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AGGLUTINATION OF MOUSE ERYTHROCYTES BY BINDING OF NON-CHOLINE PHOSPHOLIPIDS TO A 70000-DALTON PROTEIN

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The structure of some phospholipids that cause agglutination of mouse erythrocytes has been studied. Haemagglutination is a property of non-choline-containing phospholipids; the phosphate group is essential and unsaturated fatty acids optimal. A protein of M_r 70000 was isolated from mouse erythrocyte membranes which completely inhibited phospholipid-mediated erythrocyte agglutination. It is proposed that this protein is the phospholipid binding site on mouse erythrocytes and the ligand for the human B-lymphocyte receptor for mouse erythrocytes. Preliminary investigations suggest that a similar inhibitor of phospholipid-mediated agglutination is found in serum. Agglutination of mouse erythrocytes by phospholipid and specific inhibition by the 70 kDa membrane protein constitute a simple system for studying the interaction of phospholipid with protein.

Introduction

The phenomenon of rosette formation by mouse erythrocytes with human B lymphocytes has stimulated particular interest because it is a property of lymphocytes in chronic lymphocytic leukaemia [1,2]. This has been interpreted as being a marker of the point of maturation arrest of the malignant lymphocytes in that disease [3,4]. After exposure to tumour-promoting phorbol esters, these cells lose the capacity to rosette with mouse erythrocytes and subsequently undergo plasmacytoid differentiation [5,6].

Understanding these phenomena requires a knowledge of the nature of the receptor for mouse erythrocytes and of the corresponding ligand on mouse erythrocytes. During investigation of the receptor it was found that certain pure phospholipids bound to mouse erythrocytes with precisely the same specificity as did trypsin-solubilized receptor. Studies of this interaction of phospholipid with the ligand on mouse erythrocytes are described here.

Materials and Methods

Cells. Erythrocytes were obtained from ox (Alsever's solution stored, Flow Laboratories), sheep (Alsever's solution stored, Commonwealth Serum Labs), humans, rats and mice (bled into citrated saline). All erythrocytes were washed in phosphate buffered saline (pH 7.4) and resuspended in this buffer to 2% (v/v) concentration. Pronase-treated mouse erythrocytes were prepared by incubating a 2% suspension of mouse erythrocytes with 0.25 vol. of pronase (1 mg/ml, Calbiochem) at 37°C for 30 min.

Phospholipids and related substances. L-α-Phosphatidylethanolamine (PE) from egg was

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DPPE, dipalmitoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine.

purchased from Sigma Chemicals and Calbiochem. PE from *E. coli* and soybean, dipalmitoylPE, dipalmitoylmonomethylPE, dipalmitoyldimethylPE, L-α-phosphatidylserine, L-α-phosphatidylglycerol, L-α-phosphatidylinositol, L-α-phosphatidic acid (egg, dioleoyl-, dipalmitoyl-, distearoyl-, dimyristoyl-), L-α-phosphatidylcholine (PC) (egg, distearoyl-, dipalmitoyl-, dimyristoyl-, diarachidoyl-), L-α-lysophosphatidylcholine (egg), sphingomyelin (bovine erythrocyte), phosphorylcholine, phosphorylethanolamine, glycerolphosphorylinositol, 1-*O*-alkyl-2-acetylglycerolphosphorylcholine and purified gangliosides type II and III were obtained from Sigma, as were the fatty acids, arachidic, behenic, heptadecanoic, lauric, linoelaidic, elaidic, nervonic, myristoleic, palmitoelaidic, palmitoleic, linolenic, stearic, palmitic and myristic. Dioleoylphosphatidyl[2-^{14}C]ethanolamine and di[1-^{14}C]oleoylphosphatidylcholine were obtained from Amersham International. Phospholipids were stored in chloroform at −20°C. For use, the solvent was evaporated and the residue was vigorously resuspended in 80% ethanol (aqueous). Further dilutions were made in buffer. In some experiments, stock solutions were made in phosphate buffer, with or without albumin (1 mg/ml crystalline, human, Sigma). Suspensions of fatty acids and phosphatidic acid were adjusted to pH 7.4 with 0.1 M NaOH before use.

Haemagglutination assay. Haemagglutination titre was determined in microtitre plates (96 well, round-bottomed, Titertek, Linbro). Serial dilutions were made in phosphate buffer. To 25 μl of dilution were added 25 μl of 2% red cells. Wells containing ethanol at concentrations of 40% and greater gave lysis. Ethanol by itself at concentrations less than 40% did not affect the red cells. Titre is expressed in reciprocal form as a power of 2. Absence of haemagglutination is expressed as zero. Haemagglutination was recognized by carpet-like settling of red cells, in contrast to button-like settling in non-agglutinated samples. Haemagglutination was confirmed by microscopy.

For haemagglutination inhibition experiments, 25 μl of serial dilution of phospholipid and 25 μl of inhibitor were pre-incubated for 20 min at room temperature before addition of 25 μl of red cells.

Thin-layer chromatography. Samples were spotted on aluminium-backed silica gel 60 sheets (No. 5553, Merck) or glass-backed silica gel 60 plates (No. 5715, Merck). For the isolation of PE, the developing solvent was chloroform/methanol/2.5 M ammonium hydroxide (70 : 20 : 3) and for analysis of the phospholipase digest of PC and PE, the developing solvent was chloroform/methanol/acetic acid/water (65 : 15 : 10 : 4). Chromatograms were developed twice and bands were eluted with chloroform/methanol (2 : 1). Bands were identified with iodine vapour or by charring in sulphuric acid.

Preparation of crude mouse erythrocyte extract. Mouse erythrocytes were lysed in distilled water containing a few drops of acetic acid. Stroma was washed extensively, homogenized and sonicated in saline and extracted with phenol (final concentration of 33%) at 64°C. The upper aqueous layer was removed, dialyzed against distilled water and freeze-dried.

Radioiodination was by the chloramine-T standard method [7].

Chromatography on Blue Sepharose. Crude mouse erythrocyte extract (prepared by the phenol/aqueous method) or normal human serum was chromatographed on Blue-Sepharose CL-6B (Pharmacia) using a K9/15 column (Pharmacia) at a flow rate of 45 ml/h. Column was equilibrated with 0.05 M Tris-HCl buffer containing 0.1 M KCl. 1-ml fractions were collected. Beginning at fraction 38, bound material was eluted with 0.05 M Tris-HCl buffer containing 1.5 M KCl. Fractions were assayed for absorbance at 280 nm and for inhibition (see Table I) of the haemagglutination of mouse erythrocytes by an aqueous solution of PE (titre 2^{10}).

SDS-polyacrylamide gel electrophoresis. Fractions from Blue Sepharose chromatography were pooled, dialysed (against distilled water) and concentrated by rotary evaporation and then by a Minicon-CS 15 concentrator (Amicon). Concentrate was analysed by electrophoresis in a vertical slab gel system on 10% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS) and β-mercaptoethanol. Gels were stained with Coomassie blue (Sigma), silver nitrate (Bio-Rad) or periodate-Schiff reagent (BDH). Where radioiodinated samples were run, gels were sliced and counted for gamma irradiation or developed by autoradiography.

Results

Species specificity of haemagglutination. A variety of commercially available phospholipids, in aqueous suspension, agglutinated mouse and rat erythrocytes but not human, ox or sheep red cells (Table I). Agglutination could be demonstrated in microtitre wells after 60 min incubation or immediately following centrifugation in plastic tubes.

Effect of pH and Ca^{2+} on haemagglutination. Lowering of the pH to 6 decreased the haemagglutination titre from 2^9 to 2^5, but neither EDTA (2 mM), EGTA (5 mM), nor added Ca^{2+} had any effect.

Effect of phospholipid structure on haemagglutination. Haemagglutination occurred at phospholipid concentrations below 1 μg/ml with PE, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid (Table II).

Diacylglycerol, which is dephosphorylated phosphatidic acid, was inactive. Thus, phosphate is essential for activity. There was a marked variation in activity of different lots of the same commercial preparation of egg PE. One batch (lot 51F-8385, Sigma) was completely inactive, but pure PE isolated from this by thin-layer chromatography was active, suggesting the presence of an inhibitor. PE from numerous sources (e.g., egg, soybean, *E. coli*) was active. No sample of PC was active, even after purification by thin-layer chromatography and regardless of fatty acid composition. The other choline-containing phospholipid, sphingomyelin, was also inactive, suggesting that choline in the headgroup is inhibitory. DPPE with one or two methyl groups on the ethanolamine, was more active than DPPE or DPPC. The reason for this is not apparent. Significantly more activity was displayed by unsaturated than by phospholipids (Table II). Thus DPPE was inactive at 50 μg/ml, while DOPE (monounsaturated) agglutinated at 1 μg/ml and highly unsaturated egg PE (unsaturation index 70) was at least 10-times more active. No activity was displayed by fatty acids or phosphorylated headgroups alone. Com-

TABLE I

SPECIFIC BINDING OF PE TO MOUSE ERYTHROCYTES

Concentration of PE in first well was 50 μg/ml. Haemagglutination and haemagglutination-inhibition assays were done as in Materials and Methods. Section A shows the specificity defined by different species of indicator erythrocytes and by the inhibition in the presence of extracts of red cells. Section B shows the relative capacity of different species of erythrocytes to absorb haemagglutinating activity from an aqueous suspension of PE (2 μg/ml). PE was incubated with an equal volume of packed red cells from mice, ox, sheep and humans for 60 min at 37°C. Supernatants were used for agglutination in the experiment.

	Indicator erythrocyte	Phospholipid	Inhibitor	Haemagglutination titre
A	Ox	PE	–	0
	Human		–	0
	Sheep		–	0
	Pronase-treated mouse		–	0
	Rat		–	16
	Mouse		–	15
			crude extract of mouse erythrocytes	1
			crude extract of ox erythrocytes	8
			crude extract of sheep erythrocytes	15
			crude extract of human erythrocytes	13
B	Mouse	PE	–	9
		PE absorbed with mouse erythrocytes	–	1
		PE absorbed with ox erythrocytes	–	5
		PE absorbed with sheep erythrocytes	–	8
		PE absorbed with pronase-treated mouse erythrocytes	–	6

TABLE II

HAEMAGGLUTINATING ACTIVITY OF PHOSPHOLIPIDS AND RELATED SUBSTANCES

Concentration of lipid was 50 μg/ml in the first well. Haemagglutination was assayed as in Materials and Methods. Haemagglutination titre is expressed in reciprocal form as a power of 2. Absence of haemagglutination is expressed as zero. Inactive substances (titre 0) also included L-α-phosphatidylcholine (egg, distearoyl-, dipalmitoyl-, dimyristoyl-, diarachidoyl-), L-α-lysophosphatidylcholine (egg, at sublytic concentrations), sphingomyelin (bovine erythrocyte), L-α-phosphatidic acid (dipalmitoyl-, distearoyl-, dimyristoyl-), phosphorylcholine, phosphorylethanolamine, glycerol phosphorylinositol and platelet-activating factor (1-*O*-alkyl-2-acetyl-glycerolphosphorycholine) and the following fatty acids: arachidic, behenic, heptadecanoic, lauric, linoelaidic, elaidic, nervonic, myristoleic, palmitoelaidic, palmitoleic, linolenic, stearic, palmitic and myristic.

Substance	Type	Haemagglutination titre
L-α-Phosphatidylethanolamine	egg type III (Lot 90F-8350)	11
	III (Lot 51F-8385)	0
	egg (Calbiochem)	20
	E. coli	8
	soybean	9
	dipalmitoyl	0
	[^{14}C]dioleoyl (Amersham)	7
L-α-Monomethylphosphatidylethanolamine	dipalmitoyl	4
L-α-Dimethylphosphatidylethanolamine	dipalmitoyl	4
L-α-Phosphatidylserine	bovine brain	7
L-α-Phosphatidylglycerol	egg	8
L-α-Phosphatidylinositol	soybean	8
L-α-Phosphatidic acid [a]	egg	10
	dioleoyl	7
Ganglioside	type III	0
	type II	3

[a] Phosphatidic acid and the fatty acids were adjusted to pH 7.3 for testing.

mercial type III ganglioside did not agglutinate mouse erythrocytes. Commercial type II did agglutinate, but activity was traced to the organic phase of a chloroform/methanol (2 : 1) partition of this, suggesting contamination by phospholipid.

Effect of phospholipase treatment of phospholipids on haemagglutination. Treatment of phospholipids with phospholipases confirmed some of the principles shown with structure-activity studies. PE or PC (egg, Sigma) was treated for 4 h at 37°C with phospholipase D (cabbage, Sigma) by the method of Comfurius and Zwaal [8], extracted in chloroform/methanol (2 : 1) and tested for activity. The titre of PE was not significantly affected by this treatment, whilst activity was induced in PC (the titre increased from 0 to 2^9). Products of phospholipase D-digested PC were separated by thin-layer chromatography, eluted and tested for activity. Isolated phosphatidic acid contained all of the activity, confirming that PC is inactive because of the presence of the choline group. Treatment of PE (100 μg in 200 μl) with 50 μg phospholipase C (*B. cereus*, Sigma) for 3 h at 37°C destroyed haemagglutinating activity, indicating that the phosphate group is essential. Diacylglycerol isolated from the digest was also inactive. Platelet aggregating factor is a phospholipid with specific binding properties [9]. This substance did not agglutinate mouse erythrocytes (Table II).

Expression of specificity is at the level of the binding. The following experiments show that specificity is expressed at the level of binding. First, agglutinating activity was absorbed from an aqueous suspension of PE (2 μg/ml) by an equal volume of packed mouse erythrocytes with a fall of titre from 2^9 to 2, but titres after absorption under the same conditions with ox and sheep erythrocytes were 2^5 and 2^8, respectively (Table I).

Treatment of mouse erythrocytes with pronase greatly reduced their capacity to absorb PE. Secondly, mouse erythrocyte agglutination by PE was completely inhibited by a crude extract of mouse erythrocyte ghosts (less than 10 μg/ml protein, aqueous phase of phenol extract), while a similar extract from ox erythrocyte ghosts was less inhibitory and one from sheep erythrocyte ghosts did not inhibit (Table I). Dioleoyl-L-α-phosphatidyl[2-^{14}C]ethan-1-ol-2-amine (DO[^{14}C]PE, 49 mCi/mmol) preferentially bound to mouse erythrocytes, although some binding was detected with pronase-treated mouse erythrocytes and with other species of erythrocytes. 500 μl of erythrocytes (2%) were incubated with 25 μl of DO[^{14}C]PE in buffer containing human albumin (crystalline, Sigma) at 1 mg/ml. After 45 min at 37°C the erythrocytes were washed, the pellet was resuspended in scintillation fluid (toluene/POPOP/PPD) and radioactivity was counted for 20 min. In a typical experiment, 3352 counts were bound to mouse erythrocytes, 489 to sheep erythrocytes, 1759 to pronase-treated mouse erythrocytes and 1467 to mouse erythrocytes in the presence of haemagglutination-inhibiting mouse erythrocyte membrane extract. 1-Palmitoyl[1-^{14}C]oleoyl-L-α-phosphatidylcholine (58 mCi/mmol) was not preferentially bound by mouse erythrocytes. Counts on mouse erythrocytes, sheep erythrocytes and pronase-treated mouse erythrocytes were 7964, 13287 and 5870, respectively.

Isolation of the phospholipid-binding protein from mouse erythrocyte membranes. An inhibitor of PE-mediated mouse erythrocyte agglutination, the putative mouse erythrocyte ligand in B lymphocyte-mouse erythrocyte rosettes [10], was isolated from the aqueous phase of the hot phenol/saline extract of mouse erythrocyte ghosts by chromatography on Blue Sepharose (Fig. 1). A single band (M_r 70000) was seen upon sodium dodecyl sulphate polyacrylamide gel electrophoresis, using radioactive-iodine-labelled eluate, or by staining the gel with Coomassie blue or silver. There was no staining with periodate-Schiff reagent. By premixing PE with an equal weight of this protein for 30 min at room temperature, mouse erythrocyte agglutination was completely inhibited. In preliminary studies, another inhibitor of PE-mediated mouse erythrocyte agglutination has been demonstrated in serum, possibly a natural ligand of the B-lymphocyte receptor for mouse erythrocytes. All three species (human, mouse and foetal calf) of sera tested inhibited haemagglutination completely. In the case of human serum, inhibitor was only detectable after removal of natural antibodies to mouse erythrocytes by absorption (equal volumes of packed red cells and serum) or by chromatography on Blue Sepharose.

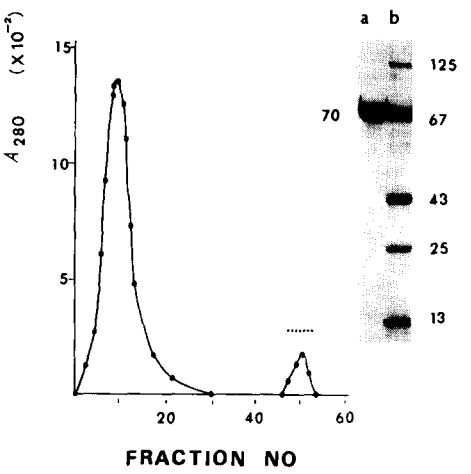

Fig. 1. Chromatography of crude mouse erythrocyte extract on Blue Sepharose. The first peak (fractions 0–30) represents the unbound material and the second peak (fractions 47–53) represents the material eluted by high salt concentration (1.5 M KCl). Fractions which gave complete inhibition of the agglutination of mouse erythrocyte by an aqueous solution of PE (titre 2^{10}) are indicated with dots (fractions 47–53). 1.5 M KCl, by itself, did not inhibit haemagglutination. Inhibiting activity remained after removal of KCl by dialysis. Active fractions were pooled and run on SDS-polyacrylamide gel electrophoresis (track (a) of inset). Track (b) contains markers: β-galactosidase (125 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa), ribonuclease (13 kDa). Gels were stained with Coomassie blue. A single band at approx. 70 kDa is seen in the chromatographed extract from mouse erythrocyte ghosts (track (a)).

Discussion

The specific interaction of a phospholipid with a red cell membrane protein, demonstrable by a simple haemagglutination assay, has not previously been reported, although it is now clear that one class of phospholipid interacts specifically with, and activates, platelets [9] while other classes

bind to phospholipid exchange proteins [11], enzymes [12] and membrane receptors [13] with considerable specificity. In the phospholipid-mouse erythrocyte system, choline is absolutely incompatible with agglutinating activity and phosphate is essential. This is shown by structure-activity relationships with a variety of commercial phospholipids and by phospholipase-digestion experiments. Choline may sterically hinder binding (because of its three bulky methyl groups). Alternatively, these structure-activity studies may reflect the conformation of the phospholipid in an aqueous environment. Thus, PE adopts a very different multilamellar structure than PC in water and unsaturated fatty acids modify conformation [14]. The significance of the requirement for phosphate is not yet known. Calcium bridging is unlikely because of the lack of effect of divalent cation chelators on the haemagglutination. Nevertheless, an electrovalent interaction between the phosphate and basic residues in the mouse red cell ligand is suggested by the inhibition at low pH.

At least four pieces of evidence support the hypothesis that the specificity of haemagglutination is expressed at the level of binding. (a) Pronase treatment of mouse erythrocytes abolished agglutination, inplicating a membrane protein as the phospholipid-binding site; (b) mouse erythrocytes preferentially absorbed haemagglutinating activity; (c) radiolabelled PE, but not PC, preferentially bound to mouse erythrocytes; (d) an extract of mouse erythrocyte ghosts was a potent inhibitor of haemagglutination.

Agglutination may be an immediate consequence of binding where phospholipid aggregates [14] act as multivalent ligands, or agglutination may be a secondary effect due to changes in the erythrocyte membrane leading to autoagglutination [15].

The pronase-sensitive mouse erythrocyte ligand which binds phospholipid was isolated from mouse and rat erythrocyte ghosts by a phenol/saline extraction. Chromatography on Blue sepharose yielded a substance which was homogeneous on SDS-gel electrophoresis and of apparent molecular weight 70000. Lack of staining with periodate-Schiff reagent suggests that it is not a glycoprotein. This material was a potent inhibitor of the phospholipid-mediated agglutination of mouse erythrocytes. Significantly, less or no inhibitor was extracted from sheep, ox or human erythrocyte ghosts.

The species specificity and inhibition by pronase and mouse erythrocyte membrane extract, shown here with phospholipid-mediated haemagglutination of mouse erythrocytes, are identical to those reported previously using trypsin-solubilized mouse erythrocyte receptor from human B cells [10]. Recent evidence indicates that the soluble receptor is a glycoprotein-phospholipid complex in which non-choline phospholipids are responsible for the binding to mouse erythrocytes (unpublished data). In this context, the finding of a potent inhibitor of haemagglutination, with properties similar to that of the mouse erythrocyte ligand, in serum suggests that the natural ligand for this human B cell receptor is a serum protein. The inhibitor in some egg yolk phospholipid preparations may be similar.

The requirements of phospholipids for optimal function of many membrane enzymes and receptors is being recognized increasingly. Analysis of the essential requirements for agglutination of mouse erythrocytes by phospholipid should complement our understanding of the nature of the interaction of phospholipids with membrane proteins.

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