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Role of a serum phospholipase A_1 in the phosphatidylserine-induced T cell inhibition

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We have previously shown that unsaturated phosphatidylserines inhibit mitogen-induced T cell activation. We now report that the inhibitory action requires a protein present in bovine and human serum. Partial purification and phospholipase assay show that this protein has phospholipase A activity on phosphatidylserine but not phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. In short incubations (1–3 h) 2-acyl lysophosphatidylserine is produced but in longer incubations the *cis*-unsaturated fatty acid is also released. Experiments on peripheral blood mononuclear cells indicate that the unsaturated fatty acid becomes the main responsible for the PS-induced inhibition and that 2-acyl lysophosphatidylserine enhances the inhibitory effect of fatty acid.

Phospholipase A_1; Phosphatidylserine; Lysophosphatidylserine (2-acyl); T cell activation; Fetal calf serum

1. INTRODUCTION

The anionic phospholipid phosphatidylserine (PS) inhibits mitogen-induced T cell activation [1,2]. Lymphocyte inhibition is also observed with other lipid compounds (e.g. cholesterol) and with lipoproteins [3,4]. Based on the antagonism by transferrin, it has been proposed that cholesterol and lipoproteins affect transferrin metabolism [3]. Since it is not known whether a direct or mediated mechanism is involved in PS-induced inhibition we have examined the action of this phospholipid on peripheral blood mononuclear cells (PBMC) in serum-free and serum-containing medium. The data show that the action of PS requires a serum factor with phospholipase activity toward this phospholipid.

2. MATERIALS AND METHODS

2.1. *Phospholipids and other materials*

PS was obtained from bovine brain and purified on Q Sepharose. Purity was assessed by thin layer chromatography. Oleic acid (34%) and stearic acid (49%) were the predominant fatty acids. 2-acyl lysoPS was prepared by the digestion of PS with adult bovine serum (2 ml/mg of PS, 3 h at 37°C), followed by differential solvent extraction [5]. To prepare [^{3}H]arachidonoyl-PS, 10^8 PBMC were incubated 30 min at 37°C with 50 μCi of [^{3}H]arachidonate and 5 μM of 1-acyl lysoPS which was obtained from PS as described previously [6]. Phospholipids were then extracted with chloroform/methanol (2:1, v/v) and PS purified by thin layer chromatography. Digestion with phospholipase A_2 from porcine pancreas showed that the tracer was exclusively incorporated in position 2 of glycerol. Other radiolabeled lipids were from Amersham. Transferrin, prostacyclin and fatty acid-free bovine serum albumin (BSA) were from Sigma; heparin (Liquemin Roche); porcine pancreas phospholipase A_2 (Boehringer), fetal calf serum (FCS, Seromed); phenyl-Sepharose CL4B and heparin-Sepharose CL6B (Pharmacia).

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2.2. *Cell cultures*

PBMC from heparinized human blood (10^5 cells/well containing 0.2 ml of RPMI medium) were cultured 72 h at 37°C in humidified air containing 5% CO_2 [1]. Phytohemagglutinin (PHA) was used at 1 μg/ml. To evaluate DNA synthesis the cells were pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]dThd) during the last 18 h of cultures.

2.3. *Assay of phospholipase activity*

The fractions obtained in the procedure developed to partially purify the serum factor enhancing the action of PS (see Results and Discussion) were tested for phospholipase activity in 0.2 ml of phosphate saline buffer (pH 7.4) containing 0.5 mM of Ca^{2+} and a sonicated dispersion of radiolabeled phospholipids (6 nmol, 3–6 × 10^3 dpm/nmol). FCS was added at 1 and 10% (v/v) corresponding to 80 and 800 μg protein/ sample. Purified fractions were added at 5 μg/ sample together with 100 μg of BSA. The incubation was 3 h at 37°C. The activity was Ca^{2+}- dependent (0.2–0.5 mM) and constant between pH 7.4 and 9. The reaction was stopped with 20 vols. of chloroform/ methanol (2:1, v/v) and the lipid extract, washed with 0.2 vols. of 0.05 M HCl, was resolved by thin layer chromatography.

3. RESULTS AND DISCUSSION

When compared to medium containing FCS, PHA-induced DNA synthesis in serum-free cultures was diminished but still detectable [7]. Transferrin substantially increased [^{3}H]dThd incorporation [8]. Under these conditions PS partially inhibited DNA synthesis in the absence of transferrin (mean value of 44% at 30 μM) but was ineffective in its presence (Fig. 1). In serum-containing medium transferrin still increased DNA synthesis [8] but did not affect the PS inhibitory effect (IC_{50} at 15 μM with and without transferrin). Similar results were obtained with increasing transferrin concentrations (up to

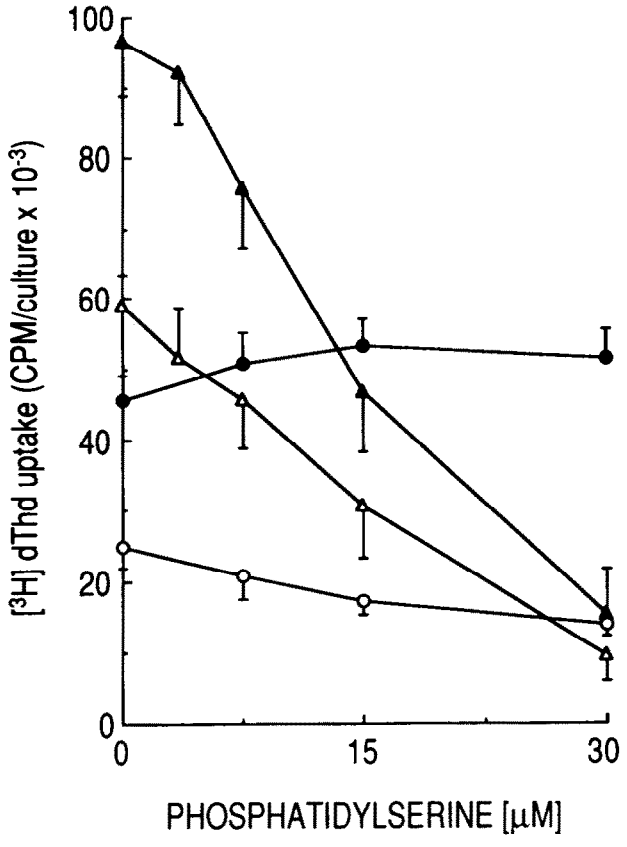

Fig. 1. Effect of fetal calf serum on the PS-induced inhibition. 10^5 PBMC were cultured in the presence of 1 μg/ml of PHA in 0.2 ml of RPMI medium. Additions: (○) 1 mg/ml of BSA; (●) BSA plus 50 μg/ml of transferrin; (△) 5% (v/v) of fetal calf serum; (▲) fetal calf serum plus transferrin. Means ± S.E.M. for 15 experiments, each in triplicate and with different donors. Without PHA [^{3}H]dThd uptake was 0.8–2.0 cpm × 10^{-3}.

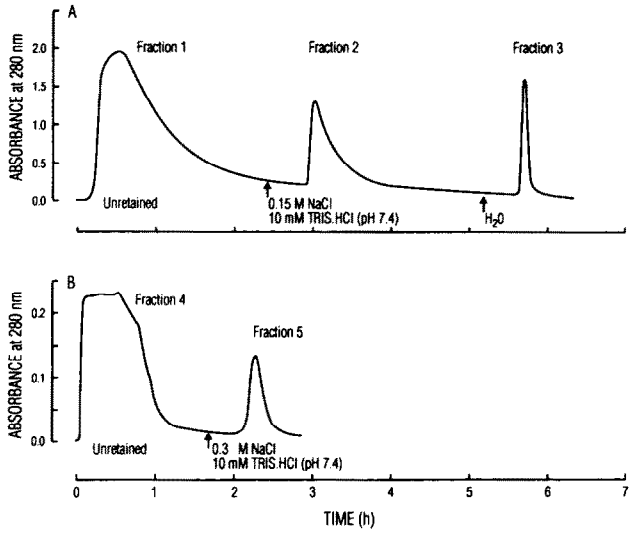

Fig. 2. Step-wise elution of serum factor in phenyl-Sepharose (A) and heparin-Sepharose (B) columns. The continuous recording of column efflux (1 ml/min) shows the elution of serum proteins in A and of proteins from fraction 3 in B. The activity was found in fractions 3 and 5 which were stored at −20°C in Tris, NaCl buffer containing 30% glycerol (v/v) after concentration with solid ammonium sulfate (fraction 3) and washing on C25 Amicon Cones.

200 μg/ml) or supplementing the medium with adult bovine or human serum (not shown). From these data we concluded that although transferrin influenced a minor component of PS action becoming manifest in serum-free medium, its effect was not sufficient to overcome the action of PS when enhanced by serum. In this respect, PS was unlike lipoproteins and cholesterol [8]. The factor enhancing the action of PS was recovered in lipoprotein-free serum (density of 1.21 g/ml) from where it could be precipitated by ammonium sulfate at 60% saturation. Partial purification was facilitated when it was found that the protein had phospholipase A activity on PS. Undialyzed lipoprotein-free FCS was applied at 4°C to a column of phenyl-Sepharose (Fig. 2A). The water-eluted fraction (fraction 3) was incubated overnight at 4°C with heparin-Sepharose in 50 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.4). After pouring in a column, proteins were eluted (Fig. 2B). Unretained proteins showed negligible phospholipase activity which was instead concentrated in fraction 5. SDS-PAGE under reducing conditions showed that fraction 5 had two main bands of 60 and 120 kDa and several minor bands when visualized by silver staining. Phospholipase activity (nmol lysoPS/mg/3 h) provided preliminary indications of purification and activity yield. The specific activity of FCS (6.3 ± 3.5, mean ± S.D., $n = 4$) increased to 180 ± 34 in fraction 3 and to 215 ± 30 in fraction 5. The yield of proteins was 3.7 ± 1.1% of serum protein in fraction 3 and about 0.4% in fraction 5. These data indicated good yield of activity in phenyl-Sepharose but loss in heparin-Sepharose. Although the reason for the activity loss needs to be investigated, the property of the enzyme to be bound to heparin-Sepharose was considered of interest since hydrophobic interaction with phenyl-Sepharose and binding to heparin-Sepharose were previously observed with lipid transfer proteins [9] and lipoprotein lipase [10]. The fractions enhancing the action of PS on PBMC had the highest phospholipase activity suggesting that the two activities were related (Table I). Kinetic experiments (Fig. 3) established that in 1–3 h of incubation phospholipase activity was primarily of the A_1 type generating 2-acyl lysoPS. In longer incubations also 2-acyl lysoPS was hydrolyzed with release of unsaturated fatty acids and glycero-*P*-serine (which was detected with [^{14}C]serine-PS). Explanations include the possibility that phospholipase A_1 had some activity on position 2 of glycerol [11] or that unsaturated fatty acid migrated to the *sn*-1 position in long incubations. In accord with a single enzyme releasing and splitting 2-acyl lysoPS, the two activities showed the same optimal pH and Ca^{2+} requirement and were similarly enriched during partial purification. Among PS

metabolites (Table II) the *cis*-unsaturated fatty acid (oleate) rather than stearate was the effective inhibitor of PHA-activated PBMC cultured in serum-free medium. 2-acyl lysoPS increased DNA synthesis but enhanced oleate-induced inhibition. Purified phospholipase A_2 releasing unsaturated fatty acids from PS, partially reproduced the action of serum factor in the absence of 2-acyl lysoPS but was fully effective in its presence. Since these results were the same in the presence and absence of transferrin (not shown) it was concluded that the inhibition by oleate and PS plus phospholipase A_2 was not mediated by interference with the action of transferrin.

Phospholipase activity on PS was detected in serum of several mammals (Fig. 4). However, only bovine and human serum showed preference for PS. Activity was also found in heparinized plasma obtained in the presence of prostacyclin to avoid platelet activation. Since inhibition by heparin [12] was not observed, the lower activity of plasma was interpreted as phospholipase activation during blood clotting. Unlike lipoprotein lipase and hepatic lipase [10,13], the enzyme from bovine and human serum did not hydrolyze PE and PC in a pH range between 7 and 10 and after the addition of deoxycholate or diethylether (not shown). Generation of lysoPI from phosphatidylinositol as well as lipase activity toward [^{3}H]triolein were not detected. Heating 30 min at 56°C partially inactivated FCS and completely inhibited fraction 5.

In conclusion, we have shown that bovine and human serum contains a phospholipase A_1 which preferentially cleaves PS in comparison to PC, PE and PI. The enzyme generates 2-acyl lysoPS which in long incubations is further hydrolyzed releasing its *cis*-unsaturated fatty acid. These metabolites become then the main responsible for PS-induced T cell inhibition. In agreement, only PS containing unsaturated acyl chains prevents T cell activation [1,2] and previous observations confirm the

Table I

Relationship between the enhancing effect of fetal calf serum (FCS) and its phospholipase activity

Exp.	Additions	[^{3}H]dThd incorporation[a]		Phospholipase activity[b]
		Without PS	30 μM PS	
1.	1 mg/ml BSA	37.2 ± 3.5	43.1 ± 4.1	–
	1% (v/v) FCS	47.9 ± 0.7	0.9 ± 0.2 (98%)	11.5
	BSA plus fraction 1[c]	29.5 ± 2.6	41.8 ± 4.4	1.3
	BSA plus fraction 2	27.5 ± 2.2	43.6 ± 10.9	1.4
	BSA plus fraction 3	24.3 ± 3.5	4.6 ± 0.3 (81%)	138
2.	1 mg/ml BSA	23.1 ± 1.1	38.8 ± 0.4	
	BSA plus fraction 4	25.2 ± 1.5	37.4 ± 5.9	7.6
	BSA plus fraction 5	30.1 ± 1.1	8.8 ± 0.2 (71%)	195

[a] 10^5 PBMC activated by 1 μg/ml of PHA were cultured in serum-free medium in the presence of 50 μg/ml of transferrin. cpm/culture ($\times 10^{-3}$). Mean ± S.D. of triplicate samples.
[b] lysoPS generation (nmol/mg of protein in 3 h of incubation).
[c] 50 μg/ml of fractions 1–3 (from phenyl-Sepharose); 10 μg/ml of fractions 4,5 (from heparin-Sepharose).

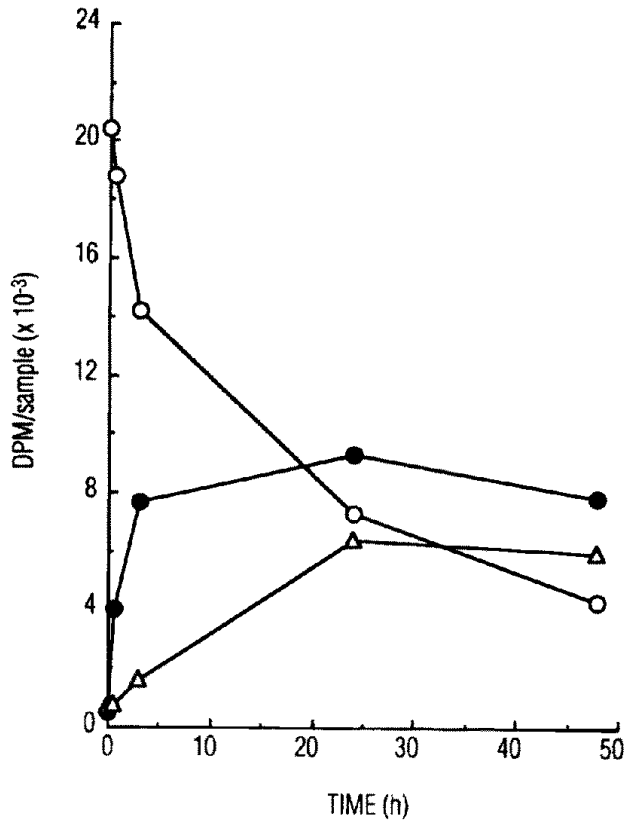

Fig. 3. Phospholipase activity with 2-[^{3}H]arachidonoyl-PS. 6 nmol of PS (24,000 dpm) were incubated with 5 μg of fraction 5 from heparin-Sepharose. (○) PS; (●) lysoPS; (△) free arachidonate.

Table II

Action of PS metabolites and pancreas phospholipase A_2 (PLA_2)

Exp.	Additions	[^{3}H]dThd incorporation (cpm/culture × 10^{-3})	
		Minus lysoPS	Plus 2-acyl lysoPS
1.	None	53.2 ± 10.5	69.1 ± 6.2
	5 μM oleate	21.0 ± 12.2 (60%)	5.4 ± 7.4 (92%)
	10 μM stearate	79.0 ± 12.2	54.3 ± 10.8 (21%)
2.	None	33.6 ± 1.9	58.8 ± 4.0
	20 ng PLA_2	53.4 ± 1.6	65.0 ± 1.8
	30 μM PS	68.1 ± 2.2	53.7 ± 1.2
	PS plus PLA_2	35.2 ± 5.3 (48%)[a]	0.8 ± 0.4 (98%)[a]

10^5 PBMC were cultured in the presence of 1 μg/ml PHA, 50 μg/ml transferrin in 0.2 ml RPMI supplemented with 1 mg/ml BSA. 10 μM 2-acyl lysoPS. Mean ± S.D. of 6 (exp. 1) or 3 (exp. 2) cultures. In parentheses the percent inhibition.
[a] with respect to PS alone.

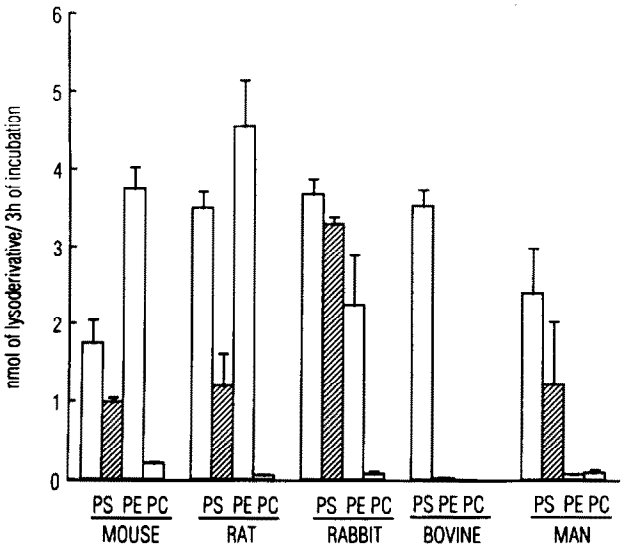

Fig. 4. Phospholipase A activity in sera or plasma (hatched bars) from mammals. 6 nmol of [^{14}C]serine-PS, phosphatidyl-[^{14}C]ethanolamine (PE), 2-[^{14}C]oleoylphosphatidylcholine (PC) incubated 3 h at 37°C in 0.2 ml of phosphate saline buffer (pH 7.4) with 10% (v/v) of serum (1–1.4 mg of protein) or plasma obtained in the presence of 20 IU/ml of heparin and 1 μM of prostacyclin. Means ± S.D. of at least 7 donors. Tests with 2-[^{3}H]arachidonoyl-PS gave the following lysoPS/free arachidonate ratio (A_1/A_2 ratio): mouse, 1.8; rat, 3.0; rabbit, 3.1; bovine, 28.3; man, 28.5.

inhibitory effect of oleate on these cells [7,14]. The detection of serum phospholipase A_1 lends support to our hypothesis that hydrolysis of PS in damaged tissues yields metabolites acting on immune cells [15]. Phospholipase A_1 acting on PS have been detected in rat liver membranes [16] and rat liver lysosomes [17].

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