Lysolecithin Induced Membrane Alterations in Thymocytes

Effects of Lysophosphatides Possessing Adjuvant and Immuno-Suppressive Activities on Cell Agglutination by Concanavalin A

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The effects of lysolecithin and of 2 synthetic ether-desoxy lysolecithin analogs, containing alkyl residues of 16 or 12 carbon atoms, on the agglutination kinetics of calf and rabbit thymocytes by concanavalin A (Con A) were investigated. Unlike the natural lysolecithin, these synthetic analogs are resistant to metabolism by membrane associated enzymes. It was found that pretreatment of thymocytes with lysolecithin or with the C_{16}-analog leads to slightly increased agglutination rates. The C_{12}-analog, in contrast, significantly inhibits thymocyte agglutination by Con A. Moreover, a comparison of these results with lysophosphatide effects on the agglutinability of erythrocytes of various species revealed that the inhibitory effect of the short-chain phosphatide is rather specific for thymocytes. The finding that long- and short-chain lysophosphatides, which have previously been shown to react as adjuvants or immunosuppressants, respectively, induce adserve alterations in thymocyte membranes indicates that these substances may affect the immune response by changing the membrane properties of immune competent cells. Concerning the nature of these membrane alterations it was shown that lysolecithin did not affect the number of Con A receptors per cell nor the affinity of lectin binding. It is therefore concluded that the lysophosphatide induced alterations of Con A agglutinability can not be caused by an uncovering or covering of lectin-receptors.

Introduction

According to an hypothesis of Munder *et al.*[1], lysolecithin may be viewed at as an adjuvant-induced endogenic mediator of immune reactions *in vivo*. Subsequently it has been found that lysolecithin itself possesses the properties of an immunological adjuvant *in vivo* as well as *in vitro*[2,3]. Synthetic lysolecithin analogs[4–7], designed to be resistant to metabolism by membrane associated enzymes such as lysophospholipase or acyl CoA : lysolecithin acyltransferase[8] exhibited a similar or even stronger adjuvanticity than the natural compound[9]. Moreover, shortening the hydrophobic chain of lysolecithin from 16 or 18 to 10 or 12 carbon atoms resulted in analogs possessing significant immunosuppressive activity[10].

The molecular and cellular events responsible for these observed effects are not yet understood. Since lysolecithin and some analogs, however, are known to be highly surface- and membrane-active substances[11,12], one might expect that the effects described above are initiated by a physical interaction of the lysophosphatide molecules with the plasma membranes of cells involved in immune reactions. In fact, it has been shown in a previous study that long- and short-chain lysolecithin analogs have different effects upon the process of junction formation between stimulated and non stimulated lymphocytes[13]. Furthermore, it has been demonstrated that at subtoxic concentrations lysophosphatides interfere with the agglutination of chicken erythrocytes by Con A, but not with that of bovine or human red cells[14,15]. It was then concluded[15] that these effects must be due to cellspecific surface alterations induced by lysolecithin. In an attempt to relate such membrane changes to the immunological activity of lysophosphatides, the present study was designed to determine whether lysolecithin altered the agglutionation behaviour also of thymus cells.

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Materials and Methods

Lysophosphatides

L-lysolecithin was purchased from C. Roth OHG, Karlsruhe, Germany, and will be called "natural lysolecithin" or "lysolecithin" in the course of this paper. Lysolecithin-analogs were synthesized according to methods published elsewhere [6]. The preparation of lysophosphatides labelled with ^{14}C in the choline methyl-groups has also been described before [7], as well as solubilization and storage conditions for these compounds [15, 16].

Buffers

Phosphate buffered saline contained 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 3.4 mM KCl, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.2. Eagle's medium was prepared as described by Smith *et al.* [17].

Cells

Thymocytes were prepared by homogenizing small pieces of calf or rabbit thymi in a loose fitting Tenbroek tissue grinder (glass) in Eagle's medium. The homogenates were filtered through nylon wool to remove aggregates and connective tissue and were then incubated for 30 min at 37 °C on a nylon fibre column (leucopac) in Eagle's medium to remove dead cells. After elution from the column the cells were centrifuged once at 1200 rpm and resuspended either in phosphate buffered saline or in Eagle's medium at a cell density of 1.5×10^8 cells per ml. The proportion of trypan blue positive cells was usually between 5 and 15%.

Erythrocytes were isolated by centrifugation of citrate stabilized whole blood which had been stored at 4 °C no longer than 5 days. After 3 washes with saline the cell sediment was diluted 1:50 in phosphate buffered saline for agglutination assays. Trypsin digestion of red cells was carried out as described earlier [15] using 0.4 mg trypsin (Boehringer/Mannheim, lyophilized) and about 5×10^8 cells per ml in phosphate buffered saline for 30 min at 37 °C. The cells were subsequently centrifuged, resuspended in saline containing 0.25 mg trypsin inhibitor (from eggwhite, Boehringer/Mannheim) per ml, washed twice with buffer, and then resuspended at a 1:50 dilution in phosphate buffered saline.

Agglutination assay

Con A agglutination experiments with thymocytes were carried out as described earlier for red cell agglutination [15], except that the test volume was reduced to 0.5 ml. In brief: The cell suspensions were kept shaking in 10 ml glass vessels in a 37 °C waterbath. At various times after the addition of Con A samples were removed and examined under a microscope. Agglutination was scored according to the number and size of agglutinates from – to ++++ (see Fig. 2). Lysophosphatides were added at various concentrations 5 min prior to the addition of Con A.

Binding of ^3H-labelled ConA to thymocytes

6×10^7 cells in 0.5 ml phosphate buffered saline were mixed with 0.5 ml of ^3H-labelled ConA (2.58×10^5 cpm/mg protein) at various concentrations. After 45 min at 23 °C the cells were centrifuged, and the sediments washed twice with 1 ml of the same buffer. The pellets were then transferred to scintillation vessels, oxidized by H_2O_2 in 1 ml of isopropanol/soluene (Packard) (1:1, v/v) at 40 °C and dissolved in 10 ml of scintigel (C. Roth, Karlsruhe, Germany). The radioactivity was determined in a liquid scintillation counter (Tricarb 3000, Packard).

Binding of ^{14}C-labelled phosphatides to cells

The detailed procedure has been described previously [16]. In short: Cells were mixed with the radioactive lipids in phosphate buffered saline under conditions identical to those used for agglutination assays. After 30 min at 37 °C, the cells were centrifuged, and the radioactivity was determined in the supernatant and in the pellet.

Concanavalin A (Con A)

Con A was obtained from Miles Yeda (2× crystallized, in saturated NaCl) and stored at 4 °C. The ^3H-labelled Con A was generous gift of Dr. M. Inbar at the Weizmann Institute of Science, Rehovot, Israel. The hapten-inhibitor α-methyl-D-mannopyranoside was purchased from C. Roth, Karlsruhe, Germany.

Results

1. Agglutination of thymocytes by concanavalin A (Con A)

Thymus cells derived from calf or rabbit were effectively agglutinated by Con A at concentrations as low as 2 μg Con A/ml. Thus, both kinds of cells were agglutinated by lower ConA concentrations and more rapidly than for instance chicken red cells or trypsinized human or bovine erythrocytes [15]. Self-agglutination of thymocytes in the absence of Con A was negligible within 15 – 20 min.

2. Lysophosphatides

Fig. 1 shows the chemical structures of the 3 lysophosphatides used during this investigation. The nomenclature ($ES_{16}-OH$, $ET_{16}-H$, $ET_{12}-H$) has been recommended by O. Westphal to simplify the identification of synthetic lysolecithin analogs. The

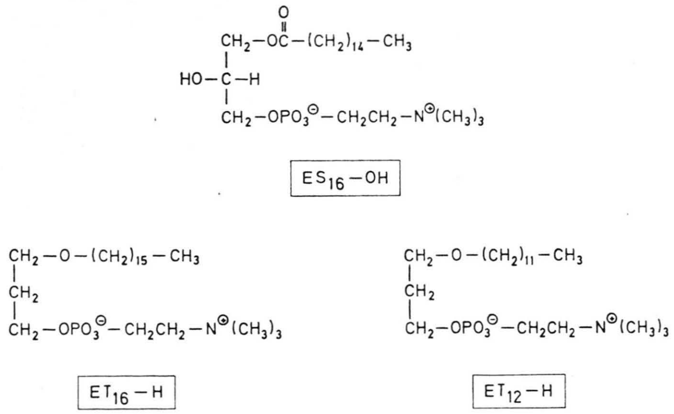

Fig. 1. Chemical structures of lysolecithin and synthetic analogs. $ES_{16}-OH$: L-palmitoyl-lysolecithin = 1-palmitoyl-*sn*-glycero-3-phosphorylcholine; $ET_{16}-H$: 1-hexadecyl-2-desoxy-lysolecithin = 1-hexadecyl-propanediol-3-phosphorylcholine; $ET_{12}-H$: 1-dodecyl-2-desoxy-lysolecithin = 1-dodecyl-propanediol-3-phosphorylcholine.

first 2 letters indicate whether the hydrophobic side chain is linked to the remainder of the molecule *via* an ester (ES) or an ether-bond (ET), whereas the number following these letters determines the number of carbon atoms in the hydrophobic residue. The following letters represent the chemical structure of the substituent attached to the central carbon atom of the glycerol backbone of lysolecithin, *i. e.* $-OH$ in "natural" 2-hydroxy-lysolecithin or $-H$ in the synthetic 2-desoxy-lysolecithin analogs, a.s.o.

Natural lysolecithin as well as the desoxy-analog $ET_{16}-H$ are highly surface active and hemolytic substances, while $ET_{12}-H$ exhibits a lower surface activity and is substantially less hemolytic towards red cells [6]. Ether-desoxy lysolecithin analogs, rather than L-lysolecithins of varying chain length in the acyl residues, were used because these phosphatides are metabolized by neither lysophospholipases nor acyltransferases. Such compounds thus remain unaltered within cellular membranes for a long period of time [15], whereas L-lysolecithin is metabolized quite readily [8]. In immunological experiments performed by Langer *et al.* [10, 18] $ET_{16}-H$ as well as natural lysolecithin have been found to possess strong adjuvant properties, in contrast to $ET_{12}-H$ and other short chain analogs, which can significantly suppress humoral immune responses in mice [10, 18].

3. *Effects of lysophosphatides on the Con A induced agglutination of thymus cells*

To determine the effects of lysophosphatides on the Con A agglutination of thymocytes, cells were preincubated for 5 min at 37 °C with various amounts of lysolecithin, $ET_{16}-H$ or $ET_{12}-H$. After this time, phosphatide adsorption to the cells had come to an equilibrium, as determined by the use of ^{14}C-labelled analogs. The toxicity of the phosphatides towards thymus cells was checked by

Cells derived from	Treated with $ES_{16}-OH$ [nmol/ml]	$ET_{16}-H$	Agglutination after minutes 3	5	10
	—	—	+	++	+++
	2.5	—	+	++	+++
calf	5.0	—	++	+++	++++
	10.0	—	++	+++	++++
	—	—	+	++	+++
	—	2.5	++	+++	++++
calf	—	5.0	+++	++++	++++
	—	11.0	++	+++	+++
	—	—	—	++	++
	2.5	—	+	++	+++
rabbit	5.0	—	+	+++	+++
	10.0	—	+	++	+++
	—	—	—	++	+++
	—	2.5	+	++	+++
rabbit	—	5.0	++	+++	+++
	—	10.0	++	+++	++++

Table I. Effect of long-chain lysophosphatides on the agglutination kinetics of calf and rabbit thymocytes. All agglutinations with 2 µg Con A/ml in 0.5 ml cell suspension (1.5×10^8 cells/ml) at 37 °C in phosphate buffered saline.

the trypan blue method. It was found that lysolecithin and $ET_{16}-H$ up to concentrations of 10 nmol/ml, and $ET_{12}-H$ up to 100 nmol/ml did not change the proportion of cells which could be stained with trypan blue.

Table I presents the kinetics of the agglutination by 2 μg Con A/ml of thymus cells pretreated with long chain lysophosphatides in comparison to untreated control cells. It may be seen that low phosphatide concentrations resulted in a slight acceleration of the agglutination rates.

In general, the effects of lysolecithin and $ET_{16}-H$ on thymocyte agglutination are rather weak when compared to their effects on Con A-agglutination of erythrocytes [15]. This may be attributed to the fact that thymocytes apparently are already close to a state of optimal agglutinability. The small differences shown in Table II thus call for a definition of our standards for agglutination readings. These standards, as they appear in dark-field microscopy, are shown in Fig. 2 *. It may be seen there, that using this visual method, a difference of one + in the readings represents about the limit of significance. Greater differences, however, are absolutely clearcut.

In contrast to the results obtained with long chain phosphatides, pretreatment of thymocytes with the short chain $ET_{12}-H$ led to highly significant – though adverse – effects on their Con A agglutinability. As is demonstrated in Table II, $ET_{12}-H$ at a concentration above 40 nmol/ml, when about 7 – 10 nmol $ET_{12}-H$ are adsorbed per 10^8 cells, inhibited almost completely the agglutination of thymocytes from both animal species. Again, these phosphatide concentrations were not toxic to the cells as determined with trypan blue. At lower concentrations $ET_{12}-H$ had virtually no effect on thymocyte agglutination.

It should be noted here, that the affinity of the C_{12}-compound to cell surfaces is significantly lower than that of the C_{16}-analog [19], so that only 20% or less of the $ET_{12}-H$ added, but more than 80% of the $ET_{16}-H$ is bound to the cells. Thus, addition of 40 nmol $ET_{12}-H$ or of 10 nmol $ET_{16}-H$ (or $ES_{16}-OH$) leads in either case to the binding of 8 – 9 nmol to the cells. With respect to lysophosphatide-binding, calf and rabbit thymocytes are indistinguishable.

The results of Tables II and III, obtained in phosphate buffered saline, were essentially the same when the agglutinations were performed in Eagle's medium with or without serum. Some data for the agglutination of rabbit thymocytes in Eagle's medium containing 10% fetal calf serum are shown in Table III. In this medium the adsorption of $ET_{16}-H$ to the cells was reduced by about 60%, that of $ET_{12}-H$ by about 30%, probably because of competing adsorption of the phosphatides to serum lipoproteins.

4. *Effects of $ET_{12}-H$ on red cell agglutination*

Experiments with 4 different species of red cells were performed to decide whether the observed inhibiting effect of $ET_{12}-H$ on thymocyte agglutination represented a general phenomenon, or if there existed differences between different cells. The results shown in Table IV clearly prove that the various cells, indeed, differ markedly in their response to the incorporation of this phosphatide. In contrast to thymocytes, the agglutinability of un-

Cells derived from	Treated with $ET_{12}-H$ [nmol/ml]	Agglutination after minutes 5	10	15	20
	–	++	++	+++	++++
	10	++	+++	+++	+++
calf	20	++	++	++	++
	40	–	–	+	++
	80	–	–	–	–
	–	+	+++	++++	++++
	10	++	+++	++++	++++
rabbit	20	++	++++	++++	++++
	40	+	++	++	++
	80	–	–	–	–

Table II. Effect of $ET_{12}-H$ on the agglutination of calf and rabbit thymocytes. All agglutinations with 2 μg Con A/ml, 0.5 ml cell suspension (1.5×10^8 cells/ml) at 37 °C in phosphate buffered saline.

* Fig. 2 see Table on page 788 a.

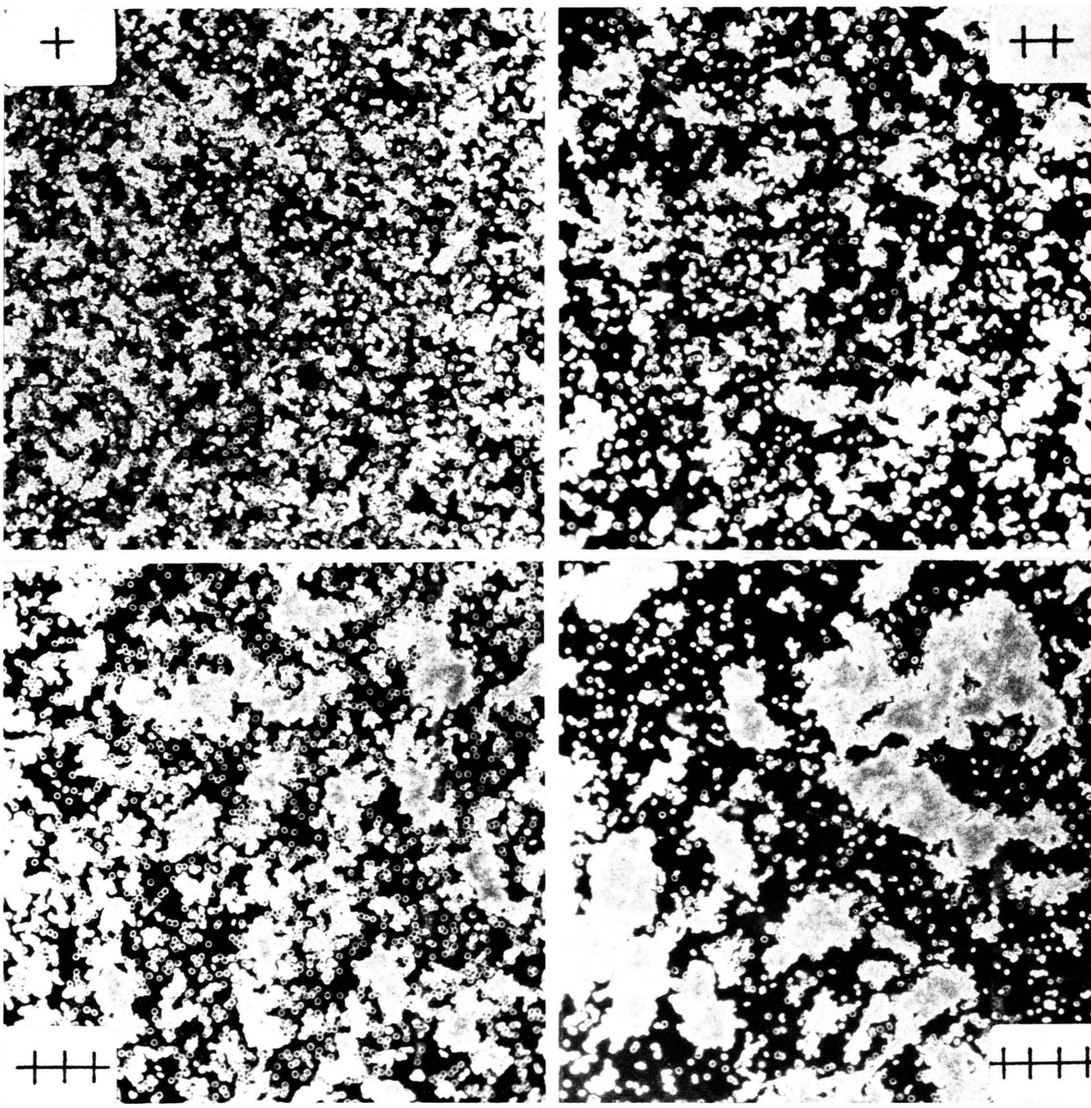

Fig. 2. Agglutination standards of 1 to 4 plus as observed in dark field microscopy. Enlargment about 40 ×.

Cell pretreated with	Amount of phosphatide added [nmol/ml]	Agglutination after minutes 3	7	12	16
	—	++	++	+++	++++
	2.5	+++	++++	++++	++++
$ET_{16}-H$	5.0	+++	++++	++++	++++
	10.0	++	+++	+++	++++
	12.5	++	+++	+++	++++
	25	+	+++	++++	++++
	—	++	+++	+++	++++
	20	++	++++	++++	++++
$ET_{12}-H$	40	++	+++	+++	++++
	80	+	+	++	+++
	100	+	+	+	+

Table III. Effect of $ET_{16}-H$ and $ET_{12}-H$ on the agglutination of rabbit thymocytes in Eagle's medium containing 10% fetal calf serum. All agglutinations with 0.5 ml cell suspension (1.5×10^8 cells/ml) at 37 °C, using 2 μg Con A/ml.

trypsinized chicken or rabbit erythrocytes was significantly enhanced by high doses of $ET_{12}-H$. Among the trypsinized red cells, chicken and rabbit cells did not respond at all, human cells responded with an acceleration, and only trypsinized bovine cells with an inhibition of their agglutinability to treatment with high concentrations of the short-chain phosphatide. The latter cell type exhibited the most complex dose response in that at lower $ET_{12}-H$ concentrations its Con A agglutination was strongly enhanced. These results are comparable to the dose dependent effects of octadecyl-benzyl-lysolecithin on trypsinized bovine erythrocytes, which have been described earlier [15].

The hemolytic activity of $ET_{12}-H$ became measurable with the most sensitive cells, *i. e.* tryp-

Table IV. Effects of $ET_{12}-H$ on the agglutination of various erythrocytes by Con A. All agglutinations at 37 °C in phosphate buffered saline, using 2% cells suspensions.

Erythrocyte species	ET_{12}-H [nmol/ml]	Con A [μg/ml]	Agglutination after minutes 4	6	8	10	15	20	30	60
	—	20		—		—		++		
	20	20		—		++		+++		
chicken	40	20		+		+++		+++		
	80	20		+++		++++		++++		
	—	0.4	++		++		++++			
chicken	20	0.4	++		+++		++++			
(trypsin-	40	0.4	++		+++		++++			
ized)	80	0.4	+		+++		++++			
	—	5		—		-	—	+	++	+++
human *	20	5		—		+	+++	+++	++++	
(trypsin-	40	5		—		++	+++	++++	++++	
ized)	80	5		—		+	++	+++	++++	
	—	20		—	—	—	+	+++		+++
bovine **	20	20		+	++	+++	++++	++++		++++
(trypsin-	40	20		—	—	+	++	+++		+++
ized)	80	20		—	—	—	—	—		—
	—	5	—	+		++	+++	++++		
	20	5	++++	++++		++++	++++	++++		
rabbit	40	5	++++	++++		++++	++++	++++		
	80	5	++++	++++		++++	++++	++++		
	—	0.1	—	++		+++	++++			
rabbit	20	0.1	—	++		+++	+++			
(trypsin-	40	0.1	—	+		++	++++			
ized)	80	0.1	—	+		++	+++			

* Untripsinized human red cells agglutinate weekly at Con A concentrations above 50 μg/ml but are not affected by lysophosphatide treatment [15].

** Untrypsinized bovine red cells are not agglutinated at all by Con A. This is not changed by treatment with lysophosphatides [15].

sinized bovine erythrocytes, a a minimal concentration of 120 nmol/ml. The effects of long chain lysolecithin analogs – including $ET_{16}-H$ on the Con A induced agglutination of various red cells has been published previously [14, 15].

5. Binding of Con A to thymocytes

The binding of tritium-labelled Con A to calf thymocytes was measured using untreated cells (control) as well as cells pretreated with either 3.3 nmol $ET_{16}-H$ or 100 nmol $ET_{12}-H$ per 1.5×10^8 cells (6×10^7 cells/ml). These are conditions which, as shown above, enhance or inhibit, respectively, the agglutination of thymocytes by Con A. The results of these experiments are shown in Figs 3 a and 3 b. Fig. 3 a represents a linear plot of specifically bound Con A, *i. e.* total binding minus Con A bound in the presence of 0.4 M α-methyl-D-mannopyranoside, *versus* the amount of Con A added. It is clear from these curves that neither phosphatide significantly affected the binding of Con A to the cells. This result is more pronounced, when the data are plotted according to Scatchard [20] (Fig. 3 b). Within the range of error, all values fit one curve, which extrapolates to a maximal binding of about 3.5×10^5 Con A molecules per cell. The affinity constant, as determined by the slope of this curve, is under all 3 conditions close to 1.1×10^7 l/mol,

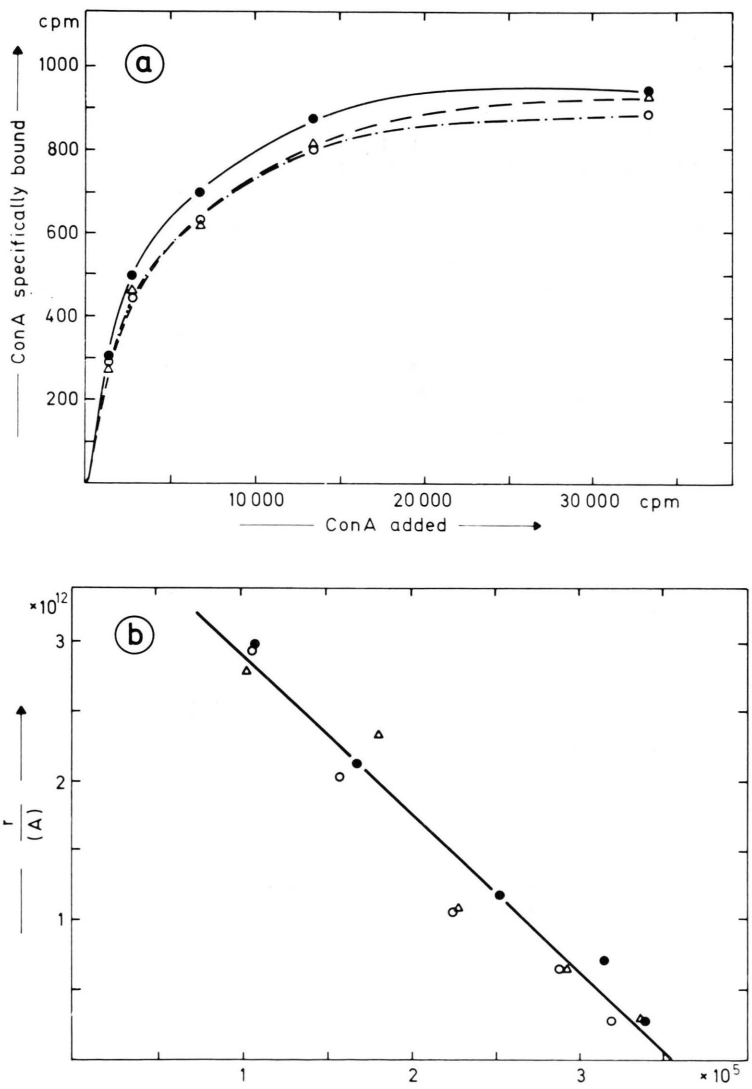

Fig. 3. Specific binding of ^{3}H-labelled Con A to calf thymocytes in the absence or presence of lysophosphatides. 6×10^7 cells per ml phosphate buffered saline were mixed with varying amounts of ^{3}H-labelled Con A at 23 °C, and binding was determined as described under Methods. Specific activity of Con A: 2.58×10^5 cpm/mg protein. ○—·—○, untreated control cells; △——△, cells pretreated with 1.3 nmol $ET_{16}-H$/ml (*i. e.* 3.3 nmol/1.5×10^8 cells); ●—●, cells pretreated with 40 nmol $ET_{12}-H$/ml (*i. e.* 100 nmol/1.5×10^8 cells). — a. Linear plot of cpm specifically bound (*i. e.* total cpm bound, minus cpm bound in the presence of 0.4 M α-methyl-mannoside) *versus* the amount of Con A added. b. Data of Fig. 3 a plotted according to Scatchard [23]. *r*, Con A *specifically* bound (molecules per cell), calculated for a molecular weight of 108 000. (A), molar concentration of free Con A (*i. e.* Con A added minus *total* Con A bound). Extrapolation of the curve to r/(A) = 0 gives the maximal number of Con A molecules bound per cell; the slope determines the affinity constant of the cells for Con A in (l/mol).

which is in good agreement with the value of 1.2×10^7 l/mol published by Bethel and van den Berg [21] for the binding constant of Con A to rat lymph-node- and thymus-cells.

Discussion

The mechanism of Con A induced cell agglutination is not yet well understood. It is apparent, however, that the agglutinability of a certain cell type is not determined simply by the number of Con A-binding sites on its surface [22, 23]. Moreover, evidence is increasing which implies that the mobility of Con A membrane receptors is a critical parameter in determining the degree of cell agglutination by this lectin [24–26], supporting the original concept of Sachs [27] and Nicolson [28]. Since lateral motion of membrane receptors is only possible in sufficiently fluid membranes [29], our previously reported finding [14, 15] that changes of the lysolecithin concentration in certain red cells result in an alteration of their Con A agglutinability, may lend further support to the idea of receptor mobility as a prerequisit for cell agglutination by Con A.

As was demonstrated by the results presented in Tables I to III, also the agglutination behaviour of thymocytes with Con A can be influenced by adsorption of lysophosphatides, most likely to their plasma membranes. Studies on the Con A-binding to thymocytes (Fig. 3), in agreement with our results on the binding of Con A to red cells [15], excluded the possibility of lysolecithin induced changes in the number and affinity of lectin-receptors as well as functional alterations of the Con A molecules. Following my previous argumentation [15], I therefore believe that also in the thymocyte system an altered receptor-mobility or -distribution, due to changes in the membrane lipid composition, presently provides the simplest explanation for the observed effects.

In this context it is important to recall that all phospholipids tested so far (see Table IV and ref. 15) exhibit different effects on different cells. This is demonstrated particularly by the results obtained with red cells and thymocytes from the same animal species (see data on rabbit thymocytes in Table II and rabbit erythrocytes, Table IV). From this it may be inferred that lysophosphatides injected into animals may well induce rather sepcific alterations in only a limited number of cell types. Moreover, the previous notion [15] that structural differences of lysolecithin analogs may be reflected in different surface alterations, has been corroborated by the results obtained here with long- and short-chain desoxy-lysolecithins. It is of particular interest, that thymocytes especially respond differently to treatment with $ET_{16}-H$ or with $ET_{12}-H$, phosphatides which have been found to act as adjuvant or immunosuppressant, respectively [10, 18].

As mentioned before, these membrane alterations may be viewed as changes in the mobility of membrane receptors. It should be stated clearly, however, that direct evidence for this latter hypothesis is yet lacking, and that other explanations such as lysolecithin induced conformational changes of membrane proteins, alterations of the net surface charge, stimulation or inactivation of membrane enzymes, and probably other factors can not be excluded at the present time.

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