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Sialic acid-binding lectins: submolecular specificity and interaction with sialoglycoproteins and tumour cells

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We examined the specificity of limulin, *Limax flavus* agglutinin (LFA) and *Sambucus nigra* agglutinin I (SNA I) at the submolecular level of sialic acid, and characterized their interactions with a panel of structurally distinct sialoglycoproteins. In haemagglutination inhibition assays NeuAc-α-glycosides were stronger inhibitors for limulin and LFA than native *N*-acetylneuraminic acid (NeuAc). The *N*-acetyl of NeuAc was crucial for binding to both lectins. N-*thio*acetylated NeuAc lost affinity for LFA, but still bound to limulin. Thus, distinct intermolecular interactions are involved in binding of sialic acid to the lectins. The glyceryl side chain was required for interaction with LFA, but not with limulin. SNA I specifically bound NeuAcα2 $\rightarrow$ 6Galβ1 $\rightarrow$ 4Glc, but not monomeric sialic acids. Limulin and LFA strongly interacted with O-chain glycoproteins, whereas SNA I preferred N-chain proteins that carry NeuAcα2 $\rightarrow$ 6 residues. The lectins were compared with those from *Cepaea hortensis* and *Tachypleus tridentatus* (TTA) and to wheat-germ agglutinin, and were then used to probe tumour cell lines for cell surface sialylation. With the exception of TTA, all lectins interacted with the tumour cells. Limulin distinguished between the low (Eb) and highly (ESb) metastatic mouse lymphoma lines by selectively agglutinating sialidase-treated ESb cells.

Keywords: lectin specificity, sialic acids, haemagglutination inhibition, sialoglycoproteins, tumour cells

Abbreviations: BSM, bovine submaxillary mucin; CHA I, Cepaea hortensis agglutinin I; LFA, Limax flavus agglutinin; NeuAc, *N*-acetylneuraminic acid; OSM, ovine submaxillary mucin; SNA I, Sambucus nigra agglutinin I; THP, Tamm-Horsfall protein; TTA, Tachypleus tridentatus agglutinin.

Introduction

Lectins are proteins or glycoproteins with the ability to bind carbohydrates in a specific and reversible manner and, by virtue of this interaction, can agglutinate cells. They are useful tools for various biological applications, including the discrimination of normal and malignant cells, the determination of blood groups, the diagnostics of pathogenic bacteria, and the purification of glycoconjugates [1–5]. Besides this, lectins are valuable probes for analysing cell surface carbohydrates by cell agglutination (6), and for studying the immunofluorescence and staining of tissue sections [3, 7]. Recently, they were assembled into distinct families of homologous proteins, and some of them subjected to extensive structural studies [4].

Sialic acids are a family of more than 30 analogues of *N*-acetylneuraminic acid (NeuAc), most of them arising from substitutions of the hydroxyl groups of NeuAc. In higher animals they are found in terminal positions of oligosaccharides. By virtue of the variety of analogues and of their differential linkage to subterminal sugars, sialic acids form oligosaccharides with an enormous structural diversity [8, 9]. Sialic acids are crucial for an array of biological processes. They are involved in the infection of cells by microorganisms, in the regulation of the immune system, in the development of the nervous system [10, 11], and in cancer metastasis [8–10, 12, 13]. In this regard, 9-*O*-acetyl-NeuAc was found in human malignant melanoma [14], and *N*-glycolylneuraminic acid

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[15] as well as $\alpha 2 \rightarrow$ 6-linked sialic acids [16, 17] were detected in human colon carcinoma tissues. Thus, it is of particular importance to characterize the specificity of sialic acid-binding lectins at the submolecular level and for the linkage of NeuAc to subterminal carbohydrates. These lectins can then be applied to further study the precise role of sialic acids in the different fields of biology.

Sialic acid-specific lectins (reviewed in [18–20]) have been isolated from the invertebrates *Limulus polyphemus* [21], *Limax flavus* [22], *Cancer antennarius* [23], *Cepaea hortensis* [24] and *Tachypleus tridentatus* [25], as well as from wheat germ [26], elderberry bark [27], seeds of *Maackia amurensis* [28] and human placenta [29].

Here, we discuss in detail the submolecular specificity of the lectins from *L. polyphemus* (limulin), *L. flavus* (LFA), *C. hortensis* (CHA I), *T. tridentatus* (TTA) and the elderberry *Sambucus nigra* (SNA I). We characterize their interaction with glycoproteins that carry sialic acids in different oligosaccharide chains and, finally, describe their diverse interaction with malignant tumour cell lines.

Materials and methods

Limulin and SNA I were obtained from Sigma (Deisenhofen, Germany). LFA was purchased from Medac (Hamburg, Germany). CHA I [24] was kindly supplied by Dr M. Wagner (Jena, Germany). WGA was purified according to Bassett [30]. Experiments with limulin and CHA I were carried out in 50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 110 mM NaCl. Assays with LFA were performed in 50 mM Tris-HCl pH 7.5, 125 mM NaCl, those with SNA I and WGA in phosphate buffered saline pH 7.4. Sialidase (E.C. 3.2.1.18) from *Vibrio cholerae* was obtained from Behringwerke (Marburg, Germany). Sialic acid analogues were synthesized at the Institute of Biochemistry II, 2-α-methyl-5-*N*-thioacetyl-Neu and *N*-thioacetyl-D-glucosamine by Dr R. Isecke [31, 32] (Heidelberg, Germany). Glycoproteins were generously donated as referred to in Brossmer *et al.* [33].

Eb cells, the methylcholantrene-induced T-cell lymphoma L5178/Eb from a DBA/2 mouse with low metastatic capacity, and its spontaneous variant ESb with high metastatic properties, were gifts from Dr V. Schirrmacher (Heidelberg, Germany). Human colon carcinoma cells HT29 were from ATCC (Rockville, MD). Cells were cultured as described [25].

Haemagglutination inhibition assay

Assays were performed as described in detail [25, 33]. Briefly, two fold serial dilutions of carbohydrate inhibitors were made on V-bottom 96-well microtitre plates. Then 4 haemagglutination units (HU) of lectin were added to each well, and the plates were incubated for 30 min at 4 °C. Then a 10% (v/v) suspension of erythrocytes was added, and plates were read after 1 h incubation at 4 °C. The lowest concentration of inhibitor showing complete inhibition was noted.

Haemagglutination assay

As previously described [25], two-fold serial dilutions of the lectins were made on V-bottom 96-well microtitre plates, and a 10% (v/v) suspension of erythrocytes was added to each well. Plates were read after 1 h incubation at 4 °C. The lowest lectin concentration showing agglutination was noted. The minimum amount of lectin protein in the well agglutinating group A erythrocytes was defined as 1 HU.

Cell agglutination assay

Two-fold serial dilutions of the lectins were made on F-bottom 96-well microtitre plates [25]. Then 1.3×10^5 tumour cells, either native or treated with 100 mU ml^{-1} sialidase, were added per well. After 1 h incubation at 4 °C plates were read under an inverted phase contrast microscope. The lowest concentration showing agglutination was noted.

Results and discussion

Haemagglutination inhibition of limulin, LFA, CHA I and TTA by sialic acid analogues

To define the structural components of the sialic acid molecule involved in binding to these lectins, we performed haemagglutination inhibition experiments with 15 sialic acid analogues (Table 1). Native NeuAc inhibited the four invertebrate lectins at very different concentrations. CHA I-mediated haemagglutination was abolished by 0.6 mM NeuAc. LFA, limulin, and TTA required 7-60- and 80-times higher amounts of inhibitor, respectively. Presence of an α-methyl- or α-benzyl-group in the NeuAc-α-glycosides promoted ligand binding to limulin, LFA and CHA I but not TTA (Table 1). A β-glycosidic substituent strongly reduced the affinity to limulin, LFA, and CHA I, but promoted binding to TTA. To elucidate the role of the equatorially oriented carboxyl of NeuAc, the group was blocked by methylester formation. This compound lost binding affinity for limulin, but still bound to CHA I and TTA (Table 1). Introduction of a methylester or an amide into the sialic acid-α-glycoside blocked the axially oriented carboxyl and decreased binding to limulin [34], LFA [35] and CHA I [33], but not TTA [25], suggesting a role of ionic interactions between the carboxyl and the lectin protein [33, 35].

To characterize the role of the *N*-acetyl group for lectin binding, three different modifications of NeuAc-α-glycosides were tested (Table 1). Removal of the *N*-acetyl yielded 2-α-benzyl-5-NH_2-Neu, and eliminated binding to the four invertebrate lectins, thus reflecting the crucial role of this group for sugar-lectin interaction.

Table 1. Inhibition of lectin haemagglutination by sialic acid analogues. The minimal concentrations required for total inhibition induced by 4 HU of lectin are shown. Values are expressed in mM and represent means of at least three experiments.

Sialic acid analogue	*Limulin*	*LFA*	*CHA I*[a]	*TTA*[b]	*SNAI*
NeuAc[c]	50	4	0.6	35	>50
2-α-Me-NeuAc	4.2	0.9	0.15	17	>50
2-α-Bzl-NeuAc	8	1.5	0.2	12.5	>50
2-β-Me-NeuAc	>50	25	0.9	12.5	>50
NeuAc-me-ester	>100	8	0.9	12.5	>50
2-α-Bzl-5-NH_2-Neu[d]	>100	>25	>50	>50	>50
2-α-Me-5-*N*-thioacetyl-Neu	3	>100	>12.5	>100	>50
2-α-Bzl-5-*N*-trifluoroacetyl-Neu	>100	>25	>50	>100	>50
2-α-Me-9-*O*-ac-NeuAc	8	3.1	0.1	12.5	>50
2-α-Bzl-8,9-*O*-isoprop-NeuAc	0.9	2.5	0.4	16	>50
2-α-Bzl-5-*N*-ac-heptulosaminic acid	12.5	>50	10	>100	>25
2,4,7,8,9-Penta-*O*-ac-NeuAc	25	ND	>25	25	ND
NeuAcα2 → 3Galβ1 → 4Glc	31	5	0.3	4	0.28
NeuAcα2 → 6Galβ1 → 4Glc	6.25	1.5	0.3	4	0.042
NeuAcα2 → $8_{n=10-12}$ (colominic acid)[e]	>100	2.5	0.8	2	>50

[a,b]values according to [33] and [25], respectively.
[c]Abbreviations: ac, acetyl; bzl, benzyl; isoprop, isopropylidene; me, methyl; Neu, neuraminic acid; ND, not determined.
[d]unsubstituted NH_2 group.
[e]denotes concentration in terms of colominic acid.

Substitution of the three hydrogens in the *N*-acetyl by fluorine also abolished binding, which may be due to the electronegativity of the halogen. To specifically assess the role of the amide oxygen it was replaced by sulfur, resulting in 2-α-methyl-5-*N*-thioacetyl-Neu [32]. This subtle change eliminated binding to LFA, CHA I and TTA, which can be explained by the lower electronegativity of sulfur and its consecutive inability to form hydrogen bonds. In sharp contrast, limulin bound this analogue with the same affinity as the unmodified NeuAc-α-glycoside. Limulin is the first sialic acid-specific lectin found to bind the *N*-thioacetyl compound. Co-crystallization of limulin with this analogue for X-ray crystallography should provide insight into the interaction of the lectin's complementary binding site with the ligand. In previous work the *N*-acetyl group was shortened by one carbon, yielding an *N*-formyl-Neu analogue. This change abolished binding to CHA I [33], LFA [35] and TTA [25]. Extension by one carbon or by introduction of a positively charged amino group, yielding 2-α-benzyl-5-*N*-propyl-Neu and 2-α-benzyl-5-*N*-aminoacetyl-Neu, respectively, disrupted the affinity to CHA I [33]. Likewise, the change from *N*-acetyl- to *N*-glycolylneuraminic acid by introduction of a hydoxyl eliminated binding to LFA [35] and CHA I [33]. Limulin was equally inhibited by the two compounds [36], but specifically bound to lipid vesicles containing *N*-glycolylneuraminic acid derivatives of gangliosides [34].

2-α-Benzyl-5-*N*-acetyl-heptulosaminic acid lacks the glyceryl side chain of sialic acid and did not bind to the lectins, but again limulin was an exception (Table 1). This can be explained by the loss in hydrogen bonding donor-acceptor pairs between sialic acid and the lectin protein [35]. Substituents at the glyceryl side chain, like the 9-*O*-acetyl or the bulky 8,9-*O*-isopropylidene group were well tolerated by limulin, CHA I and TTA (Table 1). CHA I and TTA could accommodate hydrophobic substituents as bulky as the 9-*O*-tosyl residue [33, 25]. On the other hand, 9-*O*-acetylation decreased interaction with LFA three (Table 1) or 20-fold [35].

The role of the linkage of sialic acid to the subterminal sugar was examined by employing sialyllactoses (Table 1). Limulin and LFA preferred NeuAcα2 → 6Galβ1 → 4Glc to NeuAcα2 → 3Galβ1 → 4Glc, whereas CHA I and TTA reacted equally with both compounds. Both sialyllactoses had a higher affinity to TTA than the monomeric NeuAc-α-glycosides. Thus, TTA differs from the other invertebrate lectins since a subterminal sugar promotes carbohydrate-lectin interaction. In agreement with this finding, the α2 → 8-linked sialic acid oligomer colominic acid was the strongest TTA-inhibitor. Knibbs *et al.* [35] reported an 11-fold weaker binding of colominic acid to LFA, compared to the NeuAcα2 → 8NeuAc disaccharide, whereas we found that LFA and CHA I interacted with colominic acid as well as with the sialyllactoses. Limulin stands out as the only invertebrate lectin with no affinity to colominic acid (Table 1).

Peracetylation converts the hydrophilic character of the NeuAc molecule to a more hydrophobic one, which abolished binding to CHA I, but not to limulin and TTA (Table 1).

Tentative models depicting the interactions of CHA I and LFA with their ligand sialic acid were suggested by Brossmer *et al.* [33] and Knibbs *et al.* [35].

Haemagglutination inhibition of limulin, LFA, CHA I and TTA by glycoproteins

The results of haemagglutination inhibition of limulin, LFA, CHA I and TTA by glycoproteins are summarized in Table 2. There are remarkable similarities in the inhibition profiles of the four lectins. The strongest inhibitors were BSM, OSM and *Collocalia* mucin B. Lack of inhibition of asialo-BSM and -OSM indicated the crucial role of sialic acid in these interactions. Both BSM and OSM contain multiple NeuAcα2 → 6GalNAc1 → OSer/Thr chains, whereas in *Collocalia* mucin B branched O-glycosidic carbohydrate chains predominate. Therefore, NeuAc in either branched or unbranched O-chains can bind to the lectins. Sialic acids linked to glycoprotein oligosaccharides had a 75-(CHA I) to 15 000-fold (TTA) higher inhibitory potential than the free NeuAc-α-glycoside. Glycoproteins with N-glycosidic chains were noninhibitory, except Tamm-Horsfall protein that showed strong inhibition. This can be explained by its strong tendency to form high molecular weight aggregates and therefore a stronger avidity effect [25, 33]. Roche and Monsigny [36] reported inhibition of limulin by submaxillary mucins of several species, but found a 50-times stronger effect of BSM over OSM.

Haemagglutination inhibition of SNA I

SNA I was not inhibited by any monomeric sialic acid analogue. It interacted with the trisaccharides NeuAc-α2 → 3Galβ1 → 4Glc and NeuAcα2 → 6Galβ1 → 4Glc, with preference for the α2 → 6-linked compound (Table 1). Lactose was 600-times less inhibitory, which confirms the requirement of sialic acid for high affinity binding. Galactose abolished lectin-mediated haemagglutination at 125 mM, but neither GalNAc nor any other simple sugar did (data not shown).

Transferrin, antithrombin III and Zn-α_2-glycoprotein contain only α2 → 6-linked NeuAc, and were among the strongest glycoprotein inhibitors, as well as fibronectin, β_2-glycoprotein I and Tamm-Horsfall protein (THP) (Table 2). THP and α_1-acid-glycoprotein, also strong inhibitors, carry about even amounts of α2 → 3- and α2 → 6-linkages. The weakest inhibitors fetuin, galactoglycoprotein and glycophorin A have mostly α2 → 3-bound NeuAc (for references on glycoproteins see Brossmer *et al.* [33]). These data reflect preferential interaction of the lectin with NeuAcα2 → 6-residues. All the potent SNA I-inhibitors contain the NeuAcα2 → 6Galβ1 → 4GlcNAcβ1 → XMan sequence. Shibuya *et al.* [27] reported that SNA I precipitates OSM and other highly sialylated glycoproteins. In contrast, we found no inhibition of SNA I-mediated haemagglutination by BSM and OSM, which may be due to employment of two different experimental systems.

Haemagglutination inhibition of WGA by N-thioacetyl compounds

NeuAc-α-glycoside and GlcNAc are the two favourite monosaccharide ligands of WGA. Therefore, we examined the interaction of their *N*-thioacetyl analogues with the lectin (Table 3). Substitution of the amide oxygen in both 2-α-methyl-NeuAc and GlcNAc by sulfur abrogated binding to WGA. This is most likely due to

Table 2. Inhibition of lectin haemagglutination by glycoproteins. The minimal concentrations required for total inhibition induced by 4 HU of lectin are shown. Values are expressed in μg protein per ml and, *in italics*, as corresponding μM NeuAc. They represent means of at least three experiments.

Glycoprotein	*Limulin*		*LFA*		*CHA I*[a]		*TTA*[b]		*SNA I*		*O/N-chains*
BSM	12	*3*	2	*0.5*	*7*	2	4	*1*	>500	*>154*	only O
Asialo-BSM	>1000		>500		320		>1000		>1000		
OSM	7	*4*	2	*1*	3	*2*	1	*0.5*	>1000	*>608*	800 O
Asialo-OSM	>1000		>1000		>500		>1000		>1000		
Fetuin	>500	*>65*	11	*2*	110	*14*	>1000	*>130*	300	*40*	3 O + 3 N
Asialo-fetuin	>1000		>1000		>500		>1000		>1000		
Collocalia mucin B	19	*8*	5	*2*	20	*8*	5	*2*	>1000	*>420*	O ≫ N
Glycophorin A	25	*26*	40	*42*	4	*4*	36	*38*	1000	*1100*	15 O + 1 N
Galactoglycoprotein	>1000	*>800*	250	*200*	60	*48*	10	*8*	660	*530*	43 O + 3 N
α_1-Acid-glycoprotein	>500	*>140*	400	*110*	320	*85*	>2000	*>540*	125	*34*	5 N
Zn-α_2-glycoprotein	>1000	*>110*	>500	*>55*	>500	*>55*	>2000	*>220*	200	*22*	3 N
Transferrin	>500	*>24*	>500	*>24*	>500	*>24*	>1000	*>48*	420	*10*	2 N
Fibronectin	>500		>500		>500		>1000		60		only N
β_2-Glycoprotein I	>500	*>70*	>500	*>70*	450	*60*	>2000	*>270*	60	*8*	5 N
Tamm-Horsfall protein	14	*2*	6	*1*	30	*4*	50	*6.5*	80	*10*	only N
Antithrombin III	>500	*>63*	>500	*>63*	>1000	*>125*	>2000	*>250*	80	*10*	4 N

[a,b] values according to [33] and [25], respectively.

Table 3. Inhibition of WGA haemagglutination by monosaccharides and their *N*-thioacetyl-analogues. The minimal concentrations required for total haemagglutination inhibition induced by 4 HU of WGA are shown.

Carbohydrate	*mM*
NeuAc	>50
2-α-Me-NeuAc	12.5
2-α-Me-5-*N*-thioacetyl-Neu	>100
GalNAc	100
GlcNAc	1.6
N-thioacetyl-D-glucosamine	>100

the difference in electronegativity between oxygen and sulfur and the altered capacity for hydrogen bonding. Interaction of WGA with sialic acids, *N*-acetylhexosamines and glycoproteins are well characterized [34, 37–39] and are therefore not discussed further.

Comparison with other lectins

The *N*-acetyl group of NeuAc is crucial for binding to limulin, LFA, CHA I, TTA, WGA [34, 37, 38] and *Tritrichomonas mobilensis* lectin [40]. The hydroxyl in *N*-glycolylneuraminic acid decreased binding to CHA I [33] and LFA [22, 35], while limulin [34] and the lectin from *Scylla serrata* [41] were reported to specifically bind this compound. The equatorially oriented carboxyl group of NeuAc plays a role in binding to limulin, whereas the axially oriented carboxyl in the sialic acid-α-glycoside is involved in binding to limulin [34], K99 fimbriae [42], CHA I [33] and LFA [35], but not to WGA [34] and TTA [25]. An axial hydroxyl at C-4 sterically hinders interaction with LFA [35]. The glyceryl side chain of NeuAc is required for binding to K99 [42], LFA, CHA I and TTA but not limulin, and impairs binding to WGA [34, 38]. Limulin, CHA I and TTA tolerate substituents like the 9-*O*-acetyl group, but binding to LFA is affected by this modification. The lectin from *Cancer antennarius* is highly specific for 4-*O*- and 9-*O*-acetyl NeuAc [23]. Limulin and LFA prefer $\alpha2 \rightarrow 6$- to $\alpha2 \rightarrow 3$-linked NeuAc, whereas the plant lectins SNA I and *Maackia amurensis* leukoagglutinin specifically bind NeuAc-$\alpha2 \rightarrow$ 6Gal/GalNAc [27] and NeuAc$\alpha2 \rightarrow$ 3Gal$\beta1 \rightarrow$ 4GlcNAc [43, 44], respectively. The NeuAc oligomer colominic acid has a high affinity for TTA, but does not bind to limulin and *Tritrichomonas mobilensis* lectin [40].

BSM is a potent inhibitor for limulin, LFA, WGA [37], *Scylla serrata* lectin [41] and *Tritrichomonas mobilensis* lectin [40]. WGA also interacts with glycophorin and α_1-acid-glycoprotein [37], whereas *Cancer antennarius* lectin exclusively binds to the *O*-acetyl sialic acid containing mucins from bovine and equine submaxillary glands [23]. Shibuya *et al.* [27] reported potent inhibition of SNA I by carbohydrate chains from thyroglobulin and fetuin.

Interaction of the lectins with erythrocytes and tumour cells

Haemagglutination and cell agglutination results are depicted in Fig. 1. Consistent with their sialic acid specificity, the invertebrate lectins and SNA I agglutinated native, but not sialidase-treated red cells. WGA, in contrast, interacted stronger with sialidase-treated than with native group A erythrocytes. The ability of WGA to bind to GalNAc residues [6, 26, 37–39], which are unmasked on blood group A cells by sialidase, explains this finding.

We also investigated the interactions between the lectins and three tumour cell lines, the mouse T-cell lymphoma line Eb with low metastatic potential, its highly metastatic subline ESb [45], and the human colon carcinoma line HT29 (Fig. 1). LFA, CHA I and SNA I agglutinated the tumour cells before, but not after removal of sialic acids from the cell surface. Interaction of ESb cells with limulin, in contrast, was not abolished by sialidase treatment (Fig. 1). Thus, these highly malignant cells carry sialidase-resistant limulin binding sites and can therefore be distinguished from the parental cells Eb. The lines Eb and ESb differ not only in their metastatic behaviour, but also in the expression of cell surface glycoconjugates [45, 46]. ESb cells carry gangliosides with NeuAc linked to internal galactose [47], which is known to be sialidase-resistant [48] and may be accessible to limulin. Interaction of limulin with non-sialic acid residues on the cell surface may also explain this finding. TTA did not interact with any of the three tumour lines (Fig. 1). Thus, the cells lack the specific residues essential for TTA binding, which are present on human erythrocytes and on O-chain glycoproteins. Agglutination of HT29 colon carcinoma cells by SNA I is consistent with reports on the detection of $\alpha2 \rightarrow$ 6-sialylated residues in human colon carcinoma tissues [16, 17]. The finding that WGA interacted more strongly with tumour cells than with erythrocytes corresponds to earlier reports that WGA preferentially binds to tumour cells [6].

In summary, the lectins discussed here are remarkably distinct in their sialic acid binding properties. SNA I differs from the invertebrate lectins in the binding of sialoglycoproteins. The results with *N*-thioacetylated compounds provide new insight into the interaction of the lectins' complementary binding site with the carbohydrate ligand. Co-crystallization of lectins in complex with these molecules should allow X-ray studies to further elucidate sugar-protein interactions. Finally, the lectins appear to be useful tools for the study of sialic acids on the surface of cancer cells.

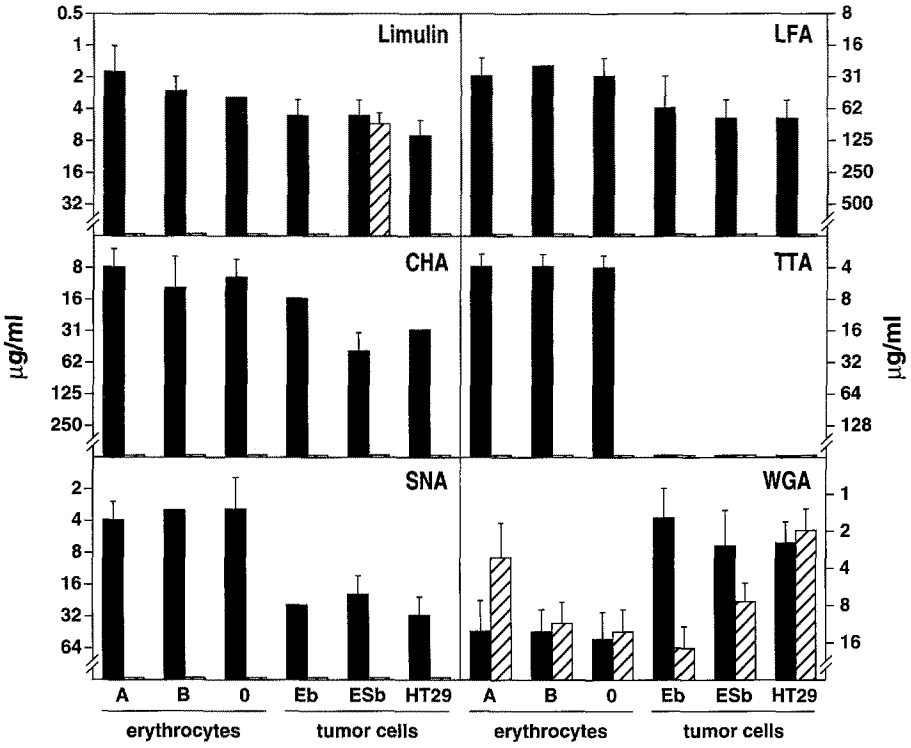

Figure 1. Agglutination of erythrocytes and tumour cells by sialic acid-specific lectins. The minimal concentrations of lectin necessary for total agglutination are depicted. Black columns represent native cells, hatched columns sialidase-treated cells. Results are means of at least three experiments. Error bars represent standard deviations. Data with TTA according to [25].

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