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SPECIFIC BINDING OF CONCANAVALIN A TO FREE INOSITOL AND LIPOSOMES CONTAINING PHOSPHATIDYLINOSITOL

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Summary: We previously reported that concanavalin A could bind specifically to liposomes containing phospholipids and lacking glycoconjugates (Biochem. Biophys. Res. Comm. 74, 208, 1977). In the present study we show that the binding of concanavalin A to the liposomes was greatly increased (up to 5 fold) by the presence of phosphatidylinositol in the liposomes. Furthermore, the binding of concanavalin A to phosphatidylinositol-liposomes was specific and could be inhibited by either α-methyl mannoside or by myo-inositol. We also found that concanavalin A-induced lymphocyte mitogenesis could be inhibited either by α-methyl mannoside or by myo-inositol. Simultaneous addition of both inhibitors to concanavalin A and liposomes showed that inhibition was non-competitive: α-methyl mannoside was more inhibitory to liposomes lacking phosphatidylinositol, and myo-inositol was more inhibitory to liposomes containing phosphatidylinositol. This suggests that the binding site for inositol might be different than that for mannose. Equilibrium dialysis and Scatchard plots revealed 4 binding sites each for inositol and mannose at neutral pH. The binding constants of concanavalin A were 0.13 x 10^4 and 0.25 x 10^4 liters/mole respectively for inositol and mannose. We conclude that concanavalin A binds specifically to the inositol portion of phosphatidylinositol.

Lectins are proteins that are characterized by the property of having binding sites for sugars. Numerous glycoconjugates, including glycoproteins (1-3) and glycolipids (4-16) can be bound by lectins. Plant lectins have been used extensively to probe mammalian membrane structures and to study lymphocyte mitogenesis and a variety of other membrane functions (reviewed in 17). Although plant lectins do interact with cells by binding to specific saccharides on the cell surface, several laboratories have reported specific binding of Con A to carbohydrate-free liposomes and lipid membranes (6, 7, 18). In the present study we demonstrate specific binding of Con A to PI in liposomes

Abbreviations: Con A, Concanavalin A; PL, phospholipid; DMPC, dimyristoylphosphatidylcholine; PI, phosphatidylinositol; α-MM, α-methyl mannoside; HBS, Hank's balanced salt solution; MEM, minimum essential medium.

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and inhibition of such binding by either myo-inositol or α-methyl mannoside. We also demonstrate that these inhibitors are capable of suppressing the mitogenic response in murine lymphocytes to Con A.

MATERIALS AND METHODS

Lipids were purchased from the following sources: DMPC and cholesterol from Calbiochem-Behring, La Jolla, CA; plant PI from Sigma Chemical Co., St. Louis, MO. Con A was purchased from Sigma. All inhibitors, including α-MM, myo-inositol, α-D-mannose, and α-L-fucose were from Sigma. Myo-[2-^{3}H]-inositol, 15.8 Ci/mmol, L-[6-^{3}H]-fucose, 16.8 Ci/mmol, D-[2-^{3}H]-mannose, 14.5 Ci/mmol, and [^{3}H]-thymidine, 2.0 Ci/mmol were from New England Nuclear, Boston, MA.

Liposomes containing PL (DMPC, or DMPC/PI) and cholesterol in molar ratios 2/1.5 were used throughout. When PI was included in the liposomes, it was in a 1/1 molar ratio with DMPC. Preparation of liposomes (multilamellar vesicles) is reported in detail elsewhere (19). Briefly, lipids (PLs and cholesterol) were dried in vacuo in pear-shaped flasks and dispersed in a small volume of 0.15 M NaCl with the aid of a few acid washed glass beads. The final PL concentration in the aqueous dispersion was 10 mM.

Studies on the binding of Con A to liposomes were performed as follows: Con A and inhibitors (if present) in the concentrations indicated in the individual figures were incubated with 100 µl of liposomes in a final volume of 1.0 ml of buffer for 45 minutes at 25°C. After incubation, liposomes were washed twice with 2 ml portions of 0.9% NaCl-0.01 M $NaHCO_3$ (pH 7.0) and collected by centrifugation at 140,000 g for 5 minutes. Bicarbonate-saline was the buffer used throughout for solution, washing and dilutions. Specific protein binding to liposomes was measured by a Lowry assay adapted to measurement of proteins on liposomes as described previously (20).

Equilibrium dialysis (microtechnique), as described by Karush et al (21). was used to determine the binding of Con A to α-D-mannose, α-L-fucose, or myo-inositol. The data were plotted according to the method of Scatchard (22).

Mitogenic determinations were made on 12 weeks old male C_3HeB/FeJ mouse (Jackson Laboratory, Bar Harbour, ME) spleen cells. The method used for cell preparation and assay of mitogenic activity is described elsewhere (23). Briefly spleen cells were extracted into HBS (HEM Research Inc., Rockville, MD), purified on a Ficoll-Hypaque solution, washed twice with HBS and suspended in a medium containing Eagles MEM with Earle's salts (GIBCO, Grand Island, NY), penicillin 100 U/ml and streptomycin 100 U/ml (GIBCO), 5 X 10^{-5} M 2-mercaptoethanol (Matheson, Coleman and Bell, Norwood, OH) and 5% fetal bovine serum (MBA, Walkersville, MD). Cultures containing 2 X 10^5 viable cells/0.2 ml were prepared in 96 wells microtiter plates (Linbro Chemical Co., New Haven, CT) containing Con A (6.0 µg Con A protein/ml culture) with either 40 µmoles/ml α-MM or myo-inositol. Cultures were incubated at 37°C in 5% CO_2 and air for 48 hours. Four hours prior to harvesting, the cells were pulsed with 1 µCi of [^{3}H]-thymidine, harvested on glass fiber filters with a cell harvester and the radioactivity determined by liquid scintillation.

RESULTS

Figure 1 demonstrates dose-dependent binding of Con A to DMPC and DMPC/PI liposomes. The binding of Con A to DMPC liposomes reached a saturation level

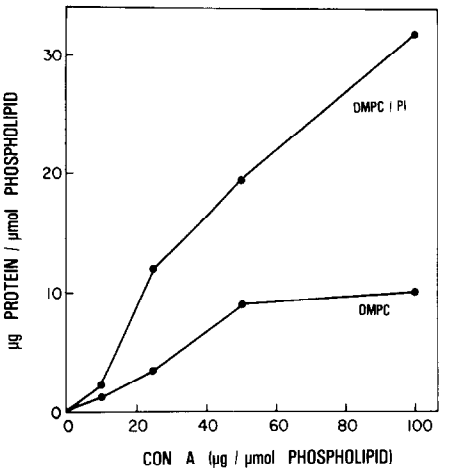


Figure 1. Con A binding to liposomes. 100 µl of liposomes containing DMPC/Cholesterol in a 2:1.5 molar ratio, or DMPC/PI/Cholesterol in a 1:1:1.5 molar ratio, were incubated with Con A in the concentrations indicated for 45 minutes at 25°C, in a final volume of 1 ml bicarbonate-saline buffer. After incubation, the liposomes were washed twice with 2 ml portions of bicarbonate-saline and specific lectin-binding to liposomes was determined. Controls without liposomes were substracted from each result to compensate for slight non-specific binding to the glass tubes.

of approximately 10 µg of protein/µmol of PL at an initial concentration of 50 µg of added Con A; however, binding of 100 µg of initially-added Con A to DMPC/PI liposomes was greatly increased compared to binding of 50 µg of initially-added Con A. Saturation of binding of Con A to the latter liposomes occurred only at levels above 100 µg of added Con A. Based on 3 experiments, total binding to DMPC/PI/CHOL reached a level of 42-50 µg of protein/µmol of PL when 200 µg of Con A was initially added (not shown). Therefore the presence of PI in the liposomes increased Con A binding by about 5 fold.

Con A binding to mannose is widely accepted as the major binding specificity for Con A, and inhibition of Con A by α-MM is often used as a criterion for specificity of binding (24). Figure 2A shows that Con A binding to either DMPC or DMPC/PI liposomes was inhibited approximately 50% by a 1.0 mM concentration of α-MM. Although the binding of Con A was inhibited to the same extent for each type of liposome when expressed as percent of control, the absolute amounts of Con A binding, as shown in Fig. 1, were at least twice as high with DMPC/PI liposomes. Since Con A binding was increased by the presen-

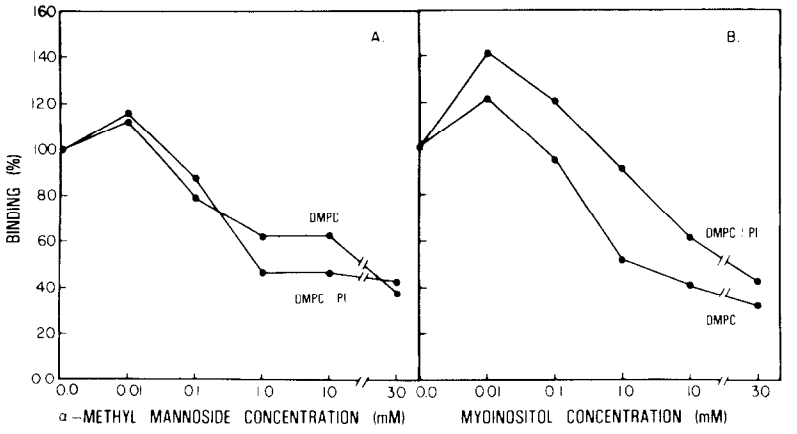
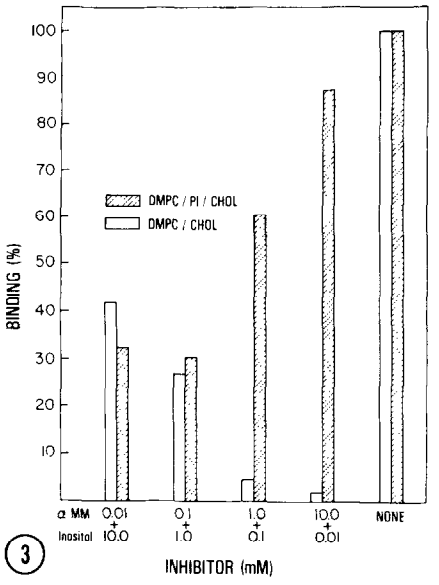


Figure 2. Inhibition of Con A binding to liposomes by α-methyl mannoside or myo-inositol. Similar incubations as those described in Fig. 1 were set up with liposomes and Con A. α-MM (A) or myo-inositol (B) were used as inhibitors in the concentrations indicated.

ce of PI in the liposomes we tried to inhibit it by adding increasing concentrations of myo-inositol as a soluble inhibitor. Figure 2B shows that myo-inositol did inhibit the binding of Con A to liposomal PLs, and although inhibition occurred in a pattern similar to that of α-MM the 50% level of inhibition was reached at a 10.0 mM concentration of myo-inositol. Furthermore, when a similar experiment was performed using increasing concentrations of α-L-fucose as an inhibitor of Con A binding to liposomes containing or lacking PI, no inhibition was observed. Therefore we conclude that the binding of Con A to PI is specific and probably involves the inositol moiety of PI.

In order to characterize further the binding of Con A to liposomal PLs, and especially to PI, we mixed varying concentrations of α-MM with varying concentrations of myo-inositol. Our reasoning was that if α-MM and myo-inositol compete for the same binding site we would record similar inhibitions to those shown in Fig. 2. The results, in Fig. 3, demonstrate that inhibition of Con A binding to liposomes containing PI occurred when the concentrations of inositol were higher than those of α-MM. The opposite occurred with liposomes lacking PI. These results suggested that myo-inositol and α-MM may have different binding sites on the Con A molecule.



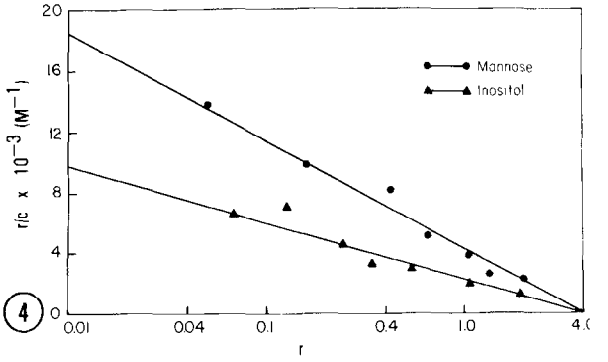


Figure 3. Inhibition of Con A binding to liposomes by mixtures of α-MM and myo-inositol. Similar incubations as those described in Fig. 1 were set up with liposomes and Con A. Myo-inositol and α-MM were mixed together as inhibitors in the concentrations indicated.

Figure 4. Equilibrium dialysis data for the binding of α-MM and myo-inositol to Con A plotted by the method of Scatchard (see text for further details).

The binding of Con A to α-D-[^{3}H]-mannose or myo-[^{3}H]-inositol in 0.01 M bicarbonate-saline, pH 7.0, at room temperature was investigated by equilibrium dialysis. The results are shown in Fig. 4 where r/c is plotted against r according to the method of Scatchard (22). The ratio of the molar concentration of the bound sugar to that of protein is represented by r, c is the molar concentration of free sugar, n is the number of binding sites and K, the binding constant. A molecular weight of 108,000 for Con A was used in the calculations since Con A is a tetramer at neutral pH and the monomer mol wt. is 27,000. Linear plots were obtained for both mannose and myo-inositol (Fig 4) with values of n = 4 which is in accordance with 1 binding site per monomer. When extrapolated to the r/c axis, binding constants of 0.13 x 10^4 l/mole and 0.25 x 10^4 l/mole were calculated for myo-inositol and D-mannose respectively. No binding could be detected for Con A with α-L-fucose under the same conditions.

TABLE 1. Suppression of Con A activation of lymphocytes mitogenesis by α- MM or myo-inositol

INHIBITOR	DPM X 10^{-3}	% SUPPRESSION
None	124 ± 10	0
α-methyl mannoside	1.3 ± 0.2	99
myo-inositol	86 ± 8	31

Cultures containing 2 x 10^5 mouse spleen lymphocytes in 0.2 ml medium were stimulated with 6 μg Con A/ml in the presence of 40 μmoles/ml of α-MM or myo-inositol and incubated for 48 h. DNA synthesis was determined by pulsing the cultures 4 h prior to harvesting with 1 μCi of [^{3}H]-thymidine. The values represent the geometric mean of quadruplicate cultures ± 1 S E.

Since Con A has been used extensively as a tool to induce biological responses such as lymphocyte mitogenesis we decided to investigate if the binding of Con A to inositol would also apply to Con A-induced lymphocyte mitogenesis. Table 1 shows that either α-MM or myo-inositol when added simultaneously with Con A suppressed Con A-mediated mitogenesis of murine lymphocytes. α-MM was a more effective inhibitor than myo-inositol, but significant inhibition was observed with either compound (Table 1). No inhibition of Con A-induced lymphocyte mitogenesis was detected with α-L-fucose (not shown).

DISCUSSION

Binding of Con A to liposomes lacking glycoconjugates has been reported by several laboratories (6, 7, 18). In this report, we show that Con A binds much more to liposomes containing PI than to liposomes lacking PI (Fig 1).

Binding of Con A to liposomes was "specific", based on the criterion that Con A binding inhibited by mannose is specific (25, 26). Inhibition studies showed that Con A binding to liposomes either lacking or containing PI was inhibited by either α-MM (Fig 2A) or by myo-inositol, a component of the PI molecule (Fig 2B). This finding coupled with the ability of myo-inositol to inhibit a biological activity of Con A, lymphocyte mitogenesis, reveals a new role for membrane-bound inositol as well as a new and generally unrecognized specificity for Con A.

The above observations suggested the possibility that myo-inositol and mannose-containing glycoconjugates each could serve as Con A receptors and that they might compete for the same binding site on the Con A molecule. However, when myo-inositol and α-MM were simultaneously used as inhibitors an unexpected inhibition pattern was observed. When PI was present in the liposomes, myo-inositol was the more potent inhibitor and high concentrations of α-MM could not provide similar inhibition; in contrast, when PI was absent from liposomes αMM was a more potent inhibitor than inositol (Fig 3). These results suggested that myo-inositol and mannose may have different binding sites. Equilibrium dialysis experiments demonstrated that both mannose and myo-inositol were bound by Con A, and there were 4 binding sites each for mannose and myo-inositol. These results are consistent with reports that Con A is a tetramer comprised of 4 identical subunits and that each subunit has one saccharide binding site (25-28). Our results are also consistent with a recent report that indicated that Con A could bind to PI (29).

We conclude that Con A binds specifically to PI and this binding probably involves the inositol moiety of PI. This therefore represents a new and generally unrecognized specificity for Con A. In view of the ubiquitous presence of PI in many cell membranes and the central role that it may play in numerous cell functions (30), it may be useful to re-evaluate the conclusions of certain studies in which Con A was employed as a cell probe.

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