Natural Antibiotic Function of a Human Gastric Mucin Against *Helicobacter pylori* Infection

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Enhancing transplant efficiency has clinical implication but is being debated. Recent reports suggest that HSCs engraft mice with absolute efficiency (*7*, *8*). One report (*7*) was heavily influenced by mathematical correction factors, and the other (*8*) addressed single-cell transplants by a subset of HSCs among competitor cells that themselves could save the lethally irradiated recipient. Contrary to this is the reality of the clinical situation (*3*) and studies in which injection of HSCs directly into BM showed enhanced engraftment compared with intravenous administering of cells (*29–31*). Removal of endogenous CD26 activity on donor HSCs increased homing and engraftment. Thus, improvement in transplant efficiency is possible. Further advancement may require more effective use of CD26 inhibitors, which may translate into the use of HSCs for clinical transplantation from sources containing limiting cell numbers, such as cord blood.

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Supporting Online Material

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Natural Antibiotic Function of a Human Gastric Mucin Against *Helicobacter pylori* Infection

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Helicobacter pylori infects the stomachs of nearly a half the human population, yet most infected individuals remain asymptomatic, which suggests that there is a host defense against this bacterium. Because *H. pylori* is rarely found in deeper portions of the gastric mucosa, where *O*-glycans are expressed that have terminal α1,4-linked *N*-acetylglucosamine, we tested whether these *O*-glycans might affect *H. pylori* growth. Here, we report that these *O*-glycans have antimicrobial activity against *H. pylori*, inhibiting its biosynthesis of cholesteryl-α-D-glucopyranoside, a major cell wall component. Thus, the unique *O*-glycans in gastric mucin appeared to function as a natural antibiotic, protecting the host from *H. pylori* infection.

Helicobacter pylori colonizes the gastric mucosa of about half the world's population and is considered a leading cause of gastric malignancies (*1–3*). However, most infected individuals remain asymptomatic or are affected merely by chronic active gastritis (*2*). Only a fraction of infected patients develop peptic ulcer, gastric cancer, and malignant lymphoma. This suggests the presence of host defense mechanisms against *H. pylori* pathogenesis.

Gastric mucins are classified into two types based on their histochemical properties (*4*). The first is a surface mucous cell–type mucin, secreted from the surface mucous cells. The second is found in deeper portions of the mucosa and is secreted by gland mucous cells, including mucous neck cells, cardiac gland cells, and pyloric gland cells.

In *H. pylori* infection, the bacteria are associated solely with surface mucous cell–type mucin (*5*), and two carbohydrate structures, Lewis b and sialyl dimeric Lewis X in surface mucous cells, serve as specific ligands for *H. pylori* adhesins, BabA and SabA, respectively (*6, 7*). *H. pylori* rarely colonizes the deeper portions of gastric mucosa, where the gland mucous cells produce mucins having terminal α1,4-linked *N*-acetylglucosamine (α1,4-GlcNAc) residues attached to core 2–branched *O*-glycans [GlcNAcα1→4Galβ1→4GlcNAcβ1→6 (GlcNAcα1→ 4Galβ1→3)GalNAcα→Ser/Thr] (*8*). Development of pyloric gland atrophy enhances the risk of peptic ulcer or gastric cancer two- to three-fold compared with chronic gastritis without pyloric gland atrophy (*3*). These findings raise the possibility that α1,4-GlcNAc–capped *O*-glycans have protective properties against *H. pylori* infection.

To test this hypothesis, we generated mucin-type glycoproteins containing terminal α1,4-GlcNAc and determined its effect on *H. pylori* in vitro. Because CD43 serves as a preferential core protein of these *O*-glycans (*8*), we generated recombinant soluble CD43 having α1,4-GlcNAc–capped *O*-glycans in transfected Chinese hamster ovary cells (*9*). Soluble CD43 without α1,4-GlcNAc was used as a control.

H. pylori (ATCC43504), incubated with the medium containing varying amounts of recombinant soluble CD43, showed little growth during the first 2.5 days, irrespective of the presence or absence of α1,4-

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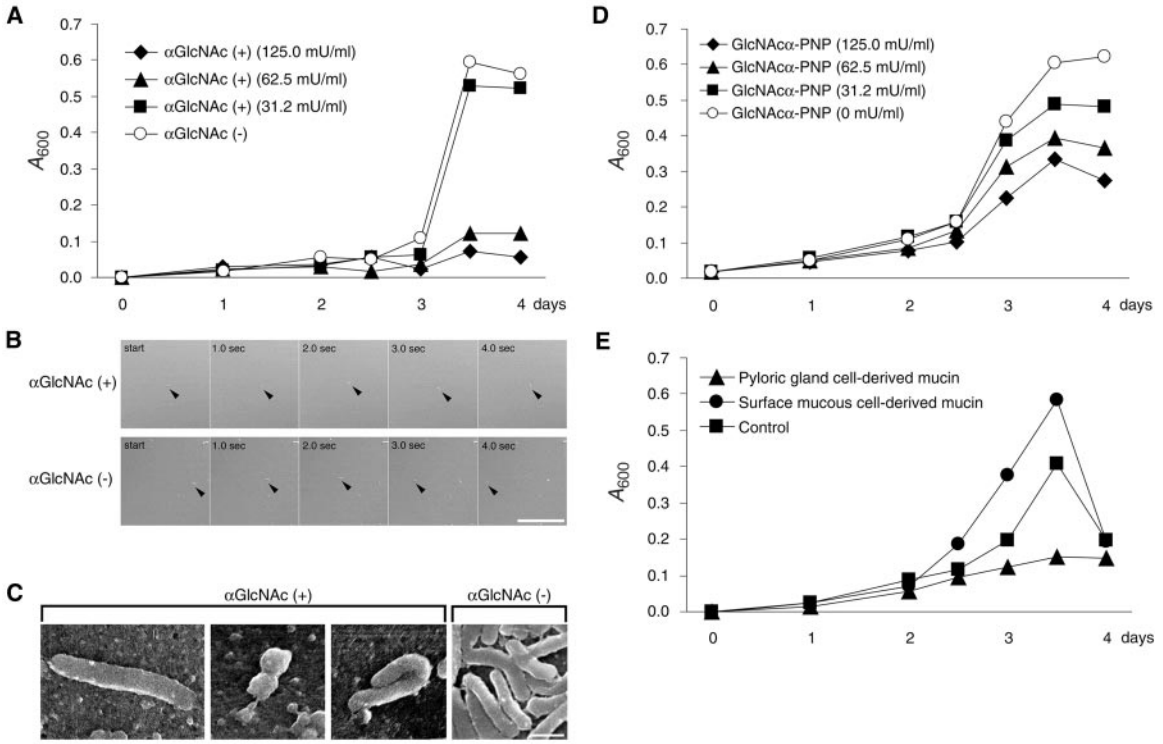

Fig. 1. α1,4-GlcNAc–capped *O*-glycans inhibit the growth and motility of *H. pylori*. (**A**) Growth curves of *H. pylori* cultured in the presence of soluble CD43 with terminal α1,4-GlcNAc [αGlcNAc (+)] or soluble CD43 without terminal α1,4-GlcNAc [αGlcNAc (–)]; the protein concentration of αGlcNAc (–) was the same as that of 125.0 mU/ml of αGlcNAc (+). One miliunit of αGlcNAc (+) corresponds to 1 μg (2.9 nmol) of GlcNAcα-PNP. A_{600}, absorbance at 600 nm. (**B**) Motility of *H. pylori* cultured with 31.2 mU/ml of αGlcNAc (+) or the same protein concentration of αGlcNAc (–) for 3 days by time-lapse recording with 1-s intervals. Representative *H. pylori* is indicated by arrowheads. The mean velocity of seven *H. pylori* cultured in the presence of αGlcNAc (+) and αGlcNAc (–) is 3.1 ± 3.5 μm/s (mean ± SD) and 21.2 ± 2.6 μm/s ($P < 0.001$). Scale bar, 50 μm. (**C**) Scanning electron micrographs of *H. pylori* incubated with 31.2 mU/ml of αGlcNAc (+) or the same protein concentration of αGlcNAc (–) for 3 days. Note abnormal morphologies such as elongation, segmental narrowing, and folding in the culture with αGlcNAc (+). All photographs were taken at the same magnification. Scale bar, 1 μm. (**D**) Growth curves of *H. pylori* cultured in the medium supplemented with various amounts of GlcNAcα-PNP. Growth of the bacteria is suppressed by GlcNAcα-PNP in a dose-dependent manner. (**E**) Growth curves of *H. pylori* cultured in the medium supplemented with pyloric gland cell-derived mucin containing 125 mU/ml of α1,4-GlcNAc or the same protein concentration of surface mucous cell-derived mucin isolated from the human gastric mucosa. The death phase started from 3.5 days, and saline instead of each mucin was supplemented as a control experiment. In (A), (D), and (E), each value represents the average of duplicate measurements.

GlcNAc–capped *O*-glycans, characteristic of the lag phase of *H. pylori* growth (Fig. 1A). After 3 days, microbes cultured in the presence of control soluble CD43 grew rapidly, corresponding to the log phase of bacterial growth. In contrast, soluble CD43 containing more than 62.5 mU/ml of terminal α1,4-GlcNAc impaired log-phase growth. Although growth inhibition was not obvious at a lower concentration (31.2 mU/ml), time-lapse images of the microbes revealed significant reduction of motility under this condition (Fig. 1B). Morphologic examination at the lower concentration revealed abnormalities of the microbe, such as elongation, segmental narrowing, and folding (Fig. 1C). These morphologic changes are distinct from conversion to coccoid form, because reduction of growth, associated with conversion from the bacillary to the coccoid form (*10*), was not apparent under these conditions. These inhibitory effects of soluble CD43 containing terminal α1,4-GlcNAc were also detected against various *H. pylori* strains, including another authentic strain, ATCC43526, and three clinical isolates with a minimum inhibitory concentration between 15.6 mU/ml and 125.0 mU/ml. By contrast, neither inhibitory growth nor abnormal morphology of *H. pylori* was observed at any concentrations of soluble CD43 lacking α1,4-GlcNAc (Fig. 1, A to C). These results indicate that α1,4-GlcNAc–capped *O*-glycans specifically suppress the growth of *H. pylori* in a manner similar to other antimicrobial agents. Similar inhibitory effects on *H. pylori* were also found in another mucin-like glycoprotein, CD34 (*11*) having terminal α1,4-GlcNAc (*12*). In addition, *p*-nitrophenyl-α-*N*-acetylglucosamine (GlcNAcα-PNP) suppressed the growth of *H. pylori* in a dose-dependent manner (Fig. 1D), although the effects were not as strong with soluble CD43 having terminal α1,4-GlcNAc (Fig. 1A). These results provide evidence that the terminal α1,4-GlcNAc residues, rather than scaffold proteins, are critical for growth inhibitory activity against *H. pylori*, and that the presentation of multiple terminal α1,4-GlcNAc residues as a cluster on mucin-type glycoprotein may be important for achieving the optimal activity.

To determine whether natural gastric mucins containing terminal α1,4-GlcNAc can also inhibit growth of *H. pylori*, subsets of human gastric mucins were prepared from the surface mucous cells and pyloric gland cells (*9*). The growth of *H. pylori* was significantly suppressed with mucin derived from pyloric gland cells at 125.0 mU/ml during the log phase (Fig. 1E). A similar inhibitory effect was also observed when the glandular mucin prepared from human gastric juice was tested (*13*). By contrast, mucin derived from surface mucous cells, MUC5AC, stimulated growth. These results support the hypothesis that natural gastric mucins containing terminal α1,4-GlcNAc, secreted from gland mucous cells, have antimicrobial activity against *H. pylori*.

The morphologic abnormalities of *H. pylori* induced by α1,4-GlcNAc–capped *O*-glycans are similar to those induced by antibiotics such as β-lactamase inhibitors, which disrupt biosynthesis of peptidoglycan in the cell wall (*14, 15*). Therefore, these *O*-glycans may inhibit cell wall biosynthesis in *H. pylori*. The cell wall of

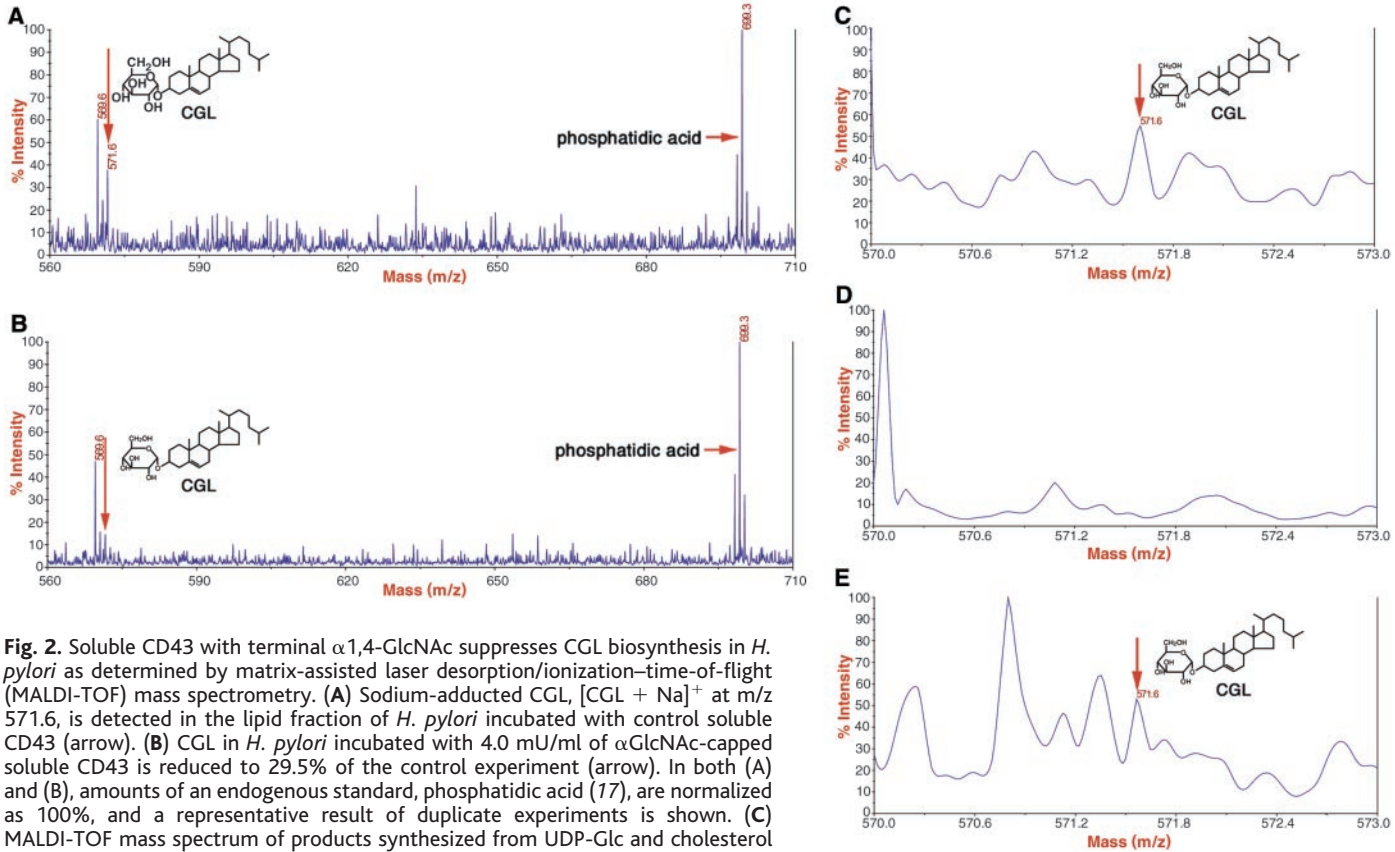

Fig. 2. Soluble CD43 with terminal α1,4-GlcNAc suppresses CGL biosynthesis in *H. pylori* as determined by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry. (**A**) Sodium-adducted CGL, $[CGL + Na]^+$ at m/z 571.6, is detected in the lipid fraction of *H. pylori* incubated with control soluble CD43 (arrow). (**B**) CGL in *H. pylori* incubated with 4.0 mU/ml of αGlcNAc-capped soluble CD43 is reduced to 29.5% of the control experiment (arrow). In both (A) and (B), amounts of an endogenous standard, phosphatidic acid (*17*), are normalized as 100%, and a representative result of duplicate experiments is shown. (**C**) MALDI-TOF mass spectrum of products synthesized from UDP-Glc and cholesterol by sonicated *H. pylori*. $[CGL + Na]^+$ at m/z 571.6 is shown. (**D** and **E**) Mass spectrum of products synthesized from UDP-Glc and cholesterol by sonicated *H. pylori* in the presence of 50.0 mU/ml of α1,4-GlcNAc–capped soluble CD43 (D) or control soluble CD43 (E). Note that CGL is not synthesized in the presence of α1,4-GlcNAc–capped soluble CD43 in (D).

Fig. 3. Absence of α-CGs including CAG, CGL, and CPG in *H. pylori* cultured without exogenous cholesterol. Total glycolipids extracted from *H. pylori* incubated with Brucella broth lacking cholesterol (lane 1) or containing 0.005% cholesterol (lane 2) were analyzed by thin-layer chromatography.

Helicobacter species characteristically contains α-cholesteryl glucosides (α-CGs), of which the major components are cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (CPG) (*16*). Mass spectrometric analysis of the cell wall components from *H. pylori* cultured with α1,4-GlcNAc–capped *O*-glycans displayed reduced lipid-extractable cell wall constituents (Fig. 2B). In particular, the levels of CGL, relative to phosphatidic acid (*17*), were significantly reduced as compared with controls (Fig. 2, A and B). These results suggest that α1,4-GlcNAc–capped *O*-glycans directly inhibit biosynthesis of CGL in vivo by *H. pylori*.

CGL is likely formed by a UDP-Glc:sterol α-glucosyltransferase, which transfers glucose (Glc) from UDP-Glc to the C3 position of cholesterol with α-linkage. Incubation of cholesterol and UDP-Glc with *H. pylori* lysates revealed substantial amounts of CGL by mass spectrometry (Fig. 2C), demonstrating the activity of UDP-Glc:sterol α-glucosyltransferase in *H. pylori*. When soluble CD43 containing terminal α1,4-GlcNAc was added to this assay, production of CGL was suppressed (Fig. 2D), whereas no effect was seen with control soluble CD43 (Fig. 2E). Considering structural similarity between α-linked GlcNAc found in the gland mucous cell–type mucin and the α-linked Glc found in CGL, these findings suggest that the terminal α1,4-GlcNAc residues could directly inhibit the α-glucosyltransferase activity through an end-product inhibition mechanism (*18*), resulting in decreased CGL biosynthesis.

Genes involved in the biosynthesis of cholesterol are not found in the genome database of *H. pylori* (*19*). Thus, *H. pylori* may not be able to synthesize CGL in the absence of exogenous cholesterol. When *H. pylori* was cultured for 5 days without cholesterol, bacterial growth was significantly reduced (table S1). In such cultures, *H. pylori* was elongated and no motile microbes were found. When *H. pylori* was further cultured without cholesterol for up to 21 days, the microbes died off completely. By contrast, when *H. pylori* was cultured with cholesterol, bacteria grew well, and no signs of abnormality were detected (table S1). *H. pylori* cultured with cholesterol (*9*) revealed a typical triplet of α-CGs including CGL (Fig. 3, lane 2), while α-CGs were not detected in *H. pylori* cultured without cholesterol (Fig. 3, lane 1). Moreover, no antibacterial effect of soluble CD43 containing terminal α1,4-GlcNAc was observed on bacterial strains lacking CGL such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, α-*Streptococcus*, and *Streptococcus pneumoniae* (*9*). These results collectively indicate that synthesis of CGL by using exogenously supplied cholesterol is required for the survival of *H. pylori* and that antimicrobial activity of α1,4-GlcNAc–capped *O*-glycans may be restricted to bacterial strains expressing CGL.

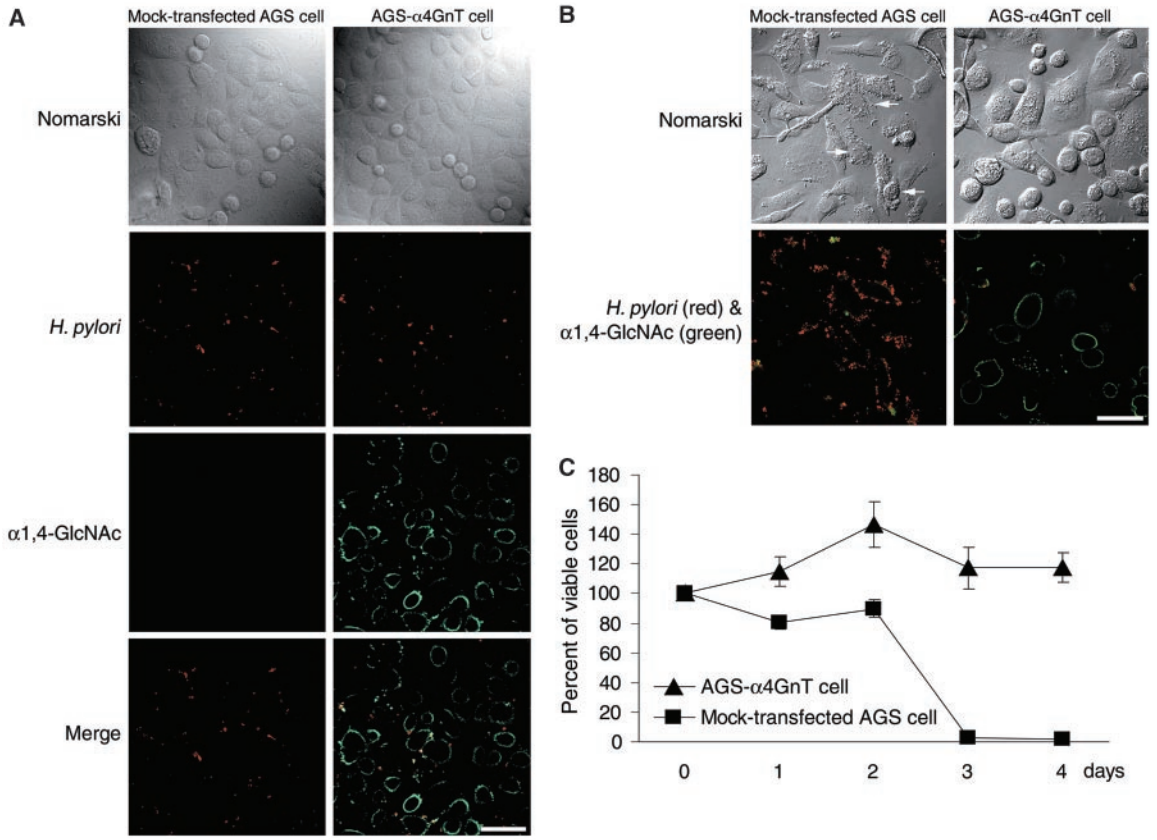

Fig. 4. α1,4-GlcNAc–capped *O*-glycans protect the host cells. AGS cells were incubated with *H. pylori* for 8 hours (**A**) or 24 hours (**B**), and doubly stained with anti-*H. pylori* antibody (red) and HIK1083 antibody specific for terminal α1,4-GlcNAc (*27*) (green). (A) Note that comparable number of *H. pylori* adhered to both mock-transfected AGS cells and AGS-α4GnT cells. (B) After 24 hours, marked damage such as cell flatness or shrinkage are noted (arrows) in mock-transfected AGS cells; no cellular damage and few attached bacteria are found in AGS-α4GnT cells. (Top) Nomarski photographs of the same field. Scale bar, 50 μm. (**C**) Viabilities of AGS cells cocultured with *H. pylori* for 4 days determined by MTS assay. Note that viability of mock-transfected AGS cells was significantly reduced after the third day, whereas AGS-α4GnT cells were fully viable for up to 4 days. The assay was done with triplicate measurements, and error bars indicate SD.

To test whether mucous cells expressing α1,4-GlcNAc–capped *O*-glycans protect themselves against *H. pylori* infection, gastric adenocarcinoma AGS-α4GnT cells stably transfected with α4GnT cDNA were cocultured with *H. pylori* (*9*). With a short-term incubation (8 hours), the microbes attached equally well to AGS-α4GnT cells and mock-transfected AGS cells. No significant damage was observed in either group of cells (Fig. 4A). Upon prolonged incubation (24 hours), mock-transfected AGS cells exhibited remarkable deterioration, such as flatness or shrinkage, with increased number of associated *H. pylori* (Fig. 4B), and the number of viable AGS cells was dramatically reduced after the third day (Fig. 4C). This cellular damage may be attributed to the perturbed signal transduction in AGS cells, where a tyrosin phosphatase, SHP-2, is constitutively activated by *H. pylori* CagA protein (*20*). By contrast, growth of *H. pylori* in cultures with AGS-α4GnT cells was markedly suppressed, and cellular damage found in mock-transfected AGS cells was barely detected in these cells (Fig. 4B). Thus, the viability of AGS-α4GnT cells was fully maintained for up to 4 days (Fig. 4C). These results indicate that α1,4-GlcNAc–capped *O*-glycans have no effect on the adhesion of *H. pylori* to AGS-α4GnT cells, but protect the host cells from *H. pylori* infection.

Glycan chains play diverse roles as ligands for cell surface receptors (*11, 21–23*) and as modulators of receptors and adhesive proteins (*24–26*). The present study reveals a new aspect of mammalian glycan function as a natural antibiotic. Because α1,4-GlcNAc–capped *O*-glycans are produced by human gastric gland mucous cells, the present study provides a basis for development of novel and potentially safe therapeutic agents to prevent and treat *H. pylori* infection in humans without adverse reactions.

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Supporting Online Material

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Materials and Methods
Table S1
References and Notes

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