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Lectin−Erythrocyte Interaction with External Transmembrane Glycophorin Saccharides Controlling Membrane Internal Cytoskeleta

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Human red blood cell (RBC, erythrocyte) membranes have internal protein skeletons that govern the cells' distinctive discocyte−echinocyte morphology (shape) changes, seen in conventional microscopy. Glycophorin, the cell's transmembrane protein, presents all of its saccharides outside the cell. The protein sector of glycophorin is linked inside to the RBC cytoskeleton, enabling lectins binding to the external saccharides to gain profound control over internal cytoskeleton behavior, expressed by governance of the visibly seen cell shape. Critical lectin binding stoichiometries (^{125}I-labeled lectins) equate to the number of glycophorin monomers per RBC, 7×10^5 copies/cell. Wheat germ agglutinin lectin (sialic acid specific) binds to glycophorin's outermost (exo) saccharides and exerts tight control over the cell's morphology. Removal of sialic acid groups (desialation) exposes the endosaccharides of glycophorin, enabling peanut agglutinin and Osage orange lectins to gain equally tight control over the RBC's morphology behavior in simple stoichiometric ratios, bound lectin molecules/glycophorin receptor. Thus, lectin specificities for saccharides are sharply in register with the glycophorin external saccharide composition, the sequence along the chains, and the number of copies of protein (stoichiometry). These relationships were determined via RBC shape change equilibria and also via shape change rates. Rate data are somewhat laborious to determine, but are exquisitely sensitive to lectin specificities and in very small lectin concentrations. Both classes of data enable these interactions to be analyzed in lectin and RBC concentrations ~100-fold smaller than agglutinating levels.

KEYWORDS: Lectins; glycophorin; erythrocytes; transmembrane signaling; saccharide specificity; analysis; rates

INTRODUCTION

Lectins specific for saccharides increasingly are used in many tasks: tissue and cell typing, isolation procedures, and many analytic methods. Often lectins can supplement and even replace expensive antibodies for these purposes, as lectin specificities become known. Frequently antibodies have quite unknown specificities if their antigenic target is not known in molecular detail. Lectin interaction toward cells and membranes has largely been monitored by agglutination of targeted cells. However, agglutination/precipitation alone reveals little of cell membrane mechanics or topology, the consequence of lectin−membrane interaction. Lectins reflect not only lectin−cell mutual binding reactions and specificities on membrane outside surfaces but also the consequences to membrane mechanics, which depend on transmembrane proteins in contact with membrane internal proteins.

In the human red blood cell (RBC), the internal proteins immediately underlying the outer membrane form the cell's internal cytoskeleton. The RBC transmembrane protein on which this paper focuses is glycophorin, diagrammed in **Figure 1**. Human RBCs normally carry 7×10^5 glycophorin monomer proteins per cell (*1*). The saccharides are all on the outside surface, 15 serine/threonine-linked oligosaccharides and 1 asparagine-linked saccharide per monomer (*2*), displaying close to 30 or 31 *N*-acetylneuraminic acid (NANA, sialic) groups, on the exo-positions. Glycophorin monomers normally are dimerized within the membrane palisade. The glycophorin internal peptides contact the cell's internal cytoskeleton and impose considerable control of the cell's overall shape (morphology), which can be easily seen. RBC reversible shape changes seen by microscopy as discocyte ⇆ echinocyte conversion reactions are illustrated in **Figure 2**.

These visible discocyte ⇆ echinocyte reactions can be moved in both directions by several convenient means. Both directions of these morphology change reactions are efficiently blockaded by lectins specific for glycophorin's external saccharides (*3*)

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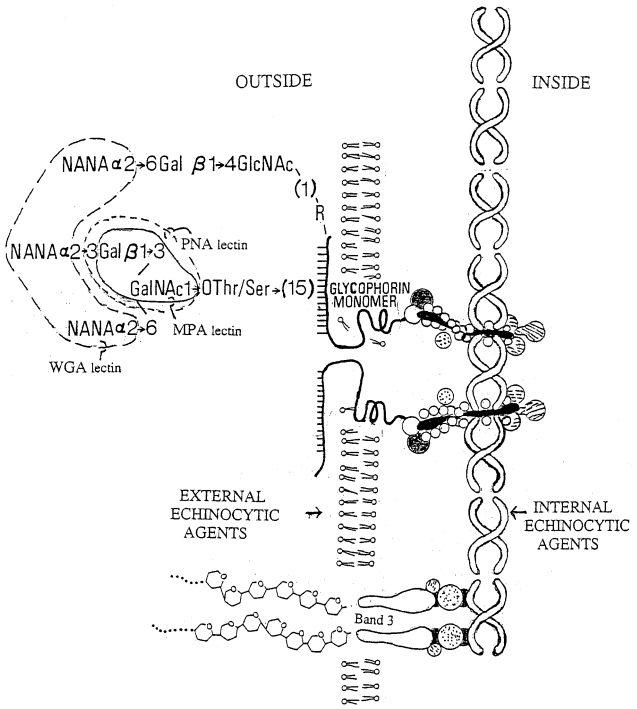

Figure 1. Erythrocyte membrane principal structural features. The internal cytoskeleton scaffold in conjunction with the lipid layers ("bilayer") provides membrane mechanical properties that determine the cell's overall morphology (shape). Transmembrane glycophorin linked to the cytoskeleton regulates cell shape. One lectin molecule specific for external glycophorin saccharides, bound per glycophorin dimer, can blockade inside cytoskeleton reactions, which are manifested as cell shape changes.

(but not by lectins specific for saccharide antigens on the RBC remote from glycophorin). A practical consequence is that the sensitivity of this lectin assay with respect to cell suspension concentrations is ~100-fold greater than conventional agglutination-based assays, besides generating a broadened perspective on how lectin−membrane mechanics operate. The molecular components of the reactions contributing to lectins affect the RBC membrane, thence, the RBC internal cytoskeleta and shape control, are outlined in **Figure 3**. Besides the equilibrium behavior expressed in discocyte ⇆ echinocyte interconversion, both conversion reactions have their characteristic rate behavior, strongly affected by glycophorin-specific lectins, in very small lectin concentrations with simple stoichiometric binding levels. Such sensitivity in rate behavior plus the specificity of lectins vis-à-vis glycophorin saccharide composition and sequence provides a set of tools for assay of lectin−membrane interaction. To quantitate numbers of lectin molecules actually bound per RBC glycophorin receptors, ^{125}I-labeled lectins were used in both categories of work, equilibrium behavior and rates behavior.

MATERIALS AND METHODS

General methods for preparing human erythrocytes in lectin blockade studies and adjusting erythrocytes to subagglutinating concentrations, 1×10^7 cells/mL in isotonic buffer, are described in our earlier papers (*3*). Desialation to remove NANA terminal carbohydrates from RBCs was effected by either *Vibrio cholerae* or *Clostridium perfringens* neuraminidase (Sigma) in pH 7.2−7.4 Tris−saline buffer, with 10 mM $CaCl_2$ in the case of the *V. cholerae* enzyme. Desialation used 1×10^9 cells/mL and 0.2 unit of neuraminidase/mL for 1 h, 37 °C, including 10 mM glucose and 5 mM inosine to maintain RBC internal metabolism and discocyte integrity. Released NANA was quantitated by thiobarbituate spectrophotometry (*4*); ~3.9×10^7 NANA residues/cell were released. The wheat germ agglutinin (WGA) lectin was prepared from raw wheat germ by using Bouchard et al.'s method (*5*) and affinity chromatographed using Marchesi's ovomucoid−Sepharose system (*6*). Peanut agglutinin (PNA) was isolated and purified according to the Terao et al. method (*7*), from raw Virginia peanuts. PNA was affinity chromatographed on lactose−Sepharose using Uy and Wold's system (*8*). Osage orange (MPA) lectin was purchased (E-Y Laboratories, San Mateo, CA). Each lectin showed one band on SDS-PAGE with a 50 μg loading. Weight absorption coefficients $E^{1\%}$ at 280 nm and molecular weights for the respective lectins were as follows: WGA, 15.0 and 36000 Da; PNA, 6.8 and 108500 Da;. MPA, 15.7 and 43000 Da. Lectin binding levels (ν_{lectin} = molecules of lectin bound per cell) were determined using ^{125}I-labeled lectins of specific activities between 10^7 and 10^8 dpm/mg, combining labeled with cold lectin. Fraker and Speck's procedure (*9*) enabled ^{125}I labeling.

For initiating one-way discocyte ⇆ echinocyte conversion, two independent means were used: (i) outside (membrane external) lipid anions, chiefly laurate anions in 100 μM concentration; (ii) inside (cell internal) agents, 0.1 mM Ca^{2+} carried inward by 5 μM A23187 ionophore. Both agents act reversibly, allowing the reverse morphology conversion, echinocyte → discocyte, to occur starting from near 100% echinocytes. Laurate was titrated away, removed, by plasma albumin, which strongly binds laurate in isotonic buffers (*10*). Calcium ionophore removal was quantitated by ^{45}Ca isotope analysis, promoted by ethylene bis(β-aminoethyl) ether chelator (EGTA) together with plasma albumin scavenging of the A23187 ionophore. Return of Ca^{2+}-induced echinocytes to discocytes was energized in erythrocytes by 5 mM glucose and 2 mM inosine (*11*).

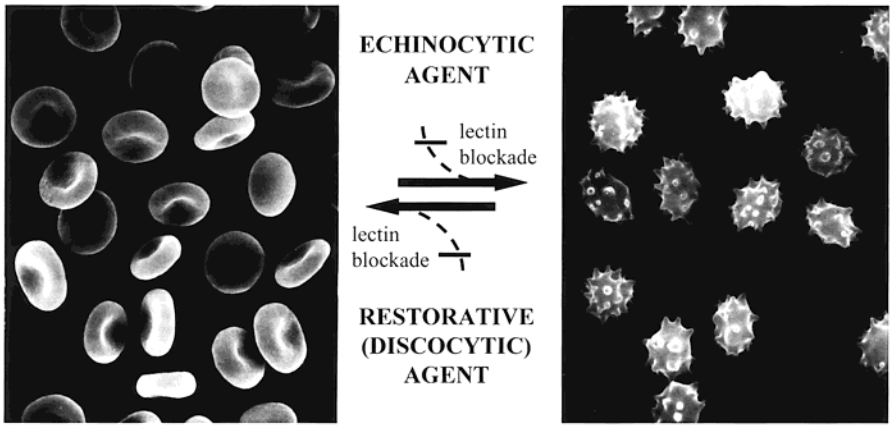

Figure 2. Erythrocyte morphology equilibria; reversible conversion of discocyte ⇆ echinocyte (×400 magnification; reproduced at 50% of the figure's original size). Both directions of morphology conversion are easily promoted, but glycophorin-specific lectins blockade morphology conversion behavior. Echinocytic agents are laurate anion (outside agent) and Ca^{2+} ionophore (inside agent). Restorative agents function to remove these, together with glucose to restore internal metabolism.

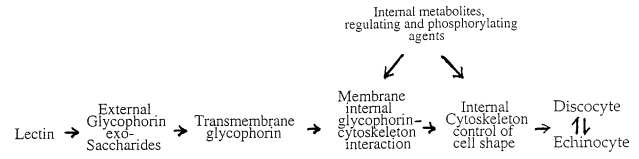

Figure 3. Functional outline of the linkage from lectin binding on external saccharides of glycophorin inward to the membrane cytoskeleton, which determines RBC morphology; discocyte ⇆ echinocyte interconversion.

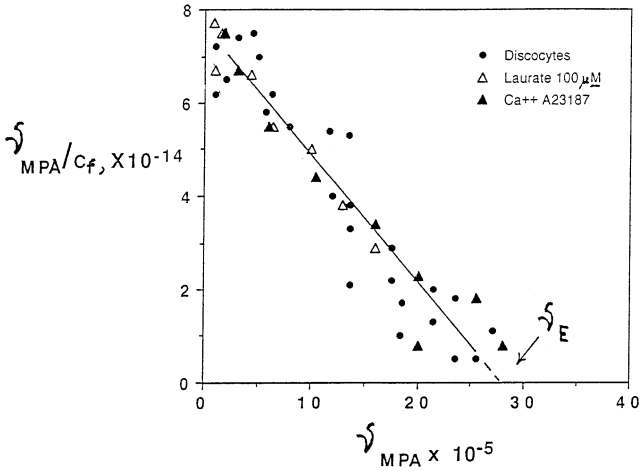

Figure 4. Scatchard plot of Osage orange (MPA) lectin, ^{125}I labeled; binding at equilibrium to saturate available sites of desialated erythrocytes at ν_E, isotonic saline, pH 7.4, 25 °C. The plot for discocytes is essentially congruent with plots for both varieties of echinocytes, generated by outside (laurate anion) and inside (Ca^{2+}-A23187) agents.

To obtain the primary rate data for the forward reaction discocyte → echinocyte modulated by lectins specific for the glycophorin saccharides on intact erythrocytes, and on asialoerythrocytes, the cells were fixed by 0.1% SEM grade glutaraldehyde (which stops all morphology change) at each time interval. The fixed samples were counted by ×400 magnification microscopy, 100−150 cells in each field to determine the fraction, denoted *r*, of cells converted in morphology (ordinate, in **Figure 5**). Velocities of conversion are equated to the slopes of *r* as a function of time *t*, multiplied by cell concentrations. They have units of cells per milliliter per second.

Lectins were added to discocytes before the echinocytic agent was added. Glycophorin-specific lectins completely block, or slow, discocyte → echinocyte conversion depending on the lectin/cells ratio. Lectins decrease morphology conversion rates, giving a range of rates, depending on ν_{lectin}, expressed as molecules of lectins bound per cell. A main goal is to find the number of lectin molecules necessary to become bound, at which morphology conversion blockade takes hold, called ν_ν. The ν_ν quantities are threshold values of ν_{lectin}, for comparing to the known number of glycophorin external saccharides, the stoichiometry. The experimental design includes using these three lectins having specificities for glycophorin saccharides outlined in **Figure 1**. They identify this transmembrane protein for mediating signals from external oligosaccharides, inward to inside cytoskeleton function, expressed as control over the cell's overall shape.

RESULTS

Lectin Binding under Equilibrium in Scatchard Plots; Measurement of Apparent Numbers of Available Sites, ν_E. Binding isotherms for all three lectins, WGA, PNA, and MPA, were determined in their equilibria with both the echinocyte and discocyte morphologies. The isotherms were determined for echinocytes produced by laurate ligands operating outside at the membrane surface and also by inside Ca^{2+} ionophore action in concentrations described above. For measurement starting with echinocytes, all cells were driven to complete echinocyte morphology before lectin was added. Such is necessary because lectins are powerful blockades for morphology changes in either direction (*1*). Discocytes were maintained by keeping them energized in glucose−inosine−isotonic buffer, noted above.

Table 1. Number of Sites per Erythrocyte Filled at Equilibrium, ν_E: Scatchard Plot Intercepts

lectin	cells, echinocytic agent	ν_E
WGA	discocytes, native	31×10^5
WGA	desialo discocytes	28×10^5
WGA	spheroechinocytes, DDS^-	29×10^5
WGA	echinocytes, Ca^{2+}-A23187	29×10^5
PNA	discocytes, native	$(0.5–5) \times 10^5$
MPA	discocytes, native	$(1–2) \times 10^5$
PNA	desialo discocytes, native	21×10^5
PNA	desialo echinocytes, laurate	21×10^5
PNA	desialo echinocytes, Ca^{2+}-A23187	21×10^5
MPA	desialo discocytes	28×10^5
MPA	desialo echinocytes, laurate	28×10^5
MPA	desialo echinocytes, Ca^{2+}-A23187	28×10^5

Representative Scatchard plots for ^{125}I-labeled MPA lectin in equilibrium binding to these three states of erythrocyte morphology, all asialoerythrocytes, are shown in **Figure 4**. The data show that the course of the binding isotherms for all three cases is essentially congruent within the scatter limits of the data points, producing the linear plot of **Figure 4**. **Table 1** lists the corresponding intercept values, ν_E, which is a measure of the apparent number of available sites on the erythrocyte receptive to MPA lectin. The MPA lectin is tetravalent and specific for the endo-disaccharide Gal β (1 → 3) Gal NAc, but in native (sialic acid complete) cells, the endo-disaccharides lie underneath the exo-, or terminal, sialic acid monosaccharides as shown in **Figure 1**, for all 15 threonine/serine O-linked glycophorin oligosaccharides. Accordingly, native sialic acid complete erythrocytes bind relatively little MPA lectin, producing $\nu_E \sim 1-2 \times 10^5$, much less than $\sim 7 \times 10^5$ glycophorin monomers and far less than the numbers of individual glycophorin oligosaccharides/cell, some 105×10^5 of them. In great contrast shown in **Table 1**, desialo discocytes and both categories of desialo echinocytes (inside and outside agents) exhibit $\nu_E \sim 28 \times 10^5$ available sites per cell for MPA when the exo NANA saccharides are all removed by neuraminidase. The disaccharides necessary for MPA binding become exposed when NANA is completely removed. Because MPA is tetravalent, $4 \times 28 \times 10^5$ or $\sim 112 \times 10^5$ glycophorin desialo-oligosaccharides are involved, essentially filling all of them, corresponding with the number of glycophorin saccharide receptors of normal cells. They evidently stay in place and function fully throughout the discocyte ⇆ echinocyte maneuvers and through the neuraminidase treatments.

Similarly, PNA binding to native (sialic acid complete) cells is sparse, because its target is the endo-positioned Gal β (1 → 3) Gal NAc dissacharide. Desialation exposes the endoglycophorin oligosaccharides to ^{125}I-labeled PNA for filling these sites. Accordingly, PNA produces equilibrium binding stoichiometries, ν_E values in **Table 1** for filling the cell's asialo glycophorin sites, congruent with MPA's stoichiometry and behavior. Few sites are available for PNA (or MPA) in native sialic acid complete discocytes, but many sites appear for these lectins, $\sim 21 \times 10^5$ per cell, when NANA is removed. Thence, the primary binding target for these lectins is the assembly of external saccharides on glycophorin for both cell morphologies however produced, consistent with PNA's tetravalency for saccharide monomers.

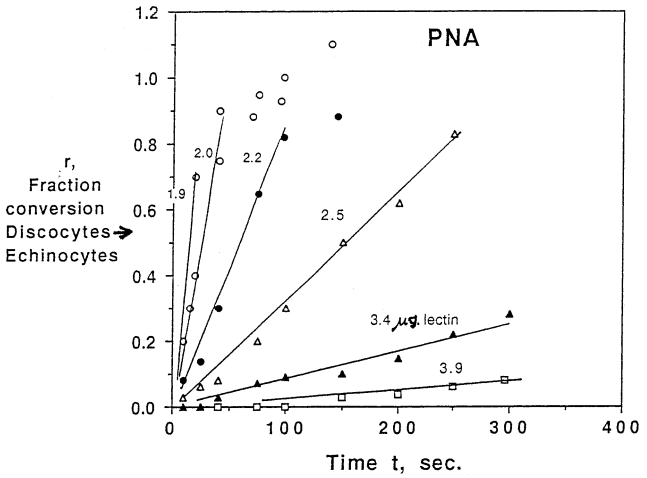

Figure 5. Initial velocities and slopes of *r* (of cells converted to echinocytes from discocytes) dependent on time, modulated by PHA lectin in microgram amounts starting with desialated discocytes.

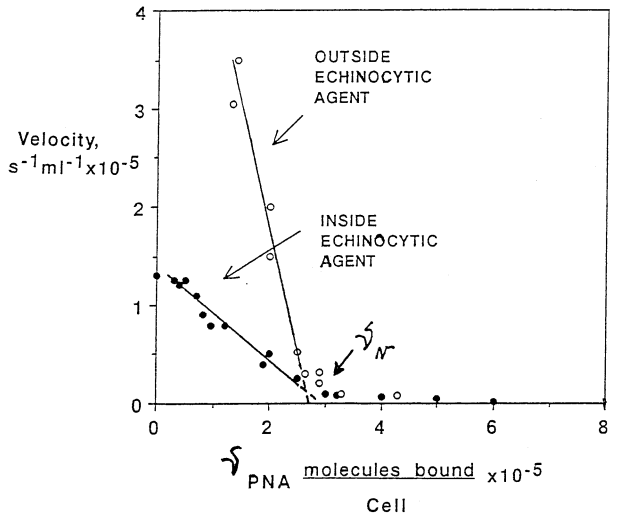

Figure 6. Velocities (ordinate) versus extent of binding of ^{125}I-labeled PNA lectin (abscissa): determination of the apparent number of erythrocyte sites ν_ν at which PNA fully blockades cell morphology conversion for both classes of echinocytogenic agents; 100 μM laurate (outside agent); Ca^{2+}-A23187 (inside agent), 25 °C.

The WGA lectin binds *N*-acetylglucosamine, but WGA has an enhanced, sharp specificity for NANA bearing oligosaccharides and antigens (*12*). **Table 1** shows WGA's binding stoichiometry as the ν_E parameter is $29-31 \times 10^5$ WGA molecules bound/cell for all three kinds of RBCs retaining their NANA groups, native discocytes, and both varieties of echinocytes produced by inside and outside acting agents. Close agreement with the number of glycophorin dimer copies is apparent.

Velocities of Erythrocyte Morphology Conversion Modulated by Lectins Specific for Glycophorin External Saccharides. For monitoring the impact of lectins on erythrocyte morphology conversion, the intensity of conditions for promoting either direction of discocyte ⇆ echinocyte changes were lowered well below those used for the equilibrium experiments. Morphology changes were slowed to take place in several minutes instead of a few seconds (as for the equilibrium studies). The two categories of measured data for rate-dependent operation were r = fraction of cells converted from an initial morphology at time zero, to a changed morphology over several time intervals, and measurement of lectin binding via ^{125}I labeling, for each set of conditions. The rate-dependent investigations were carried out with intact (NANA complete) and asialoerythrocytes, with a number of levels of echinocytic agents, largely laurate anions, but dodecyl sulfate is also usable and controllable. The reverse reaction, the conversion echinocyte → discocyte, has a more limited range of accessible velocities because it depends on the cell's cytoplasmic metabolism in response to carbon sources. It is generally slower than most of the discocyte → echinocyte conversion reactions. Velocities of the return conversion, echinocyte → discocyte, also are dependent on plasma albumin concentrations, used to strip away laurate anion and remove A23187 ionophore.

Figure 5 plots illustrative rate data, the fraction of conversion of discocytes → echinocytes (*r* ordinate), depending on the concentration of lectin. The same samples were counted via ^{125}I-labeled lectins for all three lectins. **Figure 5** pertains to MPA lectin. Thence, the initial rates of conversion were determined with respect to amounts of lectin actually bound and with respect to the status of glycophorin (asialo-, intact with normal NANA content), in accord with each lectin's specificity for its glycophorin saccharides.

These velocities, together with their corresponding amounts of bound lectin appropriate to the status of glycophorin, were then used for plotting **Figure 6**, in PNA's case. Plots of this kind, in a very short extrapolation to the abscissa, arrive at the quantity ν_ν, which is the threshold for lectin binding where blockade is evidently complete. The velocity of morphology conversion slows to zero; the cell membrane is locked down. The ν_ν quantity is the focus of most interest, for comparison with the number of glycophorin receptor assemblies on the surface of the cell—the critical stoichiometry of cell surface sites providing apparently complete blockade of conversion. **Figure 6** shows that the velocities of morphology conversion by inside echinocytic versus outside echinocytic agents are somewhat different from one another, but the plots intercept at virtually the same point, the same ν_ν value. These ν_ν intercepts, for the three lectins, related to the status of glycophorin and its susceptibility to lectin specificity, are gathered in **Table 2**.

Table 2. Lectin Binding Sites per Erythrocyte, ν_ν, at Blockade Limit of Velocities of Morphology Conversion

lectin	cells, echinocytic agent	ν_ν intercept at blockade
	A. Discocyte → Echinocyte Conversion	
WGA	native, laurate	16×10^5
PNA	desialo-, laurate	2.7×10^5
PNA	desialo-, Ca^{2+}-A23187	2.8×10^5
MPA	desialo-, laurate	3.2×10^5
	B. Echinocyte → Discocyte Complete Conversion	
WGA	native, laurate	16×10^5
PNA	desialo-, laurate	2.3×10^5
MPA	desialo-, laurate	2.4×10^5
	C. Echinocyte → Discocyte Partial Conversion	
PNA	desialo, laurate, 70% return	0.7×10^5
PNA	desialo, laurate, 50% return	0.8×10^5

There is some dependency on how severely the morphology conversion reactions are driven: "softer" driving conditions as in **Table 2C** produce lower ν_ν (velocity intercept sites) than more severe conditions as are involved with larger levels of outside agent, as with laurate anion. A large number of such plots was generated, to analyze the extent to which erythrocyte surface receptors, glycophorin in particular and glycophorin in conjunction with lectins specific for discrete saccharides on glycophorin, exercise control over the cell's shape (*13*).

DISCUSSION

Human erythrocytes as targets for lectin reactivity provide an interesting, useful basis for analysis of lectin specificity and stoichiometry, that is, by monitoring the cells' morphology (shape) changes, which are distinct, easily seen, and counted. Very small erythrocyte concentrations are needed: only $\sim 1 \times 10^7$ cells/mL in normal saline (*11*), $\sim$10−100-fold less than most assay techniques based on agglutination. Human erythrocyte morphology changes are quite reversible if overt membrane destruction has not occurred. Thence, they can be maneuvered in both directions of the discocyte $\leftrightarrows$ echinocyte conversion for evaluating cell membrane response toward lectins. Lectins specific for erythrocyte glycophorin saccharides provide well-focused tools for characterization and analysis of outer membrane−inner cytoskeleton reactivity through the transmembrane linkage outlined in **Figure 3**. This is determined by measurement of the "stoichiometry", the number of lectin molecules bound per cell in relation to the number of receptor sites (here, glycophorin) carried by the cell, at critical stages of the morphology changes.

The erythrocytes' 7×10^5 glycophorin monomers per cell are normally dimerized in the membrane, displaying $\sim 3.5 \times 10^5$ external sites, each of which carries $\sim$60 NANA groups on the outermost domain of the glycophorin dimer. The WGA lectin in neutral pH normally also is a dimer of two polypeptide chains, with no saccharides of its own. WGA has two primary strong binding sites for NANA groups (plus perhaps two weaker ones) if they are terminal, exosaccharides. The ν_E quantities for WGA of **Table 1** thus indicate close to one WGA dimer binding externally to each glycophorin dimer suffices for complete blockade of the cell's morphology change, in both directions, in any of the means for setting changes in motion to reach a new equilibrium. Some 60 NANA saccharides provided by each glycophorin dimer provide an excess of ligands to strongly bind one WGA dimer, inducing the transmembrane molecular linkage actions outlined in **Figure 3**.

Whereas the NANA groups are in place on the intact cell, the other two lectins, MPA and PNA, bind very poorly in the equilibrium mode, regardless of how the morphologies were derived (native discocytes, inside agent and outside agent echinocytes), seen in their scanty ν_E binding levels in **Table 1**. However, when NANA groups are removed, for both asialo-discocytes and echinocytes, the 3 Gal-β 1 $\rightarrow$ 3 Gal NAc disaccharides, rendered exposed by removal of the sialic acid groups, essentially duplicate WGA's control in all these respects and in the same stoichiometric relationship to the number of glycophorin dimer sites available for equilibrium binding. Thus, **Table 1** lists $21-28 \times 10^5$ MPA and PNA lectin molecules bound in their ν_E parameter, for the various conditions of discocyte echinocyte status, for all desialo forms of the cell. Summarizing so far, the three lectins quite accurately display glycophorin-directed specificities in saccharide composition of glycophorin, in the sequence of saccharides, and in the simplest stoichiometric sense. That is, one lectin is bound per one glycophorin dimer for promoting complete blockade of either direction of the cell's morphology conversion at full equilibrium in the lectin−erythrocyte interaction.

Rate or velocity studies that reflect lectin control of discocyte $\rightarrow$ echinocyte and echinocyte $\rightarrow$ discocyte conversion mediated through glycophorin provide another means for evaluating lectin−erythrocyte behavior using very small amounts of reactants, lectins and erythrocytes. The general method is rather laborious: counting morphologies to determine fractions of conversion dependent on time and lectin concentration, in conjunction with determination of the degree of lectin binding via ^{125}I-labeled lectins. Cell morphology conversions were propelled by conditions parallel to the foregoing equilibrium studies. However, the velocity-based method is basically simple and very sensitive to lectins addressed to glycophorin.

When conditions for driving morphology conversion are slackened, to observe rates at lesser degrees of conversion (<100%), the ν_ν parameters as in **Table 2C** indicate that considerably fewer lectin molecules, PNA or MPA, per glycophorin dimer suffice to exert profound rate control. In contrast, WGA's ability to govern rates in the case of native (NANA complete) cells, to slow conversion virtually to a stop, requires $\sim 16 \times 10^5$ WGA/cell. This is provisionally interpreted as one WGA molecule required per glycophorin dimer. Cleavage of NANA from glycophorin is required to enable PNA and MPA to get a grip on the cell, but evidently these desialated cells can be made to respond with ν_ν parameters to their appropriate lectins at even lower levels using only $\sim$10% of the known numbers of external glycophorin sites.

The most accessible human cell is the erythrocyte. Cells from less than five drops of blood (washed, isotonic) are quite sufficient for many measurements of the kinds described above. The focal point for these lectins is glycophorin, using intact cells (NANA complete) for WGA and desialated cells for MPA and PNA. It shall be interesting and useful to deploy these lectins for detecting and evaluating genetic variants of erythrocytes where there occur glycophorin deficiencies or abnormalities. The prism through which lectin−erythrocyte interaction is expressed is the saccharide character and transmembrane character of glycophorin embedded in the membrane with all its saccharides exposed on the surface.

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