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Stimulation of Interleukin-8 Production in Epithelial Cell Lines by *Helicobacter pylori*

JINZHOU HUANG, PAUL W. O'TOOLE,† PETER DOIG, AND TREVOR J. TRUST*

Canadian Bacterial Diseases Network and Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada

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Following exposure to *Helicobacter pylori* cells, epithelial cell lines secreted interleukin-6 (IL-6) and IL-8 but not tumor necrosis factor alpha. Purified IL-6 alone did not stimulate IL-8 production from the cell lines tested, indicating that IL-6 was not an intermediary in IL-8 induction. Enhanced IL-8 secretion occurred in a time- and dose-dependent manner. None of 12 antibiotics tested exhibited a significant effect on IL-8-inducing activity, suggesting that preformed antigens were responsible for stimulating IL-8 secretion in vitro. Live bacterial cells caused the highest level of stimulation. Proteinase-digested and heated (56 or 100°C) cells had significantly reduced stimulatory activities. Purified *H. pylori* lipopolysaccharide, but not exopolysaccharide, stimulated low-level secretion of IL-8, but only at high concentrations, while a water-extracted *H. pylori* antigen preparation was strongly stimulatory for HEp-2 cells. No reduction in IL-8-stimulatory activity was observed for *H. pylori* mutants negative for urease activity, production of a major lipoprotein, and motility. The noncytotoxic strain CCUG 915 stimulated lower IL-8 levels than other isolates. However, the otherwise isogenic cytotoxin-negative mutant 17874Δ*vacA* (S. H. Phadnis, D. Ilver, L. Janzon, S. Normark, and T. U. Westblom, Infect. Immun. 62:1557–1565, 1994) had the same IL-8-stimulatory ability as the parent strain, suggesting that surface proteins other than the vacuolating cytotoxin are involved in IL-8 stimulation.

Helicobacter pylori infection is now recognized as the primary cause of active chronic gastritis in humans (10, 33, 41). The organism also appears to play a pivotal role in peptic ulcer disease, and epidemiological data have also suggested that persistent infection with this bacterium is a risk factor for the development of gastric carcinoma (9, 36, 40).

In studies directed at elucidating the mechanism by which *H. pylori* recruits inflammatory cells, Mai et al. (31) have shown that extracted antigens from *H. pylori* have chemotactic activity in vitro for monocytes and neutrophils, and they have implicated urease as a possible chemotactic modulator. The urease antigen was detected in vivo in the lamina propria of gastric biopsies from patients infected with *H. pylori*. Immunomodulatory substances secreted in response to *H. pylori* infection have also been examined. For example, recent studies have demonstrated that increased levels of gastric interleukin-8 (IL-8) (16, 35) and mucosal anti-IL-8 immunoglobulin A autoantibodies (13) are present in *H. pylori*-associated gastritis and that *H. pylori* components such as the 31-kDa porin (46) and lipopolysaccharide (LPS) (5) can stimulate cytokines in polymorphonuclear leukocytes. Cytotoxic strains of *H. pylori* have been reported to induce significantly higher levels of IL-8 secretion in cultured gastric cell lines than cytotoxin-negative strains (12), and an association between infection with cytotoxin-positive strains and increased polymorphonuclear leukocyte infiltration has been suggested (11).

IL-8, a small polypeptide, is secreted by several cell types, including monocytes, fibroblasts, endothelial cells, and epithelial cells (3). The primary function of IL-8 is thought to be to serve as a potent inflammatory mediator attracting and activating polymorphonuclear leukocytes, neutrophils in particular (8, 23, 34). Since *H. pylori* appears to contact only the surface of the gastric epithelium, the secretion of IL-8 by the gastric epithelium as a result of the interaction between the bacterium and the epithelium may be important in initiating and regulating inflammatory and immune processes in response to this bacterium. The objective of the present study was to investigate and assess the activities of potential components of *H. pylori* in induction of IL-8 secretion in cultured epithelial cells. These data indicate considerable variability in the ability of *H. pylori* to stimulate IL-8 secretion in different epithelial cell lines. Furthermore, cell-associated proteins appear to be responsible for the majority of IL-8-stimulatory activity in vitro. Importantly, however, this stimulation appears to be independent of both an active urease enzyme and the production of the vacuolating cytotoxin VacA.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. pylori* strains used in this study were CCUG 915 (Culture Collection of the University of Gothenburg, Sweden isolate) and CCUG 17874 (equivalent to NCTC 11637, the type strain of *H. pylori*). Strain 5155 is from the culture collection of the University of Victoria and is 20-fold less adhesive for cultured cells than strain CCUG 17874 in in vitro binding assays (20). Genetically defined mutants defective in the flagellar hook protein FlgE (37); a major cell envelope lipoprotein, Lpp20 (27); the vacuolating cytotoxin VacA (42); and the putative adhesin HpaA (24) were cultured in a selective medium supplemented with 20 μg of kanamycin per ml for cultivation of the *flgE*, *vacA*, and *hpaA* mutants and with 10 μg of chloramphenicol per ml for cultivation of the *lpp20* mutant. *H. pylori* cells were grown in brucella broth supplemented with 2% (vol/vol) fetal calf serum at 37°C for 18 h in a microaerophilic atmosphere with shaking at 150 rpm.

Cell lines and stimulation. The human cell lines KATO III (gastric carcinoma, ATCC HTB 103), AGS (gastric adenocarcinoma, ATCC CRL 1739), HEp-2 (epidermoid carcinoma, ATCC CCL 23), and Int 407 (embryonic intestine, ATCC CCL 6) were obtained from the American Type Culture Collection, Rockville, Md. Cells were grown in a medium consisting of 20% (vol/vol) Ham's F12 medium, 35% (vol/vol) Dulbecco's modified Eagle's medium, 35% (vol/vol) RPMI 1640 medium, and 10% (vol/vol) fetal calf serum and containing 100 U of penicillin per ml and 100 μg of streptomycin per ml.

Cells were seeded in 24-well tissue culture plates at 2×10^5 cells per well in a volume of 1 ml per well and cultured for 2 days. Prior to stimulation, each well was washed twice with 1 ml of fresh tissue culture medium containing no antibiotic. Bacterial cells were harvested from a broth culture by centrifugation,

* Corresponding author. Phone: (604) 721-7079. Fax: (604) 721-8855. Electronic mail address: ttrust@sol.uvic.ca.

† Present address: Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

resuspended in antibiotic-free tissue-culture medium, and added to the cultured cells at a bacterium/cell ratio of 100:1 in a 1-ml volume. Supernatants (50 μl) were collected at various time intervals.

Cytokine ELISA. Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight with mouse monoclonal antibodies against human IL-6, IL-8, or tumor necrosis factor alpha (TNF-α) (R&D System, Minneapolis, Minn.) and then were washed three times with 10 mM phosphate-buffered saline (pH 7.4) (PBS)–0.05% (vol/vol) Tween 20 and blocked with tissue culture medium containing 10% (vol/vol) fetal calf serum at 37°C for 90 min. After removal of the medium, supernatants from the stimulation experiments were diluted in tissue culture medium and were added in duplicate to the wells. The plates were incubated at 37°C for 90 min. After the wells were washed as described above, diluted goat polyclonal immunoglobulin G specific for the cytokine to be tested (R&D System) was added to each well and the plates were incubated at 37°C for 60 min. The wells were washed as described above, and alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G (Southern Biotechnology Associates, Birmingham, Ala.) was added. After the plates were incubated at 37°C for 60 min, the wells were washed as described above. A substrate solution consisting of 1 mg of disodium *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) per ml in 1 M diethanolamine buffer (pH 9.5) containing 0.5 mM $MgCl_2$ was added to each well (100 μl), and the plates were incubated at room temperature for 10 to 15 min. The reaction was terminated by the addition of 3 M NaOH. The A_{405} of each well was determined in an ELISA reader. Purified human recombinant IL-6, IL-8, and TNF-α (R&D System) were used as standards. The detection limits of the ELISA for IL-6, IL-8, and TNF-α were 35, 80, and 70 pg/ml, respectively.

Chemical and physical treatments of *H. pylori* cells prior to stimulation. *H. pylori* CCUG 17874 cells were harvested from broth culture by centrifugation and resuspended in PBS (ca. 5×10^{10} cells per ml). The bacterial suspensions were treated either with proteinase K (final concentration, 50 μg/ml) at 37°C for 1 h or with formaldehyde (1%) at 4°C overnight. Bacteria were then collected by centrifugation and washed with PBS (twice in the case of proteinase K treatment and four times in the case of formaldehyde treatment). The bacteria were then resuspended in tissue culture medium for the stimulation assay. Bacterial suspensions were also subjected to heat treatment at 56°C for 30 min or 100°C for 10 min or to storage at 4°C overnight.

Induction of IL-8 in the presence of antibiotics. All antibiotics were obtained from Sigma Chemical Co. Stimulation of HEp-2 and KATO III cells by *H. pylori* CCUG 17874 cells or LPS was carried out in the presence of ampicillin (150 μg/ml), chloramphenicol (200 μg/ml), gentamicin (100 μg/ml), kanamycin (100 μg/ml), novobiocin (100 μg/ml), penicillin (100 U/ml), polymyxin B (100 μg/ml), polymyxin E (100 μg/ml), rifampin (40 μg/ml), spectinomycin (100 μg/ml), streptomycin (100 μg/ml), and tetracycline (40 μg/ml). IL-8 levels were measured by ELISA. Controls included stimulation in the absence of antibiotics, addition of antibiotics to epithelial cells without bacterial stimulation, and use of epithelial cells alone.

Cytotoxicity assay. The cytotoxicity assay was performed essentially as described by Leunk et al. (29). The supernatant separated from *H. pylori* broth culture by centrifugation was concentrated 30-fold by using a microconcentrator with a 30-kDa-cutoff membrane (Centricon YM30; Amicon, Danvers, Mass.). The concentrate was added to cultured Int 407 cells. The vacuolization of Int 407 cells was examined microscopically after 12 to 24 h of incubation.

Electrophoresis and Western blotting (immunoblotting). Protein samples were electrophoresed in 12.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (28) in a minigel format (separating gel, 8 by 5 cm) or in 8 to 25% gradient Phast gels (Pharmacia, Uppsala, Sweden). When required, separated proteins were transferred from a polyacrylamide slab gel to nitrocellulose paper by the methanol Tris-glycine system described by Towbin et al. (45). Electroblotting and subsequent steps were carried out by standard methodology (38). The reactive bands were visualized by the method of Blake et al. (6) by using 5-bromo-4-chloro-3-indolylphosphate (Boehringer, GmbH) as the alkaline phosphatase substrate and Nitro Blue Tetrazolium (Sigma Chemical Co.) as the color development reagent.

Preparation of bacterial extracts. LPS was isolated from *H. pylori* CCUG 17874 by the method described by Westphal and Jann (49), using phenol extraction, and by the proteinase K digestion method of Darveau and Hancock (17). Exopolysaccharide (EPS) was prepared essentially as described by Johnson et al. (25) and quantitated by dry weight measurement. LPS was examined by using SDS-polyacrylamide gels stained with silver as described by Hitchcock and Brown (22). The protein contents of the LPS and EPS preparations were determined with the Bradford protein assay (7) with bovine serum albumin as a standard and by Coomassie brilliant blue staining of SDS-polyacrylamide gels. Water-extracted antigen was prepared as described by Mai et al. (32). A smooth form of LPS from *Escherichia coli* serotype O26:B6 and a rough form of LPS from *E. coli* EH100 (Ra mutant) were purchased from Sigma Chemical Co.

Analysis of mRNA. Total RNA from cultured epithelial cells was extracted by using the Ultraspec RNA isolation reagent as recommended by the manufacturer (Biotecx Laboratories, Houston, Tex.). The production of IL-6, IL-8, and TNF-α mRNAs was analyzed by reverse transcriptase PCR (RT-PCR) and Northern (RNA) blotting with synthetic oligonucleotide primers specific for the relevant genes and transcripts, as described by Yamamura et al. (50).

TABLE 1. RT-PCR analysis of production of IL-6, IL-8, and TNF-α mRNAs by various cell lines in response to stimulation by cells of *H. pylori* CCUG 17874

Cytokine	Production in the following cell line[a]:			
	HEp-2	Int 407	KATO III	AGS
IL-6	+	−	−	ND
IL-8	+	+	+	+
TNF-α	−	−	−	−

[a] +, mRNA production in three independent experiments; −, no mRNA production; ND, not determined.

Chemical mutagenesis of *H. pylori*. A 10-ml culture of *H. pylori* CCUG 17874 was incubated for 16 h at 37°C, divided into five 2-ml aliquots, and treated with a dilution series of nitrosoguanidine (NTG). The final concentrations were 0, 12.5, 25, 62.5, and 125 μg/ml, from a stock solution of 2.5 mg/ml in absolute ethanol. The cell suspensions with NTG were incubated for 10 min at 37°C, washed twice with 10 ml of brain heart infusion broth by brief centrifugation and resuspension, and then transferred to 10 ml of fresh brain heart infusion broth. Following a 12-h incubation at 37°C with shaking, the turbidity was determined by A_{600}. The sample treated with 25 μg of NTG per ml showed approximately a 50% reduction in viability relative to the untreated control and was frozen at −70°C in 50% glycerol as a mutant stock.

Isolation of urease and adhesion mutants. Dilutions of the NTG mutant stock were plated and cultured for single colonies, which were then individually transferred with toothpicks into urease indicator medium (2). Clones which were phenotypically urease negative were further characterized by Western immunoblotting with monoclonal antibodies specific for the 31- and 66-kDa urease subunits (19). Nonadhesive mutants were enriched by growth of the mutant *H. pylori* stock in brain heart infusion broth over a monolayer of Int 407 cells. After 2 h, the supernatant was removed and nonadhesive mutants were amplified by four further rounds of enrichment. Finally, the suspension was plated in serial dilution for single colonies, which were individually tested for adhesion in a standard assay (18) and in a hemagglutination assay (18).

RESULTS

Cytokine production stimulated by *H. pylori* cells. Four cell lines were initially tested for enhanced production of IL-6, IL-8, and TNF-α following exposure to *H. pylori* CCUG 17874. An RT-PCR assay with primer pairs specific for each cytokine was employed. The results (Table 1) indicated that messages for IL-6 and IL-8 were produced in response to exposure to *H. pylori* cells but that messages for TNF-α were not produced. The ability of *H. pylori* cells to stimulate IL-8 production was chosen for further study.

To further examine the elevated levels of IL-8-specific message, RT-PCR with a specific oligonucleotide primer was employed. Figure 1 shows the increase in IL-8-specific message production following stimulation with *H. pylori* cells, relative to the constant level of the β-actin message. This was confirmed by Northern blotting (data not shown). RT-PCR (Fig. 1, lane 1) indicated a constitutive, but very low-level, production of

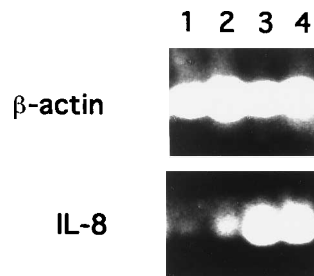

FIG. 1. Detection of IL-8 mRNA expression in HEp-2 cells by RT-PCR at 0, 3, 10, and 16 h (lanes 1, 2, 3, and 4, respectively) after stimulation with *H. pylori* CCUG 17874 cells. β-Actin was used as a control.

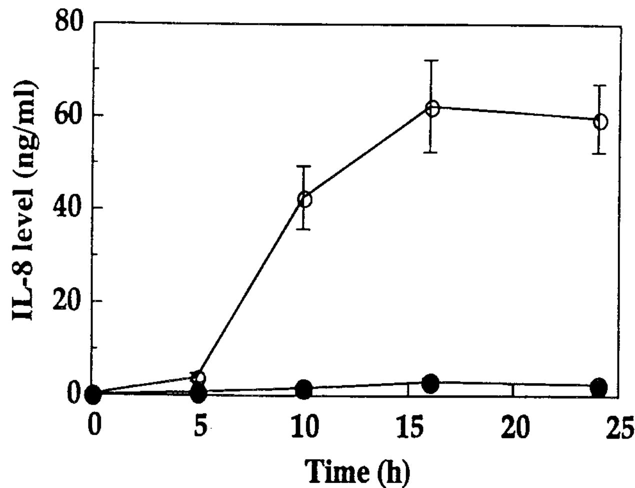

FIG. 2. Kinetic effects of TNF-α (○) (50 ng/ml) and IL-6 (●) (50 ng/ml) on IL-8 induction in HEp-2 cells. The error bars indicate the standard deviations from three determinations.

IL-8-specific message in unstimulated cells. For rapid and quantitative estimation of IL-8 production, an ELISA protocol with a monospecific monoclonal antibody to IL-8 was established. The IL-8-inducing capabilities of purified TNF-α and IL-6 on HEp-2, Int 407, and KATO III cells were also examined by IL-8-specific ELISA. This analysis (represented for HEp-2 cells in Fig. 2) indicated that while TNF-α was capable of stimulating IL-8 production, IL-6 was not.

Properties of the IL-8 response. When stimulated with *H. pylori* cells, epithelial cells secreted IL-8 in a time (Fig. 3)- and dose (Fig. 4)-dependent manner. Although IL-8 secretion could be detected in the culture supernatant within 3 h of addition of bacterial cells, the most active secretion occurred during the period from 3 to 15 h. For two cell lines tested, HEp-2 and KATO III, the number of bacterial cells per cultured cell that gave maximal IL-8 secretion was approximately 50. A ratio of 100 bacterial cells per cultured cell was routinely employed. This ratio was chosen so that more bacterial cells than were needed for maximal stimulation were available. Of the four cell lines tested, HEp-2 and Int 407 produced significantly higher levels of both induced and constitutive IL-8 than the gastric carcinoma cell lines KATO III and AGS (Fig. 5).

Compared with that by freshly prepared bacterial cells, IL-8 secretion by stimulated KATO III and HEp-2 cells was reduced when the bacteria were pretreated with proteinase K, with heating at 56 or 100°C, or with storage at 4°C overnight (Table 2). Bacterial cells treated with 1% formaldehyde showed no induction of IL-8 (Table 2). None of 12 antibiotics tested had a significant effect on the IL-8-inducing activity of *H. pylori* CCUG 17874 cells (data not shown).

Induction of secretion of IL-8 by *H. pylori* strains and mutants with various phenotypes. Three wild-type strains were tested for IL-8 stimulation. Strain 5155 is distinguished by its poor binding to cultured cells but, like CCUG 17874, produces the vacuolating cytotoxin. Neither of these strains is highly motile, in contrast to CCUG 915 (37). Strains CCUG 17874 and 5155 elicited high levels of IL-8 secretion, while strain CCUG 915 caused comparatively low levels (Table 3). During other studies of *H. pylori* surface proteins, a number of defined mutants defective in production of individual antigens had been created. Examination of the IL-8 stimulation induced by these mutants showed that loss of motility (*flgE* mutant), a

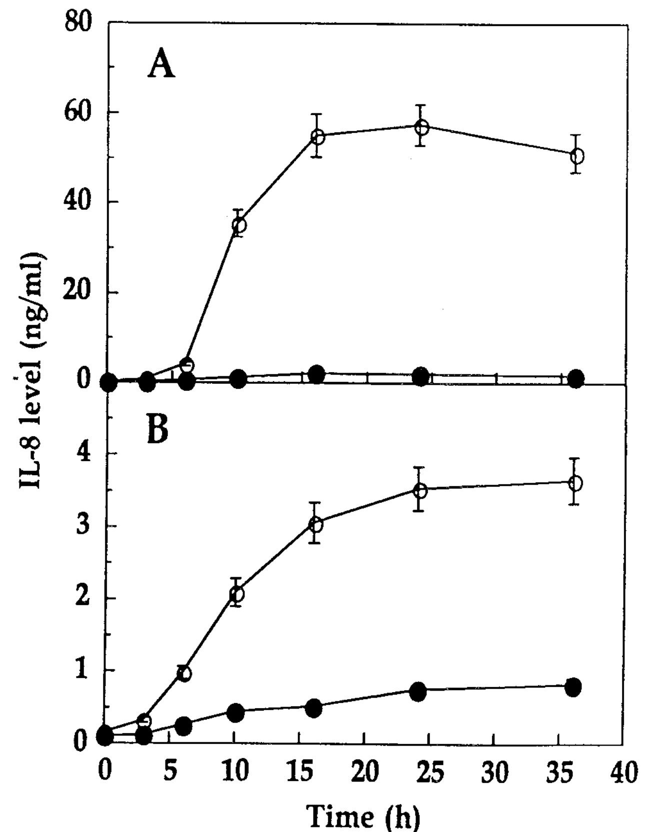

FIG. 3. Kinetics of IL-8 induction in HEp-2 (A) and KATO III (B) cells by *H. pylori* CCUG 17874 cells (○) (bacterium/cell ratio of 100:1) or a control (●) (no bacterial cells added). The error bars indicate the standard deviations from three determinations.

major cell envelope lipoprotein (*lpp20* mutant), and the presumptive adhesin HpaA had no significant effect on stimulatory activity (Table 3). Among five NTG-induced adhesion mutants, only one (M13) showed an appreciable reduction in IL-8 stimulation. In contrast to the other chemically induced mutants isolated, this variant was defective in hemagglutination, and it was virtually nonadhesive to the cell lines examined. Other phenotypic properties tested for mutant M13 were normal including motility, urease production, and cytotoxicity (data not shown).

Since urease has been implicated previously as a chemotactic substance for leukocytes (31), NTG-generated mutants impaired in urease activity were isolated and tested for their abilities to cause IL-8 secretion. Two of these continued to produce both of the major urease subunits, while a third, U24, failed to produce the larger 66,000-M_r subunit. However, all three mutants elicited IL-8 at levels similar to those elicited by the parent strain (Table 3).

Effect of the vacuolating cytotoxin on IL-8 secretion in vitro. Among the *H. pylori* strains and mutants with various phenotypes, it was apparent that the noncytotoxic strain CCUG 915 (and mutant derivatives) showed a significantly lower level of IL-8-inducing activity than the vacuolating cytotoxic strains CCUG 17874 and 5155 (Table 3). To directly test the role of the vacuolating cytotoxin VacA, a strain genetically constructed with a kanamycin resistance cassette inserted in the *vacA* gene was employed. The cytotoxin-negative mutant 17874Δ*vacA* (42) induced IL-8 at levels similar to those induced by the parent strain (Table 3).

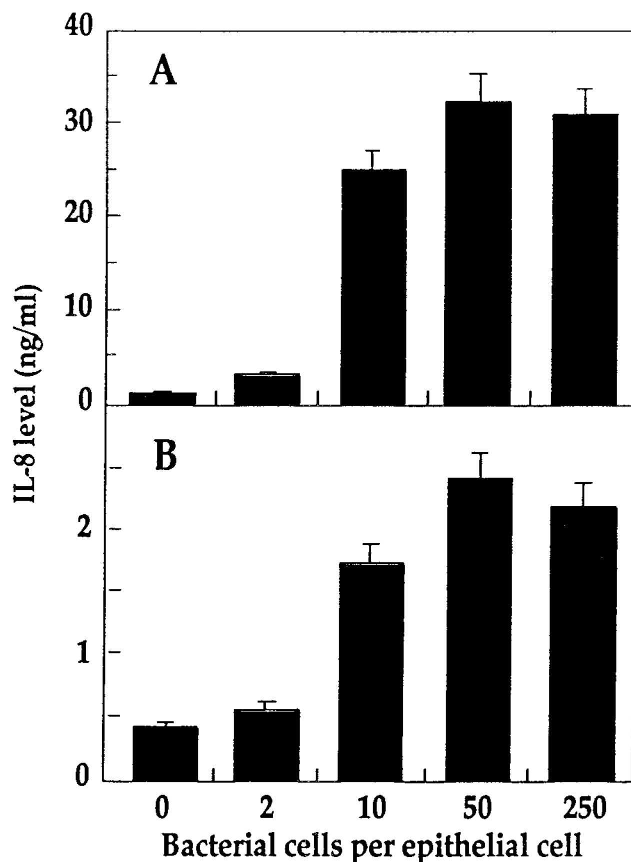

FIG. 4. Effect of *H. pylori* CCUG 17874 cell dose on IL-8 induction by the HEp-2 (A) and KATO III (B) cell lines (incubation was for 12 h). Values represent the means of three determinations ± standard deviations.

IL-8-stimulatory activity in culture supernatants. *H. pylori* broth culture supernatants which had been concentrated to 1/30 of the original volume were also capable of stimulating IL-8 production from epithelial cell lines in a time- and dose-dependent manner, and this activity was also sensitive to heat

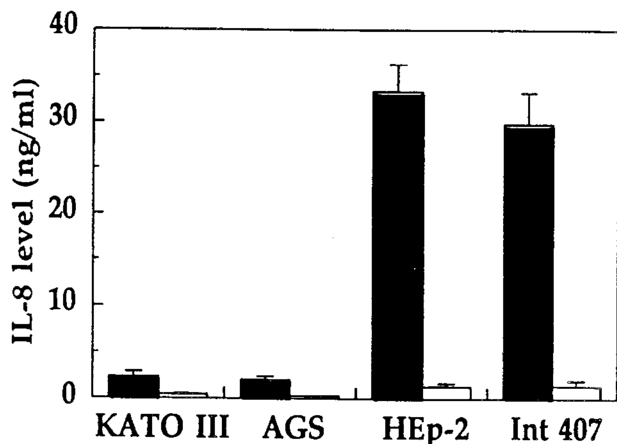

FIG. 5. Effect of *H. pylori* CCUG 17874 cells (■) (bacterium/cell ratio, 100:1; incubation was for 12 h) or a control (□) (no bacterial cells added) on IL-8 induction in various cell lines. Values represent the means of three determinations ± standard deviations.

TABLE 2. Effect of treatment of *H. pylori* CCUG 17874 cells on induction of IL-8 in cultured KATO III cells

Treatment[a]	Relative production of IL-8 (%)[b]
None	100 ± 9
Storage at 4°C overnight	69.9 ± 8.8
Proteinase K[c]	50.0 ± 7.0
56°C, 30 min	77.5 ± 6.5
100°C, 10 min	65.0 ± 5.7
1% formaldehyde	17.3 ± 2.0
Control (no bacterial cells added)	19.2 ± 2.4

[a] Bacterium/KATO III cell ratio, 100:1; incubation was for 12 h.
[b] 100% = 2,521 pg of IL-8 per ml. Results are expressed as means ± standard deviations for three determinations.
[c] Proteinase K was used at 50 μg/ml; treatment was at 37°C for 1 h.

treatment (data not shown). Since in previous studies (12) cytotoxicity had been implicated in IL-8 stimulation, the noncytotoxic mutant 17874Δ*vacA* was examined. Comparison of the supernatants derived from strains CCUG 17874, CCUG 915 and 17874Δ*vacA* indicated that the *vacA* mutant had the same level of stimulatory activity as the parent strain, while CCUG 915 (cytotoxin negative) exhibited a remarkably low level of activity (Fig. 6). The low level of IL-8 stimulation caused by culture supernatants of strain CCUG 915 was similar to that of live cells from the same strain.

IL-8-stimulatory activities of *H. pylori* surface carbohydrates. The significant but incomplete reduction of IL-8 secretion by proteinase digestion and heat pretreatment of *H. pylori* cells suggested that a nonproteinaceous component might act as an IL-8 stimulator. To evaluate this possibility, the two major surface carbohydrate components, LPS and EPS, were purified from *H. pylori* CCUG 17874. The purified materials were judged to be free of protein by the Bradford protein assay and by Coomassie brilliant blue staining of SDS-polyacrylamide gels. EPS contained no detectable LPS when analyzed by silver-stained polyacrylamide gel electrophoresis. LPS stimulated IL-8 production only at high concentrations (10 μg/ml or greater). No stimulation was noted with EPS (Fig. 7), even though concentrations as high as 500 μg of EPS per ml were used in the assay. Compared with *E. coli* LPS, *H. pylori* LPS was a slightly less potent stimulant (Fig. 7). Polymyxins B and E, which bind to LPS, had no inhibitory effect on the IL-8-stimulatory activity of whole bacterial cells and only a modest effect on purified LPS (Table 4). In contrast to LPS, water-extracted antigen, which is reported to contain mainly surface proteins (32), was strongly stimulatory for HEp-2 cells but weakly stimulatory for KATO III cells (Fig. 7).

DISCUSSION

In order to investigate which components of *H. pylori* might play a role in cytokine stimulation, an in vitro assay with epithelial cell lines was developed. Three separate assays (RT-PCR, Northern blotting, and ELISA) to detect and/or quantify cytokines were established. These techniques were employed in initial screening for three cytokines, i.e., TNF-α, IL-6, and IL-8. None of the techniques employed in this study indicated that *H. pylori* stimulated production of TNF-α in epithelial cell lines. The ELISA method of detection demonstrated that *H. pylori* induced the secretion of IL-6 and IL-8. The levels of IL-6 produced by the cell lines examined were significantly lower than those of IL-8 (data not shown), in agreement with results of *H. pylori* stimulation of monocytes (46). In agreement with previous studies (14), IL-8 stimulation by *H. pylori* was also

TABLE 3. IL-8-inducing activities of wild-type and mutant strains

Strain[a]	Relevant feature(s)[b]	Vacuolating cytotoxicity	IL-8 (pg/ml)[c] with:	
			KATO III cells	HEp-2 cells
Parental strains				
5155	Wild type, low-level adhesion	+	3,683 ± 267	35,970 ± 2475
CCUG 915	Wild type, highly motile	−	505 ± 23	1,665 ± 98
CCUG 17874	Wild type	+	2,450 ± 206	32,270 ± 2945
Isogenic mutants				
17874Δ*vacA*	Cytotoxin defect	−	2,765 ± 233	34,307 ± 1866
17874Δ*lpp20*	20-kDa lipoprotein defect	+	2,636 ± 230	33,580 ± 2607
17874Δ*hpa*	25-kDa adhesin defect	+	3,443 ± 255	41,460 ± 3658
915Δ*flgE*	915 mutant, nonmotile	−	678 ± 39	2,120 ± 222
17874 NTG-generated mutants				
M3	Low-level adhesion, HA^+	+	2,259 ± 188	30,310 ± 2340
M4	Low-level adhesion, HA^+	+	2,508 ± 185	30,780 ± 2083
M9	Low-level adhesion, $HA^{\pm}$	+	2,735 ± 211	33,230 ± 3103
M10	Low-level adhesion, $HA^{\pm}$	+	2,266 ± 187	29,370 ± 2194
M13	Nonadhesive, HA^-	+	1,480 ± 127	17,870 ± 1344
U1	Urease activity$^-$, 66-kDa$^+$, 31-kDa$^+$	+	1,809 ± 148	27,720 ± 1708
U18	Urease activity$^-$, 66-kDa$^+$, 31-kDa$^+$	+	2,855 ± 222	29,880 ± 2400
U24	Urease activity$^-$, 66-kDa$^-$, 31-kDa$^+$	+	2,008 ± 162	37,430 ± 3404
Control (no bacteria added)			473 ± 38	1,122 ± 115

[a] Δ indicates insertional inactivation of the gene encoding the respective protein.
[b] HA, hemagglutination. The 66- and 31-kDa proteins are the two subunits of *H. pylori* urease.
[c] Expressed as means ± standard deviations for three to five determinations. Induction of IL-8 was carried out for 12 h.

shown to be independent of IL-6 production. With KATO III cells, levels of IL-8 production similar to those reported by Crabtree et al. (12) for the same cell line were induced. However, significantly higher levels of IL-8 were produced by the nongastric cell lines HEp-2 and Int 407, suggesting a potentially important difference between cells of gastric and nongastric origins. Indeed, IL-8 stimulation by *H. pylori* and its cellular components was found to be cell line dependent in some cases. Despite the differences in the amounts of IL-8 produced, the bacterial dose responses and kinetic responses in IL-8 induction were similar regardless of cell line origin. Whereas similar results were obtained with the other assay systems, RT-PCR failed to detect IL-6 production in two cell lines judged positive by ELISA. Thus, the ELISA system was chosen as the method to quantify IL-8 in this study because of its high level of sensitivity, its reproducibility, and the methodological ease by which it might be performed.

Stimulation of IL-8 by *H. pylori* could be due to the release of soluble bacterial extracellular factors or to direct contact with bacterial surface components. Indeed, a role for direct contact and/or adhesion in IL-8 stimulation has been demonstrated with a variety of gram-negative bacteria, including *Salmonella* spp. (21), *Listeria moncytogenes* (21), and *E. coli* (1, 30). Three strains of *H. pylori* that are altered in their adhesive phenotypes were employed to examine the possible role of cell-cell contact in the stimulation of IL-8 secretion. Strain 5155, which is an isolate with a low level of binding, and a mutant negative for expression of the putative fibrillar adhesin HpaA were both potent stimulators of IL-8 production, as

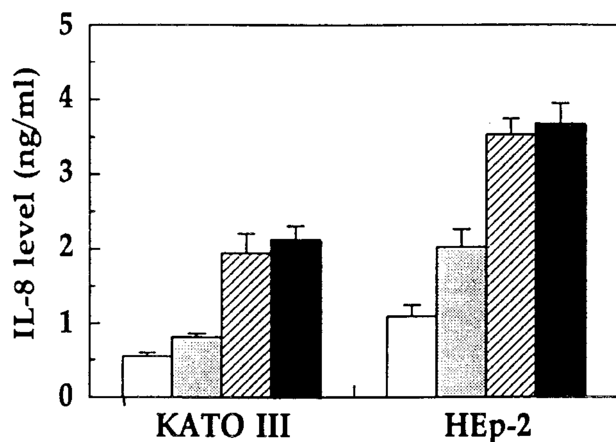

FIG. 6. Assessment of IL-8-stimulatory potentials of concentrated supernatants prepared from broth cultures of *H. pylori* CCUG 915 (▒), CCUG 17874 (▨), and 17874Δ*vacA* (■) or uninoculated broth (□) (bacterium/cell ratio, 100:1; incubation was for 12 h) on the KATO III and HEp-2 cell lines. Values represent the means of three determinations ± standard deviations.

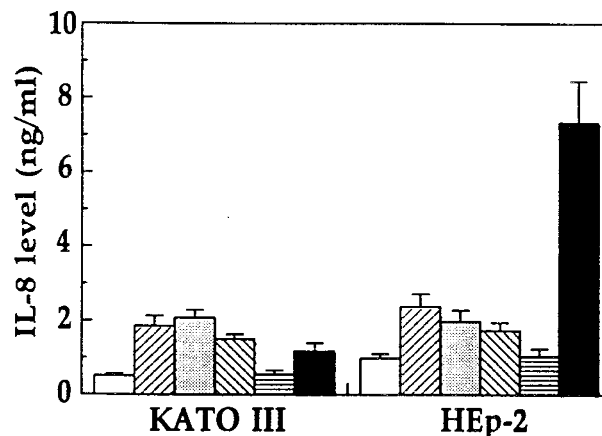

FIG. 7. Assessment of IL-8-stimulatory potentials of various bacterial cell surface components. LPS (▧) (10 μg/ml), EPS (▤) (500 μg/ml), and water-extracted antigen (■) (50 μg of protein per ml) (all prepared from *H. pylori* CCUG 17874), commercially available rough (▨) and smooth (▒) LPS (10 μg/ml) from E. coli, and tissue culture medium (□) were used. Values represent the mean of three determinations ± standard deviations.

TABLE 4. Effects of polymyxin on IL-8-inducing activities of *H. pylori* CCUG 17874 cells and LPS[a]

Stimulus	Relative IL-8 stimulation (%) with:	
	KATO III cells	HEp-2 cells
Live cells	100 ± 10	100 ± 12
+Polymyxin B	123 ± 9	115 ± 9
+Polymyxin E	115 ± 5	106 ± 11
LPS	69.4 ± 6.6	5.91 ± 0.72
+Polymyxin B	52.1 ± 3.5	4.78 ± 0.37
+Polymyxin E	45.5 ± 4.6	3.81 ± 0.34
PBS	23.5 ± 2.5	3.35 ± 0.41
+Polymyxin B	26.7 ± 1.6	3.49 ± 0.50
+Polymyxin E	27.9 ± 2.3	3.67 ± 0.41

[a] Induction of IL-8 was carried out for 12 h as described in the text. Results are expressed as the relative levels (means ± standard deviations) for three determinations. Final concentrations: LPS, 10 μg/ml; polymyxins, 100 μg/ml. LPS was incubated with polymyxin B or polymyxin E at 37°C for 1 h prior to stimulation.

were all but one of the NTG-generated reduced-binding mutants of CCUG 17874. The NTG-generated reduced-binding mutant M13 exhibited a reduction of approximately 50% in the level of IL-8 stimulated. The apparent contradiction of these data may be explained if direct contact is necessary for stimulation but small numbers of adherent bacteria are capable of causing such stimulation. In this case, the mutant M13 fails to bind at this threshold level, whereas the other strains do. However, it must be recalled that the lesion responsible for this phenotypic change in M13 has not yet been identified and that such a lesion may have effects on other bacterial components that are not involved in adhesion.

In addition to examining the role of cell-cell contact in stimulation, we examined the effects of various bacterial components on IL-8 stimulation. Water-soluble extracts of *H. pylori* were prepared and found to stimulate IL-8 expression. These extracts contain a large number of bacterial proteins, such as urease, GroEL, flagella, and various outer membrane proteins (data not shown) such as the IL-6-stimulating porin reported by Tufano et al. (46). The extracts also contained LPS and EPS. A reduction of IL-8-stimulating ability by heat or proteinase K treatment prior to the stimulation assay implicated a proteinaceous component. Live bacteria had a much stronger stimulating ability than cells made nonviable by storage or chemical treatment, suggesting that either the IL-8-stimulating component was labile or there was a need for active bacterial metabolism. To specifically disrupt bacterial metabolism, antibiotics were employed, but none had an effect on IL-8 stimulation, suggesting that the bacterial component was preformed. Crabtree et al. (12) noted a decrease in stimulation caused by gentamicin. In addition to inhibiting protein synthesis, this antibiotic has been shown to disrupt the outer membrane by an LPS-dependent mechanism (26). It is possible that the effect that Crabtree et al. observed was due to disruption of the outer membrane and cellular lysis rather than to the antimetabolic effect of the antibiotic. The strain used in the present study, CCUG 17874, has rough-form LPS rather than smooth-form LPS, possibly reducing the outer-membrane-disrupting effect of gentamicin.

The LPS of pathogenic bacteria has been shown to be a potent IL-8 stimulus in monocytes (43, 44, 48). It has been shown also that *H. pylori* LPS induces levels of IL-6 in mononuclear leukocytes comparable to those induced by *E. coli* and *Campylobacter jejuni* but that it induces much lower levels of IL-1 and TNF-α (5). The present study shows that LPS purified from *H. pylori* CCUG 17874 has poor IL-8-inducing activity and that polymyxins have no effect on stimulation by live cells, making it unlikely that LPS is an important component in stimulating IL-8 secretion in the assay system employed. Other bacterial polysaccharides have been shown to stimulate cytokines (4, 39), but EPS purified from CCUG 17874 had no effect on IL-8 secretion.

Soluble extracts enriched with the urease of *H. pylori* have previously been shown to activate monocytes (32). The mechanism of activation was not elucidated by those authors, but their results suggest a role for IL-8. Among the phenotypically urease-negative mutants isolated in the present study, two continued to produce both subunits of the enzymatic complex, indicating that the defect was in either in the known ancillary proteins required for urease function or possibly in other, yet unidentified, proteins required for assembly or transport. Regardless, functional urease was not necessary for IL-8 stimulation in the cell lines tested here. Although we have not ruled out the possibility that the 31-kDa subunit may stimulate IL-8 secretion, it appears that the 66-kDa subunit would not be necessary.

The soluble extracts that we employed also contained a number of other proteins, as described above. We tested the abilities of a nonmotile flagellum (FlgE) mutant and a lipoprotein (Lpp20) mutant to induce IL-8 production. Neither mutant differed from the parental strain with respect to IL-8 stimulation. The inability of the flagellum mutation to affect IL-8 stimulation suggests that motility is not important in induction. However, it must be noted that the assay employed here does not mimic the in vivo situation, in which a mucus layer covers the gastric epithelium. In the in vivo case, motility might be important to bring the bacterium through the gastric mucin into close proximity with the gastric epithelium.

The cytotoxin produced by *H. pylori* has previously been implicated as an IL-8 inducer in gastric cell lines (12). To unequivocally determine the role of the cytotoxin of IL-8 stimulation, we tested a genetically constructed *vacA* mutant of strain CCUG 17874. Surprisingly, this strain was not significantly altered in the level of IL-8 it elicited, thus ruling out the cytotoxin as the stimulatory agent. A possible candidate for the stimulatory antigen is the CagA protein (15), which correlates with cytotoxin production (47). However, CagA might simply be a marker for another bacterial component required for IL-8 stimulation, as appears to be the case with the cytotoxin. Further studies must be conducted in order to identify the IL-8-stimulatory components of *H. pylori.*

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REFERENCES

1. **Agace, W., S. Hedges, U. Andersson, J. Andersson, M. Ceska, and C. Svanborg.** 1993. Selective cytokine production by epithelial cells following exposure to *Escherichia coli*. Infect. Immun. **61:**602–609.
2. **Austin, J. W., P. Doig, M. Stewart, and T. J. Trust.** 1991. Macromolecular structure and aggregation states of *Helicobacter pylori* urease. J. Bacteriol. **173:**5663–5667.
3. **Baggiolini, M., A. Waltz, and S. L. Kunkel.** 1989. Neutrophil-activating peptide-1/interleukin-8, a novel cytokine that activates neutrophils. J. Clin. Invest. **84:**1045–1049.
4. **Benabdelmoumene, S., S. Dumont, C. Petit, P. Poindron, D. Wachsmann, and J. P. Klein.** 1991. Activation of human monocytes by *Streptococcus mutans* serotype f polysaccharide: immunoglobulin G Fc receptor expression and tumor necrosis factor and interleukin-1 production. Infect. Immun. **59:**3261–3266.
5. **Birkholz, S., U. Knipp, C. Nietzki, R. J. Adamek, and W. Opferkuch.** 1993.

Immunological activity of lipopolysaccharide of *Helicobacter pylori* on human peripheral mononuclear blood cells in comparison to lipopolysaccharides of other intestinal bacteria. FEMS Immunol. Med. Microbiol. **6**:317–324.

6. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Colditz, I., R. Zwahlen, B. Dewald, and M. Baggiolini.** 1989. In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. Am. J. Pathol. **134**:755–760.
9. **Correa, P.** 1992. Human gastric carcinogenesis: a multistep and multifactorial process. Cancer Res. **52**:6735–6740.
10. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Ann. Rev. Med. **43**:135–145.
11. **Cover, T. L., P. Cao, C. D. Lind, K. T. Tham, and M. J. Blaser.** 1993. Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates in vitro and in vivo. Infect. Immun. **61**:5008–5012.
12. **Crabtree, J. E., S. Farmery, I. J. D. Lindley, P. Peichl, and D. S. Tompkins.** 1993. Cytotoxic strains of *Helicobacter pylori* induce IL-8 production by gastric epithelial cells. Acta Gastro-Enterologica Belgica **56**(Suppl.):48.
13. **Crabtree, J. E., P. Peichl, J. I. Wyatt, U. Stachl, and I. J. D. Lindley.** 1993. Gastric interleukin-8 and IgA IL-8 autoantibodies in *Helicobacter pylori* infection. Scand. J. Immunol. **37**:65–70.
14. **Crabtree, J. E., S. Pulsa, S. Farmery, P. Peichl, and I. J. D. Lindley.** 1993. Cytokine regulation of interleukin-8 secretion by gastric epithelial cell lines. Acta Gastro-Enterologica Belgica **56**(Suppl.):52.
15. **Crabtree, J. E., J. D. Taylor, J. I. Wyatt, R. V. Heatley, T. M. Shallcross, D. S. Tompkins, and B. J. Rathbone.** 1991. Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration and gastric pathology. Lancet **338**:332–335.
16. **Crabtree, J. E., J. I. Wyatt, L. K. Trejdosiewicz, P. Peichl, P. H. Nichols, N. Ramsay, J. N. Primrose, and I. J. D. Lindley.** 1994. Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. J. Clin. Pathol. **47**:61–66.
17. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
18. **Doig, P., J. W. Austin, M. Kostrzynska, and T. J. Trust.** 1992. Production of a conserved adhesin by the human gastroduodenal pathogen *Helicobacter pylori*. J. Bacteriol. **174**:2539–2547.
19. **Doig, P., and T. J. Trust.** 1994. Identification of surface-exposed outer membrane antigens of *Helicobacter pylori*. Infect. Immun. **62**:4526–4533.
20. **Doig, P., and T. J. Trust.** 1995. Unpublished observations.
21. **Eckmann, L., M. F. Kagnoff, and J. Fierer.** 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect. Immun. **61**:4569–4574.
22. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
23. **Huber, A. R., S. L. Kunkel, R. F. Todd, and S. J. Weiss.** 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science **254**:99–102.
24. **Janzon, L., and M. Wickman.** 1993. Construction of deletion mutants in the *hpa*A gene encoding the binding subunit of fibrillar adhesin of *Helicobacter pylori*. Acta Gastro-Enterologica Belgica **56**(Suppl.):103.
25. **Johnson, J. A., P. Panigrahi, and J. G. Morris.** 1992. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance, and virulence in mice. Infect. Immun. **60**:864–869.
26. **Kadurugamuwa, J. L., A. J. Clarke, and T. J. Beveridge.** 1993. Surface action of gentamicin on *Pseudomonas aeruginosa*. J. Bacteriol. **175**:5798–5805.
27. **Kostrzynska, M., P. W. O'Toole, D. E. Taylor, and T. J. Trust.** 1994. Molecular characterization of a conserved 20-kilodalton membrane-associated lipoprotein antigen of *Helicobacter pylori*. J. Bacteriol. **176**:5938–5948.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26**:93–99.
30. **Linder, H., I. Engberg, H. Hoschutzy, I. Mattsby-Blatzer, and C. Svanborg.** 1991. Adhesion-dependent activation of mucosal interleukin-6 production. Infect. Immun. **59**:4357–4362.
31. **Mai, U. E. H., G. I. Perez-Perez, J. B. Allen, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1992. Surface proteins from *Helicobacter pylori* exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J. Exp. Med. **175**:517–525.
32. **Mai, U. E. H., G. I. Perez-Perez, L. M. Wahl, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1991. Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. J. Clin. Invest. **87**:894–900.
33. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **i**:1311–1315.
34. **Matsushima, K., and J. J. Oppenheim.** 1989. Interleukin 8 and MCAF: novel inflammatory cytokines inducable by IL 1 and TNF. Cytokine **1**:2–13.
35. **Noach, L. A., N. B. Bosma, J. Jansen, F. J. Hoek, S. J. H. Vandeventer, and G. N. J. Tytgat.** 1994. Mucosal tumor necrosis factor-α, interleukin-1β, and interleukin-8 production in patients with *Helicobacter pylori* infection. Scand. J. Gastroenterol. **29**:425–429.
36. **Nomura, A., G. N. Stemmermann, P. H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. **325**:1132–1136.
37. **O'Toole, P. W., M. Kostrzynska, and T. J. Trust.** 1994. Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. Mol. Microbiol. **14**:691–703.
38. **O'Toole, P. W., S. M. Logan, M. Kostrzynska, T. Wadström, and T. J. Trust.** 1991. Isolation and biochemical and molecular analyses of a species-specific protein antigen from the gastric pathogen *Helicobacter pylori*. J. Bacteriol. **173**:505–513.
39. **Otterlei, M., A. Sundan, G. Skjak-Braek, L. Ryan, O. Smidsrod, and T. Espevik.** 1993. Similar mechanisms of action of defined polysaccharides and lipopolysaccharides: characterization of binding and tumor necrosis factor alpha induction. Infect. Immun. **61**:1917–1925.
40. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325**:1127–1131.
41. **Paull, G., and J. H. Yardley.** 1989. Pathology of *C. pylori*-associated gastric and esophageal lesions, p. 73–98. *In* M. J. Blaser (ed.), *Campylobacter pylori* in gastritis and peptic ulcer disease. Igaku Shoin Medical Publishers. New York.
42. **Phadnis, S. H., D. Ilver, L. Janzon, S. Normark, and T. U. Westblom.** 1994. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. Infect. Immun. **62**:1557–1565.
43. **Strieter, R. M., S. W. Chensue, M. A. Basha, T. J. Standiford, J. P. Lynch, M. Baggiolini, and S. L. Kunkel.** 1990. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor-α, lipopolysaccharide and interleukin-1b. Am. J. Respir. Cell Mol. Biol. **2**:321–326.
44. **Sylvester, I., J. A. Rankin, T. Yoshimura, S. Tanaka, and E. J. Leonard.** 1990. Secretion of neutrophil attractant/activation protein by lipopolysaccharide-stimulated lung macrophages determined by both enzyme-linked immunosorbent assay and N-terminal sequence analysis. Am. Rev. Respir. Dis. **141**:683–688.
45. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **74**:4350–4354.
46. **Tufano, M. A., F. Rossano, P. Catalanotti, G. Liguori, C. Capasso, M. T. Ceccarelli, and P. Marinelli.** 1994. Immunobiological activities of *Helicobacter pylori* porins. Infect. Immun. **62**:1392–1399.
47. **Tummuru, M. K. R., T. L. Cover, and M. J. Blaser.** 1993. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect. Immun. **61**:1799–1809.
48. **Walz, A., P. Peveri, H. Aschauer, and M. Baggiolini.** 1987. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. Biochem. Biophys. Res. Commun. **149**:755–761.
49. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry. Academic Press, Inc. New York.
50. **Yamamura, M., X. Wang, J. D. Ohmen, K. Uyemura, T. H. Rea, B. R. Bloom, and R. L. Modlin.** 1992. Cytokine patterns of immunologically mediated tissue damage. J. Immunol. **149**:1470–1475.