Carbohydrate Research, 178 (1988) 271–292
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

BLOOD GROUP Tn-ACTIVE MACROMOLECULES FROM HUMAN CARCINOMAS AND ERYTHROCYTES: CHARACTERIZATION OF AND SPECIFIC REACTIVITY WITH MONO- AND POLY-CLONAL ANTI-Tn ANTIBODIES INDUCED BY VARIOUS IMMUNOGENS*,†

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(Received September 8th, 1986; accepted for publication in revised form, August 28th, 1987)

ABSTRACT

In contrast to healthy and noncarcinoma-diseased tissues, >80% of all carcinomas (CAs) tested express *immunoreactive* *O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-serine/threonine [α-D-Gal*p*NAc-(1→3)-Ser/Thr] in their glycoproteins. CA cells shed, into the tumor's environment, Tn, which is involved in cancer pathogenesis as adhesion molecule and as autoimmunogen. An increase in density of Tn on primary CA frequently parallels augmented CA aggressiveness. Tn-Active glycoproteins of culture-grown human breast CA DU 4475 cells were isolated from cytoplasm and from spent growth medium, and erythrocyte (RBC) Tn antigen was prepared by (1→3)-β-D-galactosidase treatment of isolated human O RBC MN glycoprotein-derived Thomsen–Friedenreich (T) antigen. Immunochemical, serological, physical, and chemical analyses showed close resemblance of CA- and RBC-derived Tn antigens. The preponderant carbohydrate in both Tn glycoproteins is the α-D-Gal*p*NAc residue, and the antigens have a qualitatively and quantitatively similar amino acid composition. Highly specific rodent monoclonal (Mo) anti-Tn antibodies (Abs) were elicited with Tn RBC and normal O RBC-derived Tn antigen, and compared with CA–anti-Tn MoAbs unwittingly evoked by others. A sensitive enzyme immunoassay (EIA) with Tn antigen as solid phase was developed. In this system, highly purified, "naturally occurring" anti-Tn antibodies, which all humans possess, were more sensitive in quantitating breast CA Tn structures than the anti-Tn MoAbs induced by Tn RBCs, and by RBC- and CA-derived Tn-active antigens. The sensitivity of anti-Tn MoAbs was higher in detecting RBC-Tn.

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*Dedicated to Professor Walter T. J. Morgan.

† Supported by U.S.P.H.S. Grant No. CA 22540, the Elsa U. Pardee Foundation, and a gift from the Sonntag Foundation.

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0008-6215/88/$ 03.50

INTRODUCTION

We showed[1,2] that the Tn epitope, α-DGal*p*NAc-(1→3)-Ser/Thr, is present in most carcinomas (CAs) in immunoreactive form and is, thus, a universal (pan) CA structure[3-8]. It apparently plays a profound role during CA pathogenesis[3,9,10]. Tn also occurs in the extremely rare, usually benign, Tn syndrome of the hematopoietic system[11,12].

Tn is the immediate precursor of T, the Thomsen–Friedenreich epitope, *O*-β-D-galactopyranosyl-(1→3)-*O*-α-D-Gal*p*NAc-(1→3)-Ser/Thr, in glycoproteins[1,2]. On ordinary MN glycoprotein (glycophorin A), both the predominant T and to a much lesser extent Tn occur covered only with a Neu5A$_c$ group[13]. In general, T and Tn's immunodominant carbohydrates are the proximate sugars for most complex carbohydrate chains[14] α-D-linked glycosidically to Ser/Thr[14-16]. Being occluded, neither Tn nor T is accessible to the immune system of healthy persons or those with nonCA diseases[17-20]. Consequently, all humans have anti-Tn and -T antibodies, stimulated predominantly by their intestinal flora[21].

Immunoreactive T may occur on human blood cells, but only transiently during microbemia[22]. The very rare manifestation of Tn on blood cells is persistent[11,20]; it is the pleiotropic result[13] of an epigenetic, autosomal, hemizygous mutation at a pluripotent stem-cell level of the hematopoietic system that affects red and white blood cells, as well as platelets, and is known as the Tn syndrome[23-26]. Immunoreactive Tn has been found in >80% of all major CA types tested thus far, particularly adenoCAs[3,5-8], and less frequently in squamous and kidney CAs.

CAs make up nearly 75% of all malignant tumors[27]. After our discovery of Tn in CAs, it was noted that Tn on RBCs may herald and be associated with different acute leukemias and myelofibrosis[28-31]. The ratio of newly detected CAs to Tn leukemia, at any given time, is >20 000:1 in the Western Hemisphere (*cf.* ref. 27 and 32); yet, only the Tn syndrome and its sequela have been carefully analyzed[3,11,12,23,28-31], and the systematic study of Tn's role in CA has just begun[10]. Uncovered, immunoreactive T generally accompanies the Tn in CAs. T is not reactive in patients with Tn syndrome, and in healthy persons and those with nonCA disease (*cf.* ref. 3).

We unwittingly produced sensitive and specific anti-Tn MoAbs with the RBC T antigen used for production of anti-T MoAbs[33] since RBC T antigen always carries some Tn epitopes[13]. Because of their high sensitivity and specificity, both anti-Tn and anti-T have proved useful in the detection of CA, including its earliest stages[10,34,35].

Hirohashi *et al.*[36] have produced and defined monoclonal anti-Tn antibodies after immunizing mice with membrane preparations from human lung CA. We have shown that the hitherto unknown minimal structure complementary to anti-CA MoAb B72.3, raised with membrane-enriched fraction from human breast CA tissue[37], encompasses the Tn epitope[10,35]. There are indications of important functions of Tn and T in cancer cell adhesion and invasiveness into healthy tissues[9,10],

and in autoantigenicity[3,5,11,12,23-26,29,30]. Tn and T are sensitive and specific immunohistochemical CA markers[10].

Glycoproteins carry the Tn epitopes in human breast CAs[38,39]. In guinea pig L-10 adenoCA, *glycolipids exclusively* have Tn- and T-activity and -immunogenicity[40-42]. Walker-Nasir *et al.*[38] have indicated that the T disaccharide β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc group is linked to Ser/Thr on glycopeptides of tissue culture-derived human breast CA cells, and Bhavanandan and Codington[43] also clearly established that in the glycoprotein I (epiglycanin) of the spontaneous murine mammary TA3 CA, the major carbohydrate chain β-D-Gal*p*-(1→3)-D-Gal*p*NAc is linked to the protein in α anomeric form[43].

We report herein (a) early chemical, physical, and more detailed biological findings on Tn-active antigen preparations derived from human breast CA cells grown *in vitro* in suspension culture; (b) a simple method of preparing highly active, purified blood group Tn antigen from O,MN RBC; and (c) highly specific inhibition studies using haptens, epitopes, and antigens that confirm the ready induction of anti-Tn MoAbs with Tn-specific antigens.

EXPERIMENTAL

Human breast CA cells and spent growth medium as sources of Tn-active antigen. — DU 4475, an anaplastic, human metastatic ductal breast CA line (EG&G Mason Res. Inst.) was used. Blood group specificities and secretor status of the donor patient are unknown. We found about twice as much Tn as T on DU 4475 CA plasma membranes[10]. Cells were grown in RPMI 1640 plus 10% fetal bovine serum (FBS) (both from Gibco Laboratories) in presence of 5% CO_2 at 37° in a water-saturated atmosphere. The cells were adapted to suspension growth and then grown on a large scale in Spinner culture, and checked periodically for Tn and T activities. The proportion of Tn to T (~ 3:1) on the CA cell membrane remained constant within the limits of experimental error. Standard procedures ascertained lack of cytomegalovirus and mycoplasma contamination. At the beginning of the plateau-phase of growth (~ 1.5×10^6 cells/mL), the cells were harvested at ~ 250*g* for 15 min at 4°; cell viability was >95% as determined by Trypan Blue exclusion. The cells were washed twice with 0.05M phosphate plus 0.10M NaCl, pH 7.4 (PBS) (10 vols.); five lots, each amounting to $>2 \times 10^{10}$ cells, were collected in glass flasks and immediately stored at −80°. After one thawing, all CA cells were intact, as determined microscopically after Hematoxylin-Eosin staining, and free of bacteria (Gram stain); they were worked up at once.

Lots (~ 20 L) of cell-free spent medium supernatant were concentrated 20-fold on a Millipore Pellicon cassette system (polysulfone filter, M_r cut-off 10 000). The concentrate after centrifugation at 100 000*g* gave a pellet with <2% of total dry matter, which consisted of cell nuclei and debris but no bacteria. Uninoculated, 20-times concentrated complete medium served as negative control. Freeze-dried fractions were stored at −80° in Mason jars over Drierite.

Washed, packed CA cells (3 g wet wt.) were used as the starting material for each set of complete experiments. The results in Table I under item II. correspond to the culture medium vol. from which ~ 3 g of DU 4475 CA cells were obtained.

Isolation of Tn-active substances from human breast CA DU 4475 cells. — Separation of cytoplasm and plasma membranes was based on the method of Brunette and Till[44]. CA cells (3 g wet wt.) were suspended in mM $ZnCl_2$ and 2mM phenylmethylsulfonylfluoride (PMSF; Sigma Chemical Co) (200 mL) for 15 min at 21–23°. Subsequent steps were carried out at 4° unless stated otherwise. The suspension was homogenized by ~ 80 strokes in an ice-cooled Dounce homogenizer, and then centrifuged at 1200*g* for 15 min. The supernatant contained the cytoplasm which was used immediately or stored at −80°.

The cytoplasm was solubilized at 4° in 1% octyl glucoside (20 mL) in PBS, continuously stirred, and then exhaustively dialyzed at 4° in the presence of a few drops of toluene and chloroform. The retentate was centrifuged at 11 500*g* for 20 min. The inactive sediment was stored at −80°. The supernatant was freeze-dried, dissolved in 0.05M Tris·HCl, pH 7.4 (Tris buffer) (~ 2 mL), and chromatographed on a TEAE-cellulose (Sigma) column (1.0 × 15.0 cm), equilibrated with the same buffer. DU 4475 CA cytoplasm fractionation was monitored by protein determination[45]. The TEAE column was eluted with Tris buffer until protein ceased to emerge; fractions (3 mL) were collected. The peak fraction ("Breakthrough") and material eluted slowly after the peak ("Retarded", see Table I) were pooled separately. Further elution with 2.0M NaCl in Tris buffer yielded an additional protein peak. Material still bound to TEAE-cellulose was eluted with 6.0M urea in 0.05M Tris·HCl – 2.0M NaCl. All fractions were dialyzed as described above and freeze-dried.

Two of the cytoplasm fractions yielded material sufficient for subfractionation: Fractions I.3. and I.4. (Table I); 2.6-mL fractions were collected. The NaCl eluate I.3, 40 mg was applied to a 1.5 × 83 cm Sephacryl S-200 (Pharmacia Fine Chemicals) column equilibrated with 0.15M NaCl in Tris buffer, which also served as eluant. Similarly, the 6.0M urea-eluted cytoplasm fraction (I.4; 40 mg) was subfractionated on the same Sephacryl S-200 column, after its equilibration with urea plus 2.0M NaCl in Tris buffer. Yield and activity units (U) of the NaCl- as well as urea-eluate subfractions were adjusted to the original weight of the parent NaCl and urea eluates, since only a part of each eluate was subfractionated; the adjusted values are presented in Table I.

The pellet, after cytoplasm recovery, was dispersed with a glass rod in the top phase of a two-phase system of Dextran 500 (Pharmacia) and poly(ethylene glycol) (PEG, M_r 6 000, Sigma, prepared as described by Brunette and Till[44]), mixed with an equal vol. of the bottom phase and centrifuged at 9 000*g* for 15 min. The pellet consisted of inactive cell nuclei and debris (determined by microscope); it was discarded. The supernatant consisting of the two-phase system (which contained the cell membranes at the interface) was remixed by inversion and centrifuged at 9 000*g* for 15 min. The interface material was diluted five-fold with de-ionized distilled

TABLE I

Tn ACTIVITY OF GLYCOPROTEIN FRACTIONS FROM HUMAN BREAST CA DU 4475 AND FROM HEALTHY BLOOD GROUP O RBC

Fractions	*Weight (Mg)*	*Tn serological activity (mg/mL)*[a]	*Tn U demonstrable*[b]	*Increase in activity over starting material (fold)*	*U recovered from starting material (%)*
I. Cytoplasm	200[c]	2.5	80		
TEAE-cellulose fractions					
1. Breakthrough material	11.2	0.8	14	3.1	18
2. Retarded fraction[d]	5.3	0.4	13	6.1	16
3. NaCl eluate	58.3	1.1	53	2.3	66
Sephacryl S-200 subfractions					
A	22.0	0.2	110	12.5	138
B	23.3	5.0	5	[e]	
C	2.9	1.6	2	1.7	3
4. Urea-NaCl eluate	56.5	2.2	26	1.2	33
Sephacryl S-200 subfractions					
A	15.1	0.6	25	4.1	31
B	21.2	0.3	71	8.4	89
II. DU 4475 spent medium		[f]			
1. HPA-agarose-bound material from spent medium	14.8	0.25	60	>300	<40
III. Human O,MN RBC-derived T antigen	0.28[g]	2.5–5	~0.07		
1. Tn-specific antigen enzymically prepared from T	0.25[h]	0.01	25	~400	

[a]Mg/mL completely inhibiting four hemagglutinating doses of pooled human sera. [b]For definition of activity U, see text. [c]Yield (dry wt. basis) from 3 g of CA cells (wet wt.). [d]Material that emerges slowly from the TEAE-cellulose column, in the wash buffer, after the sharp emergence of the "breakthrough" fraction; for definition see text. [e]No increase. [f]Not measurable (see text). [g]Yield (dry wt. basis) from 3 mL of packed RBC (wet wt.). [h]Yield from 280 μg of T antigen; for experimental details, see text. [i]Not applicable.

water, and centrifuged at 1200*g* for 15 min. The sedimented CA cell membranes were still heavily contaminated with Dextran–PEG and not suitable for analysis.

Isolation of Tn-active material from CA DU 4475 spent medium by Helix pomatia *agglutinin (HPA)-agarose affinity chromatography.* — Ultrafiltrate of spent medium (~150 mL, ~13.5 g dry wt.), because of its large quantity of FBS, was applied directly to an HPA–agarose (E-Y Labs.) affinity column (1.2 × 6.0 cm), extensively prewashed and equilibrated with 0.01M KH_2PO_4–K_2HPO_4, pH 6.8, in 0.15M NaCl and 0.1% NaN_3. After sample passage, the column was washed with the equilibrating buffer until the effluent was protein-free. Elution with 0.1M GalNAc in the same buffer followed; fractions (3 mL) were collected and 0.1 mL of each assayed for protein. The "Breakthrough" eluate was refractionated four times on the same column. The GalNAc-eluted peak fractions were pooled, thoroughly dialyzed, flash-evaporated to a small vol., redialyzed, and freeze-dried to give Tn-active proteinaceous material (~15 mg). An equivalent amount of ultrafiltrate of the *un*inoculated FBS-containing medium, under these conditions, yielded ~ 2 mg of Tn-inactive dry matter.

Preparation of Tn-specific antigen from human blood type O,MN RBC-derived T antigen. — The T antigen was prepared by neuraminidase treatment of MN glycoprotein isolated from O,MN RBC under gentle conditions[4,46]; all T antigen preparations had <2% NeuAc. In a typical experiment, T antigen (40 mg dry wt.), was dissolved in acetate buffer (0.05M sodium acetate + 0.15M NaCl + 0.02% NaN_3), pH 4.6, and treated for 62 h at 37° with bovine testis (1→3)-β-D-galactosidase, kindly donated by Drs. J. Distler and G. Jourdian[47]. Two 0.06 unit aliquots of the enzyme were added per mg of T antigen, one at the outset and another after 18 h of incubation; the final substrate concentration was 9.7 mg/mL. The incubated sample was then adjusted to pH 7.0 with M Tris·HCl buffer, pH 8.6, heated in a boiling water bath for 10 min, exhaustively dialyzed, freeze-dried, and dried to constant wt. (see Table I, III.1.). For comparison with CA-derived Tn-active antigen, the RBC-Tn recovery was extrapolated to 3 mL of washed, packed RBC as starting material (see Table I).

Polyacrylamide gel electrophoresis (PAGE) of CA- & RBC-Tn antigens. — PAGE was performed as described by Weber and Osborn[48] in a Canalco Model 1200 apparatus (Canalco Inc.), but with 25mM Tris -192mM glycine buffer, pH 8.4[49]; 10% acrylamide cross-linked with 0.18% bis(acrylamide) was used. PAGE was performed either with or without 0.1% sodium dodecyl sulfate. For SDS-PAGE, the sample (250 μg) in water (25 μL) was mixed with the "preparation buffer" (25 μL)[49]. The mixture was heated for 3 min in a boiling-water bath, cooled to 21–23°, and applied to the gel; a constant current of 1.5 mA per gel was maintained for 3–5 h. Fetuin[50] (Gibco) and carcinoembryonic antigen (CEA)[51] were the M_r markers. Staining was with the periodic acid–Schiff (PAS)[52] and Coomassie Brilliant Blue (CMB)[48] reagents.

For preparative PAGE, the samples (570 μg each) were applied to seven (0.5 × 11.0 cm) cylindrical gels [7% acrylamide + 0.18% bis(acrylamide)] and subjected to

electrophoresis as described above but without sodium dodecyl sulfate. One gel, used as reference, was stained with Coomassie Brilliant Blue; it showed two major bands, one sharp near the origin (1), and the other diffuse (2) 1 cm towards the anode. Gel slices corresponding to each band were cut from the other six gels, suspended in phosphate buffered saline, pH 6.8 (20 mL), and dialyzed for 48 h at 4° against this buffer containing 0.1% NaN_3 (3 vols. of 2 L each), and then for 48 h at 4° against de-ionized distilled water + 0.1% NaN_3. The contents of the dialysis bags were filtered through prewashed glass wool to remove most of the acrylamide gel. The filtrates were concentrated to 2 mL, dialyzed as described above against de-ionized, distilled water, lyophilized, and dried to constant weight at 21–23° in a vacuum desiccator over P_2O_5.

Analytical procedures. — All reagents were of analytical or higher grade. Wet ash (with H_2SO_4), determined by Huffman Microanalytical Laboratories, was $<2.5\%$ by wt. throughout. Tn-active fractions were analyzed for their neutral hexose content by the orcinol[53] and anthrone[54] methods with D-galactose as the standard. Determination of D-galactose was by D-galactose dehydrogenase from *Pseudomonas fluorescens*[55] (Sigma), after hydrolysis of 0.2% samples (w/v) in M HCl for 4 h at 100° under N_2, and acid removal on a Dowex 1(HCO_3^-)column (2 mL). Loss of D-galactose in CA-Tn was corrected for the decrease in hexoses of the sample due to hydrolysis, as determined by the anthrone method. Sialic acid was determined as described by Jourdian *et al.*[56]. For hexosamine determination, each sample (2 mg) was hydrolyzed in 6M HCl (1 mL) for 4 h at 100°, and then dried *in vacuo* over NaOH pellets. Total hexosamine and GalNAc were determined by the methods of Ludowieg and Benmaman[57]; the content of GlcNAc was determined indirectly by subtracting the second value from the first. Hexosamines were also determined with an amino acid analyzer after the sample (100 μg) was hydrolyzed with 4M HCl for 8 h at 100° (Waters Picotag station). Amino acids were determined with the same analyzer after sample hydrolysis for 20 h in 6M HCl at 110°.

Erythrocytes, polyclonal monospecific, and monoclonal antibodies. — O,Tn RBC (containing $\geq 90\%$ Tn RBC) were the generous gift of Drs. M. Beck and P. Lalezari; they were used either fresh or after preservation at −80° in glycerol. Collection, storage and use of O,MN; O,S; O,Rh_o(D); A_1; A_2 and B RBC were as described earlier[58].

Pools of sera were from healthy, not intentionally immunized adult donors. The pools were monospecific with respect to the blood-group antigens studied[46,59]. Sera were heat-inactivated for 10 min at 56°, and used either fresh or after being stored once at −80°. Polyclonal monospecific anti-Tn and -T antibodies were isolated by affinity purification by use of a modified Landsteiner-Miller procedure, as delineated earlier[59,60]; elution was at 48°.

MoAbs in rats were raised as described by Galfrè *et al.*[61]. Human group O RBCs on which the T antigen had been maximally uncovered by neuraminidase[62] served as immunogen and resulted in anti-T and, unwittingly, some anti-Tn MoAbs[33]. We have since demonstrated that small quantities of Tn are regularly

uncovered by specific desialylation of normal group O RBC and isolated O,MN glycoproteins[13].

For intentional production of monoclonal anti-Tn antibodies, BALB/c mice were immunized with either Tn RBC or isolated Tn glycoprotein, in immunization schedule B for the isolated Tn antigen, as described by others[63]. Isolation of hybrid cell lines producing MoAbs was as delineated by Nowinski *et al.*[64]; fusion and growth in flasks was as described by Köhler[65]. All MoAbs used in the present study were anti-Tn-specific and of high avidity. All but B72.3 (ref. 37) were flask-grown. The only purification steps were absorption with A_1B RBC, followed by removal of RBC by centrifugation, and recentrifugation of the supernatant at ~ 4000*g* for 30 min at 4°. The antibodies were then stored in 0.5-mL aliquots in liquid N_2. Two murine MoAbs induced with human lung CA tissue by Hirohashi *et al.*[36], were also studied; both had been characterized and identified as anti-Tn antibodies[36]. In agglutination tests, none of the MoAbs used reacted with blood group A_1, A_2, B, H(O), $Rh_o(D)$, or S antigens.

Hemagglutination and hemagglutination inhibition assays. — These tests were performed as described before[59,60], except that the volume of each reactant was 0.03 mL. All tests were performed at least twice, and the results were averaged. An activity unit (U) is defined as equal to the activity of the least active substance that, at 10 mg/mL, completely and specifically inhibited 4 hemagglutinating doses of the antibody.

Determination of Tn activity of CA-derived glycoproteins by solid-phase enzyme immunoassay. — All steps were carried out at 18–20°; incubations were on a rotary shaker (60 r.p.m.). Incubation and wash vols. were 100 μL and 250 μL per well, respectively, unless stated otherwise. Gelatin (Bio-Rad Laboratories) coated wells were used to measure nonspecific binding; gelatin also served as blocking agent and as component of the primary antibody diluent to reduce nonspecific binding. Tn-active antigens (RBC-Tn, 0.1–10 ng and CA-Tn, 20–1000 ng/well) and gelatin (1000 ng/well) in 0.05M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6) were incubated for 16 h in flat-bottom Immunlon 2 microtiter wells (Dynatech Laboratories, Inc.) to obtain the respective solid phases. The wells were then washed once with buffer *A* (0.01M phosphate + 0.15M aq. NaCl + 0.02% NaN_3, pH 7.4, and 0.05% Tween-20), blocked for 1 h with 1% gelatin in 0.01M phosphate buffered saline, pH 7.4 (250 μL/well), and washed once more with buffer *A*. Primary human, mouse, or rat anti-Tn antibody (20–200 ng/mL in PBS plus 0.1% gelatin) was then added to the wells; they were allowed to react for 2 h and washed twice with buffer *B* (0.02M Tris + 0.15M NaCl, pH 7.5 (TBS) + 0.05% Tween-20). The wells were then incubated with biotin-labeled anti-primary antibody Ig type heavy chain (Zymed Laboratories) in buffer *B* (~100 ng/mL) for 75 min, washed twice, and incubated for 1 h with a 1:3000 dilution of avidin-horseradish peroxidase (Bio-Rad) in this buffer. Thereafter, the wells were washed once with buffer *B* and then twice with TBS, followed by color development for 1 h with 3,3′, 5,5′-tetramethylbenzidine (TMB) reagent (200 μL/well) freshly prepared according to the instructions of the

manufacturer [ICN Immunobiologicals] (*cf.* ref. 66). The reaction was stopped by addition of 2M H_2SO_4 (50 μL per well), and after 10 min absorption was read at 450 nm in a Microplate reader (Bio-Tek Instruments).

RESULTS

Tn-active antigens were prepared from a human ductal breast CA, known to express Tn-specific epitopes[10], and from O,MN RBC-derived T antigen. Characterization and comparison of the biological, chemical, and some physical properties of these glycoproteins of widely different origin are presented. Anti-Tn MoAbs induced by Tn RBC, RBC-Tn, lung CA[36], and breast CA[37] were compared with one another and with "naturally occurring" human anti-Tn.

Breast CA DU 4475 Tn-active glycoproteins from cytoplasm fractions and spent medium. — Tn-active glycoproteins were obtained from CA cell cytoplasm and from the spent medium supernatant into which the exponentially growing CA cells had shed, predominantly from the cell membranes, Tn-active glycoprotein(s) (*cf.* refs 67,68). Table I shows that 200 mg of cytoplasm (dry wt.) was recovered from 3 g of packed CA cells (wet weight). The cytoplasm was fractionated on a TEAE-cellulose column; recoveries and activities are presented in Table I. The major fractions 1.–4. of I. cytoplasm contained 106 activity Us in 131 mg of recovered material. Approximately 75% of these activity Us were recovered in the NaCl and urea eluates, Fractions I.3. and I.4.

Sephacryl S-200 column chromatography of the NaCl eluate yielded two large cytoplasm subfractions (Table I: Fractions I.3.A and I.3.B). Fig. 1 depicts their protein elution profile, based on absorption at 595 nm by use of the microCMB method[45]. Subfraction I.3.A contained the most highly Tn-active glycoprotein isolated so far from breast CA DU 4475. It was 13 times as active as the unfrac-

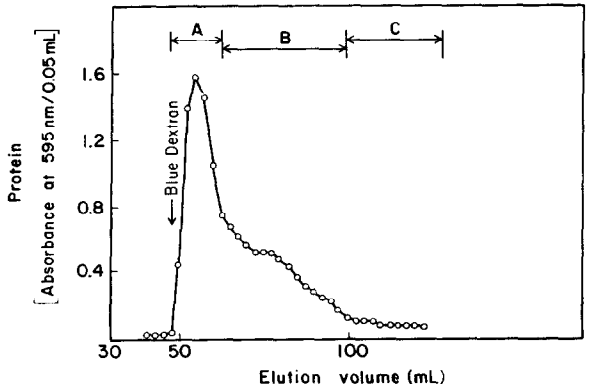

Fig. 1. Chromatography of the NaCl-eluted material of human breast CA DU 4475 cytoplasm on a Sephacryl S-200 column. The NaCl eluted material (40 mg) from TEAE cellulose was dissolved in water (1 mL) and applied to a Sephacryl S-200 column (1.5 × 83 cm) equilibrated and eluted with 0.05M Tris·HCl (pH 7.4)– 0.15M NaCl.

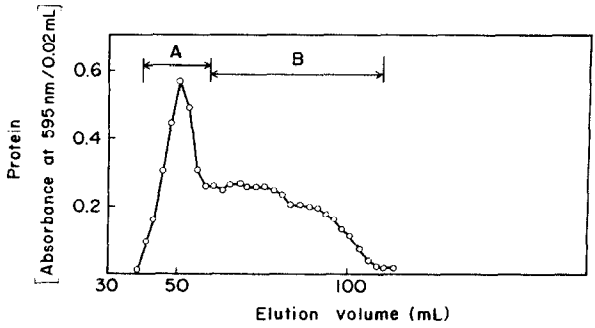

Fig. 2. Chromatography of the urea eluted material of human breast CA DU 4475 cytoplasm on Sephacryl S-200 column. The urea-eluted material (40 mg) from TEAE cellulose was dissolved in 6M urea - 2M NaCl - 0.05M Tris·HCl (pH 7.4) (1 mL) and applied to a Sephacryl S-200 column (1.5 × 83 cm), equilibrated and eluted with the same buffer. Details given in the EXPERIMENTAL section.

tionated cytoplasm and had 1.4 times as many activity Us. Subfraction I.3. B had a weight similar to Subfraction A but only 4% of its activity; the quantity and activity of subfraction C were negligible.

Some mucin-like material was too tightly bound to TEAE-cellulose to be eluted by buffered aqueous sodium chloride; however, after addition of 6M urea to the buffer, significant quantities of Tn-active material were eluted (Table I; Fraction I.4.). The urea–sodium chloride eluate was subfractionated on Sephacryl S-200 into Subfractions I.4.A and B (see Fig. 2) to yield 96 Us of Tn-active glycoprotein, with an average wt. of <0.4 mg/U; Subfraction I.4. B had the second highest activity among cytoplasm fractions. Subfractions I.4.A plus B had 3.7 times more activity U than the starting urea fraction.

Tn-active material from the spent growth medium (II. in Table I) was isolated by HPA-agarose affinity column chromatography. A volume of growth-medium supernatant corresponding to 3 g of packed DU 4475 CA cells was used. Fraction II.1 (Table I) showed that this procedure yielded a modest quantity, 60 U, of highly activeactive material. This corresponds to ~ 55% of the activity Us of the most cytoplasm fraction (I.3A).

An equal volume of uncultured control medium, when subjected to an identical HPA-affinity chromatography, yielded 1.7 mg of HPA-bound material, *i.e*, < 12% by weight of the HPA-bound substances isolated from the spent medium; this material had neither Tn nor T activity (results not shown in Table I).

Cytoplasm subfractions I.3.A, I.4.B, and the spent medium Fraction II.1. had similar activities. Subfractions I.3.A and I.4.B were similar in activity and quantity (Table I).

All highly Tn-active, DU 4475-derived preparations also had some T activity in hemagglutination inhibition assays (not shown in Table I). T activity, on a weight basis, amounted to between 4 and 15% of the Tn activity among the most highly Tn-active cytoplasm-derived fractions; and to ~ 8% for the spent medium fraction.

While no blood group A activity was found in the cytoplasm fractions, some A_1 and A_2 activities were present in Fraction II.1., the fraction eluted with GalNAc from the HPA-agarose column. These activities amounted to 4 and 14% of those of the blood group A_1 and A_2 glycoproteins from A meconium[46] used as standard. Similar A activities were found for uninoculated complete growth medium (see DISCUSSION). No B- or H(O)-active substances were detected in any preparation derived from breast CA DU 4475.

Tn-specific glycoprotein from O,MN *RBC-derived T antigen.* — The Tn-active RBC glycoprotein was obtained by enzymic uncovering of Tn-specific epitopes on O,MN-derived T glycoprotein with bovine testis β-D-galactosidase specific for (1→3) linkage. More than 90% of the Tn-specific glycoprotein, free of T activity, was recovered after correction for released Gal (~ 90% of total Gal) and enzyme. The RBC-derived Tn antigen was highly active; when measured with human anti-Tn antibodies, it was more active than the parent T antigen (weight basis), measured with homologous anti-T antibodies. At a concentration of 10 μg/mL (~ 0.02 nmol/mL), the RBC-derived Tn antigen inhibited 4 hemagglutinating doses of corresponding "naturally occurring" human anti-Tn antibodies (see Table I, Fraction III.1.). Like naturally occuring Tn RBCs, Tn-specific antigen derived from ordinary O,MN RBC reacted well with monospecific polyclonal human anti-Tn antibodies. The O RBC-derived Tn glycoprotein had no A_1 or A_2 activities. On a weight basis, measured with human polyclonal anti-Tn antibodies, the RBC-Tn-specific preparations were 20 times as active as the most active breast CA glycoprotein (see Table I, Fraction I.3.A).

PAGE of breast CA DU 4475 Tn-active fractions and O,MN RBC-derived Tn glycoprotein. — Fig. 3 shows the PAGE patterns of duplicate samples each of the

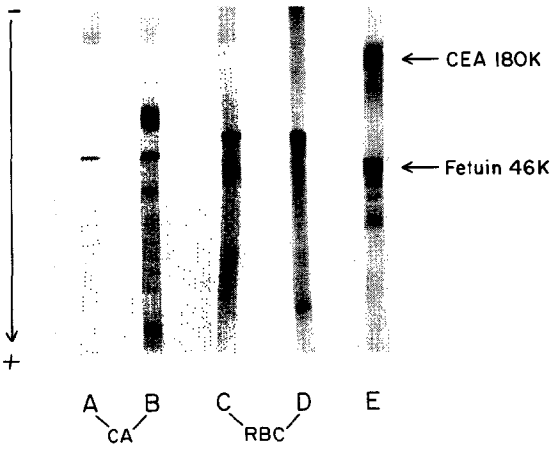

Fig. 3. SDS-polyacrylamide gel electrophoresis of human breast CA DU 4475 (A & B) and O,MN RBC-derived Tn-specific glycoproteins (C & D) (250 μg each). Staining, gels A, C, and E: periodic acid-Schiff reagent; gels B and D: Coomassie Brilliant Blue. Gel E: 110 μg each of fetuin and carcinoembryogenic antigen.

breast CA DU 4475 Fraction II.1. and O,MN RBC-derived Fraction III.1. Tn-active glycoproteins, one PAS- and the other CMB-stained as representative of all highly active fractions investigated in this study.

SDS-PAGE and subsequent PAS staining showed for the breast CA-derived fraction (gel A) one major sharp band that amounted to >85% of all stained material. Gel C showed the PAS pattern of the RBC-derived Tn-active material, and the major band amounted to ~ 70% of all PAS-positive material and was located nearer to the origin in the gel than the CA-derived Tn-active material in gel A. Gel C revealed that RBC-derived Tn had, in addition to the major PAS-positive band, three minor readily discernible diffuse bands. The fetuin and CEA standards (gel E) showed, in addition to the major band of each, several minor ones on PAS staining.

Protein staining by CMB after SDS-PAGE of the CA- and RBC-derived Tn fractions is shown in gels B and D; while both gels confirmed the location of the major band in the two specimens shown by PAS staining, they also indicated a substantial number of additional minor components.

Upon PAGE without sodium dodecyl sulfate (not shown in Fig. 3), the Tn-active fraction from CA showed a sharp band near the origin and an adjacent, slightly faster-moving broad band when stained for both carbohydrate and protein. RBC-Tn gave a broad band at the origin with both PAS- and CMB-stains under these conditions.

Preliminary preparative PAGE was carried out with the highly active CA-Tn preparation used for analytic PAGE (see Fraction II.1. in Table I). The staining of one gel used as reference revealed two predominant bands: No. 1 was sharp and located at the origin; band No. 2 was diffuse and had migrated about 1 cm towards the anode. The corresponding sections of the gels proper were eluted; 75% of the applied Tn activity Us was recovered from band No. 2. Neither band had blood group A activity.

On reelectrophoresis of band 2 in the presence of sodium dodecyl sulfate, only one sharp band with an $M_r \sim 5 \times 10^4$, based on the reference glycoproteins, CEA and fetuin, was found after staining with PAS as well as CMB; under the conditions used, this fraction appeared to be homogeneous.

Carbohydrate and amino acid analyses of the most active breast CA- and RBC-derived Tn glycoproteins. — The highly active CA-Tn Fraction II.1. and RBC-Tn Fraction III.1. (Table I) that had been studied by SDS-PAGE (Fig. 3) were analyzed for their carbohydrate and amino acid composition. Values given for carbohydrates (except hexoses) and amino acids are not corrected for hydrolytic destruction. They are listed as rehydrated molecules in Table II. Both breast CA- and RBC-derived Tn-specific antigens are glycoproteins. The breast CA-derived glycoprotein had 46.4% of carbohydrate and 45.3% of protein, and the RBC-derived Tn-active preparation 29.4% of carbohydrate and 60.7% of protein. Both preparations contained 7–11% of water readily removable at 110° *in vacuo* (13 Pa over P_2O_5) (*cf.* ref. 46).

Table II shows that GalNAc is the preponderant carbohydrate in both breast

TABLE II

COMPOSITION OF Tn-SPECIFIC GLYCOPROTEIN FRACTION FROM SPENT MEDIUM OF HUMAN BREAST CA DU 4475 CELLS AND Tn-SPECIFIC ANTIGEN PREPARED FROM HEALTHY O,MN RBC

Carbohydrates and amino acids	*Tn Glycoproteins from human*					
	DU 4475 Breast CA			*O RBC*		
	nmol/mg	*Wt.%*	*Residues/ 100 residues*	*nmol/mg*	*Wt.%*	*Residues/ 100 residues*
GalNAc[a]	633	14.0	11.2	511	11.3	8.3
GlcNAc[a]	443	9.8	7.8	385	8.5	6.3
NeuAc[b]	385	11.9	6.8		<1.0	
Gal[c]	397	7.1	7.0		<1.0	
Hexose[d]	593	10.7[e]	10.5	477	8.6[f]	7.8
Asp/Asn	273	3.6	4.8	300	4.0	4.9
Thr	380	4.5	6.7	495	5.9	8.1
Ser	396	4.2	7.0	466	4.9	7.6
Glu/Gln	430	6.3	7.6	394	5.8	6.4
Pro	349	4.0	6.2	348	4.0	5.7
Gly	232	1.7	4.1	266	2.0	4.3
Ala	286	2.5	5.0	337	3.0	5.5
Half Cys		[g]			0.0	0.0
Val	215	2.5	3.8	358	4.2	5.8
Ile	102	1.3	1.8	263	3.4	4.3
Leu	327	4.3	5.8	427	5.6	6.9
Tyr	65	1.2	1.1	132	2.4	2.1
Phe	121	2.0	2.1	151	2.5	2.5
Lys	176	2.5	3.1	219	3.2	3.6
His	69	1.1	1.2	187	2.9	3.0
Arg	156	2.8	2.8	200	3.5	3.3
Met	36	0.6	0.6	228	3.4	3.7
Try		[g]			0.0	0.0

[a]Amino acid analyzer. [b]Resorcinol[56]. [c]D-Galactose dehydrogenase[55]. [d]Orcinol[53]. [e]~33% Man. [f]~85% Man[5]. [g]Not done.

CA- and RBC-derived Tn antigens. The preponderant hexose is Gal in CA-Tn and Man in RBC-Tn. Both glycoproteins are rich in hydroxyamino acids Ser and Thr; together they constitute >20% of all amino acids in the CA-derived Tn-active and the RBC-derived Tn glycoproteins. Other amino acids shared by both antigens as major components are Asp(Asn), Glu (Gln), Pro, Ala, Val, and Leu. The aromatic amino acids Tyr and Phe were present in only small proportions. As shown by SDS-PAGE (Fig. 3), neither of the glycoproteins was homogeneous.

Anti-Tn antibodies and their fine specificities. — The O,Tn RBC of both our donors adsorbed anti-Tn antibodies completely and specifically. The antibodies were extensively eluted without demonstrable loss of their anti-Tn activity.

Table III lists examples of antigens, from entirely different sources, that induced anti-Tn antibodies. The glycoproteins and the haptens that specifically

TABLE III

SPECIFIC INHIBITION OF Tn ERYTHROCYTE AGGLUTINATION BY ANTI-Tn SPECIFIC ANTIBODIES

		Monospecific anti-Tn antibodies[a]			
	Species:	*Human*		*Rat*	
	Antigen:	*Intestinal flora*		*O,T RBC*	
Inhibitor[b]		*Polyclonal pool La-Mi '84 predominantly IgM*		*Monoclonal T9 2c [A6] IgM*κ	
	Mass (Mr)	mg[b]	*nmol*[b]	*μg*	*nmol*
AS-glycoproteins and AS-glycopeptides[c]*:*					
AS-OSM	620 000	1	< 0.002	3	< 0.005
Tn antigen	490 000	8	0.016	10	0.02
AS-LS-α_1 MN GP	~ 12 000	~ 30	~ 2.5	~ 30	~ 2.5
AS-PII MN GP	~7 000	~ 30	~ 4	~ 30	~ 4
Carbohydrates:					
α-D-Gal*p*NAcOMe	235	4.5	19	3	13
β-D-Gal*p*NAcO-C_6H_4-NO_2 (*p*)	342	9	26	15	44
D-GalNAc	221	4.5	20	9	41

[a]All MoAbs were culture fluids, except B72.3 which was ascites-derived; all antigens, except "intestinal flora", are human tissue-derived. Results listed are the arithmetic averages of two to three different experiments. [b]Smallest quantity completely inhibiting 2–4 hemagglutinating doses; final vol., 90 μL. Inactive (at ~90 μg) were: blood group A_1, B and H(O) ovarian cyst glycopro-

inhibit anti-Tn characterize them positively [as do related but inactive substances in a negative way; see Table III, footnote[b] (*cf.* ref. 9,69)]. The specifically Tn-active glycoproteins, asialo-ovine submaxillary mucin (AS-OSM) and Tn antigen, have nonreducing α-D-linked Gal*p*NAc residues as dense clusters at the molecular level.

In hemagglutination inhibition tests, Tn antigen was the most potent inhibitor (weight basis) of anti-Tn antibodies elicited by O,Tn RBC, by RBC-derived Tn-specific antigen, and by lung CA; in all three instances, full activity was exhibited by < 0.1 pmol of Tn. Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside was 4 to 10 times as active (molar basis) as free 2-acetamido-2-deoxy-D-galactose with these three antibodies. With the three remaining anti-Tn antibodies, human polyclonal, rat MoAb T9 2c[A6], and mouse MoAb B72.3, AS-OSM was 3 to 8 times more active than RBC-derived Tn-specific antigen, and both B72.3 and rat anti-Tn MoAb had the lowest reactivity with Tn antigen and AS-OSM. These same antibodies showed very little or no differences in inhibitability by methyl 2-acetamido-2-deoxy-α-D-galactopyranoside and free 2-acetamido-2-deoxy-D-galactose (0- to 3-fold).

Fig. 4 depicts the EIA results with O RBC-derived Tn-active antigen as solid-phase that binds rodent MoAbs induced by the human antigens listed in the

Mouse							
O,Tn isolated antigen		*O,Tn RBC*		*Lung CA*		*Breast CA*	
6E6-H5-G9, IgMκ		*12H5-C9-G11, IgMκ*		*NCC-LU-81, IgMκ*		*B72.3, IgG_1*	
μg	*nmol*	*μg*	*nmol*	*μg*	*nmol*	*μg*	*nmol*
0.08	<0.0002	0.04	<0.0001	0.04	<0.0001	3	<0.005
0.02	≪0.0001	< 0.02	≪0.0001	0.04	<0.0001	10	0.02
n.d.[d]		>90		n.d.		~30	~2.5
n.d.		>90		n.d.		~30	~4
1	4	3	13	3	13	10	43
n.d.		*n.d.*		*n.d.*		15	44
9	41	12	54	12	54	30	136

teins; O RBC T antigen; AS-orosomucoid; β-D-Galp-(1→3)-D-Gal*p*NAc (had trace activity with B27.3), lactose, ManNAc, GlcNAc, Man, Glc, Gal (all D isomers). [c]Abbreviations: AS, asialo; GP, glycopeptide, for AS-glycoproteins and AS-glycopeptides, the mass was calculated on the basis of M_r of the parent compounds[9]. [d]Not done.

Fig. 4 legend. One hundred μL of antigen solution was used per well, and the data are presented as amount of antigen per mL. Binding of fixed concentrations of different MoAbs to 0.1–10 ng of RBC-Tn antigen gave similar sigmoidal curves, regardless of the origin of the immunogen used for their induction. The quantity of MoAb bound increased linearly from ~ 0.8 to ~ 5 ng of Tn antigen and then reached a plateau. In similar experiments, "naturally occurring" pools of human, polyclonal monospecific anti-Tn antibody that had been purified by affinity chromatography gave flatter binding curves (not shown in Fig. 4), whose linear portions were located beyond 5 ng of Tn antigen per well.

Some of the breast CA DU 4475-derived fractions listed in Table I were used as solid phase to compare their anti-Tn antibody binding capacity with that of RBC-Tn glycoproteins. By use of affinity-purified human anti-Tn, 50 ng of spent medium-derived CA Fraction II.1. (Table I), purified by PAGE, was as active as 1 ng RBC-Tn, and more active than any other breast CA-Tn glycoprotein tested. Fraction II.1., prior to preparative PAGE, has been used in all other analytical studies reported here. All rodent anti-Tn MoAbs exhibited lower binding activity towards these breast CA fractions than did the human anti-Tn Abs.

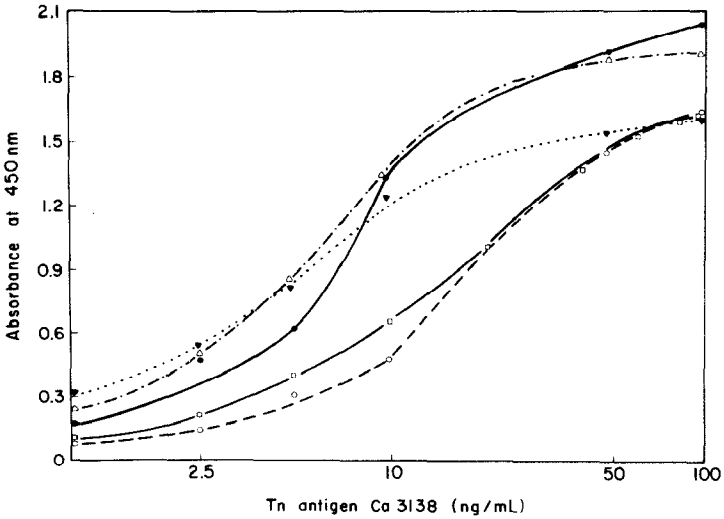

Fig. 4. Binding of rodent anti-human RBC- and CA-Tn-specific MoAbs to highly purified human O RBC-derived Tn antigen (100 μL/well) as determined by solid-phase immunoperoxidase assay, with TMB for color development. Ab designations and dilutions are given in parentheses. Immunogens used for MoAb induction: (●——●) desialylated ordinary O RBC (T9 3[C1]CTN2, 1:1500); (Δ—·—Δ) Tn antigen prepared from healthy O RBCs (6E6-H5-G9, 1:1000); (▼·····▼) intact O,Tn RBC (12A8-C7-F5, 1:2,500); (○— —○) lung CA LU65 membranes (NCC-LU-35, 1:500); and (□——□) breast CA metastasis membranes (B72.3, 1:2500).

DISCUSSION

The Tn epitope, α-D-Gal*p*NAc-(1→3)-Ser/Thr, is the most frequent direct "linker" of mucin-type heterosaccharide chains to proteins and peptides[14,16]. In nonCA tissues, it is occluded from an individual's own immune system. There are, however, two exceptions to this occlusion of Tn epitopes, both appear to be due to somatic mutation.

Firstly, immunoreactive Tn was present in >80% of the major human CAs studied so far; the Tn may be less frequent in kidney and some squamous CAs.

Our original observations[7,8] are apparently the first unequivocal demonstration of incomplete carbohydrate chains in human cancer cell membranes, and define Tn and T as universal ("pan") CA antigens that arise early on and persist throughout the CA's course[3,8,10,36,70,71]. Shortly thereafter, we pointed to their possible contribution to some of the specific properties of cancer cells[4,5,9] and the lethal effect of corresponding preexisting cytotoxic anti-T Abs[72] on CA cells *in vitro*.

Secondly, the appearance of Tn (but not T) is the most conspicuous phenomenon of the rare Tn syndrome, a disorder affecting the major cells of the hematopoietic system[11,12,22–26]. Sometimes, the Tn syndrome heralds Tn leukemia–lymphoma[28–31]. Phenomenology and some steps of pathogenesis of the Tn syndrome, and Tn leukemias, have been previously described[11,12,20,23–26,28–31]. However, little is known

of Tn's distribution, and its possible role in CAs[10,32,35,36]. One major reason for this paradox seems to be the considerable knowledge of the RBC plasmalemma in comparison to other cell membranes. T epitope location in the RBC cell membrane has been known for some time; Tn is the immediate precursor of T[1,2,58,73-75]. Clusters of occluded T epitopes are concentrated in the amino-terminal region of the RBC MN glycoproteins[9,74]; in the Tn syndrome, the promixate Gal residue has not been added by the appropriate glycosyltransferase[73,75].

Immunoreactive Tn epitopes are highly sensitive and specific immunohistopathologic markers that, on epithelia, permit the diagnosis, "CA." CA cell surface glycoproteins carrying Tn, occluded or uncovered, are likely to have considerable lateral mobility in the epithelial plasma membrane bilayers and microvilli[76,77], in contrast to the rigidity of such structures in RBC. Our work and that of others indicated that the uncovered Tn structures on CA cells are associated with fundamental functional changes observed in CA cells (*cf.* refs. 3,9,10).

We have uncovered three functions which should be of prime importance in advancing understanding of patient-CA interactions (*i.e.*, CA pathogenesis) at an early stage of the dyscrasia: (a) Tn-specific irreversible adhesion of CA cells to healthy epithelia. These adhesion events were specifically inhibited, prior to the irreversible steps, by minute quantities of Tn antigen. The extent of clustering of cell surface Tn epitopes is crucial for this adhesion[9]. (b) The strong circumstantial evidence that CA's surface Tn plays a decisive role in *in vivo* proliferation and metastasis, *i.e.*, a positive correlation between increased CA aggressiveness and density of Tn on CA cells[10,70,71]. These findings are very important for clinical prognosis and the choice of therapeutic approach[70,71]. We found support for these clinical observations in strongly invasive and metastatic sublines of two murine cancers that had much more Tn than the poorly invasive parent tumors[10]. (c) Autoimmunogenicity of blood cell Tn led to discovery of the Tn syndrome[11,20]. However, this important aspect of Tn in CA has not yet been investigated adequately. Recently, strong and specific cellular immune responses to Tn were demonstrated *in vitro* in breast CA patients by use of leukocyte-adherence inhibition[78].

The success of cellular and of humoral anti-Tn responses may be confined to early CAs before they show any clinical symptoms; Tn leukemias are usually of the acute type and kill rapidly[28,29]; likewise, many CAs on whose cell surfaces Tn epitopes predominate run a rapid course[3,10].

This is the first report, to our knowledge, on isolation, partial purification, physical and chemical analyses, and biological characterization of strictly Tn-specific glycoproteins from human breast CA and from ordinary O,MN RBC. Although the RBC T antigen used to prepare Tn-active antigen in these studies was of the highest activity[9], it was microheterogeneous owing partly to omission of the last major purification step of MN antigen (see ref. 46, Fig. 1).

For isolation and purification of Tn-active glycoprotein fractions from breast CA cells, we adopted methods used by others[44]. We obtained an adequate yield of cytoplasm, but not of cell membranes which were inextricably associated with

Dextran–PEG. The spent DU 4475 growth medium, which would be expected to contain preponderantly Tn-active substances shed from CA cell surface[67,68], yielded highly Tn-active material in modest quantity, possibly due to the difficulty in separating it from exorbitant amounts of FBS components. Table I shows that substances with similar high activities (Fractions I.3.A and I.4.B) were obtained from cytoplasm, and from the spent medium (Fraction II.1.).

In contrast to all other Tn-active fractions obtained from breast CA DU 4475, the fraction isolated from the spent medium had some blood group A_1 and A_2 activities; these were also found in uninoculated complete growth medium. Tn and A substances share the immunodominant α-D-Gal*p*NAc group[14,16], and HPA–agarose columns would be expected to bind any structure having a nonreducing α-D-Gal*p*NAc-terminal group. Cattle are known to possess water-soluble blood group A-active substances[79], and the observed A activities in the medium-derived Tn-active fraction are likely due to FBS.

Table I shows that, upon purification, the Tn activity of cytoplasm increased up to >10-fold on a dry weight basis (*e.g.*, cytoplasm I. to Fraction I.3.A). Remarkably, the total number of activity Us of cytoplasm-derived material increased upon fractionation and especially subfractionation: while cytoplasm, I., had 80 activity Us, the combined Subfractions I.3.A–C, and 4.A and B had 213 activity Us, *i.e.*, ~ 2.7 times the Us present in the starting material. These results may possibly be due to spatial rearrangement of the Tn-active groupings or more likely to removal of inhibitory substances during fractionation. On a dry weight basis, the recovery of active material was ~ 67%.

RBC-Tn glycoprotein (Table I, Fraction III.1) was obtained differently than the CA-Tn antigens, namely by enzymic removal of the β-D-Gal*p* group from T antigen. Measured with human anti-Tn it had 20 times the activity of the most active CA-Tn fraction (Table I; Fraction I.3.A); on a dry weight basis, the recovery of RBC-Tn was >90%.

The chemical similarity, Neu5Ac excepted, between CA- and RBC-Tn preparations is striking, and the amino acid composition is reminiscent of the human blood group A,B,H(O), and Le^a ovarian cyst glycoproteins (*cf.* ref. 14). However, definitive quantitative interpretations must await homogeneous Tn preparations.

The necessity for homogeneity of the Tn glycoproteins may not apply as strictly to the determination of biological activities as measured by semiquantitative hemagglutination inhibition tests, since the GalNAc contents were similar (for the Tn-active fractions assayed), and *N*-acetyl-α-D-galactosaminidase, free of any protease and of any other glycosidase, destroyed all anti-Tn Ab binding ability of the RBC Tn antigen (refs. 69 and 80).

The considerably lower *in vitro* activity of CA-Tn, compared to that of RBC-Tn, is not necessarily an indicator of the *in vivo* situation[59], because clustering, or polarization of Tn epitopes, on a macromolecular level is likely to occur in the cell membrane of CAs (see above) but not in RBC membranes.

Both Tn RBC, which has ~ 50% of ordinary RBC's Neu5Ac, and RBC-Tn, a

desialylated antigen, were powerful and reliable in eliciting anti-Tn-specific MoAbs. We present and compare herein, for the first time quantitative findings on anti-Tn elicited intentionally with Tn of RBC origin and unwittingly with CA-Tn.

Table III shows that the precondition for specific inhibition of Tn RBC agglutination is the presence of D-Gal*p*NAc groups, preferably in α-linked clusters, like in AS-OSM, and in the amino-terminal region of RBC-Tn (see also footnote [b], Table III). The first antibody listed in Table III is human polyclonal anti-Tn, purified by affinity from a pool of blood group A_1B donors' sera. By use of this "naturally occurring" anti-Tn as baseline in inhibition assay, the MoAbs may be categorized in two groups:

(*a*) The MoAbs whose inhibition pattern is closely similar to that of microbe-induced human anti-Tn, *i.e.*, the most active glycoprotein is AS-OSM and, among haptens, there is a 0–3-fold difference of inhibitory activity between 2-acetamido-2-deoxy-D-galactose and methyl 2-acetamido-2-deoxy-D-galactopyranoside . This group consists of "naturally occurring" human anti-Tn, the O,T RBC induced rat anti-Tn MoAb T9 2C[A6], and the breast CA-induced MoAb B72.3.

(*b*) Inhibition patterns closely akin to that of the RBC-Tn-induced MoAb, 6E6-H5-G9, *i.e.*, Tn antigen would inhibit as well as or better than AS-OSM, and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside would inhibit ~4–9 times better than 2-acetamido-2-deoxy-D-galactose. The O,Tn RBC-induced MoAb, 12H5-C9-G11, and the human lung CA-induced NCC-LU-81 also belong to this category.

Thus, there is a difference in antibody-combining site between the two types of anti-Tn Abs described. The type (*b*) Abs distinguished GalNAc better from its methyl α-D-glycopyranoside, and also react preferentially with the Tn antigen, whose epitope clusters, although dense, have free interspaces[74] in contrast to those of AS-OSM[81].

In addition, B72.3 (category *a*) was among the two Abs least inhibitable by Tn and AS-OSM (Table III). Also, some breast CAs adsorbed less B72.3 than any other anti-Tn Abs[10], and immunohistochemically healthy tissues reacted more readily with B72.3 than with other anti-Tn[82]. This suggests a larger antibody-combining site for B72.3 which may require, for optimal reactivity, an additional structure adjoining α-D-Gal*p*NAc residue that may augment binding. B72.3 was relatively weakly inhibited by Tn haptens, and it reacted equally well with C.L. and C.C. Tn RBC before and after maximal removal of Neu5Ac by neuraminidase (*cf.* 40).

The EIA results, depicted in Fig. 4, showed the very similar binding of rodent MoAbs to RBC-Tn antigen, regardless of the immunogen used. It may be possible to distinguish between these MoAbs by EIAs using other Tn-active substances as solid phase, and also by inhibition of binding observed in EIAs presented here. B72.3, the only IgG Ab, was also the only ascites-grown MoAb used; its binding curve and dilution range was akin to those of other MoAbs (culture supernatants), suggesting lower affinity of B72.3 for RBC-Tn; the flatter binding curves with affinity-purified human polyclonal anti-Tn may be similarly explained. Human anti-Tn bound more readily to the CA-Tn fractions than any of the anti-Tn MoAbs

studied, indicating the latters' greater rigidity in recognizing differences in spatial orientation of the Tn epitopes, as we have reported earlier in immune adsorption studies[10].

ACKNOWLEDGMENTS

The authors are grateful to Drs. J. Distler and G. Jourdian for highly purified bovine testis β-D-galactosidase; Drs. S. Hirohashi, H. Clausen, and S.-I. Hakomori for donation of MoAbs NCC-LU-35 and NCC-LU-81; and Drs. J. Schlom and D. Colcher for MoAb B72.3 and for permission to publish the results. They are most grateful to Dr. M. van der Rest for the analysis of amino acids and hexosamines with an amino acid analyzer, and to Ms. Jean Severin and Mr. C. Grannum for expert technical assistance.

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