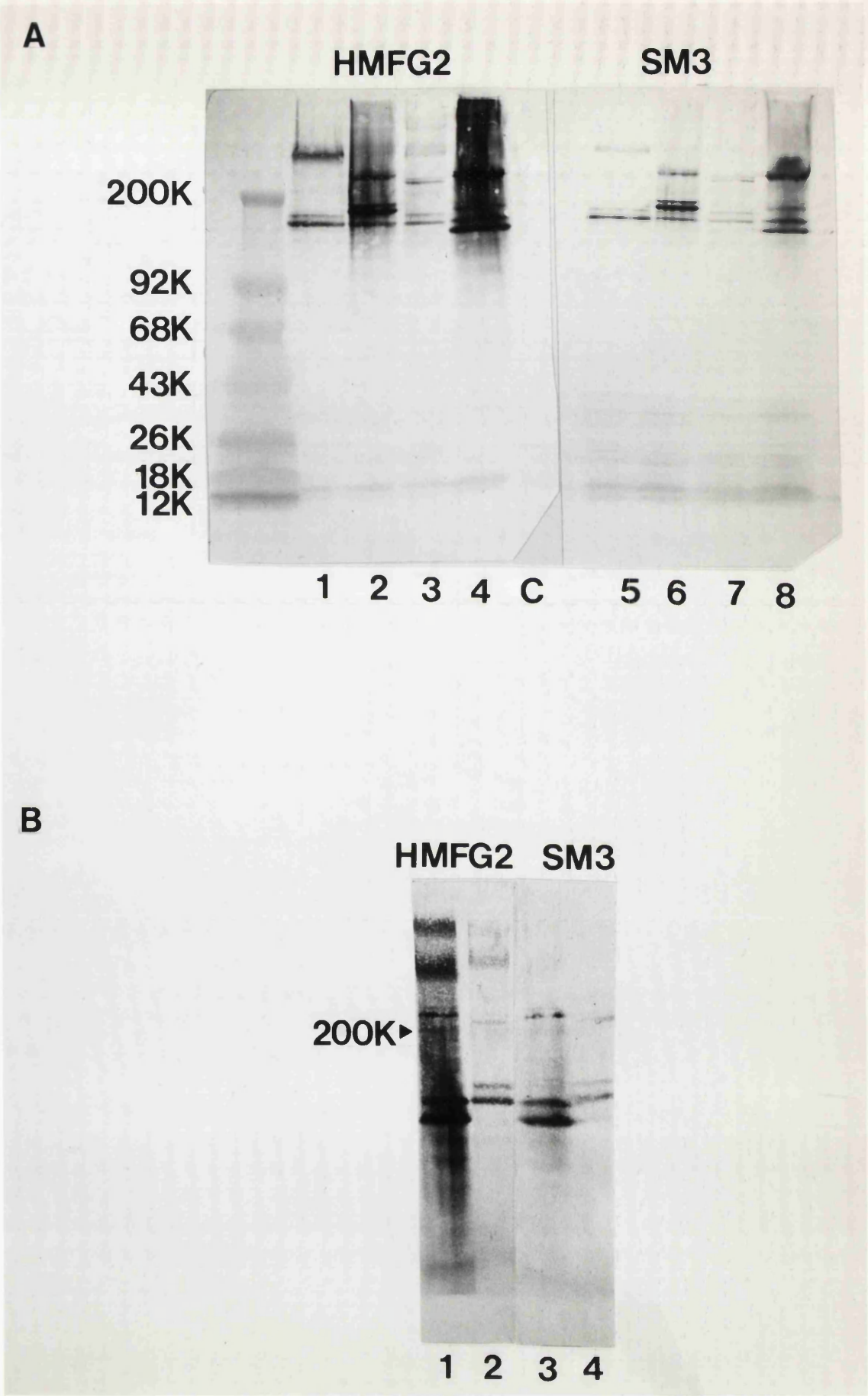
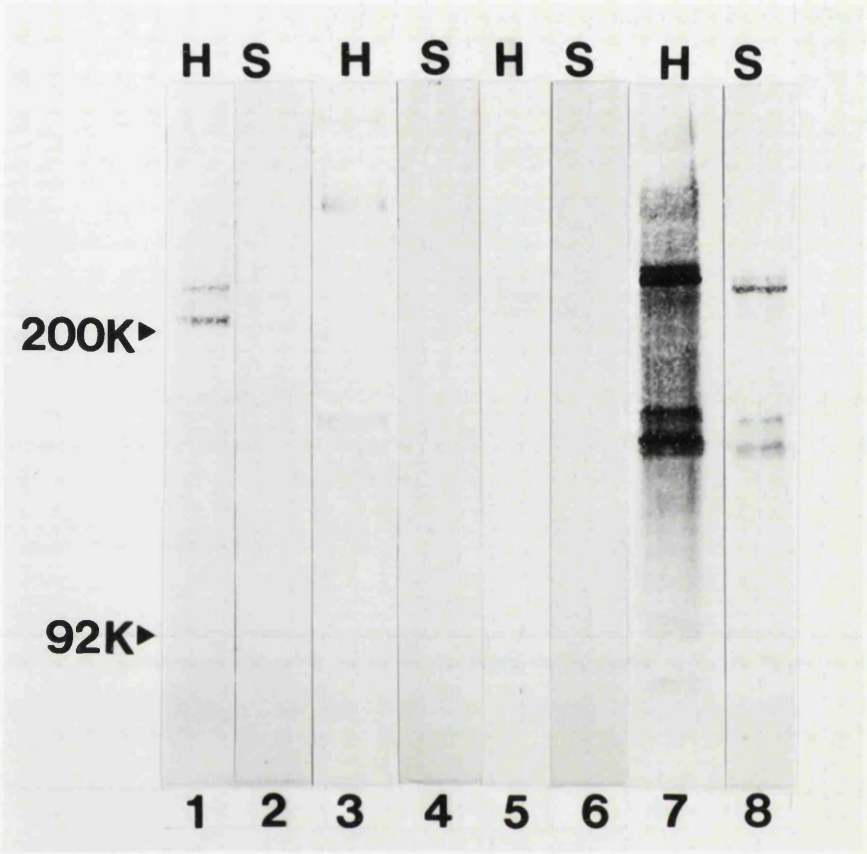

A
HMFG2
SM3
200K
92K
68K
43K
26K
18K
12K
1 2 3 4 C 5 6 7 8
B
HMFG2 SM3
200K
1 2 3 4

Figure 4.2 Immunoblotting studies: cytosols from primary mammary carcinomas. The figure illustrates an immunoblot (a 5% gel) in which cytosols prepared from three different primary mammary carcinomas (tumour 1 in tracks 1 and 2, tumour 2 in tracks 3 and 4 and tumour 3 in tracks 5 and 6) were reacted with HMFG2 (H) (tracks 1, 3 and 5) and SM3 (S) (tracks 2, 4 and 6). Results in a lysate prepared from T47D cells (tracks 7 and 8) are shown for comparison.


H S H S H S H S
200K▸
92K▸
1 2 3 4 5 6 7 8

4.3 DISCUSSION

It appears, therefore, that both in vivo (tumour cytosols) and in vitro (lysates from breast cancer cell lines), HMFG2 reacts with components of both high and low molecular weight whereas SM3 reactivity is seen predominantly with the latter (molecules of around 200 KD or less). The intensity of reactions with the tumour cytosols is, however, much less than with the lysates (compare tracks 1-6 in Figure 4.2 with tracks 7 and 8). The reason for this weak reactivity is not clear; it is possible that the total protein content of the cytosols was less than that in the lysates.

The detailed mapping of the epitopes recognised by SM3 and HMFG2 was described in the previous chapter. The exposure of the SM3 epitope in incompletely glycosylated mucin molecules produced by malignant cells was also discussed. The immunoblotting studies described here showing that the SM3 epitope is expressed predominantly on low molecular weight components in malignant cells (T47D and MCF7s and tumour cytosols) are in keeping with this concept. The smaller HMFG2 epitope is present both on high molecular weight mucins (more completely glycosylated - diffuse bands) and low molecular weight precursors (incompletely glycosylated - sharp bands) whereas the SM3 epitope is carried predominantly on the latter. In BT20 and ZR75-1 cells, the SM3 epitope is present on both high and low molecular weight bands, the pattern of reactivity being very similar to that with HMFG2. This suggests that the more fully processed (higher molecular weight) mucin molecules produced by these two cell lines have shorter and/or less branched carbohydrate side chains than those produced by T47D and MCF7 cells.

In conclusion, therefore, the immunoblotting data is in keeping with the concept that the processing of mucins in breast carcinomas and some cell lines derived from them is abnormal leading to exposure of the mucin core protein and of the SM3 epitope which is masked in more fully glycosylated mucin molecules.

Chapter 5

IMMUNOLOCALISATION STUDIES

5.1 INTRODUCTION: THE USE OF MONOCLONAL ANTIBODIES IN IMAGING

I BACKGROUND

The significance of axillary lymph node involvement in breast cancer was discussed in Chapter 1. This remains the single most important prognostic factor and also has a significant influence on the treatment of patients as, in most centres, until recently, adjuvant therapy has been given only to node positive patients.

Clinical examination is a notoriously inaccurate way of assessing nodal status (Wallace and Champion 1972). Sampling techniques have proved only slightly more accurate (Davies, Millis and Hayward 1980). Axillary dissection and histological examination of lymph nodes is therefore mandatory to obtain maximum data as it is the pathological node status rather than the clinical assessment of nodal involvement which is the best indicator of prognosis. It would clearly be desirable to be able to obtain this information by non-invasive means. Although various techniques are available for imaging lymph nodes none is entirely satisfactory for detecting metastases. CT scanning can detect large nodes but not metastases in nodes of normal size (Husband et al 1979). MRI can demonstrate increased nodal size but its role in the assessment of metastases has not yet been fully evaluated. The techniques of lymphography by which contrast medium is injected into the finger web spaces, and lymphoscintigraphy using injection of labelled colloid both depend on impaired uptake in involved nodes, and identification of these areas as foci of reduced uptake. Lymphography, however, has proved no more sensitive than clinical examination in predicting nodal involvement.

In theory, the use of antibodies which specifically bind to tumour associated antigens on malignant but not on normal cells should provide a highly sensitive means of detecting metastases.

The concept of a "magic bullet" which could target sites of disease in the body was first suggested by Ehrlich (Ehrlich 1906). It was applied to tumour imaging by Pressman and colleagues who pioneered the technique of immunoscintigraphy (Pressman and Korngold 1953).

The identification of tumour associated antigens such as carcinoembryonic antigen (CEA) (Gold and Freedman 1965) led to attempts to localise human xenografts using polyclonal anti-CEA sera (Primus et al 1973). The first in vivo studies in humans used polyclonal anti-CEA sera to image tumours of the gastrointestinal tract, ovary, cervix, endometrium, breast and bronchus (Goldenberg et al 1978). Subtraction techniques were used to overcome the problem of background blood pool reactivity. The initial promising results, however, could not be reproduced by others (Mach et al 1981).

The most important factor influencing immunolocalisation is the target to background ratio of labelled antibody uptake which appears to be limited using polyclonal antisera. Monoclonal antibodies, seem to provide the ideal solution as, in theory, tumour specific antibodies can be produced which do not cross react with other tissues and there is no need for affinity purification. Although impressive results have been obtained both in vitro and in animal experiments, studies in human subjects have been disappointing. There are several reasons for this. Firstly, the specificity of monoclonal antibodies may reduce the total accumulation in a tumour if the quantity of antigen is limited, especially if an antibody only recognises one epitope per antigen molecule. Secondly, tumour <u>specific</u> antigens have not yet been discovered. Thirdly, a so-called "specific" antibody may in fact unexpectedly bind to similar determinants on other antigens. Fourthly, the affinity of an antibody may have to be high; this is not always easy to achieve. Finally false positive localisation may occur due to interaction of mouse immunoglobulins with human Fc receptors (Bradwell et al 1985).

Initial immunolocalisation studies with monoclonal antibodies used the intravenous route of injection. This method, however, results in antibody dilution. This may be overcome by immunolymphoscintigraphy, a technique in which labelled antibody is injected locally and taken up into the lymphatics and thence to the regional lymph nodes. (In contrast to lymphography and lymphoscintigraphy, areas of antibody reactivity are identified as areas of increased uptake). In theory, immunolymphoscintigraphy has several other advantages over the intravenous injection of antibody. These include 1) The imaging of smaller metastatic deposits, 2) The minimisation of binding of antibodies to circulating tumour antigens and to cross reactive antigens on normal tissues, 3) Lower doses can be used for diagnosis or treatment, 4) Images appear more quickly as there is no need to wait for blood stream clearance (Weinstein et al 1983).

In the studies with SM3 described in this chapter, antibody has been given by both intravenous and local injection.

II IMAGING STUDIES IN BREAST CANCER

Experiments in mice bearing human breast tumour xenografts have shown tumour localisation with iodinated monoclonal antibody or antibody fragments (Colcher et al 1983).

Human studies have involved the use of a number of different antibodies, injected either intravenously or subcutaneously. Intravenous injections of iodinated HMFG1 and HMFG2 have been used to image primary and metastatic breast cancer and other tumours (Epenetos et al 1982a, Griffiths et al 1986) and the antibody M8 (see Table 1.9) to detect bony metastases in breast cancer patients (Rainsbury et al 1983).

Using immunolymphoscintigraphy (subcutaneous antibody injection) there have been a number of reports of imaging axillary node metastases. Using the monoclonal antibodies 3E1.2 and RCC-1 (raised against a human breast carcinoma and the MCF7 breast cancer cell line respectively) initial findings were encouraging but later studies showed that this technique was no more sensitive than clinical examination (Thompson et al 1984, Tjandra et al 1989a). The same group has recently reported an improvement in the specificity and sensitivity of their results using a higher dose of antibody and a cold "blocking" antibody (Tjandra et al 1989b). In similar studies, the antibodies HMFG1, HMFG2 and 3C6F9* have been used; results were encouraging with HMFG2 and 3C6F9 but not with HMFG1 (Mandeville et al 1986).

The immunohistochemical studies described in Chapter 3 have shown that the SM3 epitope is strongly tumour associated, but absent or present at only a very low level in normal tissues. SM3 would therefore appear to be a potential candidate for use in in vivo imaging studies.

In this chapter, results of a pilot imaging study will be presented. This was designed to assess the use of radiolabelled SM3 in the detection of axillary lymph node metastases in patients with breast cancer. Antibody was administered by both the subcutaneous and the intravenous routes and the merits of each assessed. Great emphasis was placed on the careful correlation of counted radioactivity and histological findings in all tissue samples (lymph nodes and breast tissue). Although previous studies have compared histological data with scan findings, none have performed the comparison in such a detailed way as that reported here.

*3C6F9 is a monoclonal antibody directed against a 37KD breast cancer associated antigen

5.2 MATERIALS AND METHODS

Details of the selection of patients and methods used have been given in Chapter 2. In brief, 25 patients with proven primary breast cancer were studied prior to definitive treatment by mastectomy with axillary clearance. SM3 antibody, labelled with either ^{123}I (9 patients) or ^{125}I (16 patients) was administered either by subcutaneous or intravenous injection. Imaging studies were carried out only in patients who had received ^{123}I labelled antibody as the photon energy of ^{125}I is too low to permit imaging. Specimens from all patients were dissected; the radioactivity in resected lymph nodes, in any residual tumour at the biopsy site and in macroscopically normal breast tissue was counted. This data was related to subsequent histological findings.

5.3 RESULTS

I IMAGING STUDIES (^{123}I ONLY) AND RADIOACTIVITY IN RESECTED NODES AND BREAST TISSUE

As described in Chapter 2 (page 110) the counted radioactivity was expressed as percentage of injected dose per gram of tissue. A vast amount of data resulted from each patient as shown in Table 5.1. From this data, the mean radioactivity in positive and negative nodes was calculated for each patient (see table 5.1). In each group of patients (^{123}I labelled SM3 subcutaneous injection, ^{123}I labelled SM3 intravenous injection etc), results were then pooled. The counts in any positive nodes from patients in a group and the counts in all negative nodes were each added together and divided by the number of samples in each category. The number of samples was less than the total number of nodes as several nodes were usually present in each sample eg. for the data shown in table 5.1 there are eight samples from a total of 20 lymph nodes. Data for radioactivity counted in breast tissue was also pooled where possible.

A. ^{123}I labelled SM3 administered by subcutaneous injection

There were four patients in this group; two had axillary node metastases and two were node negative. Imaging showed similar results in all patients. Immediate uptake of antibody was seen into the lymphatics of the hand (Figure 5.1A) and there was activity in the

Table 5.1

IMMUNOLOCALISATION DATA OBTAINED FROM A REPRESENTATIVE PATIENT - SUBCUTANEOUS INJECTION OF ^{125}I LABELLED SM3

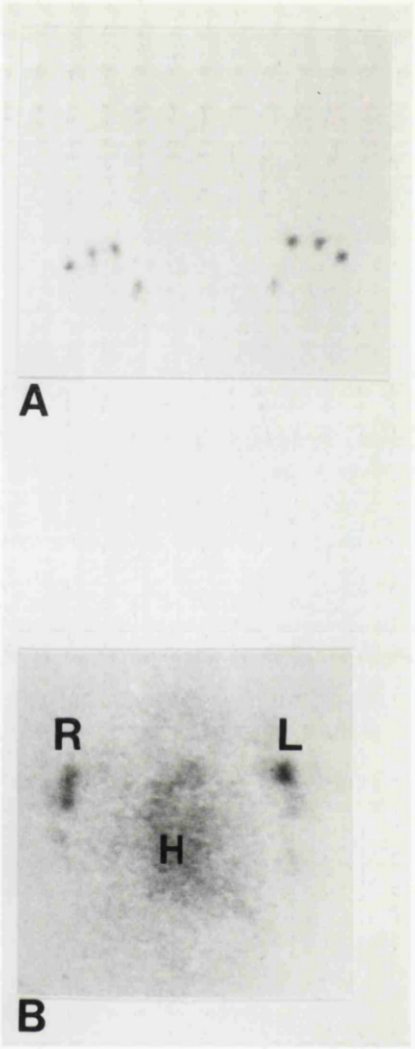
Type of Specimen**	Weight (g)	Nodal Status	Counted Radioactivity* % Inj/g	Counted Radioactivity* % Inj
Tumour	0.84	N/A	0.0016248	0.0013648
Normal breast	1.34	N/A	0.0010094	0.0013526
Lymph nodes A5	0.12	-	0.0051824	0.0006219
B3	0.42	+	0.0021328	0.0008958
C9	1.06	+	0.0027598	0.0029254
C1	1.11	+	0.0025935	0.0028787
C½ (I)	0.56	-	0.0131285	0.0073520
C½ (II)	1.16	-	0.0077481	0.0089878
C½ (III)	0.88	+	0.0055668	0.0048988
C½ (IV)	1.09	+	0.0064542	0.0065188
Total 20		mean+ =	0.0039014	Total = 0.0350792
		mean- =	0.0086863	(nodes)

* Expressed as either percentage of injected dose per gram of tissue or as percentage of total injected dose

** A, B and C indicate high, middle and low axillary lymph nodes

N/A = not applicable

Figure 5.1 Imaging studies: ^{123}I labelled SM3 - subcutaneous injection. After subcutaneous injection of ^{123}I labelled SM3, activity was seen in the web spaces of the hands at the sites of injection (A). Activity was seen in the axilla at four hours after injection (B); this was symmetrical with no apparent difference between the left (L) test and the right (R) control side. Blood pool activity in the heart (H) was also seen.


A
R
L
H
B

axilla at four hours after injection. This was symmetrical in both axillae; uptake was seen in node negative as well as node positive patients. There was no apparent quantitative difference between the test (potentially involved) and the control (non-involved) side (Figure 5.1B). Imaging of the mastectomy specimen showed uptake in all node groups. It appears therefore that ^{123}I labelled antibody given by the subcutaneous route is taken up equally by both involved and non-involved axillary nodes.

Furthermore, as shown in Table 5.2 there was no consistent quantitative difference between the mean amount of radioactivity counted in involved (patients 2 and 4) and non-involved (patients 1 and 3) nodes. In patient 4, the amount of radioactivity is slightly greater in the involved nodes than the non-involved, whereas the reverse is true for patient 2. When the data were pooled, the mean radioactivity in positive nodes (expressed as % injected dose per gram of tissue) was 0.0068, and in negative nodes 0.0051. There is no significant difference between these figures (Students t-test, $p > 0.1$).

The amount of radioactivity in residual tumour and normal breast tissue was not measured in this group of patients.

B. ^{123}I labelled SM3 administered by intravenous injection

Of the five patients in this group, three had axillary nodal metastases and two were node negative. Imaging in all patients at 24 hours after injection showed activity in the blood pool including heart, lungs and blood vessels as well as the liver, spleen, kidneys and bladder (Figure 5.2). As with subcutaneous injection of antibody, axillary nodal uptake was symmetrical with no increased uptake on the involved side. Again, imaging of the mastectomy specimen showed uptake in all node groups and in some cases activity in the breast at the site of the primary tumour (Figure 5.3).

When comparing the mean amounts of radioactivity counted (Table 5.3), the amounts were slightly greater in the involved than the non-involved nodes in patients 6 and 8, but the reverse was true in patient 7. When the data were pooled, the mean radioactivity in positive nodes was 0.0036 and in negative nodes 0.0037. Again there is no significant difference between these figures (Student's t-test $p > 0.1$).

Table 5.2

RADIOACTIVITY COUNTED IN TISSUE FROM PATIENTS INJECTED WITH ^{123}I LABELLED SM3: SUBCUTANEOUS ROUTE

Patient Number	Pathological Nodal Status (number positive and total number)	COUNTED RADIOACTIVITY (% injected dose per gram) Nodes (mean values) +	Nodes (mean values) -	Normal Breast	Residual Tumour
1	Negative (35)	None	0.0040	NC	NC
2	Positive (1 of 27)	0.0011	0.0022	NC	NC
3	Negative (16)	None	0.0123	NC	NC
4	Positive (6 of 33)	0.0079	0.0056	NC	NC

NC = not counted

Figure 5.2. Imaging studies: ^{123}I labelled SM3 - intravenous injection. Imaging at 24 hours after injection shows blood pool activity (A, B and C) in the heart (H), lungs (L) and vessels (V) as well as antibody uptake by the liver (LI), spleen (S), kidneys (K) and bladder (B) (C and D).
(A and C anterior views, B and D posterior views).

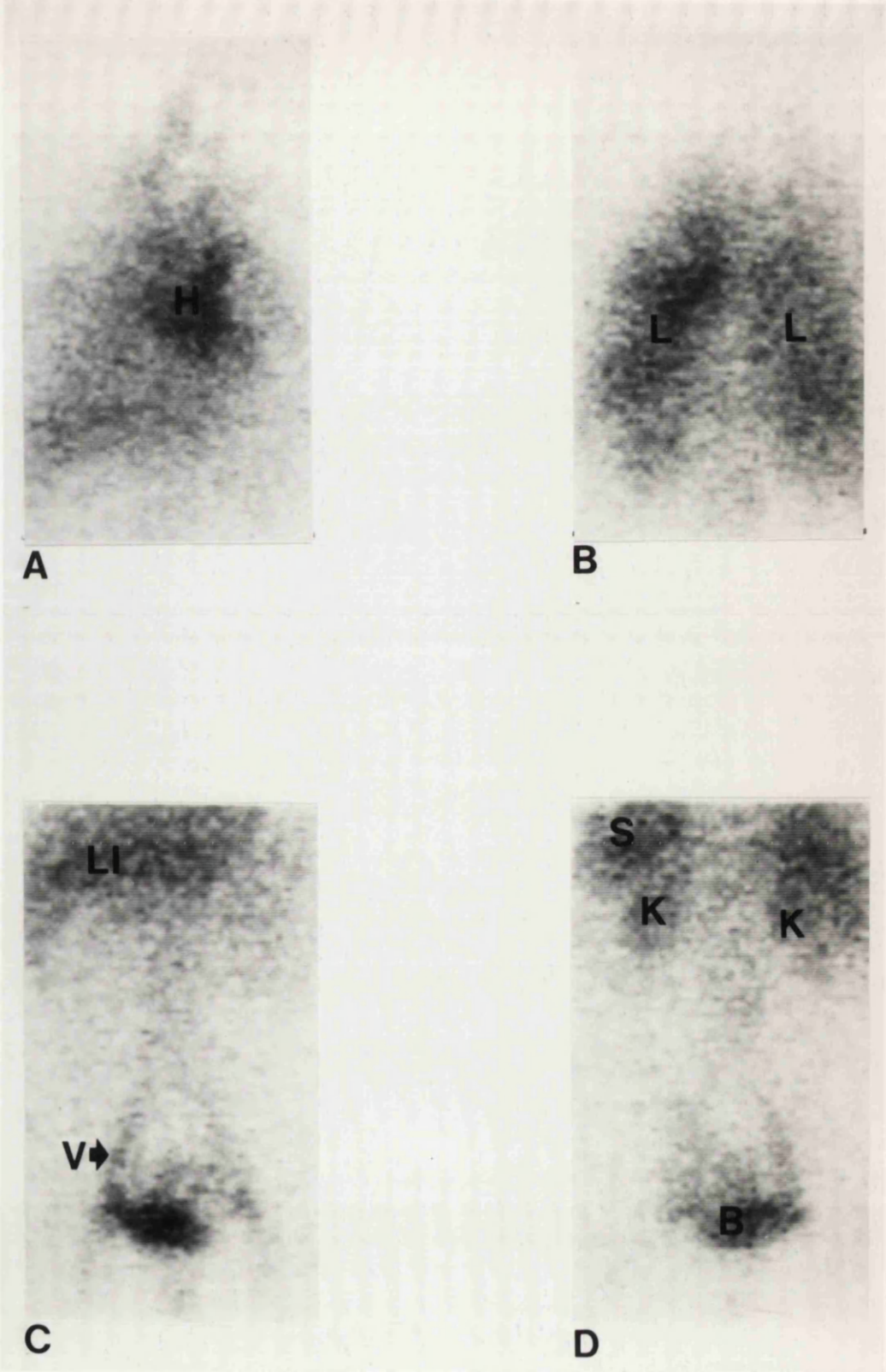

H
A
L
L
B
LI
V
C
S
K
K
B
D

Figure 5.3. Imaging studies: ^{123}I labelled SM3 - mastectomy specimen. Antibody uptake is identified in low (L) middle (M) and high (H) groups of axillary lymph nodes, as well as in the breast itself (BR).

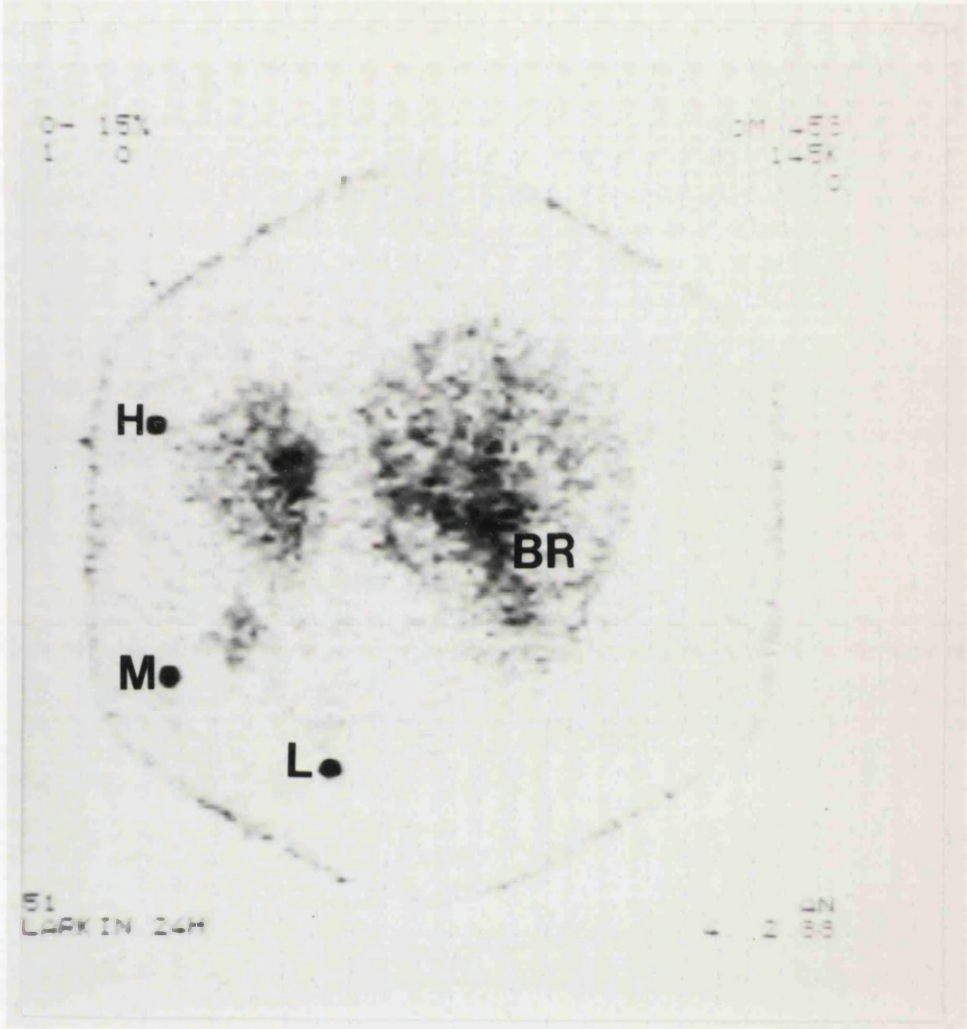

He
BR
Me
Le

Table 5.3

RADIOACTIVITY COUNTED IN TISSUE FROM PATIENTS INJECTED WITH ^{123}I LABELLED SM3: INTRAVENOUS ROUTE

Patient Number	Pathological Nodal Status (number positive and total number)	COUNTED RADIOACTIVITY (% injected dose per gram) Nodes (mean values) +	Nodes (mean values) -	Normal Breast	Residual Tumour
5	Negative (21)	None	0.0024	0.0051	NP
6	Positive (14 of 23)	0.0069	0.0038	NC	NC
7	Positive (1* of 18)	0.0035	0.0098	0.0118	NP
8	Positive (27 of 34)	0.00158	0.00094	0.00079	0.00203
9	Negative (28)	None	0.00147	0.00040	NP

NC = not counted
NP = not present
* tiny metastasis in lymphatic channel adjacent to the node

In this group, there was only one specimen of breast tissue which contained residual tumour. In this case (patient number 8) radioactivity was higher in tumour than in the corresponding normal breast tissue (Table 5.3). As there was only one tumour sample, statistical analysis was not possible in this group.

In summary therefore, using ^{123}I labelled SM3 administered by either the subcutaneous or the intravenous route, we have been unable to distinguish between involved and non-involved nodes, either by imaging or counting of radioactivity. Neither have we been able to detect a difference between the amount of antibody taken up by residual tumour and normal breast tissue.

The half life of ^{123}I (13 hours) allows assessment of early uptake of antibody. It is possible, however, that activity which occurs early may be non-specific and that specific antibody binding to tumour is delayed. It was therefore decided to use SM3 labelled with ^{125}I which due to its longer half life (60 days) is suitable for assessment of late uptake.

C. ^{125}I labelled SM3 administered by subcutaneous injection

Of the eight patients in this group, six had axillary node metastases (Table 5.4). Although the mean radioactivity in involved nodes is marginally higher than in non-involved in one patient (number 13), in patient 11 the figures are similar and in patients 14,15,16 and 17 the reverse is true. The pooled data shows mean figures of 0.0042 for involved and 0.0064 for non-involved nodes i.e. no significant difference (Student's t-test $p > 0.1$). In the breast tissue, in patients 13, 16 and 17 the radioactivity in tumour is greater than that in the normal tissue and in patient 14 the results are very similar; the pooled data gives figures of 0.00159 (tumour) and 0.00106 (normal). There is no significant difference between the two (Student's t-test $p > 0.1$).

Table 5.4

RADIOACTIVITY COUNTED IN TISSUE FROM PATIENTS INJECTED WITH ^{125}I LABELLED SM3: SUBCUTANEOUS ROUTE

Patient Number	Pathological Nodal Status (number positive and total number)	COUNTED RADIOACTIVITY (% injected dose per gram) Nodes (mean values) +	Nodes (mean values) -	Normal Breast	Residual Tumour
10	Negative (23)	None	0.0044	NP*	0.0018
11	Positive (1 of 19)	0.0105	0.013	0.0042	NP
12	Negative (36)	None	0.0107	NC	NC
13	Positive (9 of 14)	0.0018	0.00087	0.000875 mean**	0.00248 mean**
14	Positive (8 of 20)	0.0039	0.0087	0.0010	0.0016
15	Positive (1 of 15)	0.0016	0.0045	0.0032	NC
16	Positive (16 of 22)	0.0027	0.0056	0.00052	0.0013
17	Positive (1 of 23)	0.0019	0.0049	0.00030	0.0018

NC = not counted
NP = not present
* specimen macroscopically thought to be normal but microscopically showed in situ carcinoma
** several pieces of tissue were counted from this case as the extent of tumour involvement was not obvious macroscopically

D. ^{125}I labelled SM3 administered by intravenous injection

Of the eight patients in this group, three had axillary nodal metastases (Table 5.5). The mean radioactivity in involved and non-involved nodes is very similar in patients 20 and 22; in patient 24 there is marginally more radioactivity in non-involved than in involved nodes. The pooled data gives figures of 0.0013 for negative nodes and 0.0014 for positive nodes. There is no significant difference between these figures (Student's t-test $p > 0.1$).

The data on normal breast tissue versus residual tumour does however show more consistent differences in individual patients. In patients 18,19, 21 and 23 there is higher uptake in tumour than in normal breast tissue (the ratios of radioactivity in tumour: normal being 1.6:1, 3.4:1, 2.3:1 and 1.7:1). The pooled data gives figures of 0.0026 and 0.0009 for tumour and normal which are significantly different (Student's t-test $p < 0.01$). The pooled ratio of antibody uptake by tumour and normal tissue is 2.9:1.

II PHARMACOKINETICS

Pharmacokinetic data obtained from patients injected with ^{123}I labelled SM3 was analysed by Sarah Allen in the Department of Nuclear Medicine, Guy's Hospital. Results showed that for the subcutaneous route of administration, 26% of the total activity of the injected SM3 was detected in the urine at 24 hours. The corresponding value for the intravenous route was 35%. The half life of SM3 in the circulation was found to be approximately 24 hours.

Table 5.5

RADIOACTIVITY COUNTED IN TISSUE FROM PATIENTS INJECTED WITH ^{125}I LABELLED SM3: INTRAVENOUS ROUTE

Patient Number	Pathological Nodal Status (number positive and total number)	COUNTED RADIOACTIVITY (% injected dose per gram) Nodes (mean values) +	Nodes (mean values) -	Normal Breast	Residual Tumour
18	Negative (22)	None	0.00090	0.00092	0.0015
19	Negative (35)	None	0.00032	0.00010	0.00034
20	Positive (1* of 24)	0.00061	0.00062	NC	0.0031
21	Negative (20)	None	0.0013	0.0015 mean**	0.0035 mean**
22	Positive (3 of 19)	0.00026	0.00021	0.0047 mean	NP
23	Negative (34)	None	0.0029	0.00075	0.0013
24	Positive (2 of 32)	0.0018	0.0024	NC	NC
25	Negative (36)	None	0.0016	0.0037	NC

NC = not counted
NP = not present
* very small deposit of tumour
** several pieces of tissue were counted from this case as the extent of tumour involvement was not obvious macroscopically

III HISTOLOGY AND IMMUNOHISTOCHEMISTRY

A. Histology

The primary tumours from the patients studied were of the following histological types: 21 infiltrating ductal, two infiltrating lobular and two mucoid carcinomas. In 14 of the patients the entire tumour had been removed macroscopically at the time of biopsy, in six the initial diagnosis had been made by incision biopsy and in five by needle-core biopsy. In 11 patients, therefore, there was a large amount of residual tumour in the breast.

In 14 of the 25 patients, metastatic carcinoma was found in the axillary lymph nodes. The average number of nodes examined per patient was 25 and the number of involved nodes varied from one to 27. The extent of involvement also varied; some nodes contained only a few clumps of malignant cells whereas others were totally replaced by tumour which sometimes extended beyond the lymph node capsule.

B. Immunohistochemistry

Immunohistochemical staining with SM3, using the indirect immunoperoxidase method described in Chapter 2 was performed on all the primary carcinomas. Methacarn fixed tissue was available for study from all but two of the primary carcinomas; in these formalin fixed tissue was used. Any lymph nodes found to contain metastases were also stained; these had all been fixed in formalin.

Twenty-three of the 25 primary mammary carcinomas stained with SM3. As in the breast tumours described in Chapter 3, both the distribution and intensity of staining varied both from one tumour to another and within the same tumour. The two cases which failed to stain with SM3 were carcinomas which had been fixed in formalin. As discussed in Chapter 3 and illustrated in Figure 3.1, staining is often very much weaker in tissue fixed in this way than when alcohol based fixatives are used, and it is possible that these two cases would have been SM3 positive if methacarn fixed or frozen material material had been available.

In eight of the 14 patients whose lymph nodes contained metastatic carcinoma, the malignant cells stained with SM3. As in the primary tumours heterogeneity of staining was marked and in general the intensity low but this was probably due to fact that the tissue had been fixed in formalin.

Using the one-stage direct immunoperoxidase method, no radiolabelled antibody was demonstrated either within metastatic tumour cells or elsewhere in the nodes. The most likely explanation for this is that the amount of antibody reaching each node is too low to be detected in this way. Alternatively, the antibody may be altered by the formalin fixation process and therefore unreactive with the rabbit anti-mouse antibody used for its detection. Unfortunately no suitable frozen material was available for use in the one-stage staining method.

IV IMMUNOREACTIVITY OF LABELLED AND UNLABELLED ANTIBODY

Work by Dr. S. Mather in the Department of Nuclear Medicine Research at St. Bartholomew's Hospital showed that the immunoreactivity of SM3 was not altered by the iodination process. In an ELISA assay to compare the activity of iodine labelled SM3 with that of unlabelled antibody identical results were seen when each was tested against stripped mucin (Mather S personal communication).

5.4 DISCUSSION

Although the results described above have proved disappointing in terms of localisation of metastatic tumour, many lessons have been learnt and an ideal model system for future immunolocalisation studies in patients with breast cancer has been established. Ideas for ways to improve the specificity and sensitivity of such studies will now be considered.

The most valuable lesson learnt from this work was the importance of careful and detailed pathological analysis of resected tissue, and correlation of this information with imaging and counting data. The value of such comparison was stressed in our study by the finding that in some cases tissue was selected for counting which although thought macroscopically to contain tumour, proved to contain no malignancy microscopically; the reverse was also true - in one case macroscopically normal tissue proved to contain extensive in situ carcinoma. Without careful microscopic examination of the counted tissue, these samples could have produced misleading results. Previous studies which have imaged axillary nodes have usually quoted false and true positives based on a comparison of imaging data with an overall pathological assessment of the axilla (Tjandra et al 1989a, Mandeville et al 1986). In our detailed protocol, comparison of counts with histological examination in either individual or small groups of nodes has improved the accuracy of the data.

In this study, although with ^{125}I labelled SM3 administered by the intravenous route there was almost a three fold greater uptake by breast tumour than by normal breast tissue, there was no significant difference in uptake in involved and non-involved axillary nodes using either isotope of iodine administered by either route of injection. Factors which limit localisation of tumours with radiolabelled antibodies are numerous (Bradwell et al 1985). Some have been mentioned in the introduction to this chapter. Those which are patient dependent e.g tumour area, tumour depth and the patient's body thickness at the site of the tumour clearly cannot be altered. Others which are amenable to alteration include properties of the antibody and properties of the isotope. Changing these may improve the ratio of uptake of antibody by tumour to that by normal tissue.

As mentioned in Section 5.1, for successful immunolocalisation, the antibody used should ideally be tumour specific. In this respect, SM3 would seem to be ideal. The epitope recognised is highly tumour associated and either undectable or present at only very low levels in

normal breast and other tissues. Furthermore, SM3 recognises an epitope which is part of the tandem repeat on the PEM core protein (See Chapter 3.7). One would expect such an epitope to be abundant and therefore easily identified.

In contrast, apparent disadvantages of SM3 include firstly the heterogeneity of expression in breast tumours and secondly the fact that in some malignant cells the antigen is expressed predominantly within the cytoplasm rather than on the cell surface (see Chapter 3.7). These two factors together may limit antibody binding to malignant cells. A third problem lies in the fact that the antibody should ideally be of high affinity and there is some evidence that this is not the case for SM3 (Gendler et al 1988b). Finally, as mentioned previously, a well recognised problem is false positive localisation of antibody (Tjandra et al 1989a). This was observed in our study in normal lymph nodes.

The problems described above which are related to the antibody itself can only be overcome by the production of new antibodies or modifications to SM3 to increase its affinity. These possibilities will be considered in Chapter 6. The problem of false positive localisation may be due to interaction of mouse immunoglobulins with human Fc receptors and this problem may be overcome either by the use of immunoglobulin fragments (without the Fc receptor) or the use of a second antibody (e.g rabbit anti-mouse immunoglobulin) to clear the circulation of non-tumour bound imaging (first) antibody. $F(ab')^2$ fragments have recently been made from SM3 and will be available for use in future studies.

Other factors affecting antibody uptake are tumour blood supply, the rate of binding of antibody to antigen and the presence of circulating antigen which may react with antibody before it reaches the tumour.

As mentioned above, previous reports suggested that local injection of antibody was superior to administration by the intravenous route. In our experience, however, subcutaneous injection of antibody produced no better results than intravenous administration. Although the intravenous route of administration in theory results in antibody dilution, axillary uptake was only marginally higher with subcutaneous than with intravenous injection. The mean totals (expressed as percentage of injected dose reaching the axillary nodes) were 0.04% (intravenous) and 0.07% (subcutaneous) using ^{123}I and 0.01% (intravenous) and 0.04% (subcutaneous) with ^{125}I. Furthermore, the values were similar in node positive and node negative patients. These figures are all very low and the small amount of antibody reaching the axilla may be another factor accounting for our inability to demonstrate differences between involved and non-involved nodes. The experience of other groups who have used SM3 in immunolocalisation studies has been similar (Granowska M, Britton K and Epenetos A, personal communication). In previous studies, groups who reported successful imaging quoted results of the order of 0.6% of injected dose reaching the tumour (Epenetos et al 1982a). In this context, it is possible that the use of a higher dose of antibody may improve results. Preliminary data using higher doses of SM3 are encouraging.

The use of different isotopes has been suggested as a means of improving immunolocalisation results. Isotopes of iodine such as those used in this study have some limitations. Although as described above iodination of SM3 results in no loss of immunoreactivity, the use of ^{123}I is limited because of its short half life and ^{125}I does not permit imaging due to its low photon energy. Indium 111 has been said to be "the most nearly perfect radiolabel" (Tjandra and McKenzie 1988). An ELISA assay performed by Dr. S. Mather has shown that the immunoreactivity of SM3 labelled with indium is somewhat greater than that of iodine labelled SM3; indium may therefore prove useful in future studies. The use of subtracting isotopes to mask the variation in blood supply to different tissues has also been reported but has not been investigated in our system.

Other theoretical methods of enhancing results include improvements in gamma cameras, the use of computerised tomography and data manipulation.

In conclusion, therefore, although we have been unable thus far to successfully use radiolabelled SM3 as an agent for localisation of lymph node metastases, we have established a good model system for evaluating immunolocalisation in breast cancer patients. In the light of the factors discussed above modifications to our initial protocol have now been made and it is hoped that more specific results will follow.

Chapter 6

SUMMARY AND SUGGESTIONS FOR FUTURE WORK

This thesis has described the evaluation of the monoclonal antibody SM3. In this chapter the results and main conclusions will be summarised and ideas for future work with SM3 and its related antigen suggested.

Immunohistochemical studies with SM3 have shown that the antibody recognises an epitope which is highly tumour associated in both breast carcinomas and tumours from other sites. In fully differentiated (secretory) breast tissue, however, the epitope is virtually undetectable. It is present at a low level in normal resting and benign breast tissue and focally present in a small number of other normal tissues.

Immunoblotting studies have shown that in both breast cancer cell lines and fresh material from primary mammary carcinomas SM3 recognises an epitope which is expressed predominantly on low molecular weight components.

As discussed in Chapters 3.7 and 4, both the immunohistochemical and immunoblotting findings are consistent with the idea that the SM3 epitope is expressed only on incompletely glycosylated mucin molecules. Although this occurs predominantly in malignancy, the low level of SM3 reactivity seen in normal resting breast tissue, benign breast lesions and a small number of normal tissues from other sites suggests that a small amount of the mucin produced in these tissues is also incompletely glycosylated.

Immunolocalisation studies with SM3 have proved disappointing in terms of developing a useful clinical test. Lessons have, however, been learnt and as discussed in Chapter 5, modifications to try and improve the sensitivity and specificity of this technique have been made. The model system described in the thesis is ideal for the future evaluation of newly developed or modified monoclonal antibodies.

As a result of the work described in this thesis we now understand more about the immunophenotype of normal and malignant mammary epithelial cells with regard to mucin expression. This information together with the cloning of the Polymorphic Epithelial Mucin (PEM) gene and the subsequent elucidation of the SM3 and HMFG2 epitopes has enabled us to understand more about the nature of PEM and its processing in normal and malignant epithelial cells. It is important to note that the core protein is the same in the mucin produced by normal and malignant mammary epithelial cells. It is, however differently glycosylated by the two cell types.

Where should future work with SM3 be directed? Can any of the lessons learnt through working with SM3 point to new areas of research?

There are three main areas in which future work with SM3 will be conducted. 1) Pathology/Immunohistochemistry, 2) Molecular Biology and 3) Imaging.

Future pathological and immunohistochemical studies fall into two main groups; studies in the breast and in tissues from other sites. In the breast there are three main questions to be addressed. Firstly, as the SM3 epitope is only revealed when glycosylation is abnormal, it is possible that SM3 staining may be of prognostic significance. Carcinomas which are SM3 negative but stain positively with other antibodies to PEM are those which produce a relatively "normal" mucin and may be associated with a good prognosis. Similarly, carcinomas which stain very strongly with SM3 may be associated with a poor prognosis. As SM3 staining appears to be independent of other pathological predictors of prognosis such as tumour grade (see Chapter 3.1 I D) this would be an interesting theory to pursue; as yet, however, we do not have a long enough follow-up period on any of the cases studied. Secondly, as discussed in Chapter 3.7, as the SM3 epitope is highly tumour associated, one might expect staining in benign breast disease to be particularly strong in lesions which are associated with an increased risk of subsequent malignancy

e.g. severe and atypical epithelial hyperplasia and multiple papillomas. Although such a clear relationship has not so far emerged, future studies in this area are warranted. Thirdly, again as mentioned in Chapter 3.7, it would be interesting to know whether areas of SM3 positivity in normal breast tissue are related in any way to proliferative activity. This question could be answered either by immunohistochemical studies with antibodies such as Ki67 or PCNA which are markers of proliferation, or by flow cytometry studies. At sites outside the breast, the number of primary carcinomas so far studied is small; further work in this area would be of interest as would the immunohistochemical evaluation of benign lesions from the colon and ovary.

Molecular biology studies are ongoing; these aim to address several specific questions. Firstly, now that the gene for the PEM has been cloned, the factors controlling its expression are being studied. Secondly, in situ hybridisation studies using an oligonucleotide probe corresponding to the tandem repeat of the PEM are planned. These will aim to discover whether the heterogeneity of staining in breast tumours is also seen at the message (RNA) level or whether it arises due to differences in efficiency of translation.

With regard to imaging studies, some modifications to improve the sensitivity and specificity of this work have been suggested in Chapter 5. These include increasing the dose of antibody, the use of antibody fragments and the use of different radioisotopes. Even with such modifications, it is possible, however, that results will not be improved until antibodies with similar tumour specificity but higher affinity than that of SM3 can be produced.

With regard to increasing the affinity of SM3 two approaches are being used at the moment. Firstly, the variable regions of the SM3 and HMFG2 antibodies have been cloned and site directed mutagenesis will be used to change specific amino acids in the SM3 sequence which might

result in changes in affinity (Allen D. personal communication). In parallel, the SM3 variable region is being tested for its ability to react as a "single domain antibody" (dAb) (Ward et al 1989). If the small polypeptide proves to be active in binding to antigen, it will be very easy to test a large number of mutated dAbs for increased affinity. Moreover, a smaller SM3-type antibody could be extremely useful for imaging, provided the affinity is high. A second approach to selecting a high affinity antibody reactive with the cancer associated mucin is to use a mouse tumour cell transfected with the human mucin gene to induce antibodies in a syngeneic host. Such transfected lines expressing PEM on the surface and as a secreted product are now available (Lalani E and Taylor-Papadimitriou J personal communication).

The confirmation of the theory of abnormal glycosylation of the PEM in malignant cells has prompted interest in the production of new monoclonal antibodies perhaps using the synthetic peptide from the PEM core protein as an immunogen. It is hoped that in the future antibodies with the specificity of SM3 will be useful in the diagnosis and possibly even in the therapy of breast and other carcinomas.

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PUBLICATIONS

Papers previously published by the author relevant to the studies described in this thesis will be found in a pocket inside the back cover. These are as follows:

Burchell J, Gendler S, Taylor-Papadimitriou J, Girling A, Lewis A, Millis R and Lamport D (1987). Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. Cancer Res 47 5476-5482.

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A CORE PROTEIN EPITOPE OF THE POLYMORPHIC EPITHELIAL MUCIN DETECTED BY THE MONOCLONAL ANTIBODY SM-3 IS SELECTIVELY EXPOSED IN A RANGE OF PRIMARY CARCINOMAS

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The monoclonal antibody (MAb) SM-3, which was raised to chemically deglycosylated milk mucin, reacts with an epitope present on the core protein of this mucin which we have referred to as PEM (polymorphic epithelial mucin). Although this mucin is abundantly expressed by both the lactating breast and breast carcinomas, the antibody SM-3 shows very little or no reactivity on the former but does react with 92% of breast carcinomas. Furthermore, SM-3 stains primary carcinomas of the lung, colon and ovary, but on the corresponding normal tissue the epitope is expressed at a much reduced level or not at all. These results indicate that an epitope masked in the normal mucin is exposed in the mucin produced by tumour cells, perhaps due to aberrant glycosylation. An extensive immunohistochemical study of other normal tissues reveals that the majority show only weak focal staining with SM-3 or none at all, the distal tubules and collecting ducts of the kidney, and sebaceous glands being the only normal tissues studied to show homogeneously positive staining.

Among the large number of MAbs in routine diagnostic use are those directed against antigens on the membranes of epithelial cells, many of which are now known to recognize different epitopes on high-molecular-weight glycoprotein molecules or mucins. As mucins are produced by both normal epithelial cells and cancers of epithelial origin from a variety of anatomical sites, MAbs to them can show reactivity with more than one tissue or tumour type. Many MAbs including HMFG-1 and HMFG-2 (Taylor-Papadimitriou *et al.*, 1981; Burchell *et al.*, 1983) recognize different, tumour-associated epitopes on a high-molecular-weight (>400 kDa) glycoprotein molecule found in human milk (Burchell and Taylor-Papadimitriou, 1989). This milk mucin has been termed PAS-0 by Shimizu and Yamauchi (1982), who first purified it, EMA (epithelial membrane antigen) by Heyderman *et al.* (1979), MAM-6 by Hilkens *et al.* (1984) and NPG (non-penetrating glycoprotein) by Ceriani *et al.* (1983). Because the mucin exhibits a high degree of polymorphism at both the protein and DNA levels (Swallow *et al.*, 1987; Gendler *et al.*, 1987) we refer to this mucin as PEM [polymorphic epithelial mucin (Taylor-Papadimitriou and Gendler, 1988)].

Immunohistochemical studies with the HMFG-1 and HMFG-2 MAbs have shown that, while they react with many mammary carcinomas, they also show strong reactivity with secretory breast tissue and benign tumours and, to a lesser degree, with resting breast and some other epithelial tissues (Arklie *et al.*, 1981; Taylor-Papadimitriou *et al.*, 1986). Other MAbs to different epitopes on the PEM mucin show similar patterns of reactivity (Foster *et al.*, 1982; Price *et al.*, 1985; Burchell and Taylor-Papadimitriou, 1989). We have recently described the production of MAbs to the chemically deglycosylated core protein of the polymorphic epithelial mucin (Burchell *et al.*, 1987), and preliminary immunohistochemical studies with one of these antibodies, SM-3, gave particularly interesting results. In marked contrast to HMFG-1 and HMFG-2, virtually no reactivity was seen in normal secretory or resting breast tissue, although 92% of primary mammary carcinomas showed positive staining with the antibody. We have now extended these preliminary observations to see whether a similar differential expression of the SM-3 epitope is seen in other epithelial tissues and tumours and how extensively the determinant is expressed on normal tissues. Here we report the results of an immunohistochemical study on staining with SM-3 of primary carcinomas of the colon, lung and ovary as well as of normal tissue from these and other sites. We also report the results of further work on primary breast carcinomas and a large number of normal and secretory breast biopsies.

MATERIAL AND METHODS

Selection of tissues

Most normal tissue was obtained at autopsy, but in some instances it was selected from surgical specimens. Autopsy tissue was obtained within hours of death to minimize autolysis. However, if any signs of autolysis were seen in preliminary examination of sections stained with haematoxylin and eosin, these specimens were not used. Obtaining well-preserved autopsy specimens from the gastro-intestinal tract and the lung was particularly difficult and these were therefore obtained as surgical specimens. In some of these latter a tumour was present and in these cases, the tissue selected was from a site as far away from the tumour as possible. Carcinoma tissue was obtained from specimens sent for routine diagnostic purposes to the Histopathology Laboratories at the following London hospitals: Guy's, The Brompton, University College and St. Mark's.

Antibody

The development and characterization of the SM-3 MAb has been described by Burchell *et al.* (1987). Briefly, it was raised against deglycosylated mucin (purified by immunoaffinity chromatography from human skimmed milk) and was selected for its reactivity with partially and totally stripped mucin and lack of reactivity with intact mucin. It is an MAb of the IgG1 class and expresses a λ light chain (Burchell, pers. commun.).

Staining method

A conventional indirect immunoperoxidase technique was used as described by Bartek *et al.* (1985). Initially, tissue was fixed in a variety of different fixatives, so that the effect of different methods of fixation could be assessed and compared to staining of unfixed frozen tissue. These studies showed that methacarn (methanol:chloroform:acetic acid 60:30:10) was the most satisfactory fixative for use with SM-3. Tissues and tumours were therefore fixed in methacarn, processed routinely, embedded in paraffin wax and cut in 3- to 4-μm sections for staining.

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In all cases, the results of staining with SM-3 were compared with those seen with HMFG-2, staining being performed in parallel. A positive control (a primary breast carcinoma showing a uniform pattern of staining with SM-3) was included with each batch of slides to ensure reproducibility of results from one occasion to another. Negative controls (in which PBS buffer replaced the primary antibody) were included for each case.

RESULTS

Fixation of tissues and tumours

No differences in the staining patterns obtained with SM-3 were observed when methacarn-fixed and frozen tissues or tumours were used. In several tumours and tissues, the effect of 3 different formalin fixatives was examined. Acid formalin containing 40% methanol (methanol: 40% formalin:acetic acid:water 8:2:1:9) was as good as methacarn in preserving the SM-3 determinant, but use of neutral buffered formalin (4% formaldehyde in 0.15 M NaCl) or acid formalin (40% formaldehyde:acetic acid:distilled water 2:1:17) resulted in a reduction in the degree of staining with SM-3. Assessment of other fixatives showed that Carnoy's and Bouin's gave comparable results to methacarn fixation. In the studies reported here, all tissues and tumours were fixed in methacarn.

Staining of breast tissues and tumours

A pilot study suggested that SM-3 was highly selective in showing positive staining of primary and metastatic breast cancers, while staining of normal breast tissue was weak or undetectable. We have extended these initial observations to include 122 primary carcinomas, 17 normal resting breast biopsies and 13 secretory breast biopsies.

Table I shows the results of staining the 122 primary carcinomas with SM-3. In keeping with previously published results (Burchell *et al.*, 1987) the overall incidence of SM-3 positivity was 92%. Tumours of different histological types and grades were examined but no difference in staining pattern was seen according to either feature. In many of the tumours the staining pattern was strong but heterogeneous. In some cases staining was concentrated on the cell membranes, although cytoplasmic staining was present to a greater or lesser extent in all cases (Fig. 1*a*). In tumours which showed glandular differentiation, the strongest staining was seen at the surface of cells lining the glandular lumina.

When compared with HMFG-2, which stained 121 of the 122 tumours, staining with SM-3 was weaker and more heterogeneous in 103 cases (84%), a similar pattern being seen in 19 cases. The staining with HMFG-2, although usually more intense, was similar in distribution to that described for SM-3. One tumour (a metaplastic carcinoma) failed to stain with either antibody.

TABLE I – PRIMARY CARCINOMAS OF THE BREAST: RESULTS OF IMMUNOHISTOCHEMICAL STAINING

Histological type	Total number	Number SM-3-positive	Number HMFG-2-positive
Infiltrating ductal			
Grade I	7	6	7
Grade II	41	37	41
Grade III	29	29	29
Infiltrating lobular	29	28	29
In situ carcinoma			
Ductal	3	3	3
Lobular	1	1	1
Special types			
Mucoid	3	1	3
Medullary	4	4	4
Tubular	2	2	2
Adenoid cystic	2	1	2
Metaplastic carcinoma	1	0	1
Total	122	112 (92%)	

Examination of normal breast tissue showed that, of 17 normal resting breast biopsies, 9 showed no staining with SM-3 and in the remaining 8 the staining observed was weak and focal. Of 13 biopsies showing secretory changes, 5 were SM-3 negative. In the remaining 8, any positivity observed was extremely weak and focal, being confined to only very small areas of the section. This pattern is in marked contrast to that seen with HMFG-2 which stains secretory tissue strongly and homogeneously, and stains 50–60% of lobules in the resting breast.

As we have previously reported, most benign breast lesions showed either no reaction or weak and focal staining with SM-3, the strongest staining pattern being seen in epithelium showing apocrine metaplasia (Burchell *et al.*, 1987).

Staining of colonic tissues and tumours

Seventeen primary tumours of the colon and rectum were studied. All were moderately differentiated adenocarcinomas and all showed some positivity with SM-3. The pattern and intensity of staining, however, showed some variation. In 6 tumours the staining was homogeneous, strong and similar to that seen with HMFG-2. In the other 11, staining with SM-3 was heterogeneous and less intense than with HMFG-2. In these cases the strongest staining with both MAbs was noted at the luminal edges of cells lining glandular structures, whereas in the uniformly staining cases the intensity of staining in the cytoplasm and at the luminal margins was similar (Fig. 1*b*).

Although staining with SM-3 of the normal mucus-secreting epithelium of the colon could be detected, this was consistently extremely weak, in marked contrast to the strong staining seen in the colonic carcinomas (Table III).

Staining of lung tissues and tumours

In the 15 primary lung tumours studied, the pattern and intensity of staining showed some variation with histological type. The strongest reactivity was seen in adenocarcinomas (Fig. 1*c*). Tumours of neuroendocrine origin either failed to stain or showed weak positivity in a few cells only, and 2 undifferentiated large-cell carcinomas showed a similar pattern, while an intermediate level of staining was seen in squamous carcinomas. The staining patterns seen in the lung tumours are summarized in Table II.

When the results with SM-3 and HMFG-2 were compared, the latter stained a greater proportion of the total number of tumours and in general the staining was stronger with HMFG-2. In many cases, however, the staining pattern with both antibodies was heterogeneous and the intensity of staining varied between different areas of the same tumour.

Examination of 5 samples of normal lung tissue showed very weak staining of the bronchial epithelium, and heterogeneous staining of pneumocytes in the alveoli. The mesothelium lining the pleura was consistently negative (see Table III).

Staining of ovarian tissues and tumours

All 5 primary carcinomas of the ovary which were examined stained positively with SM-3 (Fig. 1*d*). Two of the tumours were poorly differentiated adenocarcinomas and, in these, staining with SM-3 was similar to staining with HMFG-2. In 1 case the pattern was heterogeneous but in the other more homogeneous. Of the other 3 tumours, one (a mesonephroid carcinoma) showed a similar staining pattern with the 2 MAbs but in the others (1 mucinous and 1 serous adenocarcinoma), stain-

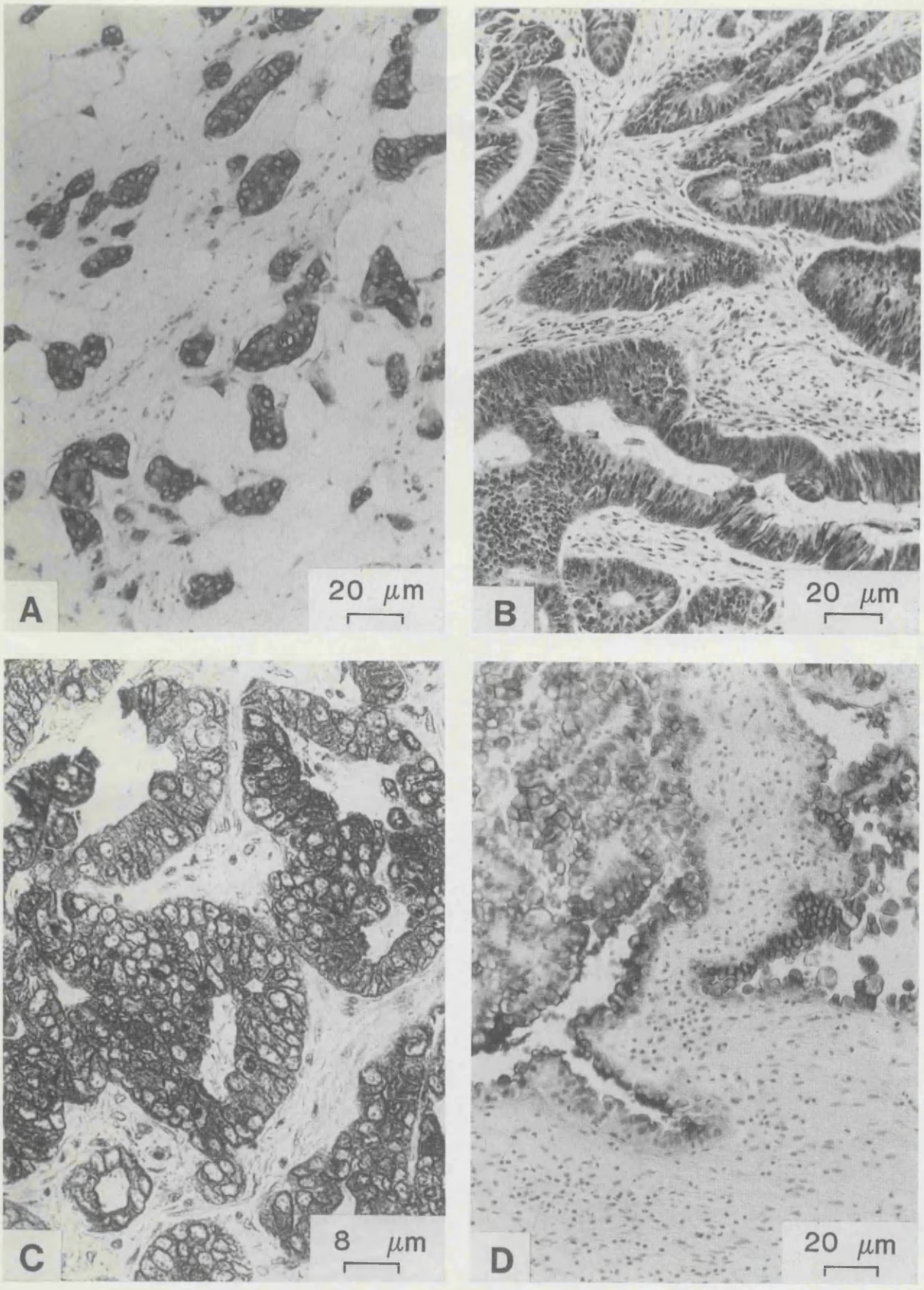

FIGURE 1 – Immunoperoxidase staining with SM-3 of primary carcinomas. *(a)* Infiltrating ductal carcinoma of breast; *(b)* adenocarcinoma of colon; *(c)* adenocarcinoma of lung; *(d)* mesonephroid carcinoma of ovary.

TABLE II – PRIMARY CARCINOMAS OF COLON, LUNG AND OVARY: RESULTS OF IMMUNOHISTOCHEMICAL STAINING

Tissue and tumor type (Total number of cases)	Number of cases	SM-3-positive	HMFG-2-positive
Colon and rectum (17)		17	17
Adenocarcinoma	17	17	17
Lung (15)		10	13
Squamous carcinoma	4	3	4
Adenocarcinoma	5	5	5
Carcinoid tumours	3	1 (weak)	2 (weak)
Large-cell undifferentiated	2	1	2
Spindle-cell	1	0	0
Ovary (5)		5	5
Adenocarcinoma	2	2	2
poorly differentiated mucinous carcinoma	1	1	1
serous carcinoma	1	1	1
mesonephroid carcinoma	1	1	1

ing with SM-3 was more heterogeneous and less intense than with HMFG-2.

The surface epithelium of normal ovarian tissues taken from 15 different individuals showed no positive staining with SM-3.

Staining of other normal tissues

The results of staining normal human tissues from a variety of sites with the antibody SM-3 are summarized in Table III. Reactivity was seen only within epithelial structures and was never observed in tissues of either mesenchymal or lymphoid origin. The only tissues showing a homogeneously positive pattern of staining were the distal tubules and collecting ducts of the kidney and the sebaceous gland. Any other positive reaction was generally focal and often weak.

DISCUSSION

The immunohistochemical study described here confirms the earlier observation that the SM-3 reactive epitope on the core protein of human polymorphic epithelial mucin is selectively unmasked in breast cancers, and only marginally detectable on the mucin produced by the normal gland. The SM-3 antibody was raised against the deglycosylated mucin which was first purified in its native form from milk by immunoaffinity chromatography using the HMFG-1 antibody. Since the SM-3 antibody was selected for lack of reaction with the fully glycosylated mucin, it is not surprising that it shows a negative reaction with the pregnant or lactating gland which produces this mucin. The positive reaction of SM-3 with breast cancers suggests that the mucin is aberrantly glycosylated in malignant cells, resulting in the unmasking of the SM-3 epitope.

Support for this idea comes from recent studies analyzing the structure of the oligosaccharide side-chains of the mucin purified from human milk (Hanisch *et al.*, 1989) and of the mucin produced by a breast cancer cell line, BT20 (Hull *et al.*, 1988); the normal mucin contains extended poly-N-acetyl-lactosamine side chains (made up of 4–14 sugars), while the BT20 mucin has short chains made up of only 3–4 sugars. Clearly, the longer chains might be expected to mask larger stretches of the core protein than would be masked by the short chains, and core protein epitopes would consequently be selectively exposed in the cancer-associated mucin.

The selective reactivity of the SM-3 antibody with breast cancers has identified a difference between the normal and malignant mammary epithelial cell in mucin processing. It was of interest, therefore, to see whether a similar difference is found between other normal and malignant epithelial cells,

TABLE III – NORMAL TISSUES: RESULTS OF IMMUNOHISTOCHEMICAL STAINING WITH SM-3

Tissue (Number of cases studied)	Pattern of staining[3]		
	Uniformly positive	Weak and/or heterogeneous	Uniformly negative
Breast			
Resting (9)			−
Resting (8)		±	
Secretory (5)			−
Secretory (8)		−/+	
Lung (5)			
Bronchial mucosa		−/+	
Alveoli (type 1 and 2 pneumocytes)		+/−	
Pleura			−
Colon (10)[1]		−/+	
Ovary (15)			−
Rete ovary (1)		+/−	
Fallopian tube		+/−	
Skin			
Interfollicular epidermis (6)			−
Sebaceous gland (6)	+		
Hair follicle (3)			−
Sweat gland (eccrine) (3)		+/−	
Endometrium (5)		±	
Endocervix (4)		±	
Ectocervix (4)			−
Vagina (1)			−
Vulva (1)			−
Placenta (1)			−
Prostate (3)		+/−	
Testis (2)			−
Epididymis (1)		−/+	
Spermatic cord (2)		+/−	
Kidney (3)			
Glomeruli			−
Proximal tubules			−
Distal tubules	+		
Collecting ducts	+		
Urinary bladder (1)			−
Ureter (1)		±	
Thymus (2)			−
Adrenal (1)			−
Thyroid (2)			−
Pancreas (3)			
Acini		±	
Ducts			−
Islets of Langerhans			−
Liver (3)			−
Gall bladder (3)		+/−	
Tongue (2)			−
Oesophagus (2)			−
Stomach[2] (3)			
Surface epithelium		−/+	
Glands		+/−	
Small intestine (1)			−
Appendix (1)			−
Salivary gland			
Parotid (5)		+/−	
Submandibular (2)		+/−	
Sublingual (4)			−
Larynx (1)			−

[1]Tumour-associated tissue in 2 cases.–[2]Tumour-associated tissue in 1 case.–[3]+ Uniformly positive; − uniformly negative; −/+ very weak, barely detectable; ± generally very weak and focal; +/− stronger foci, but very heterogeneous in distribution.

Development and Characterization of Breast Cancer Reactive Monoclonal Antibodies Directed to the Core Protein of the Human Milk Mucin

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ABSTRACT

A mucin molecule, which has a molecular weight of greater than 400,000 and which carries tumor associated epitopes recognized by monoclonal antibodies HMFG-1 and HMFG-2, has been purified from human skimmed milk by affinity chromatography followed by passage through a size exclusion column. While treatment of the mucin with hydrogen fluoride for 1 h at 4°C removed the peripheral oligosaccharides, treatment with HF for 3 h at room temperature removed all of its lectin binding ability and revealed a dominant polypeptide of about 68,000. This appears to be the size of the mucin core protein. Monoclonal antibodies have been developed that react with the stripped and partially stripped molecule but not with the intact mucin. From the initial screening on histological sections one of these antibodies, SM-3, reacts with 91% of breast carcinomas but shows little or no reactivity on benign mammary tumors, normal resting, pregnant, or lactating breast. It appears that this monoclonal antibody is reacting with an epitope that is usually masked by oligosaccharide moieties in normal cells but which is exposed, perhaps due to aberrant glycosylation, in malignant cells.

INTRODUCTION

Many of the monoclonal antibodies raised against carcinoma cells or human milk fat globule membranes have been shown to react with epitopes present on large molecular weight glycoproteins (1–7). It has recently been shown that many of these antibodies react with the same group of molecules (8), which appear to be mucins. One such component, which contains about 50% carbohydrate oxygen linked to serine and threonine and has a molecular weight of greater than 400,000, is found in large amounts in the human milk fat globule membrane and is secreted into milk (9, 10). These mucin glycoproteins are also secreted by a number of other normal epithelial cells (11, 12).

The monoclonal antibody HMFG[1]-1 (13) (original nomenclature 1.10.F3) is highly reactive with the milk mucin, and evidence suggests that the epitope recognized by this antibody is abundant on the fully processed mucin, characteristic of normal differentiation (14, 15). In tumors, the molecular weight of the molecules carrying the antigenic determinants differs among individual tumors and in the case of the components recognized by the HMFG-2 antibody (13) (original nomenclature, 3.14.A3) can range from 80,000–400,000 (1). Although it appears that the differences observed in the mobility of the high molecular weight bands are due to genetic polymorphism (12), this probably does not explain variations in the size of the lower bands observed. It has been proposed that these may be the result of aberrant processing occurring in the tumor cell possibly within the glycosylation pathways (1).

For the majority of the monoclonal antibodies reacting with this group of molecules, the exact nature of the antigenic epitopes remains undefined, although circumstantial evidence has suggested that carbohydrate may at least be partly involved in many of the epitopes. Moreover, from data so far available it is unknown whether the mucin found in the normal differentiated cells and that observed in the tumors contain the same core protein or merely carry common carbohydrate determinants.

The purpose of the present study was to develop monoclonal antibodies to the milk mucin core protein with a view to using the antibodies as tools to study processing of the mucins and to isolate the gene coding for the core protein from an expression library. The question as to whether the mucins produced by many epithelia and by cancers of epithelial origin are coded for by a single gene or by a family of genes could then be addressed. In addition, if aberrant glycosylation of the normal mucin is occurring in the tumor cells, areas of the core may become exposed during transformation, thus unmasking epitopes that may be recognized by antibodies to the core protein.

To this end the mucin was purified from skimmed milk using an HMFG-1 affinity column and stripped of its carbohydrate by treatment with hydrogen fluoride. This preparation was then used as the immunogen for the generation of monoclonal antibodies to the mucin core protein.

MATERIALS AND METHODS

Purification of the Milk Mucin. The milk mucin was purified from human skimmed milk by passage through an HMFG-1 affinity column followed by size exclusion chromatography. The HMFG-1 monoclonal antibody was purified from tissue culture supernatant using a Protein A column (1). The purified antibody was coupled to cyanogen bromide activated Sepharose (Pharmacia) as described in the manufacturer's instructions. Human skimmed milk was passed in batches of 100 ml through the antibody column followed by extensive washing with phosphate-buffered saline (153 mM NaCl-3 mM KCl-10 mM Na_2HPO_4-2 mM KH_2PO_4, pH 7.4). Bound antigen was eluted from the column using 0.1 M glycine, pH 2.5, and the fractions registering absorbance at 280 nm were pooled, dialyzed against 0.25 M acetic acid, and lyophilized. Batches of about 20 mg were dissolved in 0.25 M acetic acid and passed through a G75 Sephadex column (1 x 100 cm) which had been previously equilibrated with acetic acid. The column was washed with 0.25 M acetic acid and 1-ml fractions were collected. The peak fractions which were eluted in the void volume were pooled, lyophilized, and the dry powder stored at 4°C. Amino acid analysis was performed using a Beckman 6300 amino acid analyzer.

Deglycosylation of the Milk Mucin. To remove the oxygen-linked carbohydrate from the milk mucin the molecule was treated with anhydrous hydrogen fluoride as described by Mort and Lamport (16), for either 1 h at 4°C which produced a partially stripped preparation or 3 h at room temperature which produced the extensively stripped mucin.

Iodination of the Milk Mucin. Iodinations using 2.5 μg of protein, of the purified mucin, and the partially or extensively stripped mucin were carried out using the Bolton and Hunter method (17). Free Bolton and Hunter reagent was removed from the reaction by passage through a G25 Sephadex column (PD10 columns; Pharmacia) previously equilibrated in phosphate-buffered saline.

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[1] The abbreviations used are: HMFG, human milk fat globule; WGA, wheat germ agglutinin; PNA, peanut agglutinin; HPA, *Helix pomatia* agglutinin.

Iodination of Lectins. WGA, PNA (Vector Labs), and HPA (Boehringer) were iodinated as described by Karlsson *et al.* (18) using the chloramine-T method.

Polyacrylamide Gels and Western Blots. Polyacrylamide gel electrophoresis and immunoblotting was performed as described (19). For lectin binding studies the Western blots were reacted with the iodinated lectins as described by Swallow *et al.* (12).

Production of Monoclonal Antibodies. A female BALB/c mouse was immunized with 5 μg of the partially stripped milk mucin in Freund's complete adjuvant and 3 months later boosted with a further 5 μg of the same preparation in Freund's incomplete adjuvant. After a further 20 days, 5 μg of the mucin extensively stripped of its carbohydrate were given i.v. in saline solution. The spleen was removed 4 days later and fused with the NS2 mouse myeloma cell line (20).

Screening of Hybridoma Supernatant and Immunoprecipitations. Screening of hybridoma supernatants and immunoprecipitations was performed according to the assay described by Melero and Gonzalez-Rodriguez (21) and modified by Shearer *et al.* (22).

Staining of Tissue Sections. Tissues from primary mammary carcinomas, benign breast biopsies, pregnant and lactating breast, normal breast (see Table 3), and a variety of other normal tissues were fixed in methacarn (methanol:chloroform:acetic acid, 60:30:10) and embedded into paraffin wax. Sections were stained with the antibodies using the indirect immunoperoxidase method as previously described (23).

RESULTS

Purification of the Milk Mucin. The milk mucin was purified from human skimmed milk on a HMFG-1 antibody affinity column. Iodination of the eluted material revealed the presence of a large molecular weight component and a M_r 68,000 band (Fig. 1, *track 5*). Precipitation of the affinity purified material with antibodies HMFG-1 and HMFG-2 (*tracks 1* and *2*) followed by gel electrophoresis showed that both the high molecular weight components and the M_r 68,000 component were precipitated by both antibodies (less effectively by HMFG-2). Although the M_r 68,000 component always copurified with the milk mucin its relationship to the high molecular weight component was unclear. It was not evident on immunoblots of the purified material probed with HMFG-1 (Fig. 2*A*) and could be precipitated by a control antibody to α-interferon and by the medium control (Fig. 1, *tracks 3* and *4*). Thus the M_r 68,000 component was removed by molecular sieve chromatography on a G75 column. The final purified product showed a major high molecular weight band, with only a trace of the M_r 68,000 component and some small molecular weight material running with the dye front (Fig. 2*B*).

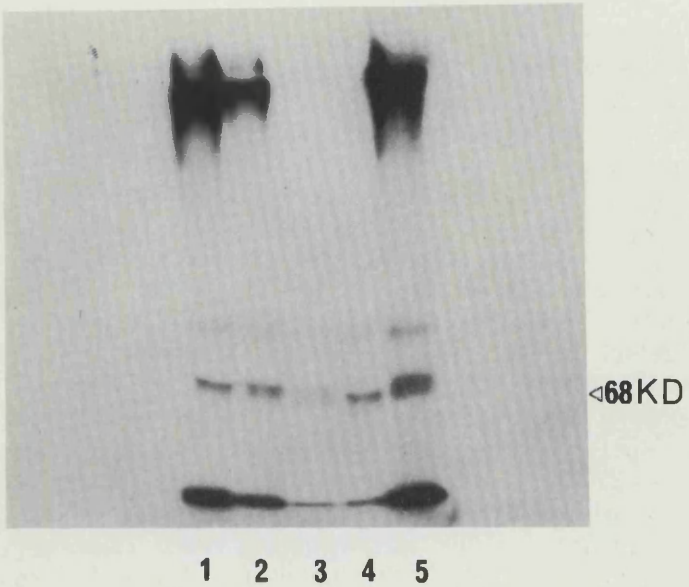

Fig. 1. Purification of the milk mucin by immunoaffinity chromatography using the antibody HMFG-1. Milks from several individuals were combined and absorbed to a HMFG-1-Sepharose column as described in "Materials and Methods." The material eluting at low pH was iodinated and subjected to 5% polyacrylamide gel electrophoresis and autoradiography (*track 5*). The iodinated material was precipitated using the Protein A method with antibodies HMFG-1 (*track 1*), HMFG-2 (*track 2*), ST254 (anti-α-interferon) (*track 3*), and RPMI 1640 plus 20% fetal calf serum (*track 4*). KD, kilodaltons, expressed as molecular weight throughout the paper.

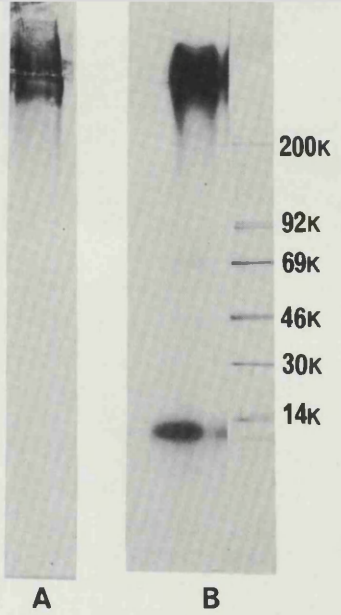

Fig. 2. Comparision of the ^{125}I-labeled purified milk mucin with immunoblot of human skimmed milk. In *A*, human skimmed milk was subjected to sodium dodecyl sulfate polyacrylamide electrophoresis using a 5–15% gradient gel, transferred to nitrocellulose paper, the blot probed with the monoclonal antibody HMFG-1, and binding detected using an enzyme-linked immunosorbent assay method. In *B*, after purification on an HMFG-1 affinity column followed by G75 Sephadex chromatography the milk mucin was iodinated by the Bolton and Hunter method and subjected to sodium dodecyl sulfate polyacrylamide electrophoresis, using a 5–15% gradient gel) and autoradiography. *K*, thousands.

Table 1 *Amino acid composition of the human milk mucin; comparison with PAS-O*

Amino acid	HMFG-1 purified milk mucin	Extensively stripped milk mucin	PAS-O (Ref. 20)
Aspartic acid	6.1	7.2	6.4
Threonine	9.4	9.7	9.8
Serine	9.1	13.0	13.1
Glutamine	6.3	9.6	8.3
Proline	14.8	14.4	12.0
Glycine	8.1	10.1	12.2
Alanine	12.3	11.9	13.0
Cysteine	Not analyzed	Not analyzed	0.5
Valine	6.0	6.3	5.3
Methionine	0.5	0.4	0.8
Isoleucine	1.6	1.7	1.9
Leucine	4.5	4.8	3.7
Tyrosine	2.0	0.9	1.6
Phenylalanine	2.0	1.6	1.7
Histidine	3.2	2.3	3.8
Lysine	2.8	3.3	2.2
Arginine	4.0	4.0	3.9

The amino acid composition of the purified HMFG-1 reactive component was determined and compared to the amino acid composition of PAS-O (periodic acid-Schiff-O), a mucin isolated from human milk fat globule membranes and which contains 50% carbohydrate in oxygen linkage (9). Table 1 shows that there is good correspondence between the two sets of data, indicating that the core proteins of PAS-O and the mucin purified here are the same.

Isolation of the Core Protein of the Milk Mucin. To remove the oxygen-linked oligosaccharides the milk mucin was treated with anhydrous hydrogen fluoride which has been shown by

pig submaxillary mucin without damaging the protein core. Amino acid analysis of the material produced after HF treatment of the milk mucin suggested that the protein core was also in this case undamaged, since the composition was very similar to that seen in the intact mucin (Table 1).

The milk mucin was exposed to hydrogen fluoride for 1 h at 4°C or for 3 h at room temperature. Fig. 3 shows an autoradiograph of the iodinated products after treatment for 1 h at 4°C (*track 2*) or 3 h at room temperature (*track 1*). It can be seen from Fig. 3 that the milder treatment results in a mixture of products made up of high molecular weight material and a number of smaller bands. After longer exposure to HF at room temperature, the high molecular weight bands disappeared resulting in polypeptide bands of about M_r 68,000 and 72,000.

To test for the presence of sugars on the intact mucin and on the components produced after the two different HF treatments, each preparation was subjected to acrylamide gel electrophoresis, transferred to nitrocellulose paper, and reacted with ^{125}I-labeled lectins. The lectins used were PNA, which reacts with galactose linked to *N*-acetylgalactosamine, WGA, reactive with *N*-acetylglucosamine, and HPA, which reacts with the linkage sugar *N*-acetylgalactosamine. Fig. 4 shows autoradiographs of the reacted blots, and it can be seen that while PNA binds strongly to the intact mucin (*track 1*) its binding to the mucin treated with HF for 1 h at 4°C is considerably reduced (*track 2*). This reduction in PNA binding is accompanied by the appearance of binding of the linkage specific lectin HPA. Thus it appears that treatment of the mucin for 1 h at 4°C partially strips the mucin of its carbohydrate, removing the peripheral oligosaccharides and so unmasking the linkage sugar. The smaller component seen in both the intact mucin (*track 1*) and in the partially stripped preparation (*track 2*) is a glycoprotein which reacts with WGA, although not with PNA. This may correspond to the component of similar molecular weight (around M_r 68,000) seen after affinity chromatography of the mucin and may represent an intermediate precursor molecule.

Fig. 4 shows clearly that the M_r 68,000 and 72,000 components produced after extensive treatment with HF (3 h at room temperature) show no reactivity with the lectins (*track 3*), including the *N*-acetylgalactosamine specific lectin HPA. This observation constitutes strong evidence that all the sugars have been removed from at least the majority of the molecules, and we will refer to this preparation as the extensively stripped mucin. It is of course formally possible that the molecule contains some nitrogen-linked sugars that would not be effected by treatment with HF; however, this seems unlikely because the extensively stripped material shows no reactivity with the wheat germ lectin.

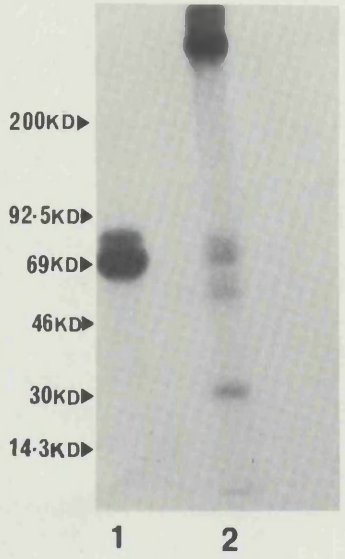

Fig. 3. Autoradiography of the iodinated milk mucin after treatment with hydrogen fluoride. The purified milk mucin was treated with HF for 3 h at room temperature (*track 1*) or 1 h at 4°C (*track 2*), and the resulting preparations were then iodinated and run on sodium dodecyl sulfate, 5–15% polyacrylamide gradient gels. *KD*, kilodaltons, expressed as molecular weight throughout the paper.

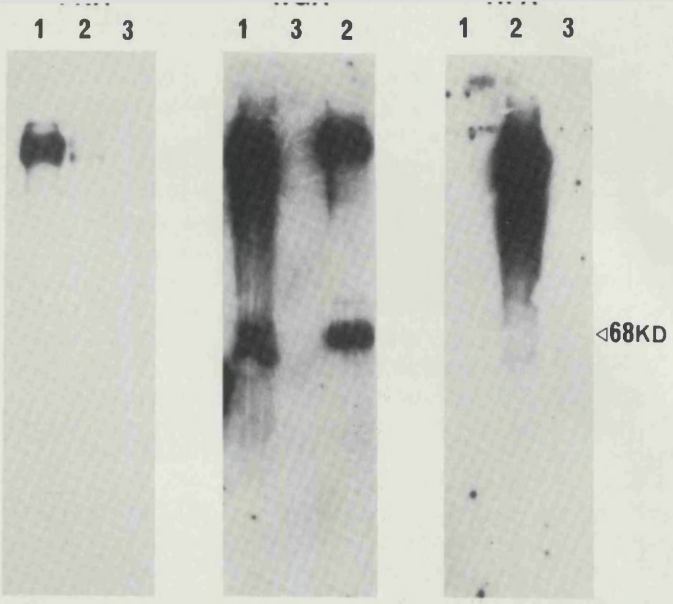

Fig. 4. Reactivity of the intact and partially or extensively stripped milk mucin with iodinated lectins. The purified intact milk mucin (*track 1*), the mucin treated with HF for 1 h at 4°C (*track 2*), and the mucin treated for 3 h at room temperature (*track 3*) were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis using a 5–15% gradient gel and then transferred to nitrocellulose paper. The paper was then probed with [^{125}I]PNA, [^{125}I]WGA, or [^{125}I]HPA *KD*, kilodaltons, expressed as molecular weight throughout the paper.

Table 2 *Reactivity of the antibodies on intact, partially, and totally deglycosylated milk mucin*

The binding of the antibodies to iodinated intact, partially, and totally deglycosylated milk mucin was assayed using the Protein A plate method as described in "Materials and Methods."

	^{125}I (cpm bound)		
Antibody	Intact molecule	Partially stripped mucin	Totally stripped mucin
9.13	525	3,000	3,328
SM-3	465	15,414	9,200
SM-4	816	16,750	9,561
HMFG-1	32,000	33,768	9,494
HMFG-2	29,500	29,230	15,832
NS2 medium	397	845	650

Generation of Monoclonal Antibodies to the Milk Mucin Core Protein. A fusion was carried out using the spleen of a mouse that had been immunized with two injections of the partially stripped milk mucin followed by a boost with the extensively stripped mucin. The clones were screened against the extensively stripped mucin, the partially stripped mucin, and the intact molecule using the Protein A plate assay (see "Materials and Methods"). Three hybridomas that secreted antibodies showing no reactivity with the intact molecule but which bound strongly to the partially and extensively stripped mucin (Table 2) were selected and cloned. These appeared to be good candidates for monoclonal antibodies to the protein core and one, SM-3 (SM for stripped mucin) selected to be characterized further, was shown to be of the IgG1 subclass.

It can also be seen from Table 2 that the HMFG-1 and HMFG-2 antibodies reacted very strongly with the mucin

stripped of its carbohydrate. These two antibodies were, in fact, developed using the intact mucin (from the milk fat globule) as immunogen and, in the case of HMFG-2, growing mammary epithelial cells (13). Their reaction with the stripped mucin was unexpected, because circumstantial evidence had previously led to the belief that carbohydrate might form at least part of their antigenic epitopes.

Molecular Weight of Molecules Carrying Antigenic Determinants. Immunoprecipitation of the extensively stripped material with SM-3 showed a reaction with the lectin unreactive M_r 68,000 component (Fig. 5*A*, *track 3*). The monoclonal antibody HMFG-2 can also be seen to immune precipitate the lectin unreactive M_r 68,000 component (*track 2*). The antibodies were reactive with antigen on immunoblots, and Fig. 5*B* shows the reaction of antibody SM-3 with the dominant M_r 68,000 band of the extensively stripped mucin (*track 2*).

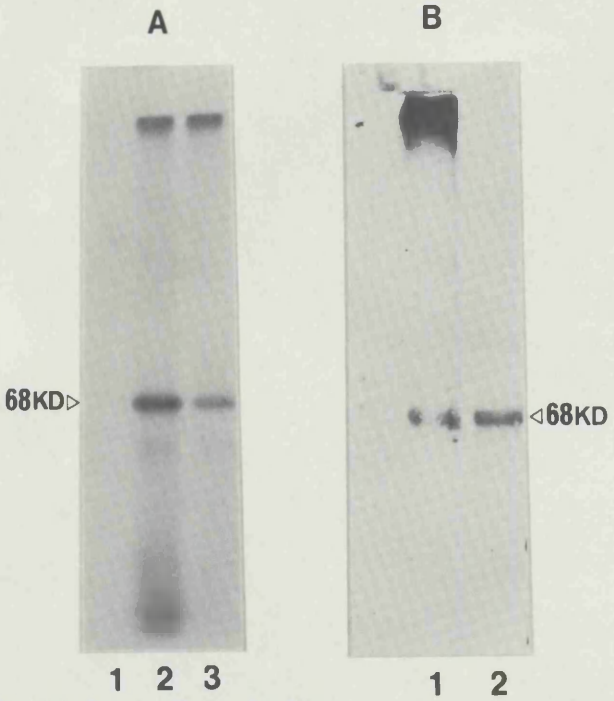

Fig. 5. Immunoprecipitation and immunoblots of the partially and extensively stripped mucin. In *A*, the ^{125}I extensively stripped mucin was immunoprecipitated with SM-3 (*track 3*), HMFG-2 (*track 2*), or NS2 medium as a control (*track 1*) by the Protein A plate method (see "Materials and Methods"). In *B*, the partially (*track 1*) or extensively (*track 2*) stripped mucin was run on sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose paper. The blot was then reacted with SM-3 and the binding detected using an enzyme-linked immunosorbent assay method. *KD*, kilodaltons, expressed as molecular weight throughout the paper.

We have previously shown that the molecular weight of the components in breast cancer cells carrying determinants found on the milk mucin is lower than 400,000 and can vary from one tumor to another (1). Reaction of antibody SM-3 with Western blots of gel separated extracts of breast tumor cells shows that this antibody reacts with components of similar molecular weight to those reactive with antibody HMFG-2 (data not shown). Because antibody SM-3 differs from antibodies HMFG-1 and 2 in that it does not react with the intact mucin processed by the lactating gland and yet reacts with molecules processed by breast cancer cells, it was appropriate to examine the reaction of SM-3 with a range of breast cancers.

Reactivity of SM-3 with Breast Tissues and Tumors. Antibody SM-3 reacted with paraffin embedded tissues provided that these were fixed in methacarn (not formalin). Using this method for preparation of tissue sections, the reaction of the antibody was compared to that of HMFG-2 on breast tissues and tumors with an indirect immunoperoxidase staining method. This analysis showed a dramatic difference in the staining pattern of SM-3 compared to that seen with HMFG-2. Thus, although a strong positive reaction was seen in 47 of 50 breast cancers stained with SM-3 (compared with 50 of 50 for HMFG-2), normal resting breast, pregnant, or lactating tissues, and most benign lesions were largely unstained with SM-3 but were stained with HMFG-2. Details of the staining are shown in Table 3, and some examples of staining patterns observed with breast tissues and tumors are illustrated in Fig. 6.

It should perhaps be noted that the intensity of staining with HMFG-2 seen with normal breast tissues and benign lesions fixed in methacarn was somewhat higher than that reported previously using formalin fixed material (11, 23).

In a preliminary study SM-3 stained 10 of 10 colon carcinomas, 2 of 3 ovarian carcinomas, and 1 of 3 lung carcinomas. However, SM-3 was shown to be negative on sections of normal liver, small intestine, lung, thymus, testes epididymis, prostate, bladder, thyroid, and skin (epidermis and sweat gland). Very weak positive staining was observed in normal pancreas, stomach, colon, ovary, and fallopian tube. Somewhat stronger staining was seen in salivary gland, sebaceous glands, and the distal tubules of the kidney.

Table 3 *Reactivity of SM-3 and HMFG-2 with breast tissue*

		SM-3			HMFG-2		
Tissue	No. of specimens	No. positive	Intensity	Type of staining when positive	No. positive	Intensity	Type of staining
Breast carcinomas	50						
Infiltrating ductal	34	32	++ → ++++	Strong cytoplasmic and membraneous staining; heterogeneous	34	+++ → ++++	Strong, cytoplasmic, and membraneous staining; in general more uniform staining than SM-3
Infiltrating lobular	13	12			13		
Carcinoma *in situ*	3	3			3		
Benign breast disease	18						
Fibroadenoma	4	3	+	Focal staining of only one or two glandular elements	4	++ → +++	Strong but heterogeneous
Papilloma	6	6	+	Weak, membraneous	6	+++	Strong, cytoplasmic, and membraneous staining
Cystic change	8	7	+	Membraneous staining; weak and heterogeneous	8	+++	Strong, cytoplasmic, and membraneous staining
Normal breast	20						
Resting	13	6	±	Extremely weak and usually confined to one or two acini/section	13	+ → ++	Heterogeneous
Pregnant and lactating	7	4	±	Very weak, staining confined to an occasional cell	7	++++	Strong homogeneous

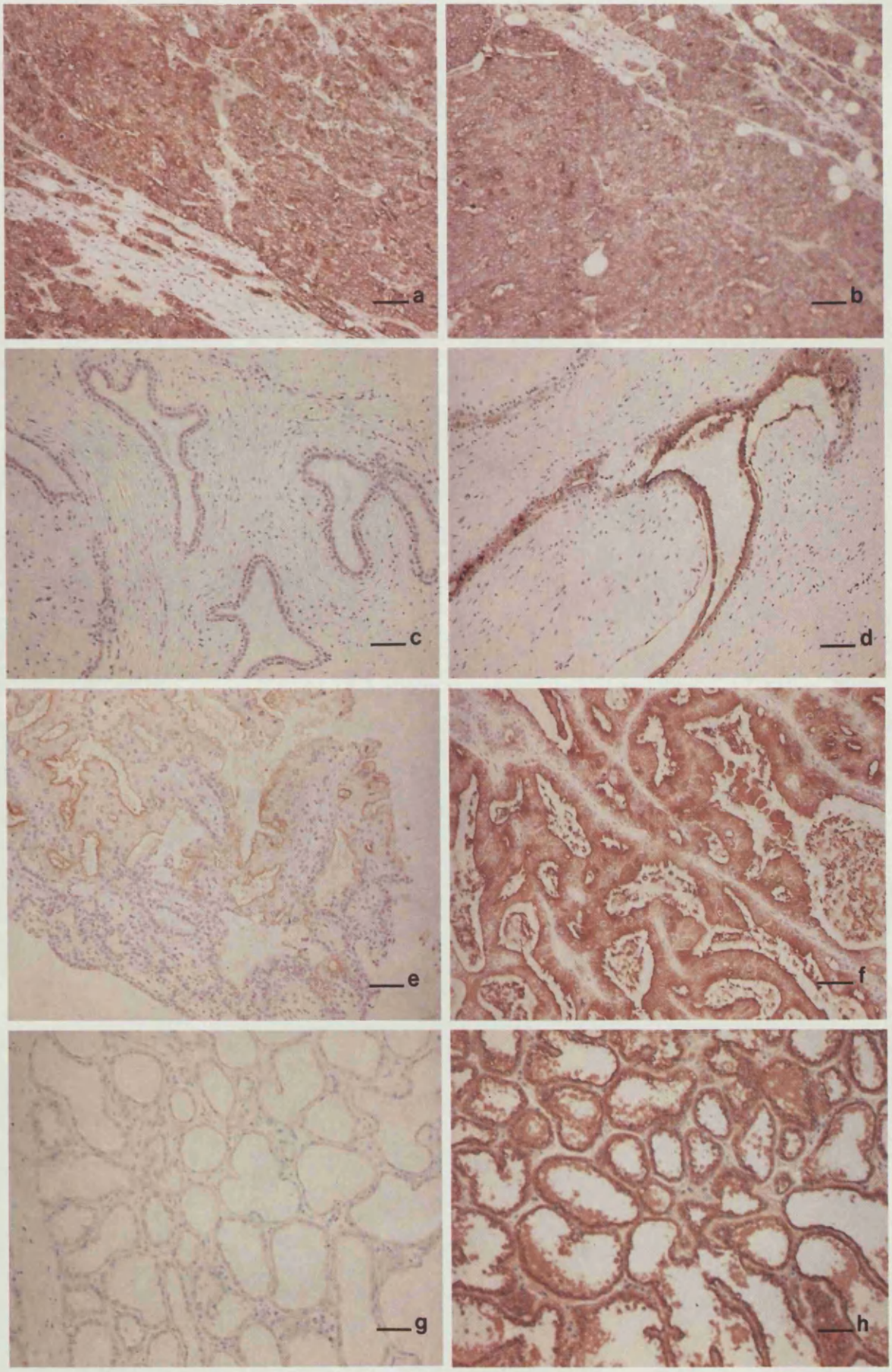

Fig. 6. Reactivity of monoclonal antibodies SM-3 and HMFG-2 with methacarn fixed breast tissue and tumor sections using an indirect immunoperoxidase staining method. Illustrated are infiltrating ductal carcinoma showing strong reactivity with both SM-3 (*a*) and HMFG-2 (*b*); fibroadenoma showing no reactivity with SM-3 (*c*) and strong heterogeneous staining of the epithelium with HMFG-2 (*d*); papilloma showing very weak reactivity with SM-3 (*e*) and strong positivity with HMFG-2 (*f*). Lactating breast (*g*) was negative when stained with SM-3, but stained positively with HMFG-2 (*h*). *Bars*, 70 μM.