Specificity of Binding of Three Soluble Rat Lung Lectins to Substituted and Unsubstituted Mammalian \(\beta\)-Galactosides*

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The function of the glycoconjugate residues on and around cells is not known. It is generally assumed that their biological roles involve interaction with complementary proteins, but thus far there is little direct evidence for this. The case that has been studied in most detail involves interaction between desialylated serum glycoproteins and the asialoglycoprotein receptor (6). This receptor is an integral membrane glycoprotein which binds to terminal galactose or \(N\)-acyetylgalactosamine residues. It is believed to play a role in the endocytosis of those circulating serum glycoproteins whose terminal sialic acids have been removed, thereby exposing penultimate galactose residues. Based on studies of the binding of this receptor with a range of known glycoconjugate structures (7, 8) it has been established that it does not react well with internal galactose or \(N\)-acyetylgalactosamine residues so that it does not bind the common mammalian glycoproteins, except after partial degradation.

In the past decade another class of proteins has been identified which could prove to be biologically significant ligands for the \(\beta\)-galactoside-containing glycoconjugates. These proteins are soluble \(\beta\)-galactoside-binding lectins known to be widely distributed in vertebrates (9). The first member of this class was discovered in soluble extracts of the electric organ of an electric eel (10), and the most extensively studied thus far are two distinct \(\beta\)-galactoside-binding lectins found in a variety of developing and adult chicken tissues (11–13). Recently, several \(\beta\)-galactoside-binding mammalian lectins have been detected (14–19), and three have been purified from rat lung and other tissues (20). As previously found in other systems (9), all three rat lectins show significant changes in levels with tissue differentiation (20), indicating that they, like the glycoconjugates to which they might bind, are developmentally regulated.

The underlying assumption of our work with soluble lectins is that they function by interaction with specific complementary glycoconjugates. To fully understand this it will be necessary to identify the specific glycoproteins and/or glycolipids bound by a given lectin in a given tissue. To guide such work we set out to determine the relative binding of each of these lectins to a library of known mammalian glycoconjugate structures which they would be expected to encounter. We found that, in contrast with the asialoglycoprotein receptor, the three soluble rat lectins can all interact with internal \(\beta\)-galactoside residues. Furthermore, the library of known mammalian oligosaccharides that was available allowed us to identify some aspects of the specificities of the binding sites of each of the three lectins. Since each can interact with distinct

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1 The carbohydrate nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature. If not stated otherwise, it is assumed that Gal, Glc, GalNAc, GlcNAc and NeuAc are in the \(\alpha\)-configuration, Fuc is in the \(\beta\)-configuration, and all sugars are in the pyranose form. The abbreviations used for the methyl glycosides are exemplified as follows. \(\alpha\)-methylgalactoside, GalαOMe. Other abbreviations used are: ASF-Sepharose, asialofetuin covalently linked to Sepharose 4B; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RL-14.5, RL-18, and RL-29, rat lectins with respective subunit \(M\), values of 14,500, 18,000, and 29,000; LAG, lactosaminoglycan. Between different tissues, mammalian species, and individuals, and between different protein types. Many of these structures are also developmentally regulated. This has been demonstrated both by chemical analysis (1–4) and with monoclonal antibodies specific for glycoconjugate epitopes (5).
groups of glycoconjugates found in and around mammalian cells, it seems likely that such selective associations would have biologically significant results.

MATERIALS AND METHODS
Purification of Rat Lectins—The established procedure (20) for isolation of the three rat lectins from lung involved co-purification of all three by affinity chromatography on ASF-Sepharose followed by their separation by ion exchange chromatography. A major problem is that RL-29, although fairly abundant in initial extracts, is selectively absorbed by dialysis tubing and materials used to stabilize it. To permit recovery of sufficient lectin for binding studies, we developed a modified procedure for purification of RL-29. We found that RL-29 was selectively extracted from lung homogenates with a relatively low ionic strength buffer consisting of 10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, and 150 mM lactose. RL-14.5 and RL-18 remained particulate in this buffer, requiring a higher salt concentration for extraction. The RL-29 was, therefore, extracted in this way, dialyzed against the same buffer without lactose, then purified by affinity chromatography on ASF-Sepharose as described previously. This gave purified RL-29 without the need for resolution from RL-14.5 or RL-18 by ion exchange chromatography. Elimination of this additional step markedly increased the final yield of RL-29.

RL-14.5 and RL-18 were prepared in the usual way (20) by extraction of rat lung homogenate with 75 mM NaCl, 75 mM NaH2PO4/KH2PO4, 4 mM 2-mercaptoethanol, 2 mM PMSF, 150 mM lactose followed by affinity chromatography on ASF-Sepharose and ion exchange chromatography, except that the latter was done on DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) instead of by high performance liquid chromatography, since this gave better recoveries.

Preparation of 125I-Lectins—First, each purified lectin (about 150 μg in 5–10 ml) was alkylated (21) by adding iodoacetamide to a final concentration of 40 mM. After 4 h at 6 °C the reaction was stopped by adding 2-mercaptoethanol, followed by dialysis against 75 mM NaCl, 75 mM NaH2PO4/KH2PO4, pH 7.2 (PBS) for 24 h. After dialysis, the lectin solution was concentrated with a Centricon 10 (Amicon, Danvers, MA) to a volume of 2.5 ml with 0.1 M sodium borate buffer, pH 8.5, and reconcentrated to 2.5 ml with 0.1 M sodium borate buffer, pH 8.5, and reconcentrated to 50 μl. This gave a concentration of lectin of about 0.7 mg/ml. The low total recovery was due to losses during dialysis and concentration.

About 15 μl of the concentrated lectin containing 10 μg of protein was added to dry Bolton-Hunter reagent containing 0.5 μCi of 125I, 2,000 Ci/mmol (Amersham Corp.) in the vial supplied by the manufacturer. The iodination reaction was allowed to proceed at 4 °C for 10 h, after which the reaction was stopped by adjusting the pH to 8.5 with 0.2 M sodium hydroxide. The iodinated lectin was dialyzed against the same buffer without lactose, then concentrated by centrifugation at 10,000 rpm for 2 min. Each 0.1 ml of this additional step markedly increased the final yield of RL-29.

Binding Assay—To study the interaction of the rat lectins with a library of known saccharides we examined their ability to inhibit lectin binding to small volumes of ASF-Sepharose. The packed ASF-Sepharose was diluted with 30 volumes of PBS, and 5 μl of this suspension was mixed with 5 μl of PBS containing 0.2% sodium azide and the appropriate concentration of saccharide to be tested in a 0.5-ml microtube (WVR Scientific, San Francisco, CA). Then 5 μl of a solution of 99I-lectin in PBS containing 2% BSA was added. This lectin eliquot contained about 2500 cpm, and the 10 mM lactose in which it had been stored was diluted at least 200–1000-fold in the assay, which was well below the concentration at which it had a significant inhibitory effect. The reactants were mixed by pipetting up and down with a micropipette and kept covered at room temperature for 3 h.

To check the total radioactive lectin that had been added, each tube was examined in a gamma counter. Then 125 μl of oil (dibutyl phthalaldehyde-2-ethylhexyl) sebacate, 90:20, density 1.02, Eastman Kodak, Rochester, NY) was added and the tubes were spun in a microfuge-12 (Beckman) at 10,000 rpm for 2 min. The ASF-Sepharose beads formed a pellet below the oil and the soluble phase formed a droplet in the upper part of the oil. After the droplet and most of the oil were removed with a pipette, the tip of the tube containing the pellet was cut off and counted in a γ counter. Binding was recorded as percentage of total counts found in the pellet.

Source of Saccharides—The saccharides we studied are referred to by name or number in the text and were obtained from the following sources. Lactose (No. 1), thiodigalactoside (No. 11), β-methylgalactoside (No. 21), α-L-arabinose (No. 22), 4-sulfoglucuronic acid (No. 23), and 4-sulfo-L-iduronic acid (No. 24) were from Sigma Chemical Co. (St. Louis, MO). Lacto-N-tetraose (No. 25) was purchased from Sigma, Cellobose (No. 19), galactose (No. 20), α-methylgalactoside (No. 22), and lactulose (No. 10) were from Pfannstiel Waukegan, IL, USA. A-tetrasaccharide (No. 4), A-heptasaccharide (No. 27), A-heptasaccharide (No. 28), and GalNAc3-3GalNAc (No. 14) were from BioCarb AB (Lund, Sweden). GalNAc3-4GalNAc (Mo) was purchased from Sigma. Cellobose (No. 19), galactose (No. 20), B-glucosamine (No. 12), Gal3-3GalNAc (No. 13), GalNAc3-3GalNAc (No. 15), GlcNAc3-2GalNAc (No. 17), Gal3-3GalNAc (No. 16), and Gal1-4Gal (No. 18) were from Beckman Instruments (Rancho, CA). Sialyllectosamine (No. 19) isolated from human milk (25) was prepared by ozonolysis (27) of the major glycolipid purified from guinea pig erythrocytes (28) and globotetraose (No. 9) was prepared by ozonolysis of the major glycolipid purified from human kidney (29). The published procedure for ozonolysis (27) was followed, except that smaller amounts were used (2 mg of glycolipid in 1 ml of methanol and the released oligosaccharide was separated from salts by an ion retardation column (AG, 11A5, Bio-Rad). The glycoconjugates and oligosaccharides (Nos. 1–28) were released from fetuin (30–32) and orosomucoid (33, 34), respectively, by hydrazinolysis (35), and the major saccharide from each protein was purified by ion exchange chromatography (37). Nos. 29 and 31 were made by treating 30 and 31, respectively, with Pronase digestion of the band 3 glycoprotein from fresh erythrocytes (38) and adult human erythrocytes (39), respectively. No. 36 was the same as No. 35 except that the peptide part was removed by hydrazinolysis.

The purity and stability to storage in PBS of the small saccharides (Nos. 1–28) was verified by thin-layer chromatography, using HPTLC silica gel plates (Merrick, Darmstadt, Germany), chloroform:methanol:water:acetic acid, 50:50:5:5 by volume as solvent, and detection with anisaldehyde reagent (40).

RESULTS
Purification and Labeling of Three Rat β-Galactoside-binding Lectins—In previous work we described the co-purification of these three lectins by affinity chromatography on a column of ASF-Sepharose and their separation by ion exchange chromatography. A major limitation of that procedure...
was that it led to considerable losses of the lectins, especially RL-29. For this reason we devised an alternative technique for purification of RL-29 which gave a much higher yield. It is based on our finding that initial extraction of rat lung with a lactose containing buffer of low ionic strength selectively solubilized RL-29. The other two lectins, RL-14.5 and RL-18 could then be solubilized from the remaining particulate fraction by re-extraction with the lactose-containing buffer of high ionic strength that we had used in the initial purification procedure (20). Because RL-29 was selectively extracted we markedly increasing its recovery.

ASF-Sepharose, precluding the need for various handling steps before and after ion exchange chromatography, and markedly increasing its recovery.

The only contaminant we found in preparing RL-29 in this way was a relatively faint band on silver-stained gels with an apparent molecular weight of 16,000. When we concentrated the RL-29 preparation for iodination, we took selective losses of RL-29 so that this contaminating band became relatively more prominent in the final iodinated product, comprising almost 20% of the iodinated protein (Fig. 1C). It remained in this proportion relative to RL-29 in the iodinated protein bound to and eluted from ASF-Sepharose in the binding assay, as determined by electrophoresis, autoradiography, and g counting of the gel bands (data not shown). Since we studied the effects of saccharide inhibitors over a range of concentrations high enough to inhibit about 80-90% of total bound lectin, the contribution of the contaminant to the studies of binding of 125I-RL-29 was not sufficient to substantially alter the results. Whether the contaminant represents yet another soluble lectin, an active degradation product, or a protein that associates with and co-purifies with RL-29 was not considered further in the course of this study.

Derivatization and Storage of Iodinated Lectins—Previous studies indicated that maintenance of the carbohydrate binding activity of RL-14.5 required storage in a reducing agent such as β-mercaptoethanol but, upon alkylation of the lectin with iodoacetamide, it was stabilized and no longer dependent on reducing agents for retention of carbohydrate binding activity (21). Presumably either the reducing agent or alkylation prevented the formation of intramolecular or intermolecular disulfide bonds which interfered with carbohydrate binding activity (21). Whatever the mechanism of the alkylation, we took advantage of this finding by routinely alkylating RL-14.5 prior to iodination with the Bolton-Hunter reagent. We found that the lectin could then be stored frozen in aliquots for months without significant loss of carbohydrate binding activity. We also tried alkylating a mixture of the three lectins with iodoacetamide, and found that binding of each to ASF-Sepharose was not affected by this treatment. Furthermore, each remained active indefinitely upon freezing. For this reason we used alkylated derivatives of all three lectins for these studies.

The radioiodinated RL-14.5 and RL-18 that we prepared for binding studies were extremely pure (Fig. 1). RL-14.5 was contaminated with about 2% of another protein (Fig. 1A), whereas RL-18 showed no significant contamination (Fig. 1B). The significant contamination of RL-29 (Fig. 1C) with a band that migrated with an apparent molecular weight of 16,000 (between RL-14 and RL-18 when they were run in parallel) has already been discussed above. As already noted, the degree of contamination was not sufficient to interfere with the interpretation of the binding studies.

Estimation of Relative Potency of Saccharide Inhibitors—
To study lectin-saccharide interactions we tested the effects of a range of concentrations of each saccharide on binding of labeled lectins to ASF-Sepharose beads. The total reaction volume was kept low (15 μl) to help conserve some of the rare compounds that we tested. For each compound examined, inhibition curves were constructed, as shown for lactose in Fig. 2. Based on these curves, we estimated the concentration for each saccharide that inhibited binding of each lectin by 50%. For comparative purposes the inhibitory activities are calculated relative to either lactose (Tables I and III) or galactose (Table II) as explained below.

The concentration of lactose that inhibited binding of 50% of RL-14.5, RL-18 and RL-29 to ASF-Sepharose was quite similar (Fig. 2). However, from the study of a wide range of saccharides, we inferred that the binding site of each lectin is distinct. To facilitate discussion of these findings, schematic drawings of several saccharides are shown in Fig. 3, showing

![Fig. 1. Autoradiograph of polyacrylamide gel after electrophoresis of 125I-labeled rat lectins.](image)

![Fig. 2. Effect of a series of concentrations of lactose on binding of the three 125I-labeled rat lectins to ASF-Sepharose.](image)
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Fig. 3. Preferred conformation of some saccharides. A schematic picture of the preferred conformations is shown. Galactose has been taken as a "reference point" and is pictured with equal orientation in all the saccharides. The glycosidic bond torsion angles are similar in all the disaccharides but the sugars to the right have a different orientation, relative to Gal, depending on the binding position of the glycosidic bond. For Nos. 1 and 12-14 the picture is based on published nuclear magnetic resonance spectroscopy studies and hard sphere exoanomeric effect calculations (41-43). For No. 10 it is based on the published crystal structure (54) with Fru in the β-pyranose form, which is the most predominant in solution (55). For No. 11 the conformation is hypothetical, assuming similar glycosidic bond torsion angles as for the other compounds.

Table I

Inhibition of binding of three rat lectins to ASF-Sepharose by a series of saccharides, expressed as inhibitory activity of each compound relative to lactose

| No. | Formula                               | Relative activity*  |
|-----|---------------------------------------|---------------------|
|     |                                       | RL-14.5  | RL-18  | RL-29  |
| 1   | Galβ1-4Glc                            | 1        | 1      | 1      |
| 2   | Galβ1-4Glc                            | 0.4      | 0.6    | 1.5    |
| 2   | Fuco1                                 |          |        |        |
| 3   | Galβ1-4Glc                            | 0.02     | <0.02  | 0.01   |
| 3   | Fuco1                                 |          |        |        |
| 4   | GalNAce1-3Galβ1-4Glc                  | 0.3      | 2.0    | 25     |
| 4   | Fuco1                                 |          |        |        |

*The inhibitory activity of a series of concentrations of each substance was tested in the ASF-Sepharose binding assay. The relative activity is the concentration (M) of lactose giving 50% inhibition divided by the concentration (M) of the test saccharide giving 50% inhibition.

Table II

Inhibition of binding of three rat lectins to ASF-Sepharose by a series of saccharides, expressed as inhibitory activity of each compound relative to galactose

| No. | Formula                               | Relative activity*  |
|-----|---------------------------------------|---------------------|
|     |                                       | RL-14.5  | RL-18  | RL-29  |
| 20  | Gal                                    | 1        | 1      | 1      |
| 21  | Galα2OMe                              | 2        | 0.5    | 1      |
| 22  | GalαOMe                               | 2        | 2      | 2      |
| 23  | L-Ara                                 | <0.3     | <0.36  | <0.1   |
| 24  | D-Fuc                                 | 0.6      | <0.05  | <0.2   |
| 1   | Galβ1-4Glc                            | 130      | 60     | 100    |
| 16  | Galα1-3Galβ2OMe                       | 5        | 18     | 6      |
| 17  | GlcNAce1-3Galβ2OMe                    | 1.3      | 4      | 1      |

*The relative activity was calculated as in Table I except that it was done in relation to galactose instead of lactose.

Their probable conformations (see legend to Fig. 3). The following sections describe the relative potency of the series of saccharides for each of the lectins studied, as tabulated in Tables I-III in a manner which leads to conclusions about the nature of the active site of each lectin. First RL-14.5 will be considered and then the other two lectins will be compared.

The Specificity of RL-14.5—The data in Table I indicate that the binding site of RL-14.5 is complementary to a determinant which includes positions 4 and 6 of Gal and position 3 of Glc in lactose, as well as the acetamido group at position 2 of GlcNAc in N-acetyllactosamine. We will first consider the evidence concerning interaction of RL-14.5 with Gal, then with Glc, and finally with these determinants as integrated into larger structures.

RL-14.5 was initially defined as a galactose-specific lectin because Gal but not Glc, Man, or other simple sugars could inhibit its activity (16, 20). Thus it should recognize the specific feature of Gal which is the orientation of the 4-hydroxyl group, being axial in Gal but equatorial in Glc. The present results strongly support this specificity since cellobiose (No. 19) was at least 1000 times less active than lactose. Cellobiose is identical to lactose except for the orientation of the 4-hydroxyl group, being axial in Gal but equatorial in Glc. Therefore, the lectin probably interacts with the 4-hydroxyl group in Gal or its vicinity. This is also indicated by the marked loss of inhibitory activity of lactose derivatives with substituents at position 4 (Nos. 5, 6, 9). Position 6 of Gal also appears to be critical since the derivative substituted at position 6 (No. 8) was virtually inactive. In contrast, lactose derivatives with substituents at positions 2 and 3 (Nos. 2, 4, 7) were not substantially less inhibitory than lactose. Although galactose itself was much less active than lactose, a comparison of some of its derivatives (Table II)
supported our inferences about the active portion of this residue. Thus methylglycosides of Gal substituted in the 3 position (Nos. 16 and 17) had about the same activity as the corresponding unsubstituted methylglycosides (Nos. 22 and 21).

The Glc of lactose contributes to interaction with the lectin since lactose was 130 times as active as free Gal. Substitution of the Glc at position 3 (No. 3) blocked activity, indicating that the lectin interacts at this position or its vicinity (upper side of lactose as drawn in Fig. 3). N-acetyllactosamine (No. 12) was 5 times as active as lactose. This indicates that the acetamido group on carbon 2 in GlcNAc (Fig. 3), which is adjacent to position 3, contributes to activity. The specific importance of the equatorial orientation of the 3-hydroxyl group was indicated from the results with compounds 13 and 14. Galβ1-3GlcNAc (No. 13) was as active as lactose. In the preferred conformation of this compound (Fig. 3) position 4 of GlcNAc takes the place of position 3 of Glc in lactose (relative to Gal, see legend of Fig. 3) and the hydroxy group there has the same orientation (equatorial) as in lactose. In contrast, Galβ1-3GalNAc (No. 14) had 25 times less activity than lactose. In the preferred conformation of this compound, position 4 of GalNAc takes the place of position 3 of Glc in lactose but the 4-hydroxyl group has the axial orientation (Fig. 3). Further support for the importance of an equatorial hydroxyl group at the position corresponding to the 3-hydroxyl of Glc in lactose was given by the activity of No. 10 and No. 11. In No. 10 the equatorial 3-hydroxyl group of Fru is found at this location (Fig. 3). In thiogalactoside (No. 11) the corresponding place is taken by the equatorial 2-hydroxyl group of one of the Gal. In addition thiogalactoside (No.11), being symmetric, has two possible lectin binding sites which could contribute in part to its greater activity than lactose.

Having identified properties of the binding site of RL-14.5 with this series of small oligosaccharides, we were interested to determine how it interacted with more complex natural structures that contained more than 1 β-galactoside residue. We therefore examined compounds with linear repeating units of β-galactosides as well as structures with β-galactoside residues on more than one branch. The structures we tested are listed in Table III along with a numerical estimate of the expected activity of each compound if all potentially active residues acted independently and with the potency predicted from their activity as simple compounds (see explanation in legend to Table III). This numerical estimate seemed to be a reasonable one, since it is known that the preferred conformation of N-acetyllactosamine is largely preserved when it is part of complex oligosaccharides or glycoproteins (44, 45).

There are two potential reasons why the complex structures we tested would have greater activity than predicted by the indicated numerical estimate. First, polyvalency might be important in interaction with a lectin, as shown with the hepatic asialoglycoprotein receptor which has a markedly greater affinity for triantennary and tetraantennary galactosides (8, 46). Another possibility is that another special structural feature of these complex compounds might interact particularly well with a lectin. The results with RL-14.5 indicate that none of the complex compounds were more active than expected for the numerical estimate. Instead, many compounds were less active than the estimate, indicating inaccessibility of certain residues to RL-14.5 in ways not explained by the data of Table I and II (e.g. No. 28).

The Specificity of RL-18—Like RL-14.5, RL-18 is complementary to a determinant on lactose which includes the 4- and 6-hydroxyl of Gal. It probably also includes the 3-hydroxyl of Glc but, in contrast with RL-14.5, both the equatorial and axial orientation of this are accepted. Another difference is that the RL-18 determinant may be extended to include part of the substituents at position 3 of Gal. Thus, changes or substitutions at position 4 (Nos. 5, 6, and 19 in Table I) and at position 6 (No. 8 in Table I and Nos. 23 and 24 in Table II) of Gal blocked activity. Substitutions of position 2 and 3 (Nos. 2, 4, 7, in Table I and Nos. 16 and 17 in Table II) did not block activity. For Nos. 2 and 4 the activity was similar to lactose and for No. 7 lower by a factor of 5. Substitution of the β-methylglycoside of Gal with GlcNAcβ (No. 17) in the 3 position increased activity 8 times compared to the unsubstituted compound (No 21). Also, substitution with Gao (compare Nos. 16 and 22 in Table II) and with GalNAca (compare Nos. 4 and 2 in Table I) significantly increased activity. Therefore, the RL-18 binding site probably extends to interact with these substituent sugars, although lack of a complete range of suitable reference compounds does not allow detailed conclusions about this interaction.

As with RL-14.5, the Glc part of lactose contributes to interaction with RL-18, since lactose was 60 times more active than galactose. The interaction probably involves the side of Glc where position 3 is found (Fig. 3). Substitution there (Nos. 3, Table I) blocked activity. Compounds No. 10-13 were all active, and, as described above for RL-14.5 and in Fig. 3, in their preferred conformation they all have an equatorial hydroxyl group, similar to lactose, at the site corresponding to position 3 in the Glc of lactose. In striking contrast to RL-14.5, RL-18 also reacted well with Galβ1-3GalNAc (No. 14) which has an axial hydroxyl group at this site (Fig. 3). GalNAcβ1-3GalOMe (No. 15) was also quite active with RL-18, but not RL-14.5. Compound No. 9 also inhibited RL-18 probably via its terminal GalNAcβ1-3Gal rather than via the internal lactose which would be blocked by the substituents, as in No. 5.

We also tested more complex structures with RL-18 (Table III). As with RL-14.5, we found no evidence that multivalency or specialized structural features of these compounds enhanced their interaction with RL-18.

The Specificity of RL-29—The specificity of RL-29 has many similarities with RL-14.5 and RL-18 in that binding to lactose involves hydroxyls 4 and 6 of Gal and hydroxyl 3 of Glc. It also includes the 2-acetamido group of GlcNAc in N-acetyllactosamine. A major difference is that incorporation of GalNAcα at position 3 of Gal markedly enhanced activity. We also found evidence for some specificity of binding to LAGs.

The arguments for interaction with positions 4 and 6 of Gal in lactose are like those for RL-14.5. Thus, substituents or changes of Gal at position 4 (Nos. 5, 6, 9, 19 in Table I) and at position 6 (No. 8) blocked or significantly decreased activity, whereas substitution at position 2 and 3 gave slightly lower (No. 7), similar (No. 2), or enhanced (No. 4) activity. The arguments concerning the interaction with Glc or the corresponding sugar in Nos. 10–14 are also similar to those described for RL-14.5, since N-acetyllactosamine (No.12) had enhanced activity, compounds Nos. 10, 11, and 13 had similar activity, and compounds Nos. 3 and 14 had much lower activity compared to lactose. However, in contrast to RL-14.5, the RL-29 determinant apparently extends to the GalNAc bound to position 3 in Gal in compound No. 4, since this had 25 times the activity of lactose and 17 times the activity of compound No. 2. GalNAc itself had only low activity (0.8 the activity of Gal). Therefore, its specific linkage to lactose or related compounds is important for the high activity.

The activity of some natural oligosaccharides (Table III) containing several RL-29 determinants was compared to the


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**TABLE III**

Inhibition of binding of three rat lectins to ASF-Sepharose by a series of oligosaccharides and glycopeptides, expressed as inhibitory activity of each compound relative to lactose

| No. | Formula* | Relative activitya |
|-----|----------|--------------------|
|     |          | RL-14.5 | RL-18 | RL-29 |
| 25  | Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 1.6 (2) | 8 (9) | 9 (4) |
| 26  | Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 1.3 (1) | 9 (9) | 15 (5) |
| 2   | Fucol | 0.5 (1) | 3 (10) | 52 (68) |
| 27  | GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc | <0.07 (1) | 0.5 (8) | 9 (2) |
| 2   | Fucol | 16 (33) | 19 (56) | 90 (59) |
| 28  | GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc | 30 (32) | 14 (32) | 34 (54) |
| 2   | Fucol | 6 (33) | 14 (56) | 120 (59) |
| 29  | Asialofetuin oligosaccharide | 3 (15) | 1.7 (3) | 3 (20) |
| 30  | Fetuin oligosaccharide | 1.2 (5) | 1.2 (1) | 6 (6) |
| 31  | Asialoorosomucoid oligosaccharide | 1.7 (20) | 1.6 (4) | 2 (28) |
| 32  | Orosomucoid oligosaccharide | 1.6 (6) | 1.2 (1) | 4 (8) |
| 33  | Granulocyte LAG glycopeptide | 16 (36) | 7 (46) | 17 (41) |
| 34  | Cord erythrocyte LAG glycopeptide | 20 (32) | 14 (32) | 34 (54) |
| 35  | Adult erythrocyte LAG glycopeptide | 16 (33) | 19 (56) | 90 (59) |
| 36  | Adult erythrocyte LAG | 6 (33) | 14 (56) | 120 (59) |

*a Compounds 29-32 and 36 are oligosaccharides released from peptides by hydrazinolysis. Nos. 33-35 are glycopeptides obtained by enzymatic digestion. The essential features of the structures of the carbohydrate residues of Nos. 29-36 are as follows: The fetuin oligosaccharide is tetraantennary, with 3 Galβ1-4GlcNAc residues linked to a mannoside core with all the terminal Gal residues sialylated (30-32); the orosomucoid oligosaccharide is tetraantennary, with 4 Galβ1-4GlcNAc residues linked to a mannoside core with all the terminal Gal residues sialylated (33, 34). The LAGs (4) all contain chains of repeating -3Galβ1-4GlcNAcβ1. The granulocyte LAG has four chains (36-37), whereas the erythrocyte LAGs have two (38, 39). The terminal N-acetyllactosamines are substituted with fucose or sialic acid. In the granulocyte LAG some of the N-acetyllactosamine residues are substituted with Fuc linked to GlcNAc. In the cord erythrocyte LAG the internal N-acetyllactosamine chains are unbranched and unsubstituted (38). In the adult erythrocyte LAG some internal N-acetyllactosamine residues are substituted with single branching N-acetyllactosamine residues bound β1-6 to Gal (39).

The critical determinant in lactose common to all three lectins is noted in this regard that these determinants contain a hydrophobic patch and that the hydroxyl groups may take part in a ring of hydrogen bonds, both favoring carbohydrate-protein interactions (47). Based on the limited data available, the bovine β-galactoside-binding lectin that resembles RL-14.5 shares this general specificity (14, 15, 48). Although they have substantial similarities, there are a

**DISCUSSION**

The results indicate both common features of the binding sites of RL-14.5, RL-18, and RL-29, which we will first summarize, as well as significant differences, which we will then review. Finally, we will consider the possible biological significance of the specificities of these three lectins.

The critical determinant in lactose common to all three lectins includes positions 4 and 6 of Gal and the 3-hydroxyl of Glc. These determinants are confined to one side of lactose (Fig. 3) and appear also to be involved in interaction with other proteins. For example, these determinants are partially included among those defined for some antibodies (47, 48). It is notable in this regard that these determinants contain a hydrophobic patch and that the hydroxyl groups may take part in a ring of hydrogen bonds, both favoring carbohydrate-protein interactions (47). Based on the limited data available, the bovine β-galactoside-binding lectin that resembles RL-14.5 shares this general specificity (14, 15, 48). Although they have substantial similarities, there are a
number of significant differences in the binding sites of the three lectins. For RL-14.5 and RL-29, the basic determinant, discussed above, extends specifically to the 3-hydroxyl of Glc which must be equatorial and also to the 2-acetamido group found on GlcNAc in N-acetyllactosamine. In contrast, for RL-18, the stereochemical requirements are less specific, since Galβ1-3GlcNAc and similar residues bind as well as lactose. With RL-18 and RL-29, the determinants of the basic structure also extend to involve sugars bound at position 3 of the Gal in lactose and other β-galactosides.

For RL-18, binding to Gal was enhanced by addition of GlcNAcβ1-3. GlcNAcβ1-3Gal is commonly found in internal residues of LAGs (4). The same modification of Gal had no enhancing effect with the other two lectins. For RL-29, activity was markedly enhanced by a GalNAcβ1-3 substitution on the Gal of lactose, whereas enhancement was very slight with RL-18, and this substitution considerably inhibited binding to RL-14.5. Therefore, despite their similarities, these three lectins can show considerable preference for natural derivatives of simple β-galactosides.

The specificity of residues in small saccharides was also apparently maintained when they were incorporated into larger glycoconjugate chains, and all three lectins could clearly associate with internal β-galactosides, as previously shown for some antibodies (5, 47, 48), bacteria (50), and enzymes (4, 51). It is notable that the more complex structures such as the LAGs did not, in general, confer additional binding affinity above what would be predicted from the sum of the combined β-galactoside residues within these complex structures. In some cases there was a detectable increase above the predicted activity. But in most cases there was actually diminution of activity compared to what would be predicted from the sum of the constituent residues, indicating that some of the internal β-galactosides were rendered inaccessible to the lectin.

The biological significance of the specificities of these three lectins is not known. Soluble lectins have been shown to be secreted (9), so they should be available for interaction with glycoconjugates on the cell surface and extracellular matrix. Since the present work shows that each of these lectins can associate specifically with natural mammalian glycoconjugates which are known to be present at such sites, it is hard to escape the conclusion that the lectins must function by interacting with them. Such functions would be regulated by the selective affinities of the given lectins as well as by the selective expression of both lectin and complementary glycoconjugate in either developing or adult cells and tissues of specific types. It is notable in this regard that both lectins (9) and specific glycoconjugate structures (1-5) show both marked developmental regulation and marked variations in expression among given cell types. For example, the structure of Galβ1-3GalNAc, which is recognized by RL-18 but only very weakly by the other lectins, is characteristic of globoseries glycolipids including the SSEA-III and SSEA-IV antigens which are specifically expressed at certain developmental stages (2, 5). Likewise RL-29 binds particularly well to β1-6 branched lactosaminoglycans, a structural feature of the I antigens which are known to be developmentally regulated and expressed in certain cell types (2, 4, 5, 39).

One way to examine the possibility of a particular lectin-glycoconjugate interaction is to determine the location of both molecules within a given tissue by histochemical techniques. For example, co-localization of chicken lactose lectin II and intestinal mucin, to which it binds, strongly supports their functional interaction (13). Evidence of concordant expression of RL-14.5 and RL-29 and “lacto-series” glycoconjugates in developing rat dorsal root ganglion and spinal cord also suggests functional interactions (52, 53). The specificities of the lectins which we have identified in the present study and the availability of monoclonal antibodies that react with residues favored by a given lectin could prove useful in functional studies, using both histochemical and more direct experimental techniques.

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