Isolation of the Receptors for Wheat Germ Agglutinin and the Ricinus communis Lectins from Human Erythrocytes Using Affinity Chromatography*

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SUMMARY

The glycoproteins of human erythrocyte ghosts were solubilized with 0.5% Triton X-100 in 56 mM sodium borate, pH 8.0. This procedure solubilized 51% of the membrane protein, 81% of the sialic acid, 89% of the receptors for the Agaricus bisporus lectin and 70 to 75% of the wheat germ agglutinin and Ricinus communis lectin receptors. The solubilized glycoproteins were then separated by affinity chromatography on lectin-Sepharose columns. Glycoproteins which adsorbed to the lectin columns were eluted with the appropriate hapten sugar and analyzed for carbohydrate composition, mobility in sodium dodecyl sulfate polyacrylamide gels, and lectin binding ability. Glycoproteins which contained the membrane binding sites for the R. communis and Abrus precatorius lectins adsorbed to ricin and R. communis agglutinin 1-Sepharose columns while the glycoproteins containing most of the receptors for the A. bisporus phytohemagglutinin (PHA), Phaseolus vulgaris erythroagglutinating (E)-PHA, Lens culinaris PHA, and wheat germ agglutinin passed through the column. The adsorbed glycoproteins were 1200 times more potent than galactose as hapten inhibitors of R. communis lectin binding to cells. The carbohydrate composition of these glycoproteins was determined to be (in residues relative to N-acetylgalactosamine): N-acetylgalactosamine (3), N-acetylgalactosamine (0.2), galactose (3.0), mannose (0.7), fucose (0.5), and sialic acid (0.3). When examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the glycoproteins could not be detected with the periodic acid-Schiff stain but were detected as several glycoprotein peaks by direct amino sugar analysis of the gels.

When Triton-solubilized material was passed over the wheat germ agglutinin-Sepharose column, only one glycoprotein adsorbed to the column. This glycoprotein was a potent hapten inhibitor of wheat germ agglutinin, A. bisporus PHA, and P. vulgaris E-PHA. On sodium dodecyl sulfate polyacrylamide gels the glycoprotein had a mobility identical with that of the major sialoglycoprotein of the erythrocyte.

Its carbohydrate composition was determined to be (in residues relative to N-acetylgalactosamine): N-acetylgalactosamine (3), N-acetylgalactosamine (7.5), galactose (6.9), mannose (1.5), fucose (0.9), and sialic acid (9.9). This composition is very similar to that of the major sialoglycoprotein. Pronase digestion of this glycoprotein reduced its lectin hapten inhibitory activity by greater than 98%.

These data demonstrate that affinity chromatography of human erythrocyte membranes on lectin-Sepharose columns results in the separation of two distinct classes of glycoproteins with different carbohydrate compositions and different lectin binding properties.

Plant lectins are carbohydrate-binding proteins which are extremely useful in the study of cell membrane glycoproteins and their oligosaccharide units (1). Over the past several years investigations in our laboratory have shown that the receptors on the human erythrocyte for the Phaseolus vulgaris erythroagglutinating phytohemagglutinin, Lens culinaris phytohemagglutinin Robinia pseudoacacia PHA,1 and Agaricus bisporus PHA are oligosaccharide chains of membrane glycoproteins (2-5). In these studies erythrocytes were treated with trypsin to release membrane glycopeptide material that contained approximately 40 to 50% of the receptors for these lectins. The oligosaccharide receptors on the released glycopeptides were then isolated and characterized. This approach has several limitations. First, it only releases receptors that are located on trypsin-sensitive glycoproteins. Second, it yields a mixture of glycopeptides which could either have been derived from a number of different membrane glycoproteins, or from different portions of the same glycoprotein. And third, trypsin treatment degrades the intact membrane glycoproteins so that it is impossible to examine the lectin receptors in their native state. For example, studies of the integration of the glycoprotein receptors into the lipid bilayer of the membrane cannot be performed. Therefore, we sought a

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1 The trivial names and abbreviations used are: PHA, phytohemagglutinin; E-PHA, erythroagglutinating phytohemagglutinin; WGA, wheat germ agglutinin; RCA I, Ricinus communis agglutinin I; PAS, periodic acid-Schiff.
membrane glycoproteins were solubilized with the detergent, Triton X-100, and then fractionated by affinity chromatography on lectin-Sepharose columns. For the initial studies lectin-Sepharose conjugates of the *Ricinus communis* lectins and wheat germ agglutinin were prepared. These lectins were selected because their binding to cell membrane receptors could be effectively blocked by simple sugar haptenes (galactose in the case of *R. communis* lectins and N-acetylglucosamine in the case of WGA) providing a convenient method for eluting glycoprotein receptors from the affinity columns. In addition, little information was available concerning the nature of the membrane receptors for these lectins.

The general procedure was to solubilize erythrocyte ghost glycoproteins with Triton X-100 and then pass the solubilized material through lectin-Sepharose columns. The adsorbed glycoprotein material was then eluted with the appropriate hapten sugar. Using this procedure we have found that the major glycoprotein of the erythrocyte contains receptors for WGA, *P. vulgaris* E-PHA, *L. culinaris* PHA, and *A. bisporus* PHA, whereas the receptors for the *R. communis* and *Abrus precatorius* lectins are on different glycoproteins which are almost devoid of sialic acid. In addition, several characteristics of the binding of WGA and the *R. communis* lectins to human erythrocytes have been determined.

**EXPERIMENTAL PROCEDURE**

**Materials**—Triton X-100 was purchased from Rohm and Haas. Na₃PO₄ (reagent grade) was a product of Mallinckrodt. Ovomucoid and wheat germ were obtained from Sigma Chemical Co. Pronase and *Vibrio cholerae* neuraminidase were from Calbiochem. Trypsin 1/250 and phytohemagglutinin P were purchased from Miles. *A. bisporus* seeds were purchased from Calbiochem. Neuraminidase and Pronase were from Calbiochem. *R. communis* lectin was prepared from outdated bank blood by a similar method of the method of Dodge et al. (22). The ghosts were extracted with Triton X-100 by the procedure of Yu et al. (23) as follows: 175 ml of ghosts were added to 800 ml of an ice-cold solution containing 2% Triton X-100 and 50 mM sodium borate buffer. The pH was adjusted to 7.4 with 2 M NaOH. After stirring for 20 min and centrifugation, the supernatant fluid was then passed through each column at room temperature. The columns were washed with 100 ml of 1 M NaCl in Buffer A followed by 100 ml of Buffer A. The *Ricinus* lectin-Sepharose columns were then eluted with 0.1 M lactose in 0.1 M NaCl and 0.1 M NaHC03 in the same buffer. The eluates were dialyzed against 300 volumes of 2 M sodium phosphate, pH 8.0, with two buffer changes. The dialyzed material was then concentrated to 5 ml on an Amicon pressure concentrator using a UM 10 membrane and stored at 4°C. When the affinity columns were used for the first time, it was possible to elute the adsorbed lectin activity with trypsin-containing Triton buffers. In these instances the columns were washed with 100 ml of 1 M NaCl in Buffer A followed by a wash with 10 ml Tris-chloride buffer, pH 8.4, until the absorption of the eluant at an optical density of 280 nm fell to less than 0.05. The adsorbed material was then eluted with 10 ml Tris-chloride buffer containing the appropriate hapten. The clear eluates were exhaustively dialyzed with 2 M sodium phosphate buffer, pH 8.0. After the lectin-Sepharose columns had been used more than once, the adsorbed membrane material could only be eluted with Triton-containing buffers.

**Assay for Lectin Receptor Activity**—The lectin receptor activity of the solubilized membrane material was determined in a standardized hapten inhibitory assay system. In this assay, receptor activity of a given lectin was estimated by the inhibition of binding of iodinated lectin to the erythrocyte membrane. The standard assay mixture contained: 50 μl of a 1:100 dilution of packed ghosts (equivalent to 5 X 10⁶ cells), 250 μg of bovine serum albumin, an amount of 125I-lectin which was in the linear range of an erythrocyte binding curve (generally 0.2 to 1 μg), an aliquot of the solubilized receptor, and 0.9% NaCl-0.01 M NaHCO₃ to a total volume of 60,000 and RCA I a molecular weight of 118,000. These values agree with the findings of other investigators (7, 8, 12, 17, 18).

1 In an attempt to alleviate confusion in the nomenclature of the *R. communis* lectins, the 60,000 molecular weight highly toxic lectin will be called ricin (10-17), whereas the 120,000 molecular weight lectin will be called RCA I after the nomenclature of Tomita et al. (7). Previously we referred to ricin as *Ricinus* PHA II and to RCA I as *Ricinus* PHA IV (9). The clear eluates were exhaustively dialyzed with 2 M sodium phosphate buffer, pH 8.0. After the lectin-Sepharose columns had been used more than once, the adsorbed membrane material could only be eluted with Triton-containing buffers.
of 0.2 ml. Erythrocyte ghosts were used in the assay since the Triton X-100 present in the solubilized receptor material lysed intact erythrocytes. The complete reaction mixtures minus ghosts were incubated at room temperature for 15 min. After this interval the ghosts were added and the incubation continued for an additional 30 min. Five milliliters of 0.9% NaCl were then added, and the diluted solution filtered by suction through Millipore filters (PHWP, 0.3 μm) which had been presoaked in 0.5% bovine serum albumin for at least 1 hour before use. The filtered ghosts were washed twice with 5-ml portions of saline solution and the filters placed in plastic counting tubes and counted in a Packard Autogamma counter. All assays were run in duplicate and compared to an identical reaction mixture in which the receptor was omitted. One I.U. is defined as that amount of receptor which gave 50% inhibition of binding in the standard assay. Care was taken to utilize an amount of receptor which gave 10 to 40% inhibition, since only in this range was the response linear. Because the purified concentrated receptors had a tendency to aggregate slowly after being stored for longer than 1 week, virtually all of the studies analyzing the hapten inhibitory activity of the membrane glycoproteins were performed within a week after isolation. In addition, a series of controls containing receptor and lectin but no ghosts was also run in each assay. Finally, the effect, if any, of the Triton X-100 on the binding of the lectin to the ghosts was also tested in each assay as a control.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—** Sodium dodecyl sulfate gel electrophoresis was performed by the procedure of Fairbanks et al. (24). Protein was stained with Coomassie blue and carbohydrate with periodic acid-Schiff reactivity by the methods of Fairbanks et al. (24).

**Analytic Methods—** Sialic acid was determined by the method of Warren (25) after hydrolysis in 1 N HCl for 2 min at 100°. Hexose was determined routinely by the phenol-sulfuric acid method (26), scaled down to ½ volume, using β-galactose as a standard. Individual hexoses and amino sugars were determined by gas-liquid chromatography as described previously (27). Protein was determined by the method of Lowry et al. (28).

### RESULTS

**Binding of the R. communis Lectins and Wheat Germ Agglutinin to Human Erythrocytes—**Since the interaction of the *R. communis* lectins and WGA with human erythrocytes had been characterized previously to only a limited extent, we initially studied the binding of these lectins to erythrocytes. Preliminary investigations revealed that the binding of RCA I, ricin, and WGA to human erythrocytes occurred very rapidly and had reached a steady state level under the conditions of the assay (30 min at room temperature). Addition of the appropriate sugar hapten at this point caused the release of greater than 98% of the bound lectin, implying that virtually all the lectin was bound to the cell surface. In addition, measurements of the initial rate of dissociation of the lectin-erythrocyte complex (in the absence of hapten) demonstrated that lectin binding to erythrocytes is a freely reversible process. Therefore, the binding data can be analyzed using the method of Scatchard (29). Typical binding curves are shown in Fig. 1. The curve for ricin binding was monophasic at the lectin concentrations tested whereas the RCA I and WGA curves were biphasic. The most likely explanation for the biphasic nature of the curves is that there are two classes of receptors for these particular lectins and that the lectins bind to these two sites with different affinities. Another possibility is that there is only one class of receptors, but the binding of the lectins exhibits negative cooperativity (30).

Using the data obtained from several binding studies with the three lectins, the number of receptor sites per cell as well as the apparent association constant for lectin binding was calculated and these data are summarized in Table I. The binding characteristics for several other lectins are also shown in Table I for purposes of comparison.

**TABLE I**

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Sites/cell</th>
<th>$K_{assay}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricin</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>RCA I</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>WGA</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>P. vulgaris E-PHA</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>A. bispora PHA</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>R. pseudocaoa PHA</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>L. culinaris PHA</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Where two numbers appear, they refer to the high and low affinity binding sites as determined from the biphasic Scatchard plots.

* Data from Reference 2.

* Data from Reference 5.

* Data from Reference 4.

* Data from Reference 3.

**Fig. 1.** Binding of lectins to human erythrocytes. The binding reactions were carried out in plastic counting tubes which had been presoaked for at least 2 hours in 2 ml of 5 mg per ml bovine serum albumin in 0.9% NaCl. The reaction mixtures contained in 0.2 ml of 0.9% NaCl-0.01 M NaHCO₃ from 1 to 20 μg of ¹²⁵I-labeled lectin, 250 μg of bovine serum albumin, and 5 × 10⁴ erythrocytes. After 30 min of incubation at room temperature, the cells were washed twice with 6 ml of 0.9% NaCl and the amount of bound ¹²⁵I-labeled lectin was determined in a Packard Auto-gamma counter. All points represent the average of duplicate samples. Appropriate corrections were made for nonspecific binding to the plastic tubes which accounted for less than 5% of the total counts bound. The data were plotted by the method of Scatchard (29). For curved plots, the equation can be solved by trial and error. $B$, micrograms of lectin bound; $F$, micrograms of lectin free; •—•, ricin; △—△, RCA I; ○—○, WGA.

When the binding of the *R. communis* lectins and WGA to trypsin-treated erythrocytes was tested, it was found that WGA binding was decreased by 20%, whereas *R. communis* lectin binding was unaffected. The latter finding is in marked contrast to our previous studies which showed that trypsin treatment of human erythrocytes decreased the binding of the lectins from *P. vulgaris, L. culinaris, R. pseudocaoa*, and *A. bispora* by
Neuraminidase treatment of the erythrocytes resulted in several different effects on lectin binding which are shown in Fig. 2 and Table II. The number of ricin binding sites decreased from $1.92 \times 10^6$ per cell to $3.72 \times 10^5$ per cell with all of the additional sites being of a high affinity type (Table II). Neuraminidase-treated erythrocytes also bound more RCA I, but in this case the enhanced binding was to the low affinity sites. In contrast, the major effect on WGA binding was to markedly decrease the apparent association constant for the high affinity sites by 15-fold.

Nature of Ricin Binding Sites Exposed by Neuraminidase—Previous investigations have established that most of the erythrocyte sialic acid is located in the major sialoglycoprotein of the membrane with the majority of these residues being found in oligosaccharides with the structure sialic acid $\alpha$-2,3 galactose $\beta$-1,3 (sialic acid $\alpha$-2,6) N-acetylgalactosamine $\rightarrow$ Ser (Thr) (31). Since both galactose and N-acetylgalactosamine are haptene inhibitors of ricin, we reasoned that the ricin binding sites exposed by neuraminidase treatment may be the Gal $\rightarrow$ GalNAc $\rightarrow$ Ser (Thr) units located on the major sialoglycoprotein. To test this, we took advantage of the fact that the A. bisporus lectin binds to these oligosaccharide units (5), and we measured the ability of this lectin to inhibit ricin binding. As shown in Table III, Experiments 1 and 2, the A. bisporus lectin had no effect on ricin binding to control erythrocytes, but it almost completely blocked the binding of ricin to the new receptors exposed after neuraminidase treatment of the cells. In addition, the binding of the A. bisporus lectin was inhibited by ricin only when desialized cells were used (Table III, Experiments 3 and 4). The 28% inhibition of A. bisporus lectin binding by a large excess of ricin was not unexpected since the number of new ricin binding sites is only 30% that of the A. bisporus lectin sites (Table I).

Isolation of R. communis Lectin Receptors by Affinity Chromatography—Yu et al. (23) have reported that treatment of erythrocyte ghosts with Triton X-100 in 50 mM sodium borate, pH 8.0, selectively releases glycoproteins and glycerolipid. In agreement with these results, we found that 0.5% Triton X-100 in 50 mM sodium borate, pH 8.0, solubilized 75 to 85% of sialic acid-containing glycoprotein material while releasing only 40 to 50% of total membrane protein (Table IV). In addition, this treatment solubilized about 68% of the membrane mannose, 50% of the galactose and amino sugars and 80% of the glucose. Compared with the intact ghosts, the material in the residue bound only 11% as much A. bisporus PHA, 25% as much WGA, 28% as much ricin, and 29% as much RCA I (Table IV). These data indicate that most of the receptors for these four lectins were solubilized by the Triton. However the treatment consistently resulted in a more complete extraction of the A. bisporus PHA receptors compared with the other lectin receptors. The nature of the receptors not solubilized by Triton is currently under investigation.

Using a quantitative haptene inhibitory assay (see “Experimental Procedure” for details), it was determined that the solubilized material had potent haptene inhibitory activity toward all lectins tested (Tables V, VI, and IX). To isolate the glycoproteins with R. communis lectin receptor activity, the Triton-solubilized material was passed through ricin-Sepharose or RCA I-Sepharose columns and the adsorbed material was eluted with 0.1 M lactose. As shown in Table V, a column containing approximately 1 μmole of RCA I adsorbed 43 mg of protein with 50% of the applied R. communis haptene inhibitory activity. The eluate also contained 41% of the haptene activity for the A. precatorius lectin which has a carbohydrate specificity which is similar to that of RCA I (7). The specificity of the column was demonstrated by the fact that only 0.9% of the A. bisporus PHA receptors adsorbed to the column. Since the receptors for A. bisporus PHA are located on the major sialoglycoprotein-(5),
this finding demonstrates that the *R. communis* and *A. precatorius* lectin receptors must be on glycoproteins which are distinct from the sialoglycoprotein. The data in Table V also show that the majority of the *P. vulgaris* and *L. culinaris* PHA receptors are present on different molecules than the *R. communis* lectin receptors. To obtain maximal yields of receptor protein from each column run and to reduce the adsorption of nonreceptor glycoproteins, the columns were deliberately overloaded. As shown in Table VI, when less receptor activity was applied to the column, the total yield was decreased and the contamination with proteins, the columns were deliberately overloaded. As shown in column run and to reduce the adsorption of nonreceptor glycoproteins, the *R. communis* receptor activity was higher. These data also demonstrate that the lectin receptor activity not bound to the affinity column appeared in the initial run-through therefore accounting for all the receptor activity. As shown in Table VI, the lactose eluted *R. communis* receptor fraction was markedly enriched in hexose compared with sialic acid. Extrapolating to 100% yield and correcting for the small contamination with the sialoglycoprotein, the *R. communis* receptor material can account for approximately 20% of the total hexose of material can account for all the receptor activity. As shown in Table VI, along with the carbohydrate composition of whole erythrocyte ghosts, the major sialoglycoprotein and affinity purified WGA receptor material. The composition of the receptor material for both the *R. communis* lectins is very similar, being particularly rich in galactose and *N*-acetylgalactosamine and almost devoid of sialic acid and *N*-acetylgalactosamine. This carbohydrate composition is strikingly different from that of the sialoglycoprotein.

Sodium dodecyl sulfate gel electrophoresis of the RCA I receptor glycoprotein was performed and the results are shown in Fig. 3. The Coomassie blue stain revealed three distinct bands plus a number of very faint bands. The total amount of staining material was less than expected, probably reflecting the fact that glycoproteins with high sugar contents stain poorly with Coomassie blue (24). Even more striking was the failure of the

**TABLE III**

*Competitive inhibition of ricin and A. bisporus lectin binding to control and neuraminidase-treated erythrocytes*

A 0.2-ml sample of 50% erythrocytes was treated with 10 units of *V. cholerae* neuraminidase for 3 hours at 37°, washed three times with 0.9% NaCl-0.01 M NaHCO₃, and diluted to 1% with the same buffer. The 125I-labeled lectins were incubated in 0.9% NaCl-0.01 M NaHCO₃ containing 250 μg of bovine serum albumin, control or treated erythrocytes (5 × 10⁸), and unlabeled lectins at the indicated amounts in a total volume of 0.2 ml. The reaction was begun with the addition of the cells and the incubations continued for 30 min at room temperature. The cells were then harvested as described in the legend of Fig. 1 and the inhibition of 125I-labeled lectin binding determined.

<table>
<thead>
<tr>
<th>Component</th>
<th>Ghosts</th>
<th>Residue</th>
<th>Extract</th>
<th>% Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg)</td>
<td>203</td>
<td>100</td>
<td>103</td>
<td>51</td>
</tr>
<tr>
<td>Sialic acid (μmoles)</td>
<td>17.1</td>
<td>3.5</td>
<td>15.2</td>
<td>81.3</td>
</tr>
<tr>
<td>Mannose (μmoles)</td>
<td>7.6</td>
<td>2.8</td>
<td>5.8</td>
<td>67.7</td>
</tr>
<tr>
<td>Galactose (μmoles)</td>
<td>37.4</td>
<td>19</td>
<td>17.4</td>
<td>48</td>
</tr>
<tr>
<td>Glucose (μmoles)</td>
<td>11.8</td>
<td>7.6</td>
<td>3.5</td>
<td>31.5</td>
</tr>
<tr>
<td>N-Acetylgalactosamine (μmoles)</td>
<td>10.8</td>
<td>8.6</td>
<td>8.2</td>
<td>49</td>
</tr>
<tr>
<td>N-Acetylgalactosaminiose (μmoles)</td>
<td>15.7</td>
<td>7.4</td>
<td>8.4</td>
<td>53</td>
</tr>
<tr>
<td>Lectin binding (ng/5 × 10⁸ ghosts)</td>
<td>301</td>
<td>106</td>
<td>72a</td>
<td></td>
</tr>
<tr>
<td>Ricin</td>
<td>952</td>
<td>278</td>
<td>71a</td>
<td></td>
</tr>
<tr>
<td>RCA I</td>
<td>1100</td>
<td>274</td>
<td>75a</td>
<td></td>
</tr>
<tr>
<td>WGA</td>
<td>830</td>
<td>90</td>
<td>89a</td>
<td></td>
</tr>
</tbody>
</table>

These values represent the difference between the lectin binding of the ghosts and residue.

**TABLE V**

*Fractionation of erythrocyte glycoproteins on RCA I-Sepharose*

Erythrocyte ghosts (77 ml) were solubilized with 0.6% Triton X-100 in 56 mM sodium borate, pH 8.0, and processed as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Sialic acid</th>
<th>Total lectin inhibitory activity of each fraction toward</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μmoles</td>
<td>RCA I</td>
</tr>
<tr>
<td>Triton extract</td>
<td>343</td>
<td>62</td>
<td>85,200</td>
</tr>
<tr>
<td>Lactose eluate</td>
<td>43</td>
<td>1.4</td>
<td>50,600</td>
</tr>
<tr>
<td>In eluate, %</td>
<td>12.5</td>
<td>2.3</td>
<td>59</td>
</tr>
</tbody>
</table>
times more potent than the sialoglycoprotein, the second best haptene inhibitor. As noted previously with other lectins (2-5), the erythrocyte receptor contained a broad band of amino sugar containing glycoproteins that were hydrolyzed in 4 N HCl for 4 hours at 100° to release the amino sugars, which were then purified on small Dowex 50 columns and measured quantitatively by the method of Reissig et al. (33). The results are shown in Fig. 4. The gels with the R. communis receptor glycoproteins contained a broad band of amino sugar containing material located on both sides of PAS 1 and probably representing several glycoproteins.

The affinity purified R. communis receptors were extremely potent haptene inhibitors of ricin and RCA I binding to erythrocytes, as shown in Table VIII. The erythrocyte receptor material was over 1200 times more potent than galactose and 25 times more potent than the sialoglycoprotein, the second best haptene inhibitor. As noted previously with other lectins (2-5),

glycoprotein material to stain with PAS stain (gels not shown). Thus there were no clearly defined PAS-positive bands even though the amount of carbohydrate applied to the gels with the R. communis lectin receptors was comparable with that applied to the gel with the erythrocyte ghosts. Similar results were obtained using the material isolated from the ricin-Sepharose column.

To visualize the R. communis glycoprotein receptors on the gels, a different approach was utilized. Three gels containing a total of 210 nmoles of amino sugar were sliced into 20 fractions each and the corresponding fractions combined. The fractions were hydrolyzed in 4 N HCI for 4 hours at 100° using sealed glass tubes. The supernatant fluid was lyophilized and the released amino sugars resuspended in 3 ml of HZO. The amino sugars were then applied to a small Dowex 50 H+ column (in a Pasteur pipette). The columns were washed with 4.5 ml of H2O and the amino sugars eluted with 4.5 ml of 1 N HCl. The eluates were evaporated to dryness and taken up in 0.2 ml of H2O. Amino sugar values were then determined by the method of Reissig et al. (33). A, erythrocyte ghost; B, R. communis receptor material. Shown at the top of each panel is a diagram of the pattern obtained on gels stained with either PAS (A) or Coomassie blue (B).

glycopeptides were better haptene inhibitors than simple sugars. The haptene inhibitory effects of simple sugars on the R. communis lectins have been studied in several laboratories (7, 8, 13, 14, 18, 34, 35).

Isolation of Wheat Germ Agglutinin Receptor by Afinity Chromatography—The glycoproteins isolated on the R. communis lectin columns were relatively poor haptene inhibitors of WGA (Table VIII) and accounted for less than 4% of the WGA haptene inhibitory activity solubilized with Triton. Since these findings suggested that the WGA receptors were located on another class of glycoproteins, we passed the Triton X-100-solubilized erythrocyte membrane material through a WGA-Sepharose column in order to isolate the glycoproteins with WGA binding sites. The results of a typical experiment are summarized in Table IX. In contrast to the previous finding with the R. comm-
munis lectin-Sepharose columns, the material eluted from the WGA-Sepharose columns with N-acetylglucosamine contained approximately an equal yield of receptor activity for the WGA, A. bisporus and P. vulgaris lectins and a much poorer yield of haptene activity for the R. communis lectins. The low percentage of recovery of lectin receptor activity reflects the fact that the column was greatly overloaded and that the capacity of the WGA columns was less than that of the R. communis columns. When examined by sodium dodecyl sulfate gel electrophoresis, the WGA receptor exhibited a single Coomassie blue staining, PAS positive band which corresponded to PAS 1 (Fig. 5). In addition, the carbohydrate composition of the WGA receptor (Table VII) was very similar to that of the major sialoglycoprotein.

As in the case of R. communis lectin receptors, the affinity purified WGA receptor was 15,000 times more potent than the haptene N-acetylglucosamine. The sialoglycoprotein purified by the lithium diidosalicylate procedure had 1/4 the haptene activity of the affinity purified material. It had been reported previously that the sialoglycoprotein had haptene inhibitory activity toward the WGA (36, 37). Treatment of the sialoglycoprotein with neuraminidase resulted in a 9-fold decrease in WGA haptene inhibitory activity. The haptene activity of the WGA receptor was also strikingly decreased by proteolytic degradation of the polypeptide portion of the molecule. As shown in Table X, Pronase treatment of the receptor decreased the WGA and A. bisporus PHA haptene activity by 98% and the P. vulgaris E-PHA activity by greater than 99%. Similar losses of haptene activity were observed when the R. communis lectin receptor proteins were degraded with Pronase.

**DISCUSSION**

The data presented in this paper demonstrate that affinity chromatography on lectin-Sepharose columns is an effective procedure for fractionating erythrocyte membrane glycoproteins which contain binding sites for different lectins. The nonionic detergent, Triton X-100, was used to solubilize the membrane glycoproteins, since, under defined conditions, this agent preferentially releases glycoprotein molecules (23) and does not sig-

![Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the WGA receptor. Gel A, erythrocyte ghosts (52 µg of protein); Gels B and C, WGA receptor (14 µg of protein, 5.8 nmoles of sialic acid). Gels A and B were stained with Coomassie blue and Gel C with PAS.](http://www.jbc.org/)

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**Table VIII**

*Lectin inhibitory activity of various carbohydrates, glycopeptides and glycoproteins*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrations of haptene to give 1.0 U.¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rin</td>
</tr>
<tr>
<td>Affinity purified ricin receptor</td>
<td>0.5</td>
</tr>
<tr>
<td>Affinity purified RCA I receptor</td>
<td>0.5</td>
</tr>
<tr>
<td>Affinity purified WGA receptor</td>
<td>N.D.⁴⁴</td>
</tr>
<tr>
<td>Sialoglycoprotein</td>
<td>18</td>
</tr>
<tr>
<td>Neuraminidase-treated sialoglycoprotein</td>
<td>4.5</td>
</tr>
<tr>
<td>Trypsin-released glycopeptide</td>
<td>230</td>
</tr>
<tr>
<td>Trypsin-released desialylated glycopeptide</td>
<td>42</td>
</tr>
<tr>
<td>IgG glycopeptide</td>
<td>50</td>
</tr>
<tr>
<td>Fetalin glycoprotein</td>
<td>108</td>
</tr>
<tr>
<td>Transferrin glycoprotein</td>
<td>185</td>
</tr>
<tr>
<td>Lactose</td>
<td>108</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>290</td>
</tr>
<tr>
<td>Galactose</td>
<td>650</td>
</tr>
<tr>
<td>Melibiose</td>
<td>580</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>280</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Mannose, glucose, L-fucose, sialic acid</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

¹ For comparison sake, the concentration of oligosaccharide haptens has been normalized to GlcNAc content in the case of the WGA assays and galactose content in the case of the ricin and RCA I assays.

⁴ Not determined.

**Table IX**

*Fractionation of erythrocyte glycoproteins on wheat germ agglutinin-Sepharose*

Erythrocyte ghosts (185 ml) were solubilized with 0.5% Triton X-100 and processed as described under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sialic acid</th>
<th>Total lectin inhibitory activity of each fraction toward</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>RCA I</td>
</tr>
<tr>
<td>Triton extract</td>
<td>180</td>
<td>168,000</td>
</tr>
<tr>
<td>GlcNAc eluate</td>
<td>5.8</td>
<td>1,600</td>
</tr>
<tr>
<td>In eluate, %</td>
<td>3.2</td>
<td>1</td>
</tr>
</tbody>
</table>
isolated from human erythrocytes by Tanner and Boxer (32). Glycoproteins may correspond to proteins E and F which were shown to band 3 of Fairbanks et al. (24) and the glycoprotein described by Bretscher (42). Tanner and Boxer (32) estimated that proteins E and F contained about 13% of the total erythrocyte ghost carbohydrate, whereas we calculate that the R. communis receptor glycoproteins contain approximately 20% of the membrane hexose and 18.0% of the total membrane carbohydrate. The finding that a significant amount of the erythrocyte membrane carbohydrate is located on glycoproteins other than the sialoglycoprotein is not unexpected, since it can be calculated that the sialoglycoprotein can account for only 30 to 45% of the total carbohydrate present in the erythrocyte ghost (31). In addition, since the sialoglycoprotein and the R. communis receptor glycoproteins account for only 49 to 64% of the membrane carbohydrate, there must be significant amounts of other glycoproteins present in the erythrocyte membrane.

The binding studies performed with the 125I-labeled R. communis lectins are consistent with the finding that these lectins bind to glycoproteins other than the major sialoglycoprotein. Thus the binding of the R. communis lectins is insensitive to trypsinization of the erythrocytes, a procedure which is known to release 40 to 50% of the oligosaccharide units of the sialoglycoprotein with a corresponding decrease in the binding of those lectins whose receptors are known to be located on the sialoglycoprotein (2-5). The results of the experiments with neuraminidase also indicate, however, that the sialoglycoprotein contains latent binding sites for ricin and perhaps for RCA I as well. Thus, neuraminidase treatment of the erythrocytes exposed 1.81 × 10^4 ricin binding sites. The binding of ricin to these newly exposed sites could be totally blocked by the A. bisporus lectin which binds to the type II carbohydrate units of the sialoglycoprotein (5). Ricin binding to untreated erythrocytes is unaffected by the A. bisporus lectin (Table III). These data indicate that the sialic acid → Gal → (sialic acid →) GalNAc → Ser (Thr) units of the sialoglycoprotein can function as ricin binding sites subsequent to the removal of the sialic acid residues. Previously, Nicolson (43) had shown that neuraminidase treatment of human erythrocytes enhanced the binding of RCA I by about 50%. The R. communis lectin receptors which were not solubilized by Triton could be located on yet another class of membrane glycoproteins or even glycolipid. The nature of these receptors is currently under investigation.

The results obtained with the WGA-Sepharose affinity columns strongly implicate the sialoglycoprotein as being a receptor for the WGA. The glycoprotein isolated from this column migrates on sodium dodecyl sulfate polyacrylamide gels the same as the sialoglycoprotein, stains strongly with PAS and has a carbohydrate composition that is very similar to that of the sialoglycoprotein. In addition, the WGA receptor glycoprotein has potent haptene inhibitory activity toward the P. vulgaris E-PHA and A. bisporus lectins which are known to bind to the sialoglycoprotein. Finally, the haptene inhibitory activity of sialoglycoprotein purified by the lithium 3,5-diodosalicylate method is similar to the activity of the affinity purified material (Table VIII). However, since the recovery of WGA receptor activity from the affinity column was only 6%, one cannot determine from these data what percentage of the total WGA receptors are located on the sialoglycoprotein. The finding that more A. viscosa receptor glycoproteins contain approximately 20% of the membrane hexose and 18.0% of the total membrane carbohydrate.
bisporus PHA receptors than WGA receptors were solubilized with Triton (89% compared with 75%) suggests that at least some of the WGA receptors are located on molecules other than the sialoglycoprotein.

Burger and Goldberg (44) noted previously that neuramidinase treatment of mouse L110 cells abolished their agglutinability by WGA, thus implicating membrane sialic acid residues in the agglutination process. Similar results have been obtained by Cuatrecasas (45) using fat cells from rats. However, the effect of this treatment on WGA binding to the cells was not determined. Our observation that neuramidinase treatment of erythrocytes decreases the apparent affinity of WGA binding by 15-fold may explain these previous results. The finding that neuramidinase treatment of the isolated sialoglycoprotein resulted in a 9-fold decrease in its haptene inhibitory activity indicates that the sialic acid residues are a component of the WGA receptor and are probably directly involved in the interaction with this lectin. Consistent with this interpretation is the demonstration by Wray and Walborg (46) that a sialoglycopeptide isolated from Novikoff ascites tumor cells has potent haptene inhibitory activity toward WGA and the equilibrium dialysis study of Greenaway and LeVine which showed that WGA can bind at least 9 moles of sialic acid (47).

When the affinity purified glycoproteins were degraded with Pronase, a dramatic fall in lectin haptene inhibitory activity was observed (Table X). This result is similar to our previous observation that trypsin treatment of the sialoglycoprotein induced a 90% loss of haptene activity toward P. vulgaris E-PHA (2). Similar findings have been noted following proteolytic degradation of glycoproteins isolated from lymphocyte plasma membranes (48). The most likely explanation for the decrease in haptene inhibitory activity is that lectin binding is enhanced when the glycoprotein receptor is multivalent in terms of oligosaccharide binding sites. Proteolytic degradation of the glycoprotein results in the conversion of a multivalent receptor to a monovalent receptor with an associated drop in binding affinity. This situation would be analogous to that observed with antibody-antigen interactions where antibody binding is much stronger toward multivalent antigens than toward univalent antigens (49).

The ability to fractionate and isolate large amounts of intact glycoproteins by affinity chromatography on lectin-Sepharose should facilitate a number of studies of the structure and function of membrane glycoproteins. The amount of material obtained by this procedure is adequate for performing structural studies on the oligosaccharide units of the isolated glycoproteins.

REFERENCES

Isolation of the Receptors for Wheat Germ Agglutinin and the *Ricinus communis* Lectins from Human Erythrocytes Using Affinity Chromatography

W. Lee Adair and Stuart Kornfeld


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