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Specificity of Reagents Directed to the Thomsen-Friedenreich Antigen and Their Capacity to Bind to the Surface of Human Carcinoma Cell Lines[1]

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ABSTRACT

The Thomsen-Friedenreich antigen (T-antigen) is a cryptic disaccharide structure on human erythrocytes and is supposed to be expressed in an unhidden form on carcinoma cells. We tested the ability of four anti-T reagents (*i.e.*, peanut agglutinin, human and rabbit anti-T antisera, and monoclonal anti-T antibodies) to agglutinate neuraminidase treated human erythrocytes and compared their capacity to bind to the surface of human carcinoma cell lines or neuraminidase treated lymphocytes. We found that all of the reagents strongly agglutinated neuraminidase treated erythrocytes. In contrast, only peanut agglutinin and the monoclonal antibody bound to the surface of carcinoma cell lines and to neuraminidase treated lymphocytes. Peanut agglutinin was inhibited by D-galactose and is known to be specific for the T-disaccharide. The determinants on erythrocytes, lymphocytes, and carcinoma cells, recognized by peanut agglutinin, are resistant to trypsin treatment. The monoclonal antibody was specifically inhibited by phenyl-β-D-galactoside. The binding sites on erythrocytes and lymphocytes for the monoclonal antibody can be removed by treatment with trypsin or Pronase. On the other hand, the binding sites on carcinoma cells are resistant to trypsin but can be removed with Pronase. In contrast to the peanut agglutinin binding sites on carcinoma cells the structures recognized by the monoclonal antibody cannot be further increased by neuraminidase treatment. Human and rabbit anti-T antisera did not bind to tumor cell surface or to neuraminidase treated lymphocytes. Hemagglutination of human anti-T could be inhibited by asialofetuin; the specificity of rabbit anti-T could not be established in this study. Hemagglutination with both antisera is resistant to trypsin but partially sensitive to Pronase treatment.

These results indicate that each of the reagents has a distinct specificity and recognizes different antigenic determinants on different molecules. Only peanut agglutinin and monoclonal anti-T antibodies are able to detect common structures on the surface of neuraminidase treated erythrocytes and carcinoma cell lines.

INTRODUCTION

The T-antigen[3] has been described as a cryptic determinant on human erythrocytes, which can be exposed by neuraminidase treatment (1). The reactive structure was found to be a carbohydrate precursor of the glycophorin A molecule on erythrocytes, which normally determines the blood group specificity of the M/N-system (2, 3). The sugar component of the T-antigen consists of the disaccharide β-D-galactosyl-(1–3)-α-*N*-acetyl-D-galactosamine (1). The immunodominant structure is located near the amino-terminus of glycophorin A and can be separated by trypsin (3, 4).

The T-antigen is supposed to be specifically expressed on carcinoma cells in an unhidden form (4). Its expression is accompanied by a depressed titer of naturally occurring anti-T antibodies and the development of a delayed-type hypersensitivity-like response following the injection of crude extracts of NDA-E (4–6). The reason why the tumor still can develop is not known. Moreover, an important role of the T-antigen in organotrophic metastasis was suggested recently (1, 8–10). For the characterization and isolation of the relevant structure on tumor cells it is therefore of interest to find reagents which specifically detect this determinant. Until now several anti-T reagents were used, including PNA, naturally occurring human anti-T antibodies, and immune rabbit anti-T antisera (10–13). Recently, monoclonal antibodies were produced against NDA-E, which resulted in two antibodies, one with high affinity for the synthetic T-disaccharide and one with high affinity for phenyl-β-D-galactoside. Only the latter was found to bind to tumor cells (14, 15).

We examined the specificity of these reagents in the hemagglutination inhibition assay and compared their capacity to bind to the surface of carcinoma cell lines. We found a heterogeneity regarding the specificity of the reagents, their tumor binding capacity, and the molecules expressing the different binding sites.

MATERIALS AND METHODS

Cells. Human carcinoma cell lines derived from breast (BT-20, MCF-7, CAMA), colon (SW-1222, SW-837), bladder (T-24, TCCSUP), lung (SK-LC-4), and ovary (2774) tissue were kindly provided by Dr. H. J. Feickert (Zentrum fuer Kinderheilkunde, Medizinische Hochschule, Hannover, Federal Republic of Germany). Cells were grown on microscope slides (in Petri dishes) in minimal essential medium (with Earle's salts) supplemented with 100 units of penicillin-streptomycin (100 μg/ml), 2 mM glutamine, 1% (v/v) non-essential amino acids, and 10% heat-inactivated fetal calf serum (Gibco, Glasgow, Scotland) for 3 to 4 days at 37°C in a humidified incubator containing 5% CO_2. All cell lines were free of *Mycoplasma*. Human lymphocytes were prepared from peripheral blood of different healthy donors by Ficoll-Hypaque density centrifugation. Human O, MM erythrocytes were drawn in heparin and washed three times in saline.

Enzyme Treatment of Cells. *Vibrio cholerae* neuraminidase was obtained from Behringwerke, Marburg/Lahn, Federal Republic of Germany. Erythrocytes were suspended as 10% (v/v) in saline containing 1 mM $CaCl_2$ and incubated with NDA (0.1 units/ml) for 2 h at 37°C with occasional shaking. Lymphocytes (10^7/ml) were incubated with NDA (0.02 units/ml), and carcinoma cells were incubated with NDA 0.02 units/slide. Cells were washed extensively after enzyme treatment.

Trypsin and Pronase Treatment. Cells were incubated with an 0.125% (w/v) solution of the enzyme in saline for 5 min at room temperature, followed by washing three times.

Antisera and Immunofluorescence. MAb anti-T (49H8), produced by immunizing mice with human NDA-E (14), was obtained from Dakopatts, Glostrup, Denmark. Cells were incubated with MAb at a dilution of 1:40, followed by an incubation with rabbit anti-mouse immunoglobulins (Dakopatts) and finally with fluorescein-conjugated swine anti-rabbit immunoglobulins (Dakopatts). Human anti-T antibodies were individually prepared from the serum of 15 different healthy donors by enrichment on NDA-E (O, MM) (16). Briefly, serum was incubated for 1 h at 4°C with untreated erythrocytes to absorb antibodies directed against normal blood group antigens. Thereafter, the serum was incubated for 1 h at 4°C with NDA-E. Erythrocytes were washed 3 times, and the adsorbed antibodies were eluted by incubation for 1 h at 37°C on a rocking plate. This procedure resulted in a 10-fold enrichment of hemagglutinating activity. Cells were incubated with 1:10 diluted hu-

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[3] The abbreviations used are: T-antigen, Thomsen-Friedenreich antigen; PNA, peanut agglutinin; MAb, monoclonal antibody; NDA, neuraminidase; NDA-E, neuraminidase treated erythrocytes.

man anti-T antibodies and then with fluorescein-conjugated rabbit anti-human immunoglobulins (Miles Scientific, Munich, Federal Republic of Germany). Rabbit anti-T antibodies were prepared by immunizing rabbits with human NDA-E (O, MM). Injections of 5×10^9 NDA-E were given in the neck and intramuscularly in the hind leg. This procedure was repeated twice in a 3-week interval. Alternatively, rabbits were immunized using the protocol of Öerntoft *et al.* (12) with 5×10^9 NDA-E i.p. at day 1 and i.v. at days 3, 5, and 7. All rabbit antisera were absorbed three times on normal human erythrocytes to eliminate antibodies directed to normal structures on erythrocytes. Incubation of cells with antisera was performed at 4°C for 30 min. Cells were washed twice in saline after each incubation step. Fluorescein-conjugated PNA (Sigma, Munich, Federal Republic of Germany) was diluted 1:20 in saline and incubated with the cells for 45 min at 4°C.

The intensity of the fluorescent stain was scored by two of the authors: 0, no staining; +, weak; ++, moderate; +++, strong; ++++, very strong staining of most of the cells.

Hemagglutination Assay. Hemagglutination of human NDA-E (O, MM) was performed in round bottom microtiter plates. PNA or antisera were serially diluted (30 μl) in saline and mixed with 30 μl of inhibitors or saline. Finally, 10 μl of a 10% erythrocyte suspension were added. The titer was determined independently by two of the authors after 1 h of incubation at room temperature.

RESULTS

Specificity of Anti-T Reagents. All of the four anti-T reagents strongly agglutinated NDA-E but not normal erythrocytes (Table 1). Hemagglutination-inhibition experiments revealed that the MAb can specifically be inhibited with phenyl-β-D-galactoside (and its amino- and nitro-derivates). For recognition a β-linked galactosyl-compound seems to be important, because α-linked galactosyl-compounds were ineffective inhibitors. β-linked glucosyl-compounds also showed no inhibition of hemagglutination (Table 2). Phenyl-β-D-galactoside did not influence the hemagglutination of the other reagents. PNA could be inhibited by a high concentration of D-galactose. Human anti-T was specifically inhibited by asialofetuin but not by any of the single sugar compounds tested. A significant specificity of rabbit anti-T could not be established in this study.

Table 1 *Hemagglutination titer of anti-T reagents after treatment with different enzymes*

Anti-T reagents	Enzyme treatment of erythrocytes			
	None	NDA	NDA/trypsin	NDA/Pronase
None	0[a]	0	0	0
MAb	0	2,048	0	0
PNA	0	6,400	25,600	51,200
Human anti-T	0	128	128	32
Rabbit anti-T	0	256	256	64

[a] Reciprocal titer of hemagglutination.

Table 2 *Fine specificity of anti-T reagents*

Inhibitors (2 mg/ml)	Anti-T reagents				
	None	MAb	PNA	Human anti-T	Rabbit anti-T
None	0	2,048[a]	800	64	256
p-Nitrophenyl-β-D-galactoside	0	0	800	32	128
p-Nitrophenyl-α-D-galactoside	0	2,048	800	32	128
Phenyl-β-D-galactoside	0	0	800	32	128
p-Nitrophenyl-β-D-glucoside	0	2,048	800	32	256
p-Nitrophenyl-α-D-glucoside	0	2,048	800	32	256
p-Aminophenyl-β-D-galactoside	0	0	800	32	128
D-Galactose	0	2,048	400	64	256
D-Galactose (16 mg/ml)	0	2,048	200	64	256
D-Glucose	0	2,048	800	64	256
D-Fucose	0	2,048	800	64	256
N-Acetyl-galactosamine	0	2,048	800	64	256
Asialofetuin (10 mg/ml)	0	2,048	800	8	256

[a] Reciprocal titer of hemagglutination.

Removal of Binding Sites by Enzyme Treatment. The binding determinants for the MAb could be removed from erythrocytes with trypsin. This procedure did not affect the hemagglutination with the other anti-T reagents; it even increased the hemagglutination of PNA. Incubation of NDA-E with Pronase also completely removed the binding structure for the MAb and did not affect binding of PNA. The binding sites for human and rabbit anti-T were partially removed by Pronase treatment (Table 1). As a control for non-transformed, nucleated cells we included human lymphocytes. None of the anti-T reagents significantly bound to normal lymphocytes (Table 3). After treatment with NDA only the MAb and PNA showed strong binding to the lymphocyte surface. The lack of human and rabbit anti-T binding was not altered by NDA-treatment of lymphocytes. The cryptic structure on lymphocytes recognized by MAb behaved like the cryptic structure on erythrocytes with respect to trypsin and Pronase sensitivity (Tables 1 and 3). The PNA binding structures differed in their Pronase sensitivity.

Binding of Anti-T Reagents to Carcinoma Cells. From the tested anti-T reagents only the MAb and PNA were found to bind to the surface of several carcinoma cell lines. PNA bound to a more or less degree to all cell lines, whereas MAb could detect binding sites only on certain cell lines (Table 4). In all cases neuraminidase uncovered more determinants for PNA. The staining intensity with MAb was unchanged after treatment of tumor cells with NDA.

Comparison of the Binding Sites on Carcinoma Cell Lines Detected by PNA and MAb. We chose the cell line with the highest binding capacity for MAb and compared the sensitivity of the MAb and PNA binding determinant to trypsin and Pronase (Table 5). The determinant for the MAb on carcinoma cells was resistant to trypsin treatment in contrast to the cryptic determinant on erythrocytes and lymphocytes. There was no difference in the sensitivity to Pronase between the cryptic and non-cryptic MAb binding structures (Tables 1, 3, and 5). Both structures to which PNA binds were found to be resistant to trypsin treatment. In contrast to the determinants on erythrocytes, the PNA binding structure on carcinoma cells and lymphocytes was reduced after Pronase treatment.

DISCUSSION

A commonly used assay for anti-T activity is the hemagglutination of neuraminidase-treated erythrocytes. All of the four anti-T reagents tested in this study strongly agglutinated NDA-E. Hemagglutination inhibition experiments, on the other hand, showed that each of the reagents has an individual specificity and therefore recognizes independent structures on erythrocytes after treatment with NDA. The MAb and PNA showed a strong carbohydrate specificity, whereas human and rabbit anti-T may be directed against non-carbohydrate determinants. The fine specificity remains to be elucidated. Treatment of erythrocytes with trypsin or Pronase revealed that the antigenic determinants recognized by the anti-T reagents also seem to be expressed on different molecules. Molecules carrying the binding site for

Table 3 *Binding of anti-T reagents to lymphocytes after treatment with different enzymes*

Anti-T reagents	Enzyme treatment			
	None	NDA	NDA/trypsin	NDA/Pronase
MAb	0[a]	+++	0	0
PNA	+/−	++++	++++	++
Human anti-T	+/−	0	0	0
Rabbit anti-T	0	0	0	0

[a] Intensity of fluorescent stain (see "Materials and Methods").

Table 4 *Comparison of anti-T reagent binding to normal and NDA-treated carcinoma cell lines*

	Anti-T reagents							
	PNA		MAb		Human anti-T		Rabbit anti-T	
Cell lines	−NDA	+NDA	−NDA	+NDA	−NDA	+NDA	−NDA	+NDA
BT-20 (breast)	+++[a]	++++	+	+	0	0	0	0
MCF-7 (breast)	+++	++++	++	++	0	0	0	0
CAMA (breast)	++++	++++	+++	+++	0	0	0	0
SW-1222 (colon)	+	++++	0	0	0	0	0	0
SW-837 (colon)	+	++++	0	0	0	0	0	0
T-24 (bladder)	+	++++	0	0	0	0	0	0
TCCSUP (bladder)	++	++++	+	+	0	0	0	0
SK-LC-4 (lung)	++	++++	+	+	0	0	0	0
2774 (ovary)	++	++++	0	0	0	0	0	0

[a] Intensity of fluorescent stain (see "Materials and Methods").

Table 5 *Binding of anti-T reagents to carcinoma cells (CAMA) after trypsin or Pronase treatment*

	Enzyme treatment		
Anti-T reagents	None	Trypsin	Pronase
MAb	+++[a]	+++	0
PNA	++++	++++	++
Human anti-T	0	0	0
Rabbit anti-T	0	0	0

[a] Intensity of fluorescent stain.

MAb were found to be sensitive to trypsin, in contrast to the binding sites recognized by all other reagents. MAb binding was also completely absent after treatment of NDA-E with Pronase. Human or rabbit anti-T binding was only partially affected by this treatment. PNA binding determinants were increased after trypsin treatment and were even more pronounced after Pronase treatment. Since glycophorin A is the most frequent glycoprotein on erythrocytes and its aminoterminal part, which carries the T-disaccharide, can be removed from erythrocytes by trypsin (20, 21), it is most likely that the MAb recognizes a structure on the desialyzed form of glycophorin A. The T-disaccharide, recognized by PNA, seems to be expressed on a variety of molecules, since PNA binding is enormously increased after trypsin or Pronase treatment.

The expression of T-antigen in an unhidden form on carcinoma cells was originally suggested by Springer *et al.* (4). Several reports confirmed or questioned these findings using different anti-T reagents (5, 6, 10–13, 19). We compared the capacity of the four anti-T reagents to bind to the surface of several carcinoma cell lines. Only the MAb and PNA reacted with carcinoma cells. Both reagents also bound to NDA-treated lymphocytes, which served as a source of non-transformed, nucleated cells. We could not detect any binding using naturally occurring human anti-T antibodies or immune rabbit anti-T, although we enriched human anti-T antibodies on NDA-E and produced rabbit anti-T antisera by different routes of immunization. This is in contrast to the findings of others, who could absorb human or rabbit anti-T on tumor tissue (4, 5) or tissue sections (10–13). It is in agreement with a recent report in which no difference of absorption of human anti-T on normal and cancerous tissue was found (19). This discrepancy could be the result of methodological differences. Tissue sections or homogenized tissue contain membrane structures as well as cytoplasmic structures, whereas vital cells expose membrane structures only. Binding sites for human or rabbit anti-T might be located within the cytoplasm and can therefore not be detected on intact cell membranes in our assays. It is unlikely that the enrichment procedure of human anti-T caused an artificial selection of antibodies because such antibody preparations were shown to react specifically with tumor tissue sections (11). Therefore, the relevance of these antisera as tumor-specific reagents needs further investigation.

PNA was found to bind to a more or less extent to all carcinoma cell lines tested. Weak binding could always be increased by prior treatment of cells with NDA. In contrast, MAb bound only to certain cell lines, and binding intensity was unchanged after NDA treatment. This may indicate that the PNA binding determinants on tumor cells exist only partially in an unhidden form. In contrast to erythrocytes and lymphocytes, MAb binding sites on carcinoma cells are completely resistant to NDA treatment. Together with the findings that the MAb binding sites on carcinoma cells cannot be uncovered by NDA and that the MAb binding sites on carcinoma and lymphocytes show different sensitivities to trypsin treatment, this suggests that the MAb binding site on carcinoma cells might not be the unhidden form of the normally cryptic binding site on lymphocytes. The inability to detect MAb binding sites on all carcinoma cell lines could reflect a tumor development-dependent expression of this antigen or a specific expression on certain carcinomas. There is one report of a second anti-T monoclonal antibody with high affinity for the T-disaccharide, which does not bind to carcinoma cells (14, 15) and therefore contrasts the binding of PNA with the same specificity. One explanation might be that the specificity of PNA is directed to the α- and β-configuration of gal(1–3)galNac as well as to galactose alone (17, 18), whereas this MAb recognizes the sugar structure in combination with a protein backbone. We conclude from these results that each of the anti-T reagents recognizes different determinants on different molecules, which are either expressed on the surface or within the cytoplasm of tumor cells. Only PNA and the MAb detect structures on the surface of carcinoma cells. These determinants are also present on erythrocytes in a cryptic form. The nature of the molecule carrying these determinants remains to be eludicated.

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