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Presentation of Binding-Epitope of Globoseries Glycolipid Receptors is Crucial in the Binding, Colonization and Infection of Escherichia coli

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Summary

Wild type *E. coli* DS 17 is a clinical isolate associated with epidemic UTI was studied. This isolate recognized selectively well Gal*α*1-4Gal sequence of globoseries glycolipids separated on thin-layer chromatogram (TLC), coated in microtiter wells and incorporated into liposomes. *E.coli* DS17, representing a class II adhesin, showed high affinity to the disaccharide Gal*α*1-4Gal sequence of the globoside glycolipid receptor in all assay systems. Weak recognition of the epitope, Galα1-4Gal, in the terminal position of globotriaosylceramide (Galα1-4Galβ4Glcβ1Cer) and also weak binding of DS17 to the disaccharide sequence within Forssman glycolipid GalNAcα3GalNAcβ3Galα1-4Galβ4Glcβ1Cer. This strain failed to recognize glycolipids missing the disaccharide sequence like gangliotetraosylceramide Galβ3GalNAcβ4Galβ4Glcβ1Cer. The modified *E. coli* DS 17 by replacing class II adhesin with class III (PrsG) type, (DS17-1), bound Gal*α*1-4Gal sequence of globoside less than Forssman glycolipid. *E. coli* DS17-8 strain, DS17 with the knocked out adhesin, revealed no binding to the glycolipids in all assay system. Using liposomes aggregation assay, *E. coli* J96, representing class I adhesin, showed strong recognition of globoside

incorporated into liposomes, weakly when Forssman glycolipid was used and very weak binding to globotriaosylceramide was detected.

Introduction

Adherence of bacteria to tissue surfaces is an important initial event in bacterial infections (1). *Escherichia coli* is so far the most common causative agent of urinary tract infections (UTI) in patients who have no predisposing conditions (32). Most of all pyelonephritogenic strains of *E. coli* express P-fimbriae, that have been recognized as a key determinant in promoting the virulence of *E. coli* in UTI (33, 34). P-fimbriae are hair-like filaments which facilitate the binding and colonization of the urinary mucosa by *E. coli* (32).In the case of *Escherichia coli*, the predominant enteric pathogen causing extraintestinal infections in man, fimbrial appendages (pili) were shown to participate in adhesion of bacteria to the target tissues. The majority of uropathogenic *E. coli* carry pili interact with specific receptors on the uroepithelium and recognize similar receptors present on human erythrocytes (2, 3). Such piliated *E. coli* cells were capable to agglutinate the erythrocytes in a mannose-resistant manner in comparison with nonpiliated bacteria (4). Genetic determinants mediating pili formation and adhesion were cloned from uropathogenic *E. coli* (5, 6). The pyelonephritic *E. coli* isolate J96 (0:4, K:6) expressed digalactoside-specific hemagglutination of human erythrocytes (7). The process that results in urinary tract

infection (UTI) was initiated by colonization in the colon of the infecting *Escherichia coli* strain (8, 9). This strain next colonizes the periurethral area and the vaginal introitus (10, 11). The clear correlation to uropathogenicity was associated with the expression of P-fimbriae because of 90% of urinary isolates from anatomically healthy children with their first acute pyelonephritis as compared with 5-10% among the commensal fecal *E. coli* flora (12, 13). On the other hand, children suffering from their first bladder infection, 50% of the urinary isolates were P-fimbriated (14). The role of P-fimbriae in colonization of the gut and the periurethral/vaginal areas is unknown. P-fimbriae are heteropolymeric fibers extending out from the bacterial surface that consist of a rigid shaft and a flexible tip fibrillum (15).

P-fimbriae are hair-like filaments which facilitate the binding and colonization of the urinary mucosa by *E. coli* (33). For *Escherichia coli* causing urinary tract infection (UTI), and especially acute pyelonephritis, adhesins were frequently expressed that give rise to a mannose-resistant (MR) hemagglutination and cause attachment to uroepithelial cells (27,30, 31). most of all pyelonephritogenic strains of *E. coli* express P-fimbriae, which are now recognized as a crusial determinant in promoting the virulence of *E. coli* in UTI (33, 34, 35). The globoside-binding specificity has been shown an association mediated by fimbrial appendages called P-fimbriae or pap pili (pili associated with pyelonephritis) (21,22, 23). *Escherichia coli* so far is the most common causative agent of urinary tract infections (UTI) in patients who have no predisposing conditions (3).

The receptor binding PapG-adhesin protein was the minor constituent of the pilus located at the distal end of the linear tip fibrillum (16, 17). P-fimbriation was the virulence factor correlated to the etiology of acute pyelonephritis, related

epidemiologically to the reported infections in over of 95% in children and 50-90% in adults (5, 10).

E. coli might express P-fimbriae with three distinctly different G-adhesins recognizing different glycolipid isoreceptors in the globoseries family (18, 19). The class I G-adhesin is rare in *E. coli* and bound preferentially to globotriaosylceramide (20), whereas *E. coli* with a class II adhesin bound strongly globoside and dominate in human UTI. P-fimbriae with a class III adhesin bound the Forssman glycolipid and to globoA but significantly less to globoside, the dominating isoreceptor in the human kidney (18, 19, 21). The class III G-adhesin was common among canine UTI isolates, which correlated with the abundance of the Forssman antigen in the dog kidney (18). In this work, we will try to show whether the environment or the surface of target tissue presenting these receptors influence the binding activity of infectious agents.

Material and Methods

Bacterial growth and labeling

Bacterial cells were metabolically labeled by addition of [^{35}S] methionine (50 µCi per plate), diluted in phosphate-buffered saline (PBS) (pH 7.4), and directly layered onto 50mm diameter GCB plates streaked with bacteria. Then the cells were harvested , washed twice with PBS and resuspended in the same buffer prior to overlay or microtiter assay.

Glycolipids

Total nonacid and acid glycolipids presented in Table 2 and Fig. 1 were prepared as described previously (10). The nonacid glycolipids were isolated by repeated latrobead column chromatography (latrobeads; 6RS-8060; latron

Laboratories Inc., Tokyo, Japan) applied for both acetylated and native derivatives using continuous gradients of chloroform-methanol (100:0 to 94:6, vol/vol) and chloroform-methanol-water (65:25:4 to 50:40:10, vol/vol/vol), respectively. Most of the individual acid glycolipids were isolated by DEAE-Sepharose column chromatography by the method of Momoi et al. (19) followed by repeated latrobead column chromatography with continuous gradients of chloroform-methanol-5 M NH3 in water (60:40:9 to 40:40:12, vol/vol/vol). The glycolipid structures were confirmed by mass spectrometry, nuclear magnetic resonance spectroscopy, and gas chromatography according to the methods described in the references in this work.

TLC binding assay

The chromatogram binding assay was conducted as described elsewhere (23, 24). Two similar glycolipid chromatograms, developed in parallel on Silica Gel 60 coated on aluminum sheets (HPTLC nanoplates; E. Merck AG, Darmstadt, Federal Republic of Germany), were used. The reference chromatogram was sprayed with anisaldehyde for visualization of the glycolipid bands. The other chromatogram was treated with 0.5% (wt/vol) of polyisobutylmethacrylate in diethyl ether (P28;Rohm, Darmstadt, Federal Republic of Germany) for 1 min and soaked in 2% bovine serum albumin in PBS for 2 h. The plate was then overlaid with 2 ml of [^{35}S]methionine-labeled bacteria (10^8 cells per ml) for 2 h, subjected to five consecutive washes, dried, and exposed to X-ray film (XAR-5, Eastman Kodak Co., Rochester, N.Y.) for 20 to 70 h. The microtiter well assay was performed as described previously (11). Serial dilutions of glycolipids in methanol (50 ul) were dried onto 96-well polyvinyl chloride plates (Cooks M24; Nutacon, Amsterdam, The Netherlands), and the wells were

incubated with 2% bovine serum albumin in PBS. Each well was then incubated with 50 µl of radiolabeled bacteria (5 x 10^6 cells and 10^5 cpm) for 4 h, washed five times with PBS, and dried, and bound radioactivity in the wells was measured with a scintillation counter.

Hemagglutination assay

Hemagglutination tests were performed on glass slide at room temperature by mixing equal volumes (10 µl) of erythrocytes (2% in PBS) and bacterial cells suspended in PBS at a concentration of 10^8 cells per ml. Positive results (i.e., hemagglutination) occurred within 1 min.

Liposome assay

According to the ״reverse phase evaporation״ procedure as previously described (22).Large unilamellar liposomes were prepared by using 20 µmol of lipids (glycolipid/ egg phosphatidylglecerol/ cholesterol/ phosphatidylcholine 0.2/0.5/10/10 (mol/mol) in chloroform were dried under nitrogen and redissolved in 15 microliter chloroform:diethel ether (1:1, v:v), thereafter 4 ml PBS was added and sonicated for 3 min at room temperature using a sonicator bath. The liposomes were formed after evaporation of the organic phase from the emulsified solution using rotary evaporator. Aggregation of bacteria was carried out in a microtiter plate by adding one part of liposome suspension to two parts of bacterial suspension($1x10^9$ CFU), mixed well and the plates were incubated at room temperature for one hour. Evaluation of bacteria-liposomes aggregation was carried out using the phase contrast microscopy.

Results

DS17 and its isogenic mutants hemagglutination activities of human and sheep eythrocytes

Strains described in Tables 1 and 4, wild type clinical isolate *E. coli* DS17, representing class II adhesin, hemagglutinated both sheep and human erythrocytes with increased hemagglutination of human erythrocytes. *E. coli* DS17-1 is a modified wild type DS17 with replaced class II adhesin with the prsG (*E. coli* J96) class III gene cluster. The class-switched mutant lost hemagglutinating activity to human erythrocytes, but showed stronger hemagglutination with the sheep erythrocytes than DS17. DS17-8, the knocked out adhesin, demonstrated clear negative binding activity to both human and sheep erythrocytes.

Binding of DS17 and its isogenic mutants to Gala4Gal-containing isoreceptors separated on thin-layer chromatograms and coated in microtiter wells

Results presented in Table 2 and Figs 1, 2, 3 showed binding data of wild type DS17 and the isogenic mutants to the various glycolipides separated on thin-layer chromatogram or coated in microtiter wells. DS17 strongly recognized globoside and less P1 antigen, Forssman glycolipid and globotriaosylceramide, whereas failed to detect gangliotetraosylceramide (GgO4) glycolipid (Table 2, Fig. 1).

DS17-1, the switched-class II adhesin to class III, showed increased binding to Forssman glycolipid, whereas binding to globoside was found to be weaker. Binding to the globotriaosylceramide was very weak. No detection was observed with GgO4 or P1 antigen using both assay systems (Table 2, Fig. 2).

Both DS17-8, the knockedout-adhesin strain, and standard negative lab-strain Hb101 failed to detect any of the tested glycolipids separated on TLC plates or those coated in microtiter wells as well (Table 2, Fig 3).

Binding of human uropathogenic E. coli DS17 isolate and isogenic G adhesin-mutants to glycolipid-containing liposomes

Three adhesin-classes of *E. coli* DS17 and *E. coli* J96 were tested in the membrane-like system(liposomes aggregation assay) regarding their binding activity to the specific receptors incorporated into liposomes (Tale 3, Fig 5). Lactosylceramide (Galβ4GlcβCer), Globotriaosylceramide (Galα4Gaβ4GlcβCer), G3, globoside (GalNAcβ3Galα4Galβ4GlcβCer) and Forssman (GalNAcα3GalNAcβ3Galα4Galβ4GlcβCer), G5.

Class I adhesin, *E. coli* J96, recognized very well the globoside, somewhat weaker the 3-sugar, G3 glycolipid, whereas selectively weak and very weak recognized Forssman, G5, and LacCer, respectively. Class II, DS17, bound moderately globoside, but weakely recognized G3 and G5. None of the tested glycolipids was detected by the DS17-8. DS17-1, the class III ahesin, showed strong affinity to the Forssman, G5 and less well to the globoside (G4). This strain bound weakly G3, whereas binding to LacCer was undetected.

Molecular modeling analysis of epitope presentation of receptor active glycolipids

The results from the liposome binding assay and microtiter wells as well as TLC showed that the selective binding of *E. coli* to one of the three globoseries glycolipids, where lactosylceramide (Galβ4Glcβ1Cer), a disaccharide (lactose molecule) as an internal part of these glycolipids located closly to ceramide, was investigated. The presence of the fatty acid of the ceramide 2-D hydroxyl group and the 4-D hydroxyl group of phytosphingosine indicated to involvement

of the fatty acids in the binding by other bacteria (not shown). They could also be indicative of a requirement for hydrogen bond interactions between the fatty acid 2-D hydroxyl group and the Glc 2-OH or 6-OH for a correct binding epitope presentation recognized by *E. coli*. In the isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer) structure having Glcβ1Cer conformation and the terminal Galα3 residue blocks the binding epitope for an adhesin approaching from above to a lesser degree, which also conformer inaccessible epitope. In globotriaosylceramide (Galα4Galβ4Glcβ1Cer) the terminal Galα4 residue does not block access to the binding epitope. Other substitutes like GalNAcβ3 (in globoside), GalNAcα3GalNAcβ3(in Forssman) did not have blocking influence either on the presentation of disaccharides Galα4Gal (black balls) binding epitope as shown in Table 2 and figure 4. Molecular modeling study of Glcβ1Cer revealed that nine different low energy conformers are obtainable by varying the dihedral angles of the Glcβ1Cer linkage, which intern can partially explain the conformers of globoseries presented in the figure 4.

Discussion

Expressions of *Escherichia coli* P-fimbriae constitutes the strongest correlation to renal pathogenicity, but is also related in the first-time to cystitis in children. As a virulence factor, P-fimbriae act as lectin-like adhesin recognizing the globoseries of glycolipids, which represents the P-blood group antigens (11, 12). The minimal binding epitope for P-fimbriae is Galα1-4Gal disaccharide in the carbohydrate chain of the glycolipids (13). We found that the way of presentation of glycolipid receptor is crucial in this binding. Due to this presentation of the binding epitope, which may become more accessible to the adhesin binding site.

The glycolipid receptors incorporated into liposomes were practically used as a model of bilayer membrane or as *in*

vivo environment to evaluate the influence of the membrane on the epitope presentation. In class I adhesin, binding was selectively moved to the disaccharide epitope in globoside (4-sugar glycolipid) than globotriaosylceramide (3-sugar glycolipid) when these glycolipids were incorporated into liposomes. This phenomenon may be correlated to either the topography of the adhesin binding site itself or to the receptor oligosaccharide sequence caring the binding epitope. Using data conformation program, the tested glycolipid receptors demonstrate clear differences in the position and accessibility of binding disaccharide Galα1-4Gal sequenc. Availability of the binding epitope of these glycolipids, to be easily recognized by bacterial adhesion, is based, among other factors, on the presentation of that epitope mostly associated with a new configuration after changing of oligosaccharide of the receptor, when the ligand is identical (adhesion).

The minimal binding epitope for P-fimbriae is Galα1-4Gal disaccharide in the carbohydrate chain of the glycosphingolipids (13). The steric interference from neighboring saccharides outside binding epitope as well as oligosaccharides outside the epitope may affect the presentation of binding epitope. In this study, the liposome-glycolipid model was demonstrated with one incorporated receptor , therefore will be useful to design a multi-incorporated glycolipids and other lipids and proteins.

P-fimbriated *E. coli* wild type DS17 strain having class II G-adhesin, binds preferentially to globoside (G4). One isogenic mutant lacking the G-adhesin *E. coli* DS17 (knockedout adhesin) designated DS17-8 failed to recognize G4 and other isoreceptors. Other isogenic mutant in which the *pap*-G class II allel , in DS17 strain, was replaced with PrsG gene cluster coding for class III adhesin from wild type J96 *E. coli*

isolate. The *E. coli* expressing class III adhesin is designated DS17-1, binds preferentially to the Forssman glycolipid (G5), which clearly indicates to a different topography of adhesin binding site based on comparison with the previous binding to G4. None of the tested glycolipids was detected by the *E. coli* DS17-8 with knocked out *pap*-G adhesin, which reveales the crucial role of adhesin in the colonization and infection.

Using the suggested model (mentioned above) in multi incorporated lipids, phospholipids, proteins and glycoproteins into liposomes, may explain the role of other factors-influence on the presentation of the receptors exposed by the membrane in comparison with binding assay of a single incorporated lipid into liposomes as well as bindings to the receptors on the artificial surfaces.

Structure of ceramide and some experimental evidence one may formulate its role in membrane stability and barrier properties determined by hydrogen bonding in amide region of ceramid. Furthermore, a natural variation in the number of hydroxyl groups (of fatty acids and long chain base) may be important for regulation of the potential hydrogen bonds.

Taken together the presented results, we can conclude from this study that the selectivity of adhesin depends on the topography of the adhesin binding site itself, the receptor oligosacharide sequence caring the binding epitope, the conformation of the oligosaccharide, steric interference from neighboring saccharides outside the binding epitope, restrictions on the epitope presentation imposed by membrane environments and effect of intrinsic membrane components.

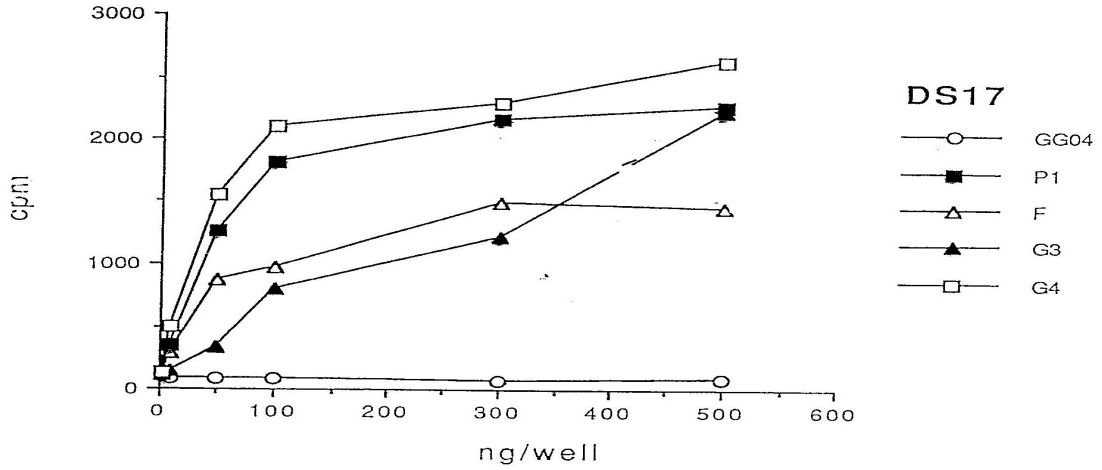


Fig 1

Binding of wild type *E. coli* DS 17 to glycolipid receptors coated in microtiter wells (GGO4: gangliotetraosylceramide, P1: P1 antigen as described in Table 2, F: Forssman glycolipide, G3: globotriaosylceramide, G4: globoside).

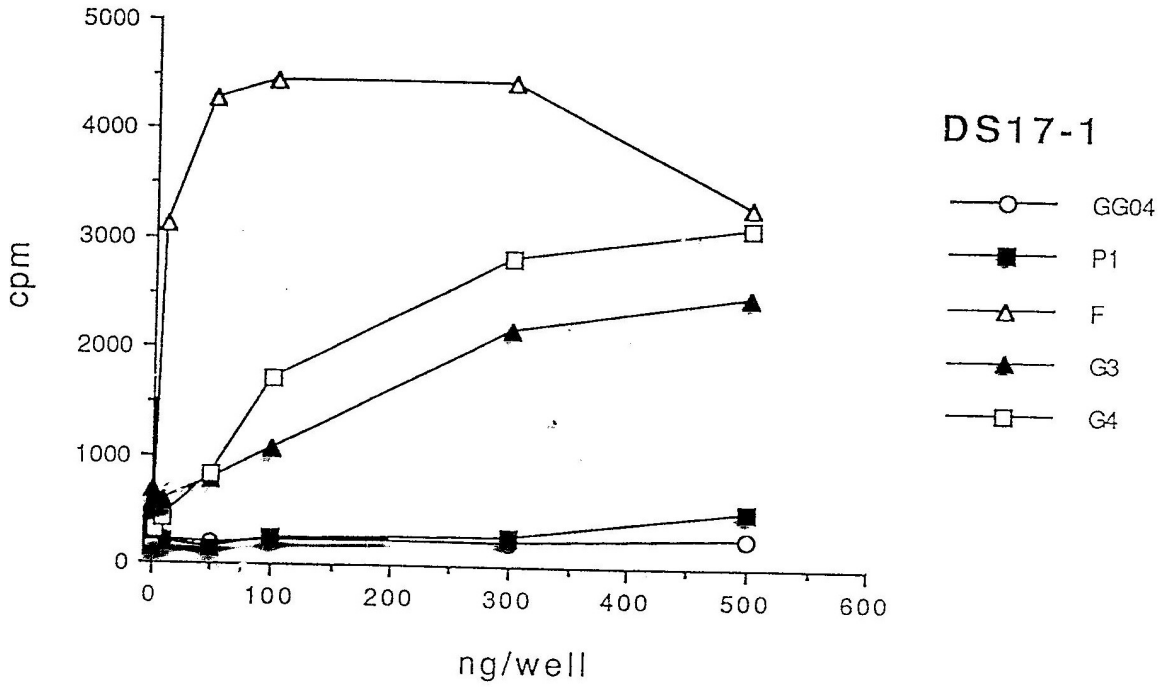


Fig 2

Binding of *E. coli* DS17-1 to glycolipid receptors coated in the microtiter wells (GGO4: gangliotetraosylceramide, P1: P1 antigen as decribed in Table 2 , F: Forssman glycolipide, G3: globotriaosylceramide, G4: globoside).

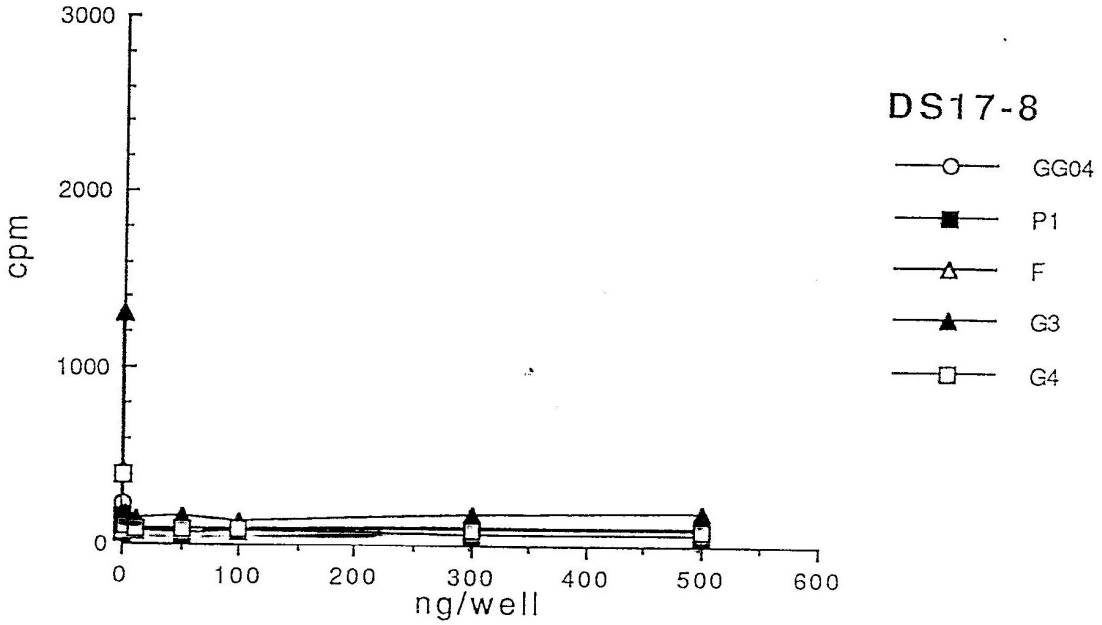


Fig 3

Binding of *E. coli* DS17-8 to glycolipid receptors coated in the microtiter wells (GGO4: gangliotetraosylceramide, P1: P1 antigen as described in Table 2, F: Forssman glycolipide, G3: globotriaosylceramide, G4: globoside).

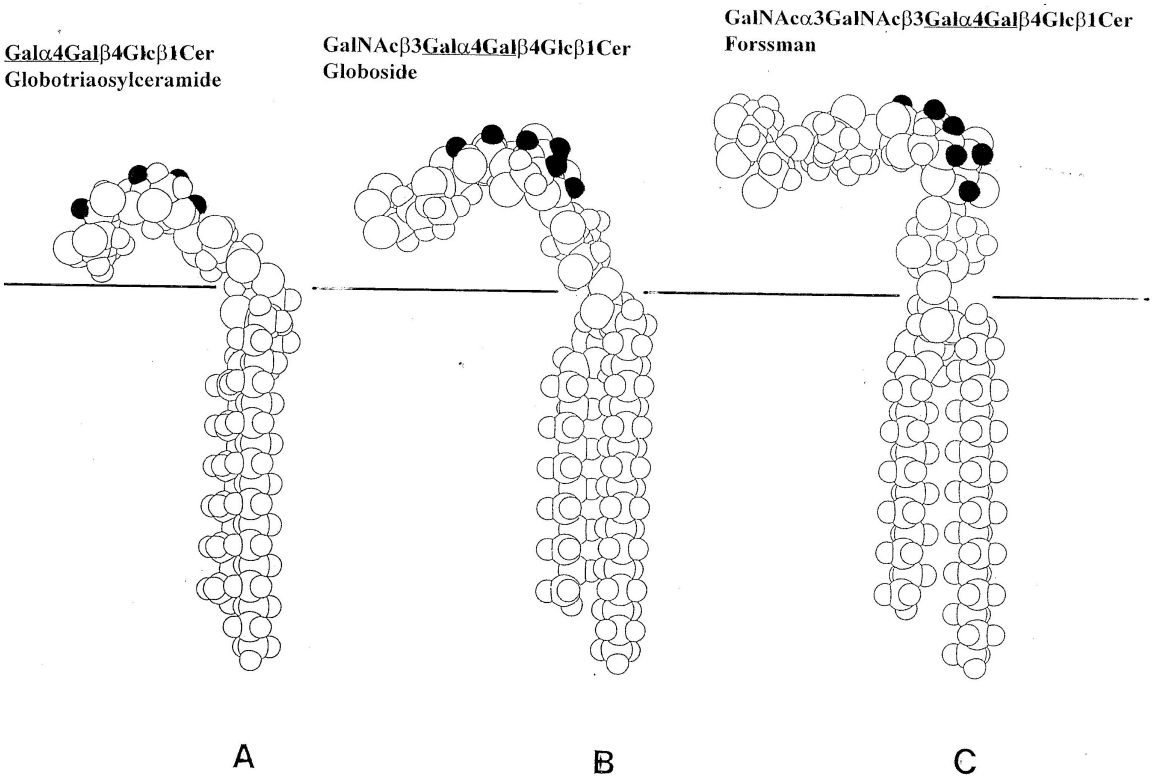
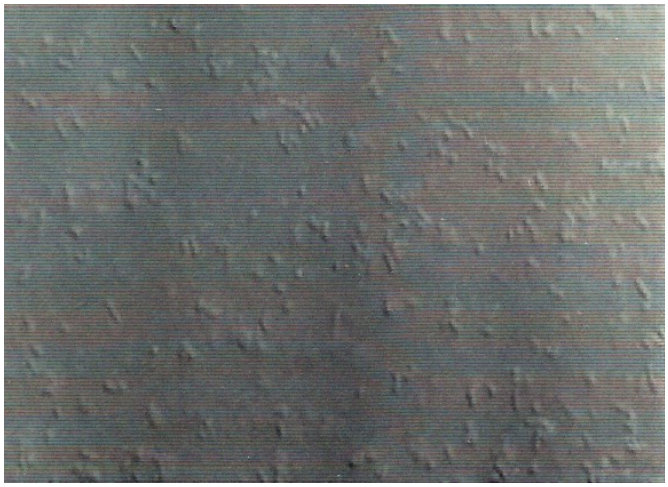


Fig 4

Molecular modeling of globoseries glycolipids demonstrates the presentation of Gala4Gal binding epitope. The black circles represent the carbon atoms demonstrating the local and the accessibility of Galα1-4Gal binding epitope.

A



B

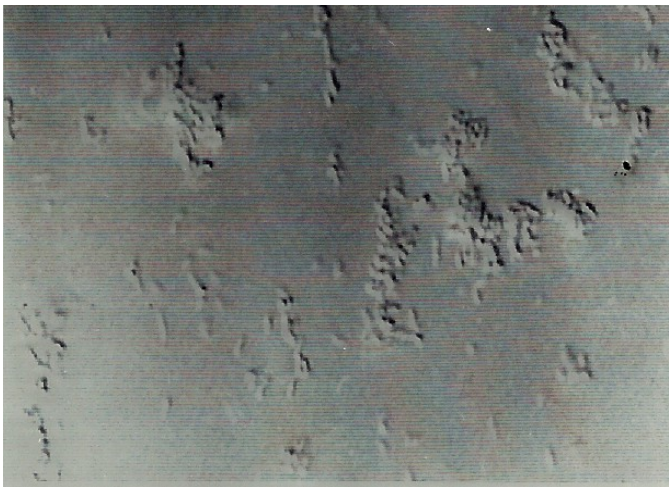


Fig. 5

Binding of bacteria to glycolipids incorporated into liposomes demonstrated by bacteria-liposome aggregations.

A. Negative control, bacterial suspension mixed with the liposomes (with no incorporated glycolipids). **B**. positive aggregation after bacterial binding to glycolipids incorporated into liposomes.

Table 1

Hemagglutination properties and antibiotic resistance of DS17 and its mutant derivatives.

Strain	MRHA-hum	MRHA-sheep
	Resistance markers	
DS17	++	+
	Amp,Cm,Tc	

DS17R	++ Amp,Cm,Tc,Rif	+
DS17-8	- Amp,Cm,Tc,	-
DS17-8R	- Amp,Cm,Tc,Rif	-
DS17-1	- Amp,Cm,Tc,	++
DS17-1R	- Amp,Cm,Tc,Rif	++

Amp: Ampicillin 100 ug/ml, Cm: Chloramphenicol 10 ug/ml, Tc:

Tetracyklin 15 ug/ml, Rif : Rifampicin 100 ug/ml.

MRHA: Mannose resistant hemagglutination ,as decribed in M&M.

Table 2

Results of binding of DS17 and its mutants to Galα4Gal-containing isoreceptors on thin-layer chromatograms and in microtiter wells

Glycosphingolipid sequence	DS17 Hb101	DS17-1 Source	DS17-8
Galα4Galβ4GlcNAcβ3Galβ4GlcβCer P1 antigen	++	- HE	- -

Galα4Galβ4GlcβCer ++ + -
- HE

P^k antigen, globotriaosylceramide, G3

GalNAcβ3Galα4Galβ4GlcβCer +++ ++ -
- HE

Globoside, globotetraosyceramide,G4

GalNAcα3GalNAcβ3Galα4Galβ4 ++ +++ -
- DK

GlcβCer, Forssman, F

Galβ3GalNAcβ4Galβ4GlcβCer - - - -
HM

Gangliotetraosylceramide,GgO4

The results from autoradiography after overlay on thin-layer chromatograms were in agreement with the curves obtained from binding in microtiter wells (figs. 1, 2, 3) as well as in liposome-assy. Strain DS17R was identical with DS 17, strain DS17-8R with DS17 and strain DS17-1R with DS17-1. The following abbreviations were used:HE: Human Erythrocytes, DK: Dog Kidney, HM: Human Meconium.

Table 3
Binding of human uropathogenic *E. coli* isolate DS17 and isogenic G adhesin mutants to glycolipid-containing liposomes

Adhesin				
			PapG $_{DS17-8}$	PrsG $_{DS17-1}$

Results presented in the table 3 demonstrate binding of bacteria to glycolipids incorporated into liposomes. Strong detection of globoside (G4) by Class I adhesin and Forssman (F) glycolipid, by Class III was shown. Weaker recognition was detected to G3 and G4 incorporated into liposomes by Class I and Class II, respectively.

Table 4

Uropathogenic *Escherichia coli* , wild type and its derivative mutants

Strain	Expressed adhesin Characterization	
1. DS17	Wild type	II
2. DS17-1	III	The papG genes for adhesin (DS17 strain) were replaced by class III PrsG(J96) gene cluster
3. DS17-8 mutant	None	Adhesin-negative

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ملخص البحث

البحث المعروض يقدم دراسه اصيله حول ا لدور المهم لطريقة عرض المستقبل (الرسبتور) الكلوبوسيد كلايكولبد (الكربوهيدرات الـدهنيه) لثنـائي- السكر- الاحادي أي كـالاكتوز- كـالاكتوز (الابيتـوب) الجـزء الخـاص لالتسـاق

ادسين بكتريا عصيات الامعاء الاشريكيا كولاي والذي يحمله الرسبتور ظمن تركيبته الكيمياويه. هذا الجزء المتخصص من المستقبل مكون من جزئين من سكر الحليب الاحادي مرتبطتين بين كاربون السكرالاول-وكاربون السكر الثاني بنظام الالفا. يختلف ثنائي- السكر-الاحادي من حيث نشاطه في الالتساق والتشخيص بالادسين البكتيري بالطريقه التي يعرض بها الرسبتور (المستقبل) هذا الابيتوب كذلك موقع ثنائي- السكر-الاحادي ظمن سلسلة كاربوهيدرات الرسبتور نفسها حيث يكون سهلا احيانا او صعبا احيانا اخرى كذلك سالبا احيانا كثيره. ادسينات بكتريا عصيات الامعاء الاشريكيا كولاي مصنفه الى ثلاث اصناف 1،2 و 3 فهيه تلتسق بالثنائي- السكري- الاحادي الابيتوب اعتمادا على نوع صنف ادسين البكتريا من جهة وعلى السطح الذي يستند عليه من جهة اخرى. صنف الادسين الثاني (رقم 2) يلتسق بقوه بالغلوبوسيد كلايكولبد رباعي السكر حيث يتمركز ثنائي- السكر- الابيتوب بموقع ماقبل نهاية السلسله الكاربوهيدراهتيه بينما يلتسق اضعف بكثير صنف الادسين الثالث (رقم 3) بالغلوبوسيد كلايكولبد. بينما يكون التساقه اقوى بالفورسمان كلايكولبد خماسي السكر حيث يشغل ثنائي- السكر- الاحادي الابيتوب موقع منتصف السلسله الكاربوهيدراتيه للرسبتور. لتعزيز هذه الفكره واهمية السطوح الساندة ودورها في عرض الابيتوب بواسطة الرسبتورات (المستقبلات) ادخلنا نظام الايبوسوم كونه يقرب صورة الغشاء الخلوي داخل جسم الكائن الحي حيث تظهر الرسبتورات بشكل مقارب للواقع مكانات الالتساق الابيتوبات مقارنة بوضعها عندما تستند على السطوح الصلبه كصفائح السيليكا الالمنيوميه اوفي الغرف البلاستيكيه لصفائح الالايسا التي اجريت عليها تجارب الالتساق الاوفرلي. صنف الادسين الاول (رقم 1) يلتسق بقوه بالمستقبل الرباعي السكر(الكلوبوسايد) في الايبوسوم على العكس تماما من تصرفه باختبار الالتساق على السطوح الصلبه. لايوجد التساق لاي مستقبل لايحتوي ثنائي السكر الاحادي كذلك البكتريا التي لاتمتلك ادسين لاتلتسق بالمستقبل على الاطلاق.