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STUDIES ON 6C3HED MURINE ASCITES TUMOR CELL RECEPTORS FOR MANNOSYL-BINDING LECTINS

HOWARD J. ALLEN and EDWARD A. Z. JOHNSON

Department of Gynecology Roswell Park Memorial Institute Buffalo, N. Y. 14263 (U.S.A.)

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SUMMARY

We have investigated the receptor site activity present on 6C3HED tumor cells for concanavalin A, fava, lentil and pea lectins. The binding of the tritiated lectins to the tumor cells was inhibited by methyl-α-D-mannoside but not by D-galactose. The number of binding sites for the lectins was $3.5 \cdot 10^6$/cell for concanavalin A, $3.3 \cdot 10^6$/cell for fava, $3.6 \cdot 10^6$/cell for lentil and $4.8 \cdot 10^6$/cell for pea. The apparent association constants were 3.6 and 1.3 μM^{-1} for concanavalin A, 3.9 μM^{-1} for fava, 4.2 μM^{-1} for lentil and 4.6 and 0.6 μM^{-1} for pea. Competitive inhibition studies showed that lentil was a good inhibitor of pea binding; concanavalin A was a poor inhibitor of pea binding; and fava was a better inhibitor than concanavalin A but not as good as lentil. Reciprocal inhibition experiments indicated that concanavalin A and pea may bind to different receptors as well as to common receptors. This was also indicated by the observation that trypsin or protease treatment of the cells decreased the binding of pea lectin by 20–40 percent whereas concanavalin A binding was unaffected.

INTRODUCTION

Two of the major problems confronting cell biologists are how cells recognize self from non-self and how recognition phenomena influence cell replication. A role in these processes for cell surface glycoproteins and glycolipids has been widely implicated [1–5]. Antigenic attenuation or amplification which occurs when some transplantable tumors are cultured in vitro may result from changes in cell surface glycoproteins or glycolipids which play a role in cellular recognition [6–8]. Despite the apparent significance of glycoproteins and glycolipids in cell-cell interactions, there are relatively scarce data on the chemistry of these components present at the surface of nucleated mammalian cells.

We have selected the 6C3HED murine ascites tumor as a model system from which to isolate and characterize the cell surface glycoproteins which may play a role in cell-cell recognition events. This tumor is an estrogen-induced lymphosarcoma

originated by Gardner et al. [9] and was converted to the ascites form by Klein [10]. The tumor has been reported to show host specificity [11]. When this tumor was cultured in vitro, it lost the ability to grow in vivo. However, the in vitro-grown tumor could induce host immunity which prevented the growth of in vivo-grown tumor [12, 13].

As part of a study to elucidate the potential role which cell surface glycoproteins play in tumor antigenicity and tumor growth in this system, and to develop procedures for their isolation and characterization, we have investigated some of the lectin-binding characteristics of the in vivo-grown tumor.

A portion of this work has been previously presented [14].

MATERIALS AND METHODS

The ascitic form of the 6C3HED tumor was obtained from Dr. G. Bekesi, Mt. Sinai, N.Y.C. The tumor was maintained in male C3H/ST.-Ha mice from West Seneca Laboratories, West Seneca, N.Y. All experiments were performed with tumor harvested 7 days after intraperitoneal injection.

The hemorrhagic tumor was freed of blood by suspending the cells in 0.83 % NH_4Cl followed by centrifugation at 4 °C for 10 min. The pellet was suspended to 15 ml with 0.15 M NaCl/0.01 M PO_4 (pH 7.2). 15 ml of deinoized water were rapidly added with mixing. After 5 s, 5 ml of 3.6 % NaCl were then added. 15 ml of the buffered saline were then added and the cell suspension was centrifuged. The cells were passed through cheese cloth if clumps were present and the cells washed 3 times with buffered saline. All steps were carried out in the cold.

Viability was determined by trypan blue exclusion and it ranged from 92–98 percent.

Trypsinization of cells was done in an ice bath with gentle stirring at $25 \cdot 10^6$ cells/ml of buffered saline 1 mM $CaCl_2$ with 100 μg/ml trypsin plus 25 μg/ml DNA-ase. The digestion was stopped by addition of a 2-fold excess of soybean inhibitor and the cells were then washed in the cold with buffered saline before use.

Protease treatment of the cells was done as above except soybean inhibitor was omitted and protease was substituted for trypsin. Lectin binding experiments were carried out in buffered saline 0.5 % bovine serum albumin in an ice bath with gentle mixing. Pyrex tubes were used for incubation. The cells were harvested by transferring them to 1 cm diameter glass fiber filters presoaked in 1 % bovine serum albumin. The filters were then washed with buffered saline 0.1 % bovine serum albumin and were transferred to Mini-Vials for liquid scintillation counting with 4 ml Aquasol as the scintillation cocktail.

Nonspecific adsorption of lectins to the glass fiber filters was determined for several different lectin concentrations by carrying out incubations in the absence of cells. Concanavalin A showed the greatest nonspecific adsorption; incubation and harvesting conditions were chosen to reduce the concanavalin A adsorption to 5 % of cell-bound counts or less. Nonspecific binding of the lectins to cells was determined by preincubating the lectins with methyl-α-D-mannoside (10 mg/ml)before addition of the cells. Nonspecific binding to cells ranged from 3 % to 10 % of cell-bound counts depending upon the lectin concentration. The Legends show when background corrections were applied.

Concanavalin A was prepared according to Agrawal and Goldstein [15]. Lentil lectin was prepared according to Howard et al. [16]. Pea lectin was prepared according to Entlicher et al. [17]. Fava lectin was prepared from ground fava beans by extraction with saline, ammonium sulfate fractionation, and adsorption to and elution from Sephadex G-150 [18]. The lentil and pea lectins were used as their mixtures of isolectins since no differences in specificities of the isolectins has been reported.

Lectins were tritiated with [^{3}H]acetic anhydride by the method of Weber [19]. The specific activities of the lectins used were 13.9 and 10.5 μCi/mg for concanavalin A, 8.8 and 6.2 μCi/mg for fava, 15.4 and 12.0 μCi/mg for lentil, and 9.0 and 7.0 μCi/mg for pea.

Stock tritiated lectins were stored at 4 °C in 1 M NaCl adjusted to pH 5.0 with dilute acetic acid. Unlabeled lectins were dissolved in the above solution 24 h before use. Lectins were diluted with buffered saline immediately before use.

DCC-treated type XI trypsin, type VI protease, type 1-S soybean trypsin inhibitor and beef pancreatic DNAase I were obtained from Sigma Chemical Co., St. Louis, Mo. Mini-Vials and Aquasol were obtained from New England Nuclear Corp., Boston, Mass. [^{3}H]acetic anhydride, 400 mCi/mM, was obtained from Schwarz/Mann, Inc., Orangeburg, N.Y. Sephadex was a product of Pharmacia Fine Chemicals, Piscataway, N.J. Bio-Gel P-150 was a product of BioRad Laboratories, Inc., Rockville, Centre, N.Y. Glass fiber filters were obtained from Reeve Angel, Inc., Clifton, N.J.

RESULTS

Purity of the lectins was checked by co-chromatography of labeled and unlabeled lectins on Bio-Gel P-150 in the presence of sucrose. The results for concanavalin A are shown in Fig. 1. The apparent molecular weight is indicated. Under these conditions concanavalin A existed as a dimer. In later experiments concanavalin A existed as a tetramer (Fig. 5 only). The tritiated lectins co-eluted with the native lectins at elution volumes corresponding to 50 000 M_r for fava lectin, 48 000 M_r for lentil lectin and 43 800 M_r for pea lectin.

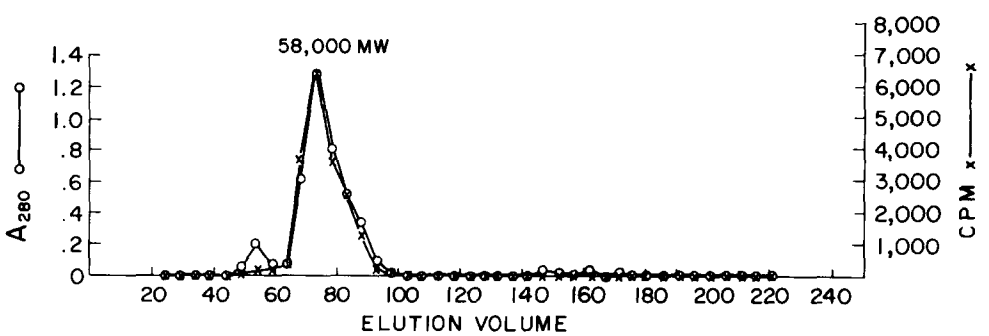

Fig. 1. Co-chromatography of tritiated and unlabeled concanavalin A on Bio-Gel P-150. 20 mg unlabeled lectin plus 50 μg tritiated lectin were dissolved in 0.15 M NaCl/0.01 M PO_4/0.05 M sucrose/0.05 % NaN_3 (pH 7.2) in a volume of 2 ml. The sample was applied to a 92.5 × 1.6 cm calibrated Bio-Gel P-150 column equilibrated with the sample buffer. Flow rate = 6.8 ml/h. Fraction volume = 5 ml. V_0 = 54 ml.

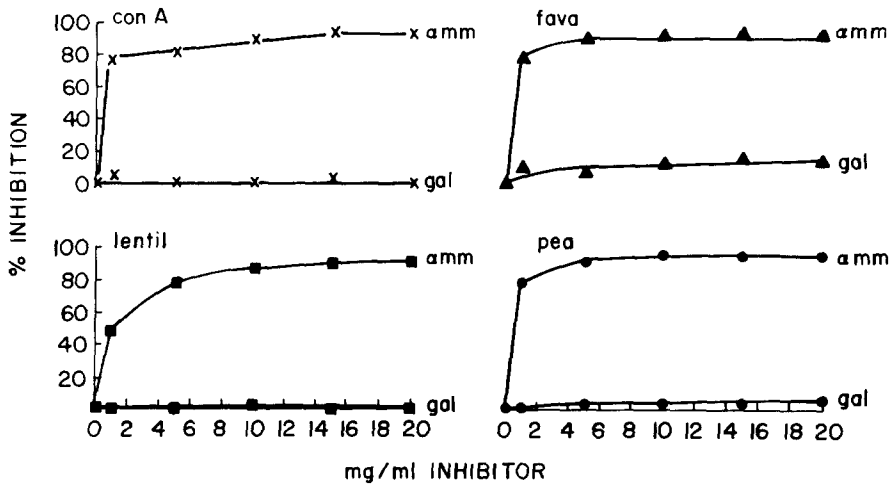

Fig. 2. Inhibition of tritiated lectin binding by methyl-α-D-mannoside and D-galactose. The lectins were preincubated for 30 min in an ice bath with varying amounts of inhibitor. Cells were then added to give 0.50 ml incubation volume. Incubation was continued for 60 min in an ice bath before harvesting of the cells. Lectin = 15 μg/ml. Cells = $5 \cdot 10^6$/ml. The data are not corrected for nonspecific adsorption to the glass fiber filters.

To determine if the tritiated lectins retained their binding specificity, the ability of D-galactose and methyl-α-D-mannoside to inhibit lectin binding was determined. It can be seen in Fig. 2 that over 90 % inhibition of binding occurs in the presence of the mannoside whereas 12 % inhibition or less occurs in the presence of galactose.

It was observed that at high lectin concentrations cell agglutination occurred. Experiments were carried out, therefore, to determine if lectin binding was a linear function of cell concentration. The results for concanavalin A and pea are shown in Fig. 3. Although agglutination occurred, binding was linear up to $50 \cdot 10^6$ cells/ml.

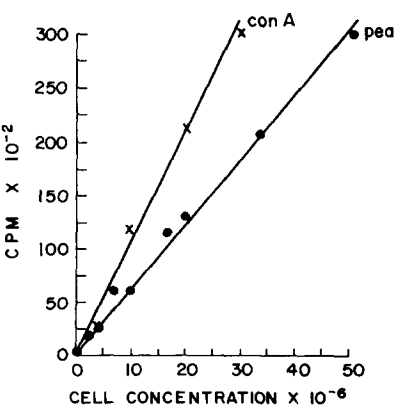

Fig. 3. Binding of concanavalin A and pea lectins versus cell concentration. Lectin was added to varying concentrations of cells to give 0.25 ml incubation volume. Binding was allowed to proceed for 10 minutes in an ice bath. Lectin = 240 μg/ml. The data were corrected for background.

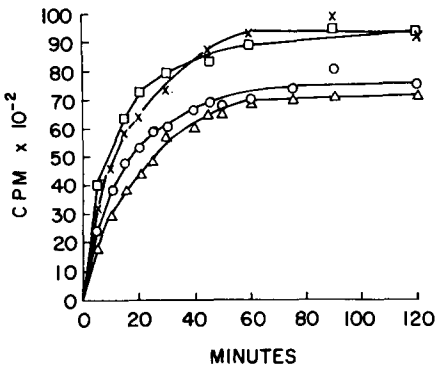

Fig. 4. Lectin binding versus time. Flasks were set up in an ice bath containing lectins at 15 μg/ml and cells at $20 \cdot 10^6$/ml. At given time intervals, 0.25 ml aliquots were removed for harvesting. The data were corrected for background. Concanavalin A, ×–×; fava, △–△; lentil, □–□; pea, ○–○.

To define conditions for binding equilibrium, the time course of lectin binding was determined for several lectin concentrations. The results are shown in Fig. 4 for a lectin concentration of 15 μg/ml. Binding equilibrium was approached by 60 min in this case and in less than 15 min at 240 μg lectin per ml. Pretreatment of the cell with azide did not change the results.

The number of receptor sites and the apparent association constants for the 4 lectins were determined. Cells were incubated with increasing concentrations of lectins

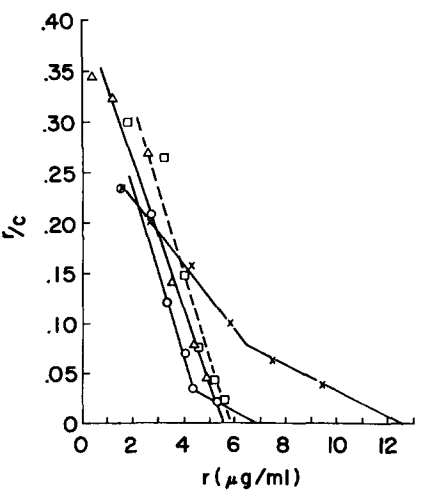

Fig. 5. Scatchard plots of lectin bound versus lectin concentration data. Varying amounts of lectins were added to $5 \cdot 10^6$ cells to give 7.8 to 250 μg/ml for concanavalin A, lentil and pea and 0.8 to 108 μg/ml for fava. Incubation volume was 0.25 ml. After 60 min in an ice bath, the cells were harvested. Background was less than 5 % of bound counts. The data are plotted according to the equation $r/c = nK - rK$, where r is lectin bound in μg/ml, c is free lectin in μg/ml, n is the amount of lectin bound at saturation, and K is the apparent association constant for lectin: receptor site binding. n was determined by extrapolation to the abscissa and K was determined by slope $= -K$. Concanavalin A, ×–×; fava, △–△; lentil, □–□; pea, ○–○.

TABLE I

NUMBER OF RECEPTOR SITES AND APPARENT ASSOCIATION CONSTANTS FOR CONCANAVALIN A, FAVA, LENTIL AND PEA LECTINS

Calculations are from data of Fig. 5.

Lectin	Receptor sites/cell	Association constant
Concanavalin A	$3.6 \cdot 10^6$	$3.6\ \mu M^{-1}$
		$1.3\ \mu M^{-1}$
Fava	$3.3 \cdot 10^6$	$3.9\ \mu M^{-1}$
Lentil	$3.6 \cdot 10^6$	$4.2\ \mu M^{-1}$
Pea	$4.8 \cdot 10^6$	$4.6\ \mu M^{-1}$
		$0.6\ \mu M^{-1}$

and Scatchard plots [20] were constructed. The results are shown in Fig. 5. The lack of linearity, as shown for concanavalin A and pea, indicated the presence of two or more different receptors with different affinities for these lectins. This is in contrast to the curves obtained for fava and lentil. For these lectins, the linear relationship obtained between r/c and r indicated the presence of one type of receptor or different receptors of similar binding affinities.

The apparent association constants and number of receptor sites were calculated for the 4 lectins. For the case of concanavalin A and pea, association constants were calculated for the two regions of the curves in Fig. 5. For this experiment, concanavalin A existed as a tetramer and a molecular weight of 108 000 was used in the calculations. For the other lectins, molecular weights used were those reported here. The results are given in Table I. The high affinity receptors for concanavalin A and pea and the receptors for fava and lentil gave similar association constants. Although nonlinearity was observed, extrapolation of the curves yielded similar numbers of receptor sites for the 4 lectins.

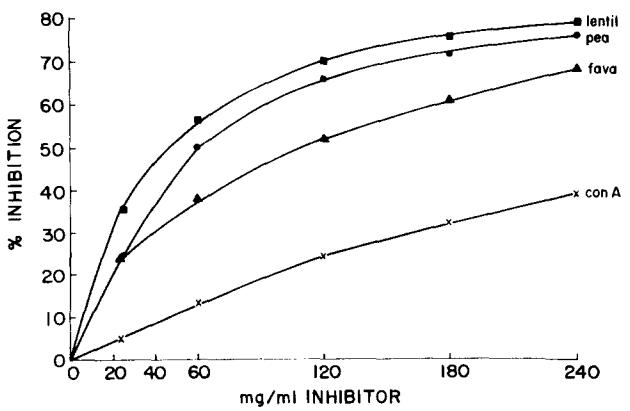

Fig. 6. Inhibition of tritiated pea binding by unlabeled Concanavalin A, fava, lentil and pea lectins. Tritiated pea lectin (24 μg/ml) was mixed with varying amounts of unlabeled lectin. $5 \cdot 10^6$ cells were added to give 0.25 ml incubation volume. After 10 min in an ice bath the cells were harvested. The data are not corrected for background.

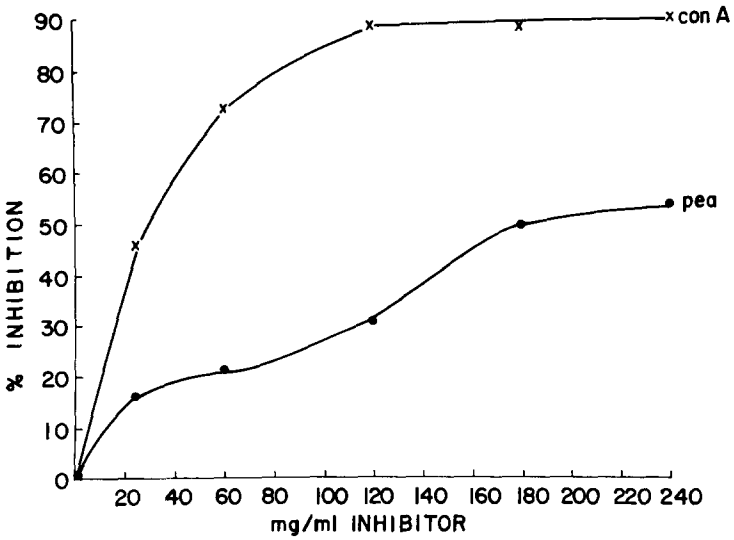

Fig. 7. Inhibition of tritiated Concanavalin A binding by unlabeled Concanavalin A and pea lectins. Details are as for Fig. 6. The data are not corrected for background.

To obtain information as to whether these lectins bound to the same receptors as was indicated by calculation of the number of receptor sites, binding inhibition studies were carried out using tritiated pea lectin and unlabeled lectins. The tritiated lectin was premixed with varying amounts of unlabeled lectins before addition of cells. The results are shown in Fig. 6. Lentil inhibited the binding of tritiated pea lectin slightly better than did unlabeled pea whereas fava was a somewhat weaker inhibitor. In contrast, concanavalin A was a poor inhibitor of pea lectin binding. Concanavalin A inhibited pea binding by 39 % under conditions where fava, lentil and

TABLE II

BINDING OF CONCANAVALIN A AND PEA TO ENZYME-TREATED CELLS

Cells were treated with trypsin or protease as described in Methods. (A) equilibrium binding assay with lectins at 15 μg/ml, $20 \cdot 10^6$ cells/ml, 0.25 ml incubated for 60 min in an ice bath. (B) non-equilibrium binding assay with lectins at 24 μg/ml, $20 \cdot 10^6$ cells/ml, 0.25 ml incubated for 10 min in an ice bath. Data are not corrected for background. Numbers in parentheses are percent of control. Values given are dpm bound.

Lectin	Control	Trypsin	Protease
	Incubation A		
con A	6021 (100)	6155 (102)	
	5812		5764 (99)
pea	3721	2856 (77)	
	4206		2736 (65)
	Incubation B		
con A	3239	3527 (109)	
	3449		3327 (96)
pea	2521	2090 (83)	
	3154		1931 (61)

unlabeled pea inhibited binding by 68 %, 79 % and 76 %, respectively. To determine if concanavalin A and pea lectins bound, at least in part, to mutually exclusive receptors, reciprocal inhibition studies were carried out with tritiated concanavalin A and unlabeled pea. The results are shown in Fig. 7. It is evident that pea lectin is a poor inhibitor of concanavalin A binding. The inhibition studies showed the presence of different receptors on the 6C3HED cell surface for concanavalin A and pea lectin. To determine if these receptors could be differentially hydrolyzed by proteolytic enzymes, binding studies were carried out with trypsin- and protease-treated cells under conditions of equilibrium and nonequilibrium binding. The results are presented in Table II. Trypsin and nonspecific protease reduced the binding of pea lectin by 20–40 % whereas the binding of concanavalin A was little affected. This was true for both equilibrium and nonequilibrium conditions. Protease was more effective in reducing pea binding than was trypsin.

DISCUSSION

Lectins have been used by numerous investigators to study the carbohydrate residues of mammalian cell surfaces [21–23] and for the affinity chromatographic isolation of cell surface receptors [24, 25]. The receptor activity of the 6C3HED ascites tumor cell for mannosyl-binding lectins has been characterized here to aid in the selection of affinity chromatographic systems for their isolation.

The tritiated lectins used retained their binding activity for over a year when stored at pH 5.0 in 1 M NaCl at 4 °C. All lectins were in their dimeric form except for later experiments where concanavalin A was unintentionaly converted to its tetrameric form by freeze-thawing. The freeze-thawed solution of concanavalin A was used for the experiment reported in Fig. 5. This solution of concanavalin A gave a doubling of radioactivity bound to cells, when compared to stock solutions which had not been frozen, at several different lectin concentrations. Because the stock lectin solutions were diluted from solutions at pH 5.0 into buffered saline at 0 °C immediately before use, we assume the cell-bound lectins were present in their dimeric form except where indicated for concanavalin A.

The data presented show that methyl-α-D-mannoside inhibited the binding of all 4 lectins as expected and at saturation, the 4 lectins occupied similar numbers of receptor sites. The number of lectin receptor sites present ($3.3–4.8 \cdot 10^6$) was within the orders of magnitude ($10^6–10^8$) reported for other cell types [26–28].

Differences in lectin binding for the 4 lectins were observed when Scatchard plots [20] were constructed from the binding versus lectin concentration data. Linearity was obtained for fava and lentil lectins whereas the curves for concanavalin A and pea were biphasic. The biphasic curves were not necessarily unusual since mammalian cell surfaces are known to contain several different glycoproteins and glycolipids [29, 30]. The Steck-Wallach plot [31] used by some authors is less sensitive to heterogeneity in association constants and hence, quite often does not indicate lectin receptor heterogeneity as found here.

The data reported showed that fava bound to a single receptor or different receptors of similar affinities. This was also true for lentil. In contrast, both concanavalin A and pea lectins bound to either two different receptors or to two sets of receptors of different average affinities. The apparent association constants for fava,

lentil and for the high affinity receptors for concanavalin A and pea are similar and are within the range found for other systems [26–28].

Reciprocal inhibition studies using labeled and unlabeled lectins confirmed the presence of receptor heterogeneity for concanavalin A and pea. While fava and lentil lectins were effective inhibitors of pea binding, concanavalin A was a poor inhibitor although some concentration-dependent inhibition did occur. The reciprocal experiment showed that pea lectin was a similarly poor inhibitor of concanavalin A binding. Since concanavalin A and pea lectins did not bind to the same receptors, these lectins have different specificities for oligosaccharide structures. The data also showed that lentil and fava lectins have binding specificities different from that of concanavalin A. This conclusion is in agreement with studies on binding of concanavalin A, lentil and pea lectins to known glycoproteins, glycopeptides and receptors [24, 32–34].

Alternative explanations are possible for these inhibition studies. For example, fava lectin may inhibit the binding of pea lectin by a steric hindrance mechanism rather than by direct binding to the pea receptors. This might occur if fava lectin receptors were close neighbors of the pea lectin receptors. The same consideration would apply to the lentil lectin and concanavalin A. Differences in binding kinetics may also influence the observed inhibition. It should be pointed out, however, that fava lectin was a good inhibitor of pea lectin binding even though the pea lectin showed somewhat faster binding kinetics at early times. Differences in binding kinetics would not account for the results in our reciprocal inhibition experiments with concanavalin A and pea lectin because each lectin was found to be a poor inhibitor of the other lectin.

The results of lectin binding to trypsin- and protease-treated cells strengthen the conclusion that concanavalin A and pea lectins bind, at least partially, to different receptors. Concanavalin A binding was unaffected whereas pea binding was reduced 20–40 % after enzyme treatment.

The isolation and characterization of the concanavalin A and pea receptor sites are in progress.

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