Equilibrium Dialysis and Cell Binding Studies on Bandeiraea simplicifolia Lectin*  

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Equilibrium dialysis studies on the binding of the blood group B-specific Bandeiraea simplicifolia lectin to methyl α-D-galactopyranoside demonstrated the existence of one carbohydrate binding site per subunit for the tetrameric protein, with an intrinsic association constant of $8.6 \times 10^4 \text{M}^{-1}$ at $2^\circ$ and $3.3 \times 10^4 \text{M}^{-1}$ at $20^\circ$. These values correspond to a free energy of binding, $\Delta G^\circ$ (pH 7.2), of $-6.21 \text{kcal/mol}$ and $-6.06 \text{kcal/mol}$ at $2^\circ$ and $20^\circ$, respectively. The sites appeared homogeneous and noninteracting.

B. simplicifolia lectin receptor sites per human B erythrocyte varied from $0.72 \times 10^9$ to $1.34 \times 10^9$ with an apparent association constant of $1.1 \times 10^4 \text{M}^{-1}$ to $2.9 \times 10^4 \text{M}^{-1}$. The binding characteristics of B. simplicifolia lectin are compared to other purified lectins.

Extracts of Bandeiraea simplicifolia seeds contain a lectin (phytohemagglutinin) that specifically agglutinates human type B erythrocytes (1). This plant lectin was recently purified by affinity chromatography, and its physical and chemical properties were studied (2). It is a glycoprotein (molecular weight, 114,000; 6.7% carbohydrate) composed of four subunits (molecular weight, 28,500) bound by nonequivalent forces. The carbohydrate binding and hemagglutinating activities of the lectin require protein-bound calcium ions and one free sulfhydryl group per subunit. Removal of metal ions or modification of the sulfhydryl group disrupts the sugar binding capacity of the B. simplicifolia lectin.

Polysaccharides and glycoproteins with terminal nonreducing α-D-galactosyl residues react with the lectin to form precipitates; this reaction is inhibited best by a α-D-galactopyranosides (2, 3).

In addition to studies on the interaction between B. simplicifolia lectin and polysaccharides (2), we have begun a detailed investigation of the binding of the lectin to simple sugars and to various cell types. The present report presents the results of equilibrium dialysis experiments with methyl α-D-galactopyranoside as ligand, and cell binding studies employing human type B erythrocytes.

MATERIALS AND METHODS  

Protein iodination grade NaI214 was purchased from New England Nuclear, Boston, Mass. Methyl α-D-galactopyranosides were products of Pfanstiehl Laboratories, Kankakee, Ill.; and bovine serum albumin was a product of Miles Chemicals, Phillipsburg, N. J. Human erythrocytes were obtained from normal donors through the University of Michigan Blood Bank courtesy of Dr. H. Oberman. Cells were stored at 4° for no more than 7 days and washed four times with PBS-Ca+ prior to use.

Bandeiraea simplicifolia lectin was purified according to Hayes and Goldstein (2) from seeds generously made available through Drs. J. Pollard and E. Schantz of Calbiochem, La Jolla, Calif.

Protein was determined by the method of Janatova et al. (4) with bovine serum albumin as standard. The phenol-sulfuric acid colorimetric assay described by DuBois et al. (5) was employed for the determination of carbohydrate.

$^1$C samples were counted as 0.5-ml aqueous aliquots in 5.0-ml scintillant (1 part Triton X-100, 2 parts toluene containing 0.4% 2,5-diphenyloxazole and 0.005% p-bis[2-(5-phenyloxazoyl)]benzene) using a Packard Tri-Carb liquid scintillation spectrometer. 125I samples were counted in a total volume of 0.5 ml of NaI (polonium) Nuclear Chicago 1185 series single channel automatic gamma counter.

Methyl α-D-[1-2$^1$C]galactopyranoside was synthesized by the method of Bollenback (6). α-D-[1-2$^1$C]galactose (0.17 mg, 52.9 mCi/mmole) was transferred to a 10-ml round bottom flask with aliquots of hot absolute methanol and evaporated four times from this solvent to remove water. Absolute methanol (5 ml) was washed, dried Dowex 50-X8 (100 mg) were added to the flask, which was then equipped with a condenser. The reaction was stirred under reflux for 24 hours. Unlabeled methyl α-D-galactopyranoside (100 mg) was added, and after stirring an additional hour, the resin was filtered and washed several times with hot absolute methanol. The filtrate and washes were evaporated to dryness, and the residue was crystallized from hot absolute ethanol. Purity of the glycoside was determined by descending paper chromatography of the crystals on Whatman No. 3MM paper in Solvent A (water-saturated n-butyl alcohol) and Solvent B ([t-pentyl alcohol/n-propyl alcohol/water, 4:1:1.5 v/v]) using authentic standards. Chromatograms were sprayed with periodate benzidine reagent (7), cut into pieces (0.5 × 1.0 cm²), and counted 5.0 ml of scintillant. Traces of methyl β-D-galactopyranoside were removed by repeated recrystallization of the α-glycoside from hot absolute ethanol.

The specific activity of methyl α-D-galactopyranoside was $9.3 \times 10^9$ cpm/mol.

Equilibrium dialysis experiments were performed in duplicate on several preparations of B. simplicifolia lectin both at 2° and at 20° as

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described by Karush (9). Dialysis casing was boiled and washed exhaustively in distilled water prior to use. Filled cells were rotated slowly on a multipurpose rotator; upon reaching equilibrium, aliquots (0.10 ml) were removed for counting.

$^{131}I$-labeled B. simplicifolia lectin was prepared by the iodooanilined coupling technique described by Hayes and Goldstein (9); specific activity varied from 1123 to 1890 cpm/μg. A new labeling technique was required because B. simplicifolia lectin, being a protein-containing oxidizable sulfhydryl group, became inactivated under the conditions employed in established procedures. For binding experiments, increasing amounts of $^{131}I$-labeled B. simplicifolia lectin were added to triplicate 12-ml conical centrifuge tubes containing PBS-Ca with 0.5 mg/ml bovine serum albumin. The reaction was initiated by the addition of a constant number of washed type B erythrocytes (counted by means of a hemocytometer). The tightly capped tubes were rotated slowly at 20° for 2 hours and then spun 15 min at top speed in an International clinical centrifuge. An aliquot (0.5 ml) of the supernatant was counted, the remainder was removed with a drawn out Pasteur pipette, and the cell pellet was dissolved in 10% Triton X-100 in water (0.5 ml) for counting. To correct for $^{131}I$-labeled B. simplicifolia lectin not specifically bound to the cell—most instances, approximately 30% of what was specifically bound—tubes containing 5.0 mM methyl α-D-galactopyranoside as inhibitor were run in parallel, and the counts per minute in these cell pellets were subtracted from the matched tubes (without inhibitor) before calculating $r$ and $[A]$ according to the derivation of Scatchard (10).

RESULTS

Scatchard plots of the binding of methyl α-D-galactopyranoside to Bandeiraea simplicifolia lectin at both 2° and 20° are shown in Fig. 1. The absence of curvature in the plots suggests that the sugar binding sites of B. simplicifolia lectin are noninteracting and homogeneous with respect to association constant. The calculated association constants are $8.6 \times 10^4$ M$^{-1}$ at 2° and $3.3 \times 10^4$ M$^{-1}$ at 20°, corresponding to a free energy of binding, $\Delta G$° (pH 7.2) of $-6.21$ kcal/mol and $-6.06$ kcal/mol at 2° and 20°, respectively. Assuming a molecular weight of 114,000 (2), the tetrameric B. simplicifolia lectin binds 4 mol of sugar/mol of protein at saturation.

Shown in Fig. 2 are the results of cell binding experiments conducted on erythrocytes of five type B donors. As in equilibrium dialysis experiments, the absence of curvature in the Scatchard plots indicates that lectin binding sites for the R determinant are independent (noninteracting) and homogeneous with respect to association constant. Association constants, calculated from the slopes of the plots in Fig. 2 and tabulated in Table I, varied from $1.1 \times 10^7$ to $2.9 \times 10^7$ M$^{-1}$. If it is assumed that all the B determinants on the erythrocyte surface are accessible to the lectin, and that at saturation 1 molecule of B. simplicifolia lectin is bound to only one B receptor site, the number of receptor sites may be calculated by extrapolation of the plots in Fig. 2. Receptor sites per erythrocyte varied from $7.2 \times 10^4$ to $13.4 \times 10^4$ sites/cell and are tabulated in Table I. Interestingly, there was a correlation between receptor density and apparent association constant; a higher density of receptors was associated with an increased binding constant. The binding of B. simplicifolia lectin to B erythrocytes was specific in that the reaction could be inhibited by methyl α-D-galactopyranoside but not by methyl α-D-mannopyranoside (2). Furthermore, the reaction was independent of cell density in the range observed. Experiments with cells of three of the five donors gave nearly identical values for $K_a$ and the number of

![Fig. 1. Equilibrium dialysis of Bandeiraea simplicifolia lectin against methyl α-D-[1-131]galactopyranoside plotted according to Scatchard. $r$, moles of sugar bound per mole of lectin (molecular weight, 114,000); $[A]$, concentration of free sugar (9). O, 2°, 72-hour incubation; □, 20°, 48-hour incubation. Experiments were performed in Karush-type dialysis cells with 1 ml of protein solution and 1 ml of ligand solution separated by dialysis casing. Protein concentration was 2.6 mg/ml of PBS-Ca; initial ligand concentration varied from 0.057 mM to 1.03 mM in PBS-Ca.](http://www.jbc.org/)

![Fig. 2. Binding of Bandeiraea simplicifolia lectin to human type B erythrocytes plotted according to Scatchard (10). $r$, moles of lectin bound per cell; $[A]$, concentration of free lectin; O, Experiment 1: 4.1 $\times 10^6$ cells/ml; □, Experiment 2: 3.9 $\times 10^6$ cells/ml; ▲, Experiment 3: 5.5 $\times 10^6$ cells/ml; ▼, Experiment 4: 3.2 $\times 10^6$ cells/ml; ▲, Experiment 5: 5.7 $\times 10^6$ cells/ml. Washed erythrocytes, labeled lectin, and PBS-Ca with 0.5 mg/ml of bovine serum albumin in a total volume of 10 ml were incubated at 20° with gentle agitation for 2 hours. Lectin concentration varied from 0.5 to 14.0 μg/ml. Cells were sedimented, an aliquot of the supernatant was counted to determine $[A]$, and the cell pellet (dissolved in 10% v/v Triton X-100 in water) was counted to determine $r$.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$10^4 \times$ lectin receptors/cell</th>
<th>$10^4 \times$ association constant M$^{-1}$</th>
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<td>1</td>
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</tr>
<tr>
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</tr>
<tr>
<td>5</td>
<td>7.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* a Calculated from the value of $r$ obtained by extrapolation of the Scatchard plots in Fig. 2 to $r/[A] \to 0$ and multiplying by $6.023 \times 10^{14}$ molecules/mol.

* b Calculated from the slope of the Scatchard plots in Fig. 2.
sites per cell, whereas the remaining two were slightly different. The lectin did not bind to type O erythrocytes nor to type B erythrocytes in the presence of 5 mM or 10 mM methyl α-D-galactopyranoside.

**DISCUSSION**

In previous studies, it was established that nonreducing terminal α-D-galactopyranosyl residues were most complementary to the carbohydrate binding sites of B. simplicifolia lectin (2, 3). A quantitative measure of the strength of this association was determined in the present investigation by equilibration dialysis. In comparing the observed B. simplicifolia lectin-methyl α-D-galactopyranoside association constant to other lectins, it is of the same order of magnitude as the binding constant for concanavalin A-methyl α-D-mannopyranoside interaction (11, 12), soybean agglutinin-N-acetyl α-D galactosamine binding (13), Ricinus communis agglutinin-lactose association (14), and Lotus tetragonolobus C lectin-l-fucose interaction (15). Of the lectins studied, all have an even number of sugar binding sites per mole of lectin, the B. simplicifolia lectin being no exception. However, in possessing one site subunit, B. simplicifolia lectin resembles concanavalin A (11). Lotus tetragonolobus C (15), the lentil lectin (16), and Helix pomatia agglutinin (17); on the other hand the carbohydrate binding sites of soybean (Glycine max) (13) and lima bean (Phaseolus lunatus) (18) lectins are formed by the interaction of two subunits.

In contrast to the intrinsic association constant of B. simplicifolia lectin for methyl α-D-galactopyranoside (3.3 x 10^4 M^-1 at 20°C), the association constant measured for binding to the multiply substituted B erythrocyte was 3 orders of magnitude greater (1.1 to 2.9 x 10^7 M^-1 at 20°C). Several other lectins exhibit similar increases in apparent binding constant for cells bearing multiple determinants compared to the intrinsic association constant for simple monovalent determinant sugar. Hammarström, working with the hexavalent Helix pomatia hemagglutinin, found the interaction between lectin and cells to be stronger by 6 orders of magnitude than that between the lectin and an A-active pentasaccharide (19). In studies on the lentil lectin, Stein et al. noted an increase of 4 orders of magnitude upon binding to erythrocytes (16, 20), and Major and Brodie, a similar difference upon binding of the lentil lectin to platelets (21). There are at least three alternative explanations for the increase in apparent association constants measured for lectin-cell interaction. Simple sugars are obviously only part of the complex receptor oligosaccharide and it is quite possible that some lectins have binding sites that are complementary to a more extensive receptor molecule, the simple sugar reflecting only a portion of the binding site. This is probably not the case for B. simplicifolia lectin inasmuch as inhibition studies suggest that the binding site is not an extended one (2, 3). A second explanation, that nonspecific noncovalent binding interactions between lectin and cell surface contribute to the higher apparent association constant, cannot be ruled out in the present study. Finally, Hornick and Karush (22) suggest the possibility that, following the initial interaction of one lectin site with one receptor, multivalent binding may occur between a molecule with multiple binding sites and a substance with several binding loci, thereby increasing the apparent association constant by 3 to 4 orders of magnitude compared to the intrinsic binding constant. We favor the latter interpretation particularly because it may explain the correlation noted in this study between receptor density and association constant.

The number of blood group B receptors per erythrocyte determined by lectin absorption in this investigation (0.72 x 10^9 to 1.34 x 10^9) is considerably lower than that reported by Economidou et al. (6.1 x 10^9 to 8.3 x 10^9) determined by absorption of 125I-labeled human anti-B antiserum to formalin-treated B cells (23). The explanation for this discrepancy may lie in the specificity of the reagent used to determine the number of receptors, i.e. B. simplicifolia lectin or immunoglobulin G antibodies. Economidou et al. (23) prepared anti-B antiserum by absorption of the immunoglobulin G fraction from two sensitized O-donor to formalin-treated B erythrocytes, and subsequent elution of the bound fraction at elevated temperature. The antibody preparation obtained may be specific for more than one type of surface receptor found on B cells. On the other hand, B. simplicifolia lectin appears to be specific for α-D-galactopyranosyl residues, and it is unlikely that it would react with receptors lacking this determinant sugar (2, 3). A larger variation in results for B cells than A cells was noted by Economidou et al. (23) who also commented that extrapolation of curvilinear Scatchard plots might well have contributed a significant error in determining receptor density. Both determinations rely on the assumption that one antibody or lectin molecule is bound to one receptor. Since immunoglobulin G is bivalent and B. simplicifolia lectin tetravalent, the possibility exists in each instance of 1 molecule reacting with more than one receptor. Thus, each estimate may theoretically be low by a factor of 2 for immunoglobulin G anti-B, and 4 in the case of B. simplicifolia lectin.

Although there is presently no other purified type B-specific lectin with which to compare the present results, type A- and type O-specific lectins have been used to quantify determinants on group A and O erythrocytes, respectively. Both Hammarström, studying Helix pomatia hemagglutinin (19), and Williams and Voak investigating Dolichos biflorus lectin (24), reported approximately 10^6 receptors/A_1 erythrocyte, which is quite close to the B receptor density determined in the present report. Most investigators have found the number of receptors on A_1 cells to be about 4-fold higher (25-29) than on A_2 cells. The results of Boyd et al. (25) with 125I-labeled lima bean lectin (Phaseolus lunatus) showing no difference in receptor density between A_1 and A_2 erythrocytes (8 x 10^6 sites/cell) are not in accord with the aforementioned investigations. Lectin receptor density on type O erythrocytes was determined to be 2 x 10^9 sites/cell by Matsumoto and Osawa (27) with the type O-specific lectins of Cytisus sessilifolius and eel serum. This is 1 order of magnitude greater than that determined for type B erythrocytes in this study.

Lectins that do not inhibit blood-type specificity have also been employed in studies of receptor density on human erythrocytes. Stein et al. (20) found 5.8 x 10^6 lentil lectin receptors/cell on both A and O erythrocytes, whereas Presant and Kornfeld (28) noted 6.8 x 10^6 Agaricus bisporus agglutinin and 5.2 x 10^6 Phaseolus vulgaris phytohemagglutinin receptors per erythrocyte. Finally, Nicolson (29) reported 8 x 10^8 sites/cell for the Ricinus communis agglutinin.

The results of this investigation on the interaction of B. simplicifolia lectin with methyl α-D-galactopyranoside and with human type B erythrocytes established that B. simplicifolia lectin is quite similar to other lectins with respect to the parameters studied. Furthermore, being the only puri-
fied and characterized phytohemagglutinin exhibiting a high degree of specificity for terminal $\alpha$-linked $\beta$-galactopyranosyl residues, this lectin offers a unique probe for the study of complex carbohydrates, both in solution and on cell surfaces.

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