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INTERACTIONS OF LECTINS WITH PLASMA MEMBRANE GLYCOPROTEINS OF THE EHRLICH ASCITES CARCINOMA CELL

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SUMMARY

Several aspects of the interaction of various lectins with the surface of Ehrlich ascites carcinoma cells are described. The order of agglutinating activity for various lectins is *Ricinus communis* > wheat germ ⩾ concanavalin A ⩾ soybean > *Limulus polyphemus*. No agglutination was noted for *Ulex europaeus*. Using ^{125}I-labeled lectins it was determined that there are 1.6 and 7 times as many *Ricinus communis* lectin binding sites as sites for concanavalin A and soybean lectins. Sodium deoxycholate-solubilized plasma membrane material was subjected to lectin affinity chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lectin receptors of the plasma membrane appeared to be heterogeneous and some qualitative differences could be discerned among the electrophoretically analyzed material, which bound to and was specifically eluted from the various lectin affinity columns. The characteristics of elution of bound material from individual lectin columns indicated secondary hydrophobic interactions between concanavalin A or wheat germ agglutinin and their respective lectin receptor molecules.

INTRODUCTION

Lectins have proven to be useful probes of cell surfaces and have been successfully employed to study the topographical arrangement of surface carbohydrate components as well as in functional studies involving these outer-surface-oriented molecules [1]. In a previous study [2] we have observed that two lectins, concanavalin A and soybean agglutinin, had significantly different effects on the net transport of Na^+ and K^+ in Ehrlich ascites carcinoma cells. The present investigation is part of an ongoing study to determine the mechanisms which underly these lectin-initiated changes in ascites cell ion transfer. In this investigation we undertook to characterize more precisely the nature of the lectin-cell surface interactions and to study the surface structures bearing lectin receptor sites. We report the results of chemical, enzymatic, immunological and structural evaluations of the purified cell surface or plasma membrane with special emphasis on the glycoprotein moieties. We also report on the agglutinability of the Ehrlich ascites carcinoma cells to a panel of lectins which

presumably react with a complete complement of the major carbohydrate constituents located on the ascites tumor cell plasma membranes, the quantitation of the number of receptor sites for each of these lectins and the isolation of the receptor molecules themselves, by affinity chromatography.

MATERIALS AND METHODS

Preparation of Ehrlich ascites carcinoma cell plasma membranes

Ehrlich ascites carcinoma cells were grown, harvested and washed as previously described [2, 3]. Washed cells were suspended in 0.001 M $ZnCl_2$ and allowed to swell. This step, which is a modification of the procedure of Warren et al. [4], was introduced by Columbini and Johnstone [5] for the Ehrlich ascites carcinoma cell. The cell suspension was then homogenized in a glass homogenizer with a teflon pestle until greater than 90 % of the cells were disrupted as determined by phase contrast microscopy. This step and all subsequent steps were performed at 0–4 °C. The homogenate was diluted with 2 vols. of 0.001 M $ZnCl_2$ and was then centrifuged at $100 \times g$ for 1 min. The pellet, which was composed mostly of nuclei with some unbroken cells and a few large membrane fragments, was discarded. The supernatant was again centrifuged at $100 \times g$ for 1 min. Again the pellet was discarded and the procedure repeated. The supernatant of the third centrifugation was centrifuged at $400 \times g$ for 15 min. The pellet, which now consisted primarily of large sheets of plasma membrane and some nuclei, was dispersed in the upper phase of a dextran 500-polyethyleneglycol two-phase aqueous polymer system described by Brunette and Till [6] and the plasma membrane was isolated according to their centrifugation procedure [6]. 2–3 such centrifugations were necessary to pellet all of the remaining nuclei and any residual cell debris. The plasma membrane fraction so obtained was then washed twice in 0.001 M EDTA (pH 7.5) and then twice with 0.01 M Tris · HCl buffer (pH 7.8). Membranes were sedimented after each wash by centrifugation at $50\,000 \times g$ for 30 min at 4 °C. The purity of all membrane preparations was established by phase contrast and electron microscopic examination. The membrane pellets from the last wash were then subjected to one of the following procedures: (a) lyophilization and storage at −20 °C, until use in chemical determinations; (b) suspension in 15 % dimethyl sulfoxide and storage at −20 °C for later use in enzymatic assays; (c) resuspension in 0.01 M Tris · HCl buffer (pH 7.8) for immediate use in enzymatic assay; (d) resuspension in the 0.01 M Tris buffer (pH 7.8) at a final concentration of 1 mg protein/ml for detergent solubilization.

Radioactive labeling of Ehrlich ascites carcinoma cells

5 μCi of [^{14}C]glucosamine (New England Nuclear, spec. act. 56.5 m Ci/nmol) were injected intraperitoneally into Swiss HA/ICR male mice bearing ascites tumor cells on the 7th–8th day after tumor inoculation. 48 h later a second injection of 5 μCi was given intraperitoneally. 24 h after the second injection the tumor cells were harvested and membrane prepared as described above. Labeled materials were counted in liquid scintillation systems using an aquasol cocktail (New England Nuclear).

Chemical characterization of Ehrlich ascites cell plasma membrane

Purified plasma membranes were assayed for the presence of succinate dehy-

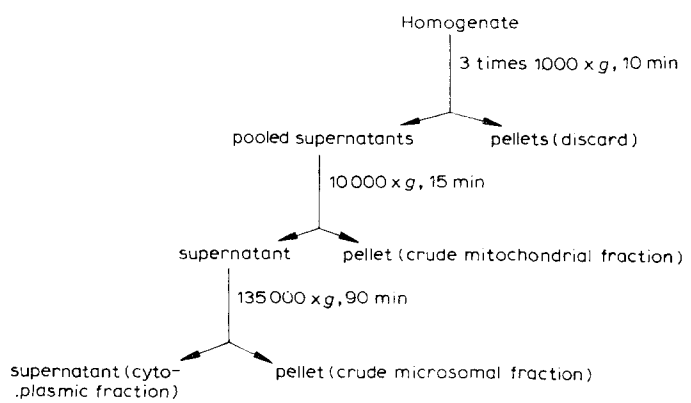
drogenase [7], NADH diaphorase [8], glucose-6-phosphatase [9], (Na^+-K^+ Mg^{2+})- and Mg^{2+}-ATPases [10] and lactate dehydrogenase [11]. For chemical determinations, protein was measured by the method of Lowry et al. [12], sialic acid determined according to Jourdian et al. [13], phospholipid levels measured as described by Ames and Dubin [14], cholesterol content by the method of Glick et al. [15] and inorganic phosphorus levels by the method of Chen [16]. DNA and RNA were isolated according to Blobel and Potter [17] and determined according to Cerrioti [18] and Fleck and Munro [19], respectively. For calculation of RNA of the Ehrlich ascites carcinoma cell, μg RNA/ml per absorbance unit at 260 nm was taken as 32, according to Molnar [20].

Immunochemistry of Ehrlich ascites carcinoma cell plasma membrane

Rabbits were immunized with freshly prepared plasma membrane suspended in Freund's complete adjuvant (Calbiochem, La Jolla, Calif.) by subcutaneous injection at multiple sites. Each animal received a series of three injections a week, each series containing 1 mg of the purified membrane, for four consecutive weeks. After the last injection the rabbits were rested for 10 days and then test bled. Antisera titers were determined for both agglutination and complement fixation of ascites tumor cells according to methods described by Campbell et al. [21]. Test bleedings of animals prior to immunization were performed routinely and yielded negative results.

Antibody to the outer surface of the ascites tumor cells was obtained essentially as described by Kedar et al. [22]. The anti-plasma membrane serum was adsorbed with glutaraldehyde-fixed intact ascites tumor cells. After washing the treated cells three times with Ringer's phosphate buffer [2], elution of the adsorbed specific antibody was achieved by treatment of the cells with 0.2 M glycine · HCl buffer (pH 2.8). After pelleting the cells the supernatant containing the adsorbed antibody was immediately neutralized by the addition of 1.0 M NaOH and dialyzed against Ringer's buffer. This preparation hereafter will be referred to as anti-outer surface antibody.

The plasma membrane fraction and several other subcellular fractions from the ascites tumor cells were then tested for their ability to adsorb out the anti-outer surface antibody so obtained. The pellet of the first aqueous two-phase system centri-



Scheme 1.

fugation used in the plasma membrane purification was called the crude nuclear pellet. Other subcellular fractions were obtained by differential centrifugation of the starting crude homogenate as indicated in the diagram on the previous page.

After purification all cell fractions were resuspended in 0.01 M Tris · HCl buffer (pH 7.8). To aliquots of these cell fractions, each containing 1 mg protein/ml, an equal amount of protein (w/w) of anti-outer surface antibody was added and after a 1-h incubation with mild shaking at 25 °C the mixtures were centrifuged at the appropriate speeds and times (see flow chart) to pellet the various membrane fractions. The supernatants obtained after centrifugation were then tested for the presence of unadsorbed anti-outer surface antibody by using serial dilution aliquots of the supernatants and testing for their ability to be adsorbed by glutaraldehyde-fixed, 0.1 M glycine and Ringer's-washed intact ascites tumor cells in an immune hemoadsorption test as described by Miyamoto and Kato [23]. The indicator system used consisted of sheep erythrocytes treated first with rabbit antisheep erythrocyte serum and then with goat anti-rabbit γ-globulin sera. γ-Globulin bound to glutaraldehyde-fixed ascites tumor cells was then detectable by the adherence of sensitized erythrocytes to the tumor cells. No sensitized sheep red cells bound to the glutaraldehyde-fixed tumor cells in the absence of specific rabbit anti-membrane antibody.

Preparation of deoxycholate-solubilized Ehrlich ascites carcinoma cell plasma membrane material for use in lectin affinity chromatography

To a solution of plasma membrane (1 mg/ml) in 0.01 M Tris · HCl buffer (pH 7.8) 0.1 vol. of a 10 % solution of sodium deoxycholate in the 0.01 M Tris · HCl buffer was added and the suspension was allowed to stand 16 h at 4 °C. The resulting slightly opalescent solution was then centrifuged at 135 000 $\times g$ for 90 min at 4 °C. The clear supernatant was found to contain 80–90 % of the protein and 85–95 % of the radioactivity of membrane which had been prepared from cells that had been prelabeled in vivo with [^{14}C]glucosamine. Exposure of membrane to sodium deoxycholate for shorter time periods resulted in less soluble material. As no differences in sodium dodecyl sulfate-polyacrylamide gel profiles or in the percentage of solubilized radioactive material binding to lectin affinity columns were observed for soluble material obtained from a shorter exposure to sodium deoxycholate, the 16-h exposure was considered optimal.

Preparation of lectins and lectin affinity columns

Concanavalin A was obtained from Miles Laboratory as a 3 times crystallized powder and was further purified by binding the agglutinin to a Sephadex G-100 column, eluting off the bound concanavalin A with 0.1 M α-methylmannoside and finally dialyzing the eluted product extensively against distilled water containing 0.001 M $MgCl_2$ and 0.001 M $CaCl_2$ to remove the sugar. Wheat-germ agglutinin was purified according to Marchesi [24]. The high molecular weight (120 000) *Ricinus communis* agglutinin with primary sugar specificity for D-galactose was purified following the procedures described by Nicolson and Blaustein [25]. Soybean agglutinin was purified according to Nachbar and Oppenheim [3]. Two distinct lectins were isolated and purified from *Ulex europaeus* extracts according to Matsumoto and Osawa [26, 27]. Using the nomenclature suggested by Chuba et al. [28] that lectin which is fucose inhibitable is referred to as *Ulex* anti-H^F while the second lectin which

is chitobiose-cellobiose inhibitable is called *Ulex* anti-H^C. *Limulus polyphemus* agglutinin was purified according to Oppenheim et al. [29].

Concanavalin A, wheat-germ agglutinin, soybean agglutinin, *Ricinus communis* agglutinin and *Ulex* anti-H^F purified lectins were covalently attached to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals), respectively, according to the Manufacturer's directions. In all cases lectin was bound in the presence of 0.1 M concentration of the sugar for which it displayed specificity. In all instances, greater than 90 % of the lectin protein was bound as determined by the absorbance measurements at 280 nm of the original protein preparation and of the subsequent washings. The final concentration of bound lectin protein per ml of settled gel were as follows: concanavalin A, 9.0 mg/ml; *Ricinus communis* agglutinin, 9.1 mg/ml; wheat-germ agglutinin, 3.3 mg/ml; soybean agglutinin, 5.0 mg/ml; and *Ulex* anti-H^F, 2.2 mg/ml. The gels were stored at 4 °C in buffers containing 0.1 M of their respective sugars and 0.25 M NaCl in 0.01 M Tris · HCl (pH 7.8).

Affinity chromatography

Chromatography of sodium deoxycholate-solubilized plasma membrane material on lectin affinity columns was performed essentially as described by Allan et al. [30]. 10 ml of the various lectin-bound resins were packed into 1×15 cm columns. Prior to use the columns were washed with 10-column volumes each of the following solutions: 0.01 M Tris · HCl buffer (pH 7.8); 1 % sodium deoxycholate in the Tris · HCl buffer made 0.1 M with respect to the sugar for which each lectin was specific (i.e. 0.1 M α-methylmannoside for concanavalin A, etc.); and 1 % sodium deoxycholate in the Tris · HCl buffer. The detergent-solubilized membrane material was passed through the column twice. The column was then washed with the 1 % sodium deoxycholate/Tris · HCl buffer until the effluent showed no absorbance at 280 nm or failed to exhibit any radioactivity. The column was then eluted with the detergent/Tris · HCl buffer containing 0.1 M of the appropriate sugar, and 3.0 ml fractions were collected. All fractions were monitored for absorbance at 280 nm and for radioactivity. Fractions with high levels of radioactivity were pooled and the eluted material was precipitated by the addition of 0.1 vol. of 2 % acetic acid and the precipitate so formed was extracted three times with 10 ml of 95 % ethanol. After the final extraction the residues were air dried with a gentle air current and solubilized immediately in 1 % sodium dodecyl sulfate in 0.01 M Tris · HCl (pH 8) for electrophoresis.

Polyacrylamide disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed using the system of Fairbanks [31] as modified by Steck and Yu [32]. The gels employed were 5.6 % (w/v) in acrylamide and 0.21 % (w/v) in the crosslinking agent *N,N'*-methylene bisacrylamide. They measured 0.6 cm in diameter and 13 cm in length. Samples were electrophoresed at 6–7 mA/column until the pyronin Y tracking dye had migrated 10 cm into the gel. Samples for electrophoresis were prepared in several ways depending on the starting physical state of the material. Lyophilized membrane or dried membrane products from lectin affinity columns were solubilized in an Na_2CO_3/sodium dodecyl sulfate/mercaptoethanol solution as described by Glossman and Neville [33]. The resulting solution was then dialyzed overnight against electro-

phoresis buffer. Membrane in Tris · HCl buffer was solubilized by the addition of sodium dodecyl sulfate and mercaptoethanol to final concentrations of 2 % and 1 %, respectively, and the solution brought to 100 °C for 3 min. The clarified solution was dialyzed overnight against electrophoresis buffer. Solutions containing sodium deoxycholate, except for the eluted materials from lectin affinity columns, were first dialyzed against several changes of distilled water for 24–48 h, then lyophilized and solubilized in sodium dodecyl sulfate as noted. After electrophoresis gels were fixed and stained with either Coomassie blue [31] to reveal protein bands or by the periodic acid-Schiff procedure [32] in order to locate carbohydrate-containing material. Stained gels were scanned in a Gilford ultraviolet-visible spectrophotometer model 240, coupled to a 20-cm gel scanner model 2520 and recorded on a model 6050 recorder, at 557 nm for Coomassie blue and 547 nm for the periodic acid-Schiff stain. Gels in which radioactive samples (e.g. [^{14}C]glucosamine labeled) had been electrophoresed were sliced and mashed into sixty 2-mm segments in a Gilson Autogeldivider and deposited into liquid scintillation vials containing 0.6 ml of distilled water. After incubation at 25 °C for 18 h, 15 ml of Aquasol cocktail was added to each vial. The vials were subsequently counted for radioactivity in a Nuclear Chicago liquid scintillation counter.

Quantitation of lectin binding

Lectins were iodinated with ^{125}I (New England Nuclear, spec. act. 490 μCi/ml) according to McFarlane [34]. Active iodinated lectins were purified by binding the labeled material to agarose gels carrying the appropriate sugar grouping and then eluting the bound material with sugar. Following elution samples were dialyzed extensively against Ringer's buffer in order to remove all traces of the sugar used in the release of the labeled lectins. The following specific activities were achieved: concanavalin A, $3.74 \cdot 10^7$ dpm/mg; *Ricinus communis* agglutinin, $5.0 \cdot 10^7$ dpm/mg; soybean agglutinin, $2.47 \cdot 10^7$ dpm/mg. Binding assays were performed according to Nicolson [35] at 0 °C with a 5-min incubation and a cell concentration of $5 \cdot 10^6$ cells/ml. It had been previously been determined that maximum binding occurred under these conditions. To calculate the number of molecules of lectin bound per 10^6 cells the molecular weights of the various lectins at pH 7.4 were assumed to be 106 000 for concanavalin A [36], 120 000 for the soybean agglutinin [37] and 120 000 for *Ricinus communis* agglutinin [25]. Non-specific binding was determined for each concentration of lectin by performing duplicate assays containing 0.1 M concentrations of the appropriate sugars. Specific binding was then calculated by subtracting non-specific binding from total binding determined in the absence of inhibitory sugar.

Agglutination assays

Agglutination assays were performed according to Chuba et al. [28]. Starting lectin concentrations were all 1 mg/ml.

RESULTS

Purification of Ehrlich ascites carcinoma cell plasma membranes

Before any serious studies can commence on the chemical organization of a specified biological membrane system the purity of that membrane, in relationship to

TABLE I

CHEMICAL CHARACTERIZATION OF PLASMA MEMBRANE PERPARATION

	Phospho-lipid*	Choles-terol*	RNA*	DNA*	Sialic acid*	$(Na^+ + K^+ + Mg^{2+})$-ATPase (μmol/mg protein per h)	Mg^{2+}-ATPase (μmol/mg protein per h)	NADH diaphorase (μmol/mg protein per min)	Glucose-6-phosphatase (μmol/mg protein per h)	Succinate dehydroge-nase (μmol/mg protein per min)
Whole cell homogenate	162	19.2	114.8	35.2	12.2	1.2	0.3	0.25	0.38	0.006
Purified plasma membrane	270	55.2	97.2	3.5	20.6	18.0	2.9	0.02	–	0.003

* μg/mg protein

other contaminating membranes, must be established. In this vein we have attempted to obtain a high degree of purity of the plasma membrane fraction from Ehrlich ascites carcinoma cells as judged by biochemical, immunological and electron microscopic evaluations.

Chemical and enzymatic composition. The chemical and enzymatic composition of the purified plasma membrane fraction as compared to a whole cell homogenate can be seen in Table I. It can be observed that the plasma membrane fraction is significantly enriched in phospholipid, cholesterol and sialic acid while showing somewhat lower levels of RNA and little if any DNA. Enzymatic activities of Mg^{2+}- and $(Na^{+}+K^{+}+Mg^{2+})$-ATPases, established plasma membrane markers [5, 38], show 10- and 15-fold increases in the purified fraction, respectively, whereas enzyme markers associated with other membrane organelles (endoplasmic reticulum, mitochondria, etc.) exhibit drastically lower levels of activity as compared to the whole cell homogenate. Both the chemical and the enzymatic data agree with other published values for the purified plasma membrane fraction for this cell [5, 38] and are indicative of a high degree of purity.

Electron microscopic evaluation. Electron micrographs of the plasma membrane fraction showed typical double-tracked membrane structures which were highly vesicularized. While the preparation was virtually free of mitochondria and reticulum structures many of the plasma membrane vesicles had some ribosomal material associated with them.

Immunological evaluation. Antiserum prepared in rabbits to the purified plasma membrane fraction gave agglutination, complement lysis and immune hemadsorption titers of 1/1000, 1/5000 and 1/1 000 000, respectively, against the ascitic cells (see Materials and Methods). Antibody to the outer surface of the cell, prepared by whole cell adsorption, was then used in immune hemadsorption tests to measure the ability of various subcellular fractions to react with that antiserum. It can be seen in Table II that the plasma membrane fraction is the most efficient fraction in adsorbing out antibody to antigenic determinants exposed at the outer surface.

TABLE II

IMMUNE HEMADSORPTION OF SERIAL DILUTIONS OF ANTI-OUTER SURFACE ANTIBODY ADSORBED BY VARIOUS FRACTIONS FROM EHRLICH ASCITES CARCINOMA CELLS

	1/2	1/4	1/8	1/16	1/32	1/64
Homogenate	++++	++++	++++	++	+	−
Membrane	++	+	−	−	−	−
Crude nuclear pellet (first two-phase pellet)	++++	++++	++	+	−	−
Mitochondria, 15 000 × *g*, 15 min	++++	++++	++++	++	+	−
Microsome, 135 000 × *g*, 90 min	++++	++++	++	+	−	−

Identification of glycoprotein components of the Ehrlich ascites carcinoma plasma membrane

Sodium dodecyl sulfate-polyacrylamide gels were prepared. Both the Coomassie blue and periodic acid-Schiff stained gels demonstrated a large number of bands. As we were primarily interested in the carbohydrate-containing components only the scans of the periodic acid-Schiff stained gels are shown (Fig. 1). Prominent peaks corresponding to molecular weights of 200 000, 170 000, 135 000, 105 000, 95 000, 90 000, 43 000 and 35 000 are seen for the scan of whole membrane. In this gel a significant band is also seen, which migrates slightly ahead of or just at the dye front and may represent glycolipid material [39–41]. When membranes were first exposed to 1 % sodium deoxycholate, followed by dialysis to remove the detergent, lyophilization, and solubilization in sodium dodecyl sulfate prior to electrophoresis the periodic acid-Schiff scan labeled 'Sodium deoxycholate membrane' in Fig. 1 is obtained. It is interesting to note that this scan is almost identical to that observed for membranes solubilized directly in sodium dodecyl sulfate ('Whole membrane'), except for an apparent shift in a band migrating at 120 000 daltons to one migrating at 135 000 daltons, and a marked reduction in the band at or just ahead of the dye front. This last difference may be explained by the loss of the low molecular weight material during the dialysis step to remove the sodium deoxycholate. The scan pattern of membrane material solubilized by sodium deoxycholate (see Materials and Methods) is

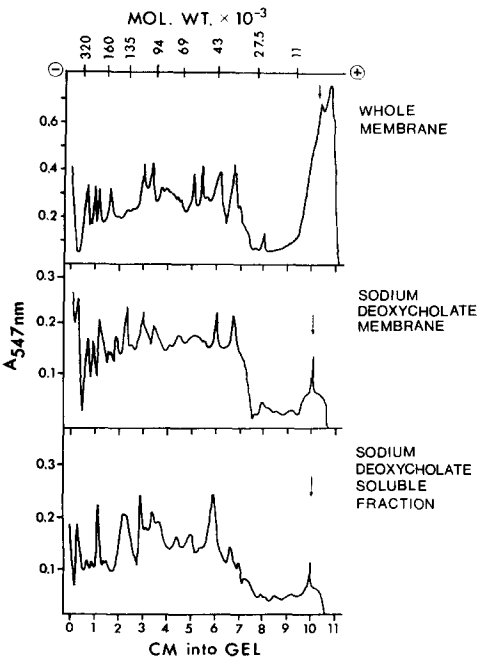


Fig. 1. Scans at 547 nm of sodium dodecyl sulfate-polyacrylamide gels stained by the periodic acid-Schiff procedure of Ehrlich ascites carcinoma cell plasma membrane (Whole membrane), plasma membrane exposed to sodium deoxycholate (Sodium deoxycholate membrane) and the 130 000 × *g*, 90 min supernatant (Sodium deoxycholate soluble fraction) following treatment of whole membrane with 1 % sodium deoxycholate. Details of preparation of samples and conditions of electrophoresis are given in the text. Arrows indicate the position of the marker dye.

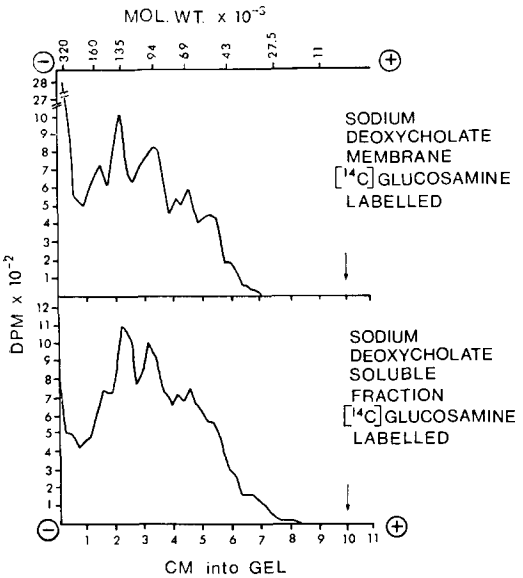


Fig. 2. Comparison of the radioactivity profiles of sodium dodecyl sulfate-polyacrylamide gels of plasma membranes from Ehrlich ascites carcinoma cells labeled in vivo with [^{14}C]glucosamine and treated with sodium deoxycholate (Sodium deoxycholate membrane [^{14}C]glucosamine labeled) and the 130 000 × *g*, 90 min supernatant following sodium deoxycholate treatment of plasma membrane (Sodium deoxycholate soluble fraction [^{14}C]glucosamine labeled). Arrows indicate the position of the marker dye. Details of preparation of samples and conditions of electrophoresis are given in text.

also shown in Fig. 1 ('Sodium deoxycholate soluble fraction') and is essentially the same as that seen for whole membrane exposed to this detergent ('Sodium deoxycholate membrane'). The most notable difference is a decrease in a glycoprotein migrating as the second major peak in the 27 500–43 000 dalton region. It is thus apparent that almost all of the major species of glycoproteins present in the plasma membrane are also represented in the detergent-solubilized material. This is an important point since this solubilized material is the source of the lectin receptors isolated by affinity chromatography (see below).

Isolation and quantitation of lectin receptors

Agglutination of Ehrlich ascites carcinoma cells by various lectins. The major plasma membrane glycoproteins may act as potential binding sites for lectins in the environment. In order to determine to which lectins the glycoproteins may bind, agglutination assays were performed. The results are shown in Table III. The order of agglutinating activity is *Ricinus communis* agglutinin > wheat germ > concanavalin A ⩾ soybean > *Limulus*. No agglutination was noted for either of the *Ulex* lectins. At concentrations of lectins yielding 1+ to just 4+ agglutination, the agglutination was reversible for *Ricinus communis* and soybean agglutinins up to 30 min after initiation of agglutination by addition of 0.1 M lactose and 0.1 M *N*-acetyl-D-galactosamine, respectively. In contrast, agglutination by concanavalin A could not be reversed by α-D-methylmannopyranoside as early as 10 min after initiation of agglutination.

TABLE III

AGGLUTINATION OF EHRLICH ASCITES CARCINOMA CELLS BY VARIOUS LECTINS

Lectin	Dilutions								
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Concanavalin A	++++	++++	++	+	−	−	−	−	−
Ricinus communis	++++	++++	++++	++++	++++	++++	++	+	−
Soybean	++++	++++	++	+	−	−	−	−	−
Wheat germ	++++	++++	++++	++++	++	+	−	−	−
Limulus	++++	++	+	−	−	−	−	−	−
Ulex anti-H^C	−	−	−	−	−	−	−	−	−
Ulex anti-H^F	−	−	−	−	−	−	−	−	−

Lectin affinity chromatography. In the previous section we determined which lectins bind to surface glycoproteins. Next we sought to isolate from the detergent-soluble material those components which would bind specifically to lectin affinity columns. Five different lectin columns were utilized (see Materials and Methods). The amounts of radioactive material applied, bound and specifically eluted, as well as the percent of material bound, eluted and un-elutable, in typical experiments for each lectin column are shown in Table IV.

As can be seen in Table IV at least 95 % of the material bound to the *Ricinus communis* agglutinin column was specifically eluted. This recovery pattern, however, did not seem to hold for other lectin columns used, with the exception of the soybean column, even when the sugar concentration was increased 10-fold or the column was eluted with 1 M NaCl in the Tris · HCl buffer. To assess more accurately the recovery of bound material by specific sugar elutions we determined the amount of radioactivity remaining on the column beads after the sugar elution. This was accomplished by either directly counting a sample of the beads or indirectly by counting a sample of supernatant following sodium dodecyl sulfate treatment of beads. In the latter method 1 ml of settled beads was resuspended in 10 ml of a sodium dodecyl sulfate solution so as to effect a final detergent concentration of 2 %. The sample was then boiled for 3 min and then centrifuged for 10 min at 50 000 $\times g$ to remove the denatured gel. After centrifugation, samples of the supernatant were measured for radioactivity. The percentages of bound material remaining in the beads are shown in the last column of Table IV. The first values are those calculated from direct counting of beads while those in parenthesis were derived from the detergent supernatants. The lower values for direct counting of concanavalin A and wheat germ agglutinin beads may be attributable to quenching by the beads. Using sodium dodecyl sulfate supernatant values it can be seen that in all instances, except for the soybean agglutinin column, 100 % of the bound radioactivity could be accounted for by adding the

TABLE IV

ANALYSIS OF THE RECOVERY OF SODIUM DEOXYCHOLATE-SOLUBILIZED, ^{14}C-LABELLED PLASMA MEMBRANE MATERIAL FROM VARIOUS LECTIN AFFINITY COLUMNS

Lectin affinity column	Radioactivity				% eluted of material bound	% bound material remaining in beads*
	Applied (total counts)	Bound (total counts)	Eluted	% bound		
Ricinus communis	630 000	508 000	485 000	80.0	95.4	0 (0)
Concanavalin A	630 000	230 000	140 000	38.5	60.9	28 (35)
Wheat germ	430 000	250 000	135 000	58.0	54.0	38 (50)
Soybean	500 000	40 000	32 000	8.0	80.0	0 (0)
Ulex anti-H^F	510 000	0	0	0	0	0 (0)

* Determined by either directly counting an aliquot of the beads or from an aliquot of the supernatant after sodium dodecyl sulfate treatment of beads (1 ml of settled beads resuspended in 10 ml of detergent solution so as to give a final detergent concentration of 2 %). The sample was then boiled for 3 min and then centrifuged for 10 min at 50 000 $\times g$ to remove the denatured gel. After centrifugation aliquots of the supernatant were measured of radioactivity.

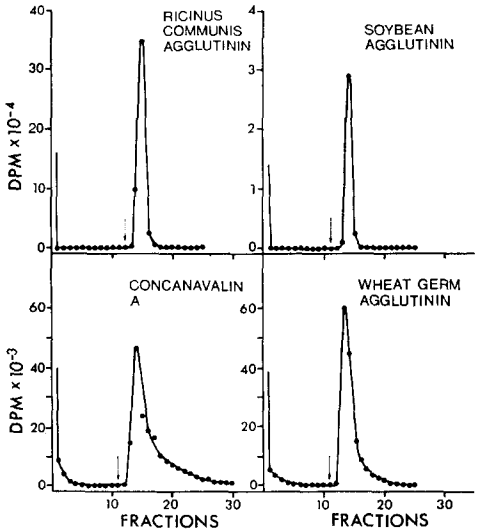


Fig. 3. Comparison of the radioactivity elution profiles from lectin-Sepharose 4B affinity columns of sodium deoxycholate solubilized material from Ehrlich ascites carcinoma cell plasma membrane labeled in vivo with [^{14}C]glucosamine. Arrows indicate the start of the elution with specific sugars.

amount remaining in the beads to that specifically eluted. It is probable that the percentage of bound material specifically eluted from the soybean agglutinin column actually was closer to 100 % rather than the 80 % figure in Table IV, since much less radioactivity was bound initially and the large volumes, relative to the radioactivity of the samples, that were encountered may have led to errors in calculation. This suggestion is supported by the failure to detect any radioactivity on the soybean agglutinin affinity beads. The order of recovery of bound material by specific sugar elution from the lectin columns then is *Ricinus communis* ≥ soybean > concanavalin A > wheat germ agglutinin. These results were consistent from experiment to experiment. However, the amount of material not specifically eluted from concanavalin A or wheat germ agglutinin columns appeared to be proportional to the amount of lectin bound per ml of gel.

The elution profiles for the individual columns are shown in Fig. 3. Noteworthy are the very sharp peaks obtained for *Ricinus communis* and soybean agglutinin columns, some tailing effect with the wheat germ agglutinin column and a very broad tail for the concanavalin A column. Analysis of material eluted in the tail showed no differences from that obtained from under the sharper part of the peak. The same two columns which showed a tailing effect were the two which failed to yield 100 % recovery of bound material following specific sugar elution (see Table IV).

Polyacrylamide gel electrophoresis of lectin receptors. The radioactivity profiles of material isolated from the lectin affinity columns are shown in Fig. 4. Once again prominent peaks are observed at approximately 170 000, 135 000 and 90 000–95 000 daltons with very broad peaks appearing at 45 000–70 000 and around 35 000 daltons. The peaks are especially apparent in the profiles of the *Ricinus communis*, concanavalin A and soybean agglutinin receptors. It is interesting to note the presence of a peak at 75 000 daltons seen in the gel profile of material off the *Ricinus communis* affinity

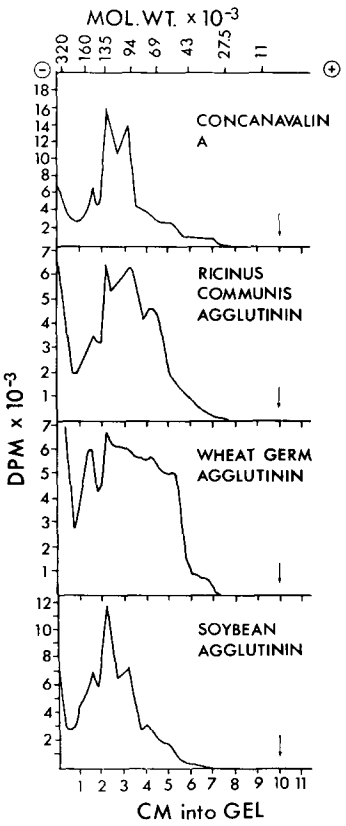


Fig. 4. Comparison of the radioactivity profiles of sodium dodecyl sulfate-polyacrylamide gels of sodium deoxycholate-solubilized [^{14}C]glucosamine-labeled plasma membrane material which bound to and was specifically eluted from various lectin-Sepharose 4B affinity columns. Arrows indicate the position of the marker dye. Details of preparation of samples and conditions of electrophoresis are given in text.

column. This peak, however, appears markedly reduced in the concanavalin A affinity released material. The wheat germ agglutinin profile also exhibits a shoulder in the 90 000–95 000 dalton region not apparent in the other profiles. Material at the origin seen in each profile is probably aggregated material and was variable from run to run. The relative amounts of radioactivity under each peak were calculated by planimetry. From 75 to 85 % of the total radioactivity which entered the gels could be accounted for by the major peaks noted above. The ratios of peaks seen for electrophoretic profiles for material released from each lectin column were consistent from run to run and were, therefore, characteristic of each column.

Determination of lectin binding sites. Information about the number of lectin receptor sites was obtained from binding studies using ^{125}I-labeled lectins. Binding curves corrected for nonspecific binding for each lectin are shown in Fig. 5. The number of binding sites for each lectin is given in Table V. It can be seen that there are 1.6 and 7 times as many *Ricinus communis* sites as sites for concanavalin A and soybean agglutinin, respectively.

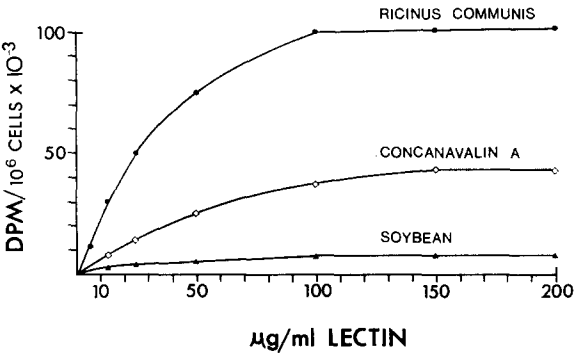


Fig. 5. The binding of ^{125}I-labeled *Ricinus communis*, concanavalin A and soybean agglutinins to the Ehrlich ascites carcinoma cell. Binding is measured in dpm of bound lectin per 10^6 cells during a 5-min incubation at 0 °C. Decays have been corrected for nonspecific binding derived from dpm of cell bound lectin in the presence of 0.1 M sugar for which each lectin displayed specificity.

TABLE V

COMPARISON OF NUMBER OF BINDING SITES ON EHRLICH ASCITES CARCINOMA CELL FOR LECTINS

Lectin	Non-specific binding (%)*	Maximum dpm/10^6 cells**	Number of binding sites per cell
Concanavalin A	11 %	43 000	$6.4 \cdot 10^6$
Ricinus communis	1 %	100 000	$10.0 \cdot 10^6$
Soybean	10 %	7 800	$1.43 \cdot 10^6$

* Gives the percent of total decays/min observed for binding in the presence of 0.1 M sugar at the concentration of lectin at which maximal binding was first noted.

** Corrected for non-specific binding.

DISCUSSION

The results of these studies allow us to assess certain aspects of lectin-cell membrane interactions. These include the relationship between the number of binding sites for a given lectin and its ability to agglutinate Ehrlich ascites carcinoma cells or to bind solubilized glycoprotein from the surface of these cells, the possible secondary interactions between the lectin and the polypeptide portion of the glycoprotein on which its receptor resides and the probable nature of the population of glycoprotein molecules functioning as lectin receptors.

As can be seen in Table V, there exist rather significant differences in the number of specific binding sites on the surface of the ascites cell for each of the various lectins tested. Such results are not unusual in that similar findings have been observed for many other types of cells [1, 42, 43]. From an analysis of Tables III and V it is apparent that while there are only 1.8 times more binding sites for *Ricinus communis* agglutinin as compared to concanavalin A on the ascites cells, on a mol or weight basis *Ricinus* agglutinin is 16 times more potent as an agglutinating agent of these cells than concanavalin A. Moreover, soybean agglutinin, for which there exists

only a quarter of the number of binding sites as for concanavalin A is, at equal concentrations, is just as effective an agglutinator of the cells. Thus, there does not appear to be any simple correlation between the number of binding sites for a given lectin and its ability to agglutinate the Ehrlich ascites carcinoma cells. However, the number of surface binding sites for a particular lectin does seem to give a good indication as to the amount of detergent solubilized membrane material that will bind to an affinity column comprised of that lectin (Tables IV and V).

It is also apparent from the results presented that the mode by which various lectins bind to their respective receptor molecules may differ. While simple sugar elution resulted in the release of almost all the glycoprotein that was initially bound to the *Ricinus* and soybean agglutinin affinity columns, only 61 % and 54 % of the material bound to the concanavalin A and wheat germ agglutinin columns, respectively, was found to be released (see Table IV). The comparatively drastic treatment needed to initiate the release of the residual bound material from these latter two columns suggests that some hydrophobic interaction must have taken place between these covalently bound, immobilized lectins and their receptor molecules. It is interesting to note that the sodium dodecyl sulfate eluted material from the concanavalin A affinity gel analyzed by polyacrylamide gel electrophoresis exhibited no major differences in Coomassie blue or periodic acid-Schiff staining patterns or in the radioactive profiles as compared to the material which was initially released by sugar elution. Thus, the apparent hydrophobic interaction that occurred was not selective but rather quantitative in nature.

The inability of sugar elution to effect a complete release of concanavalin A receptors found in the solubilized plasma membrane material from the affinity column does not appear to be due to any unique properties in the glycoprotein complement in our preparations. Similar experiences have been either reported or alluded to by some investigators attempting to isolate concanavalin A receptors from a variety of different membrane preparations [30, 44, 45]. A notable exception has recently been reported by Davey et al. [46] who have achieved 100 % recovery of human interferon bound to a concanavalin A affinity column when sugar elution was performed in the presence of ethyleneglycol, an agent which suppressed hydrophobic interactions, in the elution buffer. It is also of interest to note that the present studies confirm the observation that agglutination reactions with concanavalin A, unlike those of other lectins, cannot be reversed by the addition of its specific monosaccharide beyond the first few minutes of the reactions [47]. From our data and the results of others, it thus appears that following attachment of this lectin to its receptor oligosaccharide moiety in the glycoprotein, the lectin molecule probably undergoes a secondary hydrophobic interaction with the polypeptide portion of the receptor molecule.

While some laboratories have reported that a particular lectin affinity column binds and subsequently releases only one major glycoprotein species out of a complement of such molecules in a solubilized membrane preparation [44, 48], we have not observed this to be the case for solubilized Ehrlich ascites carcinoma cell plasma membrane material which was bound to and released from any of four different lectin affinity columns. Material released from each column showed an electrophoretic profile which indicated that each of the major glycoprotein bands present in whole membrane was well represented. A possible major exception to the last observation is the marked reduction in material migrating as a 75 000 dalton glycoprotein seen in

gel profiles of the concanavalin A receptors (Fig. 4). Since results in other systems clearly indicate that some receptors for a given lectin may be unique [44, 48], these differences in electrophoretic profiles suggest but by no means prove that such a situation may exist for some lectin receptors of the surface of the Ehrlich ascites carcinoma cell.

Our results also indicate that there is a certain amount of overlap among the receptor molecules for the lectins as determined by analysis of the data given in Table IV. If in fact each lectin receptor were represented on only one distinct glycoprotein molecule, addition of the percentage of radioactively labeled solubilized material which bound to each of the different affinity columns would yield a value approaching but not exceeding 100 %. As can be seen, this was not the case. Addition of the percentage of material bound to the *Ricinus communis* column with that bound to the concanavalin A or wheat germ agglutinin columns far exceeds 100 %, indicating that these lectins share receptors with one another.

We have previously reported that distinct differences exist in the ability of concanavalin A and soybean agglutinin to alter net transport of Na^+ and K^+ in the Ehrlich ascites carcinoma cell [2]. The observed effects could have been related to the number of sites involved, to secondary effects of the lectins or to the ability of a transport related glycoprotein to discriminate in its binding between these two lectins. The present study indicates that all three proposed mechanisms are possible explanations. Elucidation of the underlying mechanism(s) should be aided by both further characterization of isolated lectin receptors and analysis of lectin effects on cation transfer taken together with the present findings on lectin-receptor site interactions. Such studies are underway.

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