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Analysis of the tumour-associated antigen TAG-12 by monoclonal antibody 7A9 in normal, benign and malignant mammary tissues

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Summary. A monoclonal antibody 7A9 was raised against the tumour-associated glycoprotein TAG-12 purified from T47-D breast carcinoma cells. In immunoblots from cytosol of T47-D cells and from sera of breast cancer patients, antibody 7A9 detects the high molecular weight mucin-like TAG-12 antigen. A series of paraffin sections of normal, benign and malignant mammary tissues have been studied with monoclonal antibody 7A9 and the immunoalkaline phosphatase method. In resting gland, proliferating gland and fibroadenoma ducts, reactivity of 7A9 was mainly restricted to luminal membranes of epithelial cells and secretions. 77/79 primary breast carcinomas including ductal, lobular and various other carcinoma types showed cytoplasmic and/or membrane-associated staining with 7A9 in most tumour cells. Metastases (31/31) from different sites were also positive. Strong immunoreactivity with single tumour cells was noted in cytological preparations from freshly resected breast cancer tissue. Thus, monoclonal antibody 7A9 seems to be very useful for the targeting of breast carcinoma cells.

Key words: Tumour-associated antigen – Monoclonal antibody – Breast carcinoma

Introduction

In the past few years, several monoclonal antibodies among them B72.3 (Colcher et al. 1981), HMFG-1 and HMFG-2 (Taylor-Papadimitriou et al. 1981), DF3 (Kufe et al. 1984), 115D8 (Hilkens et al. 1984), and 3E1.2 (Stacker et al. 1985), have been raised against human mammary carcinoma-associated antigens. Most of the breast-carcinoma associated antigens have been characterized as high molecular weight glycoproteins (Sekine et al. 1985; Johnson et al. 1986; Abe and Kufe 1987; Griffith et al. 1987; Stacker et al. 1989). Recently, monoclonal antibody 12H12 was generated against a human breast carcinoma xenograft (Bastert et al. 1987; Kaul et al. 1990). The antigen (TAG-12) detected by antibody 12H12 is secreted by tumour cells, and the measurement of circulating TAG-12 by competition ELISA with antibody 12H12 has proved to be a useful tumour marker test for the follow up of breast cancer patients (Breitbach et al. 1987). For the development of a more sensitive sandwich ELISA, a new monoclonal antibody, 7A9, was established against purified TAG-12 antigen (Werner et al. 1988). Biochemical analyses of purified TAG-12 with monoclonal antibodies 7A9 and 12H12 revealed data indicating that TAG-12 is a high molecular weight glycoprotein (Werner et al. 1988).

In this study, we present the immunodetection of TAG-12 antigen with monoclonal antibody 7A9 in a wide range of breast carcinomas and their metastases. Furthermore, the TAG-12 content was evaluated in normal mammary gland and benign tumours of the breast. The relationship of TAG-12 to other breast carcinoma-associated glycoproteins is discussed in detail.

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Materials and methods

The monoclonal antibody 7A9 has been raised against purified TAG-12 antigen (Werner et al. 1988). Briefly, for the establishment of second generation antibodies against TAG-12 cytosol of T47-D breast carcinoma cells (Keydar et al. 1979) was fractionated by fast protein liquid chromatography with Superose-6 (Pharmacia) and loaded onto a 12H12-affinity chromatography column. Purified TAG-12 antigen was used to immunize mice. Hybridoma cells producing monoclonal antibody 7A9 were injected intraperitoneally into BALB/c mice and ascitic fluid was collected. Immunoglobulin was purified with a protein A column, dialyzed against phosphat-buffered saline (PBS), and the protein concentration was determined by the method of BIO-RAD.

PAGE was performed using the method of Laemmli (1970). Cytosol from T47-D breast cancer cells and sera of patients with breast carcinoma were separated under reducing conditions in a 7.5% running gel overlaid with a 6% stacking gel. Protein transfer was carried out according to the method of Towbin et al. (1979).

The blotted nitrocellulose sheet was blocked with 10% non-fat dry milk for 4 h at 37° C. This was followed by an incubation with monoclonal antibody 7A9 (0.2 µg/ml) for 2 h at 37° C. After washing, the nitrocellulose sheet was treated with biotinylated horse anti-mouse immunoglobulin for 30 min at 37° C and then incubated with avidin biotin-peroxidase complex for 30 min at 37° C (Vectastain). Peroxidase activity was demonstrated using 4-chloro-1-naphtol as substrate.

Surgical specimens of resected mammary tissue were fixed in 10% formalin and embedded in a routine manner. Cryostat sections (5 µm) were fixed in acetone and stored at −20° C. Imprint preparations were obtained by pressing freshly resected tissue on untreated slides, fixed in methanol/acetone (1:1), and stored at −20° C in a phosphate buffered solution containing 42.8 g sucrose, 0.7 g MgCl and 250 ml glycerin per 500 ml. From each tumour examined, histopathological grading was determiend as proposed by Bloom and Richardson (1957).

Paraffin sections (3 µm) were deparaffinized in xylene and rinsed in ethanol. After rinsing in PBS for 10 min, the preparations were incubated with PBS containing 1% bovine serum albumin (BSA) and normal horse serum (Vectastain) at a dilution of 1:80 for 30 min at room temperature. All subsequent reagents were diluted in PBS/1% BSA and incubated at room temperature. The pretreatment serum was removed and 0.2 µg of purified monoclonal antibody 7A9 was added for 60 min. After rinsing in PBS, the sections were incubated for 30 min with biotinylated horse anti-mouse antisera at a dilution of 1:200 (Vectastain). The sections were rinsed in PBS, and avidin:biotinylated alkaline phosphatase complex (Vectastain) was added. After another rinse in PBS, reactivity was visualized with New Fuchsin as substrate. The preparations were counterstained with haematoxylin and mounted with Kaiser's gelatine (Merck). Cryostat sections and imprint preparations were brought to room temperature, blocked and immunostaining with monoclonal antibody 7A9 was performed as described above.

Results

Cytosol from T47-D breast carcinoma cells and 3 sera from patients with breast carcinomas were separated on SDS:PAGE and blotted on a nitrocellulose sheet. TAG-12 was detected by immunoblotting with monoclonal antibody 7A9 (Fig. 1). High molecular weight bands in the range of more than 200000 D were obtained with 7A9 in T47-D cytosol (lane 1) and the sera of 3 breast cancer patients (lanes 2, 3, 4). There was some heterogeneity in the banding pattern of TAG-12 obtained in immunoblots from different sources.

To exclude the possibility of TAG-12 denaturation by routine formalin fixation and paraffin embedding, 5 µm cryostat sections of benign ($n=4$) and malignant ($n=8$) mammary tissues were fixed in acetone for 10 min and stained with monoclonal antibody 7A9 (0.2 µg/slide). There was no difference in the detection of TAG-12 antigen in frozen and formalin fixed, paraffin embedded tissues. Ductal and lobular mammary carcinomas were strongly reactive and exhibited the same staining pattern in both frozen and formalin fixed sections.

TAG-12 antigen was evaluated with monoclonal antibody 7A9 in sections of formalin fixed, paraffin embedded normal and benign mammary tissues (Table 1). The antigen could be detected mainly in luminal membranes of epithelial cells and secretions. Both in resting and in proliferating breast acini only luminal membranes of few epithelial cells were stained (Fig. 2e). Occasionally, only the edges of secreted materials were positive with 7A9. In epitheliosis and the ducts in fibroadenoma, most luminal membranes and secretions were stained (Fig. 2f). Diffuse cytoplasmic staining was obtained in less than 1–5% of epithelial cells in 3/11 fibroadenomas and ductules of one normal mammary tissue. Several desquamated cells of the epithelial lining in benign mammary tissues showed staining of the surrounding cell

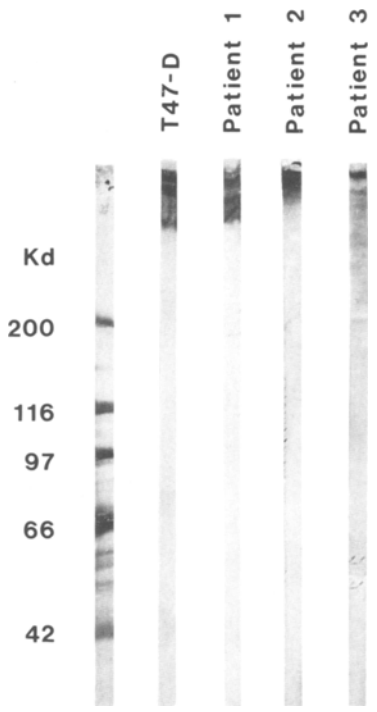


Fig. 1. TAG-12 antigen from T47-D mammary carcinoma cells (Lane 1) and sera of 3 patients with breast carcinoma (Lanes 2, 3, 4) were analysed by SDS-PAGE and immunoblotting with monoclonal antibody 7A9. The TAG-12 antigen detected by 7A9 has a molecular weight of more than 200 kD

Table 1. Reactivity of monoclonal antibody 7A9 with paraffin sections of normal and benign mammary tissues

	Secretions	Luminal membranes	Epithelial cells
Resting breast acini	6/8*	7/8	0/8
Resting ductules	6/8	8/8	1/8
Proliferating breast acini	0/1	1/1	0/1
Proliferating ductules	3/3	3/3	0/3
Fibroadenoma ducts	8/11	11/11	3/11
Cyst epithelium in fibrocystic disease	4/4	4/4	0/4

* Number of sections positive/number of sections examined

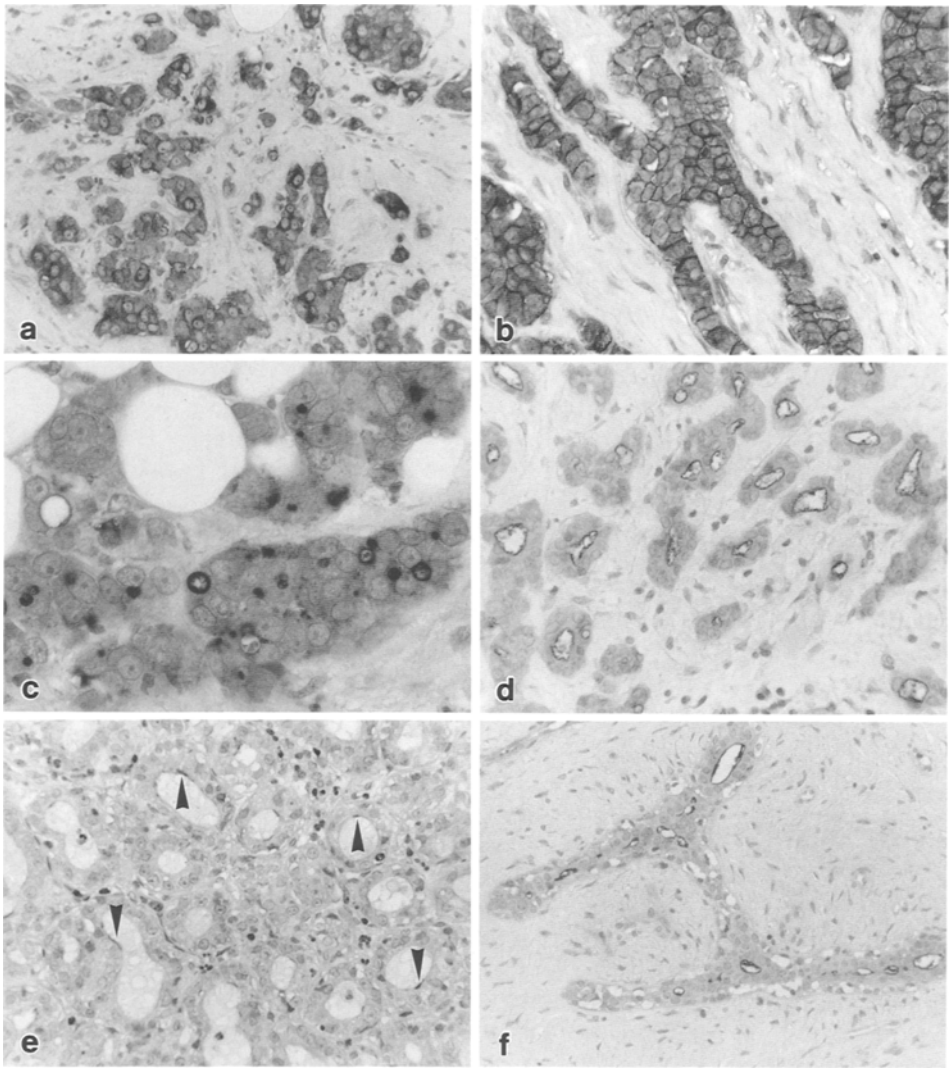


Fig. 2. Immunoalkaline phosphatase staining of formalin fixed mammary tissues with monoclonal antibody 7A9; **a** duct carcinoma; diffuse cytoplasmic staining with variable intensity of the tumour cells, the stroma is negative, ×320; **b** lobular carcinoma; tumour cells show strong cell membrane associated and weak cytoplasmic reaction, ×400; **c** ductal carcinoma; focal cytoplasmic staining next to the nucleus, ×460; **d** tubular carcinoma; reaction is restricted to the apex of tumour cells, ×320; **e** proliferating mammary gland; few epithelial cells show staining of the apical membrane (*arrows*); secretions are negative, ×400; **f** fibroadenoma duct; reactivity of apical membranes of the epithelial lining, ×320

Table 2. Reactivity of monoclonal antibody 7A9 with paraffin sections of mammary carcinomas

	n/N
Ductal Ca	40/40*
Lobular Ca	17/17
Tubular Ca	7/7
Mucinous Ca	7/9
Medullary Ca	4/4
Papillary Ca	2/2

* Number of positive sections/number of sections examined

membrane. Reactivity of myoepithelial or stromal cells with monoclonal antibody 7A9 was not observed.

A total of 79 tissue sections of primary breast carcinomas including ductal and lobular carcinomas as well as other breast tumour types were stained with monoclonal antibody 7A9 (Table 2). There was strong reactivity of 7A9 with the majority of tumour cells in nearly all carcinomas examined. The cells showed varying staining patterns with membrane and/or cytoplasmic staining (Figs. 2a–d, 3a). Membrane staining was apparent in the surrounding cell membrane (Fig. 2b) and at luminal membranes (Fig. 2d). In some cells only the edges of the cell membrane including outer parts of the cytoplasm were reactive with 7A9. Staining was diffuse in cytoplasm (Fig. 2a) or focal in the form of spots of varying size localized next to the nucleus (Fig. 2c). Tumour cell groups in some tissues showed marginal staining. In a few sections, there were areas with a high percentage of positive tumour cells close to tumour cell groups with only a few positive cells.

TAG-12 could be detected in 40/40 ductal carcinomas (Table 2). Diffuse and focal cytoplasmic staining was predominantly observed (Fig. 2a and 2c). The percentage of positive tumour cells was often lower in in-situ components compared to areas of invasion. The number of cells reactive in each tumour was correlated with the histological grading (Table 3). Specimens with the highest numbers of positive cells were found in duct carcinomas with histological grades of II and III. How-

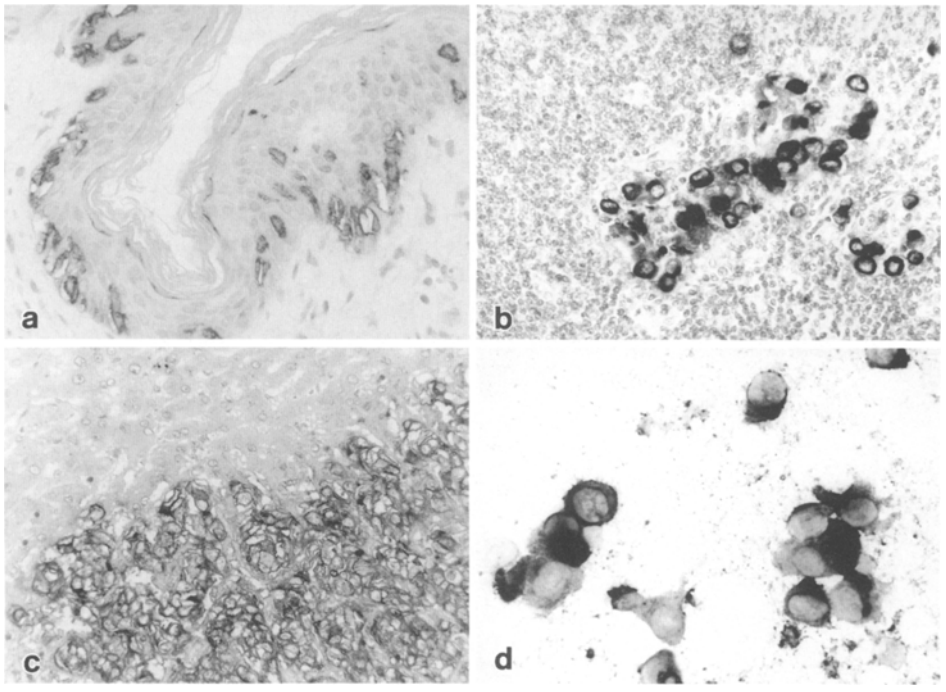
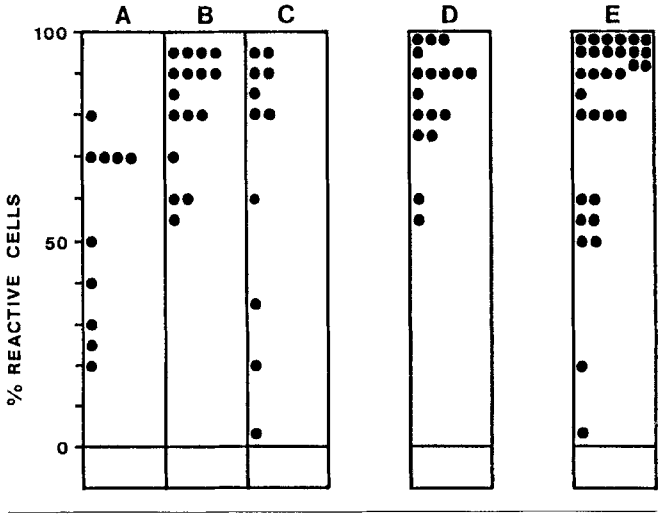


Fig. 3. Immunoalkaline phosphatase staining of formalin fixed tissues (**a, b, c**); **a** Paget's disease, cytoplasmic staining of the tumour cells, ×400; **b** lymph node metastases of a duct carcinoma, ×400; **c** liver metastases of a duct carcinoma, ×320; **d** cytology preparation of a duct mammary carcinoma, staining of single tumour cells, ×540

Table 3. Percentage of tumour cells reactive with monoclonal antibody 7A9 in paraffin sections from ductal mammary carcinomas of histological grade I (A), grade II (B) and grade III (C); lobular mammary carcinomas (D); metastases of mammary carcinomas from lymph nodes and distant sites (E)



ever, 3 cases with relatively low numbers of positive cells (less than 40%) were in the group of histological grade III. Interestingly, in duct carcinomas with histological grade I, only 40–70% of the tumour cells were reactive with monoclonal antibody 7A9.

In all lobular carcinomas (Table 3), the majority of tumour cells was reactive, with 7A9 preferentially staining the surrounding cell membrane and/or the cytoplasm of the tumour cells (Fig. 2b). Other patterns such as focal cytoplasmic staining were noticed as well, but only rarely. When compared with the histological grade, there was no difference in the percentage of tumour cells positive for TAG-12. Tumour cells of other breast carcinoma types (Table 2) showed reactivity of antibody 7A9 mainly with luminal membranes (Fig. 2d). Only medullary carcinomas exhibited staining of the cytoplasm and the surrounding cell membrane. Paget cells involving the epidermis are shown in Fig. 3a.

A series of metastatic lesions of primary mammary carcinomas ($n=31$) including metastases from lymph nodes and from distant sites (liver, skin, ovary, bone marrow) were studied with monoclonal antibody 7A9 (Table 3). 16/31 metastases had increased, 10/31 nearly the same and 5/31 had a lower rate of positive tumour cells when compared with specimens from the primary sites. In one case, the metastatic cells of a duct carcinoma with marginal staining were only reactive with 7A9 in less than 2% of cells. The individual staining pattern of the primary tumour, that is to say the predominant type of cytoplasmic or membrane staining, was conserved in most metastases. Figure 3b shows a small metastatic tumour cell group of a duct carcinoma in a lymph node. Metastases from distant sites were also strongly positive for TAG-12 (Fig. 3c).

The results of immunocytology with imprint preparations confirmed the data obtained with paraffin sections; in 26 preparations from ductal and lobular carcinomas most cells reacted with monoclonal antibody 7A9 (Fig. 3d).

Discussion

Most breast carcinoma-associated antigens defined by monoclonal antibodies like B72.3, DF3, HMFG 1 HMFG 2, 115D8 and 3E1.2, have been characterized as high-molecular-weight mucin-type glycoproteins

(Sekine et al. 1985; Johnson et al. 1986; Abe and Kufe 1987; Griffith et al. 1987; Stacker et al. 1989). Mucins are components of cellular secretions and epithelial cells of normal or lactating mammary gland showed also reactivity with monoclonal antibodies against breast carcinoma-associated glycoproteins (Thor et al. 1986; Stacker et al. 1985; Hilkins et al. 1984). Furthermore, breast carcinoma-associated glycoproteins were found to share a number of common biochemical features with components of human milk such as high molecular weight of more than 300000, O-glycosidic linkages of carbohydrates, binding of specific lectins and digestion by enzymes catalysing variable peptide bonds (Sekine et al. 1985; Johnson et al. 1986; Stacker et al. 1989; Burchell et al. 1983; Ormerod et al. 1985). Thus, mammary-carcinoma associated glycoproteins and molecules found on human milk fat globule or in human milk, like epithelial membrane antigen (EMA), may form a family of related molecules.

The antigen (TAG-12) used for the generation of monoclonal antibody 7A9 originaed from the permanent human breast cancer cell line T47-D (Werner et al. 1988). TAG-12 was detected in breast carcinomas and, at low concentrations, in normal and benign mammary gland suggesting that the antigen bound by 7A9 may be related to the family of other high-molecular-weight glycoproteins identified in breast carcinomas. Additionally, TAG-12 was found to share similar biochemical features with these molecules like a high molecular weight (more than 200000 D), high amount of terminal sialic acid and the presence of specific carbohydrates as shown by neuraminidase digestion and lectin binding assays (Werner et al. 1988). After treatment of TAG-12 with hyaluronidase and chondroitinase, binding of monoclonal antibody 7A9 was not affected but the antigen was sensitive to digestion by different proteases (Werner et al. 1988). Like most other breast carcinoma associated high-molecular-weight glycoproteins, TAG-12 is secreted by the tumour cells and circulating antigen can be determined in the serum of tumour patients (Breitbach et al. 1987; Paterson et al. 1986; Hayes et al. 1985; Stacker et al. 1985; Tjandra et al. 1988).

The detection of TAG-12 in epithelial cells of normal and benign mammary tissues was mainly restricted to the luminal membrane, whereas cells of ductal and lobular breast carcinoma showed varying staining patterns associated with the cell membrane and/or the cytoplasm. 57/57 ductal and lobular mammary carcinomas were positive for TAG-12 and the percentage of tumour cells reacting with 7A9 was relatively high. Interestingly, the number of positive cells was lower in histological grade I than in grade II and most of grade III ductal carcinomas. Occasionally, in situ components of ductal carcinomas exhibited only a few positive cells. All metastases except one were stained by 7A9, frequently displaying the appropriate staining pattern of the primary tumour. Only two mucinous carcinomas were negative and one ductal carcinoma as well as one metastatic lymph node exhibited merely few positive cells. The wide range of positive tumours and the high number of tumour cells reacting with 7A9 in tissue sections and cell preparations demonstrated that TAG-12 is a useful marker for targeting breast carcinoma cells. However, immunoreactivity of monoclonal antibody 7A9 was also noticed in several carcinomas not originating from the mammary gland (in preparation). The immunodetection of TAG-12 by 7A9 corresponded to the results obtained with monoclonal antibody 12H12 (A. Schauer, personal communication). This previously established monoclonal antibody (Bastert et al. 1987; Kaul et al. 1990) has been shown to detect a different epitope from 7A9 (Werner et al. 1988).

Despite the similarity of most characterized breast carcinoma associated antigens with TAG-12, there are some differences concerning their tissue distribution. For instance, monoclonal antibody B72.3 reacted only with 46% of mammary carcinomas and 62% of metastatic lesions examined (Nuti et al. 1982). Additionally, monoclonal antibodies 7A9 and B72.3 showed differences in the distribution of positive cells among serial sections of the same tumours (data not shown). Monoclonal antibodies HMFG-1 and HMFG-2 and other antibodies raised against human milk-fat globules seem to recognize those differentiation antigens of the mammary gland and its tumours that are mainly detected in better differentiated carcinomas (Arklie et al. 1981; Hilkens et al. 1984; Helle et al. 1988). The mucin-like glycoprotein MSA defined by antibody 3E1.2 was found in all tumours examined (Stacker et al. 1984). In contrast to 7A9, however, lobular carcinomas showed a predominantly cytoplasmic staining with 3E1.2 (Stacker et al. 1984).

Other authors have shown that epitopes of a series of monoclonal antibodies against breast carcinoma-associated glycoproteins are detectable in a family of peanut lectin binding urinary mucins (PUM) which exhibit genetic polymorphism (Swallow et al. 1986) as well as on antigens purified by monoclonal antibodies HMFG-2 and NCRC-11 (Griffith et al. 1987; Price et al. 1985, 1986). Monoclonal antibodies F36/22 and Ca1 bound to the DF3 antigen (Abe and Kufe 1987) and the epitopes of monoclonal antibodies DF3 and BC3 were shown to be present on MSA (Stacker et al. 1989). These data indicate that the mucin-like breast carcinoma associated glycoproteins may be a family of related molecules sharing the same or similar epitopes. The question of the relationship of the TAG-12 antigen bound by monoclonal antibodies 7A9 and 12H12 (Kaul et al. 1988; Werner et al. 1988) to this family of high molecular weight glycoproteins cannot be resolved by immunohistology. Initial biochemical analyses have demonstrated the similarity of TAG-12 with most of the characterized breast carcinoma associated glycoproteins and further studies comparing the epitopes of antibodies 7A9 and 12H12 with other antibodies against mucin-like glycoproteins associated with mammary carcinomas are recommanded.

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