Different Modes of Ligand Binding to the Hepatic Galactose/*N*-Acetylgalactosamine Lectin on the Surface of Rabbit Hepatocytes†

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ABSTRACT: A study of the binding of three different ^{125}I-labeled, galactose-terminated ligands to the hepatic galactose/*N*-acetylgalactosamine-specific lectin found on the surface of rabbit hepatocytes revealed that the different ligands manifest different physical parameters of binding. Asialoorosomucoid (^{125}I-ASOR) binding was best described as involving two independent classes of binding sites on rabbit hepatocytes, with 161 000 sites/cell with a dissociation constant of 0.44 nM and 292 000 sites/cell with a K_d of 9.7 nM. Asialotriantennary glycopeptide purified from human α-1 protease inhibitor and modified with tyrosine at the N-terminus to permit radioiodination (TRI) [Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202] was also found to bind to two apparent classes of binding sites but with different binding parameters: 292 000 sites/cell of K_d = 1.47 nM and 982 000 sites/cell of K_d = 25.3 nM. A synthetic ligand, α,β-diaspartamide of tris[(β-lactosyloxy)methyl](6-aminohexanamido)methane (di-tris-lac) containing six nonreducing galactose residues [Lee, R. T., Lin, P., & Lee, Y. C. (1984) *Biochemistry 23*, 4255–4261], was found to bind to 817 000 sites/cell of K_d = 0.63 nM and 1.23 × 10^6 sites/cell of K_d = 25.3 nM. Thus, there were many more total binding sites for TRI or di-tris-lac on the surface of rabbit hepatocytes than there were for asialoorosomucoid, although the dissociation constants were similar for all three ligands. The nature of the additional TRI or di-tris-lac binding sites was investigated by measuring the binding of TRI or di-tris-lac to rabbit hepatocytes that had their high-affinity ASOR sites occupied by unlabeled ASOR (ASOR-loaded cells). ASOR-loaded cells were found to bind ASOR or TRI to a single class of remaining low-affinity sites (K_d = 38.0 nM for ASOR and K_d = 10.2 nM for TRI), with an approximate stoichiometry equivalent to three TRI sites per ASOR site (171 000 ASOR sites/cell vs. 495 000 TRI sites/cell). Di-tris-lac, however, was still observed to bind with high affinity (K_d = 1.2 nM) to 87 900 sites/cell, as well as to 429 000 low-affinity (K_d = 50.0 nM) sites/cell. The high-affinity di-tris-lac sites appear to be unavailable for high-affinity TRI or ASOR binding. We concluded that the sites that accommodate one galactose residue are clustered on the cell surface, permitting ASOR to bind to or eclipse about nine galactose-combining sites. However, ASOR does not delineate all of the high-affinity binding sites on the cell surface, as defined by the small ligands TRI and di-tris-lac. We explained these observations by considering that ligands bind to a lattice of galactose-combining sites on the cell surface and that both the apparent number and affinity of binding sites are a function of the ligand utilized.

The Gal/GalNAc[1] lectin on the surface of mammalian hepatocytes can bind a variety of ligands with vastly different affinities (Baenziger & Fiete, 1980, Lee et al., 1983; Lee, Y. C., et al., 1984). Considerable differences in the binding properties of the Gal/GalNAc lectin on rat hepatocytes have been reported. In most cases, ^{125}I–asialoorosomucoid (ASOR)[2] has been used to characterize the binding properties (i.e., the dissociation constants and binding site numbers). Measured dissociation constants for the rat hepatocyte surface lectin/ASOR complex have ranged from 89 nM (Warren & Doyle, 1981) to 0.42 nM (Weigel, 1980) for "high-affinity" ASOR binding. The reported number of ASOR binding sites on rat hepatocytes has also covered a large range (Schwartz et al., 1980). The differences in the number of ASOR binding sites are partly due to variation of assay conditions between experiments (Schwartz et al., 1980) and the thermal history of the isolated hepatocyte preparations (Wiegel & Oka, 1983). The difficulty in precisely determining receptor concentrations by graphical or linear least-squares analysis of Scatchard

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[1] All sugars are of D configuration and are in pyranose form.

[2] Abbreviations: ASOR, asialoorosomucoid radiolabeled with ^{125}I; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MDE, modified Dulbecco's Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; *t*-Boc, *tert*-butyloxycarbonyl; K_d, the dissociation constant for the ligand–receptor complex; *R*, the concentration of ligand binding sites; *N*, the limiting [B]/[F] ratio of nonspecific ligand binding; *C*, the correction constant used to normalize data from experiments of different binding site concentrations to a reference concentration; TRI, ^{125}I asialotriantennary glycopeptide purified from human α-1 protease inhibitor and modified with tyrosine; di-tris-lac, α,β-diaspartamide of tris[(β-lactosyloxy)methyl](6-aminohexanamido)-methane.

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transformations of binding data has been discussed (Munson & Rodbard, 1980; Klotz, 1982, Stankowski, 1983), and determination of binding parameters via Scatchard transforms can certainly be misleading in the case of multiple classes of ligand–receptor interaction. The existence of a single class of sites or multiple classes of binding sites of differing apparent affinity is also controversial. Further, determination of the total number of "binding sites" can be quite difficult, unless one deals with the reversible binding of a homogeneous, monovalent ligand to a single, noninteracting class of monovalent binding sites and nonspecific binding has been completely and exactly corrected (Munson & Rodbard, 1980; Stankowski, 1983). In the specific case of the Gal/GalNAc lectin, the number of measured binding sites for a Gal-terminated ligand might be expected to vary according to the number of Gal residues per ligand molecule. Recently, we (Townsend et al., 1983) and others (Fiete et al., 1983) have shown that there are approximately 3 times more sites on the surface of rabbit or rat hepatocytes, respectively, available for desialylated triantennary glycopeptide binding than for ASOR binding. Interestingly, the number of binding sites on rabbit alveolar macrophages for a series of mannose-containing bovine serum albumin ligands has recently been found to depend on the average number of mannose residues linked to bovine serum albumin (Hoppe & Lee, 1983).

It should also be noted that the structure of the ligand appears to be of considerable importance in determining the course of subcellular events in the process of receptor-mediated endocytosis subsequent to surface binding. For example, asialotransferrin (Tolleshaug et al., 1981) and a tris(galactoside) (Connolly et al., 1982) are internalized by isolated rat hepatocytes or isolated rabbit hepatocytes, respectively, but subsequently released undegraded into the medium. Asialoorosomucoid and an asialotriantennary glycopeptide isolated from human α-1 protease inhibitor have been found to be degradable in the isolated, perfused rat liver (Townsend et al., 1984) but were found to be returned, undegraded, to the cell surface to differing extents. Further characterization of the cell surface binding of these ligands is now required to determine the effects of ligand structure on cellular processing.

In the present study we have rigorously examined the binding of three structurally different ligands, ASOR, TRI, and di-tris-lac [a synthetic ligand containing six nonreducing terminal Gal residues (Lee, R. T., et al., 1984)], to suspensions of rabbit hepatocytes prepared under controlled conditions of perfusion and temperature of purification. We found that all three ligands possess affinities (expressed as dissociation constant) on the order of 1 nM for the Gal/GalNAc lectin on the surface of rabbit hepatocytes. Special attention was paid to covering the complete range of the saturation function of the lectin (Klotz, 1982), as well as to systematic determination of the nonspecific component of the measured binding (Munson & Rodbard, 1980). Analysis of direct binding of all three ligands revealed that a model describing two independent classes of sites was necessary to describe the data. There were approximately 3–4 times more total binding sites for TRI or di-tris-lac on rabbit hepatocytes than there were for ASOR. We found that ASOR could neither saturate all of the Gal-binding sites on the surface of rabbit hepatocytes nor prevent the high-affinity binding of the low molecular weight ligand di-tris-lac.

Materials and Methods

The amino acids and vitamins used in the preparation of modified MDE (Schnaar et al., 1978) were obtained from Calbiochem (San Diego, CA). HEPES was from Research Organics (Cleveland, OH). All salts used were of reagent-grade quality and were from J. T. Baker (Phillipsburg, NJ). Trypan blue, crystal violet, light mineral oil, and EGTA were from Sigma Chemical Co. (St. Louis, MO). Silicone oil (DC 550) was from Accumetric (Elizabethtown, KY). Carrier-free $Na^{125}I$ for protein and glycopeptide labeling was from Amersham (Arlington Heights, IL). Orosomucoid was kindly donated by Dr. M. Wickerhauser of the American Red Cross National Fractionation Center (Bethesda, MD) and was desialylated as previously described (Connolly et al., 1981). Human α-1 protease inhibitor was the gift of Dr. R. Glew (Department of Biochemistry, University of Pittsburgh, PA). TRI was prepared, modified with L-tyrosine and radioiodinated as described (Lee et al., 1983). Di-tris-lac was provided by Dr. Reiko T. Lee, modified with *p*-nitrophenyl *tert*-butyloxycarbonyl-L-tyrosinate, and radioiodinated by a modified (Lee et al., 1983) chloramine T method (Greenwood et al., 1963).

Isolation of Rabbit Hepatocytes. Rabbit hepatocytes were prepared by a modification of the collagenase perfusion method (Seglen, 1976). To prepare morphologically intact cells more consistently, it was found that the initial flow rate for buffer I (80 mL/min) had to be decreased by 2 mL/min every 2 min during buffer I perfusion (total buffer I perfusion time was 10 min). The collagenase solution (0.025%) was perfused for 3–5 min. After collagenase perfusion, the liver was immediately immersed in medium at 2 °C (Seglen, 1976). All subsequent cell purification procedures were performed at 2 °C.

Kinetics of Labeled Ligand Binding. Freshly isolated rabbit hepatocytes were suspended (2.5×10^6 cells/mL) in ice-cold MDE containing the desired dose of labeled ligand and placed in capped polystyrene test tubes of suitable size. The cell suspensions were incubated by end-over-end rotation at 1 rpm at 2 °C in an ice–water bath. At the indicated times, duplicate 100-μL aliquots of the cell suspensions were removed from the tubes and pipetted into 400-μL microfuge tubes containing ~150 μL of a silicone–mineral oil mixture (4:1 v/v) at 2 °C, after which the tubes were immediately centrifuged in an Eppendorf microfuge for 15 s. The tips of the tubes, containing the cell pellets, were snipped off and counted for radioactivity in a Packard PRIAS auto gamma counter. Nonspecific binding at each time point was determined by pipetting duplicate 100-μL aliquots of the cell suspension into 12 × 75 mm polystyrene tubes containing 300-μL aliquots of chilled isotonic HEPES buffer, pH 7.5, containing 8 mM EGTA, and incubating for exactly 10 min at 2 °C. The cells were then resuspended, and a 200-μL aliquot of each suspension was pipetted into oil-containing microfuge tubes and centrifuged as above. In some experiments, duplicate 100-μL aliquots of the cell suspension were added to 5 μL of 0.5 M EDTA, pH 7.8, in chilled polystyrene tubes and incubated for 10 min at 2 °C. A 50-μL aliquot was then removed from each tube and pelleted as described. Duplicate 100-μL aliquots of the cell suspensions were counted for radioactivity to determine the specific activity for each dose.

Direct Binding Assay. Stock solutions of ASOR and TRI were prepared in water. The concentration of the ASOR stock was determined by measuring its absorbance at 280 nM with an extinction coefficient of 0.893 mL mg^{-1} cm^{-1} (Schmid, 1975). The concentration of TRI was determined by analysis of the component neutral sugars by automated liquid chromatography (Lee, 1972). Binding assays were carried out in capped 12 × 75 mm polystyrene test tubes. Each tube contained about 2.5×10^6 rabbit hepatocytes/mL, in a total

volume of 2 mL of MDE. In all cases, the actual cell concentration was determined after the binding assay by counting the crystal violet stained nuclei as described (Connolly et al., 1983). The cell suspensions were maintained for 2 h at 2 °C with end-over-end rotation at 1 rpm, after which time different doses of the ^{125}I-labeled ligands were added to each tube in less than 10% of the incubation volume of MDE. The suspensions were then incubated for exactly 3 h at 2 °C, and the cell-associated radioactivity was determined as described above. Determination of nonspecific ligand binding by EGTA release was as described. For all ligands, the level of nonspecific binding was less than 10% of the total binding even at the maximum dose (600 nM).

Binding of Labeled Ligands to Hepatocytes Treated with 20 nM Unlabeled ASOR. Freshly prepared rabbit hepatocytes were suspended at a concentration of approximately 2.5 × 10^6 cells/mL in MDE containing 20 nM unlabeled ASOR and incubated at 2 °C for 2 h. After this incubation, varying doses of labeled ligands were added to the cell suspensions in small volumes of MDE (less than 10% of the total volume), and the incubation was continued for exactly 3 h at 2 °C. Determination of ligand binding was as described for the direct binding experiments.

Determination of Ligand Binding Parameters. For each experiment, initial estimates of dissociation constants and receptor concentrations were made by inspection of plots of [bound]/[free] vs. [bound] (Scatchard, 1949). The choice of the best model to describe the ligand binding data for each experiment and the best fit binding parameters derived from them were determined by the nonlinear multiparameter modeling program SCATFIT (Munson & Rodbard, 1980). Analysis of binding data corrected for nonspecific binding by subtraction of a blank representing calcium-independent binding consistently resulted in an underestimation of the nonspecific, nonsaturable contribution to the total binding (i.e., SCAFIT best fit N parameter > 0), due to the dilution of the binding system in the isotonic EGTA solution used in determination of the blank. The total binding data, uncorrected for nonspecific, were used for nonlinear parameter estimation by the SCAFIT program, as described by Munson & Rodbard (1980). For graphical display of the binding data and the SCAFIT-calculated binding isotherms, the N parameters produced by SCAFIT for the best fit binding curves were used to calculate the level of nonspecific binding at each ligand dose, and this was subtracted from the total binding to determine the specific binding. In cases where binding for a given ligand obtained from multiple cell preparations were to be fit together to a single set of binding parameters, the C parameters (receptor concentration correction factors) were allowed to float in the SCAFIT program, as described by Munson & Rodbard (1980). A modified version of SCAFIT was implemented on a VAX 11/780 minicomputer at the Johns Hopkins University Engineering Computing Facility.

RESULTS

Binding of Labeled Ligands to Isolated Rabbit Hepatocytes at 2 °C. Binding of ASOR to the surface of rabbit hepatocytes at 2 °C has been shown to reach an apparent equilibrium in less than 2 h (Connolly et al., 1983). The time courses of TRI and di-tris-lac binding, however, are somewhat different, as shown in Figure 1. At higher doses of these ligands, approximately 3 h was required to achieve an apparent binding equilibrium, and even from 3 to 5 h of incubation, there was an increase of approximately 15% in the specific binding of the low molecular weight ligands. A 3-h incubation was used in all subsequent ligand binding experiments, with special precautions taken so that all experimental points were incubated for exactly the same time.

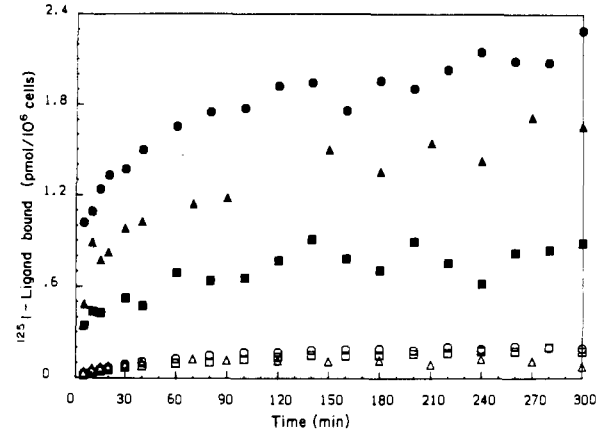

FIGURE 1: Kinetics of labeled ligand binding to rabbit hepatocytes at 2 °C. Freshly isolated rabbit hepatocytes at a concentration of (2.3–3.0) × 10^6 cells/mL were incubated at 2 °C in MDE containing either 1 (open symbols) or 200 nM (filled symbols) amounts of either ASOR (□, ■), TRI (○, ●), or di-tris-lac (△, ▲). At the indicated times, the specific binding of ligands was determined as described under Materials and Methods.

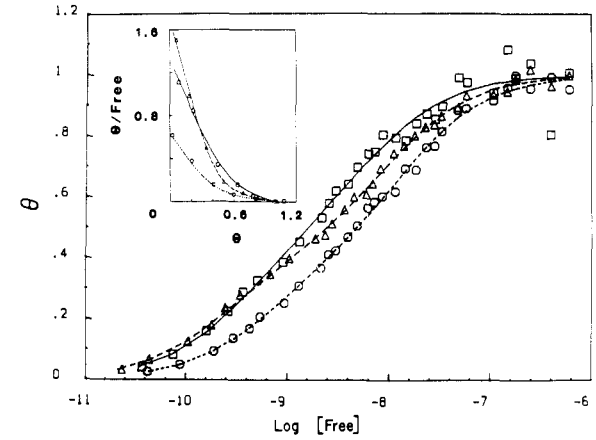

FIGURE 2: Binding of ASOR to rabbit hepatocytes at 2 °C. Freshly isolated rabbit hepatocytes [(4.4–8.0) × 10^6 cells in 2.0 mL] were incubated for 2 h at 2 °C, after which time varying doses of ASOR were added in small volumes (less than 200 μL) of cold MDE. Ligand was allowed to bind to the cells for exactly 3 h at 2 °C, after which time both total cell-associated and specifically bound ASOR were determined as described under Materials and Methods. The symbols (□, ○, △) represent the experimental binding data from three different cell preparations, normalized to the maximum level of bound ligand calculated by SCAFIT. Data have been corrected for nonspecific binding with the N parameter calculated by SCAFIT analysis. Data are plotted as the fractional saturation of the binding sites (θ) vs. the common logarithm of the free ligand concentration (Klotz, 1982). The lines represent the SCAFIT-calculated two-site binding isotherms for each cell preparation. The inset shows the Scatchard transformation of some representative data points and the transform of the best fit binding curves.

The binding isotherms, plotted as the fraction of sites saturated vs. the free ligand concentration (Klotz, 1982), are shown for ASOR, TRI, and di-tris-lac, respectively, in Figures 2–4. Both calculated and experimental data in the figures have been corrected for nonspecific binding by using the parameter N calculated by SCAFIT, as described under Materials and Methods. Even after treatment of nonspecific binding in this manner, the Scatchard transformations were curvilinear (Figures 2–4, insets), and the data fit best (i.e., F tests were highly significant) to a model describing two classes of noninteracting binding sites. The parameters thus obtained are summarized in Table I.

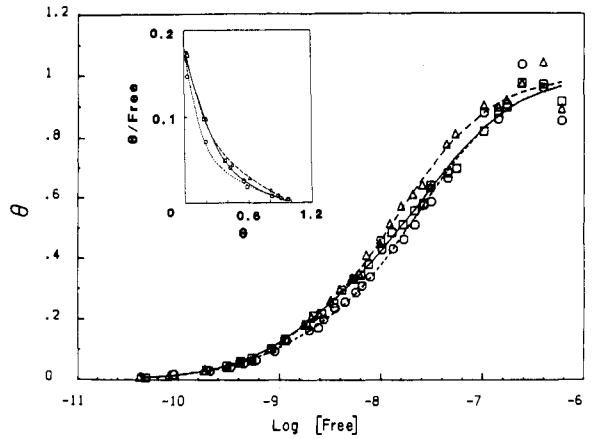

FIGURE 3: Binding of TRI to rabbit hepatocytes at 2 °C. Conditions of the experiment are as described in the legend of Figure 2. Both the saturation function (fractional saturation of the hepatocyte surface as a function of free ligand concentration) and the Scatchard transformation (inset) are shown.

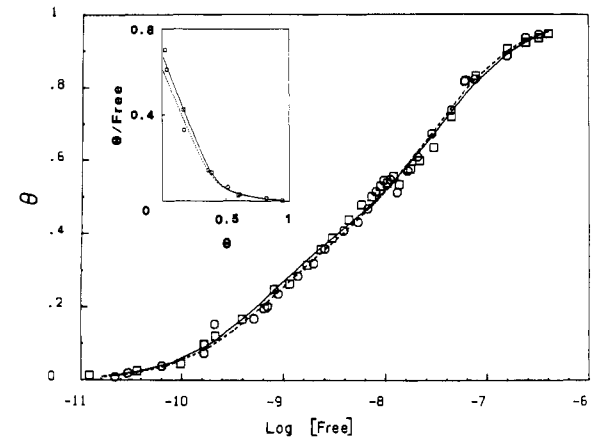

FIGURE 4: Binding of di-tris-lac to rabbit hepatocytes at 2 °C. Conditions of the experiment are as described in the legend of Figure 1. Data from two different cell preparations are shown (□, ○). The inset shows the Scatchard transformation.

From experiments using three separate cell preparations, ASOR was determined to bind to a total of 311 000–380 000 sites/hepatocytes with 147 000–183 000 sites of higher affinity (K_d = 0.25–0.62 nM) and 195 000–259 000 sites/hepatocyte of lower affinity (K_d = 6.4–13 nM). By use of TRI, a ligand structurally equivalent to a type of oligosaccharide linked to ASOR (Fournet et al., 1978; Schmid et al., 1979), approximately 3-fold more total binding sites were detected [(1.06–1.60) × 10^6 sites/cell]. A synthetic ligand containing six Gal residues, di-tris-lac (Figure 4), exhibited (1.75–2.34) × 10^6 binding sites/cell. TRI and di-tris-lac binding data also fit best to a two-site model with similar dissociation constants for both classes of sites (Table I).

Variability of Ligand Binding to Different Cell Preparations. Since a wide range of affinities (Weigel, 1980; Warren & Doyle, 1981) and lectin site numbers (Schwartz et al., 1980) have been reported for isolated rat hepatocytes, we investigated the contribution of different hepatocyte preparations to the measured binding parameters. We investigated whether the dissociation constants and the ratio R1:R2:N (i.e., the distribution of ligand into both specific and nonspecific binding sites) were constant between cell preparations for each ligand. For any one ligand, simultaneous fitting of binding data from different cell preparations to a single set of binding parameters always resulted in a significant value for the runs test for one or more of the experiments, indicating the unsuitability of describing data from different cell preparations by a single set of binding parameters (Munson, 1981). Despite this, the values of binding parameters for different experiments, when analyzed separately, were similar; K_d values varied only 2–3-fold for each ligand, and the total number of receptor sites per hepatocyte varied less than 30% for each of the three ligands (Table I) among the three cell preparations. However, the slight but significant differences in the binding parameters could not be accounted for on the basis of random error, and thus the data for each cell preparation are presented separately in Table I.

Table I: Best Fit Binding Parameters for Binding of ^{125}I-Labeled Ligands at 2 °C

ligand	parameter[a]	experiment[b] 1	2	3
ASOR	K_{d1}[c]	0.44 (±19)	0.62 (±9)	0.25 (±8)
	K_{d2}	6.37 (±36)	13.2 (±17)	9.43 (±12)
	R_1[d]	185 (±14)	152 (±7)	147 (±5)
	R_2	195 (±11)	259 (±4)	209 (±3)
TRI	K_{d1}	2.00 (±12)	1.10 (±22)	1.31 (±28)
	K_{d2}	32.5 (±24)	26.0 (±25)	17.4 (±24)
	R_1	499 (±12)	187 (±21)	196 (±31)
	R_2	1100 (±7)	978 (±9)	867 (±6)
di-tris-lac	K_{d1}	0.65 (±15)	0.61 (±13)	
	K_{d2}	30.4 (±44)	34.5 (±42)	
	R_1	914 (±14)	720 (±7)	
	R_2	1422 (±11)	1028 (±4)	

[a] Determined by SCAFIT. [b] Standard errors of the means (%) shown are approximate. [c] Dissociation constants expressed in units of nM. [d] Concentration of binding sites expressed in units of 10^3 sites/cell

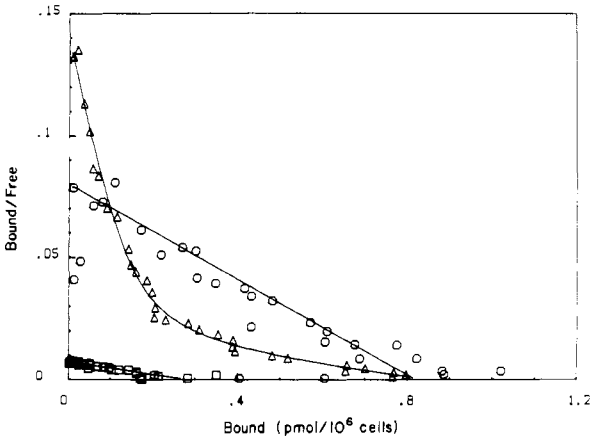

FIGURE 5: Binding of labeled ligands to rabbit hepatocytes in the presence of 20 nM unlabeled ASOR. Freshly isolated rabbit hepatocytes (4.4 × 10^6 cells) were suspended in 2.0 mL of MDE containing 20 nM ASOR and incubated for 2 h at 2 °C, after which time varying doses of labeled ASOR (□), TRI (○), or di-tris-lac (△) were added, and binding was allowed to occur for 3 h at 2 °C. Both total and specifically bound ligand were determined as described under Materials and Methods. The Scatchard transformation of the SCAFIT-corrected specific binding data is shown. The lines represent the SCAFIT best-fit binding isotherms for each ligand.

Binding of Ligands to 20 nM ASOR-Loaded Cells. Finding significantly more sites for TRI or di-tris-lac than for ASOR on the surface of rabbit hepatocytes led us to investigate the ability of ASOR to saturate all of the high-affinity ligand binding sites on the hepatocyte surface. We investigated whether ASOR, once bound to the cell surface at high-affinity binding sites, exchanges with free ligand. On the basis of the experimentally determined binding parameters (Table I), the high-affinity binding sites of rabbit hepatocytes were saturated with labeled ASOR (20 nM dose). Cell-associated label did not decrease over 3 h at 20 °C in the presence of a 500 nM dose of either unlabeled ASOR, TRI, or di-tris-lac. Thus, we

Table II: Binding Parameters for ^{125}I-Labeled Ligands to Rabbit Hepatocytes Treated with 20 nM ASOR at 2 °C

^{125}I-labeled ligand		affinity (nM)		^{125}I-labeled ligand bound (1000 sites/cell)
ASOR		38.0 (±12)[b]		171 (±12)[b]
TRI		10.2 (±23)		495 (±16)
di-tris-lac[a]	K_{d1}	1.2 (±11)	R_1	87.9 (±9)
	K_{d2}	50.0 (±23)	R_2	429 (±13)

[a] A two-site model was found to fit the observed di-tris-lac binding data significantly better than the one-site model ($F = 113.7$, $p < 0.01$). [b] Standard errors of the mean (%).

could assay binding of labeled ligands to any remaining surface Gal binding sites not occupied (or eclipsed) by the bound, unlabeled ASOR, as shown in Figure 5. Cells treated for 2 h at 2 °C with 20 nM unlabeled ASOR to saturate high-affinity sites were designated ASOR-loaded cells.

As shown in Table II, ASOR binding to ASOR-loaded cells resulted in the expected filling of 171 000 low-affinity sites (K_d = 38.0 nM). TRI bound to 495 000 sites/cell with an intermediate affinity (K_d = 10.3 nM) compared to the two sites detected by direct binding to untreated hepatocytes. Both ASOR and TRI delineate a single class of sites on ASOR-loaded cells. However, the calculated dissociation constants describing di-tris-lac binding to ASOR cells were essentially unaffected by the presence of unlabeled ASOR. Compared to the binding of di-tris-lac to the surface of untreated rabbit hepatocytes, there were less binding sites for di-tris-lac on ASOR-loaded cells. There were 87 900 high-affinity sites/cell for di-tris-lac and 429 000 low-affinity sites/cell. This latter number is similar to the number of TRI binding sites on ASOR-loaded cells.

Discussion

It has been shown that clustering of galactoses in branched oligosaccharides results in an approximately 1000-fold increase in affinity per Gal residue added, reaching a limiting K_d of 10^{-9} M for a three-Gal cluster (Lee et al., 1983; Lee, Y. C., et al., 1984). The observation that the K_d's for the present ligands are in the nanomolar range implies that at least a three-Gal cluster is involved in the interaction of ASOR with the lectin, even though ASOR contains approximately 14–25 Gal residues (Jeanloz, 1972); di-tris-lac contains six, and TRI contains three.

As has been shown previously (Connolly et al., 1983), the saturable binding of ASOR to the surface of rabbit hepatocytes is a complex function of the free ASOR concentration. The present studies show that not only ASOR but also the low molecular weight ligand TRI, which is naturally derived, and the synthetic di-tris-lac display this complex binding behavior. Although we have modeled the binding by using two classes of noninteracting sites, other mechanisms may be equally plausible. For example, negative cooperativity has been proposed to explain Scatchard transforms that are concave upward. The kinetic analysis of this phenomenon as tested in the insulin receptor system (De Meyts et al., 1976) is not applicable in our case, due to the multivalent nature of ligand binding to the hepatic Gal/GalNAc lectin (DeLean and Rodbard, 1979). Molecular heterogeneity, not of the receptor (as commonly supposed) but of the ligand, might be responsible for the different classes of ligand–lectin interaction. The known heterogeneity of oligosaccharides linked to ASOR (Fournet et al., 1978; Schmid et al., 1979) could certainly account for the observed complexity of hepatic lectin–ASOR interaction, even if the hepatic lectin sites themselves were all identical. TRI, though simpler in structure, may still be heterogeneous with respect to fine details of its constituent oligosaccharide structures [for example, the attachment site of the third *N*-acetyllactosamine branch has not been determined (Hodges et al., 1979; Mega et al., 1980)]. Also, a recent report (Bayard et al., 1982) indicates that some TRI preparations from human α-1 protease inhibitor may also contain biantennary structures with GlcNAc linked β1→4 to the β-linked Man. The observed heterogeneity of TRI binding might be due to such a contaminant. We have, however, recently found that a synthetic oligosaccharide with the same branched structure of this reported glycopeptide contaminant of TRI possesses a K_d of ca. 40 μM for the cell surface lectin (data not shown). This predicts that binding of this species is negligible across the range of TRI doses (0.1–600 nM) examined. The chemically homogeneous synthetic ligand di-tris-lac was also found to show more than one mode of binding to the lectin, which, while it could reflect different classes of lectin sites, could also be due to the binding of different conformers of di-tris-lac, although the existence of such conformers has not been shown explicitly.

Additional evidence indicates that ligand heterogeneity is not sufficient to explain all facets of ligand–hepatic lectin interaction. There is a small but significant difference in affinity profiles between cell preparations for all three ligands in the present study by certain statistical criteria (Figures 2–4). Further, there is a dramatic change in affinity toward small ligands upon receptor solubilization (Baenziger & Maynard, 1980; Connolly et al., 1982; Lee, R. T., et al., 1984). It has been shown that the binding profile for fetuin glycopeptide to the surface of rat hepatocytes is converted from an apparent two classes of binding sites to a single class with a 3-fold increase in number of total sites depending upon the thermal manipulation of the cells (Fiete et al., 1983). The apparent number of ASOR binding sites can be reversibly increased 3-fold by temperature shift (Weigel & Oka, 1983). Since the binding properties (dissociation constant and total site number) of the lectin on the surface of rat hepatocytes are not uniform and invariant, we undertook the study of the binding of three structurally distinct ligands with similar affinities to rabbit hepatocytes.

The results indicate that rabbit hepatocyte preparations kept at 2 °C from the time of preparation (Materials and Methods) can yield reproducible binding parameters for each of these ligands (Table I, Figures 2–4). It should be noted that the total number of TRI or di-tris-lac binding sites is about 3–4 times the total number of ASOR sites. This same relationship between the number of binding sites for ASOR and fetuin glycopeptide on the surface of rat hepatocytes has been described (Fiete et al., 1983) but required prior treatment of the rat hepatocytes at 37 °C to increase the number of sites for fetuin glycopeptide in the binding assay. Rabbit hepatocytes strictly maintained at 2 °C after collagenase perfusion always displayed 3–4-fold more binding sites for TRI and di-tris-lac compared to ASOR. It is also evident that there are many more high-affinity di-tris-lac binding sites on rabbit hepatocytes than either high-affinity TRI or high-affinity ASOR sites (the number of high-affinity sites for ASOR and TRI are comparable). In terms of low-affinity sites, there are an average of 221 000 ± 38 000 sites/cell of K_d = 9.7 ± 3.5 nM for ASOR but 982 000 ± 115 000 sites/cell of K_d = 25.3 ± 7.9 nM for TRI and 1.23×10^6 ± 197 000 sites/cell of K_d = 32.4 ± 2.4 nM for di-tris-lac.

In an attempt to relate the additional binding sites of TRI and di-tris-lac to the ASOR binding sites, we first titrated the

high-affinity ASOR sites with unlabeled ASOR. These ASOR-loaded cells were then allowed to bind labeled ligand (either ASOR, TRI, of di-tris-lac) over a range of doses from 0.1 to 600 nM (Figure 5 and Table II). Only a single class of binding sites was available for ASOR or TRI binding on ASOR-loaded cells, but high-affinity binding sites for di-tris-lac remained.

If it is assumed that, for all three ligands, one ligand binds to one lectin molecule (i.e., that the saturating concentration of bound ligand is exactly equal to the concentration of binding sites), there would have to be about 1.06×10^6 TRI binding sites on one hepatocyte distinct from the two classes of ASOR binding sites (382 000 sites/cell). If this were so, one would expect to see binding of 1.06×10^6 molecules of TRI to ASOR-loaded cells. The experimental result, 495 000 molecules of TRI bound to a single class of binding sites on ASOR-loaded cells, does not account for 608 000 of these putative distinct TRI sites. Binding of di-tris-lac to ASOR-loaded cells revealed only 87 900 high-affinity sites/cell instead of the predicted 656 000 high-affinity sites (high-affinity di-tris-lac sites–high-affinity ASOR sites). The remaining low-affinity sites on ASOR-loaded cells available to di-tris-lac paralleled the finding with TRI: 429 000 sites/cell. The experimentally determined figure for di-tris-lac binding sites is thus also at variance with that predicted from the data for binding of ASOR and di-tris-lac to untreated hepatocytes (about 1.05×10^6 low-affinity sites/cell).

A reasonable model to account for the numbers of binding sites and the binding behavior of these ligands is to consider that not only are the Gal-combining sites arranged within the span of an oligosaccharide (Lee et al., 1983; Lee, Y. C., et al., 1984) but also high-affinity oligosaccharide binding units (presumably equivalent to TRI binding sites) are arranged in clusters on the cell surface (Figure 6). By this argument, it is not necessary to invoke many different kinds of sites available only to certain ligands to account for quantitative differences in the binding of the three ligands. Since 495 000 TRI or 429 000 di-tris-lac molecules bind to 171 000 ASOR sites on the ASOR-loaded cells (Table II), it can be inferred that one ASOR site represents two to three small ligand sites. In ASOR-loaded cells, ASOR may cover three TRI binding sites (nine Gal-combining sites), but it may not be necessary for ASOR to actually occupy sites to prevent additional ligand binding. Perhaps its relative size sterically shields these sites either from other ASOR molecules or from some TRI or di-tris-lac added to ASOR-loaded cells. This may explain the presence of high-affinity (K_d = 1.2 nM) di-tris-lac binding sites on cells in which the high-affinity ASOR sites have been blocked. These sites, inaccessible to ASOR or TRI (at least at the concentrations of ligand used), might be available to di-tris-lac due to its conformational flexibility or to the presence of three additional Gal residues (Lee, R. T., et al., 1984). These properties of di-tris-lac might also explain the much greater number of high-affinity sites for this ligand on the surface of rabbit hepatocytes. The stoichiometric argument further implies that at least three TRI binding units would have to be clustered together on the surface of rabbit hepatocytes on the order of the span of an ASOR molecule, as depicted in Figure 6.

The shape of the binding isotherm can also be accounted for by considering an array (lattice) of Gal binding units. Lattices entropically resist being saturated by a multivalent ligand because the ligand tends to exclude itself from binding, due to its multiple attachment points, as the fractional saturation of the lattice increases. Therefore, the apparent K_d

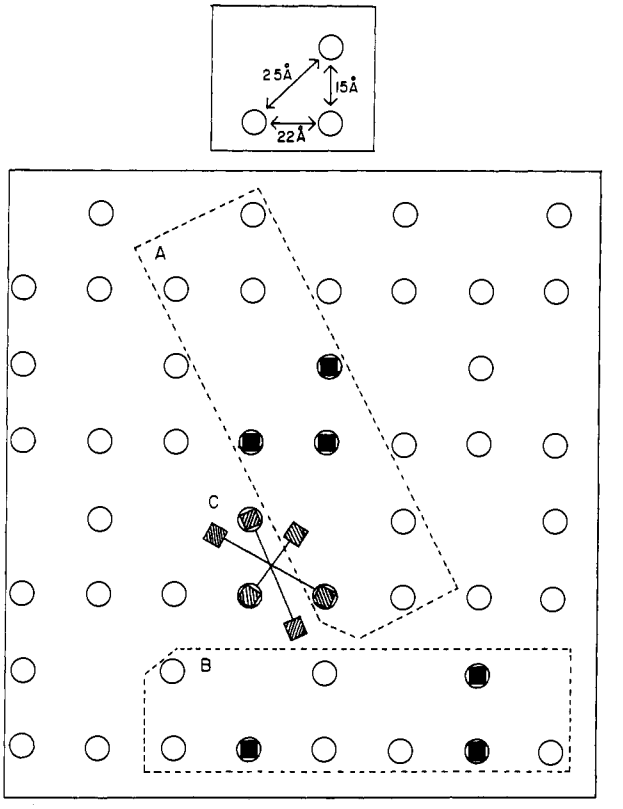

FIGURE 6: Model of binding of different ligands to surface of rabbit hepatocytes. The open circles represent unoccupied Gal-combining sites. The inset shows the high-affinity oligosaccharide binding unit (three Gal-combining sites, possibly all identical in physical properties) postulated from biochemical studies (Lee et al., 1983; Lee, Y. C., et al., 1984) on the binding of synthetic oligosaccharides to rabbit hepatocytes. This unit is presumably identical with a TRI binding site. The site–site distances shown were calculated from physical measurements (Bock et al., 1983) of the oligosaccharides displaying highest affinity. The model depicts a portion of a two-dimensional array of the high-affinity oligosaccharide binding units on the hepatocyte surface. When the saturation of this lattice of sites is low, an ASOR molecule (area A) could bind to one high-affinity oligosaccharide binding unit but, due to its bulk, render three units unavailable for ligand binding. On a crowded lattice, additional binding of ASOR could occur to clusters of sites with a less favorable conformation (area B), accounting for the lower affinity "class" of ASOR binding sites revealed at high fractional saturation. The presence of high-affinity binding sites for di-tris-lac even on cells loaded with ASOR might be due to the greater conformational flexibility of this ligand with six Gal residues, allowing it to bind to unoccupied Gal-combining sites eclipsed by ASOR with respect to either TRI or further ASOR binding (area C).

describing the binding decreases with increasing saturation, giving rise to Scatchard plots that are concave upward, even without heterogeneity of either site or ligand nor negative interaction (cooperativity) between sites. The concept of a lattice of surface lectin sites is certainly attractive given the morphological evidence, albeit with much coarser resolution, that at least some of the lectin sites are clustered in coated pits on isolated rat hepatocytes (Kolb-Bachofen, 1981; Zeitlin & Hubbard, 1982).

Theoretical and experimental bases exist for statistical treatment of the problem of polyvalent ligands binding to one-dimensional lattices of identical binding sites (McGhee & von Hippel, 1974; Epstein, 1978). A theoretical model of the binding of large ligands to two-dimensional lattices of binding sites has been recently developed (Stankowski, 1983). Qualitatively, the entropic effects in the two-dimensional case are similar to the one-dimensional case but are further complicated in that the geometry of the lattice and the shape of

the ligand, as well as its valence, effect the shape of the binding curve. Therefore, more information on the geometry of the system is needed to permit analyses of the present data in terms of this model. Definition of the cell surface arrangement of high-affinity oligosaccharide units will require binding studies using defined ligands with known distances between three Gal clusters.

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