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IMMUNOCHEMICAL STUDIES ON THE COMBINING SITE OF THE BLOOD GROUP A-SPECIFIC LIMA BEAN LECTIN*

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ABSTRACT

The combining site of the lima bean (*Phaseolus lunatus*) lectin (LBL) was studied by quantitative precipitin and precipitin-inhibition assays. The lectin precipitated best with hog gastric mucosa and human ovarian cyst blood group A_1 substances and moderately with A_2 substances. B substances precipitated very poorly and H, Le^a, Le^b, and precursor I substances did not react. Blood group A_1 and A_2 substances reacted to varying extents and these differences are attributable to heterogeneity resulting from incomplete biosynthesis of carbohydrate chains. By inhibition of precipitation of LBL with A_1 blood group substance, the lectin was found to be most specific for fucose-containing oligosaccharides having the A trisaccharide, DGalNAcα1→3[L-Fucα1→2]DGal determinant. The best inhibitor, an A-specific hexasaccharide, DGalNAcα1→3[LFucα1→2]DGalβ1→3DGlcNAcβ1→3-DGalβ1→4DGlc, was 11 times more active than the A trisaccharide. A difucosyl oligosaccharide with a second fucose linked α1→3 to the DGlcNAc is less active; fucose linked α1→4 to DGlcNAc was completely inactive. These results suggest that specific interactions with the subterminal sugars may be important in the binding, and that the specificity of the lectin combining site involves at least the nonreducing terminal four and probably five sugars of the hexasaccharide. Thus LBL has a more-extended binding site than was inferred previously and is in the upper range of antibody combining-site sizes.

INTRODUCTION

Lectins are carbohydrate-binding proteins and glycoproteins of nonimmune origin which have been isolated from a variety of plant and animal sources[1–3]. Their main characteristic is their ability to bind sugars specifically, and thus they may

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agglutinate erythrocytes, lymphocytes, and tumor cells and react with certain blood group substances, glycolipids, or glycoproteins to form precipitates analogous to those of antigen–antibody reactions. They are valuable reagents for the detection, isolation, and characterization of glycoconjugates and polysaccharides[4], in blood grouping, and in the determination of secretor status[5,6]. Lectins are used extensively to probe functions of cell-surface carbohydrates[1–3], to isolate subpopulations of lymphocytes[7–9] to evaluate the distribution and movement of cell-surface receptors[10], and to clarify mechanisms of membrane-mediated T-lymphocyte activation[11,12].

Lima bean lectin (LBL) is an anti-A agglutinin which binds glycoconjugates containing terminal nonreducing αDGalNAc residues[13–16]. It was purified in two active forms, designated components II (M_r = 247,000) and III (M_r = 124,000)[16–18]. A single subunit (M_r = 31,000) contained 6–7 moles of mannose, 2 of glucosamine, 1 fucose, and traces of pentose[16,17]; four or eight subunits are combined in components III and II, respectively[18,19]. Component I, of even higher molecular weight, also has the same subunit[20]. In addition to the sugar-binding site, LBL has receptors[16,17,21] for Ca^{2+} and Mn^{2+} and also binds nonpolar ligands[22], including 1,8-anilinonaphthalenesulfonate, 2,6-toluidinylnaphthalenesulfonate, and *N*-phenyl-1-naphthylamine. Carbohydrate is not bound unless the sites for divalent cations are occupied[16,17].

Most lectins from legumes are poor in sulfur-containing amino acids[2] and usually have no cysteine, but LBL contains two cysteine residues per subunit[16,18]; one forms an intersubunit disulfide bond[18], the second is free and is required for carbohydrate binding[19]. Several sulfhydryl reagents namely dithiobis(2-nitrobenzoic acid) (Nbs_2), *N*-ethylmaleimide, mercurials, and Cu^{2+} inactivated the lectin[16,19]. Inactivation of LBL by Nbs_2 and by *N*-ethylmaleimide was inhibited by DGalNAc but not by DGlcNAc[19]. The location of and amino acid sequence surrounding the free cysteine required for carbohydrate binding in LBL were also determined[23]. Among all the carbohydrate ligands used, a blood group type A trisaccharide with a linker arm, DGalNAcα1→3[LFucα1→2]DGalβ1→$O(CH_2)_8$-CO_2Me was[24] the most potent inhibitor of thiol modification by Nbs_2. Although the combining site of the LBL has been studied[16,24,25], the fine specificity of its carbohydrate-binding site is still not precisely defined. In the present study, this was explored further by quantitative precipitation of the lectin with A_1, A_2, B, H, Le^a, Le^b, and precursor I and i blood group substances and by inhibition of precipitation using monosaccharides, glycosides, and various oligosaccharides, particularly blood group A tri- to hepta-saccharides containing fucose.

EXPERIMENTAL

Materials and methods. — Lectin. Lima bean lectin (components I, II, and III) from green lima beans (*Phaseolus lunatus*, Thorogreen or Sieva varieties) was prepared as described previously[20] by affinity chromatography on a blood group

A-active trisaccharide (DGalNAcα1→3[LFucα1→2]DGalβ1→) coupled to Synsorb (Synsorb A), followed by elution with 0.1M acetic acid or 0.4M GalNAc. The preparation used in the present study is a mixture of components II (10%) and III (90%), both having the same specificity. All buffers were degassed, saturated with N_2, and the lectin solution in PBS was also maintained under an atmosphere of N_2, to avoid oxidation of the free thiol groups.

Blood group substances. The purified blood group A, B, H, Le^a, Le^b, and precursor Ii substances used were prepared in the laboratory from human ovarian cyst fluid or saliva and from horse, bovine, or hog gastric mucosa[26–31]. The P1 fraction of A_1 substance McDon 15% (ref. 32) constitutes the non-dialyzable portion of the blood group substance after mild acid hydrolysis at pH 1.5 to 2.0 for 2 h at 100°; this removed most of the α-L-fucosyl end groups as well as some of the group A-active side chains[27,33,34].

Mono- and oligo-saccharides as inhibitors. Various monosaccharides were from Sigma Chemical Company (St. Louis, Mo.), Eastman Organic Chemical Company (Rochester, N.Y.), and Nutritional Biochemicals (Cleveland, Ohio). Methyl 2-acetamido-2-deoxy-α- and β-D-galacto-pyranosides and -furanosides were available in the laboratory[35].

DGalNAcα1→3DGalNAcβ-O$(CH_2)_8CO_2$Me and Forssman trisaccharide aglycon, DGalNAcα1→3DGalNAcβ1→3DGalα-O$(CH_2)_8CO_2$Me were synthesized by Professor R. U. Lemieux, University of Alberta, Canada (R. U. Lemieux, P. Hermentin, and R. M. Ratcliffe, unpublished experiments). DGalNAcα1→3-DGal (R_L 1.34), DGalNAcα1→3DGalβ1→3DGlcNAc (A_5II), DGalNAcα1→3[L-Fucα1→2]DGal and DGalNAcα1→3[LFucα1→2]DGalβ1→4DGlcNAcβ1→6R (MSM AR_L 0.56) used were purified and isolated in the laboratory from blood group A substances as described previously[32,36–39]. The O1 trisaccharide was from Dr. Donald Baker[40]. The group A tetra-, A penta-, A hexa-, and A hepta-saccharides were gifts from Dr. A. Lundblad[41,42].

Immunochemical assays. Quantitative precipitin and precipitin-inhibition assays were performed by a microtechnique[43] using 12.6 μg of lectin N (nitrogen) in a total volume of 200 μL with saline (0.15M NaCl) for each determination. The tubes were incubated for 1 h at 37° and then kept for one week at 4° with daily mixing. Total nitrogen in the washed precipitate was determined by the ninhydrin method[37].

RESULTS

Quantitative precipitin assays. — Precipitin curves, showing the total N precipitated by various amounts of A, B, H, Le^a, Le^b, and precursor Ii blood group substances are shown in Fig. 1 and Table I. The lectin reacts best with A_1 blood group substances, particularly all of the hog gastric mucosa preparations, precipitating 6.2–7.3 μg of lectin N; 3.3–6.7 μg of these blood group substances are required for 50% of maximum precipitation. An amount of 7.5 μg of N is precipitated by hog A + H with 15.5 μg precipitating 50% of maximum.

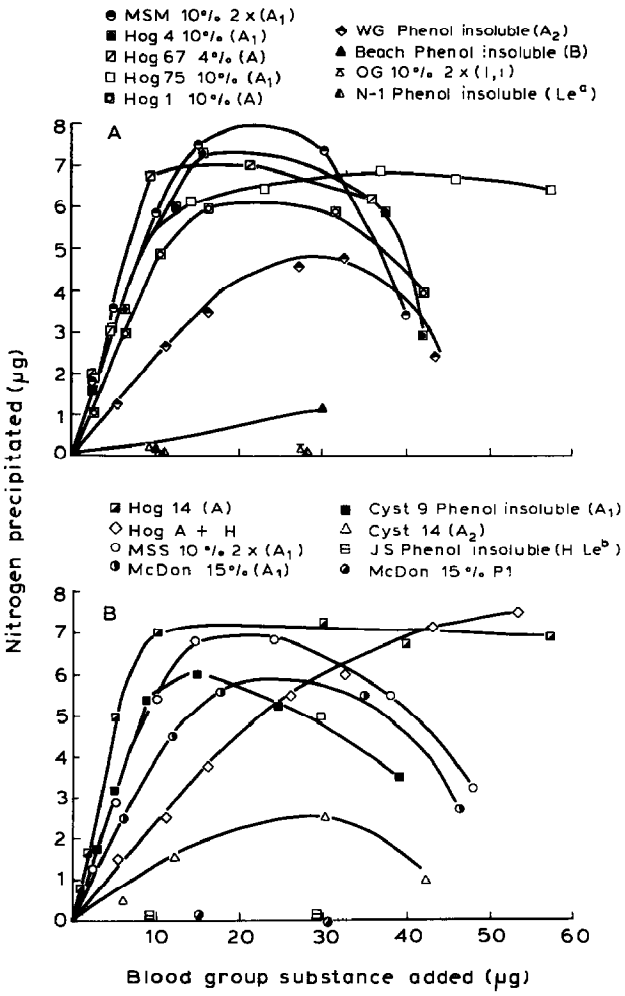


Fig. 1. Quantitative precipitin curves of lima bean lectin (12.6 μg of N) with various blood group substances; total volume 200 μL.

Human A_1 blood group substances, cyst MSM 10% 2X, and cyst MSS 10% 2X react strongly with this lectin, precipitating 7.8 and 6.9 μg N, respectively, and their respective amounts for 50% of maximum precipitation are 5.8 and 6.0 μg. Cyst 9 phenol-insoluble, A_1 specific, precipitates 5.9 μg N and 4.5 μg is required for 50% of maximum precipitation. Another A_1 substance, McDon 15%, reacts moderately with the lectin, precipitating 5.9 μg N; but the corresponding P_1 fraction failed to react with the lectin. The lectin has somewhat weaker activity with two blood group A_2 substances; each reacted to a different extent. WG phenol-insoluble precipitates 4.9 μg of N and cyst 14 phenol-insoluble precipitates 2.5 μg of lectin N, with 10.5 and 9.3 μg, respectively, giving 50% of maximum precipitation.

TABLE I

BLOOD GROUP SUBSTANCES USED IN QUANTITATIVE PRECIPITATION OF LIMA BEAN LECTIN AS SHOWN IN FIG. 1

Figure	*Symbol*	*Blood group substance*	*Maximum N precipitated (μg)*	*Blood group substance required for 50% of maximum precipitation (μg)*
1A				
	◓	Cyst MSM 10% 2X (A_1)	7.8	5.8
	◙	Hog 4 10% (A_1)	7.3	6.3
	◪	Hog 67 4% (A)	7.0	4.5
	□	Hog 75 10% (A_1)	7.0	5.0
	⊡	Hog 1 10% (A)	6.2	6.7
	◆	Cyst WG phenol-insoluble (A_2)	4.9	10.5
	▲	Cyst Beach phenol-insoluble (B)	1.1	15.3
	⍓	Cyst OG 10% 2X (Ii)		
	◭	Cyst N-1 Phenol-insoluble (Le^a)		
1B				
	◪	Hog 14 (A)	7.3	3.3
	◇	Hog A + H	7.5	15.5
	○	Cyst MSS 10% 2X (A_1)	6.9	6.0
	◑	Cyst McDon 15% (A_1)	5.9	7.0
	■	Cyst 9 phenol-insoluble (A_1)	5.9	4.5
	△	Cyst 14 phenol-insoluble (A_2)	2.5	9.3
	⊟	Cyst JS phenol-insoluble (H, Le^b)		
	◕	Cyst McDon 15% P1		

The lectin has very poor activity for blood group B substances. Beach phenol-insoluble precipitates only 1.1 μg of N, with 15.3 μg needed for 50% precipitation. No precipitate was obtained with JS phenol-insoluble (H, Le^b), N-1 phenol-insoluble (Le^a), and OG 10% 2X (Ii) preparations.

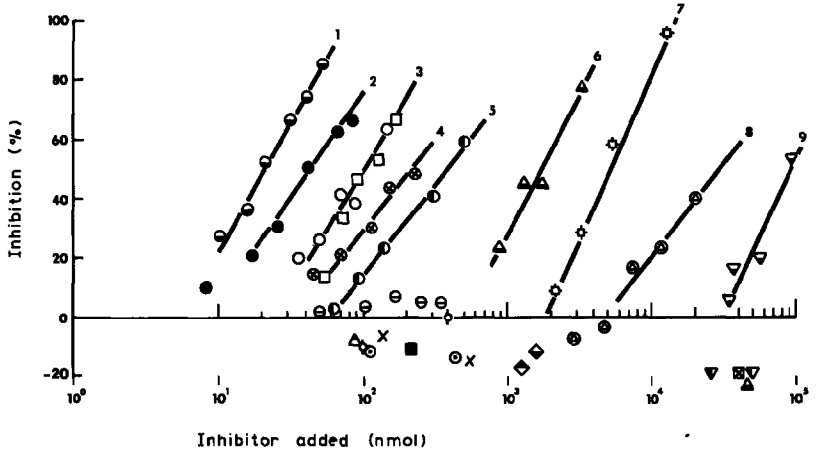


Fig. 2. Inhibition by monosaccharides, glycosides and oligosaccharides of precipitation of lima bean lectin (12.6 μg of N) with blood group A_1 substance hog 75 10% (15 μg); total volume 200 μL. Symbols and structures of inhibitors are shown in Table II.

TABLE II

INHIBITION OF THE PRECIPITATION OF LIMA BEAN LECTIN BY THE A_1 ACTIVE BLOOD GROUP SUBSTANCE (HOG 75 10% AS SHOWN IN FIG. 2) AND CHANGES IN THE FREE ENERGIES OF BINDING ($\Delta\Delta G°$) CAUSED BY STRUCTURAL MODIFICATIONS

Line No.	*Symbol*	*Inhibitor*	*Quantity giving 50% inhibition (nmol)*	*ΔΔG° (Kcal/mol)*	K_I[a] *(mM)*	K_I	
						Relative values with respect to D-*GalNAc*	*Precipitin inhibition*[b]
1	◒	DGalNAcα1→3[LFucα1→2]DGalβ1→3DGlcNAc-β1→3DGalβ1→4DGlc (A Hexa-)	21	−1.30			
2	●	DGalNAcα1→3[LFucα1→2]DGalβ1→4DGlc (A Tetra-)	43	−0.91			
3	○	DGalNAcα1→3[LFucα1→2]DGalβ-O$(CH_2)_8CO_2Me$ (O1)	110	−0.40	0.28	0.023	0.021
3	□	DGalNAcα1→3[LFucα1→2]DGalβ1→4DGlcNAc-β1→6R (MSM AR_L 0.56) (R = -CH_2OH-CHOH-CH=CH-CHOH-CH_2OH)	110	−0.40			
4	⊗	DGalNAcα1→3[LFucα1→2]DGal (A Tri-)	230	0.00			
5	◐	DGalNAcα1→3[LFucα1→2]DGalβ1→4[LFuc-α1→3]DGlc (A Penta-)	380	+0.27			
6	▲	MeαDGal*p*NAc	1750	+1.10	3.50	0.29	0.34
7	⊡	DGalNAc	5100	+1.67	12.10	1.00	1.00
8	⊘	MeβDGal*p*NAc	30,000	+2.63	21.00	1.7	5.8
9	▼	DGal	90,000	+3.23			
	⊖	DGalNAcα1→3[LFucα1→2]DGalβ1→3[LFuc-	350				

	α1→4]DGlcNAcβ1→3DGalβ1→4DGlc (A Hepta-)	(5% inhibition)			
×	DGalNAcα1→3DGalNAcβ1→3DGalα-O$(CH_2)_8CO_2Me$ (Forssman trisaccharide)	550 (inactive)			
○	DGalNAcα1→3DGalβ1→3DGlcNAc (A_5 II)	400 (inactive)			
⊙	DGalNAcα1→3DGalNAcβ-O$(CH_2)_8CO_2Me$	450 (inactive)	4.3	0.36	>0.09
■	DGalNAcα1→3DGal (R_L 1.34)	220 (inactive)	5.7	0.47	>0.04
◆	MeαDGal*f*NAc	1250 (inactive)			
◆	MeβDGal*f*NAc	1600 (inactive)			
⊠	DGlcNAc	40,000 (inactive)			
▲	DManNAc	47,000 (inactive)			
▼	LFuc	50,000 (inactive)			

[a]Inhibition constant in the thiol-protection assay (ref. 24). [b]Ratio of nmol giving 50% inhibition in the present study.

Quantitative precipitin inhibition assays. — The abilities of various monosaccharides, glycosides, and blood group oligosaccharides to inhibit precipitation of the lima bean lectin by A_1 blood group substance hog 75 10% are shown in Fig. 2. The structures of the oligosaccharides and the amounts giving 50% inhibition are given in Table II.

The best inhibitor was a blood group A-active monofucosyl oligosaccharide, A hexa- (line 1), 21 nmol giving 50% inhibition, followed by A tetra- (line 2), which is half as active as A hexa-; O1, a fucosylated A trisaccharide with the aglycon $O(CH_2)_8CO_2Me$, and MSM AR_L 0.56 are equally potent (line 3) and inhibited 50% at 110 nmol, 5.2 times poorer than A hexa-. The A trisaccharide, DGalNAcα1→3[LFucα1→2]DGal (A tri-, line 4), which is also the non-reducing portion of A hexa-, A tetra-, O1, and MSM AR_L 0.56, gives 50% inhibition at 230 nmol, 11 times less active than the best inhibitor. The A pentasaccharide (A penta-, line 5) containing a second fucosyl residue linked α1→3 to the DGlc of the A tetra-, decreases the activity to about 1/9 of the A tetra- and 1/18 that of the A hexa-, 380 nmol giving 50% inhibition. The A heptasaccharide (A hepta-), composed of A hexa-, with an additional LFuc linked α1→4 to DGlcNAc, gave only 5% inhibition at 350 nmol. Thus substitution of an α1→4 linked LFuc on the DGlcNAc adjacent to α1→2 linked LFuc completely blocked the activity.

Oligosaccharides lacking fucose, such as the Forssman trisaccharide with the aglycon $O(CH_2)_8CO_2Me$, DGalNAcα1→3DGalβ1→3DGlcNAc(A_5II), DGalNAcα-1→3DGalNAcβ-$O(CH_2)_8CO_2Me$, and DGalNAcα1→3DGal (R_L 1.34) were inactive over the range studied, although they have DGalNAc at the non-reducing end. Of the monosaccharides tested, DGalNAc (line 7) and DGal (line 9) are the only ones with activity, 5100 and 90,000 nmol respectively, giving 50% inhibition. DGlcNAc, DManNAc, and LFuc were not inhibitory even at 4–5 × 10^5 nmol.

Of the methyl glycosides of DGalNAc tested, only those in the pyranose form were active, MeαDGal*p*NAc giving 50% inhibition at 1750 nmol, whereas MeβDGal*p*NAc requires 30,000 nmol. The α anomer is 3 fold more potent than DGalNAc and 17 times more active than the β anomer. No inhibition was obtained with MeαDGal*f*NAc or MeβDGal*f*NAc at 1250 and 1600 nmol, respectively.

DISCUSSION

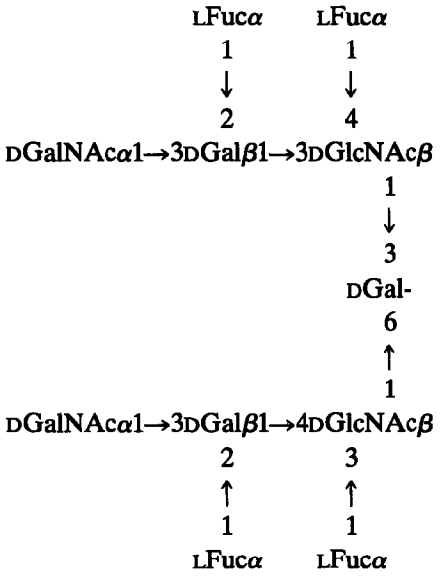
In the initial characterization of lima bean lectin, it was reported[13,16,17] that the LBL agglutinates human blood group A erythrocytes very strongly, B slightly, and O not at all. The purified active components II and III of LBL also precipitated human ovarian cyst A or hog mucin A substance, but were less reactive with human A_2 and very weakly reactive with B substances, and were not precipitable with hog mucin H substance[16]. Since components II and III gave similar precipitin curves with blood group A substance[16], an LBL preparation containing 10% of component II and 90% of component III was used for the present study. The best inhibitors of the lectin were reported[16] to be DGalNAc and its glycosides; DGalNAc was

required for binding and the combining site was assumed to be complementary to a monosaccharide[25]. In a recent study[24] in which inhibition of thiol modification was used as an assay (LBL has free sulfhydryl groups which are involved in carbohydrate binding, ref. 19) the best inhibitor was found to be the oligosaccharide

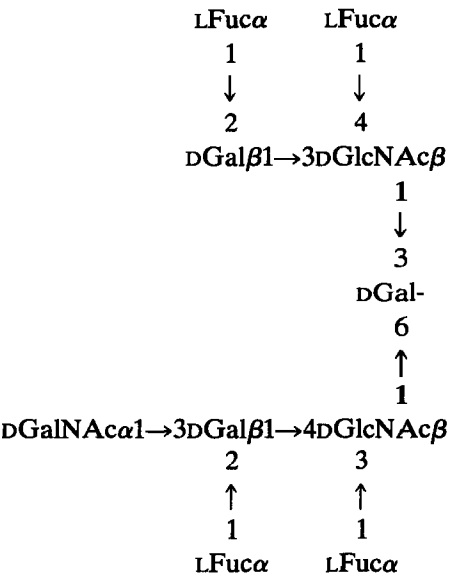
DGalNAcα1→3[LFucα1→2]DGalβ-O$(CH_2)_8CO_2Me$.

The observation that this oligosaccharide was 20-fold more potent than the disaccharide DGalNAcα1→3DGal, indicated that LBL has a more-extended binding site. It thus seemed important to study precipitation of the lectin by various water-soluble blood group A_1, A_2, B, H, Le^a, Le^b, and precursor I substances and to map its combining site by inhibition of precipitation by using additional fucose-containing blood group A oligosaccharides.

LBL reacted best with blood group A oligosaccharides containing fucose, the best inhibitor of precipitation being a monofucosyl hexasaccharide (A hexa-), DGalNAcα1→3[LFucα1→2]DGalβ1→3DGlcNAcβ1→3DGalβ1→4DGlc, having a type 1 chain (DGalβ1→3DGlcNAc) (Fig. 2 and Table II). LBL precipitates better with A_1 than with A_2 substances, in agreement with earlier data[16]. As several different determinants on blood group A substances have been found, a basis for a structural difference between A_1 and A_2 exists[44,45]; all A determinants have the structure DGalNAcα1→3[LFucα1→2]DGal, but differ in that this trisaccharide may be linked β1→3 or β1→4 to DGlcNAc to give what have have been termed type 1 and type 2 blood group A determinants. A second LFuc linked α1→4 or α1→3 to the DGlcNAc also gives two additional A determinants.



(a)



(*b*)

Earlier studies[44,45] on human ovarian cyst blood group substances showed that A_2 substances precipitated only a fraction of the anti-A precipitable by A_1 substances, and these findings were confirmed by using polyleucyl A_1 and A_2 substances as insoluble adsorbents. Mohn *et al.*[46] also showed that specific antibodies produced by immunizing rabbits with human group A_1 secretor saliva reacted only with A_1 but not with A_2 erythrocytes in agglutination, as well as in gel diffusion with A_1 erythrocytic stromal particles. These findings clearly established a qualitative difference between A_1 and A_2 substances. In terms of the then-known blood group A structures, A_1 substances were considered to have both type 1 and type 2 determinants (structure *a*), and A_2 substances to lack type 1 determinants (structure *b*). This conclusion remained controversial[47–49] for many years, largely because type 1 determinants were not isolated from A_1 erythrocytes.

Unequivocal evidence confirming a qualitative distinction between A_1 and A_2 determinants was obtained from recent studies on blood group glycolipids. Clausen *et al.*[50,51] isolated and characterized two new types of blood group A determinants

DGalNAcα1→3[LFucα1→2]DGalβ1→3DGalNAcα1→3[LFucα1→2]-
DGalβ1→4DGlcNAcβ1→3DGalβ1→4DGlc

(Type 3 chain) (*c*)

DGalNAcα1→3[LFucα1→2]DGalβ1→3DGalNAcβ1→3DGalα1→4DGalβ1→4DGlc

(Type 4 chain) (*d*)

from human A_1 erythrocytes; and the type 4A chain was also isolated from human blood group A kidney[52]. These structures were characteristic of A_1 erythrocytes and present only in trace amounts in A_2 erythrocytes. The precursor H [structures (*c*) and (*d*) without the non-reducing terminal DGalNAc] were present in greater quantity in A_2 than in A_1 erythrocytes. The relation of the blood group A_1 and A_2 glycoproteins to the type 3 and type 4 chains requires further study. The various blood group A_1 and A_2 substances used for LBL, when used as inhibitors of A_1 hemagglutination[53] with an A_1-specific monoclonal antibody TH-1 (ref. 50) directed to the type 3 chain (structure *c*), the A_1 substances Cyst 9, MSS 10% 2X, hog 75 10% and McDon 15% were found to be strongly active. Of the two A_2 substances studied, WG phenol-insoluble was inactive but the other, Cyst 14 phenol-insoluble, was as active as several A_1 substances. The type 1 and type 2 A_1 oligosaccharides have not been tested for their ability to inhibit agglutination of A_1 cells by anti-TH-1. Quantitative precipitin tests with anti-TH-1 and the various A_1 and A_2 substances may help to define more precisely the proportions of these various determinants in blood group A_1 and A_2 glycoproteins. It is not established whether the type 3 and type 4 chains occur on blood group A glycoproteins or whether the reactivity with anti-TH-1 is due to a cross reaction of the type 1 A determinants. Recently Clausen *et al.*[54] reported the isolation of two monoclonal antibodies, one (AH21) reacting with monofucosyl type 1 A chain and the other (HH3) reacting with difucosyl type 1 A chain as well as two glycolipids from A_1 erythrocytes, one having the A hexa- and the other the A hepta- structure (Table II) linked to ceramide. The A hexa- occurred in Le^{a-b-} and not in Le^{a+b-} or Le^{a-b+} erythrocytes and the A hepta- chain occurred only in Le^{a-b+} and not in Le^{a+b-} and Le^{a-b-} erythrocytes. Thus within the A_1 subgroup there is also an association with Lewis subgroups, which is independent of A_2. The A hexa- glycolipid from an A_1Le^{a-b-} secretor plasma was also isolated (Jovall *et al.*, 1984, cited in ref. 54). Thus the best inhibitor of lima bean lectin and of the monoclonal anti-A_1 hexa- glycolipid AH21 is the A hexa- oligosaccharide (Fig. 2, Table II).

In agreement with previous results[24], lima bean lectin is the most specific of the characterized DGalNAc binding lectins for fucose-containing type A blood group substance. This is best supported by the finding that the various A-active fucosylated oligosaccharides are active in the inhibition of precipitation, whereas oligosaccharides without fucose, such as Forssman trisaccharide, A_5II, R_L 1.34 (Fig. 2 and Table II) are inactive, even though they have terminal DGalNAc. Thus an LFucα1→2 linkage to the subterminal DGal is essential.

The A tetra-, DGalNAcα1→3[LFucα1→2]DGalβ1→4DGlc, was found to be 9 times more active than A penta-, DGalNAcα1→3[LFucα1→2]DGalβ1→4[LFucα-1→3]DGlc, indicating that a second LFuc linked α1→3 decreases the binding. The most striking finding of the present study of LBL specificity is that the best inhibitor of precipitation is the monofucosyl hexasaccharide, A hexa-, but a difucosyl heptasaccharide (A hepta-), DGalNAcα1→3[LFucα1→2]DGalβ1→3[LFucα1→4]D-GlcNAcβ1→3DGalβ1→4DGlc, having the structure of the A hexa- except for one

additional LFuc linked α1→4 to DGlcNAc, is inactive; the LFuc1→4 linkage probably changes the conformation or topography of the molecule and thus completely blocks the access to the lectin-combining site as has been shown[55,56] for interactions of other blood group oligosaccharides with antibodies and lectins. This is unlike the Le^b-specific lectin *Griffonia simplicifolia* IV[57], in which LFucα1→4 is favored over the LFucα1→2 linkage. The specificity of LBL may also be compared with other GalNAc-binding lectins. *Dolichos biflorus* lectin[58] has some specificity (1.5 fold) for fucose-containing oligosaccharides over those lacking fucose, whereas soybean agglutinin[59] and *Griffonia simplicifolia* I-A_4 lectin[60] exhibit a preference for oligosaccharides without fucose.

McDon 15%, an A_1 substance, reacts well with LBL, whereas its P_1 fraction, which lacked most of the αL-fucosyl end groups and some of the group A-active side chains, was found to be inactive (Fig. 1, Table I). These data add further support to the specificity of the lima bean lectin for fucose-containing determinants.

Hydrophobic forces have been inferred[55,61,62] to be important for the binding of fucose-containing oligosaccharides to antibodies and lectins. The interaction of fucose on the A oligosaccharides with LBL may also have hydrophobic character, as it enhances binding[24] by increasing $\Delta S°$. In an attempt to evaluate more precisely the role of hydrophobic interactions, we consider it useful to assess selected differences in the potencies of inhibitors in terms of differences in free energies of binding, $\Delta\Delta G°$ values, as presented in Table II, to provide an appreciation of the structure–activity relationships in terms of differences in the driving forces for the reaction. This is calculated from the expression[63–66], $\Delta\Delta G° = 273\ \mathrm{Rln}(x/y)$, where x is the amount of inhibitor in nmol giving 50% inhibition at 0°, and y is the amount of A trisaccharide giving 50% inhibition. This permits ready comparisons of all compounds in the changes of their free energies of binding caused by structural modifications with respect to the trisaccharide DGalNAcα1→3[LFucα1→2]DGal, which was isolated from a blood group A substance and for which $\Delta\Delta G°$ is assumed to be zero. If the trisaccharide is linked β1→3 to DGlcNAcβ1→3DGalβ1→4DGlc to give A hexa-, the differences in $\Delta\Delta G°$ between the A tri- and A hexa- becomes −1.3 Kcal/mol, indicating tighter binding of A hexa- in the lectin-combining site and establishes that LBL has a more-extended combining site clearly involving a tetra- and probably a penta-saccharide. The A tetra- with a reducing DGlc instead of DGlcNAc and lacking the subterminal residue β1→3DGalβ1→4DGlc of A hexa-, showed a decrease in $\Delta\Delta G°$ values by 0.4 Kcal/mol, indicating that the subterminal structure and the DGlcNAc also contribute to the binding affinity. The hydrophobic contribution of the aglycon $-O(CH_2)_8CO_2Me$ in O1, and the β1→6 linked hexenetetrol (R) in MSM AR_L 0.56 (Table II) contribute equally (−0.4 Kcal) to the $\Delta\Delta G°$. Substitution of a second fucose by an α1→3 linkage to the type 2 chain of the A tetra- (to give A penta-) decreased binding affinity (+1.18 Kcal/mol), but if the second fucose was linked α1→4 to the type 1 chain of A hexa- to give A hepta-, binding was completely abolished.

It has been reported[16,18] earlier that LBL contains two cysteine residues per

subunit; one forms an intersubunit disulfide bond[18]; the sulfhydryl group of the other remains free and is required for carbohydrate binding[19]. In a recent inhibition study[24] using free-sulfhydryl modification as an assay, the best inhibitor was found to be O1, DGalNAcα1$\rightarrow$3[LFucα1$\rightarrow$2]DGalβ-O$(CH_2)_8CO_2Me$. Our precipitin-inhibition data for some monosaccharides and oligosaccharides are comparable (Table II), confirming further the involvement of free thiol groups in LBL in carbohydrate binding.

The present study further demonstrates the importance of establishing the fine structure of lectin-combining sites.

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