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Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining

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Helicobacter pylori has been identified as a causative agent in active chronic gastritis. The receptor for this bacteria, however, is not known. It is likely that the receptor molecules may be glycosphingolipids* as shown in the cases of other bacteria. We explored this possibility by a thin-layer chromatography (TLC)-immunostaining method. Among glycosphingolipids extracted from human gastric mucosa, intact *Helicobacter pylori* specifically bound to I^3SO_3-GalCer and II^3NeuAc-LacCer, whereas no specific binding to neutral glycosphingolipids, which share the same ceramide moiety with I^3SO_3-GalCer or II^3NeuAc-LacCer, was demonstrated. Sonicated bacteria could still bind to II^3NeuAc-LacCer with comparable affinity. In contrast, the binding of bacteria to I^3SO_3-GalCer was greatly diminished upon sonication. These results suggest that each of the oligosaccharide moieties of II^3NeuAc-LacCer and I^3SO_3-GalCer may be specifically recognized by different ligand molecules of *Helicobacter pylori*.

Helicobacter pylori; Gastric mucosa; Glycosphingolipid; Bacterial adhesion

1. INTRODUCTION

Recent studies have shown a close association of *Helicobacter pylori* (*H. pylori*) on the gastric mucosa with the development of chronic gastritis or peptic ulcers [3–6]. So far, *H. pylori* has been isolated solely from gastric mucosa and no colonization of *H. pylori* on intestinal mucosa was demonstrated. The precise mechanism of the selective colonization on the gastric mucosa has not been clarified yet. Recently, it was reported that attachment of *H. pylori* to the gastric mucosa is mediated possibly by specific recognition of the sialic acid-bearing carbohydrate structures on the bacterial cell surface [7,8]. Other reports suggested the existence of specific sulfate-containing glycolipid receptors for *H. pylori* on the gastric mucosa [9,10]. In this report, we tried to characterize the glycolipid receptors in human gastric mucosa with a solid-phase overlay approach which has been useful for elucidating glycolipid receptors for several pathogens [11–14].

2. MATERIALS AND METHODS

2.1. *Bacteria*

Helicobacter pylori was isolated from the antral mucosal biopsy specimen at the time of endoscopic examination and cultivated in Belo-Horizonte medium with the selective supplement of Skirrow (Nikken Biomedical Laboratory, Kyoto) on agar plates under a microaerophilic atmosphere at 37°C for 4 days. The colony formed was transferred to new plates and cultivated under the same conditions for 3 days. After bacteriological identification as Gram-negative organisms, and determination of oxidase, catalase and urease activities, the bacteria were transferred to a liquid medium (Multiply Broth No. 1 from Nikken Biomedical Laboratory, Kyoto) supplemented with 2.5% fetal bovine serum.

2.2. *Preparation of antibody to* Helicobacter pylori

2.5 mg of *H. pylori* and 0.5 mg of bovine serum albumin in 1 ml of phosphate-buffered saline (pH 7.4) were mixed with 1 ml of Freund's complete adjuvant to prepare a water-in-oil emulsion. The emulsion obtained was injected intradermally into the footpads of a rabbit. In the case of a booster injection, the same emulsion was injected subcutaneously into the back 4 weeks after the initial injection. The antibody titer was monitored by an enzyme-linked immunosorbent assay (ELISA) with a bacterium as the antigen. The antiserum with the titer of 1:12800 was used for TLC-immunostaining.

2.3. *Preparation of glycolipids*

Glycolipids were extracted from the mucosa of human gastric antrum obtained at autopsy, and were fractionated into neutral and acidic glycolipids by DEAE-Sephadex (A-25, acetate form, Pharmacia Fine Chemicals, Sweden) column chromatography. Purification of neutral or acidic glycosphingolipids from each fraction was performed according to the method reported previously [15].

2.4. *TLC-immunostaining*

Glycosphingolipids were developed on a silica gel-coated plate (Polygram, Sil G, Macherey-Nagel, Germany) with chloroform/methanol/water (65:35:8, v/v), and then the plate was incubated with blocking buffer (1% polyvinylpyrrolidone (PVP), 1% ovalbumin and 0.02% NaN_3 in phosphate-buffered saline (PBS)) at 37°C for 1 h, followed by incubation with intact or sonicated *H. pylori* (approximately 10^8 cells/ml) in the medium at 37°C for 1 h. The plate was washed 3 times with washing buffer (0.1% Tween-20 in

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* The nomenclature used for glycosphingolipids followed the recommendation of the IUPAC-IUB Commission [1], and the nomenclature of gangliosides of Svennerholm [2] was partly used

PBS), incubated with the blocking buffer at 37°C for 15 min, and washed twice with PBS. Then the plate was reacted with rabbit anti-*H. pylori* antiserum diluted to 1:200 with dilution buffer (3% PVP in PBS) at 37°C for 1 h, followed by a peroxidase-conjugated anti-rabbit IgG antibody (Kirkegaard & Perry Lab., Gaithersburg, MD, USA) diluted to 1:500 for 1 h at 37°C. The enzyme activity remaining on the plate was visualized by incubation with 0.1% H_2O_2 and 3.4 mM 4-chloro-1-naphthol in Tris-HCl buffer (50 mM, pH 7.4) containing 200 mM NaCl at 37°C for 15 min. The intensity of the spots was determined by means of a dual-wavelength TLC densitometer (CS-9000, Shimadzu, Kyoto) at a sample wavelength of 580 nm.

3. RESULTS

3.1. *Glycosphingolipids in human gastric mucosa*

As shown in Fig. 1A, glycosphingolipids of the human antral mucosa occupied the same positions on TLC as ceramide monohexoside (CMH), LacCer, Gb_3Cer and Gb_4Cer as the neutral glycosphingolipids, and I^3SO_3-GalCer (sulfatides) and II^3NeuAc-LacCer as the acidic glycosphingolipids. In the acidic glycosphingolipids, sulfatides were more predominant than gangliosides. The molar ratios of sulfatides/gangliosides in the fundic and antral mucosa were 2.1 and 3.5, respectively (Table I). The structure of sulfatides from human gastric mucosa was confirmed to be I^3SO_3-GalCer with α-hydroxy fatty acids by GLC and negative ion fast atom bombardment mass spectrometry.

3.2. *Binding of* H. pylori *to glycosphingolipids from the antral mucosa of human stomach*

Direct binding of *H. pylori* to glycosphingolipids from the antral mucosa of the human stomach was examined by TLC-immunostaining with rabbit anti-*H. pylori* antiserum. As shown in Fig. 1, the bacteria principally bound to I^3SO_3-GalCer and II^3NeuAc-LacCer, but no binding to neutral glycosphingolipids was

Table I

Concentration of glycosphingolipids in the fundic and antral mucosa of human stomach

Glycosphingolipids	Fundus (nmol/g, dry weight)	Antrum (nmol/g, dry weight)
GlcCer	284	316
GalCer	570	775
LacCer	716	420
Gb_3Cer	882	551
Gb_4Cer	767	376
II^3NeuAc-LacCer	92	122
II^3NeuAc_2-LacCer	106	141
I^3SO_3GalCer	416	934
I^3SO_3GalCer/gangliosides	2.1	3.5

Carbohydrate composition of CMH (GlcCer or GalCer) was determined by gas-liquid chromatography (GLC) on 3% OV-101 coated on Chrolite with a programmed temperature rise 2°C/min from 150 to 200°C as the *N*-acetyl-*O*-trimethylsilyl derivatives.

Table II

Binding of *H. pylori* to glycosphingolipids purified from the antral mucosa

	I^3SO_3-GalCer	II^3NeuAc-LacCer	Ratio (a/b)
	Peak area		
	(a)	(b)	
Viable *H. pylori*			
Antral mucosa 1	27861	3438	8.1
Antral mucosa 2	27862	5199	5.4
Sonicated organisms			
Antral mucosa 1	5797	6426	0.9
Antral mucosa 2	3015	5796	0.5

Peak areas (a) and (b) represent values obtained from the same TLC plate by electric integrator (μV). Antral mucosa 1 and 2 were from patients 1 and 2, respectively.

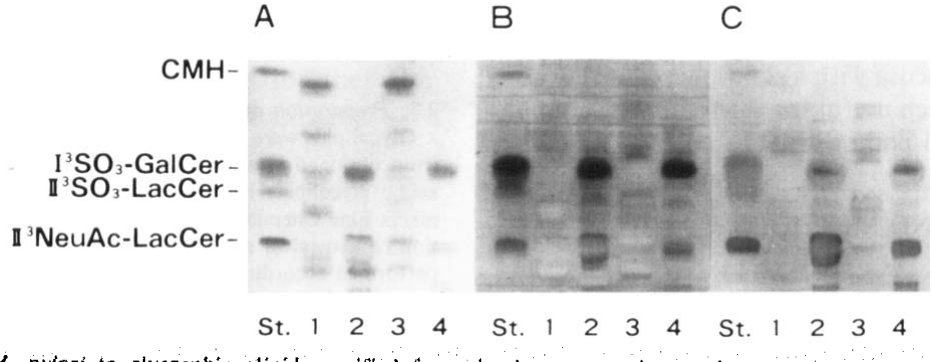

Fig. 1. Binding of *H. pylori* to glycosphingolipids purified from the human gastric antral mucosa on a TLC plate. The amount of glycosphingolipids applied on each lane corresponds to about 5 mg dry weight of the tissue and the TLC plate was developed in the solvent system with chloroform/methanol/water (65:35:8, v/v). (A) Glycosphingolipids visualized by orcinol/H_2SO_4 reagent. (B and C) The plates were incubated with cultured *H. pylori* (B) or sonicated organism (C) and then stained by TLC-immunostaining according to the method described in the text. (Lanes 1 and 2) Neutral and acidic glycosphingolipids, respectively, from the antral mucosa of patient 1. (Lanes 3 and 4) Neutral and acidic glycosphingolipids, respectively, from the antral mucosa of patient 2. Lane St = standard glycosphingolipids (1 μg each of GalCer(CMH) from human brain, I^3SO_3-GalCer from human brain, II^3SO_3-LacCer from SNG II cells derived from human uterine cancer and II^3NeuAc-LacCer from bovine brain).

observed. Also, the binding disappeared when we omitted the incubation step with *H. pylori*. Therefore, the interaction of bacteria with I^3SO_3-GalCer and II^3NeuAc-LacCer was considered to be specific. The binding to these acidic glycosphingolipids on the TLC plate, after preincubation of *H. pylori* with I^3SO_3-GalCer or II^3NeuAc-LacCer, was found to be completely abolished. As shown in Table II, the binding affinity of *H. pylori* to I^3SO_3-GalCer was estimated to be 5–8-fold higher than that to II^3NeuAc-LacCer. On the other hand, the binding to I^3SO_3-GalCer was diminished when the bacterial cells were disrupted by freeze-thawing or sonication. However, under the condition, the binding of *H. pylori* to II^3NeuAc-LacCer was maintained.

4. DISCUSSION

Evans et al. have demonstrated that the hemagglutinin from *H. pylori* recognized *N*-acetylneuraminyl-lactose, which is the oligosaccharide moiety of GM_3 [7]. Slomiany et al. reported a strong inhibition by II^3NeuAc-LacCer and sulfated lactosylceramide of the hemagglutination reaction of *H. pylori* [10]. The effectiveness of a solid-phase overlay approach in determining the glycolipid receptors for various bacteria was recently well documented [11–14]. Using a TLC-immunostaining method, Lingwood et al. found that *H. pylori* specifically bound to a sulfated alkylacylglycerolipid from pig stomach [9], though the precise structure of the lipid was not elucidated. In this study, we also applied TLC-immunostaining to elucidate the glycosphingolipids specially recognized by *H. pylori* and successfully demonstrated direct binding of *H. pylori* to II^3NeuAc-LacCer(GM_3) and sulfatide. Our preliminary results have also shown that freeze-thawing or sonication differentially influenced *H. pylori* binding to GM_3 and sulfatide, indicating the possible involvement of different proteins in binding of each glycosphingolipid. In fact, the glycolipid-binding adhesins locating on either the pilus fiber or cell surface have been demonstrated in *N. gonorrhoea* and *E. coli* [16]. On the other hand, our studies showed that sulfatides were the most predominant acidic glycosphingolipids in the human stomach. Taken together, the results indicate that sulfatides might more likely serve as a receptor for *H. pylori* in situ. Further study will be necessary to clarify the structure and property of these adhesins responsible for the binding to glycolipid receptors.

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