Sugar Binding Specificities of Anti-H(O) Lectins Disclosed by Use of Fucose-Containing Human Milk Oligosaccharides as Binding Inhibitors

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The binding to normal and sialidase-treated human erythrocytes of six ^{125}I-labeled lectins [*Ulex europeus* lectin I (UEA-1) and II (UEA-II), *Laburnum alpinum* lectins I (LAA-I) and II (LAA-II), and *Cytisus multiflorus* lectins I (CMA-I) and II (CMA-II)], was studied in detail. Quantitative inhibition assays of the lectin binding to the cells were also performed with various human milk oligosaccharides as inhibitors. Based on a comparison of the inhibition constants of the inhibitors thus obtained with the association constants of the lectins to the cells, the relative activities of cell surface blood group antigens toward the lectins are discussed.

Keywords lectin; anti-H(O) lectin; milk oligosaccharide; sugar binding specificity

So far, more than a hundred plant lectins have been isolated and characterized with respect to their biochemical properties and molecular structures, and found to be a heterogeneous class of proteins or glycoproteins that have in common the ability to recognize specifically certain sugars or sugar-containing macromolecules.[1] We have previously isolated and characterized several anti-H(O) lectins, namely, *Ulex europeus* lectins I and II (UEA-I and II),[2-5] *Laburnum alpinum* lectins I and II (LAA-I and II)[6] and *Cytisus multiflorus* lectins I and II (CMA-I and II).[7] In order to apply them effectively to the detection of sugar moieties on the cell surface, it is important to define the precise carbohydrate binding specificities of these lectins, which have generally been studied by hemagglutination inhibition assays using simple sugars as hapten inhibitors. Since quantitative comparison of the inhibitory activities of the haptenic sugars is almost impossible in the case of hemagglutination inhibition assays, binding inhibition assays using various oligosaccharides and glycopeptides as inhibitors were utilized as a more quantitative method for the elucidation of the sugar-binding specificities of lectins.[8] We present here the results of quantitative binding inhibition studies on the *Ulex*, *Laburnum* and *Cytisus* anti-H(O) lectins using various simple sugars, milk oligosaccharides and chitin oligosaccharides as inhibitiors, and on the basis of a comparison of their inhibition constants with the association constants of lectins to the cell surface, we discuss the structure of cell surface binding sites for the lectins.

Materials and Methods

Materials *Ulex europeus* lectin I (UEA-I) was purified from *Ulex europeus* seeds (purchased from F.W. Schumacher, Sandwich, Mass., U.S.A.) according to the specific method of Gürtler.[9] *Ulex europeus* lectin II (UEA-II), *Laburnum alpinum* lectins I and II (LAA-I and II), and *Cytisus multiflorus* lectins I and II (CMA-I and II) were specifically purified from *Ulex europeus* seeds, *Laburnum alpinum* seeds and *Cytisus multiflorus* seeds (all purchased from F. W. Schumacher, Sandwich, Mass., U.S.A.), respectively, by the methods previously described.[5-7] The homogeneity of these purified lectins was ascertained by electrophoresis on polyacrylamide gel. Sialidase for the treatment of human O erythrocytes was obtained from Nakarai Chemicals Ltd. (Kyoto). Tri-*N*-acetylchitotriose and di-*N*-acetylchitobiose used for the inhibition assays as inhibitors were prepared by the method of Rupley.[10] Lacto-*N*-fucopentaose I (LNF-I), lacto-*N*-difucohexaose I (LND-I), lacto-*N*-tetraose (LNT) and 2′-α-fucocyllactose (2′-FL) were isolated from human colostrum by the method described previously.[11] Lacto-*N*-fucopentaose II (LNF-II) was kindly provided by Prof. A. Kobata, Institute of Medical Sciences, University of Tokyo, Tokyo. *N*-Acetyllactosamine, 3′-β-fucosyllactose (3′-FL) and 6′-α-fucosyllactose (6′-FL) were kindly provided by Prof. S. Tejima, Nagoya City University, Nagoya. Figure 1 shows the structures of these oligosaccharides.

Iodination of Lectins The purified lectins were iodinated with ^{125}I by the chloramine-T method of Hunter[12] by using a 30-s exposure to chloramine-T, and the labeled lectins were freed from excess reagents by passage over Sephadex G-50. This procedure did not affect the hemagglutinating activity of the lectins. The specific radioactivity was 0.01—4.5×10^6 cpm/μg of protein.

Sialidase Treatment of Human O Erythrocytes Sialidase treatment of cells was performed according to the method previously described.[6]

Binding Studies Binding reactions were carried out in microtiter V plates presoaked overnight with 10 mM sodium phosphate (pH 7.3) containing 0.15 M NaCl–0.25% bovine serum albumin (PBS–BSA) according to the method previously described.[13] The reaction mixture contained 5×10^6 erythrocytes and 0.2 to 4 μg of ^{125}I-labeled lectin in a final volume of 0.15 ml of PBS–BSA solution. In binding inhibition assays with simple sugars or human milk oligosaccharides, various amounts (0.60—

lacto-*N*-tetraose (LNT)
Galβ1→3GlcNAcβ1→3Galβ1→4Glc

lacto-*N*-fucopentaose I (LNF-I)
Galβ1→3GlcNAcβ1→3Galβ1→4Glc
2
↑
α1
Fuc

lacto-*N*-fucopentaose II (LNF-II)
Galβ1→3GlcNAcβ1→3Galβ1→4Glc
4
↑
α1
Fuc

lacto-*N*-difucohexaose I (LND-I)
Galβ1→3GlcNacβ1→3Galβ1→4Glc
2 4
↑ ↑
α1 α1
Fuc Fuc

2′-α-fucosyllactose (2′-FL)
Fucα1→2Galβ1→4Glc

3′-β-fucosyllactose (3′-FL)
Fucβ1→3Galβ1→4Glc

6′-α-fucosyllactose (6′-FL)
Fucα1→6Galβ1→4Glc

Fig. 1. Structures of Oligosaccharides Used in the Present Experiment

29.23 μmol/ml) of the sugars or the oligosaccharides were mixed with a ^{125}I-labeled lectin 60 min before the addition of human group O erythrocytes. After incubation at 25 °C for 60 min, the cells were washed seven times with 0.15 ml of PBS–BSA solution, and the amount of bound ^{125}I was determined in an Aloka autogamma counter. When the radioactivity of the supernatant of washings was checked, the dissociation of ^{125}I during the washing process was found to be negligible. Appropriate corrections were made for nonspecific binding of the lectins to the plates.

Results and Discussion

Estimation of Binding Constants and Number of Binding Sites Studies of the binding of ^{125}I-labeled lectins to normal and sialidase-treated human erythrocytes were carried out, and the data obtained were plotted according to the method of Steck and Wallach[14] as shown in Fig. 2. The binding of the ^{125}I-labeled lectins gave biphasic plots in some of these cases, as in the cases of *Maackia amurensis* lectins reported by Kawaguchi and Osawa[8] and Kawaguchi *et al.*[13] Since these lectins were purified by affinity chromatography and found to be homogeneous in terms of polyacrylamide gel electrophoresis, it seems that two kinds of binding sites exist on the cell surface for each of these lectins, namely the major binding sites to which the lectin binds preferentially and the minor binding sites to which the lectin binds only at high concentrations. The apparent association constants for the major binding sites (K_0) and the average number of major binding sites per cell (n) were calculated. These values are listed in Table I. Sialidase treatment of the cells caused significant increases in n values for LAA-I, CMA-I and CMA-II, though the K_0 values for these lectins were not significantly changed by this treatment. In the case of LAA-II, effective binding of the lectin was observed only after the sialidase treatment. These results suggest that sialic acid reidues effectively mask the binding sites for LAA-I, LAA-II, CMA-I and CMA-II.

Inhibition of Lectin Binding to Cell Surface by Various Sugars and Oligosaccharides The inhibition constants of various human milk oligosaccharides such as lacto-*N*-fucopentaoses I (LNF-I) and II (LNF-II), lacto-*N*-tetraose (LNT), lacto-*N*-difucohexaose I (LND-I), 2′-α-fucosyllactose (2′-FL), 3′-β-fucosyllactose (3′-FL) and 6′-α-fucosyllactose (6′-FL), and such simple sugars as tri-*N*-acetylchitotriose, di-*N*-acetylchitobiose, *N*-acetyllactosamine, lactose, D-galactose and L-fucose against ^{125}I-labeled lectin binding to the major cell surface binding sites were calculated by the method of Kawaguchi and Osawa[8] according to the equation

$$\log[([L_0]-[LR])([R_0]-[LR])/[LR]-1/k_0]=m\log[I]+\log(k_I/k_0)$$

where $[L_0]$ = input ^{125}I-labeled lectin concentration, $[LR]$ = concentration of ^{125}I-labeled lectin–receptor complex, $[R_0]$ = input receptor concentration calculated as input cell number (per liter) × n (listed in Table I)/Avogadro's number, and $[I]$ = free inhibitor concentration. This equation is of the form $y=ax+b$. From the binding inhibition data, values of $\log[([L_0]-[LR])([R_0]-[LR])/[LR]-1/k_0]$ were calculated and are shown as a function of $\log [I_0]$ ($[I_0]$ is an input inhibitor concentration which is approximately equal to [I]) in Fig. 3. The slope will give m and the intercept on the ordinate will give $\log (k_I/k_0)$. The K_I values were then calculated and are listed in Table II.

It has previously been shown that L-fucose specifically

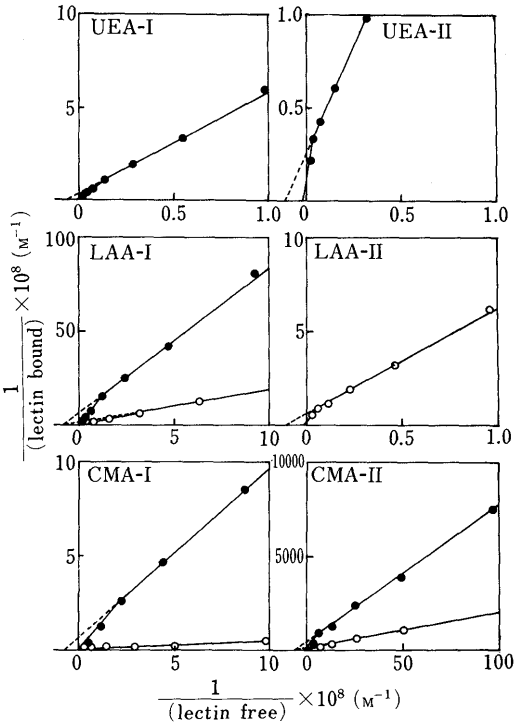

Fig. 2. Binding of ^{125}I-Labeled Lectin to Normal (●—●) and Sialidase-Treated (○—○) Human Group O Erythrocytes

TABLE I. Association Constants of Lectins to Human Group O Erythrocytes

Lectin	Normal		Sialidase-treated	
	K_0 [a]	n [b]	K_0 [a]	n [b]
UEA-I	7.0×10^6	3.9×10^5	n.d.	n.d.
UEA-II	1.2×10^7	4.2×10^5	n.d.	n.d.
LAA-I	8.5×10^7	3.1×10^4	8.0×10^7	1.0×10^5
LAA-II	n.d.	n.d.	1.1×10^7	4.0×10^5
CMA-I	8.0×10^7	3.7×10^3	1.0×10^8	4.4×10^4
CMA-II	7.0×10^8	3.0×10^2	6.0×10^8	1.4×10^3

a) Apparent association constant (M^{-1}) for major binding sites. Average value of duplicate experiments. *b*) Number of major binding sites. Average value of duplicate experiments. n.d.: not determined.

inhibits the binding of UEA-I to human group O erythrocytes by hemagglutination inhibition assays.[2-5] In the present study, this was confirmed by the binding inhibition assay of ^{125}I-UEA-I (Table II). The K_I value of 2′-FL, which is similar in structure to the Fucα(1—2)Galβ(1—4)GlcNAc-sugar sequence (type 2) in the H-determinant chain on the cell surface, was highest, and that of LNF-I, which has the same structure as the type 1 sugar sequence in the H-determinant, was relatively high. These results suggest that the fucose residues are essential for the binding of UEA-I, and that this lectin mainly interacts with type 2 sugar chains on human O group erythrocytes but also possibly interacts with type 1 sugar chain less strongly. Moreover, since the K_I values of 2′-FL and LNF-I were higher than those of LNF-II and LND-I, Fucα(1—2)Galβ(1—3 or 4)GlcNAc-sugar chain seemed to be preferable to Galβ(1—3)[Fucα(1—4)] GlcNAc-sugar chain for the binding of UEA-I, and the fucose residue attached to the GlcNAc residue rather decreases the affinity of the lectin to

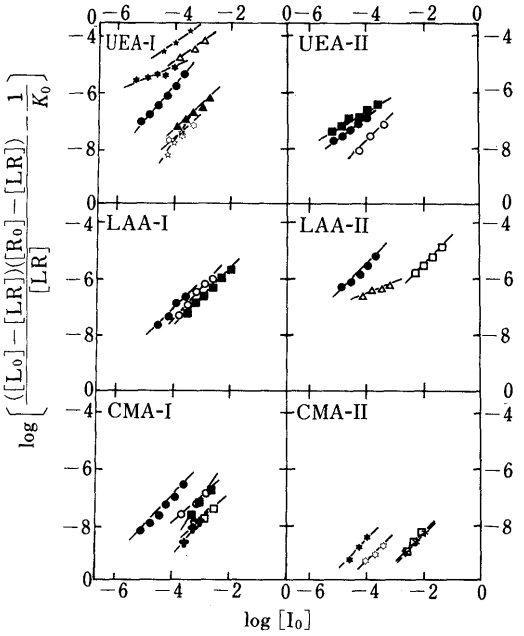

Fig. 3. Effect of Various Sugars and Oligosaccharides on ^{125}I-Labeled Lectin Binding to Human Group O Erythrocytes

Binding of ^{125}I-UEA-I, ^{125}I-UEA-II, ^{125}I-LAA-I, ^{125}I-CMA-I and ^{125}I-CMA-II to normal erythrocytes and ^{125}I-LAA-II to sialidase-treated erythrocytes was carried out in the presence of various concentrations of inhibitors. From the binding inhibition data $\{([[L_0]-[LR])([R_0]-[LR])]/[LR]-(1/K_0)\}$ values were calculated and are shown as a function of $\log[I_0]$. K_I values determined are presented in Table II. (★), 6′-FL; (△), 3′-FL; (✱), LND-I; (●), 2′-FL; (▲), Fuc; (☆), LNF-I; (✲), LNF-II; (■), $(GlcNAc)_2$; (○), $(GlcNAc)_3$; (□), Lac; (✤), LacNAc; (∗), Gal.

TABLE II. K_I Values of Various Mono- and Oligosaccharides toward Lectin Binding to Human Group O Erythrocytes

Inhibitors	K_I values[a] in the competitive binding with [^{125}I]lectins					
	UEA-I	UEA-II	LAA-I	LAA-II[b]	CMA-I[b]	CMA-II[b]
Lacto-*N*-fucopentaose I	7×10^4	n.i.[c]	n.i.[c]	n.i.[c]	n.i.[c]	n.i.[c]
Lacto-*N*-fucopentaose II	7×10^2	n.i.[d]	n.i.[d]	n.i.[d]	n.i.[d]	3×10^3
Lacto-*N*-difucohexaose I	3×10^3	n.i.[e]	n.i.[e]	n.i.[e]	n.i.[e]	2×10^4
Lacto-*N*-tetraose	n.i.[f]	n.i.[f]	n.i.[f]	n.i.[f]	n.i.[f]	n.i.[f]
2′-α-Fucosyllactose	7×10^5	2×10^4	8×10^4	4×10^5	3×10^5	n.i.[g]
3′-β-Fucosyllactose	7×10^5	n.i.[h]	n.i.[h]	1×10^3	n.i.[h]	n.i.[h]
6′-α-Fucosyllactose	2×10^5	n.i.[i]	n.i.[i]	n.i.[i]	n.i.[i]	n.i.[i]
Tri-*N*-acetylchitotriose	n.i.[j]	2×10^3	8×10^4	n.i.[j]	1×10^4	n.i.[j]
Di-*N*-acetylchitobiose	n.i.[k]	9×10^2	2×10^4	n.i.[k]	8×10^3	n.i.[k]
N-Acetyllactosamine	n.i.[l]	n.i.[l]	n.i.[l]	n.i.[l]	3×10^4	n.i.[l]
Lactose	n.i.[m]	n.i.[m]	n.i.[m]	4×10^3	2×10^3	3×10^2
L-Fucose	2×10^3	n.i.[n]	n.i.[n]	n.i.[n]	n.i.[n]	n.i.[n]
D-Galactose	n.i.[o]	n.i.[o]	n.i.[o]	n.i.[o]	n.i.[o]	3×10^2

a) Average values of duplicate experiments. *b*) Sialidase-treated cells were used. *c*) Not inhibitory (n.i.) at a concentration of 1.52 μmol/ml. *d*) n.i. at 1.17 μmol/ml. *e*) n.i. at 0.60 μmol/ml. *f*) n.i. at 0.70 μmol/ml. *g*) n.i. at 0.72 μmol/ml. *h*) n.i. at 3.69 μmol/ml. *i*) n.i. at 2.46 μmol/ml. *j*) n.i. at 7.97 μmol/ml. *k*) n.i. at 11.79 μmol/ml. *l*) n.i. at 2.62 μmol/ml. *m*) n.i. at 29.33 μmol/ml. *n*) n.i. at 6.45 μmol/ml. *o*) n.i. at 27.78 μmol/ml.

the fucose residue attached to the galactose residue.

As shown previously,[5-7] the sugar binding specificities of UEA-II, LAA-I and CMA-I were similar. The binding of these lectins was inhibited by di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and 2′-FL, and the K_I value of 2′-FL was highest among the oligosaccharides tested. Basides di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and 2′-FL, *N*-acetyllactosamine and lactose were also inhibitory for the binding of CMA-I. For LAA-II, 2′-FL was a potent inhibitor; lactose and 3′-FL were also inhibitors but their K_I values were much lower than that of 2′-FL. However, the inhibitory activity of chitin oligosaccharides was found to be very weak. As mentioned above, though the binding specificities of UEA-II, LAA-I, LAA-II and CMA-I for sugar sequences were somewhat different from each other, the binding of all of these lectins was strongly inhibited by 2′-FL but not by LNT, which lacks a fucose residue. These observations correspond to the fact that these lectins could not agglutinate human group O erythrocytes treated with purified H-decomposing enzyme (α-L-fucosidase) from *Bacillus fulminans*.[3] Furthermore, the fact that UEA-II, LAA-I, LAA-II and CMA-I were strongly inhibited by 2′-FL but not by LNF-I or LND-I suggests that these lectins interact with type 2 chain in the H-determinant chain on the cell surface but not with type 1 chain.

In the previous paper[7] we demonstrated that CMA-II does not have anti-H(O) specificity. Actually, this lectin was not inhibited by 2′-FL and LNF-I, but it was strongly inhibited by LND-I, which is a determinant structure for the Le^b blood group. Although CMA-II was also inhibited by LNF-II, which is a determinant of Le^a blood group, its reactivity was less than that of LND-I. Moreover, lacto-*N*-fucopentaose III (kindly provided by Prof. T. Muramatsu, Kagoshima Univ. Medical School), which has a type 2 chain and is a determinant of Le^x blood group, was found to be not inhibitory toward CMA-II (data not shown). Therefore, CMA-II was supposed to be most specific for type 1 chains containing two α-L-fucosyl groups on C-2 of the D-galactosyl residue and C-3 of the D-GlcNAc residue.

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