Complex N-glycans are the major ligands for galectin-1, -3, and -8 on Chinese hamster ovary cells

Santosh Kumar Patnaik², Barry Potvin², Susanne Carlsson³, David Sturm², Hakon Leffler³, and Pamela Stanley¹,²

¹Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461; and ²Section MIG (Microbiology, Immunology, Glycobiology), Institute of Laboratory Medicine, Lund University, PO Box 124, SE-221 00, Lund, Sweden

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Galectins are implicated in a large variety of biological functions, many of which depend on their carbohydrate-binding ability. Fifteen members of the family have been identified in vertebrates based on binding to galactose (Gal) that is mediated by one or two, evolutionarily conserved, carbohydrate-recognition domains (CRDs). Variations in glycan structures expressed on glycoconjugates at the cell surface may, therefore, affect galectin binding and functions. To identify roles for different glycans in the binding of the three types of mammalian galectins to cells, we performed fluorescence cytometry at 4°C with recombinant rat galectin-1, human galectin-3, and three forms of human galectin-8, to Chinese hamster ovary (CHO) cells and 12 different CHO glycosylation mutants. All galectin species bound to parent CHO cells and binding was inhibited >90% by 0.2 M lactose. Galectin-8 isoforms with either a long or a short inter-CRD linker bound similarly to CHO cells. However, a truncated form of galectin-8 containing only the N-terminal CRD bound only weakly to CHO cells and the C-terminal galectin-8 CRD exhibited extremely low binding. Binding of the galectins to the different CHO glycosylation mutants revealed that complex N-glycans are the major ligands for each galectin except the N-terminal CRD of galectin-8, and also identified some fine differences in glycan recognition. Interestingly, increased binding of galectin-1 at 4°C correlated with increased propidium iodide (PI) uptake, whereas galectin-3 or -8 binding did not induce permeability to PI. The CHO glycosylation mutants with various repertoires of cell surface glycans are a useful tool for investigating galectin–cell interactions as they present complex and simple glycans in a natural mixture of multivalent protein and lipid glycoconjugates anchored in a cell membrane.

Key words: CHO/galectin/glycan recognition/glycosylation mutants/lectin

Introduction

Animal lectins belonging to the galectin family possess at least one structurally conserved carbohydrate-recognition domain (CRD) that specifically binds to β-galactoside-containing glycoconjugates (Barondes et al., 1994). To date, 15 different galectins (galectin-1 to -15) have been identified in mammals by galactose (Gal) binding (Houzelstein et al., 2004). Galectins function at the cell surface in facilitating cell adhesion to the extracellular matrix, cell migration, and intercellular interactions (reviewed in Ochieng et al., 2004), as well as in intracellular processes such as pre-mRNA splicing and apoptosis (reviewed in Liu et al., 2002; Wang et al., 2004), and they have been shown to modulate immune responses and tumor progression (reviewed in Liu, 2005; Liu and Rabinovich, 2005).

Many of the functions of galectins depend on their binding to Gal in the context of glycans associated with glycoconjugates. The galectin CRD of ~130 amino acids forms a bent β-sandwich, with the concave side forming a groove long enough to hold a linear oligosaccharide made of up to four monosaccharide units (Loris, 2002; Leffler et al., 2004). It is convenient to describe the groove as consisting of four subsites (A–D). A fifth subsite, E, at the reducing end accounts for interactions with the structure to which the oligosaccharide is attached (protein, lipid, or additional saccharides). Subsite C, the defining feature of galectins, is built from the side chains of conserved amino acids that constitute the motif characteristic of galectins (e.g., galectins-5, -1, and -3, respectively). Dimerization/oligomerization appears to be required for biological activity, because monomeric forms of galectin are less active than dimeric and oligomeric forms, and they are more sensitive for variations in specificity between galectins. Some galectins have subunits with a single CRD and are found as monomers, noncovalent dimers, or multimers (e.g., galectins-5, -1, and -3, respectively). Dimersization/oligomerization appears to be required for biological activity, because monomeric forms of galectin-1 (Dias-Baruffi et al., 2003) and galectin-3 (Karlsson et al., 1998) did not induce signals despite binding cells with similar or only slightly lower affinity and, in the case of galectin-3, could even be used as a dominant negative inhibitor (John et al., 2003). Other galectins have subunits with two different CRDs (i.e., galectins-4, -8, -9, and -12). The fine specificity of the two CRDs may differ (Hirabayashi et al., 2002; Iedo et al., 2003). For example, a glutamine residue in subsite B (eight amino acids upstream of the galectin signature motif) in the N-terminal CRD of galectin-8, but not the C-terminal CRD, allows the former, but not the latter, to bind α2,3-sialylated or 3-O-sulfated Gal (Iedo et al., 2003). Both CRDs of these galectins appear to be required for the

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few biological activities studied so far (Hirashima et al., 2004; Zick et al., 2004).

The sugar-binding specificity of galectins has primarily been studied in vitro, using a multitude of methods including binding to simple oligosaccharides, neoglycoproteins or glycolipids (Hirabayashi and Kasai, 1993), surface plasmon resonance (Dettmann et al., 2000), isothermal titration calorimetry (Gupta et al., 1996; Bachhawat-Sikder et al., 2001; Brewer, 2004), fluorescence polarization (Sorme et al., 2004), frontal affinity chromatography (Hirabayashi et al., 2002), and fluorescence- or enzyme-based lectinosorption (Blixt et al., 2004; Leppanen et al., 2005). Although these assays have been most useful in identifying galectin-binding specificities, a drawback is that the carbohydrate structures examined are neither in their natural context, nor conjugated to cell membrane-associated glycoproteins and glycolipids, nor present together as a mixture. Physical conditions may affect galectin binding as shown by the observation that human galectin-1 binds to immobilized extended N-acetyllactosamine (LaCNAc) structures with affinity in the \( K_d \) 2–10 \( \mu M \) range, but binds the same structures free in solution with about 10 times lower affinity (\( K_d \) in the 20–70 \( \mu M \) range; Leppanen et al., 2005). Similarly, immobilized GM1 ganglioside was reported to be bound by rat galectin-5 in a solid-phase assay, but not when presented on the surface of neuroblastoma cells (Andre S., Kaltner H., et al., 2005). Multivalency of glycan structures, and of galectins, serves to strengthen galectin–carbohydrate interactions which otherwise are relatively weak (Hirabayashi et al., 2002). Furthermore, multivalent carbohydrate–galectin interactions may form cross-links to generate two-and three-dimensional lattices (Brewer et al., 2002). Such lattices have been proposed to occur during galectin-1-induced apoptosis of T cells (Pace et al., 1999) and to affect signaling by growth factors and cytokines by inhibiting endocytosis of their cell surface receptors (Partridge et al., 2004).

Here, we investigate binding at 4°C of the three types of mammalian galectins as defined by their CRD organization (Hirabayashi and Kasai, 1993) to Chinese hamster ovary (CHO) cells and a panel of CHO glycosylation mutants by flow cytometry. Galectin-1 is a single CRD-bearing galectin that acts as a dimer (Bourne et al., 1994; Liao et al., 1994). Galectin-3, also known as carbohydrate-binding protein 35 (CBP35) and Mac-2, has a C-terminal CRD and an N-terminal collagen-like domain that contains repeats rich in glycine, proline, and tyrosine and is required for multimerization (Ahmad et al., 2004). Galectin-8, also known as prostate carcinoma tumor antigen 1 (PCTA-1) and Po66-CBP, is a member of the tandem-repeat (bi-CRD) galectin subfamily with two CRDs of different specificity separated by a linker within one polypeptide chain. In humans, six galectin-8 mRNA isoforms arising from alternate splicing produce protein variants with one or both CRDs, the latter with either a short or a long (32 aa longer) linker region (Bidon-Wagner and Le Pennec, 2004). Our binding studies show that complex N-glycans are the major ligands for each galectin. In their absence, Gal residues on other glycoconjugates including mucin O-glycans, GM3, or proteoglycans were not able to bind significant amounts of these galectins at the cell surface. However, the single N-terminal CRD of galectin-8 (galectin-8N) did not require complex N-glycans for binding.

**Results**

**Binding to parent CHO cells**

Recombinant rat galectin-1 (14.5 kDa), human galectin-3 (31 kDa), and human galectin-8 isoforms were produced in *Escherichia coli* and purified by affinity chromatography using a lactosyl-sepharose column. Each galectin was conjugated to fluorescein succinimide in the presence of lactose and stored at 4°C in phosphate-buffered saline (PBS) with sodium azide and 1 mM \( \beta \)-mercaptoethanol. Coomassie blue staining of the preparations electrophoresed on a denaturing polyacrylamide gel showed them to be \( \sim 90\% \) pure (Figure 1A). Four different forms of galectin-8 were produced as thioredoxin fusion proteins—8S or isoform b (53 kDa), 8L or isoform a with a longer inter-CRD linker region (58 kDa), and 8C (35 kDa) and 8N (33 kDa), isoforms containing, only the N- or C-terminal CRD, respectively (Figure 1B).

The binding of galectins to the surface of parent CHO cells was determined at 4°C by flow cytometry as described in Materials and methods. CHO cells secrete galectin-1 and bind it to the cell surface (Cho and Cummings, 1995). Cells were, therefore, washed with 0.5 M lactose to remove bound endogenous galectins before preincubation in 2% bovine serum albumin (BSA) in Hank’s balanced salt solution (HBSS) at 4°C to reduce nonspecific binding. Cells were subsequently incubated with galectin (20 \( \mu g \) per 5 \( \times 10^6 \) cells per mL, i.e., 0.3–1.5 \( \mu M \)) for an hour at 4°C in HBSS containing 1 mM \( \beta \)-mercaptoethanol. After washing at 4°C, bound galectin was measured as fluorescein fluorescence in a flow cytometer. Damaged cells with a permeable membrane were detected by propidium iodide (PI) uptake and were excluded from the analysis.

All six fluoresceinated galectin preparations bound to parent CHO cells but to widely varying degrees (Figure 1C). Mean of fluorescence intensities (MFI) was reduced by \( \sim 50\% \) when galectin-1 and -3 concentrations were reduced by half to 10 \( \mu g \) per 5 \( \times 10^6 \) cells per mL, and cell agglutination was observed when these galectins were used at a 2- or 4-fold higher concentration (data not shown). An \( \sim 30-40\% \) reduction in galectin-1 and -3 binding was observed for cells grown in monolayer instead of suspension culture (data not shown). The highest MFI was detected with galectin-8S and the lowest with galectin-8C. In some experiments, galectin-3 exhibited greater MFI values, the maximum being 1312. The differences in MFI mainly reflect differences in the amount of galectin bound, because the efficiency of fluoresceination was similar (within a factor of two) in all preparations. The specificity of binding was determined by incubation in the presence of 0.2 M lactose (Figure 1C, dark grey profiles). The presence of lactose reduced MFI values to about 10 for all the galectins, most likely representing nonspecific binding by other fluoresceinated proteins. The specific binding above the background that was lactose inhabitable was high for galectin-1, -3, -8S, and -8L, but moderate for galectin-8N and low for galectin-8C which was not analyzed further.
Galectin binding to CHO glycosylation mutants

Fig. 1. (A) Galectin preparations. Purified recombinant galectins (2 μg each) were electrophoresed on a reducing and denaturing polyacrylamide gel and stained with Coomassie blue. (B) Galectin-8 forms used in this study. The domain organization of the various forms of galectin-8 used in this study is illustrated. Gal-1, rat galectin-1; Gal-3, human galectin-3; Gal-8S, human galectin-8, short linker form; Gal-8L, human galectin-8, long linker form; Gal-8C, C-terminal lectin domain of human galectin-8; galectin-8N, N-terminal lectin domain of human galectin-8. (C) Binding of galectin-1, -3, and -8 to CHO cells. Binding of recombinant rat or human galectins to parent CHO cells was assessed by fluorescence cytometry, as described in Materials and methods. Galectin-binding profiles are shaded black. Binding in the presence of 0.2 M lactose is shaded in dark grey, and light grey profiles indicate background fluorescence in the absence of galectins.
CHO cells synthesize a range of complex and oligomannosyl N-glycans with few hybrid structures (Lee et al., 2001), mucin O-glycans that are maximally a tetrasaccharide (Sasaki et al., 1987), and O-fucose (Moloney et al., 2000), O-glucose (Moloney et al., 2000), and O-mannose (Patnaik and Stanley, 2005) glycans, as well as polysialic acid on a minor subset of glycoproteins (Muhlenhoff et al., 1996; Hong et al., 2004). The major glycolipid in CHO cells is GM_3 (Stanley et al., 1980; Warnock et al., 1993). CHO cells also contain heparan sulfate and chondroitin-sulfate complexes and hybrid mutant (Chen and Stanley, 2003), and Lec1 cells lack both galactosylation, sialylation, or the synthesis of complex N-glycans. Binding to CHO glycosylation mutants defective in glycosyltransferases that transfer colipids or sulfated sulfotransferase activities required to generate sulfated glycosyltransferases (Campbell and Stanley, 1984). CHO cells also lack α1,2-, α1,3-, or α1,4-linked fucose (Howard et al., 1987), β1,6-linked N-acetylgalcosamine (GlcNAc) to form core 2 O-glycans (Sasaki et al., 1987; Bierhuizen and Fukuda, 1992), sialic acid α2,6-linked to Gal (Sasaki et al., 1987), or the bisecting GlcNAc (Campbell and Stanley, 1984). CHO cells also lack sulfotransferase activities required to generate sulfated glycolipids or sulfated N- or O-glycans (Brockhausen et al., 2001). However, gain-of-function CHO mutants in which some of these structures are expressed are available (Stanley et al., 1996) and were used in this study. In addition, a panel of well-characterized, loss-of-function CHO glycosylation mutants were compared for binding of the three galectins. Table I summarizes the effect of each glycosylation mutation on the repertoire of glycan structures expressed by the CHO mutants examined here.

The binding of each galectin to CHO cells and Lec8, Lec2, Lec20, and Lec1 CHO mutants is compared in Figure 2. The Lec8 loss-of-function mutant has a deletion mutation in the Golgi UDP-Gal transporter (Oelmann et al., 2001) and has few galactosylated glycoconjugates (Stanley et al., 1980; Kawar et al., 2005). The Lec2 mutant has a CMP-sialic acid transporter mutation (Eckhardt et al., 1998) and has almost no sialylated glycoconjugates (Stanley et al., 1980). The Lec20 mutant has no β1,4-galactosyltransferase I activity and has significantly reduced amounts of Gal on complex N-glycans, although some fully galactosylated N-glycans are made owing to the presence of other β1,4-galactosyltransferases (Lee et al., 2001). The Mgst1 gene that encodes β1,2-N-acetylglucosaminyltransferase I (GlcNAc-TI) is mutated in the Lec1 loss-of-function CHO mutant (Chen and Stanley, 2003), and Lec1 cells lack both complex and hybrid N-glycans (Robertson et al., 1978; Chaney and Stanley, 1986). Representative N- and mucin O-glycans synthesized by these mutant cells are illustrated at the top of Figure 2. Glycolipids (Stanley et al., 1980), O-fucose glycans (Moloney et al., 2000), O-mannose glycans (Patnaik and Stanley, 2005), and proteoglycans (Esko et al., 1985) are also affected by the glycosylation defect in Lec2 and/or Lec8 cells, respectively.

Binding of the different galectins to each glycosylation mutant was examined at least twice with different galectin preparations. Duplicate samples examined on the same day with the same preparation gave indistinguishable profiles. Flow-cytometry profiles were unimodal like those for parent CHO cells in Figure 1. Hence, the geometric MFI (GMFI) was used to compare galectin binding and is represented by histograms. Figure 2A shows GMFI values for the binding of galectin-1, -3, -8S, and -8L to CHO cells and the glycosylation mutants Lec8, Lec20, Lec1, and Lec2. Specific binding of galectin-8C was not significantly above background to these or other glycosylation mutants and is not shown. Figure 2B compares the binding of each galectin to mutant CHO lines relative to parent CHO (100%) and gives the range of GMFI values obtained after the subtraction of background GMFI for CHO in lactose tested in each experiment with the different galectin preparations.

The least binding was observed to Lec8 cells for all galectins (Figure 2). This is consistent with the fact that Lec8 cells have little or no Gal on all cell surface glycoconjugates. The results show that binding to CHO cells is mediated entirely through the CRD of each galectin and that background binding owing to contaminating proteins (Figure 1A) is negligible. The binding of galectin-8L and -8S, therefore, is because of the combined actions of their two component CRDs, resulting in a stronger binding than for either CRD alone (galectin-8N and -8C). In contrast to Lec8, Lec1 cells lack Gal residues solely on their N-glycans. Nevertheless, despite the presence of Gal in glycolipids, in all O-glycans and in proteoglycans of Lec1 cells, the binding of galectin-1, -3, -8S, and -8L were at background levels (Figure 2). The mono-CRD galectin-8N was an interesting exception. Although GMFI values with galectin-8N were much lower

### Table I. CHO glycosylation mutants used in this study

<table>
<thead>
<tr>
<th>CHO mutant</th>
<th>Biochemical phenotype</th>
<th>Glycans altered</th>
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<tbody>
<tr>
<td>Lec1</td>
<td>Loss of GlcNAc-TI</td>
<td>No complex or hybrid N-glycans</td>
</tr>
<tr>
<td>Lec2</td>
<td>Loss of CMP-SA</td>
<td>Few sialylated glycoconjugates</td>
</tr>
<tr>
<td>Lec4</td>
<td>Loss of GlcNAc-TV</td>
<td>No β1,6-linked arm on complex N-glycans</td>
</tr>
<tr>
<td>Lec8</td>
<td>Loss of UDP-Gal</td>
<td>Few galactosylated glycoconjugates</td>
</tr>
<tr>
<td>Lec10</td>
<td>Gain of GlcNAc-TIII</td>
<td>Bisecting GlcNAc on complex N-glycans</td>
</tr>
<tr>
<td>Lec12</td>
<td>Gain of Fuc-TIX</td>
<td>α1,3-fucosylated Le^a_ and VIM-2 structures on N-glycans</td>
</tr>
<tr>
<td>Lec13</td>
<td>Loss of GDP-mannose-4,6-dehydratase activity</td>
<td>Few fucosylated glycoconjugates</td>
</tr>
<tr>
<td>Lec14</td>
<td>Gain of GlcNAc-TVII</td>
<td>β1,2-linked GlcNAc on core N-glycans</td>
</tr>
<tr>
<td>Lec18</td>
<td>Gain of GlcNAc-TVIII</td>
<td>α1,6-linked GlcNAc on core GlcNAc of N-glycans</td>
</tr>
<tr>
<td>Lec20</td>
<td>Loss of Gal-TI</td>
<td>Reduced β1,4-linked galactose on N-glycans</td>
</tr>
<tr>
<td>Lec29</td>
<td>Gain of Fuc-TIX</td>
<td>Low level of α1,3-fucosylated Le^a_ structures on N-glycans</td>
</tr>
<tr>
<td>CHO/Fut7</td>
<td>Gain of Fuc-TVII</td>
<td>α2,3-fucosylated and α1,3-, fucosylated sialyl-Le^a_ structures on N-glycans</td>
</tr>
</tbody>
</table>

References are given in the text.

*α*-fucose and *O*-mannose glycans, that are present in an extremely low abundance, may acquire Le^a_ or sialyl-Le^a_ determinants.
Lute GMFI values obtained in a typical, internally controlled experiment represent monosaccharides (GalNAc, to Lec20 cells are from one experiment. Different galectin preparations are shown. The data for galectin-3 binding mean and range of values from two independent experiments that used subtracting background fluorescence in the presence of 0.2 M lactose.

For each mutant line as a percentage of GMFI for parent CHO after galactosylation, sialylation, or the synthesis of complex and hybrid glycans. Predicted structures of complex N- and mucin O-glycans synthesized in the different cell lines are shown at the top along with symbols used to represent monosaccharides (GalNAc, N-acetylgalactosamine) (A) Absolute GMFI values obtained in a typical, internally controlled experiment are plotted in a histogram. (B) Binding of recombinant rat or human galectins to cells determined by fluorescence cytometry is shown as GMFI for each mutant line as a percentage of GMFI for parent CHO after subtracting background fluorescence in the presence of 0.2 M lactose. Mean and range of values from two independent experiments that used different galectin preparations are shown. The data for galectin-3 binding to Lec20 cells are from one experiment.

Fig. 2. Binding of galectin-1, -3, and -8 to CHO mutants defective in galactosylation, sialylation, or the synthesis of complex and hybrid N-glycans. Predicted structures of complex N- and mucin O-glycans synthesized in the different cell lines are shown at the top along with symbols used to represent monosaccharides (GalNAc, N-acetylgalactosamine) (A) Absolute GMFI values obtained in a typical, internally controlled experiment are plotted in a histogram. (B) Binding of recombinant rat or human galectins to cells determined by fluorescence cytometry is shown as GMFI for each mutant line as a percentage of GMFI for parent CHO after subtracting background fluorescence in the presence of 0.2 M lactose. Mean and range of values from two independent experiments that used different galectin preparations are shown. The data for galectin-3 binding to Lec20 cells are from one experiment.

For parental CHO and all mutants compared with the other galectins (Figures 1C and 2A), and therefore small differences may appear magnified, the data suggest that galectin-8N binds well to glycans that are not N-linked. Thus, galectin-8N bound well to Lec1 cells that have wild-type levels of GM3 and sialylated O-glycans. The bi-CRD galectin-8 preparations behaved similarly to galectin-8N in one experiment in which they had an ∼3-fold higher GMFI with Lec1 than with Lec8. In addition, galectin-8N appears to require sialic acid for binding to CHO. Thus, galectin-8N exhibited no binding above background to Lec2 cells which have little sialic acid on all glycoconjugates. Consistent with these data, Ideo et al. (2003) found that galectin-8N binds preferentially to GM3. Interestingly, galectin-8C had a GMFI ∼2-3-fold better with Lec2 cells than with parent CHO cells plus lactose (data not shown). Therefore, bi-CRD galectin-8 binding reflects a combination of the different 8N- and 8C-binding specificities.

Lec20 mutant cells have a comparatively small reduction in Gal that affects only N-glycans. Compared with CHO, binding to Lec20 cells was significantly decreased for galectin-1, but only moderately reduced for galectin-3, -8L, and -8S (Figure 2). The comparatively greater effect on galectin-1 binding may reflect a different requirement for spacing of terminal LacNAc residues for cross-linking (Leppanen et al., 2005). However, binding to Lec20 cells was not reduced for galectin-8N (Figure 2), consistent with the fact that levels of GM3 are not affected by the Lec20 mutation (Lee et al., 2001).

Both galectin-1 and -3 showed increased binding to Lec2 cells (Figure 2), presumably reflecting the increase in terminal Gal residues in Lec2 glycoconjugates. However, the increase was not very marked, indicating that galectin-1 and -3 bind to α2,3-sialylated and nonsialylated Gal termini with similar affinity, as observed in other studies (Lefler and Barondes, 1986; Brewer, 2004; Leppanen et al., 2005). Binding of galectin-8N to Lec2 cells was reduced almost to background (Figure 2), whereas the binding of galectin-8L and -8S to Lec2 cells was only slightly decreased compared with parental CHO cells. This is surprising considering that the bi-CRD species should have lost most of the binding of the N-terminal CRD (galectin-8N) that did not bind to Lec2 cells, and the other CRD (galectin-8C) did not bind well to begin with. Possibly residual binding of galectin-8N, along with enhanced binding of galectin-8C (which did bind better to Lec2 than to CHO cells; data not shown) and/or more opportunities for bivalent interactions on Lec2 cell surface glycoconjugates can account for the observed binding of galectin-8N and -8L.

Binding to CHO glycosylation mutants with altered fucosylation

A stable transfec tant CHO line expressing α1,3-fucosyltransferase VII (CHO/Fut7) and three CHO glycosylation mutants were used to study the effects of altered fucosylation on the binding of the galectins. Lec13 cells are defective in GDP-mannose-4,6-dehydratase, an enzyme required to generate GDP-fucose from GDP-mannose, and have very small amounts of fucosylated glycoconjugates (Ripka et al., 1986; Ohyama et al., 1998; Sullivan et al., 1998; Chen et al., 2001). The gain-of-function mutants LEC12 and LEC29 express α1,3-fucosyltransferase IX which is not expressed by parent CHO cells (Patnaik et al., 2000). However, LEC12 cells have higher levels of the enzyme and express more glycans terminating in Lewisα [Leα; Galβ1,4-(Fucα1,3)-GlcNAc] than LEC29 cells. LEC12 cells also express internally fucosylated, poly-LacNAc structures such as VIM-2 [Sα2,3-Galβ1,4-GlcNAcβ1,3-Galβ1,4-(Fucα1,3)-GlcNAc] that are essentially absent from LEC29 cells (Potvin and Stanley, 1991; Patnaik et al., 2004). CHO/Fut7 cells stably express the Chinese hamster Fut7 gene in CHO cells and generate termini with sialyl-Leα [Sα2,3-Galβ1,4-(Fucα1,3)-GlcNAc], but do not express VIM-2 or nonsialylated, α1,3-fucosylated glycans such as Leα (data not shown). Representative complex N- and mucin O-glycans of CHO cells and the fucosylation mutants are illustrated at
the top of Figure 3. Figure 3A shows a comparison of GMFI for binding of the five galectin species to CHO cells and the fucosylation mutants. Figure 3B shows the comparative GMFI normalized to CHO (100%) and the range of values obtained with the different galectin preparations.

LEC13 cells that essentially lack fucose in the core of complex N- and mucin O-glycans, and O-fucose glycans showed no significant difference in the binding of any galectin compared with CHO cells (Figure 3). Thus the absence of core α1,6-fucosylation of N-glycans has no apparent effect on the binding of these galectins. By contrast, all five galectins bound markedly less to LEC12 cells compared with CHO cells (Figure 3). This is consistent with the fact that LEC29 cells express much less α1,3-fucosyltransferase IX activity than LEC12 cells and have fewer α1,3-fucosylated N-glycans. Interestingly, galectin-8N bound to LEC29 cells slightly better than to CHO cells, again consistent with significant binding of galectin-8N, in contrast to bi-CRD galectin-8 forms, to α2,3-sialylated glycans unaffected by the LEC29 mutation such as GM3. In the CHO transfectant expressing sialylated-LeX termini on N-glycans (CHO/Fut7), binding of each galectin was reduced compared with CHO cells. However, the greatest reduction was only ~50% for galectin-1, and each galectin bound significantly more to CHO/Fut7 cells than to LEC12 cells that express non-fucosylated LeX termini. Therefore, for each galectin, binding was reduced if fucose was present on the GlcNAc near a terminal Gal or α2,3-sialylated Gal. This could be because of an increased proportion of fucosylated termini in LEC12 compared to CHO/Fut7 cells, or to the distribution of unfucosylated termini in the two lines, or to an enhancement of binding by the presence of the sialic acid in sialylated-LeX termini.

**Binding to CHO glycosylation mutants with alterations in N-glycan branching**

The Lec4 loss-of-function CHO glycosylation mutant lacks β1,6-N-acetylglucosaminyltransferase V (GlcNAc-TV) activity because of a deletion in the Mgat5 gene, and N-glycans, consequently, do not possess an upper β1,6 branch in Lec4 cells (Stanley et al., 1984; Chaney et al., 1989; Weinstein et al., 1996). The LEC10 gain-of-function glycosylation mutant expresses β1,4-N-acetylglucosaminyltransferase III (GlcNAc-III) because of an activated Mgat3 gene which is silent in parent CHO cells (Campbell and Stanley, 1984; Stanley et al., 2005). The LEC14 and 18 gain-of-function glycosylation mutants express N-glycans that have a GlcNAc attached to the β-linked core mannos at C2 in the case of LEC14 (Raju and Stanley, 1996) or to the core GlcNAc at C6 in the case of LEC18 (Raju et al., 1995). It is known that the LEC14 core structure can be galactosylated in vitro (Prahl and Unverzagt, 2002), and thus it is possible that the LEC14 and/or LEC18 GlcNAc branch is extended with Gal in vivo. Gal is not added to the bisecting GlcNAc in CHO cells (Stanley et al., 2005). Representative N- and mucin-type O-glycans of CHO mutants with altered GlcNAc branching are illustrated at the top of Figure 4.

LEC4 cells had slightly decreased binding of galectin-1, -3, and -8S compared with parent CHO, but similar binding to galectin-8L and increased binding to galectin-8N, respectively (Figure 4). This mutant lacks the upper (β1,6-GlcNAc) branch, where poly-LacNAc units are thought to be expressed preferentially (Li et al., 1980), which apparently had only a slight effect on galectin-1 binding, consistent with recent results showing its preference for terminal but not repeating LacNAc residues (Leppanen et al., 2005). It is interesting that galectin-8N binding is significantly increased by the absence of this branch.

Binding of all five galectins was somewhat reduced by the presence of the bisecting GlcNAc in LEC10 cells. The bisecting GlcNAc is known to change the recognition of Gal-binding plant lectins such as ricin and the erythroagglutinin.
Galectin binding to CHO glycosylation mutants

from Phaseolus vulgaris (E-PHA). It increases the binding of E-PHA but decreases binding of ricin to Gal-containing N-glycans (Green and Baenziger, 1987). In LEC10 CHO cells, most complex N-glycans carry the bisecting GlcNAc (Stanley et al., 2005). LEC10 glycoproteins bind little ricin and exhibit enhanced binding of E-PHA (Bhattacharyya et al., 2002). The five galectin preparations behaved similarly to ricin, except their binding was only moderately reduced (by <40%) compared with the large reduction in binding that the bisecting GlcNAc causes for ricin.

The only galectin binding to be affected by the presence of the LEC14 core was galectin-1, which was moderately decreased (Figure 4). Galectin-1 binding to the LEC18 core was also reduced. By contrast, the binding of galectin-8S and -8L was significantly increased.

PI uptake is induced by galectin-1 binding at 4 °C

In all experiments shown above, fluorescence arising from bound galectins was determined after the exclusion of damaged cells identified by their permeability to PI at the time of flow cytometry. Generally only –10–15% of cells took up PI after incubation with galectin-3 or any of the four forms of galectin-8 (Figure 5B). However, a wide variation was observed in the proportion of cells that took up PI in the case of galectin-1. CHO parent cells and the glycosylation mutants that bound most galectin-1 had a markedly higher proportion of PI-positive cells compared with cells that bound little galectin-1 (Figure 5A). Simple linear regression analysis showed a significant correlation between the degree of binding and PI uptake ($r^2 = 0.71$). Binding of galectin-1 and PI in the presence of 0.2 M lactose is shown for CHO by the rectangle. (B) Absence of correlation between galectin-3 or -8 binding and PI uptake. Data from one of two independent experiments for each of galectin-3, -8S, and -8L are plotted as a scatter plot.
Discussion

These studies define some important basic parameters of the binding of galectins to a cell surface. The lack of binding of all three galectins to the Lec8 CHO mutant shows their absolute requirement for Gal residues (Figure 2). This is important, as it rules out the presence of non-Gal-containing ligands for these galectins at the cell surface, as predicted for intracellular galectins (Yang et al., 1996; Wang et al., 2004; Liu and Rabinovich, 2005) and also suggested for extracellular galectin ligands (Ochieng et al., 2004). The lack of galectin-1 and -3 binding to the Lec1 mutant shows that they bind essentially exclusively to complex N-glycans (Figure 2). This is consistent with their known specificity combined with the known major carbohydrate structures of CHO cells. The major glycoproteins of CHO are built on the lactosyl-ceramide core, which is not expected to bind galectin-1 or -3 (Solomon et al., 1991; Feizi et al., 1994). The major O-glycans are built on core 1 (Galβ1-3GalNAc), which is a poor ligand for galectin-1 and -3 (Leffler and Barondes, 1986; Sparrow et al., 1987). Moreover, recent mass spectrometric analyses suggest that mucin type O-glycans are much less abundant than N-glycans on CHO cell glycoproteins (Haslam et al., manuscript in preparation). This makes the LacNAc sites present on N-glycans the major galectin-1, -3, and -8 binding sites on CHO cells. Other glycans on CHO cells may also interact with the galectins. O-linked fucose, glucose, or mannose glycans and the xylose core of proteoglycans may all have extensions containing Gal residues that could potentially bind galectins. However, binding to them in the Lec1 mutant may not have been detected, because they are not accessible (e.g., the core of proteoglycans) and/or much less abundant.

The remaining CHO mutants examined here have more subtle glycosylation changes that could influence galectin binding either by changing a single galectin binding site (e.g., by sialylation or fucosylation), or by changing the spacing of multiple binding sites (e.g., by modification of the N-glycan core or branching structures) and the possibility for multivalent interaction. The mutants that report on how modifications of N-glycan branching or core structures, in which the spatial arrangement of LacNAc-containing antennae may affect binding (Rice et al., 1993; Stubbs et al., 1996), are Lec4, Lec10, Lec13, LEC14, and LEC18. Effects on galectin binding will depend on how well the spacing of antennae or branching of the N-glycan matches the spacing of carbohydrate-recognition sites in the di- or oligomeric galectins. LEC10, LEC14, and LEC18 show a partial, but significant, decrease (40%) in galectin-1 binding, whereas binding of galectin-3 to these mutants was reduced less (Figure 4). If the extra core GlcNAc in those mutants were extended with a Gal, as has been shown in vitro for the LEC14 N-glycan core (Prahl and Unverzagrt, 2002; Andre S., Kojima S., et al., 2005), a new galectin-binding site might be created, but there was no increased binding of galectin-1 or -3 to these cells. The stronger effect of core modifications on galectin-1 may be because the carbohydrate-binding sites in the two CRDs in each dimer have a precise relative orientation and distance from each other (Loris, 2002; Leffler et al., 2004). In contrast, galectin-3 forms oligomers through its flexible N-terminal domain upon ligand encounter (Ahmad et al., 2004), and the spacing of carbohydrate-recognition sites may adapt more easily to changed spacing of carbohydrate antennae. In solid phase assays using N-glycans having different arrangements of their antennae, the affinities of galectin-1 and -3 have been found to vary, but by a factor of less than three as long as at least two antennae are available for binding (Hirabayashi et al., 2002; Unverzagrt et al., 2002; Andre S., Kaltner H., et al., 2005). The effect of adding a bisecting GlcNAc to N-glycans in LEC10 mutants is a rather mild reduction for galectin binding compared with the marked inhibition previously observed for ricin binding or the increased binding of E-PHA (Bhattacharyya et al., 2002). In Lec4, the β1,6-GlcNAc antenna is lost, which is proposed as the main carrier of poly-LacNAc chains with repeating LacNAc units in CHO cells (Li et al., 1980). These are not better ligands compared with LacNAc alone for galectin-1, which binds only terminal (or sialylated) LacNAc in subsites C–D because it cannot accommodate LacNAc extensions into subsites A–B (Stowell et al., 2004; Leppanen et al., 2005). By contrast, repeating LacNAc is a better ligand than terminal LacNAc for galectin-3 (Leffler and Barondes, 1986; Hirabayashi et al., 2002). The very slight decrease in binding of galectin-1 and -3 to Lec4 cells (Figure 4) indicates that the β1,6GlcNAc branch is not a major binding site for either galectin in CHO cells.

Terminal LacNAc residues may be α2,3-sialylated in CHO cells, and both galectin-1 and -3 are expected to tolerate this modification (in subsite B) making their affinity for NeuAcα2,3LacNAc similar to (Sparrow et al., 1987; Leppanen et al., 2005) or slightly worse for rat galectin-1 used here (Leffler and Barondes, 1986), compared with LacNAc. The very slightly increased binding of galectin-1 and -3 to the Lec2 mutant that has little sialic acid on cell surface glycans is consistent with this (Figure 2). The larger increase of galectin binding after neuraminidase treatment of other cell types (Dias-Baruffi et al., 2003) may be because of removal of α2,6-linked sialic acid which is not tolerated in subsite C by any galectin studied so far (Leffler et al., 2004) or to the fact that more N-glycans carry sialic acid in that cell type. The most abundant complex N-glycans in CHO cells are nonsialylated, and none of the N-glycans is completely sialylated (Lee et al., 2001).

For subsite D, fucosylation on a glucose or GlcNAc also blocks binding of all galectins studied to date (Leffler and Barondes, 1986; Leffler et al., 2004). This is presumably because, like the α2,6-linked sialic acid on Gal, a fucose in this position would be on the side of the saccharide directly interacting with the galectin and would sterically hinder that interaction (Loris, 2002; Leffler et al., 2004). A fucosylated GlcNAc in site B may be tolerated however, if sites C–D bind an internal, unfucosylated LacNAc residue. Consistent with this specificity, the LEC12 mutant with the highest fucosylation of both terminal and internal LacNAc residues by α1,3-fucosyltransferase IX (Potvin and Stanley, 1991; Patnaik et al., 2000, 2004) bound much less of both galectin-1 and -3 than CHO cells (Figure 3). The LEC29 and CHO/Fut7 cells with less fucosylation of GlcNAc and restricted to terminal sialylated LacNAc residues had a partial decrease in galectin-1 and -3 binding, somewhat more
pronounced for the former (Figure 3). The residual binding may be because of remaining unfucosylated terminal Lac-NAc and α2,3-sialylated LacNAc residues and also to internal LacNAc residues for galectin-3.

Binding of galectin-8 to ligands is the result of the combined action of its two CRDs (galectin-8N and -8C). In contrast to galectin-1 and -3, galectin-8 isoforms do not prefer LacNAc over lactose in their core-binding site, and galectin-8N actually prefers lactose. The N-terminal CRD of galectin-8N does not require complex N-glycans for binding to CHO cells because it bound the Lec1 mutant as well as parent CHO cells (Figure 2), and, unlike the other galectins, also bound well to the Lec23 loss-of-function glycosylation mutant that expresses predominantly oligomannosyl N-glycans because of a defect in α-glucosidase I (Hong et al., 2004; data not shown). This is consistent with its strong preference for 3′-sulfated or α2,3-sialylated Gal, as in GM3 ganglioside (Ideo et al., 2003) found in CHO cells. However, galectin-8N bound at much lower levels to CHO cells than galectin-1 and -3 at equivalent concentrations. This is perhaps because it binds monovalently at the cell surface, whereas the other galectins act bi- or oligovalently. Bi-CRD galectin-8 isoforms bound at much higher levels to CHO cells than galectin-8N and they required complex N-glycans (Figure 2). This indicates that galectin-8C may bind complex N-glycans on CHO cells. Even if the binding of galectin-8C alone was too weak to give reliable results in this study, it may easily be enough to “help” the N-terminal CRD in bivalent cooperation. A requirement for complex N-glycans in the binding of galectin-8C to CHO cells would be consistent with previous studies showing its lack of binding to α2,3-sialylated Gal residues, and its reasonable, though not strong, binding to LacNAc (Ideo et al., 2003). The combined data suggest the interesting possibility that galectin-8 may form a cross-link between a glycolipid (through the N-terminal CRD) and an N-glycan (through the C-terminal CRD), and thereby, like the bi-CRD galectin-4, be involved in lipid rafts (Danielsen and Hansen, 2003; Delacour et al., 2005).

Given the strong preference of galectin-8N for α2,3-sialic acid (Ideo et al., 2003), it is not surprising that it did not bind to the Lec2 mutant that mostly lacks cell surface sialic acid. However, it is surprising that the binding of bi-CRD galectin-8S and -8L was almost the same (≥75%) to Lec2 as to CHO cells (Figure 2). If all binding of N-terminal CRD of galectin-8 had been lost, Lec2 should have bound through the C-terminal CRD only. In fact, galectin-8C had increased binding to the Lec2 mutant (data not shown), but at a very low level compared with that of intact galectin-8S. Thus, binding of the N-terminal CRD of galectin-8 to non-sialylated structures must also contribute to the overall binding of bi-CRD galectin-8 to Lec2. The partial decrease in binding of the galectin-8 N-terminal CRD to Lec2 cells may be compensated by an increased binding of the C-terminal CRD. This may reflect a shift from glycolipid-glyco-protein cross-linking by galectin-8 in binding to Lec1 cells, to glycoprotein-glycoprotein cross-linking in binding to Lec2, which opens interesting possibilities for the regulatory role of sialylation.

The binding studies described here were performed at 4°C. Considering that incubation with galectins was for only an hour, it was intriguing to observe the induction of PI staining of cells specifically by galectin-1 (Figure 5). PI staining induced by galectin-1 was dose dependent, correlated with the amount of galectin-1 bound, and was inhibited by lactose. Like annexin V staining (Koopman et al., 1994), PI staining is routinely used to identify cell death, including that mediated by galectins (Vespa et al., 1996; Pace et al., 2000). The short duration of exposure to galectin-1, early onset of PI staining, occurrence at 4°C, and the reduction in PI staining when CHO cells were incubated with galectin-1 at 37°C (and bound ∼1.5- to 2-fold more galectin-1; data not shown) suggest that the increase in PI staining is not a prelude to apoptosis. It seems likely that carbohydrate-dependent galectin-1 binding at 4°C perturbs the cell membrane resulting in increased permeability to PI. Such perturbations leading to phosphatidylinerine exposure and binding by annexin V have been shown in HL-60 cells and in activated human neutrophils treated with galectin-1 for an hour (Dias-Baruffi et al., 2003). This effect of galectin-1 could also be blocked by lactose. Furthermore, cells were shown not to be undergoing cell death, and annexin V binding was reversible. These observations are important, as galectins are often studied in the context of cell death, and PI staining is often interpreted as an indicator of apoptosis. The finding that galectin-1, but not galectin-3 or -8 that bind well to CHO cells, induces PI uptake at 4°C is intriguing for membrane biology. It may also be of practical use as a way in which to rapidly load cells with desired compounds.

In considering the combined results of galectin binding to CHO cells with respect to other cell types and to the biological functions of galectins, two factors should be remembered. First, the binding shown here is the average for a population of cells. Alterations in glycosylation may be much more pronounced for a single glycoprotein or group of glycoproteins. Thus, even a small change in total binding (e.g., as for both galectins-8 to Lec2 cells) might reflect a change in important functional ligands. Second, additional galectin ligands may be present in other cell types. For example, the expression of core-2 GlcNAc-T in CHO cells would be expected to generate galectin binding sites on O-glycans (Sparrow et al., 1987). Conversely, the expression of α2,6-sialyltransferase, which is also not expressed by CHO cells, would be expected to block large numbers of galectin binding sites on N-glycans. Therefore, changes of glycosylation that occur such as in the immune system may have dramatic effects on the expression of binding sites for galectins (Galvan et al., 2000; Amano et al., 2003), although this may not always be the case (Carlow et al., 2003). Finally, the different CHO glycosylation mutants should be very useful in future studies of galectin-specific effects on cell-signaling events (Partridge et al., 2004).

Materials and methods

Cell lines and cell culture

Parent Pro-5 CHO cells, the loss-of-function CHO mutants derived from Pro-5 CHO and named Lec1 (clone 3C; Stanley et al., 1975), Lec2 (clone 6A; Deutscher et al., 1984), Lec4 (clone 7B; Chaney et al., 1989; Weinstein et al., 1996), Lec8...
and binding of anti-sialyl-Le^a antibody by flow cytometry. were transferred to coupling buffer (100 mM KH₂PO₄, pH 8.2) in a total volume of 1 mL. A stock solution of succinimide-coupled fluorescein (20 mM) was made in dimethyl sulfoxide, added to the protein solution in a molar ratio of 10:1 (fluorescein : protein) and incubated for 1 h at 37°C. After incubation, nonreacted succinimide was quenched by buffer change to PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4). All cells were grown and maintained in alpha-modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) in suspension or on plates in 5% CO₂ at 37°C. 

Galecrtins

Recombinant human galectin-3 and rat galectin-1 were produced in E. coli and purified by affinity chromatography on lactosyl-sepharose as previously described in Massa et al. (1993) and Cooper et al. (1991), respectively. Human galectin-8 expression constructs were generated in the vector pET-32 Ek/LIC Vector (Novagen, Madison, WI) to produce N-terminal thioredoxin fusion proteins containing two forms of intact protein differing in length of linker between the CRDs: galectin-8L (359 aa) and 8S (317 aa), and each CRD, galectin-8N (aa 1–156) and -8C (aa 182–317) (Figure 1B). The proteins were expressed in E. coli host BL21 Star (DE3) (Invitrogen, San Diego, CA) and purified on lactosyl-sepharose in principle as described for galectins-1 and -3. Galectin-8C interacted only weakly with lactosyl-sepharose, was first purified using the histidine (His×6)-tag of the fusion construct, and then by lactosyl-sepharose where it did not bind but was retarded enough to be separated from contaminants. All the proteins had carbohydrate-binding activity in a fluorescence polarization assay (Sorme et al., 2004). The details will be given elsewhere (Carlsson et al., manuscript in preparation). For fluorescein labeling, 2 mg of each recombinant proteins were transferred to coupling buffer (100 mM KH₂PO₄, pH 8.2) in a total volume of 1 mL. A stock solution of succinimide-coupled fluorescein (20 mM) was made in dimethyl sulfoxide, added to the protein solution in a molar ratio of 10:1 (fluorescein : protein) and incubated for 1 h at 37°C. After incubation, nonreacted succinimide was quenched with 100 μL of 1 M Tris–HCl for 30 min at room temperature. Labeled protein was separated from free fluorescein by buffer change to PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4). All changes of buffer were performed using PD10 desalting columns (Amersham Biosciences, Piscataway, NJ), according to the manufacturer’s instructions and, if necessary, followed by a concentration step using Centriprep YM-10 (Millipore, Billerica, MA). Fluorescein per mg protein was equivalent as estimated by measurements on fluorometer (PolarStar BMG, Offenburg, Germany) of different concentrations of the fluorescein-labeled galectins. Fluoresceinated galectins were shipped (Lund, Sweden to New York), in PBS at 4°C. The purity of galectins was assessed as >90% by Coomassie blue staining of the preparations electrophoresed on a denaturing polyacrylamide gel. The galectins were used within 2–3 weeks of their preparation.

Flow cytometry

All cells were grown in suspension culture to a density of 5–8 x 10⁵ cells/mL. Harvested cells were centrifuged and resuspended in 10 mL of cold HBSS (0.4 g/L KCl, 0.06 g/L K₂HPO₄, 8 g/L NaCl, 0.048 g/L Na₂HPO₄, 1 g/L d-glucose, pH 7.4) with 0.5 M lactose (Sigma, St. Louis, MO) to remove bound endogenous galectins. After centrifugation, the cells were resuspended in 10 mL of cold HBSS with 2% BSA (Sigma) and incubated at 4°C for an hour with gentle agitation. Cells were then washed once with 1 mL cold HBSS/BSA and resuspended in HBSS/BSA with 2 mM β-mercaptoethanol at 1 x 10⁶ cells/mL. Fifty microliters of cold HBSS/BSA containing 2 μg galectin was added to 50 μL of the suspended cells (5 x 10⁶ cells). After 1 h with gentle agitation at 4°C, cells were washed twice with 1 mL HBSS and resuspended in 0.5 mL HBSS. All washes were performed by resuspending cells in cold HBSS followed by centrifugation at 500 x g. Fluorescence cytometry was performed on a FACSScan or FACSCalibur machine (Becton Dickinson, San Jose, CA), and data analyzed using Cell Quest software (Becton Dickinson). Fluorescence data were collected using logarithmic amplification on 10,000 light scatter-gated events (cell counts). PI (Sigma) was added at a final concentration of 1–2 μg/mL just before cytometry to identify permeable cells. Parent CHO cells were used as controls in every experiment to normalize for variations in instrument sensitivity and sample handling. Negative controls were either cells that were not incubated with galectin or cells that were incubated with both galectin and 0.2 M lactose to inhibit galectin binding. At least two binding experiments using different galectin preparations were performed for each cell line.

Data analysis

Cell Quest software (Becton Dickinson) was used to determine the GMFI of sample cell populations. Permeable cells as defined by high PI staining were excluded from analysis. Absolute GMFI were plotted in histograms or, for comparisons between experiments and cell lines, background fluorescence (GMFI values for CHO cells incubated with galectin in the presence of 0.2 M lactose) was subtracted from absolute GMFI values, means were determined as a percent of the lactose-corrected GMFI obtained for CHO cells, and plotted as a histogram and range of values. The correlation between PI staining and the degree of galectin binding was analyzed using the least-squares method for simple linear regression. Prism software (GraphPad Software, San Diego, CA) was used to analyze and plot the values.

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Abbreviations

BSA, bovine serum albumin; CHO, Chinese hamster ovary; CRD, carbohydrate-recognition domain; E-PHA, erythroagglutinin from Phaseolus vulgaris; Gal, galactose; GlcNAc, N-acetylgalcosamine; GMFI, geometric mean of fluorescence intensities; HBSS, Hank’s balanced salt solution; LacNAc, N-acetyllactosamine; Le\(^\alpha\), Lewis\(^\alpha\); PBS, phosphate-buffered saline; PI, propidium iodide.

References


