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Role of Gamma Interferon in *Helicobacter pylori* Induction of Inflammatory Mediators during Murine Infection

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Gamma interferon (IFN-γ) has been proposed to play an important role in *Helicobacter*-related gastritis. Using the IFN-γ gene knockout (IFN-$\gamma^{-/-}$) mouse model and a murine gastric epithelial cell line, GSM06, we demonstrated that *Helicobacter pylori* maximally induced macrophage inflammatory protein-2 (MIP-2) and inducible nitric oxide synthase (iNOS) mRNA only in wild-type mice. MIP-2 and iNOS mRNA were also induced by *H. pylori* in GSM06 cells. Induction of cyclooxygenase 2 mRNA through IFN-γ was demonstrated in GSM06 cells. These data indicate that IFN-γ mediates the induction of MIP-2 and iNOS mRNA expression by *H. pylori* in mice.

Helicobacter pylori colonizes the human gastric epithelium, causing chronic gastritis, peptic ulcer disease, and increased risk of cancer. Recent studies indicate that the host immune response contributes to the disease during infection with this organism (6, 17). Numerous studies have described a predominantly Th1 response with increased gamma interferon (IFN-γ) production in infections with *Helicobacter* species (1, 5, 14, 17). IFN-γ activates macrophages and natural killer cells and has been detected in *H. pylori*-positive gastric tissues in both humans and mice (1, 5, 14, 25). Several studies have shown that interleukin 8 (IL-8), a potent chemoattractant for polymorphonuclear leukocytes and T lymphocytes (4, 20), is stimulated by *H. pylori* in human gastric epithelial cell lines (12, 26) and, in vivo, in *H. pylori*-infected gastric biopsy specimens (8, 22, 33). Murine infection with *H. pylori* provides a model to study the host response to this organism, and less severe gastritis in mice deficient in IFN-γ has been reported (25, 27). Macrophage inflammatory protein-2 (MIP-2), a mouse counterpart of IL-8 (21, 29), has not been investigated with *H. pylori* infection.

In addition to these effectors, inducible forms of cyclooxygenase 2 (COX-2) and nitric oxide synthase (iNOS) have been shown to be upregulated during *H. pylori* gastritis (9, 10, 18, 24). Induction of COX-2 may contribute to either mucosal protection or inflammation during bacterial infection (9, 32). Likewise, the nitric oxide synthesized by NOS is cytoprotective at low doses but causes direct mucosal damage at high concentrations (2).

We postulated that the Th1 host response mediated by IFN-γ potentiates expression of inflammatory mediators (MIP-2, iNOS, and COX-2) in the murine gastric mucosa. Our findings suggest that IFN-γ is crucial to the maximal induction of iNOS and MIP-2 expression. To our knowledge, this is the first paper to show induction of MIP-2 by *H. pylori* in mice and murine gastric epithelial cells.

H. pylori infection studies in vitro were performed with the murine gastric epithelial cell line GSM06 (28). GSM06 cells, maintained in Dulbecco's modified Eagle's medium-F12 (Cellgro, Herndon, Kans.) supplemented with 10% fetal calf serum, were seeded into collagen-coated six-well tissue culture plates and grown to confluence (approximately 1.7×10^6 cells/ml) at 33°C in a CO_2 pouch (Becton Dickinson, Cockeysville, Md.). *H. pylori* SS1 (1.7×10^9 organisms/ml) was added, and the cells were incubated for 24 h. GSM06 cells were also stimulated with 10 ng of IFN-γ per ml prior to the addition of *H. pylori* (13, 31). All experiments were done in triplicate for all treatments. Cells were lysed by the addition of TRIzol reagent (Life Technologies, Grand Island, N.Y.), and total RNA was extracted as described in the TRIzol extraction protocol, followed by DNase treatment. For competitive reverse transcription (RT)-PCR of COX-1, COX-2, and MIP-2, plasmids were constructed to generate internal RNA competitors in vitro and mRNA was quantified as previously described (34). The amount of internal RNA competitors was determined by absorbance at 260 nm. The number of internal RNA competitor molecules per microliter was calculated based on the amount and size of the internal RNA competitor. To determine the amount of target RNA, a known amount of COX-1, COX-2, or MIP-2 internal RNA competitor was reverse transcribed in parallel with a constant amount of target total RNA from GSM06 cells. Following PCR amplification, target and competitor bands were compared on an agarose gel. Quantitation of iNOS mRNA was performed at the PCR step using an iNOS competitive kit from Maxim Biotech Inc. (San Francisco, Calif.). According to the manufacturer, this kit has a detection limit of 1.2 molecules, with a faint band visible on a 2% gel after 40 amplification cycles. *Taq* DNA polymerase (Qiagen Inc., Valencia, Calif.) was used for PCR with oligonucleotide primers shown in Table 1. PCR products were analyzed on a 2% agarose gel. Representative gels are shown in Fig. 1, with similar trends for all experiments.

iNOS mRNA was detected only in infected GSM06 cells (Fig. 1A, lanes 7 and 8) at 24 h. Cells stimulated with IFN-γ before addition of *H. pylori* expressed fourfold more COX-2 mRNA molecules (2×10^{11}) than the other treatment groups at both 12 h (data not shown) and 24 h (Fig. 1B, lane 19).

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TABLE 1. Oligonucleotide primers for PCR

mRNA	Sense and antisense primer sequences	PCR annealing temp, time, and no. of cycles	Size of PCR product (bp)	
			Standard	Target
COX-1[a]	AGTCGAAGGAGTCTCTCGCTCTGG CAGGAAATGGGTGAACGAGGGGCT	70°C, 30 s, 32	127	279
COX-2[a]	GCCCACCCCAAACACAGTGCAC GAGCCTTGGGGGTCAGGGATGAAC[d]	70°C, 30 s, 32	463	336
MIP-2[a]	CTGTTGTGGCCAGTGAACTGCG GGCTCCTCCTTTCCAGGTCAGT	62°C, 1 min, 30	300	238
iNOS[b,c]		56°C, 1 min, 30	200	231
GAPDH[b,c]		58°C, 2 min, 30	467	532

[a] Competition at the RT step. COX-1 and COX-2 primers were originally published by Mizuno et al. (19).
[b] Competition at the PCR step using a quantitative competitive PCR kit from Maxim Biotech (San Francisco, Calif.).
[c] Proprietary primer sequence.
[d] This sequence is different from that published (19), which was presented as the sense strand written in reverse.

COX-2 mRNA levels (5×10^{10}) were not altered in cells infected without stimulation (Fig. 1B, lane 15) or uninfected cells (Fig. 1B, lanes 3 and 9). Expression of COX-1 mRNA was not influenced by stimulation with IFN-γ and/or infection with *H. pylori* at 12 h (data not shown) or 24 h (Fig. 1C, lanes 1, 7, 13, and 19).

Infection of unstimulated cells with *H. pylori* resulted in a fourfold increase of MIP-2 mRNA at 12 h (data not shown) and a twofold increase at 24 h (Fig. 1D, lane 17) over levels in uninfected cells. However, stimulation with IFN-γ before addition of *H. pylori* resulted in a 32-fold increase in MIP-2 mRNA expression at both 12 h (data not shown) and 24 h (Fig. 1D, lane 19) compared to uninfected cells. No difference was observed in MIP-2 mRNA levels in uninfected cells at 12 h (data not shown) or 24 h (Fig. 1D, lanes 6 and 12). As a positive control, glyceraldehyde-3-phosphate dehydrogenase

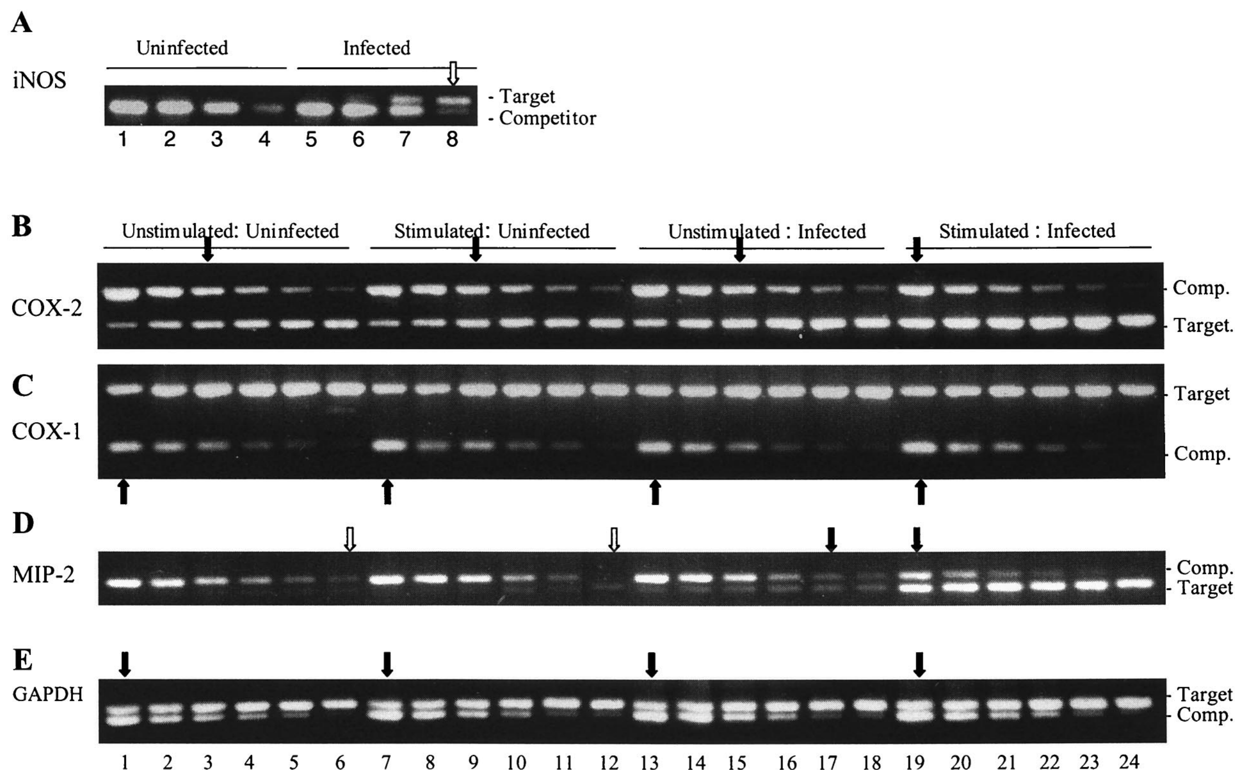

FIG. 1. Representative gels showing competitive RT-PCR of GSM06 cells infected with *H. pylori* for 24 h. (A) Tenfold serial dilutions of iNOS competitor (1.2×10^4 to 1.2×10^1 molecules) were amplified in parallel with target cDNA from uninfected (lanes 1 to 4) and infected (lanes 5 to 8) cells. In addition, GSM06 cells were stimulated with IFN-γ before addition of *H. pylori* to the cell monolayer (B to E). A twofold serial dilution of competitor and constant amount of target from unstimulated and uninfected cells (lanes 1 to 6), stimulated and uninfected cells (lanes 7 to 12), unstimulated and infected cells (lanes 13 to 18), and stimulated and infected cells (lanes 19 to 24) is shown for COX-2, COX-1, MIP-2, and GADPH (B to E, respectively). The starting concentrations for the competitor were 2×10^{11} molecules for COX-2 (B) and COX-1 (C), 1.5×10^{10} molecules for MIP-2 (D), and 6×10^4 molecules for GADPH (E). Closed arrows indicate equivalent competitor and target mRNA levels. Open arrows indicate the lowest dilution of competitor used for RT-PCR.

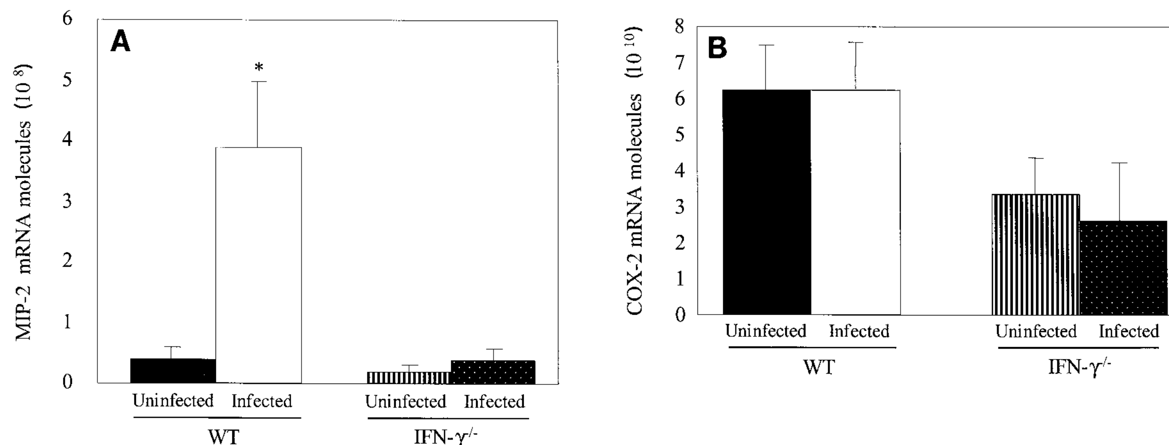

FIG. 2. Quantitation of MIP-2 (A) and COX-2 (B) mRNA expression in mouse stomach tissues by competitive RT-PCR. Results are means ± standard errors for four mice per treatment group. *, $P < 0.05$ compared to results for uninfected mice. For ease of comparison between treatment groups, the values correspond to mRNA molecules present in the total RNA used for competitive RT-PCR. For any one mRNA species tested, the same amount of total RNA was used for RT-PCR quantification in all mice. Five micrograms of total RNA was used to quantitate MIP-2, and 1 μg was used for COX-2.

mRNA expression was not influenced by infection with *H. pylori* or stimulation of GSM06 cells with IFN-γ (Fig. 1E).

To corroborate our in vitro studies, C57BL/6 wild-type (WT) and IFN-$\gamma^{-/-}$ mice obtained from Jackson Laboratory (Bar Harbor, Maine) were divided into two groups: uninfected mice ($n = 4$) and mice infected with *H. pylori* strain SS1 ($n = 4$) (15) as previously described (6). Sixteen weeks postinoculation, mice were euthanized, and the stomachs were removed and divided into three longitudinal sections. Serial dilutions of a weighed and homogenized longitudinal section of mouse stomach were inoculated on Columbia blood agar plates (3) containing antibiotics (5× Skirrow supplement; Oxoid, Basingstoke, Hampshire, England). All *H. pylori*-infected mice were colonized, as confirmed by colony morphology, microscopic examination, and urease assay. Colonization ranged from 1.8×10^4 to 3.9×10^5 CFU/g of gastric tissue, and no major differences were observed between WT and IFN-$\gamma^{-/-}$ mice. A longitudinal section was processed for histology, pathological changes were scored by a method described by Eaton et al. (6), and differences in histology between treatment groups were analyzed by the Mann-Whitney *U* test. Infected mice had a tendency toward higher levels of gastritis and adenitis than uninfected mice. All animal procedures were approved by the veterinary medical unit of the Veterans Affairs Hospital, San Diego, Calif.

For RNA extraction, a longitudinal stomach section was homogenized in TRIzol reagent and total RNA processed for RT-PCR. Differences in mRNA of inflammatory effectors between treatment groups were analyzed by the Mann-Whitney *U* test. A *P* value of <0.05 was considered statistically significant. mRNA molecules for MIP-2 and COX-2 in gastric tissue were averaged per treatment group (four mice), and the results are shown in Fig. 2. Infection of WT mice with *H. pylori* resulted in over 10 times more MIP-2 mRNA expression (3.88×10^8) than in uninfected mice (3.25×10^7 molecules, $P < 0.05$) (Fig. 2A). Infected IFN-$\gamma^{-/-}$ mice had approximately twice the amount of MIP-2 mRNA in uninfected IFN-$\gamma^{-/-}$ mice, but this difference was not statistically significant in this experiment. It is possible that use of a larger number of mice could have shown minor differences.

Infection with *H. pylori* did not alter COX-2 mRNA levels in either WT or IFN-$\gamma^{-/-}$ mice (Fig. 2B). Although IFN-$\gamma^{-/-}$ mice had lower COX-2 mRNA levels, they were not significantly different from those in WT mice. Concomitantly, no difference was noted in COX-1 message levels between uninfected and infected mice in both WT and IFN-$\gamma^{-/-}$ mice (data not shown).

Because of lower expression of iNOS mRNA in these mice, the total RNA from mouse stomachs in similar treatment groups was pooled. iNOS mRNA was detected only in samples from *H. pylori*-infected WT mice (Fig. 3, lanes 8 and 9). IFN-$\gamma^{-/-}$ mice, irrespective of infection, did not have detectable iNOS mRNA (Fig. 3). These results suggest that IFN-γ is

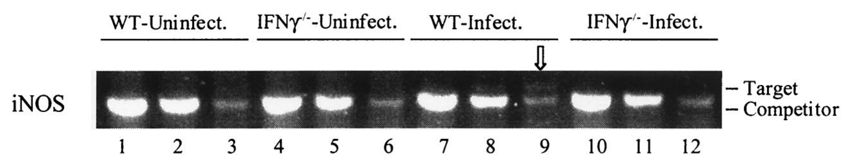

FIG. 3. Quantitation of iNOS mRNA expression in mouse stomach tissue. The gel shows RT-PCR data from pooled samples from each treatment group. Tenfold serial dilutions of competitor cDNA starting with 1.2×10^3 molecules per sample were amplified in parallel with target cDNA from uninfected WT (lanes 1 to 3), uninfected IFN-$\gamma^{-/-}$ (lanes 4 to 6), infected WT (lanes 7 to 9), and infected IFN-$\gamma^{-/-}$ (lanes 10 to 12) mice. The arrow indicates the lowest dilution of competitor used for RT-PCR.

important for induction of both MIP-2 and iNOS by *H. pylori* in mice.

In summary, the mRNA expression pattern of MIP-2, COX-1, and iNOS was similar in in vivo and in vitro studies. Expression of MIP-2 mRNA in both infected mice and infected GSM06 cells was higher than in uninfected samples. In both systems, maximal expression of MIP-2 was dependent on IFN-γ. COX-1 remained constitutively expressed in all treatment groups, both in mice and in GSM06 cells. In contrast, in both in vivo and in vitro studies, iNOS mRNA was detected only in infected samples. COX-2 mRNA was induced in infected GSM06 cells, but C57BL/6 mice constitutively expressed high levels of COX-2 mRNA.

In this study, we hypothesized that IFN-γ, a major mediator of the Th1 immune response, induces proinflammatory responses in the gastric mucosa during *H. pylori* infection. In support of this proposal, we found that IFN-γ greatly enhanced expression of a murine IL-8 analogue, MIP-2, in response to *H. pylori* incubation with cultured gastric epithelial cells and during extended gastric infection in vivo. A number of studies have focused on the ability of *H. pylori* to stimulate IL-8 expression in human gastric epithelial cell lines (12, 26) and in gastric tissue from biopsy specimens (8, 22, 33). However, van Doorn et al. (30) reported a lack of IL-8 induction by *H. pylori* strain SS1 in human gastric epithelial (KATO III) cells. To observe a significant induction of MIP-2 mRNA by *H. pylori* in the murine gastric cell line, cells required stimulation with IFN-γ prior to infection. Strain SS1 is commonly used in murine models of *H. pylori* infection and induces inflammatory changes in the gastric mucosa. Since murine gastric epithelial cells also produce MIP-2 upon costimulation with IFN-γ and *H. pylori* SS1, the findings also suggest that the epithelium could be a significant source of proinflammatory signaling during infection.

We did not observe a difference in either COX-1 or COX-2 mRNA levels between infected and uninfected mice, irrespective of IFN-γ status. These results are in agreement with a previously unpublished experiment in which we studied the effect of indomethacin treatment on COX mRNA expression. Indomethacin is a synthetic nonsteroidal anti-inflammatory inhibitor for both forms of COX (7). No difference was observed in either COX-1 or COX-2 mRNA between uninfected and infected mice or between mice that received indomethacin and those that did not (data not shown). In contrast to our in vivo studies, *H. pylori* induced COX-2 fourfold when GSM06 cells were stimulated with IFN-γ. These results are similar to those reported for human gastric cell lines. Romano et al. (23) reported a fivefold upregulation of COX-2 in *H. pylori*-infected human gastric MKN28 cells. IFN-γ is known to be upregulated during *H. pylori* infection (1, 5, 14, 25). In our in vitro study, addition of IFN-γ resulted in the induction of COX-2 mRNA by *H. pylori*. We therefore expected to find higher levels of COX-2 in infected WT mice. Our studies highlight the importance of doing both in vivo and in vitro analyses. Perhaps COX-2 mRNA is already maximally expressed in C57BL/6 mice. Alternatively, the upregulation of COX-2 occurs only in epithelial cells in vivo, and thus, the mRNA levels may have been diluted out by those from other cells in the stomach. A recent study indicates that COX-2 regulation in murine peritoneal macrophages occurs at the posttranscriptional level (16). In any case, these data indicate that COX-2 mRNA expression in the murine gastric mucosa is not dependent on IFN-γ.

In both in vitro and in vivo studies, except in IFN-$\gamma^{-/-}$ mice, infection with *H. pylori* resulted in expression of iNOS mRNA. Induction of iNOS in infected WT but not IFN-$\gamma^{-/-}$ mice suggests that IFN-γ is important for iNOS induction in gastric tissues. Guo et al. (11) reported induction of iNOS expression by IFN-γ in vitro using human epithelial cells. iNOS mRNA induction by *H. pylori* in vitro and in *H. pylori*-positive gastritis biopsies has been reported (9, 10, 24). In addition, since COX-2 mRNA expression was unchanged in all treatment groups, it appears that iNOS and COX-2 are not coregulated at the transcriptional level in gastric tissue.

H. pylori is a mucosal pathogen that induces chronic inflammation, in part by stimulating an inappropriate Th1-type immune response that is insufficient to clear the organism. Our results provide a mechanistic link in the mouse between IFN-γ, a key Th1 cytokine, and the production of MIP-2, a potent proinflammatory chemokine that is coinduced by *H. pylori* and IFN-γ, both in vitro and in vivo. Because of the central role of IFN-γ in regulating these inflammatory mediators, this study supports the role of the Th1 immune response in mediating chronic gastric inflammation during *H. pylori* infection. Our results also downplay the importance of COX-2 expression during gastritis in this animal model. While expression of iNOS mRNA was detected only in infected WT mice and GSM06 cells, the increases were not sufficient to allow conclusions to be drawn on their own merit. However, our data corroborate other numerous studies in humans that show upregulation of iNOS by *H. pylori* infection (9, 10, 24).

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