Inhibition of *Escherichia coli* heat-labile enterotoxin by neoglycoprotein and anti-lectin antibodies which mimic GM1 receptor

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Received 20 March 2002; received in revised form 2 September 2002; accepted 3 September 2002

First published online 5 October 2002

Abstract

*Escherichia coli* producing heat-labile enterotoxin is responsible for numerous cases of diarrhea worldwide, leading to considerable morbidity and mortality. The B subunits of this toxin are responsible for the binding to the receptor, the complex ganglioside GM1 which has galactose as its terminal sugar. In this study we showed that analogs of galactose (gal) and N-acetylgalactosamine (GalNAc) interfere with the binding of heat-labile toxin to GM1. Antibodies to lectins which mimic sugar structures and neoglycoprotein were employed. These compounds were able to inhibit heat-labile toxin activity efficiently in Vero cells: 37 \( \mu \)g of IgG-enriched fraction from an antiserum inhibited up to 70% of this activity, and 50% of the binding of heat-labile toxin to GM1. Neoglycoprotein was more efficient than antibodies, since 2.5 \( \mu \)g of this ligand completely abolished the activity of heat-labile toxin on Vero cells. These data suggest that these molecules could be developed for prophylaxis and diagnosis of diarrhea caused by heat-labile toxin.

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Keywords: GM1 receptor; Heat-labile enterotoxin; Neoglycoprotein; Anti-lectin antibody; *Escherichia coli*

1. Introduction

Strains of *Escherichia coli* producing heat-labile enterotoxin (LT-I) are important pathogens responsible for traveler’s diarrhea and the cause of more than 700,000 child deaths per year due to acute diarrhea in developing countries [1]. This toxin belongs to a structurally and functionally related AB\(_5\) enterotoxin family, in which the A polypeptide assembles non-covalently with five identical B polypeptides to form the holotoxin. After being released into the jejunum, LT first binds to intestinal epithelial cells through its B pentameric subunits [2,3]. The A subunit of LT is then translocated across the cell membrane. Inside the intestinal cell, A subunit modifies the \( \alpha \) subunit of the trimeric protein G\(_s\) so that G\(_{sa}\) loses its GTP\(_\beta\)ase activity and remains constitutively in its GTP-bound state. This causes a continuous stimulation of adenylate cyclase and consequently increases the level of cyclic AMP in the cell, leading to massive loss of fluid and ions, resulting in osmotic diarrhea [4,5].

The pentameric B subunit of LT exhibits lectin-like activity and recognizes the oligosaccharide portion of GM1 ganglioside molecules on the surface of the epithelial cells with high affinity and stability [6]. GM1 has the ceramide moiety firmly embedded in the outer layer of plasma membrane and the oligosaccharide portion is a branched pentasaccharide with the sequence galβ1-3-galNAcβ1-4(Neu5Acα2-3)galβ1-4glc. These sugars are in the D configuration and the glucose moiety is covalently linked to ceramide [7,8].

Currently, there is no prophylaxis and only very labor-intensive therapy against diarrhea caused by LT. Therefore, the search for inhibitors that can block LT is very important for the development of effective drugs for prevention and treatment of LT-induced diarrhea [9,10]. Recently, a chemical library targeting a novel hydrophobic pocket in the receptor-binding site of LT was constructed based on galactose derivatives and screened for high affinity to the receptor-binding site of LT [9]. These authors
identified compounds that have higher affinity toward the receptor-binding site of LT than the parent compound.

In the present study, we examined the inhibition of binding of LT with Vero cells and GM1 using the IgG-enriched fraction of anti-lectin antibodies and neoglycoprotein.

2. Materials and methods

2.1. Bacterial culture conditions

Enterotoxigenic E. coli (ETEC) strain H10407 producing LT-I [11] was grown in LB broth at 37°C for 18 h under constant shaking (200 rpm). The bacterial growth was centrifuged at 5000 × g and the supernatant was filtered through a 0.45 μm membrane. These conditions were employed in all experiments.

2.2. GM1 analogs

2.2.1. Anti-lectin antibody

New Zealand rabbits were immunized with 2 ml of a 1 mg ml⁻¹ solution of each lectin (Table 1) in complete Freund’s adjuvant. After 21 days, the rabbits were given booster injections three times at 10 days intervals using the same protein concentration in incomplete Freund’s adjuvant.

2.2.2. Neoglycoprotein

N-acetylated galactosamine (β-GalNAc), N-acetylated glucosamine (β-GlcNAc), manno-β-pyranoside (α-man) sugars were conjugated with bovine serum albumin (BSA). These chemical compounds were purchased from Sigma Chemical Co., and were employed for inhibition assays.

2.3. LT antisera

Mice were immunized intraperitoneally with 2 μg of LT (Sigma) in complete Freund’s adjuvant. The animals were given booster injections two times using the same protein concentration in incomplete Freund’s adjuvant at intervals of 14 days and sera were subsequently titrated by enzyme-linked immunosorbent assay (ELISA) [12].

2.4. IgG-enriched fraction of antisera

The IgG-enriched fractions were obtained from rabbit antisera after being submitted to caprylic acid and ammonium sulfate precipitation [13]. The purity of this fraction was analyzed in a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [14,15] gel stained with Coomassie blue R-250.

2.5. Inhibition of LT-I activity in Vero cells

Vero cells seeded at 1 × 10⁵ cells per well in a 96-well tissue culture microplate were incubated with test samples for 24 h at 37°C in an atmosphere of 5% CO₂/95% air. Cell damage was determined by measuring the absorbance at 620 nm after staining the cells with 0.2% of crystal violet in 20% methanol [16]. Test samples consisted of supernatants of ETEC strain H10407 preincubated with analogs for 2 h at room temperature. All experiments were carried out in quadruplicate in three independent experiments.

2.6. LT GM1 ELISA

Microtiter plates (C96 Maxisorp-NUNC) were incubated at 37°C for 16 h with 10 μg ml⁻¹ ganglioside GM1 in phosphate-buffered saline, pH 7.2 (PBS). Unattached ganglioside was removed by washing the wells two times with PBS. Additional binding sites on the plate surface were blocked by incubating the wells with 1% bovine serum albumin (BSA)-PBS solution for 30 min at 37°C and then washing three times with PBS. The test samples were diluted in 0.1% BSA-PBS, added to the microplates and incubated for 30 min at room temperature. Unbound toxin was removed from the wells by washing them three times with PBS. Toxin bound to GM1 was then detected as described by Minke et al. [10]. Test samples consisted of supernatants of ETEC H10407 preincubated with analogs for 2 h at room temperature. All experiments were carried out in quadruplicate in three independent experiments.

2.7. Data analysis

ELISA optical densities and cell damage data were analyzed by mean and standard error using GraphPad Prism 3.00®. Analysis of the correlation between data was performed using the Student’s t-test. Differences were considered significant when the probability of equality was less than 0.05 (P < 0.05).

3. Results and discussion

The LT B subunit binding to glycoconjugate receptors on the small intestinal epithelial cells is a prerequisite for toxin action leading to diarrhea [2]. This subunit exhibits

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Abbreviation</th>
<th>Sugar specificity</th>
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<tbody>
<tr>
<td>Arachis hypogaea</td>
<td>PNA</td>
<td>β-gal(1-3)GalNAc</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>ConA</td>
<td>α-man, α-glc</td>
</tr>
<tr>
<td>Enomysurus europaeus</td>
<td>EE</td>
<td>α-gal(1-3)gal</td>
</tr>
<tr>
<td>Triticum vulgaris</td>
<td>WGA</td>
<td>(GlcNAc)₂, NeuNAc</td>
</tr>
<tr>
<td>Wisteria floribunda</td>
<td>WFA</td>
<td>GalNAc</td>
</tr>
</tbody>
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aCommercially obtained from Sigma (USA).
Lectin-like activity and its natural cell surface receptor is the ganglioside GM1, which has galactose as its terminal sugar [7,8]. N-acetyllactosamine-terminated glycoconjugates, gangliotetraosylceramide and the GD1b ganglioside have also been reported to present carbohydrate-binding specificity for the B subunit of LT [6,17-19]. In this study we investigated the role of neoglycoproteins and IgG-enriched fractions of anti-lectin antibodies, which presented galactose and N-acetylgalactosamine as carbohydrate structures, in the inhibition of LT–Vero cell and LT–GM1 interactions.

These synthetic carbohydrate ligands were employed in this study because they are important tools to recognize lectins, and contain multiple copies of monosaccharide determinants for a specific lectin. Therefore, the affinity of ligand often increases geometrically with increasing sugar valency [20]. Anti-lectin antibodies were also used since they present specific binding sites for their corresponding sugars, which mimic carbohydrate structures [21–23].

IgG-enriched fractions of the anti-lectin antibodies inhibited LT activity efficiently in Vero cells (Table 1). The inhibition was 70% when anti-WFA was used, followed by inhibition of 63 and 64% when supernatants of ETEC producing LT were incubated with the same fraction of anti-PNA and anti-EE antibodies, respectively (Fig. 1). To ascertain the anti-lectin antibody specificity, the corresponding sugars were incubated with anti-WFA, anti-PNA and anti-EE IgG-enriched fractions. These antibody–sugar interactions were not able to block LT binding to Vero cells indicating specificity of inhibition (data not shown). As negative controls, IgG-enriched fractions of rabbit non-immune antiserum and the non-related antiserum anti-ConA and anti-WGA were employed.

When neoglycoproteins were compared with IgG for inhibition of LT, GalNAc-BSA was more efficient than anti-WFA, anti-PNA and anti-EE antibodies; 2.5 μg of this neoglycoprotein abolished 95% of the activity of LT in Vero cells. GlcNAc-BSA and Man-BSA were less inhibitory for the LT–Vero cell interaction (Fig. 2). This could be explained by the size of these molecules, which caused steric hindrance and by the fact that the lectin-like binding property of LT is directed against different receptors on mammalian cells [6,25].

The inhibition of LT binding to GM1 was 30% and no effect was observed when non-related neoglycoproteins were used. The inhibition of cell damage by IgG-enriched fractions incubated with supernatant of ETEC producing LT before interaction with Vero cells. Cell damage was determined at 620 nm after staining with crystal violet. Toxin, Vero cell incubated with supernatant of ETEC producing LT; non-immune serum, rabbit antiserum before immunization. Bars represent 95% confidence interval.
and BSA itself were assayed, regardless of the concentration employed (data not shown).

The inhibitory ability of IgG-enriched fraction of anti-WFA, anti-PNA, anti-EE antibodies and GalNAc-BSA was observed, only when these compounds were incubated with supernatant of the LT-producing ETEC strain H10407. When Vero cells were incubated with these same compounds, the effect was not observed (data not shown). Therefore, our data suggest that these analogs bind to LT, blocking the binding with its receptor.

The IgG-enriched fraction of anti-WFA was the most efficient antibody in inhibiting the binding of LT to GM1 (Fig. 3), showing affinity of LT for its efficient antibody in inhibiting the binding of LT to GM1 receptor. Similar findings were reported by other authors.[6,24] The corresponding sugars, galactose and N-acetylgalactosamine, were incubated with IgG-enriched fractions of anti-lectin antibodies. These antibody–sugar interactions were not able to block LT binding to Vero cells and GM1 receptor (data not shown).

The glycosylated proteins evaluated in this study proved to be effective inhibitors of LT and are a promising possible prophylaxis for ETEC infections. However, in vivo studies must be conducted to confirm this proposal. Also, since the glycosylated proteins studied specifically recognized LT, they could be also employed in the diagnosis of diarrhea caused by LT.

Acknowledgements

We thank Dr. Waldir P. Elias Jr. and Dr. Rogéria Keller and Dr. Lothar Beutin for critically reading the manuscript. This work was supported by FAPESP (grant 99/09458-0 to R.M.F.P., and fellowship to C.A.M.).

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