Acta Chemica Scandinavica B 36 (1982) 337–350

Short Communications

Aggregation of Lipid Vesicles (Liposomes). A Versatile Method to Study Sugar Exposure on Biological Membranes and Sugar Affinity of Bacteria *

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Sugar ligands exposed on biological membranes are important for recognition by bacteria, viruses, toxins and hormones.[1] Thus, the GM_1-ganglioside has been shown to be the receptor for Vibrio cholerae toxin,[2] and a GM_2-like glycoconjugate the receptor for the adhesins CFA/I and K99 of *Escherichia coli*.[3] In the urinary tract the sugar moiety, α-D-Gal-(1→4)-β-D-Gal-, of the P-blood group antigen has been recognized as the binding site for uropathogenic *E. coli*.[4,5] We have employed aggregation of liposomes to characterize (a) sugar exposure on glycolipids of rat intestinal membranes, by using different lectins, and (b) sugar affinity of *E. coli* and *Actinomyces* bacteria, by using appropriate glycolipid-containing liposomes.

Experimental. Determination of sugar exposure on rat intestinal glycolipids. The mucosa of distal ileum in Spraque-Dawley rats (♀; 200 g), was gently scraped off with a curette and put in ice-cold deionized water. It was then freeze-pressed with the X-press[6,7] at −25 °C and about 250 MPa, freeze-dried and extracted according to Karlsson *et al.*[8] The alkaline and neutral lipids were used to prepare lipid vesicles (liposomes) using the reverse phase evaporation method.[9,10] 2 ml lipid extract (≈20 μmol) was mixed with 50 μl 0.1 mM fluorescent lipid *N*-(4-nitrobenzo)-2-oxa-1,3-diazole (NBD)-phosphatidylethanolamine and dried in a rota-evaporator and then dissolved in 15 ml chloroform–diethylether, 1:1, and an emulsion made with 4 ml phosphate-buffered (0.01 M) saline, pH 7.3 using an MSE-sonicator. The vesicles were then formed by evaporation of the organic solvent under vacuum, followed by brief ultrasonic treatment (2 × 30 s) to achieve sonicated unilamellar vesicles (SUVs).[9] To characterize the sugars exposed on the vesicles prepared from intestinal glycolipids, 10 μl lectin (0.25 mg/ml) was mixed with 50 μl liposome suspension at room temperature, and investigated under the epifluorescence microscope with excitation around 480 nm and emission around 517 nm.

Sugar affinity of bacteria. (a) *Aggregation of bacteria with glycolipid-containing fluorescent liposomes.* Unilamellar large liposomes (5 μmol per ml PBS) were prepared by the reverse phase evaporation method (REVs).[9] The vesicles had the following composition: maltobionamide (MB)-vesicles, hexadecylmaltobionamide[11] – phosphatidylglycerol – egg phosphatidylcholine – cholesterol – NBD – phosphatidylethanolamine, 0.7 (or 4 or 10):1:9:9:0.1, and lactosylceramide (LC)-vesicles, *N*-stearoyl-dihydrolactocerebroside – phosphatidylglycerol – egg phosphatidylcholine – cholesterol – NBD-phosphatidylethanolamine, 1:0.5:10:10:0.1. The MB-vesicles were agglutinad only with concanavalin A (ConA, mannose-specific), which was inhibited with α-methyl-mannoside (2 % w/v), LC-vesicles were aggregated with *Ricinus communis* agglutinin -I (D-gal and lactose specific) but not with conA. Aggregation of bacteria with the vesicles was performed at room temperature in microtiter plates on 200 μl of bacteria (10^9 per ml) and 10–100 μl of lipid vesicles (5 μmol per ml). To assess the effect of inhibiting sugars 100 μl bacteria (10^9 per ml) were incubated together with 100 μl PBS containing 2 % appropriate sugar, and 25 or 50 μl lipid vesicles (5 μmol per ml). Spontaneous aggregation of bacteria was studied with 100 μl bacteria (10^9 per ml) and 100 μl PBS. The aggregation was scored after 1 h; ++ = significant agglutination, + weak agglutination, and 0 = no agglutination.

(b) *Agglutination of bacteria and guinea-pig erythrocytes* was studied at room temperature on glass slides using a 3 % (v/v) guinea-pig erythrocyte suspension and 10^9 bacteria per ml PBS (pH 7.2) or two-fold dilutions of this concentration.[12]

(c) *Agglutination of bacteria and yeast cells*

*Communication at the Meeting of the Swedish Biochemical Society in Stockholm, 26–27th November, 1981.

0302-4369/82/050337-04$02.50

Table 1. Aggregation of fluorescent intestinal glycolipid vesicles with different lectins.

Lectin[a]	Sugar specificity	Aggregation
RCA-1	β-D-Gal	+
SBA	MD-Gal, α-NAcGal	(+)
WGA	$[\beta(1\rightarrow4)$-D-GlcNAc$]_2$ (sialic acid)	++
UEA-1	α-L-fucose	+
PNA	Gal-$\beta(1\rightarrow3)$-GalNAc	(+)
Control	–	0

[a] RCA-1 = *Ricinus communis* agglutinin I;
SBA = *Glycine max* agglutinin = soybeen agglutinin;
WGA = Wheat germ agglutinin = *Triticum vulgaris* agglutinin;
UEA-1 = *Ulex europaeus* agglutinin I;
PNA = Peanut agglutinin = *Arachis hypogaea* agglutinin.

(Saccharomyces cerevisiae) was done at room temperature in microtiterplates on a 1 % (v/v) yeast cell suspension incubated for 2 h with 10^9 bacteria per ml or two-fold dilutions of this concentration.[12]

Hydrophobic interaction chromatography (HIC). HIC was performed on Octyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by *Öhman et al.*[13]

Bacteria. E. coli bacteria isolated from urinary tract infections were grown in suspension for 48+48+16 h at 37 °C in Nutrient Broth (NB; Oxoid No. 2) without and with 0.75 % (w/v) glucose to promote and depress type I-fimbriae formation, respectively.

Actinomyces naeslundii and *A. viscosus* bacteria were grown in Brain Heart Infusion Broth (BHI; Oxoid) for 16 h. The bacteria were washed twice in PBS before being analyzed.

Chemicals. N-Stearoyldihydrolactocerebroside, phosphatidylglycerol, egg-yolk phosphatidylcholine, cholesterol, galactose, α-methylmannoside, mannose and maltose were obtained from Sigma (St. Louis, Mo.). The fluorescent lipid, NBD–phosphatidylethanolamine, was obtained from Polar Lipids, Inc. (Birmingham, Ala.). The lectins were a product of E.Y.Lab.s (San Mateo, Ca.), and concanavalin A of Pharmacia Fine Chemicals (Uppsala, Sweden) Hexadecylmaltobionamide was a generous gift from Dr. Francis Szoka (School of Pharmacy, UCSF, San Fransisco). It was synthetized as described by Williams *et al.*[11]

Results. Sugar exposition on rat intestinal glycolipids. The agglutinability of intestinal lipid vesicles with different lectins is shown in Table 1. Assuming that the degree of aggregation is proportional to the amount of sugar presented as glycolipid on the liposomal membrane, *N*-acetylglucosamin, galactose and fucose should be the dominant sugars.

Sugar affinity of bacteria. The mannose-sensitive agglutination of type 1 fimbrated *E. coli* is shown in Table 2, along with the tendency to hydrophobic interaction with Octyl-Sepharose. It is evident that glucose (a) reduces the hydrophobicity, and (b) abolishes the affinity for mannose-residues presented on MB-vesicles, yeast cells or guinea-pig erythrocytes. When the percentage of the *E. coli* retained in the gel (*y*-value) was plotted against the $-{}^2\log$ of the maximum bacterial dilution (*x*-value) yielding positive guinea-pig hemagglutination, there was a linear relationship, $y=12.6x+16.7$ with $r=0.82$.

When increasing the relative amount of hexadecylmaltobionamide (=mannose-equivalent) in the MB-vesicles from 0.7 to 4 on 10 on molar basis, which corresponds to about 2, 17 and 34 % of the total lipid content, it was found that the intermediate concentration agglutinated most efficiently the type 1 fimbrated *E. coli* bacteria.

When the concentration of MB-vesicles in the assay was varied, agglutination was most apparent in the system with 100 μl bacteria (10^9 per ml PBS), 100 μl PBS (or PBS with 2 % (w/v) mannose or maltose for inhibition and 25 μl MB-vesicles (5 μmol per ml PBS) containing about 17 % hexadecylmaltobionamide.

No aggregation of the *E. coli* bacteria was observed with LC-vesicles.

For the *Actinomyces* bacteria the sugar affinity was done only on liposome-bound ligands (Table 3). Some of the *Actinomyces* bacteria showed some spontaneous aggregation, which, however, could be diminished by dilution of the bacteria from 10^9 to 10^8 per ml. It is apparent that the LC-vesicles increased the aggregation of the *Actinomyces* bacteria, whereas MB-vesicles had no effect.

Discussion. Host-parasite interaction mediated *via* sugar ligands on the animal cell and lectin-like

Table 2. Mannose-sensitive agglutination *E. coli* bacteria with MB-vesicles, yeast cells, and guinea-pig erythrocytes, and tendency to hydrophobic interaction with Octyl-Sepharose.

Bacterial strain	% bacteria retained in column	Mannose-sensitive agglutination		
		MB-vesicles	Yeast cells	Guinea-pig erythrocytes
Culture condition: −glucose				
PN3	67	++	++	++
PN7	64	++	++	++
PN8	69	++	++	++
PN12	72	++	++	++
CU10	72	++	++	++
CU15	40	+	+	+
Culture condition: +glucose				
PN3	3	0	0	0
PN7	5	0	0	0
PN8	26	0	0	0
PN12	10	0	0	0
CU10	10	0	0	0
CU15	42	(0)	(0)	(0)

Table 3. Aggregation of *Actinomyces* bacteria with LC-vesicles (MB-vesicles controls).

	Exp 1		Exp 2	
Bacteria	Bacteria	Bacteria+ liposomes	Bacteria (1:10)	Bacteria (1:10) +liposomes
A. viscosus				
11B2	(0)	(+)	0	+
Be66	++	++	(+)	++
B236	(0)	+	(+)	++
W1053	(0)	(+)	0	++[a]
A. naeslundii				
W752	(+)	++	0	++[a]
B74	(0)	+	(0)	+
12104	0	+	0	++
398A	0	++	0	++[a]
A14	0	+	0	++[a]
A18	(0)	++	(+)	++

[a] No aggregation with MB-vesicles visible in wells, or in the microscope.

appendages (fimbriae) on the microorganism have recently come into focus.[1,14] There is, therefore, a need for simple assays that enable characterization of sugars presented, and of the sugar specificity of the bacterial fibriae. Carbohydrate receptors occur on two classes of molecules, glycolipids and glycoproteins. The sugar affinity is generally tested by using inhibiting sugars. We have used liposomes as vehicle carrying, (i) an unknown glycolipid from rat intestine, and directly tested the sugar exposure as aggregation with well-defined lectins, or (ii) a known glycolipid, and assayed the bacterial affinity for the carbohydrate moiety with the agglutination.

It is evident that a variety of sugars are presented on the intestine (Table I), possibly enabling bacteria with different sugar-binding properties to attach to mucosal membranes. Studies are in progress comparing fluorescent-lectin binding to sections of

intestinal mucosa, and aggregation of liposomes prepared from intestinal glycolipids.

The affinity of type 1 fimbriae from *Escherichia coli* for mannose-residues,[14] and of *Actinomyces* fibrilliae for galactose, or lactose residues[15] is confirmed using the liposome-aggregation assay (Tables 2 and 3). Compared to other assayes available,[12] embedding of defined glycolipids into liposomal membranes focuses on one type of ligand at a time, while keeping other parameters unchanged. Using natural, as well as synthetic glycolipids and fluorescent lipid analogs, for preparation of liposomes of varying size offers a very versatile, and inexpensive, method to study sugar-dependent host–parasite interaction, or cell–cell recognition in general.

Acknowledgements. Dr. Richard P. Ellen, Toronto, is gratefully thanked for the supply of *Actinomyces* bacteria, and Dr. Francis Szoka, Jr., for the preparation of hexadecylmaltobionamide. This research was supported by the Swedish Medical Research Council (Project No. 6251), and *Kung Gustav V:s 80-årsfond.*

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Received February 2, 1982.