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THE ACYL CHAIN REQUIREMENT OF PHOSPHATIDYL CHOLINE IN THE INHIBITION CONCANAVALIN-A STIMULATED BLASTOGENESIS

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SUMMARY: The structural requirement of phosphatidyl choline in the inhibition of concanavalin-A (Con A) stimulated blastogenesis in mouse lymphocytes was studied. Lecithin isolated from egg, bovine liver and soy bean were active and that isolated from bovine brain was inactive. Moreover, synthetic dimyristoyl-, dipalmitoyl-, dioleoyl- and 1-palmitoyl-2-oleoyl-phosphatidyl choline were inactive. Egg lecithin was fractionated by high pressure liquid chromatography and the components thus obtained tested for inhibitory activity. Two fractions showing substantial activity were obtained. One contained no detectable phosphorous and it was not further analyzed. The other contained phosphorous and when it was analyzed for fatty acid composition, a high content of palmitate and linoleate was observed. In contrast, other phosphorous containing fractions with low inhibitory activity had none or low linoleate content. Based on these results, linoleate appears to be a crucial structural component for mediating the inhibition of blastogenesis. This hypothesis was confirmed when synthetic dilinoleoyl-phosphatidyl choline was shown to have a higher potency than egg lecithin in the inhibition of blastogenesis.

INTRODUCTION

The mechanism of how cells are triggered to divide is poorly understood. The use of mitogenic lectins to stimulate resting lymphocytes to go into division has provided a simple and direct mean to study events associated with cell division. Several lines of evidence have provided support that the plasma membrane may play an important role at the initial stage of lectin-stimulated transformation (1-3). Based on studies performed using lectins immobilized on agarose beads, it has been shown that the binding of the lectin on the outer membrane is enough to trigger internal cellular mechanisms leading finally to DNA synthesis and cell division (4,5).

Abbreviations: Con A, concanavalin A; GLC, gas liquid chromatography; HPLC, high pressure liquid chromatography; IC_{50}, inhibitory concentration at 50%; PC, phosphatidyl choline.

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Previously, we as well as others have observed that lectin mediated blastogenesis can be abrogated by treatment with dispersions of phosphatidyl choline (6-8). This inhibition was time dependent and once the cells are committed to division further treatment with phospholipid is ineffective (6). Taken together, these results suggest that the inhibitory effect of phosphatidyl choline is exerted at a rather early stage of the cell cycle and that it may involve an alteration of membrane fluidity as exogenous phospholipids have been shown to exchange with and promote the transfer of lipid components between the cell membrane and its outer environment.

In the initial studies (6), we have used egg lecithin as a convenient source of phospholipid. Unfortunately, egg lecithin is heterogenous with respect to its acyl chain composition and because of this, exact identification of the structural requirement for inhibition was not possible. The aim of the present study is to identify the active component in the egg lecithin mixture responsible for the inhibition of lymphocyte transformation. In this respect, we have taken two approaches. One is to test the ability of synthetic lecithin to inhibit blastogenesis. Another is to fractionate egg lecithin by high pressure liquid chromatography (HPLC) and to examine the relative potencies of the components.

MATERIALS AND METHODS

Concanavalin A (Con A) and all the phosphatidyl cholines, except 1-palmitoyl-2-oleoyl-PC and dilinoleoyl-PC, used in this study were purchased from Sigma Chemical Co., St. Louis, Mo. 1-palmitoyl-2-oleoyl-PC, dilinoleoyl-PC and fatty acid methyl ester standards were from Supelco, Bellefonte, Pa. Aqueous dispersions of the phospholipids were prepared by sonication under nitrogen as previously described (6).

Lymphocytes isolated from the spleen of CBA mice were used throughout this study. Stimulation was measured as previously described (6). Briefly, lymphocytes were cultured in the presence of Con A (4 μg/ml) for 72 hours and then labeled with [^{3}H]-thymidine (1 μCi/ml, specific activity 200 mCi/mmol, Radiochemical Centre, Amersham, U.K.) in the last 8 hours. Cells were harvested with a cell harvester (Minimash, AM 78, Dynatech) and the amount of radioactivity incorporated determined. Cultures were performed in quadruplicates and the mean of the radioactivity incorporated was used for the calculation of IC_{50}.

Egg lecithin (10 mg) was fractionated by HPLC on a u-bondapak C-28 (Waters Associate, Milford, Mass) column using a solvent system of MeOH:$CHCl_3$:H_2O (100:10:10, ref 9). Flow rate was maintained at 2 ml/min and 2 ml fractions were

collected. One fourth of the collected fractions were taken for phosphorous determination (10) and the rest were used either for testing inhibitory activity or the determination of fatty acid composition.

The fatty acid composition of the various synthetic and natural PC and the HPLC fractions were determined by gas liquid chromatography (GLC). Phospholipids were first transesterified in a MeOH-acid solution according to the procedure outlined in reference 11. The methyl esters thus prepared were chromatographed at 180°C on a column (1/8" x 6') packed with 10% SP 2300 on Supelcoport (Supelco, Bellefonte, Pa.). The elution profile was monitored by flame-ionization (Model 3200 chromatograph, Varian, Palo Alto, Ca.).

RESULTS AND DISCUSSIONS

To investigate the effect of different PC on lymphocyte stimulation, cells prepared from the spleen of freshly killed CBA mice were incubated at the optimal concentration of Con A in the presence and absence of different lipid concentrations (range, 1 to 100 μg/ml). The difference between [^{3}H]-thymidine incorporation by these cultures measured 72 hours later and that of the control cultures not treated with mitogen but otherwise similarly set up represents lymphocyte stimulation due to mitogen. Stimulation observed in the presence of lipids was compared and expressed as percent of that by the culture which received the mitogen alone. The percent stimulation thus obtained was plotted against the lipid concentration and the amount of lipid required to produce a 50% inhibition (IC_{50}) was determined from these data by the best fitted curve using a standardized computer program.

Table 1 summarizes the potencies of the different types of PC in the inhibition of lymphocyte stimulation. Of the four natural lecithins tested, only the one from bovine brain was inactive. Compared with egg lecithin, the IC_{50} of soy bean and bovine liver lecithin were approximately the same. In a previous study, using human lymphocytes, we observed that bovine brain lecithin was as effective as the other sources of lecithin (12). The reason for this discrepancy between the mouse and human lymphocytes is uncertain at the moment. In contrast to the lecithins isolated from the natural sources, none of the synthetic ones tested had a significant inhibitory activity.

Table 1

Effect of Different Phosphatidyl Cholines on the Inhibition of Con A Mediated Blastogenesis

Type of PC	IC_{50} (μg/ml)	Cell viability (%)[1]
Natural PC		
Egg	29.8	63
Soy bean	31.1	62
Bovine brain	>100	73
Bovine liver	28.9	66
Synthetic PC[2]		
Dimyristoyl	>100	68
Dipalmitoyl	>100	93
Dioleoyl	>100	79
1-palmitoyl-2-oleoyl	>100	91

[1]Determined by trypan blue exclusion at the highest PC concentration used. In the absence of lipid the viability was 78%.

[2]Distearoyl PC was omitted in this series because of its insolubility.

The acyl groups of egg lecithin are heterogenous and consist of four major fatty acid chains; viz., palmitate, stearate, oleate and linoleate. According to this composition there are sixteen possible arrangements by which these acyl groups can pair. Since preparation of PC having all of the sixteen possible combinations was technically not feasible in our laboratory, we decided to fractionate egg lecithin by a recently reported HPLC procedure (9) and test the fractionated materials for inhibitory activity.

Fig. 1 represents a typical chromatogram when 10 mg of egg lecithin was fractionated on a Waters u-bondapak C-18 column. Altogether, five decernable phosphorous containing components were detected. In contrast to the data reported by Porter *et al.* (9), the two major components of our chromatogram were only partially resolved (i.e., peaks at fraction 11 and 13). Other than the phosphorous containing components, a number of UV absorbing (280 mu) peaks were also observed between fractions 2 to 12. The nature of these peaks are unknown and none of their positions corresponded to the phosphorous peaks. When the fractions were scanned for lymphocyte stimulation inhibitory activity, the most active components were detected at fractions 2 and 10 (Table 2). Although

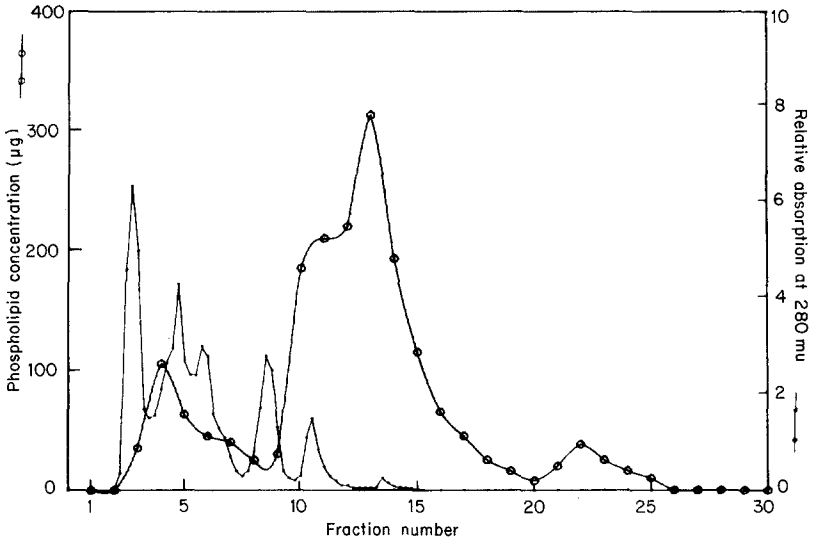

Fig. 1 Fractionation of egg lecithin by HPLC. Open circles, phosphorous.

fraction 13 is the major phosphorous containing component, its activity was the lowest. Since fraction 2 contained no phosphorous, its chemical nature remains to be determined.

Table 2

Comparison of the Potencies of Selected HPLC Fractions in the Inhibition of Con A Stimulated Blastogenesis

Fraction Number[1]	Relative Potency
Egg PC (starting material)	100
#2	1/27 (see note 2)
#4	30
#10	85
#12	53 (see note 3)
#13	4
#15	14
#22	28

[1]Based on several orientation experiments, these fractions were selected for either their high inhibitory activity (#2 and #10) or their correspondence to the major phosphorous peaks (#4, #10, #12, #13, #15 and #22). The amount of material in fraction 7 was too low to give an accurate estimate of IC_{50}. Cell viability in the presence of lipid was comparable to that of the control (approx. 70%)

[2]Since this fraction contained no phospholipid, a dilution factor producing 50% inhibition is given. For comparison purpose, the dilution factor for fraction 10 of the same chromatogram was also 1/27.

[3]The high activity in this fraction was probably due to incomplete separation of the active component from fraction 10.

Table 3

Fatty Acid Compositions of the HPLC Fractions

Fraction	Major fatty acid components (wt %)				
	C16:0 (2.95)[1]	UK (3.4)[1]	C18:0 (5.2)[1]	C18:1 (6.0)[1]	C18:2 (7.4)[1]
Egg PC	33	nd	18	34	15
Fx 4	34	nd	38	28	nd
Fx 10	35	12	5	12	36
Fx 12	43	nd	nd	45	12
Fx 13	40	nd	nd	60	nd
Fx 15	20	nd	25	47	8

[1]Values in brackets are retention times in minutes. nd, not detectable, UK, unknown peak.

To further analyze the structural requirement of PC in the inhibition of Con A stimulated blastogenesis, the fatty acid compositions of fraction 10 and other selected fractions were determined and compared. In contrast to the native egg lecithin and the other fractions, the fatty acid composition of fraction 10 showed a much higher content of linoleate (Table 3). Of the five fractions examined, fractions 4 and 13 contained no linoleate (Table 3) and their inhibitory activities were three to twenty times lower than that of fraction 10, respectively (Table 2). Fraction 15 contained only 8% linoleate by weight and showed a relative potency of 14. Other than a higher weight percent of linoleate, fraction 10 also contained an unidentified component with a retention time of 3.4 min. Whether this component is essential for inhibitory activity is uncertain. In view of the fact that fraction 12 contained no such a component and yet still showed a relatively high inhibitory activity, it would seem unlikely that this compound is critical.

Based on the data presented in Table 3, one of the basic requirements for inhibitory activity appears to be the presence of linoleate in the structure of PC. According to the data reported by Porter *et al.* (9), fractions 10 and 13 correspond to 1-palmitoyl-2-linoleoyl-PC and 1-palmitoyl-2-oleoyl-PC, respectively. In light of this, the inability of fraction 13 to inhibit Con A stimulated blastogenesis accords with our finding on synthetic 1-palmitoyl-2-oleoyl-PC

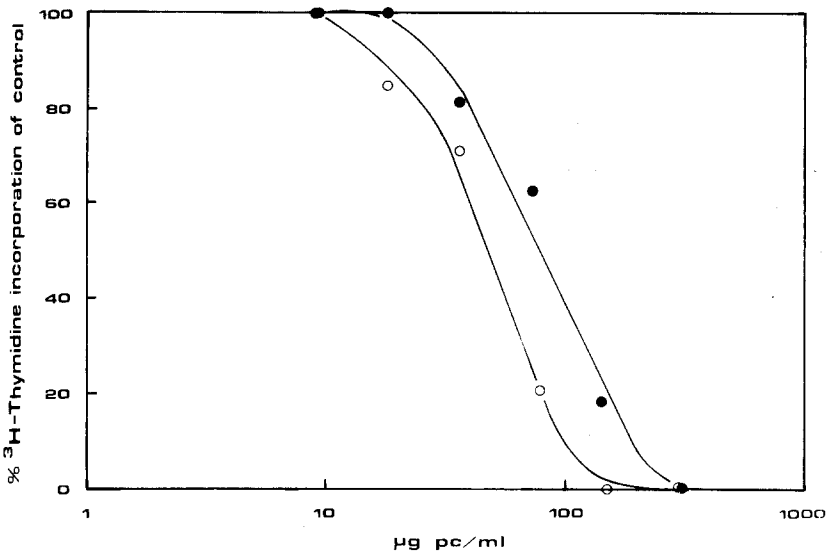

Fig. 2 Inhibition of Con A stimulated blastogenesis by dilinoleoyl-PC and egg-PC. Open circles, dilinoleoyl-PC; close circles, egg-PC. Each point represents the average of four independent measurements.

(Table 1). To confirm the role of linoleate in the inhibition of blastogenesis, the ability of synthetic dilinoleoyl-PC to inhibit Con A stimulated incorporation of [^{3}H]-thymidine was evaluated. As depicted in Fig. 2, both dilinoleoyl-PC and egg-PC inhibited blastogenesis in a dose dependent manner and that the potency of the synthetic phospholipid was approximately twofold higher. A similar experiment was set up to determine the inhibitory effect of dilinoleoyl-PC on human lymphocytes and the results obtained were essentially identical to that of the mouse (results not shown). Clearly, these results provide additional proof that linoleate is a crucial component for inhibitory activity.

The requirement of linoleate in the PC structure for the inhibition of Con A mediated blastogenesis implies a highly specific interaction between the exogenous phospholipid and the cells. Chen and Keenan (7) have proposed that PC treatment may abrogate blastogenesis by depleting cholesterol from the plasma membrane. This hypothesis was tested in our previous study (12) and found to be inadequate. In view of the specific structural requirement as demonstrated in our present

study, the mechanism underlying phospholipid inhibition may be more specific than just a change of membrane fluidity. Thus, it is possible that a specific PC may be required to interact with Con A receptors on the cell surface in order to bring about an inhibition of transformation. Partial support for this contention comes from a preliminary observation in our laboratory (Ng and Ng, unpublished results) which indicated that pretreatment of lymphocytes with egg lecithin can alter their binding characteristics with [^{125}I]-Con A.

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