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No direct binding of the heat-labile enterotoxin of *Escherichia coli* to *E. coli* lipopolysaccharides

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Abstract A novel carbohydrate binding site recognizing blood group A and B determinants in a hybrid of cholera toxin and *Escherichia coli* heat-labile enterotoxin B-subunits (termed LCTB*K*) has previously been described, and also the native heat-labile enterotoxin bind to some extent to blood group A/B terminated glycoconjugates. The blood group antigen binding site is located at the interface of the B-subunits. Interestingly, the same area of the B-subunits has been proposed to be involved in binding of the heat-labile enterotoxin to lipopolysaccharides on the bacterial cell surface. Binding of the toxin to lipopolysaccharides does not affect the GM1 binding capacity. The present study aimed at characterizing the relationship between the blood group A/B antigen binding site and the lipopolysaccharide binding site. However, no binding of the B-subunits to *E. coli* lipopolysaccharides in microtiter wells or on thin-layer chromatograms was obtained. Incubation with lipopolysaccharides did not affect the binding of the B-subunits of heat-labile enterotoxin of human isolates to blood group A-carrying glycosphingolipids, indicating that the blood group antigen site is not involved in LPS binding. However, the saccharide competition experiments showed 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.

Keywords Carbohydrate binding · *E. coli* heat-labile toxin · lipopolysaccharide binding · blood group A/B binding site · GM1 binding site

Introduction

Cholera toxin (CT[1]) and heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* (ETEC) are causative agents of diarrheal diseases, cholera and traveller's diarrhea, respectively. The toxins are structurally as well as functionally and immunologically related and consist of one enzymatically active A-subunit non-covalently anchored in the centre of a homo B-pentamer. The A-subunit has ADP ribosyl-transferase activity and the B pentamers, CTB and LTB, respectively, carry carbohydrate binding domains responsible for binding the toxin to target cell receptors. The primary receptor for both toxins is the GM1 ganglioside Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer [1, 2]. The B-pentamers share about 85% sequence identity and the GM1 binding site is largely conserved. While CTB almost exclusively recognizes the

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[1] The abbreviations used are: CT, cholera toxin; CTB, cholera toxin B-subunits, ETEC, enterotoxigenic *Escherichia coli*; hLTB, heat-labile enterotoxin B-subunits from human isolates of *E. coli*; Kdo, 3-deoxy-D-*manno*-octulosonic acid; LPS; lipopolysaccharides; LT, heat-labile enterotoxin from *E. coli*.

The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: *Eur J Biochem* (1998) **257**, 293). It is assumed that Gal, Glc, GlcNAc, GalNAc and NeuAc are of the D-configuration, Fuc of the L-configuration, and all sugars are present in the pyranose form.

GM1 ganglioside, LTB has been shown to also interact with several other carbohydrate sequences on glycoproteins and glycosphingolipids [3–6].

We have previously constructed a CTB/LTB hybrid, termed LCTB*K,* which binds to blood group A and B type 2 determinants [7]. In the crystal structure of this hybrid, a novel binding site interacting with blood group A and B determinants was identified at the top of the B-subunit interfaces [8]. The native B-subunits from human isolates of *E. coli* (hLTB) also binds to blood group A/B antigens, but the binding affinity is considerably lower than for LCTB*K*. Still, a blood group A/B antigen binding site with the same location as in LCTB*K* was identified in the crystal structure of native hLTB in complex with blood group A pentasaccharide [9].

A recent series of studies have shown that the heat-labile enterotoxin and cholera toxin both bind to lipopolysaccharides (LPS) on the surface of *E. coli* bacterial cells [10–13]. The LPS-bound toxins have retained GM1 binding capacity, and LT attached to LPS is proposed to function as an adhesin, mediating the delivery of LT-containing outer membrane vesicles to target cells. Interestingly, the area harboring the blood group A/B binding site, located at the interface of the B-subunits, has also been proposed to be involved in the LPS-binding of LT [12]. The minimal structure of LPS bound by the toxin is the inner core Kdo sugar, 3-deoxy-D-*manno*-octulosonic acid, although full-length LPS is bound with higher affinity [12].

In this study a number of studies of B-subunit (hLTB, CTB and LCTB*K*) binding to glycosphingolipids, LPS and LPS-derived compounds were performed, along with saccharide inhibition studies, with the aim of defining the relationship between the hLTB blood group A/B binding site and the LPS binding site. However, no signal was obtained when direct binding of B-subunits to *E. coli* lipopolysaccharides, Kdo-Lipid A or Lipid A in microtiter wells or on thin-layer chromatograms was tested. The binding of hLTB to blood group A-carrying glycosphingolipids was not affected by incubation with LPS or Kdo saccharide, indicating that the blood group antigen site is not involved in LPS binding. Furthermore, saccharide competition experiments showed that binding of hLTB to GM1 reduced the affinity for blood group A determinants, suggesting that a concurrent occupancy of the two binding sites does not occur.

Materials and methods

Protein expression and purification

Recombinant human LTB was expressed in *Vibrio cholerae* strain JS1569 [14] carrying the relevant expression plasmid pML-hLTB*tac*. The *eltB* gene was derived from *E. coli* strain H4-114 and was subcloned from the expression vector pMMB68 [15]. The genes were expressed constitutively from the *tac* promoter. Cultures for production of hLTB were grown in 2 l flasks containing 500 ml modified syncase [16] at 37° C with shaking (180 rpm) for 24 h. Assembled pentameric hLTB was secreted from the bacteria and accumulated in the growth medium. The cells were removed by centrifugation followed by passage of the growth medium through a 0.2 micron filter. Then hLTB was precipitated with sodium hexametaphosphate as previously described [17]. The protein was further purified by ion exchange chromatography [17], followed by gel filtration. Finally, the purified protein was extensively dialyzed against 10 mM Tris-HCl, pH 7.5 and concentrated to an appropriate concentration.

Production and purification of LCTB*K* and CTB was done as described [7].

Radiolabeling

Aliquots of 100 μg of each B-subunit preparation, and antibodies, were labeled with ^{125}I by the Iodogen method [18].

Reference glycosphingolipids

Glycosphingolipids were isolated and characterized by mass spectrometry, ^{1}H NMR, and degradation studies, as described [19]. The A9 type 2 glycosphingolipid (GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) was from cat intestine [7], the GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer) from human brain [20], the A7 type 2 glycosphingolipid (GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) from dog intestine [21], and glucosylceramide (Glcβ1Cer) was from porcine kidney [22].

Binding to glycosphingolipids in microtiter wells

Binding of radiolabeled B-subunits to glycosphingolipids in microtiter wells was performed as previously described [7]. In short, 50 μl of serial dilutions (each dilution in triplicate) of pure glycosphingolipids in methanol were applied to microtiter wells (Falcon 3911, Becton Dickinson Labware, Oxnard, CA). When the solvent had evaporated, the wells were blocked for 2 h with 200 μl of phosphate-buffered saline (PBS), pH 7.2, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) NaN_3 (Sol. A). Thereafter, 50 μl of radiolabeled B-subunits (diluted in Sol. A to approximately 2×10^3 cpm/μl) were added per well and incubated for 4 h at room temperature. After washing six times with PBS, the

wells were cut out and the radioactivity counted in a gamma counter.

Binding to lipopolysaccharides, Kdo2-lipid A and lipid A in microtiter wells

Serial dilutions of *E. coli* O55:B5 LPS (Sigma, St. Louis, MO), *E. coli* Ra LPS (Sigma) in distilled water, Kdo2-lipid A (Avanti Polar Lipids, Inc., Alabaster, AL), mono- and diphosphoryl lipid A from *E. coli* (Sigma) in methanol, were applied in Falcon 3911 microtiter wells (50 μl/well, each dilution in triplicate), and left at room temperature until the solvent had evaporated. Thereafter, binding of ^{125}I-labeled B-subunits was done as described above. In control binding assays LPS adsorbed in microtiter wells was detected with rabbit anti-*E. coli* O55 monospecific antiserum (Statens Serum Institute, Copenhagen, Denmark), diluted 1:100, and ^{125}I-labeled polyclonal swine anti-rabbit secondary antibodies (DakoCytomation Norden AB, Solna, Sweden), essentially as described by Urbina *et al*. [23].

Inhibition studies

For inhibition experiments, 10 μg of ^{125}I-labeled hLTB in 0.5 ml Sol. A (4×10^3 cpm/μl) was incubated with *E. coli* O55:B5 LPS 1 mg/ml, *E. coli* Ra LPS 1 mg/ml, Kdo saccharide (Sigma) 10 mM, or GM1 pentasaccharide (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glc; IsoSep, Tullinge, Sweden) 1 mM, for 2 h at room temperature. The solutions were then diluted to 1×10^3 cpm/μl and used for binding to the A9 type 2 glycosphingolipid in the microtiter well binding assay, as above.

In a separate series of experiments, 10 μg (1.2 nmol) of ^{125}I-labeled LCTB*K* in 100 μl Sol. A (4×10^3 cpm/μl) was incubated with 1 or 10 mM GM1 pentasaccharide, or 1 or 10 mM A type 2 pentasaccharide (GalNAcα3(Fucα2)Galβ4(Fucα3)Glc; IsoSep), for 2 h at room temperature. Thereafter, the solutions were diluted to 1×10^3 cpm/μl and used for binding to the GM1 ganglioside, the A9 type 2 glycosphingolipid or the A7 type 2 glycosphingolipid in the microtiter well binding assay, as described above.

Thin-layer chromatogram binding assay

Binding of ^{125}I-labeled B-subunits to glycosphingolipids, LPS, Kdo2-lipid A and lipid A on thin-layer chromatograms was performed according to Alaniz *et al*. [24], with minor modifications. In short, reference GM1 ganglioside (0.1 μg), reference glucosylceramide (4 μg), *E. coli* O55:B5 LPS (10–40 μg), *E. coli* Ra LPS (10–40 μg), Kdo2-lipid A (5–20 μg), mono- and diphosphoryl lipid A (5–20 μg each) were separated on aluminium-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using 2-propanol/7% $NH_4(OH)$ (6:4, by volume) as solvent system. One chromatogram was stained with anisaldehyde [25], while the other chromatograms were dipped for 1 min in diethylether/*n*-hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich Chem. Comp. Inc., Milwaukee, WI). After drying, the chromatograms were soaked in Sol. A for 2 h at room temperature. A suspension of ^{125}I-labeled B-subunits (2×10^3 cpm/μl) diluted in Sol. A was thereafter gently sprinkled over the chromatograms, followed by incubation for 2 h at room temperature. Finally, the plates were washed six times with PBS. Dried chromatograms were autoradiographed for 12–48 h using XAR-5 X-ray films (Eastman Kodak, Rochester, NY) and an intensifying screen.

Results

Binding of B-subunits to blood group A-terminated glycosphingolipids

The microtiter well assay showed that the three B-pentamers bound to GM1 with similar affinities (Fig. 1). In addition, hLTB and the CTB/hLTB hybrid LCTB*K* bound to the A9 type 2 glycosphingolipid, while no binding of CTB to this compound occurred. However, the blood group A binding obtained with hLTB was of lower affinity than for LCTB*K*, when compared with the GM1 binding affinity.

Lipopolysaccharide binding assays

In parallel with the glycosphingolipid binding experiments, the binding of hLTB, CTB and LCTB*K* to LPS from *E. coli* O55:B5, Kdo2-lipid A and lipid A adsorbed in microtiter wells was examined. However, despite the normal GM1 binding, no binding of the B-subunits to these compounds was obtained (Fig. 1). In addition, hLTB binding to LPS from *E. coli* Ra was tested but gave no signal (data not shown). To ensure that the LPS was indeed adsorbed in the microtiter wells control experiments using rabbit anti-*E. coli* O55 monospecific antiserum and ^{125}I-labeled secondary anti-rabbit antibodies were performed (Fig. 1d).

Binding of the B-subunits to LPS was also evaluated in the thin-layer chromatogram binding assay. The major part of the LPS from *E. coli* O55:B5 (Fig. 2, lane 1) and LPS from *E. coli* Ra (Fig. 2, lane 2) migrated just above the application line. This slow-migrating material was stained green by anisaldehyde, and thus carbohydrate-containing. Both LPS preparations also contained bands migrating close to the solvent front. These bands co-migrated with reference phosphoethanolamine, and disappeared upon mild

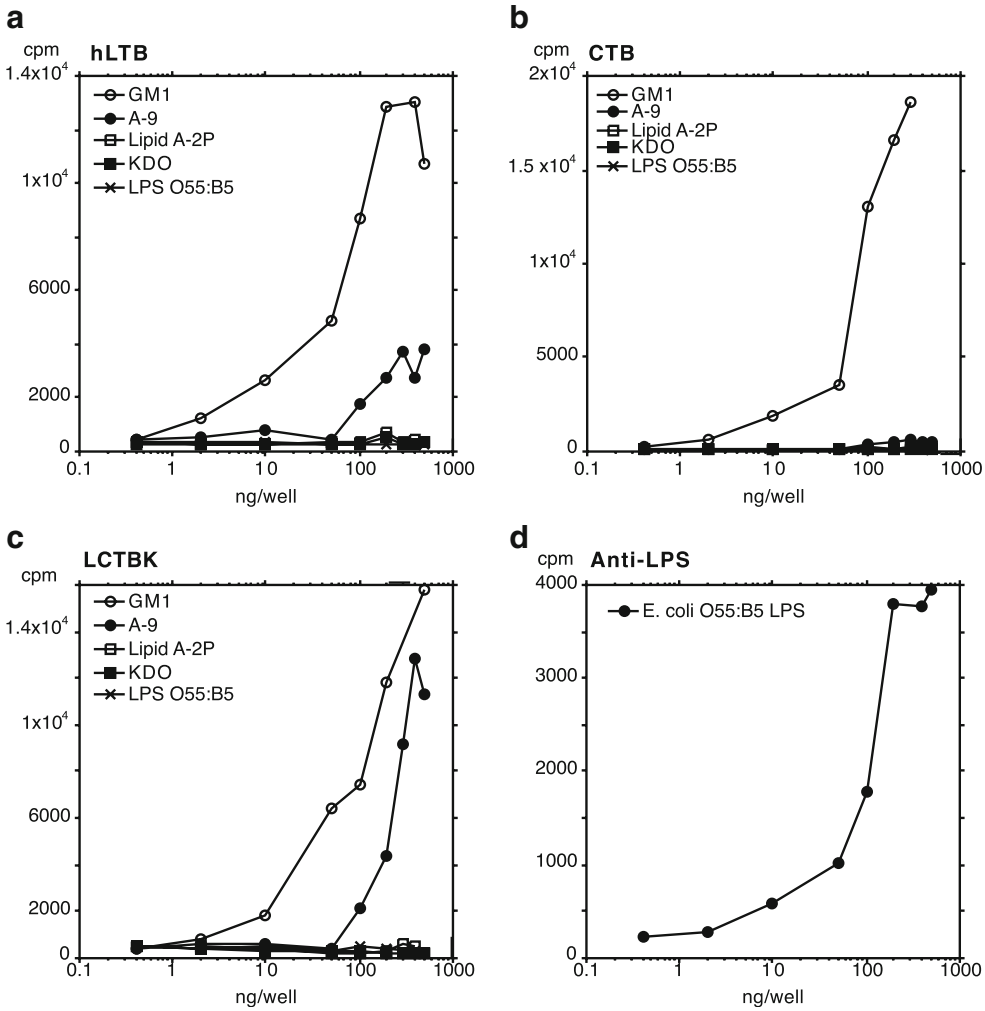

Fig. 1 Binding of ^{125}I-labeled B-subunits and anti-LPS antibodies to glycosphingolipids, lipopolysaccharides, Kdo2-lipid A and lipid A in microtiter wells. (A) binding of heat-labile enterotoxin B-subunits from human isolates of *E. coli,* (B) binding of cholera toxin B-subunits, (C) binding of the cholera toxin/heat-labile enterotoxin B-subunit hybrid LCTB*K*, and (D) binding of rabbit anti-*E. coli* O55 monospecific antiserum. Serial dilutions of the GM1 ganglioside, the A9 type 2 glycosphingolipid, *E. coli* O55:B5 LPS, Kdo2-lipid A and lipid A were adsorbed in microtiter wells, and the binding assay was performed as described in the Experimental procedures section. Data are expressed as mean values of triplicate determinations, after subtraction of background values. GM1, GM1 ganglioside (Galβ3GalNAcβ4 (NeuAcα3)Galβ4Glcβ1Cer); A9, A9 type 2 glycosphingolipid (GalNAcα3(Fucα2)Galβ4 (Fucα3)GlcNAcβ3Galβ4Glc NAcβ3Galβ4Glcβ1Cer); Lipid A-2P, diphosphoryl-lipid A; LPS O55:B5, LPS from *E. coli* O55:B5

alkaline hydrolysis (data not shown). Thus, these fast-migrating bands most likely were glycerol-based phospholipid contaminants. No binding of hLTB, CTB or LCTB*K* to any of these compounds occurred, not even when high amounts of LPS (40 μg) from *E. coli* O55:B5 and *E. coli* Ra were applied on the thin-layer plates (exemplified in Fig. 2, lanes 1 and 2). Still, the GM1 ganglioside (0.1 μg) was readily recognized (Fig. 2, lane 4). Binding of B-subunits to Kdo2-lipid A and lipid A on thin-layer chromatograms was also tested, but no binding was observed (data not shown).

The results from binding of B-subunits to the different compounds are summarized in Table 1.

Inhibition studies

Since the blood group A/B binding site of hLTB has been proposed to be involved in the LPS-binding of LT [12], the effect of LPS on blood group A/B antigen binding was next examined. hLTB was incubated with *E. coli* O55:B5 LPS, *E. coli* Ra LPS, Kdo saccharide, or GM1 pentasaccharide, before binding to the A9 type 2 glycosphingolipid in microtiter wells. However, no effect of incubation with LPS or Kdo saccharide on the binding of hLTB to this blood group A-terminated glycosphingolipid was obtained (Fig. 3).

However, a further observation from these studies was that the binding of hLTB to the the A9 type 2 glycosphingolipid was decreased by the GM1 pentasaccharide (Fig. 3). This inhibitory effect of the GM1 pentasaccharide upon blood group A binding of hLTB was unexpected since the blood group binding site of hLTB is located at the top of B-subunit pentamer interfaces, distinct from the GM1 binding site [9]. Inspection of crystal complexes of hLTB and A-pentasaccharide [9], and of LCTB*K* and A-pentasaccharide (Fig. 4) [8], showed that in both cases a number of amino acid residues involved in GM1 binding also interacts with the blood group A pentasaccharide, suggesting that a simultaneous occupancy of the two binding sites may be not possible. To test this hypothesis a series of further competition experiments were performed, using the hybrid LCTB*K* that binds to blood group A/B

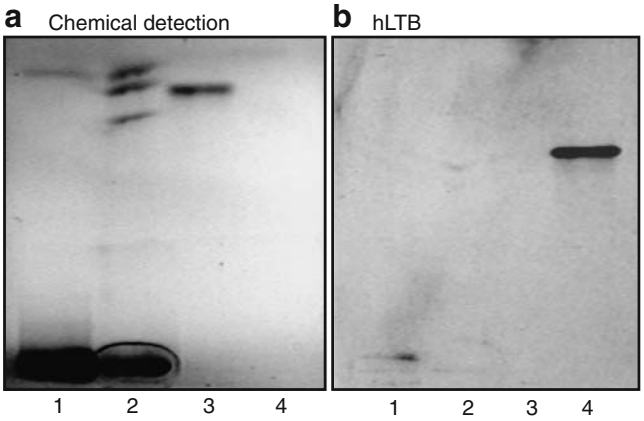

Fig. 2 Binding of hLTB to lipopolysaccharides and glycosphingolipids on thin-layer chromatograms. The left panel (A) is a chromatogram stained with anisaldehyde, while the right panel (B) is an autoradiogram obtained after binding of ^{125}I-labeled hLTB. The lanes were: Lane 1, lipopolysaccharides from *E. coli* O55:B5, 40 μg; Lane 2, lipopolysaccharides from *E. coli* Ra, 40 μg; Lane 3, glucosylceramide (Glcβ1Cer), 4 μg; Lane 4, GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer), 0.1 μg. The chromatogram binding assay was done as described under Experimental procedures. Autoradiography was for 12 h

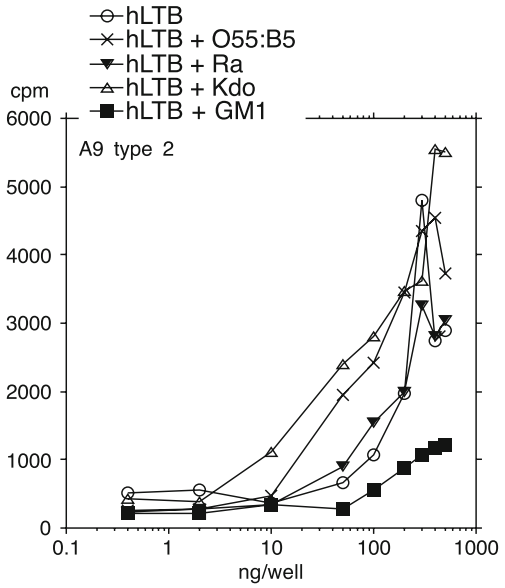

Fig. 3 Effects of incubation of hLTB with lipopolysaccharides and saccharides on binding to the A9 type 2 glycosphingolipid GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4 Glcβ1Cer. Radiolabeled hLTB was incubated with *E. coli* O55:B5 LPS, *E. coli* Ra LPS, Kdo saccharide or GM1 pentasaccharide in PBS for 2 h at room temperature. Thereafter, the suspensions were utilized in the microtiter well binding assay, as described in the Experimental procedures section. hLTB, hLTB alone; hLTB+O55:B5, hLTB incubated with *E. coli* O55:B5 LPS (1 mg/ml); hLTB+Ra, hLTB incubated with *E. coli* Ra LPS (1 mg/ml); hLTB+Kdo, hLTB incubated with Kdo saccharide (10 mM); hLTB+GM1, hLTB incubated with GM1 pentasaccharide (1 mM)

determinants with higher affinity than hLTB does. As shown in Fig. 5, LCTB*K* binding to the GM1 ganglioside is partly inhibited by incubation with 10 mM blood group A pentasaccharide, and binding to the A-7 type 2 glycosphingolipid is decreased by incubation with 10 mM GM1 pentasaccharide. Upon using 1 mM saccharide only inhibition of the binding of LCTB*K* to the blood group A-terminated glycosphingolipid by the GM1 pentasaccharide was obtained, while the A pentasaccharide at this concentration did not affect the GM1 ganglioside binding (data not shown).

Discussion

Recent evidence indicates that LT after secretion via the general secretory pathway becomes bound to outer membrane vesicles through interactions with *E. coli* lipopolysaccharides [11, 12]. The blood group A/B binding site of LTB has been proposed to be involved in LPS binding, although the reasons given for this proposal are unclear and not supported by any experimental data [12]. Binding of CTB to *E. coli* LPS has also been described [12], although this B-subunit is devoid of blood group A/B binding capacity.

Our initial ambition was to determine the relationship between the LPS binding site and the blood group A/B binding site. In the studies by Horstman *et al*. LPS from *E. coli* O55:B5 and *E. coli* Ra were shown to interfere with

Table 1 Summary of results from binding of B-subunits to glycosphingolipid, lipopolysaccharides, and lipopolysaccharide-derived compounds in microtiter wells and on thin-layer chromatograms

	GM1	Blood group A	LPS[a]	Kdo2-lipid A	Lipid A[b]
CTB	+	−	−	−	−
hLTB	+	+	−	−	−
LCTBK	+	+	−	−	−

[a] LPS from *E. coli* O55:B5 and *E. coli* Ra

[b] Mono- and diphosphoryl lipid A from *E. coli*

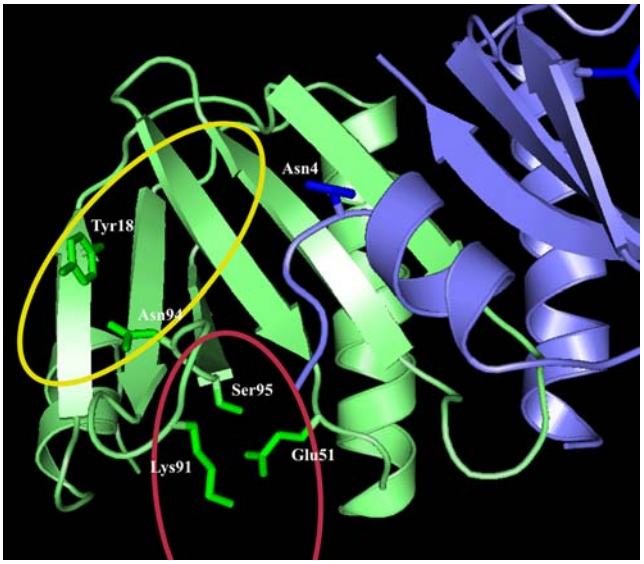

Fig. 4 Communication between the GM1 binding site and the blood group A/B binding site of LCTB*K*. This figure shows one of the subunit interfaces of the LCTB*K* pentamer displaying some of the amino acid side chains directly or indirectly involved in binding of either the A5 pentasaccharide (Asn4, Tyr18 and Asn94) or the terminal Galβ3 residue of the GM1 pentasaccharide (Glu51, Gln56, Lys91 and Ser95). The A5 binding site is approximated by the yellow ellipse, whereas the GM1 binding site is shown as a red ellipse. In the former site the Tyr18 and Asn94 side chains hydrogen bond each other and are also involved in hydrogen bonding to several structural water molecules, some of which in turn hydrogen bond to each other as well as to sugar residues [8]. The GM1 site also reveals an intricate hydrogen bonding network in which the Ser95 side chain bonds to the one of the oxygens of the Glu51 sidechain while the other oxygen is involved in bifurcated interactions with the Gln56 and Lys91 sidechains. The sidechains just mentioned all interact with the terminal Galβ3 residue of GM1 [29]. Structural perturbations originating from saccharide binding in either site is transmitted to the other site mainly via Asn94 — Ser95 bridging the two sites

binding of LT to the cell surface of *E. coli* [11, 12]. However, when direct binding of hLTB to full-length LPS from *E. coli* O55:B5 and *E. coli* Ra, Kdo2-lipid A and lipid A on thin-layer chromatograms, or coated in microtiter titer wells, was attempted, no binding was obtained. Furthermore, incubating hLTB with a large excess of LPS or Kdo saccharide had no inhibitory effect on the binding to blood group A-terminated glycosphingolipids, indicating that the blood group antigen binding site of hLTB is not involved in LPS binding.

LPS are amphiphilic molecules 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 [26]. A number of complex three-dimensional supramolecular structures formed by LPS, as *e.g.* lamellar, cubic and the hexagonal H_{II} phase, have been defined, and structural transitions may be obtained by small changes of ambient conditions as *e.g.* water content, pH, temperature and concentration of divalent cations [27, 28]. The aggregation behavior of LPS from *E. coli* O55:B5 has been studied by dynamic light scattering, steady-state fluorescence, NMR diffusometry and cryo-TEM, demonstrating a great variation in the size of the amphiphilic head group and a concomitant broad molecular weight distribition [29]. In PBS buffer, ph 7.2, the aggegation of this LPS starts at 10 μg/ml and proceeds up to 300 μg/ml. 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* O55:B5 or Ra LPS in a buffer [11, 12]. However, since LPS may form different three-dimensional supramolecular structures, the interpretation of such inhibition studies is difficult.

Unexpectedly, during the course of these studies we found that the GM1 pentasaccharide had an inhibitory effect on the binding of hLTB to blood group A-terminated glycosphingolipids, although the GM1 binding site and the blood group A/B binding site of hLTB are at distinct locations [9]. To further explore this finding a number of binding studies were done using LCTB*K*, a hybrid between CTB and hLTB, having a binding site recognizing blood group A and B determinants at a higher affinity than the native hLTB [7, 8]. By inspection of the crystal complexes of LCTB*K* and A-pentasaccharide [8], and hLTB and A-pentasaccharide [9], we found that the GM1 binding site [30] and the blood group A/B binding site are physically connected. This communication between the binding sites starts with residues Tyr18 and Asn94 at the A/B site *via* the 90–95 loop to residues Ser95, Glu51, Gln56 and Lys91 at the GM1 site, with Asn94 — Ser95 bridging the two sites (Fig. 4). The proposed communication between the two binding sites was verified by competition experiments showing that at high concentrations of oligosaccharides the binding of LCTB*K* to the GM1 ganglioside was reduced by the blood group A pentasaccharide, and binding to blood group A-terminated glycosphingolipids decreased by GM1 pentasaccharide. At lower concentrations only inhibition of binding to the blood group A-terminated glycosphingolipid by the GM1 saccharide was obtained. Thus, a simultaneous binding to the GM1 ganglioside and blood group A/B antigens is not likely, and given the higher affinity for the GM1 ganglioside it will be the preferred ligand. Although not possible to verify experimentally (see above) this indicates that accomodation of LPS into the blood group antigen binding site with retained GM1 binding capacity is not likely.

Binding of LTB, but not CTB, to *N*-acetyllactosamine-terminated glycoconjugates has also been reported [5, 6]. The *N*-acetyllactosamine sequence is found in *E. coli* LPS [31], and thus this carbohydrate sequence is a also a

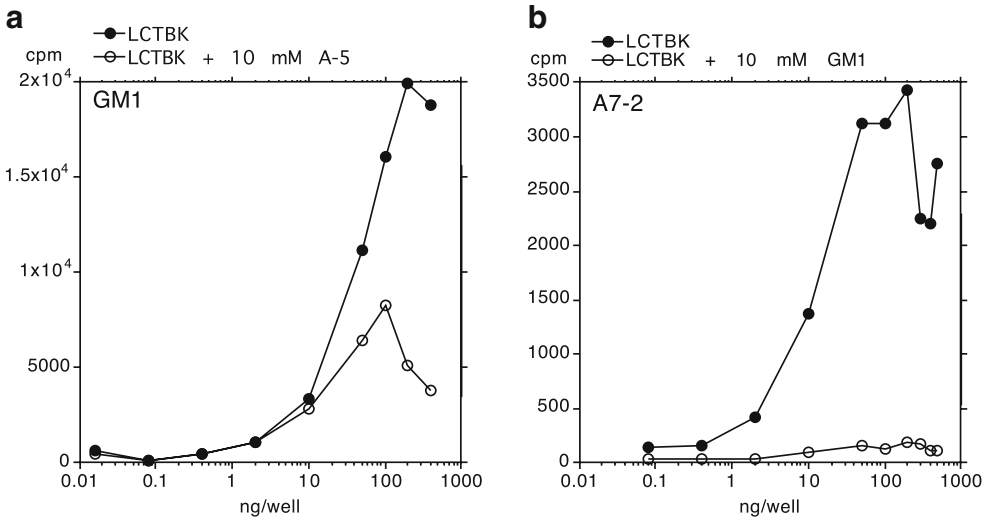

Fig. 5 Effects of incubation of LCTB*K* with oligosaccharides. Radiolabeled LCTB*K* was incubated with GM1 pentasaccharide or A5 type 2 pentasaccharide in PBS for 2 h at room temperature. Thereafter, the suspensions were utilized in the microtiter well binding assay, as described in Experimental procedures. (A) Binding of LCTB*K*, and LCTB*K* incubated with 10 mM A5 type 2 pentasaccharide (A-5), to the GM1 ganglioside (Galβ3GalNAcβ4(NeuAcα3) Galβ4Glcβ1Cer); (B) Binding of LCTB*K*, and LCTB*K* incubated with 10 mM GM1 pentasaccharide, to the A7 type 2 glycosphingolipid (Galα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3 Galβ4Glcβ1Cer)

candidate for mediating hLTB-LPS interactions. However, molecular modeling and docking studies indicate that the *N*-acetyllactosamine-terminated carbohydrates are accomodated within the GM1 binding site [32]. Since the GM1 binding is not compromised by the reported binding of LTB to LPS, and since also CTB which is devoid of *N*-acetyllactosamine is reported to bind LPS, it is not likely that this carbohydrate sequence is involved in the LPS binding of CTB or hLTB.

Recently, there have been several reports about carbohydrate structures belonging to the ABO blood group system being recognized by the heat-labile enterotoxin from human isolates [33–38], and a reduction of hLT-induced fluid accumulation in GM1-blocked HT-29 cells upon additional blockage of blood group ligands has been reported [37, 38].

However, the relationship between the blood group ABO phenotype of the infected individuals and the severity of the diarrheal diseases induced by *V. cholerae* and enterotoxigenic *E. coli* is still a confusing issue. For cholera there is a strong correlation between the severity of the infection and the blood group ABO phenotype of infected individuals, with blood group O individuals being more prone to develop severe diarrhea upon contracting *V. cholerae* infection than individuals with blood groups A, B or AB [39, and references therein]. Still, CTB does not bind to the blood group A determinant (7; Fig. 1b). This lack of CTB binding to blood group A type 2-terminated glycoconjugates is most likely due to the cumulative effect of three structural differences, as revealed by structural comparisons between LCTB*K* and hLTB, on the one hand, and CTB, on the other [8, 30]. Two of these involve loss of bridging water molecules between Glu7# (Asp in CTB) and the GalNAcα3 acetamido group, and between Asn94 (His in CTB) and the GlcNAcβ 6-OH. The third effect involves perturbation of several water molecules close to Tyr18 (His in CTB) in close proximity to the reducing GlcNAcβ residue.

In contrast, there is no clearly established association between severity of the diarrheal disease and ABO blood group for ETEC-associated diarrhea. One provocation study using LT-producing *E. coli* reported a significantly higher attack rate in blood group O volunteers [40], but the same correlation was not found in a later case-control study [41]. However, recent epidemiological evidence show that individuals with blood group A are more susceptible to ETEC diarrhea than blood group O individuals [42], *i.e.* the relationship between the blood group ABO phenotype of the infected individuals and the severity of the diarrhea is the reverse of that seen in cholera.

However, the relevance of the blood group A/B binding capacity of hLT for toxin action still remains to be shown, and further studies are needed.

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