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Studies on the binding substances on human erythrocytes for the heat-labile enterotoxin isolated from chicken enterotoxigenic *Escherichia coli*

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To study the predominant binding substance for the heat-labile enterotoxin (LT_c) isolated from chicken enterotoxigenic *Escherichia coli*, competitive binding assays were performed with neuraminidase-treated human type B erythrocytes and ^{125}I-labeled B subunit of LT_c (LT_c-B). Of all inhibitors used, the ganglioside G_{M1} was the most effective in inhibiting the binding of ^{125}I-labeled LT_c-B to the erythrocytes. The other gangliosides used as inhibitors, gangliosides G_{D1b}, G_{D1a}, G_{M2}, G_{T1b} and G_{M3}, were about 24, 166, 250, 440 and at least 440 times less reactive than ganglioside G_{M1}, respectively. With glycoproteins as inhibitors, on the other hand, hog A + H, porcine thyroglobulin and bovine salivary mucin were over 10^4 times less potent. No inhibition was obtained by other mono-, di- and polysaccharides at the highest concentrations used. These findings suggest that the predominant binding substance on neuraminidase-treated human type B erythrocytes for the LT_c-B is ganglioside G_{M1} and that the combining site of LT_c-B may be specific for the terminal disaccharide (galactose-*N*-acetyl-D-galactosamine)-linked portion of ganglioside G_{M1}.

Introduction

Escherichia coli from different sources are known to produce various bacterial lectins [1–6]. In addition to these lectins, the classical type I heat-labile enterotoxins (LT-I) produced by enterotoxigenic *E. coli* have been recently demonstrated to be also one of lectins produced by *E. coli* [7–9]. LT-I are structurally, biologically, immunologically and functionally similar to cholera toxin (CT) [1,10–15]. They are composed of A and B subunits. The A subunit is responsible for the biological effects of these toxins, whereas the B subunit is associated with the binding of toxins to target cells. The receptor for CT is established to be ganglioside G_{M1} [1,16–19]. Although CT and LT-I have shown similar specificities to different gangliosides [20], the receptor substance for LTs is not clearly identified since LT-I has been demonstrated to bind to glycoprotein(s) on rat and human intestinal brush-border membranes not recognized by CT [21–23] in addition to ganglioside G_{M1} [24,25]. Furthermore, ganglioside G_{M1} has been shown to inactivate CT much better than LT-I [24]. The difference between ganglioside G_{M1} and other gangliosides in neutralization tests has been shown to be also more distinct for CT than for LT-I [24]. LT-I bound tightly to agarose polymer Bio-Gel A-5M [26–28], whereas CT bound weakly to the same agarose [29]. The B subunit of heat-labile enterotoxin (LT) isolated from chicken enterotoxigenic *E. coli* (LT_c-B) has been recently shown to be antigenically similar to that of LT isolated from human enterotoxigenic *E. coli* (LT_h-B). LT-I and LT_c-B have been demonstrated to strongly agglutinate neuraminidase-treated human and animal erythrocytes [7–9]. Since less is known about the binding substances for LT_c, attempts were made to identify the predominant binding substance on human erythrocytes for LT_c-B by competitive binding assays.

Materials and Methods

Toxin. LT_c produced by chicken enterotoxigenic *Escherichia coli* strain JT-21d and its B subunit were isolated by the methods reported previously [30–32].

Enzyme. Clostridium perfringens neuraminidase type V was obtained from Sigma Chemicals Co.

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Inhibitors. Mono-, di- and polysaccharides were purchased from Nakarai Chemicals Ltd. Gangliosides G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{D1b}, and G_{T1b} were obtained from Bachem Fine Chemicals, Biosynth AG, and Trans Busan S.A. Glycoproteins (porcine thyroglobulin, bovine salivary mucin, fetuin and hog A + H) were purchased from Sigma Chemical Co. Glycophorin was prepared from the lyophilized ghosts of human erythrocytes by the methods reported previously [33,34].

Labeling of LT_c-B. LT_c-B was iodinated with the Bolton-Hunter reagent [*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl]propionate (Amersham International) by the methods reported previously [35]. Briefly, 20 μg of LT_c-B in 10 μl of 0.1 M borate buffer (pH 8.5) was reacted with 75 μCi of ^{125}I-labeled Bolton-Hunter reagent for 15 min with frequent mixing on ice. The reaction was quenched by addition of 0.5 ml of 0.2 M glycine in 0.1 M borate buffer (pH 8.5). The labeled LT_c-B was separated by gel filtration on Sephadex G-25 equilibrated with 0.01 M phosphate buffer (pH 7.2) containing 0.5% gelatin. LT_c-B labeled by this procedure had a specific activity of $(1-2) \cdot 10^5$ cpm/μg protein.

Competitive binding assays. For competitive binding assays, human type B erythrocytes and glycoproteins were treated with neuraminidase by the methods reported previously [6–9,36,37]. Competitive binding assays were carried out in a total volume of 400 μl using 0.01 M phosphate buffer (pH 7.2) containing 0.5% bovine serum albumin as a diluent. In competitive binding assays with ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes, 100 μl of 2% treated erythrocytes was sufficient to bind 50–60% of 4000 cpm of ^{125}I-labeled LT_c-B (40 μl of 1 : 5 dilution of ^{125}I-labeled LT_c-B). A mixture of labeled or unlabeled LT_c-B, or other inhibitor was added to the erythrocyte suspension. The tubes containing ^{125}I-labeled LT_c-B were mixed by end-over-end rotation at 4 C for 4 h. Separation of bound labeled LT_c-B from free one was performed by centrifugation. 360 μl of the supernatants were counted for ^{125}I. All determination were set up in triplicate.

The data are expressed graphically as percentage inhibitor of the binding of LT_c-B against micromoles or micrograms of mono-, di- and polysaccharides, glycoproteins and gangliosides. The formula used to calculate percentage inhibition is:

$$\left(1 - \frac{\text{total cpm added} - \text{cpm in supernatant with inhibitor}}{\text{total cpm added} - \text{cpm in supernatant without inhibitor}}\right) \times 100$$

Results

Binding assays

Since neuraminidase-treated human erythrocytes have been more strongly agglutinated by LT_c-B than untreated ones [9], the binding ability of ^{125}I-labeled LT_c-B was compared with neuraminidase-treated and untreated human type B erythrocytes. With neuraminidase-treated human type B erythrocytes, 1.5–2.0-fold enhancement was found in the binding of ^{125}I-labeled LT_c-B to these erythrocytes.

Competitive binding assays

To determine the predominant binding substance for the LT_c-B on erythrocytes, competitive binding assays were performed with ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes using various inhibitors. The inhibitory activities of these substances are shown in Figs. 1 and 2 and their minimum amounts (μg) to give 50% inhibition are summarized in Tables I and II. Ganglioside G_{M1} was the most potent inhibitor among mono-, di-, and polysaccharides, gangliosides and glycoproteins used (Tables I and II). As presented in Table I, ganglioside G_{M1} was a most potent inhibitor among gangliosides used. It was

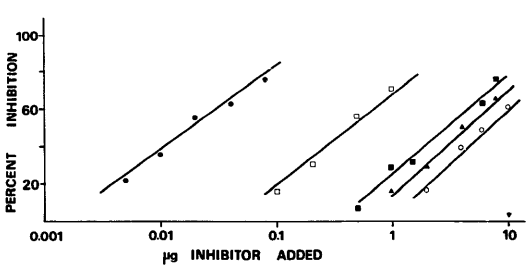

Fig. 1. Competitive binding assays by gangliosides of the binding of ^{125}I-labeled LT_c-B to neuraminidase-treated human type B erythrocytes. Inhibitors used were ganglioside G_{M1} (●), G_{D1a} (■), G_{D1b} (□), G_{M2} (▲), G_{M3} (▼) and G_{T1b} (○).

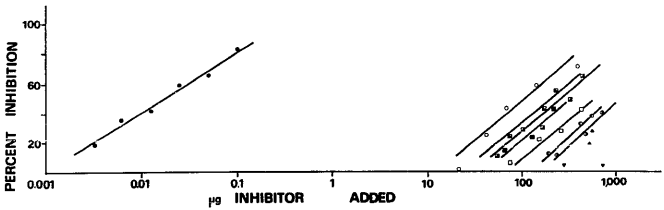

Fig. 2. Competitive binding assays by ganglioside G_{M1} and glycoproteins of ^{125}I-labeled LT_c-B to neuraminidase-treated human type B erythrocytes. Inhibitors used were ganglioside G_{M1} (●), hog A+H (○), bovine salivary mucin (□), asialo-bovine salivary mucin (■), fetuin (◐), asialo-fetuin (◑), thyroglobulin (◪), asialo-thyroglobulin (◧), glycophorin (▲), and asialo-glycophorin (▼).

24, 166, 250, 444 and over 444 times more active than gangliosides G_{D1b}, G_{D1a}, G_{M2}, G_{T1b} and G_{M3}, respectively. On the other hand, mono-, di- and polysaccharides were at least 10^4 times less potent (Table II). With glycoproteins as inhibitors, hog A + H, thyroglobulin, intact and asialo-bovine salivary mucin were about 10^4 times less potent and other glycoproteins were much less (Table II).

To study which carbohydrate sequence of ganglioside G_{M1} is specific for the combining site of LT_c-B, different lectins with well-defined carbohydrate specificities were used as inhibitors. Less than 10% inhibition was found by lectins at up to 100 μg such as *Erythrina crystagalli* agglutinin, ricin, *Wistaria floribunda* agglutinin, *Sophora japonica* agglutinin, peanut agglutinin, soybean agglutinin, *Ulex europaeus*-I agglutinin, wheat germ agglutinin, *Bauhinia purpurea* agglutinin, *Maclura pomifera* agglutinin, concanavalin A, *Dolichos biflorus* agglutinin, *Griffonia simplicifolia*-I A_4 and B_4 agglutinins, *Ricinus communis* agglutinin and lentil lectin although neuraminidase-treated human type B erythrocytes were strongly agglutinated by all of these lectins.

TABLE I

Inhibitory activities of different gangliosides to the binding reaction between ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes

Inhibitor	μg to give 50% inhibition
G_{M1}	$1.8 \cdot 10^{-2}$
G_{M2}	4.5
G_{M3}	> 10.0 (3%) [a]
G_{D1a}	3.0
G_{D1b}	$4.4 \cdot 10^{-1}$
G_{T1b}	8.0

[a] Number in parenthesis is maximum inhibition obtained by inhibitor at the highest concentration used.

Discussion

The B subunit(s) of LT-I are known to interact with gangliosides [24,25], galactoproteins [21–23] and agarose polymer Bio-Gel A-5M [26–28], suggesting that the B subunit may be reactive with all or a part of carbohydrate side chains of complex carbohydrates on cell

TABLE II

Inhibitory activities of different inhibitors to the binding reaction between ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes

Numbers in parentheses are maximum inhibition obtained by inhibitors at the highest concentrations used.

Inhibitor	Minimum amount of inhibitors to give 50% inhibition	
	μmol	μg
Ganglioside G_{M1}	$(1 \cdot 10^{-5})$	$1.6 \cdot 10^{-2}$
Methyl α-D-galactopyranoside	> 15.6 (1%)	> 3026
Galactose	> 25.0 (26%)	> 4500
Lactose	> 23.5 (0%)	> 8037
Melibiose	> 23.5 (0%)	> 8037
N-Acetyl-D-galactosamine	> 25.3 (0%)	> 5591
N-Acetyl-D-glucosamine	> 45.2 (0%)	> 9989
Glucose	> 49.6 (0%)	> 8928
Mannose	> 22.0 (12%)	> 3960
L-Fucose	> 15.6 (15%)	> 2820
L-Arabinose	> 35.0 (12%)	> 5250
N-Acetylneuraminic acid	> 1.9 (0%)	> 587
Hog A+H		105
Thyroglobulin		200
Asialo-bovine salivary mucin		210
Bovine salivary mucin		380
Fetuin		> 720 (40%)
Asialo-fetuin		> 550 (38%)
Glycophorin		> 570 (28%)
Asialo-glycophorin		> 720 (18%)
Galactan		> 500 (19%)
Mannan		> 560 (23%)

surfaces. The present results suggest that glycoproteins on human type B erythrocytes may not be the predominant binding substance for LT_c-B since different glycoproteins used were at least 10^4 times less reactive than a most potent inhibitor ganglioside G_{M1} in competitive binding assays with ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes. Although this is different from the previous findings [21–25] that LT-I (LT_p) bound to both gangliosides and glycoproteins on human and animal intestinal epithelial cells, it may be consistent with the present findings that none of lectins used in this study, which are known to be specific for carbohydrate side chains glycoproteins [38,39], effectively inhibited the binding of ^{125}I-labeled LT_c-B to neuraminidase-treated human type B erythrocytes. The poor inhibitory activities of *Dolichos biflorus*, *Ulex europaeus*-I, and *Griffonia simplicifolia*-I A_4 and B_4 agglutinins specific for A, B, and H determinants [38,39] also suggest that LT_c-B may not be reactive with the blood-group determinants although human type B erythrocytes were most strongly agglutinated by LT_c-B [9]. Thus, the predominant binding substance on human erythrocytes for LT_c is ganglioside GM_1 as found for LT-I [20,24,25].

TABLE III

Chemical structures of different gangliosides

Abbreviations used are: NANA, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, glucose; Cer, ceramide.

Name [a]	Structure
G_{M3}	$Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $(3 \leftarrow 2\alpha NANA)$
G_{M2}	$GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $(3 \leftarrow 2\alpha NANA)$
Asialo-G_{M2}	$GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$
G_{M1}	$Gal\beta1 \rightarrow 3GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $(3 \leftarrow 2\alpha NANA)$
Asialo-G_{M1}	$Gal\beta1 \rightarrow 3GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$
G_{D1a}	$Gal\beta1 \rightarrow 3GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $\vert$ $(3 \leftarrow 2\alpha NANA)$ $(3 \leftarrow 2\alpha NANA)$
G_{D1b}	$Gal\beta1 \rightarrow 3GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $(3 \leftarrow 2\alpha NANA\text{-}8 \leftarrow 2\alpha NANA)$
G_{T1b}	$Gal\beta1 \rightarrow 3GalNAc\beta1 \rightarrow 4Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$ $\vert$ $\vert$ $(3 \leftarrow 2\alpha NANA)$ $(3 \leftarrow 2\alpha NANA\text{-}8 \leftarrow 2\alpha NANA)$

[a] According to Svennerholm (1963).

To determine the carbohydrate specificity of the combining site of LT_c-B, different gangliosides were used as inhibitors in competitive binding assays with ^{125}I-labeled LT_c-B and neuraminidase-treated human type B erythrocytes. Ganglioside G_{M1} was a most potent inhibitor and was 250 times more reactive than ganglioside G_{M2}. This suggests that nonreducing terminal galactose residue of ganglioside G_{M1} may be important role(s) in the binding according to the chemical structures of different gangliosides presented by the product information based on Svennerholm [40] (Table III). Ganglioside G_{M1} was 24 times more potent than ganglioside G_{D1b} which was more reactive than gangliosides G_{D1a} and G_{T1b} (Table I). These suggest that the terminal sialic acid linked to penultimate galactose found in gangliosides G_{D1b} and G_{T1b} may inhibit the binding of these gangliosides to the LT_c-B as found for CT and LT_h-1 (LT-I) [20]. Another terminal sialic acid linked to penultimate sialic acid found in gangliosides G_{D1b} and G_{T1b} may also inhibit the binding. These results are similar to those with LT-I produced by porcine and human enterotoxigenic *E. coli* (LT_p and LT_h-B) [41]. From these findings, LT_c may be immunologically and functionally similar to LT-I and may be reactive with the terminal disaccharide (galactose-*N*-acetyl-D-galactosamine)-linked portion of ganglioside G_{M1} as was found to be the case with LT-I and CT [20].

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