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Reactivity of a monoclonal antibody recognicing an estrogen receptor regulated glycoprotein in relation to lectin histochemistry in breast cancer

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Summary. We have raised monoclonal antibodies against human milk fat globule membrane antigens and previously shown that one of them, called III D 5, recognises a glycoprotein associated with estrogen receptor activity of breast cancer. In immunoblotting it was shown that the molecule in human milk exclusively stained with III D 5 also binds peanut agglutinin (PNA) and Ricinus communis. In this study we correlate the staining of III D 5 and binding of lectins to tissue sections fixed in formalin and embedded in paraffin. Similar rections were seen only with III D 5 and PNA. Our results suggest that III D 5 and PNA detect overlapping antigenic epitopes in mammary carcinoma. This is in keeping with previous results that PNA or III D 5 reactivity is correlated with estrogen receptor status of breast cancer.

Key words: Monoclonal antibody – Estrogen receptor – Lectins – Breast cancer

Introduction

Milk, the apocrine secretion of lactating breast, contains fat globules, (HMFG), surrounded by a bi-layer derived from the apical plasma membrane (Anderson and Cawston 1975; Patton and Keenan 1975). The specific carbohydrate residues on the HMFG can be identified with different lectins (Newman and Uhlenbruck 1976; Horisberger et al. 1977; Farrar et al. 1979; Murray et al. 1979; Farrer et al. 1980) and some of them may act as antigens to monoclonal or heterologous HMFG antibodies.

Antibodies generated against HMFG membrane have been shown to react to normal and lactating breast and with mammary carcinomas and a variety of other malignancies (Ceriani et al. 1977; Arklie et al. 1981; Foster et al. 1982; Hilkens et al. 1984; Krohn et al. 1985). Several studies on the structure of the antigens detected by these antibodies have previously been published. The monoclonal antibody HMFG1 recognizes a determinant which is present in large glycoprotein molecules with complex carbohydrate side chains and the monoclonal antibody HMFG2 a determinant in smaller molecules where glycosylation may be incomplete (Burchell et al. 1983). Monoclonal antibody LICR-LON-M18 identifies an antigenic structure which constitutes the major portion of the I(Ma) cell-surface antigen and is very similar to the determinant recognized by peanut agglutinin (Foster and Neville 1984).

We have raised monoclonal antibodies against human milk fat globule membranes and characterized them previously (Krohn et al. 1985; Ashorn et al. 1985). The lectin binding affinities of antigens recognized by these were further characterized by immunoblotting (Ashorn et al. 1985). Most of the antigens reacting with anti-HMFG-antibodies contain several sugars. The antigens exclusively stained with one of monoclonal antibodies, called III D 5, binds only to PNA and Ricinus communis (RCA) lectins.

Antibody III D 5 recognizes a cytoplasmic mammary carcinoma antigen that has a highly significant correlation with the estrogen receptor (ER) status of the tumour. Positive staining of breast cancer cells with PNA has previously also been shown to correlate significantly with the presence of estrogen receptor and also with better responsiveness to hormonal therapy (Klein et al. 1983; Böcker et al. 1984).

The aims of this study were to investigate the

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Table 1. List of lectins used, with corresponding specific inhibitors

Lectin	Carbohydrate inhibitor
Peanut agglutinin (PNA)	D-galactose
Wheat germ agglutinin (WGA)	N-acetyl-D-glucosamine
Ricinus communis agglutinin I (RCA)	Lactose
Soy bean agglutinin (SBA)	N-acetylgalactosamine
Dolichos biflorus agglutinin	N-acetylgalactosamine
Ulex europeus agglutinin I (UEA)	L-fucose
Concanavalin A (ConA)	D-mannose

occurence of lectin receptors in breast cancer in relation to the antigen reactivity with monoclonal HMFG antibody III D 5, and to discuss the structure of antigens detected by III D 5.

Material and methods

The generation of monoclonal III D 5 antibody has been described elsewhere (Krohn et al. 1985). The antibody was derived from a fusion of spleen cells from HMFG immunized BALB/c mice with a HAT sensitive Sp-2 myeloma line. After double cloning with limiting dilution, cells were grown in the peritoneal cavity of 3 months old BALB/s mice and the ascites fluid was collected after 10–14 days and used for immunohistochemistry.

Surgical specimens were obtained from operations performed at Mikkeli Central Hospital, fixed in neutral formalin for up to 24 hours and embedded in paraffin. Sections were stained with HE, different lectins and the monoclonal antibody. Thirty cases of breast cancer were selected, 16 were III D 5 positive and 14 negative. All cases were derived from a previous study where the correlation between III D 5 and steroid receptors were shown (Krohn and Helle 1985).

Immunohistochemistry. Endogenous peroxidase was blocked with 3% hydrogen peroxidase. After inhibition of nonspecific binding of secondary antibody the sections were stained with the monoclonal antibody III D 5 in mouse ascites fluid, diluted 1:100. The secondary antibody was biotinylated anti-mouse immunoglobulin and avidin-biotin-peroxidase complex (ABC, Vectastain, Vector, CA, USA) was used to develop the sections in a solution of 3-ethylene-9-aminocarbazole and hydrogen peroxide. Counterstaining was with haemalum. In control sections, the primary antibody was substituted either by phosphate buffered saline (PBS) or with monoclonal anti-streptococcal antibody. The various lectins used in this study are listed in Table 1. Sections were incubated at room temperature with biotin-conjugated lectins (Vector Laboratories, CA, USA) for 20 min washed in PBS and incubated for additional 20 min with avidin. After a second PBS wash, peroxidase-conjugated biotin was added for 20 min. (Vector Laboratories, CA, USA). The sections were developed in a solution of 3-ethylene-9-aminocarbazole and hydrogen peroxide. In control sections the lectins were substituted with PBS, or the lectins were absorbed with appropriate carbohydrate inhibitors (Table 1) as described by Franklin (1983).

Neuraminidase or trypsin digestion did not change the staining results with monoclonal antibody III D 5 and the digestion was not used in the lectin experiments.

In blocking the staining of the III D 5 the sections were incubated with different lectins at dilution of 1:20 for 20 min before staining with III D 5. The blocking of lectin binding with III D 5 was studied by pretreatment of the slides with III D 5 before lectin histochemistry.

Results

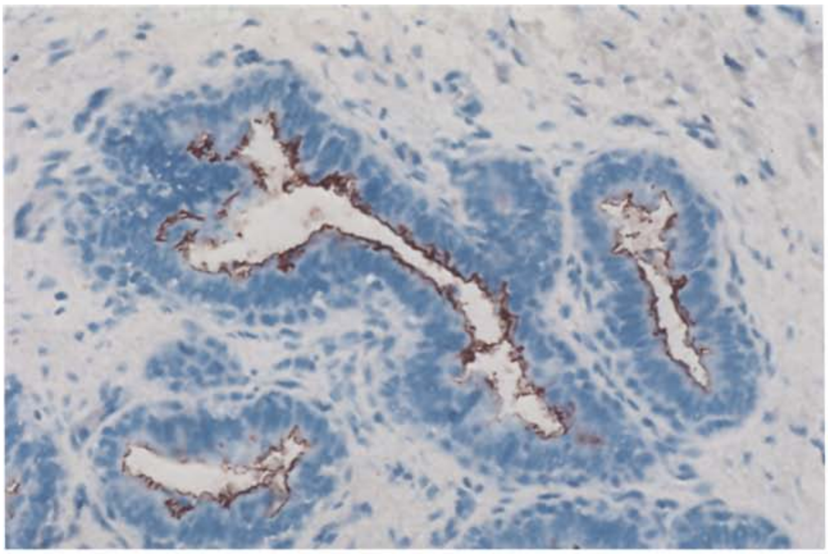
In normal breast and benign proliferations the staining reaction with III D 5 was always along the luminal plasma membrane (Fig. 1). Myoepithelial cell binding was not seen and the stroma was negative. In papillomas and fibroadenomas the antibody showed plasma membrane staining but the staining was not entirely limited to luminal border. Intracytoplasmic staining was never seen.

PNA (Fig. 2), WGA and RCA showed strong apical membrane staining. WGA stained also some myoepithelial cells. The staining with SBA and DBA was weak and there was variation in the intensity of staining. Some acini were totally negative. UEA and ConA showed very weak membrane staining of some cells.

The staining with III D 5 and lectins was always heterogenous. Variable degrees of focal staining were observed and intensity of staining varied between different cells. In 90 percent of the cases identical localization of staining with III D 5 and PNA was observed.

In those cases of breast cancer examined 14 were III D 5 negative. Thirteen of these were also ER negative, measured by biochemical methods. The remaining cases (16) were III D 5 and ER positive. Staining with III D 5 was generally stronger than in benign disease but it also differed qualitatively from benign lesions. Plasma membranes were occasionally stained but the strongest reaction was always intracytoplasmic (Fig. 3). The staining was mostly granular and in some cases the secretory material outside the cancer cells was also positive. The staining showed heterogenous pattern with variations both in the percentage of tumour cells stained and in the staining intensity.

PNA showed strong binding to carcinoma in 15 of cases. The staining was mostly granular but some luminal staining was also observed (Fig. 4). WGA binding was seen in all 30 cases, as a coarsely granular cytoplasmic positivity. RCA showed positive staining in 28 cases with a mainly granular cytoplasmic pattern but luminal binding was also often strong in the cells. Other lectins DBA, SBA,



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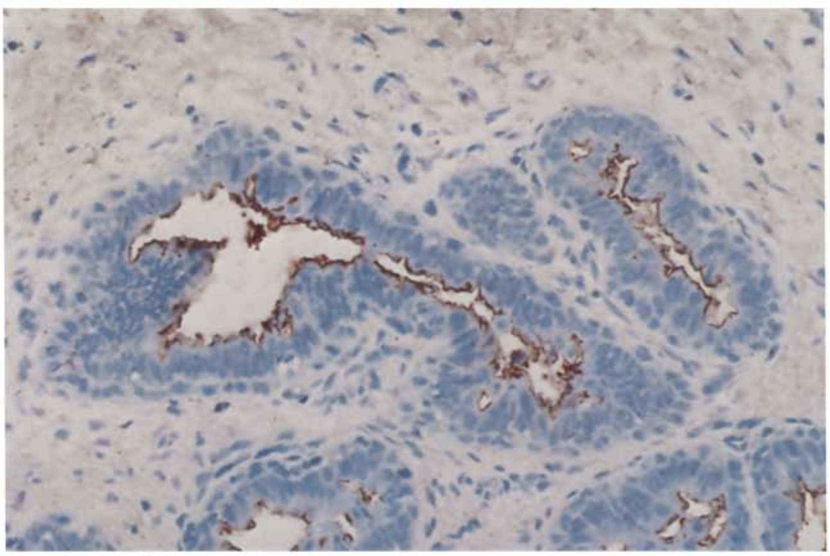

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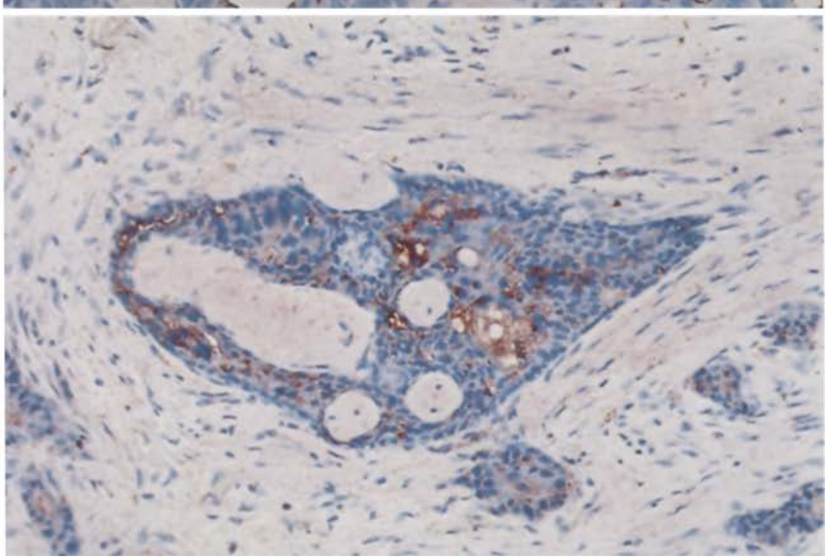

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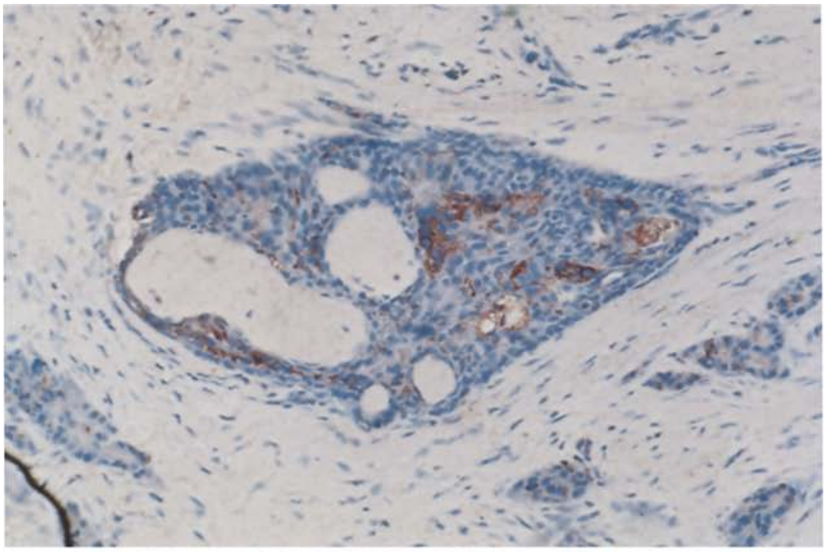
Figs. 1, 2. Staining of normal breast epithelium showing reactivity along the luminal plasma membrane. (**1**) monoclonal antibody III D 5, (**2**) PNA. Immunoperoxidase, counterstained with haemalum. 85 ×

Figs. 3, 4. Staining of infiltrating mammary carcinoma showing heterogenous intracytoplasmic reactivity and reactivity in extracellular secretory material. (**3**) III D 5, (**4**) PNA. 140 ×



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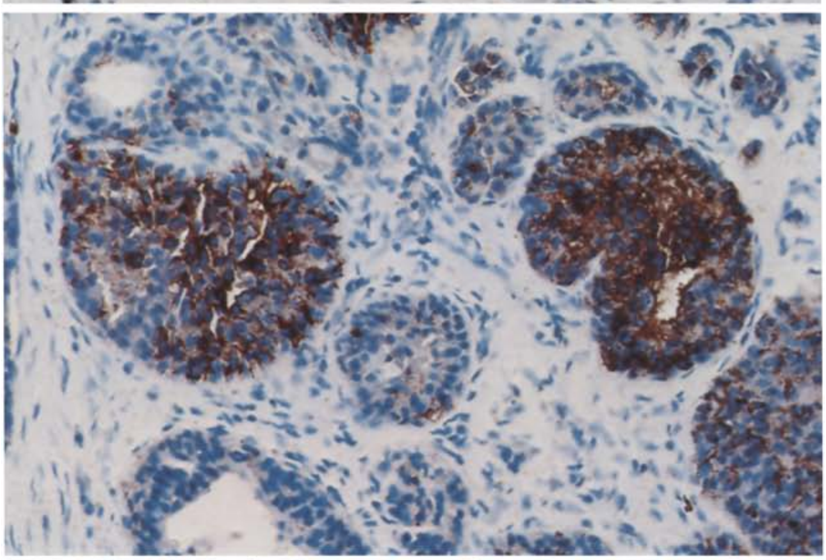

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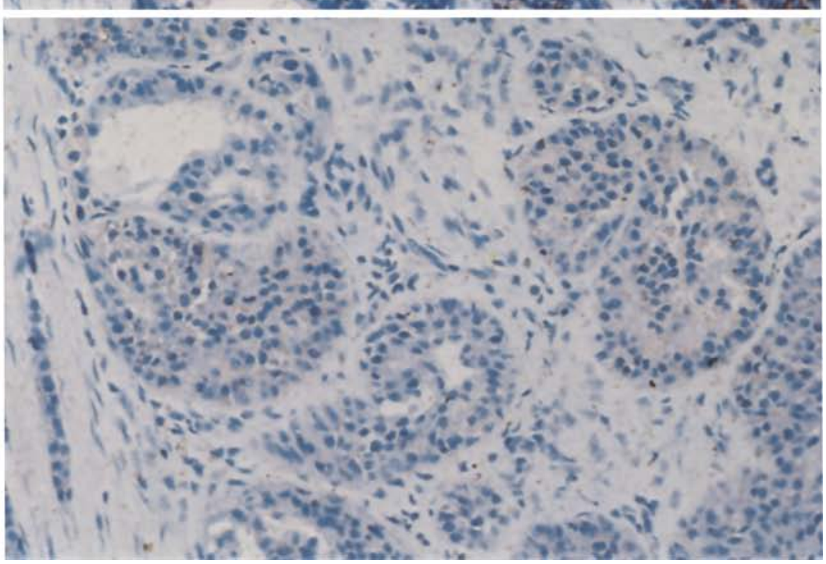

5b

Fig. 5a, b. Staining of infiltrating mammary carcinoma showing the influence of PNA pretreatment to the staining with III D 5. (**a**) III D 5, (**b**) PNA pretreatment. 110 ×

UEA, and ConA showed very heterogenous staining; this was mostly cytoplasmic but some luminal membrane staining was observed. Most of the cases were only weakly stainable or totally negative with these lectins.

Comparition of the reactivity with III D 5 and lectins showed similarities in staining only between III D 5 and PNA. The staining pattern was same in 87% of cases. Thus 13 were almost totally negative with both probes and in 13 cases the positive staining was very similar, including identical heterogenity in staining. The staining pattern with all other lectins was totally different from that of III D 5.

Blocking experiments

When the slides were incubated with lectins before staining with III D 5 only PNA had an effect on the staining reaction. In III D 5 positive cases PNA abolished the intensity of staining significantly (Fig. 5). A weak reaction with III D 5 disappeared when the slides were preincubated with PNA and strong reactions became much weaker. The inhibitory effect of PNA was approximately 80% using a semiquantitative estimation. Pretreatment of slides with III D 5 before lectin staining had only a very weak effect on staining with PNA and RCA. In most cases this pretreatment had no effect on binding of lectins.

Discussion

It has been postulated that tumour associated antigenic epitopes are not proteins but saccharide structures of glycoproteins or glycolipids (Gooi et al. 1983; Feizi 1984). It has been shown that HMFG which is used as antigenic material for different monoclonal antibodies (Arklie et al. 1981; Foster et al. 1982; Hilkens et al. 1984; Krohn et al. 1985) contains different carbohydrate moieties on the surface, both glycolipids and glycoproteins being present (Newman and Uhlenbruck 1976; Horisberger et al. 1977; Farrer et al. 1979; Murray et al. 1979; Farrer et al. 1980). Thus, it should be possible to characterize antigenic epitopes of monoclonal HMFG antibodies with the aid of lectins.

It has been previously shown that the 42–57 kDa molecule, an antigen probably relating to estrogen receptor and detected with monoclonal antibody III D 5, contains galactose or N-acetylgalactosamine (Ashorn and Krohn 1985). Further studies with immunoblotting have shown that this molecule can only be detected with PNA or RCA lectins (Ashorn et al. 1985).

In this study PNA was the only lectin that was similar in staining in normal or benign breast lesions and breast cancer. All other lectins studied, WGA, RCA, SBA, DBA, UEA and ConA gave different staining reactions although there was always some cells which reacted both with III D 5 and these lectins. If the slides were incubated with lectin before staining with III D 5 only, PNA could inhibite staining reaction although not completely. When the incubation with III D 5 was made before lectin staining weak inhibition was observed in PNA staining and very weak effect with RCA staining.

PNA binding in benign lesion was localized mainly to the luminal membrane and the main difference with malignant cells was the transition from luminal to cytoplasmic staining. This binding pattern act as a marker for cell differentiation (Newman et al. 1979; Howard et al. 1981; Franklin 1983; Leathem et al. 1983; Louis et al. 1983; Foster and Neville 1984). A significant correlation with PNA binding and histological grade of breast cancer was demonstrated by Walker (1985). Böcker et al. (1984) showed higher PNA positivity in grade I and II tumours than in grade III but the difference was not significant. The relationship of PNA to histological grade of mammary carcinoma is in keeping with similar staining of III D 5 as III D 5 has been shown to correlate to histological differentiation of breast cancer (Helle and Krohn, unpublished).

In normal rat mammary tissues, estrogen controls the binding sites of PNA. In experimental breast cancer the number of PNA binding sites were lower in hormone-independent than in hormone dependent tumours (Vierbuchen et al. 1983). In human breast cancer the binding of PNA correlates with responsiveness to endocrine treatment and to the estrogen receptor content of the tumour (Klein et al. 1983). Böcker et al. (1984) found similar results between PNA positivity and steroid receptor positivity of tumours. This is in agreement with our results with III D 5. We have previously shown that III D 5 positivity correlates highly significantly with estrogen and progesterone receptor positivity in breast cancer (Krohn and Helle 1985).

PNA reacts with terminal beta-galactosyl groups and it has a high affinity for D-galactosyl-(1–3)-N-acetyl-D-galactosamine (Lotan et al. 1975). RCA, specific to beta-galactose, has different staining reaction when compared with III D 5. SBA and DBA react with N-acetyl-D-galactosamine groups but the staining with these lectins differs from staining with III D 5. Thus, it is difficult to identify the special carbohydrate moieties recognized by III D 5, especially as lectins generally react with more than one mono- or disaccharide. The results from this and our previous study (Ashorn et al. 1985) suggest, however, that the antigen detected by III D 5 is a mono- or di-galactosyl saccharide. III D 5 probably detects overlapping epitopes in the glycoprotein which also binds to PNA as the pretreatment of the slides with PNA reduced the binding of III D 5. On the other hand, as III D 5 can not totally inhibit PNA binding there must be additional galactosyl-groups detected by PNA which do not belong to the epitope of III D 5.

It has been shown that estrogen will induce the synthesis of galactosyltransferase in rats (McGuire 1969). Bolander and Topper (1980) confirmed the stimulation of galactosyltransferase activity by estradiol (E2). Furthermore it has been demonstrated that exogenous estradiol stimulates galactosyltransferase activity in ER positive mammary carcinoma of the rat (Ip and Dao 1978). Sera of patients with breast cancer were also found to have elevated levels of galactosyltransferase (Paone et al. 1980). Thus, our results showing galactosyl-groups in the antigen recognized by III D 5 is further indirect evidence for the dependence of activation of galactosyl transferase enzyme for functional estrogen receptor activity in human mammary carcinomas.

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