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

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The binding substance for the heat-labile enterotoxin (LT_p) isolated from porcine enterotoxigenic *Escherichia coli* was studied by competitive binding assays. The binding of ^{125}I-labeled LT_p to neuraminidase-treated human type A erythrocytes was most effectively inhibited by ganglioside G_{M1} among inhibitors used. Mono-, di- and polysaccharides, glycoproteins and lectins were over 10^4-times less potent inhibitors. Similar results were also obtained in competitive binding assays with ^{3}H-labeled ganglioside G_{M1} and LT_p-coupled Sepharose 4B. On the other hand, hemagglutination of neuraminidase-treated human type A erythrocytes by LT_p was inhibited by methyl α-D-galactopyranoside, galactose, melibiose and some glycoproteins, but not effectively inhibited by ganglioside G_{M1} at the highest concentration used. Preincubation of LT_p with an appropriate amount of ganglioside G_{M1} resulted in much higher hemagglutination than LT_p alone. Although these findings show that there may be fundamental differences between interactions with ganglioside G_{M1} in hemagglutination compared to interactions with ganglioside G_{M1} in binding, the predominant binding substance for LT_p on neuraminidase-treated human type A erythrocytes is suggested to be ganglioside G_{M1}.

Introduction

E. coli from different sources are known to produce various bacterial lectins [1–7]. These lectins have been shown to play important roles in attachment and proliferation of *E. coli* at the early stages of infection. In addition to these lectins, the heat-labile enterotoxin (LT_p) produced by porcine enterotoxigenic *E. coli* has been recently demonstrated to be also one of lectins produced by *E. coli* [8]. Heat-labile enterotoxins are structurally, biologically, immunologically and functionally similar to cholera toxin (CT) [1,9–14]. 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. Although ganglioside G_{M1} is widely accepted as the receptor for CT on cell surfaces [1,15–18], the nature of the receptor substance(s) for heat-labile enterotoxins is still inconclusive, since heat-labile enterotoxins have been demonstrated to bind to glycoprotein(s) on rat and human intestinal brush-border membranes not recognized by CT [19–21] in addition to ganglioside G_{M1} [22,23]. Furthermore, ganglioside G_{M1} has been shown to inactivate CT much better than LT_p [22]. 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_p [22]. Heat-labile enterotoxin bound tightly to agarose polymer Bio-Gel A-5M [24–26], whereas CT bound weakly to the same agarose [27]. These findings suggest that the receptor substance(s) for heat-labile enterotoxins is different from that of CT. To identify the binding substance for LT_p, therefore, the present studies were undertaken to study the binding and hemagglutinating properties of LT_p to neuraminidase-treated human type A erythrocytes by competitive binding assays and hemagglutination inhibition.

Materials and Methods

Toxin. LT_p produced by porcine enterotoxigenic *E. coli* strain WT-1 was isolated by the methods reported previously [28].

Abbreviations: LT_p, porcine heat-labile enterotoxin; CT, cholera toxin.

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Enzyme. Clostridium perfringens neuraminidase type V, pronase and galactose oxidase were obtained from Sigma, Kaken and Worthington, respectively.

Inhibitors. Mono-, di- and polysaccharides were purchased from Nakarai. Ganglioside G_{M1} and glycoproteins were obtained from Bachem and Sigma, respectively. Pure lectins used in this study were the products of E.Y. Laboratories, Sigma and Seikagaku Kogyo.

Isolation of glycophorin from human erythrocytes. Glycophorin was prepared from the lyophilized ghosts of human erythrocytes by treatment with 0.3 M 3,5-diiodosalicylic acid lithium salt (Nakarai) according to the methods described previously [29,30]. The fractions containing glycophorin were collected and finally extracted with chloroform/methanol under the condition described by Folch et al. [31]. The treated glycophorin was used as glycophorin.

Labeling of glycoproteins, LT_p and ganglioside G_{M1}. LT_p was iodinated with the Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl-)propionate (Amersham International) by the methods reported previously [32]. LT_p labeled by this method had a specific activity of $(1–2) \cdot 10^6$ cpm/μg protein.

For labeling of glycoproteins with NaB^3H_4 (Amersham International), glycoproteins were treated at 37°C for 4 h with neuraminidase in 0.1 M acetate buffer (pH 5.8)/1 mM $CaCl_2$, according to the methods described previously [33,34]. 1 mg of desialized glycoprotein was incubated with 21 units of galactose oxidase at 37°C for 4 h in 0.01 M phosphate buffer (pH 7.2). Then, oxidized glycoprotein was mixed with 800 μCi NaB^3H_4 and 1 mg unlabeled $NaBH_4$ at room temperature for 30 min. The labeled glycoproteins had specific activities of $(2–3) \cdot 10^3$ cpm/μg.

For labeling of ganglioside G_{M1}, 1 mg of ganglioside G_{M1} was oxidized with 2,3-dichloro-5,6-dicyanobenzoquinone at 37°C for 40 h by the methods described previously [35]. The oxidized ganglioside G_{M1} was reduced with 1 mCi NaB^3H_4 and 2 mg of unlabeled $NaBH_4$ at room temperature for 1 h. The pH of the mixture was adjusted to 6.0 with 0.1 M acetic acid and evaporated at 37°C. Ganglioside G_{M1} labeled by this method had a specific activity of $4 \cdot 10^4$ cpm/μg.

Hemagglutination and hemagglutination inhibition. Human erythrocytes were obtained from the fresh blood from healthy volunteers. Sheep erythrocytes were obtained from Nippon Biotest Laboratories. These erythrocytes were treated with neuraminidase and pronase as reported previously [8]. Hemagglutination and hemagglutination inhibition were carried out at room temperature with a 96 U-bottomed microtitrator (Becton Dickinson Labware) using saline as a diluent. For inhibition assays, 25 μl of 2-fold diluted inhibitor was mixed with 25 μl of LT_p (0.5 μg) containing 4 hemagglutinating units at room temperature for 30 min. When the hemagglutinating activity of the ganglioside G_{M1}-LT_p complex was being studied, 25 μl of LT_p (0.5 μg) containing 4 hemagglutinating units were incubated for 1 h at room temperature with 25 μl of ganglioside G_{M1} containing 25 μg, 2.5 μg, 250 ng, 25 ng and 2.5 ng, respectively. After that, the mixture was diluted 2-fold. Then, 25 μl of 2% washed erythrocytes were added to the mixture. The hemagglutinating titer was usually determined after 1 h of incubation.

Competitive binding assays. 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 LT_p and ^{3}H-labeled ganglioside G_{M1}, 25 μl of LT_p-coupled Sepharose 4B (12% gel containing 20 μg LT_p/ml), prepared by the methods described previously [36], was sufficient to bind 50–60% of approx. 5000 cpm of ^{3}H-labeled ganglioside G_{M1} and ^{3}H-labeled glycoproteins. A mixture of labeled and unlabeled ganglioside G_{M1}, or other inhibitor, was added to LT_p-coupled Sepharose 4B. In competitive binding assays with ^{125}I-labeled LT_p and neuraminidase-treated human type A erythrocytes, on the other hand, 100 μl of 2% treated erythrocytes was sufficient to bind 50–60% of 4000 cpm of the ^{125}I-labeled LT_p (40 μl of 1 : 20 dilution of ^{125}I-labeled LT_p). A mixture of labeled and unlabeled LT_p, or other inhibitor was added to the erythrocytes suspension. The tubes containing ^{3}H-labeled ganglioside GM_1, labeled glycoprotein, or ^{125}I-labeled LT_p were mixed by end-over-end rotation at 4°C for 4–12 h. Separation of bound labeled ganglioside G_{M1}, glycoprotein, or LT_p from free G_{M1} was done by centrifugation. 360 and 375 μl of the supernatants were assayed for ^{3}H in 5 ml of Clear Sol I (Nakarai Chemicals) and for ^{125}I, respectively. All determinations were performed in triplicate.

The data are expressed graphically as percentage inhibition of the binding of labeled ganglioside G_{M1}, or LT_p against micromoles or micrograms of mono- and disaccharides, glycoproteins or polysaccharides. 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

To study the binding abilities of LT_p to erythrocytes, glycoproteins and ganglioside G_{M1}, the binding ability of ^{125}I-labeled LT_p was determined. About 2-fold enhancement was found in the binding of ^{125}I-labeled LT_p to sheep and human type A erythrocytes before and after neuraminidase treatment. LT_p-coupled Sepharose 4B bound to ^{3}H-labeled ganglioside G_{M1} but did not

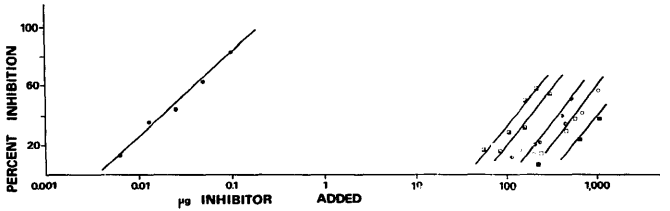

Fig. 1. Competitive binding assays by ganglioside G_{M1} and glycoproteins of the binding of ^{125}I-labeled LT_p to neuraminidase-treated human type A erythrocytes. Inhibitors used were ganglioside G_{M1} (●), hog A + H (○), bovine salivary mucin (□), asialo-bovine salivary mucin (■), fetuin (◑), asialo-fetuin (◐), thyroglobulin (◪), asialo-thyroglobulin (◧), glycophorin (▲) and asialo-glycophorin (▼).

bind to ^{3}H-labeled glycoproteins such as fetuin, thyroglobulin hog A + H, bovine salivary mucin and glycophorin.

Competitive binding assays

To determine the binding substance for LT_p on erythrocytes, competitive binding assays were performed with ^{125}I-labeled LT_p and neuraminidase-treated human type A erythrocytes using various inhibitors. The inhibitory activities of these substances are shown in Fig. 1 and their minimum amounts (μg) to give 50% inhibition are summarized in Table I. Ganglioside G_{M1} was a most potent inhibitor among inhibitors used. Other inhibitors were at least 10^4-times less potent (Table I). Similar results were also obtained in competitive binding assays with ^{3}H-labeled ganglioside G_{M1} and LT_p-coupled Sepharose 4B using different inhibitors. The results are shown in Fig. 1 and summarized in Table II. As shown in Fig. 2 and Table II, only unlabeled ganglioside G_{M1} effectively inhibited the binding of ^{3}H-labeled ganglioside G_{M1} to LT_p.

To determine which carbohydrate portion of ganglioside G_{M1} is specifically reactive with the combining site of LT_p, lectins with well-defined specificities were used as inhibitors. Although neuraminidase-treated human type A erythrocytes were strongly agglutinated by lectins at amounts up to 100 μg, such as *Erythrina crystagalli* agglutinin, ricin, *Wistaria floribunda*, *Sophora japonica* agglutinin, peanut agglutinin, soybean agglutinin, *Ulex europaeus-1* agglutinin, wheat germ agglutinin, *Bauhinia purpurea* agglutinin, *Maclura pomifera* agglutinin, *Griffonia simplicifolia-1* A_4 and B_4 agglutinins, concanavalin A, *Dolichas biflorus* agglutinin, *Ricinus communis* agglutinin, lentil lectin, the binding of ^{125}I-labeled LT_p to the erythrocytes was not at all inhibited by these lectins. Similar results were also obtained in competitive binding assays with ^{3}H-labeled ganglioside G_{M1} and LT_p-coupled Sepharose 4B.

Hemagglutination inhibition

To ascertain whether or not ganglioside G_{M1} is associated with induction of hemagglutination by LT_p,

Fig. 2. Competitive binding assays by unlabeled ganglioside G_{M1} and mono- and disaccharides of the binding of ^{3}H-labeled ganglioside G_{M1} to LT_p-coupled Sepharose 4B. Inhibitors used were unlabeled ganglioside G_{M1} (●), galactose (○), lactose (▲), melibiose (▼), glucose (◑), mannose (◐), *N*-acetyl-D-galactosamine (□), *N*-acetyl-D-glucosamine (■), L-fucose (▤) and L-arabinose (▥).

TABLE I

Inhibitory activities of different substances to the binding reaction between ^{125}I-labeled LT_p and neuraminidase-treated human type A erythrocytes

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

Inhibitor	Minimum amount to give 50% inhibition	
	μmol	μg
Ganglioside G_{M1}		$2.5 \cdot 10^{-2}$
Methyl α-D-galacto-pyranoside	8.2	1590
Galactose	> 31.3 (29%)	> 5634
Lactose	> 26.8 (7%)	> 9165
Melibiose	> 26.8 (13%)	> 9165
N-Acetyl-D-galactosamine	> 28.0 (0%)	> 6188
N-Acetyl-D-glucosamine	> 56.5 (5%)	> 12486
Glucose	> 100.0 (30%)	> 18000
Mannose	> 28.0 (28%)	> 5040
L-Fucose	> 19.5 (20%)	> 3198
L-Arabinose	> 40.0 (25%)	> 6000
N-Acetylneuraminic acid	> 2.4 (0%)	> 742
Thyroglobulin		170
Fetuin		490
Asialo-fetuin		> 690 (35%)
Asialo-bovine salivary mucin		> 1060 (40%)
Bovine salivary mucin		> 594 (37%)
Hog A + H		650
Asialo-thyroglobulin		240
Glycophorin		> 515 (27%)
Asialo-glycophorin		> 675 (17%)
Galactan		> 400 (0%)
Mannan		> 400 (11%)

TABLE II

Inhibitory activities of different substances to the binding reaction between ^3H-labeled ganglioside G_{M1} and LT_p-coupled Sepharose 4B

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

Inhibitor	Minimum amount to give 50% inhibition	
	μmol	μg
Ganglioside G_{M1}		0.92
Methyl α-D-galacto-pyranoside	> 11.0 (15%)	> 2134
Galactose	> 31.3 (5)	> 5634
Lactose	> 26.8 (5%)	> 9165
Melibiose	> 26.8 (14%)	> 9165
N-Acetyl-D-galactosamine	> 20.1 (0%)	> 4442
N-acetyl-D-glucosamine	> 56.6 (0%)	> 12509
L-Fucose	> 19.5 (0%)	> 3198
L-Arabinose	> 40.0 (0%)	> 6000
Glucose	> 61.9 (0%)	> 11142
Mannose	> 27.6 (0%)	> 4968
N-Acetylneuraminic acid	> 2.4 (0%)	> 742
Hog A + H		> 250 (0%)
Bovine salivary mucin		> 250 (0%)
Asialo-bovine salivary mucin		> 424 (0%)
Fetuin		> 517 (0%)
Asialo-fetuin		> 575 (0%)
Thyroglobulin		> 542 (0%)
Asialo-thyroglobulin		> 532 (0%)
Glycophorin		> 515 (0%)
Asialo-glycophorin		> 481 (0%)
Galactan		> 500 (0%)
Mannan		> 560 (0%)

hemagglutination inhibition was carried out using different inhibitors. Hemagglutination of neuraminidase-treated human type A erythrocytes was inhibited by methyl α-D-galactopyranoside, galactose and melibiose, but not by *N*-acetyl-D-galactosamine, lactose and ganglioside G_{M1} at the highest concentrations used (Table III). With glycoproteins as inhibitors, hog A + H, asialo-bovine salivary mucin, intact and asialo-porcine thyroglobulin showed inhibition, whereas no inhibition was found by other glycoproteins at the highest concentrations used (Table III).

To confirm the poor inhibitory activity of ganglioside G_{M1} in hemagglutination inhibition, hemagglutinating activities of ganglioside G_{M1} alone and the ganglioside G_{M1}-LT_p complex were determined. Although ganglioside G_{M1} alone showed no hemagglutination, preincubating ganglioside G_{M1} with LT_p to form the complex gave significant enhancement in hemagglutination. About 4–250-fold enhancement was found by such preincubation. Preincubation with either 2.5 μg or 250 ng of ganglioside G_{M1} showed the highest hemagglutinating activity, while weak or almost the same activity

TABLE III

Inhibitory activities of different sugars and glycoproteins to hemagglutination of neuraminidase-treated human type A erythrocytes induced by LT_p

Inhibitor	Minimum amount of sugars and glycoproteins to inhibit hemagglutination	
	mg/ml	μg/ml
Methyl α-D-galactopyranoside	4.3	
Galactose	11.3	
Melibiose	18.3	
Ganglioside G_{M1}	> 2.0	
N-Acetyl-D-galactosamine	> 25.4	
Lactose	> 36.7	
Hog A + H		87.5
Bovine salivary mucin		> 2980
Asialo-bovine salivary mucin		440
Fetuin		> 2070
Asialo-fetuin		> 1640
Thyroglobulin		271
Asialo-thyroglobulin		1500
Glycophorin		> 2060
Asialo-glycophorin		> 2060

was found by preincubation with more than 50 μg or less than 250 ng of ganglioside G_{M1}. Such preincubation also showed significant enhancement in hemagglutination of neuraminidase-treated sheep and intact human type A erythrocytes. On the other hand, preincubation of LT_p with glycoproteins (such as hog A + H, thyroglobulin and fetuin) showed no enhancement in the hemagglutinating activity.

Discussion

LT_p has been shown to bind most strongly to ganglioside G_{M1} in vitro [22,23]. This is confirmed by the present results that ganglioside G_{M1} was more than 10^4-times more reactive with LT_p than glycoproteins in competitive binding assays. This is also supported by other findings that the binding of ^{3}H-labeled ganglioside G_{M1} to LT_p-coupled Sepharose 4B was effectively inhibited by only unlabeled ganglioside G_{M1}. Although LT_p has been recently reported to also react with glycoprotein(s) exposed on intestinal epithelial cells [19–21], LT_p-coupled Sepharose 4B bound to ^{3}H-labeled ganglioside GM_1 but not to ^{3}H-labeled glycoproteins. None of the lectins used in this study, which are specific for carbohydrate side-chains of glycoproteins [37,38], inhibited both binding of ^{125}I-labeled LT_p to neuraminidase-treated human type A erythrocytes and of LT_p-coupled Sepharose 4B to ^{3}H-labeled ganglioside G_{M1}. Thus, proteins on human erythrocytes may not be the predominant binding substances for LT_p. The poor inhibitory activities of *D. biflorus, U. europaeus-I* and *G. simplicifolia-I* A_4 and B_4 agglutinins specific for A, B, and H determinants [37,38] also suggest that LT_p may not be reactive with the blood-group determinants, although LT_p most strongly agglutinated human type A erythrocytes [8]. From these findings, the predominant binding substance for LT_p on human type A erythrocytes is strongly suggested to be ganglioside G_{M1}.

Ganglioside G_{M1} was a good inhibitor in competitive binding assays, whereas it was a poor inhibitor in hemagglutination inhibition. This suggests that there may be fundamental differences between interactions of LT_p with ganglioside G_{M1} in the binding compared to interactions of LT_p with ganglioside G_{M1} in hemagglutination, as found for the case with CT [39]. The possibility may be supported by the present results that hemagglutination induced by LT_p was inhibited by galactose, melibiose and hog A + H but not by ganglioside G_{M1}, whereas the binding of ^{125}I-labeled LT_p was inhibited by ganglioside G_{M1} but not by other substances. This is also supported by the finding that sheep erythrocytes were bound to LT_p but not agglutinated by LT_p [8]. Although the poor inhibitory activity of ganglioside G_{M1} in hemagglutination inhibition was in part accounted for by the finding that preincubation of LT_p with ganglioside G_{M1} to form the complex showed much higher hemagglutinating activity, little is known about the mechanism to enhance hemagglutination by preincubating LT_p with ganglioside G_{M1}. Gangliosides are known to aggregate into micelles in aqueous medium [40–47] and to spontaneously associate with cell membranes [43,44]. CT has been shown to agglutinate phospholipid vesicles containing ganglioside G_{M1} [39,45]. In addition, the CT-ganglioside G_{M1} complex has been shown to bind to cellular protein(s) [46,47]. From these previous findings, the high hemagglutinating activity of the LT_p-ganglioside G_{M1} complex may be due to the possibility that the complex may bind to neuraminidase-treated erythrocytes. Another possibility is that free aggregated ganglioside G_{M1} in diluent might be incorporated into the erythrocytes during incubation. Furthermore, the LT_p-ganglioside G_{M1} complex might be similarly hydrophobic in saline to give higher hemagglutination, since the CT-ganglioside G_{M1} complex has been suggested to be much more hydrophobic than the individual molecules [48]. In any case, the zeta-potential on erythrocyte surfaces was altered by the presence of the LT_p-ganglioside G_{M1} complex or coexistence of these two substances, resulting in higher hemagglutination.

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