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Solid-Phase Assays for Study of Carbohydrate Specificity of Galectins

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Abstract—We have recently shown that the carbohydrate-binding pattern of galectins in cells differs from that determined in artificial (non-cellular) test-systems. To understand the observed discrepancy, we compared several test-systems differing in the mode of galectin presentation on solid phase. The most representative system was an assay where the binding of galectin (human galectins-1 and -3 were studied) to asialofetuin immobilized on solid phase was inhibited by polyacrylamide glycoconjugates, Glyc-PAA. This approach permits us to range quantitatively glycans (Glyc) by their affinity to galectin, i.e. to study both high and low affinity ligands. Our attempts to imitate the cell system by solid-phase assay were not successful. In the cell system galectin binds glycoconjugates by one carbohydrate-recognizing domain (CRD), and after that the binding to the remaining non-bound CRD is studied by means of fluorescein-labeled Glyc-PAA. In an "imitation" variant when galectins are loaded on adsorbed asialofetuin or Glyc-PAA followed by revealing of binding by the second Glyc-PAA, the interaction was not observed or glycans were ordered poorly, unlike in the inhibitory assay. When galectins were adsorbed on corresponding antibodies (when all CRDs were free for recognition by carbohydrate), a good concentration dependence was observed and patterns of specificities were similar (though not identical) for the two methods; notably, this system does not reflect the situation in the cell. Besides the above-mentioned, other variants of solid-phase analysis of galectin specificity were tested. The results elucidate the mechanism and consequence of galectin CRD *cis*-masking on cell surface.

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Key words: galectins, glycoconjugates, masking, solid-phase assay, oligosaccharides, carbohydrate specificity

Galectins are a family of β-galactoside-binding proteins homological in amino acid sequence of their carbohydrate-recognizing domain (CRD) [1-3]. The interest in galectin research is due to the fact that these proteins are involved in many processes connected with vital activity of the cell, such as regulation of the cell cycle, cell–cell and cell–extracellular matrix adhesion, and intercellular signal transmitting. It is now known that mammals have 14 representatives of this family, and the best-studied are galectin-1 and -3 [2-7]. Galectin-1 is related to galectins of the "prototype", namely, a noncovalent dimer of two similar subunits of 14 kDa, each of which has one CRD [6]. Galectin-3 is the only representative of "chimera type" galectins, i.e. it consists of a regulatory collagen-like N-terminal domain and a CRD [7].

The minimal structure recognized by galectins is the disaccharide Galβ1-4GlcNAc (LN). More complicated glycans that bind to galectins with higher affinity than LN were revealed using different methods from hemagglutination [8] to microarray solid-phase assay [9, 10]. The most complete characteristics of galectin specificity were obtained by solid-phase assay, plasmon resonance, fluorescence polarization, and frontal affinity chromatogra-

Abbreviations: AP, alkaline phosphatase; ASF, asialofetuin; BSA, bovine serum albumin; biot, biotin; CRD, carbohydrate-recognizing domain; Glyc-PAA, polyacrylamide glycoconjugate; PBA, PBS containing 0.3% BSA, pH 7.2; PBS, phosphate buffered saline, pH 7.2; PBS-Tw, PBS containing 0.1% Tween-20; PO, horseradish peroxidase; Str, streptavidin; TBA, TBS containing 0.2% BSA; TBS, buffer containing 50 mM Tris-HCl and 150 mM NaCl, pH 7.5; TBS-Tw, TBS containing 0.25% Tween-20.

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phy [11-15]. The main conclusion is that galectins recognize the fragment Galβ1-4/3GlcNAc, where hydroxyl groups at the C-4 and C-6 atoms of the Gal residue and the C-3 or C-4 of the GlcNAc residue play an essential role in formation of the complex with the ligand. If there are substituents on the 2-OH and 3-OH groups of the galactose residue, they can also take part in interaction with galectins. So the affinity of trisaccharide Galα3′LN to galectin-3 far exceeds that of lactosamine [15]. Sulfation of 3-OH of galactose residue increases affinity of ligand to galectin-1 by an order of magnitude, but sulfation of the 4- or 6-OH abolishes the binding [11]. 3-Sialylated by the Gal residue oligolactosamines retain ability to bind to galectin-1 despite large size of the substituent, whereas 6-sialylated analogs do not [11, 12].

Of special interest is the study of galectins on cell membranes. In this case, approximation by the test systems described below to the real situation, i.e. when galectin is situated on the cell surface, remains unknown. Galectins mediate intracellular adhesion and adhesion of cells to extracellular matrix; but before galectin of the cell surface binds to glycoconjugate of another one, it should anchor to the ligands of the first cell because these small proteins have no other way to anchor. Moreover, cellular galectins can interact with glycoproteins of the intracellular space or can be masked with glycans of the same cells [16, 17]. We supposed that common assays did not reproduce the features of interaction between galectin and cellular glycans, so a new method for studying carbohydrate specificity of cellular galectins was developed. This assay can be used to study specificity of galectins in the cell context; for this purpose, galectins were loaded onto cells and then their binding to fluorescein-labeled glycoconjugates Glyc-PAA-fluo [17] was studied. The results have revealed a number of features that were not found using common model methods. The determined differences can be partially explained by participation of endogenous cellular glycoconjugates that compete with glycoprobe for binding to lectin [17]. However, some questions remained unanswered, necessitating a return to solid-phase assays, but taking into consideration the experience of studying specificity of galectins on the cell membrane. In this report, we compare several solid-phase assays for galectin-1 and -3 that have revealed some new features of their interaction with glycans.

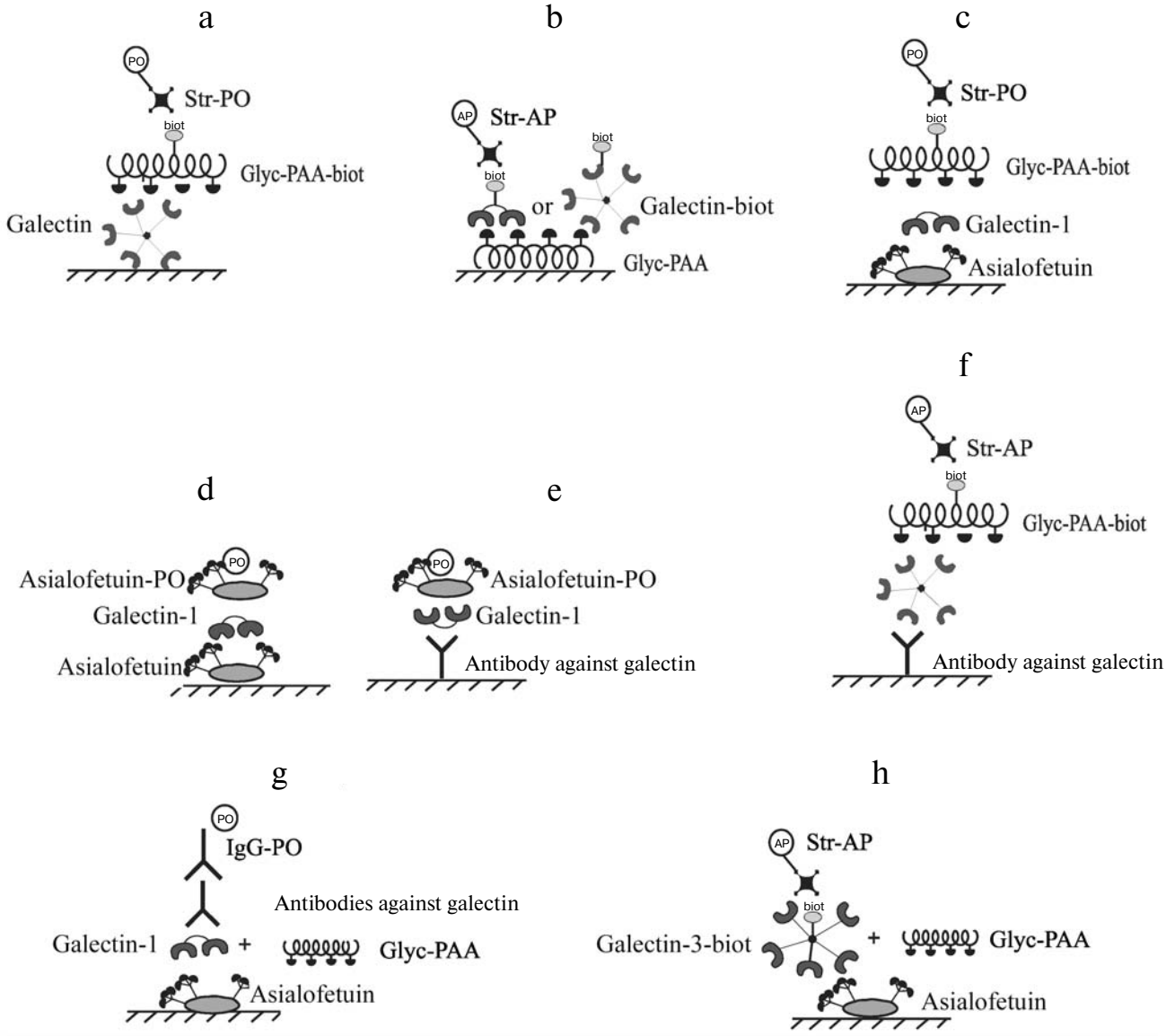
MATERIALS AND METHODS

Materials. Synthetic glycoconjugates (Glyc-PAA and Glyc-PAA-biot, where PAA is soluble polyacrylamide with molecular weight ~30 kDa [18]) were purchased from Lectinity (Russia); HNK-1 derivatives were kindly provided by Dr. N. E. Nifant'ev (Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences). Samples of recombinant galectins-1 and -3 and rabbit polyclonal antibodies against these galectins were obtained as described earlier [19, 20]. The following reagents have been used in the study: asialofetuin, *p*-nitrophenyl phosphate, *o*-phenylenediamine, and Tween-20 (Sigma, USA); bovine serum albumin (BSA) (Merck, Germany); conjugates of streptavidin (Str) with alkaline phosphatase (AP) or horseradish peroxidase (PO) Str-AP and Str-PO (Amersham, Great Britain). Conjugate of PO with mouse antibodies against rabbit immunoglobulins (IgG-PO) was purchased from Gamaleya Research Institute for Epidemiology and Microbiology. Conjugate of asialofetuin (ASF) with PO, ASF-PO, was synthesized as described [21]. Other reagents were obtained from Reakhim (Russia). Maxisorb polystyrene 96-well plates were from Nunc (Denmark).

Sorption of galectins on plastic and binding control with antibodies. Galectin-1 or galectin-3 (20 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, was coated on plate wells. The plate was incubated at 4°C overnight. After incubation of the plate with phosphate buffered saline (PBS) containing 2% BSA for an hour, two-fold dilutions of antibodies against galectin in PBA (PBS containing 0.3% BSA, pH 7.2) were added to the wells in the range of concentrations from 1 to 100 μg/ml. Then the plate was incubated at 37°C for an hour followed by washing three times with PBS-Tw (PBS containing 0.1% Tween-20), incubation with IgG-PO conjugate (1 : 1000 dilution) for an hour at 37°C, washing with PBS-Tw, and detection with *o*-phenylenediamine (0.4 μg/ml) in the buffer containing 0.01 M Na_2HPO_4 and 0.05 M citric acid (pH 5.0) supplemented with 30 μl 30% H_2O_2. The reaction was stopped by addition of 50 μl 1 M H_2SO_4, and absorbance was read at 492 nm using a Wallac 1420 Multilabel Counter (Perkin Elmer, USA).

Binding of Glyc-PAA-biot to galectin adsorbed on plastic (Scheme, panel (a)). Galectin-1 or galectin-3 (20 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, was coated on plate wells. The plate was incubated at 4°C overnight. After the incubation of the plate with PBS containing 2% BSA for an hour, two-fold dilutions of Glyc-PAA-biot in PBA were added to the wells in the range of concentration from 100 ng/ml to 500 μg/ml. The plate was incubated at 37°C for an hour, three times washed with PBS-Tw, and then Str-PO conjugate (1 : 4000 dilution in PBA) was added. The plate was incubated at 37°C for an hour, washed with PBS-Tw, and detected with solution of *o*-phenylenediamine as described.

Binding of glycoconjugates immobilized on plastic to galectins (Scheme, panel (b)). Glyc-PAA (40 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, was coated on plate wells. The plate was incubated at 37°C for 3 h and then at 4°C overnight. After 1 h of incubation with TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 2% BSA, the plate was washed with TBS-Tw (TBS containing 0.25% Tween-20), and then two-fold dilutions of biotinylated galectin in TBA (TBS containing 0.2% BSA) were added



Schemes of test systems of galectin solid-phase assay: design and sequence of stages

to the wells in the range of concentrations from 0.2 to 30 μg/ml. The plate was incubated at 37°C for 2 h, washed three times with TBS-Tw, and then it was incubated with Str-AP conjugate (1 : 1000 dilution in TBA) at 37°C for an hour, washed with TBS-Tw, and after the addition of *p*-nitrophenyl phosphate (1 mg/ml) in buffer containing 200 mM diethanolamine and 2 mM $MgCl_2$ (pH 10.5) absorbance was read at 405 nm.

Interaction of Glyc-PAA-biot with galectin-1 adsorbed on asialofetuin (Scheme, panel (c)). Asialofetuin (10 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, was coated on plate wells. The plate was incubated at 37°C for 3 h, then at 4°C overnight. After 1 h of incubation with PBS containing 2% BSA, the plate was washed with PBS-Tw and galectin-1 (10 μg/ml) in PBA was added. The plate was incubated at 37°C for 1 h, washed three times with PBS-Tw, followed by incubation with biotinylated glycoconjugates in the range of concentrations from 100 ng/ml to 500 μg/ml in PBA at 37°C for 1 h, washing with PBS-Tw, and incubation with Str-PO conjugate (1 : 4000 dilution) at 37°C for 1 h. Then the plate was washed with PBS-Tw and detected with *o*-phenylenediamine (0.4 mg/ml) as described above.

Interaction between ASF-PO and galectin-1 (Scheme, panels (d) and (e)). Asialofetuin (10 μg/ml, panel (d)) or antibodies against galectin-1 (10 μg/ml, panel (e)) in 0.1 M $NaHCO_3$ buffer, pH 9.6, were coated on plate wells. The plate was incubated at 4°C overnight, washed with PBS-Tw, and then it was incubated with PBS containing 2% BSA at 37°C for 1 h and the solution of

galectin-1 (10 μg/ml) was added. The plate was incubated at 37°C for 1 h and washed, followed by incubation with two-fold dilutions of ASF-PO conjugate in PBA in the range of dilutions from 1 : 10 to 1 : 320 at 37°C for 1 h. Then the plate was washed three times with PBS-Tw and detected with *o*-phenylenediamine solution.

Interaction of Glyc-PAA-biot with galectins adsorbed on antibodies (Scheme, panel (f)). Antibodies against galectin-1 or -3 (10 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, were coated on plate wells. The plate was incubated at 37°C for 3 h, and then at 4°C overnight. After incubation with TBS containing 2% BSA for 1 h, the plate was washed with TBS-Tw and the solution of galectin (20 μg/ml) in TBA was added. The plate was incubated at 37°C for 1 h, washed three times with TBS-Tw, followed by incubation with biotinylated glycoconjugates in TBA in the range of concentrations from 75 ng/ml to 100 μg/ml at 37°C for 1 h, and washing with TBS-Tw. Then the plate was incubated with Str-AP conjugate (1 : 1000 dilution) at 37°C for an hour, washed with TBS-Tw, and developed with *p*-nitrophenyl phosphate (1 mg/ml) as described above.

Inhibitory assay, galectin-1 (Scheme, panel (g)). Different concentrations of Glyc-PAA glycoconjugates (1.5-100 μg/ml) in PBA were incubated with galectin-1 (20 μg/ml) at 37°C for 2 h and then added into plate wells with immobilized asialofetuin as described above. The plate was incubated at 37°C for 2 h, washed with PBS-Tw, and incubated with antibodies against galectin-1 (10 μg/ml) at 37°C for 1 h. Then the plate was washed with PBS-Tw and incubated with IgG-PO conjugate (1 : 1000 dilution) at 37°C for 1 h. After the termination of reaction, the plate was washed with PBS-Tw and detected with *o*-phenylenediamine solution.

Inhibitory assay, galectin-3 (Scheme, panel (h)). Different concentrations of Glyc-PAA glycoconjugates (1.5-100 μg/ml) in TBA were incubated with biotinylated galectin-3 (10 μg/ml) at 37°C for 2 h, and then added into plate wells with immobilized asialofetuin as described above. The plate was incubated at 37°C for 2 h, washed with TBS-Tw, and incubated with Str-AP conjugate (1 : 1000 dilution in TBA) at 37°C for 1 h. Then the plate was detected with *p*-nitrophenyl phosphate as described above.

RESULTS

The following solid-phase test systems have been suggested for studying carbohydrate specificity of galectins-1 and -3 (Scheme).

Direct binding methods. *Binding of glycoconjugates to galectins adsorbed on plastic* (Scheme, panel (a)). Galectins-1 and -3 were coated directly on polystyrene; the fact of adsorption was monitored using antibodies against galectins. Though galectins really sorbed on plastic (Fig. 1), there was no binding of biotinylated glycoconjugates to them (data not shown).

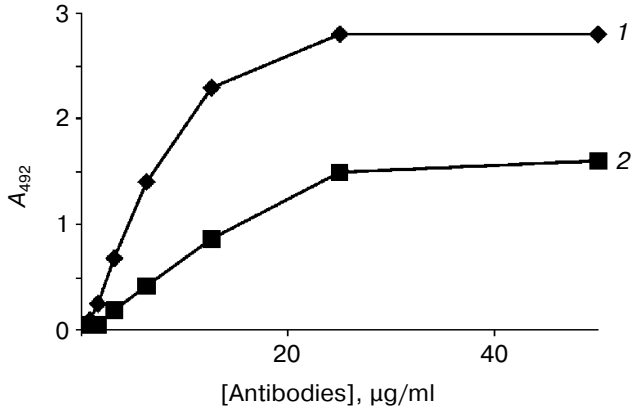


Fig. 1. Binding of galectins-1 (*1*) and -3 (*2*) to antibodies. Galectins-1 and -3 were coated on a 96-well plate, and after washing corresponding antibodies were added.

Binding of galectins to Glyc-PAA glycoconjugates adsorbed on plastic. Glyc-PAA were coated on the plate and their binding to biotinylated galectin-1 and -3 (Scheme, panel (b)) was studied. Though interaction of immobilized glycoconjugates with galectins took place, a small difference between ligand binding, which was expected to be more significant, demonstrates the inability to use this "configuration" of the components (Fig. 2).

Interaction of glycoconjugates with galectin-1 adsorbed on asialofetuin. In this variant of assay, asialofetuin was coated on plates, then galectin-1 followed by Glyc-PAA-biot glycoprobes were added (Scheme, panel (c)). Binding of probes to galectin-1 was not observed, though lectin adsorbed on asialofetuin, which was proved by its detection with corresponding antibodies (data not shown).

Interaction of conjugate ASF-PO with galectin-1 adsorbed on asialofetuin. As in the previous case, galectin-1 was coated on asialofetuin, but then it was incubated with ASF-PO conjugate (Scheme, panel (d)). Specific binding in this type of experiment was again not detected (Fig. 3, curve *1*). However, if lectin was adsorbed via antibodies instead of ASF (Scheme, panel (e)), its further interaction with ASF-PO took place and was dose-dependent (Fig. 3, curve *2*).

Interaction of glycoconjugates with galectins adsorbed on antibodies. In this variant of assay, antibodies against galectin-1 (see previous section) or -3 were coated on plates, then galectins were added, followed by Glyc-PAA-biot glycoprobes (Scheme, panel (f)). Curves differed well, in particular, 3′OSuLe^c exhibited the highest affinity to galectin-1, whereas binding of its isomer 3′OSuLN was two-fold weaker, and LN_3 was three-fold weaker (Fig. 4a). The other glycoconjugates including LN, Le^c, LNnT, LN_2, and Galα3′LN did not bind to galectin-1. In the

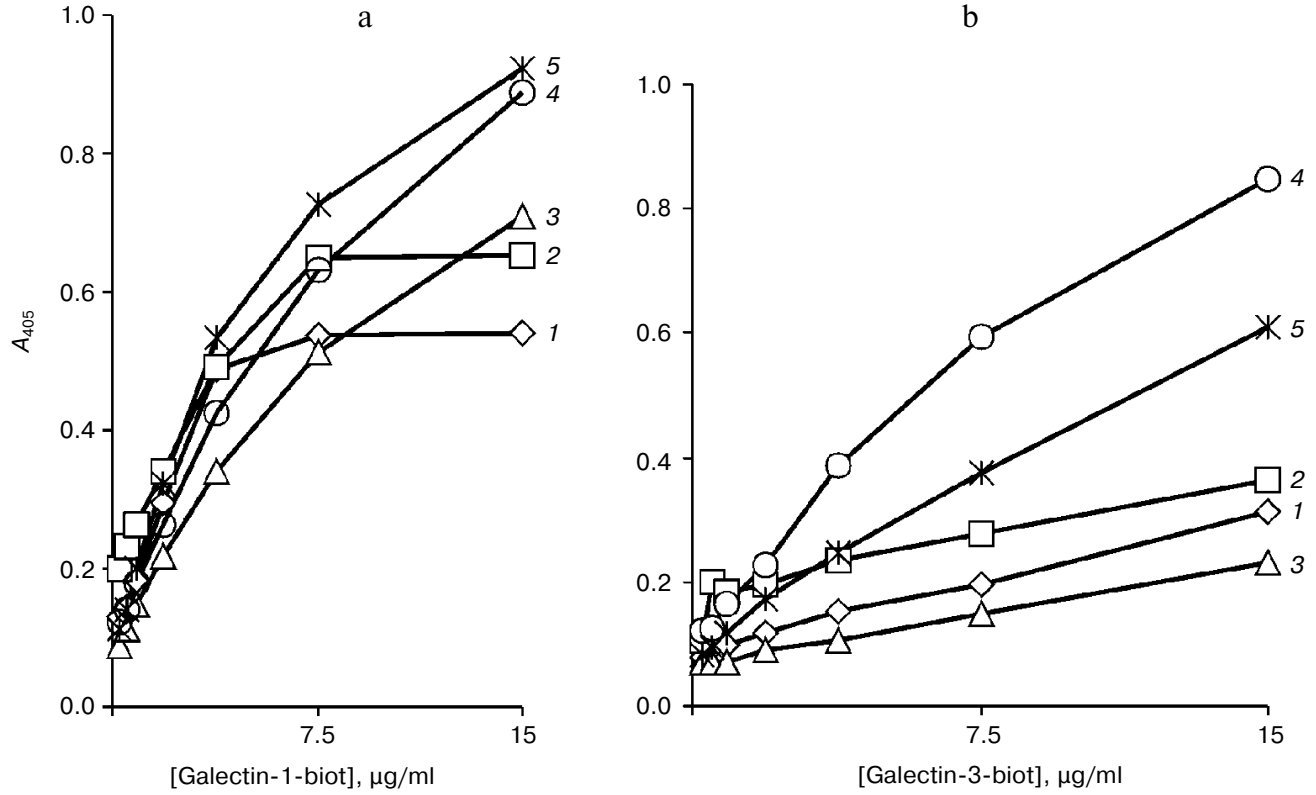


Fig. 2. Binding of glycoconjugates adsorbed on plastic to biotinylated galectins-1 (a) and -3 (b). *1*) LN; *2*) 3′OSuLN; *3*) 3′OSuLe^c; *4*) LN_3; *5*) Galα3′LN.

case of galectin-3, hexasaccharide LN_3 exhibited the highest affinity, and binding levels of the other studied glycoconjugates including sulfated ones were significantly lower (Fig. 4b).

Inhibitory tests. Mixture of galectin and Glyc-PAA was added into plate wells with precoated asialofetuin

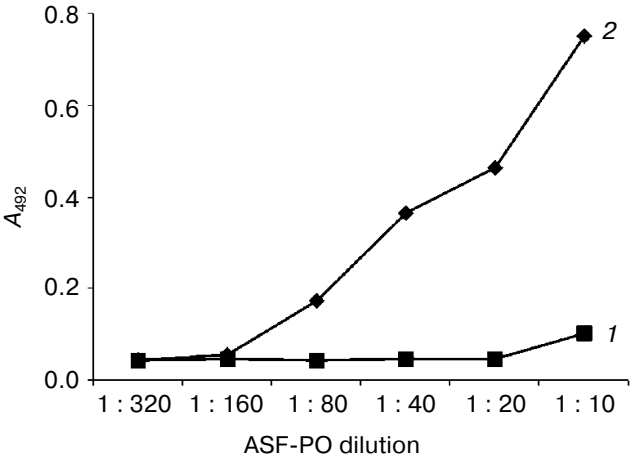


Fig. 3. Binding of galectin-1 with ASF-PO conjugate (*1*) or anti-galectin-1 antibodies (*2*). Galectin was coated on asialofetuin previously immobilized on a plate.

(Scheme, panel (g)). Bound galectin-1 was detected using corresponding antibodies. In the case of galectin-3, an analogous inhibitory system was used with the only difference that biotinylated lectin was taken (Scheme, panel (h)).

Specificity of galectins-1 and -3 was studied using the group of glycoconjugates-inhibitors presented in the table.

Specificity of galectin-1. LN and Le^c weakly inhibited binding of galectin-1 to asialofetuin (Fig. 5a), and the inhibitory activity increased with the expanding of the number of LN units in the oligolactosamine chain, namely, LN_2 bound to galectin-1 two-fold stronger, and LN_3 3.5-fold stronger than LN. Strong interaction was exhibited by sulfated derivatives of LN containing sulfate group at the 3-OH galactose residue, such as 3′,6OSu_2Lac (No. 16), 3′OSuLN (No. 18), and 3′OSuLe^c (No. 24), while inhibition by corresponding non-sulfated disaccharides was by an order of magnitude weaker (Fig. 5b). Their analogs, for example, one with sulfate in 4′ (No. 20) or 6′ position (No. 19), did not inhibit binding of galectin-1 to asialofetuin, i.e. the contribution of sulfate in these positions is negative (data not shown). Sulfated monosaccharides (Nos. 1-6; table) and oligosaccharide containing sulfate as part of a GlcA residue in pentasaccharide HNK-1 (No. 44) did not interact with galectin-1.

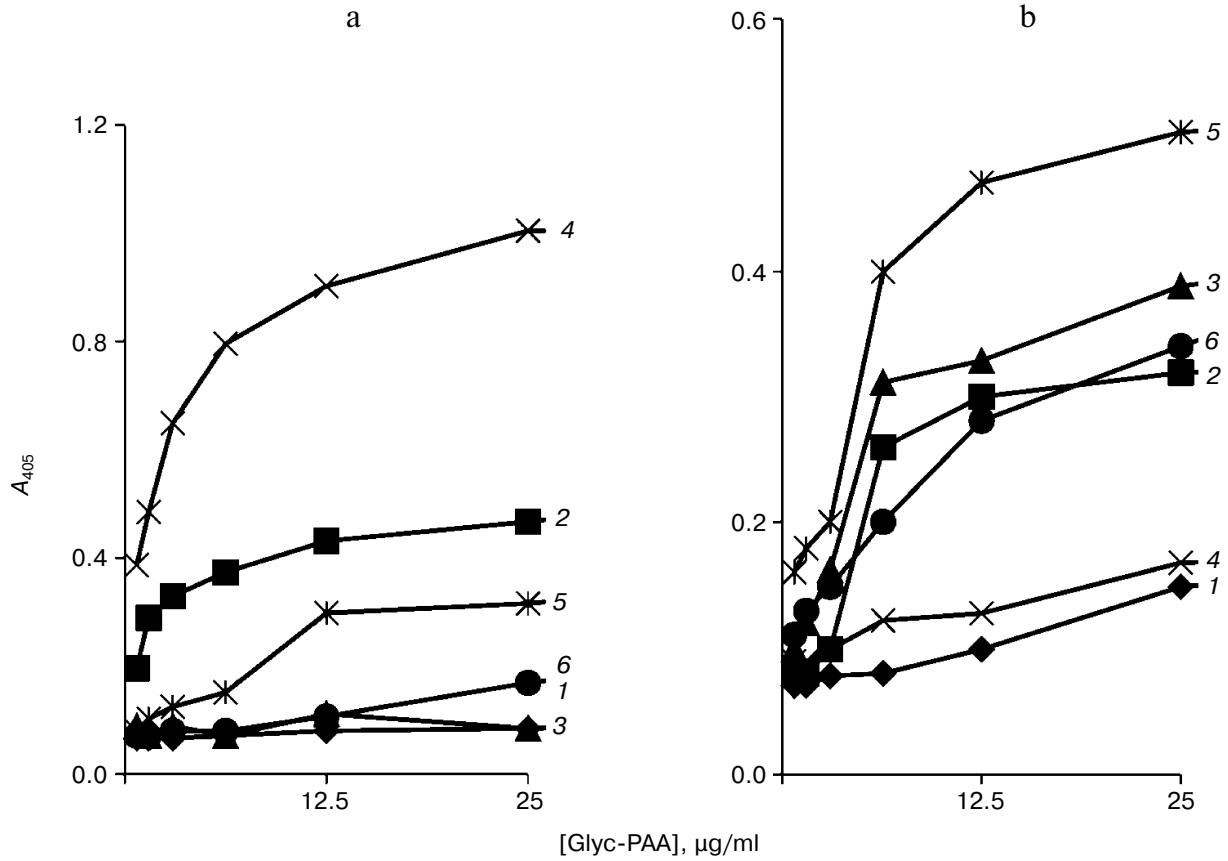


Fig. 4. Binding of glycoconjugates to galectin-1 (a) and -3 (b) adsorbed on antibodies. *1*) LN; *2*) 3′OSuLN; *3*) Le^c; *4*) 3′OSuLe^c; *5*) LN_3; *6*) Galα3′LN.

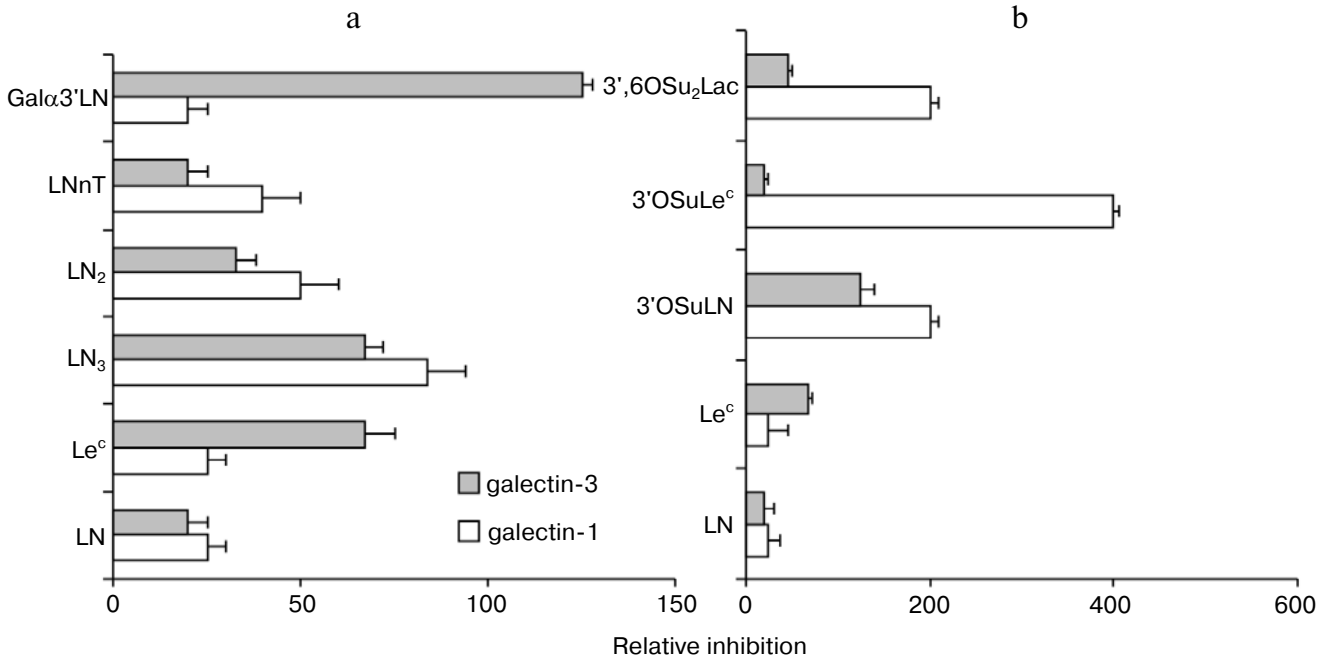


Fig. 5. Comparative carbohydrate specificity of galectins-1 and -3 in the inhibitory assay (Scheme, panels (g) and (h)). Relative inhibitory activity was calculated as: $(1/IC_{50}^{Glyc\text{-}PAA}) \times 100\%$, where $IC_{50}^{Glyc\text{-}PAA}$ is concentration of Glyc-PAA giving 50% inhibition.

List of synthetic oligosaccharides

No.	Structure of saccharide	Abbreviated name
	Monosaccharides	
1	3-O-Su-GlcA	3OSuGlcA
2	3-O-Su-Gal	3OSuGal
3	6-O-Su-GlcNAc	6OSuGlcNAc
4	3-O-Su-GalNAc	3OSuGalNAc
5	4-O-Su-GalNAc	4OSuGalNAc
6	6-O-Su-GalNAcα	6OSuGalNAcα
	Disaccharides	
7	Galβ1-4Glc	Lac
8	Galβ1-4GlcNAc	LN
9	GalNAcβ1-4GlcNAc	LacdiNAc
10	Galβ1-3GalNAcα	TF
11	Galβ1-3GlcNAc	Le^c
12	Galα1-3GalNAcβ	$T_{\alpha\beta}$
13	Galβ1-3GalNAcβ	$T_{\beta\beta}$
14	3-O-Su-Galβ1-4Glc	3'OSuLac
15	6-O-Su-Galβ1-4Glc	6'OSuLac
16	3-O-Su-Galβ1-4(6-O-Su)Glc	3',6OSu_2Lac
17	6-O-Su-Galβ1-4(6-O-Su)Glc	6,6'OSu_2Lac
18	3-O-Su-Galβ1-4GlcNAc	3'OSuLN
19	6-O-Su-Galβ1-4GlcNAc	6'OSuLN
20	4-O-Su-Galβ1-4GlcNAc	4'OSuLN
21	4,6$(O\text{-}Su)_2$-Galβ1-4GlcNAc	4',6'OSu_2LN
22	GalNAcβ1-4(6-O-Su)GlcNAc	6OSuLacdiNAc
23	3-O-Su-Galβ1-3GalNAcα	3'OSuTF
24	3-O-Su-Galβ1-3GlcNAc	$3'OSuLe^c$
25	3-O-Su-GlcAβ1-3Gal	3'OSuGlcAβ3'Gal
	Trisaccharides	
26	Galα1-3Galβ1-4GlcNAc	Galα3'LN
27	Fucα1-2Galβ1-3GalNAcβ	H type 4
28	Galα1-3(Fucα1-2)Gal	B_{tri}
29	3-O-Su-Galβ1-3 Fucα1-4) GlcNAc	$3'OSuLe^a$
30	3-O-Su-Galβ1-4(Fucα1-3) GlcNAc	$3'OSuLe^x$
31	Neu5Acα2-3Galβ1-4GlcNAc	3'SiaLN
32	Neu5Acα2-6Galβ1-4GlcNAc	6'SiaLN
33	Neu5Acα2-3Galβ1-4(6-O-Su)GlcNAc	6OSu3'SiaLN
34	Neu5Acα2-6Galβ1-4(6-O-Su)GlcNAc	6OSu6'SiaLN
35	Neu5Acα2-Galβ1-3(6-O-Su)GalNAcα	6OSu3'SiaTF
36	Neu5Acα2-3Galβ1-3(6-Su)GlcNAc	$6OSu3'SiaLe^c$
	Tetrasaccharides	
37	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	LNT
38	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	LNnT
39	Galβ1-3GalNAcβ1-4Galβ1-4Glc	aGM1
40	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	LN_2
41	Neu5Acα2-3(6-O-Su)Galβ1-4 (Fucα1-3)-GlcNAc	$6'OSuSiaLe^x$
42	Neu5Acα2-3Galβ1-4(Fucα1-3)6-O-Su-GlcNAc	$6OSuSiaLe^x$
	Higher oligosaccharides	
43	Neu5Acα2-6(Galβ1-3)GlcNAcβ1-4Galβ1-4Glc	LSTb
44	3-O-Su-GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	HNK-1
45	GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	desulfo-HNK-1
46	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	LN_3
47	$(Galβ1\text{-}4GlcNAcβ1\text{-}2Manα)_2$-3,6-Manβ1-4GlcNAcβ1-4GlcNAc	9-OS
48	$(Neu5Acα2\text{-}6Galβ1\text{-}4GlcNAcβ1\text{-}2Manα)_2$-3,6-Manβ1-4GlcNAcβ1-4GlcNAc	11-OS

Oligosaccharides containing fragment Galβ1-3GalNAc (Nos. 10, 13, 39) and also Fs-disaccharide (No. 12) did not interact with galectin-1 or were very weak inhibitors. The other studied Glyc-PAA (table) such as B_{tri} (No. 28), LacdiNAc (No. 9), 9-OS (No. 47), 11-OS (No. 48), and LSTb (No. 43) did not interact with galectin-1.

Specificity of galectin-3 (Fig. 5). Comparing only oligolactosamine patterns of galectin-3 and galectin-1 specificity, they are close to each other, namely the LN_3 was most potent, while affinity of LN_2 and LNnT was two-fold lower (Fig. 5). The fundamental difference between galectin-1 and -3 was that Galα3′LN and Le^c exhibited affinity only to galectin-3. It should be noted high binding of Le^c (similar to LN_3) and significant binding of 3′OSuLN (but not 3′OSuLe^c) to galectin-3 (though somewhat lower than to galectin-1). The other oligosaccharides presented in the table did not bind to galectin-3.

DISCUSSION

As already mentioned above while studying galectin specificity, various laboratories used methods that often differed crucially. First of all, differences took place in presentation of glycans [9-15], which can influence the mobility of the glycans, availability of their key sites, and clusterization capability. Second, the cited approaches differed in immobilization "polarity": either lectin or saccharide is immobilized on the solid surface, whereas in fluorescence polarization assay both components are dissolved. Third, both direct binding and inhibitory test were used in solid-phase assay. Finally, both mono- and multivalent glycans were used. Published results basically agree in binding of lectins to Galβ1-4/3GlcNAc derivatives; however, they vary in fine oligosaccharide specificity.

Galectins are capable of recognizing both unsubstituted oligolactosamine chains and chains modified with Neu5Ac, Fuc, or Galα residues [9-15], i.e. specificity of these lectins is broader than nominally follows from their classical definition as "β-galactoside-binding proteins" [1, 2]. Till now it remains unknown how the pronounced selectivity of lectin-mediated recognition *in vivo* can be achieved with such broad galectin specificity (observed *in vitro*). The results of recent publications suggest that cellular galectins can selectively bind only to some ligands. So 3′-sialylated dilactosamine Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc exhibits affinity to galectin-1 in solution, but does not bind to cell-associated galectin-1 [11]. Another example is that oligolactosamines bind to galectin-2 expressed on the cell surface, but do not interact with the same protein in a solid-phase assay [11]. These and other examples suggest that artificial test systems do not always reflect the cell situation adequately. It is ultimately important that lectin on cells can be masked with closely located *cis*-ligands, and this prevents its binding to complementary ligands of other cells (*trans*-ligands) [22]; it seems impossible to reproduce such a situation adequately in a solid-phase assay.

There has been no systematic investigation of specificity of galectins expressed on the cell surface until now; this can be explained by the absence of a suitable experimental model. Therefore, we developed a method that allowed us to investigate specificity of galectins associated with cells; for this purpose galectins were loaded to the cells, and probed with glycoconjugates Glyc-PAA-fluo [17]. This approach confirmed many features previously found using artificial systems, and also demonstrated some fundamental qualitative differences between cellular and non-cellular methods. Obviously, to interpret the results and to explain revealed differences it is necessary to compare several artificial systems differing in galectin presentation, but corresponding in the structure of carbohydrate probes (in our case all of them are Glyc-PAA, labeled or not).

In this work, we used three variants of immobilization, namely, when galectin is adsorbed on the surface through Glyc-PAA, asialofetuin, or antibodies. When galectins were adsorbed directly on plastic (Scheme, panel (a)), glycoconjugates did not interact with them, i.e. their carbohydrate-recognizing domains (CRD) became inaccessible for interaction with glycoprobes. In reverse order (Scheme, panel (b)) binding was observed, but the curves of various glycoconjugates slightly differed from each other, which points to "non-optimal" condition (presentation) of the CRD. Therefore, this test system is also unsuitable for investigation of galectin specificity. When the proteins were loaded to adsorbed asialofetuin (Scheme, panel (c)), binding to galectin-3 (but not to galectin-1) was observed. We explain this result as follows: the symmetry of bivalent galectin-1 is such that carbohydrate-recognizing domains are located on the one side of the dimer [23], and that is why they are involved in binding to glycoprotein carbohydrate chains of glycans; thus galectin-1 loaded on asialofetuin has no valences for binding to the second ligand. It is interesting that the same glycoconjugates bind to galectin-1 loaded onto cells [17], where a single CRD of galectin appears to be involved in bonding to the cell and the second remains free for binding to exogenous glycoconjugate, or both CRD are bound to cell glycans but with different affinity (by reason of cell surface heterogeneity). If the last statement is correct, added glycoconjugate competes with low affinity cellular *cis*-ligand, supersedes it, and binds to galectin. At the same time, it is not observed in artificial test system where chemically and "architecturally" identical glycoligands are bound to galectin to the same extent.

In contrast to dimeric galectin-1, galectin-3 is a multimer (probably pentamer [24]), so even if some of its CRD are bound to sorbed asialofetuin or Glyc-PAA, it must retain the ability to bind to the added glycoconju-

gate, and that was confirmed experimentally (Scheme, panels (b) and (c)).

When galectins were immobilized on plastic via antibodies, glycoconjugates bound to them; in this system good resolution between curves of various glycans was observed, and the results agreed in general with already known data, though they differed in details. Thus, using a glycoarray it was shown 3′OSuLN was the most potent ligand for galectin-1, while its isomer 3′OSuLe^c and LN_3 were significantly weaker [9]; in the used test system 3′OSuLe^c conversely exhibited the highest affinity to galectin-1 whereas 3′OSuLN and LN_3 bound to galectin-1 much more weakly. LN_3 exhibited high affinity to galectin-3, and this agrees with glycoarray data [9]. Moreover, Galα3′LN, Le^c, and 3′OSuLN bound to galectin-3, that were also detected as its affinity ligands with plasmon resonance assay [11]. So only direct solid-phase assays where galectins are immobilized via antibodies allow investigation of carbohydrate specificity of galectins, while other described models have a variety of problems. However, this test system certainly cannot reflect the cell situation, because galectins are anchored on the cell surface via the binding of their CRD to the cell, i.e. they are involved in carbohydrate–protein interaction instead of protein–protein interaction as in the case of antibodies. That is why specificity of galectins was then studied only using the inhibitory test, namely, their binding to adsorbed asialofetuin in the presence of Glyc-PAA as inhibitor was investigated. It should be noted that inhibitory test was used earlier [25, 26] for studying specificity of prototype galectins (galectins -1, -5).

Polyacrylamide glycoconjugates provide the multivalent binding and minimize nonspecific interactions [27]. Oligosaccharides in Glyc-PAA (table) were chosen to estimate the influence of substitutions (sulfate, neuraminic acid, and others) on the affinity of the "core" fragment (Galβ1-4GlcNAc or Galβ1-3GlcNAc) binding. Comparison of direct (through the antibodies) method and the inhibitory test gave good correlation of data, but not their complete match. Indeed, 3′OSuLe^c and 3′OSuLN exhibited the highest affinity to galectin-1, and, moreover, the affinity of the latter was two-fold lower (as in direct method); affinity of LN_3 to galectins-1 and -3 was higher than affinity of LN_2 and LNnT, whereas Le^c, 3′OSuLN, and Galα3′LN were the most potent ligands for galectin-3. Although the results for galectin-1 practically coincide, for galectin-3 some inconsistencies were revealed, i.e. LN_3 possesses the highest affinity to galectin-3 in direct binding but yields to trisaccharide Galα3′LN in the inhibitory system, and the affinity of Le^c relative to LN_3 differs by about 40% in these two test systems.

Inhibition of binding of galectin-3 to LN_3-PAA (instead of asialofetuin) adsorbed on plastic with glycoconjugates displayed practically the same pattern of specificity (data not shown), i.e. in this case the nature of primary ligand-"anchor" influences the result only slightly.

Summing up, the making of an "ideal" solid phase model, suitable for all galectins (galectins of prototype, chimera, and tandem types) seems to be impossible. So, although the results of inhibitory and direct binding (through antibodies) for galectin-1 coincide, there are some differences in binding of Le^c, LN_3, Galα3′LN for galectin-3 (Figs. 4 and 5). It seems to be impossible to make an "ideal" test system that would reflect all features of intracellular interactions to the full extent. In fact, comparison of galectin specificity data obtained in solid-phase and cellular systems proves that the pattern of the ligands with highest affinity to galectins in the cell system is significantly narrower than in the artificial one, for example, already mentioned sulfated glycans practically do not bind to galectins. We explain this fact by the possibility of galectin masking on the cell with *cis*-ligands [17]; it is very difficult if possible at all to reproduce such a situation in the solid-phase assay. At the same time, this work allowed us to investigate some aspects of binding of galectins to their ligands.

First of all, bivalent galectin binds to ligands with two CRDs. This is confirmed by the fact that we could not detect galectin-1 immobilized on asialofetuin using biotinylated glycoconjugates or ASF-PO conjugate; in this case lectin bound to glycans was detected with antibodies. Besides that, it is possible to visualize galectin with biotin [25] or fluorescein [12, 13] label directly, as it was shown previously.

Second, bivalent galectins bind to both high- and low-affinity ligands by means of two CRDs. In fact, binding of galectin-1 to immobilized LN was similar to 3′OSuLe^c as detected with antibodies (data not shown).

Finally, if bivalent galectin is anchored on the cell surface with both affinity (the first CRD) and weak (the second CRD) ligands, which is ultimately close to the situation in a cell, the strongest ligand can replace the weakest one. This situation occurs in the inhibitory test, when glycoprobes-inhibitors compete with asialofetuin for binding to galectin. In the cell assay *trans*-ligands compete with *cis*-ligands for binding to lectin by the same mechanism, and this provides for regulation of functional activity of galectins; if "external" glycan exhibits significantly higher affinity, it replaces *cis*-ligand. In other words, *cis*-ligands can control the binding of "external" ligands to cellular galectin.

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