FEBS Letters 517 (2002) 32–36 FEBS 25976

Carbohydrate carriers affect adhesion of *H. pylori* to immobilized Le^b-oligosaccharide

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Received 31 January 2002; revised 26 February 2002; accepted 1 March 2002

First published online 18 March 2002

Edited by Guido Tetamanti

Abstract The present study involved comparison of adhesion of *Helicobacter pylori* KH202 to immobilized Le^b-oligosaccharide carried on different carriers, i.e. Le^b-oligosaccharide conjugated with polyacrylamide, bovine serum albumin, and dipalmitoylphosphatidylethanolamine (Le^b-PAA, Le^b-BSA, and Le^b-DPPE). All of the Le^b-oligosaccharide-carrying neoglycoconjugates served as ligands for *H. pylori*. However, *H. pylori* required 10-fold and 100-fold quantities of Le^b-antigen to adhere to Le^b-PAA and to Le^b-DPPE in comparison to the quantity of Le^b-antigen needed to adhere to Le^b-BSA, respectively. *H. pylori* adhesion to Le^b-PAA and Le^b-DPPE was clearly inhibited by Le^b-oligosaccharide, but adhesion to Le^b-BSA was hardly inhibited by the oligosaccharide. Therefore, the carbohydrate carrier affects the affinity of *H. pylori* KH202 toward Le^b-antigen, although the bacteria recognize Le^b-antigen regardless of the carbohydrate carrier.

Key words: Lewis b; Neoglycoprotein; Neoglycolipid; Bacterial adhesion; *Helicobacter pylori*

1. Introduction

Helicobacter pylori have been shown to be the primary cause of active chronic gastritis, are associated with the development of gastric and duodenal ulcers, and may be associated with gastric cancer [1–5]. Recognition of cell surface carbohydrates plays an essential role for colonization and initiation of infection of *H. pylori*. It has been suggested that several sialic acid-containing oligosaccharides, sulfated glycans, and glycosphingolipids served as possible receptor molecules for *H. pylori* [6–12]. Recent reports suggested that *H. pylori* recognized the Le^b-antigen (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ-R) expressed on the gastric mucosa, since neoglycoproteins or neoglycolipids containing Le^b-oligosaccharide and an antibody against Le^b-antigen inhibited the bacterial adhesion to gastric mucosa [13–15]. In addition, it has been shown that *H. pylori* adhere to a glycoprotein or a glycolipid containing Le^b-oligosaccharide [14]. These facts strongly suggest that *H. pylori* recognize only the Le^b-antigen of these glycoconjugates without regard to the carbohydrate carrier. However, the effect of carbohydrate carrier of Le^b-antigen in terms of bacterial adhesion remains unclear.

To address the possible role of carbohydrate carriers in bacterial adhesion to carbohydrates, the present study involved analysis of the direct adhesion of *H. pylori* to three types of neoglycoconjugates carrying Le^b-oligosaccharide immobilized on plates, i.e. Le^b-oligosaccharide conjugated with polyacrylamide (PAA), bovine serum albumin (BSA), and dipalmitoylphosphatidylethanolamine (DPPE) (Le^b-PAA, Le^b-BSA, and Le^b-DPPE).

2. Materials and methods

2.1. Materials

Le^b-hexasaccharide (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc), Le^b-hexasaccharide conjugated with BSA (Le^b-BSA), lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc; H-type 1), and lacto-*N*-fucopentaose I-BSA (H-type 1-BSA) were purchased from Funakoshi (Tokyo, Japan). The average carbohydrate content of Le^b-BSA and H-type 1-BSA is about 8 mol per BSA. The Le^b-neoglycolipid (Le^b-DPPE) constructed with DPPE and Le^b-hexasaccharide and H-type 1-neoglycolipid (H-type 1-DPPE) constructed with DPPE and lacto-*N*-fucopentaose I were synthesized and purified in our laboratory as described previously [16]. It should be noted that the hemiacetal ring of glucose residue at the reducing end of the oligosaccharide of each neoglycolipid was unclosed by reductive amination. Purity of the Le^b-DPPE and H-type 1-DPPE was confirmed using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. The biotin-labelled PAAs conjugated with Le^b-tetrasaccharide (Fucα1-2Galβ1-3(Fucα1-4)Glc) (Le^b-PAA-biotin) and those conjugated with other histo-blood group antigen oligosaccharides [17] were from Seikagaku Kogyo Co. (Tokyo, Japan). Molecular weight of each oligosaccharide-PAA-biotin probe is about 30 to 40 kDa and the carbohydrate content is 20 mol%. An anti-Le^b monoclonal antibody (IgG1) was obtained from Chemicon International Inc. (Temecula, CA, USA).

2.2. Bacterial strain and growth condition

H. pylori strain KH202 obtained from the Central Research Laboratories of Kaken Pharmaceutical Co. Ltd (Kyoto, Japan) was cultured on brain-heart infusion agar with defibrinated horse blood (7% v/v) under a microaerophilic condition at 37°C for 3 days [12]. Subsequently, the bacteria were transferred to a liquid medium of brucella broth (Difco, Detroit, MI, USA) containing 7% fetal calf serum under microaerophilic condition 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 oligosaccharides on solid phase

The Le^b-DPPE was dissolved in methanol at a concentration of 0.1 mg/ml. The solution (50 μl) was placed in wells of a 96-well microtiter

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Abbreviations: BSA, bovine serum albumin; DPPE, dipalmitoylphosphatidylethanolamine; PAA, polyacrylamide; Le^b-BSA, Le^b-hexasaccharide-conjugated BSA; Le^b-DPPE, neoglycolipid constructed with Le^b-hexasaccharide and DPPE; Le^b-PAA, Le^b-tetrasaccharide-conjugated PAA

PII: S0014-5793(02)02571-1

plate and dried overnight at 37°C; then, the wells were blocked overnight with PBS containing 3% BSA at 4°C. For immobilization of Le^b-BSA, the solution (50 μg/ml) was placed in wells of a 96-well microtiter plate, kept overnight at 4°C, and the wells were extensively washed with PBS. Then, wells were blocked overnight with PBS containing 3% BSA at 4°C. For immobilization of Le^b-PAA-biotin, wells of a 96-well plate were first coated with 50 μl of streptavidin (50 μg/ml) overnight at 4°C, washed with PBS extensively, and then 50 μl of the oligosaccharide-PAA-biotin probes (50 μg/ml) were placed in wells. After the wells had been incubated for 1 h, they 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. As the control, wells coated with streptavidin were used after masking with biotin. The amount of Le^b-antigen coated on the solid phase was determined with ELISA using anti-Le^b monoclonal antibody followed by peroxidase-conjugated anti-mouse IgG antibody. The absorbance at 415 nm was determined by means of a Model 450 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). When 50 μg/ml of these glycoconjugates were added to wells and the solid phase was coated, the wells coated with Le^b-BSA, Le^b-PAA, and Le^b-DPPE exhibited the absorbance at 415 nm of 0.32, 1.65, and 2.5, respectively.

2.4. Direct adhesion experiment of H. pylori

Individual assays were performed in triplicate at least twice. The suspension (100 μl) of *H. pylori* in culture medium was added to the wells of a 96-well plate coated with glycoconjugates, and the plate was incubated under microaerobic conditions at 37°C for 90 min. After incubation, non-adherent bacteria were washed three times with PBS, the wells were allowed to react with mouse anti-*H. pylori* antiserum (1:5000 dilution) in PBS containing 1% BSA at 37°C for 1 h, followed by peroxidase-conjugated goat anti-mouse IgG antibody (1:1000 dilution) in PBS containing 1% BSA at 37°C for 1 h. After the wells were washed with PBS containing 0.05% Tween-20, the remaining enzyme activity in the well was visualized by incubation with 0.1% H_2O_2 and 2,2′-azino-di-[3-ethylbenzothianozoline-6-sulfonic acid] (ABTS) at room temperature for 30 min. The absorbance at 415 nm was determined by means of a Model 450 Microplate Reader. Specific adhesion of *H. pylori* to immobilized glycoconjugates was determined from the difference in absorbance with respect to the glycoconjugate-immobilized wells and non-coated wells (for Le^b-BSA and Le^b-DPPE), or biotin-coated wells (for oligosaccharide-conjugated PAA).

2.5. Inhibition of bacterial adherence

For inhibition of *H. pylori* adhesion to immobilized neoglycoconjugates by soluble inhibitors, the inhibitor was added to the suspension of *H. pylori* at the indicated concentrations, and the bacteria were incubated under microaerobic conditions at 37°C for 60 min. Then the suspension was added to the wells of a 96-well plate coated with Le^b-oligosaccharide-carrying glycoconjugates, and the plate was incubated in a microaerobic atmosphere at 37°C for 90 min.

3. Results

3.1. Adhesion of H. pylori to immobilized histo-blood group antigen oligosaccharides

It has been shown that *H. pylori* adhere to some blood group antigens such as Le^b and H-type 1 antigens [13–15]. To investigate the specificity of recognition of histo-blood group antigen oligosaccharides by *H. pylori* strain KH202, oligosaccharides conjugated with biotin-labelled PAA were immobilized on the solid phase that had been pre-coated with streptavidin, and the direct adhesion of *H. pylori* to the immobilized oligosaccharides was compared. As shown in Fig. 1, *H. pylori* adhered to only the Le^b-PAA-immobilized plate. In contrast, PAA derivatives of other histo-blood group antigens did not serve as ligands for *H. pylori*. Therefore, *H. pylori* strain KH202 appeared to specifically recognize Le^b-antigen among the histo-blood group antigen oligosaccharides.

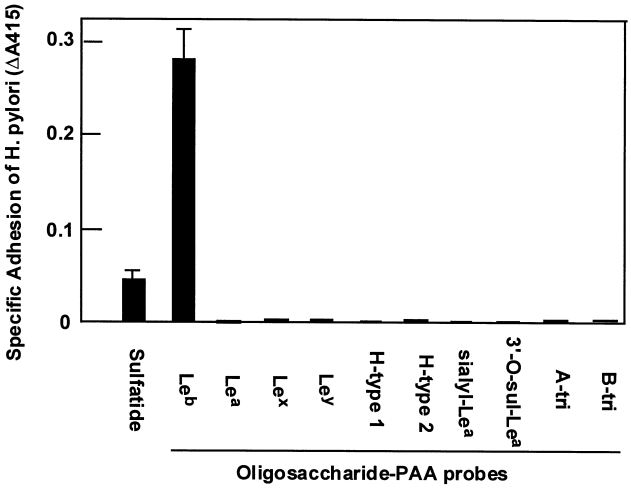

Fig. 1. Adhesion of *H. pylori* to immobilized oligosaccharide-PAA. The PAA conjugated with the following histo-blood group antigen oligosaccharides and biotin (oligosaccharide-PAA-biotin) (50 μg/ml) was added to wells of a 96-well plate and coated, as described in Section 2: blood group type A-trisaccharide (A-tri), blood group type B-trisaccharide (B-tri), 3′-sulfo-Le^a-tetrasaccharide (3′-0-sul-Le^a), sialyl-Le^a, blood group H-type 1-trisaccharide (H-type 1), blood group H-type 2-trisaccharide (H-type 2), Le^y-tetrasaccharide, Le^x-trisaccharide, Le^b-tetrasaccharide, and Le^a-trisaccharide. Wells coated with streptavidin and biotin were used as non-coated wells. The suspension (100 μl) of *H. pylori* was then added to the wells of a 96-well plate coated with glycoconjugates, and the plate was incubated under microaerobic conditions at 37°C for 90 min. The specific adhesion of *H. pylori* to immobilized glycoconjugates was obtained from the difference in absorbance with respect to the glycoconjugate-immobilized wells and non-coated wells ($A_{415} = 0.03$). Adhesion of *H. pylori* to sulfatide was used as a positive control.

3.2. Adhesion of H. pylori to different types of Le^b-carrying neoglycoconjugates

To investigate whether *H. pylori* strain KH202 recognizes only the carbohydrate portion of Le^b-carrying glycoconjugates regardless of the carbohydrate carrier, several synthetic neoglycoconjugates carrying Le^b-oligosaccharides were added to wells in the same amounts (20 μg/ml) and coated, and then *H. pylori* adhesion to the Le^b-oligosaccharide-coated solid phase was examined. All of the neoglycoconjugates carrying Le^b-oligosaccharides, i.e. Le^b-PAA, Le^b-BSA, and Le^b-DPPE, served as ligands for *H. pylori* (Fig. 2). In contrast, lacto-*N*-fucopentaose I-BSA (H-type 1-BSA) and H-type 1-DPPE as well as H-type 1-PAA did not serve as receptors for *H. pylori* (Fig. 2). These results suggest that *H. pylori* strain KH202 specifically recognizes Le^b-oligosaccharide, regardless of the carbohydrate carrier.

Next, the relationship between *H. pylori* binding to the immobilized Le^b-oligosaccharide-carrying synthesized glycoconjugates and quantities of Le^b-antigen immobilized in the wells was examined. The quantity of Le^b-antigens immobilized on the plates was determined from the reactivity to the anti-Le^b monoclonal antibody. As shown in Fig. 3, the adhesion of *H. pylori* to the immobilized Le^b-carrying synthesized glycoconjugates increased with respect to the increased quantity of Le^b-antigen on the plate. However, the quantity of Le^b-antigen required for the adhesion differed among the Le^b-BSA, Le^b-PAA, and Le^b-DPPE. The adhesion of *H. pylori* to immobilized Le^b-BSA was clearly observed with absorbance of 0.02 at 415 nm as Le^b-antigen quantity, and reached a plateau at absorbance of 0.05. In contrast, the adhesion of

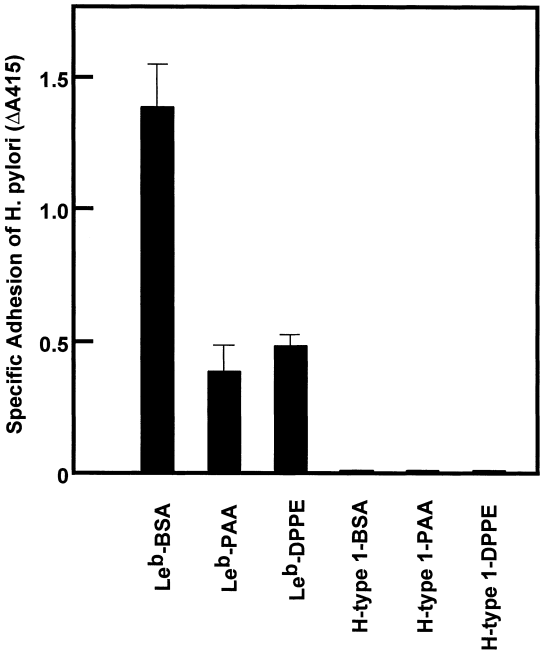

Fig. 2. Adhesion of *H. pylori* to different types of Le^b-neoglycoconjugates. The synthetic neoglycoconjugates carrying Le^b-oligosaccharide (Le^b-BSA, Le^b-PAA, and Le^b-DPPE, each 20 μg/ml) were added to wells of a 96-well plate and coated, as described in Section 2.

H. pylori to immobilized Le^b-PAA was not observed with absorbance of 0.2 at 415 nm with respect to the Le^b-antigen quantity, the point of the strongest adherence of *H. pylori* to the Le^b-BSA-coated plate. Past absorbance of 0.2 at 415 nm, the adhesion of *H. pylori* to the Le^b-PAA-coated plate increased in tandem with the increased quantity of Le^b-antigen, and reached a plateau at the absorbance of 1.5. In addition,

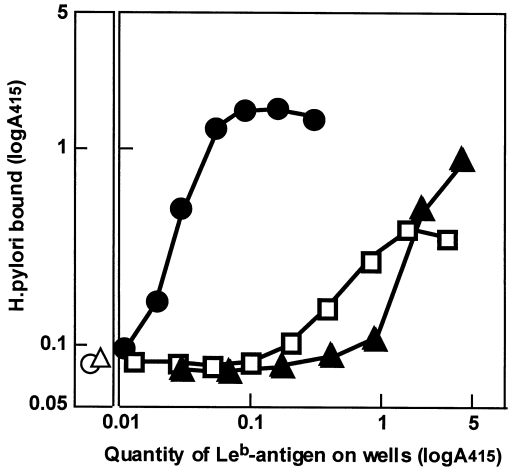

Fig. 3. Adhesion of *H. pylori* to different amounts of neoglycoconjugates carrying Le^b-antigen. 100 μg/ml of Le^b-BSA (●), Le^b-PAA (□), or Le^b-DPPE (▲) was two-fold serially diluted, and the neoglycoconjugate carrying Le^b-antigen (100 μl) was added to wells of two 96-well plates at the different concentration and coated on wells as described in Section 2. The immobilized Le^b-antigen in wells was determined from the reactivity of the wells of one plate against an anti-Le^b monoclonal antibody (indicated in abscissa), as described in Section 2. Non-coated wells were used as the control for Le^b-BSA- or Le^b-DPPE-coated wells (○), and streptavidin-biotin-coated wells were used as the control for Le^b-PAA-coated wells (△).

the maximum binding of *H. pylori* to Le^b-PAA was about one-third of that to Le^b-BSA (Fig. 3). A greater quantity of Le^b-antigen was required on the plate (beyond absorbance of 1.0 at 415 nm) for adhesion to the Le^b-DPPE-coated plate (Fig. 3). Therefore, the quantity of Le^b-antigen in the solid phase needed for *H. pylori* adhesion differed among the carriers that bind Le^b-antigen.

3.3. Inhibition of *H. pylori* adhesion to Le^b-neoglycoconjugates by Le^b-oligosaccharide

To confirm that *H. pylori* preferentially recognized Le^b-antigen carried on BSA rather than on lipids or PAA, three kinds of neoglycoconjugates were placed in the solid phase at the concentration with the strongest adherence of *H. pylori* (see Fig. 3), the solid phase was coated, and the adhesion of *H. pylori* to Le^b-carrying glycoconjugate-coated plates was inhibited by the Le^b-oligosaccharide (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc). As shown in Fig. 4, the

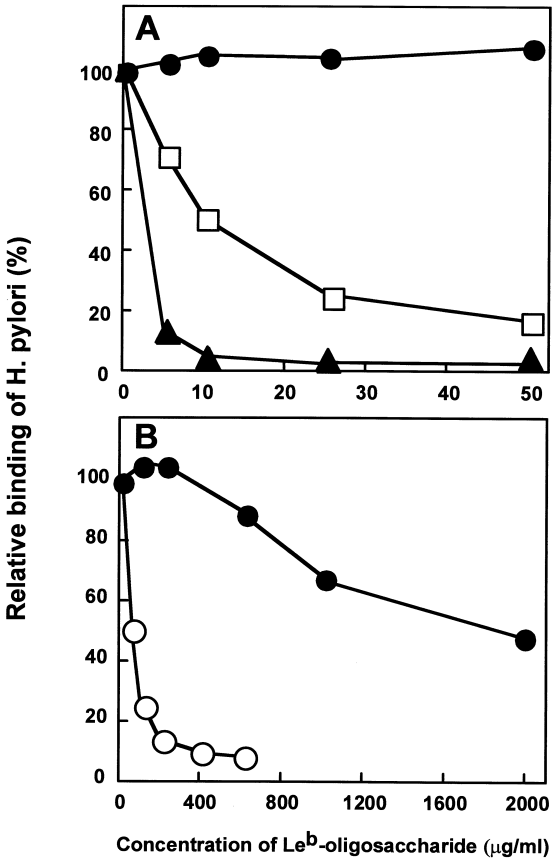

Fig. 4. Inhibition of *H. pylori* adhesion by Le^b-oligosaccharide. 50 μg/ml of Le^b-BSA and Le^b-PAA, and 100 μg/ml of Le^b-DPPE, where adhesion of *H. pylori* to wells coated with Le^b-BSA, Le^b-PAA, and Le^b-DPPE was the strongest as shown in Fig. 3, were coated on wells of a 96-well plate. A: Different concentrations (0–50 μg/ml) of Le^b-oligosaccharide were added to the suspension of *H. pylori*, and incubated at 37°C for 60 min under microaerobic conditions. (●) Le^b-BSA-coated wells; (□) Le^b-PAA-coated wells; (▲) Le^b-DPPE-coated wells. The ordinate represents the relative adhesion with respect to the adhesion of *H. pylori* without the oligosaccharide. Adhesion to Le^b-BSA, Le^b-PAA, and Le^b-DPPE without the oligosaccharide was 1.17, 0.52, and 0.87, respectively, at A_{415}. B: Represents the inhibition of *H. pylori* adhesion to Le^b-BSA-coated wells by a high concentration of Le^b-oligosaccharide (●) or Le^b-BSA (○).

Table 1
Inhibition of *H. pylori* adhesion to Le^b-oligosaccharide-coated plate by neoglycoconjugates

Inhibitor[a]	Relative adhesion to immobilized Le^b-oligosaccharide[b] (% of control)		
	Le^b-BSA	Le^b-PAA	Le^b-DPPE
Le^b-hexasaccharide	102.5	21.3	5.1
Le^b-PAA	93.1	6.6	1.3
Le^b-BSA	51.8	2.6	0.5
H-type 1-BSA	101.5	98.7	92.3
BSA	103.1	101.6	99.5

[a]Each inhibitor was used at a concentration of 100 μg/ml.
[b]Adhesion of *H. pylori* without an inhibitor was used as the control.

adhesion of *H. pylori* to the Le^b-DPPE-coated plate was almost completely inhibited by Le^b-oligosaccharide at a concentration of 10 μg/ml. The same concentration of Le^b-oligosaccharide (10 μg/ml) inhibited about 50% of adhesion to the Le^b-PAA-coated plate. The inhibition of bacterial adhesion to Le^b-PAA- and Le^b-DPPE-coated plates by the soluble oligosaccharide appeared in a concentration-dependent manner. On the other hand, the inhibition of adhesion to the Le^b-BSA-coated plate by the oligosaccharide was almost negligible even when 50 μg/ml of inhibitor was used (Fig. 4A). With much higher concentrations of Le^b-oligosaccharide, the adhesion of *H. pylori* to Le^b-BSA-coated plate was inhibited by Le^b-oligosaccharide in a concentration-dependent manner, but 2 mg/ml of the oligosaccharide inhibited only 50% of the adhesion (Fig. 4B). Le^b-PAA (100 μg/ml) inhibited the adhesion to Le^b-PAA and Le^b-DPPE to a greater degree than Le^b-oligosaccharide but did not inhibit adhesion to Le^b-BSA (Table 1). In contrast, 100 μg/ml of Le^b-BSA inhibited *H. pylori* adhesion to the Le^b-BSA-coated plate by about 50% (Fig. 4B) and almost completely inhibited adhesion to the Le^b-DPPE- or Le^b-PAA-coated plate (Table 1). The same concentration of H-type 1-BSA or BSA did not inhibit *H. pylori* adhesion to the Le^b-BSA-coated plate as well as to the Le^b-PAA- and Le^b-DPPE-coated plates (Table 1). The concentration of Le^b-oligosaccharide required for 50% inhibition of the bacterial adhesion to Le^b-DPPE-, Le^b-PAA-, and Le^b-BSA-coated plates was about 2, 10, and 2000 μg/ml, respectively. These results indicate that *H. pylori* KH202 adheres to Le^b-BSA with the highest affinity among the different types of Le^b-carrying neoglycoconjugates. Therefore, a certain configuration of glycoconjugates may be required in addition to the specific carbohydrate structure when the carbohydrates serve as high affinity receptors for *H. pylori*.

4. Discussion

The studies of carbohydrate receptors for *H. pylori* have thus far focused on the structure of carbohydrates, and several candidate carbohydrates including Le^b-antigen have been demonstrated [6–15]. However, no research has explored the role of carbohydrate carriers in bacterial adhesion. The present study involved comparison of the direct adhesion of *H. pylori* strain KH202 to solid phase-coated Le^b-oligosaccharide carried on different types of carriers, i.e. protein, lipid, and PAA, in order to clarify the possible role of the carbohydrate carrier in the adhesion of *H. pylori* to carbohydrates. With direct adhesion and inhibition studies, the results demonstrate that the carbohydrate carrier significantly affects the affinity of *H. pylori* adhesion to Le^b-oligosaccharide, although *H. pylori* recognize Le^b-antigen regardless of the carbohydrate carrier, as indicated previously [13–15].

Le^b-PAA, Le^b-BSA, and Le^b-DPPE served as ligands for *H. pylori* KH202, indicating that the Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ-R structure (Le^b-antigen) is recognized by this strain regardless of the carbohydrate carriers. However, the quantity of Le^b-antigen required on the solid phase for adhesion of *H. pylori* clearly differs among the glycoconjugates carrying Le^b-oligosaccharide. When Le^b-BSA was coated on the solid phase, *H. pylori* KH202 adhered strongly to the solid phase only with small quantities of Le^b-antigen (Le^b-antigen quantity in the solid phase of $A_{415} = 0.02$ as determined by anti-Le^b monoclonal antibody and shown in Fig. 3). In contrast, these bacteria required 100 times the quantity of Le^b-antigen ($A_{415} = 2.0$) to adhere to the Le^b-DPPE-coated plate than they did to adhere to the Le^b-BSA-coated plate. It should be noted that the oligosaccharide portions of both Le^b-BSA and Le^b-DPPE are the same Le^b-hexasaccharide (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc), although the hemiacetal ring of glucose residue at the reducing end of the oligosaccharide of Le^b-DPPE was unclosed by reductive amination, indicating that the difference in the quantity of Le^b-antigen on the plate required for the adhesion between these two Le^b-glycoconjugates is primarily due to the differences in the carrier of the oligosaccharide (i.e. protein or lipid). Similarly, the bacteria required 10 times the quantity of Le^b-antigen ($A_{415} = 0.2$) to adhere to the Le^b-DPPE-coated plate than they did to adhere to the Le^b-BSA-coated plate. The difference in the binding affinity of *H. pylori* to these glycoconjugates was confirmed by the inhibition of *H. pylori* adhesion by Le^b-oligosaccharide. The concentration of Le^b-oligosaccharide required for 50% inhibition of the bacterial adhesion to immobilized Le^b-carrying neoglycoconjugates ranked in the order from Le^b-BSA $\ggg$ Le^b-PAA $>$ Le^b-DPPE. It is notably that the adhesion of *H. pylori* to Le^b-BSA-coated plates is hardly inhibited by Le^b-oligosaccharide or Le^b-PAA, but clearly inhibited with Le^b-BSA.

An established finding is that the carbohydrate binding affinity of lectin is enhanced by multivalent carbohydrates [18]. The carbohydrate content of Le^b-BSA is about 10 wt% and that of Le^b-PAA is about 65 wt%, indicating that the oligosaccharides are more multivalent in Le^b-PAA than in Le^b-BSA. However, *H. pylori* adhered to Le^b-BSA with higher affinity than Le^b-PAA. Therefore, the high affinity of *H. pylori* to Le^b-BSA may not be simply explained by the multivalence of the carbohydrates. Further studies are required regarding the relationship between the binding affinity of *H. pylori* to carbohydrates and the multivalence of the carbohydrates.

We have recently shown that *H. pylori* have a stronger recognition for glycosphingolipids with α-hydroxyl fatty acid than those with non-hydroxyl fatty acid, suggesting that a specific configuration is required for the recognition of glycosphingolipids by *H. pylori* [12]. As shown in the present study, the carrier also significantly affects adhesion of *H. pylori* to Le^b-antigen. Therefore, carbohydrate carriers as well as carbohydrate structures are important factors in understanding the adhesion of *H. pylori* to carbohydrate receptors.

Acknowledgements: We thank Dr. Hisato Senda (Central Research

Laboratories, Kaken Pharmaceutical Co., Ltd.) to provide us the anti-*H. pylori* antibody. This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 12680619) from the Ministry of Education, Science, Sports, and Culture of Japan, and by the Proposal-Based New Industry Creative Type Technology R&D Promotion Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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