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Gastric Mucus Alterations Associated With Murine *Helicobacter* Infection

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SUMMARY The C57BL/6 mouse has been shown to develop gastric adenocarcinoma after *Helicobacter felis* infection. This model was used to determine whether mucin and trefoil factor (TFF) expression after infection was altered in a similar fashion to the changes seen in the protective gastric mucus layer of the human stomach after *H. pylori* infection. Our results indicate that this mouse model mimics many of the changes seen after human *H. pylori* infection, including increased expression of *muc4* and *muc5b* and loss of *muc5ac*. These alterations in mucin expression occurred as early as 4 weeks postinfection, before the development of significant mucous metaplasia or gastric dysplasia. The decrease in *muc5ac* expression occurred only in the body of the stomach and was not secondary to the adaptive immune response to infection, because a similar decrease in expression was seen after infection of B6.Rag-1^{-/-} mice, which lack B and T cells. Intriguingly, the increased expression of *Muc4* and *Muc5b* in infected C57BL/6 mice was not seen in the infected B6.Rag-1^{-/-} mice. Because B6.Rag-1^{-/-} mice do not develop gastric pathology after *H. felis* infection, these findings point to the potential role of *Muc4* and *Muc5b* in disease progression. This manuscript contains online supplemental material at <http://www.jhc.org>. Please visit this article online to view these materials. (J Histochem Cytochem 57:457–467, 2009)

KEY WORDS

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GASTRIC ADENOCARCINOMA is the second leading cause of cancer death worldwide and is associated with *Helicobacter pylori* infection (Vanagunas 1998; Parkin et al. 2005). Based on the Laurén system, there are two types of gastric adenocarcinoma: diffuse and intestinal (Lauren 1965). The diffuse type is characterized by a poorly differentiated epithelium and has transmural invasion with lymphatic spread. It is more commonly seen in younger people and affects women more than men. The intestinal type is more common with increasing age and in men. It is associated with environmental exposures, including *H. pylori* infection and diets high in nitrates and has been proposed to develop in a stepwise progression from normal gastric epithelium to gastritis, atrophy, intestinal metaplasia, dysplasia, and

finally adenocarcinoma (Correa 1988). The initial gastritis consists of both active (neutrophilic) and chronic (monocytic and lymphocytic) inflammation in the stomach, which is triggered by the gastric *Helicobacter* infection. This is often followed by gastric atrophy, where the specialized cells, such as parietal and zymogenic cells, are lost resulting in an increased pH in the stomach. In a small number of susceptible patients, this is followed by intestinal metaplasia, which is defined by alterations in mucin carbohydrate modifications and mucin expression, including increased expression of MUC2 (Reis et al. 1999). The final stage before adenocarcinoma development is dysplasia, which is marked by abnormal growth or development of cells. Early gastric adenocarcinomas are confined to the gastric mucosa and submucosa, whereas advanced cancers extend into the muscularis (Fox et al. 1993; Leung and Sung 2002). The C57BL/6 mouse model of disease uses the closely related gastric *Helicobacter*, *H. felis*, to initiate gastritis and subsequent gastric pathology (Lee et al. 1990; Mohammadi et al. 1996; McCracken et al. 2005). Cai et al. (2005) have reported that, in C57BL/6 mice in-

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ected for 15 months, 100% of infected mice have progressed to gastric adenocarcinoma.

One of the hallmarks of human gastric adenocarcinoma is the alterations in the types of mucins expressed (Correa 1988). Mucus, a gel-like substance that covers the mammalian epithelial surfaces of tissues, is composed of mucin glycoproteins and trefoil factors (TFFs) (Kaneko et al. 2003; Chen et al. 2004). Mucus acts as both a lubricant and as a protective barrier between the contents of the stomach and the mucosal epithelial surface (Shirazi et al. 2000). In humans, 21 mucins have been identified in tissues such as lung, nose, salivary glands, and gastrointestinal tract (Chen et al. 2004; Higuchi et al. 2004). Seven mucins have been identified in mice (muc1, 2, 3, 4, 5a, 5b, and 6) (Spicer et al. 1991; Shekels et al. 1995; van Klinken et al. 1999; Machado et al. 2000; Chen et al. 2001; Desseyn et al. 2002; Shekels and Ho 2003). The murine muc3 is the ortholog of human MUC17, whereas all other murine mucins are numbered similar to their human orthologs (Moniaux et al. 2006; Malmberg et al. 2008). Mucins consist of a protein backbone with tandem repeats of serine, threonine, and proline. The mucins are heavily glycosylated and are thought to play a role in the bacterial colonization of the gastric mucosa (de Bolos et al. 2001). There are two types of mucins: membrane bound (muc1, 3, and 4) and secreted (muc2, 5a, 5b, and 6) (Ringel and Lohr 2003; Kawakubo et al. 2004). The secreted mucins are conserved between the human and mouse forms (Escande et al. 2004). In normal human gastric mucosa, MUC1 and MUC5AC are expressed in the superficial epithelium, whereas MUC6 is localized to the deep glands and the mucus neck cells. MUC2, MUC4, and MUC5B are not normally expressed in the human gastric mucosa (Ho et al. 1995; Pinto-de-Sousa et al. 2004; Babu et al. 2006).

In humans and mice, there are three TFFs: TFF1/pS2, TFF2/spasmolytic polypeptide (SP), and TFF3/intestinal trefoil factor (ITF). Trefoil factors are small, soluble peptides with trefoil or P domains. The trefoil domains are made up of six cysteine residues (Hoffmann and Hauser 1993; Katoh 2003). TFFs are secreted from the granules in the mucus-secreting cells and are believed to act as scaffolding for the mucins within the stomach, with specific TFFs cross-linking with mucins to help form the gel layer in the stomach (Shirazi et al. 2000; Clyne et al. 2004). TFF1 is normally found in the superficial cells of the body and antral mucosa of the stomach, whereas TFF2 is found in the mucus neck cells of the body and antral glands in the stomach. TFF3 is normally not expressed in the stomach but is expressed in the intestine and the salivary glands (Wong et al. 1999). Previous studies in humans show that TFF1 interacts with MUC5AC, TFF2 interacts with MUC6, and TFF3 interacts with MUC2 (Clyne et al. 2004; Ruchaud-Sparagano et al. 2004; Thim and May 2005).

Changes have been seen in the expression of mucins and TFFs in gastric adenocarcinoma. MUC1 has been shown to be expressed early in the infection process but is decreased during the metaplastic stage (Wang and Fang 2003). MUC2, MUC4, MUC5B, and TFF3 are not expressed in the normal human stomach but are expressed in gastric adenocarcinoma biopsies (Ho et al. 1995; Wang and Fang 2003; Pinto-de-Sousa et al. 2004; Roessler et al. 2005; Babu et al. 2006). This contrasts with MUC5AC, which is expressed in a normal stomach but not in gastric adenocarcinoma (Ho et al. 1995; Wang and Fang 2003; Pinto-de-Sousa et al. 2004; Dhar et al. 2005; Marques et al. 2005; Roessler et al. 2005; Babu et al. 2006). MUC6 is expressed at high levels in a normal human stomach in the mucus neck cells and the antrum but is absent in gastric epithelium altered by gastric cancer (Ho et al. 1995; Pinto-de-Sousa et al. 2004). TFF1 has been shown to be lost in 50% of gastric carcinomas (Muller and Borchard 1993; Wong et al. 1999). TFF2 expression was detected by IHC in human stomach biopsies showing gastritis and atrophy but not during intestinal metaplasia and gastric carcinoma (Hu et al. 2003; Dhar et al. 2005). TFF3 is found in the stomach as it progresses through the intestinal metaplasia stage and is conserved in gastric cancer (Taupin et al. 2001). In the aforementioned studies, MUC1, MUC2, and MUC6 expression was characterized by both RNA expression and IHC staining. MUC4 expression was characterized by RNA. MUC5AC, MUC5B, TFF1, TFF2, and TFF3 were characterized only by IHC.

H. pylori has at least two surface adhesion proteins, BabA and SabA, which facilitate bacterial binding to gastric mucus (Linden et al. 2008b). Previous studies have shown that *H. pylori* BabA will bind to only MUC5AC under the low pH conditions of a normal stomach, whereas under neutral pH conditions, BabA⁺ strains can bind both MUC5AC and MUC1 (Linden et al. 2004). The importance of mucin expression in *Helicobacter* infection has been shown through the infection of *muc1*^{-/-} mice (McGuckin et al. 2007). *H. pylori* adhered tighter to *muc1*^{-/-} gastric epithelial cells and induced a more severe gastritis, leading to the hypothesis that the normally secreted muc1 might bind *H. pylori* and serve as a "decoy" to keep the bacteria from directly interacting with the gastric epithelial cell.

One previous study investigated the mucin alterations in Rhesus monkeys after *H. pylori* infection. There was an overall loss of mucosal fucosylation and an increase in mucosal sialylation; however, individual mucin or TFF expression was not evaluated (Linden et al. 2008a). As we previously showed, the *H. felis*-infected C57BL/6 mouse is an excellent model of the effects of adaptive immunity on the development of gastric pathology (Roth et al. 1999; McCracken et al. 2005). Therefore, we initiated experiments to determine how this

infection and the subsequent immune response alter the protective gastric mucus layer. Previous studies concentrated on the expression of TFFs, specifically *tff1* and *tff2*, in the mouse model. Spasmolytic polypeptide (TFF2)-expressing metaplasia (SPEM) is observed in mucosa adjacent to human gastric cancer and in fundic glands showing oxyntic atrophy in *H. felis*-infected mice (Nomura et al. 2004; Kurt-Jones et al. 2007). However, there has been no comprehensive analysis of all the murine mucins and TFFs over the course of the disease. We studied the alterations in gastric mucins and TFFs as the murine disease progresses from gastritis through dysplasia and metaplasia to gastric carcinoma by both IHC and RNA expression.

Materials and Methods

Murine Model of *H. felis* Infection

C57BL/6 (B6) and C57BL/6J-*Rag-1^{tm1Mom}* (B6.*Rag-1^{-/-}*) strains were originally purchased from The Jackson Laboratory (Bar Harbor, MA) and were bred and maintained under specific pathogen-free (SPF) conditions. SPF conditions at University of Alabama at Birmingham (UAB) include absence of the following organisms, as determined by serological screening: mouse parvoviruses, including MPV-1, MPV-2, and minute virus of mice; mouse hepatitis virus; murine norovirus; Theiler's murine encephalomyelitis virus; mouse rotavirus (epizootic diarrhea of infant mice); Sendai virus; pneumonia virus of mice; reovirus; *Mycoplasma pulmonis*; lymphocytic choriomeningitis virus; mouse adenovirus; ectromelia (mousepox) virus; K polyoma virus; and mouse polyoma virus. Testing and other methods were as described at <http://main.uab.edu/Sites/ComparativePathology/surveillance/>.

These mice were between 6 and 10 weeks of age at the time of initial infection and were fed autoclaved rodent chow (NIH-31; Harlan Teklad, Madison, WI) and water ad libitum and maintained on a 12:12-hr light-dark schedule. Animal procedures and protocols were conducted in accordance with the Institution Animal Care and Use Committee at the UAB (Birmingham, AL). For each point postinfection, mice were mock-infected or infected with *H. felis* as described previously (Roth et al. 1999). Briefly, the mice were infected by oral gavage three times over a 7-day period with 5×10^7 CFU *H. felis* (ATCC 49179) suspended in 1:1 brain heart infusion broth (BHI)/glycerol. One OD₄₅₀ corresponds to 10^9 bacteria. This results in a ~100% infection efficiency in our laboratory (based on *Helicobacter* colonization scores; see below, data not shown). The mock-infected mice were inoculated with the sterile BHI/glycerol mixture without the bacteria. Mice were euthanized at multiple times postinfection (4–52 weeks). Three separate infections were used to generate all of the data shown.

Tissue Preparation

The mice were euthanized using isoflurane inhalation followed by cervical dislocation. The stomachs were rapidly removed and divided longitudinally along the greater and lesser curvature, with one half quick frozen in liquid nitrogen for total RNA isolation and the other half immersion fixed in Carnoy's solution (6:3:1 of 100% ethanol, chloroform, and glacial acetic acid) for histological and immunofluorescence analysis. The tissue was fixed for 4 hr at 4°C and changed to an ethanol wash for 18–24 hr before paraffin embedding. Five- μ m sections were cut on a microtome and attached to pre-cleaned microscope slides (Snowcoat X-tra; Surgipath, Richmond, IL).

Total RNA was isolated by the phenol and guanidine isothiocyanate method using Trizol (Invitrogen; Carlsbad, CA) (Chomczynski and Sacchi 1987). Genomic DNA was removed from the extracted total RNA using the Turbo DNase kit (Ambion; Austin, TX). cDNA was made with equal amounts of mRNA (2 μ g), using the Transcriptor First Strand cDNA Synthesis Kit (Roche; Pensberg, Germany). Quantitative real-time RT-PCR (qRT-PCR) was performed on the samples using Applied Biosystems Assays-On-Demand primer/probe sets and TaqMan Universal PCR Mix (PE Applied Biosystems; Foster City, CA) (Supplemental Table ST1). The samples were analyzed on the Stratagene MX3000P real-time PCR Machine (Cedar Creek, TX). The fold change was determined as described in the Applied Biosystems manufacturer's instructions (4371095 Rev A; PE Applied Biosystems). Briefly, the average crossing threshold (Ct) of the target genes for each group minus the average housekeeping gene (18S rRNA) Ct was used to determine the relative expression (Δ Ct). The 18S housekeeping gene was used as the housekeeping gene in this study because published results indicate it is expressed with relatively stability under inflammatory conditions (Bas et al. 2004; Ropenga et al. 2004; Rubie et al. 2005). The average Δ Ct of the experimental animals (*Helicobacter* infected) was subtracted from the average control (mock-infected) Δ Ct to determine the $\Delta\Delta$ Ct. The $\Delta\Delta$ Ct was used in the formula $2^{\Delta\Delta Ct}$ to determine the fold change in mRNA expression. The upper and lower limits of fold change were determined by taking the averaged SDs of each experimental group through the above calculations (Heid et al. 1996; Bas et al. 2004).

Histological and Immunofluorescent Scoring Systems

All histological and immunofluorescent scores were determined by an observer blinded to the experimental groups. One well-oriented gastric tissue section from each mouse was stained with hematoxylin–eosin for histological analysis. The scoring system was a 0–9 scale (0, no inflammation or epithelial changes; 9, severe in-

flammation and extensive epithelial abnormalities) with subscores of 0–3 in each of the following three areas: longitudinal extent of inflammation, vertical extent of inflammation, and histological changes (Roth et al. 1999). The scores were averaged for each animal and graphed individually with the horizontal line indicating the median. Periodic acid-Schiff (PAS) staining was performed on the sections by the UAB Comparative Pathology Laboratory.

Gastric tissue sections from each mouse were stained with the rabbit polyclonal anti-*H. pylori* antibody, which cross-reacts with *H. felis* (unpublished observations; SIG-3431; Convance, Emeryville, CA). Briefly, sections are deparaffinized by successive immersions in Citrisolv (Fisher Scientific; Pittsburgh, PA) and isopropanol, and rehydrated with PBS. The tissues were pretreated with 0.25% pepsin in PBS for 10 min at room temperature and blocked with PBS-blocking buffer (PBS-BB; 1% BSA and 0.3% Triton) for 15 min to block nonspecific binding and to increase antibody access to cell surface and internal antigens. The slides were incubated with undiluted anti-*Helicobacter* antibody for 1 hr at room temperature, washed in PBS, and incubated with Cy3 donkey anti-rabbit IgG (24 ng/ml final concentration; cat. 711-165-152; Jackson ImmunoResearch, West Grove, PA) for 1 hr. The sections were counterstained with Hoechst dye (1 ng/ml; bisbenzimidazole H 33258, cat. B2883; Sigma, St. Louis, MO) to visualize nuclei. Colonization was scored in a semiquantitative system, with a range of 0–4 (0, no bacteria per crypt; 1, 1–2 bacteria per crypt; 2, 3–10 bacteria per crypt; 3, 11–20 bacteria per crypt; 4, >20 bacteria per crypt). Each individual mouse colonization score is graphed.

Mucin Immunofluorescence

All slides were deparaffinized, and those stained for muc3 or muc4 were taken through sequential 15-min avidin and biotin blocks (cat. SP-2001; Vector Laboratories, Burlingame, CA) with a PBS wash in between. All stains needed a 15-min blocking step with PBS-BB after which the slides were incubated overnight with one of the following antibodies: rabbit polyclonal anti-muc1 (2 µg/ml final concentration; cat. 15481; Abcam, Cambridge, MA), chicken anti-mouse muc3 (1:200 dilution, HO29; Dr. Samuel Ho, San Diego, CA), or rabbit anti-mouse muc4 (1:200 dilution, HO4-2; Dr. Samuel Ho). HO29 is a chicken polyclonal antibody raised against a recombinant protein corresponding to the extracellular epidermal growth factor 1 domain of the mouse muc3 mucin, as described previously (Ho et al. 2006 and unpublished observations). The Muc4-2 antibody was raised against the extracellular domain of the rodent muc4 mucin (WNDNPEDDFRMPNGST). This antibody recognizes a subgroup of mouse gastric epithelial cells and apical membrane proteins of mouse small intestine and a subgroup of colon goblet cells (unpub-

lished observations). Secondary detection for muc1 used Cy3 donkey anti-rabbit IgG. All other antibodies went through an additional biotin labeling step: biotin rabbit anti-chicken IgG (0.075 µg/ml final concentration; cat. 61-3140; Zymed, San Francisco, CA) for muc3 and biotin donkey anti-rabbit IgG (1.4 µg/ml final concentration; cat. 711-065-152; Jackson ImmunoResearch, West Grove, PA) for muc4. Detection for these antibodies was with Cy3 streptavidin (18 µg/ml final concentration; cat. 016-106-084; Jackson ImmunoResearch), followed by nuclear visualization with Hoechst dye.

Muc5ac Immunofluorescence

Muc5ac expression was evaluated in the gastric epithelium through the use of a “mouse on mouse” protocol (Brown et al. 2004). Briefly, the sections were deparaffinized, rehydrated, and blocked as described above. Mouse monoclonal IgG₁ anti-muc5ac (clone 45M1; 200 µg/ml, cat. MS-145-PO; Thermo Scientific, Fremont, CA) and the Cy3 Fab goat anti-mouse secondary antibody (1.5 mg/ml; cat. 115-167-003; Jackson ImmunoResearch) were incubated together at a 1:2 ratio (w/w) in Triton-free PBS-BB for 40 min at room temperature. Unbound Fab fragments were bound with excess mouse serum for 10 min at room temperature before placed on the slide for 1 hr (1:100 final dilution). Visualization of the nuclei was by Hoechst staining. The slides were scored as described above, with the exception that the scoring was on a 0–3 scale (0, no staining; 3, maximal staining). Each stomach section was scored in both the body and the antrum. Because we were studying the loss of muc5ac expression, the antral staining was used as an internal positive control, because *H. felis* infection in the murine model only alters expression of muc5ac in the body of the mouse stomach (data not shown). Only slides that had well-oriented body and antral tissue were included in the data analysis, and one slide per mouse was analyzed.

Microscopy

All histological and fluorescent photomicrographs were taken using an automated operated Zeiss Axioskop 2 (Thornwood, NY) with an Axiocam HRC camera. The software used was Axiovision Release 4.6.3 (Zeiss). For fluorescent photomicrographs, the exposure settings for the microscope and camera used for the stained mock stomach were recorded, and the same settings were used for all subsequent exposures of *H. felis*-infected stomachs. All photomicrographs shown were taken at the same magnification (within each figure) and are representative of the staining seen in all sections stained.

Graphic and Statistical Analysis

Graphs were made using GraphPad Prism 4 (San Diego, CA). All the qRT-PCR graphs are horizontal with the

y-axis set at 1 to represent the baseline expression of each gene in mock-infected animals for comparison. The mean fold change and the upper and lower limits of the range of the fold change are graphed. Statistics were performed on all data using GraphPad InStat 3 (San Diego, CA) using unpaired *t*-tests for continuous data. For the qRT-PCR statistical analysis, the unpaired *t*-test was performed on the $\Delta\Delta\text{CT}$ and SD. For categorical data, statistics were performed using the Mann Whitney *U* test. $p < 0.05$ was considered significant.

Results

H. felis Infection of C57BL/6 Mice Results in Chronic Active Gastritis and Gastric Adenocarcinoma

To study the timing of progression to gastric adenocarcinoma, female C57BL/6 mice were infected with *H. felis* and sacrificed at multiple time points over a 1-year period (4, 8, 12, 16, 20, 24, and 52 weeks). Over the 1-year time course, gastric inflammation and mu-

cous metaplasia were increased, whereas the number of parietal cells and zymogenic cells were decreased, with maximal histological scores seen 16 weeks into the infection (Figure 1A). These histological changes were paralleled by a subsequent reduction in *Helicobacter* colonization, which is also reported in human disease (Figure 1B) (Karnes et al. 1991; Kikuchi 2002). Figures 1C–1G show the representative histology at each time point (in comparison to a mock control). Figure 1C is a B6 mock-infected stomach, showing evidence of the normal glandular organization in the parietal zone of the murine stomach and no evidence of inflammation. Figure 1D is representative of gastritis in the mouse stomach, when the inflammatory infiltrates are first evident; this is 4 weeks into the infection. The next panel, Figure 1E, is representative of a stomach with gastric atrophy, which occurs 12–16 weeks after the initial infection in our mouse model. At this stage, the parietal and zymogenic cells are lost, whereas significant immune infiltrates are evident. Figure 1F is representa-

Figure 1 Disease progression during *Helicobacter felis* infection in C57BL/6 mice. (A) Histological scores of *H. felis* infected B6 stomachs postinfection. * $p < 0.05$ compared with B6 mock controls. (B) Colonization scores over the course of the infection. * $p < 0.05$ compared with 4-week B6 *H. felis*-infected stomachs. In these two graphs, the horizontal line represents the median score, and the circles represent individual animals and indicate the numbers of animals used in the experiments in this study. Representative hematoxylin-eosin stains of infected stomachs over time are shown in C–G. (C) A mock-infected mouse showing the normal glandular structure of the stomach (histologic score 0). (D) Four weeks postinfection the stomach shows significant gastritis while maintaining normal gastric glandular architecture (histologic score = 4). (E) A stomach infected for 16 weeks shows significant gastric atrophy, whereas a 52-week infected animal shows intestinal metaplasia (histologic scores of 8 and 9, respectively) (F). (G) A 52-week infected stomach that has gastric carcinoma in situ, as indicated by the invasion of the abnormal gastric glands through the muscularis layer (histologic score = 9). Periodic acid-Schiff (PAS) stains of infected stomachs over time are shown in H–L. The mock-infected shows the normal magenta-stained neutral mucus distribution of the stomach (H). These neutral mucins are not significantly altered 4 weeks after infection (I) but are lost as the infection proceeds and dysplasia and gastric adenocarcinoma develop (J–L). Bar = 50 μm .

tive of a stomach with intestinal mucous metaplasia, which usually occurs late in the infection (pictured is 24 weeks postinfection). This is when the stomach mimics an intestinal morphology, with the appearance of goblet-like cells. Figure 1G is representative of the gastric adenocarcinoma seen after *H. felis* infection of B6 mice and shows invasion of the dysplastic glands through the muscularis. In our facility, 58% of our mice progress to gastric adenocarcinoma after 52 weeks of infection. Figures 1H–1L represent the same stomach sections stained by the PAS staining method to allow for visualization of the mucus layer. The mock-infected B6 stomach shown in Figure 1H has a superficial layer of magenta-stained neutral mucins. As the gastric morphology is altered after *H. felis* infection, so is the PAS-positive material, with a loss of the PAS-positive neutral mucin component (Figures 1I–1L).

mRNA Expression of Mucins and tffs Are Altered After *H. felis* Infection

qRT-PCR was performed on total gastric RNA samples taken at multiple times after infection to determine the expression of mucins and tffs. At each time, the mRNA expression level in mock-infected gastric RNA is assigned a fold change of 1. To determine the baseline expression of mucins and tffs over time, the fold change analysis was done on the mocks from the different time points compared with the 4-week mock control. There was no significant change in the expression of mucins or tffs in the mock animals over the duration of the experiment; however, there was a trend toward an increase in mRNA expression of *muc3* and *tff3*, whereas *muc4* and *tff1* expression decreased (data not shown). *H. felis* infection of C57BL/6 mice for 16–52 weeks caused no significant change in the mRNA expression of most mucins, including *muc1*, *muc2*, *muc3*, and *muc6* and all *tffs* (Table 1). However, there was a significant increase in *muc4* and *muc5b* over the course of the infection, even as early as 4 weeks post-

innoculation (Table 1). Surprisingly, the expression of *muc5ac* by qRT-PCR in the total gastric RNA showed either no change or only a minor decrease postinfection (Table 1). Because IHC expression of this mucin has been shown to be almost completely lost in human biopsies, we extended our studies to include immunofluorescent analysis of gastric expression of murine mucins.

Mucin Glycoprotein Expression After *H. felis* Infection

Because the majority of reports studying mucin and TFF alterations in human gastric pathology focus on glycoprotein expression by IHC, we evaluated the expression of selected mucins in our murine model of *Helicobacter*-associated gastric dysplasia and adenocarcinoma. The mucins analyzed were chosen based on the availability of antibodies recognizing the murine mucins. By immunofluorescent analysis, there was no change in the expression of *muc1* or *muc3* at 16 or 52 weeks (Figure 2), a finding consistent with the mRNA expression analysis. There was a clear increase in the IHC expression of *muc4* (Figures 2C, 2G, and 2K) after 16 and 52 weeks of *H. felis* infection. This correlates with the RNA expression shown in Table 1. There is an almost complete loss of *muc5ac* expression in the body of the 16- and 52-week *H. felis* infected stomach (Figures 3D, 3H, and 3L), which correlates with the lack of expression seen in human adenocarcinoma biopsies. These changes in immunoreactive *muc4* were only seen in areas of mucous metaplasia, whereas changes in *muc5ac* were seen in areas of significant gastric inflammation and sites of mucous metaplasia. Although this loss of *muc5ac* immunoreactivity does not correlate well with the expression data from total gastric RNA, this discrepancy is probably caused by the observation that *muc5ac* immunoreactivity is only lost in the gastric body but not the antrum of mice infected with *H. felis* (data not shown). As our RNA expression data are for the total stomach, the technique may not be sensitive enough to pick up this region-specific loss of mucin expression. Because our qRT-PCR analysis could not accurately evaluate the timing of the decrease in *muc5ac* expression after *H. felis* infection, we developed a semi-quantitative histological scoring system to analyze *muc5ac* immunoreactivity at 4-, 16-, and 52-week post-infection times. Our data indicated that *muc5ac* was lost in the gastric body as early as 4 weeks after infection, with complete loss seen by 16 weeks (Figure 3). This decreased *muc5ac* expression correlates with gastritis but occurs before significant epithelial alterations such as atrophy, mucous metaplasia, or dysplasia.

Role of Adaptive Immunity in Mucin Alterations Seen After *H. felis* Infection

We have previously shown that CD4⁺ T cells are crucial to the gastric pathology induced by *H. felis* infec-

Table 1 Average fold change of mucin and TFF genes after *H. felis* infection

	4 weeks	16 weeks	52 weeks
<i>muc1</i>	1.45 (0.62–3.37) ^a	0.41 (0.12–1.35)	1.31 (0.18–9.63)
<i>muc2</i>	0.36 (0.08–1.71)	0.10 ^b (0.025–0.43)	0.22 (0.03–1.57)
<i>muc3</i>	2.5 (1.28–4.97)	2.33 (1.16–4.68)	0.35 (0.02–5.87)
<i>muc4</i>	5.99 (2.38–15.09)	26.22 ^b (13.15–52.29)	3.67 (0.34–39.31)
<i>muc5ac</i>	0.41 (0.07–2.39)	0.22 (0.08–0.65)	0.24 (0.03–1.92)
<i>muc5b</i>	15.70 ^b (8.08–30.51)	25.90 ^b (12.21–54.96)	4.22 (0.56–31.95)
<i>muc6</i>	0.68 (0.25–1.87)	0.21 (0.05–0.79)	1.69 (0.2–13.93)
<i>tff1</i>	1.1 (0.44–2.77)	0.6 (0.27–1.33)	0.4 (0.1–1.65)
<i>tff2</i>	2.44 (1.3–4.57)	0.28 (0.08–0.97)	2.21 (0.56–8.69)
<i>tff3</i>	1.01 (0.34–3.01)	0.15 (0.03–0.67)	Not tested

^aLower and upper limits of range of fold change.

^b*p* < 0.05.

expression after *H. felis* infection. Because B6.RAG-1^{-/-} mice are deficient in B or T cells, they are a good model to use to analyze the effects of *H. felis* infection in the absence of an adaptive immune system (Mombaerts et al. 1992). As previously published, at 4 and 16 weeks after *H. felis* infection, the B6.RAG-1^{-/-} mice showed no significant histological alterations while maintaining a high level of *H. felis* colonization (data not shown). Intriguingly, these mice still lose muc5ac expression in the body as early as 4 weeks after *H. felis* expression, implying that the adaptive immune response is not responsible for this expression change (Figure 4A). However, the increased expression of *muc4* and *muc5b* was critically dependent on the presence of an adaptive immune response, because expression of *muc4* and *muc5b* is not altered after *H. felis* infection of B6.RAG-1^{-/-} (Figure 4B). These alterations in mRNA expression were confirmed by immunofluorescence analysis. *H. felis*-infected B6.RAG-1^{-/-} mice showed no change in muc1, muc3, or muc4 immunoreactivity, whereas muc5ac immunoreactivity was still decreased in the body of the stomach (Figure 5).

Figure 4 Mucin expression in C57BL/6 and B6.RAG-1^{-/-} *H. felis*-infected mice. (A) Levels of muc5ac immunoreactivity decrease in both the B6 and B6.RAG-1^{-/-} stomach after *H. felis* infection. Mock animals from all times after infection are combined. **p* < 0.05 compared with respective mocks. (B) Fold change in RNA expression in *H. felis*-infected B6.RAG-1^{-/-} and B6 stomachs 16 weeks postinfection. **p* < 0.05 compared with respective mocks, and the number of mice used in the RNA analysis are as shown by the individual symbols in A.

Discussion

Many studies have shown alterations in the expressions of mucins and TFFs in human gastric atrophy and adenocarcinoma; however, it is unclear how murine models of disease correlate with these observations. Because C57BL/6 infection with *H. felis* resembles the human disease histologically, we designed experiments to examine whether mucin and tff expression would be altered in a way that resembled the human disease. The histological changes and the degree of *H. felis* colonization were inversely proportional in our mouse model, which is similar to what is seen in the human disease (Correa 1988; Karnes et al. 1991; Kikuchi 2002). As the histological scores increase (indicating an increase

in both inflammation and epithelial alterations), the colonization of *H. felis* is decreased. Our RNA expression data showed that *muc4* and *muc5b* resemble what has been seen in biopsies of human gastric adenocarcinoma, because both increase as the murine gastric epithelium progresses through the stages of gastric pathology seen after *H. felis* infection. This similarity to human expression after infection was also seen for *muc5ac*. The expressions of the other mucins and tffs are essentially unchanged, which is different from what is reported in human disease (Supplemental Table ST2). Because the majority of studies on human mucin expression rely on IHC visualization and because mucins are complex glycoproteins, their antibody epitopes are al-

Figure 5 Comparison of gastric body mucin expression in B6.RAG-1^{-/-} *H. felis*-infected or mock-control mice. All photomicrographs shown are representative of the staining seen in all stomachs examined. (A,E) Muc1, (B,F) muc3, (C,G) muc4, and (D,H) muc5ac. A–D are mock-control stomachs. E–H are 16 weeks after *H. felis* infection. Note that immunoreactivity in B6.RAG-1^{-/-} mice for muc1, 3, and 5ac is similar to that seen in B6 mice with *H. felis* infection, whereas muc4 immunoreactivity does not increase in infected B6.RAG-1^{-/-} stomachs. This is in contrast to the increase in muc4 seen after *H. felis* infection of B6 mice (see Figure 2). Bar = 50 μ m.

most certainly made up of both specific amino acid sequences and the specific sugar residues attached to this protein backbone (de Bolos et al. 2001; Brockhausen 2003). Our RNA expression data cannot evaluate any differences in glycoprotein expression secondary to alterations in glycosylation and may account for some of the difference seen in our study vs biopsies of human disease. However, our results may also indicate that the murine model does not absolutely mimic the human infection for all components of the mucous layer.

The absence of a change in *tff2* does not correlate with the previously reported change in *tff2* immunoreactivity (spasmolytic polypeptide expression metaplasia) in the gastric antrum of mice after *H. felis* infection (Nomura et al. 2004). One potential explanation is that this study specifically evaluated changes in the antrum, whereas our study investigated global changes in gastric gene expression.

The loss of *muc5ac* has been proposed to be a key alteration in the progression to gastric adenocarcinoma. By losing a component of the mucin layer that interacts with the TFFs, the tissue of the stomach may have an increased exposure to dietary carcinogens and may show decreased repair in response to injury. This potential mechanism in gastric tumorigenesis is supported by the study that mice lacking *tff1* (which interacts with *muc5ac*) spontaneously develop antropyloric adenomas and carcinomas (Lefebvre et al. 1996). However, our data from the *H. felis*-infected immunodeficient B6. RAG-1^{-/-} mouse would indicate that altered *muc5ac* expression, in the continuing presence of *Helicobacter* colonization, is not sufficient for progression to gastric adenocarcinoma. Some component of the adaptive immune response must also be crucial to disease progression. Because it has recently been shown that *H. pylori* alone can alter mRNA expression of *MUC5AC* in gastric carcinoma cells, the decrease of immunoreactive *muc5ac* in our model may be caused by a direct effect of *H. felis* (Matsuda et al. 2008).

The requirement for components of the adaptive immune response in the alteration of expression of *muc4* and *muc5b* implies that these two mucins may play a crucial role in progression to gastric cancer, because the adaptive immune response is needed for this progression. *MUC4* is known to encode a membrane-bound mucin that is overexpressed in pancreatic adenocarcinomas (Jonckheere et al. 2004). In addition, overexpression of *MUC4* in A375 human melanoma cells promotes spontaneous metastasis (Carraway et al. 2000; Komatsu et al. 2000). The molecular mechanisms for this involvement in tumorigenesis may be caused by the EGF-like domain in *MUC4* that is known to interact with ErbB2 and alter epithelial cell growth. *MUC4* has been shown to be upregulated by interleukin 6 (IL-6) and transforming growth factor β (TGF β) in gastric and pancreatic cell lines (Jonckheere et al. 2004;

Mejias-Luque et al. 2008); therefore, the role of these cytokines in gastric tumorigenesis should be further studied. *MUC5B* is predominantly found in both the saliva and the respiratory tract and has been shown to bind SabA adhesion expression strains of *H. pylori* at neutral pH (Linden et al. 2008b). As parietal cells (and therefore the low pH) of the stomach are lost in chronic gastritis, this upregulation of *MUC5B* may allow for increased binding of *Helicobacter* and/or other bacteria. This potential colonization by bacteria in the stomach may help drive the continuing chronic inflammation seen in both our mouse model and in the human disease. One recent study has shown that the gastric microbiota of BALB/c mice infected with *H. pylori* does change from being predominately lactobacilli to including *Clostridia*, *Bacteroides/Prevotella* spp., *Eubacterium* spp., *Ruminococcus* spp., *Streptococci*, and *E. coli* (Aebischer et al. 2006). The importance of this increased microbiological diversity on the progression to gastric adenocarcinoma has not been evaluated.

These findings strengthen the approach of using the *H. felis* mouse model, because multiple aspects of the disease process can be manipulated, and the disease progression can be closely monitored. The model can now be used to determine whether these changes in mucin expression are a consequence of the progression to gastric cancer or whether they play a causal role. Factors evident in the model before progression to gastric adenocarcinoma may lead to the development of novel detection or treatment strategies. To our knowledge, a *muc4*- or *muc5b*-deficient mouse has not been generated, but once available, such models could be used to directly determine whether these mucins do play a crucial role in progression to gastric adenocarcinoma.

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