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GLYCOCONJUGATES WITH NeuAc-NeuAc-Gal-Glc ARE MORE EFFECTIVE AT PREVENTING ADHESION OF Helicobacter pylori TO GASTRIC EPITHELIAL CELLS THAN GLYCOCONJUGATES WITH NeuAc-Gal-Glc

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Helicobacter pylori (H. pylori) adheres to human gastric epithelial cells, eliciting various gastroduodenal diseases. Gangliosides play a critical role in bacterial adhesion to cell surfaces. The present study examined how residues of gangliosides are important for inhibition of adhesion of H. pylori to MKN-45 cells. We measured adhesion or detachment effects of gangliosides on the interaction between MKN-45 cells and H. pylori, as well as interleukin-8 production. Among the gangliosides, O-Ac-GD₃, GT₁b, GD₁a, GD₁b, GT₁a, and GD, had potent dose dependent inhibitory effects on adhesion of H. pylori to MKN-45 cells, interleukin-8 production, and vacuole formation induced by H. pylori toxin binding to Vero cells. GD₃ also accelerated bacterial detachment of MKN-45 cells with adherent H. pylori in a dose dependent manner. Such results strongly suggest that the mechanism involved in the inhibition of H. pylori adhesion is mediated by the variations of the residues of the NeuAc-NeuAc-Gal-Glc chain of gangliosides. NeuAc-NeuAc-Gal-Glc exhibits a more inhibitory effect on adhesion than the NeuAc-Gal-Glc chain. Such gangioside and oligosaccharide sequences appear to have therapeutic importance for prevention of H. pylori adhesion, as well as reduction of both inflammation and gastric mucosal injuries.

Key words: gangliosides, Helicobacter pylori, adhesion, interleukin-8, vacuolation

INTRODUCTION

Infection of human gastric mucosa with Helicobacter pylori (H. pylori) causes chronic gastritis and increases the risk of gastric diseases such as peptic ulceration and gastric cancer (1-4). As a matter of fact, animal studies provided
evidence that *H. pylori* could induce gastritis, gastric ulcers, intestinal metaplasia, and gastric cancer-like mucosal changes (5-8). Adhesion of *H. pylori* to gastric epithelial cells represents an important first step in the pathogenesis of such various lesions, as well as a novel therapeutic target for treatment and prevention of *H. pylori* infection (9-14). In general, adhesion is mediated by binding of bacterial surface proteins, i.e. colonization factor antigens, to receptors of host epithelial cells (11, 15, 16). Receptors typically consist of carbohydrate residues of glycoprotein and glycolipid located on the apical cell membrane (9, 17-20). Gangliosides are sphingoglycolipids that contain sialic acid residues in the oligosaccharide chain. Sphingoglycolipids localize to the outer side of the surface membrane bilayer. Their hydrophobic moiety, namely ceramide, embeds in the lipid bilayer, whereas the carbohydrate moiety extends to the outside. Alterations in sphingoglycolipid composition have been observed in cell differentiation, proliferation, and transformation (21). In human gastric mucosa, sulfatide, ganglioside GM₃, GM₁, GD₃, and GD₁α are present in gangliosides (22).

It has been reported that gangliosides present in milk inhibit *Vibrio cholerae* enterotoxin and *Escherichia coli* heat-labile enterotoxin (23). Idota et al. (24) suggested that milk gangliosides and their derivatives affect adhesion of enterotoxigenic and enteropathogenic *Escherichia coli* to Caco-2 cells. Some human milk oligosaccharides exhibit the same structures as receptors found on cellular surfaces and are thus regarded as potential inhibitors of infection, since they represent soluble receptor analogues for pathogens. Sialylated components in human milk may assist in preventing infection from pathogens by acting as soluble receptor analogues (17, 25).

Given the above, the present study investigated (1) the effects of gangliosides present in milk on the adhesion of *H. pylori* to epithelial cells to elucidate what kind of ganglioside is important in inhibition of adhesion and (2) the effects of gangliosides on the factors involved in the pathogenesis of *H. pylori*-associated mucosal damage, namely the interleukin-8 (IL-8) production, and vacuole formation.

**MATERIALS AND METHODS**

**Compounds**

Gangliosides GD, and GM₁ produced by Snow Brand Milk Products Co., Ltd. were purchased from Nakarai tesque Co. (Kyoto, Japan). Neuraminidase extracted from *Clostridium per* and sulfatide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Endoglycoceramidase extracted from Rhodococcus sp. was purchased from Seikagaku Co. (Tokyo, Japan). Gangliosides GM₁, GM₁α, GM₁ inner ester, GD₁α, GD₁β, GT₁α, GT₁β, GQ₁β, and asialo GM₁ were purchased from Funakoshi Co. (Tokyo, Japan). These chemicals were dissolved in PBS and used for the experiments.

**Bacterial strains and cultures**

A standard strain of *H. pylori*, NCTC 11637 [CagA⁺/Tox⁻], was purchased from American Type Culture Collection (USA) and cultured in Brain Heart Infusion agar (Nissui Co., Tokyo,
Japan) plates containing 7% defibrinized horse blood in a microaerobic atmosphere at 37°C for 3 to 4 days.

Cell cultures

MKN-45 cells, i.e. poorly differentiated human gastric adenocarcinoma cell lines (Immunobiological Laboratories), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in polystyrene tissue culture flasks at 37°C for 2 days in an atmosphere of 5% CO₂. Confluent cultures were treated with 0.25% trypsin to detach the cells that were subsequently used to prepare monolayers grown without antibiotics. For the adhesion assay, 96-well tissue culture plates were seeded with 10⁴ cells in 100 µl of culture medium/well and allowed to attach at 37°C for 2 days in an atmosphere of 5% CO₂.

Vero cells, i.e. African green monkey kidney cells (Human Science Research Resources Bank), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in polystyrene tissue culture flasks at 37°C for 2 days in an atmosphere of 5% CO₂. Confluent cultures were treated with 0.25% trypsin to detach the cells that were subsequently used to prepare monolayers grown without antibiotics. For the cytotoxicity assay, 96-well tissue culture plates were seeded with 10² cells in 100 µl of culture medium/well and allowed to attach at 37°C for 2 days in an atmosphere of 5% CO₂.

Bacterial extracts

*H. pylori* strains were cultured at 37°C in Brucella broth (Becton Dickinson, USA) containing 7% fetal bovine serum. Broth cultures were incubated in an atmosphere of 7.5% CO₂ - 7.5% H₂ - 5% O₂ - 80% N₂ on a shaker at 100 rpm for 48 hr. The cultures were centrifuged at 16,000 x g for 20 min and protein present in the supernatant were precipitated with a 50% saturated solution of ammonium sulfate. After centrifugation at 16,000 x g for 15 min the pellets were resuspended in phosphate-buffered saline (PBS) and dialyzed overnight. This solution was then sterilized by filtration through 0.22 µm cellulose filters.

In vitro adhesion assay

The enzyme-linked immunosorbent assay (ELISA) adhesion assay used for the present study was based on a slight modification of the method used by Huesca et al. (26). Round-bottom welled microtiter plates (IWAKI 3882-096, Iwaki Glass, Tokyo, Japan) were coated with 1 µg of sulfatide, GD₃, and GM₁ and evaporated at room temperature. Nonspecific-binding sites were blocked with 2% gelatin and 2% albumin in 100mM tricine buffered saline (TBS) for 1 hr at room temperature. After washing, wells were incubated in a microaerobic atmosphere at 37°C for 1 hr with a 100 µl suspension (10⁸ CFU/ml) of *H. pylori* that had been pre-incubated for 30 min with the test compounds. The plates were then washed and incubated at 37°C for 1 hr with mouse anti-*H. pylori* antiserum. The plates were washed again and incubated with goat-affinity purified antibody to mouse immunoglobulins (CAPPEL, USA) conjugated with peroxidase for 1 hr at room temperature. After washing, bound peroxidase was colorimetrically detected using the TMB Peroxidase Substrate Kit (BIO-RAD, USA). The enzyme reaction was stopped with 1N-sulfuric acid and optical density was measured at 450nm using a microplate ELISA reader (BIO-RAD). The inhibitory concentration was calculated from the optical density readings by the following formula: 

% inhibition = [(OD experimental - OD negative) / (OD positive - OD negative)] x 100. 

With this formula, negative wells contain no bacteria, positive wells contain bacteria without added inhibitors, and OD represents optical density. Each determination was performed in four wells and all experiments were conducted on at least two separate days.
MKN-45 cell adhesion assay

The ELISA adhesion assay was conducted as follows. Flat-bottom welled microtiter plates (IWAKI 3860-096, Iwaki Glass) seeded with MKN-45 cells were incubated in a microaerobic atmosphere at 37°C with a 100 µl suspension (10^8 CFU/ml) of H. pylori that had been preincubated for 30 min with test compounds. Incubation was typically 1 hour in duration, but for measurement of IL-8 production a duration of 4 hours was utilized. Culture supernatants were centrifuged at 10,000 x g for 5 min and the resulting supernatants were then frozen at -70°C until assayed. The plates were subsequently washed and fixed in 3.7% formalin at room temperature for 1 hr. After washing, the plates were then incubated at 37°C for 1 hr with mouse anti-H. pylori antiserum. The plates were washed again and incubated with goat-affinity purified antibody to mouse immunoglobulins conjugated with peroxidase for 1 hr at room temperature. After washing, bound peroxidase was colorimetrically detected using a TMB Peroxidase Substrate Kit. The enzyme reaction was stopped with 1N-sulfuric acid and optical density was measured at 450nm using a microplate ELISA reader.

IL-8 enzyme immunoassay

The IL-8 level in culture supernatants was determined by utilizing a sandwich-type IL-8 ELISA kit (R&D systems, USA); results were expressed as pg/ml.

Detachment assay

To measure detachment, H. pylori was first allowed to bind to MKN-45 cells for 30 min. After washing, test compounds were applied with 10% RPMI 1640 medium at 37°C for 1 hr. The plates were then washed and fixed in 3.7% formalin at room temperature for 1 hr. After washing, the plates were incubated at 37°C for 1 hr with mouse anti-H. pylori antiserum. Plates were washed again and incubated with goat-affinity purified antibody to mouse immunoglobulin conjugated with peroxidase for 1 hr at room temperature. After washing, bound peroxidase was colorimetrically detected using a TMB Peroxidase Substrate Kit. The enzyme reaction was stopped with 1N-sulfuric acid and optical density was measured at 450 nm using a microplate ELISA reader.

Cytotoxicity assay

The cytotoxicity assay was conducted after slightly modifying the method utilized by Cover et al. (27). Cultured Vero cells were seeded on a 96-well tissue-culture plate (IWAKI 3860-096, Iwaki Glass, Tokyo, Japan) and incubated in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum at 37°C for 24 hr in an atmosphere of 5% CO₂. Bacterial extract was added to the wells and cultured for 24 hr. After washing, the Vero cells were stained with methylene blue and examined for the presence of intracellular vacuolation under a microscope (28). Estimation of vacuole formation was performed as follows. Degree of vacuole formation was divided into 4 groups: no vacuolating cells, 0; <10% of vacuolating cells, +1; 10-50% of vacuolating cells, +2; and >50% of vacuolating cells, +3.

Statistical Methods

Results were expressed as means ± S.D. Differences between control and experimental subgroups were analyzed by the one-way ANOVA and Dunnett's test. A p value of <0.05 was considered statistically significant. IC₅₀ values for gangliosides were calculated by linear correlation analysis.
RESULTS

In vitro adhesion assay

The effects of the test compounds (0, 125, 250, 500 µg/ml) on *H. pylori* adhesion were examined. *H. pylori* strongly adhered to the polystyrene coated with sulfatide, ganglioside GD₃, and ganglioside GM₃. *H. pylori* adhesion was significantly inhibited by the test chemicals in a dose-dependent manner. Sulfatide, GD₃, and GM₃ significantly inhibited *H. pylori* adhesion to sulfatide. GM₃ and GD₃ significantly inhibited *H. pylori* adhesion to GM₃. GD₃ and GM₃ significantly inhibited *H. pylori* adhesion to GD₃ (*Table 1*). GD₃ markedly inhibited *H. pylori* adhesion to each glycolipid-coated plate. Such results suggest that *H. pylori* adhered with greater affinity to GD₃ than GM₃ or sulfatide.

Neuraminidase is an enzyme that ligates α₂→3, α₂→6, and α₂→8 bonds between sialic acid and oligosaccharides. GD₃ possesses two neuraminic acids and α₂→3 bonds, but sulfatide exhibits neither of the above. Sulfatide and GD₃ significantly inhibited *H. pylori* adhesion to sulfatide beginning with a dose of 125 µg/ml (*Fig. 1*). The effects of GD₃ on *H. pylori* adhesion to sulfatide were prevented with pre-treatment of the enzyme with a compounds-*H. pylori* mixing solution. Nonetheless, the effects of sulfatide on *H. pylori* adhesion to sulfatide were not prevented with pre-treatment with neuraminidase. Such results suggest that neuraminic acids play a role in GD₃ adhesion to *H. pylori*.

MKN-45 cell adhesion assay

The inhibitory effects of gangliosides on *H. pylori* adhesion were examined with MKN-45 cells. GD₃ significantly inhibited *H. pylori* adhesion to cells at doses ≥ 125 µg/ml (*Fig. 2*). In terms of inhibition potency, GD₃ was stronger than

*Table 1*. Inhibition of *H. pylori* adhesion to glicolipids coating plates (500µg/ml).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfatide_coating</td>
<td></td>
</tr>
<tr>
<td>Sulfatide</td>
<td>96.7 **</td>
</tr>
<tr>
<td>GD₃</td>
<td>85.5 **</td>
</tr>
<tr>
<td>GM₃</td>
<td>39.7 **</td>
</tr>
<tr>
<td>GD3_coating</td>
<td></td>
</tr>
<tr>
<td>Sulfatide</td>
<td>22.9</td>
</tr>
<tr>
<td>GD₃</td>
<td>92.0 **</td>
</tr>
<tr>
<td>GM₃</td>
<td>52.9 **</td>
</tr>
<tr>
<td>GM3_coating</td>
<td></td>
</tr>
<tr>
<td>Sulfatide</td>
<td>32.9</td>
</tr>
<tr>
<td>GD₃</td>
<td>84.9 **</td>
</tr>
<tr>
<td>GM₃</td>
<td>91.8 **</td>
</tr>
</tbody>
</table>

n=4, ** p<0.01, Dunnett
GM₃, which was stronger than sulfatide (Table 2). Such results were consistent with the in vitro adhesion assay in which GD₃ demonstrated the highest adhesion affinity to *H. pylori*. The ability of GD₃ to inhibit *H. pylori* adhesion to cells was prevented with pretreatment with a compound-*H. pylori* mixing solution with neuraminidase. In contrast, the inhibitory effect of sulfatide was not prevented by pretreatment with the enzyme (Fig. 2). Endoglycoceramidase acted on GD₃, but...
failed to exert an effect on sulfatide. Nonetheless, the inhibitory effects of GD$_3$ and sulfatide on *H. pylori* adhesion were similar after pretreatment with this enzyme (Fig. 2). The primary site of ganglioside inhibition was an oligosaccharide chain located on the gangliosides.

In a 4 hr assay protocol, sulfatide and GD$_3$ significantly inhibited cellular adhesion to *H. pylori* at doses $\geq$125 $\mu$g/ml (Fig. 3A).

![Graph A](image1)

![Graph B](image2)

*Fig. 3. (A) The inhibitory effect of sulfatide (■) and ganglioside GD$_3$ (●) against *H. pylori* adhesion to MKN-45 cell-coated plates after a 4hr incubation. (B) The inhibitory effect of sulfatide (□) and ganglioside GD3 (○) against IL-8 production by MKN-45 cells after a 4 hr incubation. Data are presented as means ± 1 S.D. for 4 tests. **, significantly different from the control group, P<0.01.*

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**Table 2. Inhibition of *H.pylori* adherence to MKN-45 cells**

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>IC50 (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asialo-GM1</td>
<td>400 $^&lt;$</td>
</tr>
<tr>
<td>ganglioside GM1</td>
<td>400 $^&lt;$</td>
</tr>
<tr>
<td>GM1 ester</td>
<td>343</td>
</tr>
<tr>
<td>GM3</td>
<td>379</td>
</tr>
<tr>
<td>GD1a</td>
<td>133</td>
</tr>
<tr>
<td>GD1b</td>
<td>161</td>
</tr>
<tr>
<td>GD3</td>
<td>191</td>
</tr>
<tr>
<td>O-Ac-GD3</td>
<td>81 $^&gt;$</td>
</tr>
<tr>
<td>GT1a</td>
<td>166</td>
</tr>
<tr>
<td>GT1b</td>
<td>114</td>
</tr>
</tbody>
</table>

$^a$IC50 is the concentration required to reduce the number of organisms bound to 50% of the control value. If binding inhibition fails to reach 50% at the dose of 400 $\mu$M of inhibitor tested, the value used in 400 $^<$.
**IL-8 ELISA assay**

Using a 4 hr assay protocol, IL-8 production in a culture medium of MKN-45 cells was determined. The experiment confirmed that MKN-45 cells linearly produce IL-8 for 24 hr (data not shown). In the presence of *H. pylori*, IL-8 production by MKN-45 cells increased by approximately 5 fold compared with production in the absence of *H. pylori*. Sulfatide and GD₃ both significantly inhibited IL-8 production at doses ≥ 125 µg/ml, but GD₃ inhibition was clearly stronger than that of sulfatide (Fig. 3B). Significant relationship was observed between *H. pylori* adhesion to MKN-45 cells and IL-8 production (data not shown).

**Detachment assay**

The detachment effects of glycolipids were examined with *H. pylori* adherent MKN-45 cells. Sulfatide inhibition reached a plateau at a dose of 500 µg/ml. GD₃ achieved significant inhibition at a dose ≥125 µg/ml, demonstrating dose-dependent inhibition up to a dose of 1000 µg/ml (Fig. 4).

**Competitive effect of ganglioside GD₃**

*H. pylori* suspensions of varying concentrations were applied to MKN-45 cells. It was found that with concentrations > 5 x 10⁷ CFU/ml maximum adhesion was achieved. Addition of GD₃ to the *H. pylori* suspension shifted the concentration-effect curve to the right, resulting in curves 96% and 91% that of baseline with doses of 50 and 100 µg/ml, respectively (Fig. 5). Such results suggest the existence of a competitive interaction between *H. pylori* and GD₃ in the presence of MKN-45 cells.

**Comparative ganglioside assay**

To elucidate the chemical structure involved in the adhesion mechanism, the present study examined the inhibitory effects of available gangliosides on *H. pylori* attachment to MKN-45 cells. Sulfatide inhibition reached a plateau at a dose of 500 µg/ml. GD₃ achieved significant inhibition at a dose ≥125 µg/ml, demonstrating dose-dependent inhibition up to a dose of 1000 µg/ml. GD₃ inhibition was clearly stronger than that of sulfatide (Fig. 3B). Significant relationship was observed between *H. pylori* adhesion to MKN-45 cells and IL-8 production (data not shown).

**Fig. 4.** The effects of sulfatide (□) and ganglioside GD₃ (○) on *H. pylori* detachment from MKN-45 cells. Data are presented as means ± 1 S.D. for 4 tests. **, significantly different from the control group, P<0.01.
pylori adhesion and \textit{H. pylori}-induced IL-8 production. In addition, the effects of gangliosides on detachment of \textit{H. pylori} from cells to which the bacteria is adhered were examined. Data are presented as IC$_{50}$ values. O-Ac-GD$_3$ was found to be most effective at inhibiting \textit{H. pylori} adhesion to MKN-45 cells compared with all other test gangliosides (Table 2). In addition, GD$_3$ was found to be most effective at inhibiting IL-8 production induced by \textit{H. pylori} in MKN-45 cells compared with all other test gangliosides (Table 3). GM$_1$ was found to have the greatest inhibitory

\textbf{Table 3.} Inhibition of \textit{H. pylori} induced IL-8 production to MKN-45 cells

\begin{tabular}{l|c}
\hline
 & IC50 (\(\mu\) M)$^a$ \\
\hline
Asialo-GM1 & 400$<$ \\
Ganglioside GM1 & 400$<$ \\
GM1 ester & 400$<$ \\
GM3 & 400$<$ \\
GD1a & 261 \\
GD1b & 199 \\
GD3 & 205 \\
O-Ac-GD3 & 320 \\
GT1a & 223 \\
GT1b & 400$<$ \\
\hline
\end{tabular}

$^a$ See Table 2, footnote a for details. If binding inhibition fails to reach 50\% at the dose of 400 \(\mu\)M of inhibitor tested, the value used in 400$<$
effect for detachment of *H. pylori* from MKN-45 cells to which the bacteria was adhered, compared with all other test gangliosides (*Table 4*).

*Table 4. Detachment of *H. pylori* adherence to MKN-45 cells*

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>IC50 (µM)</th>
</tr>
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<tbody>
<tr>
<td>GM1</td>
<td>160</td>
</tr>
<tr>
<td>GM1b</td>
<td>355</td>
</tr>
<tr>
<td>GM3</td>
<td>&lt;800</td>
</tr>
<tr>
<td>GD1a</td>
<td>523</td>
</tr>
<tr>
<td>GD1b</td>
<td>512</td>
</tr>
<tr>
<td>GD3</td>
<td>524</td>
</tr>
<tr>
<td>GQ1b</td>
<td>&lt;800</td>
</tr>
</tbody>
</table>

*See Table 2, footnote a for details. If binding inhibition fails to reach 50% at the dose of 800 µM of inhibitor tested, the value used is <800.*

*Fig. 6. Picture shows the reversal of *H. pylori* vacuolized cells by ganglioside GM1, in Vero cells. Intense vacuolization caused by *H. pylori* NCTC strain was observed in A. The density of the vacuole decreased in order of A, B, C, D, showing that higher concentration of GM, inhibit vacuole formation by *H. pylori*. A: Intense vacuolization caused by the NCTC strain, B: 250 µg/mL of ganglioside GM1, C: 500 µg/mL of ganglioside GM1, D: 1000 µg/mL of ganglioside GM1.*
**Cytotoxicity assay**

Gangliosides inhibited vacuole formation induced by *H. pylori* cytotoxin in a dose-dependent manner. GM₃ and GT₁b exhibited a marked inhibitory effect against vacuole formation in Vero cells induced by *H. pylori* compared with all other test gangliosides (Figs. 6, 7). GM₁, GM₃, GT₁b, and GQ₁b prevented *H. pylori* -induced vacuole formation in Vero cells at a dose of 1000 µg/ml (Table 5). Judging from microscopic observations, the morphology of Vero cells exposed to gangliosides appeared to be equivalent to negative controls.

**DISCUSSION**

*H. pylori* adhesion to the gastric mucosa represents the initial contact between the bacteria and its host. In general, adhesin receptors are carbohydrate moieties on glycoproteins or glycosphingolipids located on cell surfaces and often covalently adhered to membrane proteins of epithelial cells that reside in the target tissue or organ. Accordingly, research in such a field will likely determine candidates for *H. pylori* anti-adhesion therapy.

*Fig. 7.* Picture shows the reversal of *H. pylori* vacuolized cells by ganglioside GT₁ in Vero cells. Intense vacuolization caused by *H. pylori* NCTC strain was observed in A. The density of the vacuole decreased in order of A, B, C, D, showing that higher concentration of GT₁ inhibit vacuole formation by *H. pylori*. A: Intense vacuolization caused by the NCTC strain. B: 250 µg/mL of ganglioside GT₁b. C: 500 µg/mL of ganglioside GT₁b. D: 1000 µg/mL of ganglioside GT₁b.
Saito et al. (29) isolated glycolipids from gastric mucosa and identified the specific gangliosides GM\(_3\) and sulfatide. Hirmo et al. (9) reported that *H. pylori* binds to specific glycolipids on α-2, 3-linked sialic acids. We also demonstrated strong binding between *H. pylori* and sulfatide, GD\(_3\), as well as GM\(_3\), all of which were coated on polystyrene for the experiment. Such binding was specifically inhibited by sulfatide, GD\(_3\), and GM\(_3\) respectively. Such in vitro data suggested that *H. pylori* can bind such gangliosides.

In contrast, *H. pylori* binding to MKN-45 cells was inhibited by GD\(_3\), but not by sulfatide or GM\(_3\). Such findings suggest that sulfatide and GM\(_3\) are not important for adhesion of *H. pylori* to MKN-45 cells. The present study clearly demonstrates that ganglioside GD\(_3\) inhibited adhesion of *H. pylori* to both MKN-45 cells and ganglioside-coated polystyrene, suggesting GD\(_3\) involvement in *H. pylori* adhesion to cells.

**Table 5. Effect of gangliosides against *H. pylori* extracts inducing vacuole formation in Vero cells**

<table>
<thead>
<tr>
<th></th>
<th>Grade (0~3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dose (µg/ml)</td>
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<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>VT</td>
<td>+3</td>
</tr>
<tr>
<td>GM(_1) + VT</td>
<td>+1 +1 0</td>
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<tr>
<td>GM(_1)a + VT</td>
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<tr>
<td>GM(_1)b + VT</td>
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<tr>
<td>GM(_3) + VT</td>
<td>+1 +1 0</td>
</tr>
<tr>
<td>GD(_1)a + VT</td>
<td>+2 +1 +1</td>
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<td>GD(_1)b + VT</td>
<td>+2 +1 +1</td>
</tr>
<tr>
<td>GD(_3) + VT</td>
<td>+3 +2 +1</td>
</tr>
<tr>
<td>GT(_1)b + VT</td>
<td>+1 +1 0</td>
</tr>
<tr>
<td>GQI(_1)b + VT</td>
<td>+1 +1 0</td>
</tr>
</tbody>
</table>

VT : vacuolating toxin (bacterial extract)
control : neither ganglioside nor VT was added to Vero cells
graded 0: no vacuolating cells +1: vacuolating cells less than 10%
+2: vacuolating cells from 10% to 50%
+3: vacuolating cells more than 50%
One possible explanation for such findings is either that GD₃ is abundant in MKN-45 cells or that GD₃ is specifically involved in the adhesion mechanism due to its chemical structure. Such GD₃ inhibition was abolished by pre-treatment with neuraminidase, but not by pre-treatment with endoglycoceramidase, suggesting that the inhibition was sialic acid dependent. It has been reported that both bacterial adhesion to epithelial cells and cell-cell adhesion is related to the number of sialic acid moieties containing oligosaccharides on glycoconjugates (30). Our data strongly suggest that a structure of sialic acids with ganglioside GD₃ may possess therapeutic potential for *H. pylori* infection.

Recently we demonstrated that milk inhibited *H. pylori* adhesion to sulfatide-coated polystyrene (31). Milk gangliosides are known to inhibit *Vibrio cholerae* enterotoxin and *Escherichia coli* heat-labile enterotoxin. In milk, gangliosides are primarily found in the fat globule membrane (32). In bovine milk, six gangliosides were detected, but, to date, only three have been identified, i.e., GD₃, GM₃, and GM₁, the foremost being present at the highest concentration among the three (17, 23-25). Commercially available milk exhibited weak, but significant inhibition of *H. pylori* adhesion to MKN-45 cells (data not shown). Such a finding might be explained by the fact that milk possesses one tenth of the concentration of GD₃ used in the present study.

Recent studies indicate that *H. pylori* infection induces IL-8 production by infected gastric epithelial cells. In vivo gastric infection with *H. pylori* induces mucosal production of various cytokines in the host (33-36). IL-8, a potent T-cell and neutrophil recruitment factor, is produced by various cell types, including macrophages, T cells, endothelial cells, keratinocytes, and epithelial cells (37, 38). Prolonged IL-8 production by gastric epithelial cells during *H. pylori* infection could result in recruitment of neutrophils and lymphocytes to infected tissues, thereby playing a role in progression of *H. pylori* infection. IL-8 represents a potent neutrophil chemoattractant, which might explain the presence of the polymorphonuclear leukocyte infiltrate in chronic active gastritis that is observed in many *H. pylori*-infected individuals.

In the present study, IL-8 production stimulated by *H. pylori* was inhibited by sulfatide and GD₃. A recent study found that *E.coli* lectins bind the glycolipid receptors of host cells, increasing ceramide release from the receptor, and consequently stimulating either cytokine production or induction of apoptosis (39). A similar mechanism is assumed to account for *H. pylori*-induced IL-8 production (40).

*H. pylori* strains that produce cytotoxin VacA are involved in development of atrophic gastritis, gastroduodenal ulcers, and gastric adenocarcinoma in humans (41-49). Oral administration of purified VacA to mice induced degeneration of gastric mucosa and recruitment of inflammatory cells (44, 50, 51). Strains of *H. pylori* with an altered VacA gene are non-cytotoxic (52, 53). Our data clarified that gangliosides inhibit vacuole formation induced by *H. pylori* VacA toxin. We presume that the underlying mechanism consists of two steps, namely, first...
gangliosides bind *H. pylori* cytotoxin proteins, which is followed by neutralization of the binding complex due to inability to invade into host cells.

Fig. 5 represents an adhesion curve of bacteria to MKN-45 cells. When the bacterial count exceeded $10^8$ CFU/ml, the adhesion curve reached a plateau. The graph shifted to the right when gangliosides were added to the protocol. The width of the shift was found to be proportionate to the dose of the added gangliosides. The maximum level of adhesion was unchanged with gangliosides addition. Such results suggest a competitive interaction between *H. pylori* and cell surface glycoconjugates.

As shown by Table 6, gangliosides that exhibited potent anti-*H. pylori* effects contained the structure NeuAc-NeuAc-Gal-Glc (54). In terms of adhesion, the

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Oligosacchalide structure</th>
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<tbody>
<tr>
<td>Ganglioside GM1(GM1a)</td>
<td>Gal β 1-3GalNAc β 1</td>
</tr>
<tr>
<td></td>
<td>NeuAc α 2-3Gal β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Ganglioside GM1b</td>
<td>NeuAc α 2-3Gal β 1-3GalNAc β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Ganglioside GM3</td>
<td>NeuAc α 2-3Gal β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Ganglioside GD3</td>
<td>NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Ganglioside GD1a</td>
<td>NeuAc α 2-3Gal β 1-3GalNAc β 1</td>
</tr>
<tr>
<td></td>
<td>NeuAc α 2-3Gal β 1-4Glc β 1-1’Cer</td>
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</tr>
<tr>
<td>Ganglioside GQ1b</td>
<td>NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>HSO3-3Gal β 1-4Glc β 1-1’Cer</td>
</tr>
<tr>
<td>Asialo GM1</td>
<td>Gal β 1-3GalNAc β 1</td>
</tr>
</tbody>
</table>

Trival names for the glycolipids are according to Svennerholm L (54)
present study found that NeuAc-NeuAc-Gal-Glc-containing glycoconjugates, eg, GD₃, were more effective in preventing adhesion of *H. pylori* to gastric epithelial cells than NeuAc-Gal-Glc-containing glycoconjugates, eg, GM₁. Pan et al. (55) demonstrated that GD₃ represents the major component in human colostrums and illustrated how GD₃ protects from bacterial infection in early infancy. The stereostructure of sialic acid is thought to be important for inhibition of adhesion, as supported by the review by Nakano *et al.* (56). In a study of *H. pylori* adhesion to human leucocytes, selective leucocyte binding sites were gangliosides with three linked sialic acids (57).

Judging from the above, a schematic representation of the mechanism of ganglioside interference of *H. pylori* adhesion to MKN-45 cells is depicted in Fig. 8. *H. pylori* adhesion to gastric epithelial cells increased the intracellular ceramide production and mediate IL-8 expression through activation of NF-κB (40, 58). Figure shows schematic representation of *H. pylori* adhesion to gastric epithelial cells and NF-κB activation with cytokine secretion (upper) and schematic representation of the mechanism of adhesion interference via interaction of *H. pylori* with gangliosides and decrease in IL-8 secretion (lower).

*Fig. 8.* Recent evidence suggests that *H. pylori* increases intracellular ceramide production and mediates IL-8 expression through activation of NF-κB (40, 58). Figure shows schematic representation of *H. pylori* adhesion to gastric epithelial cells and NF-κB activation with cytokine secretion (upper) and schematic representation of the mechanism of adhesion interference via interaction of *H. pylori* with gangliosides and decrease in IL-8 secretion (lower).
concentration of ceramide, activating NF-κB, and subsequently promoting IL-8 expression by inducing mRNA production (40). Given the above, the present study suggests that gangliosides that contain NeuAc-NeuAc-Gal-Glc are capable of suppressing both the inflammatory response and cell death of epithelial cells.

REFERENCES


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