Interactions between Inducible Nitric Oxide and Other Inflammatory Mediators during Helicobacter pylori Infection

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ABSTRACT

Background. Recent studies in both humans and animal models strongly suggest the contribution of the host immune response to Helicobacter pylori-related disease. Inducible nitric oxide synthase has been shown to be up-regulated in the gastric epithelium during H. pylori gastritis, suggesting a role in inflammation.

Materials and Methods. C57BL/6 wild-type and inducible nitric oxide synthase gene knockout mice were infected with H. pylori strain SS1. Expression of macrophage inflammatory protein-2 (MIP-2), interleukin-1 beta (IL-1β), Th1 (IL-2 and gamma interferon) and Th2 (IL-4 and IL-10) cytokines, and inducible cyclooxygenase mRNA in mice was determined in mouse gastric tissues and quantified using either competitive reverse transcription–polymerase chain reaction or competitive polymerase chain reaction following reverse transcription.

Results. The Th1 cytokine gamma interferon was only detected in wild-type and inducible nitric oxide synthase gene knockout infected mice, while a Th2 (IL-4) response was not detected. H. pylori induced MIP-2 and IL-1β mRNA in mice.

Conclusions. Because similar levels of inflammatory mediators were noted in both wild-type and nitric oxide synthase gene knockout infected mice, our data suggest that inducible nitric oxide synthase does not influence expression of these inflammatory mediators in the early stages of H. pylori infection in mice.

H. pylori infection is the most common chronic bacterial infection in humans, causing chronic gastritis, peptic ulcer disease and increased risk of cancer. The host immune response contributes to the disease during infection with H. pylori [1,2], which is characterized by the infusion of inflammatory cells in the gastric mucosa leading to the generation of mucosal cytokines [3,4]. Nitric oxide produced by constitutively expressed nitric oxide synthase is important for normal functioning of the gastric mucosa [5,6]. H. pylori has been shown to up-regulate inducible nitric oxide synthase (iNOS) in humans both in vivo, in gastric biopsy specimens [7–9], and in vitro, in gastric epithelial cell lines [10,11]. In addition, we recently demonstrated that H. pylori induces murine iNOS mRNA both in gastric epithelial cells and in mice [12].

A bias toward a Th1 [interleukin-2 (IL-2) and gamma interferon (IFN-γ)] response during infection with Helicobacter species has been reported [2,13–15]. IFN-γ activates both macrophages and natural killer cells and has been demonstrated in H. pylori positive gastric tissues in both humans and mice [13–16]. We recently demonstrated that macrophage inflammatory protein-2 (MIP-2), a mouse counterpart of IL-8 [17,18], is induced by an IFN-γ-dependent mechanism during H. pylori infection in both mice and cultured murine gastric epithelial cells [12].

In addition to these cytokines and chemokines, we [12] and others [19] have shown that inducible cyclooxygenase (COX-2) is induced by H. pylori in vitro. COX-2 has also been shown to be up-regulated in H. pylori positive biopsies [7,8,20,21]. Because of its role in the synthesis of prostaglandins, which regulate acid levels in the stomach [22], induction of COX-2 may contribute to either mucosal protection or inflammation during bacterial infection [7,23].

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Studies involving a protozoan parasite, *Leishmania*, indicate a possible role of iNOS in regulating the innate cytokine response, particularly the Th1 response [24,25]. We hypothesized that iNOS regulates the *H. pylori*-associated inflammatory response either directly, by regulating inflammatory mediators (MIP-2, COX-2 and IL-1β), or indirectly, through regulation of the Th1 response, as in the case of *Leishmania* infection. Therefore, we designed this study to evaluate the interactions between iNOS and MIP-2, COX-2, and IL-1β in *H. pylori*-infected mice. The influence of iNOS on the expression of the Th1/Th2 cytokines in mice was also evaluated. C57BL/6 wild-type (WT) and iNOS gene knockout (iNOS−/−) mice were infected with *H. pylori* and the induction of mRNA of these effectors was compared. This work represents the first quantitative analysis of COX-2, MIP-2 and cytokine expression in WT and iNOS−/− mice infected with *H. pylori*.

**Materials and Methods**

**Bacteria and Growth Conditions**

The *H. pylori* mouse-adapted strain SS1 [26] was used for all infection studies. For continuous growth, *H. pylori* was grown on Columbia agar supplemented with 5% laked blood and 2% Isovitalex (Becton Dickinson, Cockeysville, MD) under microaerophilic conditions at 37 °C, as described previously [27]. For infections, bacteria were grown overnight in brain heart infusion broth (BHI) supplemented with 5% fetal calf serum and incubated at 37 °C under microaerophilic conditions on a reciprocal shaker. Bacteria used for infections were in the logarithmic phase of growth.

**Experimental Animals and Infections with *H. pylori* SS1**

Twenty 6-week-old female C57BL/6 WT and iNOS−/− mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were provided with food and water ad libitum. Ten WT and 10 iNOS−/− mice were equally divided between two treatment groups: uninfected and infected. Mice to be infected were inoculated with 300 µl of a 2×10⁶–1×10⁹ bacterial suspension in BHI broth by oral gavage three times at 2-day intervals. Sixteen weeks post-inoculation, mice were euthanized and the stomachs were removed under aseptic conditions. The stomach tissue was divided into longitudinal sections and used for recovery of bacteria, RNA extraction, and histology. For histologic evaluation, longitudinal sections of mouse stomach tissue were fixed in 10% formalin and embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin. The stained slides were then scored using the 0–5 scale recently described by Garhart et al. [28]. Stomach tissue used for bacterial recovery was weighed and homogenized in 1 ml of BHI broth supplemented with 5% fetal calf serum, and serial dilutions were inoculated on Columbia blood agar plates containing 5X Skirrow supplement (vancomycin, polymyxin B, and trimethoprim lactate; Oxoid, Hampshire, UK). Cultures were incubated at 37°C under microaerobic conditions for 5 days. Infections were confirmed by colony and bacterial morphology, in addition to a positive urease test. All animal procedures were approved by the veterinary medical unit of the Veteran’s Affairs San Diego Healthcare System.

**RNA Extraction**

Stomach tissue for RNA extraction was snap-frozen at −70°C. The gastric tissue (30–50 mg) was homogenized using a glass tissue grinder in 1 ml of TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA was extracted by the guanidinium isothiocyanate-phenol-chloroform method, as described in the TRIzol extraction protocol, followed by DNAase treatment. The quality and amount of RNA extracted was determined by absorbance at 260/280 nm.

**Construction and Use of Internal RNA Competitors**

For competitive reverse transcription–polymerase chain reaction (RT-PCR) of COX-2 and MIP-2, plasmids were constructed to generate in vitro internal RNA competitors. The general procedures for quantifying mRNA have been described [29]. Total RNA from *H. pylori*-infected stomach tissue of mice was extracted using TRIzol reagent, as described above. Total RNA was reverse-transcribed using random hexamer primers. The resulting cDNA was amplified with primers for the specific mRNA species of interest. The PCR products were cloned into PCR-Script (Stratagene, La Jolla, CA). The cloned DNA was either increased or reduced in size by inserting a small DNA fragment or deleting a few base pairs, respectively. The plasmid was linearized with NotI (New
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England Biolabs Inc., Beverly, MA) and transcribed using T7 RNA polymerase (Life Technologies) to yield the internal RNA competitor. The reaction was treated with DNase, extracted with phenol/chloroform, precipitated and resuspended in 10 mmol/l Tris and 1 mmol/l EDTA. After DNase treatment, the number of internal RNA competitor molecules per microliter was calculated based on the size and amount of the internal RNA competitor. To determine the amount of target RNA, a known amount of COX-2 or MIP-2 internal RNA competitor in decreasing concentrations was reverse-transcribed in parallel with a constant amount of target total RNA from mouse stomach tissue. Following PCR amplification, target and competitor bands were compared on an agarose gel. The sizes of the PCR products for the competitors were 463 and 300 bp for COX-2 and MIP-2, respectively.

cDNA Preparation

Aliquots (2 µg for COX-2 or 5 µg for MIP-2) of total RNA from mouse stomachs were reverse-transcribed together with the appropriate competitor RNA, followed by PCR. For competition between target and internal RNA competitor, a dilution series (1:2 or 1:5) of the competitor was reverse-transcribed in parallel with a constant amount of target total RNA from mouse stomach tissue. Following PCR amplification, target and competitor bands were compared on an agarose gel. The sizes of the PCR products for the competitors were 463 and 300 bp for COX-2 and MIP-2, respectively.

Oligonucleotide Primers and PCR Conditions

Mouse COX-2 was amplified using oligonucleotides previously published by Mizuno et al. [30], with modifications in the antisense strand, which was previously published as the sense strand written in reverse. In addition, we modified the PCR conditions to suit our application. For COX-2, the amplification conditions consisted of a hot start at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 1 minute. The cDNA was amplified for 32 cycles, yielding a 336-bp PCR product. For MIP-2, PCR primers used were 5'-CTGTTGTGGCCTGTAACGTGCG-3' and 5'-GGCTCCTCCTTTCCAGGTTCAGT-3' (238 bp). The reaction consisted of a hot start at 95°C for 3 minutes, followed by 30 amplification cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute. For amplification of IL-1β, a quantitative competitive kit (Maxim Biotech Inc., San Francisco, CA) which included a positive control was used. The competition reaction mixture consisted of a decreasing amount of competitor and a constant amount of target product. For Th1/Th2 cytokines, a multiple PCR kit (Maxim Biotech Inc.), which included a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and positive controls of the Th1/Th2 cytokines, was used. Taq DNA polymerase (Qiagen Inc., Valencia, CA) was used for amplification and the PCR products analyzed on a 2% agarose gel in the presence of ethidium bromide.

Statistical Analysis

Where appropriate, data were analyzed using the Mann–Whitney U-test. A p-value of < .05 was considered statistically significant.

Results

All mice inoculated with H. pylori were colonized, as confirmed by colony morphology, microscopic evaluation, and urease assay. All H. pylori-infected mice had a significantly higher gastritis score than uninfected mice (Fig. 1). There was no significant difference between WT and iNOS−/− infected mice. Figure 2 shows cytokines induced by H. pylori in mouse gastric tissues. H. pylori infection induced the Th1 cytokine IFN-γ in both WT and iNOS−/− mice (Fig. 2A), while a Th2 (i.e. IL-4) response was not detected. Both WT and iNOS−/− infected mice expressed very low levels of IL-10.
MIP-2 and COX-2 expression were quantified by competitive RT-PCR. Representative gels for MIP-2 and COX-2 mRNA expression are shown in Fig. 3. Averages of mRNA molecules for MIP-2 and COX-2 in gastric tissue per treatment group are also shown. In both WT and iNOS−/− mice, MIP-2 mRNA was expressed at higher levels in infected (Fig. 3A, lanes 7 and 8) than in uninfected (Fig. 3A, lanes 3 and 4) mice. COX-2 mRNA levels were similar between infected and uninfected mice in both WT (Fig. 3B, lanes 3 and 8) and iNOS−/− (Fig. 3B, lanes 3 and 8) mice. Averages of MIP-2 and COX-2 mRNA molecules per treatment group (five mice) are shown in Fig. 3C. Both WT and iNOS−/− infected mice had significantly more MIP-2 mRNA molecules than their counterpart uninfected mice (p < .05). However, no significant difference was noted in MIP-2 mRNA molecules between WT and iNOS−/− infected mice (p > .05). There were no significant differences in COX-2 mRNA levels between treatment groups.

**Discussion**

We had previously shown that iNOS is up-regulated during infection with *H. pylori* [12], which led us to investigate the role of iNOS in *H. pylori* infection using the iNOS−/− mouse model. In the present study, we observed that all mice infected with *H. pylori* had gastritis and their gastric tissue expressed IFN-γ, a Th1 cytokine. These results are in agreement with published data that show *H. pylori* infections to be biased toward a Th1 response [2,13–15]. IL-2, also indicative of a Th1 response, was not detected in infected mice, consistent with our previous observations and those of others [31]. However, we could detect IL-2 mRNA in the mouse liver using the same technique and primers, suggesting that IL-2 mRNA transcripts in the mouse stomach are at levels too low to be detected by RT-PCR. While IL-4 (Th2 response) was not detected, we observed very low levels of IL-10 mRNA expression in both infected WT and iNOS−/− mice. However, because the expression levels were very low, we cannot draw significant conclusions on the effect of *H. pylori* on IL-10 expression in mice. IL-10 has been detected in human biopsies positive for *H. pylori* gastritis [31]. Therefore, if IL-10 plays a significant role in the induction of gastritis in mice, as suggested by Berg et al. [32], it is effective at very low mRNA levels. Similarly to iNOS, the...
Figure 3  Quantification of MIP-2 and COX-2 mRNA expression by competitive RT-PCR. Representative gels are shown for WT and iNOS−/− mice. (A) MIP-2 and (B) COX-2 mRNA levels from uninfected (lanes 1–5) and infected (lanes 6–10) mice are shown. The starting concentration for MIP-2 competitor was $1 \times 10^9$ molecules and $5 \times 10^{10}$ molecules for COX-2 mRNA. (C) Results per treatment group represented in graph form, expressed as mean ± standard error for data obtained from five mice each. *p < .05 compared with results for uninfected mice.

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proinflammatory cytokine IL-1β is up-regulated during human *H. pylori* infection. We observed induction of IL-1β only in *H. pylori*-infected mice. Although IL-1β mRNA was expressed at slightly higher levels in WT than in iNOS−/− infected mice, we cannot comment on the significance of this relatively modest difference, because we did not measure the levels of the IL-1β peptide. Nevertheless, our result is in agreement with those of previous studies that showed up-regulation of IL-1β by *H. pylori* [31,33].

Our present data suggest that iNOS does not significantly influence MIP-2 expression during *H. pylori* infection in mice. We have previously demonstrated induction of MIP-2 mRNA, a mouse counterpart of IL-8 [17,18], by *H. pylori* in both gastric epithelial cells and mice [12]. IL-8 is a potent chemoattractant for polymorphonuclear leukocytes [34,35] and T lymphocytes [3], and its expression has been shown to be more closely correlated with the severity of *H. pylori*-related gastritis [36,37]. In the present study, both *H. pylori*-infected C57BL/6 WT and iNOS−/− mice had more gastritis than uninfected mice; however, we did not find histologic differences between WT and iNOS−/− infected mice, an observation also noted by Gobert et al. [38]. A recent study by Garhart et al. [39] also showed no differences in the gastritis score between C57BL/6 WT and iNOS−/− mice infected with *H. pylori* SS1. Lack of significant histologic differences between WT and iNOS−/− mice infected with *H. pylori* is consistent with the lack of influence of iNOS on MIP-2 expression in mice.

For COX-2, similar mRNA levels were observed between infected and uninfected mice, confirming our previous findings that COX-2 is constitutively expressed at high levels in C57BL/6 mice [12]. However, we [12] and others [19] have reported an up-regulation of COX-2 message by *H. pylori* in murine and human gastric epithelial cell lines. It is possible that up-regulation of COX-2 occurs in epithelial cells in vivo, but the overall mRNA levels may have been diluted by those from other cells in the stomach. COX-2 expression has also been reported to be up-regulated in human *H. pylori* positive stomach biopsies [7,20,21]. Nevertheless, our results indicate that iNOS does not influence the expression of COX-2 in C57BL/6 mice at the transcriptional level. It is still possible that iNOS regulates COX-2 at the post-transcriptional level, as reported by Marnett et al. [40].

We and others have shown that iNOS is up-regulated during infection with *H. pylori* [7–9,12], which led us to investigate the role of iNOS in *H. pylori* infection using the iNOS−/− mouse model. In the present study, we have shown that iNOS did not influence expression of some of the inflammatory mediators thought to be important for induction of gastritis. A study by Son et al. [41] demonstrated significantly higher iNOS expression in patients with gastric cancer, a later stage in the multistep process of *H. pylori* infection, than in patients with gastritis. This may suggest that iNOS exerts its effects in the later stages of *H. pylori* infection. Therefore, iNOS expression may be associated with more severe peptic ulcers that have no mouse model and occur after initial gastritis.

Our results clearly show that iNOS expression does not mediate the expression of proinflammatory (MIP-2 and IL-1β) and Th1/Th2 cytokines at the transcriptional level in mice. In addition, COX-2 mRNA levels were not affected by iNOS. We conclude that iNOS is not important for the induction of either histologic gastritis or proinflammatory mediators during the initial stages of *H. pylori* infection in C57BL/6 mice.

These data were presented in part at the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), September 2002, San Diego, CA (Abstract B-1424). This study was supported by PHS grants R01 DK53649 and DK35108, and NIH/NCI grant 1K01 CA96709.

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