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LIPID-MEDIATED HEMAGGLUTINATION AND ITS RELEVANCE TO LECTIN-MEDIATED AGGLUTINATION

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Summary

Oleic acid and dioleoyl phosphatidic acid at low concentrations (20 and 0.5 μg/ml, respectively) agglutinate rabbit and rat erythrocytes, while dioleoyl phosphatidylcholine is not hemagglutinating up to 0.5 mg/ml. Palmitic acid is not a hemagglutinin and dipalmitoyl phosphatidic acid is a very poor one. A polar lipid fraction obtained from calf thymocytes and a commercial preparation of gangliosides also exhibit pronounced hemagglutinating activity.

Modification of the erythrocytes by either trypsin or neuraminidase causes a marked increase in agglutination only with oleic acid, whereas glutaraldehyde fixation of the cells significantly decreases agglutination with oleic acid, dioleoyl phosphatidic acid and calf thymocyte lipids. None of the lipids tested agglutinate freshly drawn human and sheep erythrocytes, but agglutination occurs following fixation of the sheep cells with glutaraldehyde.

Lipid-mediated hemagglutination is strongly inhibited by fetuin and bovine submaxillary mucin (0.5 mg/ml). Defatted bovine serum albumin, also at 0.5 mg/ml, inhibits agglutination by oleic acid, whereas agglutination by other lipids is only poorly inhibited if at all. Monosaccharides at concentrations up to 0.25 M do not inhibit the hemagglutinating activity of the lipids. Comparison of the hemagglutinating properties of lipids and lectins raises the possibility that the agglutinating activity of crude biological extracts which is not inhibited by mono- or oligosaccharides may be due to lipid constituents. Since agglutination by lipids is species specific, they may serve as mediators in intercellular recognition.

The mechanism of lipid-mediated hemagglutination is discussed in terms of current concepts of the fusogenic activity of these compounds.

Introduction

Agglutination of a homogeneous population of cells and adhesions of heterologous cell types are aggregation phenomena commonly mediated by polyvalent proteins, such as antibodies and lectins, capable of simultaneous binding to specific molecules on the surfaces of apposing cells. Aggregation can also be induced by non-specific agents; for example, charged polymers may link cells together by virtue of their electrical properties.

There are scattered reports in the literature on the ability of lipids to induce agglutination or cell adhesion. Agglutination of avian erythrocytes mediated by phospholipids was described as early as 1946 [1]; recently, incorporation of various glyco- and phospholipids into erythrocyte membranes was shown to cause adhesion of these cells to HeLa cells [2]. However, no mention has been made in the literature of the possible relevance of these findings to studies of agglutinins such as lectins present in extracts of various biological materials.

We have investigated the hemagglutinating activities of various amphipathic lipids as well as a lipid fraction extracted from calf thymocytes. This activity is compared with that of two lectins, concanavalin A (specific for glucose * and mannose) and soybean agglutinin (specific for *N*-acetylgalactosamine and galactose) [3]. We show that erythrocyte agglutination by lipids is indistinguishable in some respects from that caused by lectins; in particular, both hemagglutination reactions exhibit cell specificity and are inhibited by certain glycoproteins.

Materials and Methods

Erythrocytes. Fresh heparinized blood was obtained from healthy donors and used not more than 2 days after withdrawal. The cells were washed four times in saline, care being taken to remove the buffy coat. The washed erythrocytes were suspended in phosphate-buffered saline (0.02 M Na_2HPO_4/NaH_2PO_4, pH 7.4, containing 0.15 M NaCl and 0.1 g/l $CaCl_2$ and 0.1 g/l $MgCl_2$). Outdated blood (stored for a period of at least 3 weeks in citrate buffer) was washed and the erythrocytes suspended as above.

Trypsin treatment was performed using the twice-crystallized enzyme (Calbiochem, A grade, 1 mg/ml) and a 4% erythrocyte suspension in phosphate-buffered saline; incubation was for 1 h at 37°C. Treatment with neuraminidase (Sigma, from *Clostridium perfringens*, type VIII, 0.1 U/ml), was performed using a 10% erythrocyte suspension in phosphate-buffered saline for 30 min at 37°C. Fixation of fresh erythrocytes was performed with 1% (v/v) glutaraldehyde (Fluka) [4]. All treated cells were washed in saline five times, suspended in phosphate-buffered saline and used within 24 h.

Lipids and lectins. Oleic acid was purchased from Baker, palmitic acid from Fluka, bovine brain gangliosides and dipalmitoyl phosphatidic acid from Sigma, and dioleoyl phosphatidylcholine was a gift from Dr. Meir Shinitzky, Weizmann Institute of Science. Desialylation of gangliosides was carried out by hydrolysis with HCO_2H [5]. Dioleoyl phosphatidic acid was synthesized

* All sugars are of the D configuration unless otherwise noted.

[6], and purified on a column (7 × 1.25 cm) of silicic acid (Baker, 100 mesh) [7].

Calf thymocyte lipids were prepared as follows: a frozen thymus (200—300 g) was thawed and cut into small pieces in ice cold phosphate-buffered saline. The suspended thymocytes were washed in phosphate-buffered saline, collected by centrifugation (300 × *g*, 10 min) and extracted overnight in about 400 ml of $CHCl_3/CH_3OH$ (2 : 1, v/v) at 4°C. After evaporation of the solvent, the lipid residue (total weight from a representative thymus was 140 mg) was taken up in 5 ml $CHCl_3$, filtered twice through filter paper (Whatman No. 2) and loaded onto a column of silicic acid, as above. The column was eluted [7] and the acetone fraction (approx. 35 mg) which contains mainly glycolipids was further fractionated on preparative silica gel plates (Merck) using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 5, v/v) as solvent. The lipids were revealed with anisaldehyde [8] and the fraction with R_f 0—0.2 (weighing 8 mg) was eluted with CH_3OH, stored in $CHCl_3/CH_3OH$ (2 : 1, v/v) and used for agglutination studies. Chemical analysis of the fraction showed 1% (w/w) free amino acids, 2% peptides, 16% neutral sugars as measured according to the phenol sulfuric acid method [9] using glucose as standard, 0.36% phosphorus [10], and 2.7% nitrogen as lipid-soluble bases after hydrolysis with 6 M HCl for 3 h [11]. The fraction withstood 90 min boiling without any detectable loss of rat erythrocyte agglutinating activity.

Concanavalin A (crystallized three times, Miles Yeda) and soybean agglutinin (prepared in this laboratory [12]) were dissolved in phosphate-buffered saline to a concentration of 1 mg/ml (w/v). Lipids were dried in vials under an N_2 stream and then dispersed in phosphate-buffered saline by vigorous shaking to a final concentration of 1 mg/ml (w/v). The fatty acids were dispersed in phosphate-buffered saline by sonication for about 5 min, also to a concentration of 1 mg/ml.

Hemagglutination assays. The serial 2-fold dilution assay in microtiter plates (Greener, V form) was used with 50-μl aliquots of the agglutinins to which 50 μl of a 2% suspension of erythrocytes were added. Agglutination was scored after the plates were left standing at room temperature for 1 h. Agglutination tests with treated erythrocytes included controls employing untreated cells from the same batch. Each test was carried out twice with the same batch of both erythrocytes and agglutinin. The results were reproducible within one well.

Inhibition of agglutination by proteins. Bovine serum albumin (Sigma, fraction V) was defatted according to the method of Chen [13]. Commercial preparations of bovine submaxillary mucin (Sigma and Worthington) and fetuin (Sigma) were used. The proteins were dissolved in phosphate-buffered saline to a concentration of 1 mg/ml.

Effect of sugars on agglutination. The following sugars were tested at a concentration of 0.25 M for their effect on agglutination: glucose (BDH), galactose (BDH), L-fucose (Sigma), *N*-acetylgalactosamine (Pfanstiehl) and GlcNAcβ1 → 4GlcNAcβ1 → 4GlcNAc (prepared from chitin [14]).

Results

Agglutination of erythrocytes by lipids is readily observed on microscope slides (Fig. 1) as well as in microtiter plates (Fig. 2) and is indistinguishable from that mediated by lectins.

Rabbit and rat erythrocytes are agglutinated by dioleoyl phosphatidic acid, oleic acid and calf thymocyte lipids (Tables I and II), but are not agglutinated by dioleoyl phosphatidylcholine and palmitic acid. Dipalmitoyl phosphatidic acid agglutinates rabbit erythrocytes slightly (data not shown) but not rat erythrocytes. With dioleoyl phosphatidic acid, agglutination of rabbit erythrocytes is observed only within a limited range of concentrations (1.5—62 μg/ml). At concentrations higher than 62 μg/ml the cells are lysed; a similar effect of dioleoyl phosphatidic acid is apparent also with native rat erythrocytes. In contrast, with native cells, fixed rabbit cells are agglutinated by dioleoyl phosphatidylcholine (125 μg/ml).

Trypsin treatment increases considerably the agglutination of rabbit erythrocytes by oleic acid (Table I) and only slightly the agglutination by dioleoyl phosphatidic acid and calf thymocyte lipids. This contrasts with the well known increase after trypsin treatment of the hemagglutination of the same cells by concanavalin A and soybean agglutinin. Neuraminidase treatment of rabbit erythrocytes increases their agglutination by oleic acid but not by dioleoyl phosphatidic acid and calf thymocyte lipids (Table I). The same treatment increases agglutination of rabbit erythrocytes by the lectins but to a lesser extent than treatment with trypsin.

Rat erythrocytes are agglutinated by dioleoyl phosphatidic acid, oleic acid and calf thymocyte lipids, as they are by lectins (Table II). These cells are not agglutinated by dioleoyl phosphatidylcholine, palmitic acid and dipalmitoyl phosphatidic acid. It is not possible, however, to examine the

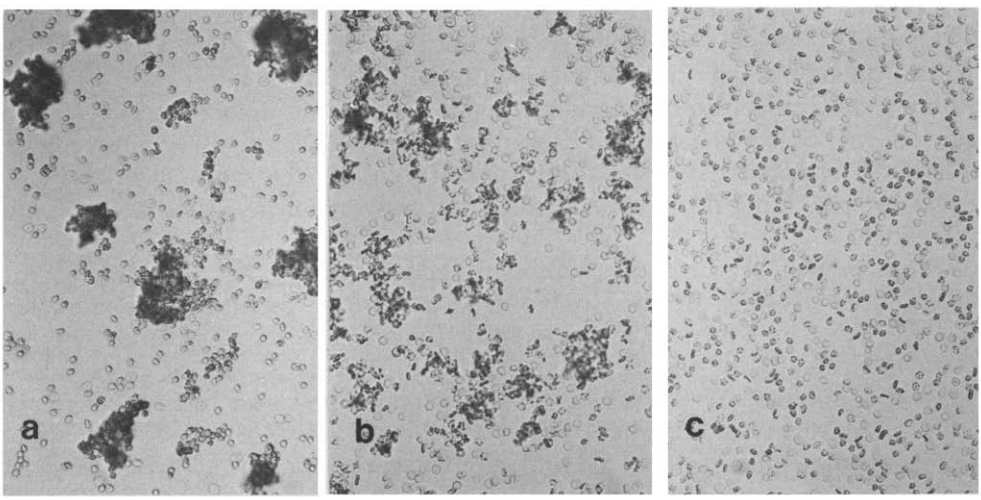

Fig. 1. Freshly drawn rat erythrocytes agglutinated by equal concentrations (250 μg/ml, w/v) of calf thymocyte lipids (a) and concanavalin A (b) by mixing erythrocyte suspensions on microscope slides for approx. 0.5 min. Control without agglutinin (c).

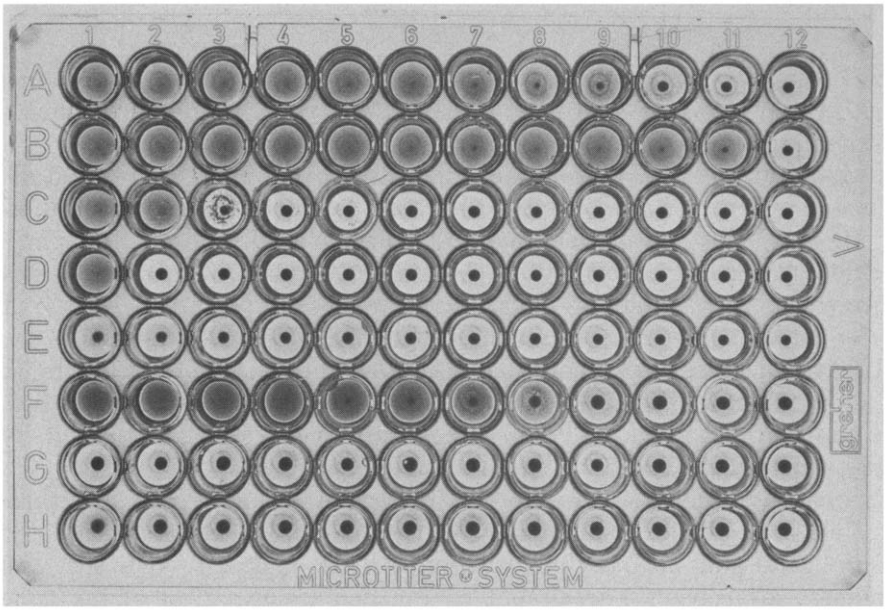

Fig. 2. Agglutination assay on microtiter plate: rat erythrocytes aged for 10 days (rows A—E) and neuraminidase-treated sheep erythrocytes (rows F—H). In each row, the first well contains 0.5 mg/ml of agglutinin which is diluted 2-fold in each successive well. The last well in each row does not contain agglutinin. Bovine submaxillary mucin (50 μg), is added to each well except the first in rows C and D. Agglutinins; *rows* A, C, F, *soybean agglutinin; rows* B, D, G, *calf thymocyte lipids; rows* E, H, *dioleoyl phosphatidylcholine.*

effect of enzymatic treatments and fixation on the agglutination of rat erythrocytes, since such treatments render the cells self-agglutinable. Both rat and rabbit erythrocytes are agglutinated by gangliosides (11 and 62 μg/ml, respectively) and less readily by desialylated gangliosides (180 and 500 μg/ml, respectively). Sheep erythrocytes are not agglutinated by any of the lipids tested, unless the cells have been fixed (Table I). With the latter cells, agglutination by dioleoyl phosphatidic acid occurs only within a narrow range of concentrations, as is the case with rabbit erythrocytes. Neither trypsin nor neuraminidase treatment of sheep erythrocytes renders the cells agglutinable by lipids (up to 0.5 mg/ml). Such treatments, however, enhance their agglutination by lectins.

Fresh human erythrocytes, although agglutinated by both lectins, are not agglutinated by any of the lipids tested even after fixation or modification by enzymes. In contrast to the freshly drawn erythrocytes, those obtained from outdated blood are agglutinated by as little as 30 μg/ml of oleic acid. The susceptibility of the other erythrocytes studied also increases with the time of storage; for example, about 32-times less calf thymocyte lipids are required to agglutinate rabbit erythrocytes stored for 2 weeks than to agglutinate fresh erythrocytes.

Inhibition studies

Bovine serum albumin (defatted), a protein devoid of covalently linked

TABLE I

AGGLUTINATION BY LECTINS AND LIPIDS OF ERYTHROCYTES BEFORE AND AFTER THEIR MODIFICATION

The maximal concentration used of lipids and lectins was 500 μg/ml. N.A., no agglutination.

Source of erythrocytes	Treatment	Minimal agglutinating concentration (μg/ml)				
		Concanavalin A	Soybean agglutinin	Dioleoyl phosphatidic acid	Oleic acid	Calf thymocyte lipids
Rabbit	None	4	4	1.5	180	22
	Fixation	1.5	0.5	10	N.A. *	N.A.
	Trypsin	$2 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	0.5	5.5	10
	Neuraminidase	0.5	2	1	31	31
Sheep	None	N.A.	500	N.A. **	N.A. *	N.A.
	Fixation	500	45	2	62	350
	Trypsin	8	62	N.A. ***	N.A. *	N.A.
	Neuraminidase	500	11	N.A. **	N.A. *	N.A.

* Lysis occurs above 250 μg/ml.
** Lysis occurs above 62 μg/ml.
*** Lysis occurs above 16 μg/ml.

sugars, effectively inhibits the agglutination of rabbit erythrocytes mediated by oleic acid. In contrast, agglutination by dioleoyl phosphatidic acid is only slightly inhibited by bovine serum albumin (Table II). No effect on agglutination by calf thymocyte lipids is observed. Lectin-mediated agglutination is only slightly inhibited by bovine serum albumin.

The inhibitory effect of glycoproteins on agglutination mediated by lipids was examined with bovine submaxillary mucin from two sources and with fetuin. Mucin (from Sigma) and fetuin substantially decrease agglutination

TABLE II

EFFECT OF A PROTEIN AND GLYCOPROTEINS ON AGGLUTINATION OF ERYTHROCYTES BY LECTINS AND LIPIDS

The maximal concentration used of lipids and lectins was 500 μg/ml. N.A., no agglutination.

Source of erythrocytes	Addition (0.5 mg/ml)	Minimal agglutinating concentrations (μg/ml)				
		Concanavalin A	Soybean agglutinin	Dioleoyl phosphatidic acid	Oleic acid	Calf thymocyte lipids
Rabbit	None	4	1	1	22	44
	Bovine serum albumin	11	1	3	500	44
	Bovine submaxillary mucin	31	62	N.A. *	500	500
	Fetuin			N.A. *	500	500
Rat	None	5.5	62	0.5	62	62
	Bovine serum albumin	11	88	2	350	31
	Bovine submaxillary mucin	62	500	N.A. **	500	500
	Fetuin			N.A. **	500	350

* Lysis occurs above 31 μg/ml.
** Lysis occurs above 125 μg/ml.

of rabbit and rat erythrocytes by dioleoyl phosphatidic acid, oleic acid and calf thymocyte lipids (Table II). Submaxillary mucin from Worthington gave the same inhibitory activity as that from Sigma (results not shown). As expected, lectin-mediated agglutination is also inhibited by the glycoproteins (Table II).

None of the sugars tested, at concentrations up to 0.25 M, inhibit lipid-mediated hemagglutination.

Discussion

In the course of our attempts to isolate lectins from lymphocyte membranes, it was found that crude Triton X-100 extracts from whole calf and rat thymocytes and from human peripheral blood lymphocytes, as well as similar extracts from membrane preparations of calf thymocytes, agglutinate rabbit and rat erythrocytes. The hemagglutinating activity was resistant to boiling for prolonged periods of time (2 h), suggesting that the agglutinins are not proteins. Because of their ready availability, calf thymocytes were used for further work. Purification of the agglutinins from calf thymocyte extracts has indicated that they are in fact polar lipids (see Materials and Methods). Different well defined amphipathic lipids were therefore tested for agglutinating activity.

The finding that both oleic acid and its acidic phosphatidyl derivative agglutinate rat and rabbit erythrocytes, whereas palmitic acid and its acidic phosphatidyl derivative do not, indicates that unsaturation of the fatty acid constituent of the lipid is required for the hemagglutinating activity. Another factor contributing to this activity of the lipids appears to be their acidity. Among the lipids examined, the acidic dioleoyl phosphatide is the most active agglutinin, both with native and treated erythrocytes (Tables I and II). On the other hand, the positively charged dioleoyl phosphatidylcholine does not agglutinate any of the untreated erythrocytes tested, although it agglutinates fixed rabbit erythrocytes. The contrast between the properties of dioleoyl phosphatidylcholine and the acidic phosphatide derivative of oleic acid is also manifested in the inability of the former to agglutinate fixed sheep erythrocytes. Consideration of these data leads to the conclusion that in glycerophospholipids, both the hydrophobic moiety and the hydrophilic head group determine the hemagglutinating properties of the lipid.

Mode of action of lipids as hemagglutinins

Oleic acid and other lipids have been reported to induce cell fusion in avian as well as in mammalian cells [15]. It was assumed that intercellular adhesion is an early event in the process leading to cell fusion. In addition to fusion by lipids, agglutination of erythrocytes by a non-fusogenic derivative of oleic acids (glyceryl dioleate) was also reported [15]. There are many similarities between lipid-induced cell fusion and lipid-mediated agglutination of native erythrocytes. Our results on the agglutinating activity of fatty acids parallels the finding that the unsaturated oleic acid is fusogenic while the saturated palmitic acid is not [15]. It was also reported that in contrast to negatively charged lipid vesicles which are fusogenic, vesicles composed of

phosphatidylcholine do not induce cell fusion [16]. The fusion of pure lipid structures was recently shown to depend on negative charges of the lipids [17], and we too find that dioleoyl phosphatidylcholine does not agglutinate non-fixed cells. The agglutinating activity of gangliosides and its decrease upon desialylation agrees with the requirement for a negatively charged hydrophilic head group. However, the decrease may also be ascribed to a decrease in solubility of the lipid.

It was assumed that lipids induce cell fusion by incorporation into the lipid bilayer of the outer membrane [18]. The fusogenic lipid destabilizes the membrane [18,19] and causes formation of protein-free patches which can interact with apposing patches of lipids free of protein [18—21], to merge and form a complex multilamellar lipid membrane [18] with ensuing cell adhesion. As the process continues, the cells eventually fuse.

Another mechanism by which lipids may agglutinate cells is through interaction of the dispersed lipid with the membranal proteins. Vesicles may thus join together apposing cells and form cell aggregates. It has indeed been suggested that proteins in solution may interact with dispersed phospholipids via the negative charges of the latter [22]. Such a mechanism may account for the agglutination of fixed cells as observed in this study, since these cells cannot undergo fusion because of the cross-links between their membranal proteins and perhaps even between lipids containing amine groups.

Effect of trypsin and neuraminidase treatment

The enhancing effect of erythrocyte trypsinization on hemagglutination by lipids is much less pronounced than with lectins, but is nevertheless significant with oleic acid (Table I). This enhancement may be due to removal of negative charges from the cell surface and a decrease in the amount of proteins that protrude from the surface and impede contact formation between the lipids of the membranes [23]. It has been suggested that the removal of repelling electrical charges enables formation of close contacts between cells, constituting an essential step in cell fusion [24]. The reason for the lesser effect (with oleic acid and phosphatidic acid) or lack of effect (with calf thymocyte lipids) of neuraminidase treatment as compared to trypsin treatment is not clear.

Protein and glycoprotein inhibition of agglutination

Defatted serum albumin was reported to inhibit cell fusion induced by oleic acid by virtue of its trapping of lipids [15]. The reason for the pronounced inhibitory activity of submaxillary mucin and fetuin on agglutination by lipids may be similar to that of serum albumin, although direct evidence for the binding of lipids to these glycoproteins is not available.

Reports in the literature describing lectins, the hemagglutinating action of which is not inhibitable by sugars, are not infrequent [25,26]. Recently, Meade et al. [27] found a plant agglutinin which is not a protein and is not inhibited by simple sugars. Our findings suggest that the presence of strong hemagglutinating activity in crude extracts from biological material which is inhibited by glycoproteins but not by simple sugars cannot be taken as proof of the existence of lectins in the extracts.

Specificity of action on different species of erythrocytes

Under the experimental conditions used, susceptibility to agglutination by lipids is clearly species dependent. This specificity is not changed by enzymatic treatment of the cells, in contrast to agglutination by lectins (Table II). Glutaraldehyde fixation of the erythrocytes, however, induces a change in specificity of the action of lipids in that it diminishes or completely abolishes agglutination of rabbit and rat erythrocytes but induces agglutinability of the otherwise unagglutinable sheep erythrocytes.

The specificity of action of lipid-mediated hemagglutination may be indicative of a role for the lipids in determining specific interactions between cells in vivo. Such interaction may be controlled by changes in lipid composition of cell membranes, or by release of lipids into the medium in which the cells are suspended. From a practical point of view, lipids may be useful to separate different populations of cells, as are lectins [28].

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