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Expression of the antigen detected by the monoclonal antibody Ca 19.9 in human breast tissues

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Summary. The incidence and significance of the expression of the antigen defined by the monoclonal antibody Ca 19.9 (Sialyl Le^a) has been assessed in human breast tissue. Frozen and formalin-fixed, paraffin embedded specimens of normal, hyperplastic, pregnant breast and carcinomas were examined using an immunoperoxidase technique.

Ductal and acinar epithelium of normal and hyperplastic tissues showed variable reactivity in frozen sections but there was a reduction in staining in comparable samples after fixation and processing, such that in many instances only focal ductal epithelium reacted. A distinctive feature in the pregnant breast was the absence of staining in acini showing differentiated secretory activity, despite a reaction in adjacent non-secretory acini and ducts.

The overall incidence of detection of the Ca 19.9 antigen in breast carcinomas was 62%, but in half of these only a small number of cells stained. A significant relationship between expression of Sialyl Le^a and poor differentiation of carcinomas was identified, but there was no correlation with local lymph node status. In contrast to the non-malignant tissue fixation and processing had little effect on the reactivity of carcinomas. It is suggested that this difference may be quantitative in nature, with malignant breast showing much greater expression, or be related to organisation of the antigen.

The observations concerning carcinomas and pregnant breast indicate that the synthesis of the Ca 19.9 antigen is related to the state of differentiation and functional activity of human breast.

Key words: Breast cancer – Monoclonal antibodies – Blood groups

Introduction

The use of monoclonal antibodies has led to the recognition that carbohydrate determinants of both glycolipids and glycoproteins can behave as

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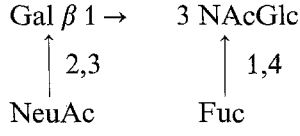
onco-developmental and tumour-associated antigens (Feizi 1985). Several of these oligosaccharide sequences are also expressed in the major blood group antigens. For example, the determinants recognised by the monoclonal antibodies Co-514 and Co-43 which were initially considered to be adenocarcinoma-associated antigens, were later shown to be components of the blood groups Le^a and Le^b respectively (Blaszczyk et al. 1984).

The monoclonal antibody Ca 19.9 detects the sequence:



which is the determinant of sialylated blood group Le^a, but is also a tumour associated antigen (Magnani et al. 1982). It has been detected in lipid extracts of colonic, gastric and pancreatic carcinomas but not in extracts from respective normal tissues (Magnani et al. 1982) and identified as a monosialoganglioside. Immunohistochemistry has demonstrated sialyl Le^a in a high percentage of gastric, pancreatic and colonic carcinomas but has shown only a restricted and limited distribution in normal tissues (Atkinson et al. 1982). Besides being a component of glycolipids it is present in glycoproteins, since it can be detected in the form of a mucin in the serum of patients with gastrointestinal cancer (Magnani et al. 1983).

Immunohistochemical studies concerned with the distribution of the antigen detected by Ca 19.9 (Atkinson et al. 1982; Arends et al. 1983a) have observed only occasional focal reactions in normal breast ducts and reactivity in one out of 18 and one out of 10 carcinomas respectively. These investigations were undertaken on fixed, paraffin embedded tissue. Olding et al. (1985) have demonstrated that the glycolipid antigen will be partly lost during the dehydration steps of tissue processing. We have studied a larger series of breast carcinomas, as well as normal and hyperplastic breast tissue, and compared frozen and fixed, processed tissue. The findings have been related to parameters of tumour behaviour such as differentiation and local lymph node metastasis.

Materials and methods

Tissues. Material was available from 20 cases of normal and hyperplastic breast tissue, two specimens of early and mid-trimester pregnancy breast and 50 carcinomas. The latter were collected consecutively between September 1984 and July 1985. The Lewis blood group status of the patients was not known. Samples from each case were frozen in liquid nitrogen, with parallel slices being fixed in 4% formaldehyde in 0.15 M sodium chloride solution. The frozen tissue was stored in liquid nitrogen. The fixed tissue was dehydrated, cleared and embedded in paraffin wax.

Haematoxylin and eosin stained sections of the fixed tissue were examined, and the carcinomas classified according to W.H.O. criteria, and assessed for degree of histological differentiation using a modification of the Bloom and Richardson grading system (Elston et al. 1982).

Antibodies. The mouse monoclonal IgG1 antibody Ca 19.9 was obtained from C.I.S. International and had been characterised as detecting sialylated lacto-N-fucopentaose II (Le^a) (Mag-

nani et al. 1982). Biotinylated anti-mouse immunoglobulin serum and avidin-peroxidase complex formed part of the kit provided with the monoclonal antibody.

Methods. Frozen sections (6–8 μm) were cut from all samples in a cryostat at −20° C, air dried for 60 min at 4° C and fixed in acetone for 10 min at room temperature.

Fixed, embedded material from the pregnant tissue and 10 cases of non-malignant breast were examined. After examination of the stained frozen sections of the carcinomas, fixed, embedded tissue from all carcinomas showing some evidence of reactivity were examined, along with such material from some tumours which failed to react on frozen section. In total fixed, embedded tissue from 25 carcinomas was assessed. For all such samples the sections were dewaxed and rehydrated. The effect of enzyme treatment on reactivity was considered by treating sections with 0.1% Trypsin (Difco 1:250) pH 7.8 at 37° C for 30 min. This step was omitted for parallel sections from each case.

Frozen and fixed tissue were then treated alike. The monoclonal antibody Ca 19.9 was applied to the sections and they were incubated for 18 h at 4° C. After thorough rinsing and washing in phosphate buffered saline pH 7.2, the biotin labelled anti mouse immunoglobulin antiserum was applied for 10 min, followed again by rinsing and washing in buffer. The sections were then treated with avidin-peroxidase complex. All reagents forming the kit were pre-diluted the Ca 19.9 antibody was further diluted 1:5. After washing, the peroxidase was developed using freshly prepared amino ethyl carbazole with a resultant red colour.

Controls were. Omission of the primary antibody; omission of the second antibody so as to check for evidence of avidin binding to endogenous biotin; staining of sections to detect endogenous peroxidase. The effect on staining of removing sialic acid from the tissues was assessed by treating sections from five carcinomas and pregnant breast with neuraminidase (Koch-Light) at pH 5.5 for 18 h at 37° C prior to application of primary antibody.

Assessment of staining. The extent of staining within carcinomas was categorised as:

+	single and/or small clusters (2–6) of cells reacting in one to three areas only
++	single and/or small groups of cells reacting throughout the section
+++	groups of cells, with not less than 20 cells per group, staining throughout the section.

Results

Benign tissues

All of the frozen sections of normal and hyperplastic tissues showed evidence of staining but the extent varied from case to case. In all instances many of the interlobular and intralobular ducts reacted but the number of cells staining differed within and between specimens; although the intensity was generally consistent. The reaction of acini was more variable. In some specimens only a small number stained in a few lobules, whilst in others many of the lobules contained acini that reacted, although they ranged in number from 2 to 10 (Fig. 1). Variation in intensity was observed. Hyperplastic acini were more likely to react and to be more intense. Only epithelial cells stained.

The fixed, embedded tissue was consistently less reactive even after treatment of the sections with trypsin (Fig. 2). The ductal epithelium was less affected although fewer cells tended to react. Only small numbers of acini in just a few specimens of normal breast stained with a weak reaction. In hyperplastic breast more acini reacted with a stronger intensity.

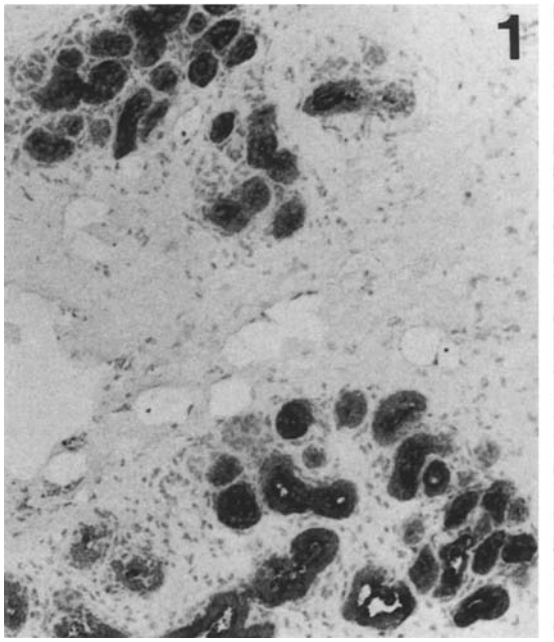


Fig. 1. Frozen section of breast tissue with minimal hyperplastic features in which there is staining of all ducts and acini within these lobules by the Ca 19.9 antibody. Magnification ×87

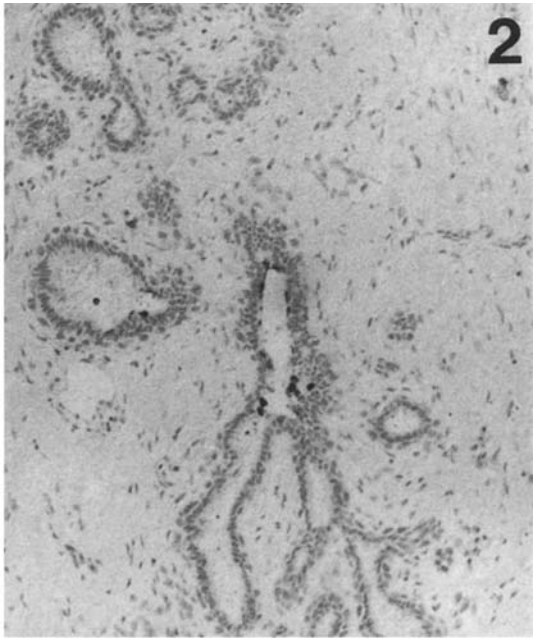


Fig. 2. Fixed, embedded tissue from the specimen illustrated in Fig. 1, showing that there is quite a marked reduction in reactivity. Magnification ×87

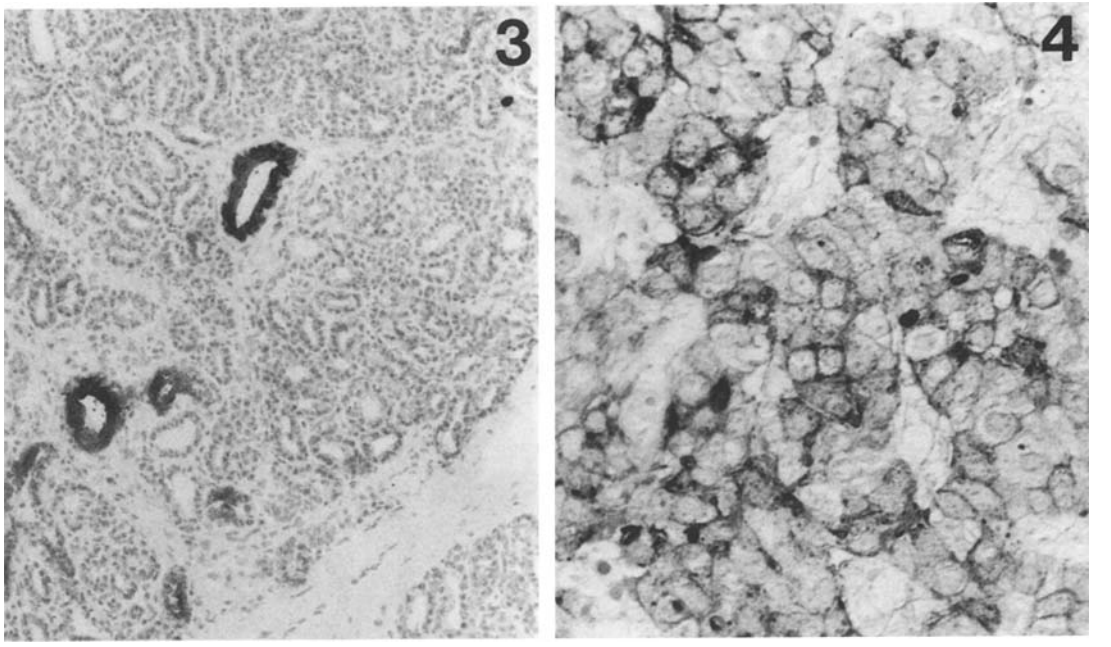


Fig. 3. Frozen section of mid-pregnancy breast in which there is staining of intralobular ducts but not of acini showing secretory changes. Magnification ×87

Fig. 4. Fixed, embedded tissue of a carcinoma in which many cells showed a granular cytoplasmic staining with the Ca 19.9 antibody. Magnification ×220

Both samples of pregnant breast behaved in a similar manner. In the frozen sections there was strong staining of interlobular ducts, weaker staining of intralobular ducts but the acini showing secretory activity typical of pregnancy were negative (Fig. 3). Occasional acini not showing this change stained weakly. The fixed, embedded tissue behaved in a similar fashion but there was a slight reduction in the number of cells staining.

Neuraminidase treatment resulted in a complete loss of staining.

Carcinomas

Thirty one of the fifty carcinomas (62%) showed evidence of reactivity for Ca 19.9 antigen when frozen sections were examined. The numbers within each of the staining categories were: +16, ++10; +++5; so that only 30% of the tumours showed significant staining.

When the fixed, embedded tissue was compared with the frozen tissue only two carcinomas showed a difference in the extent of reactivity decreasing from ++ to +, despite enzyme treatment. In some sections that included normal breast as well as tumour the reduction in the proportion and intensity of staining of normal tissues in the processed tissue was obvious whereas there was little difference in the reactivity of the tumour cells.

The reaction within individual cells was similar in both frozen and fixed tissue, being granular cytoplasmic with occasional peripheral accentuation (Fig. 4). Staining of inter cytoplasmic luminal secretions was occasionally seen.

Neuraminidase treatment resulted in complete loss of staining.

Relationship to classification

Forty three were infiltrating duct carcinomas, five infiltrating lobular, one a mucinous carcinoma and one an intraduct carcinoma. Two of the infiltrating lobular tumours had no evidence of staining and the other three had only a few cells reacting (+), as did the mucinous and intraduct carcinoma.

Relationship to differentiation

There were three well differentiated carcinomas, 26 moderately differentiated tumours and 20 poorly differentiated carcinomas. The intraduct carcinoma was not graded. The relationship between histological differentiation and staining pattern is shown in Table 1. Of the 15 tumours showing significant (++ and +++) staining 12 were poorly differentiated. There is a significant correlation between the presence of the Ca 19.9 antigen and poorer differentiation of carcinomas ($\chi^2 = 14.58$; $P < 0.001$).

Relationship to node status

Information concerning the presence or absence of axillary lymph node metastasis was known for 37 of the carcinomas and its relationship to staining pattern is shown in Table 1. Just under half of the tumours which had not metastasized to lymph nodes showed significant staining compared

Table 1. Relationship between histological differentiation, lymph node status and reactivity of carcinomas for the Ca 19.9 antigen

Staining pattern	Grade			Lymph node metastasis	
	I	II	III	Present	Absent
Neg	2	13	4	11	5
+	1	10	4	6	3
++	0	2	8	3	6
+++	0	1	4	4	1

to 29% of those with metastases. However there was no significant correlation ($\chi^2 = 2.34$; $0.5 > P > 0.1$) between staining and the presence or absence of metastasis.

Discussion

We have demonstrated the antigen detected by the Ca 19.9 antibody in 62% of breast carcinomas, with more extensive staining occurring in 30% of tumours. This is a higher incidence that found in other immunohistochemical studies. It probably reflects the number and range of carcinomas assessed, rather than factors such as fixation and methodology. Overall, little difference was observed between frozen and fixed, embedded tissues, the latter being the material used in other, more limited studies (Atkinson et al. 1982; Arends et al. 1983a). The avidin-biotin complex method, as used in this study, has been shown to be highly sensitive (Hsu et al. 1981) but Atkinson et al. (1982) used a four-layer immunoperoxidase technique which should be a comparable amplification method.

Differences have been observed between non-malignant and malignant breast in the expression of the Ca 19.9 antigen after fixation and processing. This may be due to differences in the nature of the glycoconjugates in the two tissue groups, but in other studies of fucosubstances of breast it has been the carcinomas rather than the non-malignant tissue that have been more likely to contain fucolipids (Walker 1984). The differences in expression are therefore probably related to the amount of antigen present, with enhanced expression occurring in the carcinomas.

It has been suggested that the antigen detected by Ca 19.9 antibody is onco-fetal or tumour-associated in nature (Magnani et al. 1982). This is based on studies of colorectal tissue in which the antigen can be demonstrated in meconium, fetal intestine, adenomas and carcinomas but only in normal tissue if adjacent to tumour (Atkinson et al. 1982; Arends et al. 1983a). However, the findings in unfixed normal breast indicate that the antigen could not be defined as oncofetal, a conclusion reached by Arends et al. (1983a). The staining pattern of hyperplastic and pregnant breast has not been considered before. Hyperplastic tissues, irrespective of tissue handling, are more reactive than normal. One of the striking features in

the study has been the behaviour of pregnant tissues, where clearly demarcated staining occurs, there being a sudden loss of reactivity once the acini show secretory differentiation.

The relationship between Sialyl Le^a and differentiation is also demonstrated by the carcinomas in that the majority of the tumours with a greater degree of reactivity are poorly differentiated. If in previous studies which had surveyed breast the tumours had been predominantly well or moderately differentiated this could account for the low incidence of detection. A relationship with differentiation has not been observed for other tumours such as colo-rectal carcinomas (Arends et al. 1983b). Other studies of breast carcinomas using polyclonal antisera against a range of potential markers, including carcinoembryonic antigen, have found poorly differentiated tumours to be generally less reactive (Walker 1982), as have investigations using lectins (Walker, 1985) so sialyl Le^a is unusual in this respect.

There appears to be no significant relationship between expression of the Ca 19.9 antigen and the absence or presence of local lymph node metastasis. This is similar to the observations for carcinoma of colon (Arends et al. 1983b).

Hakomori (1985) has suggested that the synthesis of substances such as sialyl Le^a within tumours is related to the activation of glycosyltransferases that are present only in small quantities in normal cells. The observations in pregnant breast suggest that the enzymes necessary for the synthesis of the Ca 19.9 antigen are not active once the cells secretory activity is directed towards the formation of milk. Regulation of the necessary transferases may be modified by alterations in hormonal state of the breast. Unfortunately, the steroid receptor status of the carcinomas studied is not available. However, in most series approximately 30% of tumours will be oestrogen receptor negative (Hawkins et al. 1980) and these will generally be the poorly differentiated carcinomas (Maynard et al. 1978). It is interesting to note that these findings parallel those of the tumours in this series which have shown significant expression of sialyl Le^a. The possibility that the Ca 19.9 antigen in breast carcinomas can act as an indicator of hormone insensitivity will be explored further.

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