

# Study of the expression of Tn antigen in different types of human breast cancer cells using VVA-B<sub>4</sub> lectin

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Received May 17, 2005; Accepted July 8, 2005

**Abstract.** VVA-B<sub>4</sub> lectin was used to investigate the differences in Tn antigen expression in tissues of different types of human breast cancer (benign lesions, carcinoma *in situ*, invasive carcinoma) and in normal tissues neighboring lobular carcinoma. Locations in which Tn antigen was expressed were identified using the avidin-biotin-peroxidase labeling system. Tissues collected during cosmetic procedures and classified as normal were completely negative, except for one case. Benign proliferative changes including fibroadenoma, apocrine and cylindrical metaplasia showed a very weak positive reaction, although strongly positive cells were also observed. The reaction in non-invasive cases of atypical hyperplasia was diversified depending on site. Intralobular hyperplasia was characterized by a particularly high percentage of labeled cells. A majority (up to 80%) of ductal and lobular carcinoma *in situ* showed very strong or moderate staining. In invasive cancers, there were conspicuous differences between stage of cancer development and tendency towards a decrease in intensely labeled cell count in the most advanced stages. In normal tissues in the direct neighborhood of carcinoma *in situ*, the cytoplasm of 40% of cells was strongly labeled. However, the findings for normal tissues in the close vicinity of invasive cancer were the most surprising, since there was either no or only very weak positive reaction. It can be concluded that glycosylation modifications during carcinogenesis, as demonstrated by the presence of Tn epitope, develop very early, before any destructive changes in proliferation/apoptosis or cell differentiation become discernible.

## Introduction

Oncofetal antigens, including T (Thomsen-Friedenreich) antigen and Tn antigen, occupy a prominent position among numerous known tumor markers (1). The structure of these antigens matches the basic core 1 structure of Golgi apparatus glycoproteins containing *O*-glycoside bond which comprise, among others, blood group antigens A, B, H and Lewis (2). T antigen structure can be identified in a molecule of glycophorine A<sup>M</sup> and glycophorine A<sup>N</sup> in the form of tetrasaccharide chains NeuAc- $\alpha$ 2-3Gal $\beta$ 1-3[NeuAc $\alpha$ 2-6]GalNac $\alpha$ 1-linked through *O*-glycoside bonds with serine and threonine of the peptide chain. The sialylated form of Tn antigen (sTn) is formed due to a lack of  $\beta$ 1-3 D-galactosyltransferase activity, while Tn antigen is created when the activities of the two enzymes -  $\beta$ 1-3 D-galactosyltransferase and  $\alpha$ 2-6 sialyltransferase, are lacking (3,4).

The appearance of a non-cryptic Tn antigen structure is observed in a short period of fetal life (5) in so-called Tn syndrome (6) and during the process of carcinogenesis (7). Its widespread presence has been noted on tumors of epithelial origin but it is absent on tumors of ectodermal and mesodermal origin (8). Studies using monoclonal antibodies and lectins have demonstrated the expression of sialylated and non-sialylated forms of Tn antigen in gastrointestinal tumors, particularly in colon cancer (9,10), pancreatic (11) and gastric (12) cancers, in ovarian (13) and uterine (14) cancers, and in numerous cases of breast cancer (14-18). Tn antigen mostly occurs in malignant breast lesions, invasive carcinomas and their metastases, but it has also been found in benign lesions. Except in very rare cases (19,20), it is not detected in normal breast tissue.

Studies into the localization of Tn antigen have widely used lectins with appropriate sugar specificity, besides monoclonal antibodies. VVA-B<sub>4</sub>, one of three isolectins occurring in *Vicia villosa* (*Fabaceae*), belongs to the group of the lectins commonly used for this purpose. This lectin (108 kDa glycoprotein built of 4 identical subunits) specifically binds to the sugar sequence containing *N*-acetylgalactosamine at the chain end, and reacts only with human Tn antigen-exposed erythrocytes or erythrocytes with Cad specificity (21).

It has been demonstrated that neoplastic transformation of the cell leads to permanent or reversible blockade of  $\beta$ 1-3

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**Key words:** Tn antigen, human breast tumors, lectin histochemistry, *Vicia villosa* lectin

Table I. Tn antigen expression in normal tissue and in different types of breast cancer.

Cancer type		No. of cases	Staining intensity <sup>a</sup>	Stained cells (%)
Normal tissues		3	(0): 2 cases (+): 1 case	0 >15
Benign changes	Cylindrical metaplasia	3	(0)→(+)	<20
	Apocrine metaplasia	7	(+)→(+++)	90
	Fibroadenoma	2	(+) (++)	5 40
Atypical hyperplasia	Intraductal hyperplasia	4	(0)→(+): 3 cases (+++): 1 case	0-5 30-50
	Intralobular hyperplasia	2	(++)	30-60
Primary cancer <i>in situ</i>	Ductal carcinoma	5	(++)	60-80
	Lobular carcinoma	2	(++)→(+++)	20-50
Invasive intraductal cancer	Grade I <sup>b</sup>	1	(+++)	70
	Grade II <sup>b</sup>	4	(++)→(+++)	25-100
	Grade III <sup>b</sup>	3	0→(+++)	40-60
Invasive lobular cancer	Lobular carcinoma	2	(++)	20-70
Cancer metastasis	Metastasis cells of ductal carcinoma	1	(++)→(+++)	100
Normal epithelial tissue neighboring ductal and lobular cancer lesions	<i>In situ</i> carcinoma	2	(+++)	40
	Invasive carcinoma	8	(0): 4 cases (+): 3 cases (++): 1 case	0 5 40

<sup>a</sup>According to a scale from + to ++++; <sup>b</sup>According to Bloom Richardson classification.

D-galactosyltransferase (22), but it is not known at which stage of this process functional changes of this enzyme occur.

In precedent study reports, we have demonstrated using direct fluorescent labeling that in pathologies considered as benign and in grade I of invasive ductal carcinomas, the binding site was essentially on the cell membrane, but that in grades II and III, the labeling involved the cytoplasm, and cell heterogeneity appeared.

In the present study, we used VVA-B<sub>4</sub> lectin to investigate differences in Tn antigen expression in tissues of different types of human breast cancer (benign lesions, carcinoma *in situ*, invasive carcinoma) and in normal tissues neighboring ductal or lobular carcinoma, using an indirect HIC labeling to accurately determine the nature and localization of the labeling cells in these tissues.

## Materials and methods

**Breast tissues.** Breast tissue specimens sampled during surgical procedures from 46 patients were provided by the Anatomical and Pathological Service of the Jean Perrin University Clinic in Clermont-Ferrand. The tissues showed benign hyperplastic changes (apocrine metaplasia, cylindrical metaplasia, and

fibroadenoma), atypical hyperplasia, carcinoma *in situ* and invasive cancer (ductal and lobular forms). The invasive ductal tumors were graded according to the Bloom and Richardson classification (23). All cases were initially assessed by an expert pathomorphologist. Normal breast tissues collected from 3 non-pregnant woman aged between 17-29 years, originated from a private clinic performing mammoplastic procedures. The study material was divided into 7 categories: normal tissues, benign changes, atypical changes, primary cancer *in situ*, invasive ductal and lobular cancers, ductal cancer metastases to lymph nodes, and normal epithelial cells in the direct vicinity of lobular cancer (Table I).

The tissues were fixed with 4% formalin solution and embedded in paraffin. The 4 µm-thick sections were simultaneously analyzed by the conventional staining method (hematoxylin and eosin) and by an indirect immunohistochemical method utilizing dual antibodies and the streptavidin-biotin-peroxidase labeling system.

## Immunohistochemical method

**Lectin.** The solution of VVA-B<sub>4</sub> lectin (Sigma) in PBS had a concentration of 2.5 µg/ml. This lectin, isolated from *Vicia villosa* (Fabaceae) seeds, shows specificity for N-acetyl-

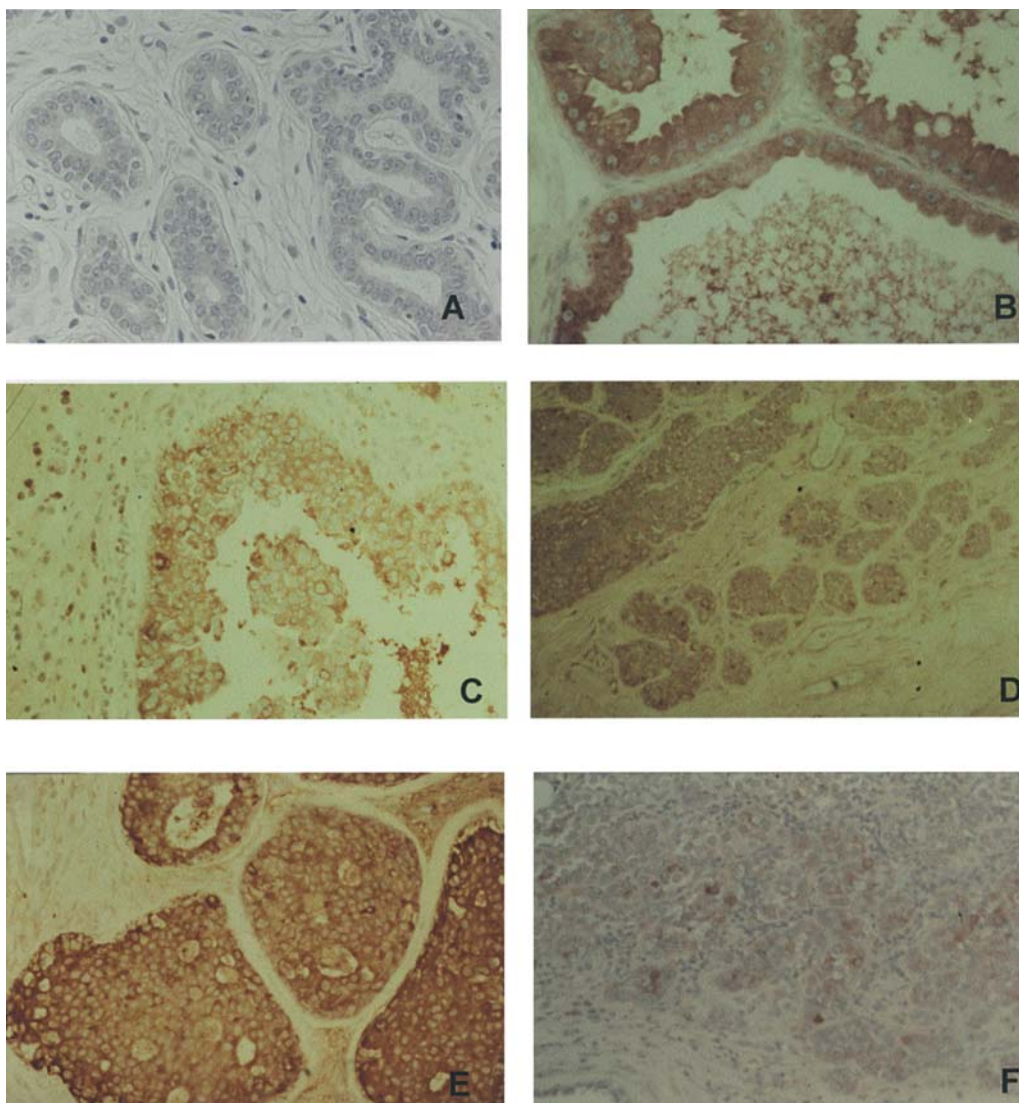


Figure 1. Tn antigen in benign changes and non-invasive primary cancer. A, Normal breast tissue from a mammoplastic procedure (x200). B, Apocrine metaplasia of three galactophores. Cells with strongly-stained cytoplasm on the apical pole (x320). C, Atypical ductal hyperplasia (x200). D, Atypical lobular hyperplasia (x200). E, Ductal *in situ* carcinoma. A majority of the cells were strongly stained (x200). F, Lobular *in situ* carcinoma. The heterogeneity of staining intensity (from + to +++) and of the number of labeled cells are clearly visible, with inflammatory infiltration negative (x200).

galactosamine (GalNAc) linked to the  $\alpha$ -O-glycoside bond with serine or threonine of the peptide chain, which corresponds to Tn antigen structure.

**Antibodies.** Rabbit anti-VVA-B<sub>4</sub> polyclonal antibodies were obtained by immunization of rabbits by systematic intravenous injections of the lectin bound with erythrocytes previously collected from the same rabbit. The appearance and specificity of the anti-lectin antibodies in the rabbit serum were monitored according to the Ouchterlony immunoprecipitation method.

Biotinylated goat anti-rabbit IgA monoclonal antibodies (Dako) were purchased as Universal LSAB 2 Kits together with the streptavidin-peroxidase system.

**Streptavidin labeling system.** Streptavidin-peroxidase complex and AEC (3-amino-9-ethylcarbazole), which is a peroxidase substrate, were purchased from Dako as ready-to-use reagent.

**PBS-BSA buffer.** Phosphate-buffered physiological saline containing 1% bovine serum albumin fraction V (MERCK)

was used to saturate unspecific lectin and antibody binding sites.

**Procedure.** Following paraffin removal and hydration, the sections were incubated in 3% hydrogen peroxide for 10 min in order to deactivate endogenous peroxidase. They were subsequently washed once in distilled water, three times in PBS and twice in PBS-BSA, and incubated with lectin solution at room temperature for 1 h. Then, the tissues washed in PBS-BSA were overlaid with rabbit serum containing anti-lectin antibodies (1-h incubation), biotinylated goat antibody (10-min incubation), streptavidin-peroxidase system (10 min) and 3-amino-9-ethylcarbazole (AEC), which is a peroxidase substrate (10 min). Before application of each reagent, the tissues were thoroughly rinsed with PBS-BSA. Finally, the sections were dipped in hematoxylin solution for 15 sec and in water with several drops of ammonia to sharpen image contrast. Double negative control was processed simultaneously. Tissues taken as control were incubated with PBS-BSA buffer instead of lectin and rabbit antiserum. Inhibition tests were used to



confirm specificity of VVA-B<sub>4</sub> binding to the sugar sequence corresponding to Tn antigen. This consisted in adding 0.1 M *N*-acetyl-D-galactosamine solution to the lectin solution before application to the tissues.

## Results

The immunohistochemical method was able to localize sites of expression of Tn antigen sugar structure in the tissues, which were visualized as reddish-brown-stained cells. Staining intensity (expressed according to a four-grade scale), staining character (granular, dispersed), localization in the cell (cytoplasmatic or membrane labeling) were analyzed. In addition, an approximate percentage of labeled cells was calculated. The following observations were made in the cases divided into 7 basic categories (Table I).

*1. Normal tissues.* Among three cases under analysis, in two cases the reaction of epithelial cells was entirely negative. In the third case, the cytoplasm of about 15% of epithelial cells was very weakly stained (Fig. 1A).

*2. Benign changes.* Specimens with benign changes, caused by disturbances in the proliferation process, showed generally weak positive reactions, with differentiated staining intensity depending on the type of change: a) Cylindrical metaplasia: staining intensity was weak and number of stained cells did not exceed 20%. b) Apocrine metaplasia: a large majority of cells (90%) showed dispersed cytoplasmic staining, and staining intensity varied greatly between the cases (Fig. 1B). c) Fibroadenoma: nearly 4% of the cells showed moderate staining intensity.

*3. Atypical changes.* a) Atypical ductal hyperplasia: among four cases under investigation, three exhibited negative or almost negative reactions. In one case, cytoplasm staining of about 40% of the cells was intense (Fig. 1C). b) Atypical lobular hyperplasia: the cytoplasm of 30-60% of the cells was weakly stained (Fig. 1D).

*4. Carcinoma in situ.* a) Ductal carcinoma: the cytoplasm of more than half (60-80% depending on the case) of the cells was moderately stained (++). Granular aggregations were sometimes clearly visible (Fig. 1E). b) Lobular carcinoma: 20-50% of the cells were strongly or moderately stained. Cytoplasm staining was noticeably heterogeneous (Fig. 1F).

*5. Invasive carcinoma.* a) Invasive ductal carcinoma: both number of stained cells and stain intensity varied depending on stage of tumor development (as per Bloom Richardson classification): Stage I, distinct strong staining of the cytoplasm of the majority of cancer cells (Fig. 2A). Stage II, number of stained cells and stain intensity differed strongly between cases. We observed that the cytoplasm of cells with a lower degree of transformation was more intensely stained (Fig. 2B). Stage III, at this stage of carcinogenesis, staining intensity became heterogeneous and there was a tendency towards lower numbers of stained cells (Fig. 2C). b) Invasive lobular carcinoma: from 20 to 70% of the cells were moderately stained (Fig. 2D).

*6. Metastases of invasive ductal carcinoma.* Specimens of axillary lymph nodes contained intensely-stained metastatic cells forming cords (Fig. 2E).

*7. Normal epithelial cells located near in situ or invasive ductal and lobular cancer lesions.* a) Primary carcinoma *in situ*: nearly half the normal epithelial cells located in close vicinity of cancer cells had intensely-stained cytoplasm (Fig. 2G and H). b) Invasive carcinoma: the reactions of the non-transformed cells varied among the 8 cases under study. In 4 cases, all normal cells presented negative reaction, in 3 cases only 5% of non-transformed cells showed weak reaction, and in 1 case, about 40% of the cells were moderately stained (Fig. 2F).

## Discussion

Immunolabeling using VVA-B<sub>4</sub> lectin and anti-lectin antibody allowed us to demonstrate the changes in the *O*-glycosylation process leading to the formation of GalNAc  $\alpha$ -*O* serine/threonine-type (Tn antigen) residues. Application of this method confirmed results obtained much earlier with procedures utilizing fluorochrome-bound lectin (FITC) (19,20). Moreover, this method provides additional information about character and labeling localization (membrane, cytoplasmic, dispersed, condensed, granular), and allows both a preliminary quantification and establishment of the nature of the labeled cells.

Tissues collected during cosmetic procedures and classified as normal were completely negative in two cases, whereas a weak positive reaction of a very small number of cells seen in one case remains puzzling and difficult to explain. The labeling could have been caused by genetically-determined changes in glycosylation which did not carry any pathological consequences, although some early stage of cell pathology cannot be ruled out. Therefore, in view of proper and widespread prevention, it seems justified to also conduct systematic studies and identification of T and Tn antigen in tissues collected during cosmetic procedures.

Benign proliferative changes such as fibroadenoma and apocrine and cylindrical metaplasia usually showed a very weak positive reaction but there was a population of strongly-labeled cells. Numerous cells with strongly-stained cytoplasm were particularly conspicuous in apocrine metaplasia (Fig. 1B). The question as to whether the population of labeled cells will evolve towards neoplastic transformation could be definitively answered when this reaction is confirmed with other methods (e.g. using specific antibodies). Therefore, modifications of the glycoconjugate structures may precede the anomalies considered to be characteristic of malignant transformation, which is consistent with the existence of intermediate stages between normal and malignant states.

In non-invasive cases of atypical hyperplasia, labeling was distinct and depended on the site where this hyperplasia occurred. The ductal hyperplasia cells, except for one case (Fig. 1C), showed weaker labeling, while lobular hyperplasia was characterized by a relatively high percentage (up to 60%) of cells showing moderate labeling (Fig. 1D).

A vast majority (up to 80%) of *in situ* ductal and lobular carcinomas exhibited very strong or medium staining (Fig. 1E

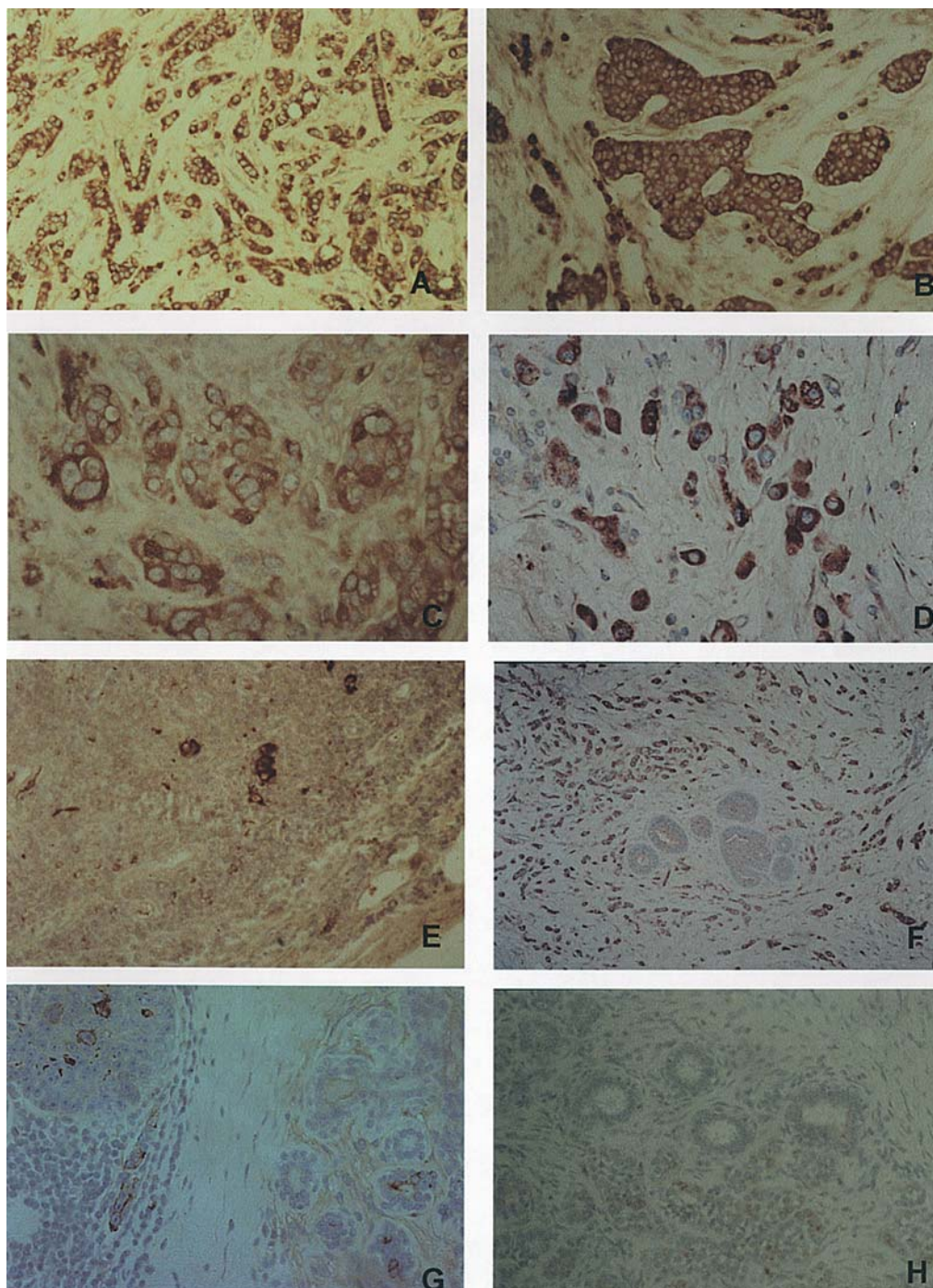


Figure 2. Tn antigen in invasive carcinoma and normal epithelial tissue neighboring ductal and lobular cancer. A, Invasive ductal cancer grade I. B, Invasive ductal cancer grade II (x200). Strong (+++) staining of almost all cells. C, Invasive ductal cancer grade III (x320). Besides strongly stained (+++) cells, negative (-) cancer cells are visible. D, Invasive lobular cancer (x450). Strong (+++), granular staining of the cytoplasm. E, Metastasis cells of ductal cancer to the axillary lymph node. Very strong cytoplasmic staining of the cancer cells (x225). F, Normal tissue in the vicinity of lobular invasive carcinoma (x200). Galactophore cells exhibit cytoplasm staining (++) in the apical region. G, Normal tissue in the vicinity of ductal *in situ* carcinoma (x200). H, Normal tissue in the vicinity of lobular *in situ* carcinoma (x200).

and F), with case-dependent labeling intensity, as was particularly noticeable in lobular carcinoma (Fig. 1F).

Labeling of the invasive cancers (ductal and lobular carcinoma) differed according to stage of cancer advancement, with a tendency towards lower numbers of cells in the most advanced stages. The heterogeneity in ductal carcinoma was seen beginning from stage II and progressing to a maximum at stage III, in which the number of labeled cells dropped to between 40 and 60% (Fig. 2B). The disappearance of all

reactivity for a large proportion of cells at stage III may be due either to a loss of polypeptidyl-GalNAc transferase, which binds GalNAc to the protein backbone, or possibly to a lack of polypeptide synthesis, as has been suggested for colonic cancer in which the cells do not react with lectins or Tn-specific antibodies (9).

In the tissues located in the direct vicinity of *in situ* carcinoma, which did not show any morphological transformations, 40% of cells had labeled cytoplasm. This can be



considered as a confirmation of a hypothesis suggesting the existence of 'an area of transformation' in which the cells receive a signal triggering neoplastic transformation which induces changes in the chemical structure of glycoconjugates. Further evidence for this hypothesis includes the observations reported by Itzkowitz *et al* (9) in colon cancer, demonstrating that Tn, sialosyl-Tn and T antigens are expressed in the transitional mucosa without cellular atypia, immediately adjacent to the cancer. The findings with 'normal' tissue directly adjacent to invasive carcinoma were surprising, since these cells either did not show any positive reaction or the reaction was very weak (Fig. 2F). This can be explained by the rapid across-tissue penetration of cancer cells which had not yet transformed.

A single example of strongly reactive metastasis cells in intraductal cancer collected from the axillary lymph node is insufficient to draw any far-reaching conclusions. However, it should be emphasized that metastatic cells have similar phenotype as primary carcinoma cells.

In all the pathological cases, the labeling appeared to be membrane and cytoplasmic. This endoplasmic localization could be explained by alteration of abnormal glycoconjugate transport from the Golgi vesicles to the plasmalemma, resulting in a cytoplasmic accumulation of the antigen. Moreover, the entire mucin-type glycoconjugate process is involved, and it is probable that the Tn antigen is likewise expressed by the internal membrane system.

It can be concluded that modifications in glycosylation manifested as the presence of Tn epitopes occur very early in neoplastic transformation, before destructive changes in proliferation/apoptosis or cell differentiation become observable. Tn antigen is therefore present in different forms of primary carcinoma and cancer metastases, while its structure disappears in very advanced stages.

The origin of Tn antigen and its influence on carcinogenesis have not yet been elucidated in detail. However, Tn antigen expression in breast carcinoma was proven not to be connected with a mutation leading to the blockade of T transferase, and it has been shown that different intermediate forms, e.g. antigen T, sTn and Tn, could be present on the same cancer cell (19).

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