Glycoconjugate Journal (1997) **14:** 45–55

Immobilized *Lotus tetragonolobus* agglutinin binds oligosaccharides containing the Le^x determinant

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A defined set of oligosaccharides and glycopeptides containing α-linked fucose were used to examine the specificity of the immobilized fucose-binding lectin *Lotus tetragonolobus* agglutinin (LTA[1]), also known as lotus lectin. Glycans containing the Lewis x determinant (Le^x) Galβ1-4[Fucα1-3]GlcNAcβ1-3-R were significantly retarded in elution from high density LTA-Emphaze columns. The lectin also bound the fucosylated lacdiNAc trisaccharide GalNAcβ1-4[Fucα1-3]GlcNAc. The lectin did not bind glycans containing either sialylLe^x or VIM-2 determinants, nor did it bind the isomeric Le^a, Galβ1-3[Fucα1-4]GlcNAc-R. Although 2′-fucosyllactose Fucα1-2Galβ1-4Glc) was retarded in elution from the columns, larger glycans containing the H-antigen Fucα1-2Galβ1-3(4)GlcNAc-R interacted poorly with immobilized LTA. Our results demonstrate that immobilized LTA is effective in isolating glycans containing the Le^x antigen and is useful in analyzing specific fucosylation of glycoconjugates.

***Keywords*: fucosylation, *Lotus tetragonolobus* agglutinin, lotus lectin, affinity chromatography, Lewis antigens**

***Abbreviations*: LTA, Lotus tetragonolobus agglutinin; UEA-1, *Ulex europaeus* agglutinin-I; LNT, AAL, *Aleuria aurantia* agglutinin; Galβ1-3GlcNAcβ1-3Galβ1-3Glc; LNnT, Galβ1-4GlcNAcβ1-3Galβ1-3Glc; Le^x, Lewis x antigen; Le^a, Lewis a antigen; GDPFuc, guanosine 5′-diphosphate-β-L-fucose**

Introduction

Plant and animal lectins are widely used in the study of complex carbohydrates from animal cells. Numerous lectins have been purified and their properties well defined [1, 2]. Lectins are used to identify and separate cells, assay for the activities of glycosyltransferases, and isolate glycopeptides by affinity chromatography [3–7]. Although lectin binding in many cases can be inhibited by simple monosaccharides such as fucose, mannose or galactose, most lectins bind with differential affinities to oligosaccharides depending on their specific structural features.

Three lectins considered to bind to fucose-containing oligosaccharides are *Lotus tetragonolobus* agglutinin (LTA[1]) (also called Lotus lectin), *Ulex europaeus* agglutinin-I (UEA-I), and *Aleuria aurantia* agglutinin (AAL) [8–10]. These lectins are significantly different, however, in their specificities for fucosylated oligosaccharides. For example, UEA-I displays high affinity interactions with H-blood group determinants [11, 12] whereas the other lectins are much less specific in that regard [9, 10, 13, 14]. Though all of these lectins have been used in affinity chromatography procedures, only the carbohydrate-binding specificity of immobilized AAL has been carefully examined [10, 13].

Early studies by Pereira and Kabat [9] suggested that oligosaccharides containing the Lewis x antigen (Le^x) Galβ1-4[Fucα1-3]GlcNAc-R are potent inhibitors of LTA. The Le^x antigen has received much attention in recent years because of evidence that Lewis antigens are important for cell adhesion involving selectins within the vascular system [15, 16]. The Le^x antigen, also recognized as the stage-specific embryonic antigen-1, is thought to be important in early events in murine embryogenesis and was one of the first developmentally-regulated carbohydrate antigens to be defined [17, 18].

To aid in characterizing oligosaccharides containing the Le^x and isomeric Lewis a (Le^a) determinants Galβ1-3[Fucα1-4]GlcNAc-R, we sought to develop a lectin affinity chromatography approach to complement existing serial lectin affinity chromatography methods. We now report our study on the interaction of immobilized LTA with a variety of purified oligosaccharides containing fucosyl residues in various positions. We also examined the effect of the

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coupling density of LTA on the ability of the lectin to discriminate among fucosylated oligosaccharides. Our results demonstrate that immobilized LTA displays specificity for Le^x determinants and is useful in separating and analyzing complex carbohydrates containing fucose.

Materials and methods

Materials

Guanosine 5′-diphosphate-β-L-fucose (GDPFuc), sodium cacodylate, L-fucose, Triton CF-54, adenosine 5′-triphosphate (ATP), L-fucosyl-agarose, fetuin, orosomucoid, Sephadex G-25, QAE-Sephadex 25, bovine milk UDPGal:GlcNAc-β-1,4-galactosyltransferase, and *Arthrobacter ureafaciens* neuraminidase were purchased from Sigma. Triton X-100 was purchased from BioRad and α-lactose was obtained from Fisher. Lacto-N-neotetraose (LNnT) and lacto-N-tetraose (LNT) were purified from human milk in our laboratory. Emphaze beads were obtained from Pierce. [6^{-3}H]-Glucosamine and guanosine 5′-diphosphate-β-L-[1^{-3}H]-fucose were purchased from American Radiolabeled Chemicals. Guanosine 5′-diphosphate-β-L-[U^{-14}C]-fucose was obtained from DuPont/ NEN. Pronase from *Streptomyces griseus* was purchased from CalBiochem and Con A-Sepharose was obtained from Pharmacia. *L. tetragonolobus* seeds were purchased from R. W. Schumacher Co.

Purification of LTA

LTA was purified by a modification of the procedures described by Yariv *et al.* (19). Dry *L. tetragonolobus* seeds (50 g) were powdered in a Waring blender and the powder was extracted using 2 l of 0.02 M sodium phosphate, 0.85% sodium chloride (pH 6.8) at 4 °C. The crude extract was centrifuged and the supernatant was fractionated by addition of solid ammonium sulfate to 30% saturation. The suspension was centrifuged at 8000 × **g** for 30 min, then the supernatant solution was brought to 60% saturation with ammonium sulfate and precipitated overnight at 4 °C, then centrifuged again. The pellet was resuspended in 40 ml of 0.05 M sodium phosphate buffer (pH 6.8) and dialysed extensively against the same buffer overnight, at 4 °C. The dialysed extract was then centrifuged and the supernatant solution was applied to a column of L-fucosyl-agarose. The column was washed extensively with 0.05 M sodium phosphate buffer, (pH 6.8) and the bound lectin was eluted with 40 mM L-fucose in the same buffer. The lectin, which was eluted in a single peak was dialysed against 0.05 M of sodium phosphate buffer (pH 6.8). Purified LTA migrated as a homogeneous molecular species with a molecular weight of about 27 000 on SDS-PAGE (data not shown). 50 g of dry seed yielded 40 mg of purified LTA. The purified LTA, at a concentration of 20 μg ml^{-1}, had potent agglutinating activity with COS7/FTIV cells.

LTA was immobilized on Emphaze beads at various densities, 6, 9, and 15 mg ml^{-1}, according to the manufacturer's instructions. A solution containing LTA was directly added to 187 mg of dry Emphaze beads (equivalent to 1.5 ml of gel), in coupling buffer, 0.05 M sodium phosphate buffer (pH 7.4) 0.6 M sodium citrate, 0.2 M L-fucose. The mixture was rotated for 3–4 h at room temperature and coupling was stopped by incubating with a solution of 3.0 M ethanolamine (pH 9.0) for 3 h at room temperature. Beads were suspended in Tris-buffered saline (TBS, 10 mM Tris, pH 8.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, and 0.02% NaN_3) and transferred to small disposable Bio-Rad columns (10 ml). Each LTA column contained approximately 1.5 ml of LTA-Emphaze resin. Efficient absorption of LTA to the Emphaze beads was monitored using the BCA assay after dialysing the uncoupled fraction to remove excess free fucose. In all cases, greater than 95% of the protein was coupled to the Emphaze resin.

Fucosyltransferase-transfected COS cells

COS7/FTIV and COS7/FucTIII cells were generated by stably transfecting monkey kidney COS7 cells with cDNAs encoding either human GDPFuc:Galβ1-4GlcNAc (Fuc to GlcNAc) α1-3 fucosyltransferase IV (FTIV) or human GDPFuc:Galβ1-4/3GlcNAc (Fuc to GlcNAc) α1-3/4 fucosyltransferase III (FTIII) as described [20]. NeoH CHO cells or CHO/FucTIII were generated by stably transfecting CHO cells with cDNAs encoding the human GDPFuc:Gal α1-2-fucosyltransferase [21] or the human FTIII [22].

Preparation of radiolabelled oligosaccharides

Cell extracts were prepared from COS7/FTIV and NeoH CHO cells as described [23]. The protein concentration of cell extracts were determined by the BCA assay. The structures of oligosaccharide standards **I–XIV** are shown in Figs 1–3. [^{14}C]-Fucose-labelled glycans were synthesized according to the following procedures. [^{14}C]-Fucose-labeled glycan **I** was prepared by incubating *N*-acetyllactosamine in 99.2 mM sodium cacodylate buffer, (pH 7.0) 40 mM $MnCl_2$, 10 mM ATP, 30 mM-L-fucose, 1% Triton CF-54 with 30 μg COS7/FTIV cell extract and 1.9 nmol GDP-[^{14}C]Fuc. [^{14}C]-Fucose-labelled glycan **III** was synthesized using the fucosyltransferase reaction as described above with 0.76 nmol GDP-[^{14}C]Fuc and LNnT in a total reaction volume of 25 μl. For the preparation of both glycans **I** and **III** the reactions proceeded overnight and the radiolabelled products were obtained in the run-through fractions of a 2 ml column of Dowex 1-X8 in water. The fucosylated glycans were further purified by descending paper chromatography. [^{3}H]-Glucosamine-labelled glycans **II, IV, V, VI, VII,** and **XII** were prepared by endo-β-galactosidase digestion of total N-glycans derived from CHO cells expressing human FucTIII, as described by Sueyosh, *et al.* [24].

Glycans **VIII, IX** and **X** were prepared according to the method of Rajan *et al.* [21]. The α1,2 fucosyltransferase reaction was performed with 25 mM of various acceptors

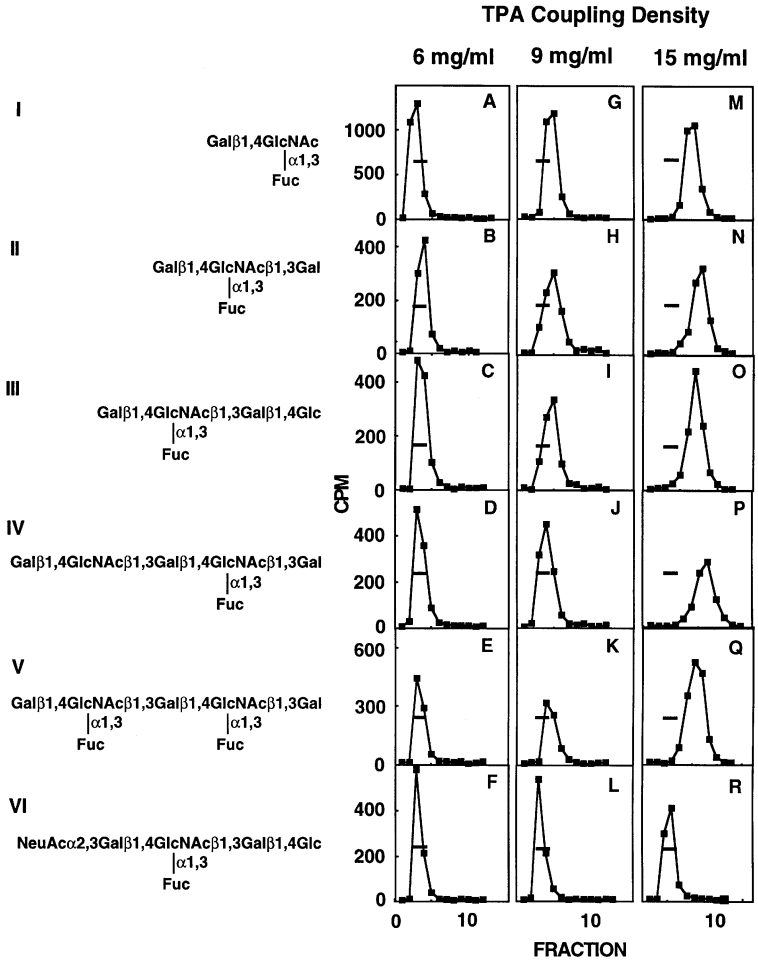

Figure 1. Chromatography of *a*1,3 fucosylated oligosaccharides on LTA-Emphaze columns. Different *a*1,3-fucosylated oligosaccharide standards **I–VI** were applied to 6 mg ml^{-1}, 9 mg ml^{-1} and 15 mg ml^{-1} LTA-Emphaze columns, as indicated. The short horizontal bar in each panel at fractions 3 and 4 indicates the elution position of oligosaccharides not interactive with the lectin.

(lactose, LNT, or LNnT), 0.67 μM GDP-[^{3}H]Fuc, and 56 μM GDPFuc, 5 mM ATP, and 30 μl of NeoH CHO cell extract (~50 μg protein). Incubations were performed at 37 °C for 2 h. The reaction mixtures were applied to QAE-Sephadex in water and the fucosylated glycans were recovered in the void volume fractions.

The Le^y-containing glycan (**XI**) was prepared in a 50 μl reaction containing 0.67 μM GDP-[^{3}H]Fuc, and 56 μM nonradiolabeled GDPFuc, 40 mM $MnCl_2$, 10 mM ATP, 30 mM L-fucose, and 1% Triton CF-54 (pH 7.0). [^{3}H]Fuc-labelled glycan **IX** was used as the acceptor and 50 μg COS/FTIV cell extract was used as the enzyme source. The reaction mixture was incubated at 37 °C for 2 h and the product was separated from GDP-[^{3}H]Fuc using QAE-Sephadex (A25). Glycan **XI** was purified from **IX** by preparative descending paper chromatography [25].

Glycan **XIII** was synthesized by fucosylating the disaccharide GalNAc*β*1,4GlcNAc using GDP-[^{3}H]Fuc and a COS/FTIV cell extract as the enzyme source, as described above. The disaccharide acceptor was synthesized using bovine milk UDPGal:GlcNAc-*β*-1,4-galactosyltransferase and nonradiolabeled UDPGalNAc as the donor in the presence of *α*-lactalbumin (8 mg ml^{-1}), as described previously [26].

Glycan **XIV** was synthesized as described [27]. Briefly, [^{3}H]-glucosamine-labelled Gal*β*1,4GlcNAc*β*1,6[Gal*β*1,3] GalNAcOH was isolated from total [^{3}H]-glucosamine-labeled HL-60 cell *β*-eliminated glycans. The tetrasaccharide was fucosylated with nonradiolabeled GDPFuc as the donor and COS/FTIV as the enzyme source, as described above.

Preparation of radiolabeled glycopeptides

COS7/FTIV cells were metabolically labeled with 0.1 mCi ml^{-1} [^{3}H]-glucosamine in DMEM, 10% foetal calf serum, 2 mM glutamine, and 400 μg ml^{-1} G418 at 37 °C for 24–48 h. The [^{3}H]-glucosamine-labelled glycopeptides were prepared by incubating the cell pellet with 10 mg ml^{-1} Pronase in digestion buffer (0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$) in a total volume of 0.5 ml at 60 °C

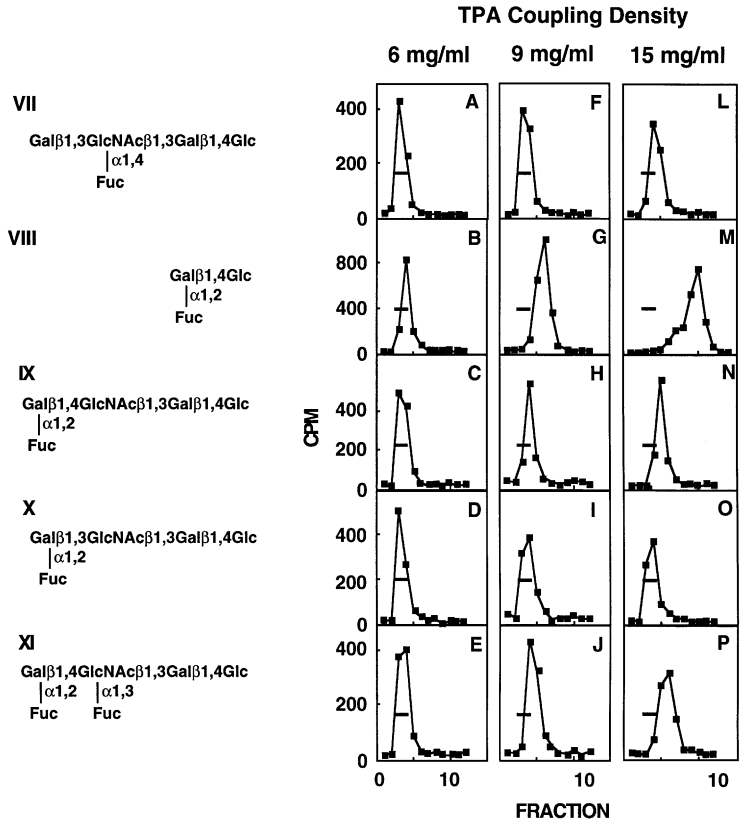

Figure 2. Chromatography of fucosylated oligosaccharides on LTA-Emphaze columns. Oligosaccharides **VII–XI** were applied to 6 mg ml^{-1}, 9 mg ml^{-1} and 15 mg ml^{-1} LTA-Emphaze columns, as indicated. The short horizontal bar in each panel at fractions 3 and 4 indicates the elution position of oligosaccharides not interactive with the lectin.

for 16–24 h [6]. The radiolabelled glycopeptides were recovered after gel filtration on a column of Sephadex G-25 (1 × 50 cm) in 7%-1-propanol. The samples were pooled and dried by lyophilization.

Di- and triantennary N-glycans terminating in multivalent Le^x determinants (di- and trifucosylated) (compounds **XV** and **XVI**) were graciously provided by Dr Kevin G. Rice, University of Michigan [28]. These glycans are modified with a single *tert*-butoxycarbonyl-tyrosine on the reducing end of each oligosaccharide that was iodinated, as described [27].

Preparation of radiolabeled fucosylated glycopeptides from fetuin and orosomucoid

Fetuin and orosomucoid (25 mg each) were incubated with 10 mg ml^{-1} Pronase in digestion buffer as described above. The recovered glycopeptides, corresponding to 1.2 μmol of fetuin carbohydrate and 3.3 μmol of orosomucoid carbohydrate, were desialylated by incubation with 5 μl (50 mU) of neuraminidase in 20 μl of 0.1 M of sodium acetate (pH 4.8) at 37 °C overnight. The desialylated glycopeptides were then fucosylated to generate the Le^x determinant using a COS7/FTIV cell extract as an enzyme source. The α1,3-fucosyltransferase reactions were carried out at 37 °C for 2 h in a 50 μl incubation mixture containing 60 μg of cell extract, 11.76 μM GDPFuc, 0.47 μM GDP-[^{3}H]Fuc, in 100 mM sodium cacodylate buffer (pH 7.0) 40 mM $MnCl_2$, 10 mM ATP, 30 mM-L-fucose, 1% Triton CF-54.

Affinity chromatography of oligosaccharides on LTA-Emphaze

One hundred μl of radiolabeled glycans (about 1000–2000 cpm) in TBS were applied to the 6, 9 and 15 mg ml^{-1} LTA-Emphaze columns (1.5 ml bed volume). The unbound glycans were eluted with TBS. In some cases glycopeptides and multivalent Le^x-containing N-glycans that were tightly bound to the column were eluted with 500 mM L-fucose in TBS. Fractions (0.5 ml) were collected directly into scintillation vials and counted in a liquid scintillation counter. The flow rate of the LTA-Emphaze columns was approximately 0.35 ml min^{-1}. All lectin chromatography experiments were conducted at room temperature. Recovery of radioactivity was routinely greater than 90% for all lectin chromatography experiments.

Glycopeptides or the di- and triantennary Le^x-containing glycans were fractionated on 2.0 ml columns (Bio-Rad minicolumn) of Con A-Sepharose equilibrated with TBS at room temperature, as described [29, 30]. The columns

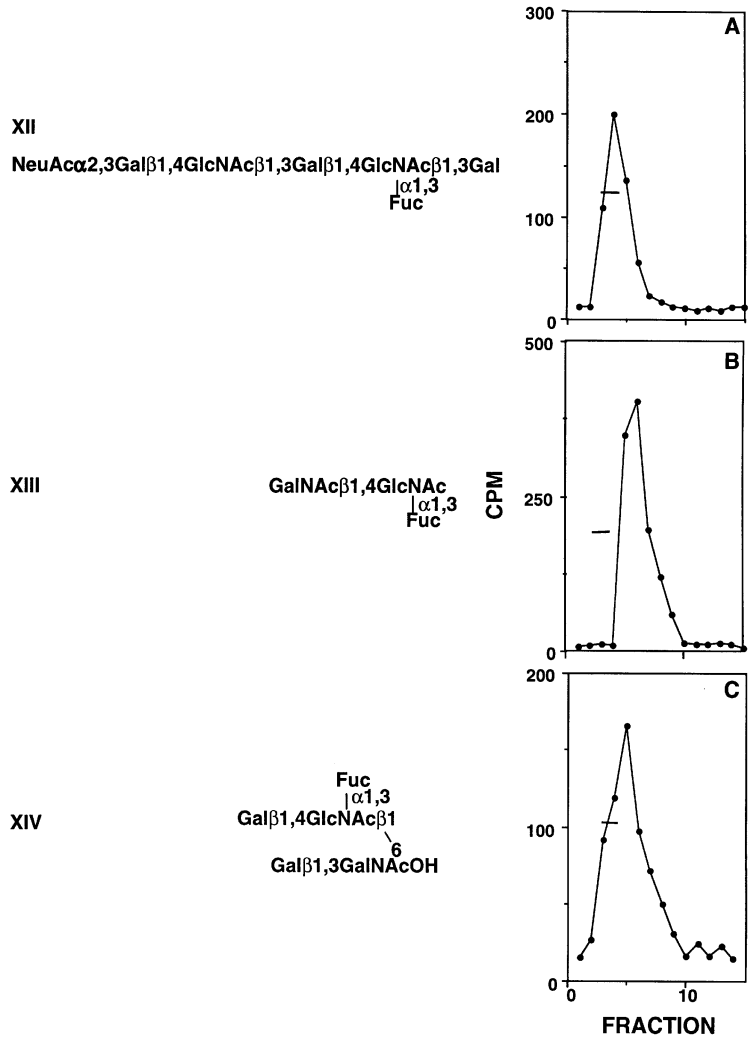

Figure 3. Chromatography of additional fucose-labelled oligosaccahrides. Oligosaccharides containing the Le^x determinant were prepared as described under Materials and Methods and applied to a 15 mg ml^{-1} LTA column. (A) VIM-2 (**XII**) isolated from [^{3}H]glucosamine-labeled CHO/FTIII cells; (B) [^{3}H]Fucose-labeled GalNAcβ1,4[Fucα1,3] GlcNAc (**XIII**); (C) [^{3}H]glucosamine-labeled fucosylated core-2 O-glycan Galβ1,4[Fucα1,3]GlcNAcβ1,6[Galβ1,3] GalNAcOH (**XIV**). The short horizontal bar in each panel at fractions 3 and 4 indicates the elution position of oligosaccharides not interactive with the lectin.

contained 2 ml of Con A-Sepharose and the flow rate was approximately 0.5 ml min^{-1}. Bound glycans were eluted with 10 mM α-methylglucosidase in TBS or 100 mM α-methylmannoside in TBS, as indicated. COS/FTIV glycopeptides were fractionated by serial lectin chromatography, first on Con A-Sepharose and then on 15 mg ml^{-1} LTA-Emphaze. The pooled fractions were dried and desalted by column chromatography on Sephadex G-25 (1 × 50 cm).

Results

Chromatography of fucosylated glycans on immobilized LTA

LTA was immobilized on Emphaze beads at three different densities, 6, 9, and 15 mg ml^{-1}. Approximately the same amount of each of the different oligosaccharide standards was applied to LTA-Emphaze columns and the results are shown in Figs 1–3.

None of the fucosylated glycans **I–XI** were retarded significantly in their elution from the 6 mg ml^{-1} LTA-Emphaze (Fig. 1A–F and Fig. 2A–E). However, glycans **I–V** which contain α1,3-linked fucose were retarded in their elution from the 9 mg ml^{-1} column (Fig. 1G–L) and more significantly retarded in elution from the 15 mg ml^{-1} column (Fig. 1M–R). In contrast, the sialylated Le^x glycan **VI** was not retarded in elution, even on the 15 mg ml^{-1} column (Fig. 1F, L and R). Glycan **IV**, which contains an α1,3-linked fucose on the innermost GlcNAc residue also bound to LTA (Fig. 1D, J and P), similar to **II** and **III** binding. The binding of glycan **V**, which has two α1,3-linked fucosyl

residues (Fig. 1E, K and Q) was no better than for glycans with single α1,3-linked fucosyl residues. These results demonstrate that LTA binds to glycans containing a single α1,3-linked fucosyl residue in the Le^x motif, and that sialylation of the Le^x determinant blocks recognition by LTA.

We then assessed the ability of LTA to bind to glycans containing fucosyl residues in other linkages to the lactosaminyl sequence. Oligosaccharide **VII**, which contains the Le^a determinant and is identical to **III** except for linkage of the galactosyl and fucosyl residues, was bound poorly by even the highest density LTA column (Fig. 2A, F and L). These results demonstrate that LTA binds the Le^x structure with higher affinity than the Le^a structure.

It was reported previously that 2′-fucosyllactose (**VIII**) inhibits LTA-mediated agglutination of cells [31]. Interestingly, this simple glycan was retarded in its elution by both the 9 mg ml^{-1} and 15 mg ml^{-1} LTA columns (Fig. 2B, G and M). This result was not anticipated, since other preliminary studies in our laboratory had indicated that LTA bound poorly to CHO cells expressing the H-antigen structure (NeoH CHO cells) compared to CHO cells expressing the Le^x-containing structures. In addition, this poor reactivity of LTA to H-antigen versus Le^x-antigen expressing cells is consistent with earlier studies by Muramatsu *et al.* [33]. We therefore prepared a larger glycan (**IX**) with a type-2 chain containing the H-antigen. Glycan **IX** was weakly bound by even the high density LTA column (Fig. 2C, H and N). The isomeric structure **X**, which has the H-antigen structure on a type 1 chain, was not recognized by immobilized LTA (Fig. 2D, I and O). Glycan **XI**, which has two fucosyl residues, was bound only weakly by even the highest density LTA column (Fig. 2E, J and P) and its elution profile was not greatly different from **IX**. These results demonstrate that LTA does not bind well to either the H- antigen, Le^a or Le^y structures relative to the Le^x structure. These data also demonstrate that LTA binds better to glycans having type-2 chain than to those having type-1 chain.

Other unique fucosylated glycans were tested for binding to the 15 mg ml^{-1} LTA column (Fig. 3). Glycan **XII**, which contains the structure of the VIM-2 antigen, was not able to interact with LTA (Fig. 3A), even though the nonsialylated analog (structure **IV**) did interact strongly with LTA immobilized at the same density. This demonstrates that sialylation interferes with LTA binding even when a sialic acid residue is not adjacent to the Le^x structure.

A modified form of the Le^x structure, **XIII**, containing a terminal GalNAc instead of Gal in the lacdiNAc motif, was tested for binding to LTA (Fig. 3B). This structure did interact with LTA, and the interaction was only slightly less than that of the related oligosaccharide **I**.

The above results indicate that all simple glycans containing the Le^x determinant can interact with LTA. However, in recent studies of O-glycans we identified some α1,3-fucosylated core-2 O-glycans that bind weakly to LTA. The Le^x structure is present on O-glycans extended from the core 2 β1,6 branched GlcNAc [34]. Therefore, we tested the minimum core-2 containing glycan that also could carry the Le^x structure (structure **XIV**) (Fig. 3C). This compound was only slightly retarded in its elution from the 15 mg ml^{-1} LTA column, suggesting that the O-glycan core-2 structure may interfere with recognition of the Le^x antigen by LTA. In addition, these data suggest that LTA may be affected its recognition of the Le^x determinant by structures at the reducing termini of glycans.

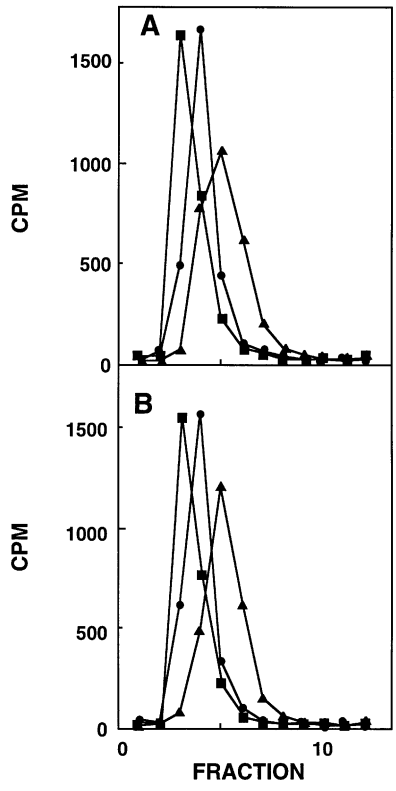

Figure 4. Chromatography of [^{3}H]fucose-labeled glycopeptides from desialylated fetuin and orosomucoid on LTA-Emphaze columns. [^{3}H]Fucose-labeled glycopeptides containing the Le^x determinant were enzymaticaly synthesized using desialylated glycopeptides derived from fetuin and orosomucoid, as described under Materials and Methods. (A) [^{3}H]fucose-labeled glycopeptides from fetuin; (B) [^{3}H]fucose-labeled glycopeptides from orosomucoid. The labeled glycopeptides were applied to the three different LTA-Emphaze columns and their elutions are shown. In each panel the triangle represents glycopeptides on the 15 mg ml^{-1} LTA-Emphaze column, the circle represents glycopeptides on the 9 mg ml^{-1} LTA-Emphaze column, and the square represents glycopeptides on the 6 mg ml^{-1} LTA-Emphaze column.

Binding of fucosylated glycopeptides from fetuin and orosomucoid

The studies described above were conducted with relatively simple glycans containing Le^x determinants. We sought to demonstrate whether immobilized LTA could be useful for

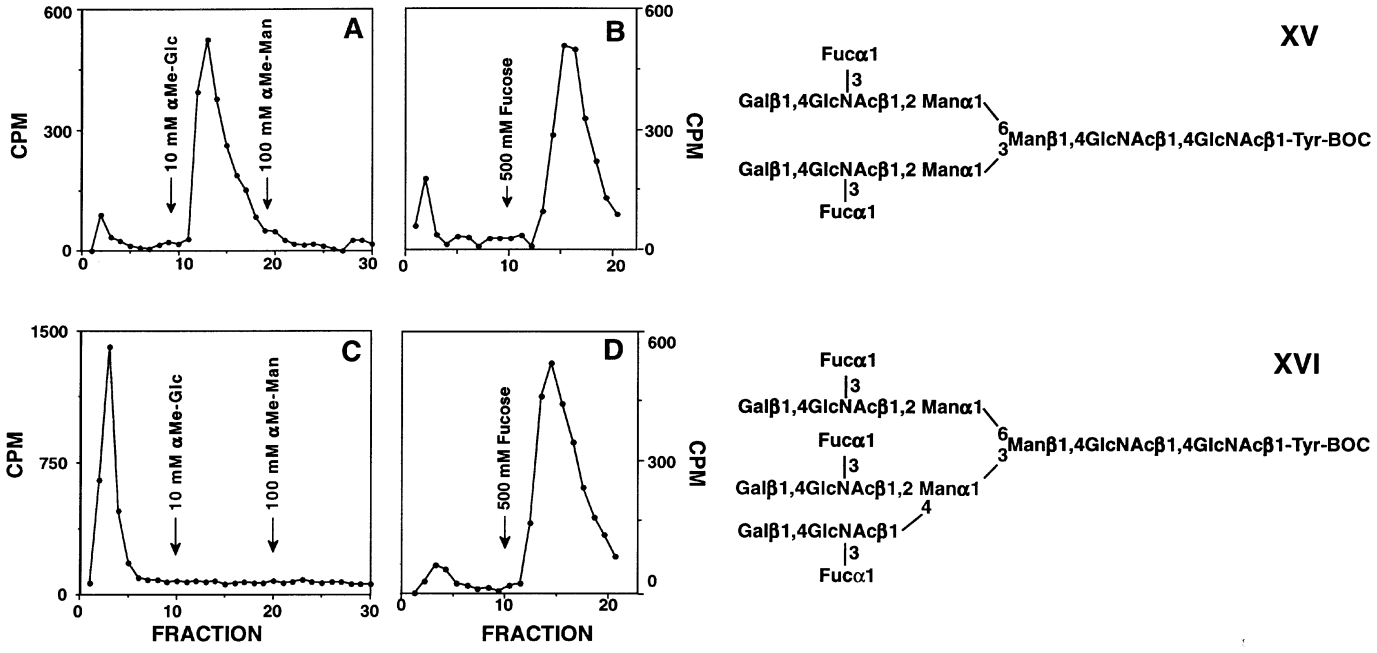

Figure 5. Lectin affinity chromatography of [^{125}I]-labeled multivalent Le^x-terminated N-glycans. [^{125}I]-labeled multivalent (difucosylated) Le^x-terminated diantennary N-glycan (**XV**) (A, B) or multivalent (trifucosylated) Le^x-terminated triantennary N-glycan (**XVI**) (C, D) were applied either to a column of Con A-Sepharose (A, C) or to a column containing 15 mg ml^{-1} LTA-Emphaze (B, D).

isolating large-sized N-glycans containing Le^x. Desialylated glycopeptides were prepared from bovine fetuin and human orosomucoid containing tri- and tetraantennary complex-type N-glycans, respectively. The glycans were fucosylated to generate the Le^x determinant by incubation with extracts of COS7/FTIV and GDP-[^{3}H]Fuc for 2 h. Based on the amount of radioactive fucose incorporated into the glycopeptides, each glycan was determined to contain, on average, a single α1,3-linked fucosyl residue. The [^{3}H]fucose-labelled glycopeptides from both fetuin and orosomucoid were retarded in their elution from the 9 and 15 mg ml^{-1} columns of LTA-Emphaze (Fig. 4A, B) in a fashion similar to that for glycans containing a single Le^x determinant. These results demonstrate that immobilized LTA can be used to fractionate complex-type N-glycans containing the Le^x determinant.

The fucosylated glycans from fetuin and orosomucoid were determined to contain only one fucose residue per oligosaccharide. To assess the binding of oligosaccharides with multivalent Le^x determinants, quantitatively-fucosylated N-glycans were used [28]. Di- and triantennary N-glycans with two and three terminal Le^x determinants, respectively, were applied to either Con A-Sepharose or LTA-Emphaze columns (Fig. 5). The difucosylated diantennary N-glycan (**XV**) bound Con A-Sepharose and was eluted with 10 mM α-methylglucoside (Fig. 5A). This structure also bound tightly to LTA and was eluted with 500 mM fucose (Fig. 5B). The trifucosylated triantennary N-glycan (**XVI**), synthesized from fetuin, did not bind Con A (Fig. 5C), but was tightly bound to LTA (Fig. 5D).

The inability of the triantennary N-glycan **XVI** to bind Con A-Sepharose was not surprising, since triantennary oligosacchardies with 2,4 disubstituted Man residues do not bind Con A-Sepharose [35]. However, it has been reported that difucosylated diantennary N-glycans similar to **XV** do not bind to Con A-Sepharose, and those with a single outer fucosyl residue bind weakly to the resin [36]. However, we have found that **XV** binds well to the commercial Con A-Sepharose obtained from Pharmacia. This preparation contains a high amount of coupled Con A (>15 mg ml^{-1}). This high density coupling may explain the apparent discrepancy. In the original study of Yamashita *et al.* [36] no information is supplied as to coupling density of the lectin, flow rates, etc.

Overall, these data demonstrate that the number of terminal Le^x determinants per glycan greatly influences the degree of interaction with LTA. These data also demonstrate that LTA is useful for separating and characterizing oligosaccharides that contain multivalent terminal Le^x structures.

Binding of glycopeptides from COS7/FTIV cells to immobilized lotus lectin

To demonstrate the usefulness of immobilized LTA for isolating glycans containing α1,3 fucosylated residues, we

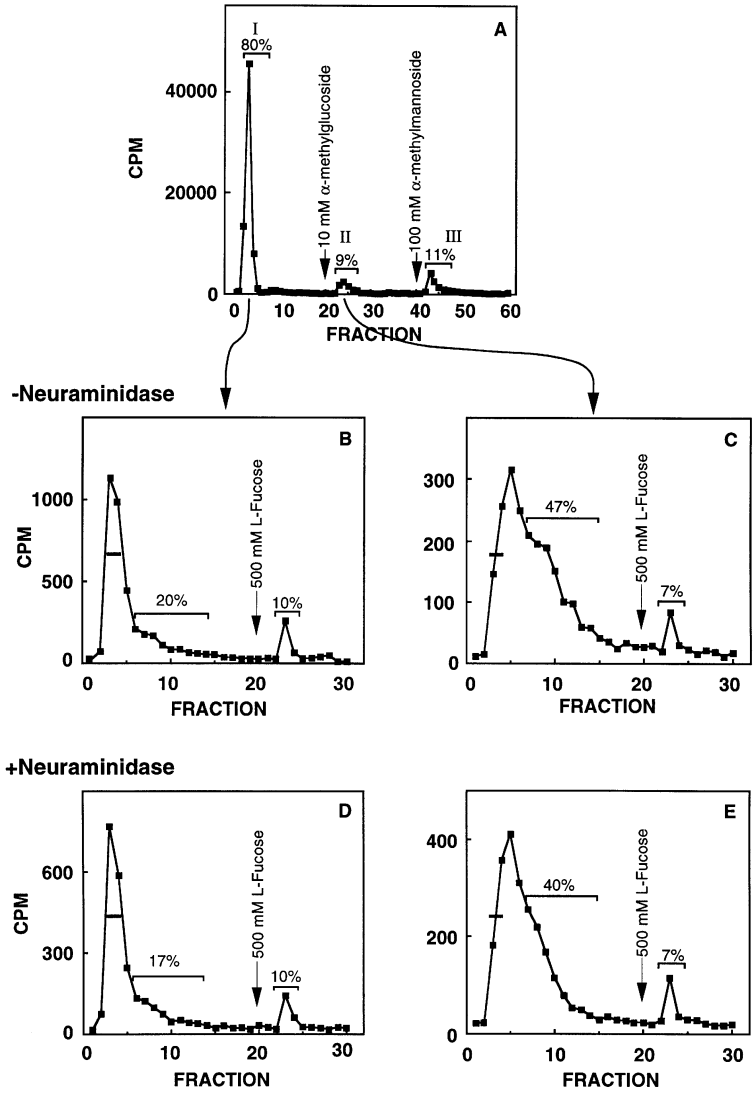

Figure 6. Serial lectin affinity chromatography of [^{3}H]-glucosamine-labeled glycopeptides from COS7/FTIV. [^{3}H]-glucosamine-labeled glycopeptides from COS7/FTIV cells were applied to a column of Con A-Sepharose, as described under Materials and Methods. The indicated fractions from the Con A-Sepharose column were pooled, dried, desalted, treated with or without neuraminidase, and applied to a column containing 15 mg ml^{-1} LTA-Emphaze. (A) Elution profiles of radiolabelled glycopeptides from COS7/FTIV cells on Con A-Sepharose; (B, C) elution profiles of peak I and peak II glycopeptides, respectively, on the LTA-Emphaze column. The short horizontal bar in each panel at fractions 3 and 4 indicates the elution position of oligosaccharides not interactive with the lectin.

prepared radiolabeled glycopeptides from both parental COS7 cells and the COS7/FTIV cells. Parental COS7 cells do not express any Lewis determinants and have been used as expression systems for studying the functions of human fucosyltransferases [22, 37]. In contrast, COS7/FTIV cells express high levels of Le^x determinants, but not sialyl Le^x [38]. It should be noted that although complex-type N-glycans from parental COS7 cells lack the Le^x determinant, oligosaccharides from these cells do contain fucosyl residues linked α1,6 to the innermost GlcNAc residues.

COS7/FTIV cells were labeled with [^{3}H]-glucosamine and the radiolabelled glycopeptides were first fractionated by chromatography on Con A-Sepharose (Fig. 6A). The glycopeptides not bound by Con A-Sepharose are expected to be highly branched N-glycans, O-glycans and glycosaminoglycans. In contrast, the glycans bound to Con A-Sepharose and eluted with 10 mM α-methylglucoside are the diantennary complex-type N-glycans. The high mannose-type and hybrid-type N-glycans were eluted from Con A-Sepharose with 100 mM α-methylmannoside [7]. Eighty per cent of the [^{3}H]-glucosamine-labeled glycopeptides from COS7/FTIV cells failed to bind to Con A-Sepharose (designated peak I), whereas 9% of the recovered glycopeptides bound to Con A-Sepharose and were eluted with α-methylglucoside (designated peak II) (Fig. 6A).

The [^{3}H]-glucosamine-labeled glycopeptides in peaks I and II were further subjected to affinity chromatography on a 15 mg m^{-1} column of LTA-Emphaze (Fig. 6B, C). Glycopeptides bound by LTA were eluted with 500 mM α-L-fucose. Twenty per cent of the peak I material was retarded in elution from the column, and 10% of the glycopeptides were bound to the lectin column and eluted with 500 mM fucose (Fig. 6B). Those glycopeptides bound to LTA-Emphaze and required fucose hapten for elution are likely to contain multiple Le^x determinants (as shown in Fig. 5). About 50% of the peak II glycopeptides, which represent complex-type diantennary N-glycans, were retarded in elution from the column, and 7% were bound and eluted with 500 mM fucose (Fig. 6C). Neuraminidase treatment of the glycopeptides prior to affinity chromatography did not appreciably enhance binding to the column (Fig. 6D, E). This result was expected, since COS7/FTIV cells cannot make sialylated Le^x structures [37, 39]. These results indicate that about 7% of the diantennary N-glycans contain at least two outer Le^x structures and are bound tightly by LTA-Emphase, whereas about 50% of the diantennary N-glycans contain a single outer fucosyl residue and are retarded in their elution from the lectin column.

In control studies radiolabelled glycopeptides from parental COS7 cells were prepared and their behaviour on a 15 mg ml^{-1} column of LTA-Emphaze was analysed. Glycopeptides were fractionated by Con A-Sepharose in a similar fashion to those from COS7/FTIV cells and the peak I and II glycopeptides were analysed. None of the peak I or II glycopeptides from parental COS7 cells interacted with immobilized LTA (data not shown).

These results demonstrate that a significant percentage of the [^{3}H]-glucosamine-labeled oligosaccharides from COS7/FTIV cells contain the Le^x determinant. This interpretation is consistent with other preliminary studies in which we prepared [^{3}H]-glucosamine-labeled glycopeptides from COS7 cells stably transfected with cDNA encoding the human *FTIII* gene. These cells produce both Le^x and sialylated Le^x [22]. Many of the glycopeptides derived from the COS7/FTIII cells do not bind to the 15 mg ml^{-1} column of LTA-Emphaze until they are first desialylated to expose the Le^x determinant (data not shown).

To determine the reliability and reproducibility of the chromatographic procedure, the [^{3}H]-glucosamine-labeled glycopeptides from COS7/FTIV cells from Fig. 6C were reanalysed by chromatography on LTA-Emphaze. Three different samples of glycopeptides were pooled as indicated in Fig. 7A and re-analysed by chromatography on LTA-Emphaze. In each case the elution pattern of the three different sets of glycopeptides was different (Fig. 7B–D) and resembled their original elution position. These results demonstrate that LTA-Emphaze provides a reliable means of separating fucosylated oligosaccharides based on subtle differences in their affinity for the lectin.

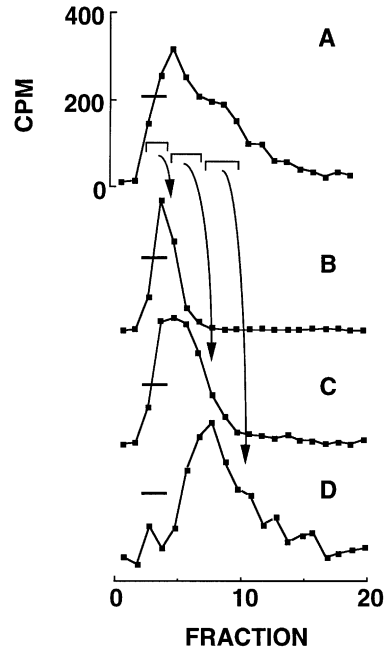

Figure 7. Rebinding of COS7/FTIV cell glycopeptides on LTA-Emphaze. COS7/FTIV Peak II glycopeptides were applied to a 15 mg ml^{-1} LTA-Emphaze column, and different fractions were pooled, and reapplied, as indicated, to a 15 mg/ml^{-1} LTA-Emphaze column. (A) COS7/FTIV peak II glycopeptides on a 15 mg ml^{-1} LTA column, as in Fig. 6C; (B) pooled fractions 3 and 4 re-applied to the LTA-Emphaze column; (C) elution profile of pooled fractions 5, 6, and 7 re-applied to the LTA-Emphaze column; (D) pooled fractions 8, 9, and 10 re-applied to the LTA-Emphaze column. The short horizontal bar in each panel at fractions 3 and 4 indicates the elution position of oligosaccharides not interactive with the lectin.

Discussion

The results of our study demonstrate that immobilized LTA (a) binds well to oligosaccharides containing the Le^x determinant; (b) binds poorly to sialylated oligosaccharides containing the Le^x structure; (c) binds poorly to oligosaccharides containing the Le^a structure or larger oligosaccharides containing the H- or Le^y antigens; and (d) can be used for isolating glycopeptides that contain the Le^x determinant in complex cell-derived mixtures. Moreover, we have defined the conditions for the coupling density of LTA to provide the most efficient means of separating the Le^x-containing oligosaccharides from those lacking the determinant.

Previous studies show that hemagglutination by LTA is inhibited by some, but not all, fucosylated oligosaccharides [9]. For example, both 2′-fucosyllactose and lacto-N-fucopentaose III (oligosaccharides **VIII** and **III**, respectively) inhibited LTA agglutination whereas lacto-N-fucopentaose I (oligosaccharide **X**) was not an inhibitor [9]. Our results are in agreement with these findings. Our results are also in accordance with those of Debray *et al.* who found that a Le^x-containing oligosaccharide was a potent inhibitor of LTA agglutination [32].

These results contribute significantly to our understanding of the specificity and utility of LTA. Previous studies did not examine the interaction of LTA with sialyl Le^x determinants, but it is clear that sialylation inhibits interaction of oligosaccharides with the lectin. We found that the Le^a oligosaccharide (**VII**) binds weakly to even the high density LTA column, whereas the Le^x-containing oligosaccharides (**I, II, III** and **V**) are highly retarded in their elution. A unique oligosaccharide containing the lacdiNAc motif (**XIII**) was also able to interact with LTA. This structure is similar to the Le^x structure, but is modified with a terminal GalNAc residue in the β1,4 linkage to GlcNAc, instead of a Gal residue as seen in the authentic Le^x determinant. This finding is interesting because this structure is present on recombinant glycoproteins expressed in 293 cells and has been shown to be a potent inhibitor of the vascular adhesion molecule, P-selectin [40]. These results demonstrate that LTA does not require a galactosyl residue in the underlying backbone and that a GalNAc residue can substitute effectively.

In addition, we found that LTA bound well to a type 2 chain oligosaccharide lacking the Le^x motif but having an internal α1,3-fucosyl residue (structure **IV**). Structure **IV** is related to the VIM-2 antigen (**XII**), which is the sialylated version of **IV** having the structure NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ-R [36]. However, sialylation of this structure (**XII**) at the nonreducing terminus completely inhibited binding to LTA. This suggests that any sialic acid residue, even if distal to the Le^x structure, prohibits LTA interaction with the Le^x determinant. Similarly, modifications at the reducing end of the oligosaccharide may influence LTA binding, since the Le^x-containing core-2 O-glycan (structure **XIV**) interacted very weakly with LTA. This interaction may be inhibited by the presence of an alcohol at the reducing end of the oligosaccharide. Alternatively, the β1,6 linkage of the GlcNAc may also prevent interaction of the Le^x determinant with LTA.

It has been observed that hemagglutination by LTA is inhibited by small mono-, di- and trisaccharides containing fucosyl residues in a variety of linkages. As examples, Fucα1-6GlcNAc, Fucα1-3GlcNAc, α-methyl-Fuc, and Fucα1-2Galβ1-4Glc are all potent and relatively equal inhibitors of LTA-induced agglutination [9, 11, 32]. This is in contrast to the inability of large-sized oligosaccharides containing even multiple fucosyl residues to substantially inhibit or bind to the lectin [9, 32 and current study]. These results suggest that the binding site for LTA can directly accommodate fucosyl residues, but when the fucose is part of a larger oligosaccharide, the sequence (e.g. type 1 versus type 2) and conformation of the oligosaccharide, in addition to the linkage of the fucose, all influence the recognition of the oligosaccharide by the lectin and generate the type of specificity we have observed.

We chose to examine the effect of coupling density of the LTA to Emphaze, following our initial observation that a coupling density of only 6 mg ml^{-1} was not sufficient to support the binding density of any test oligosaccharides to the immobilized LTA. This indicates that LTA has relatively low affinity for fucosylated oligosaccharides, which is consistent with previous inhibition studies indicating that millimolar concentrations of 2′-fucosyllactose are needed to obtain 50% inhibition of hemagglutination by LTA [9]. Oligosaccharides containing multiple terminal Le^x structures (**XV** and **XVI**) are bound with higher affinity by LTA, as we observed for the multivalent Le^x-terminating N-glycans and the cell-derived glycopeptides. Our studies show that for isolating glycans containing a single Le^x moiety it is necessary to use high densities of immobilized LTA. The densities of LTA used in our study far exceed those of most commercially available LTA supports, where the density of lectin conjugation is in the range of 1-5 mg ml^{-1}. We were able to reuse the columns prepared in this study for dozens of analyses over a period of months, without appreciable loss of lectin activity.

Serial lectin affinity chromatography based on the differential affinity of oligosaccharides for a series of immobilized lectins has proven to be useful in fractionating complex mixtures of oligosaccharides [4–6]. Our study demonstrates that LTA-Emphaze can now also be used efficiently within the overall scheme to isolate oligosaccharides containing Le^x determinants.

Acknowledgements

This work was supported by NIH Grant CA37626 to R.D.C. The authors thank Drs Qun Zhou, Kwame Nyame, and Jiunn-Chern Yeh for helpful discussion and critical reading of the manuscript and Ms Judy Gaar for preparation of the manuscript.

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Received 7 May 1996, accepted 27 May 1996