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Monoclonal Antibodies to the Human Mammary Gland

II. Distribution of Determinants in Breast Carcinomas

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Summary. A range of primary and metastatic human breast carcinomas has been examined with respect to the staining by four monoclonal antibodies which were raised to the human milk fat globule membrane. Within the normal breast the luminal epithelial cells expressing the antigens detected by the monoclonal antibodies were heterogeneous in their distribution. The heterogeneity was not only confined to single cells, but also to regions within the breast. The breast carcinomas also expressed the antigens in a variable manner. Morphological differentiation and functional differentiation, defined by the monoclonal antibodies, were not invariably coincident. Lymph node metastases gave similar results to the primary carcinomas.

The monoclonal antibodies have revealed a heterogeneity, with respect to surface antigenic expression, within the normal and neoplastic breast epithelium. This cellular heterogeneity of breast carcinomas, may have significant prognostic and therapeutic implications in the management of primary breast cancer.

Key words: Monoclonal antibodies – Milk fat globule membranes – Human breast carcinomas – Differentiation markers

Introduction

The purely morphological classifications of breast carcinomas have long been inadequate in the histological assessment of tumour-cell differentiation (Foster and Neville 1981). Although the analysis of breast carcinomas with respect to cytological appearance, stromal infiltration or hormone receptors has enabled the general behaviour of many histologically distinct types of breast carcinoma to be defined, it is still not possible to predict the behaviour of a *specific* tumour using these criteria. In an attempt to gain a better insight into the biology of the normal breast and breast carcinomas, we have raised, and are evaluating, a panel of monoclonal antibody probes. We chose, for the antigen,

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human milk fat globule membrane as a heterogeneous product of functionally differentiated breast epithelial cells. In the normal breast the antigens identified by three of the antibodies are restricted to the luminal membranes of alveolar and duct epithelial cells (Foster et al. 1981). In addition, the fourth antibody identifies a determinant also expressed by myoepithelial cells.

In this paper, the distribution of staining produced by these four monoclonal antibodies on breast carcinomas is described, and compared and contrasted with the antiserum to the Epithelial Membrane Antigen. This is a conventional heteroantiserum reacting with multiple determinants within the milk fat globule membrane and has been shown to be a useful marker in the detection of breast carcinoma cells (Sloane et al. 1980). We have also compared the monoclonal antibody staining of the cells of both primary and metastatic breast carcinomas with peanut agglutinin which specifically combines with terminal galactosyl residues (Lotan et al. 1975) and which has also been suggested (Springer et al. 1976; Howard and Batsakis 1980) as a potential marker of breast carcinoma.

Our results indicate that monoclonal antibodies to determinants present on selected products of differentiated breast epithelial cells may assist in a functional analysis of human breast carcinoma. They may afford significant advantages over the use of conventional antisera and other cell-surface probes in the analysis of the biology of breast cancer.

Materials and Methods

1. Preparation and Selection of Monoclonal Antibodies. These procedures have been reported in detail in the accompanying paper (Foster et al. 1982).

2. Tumour Immunocytochemistry with Monoclonal Antibodies. Two groups of tumours were examined. The first group consisted of 20 primary, infiltrating, duct carcinomas and their associated lymph nodes. These carcinomas were all Grade II (Bloom and Richardson 1957) and of the type NOS (Fisher et al. 1975). Any uncommon histological types were specifically excluded. The second group comprised typical examples of three of the more unusual histological types of breast carcinomas (medullary, colloid and infiltrating lobular).

All tumours used in this study were removed surgically and were fixed in neutral formalin, routinely processed to be embedded in paraffin wax.

The immunohistochemical technique was identical to that previously described (Foster et al. 1982) with the exception that endogenous tissue alkaline phosphatase was blocked with 1 mM levamisole (Borgers 1973). Nuclei were counterstained with Meyer's haemalum and the sections were mounted in glycerin jelly.

3. Epithelial Membrane Antigen. The method of staining with the conventional antiserum to the Epithelial Membrane Antigen was identical to that for the monoclonal antibodies, except that being a rabbit antiserum, it required a different second-antibody-alkaline phosphatase conjugate to that used to detect the mouse monoclonal antibodies (Heyderman et al. 1979). The blocking of endogenous alkaline phosphatase was performed by the addition of 1 mM levamisole at the final stage of procedure. Nuclei were counterstained with Meyer's haemalum and the sections were mounted in glycerin jelly.

4. Peanut Agglutinin. The lectin was conjugated to horseradish peroxidase (Sigma, type VI) using the method of Carlsson et al. (1978). Endogenous peroxidase within the tissue sections was not blocked before addition of the lectin conjugate. Instead control staining of adjacent sections from the same tissue blocks was performed in the presence of 2% D-galactose. The subsequent tissue-

distribution of the lectin-peroxidase conjugate was visualised following the polymerisation of diaminobenzidine (Sigma) in the presence of hydrogen peroxide (Heyderman and Neville 1976). Nuclei were counterstained in Meyer's haemalum. The sections were mounted in XAM.

Results

I. Primary Infiltrating Duct Carcinomas

A detailed analysis of the findings is presented in Tables 1 and 2. The data in these tables are restricted to the infiltrative components of the primary tumours.

a) Grade II Carcinomas with Lymph Node Metastases

All the primary tumours were stained by at least one of the monoclonal antibodies. However, all four of these antibodies demonstrated a wide variation in their staining of individual tumour cells. The heterogeneity was not only seen between various regions of the infiltrating component of the tumours, but also between adjacent cells in the in situ components. The overall pattern of staining exhibited by a primary tumour was generally exhibited in the lymph node metastasis.

Monoclonal Antibody LICR LON/M3. This antibody usually stained membranes only in the presence of strong cytoplasmic staining. The appearance of the

Table 1. Distribution of MFGM monoclonal antibody staining of 20 primary infiltrating duct carcinomas (Grade II) and lymph node metastases at the time of primary surgery. The distribution of monoclonal antibody staining is compared to that obtained with peanut agglutinin and a conventional antiserum to the Epithelial Membrane Antigen

Antibody		M3		M8		M18		M24		PNA		EMA	
Tumour	Total	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Primary	20	17	3	20	0	3	17	15	5	15	5	20	0
Metastasis	20	12	8	20	0	2	18	15	5	11	9	20	0

Table 2. Primary infiltrating ductal carcinomas of varying grades (Bloom and Richardson 1957) and morphological appearances stained with MFGM monoclonal antibodies

Antibody		M3		M8		M18		M24	
Grade	Total	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
I	3	3	0	3	0	0	3	1	2
II	5	3	2	5	0	2	3	1	4
III	6	5	1	6	0	0	6	1	5
Total	14	11	3	14	0	2	12	3	11

cytoplasmic staining was always finely granular, and distributed uniformly throughout the cytoplasm. All but one of the primary tumours were stained, however, three of the metastases from positive primary tumours and the one metastasis from a negative primary did not stain. These tumours were either a 'poor' grade II or showed very scanty staining with the antibody. Although staining of luminal surfaces was generally strong with this antibody, this was not invariable. In several cases cells having strong cytoplasmic staining did not show staining of free luminal faces, although these were present. Even in regions showing tubule formation, little nuclear pleomorphism and no mitoses, staining was not always observed. In other situations good luminal staining was observed in regions of poor differentiation.

Monoclonal Antibody LICR LON/M8. All primary tumours and lymph node metastases of this section showed some staining with this antibody. However, not all tumour cells stained. Unlike the other three monoclonal antibodies staining with LICR LON/M8 was more directly related to cell polarity. Thus in those situations where free luminal surfaces occurred, the antibody stained the luminal membrane strongly. Whenever such a free boundary occurred above groups of cells forming a multi-layered structure, the cells below the uppermost layer showed little, if any, staining (Fig. 1). In single cells, or clusters of cells not containing a lumen, the staining was always intracytoplasmic. Unlike the other monoclonal antibodies, the appearance was that of coarse granules or vesicles which often coalesced into a single unit within the cytoplasm.

Monoclonal Antibody LICR LON/M18. Staining of infiltrating breast carcinoma cells was hardly detectable with this antibody. Only two of the primary infiltrating tumours showed any staining. In both cases the staining was extremely scanty, but very strong where present. In one example, the cytoplasmic component in a few of the cells was moderately granular. These cells tended to show no membrane localisation of the staining. Three of the lymph node metastases also stained strongly in some foci with this reagent. One exceptional case was that in which a focus of metastatic tumour appeared morphologically more differentiated than the primary tumour. In this example, antibody LICR LON/M18 also stained the metasatic tumour, without staining having been detected in the primary tumour.

Monoclonal antibody LICR LON/M24. Of the 20 primary carcinomas, only 12 stained. The staining of primary and metastatic breast carcinoma cells by antibody LICR LON/M24 was more uniform than with the other monoclonal antibodies. The usual distribution was uniform pale and cytoplasmic with no accentuation of staining the membranes in the primary tumours examined. This was independent of the position of the individual tumour cells and independent of the presence of a luminal face. In each instance the majority of the tumour cells stained positively. Much less heterogeneity was observed either between adjacent cells, or between adjacent regions than was observed with the other monoclonal antibodies. In one instance, plasma membrane localisation

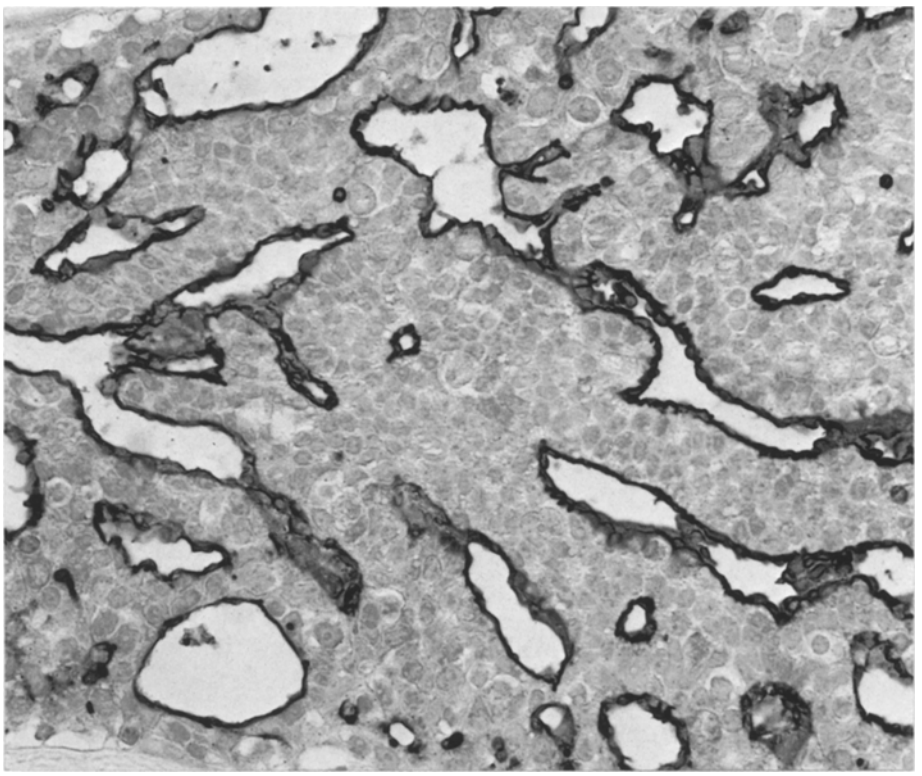

Fig. 1. In situ primary breast carcinoma stained by monoclonal antibody LICR LON/M8. The antibody is located to the surface membranes of those cells bordering lumina within the tumour. Cells without a luminal face do not stain. (×375)

of staining was observed in a particularly well differentiated portion of a metastasis. Staining of the primary tumour in this case, although strong, had nevertheless, been entirely cytoplasmic and scanty. The distribution, and the nature, of the staining observed with LICR LON/M24 was very similar to that obtained with the peanut agglutinin (Table 1). The possible relationship between the specificities of the monoclonal antibody LICR LON/M24 and the peanut agglutinin is presently being investigated.

b) Infiltrating Duct Carcinomas of Various Morphologies

Despite good tubular differentiation in all three examples of the Grade I carcinomas, antibody LICR LON/M3 gave luminal membrane staining in only one of the cases. This staining was both weak and focal. Generally the staining was rather poor and extended throughout the cytoplasm of most of the tumour cells (Fig. 2). Conversely, strong plasma-membrane staining was observed in two of the Grade III tumours.

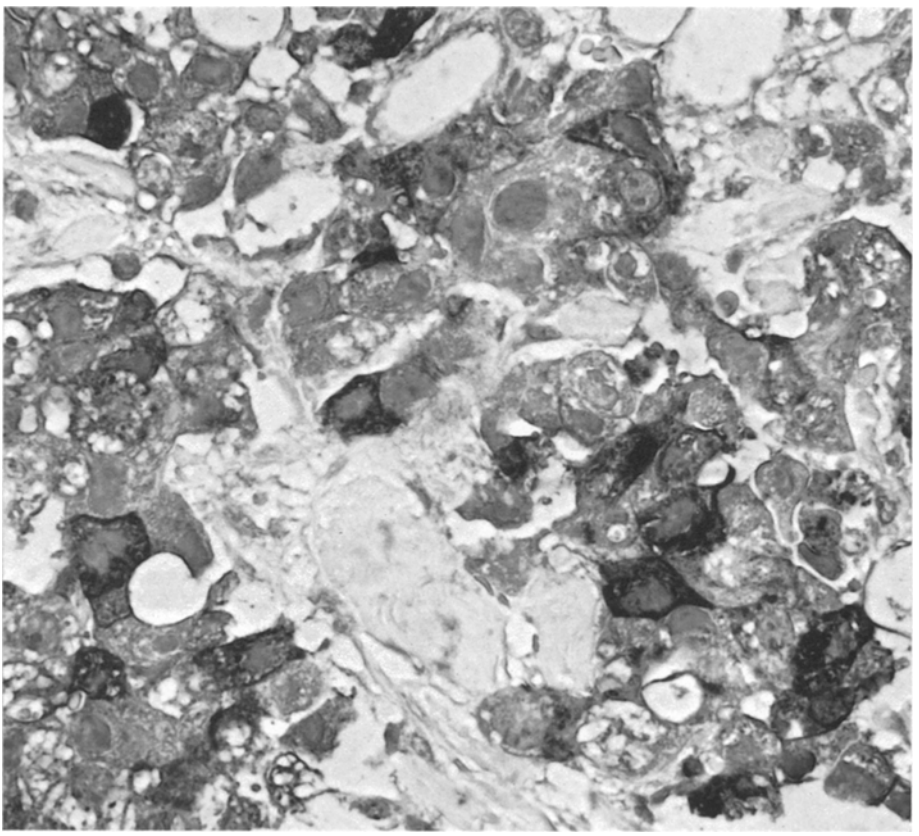

Fig. 2. Primary Infiltrating breast carcinoma stained by monoclonal antibody LICR LON/M3. Heterogeneity of staining is marked. Some of the tumour cells show intense cytoplasmic staining with the antibody. Localisation of the antibody to the plasma membranes of the tumour cells is not seen. (×450)

Antibody LICR LON/M8 gave strong membrane staining in all but three of the carcinomas. One of the three was a Grade I tumour with good tubular differentiation. The staining of this tumour was strong, and throughout the cytoplasm of almost all of the cells. Those tumours staining weakly with LICR LON/M8 were, with one exception, Grade III.

Despite being histologically 'low grade', with goods tubule formation, the three Grade I carcinomas failed to show staining of the free luminal membranes with LICR LON/M18. However, staining of luminal membranes with this antibody was observed in two of the Grade II tumours. In each case, tubule formation was of only intermediate grade.

No correlation was observed between the histological grade or tubule formation and the staining of the tumours by LICR LON/M24 (Fig. 3). The appearances of the staining with this antibody were identical to those reported in Section (*a*).

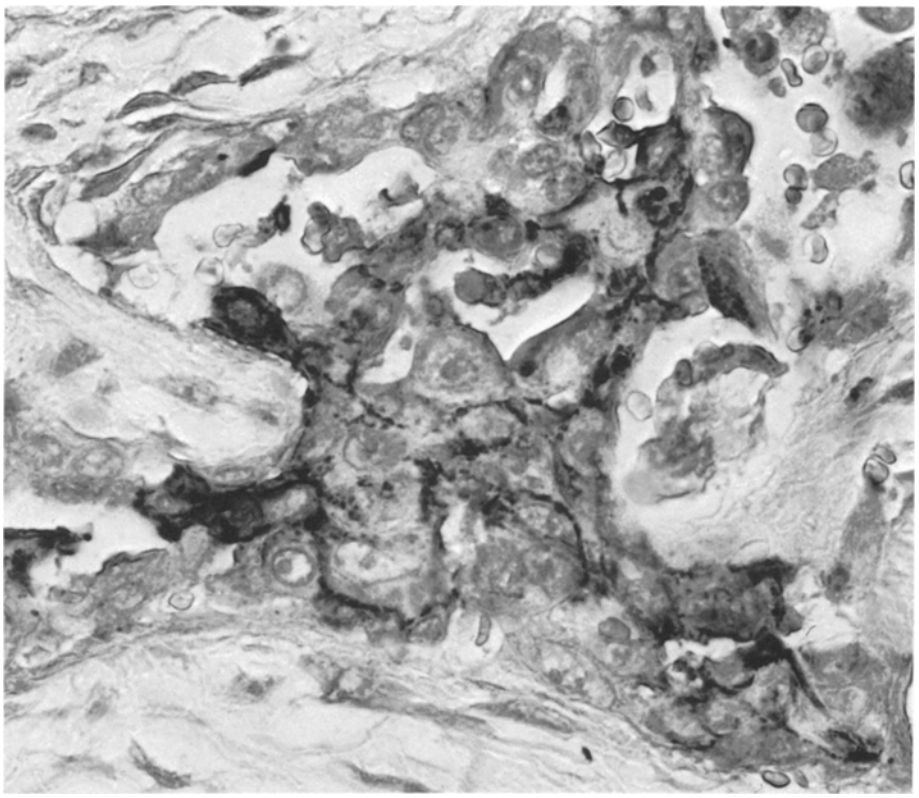

Fig. 3. Primary infiltrating breast carcinoma stained by monoclonal antibody LICR LON/M24. In this section some of the tumour cells stained intensely and uniformly. In other cells, granular staining was observed along intercellular membranes. Cell membranes bordering lumina did not necessarily stain. (×450)

Table 3. Distribution of MFGM monoclonal antibody staining of primary breast carcinoma of unusual histological types

Antibody		M3		M8		M18		M24	
Tumour	Total	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Medullary	4	2	2	3	1	0	4	2	2
Colloid	5	3	2	4	1	1	4	1	4
Lobular	4	4	0	4	0	0	4	4	0

II. Medullary, Colloid and Lobular Carcinomas

An analysis of the data is presented in Table 3. Although histologically similar, the four medullary carcinomas were, as a group, quite heterogeneous in their response to staining with the monoclonal antibodies. For example, one of the

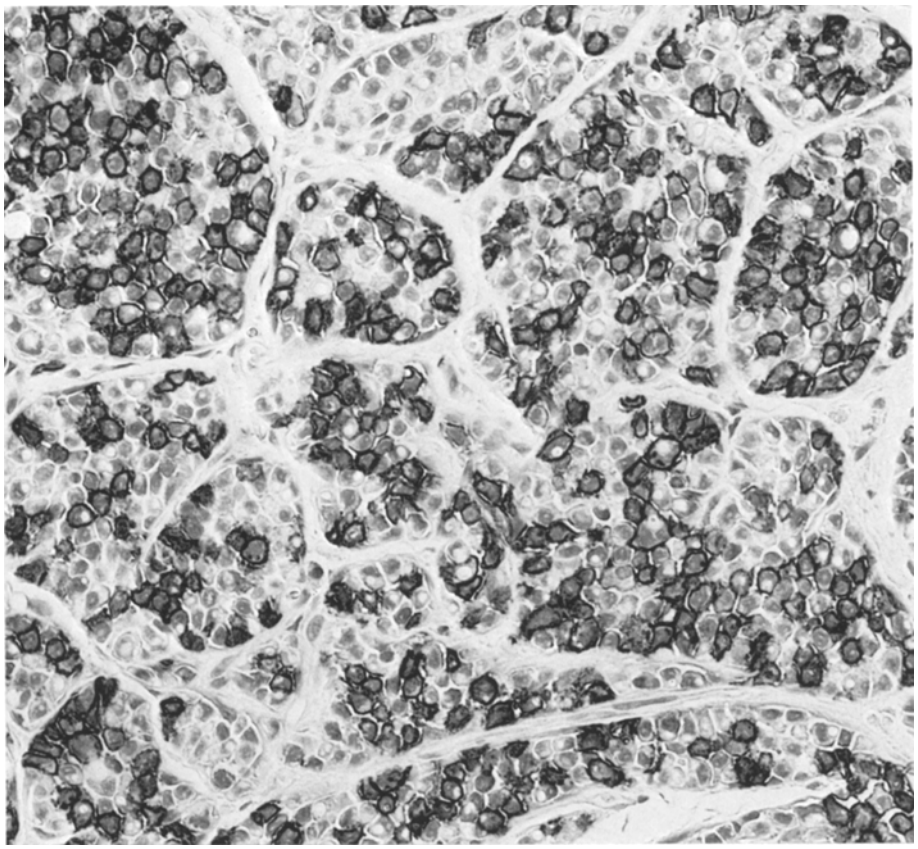

Fig. 4. In situ lobular breast carcinoma stained by monoclonal antibody LICR LON/M3. This figure emphasises the degree of heterogeneity within primary breast carcinomas, which is defined by monoclonal antibodies. (× 300)

tumours staining with LICR LON/M24 did not stain with LICR LON/M8. This was one of the only two breast carcinomas in which staining by LICR LON/M8 has not been obtained; the other was one of the five colloid carcinomas. No plasma membrane staining of the medullary carcinomas was observed.

The colloid carcinomas were also a heterogeneous group with respect to monoclonal antibody staining. Staining was observed in the colloid stroma of two of the tumours. In each tumour it was by a different monoclonal antibody. In both instances the colloid staining was vesicular. The appearances were interpreted as membranes containing the antigen shed from the tumour cells and trapped within the colloid.

The lobular carcinomas were a more homogeneous group than the previous types of primary breast carcinomas. Monoclonal antibody LICR LON/M3 was localised to the plasma membrane of at least some of the tumour cells in each example (Fig. 4). LICR LON/M24 was similarly localised to the plasma membranes in two of the four cases. LICR LON/M8 failed to produce unequivo-

cal plasma-membrane staining of infiltrating tumour cells in one instance. In this case the observed pattern was extremely focal, cytoplasmic and very intense.

Discussion

The four monoclonal antibodies which have been described here fulfil many of the criteria required of probes to study aspects of the *functional* status of breast carcinomas: (1) Each antibody is directed towards a unique and separate determinant; (2) they may also be used in formalin-fixed and paraffin-wax embedded material. Finally, (3) the different antibodies stain different breast carcinomas in patterns which are not apparently related to the overt morphology of the tumours, thus providing independent parameters of functional differentiation. Consequently, the information concerning the differentiation of breast carcinomas using these probes acts as a valuable adjunct to that obtained from morphology alone. McIlhinney (1981) has already demonstrated the value of monoclonal antibodies to cell surface antigens in the study of another group of human malignancies, the teratomas. In comparison with the other two types of reagents used in this paper, a conventional hetero-antiserum offers no advantage, with respect to the detection of breast carcinomas, over a monoclonal antibody having the appropriate specificity and affinity. Rather, the conventional antiserum is less useful as a biological probe since it is usually directed to many diverse and uncharacterised determinants simultaneously. Lectins may be useful in specific and restricted circumstances. They are monospecific and directed to particular sugar residues. Provided that the determinants can be shown to comprise a part of those molecules having a defined relationship to the functional or differentiation status of the cells, then information derived from their use may be helpful. Monoclonal antibodies offer the advantage that they are not only monospecific, but that they are also dependent upon the appropriate structural configuration of the determinant to which they are directed. Hence, the information provided by their use is much more specific.

We have demonstrated variable antigenic expression to occur within in situ primary infiltrating and metastatic breast carcinomas, using monoclonal antibodies to the milk fat globule membrane. We have demonstrated that the heterogeneity of antigenic expression, as defined by monoclonal antibody staining of tissue sections, is generally conveyed from the primary tumour to the lymph node metastases. We have also shown that, in some circumstances the lymph node metastases may express functional determinants which are common to normal breast epithelial cells but not observed in the original primary tumour.

The heterogeneity observed in the breast tumours reflects that already identified in the normal and lactating breast (Foster et al. 1982) and in extramammary epithelia. The variable expression of antigens identified by the monoclonal antibodies may indicate a variable state of functional differentiation or it may define specific sub-populations of normal breast and breast carcinoma cells. For example, the distribution of antibody LICR LON/M8 correlates well with tumour morphology, particularly the topographical location of surface cells in a multilayer. Nevertheless, the simultaneous absence of staining from these

cells by M18 and M24 indicates a marked functional loss which is not evident from the morphology alone.

The heterogeneous expression of surface antigens, by normal and malignant breast epithelial cells is important in understanding the pathobiology of metastatic malignant disease. Dexter et al. (1978) demonstrated heterogeneity with respect to tissue culture morphology, in vitro growth properties, expression of murine mammary tumour virus antigen and karyotype of tumour cells derived from a single mouse mammary tumour. An analysis of immunologic status of the same tumours showed a marked variation in the expression of surface determinants between the sub-populations (Miller and Heppner 1979). Heppner et al. (1978) examined the drug sensitivities of these tumour cell sub-populations and it was clearly shown that the effects of the chemotherapeutic agents differed widely in their effects on the individual sub-populations.

Fidler and Kripke (1977) have shown that clones of murine melanoma cells derived from a single primary tumour varied significantly in their ability to produce metastatic colonies in the lungs. Thus, by these or other monoclonal antibodies, it may be possible to identify, early, cells having the potential to metastastise in specific patterns.

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