Studies of Carbohydrate-Binding Proteins from Enterotoxigenic *Escherichia coli*

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Abstract

Knowledge about the carbohydrate binding specificity of pathogens can give an increased understanding of their virulence strategies, and is also of importance for the development of anti-adhesive therapies and vaccines. Enterotoxigenic *Escherichia coli* (ETEC) is a well-known causative agent of diarrheal disease. The first step in the pathogenesis of ETEC infections is adhesion of the bacterium to the small intestinal epithelium, mediated by colonization factors (CFs). Two of the most common CFs are CFA/I and CS6. CFA/I is a fimbriae with a major subunit, CfaB, and a minor subunit, CfaE. CS6 is non-fimbrial and composed of two major subunits, CssA and CssB.

The potential carbohydrate recognition by CFA/I was investigated by binding CFA/I-fimbriated bacteria and purified CFA/I fimbriae to variant glycosphingolipids on thin-layer chromatograms. Both with CFA/I bacteria and purified fimbriae binding to a number of non-acid glycosphingolipids was obtained. These compounds were also recognized by CFA/I in the absence of CfaE, demonstrating that the glycosphingolipid-binding of CFA/I resides within CfaB. Using CS6-expressing *E. coli*, and purified CS6 protein, a highly specific binding to sulfatide (SO_3-3Galβ1Cer) was obtained. The binding of the CS6 protein and CS6-bacteria to sulfatide was inhibited by dextran sulfate, but not by dextran, heparin, galactose 4-sulfate or galactose 6-sulfate, suggesting that sulfate in position 3 is of importance for binding of CS6. When using recombinantly expressed and purified CssA and CssB, sulfatide binding was obtained with CssB, demonstrating that this subunit carries the glycolipid binding capacity. CS6-binding sulfatide is present in the small intestine of species susceptible to CS6-mediated infection, as humans and rabbits, but lacking in species not affected by CS6 ETEC, as mice. The ability of CS6-expressing ETEC to adhere to sulfatide in target small intestinal epithelium may thus contribute to virulence.

The heat-labile enterotoxin of ETEC is an AB_5 toxin, with a pentamer of receptor-binding B-subunits. B-subunits from porcine and human isolates of ETEC (pLTB and hLTB, respectively) and the B-subunit of cholera toxin (CTB) are highly similar, and all of them bind to the GM1 ganglioside. pLTB also binds neolacto sequences and hLTB blood group A type 2 sequences. A B-subunit (CFXB), with 73% sequence identity to CTB, is found in *Citrobacter freundii* and some ETEC strains. Binding studies showed that CFXB binds with high affinity to GM1, and to both neolacto and blood group A sequences. The blood group antigen A binding site of hLTB has been proposed to be involved in binding of hLTB to lipopolysaccharides (LPS). Our study aimed at defining the relationship between the blood group A/B binding site and the LPS binding site. However, no binding of the B-subunits to *E. coli* LPS was obtained, and incubation with LPS did not affect the binding of hLTB to blood group A-determinants, indicating that the blood group A binding site is not involved in LPS binding. Incubation with blood group A saccharide inhibited the binding of hLTB to GM1, and *vice versa*, suggesting that a concurrent occupancy of the two binding sites does not occur.

Key words: Carbohydrate recognition/ETEC colonization factors/CFA/I fimbriae/CS6 protein/heat-labile enterotoxin

Populärvetenskaplig sammanfattning

Enterotoxigen *Escherichia coli* (ETEC) är en bakterie som varje år orsakar ca 650 miljoner fall av magsjuka varav 800 000 med dödligt utfall, främst hos barn upp till fem års ålder. ETEC är vanligast förekommande i utvecklingsländer och är också orsaken till turistdiarré.

För att kunna orsaka sjukdom måste ETEC få fäste i tarmen. Detta gör bakterien med hjälp av kolonisationsfaktorer som känner igen och greppar tag om särskilda kolhydratstrukturer som finns på tarmcellerna. Olika ETEC-bakterier kan ha olika kolonisationsfaktorer och det finns mer än tjugo olika varianter av dessa faktorer. Två av de vanligaste kolonisationsfaktorerna är CFA/I och CS6.

CFA/I fungerar som en arm som sticker ut från bakterien, en fimbrie. Den är uppbyggd av två olika byggstenar, CfaB och CfaE. Vi har kartlagt vilka kolhydratstrukturer som CFA/I känner igen och även visat att det är via CfaB som igenkänningen sker.

CS6 är inte en fimbrie. Dock kan även den känna igen och haka fast vid särskilda kolhydratstrukturer på tarmcellerna. CS6 är också uppbyggd av två olika enheter, CssA och CssB. Vad gäller CS6 har vi kunnat visa att det är CssB som känner igen och binder till vissa kolhydratstrukturer på tarmcellerna.

När ETEC-bakterierna väl sitter fast på tarmcellerna kan de börja kolonisera tarmen. Därefter utsöndrar de ett gift, ett toxin, som skickas in i tarmcellerna och gör att tarmcellerna minskar upptaget av vätska från tarmen och dessutom utsöndrar ytterligare vätska till tarmen. Resultatet blir att man får diarré. ETEC kan producera två olika toxiner, värmelabilt toxin (LT) och värmestabilt toxin (ST). Vi har studerat LT som är mycket likt koleratoxinet (CT). Båda dessa toxiner binder till kolhydratstrukturer på tarmcellerna. Vi har jämfört kolhydratbindningen för det traditionella värmelabila toxinet och ett nyupptäckt toxinliknande protein som finns hos bakterien *Citrobacter freundii*, och även hos en del ETEC-stammar.

I nuläget finns inget vaccin mot ETEC-infektion. Vaccinet Dukoral mot kolera skyddar även delvis mot ETEC-infektion, men bara delvis. Tidigare studier har visat att ETEC-bakterier utan kolonisationsfaktorer inte kan orsaka sjukdom. Våra studier av olika kolonisations-faktorer och LT är ett steg i utvecklingen av ett vaccin som ska hindra kolonisationsfaktorerna från att få fäste, göra toxinet overksamt och därmed oskadliggöra bakterien.

Papers included in this thesis

This thesis is based on the following papers:

I. The major subunit, CfaB, of colonization factor antigen I from enterotoxigenic *Escherichia coli* is a glycosphingolipid binding protein. Lena Jansson, Joshua Tobias, Michael Lebens, Ann-Mari Svennerholm, and Susann Teneberg. 2006. Infect. Immun.74, 3488-3497.

II. Sulfatide recognition by colonization factor antigen CS6 from enterotoxigenic *Escherichia coli*. Lena Jansson, Joshua Tobias, Catharina Jarefjäll, Michael Lebens, Ann-Mari Svennerholm, and Susann Teneberg. 2009. PLoS ONE 4: e4487.

III. No direct binding of the heat-labile enterotoxin of *Escherichia coli* to lipopolysaccharides. Lena Jansson, Jonas Ångström, Michael Lebens, and Susann Teneberg.
Submitted manuscript

IV. Carbohydrate binding specificities of the cholera toxin-like B-subunit from *Citrobacter freundii*. Lena Jansson, Michael Lebens, and Susann Teneberg.
Manuscript

The papers are referred to in the text by their Roman numerals.

Contents

Abbreviations

BSA	bovine serum albumin
Cer	ceramide
CF	colonization factor
CFA/I	colonization factor antigen I
CFXA	putative A-subunit from *Citrobacter freundii*
CFXB	B-subunit from *Citrobacter freundii*
CS6	coli surface antigen 6
CT	cholera toxin
CTB	cholera toxin B-subunit
ETEC	enterotoxigenic *Escherichia coli*
Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
hLTB	B-subunits of heat-labile enterotoxin from ETEC pathogenic to humans
LPS	lipopolysaccharides
LT	heat-labile enterotoxin from ETEC
NeuAc	*N*-acetyl neuraminic acid
NeuGc	*N*-glycolyl neuraminic acid
pLTB	B-subunits of heat-labile enterotoxin from ETEC pathogenic to pigs
ST	heat-stable enterotoxin from ETEC
TLC	thin-layer chromatography

Background

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrheal disease. Two major virulence factors of ETEC are the colonization factors and the enterotoxins. The colonization factors are proteins, mediating the initial attachment of the bacteria to the epithelial cells of the small intestine of the host by binding to carbohydrate structures on the cell membrane. The enterotoxins produced by the bacterium are responsible for most of the diarrheal symptoms, and the primary receptor for one of these toxins, the heat-labile enterotoxin is the GM1 ganglioside, *i.e*. a glycosphingolipid. Thus, protein-carbohydrate interactions are involved in at least two steps in the pathogenesis of diarrheal disease by ETEC and charcterization of the recognition mechanisms between proteins and carbohydrates are of importance for the understanding of pathogenic mechanisms of ETEC-induced diarrheal disease.

Microbial adhesion

Adhesion of microbes to the target tissue is an important determinant for successful initiation, establishment and maintenance of infection. The majority of known attachment sites for bacteria, viruses and bacterial toxins on host cells are glycoconjugates. Glycoconjugates exhibit a characteristic and specific pattern of expression, which is dependent on the animal species, individual and cell type, thus explaining the phenomenon of tropism of infection. A large number of glycoconjugate receptors for microbes are known (Karlsson 1989; Ofek and Doyle 1994; Varki et al. 1999), although a detailed characterization including recognized epitopes in most cases is lacking. One of the best characterized examples of bacterial carbohydrate recognition is the binding of P-fimbriated uropathogenic *E. coli* to Galα4Gal-containing glycosphingolipid receptors, where evidence from a monkey model of urinary tract infection show that the expression of the Galα4Gal-binding PapG adhesin is essential for the ability of the bacteria to cause pyelonephritis (Roberts et al. 1994).

At a structural level, noteworthy results are the first X-ray structures of some *E. coli* fimbrial adhesins; the mannose-binding FimH adhesin, the Galα4Gal-binding PapG adhesin and the GlcNAc-binding F17-G adhesin (Choudhury et al. 1999; Dodson et al. 2001; Buts et al. 2003). Interestingly, these proteins all have an immunoglobulin-like (Ig-like) fold, although they have little to no sequence identity. Crystal complexes are also known for bacterial toxins and receptor saccharides, including cholera toxin B subunits and GM1 pentasaccharide (Merritt et al. 1994), and the heat-labile enterotoxin of *E. coli* and lactose (Sixma et al. 1991).

Carbohydrates

Carbohydrates are one of the most abundant types of molecules on earth, and have a variety of different functions as *e.g.* energy storages and structural building blocks. Other important roles of carbohydrates on the mammalian cell surface are as recognition molecules, involved in cell signaling and microbial adhesion (Varki and Sharon 2008).

Carbohydrates are either monosaccharides (*e.g.* glucose), disaccharides (*e.g.* lactose) or poly-/oligosaccharides. Due to variations of the constituent monosaccharides, carbohydrate sequence, binding position, glycosidic configuration, ring size and branching the diversity of the oligosaccharides is almost infinite.

The most common monosaccharides found in mammalian glycoproteins and glycolipids are mannose (Man), galactose (Gal), glucose (Glc), fucose (Fuc), *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc), *N*-acetyl-neuraminic acid (NeuAc), and *N*-glycolylneuraminic acid (NeuGc). The monosaccharides are either aldoses or ketoses. In vertebrates most monosaccharides have D configuration. One exception is fucose, which has L configuration (Bertozzi and Rabuka 2008).

Glycoconjugates on the mammalian cell surface

Three major types of membrane-linked carbohydrates can be found on the surface of animal cells. These are proteoglycans, glycoproteins and glycolipids (Varki and Sharon 2008). The proteoglycans are macromolecules with a central core protein, to which a number of glycosaminoglycan chains are attached by xylosyl-serine linkages. Although proteoglycans are mainly found in the extracellular matrices of connective tissues, they may also be

membrane-inserted. The presence of *e.g.* heparan sulfate, anchored in the plasma membrane by a hydrophobic region in the protein core, has been demonstrated in a variety of mammalian cells (Höök et al. 1984). The protein part of the glycoproteins is also integrated in or through the lipid bilayer. In addition, glycoproteins may be anchored to the membrane via phosphatidylinositol-containing glycolipids. The glycosylation of glycoproteins mainly occurs at asparagine residues by linking of *N*-acetylglucosamine to the amino group (*N*-linked), or at serine or threonine by linking of *N*-acetylgalactosamine to the oxygen atom of the hydroxyl group of these residues (*O*-linked). The carbohydrates of the glycolipids are found linked to glycerolipid or sphingolipid, with the lipid part inserted in the outer leaflet of the plasma membrane.

The main oligosaccharide chains of proteoglycans, consisting of linear repeating disaccharide units, which are highly substituted with carboxyl and/or sulfate ester groups, differ significantly from the carbohydrates of glycoproteins and glycolipids. In contrast similarities between the oligosaccharide chains, in particular the terminal sequences, of glycoproteins and glycosphingolipids are common (Finne et al. 1989). However, there are also important differences. Mannose, which is a common constituent of *N*-linked oligosaccharide chains of glycoproteins, has not as yet been described in glycosphingolipids of higher animals. On the other hand, there are carbohydrate sequences that so far only have been identified in glycosphingolipids, as *e.g.* the lactose sequence (Galβ4Glcβ) and the Galα4Gal sequence (Yang et al. 1994).

Glycosphingolipids

Glycosphingolipids are present on all mammalian cell plasma membranes. They are amphipatic molecules, which have one hydrophobic part and one polar part. The hydrophobic part is the ceramide, built up of a long-chain base (sphingosine, sphinganine or phytosphingosine) and an amide linked fatty acid (Chester 1999; Schnaar et al. 2008). Variations of the number of carbon atoms, double bonds, methyl branches and hydroxyl groups in the fatty acid and the long-chain base result in a large number of molecular species. Sphingosine is named after the Egyptian Sphinx, since the structure was a bit of a mystery when first isolated (Thudichum 1884). The polar part of the glycosphingolipids is formed by one or more monosaccharides. In most cases the carbohydrate moiety contains one to ten monosaccharide units, but glycosphingolipids with more than forty saccharide residues

(polyglycosylceramides) have also been described (Karlsson et al. 1999). More than 500 different glycosphingolipids have been found so far (LipidBank 2007).

In 1884 the German biochemist J. L. W. Thudichum described the first glycosphingolipid, galactosylceramide (Galβ1Cer) (Thudichum 1884). It is one of the simplest glycosphingolipids, consisting of the ceramide and only one monosaccharide, galactose.

The most common core structures of mammalian glycosphingolipids are lacto-, neolacto-, ganglio-, globo- and isoglobo-series (Table 1). In humans glycosphingolipids the lacto-series are found in *e.g.* the gastrointestinal tract, neolacto-series in hematopoietic cells, ganglio-series in the brain and globo-series in erythrocytes (Schnaar et al. 2008).

Table 1. Tetraglycosylceramides based on the different core structures. The carbohydrate sequences defining the different core structures are in bold.

Series	Structure
Lacto	**Galβ3GlcNAc**β3Galβ4Glcβ1Cer
Neolacto	**Galβ4GlcNAc**β3Galβ4Glcβ1Cer
Ganglio	Galβ3**GalNAcβ4Gal**β4Glcβ1Cer
Globo	GalNAcβ3**Galα4Gal**β4Glcβ1Cer
Isoglobo	GalNAcβ3**Galα3Gal**β4Glcβ1Cer

The expression of glycosphingolipids varies both quantitatively and qualitatively between different species, individuals of the same species, organs, and cells. The composition of the glycosphingolipids of a certain cell is dependent on the glycosyltransferases expressed by the cell in question. The ceramides are produced at the cytosolic face of the endoplasmic reticulum, and after transfer to the luminal side, transported to the lumen of the early Golgi apparatus (Snook et al. 2006; Schnaar et al. 2008). There, a stepwise glycosylation of the ceramide is catalyzed by a number of glycosyltransferases (Schnaar et al. 2008).

Sulfatide is a simple glycosphingolipid (Figure 1), which in mammalians is expressed in large quantities in the myelin-producing oligodentrocytes of the central nervous system, and in the

epithelial cells of the gastrointestinal tract. The creation of sulfatide starts with synthesis of galactosylceramide (Galβ1Cer) on the lumenal face of the endoplasmic reticulum. Galactosylceramide is sulfated in position 3 by 3'phosphoadenosine-5'-phosphosulfate: cerebroside sulfotransferase, giving SO_3-3Galβ1Cer, during the passage through the Golgi (Eckhardt 2008).

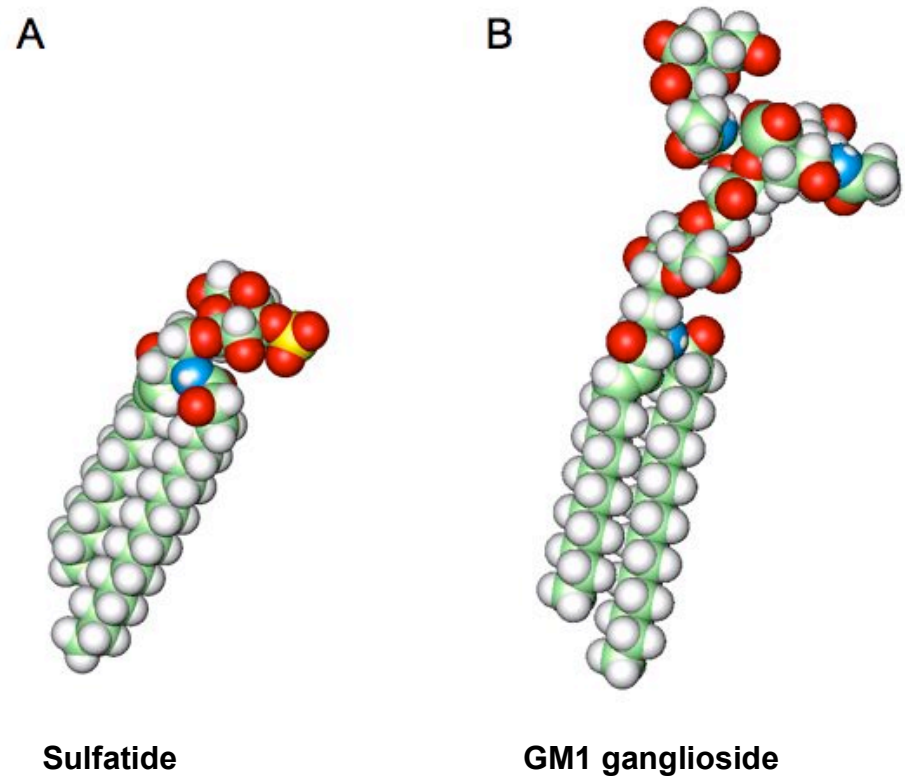

Figure 1. Molecular structures of sulfatide (SO_3-3Galβ1Cer) (A) and GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer) (B).

Gangliosides have one or more sialic acids. The complex gangliosides are built mainly on a ganglio- or neolacto core structures (Proia 2003). The GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer) (Figure 1) is of great biological importance since it is the primary receptor for cholera toxin from *V. cholerae* (Holmgren and Svennerholm 1977; Fishman 1982) and heat-labile enterotoxin from enterotoxigenic *E. coli* (Fishman 1982; Lencer and Tsai 2003).

Protein-carbohydrate interactions

Investigations of protein-carbohydrate interactions at the molecular level may be done by x-ray crystallography and nuclear magnetic resonance (NMR). By comparison of crystal structures of a large set of protein-carbohydrate complexes, some general principals that underlie specific carbohydrate binding by proteins have emerged (reviewed in (Weis and Drickamer 1996; Cummings and Esko 2008). Thus, the selective binding is obtained by a combination of hydrogen bonding to the hydroxyl groups of the carbohydrate, and van der Waals interactions, often due to stacking of hydrophobic surfaces of the carbohydrates on aromatic side chains of amino acids. Indirect interactions, as water-mediated interactions between the protein and the carbohydrate ligand, also contribute to ligand binding.

Escherichia coli

E. coli is a gram-negative bacterium belonging to enterobacteriaceae. This bacterium was discovered by the German-Austrian bacteriologist Theodor Escherich in 1885 and named after him. Most *E. coli* strains are harmless and some strains live in the intestines and are part of the normal flora. Some of their tasks are to produce vitamin K and to stop pathogenic *E. coli* from colonizing the intestines. However, certain strains of *E. coli* may cause diseases, as *e.g*. sepsis/meningitis, urinary tract infections and enteric diseases (Nataro and Kaper 1998). The different forms of *E. coli,* which cause enteric diseases are enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC) (Nataro and Kaper 1998; WHO 2009).

Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) is one of the major causes of diarrheal diseases. There are approximately 650 million cases annually, and 800 000 deaths due to ETEC infections each year (Gaastra and Svennerholm 1996). ETEC infections are most common in developing countries and are lethal mostly for children under the age of five. ETEC is also responsible for traveler's diarrhea.

Virulence factors of ETEC

The host has a defense system to protect itself against infections. The defense against diarrheal diseases compiles e.g. peristalics, normal flora, acid, mucus, bile salts and secretory IgA (Gaastra and Svennerholm 1996). In order to cause infection ETEC need to adhere strongly, and with high specificity, to the target cells of the host, otherwise there is evident risk that it is put out of action by the host's defense mechanisms. Hence, ETEC have a large number of virulence factors. The major groups of virulence factors of ETEC are colonization factors and enterotoxins.

I. Colonization factors

The colonization factors mediate adherence of the bacteria to the host small intestinal epithelium. About twenty different colonization factors have been described so far (Gaastra and Svennerholm 1996; Qadri et al. 2005). These colonization factors are divided into different groups according to their structure (Table 2). The CFA/I-like group, the CS5 group, type-IV-like group and a few do no have enough similarity to other CFs and therefore not belonging to any group. The CFA/I-like group compiles CFA/I, CS1, CS2, CS4, CS14, CS17, CS19 and PCFO71 (Gaastra and Svennerholm 1996; Nataro and Kaper 1998; Qadri et al. 2005). CS6 is an individual colonization factor.

Table 2. ETEC colonization factors in their groups. The last column contains CFs which do not belong to any group.

CFA/I-LIKE	CS5	TYPE-IV-LIKE	DISTINCT
CFA/I	CS5	CS8 (CFA/III)	CS3
CS1	CS7	CS15	CS6
CS2	CS13	CS21	CS10
CS4	CS18		CS11
CS14	CS20		CS12
CS17			
CS19			
PCFO71			

Almost one third of the ETEC strains express CFA/I (Wolf 1997). Of these strains two thirds express the heat stable enterotoxin (LT), and one third both LT and the heat-stable enterotoxin (ST).

The CFA/II group is defined by the expression of CS3 and/or CS1 or CS2 (Cassels and Wolf 1995; Wolf 1997; Torres et al. 2005), and is found on 23 % of the strains. CFA/II strains usually express both ST and LT. 21 % of the strains belong to CFA/IV group, which means that they express CS6 and/or CS4 or CS5 (Cassels and Wolf 1995; Wolf 1997; Torres et al. 2005). These strains usually express ST.

CFA/I

Colonization factor antigen I (CFA/I) was the first described CF, and is also one of the most common CFs in ETEC strains (Gaastra and Svennerholm 1996; Wolf 1997; Qadri et al. 2005). CFA/I belongs to a family of eight CFs (Table 2). These are divided into three subgroups; CFA/I, CS4 and CS14, CS1, CS17, CS19 and PCFO71, and CS2. CFA/I is a fimbriae made of a major subunit, CfaB, and a minor subunit, the tip protein CfaE. The operon contains *cfaA*, *cfaB*, *cfaC* and *cfaE* (Jordi et al. 1992). *cfaA* encodes a chaperone-like protein, and the *cfaC* gene product is a protein involved in transport of the fimbriae across the outer membrane.

CS6

Coli surface antigen 6 (CS6) is also one of the most common CFs (Qadri et al. 2005). It is non-fimbrial and the structure has not yet been defined. However, CS6 is made of two different subunits, CssA and CssB. The operon contains *cssA*, *cssB*, *cssC* and *cssD* (Wolf et al. 1997). CssC is a chaperone and CssD is an usher that transports CssA and CssB across the outer membrane.

II. Enterotoxins

ETEC can produce at least two different enterotoxins, the heat-stable enterotoxin (ST) and/or the heat-labile enterotoxin (LT). ST is a peptide of twenty amino acids and has no resemblance to LT. There are two subtypes of the heat-stable toxin, designated STa and STb. The receptor for STa, which is associated with diarrhea in humans and animals, is the extracellular domain of a trans-membrane guanylate cyclase type C protein (Schulz et al.

1990). Binding to the extracellular domain of this protein activates the intracellular activity leading to raised levels of cGMP, which ultimately results in secretory diarrhea.

The heat-labile enterotoxin (LT) (Figure 2) and is related to cholera toxin (CT), and both belong to the AB_5 toxins, which also include shiga toxin and pertussis toxin and the recently characterized subtilase cytotoxin from *E. coli* (Fan et al. 2000; Paton et al. 2004; Wang et al. 2007). These toxins are formed of one A-subunit and five B-subunits. The B-subunits form a pentamer, and binds to cell surface receptors. The actual pathogenic property resides within the A-subunit, which, in the case of LT and CT, is a potent stimulator of adenylate cyclase and cause the intestine to secrete watery fluid rich in sodium, bicarbonate and potassium, in volumes far exceeding the intestinal absorptive capacity (reviewed in (Sack et al. 2004).

The molecular weight of LT is 86 kDa. The A subunit is 28 kDa and the five B subunits are 11,5 kDa each (Nataro and Kaper 1998).

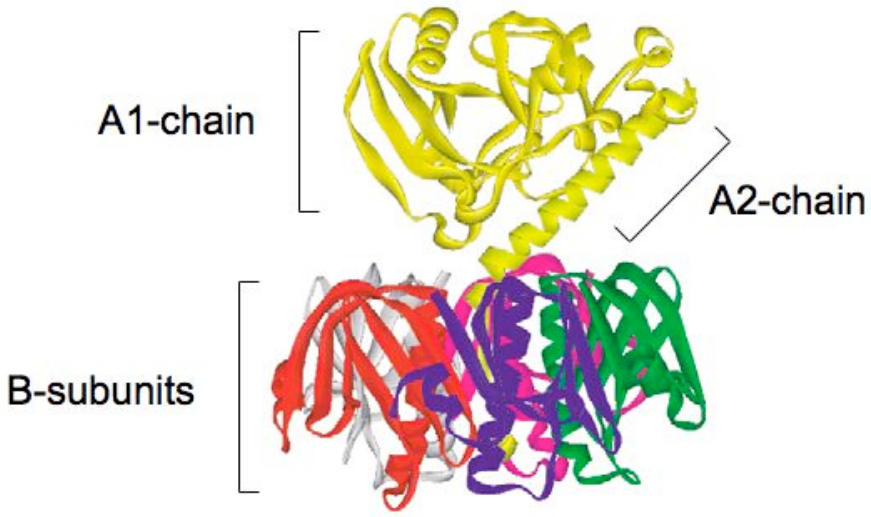

Figure 2. Crystal structure of enterotoxigenic *Eschericha coli* heat-labile enterotoxin (LT). Reprinted from (Lencer and Tsai 2003), with permission from Elsevier.

The B-subunit of the heat-labile enterotoxin from *Escherichia coli* (LTB) and cholera toxin B-subunit (CTB) has a sequence identity of 83 %. In both cases five B-subunits form a pentamer which primary receptor is the ganglioside GM1. While CTB almost exclusively recognizes this one receptor, LTB is more promiscuous than CTB, and in addition to GM1, LTB from piglet pathogenic ETEC (pLTB) binds to glycolipids and glycoproteins with *N*-

acetyllactosamine- (Galβ4GlcNAc-) terminated carbohydrate chains, such as neolacto-tetraosylceramide (Galβ4GlcNAcβ3Galβ4GlcβCer) (Orlandi et al. 1994; Teneberg et al. 1994; Ångström et al. 1994), while LTB from human pathogenic ETEC (hLTB) recognize glycoconjugates carrying blood group A and B type 2 epitopes (Ångström et al. 2000; Holmner et al. 2007). hLTB and pLTB only differ in four amino acids. These are the amino acids in position 4, 13, 46 and 102. In these positions hLTB have a serine, histidine, alanine and glutamate while pLTB have a threonine, arginine, glutamate and a lysine.

Therapeutic strategies

It is of great interest to develop a vaccine against ETEC infection since it is a major cause of diarrheal disease worldwide. One of the problems is that there are a large number of ETEC strains with different properties and virulence factors. However, one possible strategy is to construct a vaccine based on the enterotoxins and the most common colonization factors or a common denominator for them. Another possible strategy is anti-adhesive therapy (Pieters 2007). This can be done in different manners. The carbohydrate receptor on the host cell can be blocked so that adhesion by the pathogen is inhibited or carbohydrate receptor analogs can be constructed which bind to the adhesins of the pathogen and put it out of action. Carbohydrates have already been tested in studies for prevention of several bacterial infections in different animals, e.g. infections caused by *E. coli* F18 fimbriae in pigs (Nollet et al. 1999) and *E. coli* P pili in mouse (Eden et al. 1982; Johnson and Berggren 1994). This suggests that it could be a possible strategy also with ETEC infection in humans.

In 1991 a cholera vaccine, Dukoral, containing killed whole cell *V. cholerae* and recombinant non-toxic cholera toxin B-subunits (rCTB) was approved in Sweden (Läkemedelsverket 2007). The approval was foremost based on studies in Bangladesh (Clemens et al. 1986; Clemens et al. 1988; van Loon et al. 1996; Ali et al. 2005) and Peru (Sanchez et al. 1994; Taylor et al. 2000). Because of similarities between CT and LT from enterotoxigenic *E. coli* the vaccine had some protection against ETEC infection as well (Clemens et al. 1988). Therefore, in 1996 the indication for Dukoral was increased to include traveler's diarrhea. Later when Dukoral was approved in the European Union, the indication only comprised cholera infection, due to that the documentation was not sufficient concerning Dukoral's protection against ETEC infection.

Methodology

Isolation of glycosphingolipids

Glycosphingolipids are excellent tools for studies of carbohydrate interactions. This is due to the fact that glycosphingolipids have one carbohydrate unit per ceramide. In contrast, glycoproteins usually carry several different carbohydrate structures, and hence it is difficult to determine which carbohydrate sequence is involved in the binding. However, a prerequisite for using glycosphingolipids for studies of protein-carbohydrate interactions is the availability of these compounds in pure form, and with well-defined structures. The method developed at this department for isolation of total acid and non-acid glycosphingolipid fractions (Karlsson 1987) starts with extraction of lyophilized tissue with mixtures of chloroform and methanol in a Soxhlet apparatus. The extract is subjected to mild alkaline hydrolysis, followed by dialysis to remove alkali-labile phospholipids. Fatty acids, cholesterol and cholesterol esters are removed by chromatography on a silicic acid column, and thereafter acid and non-acid glycolipids are separated on a DEAE-cellulose column. The fraction containing the non-acid glycolipids is acetylated, and separated on a second silicic acid column in order to remove alkali-stable phospholipids (mainly sphingomyelin). After deacetylation and dialysis, final purification is done on DEAE-cellulose and silicic acid columns. Isolation of individual glycosphingolipids is done by chromatography on Iatrobeads silica gel columns, or by preparative HPLC.

For structural characterization of the isolated compounds a variety of methods is utilized, mainly mass spectrometry (Samuelsson et al. 1990), proton NMR spectroscopy (Koerner et al. 1983), methylation analysis using gas chromatography-mass spectrometry (Levery and Hakomori 1987; Hellerqvist 1990), stepwise exoglycosidase digestion and binding of specific ligands on thin-layer chromatograms.

Expression and purification of B-subunits

A very efficient over-expression system allowing production of large amounts of B-subunits in *V. cholerae* has been developed at the Department of Medical Microbiology and Immunology, Göteborg (Sanchez and Holmgren 1989; Lebens et al. 1993). Thus,

recombinant B-subunits were expressed in *Vibrio cholerae* strain JS1569 (Sanchez and Holmgren 1989), carrying the relevant expression plasmids. The cultures were grown overnight at 37°C in modified syncase medium (Lebens et al. 1993). The B-subunits were secreted from the bacteria and accumulated in the growth medium. The bacteria were then removed by centrifugation and filtration. The B-subunits were precipitated with hexametaphosphate (Lebens et al. 1993). The precipitate was dissolved in 50 mM Tris, pH 8 and dialyzed against phosphate buffer. The B-subunits were further purified by ion exchange chromatography (Lebens et al. 1996). The B-subunit from *Citrobacter freundii* (CFXB) was purified using a hydroxyapatite column. The purity of the B-subunits was checked with SDS-PAGE and the gels were stained with Coomassie Brilliant Blue R-250. During the production and purification of different B-subunits their ability to bind to GM1 was tested in GM1-ELISA.

Thin-layer chromatography

Thin-layer chromatography (TLC) is both a qualitative and quantitative separation method that in a physical chemistry perspective is very similar to column liquid chromatography. A thin layer of a stationary phase is coated on a glass, metal or plastic plate. The plate is put in a tank with a mobile phase in the bottom. The mobile phase move up in the plate due to capillary forces. Staining with orcinol (Klenk and Langerbeins 1941) or anisaldehyde (Waldi 1962) may be used for detection of glycosphingolipids, while the resorcinol reagent is used for detection of gangliosides (Svennerholm 1963). TLC is very useful because it is a fast method with high capacity and easy to perform.

Different compounds migrate with different velocity depending on their polarity and size. In principal, a hydrophobic compound migrates faster than a polar compound. The polar compound interacts with the stationary phase and is slowed down. The separation of glycosphingolipids depends both on the carbohydrate sequence (with a large number of possible isomers), and is influenced by heterogeneities in the ceramide moiety. Thus, a seemingly pure spot on the chromatogram may still contain several glycosphingolipids with different structures.

TLC binding assay

For binding assays (Magnani et al. 1980; Hansson et al. 1985) the TLC plates were prepared as described above. Dried chromatograms were dipped polyisobutyl-methacrylate suspension for 1 min, and dried. In theory, the coating of polyisobutylmethacrylate is supposed to mimic a cell membrane, where the hydrophobic part of the glycosphingolipid is hidden and the glycan faces the outside. However, there are also practical reasons for this plastic treatment: if it is not done the silica gel will fall off during the ensuing incubations. The chromatogram was then blocked with phosphate-buffered saline, pH 7.2, containing bovine serum albumin to prevent unspecific binding. Thereafter, the plates were incubated with ^{125}I-labeled proteins or ^{35}S-labeled bacteria, and finally the plates were washed and subjected to autoradiography.

Inhibition assay

Inhibition assays were performed by preincubation of 100 µl of radiolabeled toxin or CF with 100 µl of the sugar suspension of interest, for two hours. The concentration of sugar was usually 1 mg/ml. Thereafter the suspension was diluted 50 times and used in TLC overlay assay, or microtiter well binding assay. The same method was used for inhibition experiments with bacteria. The bacteria were washed and spinned down and dissolved in a small volume of PBS and then incubated with the sugar of interest.

Microtiter well binding assay

For binding of radiolabeled B-subunits and CS6 to glycosphingolipids in microtiter wells serial dilutions (each dilution in triplicate) of pure glycosphingolipids in methanol were initially applied to the wells. When the solvent had evaporated, the wells were blocked and thereafter, incubated with ^{125}I-labeled proteins. After washing, the radioactivity was counted in a gamma counter.

Culture and labeling of bacteria

The recombinant colonization factor antigens were expressed in the *E. coli* strain TOP10 (Tobias et al. 2008). The different TOP10 strains were grown in CFA broth with ampicillin added, and shaken over night. The bacteria were metabolically labeled using ^{35}S-methionine. 100 µl of bacterial suspension and 10 µl ^{35}S-methionine was added to 10 ml CFA broth and

grown at 37 °C for two hours with shaking. To induce expression of the recombinant proteins, isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM was added, and the bacteria were incubated at 37 °C over night with shaking.

Radiolabeling

Proteins were labeled with ^{125}I by using Na-^{125}I according to the IODO-GEN protocol of the manufacturer (Pierce, Rockford, IL). Thereby the ^{125}I gets incorporated into the tyrosine side chains of the protein. By passing the material through a gel filtration column the radiolabeled protein is separated from unbound iodine and collected.

Aims

- Investigate the potential carbohydrate recognition of ETEC colonization factors.
- Determine the relationship between the blood group A/B antigen binding and lipopolysaccharide binding of the *E. coli* heat-labile enterotoxin.
- Determine the carbohydrate binding specificity of the cholera toxin-like B-subunit CFXB from *Citrobacter freundii*.

Results and Discussion

Paper I

CFA/I is a fimbriae formed by repetitions of a major subunit, CfaB, which form a rod, and a minor subunit, CfaE, also called the tip subunit. In this study, the potential carbohydrate recognition of CFA/I was first investigated by binding CFA/I-fimbriated bacteria and purified CFA/I fimbriae to mixtures of glycosphingolipids using the thin-layer chromatogram binding assay. This showed that CFA/I seemed to have affinity for non-acid glycosphingolipids in a manner reminiscent of lactosylceramide-binding bacteria (Karlsson 1989). However, CFA/I also bound to additional glycosphingolipids not recognized by lactosylceramide-binding bacteria. No binding was seen to acid glycosphingolipid fractions, which suggest that no binding to sialic acid-containing glycosphingolipids occurs. To further define the binding properties of the CFA/I fimbriae, a large number of pure glycosphingolipids at defined concentrations were tested in the binding assay. The binding patterns of CFA/I-fimbriated bacteria **(**Fig. 3D) and purified CFA/I fimbriae (Fig. 3B) were identical, *i.e.* both ligands bound to lactosylceramide with hydroxy long chain base and/or fatty acids, isoglobotriaosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, neolactotetraosylceramide, the Le^a pentaglycosylceramide, the Le^x pentaglycosylceramide, the Le^y hexaglycosylceramide and the H5 type 2 pentaglycosylceramide (see Appendix I for glycosphingolipid structures).

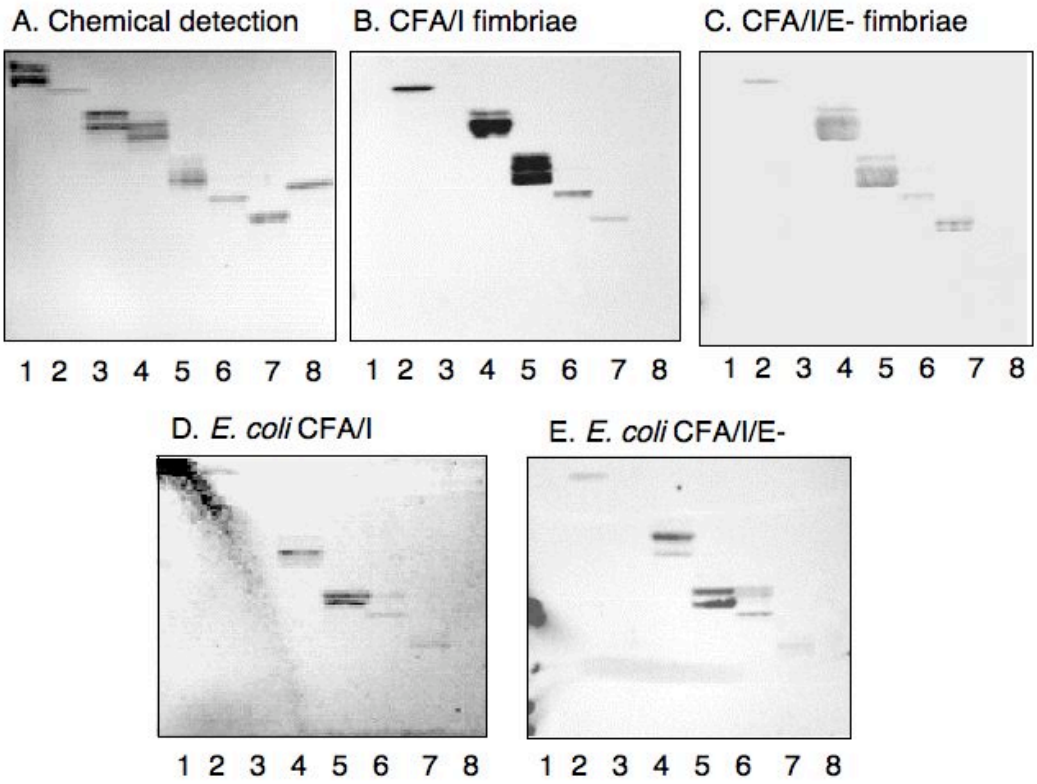

Figure 3. Binding of CFA/I fimbriae, CFA/I/E- fimbriae, and recombinant bacterial cells expressing CFA/I fimbriae and CFA/I/E- fimbriae to pure glycosphingolipids on thin-layer chromatograms. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of CFA/I fimbriae (B), CFA/I/E- fimbriae (C), CFA/I-expressing *E. coli* (D) and *E. coli* with CFA/I/E- fimbriae (E). The lanes were: Lane 1, galactosylceramide (Galβ1Cer), 2 μg; Lane 2, glucosylceramide (Glcβ1Cer), 2 μg; Lane 3, lactosylceramide (Galβ4Glcβ1Cer) with d18:1-16:0-24:0, 2 μg; Lane 4, lactosylceramide (Galβ4Glcβ1Cer) with t18:0-h16:0-h24:0, 2 μg; Lane 5, isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer), 2 μg; Lane 6, neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; Lane 7, Le^a pentaglycosylceramide (Galβ3(Fucα4)GlcNAcβ3Galβ4 Glcβ1Cer), 2 μg; Lane 8, globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer), 2 μg.

The next step was to investigate if both the major subunit and the minor subunit of the CFA/I fimbriae were essential for binding, or if the binding resided within just one of the subunits. Hence, a CfaE deletion mutant was constructed, and the glycosphingolipid recognition by both CFA/I/E- fimbriated *E. coli* (Fig. 3D) and purified CFA/I/E- fimbriae (Fig. 3C) was studied. These experiments showed that the CfaE deletion mutant had identical binding patterns to the native CFA/I fimbriae. Thus CfaB is the glycosphingolipid binding protein, while the minor subunit CfaE is not involved in CFA/I glycosphingolipid recognition.

It has previously been reported that CFA/I can cause hemagglutination (Cravioto et al. 1982) and that it is due to the CfaE tip protein (Sakellaris et al. 1999). A point mutation (R181A) in CfaE resulted in the loss of hemagglutination and adherence to CaCo-2 cells and biopsies

from human small intestine (Sakellaris et al. 1999; Li et al. 2007; Baker et al. 2009). So even if CfaE is not involved in recognition of the CFA/I binding glycosphingolipids defined in this study, it might have other important adherence properties. Still, the cellular receptor for CfaE is not yet defined.

In order to evaluate the importance of the glycosphingolipid recognition by CFA/I we compared CFA/I to other CFs, *i.e.* CS1, CS4 and CS7. CS1 and CS4 belong to the same family as CFA/I, the CFA/I group. CS4 and CFA/I also belong to the same subgroup in the family, while CS1 belongs to another subgroup. CS7 belongs to the CS5 group. The major subunit of CS4, CsaB, and CfaB have a sequence identity of 67 %. For the major subunit of CS1, CooA, and CfaB the sequence identity is 54 %. The binding patterns of these CFs were compared in a binding assay with glucosylceramide, lactosylceramide, isoglobotriaosyl-ceramide, gangliotetraosylceramide, neolactotetraosylceramide and Le^a pentaglycosyl-ceramide. All these CFA/I-binding glycosphingolipids were also recognized by CS1 and CS4. CS7 on the other hand, which major subunit, CsvA, has a sequence identity of 19 % with CfaB, showed weak or no binding to these glycosphingolipids.

The binding of CFA/I to lactosylceramide, Le^a pentaglycosylceramide, isoglobotriaosyl-ceramide and neolactotetraosylceramide was inhibited by preincubation of CFA/I with Le^a pentasaccharide or lactose.

To assess the potential role of glycosphingolipid recognition by CFA/I in the target tissue adherence, the binding of CFA/I fimbriae to non-acid glycosphingolipid fractions from human small intestine was studied. Binding of CFA/I to a compound co-migrating with Le^a pentaglycosylceramide was thereby obtained. Also monoclonal antibodies directed against Le^a determinant bound in this region. In the epithelium of human small intestine monohexosylceramides (galactosylceramide and glucosylceramide) and blood group ABH (type 1 chain) and Lewis glycolipids with 5-7 sugar residues are the major compounds (Björk et al. 1987). There are also trace amounts of type 2 carbohydrate chain glycosphingolipids, like Le^x- and Le^y-terminated compounds. The expression of the major blood group glycosphingolipids in the intestine is in agreement with the ABO, Lewis, and secretor phenotype of the individuals. Several of the CFA/I-binding compounds, like glucosylceramide, Le^a-, Le^x- and Le^y-terminated glycosphingolipids may thus function as targets for CFA/I-mediated adherence.

In a recent follow-up study the Lewis antigen expression was determined in 179 Bangladeshi children from a prospective birth cohort study in urban Dhaka, in which diarrhea caused by ETEC expressing major CFs during the first two years of life was assessed (Ahmed et al. in press). These studies showed that Le a+b- children more often had symptomatic than asymptomatic ETEC infections ($P<0.001$), whereas symptomatic and asymptomatic ETEC infections were equally frequent in Le a-b+ children. Children with Le a+b- type also had significantly higher incidence of diarrhea caused by ETEC expressing fimbriae of the CFA/I group than Le a-b+ children ($P<0.001$). In contrast, no association between Lewis blood group phenotype and diarrhea caused by ETEC expressing CS6, or by rotavirus, was found.

Paper II

Coli surface antigen 6 (CS6) is a non-fimbrial colonization factor antigen from enterotoxigenic *E. coli*. The structure of CS6 has not yet been defined. CS6 is formed by two major subunits, CssA and CssB (Wolf et al. 1997). A previous study has shown that treatment with meta-periodate abolished the binding of CS6 expressing *E. coli* to rabbit and human intestine (Helander et al. 1997), which suggest that carbohydrates are involved in the binding process.

To investigate the potential carbohydrate recognition of CS6 screening by binding of CS6-expressing *E. coli* and purified CS6 to mixtures of glycosphingolipids was performed in the same manner as with CFA/I. These assays showed binding to a fast-migrating compound in some of the mixtures containing acid glycosphingolipids (Fig. 4B, lanes 2, 5 and 6). The acid fractions comprise sialic acid-containing glycosphingolipids (gangliosides) and sulfated glycosphingolipids. No binding to more slow-migrating compounds in the acid fractions or to non-acid glycosphingolipids was obtained. Ensuing binding assays with pure glycosphingolipids at defined concentrations showed binding to sulfatide. In addition, the CS6 expressing *E. coli* and purified CS6 protein bound to sulfolactosylceramide, but not to sulfogangliotetraosylceramide.

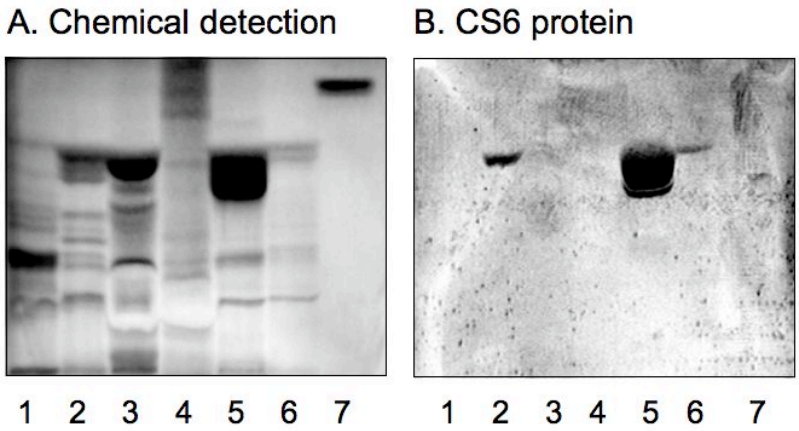

Figure 4. Binding of CS6 adhesin to mixtures of glycosphingolipids on thin-layer chromatograms. Chemical detection by anisaldehyde (A), and autoradiogram obtained by binding of ^{125}I-labeled CS6 protein (B). The lanes were: Lane 1, acid glycosphingolipids of human hepatoma, 40 μg; Lane 2, acid glycosphingolipids of human small intestine, 40 μg; Lane 3, acid glycosphingolipids of guinea pig erythrocytes, 40 μg; Lane 4, acid glycosphingolipids of guinea pig stomach, 40 μg; Lane 5, acid glycosphingolipids of human meconium, 40 μg; Lane 6, acid glycosphingolipids of human colon cancer, 40 μg; Lane 7, glucosylceramide (Glcβ1Cer), 4 μg.

Binding of CS6 expressing *E. coli* and purified CS6 protein to sulfatide was inhibited by dextran sulfate, but not with dextran, heparin, galactose-4-sulfate or galactose-6-sulfate (exemplified in Fig. 5). Sulfatide has a sulfate group in 3-position on the galactose, which seems to be important for the interaction with CS6, since galactose-4-sulfate and galactose-6-sulfate did not inhibited binding of CS6 to sulfatide.

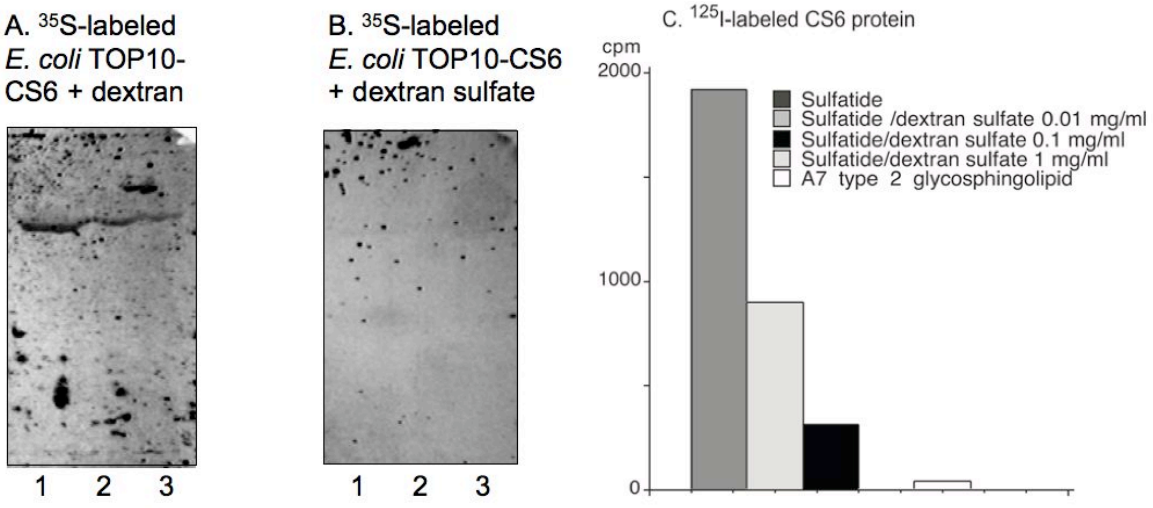

Figure 5. Effects of incubation of CS6 protein and recombinant CS6-expressing *E. coli* with dextran and dextran sulfate. Radiolabeled CS6 protein and recombinant CS6-expressing bacteria were incubated with dextran or dextran sulfate (0.01-1 mg/ml) for 1 h at room temperature. Thereafter the suspensions were utilized in the chromatogram binding assay or the microtiter well assay. Autoradiograms obtained by binding of ^{35}S-labeled *E. coli* TOP10-CS6 strain incubated with dextran (A), and with dextran sulfate (B). The lanes were: Lane 1, sulfatide (SO_3-3Galβ1Cer), 4 μg; Lane 2, sulfatide, 2 μg; Lane 3, sulfatide, 1 μg. Binding of CS6 protein, and CS6 protein incubated with dextran sulfate, to pure glycosphingolipids in microtiter wells (C).

To more thoroughly evaluate the roles of the two major subunits, CssA and CssB were expressed separately. The CssB subunit showed a very distinct binding to sulfatide, while CssA bound in an unspecific manner to both acid and non-acid glycosphingolipids. Thus, the affinity for sulfatide resides within the CssB subunit.

Interestingly, a recent study has demonstrated that the CssA subunit binds to fibronectin, and suggested a role for fibronectin in matrix adherence of CS6 expressing ETEC (Ghosal et al. 2009).

To be able to purify the subunits efficiently they were produced with affinity tags, and the binding studies were performed with these tags remaining on the subunits. In order to purify native CS6 an affinity system based on a sulfate-carrying polymer has now been constructed. On this column the CS6 protein and native CssB, but not native CssA, were retained on this column, verifying that the CssB subunit is responsible for sulfatide recognition (to be published).

Sulfatide is present in the small intestine of species susceptible to CS6-expressing *E. coli* infections, as humans and rabbits (Falk et al. 1979; Breimer et al. 1983), but not in the small intestine of species not susceptible to infection with CS6-expressing *E. coli,* as mice (Leffler et al. 1986). This suggests that the ability of CS6-expressing ETEC to adhere to sulfatide in target small intestinal epithelium may contribute to virulence.

CFA/I and CS6 are two of the most common colonization factors from enterotoxigenic *E. coli*. The binding studies with these two CFs demonstrate that they have very different carbohydrate binding patterns. CFA/I only binds to non-acid glycosphingolipids, while CS6 only binds to a few sulfate-carrying glycosphingolipids. Furthermore, for the major part of the large repertoire of CFs expressed by ETEC, the attachment sites have not yet been defined. The mechanisms of adherence mediated by the variant CFs of ETEC thus merits further studies.

Paper III

Lipopolysaccharides (LPS), which form the outer layer of the outer membrane of most gram negative bacteria is composed of a covalently-linked N,O-polyacylated disaccharide of glucosamine with two negatively charged phosphates (lipid A), a core oligosaccharide and an O-specific polysaccharide antigen (Freudenberg and Galanos 1990). Using a series of commercial LPS preparations from *E. coli*, Horstman *et al*. have reported that the heat-labile enterotoxin of ETEC, after secretion via the general secretory pathway becomes bound to outer membrane vesicles through interactions with *E. coli* LPS (Horstman and Kuehn 2000; Horstman and Kuehn 2002; Horstman et al. 2004). This binding occurs without disturbing the binding of LTB to its primary GM1 ganglioside receptor. For unclear reasons the blood group A/B binding site of LTB has been proposed to be involved in LPS binding. The aim of this study was therefore to determine the relationship between the blood group A/B binding site and the LPS binding site of LTB.

To perform this task we used a battery of B-subunits (CTB, hLTB and the CTB-hLTB hybrid LCTB*K* (Ångström et al. 2000)) in binding assays with different glycosphingolipids, LPS and LPS-derived compounds, and inhibition studies. The same commercial *E. coli* LPS preparations as in the previous studies by Horstman *et al*. were used. However, none of these studies showed any binding between the B-subunits and LPS (exemplified in Figure 6). Preincubation of B-subunits with LPS before incubation with blood group A terminated glycosphingolipids did not affect the binding to these glycosphingolipids. To eliminate the possibility that LPS was washed away during the incubation steps, control experiments with antibodies against LPS were done.

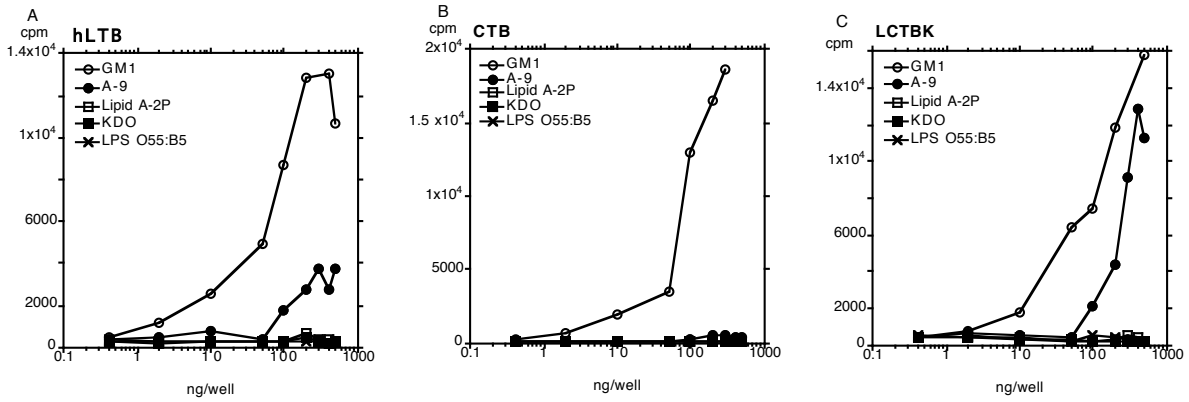

Figure 6. Binding of ^{125}I-labeled B-subunits to glycosphingolipids, lipopolysaccharides, Kdo2-lipid A and lipid A in microtiter wells. GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer); A9 type 2 glycosphingolipid (GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer); Lipid A-2P, diphosphoryl-lipid A; LPS O55:B5, LPS from *E. coli* O55:B5.

During the course of these inhibition studies we found that GM1 binding reduced the affinity for blood group A determinants and *vice versa*, suggesting that a concurrent occupancy of the two binding sites does not occur. The latter finding is related to a connection between the blood group antigen binding site and the GM1 binding site through residues interacting with both ligands.

LPS are amphiphilic molecules and easily form micelles. In the studies by Hortsman *et al.* LPS in buffer solutions were utilized. Small changes in water content, pH, temperature, cation concentration, can affect the supramolecule structure of LPS (Brandenburg et al. 1990; Brandenburg 1993). The evidence for association of LT to the bacterial cell surface through interactions with LPS is indirect, *i.e.* inhibition of binding of exogenously added LT to the bacterial surface by preincubation of the toxin with *e.g. E. coli* LPS in a buffer. However, since LPS may form different three-dimensional supramolecular structures, the interpretation of such inhibition studies is difficult.

Paper IV

Karasawa *et al*. have reported that *Citrobacter freundii* produces a cholera toxin-like B-subunit (CFXB) with high sequence identity (73 %) to CTB and LTB (Figure 7) (Karasawa et al. 2002). A CFXB-like B-subunit was also detected in some ETEC strains.

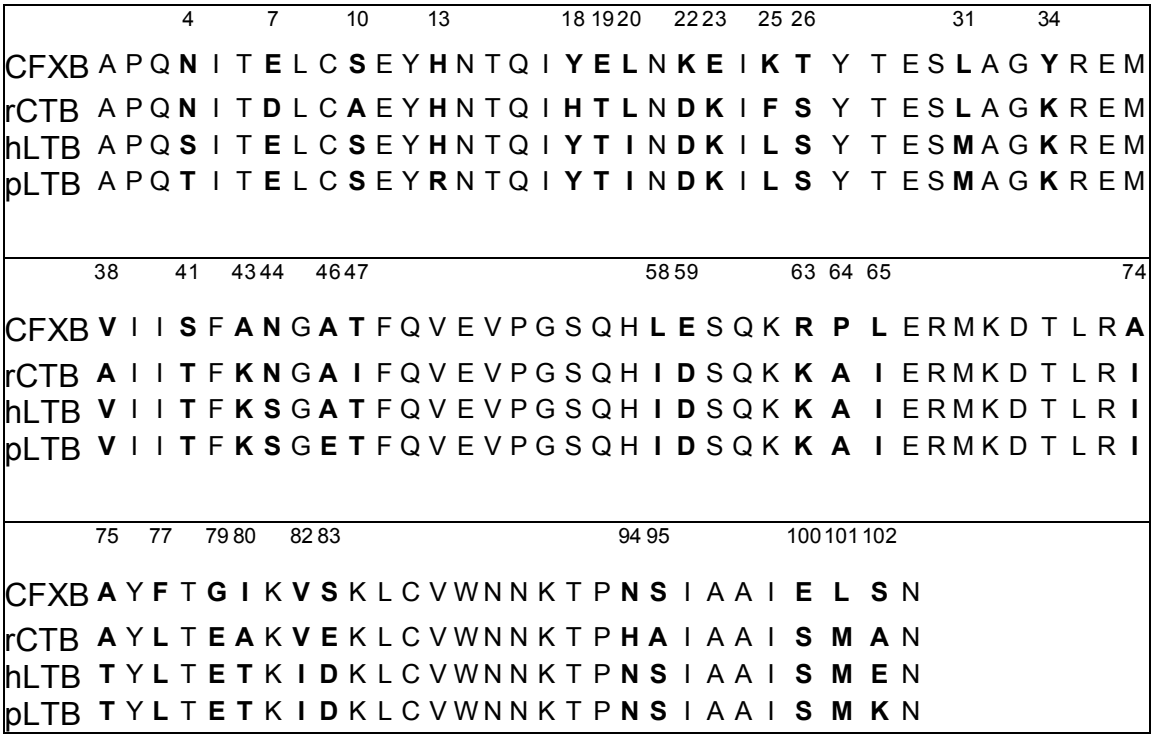

Figure 7. Alignment of amino acid sequences of CFXB, rCTB, hLTB and pLTB. Residues highlighted in bold differ between the B-subunits. The recombinant CTB has an alanine instead of a threonine in its first position (Sanchez and Holmgren 1989).

In order to determine the carbohydrate binding specificity of CFXB, this B-subunit was expressed in *V. cholerae*, and isolated in high yields (20 mg of protein obtained from 0.5 l culture medium). Glycosphingolipid binding studies show that CFXB binds to the GM1 ganglioside with high affinity (Figure 8). In addition, CFXB has high affinity for both neolacto-terminated and blood group A type 2-terminated glycoconjugates.

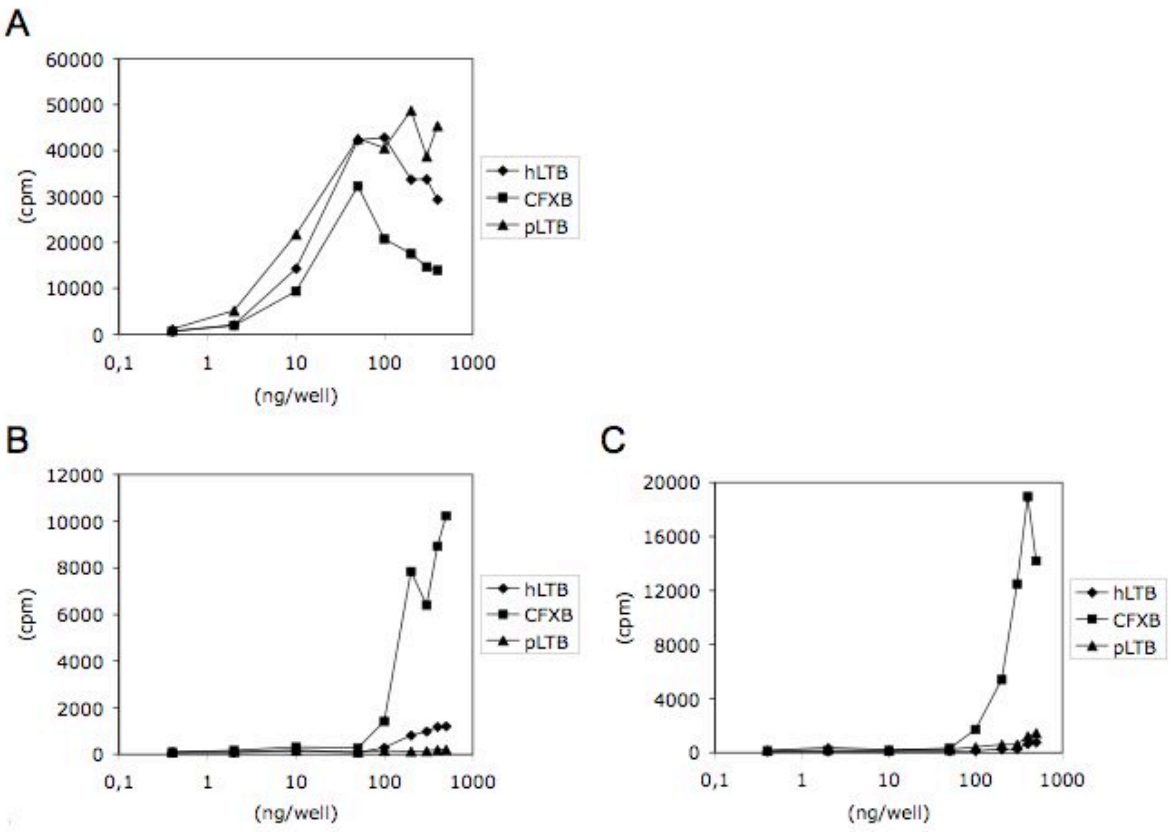

Figure 8. Binding of ^{125}I-labeled B-subunits (CFXB, hLTB and pLTB) to serial dilutions of the GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer) (A), blood group A-9 type 2 glycosphingolipid (GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (B) and neolactotetraosyl-ceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (C), adsorbed in microtiter wells.

While pLTB binds to neolacto-terminated glycoconjugates, hLTB does not recognize these carbohydrate structures. One of the differences between hLTB and pLTB is amino acid number 13, which is a histidine in hLTB and an arginine in pLTB. A hLTB H13R mutant has been constructed and this mutant received affinity for neolacto-terminated structures. Hence, the arginine in position 13 is of importance for the neolacto binding. However, CFXB also has a high affinity for neolacto-terminated glycoconjugates despite having a His at position 13. Thus, other factors must be involved in giving neolacto-specific binding, which merits further investigations.

A hybrid of CT and hLT B-subunits (LCTB*K*) with an Asn (as in CTB) at position 4, instead of Ser (as in hLTB), and having a gain-of-function mutation resulting in recognition of blood group A and B type 2 determinants has previously been constructed (Ångström et al. 2000). In the crystal structure of LCTB*K*, a novel binding site interacting with blood group A/B

determinants was identified at the top of the B-subunit interfaces (Holmner et al. 2004). The native hLTB molecule also binds to blood group A/B antigens, but with much lower affinity than LCTB*K*. However, an increased binding affinity for blood group A determinants was created by introducing a single Ser to Asn substitution at position 4 in hLTB. In the native CFXB, the residue at position 4 is also an Asn, which may contribute to the high affinity binding of this B-subunit for blood group A type 2 determinants.

One difference between *C. freundii* and *V. cholerae*/enterotoxigenic *E. coli* is that the B-subunit of *C. freundii* does not seem to have a "classical" A-subunit. The *C. freundii* gene encoding the CFXB (*cfxB*) is located downstream of an 852-nucleotide open reading frame (denoted *cfxA*) (Karasawa et al. 2002). There is no homology between the deduced polypeptide (named CFXA) and the A-subunits of CT or LT. In fact, the CFXA sequence does not resemble any other known protein sequence. However, when performing a BLAST search one find low sequence similarity with metallopeptidases, and a closer look reveals that CFXA contains a zinc binding region, the sequence HEXGHXXGXXH, as in the zinc binding region in the catalytic domain of bacterial metalloproteinases (Bode et al. 1999). Further studies are needed to investigate if there is any association between CFXA and CFXB. Still it is very tempting to speculate about this being a novel type of toxin. It should, however, be noted that no toxic effects in terms of CHO cell elongation, or fluid accumulation in the suckling mouse assay, were obtained by culture supernatants of CFXB-producing *C. freundii*.

Concluding remarks

To summarize, the investigations of carbohydrate recognition by ETEC virulence factors, reported in this thesis, have led us to conclude the following

- CFA/I and CS6, two of the most common colonization factors of ETEC, have distinctly different the carbohydrate binding preferences. CFA/I binds to a set of non-acid glycosphingolipids, while CS6 to sulfate-carrying compounds. Further studies are needed to define the potential carbohydrate binding of other CFs within the large repertoire of variant CFs expressed by ETEC.
- We found no evidence to support an involvement of the blood group A/B antigen binding site of the heat-labile enterotoxin in binding to lipopolysaccharides. Inhibition studies showed that GM1 binding led to a reduced binding of blood group A determinants, and *vice versa*, indicating that a concurrent occupancy of the GM1 binding site and the blood group A antigen binding site, is not likely.
- CFXB binds to the GM1 ganglioside, neolactotetraosylceramide and blood group A type 2-terminated glycosphingolipids, *i. e*. the carbohydrate binding preferences of this B-subunit has traits in common with both hLTB and pLTB.

There is an urgent need to develop new therapeutic strategies against ETEC-induced diarrheal disease in developing countries. One possible strategy is to interfere with the initial attachment of the bacteria to the target tissue using synthetic analogues of the binding-active carbohydrates (Ofek et al. 2003; Pieters 2007). However, given the different carbohydrate recognition profiles of CFA/I and CS6, and the fact that binding specificities of the major part of the ETEC CF repertoire are undefined, further studies are needed before an anti-adhesive approach to prevent ETEC-induced diarrhea is possible.

The binding of the B-subunits of LT to receptor glycoconjugates on the small intestinal epithelial cells is a prerequisite for the following steps in toxin action leading to diarrhea. In addition, LT elicit a strong immune responses, and is, along with CT, among the most potent mucosal adjuvants identified. The immunogenicity, and to some extent the adjuvant activity, are also dependent on receptor binding. An important further step to will be to investigate whether the ”multi-binding” mode of the B-subunit CFXB, with binding to carbohydrate receptors different from the GM1 ganglioside, will influence the immunogenic capacity. Comparison of the immunogenicity of CTB, LTB and CFXB may lead to novel insights into

how the biological activities of these B-subunits are related to recognition of different carbohydrate receptors.

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Appendix I. Summary of glycosphingolipid binding specificities of the colonization factors (CFA/I and CS6) and enterotoxin B-subunits (CFXB, CTB, hLTB and pLTB)

Trivial name	Structure	Protein
Simple compounds		
Galactosylceramide	Galβ1Cer	
Glucosylceramide	Glcβ1Cer	CFA/I[a]
Sulfatide	SO_3-Galβ1Cer	CS6
LacCer (d18:1-16:0-24:0)[b]	Galβ4Glcβ1Cer	
LacCer (t18:0-h16:0-h24:0)	Galβ4Glcβ1Cer	CFA/I
Sulf-LacCer	SO_3-3Galβ4Glcβ1Cer	CS6
Ganglioseries		
GgO3	GalNAcβ4Galβ4Glcβ1Cer	CFA/I
GgO4	Galβ3GalNAcβ4Galβ4Glcβ1Cer	CFA/I
Fuc-GgO4	Fucα2Galβ3GalNAcβ4Galβ4Glcβ1Cer	
Sulf-GgO4	SO_3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer	
Neolactoseries		
Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	CFA/I, pLTB, CFXB
H5-2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	CFA/I
Le^X-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	CFA/I
B5	Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	CFA/I

Le^{y}-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	CFA/I
B6-2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	
A6-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	
A7-2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	CFXB
A9-2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	CFXB, hLTB
Neolactohexa	Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	
Lactoseries		
Le^{a}-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	CFA/I
Le^{b}-6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	
A7-1	GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	
B7-1	Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	
A8-1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	
A9-1	GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer	
Globoseries		
Globotri	Galα4Galβ4Glcβ1Cer	
Isoglobotri	Galα3Galβ4Glcβ1Cer	CFA/I
Globotetra	GalNAcβ3Galα4Galβ4Glcβ1Cer	
Isoglobotetra	GalNAcβ3Galα3Galβ4Glcβ1Cer	
Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer	
A7-4	GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	
	Galα3Galα3Galβ4Glcβ1Cer	CFA/I

Gangliosides		
NeuAc-GM3	NeuAcα3Galβ4Glcβ1Cer	
GM2	GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	
GD2	GalNAcβ4(NeuGcα8NeuGcα3)Galβ4Glcβ1Cer	
GM3	NeuAcα3Galβ4Glcβ1Cer	
GD3	NeuAcα8NeuAcα3Galβ4Glcβ1Cer	
GM1	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	CTB, hLTB, pLTB, CFXB
GD1a	NeuAcα3Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	
GD1b	Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glcβ1Cer	
NeuAcα3SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	
NeuGcα3SPG	NeuGcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	
NeuAcα6SPG	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	
NeuAcα- Le^a	NeuAcαGalβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	
NeuAcα- Le^x	NeuAcαGalβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	
	Galβ4GlcNAcβ6(NeuAcα6Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	

[a] CFA/I, colonization factor antigen I of enterotoxigenic *Escherichia coli* (ETEC); CFXB, B-subunits from *Citrobacter freundii;* CS6, ETEC coli surface antigen 6; CTB, cholera toxin B-subunits; hLTB, B-subunits of heat-labile enterotoxin from ETEC pathogenic to humans; pLTB, B-subunits of heat-labile enterotoxin from ETEC pathogenic to pigs.

[b] In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length and the number after the colon gives the total number of double bonds in the molecule. Fatty acids with a 2-hydroxy group are denoted by the prefix h before the abbreviation *e.g.* h16:0. For long chain bases, d denotes dihydroxy and t trihydroxy. Thus d18:1 designates sphingosine (1,3-dihydroxy-2-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecene).