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Mechanisms Involved in *Helicobacter pylori*-Induced Interleukin-8 Production by a Gastric Cancer Cell Line, MKN45

MIKI AIHARA,[1]* DAISUKE TSUCHIMOTO,[1] HISAO TAKIZAWA,[1] ATSUSHI AZUMA,[1] HIROKAZU WAKEBE,[1] YASUKAZU OHMOTO,[1] KENICHI IMAGAWA,[1] MIKIO KIKUCHI,[1] NAOFUMI MUKAIDA,[2] AND KOUJI MATSUSHIMA[2]

Microbiological Research Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima 771-01,[1] and Department of Pharmacology, Cancer Research Institute, Kanazawa University, Kanazawa 920,[2] Japan

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Accumulating evidence suggests an important role of interleukin-8 (IL-8) in *Helicobacter pylori* infection-associated chronic atrophic gastritis and peptic ulcer. We observed in this study that a gastric cancer-derived cell line, MKN45, produced a massive amount of IL-8 upon coculture with live *H. pylori* but not with killed *H. pylori*, *H. pylori* culture supernatants, or live *H. pylori* separated by a permeable membrane, indicating that IL-8 production requires a direct contact between the cells and live bacteria. Moreover, the tyrosine kinase inhibitor herbimycin but neither a protein kinase C inhibitor (staurosporine) nor a protein kinase A inhibitor (H89) inhibited IL-8 production by MKN45 cells cocultured with live bacteria, suggesting the involvement of a tyrosine kinase(s) in *H. pylori*-induced IL-8 production. In addition, coculture of *H. pylori* induced IL-8 mRNA expression in MKN45 cells and an increase in luciferase activity in cells which were transfected with a luciferase expression vector linked with a 5′-flanking region of the IL-8 gene (bp −133 to +44), indicating that the induction of IL-8 production occurred at the transcriptional level. This region contain three *cis* elements important for induction of IL-8 gene expression: AP-1 (−126 to −120 bp), NF-IL6 (−94 to −81 bp), and NF-κB (−80 to −70 bp) binding sites. Mutation of the NF-κB binding site abrogated completely the induction of luciferase activity, whereas that of the AP-1 site partially reduced the induction. However, mutation of the NF-IL6 binding site resulted in no decrease in the induction of luciferase activity. Moreover, specific NF-κB complexes were detected in the nuclear proteins extracted from MKN45 cells which were infected with *H. pylori*. Collectively, these results suggest that *H. pylori* induced the activation of NF-κB as well as AP-1, leading to IL-8 gene transcription.

The microaerophilic bacterium *Helicobacter pylori*, as a causal agent of gastritis and peptic ulcer in humans (3, 17, 35), adheres to gastric epithelium without invading the epithelium (14, 19). Hence, it is presumed that the interaction between bacteria and host epithelium cells may be required for *H. pylori*-induced inflammation consisting of leukocyte infiltration and epithelium damage.

Mai et al. reported that an extract from *H. pylori* itself exhibited in vitro chemotactic activity for monocytes and neutrophils (24). In contrast, it is also known that *H. pylori* components such as porin and lipopolysaccharide (LPS) stimulate the production of cytokines and chemokines by polymorphonuclear leukocytes (PMNC) (2, 42). Several independent groups, including ours, observed that human gastric cancer cells produced a massive amount of a potent leukocyte chemotactic and activating factor, interleukin-8 (IL-8), in vitro upon coculture with *H. pylori* (1, 8, 9, 21, 39). Moreover, the association of IL-8 with *H. pylori* infection was further suggested by increased IL-8 contents in gastric epithelial cells in vivo (7) and in tissue homogenates of mucosal biopsy specimens (16, 28), IL-8 secretion from in vitro-cultured biopsy specimens (33), and the presence of anti-IL-8 immunoglobulin A (IgA) autoantibodies (6) in *H. pylori*-associated gastritis and peptic ulcer patients.

The ability to induce IL-8 production has been proposed to be associated with several gene products of *H. pylori*. *cagA*$^+$ strains can enhance IL-8 production by gastric epithelial cells compared with *cagA* strains (8, 21), although the involvement of other component(s) has been also suggested (10). This notion was supported by the observation that *picA* and *picB* gene products were strongly associated with the induction of IL-8 in gastric epithelial cells (32). Determination of chromosomal structure of *cag* genes demonstrated that *cagA* gene is part of 40-kb pathogenicity island consisting of at least 18 *cag* genes (4). Moreover, the disruption of *cagE*, *cagG*, *cagH*, *cagI*, *cagL*, and *cagM* but not *cagA* diminished the ability to induce IL-8 secretion from gastric epithelial cells (4). However, the signal pathways involved in *H. pylori*-induced IL-8 production are not known.

Various types of inflammatory stimuli, including LPS, IL-1, and tumor necrosis factor (TNF), can activate IL-8 gene transcription in a wide variety of cells. Moreover, our previous studies revealed that IL-8 gene transcription requires the activation of the combination of transcription factor NF-κB and AP-1 or that of NF-κB and NF-IL6, depending on the types of cells or stimuli (26, 29, 30). We previously reported that the activation of both NF-κB and AP-1 was indispensable for IL-8 gene transcription in gastric cancer-derived MKN45 cells stimulated with TNF-α alone or TNF-α plus gamma interferon (IFN-γ) (46). However, it remains to be investigated whether *H. pylori* induce IL-8 production through a similar transcriptional regulation.

In this investigation, we studied the role of adhesion of *H. pylori* to a gastric cancer-derived cell line, MKN45, to induce IL-8 production and the involvement of protein kinases, and

* Corresponding author. Mailing address: Microbiological Research Institute, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan. Phone: 81-886-65-2126. Fax: 81-886-65-6286. E-mail: m_aihara@research.otsuka.co.jp.

TABLE 1. IL-8 induction in MKN45 cells by clinical isolates and type strain of *H. pylori*

Strain	Clinical setting	Phenotype			IL-8 induction (mean protein concn [pg/ml] ± SE [$n = 3$])[b]
		CagA[a]	VacA[a]	Tox[c]	
OHPC0001	Chronic gastritis	+	+	−	5,227.3 ± 171.6
OHPC0002	Chronic gastritis	−	+	−	54.5 ± 7.2
OHPC0003	Chronic gastritis	+	+	+	4,820.8 ± 246.6
OHPC0004	Gastric ulcer	+	+	+	3,841.9 ± 185.2
OHPC0005	Gastritis	+	+	+	1,271.3 ± 18.6
OHPC0016	Duodenal ulcer	+	+	+	3,687.5 ± 134.7
NCTC11637		+	+	+	2,258.8 ± 144.7

[a] As detected by reverse transcription-PCR.
[b] IL-8 contents in supernatants were determined as described in Materials and Methods. Representative results are shown as mean ±1 SE calculated from the results of three independent experiments.
[c] Bacterial cells were assessed for cytotoxicity against HeLa cells in vitro.

we analyzed the transcriptional regulation of IL-8 gene in MKN45 cells upon coculture with *H. pylori.*

MATERIALS AND METHODS

Bacteria. The *H. pylori* strain used in most of this study (OHPC0016) was isolated from a duodenal ulcer patient and was a kind gift of T. Fujioka (Oita Medical University, Oita, Japan). Other clinical strains, isolated from chronic gastritis and ulcer patients, were kind gifts of T. Kitahora (Ohkura Hospital, Tokyo, Japan) and M. Karita (Yamaguchi University, Yamaguchi, Japan). NCTC 11637 was obtained from the American Type Culture Collection (Rockville, Md.). The presence of *cagA* and *vacA*, or of Tox (vacuolating cytotoxin) in these strains was determined by reverse transcription-PCR using specific sets of primers or by cytotoxin assay against HeLa cells in vitro, respectively (Table 1). After these strains were cultured at 37°C for 18 h under microaerobic conditions in brucella broth (Life Technologies, Grand Island, N.Y.) supplemented with 7% heat-inactivated fetal bovine serum (FBS), bacteria were harvested from a broth culture by centrifugation and were resuspended at the indicated concentration in antibiotic-free RPMI 1640 medium supplemented with 5% FBS. At this time point, bacteria grew to give rise to about 0.05 unit of optical density at 660 nm; they reached a concentration of 5×10^7 CFU/ml and were in the early log phase with good motility. LPS was purified from *H. pylori* by Westphal and Jann's phenol-water extraction method (45) or by the proteinase K digestion method of Darveau and Hancock (12).

Cell culture. Human gastric cancer cell lines MKN45, MKN1, MKN28, MKN74 (20), and KATO III (38) were maintained in complete medium, consisting of RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U of penicillin G per ml, and 100 mg of streptomycin per ml. These cell lines were obtained from the Japanese Cancer Research Resources Bank (Tsukuba, Japan). MKN45 was established from a poorly differentiated adenocarcinoma metastasized to liver. MKN1 was established from an adenosquamous carcinoma in stomach, while MKN28 and MKN74 were from moderately differentiated tubular adenocarcinomas in stomach. KATO III originated from a signet ring cell gastric carcinoma disseminated as ascites.

Determination of IL-8 production in gastric cancer cells. Cells were cultured in complete medium in 24-well plates. After the cells reached subconfluency, recombinant human IL-1β (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) was added to a final concentration of 10 ng/ml. Live *H. pylori* cells were added to a final concentration of 1.5×10^7 CFU/ml, since higher concentrations of live bacteria induced the death of gastric cancer cell lines as determined by a morphological analysis. In the same series of experiments, MKN45 cells were cocultured with culture supernatants from live *H. pylori* or heat-killed or glutaraldehyde-fixed *H. pylori* of the same colony number as the live bacteria used. In some experiments, the same concentration of live bacteria was added to the upper well of a transwell plate (Costar; Toyobo, Osaka, Japan) whose lower well contained subconfluent MKN45 cells. After 24 h at 37°C in 5% CO_2, the cell supernatants were collected after centrifugation at 10,000 rpm for 3 min to remove bacteria and stored at −20°C until the measurement. In another series of experiments, the cells were preincubated for 1 h with the indicated concentrations of staurosporine, herbimycin (Sigma Chemical, St. Louis, Mo.), H89 (BIOMOL Research Laboratories Inc., Plymouh Meeting, Pa.), or an anti-human CD14 monoclonal antibody (3C10; ATCC TIB 228) before the addition of stimuli. IL-8 contents in the culture supernatants were determined by using a specific enzyme-linked immunosorbent assay against human IL-8 as previously described (30, 46) except that the formed immune complexes were detected by peroxidase-conjugated goat anti-rabbit Ig (Biosource, Camarillo, Calif.) diluted to 1/10,000. The enzyme activity was determined by using 200 μl of *o*-phenylenediamine at a concentration of 1 mg/ml in 0.1 M citric acid with 0.2 M Na_2HPO_4 and 0.015% (vol/vol) H_2O_2, and the reaction continued for 10 min. The enzyme reaction was terminated by addition of 50 ml of 2 N H_2SO_4, and the optical density at 490 nm was determined. The detection limit of this assay was about 20 pg/ml.

Northern blotting analysis. MKN45 cells were cocultured with *H. pylori* for the indicated time intervals. Total cellular RNA was extracted from the cells with guanidium isothiocyanate, and the amount of the total RNA was determined by measuring the absorbance at 260 nm. Twenty micrograms of total RNA was electrophoresed on a 1% agarose gel containing 6.5% formaldehyde and was transferred onto a nylon filter. Human IL-8 cDNA corresponding to its coding region and human β-actin cDNA (Toyobo) were radiolabeled with [α-^{32}P]dCTP by a random primer DNA labeling kit (Takara Shuzo, Kyoto, Japan). The filters were hybridized with the labeled probe for 20 h at 42°C in 6× SSC (1× SSC consists of 0.15 M NaCl and 0.015 sodium citrate [pH 7.0]) containing 50% formamide, 1% blocking agent (Amersham, Buckinghamshire, England), and 50 mg of salmon sperm DNA per ml. After being washed sequentially in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and 0.2× SSC–0.1% SDS, filters were exposed overnight at −80°C to Kodak X-Omat AR film (Kodak, Rochester, N.Y.).

Luciferase assay. The 5′-flanking region of the IL-8 gene spanning from bp −133 to +44 was subcloned into a luciferase expression vector as previously described (22). Site-directed mutagenesis of the IL-8 AP-1, NF-IL6, and NF-κB binding sites was carried out as previously described (22, 23). For the luciferase assay, 3×10^6 MKN45 cells were transfected with 10 mg of each luciferase vector along with 2 mg of β-galactosidase expression vector as an internal control, using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. After 24 h, the transfected cells were divided into three parts, and live *H. pylori* (final concentration, 3×10^7 CFU/ml) or IL-1β (final concentration, 10 ng/ml) was added separately into each part. After an additional 24 h of incubation, cell lysates were prepared using Pica Gene kit (Toyo Ink Co., Tokyo, Japan), and their protein concentrations were measured by using a Bio-Rad protein assay kit, with bovine serum albumin as a standard, according to the manufacturer's instructions. The light intensities were measured on 20 μg of cell lysates by using a Pica Gene kit and a model BLR-301 Luminescence Reader (Aloka Co., Ltd., Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted from MKN45 cells incubated in the presence or the absence of live *H. pylori* (3×10^7 CFU/ml) for 4 h as described by Dignam et al. (13). Four-microgram aliquots of nuclear proteins were incubated for 30 min at 25°C with 20 pg of ^{32}P-labeled NF-κB binding site of the Igκ gene (GATCGAGGGGGACTTTCCCTAGC; Stratagene, La Jolla, Calif.) and 1 mg of poly(dI-dC)–poly(dI-dC) in 5 ml of solution consisting of 20 mM HEPES (pH 7.9), 4 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 4% glycerol. In some experiments, nuclear proteins were preincubated with the indicated amounts of antibodies to p65, p50, p52, or c-Rel (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) or 100-fold-excess amounts of unlabeled oligomers for 1 h at 4°C before addition of the labeled probe. The mixtures were loaded onto a 4% polyacrylamide gel with 0.25× Tris borate electrophoresis buffer. After electrophoresis, gels were dried and exposed to X-ray film (X-Omat AR; Kodak).

Statistical analysis. All data in each experiment were expressed as mean ± 1 standard error (SE). Statistical significance of the effects of various protein inhibitors on IL-8 production (Fig. 3) and of various concentrations of herbimycin on IL-8 production (Fig. 4) was evaluated by using a two-way analysis of variance and Tukey's test and one-way analysis of variance and Dunnett's test, respectively.

RESULTS

***H. pylori*-induced IL-8 production in human gastric cancer cell lines.** We first examined the capacity of several gastric cancer-derived cell lines to produce IL-8 upon coculture with live *H. pylori*. MKN1 and KATO III cells produced constitutively a marked amount of IL-8, whereas MKN45, MKN28, and MKN74 cells did not (Fig. 1). Coculture with live *H. pylori* enhanced significantly IL-8 production by all gastric cancer cell lines except MKN74 (Fig. 1). Since the *H. pylori*-induced ratio of enhancement of IL-8 production was greatest in MKN45 cells, we used MKN45 cells in the following experiments. We next examined the time kinetics of *H. pylori*-induced IL-8 production by MKN45 cells. MKN45 cells started to produce a significant amount of IL-8 at 6 h after the initiation of coculture with the bacteria (Fig. 2a). Neither *H. pylori* culture supernatants nor live bacteria separated by a permeable membrane induced IL-8 production by MKN45 cells (Fig. 2b). Moreover, heat-killed or glutaraldehyde-fixed bacteria failed to induce IL-8 production (Fig. 2b). These results suggested

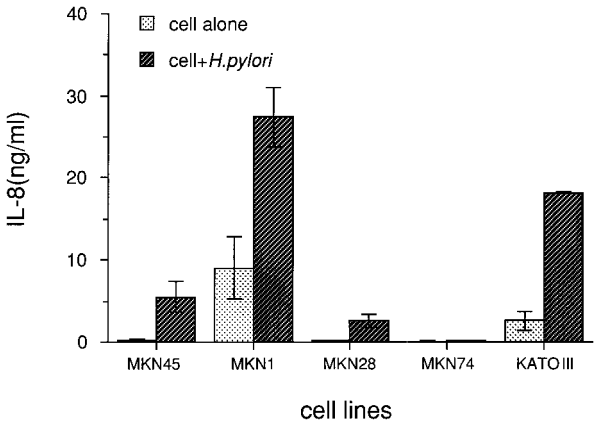

FIG. 1. IL-8 production by gastric cancer-derived cell lines. Gastric cancer-derived cells were incubated in quadruplicate in the presence or the absence of live *H. pylori* for 24 h, and IL-8 contents in the culture supernatants were determined as described in Materials and Methods. Representative results are shown as the mean ± 1 SE calculated from the results of three independent experiments.

that the interaction with live bacteria is required for IL-8 production by MKN45 cells.

Capacities of several *H. pylori* strains to induce IL-8 production. We next examined the capacities of other *H. pylori* strains to induce IL-8 production. All isolates were positive for *vacA* irrespective of the ability to induce IL-8 production (Table 1). Moreover, strain OHPC0001 induced IL-8 production despite the absence of Tox. Furthermore, strain OHPC0002, devoid of the *cagA* gene, failed to induce IL-8 production, whereas other strains significantly enhanced IL-8 production in MKN45 cells. These results support the previous observation that a $cagA^+$ strain was able to induce IL-8 production (8, 9). Since several genes adjacent to the *cagA* gene are involved in the induction of IL-8 production (4), we cannot exclude the possibility that OHPC0002 failed to enhance IL-8 production due to the lack of these genes.

Involvement of a tyrosine kinase(s) in *H. pylori*-induced IL-8 production by MKN45 cells. We next examined the roles of protein kinases in *H. pylori*-induced IL-8 production. A protein kinase A (PKA) activator, forskolin, and a PKA inhibitor, H89, changed neither constitutive nor *H. pylori*-induced IL-8 production by MKN45 cells (Fig. 3a). Moreover, a protein kinase C (PKC) inhibitor, staurosporine, inhibited phorbol myristate acetate (PMA)-induced but not *H. pylori*-induced IL-8 production (Fig. 3b). These results suggested that neither PKA nor PKC was involved in *H. pylori*-induced IL-8 production by MKN45 cells. In contrast, a tyrosine kinase inhibitor, herbimycin, inhibited *H. pylori*-induced IL-8 production (Fig. 3c), suggesting the involvement of a tyrosine kinase(s) in *H. pylori*-induced IL-8 production. Moreover, herbimycin inhibited *H. pylori*-induced IL-8 production by MKN45 cells more effectively than IL-1β-induced production (Fig. 4). Thus, a tyrosine kinase-mediated pathway might play a more important role in *H. pylori*-induced IL-8 production than in IL-1β-induced production.

Roles of *H. pylori*-derived LPS in IL-8 production. *H. pylori* contains LPS, a potent inducer of IL-8 production. However, *H. pylori*-derived LPS at up to 100 μg/ml induced a marginal level of IL-8 production by MKN45 cells compared with live *H. pylori* or IL-1β (Fig. 4). Moreover, an anti-CD14 antibody failed to reduce *H. pylori*-induced IL-8 production by MKN45 cells (data not shown). These results suggested that the interaction between *H. pylori*-derived LPS and CD14 played little if any role in the processes.

IL-8 mRNA expression induced by coculture with *H. pylori*. We next examined the effect of coculture with *H. pylori* on IL-8 mRNA expression by MKN45 cells by Northern blotting analysis. MKN45 cells started to express IL-8 mRNA rapidly within 1 h after the coculture with the bacteria (Fig. 5), indicating that *H. pylori*-induced IL-8 production was preceded by an increase in IL-8 mRNA expression.

***H. pylori*-induced transcriptional regulation of the IL-8 gene.** Since most types of cells exhibited simultaneously an increase in IL-8 mRNA expression and an increase in gene transcription in response to various stimuli, we next examined the effects of *H. pylori* on IL-8 gene transcription. Coculture with *H. pylori* enhanced markedly luciferase activities in MKN45 cells which were transfected with luciferase expression vectors containing a minimally essential promoter region of the IL-8 gene (bp −133 to +44) (Fig. 6), and *H. pylori*-induced enhancement was greater than IL-1β-induced enhancement. Since this promoter region contains three important *cis* elements for IL-8 gene transcription, AP-1, NF-IL6, and NF-κB binding sites, we next examined the relative contribution of each element to *H. pylori*-induced IL-8 gene transcription. Mutation of the NF-IL6 binding site failed to reduce *H. pylori*-induced enhancement in luciferase activities, whereas that of

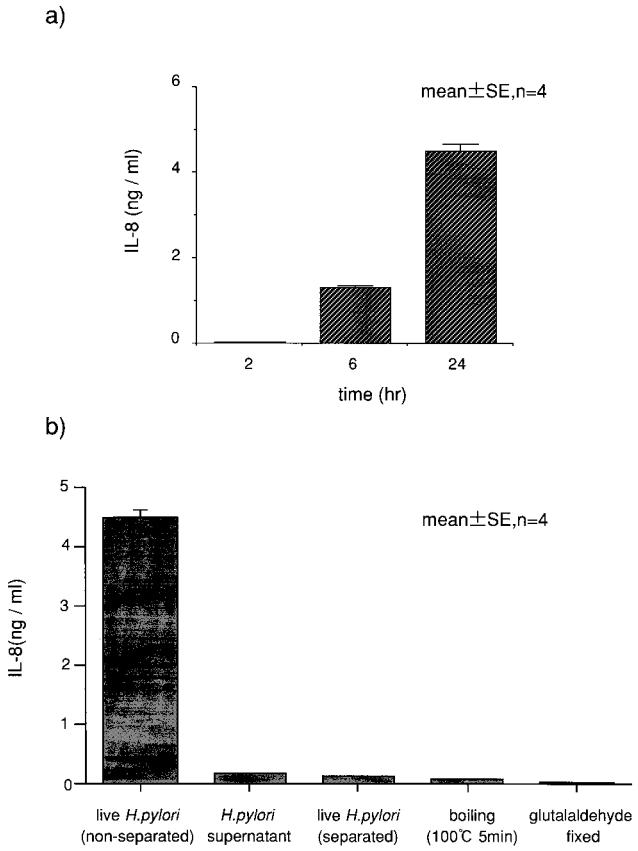

FIG. 2. Time kinetic study of IL-8 production by MKN45 cells incubated with live *H. pylori* (a) or incubated with *H. pylori* treated under various conditions (b). The cells were incubated with live bacteria for the indicated time intervals (a) or were incubated for 24 h with live *H. pylori*, *H. pylori* culture supernatants, *H. pylori* separated by a membrane, or heat-killed or glutaraldehyde-fixed *H. pylori* (b). IL-8 contents in the culture supernatants were determined as described in Materials and Methods. Representative results are shown as the mean ± 1 SE calculated from the results of two independent experiments.

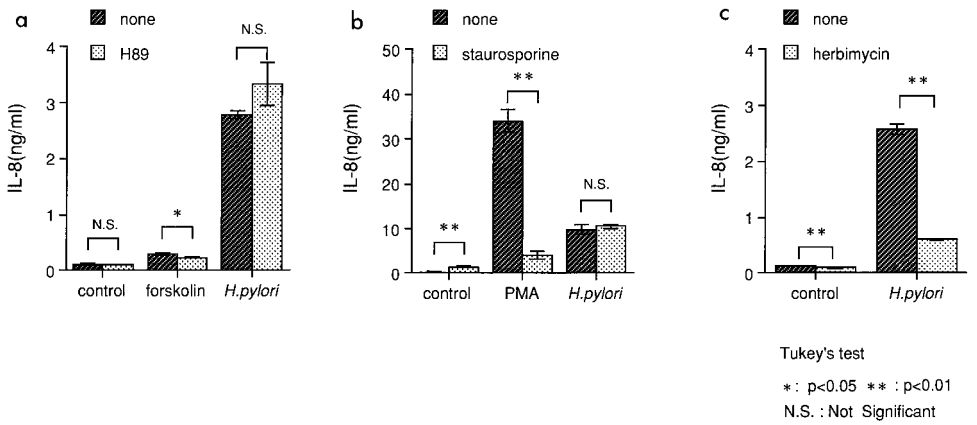

FIG. 3. Effects of protein kinase inhibitors on *H. pylori*-induced IL-8 production by MKN45 cells. MKN45 cells were incubated for 24 h with live *H. pylori*, forskolin (10 μM), or PMA (10 ng/ml) after 1 h of preincubation with either H89 (20 μM), staurosporine (100 nM), or herbimycin (500 nM). IL-8 levels in supernatants were determined as described in Materials and Methods. Representative results are shown as the mean ± 1 SE calculated from the results of three independent experiments. Statistical analysis was performed by using a two-way analysis of variance and Tukey's test.

the AP-1 binding site decreased the enhancement by more than 70% (Fig. 6). Moreover, mutation of the NF-κB binding site completely abrogated the enhancement induced by *H. pylori* as well as IL-1β (Fig. 6). These results suggested that the NF-κB binding site and to a lesser degree the AP-1 binding site were involved in *H. pylori*-induced IL-8 gene transcription similarly as TNF-α–IFN-γ-induced IL-8 gene activation in the same MKN45 cells (46).

We then evaluated the effects of coculture with *H. pylori* on NF-κB complex formation by an EMSA. Nuclear proteins extracted from unstimulated cells did not give rise to any complex formation (Fig. 7). In contrast, coculture with live *H. pylori* induced the formation of two complexes, and both were abolished by a specific oligomer but not by an unrelated one. Moreover, specific antibodies to p50 and p65 retarded both of these complexes and a slower-migrating one, respectively. In contrast, antibodies to c-Rel and p52 failed to affect complex formation. These results suggested that coculture with *H. pylori* induced NF-κB complexes which were found immunochemically to be composed of p65 plus p50 or p50 plus p50.

DISCUSSION

The presence of *H. pylori* in gastric antrum is, in most cases, associated with mucosal inflammatory changes consisting of infiltration of a large number of polymorphonuclear and mononuclear phagocytes (17). *H. pylori* resides in the mucus layer overlying the epithelium and does not invade epithelial cells. However, accumulating evidence indicates that gastric infection with *H. pylori* induces the expression of several proinflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, in gastric mucosa (5–7, 16, 28, 33). Since IL-8 has been documented as a factor crucial for inducing several types of neutrophil-mediated inflammation (27), IL-8 may have a pivotal role in inducing leukocyte infiltration also in *H. pylori*-associated diseases. However, it is not known how a noninvasive bacterium, *H. pylori*, induces production of proinflammatory cytokines, particularly IL-8.

Most human gastric cancer-derived cell lines that we examined could produce IL-8 in vitro upon coculture with *H. pylori*. Of the five cell lines tested, MKN1 and KATO III constitutively produced high levels of IL-8. However, MKN45 cells produced a marginal level of IL-8 without any stimuli as previously described (46), and *H. pylori* infection enhanced the production markedly, in contrast to the report by Crabtree et al. that MKN45 constitutively expressed IL-8 mRNA (7). The discrepancy may be explained by the differences in clones used and culture conditions.

Consistent with previous reports (8, 39) direct contact with live *H. pylori* was indispensable for IL-8 production by a gastric cancer cell line, MKN45. Moreover, live bacteria could not induce the production of potent IL-8-inducing cytokines such as IL-1 or TNF-α by MKN45 cells (our unpublished data). Thus, MKN45 cells produced IL-8 only through direct contact with an *H. pylori*-derived cell component(s) which is lost by heat or glutaraldehyde treatment. Moreover, the difference in responsiveness of gastric cancer cell lines may reflect a difference in the number of *H. pylori*-binding molecules on the cell surface or in the magnitude of the intracellular signal gener-

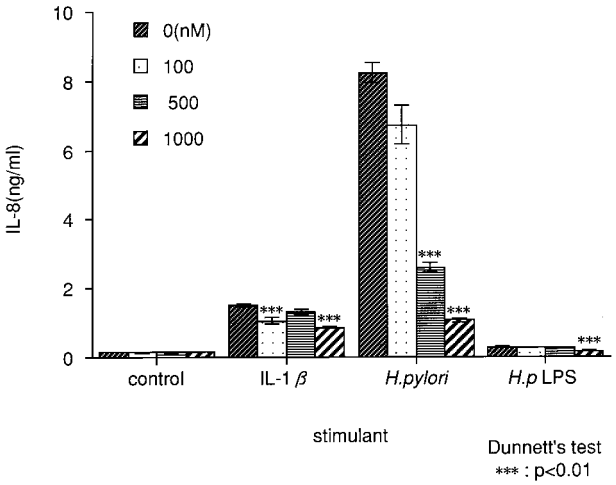

FIG. 4. Effects of herbimycin on *H. pylori*-induced IL-8 production by MKN45 cells. MKN45 cells were incubated for 24 h with IL-1β, live *H. pylori*, or *H. pylori*-derived (*H.p*) LPS (100 μg/ml) after 1 h of preincubation with the indicated concentrations of herbimycin. IL-8 levels in supernatants were determined as described in Materials and Methods. Representative results are shown as the mean ± 1 SE calculated from the results of two independent experiments. Statistical analysis was performed by using a one-way analysis of variance and Dunnett's test.

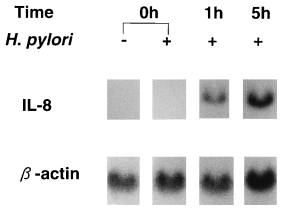

FIG. 5. *H. pylori*-induced IL-8 mRNA expression by MKN45 cells. Total RNA was extracted from the cells cocultured with live *H. pylori* for the indicated time intervals and used for Northern blotting analysis as described in Materials and Methods.

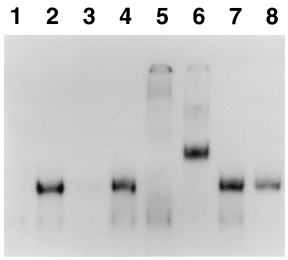

FIG. 7. Induction of specific NF-κB complex formation by coculture with live *H. pylori*. Nuclear proteins were extracted from MKN45 cells cocultured for 4 h without (lane 1) or with (lanes 2 to 8) live *H. pylori*. Binding reactions were performed, as described in Materials and Methods, after preincubation with a specific NF-κB oligomer (lane 3), an unrelated SP-1 oligomer (lane 4), antibodies to p65 (lane 5), p50 (lane 6), p52 (lane 7), or c-Rel (lane 8), or no reagents (lanes 1 and 2). EMSA was performed as described in Materials and Methods, and representative results from three independent experiments are shown.

ated by exposure to an *H. pylori*-derived IL-8-inducing component(s).

In contrast, Huang et al. reported that a water-extracted *H. pylori* antigen preparation and, to a less degree, proteinase-digested cells and heated cells induced a human epithelial cell line, HEp-2, to produce IL-8 (21). However, significant IL-8 production by MKN45 cells was obtained only by the preparation extracted in water from more than 10^{10} CFU of *H. pylori* NCTC11637. The discrepancy may be due to the differences in cells and bacteria strains used in our studies and theirs.

Consistent with previous observations that *H. pylori* did not invade gastric cell lines in vitro and gastric mucosa in vivo, our *H. pylori* strain could not invade MKN45 cells (our unpublished data). However, we cannot completely exclude the possibility that an undetectable level of *H. pylori* invasion may occur and facilitate IL-8 production as Crowe et al. claimed (11).

Gram-negative bacterium-derived LPS, particularly LPS from *Escherichia coli*, generally induces the production of several proinflammatory cytokines, including IL-8, by human PBMC (40, 41). However, *H. pylori*-derived LPS weakly induced IL-8 production by human PBMC (our unpublished data) as well as MKN45, consistent with the previous report that *H. pylori*-derived LPS exhibited lower biological activities compared with *E. coli*-derived LPS (21, 34). Several lines of evidence indicate that CD14, previously identified as an LPS receptor on host cells, is involved in the recognition for other components of microorganisms. However, an anti-CD14 monoclonal antibody failed to reduce *H. pylori*-induced IL-8 production by MKN45 cells. Collectively, these results implied that neither LPS nor CD14 was involved in *H. pylori*-induced IL-8 production.

A tyrosine kinase inhibitor, herbimycin, but not a PKC inhibitor or a PKA inhibitor abrogated *H. pylori*-induced IL-8 production by MKN45 cells. Although staurosporine can inhibit broader ranges of kinases other than PKC, it inhibited PMA-induced but not *H. pylori*-induced IL-8 production, negating the involvement of PKC in *H. pylori*-induced IL-8 production. In contrast, herbimycin at up to 4 μM inhibited *H. pylori*-induced IL-8 production but failed to affect the growth of *H. pylori* in vitro. Since herbimycin inhibits tyrosine kinases, particularly Src-type receptor tyrosine kinases, these results suggest that *H. pylori* activated a tyrosine kinase(s). Accumulating evidence indicates that infection with several types of bacteria induces tyrosine phosphorylation of host cellular proteins. The invasion of *Salmonella typhimurium* induced tyrosine phosphorylation of epidermal growth factor receptor in Henle-407 cells, and its *invA* gene was presumed to be indispensable for the tyrosine phosphorylation (15). Moreover, the *cfm* gene of enteropathogenic *E. coli* induced tyrosine phosphorylation of host cellular proteins, resulting in cytoskeletal rearrangement and uptake of bacteria (36). Furthermore, the binding of *H. pylori* to AGS cells caused tyrosine phosphorylation of two proteins with molecular masses of 145 and 105 kDa, leading to cytoskeletal rearrangements (37). Thus, the attachment of *H. pylori* to gastric mucosal cells may induce phosphorylation of host cellular proteins and may change the shape and membrane integrity of these cells, leading to tissue damage.

Herbimycin inhibited IL-1β-induced IL-8 production by MKN45 cells only partially at the highest concentration used, suggesting that the signals required for *H. pylori*-induced IL-8 production are not be completely identical with those for IL-1β-induced production. It was also reported that herbimycin inhibited IL-8 production by a colon epithelial cell line, HT-29, stimulated with IL-1β or TNF-α (18). The activation of a tyrosine kinase(s) (PTK) has been presumed to be involved in LPS-induced activation of murine macrophage cell lines (43, 44). We also demonstrated that herbimycin inhibited LPS-induced activation of NF-κB, a transcription factor indispensable for IL-8 gene transcription (22). Thus, it is tempting to speculate that *H. pylori* activated PTK more efficiently than IL-1β and that PTK induced the activation of NF-κB, leading to IL-8 gene transcription.

IL-8 gene transcription requires the activation of the com-

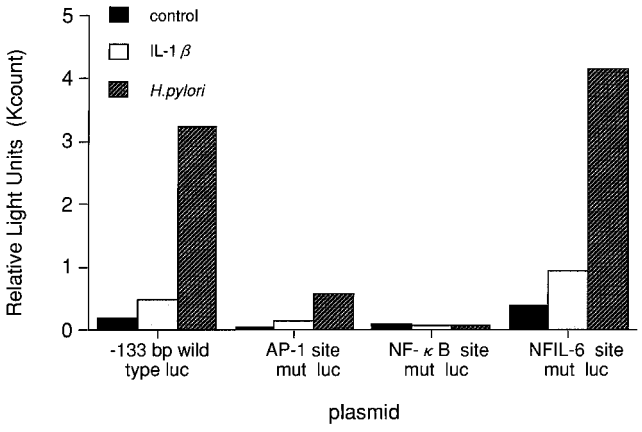

FIG. 6. *H. pylori*-induced IL-8 gene transcription through activating NF-κB and AP-1 but not NF-IL6. The cells were transfected with various luciferase (luc) expression vectors and were stimulated with either live *H. pylori* or IL-1β as described in Materials and Methods. Intracellular luciferase activities were determined and normalized to β-galactosidase activities as an internal control. Similar experiments were repeated three times, and the results from one representative experiment are shown. mut, mutant.

bination of NF-κB with either AP-1 or NF-IL6, depending on the types of cells (22, 26, 29, 46). These observations prompted us to investigate the contribution of these transcription factors to *H. pylori*-driven IL-8 gene transcription. As in the case of TNF-α–IFN-γ-induced IL-8 gene transcription in MKN45 cells (46), we identified NF-κB and, to a lesser degree, AP-1 binding sites as an essential *cis* elements involved in IL-8 gene transcription. Since we could not detect NF-IL6 complex formation in MKN45 cells incubated with various stimuli (31), AP-1 may substitute for NF-IL6 in MKN45 cells to induce IL-8 gene transcription in collaboration with NF-κB.

We observed that coculture with live *H. pylori* induced the formation of NF-κB complexes which were immunochemically identified as p50-p50 and p65-p50 complexes. We also observed that various types of inflammatory stimuli such as IL-1, TNF-α, and LPS induced NF-κB complex formation, culminating in IL-8 gene transcription. In addition to these soluble mediators, intact respiratory syncytial virus induced IL-8 gene transcription by activating mainly NF-κB (25). Thus, it is likely that the interaction with bacteria or virus could generally induce the activation of NF-κB. This may, in turn, cause the transcription of genes involved in inflammatory responses, such as proinflammatory cytokines, adhesion molecules, and acute-phase reactant proteins. Thus, the elucidation on the molecular mechanism of *H. pylori*-induced NF-κB activation may unravel the pathogenesis of *H. pylori*-associated gastric diseases with a respect to the activation of proinflammatory cytokine genes and may eventually be useful in the development of a novel type of drug for these diseases.

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