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The Monoclonal Antibody, Anti-Asialoglycophorin from Human Erythrocytes, Specific for β-D-Gal-1-3 -α-D-GalNAc-Chains (Thomsen-Friedenreich Receptors)

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The monoclonal antibody 22.19 of IgM class obtained after immunization of BALB/c mice with asialoglycophorin of human erythrocyte membranes is described. The specificity of this antibody for β-D-Gal-1-3-α-D-GalNAc- disaccharide chains (Thomsen-Friedenreich receptors) was established by studying its reactivity against various erythrocytes, glycoproteins and oligosaccharides and by comparison with two lectins, peanut agglutinin and *Vicia graminea* lectin, which recognize these disaccharide chains.

The sialylated β-D-Gal-1-3-α-D-GalNAc-*O*-glycosidic chains are present in many glycoproteins [1]. Glycophorins of human and some animal erythrocytes contain multiple chains of this type [2-5]. Vertebrate sera contain common antibodies agglutinating neuraminidase-treated red cells. The cryptic structures recognized by these antibodies, called Thomsen-Friedenreich receptors (T or TF receptors), were identified as the above mentioned disaccharide chains which are uncovered by desialylation [6]. These disaccharide chains are also receptors for peanut agglutinin (PNA), which is also called anti-T lectin for this reason [7, 8]. The unsubstituted Galβ1-3GalNAc- chains occur rarely, but have been found on some cancer cells [9, 10] and subsets of immature lymphocytes [11]. In this respect they are interesting as markers of cell differentiation and malignant transformation, which has awakened interest in reagents suitable for recognition of these structures. So far, PNA has been used widely [11], since its main specificity is the Galβ1-3GalNAc- structure, but this lectin also reacts, albeit more weakly, with other oligosaccharide chains terminated with galactose residues [8, 12].

Abbreviations: PNA, peanut agglutinin; VgL, *Vicia graminea* lectin; TF, Thomsen-Friedenreich; HSA, human serum albumin; MoAb, monoclonal antibody.

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The commonly available anti-TF agglutinins are present in sera at low concentrations and represent a wide spectrum of polyclonal antibodies which may differ in their fine specificity. Two monoclonal anti-TF antibodies obtained recently by immunization of mice with asialoerythrocytes differed in their reactions with low molecular weight haptens and cells [13, 14], which confirmed that specificity differences can exist in reagents of this type. Therefore, the application of monoclonal anti-TF antibodies should be not only useful for the detection of Galβ1-3GalNAc- chains but also should provide more detailed information about the antigens carrying these disaccharides.

In this report we describe a monoclonal antibody obtained after immunization of mice with asialoglycophorin from human erythrocyte membranes. The evidence is presented that this antibody is specific for desialylated *O*-glycosidic chains of glycophorin, i.e. TF receptors.

Materials and Methods

Erythrocytes

Human blood group MM and NN, horse, dog, guinea pig, porcine, rabbit, murine and bovine blood samples were collected into an anticoagulant solution and stored at 4°C for up to several days. Erythrocytes were washed and treated with neuraminidase directly before use.

Glycoproteins and Glycopeptides

Sialoglycoproteins (glycophorins) were obtained from erythrocyte membranes by the selective extraction with 45% aqueous phenol at 65°C [15]. The aqueous phase was exhaustively dialyzed against water, concentrated by lyophilization, centrifuged at 30 000 × *g* for 30 min, and lyophilized. The term glycophorin without more detailed specification is used for the unfractionated preparations which usually are mixtures of two or more glycophorins of a given species. The tryptic glycopeptides T1/T2 and T3 (amino acid residues 1-30/31 + 1-39, and 40-61, respectively) and CNBr-fragments (amino acid residues 1-8, and 9-30/31 + 9-39) of human glycophorin A were prepared as described previously [16-18]. Epiglycanin from TA3-Ha murine ascites tumor cells [10] was kindly provided by Dr. J.F. Codington (Boston, MA). Orosomucoid (α_1-acid glycoprotein) was a product of Calbiochem AG (Lucerne, Switzerland).

Oligosaccharides and Monosaccharides

The disaccharide β-D-Gal-1-3-D-GalNAc was obtained from products of digestion of T1/T2 glycopeptide of glycophorin A with *Diplococcus pneumoniae* glycosidases [19]. The disaccharide was separated from other sugars by Bio-Gel P-4 (-400 mesh) chromatography (Bio-Rad, Richmond, CA, USA), preparative high voltage paper electrophoresis and Sephadex G-10 gel filtration (Pharmacia, Uppsala, Sweden) as previously described [19, 20]. Lactose was from Sigma Chemical Co., St. Louis, MO, USA; galactose from Serva, Heidelberg, W. Germany; and *N*-acetylgalactosamine from Koch-Light, Colnbrook, UK.

Desialylation

Erythrocytes (0.4 ml 50% suspension in 10 mM sodium barbital, 0.15 M NaCl, 4 mM $CaCl_2$, pH 7.0) were treated with 30 μl (3×10^{-3} U) neuraminidase from *Vibrio cholerae* (Serva) for 1 h at 37°C. Glycoproteins and glycopeptides were desialylated by hydrolysis of 0.5 - 1.0% solutions in 0.025 M sulfuric acid for 4 h at 60°C [21], followed by neutralization, dialysis and lyophilization.

Immunization and Production of Hybridomas

BALB/c-J Boy/JIW mice, 6-8 weeks old, were injected subcutaneously with 100 μg human asialoglycophorin in complete Freund adjuvant (Difco Labs., Detroit, MI, USA). Three booster injections (100 μg) were given intraperitoneally at weekly intervals after disappearance of local subcutaneous swelling, and were followed by one intravenous injection of 100 μg asialoglycophorin. Three days after the last injection, the fusion of spleen cells from immunized mice with SP2/O-Ag14 hybrid plasmacytoma cells was performed according to the method of Köhler and Milstein [22], as modified by Dippold *et al.* [23]. The fused cells were grown in selective HAT (hypoxanthine-aminopterin-thymidine) medium in tissue culture plates (Costar, Cambridge, MA, USA; No. 3524, 24 wells/plate) at a density of 1.6×10^6 cells/well (1 ml), without a feeder layer. The samples of medium from wells with growing clones were screened for hemagglutination of untreated and neuraminidase-treated human erythrocytes. Cells from wells producing antibody specific for asialoerythrocytes were subcloned at least three times by limiting dilution on a feeder layer of $1\text{-}3 \times 10^5$ mouse peritoneal cells in 96-well tissue culture plates (Costar No. 3596). Selected clones were expanded *in vitro*, and then grown intraperitoneally in BALB/c mice. Sera of ascites fluids from these mice and hybridoma cells after each step of the procedure were stored in liquid nitrogen.

The immunoglobulin class of the monoclonal antibodies was determined by double diffusion in agarose gel, using monospecific antimouse-(Ig heavy chains) sera, purchased from Bionetics (Kensington, MD, USA).

Lectins

PNA and *Vicia graminea* lectin (VgL) were purified by affinity chromatography on immobilized human asialoglycophorin, as described previously [24-26].

Hemagglutination and Hemagglutination Inhibition

Agglutination of erythrocytes by monoclonal antibodies and lectins was assayed at 20°C on U-well microplates in phosphate buffered saline (0.075 M sodium phosphate buffer, pH 7.4 and 0.075 M NaCl; PBS) containing 0.1% human serum albumin (HSA). Serially diluted 20 μl samples of agglutinin were mixed with 20 μl 2% erythrocyte suspension and agglutination was read after 30 min (antibodies) or 1 h (lectins). For hemagglutination-inhibition studies, 20 μl portions of antibody or lectin diluted to the titer 1/4 - 1/8 were incubated with 20 μl samples of serially diluted inhibitor for 30 min, then 20 μl of a 2% suspension of human asialoerythrocytes were added to each well and agglutination was read as described above. The inhibitory activity is expressed as the minimal

concentration of inhibitor required to inhibit the hemagglutinating activity of an equal volume of antibody or lectin solution of the titer given above.

Radioiodination of Lectins and Anti-mouse IgM Antibody

Iodination was performed by the Iodogen method [27]. The PNA or VgL solution (60 μg/400 μl PBS) was incubated in a tube coated with 20 μg Iodogen (Sigma) with 0.5 mCi $Na^{125}I$ (Center for Production and Distribution of Isotopes, Poland) for 20 min in an ice-bath, with shaking. The excess iodine was separated by placing the sample on the Sephadex G-25 (coarse) minicolumn (Pharmacia) at a concentration of 100 μl sample/ml Sephadex and centrifuging for 1 min at 650 $\times$ *g*. The labeled lectins present in the eluates were additionally purified by affinity chromatography on Concanavalin A-Sepharose (for VgL) [24] or asialoglycophorin A-Sepharose (for PNA) [28]. The agglutinating activity of the radiolabeled lectins was unchanged and the total radioactivity was 4×10^7 cpm (Beckman Gamma 9000 counter).

Goat anti-mouse IgM antibody (Kirkegaard and Perry Labs., Gaithersburg, MD, USA) was dissolved in 0.3 M sodium phosphate buffer pH 7.4 (100 μg/400 μl) and incubated with 1 mCi $Na^{125}I$ in a tube coated with 20 μg Iodogen for 10 min in an ice-bath with shaking. The labeled antibody was separated from the excess iodine on the Sephadex G-25 minicolumn as described above. The total radioactivity bound to antibody was 3.8×10^8 cpm.

Identification of Erythrocyte Membrane Electrophoretic Fractions Reacting with Monoclonal Antibody and Lectins

Erythrocyte membranes were dissolved in 0.2% sodium dodecyl sulfate and aliquots corresponding to 10 μg membrane neutral sugars [29] were fractionated by electrophoresis in 10% polyacrylamide in the system of Laemmli [30]. Electrophoretic transfer of membrane fractions to nitrocellulose (Schleicher-Schuell BA85, Dassel, W. Germany) was performed under the conditions reported by Towbin *et al.* [31], using a current density of 6.5 mA/cm^2 for 6 h. The nitrocellulose sheets (4.5 $\times$ 8 cm) were washed with PBS, and non-specific protein-binding sites were blocked by incubating for 2 h at 40°C with PBS containing 5% HSA. The sheets were then incubated for 12 h at 37°C in 20 ml PBS containing 0.1% HSA, 0.02% NaN_3 and 0.02 U *Vibrio cholerae* neuraminidase in order to desialylate the glycoproteins, followed by washing three times with PBS containing 0.1% HSA and 0.05% Tween 80.

For detection of lectin-binding components, the nitrocellulose sheets were incubated for 12 h at 20°C in 25 ml PBS containing 5% HSA, 0.05% Tween 80 and radiolabeled PNA or VgL (1.7×10^7 cpm/25 ml). In order to detect the components reacting with monoclonal antibody, the sheets were reacted under the same conditions with ascitic fluid diluted 1:100 with PBS-5% HSA-0.05% Tween. This was followed by three washings with PBS-0.05% Tween and by incubation with the radiolabeled goat antibody against murine IgM (1.4×10^7 cpm/25 ml PBS-5% HSA-0.05% Tween) for 6 h at 20°C. The sheets overlayed with lectins or antibodies were washed five times for 1 h with PBS-0.05% Tween, dried between celophane sheets and autoradiographed at -20°C, using Kodak X-OMAT AR film.

Results

The fusion of spleen cells from mice immunized with asialoglycophorin with SP2/O-Ag14 plasmacytoma cells resulted in growing hybridoma clones in 96 out of 144 wells. The media from eight wells agglutinated both untreated and desialylated human erythrocytes, whereas in two wells only the agglutination of asialoerythrocytes was observed. The cells from one of these two wells were subcloned twice and a cell line (BM 22.19) producing agglutinins specific for asialoerythrocytes was established. This line was submitted to two further subclonings and two hybridoma sublines (BM 22.19.20.7 and BM 22.19.14.34) were obtained which produced IgM agglutinins with identical properties. Therefore, the results obtained with the monoclonal antibody (MoAb) produced by the BM 22.19.20.7 cell line, subsequently designated 22.19, are presented.

The ascitic fluid containing MoAb 22.19 agglutinated human asialoerythrocytes at the titer 1/30 000 - 1/60 000, but did not agglutinate untreated erythrocytes. Hemagglutination could be inhibited by human asialoglycophorin, which indicated that MoAb 22.19 reacts with an epitope containing desialylated oligosaccharide chains of glycophorin.

In order to elucidate whether MoAb 22.19 is specific for a particular glycosylated fragment of human asialoglycophorin or only for the *O*-glycosidic disaccharide chains, the antibody was tested against a panel of animal erythrocytes and various well characterized glycoproteins. The red cells selected for these experiments have glycophorins with different *O*-glycosidic chains. Horse, dog and rabbit erythrocyte glycophorins contain sialylated Galβ1-3GalNAc- chains [4, 5, 32], whereas porcine and bovine erythrocyte glycophorins have more complex, GlcNAc-containing *O*-glycosidic chains [33, 34]. Murine (BALB/c) erythrocytes contain at least two glycophorins [35, 36] carrying sialylated Galβ1-3GalNAc- chains (H. Krotkiewski *et al.*, unpublished results). However, it is difficult to desialylate erythrocytes of BALB/c mice because they containmainly the 9-*O*-acetyl derivative of neuraminic acid [37] which is not released by commonly used neuraminidases [38]. For this reason only murine glycophorin desialylated by mild acid hydrolysis was tested.

The activity of MoAb 22.19 was compared with that of PNA and VgL, both of which are lectins specific for Galβ1-3GalNAc- chains [8, 12, 39]. The latter lectin has a more complex specificity, reacting with glycoproteins having clusters of the disaccharide chains. The reaction is strongly enhanced by an adjacent hydrophobic residue, the presence of which diminishes the interaction of VgL with sialylated glycoproteins [39]. Because of this hydrophobic interaction VgL shows anti-N specificity, since glycophorin A of blood group N has a hydrophobic Leu residue at position 1 of the polypeptide chain (the cluster of 3 disaccharide chains is located on amino acid residues 2, 3 and 4), instead of the hydrophilic Ser residue found in M glycoprotein [39].

MoAb 22.19 agglutinated human asialoerythrocytes, independent of MM or NN blood group. Horse, dog and rabbit asialoerythrocytes were also agglutinated (the latter weakly) but porcine and bovine asialoerythrocytes were not agglutinated, nor were any of the untreated cells (Table 1). This showed that the agglutinating activity of MoAb 22.19 is correlated with the presence of the disaccharide chains (TF receptors) on the cell surface. Moreover, MoAb 22.19 showed selectivity in agglutination similar to PNA and VgL.

Table 1. Agglutination of human and animal erythrocytes by the MoAb 22.19, PNA and VgL.

Erythrocytes		Presence of TF receptors	Agglutinating titer		
			MoAb 22.19	PNA	VgL
Human O NN	u[a]		—[c]	—	1/128
	d[b]	yes	1/500	1/250	1/250
Human O MM	u		—	—	1/16
	d	yes	1/500	1/250	1/128
Horse	u		—	—	1/1000
	d	yes	1/1000	1/2000	1/16000
Dog	u		—	—	1/32
	d	yes	1/128	1/128	1/250
Guinea pig	u		—	—	—
	d	unknown	1/64	1/128	1/256
Rabbit	u		—	1/4	—
	d	yes	1/8	1/64	1/8
Porcine	u		—	1/1	—
	d	no	—	1/32	—
Bovine	u		—	—	—
	d	no	—	—	—

[a] Untreated.
[b] Desialylated.
[c] Lack of agglutination.

The structure of the oligosaccharide chains of guinea pig glycophorin is not known, but agglutination of guinea pig asialoerythrocytes by MoAb 22.19 and both lectins (Table 1) strongly suggested that these red cells express sialylated Galβ1-3GalNAc- chains on their surface.

In order to compare the dependence of agglutination by MoAb 22.19 and PNA on the density of receptors on the cell surface, the human erythrocyte samples were desialylated to different degrees and their agglutinability was tested (Fig. 1). PNA showed a linear relationship between the agglutinating titer and the degree of erythrocyte desialylation. With MoAb 22.19 a threshold effect was observed, the agglutination began after 50 μg sialic acid was released from 1 ml red cells and the titer increased 64-fold when double the amount of sialic acid was released (Fig. 1). A similar threshold effect, but in the opposite direction, was found for MoAb 179K which is an IgM type with anti-N specificity and requires the sialylated oligosaccharide chains of the antigen [18] (Fig. 1). Agglutination of erythrocytes by MoAb 22.19 was not dependent on pH within the range 6-8.

Hemagglutination-inhibition assays showed that MoAb 22.19 was inhibited by human, horse, dog and murine asialoglycophorins (Table 2), as could be expected from its ability to agglutinate the same red cells and/or from the content of Galβ1-3GalNAcα- chains.

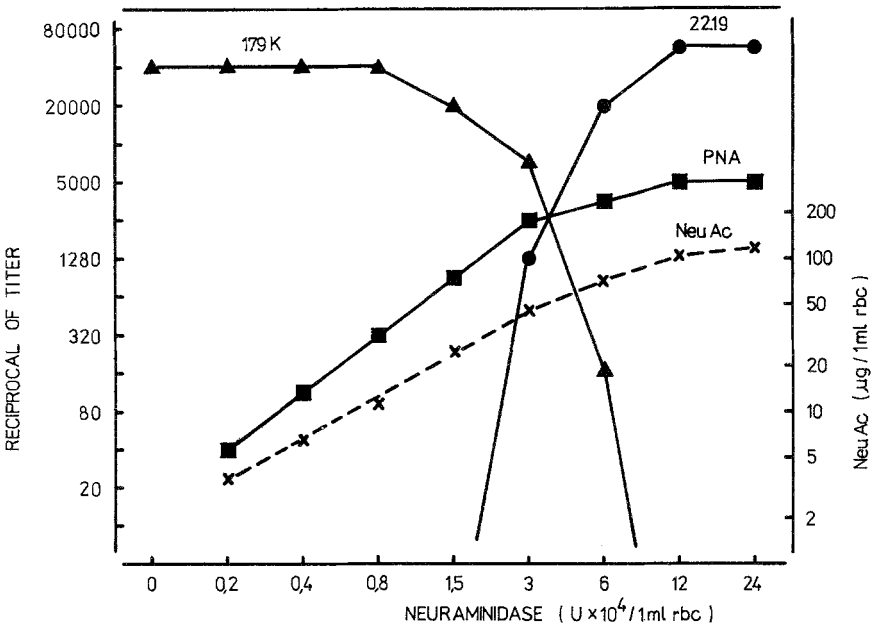

Figure 1. Dependence of agglutination of human O NN erythrocytes by MoAb 22.19 (●), anti-N MoAb 179K [18] (▲) and PNA (■) on the degree of red cell desialylation. Erythrocytes were treated with increasing amounts of neuraminidase for 1 h at 37°C, washed and tested in the agglutination assay as described under Materials and Methods. Released sialic acid was determined by the Warren method [40]. rbc = red blood cell.

Table 2. Hemagglutination-inhibition tests. Concentrations of glycoproteins which inhibit agglutination of human asialoerythrocytes by MoAB 22.19, PNA and VgL.

Glycoprotein	Inhibitor concentration (µg/ml)		
	MoAb 22.19	PNA	VgL
Asialoglycophorins from erythrocytes			
Human	40	5	40
Horse	300	300	20
Dog	60	80	80
Murine (BALB/c)	600	600	300
Bovine	>5000[a]	1250	>5000
Epiglycanin			
Untreated	15	4	30
Desialylated	15	2	15
Asialo-orosomucoid	>2500	300	>2500

[a] > = Lack of inhibition at the concentration given.

Table 3. Inhibition of agglutination of human asialoerythrocytes by MoAb 22.19 and PNA with glycopeptides, disaccharides and monosaccharides.

Inhibitor	Inhibitor concentration (mM)[a]	
	MoAb 22.19	PNA
Asialoglycopeptides of human glycophorin A		
(1-30/31 + 1-39)[b]	6.2	1.2
(40-61)	10.2	2.4
(9-30/31 + 9-39)	5.1	1.2
(1-8)	15.0	0.9
β-D-Gal-(1-3)-D-GalNAc	50	2.0
Lactose	⩾200[c]	25
Galactose	>200	25
N-acetylgalactosamine	>200	>200

[a] Inhibitory concentration is expressed as mmol/l of a disaccharide or monosaccharide.
[b] Numbers denote amino acid residues of glycophorin A polypeptide chain.
[c] > or ⩾ denote lack of inhibition or partial inhibition, respectively, at the concentration given.

MoAb 22.19 was also inhibited by untreated and desialylated epiglycanin from TA3-Ha mammary carcinoma ascites cells in which about 60% of the oligosaccharide units are unsubstituted Galβ1-3GalNAcα- chains [10]. Neither bovine asialoglycophorin nor asialo-orosomucoid inhibited MoAb 22.19. VgL showed a pattern of inhibition similar to the MoAb, whereas PNA reacted with all glycoproteins tested (Table 2), thus demonstrating the broader specificity of the latter lectin.

MoAb 22.19 reacts preferentially with antigens of high mol wt, such as aggregated erythrocyte asialoglycophorins or epiglycanin (M_r ~ 40 000). The non-aggregated glycopeptides of human glycophorin A and the free disaccharide, Galβ1-3GalNAc, were weak inhibitors of the antibody and a several-fold higher concentration was required to inhibit the MoAb in comparison to PNA (Table 3). The glycopeptides with amino acid residues 1-8 and 40-61 both have three disaccharide chains. However, the oligosaccharides in the glycopeptide (1-8) are located on adjacent amino acid residues and this glycopeptide is an inhibitor of VgL, whereas this lectin is not inhibited by glycopeptide (40-61) with dispersed oligosaccharide chains [39]. The similar (weak) inhibition of MoAb 22.19 by both these glycopeptides suggested that clusters of the disaccharide chains are not important for the reaction with the MoAb.

Human, horse, dog and guinea pig erythrocyte asialoglycoproteins which react with MoAb 22.19 were identified on neuraminidase-treated nitrocellulose blots of membrane fractions separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). Comparison of binding of the antibody, PNA and VgL to electrophoretic fractions showed that MoAb 22.19 and both lectins were bound essentially to the same glycoproteins within each species, but differences in intensity of binding to some bands

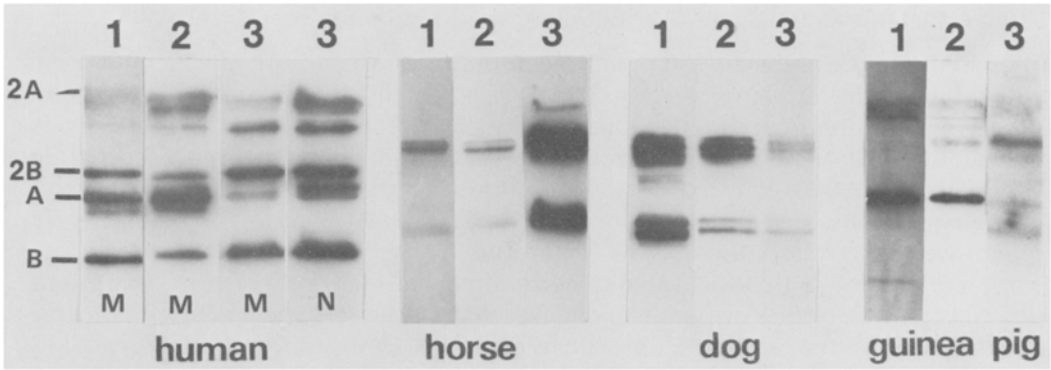

Figure 2. Comparison of membrane components of human (M or N), horse, dog and guinea pig erythrocytes reacting with MoAb 22.19 (1), PNA (2) and VgL (3). Electrophoretically separated membrane fractions were transferred to nitrocellulose, the blots were treated with neuraminidase and overlayed with radiolabeled lectins or with MoAb 22.19 and radiolabeled anti-mouse IgM antibody. The blots were autoradiographed for 24 h (antibodies) or 48h (lectins) except guinea pig membranes treated with VgL, which was autoradiographed for four days. The positions of human glycophorin A and B dimers and monomers are shown on the left. Other details are described under Materials and Methods.

were seen (Fig. 2). Guinea pig asialoglycophorins showed the most distinct differences, binding less VgL than PNA and antibody. No bands binding MoAb 22.19 and the lectins were detected in rabbit erythrocyte membranes under the conditions used (not shown), despite a weak agglutination of rabbit asialoerythrocytes by these reagents.

Discussion

The disaccharide chain Galβ1-3GalNAcα-, widespread in the cryptic form, is immunogenic in all vertebrates. This is shown by the common existence of anti-TF antibodies in human and animal sera and by the possibility of obtaining these antibodies by immunization. Injection of rabbits with human asialoglycophorins of blood group M and N gave rise to polyclonal antisera which contained mostly anti-carbohydrate antibodies [41]. These antisera precipitated asialoglycophorins and did not react with the untreated glycoproteins or with asialoglycophorins after Smith degradation [42]. Some of the polyclonal antibodies had a specificity restricted to definite glycopeptide fragments of glycophorin A, e.g. to desialylated blood group M or N determinants [41]. The polyclonal antibodies with anti-TF specificity were obtained by immunization of rabbits with polymerized T3 glycopeptide of human glycophorin A [43]. Immunodominant structures in the epitopes for anti-TF antibodies are the Galβ1-3GalNAcα- disaccharide chains, and the contribution of other fragments of the antigen to the immunological reaction should be of minor importance. However, this cannot be entirely excluded since anti-TF antibodies differ in their fine specificity, which may result from some additional restrictions concerning the structure of the antigenic determinant. The diversity of human "natural" anti-TF antibodies has been recently shown in studies of the reactions of the

sera with different synthetic antigens carrying TF receptors [44]. Thus, it should be considered that monoclonal anti-TF antibodies may differ in fine specificity.

The MoAb 22.19 described in this report meets the criteria for anti-TF specificity. It reacts with different glycoproteins carrying Galβ1-3GalNAcα- disaccharide chains, despite the different structures of their polypeptide chains and different distribution of glycosylation sites [45, 46]. Moreover, the antibody is weakly inhibited by the free disaccharide. Comparison of MoAb 22.19 to PNA and VgL shows that these three reagents are similar in their recognition of glycoproteins with Galβ1-3GalNAcα- chains, but some differences were also found. MoAb 22.19 is more similar in specificity to PNA than to VgL, since it reacts only with desialylated glycoproteins and does not seem to follow the additional restrictions (the presence of oligosaccharide clusters and a hydrophobic residue) required for the reaction of VgL with its receptors. However, the specificities of MoAb 22.19 and PNA are not identical, as is shown by hemagglutination and hemagglutination-inhibition assays and by binding to electrophoretic fractions of erythrocyte membranes.

It is difficult to compare the antibody described here with two other anti-TF MoAbs of the IgM class described recently by Longenecker *et al.* [14], because different methods were used for their characterization. However, the specificity of the two antibodies obtained by these authors differed significantly. One of them, 49H.24, was highly specific for the Galβ1-3GalNAcα- structure, whereas the other, 49H.8, did not differentiate between the α or β anomeric configuration of the *N*-acetylgalactosamine residue in the hapten and was best inhibited by hydrophobic (nitrophenyl- or phenyl-) glycosides of β-galactose. Surprisingly, only 49H.8 reacted with different cancer cell lines, for example with TA3-Ha murine carcinoma cells [14], despite the α configuration of *N*-acetylgalactosamine residues in the oligosaccharides of epiglycanin from these cells [47]. Our MoAb, 22.19, reacted with epiglycanin, as well as with other glycoproteins containing Galβ1-3GalNAcα- chains. It was shown to be a suitable reagent for studies on glycoconjugates carrying these structures.

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