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Carbohydrate-Dependent Defense Mechanisms Against *Helicobacter pylori* Infection

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Abstract

Helicobacter pylori is a Gram-negative bacterium that infects over 50% of the world's population. This organism causes various gastric diseases such as chronic gastritis, peptic ulcer, and gastric cancer. *H. pylori* possesses lipopolysaccharide, which shares structural similarity to Lewis blood group antigens in gastric mucosa. Such antigenic mimicry could result in immune tolerance against antigens of this pathogen. On the other hand, *H. pylori* colonize gastric mucosa by utilizing adhesins, which bind Lewis blood group antigen-related carbohydrates expressed on gastric epithelial cells. In chronic gastritis, lymphocytes infiltrate the lamina propria, and such infiltration is facilitated by 6-sulfo sialyl Lewis X-capped *O*-glycans, peripheral lymph node addressin (PNAd), on high endothelial venule (HEV)-like vessels. The number of HEV-like vessels increases as chronic inflammation progresses. Furthermore, PNAd formed on HEV-like vessels disappear once *H. pylori* is eradicated. These results indicate that PNAd plays an important role in *H. pylori*-associated inflammation. *H. pylori* barely colonizes gland mucous cell-derived mucin where α1,4-GlcNAc-capped *O*-glycans exist. *In vitro* experiments show that α1,4-GlcNAc-capped *O*-glycans function as a natural antibiotic to inhibit H. pylori growth. We recently identified cholesterol α-glucosyltransferase (CHLαGcT) using an expression cloning strategy and showed that this enzyme is specifically inhibited by mucin-type *O*-glycans like those present in deeper portions of the gastric mucosa. These findings show that a battery of carbohydrates expressed in the stomach is closely associated with pathogenesis and also prevention of H. pylori-related diseases.

Keywords

Helicobacter pylori; lipopolysaccharide; Lewis blood group antigen; adhesin; 6-sulfo sialyl Lewis X-capped *O*-glycan; α1,4-GlcNAc-capped *O*-glycan; cholesteryl-α-D-glucopyranoside; cholesterol α-glucosyltransferase

1. INTRODUCTION

1.1. Impact of *H. pylori* Discovery

Spiral microorganisms in the stomach had been observed in the 1930's and 1940's [1,2], but little attention was paid to gastric microorganisms. In 1983, Marshall and Warren in Australia

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first isolated and succeeded in culturing the bacterium *Helicobacter pylori*, originally named *Campylobacter pyloridis*, from the gastric mucosa of patients with chronic gastritis [3,4]. Surprisingly, Marshall himself drank a culture of *H. pylori* to prove that the bacteria could infect a healthy person and cause gastritis [5]. Their epoch-making discovery revealed that *H. pylori* is associated with various gastric diseases such as chronic gastritis, peptic ulcer, and malignant tumors including gastric carcinoma and malignant lymphoma, and the eradication of this microorganism prevents such gastric disorders. For their achievement, Marshall and Warren won the Nobel Prize in Physiology or Medicine in 2005 [6].

1.2. Specialized Traits of *H. pylori*

H. pylori is a spiral-shaped, Gram-negative, and microaerophilic bacterium, measuring approximately 3–5 μm in length. *H. pylori* is a member of a genus of bacteria that have adapted to the ecological niche provided by gastric mucus, where there is little competition from other microorganisms [7]. Many specialized traits allow this organism to flourish in the harsh environment of the stomach. First, *H. pylori* elaborates a large amount of urease (10%–15% of total proteins by weight), which produces ammonia and carbon dioxide resulting from hydrolysis of endogenous urea, thereby buffering (neutralizing) gastric acid in the immediate vicinity of the organism. *H. pylori* also possesses numerous long flagella, the flailing movements of which allow them to swim through viscous gastric mucus with forceful screw-like movements, much like the spinning of a drill bit [8]. Finally, *H. pylori* binds to gastric epithelial cells via bacterial adhesins: the bacterium colonizes the gastric mucosa by adhering to mucous epithelial cells and the mucus layer lining the gastric epithelium. *H. pylori* possesses adhesins that enhance adhesion with gastric epithelial cells by recognizing specific carbohydrate structures, such as the Lewis b blood group antigen and sialyl dimeric Lewis X (see section 2.3. for detail).

1.3. Epidemiology of *H. pylori* Infection

H. pylori infection occurs worldwide and affects over 50% of the world's population, but the prevalence of infection varies greatly from country to country. The overall prevalence is highly correlated with socioeconomic status measured by household crowding and parental income [9,10]. Prevalence among adults is approximately 80% in many developing countries and 50% in industrialized countries [11]. The prevalence of infection increases with advancing age. In some populations, a disproportionately high rate of *H. pylori* infection is observed in people over 40. This seems to reflect a birth cohort effect: transmission of this chronic infection was more common in the past than it is today [12–14].

The mode of transmission has not yet been fully defined; however, it is widely believed that the organism is transmitted directly from person to person by human feces (fecal-oral spread) or gastric contents (gastric-oral spread). It is now generally accepted that most individuals acquire *H. pylori* infection in childhood [15]. Once the stomach is colonized and left untreated, the organism persists for decades, if not for a lifetime [16]. Frequently children are infected by a strain with a genetic fingerprint identical to that of each parent. Husbands and wives do not exchange strains, and infection is rarely transmitted to an uninfected partner [17].

1.4. *H. pylori* and Associated Diseases

1.4.1. Chronic Gastritis—Following *H. pylori* infection, a chronic, usually lifelong mucosal inflammation (gastritis) develops with concomitant appearance of serological responses against the bacterium. However, *H. pylori* is resistant to innate and acquired immune responses, and the immune system fails to remove the organism effectively [18]. Chronic gastritis leads eventually to mucosal atrophy characterized by a decrease in the proper gastric glands, and intestinal metaplasia marked by the replacement of gastric epithelial cells with other epithelial cells such as columnar absorptive cells and goblet cells of intestinal morphology [19].

Intestinal metaplasia has been categorized into two major types: one is the complete type, which is characterized by the presence of absorptive cells, Paneth cells, and goblet cells secreting sialomucins and corresponds to the small intestine phenotype, and the other is the incomplete type, which is characterized by the presence of columnar and goblet cells secreting sialo and/or sulfomucins [20]. These two types of intestinal metaplasia can be distinguished also by altered mucin expression patterns. While the intestinal mucin MUC2 is expressed in goblet cells of both types of intestinal metaplasia (normal gastric mucosa does not express MUC2 [21]), MUC1, MUC5AC and/or MUC6 is expressed in the incomplete type but not in the complete type [20].

1.4.2. Peptic Ulcer—Peptic ulcers are chronic, often solitary lesions that occur in gastroduodenal mucosa exposed to aggressive action of acid-peptic juices. These lesions appear to be produced by an imbalance between mucosal defense mechanisms and damaging forces. The pathogenesis of peptic ulcers appears to be multi-factorial, and the apparent role of *H. pylori* in peptic ulcers cannot be overemphasized. However, *H. pylori* infection is present in virtually all patients with duodenal ulcers and about 70% of those with gastric ulcers. Furthermore, antibiotic treatment of *H. pylori* infection promotes healing of ulcers and tends to prevent their recurrence [7].

1.4.3. Gastric Adenocarcinoma—Gastric adenocarcinoma is the fourth most common cancer and second leading cause of cancer-related death worldwide [22]. Gastric adenocarcinoma can be divided into two distinct histological subtypes [23], each with different epidemiological and clinicopathological features. One subtype is intestinal-type adenocarcinoma, which usually occurs at a later age and progresses through a relatively well-defined series of histological steps, namely, chronic gastritis, atrophy of pyloric glands, intestinal metaplasia, and dysplasia [24]. The other subtype is diffuse-type adenocarcinoma, which more commonly affects younger people and is not associated with intestinal metaplasia [24].

1.4.4. MALT Lymphoma—Most lymphomas of the stomach are mucosa-associated lymphoid tissue (MALT) lymphoma, a low-grade B cell lymphoma. This type of lymphoma arises in MALT, hence the name. B cells that give rise to MALT lymphomas normally reside in the marginal zones of lymphoid follicles and are increased in response to various types of chronic inflammation, including chronic gastritis due to *H. pylori* infection [25]. It is generally accepted that chronic infection with *H. pylori* leads to generation of *H. pylori*-reactive T cells, which, in turn, activate a polyclonal population of B cells by secreting soluble factors. In time, a monoclonal but T cell-dependent population of proliferating B cells emerges. Presumably, such monoclonal B cell proliferation subsides when the antigenic stimulus for T cells is removed by antibiotic treatment. However, if untreated, genetic mutations accumulate in these proliferating B cells, and they eventually become T cell-independent [26].

1.5. Virulence Factors

1.5.1. CagA—In the industrialized world, 60–70% of *H. pylori* strains possess the cytotoxin-associated antigen A (CagA), a 120–145 kDa protein [27]. The *cagA* gene is localized at one end of the *cag* pathogenicity island (*cag*-PAI), a 37-kb genomic fragment containing 31 genes [28,29]. Several of these are homologous to genes encoding the type IV secretion apparatus [17]. Upon direct contact of *H. pylori* with gastric epithelial cells, CagA is injected from the bacterium into the host cell via the type IV secretion system [30–33]. After entering an epithelial cell, CagA is phosphorylated and binds to Src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2), leading to a growth factor-like cellular response and cytokine production [34]. Deregulation of SHP-2 by CagA is an important mechanism by which CagA promotes gastric epithelial carcinogenesis. Recently, Ohnishi and co-workers generated CagA

transgenic mice and found that CagA induces abnormal proliferation of gastric epithelial cells and hematopoietic cells, followed by the development of gastrointestinal carcinomas and leukemias/lymphomas in a tyrosine phosphorylation-dependent manner, revealing *H. pylori* CagA is the first bacterial oncoprotein that acts in mammals. [35]. CagA also elicits junctional and polarity defects in epithelial cells by interacting with and inhibiting partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK) independently of CagA tyrosine phosphorylation [36].

Very recently, Marcos and co-workers reported that *cag*-PAI$^+$ high pathogenic *H. pylori* strains induce expression of several genes involved in glycan biosynthesis, in particular that encoding β1,3-*N*-acetylglucosaminyltransferase 5 (β3GnT5) [37], a GlcNAc transferase essential for the biosynthesis of Lewis antigens on glycolipids [38]. This induction is dependent on *cagA* and *cagE*, most probably through the TNF/NF-κB pathway [38]. The study identified a novel mechanism by which *H. pylori* modulates the biosynthesis of the sialic acid-binding adhesin (SabA) ligand in gastric cells, thereby strengthening the epithelial attachment necessary to achieve successful colonization (see also section 2.4.2. for detail).

1.5.2. VacA—Vacuolating toxin (VacA) is a major virulence factor secreted by *H. pylori* and is a key component in the pathogenesis of gastric diseases [39]. Approximately 50% of *H. pylori* strains express the VacA protein, and that expression is correlated with expression of CagA. The most established activity of VacA is cellular vacuolation in mammalian cells [39–41]. Although the precise mechanism of VacA-induced vacuole formation is not fully understood, it involves binding and internalization of toxin. It has been proposed that vacuolation is a consequence of anion-selective channel formation in late endosomal compartments [42–45]. In addition to its vacuole formation activity, VacA causes numerous cellular events, including depolarization of the membrane [44,46], apoptosis [47–50], interference with epithelial cell attachment [51], and inhibition of T lymphocyte activation [52].

2. GLYCOCONJUGATES ASSOCIATED WITH *H. PYLORI*

2.1. Glycan Structure of *H. pylori* LPS

The cell wall of all Gram-negative bacteria is composed of two phospholipid bilayers with a peptidoglycan layer sandwiched between them. Lipopolysaccharide (LPS) is a structural component of the outer cell wall. LPS is composed of a long-chain fatty acid anchor called lipid A, a core sugar chain, and a variable carbohydrate chain designated O antigen, which is attached to the core sugar [53]. Thus, the O antigen has the potential to exhibit enormous structural variability and is the domain determining the serological specificity of LPS [54].

Clinical isolates of *H. pylori* produce O antigen of a relatively constant chain length [55]. It is this region of *H. pylori* LPS that shares structural homology with Lewis blood group antigens in the gastric mucosa, predominantly Lewis X and Lewis Y antigens bearing type 2 blood group determinants. Serologically, 80–90% of *H. pylori* strains have been found to contain Lewis X and/or Lewis Y epitopes.

2.2. Putative Consequence of Lewis Expression by *H. pylori*

Lewis blood group antigens are present in normal human gastric mucosa, and the expression of these antigens on *H. pylori* LPS has important biological implications. Molecular mimicry mediated by *H. pylori* LPS has been suggested to camouflage the bacterium and facilitate initial colonization [56].

Additionally, *H. pylori* Lewis antigens undergo phase variation: specifically, random, reversible high-frequency switching of phenotype contributes to virulence. The molecular

mechanisms involved in phase variation are slipped-strand mispairing in poly-C tracts and translational frameshifting by ribosomal slippage [57]. At least 5 glycosyltransferase genes are involved in generating phase variants: the genes encoding α3-fucosyltransferase (of which there are two similar but non-identical copies), α2-fucosyltransferase, β3-galactosyltransferase and β3-*N*-acetyl-D-glucosaminyltransferase [58]. Each of these genes can be either "on" or "off", and thus, in any *H. pylori* cell population, at least 32 different glycosyltransferase gene "on-off" combinations and potentially the same number of LPS phenotypes are present [58]. Thus, any *H. pylori* strain can potentially express any LPS Lewis phenotype.

This antigenic mimicry may result in immune tolerance against antigens of the pathogen or in induction of autoantibodies that recognize gastric epithelial cells, which is frequently observed in patients with chronic active gastritis.

2.3. Adhesion of *H. pylori* to Gastric Epithelial Cells

Attachment is a prerequisite for microbial colonization of epithelial surfaces and is mediated by molecules on the bacterial surface, adhesins, which recognize proteins or glycoconjugates on the surface of eukaryotic cells. The specificity of this interaction and the limited distribution of receptors often result in a restricted range of hosts and tissues utilized for colonization, a phenomenon known as tropism. Bacteria, which are unable to adhere to epithelia, tend to be rapidly removed by shedding from surface cells and the mucus layer.

H. pylori expresses adhesins that confer intimate adherence to the gastric epithelium where the bacteria can gain easy access to nutrients from host tissues [59]. These adherence properties protect the bacteria from the extreme acidity of the gastric lumen and displacement from the stomach by forces such as those generated by peristalsis and gastric emptying [60]. Two carbohydrate structures in surface mucous cells serve as specific ligands for *H. pylori* adhesins: Lewis b, which binds to blood group antigen-binding adhesin (BabA), and sialyl dimeric Lewis X-bearing glycosphingolipid, which binds to sialic acid-binding adhesin (SabA). In addition, attachment of *H. pylori* to gastric epithelial cells can induce pedestal formation [61]. Pedestal formation describes the creation of an upright support, constructed of host cell material, beneath an attached bacterium.

2.4. *H. pylori* Adhesins

2.4.1. BabA—The best defined *H. pylori* adhesin-receptor interaction characterized to date is that between BabA, a member of a family of *H. pylori* outer membrane proteins, and Lewis b, H, and related ABO antigens [60]. These fucose-containing blood group antigens are found on red blood cells and in the gastrointestinal mucosa. Blood group O individuals suffer disproportionately from peptic ulcer disease [62], suggesting that bacterial adherence to H and Lewis b antigens influences severity of infection. The human population of South American Amerindians dominantly express blood group O antigen. Interestingly, BabA from this population binds blood group O antigen more efficiently than other blood group antigens [59]. BabA has 2 isoforms, babA1 and babA2. The product of *babA1* gene, in contrast to that encoded by the *babA2* gene, cannot interact with Lewis b; thus it does not enhance *H. pylori* colonization of the surface epithelium [60,63].

2.4.2. SabA—The *SabA* gene encodes a 651 amino acid protein of 70 kDa and belongs to the large hop family of *H. pylori* outer membrane protein genes, which also includes the *babA* gene [64]. Sialyl dimeric Lewis X glycolipid is rarely expressed in normal gastric mucosa. However, the gastric mucosa infected by *H. pylori*, particularly *cag*-PAI$^+$ strains, newly expresses this unique glycolipid in surface mucous cells partly facilitated by increased expression of β3GnT5 [37,38], and its expression level is increased as inflammation progresses. The adhesion mediated by SabA binding to sialyl dimeric Lewis X glycolipid contributes to

persistent *H. pylori* infection. Sialyl dimeric Lewis X is also expressed in leukocytes, but an "on-off" frameshift mutation of the *SabA* gene allows *H. pylori* to escape intimate contact with these inflammatory cells. Such adaptive mechanisms play an important role in the extraordinary chronicity of *H. pylori* infection in human gastric mucosa.

3. INDUCTION OF PNAD IN GASTRIC MUCOSA INFECTED BY *H. PYLORI*

3.1. Role of PNAd in Secondary Lymphoid Organs and Chronic Inflammatory Sites

In chronic inflammatory states, L-selectin and its ligands are implicated in lymphocyte recruitment in those diseases in which peripheral lymph node addressin (PNAd) is induced on high endothelial venule (HEV)-like vessels [65,66]. Such HEV-like vessels have been observed in rheumatoid arthritis, lymphocytic thyroiditis, and inflammatory bowel diseases [67–71]. In these studies, the induction of PNAd is detected by the MECA-79 antibody [72], which decorates PNAd on HEV-like vessels. MECA-79-positive HEVs in secondary lymphoid organs play a major role in lymphocyte circulation [65]. The MECA-79 epitope has been shown to be 6-sulfo *N*-acetyllactosamine attached to extended core 1 *O*-glycans, Galβ1→4(SO_3→6) GlcNAcβ1→3Galβ1→3GalNAcα1→Ser/Thr [73]. Moreover, MECA-79 antibody can also bind to its sialylated and fucosylated form that constitutes PNAd [73]. Structural studies also show that 6-sulfo sialyl Lewis X on core 2 branched *O*-glycans, sialic acidα2→3Galβ1→4 [Fucα1→3(SO_3→6)]GlcNAc β1→6(Galβ1→3)GalNAcα1→Ser/Thr, is present as a major L-selectin ligand on HEVs [73,74].

3.2. HEV-Like Vessels Are Induced in *H. pylori*-Induced Inflammation

Because it has been reported that *de novo* formation of HEV-like vessels, which express PNAd, is associated with various chronic inflammatory diseases, we determined whether chronic inflammation caused by *H. pylori* infection is associated with formation of HEV-like vessels [75]. To do so, gastric mucosa from patients infected with *H. pylori* was stained with MECA-79 antibody and HECA-452 antibody, which reacts equally well with sialyl Lewis X and 6-sulfo sialyl Lewis X capped structure on extended core 1 and core 2 branches. Gastric mucosa derived from *H. pylori*-infected patients displayed HEV-like vessels expressing MECA-79 and HECA-452 antigens as well as CD31 and CD34, which are markers of vascular endothelial cells. These HEV-like vessels can potentially recruit L-selectin-expressing lymphocytes, because L-selectin•IgM chimeric protein bound to the same vessels in a calcium-dependent manner [75]. These results indicate that *H. pylori*-induced inflammation is associated with formation of PNAd present on HEV-like vessels.

These results demonstrate that 6-sulfo sialyl Lewis X attached to extended core 1 *O*-glycans is present on HEV-like vessels, based on positive staining by MECA-79 and HECA-452 antibodies. To elaborate further the chemical nature of L-selectin ligands on these vessels, the NCC-ST-439 monoclonal antibody was used. NCC-ST-439 antibody binding has been verified for sialyl Lewis X-capped structure on Galβ1→4GlcNAcβ1→6GalNAcα1→R but not on natural core 2 branched *O*-glycan Galβ1→4GlcNAcβ1→6 (Gal β1→3)GalNAcα1→R [76]. Moreover, it has not been determined whether 6-sulfo sialyl Lewis X is also recognized by this antibody. To test these possibilities, we made CHO cells expressing various types of *O*-glycans and stained cells with NCC-ST-439 antibody. NCC-ST-439 antibody binds to CHO cells expressing non-sulfated and 6-sulfo sialyl Lewis X on core 2 branched *O*-glycans but barely to CHO cells expressing those capped structures on extended core 1 *O*-glycans. NCC-ST-439 antibody can also stain HEV-like vessels formed in the gastric mucosa. These combined results suggest that PNAd induced by *H. pylori* infection expresses 6-sulfo sialyl Lewis X on both extended core 1 and core 2 branched structures in the same manner as PNAd expressed in secondary lymphoid organs [73].

3.3. Increased Formation of HEV-Like Vessels Is Correlated with Progression of Inflammation

Based on the updated Sydney system, progression of inflammation initiated by *H. pylori* infection is ranked in four stages from least to most severe: normal, mild, moderate, and marked [77]. In moderate and marked stages, intestinal metaplasia frequently occurs, indicating an advanced stage of the disease.

Fig. (1) A (lower panels) indicates a marked stage of inflammation in which recruitment of mononuclear cells obscures proper glands in the gastric mucosa, which contrasts with glands visible in mucosa at the mild stage (Fig. (1) A, upper panels). These observations demonstrate that lymphocyte infiltration is more prominent when HEV-like vessels are more abundant.

After examining over 140 human specimens, we found that the number of HEV-like vessels, as detected by MECA-79 or HECA-452 antibody, correlates positively with the progression of inflammation (Fig. (1) B). Fig. (1) C illustrates that more patients display HEV-like vessels as inflammation progress. *H. pylori* was detected in 0%, 21%, 82% and 87% of patients in normal, mild, moderate, and marked stages of inflammation, respectively. Overall, HEV-like vessels were found in 79.2% of *H. pylori* infected patients.

3.4. Formation of HEV-Like Vessels Requires Continuous *H. pylori* Infection

To determine whether formation of HEV-like vessels is correlated with *H. pylori* infection, gastric biopsies were obtained from 17 patients with chronic active gastritis before and after eradication of *H. pylori* by treatment with antibiotics and a proton pump inhibitor. Patients with moderate inflammation displayed both *H. pylori* and HEV-like vessels detected by MECA-79 and HECA-452 antibodies (Fig. (2) A). After eradication of *H. pylori*, the gastric mucosa of all patients no longer displayed HEV-like vessels as assessed by MECA-79 and HECA-452 staining and showed minimum lymphocyte infiltration (Fig. (2) B). These results indicate that continuous infection of *H. pylori* is necessary for formation and maintenance of HEV-like vessels expressing PNAd. It is tempting to speculate that bacterial components such as LPS acting through Toll-like receptor-dependent pathways in the gastric epithelium, stimulate the release of cytokines, i.e., lymphotoxin α [78]. This effect might in turn modulate gene expression in postcapillary venules in ways that could cause their biochemical, functional, and morphological transformation by up-regulating chemokines, such as CCL19 and CCL21 that act on CCR7 receptors.

3.5. HEV-Like Vessels in NSAID-Induced Gastritis

It is well established that continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) results in chemical gastritis [79]. To determine whether HEV-like vessels are induced in chronic inflammatory responses caused by factors other than *H. pylori* infection, gastric mucosa obtained from long-term rheumatoid arthritis patients taking NSAIDs was examined. Most of the 20 patients examined exhibited chemical gastritis phenotypes and were devoid of HEV-like vessels. HEV-like vessels were found in specimens from six of the 20 patients, but three of these were also infected with *H. pylori*. Those three patients also had lower scores for chemical gastritis, and HEV-like vessels were likely formed by inflammation caused by *H. pylori* infection. In three *H. pylori*-free patients, HEV-like vessels were found only in 0.68%, 0.67% and 0.21% of CD34-positive vessels, and two patients displayed intestinal metaplasia, suggesting a possible prior *H. pylori* infection. The other patient displayed both a chemical gastritis phenotype and lymphocyte recruitment. Interestingly, MECA-79 staining was much less intense in tissues from this patient than in tissues from patients infected with *H. pylori*. These results indicate that chemical gastritis induces PNAd at a very low level in the gastric mucosa.

4. ANTIBIOTIC FUNCTION OF α1,4-GLCNAC-CAPPED *O*-GLYCANS AGAINST *H. PYLORI*

4.1. Two Types of Mucins Present in Gastric Mucosa

As described in section 1.3., over half the world's population harbor *H. pylori*, but only a fraction of those infected develop diseases such as peptic ulcer disease, gastric adenocarcinoma, and MALT lymphoma. This observation suggests the presence of host defense mechanisms against *H. pylori* pathogenesis.

Gastric mucins are classified into two types based upon their histochemical properties [80]; one is a surface mucous cell-derived mucin displayed on MUC5AC core protein [21], and the other is a mucin displayed on MUC6 core protein secreted by gland mucous cells, including cardiac gland cells, mucous neck cells, and pyloric gland cells [81]. These two types of mucins form the surface mucous gel layer (SMGL), which shows an alternating laminated array.

H. pylori is associated exclusively with surface mucous cell-derived mucins and rarely colonizes deeper portions of gastric mucosa, where gland mucous cells produce mucins having terminal α1,4-linked-GlcNAc residues attached to core 2 branched *O*-glycans [GlcNAcα1→4Galβ1→4GlcNAcβ1→6(GlcNAcα1→4 Gal β1→3)GalNAcα→Ser/Thr] (α1,4-GlcNAc-capped *O*-glycans) [82]. These findings raise the possibility that α1,4-GlcNAc-capped *O*-glycans protect against *H. pylori* infection.

4.2. Effect of α1,4-GlcNAc-Capped *O*-Glycans on *H. pylori* Growth

To test the above hypothesis, we generated α1,4-GlcNAc-capped *O*-glycans and determined their effect on *H. pylori in vitro* [83]. *H. pylori* was cultured in the presence of recombinant soluble CD43 with or without α1,4-GlcNAc-capped *O*-glycans secreted from transfected Lec2 cells, a mutant of Chinese hamster ovary cells defective in the CMP-sialic acid transporter [84]. As shown in Fig. (3), *H. pylori* cultured in the presence of control soluble CD43 lacking α1,4-GlcNAc-capped *O*-glycans grew rapidly, whereas soluble CD43 having α1,4-GlcNAc-capped *O*-glycans inhibited growth of *H. pylori* in a dose dependent manner. Such inhibitory effects were also demonstrated when CD34 was used as a scaffold protein instead of CD43. Moreover, GlcNAcα-*p*-nitrophenyl (PNP) and gastric gland mucins containing α1,4-GlcNAc-capped *O*-glycans also suppressed *H. pylori* growth. In addition to growth suppression, significant reduction of motility as well as abnormal morphology such as elongation, segmental narrowing, and folding were seen in bacteria incubated with soluble CD43 having α1,4-GlcNAc-capped *O*-glycans (Fig. (4)), supporting the idea that α1,4-GlcNAc-capped *O*-glycans have anti-*H. pylori* activity.

4.3. α1,4-GlcNAc-Capped *O*-glycan-Mediated Reduction of a Cell Wall Component, CGL

The morphological abnormalities seen in *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 [85]. Therefore, these *O*-glycans may inhibit cell wall biosynthesis in *H. pylori*. The cell wall of *Helicobacter* spp. characteristically contains α-cholesteryl glucosides, including cholesteryl-α-D-glucopyranoside (CGL) [86]. Mass spectrometric analysis of cell wall components, particularly CGL, from *H. pylori* cultured with α1,4-GlcNAc-capped *O*-glycans showed significant reduction of CGL compared to controls in which *H. pylori* was incubated with soluble CD43 without α1,4-GlcNAc-capped *O*-glycans [83]. Similar results were seen when sonicated *H. pylori* was used as an enzyme source of UDP-Glc:sterol α-glucosyltransferase, which is responsible for the biosynthesis of CGL. These results suggest that α1,4-GlcNAc capped *O*-glycans directly inhibit biosynthesis of CGL by *H. pylori*.

4.4. Role of CGL in *H. pylori* Viability

CGL is formed by an UDP-Glc:sterol α-glucosyltransferase, which transfers glucose (Glc) from UDP-Glc to the C3 position of cholesterol with an α-linkage. Since genes involved in cholesterol biosynthesis are not found in its genome, *H. pylori* cannot synthesize CGL in the absence of exogenous cholesterol. We thus created *H. pylori* deficient in CGL by culturing it without cholesterol for 5 days and found that growth and motility of bacteria were dramatically suppressed compared with *H. pylori* cultured in the presence of cholesterol [83]. Abnormal morphology was also noted in the culture, and bacteria died after 21 days in culture, indicating that CGL is crucial for *H. pylori* survival.

4.5. Antibiotic Function of α1,4-GlcNAc Residues

Finally, to determine whether mucous cells expressing α1,4-GlcNAc-capped *O*-glycans are protected against *H. pylori* infection, gastric adenocarcinoma AGS cells stably transfected with α1,4-*N*-acetylglucosaminyltransferase (α4GnT) (AGS-α4GnT) [87] were co-cultured with *H. pylori* [83]. With a short-term incubation of 8 hours, the microbes attached equally well to AGS-α4GnT cells and mock-transfected AGS cells. No significant morphological changes were observed in either group of cells at this time point. However, after a 24-hour incubation, mock-transfected AGS cells exhibited remarkable deterioration, such as flat morphology or shrinkage, and showed increased numbers of associated *H. pylori*. After three days in culture, the number of viable AGS cells was dramatically reduced. By contrast, growth of *H. pylori* in cultures with AGS-α4GnT cells was markedly suppressed, and evidence of cellular damage seen in mock-transfected AGS cells was barely detected in these cells. The viability of AGS-α4GnT cells was fully maintained for up to 4 days. These findings indicate that α1,4-GlcNAc-capped *O*-glycans have no effect on adhesion of *H. pylori* to GAS-α4GnT cells but protect the host cells from *H. pylori* infection.

Additionally, it should be noted that *H. pylori* is rarely observed in metaplastic glands: development of gastric intestinal metaplasia creates a microenvironment that is hostile to *H. pylori* colonization and generally leads to clearing of *H. pylori* from metaplastic glands [88, 89]. However, in contrast to the observation in normal gastric mucosa, α1,4-GlcNAc-capped *O*-glycans are scarcely detected in metaplastic glands, indicating that this *O*-glycan is not implicated in the reduction/absence of *H. pylori* in this particular pathologic lesion [90].

5. EXPRESSION CLONING OF CHOLESTEROL α-GLUCOSYLTRANSFERASE FROM *H. PYLORI*

5.1. Expression Cloning of *H. pylori* and *H. felis* CHLαGcT

As described in section 4.3., α-glucosyl cholesterol and its derivatives are major components of the cell wall of *Helicobacter* spp. and constitute more than 25% of total cell wall lipids of *H. pylori*, reaching almost 130 μM [86,91]. In addition, it has been reported that α-glucosyl cholesterol abrogates phagocytosis of *H. pylori* and compromises subsequent T cell activation directed toward *H. pylori* [92]. Conversely, the increased amount of cholesterol resulted in increased phagocytosis of *H. pylori* and increased T cell responses toward *H. pylori*. These results demonstrate that α-glucosylation of cholesterol in *H. pylori* facilitates both infectivity and pathogenicity of *H. pylori*.

Recently, we and Lebrum *et al*. independently cloned genomic DNA encoding cholesterol α-glucosyltransferase (CHLαGcT) (HP0421) [93,94]. Since a BLAST search did not reveal genomic sequences homologous to sterol β-glucosyltransferase [95] and other bacterial glucosyltransferases, we undertook expression cloning to identify a genomic DNA encoding CHLαGcT. Following expression of genomic fragments in *Escherichia coli*, we identified a pool of colonies directing [^{3}H]glucose incorporation from UDP-[^{3}H]Glc to cholesterol and

eventually identified a single plasmid harboring genomic sequences for two open reading frames (designated HP0420 and HP0421 in the *H. pylori* genome [28]). HP0421 was responsible for CHLαGcT activity and deletion of HP0420 did not impair expression of CHLαGcT activity. *H. felis* CHLαGcT was identified by similar approach.

The amino acid sequences of *H. pylori* and *H. felis* CHLαGcT are shown in Fig. (5). Corresponding sequences for *H. hepaticus* [96], *H. mustelae* [97], and *H. acinonychis* [98], based on their whole or partial genome sequences, are also shown. CHLαGcT from *H. pylori* has 52–93% identity with that from other *Helicobacter* spp. CHLαGcT does not resemble a typical eukaryotic glycosyltransferase since it lacks DXD motif, which is found all the eukaryotic glycosyltransferases cloned to date [99]. A BLAST search did not identify other proteins exhibiting significant homology to the cloned CHLαGcT, strongly suggesting that the identified enzyme is solely responsible for α-glucosylation of cholesterol in *H. pylori* and other *Helicobacter* spp.

5.2. Expression of *H. pylori* CHLαGcT

To characterize *H. pylori* CHLαGcT, the enzyme was expressed as a fusion protein with a C-terminal 6x His-tag. CHLαGcT expression in *E. coli* was driven by an amylolytic enzyme promoter from *Bacillus licheniformis*. After bacterial cells were treated with lysozyme and sonicated, approximately half of the enzyme was released into the soluble fraction (Fig. (6) B) and purified using a Ni-NTA column (Fig. (6) A, lane 4). The apparent molecular weight of the purified protein was slightly smaller than the calculated molecular weight (46,030 Da), indicating that the enzyme exhibits anomaly mobility upon SDS-gel electrophoresis.

A product obtained using the expressed CHLαGcT and cholesterol was susceptible to α-glucosidase but not β-glucosidase, confirming that the product was α-glucosyl cholesterol.

5.3. Inhibition of CHLαGcT Activity by Mucin-Type *O*-Glycans

As described in section 4.2., *H. pylori* growth is inhibited by mucin-type *O*-glycans, particularly those containing α1,4-GlcNAc capped structures [83]. To determine if CHLαGcT activities reflect *in vivo* effects of oligosaccharides, inhibition by mucin-type *O*-glycans was tested (Table 1). *O*-glycans resembling those present in gastric mucin were potent inhibitors for CHLαGcT activity, while non-sialylated *O*-glycans were relatively weak inhibitors. The results also indicate that the α1,4-GlcNAc capped structure is the most efficient inhibiting structure as predicted from inhibition of *H. pylori* grown in culture [83].

5.4. CHLαGcT Acts in an Ordered Bi-Bi Manner

To determine how CHLαGcT acts on acceptor and donor substrates, enzyme activity was measured at various UDP-Glc concentrations, while fixed amounts of UDP or cholesterol were added as inhibitors [100]. The Lineweaver-Burk plot of the inhibition profile of UDP in the presence of UDP-Glc intersected at the Y-axis, indicating competitive inhibition. In contrast, cholesterol did not exhibit competitive inhibition in the presence of UDP-Glc and showed mixed-type inhibition. We then measured CHLαGcT activity at different fixed concentrations of cholesterol. Cholesterol affected CHLαGcT activity in a mixed-type manner, consistent with the above results.

To delineate further how the enzyme acts on donor and acceptor substrates, inhibition of CHLαGcT by α-glucosyl cholesterol was examined. CHLαGcT activity was measured at various UDP-Glc concentrations in the presence of 5 μM cholesterol. α-Glucosyl cholesterol was then added as an inhibitor. The Lineweaver-Burk plot showed that α-glucosyl cholesterol inhibited CHLαGcT in a non-competitive, mixed-type manner. Similarly, α-glucosyl cholesterol inhibited CHLαGcT activity in a mixed type manner when the cholesterol

concentration was varied. These results combined indicate that CHLαGcT acts in an ordered Bi-Bi manner, and strongly suggest that cholesterol is added to the enzyme/substrate complex after a complex forms between the enzyme and UDP-Glc (Fig. (7)). By this mechanism, the enzyme-UDP-Glc complex presumably induces a conformational change such that flexible loops form to accommodate more favorable binding to an acceptor substrate.

5.5. α1, 4-GlcNAc-Capped Core 2 *O*-Glycan Inhibits *H. pylori* Growth

As described in section 4.2., recombinant CD43 expressing α1,4-GlcNAc residues inhibits *H. pylori* growth [83]; however, we had not determined whether oligosaccharides containing α1,4-GlcNAc residues inhibit *H. pylori* growth. We thus evaluated the effect of α1,4-GlcNAc-capped core 2 *O*-glycan, sialylated core 2 *O*-glycan, monosaccharides, or α-glucosyl cholesterol on *H. pylori* growth. Monosaccharide GlcNAc had no effect on *H. pylori* growth, but higher concentrations of GlcNAcα-PNP inhibited microbial growth (Fig. (8) B and C). *H. pylori* growth was significantly inhibited by 0.5 mM α1,4-GlcNAc-capped core 2 *O*-glycan (Fig. (8) E). Inhibition by α1,4-GlcNAc-capped core 2 *O*-glycan was more robust than that by α-glucosyl cholesterol, the product of CHLαGcT (Fig. (8) F). We also tested sialylated core 2 *O*-glycan, since this *O*-glycan is the second best inhibitor for CHLαGcT *in vitro* among the oligosaccharides tested [93], and represents a major *O*-glycan in mucins [101,102]. Interestingly, sialylated core 2 *O*-glycan without an α1,4-GlcNAc-capped structure (disialo-core 2) had no inhibitory activity (Fig. (8) D). These combined results indicate that an α1,4-GlcNAc-capped core 2 *O*-glycan most efficiently inhibits *H. pylori* growth, and an α1,4-GlcNAc residue is essential for that inhibition.

The discovery of CHLαGcT inhibitors will be important because of their function as antibiotics against *H. pylori*. Since CHLαGcT is present only in *Helicobacter* spp., inhibition of this enzyme acts as a *Helicobacter*-specific antibiotic, therefore, with minimal side effects. Future studies are important to develop α1,4-GlcNAc-capped core 2-based drug and identifying an inhibitor of low molecular weight for the treatment of *H. pylori* infection.

6. CONCLUSION

In this review, we have shown that a battery of *O*-glycans such as 6-sulfo sialyl Lewis X and α-1,4-GlcNAc-capped *O*-glycans expressed in the HEV-like vessels and gland mucous cells, respectively, play pivotal roles on pathogenesis of chronic active gastritis and on protection of the gastric mucosa from *H. pylori*, respectively. These discoveries allow us to not only understand the pathogenesis of chronic active gastritis but also to develop new carbohydrate-based therapy or prevention to *H. pylori* infection.

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ABBREVIATIONS

α4GnT
: α1,4-*N*-acetylglucosaminyltransferase

BabA
: Blood group antigen-binding adhesin

β3GnT5

β1,3-*N*-acetylglucosaminyltransferase 5

CagA
Cytotoxin-associated antigen A

CGL
Cholesteryl-α-D-glucopyranoside

CHLαGcT
Cholesterol α-glucosyltransferase

DMSO
Dimethyl sulfoxide

HEV
High endothelial venule

LPS
Lipopolysaccharide

MALT
Mucosa-associated lymphoid tissue

MARK
Microtubule affinity-regulating kinase

NSAID
Non-steroidal anti-inflammatory drug

PAR1
Partitioning-defective 1

PNAd
Peripheral lymph node addressin

PNP
p-nitrophenyl

SabA
Sialic acid-binding adhesin

SHP-2
Src homology 2 domain-containing tyrosine phosphatase 2

SMGL
Surface mucous gel layer

VacA
Vacuolating toxin

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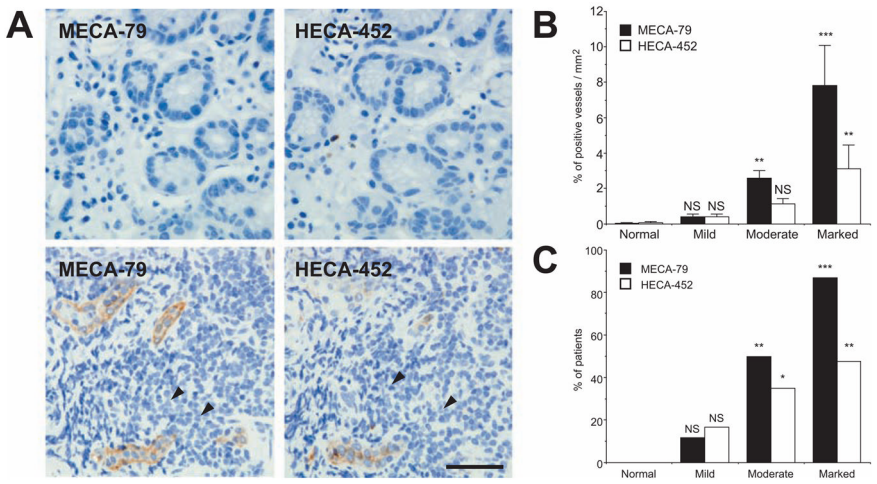

Fig. (1).

Gastric mucosa of different degrees of chronic inflammation and association of HEV-like vessels with progression of inflammation. **(A)** (Upper) Gastric mucosa at a mild stage barely expresses HEV-like vessels with minimum recruitment of lymphocytes. (Lower) Gastric mucosa at a marked stage expresses a significant number of recruited lymphocytes (arrowheads) around HEV-like vessels. **(B)** The number of MECA-79$^+$ or HECA-452$^+$ vessels is positively correlated with the progression of chronic inflammation. Each group consists of 11 (normal), 42 (mild), 67 (moderate), and 23 (marked) patients. **(C)** The number of patients exhibiting greater than 1% MECA-79$^+$ or HECA-452$^+$ vessels is highly correlated with progression of chronic inflammation. *, P<0.05; **, P<0.01;, P<0.001; NS, not significant. Bar = 50 μm. Adapted with permission from Kobayashi, M.; Mitoma, J.; Nakamura, N.; Katsuyama, T.; Nakayama, J.; Fukuda, M. *Proc. Natl. Acad. Sci. U. S. A.*, **2004**, *101*(51), 17807–17812, Copyright 2004 National Academy of Science, USA.

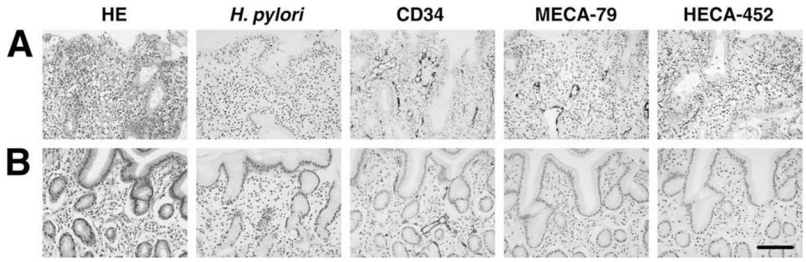

Fig. (2).

Disappearance of HEV-like vessels in the gastric mucosa after eradication of *H. pylori*. Gastric mucosa infected with *H. pylori* was examined before and 2 months after treatment to eradicate *H. pylori*. **(A)** Before treatment, HEV-like vessels detected by MECA-79 and HECA-452 antibodies were abundant, and large numbers of mononuclear cells (lymphocytes) were present around these vessels. **(B)** After eradication of *H. pylori*, HEV-like vessels were no longer present and very few mononuclear cells were present. CD34 was used for a marker of vascular endothelial cells. HE, hematoxylin and eosin, Bar = 100 μm. Adapted with permission from Kobayashi, M.; Mitoma, J.; Nakamura, N.; Katsuyama, T.; Nakayama, J.; Fukuda, M. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*(51), 17807–17812, Copyright 2004 National Academy of Science, USA.

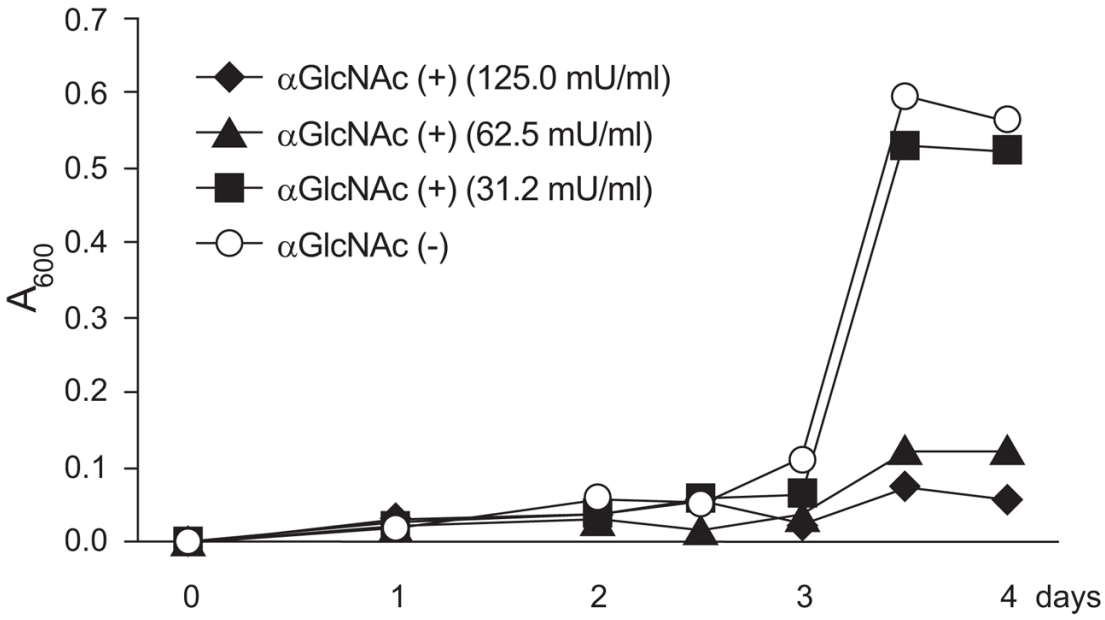

Fig. (3).

Growth curve of *H. pylori* incubated with soluble CD43 having α1,4-GlcNAc-capped *O*-glycan (αGlcNAc (+)) and soluble CD43 lacking this *O*-glycan (αGlcNAc (−)). One milliunit (mU) of αGlcNAc (+) is defined as 1 μg of GlcNAcα-PNP. The protein concentration of αGlcNAc (−) is the same as that of 31.2 mU/ml of αGlcNAc (+). Adapted with permission from Kawakubo, M.; Ito, Y.; Okimura, Y.; Kobayashi, M.; Sakura, K.; Kasama, S.; Fukuda, M.N.; Fukuda, M.; Katsuyama, T.; Nakayama, J. *Science*, **2004**, *305*(5686), 1003–1006.

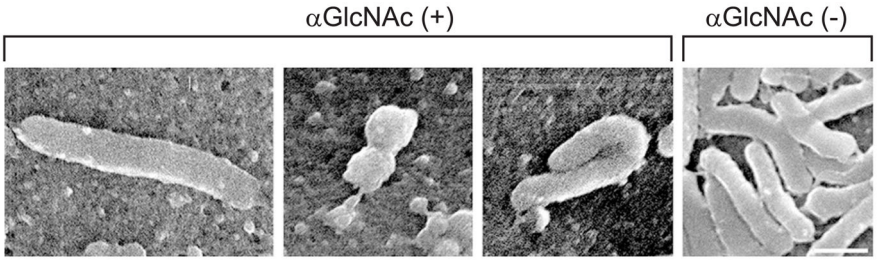

Fig. (4).
Morphology of *H. pylori* incubated with 31.2 mU/ml of soluble CD43 having α1,4-GlcNAc-capped *O*-glycan (αGlcNAc (+)) and the same protein concentration of soluble CD43 lacking this *O*-glycan (αGlcNAc (−)). Bar = 1 μm. Adapted with permission from Kawakubo, M.; Ito, Y.; Okimura, Y.; Kobayashi, M.; Sakura, K.; Kasama, S.; Fukuda, M.N.; Fukuda, M.; Katsuyama, T.; Nakayama, J. *Science*, **2004**, *305*(5686), 1003–1006.

Fig. (5).

Comparison of amino acid sequences of cloned *H. pylori* CHLαGcT (Hp), *H. felis* CHLαGcT (Hf), and putative CHLαGcTs from *H. hepaticus* (Hh), *H. mustelae* (Hm), and *H. acinonychis* (Ha). Identical amino acid residues are indicated by closed boxes. Adapted from Lee, H.; Kobayashi, M.; Wang, P.; Nakayama, J.; Seeberger, P.H.; Fukuda, M. *Biochem. Biophys. Res. Commun.*, **2006**, *349*(4), 1235–1241.

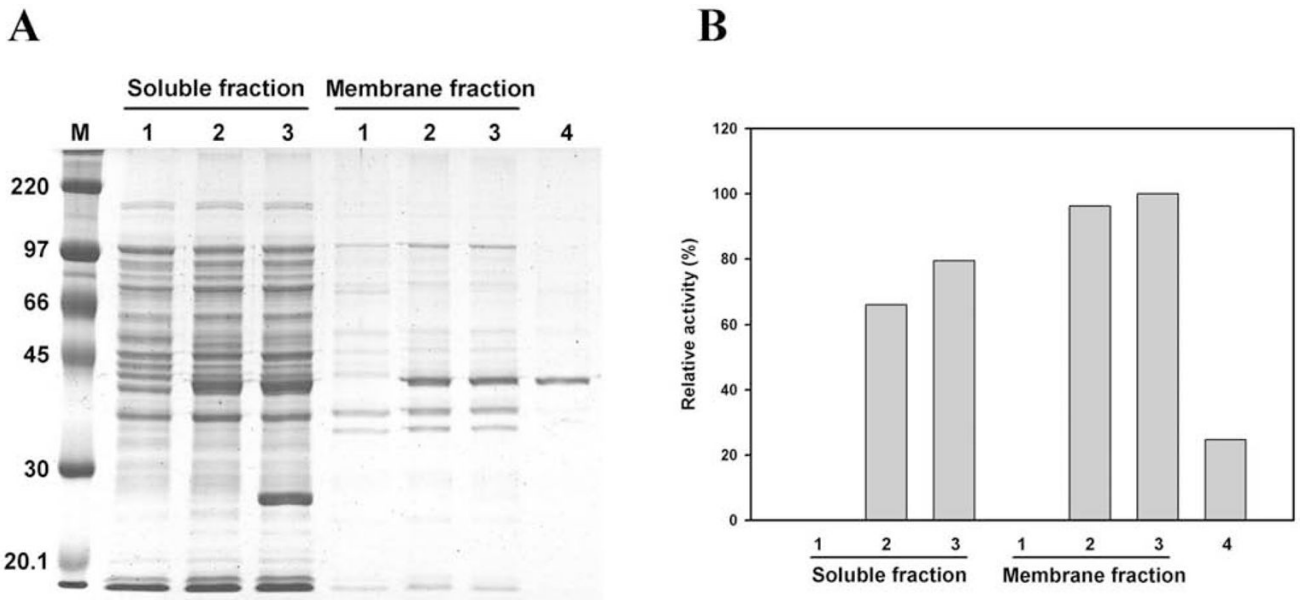

Fig. (6).

Expression and purification of CHLαGcT. **(A)** SDS-polyacrylamide gel electrophoresis of proteins in both soluble and membrane fractions (lanes 1–3: pTKNd6xH vector only; 2, pTKNd6xH-CHLαGcT; 3, pTKNd6xH-CHLαGcT + pRARE (Novagen), and of protein purified from the soluble fraction using Ni-NTA column (lane 4). **(B)** Distribution of CHLαGcT activity in samples shown in (A). The amount of protein in lane 4 was approximately one-third of that in lane 3 of the membrane fraction. Adapted from Lee, H.; Kobayashi, M.; Wang, P.; Nakayama, J.; Seeberger, P.H.; Fukuda, M. *Biochem. Biophys. Res. Commun.*, **2006**, *349*(4), 1235–1241.

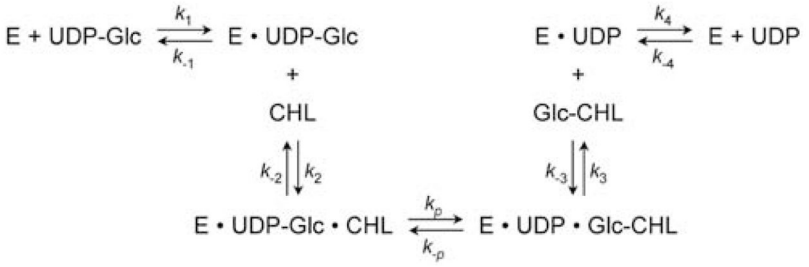

Fig. (7).

Catalytic mechanisms of CHLαGcT. The kinetic data are consistent with an ordered Bi-Bi reaction mechanism. UDP-Glc binds to CHLαGcT (E) prior to cholesterol binding, and α-glucosyl cholesterol (Glc-CHL) is released prior to UDP release from the enzyme-UDP complex. The arrows indicate the directions of reactions. k_p represents a kinetic constant to form a product, Glc-CHL. Adapted from Lee, H.; Wang, P.; Hoshino, H.; Ito, Y.; Kobayashi, M.; Nakayama, J.; Seeberger, P.H.; Fukuda, M. *Glycobiology*, **2008**, *18*(7), 549–558.

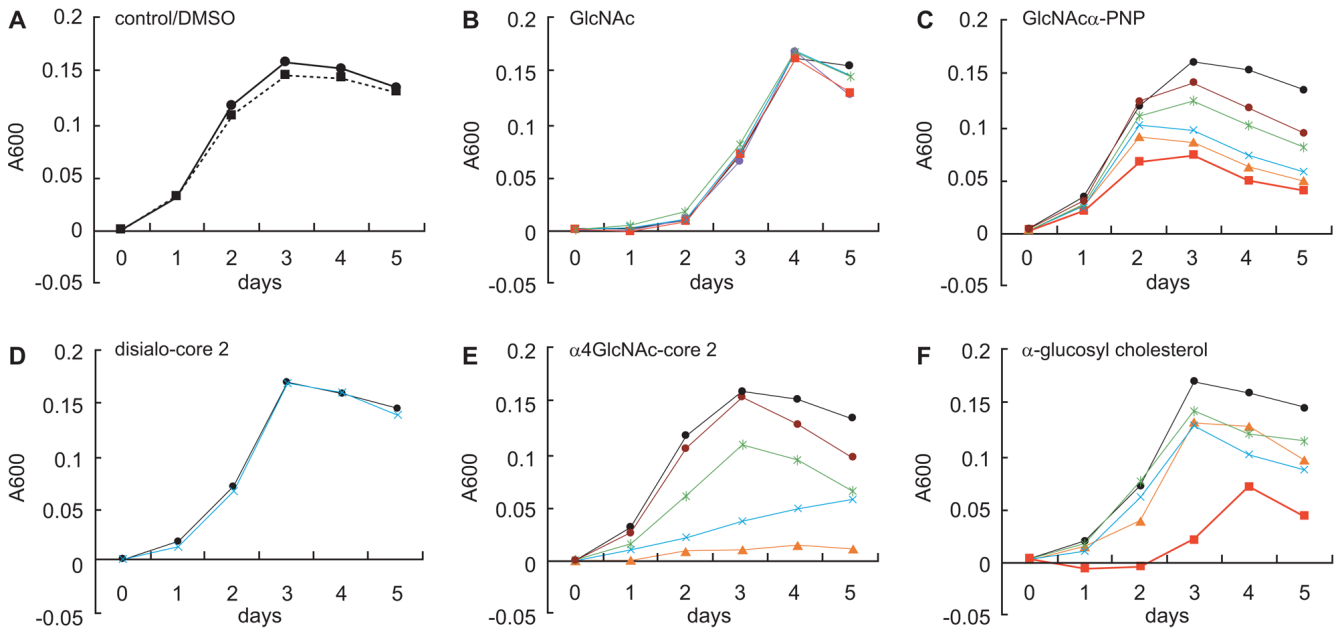

Fig. (8).

Inhibition of *H. pylori* growth by synthetic oligosaccharides and monosaccharides. *H. pylori* was cultured for 5 days in Mueller-Hinton broth supplemented with 5.5% horse serum containing various amounts of synthetic oligosaccharides and monosaccharides. Bacterial growth was measured at O.D. 600 nm, and the absorbance for control experiments at time 0 was subtracted from absorbance at later time points. Oligosaccharide and monosaccharide concentrations are 1 mM (red), 0.75 mM (orange), 0.5 mM (blue), 0.25 mM (green), 0.125 mM (brown), and control (closed circle). Two mM GlcNAc was also added in B (magenta). Oligosaccharides and monosaccharides were initially dissolved in DMSO, and the final DMSO concentration in the culture medium was 1%. The growth curve in the absence of DMSO is shown as a dotted line (A). Adapted from Lee, H.; Wang, P.; Hoshino, H.; Ito, Y.; Kobayashi, M.; Nakayama, J.; Seeberger, P.H.; Fukuda, M. *Glycobiology*, **2008**, *18*(7), 549–558.

Table 1

Inhibition of Cholesterol α-Glucosyltransferase by Various *O*-Linked Oligosaccharides. The Concentration of UDP-Glc and Cholesterol was 3.6 μM and 400 μM, Respectively

Adapted from Lee, H.; Wang, P.; Hoshino, H.; Ito, Y.; Kobayashi, M.; Nakayama, J.; Seeberger, P.H.; Fukuda, M. *Glycobiology*, **2008**, *18*(7), 549–558.

***O*-linked oligosaccharide**	**IC50 (mM)**
Galβ1→3GalNAcα1→*p*-nitrophenol	7.49
GlcNAcβ1 ↓ 6 Galβ1→3GalNAcα1→*p*-nitrophenol	2.21
NeuAcα2→3Galβ1→3GalNAcα1→octyl	1.09
NeuAcα2→3Galβ1→4GlcNAcβ1 ↓ 6 Galβ1→3GalNAcα1→octyl	0.75
GlcNAcα1→4Galβ1→4GlcNAcβ1 ↓ 6 Galβ1→3GalNAcα1→octyl	0.47