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THE BINDING OF LECTINS TO COMPONENTS OF PLASMA MEMBRANES FROM PORCINE SUBMAXILLARY LYMPH NODE LYMPHOCYTES

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Summary

By sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis the plasma membranes from porcine lymphocytes contain at least 30—35 glycopolypeptides and one or more glycolipids to which one or more of 12 purified lectins bind. The specificities of binding generally followed the same pattern as those of the reaction of the lectin with intact pig lymphocytes. Some lectins (e.g., the isolectin pair, *Agaricus bisporus* lectins A and B and a group consisting of the *Lens culinaris* A and B isolectins and the closely related *Pisum sativum* lectins) bind to almost identical populations of plasma membrane components and compete with each other for all their binding sites. Others (e.g., Concanavalin A and the *Lens culinaris-Pisum sativum* group and a group consisting of phytohemagglutinin-L, *Ricinus communis* lectin-60 and *Ricinus communis* lectin-120 bind in a cross reactive manner to some common binding moieties but, in addition, to certain nonshared ones. Still others (e.g., soybean agglutinin, peanut agglutinin and wheat germ agglutinin) do not share any common binding moieties with the other lectins. The amount of lectin binding and the number of membrane components to which a lectin binds is directly related to the K_a of binding of the lectin to the intact lymphocyte. Those with high K_a (Cocanavalin A *Lens culinaris* lectins, *Pisum sativum* lectins, phytohemagglutinin-L), bind to 20—30 different components giving very complex binding patterns while those with lower K_a (*Agaricus bisporus* lectins, wheat germ agglutinin, peanut agglutinin, and soybean agglutinin) bind to 8—13 components with easily distinguishable patterns. Soybean agglutinin binds almost exclusively to a glycolipid fraction while for the others one or more glycopoly-

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peptides served as the major lectin-binding molecule. The *Ricinus* lectins, two lymphocyte toxins, bind to essentially every plasma membrane component to which the mitogen phytohemagglutinin-L binds, in fact competing for most of those plasma membrane moieties which bind phytohemagglutinin-L.

Introduction

A very interesting problem in stimulation of mitosis in lymphocytes is how mitogens initiate their biological effects without entering the cell [1—3]. An important step in understanding the process is the identification of those surface molecules which act as receptors for mitogens and for nonmitogens. In this paper we will report our studies on the binding of various lectins to dissociated plasma membranes from pig lymphocytes.

Materials and Methods

Materials. Phytohemagglutinin L was purchased from Pharmacia. The *Agaricus bisporus* lectins were purified by a modification of a published procedure [4]. *Lens culinaris* lectins were purified by affinity and ion exchange chromatography [5]. *Ricinus communis* lectins were purified by affinity and molecular seive chromatography [6]. Concanavalin A was purified by affinity chromatography [7] or purchased from Pharmacia. A mixture of *Pisum sativum* lectins was purified by affinity chromatography [8]. Wheat germ agglutin, soybean agglutinin and peanut agglutinin were purified by a modification of the method of Vretblad [9]. Except for *Pisum sativum* lectin all the proteins were homogeneous by cellulose acetate electrophoresis. Lectins were labelled with ^{125}I by a modification of a published method [10]. Submaxillary lymph nodes were obtained at a local slaughter house, trimmed, cleaned, and washed with Hank's balanced salt solution (Grand Island Biological Company) within four hours of slaughter. For studies requiring high purity lymphocytes, cells from the nodes of 5—10 animals were teased with surgical needles into Hank's solution, filtered through cotton wool, centrifuged at 200 × *g* for 8 min and suspended in Hank's solution. The resultant suspensions contained 96—97% lymphocytes, less than 3% red blood cells and were >90% viable (by vital dye exclusion). On an average the nodes of one animal yielded $3—5 \cdot 10^9$ cells. The cells were frozen as a suspension (approx. 10^9 cells/ml) in Hank's balanced salt solution at —20° C or —120° C. For large scale preparations the cleaned nodes were frozen in bulk at —20° C or —120° C with a small amount of Hank's balanced salt solution. Typically the nodes of approx. 60 animals, with a weight of approx. 600 g, were frozen in plastic beakers in a total volume of approx. 900 ml.

Plasma membrane preparations. Plasma membranes were prepared by subjecting freeze-thawed cells to mild osmotic shock, removing the organelles and cytoplasm by differential centrifugation and purifying the resultant membranes by sucrose density centrifugation. Our procedure is a combination of methods published by other laboratories [11—14], utilizing 0.01 M Tris pH 7.6 as the standard buffer solution (buffer T) with all operations carried out at 4° C unless noted. Frozen nodes (approx. 600 g) were broken up into small chunks,

chopped into smaller fragments in a meat grinder and pressed through a food mill using a small amount of 0.15 M NaCl-Buffer T at room temperature. Phase contrast microscopic examination of the cells contained in the slurry revealed very few free nuclei, most cells looking like lymphocytes with intact organelles. Yield approx. 1 liter of cell slurry. After melting, the suspension was mixed with two volumes of Buffer T for 10 min according to Ladoulis et al. [13] and enough (approx. 300 ml) 1.5 M NaCl/2 M sucrose/Buffer T added to make the final concentration 0.15 M NaCl/0.2 M sucrose. The suspension was blended for 5 s at high speed followed by 5 s at low speed in a Waring blendor, filtered through several layers of cotton gauze to remove fatty tissue and debris, and centrifuged at 2900 × *g* for 30 min. The supernatant (membrane fraction IA) was reserved and the pellet (unlysed cells, nuclei, trapped membrane, etc.) was suspended in 200—400 ml of 0.15 M NaCl/2 M sucrose/Buffer T, homogenized briefly (10 strokes) in a large loose-fitting Dounce homogenizer, and centrifuged as above. The supernatant (membrane fraction IB) was pooled with membrane fraction IA, and the pellet discarded. The pooled membrane fraction I was centrifuged as above to remove any residual cells, nuclei, etc., and the supernate filtered through cotton gauze to remove the fat layer. The filtrate was centrifuged at 27 000 × *g* for 90 min and the pellet suspended in 200—250 ml of Buffer T using a tight fitting Dounce homogenizer (10 strokes). The homogenate was centrifuged at 28 000 rev./min for 1 h in a Spinco Model L-2 centrifuge using a No. 30 rotor, and the pellet suspended in 10 ml of Buffer T using a tight fitting Dounce homogenizer as above. The homogenate (membrane fraction II) was either frozen and stored at −20, at −120°C, or used directly. To membrane fraction II enough Buffer T and Buffer T + 40% sucrose were added to make the final volume 264 ml and the concentration 25% sucrose. Aliquots (11 ml) of the suspension were layered over 15 ml of 40% sucrose in Buffer T contained in centrifuge tubes, and spun at 28 000 rev./min for 8—16 h, the plasma membranes forming a band at the interface. The plasma membrane fraction was collected by aspiration, diluted with two volumes of Buffer T, and pelleted by centrifugation at 28 000 rev./min for 1 h. The pellet was homogenized with 20 ml of Buffer T in a tight-fitting dounce homogenizer and either frozen at −20°C or −120°C, or used directly. Plasma membranes were characterized by having a 2—5 fold elevation in specific 5′-nucleotidase activity over the membrane fraction II similar to values published for similarly prepared membranes from rabbit and bovine lymphocytes and thymocytes [14, 15]. For plasma membrane stability studies, high purity lymphocytes were frozen overnight, processed exactly as for the bulk preparations and the membranes either immediately dissociated in SDS or stored at −20°C or −120°C (see below).

Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of membranes. Membranes were dissociated in Na_2CO_3/sodium dodecyl sulfate/mercaptoethanol and dialyzed against J4179 upper gel containing 0.1% sodium dodecyl sulfate and 0.05% dithiothreitol as described by Neville and Glossman [16]. Protein concentration was roughly estimated by ultraviolet absorbance at 280 nm, and the amount of sample applied to a gel measured in A_{280} units. Typically, a high purity preparation yielded approx. 2 A_{280} units while a bulk preparation yielded 25—50 times as much. A tracking dye (Pyronin Y) and

glycerol (final concentration 10%) were added, and electrophoresis was done on a Hoefer slab gel electrophoresis apparatus (gel size 12 cm long × 17 cm wide × 0.75 mm thick) using system J4179 with both a stacking and separating gel as described by Neville and Glossman [16] for disc gel electrophoresis. The sample(s) was applied either in performed wells or as a continuous band on top of the stacking gel, electrophoresed through the stacking gel at 15 mA and through the separating gel at 25 mA. In most experiments a continuous band was used and 0.7—1.0 A_{280} units of dissociated membranes was applied. Polypeptides were identified by staining the gel with 0.1% Coomassie G-250 in methanol/acetic acid/H_2O (45 : 10 : 45, v/v) and carbohydrate identified by periodate-Schiff staining [17] or by Alcian blue staining [18].

Binding of ^{125}I-labeled lectins to pm components. The procedure we have adopted is a modification of published procedures [19,20]. From both outer edges of the sample-containing gel was cut a 2 cm vertical strip. Both strips were stained with Coomassie G-250, aligned adjacent to each other on a filter paper or a clear membrane (Hoefer) and dried with a Hoefer gel slab drier. The stained strips were examined to assure that all the bands were in register, and the intensity and R_F of each band estimated visually. The remaining gel was fixed for 2 h at 25°C with 0.05% glutaraldehyde in 50% methanol/45% H_2O/5% acetic acid, soaked overnight at 25°C in 0.002% $NaBH_4$ in phosphate buffered saline, pH 7.3, and soaked twice for 3 h in phosphate-buffered saline. It was then cut into vertical strips 1.5—2.0 cm wide and each strip soaked in 5 ml of 0.1 mg/ml ^{125}I-labeled lectin in buffered saline containing 0.02% NaN_3 for 24 h at room temperature. In most experiments the specific ^{125}I activity was 88 000 cpm/μg of lectin and in experiments where the binding of different lectins were compared the specific activities and protein concentrations were always adjusted to be equal. For competition experiments, strips were soaked in mixtures containing 0.1 mg/ml of ^{125}I-labeled lectin and 1 to 10 mg/ml concentrations of unlabeled lectins. After incubation each strip was soaked in 300—400 ml of buffered saline for 2 hours to remove unbound lectin. The strips were aligned in parallel on a filter paper and dried as above. The mounted gels were placed on Kodak Royal X-Omat films in Kodak Royal X-ray exposure film holders and developed after 2—14 days. With each set several different exposure times were used and examined visually. Selected films and stained strips were scanned with a Joyce-Loebl microdensitometer at the highest sensitivity. Because many of the autoradiograms showed closely spaced bands and/or high "backgrounds" typically a gel pattern was aligned in the microdensitometer and 2—4 scans were made through contiguous areas in the center of the gel with the traces being made in register on the same paper. The patterns shown in this paper are hand drawn traces of "average" scans.

Results

Fig. 1 shows a densiometer scan of a typical sodium sulfate-polyacryamide gel electrophoresis pattern of membranes from high purity lymphocytes stained with a Coomassie G-250. By visual inspection some 37 bands were observed, each band indicated by an arrow in Fig. 1. Although the trace is very complex, each band corresponds to a peak or shoulder in the trace. Very similar

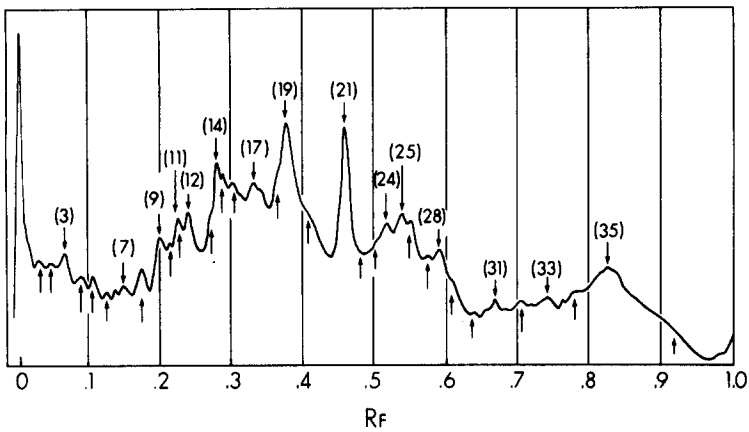

Fig. 1. Densitometric tracing of a typical membrane preparation analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie G-250. Arrows represent positions of polypeptide bands as determined by visual inspection.

patterns were seen with other membrane preparations from high purity lymphocytes and from bulk stored lymph nodes, but the relative amount of staining for each band varied somewhat from experiment to experiment. The top line in Table I shows an analysis of 23 gels obtained with 4 different membrane preparations. Most gels showed 30—37 components of which 8 were present in every run as bands of medium to very heavy intensity with characteristic R_F values (bands 9, 11, 12, 15, 19, 21, 24, and 27). These were used as internal markers to locates other components. Another nine were present in twenty or more of the gels in low to medium intensity (bands 3, 5, 17, 20, 25, 28, 30, 31, and 32). In addition certain characteristic features were present in all gels involving one or more weakly stained bands such as a quadruplet of bands 9, 10, 11, and 12 and the triplet of bands 14, 15, and 16. Generally the greatest variation in band intensity was seen in components with high R_F (0.65—0.85). Indeed bands 30 and 31 were very intense in some gels and weakly stained in others. Bands 33—37 were the most difficult to assess, particularly 33, 36 and 37 which appeared (by Coomassie G-250 staining) in 25—35% of the gels. However, all of these bands showed appreciable binding of ^{125}I-labeled peanut agglutinin in gels where very little Coomassie G-250 staining of these bands was seen (see Table I). When gels were stained for carbohydrate, a broad indistinct stain was seen over the entire length of the gel (not shown) indicating that most, if not all, of the bands had some carbohydrate.

By visually examining films exposed for different times, using 2- to 5-fold magnification, lectin "maps" could be constructed in which the binding of an individual lectin to its various membrane "receptors" was semiquantitatively rated. Table I shows the binding patterns observed.

The only portion of the gel which showed appreciable binding of ^{125}I-labeled soybean agglutinin was at an R_F of 0.9—0.95, a region which does not stain with Coomassie G-250. This component does stain for carbohydrate and with Sudan black, indicating that it is probably a glycolipid-containing fraction. In addition 5 or 6 Coomassie G-250 staining bands gave barely detectable binding

TABLE I

BINDING * OF COOMASSIE G-250 AND ^{125}I-LABELED LECTINS TO MEMBRANE COMPONENT

Binding material	Component number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Coomassie G-250	1	1	2	1	3	1	1	1	4	1	4	4	1	1	5	1	3	1	6	2
L. Culinaris (A and B)	1	8	8		3		1	1	8	1	1	1	1	1	3		3	5		
P. Sativum	1	8	8		3		1		8	1	1	1	1	1	3		3	5		2
Concanavalin A		4	4	5	3		1		3		1	4	1	1	3			4		4
Phytohemagglutinin	3	8	8	5	3		1	5	7	1		1	3		1			1	3	4
R. Communis 60	1	7	7		1		1	3	7	1	3	3	1		2			3	3	3
R. Communis 120	1	8	8	3	3		1	3	8	3		3	1		1			3		3
Wheat germ agglutinin		3		3		1		3					2	2	2		1		1	
A. Bisporus (A and B)		8		8			3		3						1			1	1	
Peanut agglutinin								1					1						3	
Soybean agglutinin											1									
R_F **	0.02	0.05	0.07	0.09	0.11	0.13	0.15	0.17	0.20	0.215	0.23	0.24	0.27	0.29	0.30	0.31	0.33	0.35	0.38	0.41

Binding material	Component number																			
	20 ***	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	GL ***	
Coomassie G-250		8	1	1	4	3	1	5	2	1	2	2	2	1	1	1	1	1		
L. Culinaris (A and B)	3	3	1	1	3		3	3	3	1		1							1	
P. Sativum	3	1	1	1	3		3	3	3	0									1	
Concanavalin A	4	3	3	3	8		3	3	1	3		3	1	1	1	3	1		7	
Phytohemagglutinin				1	3		1		4	3									3	
R. Communis 60		8		3	3			8		1	1						1		2	
R. Communis 120		4		1	3		1	1	4		1	1				1			1	
Wheat germ agglutinin	3	3	3	2	5		3		4				1		2			1		
A. Bisporus (A and B)					1						1	3	1							
Peanut agglutinin			2				5	5			3	5	5	3	5	3	3	2		
Soybean agglutinin		1								1			1				1	1	8	
R_F **	0.43	0.45	0.48	0.50	0.52	0.535	0.55	0.57	0.59	0.62	0.65	0.68	0.71	0.74	0.77	0.80	0.83	0.87	0.95	

* Intensity of binding estimated visually and assigned an arbitrary numerical value. Low intensity = 1; medium intensity = 3; high intensity = 5; very high intensity = 8. If there was no binding the space is left blank.

** R_F is relative to dye front. Standard molecular weight markers, cytochrome *c* (12 000, R_F = 0.89), immunoglobulin L chain (24 000, R_F = 0.69), lactate dehydrogenase (36 000, R_F = 0.52), ovalbumin (45 000, R_F = 0.49), immunoglobulin H chain (50 000, R_F = 0.45), bovine serum albumin (69 000, R_F = 0.36), phosphorylase *a* (94 000, R_F = 0.21).

*** Components 20b and glycolipid (GL) do not stain with Coomassie G-250.

of this lectin. When membranes were extracted with chloroform-methanol according to a published procedure [21] and the organic soluble material, analyzed as above the only ^{125}I-labeled soybean agglutinin binding component migrated with the same R_F and showed the same carbohydrate and lipid staining properties as the major binding component above. The amounts of this material vary greatly from experiment to experiment and other ^{125}I-labeled lectins (e.g., phytohemagglutinin-L, the *Ricinus* lectins and particularly Concanavalin A) also bind in this region. High concentrations of unlabelled soybean agglutinin completely inhibit the binding of ^{125}I-labeled soybean agglutinin while none of the other lectins do. Concanavalin A precipitates with ^{125}I-labeled soybean agglutinin and could not be tested for inhibition of binding.

The binding patterns of the ^{125}I-labeled *Agaricus bisporus* lectins are identical and relatively simple (Table I). Five bands show heavy (Nos. 2 and 4) or medium (Nos. 7, 9, and 31) binding, the predominant binding being to low R_F components. Interestingly three of these bands (Nos. 2, 4, and 7) are among the weakest staining with Coomassie G-250. There was also weak binding to ten other components. High concentrations of either unlabelled *Agaricus bisporus* lectin completely inhibited the binding of either ^{125}I-labeled lectin while no other lectin tested interfered with the binding of either ^{125}I-labeled isolectin. Concanavalin A also precipitates the *Agaricus bisporus* lectins. In contrast to the *Agaricus bisporus* lectins ^{125}I-labeled peanut agglutinin binds appreciably to 13 high R_F components from bands 19—37, the greatest binding being to bands 25, 27, 31, 32, and 34. In addition, two or three bands show weak binding. Only band 31 binds both the *Agaricus bisporus* lectins and peanut agglutinin appreciably. But the peanut agglutinin and *Agaricus bisporus* lectins do not compete for any membrane component nor does any other tested lectin compete with the peanut agglutinin. Wheat germ agglutinin also shows a relatively simple binding pattern which is distinctly different than the *Agaricus bisporus* lectins and peanut agglutinin (Table I). The most intensely binding component was band 24 and four bands bound both *Agaricus bisporus* lectin and wheat germ agglutinin well (Nos. 2, 4, 7, and 9).

The *Lens culinaris* isolectins always gave complicated indistinguishable binding patterns with slight differences in the *Pisum sativum* binding patterns (Table I). The major binding components for these lectins were bands 2, 3, and 9. In competition experiments, the three lectins competed with each other similarly for all of the components to which ^{125}I-labeled *Lens culinaris* lectin A (Fig. 2) and ^{125}I-labeled *Pisum sativum* lectin (not shown) bound. This strongly indicates that they bind to the same membrane components with similar affinities. Concanavalin A showed appreciable inhibition of binding of either ^{125}I-labeled lectin to most of the ^{125}I-labeled *Lens culinaris* lectin A (Fig. 2) and ^{125}I-labeled *Pisum sativum* lectin (not shown) binding components.

Although Concanavalin A binds to many of the same components as the *Lens culinaris* and *Pisum sativum* Lectins, it shows a clearly distinguishable pattern. In every experiment Concanavalin A showed the densest autoradiograms and bound to the greatest number of membrane components of all the lectins. It showed very avid binding to the glycolipid containing component (Table I and Fig. 3) and showed the highest binding to band 24 (a component to which the *Lens culinaris* and *Pisum sativum* lectins bind relatively weakly). Fig. 3

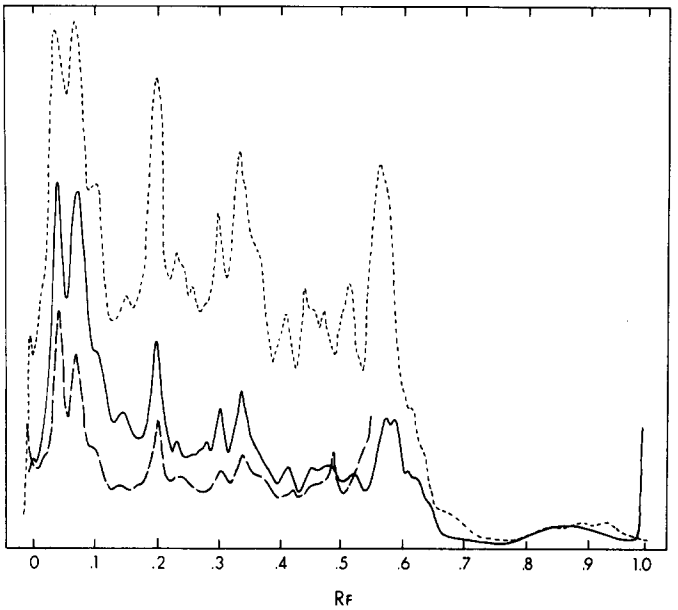

Fig. 2. Binding of ^{125}I-labeled *Lens culinaris* lectin A to membrane components in the presence and absence of unlabeled *Lens culinaris* lectin A or Concanavalin A. No added lectin (- - - - - -); added *Lens culinaris* lectin A, 1 mg/ml (— — —); added Concanavalin A, 1 mg/ml (———). Densitometer trace of radioautograms.

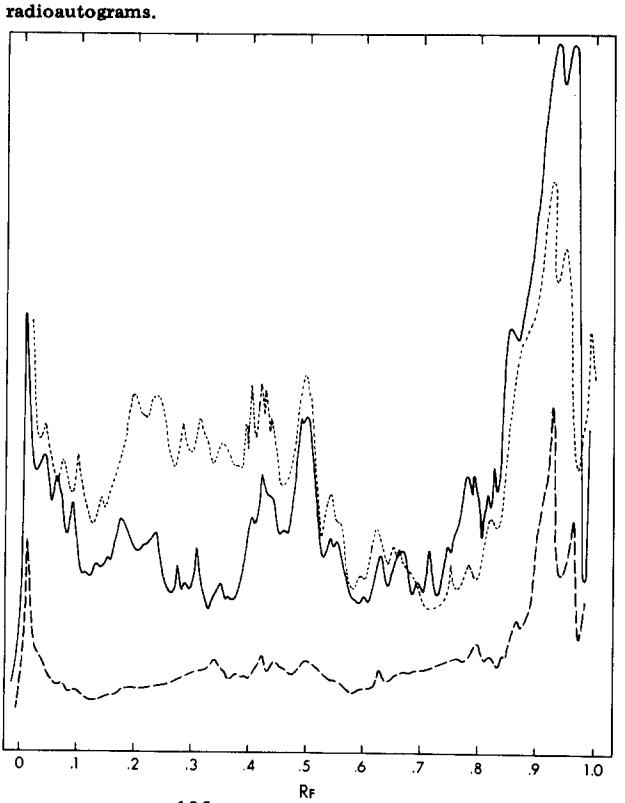

Fig. 3. Binding of ^{125}I-labeled Concanavalin A to membrane components in the absence and presence of unlabeled Concanavalin A or *Pisum sativum* lectins. No added lectin (- - - - - -); added Concanavalin A, 1 mg/ml (— — —); added *Pisum sativum* lectins, 1 mg/ml (———). Densitometer trace of radioautograms.

shows that Concanavalin A was much more effective in inhibiting the binding of ^{125}I-labeled Concanavalin A than was *Pisum sativum* Lectin and that Concanavalin A inhibited the binding of ^{125}I-labeled Concanavalin A to all of the binding components about equally well. In contrast *Pisum sativum* (Fig. 3) and *Lens culinaris* lectins (not shown) effectively inhibited the binding of ^{125}I-labeled Concanavalin A to some membrane components (e.g., most of the bands from R_F 0.1-0.35 and from R_F 0.75—0.80), but was almost ineffective in inhibiting binding to components of R_F 0—0.1 and 0.4—0.75. In particular, the binding of ^{125}I-labeled Concanavalin A to its major binding component (band 24) was not detectably inhibited by *Pisum sativum* lectin. Another interesting feature is that in spite of the fact that *Pisum sativum* lectin binds very well to bands 2 and 3 (Table I), it does not effectively inhibit the binding of ^{125}I-labeled Concanavalin A to these same bands.

The binding pattern of ^{125}I-labeled phytohemagglutinin-L is different from those of *Lens culinaris*, *Pisum sativum* or concanavalin A, although many of the components which bind the latter lectins also bind phytohemagglutinin-L (Table I). The binding of ^{125}I-labeled phytohemagglutinin-L is inhibited by unlabeled phytohemagglutinin-L, partly inhibited by *Ricinus communis* Lectin 60 (Fig. 4) and *Ricinus communis* lectin 120 (not shown) but by none of the other lectins (Concanavalin A precipitates phytohemagglutinin-L and couldn't be tested). Fig. 4 shows that a 100-fold excess of RcA60 effectively competes for all ^{125}I-labeled phytohemagglutinin-L binding molecules except for some components in the R_F range 0.25 to 0.5. The binding pattern of ^{125}I-labeled

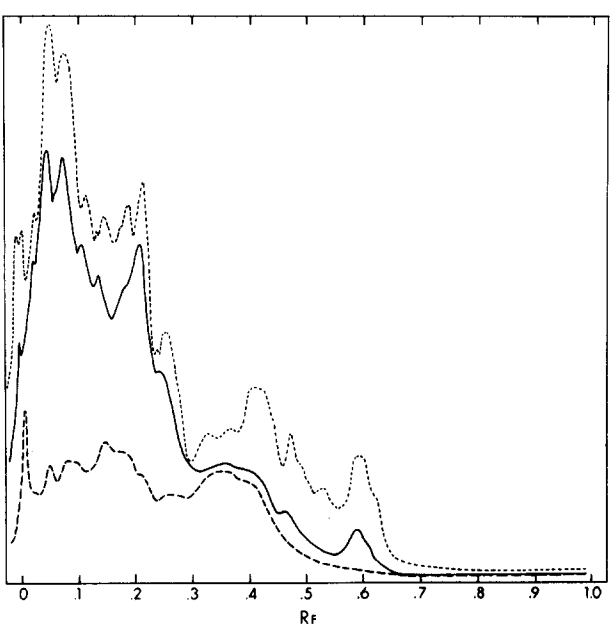

Fig. 4. Binding of ^{125}I-labeled phytohemagglutinin-L to membrane components in the absence and presence of unlabeled phtohemagglutinin-L or *Ricinus communis* lectin 60. No added lectin (- - - - -); added phytohemagglutinin-L, 1 mg/ml (———); Added *Ricinus communis* lectin 60, 10 mg/ml (— — —).

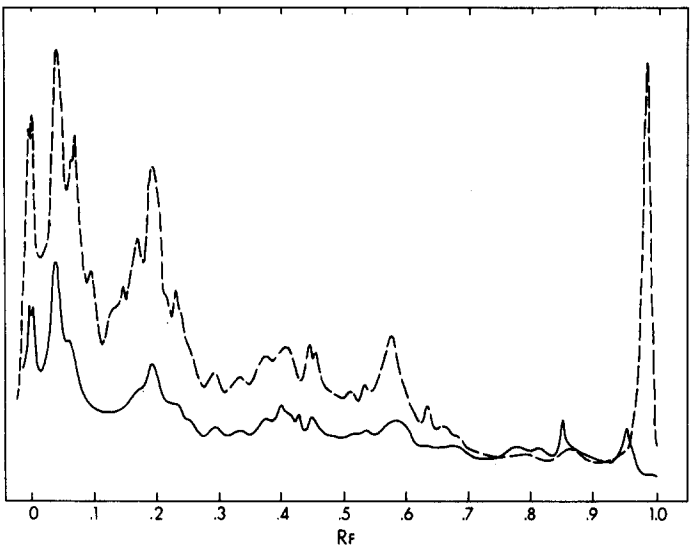

Fig. 5. Binding of ^{125}I-labeled *Ricinus communis* lectin 120 in the absence and presence of unlabeled *Ricinus communis* lectin 60. No added lectin (------); added *Ricinus communis* lectin 60, 1 mg/ml (———).

Ricinus communis lectin 60 (Table I) is different than ^{125}I-labeled phytohemagglutinin-L. While the latter binds most avidly to low R_F (0—0.2) components, the former binds avidly with various components with R_F values from 0 to 0.57. ^{125}I-labeled *Ricinus communis* lectin 120 shows a pattern very similar to ^{125}I-labeled *Ricinus communis* lectin 60 (Table I), and the latter effec-

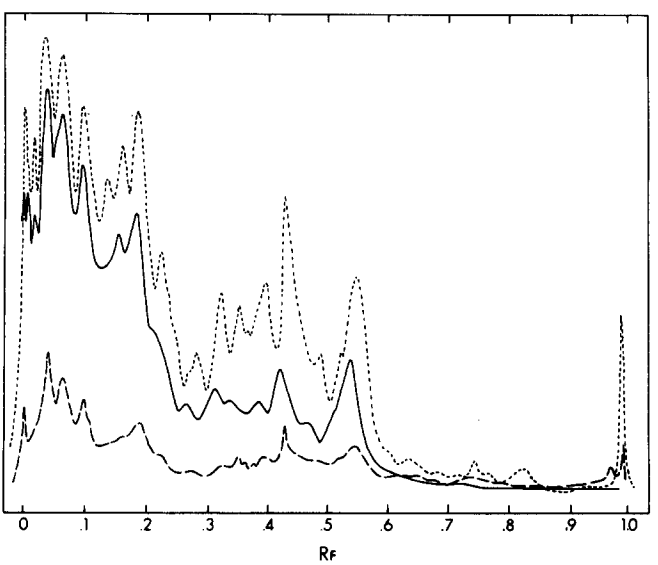

Fig. 6. Binding of ^{125}I-labeled *Ricinus communis* lectin 60 in the presence and absence of unlabeled *Ricinus communis* lectins 60 and 120. No added lectin (------); added *Ricinus communis* lectin 60, 1 mg/ml (— — —). Added *Ricinus communis* lectin 120, 1 mg/ml (———).

tively blocks binding of ^{125}I *Ricinus communis* lectin 120 to most of its membrane binding components (Fig. 5). *Ricinus communis* lectin 120 was not as effective in inhibiting the binding of ^{125}I-labeled *Ricinus communis* lectin 60 (Fig. 6) as vice versa (Fig. 5), showing a similar type of cross reaction as above for the *Ricinus communis* lectin 60 inhibition of ^{125}I-labeled phytohemagglutinin and the Concanavalin A-*Lens culinaris* lectin system.

Discussion

The membranes we have examined contain a very complex mixture of glycopolypeptides and at least one lectin-binding glycolipid. Similarly complex polypeptide-staining patterns have been reported by Chavin and coworkers [22, 23], by Crumpton and coworkers [11,24], and by Wallach and his coworkers [15,25]. In each case some 14—18 major polypeptides have been identified with similar R_F values (or positions relative to known molecular weight protein standards). For our purposes the location of the less heavily-stained components was as important as the major bands, and it was critical that the bands of every gel in particular experiment be in register and identifiable relative to the major and minor Coomassie G-250 staining components. In our hands the methods we have adopted yielded better resolution than those previously reported particularly with the radioautographic studies. The complexity of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns is not very likely due to appreciable contamination of lymphocytes with other cell types, and none of the glycopolypeptides appears to be an artifact of long term (up to six months) frozen storage of membranes or lymph nodes. If membranes were stored frozen and periodically dissociated with 1—10% sodium dodecyl sulfate containing 0.1 M mercaptoethanol (with or without boiling), electrophoresis experiments showed a gradual disappearance of several high R_F components with a simultaneous increase in aggregates which did not enter the analytical gel, and, to a much lesser extent, increased amounts of bands 9 and 15 (Livingstone, L.R. and Sage, H.J., unpublished observation). However, these changes were completely reversed by the dissociation procedure of Neville and Glossman [16]. Membranes prepared from cells frozen only overnight and immediately dissociated in the same solvents could be stored indefinitely at 4° C without any detectable changes in electrophoretic patterns. Two other possible sources of complexity are appreciable contamination with intracellular membranes and heterogeneity of the lymphocytes. We have identified our plasma membrane preparations by a 5′-nucleotidase marker enzyme [14,26] and sedimentation characteristics. However, our procedure for isolating the final membranes and heterogeneity of the lymphocytes. We have identified our plasma sented better evidence that such membranes are reasonably pure plasma membranes. In addition we have examined some of our preparations for membrane bound marker enzymes associated with intracellular organelles. The preparation we have examined contained essentially undetectable amounts of a lysosome marker acid phosphatase [27], a microsomal marker NADH diaphorase [28], and a mitochondrial marker succinic dehydrogenase [29]. This suggests that our preparations are not grossly contaminated with membranes from these organelles, but we cannot rule out the presence of membranes from other

organelles (e.g. nuclear membranes). The possibility that lymphocytes are a heterogenous population of cells each with a smaller number of varying glycopolypeptides is a viable one, although Chavin has observed no obvious differences in the sodium dodecyl sulfate-polyacrylamide cell electrophoresis patterns of membranes from lymph node and thymus cells [22]. It is also possible that some of the glycopolypeptides are not true membrane components but absorbed into the plasma membrane from the lymph or other fluids, as suggested for some high R_F components by Schmidt-Ullrich at al. [15].

In general there was no direct relationship between Coomassie G-250 staining and ^{125}I-labeled lectin binding. In some cases minor components (e.g. bands 2, 3, 4, and 18) gave very appreciable binding with most of the lectins. There is also a macromolecule (component 20b Table I) which binds *Pisum sativum* lectin, *Lens culinaris* lectins, Concanavalin A and wheat germ agglutinin (which all react with GlcNAc) and gave no detectable Coomassie G-250 staining. The amount of ^{125}I-labeled lectin binding to a particular band is a function of both the amount of binding material in the band and the K_a of binding. In principle it is possible to measure these values by quantitating Coomassie G-250 and ^{125}I-labeled lectin binding. Presently this is not fruitful because the resolution of plasma membrane components is not good enough. Even if the resolution were satisfactory, accurate values of K_a are precluded by the anamalous molecular weights given by sodium dodecyl sulfatepolyacrylamide gel electrophoresis for glycoproteins [30—32]. Except for soybean agglutinin every lectin binds to several membrane components with widely varying ^{125}I-labeled lectin/Coomassie G-250 binding ratios. Hence it appears that each lectin binds to the different components with different K_a values.

Except for soybean agglutinin the overall density of ^{125}I-labeled lectin binding and the number of components to which a particular ^{125}I-labeled lectin bound was directly related to its K_a of binding to viable pig lymphocytes. We have observed (Sage, H.J. and Horton, C.B., unpublished observations) that the binding constants for the five mitogens (Concanavalin A, *Lens culinaris* lectins A and B, *Pisum sativum* lectin, and phytohemagglutinin-L) were remarkably constant with a K_a of $6.6 \pm 1.6 \cdot 10^6$ l/mol (Concanavalin A being the most avid of the group), while K_a values for the nonmitogens (wheat germ agglutinin, soybean agglutinin, peanut agglutinin, and *Agaricus bisporus* lectins A and B) were 10 to 50 fold lower, the peanut agglutinin being the weakest by far. The *Ricinus* lectins showed binding constants which were intermediate between the mitogens and nonmitogens.

Soybean agglutinin doesn't bind appreciably to any of the glycopolypeptides but essentially only to a glycolipid-containing fraction. Cuatrecasas [21,33] has shown that a similar fraction prepared from liver microsomes prevents the attachment of cholera vibrio toxin to fat cells and liver microsomes, and that the most potent inhibiting agent was a ganglioside, GM1. Peanut agglutinin, the weakest binding of the lectins to intact pig lymphocytes, nevertheless showed very good binding to several high R_F glycopolypeptides. In this respect it was unique since, in general, the high R_F bands showed little binding with most of the other lectins. Together with *Agaricus bisporus* lectins, soybean agglutinin and peanut agglutinin are a group which reacts strongly with sialidase-treated submaxillary mucins from pigs and sheep (Sage, H.J. and Horton, C.B., unpub-

lished observations). Soybean agglutinin has been shown to bind to GalNAc containing saccharides, but that substitution on the C-6 hydroxyl blocks binding [34]. Similarly GalNAc residues in bovine and porcine submaxillary mucins with sialic acid on the G-6 position do not react well with soybean agglutinin and *Agaricus bisporus* lectins (Sage, H.J. and Horton, C.B., unpublished observations). Polymeric soybean agglutinin mitogenically stimulates pig lymphocytes while the monomeric form M_r = 128 000) does not [35]. If human, rat, mouse, or guinea pig lymphocytes are sialidase treated they are mitogenically stimulated by monomeric soybean agglutinin [36] and peanut agglutinin is reported [37] to have a similar effect on sialidase treated human, rat and possibly pig lymphocytes.

This suggests that one or more of the membrane components to which these lectins do not normally bind appreciably contains a sialidase-blocked binding site for one or more of these lectins, and is possibly a mitogenic receptor. We are presently doing experiments with sialidase-treated cells to see whether the K_a of binding for these lectins (and number of receptors) is increased and whether new lectin-binding membrane components are revealed.

The most complex patterns were those of the five mitogens and the *Ricinus* lectins. The *Ricinus* lectins bind to most of the same receptors to which phytohemagglutinin-L binds. Only in the R_F range 0.3—0.4 are there receptors for which phytohemagglutinin-L and *Ricinus communis* 60 do not compete. However the bands in this region which bind phytohemagglutinin-L all bind the *Ricinus* lectins as strongly. This parallels their reactions with intact pig lymphocytes (Sage, H., unpublished) where each of the three lectins partially inhibits the binding of the other two. The *Ricinus* lectins are more effective in inhibiting the binding of phytohemagglutinin L than vice versa. In contrast to the *Lens culinaris* and *Agaricus bisporus* isolectins the *Ricinus* isolectins do not share an identical specificity either for galactose and *N*-acetyl galactosamine [6] or for intact pig lymphocytes (Sage, H., unpublished). *Ricinus communis* lectin 60 and perhaps *Ricinus communis* lectin 120 are potent intracellular toxins for a variety of cells [38] including lymphocytes [39]. It is an intriguing possibility that the initial event in the toxic effect (binding of the lectin to the pig lymphocyte surface) involves the same receptors as the initial event in phytohemagglutinin-L stimulation of the same lymphocytes. The identical specificities of the *Lens culinaris* isolectins and *Pisum sativum* for membrane components also parallels their binding to intact pig lymphocytes (Sage, H., unpublished). Concanavalin A shows the same kind of unequal cross reactive competition with this group (Sage, H., unpublished) that the *Ricinus* lectins show with phytohemagglutinin (i.e., Concanavalin A is more effective in inhibiting the binding of the other lectins in the group to intact lymphocytes and membrane components than vice versa). An examination of the binding patterns of the lectins to the membrane components reveals several molecules which bind all or most of the mitogens significantly better than most or all of the non-mitogens (e.g. bands 3 and 5). However in view of the similar specificies of *Pisum sativum* lectins, *Lens culinaris* lectins and Concanavalin A and the small number of mitogens and nonmitogens examined, it is premature to consider any of these as good candidates for a mitogenic receptor. In addition it is possible that better resolving techniques will separate some of the bands into more than one component generating more such candidates.

It is interesting that such a variety of binding patterns are seen. Few, if any, of the bands show the same binding pattern for all the lectins. Assuming that each band represents a unique glycopolypeptide, this suggests that there are an appreciable variety of carbohydrate structures on the various components (as opposed to a few common carbohydrates on polypeptides of different size). However it is still an open question as to whether all of the lectins-binding membrane components are present on the lymphocyte surface.

An alternative method for identifying lectin-binding components in lymphocyte plasma membranes has been taken by Crumpton and his coworkers [40, 41]. These workers have dissociated plasma membranes in nonionic detergents, subjected the dissociated membranes to affinity chromatography on insolubilized lectin columns, transferred the lectin-binding material to sodium dodecyl sulfate and analyzed it by sodium dodecyl sulfate disc gel electrophoresis. Affinity chromatography of dissociated pig lymphocyte plasma membrane on a *Lens culinaris* lectin column yielded at least 10 major glycopolypeptides [40] with R_F values ranging from 0 to approx. 0.5. In our experiments described above, there were 12 bands which showed a medium to very heavy staining with ^{125}I-labeled *Lens culinaris* in the same R_F region. Similarly Allen et al. [41] have subjected pig lymphocytes to affinity chromatography on Concanavalin A-Sepharose and have observed a very complex mixture of glycopolypeptides which bind to this lectin. These glycopolypeptides have widely variant affinities for the lectin column and a greater proportion of the dissociated plasma membrane adhered to Concanavalin A columns than to *Lens culinaris* lectin columns. Our observation that ^{125}I-labeled Concanavalin A binds to more components and with a greater affinity than ^{125}I-labeled *Lens culinaris* lectin is consistent with this observation. ^{125}I-labeled Concanavalin A binds with medium to very high intensity to 19 different glycopolypeptides. Schmidt-Ullrich et al. have suggested [42] that the major Concanavalin A receptor on rabbit thymocytes is a glycopolypeptide with an equivalent molecular weight of 55 000. In contrast, our findings above show that there is a Concanavalin A binding component at an R_F corresponding to 55 000 daltons (either band 20 or 21) but that, by far, the most avidly Concanavalin A binding glycopolypeptide was band 24 with an R_F of 0.52 corresponding to an approximate molecular weight of 35 000. Of course we are examining different lymphocytes from different species and have used entirely different methods of membrane preparation and analysis. Studies are in progress to see if the two methods of analysis are equivalent.

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