INTERACTION BETWEEN VESICLES CONTAINING GANGLIOSIDES AND LECTINS

Limulin and wheat-germ agglutinin

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1. Introduction

Limulin (*Limulus polyphemus* agglutinin) is a lectin whose agglutinating and mitogenic properties are inhibited by sialyl-containing glycans [1,2]. Because limulin agglutinates preferentially horse erythrocytes and since these bear an unusually high level of sialoglycolipids [3], we were interested to study the interaction between limulin and sialoglycolipids. Furthermore, because it has been claimed [4–8] that wheat-germ agglutinin (WGA) is able to bind specifically sialic acids, we investigated also the activity of this lectin.

2. Materials and methods

Bovine submaxillary mucin, bovine brain gangliosides type III were purchased from Sigma, sialyllactose from Boehringer and fetuin from Koch-Light. Thyroglobulin and wheat-germ agglutinin [9] were supplied by Industries Biologiques Françaises, Gennevilliers, France. Pronase was purchased from Calbiochem. Limulin, the pure agglutinin of *Limulus polyphemus*, was obtained by affinity chromatography as previously described [1]. Cholesterol from Prolabo was recrystallized twice from methanol. Phosphatidylcholine was isolated from egg yolk according to Singleton et al. [10].

Membrane fractions of horse erythrocytes were prepared as described by Yamakawa and Suzuki [11]. Horse erythrocyte glycopeptides were isolated by gel filtration after three pronase digestions [12] of the membrane fractions. Crude extracts of gangliosides were obtained by Svennerholm's method [13], as modified by Weinstein et al. [14] from membrane fraction (HEGN) or from the insoluble pellet after pronase digestion of membrane fractions (HEGP).

Phospholipid content of vesicles was determined as lipid bound phosphorus by the method of Chen et al. [15], cholesterol by the method of Kingsley and Schaffert [16], and sialic acids (NANA) by Aminoff's method [17] after acid hydrolysis (0.1 N H_2SO_4, 60 min, 80°C). As pure phospholipids gave a color with Aminoff's method, the actual concentrations of sialic acids were calculated after substrating the contribution of phospholipids.

2.1. *Preparation of vesicles*

Liposomes were generated by dispersing the dry lipids in buffer solution (0.1 M NaCl, 0.05 M Tris–HCl, pH 8.5) followed by sonication [18] for 10 min under pure ntirogen. The liposome suspension was further centrifuged at 120 000 × *g* for 60 min. Vesicles were isolated after gel filtration on a Sepharose 4B column (2 × 40 cm). The homogeneity of vesicles was checked by electron microscopy [18], by Dr J. Schrevel, Poitiers. The concentration of vesicles in suspension was adjusted to 1 mg phospholipid/ml. The molar ratio of phosphatidylcholine and cholesterol was 4:1.

2.2. *Vesicle aggregation*

The turbidity of vesicle dispersion kept at 30°C was measured at 500 nm, i.e., about 20 times the vesicle diameter. The NANA:lipid–P molar ratios

were varied in the range 0.012–0.125. The lectin solution was added directly to the vesicle dispersion in quartz cells. For studies with Limulin, $CaCl_2$ was added to 0.01 M final concentration.

2.3. *Inhibition of erythrocyte agglutination*

Hemagglutination inhibition experiments were done in the conditions previously described [19] using 3% horse erythrocyte suspension in 0.1 M NaCl, 0.05 M $CaCl_2$.

3. Results

3.1. *Preparation and stability of vesicles*

The liposome preparation was a mixture of multilamellar and unilamellar vesicles. The small proportion (<10%) of multilamellar liposomes was removed by gel filtration. The vesicles containing phospholipids and cholesterol appeared to be quite stable. No increase of turbidity was observed during the first ten hours after isolation from the gel filtration column. Vesicles containing gangliosides exhibited a similar behavior.

3.2. *Inhibition of horse erythrocyte agglutination by vesicles containing gangliosides*

The vesicles containing brain gangliosides were not able to inhibit the action of limulin or concanavalin A but inhibited the action of WGA (table 1). Vesicles containing horse erythrocyte gangliosides (HEGN) strongly inhibited the effect of limulin and WGA. With limulin, the activity also depended on the ganglioside/phospholipid ratio. Vesicles containing HEGP had an inhibitory power as high as the HEGN-vesicles towards limulin but had not activity towards WGA, although the sialic acid content was identical in both preparations.

3.3. *Aggregation of vesicles containing gangliosides by lectins*

Absorbance of a dispersion of vesicles containing horse erythrocyte gangliosides (HEGN) increased upon addition of limulin (fig.1). The enhancement of absorbance did not follow simple kinetics (fig.2). The function log $[(A_\infty - A_t)/(A_\infty - A_0)]$ versus t is at least bimodal, a fast process at first and then a much slower one (A_∞ and A_t are the absorbance of the dispersion at times $t = \infty$ and t). The slow process

Table 1
Inhibition of horse erythrocyte agglutination by vesicles containing gangliosides [a]

Vesicles NANA/phospholipid ratio (mol/mol)	Concentration of gangliosides required to inhibit one unit of agglutination (expressed as sialic acid μM)	
	Limulin	WGA
Brain gangliosides		
0.05	> 2000 (d)	160
HEGN [b]		
0.125	50	n.d.
0.09	50	20
0.05	130	20
HEGP[c]		
0.09	60	> 240[d]

[a] In each experiment, the phospholipid concentration was 1 mg/ml. Vesicles without gangliosides had no inhibitory effect
[b] HEGN, horse erythrocyte glycolipids extracted from stroma
[c] HEGP, horse erythrocyte glycolipids extracted from stroma after three pronase digestions
[d] No inhibition at that concentration

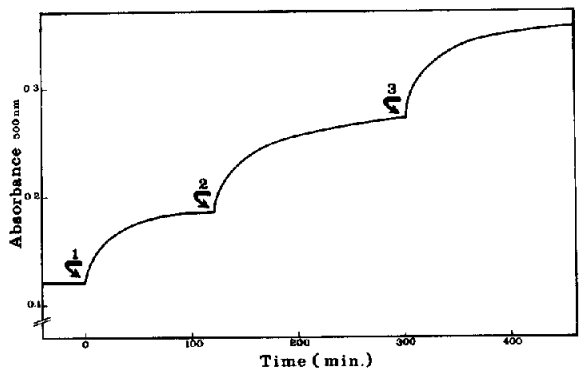

Fig.1. Change in turbidity at 500 nm of the vesicle suspension after addition of limulin. Final limulin concentration: 14 μg/ml (arrow 1), 27 μg/ml (arrow 2) and 38.5 μg/ml (arrow 3). The NANA:phosphatidylcholine molar ratio was R 0.125. The absorbance of a sample containing vesicles and lectin was compared to that of a reference containing only vesicles.

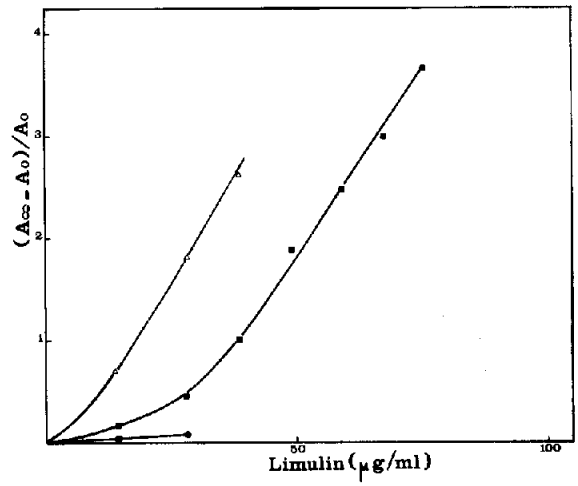

Fig.3. Maximal turbidity of vesicles containing HEGN gangliosides upon addition of limulin. Each point is the calculated value at time $t = \infty$. (—△—) R 0.125, (—■—) R 0.09, (—●—) R 0.0125.

appeared to be a pseudo-first order phenomenon. A rate constant of 2×10^{-4} s^{-1} was calculated, for R 0.125. The maximal increase of turbidity for a constant ganglioside concentration was dependent on lectin concentration. For a constant lectin concentration, it was dependent on ganglioside concentration (fig.3). No increase of absorbance was obtained when limulin was added to a suspension of vesicles free of gangliosides or to a suspension of vesicles containing mixed bovine brain gangliosides. Vesicles containing erythrocyte gangliosides from stromas treated with pronase (HEGP) displayed an increase of absorbance upon addition of limulin, as vesicles containing HEGN did.

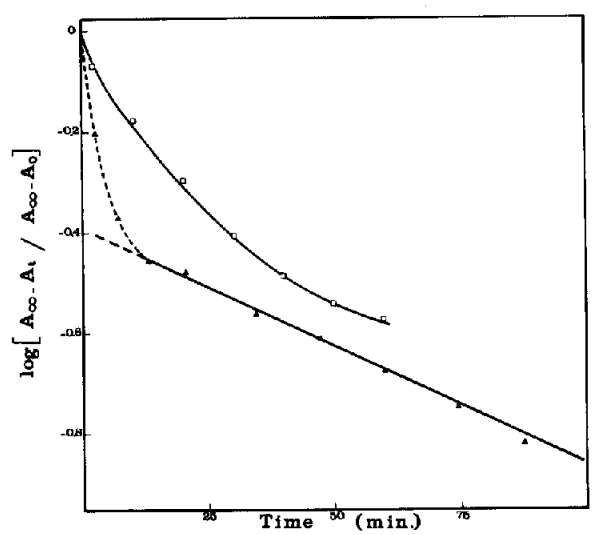

Fig.2. Kinetics of aggregation of vesicles containing HEGN gangliosides, after addition of lectins: plot of log $[(A_\infty - A_t)/(A_\infty - A_0)]$ versus time. (—□—) WGA (10.5 μg/ml); (—▲—) limulin (14 μg/ml); R 0.05.

Limulin I (1–200 μg/ml) which has many molecular properties similar to those of the *Limulus polyphemus* lectin except the binding to sialylglyco-conjugates [1] did not increase the turbidity.

Concanavalian A (8–25 μg/ml) had no effect on vesicles free of gangliosides or on vesicles HEGN or HEGP. Conversely, wheat-germ agglutinin (up to a concentration of 30 μg/ml) had no effect on vesicles free of gangliosides nor on vesicles containing mixed brain gangliosides but had a very marked effect on vesicles containing HEGN. WGA had no effect on vesicles containing HEGP. This behaviour contrasted with that of limulin which increased the absorbance of vesicles containing HEGP as well as of vesicles containing HEGN.

3.4. *Reversibility of the aggregation process*

When aggregation occurred, addition of specific inhibitors reversed the process (fig.4). Ethylene diamine

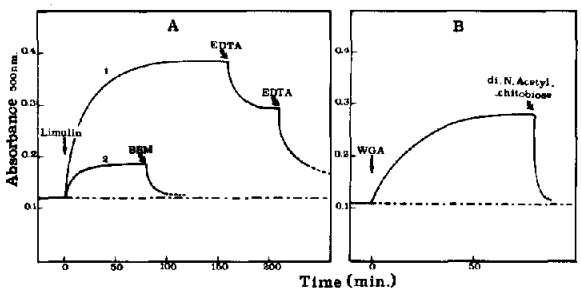

Fig.4. Reversibility of vesicles aggregation. (A) Limulin: Curve 1; *R* 0.125, the limulin concentration was 38.5 μg/ml, concentration of EDTA was 0.01 M and 0.02 M. Curve 2; by submaxillary mucin (BSM) *R* 0.05, the limulin concentration was 27 μg/ml. The BSM concentration was 30 μg/ml (10 μM expressed as sialic acid). (B) Wheat-germ agglutinin; R 0.05, the WGA concentration was 105 μg/ml, the acetyl chitobiose concentration was 100 μg/ml.

tetracetate, which is a strong inhibitor of limulin, since it requires Ca^{2+} for activity [1], had a very marked effect on the aggregation process. Bovine submaxillary mucin was also able to reverse the aggregation effect of limulin. Similar results were obtained when di-*N*-acetylchitobiose was used to reverse the effect of WGA (fig.4B).

4. Discussion

Early work with hemolymph of *Limulus polyphemus* showed that the agglutinability of erythrocytes was very dependent on the species from which the erythrocytes were obtained. These differences could be related to the total sialic acid content or to the sialoglycolipid content (table 2).

The direct demonstration of a specific interaction between limulin and sialoglycolipid could not be obtained by conventional hemagglutination inhibition experiments, for sialoglycolipid dispersions were taken up by cell membranes and agglutination occurred with lower concentrations of lectins . However, when sialo-gangliosides were incorporated in phospholipid/cholesterol vesicles, they inhibited erythrocytes agglutination. This inhibition can be related to some extent to the concentration of sialic acids, but as previously stated [1], the specific binding of limulin involved both the molecule of *N*-acyl neuraminic acid and the sugar residues on which NANA is covalently bound (*N*-acetylgalactosamine, for instance). This may explain the lack of effect displayed by brain gangliosides.

It is noteworthy that the inhibition of the agglutination capacity of WGA was not related to that of limulin. When gangliosides were extracted after an exhaustive treatment with pronase, limunin was inhibited while WGA was not. These results demonstrate that WGA does not bind the sialo-glycolipids which are specific of limulin. These findings were emphasised by hemagglutination inhibition experiments using native bovine submaxillary mucin (native BSM) and desialylated mucin (asialo-BSM).

Table 2
Agglutination titers and sialic acid content of erythrocytes of some animals

	Agglutination titers		Molecules of sialic acids/cells surface[c]	%Sialic acids in glycolipids[d]	
	Limulin[a]	WGA[b]		e	f
Horse	1/2000	1/64	5.9×10^6	0	20
Cat	1/2000	–	–	3	38
Man	1/128	1/64	24×10^6	0	0
Sheep	1/16	–	–	0	0
Rabbit	1/4	1/256	–	0	0

[a] Initial limulin III concentration, 20 μg/ml
[b] Initial WGA concentration, 1 mg/ml
[c] From Eylar et al. [20]
[d] From Yamakawa et al. [3]
[e] Glycolipids extracted with methanol/ether
[f] Glycolipids extracted with chloroform/methanol

Native-BSM (10 μg/ml) inhibited agglutination by limulin but asialo-BSM (500 μg/ml) had no inhibitory effect. Conversely, native-BSM and asialo-BSM (30 μg/ml) had the same inhibitory effect on agglutination induced by WGA.

The experiments on aggregation of vesicles containing gangliosides were carried out with lectins in concentrations ranges (0–50 μg/ml) which are used to agglutinate cells. The concentrations of glycolipid-sialic acids were between 10–100 μM. The total concentration of bound sialic acids in a 3% suspension of horse erythrocytes is about 40 μM. Even with these minimal conditions, the effect of limulin was quite important (fig.1). In this lectin concentration range (0.05–1 μM) no aggregation occurred with vesicles free of glycolipids. These conditions gave clear-cut results, and allowed kinetic studies. At least, two processes occurred with low concentration of limulin and of WGA; the first step with limulin was much faster than with WGA. These results contrast with those obtained by Surolia et al. [21] using liposomes containing GM_1 and RCA_1 (*Ricinus communis* agglutinin) when only one step could be detected. Surolia et al. worked with liposomes containing only one type of ganglioside, while we worked with a mixture of gangliosides; then the two processes, we found, could be related to the presence of two types of ligands with high and low affinities.

The increase of turbidity upon addition of limulin or WGA could be reversed by their specific ligands. Similar results were obtained by Rendi et al. [22] using rabbit erythrocyte lipids and WGA or SBA. This reversibility supports strongly a mechanism of interaction similar to the agglutination process but disagrees with a fusion mechanism as described by several authors using high concentrations of lectins [22–25].

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