J Mol Med (1997) 75:594–602

REVIEW

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Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy

Received: 18 February 1997 / Accepted: 18 April 1997

Abstract Aberrant glycosylation is a hallmark of cancer cells. The blood group precursors T (Thomsen-Friedenreich) and Tn epitopes are shielded in healthy and benign-diseased tissues but uncovered in approx. 90% of carcinomas. T and Tn glycoproteins are specific, autoimmunogenic pancarcinoma antigens. These antigens may also be found in neoplastic blood cells (and on LTV-II infected T lymphocytes). Fundamental chemical and physical aspects of these glycoproteins of primary carcinomas are discussed first. Tn and T epitopes are cell and tissue adhesion molecules, essential in invasion by and metastasis of carcinoma which includes adherent and proliferative phases. These properties are then delineated next, followed by consideration of pathophysiological and clinical aspects of these antigens. Immunohistochemical studies of the extent of Tn antigen expression in primary breast carcinoma demonstrate its highly significant correlation with clinicopathological tumor stages, and hence its value as a reliable prognosticator. On the other hand, there is no significant, prognostically useful association between T antigen expression and clinical disease course. Everyone has "preexisting" anti-carcinoma anti-Tn and anti-T antibodies, induced predominantly by the intestinal flora, while cellular immune responses to T and Tn epitopes are evoked only by carcinomas and some lymphomas. Carcinoma dedifferentiation leading to predominance of Tn over T epitopes is described, as is the role of Tn and T epitopes in very early, including preclinical, carcinoma detection. The highest sensitivities in carcinoma detection are for *pre*clinical and the earliest clinical stages. Obviously, *pre*clinical carcinoma detection is of practical importance. T/anti-T tests detected *pre*clinical carcinoma in 77% of 48 patients long ($\bar{x}$ 6 years) before their biopsy/X-ray results became positive. There were no false predictions of carcinoma in 38 control persons with benign diseases (observation average 4.8 years). These findings open a novel window for both curative approaches and pathophysiological studies. The autoimmunogenicity of carcinoma T/Tn antigen led us more than two decades ago to begin intradermal vaccination of patients with advanced breast carcinoma of stages IV–IIb, predominately after modified radical mastectomy and sometimes lumpectomy plus axillary dissection always followed by adjuvant radio/chemotherapy. The vaccine consists of human group O red blood cell membrane derived, HLA-free T/Tn antigen containing as adjuvant $Ca_3(PO_4)_2$ plus a trace of phosphoglycolipid A hyperantigen, i.e., *S. typhi* vaccine (USP), which itself has T and Tn specificities. Our efforts have now for up to 20 years remained successful in a large majority of the 32 patients. All 32 patients survived at least 5 years; 10-year survival was statistically highly significantly improved (5-year survival: $P<1\times10^{-7}$; 10-year survival: $P<1\times10^{-5}$) compared to statistics of the United States National Cancer Institute. Because these vaccinations are successful, their extension to large populations with major types of carcinomas should be considered, and even immunological carcinoma prevention may be contemplated.

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Key words T/Tn pancarcinoma autoimmunogens · β-Gal1→3GalNAc-α_1-O-Ser/Thr and α_1-GalNAc-O-Ser/Thr epitopes · Immune responses to T and Tn antigens · T/Tn epitopes in carcinoma pathogenesis · Preclinical carcinoma detection by T assays · T/Tn epitopes in cell adhesion and delayed-type hypersensitivity skin reactions · Erythrocyte-derived T/Tn antigen, efficacious carcinoma vaccine

Abbreviations *CA* Carcinoma · *DTHR-T* Delayed-type skin hypersensitivity reaction to T antigen · *LAI* leukocyte adherence inhibition assay · *RBC* Red blood cells

Introduction

T (Thomsen-Friedenreich) and Tn epitopes are progenitors of fully differentiated normal epithelial and blood cell surface structures. T (Gal-β_1→3GalNAc-α_1-*O*-Ser/Thr) is terminal and Tn (GalNAc-α_1-*O*-Ser/Thr) is penultimate [1–4] on T-specific glycoproteins and is covered with only one or two *N*-acetyl-neuraminic acids [1–4] to form the histo-blood group M, N antigens [1, 4], or these precursors link complex heterosaccharide chains O-glycosidically [5, 6]. T and Tn epitopes are occluded in healthy and benign-diseased tissues but are immunoreactive in approx. 90% of all carcinomas (CAs) tested, as found first by us [1, 7, 8] and soon confirmed by numerous others (e.g., [9–12]].

Specific removal of β-Gal1→3 with bovine testicular β-D-galactosidase transforms T to Tn glycoprotein [13]. Conversely, human serum β_1→3-Gal transferase in the presence of UDP-Gal synthesizes T from Tn [2, 3]. The two immunodeterminant carbohydrates of T Ag are Gal and GalNAc [2–4, 13]. The predominant amino acids (>4%) in descending order of abundance for two typical preparations of complete T Ag from our laboratory are Thr, Glu, Leu, Ser, Val, and Pro [14, 15] (see Figs. 1, 2).

We have shown the majority of Enterobacteriaceae to possess highly immunogenic T and Tn specificities [8, 16]. The origin for at least the majority of anti-T and anti-Tn antibodies, which all humans have, was confirmed in experiments with germ-free and ordinary chicks and human infants and adults. Germ-free chicks produced no anti-blood group T and Tn antibodies, in contrast to chicks from the same hatch raised under ordinary conditions. Feeding germ-free chicks live blood group T/Tn active bacteria elicited high anti-T and anti-Tn antibody titers. Similarly, anti-T and anti-Tn antibodies were elicited in human infants and adults following feeding or inhalation of live or killed blood group active bacteria [16].

Monoclonal rodent anti-T and anti-Tn antibodies were first elicited with T red blood cell (RBC) antigens [17] and a lung CA [18], and anti-Tn also by us with O Tn RBC and CAs [19], and by others (e.g., [20, 21]).

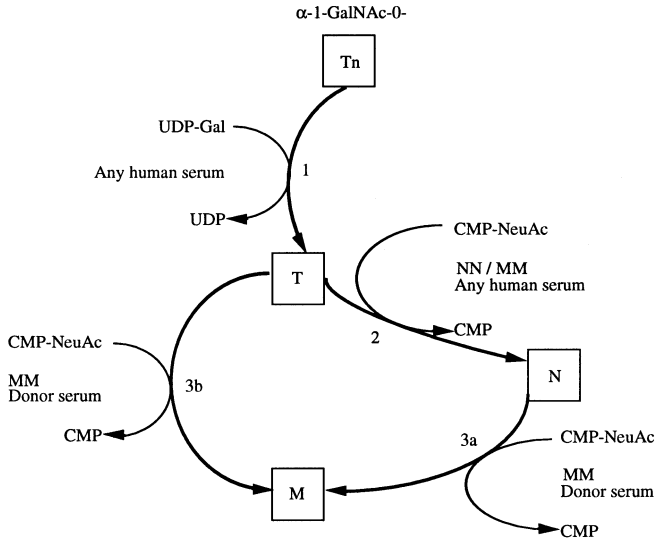

Fig. 1 Pathway depicting biosynthesis of the T immunodeterminant structures of human histo-blood group M and N glycoproteins, by preexisting human serum glycosyltransferases. Tn is transformed to (*1*) T by GalNAc-β-3-D-Gal-transferase in the presence of UDP-Gal plus ATP and $MnCl_2$ and $MgCl_2$ as activators. T is transformed to (*2*) N by α-*N*-acetyl-neuraminic acid (*NeuAc*) transferase present in sera of donors of all MN genotypes. M is synthesized either from (*3a*) N or from (*3b*) T by α-NeuAc transferase (present *only* in human donors who possess the M gene) in the presence of CMP; activators are not needed [3]

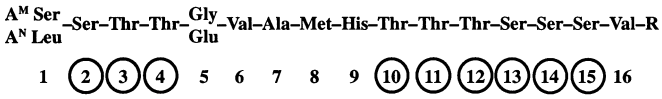

Fig. 2 T glycoprotein: amino-terminal sequence. *Circles*, O-glycosylation positions (approx. 90% T and 10% Tn haptens). Note cluster formations [73, 74]

Tn and T epitope expression in CA prognostication

It is of paramount importance to establish whether the degree of pan-CA T and Tn epitope expression is correlated with the prognosis of primary breast and other CAs. We determined the prognostic value of the extent of Tn and T epitope expression quantitatively in 55 primary invasive breast ductal CA tissues of stages I–IV [22]. Tissue sections from paraffin blocks were reacted with pools of rodent monoclonal anti-Tn and anti-T as primary antibodies, followed by streptavidin-biotin-peroxidase, then stained with diaminobenzidine tetrahydrochloride and counterstained with methyl green. Immunostained Tn and T epitopes were quantitated by computerized image analysis. Classical pathological and histological prognostic indicators associated with survival were subdivided into favorable and unfavorable categories by standard critera.

Of the 55 CAs 51 strongly expressed Tn and T, and 4 had traces. As shown in Table 1, high extent of Tn epitope expression was statistically significantly associated

Table 1 Prognostic factors associated with the mean percentage of Tn epitope immunostained on primary breast CA cells of the 55 patients studied (*high* ≥33% of immunostained areas, *low* <33%)

	n	Tn epitope			
		High	Low	Association	*P*
All cases, primary CA	55	31	24		
pTNM stage				0.24	0.027
I and IIA	31	13	18		
IIB–IV	24	18	6		
Size of primary CA				0.39	0.109
<2.0 cm	25	11	14		
≥2.0 cm	30	20	10		
Lymph node				0.21	0.013
Negative	30	12	18		
Positive	25	19	6		
Combined histological grade				0.14	0.001
I and II	27	9	18		
III	28	22	6		
Disease-free interval				0.13	0.006
≥5 years	40	18	22		
<5 years	15	13	2		

with shortened 5-year disease-free interval, higher pTNM stages, positive lymph node status, and increased combined histological grades, by both determination of probability values and odds ratios. There was only a weak association with tumor size. On the other hand, T antigen expression showed no significant association with prognostic factors. Our results suggest that quantitative immunohistochemical image analysis of Tn epitopes of primary breast CAs aids importantly in prognostication of the disease (a finding that is in accord with our semiquantitative absorption assays using live breast CA tissues [23]). Immunohistochemical analysis permits rather precise location of Tn- and T-active structures and uses considerably less tissue or cultured cells than are needed in specific antibody absorption experiments. On the other hand, however, the latter have the advantage of permitting the study of live cells [23]. It is relevant for immunochemical studies that antibody Fc receptors have been shown not to react with breast CA cells (negative in 97 of 97 cases [24]).

T and Tn epitopes in cell adhesion invasion and metastasis cellular immune responses

It was first suggested some 45 years ago that human CAs possess characteristic antigenic structures capable of eliciting autoimmune responses [25]. This allows patient tumor-interactions to be probed by means of delayed-type skin hypersensitivity responses to CA tissue but permits in vivo testing only with autologous tumor preparations to avoid transfer of potential tumor viruses and allogeneic "background" reactivity [25].

Cell-mediated autoimmune responses to O RBC MN glycoprotein-derived, sterile and HLA-free T/Tn antigen were successfully measured in vivo by delayed-type skin hypersensitivity reactions (DTHR-T) [8, 26]. The results obtained are listed in Table 2 for patients with various CAs of all stages and for non-CA control test subjects. DTHR-T was positive in 85% of 461 CA patients tested and in 3 of 12 lymphoma/leukemia patients. The great majority (approx. 60%) of the lung CA patients had adeno-CAs (78 bronchial and 8 bronchioloalveolar); 89.5% of these 86 lung adeno-CA patients had positive DTHR-T results. Among the 21 patients with small-cell lung CA 90.5% were DTHR-T positive, while 80% of 25 squamous-cell lung CA patients tested positive. Positive DTHR-T results were shown by 85%–95% of breast, pancreas, and colon CA patients and also by approx. 80% of patients with CAs of other major organs.

DTHR-T was negative in 58 of 64 benign lung disease patients (Table 2). All 14 patients with lobar, lobular and acute pneumonias, granulomatous fungal disease, cysts, effusive tuberculosis, silicosis, chronic interstitial fibrosis, emphysema, and/or benign lung tumors reacted negatively, as did 37 of 40 patients with chronic obstructive pulmonary disease, severe chronic indurating pneumonia, and/or abscess, two of four patients with sarcoidosis, and five of six with hamartomas. The positive DTHR-T in two of the six patients with benign disease, one with pneumonia and one with sarcoidosis, became negative in less than 1 year. In the former of these the positive reaction was shown to be due to uncovering of T antigen in lung tissue by neuraminidase, elaborated by β-hemolytic streptococci [26].

The DTHR-T was also negative in all 66 patients with benign diseases of organs other than lung and breast (Table 2). This population included those suffering from pancreatitis, ulcerative colitis, diverticulitis, various benign adenomas, ovarian cysts, cardiovascular diseases, diabetes mellitus, alcoholism with liver disease, myasthenia gravis, chronic ear, nose, throat infections, parkinsonism, Wilson's disease, tracheal stenosis, lymphoblastic granulomatosis, post-Guillain-Barré syndrome, and hypothyroidism. Altogether only 8% of 328 patients with

Table 2 Carcinoma detection by DTHR-T at initial patient visit

Category	Total tested	DTHR-T positive	% Positive[a]
Carcinoma	461	391	85
Lung	144	127	88
Breast	199	166	83
Pancreas	26	23	89
Upper aerodigestive tract	21	16	76
Gastrointestinal tract	20	17	85
Genitourinary tract	38	32	84
Melanoma	12	9	75
Thymoma	1	1	(100)
Non-CA cancer	26	3	12
Leukemia/lymphoma	12	3	25
Sarcoma	5	0	(0)
Central nervous system	9	0	(0)
Benign diseases	328	25	8
Lung	64	6	9
Breast	198	19[b]	10
Other	66	0	0
Healthy	126	0	0

[a] Cohorts of fewer than10 persons in parentheses
[b] 17/19 had premalignant hyperplastic disease

non-CA disease were DTHR-T positive. None of the 126 healthy persons had a positive DTHR-T reaction.

The high sensitivity and high specificity of the DTHR-T in CA detection makes it a valuable diagnostic test, and as we found [26, 27], especially of clinically incipient and even *pre*clinical CA.

Cell-mediated autoimmune responses of CA patients to T epitopes, when measured in vitro with leukocyte adherence inhibition assays (LAI) [28], in which the mean number of nonadherent cells was counted by computerized image analysis and expressed as a percentage of nonadherence, showed a positive reaction in 78% of 76 breast, lung, and colon CA patients and in fewer than 5% of 65 patients with benign diseases, including benign breast disease, diverticulitis, and pneumonitis. These findings are in accord with those reported by Ichinose et al. [29]. LAI also using T antigen was much less sensitive, in our hands, although it had about the same specificity as both LAI-T and DTHR-T in the 150 CA patients and 199 non-CA subjects studied [26].

It is important in this connection that Black et al. [30] observed that a strong anti-CA cell-mediated autoimmune response in patients with prior, current, or subsequent in situ breast CA significantly improves survival time in patients with invasive breast CA; their study encompassed 129394 patients.

Humoral anti-CA-T and anti-Tn responses

After confirming the specificity of anti-T and anti-Tn antibodies by absorption and inhibition studies with antigens, epitopes, and haptens [4, 27, 31], anti-T antibody levels were assessed by in vitro studies of the relevant serum antibodies. In healthy persons anti-T antibody levels are rather constant throughout adult life [32]. We measured them initially by hemagglutination assays using RBCs on which T antigen had been maximally uncovered by neuraminidase [1] to determine the anti-T antibody levels semiquantitatively in the test subjects' sera by standard serological titration using twofold geometrical dilution steps [14, 33]. Among 287 consecutive *pre*operative patients who turned out to have primary CA of breast, respiratory, gastrointestinal, and urogenital tracts, 41% had abnormal anti-T titer scores, whereas normal anti-T agglutinin levels were found in 91% of 309 nonpurposely selected patients with benign diseases and 92% of 200 putatively healthy persons ($P<0.001$ for CA patients versus non-CA groups) [14, 26, 33]. Depression of anti-T antibodies in CA patients is specific, anti-AB0 antibodies and anti-sheep RBC hemolysin levels did not change along with anti-T [12, 34].

Thatcher et al. [35] compared 55 patients with untreated, disseminated melanoma who had significantly depressed serum anti-T agglutinin levels with 609 healthy persons ($P<0.0005$). Significant increase in anti-T antibodies and improved survival followed a single intravenous infusion of *Corynebacterium parvum* (which has T antigen), given at a concentration of 2 mg/m^2 body surface into all of 14 melanoma patients, indicating immunotherapeutic relevance of *C. parvum*; there was no anti-T increase using bacille Calmette-Guérin (no T antigen) injected in nine other melanoma patients. Also, pretreatment anti-T agglutinin titers were statistically significantly higher among the 21 melanoma patients who responded to chemoimmunotherapy; their 50% survival time was approx. 120 weeks compared to 20 weeks for the 34 nonresponders. Hence the anti-T titers had prognostic meaning, while tumor burden was irrelevant.

Bray et al. [12] showed that all 40 normal human sera tested had anti-T antibodies that specifically lysed O, T RBCs in the presence of complement; 83% of 18 postoperative (>3 months) patients with massive metastatic gastrointestinal CA had depressed lytic anti-T levels, correlated with disease burden, while 11 patients with nonde-

monstrable or minimal disease had no decrease in anti-T hemolysin ($P<0.001$).

There has been an epidemic of esophageal CA for over 40 years among black men in Transkei, South Africa [36]. Among more than 500 children in geographic areas with and without high risk for esophageal CA, anti-T titers, as measured with serological titration assays, were highly elevated exclusively in the high-risk areas. Vos et al. [36] concluded that the T epitopes themselves may be a decisive factor in the development of CA, via cellular defects exposing them, and thus facilitating cell entry by carcinogenic viral or other agents.

We introduced quantitative, solid-phase anti-T serum immune assays for CA detection (see [37]). These tests more than doubled the sensitivity of CA detection compared to the hemagglutinin titration tests. These assays determine anti-T IgM and total IgM in sera using isolated, highly purified T antigen, and anti-human IgM antibodies both fluorescently labeled as solid phases; later we introduced an enzyme-linked immunoassay using 3,3′ 5,5′ tetramethyl benzidine for color development [37]. The results with the two assays were very similar. The sensitivity of differentiation between sera of CA patients and controls was greater than that obtained by anti-T determination alone when the total IgM values of any given serum were included (by relating anti-T IgM to total IgM and expressing it as the quotient, Q_M), by using the formula: $Q_M=100$ [(mg/dl anti-T IgM)^2/mg/dl total IgM]. The average percentage variation in Q_M values of the same samples determined on different days was less than ±3% [37].

Table 3 lists the results of the two procedures combined. The assay identified 88% of 360 CA patients with CAs of major organs and was correctly negative in 90% of 124 patients with benign disease. There were false-positive results in three of four breast disease patients with clearly premalignant breast conditions. Importantly, the assays were especially sensitive for cases of incipient CA, stages 0 and I. Specificity was 92% in 287 benign-diseased and healthy persons [31, 37]. There were 154 CA patients studied 0–14 days prior to surgery; the reactions were positive. False-positive reactions were observed in 10% of 124 patients.

Table 3 CA detection with quantitative serum anti-T antibody immunoassay at initial patient visit (solid-phase immunoassay to determine anti-T IgM antibodies in relation to total IgM)

Category	Total tested	No. positive	% Positive[a]
Carcinoma	360	315	88
Breast	216	184	85
Lung	96	89	93
Pancreas	20	19	95
Genitourinary tract	18	15	83
Other CAs	10	8	80
Benign diseases	124	13	10
Breast	75	11[b]	15
Lung[c]	32	2	6
Pancreas	5	0	(0)
Other non-CA diseases	12	0	0
Healthy	163	11	7

[a] In parentheses: cohorts of fewer than 10 persons
[b] 3/11 had premalignant atypically hyperplastic disease
[c] 1/2 carcinoid and 1/5 chronic obstructive pulmonary disease patients

Combined measurements of cellular and humoral anti-CA responses compared with other procedures are predictive of CA

Table 4 demonstrates both the superior sensitivity and specificity of T antigen tests in preoperative detection of CA in patients with uncertain diagnosis, by cell-mediated autoimmune and humoral responses compared to the carcinoembryonic antigen, where we assessed these responses immediately prior to operation. Both types of anti-T responses were close to 100% correctly positive for patients subsequently shown to have pancreatic CA and negative for those who had chronic inflammation, while carcinoembryonic antigen had a failure rate of nearly 50% for both CA and inflammation [31].

It is of true clinical importance that positive anti-T tests predicted CA months to years *before* biopsy and X-ray did [27, 31, 33, 38]. The value of anti-T tests in CA prediction is shown in Fig. 1. In view of negative biopsy and X-ray results, to the clinicians 37 of 48 (77%) patients were persistently falsely positive in respect to our positive anti-T tests between 2 months and 12 years (tested at 8- to 12-month intervals) before their negative biopsy or X-ray findings also became positive for CA. As of now 11 patients have not yet developed CA. Thus positive T reactions predicted with greater than 77% accuracy clinical CA within approx. 10 years in the patients studied, while their biopsy/X-ray remained negative. However, of these 48 patients 7 (15%) were lost to follow up after an average of 5.3 years. Hence the number of patients that developed CA within 10 years may be greater than 77%. Of 38 women with suspicious breast lesions who had consistently negative anti-T reactions none developed CA during an average observation period of 5.4 years [27, 31, 33]. Our correct *pre*clinical prediction of CA opens a new avenue for CA prophylatic treatment and pathogenesis studies. Remarkably, one patient diagnosed unequivocally by clinicians as having pancreatic CA had preoperatively negative anti-T tests. Surgery revealed a normal pancreas but a duodenal volvulus [27].

Table 4 Cellular and humoral immune responses to T antigen of patients with pancreatic disease compared with reactivity to CEA

Disease	Positive/total tested		
	DTHR-T	anti-T Q_M	CEA
Adenocarcinoma	22/23	18/18[a]	12/23
Chronic inflammation	0/11	0/6	4/9

[a] One carcinoma patient was borderline positive

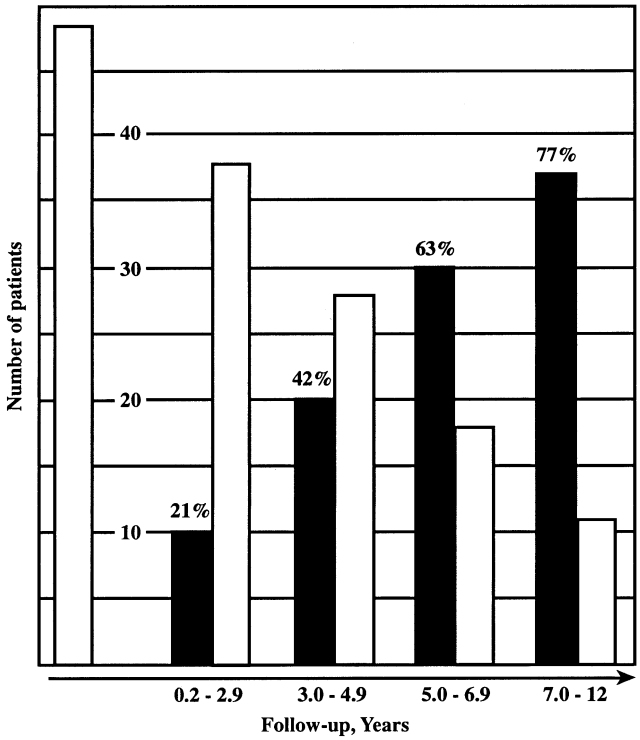

Fig. 3 Prediction of CA. *Open bars*, confirmation by positive anti-T tests in persons with negative biopsies; *closed bars*, confirmation by X-rays and biopsy in persons who developed clinically verified carcinoma long thereafter.

Importantly, a human macrophage cell surface lectin that specifically recognizes CA-associated Tn epitopes was recently shown to locate preferentially within lung metastases of mouse ovarian CA. It reacted well with tetraantennary complex type carbohydrate Tn chains [39].

Clustered carcinoma T- and Tn epitopes are powerful adhesion arrays

At the molecular level we assessed adhesion functions of T and Tn epitopes using live cancer cells in in vitro models. Cultured breast CA DU 4475 cells and T RBC bound specifically to the Gal/GalNAc receptors of rat Kupffer cells and of hepatocytes [40]. Kupffer cells were chosen not only because they represent the reticuloendothelial system, but also because the liver is a frequent target for tumor metastasis, and Kupffer cells are the first barrier encountered by tumor cells; their Gal/GalNAc ligands are membrane-associated. Hepatocytes were studied for comparison; in contrast to macrophages, their ligands are transmembrane molecules, M_r 180 000, that have fewer and differently spaced combing sites [41]. Cell attachment was specifically inhibited by T and Tn antigens in a concentration-dependent manner: 50% of the T RBC Kupffer cell contacts were inhibited at 8.5×10^{-6} mM T and 8.5×10^{-5} mM Tn antigen concentrations; for hepatocytes these figures were 6×10^{-6} mM T and 1.2×10^{-6} mM Tn antigen. Amino-terminal cleavage products of the T glycoprotein, possessing *clusters* terminating in nonreducing Gal/GalNAc, inhibited T RBC-binding to Kupffer cells and hepatocytes by at least 50% at 10^{-5}–10^{-7} mM [41]. Free GalNAc, galactose, and galactose glycosides had only trace inhibitory activities (1–10 mM). Galactose-unrelated carbohydrates were inactive at concentrations of 50 mM or higher.

Electron microscopy of gold-labeled T and Tn antigens revealed their specific binding to macrophages; this was followed by their uptake via the coated pit/vesicle pathway of receptor-mediated endocytosis. Preincubation of the Kupffer cells with GalNAc and GalNAc-BSA, but not GlcNAc or GlcNAc-BSA, specifically blocked binding of the T and Tn glycoproteins [40].

In a different system the specific adhesion between invasive, metastatic murine ESb T-lymphoma cells and syngeneic hepatocytes was measured [42]. Direct molecular binding of the ESb cells to the corresponding ligands is the principal first step after recognition of hepatocyte ligands. ESb cells strongly express T and Tn epitopes on their cell surfaces [42]. Binding of ESb tumor cells to isogenic hepatocytes was inhibited in a dose dependent manner by T antigen and NH_2 terminal peptides cleaved from them. The extent of inhibition increased exponentially with the number of clusters and epitopes per clusters [42].

Role of T and Tn epitopes in the cancer metastatic process

T and Tn epitopes are promising detectors of CA dissemination (metastasis) in borderline lesions, including a single CA cell in regional lymph nodes [43, 44]. We recently established a highly statistically significant association between T antigen density of primary human colon CA and metastasis to the liver [45]. Primary melanomas with high density of T epitopes are the most likely ones to metastasize [46]. Namada et al. [47] and Hirao et al. [48] have shown a close relationship between the expression rate of Tn antigen in uterine cervival cancer and metastasis.

Immunotherapy using T/Tn autoantigens

The autoimmunogenicity of CA-T and anti-Tn epitopes on CA glycoproteins and their abundance, in covered form, on RBC glycoproteins [4, 8, 31], prompted us 23 years ago to attempt intradermal vaccination with human O RBC derived T/Tn antigen against recurrence of human breast CAs [38, 49–51], despite the then prevailing general opinion that vaccination against cancer amounted to “black magic” [52]. Initially we were permitted to treat only few patients and had to severely restrict the entrance in our protocol. The number of those engaged in developing vaccines against cancer in humans using purified antigens has substantially increased in the early 1990s [53–56].

We therapeutically vaccinate patients with advanced breast CA to prevent its clinical recurrence. T/Tn antigen vaccine is safe and specific and affords long-term effective protection for up to at least 20 years (March 1997) against recurrence of advanced breast CA stages II–IV. Intradermal vaccination is ad infinitum and repeated on various body sites every 6–20 weeks, depending on the size of the resulting delayed-skin hypersensitivity reaction, given by the adjuvant-containing vaccine. The adjuvant is necessary for persistence in the tissue of the antigen to maintain $CD8^+$ T cell-mediated cytotoxic immunity (see [57]). T/Tn antigens are HLA-allele-free, apyrogenic, and the vaccine is safe and specific and affords long-term effective protection against recurrence of CA, now up to 20 years [49–51]. Initially 32 patients with ductal breast CA (13 stage IV, 6 stage III, 13 stage II) were vaccinated after modified radical mastectomy or lumpectomy with axillary dissection or after first CA recurrence, provided they were of AJCC host performance scale H0. Patients with more than one recurrence in time were not treated. All patients received first chemo- or radiotherapeutical adjuvant treatment. Median interval from surgery to beginning of vaccination was 1.5 years. Of the 32 patients 24 were less than 50 years old and 8 were older than 50. All patients survived longer than 5 years. Of the 32 patients 14 (44%) survived longer than 10 years (10.2–20.1 years), 11 (34%) died of their CA within 5–10 years, and 7 (22%), all but one, without evidence of disease, have not yet reached the 10-year survival time [31, 38, 49–51].

The survival period was measured from day of operation; TNM staging was pathological and, where applicable, r_1TNM. Controls were the national survival rates as reported by the Biostatistics Branch of the United States National Cancer Institute [58]. The probability that our survival statistics of these 32 originally treated patients are due solely to chance is for all three stages taken together: 5-year survival, $P<1\times10^{-7}$; 10-year survival, $P<1\times10^{-5}$.

An additional 20 ductal breast CA patients of stages IV, III, and IIb who had either lumpectomy, simple mastectomy, or modified radical mastectomy were vaccinated postoperatively for 3–5 years (March 1997); 15 have no evidence of disease and 5 have distant metastases with optimal host performance scale (AJCC-H0; unpublished).

T/Tn vaccine is chemically and physically fully defined, sterile, and free of any known viruses, including human immunodeficiency virus (P.M. Feorino and M.S. Favero, Centers for Disease Control, 1986). Adjuvants are $Ca_3(PO_4)_2$ and traces of typhoid vaccine, which has a polysaccharide phosphoglycolipid A "hyperantigen" coat [59] and T and Tn specificities [8, 31]. Our active, specific immunization approach is successful, and expansion of such studies should be considered for treatment with this or similar vaccines on large populations with major types of CAs, and even immunological prevention of CA with such vaccines should be considered.

As described here, uncovered T and Tn epitopes are pan-CA adhesion, invasion, metastasis, and autoimmunogenic epitopes. T and Tn epitopes are occluded in healthy and benign-diseased tissues except in early embryonal stages (see [31]). They do occur in lymphomas and leukemias [23] but have not been found in other mesodermal malignancies. In vitro, isolated glycoproteins and glycopeptides carrying clusters of T and/or Tn epitopes are potent inhibitors of cancer cell adhesion to healthy tissue [41, 42]. The density of Tn epitope expression allows the prediction of a primary CA aggressiveness [27]. Increased density of Tn over T is an indicator of rapid CA progression and high metastatic potential [22, 60]. The T and Tn epitopes represent classical examples of aberrant glycosylation which is commonplace among malignantly transformed cells. It had been shown long before the introduction of monoclonal antibody in 1975 [61] that such epitopes, i.e., carbohydrate structures, are frequently terminal on antigens [62, 63]. Cell adhesion is frequently carbohydrate dependent (see above). Glycoproteins confer specificity to cell recognition and to cell adhesion [64]; tumor-associated antigens are preeminently involved in these processes [65–69].

Anti-CA autoimmune responses, among which those against T and Tn are very important, are likely to eliminate most CAs at nascence [70, 71]. The strong anti-T autoimmune responses elicited to CA are cellular and humoral and are readily measurable in about 90% of incipient and established cases of CA. In our studies the sensitivity and specificity of these reactions was on the order of 90% among more than 500 CA patients, approx. 350 with benign diseases and 176 healthy subjects [8, 14, 26, 27, 31]. Remarkably, in a high percentage of persons, positive anti-T responses detected *pre*clinical CA months to many years (mean 6 years) prior to positive biopsy/X-ray [31, 38]; hence measurement of these responses is of great practical clinical import.

Investigation of immune responses to CA-Tn antigen has lagged, and their evaluation is of immediate concern, especially because of the close association of Tn with the most malignant CAs [22, 60]. Among antibodies other anti-T and anti-Tn antibody classes than IgM need study. Our preliminary findings indicate a role for IgG subclasses in CA. In contrast to anti-T and anti-Tn antibodies, which everyone has due to humoral immune response to common Enterobacteriacae, there is no preexisting cellular immune response to CA-T and anti-Tn antigens. In fact, any measurable response in clinically CA-free individuals indicates evolving CA [31].

The role of both T and Tn in CA pathogenesis needs to be more firmly established. The complex interactions of CA T and Tn epitopes with the patient's microenvironment requires elucidation close to the cancer's monoclonal origin, beginning with intercellular recognition of CA and its adhesion to healthy tissue, followed by invasion and proliferation, prior to metastasis.

The clinical studies are to be expanded, especially in regards to very early diagnosis of preinvase phases of CA, which allows consideration of the use of isolated T/Tn epitopes preventing clinical CA manifestation and/or locoregional blocking procedures together with

other therapeutic agents. The principles established largely with breast CA are likely to apply to all CAs and contribute importantly to solution of the cancer problem, which show that in addition to carbohydrates [68, 69] protein conformation is importantly involved [31, 67, 72].

Acknowledgements The author's own work reported in this manuscript was supported by the U.S. Public Health Service, National Cancer Institute grants CA 19083 and CA 22540 and the Heather M. Bligh Cancer Fund. All studies on patients and volunteers were carried out in compliance with the recommendations of the Declaration of Helsinki and were approved by the Institutional Review Boards (local ethics committees) of the Evanston Hospital (Northwestern University) and the Chicago Medical School.

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