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Specific Role of T and Tn Tumor-associated Antigens in Adhesion between a Human Breast Carcinoma Cell Line and a Normal Human Breast Epithelial Cell Line

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The possibility that tumor-associated antigens T and Tn act as adhesion molecules between normal and malignant breast epithelial cells at the early stages of recognition in the metastatic pathway was examined *in vitro*. The adhesive specificity of the antigens was assessed by means of *in vitro* adhesion tests between a carcinomatous breast cancer cell line (ZR75-30) and a normal epithelial breast cell line (HLB100) using both monoclonal antibodies and lectins specific as well as nonspecific for each antigen. Adhesion assay was performed using monolayers of the normal cell line prepared on plastic culture plates and the tumor cell line labeled with a fluorescent dye as a probe. The adhesion between the two cell types occurred with significant specificity via T and Tn antigens ($P<0.001$), and was temperature-dependent. The results suggest that at the early stages of recognition by tumor cells in the metastatic process, T and Tn antigens play a role as adhesion molecules between the tumor cells and adjacent normal cells.

Key words: Breast carcinoma — Cell line — Adhesion — T and Tn antigens

Cell-cell interactions are of obvious importance not only in normal physiological processes, but also in the processes of proliferation and invasion of carcinoma (CA) cells,[1–3] and metastasis.[4] Several steps in cancer invasion have been investigated. The first step is considered to be the release of cancer cells from the primary cancer lesion due to the regulatory effects of E-cadherin-related systems.[5, 6] In the second step, the released cancer cells make contact with nearby normal epithelial cells before reaching the extra-cellular matrix or vascular endothelial cells. This step may be one of the most important in the very early stage of the metastatic processes, such as in the intraductal invasion of breast cancer cells. Little attention, however, has been paid to this event, i.e., the interaction or the binding of malignant cells to healthy (normal) cells immediately after release from the original cancer lesion.

Among the tumor-associated antigenic glycoproteins, T [Gal(β1-3)GalNAc(α1-3)Ser/Thr] and Tn [GalNAc(α1-3)-Ser/Thr] antigens were first described as cryptic determinants on human erythrocytes[7] that were exposed by neuraminidase treatment. These pan-CA auto-antigens[8–10] play a profound role in cancer pathogenesis.[11] Specific adhesion between invasive, metastatic murine Esb T-lymphoma cells and syngeneic hepatocytes has been demonstrated[12, 13]; direct molecular binding of the Esb cells, which were later confirmed to strongly express T and Tn antigens on their cell surfaces,[14] to the corresponding ligands was the first step after the recognition of hepatocyte ligands. Moreover, cultured breast CA DU4475 cells and desialylated, isologous erythrocytes [TRBC] bound specifically to the Gal/GalNAc receptors of rat Kupffer cells and of hepatocytes.[15, 16] These findings suggest that T and/or Tn antigens on released cancer cells might have a role in adhesion of these cells to normal cells in the early stage of metastasis.

In this study, the specific role of T and Tn tumor-associated antigens in adhesion between a breast cancer cell line and a normal breast epithelial cell line was examined *in vitro*.

MATERIALS AND METHODS

Cell culture The human breast cancer cell line ZR75-30 (Flow Laboratories, Inc., McLean, VA; purchased from Dai-Nippon Seiyaku, Osaka) was cultured in RPMI 1640 medium with 10% fetal bovine serum. The normal human breast epithelial cell line HBL100 (Riken Cell Bank, Saitama) was cultured in modified Dulbecco's medium with 10% fetal bovine serum as recommended by the manufacturer.

Immunohistochemistry and electron microscopy Expression of T and Tn antigens on the cells was deter-

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mined by immunohistochemistry and electron microscopy using HT-8 (IgG, 1:100 dilution)[17] and BaGS-3 (IgM, 1:40 dilution)[18] monoclonal antibodies (Mabs) respectively, and a streptavidin-biotin-peroxidase method described previously.[19]

Preparation of monolayer cells Monolayers of HBL100 cells used in the heterotypic cell adhesion study were prepared on polyvinyl slides with 8 flat-bottomed chambers (tissue culture chamber/slides; Lab-Tek Products, Miles Laboratories Inc., Naperville, IL) by the method of McClay *et al.*[20] with a slight modification. The plates were pre-treated with *Mr* 60,000 poly-L-lysine in distilled water (50 μg/ml; Sigma, St. Louis, MO) for 1 h at 24°C, and washed with distilled water. The HBL100 cell suspension, 0.2 ml of culture medium containing 10^6 cells, was added to each well. The cells were then centrifuged onto the poly-L-lysine coated bottoms (50*g*; 3 min). The plates were incubated for 30 min at 37°C in a CO_2 incubator.

Preparation of probe cells Probe cells (tumor cells) were prepared based on the method of Takeuchi *et al.*[21] The tumor cells were labeled by incubation with 3 μM BCECF AM [2′,7′-bis{carboxyethyl}carboxy-fluorescein tetraacetoxy-methyl ester; Dojin, Kumamoto] in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, St. Louis, MO) at 37°C for 40 min. The labeled cells were then washed three times with phosphate-buffered saline (PBS), pH 7.4.

Heterotypic adhesion assay Adhesion assays of the carcinoma cells to HBL100 cells were performed according to the method of McClay *et al.*[20] The monolayers of HBL100 cells were washed first with culture medium containing *Mr* 30,000 poly-L-glutamic acid (10 μg/ml; Sigma) which prevented the binding of probe cells to the cell-free poly-L-lysine-treated plate surface, and then with culture medium. The probe cells (10^6 cells/0.2 ml medium) were added to the monolayer cells in each well, then centrifuged at 10*g* (10 min; 4°C) to provide contact with the monolayer cells. The plates were incubated for 15 min at 4°C or at 37°C, then wrapped in Parafilm (American National Can, Greenwich, CT), inverted, and centrifuged at 400*g* for 10 min at 4°C (these conditions were chosen based on the results obtained by McClay *et al.*).[20] The inhibition assays of heterotypic adhesion were performed by pre-treating the CA cells with either antibodies specific for each of the antigens or peanut agglutinin (PNA) and *Helix pomatia* agglutinin (HPA) (E. Y. Laboratories, San Mateo, CA), the lectins specific for T and Tn antigens respectively. Dose-response of the inhibitory lectins was tested at 0.01–0.50 μM. A number of other lectins nonspecific for T and Tn antigens including concanavalin A (Con A), wheat germ agglutinin (WGA), and *Griffonia simplicifolia* (GS-II) (all from E. Y. Laboratories, Inc.) were tested for reactivity with ZR-75-30 cells, and among them, GS-II was strongly reactive. GS-II lectin was therefore used as a control in inhibition assays. In addition, a control antibody (anti-pancytokeratin) was used as the antibody control. All attached cells were photographed under a phase-contrast microscope. Probe cells in the same areas were photographed under a fluorescence microscope. Prints were prepared from the negative films, and all cells, both adhering and non-adhering were counted. The number of probe cells observed as adhering to the HBL100 cells was counted and the percentage of the adhering cells with respect to total HBL100 cells was calculated. The experiments were repeated three times for each antigen.

Homotypic adhesion In a similar monolayer cell adhesion assay, fluorescence-labeled ZR75-30 cells were overlaid on the monolayer of ZR75-30 cells. The inhibition test was performed in the same fashion as in the heterotypic adhesion assay.

Statistical analysis The rates of adhesion, dependent on T or Tn antigen, were determined as the percentage of probe cells that adhered to the monolayer of counterpart cells, evaluated by comparing the number observed upon incubation in the presence of antibody specific to T or Tn antigen, or the specific or nonspecific agglutinin, with that observed in the absence of these reagents. Comparison between groups with and without antibodies or agglutinins was performed using Student's *t* test. A *P* value of less than 0.05 was considered as significant.

RESULTS

Immunostaining of ZR75-30 breast CA cells and HBL100 normal breast epithelial cells Fig. 1, A and B show light micrographs of ZR75-30 cells immunostained with anti-T (A) or anti-Tn (B) antibody. Compared with the negative control staining (C), the cells were clearly positive for T and Tn antigens. Overall, 60% of the cells expressed T, and 75% Tn antigens. Fig. 1D shows a light micrograph of HBL100 cells immunostained with anti-T antibody. The normal breast epithelial cells did not display these antigens.

Electron micrographs of ZR75-30 CA cells immunostained with anti-T (Fig. 2A) or anti-Tn (Fig. 2B) antibody showed that both of these antigens are expressed on the cell surface.

Study of the adhesion of ZR75-30 breast CA cells to HBL100 normal breast epithelial cells Fig. 1E shows a typical phase-contrast photomicrograph of HBL100 cells to which many of the probe ZR75-30 cells adhered, as revealed under the fluorescence microscope (Fig. 1F).

Temperature dependency of heterotypic adhesion Fig. 3 shows that heterotypic adhesion amounted to 45% at 37°C (first column) but was negligible (3%) at 4°C (second column). This result is consistent with the fact that at 4°C, the binding force of adhesion is very weak.[20]

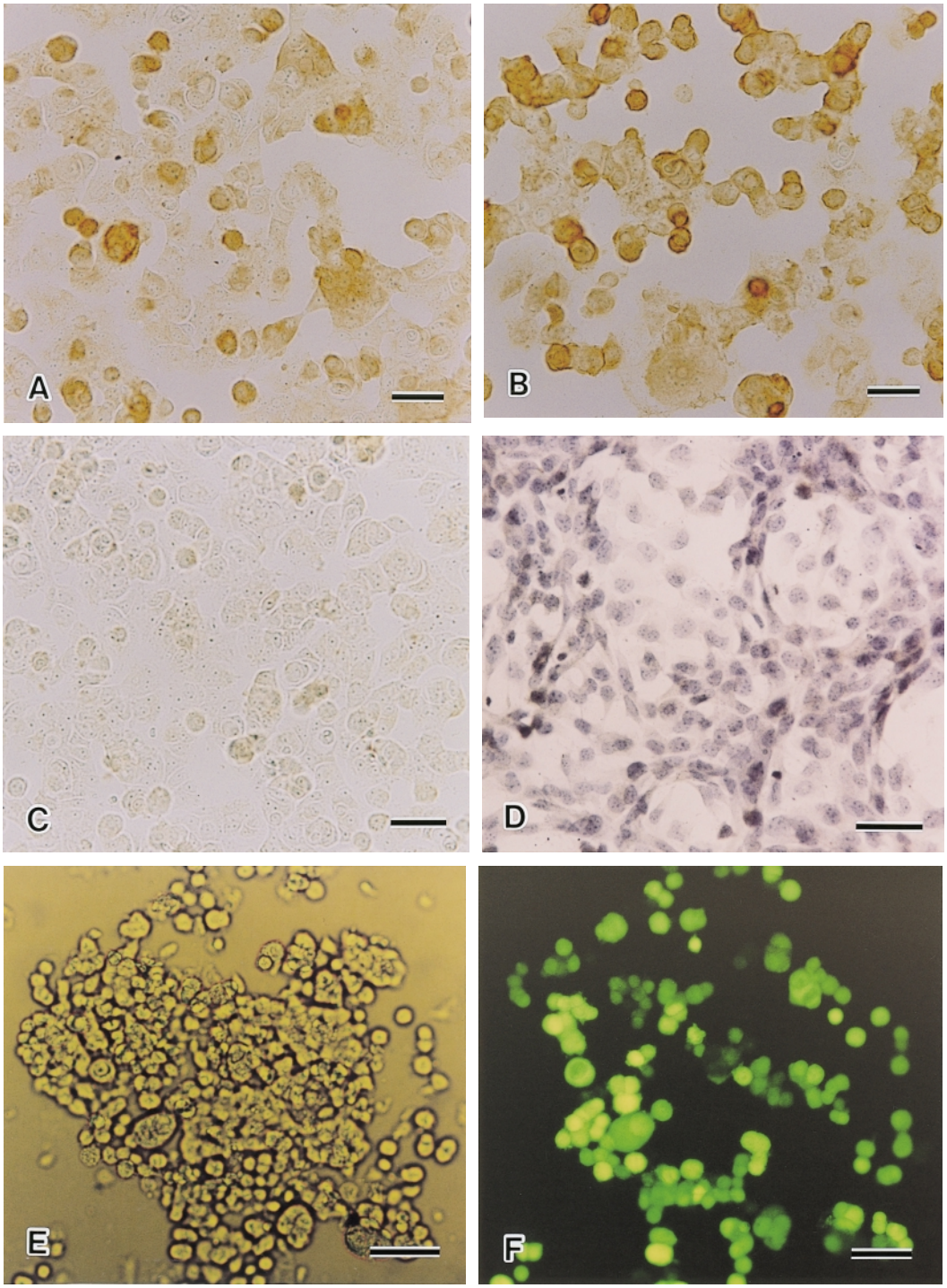

Fig. 1. (A) Light micrograph of ZR75-30 cells immunostained with anti-T antibody, (B) with anti-Tn antibody, and (C) negative control staining. (D) Light micrograph of HBL100 cells immunostained with anti-T antibody shows no reaction. (E) Light micrograph of cells after adhesion assay shows normal breast epithelial cells on which breast cancer cells (probe cells) adhered. (F) The same view as observed by fluorescence microscopy confirms that probe cells adhered to the normal cells. By comparing the two micrographs, the ratio of probe cells adhering to the counterpart normal cells can be calculated (bar=10 μm, A–C; bar=20 μm, D–F).

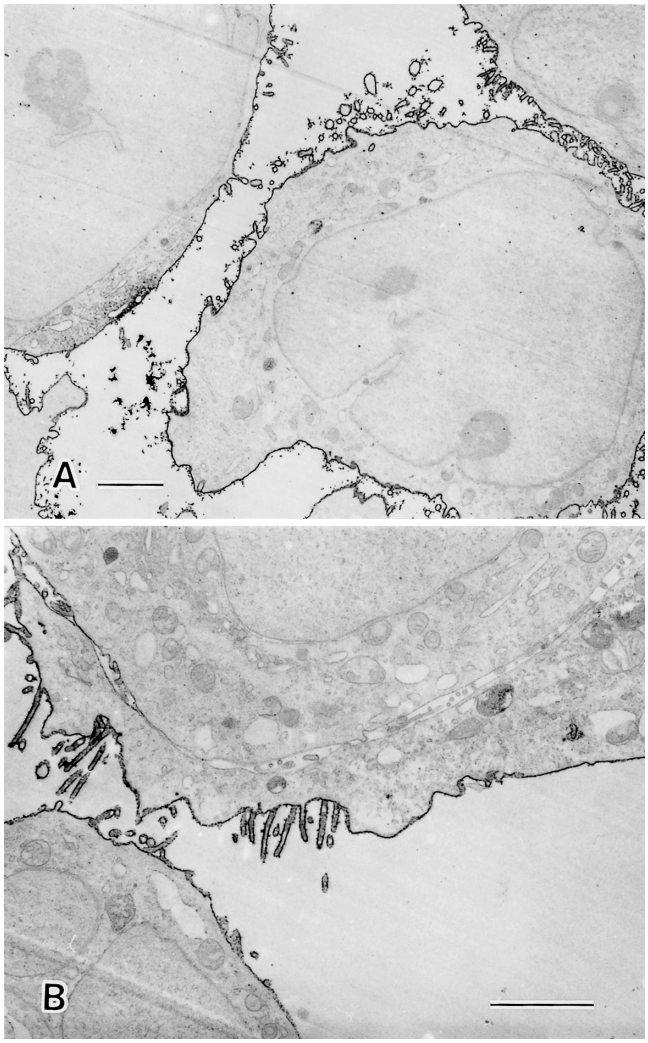

Fig. 2. Electron micrograph of ZR75-30 cells immunostained with anti-T (A) or anti-Tn (B) antibody. A strong positive reaction for these antigens is exhibited on the cell membrane and microvillous surfaces (bar=2 μm).

Antigen specificity of adhesion of ZR75-30 cells to HBL100 cells at 37°C The adhesion of the tumor cells to HBL100 cells was significantly ($P<0.001$) inhibited by pretreatment of the cells with anti-Tn antibody (Fig. 4, second column) compared to that without pretreatment (Fig. 4, control). A similar result was obtained in the inhibition assay with HPA at 0.05 μM, beyond which no increase in inhibition rate was observed (Fig. 4, third column). The inhibition of adhesion by anti-Tn antibody and HPA agglutinin was not additive (Fig. 4, fourth column), indicating that the adhesion was due to Tn antigen.

The adhesion of the tumor cells to HBL100 cells was also markedly ($P<0.001$) inhibited by pretreatment of the

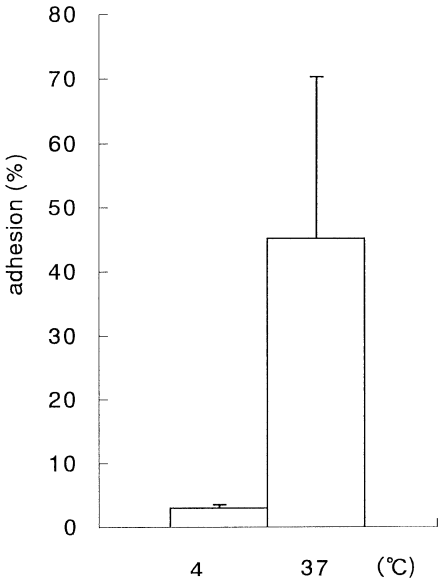

Fig. 3. Adhesion between ZR75-30 cells and HBL100 cells is significantly different at 37°C and 4°C ($P<0.05$, $n=10$). The ratio of probe cells adhering to the counterpart cells is expressed as a percentage of total HBL100 cells.

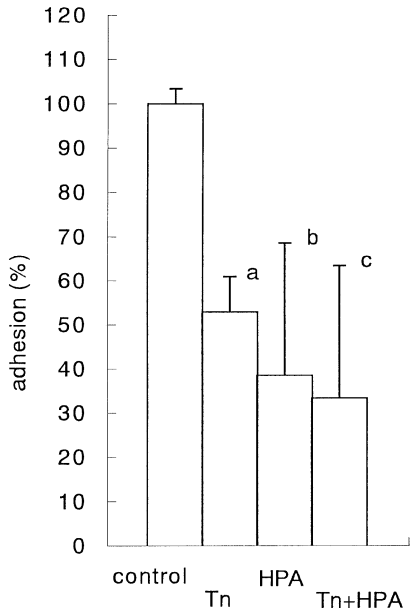

Fig. 4. Comparison of rates of adhesion of the tumor cells (ZR75-30 cells) to HBL100 cells after no treatment (control as 100%, first column), or treatment with anti-Tn antibody (Tn, second column), HPA (third column), or anti-Tn antibody plus HPA (fourth column). Bar=SD. Differences from the control value were statistically significant at (a) $P<0.001$ ($n=10$), (b) $P<0.02$ ($n=10$), and (c) $P<0.05$ ($n=10$), as assessed by Student's t test. There were no significant differences between any of the treatment groups.

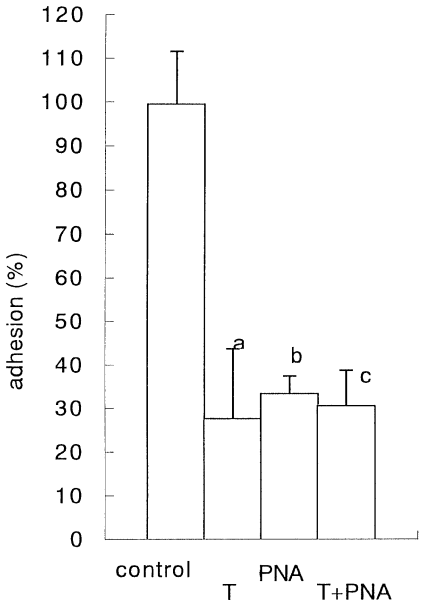

Fig. 5. Comparison of rates of adhesion of the tumor cells (ZR75-30 cells) to HBL100 cells after no treatment (control as 100%, first column), or treatment with anti-T antibody (T, second column), peanut agglutinin (PNA, third column), or anti-T antibody plus PNA (fourth column). Bar=SD. Differences from the control value were significant at (a) $P<0.001$ ($n=10$), (b) $P<0.001$ ($n=10$), and (c) $P<0.001$ ($n=10$), as assessed by Student's t test. There were no significant differences between any of the treatment groups.

cells with anti-T antibody (Fig. 5, second column) compared to that without pretreatment (Fig. 5, control, first column). A similar result was obtained in the inhibition assay with PNA specific for T antigen at 0.05 μM, beyond which no increase in inhibition rate was observed (Fig. 5, third column). The inhibition of adhesion by anti-T antibody and PNA was not additive (Fig. 5, right column), indicating that the adhesion was due to T antigen. The control lectin and antibody (GS-II and anti-pancytokeratin), that reacted with ZR75-30 cells, did not inhibit the cell adhesion (adherence rates: 65% with and 64% without their addition).

Effect of T or Tn antibody on homotypic aggregation (adhesion) of the tumor cell line In a similar monolayer cell adhesion assay using fluorescence-labeled ZR75-30 cells overlaid on a monolayer of ZR75-30 cells treated with or without T or Tn antibodies, the percentage of T or Tn antigen-bearing tumor cells attached homotypically to each other was determined and compared with that in the case of cells pretreated with the antibodies or lectins specific to each antigen. There was no significant difference (Student's t test, $P<0.9$).

DISCUSSION

Cell contact usually involves mutual recognition prior to adhesion, and this may be mediated by specific receptor molecules on the cells. Specific cell adhesion is a multistep phenomenon, with specific recognition being only the initial event. Umbreit and Roseman[22] demonstrated that there are at least two separate steps in cell-cell adhesion. Initial weak adhesion was followed by a second step, which required metabolic energy, during which the cells became more firmly attached to each other. McClay *et al.*[20] and Guarmacci and Schnaar[23] reported a method to measure directly the force of cell-cell adhesion and they quantitatively examined the strengthening event. The initial adhesion was cell-specific and occurred at low temperature or in the presence of metabolic inhibitors. The first cell-cell recognition step, as monitored at 37°C, was reversible, while the second step, the strengthening of adhesion, occurred within a few minutes and reached a maximum after 10 min. This step was blocked by metabolic inhibitors. We followed the method of McClay *et al.*,[20] which seemed suitable to elucidate the role and specificity of T or Tn antigens in the adhesion of tumor cells to normal cells in the early step, whether or not it is metabolically dependent. In order to allow complete reaction of the antigen-bearing cells with the counterpart cells, we chose experimental conditions of 15 min at 37°C or 4°C. We obtained clear evidence of the specific involvement of T and Tn antigens in the interaction between the breast cancer cells and the HBL100 cells. We found that T and Tn antigen-associated adhesion occurred at 37°C, but not at 4°C, indicating temperature and metabolic energy dependency of adhesion between the two types of cells, as reported previously.[20] The adhesion and anchorage of released cancer cells to nearby normal cells[1, 24] is likely to be a key step in the spread of cancer.

ZR75-30 cells exhibited both T and Tn antigens, and the adhesion to normal human breast cell line HBL100 was specifically dependent upon these antigens. Further studies are in progress to examine whether the effect of anti-T and anti-Tn antibodies was additive or not, in order to provide information on the possible existence of a receptor on HBL100 cells for T and Tn antigens.

The normal breast epithelial cell line HBL100 did not express T or Tn antigen, both of which seldom appear in normal breast epithelium or other tissues[10, 11] except in the healthy colon, and uterine cervix.[25] The T and Tn antigens appear on the surface of undeveloped epithelial cells, such as cancer cells, in which glycosyl-transferases are absent or abnormal.[26]

Hull *et al.*[27] have reported the presence of sialyl-Tn on the surface of cells of the breast cancer cell line BT-20. Also, sialyl-Tn has been detected on the surface of gastrointestinal CAs[28–30] and ovarian CAs.[19, 31] It was reported that sialylation of asparagine-linked oligosaccha-

rides was more evident on the surface of highly metastatic colon CA cells than on the surface of less metastatic cells.[32] Thus, sialyl-Tn might also have a role in adhesion processes between normal and cancer cells.

In conclusion, the results of our study strongly suggest that the T and Tn antigens expressed on the surface of breast cancer cells have an important role in adhesion of these cells to normal epithelial mammary cells in the early stages of invasion and metastasis. Further studies are in progress to clarify the role of sialyl-Tn antigen in such adhesion processes.

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The authors wish to dedicate this work to Professor Georg F. Springer, who passed away on 10th March 1998, leaving an unforgettable memory of a powerful intellect and scientific mind. He was deeply involved in the investigation of T, Tn, and sialyl-Tn antigens and breast carcinoma vaccines. He deserves our lasting respect.

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