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***Helicobacter pylori* induces an antimicrobial response in rhesus macaques in a Cag Pathogenicity Island-Dependent Manner**

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Abstract

Background and Aims—We used the rhesus macaque model to study the effects of the *cag* pathogenicity island (*cag* PAI) on the *H. pylori* host-pathogen interaction.

Methods—*H. pylori* Specific pathogen free (SPF) monkeys were experimentally challenged with wild type *H. pylori* strain J166 (J166WT, n=4) or its *cag* PAI isogenic knockout (J166Δ*cag* PAI, n=4). Animals underwent endoscopy before and 1, 4, 8, and 13 wks after challenge. Gastric biopsies were collected for quantitative culture, histopathology, and host gene expression analysis.

Results—Quantitative cultures showed that all experimentally challenged animals were infected with J166WT or its isogenic J166Δ*cag* PAI. Histopathology demonstrated that inflammation and expansion of the lamina propria were attenuated in animals infected with J166Δ*cag* PAI compared to J166WT. Microarray analysis showed that of the 119 up-regulated genes in the J166WT-infected animals, several encode innate antimicrobial effector proteins, including elafin, siderocalin, DMBT1, DUOX2, and several novel paralogues of human β-defensin2. Quantitative RT-PCR confirmed that high level induction of each of these genes was dependent upon the presence of the *cag* PAI. Immunohistochemistry confirmed increased β-defensin2 epithelial cell staining in animals challenged with J166WT compared to either J166Δ*cag* PAI-challenged or uninfected control animals.

Conclusion—We propose that one function of the *cag* PAI is to induce an antimicrobial host response that may serve to increase the competitive advantage of *H. pylori* in the gastric niche and could even provide a protective benefit to the host.

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Introduction

Helicobacter pylori infects the gastric mucosa of approximately half the world's population and causes chronic active gastritis, which in 10 to 15% of patients will progress to peptic ulcer disease or gastric adenocarcinoma¹. Treatment of all those infected with *H. pylori* is not practical and may even be undesirable, since controversial evidence suggests that *H. pylori* may protect against gastric esophageal reflux disease, asthma, and cancers of the proximal stomach and distal esophagus^{2, 3}. Therefore, considerable work has been devoted to understanding the host, bacterial, and environmental factors associated with the development of *H. pylori*-related diseases.

The best studied bacterial factor associated with development of gastric cancer and peptic ulcer is the *cag* pathogenicity island (*cag* PAI). The 40 kb *cag* PAI consists of approximately 27 genes, several of which encode a Type IV secretion system that exploits integrins to translocate CagA into gastric epithelial cells⁴. Intracellular CagA is phosphorylated by Src family and other kinases⁵, which disrupts host cell signaling and causes morphologic changes and disruption of tight junctions. The Type IV secretion system also mediates the translocation of *H. pylori* peptidoglycan into host cells and induction of the proinflammatory cytokine interleukin-8 (IL-8)⁶.

While the cell biology of the *H. pylori* *cag* PAI has been studied extensively *in vitro*, its effects *in vivo* are poorly understood. Studies in *H. pylori*-infected humans have confirmed that CagA is translocated into the gastric epithelium where it is tyrosine phosphorylated and binds SHP-2, similar to what is observed in cultured epithelial cells⁷. However, the results from experimental infection in animal models have been inconsistent. Deletion of the entire *cag* PAI or interruption of the *virB10* or *virB11* homolog had no effect on bacterial colonization, gastritis, or neutrophilic infiltration in germ free piglets or in conventional C57BL/6 mice⁸. In contrast, studies in the Mongolian gerbil showed that infection with wild type *H. pylori* induced more intense inflammation, atrophic gastritis, and pro-inflammatory cytokine expression compared to partial or complete *cag* PAI deletion mutants^{9, 10}.

The rhesus macaque represents an alternative model of *H. pylori* that is tractable and more closely resembles human infection. Socially housed macaques are naturally infected with *H. pylori* that is indistinguishable from human isolates¹¹. Experimental infection of animals reared under pathogen free conditions produces a histologic gastritis similar to that seen in humans¹². Here we report the results of histopathology and microarray analysis of experimental infection of rhesus macaques with J166 wild type *H. pylori* (J166WT) or its isogenic *cag* PAI knockout (J166Δ*cag* PAI). The results suggest that J166WT induces a *cag* PAI-dependent inflammatory response that is remarkable for an increased expression of antimicrobial molecules in the gastric mucosa. We speculate that the *cag* PAI-dependent innate immune response plays a role in the relative fitness of *H. pylori* in the gastric ecology, and may protect the host against enteric pathogens.

Materials and Methods

Animals and experimental design

Colony bred SPF male and female rhesus macaques between the ages of 2 and 3 years (3–6 kg) were housed at the California National Primate Research Center (CNPRC) and derived as reported previously¹³. Two experimental groups of four SPF animals were challenged with either J166WT or an isogenic J166Δ*cag* PAI. A third group of four SPF animals served as uninoculated controls. All experiments were approved by the Research Advisory Committee of the CNPRC and the Institutional Animal Care and Use Committee at the University of California.

Bacterial strains and culture

J166WT is a strain from a patient with peptic ulcer disease that by DNA microarray analysis contains all the genes of the *cag* PAI (unpublished observations), and was previously shown to effectively colonize rhesus macaques¹². Inoculation with J166WT was performed with six single colony isolates that were rhesus passaged once or twice and then each isolated from a different experimentally infected macaque after 2 to 17 weeks of colonization. The original human strain of J166 and all rhesus passaged J166 isolates were indistinguishable (data not shown) by repetitive palindromic PCR performed as described¹². A mixture of six J166WT strains was chosen because inoculation with a single colony isolate was found to colonize macaques less efficiently (unpublished observations). Plate-grown bacteria were cultivated using routine methods¹². A complete deletion of the *cag* PAI was made in each of the six J166WT isolates using allelic exchange methods similar to those previously described¹⁴ and detailed in SI. PCR of the *cag* PAI empty site was performed as described¹⁵ to confirm replacement of the *cag* PAI by the CAT cassette (SI Fig 7). PCR was also used to confirm that *cag* PAI flanking genes were unaffected during construction of the J166 Δ *cag* PAI strains (unpublished results). Each of the six individual J166WT or J166 Δ *cag* PAI isolates was combined to a total concentration of approximately 10⁹CFU/2ml and inoculated by gavage as described¹².

Functional analysis of the *cag* PAI

H. pylori J166 strains were assayed for induction of IL-8 secretion by AGS cells essentially as described¹⁶, with modifications detailed in SI Materials and Methods.

Endoscopy, gastric biopsy, and quantitative culture

Eight antrum and five corpus biopsies of the stomach were obtained by endoscopy under ketamine (10mg/kg IM) anesthesia. Biopsies were collected 1 week prior to *H. pylori* inoculation and 1, 4, 8, and 13 wks post inoculation (PI). Three biopsies of the antrum were collected in Brucella broth for *H. pylori* quantitative culture by serial dilution and plating as described¹². The remaining antrum and corpus biopsies were processed for total RNA isolation. One additional antrum biopsy was used for histopathology and immunohistochemical analysis 8 wks PI (J166WT) and 13 wks PI (J166 Δ *cag* PAI).

Histology and immunohistochemistry

Gastric biopsies from the antrum of macaques infected with *H. pylori* J166WT (8 wks PI) and J166 Δ *cag* PAI (13 wks PI), and from uninoculated controls, were paraffin imbedded and sectioned. Slides stained with hematoxylin and eosin were analyzed for inflammation (mononuclear cells) and activity (PMNs) by a primate pathologist without knowledge of the experimental condition. Scores were generated as a composite of inflammation and activity according to the modified Sydney system¹⁷. Immunohistochemical staining was conducted according to methods reported previously using polyclonal anti-human BD2 antibody¹⁸. Gastric mucosa sections incubated with anti-laminin (Sigma, St. Louis, MO) primary antibody served as immunohistochemistry controls.

RNA isolation and microarray analysis

Ten gastric biopsies (5 antrum and 5 corpus) in TRIzol were pooled, ground with a glass pestle, and used for RNA extraction with methods provided by the manufacturer (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I (Roche Applied Science, Mannheim, Germany), purified with RNeasy (Qiagen, Valencia, CA), and hybridized to the Affymetrix Human Focus Array according to recommendations of the manufacturer (Affymetrix, Santa Clara, CA) and methods reported previously¹⁹. Gene expression without *H. pylori* infection was determined with RNA isolated from experimental animals sampled once before challenge (n=8) and from

control animals (n=4) sampled twice during the time of the experiment. These results were compared to gene expression determined from RNA isolated 1 and 13 wks after challenge with J166WT (n=4) or J166 Δ cag PAI (n=4). Data were collated and scaled in Microarray Suite 5.0 (MAS 5.0, Affymetrix, Santa Clara, CA). Of 8,793 probe sets, approximately 5,000 passed filtering and were analyzed with BRB Array Tools (version 3.3). Triple comparisons were performed separately using an F statistic ($p < 0.01$) for 1 versus 13 wks PI (Control, J166WT, J166 Δ cag PAI) and J166WT versus control (control, 1 week PI, 13 wks PI).

Real time quantitative RT-PCR

Total RNA was reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA), purified, diluted to 10ng RNA/ul, and analyzed on the Roche LightCycler 2.0 with the FastStart DNA Master SYBR Green I detection kit (Roche Diagnostics, Mannheim, Germany). Absolute mRNA copy number was determined as described²⁰ by comparison of crossing points of experimental cDNA to standard curves generated from plasmids that contained partial cDNA encoding BD2, elafin, siderocalin, DMBT1, or DUOX2. Plasmids were generated using gene-specific PCR primers (SI Table 1) and first-strand cDNA from *H. pylori*-infected gastric mucosa, except that for β -actin, which was purchased (Invitrogen). Melting point analysis of the RT-PCR product was used to confirm that a single product was generated.

Cloning of BD2-like cDNA

Initial experiments to quantitate BD2 expression in *H. pylori*-infected gastric mucosa used primers (5'-GTCTTGATCTCCTCTTCTCG-3' and 5'-CACAGGTGCCAATTTGTTTAT-3') for quantitative RT-PCR that were based on the annotated rhesus BD2 sequence (NM_001032827), which differs from the cDNA encoding human BD2 (NM_004942) at only one nucleotide. However, sequence analysis of the PCR product revealed nine nucleotide differences compared to rhesus BD2. Complete sequence for this apparently novel BD2 cDNA was determined using a combination of conventional and 3'-RACE PCR as described in SI Materials and Methods.

Statistical analysis

Quantitative RT-PCR data were log transformed and analyzed (SAS) by one-way ANOVA (control, J166WT, J166 Δ cag PAI) and repeated measures 2-way ANOVA, with main effects of group (J166WT versus J166 Δ cag PAI) and time. Bacterial density (CFU/g) in J166WT- and J166 Δ cag PAI-infected animals was log transformed and analyzed by ANOVA with repeated measures. Pairwise comparisons were performed using student's *t* test with the Bonferroni correction. Histologic analysis of gastric biopsies from uninfected, J166WT-, and J166 Δ cag PAI-infected animals was performed with the Kruskal-Wallis test, followed by pairwise comparisons using the Mann-Whitney U test. Two-tailed *p* value for statistical significance was 0.05 except for analysis of the microarray data (described separately), where it was 0.01.

RESULTS

Colonization of rhesus macaques with J166WT and J166 Δ cag PAI

Two groups of 4 SPF rhesus macaques were inoculated with rhesus passaged J166WT (7.2×10^8 CFU) or J166 Δ cag PAI (1.2×10^9 CFU). Quantitative cultures of three antrum biopsies at 1, 4, 8, and 13 wks PI showed that all animals were chronically infected, typically with 10^5 to 10^7 CFU/g of gastric tissue (Fig. 1). There were no significant effects of group or time on bacterial density, though the group-time interaction approached significance ($p < 0.07$). Colonization density was initially lower (10^3 CFU/g) in the J166 Δ cag PAI-infected animals at 1 week PI, but increased to the levels in J166WT-infected animals at 8 wks PI. Lower initial colonization in J166 Δ cag PAI-infected animals may reflect laboratory adaptation that occurred

during construction of the J166 Δ cag PAI strain. This is supported by the observation that subsequent challenge of macaques with J166 Δ cag PAI strains recovered from these animals yielded a bacterial density similar to that seen in animals infected with J166WT (unpublished observations). Colonization density was greater in animals infected with J166 Δ cag PAI than in those infected with J166WT at 13 wks PI ($p<0.05$), but the differences were not statistically significant at other time points. Increased colonization density has also been described using the gerbil model in strains with isogenic deletions in *cagA* or *cagY* compared to wild type controls¹⁰. *H. pylori* was never isolated from uninoculated controls.

IL-8 induction by J166WT and J166 Δ cag PAI

As a functional measure of the *cag* PAI we examined the induction of IL-8 in AGS cells by six single colony J166WT isolates, each derived from a different experimentally infected macaque and used together as the J166WT inoculum, and compared the results to their isogenic J166 Δ cag PAI. Among the six input J166WT strains, three induced IL-8 at a high level (mean=2632 pg/ml; SE=126 pg/ml) and three did not (mean=322 pg/ml; SE=121 pg/ml), although the *cag* PAI was detected by empty site PCR (SI Fig. 7) and DNA microarray (unpublished observations) in both. Similar results have been reported by others²¹. As expected, the six input J166 Δ cag PAI strains showed no significant IL-8 induction (mean=240 pg/ml; SE=17 pg/ml), which was similar to broth controls (276pg/ml; SE=6.7pg/ml). Since only some of the input J166WT strains appeared to have a functional *cag* PAI, we next examined IL-8 induction in *H. pylori* isolates recovered from the J166WT-infected animals, in order to better assess the function of the *cag* PAI *in vivo*. The results showed a non-overlapping, bimodal distribution, in which some isolates ($n=33$) induced high levels of IL-8 (mean=2,987 pg/ml; SE=847 pg/ml) and others ($n=25$) induced low levels (mean=342 pg/ml; SE=73 pg/ml), both of which were isolated throughout the course of infection in all animals (SI Fig. 8). These results are consistent with studies that suggest that natural *H. pylori* infection in humans may represent colonization with *cag* PAI-positive strain variants that differ widely in their induction of IL-8, or even with a mixed population of *cag* PAI-positive and *cag* PAI-negative strains^{21, 22}. Similar to other reports, we were unable to detect induction of IL-8 expression by microarray²³. Together, these results confirm that animals challenged with J166WT, but not those challenged with J166 Δ cag PAI, were infected with a mixed population of *H. pylori* that contained a functional *cag* PAI.

Histologic analysis of gastric biopsies from J166WT- and J166 Δ cag PAI-infected rhesus macaques

Representative sections of gastric antrum from J166WT-infected animals (8 wks PI) showed increased inflammation compared to J166 Δ cag PAI-infected animals (13 wks PI) and uninfected controls (Fig. 2ABC). Blind scoring of sections using the modified Sydney system confirmed that J166WT-infected animals had more gastritis than either J166 Δ cag PAI-infected or control animals (Fig. 2D). Non-parametric ANOVA demonstrated significant differences among the groups ($p=0.02$), which was supported by pairwise comparisons between J166WT versus control ($p=0.03$) and J166WT versus J166 Δ cag PAI ($p=0.03$). Although the tissue sections were not obtained from the J166WT- and J166 Δ cag PAI-infected animals at the same time PI (due to inadequate sampling), greater inflammation in animals infected with J166WT (8 wks PI) compared to J166 Δ cag PAI (13 wks PI) is most likely a strain effect, since bacterial loads were nearly identical (Fig 1.).

Microarray analysis

Microarray analysis was performed on gastric mucosa from infected animals before and 1 and 13 wks PI, and from uninfected control animals. Data were analyzed with a univariate ANOVA that compared the composite controls to J166 Δ cag PAI and J166WT (separately for 1 and 13

wks PI), or to 1 and 13 wks PI (separately for J166WT and J166 Δ cag PAI). To examine temporal changes, we first analyzed the overall pattern of gene expression in J166WT-infected animals at 1 wk compared to 13 wks PI. Among the 119 genes that were differentially expressed in the J166WT-infected animals, the absolute fold-change was significantly greater (two tailed *t* statistic, $p < 0.0001$) at 1 wk (mean=2.32) than at 13 wks (mean=1.82) PI (SI Fig. 6A). Similar results were seen among the 200 genes that were differentially expressed in the J166 Δ cag PAI-infected animals (SI Fig. 6B). This was also supported by the observation that 206 genes were differentially expressed 1 wk PI, while expression was altered for only 63 genes at 13 wks PI. These results suggest that a cellular dysregulation occurs early during *H. pylori* infection, and then partially resolves. Gene expression results have been deposited to the Gene Expression Omnibus (GEO) database and summary tables can be found in SI Table 2 and 3.

One particularly prominent observation was the induction of β -defensin-2 (BD2), which was induced 10.9-fold at 13 weeks PI (SI Fig. 6A). This effect was dependent upon the presence of the cag PAI, since no change in BD2 expression was detected in J166 Δ cag PAI-infected animals at any time point. Because cag PAI-dependent induction of BD2 was such a striking observation, we examined the data for changes in expression of other genes that encode antimicrobial defense molecules. The results showed evidence of cag PAI-dependent induction of four additional genes (elafin, siderocalin, DMBT1, DUOX2), which are expressed at epithelial surfaces and encode innate immune effector molecules that protect against Gram-negative and Gram-positive pathogens. Although counterintuitive, these results suggest that one function of the cag PAI during *H. pylori* infection is to mediate induction of a collection of innate immune antimicrobial effector molecules.

Quantitative RT-PCR analysis

To further examine this hypothesis, two-step quantitative RT-PCR was performed on RNA isolated from J166WT, J166 Δ cag PAI, and uninfected control animals at each time point using sequence-validated primers (SI Table 1). Cloned partial cDNA of each gene was used to construct standard curves, and the data were expressed as mRNA copy number per 10 ng of total RNA. The results demonstrated a dramatic cag PAI-dependent increase in expression of each of the five antimicrobial genes (Fig. 3), with peak levels observed at 4 to 8 wks PI. One-way ANOVA and pairwise *t* tests demonstrated that antimicrobial gene expression was significantly greater in the J166WT- and J166 Δ cag PAI-infected animals compared to uninoculated controls. Statistical analysis for each gene was then performed by two-way ANOVA, using time and group (J166WT versus J166 Δ cag PAI) as main effects. Of particular interest were the group effects, which demonstrated a significant difference for BD2 ($p < 0.01$) and elafin ($p < 0.05$). Owing to small sample size, group effects for the other genes did not achieve statistical significance (DMBT1, $p = 0.14$; siderocalin, $p = 0.22$; DUOX2, $p = 0.14$). There were no differences in expression of β -actin among the three groups, which served as a control for amount and quality of RNA.

Identification of novel rhesus BD2 paralogues

Initial experiments to validate *H. pylori*-induced expression of BD2 were performed using primers based on annotated human (NM_004942) and rhesus (NM_001032827) BD2 sequences, which differ by only 1 of 195 nucleotides. The results showed that this BD2 was not expressed in the gastric mucosa of experimental animals to any appreciable level (unpublished observations). Rather, PCR and 3'-rapid amplification of cDNA ends (RACE) revealed that a group of five novel rhesus genes with marked similarity to rhesus BD2 was in fact highly induced. The qRT-PCR assay using primers designed to detect all the novel BD2-like transcripts (and the annotated rhesus BD2) confirmed the microarray analysis, and demonstrated that this group of β -defensins was induced to high levels in a cag PAI-dependent manner (Fig. 3). These peptides were about 77% identical to human and rhesus BD2 (Fig. 4)

and we therefore designate them as rhesus BD2-like 1–5 (BD2-L1-5). Since we were able to detect a PCR product in rhesus genomic DNA using an antisense primer (5'-TGGCTTTTTCAGCATTAT-3') designed to amplify both BD2 and BD2-L1-5, but not using a primer that should amplify BD2 only (5'-CTTTTTCAGCATTGT TCCAGGGAG-3'), the annotated rhesus BD2 allele appears to be absent in these animals (unpublished observations).

Detection of BD2 paralogues in gastric epithelial cells by immunohistochemistry

Immunohistochemical analysis was used to determine if the increase in rhesus BD2-like mRNA correlated with an increase in expression of rhesus BD2-like peptides. Biopsies from animals inoculated with J166WT (8wks PI), J166 Δ cag PAI (13wks PI), and uninoculated controls were sectioned, deparaffinized, and stained with anti-human BD2 polyclonal antibody that is specific for human BD2¹⁸. Sections from uninoculated controls showed only minimal levels of BD2-like proteins expressed at the epithelial surface lining the gastric pits (Fig. 5A). A moderate increase in BD2-like staining was seen in sections from the J166 Δ cag PAI-infected animals compared to the uninfected control (Fig. 5B). Intense BD2-like staining was seen at the epithelial surface and in the gastric pits from the J166WT-infected animal (Fig. 5C), suggesting an increase in expression when compared to the J166 Δ cag PAI-infected and uninoculated controls. These results support the RT-PCR data, and suggest that expression of rhesus BD2-like peptides at the gastric epithelial surface is induced by J166WT infection in a cag PAI-dependent manner.

Discussion

Elegant studies in cell culture have demonstrated that one key function of the *H. pylori* cag PAI is to encode a Type IV secretion system through which CagA and peptidoglycan are injected into host cells, redirecting host cell signaling. However, the broader function of the cag PAI in the complex environment of the gastric mucosa is poorly understood. Here we demonstrate using the rhesus macaque model, which arguably is most relevant to human infection, that *H. pylori* induces a cag PAI-dependent increase in mucosal inflammation that is accompanied by expression of an array of antimicrobial host defense molecules. This inducible response includes both a small group of novel peptides that are closely related to human BD2, and also several additional innate host defense proteins that have antimicrobial activity at mucosal surfaces.

Previous studies in cultured human gastric epithelial cells have demonstrated that *H. pylori* induces expression of BD2^{24, 25}. The cag PAI-encoded Type IV secretion system (but not CagA) is required for *H. pylori* induction of BD2 in gastric cell culture, and it is dependent upon signaling through the intracellular pathogen-recognition receptor, NOD1²⁶, whose cognate ligand is peptidoglycan. Similar findings with *H. pylori* peptidoglycan have demonstrated NOD1-dependent induction of IL-8⁶. Studies of human clinical samples also suggest that *H. pylori* induces BD2^{25, 27, 28}. We previously reported a microarray analysis that demonstrated *H. pylori* induction of BD2 in macaques¹⁹. However, the role of the cag PAI has not been systematically studied in humans or in animal models. The results reported here extend these observations and demonstrate for the first time *in vivo* that *H. pylori* induction of BD2-like peptides is dependent upon a functional cag PAI. Furthermore, our discovery of the expression of a family of novel BD2-like peptides is consistent with the well-known gene duplication and heterogeneity at the incompletely characterized defensin gene locus²⁹. Since polymorphisms in gene-copy number and quantitative differences in defensin levels have clinical relevance in the inflammation of colonic Crohn's disease³⁰, variation in BD2 alleles and gene-copy number may also affect susceptibility to *H. pylori*-related diseases.

We also identified four additional innate antimicrobial proteins that are strongly induced by *H. pylori* in a *cag* PAI-dependent manner: siderocalin, elafin, DUOX2, and DMBT1. The mechanisms by which this heterogeneous group of proteins participate in defense of epithelial surfaces are varied, and with the exception of BD2 and DMBT1, the effects on *H. pylori* are unknown. Siderocalin limits the growth of Gram-negative bacteria by binding enterobactin and interfering with siderophore-mediated iron acquisition³¹. Elafin (previously SKALP) is a 9.9 kDa serine anti-protease that is active against both Gram-positive and Gram-negative bacteria³². Although the mechanism of its antimicrobial activity is not fully established, the cationic nature of elafin may allow it to disrupt the anionic bacterial cell membrane in a manner similar to defensins and other antimicrobial peptides³³. The antimicrobial activities of DMBT1 and DUOX2 are likely to be indirect. DUOX2 is a member of the NOX family of NADPH oxidases³⁴, which generates reactive oxygen species and may have a role in protection from enteric infection with *Escherichia coli*³. DMBT1 belongs to the scavenger receptor cysteine-rich family that is implicated in innate immune defense³⁵. A splice variant of DMBT1 (salivary agglutinin) interacts with the defense collectins, surfactant proteins A and D³⁶. It is well known to bind the antigen I/II family of streptococcal adhesins³⁷, and also has been shown to aggregate *H. pylori*³⁸. Whether DMBT1 facilitates bacterial adherence, or aggregation and subsequent clearance, is unknown. Since *H. pylori* can regulate binding to surfactant protein D by phase variation in LPS³⁹, both functions might be active under different conditions.

Although the expression of an antimicrobial host response was most pronounced in animals infected with J166WT, the J166 Δ *cag* PAI strain also showed significant, but much attenuated induction of BD2, siderocalin, elafin, and DUOX2 when compared to controls. Since expression of β -defensins^{25, 40} and other antimicrobial molecules^{41, 42} can be induced by cytokines such as IL-1, TNF- α , and others, induction of an antimicrobial response by the J166 Δ *cag* PAI strain may be mediated by the inflammatory response, rather than by a direct effect of peptidoglycan or other bacterial product. Although IL-1 is not required for the *H. pylori* induction of BD2 *in vitro*²⁶, it is likely that IL-1 (or other cytokines) contribute to induction of BD2 *in vivo*⁴³. Alternatively, or in addition, it may be that peptidoglycan from J166 Δ *cag* PAI can gain access to the cytosol of epithelial cells through mechanisms other than Type IV secretion. For example, it was recently demonstrated that peptidoglycan from *Haemophilus influenzae* can enter epithelial cells via pores formed by pneumolysin from concomitant infection with *Streptococcus pneumoniae*⁴⁴.

It seems paradoxical that *H. pylori* has horizontally acquired a *cag* PAI that serves, at least in part, to induce an antimicrobial host innate immune response. It may be that the *cag* PAI mediates an increased inflammatory response that is important for colonization, similar to what was described recently for *Salmonella*⁴⁵. Or perhaps the antimicrobial proteins induced in a *cag* PAI-dependent manner are simply not active against *H. pylori*, which has been studied in only a limited way for BD2²⁷ and DMBT1³⁸. Alternatively, these antimicrobial proteins may be active against *H. pylori*, but less so than against other microbiota that compete for the same gastric niche. While previously viewed as sterile except for *H. pylori*, recent evidence based on broad range 16S rDNA libraries suggests that the microbiota of the human stomach has considerable diversity⁴⁶. *H. pylori* bearing the *cag* PAI may induce an antimicrobial response that is active against some of these organisms, and so may help *H. pylori* compete effectively. To the extent that some of these mucosal bacteria are potential pathogens that briefly transit the stomach, *H. pylori* could even provide a benefit to the host. This suggestion is analogous to the recent observation that normal colonization of the vagina by *Lactobacillus* is associated with increased expression of BD2, which may protect against colonization with *Gardnerella* and other agents of bacterial vaginosis⁴⁷. Viewed in this way, *H. pylori* could be considered “almost normal” biota of the stomach that sometimes has a symbiotic or even mutualistic relationship with its human host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quantitative *H. pylori* cultures (mean $\pm$ SE log₁₀ CFU/g) from gastric antrum biopsies of rhesus macaques inoculated with J166WT (open circles) or J166 Δ cag PAI (closed circles). Biopsies were obtained 1, 4, 8, and 13 wks PI.

Figure 2.

Representative photomicrographs of hematoxylin and eosin stained biopsies of the gastric antrum collected from a control macaque (A) and after challenge with J166Δcag PAI (B) or J166WT (C) at 13 and 8 wks PI, respectively. Compared to J166Δcag PAI-infected animals and uninfected controls, J166WT-infected animals showed an increase in mononuclear and neutrophilic infiltration, and marked expansion of the lamina propria, with scattered neutrophils, plasma cells, and histiocytes. Bar=100μm. Lymphoid follicles were occasionally seen in both groups of *H. pylori* infected macaques (B). Mean (SE) composite gastritis score (inflammation and activity) in J166WT, J166Δcag PAI, and uninfected controls was quantitated using the modified Sydney system (D).

Figure 3.

Quantitative RT-PCR analysis (Mean $\pm$ SE) of five antimicrobial proteins that were shown by microarray analysis to be induced by *H. pylori* infection in a *cag* PAI-dependent manner. RNA was isolated from antrum and corpus biopsies obtained from rhesus macaques before and 1, 4, 8, and 13 wks after infection with J166WT (black) or J166Δ*cag* PAI (white).

Figure 5. Immunohistochemical analysis of rhesus macaque gastric biopsies stained with antibody to human BD2. Boxes highlight representative areas of positive (brown color) BD2-like staining. Tissue was obtained from an uninfected control animal (A) and from animals infected with J166 Δ cag PAI at 13 wks PI (B) or J166WT at 8 wks PI (C). Anti-laminin staining of gastric tissue from an uninfected macaque (D) is shown as a control to demonstrate specificity. Bar=100 μ m.