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Immunochemical studies on the combining site of the A + N blood type specific *Moluccella laevis* lectin *,**

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ABSTRACT

The specificity of the anti A+N lectin of *Moluccella laevis* (MLL) was examined by hemagglutination experiments with enzyme-modified human erythrocytes and by inhibition of hemagglutination. In addition, binding to various glycoproteins and inhibition by different sugars and glycoproteins were examined by enzyme immunoassay with antibodies to the lectin. Treatment of A^{MM} erythrocytes with proteolytic enzymes increased their agglutinability by MLL 4–16-fold; similar treatment of O^{NN} cells decreased their agglutinability 8–16-fold. This is in line with the known location and enzyme sensitivity of A and N specificity determinants. Treatment of the erythrocytes with sialidase increased their agglutinability and abolished the distinction between N and M cells. Hapten inhibition of hemagglutination of A^{MM} and O^{NN} erythrocytes by the lectin, and its binding to glycoproteins measured by enzyme immunoassay, confirmed the high specificity of MLL for *N*-acetyl-D-galactosamine (200–500 times more than for D-galactose) and suggested the presence of hydrophobic interactions around HO-2 of the D-galactose unit. The methyl α-glycosides of D-galactose and of *N*-acetyl-D-galactosamine were better inhibitors than the corresponding β-glycosides; this preference was abolished, and sometimes reversed, when the *p*-nitrophenyl glycosides of the same monosaccharides were tested, stressing again the importance of hydrophobic interactions in the binding of carbohydrates to MLL. The lectin reacted well with O^{NN} substance and with glycophorin A of the N phenotype (GPA_N), but did not react with O^{MM} substance or GPA_M. The strongest inhibitor was asialo ovine submaxillary mucin, which contains many unsubstituted α-D-Gal*p*NAc-(1 → 3)-Ser/Thr residues; calculated per *N*-acetyl-D-galactosamine residue, it was 1500 stronger than free *N*-acetyl-D-galactosamine. In accordance with this result, it was found that the lectin strongly agglutinates Tn cells. The specificity of MLL can, thus, be defined as

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anti-Tn, crossreactive with blood types A and N, and with sialosyl-Tn. The N-specificity can best be explained by assuming that GPA_N contains a small number of unsubstituted or partially sialylated α-D-Gal*p*NAc-(1 → 3)-Ser/Thr residues, which are present in smaller proportions, if at all, in GPA_M.

INTRODUCTION

Affinity purified *Moluccella laevis* lectin (MLL) exhibits an unusual, dual blood-type specificity, in that it agglutinates both A type and N type erythrocytes[1–3]. Both reactions are specifically inhibited by low concentrations of *N*-acetylgalactosamine *, as well as by a much higher (200–500-fold) concentration of galactose. MLL consists of three types of subunit, one of 67 kDa, made up of two polypeptides of 46 and 28 kDa held together by S–S bonds, and two noncovalently-linked subunits of 42 and 26 kDa (ref. 3). To clarify the molecular basis of the unusual specificity of MLL, we have examined the interaction of the lectin with native and modified glycophorins from human erythrocytes of blood type M and N, with other blood group-reactive and nonreactive glycoproteins as well as with Tn erythrocytes. Data on inhibition of the lectin by a wide range of simple saccharides are also reported.

EXPERIMENTAL **

Lectin.—MLL was prepared from ground, defatted *Moluccella laevis* seeds by affinity chromatography on Sepharose-bound galactose[3].

Glycophorins and glycophorin-derived glycopeptide fragments.—Crude human erythrocyte glycophorin A (GPA) was prepared by phenol extraction of membranes of outdated blood group M or N red blood cells[4], and further purified by filtration on an Ultrogel AcA44 column in the presence of sodium dodecyl sulfate[5] to give GPA_M and GPA_N, respectively. Glycophorin preparations from O^{MM} and O^{NN} erythrocytes, designated as O^{MM} and O^{NN} substances, were a gift from Dr. G.F. Springer (Chicago Medical School, IL). Fresh horse erythrocytes served as a source of crude horse glycophorin, which was obtained as described above and used without further purification. Tryptic peptides were obtained from GPA according to published procedures[5,6]. A mixture of two glycopeptides, corresponding to amino acid residues 1–39 and 1–30/31, was designated as fraction T1/T2, and the peptide-containing residues 40–61 as fraction T3.

Other glycoproteins and sugars.—Ovine submaxillary mucin (OSM) was obtained from a water extract of sheep submaxillary glands by fractionation with hexadecyltrimethylammonium bromide and ethanol[7]. The A, B, and H blood group

* All sugars discussed have the D configuration.

** Since the work was done in three laboratories, different modifications of the same technique were occasionally used for similar purposes. When relevant, this is mentioned in the text.

glycoproteins were isolated from human ovarian cyst fluids either by the method of Morgan[8] or according to Kabat[9]; the latter method was also used to prepare Le substances from human saliva and various blood group substances from horse, bovine, or hog gastric mucosa. The P1 fractions of A and B substances were prepared by mild acid hydrolysis, pH 1.5–1.8, at 100° for 2 h of the parent compounds[10]. Epiglycanin, a glycoprotein from murine adenocarcinoma TA3-Ha cells[11], was a gift from Dr. J.F. Codington (Boston Biomedical Research Institute, Boston, MA).

The disaccharide, β-Gal*p*-(1 → 3)-GalNAc, was obtained from human asialoglycophorin by treatment with *O*-glycanase (Genzyme, Boston, MA), followed by gel filtration on a Bio-Gel P-4 column; its purity was confirmed by TLC and determination of monosaccharide constituents. The *N*-substituted derivatives of galactosamine and lactosamine were a gift from Dr. W. Kinzy[12]. All other sugars were from commercial sources, of the highest purity available.

Modified glycoproteins.—Removal of sialic acid from glycoproteins was achieved by hydrolysis in 25 mM H_2SO_4 for 4 h at 60°. Asialoagalactoglycophorin was obtained from asialoglycophorin by a one-step Smith degradation. For this purpose, a 0.5% solution of the glycoprotein in 0.05 M acetate buffer, pH 4.5, containing 0.05 M $NaIO_4$, was kept for 18 h at 4° and dialyzed, first against 0.1 M NaCl, then against water. Solid $NaBH_4$ was added to a final concentration of 0.15 M and, after 1–2 h at room temperature, the solution was dialyzed exhaustively against water, H_2SO_4 was added to 25 mM concentration, and the glycoprotein was hydrolyzed for 1 h at 80°, neutralized, dialyzed again, and lyophilized.

O-Deglycosylation of GPA attached to wells of ELISA plates was carried out by treatment in situ with different dilutions of a mixture of *Diploccocus pneumoniae* glycosidases for 6 days at 37° as described [13,14]. *N*-Acetylation, release of N-terminal amino acids by Edman degradation and modification of carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was done according to described procedures[4,5,15,16].

Rabbit anti-MLL serum.—Rabbits were immunized by repeated injections of a solution of MLL (500 μg/mL) in 50 mM phosphate buffer–0.15 M NaCl, pH 7.4 (0.05-PBS) mixed 1:1 with complete Freund's adjuvant and incubated for 15 min in an ultrasonic bath. The rabbits were bled on day 41, the blood was incubated for 30 min at 37° and overnight at 4°, and then centrifuged. The serum was incubated at 56° for 1 h and stored frozen. It had a titer of 80–160 when tested by double immunodiffusion against MLL (1 mg/mL). The IgG-containing fraction was obtained from the antiserum by precipitation with $(NH_4)_2SO_4$ at 40% saturation. The precipitate was dissolved in 0.05-PBS and dialyzed extensively against the same buffer. The final volume was ~ 50% of the original serum volume.

Monoclonal antibodies (MAb).—MAb 22.19 (IgM) specific for β-Gal*p*-(1 → 3)-GalNAc (Thomsen–Friedenreich antigen) has been described[17]. Anti-Tn specific MAb Tn5 (IgG1) was raised by immunization of mice with asialoagalactoGPA[18];

this MAb reacts with asialoagalactoGPA, but not with GPA or asialoGPA, and agglutinates Tn erythrocytes only.

Enzyme-linked immunosorbent assay (ELISA).—Two modifications of the ELISA technique were used to measure the binding of MLL to different glycoproteins and in inhibition experiments.

Modification 1. Wells of the ELISA plates (Dynatech, Plochingen, Germany) were coated with GPA in 50 mM $NaHCO_3$ buffer, pH 9.6, (1 μg/50 μL/well) overnight at 4°. All reagents (except the substrate) were diluted with PBS–T (0.05-PBS containing 0.05% Tween 20), which was also used for washing the plates between the incubations; 50-μL aliquots per well were used. The binding of MLL to GPA-coated wells was determined by incubation for 1 h at room temperature with different concentrations of MLL, followed by a 1:250 dilution of rabbit anti-MLL IgG, and goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA), under the same conditions as described above. The plates were then incubated for 30 min with 4-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, 104 5-mg phosphate substrate tablets, one tablet dissolved in 5 mL of 50 mM H_2CO_3 buffer, pH 9.6, containing mM $MgCl_2$) and absorbance at 405 nm was read in a microtiter-plate scanner. Binding of MAb's to GPA-coated wells was detected with alkaline phosphatase-conjugated rabbit anti-mouse Ig antibodies (Dakopatts, Denmark). In inhibition experiments, the serially diluted (with PBS–T) inhibitor samples were incubated with an equal volume of MLL solution (0.5 μg/mL PBS–T) for 1 h at room temperature, and the binding to asialoGPA-coated plates was measured as described above. All determinations were carried out in duplicate; variations between the duplicates were usually within 5%.

Modification 2. Wells of ELISA plates (Corning Glass Works, Corning, NY) were coated with serial dilutions of various blood group substances [100 μL of 1–10 μg/mL in 0.01 M phosphate buffered saline solution, pH 7.2 (0.01-PBS)] for 2 h at 37°, and blocked with 1% bovine serum albumin in the aforementioned buffer for 2 h at room temperature. After each incubation (also in the following steps), the wells were washed thrice with 0.05% Tween 20 and 0.02% NaN_3 in 0.01-PBS. The plates were then incubated for 2 h with the lectin (100 μL of 1–5 μg/mL in 0.01-PBS), followed by incubation for 90 min at 37° with a 1:500 dilution of rabbit anti-MLL antiserum. A 1:1000 dilution of goat anti-rabbit IgG, coupled to alkaline phosphatase (Sigma), was then added for 90 min at 37°. The wells were incubated for 30 min with 4-nitrophenyl phosphate (100 μl; 30 mg in 50 mL diethanolamine buffer, pH 9.8) and absorbance at 405 nm was read in a microtiter-plate scanner. In inhibition experiments, wells were coated with McDon P1 (ref. 10) (100 μL; 5 ng/mL). Serial dilutions of inhibitors in 50 μL were then incubated with MLL (50 μL; 2 μg/mL) in the coated wells, followed by rabbit anti-MLL antiserum and IgG-alkaline phosphatase, as described above.

Agglutination.—Typed human M and N erythrocytes were obtained from the Blood Bank of Sheba Hospital (Tel Hashomer, Israel), from the Blood Bank in

Wroclaw, or from Dr. Celso Bianco of the New York Blood Center. Hemagglutination tests, using untreated or modified human erythrocytes of known blood types, were done by the double-dilution technique in microtiter plates. Agglutination was scored after 1 h at room temperature. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination. To examine the anti-Tn activity of MLL, hemagglutination was carried out in test tubes and scored both macroscopically and microscopically[19]. The inhibitory activity of sugars was measured by mixing serial dilutions of the inhibitor with four units of the lectin before addition of erythrocytes and determining the lowest concentration giving full inhibition of agglutination.

For protease treatment, erythrocytes were incubated for 2 h at 37° with TPCK-trypsin or TLCK-chymotrypsin (1 mg; Sigma)–50% red blood cell suspension in 0.05-PBS (1 mL), or with papain (5 mg; Merck) in phosphate buffer (pH 6.3) containing 0.3% cysteine/mL of 50% red blood cell suspension. For enzymic desialylation, a 20% suspension of erythrocytes in 0.05-PBS (1 mL) was treated with *Vibrio cholerae* sialidase (5×10^{-3} units; Behringwerke AG, Marburg, Germany) for 1 h at 37°, and the cells were washed 4–5 times with 0.05-PBS.

RESULTS AND DISCUSSION

As previously reported[1–3], MLL exhibits dual blood type specificity in that it agglutinates both A^{MM} and O^{NN} erythrocytes at concentrations much lower than those needed for the agglutination of B^{MM} or O^{MM} cells (Table I). The data in Table I show, in addition, that desialylation of the erythrocytes of any blood type increased their agglutinability and abolished the distinction between N and M cells, as also found for the N specific *Vicia graminea* lectin[20]. Treatment of A^{MM} erythrocytes with proteolytic enzymes increased their susceptibility to agglutination by MLL up to 16-fold; similar treatment of O^{NN} cells decreased their susceptibility 8–16-fold. This is in line with the known localization and enzyme sensitivity of the N and A blood type determinants[21,22]; N specificity is located on glycophorin, which is digested by proteolytic treatment of erythrocytes, thus decreasing the susceptibility of O^{NN} cells to agglutination by MLL; A type determinants are

TABLE I

Agglutination of untreated and enzymically modified human erythrocytes by MLL

Enzymic treatment	Minimal agglutinating conc. (μg/mL)			
	A^{MM}	B^{MM}	O^{NN}	O^{MM}
None	31	> 500	31	> 500
Sialidase	4	8	2	8
Trypsin	2	[a]	250	> 500
Chymotrypsin	8	[a]	250	[a]
Papain	2	> 500	> 500	[a]

[a] Not determined.

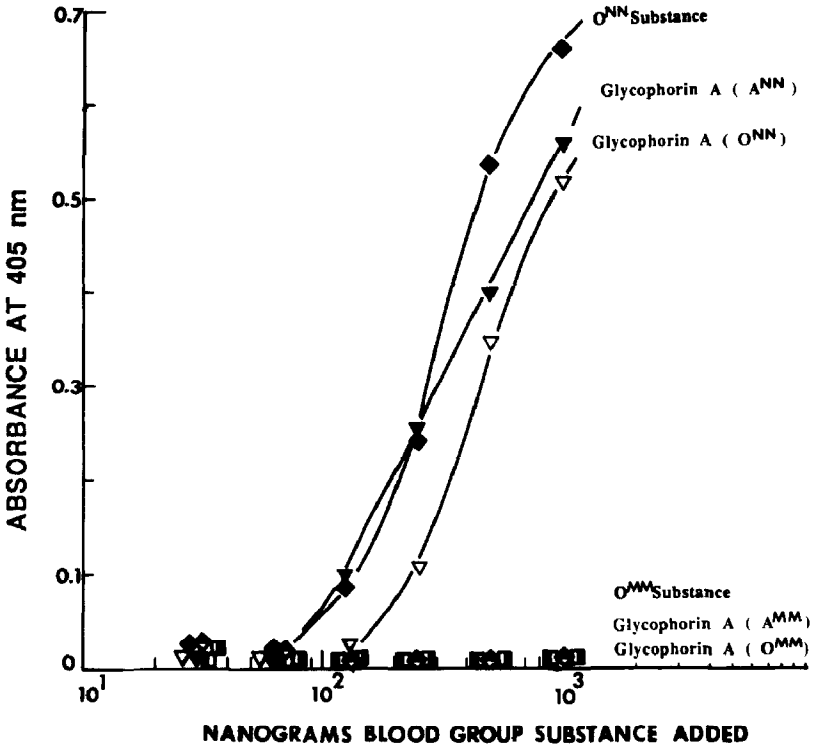

Fig. 1. Interaction of blood group M and N substances with MLL (500 ng) measured by ELISA, as described in the Experimental section (modification 2).

carried by glycoproteins other than glycophorin, predominantly Band 3 (the blood type activity of which is protease resistant) and by glycolipids, which become exposed after removal of protease-sensitive protein chains from the erythrocytes, thus resulting in increased agglutinability of A^{MM} cells.

Agglutination of A^{MM} or A^{NN} red blood cells (at a lectin concentration of 12.5 μg/mL) was inhibited by 62.5 μg/mL GPA_N from A^{NN} erythrocytes, while 250 μg/mL of GPA_N from O^{NN} cells was required for inhibition of the same reaction. Agglutination of O^{NN} cells (at a lectin concentration of 25 μg/mL) was inhibited by 250 μg/mL of GPA from A^{NN} cells, while 500 μg/mL of GPA from O^{NN} erythrocytes was required for inhibition in the same assay. GPA from A^{MM} or O^{NN} erythrocytes was not inhibitory, even at a concentration of 1000 μg/mL, in any of these assays. Hemagglutination of A^{MM} and O^{NN} erythrocytes (at 100 μg MLL/mL) was also inhibited by 20–40 μg/mL of asialoGPA_N and 80 μg/mL of asialoGPA_M.

In ELISA, strong reaction of the lectin with O^{NN} substance, as well as with GPA_N was observed, but no reaction at all with O^{MM} substance or GPA_M (Fig. 1). Desialylation of the GPA of either phenotype greatly increased their interaction with the lectin and abolished the difference between GPA_M and GPA_N (not shown).

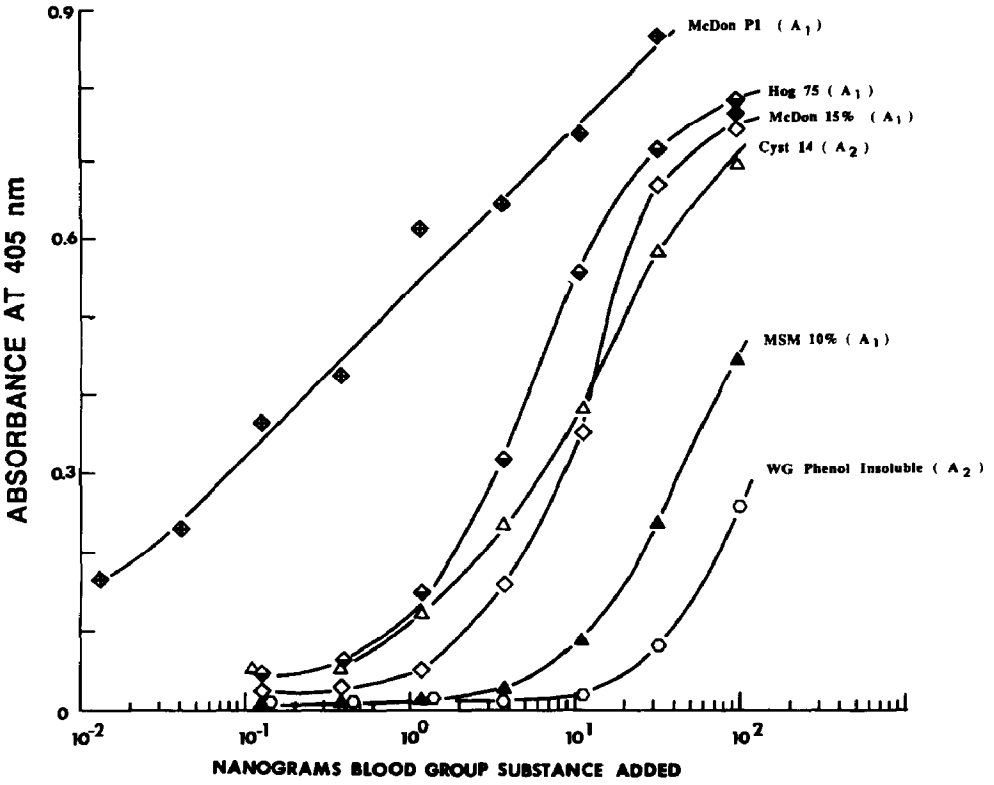

Fig. 2. Interaction of blood group A_1, A_2 and AP1 substances with MLL (100 ng) measured by ELISA, as described in the Experimental section (modification 2).

Figures 2–4 show the binding curves of the lectin for blood group A, B, H, Le^a, and Le^b substances, respectively. The lectin reacted well with most of the blood group substances tested, but it appears that it recognizes the partially hydrolyzed P-1 substances (e.g., McDon P1 and Beach P1) better than their nonhydrolyzed precursors. These data indicated that MLL probably recognizes some internal sugar structures in the blood group active substances. The variation in activity of the intact blood group substances with the lectin further indicated their well-known heterogeneity with respect to numbers of determinants and variations due to incomplete biosynthesis, as established by alkaline borohydride degradation[23].

To obtain additional information on the carbohydrate specificity of MLL, we examined the hemagglutination inhibitory activity of a wider range of sugars than previously reported[3]; these included various *N*-substituted derivatives of galactosamine and lactosamine (Table II). By far the best inhibitor (15 000 times better than galactose and 30 times better than *N*-acetylgalactosamine) was methyl 2-(4-azidosalicylamino)-2-deoxy-α-D-galactopyranoside, indicating strong hydrophobic interactions around HO-2 of the galactose unit and raising the possibility that this saccharide could be used as an active site-directed, irreversible inhibitor of the lectin.

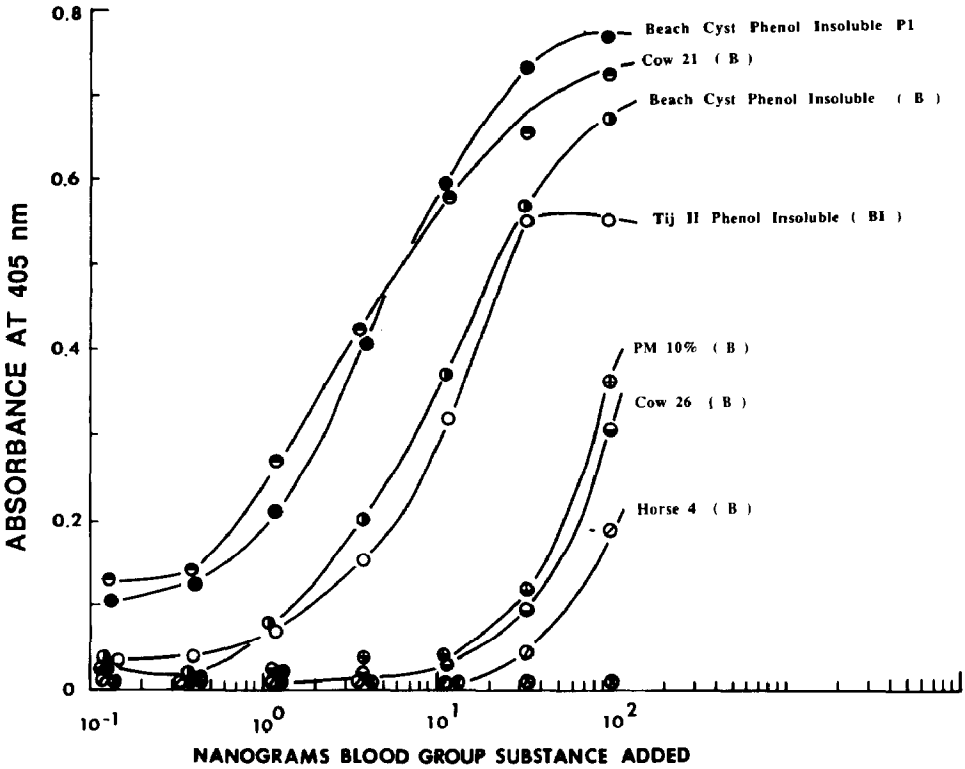

Fig. 3. Interaction of blood group B and BP1 substances with MLL (100 ng) measured by ELISA, as described in the Experimental section (modification 2).

Inhibition was also tested by ELISA, which is more sensitive than inhibition of hemagglutination (Table II). *N*-Acetylgalactosamine was 200–400 times more active than galactose, as previously found by hapten inhibition of hemagglutination. The methyl α-glycosides, both of galactose and of *N*-acetylgalactosamine, were significantly better inhibitors of MLL than the corresponding β-glycosides, both by inhibition of hemagglutination and by microtiter-plate ELISA. This preference was abolished, and in some cases reversed, when the 4-nitrophenyl glycosides of these sugars were tested. Such an apparent change in specificity with the introduction of a hydrophobic aglycon has been observed with several other lectins, stressing the importance of hydrophobic interactions in the binding of carbohydrates to lectins[24].

Among the oligosaccharides tested, α-Gal*p*NAc-(1 → 3)-Gal was the most potent inhibitor, 240 times better than galactose, and was in the inhibitory range of *N*-acetylgalactosamine, but considerably less active than methyl 2-acetamido-2-deoxy-α-D-galactopyranoside. Oligosaccharides having an α-D-galactosyl group at the nonreducing end were 10–20 times stronger inhibitors than galactose. Interestingly, stachyose was a three-fold better inhibitor than raffinose. Lactose, which has a β-linked D-galactopyranosyl group at the nonreducing end, was practically

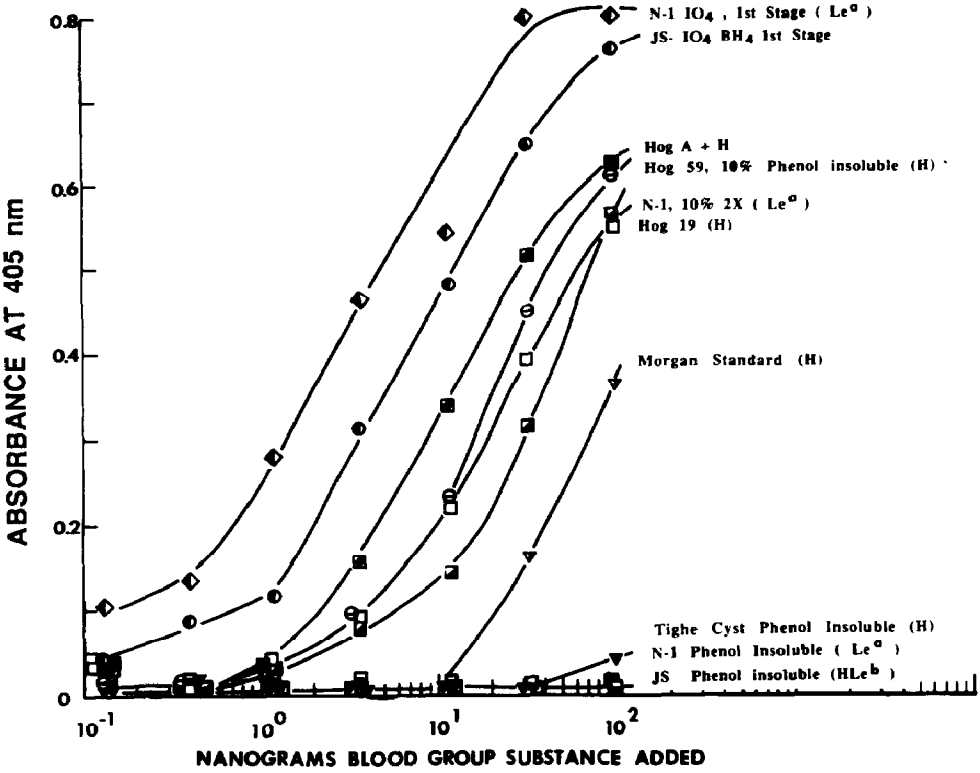

Fig. 4. Interaction of blood group H, Le^a and Le^b substances with MLL (100 ng) measured by ELISA, as described in the Experimental section (modification 2).

noninhibitory. The disaccharide β-Gal-(1 → 3)-GalNAc was as active as galactose, suggesting that MLL reacts poorly with *N*-acetylgalactosamine substituted at O-3.

Distinctly lower concentrations of GPA_N and GPA_N-derived N-terminal glycopeptides were required to inhibit the binding of MLL to asialoGPA_N-coated plates than of GPA_M and GPA_M-derived glycopeptides (Table III and Fig. 5). AsialoGPA's were much better inhibitors than the native glycoproteins (500-fold increase for GPA_M and 100-fold for GPA_N), but with a decrease in the difference between N and M glycoproteins. The strongest inhibitor among the modified GPA's was the asialoagalacto derivative, being 100–300 times better than asialoGPA's. The blood group N and M specific amino acids in positions 1 (Leu in GPA_N vs. Ser in GPA_M) and 5 (Glu vs. Gly) do not appear to be directly involved in the N-specific binding of MLL, since neither modification of the carboxyl groups of GPA_N, nor *N*-acetylation of GPA's N and M, affected their inhibitory activities. Removal of the N-terminal amino acid from either GPA increased its inhibitory activity, most probably owing to partial desialylation occurring during this procedure. These results are different from those obtained with the *Vicia graminea* lectin, whose anti-N specificity is clearly dependent on the hydrophobic character of residue 1 (Leu) in GPA_N[20].

TABLE II

Carbohydrate specificity of MLL determined by various methods [a]

Sugar [b]	Inhibition of MLL assayed by:		
	Hemagglutination	ELISA with asialoGPA$_N$	ELISA with McDon P1
Gal	1	1	1
GalNAc	500	400	200
αGal*p*OMe	8		7
βGal*p*OMe	1		1.3
αGal*p*NAcOMe			1850
βGal*p*NAcOMe			28
αGal*p*O4NP	7	3	2.4
βGal*p*O4NP	14	4.5	5
αGal*p*NAcO4NP	2100	750	630
βGal*p*NAcO4NP		900	530
αGal*p*-(1 → 6)-Glc	10		20
αGal*p*-(1 → 6)-αGlc*p*(1 → 2)-βFru*f* (raffinose)	10		3
αGal*p*-(1 → 6)-αGal*p*(1 → 6)-αGlc*p*-(1 → 2)-βFru*f* (stachyose)	18		9
2-DeoxyGal	0.4		1
GalN	0.6		
βGal*p*-(1 → 4)-Glc	0.05		
αGal*p*-(1 → 4)-Fuc	18		
αFuc*p*-(1 → 4)-Gal	1		
αGal*p*-(1 → 4)-Gal	20		
βGal*p*-(1 → 3)-GalNAc	2	1.2	
αGal*p*NAc-(1 → 3)-Gal			240
α-Gal*p*N(4Azsal)OMe	15000		
βGal*p*N(4Azsal)OMe	2250		
(2Az)βGal*p*O4NP	24		
βGal*p*-(1 → 4)-βGlc*p*NAcOMe	0.8		
βGal*p*-(1 → 4)-βGlc*p*NDsOMe	3		
βGal*p*-(1 → 4)-βGlc*p*N[$CH_3(CH_2)_{10}CO$]	1		
βGal*p*-(1 → 4)-βGlc*p*NBzOMe	0.1		
βGal*p*-(1 → 4)-βGlc*p*NBut	0.1		

[a] All numbers are given as relative inhibitory activity compared to galactose, which was arbitrarily set to 1.0. The minimal inhibitory concentration of galactose in the hemagglutinating inhibition experiment (for complete inhibition of 4 hemagglutinating units of the lectin) was 7 mM and in ELISA (for 50% inhibition of binding) was 3.7 mM with asialoGPA$_N$ and 2.4 mM with McDon P1 (a modified blood group glycoprotein). [b] All sugars have the D configuration. Abbreviations: 4NP, 4-nitrophenyl; 4Azsal, 4-azidosalicyl; Az, azido; Ds, dansyl; and But, butyryl (butanoyl).

Inhibition of MLL by blood group glycoproteins from ovarian cyst fluids was strongest with type A substance, followed by type B (~ 10 times weaker) and type H (300 times weaker) (Table III). The relatively small difference in the inhibitory activities of type A and type B substances would suggest cross-reactivity of the lectin with the B antigen; however, B erythrocytes were not agglutinated by the lectin, even after treatment with proteases. It is possible that the reaction of MLL with the cyst glycoproteins depends not only on the A determinant, but also on the

TABLE III

Inhibition of MLL by native and modified glycoproteins assayed by ELISA

Compound	Blood group	I_{50} [a]
Glycophorin A		
untreated	M	3550
	N	200
asialo	M	7.1
	N	2.3
asialoagalacto	M	0.025
asialo, *N*-acetylated	M	9.5
	N	2.0
asialo, carbodiimide-treated	N	3.4
Edman-degraded, *N*-acetylated	M	96
	N	43
Tryptic glycopeptides of GPA		
T1/T2 (a.a. 1–39 + 1–30/31) untreated	M	1860
	N	250
asialo	M	9.1
	N	10.3
T3 (a.a. 40–61) untreated	M	4000
	N	4500
asialo	M	36
	N	51
Horse glycophorin		
untreated		2000
asialo		33
asialoagalacto		0.06
Ovarian cyst blood group glycoproteins		
	A	1.7
	B	13
	H	515
Ovine submaxillary mucin		
untreated		0.1
asialo		0.003
Epiglycanin		
untreated		0.21
asialo		0.27

[a] Concentration of inhibitor (μg/mL) giving 50% inhibition of MLL binding to asialoGPA_N.

presence of unsubstituted 2-acetamido-2-deoxygalactopyranosyl groups, attached to Ser/Thr, which may occur in different proportions in various preparations.

The inhibitory activity of horse glycophorin, whether intact or modified (asialo and asialoagalacto), was of the same order of magnitude as that observed with human native and modified GPA_M, respectively. Epiglycanin, which carries β-Gal*p*-(1 → 3)-GalNAc and GalNAc units and is heterogeneous and poorly sialylated, was a good inhibitor and its inhibitory activity was not affected by desialyla-

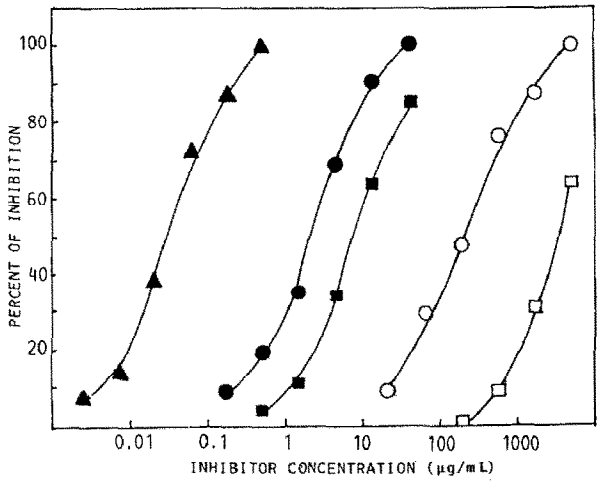

Fig. 5. Inhibition by glycophorins of MLL binding (25 ng) to asialoGPA$_N$ measured by ELISA, as described in the Experimental section (modification 1). (□) GPA$_M$, (○) GPA$_N$, (■) asialoGPA$_M$, (●) asialoGPA$_N$, (▲) asialoagalactoGPA$_M$. The results are presented as % inhibition of control (MLL with buffer).

tion. OSM, which carries multiple α-NeuAc-(2 → 6)-GalNAc units[25], was as inhibitory as epiglycanin and 2000 times more inhibitory than GPA$_N$. It is noteworthy that, after desialylation, it showed the highest activity of all glycoproteins tested, i.e., 3 ng of asialoOSM inhibited by 50% the binding of 500 ng of MLL to asialoagalactoGPA-coated plates, as compared to 2.3 μg of asialoGPA$_N$ and 25 ng of asialoagalactoGPA. Calculated per *N*-acetylgalactosamine residue, asialoOSM is inhibitory at a concentration of 6 nM, and asialoagalactoGPA at 20 nM, as compared to free *N*-acetylgalactosamine, which is inhibitory at 9 μM concentration. This may be due to an avidity effect, resulting from the high density of unsubstituted 2-acetamido-2-deoxygalactopyranosyl groups in the two glycoproteins.

The strong inhibitory activity of asialoOSM and of asialoagalactoGPA suggested that the reaction of MLL with glycophorins is based on interactions with unsubstituted 2-acetamido-2-deoxypyranosyl groups linked directly to the protein. To substantiate this suggestion, the binding of MLL to GPA$_N$-coated microtiter wells, treated with increasing concentrations of a mixture of *Diplococcus pneumoniae* glycosidases, was measured (Fig. 6). The only enzymes in the mixture which act on *O*-glycans are sialidase and *O*-glycanase. The absence of *N*-acetyl-α-D-galactosaminidase was confirmed previously, when tryptic glycopeptides of GPA were treated with this mixture and no free *N*-acetylgalactosamine was detected among the reaction products[13]. The lack of release of galactose was demonstrated by the lack of binding to the treated GPA of the anti-Tn MAb Tn5, which reacts only with asialoagalactoGPA (Fig. 6).

The binding of anti-β-Gal*p*-(1 → 3)-GalNAc MAb 22.19 initially increased with increasing enzyme concentrations, presumably due to the action of the sialidase; at

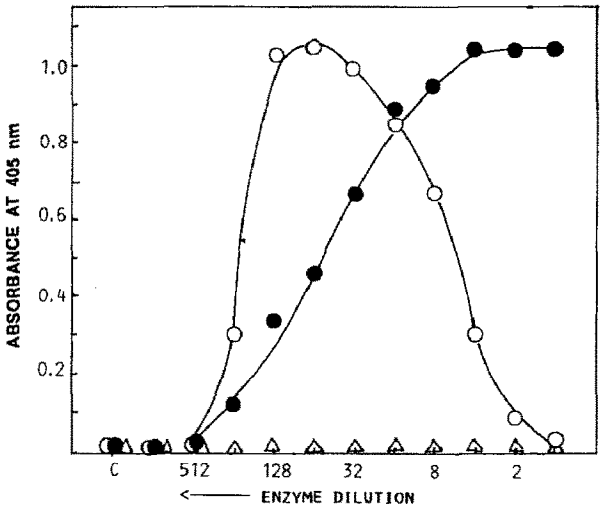

Fig. 6. Binding of MLL (50 ng) (●), anti-TF MAb 22.19 (○) and anti-Tn MAb Tn5 (△) to wells of ELISA plates coated with asialoGPA$_N$ (modification 1) and treated with serial dilutions of a mixture of *Diplococcus pneumoniae* glycosidases as described in the Experimental section.

higher enzyme concentrations, binding was gradually abolished, indicating the removal of β-Gal*p*-(1 → 3)-GalNAc by *O*-glycanase. Binding of MLL to identically treated, GPA$_N$-coated wells also initially increased as a result of desialylation of the glycoprotein, but remained constant at the high level attained, even at very high enzyme concentrations. These results strongly suggested that MLL reacts with asialoGPA$_N$ via minor, nongalactosylated *N*-acetylgalactosamine residues, which in native GPA$_N$ are at least partially substituted at O-6 with an *N*-acetylneuraminyl group. On the basis of these findings, the specificity of MLL can be defined as anti-Tn, crossreactive with blood types A and N, and with sialosyl-Tn. Indeed, agglutination experiments have shown that the lectin is ~ 100-fold more active with O^{MM}Tn erythrocytes than with non-Tn O^{MM} cells. MLL differs from the two Tn specific lectins isolated to date, i.e., the B$_4$ lectin from *Vicia villosa*[26] and that from *Salvia sclarea*[27]. These lectins, although similar to MLL in their pronounced specificity for *N*-acetylgalactosamine and the disaccharide, α-GalNAc-(1 → 3)-GalNAc, do not agglutinate non-Tn erythrocytes of either A, B, or O blood type, nor do they recognize α-Gal*p*NAc groups of blood group A structures.

Our results suggest that both the A and N specificity of MLL, and in particular its Tn specificity, may be ascribed to the high affinity of the lectin for a 2-acetamido-2-deoxy-α-D-galactopyranosyl group. To explain the stronger reaction of MLL with GPA$_N$ than with GPA$_M$, it is tempting to assume that the former glycoprotein has higher levels of incomplete (nongalactosylated) *O*-glycans than the latter, or that the incomplete chain occurs with significantly higher frequency in a particular part of GPA$_N$, e.g., close to the N-terminus which is different from that of GPA$_M$. Alternately, the difference between GPA$_N$ and GPA$_M$ may be due

to different degrees of sialylation of the nongalactosylated chains. The slight differences in glycosylation of the glycophorins may be controlled by the differences in amino acids at their N-terminus.

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REFERENCES

1 G.W.G. Bird and J. Wingham, *Vox Sang.*, 18 (1970) 235–239.
2 G.I. Pardoe, G.W.G. Bird, G. Uhlenbruck, I. Sprenger, and M. Heggen, *Z. Immunitaetsforsch. Allerg. Klin. Immunol.*, 140 (1970) 374–394.
3 H. Lis, H. Latter, R. Adar, and N. Sharon, *FEBS Lett.*, 233 (1988) 191–195.
4 E. Lisowska, L. Messeter, M. Duk, M. Czerwinski, and A. Lundblad, *Mol. Immunol.*, 24 (1987) 605–613.
5 K. Wasniowska, C.M. Reichert, M.H. McGinniss, K.R. Schroer, D. Zopf, E. Lisowska, L. Messeter, and A. Lundblad, *Glycoconjugate J.*, 2 (1985) 163–176.
6 M. Tomita, H. Furthmayr, and V.T. Marchesi, *Biochemistry*, 17 (1978) 4756–4769.
7 Y. Hashimoto, S. Hashimoto, and W. Pigman, *Arch. Biochem. Biophys.*, 104 (1964) 282–291.
8 W.T.J. Morgan, *Methods Immunol. Immunochem.*, 1 (1967) 75–81.
9 E.A. Kabat, *Blood Group Substances – Their Chemistry and Immunochemistry*, Academic Press, New York, 1956.
10 P.Z. Allen and E.A. Kabat, *J. Immunol.*, 82 (1959) 340–357.
11 D.H. van den Eijnden, N.A. Evans, J.F. Codington, V. Reinhold, C. Silber, and R.W. Jeanloz, *J. Biol. Chem.*, 254 (1979) 12 153–12 159.
12 W. Kinzy, H. Lis, and N. Sharon, *Abstr. Int. Symp. Carbohydr. Chem., 14*th (1988) 358.
13 Z. Drzeniek, H. Krotkiewski, D. Syper, and E. Lisowska, *Carbohydr. Res.*, 120 (1983) 315–321.
14 E. Jaskiewicz, E. Lisowska, and A. Lundblad, *Glycoconjugate J.*, 7 (1990) 255–268.
15 A.M. Weiner, T. Platt, and K. Weber, *J. Biol. Chem.*, 247 (1972) 3242–3251.
16 R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
17 I. Steuden, M. Duk, M. Czerwinski, C. Radzikowski, and E. Lisowska, *Glycoconjugate J.*, 2 (1985) 304–314.
18 M. Duk, I. Sterden, D. Dus, C. Radzikowski, and E. Lisowska, *Glycoconjugate J.*, 9 (1992) 148–153.
19 W.L. Marsh, *Transfusion*, 12 (1972) 352.
20 M. Duk, E. Lisowska, M. Kordowicz, and K. Wasniowska, *Eur. J. Biochem.*, 123 (1982) 105–112.
21 E. Lisowska, *Adv. Exp. Med. Biol.*, 228 (1988) 265–315.
22 R.A. Laine and J.S. Rush, *Adv. Exp. Med. Biol.*, 228 (1988) 331–347.
23 A.M. Wu, E.A. Kabat, B. Nilsson, D.A. Zopf, F.G. Gruezo, and J. Liao, *J. Biol. Chem.*, 259 (1984) 7178–7186.
24 H. Lis and N. Sharon, in V. Ginsburg and P.W. Robbins (Eds.), *Biology of Carbohydrates*, Wiley, New York, Vol. 2, 1984, pp 1–85.
25 H.D. Hill, Jr., J.A. Reynolds, and R.L. Hill, *J. Biol. Chem.*, 252 (1977) 3791–3798.
26 S.E. Tollefsen and R. Kornfeld, *J. Biol. Chem.*, 258 (1983) 5172–5176.
27 V. Piller, F. Piller, and J.-P. Cartron, *J. Biol. Chem.*, 261 (1986) 14069–14075.