LECTINS IN LYMPHOCYTE MEMBRANES

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1. Introduction

Sugar binding and cell agglutinating proteins known as lectins have been extensively studied recently (reviewed [1]). Although lectins have been mostly isolated from plant seeds, they are also present in other parts of plants [2,3], in fishes [4], in birds [5,6] and in mammals [4,7–9]. In an increasing number of cases, it has been shown that lectins are present in membranes so it has been proposed that lectins may be membrane components, whose capacity to reversibly bind carbohydrates could enable them to play a role in both intracellular and intercellular recognition [2,8–12]. In this report, we present data showing that lectins are found in lymphocyte membranes.

2. Materials and methods

Lymphocytes isolated from thymuses and spleens of ~ week 6 male and female BALB/c mice by the method in [13] were broken by nitrogen cavitation using a French press (Artisan Metal Products, Waltham, MA) with 50 atm. for 15 min at 0°C with constant gently stirring. A microsomal membrane fraction was prepared at 4°C from the homogenate by differential centrifugation as follows: cell debris were removed by centrifugation at 850 × *g*, for 10 min and large particles and mitochondria by centrifugation of 10 000 × *g* for 20 min. The supernatant was further centrifuged at 150 000 × *g* for 90 min (SW 50 rotor type, Spinco ultracentrifuge) to give a microsomal pellet and freely soluble cytoplasmic components. The microsomal pellet was either sonicated (MSE sonicator) (10 s × 5) in phosphate buffered saline (0.15 M NaCl, 0.01 M sodium phosphate (pH 7.4) PBS) to give a suspension of membrane vesicles, or extracted in a variety of ways to determine how the lectin was attached to the membrane. These included:

(i) Sonication in high ionic strength buffer (18 mM NaCl in Hanks medium).
(ii) Sonication in low ionic strength buffer (1 mM $MgCl_2$, 1 mM Tris–HCl, pH 8.0).
(iii) Sonication in 0.5% Triton X-100, 0.5% dimethyldodecylglycine (Empigen BB) or 0.1% deoxycholate.

In addition, *n*-butanol–acetone powder was prepared from the microsomal pellets according to [14]. The acetone powder obtained from about 2×10^9 thymocytes or splenocytes was suspended in 1 ml 0.1% Triton X-100, 4 mM mercapto-2-ethanol in PBS and homogenized by sonication at 4°C (30 s × 10). The suspension was then kept for 1 h at 4°C, centrifuged and the supernatant tested for agglutinating activity.

2.1. *Protein determination*

Protein was determined according to [15], using bovine serum albumin (Pentex, fraction V) as standard.

2.2. *Agglutination assays*

The assays were routinely performed with a 3% erythrocytes suspension in PBS or in PBS containing 100 μg bovine serum albumin/ml, or with lymphocyte suspensions (10^7 cell/ml) from thymus or spleen. Trypsinization was done according to [16] using

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2 X crystallized trypsin (Calbiochem). In some cases, the cells were treated with *Vibrio cholerae* neuraminidase (Calbiochem) [13,17]. To 2-fold serial dilutions of 50 μl extracts in microtiter U-plates (Cooke Engineering) were added 50 μl aliquots of a cell suspension. After 1 h incubation at room temperature the degree of agglutination was assessed using a serological scale (0+ to 4+) according to [18]. One agglutinating or hemagglutinating unit is the amount of material causing half-maximal agglutination of the cells.

The agglutinating activity was also tested with glutaraldehyde-fixed cells (native or enzyme treated). For this purpose, the cells were suspended in glutaraldehyde (Ladd Research Industries, Burlington, Vermont; 0.1% in PBS). After incubation (1 h, 25°C) the cells were washed twice with 5 vol. 0.1 M L-lysine in PBS (pH 7.4) and twice with PBS.

2.3. *Inhibition of hemagglutination assays*

The inhibition tests were performed with two hemagglutinating units as in [19]. The inhibitory activity is given in terms of concentration of inhibitor needed to inhibit one hemagglutinating unit of lectin. The following glycoproteins were tested as inhibitors: bovine submaxillary mucin (BSM); fetuin; porcine thyroglobulin; human transferrin (obtained from Miles); and their asialo derivatives: asialo-BSM; asialofetuin; asialothyroglobulin; asialotransferrin. The glycoproteins were desialylated by mild acid hydrolysis (H_2SO_4 0.1 N, 80°C, 1 h). Sugars and oligosaccharides used were obtained from Sigma. ε-Aminocaproyl α- and β-D-galactopyranosylamines were a gift of I. Jacobson from this Department. The glycopeptides from asialothyroglobulin: asialoglycopeptide B [20] and the mannose-rich glycopeptide A [21] were prepared by exhaustive pronase (Calbiochem) digestion of asialothyroglobulin [22]. These glycopeptides were separated on a column of Sepharose 4B (Pharmacia) substituted with peanut agglutinin [23] on which the asialoglycopeptide B was retained while the glycopeptide A was not.

2.4. *Adsorption of lectins on glutaraldehyde-fixed lymphocytes*

Lymphocytes from mouse spleens and thymus were fixed by glutaraldehyde as described above. The glutaraldehyde-fixed splenocytes and thymocytes (2×10^8 cells) were incubated with 2 ml extract for 1 h at room temperature with gently shaking. The cells were collected by centrifugation (3000 rev./min, 10 min) and the supernatants tested for hemagglutinating activity against rabbit erythrocytes. The cells were then incubated for 1 h in a sugar solution to release the sugar specific proteins bound to the surface of the lymphocytes. The following sugars (0.1 M in PBS, 1 ml) were used: D-galactose, *N*-acetyl-D-galactosamine, lactose, D-mannose, methyl α-D-mannoside, *N*-acetyl-D-glucosamine and D-glucose. The cells were removed, and after extensive dialysis (12 h X 2 against 4 l PBS) and vacuum dialysis to concentrate the eluates to 1 ml, the hemagglutinating activity of each sample was tested.

3. Results and discussion

3.1. *Localization of the lectin activity*

Lectin activity is not found in the cytoplasmic fraction but is associated with the membrane vesicles. Solubilization of hemagglutinating activity from the membranes is only possible with the aid of Triton X-100 as a detergent which does not inactivate this activity. Sonication of the vesicles in high salt or low salt does not release any significant activity to the supernatants, nor do detergents as deoxycholate or empigen BB.

In a typical experiment the Triton extracts of different membrane preparations obtained from 20 mice, had the following titers (in hemagglutinating units/50 μl extracts): with untreated rabbit erythrocytes: thymocyte extracts (320 μg protein/ml) using microsomal pellets, 128 hemagglutinating units; using acetone powder, 512 hemagglutinating units; splenocyte extracts (400 μg protein/ml) using microsomal pellets, 64 hemagglutinating units; using butanol–acetone powder, 128 hemagglutinating units. The lectins described here are not related to LETS, since they are readily solubilized by Triton X-100, in contrast to LETS [24] nor are they extracted from the cell surface with urea [25].

3.2. *Cell specificity of the lectins*

The active Triton extracts from microsomal pellets and from butanol–acetone powders of thymocytes (320 μg protein/ml) and of splenocytes (400 μg protein/ml) were tested on native and enzyme-treated

Table 1
Hemagglutinating activity of extracts towards various types of erythrocytes (values in hemagglutinating units/50 μl extracts)[a]

Extracts	Treatment of erythrocytes	Erythrocyte source		
		Rabbit II	Rat II	Mouse I
Thymus	Native	512	128	32
Spleen		128	32	16
Thymus	Trypsin	1024	128	64
Spleen		512	32	32
Thymus	Neuraminidase	512	1024	n.d.[b]
Spleen		128	64	n.d.
Thymus	Neuraminidase + galactose oxidase	64	64	n.d.
		32	16	n.d.

[a] No agglutination was observed with human erythrocytes from A, B, or O red blood cell groups, either before or after their treatment with enzymes
[b] n.d., not determined

Triton extracts from microsomes (I) and butanol–acetone powders (II) of thymocytes (protein 16 μg/50 μl) and of splenocytes (protein 20 μg/50 μl)

Table 2
Agglutinating activity of membrane extracts towards mouse thymocytes and splenocytes (10^7 cell/ml) native and glutaraldehyde-treated (values in agglutinating units/50 μl extract

Cells tested	Agglutinating activity in extracts of			
	Thymocytes		Splenocytes	
	I	II	I	II
Native thymocytes	32	64	4	8
Glutaraldehyde-fixed thymocytes	2	2	0	0
Native splenocytes	8	8	32	32
Glutaraldehyde-fixed splenocytes	0	0	2	2

Triton X-100 extracts from microsomes (I) and acetone powders (II) of thymocytes (protein 16 μg/50 μl) and of splenocytes (protein 20 μg/50 μl)

erythrocytes from different animals. In any cases, the Triton extracts from butanol–acetone powders had a twice-higher activity than the Triton extracts from microsomal pellets and the specificity of agglutination was the same. The results summarized in table 1 show that trypsinized rabbit erythrocytes and neuraminidase-treated rat erythrocytes are the most easily agglutinated by the extracts. No activity is observed with human erythrocytes either before or after enzymatic treatments and a very low activity is found towards mouse erythrocytes with extracts from microsomal pellets. Neuraminidase treatment of rat erythrocytes strongly enhances their agglutination by the thymocyte extracts and to a lesser extent with the splenocyte extracts. Thus the extracts probably contain a lectin specific for asialoglycoproteins. This assumption is supported by the finding that galactose oxidase treatment of the neuraminidase-treated rat and rabbit erythrocytes markedly reduced their agglutinability by the extracts. The agglutinating activity was also tested with mouse thymocytes and splenocytes. Table 2 shows that extracts from spleen cells are more active on splenocytes and extracts from thymus cells are more active on thymocytes and that, in both cases, glutaraldehyde-fixed splenocytes and thymocytes are not (or very weakly) agglutinated by the extracts.

3.3. *Inhibition of agglutination*

D-Galactosides, *N*-acetyl-D-galactosamine and methyl α-D-mannoside are inhibitory, in most cases at 0.1–0.15 M (table 3). Other simple sugars tested are not inhibitory even at 0.2 M. Glycoproteins are much more inhibitory and their activity is greatly enhanced after desialylation. However, porcine thyroglobulin is very active even before desialylation (table 3). This result is not surprising for thyroglobulin contains two types of carbohydrate moieties: mannose-rich glycopeptides and complex glycopeptides. The complex glycopeptides contain two chains with a NANA–Gal–GlcNAc sequence and one chain with a Gal–GlcNAc sequence [26].

These results suggest that the lectin activity in the lymphocyte extracts is specific for glycoconjugates containing galactose in a terminal non-reducing position. Furthermore, mannose-rich glycopeptides and asialocomplex glycopeptides from porcine thyroglobulin inhibit hemagglutination only poorly when they are tested separately and are strong inhibitors when they they are used together. These findings suggest that two types of lectins are present in the extracts; one binding complex glycopeptides with terminal β-galactosides and another one binding

Table 3
Inhibition of hemagglutination of rabbit red blood cells with Triton X-100 extracts of butanol–acetone powder from mouse thymocytes and splenocytes by simple sugars, oligosaccharides and glycoproteins[a]

Inhibitor	Minimal inhibitor conc. with extract of	
	Thymocytes	Splenocytes
Lactose	100 mM	150 mM
Methyl α-D-galactoside	100 mM	150 mM
Methyl β-D-galactoside	100 mM	150 mM
Methyl α-D-mannoside	150 mM	100 mM
N-acetyl-D-galactosamine	150 mM	100 mM
ε-Aminocaproyl α-D-galactopyranosylamine	12.5 mM	50 mM
ε-Aminocaproyl β-D-galactopyranosylamine	100 mM	100 mM
Lactose + methyl α-D-mannoside (1:1)	5.0 mM	10 mM
Fetuin	1.25 mg/ml	3.50 mg/ml
Asialofetuin	0.04 mg/ml	0.30 mg/ml
Bovine submaxillary mucin	1.25 mg/ml	2.50 mg/ml
Asialo-bovine submaxillary mucin	0.02 mg/ml	0.02 mg/ml
Thyroglobulin (porcine)	0.08 mg/ml	0.04 mg/ml
Asialothyroglobulin	0.02 mg/ml	0.02 mg/ml
Transferrin (human)	2.50 mg/ml	2.50 mg/ml
Asialotransferrin	0.30 mg/ml	0.60 mg/ml
Thyroglobulin mannose-rich glycopeptide A	9.0 mg/ml	4.0 mg/ml
Asialothyroglobulin glycopeptide B	2.0 mg/ml	3.0 mg/ml
Glycopeptides A + B (1:1)	0.2 mg/ml	0.2 mg/ml

[a] No inhibition is observed with D-glucose, D-galactose, D-mannose and *N*-acetyl-D-glucosamine up to 200 mM

Minimal inhibitor concentrations required to inhibit one hemagglutinating unit

mannose-rich glycopeptides. This is also supported by the observation that lactose and methyl α-D-mannopyranoside were more active when they were used as a mixture than when they were independently used. For example the minimal concentrations of methyl α-D-mannopyranoside and of lactose required to inhibit one hemagglutinating unit are 100 mM and 150 mM, respectively, while the mixture of these two sugars is inhibitory at a much lower concentration (5–10 mM, combined). This synergistic action may indicate the presence of a lectin with two combining sites, for both galactose and mannose, that interact with each other in some unknown way, perhaps by being adjacent or overlapping. In addition to the lectin specificities towards α-D-mannosides and β-D-galactosides, a specificity towards α-D-galactoside and (or) *N*-acetyl-D-galactosaminide was suggested by the inhibitory activity of asialo bovine submaxillary mucin, free *N*-acetyl-D-galactosamine and ε-aminocaproyl α-D-galactopyranosylamine.

In all cases, the concentrations of glycoproteins required to inhibit cell agglutination were much lower than those of simple sugars or free glycopeptides, even when they were used as a mixture. This result is related to the increase of the apparent binding constant of a ligand upon its oligomerization [27]. For example, the concentration of a *p*-nitrophenyl glycoside required to inhibit cell agglutination by a lectin is several orders of magnitude higher than the sugar concentration of serum albumin substituted with the same glycoside (Monsigny and Delmotte, unpublished data).

3.4. *Adsorption of lectins on glutaraldehyde-fixed lymphocytes*

Incubation of 2×10^8 glutaraldehyde-fixed thymocytes and splenocytes with 2 ml butanol–acetone powder extracts of thymocytes (320 μg protein/ml,

Table 4
Sequential elution of lectins adsorbed on glutaraldehyde-treated lymphocytes

Sugars	Hemagglutinating units in extracts of	
	Thymocytes	Splenocytes
D-Galactose	256	8
Lactose	512	32
Methyl α-D-mannoside	16	128
N-Acetyl-D-glucosamine	32	32
N-Acetyl-D-galactosamine	128	64
D-Glucose	0	0

Triton X-100 extracts of butanol–acetone powders from thymocytes (640 μg protein in 2 ml, 512 hemagglutinating units/50 μl) and from splenocytes (800 μg protein in 2 ml, 128 hemagglutinating units/50 μl) were adsorbed by glutaraldehyde-treated thymocytes and splenocytes (2×10^8 cells), respectively. Sequential elution was carried out by the use of 1 ml PBS containing one of the listed sugars (sugar 0.1 M). Eluted fractions were dialyzed against sugar-free buffer, concentrated to 1 ml and 50 μl were assayed for agglutinating activities using rabbit red blood cells

512 hemagglutinating units/50 μl) showed that the lectin activities disappeared from the supernatants. After washings with PBS, the cells were sequentially incubated with several 0.1 M sugar solutions in order to release the lectins from the cell surface. The data shown in table 4 support the proposal that at least three lectins specificities are present in the spleen and thymus cell membranes: lectin specificities towards β-D-galactosides and *N*-acetyl-D-galactosamine are mainly present in the thymus extracts, and towards α-D-mannosides in the spleen extracts. Moreover the fact that binding and agglutination is observed in a homologous system (e.g., mouse thymocytes and mouse thymocyte membrane extract) clearly show that the lectins described are constituents of the membranes tested, and not of erythrocytes, as could have been concluded from the hemagglutination experiments.

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