Requirement of ceramide for adhesion of *Helicobacter pylori* to glycosphingolipids

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Abstract Direct adhesion of *Helicobacter pylori* to immobilized glycosphingolipids (GSLs) was compared to that of their corresponding oligosaccharide-conjugated neoglycoconjugates in order to clarify the roles of the carbohydrate and lipid portions of GSLs in *H. pylori* adhesion. These bacteria were found to adhere to sulfatide, GM3, GalCer and LacCer, but not to ceramide, sphingomyelin, or polyacrylamides conjugated with β-galactose, lactose, 3′-sialyllactose or 3′-sulfo-β-galactose. Furthermore, neoglycolipids or bovine serum albumin derivatives with corresponding oligosaccharides were unable to serve as the ligands. *H. pylori* adhesion to GalCer with α-hydroxyl fatty acid was much stronger than GalCer with the non-hydroxyl fatty acid. These results suggest that *H. pylori* recognize the conformation of GSL with α-hydroxyl fatty acid on solid phase.

Key words: Glycosphingolipid; Neoglycolipid; Neoglycoprotein; Neoglycoconjugate; Bacterial adhesion; *Helicobacter pylori*

1. Introduction

Recognition of cell surface carbohydrates is essential for the colonization and initiation of infection by bacteria. *Helicobacter pylori* is thought to be the primary cause of active chronic gastritis, to be involved in the development of gastric and duodenal ulcers, and to have a possible association with gastric cancer [1–4]. Many studies have suggested that one putative group of receptor molecules for *H. pylori* consists of glycosphingolipids[1] (GSL) expressed on the epithelial surface of the gastric mucosa, including sulfatide (I^3SO_3-GalCer) and GM3 (II^3NeuAc-LacCer) [5–7]. However, it remains unclear whether *H. pylori* recognize only the carbohydrate portion of GSL or whether both carbohydrate and lipid portions are required for recognition of GSL by *H. pylori*. In addition, there is only limited information regarding the effect of the glycoform in the adhesion of *H. pylori*.

For a better understanding of *H. pylori* adhesion to GSLs, in the present study, direct adhesion of these bacteria to immobilized GSLs was compared with that to neoglycolipids, neoglycoproteins, and oligosaccharide-conjugated polyacrylamides (PAAs) with oligosaccharide moieties corresponding to those of the GSLs.

2. Materials and methods

2.1. Materials

Sulfatide, GM3, GalCer, LacCer, sphingomyelin, ceramide from bovine brain and lactose-BSA (bovine serum albumin) were purchased from Sigma-Aldrich (Tokyo, Japan). 3′-Sialyllactose-BSA was from Funakoshi (Tokyo, Japan). GalCer and ceramide with α-hydroxyl fatty acid and GalCer and ceramide with non-hydroxyl fatty acid were also obtained from Sigma-Aldrich. Neoglycolipids constructed with dipalmitoylphosphatidylethanolamine (DPPE) and oligosaccharides were synthesized by reductive amination and purified in our laboratory, as described previously [8]. The biotin-labeled PAA-conjugated oligosaccharides (oligosaccharide-PAA-biotin) [9] were from Seikagaku Kogyo Co. (Tokyo, Japan) and Syntesome (Munich, Germany). Molecular weight of each oligosaccharide-PAA-biotin probe is about 30–40 kDa and the carbohydrate content is 20 mol%. Anti-*H. pylori* mouse polyclonal antibody was produced by injecting BALB/c mice with *H. pylori* strain KH202 as an antigen. *Ricinus communis* agglutinin 120 (RCA120) was purchased from Seikagaku Kogyo Co.

2.2. Bacterial strain and growth conditions

H. pylori strain KH202, which is a clinical isolate from a patient with gastritis, was obtained from Amagasaki Hospital, and maintained in the Central Research Laboratories of Kaken Pharmaceutical Co., Ltd. (Kyoto, Japan). *H. pylori* KH202 was cultured on brain–heart infusion agar with defibrinated horse blood (7% v/v) under microaerophilic conditions at 37°C for 3 days. Subsequently, the bacteria were transferred to a liquid medium of brucella broth (Difco) containing 7% fetal calf serum and kept under microaerophilic conditions at 37°C for 24 h. A bacterial suspension with an absorbance of about 0.5 at 540 nm was used for the experiments.

2.3. Immobilization of glycoconjugates

GSLs and neoglycolipids were dissolved in methanol at a concentration of 0.1 mg/ml. Each solution (50 μl) was placed in wells of a 96-well microtiter plate and dried overnight at 37°C. For immobilization of neoglycoproteins, a solution of these glycoconjugates was placed in wells of a 96-well microtiter plate and kept overnight at 4°C. The wells were washed thoroughly with phosphate-buffered saline (PBS) and then blocked with PBS containing 3% BSA. For immobilization

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Abbreviations: BSA, bovine serum albumin; DPPE, dipalmitoylphosphatidylethanolamine; PAA, polyacrylamide; RCA, *Ricinus communis* agglutinin; GSL, glycosphingolipid

[1] The nomenclature used for glycosphingolipids was according to the recommendations of the IUPAC-IUB [15] and Svennerholm [16].

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of oligosaccharide-PAA-biotin, wells of a 96-well plate were first coated with 50 μl of streptavidin (50 μg/ml), washed thoroughly with PBS, and then treated with 50 μl of the oligosaccharide-PAA-biotin probes. After a 1-h incubation, the wells were washed, and the excess of streptavidin coated on the wells was masked with 0.1 mg/ml of biotin at room temperature for 1 h, followed by blocking with 3% BSA in PBS. Immobilization of GSLs, neoglycolipids, neoglycoproteins, and oligosaccharide-PAA-biotin probes was determined by the binding of peroxidase-conjugated lectins, e.g. RCA120, followed by visualization with 0.1% H_2O_2 and 2,2′-azino-di-[3-ethylbenzthianozoline-6-sulfonic acid]. The absorbance at 415 nm was determined using a Model 450 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). As shown in Fig. 1, LacCer, lactose-DPPE, lactose-PAA, and lactose-BSA were immobilized on wells in a concentration-dependent manner in accordance with these procedures. It should be noted that the wells coated with LacCer, lactose-DPPE, and lactose-PAA exhibited similar absorbance at 415 nm when the same concentration of glycoconjugates was added and immobilized in wells, indicating that almost the same quantities of lactose residues are present in these coated wells. These results suggest that equivalent quantities of the oligosaccharide residues were immobilized when other GSLs, neoglycolipids, and PAA-biotin probes were used, although the actual quantities of the other oligosaccharide residues in wells were not determined.

2.4. Direct adhesion of H. pylori

Each assay was performed in triplicate at least twice. The suspension (100 μl) of *H. pylori* was added to the wells of a 96-well plate coated with glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. After incubation, non-adherent bacteria were removed by washing with PBS and the wells were allowed to react with mouse anti-*H. pylori* antiserum in PBS containing 1% BSA. The plates were then treated at 37°C for 1 h with peroxidase-conjugated goat anti-mouse IgG antibody in PBS containing 1% BSA. After they were washed with PBS containing 0.05% Tween 20, the remaining enzyme activity in the wells was visualized by incubation with 0.1% H_2O_2 and 2,2′-azino-di-[3-ethylbenzthianozoline-6-sulfonic acid] at room temperature for 30 min. Absorbance at 415 nm was determined using a Model 450 Microplate Reader (Bio-Rad). Specific adhesion of *H. pylori* to the immobilized glycoconjugates was estimated from differences in absorbance at 415 nm with respect to the glycoconjugate-coated wells and uncoated wells (for glycolipids and glycoproteins) or biotin-coated wells (for oligosaccharide-conjugated PAAs).

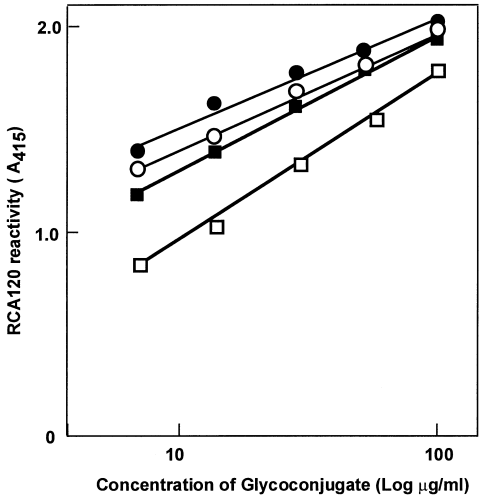

Fig. 1. Immobilization of various glycoconjugates with lactose on solid phase. 50 μl of different concentrations (5, 10, 25, 50, and 100 μg/ml) of LacCer (■), lactose-PAA (●), lactose-DPPE (○), or lactose-BSA (□) were added to wells of 96-well plates, and immobilized according to the procedures described in Section 2. After the wells had been washed and covered with BSA, the remaining lactose residues on wells were determined with peroxidase-conjugated RCA120 (1 μg/ml). Absorbance at 415 nm was measured.

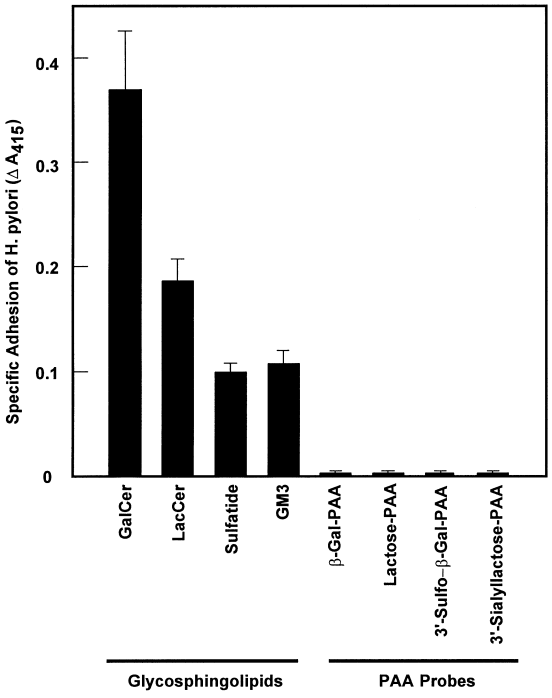

Fig. 2. Adhesion of *H. pylori* to GSL-coated and oligosaccharide-PAA-coated plates. GalCer, LacCer, GM3 and sulfatide (100 μg/ml), as well as biotinylated β-galactoside-PAA, lactose-PAA, 3′-sialyllactose-PAA, and 3′-sulfo-β-galactoside-PAA (100 μg/ml), were coated on other wells of the same 96-well plate, as described in Section 2. With LacCer and lactose-PAA, the level of immobilization on wells was determined by RCA120 ($A_{415nm} = 2.03$ for a LacCer-coated well and $A_{415nm} = 1.96$ for a lactose-PAA-coated well). A suspension of *H. pylori* was added to wells of the 96-well plate coated with the glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. The adherent *H. pylori* were detected with the anti-*H. pylori* polyclonal antibody. The specific adhesion of *H. pylori* to the immobilized glycoconjugates was obtained from differences in absorbance with respect to the glycoconjugate-coated wells and uncoated (for GSLs, $A_{415nm} = 0.07$) or biotin-coated wells (for oligosaccharide-PAA, $A_{415nm} = 0.086$). Bars represent the means+S.D. of three experiments.

3. Results

3.1. Comparison of H. pylori adhesion to GSL and oligosaccharide-conjugated PAA

It has been shown that *H. pylori* preferentially recognize sulfatide (I^3SO_3-GalCer) and GM3 (II^3NeuAc-LacCer) [5–7]. In the direct adhesion assay used in the current research, *H. pylori* strain KH202 clearly adhered to the sulfatide and GM3 immobilized on the plates (Fig. 1). However, *H. pylori* bound to immobilized GalCer and LacCer much more strongly than to sulfatide and GM3 (Fig. 2). These results suggest that GalCer and LacCer serve as better ligands for *H. pylori* than sulfatide and GM3, which have both been proposed as GSL ligands for the bacteria. In order to investigate whether *H. pylori* recognized only carbohydrate regions of the GSLs, the same concentration of PAA-conjugated derivatives with the corresponding carbohydrate moieties to sulfatide, GM3, GalCer and LacCer, i.e. 3′-sulfo-β-galactose-PAA, 3′-sialyllactose-PAA, β-galactose-PAA, and lactose-PAA, were added and immobilized in the plates. The adhesion of *H. pylori* to these immobilized oligosaccharides was then compared to that to the corresponding immobilized GSLs. The quantities of the immobilized lactose residues of LacCer- and lactose-PAA-

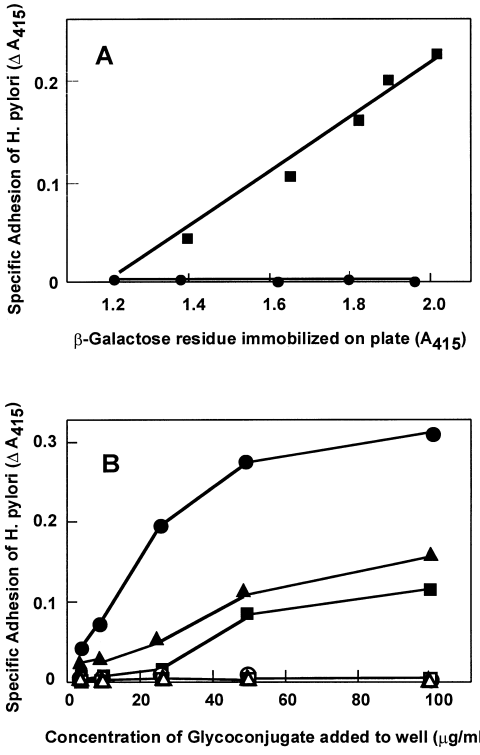

Fig. 3. Adhesion of *H. pylori* to different amounts of immobilized GSLs and oligosaccharide-PAA probes. A: Different concentrations (5, 10, 25, 50, and 100 μg/ml) of LacCer (■) or lactose-PAA (●) were coated onto wells of two 96-well plates. The level of immobilized LacCer and lactose-PAA were measured as the amount of β-galactose residues remaining on the wells of one plate (indicated on the abscissa) using the reactivity of the glycoconjugate-coated wells with peroxidase-conjugated RCA120. B: Different concentrations (5, 10, 25, 50, and 100 μg/ml) of GalCer (■), sulfatide (●), and GM3 (▲), as well as PAA probes containing their corresponding oligosaccharides (respective open symbols), were added to wells of 96-well plates and coated. A suspension of *H. pylori* was added to wells of the 96-well plate coated with the glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. The adherent *H. pylori* were detected with the anti-*H. pylori* polyclonal antibody.

coated plates were almost equivalent, as determined by the reactivity of the wells with RCA120 ($A_{415nm} = 2.03$ for a LacCer-coated well and $A_{415nm} = 1.96$ for a lactose-PAA-coated well; Fig. 1). *H. pylori* did not bind to immobilized lactose-PAA, but did bind to immobilized LacCer (Fig. 2). The increase in *H. pylori* binding to the LacCer-coated plate paralleled the increase in the amounts of immobilized LacCer (estimated from the reactivity with RCA120 on the coated plate). However, no binding to the lactose-PAA-coated plate was observed even when the same quantity of lactose residue from LacCer was immobilized on a plate (Fig. 3A). Similarly, the levels of adhesion of *H. pylori* to GalCer, GM3, and sulfatide were also dependent on the concentration of GSLs added to wells, but adhesion of *H. pylori* to β-galactoside-PAA, 3′-sialyllactose-PAA, and 3′-sulfo-β-galactoside-PAA was not observed, even when the concentration of oligosaccharide-PAA increased (Figs. 2 and 3B). These results indicate that the lipid portion of GSL is required for adhesion of *H. pylori*. In contrast, the recognition specificity of the carbohydrate portions in the GSLs seemed to be rather broad.

3.2. Ceramide is essential for recognition of GSLs

The adhesion of *H. pylori* to GSLs was then compared to that with neoglycolipids composed of DPPE oligosaccharides in order to investigate the requirement of a lipid portion for bacterial adhesion. When the equivalent quantities of lactose-DPPE and LacCer were coated on wells as lactose ($A_{415nm} = 2.03$ for a LacCer-coated well and $A_{415nm} = 1.99$ for a lactose-DPPE-coated well; Fig. 1), *H. pylori* did not adhere to lactose-DPPE, whereas they clearly adhered to LacCer

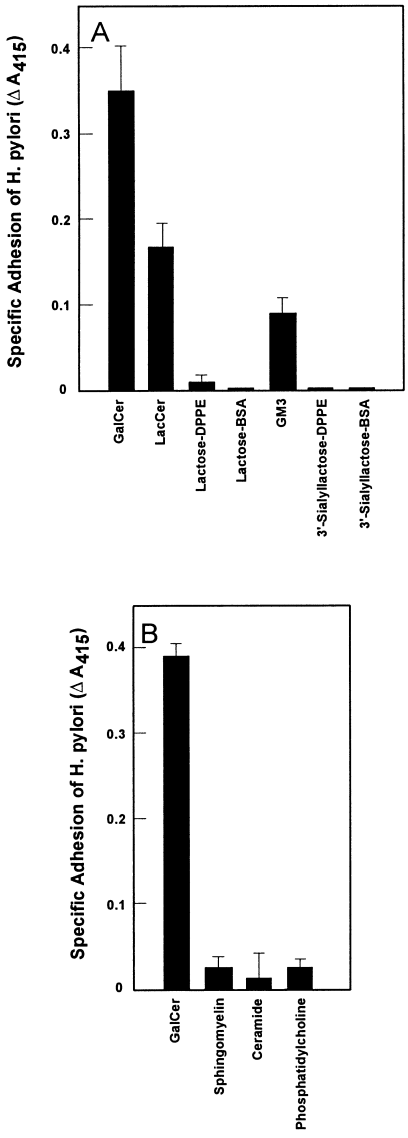

Fig. 4. Adhesion of *H. pylori* to GSL-coated plates and corresponding neoglycolipid- or neoglycoprotein-coated plates. A: GalCer, LacCer, GM3, lactose-DPPE, 3′-sialyllactose-DPPE and BSA derivatives (100 μg/ml) were coated on wells of a 96-well plate. The lactose residue remaining on wells coated with LacCer, lactose-DPPE, and lactose-BSA was determined with peroxidase-conjugated RCA120 ($A_{415nm} = 2.03$, 1.99, and 1.38 for LacCer-, lactose-DPPE-, and lactose-BSA-coated wells, respectively). B: GalCer, ceramide, sphingomyelin, and phosphatidylcholine (100 μg/ml) were coated on wells of a 96-well plate. A suspension of *H. pylori* was added to wells of the 96-well plate coated with the glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. The adherent *H. pylori* were detected with the anti-*H. pylori* polyclonal antibody. Bars represent the means+S.D. of three experiments.

(Fig. 4A). They also adhered to an equivalent amount of GalCer. Notably, lactose-DPPE, LacCer, and GalCer all have a β-galactoside residue at their non-reducing termini, but their lipid portions differ, i.e. lactose-DPPE has diglyceride as its lipid and GalCer and LacCer have ceramide. In addition, 3′-sialyllactose-DPPE did not serve as a ligand, whereas GM3 did (Fig. 4A). Furthermore, lactose-BSA and 3′-sialyllactose-BSA did not serve as ligands, although the quantities of the immobilized oligosaccharide residues on BSA-derivative-coated wells were much lower than those of the corresponding GSL-coated wells ($A_{415nm} = 2.03$ for a LacCer-coated well and $A_{415nm} = 1.58$ for a lactose-BSA-coated well; Fig. 1). These results suggest that the ceramide contained in GSL is essential for adhesion of *H. pylori* to glycolipids.

Since all of the GSLs tested in this study served as ligands for *H. pylori*, while none of the synthesized neoglycoconjugates did, regardless of the carbohydrate portion, we considered whether ceramide itself acts as the ligand for *H. pylori* and whether the carbohydrate portion may be not involved in the adhesion of *H. pylori* to GSLs. Therefore, we examined the adhesion of *H. pylori* to ceramide and sphingomyelin, which are sphingolipid derivatives lacking carbohydrates. As shown in Fig. 4B, the adhesion of *H. pylori* to sphingomyelin and ceramide was almost negligible when the same amount of sphingomyelin, ceramide, and GalCer were immobilized on the solid phase. These results strongly indicate that carbohydrate moieties are also essential for recognition of GSL by *H. pylori*, regardless of the carbohydrate structure.

The binding of *H. pylori* to GalCer with α-hydroxyl fatty acid was 20-fold higher than to that with non-hydroxyl fatty acid (Fig. 5). On the other hand, adhesion of the bacteria to ceramide with or without α-hydroxyl fatty acid is very low. Although the specific adhesion of ceramide mixture in Fig. 3 is much lower than that of ceramide with or without α-hydroxyl fatty acid in Fig. 4, this difference is due to the difference in the background level. When wells were coated with GalCer in the same quantities, but each well contained different ratios of the two fatty acid forms, the increase observed in the adhesion of *H. pylori* paralleled the concentration of GalCer with α-hydroxyl fatty acid (Fig. 6). On the other hand, ceramide with α-hydroxyl fatty acid did not serve as a ligand for *H. pylori*. These results suggest that both the carbohydrate portion and ceramide with α-hydroxyl fatty acid are essential for *H. pylori* recognition of GSLs.

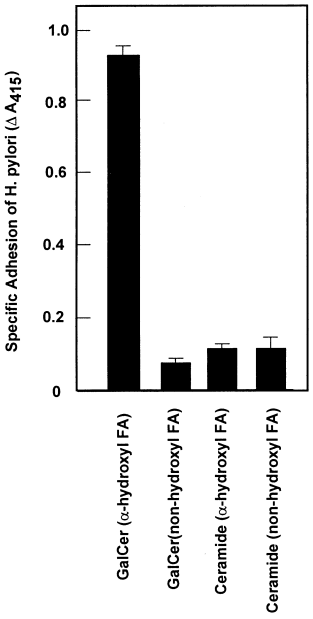

Fig. 5. Adhesion of *H. pylori* to GalCer with α-hydroxyl fatty acid or non-hydroxyl fatty acid. GalCer with α-hydroxyl fatty acid, GalCer with non-hydroxyl fatty acid, ceramide with α-hydroxyl fatty acid, and ceramide with non-hydroxyl fatty acid (100 μg/ml) were coated on wells of a 96-well plate, as described in Section 2. A suspension of *H. pylori* was added to wells of the 96-well plate coated with the glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. The adherent *H. pylori* were detected with the anti-*H. pylori* polyclonal antibody. Bars represent the means+S.D. of three experiments.

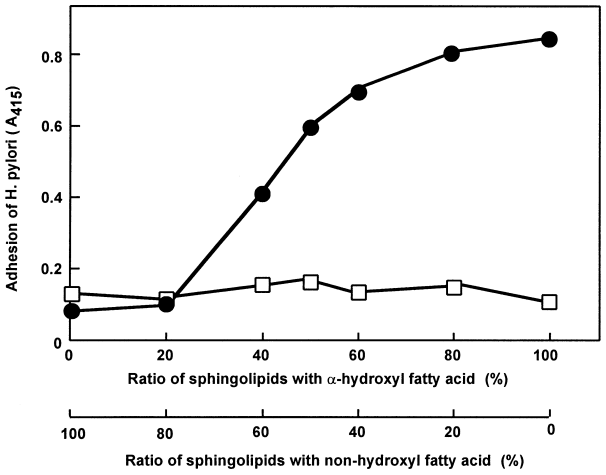

Fig. 6. Adhesion of *H. pylori* depends on the density of GalCer with α-hydroxyl fatty acid. GalCer with α-hydroxyl fatty acid and GalCer with non-hydroxyl fatty acid were mixed at the appropriate ratio (indicated in the abscissa) at the final concentration of 100 μg/ml. The ceramide with α-hydroxyl fatty acid and the ceramide with non-hydroxyl fatty acid were also mixed at the appropriate ratio at the final concentration of 100 μg/ml. Fifty microliters of the mixed GalCer (●) or ceramide (□) (each 100 μg/ml) was added in the wells of a 96-well plate and coated on the wells, as described in Section 2. The suspension of *H. pylori* was added to the wells of a 96-well plate coated with glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min. The adherent *H. pylori* were detected with the anti-*H. pylori* polyclonal.

4. Discussion

At present, several candidate carbohydrates have been proposed as adhesion receptors for *H. pylori* based on the inhibition of *H. pylori* adherence by glycoconjugates. For instance, GM3 and glycoproteins containing the Siaα2-3Gal structure, such as fetuin, inhibit the adhesion of these bacteria [5,10,11]. It was also demonstrated that sulfated lactosylceramide (II^3SO_3-LacCer), sulfatide (I^3SO_3-GalCer), and several glycosaminoglycans or sulfated polysaccharides all had the ability to inhibit *H. pylori* adhesion [5,7,12–14]. These previous inhibition studies indicated that *H. pylori* recognized glycoconjugates. They also suggested that carbohydrate ligands for *H. pylori* were shared in several glycoforms such as GSLs, glycoproteins, and glycosaminoglycans, and that *H. pylori* appeared to recognize only specific carbohydrate moieties carried on these glycoconjugates, regardless of the carrier to which the carbohydrates were bound. However,

no experiment has compared the direct adhesion of *H. pylori* with respect to different types of glycoconjugates with the same carbohydrate portions using the same conditions at the same time. Therefore, whether *H. pylori* recognize only carbohydrate portions of glycoconjugates or not remains unclear. In order to elucidate the effect of the carbohydrate carrier on recognition of glycoconjugates by *H. pylori*, direct adhesion of the bacteria to different types of glycoconjugates with the same carbohydrate portion should be compared.

In the present study, we analyzed the direct adhesion of *H. pylori* to GSLs and glycoconjugates with the corresponding carbohydrates of GSLs in order to clarify the role of the lipid portion in recognition of GSLs by *H. pylori*. The following findings strongly indicate that not only the carbohydrate portion, but also the ceramide portion, particularly ceramide with α-hydroxyl fatty acid, is required for recognition of GSLs by *H. pylori*, although the recognition of the carbohydrate portions in the GSLs seems to be not specific; (1) *H. pylori* clearly bound to several immobilized GSLs such as GalCer, sulfatide, LacCer and GM3, but they did not bind at all to the corresponding oligosaccharide-PAA probes, neoglycolipids, or BSA derivatives, (2) sphingomyelin or ceramide itself did not serve as a ligand for *H. pylori*, indicating that carbohydrate moieties are also essential for adhesion of *H. pylori* to GSLs, and (3) GalCer with α-hydroxyl fatty acid was preferentially recognized by *H. pylori* rather than that with non-hydroxyl fatty acid. Therefore, consideration of the carbohydrate carrier is important in understanding the specific carbohydrate ligand for *H. pylori*. In the case of GSLs, *H. pylori* may recognize the specific conformation of GSLs consisting of both carbohydrate portions and ceramide with α-hydroxyl fatty acid.

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