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INTERACTIONS OF MULTILAMELLAR PHOSPHOLIPID VESICLES WITH BOVINE LYMPHOCYTES: EFFECTS OF α-TOCOPHEROL ON LYMPHOCYTE BLASTOGENESIS

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Abstract—Multilamellar lipid vesicles (MLV) composed of egg yolk lecithin (EYL) suppressed the response of bovine peripheral blood lymphocytes (BPBL) to phytohemagglutinin (PHA). EYL contains 18:1 and 18:2 as the major unsaturated phospholipids. Dioleoyllecithin (DOL; *cis* 9) MLV did not suppress BPBL blastogenesis. Dilinoeyllecithin (DLL; *cis, cis* 9, 12) MLV suppressed BPBL blastogenesis. The suppressive effect could be reversed by increasing the MLV DML concentration. The addition of α-tocopherol (α-T) at 10 mole% into MLV containing DLL reversed blastogenic suppression of BPBL. MLV composed of mixed saturated phospholipids (dimyristoyllecithin and dipalmitoyllecithin) and α-T enhanced the BPBL blastogenic response to PHA. BPBL incubated with varying PHA concentrations (11.82–375 mg/ml) and a constant concentration (2 μmoles/ml) of MLV composed of EYL remained suppressed either when PHA and MLV were added simultaneously or when MLV were incubated for 1 hr prior to the addition of PHA. This suggests that α-T may act as an immunomodulator in the blastogenic response to PHA. Results suggest that α-T reversion of EYL suppression of BPBL blastogenesis may be due to interactions of α-T with unsaturated acyl chains in EYL phospholipids.

INTRODUCTION

The use of phospholipid vesicles as the carrier for a variety of entrapped compounds, including antigenic molecules, has been demonstrated (Kinsky, 1972; Gregoriadis & Davis, 1977; Fountain *et al.*, 1981; Inoue, 1974; Szoka & Papahadjopoulos, 1978). Vesicles composed of phosphatidylcholine were shown to decrease the cholesterol content of cells with which they were incubated and, by a similar extraction of cholesterol, were shown to suppress lymphocyte blastogenesis (Chen & Keenan, 1977). Extraction of cellular cholesterol in the presence of phospholipid vesicles is believed to control the successful blastogenesis of lymphocytes by retarding the generation of new membranes, thus regulating cell growth and division (Chen & Keenan, 1977; Inbar & Shinitzky, 1974). Cholesterol acts on the membrane by modifying the fluidity of the hydrocarbon core (Engleman & Rothman, 1972). Alterations in membrane fluidity significantly affect the mobility of cell surface components involved in lymphocyte blastogenesis (Dunnick *et al.*, 1976).

In vitro studies have shown that α-tocopherol (α-T)§ can modulate immune responses but the mechanism is unclear (Fountain & Schultz, 1981, 1982*a*; Campbell *et al.*, 1974; Sheffy & Schultz, 1979. The action of α-T in the stabilization of cell membranes by altering or maintaining the lymphocyte membrane fluidity (Diplock, 1974) may directly influence the mobility of membrane components similar to the mechanism proposed for membrane-associated cholesterol (Lucy, 1972; Spallholz *et al.*, 1973; Tengerdy & Brown, 1977; Tengerdy *et al.*, 1973; Nes, 1974).

Our previous studies (Fontain & Schulz, 1981, 1982*b*) demonstrated the ability of α-T to reverse the suppression of the blastogenic response to PHA when BPBL were incubated with liposomes composed of EYL. MLV incubated with BPBL prior to addition of PHA exhibited a time-dependent suppression of blastogenesis which was reversible for MLV composed of EYL:α-T (1:1) but not for MLV composed of EYL alone. Incubation of BPBL with PHA prior to the addition of MLV caused a time-dependent reversible suppress-

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§ Abbreviations: DOL, 9-*cis*-dioleoylphosphatidylcholine; DLL, 9,12-*cis, cis*-dilinoeylphosphatidylcholine; DPL, dipalmitoylphosphatidylcholine; α-T, α-tocopherol; DML, dimyristoylphosphatidylcholine; [^{3}H]TdR, [^{3}H]thymidine; EYL, egg yolk lecithin; PHA, phytohemagglutinin; BPBL, bovine peripheral blood lymphocytes; FBS, fetal bovine serum; MLV, multilamellar lipid vesicles.

of blastogenesis for EYL:α-T (1:1) but not for MLV composed of EYL alone. A concentration dependence of the suppression of BPBL blastogenesis occurred over the range from 0.5 to 3 μmoles of EYL phospholipid per culture. Maximum suppression occurred at 2 μmoles of EYL phospholipid per culture (Fountain & Schultz, 1982).

The present study was designed to determine if MLV composed of EYL, DPL, DML, DOL, DLL and α-T would enhance or suppress the response of BPBL to PHA and to determine the component of EYL responsible for suppression of blastogenesis. This approach was used to elucidate the relationship between liposome membrane lipid composition, presence or absence of α-T and the lymphocyte cell surface properties.

MATERIALS AND METHODS

Liposome preparations

EYL, DPL, DML, DOL and DLL (purity ≥ 98%) were obtained from Avanti Polar Lipids, Birmingham, Alabama. α-T was obtained from Sigma Chemical Co., St. Louis, Missouri. Liposomes were prepared as described by Fountain & Schultz (1982*a*). In the case of α-T incorporation into liposomes, α-T was added prior to drying of the lipid film. After allowing the liposomes to swell for 30 min at 45°C in Hanks' Balanced Salt Solution (HBSS), pH 7.4, the liposomes were eluted over a Sephadex G-50 column equilibrated with HBSS. Multilamellar liposomes collected in the void volume from the column were used for incubation with bovine lymphocytes. Vesicles containing equimolar mixtures of EYL:α-T (mol. wt of EYL based on palmitoyl form), DPL:α-T and DML:α-T were prepared. Vesicles containing 1:2 and 2:1 molar mixtures of EYL:α-T, DML:α-T and DPL:α-T; a 5:1 molar mixture of DML:α-T; and 2:1:1, 2:1:3 and 2:1:6 molar mixtures of DPL:DML:α-T were prepared. Vesicles containing varying concentrations of DML:DOL and DML:DLL with and without α-T were also prepared. All vesicle suspensions were maintained at 22–25°C under nitrogen until used, which was usually within less than 4 hr from the time they were prepared. The total lipid concentration in the vesicle was 2 μmoles of phospholipid per culture.

Lymphocyte blastogenesis

The lymphocyte blastogenesis assay was adapted directly from Schultz & Adams (1978). Lymphocyte cultures were established in 96-well, flat-bottom plates (Costar, Cambridge, Massachusetts). The medium consisted of RPMI-1640 (Gibco, Grand Island, New York) containing 10 units of penicillin, 10 μg of streptomycin (Gibco, USA), 10 μg of Gentocin (Schering Corporation, Kenilworth, New York) per milliliter of medium and 10% heat-inactivated FBS. PHA (Gibco) was used at a final concentration of 15 μg/ml.

Heparinized blood was placed on Ficoll–Hypaque (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) and centrifuged at 1300 *g* for 20 min as described by Boyum (1966). The mononuclear cell layer at the plasma–Ficoll interface was removed and the cells were placed in an appropriate volume of RPMI-1640. Approximately 5×10^6 cells/ml with a viability of not less than 95% as determined by trypan blue exclusion were obtained with this procedure. One-tenth of a milliliter of test material containing PHA, PHA and liposomes, or liposomes and 50 μl of cell suspension were added to each well. Plates were incubated at 39°C in 5% CO_2 and 70% r.h. After 72 hr, 1 μCi of [^{3}H]TdR (2.0 Ci/mmole) (New England Nuclear, Boston, Massachusetts) in 0.1 ml of RPMI-1640 was added to each well and the plates were incubated for an additional 18 hr. Cells were harvested onto glass fiber filter pads using an automated cell harvester (Mash II, Microbiological Associates, Bethesda, Maryland) as described by Hartzman *et al.* (1972). Filter paper discs were placed in glass vials containing 5 ml of liquid scintillation fluid (Eastman-Kodak, Rochester, New York) and the radioactivity of the vials was determined in a liquid scintillation spectrometer (LS7000) (Beckman Instruments, Palo Alto, California). A blank consisting of liquid scintillation fluid and a filter pad disc was used.

Lipid composition of liposome preparations

Derivatization of fatty acids. Fatty acids were released from their respective phosphatidylglycerocholines by mild alkaline hydrolysis as described by Kates (1975). The fatty acids were converted to methyl ester derivatives with boron trifluoride in methanol according to the procedures described by Metcalf & Schmitz (1961).

Gas chromatographic analysis. Fatty acid methyl esters were analysed using a Hewlett-Packard Model 5710 gas chromatograph linked to a Hewlett-Packard 3380 recorder-integrator. The gas chromatograph was equipped with a flame ionization detector and a 30-m glass capillary column coated with SP-2330 (Supelco Inc., Bellefonte, Pennsylvania). The oven temperature was programmed from 120 to 220 at 4 C min. The maximum oven temperature was held for 8–20 min. The detector temperature was 250 C and the injection temperature was 300 C. The carrier gas (helium) flow rate was approximately 1 ml/min.

PHA concentration effects of lymphocyte blastogenesis

Varying PHA concentrations were prepared by dilution of PHA stock solution (750 μg/ml) with RPMI-1640. PHA concentrations were 375, 187.5, 93.73, 46.88, 23.44 and 11.82 μg/ml. Lymphocytes were incubated with and without liposomes (EYL) at the various PHA concentrations. Liposomes (EYL) were either preincubated for 1 hr prior to or added simultaneously into BPBL cultures containing PHA.

Effects of α-T concentration on lymphocyte blastogenesis

Varying α-T concentrations were prepared in 0.07% ethanol:RPMI-1640. These included: 20 and 2 mg/ml; 200, 20 and 2 μg/ml; and 200, 20 and 2 ng/ml. Liposomes composed of DPL:DML (1:1) with α-T concentrations equivalent to those earlier were prepared. Control cultures were prepared that lacked liposome α-T. Control cultures were also prepared that contained only DPL:DML (1:1) liposomes without α-T.

Effect of unsaturation of liposome phospholipids on lymphocyte blastogenesis and the effects of α-T on liposomes containing DOL or DLL

Liposomes containing either DML and DOL or DML and DLL at mole ratios (DML:DLL) of 0:1, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:0 were prepared. Liposomes containing DML, DOL and DLL alone at these mole ratios and liposomes with 10 mole% α-T and the earlier liposome compositions were also prepared. Liposomes were placed in lymphocyte cultures at phospholipid concentrations of 2 μmoles culture.

Statistical methods

Statistical evaluation of data was computed using the Students' *t*-test. Confidence intervals were calculated at $-t_{0.99}$, $-t_{0.95}$ and $-t_{0.90}$. Any data groups below $-t_{0.90}$ were considered not significant. Lymphocyte cultures for all treatment groups were performed with eight cultures per group. Variance was less than 10% per treatment group.

RESULTS

The fatty acid compositions of phospholipids used in the formation of MLV are given in Table 1. EYL contained $C_{16:0}$ and $C_{18:0}$ as the major saturated phospholipids and $C_{18:1}$ and $C_{18:2}$ as the major unsaturated phospholipids. Minor (<5%) unsaturated phospholipids occurred such as $C_{16:1}$ and $C_{20:4}$. DML contained greater than 95% $C_{14:0}$ with a minor (<2%) quantity of $C_{16:0}$. No detectable unsaturated phospholipids were found in DML. DOL contained greater than 94% ($C_{18:1}\Delta^{9c}$). Minor components (<2%) in DOL were $C_{18:0}$, $C_{16:0}$ and $C_{14:0}$. DLL contained greater than 95% of $C_{18:2}\Delta^{9c,12c}$ with $C_{18:1}$, $C_{18:0}$ and $C_{16:0}$ occurring as minor (<2%) components.

BPBL cultured with EYL lipid vesicles in a FBS (10%) enriched medium containing a mitogenic dose of PHA (15 μg/ml) were significantly ($P < 0.01$) suppressed when compared to control cultures (Table 2). The addition of α-T to the MLV (EYL) at 1:1 EYL:α-T or 1:2 EYL:α-T vesicles reversed the lymphocyte suppression caused by PHA (Table 2).

BPBL which were cultured with multilamellar DML, 2:1 DML:α-T, 1:1 DML:α-T or 1:2 DML:α-T vesicles in the absence of PHA in every case exhibited significant ($P < 0.1$) enhancement of the incorporation of [^{3}H]TdR when compared to control, untreated cultures (Table 2).

BPBL cells cultured for 72 hr in the presence of PHA containing 1:1 DML:α-T or 1:2 DML:α-T lipid vesicles exhibited no significant change in the incorporation of [^{3}H]TdR when compared to PHA-stimulated control cultures (Table 2). BPBL cells cultured for 72 hr in medium containing DML or 2:1 DML:α-T lipid vesicles in the presence of PHA exhibited a significant enhancement ($P < 0.05$) of the [^{3}H]TdR incorporation when compared to control, PHA-stimulated cultures (Table 2).

BPBL cells which were cultured for 72 hr in medium containing DPL, 2:1 DPL:α-T, 1:1 DPL:α-T or 1:2 DPL:α-T lipid vesicles in the absence of PHA exhibited significant

Table 1. Analysis of phospholipids used in liposome preparations

	Per cent composition								
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:0	20:4
EYL	0	0	35.52	1.57	11.54	28.38	16.82	0	3.88
DML	95.81	0	1.03	0	0	0	0	0	0
DOL (9-*cis*-DOL)	1.70	0	1.28	0	1.50	94.03	0	0	0
DLL (9,12-*cis*, *cis*-DLL)	0	0	1.46	0	1.25	1.00	95.10	0	0

($P < 0.05$) enhancement of the incorporation of [^{3}H]TdR when compared to control cultures (Table 2). The BPBL cells which were cultured with 1:1 DPL:α-T lipid vesicles for 72 hr in the presence of PHA showed no significant change in the incorporation of [^{3}H]TdR when compared to PHA-stimulated control cultures (Table 2).

Cultures containing BPBL cells and 2:1 DPL:DML, 2:1:1 DPL:DML:α-T, 2:1:3 DPL:DML:α-T or 2:1:6 DPL:DML:α-T lipid vesicles in the absence of PHA in all cases demonstrated a significant enhancement ($P < 0.05$) for 2:1 DPL:DML and $P < 0.01$ for 2:1:1, 2:1:3 and 2:1:6 DPL:DML:α-T) when compared to control cultures (Table 2). BPBL cultured with 2:1:1 DPL:DML:α-T lipid vesicles were not significantly stimulated to incorporate [^{3}H]TdR when compared to PHA-stimulated control cultures (Table 2). The

Table 2. The response (cpm) of bovine peripheral blood lymphocytes cultured with and without liposome preparations in the presence and absence of PHA

		Mole ratio of phospholipid:α-tocopherol			
Liposome		2:0[a]	2:1	1:1	1:2
Control	554 ± 170[c]				
Control + PHA[b]	25.125 ± 2815				
α-Tocopherol[d]	746 ± 90				
α-Tocopherol + PHA	25.263 ± 3800				
EYL		470 ± 78	570 ± 162	681 ± 59	614 ± 182
EYL + PHA		2825 ± 242	15.829 ± 1347	28.106 ± 2858	28.516 ± 2025
DML		1039 ± 286	1614 ± 121	1512 ± 51	1102 ± 324
DML + PHA		47.989 ± 3757	41.137 ± 6895	27.027 ± 5106	25.894 ± 5036
DPL		2198 ± 194	2022 ± 179	1513 ± 121	2201 ± 223
DPL + PHA		33.086 ± 1301	21.296 ± 1043	20.017 ± 3518	18.966 ± 1855
DPL:DML		1918 ± 429	5865 ± 829	5206 ± 640	4296 ± 984
DPL:DML:PHA		31.788 ± 4083	29,992 ± 2193	34.811 ± 3800	53.552 ± 5377

[a] 2 μmoles of phospholipid per culture.
[b] 15 μg/ml.
[c] Values are means ± SDs of eight replicates per culture.
[d] 2 μmoles of α-tocopherol per culture.

Table 3. The effect of incubation of bovine peripheral blood lymphocytes with liposomes[a] prior to addition of PHA[b] on the response to various PHA concentrations

PHA concentration[c]	Control[d]	Preincubation[e]	Coincubation[f]
375	6833 ± 579[g]	40 ± 6	20 ± 2
187.5	7179 ± 1249	22 ± 1	35 ± 13
93.73	5168 ± 604	48 ± 16	35 ± 9
46.88	11.360 ± 675	45 ± 11	23 ± 2
23.44	14.929 ± 2602	70 ± 9	17 ± 2
11.82	22.540 ± 2833	40 ± 6	37 ± 13

[a] EYL liposomes (2 μmoles of phospholipid per culture).
[b] Phytohemagglutinin.
[c] Concentration in culture (μg/ml).
[d] Without liposomes.
[e] 1-hr preincubation of cultures prior to addition of PHA.
[f] All culture components added simultaneously.
[g] Means ± SD of triplicate cultures.

cultures containing BPBL cells and 2:1 PL:DML, 2:1:3 DPL:DML:α-T or 2:1:6 DPL:DML:α-T lipid vesicles incubated for 72 hr exhibited significant ($P < 0.05$) enhancement of the incorporation of [^{3}H]TdR when compared to PHA-stimulated control cultures.

From the information presented in Table 2 an apparent order of enhancement of the incorporation of [^{3}H]TdR by BPBL cells cultured with the various lipid vesicle compositions in the absence of PHA may be made. This order is as follows: 2:1:1 DPL:DML:α-T > 2:1:3 DPL:DML:α-T > 2:1:6 DPL:DML:α-T > 1:2 DPL:α-T > DPL > 2:1 DPL:α-T > 2:1 DPL:DML > 2:1 DML:α-T > 1:1 DPL:α-T > 1:1 DML:α-T > 1:2 DML:α-T > DML > α-T > 1:1 EYL:α-T > 1:2 EYL:α-T > 2:1 EYL:α-T > control > EYL.

From the information presented in Table 2 an apparent order of enhancement/suppression of the incorporation of [^{3}H]TdR by BPBL cells cultured with the various lipid vesicle compositions in the presence of PHA may be made. This order is as follows: 2:1:6 DPL:DML:α-T > DML > 2:1 DML:α-T > 2:1:3 DPL:DML:α-T > DPL > 2:1 PL:DML > 2:1:1 DPL:DML:α-T > 1:2 YL:α-T > 1:1 EYL:α-T > 1:1 DML:α-T > 1:2 DML:α-T > α-T > control > 2:1 DPL:α-T > 1:1 DPL:α-T > 1:2 DPL:α-T > 2:1 EYL:α-T > EYL.

Increasing the concentrations of PHA present in BPBL cultures incubated with EYL liposomes had no effect in reversing the suppression caused by EYL (Table 3). Preincubation of EYL liposomes with BPBL for 1 hr prior to the addition of PHA was also unable to reverse the suppression of blastogenesis. The simultaneous addition of PHA and EYL liposomes had no reversing effect when PHA was increased from 11.82 to 375 μg/ml.

Various α-T concentrations were added into BPBL cultures as liposome-entrapped α-T (DPL:DML, 1:1) or free α-T (0.07% ethanol:RPMI-1640). The maximum effect of free α-T occurred between 20 and 0.2 μmoles/ml (Table 4). Increasing the free α-T concentration beyond 20 μmoles/ml suppressed blastogenesis of BPBL. Decreasing the free α-T concentration below 0.2 μmoles/ml to 2×10^{-4} μmoles/ml had no significant ($P < 0.05$) effect on blastogenesis. Mixtures of liposomes with entrapped and excess free α-T did not significantly ($P < 0.05$) suppress BPBL blastogenesis with between 2×10^3 and 2×10^4 μmoles/ml α-T (Table 4).

Table 4. The effect of α-tocopherol concentration in cultures with bovine peripheral blood lymphocytes on response to PHA[a]

	α-Tocopherol[b] (μmoles)						
	2000	200	20	2	0.2	0.02	0.002
Control[c]	2.3 ± 0.28[d]						
Liposomes[e]	3.4 ± 0.48						
α-Tocopherol Liposome[f] + α-tocopherol	0.01	0.01	1.1 ± 0.28	4.7 ± 0.39	3.0 ± 0.24	2.7 ± 0.51	2.4 ± 0.10
	6.4 ± 0.22	4.2 ± 0.43	2.9 ± 0.24	3.5 ± 0.43	2.9 ± 0.17	3.1 ± 0.27	2.4 ± 0.22

[a] Phytohemagglutinin (15 μg/ml).
[b] Concentration (μmoles/ml) of total α-tocopherol added per culture either as free or a mixture of liposome-associated and free.
[c] Without liposome or α-tocopherol.
[d] Mean ± SD of triplicate cultures (1×10^{-4} cpm).
[e] 2 μmoles of phospholipid per culture (composition of DPL:DML 1:1).
[f] Free α-tocopherol suspended in 0.07% ethanol.

Table 5. The effect of α-tocopherol (α-T) on the response (cpm) of bovine peripheral blood lymphocytes cultured with liposomes[a] containing varying concentrations of DOL[b] or DLL[c] and PHA[d]

		Mole ratio of DML:unsaturated phospholipid					
Liposome	Alone	1:1	2:1	4:1	8:1	16:1	32:1
Control[d]	6.6 ± 0.4[e]						
α-T[f]	6.2 ± 0.2						
DML	7.1 ± 0.1						
DML:α-T	9.0 ± 0.8						
DOL	7.2 ± 0.5	8.9 ± 0.3	7.9 ± 0.6	8.2 ± 1.2	7.9 ± 1.4	8.8 ± 0.2	6.8 ± 0.7
DOL:α-T	6.8 ± 0.9	7.3 ± 0.7	6.7 ± 1.0	6.6 ± 1.5	6.6 ± 0.2	6.2 ± 0.5	6.8 ± 0.1
DLL	0.01	0.3 ± 0.0	2.4 ± 0.2	3.0 ± 0.5	3.4 ± 0.2	5.1 ± 1.1	6.8 ± 0.8
DLL:α-T	8.2 ± 0.3	8.0 ± 1.4	6.6 ± 0.6	6.0 ± 0.4	4.9 ± 0.4	6.1 ± 0.5	6.3 ± 0.6

[a]Concentration of phospholipid was 2 μmoles per culture.
[b]9-cis-Dioleoylphosphatidylcholine.
[c]9.12-*cis*, *cis*-Dilinoeylphosphatidylcholine.
[d]Without liposomes.
[e]Mean ± SD of triplicate cultures (1 × 10^{-4} cpm).
[f]Concentration 0.2 μmoles per culture.

The effects of the unsaturation of phospholipids on BPBL blastogenesis were examined (Table 5). MLV composed of DOL, mixtures of DML and DOL and DML alone were not suppressive at mole ratios (DML:DOL) from 0:1 to 1:0 (Table 5). The addition of 10 mole% α-T in MLV containing DOL had no significant ($P < 0.01$) effect on BPBL blastogenesis. MLV composed of DLL and mixtures of DML and DLL exhibited a suppression of BPBL blastogenesis that was directly related to the concentration of DLL present in the MLV. The suppressive effect occurred between mole ratios (DML:DLL) of 0:1 and 8:1. The addition of DML in MLV (DML:DLL) at concentrations between 16:1 and 32:1 reversed suppression of BPBL blastogenesis. The addition of α-T at 10 mole% into MLV composed of mixtures of DML and DLL reversed suppression of BPBL blastogenesis at all DML:DLL concentrations between 32:1 and 1:1. MLV composed of DML with 10 mole% α-T enhanced ($P < 0.05$) the blastogenic response of BPBL to PHA.

DISCUSSION

The results of this study provide evidence that BPBL, incubated for 72 hr with various vesicles of saturated lecithin and α-T, show a general increase in the incorporation of [^{3}H]TdR in the presence and absence of PHA. The degree of enhancement varied depending on the type of lipid used with vesicles composed of 2:1 DPL:DML always producing the most pronounced enhancement when α-T was present at a 2:1:6 DPL:DML:α-T mole ratio. EYL and DLL caused the most pronounced suppression as as measured by incorporation of [^{3}H]TdR. The order of enhancement in the presence of PHA was 2:1 DPL:DML > DPL > DML > DOL ⩾ unstimulated control cultures > EYL ⩾ DLL. In vesicles composed of DPL or DML increasing the α-T concentration gradually decreased the ability of BPBL to incorporate [^{3}H]TdR. In vesicles composed of 2:1 DPL:DML or EYL increasing the α-T concentration increased the ability of BPBL cells to incorporate [^{3}H]TdR. A previous study (Ozato *et al.*, 1977) demonstrated that the enhancement was not confined to a particular part of the mitogenic period but was observed at all times during a 5-day culture period, but it should be noted that they utilized only a brief stimulation of cells with liposomes. It is possible that the continuous exposure of lipid vesicles, possibly absorbed onto the surface of BPBL cells, may induce or prevent a large number of cells to enter the proliferative cycle. The present study seems to be in agreement with previous studies (Alderson & Green, 1975; Chen & Keenan, 1977) which demonstrated a suppressive effect with EYL lipid vesicles on the incorporation of [^{3}H]TdR after incubation times longer than those described by Ozata *et al.* (1977).

A difference appears to exist between DOL:DML, DPL and DML and EYL lipid vesicles with respect to their effect on the incorporation of [^{3}H]TdR. The mode of interaction, stable absorption (DPL:DML, DPL and DML) vs lipid exchange (EYL) of vesicles with cells, may be due to differences in the mechanisms of vesicle–cell membrane interactions that modify the mitogenic response of the BPBL cells (Ozato *et al.*, 1977).

EYL contains both saturated and unsaturated phospholipids. Saturated fatty acids include palmitate and stearate. Unsaturated fatty acids include oleate, linoleate and arachidonate (Table 1). Suppression of blastogenesis resulting from exposure of lymphocytes to MLV containing EYL has been demonstrated (Fountain & Schultz, 1982*a*, *b*) but no explanation of the suppressive component of EYL has been offered. Changes in the fluidity of the lymphocyte membrane by MLV (EYL) have been suggested as affecting lymphocyte responses to mitogen (Pagano & Huang, 1975).

Previous studies (Pagano & Huang, 1973; Sandra & Pagano, 1979; Pagano *et al.*, 1980) have demonstrated that liposomes composed of EYL or DOL interact with Chinese hamster fibroblasts and mouse lymphocytes by lipid transfer or lipid exchange. The exchange of lipid from liposome to cell occurs without transfer of aqueous contents of liposomes (Pagano & Huang, 1975). Lipid exchange between DOL, DLL or EYL liposomes and cells appears to be the dominant mechanism of vesicle-cell interaction between liposomes and mouse lymphocytes (Pagano *et al.*, 1980). In lipid exchange of fluorescent-probe derivatives of EYL, those phospholipids transferred to the cell surface by vesicle-cell lipid exchange are properly integrated into the plasma membrane. Within 60 min of incubation of liposomes with mouse lymphocytes at 37 C the fluorescent probe of EYL was internalized within the lymphocyte by a temperature-dependent capping of lipids introduced after vesicle-lipid exchange (Pagano *et al.*, 1980).

The capping appears to occur with maximal effectiveness after 20–30 min followed by subsequent internalization of exogenous lipids within 1 hr (Sandra & Pagano, 1979).

We present evidence (Table 5) that the suppressive component of EYL may be linoleic acid derivatives of phospholipids. Linoleic acid could be utilized by cells to synthesize PGE_1 and PGF_1. The resulting suppression of BPBL blastogenesis by EYL and DLL MLV may result from increased production of PGE_1 which suppresses the lymphocyte response to PHA (Novogrodsky *et al.*, 1979) or by alteration in lymphocyte membrane fluidity by introduction of highly fluid exogenous unsaturated phospholipids. The alteration of membrane fluidity could affect PHA receptor mobility resulting in a decrease in the effectiveness of PHA to stimulate lymphocytes to undergo blastogenesis.

α-T interacts with unsaturated phospholipids and stabilizes bilayers (Diplock *et al.*, 1977). α-T incorporated into liposomes composed of EYL reversed suppression of blastogenesis (Fountain & Schultz, 1982*a*). We demonstrated that α-T specifically reversed the suppression induced by DLL vesicles. We believe that α-T may stabilize EYL, DOL and DLL vesicles and alter their mode of interaction with BPBL. As lipid exchange occurs in fluid vesicles, the presence of α-T may decrease the fluidity of the vesicles and prevent exchange of unsaturated phospholipids such as DOL or DLL into lymphocyte plasma membranes (Pagano *et al.*, 1980).

We demonstrated that BPBL incubated with EYL vesicles prior to the addition of PHA caused maximal suppression after between 0.5 and 1 hr and that reversal, when α-T was present, began to occur after 4 hr with complete reversion after 12 hr (Fountain & Schultz, 1982*b*). The suppressive effect may be modulated by the ratio of DLL to saturated phospholipid (DML) present in the liposome and by the presence of α-T (Table 5).

The enhancement is seen in the presence and absence of mitogen, but was most pronounced in the case of 2:1:6 DPL:DML:α-T lipid vesicles in the presence or absence of PHA.

A mechanism similar to that reported for cholesterol exchange (Chen & Keenan, 1977) from EYL:cholesterol vesicles may be occurring between EYL lipid vesicles and lymphocytes. This may be due to a cellular requirement of cholesterol for successful lymphocyte transformation. Also, α-T may be essential for lymphocyte blastogenesis (Fountain & Schultz, 1982*a*). α-T interacts with the acyl chains of phospholipids through the association of the phytol side chain with the acyl chain and chromanol ring with the polar head group of the lecithin molecule (Lucy, 1971). This may directly affect the membrane fluidity (Barber *et al.*, 1977; Spallholz *et al.*, 1973; Tengerdy *et al.*, 1973) and thus the expression or function of receptors. We propose a possible modulating effect of α-T (either at the liposome or lymphocyte membrane lvel) on immune responsiveness as an explanation of the increased lymphocyte blastogenic response in the presence of α-T delivered by DPL:DML, DPL and DML lipid vesicles. In addition to preventing the autooxidation of unsaturated acyl chains of the lymphocyte membrane during blastogenesis, the α-T molecule may play a role in stabilizing the fluidity of the lymphocyte membrane.

Studies are currently under way to evaluate possible implications of pathologic conditions of vitamin E deficiency (Horwitt, 1965). The information gained from this study forms the basis for studies on the possible enhancement of antigens presented by DPL:DML, DPL and DML lipid vesicles containing α-T *in vivo*.

REFERENCES

Alderson J. C. E. & Green C. (1975) Enrichment of lymphocytes with cholesterol and its effect on lymphocyte activation. *FEBS Lett.* **52**, 208–211.

Boyum A. (1966) Ficoll–Hypaque method for separating mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest.* Suppl. 77–89.

Campbell P. A., Cooper H. R., Heinzerling H. R. & Tengerdy R. P. (1974) Vitamin E enhances *in vitro* immune response by normal and nonadherent spleen cells. *Proc. Soc. exp. Biol. Med.* **36**, 465–472.

Chen S. S. & Keenan R. M. (1977) Effect of phosphatidylcholine liposomes on the mitogen-stimulated lymphocyte activation. *Biochem. biophys. Res. Commun.* **79**, 852–858.

DeGier J., Manderslott J. G. & Van Deenen L. L. M. (1969) The role of cholesterol in lipid membranes. *Biochim. biophys. Acta* **173**, 143–145.

Diplock A. T. (1974) Vitamin E, selenium and the membrane associated drug-metabolizing enzyme system of rat liver. *Vitams Horm.* **32**, 445–462.

Diplock A. T., Lucy J. A., Verrinder M. & Zieleniewski A. (1977) α-Tocopherol and the permeability of glucose and chromate of unsaturated liposomes. *FEBS Lett.* **82**, 341–344.

Dunnick J. K., Rooke J. D., Aragon S. L. & Kriss J. P. (1976) Alteration of mammalian cells by interaction with artificial lipid vesicles. *Cancer Res.* **36**, 2385–2389.

Edwards P. A. (1975) Effect of plasma lipoproteins and lecithin–cholesterol dispersions on the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase of isolated rat hepatocytes. *Biochim. biophys. Acta* **309**, 39–50.

Engleman D. E. & Rothman J. E. (1972) The planar organization of lecithin–cholesterol bilayers. *J. biol. Chem.* **247**, 2694–2697.

Fountain M. W., Dees C. & Schultz R. D. (1981) Effect of phosphatidylcholine liposomes containing *Brucella abortus* soluble antigen on the response of bovine lymphocytes to phytohemagglutinin. *Curr. Microbiol.* **6**, 61–64.

Fountain M. W. & Schultz R. D. (1981) Regulation of response of bovine lymphocytes to phytohemagglutinin by liposomes containing α-tocopherol. *Fedn Proc. Fedn Am. Socs exp. Biol.* **40**, 569.

Fountain M. W. & Schultz R. D. (1982*a*) Effects of enrichment of phosphatidylcholine liposomes with cholesterol or α-tocopherol on the response of lymphocytes to phytohemagglutinin. *Molec. Immun.* **19**, 59–64.

Fountain M. W. & Schultz R. D. (1982*b*) Effects of mitogen and liposome phospholipid concentration on the blastogenic response of bovine lymphocytes to phytohemagglutinin. *Immunobiology*. In press.

Gregoriadis G. & Davis C. (1979) Stability of liposomes *in vivo* and *in vitro* is promoted by their cholesterol content and the presence of blood cells. *Biochem. biophys. Res. Commun.* **89**, 1287–1293.

Grunze M. & Deuticke B. (1974) Changes of membrane permeability due to extensive cholesterol depletion in mammalian erythrocytes. *Biochim. biophys. Acta* **356**, 125–130.

Hartzman R., Buch M. & Thurman G. (1972) Precipitation of radioactively labeled samples, a semi-automatic multiple sample harvester. *Cell. Immun.* **4**, 182–186.

Horwitt M. K. (1965) Role of vitamin E, selenium and polyunsaturated fatty acids in clinical and experimental muscle disease. *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 68.

Huang C. (1969) Studies on phosphatidylcholine vesicles: formation and physical characteristics. *Biochemistry* **8**, 344–351.

Inbar M. & Shinitzky M. (1974) Increase of cholesterol level in the surface membrane of lymphoma cells and its inhibitory effect on ascites tumor development. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2128–2130.

Inoue K. (1974) Permeability properties of liposomes prepared from dipalmitoyllecithin, dimyristoyllecithin, egg lecithin, rat liver lecithin and beef brain sphingomyelin. *Biochim. biophys. Acta* **339**, 390–402.

Kates M. (1975) In *Techniques of Lipidology* (Edited by Work T. S. & Work E.). pp. 558–559. Elsevier, New York.

Kinsky S. C. (1972) Antibody-complement interaction with lipid model membranes. *Biochim. biophys. Acta* **265**, 1–23.

Lucy J. A. (1972) Functional and structural aspects of biological membranes: a suggested structural role for vitamin E in the control of membrane permeability and stability. *Ann. N.Y. Acad. Sci.* **203**, 4–11.

Metcalf L. D. & Schmitz A. A. (1961) Rapid preparation of fatty acid methyl esters for gas chromatography. *Analyt. Chem.* **33**, 363–364.

Nes W. R. (1974) Role of sterols in membranes. *Lipid* **9**, 596–609.

Novogrodsky A., Rubin A. L. & Stenzel K. H. (1979) Triggering signals for T-cell activation. In *Cell Biology and Immunology of Leukocyte Function* (Edited by Quastel M. R.). pp. 11–25. Academic Press, New York.

Ozato K., Huang L. & Pagano R. E. (1977) Interactions of phospholipid vesicles with murine lymphocytes. II. Correlation between altered surface properties and enhanced proliferative response. *Membrane Biochem.* **1**, 27–39.

Pagano R. E. & Huang L. (1975) Interaction of phospholipid vesicles with mammalian cells. II. Studies of mechanism. *J. Cell Biol.* **74**, 531–546.

Pagano R. E., Struck D. K. & Schroit A. J. (1980) Introduction of phospholipids into mammalian cell surfaces via lipid vesicles. In *Liposomes and Immunobiology* (Edited by Tom B. & Six H.). pp. 193–209. Elsevier North-Holland, New York.

Sandra A. & Pagano R. E. (1979) Liposome-cell interactions. Studies of lipid transfer using isotopically asymmetric vesicles. *J. biol. Chem.* **254**, 2244–2249.

Schultz R. D. & Adams L. S. (1978) Immunologic methods for the detection of humoral and cellular immunity. In *Symposium on Basic and Clinical Aspects of Veterinary Immunology* (Edited by Schultz R. D.). *Vet. Clin. N. Am.* **8**, 721–741.

Sheffy B. E. & Schultz R. D. (1979) Influence of vitamin E and selenium on immune response mechanisms. *Fedn Proc. Fedn Am. Socs exp. Biol.* **38**, 2139.

Spallholz J. E., Martin J. L., Gerlach M. L. & Heinzerling R. H. (1973) Immunologic responses of mice fed diets supplemented with selenite selenium. *Proc. Soc. exp. Biol. Med.* **143**, 685–689.

Szoka F. & Papahadjopoulos D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4194–4198.

Tengerdy R. P. & Brown J. C. (1977) Effect of vitamin E and A on humoral immunity and phagocytosis in *E. coli* infected chickens. *Poult. Sci.* **56**, 957–963.

Tengerdy R. P., Heinzerling R. H., Brown G. L. & Bathias M. M. (1973) Enhancement of humoral immune response by vitamin E. *Int. Archs Allergy appl. Immun.* **44**, 221–229.