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Studies of the binding specificity of the soluble 14 000-dalton bovine heart muscle lectin using immobilised glycolipids and neoglycolipids*

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ABSTRACT

The aim of the present study has been to investigate the binding specificity of the soluble 14 000-dalton lectin of bovine heart muscle towards immobilised oligosaccharides in clustered form. To this end, chromatogram overlay assays and quantitative plastic-microwell-binding assays have been performed using several natural glycolipids and neoglycolipids containing one or more of the disaccharide units, β-D-Gal*p*-(1→4 or 3)-D-GlcNAc or β-D-Gal*p*-(1→4)-D-Glc and related structures. The microwell assay gave the most consistent results. It was observed that for binding by the soluble lectin the optimal sequence, which is β-D-Gal*p*-(1→4 or 3)-D-GlcNAc, must occur at the nonreducing end of longer oligosaccharides when linked to lipid. These oligosaccharides may be of poly(*N*-acetyllactosamine) type or they may be mono- or multi-antennary, complex-type chains in which the disaccharide is joined directly to a trimannosyl core. The lectin bound to such immobilised lipid-linked oligosaccharides on which the terminal D-galactosyl groups are substituted with α-L-Fuc*p*-(1→2), α-D-Gal*p*-(1→3), or α-NeuAc-(2→3) groups. However, no binding was detected if the terminal D-galactosyl groups were substituted with an α-NeuAc-(2→6) group or the subterminal *N*-acetylglucosamine units with an α-L-Fuc*p*-(1→3 or -4) group. Internally located *N*-acetyllactosamine units where the D-galactose units are disubstituted by β-D-GlcNAc*p*-(1→3) and -(1→6) units, as in branched poly(*N*-acetyllactosamine) backbones were not bound by the bovine lectin. These results are in accord with previous observations on the bovine lectin and the corresponding human and rat lectins, using structurally defined oligosaccharides as inhibitors of binding. The results of comparative binding experiments using paragloboside and ceramide hexasaccharide which contain one and two *N*-acetyllactosamine units, respectively, joined in linear sequence to the lactosylceramide core, were equivocal with respect to the availability of the internal *N*-acetyllactosamine units for binding by the bovine lectin.

INTRODUCTION

Bovine heart muscle contains a soluble β-D-galactoside-binding lectin with a subunit molecular weight of approximately 14 000 (refs. 1–3) which is a member of a unique family of proteins widely distributed in animal tissues[4,5]. The lectin occurs predominantly as a soluble cytosolic protein consistent with its lack of a signal sequence[3]; a membrane-associated form dissociable with lactose has also been described[1].

* Dedicated to Professors Toshiaki Osawa and Nathan Sharon.

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The *in vivo* oligosaccharide ligands for the lectin and their subcellular locations are not yet known. The ligands potentially include oligosaccharide components of glycoconjugates that are soluble, or membrane-associated, or associated with the extracellular matrix. The type and presentation of oligosaccharide ligands for the lectin may differ in these subcellular locations; they may be sparsely distributed or clustered. Hence, it is desirable to know the reactivities of the lectin both with soluble and immobilised glycoconjugates, and under different assay conditions. To date, three assay procedures have been used: (a) Binding of the soluble lectin to erythrocytes and to immobilised glycoproteins, (b) inhibition of lectin binding by glycoproteins and oligosaccharides, and (c) binding of glycopeptides to immobilised lectin. Specificity for terminal D-galactosyl groups was clearly indicated by lectin binding to immobilised asialofetuin and enhanced lectin binding to erythrocytes after their treatment with sialidase[1]. Similarly, asialo-erythrocyte glycoproteins and oligosaccharides were stronger inhibitors of lectin binding than their sialylated forms, and β-D-galactosidase treatment of asialo-oligosaccharides impaired their inhibitory activity[1]. Inhibition of binding assays with structurally defined oligosaccharides[1,6,7] have indicated that *N*-acetyllactosamine, β-D-Gal*p*-(1→4)-D-GlcNAc and its isomer/β-D-Gal*p*-(1→3)-D-GlcNAc, which are more potent inhibitors than D-galactose and lactose [β-D-Gal*p*-(1→4)-D-Glc] are the optimal recognition structures for the combining site of the lectin. Substitution of the terminal D-galactose units with α-L-Fuc*p*-(1→2) or α-D-Gal*p*-(1→3) groups diminished the inhibitory activity by approximately 50%, and with α-NeuAc-(2→3) groups by 60%, while substitution with α-NeuAc-(2→6) or α-Gal*p*NAc-(1→3) groups abolished inhibitory activity, as did substitution of *N*-acetyl-D-glucosamine with α-L-Fuc*p*-(1→4 or -3) groups[7]. The common feature of the nonreactive oligosaccharides (as discussed in ref. 7) is that the substitutions mask the axial OH-4 of the D-galactose, the CH_2OH-6 of the D-galactose, and the equatorial OH-3 or -4 of the *N*-acetyl-D-glucosamine and D-glucose units. These groups have therefore been proposed as the major determining factors in recognition by the bovine heart lectin, a feature shared with soluble β-D-galactoside-binding lectins of rat and human lung[8,9]. When the lectin was immobilised on Sepharose beads, it was observed to bind with high affinity to Pronase glycopeptides rich in poly(*N*-acetyllactosamine) sequences, derived from Chinese hamster ovary (CHO) cells and from BW 5147 mouse lymphoma cells[10]. Residual binding after sequential treatments of the CHO cell glycopeptides with mild acid (to remove sialic acid) and exo-β-D-galactosidase was interpreted as lectin binding to certain internal D-galactose units, in addition to terminal, unsubstituted D-galactosyl groups on these glycopeptides. Here, we describe a fourth approach to understanding the reactivities of the bovine heart muscle lectin, namely, binding of the soluble lectin to structurally defined, lipid-linked oligosaccharide sequences in the form of natural glycolipids and neoglycolipids immobilised on silica gel chromatograms and in plastic microwells.

TABLE I

Reactivity of glycolipids with the soluble bovine lectin in the t.l.c.-overlay and in the plastic-microwell assays

Glycolipid	*Binding*[a]	
	T.l.c.	*Wells*
Galactosylceramide (**1**)	–	[b]
Glucosylceramide (**2**)	[b]	–
Lactosylceramide (**3**)	–	–
Trihexosylceramide (**4**)	–	[b]
Globoside (**5**)	–	[b]
Forssman (**6**)	–	[b]
Paragloboside (bovine) (**7**)	+	+
Ceramide 5 (**8**)	tr	+
Ceramide 6 (**9**)	+	+
Ceramide 7 (**10**)	–/+	+
Ceramide 9 (**11**)	–	–
Ceramide 12 (**12**)	+	+
Ceramide 15 (**13**)	+	+
Ceramide 8 (**14**)	[b]	+
Ceramide 10 (**15**)	[b]	+

[a] Symbols +, –, and tr indicate binding, lack of binding, or negligible binding, respectively. –/+ Indicates variable results. [b] Not tested.

EXPERIMENTAL

Materials. — Bovine heart lectin. Lectin was isolated from a homogenate of bovine heart[2] by affinity chromatography on a column of lactose coupled to Sepharose 6B *via* a divinyl sulphone bridge according to Fornstedt and Porath[11]. Aprotinin (0.1 trypsin inhibitory units/mL) and 0.1 mM phenylmethylsulphonyl fluoride were incorporated[12] as protease inhibitors during tissue homogenisation. The lectin was stored frozen at −70°.

Glycolipids. The glycolipids investigated are shown in Table I (see Scheme 1).

βGal→Cer (**1**) βGlc→Cer (**2**) βGal→4βGlc→Cer (**3**)

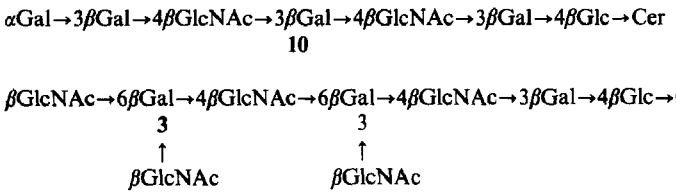
αGal→4βGal→4βGlc→Cer (**4**) βGalNAc→3αGal→4βGal→4βGlc→Cer (**5**)

αGalNAc→3βGalNAc→3αGal→4βGal→4βGlc→Cer (**6**)

βGal→4βGlcNAc→3βGal→4βGlc→Cer (**7**)

αGal→3βGal→4βGlcNAc→3βGal→4βGlc→Cer (**8**)

βGal→4βGlcNAc→3βGal→4βGlcNAc→3βGal→4βGlc→Cer (**9**)



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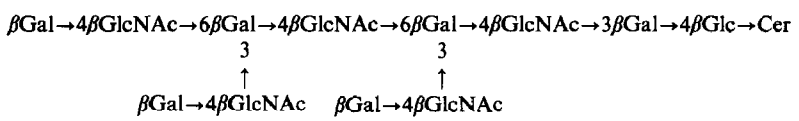

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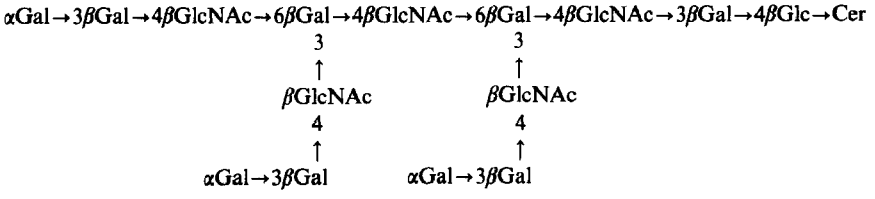

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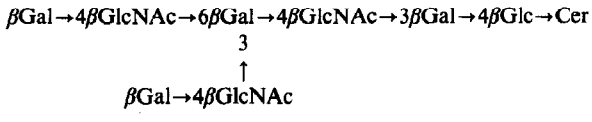

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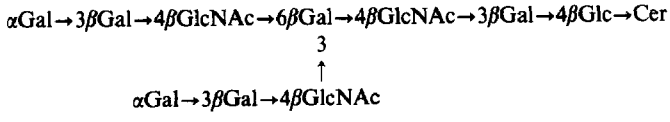

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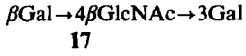

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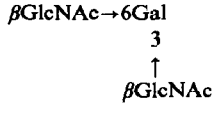

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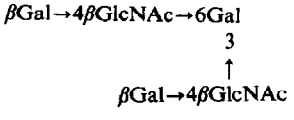

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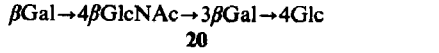

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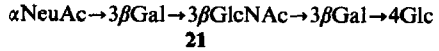

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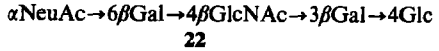

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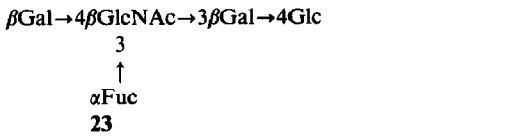

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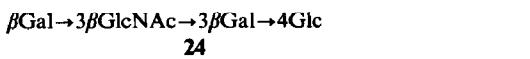

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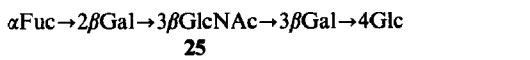

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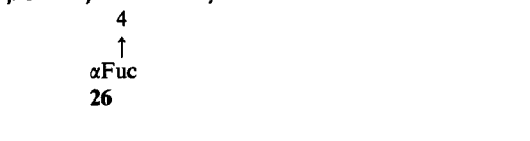

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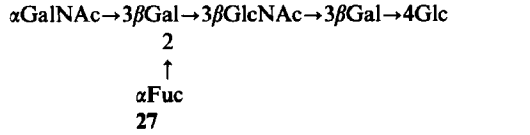

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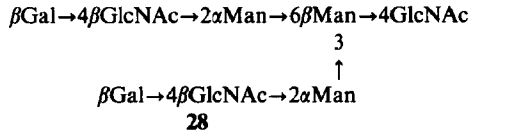

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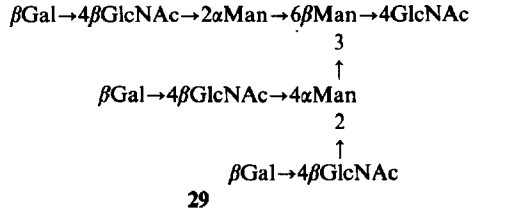

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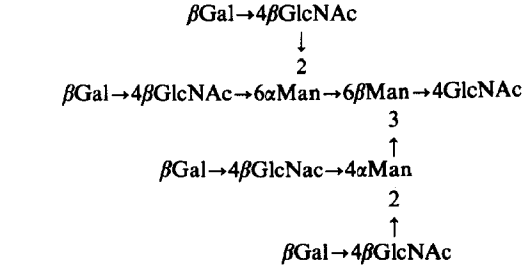

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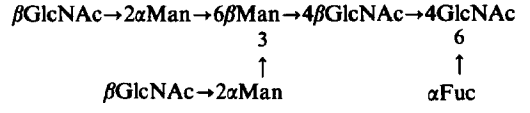

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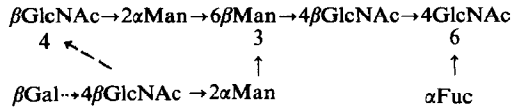

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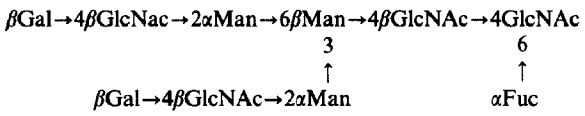

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Scheme 1. Structures of glycolipids and neoglycolipids investigated. Glycosyl linkage at O-1 (O-2 for NeuAc), D configuration (L for fucose), and pyranose form are assumed.

These consisted of glucosylceramide (**2**) (glucocerebrosides) from human Gaucher's spleen (Sigma Chemical Co., Poole, Dorset, UK); a mixture of the neutral glycolipids galactosylceramide (**1**), lactosylceramide (**3**), trihexosylceramide (**4**), globoside (**5**), and

TABLE II

Reactivities of neoglycolipids derived from oligosaccharides[a]

Neoglycolipid derived from oligosaccharide	*Binding*	
	T.l.c.	*Wells*
Lactose	–	–
3-Sialyllactose	–	[b]
6-Siallylactose	–	[b]
N-Acetyllactosamine	–	[b]
04 (**16**)	–	–
07 (**17**)	–	–
01 (**18**)	–	[b]
08 (**19**)	+	–
Chitotriose	–	[b]
LNNT (**20**)	+	+
S1 (**21**)	–/+	+
S3 (**22**)	–	–
LNFP-III (**23**)	–	[b]
LNT (**24**)	+	+
LNFP-I (**25**)	+	+
LNFP-II (**26**)	–	–
A Hexasaccharide (**27**)	–	[b]
GM_1-A (**28**)	+	+
GM_1-B (**29**)	+	+
GM_1-C (**30**)	+	+
Agal-IgG (**31**)	–	[b]
Monogal-IgG (**32**)	+	[b]
Digal-IgG (**33**)	+	[b]

[a] See footnote to Table I. [b] Not tested.

Forssman glycolipid (**6**) (Biocarb, Lund, Sweden); paragloboside (**7**) prepared by treatment of sialosylparagloboside from bovine erythrocytes[13] with *Vibrio cholerae* neuraminidase and ceramide 5 (**8**) from bovine erythrocytes[13] (gifts of Dr. K. Uemura); ceramides 7 (**10**), 10 (**15**), and 15 (**13**) derived from rabbit erythrocytes[14,15]; ceramides 6 (**9**), 8 (**14**), and 12 (**12**) prepared by α-D-galactosidase treatment of ceramides 7 (**10**), 10 (**15**), and 15 (**13**), respectively; and ceramide 9 (**11**) prepared by Smith degradation of ceramide 12 (**12**). A preparation of paragloboside isolated from human myeloid cells (gift of Dr. B. Macher) was also used.

Neoglycolipids. Table II shows the structures of the oligosaccharides used for preparing neoglycolipids[16] by conjugation to the lipid phosphatidylethanolamine dipalmitoate as described by Stoll *et al.*[17]. These consisted of lactose, lacto-*N*-tetraose (LNT, **24**), lacto-*N*-neotetraose (LNNT, **20**), lacto-*N*-fucopentaose I (LNFP-I, **25**), lacto-*N*-fucopentaose II (LNFP-II, **26**), lacto-*N*-fucopentaose III (LNFP-III, **23**), blood group A hexasaccharide (**27**), urinary oligosaccharides GM_1-A (**28**), GM_1-B (**29**), and GM_1-C (**30**) (for descriptions, see refs. 17 and 18), desialylated human IgG oligosaccharides (**31–33**), and chitotriose (prepared as described in refs. 19 and 20, respectively). Neoglycolipids were also prepared[17] from the sialyloligosaccharides S1 (**21**) and S3 (**22**) (gifts of Dr. A. S. R. Donald, Research Sugars, Aylesbury, Buckinghamshire, UK); maltopentaose (Sigma); 3- and 6-sialyllactoses (see ref. 21); and the chemically synthesised oligosaccharides *N*-acetyllactosamine, 01 (**18**), 04 (**16**), 07 (**17**), and 08 (**19**) (gifts of Dr. A. Veyrières).

For microwell-binding assays (see below), the neoglycolipids were separated from free lipid and other components in the conjugation mixture using a C_{18} Bond Elut column (100 mg) (Analytichem International, USA, purchased from Jones Chromatography, Hengoed, Glamorgan, UK). The column was washed with 60:35:8 (v/v) chloroform–methanol–water (solvent *A*) and equilibrated with 3:14:6 (v/v) chloroform–methanol–water, (solvent *B*). The neoglycolipid-conjugation mixture (containing 50–400 μg of oligosaccharide) was dried under N_2, dissolved in solvent *B* (0.25–1 mL) and applied to the column. The fall through, followed by a wash of solvent *B* (400 μL) was collected and a further 16 fractions (400 μL each) were collected, four using solvent *B*, ten using 2:7:3 (v/v) chloroform–methanol–water, and two using solvent *A*. A sample (1 μL) of each fraction was applied for t.l.c.[17] using aluminium-backed silica gel plates and solvent *A*. Lipid was detected with primulin and hexose with orcinol stain. The fractions containing uncontaminated neoglycolipid (reacting to both reagents) were pooled, evaporated under N_2, and taken up in solvent *B* or 19:19:2 (v/v) chloroform–methanol–water. The derivatives were quantified with a Shimadzu CS-9000 scanner following t.l.c. in solvent *A* and staining with orcinol or primulin, using the neoglycolipid derivative of maltopentaose as a standard.

Binding of lectin to glycolipid and neoglycolipids. — Chromatogram overlay assays[22] were performed at ambient temperature (20–25°) as described in ref. 17 with minor modifications. In brief, t.l.c. of glycolipids and neoglycolipids (1–2 μg of carbohydrate per lane) was performed on aluminium-backed silica gel plates with solvent *A* or 105:100:28 (v/v) chloroform–methanol–water (solvent *C*). Chromatograms were first

soaked in Plexigum P28 for 60 s (Cornelius Chemical Co., Romford, Essex, UK) and then in 20mM phosphate buffered saline (PBS), pH 7.2, containing 5% (w/v) bovine haemoglobin (BDH, Poole, Dorset, UK) for 2 h, and overlaid for 1 h with bovine lectin (100 μg/mL) in PBS containing 1% (w/v) haemoglobin and mM dithiothreitol (DTT) (from Boehringer Mannheim GmbH, Germany), or with the diluent alone as a control. They were then washed with PBS–mM dithiothreitol and overlaid for 2 h with a 1:50 dilution of a rabbit antiserum to the bovine lectin[2] in PBS, washed, and overlaid for 1 h with ^{125}I-labeled[23] (5×10^5 c.p.m.) Staphylococcal protein A (Sigma) per mL of PBS containing 1% (w/v) bovine haemoglobin. After washing, binding was detected by autoradiography[17] for 1 to 8 days. Unless otherwise stated, chromatograms of the glycolipids and neoglycolipids shown after chemical staining (orcinol) were the same as those used for the binding experiments. Binding was not detected in control experiments where the lectin was applied in the presence of a saccharide inhibitor (50mM thiodigalactoside) of the lectin. Also, where the lectin was omitted, no binding was detected to any of the glycolipids or neoglycolipids.

In a second assay system, glycolipids and the purified neoglycolipids were dried by evaporation (except paraglobosides and ceramides 5 to 15 (**8–15**), dried by lyophilization) and serially diluted in methanol containing 4 μg/mL each of cholesterol and egg lecithin; 30 μL of the dilutions were applied in duplicate to Immulon 1 unirradiated U-bottom plastic microwells (Dynatech Laboratories Limited, Billingshurst, UK) and dried overnight by evaporation at 37°; all further incubations were at room temperature. Wells were washed twice with PBS, after which PBS containing 5% (w/v) bovine haemoglobin (50 μL) was added to each well and incubated for 2 h. After washing twice with PBS, bovine lectin [30 μL; 100 μg/mL in PBS containing 1% (w/v) haemoglobin and mM dithiothreitol] was added and incubated for 1 h. Wells were washed three times with PBS–mM dithiothreitol and rabbit antiserum to bovine lectin at 1:100 dilution in PBS (30 μL) was added and incubated for 2 h. After washing three times with PBS, ^{125}I-labeled Staphylococcal protein A ($4–8 \times 10^4$ c.p.m.) in PBS containing 1% (w/v) haemoglobin (30 μL) was added to each well and incubated for 1 h. After washing five times with PBS, the radioactivity counts bound per well were determined by use of a Nuclear Enterprises 1600 gamma counter. Baseline radioactivity counts were those bound to wells coated with diluent containing carrier lipids only. The means of the radioactivity counts bound are indicated for each assay. When lectin was omitted or when lectin was applied to glycolipids or neoglycolipids in the presence of 50mM thiodigalactoside, binding was always at baseline.

RESULTS

Influence of oligosaccharide chain length on lectin binding. — Oligosaccharide chain length is clearly an important factor for lectin binding to lipid-linked oligosaccharides on the chromatogram surface, since the lectin bound strongly to bovine paraglobosides and to neoglycolipids derived from the tetrasaccharides LNNT (**20**) and LNT (**24**) but not to neoglycolipids derived from *N*-acetyllactosamine and the trisaccharides

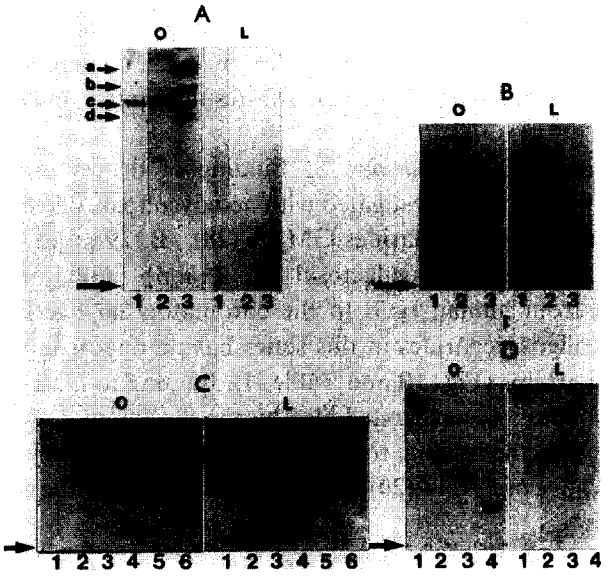

Fig. 1. Binding of the soluble bovine heart lectin to natural glycolipids and neoglycolipids on silica gel chromatograms. Chromatogram overlay assays were performed, as described in the Experimental section, using 1 μg of carbohydrate for each glycolipid or neoglycolipid per lane (lane 5, part C contained approx. 0.5 μg carbohydrate; the same result was obtained by use of 1 μg of carbohydrate in a separate experiment). Chromatography was in solvent *A*. Large arrows indicate origin. Binding of lectin was detected by autoradiography (panels L); glycolipid and neoglycolipid bands were then detected by orcinol stain of the same chromatograms (panels O). Autoradiography was for 3 d (parts A, B, D) or 6 d (Part C). Part A: Lanes 1 and 2 contained neoglycolipids derived from lactose and *N*-acetyllactosamine, respectively, and lane 3, a mixture of the glycolipids lactosylceramide (**3**) (duplex band a), trihexosylceramide (**4**) (band b), globoside (**5**) (band c), and Forssman glycolipid (**6**) (band d). Part B: Lanes contained neoglycolipid derived from LNNT (**20**) (lane 1), oligosaccharide 07 (**17**) (lane 2), and oligosaccharide 08 (**19**) (lane 3). Part C: Lanes contained neoglycolipids derived from LNT (**24**) (lane 1), LNFP-I (**25**) (lane 2), LNFP-II (**26**) (lane 3), LNFP-III (**23**) (lane 4), LNNT (**20**) (lane 6). Lane 5 contained ceramide 5 (**8**). Part D: Lane 1 contained bovine paragloboside (**7**), and lanes 2, 3, and 4 contained neoglycolipids derived from LNNT (**20**), S1 (**21**), and S3 (**22**).

04 (**16**) and 07 (**17**) (Fig. 1, and Tables I and II). Lactosylceramide and neoglycolipids derived from lactose and 3- and 6-sialyllactoses were also not bound in chromatogram overlay assays (Fig. 1A, and Tables I and II), nor were galactosylceramide (**1**), trihexosylceramide (**4**), globoside (**5**), and Forssman glycolipid (**6**) (Table I and Fig. 1A). The results of plastic microwell-binding assays performed with bovine paragloboside (**7**), lactosylceramide (**3**), and neoglycolipids derived from LNNT (**20**), LNT (**24**), trisaccharides 04 (**16**) and 07 (**17**), and lactose were in good accord with those of the chromatogram overlay assays [results with bovine paragloboside (**7**), lactosylceramide (**3**), and LNNT (**20**) are shown in Fig. 2A]; in both assay systems, the radioactivity counts bound to this preparation of paragloboside were consistently fewer than those bound to the neoglycolipid derived from LNNT (**20**). LNT (**24**) gave similar results to LNNT (**20**) in separate microwell binding experiments (not shown). There is an apparent discrepancy in results with the two assay systems using the neoglycolipid derived from the branched

oligosaccharide 08 (**19**) which has two accessible β-D-galactosyl groups. The lectin clearly bound to this neoglycolipid in the chromatogram overlay assay (1 μg of the derivative was applied), but there was no detectable binding on the plastic microwell assay at the highest concentration tested (200 pmol, 0.2 μg). This result requires further investigation.

Lectin binding to complex-type oligosaccharides. — Binding of the lectin to clustered, complex-type oligosaccharides was investigated with neoglycolipids derived from the bi-, tri-, and tetra-antennary oligosaccharides GM_1-A (**28**), -B (**29**), and -C (**30**), and with sialidase-treated biantennary agalactosyl (**31**), mono- (**32**), and di-galactosyloligosaccharides (**33**) from human IgG. In the chromatogram overlay assay, the lectin bound to all the oligosaccharides in this series having one or more nonreducing terminal D-galactosyl groups (Fig. 3B and Table II). Binding to GM_1-A (**28**), -B (**29**), and -C (**30**) neoglycolipids was evaluated also by the plastic microwell assay (Fig. 2B). Per mole of derivative, the binding to each of the three complex-type chains was greater than to the derivative of LNNT (**20**). However, the binding to the tri-

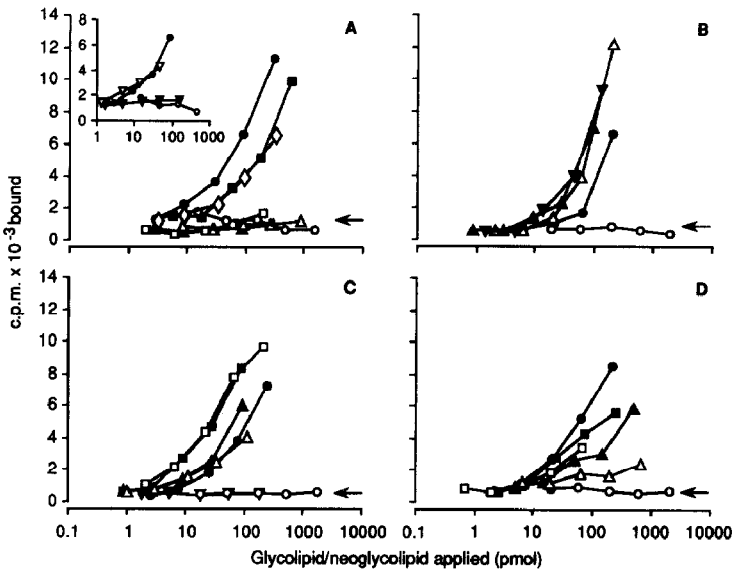

Fig. 2. Binding of soluble bovine heart lectin to natural glycolipids and neoglycolipids immobilized in microwells of plastic plates. Microwell binding assays were performed as described in the Experimental section. Each panel shows the results of a separate experiment where the horizontal arrows indicate radioactivity counts bound to wells coated with the carrier lipids only. In all panels, the symbols ○ and ● were for glucosylceramide (**2**) (used as a negative control) and the neoglycolipid from LNNT (**20**), respectively. In a separate experiment (not shown), the neoglycolipid of LNT (**24**) gave a comparable binding to that of LNNT (**20**). Other symbols are as follows. Panel A: ■, paragloboside from bovine erythrocytes (**7**); △, lactosylceramide (**3**); ▲ neoglycolipid from lactose; □, neoglycolipid from oligosaccharide 08 (**19**); ◇ ceramide 5 (**8**), not shown here are neoglycolipids from oligosaccharides 04 (**16**) and 07 (**17**) which gave no binding above background. Panel A inset: ▽ and ▼, neoglycolipids from oligosaccharides S1 (**21**) and S3 (**22**), respectively. Panel B: △, ▲, ▼, neoglycolipids from oligosaccharide GM_1-A (**28**), GM_1-B (**29**), and GM_1-C (**30**) respectively. Panel C: ▲, △, ▼, ■, □, ceramides 8 (**14**), 10 (**15**), 9 (**11**), 12 (**12**) and 15 (**13**), respectively. Panel D: ▲, △, paragloboside (**7**) prepared from bovine erythrocytes and from human myeloid cells, respectively; □, ■, ceramides 6 (**9**) and 7 (**10**), respectively.

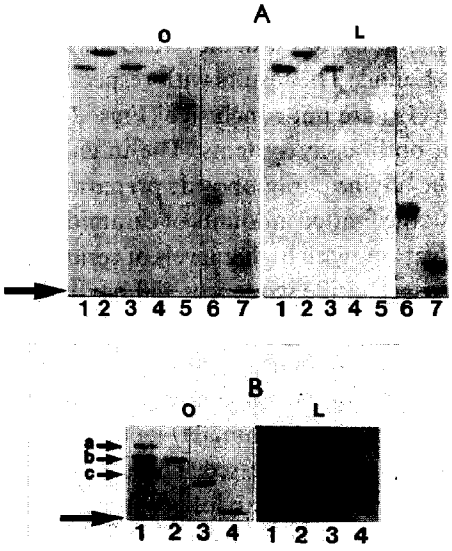

Fig. 3. Binding of soluble bovine heart lectin to natural glycolipids and neoglycolipids on silica gel chromatograms. Procedures and designations of panels are as described in legends to Fig. 1 and approximately 1 μg carbohydrate for each glycolipid or neoglycolipid was applied, except bands b and c in panel B, lane 1 ($\sim$ 2 μg of each). Chromatography was in solvent *C* (part A) and solvent *A* (part B). Autoradiography was for 3 days. Part A: Lane 1 contained neoglycolipid derived from LNNT (**20**); other lanes contained natural glycolipids: bovine paragloboside (**7**) (lane 2), ceramide 6 (**9**) (lane 3), ceramide 7 (**10**) (lane 4), ceramide 9 (**11**) (lane 5), ceramide 12 (**12**) (lane 6), and ceramide 15 (**13**) (lane 7). The orcinol stained lanes 6 and 7 (panel O) are not those used in panel L, hence the difference in mobilities of the glycolipid bands. Part B: Lane 1 contained neoglycolipids derived from IgG oligosaccharides (biantennary agalactosyl) structures (**31**), band a; monogalactosyl structures (**32**), band b; digalactosyl structures (**33**), band c). Lanes 2, 3, and 4 contained neoglycolipids derived from oligosaccharides GM_1-A (**28**), -B (**29**), and -C (**30**), respectively.

and tetra-antennary structures was no greater than to the biantennary structure. This result suggests that the lectin binds to only two of the outer chains on the tri- and tetra-antennary oligosaccharides. Purified monoantennary, complex-type chain was not available for comparison in the microwell assay. Nevertheless, the results showed that the lectin does not require a repeating β-D-Gal*p*-(1$\rightarrow$3 or 4)-β-D-Glc*p*NAc-(1$\rightarrow$3)-β-D-Gal*p*-(1$\rightarrow$4)-D-GlcNAc or -D-Glc sequence for binding, but can also bind to clustered oligosaccharides that contain the nonreducing terminal β-D-Gal*p*-(1$\rightarrow$4)-β-D-Glc*p*NAc-(1$\rightarrow$2)-α-D-Man*p*-(1$\rightarrow$6 or 3)-D-Man sequence as on *N*-linked oligosaccharides.

*Lectin binding to oligosaccharides of poly(*N*-acetyllactosamine) type.* — The influence of a poly(*N*-acetyllactosamine) backbone structure on the intensity of lectin binding was investigated by comparing the binding to paragloboside (**7**) and to ceramides 6 (**9**), 9 (**11**), and 12 (**12**). Conclusive results were obtained only with respect to the branched poly(*N*-acetyllactosamine) backbones (Table I, Figs. 2C and 3A). In both assays, the lectin bound to ceramide 12 (**12**) but not to ceramide 9 (**11**), which is derived

from ceramide 12 (**12**) by removal of the terminal D-galactosyl groups. Thus, not only is the 3-substituted D-galactosyl residue near the glycolipid core not accessible for lectin binding, but the two *N*-acetyllactosamine units at branch points in the backbone having 3,6-*O*-disubstituted galactose units are also not bound. The results with respect to the internal *N*-acetyllactosamine unit in ceramide 6 (**19**) are not conclusive (Figs. 2D and 3A); the lectin bound well to this glycolipid in both assay systems. The intensity of binding was comparable to that observed with the bovine paragloboside preparation at the levels tested in the plastic microwell assay, but the limited amounts of ceramide 6 (**9**) precluded assays at higher levels. Further investigations with higher levels of ceramide 6 (**9**) and with ceramides having longer linear oligosaccharide sequences will be required to evaluate lectin binding to internal *N*-acetyllactosamine units having 3-*O*-linked galactose units within the poly(*N*-acetyllactosamine) backbone.

Effects of substitution at D-*galactose and* N-*acetyl*-D-*glucosamine residues on lectin binding.* — The effects on lectin binding of substitution of the terminal D-galactosyl and subterminal *N*-acetyl-D-glucosamine residues were further addressed. There was a lack of lectin binding to the neoglycolipids derived from the 6-*O*-sialyloligosaccharide S3 (**22**) and the 4-*O*-L-fucosyloligosaccharide LNFP-II (**26**) in both assay systems, and to the 3-*O*-L-fucosyloligosaccharide LNFP-III (**23**) and the blood group A hexasaccharide (**27**) tested by the chromatogram overlay assay only (Table II); results of the chromatogram overlay assay are shown in Figs. 1C and D. These results are in accord with the lack of inhibitory activity of the corresponding free oligosaccharides[7]. Also in accord with oligosaccharide inhibition data[7] was the binding of the lectin in both assay systems to the lipid-linked 2-*O*-L-fucosyloligosaccharide LNFP-I (**25**) (Table I); the chromatogram overlay result is shown in Fig. 1C.

Lectin binding to the 3-*O*-sialyloligosaccharide S1 (**21**) was variable in the chromatogram overlay assay; in two out of four experiments, there was no detectable binding to the neoglycolipid derived from this oligosaccharide as in Fig. 1D; binding was detectable in the other experiments (not shown). In the plastic microwell assay, the lectin bound to the S1 neoglycolipid (**21**) as strongly as to the neoglycolipid derived from LNNT (**20**) (Fig. 2A, inset).

Substitution of the β-D-Gal*p*-(1→4)-D-GlcNAc sequence with an α-D-Gal*p*-(1→3) group has a more marked effect on lectin binding under the conditions of the chromatogram overlay assay: lectin binding was not detected with ceramide 5 (**8**) under the standard assay conditions, *i.e.*, 1–3 days autoradiography (Fig. 1C); only a very weak binding was detected after prolonged autoradiography (8 days, results not shown). With ceramide 7 (**10**), lectin binding was observed in one of two experiments (Table I). Similarly, with ceramide 15 (**13**), which tended to chromatograph as a diffuse band, binding was observed in one of two chromatogram-binding experiments (Fig. 3A and Table I). In contrast, in the plastic microwell assay, the intensity of lectin binding to bovine paragloboside (**7**) and to ceramide 5 (**8**) was comparable (Fig. 2A); similarly the binding to the ceramides 6/7 (**9**/**10**) pair was comparable (Fig. 2D) as was binding to the ceramides 12/15 (**12**/**13**) pair (Fig. 2C). Limited amounts of the ceramides 8/10 (**14**/**15**) pair were also available for the microwell binding assay; lectin binding to both prepa-

rations was observed, though fewer radioactivity counts were bound to ceramide 10 (**15**) (Fig. 2C). Taken together, the results showed that the bovine lectin can bind to the terminal *N*-acetyllactosamine unit that is substituted with an α-D-Gal*p*-(1→3) group, and that the presentation in the microwell assay is better for lectin binding.

Influence of ceramide composition on lectin binding to glycolipids. — During the review stage of this manuscript, we have noted that the ceramide component may influence the intensity of lectin binding to natural glycolipids, since a second preparation of paragloboside (derived from myeloid cells) with a different ceramide composition (unpublished observations) gave negligible lectin binding, compared with the binding observed with paragloboside derived from bovine erythrocytes (Fig. 2D). Thus, comparisons of lectin binding within ceramides pair 6/7 (**9**/**10**), ceramides pair 8/10 (**14**/**15**), and within the set of ceramides 9/12/15 (**11**/**12**/**13**) are valid, since ceramides 7 (**10**), 10 (**15**), and 15 (**13**) were the starting compounds for the three sets of glycolipids, respectively. However, the glycolipids cannot be compared between sets without detailed knowledge of their ceramide components.

DISCUSSION

The salient conclusions from the present study are, first, that among the sequences investigated in lipid-linked form, the β-D-Gal*p*-(1→4 or 3)-D-GlcNAc unit is optimal for binding by the soluble lectin, but the disaccharide must occur at the nonreducing terminal part of longer oligosaccharides. These may be of poly(*N*-acetyllactosamine) type or they may be mono- or multi-antennary, complex-type chains in which the disaccharide is joined directly to the tri-D-mannosyl core without intervening *N*-acetyllactosamine sequences. Second, on natural glycolipids, the lactosyl sequence (a potential ligand for the lectin) adjacent to the ceramide component is not available for binding. Third, lectin binding is prevented by α-NeuAc-(2→6) substitution of the terminal D-galactose units, and by α-L-Fuc*p*-(1→3) or -(1→4) substitution of the subterminal *N*-acetylglucosamine unit. Fourth, the lectin binds in the presence of α-L-Fuc*p*-(1→2), α-D-Gal*p*-(1→3), or α-NeuAc-(2→3) substitution of the terminal D-galactose unit; binding to the latter two is detected best by the microwell assay. Fifth, the lectin does not bind to the *N*-acetyllactosamine sequence where the terminal D-galactose unit is disubstituted by β-D-Glc*p*NAc-(1→3) and -(1→6) groups, nor does it bind to internal *N*-acetyllactosamine sequences where the galactose units are similarly 3,6-*O*-disubstituted, as in branched poly(*N*-acetyllactosamine) backbone sequences. All of these results are consistent with the oligosaccharide inhibition data available for the 14 000-dalton bovine lectin[1,6,7], and those for the corresponding rat[8] and human[9] lectins. The question of the extent of contribution to lectin binding of internal *N*-acetyllactosamine units in unbranched backbones of poly(*N*-acetyllactosamine) type, where the D-galactosyl residues are monosubstituted by β-D-Glc*p*NAc-(1→3) residues, has not been resolved in the present study. Further investigations with a series of linear hexa-, octa-, and deca-saccharides in this series will be required to resolve this point. It should be noted, however, that per mole of oligosaccharide, the inhibitory activity of the

tetrasaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, towards the bovine heart lectin is no greater than that of *N*-acetyllactosamine[7]. This result suggested that the internally located disaccharide unit may not contribute to lectin binding.

Glycoconjugates with I and i antigen activities (*i.e.*, rich in unsubstituted poly(*N*-acetyllactosamine) sequences[25], isolated from bovine heart muscle, were shown previously to be potent inhibitors of lectin binding[6]. These observations, together with the glycopeptide-binding data using the bovine lectin immobilised on Sepharose beads[10], indicated that bovine heart muscle tissue, CHO cells, and BW 5147 mouse lymphoma cells contain oligosaccharides of poly(*N*-acetyllactosamie) type that are rich in recognition structures for the bovine heart lectin. Detailed structural studies of the purified oligosaccharides from these sources will be required to identify precisely the recognition structures on these glycoconjugates. Asialo, triantennary, complex-type glycopeptides from fetuin, which have the same D-galactose-terminated outer chains as the oligosaccharide GM_1-B (**29**) used in the present study, did not bind to the immobilised bovine lectin[10]; the authors commented that this is probably because the lectin was coupled at a density too low for binding to these glycopeptides. The results in the present study showed that when the bi-, tri-, or tetra-antennary oligosaccharides of complex-type are presented in clustered form, the soluble lectin binds strongly.

Although in the present study with lipid-linked oligosaccharides, the soluble lectin showed no binding to oligosaccharides shorter than tetrasaccharides, the lectin binds tightly to lactose that has been coupled to Sepharose *via* a divinyl sulphone bridge (see Experimental section); this was shown earlier with the chick skeletal muscle lectin[26]. This result suggested that at the surface of both the silica gel and the plastic microwell, the lipid components exert a steric hindrance on the adjoining oligosaccharide domain. With neoglycolipids, an additional consideration is that there is a modification (ring opening)[17] of the monosaccharide unit linked to the lipid residue. However, such ring opening does not interfere with the binding of the trisaccharide-specific monoclonal antibody, anti-I Ma, as the neoglycolipid derived from a trisaccharide hapten for this antibody (04, **16**) is bound by the antibody both in the chromatogram and the plastic microwell assays[16,27]. Binding studies with lipid-linked oligosaccharides incorporated into cell membranes will be required to determine the minimum chain length of oligosaccharides linked to lipid to which the lectin can bind on intact cellular membranes. The observation with the natural glycolipid, paragloboside, that the composition of the ceramide components may substantially influence the extent of lectin binding means that such investigations will need to take into account structures of both the carbohydrate and the ceramide units of glycolipids. The advantage of the neoglycolipids is that lectin binding to defined oligosaccharides attached to the same lipid can be readily compared.

Recent immunohistochemical experiments[28] strongly suggested that the 14000-dalton lectin of cultured murine muscle cells is translocated to the cell exterior under certain conditions. This work was carefully performed[28] to minimise spurious antigenic cross-reactions with other proteins, which is a major concern[3] in immunochemical work

with the 14000-dalton lectin. The present study goes some way to reveal several potential ligands for the soluble lectin on the sugar chains of glycoproteins and glycolipids of the types that occur at locations outside the cell cytosol.

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