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IMMUNOCHEMICAL STUDIES ON THE *N*-ACETYLLACTOSAMINE β-(1→6)-LINKED TRISACCHARIDE SPECIFICITY OF *Ricinus communis* AGGLUTININ*,†

ALBERT M. WU‡, SHUNJI SUGII‡

Department of Veterinary Pathology, Texas A & M University, College of Veterinary Medicine, College Station, Texas 77843 (U.S.A.)

FLAVIO G. GRUEZO, AND ELVIN A. KABAT§

Departments of Microbiology, Human Genetics and Development, and Neurology, and the Cancer Center/Institute for Cancer Research, Columbia University of Physicians and Surgeons, New York, 10032 (U.S.A.)

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ABSTRACT

The combining site of *Ricinus communis* agglutinin (RCA_1) was studied by quantitative precipitin and precipitin inhibition assays. Of 31 complex carbohydrates tested, all except active and inactive antifreeze glycoproteins, Streptococcus group C polysaccharide, and native rat salivary glycoprotein, reacted strongly, and 22 completely precipitated the lectin, indicating that RCA_1 has both a broad range of affinity and a low solubility of its carbohydrate-bound complex. Of the monosaccharides and glycosides tested for inhibition of precipitation, *p*-nitrophenyl β-D-galactopyranoside was the best. It was about 6.4 times better than methyl β-D-galactopyranoside. The β anomer of glycosides of D-galactose was much more potent than the corresponding α anomer. Among the oligosacharides tested, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal was the best inhibitor, which was ~2/3 as active as *p*-nitrophenyl β-D-galactopyranoside. It was ~1.4 times as active as β-D-Gal-(1→4)-D-GlcNAc (*N*-acetyllactosamine), twice as active as β-D-Gal-(1→3)-D-GlcNAc, and 4.5 times more active than lacto-*N*-tetraose. From the results, it can be concluded that; (a) hydrophobic interaction is important for binding; (b) the combining site of this lectin is at least as large as a trisaccharide; and (c) of the compounds studied, the trisaccharide β-D-Gal*p*-(1→4)-β-D-

*Dedicated to Professor Walter T. J. Morgan.

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‡For reprint request. Present address: Kitasato University, Department of Serology and Immunology, Kitasato, Sagamihara, Kanagawa, Japan.

§To whom correspondence should be addressed.

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Glc*p*NAc-(1→6)-D-Gal was the most complementary to the human blood group I Ma determinant β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal.

INTRODUCTION

Ricinus communis agglutinin (RCA_1), which agglutinates human erythrocytes of the four ABO blood groups, is a lectin specific for a linked β-D-galactopyranosyl group[1–6]. It has been widely used as a tool for studying the carbohydrate structures of the membrane surface of mammalian cells[7–11]; viruses[12], and glycolipids[13–17]. RCA_1 has a molecular weight of 120 000 consisting of two A chains (M_r 29 500) and two B chains (M_r 37 000) (ref. 11). During the past decade, the carbohydrate specificity of the agglutinin and its affinity properties have been extensively studied[1–6]. It was found to be specific for *N*-acetyllactosamine. Branched oligosaccharides having β-D-Gal*p*-(1→ linked at nonreducing ends enhanced the binding reactivity[1–6]. However, some of the binding properties of the agglutinin, as well as its combining-site size for carbohydrate, have not been well defined. The purpose of the present communication is to demonstrate the affinity of RCA_1 for various glycoproteins and polysaccharides, effects of volume on the precipitability of lectin–glycoconjugate complexes, and also to report a well-defined carbohydrate specificity of RCA_1 for human blood group I Ma determinants and related compounds.

EXPERIMENTAL

Lectin. — The *Ricinus communis* agglutinin, purchased from Boehringer (Mannheim GmbH, West Germany) was diluted with phosphate-buffered saline solution (PBS, pH 6.8) to 2.0 mg/mL and centrifuged to remove insoluble material.

Glycoproteins and polysaccharides. — The purified blood group substances used were prepared from human ovarian cyst fluid or saliva, and from horse, cow, or hog gastric mucosa[18–24]. The blood group substances were purified from human ovarian cyst fluid by digestion with pepsin and precipitation with ethanol; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being given after the name of the blood group substance (e.g., Cyst Beach *Φ*-OH) insoluble). The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations. The designation 10 or 20% (ppt.) denotes a fraction precipitated from phenol at an ethanol concentration of 10 or 20%; 2X signifies that a second phenol extraction and ethanol precipitation were carried out (e.g., Cyst OG 20% 2X). Regardless of their A, B, H, or Le^a activity, the purified water-soluble blood group substances have a similar overall structure. They are polydispersed macromolecules (M_r 200 000–1 000 000) of similar composition (75 to 85% of carbohydrate, 15 to 20% protein). They consist of multiple heterosaccharide branches attached by glycosidic linkages at their internal reducing ends to serine or threonine of the polypeptide backbone[18,19,21–27].

In general, the P*1* fractions represent the nondialyzable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0 for 2 h which removed most of the L-fucopyranosyl end groups, as well as some blood group A and B active oligosaccharide side-chains[24,25,28,29]. The 1st- and 2nd-Smith-degraded products of blood group A active substances (MSS 10 percent 2X), in which almost all of the sugar groups at the nonreducing ends were removed[26], were prepared by procedures described earlier[18,27,30]. The Tamm and Horsfall glycoprotein was from Dr. W. M. Watkins[31].

The pneumococcus type XIV polysaccharide (Squibb No. 227, Lot. 80320) was prepared as previously[32,33]. It has repeating unit **1** (refs. 32, 33).

The inactive and active antifreeze glycoproteins were provided by Dr. R. E. Feeney through Professor Michael Heidelberger.

The active antifreeze glycoprotein from the Antarctic fish is composed[34] of repeating units of the diglycosyltripeptide **2**. The mol. wts. of these glycoproteins vary[35] from 10 500 to 21 000. The inactive antifreeze glycoproteins showed[35] a mol. wt. range from 2600 to 3800. Their structures are similar to those of the active preparation except that proline is present[35]. The inactive glycoproteins were initially thought not to lower the freezing point, but in later work[35–37], they were reported to have some activity.

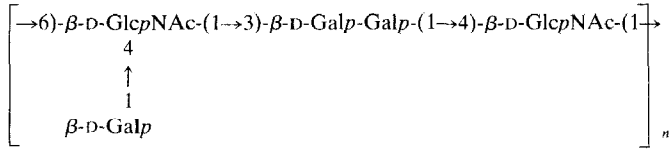
Streptococcal group C polysaccharides were from Dr. R. M. Krause[38]. They have terminal nonreducing D-Gal*p*NAc groups linked to a rhamnose backbone[38].

Fetuin was from Gibco (Grand Island, New York). It is the major glycoprotein in fetal calf serum[39] with a mol. wt. of 48 400 (ref. 40), and is composed of 78% amino acids, 8.7% sialic acid, 6.3% hexosamine, and 8.3% neutral sugar[40]. It has three oligosaccharide side-chains of two structures (**3** and **4**) per molecule, *O*-glycosyl-linked to Ser or Thr residues of the protein core, as well as three identical carbohydrate side-chains (**5**) per molecule, *N*-glycosyl-linked to asparagine[41].

The rat sublingual glycoprotein was prepared by the method of Moschera and Pigman[42]. It has a mol. wt. of $2.2 \cdot 10^6$ and is composed of 17% protein and 81% carbohydrate[42]. The carbohydrate side-chains, which are *O*-glycosyl-linked to the Ser or Thr residues of the protein core, have 9, 10, 12, 13, and 15 sugar residues with 4-*O*-acetyl- and 4,7-di-*O*-acetyl-NeuNAc, and GlcNAc groups at nonreducing ends[14] and a repeating unit, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-, as carbohydrate core structure[43].

For desialylation, a sample of glycoprotein was hydrolyzed with 0.01M HCl for 90 min at 80°, and dialyzed against water.

Sugar inhibitors. — Mono- and di-saccharides, melibiose, raffinose, and stachyose, were purchased from Eastman Organic Chemical Co., (Rochester, New York), Sigma Chemical Co., (St. Louis, Missouri), and Nutritional Biochemicals (Cleveland, Ohio). The methyl α-D- and β-D-galactopyranosides were from Dr. A. B. Pardee, Harvard University Medical School (Boston, Massachusetts). Lacto-*N*-tetraose was obtained from the late Dr. Richard Kuhn (Max Planck Institute for Biochemistry, Heidelberg, Germany). β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-α-D-



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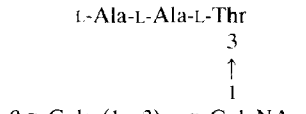

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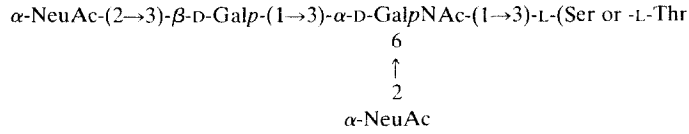

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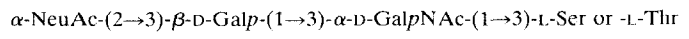

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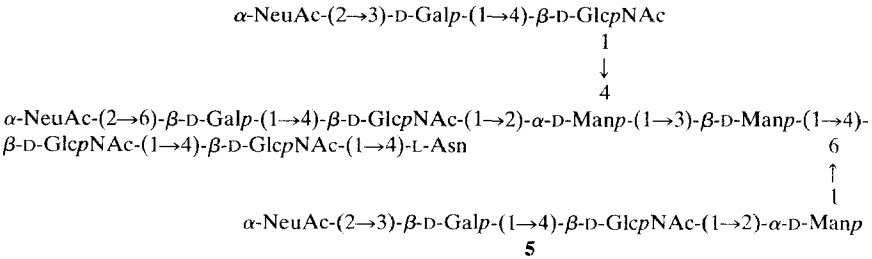

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Scheme 1

GalpNAcO$(CH_2)_8CO_2Me$ was synthesized by Dr. R. U. Lemieux. β-D-Gal-(1→4)-D-GlcNAc was from Dr. F. Zilliken (cf. ref. 44).

Immunochemical assays. — Quantitative precipitin and precipitin inhibition assays were performed by a microprecipitin technique[45] using 5.4 μg of protein N for each determination; total N in the washed precipitates was estimated by the ninhydrin method[46].

RESULTS

Effects of volume on the precipitation of glycoproteins and polysaccharides by RCA_I. — Fig. 1 shows the effect of volume on the ability of the lectin to precipitate

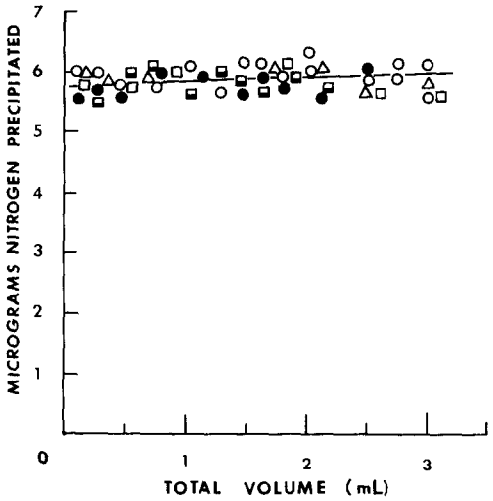

Fig. 1. Effect of volume on the precipitation of glycoprotein by *Ricinus communis* agglutinin; 5.8 mg N *Ricinus communis* agglutinin, ○—○ +21 μg Cyst MSS 1st Smith degraded glycoprotein; ●—● +6.4 μg Pneumococcus type XIV polysaccharides; ◘—◘ + 15 μg Cyst OG 20% from 10% (I); △—△ +11 μg Cyst Beach P1; □—□ +20 μg Saliva PM (B).

glycoproteins, with or without blood group activity, and polysaccharides. Among five glycoproteins tested, all of the lectin–glycoconjugate complexes were unusually stable to changes in volume, indicating a low solubility. No significant change was found with an increase in volume to 3.0 mL.

Quantitative precipitin assays. — Quantitative precipitin curves of RCA_1 with glycoproteins with and without blood group activity, and their Smith or mild acid degraded products are shown in Fig. 2. Of 31 complex carbohydrates tested, all, except active and inactive antifreeze glycoproteins, Streptococcus group C polysaccharide, and rat salivary glycoprotein reacted strongly, and 22 completely precipitated the agglutinin. The most reactive glycoproteins, such as Pneumococcus type XIV polysaccharide, mild acid-hydrolyzed rat salivary glycoprotein, cow 21 phenol insoluble, and Cyst Beach phenol insoluble, completely precipitated the lectin with less than 3.0 μg required for 50% precipitation (Table I). These results indicate that RCA_1 has a broad range of affinity and a low solubility of its complexes with blood group glycoproteins.

Quantitative precipitin-inhibition assays. — The abilities of various sugars to inhibit the precipitation of RCA_1 lectin by Smith-degraded human blood group A active glycoprotein (Cyst MSS 1st Smith degraded) are shown in Fig. 3, and Tables II and III. The quantities (μmols.) required for 50% inhibition were estimated from Fig. 3 and shown in Table II. Among the monosaccharides and their glycosides tested, *p*-nitrophenyl β-D-galactopyranoside (curve 1) was the best inhibitor, with 0.22 μmol being required for 50% inhibition, and was 14.6 times as active as D-galactose (curve 10). It was also ~6.4 times better than methyl β-D-galactopyranoside (curve 7), indicating that the hydrophobic interaction is important for binding. The β anomer of D-galactopyranosides was more potent than the corre-

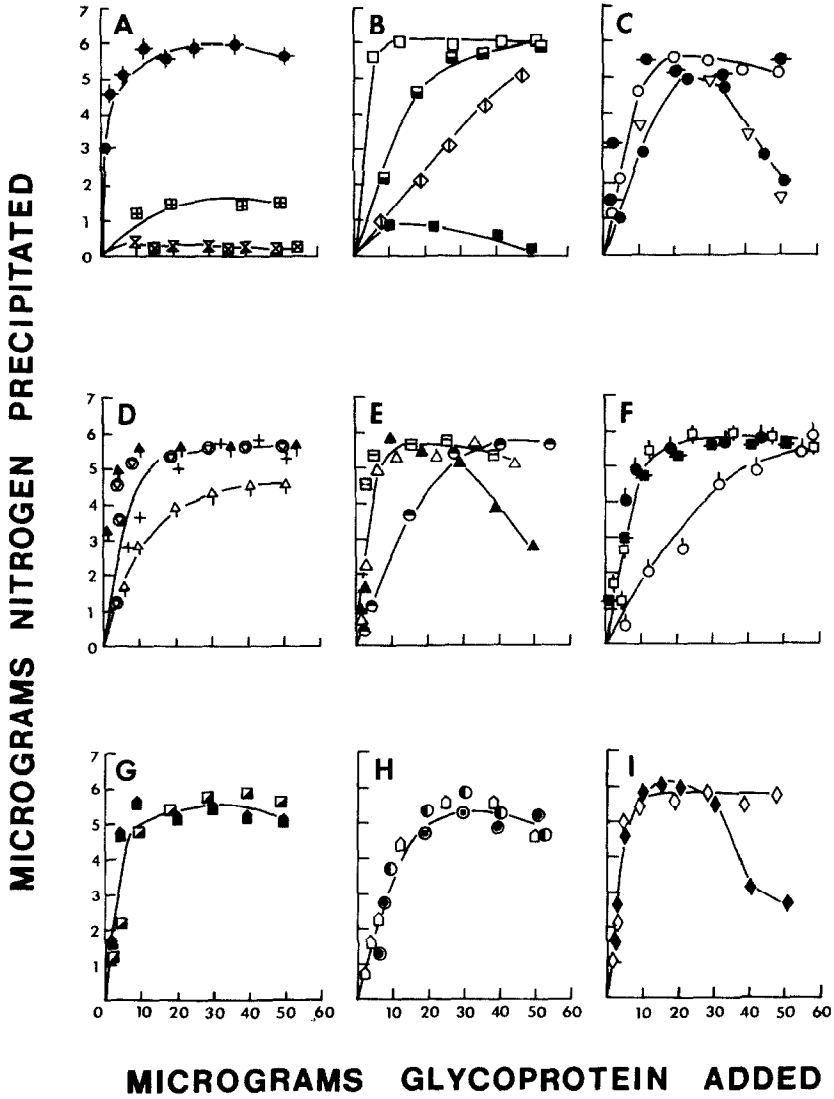

Fig. 2. Quantitative precipitation curves of *Ricinus communis* agglutinin with various glycoproteins with or without blood group activity of their Smith or mild acid-degraded products. See conditions and symbols in Table I.

sponding α anomer (curves 1A *vs.* 12, and 7 *vs.* 11). Among the oligosaccharides tested, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal, the human blood group I Ma specific trisaccharide (curve 1), was the best inhibitor, and was ~2/3 as active as *p*-nitrophenyl β-D-galactopyranoside. It was ~1.14, 1.40, and 2.0 times as active as β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man (curve 2), β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyllactosamine, curve 3), and β-D-Gal*p*-(1→3)-D-GlcNAc (curve 6), respectively. It was also 4.5 times more active than lacto-*N*-tetraose (curve 8),

TABLE I

COMPARISON OF PRECIPITATING ACTIVITIES OF *Ricinus communis* AGGLUTININ WITH VARIOUS GLYCOPROTEINS AND POLYSACCHARIDES[a]

Symbol	*Curve in Fig. 2*	*GP or PS*[a,b]	*Maximum μgN precipitated*[c]	*GP or PS giving 50% precipitation*[a]
⊞	A	Active antifreeze GP	1.5 (28)	
⊠	A	Inactive antifreeze GP	0.2 (4)	
⊕	A	Pneumococcus type XIV PS	5.9 (109)	1.0
⧖	A	Streptococcus group C PS	0.2 (4)	
◈	B	Porcine thyroglobulin	5.2 (96)	23.5
■	B	Rat sublingual GP (RSG)	0.8 (15)	
□	B	Desialized RSG	6.0 (111)	3.0
▬	B	Tamm-Horsfall (TH-4) Watkins	6.0 (111)	10.0
▽	C	Cyst MSM 10% ppt (A_1)	5.0 (93)	7.0
●	C	Cyst 9 Φ-OH[a] insoluble (A_1)	5.0 (93)	7.0
○	C	Cyst 14 10% Φ-OH insoluble (A)	5.5 (102)	4.0
⊕	C	Hog 32 10% ppt (A + H)		
		P1 (mild acid hydrolyzed)	5.5 (102)	3.5
ⓥ	D	Cyst McDon 15% ppt (A)		
		P1 (mild acid hydrolyzed)	5.5 (102)	4.0
+	D	Cyst MSS 10% 2X (A_1)	5.6 (104)	5.0
▲	D	MSS 1st Smith degraded	5.5 (102)	1.0
△	D	MSS 2nd Smith degraded	4.5 (83)	10.0
▲	E	Cyst Beach Φ-OH insoluble (B)	5.8 (107)	3.0
△	E	Beach P1 (mild acid hydrolyzed)	5.6 (104)	2.5
◓	E	Cow 21 Φ-OH insoluble (B)	5.6 (109)	13.0
⊟	E	Cow 21 P1 (mild acid hydrolyzed)	5.4 (100)	1.0
■	F	Salivary PM Φ-OH insoluble (B)	5.6 (104)	5.0
⊡	F	Horse 4 25% ppt (B)	5.6 (104)	5.0
○	F	Fetuin	5.6 (104)	16.0
●	F	Asialofetuin	5.8 (107)	3.0
◪	G	Cyst N-1 Φ-OH insoluble (Le^a)	5.8 (107)	4.0
⬟	G	Cyst N-1 10% 2X (Le^a)	5.5 (102)	3.0
⬠	H	Cyst Tighe Φ-OH insoluble (H)	5.5 (102)	8.0
◉	H	Cyst JS Φ-OH insoluble (H)	5.2 (96)	8.0
◐	H	Morgan standard (H)	5.7 (106)	7.5
◇	I	Cyst OG 10% from 20% (I)	5.5 (102)	3.5
◆	I	Cyst OG 20% from 10% (I)	5.5 (102)	2.5

[a]Abbreviations: GP, Glycoproteins; PS, polysaccharide; ppt, precipitate; and Φ-OH, phenol. [b]The symbol in parentheses indicates the human blood group activity. [c]The value in parentheses indicates the % of μgN precipitated at maximum when the amount of lectin added is expressed as 100% (= 5.4 μgN).

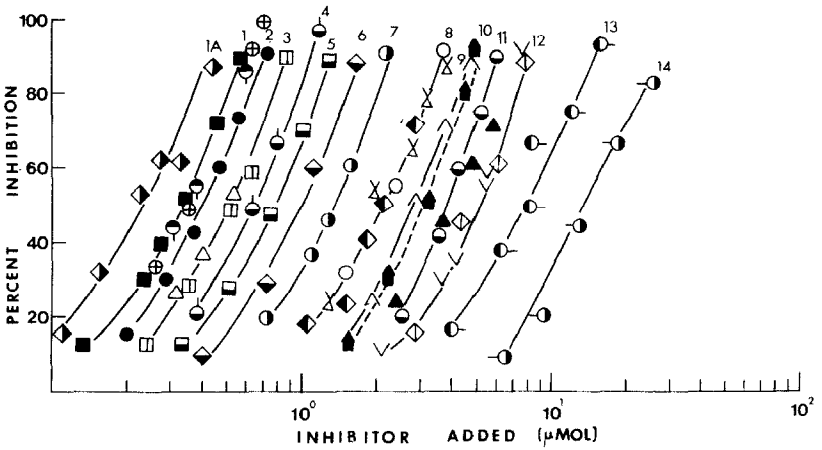

Fig. 3. Inhibition by monosaccharides, glycosides, and oligosaccharides, of precipitation of *Ricinus communis* agglutinin by Smith-degraded human blood group A active glycoprotein (Cyst MSS 1st Smith degraded); 5.4 μg of lectin plus 20 μg of Smith degraded glycoprotein; total volume 250 μL: (1A) *p*-nitrophenyl β-D-galactopyranoside (◑); (1) *O*-nitrophenyl β-D-galactopyranoside (○), phenyl β-D-galactopyranoside (◓), and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal (■); (2) β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man (●); (3) β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcO$(CH_2)_8CO_2Me$ (△); β-D-Gal*p*-(1→4)-D-GlcNAc (lactosamine) (◫); (4) β-D-Gal*p*-(1→6)-D-Gal (◒); (5) β-D-Gal*p*-(1→4)-D-Glc (lactose) (◘); (6) β-D-Gal*p*-(1→3)-D-GlcNAc (◆); (7) methyl β-D-galactopyranoside (◑); (8) (Lacto-*N*-tetraose) (◧), α-D-Gal*p*-(1→3)-D-Gal (○), and α-D-Gal*p*-(1→6)-D- β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-D-Glc (melibiose) (△); (9) (raffinose) (△); (10) D-galactose (▲); (11) methyl α-D-galactopyranoside (◒); (11A) L-rhamnose (▲); (12) α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-D-Fru*f* (stachyose) (▽) and *p*-nitrophenyl α-D-galactopyranoside (◧); (13) D-fucose (◐)-; and (14) L-arabinose -(◑).

demonstrating that the combining site of the lectin is at least as large as a trisaccharide and, of the compounds studied, it was most complementary to the human blood group I Ma determinant (curve 1).

DISCUSSION

In the present study, it was demonstrated that RCA_1 is most specific to oligosaccharides containing a β-D-Gal*p*-(1→4)-D-GlcNAc group at the nonreducing end, β-linked to a third sugar residue. Among the oligosaccharides tested, human blood group I Ma active trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal was the best inhibitor. As summarized with previous reports (Table IV) (refs. 4–6), it can be concluded that β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2 or 6)-D-Man were the best, which are ~1.25 times more active than *N*-acetyllactosamine. When the relative potency of monosaccharides were compared (Table IV), it was found that *p*-nitrophenyl β-D-galactopyranoside is the most potent monosaccharide, which is ~15 times more active than D-galactose. The inhibition profiles obtained from precipitin-inhibition assay were in general agreement with earlier studies[4–6]. Differences in the relative inhibitory powers found by various laboratories are within experimental error.

TABLE II

AMOUNT OF VARIOUS SUGAR GROUPS OF 50% INHIBITION OF PRECIPITATION OF *Ricinus communis* AGGLUTININ BY SMITH-DEGRADED BLOOD GROUP A ACTIVE (CYST MSS) GLYCOPROTEIN[a]

Curve number in Fig. 3	*Inhibitor*	*Quantity giving 50% inhibition (μmol)*	*Relative potency[b]*
1A	β-D-Gal*p*OC$_6$H$_4$NO$_2$(*p*)	0.22	14.6
1	β-D-Gal*p*OC$_6$H$_4$NO$_2$(*o*)	0.35	9.1
1	β-D-Gal*p*OPh	0.35	9.1
1	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal (Human blood group I Ma specific trisaccharide)	0.35	9.1
2	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man	0.40	8.0
2A	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcO(CH$_2$)$_8$CO$_2$Me	0.50	6.4
3	β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyllactosamine)	0.50	6.4
4	β-D-Gal*p*-(1→6)-D-Gal	0.63	5.1
5	β-D-Gal*p*-(1→4)-D-Glc (lactose)	0.80	4.1
6	β-D-Gal*p*-(1→3)-D-GlcNAc	1.00	3.2
7	β-D-Gal*p*OMe	1.40	2.3
8	β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-D-Glc (Lacto-*N*-tetraose)	2.20	1.5
8	α-D-Gal*p*-(1→3)-D-Gal	2.20	1.5
8	α-D-Gal*p*-(1→6)-D-Glc (melibiose)	2.20	1.5
9	α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-D-Fru*f* (raffinose)	2.80	1.1
10	D-Galactose	3.20	1.0
11	α-D-Gal*p*OMe	3.90	0.82 (1.2)
11A	L-Rhamnose	4.00	0.80 (1.25)
12	α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-D-Fru*f* (stachyose)	5.20	0.62 (1.6)
12	α-D-Gal*p*OC$_6$H$_4$NO$_2$(*p*)	5.20	0.62 (1.6)
13	D-Fructose[c]	8.20	0.39 (2.7)
14	L-Arabinose[c]	14.00	0.23 (4.3)

[a]5.4 μg of lectin + 20 μg of MSS 1st Smith-degraded glycoprotein. Total volume 250 μL. [b]Reciprocal of relative potency in parentheses. [c]L-Fucose and D-arabinose gave no inhibition of precipitation of *Ricinus communis* agglutinin, as shown in Table III.

TABLE III

MAXIMUM QUANTITIES OF VARIOUS SUGARS GIVING NEGLIGIBLE INHIBITION OF PRECIPITATION OF *Ricinus communis* AGGLUTININ BY SMITH DEGRADED BLOOD GROUP A ACTIVE GLYCOPROTEIN CYST MSS)[a]

Inhibitor	*Maximum amount of inhibitor used (μmol)*	*Percentage inhibition*
α-D-Gal*p*NAcOC$_6$H$_4$NO$_2$(*p*)	4.2	−5
β-D-Gal*p*NAcOC$_6$H$_4$NO$_2$(*p*)	1.2	−7
2-Acetamido-2-deoxy-D-galactose	84.8	0
L-Fucose	127.0	1
D-Arabinose	102.0	−2
Maltose	106.0	−5
D-Mannose	113.0	6

TABLE IV

COMPARISON OF RECIPROCAL OF RELATIVE POTENCY OF MONOSACCHARIDES AND OLIGOSACCHARIDES TO D-GALACTOSE IN QUANTITATIVE PRECIPITIN INHIBITION AND HEMAGGLUTINATION INHIBITION

Inhibitor	*Precipitin inhibition*[a]	*Hemagglutination inhibition*		
		Ref. 3	*Ref. 4*	*Ref. 5*
D-Galactose and D-galactopyranosides				
D-Galactose	1.00	1.00	1.00	1.00
Methyl α-D-galactopyranoside	0.82	1.34	0.54	
Methyl β-D-galactopyranoside	1.40	2.44	2.15	
Phenyl α-D-galactopyranoside			<0.28	
Phenyl β-D-galactopyranoside	9.10		6.08	
p-Nitrophenyl α-D-galactopyranoside	0.62	1.50		
p-Nitrophenyl β-D-galactopyranoside	14.60	15.00		
β-D-Galactopyranosyl as terminal nonreducing group β-(1→4)-linked to D-Glc or D-GlcNAc as subterminal residue				
β-D-Gal*p*-(1→4)-D-Glc (Lactose)	4.10	7.80		7.93
β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyllactosamine)	6.40		8.43	16.1
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man	8.00		12.10	
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-D-Man	9.10			
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal	6.40			
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcO(CH$_2$)$_8$CO$_2$Me				

β-D-Galactopyranosyl as terminal nonreducing group β-(1→3)-linked to D-GlcNAc or D-GalNAc as subterminal residue				
β-D-Gal*p*-(1→3)-D-GlcNAc	3.20			
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-D-Glc (Lacto-*N*-tetraose)	1.50			
β-D-Gal*p*-(1→3)-D-GalNAc				1.00
β-D-Gal*p*-(1→3)-β-D-GalNAcOBzl				1.00
β-D-Galactopyranosyl as terminal nonreducing group β-(1→6)-linked to D-GlcNAc or D-Gal				
β-D-Gal*p*-(1→6)-D-GlcNAc				7.93
β-D-Gal*p*-(1→6)-D-Gal	5.10			
α-D-Galactopyranosyl as terminal nonreducing group and α-(1→6)-linked to D-Glc as subterminal residue				
α-D-Gal-(1→6)-D-Glc (melibiose)	1.50	1.22		0.55
α-D-Gal-(1→6)-β-D-Glc*p*-(1→2)-D-Fru*f* (raffinose)	1.10	1.14		
β-D-Galactopyranosyl as terminal nonreducing group α-(1→3 or 6)-linked to D-Gal as subterminal residue				
α-D-Gal*p*-(1→3)-D-Gal (blood group B-specific)	1.50			
α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-D-Fru*f* (stachyose)	0.62			
2-Acetamido-2-deoxy-β-D-glucopyranosyl as terminal nonreducing group				
β-D-Glc*p*-(1→2)-D-Man			0.58	

The inhibitory potency of Gal was taken as 1.0. Blanks occur where testing was not done. The relative potency of different monosaccharide, glycosides, and oligosaccharides were calculated from Fig. 3 and Table 2 for RCA_I. Their relative potencies obtained by hemagglutination inhibition were calculated from the minimum concentration (μM) giving complete inhibition of three or four hemagglutinating doses (4–6).

[a]See Table II.

Consistent with these precipitin-inhibition results (Table I), RCA_I did not show blood group specificity and precipitated all of blood substances with A, B, H, Le^a, Le^b, and precursor I activities, all of which contain incomplete carbohydrate side-chains with β-D-Gal*p*-(1→4 or 3)-D-GlcNAc groups at nonreducing ends as well as many such structures in the core of carbohydrate side-chains. These findings were[11,47] further supported by the nonspecific agglutinability of RCA_I with human erythrocytes and good precipitability of the mild acid-hydrolyzed and Smith-degraded products of blood group substances, in which all A, B, H, Le^a, and Le^b group determinants were abolished and β-D-Gal*p*-(1→4 or 3)-D-GlcNAc residues in the core of carbohydrate side-chains were exposed at nonreducing ends. Thus, it can be assumed that the interaction of RCA_I with blood group glycoproteins is mainly due to β-D-Gal*p*-(1→4 or 3)-D-GlcNAc groups of incomplete carbohydrate side chains. The ability of RCA_I to recognize the internal β-D-Gal-(1→ linked residues has not been well established. However, the results of the shielding effects, obtained by both quantitative precipitin (cow 21 *vs.* cow 21 P-1 in Fig. 2, and rat salivary mucus glycoprotein (RSG) *vs.* desialylated RSG in Fig. 2B) and others[1-3] indicated that the contribution of internal β-D-Gal*p*-(1→4 or 3)-D-GlcNAc residues for binding in these circumstances must be minor or limited. The best precipitinogens were the mild acid-hydrolyzed products of Beach phenol insoluble (Fig. 2E), Cow 21 phenol insoluble (Fig. 2E), pneumococcus type XIV polysaccharide (Fig. 2F), and RSG (Fig. 2B), which gave 50% of maximum precipitation at <2.5 μg. Pneumococcus type XIV polysaccharide shares human blood group I Ma activity[48]. Its good reactivity with RCA_I was consistent with high specificity of RCA_I for the I Ma-active trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal. Porcine thyroglobulin and asialofetuin were also precipitated well by RCA_I, owing also to the branched oligosaccharide side-chains with similar carbohydrate structures containing β-D-Gal*p*-(1→4)-GlcNAc residues[49-53]. Although RCA_I is recognized as specific for terminal β-D-Gal*p* groups, the disaccharide of mucin type sequence, β-D-Gal*p*-(1→3)-D-GalNAc was reported to be as active as D-galactose, which is ~1/6 as active as *N*-acetyllactosamine (Table IV). However, two β-D-Gal*p*-(1→3)-D-GalNAc groups linked by peptides enhanced some activities[1]. RCA_I did not precipitate well with active and inactive antifreeze glycoproteins, which are composed of repeat units of β-D-Gal*p*-(1→3)-D-GalNAc. The poor precipitability may be mainly due to the high solubility of the lectin–glycoprotein complex[54,55].

Recently, it was reported[56,57] that both *Erythrina* and *Datura* lectins are also specific for β-D-Gal*p*-(1→4)-D-GlcNAc. Their reactivities towards linear and branched oligosaccharides containing *N*-acetyllactosamine are summarized in Table V. All branched oligosaccharides with β-D-Gal*p*-(1→4)-D-GlcNAc at nonreducing ends tested were more active than the corresponding linear oligosaccharides. Especially, *Datura* lectin showed a >480-fold higher specificity for a biantennary 2,6-linked pentasaccharide, which contains two groups of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-D-Man at nonreducing ends, than for one of its branched trisaccharides. This is one of the examples of the cooperative binding effects of branched oligosaccharides or

TABLE V

COMPARISON OF RECIPROCAL OF RELATIVE POTENCY OF LINEAR OLIGOSACCHARIDES AND BRANCHED OLIGOSACCHARIDES HAVING β-D-Gal*p*-(1→4)-D-Glc*p*NAc NONREDUCING END-GROUPS TO β-D-Gal*p*-(1→4)-D-GlcNAc[a]

Inhibitors	*DSA*[b] (*PIA*[c])	*ECS*[d] (*PIA*)	*RCA_I*		
			PIA[e]	*HIA-I*[f] (4)	*HIA-II*[f] (2)
Branched oligosaccharide of polysaccharide with N-acetyllactosamine as nonreducing end-group					
Biantennary					
Penta-(2,6)	480	2.9			
Penta-(2,4)	19				
Hexa-(3,6)	11				
Hepta	3				
Octa					(7.6)[f]
Triantennary					
(2 and 4)		2.9			
Nona II	260				
Nona I	1				
Tetraantennary					
Undeca	188	3.4			
Linear oligosaccharide with N-*acetyllactosamine as nonend-group linked to*					
β-(1→6)-D-Gal		1.5	1.4		
β-(1→6)-D-Man	0.6	1.2	1.3		
β-(1→2)-D-Man	0.18			1.5	
β-(1→6)-α-D-Gal*p*NAcO$(CH_2)_8CO_2Me$		1.5	1.0		
(*N*-Acetyllactosamine)[g]	1.0	1.0	1.0	1.0	1.0[h]

[a]The inhibitory potency of β-D-Gal*p*-(1→4)-D-GlcNAc was taken as 1.0. [b]DSA, *Datura stramonium* agglutinin, which also has specific oligosaccharides having a β-D-Glc*p*NAc-(1→4) group at nonreducing end (56). [c]PIA, Precipitin inhibition assay. [d]ECS, *Erythrina crystagalli* (58). [e]From Fig. 3 and Table II. [f]HIA, Hemagglutination-inhibition assay. [g]Free sugar taken as base unit. [h]Calculated value from asialo form of sialoside 9 in Table IV, ref. 12. It is assumed that *N*-acetyllactosamine is 6.4 times more active than D-galactose.

polysaccharides. Some of such effects are also found[1] in RCA_1, since a biantennary 2,6-linked pentasaccharide is ~49 times more active than D-galactose which is estimated at ~7.6 times more active than *N*-acetyllactosamine. The cooperative binding effect to RCA_1 could not be confirmed in this study because of unavailability of branched oligosaccharides containing β-D-Gal*p*-(1→4)-D-GlcNAc groups. However, in combination with previous reports[1–6], it can be concluded that the combining site of RCA_1 is most complementary to human blood group I Ma-active trisaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal, and branched oligosaccharides with β-D-Gal*p*-(1→4)-D-GlcNAc groups at nonreducing ends are more potent than a linear human blood group I Ma-active trisaccharide.

RCA_1 differs sharply in specificity from *Sophora japonica* lectin in that 2-

acetamido-2-deoxy-D-galactose and its *p*-nitrophenyl α- and β-glycosides are inactive with RCA_1, whereas they were the best inhibitors of *Sophora japonica* lectin[58].

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