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High and low affinity carbohydrate ligands revealed for murine SIGN-R1 by carbohydrate array and cell binding approaches, and differing specificities for SIGN-R3 and langerin

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Abstract

The number of receptors of the 'C-type' lectin family is greater than previously thought with a considerable proportion on cells (dendritic cells and macrophages) critical for innate immunity. Establishing that they bind carbohydrates, unravelling and comparing details of their ligands is crucial for understanding the molecular basis of the cell–cell and cell–pathogen interactions that they mediate. Here we use carbohydrate arrays as a new approach to discovering the ligands of three recently described C-type lectin-type receptors on antigen-presenting cells: murine SIGN-R1, SIGN-R3 and langerin. The arrays encompass an extensive panel including polysaccharides, glycoproteins, oligosaccharides and monosaccharides. These are probed with soluble forms of the receptors (IgG–Fc chimeras). The dominant specificities found for SIGN-R1 and SIGN-R3 are mannose- and fucose-related, as expressed on high mannose type *N*-glycans and Lewis$^{a/b}$/Lewis$^{x/y}$-type sequences, respectively, with subtle differences between the receptors. The dominant specificity for langerin is unique so far: a Lewis^{x}-related sequence with sulfate at position 6 of the terminal galactose. The polysaccharide dextran, known from classical studies to elicit a T-independent response, and whose cellular uptake has been shown recently to be mediated by membrane-associated SIGN-R1, gave no binding signals with the soluble form of the protein. We highlight here the additional need for cell-based assays for detecting biologically relevant low affinity ligands, for we show with SIGN-R1-transfected cells that dextran is such a low affinity ligand for SIGN-R1 that binding is detectable only with the cell membrane-associated receptor. But there is a close relationship between dextran recognition and mannose/fucose recognition, with dextran- and mannose-conjugates co-localizing in intracellular compartments.

Introduction

Carbohydrate-binding proteins of the 'C-type' lectin and Siglec families are intimately involved in mechanisms of cell trafficking in inflammation, immune cell activation, pathogen recognition and innate immunity (1–5). A sub-family of C-type lectins, newly discovered on antigen-presenting cells, has become the subject of exciting research at the interface of innate and acquired immunity (6,7). In the human, these include the receptor known as DC-SIGN expressed mainly in immature dendritic cells (DCs) (8,9), langerin in Langerhans cells (10), and L-SIGN in endothelial cells (11).

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Information is accumulating on the carbohydrate ligands for the new family of lectins on antigen presenting cells. In the human, DC-SIGN, L-SIGN and langerin have been shown to recognize oligosaccharides with terminal mannose or fucose (12–16), and to variously exhibit carbohydrate-mediated interactions with glycoconjugates of the host and of microbial pathogens (7,17). Among the proposed outcomes of DC-SIGN interactions with counter-receptors in the host are the regulation of transendothelial migration of DCs through interactions with ICAM-2 (18), and a contribution to T-cell activation and proliferation through interactions with ICAM-3 (8). Among sequelae of DC-SIGN–pathogen interactions are the subversion of DC functions for pathogen escape from immune surveillance (19). Examples include the retention of HIV by DCs without killing, and the subsequent transmission of the virus to susceptible T cells (20,21) and promotion of persistence of *Mycobacterium tuberculosis* in DCs (22).

In the mouse, six related proteins have been cloned: DC-SIGN (23) and langerin (24) predominantly in DCs, and four SIGN-related genes, SIGN-R1, SIGN-R2, SIGN-R3 and SIGN-R4, in other cell types of the immune system (23). SIGN-R1 is expressed on medullary and subcapsular macrophages in lymph nodes (25) and on marginal zone macrophages (MZMs) (25,26). In classical studies, more than a decade before the discovery of SIGN-R1, and its assignment as a lectin-type molecule, it was recognized that bacterial polysaccharides such as dextran elicit T-cell independent antibody responses (27–30). It is now established that SIGN-R1 is a key receptor that mediates the uptake of dextran. Now that other receptors of this family have been described on antigen presenting cells, it is important to unravel and compare details of their carbohydrate ligands. This is a prerequisite for understanding the molecular basis of the cell–cell as well as cell–pathogen interactions that they mediate, thereby influencing innate and acquired immunity. This could open the way to manipulating these interactions therapeutically. The pinpointing of oligosaccharide ligands, however, remains a challenging area of cell biology (31). This is because ligands that are oligosaccharides cannot be readily cloned, each being the product of multiple glycosyltransferases. Thus there is a need for sensitive and high throughput technologies to perform analyses of carbohydrate–protein interactions in order to detect and characterize the oligosaccharide sequences bound.

Microarray approaches, analogous to those developed for DNA (32), and being developed for proteins (33), are ideal for addressing this need. Only small amounts of products are required for generating microarrays and many compounds can be screened in parallel in a single operation. Indeed, array technology is a new development in glycobiology and immunology. The special advantage is that it enables diverse carbohydrates (polysaccharides, glycoproteins, monosaccharides as well as defined oligosaccharide sequences) to be probed simultaneously with carbohydrate-binding proteins (34). Recent reports of 'proof of concept' experiments with known carbohydrate-recognizing proteins have shown there is selectivity of binding to arrayed carbohydrates. Thus, the predicted binding signals have been recorded with well characterized carbohydrate-recognizing antibodies (35–37), the selectins, a cytokine, a chemokine (36) and several plant lectins (38–40). A carbohydrate array system consisting of lipid-linked oligosaccharide probes derived from natural and chemically synthesized oligosaccharides (36) has shown promise as a powerful means of generating large repertoires of oligosaccharide sequences both for detecting protein–carbohydrate interactions and assigning the sequences recognized.

Here we describe the use of carbohydrate arrays to identify and compare the carbohydrate ligands for three recently described receptors of the immune system, murine SIGN-R1, SIGN-R3 and langerin. This was carried out by probing recombinant soluble forms of the proteins, IgG Fc chimeras, with arrays of monosaccharides, polysaccharides, glycoproteins and defined oligosaccharide sequences. In the course of this work, we have found no detectable dextran binding by the soluble form of the SIGN-R1 (nor of SIGN-R3 and langerin) although it had been clearly demonstrated previously, using SIGN-R1-transfected cells, that this receptor mediates the cellular uptake of the polysaccharide dextran (25,26). We have therefore carried out detailed carbohydrate-binding studies with cell transfectants expressing the membrane-associated protein. The results provide the explanation that, compared with other carbohydrate ligands with mannose and fucose termini, dextran has a very low affinity for SIGN-R1. Thus, the cell-based approach complements the array approach for ligand assignment, and may be necessary for detecting biologically relevant, low affinity, ligands that cannot be detected with the soluble receptor, even after multimerization.

Methods

Carbohydrate materials

The monosaccharides mannose, galactose and fucose, and a FITC-labelled neoglycoprotein, galactose linked to BSA, 15–20 moles monosaccharide per mole of albumin, were from Sigma (Dorset, UK). Biotinylated neoglycoconjugates of mannose and galactose linked to polyacrylamide (PAA), M_r ~30 000, were from Syntesome (Moscow, Russia), and are said to contain, on average, 20% mol monosaccharide. The following BSA–monosaccharide conjugates were gifts of Professor Y.C. Lee (Department of Biology, Johns Hopkins University, Baltimore, MD): mannose (BSA–Man_{23}), glucose (BSA–Glc_{25}), fucose (BSA–Fuc_{23}), galactose (BSA–Ga_{23}), *N*-acetylglucosamine (BSA–$GlcNAc_{23}$) and *N*-acetylgalactosamine (BSA–$GalNA_{23}$); the subscripts indicate the number of moles of the monosaccharides per mole of BSA. The glycoproteins ribonuclease B, invertase, soybean agglutinin, ovalbumin, fetuin and asialofetuin, and the polysaccharides heparan sulfate, heparin, fucoidan, Ficoll, yeast mannan, dextrans of 10 kDa, 70 kDa and 2000 kDa, and dextran sulfate were from Sigma. Bovine articular cartilage keratan sulfate was from Dr Gavin Brown (Department of Biological Sciences, Lancaster University, UK). The polysaccharide of *Escherichia coli*, serotype K5 was from Camilla Westling (Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden). FITC-conjugated dextrans of molecular mass 10 kDa, 70kDa, and 2000 kDa (dextrans 10K-F, 70K-F and 2000K-F), Texas-red conjugated dextran of molecular mass

Table 1. Designations and locations on the array of the monosaccharide-conjugates, glycoproteins and polysaccharides investigated, and semi-quantitative scores of the binding by soluble SIGN-R1, SIGN-R3 and langerin

	Samples	Type[a]	Location[b]	Main or representative sequences[c]	SIGN-R1[d]	SIGN-R3	Langerin
1	BSA-Man	NGP	a1	Ma	++	+/–	–
2	BSA-Glc	NGP	a2	Gc	–	–	–
3	BSA-Fuc	NGP	a3	Fu	+	+	–
4	BSA-Gal	NGP	a4	Ga	–	–	–
5	BSA-GlcNAc	NGP	a5	GcN	+/–	–	–
6	BSA-GalNAc	NGP	b1	GaN	–	–	–
7	PAA-Gal	NGC	b2	Ga	–	–	–
8	PAA-Man	NGC	b3	Ma	+	+/–	+/–
9	RNAse B	GP	b4	Maα-6(Maα-3)Maα-6(Maα-3)Maβ-4GcNβ-4GcN	–	–	+/–
10	Invertase[e]	GP	b5	-6(Ma-2)Ma-6(Ma-2Ma-2)Ma-6(Ma-3Ma-2)Ma-6(Ma-3Ma-2Ma-2)Ma-GcN-4GcN	+++	+	–
11	Soybean agglutinin	GP	c1	Maα-2Maα-6(Maα-3)Maα-6(Maα-2Maα-2Maα-3)Maβ-4GcNβ-4GcN	++*	++*	+/–*
12	Ovalbumin	GP	c2	Maα-6(Maα-3)Maα-6(Maα-2Maα-3)Maβ-4GcNβ-4GcN	+	–	+/–
13	Fetuin[e]	GP	c3	SA-3/6Ga-4GcN-2(SA-3/6Ga-4GcN-4)Ma-3(SA-3/6Ga-4GcN-2Ma-6)Ma-4GcN-4GcN	–	–	–
14	Asialofetuin	GP	c4	Gaβ-4GcNβ-2(Gaβ-4GcNβ-4)Maα-3(Gaβ-4GcNβ-2Maα-6)Maβ-4GcNβ-4GcN	–	–	–
15	HS	PS	c5	-[4GcAβ-4GcN(6SU)α]$_n$, -[4IdA(2SU)α-4GcN(2,6SU)α]$_m$-	–	–	–
16	K5	PS	d1	-[4GcAβ-4GcNα]$_n$-	–	–	–
17	Fucoidan	PS	d2	-[3(Fuα-4)Fuα-3Fuα-3Fuα(4SU)-3Fuα-3(Fuα-2)Fu(4SU)α]-	–	–	+/–
18	Ficoll	PS	d3	(Gcα-2Frα-)$_n$	–	–	–
19	Yeast mannan[e]	PS	d4	-[6(Ma-2)Ma-6(Ma-2Ma-2)Ma-6(Ma-3Ma-2)Ma-6(Ma-3Ma-2Ma-2)Ma]-	+	–	–
20	Dextran 2000K	PS	d5	-[6Gcα-6Gcα]$_n$-	–	–	–
21	Dextran 70K	PS	e1	-[6Gcα-6Gcα]$_n$-	–	–	–
22	Dextran 10K	PS	e2	-[6Gca-6Gcα]$_n$-	–	–	–
23	Dextran 2000K-F	PS	e3	-[6Gcα-6Gcα]$_n$-	–*	–*	–*
24	Dextran 70K-F	PS	e4	-[6Gcα-6Gcα]$_n$-	–	–	–
25	Dextran 10K-F	PS	e5	-[6Gcα-6Gcα]$_n$-	–	–	–
26	Dextran 70K-TR	PS	f1	-[6Gcα-6Gcα]$_n$-	–*	–*	–*
27	Dextran SU-500K	PS	f2	-[6Gcα-6Gcα]$_n$-, sulfated	++	++	++

[a]Abbreviations for glycoconjugates and their fluorescent tags: F, FITC; GP, glycoprotein; NGP, neoglycoprotein; NGC, neoglycoconjugate; PS, polysaccharide; TR, Texas red.
[b]Refers to location on array.
[c]Abbreviations for monosaccharide residues and substituents: Fr, fructose; Fu, fucose; Ga, galactose; GaN, *N*-acetylgalactosamine; Gc, glucose; GcA, glucuronic acid; GcN, *N*-acetylglucosamine; IdA, iduronic acid; Ma, mannose; SA, sialic acid; SU, sulfate.
[d]Semi-quantitative visual scores for binding signals detected: +++, very strong; ++, strong; + moderate; +/–, weak; –, not detected. * = scores of binding signals above those elicited with the control protein, mouse Ig Fc.
[e]Due to space limitation, anomeric configurations of monosaccharide residues are not shown for these glycans.

70 kDa (dextran-70K-TR) and biotinylated dextran molecular mass 500 kDa were from Molecular Probes (Leiden, The Netherlands). The main carbohydrate sequences on the arrayed glycoconjugates are described in Table 1.

The lipid-linked oligosaccharides investigated are listed in Table 2. Unless otherwise stated, they are the compounds described in (36). The Lewis^x (Le^x) glycolipid (GL), hexaglycosyl-ceramide with sialic acid at position 6 of galactose, 6′-SA-Le^x-5 (oligosaccharide no. 63) was chemically synthesized (41). The high mannose *N*-glycans Man3, Man4a, Man4b, Man5, Man6, Man7D1, Man7D3, Man8, Man9, Man3F, and complex-type biantennary NA2, NGA2 and NA3-Le^x were gifts from Dr Vladimir Piskarev (Nesmeyanov Institute of Organoelement Compounds, Moscow, Russia). The blood group A trisaccharide (A-tri) was from Dextra laboratories (Reading, UK) and blood group B-pentasaccharide (B-penta) from Oxford GlycoSystems (Abingdon, UK). Dextran fragments tetra-, penta-, hexa- and heptasaccharides were isolated from an acid hydrolysate of dextran. Chitotriose was from Sigma. The Le^x pentasaccharides with sulfate at position 6 of galactose, 6′-SU-Le^x-5 and with sulfate at positions 3 and 6 of galactose, 3′,6′-SU-Le^x-5 (oligosaccharides no. 62 and 61; Table 2) were chemically synthesized and were gifts from Professor Andre Lubineau (Department of Chemistry, University of Orsay, France). These reducing oligosaccharides were conjugated to 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine to form neoglycolipids (NGLs) as described (42). The NGL of 6′-SU-LNNT (no. 64) was prepared by mild acid treatment of the NGL of 6′-SU-Le^x-5.

Recombinant proteins

The extracellular portions of murine SIGN-R1, SIGNR3 and langerin were expressed as soluble proteins fused to the

Table 2. Designations and locations of the carbohydrate-containing molecules investigated, and semi-quantitative scores of the binding by the soluble SIGN-R1, SIGN-R3 and langerin

	Oligosaccharides	Type[a]	Location[b]	Sequences or compositions[c]	SIGN-R1[d]	SIGN-R3	langerin
28	*N*-glycan: Man3	NGL	g1	Maα-6(Maα-3)Maβ-4GcNβ-4GcN	+	–	–
29	*N*-glycan: Man4a	NGL	g2	Maα-3Maα-6(Maα-3)Maβ-4GcNβ-4GcN	++	–	–
30	*N*-glycan: Man4b	NGL	g3	Maα-6(Maα-3)Maα-6Maβ-4GcNβ-4GcN	++	–	–
31	*N*-glycan: Man5	NGL	g4	Maα-6(Maα-3)Maα-6(Maα-3)Maβ-4GcNβ-4GcN	+++	+	–
32	*N*-glycan: Man6	NGL	g5	Maα-6(Maα-3)Maα-6(Maα-2Maα-3)Maβ-4GcNβ-4GcN	++	+	–
33	*N*-glycan: Man7D1	NGL	h1	Maα-6(Maα-3)Maα-6(Maα-2Maα-2Maα-3)Maβ-4GcNβ-4GcN	++	+	–
34	*N*-glycan: Man7D3	NGL	h2	Maα-2Maα-6(Maα-3)Maα-6(Maα-2Maα-3)Maβ-4GcNβ-4GcN	+++	+	–
35	*N*-glycan: Man8	NGL	h3	Maα-2Maα-6(Maα-3)Maα-6(Maα-2Maα-2Maα-3)Maβ-4GcNβ-4GcN	++	+	–
36	*N*-glycan: Man9	NGL	h4	Maα-2Maα-6(Maα-2Maα-3)Maα-6(Maα-2Maα-2Maα-3)Maβ-4GcNβ-4GcN	+++	++	–
37	*N*-glycan: Man3F	NGL	h5	Maα-6(Maα-3)Maβ-4GcNβ-4(Fuα-6)GcN	+	–	–
38	*N*-glycan: A2	NGL	i1	SAα-6Gaβ-4GcNβ-2Maα-6(SAα-6Gaβ-4GcNβ-2Maα-3)Maβ-4GcNβ-4GcN	–	–	+/–
39	*N*-glycan: NA2	NGL	i2	Gaβ-4GcNβ-2Maα-6(Gaβ-4GcNβ-2Maα-3)Maβ-4GcNβ-4GcN	–	–	–
40	*N*-glycan: NGA2	NGL	i3	GcNβ-2Maα-6(GcNβ-2Maα-3)Maβ-4GcNβ-4GcN	+	–	–
41	*N*-glycan: NA3-Le^x	NGL	i4	Fuα{Gaβ-4GcNβ-2Maα-6[Gaβ-4GcNβ-2(Gaβ-4GcNβ-4)Maα-3]Maβ-4GcNβ-4GcN}	+	–	–
42	*O*-glycan: fucosylated	NGL	i5	$(Fu)_2.(Hx)_2.(HxN)_4$ (BSM fraction N6)	++	+	+/–
43	*O*-glycan: SA-fucosyl	NGL	j1	SA.Fu.Hx.$(HxN)_3$ (BSM fraction A6)	–	–	+/–
44	*O*-glycan: sialyl	NGL	j2	SA.$(HxN)_2$ (BSM fraction A4)	–	–	+/–
45	LNT	NGL	j3	Gaβ-3GcNβ-3Gaβ-4Gc	–	–	–
46	LNnT	NGL	j4	Gaβ-4GcNβ-3Gaβ-4Gc	–	–	+/–
47	A-tri	NGL	j5	GaNα-3(Fuα-2)Ga	+	–	+/–
48	B-penta	NGL	k1	Gaα-3(Fuα-2)Gaβ-4(Fuα-3)Gc	+	–	–
49	H (LNFP I)	NGL	k2	Fuα-2Gaβ-3GcNβ-3Gaβ-4Gc	–	–	+/–
50	Le^a (LNFP II)	NGL	k3	Gaβ-(Fuα-4)3GcNβ-3Gaβ-4Gc	++	++	–
51	Le^x (LNFP III)	NGL	k4	Gaβ-(Fuα-3)4GcNβ-3Gaβ-4Gc	++	+	+/–
52	Le^b (LNDFH I)	NGL	k5	Fuα-2Gaβ-(Fuα-4)3GcNβ-3Gaβ-4Gc	++	+++	–
53	Le^y (LNnDFH I)	NGL	l1	Fuα-2Gaβ-(Fuα-3)4GcNβ-3Gaβ-4Gc	++	+	–
54	3′-SA-Le^a-5	NGL	l2	SAα-3Gaβ-3(Fuα-4)GcNβ-3Gaβ-4Gc	+	+	–
55	3′-SA-Le^x-5	NGL	l3	SAα-3Gaβ-4(Fuα-3)GcNβ-3Gaβ-4Gc	–	–	–
56	3′-SU-Le^a-5	NGL	l4	Ga(3SU)β-3(Fuα-4)GcNβ-3Gaβ-4Gc	+	+	–
57	3′-SU-Le^x-5	NGL	l5	Ga(3SU)β-4(Fuα-3)GcNβ-3Gaβ-4Gc	++	+	–
58	3′,6-SU-Le^x-5	NGL	m1	Ga(3SU)β-4(Fuα-3)GcN(6SU)β-3Gaβ-4Gc	+	–	–
59	6-SU-3′-SA-Le^x-5	GL	m2	SAα-3Gaβ-3(Fuα-4)GcN(6SU)β-3Gaβ-4Gc	–	–	–
60	6′-SU-3′-SA-Le^x-5	GL	m3	SAα-3Ga(6SU)β-3(Fuα-4)GcNβ-3Gaβ-4Gc	–	–	++
61	3′,6′-SU-Le^x-5	NGL	m4	Ga(6SU)β-(Fuα-3)4GcN(3SU)β-3Gaβ-4Gc	+	+	+
62	6′-SU-Le^x-5	NGL	m5	Ga(6SU)β-(Fuα-3)4GcNβ-3Gaβ-4Gc	–	–	++
63	6′-SA-Le^x-5	GL	n1	Ga(6SA)β-(Fuα-3)4GcNβ-3Gaβ-4Gc	–	–	–
64	6′-SU-LNnT	NGL	n2	Ga(6SU)β-4GcNβ-3Gaβ-4Gc	+	–	++
65	HNK-1	GL	n3	GcA(3SU)β-3Gaβ-4GcNβ-3Gaβ-4Gc	-	–	–
66	Man5-phosphate	NGL	n4	Ma(6PA)α-3Maα-3Maα-3Maα-2Ma	++	–	–
67	CSA 2mer	NGL	n5	ΔUA-3GaN(4SU)	–	–	–
68	CSA 14mer	NGL	o1	ΔUA-[3GaN(4SU)β-4GcAβ]$_6$-3GaN(4SU)	–	–	–
69	CSB 2mer	NGL	o2	ΔUA-3GaN(4SU)	+	+	+/–
70	CSB 14mer	NGL	o3	ΔUA-[3GaN(4SU)β-4IdAα]$_6$-3GaN(4SU)	–	–	+/–-
71	CSC 2mer	NGL	o4	ΔUA-3GaN(6SU)	++	+	–
72	CSC 14mer	NGL	o5	ΔUA-[3GaN(6SU)β-4GcAβ]$_6$-3GaN(6SU)	–	–	–
73	HS/HEP 2mer	NGL	p1	ΔUA(2SU)-4GcNSU(6SU)	–	–	–
74	HS/HEP 8mer	NGL	p2	UA-[GcN-UA]$_3$-anMa	–	–	–
75	KS 4mer (C4U)	NGL	p3	SAα-3Gaβ-4(Fuα-3)GcN(6SU)β-3Ga(6SU)β-4GcN(6SU)	–	–	–
76	dextran-Glc4	NGL	p4	Gcα-6Gcα-6Gcα-6Gc	–	–	–
77	dextran-Glc5	NGL	p5	Gcα-6Gcα-6Gcα-6Gcα-6Gc	–	–	–
78	dextran-Glc6	NGL	q1	Gcα-6Gcα-6Gcα-6Gcα-6Gcα-6Gc	–	–	–
79	dextran-Glc7	NGL	q2	Gcα-6Gcα-6Gcα-6Gcα-6Gcα-6Gcα-6Gc	–	–	–
80	Chitotriose	NGL	q3	GcNβ-4GcNβ-4GcN	+	–	+/–

[a]Abbreviations for type of lipid-linked oligosaccharides: NGL, neoglycolipid; GL, glycolipid.
[b]Refers to location on array.
[c]Abbreviations for monosaccharide residues and substituents: anMa, anhydro-mannose; Fu, fucose; Ga, galactose; GaN, *N*-acetylgalactosamine; Gc, glucose; GcA, glucuronic acid; GcN, *N*-acetylglucosamine; Hx, hexose; HxN, *N*-acetylhexosamine; IdA, iduronic acid; Ma, mannose; PA, phosphate; SA, sialic acid; SU, sulfate; UA, hexuronic acid; ΔUA, 4,5-unsaturated hexuronic acid. Oligosaccharides 42–44 and the glycosaminoglycan oligosaccharide fractions 67–75 are heterogeneous. Compositions (given with a dot '.' inserted between monosaccharide residues) or sequences of the main components are shown here.
[d]Semi-quantitative visual score for binding signals detected: +++, very strong; ++, strong; +, moderate; +/–, weak; –, not detected.

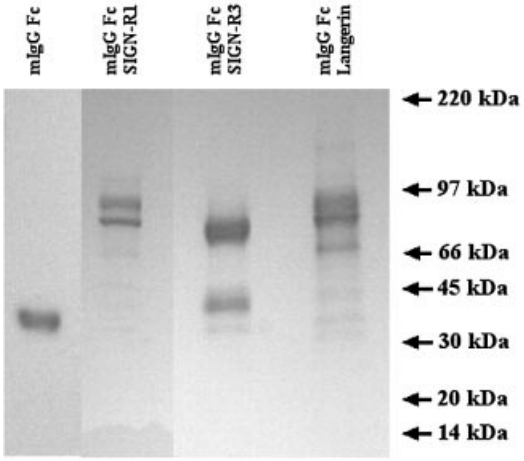

Fig. 1. Coomassie blue stains of the purified recombinant soluble proteins. The purified samples of the four soluble mouse (m) IgG Fc fusion proteins were analysed in a 4–15% gradient SDS–PAGE gel after boiling for 5 min in 2-mercaptoethanol containing SDS–PAGE buffer.

C-terminus of murine IgG Fc (43). The fused cDNA constructs were inserted into the pCMV expression vector (BD Biosciences Clontech, Palo Alto, CA) and transfected onto mammalian cell lines such as 293 and CHO cells. The transfected cells were selected to stably express and secrete the soluble fusion proteins under G418 selection (1 mg/ml). These soluble C-type lectin–IgG fusion proteins were affinity purified (Fig. 1) from the culture medium of mammalian cells, as previously described (43). The full length murine SIGN-R1 was expressed in CHO cells as previously described (26). Murine IgG–Fc and CHO cells transfected with pCMV vector alone (CHO-Neo) served as controls.

Binding of recombinant soluble proteins to carbohydrates arrayed on nitrocellulose

Polysaccharides, glycoproteins and neoglycoproteins, 0.5 μg of each (in water/methanol 1:1), and lipid-linked oligosaccharides, 10 pmol of each (in chloroform/methanol/water 25:25:8 by volume) were applied onto nitrocellulose membranes, and binding experiments were performed at ambient temperature as described previously (36). In brief, the membranes were blocked for 1 h with 1% casein w/v in 10 mM Tris HCl buffer pH 7.4 containing 50 mM calcium chloride and 150 mM NaCl, and overlaid for 2 h with the recombinant proteins, 1 μg/ml, precomplexed (44) by incubation for 1 h with biotinylated anti-mouse IgG from Jackson laboratories (1:3 ratio of protein to antibody w/w). Binding was detected by colour development with streptavidin-conjugated horseradish peroxidase (Sigma), followed by FAST-diaminobenzidine (Sigma).

Binding of soluble proteins to lipid-linked oligosaccharides immobilized in microwells

Lipid-linked oligosaccharides, 10–80 pmoles per well (in methanol containing 4 μg egg lecithin and cholesterol/ml), were dried down in 96-well microwell plates (Falcon 3912, Marathon Labs, London, UK) overnight at 37°C. Binding experiments were performed at ambient temperature as described previously (44). In brief, wells were blocked for 1 h with 1% casein w/v in 10 mM Tris HCl buffer pH 7.4 containing 2 mM $CaCl_2$ and 150 mM NaCl and recombinant proteins, 50 ng/well, precomplexed (44) with biotinylated anti-mouse IgG, (protein to antibody ratio 1:3 w/w), were added to the wells. Incubation was for 2 h. Binding was detected by colour development with streptavidin-conjugated horseradish peroxidase, followed by *O*-phenyldiamine (both from Sigma).

Inhibition of microwell binding of soluble SIGN-R1 to mannose-BSA by monosaccharide conjugates and polysaccharides

BSA-Man, or BSA-Gal as a negative control (0.5 μg/well) was dried down overnight at 37°C in 96-well microwell plates, and binding experiments with pre-complexed soluble recombinant SIGN-R1were performed at ambient temperature as described above. For inhibition experiments, binding of the SIGN-R1 was carried out in the presence of varying concentrations of BSA–monosaccharide conjugates, or the polysaccharides mannan, Ficoll and dextran 2000 kDa, or diluent alone. The percentage of inhibition of binding in the presence of inhibitors was determined as follows:

$$\%\text{Inhibition} = [(\text{OD with no inhibitor} - \text{OD negative control}) - (\text{OD with inhibitor} - \text{OD negative control})] / [(\text{OD no inhibitor} - \text{OD negative control})] \times 100$$

Staining of transfected CHO cells with monosaccharide- and polysaccharide-conjugates

For flow cytometry experiments, cells were suspended at 2×10^6 cells/ml in flow cytometry buffer consisting of 20 mM Hepes-buffered saline (HBS) containing 2 mM $CaCl_2$ and 0.1% w/v BSA; all dilutions and washing steps were carried out using this buffer. Twenty microlitres of cell suspension and 50 μl of FITC–dextran 2000 kDa, 24 μg/ml (final concentration of 10 μg/ml) or mannose–PAA biotin or galactose–PAA–biotin, 120 μg/ml (final concentration of 50 μg/ml) and 50 μl of flow cytometry buffer were added to Falcon 2052 tubes and incubated for 1 h on ice or at 37°C as indicated. The cells were washed twice at 4°C (all subsequent washing steps were carried out at 4°C). In the experiments on ice, cells that had been incubated with mannose- or galactose–PAA–biotin were further incubated for 30 min with 50 μl of FITC-conjugated streptavidin, 10 μg/ml (Becton Dickinson, Oxford, UK) and washed twice. In experiments carried out at 37°C with cells that had been incubated with mannose- or galactose–PAA–biotin, the cells were fixed and permeabilized with 100 μl Leukoperm reagent (Serotec, Kidlington, UK) and washed once before the addition of the streptavidin–FITC. Staining of cells was detected using a FACvantage cell sorter (Becton Dickinson). For inhibition experiments, cells were first incubated for 30 min with 50 μl of 20 mM EDTA or 10 μg/ml ERTR9 antibody, or varying concentrations of monosaccharide, oligosaccharide or polysaccharide inhibitors (at 4°C or 37°C as appropriate) before addition of 50 μl FITC–dextran or mannose–PAA–biotin. Oligosaccharide inhibitors were tested as lipid-linked compounds displayed on liposomes (45). Liposomes displaying galactosyl ceramide (Gal-ceramide), or no oligosaccharide (empty liposomes) were included as

negative control inhibitors. The percentage of inhibition of binding in the presence of inhibitors was determined as follows:

$$\% \text{ Inhibition} = [(\text{MF of cells without inhibitors} - \text{MF of cells with inhibitors}) / \text{MF of cells stained without inhibitors}] \times 100$$

where MF = median fluorescence.

Scatchard type analyses of the binding of dextran- and mannose-conjugates to transfected CHO cells

For Scatchard type analyses, SIGN-R1-transfected CHO cells, or CHO-Neo cells as controls, were suspended at 2×10^6 cells/ml in flow cytometry buffer. Twenty microlitres of cell suspensions were placed in Falcon 96 well U-shaped tubes, and incubated for 1 h on ice in the presence of the following: 50 μl of biotinylated dextran at concentrations from 360 to 3.8 μg/ml (final concentrations of 150–1.6 μg/ml) or biotinylated mannose–PAA, 240 μg/ml to 1.9 μg/ml (final concentration of 100–0.8 μg/ml) and 50 μl of flow cytometry buffer. Total reaction volume was 120 μl. The plates were spun at 300 *g* for 5 min, and the supernatants harvested. The concentrations of the total conjugates added, and of the free conjugates after incubation were assayed by determining biotin concentrations, using a sensitive competitive inhibition assay (46), and extrapolating from standard curves that we constructed for the two biotinylated conjugates. The bound values for the SIGN-R1 transfectants were calculated after subtracting bound values for the CHO-Neo cells. The values were converted to molar concentrations of monosaccharides for Scatchard analyses to calculate the dissociation constants (Kds) for the two conjugates, taking the carbohydrate content of the dextran polysaccharide as 99% and of the mannose-PAA as 20%.

Staining of cell monolayers

For experiments with cell monolayers, 2×10^5 CHO-SIGN-R1 cells or CHO-Neo cells in 1 ml of culture medium (DMEM containing 10% v/v foetal bovine serum, 100 μg of antibiotic–antimycotic solution, and 1 μg of geneticin; all from Invitrogen, Paisley, UK) were seeded in Nunc 4-well chamberslides (Fisher Scientific, UK) and cultured for 2–3 days to reach 70–80% confluency. Medium was removed from wells and cells were washed twice; all dilutions and subsequent washing steps were carried out with this buffer. For dual staining with mannose conjugate and dextran, cells were incubated for 1 h at 37°C with mannose–PAA–biotin, and FITC–dextran at 50 and 10 μg respectively per ml. Cells were washed twice, and fixed with 1 ml of 1% v/v paraformaldehyde for 15 min at 4°C. Cells were permeabilized by incubating with 1 ml of 0.2% v/v Triton X-100 for 20 min at 4°C, and washed twice. Cells were then incubated for 30 min at 4°C with Cy3 labelled streptavidin, 10 μg/ml, and washed twice. Cells were mounted with Vector Hardset fluorescent mountant, for microscopic visualization of staining using a Leica TCS NT confocal microscope with a 100× objective (magnified digitally to 1000×).

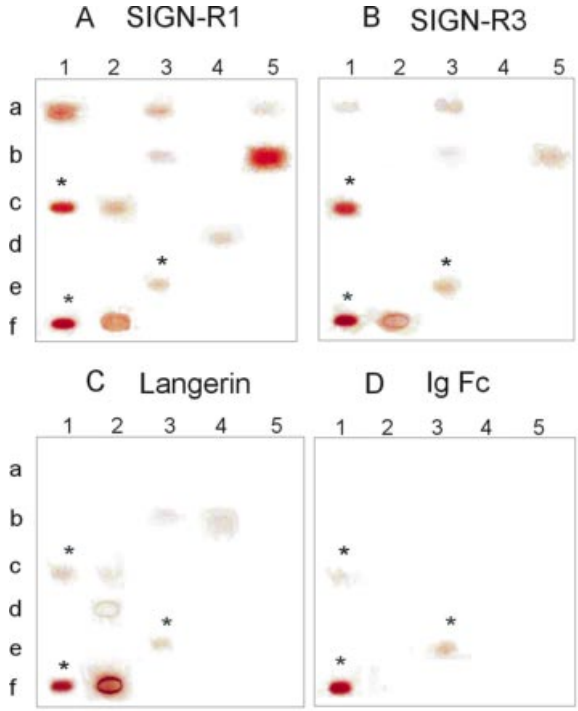

Fig. 2. Binding of recombinant soluble proteins to monosaccharide-conjugates, glycoproteins and polysaccharides immobilized on nitrocellulose membranes. The glycoconjugates (Table 1) were spotted onto nitrocellulose membranes and overlaid with the recombinant soluble fusion proteins, SIGN-R1 (A), SIGN-R3 (B) and langerin (C), and as a control, mouse IgG Fc (D), which had been pre-complexed with biotinylated anti-mouse-IgG; binding was detected as described in Methods. The three spots, those of soybean agglutinin (c1) fluorescein-labelled and FITC- and TR-conjugated dextrans (e3 and f1, respectively) showing binding with the control protein, IgG Fc, as well as the chimeric proteins are asterisked; of these only soybean agglutinin was bound by the three receptors more strongly than by the control protein.

Results

Binding of the soluble SIGN-R1 protein to glycoconjugates arrayed on membranes

The soluble SIGN-R1 gave binding signals with the monosaccharides mannose, fucose and *N*-acetylglucosamine conjugated to BSA (Table 1 and Fig. 2). Among the six glycoproteins arrayed, ribonuclease B, invertase, soybean agglutinin, ovalbumin, fetuin and asialo-fetuin (Table 1), four are known to have high mannose chains. SIGN-R1 gave binding signals with three of these: invertase, soybean agglutinin and ovalbumin (Fig. 2A, b5, c1 and c2, respectively). Of the polysaccharides tested, yeast mannan was bound; but the neutral dextrans and Ficoll were notable by their lack of binding signals with SIGN-R1 (Fig 2A). Only the dextran sulfate was strongly bound; another acidic polysaccharide, heparan sulfate, was not bound.

The recognition of mannose-, fucose- and *N*-acetylglucosamine-terminating oligosaccharides was also apparent for SIGN-R1 in the binding detected with the arrayed lipid-linked oligosaccharides (Table 2, Fig. 3A). With the high mannose type *N*-glycans that were tested in the array, the Man3 (g1)

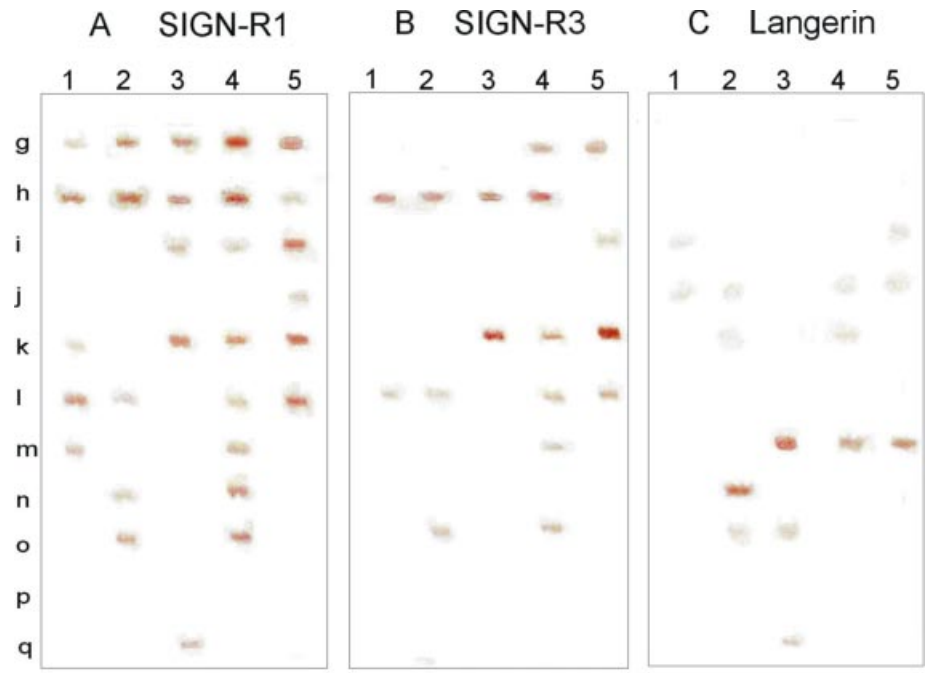

Fig. 3. Binding of recombinant soluble proteins to lipid-linked oligosaccharides immobilized on nitrocellulose membranes. Lipid-linked oligosaccharides (Table 2) were spotted onto nitrocellulose membranes and overlaid with the recombinant soluble fusion proteins SIGN-R1 (A), SIGN-R3 (B) and langerin (C), which had been pre-complexed with biotinylated anti-mouse IgG; binding was visualized as described in Methods.

gave a weaker signal than Man4–Man9 (g2-h4, respectively). With the fuco-oligosaccharides, the linkage position of the fucose residue was important: binding was not observed to the blood group H type oligosaccharide with fucose 2-linked to galactose (k2), whereas strong binding was detected to the neutral oligosaccharides of $Le^{a/b}$ and $Le^{x/y}$ types with fucose 3- or 4-linked to *N*-acetylglucosamine (k3, k4, k5 and l1). Sialylation at position 3 or 6 of galactose or sulfation of the Le^a and Le^x sequences at position 3 or 6 of galactose or position 6 of *N*-acetylglucosamine (l2 to n1) did not enhance the SIGN-R1 binding; the binding was either weakened or unaffected.

Binding was also examined to arrayed oligosaccharides derived from dextran: 4-, 5-, 6-, and 7-mers. No binding was detected in accord with the lack of binding to preparations of the intact polysaccharide (Fig. 3A, locations p4 to q2 respectively).

Binding of the soluble SIGN-R1 protein to oligosaccharides immobilized in microwells

The microwell binding experiments validated results with the arrayed oligosaccharides (Fig. 4AA′). They showed, in addition, a clear correlation between binding signal and mannose number in the *N*-glycans tested. The results also showed that SIGN-R1 binding to the neutral Le^a- and Le^x-type oligosaccharides was of the same order as to the high mannose *N*-glycans Man7 and Man9. There was little binding to the 6-mer, dextran oligosaccharide.

The lack of detectable interaction of the soluble SIGN-R1 with dextran and Ficoll, was corroborated by inhibition of binding experiments in microwells (Fig. 5). Dextran and Ficoll gave no inhibition of SIGN-R1 binding to the mannose–BSA conjugate (Fig. 5A), whereas mannan was inhibitory.

Comparisons of the binding of the soluble SIGN-R3 and langerin proteins with SIGN-R1

Comparisons of the saccharide binding of SIGN-R3 and langerin with that of SIGN-R1 showed only subtle differences with SIGN-R3 but striking differences with langerin as follows. SIGN-R3 showed binding to the monosaccharide mannose- and fucose-conjugates, and among the glycoproteins, there was binding to invertase and soybean agglutinin (Fig. 2B, locations b5 and c1 and Table 1). Among the polysaccharides, as with SIGN-R1, there was SIGN-R3 binding to the sulfated dextran but not the neutral dextrans. SIGN-R3 showed stronger binding to $Le^{a/b}$-related sequences than to $Le^{x/y}$ (Fig. 3B and 4B).

With langerin, there was binding to the dextran sulfate. Binding to the mannosyl probes was barely detectable in the arrays (Figs 2C and 3C; Table 1), and not in the microwell assay (Fig. 4C). The non-acidic Lewis-related sequences also gave little or no binding (Figs 3C and 4C′). The striking difference of langerin from SIGN-R1, and also SIGN-R3, was a clear preference of langerin for binding to sulfated Le^x-related sequences (oligosaccharides 60–62) with sulfation at position 6 of galactose, designated 6′-sulfated Le^x (Fig. 3C, m3 to m5 and Fig. 4C′). This indicates that where there is a terminal 6′-sulfated galactose, the fucose residue is not essential for langerin binding.

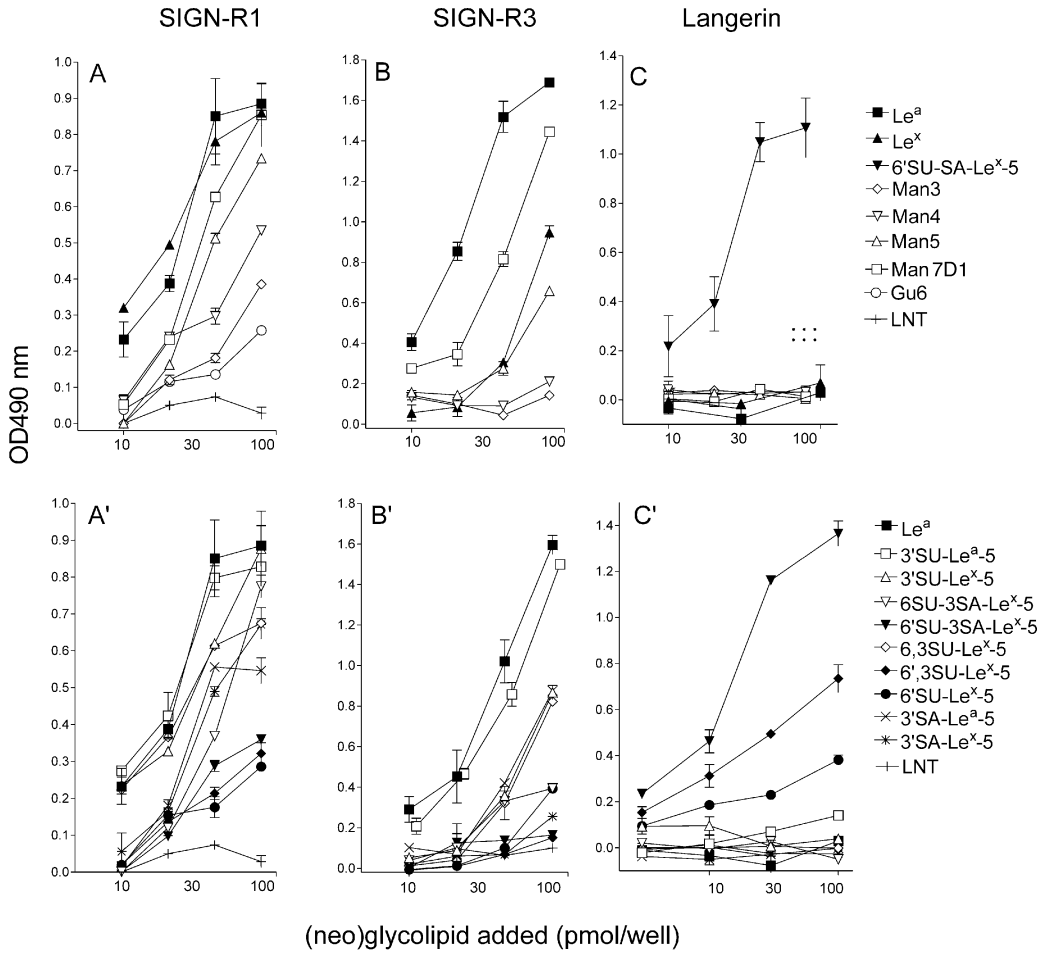

Fig. 4. Binding of recombinant soluble proteins to lipid-linked oligosaccharides immobilized in microwells. Varying amounts of lipid-linked oligosaccharides were dried down in microwells and probed with recombinant fusion proteins SIGN-R1 (A and A′), SIGN-R3 (B and B′), and langerin (C and C′), which had been pre-complexed with biotinylated anti-mouse-IgG; binding was detected as described in Methods. Results are expressed as the means of duplicate wells with the range indicated by error bars. A and A′ are results of a single experiment; B, B′, C and C′ are results of different experiments.

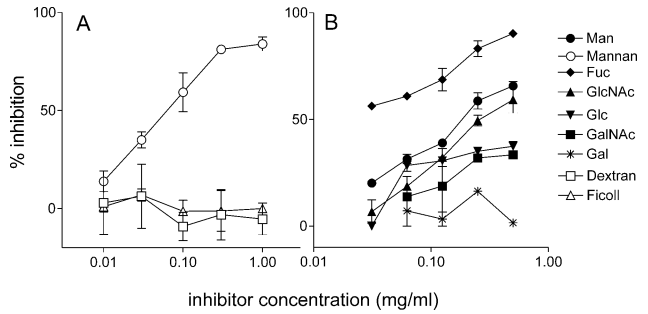

Fig. 5. Inhibition of binding of recombinant soluble SIGN-R1 to mannose–BSA by mannan and monosaccharide conjugates. Mannose–BSA, 10 μg/ml, was dried down in microwells and probed with soluble SIGN-R1 (precomplexed with biotinylated anti-mouse IgG) in the presence of varying concentrations of mannan, Ficoll and dextran 2000 kDa (A) and monosaccharide conjugates (B). Binding was detected as described in Methods. Results are expressed as the means of duplicate wells with the range indicated by error bars.

Binding experiments with mannose conjugate and fluorescein labelled dextran and cells transfected to express SIGN-R1

Having detected no dextran binding by the soluble SIGN-R1 protein, it was important to reconcile with the earlier observations (26) on the membrane-associated form of the receptor mediates dextran. We examined by flow cytometry CHO cells that had been transfected to express the membrane associated SIGN-R1. There was staining of the SIGN-R1-transfected cells with FITC–dextran not only at 37°C as described previously (26) but also at 4°C (Fig. 6A, panels i and ii). Another fluorescein-conjugated probe, fluorescein-conjugated galactose–BSA, gave no staining (Fig. 6A, vii and viii). The SIGN-R1-CHO cells gave staining also with mannose–PAA conjugate (Fig. 6B, i and ii), but not with the galactose analog (Fig. 6B, vii and viii). Specificity of FITC–dextran and mannose conjugate staining was further corroborated by lack of staining of the CHO-Neo cells, transfected with the vector only (Fig. 6B, ix and x).

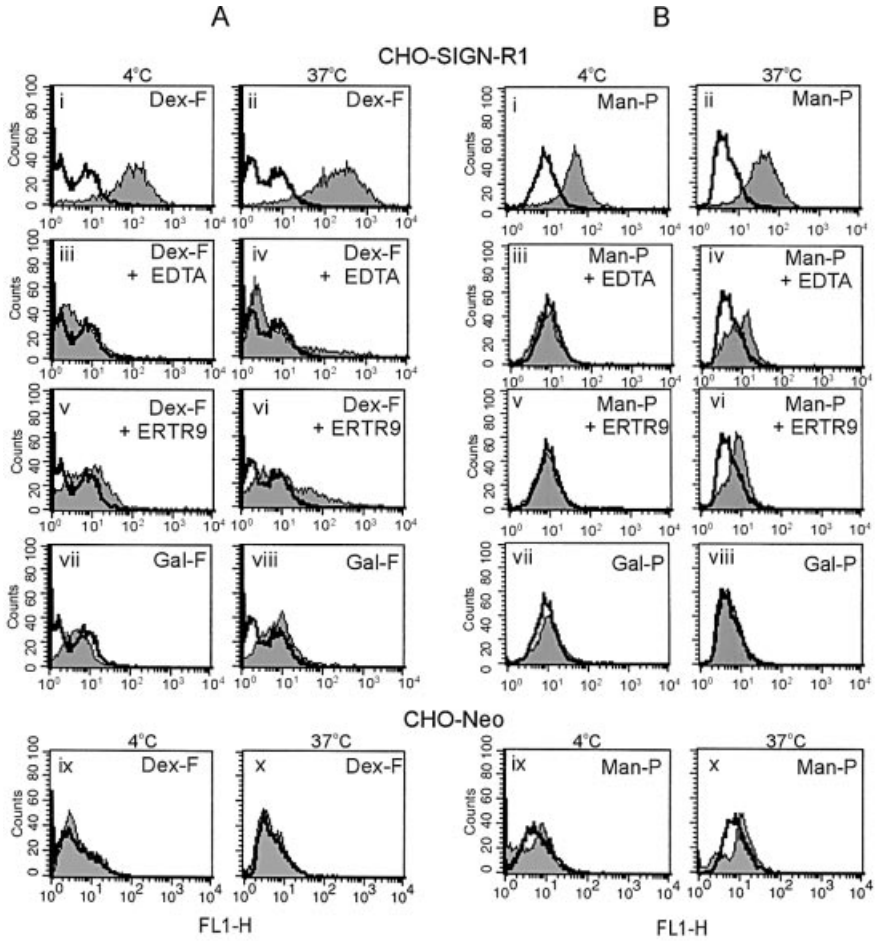

Fig. 6. Flow-cytometric analyses of the staining of transfected CHO cells with monosaccharide- and dextran-conjugates. CHO-SIGN-R1 cells were incubated with FITC–dextran 2000 kDa (Dex-F) (A) or with mannose–PAA–biotin conjugate (Man-P) (B) at 4°C or at 37°C in the absence or presence of 20 mM EDTA or 10 μg/ml of anti-SIGNR1 antibody (ERTR9). As negative controls, CHO-SIGN-R1 cells were incubated with galactose–PAA–biotin (Gal-P) or FITC–galactose BSA conjugate (Gal-F) and CHO-Neo cells with Man-P or Dex-F.

The staining both with the dextran- and mannose-conjugates was cation dependent, being inhibited in the presence of EDTA, as predicted for a C-type lectin. The staining with the mannose- as well as the dextran-conjugates was inhibited in the presence of the SIGN-R1 antibody, ERTR9 (Fig. 6A and B, iii–vi).

To gain further insights into the relationship between the dextran and mannose binding to the SIGN-R1-transfected cells, the cells were incubated with dextran- and mannose-conjugates at 37°C, and their distribution examined by confocal microscopy. The staining was predominantly intracellular, and the majority of the staining with the two conjugates was concordant (Fig. 7). There was negligible staining in the non-transfected cells (data not shown).

A series of inhibition experiments was performed with mono-, oligo- and polysaccharides (Fig. 8). Both the dextran and mannose conjugate staining were inhibitable by mannan and other carbohydrate ligands identified for the soluble SIGN-R1. These include the monosaccharides mannose and fucose, a high mannose *N*-glycan, Man-7D1, the Le^a- and Le^x-related sequences, and dextran sulfate. Other charged polysaccharides, heparin and heparan sulfate, were not inhibitory. However, only the FITC–dextran staining, but not the mannose staining, was inhibited to a significant extent by the neutral dextran 2000 kDa.

Collectively, the concordant intracellular staining with the mannose and dextran conjugates, the inhibitory activities of the anti-SIGN-R1 antibody shown in Fig. 6, together with the IC_{50} values (concentrations giving 50% inhibition of binding) summarized in Table 3, indicate that both the mannose and dextran binding are mediated by the cell-associated SIGN-R1, but that the dextran binding is more readily inhibitable than the mannose binding: mannan is over 10 000-fold more active than dextran at inhibiting FITC–dextran binding.

Scatchard type analysis of the mannose- and dextran-binding to SIGN-R1 transfected cells

At the suggestion of one of the reviewers of our manuscript, we performed additional cell binding experiments for Scatchard type analyses, to compare the avidities of binding of the mannose–PAA conjugate and dextran to SIGN-R1-transfected cells. We have duly carried out Scatchard analyses using the biotinylated mannose–PAA and a biotinylated dextran (Fig. 9), although it must be noted that the carbohydrate presentation

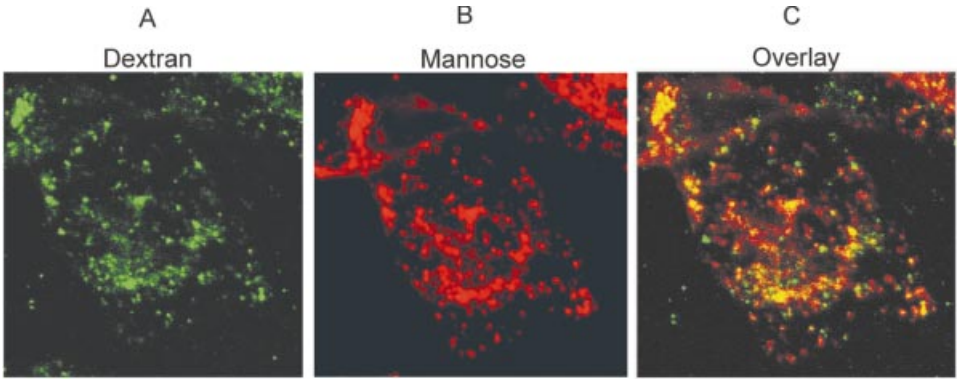

Fig. 7. Confocal microscopic analysis of the intracellular distribution of mannose- and dextran-conjugates taken up by SIGN-R1-transfected cells. To examine intracellular distribution of the mannose- and dextran-conjugates, monolayers of SIGN-R1 transfected cells were stained for 1 h with FITC–dextran, 10 μg/ml (A) and mannose–PAA–biotin, 50 μg/ml (B) at 37°C. An overlay of the cellular distribution of the two conjugates is shown in (C).

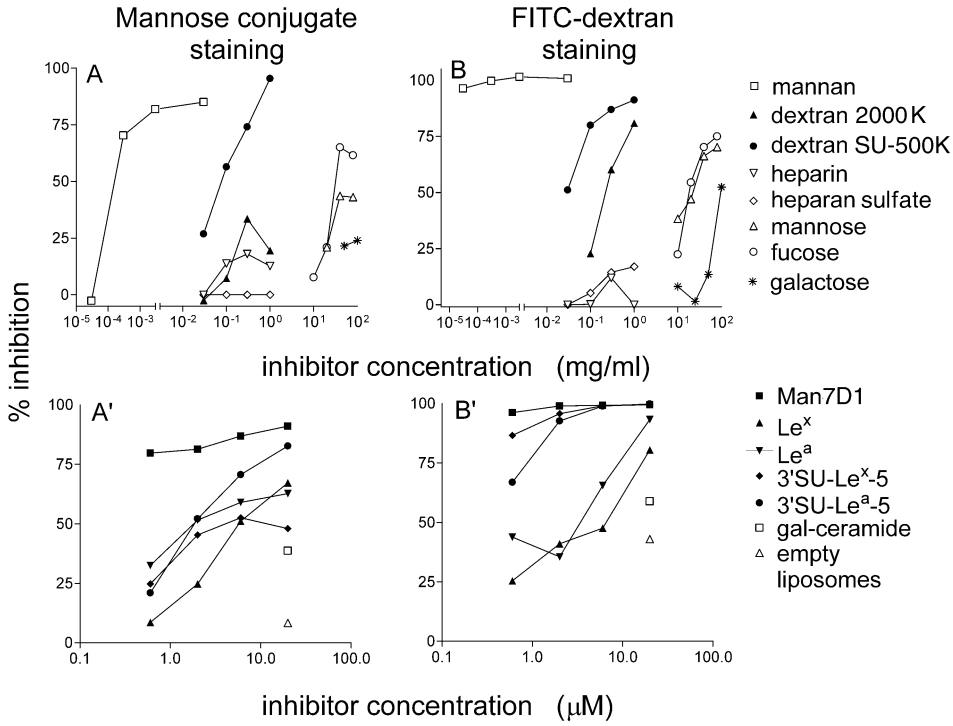

Fig. 8. Flow-cytometric analyses of inhibition of staining of SIGN-R1-transfected CHO cells with mannose- and dextran-conjugates using as inhibitors, monosaccharides, and polysaccharide and lipid-linked oligosaccharides. For inhibition experiments, CHO-SIGN-R1 cells were incubated for 30 min with the monosaccharides, mannose, fucose or galactose, at final concentrations of 10–100 mg/ml or with the polysaccharides mannan, dextran or dextran-sulfate polysaccharides, at final concentrations of 0.03–1 mg/ml (A and B) or with liposomes displaying Man-7D1, Le^x, Le^a, $SuLe^x$ or $Su\text{-}Le^a$ oligosaccharides, final concentrations of 0.3–30 μM, (A′ and B′). Staining with mannose–PAA–biotin, final concentration 50 μg/ml or with FITC–dextran 2000 kDa, 10 μg/ml was then performed at 4°C as described in Methods. Results are expressed as percentage inhibition of staining by the mannose conjugate (A and A′), or dextran conjugate (B and B′). The four panels are each the results of separate experiments.

is different in the two glycoconjugates. The dissociation constants thus generated for the mannose and the dextran conjugates are 9×10^{-6} M and 5×10^{-3} M, respectively. These results support our conclusion that the avidity of interaction of dextran with the membrane-associated SIGN-R1 is low, and it is substantially lower than that of the mannose conjugate.

Discussion

We describe here the use of arrays of an extensive panel of carbohydrates for discovering ligands of three novel receptors of the immune system. The arrays encompass glycoproteins, polysaccharides, monosaccharides and oligosaccharides,

Table 3. Inhibitory activities of monosaccharides, oligosaccharides and polysaccharides toward the staining of CHO-SIGN-R1 cells with mannose- and dextran-conjugates

Inhibitors	Staining with mannose-conjugate	Staining with dextran-conjugate
	IC_{50} (μg/ml)	
Monosaccharides		
Mannose	n.i.[a]	23 000
Fucose	33 000	18 000
Galactose	n.i.	~100 000
Oligosaccharides (NGLs)		
Man7D1	<0.69 (< 0.6 μM)	<0.69 (< 0.6 μM)
Le^x	4.95 (5.8 μM)	5.79 (7 μM)
Le^a	1.54 (1.8 μM)	3.41 (4 μM)
3′-SU-Le^x-5	4.29 (4.6 μM)	<0.56 (0.6 μM)
3′-SU-Le^a-5	1.77 (1.9 μM)	<0.56 (0.6 μM)
Polysaccharides		
Mannan	0.25	<0.03
Dextran 2000K	n.i.	560
Dextran SU-500K	85	30
Heparin	n.i.	n.i.
Heparan sulfate	n.i.	n.i.
Negative controls		
Gal-ceramide	n.i.	n.i.
Empty liposomes	n.i.	n.i.

[a]n.i. = no inhibition or <50% inhibition at the highest concentration tested: 100 mg/ml for monosaccharides, 1 mg/ml for polysaccharides and 30 μM for the galactosylceramide, and empty liposomes, in which DHPE was used instead of NGL or glycolipid.

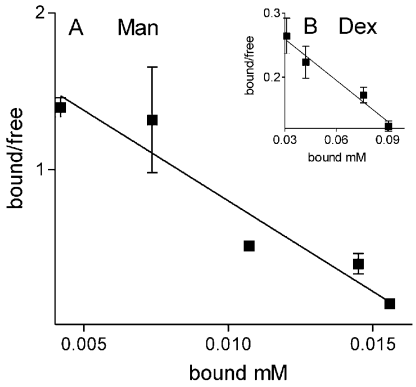

Fig. 9. Scatchard type plot of the interactions of biotinylated dextran and mannose and mannose–PAA to SIGN-R1 transfected cells. To determine dissociation constants for the interactions of biotinylated dextran and mannose–PAA with SIGN-R1 transfected CHO cells, different concentrations of the biotinylated conjugates were incubated with the CHO-SIGN-R1 and CHO-neo transfectants for 1 h on ice, and the cell supernatants harvested for assays of biotin concentration, as described in Methods. From the biotin concentrations added to the cells and the concentrations of free biotin in the cell supernatants, the amounts of bound and free conjugates were calculated, after subtraction of the non-specific binding of the conjugates to CHO-neo cells. Results are expressed as Scatchard type plots for biotinylated mannose–PAA (A) and the biotinylated dextran (B). The conjugates bound are given on the *x*-axis as mM of monosaccharides, and the ratios of bound/free, on the *y*-axis; using the mean values from quadruplicate wells, except for the 0.004 mM bound concentration that was from duplicate wells.

including an extensive list of *N*-glycans. Quantitative ELISA-type binding and inhibition experiments corroborate the readout in the arrays.

The salient conclusions, with the soluble recombinant forms of the three murine receptors, SIGN-R1, SIGN-R3 and langerin, are first, that both SIGN-R1 and SIGN-R3 exhibit mannose- and fucose-related recognition. This is in common with several other C-type carbohydrate-binding proteins of the innate immune system (3,13,14,16). The mannose recognition is manifest by their interactions with invertase, soybean agglutinin and with the high mannose *N*-glycans; the fucose recognition is manifest with the Le^a- and Le^x-related sequences, with SIGN-R3 showing a clear preference for Le^a over Le^x. The two receptors contrast with the selectins (4,44,47–51) in that they do not show enhanced binding to sialyl and sulfated forms of the Le^a and Le^x. Moreover, mannose specificity is not a feature of the selectins (36). These receptors also differ from the macrophage C-type lectin (MGL) on monocyte-derived immature dendritic cells (52), which has specificity for oligosaccharides that terminate in galactose or *N*-acetyl-galactosamine. Rather, the binding specificities we observe for the soluble forms of SIGN-R1 and SIGN-R3 resemble that of the human DC-SIGN (14) and partially overlap with those of the collectins (53), which were assigned using a limited number of oligosaccharide probes. The second major finding in the array experiments is the specificity of langerin toward Le^x-type sequences that are sulfated at position 6 of the outer galactose, particularly oligosaccharide compound 60 (6′-SU-3′-SA-Lex-5). This specificity is unique so far among the C-type lectins, and contrasts very markedly with the selectins, which bind to the Le^x analog that has sulfate at position 3 of galactose, but not the analog with sulfate at position 6 of galactose (36,49,54). The 6′-SU-3′-SA-Le^x-5 was strongly bound by the soluble form of langerin. High-mannose glycans were weakly bound, as shown for the soluble form of the receptor in earlier studies (16). Thirdly, the three proteins, in soluble form, show binding to dextran-sulfate, but not at all to unmodified dextran.

The eukaryotically expressed soluble receptors of the type used here, IgG Fc chimeras, have the advantage over bacterially expressed proteins in that refolding and enrichment by ligand affinity chromatography is not usually a prerequisite and the carbohydrate arrays can thus be probed without the need to predict binding activity or specificity. An additional advantage of IgG Fc chimeras is that the dimeric proteins can be readily rendered oligomeric in the presence of anti-Fc, a system first introduced for the selectins, e.g. (55). This strategy may not be a complete substitute, however, for multimeric presentation of the receptors at the surface of cells (26), with an optimally orientated presentation that would allow interactions with low affinity ligands. This is illustrated here with SIGN-R1-transfected CHO cells, and in preliminary studies with SIGN-R3- and langerin-transfected cells (data not shown). Interactions with dextran could not be detected at all with the soluble proteins either by binding or inhibition of binding experiments, but could be detected when the proteins were expressed at the surface of cells. Dextran uptake by cells transfected with SIGN-R3 and langerin has been shown by independent experiments (56). There was weak binding of

soluble langerin-IgG to glycoconjugates with high-mannose glycans, whereas langerin-transfected cells bound strongly to a mannose conjugate and ovalbumin (our data not shown) (56). Thus, the two experimental approaches with the soluble and the cell-associated proteins complement one another; the soluble proteins lending themselves to surveys of binding to large numbers of carbohydrate probes, and the cell-associated proteins enabling detection of ligands of very low avidity that are not detectable with the soluble proteins even after artificial oligomerization.

The cell staining experiments with the SIGN-R1-transfectants in the present investigation have shown that the monosaccharide ligands, as well as oligosaccharide and polysaccharide ligands of this receptor can inhibit the dextran binding. Thus the dextran binding is likely to be mediated by the site that also binds mannose and fucose. The cytochemical experiments with SIGN-R1 transfectants to visualize and compare the uptake at 37°C of dextran- and mannose-conjugates, also showed the relatedness of the SIGN-R1 interactions with the two conjugates. At 1 h after incubation, the two conjugates were largely co-localized in intracellular compartments. Collectively, these data, and the ease of inhibition of dextran binding compared with inhibition of mannose-conjugate binding, as well as the results of Scatchard analyses lead to the conclusion that dextran is a ligand for SIGN-R1, but that its avidity is low.

Knowledge of the oligosaccharide sequences recognized by the murine receptors on antigen presenting cells now opens the way to examining experimentally the immunological sequelae of the interactions of the receptors with defined ligands of differing affinities. The way is also open to the inhibition of lectin-mediated uptake of pathogens, whereby they reach protected cell compartments and evade immune recognition and clearance. The overlaps in carbohydrate structures recognized by several of the receptors should not preclude such studies, as it is possible to design ligand presentations that are preferred by individual receptors (15,57). There is precedent for striking differences between carbohydrate binding receptors in recognition of a given oligosaccharide ligand (a high-mannose type *N*-glycan, Man9 for example) presented on different carrier proteins and also on the same proteins such as ribonuclease B before and after its denaturation (58,59). Two of the serum proteins of the innate immune system, conglutinin and mannan binding protein, bind avidly to the clustered Man9 *N*-glycan in the absence of carrier proteins (60,61). However, only conglutinin binds to the complement glycopeptide, iC3b, a proteolytic fragment of the glycoprotein C3, which bears a high-mannose *N*-glycan, predominantly Man9, at a single glycosylation site (58). The carrier protein has been shown to have a major influence on the availability of the *N*-glycan, as conglutinin does not bind to the parent C3 glycoprotein. Conglutinin can only bind after a proteolytic cleavage occurs of the glycoprotein in the complement cascade and conversion into iC3b.

With refinements in carbohydrate ligand presentation, in conjunction with gene deletions of the individual receptors, it will be possible to address the roles of the individual C-type lectins in the immune response, and above all to revisit and dissect pathways in the orchestration of T-independent antibody responses, this time using defined oligosaccharides as immunogens.

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Abbreviations

DC-SIGN	dendritic cell specific intercellular adhesion molecule grabbing non-integrin
GL	glycolipid
HBS	HEPES-buffered saline
Le^x	$Lewis^x$
NGL	neoglycolipid
PAA	polyacrylamide
PE	phycoerythrin

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