Phaseolus vulgaris Isolectin Binding to Human Erythrocytes*

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MATERIALS AND METHODS

The Phaseolus vulgaris isolectins Ld, Ls, El, L2E2, L3E3, and E4 were isolated by affinity and ion exchange chromatography. Pure isolectins were radiolabeled by the chloramine-T method with Na\(^{125}I\), and their binding to human erythrocytes was studied. A normal erythrocyte has approximately 8 x 10\(^7\) receptor sites for each isolectin; however, the association constants (\(K_a\)) of the binding increased from 1.1 x 10\(^7\) M\(^{-1}\) to 3.8 x 10\(^8\) M\(^{-1}\), with increasing number of E subunits per tetrameric isolectin molecule. Isolectin to erythrocyte binding reached equilibrium rapidly and was reversed by feutin. All isolectins competed with \(^{125}I\)-E\(_4\) for erythrocyte binding sites, with a constant (\(K_a\)) similar to the \(K_a\) calculated for each respective radiolabeled isolectin. When isolectin binding at 0°C, 4°C, or 8°C was compared to that at 25°C, there was no reduction in the number of binding sites per cell, but the \(K_a\) of E\(_1\) was reduced to 3 x 10\(^7\) M\(^{-1}\). Fixed erythrocytes displayed similar isolectin binding characteristics.

The interaction of plant lectins with cells has revealed many characteristics of cell membranes (1-4). In the case of the phytohemagglutinin from red kidney bean, Phaseolus vulgaris, previous research established that this mixture of protein interacts with erythrocytes, lymphocytes (5-7), and serum glycoproteins (8). Subsequently, the erythroagglutinating and mitogenic activities of PHA\(_1\) were shown to reside in different protein fractions (9, 10) and the binding of these fractions to the cellular (11-15) and soluble components of blood (16, 17) was characterized. Since these studies, we have isolated and characterized five PHA isolectins from \(P.\) vulgaris (18-20). These tetrameric protein molecules have been designated as L\(_1\), L\(_2\)E\(_2\), L\(_3\)E\(_3\), L\(_4\)E\(_4\), and E\(_5\) based on the number of leukosagglutinating (L) and erythroagglutinating (E) polypeptide subunits per molecule. Other studies in our laboratory have characterized the physical interaction of these proteins with lymphocytes and platelets and the cellular physiological consequences of these interactions (18-21). We have characterized the binding of the PHA isolectins to human erythrocytes in an attempt to understand the nature of this interaction and to relate this information to isolectin and membrane structure.

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1 The abbreviation used is PHA, phytohemagglutinin.


3 Schmukler, M., Egorin, M., Felsted, R., and Bachur, N., manuscript in preparation.

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were incubated at 8°C in a humidified chamber for at least 24 h before examination for precipitin bands.

**Gel Filtration of Isolectin-Protein Mixtures**—18 μg of 125I-E, (70,000 dpm) were mixed with 2 mg of either L, E, porcine thyroglobulin (Sigma), or crystalline bovine serum albumin in 0.5 ml of phosphate-buffered saline. The 125I-E-protein mixtures were applied to a column (1.5 x 28 cm) of Bio-Gel P-200 (Bio-Rad), equilibrated with the same buffer. Fractions (1.4 ml) were collected at a flow rate of 10 ml/h at room temperature, and the radioactivity of each fraction was determined. The void volume of the column was determined with blue dextran ($M_v = 2,000,000$).

**RESULTS**

**Time Course and Reversibility of Isolectin Binding to Human Erythrocytes**—PHA isolectins bind to human erythrocytes rapidly. Equilibrium of 125I-E, binding was attained by 20 min and remained stable for at least 120 min. Similar results were obtained with the radioiodinated isolectins L, E, L, E, and L, E. The 125I-E, isolectin-erythrocyte complex was rapidly dissociated by fetuin (Fig. 1), a soluble glycoprotein possessing oligosaccharide moieties similar to those bound by PHA on the erythrocyte membrane.

**Estimation of Binding Constants and Number of Receptor Sites**—Addition of increasing amounts of 125I isolectin to erythrocytes resulted in increasing amounts of isolectin bound, until saturation was attained (Fig. 2). When the association constants ($K_a$) and the number of lectin receptor sites on a red cell ($N$) were estimated according to the method of Steck and Wallach (27) (Fig. 3), a family of straight lines was generated, all having the same $y$ intercept ($N^{-1}$) but with different slopes ($K_a^{-1}N^{-1}$). These results indicate the same number of receptor sites per red cell for each isolectin and that each isolectin binds to the receptor sites with a uniform affinity. The association constants of the isolectins to erythrocytes, however, vary greatly, depending on the number of E subunits per tetrameric protein molecule. Although some variation in the association constant for each isolectin was observed among individual experiments, the overall relationship of $K_a^{-1}_E > K_a^{-1}_E > K_a^{-1}_E > K_a^{-1}_E$ remained constant throughout all experiments (Table I). That red cells have the same number of receptor sites for each isolectin suggests that the isolectins all bind to the same receptors, but with different affinities.

**Competitive Binding of Isolectins**—Addition of increasing amounts of any isolectin to a binding reaction of erythrocytes...
TABLE I

Binding constants for PHA isolecitins and human erythrocytes

<table>
<thead>
<tr>
<th>Isolectin</th>
<th>N0</th>
<th>K0-1</th>
<th>KI</th>
<th>M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>7.3 ± 3.1 x 10^6</td>
<td>3.8 ± 0.3 x 10^6</td>
<td>3.1 ± 0.3 x 10^6</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>L1E2</td>
<td>7.8 ± 2.5 x 10^6</td>
<td>2.9 ± 1.8 x 10^6</td>
<td>7.1 ± 2.4 x 10^6</td>
<td>1.1 ± 0.12</td>
</tr>
<tr>
<td>L2E2</td>
<td>7.8 ± 2.5 x 10^6</td>
<td>6.5 ± 2.4 x 10^6</td>
<td>1.7 ± 0.3 x 10^6</td>
<td>0.94 ± 0.27</td>
</tr>
<tr>
<td>L3E1</td>
<td>8.6 ± 2.0 x 10^6</td>
<td>1.1 ± 0.6 x 10^6</td>
<td>4.9 ± 3.5 x 10^6</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>L4</td>
<td>7.8 ± 2.5 x 10^6</td>
<td>2.5 ± 1.8 x 10^6</td>
<td>1.1 ± 0.6 x 10^6</td>
<td>0.90 ± 0.03</td>
</tr>
</tbody>
</table>

a Number of 125I-iodinated isolecitin molecules bound per red cell as calculated from Steck-Wallach (27) plots. Values represent the mean ± S.D. of from 3 to 16 duplicate experiments.

b Association constants of isolecitins to erythrocytes calculated from Steck-Wallach (27) plots. Values represent the mean ± S.D. of from 3 to 16 duplicate experiments.

c Inhibition constant determined by competitive binding reaction of noniodinated isolecitin against 125I-E4. Values were derived according to Harris and Pestka (28) and represent the mean ± S.D. of five to six duplicate experiments.

d The number of noniodinated isolecitin molecules displacing 1 molecule of 125I-E4. Values represent the slopes of lines derived according to Harris and Pestka (28) and represent the mean ± S.D. of five or six duplicate experiments.

These values for L4 could not be calculated since the binding of L4 and erythrocytes could not be measured directly.

and a fixed amount of 125I-E4 produced a reduction in the amount of 125I-E4 bound (Fig. 4A). The ability of each isolecitin to compete with 125I-E4 was proportional to the number of E subunits per tetrameric molecule. In these experiments L4 competed with 125I-E4 for red cell receptor sites, even though high concentrations of L4 were required. Using the KI of 125I-E4 as a reference, we calculated the inhibition constant (KI) for each isolecitin by analyzing these competitive binding experiments according to the method of Harris and Pestka (28). By this method (Fig. 4B), the slope of each line was approximately one (Table I), implying that 1 molecule of 125I-E4 is displaced by 1 molecule of any of the isolecitins; and, therefore, that all isolecitins bind to a single type of binding site. The KI for each isolecitin, as calculated from these studies with unlabeled lectins, was proportional to the number of E subunits per isolecitin and was comparable to the KI for that respective 125I labeled isolecitin as determined from the direct binding studies described earlier (Table I).

Interaction between Isolecitins—In order to conclude from competitive binding studies that the isolecitins all bind to the same red cell receptor, it is necessary to prove that isolecitin molecules do not bind to one another. If 125I-E4 was mixed with unlabeled E4, L4, or bovine serum albumin and chromatographed on a Bio-Gel P-200 column, all radioactivity eluted as a single component compatible with free E4, and no radioactivity appeared in the void volume (Fig. 5). On the other hand, when a mixture of 125I-E4 and porcine thyroglobulin was chromatographed in a similar fashion, all radioactivity appeared in the void volume of the column, indicating a stable 125I-E4-thyroglobulin complex.

When the PHA isolecitins were allowed to interact in Ouchterlony double radial diffusion experiments, no precipitable complexes were observed as a result of interaction. However, the positive control, E4-fetal bovine serum interaction, produced precipitin bands.

Effect of Fixation of Erythrocytes on Isolecitin Binding—125I-E4 bound less tightly to fixed erythrocytes than to unfixed cells, but there was no increase or decrease in the
Effects of erythrocyte fixation and reaction temperature on the binding of \(^{125}\)I PHA isolectins to erythrocytes

Isolectins were purified and radiolabeled as described under "Materials and Methods." Binding reactions were performed as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Reaction temperature</th>
<th>Isolectin</th>
<th>Unfixed cells</th>
<th>Fixed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction temperature</td>
<td>N (^{-6})</td>
<td>K(_d) (\text{M}^{-1})</td>
<td>N (\text{M}^{-1})</td>
</tr>
<tr>
<td>0°C</td>
<td>E(_4)</td>
<td>8.5 (\pm) 2.9 (\times) 10(^{-6})</td>
<td>2.99 (\pm) 1.84 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>4°C</td>
<td>E(_4)</td>
<td>9.4 (\pm) 5.4 (\times) 10(^{-6})</td>
<td>4.83 (\pm) 4.7 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>8°C</td>
<td>E(_4)</td>
<td>6.8 (\pm) 1.7 (\times) 10(^{-6})</td>
<td>4.92 (\pm) 3.2 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>25°C</td>
<td>E(_4)</td>
<td>7.3 (\pm) 3.1 (\times) 10(^{-6})</td>
<td>3.80 (\pm) 0.31 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>37°C</td>
<td>E(_4)</td>
<td>3.2 (\pm) 7.5 (\times) 10(^{-6})</td>
<td>8.20 (\pm) 5.50 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>0°C</td>
<td>E(_4)</td>
<td>8.6 (\pm) 0.8 (\times) 10(^{-6})</td>
<td>7.03 (\pm) 2.15 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>25°C</td>
<td>L(_2)E(_1)</td>
<td>8.6 (\pm) 2.0 (\times) 10(^{-6})</td>
<td>1.11 (\pm) 0.57 (\times) 10(^{-6})</td>
</tr>
</tbody>
</table>

\(^{a}\) Number of iodinated isolectin molecules bound per red cell as calculated from Steck-Wallach (27) plots. Values represent the mean \(\pm\) S.D. of from 3 to 10 duplicate experiments.

\(^{b}\) Association constant calculated from Steck-Wallach (27) plots. Values represent the mean \(\pm\) S.D. of from 3 to 10 duplicate experiments.

**Discussion**

Whereas previous investigators have described *P. vulgaris* PHA as a mixture of active components and have correctly postulated the tetrameric subunit structure of this family of plant lectins (9, 10), characterization of the interaction of the individual isolectins with cellular and serum glycoproteins required the isolation of the PHA isolectin components. Using purified L\(_4\) and a mixture of erythroagglutinating isolectins, previous workers proposed that L\(_4\) did not bind to erythrocytes (24, 30, 31); that only proteins containing an L subunit agglutinated erythrocytes (10, 24) and did so with an affinity proportional to the number of E subunits per tetrameric molecule (32); that lymphocyte mitogenicity might be limited to those containing an L subunit (10); and that each peptide subunit bears a single cell receptor binding site (24).

With the resolution and purification of all five individual PHA isolectins, the opportunity arose to examine the interaction of these important proteins with erythrocytes and hopefully to understand the nature of both the binding site on the isolectin molecule and the receptor sites on the erythrocyte surface. Our studies were undertaken to test the hypothesis that L\(_4\) did not bind to red cells. If this were confirmed, the interaction with erythrocytes of L\(_2\)E\(_1\), L\(_2\)E\(_2\), L\(_4\), and E\(_4\) could then be used to address the question of whether each isolectin subunit possessed a red cell binding site or whether the isolectin's binding site resulted from a cooperative configuration of two or more polypeptide subunits.

Our determination of the K\(_d\) values proved the earlier prediction that the affinities of the E-containing isolectins for erythrocytes increases with an increasing number of E subunits per tetramer (30, 31). Moreover, the fact that analysis of the data by the method of Steck and Wallach (27) yields a family of straight lines, all with the same y intercept argues that each isolectin interacts with only one type of receptor site on erythrocytes and that the number of receptor sites per erythrocyte is the same for each isolectin. These studies support the earlier claim that the binding of L\(_4\) to erythrocytes cannot be measured directly, implying either no association of L subunits with red cell membranes (32) or relative insensitivity of the assay procedure.

Our observation of increasing K\(_d\) values with increasing E subunits per isolectin could be explained either by an increasingly spatially favorable isolectin binding site or by multipoint attachment with the interaction of an increasing number of erythrocyte binding sites on each isolectin molecule.
subunits per tetrameric lectin with red cell receptors. The fact that erythrocytes have the same number of receptor sites for each isolectin argues against the idea of multipoint attachment. However, steric or other hindrance preventing the binding of multiple LE molecules to the same receptor areas as 1 E subunit molecule would produce varied $K_v$ values due to multipoint attachment, but no variation in the number of isolectin molecules bound per erythrocyte. These hypotheses assume no interaction of L subunits with red blood cell membranes. However, our competitive binding studies clearly show the ability of $L_q$, albeit at high concentrations, to compete with $E_q$ for erythrocyte receptor sites. The fact that the $K_v$ values for $L_qE_1$, $L_qE_2$, $L_qE_3$, and $E_1$ calculated from competitive binding experiments are so similar to the $K_v$ values calculated from direct binding studies with these same molecular species argues that the $K_v$ value determined for $L_q$ is a reasonable estimate of its $K_v$ value. The behavior of $E_q$, $L_q$, and $E_q$-thioglycolubin mixtures on a Bio-Gel P-200 column and the absence of precipitable complexes in agarose double radial diffusion experiments eliminates $L_qE_1$ interaction as the cause for reduced $E_q$ binding to red cells in the presence of $L_q$. Since $L_q$ subunits as well as E subunits may interact with red cell receptors, our observations with regard to $K_v$ and receptor site values for the PHA isolectins may be reconciled very adequately with the concept of each isolectin subunit possessing a binding site. That is, the increased $K_v$ values observed could reflect a progressively greater number of high affinity (E) interactions between erythrocyte and isolectin replacing lower affinity (L) interactions.

Our competitive binding studies prove another point. When the ability of each isolectin to compete with $E_q$ for binding was analyzed by the method of Harris and Pestka (28), the slope of each derived line was unity. This is consistent with the stoichiometry of 1 unlabelled isolectin molecule displacing a single molecule of $^{125}$I-E from its receptor. Thus, all of the isolectins bind to a single population of erythrocyte receptors. This is quite different from the interaction of these molecules with lymphocytes and platelets which possess separate classes of receptor sites for $L_q$ and $E_q$.

Binding reactions at reduced temperature or with aldehyde-fixed red cells shed some light on the nature of the erythrocyte receptors. Both fixation and reduced temperature cause a similar decrease in $K_v$ for $125$I-E$_1$ without a change in the number of receptors (Table II). This suggests that cold and fixation alter the receptor site, possibly preventing the red cell receptor from assuming a configuration that is optimal for binding. It is unlikely that the fixation affects the isolectin-binding site, since the fixation is removed before the $125$I-E$_1$ is added. In contrast to $^{125}$I-E$_1$, the binding of $^{125}$I-$L_qE_1$ is unchanged by cold or fixation (Table II). Since the number of receptors, the $K_v$, and the linearity of the Steck-Wallach (27) transformation are all unchanged for $125$I-$L_qE_1$, this suggests that $125$I-$L_qE_1$ binds to only a part of the receptor recognized by $125$I-E$_1$. The availability of this part for binding of $125$I-$L_qE_1$ is not affected by temperature or fixation.

Since cellular agglutination is not simply a function of isolectin attachment to cell membranes (1), differences in the ability of the isolectins to agglutinate red cells may reflect differences in the interaction of these proteins with erythrocytes (Table III). Although an overestimate of $K_v$ especially for $L_q$ may explain the apparent necessity for a higher proportion of receptor occupancy for equivalent agglutination, it is attractive to speculate that $L_q$ and $E_q$ bind to a portion of the red cell receptor that is less favorably oriented for agglutination.

Therefore, our experiments demonstrate that red cell membranes possess only one class of PHA receptor and show that the strength of the interaction of these receptors with PHA isolectins is related to the number of E subunits per isolectin molecule. Our data also show that each isolectin contains one homogeneous class of binding site and imply that the binding site of some isolectins may recognize and attach to only a portion of the receptor bound by other isolectin molecules. Our studies do not definitively establish the valency of the isolectins or show whether or not the PHA molecules interact with red cells via single or multipoint attachment. Resolution of these problems will require the discovery of a simple hapten inhibitor or the use of radiolabeled E and L subunits. However, this latter approach is hampered at present by the rapidity and avidity with which the subunits spontaneously recombine and the rigorous conditions required to prevent this spontaneous association (20).

REFERENCES

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