A Novel In Vitro Infection Model of Helicobacter pylori Using Mucin-Producing Murine Gastric Surface Mucous Cells

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ABSTRACT

Background. Helicobacter pylori is found within the gastric surface mucous gel layer and in the epithelial surface. Gastric cancer cells have been used in experimental H. pylori infection in vitro, although cancer cells have some abnormalities in cellular properties. The aim of this study was to develop an in vitro H. pylori infection model using normal gastric surface cells that produce gastric mucin.

Materials and methods. Normal murine gastric surface mucous cells (GSM06) were cultured by the liquid interface method using a serum-free medium and a collagen gel containing a fibroblast cell line (L929) and infected with H. pylori. Infection by H. pylori was assessed by enumerating the colony-forming units (CFU) of H. pylori adhered to GSM06 cells and by transmission electron microscopy. The production of mucin was determined by a lectin binding assay, sugar analysis, and MUC5AC gene expression.

Results. GSM06 cells cultured under these conditions produced mucin containing N-acetylgalactosamine and MUC5AC as the core protein. Significantly higher numbers of H. pylori adhered to GSM06 cells under mucin-producing conditions than under nonproducing conditions. Microscopic observation showed a filamentous structure resembling a type IV secretion system apparatus formed between the surface of GSM06 cells and H. pylori.

Conclusions. This study demonstrates a novel in vitro H. pylori infection model using mucin-producing murine GSM06 cells for early stages of infection.

Keywords. Helicobacter pylori, mucin, GSM06, infection model.

Chronic infection with Helicobacter pylori has been shown to be one of the major causes of gastritis resulting in various disease states, including peptic ulcers, gastric adenocarcinoma and mucosal associated lymphoid tissue lymphoma [1]. H. pylori is found within the gastric surface mucous layer and the epithelial surface. The gastric mucous layer consists of a multilamellar structure in which two types of mucin, MUC5AC and MUC6, are produced. MUC5AC is produced by the surface mucous cells and MUC6 by the mucous gland [1,2]. In patients positively diagnosed with H. pylori infection, more than 99% of the bacteria are associated with extracellular MUC5AC or the apical domain of MUC5AC-producing cells, making MUC5AC a likely candidate for bacterial colonization in the gastric mucosa [3].

Several in vitro model systems for H. pylori infection have been employed, using gastric cancer cells such as AGS or MKN cells. Cancer cells have been shown to affect mucin production in gastric cancer cells [4], in addition to affecting cell surface antigens and intracellular signaling. However, little information is available on the possible role mucins may play in H. pylori infections involving normal gastric surface mucous cells. Because H. pylori is closely associated with the extracellular secretion of MUC5AC and gastric epithelial cells of the stomach wall [3], an in vitro infection model reflecting in vivo conditions would necessitate experiments on normal mucin-producing gastric mucous cells. The mouse gastric surface cell line GSM06 has been established from a primary culture of gastric mucosal cells of transgenic mice harboring a temperature-sensitive simian virus 40 (tsSV40) large T-antigen gene [5].
GSM06 cells have been shown to retain many of the characteristics of normal gastric surface mucus cells [6]. However, when GSM06 cells are cultured in plastic culture ware, periodic acid Schiff-positive mucus-like substances such as hyaluronan and not mucin were secreted [7]. One possibility is that GSM06 cells in the above conditions are not fully differentiated and therefore are not able to produce mucin. Therefore, establishing GSM06 cells from normal gastric surface mucosa could provide the means for complete differentiation and the ability to secrete mucin.

Because there are no studies that show that gastric mucin promotes \textit{H. pylori} adhesion to the gastric epithelium, an in vitro infection model using MUC5AC mucin-secreting normal gastric epithelial cells would be useful. Such a model could provide useful information on the mechanism of \textit{H. pylori} infection and a method of screening for other substances promoted by infection. This study describes the conditions for culturing gastric surface mucin-producing GSM06 cells and an in vitro model that closely resembles in vivo conditions leading to infection by \textit{H. pylori}.

**Methods**

**Cell culture**

The gastric mucosal cell line GSM06, which was established from transgenic mice harboring the tsSV40 large T-antigen gene [5], was kindly provided by Dr T. Horiuchi (Daiichi Pharmaceutical, Tokyo, Japan). The large T-antigen is rapidly degraded at nonpermissive temperatures (≥39°C) but functions at the permissive temperature (33°C) [5].

The murine fibroblast cell line L929 was from Dr M. Hayashi (Kitasato University, Tokyo, Japan). Both GSM06 and L929 cells were cultured in Dulbecco’s modified minimum essential medium (DMEM)/Ham’s F-12 medium (Sigma Chemical Co., St. Louis, MO) containing 1% SITE (10 mg/ml insulin, 5.5 mg/ml transferrin, 5 mg/ml selenium and 2 mg/ml ethanolamine) and 10 ng/ml epidermal growth factor (EGF) (Sigma) supplemented with [DMEM/F-12/fetal bovine serum (FBS)] or without (DMEM/F-12) 10% FBS at 37°C in a humidified atmosphere of 5% CO$_2$ in air.

**Radiolabeling and preparation of macromolecules**

Cells were cultured at 39°C for 2 weeks under the various conditions listed in Table 1, and cultured further for 48 hours in the presence of 92.5 kBq/ml D-[1,6-3H]-glucosamine (814 GBq/mmol; Dupont-New England Nuclear, Boston, MA). The 3H-labeled macromolecules of GSM06 were extracted with 6 mol/l guanidine hydrochloride (GuHCl), pH 7.4, containing 2% Triton X-100. The extracts were dialyzed against water at 4°C, and the nondialyzable fraction was lyophilized. The lyophilizate was dissolved in

**Culture of GSM06 cells using the air-liquid interface (ALI) method**

GSM06 cells were cultured using an air-liquid interface (ALI) method [8]. Briefly, 200 µl of type-I collagen gel solution (Nitta Gelatin, Osaka, Japan) with or without L929 cells (1 × 10⁵ cells/ml) was transferred to a 8-mm inner cup with a nitrocellulose bottom (Intercell®; Kurabo, Osaka, Japan) placed in the well of a 24-well culture plate and incubated at 37°C in a humidified atmosphere for 20 minutes to solidify the gel. Then 400 µl of GSM06 cell suspension (1 × 10⁶ cells/ml) in DMEM/F-12/FBS was added to the collagen gel in the inner well, and cultured for 3 days until the cells became confluent at 37°C in a humidified atmosphere of 5% CO$_2$ in air. The medium in the inner cup was removed by decantation, and the cells washed three times with 400 µl of phosphate-buffered saline (PBS) and exposed to air in a humidified atmosphere supplemented with 5% CO$_2$. The culture medium with or without retinol acetate (Sigma) and/or FBS was then applied to the outer well. Incubation was carried out over a period of 2 weeks at 39°C in a humidified atmosphere of 5% CO$_2$, and the medium in the outer well was changed every 3 days. The culture conditions tested in this study are listed in Table 1.

**Table 1**

<table>
<thead>
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<th>Culture conditions</th>
<th>L929 cells*</th>
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Basal medium (Dulbecco’s modified minimal essential medium/F-12) containing 1% SITE (10 mg/ml insulin, 5.5 mg/ml transferrin, 5 mg/ml selenium and 2 mg/ml ethanolamine) and 10 ng/ml murine epidermal growth factor was tested.

* L929 cells were suspended in a collagen gel (1 × 10⁵ cells/ml) before solidification.
† Fetal bovine serum was 10% in the culture medium.
‡ Retinol acetate was 1 µmol/l in the culture medium.

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1 ml of 4 mol/l GuHCl, pH 7.4, containing 0.5% Triton X-100, and the insoluble materials were removed by centrifugation (10,000 x g for 10 minutes). The resulting supernatant was applied to a column (3 x 40 cm) of Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) which had been previously equilibrated with 4 mol/l GuHCl, pH 7.4, containing 0.5% Triton X-100, and eluted with the same buffer. The radioactivity of each fraction (2.25 ml/tube) was measured, and 3H-labeled macromolecules were obtained as excluded fractions.

**Component sugar analysis**

Total hexose contents of the macromolecules were determined by the phenol-sulfuric acid method using glucose as the standard [9]. Component sugars of the macromolecular fractions were hydrolyzed with 4 mol/l trifluoroacetic acid at 100°C for 4 hours [10]. Neutral sugars and amino sugars in the hydrolysate were converted into corresponding alditol acetates [11,12]. Gas liquid chromatography (GC) and electron impact mass spectrometry (EIMS) were performed on a Hewlett-Packard model 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) on a DB-1 capillary column (0.2 µm film thickness, 0.25 mm i.d. x 30 m; Agilent, Wilmington, DE) equipped with an HP-5970B mass selective detector (ionizing voltage 70 eV; Hewlett-Packard) by splitless mode. The carrier gas was He (0.9 ml/minute). Sugar derivatives were analyzed by total ion monitoring and selective ion monitoring techniques. The temperature programs were 60°C for 1 minute, 60→150°C (30°C/minute), 150→220°C (3°C/minute), and 220°C for 5 minutes. Injector and detector temperatures were set at 250°C. Alditol acetate derivatives were identified by their relative retention times and fragment ions by gas liquid chromatography–mass spectrometry (GC–MS) using known sugar derivatives as reference standards.

**Lectin blot analysis**

A polyvinylidene difluoride membrane (Hybond™-P; Amersham, Buckinghamshire, UK) was assembled in the slot blot apparatus connected to a water suction vacuum, and the macromolecules (100 ng as hexose) were blotted onto the membrane. After blocking in 0.1 mol/l Tris-HCl, pH 7.5, and 0.15 mol/l NaCl containing 1% bovine serum albumin (BSA) (TBS-BSA) overnight at 4°C, the membranes were incubated with the biotinyl lectin in TBS-BSA, followed by incubation with a horseradish peroxidase-conjugated streptavidin in TBS-BSA. The enzyme activity was detected by an enhanced-chemiluminescence method. The membrane was then washed, incubated with chemiluminescence detection reagents (Perkin-Elmer Life Sciences, Boston, MA), and exposed to Hyperfilm™ECL™ film (Amersham).

**Bacterial strain, media and growth conditions**

*H. pylori* SS1 was provided by Dr A. Kai (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan). SS1 is a mouse-adapted strain originally isolated from a patient with peptic ulcer disease [13]. All the experiments except study of adherence of *H. pylori* to GSM cells were performed using *H. pylori* SS1. *H. pylori* NCTC11637 was stock culture from the Institute of Medical Science, University of Tokyo. *H. pylori* RC-1 was isolated and established from a human gastric biopsy specimen obtained from the Kitasato Institute Hospital, Tokyo, Japan. RC-1 is a cagA- and vacA-positive strain.
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characterized by PCR (data not shown). The bacteria were grown on brain heart infusion (BHI; Difco Laboratories, Detroit, MI) agar plates supplemented with 5% defibrinated horse blood, 1 mg/ml glucose, 250 µg/ml BSA, and 1 vial/l skimrow supplement (Oxoid, Basingstoke, UK), and were incubated for 4 days in a microaerobic gas environment (15% CO₂, 5% O₂ and 80% N₂). The colonies were harvested from the plates, suspended in 20 ml BHI broth containing 10% FBS and 1 mg/ml glucose, and incubated at 37°C overnight with agitation on a rotary shaker at 100 r.p.m. in microaerobic gas conditions. After the culturing, 2 ml of the culture broth was transferred to 200 ml of the same medium, and incubated further for 48 hours at 37°C. The cultures were checked by phase contrast microscopy to ensure their purity, and were centrifuged at 10,000 × g for 10 minutes. The resulting H. pylori cells were resuspended in DMEM/F-12. Preliminary experiments showed that a suspension of cells yielding an optical density at 600 nm of 0.25 corresponded to 1 × 10⁸ colony-forming units (CFU)/ml of living bacteria.

Transmission electron microscopy

Ruthenium red en bloc staining was performed by a prescribed method [14]. Briefly, GSM06 cells cultured in the absence of FBS were fixed with the mixed solution of 4% formaldehyde and 1% glutaraldehyde for 12 hours. After exchanging the fixative for cacodylate buffer (pH 7.2), the sample was treated with Ruthenium red by an en bloc staining method [14]. Then, the samples were treated with graded concentrations of ethanol and QY-1® (NEM, Tokyo, Japan), and embedded in Epon. Staining with osmic acid and uranyl acetate was as follows. GSM06 cells cultured in the absence of FBS were incubated with 100 µl of the suspension of H. pylori (4 × 10⁸ CFU/ml) in DMEM/F-12 for 24 hours. The cells were fixed with Zamboni’s solution [18] for 24 hours at 4°C. Four-µm-thickness cryostat sections were mounted on glass slides and incubated for 12 hours with murine monoclonal anti-CagA antibody (clone 2I2/G10; Austral Biologicals, San Ramon, CA). After washing with PBS, sections were incubated with Alexa Fluor 488-labeled antimouse IgG (Molecular Probes, Eugene, OR) for 3 hours, and counterstained with Alexa Fluor 594-labeled phallolidin (Molecular Probes) and 4′,6 diamidino-2-phenylindole (DAPI) (Sigma) for 30 minutes. The resulting sections were washed with PBS, and observed with a fluorescence microscope.

Fluorescence microscopy

GSM06 cells cultured in the absence of FBS were incubated with 100 µl of the suspension of H. pylori (4 × 10⁸ CFU/ml) in DMEM/F-12 for 24 hours. The cells were fixed with Zamboni’s solution [18] for 24 hours at 4°C. Four-µm-thickness cryostat sections were mounted on glass slides and incubated for 12 hours with murine monoclonal anti-CagA antibody (clone 2I2/G10; Austral Biologicals, San Ramon, CA). After washing with PBS, sections were incubated with Alexa Fluor 488-labeled antimouse IgG (Molecular Probes, Eugene, OR) for 3 hours, and counterstained with Alexa Fluor 594-labeled phallolidin (Molecular Probes) and 4′,6 diamidino-2-phenylindole (DAPI) (Sigma) for 30 minutes. The resulting sections were washed with PBS, and observed with a fluorescence microscope.

Adherence of H. pylori to GSM06 cells

GSM06 cells cultured in the absence of FBS on a collagen gel containing L929 cells were incubated with 100 µl of the suspension of H. pylori strains SS-1, NCTC11637 and RC-1 (4 × 10⁷ CFU/ml) in DMEM/F-12. After incubation for 24 hours at 39°C in a humidified atmosphere of 5% CO₂ in air, the suspension of H. pylori was discarded by decantation, and the surfaces of GSM06 cells were washed three times with PBS containing 0.01% gelatin. The resulting GSM cells were homogenized using a mortar under sterile conditions, and the number of
adherent bacterial CFUs of *H. pylori* were enumerated by diluting and plating using triplicate sampling of each infected well.

**Results**

*Reactivity of lectins to macromolecules secreted from GSM06 cells*

The various conditions in which GSM06 cells pulse-labeled with D-[1,6-3H]-glucosamine were cultured are shown in Table 1. The macromolecules which incorporated radioactivity under the various conditions were separately chromatographed on a Sepharose CL-4B column and the excluded fractions eluted and collected. Because all mucins are eluted in the excluded fractions, the carbohydrate moieties associated with the macromolecules can be analyzed by lectin-specific blotting techniques using lectins, soybean agglutinin (SBA), wheat germ agglutinin (WGA), and ulex europaeus agglutinin I (UEA-I). The excluded fraction from GSM06 cells that were cultured in FBS-free medium on collagen gels containing L929 cells showed the highest reactivity towards the *N*-acetylgalactosamine (GalNAc) binding lectin, SBA (Fig. 1A). Significant decreases in reactivities to SBA were observed in macromolecules secreted by cells supplemented with FBS or retinol or by removing L929 cells from the collagen gel (Fig. 1A, conditions 4, 5, and 6). Alpha-fucose (α-Fuc) binding lectin, UEA-I, showed similar reactivity to SBA (Fig. 1B). In contrast to SBA and UEA-I, the *N*-acetylglucosamine (GlcNAc) binding lectin, WGA, reacted strongly with macromolecules secreted by GSM06 cells in a medium supplemented with FBS on collagen gels containing L929 cells (Fig. 1C). Because GalNAc is a characteristic saccharide for *O*-glycosylated glycoproteins such as mucin, these results suggest that GSM06 cells cultured in FBS-free medium on collagen gels containing L929 cells produce significant amounts of mucin.

*Component sugar analysis*

The Sepharose CL-4B excluded macromolecular fractions from GSM06 cells cultured on collagen gels containing L929 cells in the presence or absence of FBS supplementation were separately hydrolyzed and component sugars analyzed by GC-MS. GSM06 cells in the presence or absence of FBS both produced macromolecules containing GlcNAc, as assessed by the components yielding peaks at m/z of 318 and a retention time of 30 minutes using standard GlcNAc, GalNAc, and neutral sugars (Fig. 2B and G). However, GalNAc (m/z 318 and retention time 31 minutes) appeared only in the hydrolysate of macromolecules derived from GSM06 cells not supplemented with FBS. These results correlate well with those of the lectin-binding experiments. Moreover, under both conditions (with or without FBS supplementation), component peaks at m/z of 170 and 115 with retention times of 26 minutes, typical of a hexose and

**Figure 1** Lectin blot analyses of macromolecules from GSM06 cells. GSM06 cells were cultured in DMEM/F-12 medium supplemented (2, 4 and 6 in Table 1) or not supplemented (1, 3 and 5 in Table 1) with 10% FBS, with (5 and 6 in Table 1) or without (1, 2, 3 and 4 in Table 1) 1 µmol/l retinol, on collagen gel with (3, 4, 5 and 6 in Table 1) or without (1 and 2 in Table 1) L929 cells for 2 weeks under 95% air, 5% CO₂ at 39°C, and then cultured for 2 days at 39°C. The fraction excluded by Sepharose CL-4B column was blotted onto a polyvinylidene difluoride (PVDF) membrane and analyzed by the lectin blotting procedure. The blotting image was analyzed by National Institutes of Health image software.
6-deoxyhexose, were detected and characterized as galactose using neutral sugar standards (Fig. 2B and G).

**Expression of the MUC5AC gene**

Gastric mucin, which is produced by surface mucous cells, contains MUC5AC as a core protein. GSM06 cells in FBS-free medium on collagen gel containing L929 cells were cultured for 14 days and the expression of the MUC5AC gene analyzed by RT-PCR. There were no detectable levels of MUC5AC expression until day 7, and significant expression of the MUC5AC gene was observed on day 14 (Fig. 3). These results suggest that there is a significant lag phase in the expression of the MUC5AC gene by GSM06 cells.

**Morphological analysis of cultured GSM06 cells**

Electron microscopic examination revealed the existence of an exocytosis and an accumulated mucous granule near the plasma membrane (Fig. 4). Exocytosis is characteristic of mucus-secreting cells. Thus, GSM06 cells cultured in
medium lacking FBS on collagen gel containing L929 cells were highly differentiated.

**Morphological analysis of H. pylori infection of GSM06 cells**

When GSM06 cells cultured in the absence of FBS were infected with *H. pylori*, adhesion of *H. pylori* cells to GSM06 cells was observed by transmission electron microscopy (TEM) (Fig. 5A). This observation was substantiated by the formation of a filamentous structure between the surfaces of GSM06 cells and *H. pylori* cells (Fig. 5B and C). In addition, the filamentous structure was immunostained with anti-Hp0532 (VirB7) antibody (Fig 5D and E), and coincided with a type IV secretory apparatus previously reported [16,19].

**Fluorescence microscopic analysis of GSM06 cells infected with H. pylori**

*H. pylori* was found adjacent to gastric surface mucous cells when GSM06 cells, which were cultured in the absence of FBS, were infected with *H. pylori*. To determine whether CagA of *H. pylori* was injected into the cytoplasm of GSM06 cells, fluorescent immunostaining with...
A novel in vitro infection model of *H. pylori*

anti-CagA antibodies was performed, and positive staining was observed in the cytoplasm (Fig. 6).

**Adherence of *H. pylori* to GSM06 cells**

When GSM06 cells cultured in the presence and absence of FBS were infected with different *H. pylori* strains, such as SS-1, NCTC11637 and RC-1, the CFUs of all the *H. pylori* cells that adhered to GSM06 cells were much higher for cells cultured in the absence of FBS than for those cultured in the presence of FBS (Fig. 7).

**Discussion**

This study describes the conditions for culturing gastric surface mucin-producing GSM06 cells and an in vitro model that closely resembles in vivo conditions leading to infection by *H. pylori*. Previous studies have shown that hamster tracheal epithelial cells both differentiate and produce mucin when cultured in a serum-free medium [20]. In this study, efforts were focused on culturing GSM06 cells in a serum-free medium on a collagen gel containing L929 cells.
Under these conditions, GSM06 cells produced mucin containing GalNAc as a carbohydrate moiety and expressed MUC5AC as the core protein. SBA and WGA bind both gastric surface mucous cells and mucous neck cells, whereas UEA-1 preferentially binds gastric surface mucous cells in mice [21]. Because the mucin from GSM06 cells cultured in the absence of FBS strongly reacted with SBA and UEA-1, it was suggested to be gastric surface mucous cell type mucin, not gland mucous or mucous neck cell type mucin.

The addition of FBS to the culture medium significantly reduces the production of mucin. The mechanism by which mucin production is suppressed in the presence of FBS is unknown. The effects of L929 cells and retinol on mucin production in GSM06 cells are also unknown. Studies showed that fibroblasts embedded in a collagen gel induced cell differentiation [22] with production of fibronectin [23]. Fibronectin increases the number of epidermal growth factor receptors (EGF-R) in mouse mammary epithelial cells [24]. Because human EGF induced both in vitro synthesis and secretion of neutral mucin in a dose-dependent manner in rabbit fundal mucosa cells [25], EGF was added to the culture medium in this study. EGF may be a crucial factor for signaling both differentiation and mucin production in GSM06 cells. In addition, EGF interaction with fibronectin from L929 cells may enhance the cellular signal of EGF with upregulation of EGF receptors in GSM06 cells. Retinol, widely known as an inducer of cell differentiation, significantly reduced the production of mucin in GSM06 cells. The reduction of mucin production in this study may be associated with the retinol suppression of EGF-associated signal cascades [26].

Fibroblasts are known to secrete various cytokines [27]. Therefore, in addition to fibronectin, other cytokines derived from the fibroblasts of L929 cells may contribute to stimulating mucin production under the culture conditions used in this study. An assumption in this study is that the observed differentiation of GSM06 cells is induced by the interactions of fibronectin and/or other cytokines derived from L929 cells with concomitant increases in mucin production. The comparatively long culturing times (greater than 7 days) observed for the expression of the MUC5AC gene in GSM06 cells may be associated with the induction of fibronectin and/or cytokine synthesis and secretion in differentiated L929 cells. Further investigations are needed to determine whether fibronectin and/or cytokines are produced by L929 cells embedded in a collagen gel and whether these substances stimulate processes within GSM06 cells.

When GSM06 cells were infected with H. pylori, a filamentous structure was formed between the cell surfaces of the host and bacterium. Previous studies have shown that H. pylori expresses the subunits of a type IV secretory apparatus coded by the homologous sequence in the cag pathogenic island, which is analogous to the sequence coding a type IV secretory apparatus in A. tumefaciens [17]. In this study, the CagA protein was detected in the cytoplasm of GSM06 cells infected with H. pylori. Assuming that the CagA protein was delivered via a type IV secretory apparatus following H. pylori infection, the filamentous structure observed by transmission electron microscopy suggested the presence of such a secretory apparatus.

The number of bacterial CFUs that adhered to GSM06 cells cultured under serum-free medium conditions (mucin-producing conditions) was significantly greater than that found in conditions using FBS supplementation (non-mucin-producing conditions). Adherence of H. pylori to GSM06 cells under mucin-producing conditions

![Figure 7](image_url)
was commonly observed among the three tested *H. pylori* strains, including a clinical isolate. Whether or not *H. pylori* adheres to gastric surface cells is probably determined by differences in the microenvironments of the cell surfaces. Studies have shown that *H. pylori* recognizes various cell surface molecules such as Leb [28], sialic acid [29], and sulfatide [30]. However, the details of the cell surface interactions between *H. pylori* and gastric mucosal cells remain unknown.

It has been reported that many *H. pylori* strains could not colonize the mouse stomach [13]. In the present study, NCTC11637, which poorly colonizes in the mouse [31], adhered to the GSM06 cells cultured under mucin-producing conditions at the same level as the mouse-colonizing strain (SS-1). This infection model may therefore be useful for investigation using many *H. pylori* strains. However, the differences in the nature of *H. pylori* adherence to several host cells, such as GSM06 cells, human cancer cells and human normal cells, are still not characterized. Further investigation is necessary.

The effect of expression of large T-antigen in GSM06 cells on *H. pylori* is currently not known. Therefore, to minimize possible effects of the large T-antigen, infection with *H. pylori* was conducted at 39°C. It should be emphasized that thermoregulation plays an important role in virulent gene expression in pathogenic bacteria including *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia* spp. Most of the genetic make-up of *H. pylori*, as revealed by its genomic sequence, shows an absence of thermoregulatory gene homologies that are otherwise present in certain gastrointestinal bacteria [32,33]. It has been reported that expressions of some virulence genes in *H. pylori* are increased by incubation at 42°C [34]. Therefore, further studies are warranted to determine whether genes of *H. pylori* are thermoregulated.

The filamentous structures observed between the surfaces of GSM06 cells and *H. pylori* cells were suggestive of a type IV secretion system apparatus, and the secretion of the CagA effector protein in the cytoplasm of GSM06 cells provided the basis for an in vitro infection model. Although the expression of both the type IV secretion system and the CagA protein was not affected by incubation at 39°C, this does not exclude the possibility that other virulence factors may be expressed at the lower temperature of 37°C. Therefore, a comparative study on the expression of virulence factors at different temperatures is necessary.

This study establishes the first *H. pylori* infection model using the normal gastric mucosal cell line GSM06. This cell line produced mucin with GalNAc as a sugar moiety and MUC5AC as the core protein. The results of this study provide the first step in the development of a useful in vitro experimental model to evaluate the complex mechanisms involving the interactions between a gastric host cell and *H. pylori* during the early stages of infection. Moreover, the model system described in this study may facilitate the development and screening of novel therapeutic agents for *H. pylori* infections.

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