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Interleukin-8 Response of Gastric Epithelial Cell Lines to *Helicobacter pylori* Stimulation In Vitro

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Gastric infection with *Helicobacter pylori* activates a mucosal inflammatory response by mononuclear cells and neutrophils that includes expression of cytokines interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha, and IL-8. In this study, we analyzed the IL-8 response of human gastric cancer cell lines (Kato III, AGS, and MKN28) to *H. pylori* infection in vitro. IL-8 mRNA expression was detected by reverse transcription-PCR amplification of RNA extracted from epithelial cells after incubation with different *H. pylori* wild-type and mutant strains, and IL-8 secretion was measured by an enzyme-linked immunosorbent assay. Exposure to viable *H. pylori* induced IL-8 mRNA and protein synthesis in all three gastric cell lines but not in nongastric epithelial cell lines. Heat-killed *H. pylori* and a crude cytotoxin preparation did not induce significant IL-8 secretion. IL-8 mRNA peaked between 2 and 4 h postinfection, and IL-8 protein production was maximal 24 h postinfection. Exposure of gastric carcinoma cells to other gastrointestinal bacteria, such as *Pseudomonas aeruginosa*, *Campylobacter jejuni*, and *Escherichia coli*, but not *Campylobacter fetus*, induced IL-8 synthesis. Wild-type strains that expressed the vacuolating cytotoxin (Tox^+) and a cytotoxin-associated gene (*cagA*) product ($CagA^+$) induced significantly more IL-8 than did $CagA^-$ Tox^- strains. However, there was no decrease in IL-8 induction by isogenic mutants of $CagA^-$, Tox^-, or Cag^- Tox^- strains or by a mutant lacking the urease subunits. These results indicate that exposure to *H. pylori* and other gram-negative organisms that do not colonize the gastric mucosa induces IL-8 production by gastric carcinoma cells in vitro. Although the $CagA^+$ Tox^+ phenotype of *H. pylori* is associated with enhanced IL-8 production by gastric cell lines, other bacterial constituents are clearly essential.

Infection with *Helicobacter pylori* leads to diverse clinical and pathological outcomes in humans. Infected persons may be asymptomatic carriers with chronic superficial gastritis, or they may develop gastric or duodenal ulceration or adenocarcinoma of the stomach (2, 4, 5, 20, 27, 39). The basis for this variation is not known. Preliminary findings indicate that both bacterial and host factors may be important in leading to particular clinical and pathological sequelae of infection (4, 5).

In infected persons, the gastric mucosa shows acute and chronic inflammation and a specific form of epithelial cell degeneration, characterized by loss of apical mucus from the gastric cells, cell dropout, spotty necrosis, and microerosions (31). A key question is how these noninvasive bacteria residing in the mucus layer overlying the epithelium produce inflammation and cause damage to the underlying tissue. Epithelial cell damage may occur as a direct effect of bacterial secretory products or result as a consequence of the chronic and acute inflammatory responses induced by *H. pylori* (4, 5, 16, 30). Several potential bacterial virulence factors that may contribute to mucosal inflammation and epithelial cell damage have been identified (4, 30). Two of these factors, which are known to differ among *H. pylori* strains and are significantly associated with peptic ulceration in *H. pylori*-infected persons (9, 16, 26, 28, 48), are the presence of high-molecular-weight proteins encoded by *cagA* (9, 11, 49) and the expression of an 87-kDa vacuolating cytotoxin (encoded by *vacA*) which is toxic to epithelial cells in vitro (10, 11, 32).

In vivo, gastric infection with *H. pylori* induces the mucosal production of various cytokines in the host, including interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) (14, 15, 17, 37). IL-8, a potent T-cell and neutrophil recruitment factor, is produced by various cell types, including macrophages, T cells, endothelial cells, keratinocytes, and epithelial cells (40), and elevated levels of IL-8 have been reported in a number of inflammatory conditions, including psoriasis, inflammatory bowel disease, rheumatoid arthritis, cystic fibrosis, septic shock, and acute meningococcal infections (47). IL-1 or TNF-α alone and gamma interferon in synergy with TNF-α can induce IL-8 production in gastric cancer cell lines (51). Thus, enhanced IL-8 production, which occurs during infection, ischemia, trauma, and other disturbances of tissue homeostasis, also may occur secondary to up-regulation of IL-1 or TNF-α, which is observed during *H. pylori* infection (15, 37). Prolonged IL-8 production by gastric epithelial cells during *H. pylori* infection could result in the recruitment of neutrophils and lymphocytes to infected tissues and therefore play a role in the immunopathogenesis of *H. pylori* infection. The goals of this study were to determine the characteristics of *H. pylori* induction of IL-8 expression in gastric epithelial cell lines and to examine whether there are substantial differences among wild-type clinical *H. pylori* isolates and defined mutants in the ability to induce IL-8 gene expression and protein secretion.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *H. pylori* clinical isolates used in this study and their origins are listed in Table 5. Five strains, 88-23 (60190), 84-183, 87-199, 87-29, and 92-25, express the vacuolating cytotoxin (i.e., are Tox^+) and possess *cagA* (10, 11, 49). Strains 88-22 (Tx-30a), 86-313, 87-203, 92-23, and 92-24 were wild-type strains that did not possess *cagA* and did not detectably express cytotoxin activity (i.e., were Tox^-) in vitro (10, 11, 49). Isogenic *cagA* or *vacA* mutants were prepared by insertion of a kanamycin resistance gene within the *cagA* or *vacA* loci, and the isogenic $CagA^-$ and $VacA^-$ double mutant of *H. pylori* was created by inserting chloramphenicol and kanamycin resistance markers, respectively, as previously described (50). As expected, these strains show no detectable presence of the products of the uninterrupted genes (12, 50). The urease mutant had been selected for resistance to 60 mM urea and does not express either the 66- or 31-kDa urease subunit (encoded by *ureB* or *ureA*, respectively), as described previously (41). For all incubations with cell cultures, bacterial cultures grown for 24 to 48 h were harvested in phosphate-buffered saline (pH 7.4) and suspended to yield a concentration of 5×10^{10} CFU/ml. For some experiments, *H. pylori* 88-23 cells were heated to 65°C for 60 min.

Campylobacter jejuni 81-176, *Pseudomonas aeruginosa* 27239, *Campylobacter fetus* 23D, and *Escherichia coli* DH5α used in this study were from the culture collection of the Vanderbilt University Campylobacter/Helicobacter Laboratory. Stock cultures were maintained at −70°C in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% glycerol. *H. pylori* isolates were cultured on 5% sheep blood agar plates in a microaerobic atmosphere (generated by Campy Pak-Plus [BBL]) at 37°C for 24 to 48 h. *E. coli*, *C. fetus*, *C. jejuni*, and *P. aeruginosa* strains were cultured on tryptic soy agar with 5% sheep blood plates (BBL).

Cell cultures. Human gastric cancer cell lines AGS (ATCC CRL 1739) (3), Kato III (ATCC HTB103) (45), and MKN28 (43) and the laryngeal carcinoma cell line HEp-2 (ATCC CCL23) (34) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; HyClone Laboratory, Logan, Utah), 20 μg of gentamicin per liter, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, and 2 mM glutamine. BEAS-2B (ATCC CRL 9609), a human bronchial epithelial cell line (42), was maintained in serum-free LHC-8 medium. AGS, Kato III, and BEAS-2B were obtained from the American Type Culture Collection (Rockville, Md.). MKN28 was kindly provided by Robert Coffey. Fifty-milliliter flasks (Falcon; Becton Dickinson, Lincoln Park, N.J.) were seeded with stock cultures of the above-mentioned cell lines taken from liquid nitrogen and incubated at 37°C in an ambient atmosphere with 5% CO_2 until cells were confluent. The cells were then removed from the flasks by 0.05% trypsin-EDTA treatment (Gibco BRL, Life Technologies, Grand Island, N.Y.) for 10 min at room temperature and then harvested by centrifugation at $200 \times g$ for 10 min, the supernatant was discarded, and the cells were resuspended in RPMI 1640 with 5% FCS. The cell suspension was diluted in fresh medium to a final concentration of 10^5 cells per ml, seeded into six-well tissue culture plates (Costar, Cambridge, Mass.), and allowed to grow 2 to 3 days to confluency. The cell density per well at confluency was between 1.5×10^6 and 2×10^6 cells.

Assay for IL-8 induction by epithelial cells. The human gastric carcinoma cell lines Kato III, MKN28, and AGS were cured of mycoplasma infection prior to these experiments. Reverse transcription (RT)-PCR was performed on cDNA from the epithelial cells and showed that all expressed IL-8 mRNA constitutively under routine growth conditions in RPMI 1640 with 5 to 10% FCS (data not shown). This finding was consistent with earlier reports showing that two gastric carcinoma cell lines, Kato III and MKN45, constitutively express IL-8 mRNA, as determined by PCR and by Northern (RNA) analysis, which is increased by stimulation with TNF-α (17, 51). We therefore established culture conditions under which the cell lines remained viable but which minimized IL-8 secretion. Confluent monolayers cultured in six-well plastic tissue culture plates in serum-free RPMI 1640 showed little IL-8 production and responded to exogenous stimuli for IL-8 production. Therefore, cells were cultured overnight in serum-free medium for all experiments. At the onset of each experiment, media were replaced with either RPMI 1640 alone, phorbol myristate acetate (PMA; 100 ng/ml) plus ionomycin (200 nM), or RPMI 1640 containing live bacteria at a concentration of 10^9/ml (bacterium-to-cell ratio of 1,000:1), a ratio at which *H. pylori* adherence to gastric epithelial cells is saturated. This ratio was based on earlier observations of cell adherence; when measured by flow cytometry, the mean fluorescence intensity values were nearly identical at bacterium-to-cell ratios of 100:1 and 1,000:1 (21). Supernatants were removed from the wells at the time intervals described for each experiment and centrifuged at $15{,}000 \times g$ before freezing at −70°C until further analysis of IL-8 protein by enzyme-linked immunosorbent assay (ELISA). The epithelial cells were harvested directly into guanidium thiocyanate lysis buffer (Tri-reagent) for preparation of mRNA and subsequent cDNA synthesis. Epithelial cell lines of non-gastrointestinal tract origin, HEp-2 (laryngeal carcinoma) and BEAS-2B (bronchial carcinoma), were used as controls; BEAS-2B cells secrete IL-8 in response to respiratory syncytial virus (38).

RNA preparation. Total cellular RNA was extracted from epithelial cells with Tri-reagent as previously described (8), and quantitation of the purified RNA was performed by measuring absorbance at 260 nm. Briefly, 1.5×10^6 to 2×10^6 cells grown in monolayers were directly lysed with Tri-reagent and then subjected to phase separation using chloroform. Following centrifugation at $12{,}000 \times g$, the RNA, which exclusively remains in the aqueous phase, was removed and precipitated with isopropanol. The RNA pellet obtained after centrifugation at $12{,}000 \times g$ was washed in 75 to 80% ethanol and subsequently centrifuged at $12{,}000 \times g$. The RNA pellet obtained was air dried and resuspended in 15 μl of diethyl pyrocarbonate-treated water at 37°C for 15 to 20 min.

TABLE 1. Effect of adding serum to culture medium on IL-8 production by AGS cells

Addition to cultures	Mean IL-8 concn (pg/ml) ± SEM (n = 3)			
	6 h		24 h	
	No serum	Serum[a]	No serum	Serum
None (medium control[b])	50 ± 18	65 ± 7	60 ± 28	123 ± 28
Viable *H. pylori* cells[c]	1,053 ± 440	1,652 ± 483[d]	2,687 ± 764	2,577 ± 837

[a] 5% FCS.
[b] RPMI 1640 medium.
[c] 10^9 cells from 48-h cultures of strain 88-23 suspended in RPMI 1640.
[d] IL-8 induction was significantly greater in the presence of serum than in serum-free medium (P = 0.004).

RT and PCR. cDNA was synthesized from 2 μg of total RNA obtained from cultured human epithelial cells, by priming with 1 μg of oligo(dT) primer, 200 nmol of each deoxynucleoside triphosphate (dNTP), 20 U of RNase inhibitor, and RNase H^- Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) at 100 U per μg of RNA in a final volume of 25 μl at 42°C for 1 h. cDNA equivalent to 80 ng of starting RNA was used for each PCR with primers for human IL-8 or control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ClonTech, Palo Alto, Calif.). Primers used were as follows. GAPDH, 5′TGAAGGTCGGAGTCAACGGATTTGGT3′ (sense) and 5′CATGTGGGCCATGAGGTCCACCAC3′ (antisense); IL-8, 5′ATGACTTCCAAGCTGGCCGTGGC3 (sense) and 5′TCTCAGCCCTCTTCAAAAACTTCTC3′ (antisense). PCRs were performed in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.2 mM dNTP–2 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a final volume of 50 μl. Primers were added at a final concentration of 0.5 μM. Reactions were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 30 (IL-8) or 39 (GAPDH) cycles, each including denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. PCR products were analyzed on ethidium bromide-stained 2% agarose gels. To control for false-positive results due to contaminating genomic DNA, the primers used for RT-PCR spanned introns, based on the published genomic DNA sequences for these genes (24, 35). In this manner, if the primers amplify genomic DNA, the resulting amplified product will be of a larger size than products amplified from cDNAs that lack the intron. In the experiments reported, the sizes of the amplified products were those predicted for amplification of cDNA and no larger products were seen. All PCR experiments included a positive control, which was a cDNA supplied by the manufacturer (ClonTech), and a negative control without template to ensure absence of contamination.

IL-8 protein assay. IL-8 protein levels in cell culture supernatants were determined by use of a human IL-8 sandwich ELISA kit (R&D Systems, Minneapolis, Minn.) and expressed as picograms per milliliter. The lower limit of detection for IL-8 was 32 pg/ml. Differences between cytokine levels were evaluated by Student's t test and were considered significant when P was ≤0.05.

RESULTS

Gastric epithelial cell expression of IL-8. As a source of human gastric epithelial cells, we used three cell lines, AGS, Kato III, and MKN28. RT-PCR performed on cDNA from these cells showed that all expressed IL-8 mRNA constitutively under routine growth conditions in RPMI 1640 with 5 to 10% FCS (data not shown), which was consistent with prior observations that show that both normal and malignant gastric epithelial cells express IL-8 in vivo and in vitro (17). Incubation of cells overnight without serum, however, minimized IL-8 expression and allowed analysis of response to exogenous stimuli. In addition, at 6 h, IL-8 secretion in *H. pylori*-stimulated cultures was significantly greater in the presence of serum than in serum-free medium (P = 0.004) (Table 1). This finding indicated that there was synergism between the bacteria and

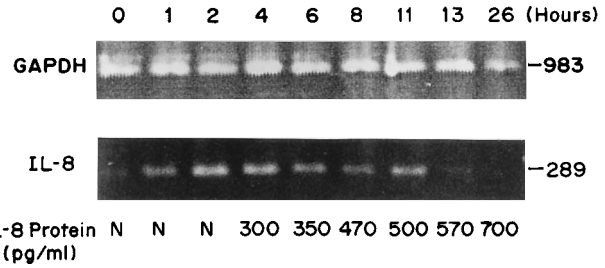

FIG. 1. Time course of IL-8 mRNA and protein induction in AGS gastric epithelial cells by live *H. pylori* 88-23 cells. Data shown are from a representative gel electrophoresis of RT-PCR amplification products of GAPDH and IL-8 mRNAs and IL-8 protein levels in culture supernatants from AGS epithelial cells after stimulation with viable *H. pylori* 88-23 at various time intervals from 0 to 26 h. Peak mRNA expression was seen between 2 and 4 h, and IL-8 secretion remained at high levels after 4 h. N, no product detected (<32 pg/ml). Background secretion of IL-8 in unstimulated AGS cultures was <32 pg/ml at 26 h. Sizes are indicated in base pairs.

serum components in IL-8 induction. However, by 24 h, there was little difference in IL-8 levels in *H. pylori*-stimulated cultures in the presence or absence of serum. In this and subsequent experiments, *H. pylori*-induced IL-8 secretion by AGS cells ranged from 11- to 45-fold stimulation over the medium control background level. This wide range was due in part to variation in background levels of IL-8 secretion from <50 to 300 pg/ml.

Using the conditions specified above, we first studied the kinetics of *H. pylori*-induced IL-8 mRNA expression in AGS cells. As detected by RT-PCR, IL-8 mRNA transcription was evident in AGS cells within an hour of exposure to *H. pylori* 88-23. Induction was maximal after 2 to 4 h of incubation and then tapered after 11 h (Fig. 1). Amplification of the same cDNA with primers for GAPDH demonstrated that expression of transcript for this constitutive enzyme was unaffected. We also examined by ELISA IL-8 protein levels in the supernatants of AGS cells exposed to *H. pylori* at various time intervals. Incubation of AGS cells with *H. pylori* 88-23 increased IL-8 production in a time-dependent manner (Fig. 1). IL-8 secretion was detectable at 4 h of exposure to *H. pylori* and continued through 24 h of exposure, reaching a peak of 700 pg/ml. These data demonstrate that incubation of AGS cells with *H. pylori* induces increased IL-8 secretion. After 24 h of exposure, the integrity of the AGS monolayer was compromised (AGS cell viability was reduced 38% as assessed by trypan blue staining), and considerable numbers of detached cells in the cultures stimulated by bacteria were evident. IL-8 induction by AGS gastric epithelial cells was dependent on the viability of the bacteria, since heat-killed preparations and a crude cytotoxin preparation did not significantly induce IL-8 secretion (Table 2).

Target cell specificity of *H. pylori* for IL-8 induction. We next analyzed the target cell specificity of IL-8 induction by examining responses of other gastric and nongastric cell lines. Stimulation of all three gastric cell lines, Kato III, AGS, and MKN28, in serum-free medium with control PMA and ionomycin or with live *H. pylori* cells induced expression of IL-8 mRNA (Fig. 2A). In contrast, in HEp-2 cells, *H. pylori* did not induce IL-8 mRNA after 2 h of incubation, whereas expression of the GAPDH housekeeping gene was unchanged (Fig. 2B). In parallel with increased IL-8 mRNA levels, IL-8 secretion in culture supernatants after exposure to *H. pylori* 88-23 also was substantially higher than in unstimulated cultures (Table 3; AGS, $P = 0.02$; Kato III, $P = 0.005$; MKN28, $P = 0.08$). In contrast, 24-h exposure to *H. pylori* did not induce IL-8 production in nongastric cell lines HEp-2 and BEAS-2B, although these cells could be stimulated to produce IL-8 by PMA and ionomycin (Table 3). The data demonstrate that *H. pylori* 88-23 shows specificity in its ability to induce IL-8 production by transformed cell lines.

TABLE 2. Stimulation of IL-8 protein production in AGS gastric epithelial cells by *H. pylori* 88-23 preparations requires viable bacteria

Expt	Addition to cultures (no. of determinations)	Mean IL-8 concn (pg/ml) ± SEM	
		6 h	24 h
A	None (medium control[a]) (4)	123 ± 74	60 ± 20
	Viable *H. pylori* cells[b] (4)	1,692 ± 558	2,117 ± 624
	Heat-killed *H. pylori* cells[c] (4)	66 ± 25	162 ± 79
	PMA + ionomycin[d] (4)	1,926 ± 316	1,899 ± 619
B	Medium control (2)	<32	<32
	PMA + ionomycin (2)	1,548 ± 172	996 ± 338
	Crude cytotoxin[e] (2)	34 ± 3	35 ± 3

[a] RPMI 1640 medium without serum.
[b] 10^9 *H. pylori* 88-23 cells from 48-h cultures suspended in RPMI 1640.
[c] Cells were heated to 65°C for 60 min.
[d] PMA, 100 ng/ml; ionomycin, 200 μM.
[e] Culture supernatants from *H. pylori* cells grown in brucella broth with 0.5% charcoal and then precipitated in 50% ammonium sulfate (90 μg/ml).

Bacterial specificity for IL-8 induction in AGS cells. To determine whether other gram-negative bacteria can induce IL-8 production by gastric cells, AGS cells were incubated with live *C. jejuni*, *C. fetus*, *P. aeruginosa*, or *E. coli* cells. Increased levels of IL-8 were observed in culture supernatants after incubation for 24 h with each of the tested bacteria, ranging from a 1.6-fold increase for *C. fetus* to a 16.7-fold increase for *H. pylori* (Table 4). These data indicate that IL-8 production by AGS cells is not specific for *H. pylori*.

Variability among *H. pylori* clinical isolates in IL-8 induc-

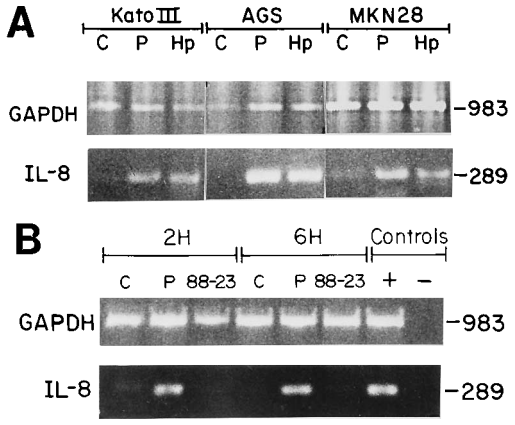

FIG. 2. (A) Live *H. pylori* 88-23 cells induce IL-8 mRNA in three gastric cancer cell lines. Data shown are from a representative gel electrophoresis of RT-PCR amplification products of GAPDH and IL-8 mRNAs for 2 h from Kato III, AGS, and MKN28 gastric cancer cell lines after stimulation with control medium alone (C), PMA plus ionomycin (P), and viable *H. pylori* (Hp) 88-23 cells (10^9 CFU/ml). (B) Live *H. pylori* 88-23 fails to induce IL-8 mRNA in a nongastric epithelial cell line (HEp-2). Data shown are from a representative gel electrophoresis for RT-PCR amplification products of GAPDH and IL-8 mRNAs from HEp-2 cells after stimulation for 2 or 6 h with control medium (C), PMA plus ionomycin (P), and viable *H. pylori* 88-23 cells (10^9 CFU/ml). +, PCR positive control cDNA preparation for GAPDH or IL-8, −, negative control. Sizes are indicated in base pairs.

TABLE 3. *H. pylori*-induced IL-8 secretion by gastric and nongastric epithelial cells[a]

Addition to cultures	Mean concn of IL-8 secreted (pg/ml) ± SEM				
	Gastric			Non-gastric	
	AGS (n = 7)	Kato III (n = 3)	MKN28 (n = 3)	HEp-2 (n = 2)	BEAS-2B (n = 2)
None (medium control)	83 ± 30	517 ± 279	3,283 ± 1,169	230 ± 50	865 ± 335
PMA + ionomycin[b]	1,950 ± 728	6,450 ± 2,050	23,267 ± 11,349	4,400 ± 200	2,300 ± 300
H. pylori 88-23	2,514 ± 964	3,933 ± 635	13,233 ± 5,881	200 ± 40	1,400 ± 300

[a] Cultures of human epithelial cells were infected with viable *H. pylori* 88-23 as described in Materials and Methods, and after 24 h in culture, the concentration of IL-8 was determined in the supernatants.
[b] PMA, 100 ng/ml; ionomycin, 200 μM.

tion. All *H. pylori* isolates are phenotypically similar except for the recognized differences in the expression of a vacuolating cytotoxin (encoded by *vacA*) and the *cagA* product (11, 49). Strain 88-23, which induced IL-8 in AGS cells, is $CagA^+$ Tox^+. To determine whether the ability to induce IL-8 was specific for the $CagA^+$ Tox^+ phenotype or common to all strains, we next examined nine other clinical isolates differing in expression of CagA and cytotoxin. All $CagA^+$ Tox^+ strains induced IL-8 mRNA and showed significantly increased levels of IL-8 protein in AGS cell culture supernatants compared with $CagA^-$ Tox^- strains (P = 0.008) (Table 5). These results suggested that CagA and cytotoxin production may be phenotypic markers for IL-8 induction.

IL-8 induction by defined mutants of strains 88-23 and 84-183. To determine directly whether the CagA protein or the vacuolating cytotoxin is required for IL-8 production by AGS cells, we tested several isogenic mutants of *H. pylori* 88-23 and 84-183. These mutants lack expression of either CagA, cytotoxin, or both. We also tested a mutant of strain 88-23 that lacked both urease subunits (41). Mutants of *H. pylori* lacking expression of CagA, cytotoxin, or urease were able to induce AGS cells to express IL-8 mRNA and protein as well as the parental wild-type strains (Fig. 3; Table 6). In a second gastric cell line (Kato III), mRNA levels detected by RT-PCR for the $CagA^-$ mutants were essentially the same as those shown for the parental strains (data not shown). Strain 84-183 and its derivatives consistently induced lower levels of IL-8 protein than did strain 88-23 and its derivatives (Table 6). These results indicate that IL-8 induction of gastric epithelial cells by *H. pylori* cells is independent of CagA, cytotoxin, and urease production.

DISCUSSION

Chemokines belonging to the CXC intercrine family of cytokines, such as IL-8, play a major role in mobilizing cellular defense mechanisms to eliminate pathogens by recruiting and activating neutrophils and T cells (40). IL-8 also has been shown to enhance the killing of intracellular mycobacteria by nonoxidative mechanisms (36). Recent studies indicate that IL-8 secreted by epithelial cells may be the initial signal for the acute inflammatory response following bacterial invasion of mucosal surfaces (23). In *H. pylori* infection, there is increased mucosal production of IL-8 (14), and by immunofluorescence, the cytokine was localized to the epithelium and lamina propria in *H. pylori*-colonized gastric mucosa (17). IL-8 thus may be an important mediator of the inflammatory response that contributes to mucosal injury in *H. pylori* infection. As *H. pylori* is a lumenal organism, detected only in the gastric mucus layer or found attached to the surface of gastric epithelial cells both in vivo and in vitro (6, 7, 21, 25, 29), we sought to develop an in vitro model for IL-8 induction in gastric epithelial cells to characterize this host-pathogen interaction.

Our results show that interaction of viable *H. pylori* with gastric epithelial cells results in increased levels of IL-8 mRNA and protein secretion, which corroborates recent reports (13, 18). The kinetics of IL-8 gene expression induced by *H. pylori* were similar to that reported for other enteric organisms such as *Salmonella dublin* in intestinal epithelial cells (23), as well as for IL-1α and TNF-α- and PMA-induced IL-8 mRNA in many cell types (40).

Interestingly, we found heterogeneity in responses to different *H. pylori* strains. Most phenotypic traits, including motility, adhesiveness, and urease production, which appear to be essential for efficient survival and colonization (5, 22, 30), are conserved among *H. pylori* strains. However, 50 to 60% of strains differ in expression of the vacuolating cytotoxin and the *cagA* product (11, 32, 49), and these are potential virulence factors that may influence the clinical outcome of *H. pylori* infection (26, 28, 48). Although expression of these two proteins in clinical isolates is usually concordant, the genes encoding these proteins map to different loci, and mutagenesis of *cagA* resulting in the loss of CagA expression does not affect cytotoxin expression, indicating that the proteins are expressed independently (50). The gene (*vacA*) encoding the toxin is present in strains regardless of whether they produce the vacuolating cytotoxin in vitro, but nucleotide and deduced amino acid sequences diverge (12). In contrast, strains that do not express the CagA protein lack *cagA* in any form (9, 49). Strains that vary in production of the cytotoxin or CagA all produce urease, a highly conserved property. In our studies, using a variety of clinical isolates, we correlated the ability to induce IL-8 mRNA and protein in gastric epithelial cells with in vitro expression of cytotoxin and the *cagA* product. The strain specificity that we observed of enhanced IL-8 induction by *H. pylori*

TABLE 4. IL-8 induction by gram-negative bacteria in AGS gastric epithelial cells[a]

Addition to cultures	Mean IL-8 concn (pg/ml) ± SEM (n = 2)
None (medium control[b])	300 ± 20
C. fetus 23D	490 ± 70
P. aeruginosa 27239	2,800 ± 400
H. pylori 88-23	5,000 ± 2,000
C. jejuni 81-176	765 ± 185
E. coli DH5α	2,350 ± 150

[a] AGS cultures were infected with various bacteria as described in Materials and Methods, and the concentration of IL-8 was determined in the supernatants after 24 h of exposure.
[b] RPMI 1640 medium without serum.

TABLE 5. IL-8 induction in AGS cells by clinical isolates of *H. pylori*

Strain	Origin	Clinical setting	Phenotype		IL-8 induction	
			Tox	CagA	mRNA[a]	Mean protein concn (pg/ml) ± SEM (n = 2–3)[b]
87-29	Colorado	Duodenal ulcer	+	+	+	318 ± 35
92-25	Colorado	Duodenal ulcer	+	+	+	135 ± 4
88-23 (60190)	England	NUD[c]	+	+	+	367 ± 148
84-183	Texas	NUD	+	+	+	153 ± 41
87-199	Colorado	NUD	+	+	+	380 ± 59
86-313	Colorado	NUD	−	−	−	79 ± 39
87-203	Colorado	NUD	−	−	−	58 ± 20
92-23	Tennessee	NUD	−	−	−	54 ± 22
92-24	Tennessee	NUD	−	−	−	55 ± 12
88-22 (T-30a)	Texas	NUD	−	−	−	51 ± 16

[a] As detected by RT-PCR.
[b] After 24-h incubation. Background secretion of IL-8 in unstimulated AGS cultures was <32 pg/ml. Differences between the five Tox^+ $CagA^+$ strains and the five Tox^- $CagA^-$ strains were statistically significant (P = 0.008).
[c] NUD, nonulcer dyspepsia.

$CagA^+$ Tox^+ strains in gastric cell lines was consistent with a recent report (13), although we used different culture conditions for both gastric cell lines (serum-free conditions) and bacterial cells (24- to 48-h versus >72-h [day 4] cultures). In our experiments, *H. pylori* cultures grown for 72 to 96 h did not induce significant IL-8 production by gastric cells (data not shown).

The use of defined bacterial mutants allowed us to further analyze whether these specific bacterial constituents were necessary for IL-8 induction. Results with isogenic mutants lacking either the *cagA* or *vacA* product or both indicated that these proteins were not directly involved. A cell-free, crude preparation of the cytotoxin also did not induce IL-8 production by gastric cells. Therefore, expression of these proteins appears to be a phenotypic marker of associated bacterial factors required for this strain-specific trait. Since urease production is a highly conserved trait, it was not surprising that urease production was not found to be a determinant of IL-8 induction. Studies are in progress to deduce the *H. pylori* factor(s) or constituent(s) that induces IL-8 in the host cells. Since our results demonstrate that $CagA^+$ Tox^+ and $CagA^-$ Tox^- clinical isolates differ in the ability to induce IL-8, while adhesion is a conserved feature among clinical isolates (19), we consider it unlikely that adherence alone accounts for the observed differences. *H. pylori* induction of IL-8 by gastric cells appears to be an active process, since heat-killed preparations did not induce IL-8, corroborating previous observations (13, 18). For other gram-negative bacteria such as *P. aeruginosa*, bacterial adhesion to airway epithelial cells and monocytes is not required for either IL-8 expression or secretion (33). However, human intestinal and cervical epithelial cells secreted IL-8 only in response to entry of shigellae, salmonellae, or *Listeria monocytogenes*, not in response to noninvasive bacteria such as *E. coli* DH5α and *Enterococcus faecium* (23). In contrast, incubation of uropathogenic *E. coli* cells, which adhere to but do not enter epithelial cells, induced increased IL-8 levels in these cells (1). These observations indicate that the mechanisms of IL-8 induction may differ among bacteria and are dependent on the host cell as well. Thus, the ability of other intestinal pathogens such as *P. aeruginosa*, *E. coli*, and *C. jejuni* to induce IL-8 in gastric epithelial cells was not surprising. Although the effects of *H. pylori* on epithelial cells are not specific, the fact that *H. pylori* can stimulate gastric epithelial cells (albeit in vitro) to secrete IL-8 may provide a link between this infection and its consequences involving inflammation.

The fact that gastric epithelial cells can participate in the network of cytokine responses and the observation that gastric epithelial cells from *H. pylori*-infected persons express HLA class II molecules (44) suggest a role for these cells in mucosal defense against *H. pylori* infection. Cytokines in the gastric milieu provide bidirectional communication between inflammatory cells such as mucosal macrophages and epithelial cells and thus may regulate the degree of inflammation and extent of epithelial cell degeneration in *H. pylori* infection (4, 5). IL-1 and TNF-α, released by activated mucosal macrophages (40),

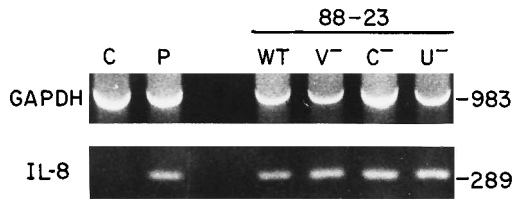

FIG. 3. Induction of IL-8 mRNA in AGS cells by *H. pylori* wild-type strain 88-23 and mutant cells. Data shown are from a representative gel electrophoresis of RT-PCR amplification products of GAPDH and IL-8 mRNAs from AGS cells 2 h after stimulation with control unstimulated medium (C), PMA plus ionomycin (P), or suspensions of cells of *H. pylori* 88-23. WT, wild-type strain; V^-, isogenic *vacA* mutant; C^-, isogenic *cagA* mutant; U^-, mutant lacking in expression of *ureA* and *ureB*. Sizes are indicated in base pairs.

TABLE 6. IL-8 protein production in AGS gastric epithelial cells stimulated by *H. pylori* wild-type and mutant strains

H. pylori phenotype[a]	Mean IL-8 concn (pg/ml) ± SEM (n = 2–3)[b]	
	Strain 88-23	Strain 84-183
U^+ $CagA^+$ $VacA^+$ (WT)	367 ± 148	153 ± 41
U^- $CagA^+$ $VacA^+$	603 ± 128	ND
U^+ $CagA^-$ $VacA^+$	513 ± 121	195 ± 44
U^+ $CagA^+$ $VacA^-$	515 ± 28	258 ± 26
U^+ $CagA^-$ $VacA^-$	490 ± 79	273 ± 23

[a] U^+, *ureA* and *ureB* products present; $CagA^+$, *cagA* product present; $VacA^+$, toxin produced in vitro; WT, wild type. Isogenic mutants were examined at the same time as strains shown in Table 5; thus, values for wild-type strains 88-23 and 84-183 are identical in the two tables.
[b] Values from 24-h incubations. Background secretion of IL-8 in unstimulated AGS cultures was <32 pg/ml. ND, not determined.

may result in increased IL-8 secretion by gastric cells (51), while agents such as IL-4 and transforming growth factor β may be important in down-regulating responses mediated by IL-8 (40). Parasitism by *H. pylori* may lead to altered homeostasis in the gastric mucosa (5), resulting in an imbalance between proinflammatory and anti-inflammatory cytokines leading to inflammation and disease. Taken together, the data we report indicate that wild-type Tox^+ $CagA^+$ strains have enhanced ability to induce gastric cells to synthesize proinflammatory cytokines such as IL-8. Up-regulation of IL-8 expression which also is observed in vivo (46) may help explain the increased incidence of peptic ulcer disease among patients infected with Tox^+ $CagA^+$ strains.

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