

# ***Helicobacter pylori* Upregulates Expression of Epidermal Growth Factor–related Peptides, but Inhibits Their Proliferative Effect in MKN 28 Gastric Mucosal Cells**

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## **Abstract**

Acute exposure to *Helicobacter pylori* causes cell damage and impairs the processes of cell migration and proliferation in cultured gastric mucosal cells in vitro. EGF-related growth factors play a major role in protecting gastric mucosa against injury, and are involved in the process of gastric mucosal healing. We therefore studied the acute effect of *H. pylori* on expression of EGF-related growth factors and the proliferative response to these factors in gastric mucosal cells (MKN 28) derived from gastric adenocarcinoma. Exposure of MKN 28 cells to *H. pylori* suspensions or broth culture filtrates upregulated mRNA expression of amphiregulin (AR) and heparin-binding EGF-like growth factor (HB-EGF), but not TGF $\alpha$ . This effect was specifically related to *H. pylori* since it was not observed with *E. coli*, and was independent of VacA, CagA, PicA, PicB, or ammonia. Moreover, *H. pylori* broth culture filtrates stimulated extracellular release of AR and HB-EGF protein by MKN 28 cells. AR and HB-EGF dose-dependently and significantly stimulated proliferation of MKN 28 cells in the absence of *H. pylori* filtrate, but had no effect in the presence of *H. pylori* broth culture filtrates. Inhibition of AR- or HB-EGF-induced stimulation of cell growth was not mediated by downregulation of the EGF receptor since EGF receptor protein levels, EGF binding affinity, number of specific binding sites for EGF, or HB-EGF- or AR-dependent tyrosine phosphorylation of the EGF receptor were not significantly altered by incubation with *H. pylori* broth culture filtrates. Increased expression of AR and HB-EGF were mediated by an *H. pylori* factor > 12 kD in size, whereas antiproliferative effects were mediated by both VacA and a factor < 12 kD in size. We conclude that *H. pylori* increases

mucosal generation of EGF-related peptides, but in this acute experimental model, this event is not able to counteract the inhibitory effect of *H. pylori* on cell growth. The inhibitory effect of *H. pylori* on the reparative events mediated by EGF-related growth factors might play a role in the pathogenesis of *H. pylori*-induced gastroduodenal injury. (*J. Clin. Invest.* 1998. 101:1604–1613.) Key words: *Helicobacter pylori* • heparin-binding EGF-like growth factor • amphiregulin • EGF receptor • gastric cell proliferation

## **Introduction**

*Helicobacter pylori* is the major causative agent of chronic superficial gastritis, and plays a central role in the etiology of peptic ulcer disease in humans (1, 2). Moreover, epidemiological studies suggest that infection with *H. pylori* increases the risk for developing adenocarcinoma of the distal stomach (3, 4). The mechanisms whereby *H. pylori* causes disease are not completely elucidated. In addition to virulence factors common to all clinical isolates (5, 6), two factors have been described that are produced by ~60% of wild-type isolates of *H. pylori*: (a) an ~90-kD cytotoxin (VacA) that causes vacuole formation in epithelial cells (7–9), and (b) a 120–140-kD immunodominant protein (CagA; references 10 and 11). Essentially, all *H. pylori* strains possess *vacA*, but those that have the cytotoxin phenotype are referred to as Tox<sup>+</sup>. In contrast, only ~60% of wild-type *H. pylori* strains possess *cagA*; all of these are CagA<sup>+</sup>. An increasing body of evidence indicates that, in western countries, *H. pylori* strains producing both CagA and cytotoxic VacA are preferentially associated with development of peptic ulcer disease and gastric cancer (9, 12, 13). We have recently shown that *H. pylori* broth culture filtrates, besides causing vacuolating damage (14), also alter the processes of cell migration and proliferation in gastric mucosal cells in vitro (15).

EGF-related peptides are polypeptides that share structural homology and a similar spectrum of biological activities exerted through interaction with a common cell surface receptor (EGFr; reference 16).<sup>1</sup> EGF-related growth factors prevent drug-induced damage to the rat gastric mucosa (17, 18) and stimulate epithelial cell migration and proliferation (19–21), the main processes involved in healing gastroduodenal ulceration (22). Moreover, increased expression of EGF-related

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1. Abbreviations used in this paper: AR, amphiregulin; B<sub>max</sub>, total number of specific binding sites; EGFr, EGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB-EGF, heparin-binding EGF-like growth factor; rh, recombinant human.

peptides and of EGFr follows gastric mucosal injury (23–25), thus supporting the concept that these peptides acting through the EGFr participate in recovery of gastric mucosal integrity after injury (26, 27). Whether *H. pylori* infection alters expression of EGF-related growth factors, and whether these peptides play any role in the host response to *H. pylori* infection have not been studied.

This study was therefore designed to determine whether *H. pylori* alters expression of any of the EGF receptor (EGFr) ligands and, if so, whether specific bacterial virulence factors were responsible for this effect. Herein, we demonstrate that *H. pylori* induces expression of amphiregulin (AR) and heparin-binding EGF-like growth factor (HB-EGF), but not TGF $\alpha$ , in MKN 28 gastric mucosal cells, effects that are independent of several known bacterial virulence factors. In addition, we demonstrate that pretreatment of cells with *H. pylori* broth culture filtrate blocks the mitogenic effect of exogenous AR or HB-EGF by a mechanism independent of EGFr down-regulation or inactivation.

## Methods

**Cell culture.** MKN 28 cells are derived from a human gastric tubular adenocarcinoma (28), and show gastric-type differentiation (29, 30). Cells were grown in DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic solution (Gibco Laboratories, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cells were grown in 100-mm dishes for RNA extraction, and in 24-well dishes for proliferation and binding studies.

**Bacterial strains and growth conditions.** We have used the urease-positive Tox<sup>+</sup> CagA<sup>+</sup> wild-type *H. pylori* 60190 strain (ATCC 49503) and isogenic mutants in which *vacA*, *cagA*, *vacA*, and *cagA*, *picA*, or *picB* genes were disrupted by insertional mutagenesis (8, 31–33). We also used the Tox<sup>+</sup> CagA<sup>+</sup> *H. pylori* strain Bx2 U<sup>+</sup> and its urease-negative mutant Bx2 U<sup>−</sup> (provided by F. Megraud, Bordeaux, France) and the CCUG 17874 (Tox<sup>+</sup> CagA<sup>+</sup>) strain (from the culture collection of the University of Goteborg, Goteborg, Sweden). Bacteria were grown in brucella broth supplemented with 5% FCS (Gibco Laboratories) for 24–36 h at 37°C in a thermostatic shaker under microaerobic conditions. As previously described (14), when the bacterial suspensions reached 1.2 OD U at 450 nm, bacteria were removed by centrifugation, and the supernatants were sterilized by passage through a 0.22- $\mu$ m pore-size cellulose acetate filter (Nalgene Co., Rochester, NY) to obtain the broth culture filtrates. Broth culture filtrates from different bacterial strains and different preparations from the same strain were standardized by growing the bacterial suspensions until they reached an OD of 1.2 U at 450 nm, which corresponds to a bacterial concentration of  $5 \times 10^8$  CFU/ml. In the experiments with dialyzed broth culture filtrates, aliquots of both *H. pylori* broth culture filtrates and control filtrates were dialyzed against HBSS for 36 h in dialysis tubing with a 12-kD cutoff (Sigma Chemical Co., Milano, Italy). The presence or absence of VacA and/or CagA in *H. pylori* cells or filtrate was verified by means of SDS-PAGE followed by immunoblotting with anti-VacA or anti-CagA polyclonal antisera (7, 34). In the experiments with bacterial suspensions, cells were incubated with bacterial preparations at a concentration of  $5 \times 10^7$  CFU/ml in DMEM supplemented with 10% FCS.

**RNA isolation and Northern analysis.** Total RNA was isolated from MKN 28 cells by the guanidinium thiocyanate acid-phenol procedure (35) and subjected to Northern analysis as described previously (36). In brief, 10  $\mu$ g of total RNA per lane was separated by electrophoresis in 1% agarose-formaldehyde gels. RNA was transferred to Hybond-N<sup>+</sup> (Amersham Corp., Milano, Italy), cross-linked (UV Stratalinker-1800; Stratagene, La Jolla, CA), and hybridized to <sup>32</sup>P-labeled cDNA probes. <sup>32</sup>P-labeled isotopes were from Amersham

Corp. The TGF $\alpha$  probe was a 1.27-kb Eco RI cDNA (37), the HB-EGF probe was a 1.102-kb Eco RI cDNA (38), the AR probe was a 0.87-kb Eco RI-HinD III cDNA of (39), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.5-kb Bam HI cDNA (40). TGF $\alpha$ , HB-EGF, and AR mRNA levels were normalized to mRNA levels of the constitutively expressed GAPDH gene. mRNA levels were quantitated by densitometric scanning of the autoradiograph using a Howtek Scanmaster-3 densitometer with RFL Print-TM software (Pharmacia Biotech, Cologno Monzese, Italy).

**Collection of conditioned medium.** Cells were grown to semiconfluency in serum-containing medium. Semiconfluent monolayers were then washed twice with PBS and incubated for 24 h with broth culture filtrate from the *H. pylori* 60190 (wild-type) strain or with control (uninoculated) filtrate diluted 1:3 in serum-free medium. At the end of the incubation period, conditioned medium was harvested, centrifuged (4,000 g for 20 min at 4°C) and concentrated using Centricon 3 microconcentrators (Amicon, Inc., Beverly, MA).

**Preparation of cell extracts and immunoprecipitation.** At the end of the incubation period, cells were washed twice with ice-cold PBS and incubated for 20 min at 4°C in lysis buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF (Sigma Chemical Co., Milano, Italy), 1% (vol/vol) Triton X-100, and 0.3  $\mu$ g/ml aprotinin (Sigma Chemical Co.). Cells were dislodged with a cell scraper. The lysates were cleared by microfuging at 14,000 rpm for 20 min at 4°C. Immunoprecipitation was as described by Auricchio et al. (41). In brief, 1 ml of lysate was diluted with lysis buffer to a final concentration of 1 mg/ml and incubated for 1 h with 5  $\mu$ g of rabbit anti-mouse IgG (Sigma Chemical Co.) and 40  $\mu$ l of protein G-Sepharose (50% suspension; Pharmacia Biotech Inc., Cologno Monzese, Italy). The samples were centrifuged, and supernatants were incubated overnight with 3  $\mu$ g/ml of anti-EGFr mouse monoclonal antibody, clone LA22 (Upstate Biotechnology Inc., Lake Placid, NY). Each sample was added to 100  $\mu$ l of protein G-Sepharose (50% suspension), and was incubated for an additional hour. The samples were centrifuged, the pellets were washed with lysis buffer, and the proteins were eluted by boiling in Laemmli sample buffer.

**Western blot analysis.** After electrophoresis, proteins were blotted by electrotransfer to BA 85 0.45- $\mu$ m protan nitrocellulose filters (Schleicher & Schuell, Inc., Dassel, Germany). After blocking with 10% BSA in TTBS (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20), filters were probed with specific antibodies, and protein was visualized with peroxidase-coupled secondary antibody using the enhanced chemiluminescence detection system (ECL; Amersham Corp., Milano, Italy). The antibodies used in our studies were as follows: 2998 rabbit polyclonal anti-human HB-EGF antibody (42) kindly provided by Dr. M. Klagsbrun (Boston, MA); a rabbit polyclonal antibody directed against residues 26–44 of human AR (Neomarkers, Fremont, CA); a mouse monoclonal antiphosphotyrosine antibody, clone 4G10 (Upstate Biotechnology Inc.); 1005 rabbit polyclonal anti-EGFr antibody (Santa Cruz Biotechnology Inc., Milano, Italy); and secondary anti-rabbit or anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Corp., Milano, Italy). In the EGFr immunoprecipitation experiments, filters were first immunodetected with antiphosphotyrosine antibody, and then stripped in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM  $\beta$ -mercaptoethanol for 30 min at 65°C, washed three times in TTBS, blocked, and reprobed with 1005 rabbit polyclonal anti-EGFr antibody.

**Determination of [<sup>3</sup>H]thymidine incorporation.**  $5 \times 10^4$  cells/well were seeded onto 24-well dishes. 12 h after seeding, medium was removed and replaced with serum-deprived medium in order to synchronize cell cycles. 12 h later, cells were incubated with test solutions. After 21 h, 1  $\mu$ Ci/ml [<sup>3</sup>H]thymidine (Amersham Corp.) was added, and incubation was continued for an additional 3 h. Cells were then washed twice with ice-cold PBS and, after trypsinization, were collected on glass fiber filter mats using an LKB automatic cell harvester before liquid scintillation counting, as described previously

(43). [ $^3\text{H}$ ]Thymidine uptake was expressed in cpm/well. Viability of cells treated with *H. pylori* broth culture filtrates and that of cells treated with the control (uninoculated) filtrate were comparable, as assessed by the Trypan blue dye exclusion test (data not shown). Data are presented as mean  $\pm$  SEM of three experiments run in quadruplicate.

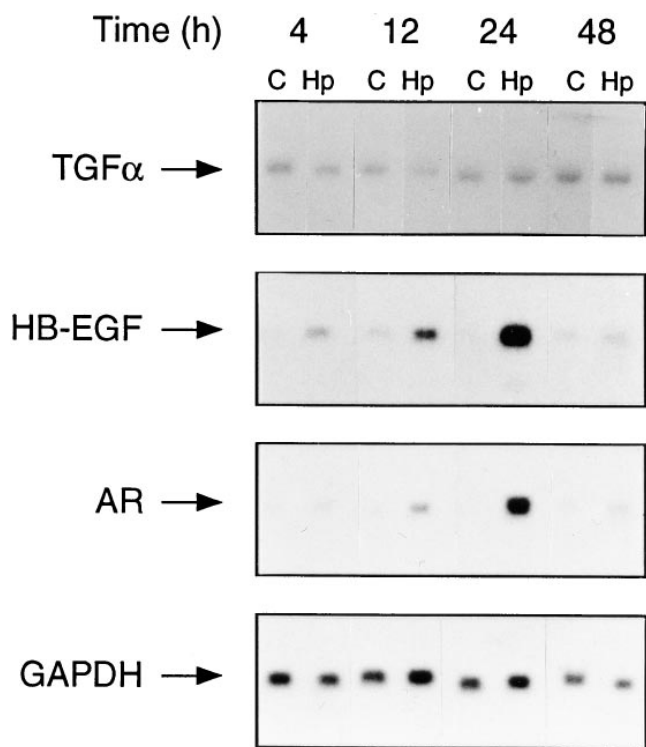
**Binding assays.** Cells were seeded onto 24-well dishes at the concentration of  $5 \times 10^4$  cells/well. When monolayers were 50% confluent, cells received serum-free medium for 24 h. Subsequently, cells were incubated with serum-free medium, uninoculated broth filtrate, or broth culture filtrate from *H. pylori* 60190 strain for 24 h. Before the beginning of each assay, the cells were washed three times with 4°C chilled binding medium that consisted of Krebs-Ringer/(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid containing 0.5% BSA and adjusted to pH 7.4. 400- $\mu\text{l}$  aliquots of the binding medium containing 0.06 nmol/liter [ $^{125}\text{I}$ ]EGF (Amersham Corp.) with different concentrations of unlabeled recombinant human (rh) EGF, rhHB-EGF (Sigma Chemical Co.), or rhAR (R&D Systems, Inc., Minneapolis, MN) were added to each well. After equilibration at 4°C for 4 h, the cells were then washed three times with 4°C binding medium to remove unbound [ $^{125}\text{I}$ ]EGF. The cultures were then solubilized by adding 1.0 ml of 1 N NaOH to each well. After 30 min, the contents of each well were withdrawn and added to tubes for counting in a Beckman Gamma 5500 counter. Nonspecific binding, determined as radioactivity bound in the presence of  $10^{-6}$  M unlabeled EGF, HB-EGF, or AR, was subtracted from total binding to obtain specific binding. All data points are expressed as mean  $\pm$  SEM of three separate experiments performed in triplicate.

**Statistics and calculations.** In the cell proliferation experiments, the significance of differences was evaluated by ANOVA followed by Duncan's multiple range test. Differences were considered significant if  $P < 0.05$ . Binding data were analyzed using the curve-fitting program EBDA/LIGAND (44).

## Results

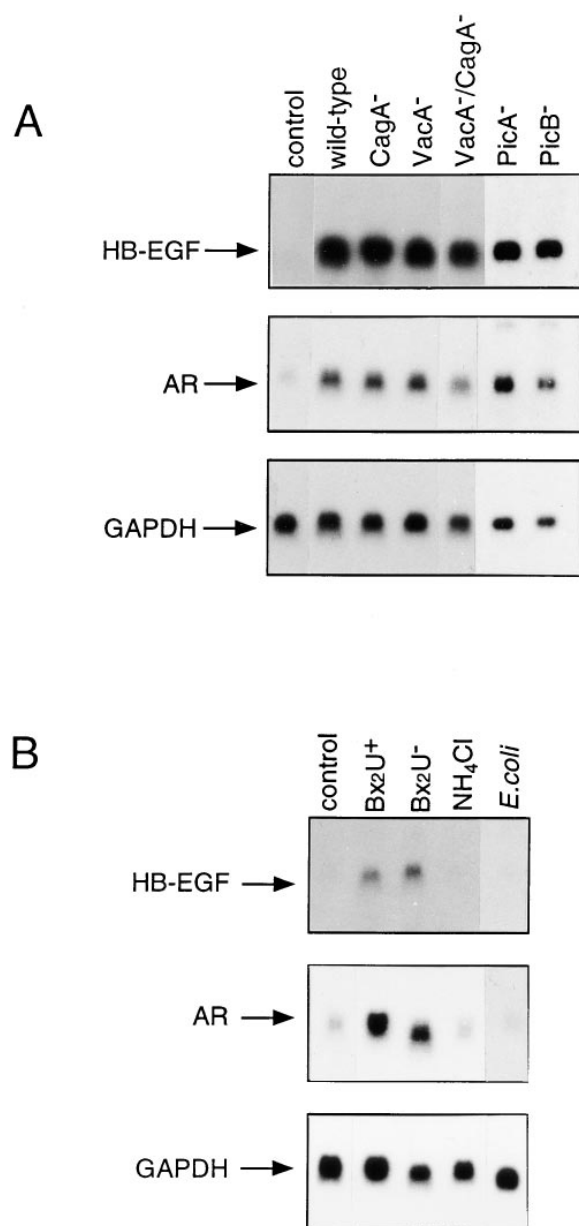
**Effect of *H. pylori* on *TGF $\alpha$* , *HB-EGF*, and *AR* mRNA expression in MKN 28 cells.** Incubating MKN 28 cells with bacterial suspensions from the *H. pylori* 60190 (wild-type) strain for 4–48 h did not have any effect on *TGF $\alpha$*  mRNA expression. There was, however, a time-dependent increase in HB-EGF and AR mRNA content in these cells in response to *H. pylori* (Fig. 1). The increase in HB-EGF and AR mRNA expression was already evident after 4 and 12 h of treatment, respectively, reached a peak at 24 h (20-fold increase vs. control), and declined at 48 h (Fig. 1). To determine whether the increase in HB-EGF and AR mRNA expression was specific for *H. pylori* 60190, we evaluated the effect of a bacterial suspension from wild-type *H. pylori* strain CCUG 17874, and obtained comparable results (data not shown). Similar effects were obtained with bacterial suspensions and with broth culture filtrates from *H. pylori* 60190 or CCUG strains (data not shown). Therefore, in the subsequent experiments, we used *H. pylori* broth culture filtrates only.

**Role of *H. pylori* virulence factors in upregulation of HB-EGF and AR mRNA expression.** To identify potential *H. pylori* virulence factors responsible for HB-EGF induction and AR mRNA expression, MKN 28 cells were incubated for 24 h with uninoculated broth filtrate (control) or with broth culture filtrates from *H. pylori* 60190 (wild-type) strain, or its isogenic mutants lacking *vacA*, *cagA*, *vacA*, and *cagA*, *picA*, or *picB*. Fig. 2 A shows that a comparable increase in HB-EGF and AR mRNA expression was observed for all of the *H. pylori* strains tested. We also investigated whether urease-dependent ammonia generation might play a role in upregulating EGF-related



**Figure 1.** Time course of *H. pylori* effect on EGFr ligand mRNA expression in MKN 28 cells. Approximately  $2 \times 10^6$  cells were incubated with DMEM (control) or with a bacterial suspension ( $5 \times 10^7$  CFU/ml) from *H. pylori* 60190 (wild-type) for up to 48 h. Total RNA was isolated, Northern blots were performed using 10  $\mu\text{g}$  of total RNA per lane, and filters were sequentially hybridized to  $^{32}\text{P}$ -labeled cDNA probes for human *TGF $\alpha$* , *HB-EGF*, *AR*, or *GAPDH*, as previously described (36). The constitutively expressed *GAPDH* transcript was used to evaluate equivalence of RNA loading and transfer. A representative autoradiograph of four separate experiments is shown. C, control; Hp, *H. pylori* 60190.

peptides. Broth culture filtrates from a urease-positive (Bx2U $^+$ ) strain and its urease-negative mutant (Bx2U $^-$ ) did not differ in their ability to induce HB-EGF or AR mRNA expression, nor did  $\text{NH}_4\text{Cl}$  (4 mM) exert any effect on mRNA expression of either growth factor (Fig. 2 B). This concentration of  $\text{NH}_4\text{Cl}$  was similar to the ammonia concentration present when MKN 28 cells were incubated with broth culture filtrates from all the urease-positive *H. pylori* strains used (15). To determine whether similar effects were induced by other Gram-negative bacteria, we studied the effect of *E. coli* on the expression of growth factors. That incubation of cells with broth culture filtrate or cell suspension from a clinical *E. coli* isolate (ATCC 25922) did not have any effect on HB-EGF or AR mRNA expression compared with control (Fig. 2 B and data not shown) suggests that the observed effects were due to unique properties of *H. pylori*. We have previously shown that the vacuolating cytotoxin and a yet unidentified low (< 12 kD) molecular mass component inhibit proliferation of gastric mucosal cells (15). To evaluate whether this low molecular mass bacterial product might be responsible for upregulating EGFr ligand mRNA expression, we incubated MKN 28 cells with dialyzed (to eliminate products < 12 kD) or undialyzed broth culture filtrates from wild-type strain 60190, or from its isogenic *vacA* $^-$  mutant. HB-EGF and AR mRNA expression was increased to

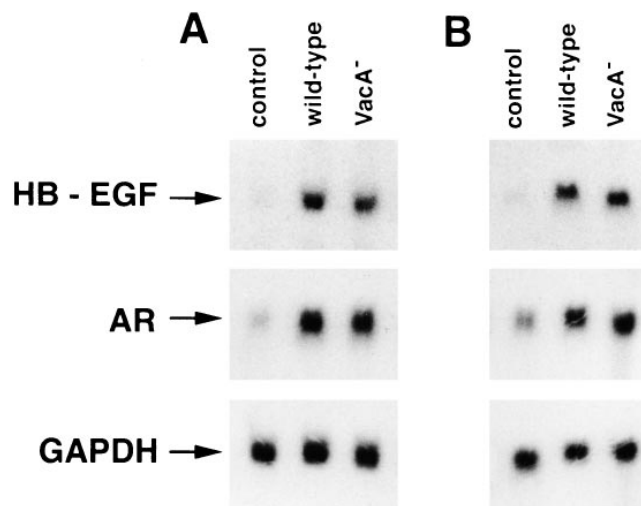


**Figure 2.** Role of *H. pylori* virulence factors in upregulating HB-EGF and AR mRNA expression in MKN 28 cells. (A) Approximately  $2 \times 10^6$  cells were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in culture medium, i.e., DMEM supplemented with 10% FCS) from *H. pylori* 60190 strain (wild-type) or its isogenic mutants (CagA<sup>-</sup>, VacA<sup>-</sup>, VacA<sup>-</sup> CagA<sup>-</sup>, PicA<sup>-</sup>, or PicB<sup>-</sup>). (B) Approximately  $2 \times 10^6$  cells were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrates from *H. pylori* Bx2U<sup>+</sup> (Urease<sup>+</sup>) strain or its urease-negative mutant (Bx2U<sup>-</sup>; each diluted 1:3 in culture medium, i.e., DMEM supplemented with 10% FCS), or 4 mM NH<sub>4</sub>Cl or with *E. coli* (ATCC 25922 strain) broth culture filtrate. Isolation of total RNA, Northern blotting, and filter hybridization were identical to that described in Fig. 1. A representative autoradiograph of four separate experiments is shown.

a similar extent by dialyzed or undialyzed broth culture filtrates irrespective of expression of the vacuolating cytotoxin (Fig. 3), suggesting that a high (> 12 kD) molecular mass component other than VacA is responsible for this effect.

**Effect of *H. pylori* on HB-EGF and AR protein release in the conditioned medium of MKN 28 cells.** To determine whether HB-EGF and AR protein production correlated with mRNA expression, conditioned medium from *H. pylori*-treated or -untreated (control) cells was analyzed by Western blot using antisera specific for HB-EGF and AR, respectively (Fig. 4). An HB-EGF immunoreactive peptide of ~ 30 kD (Fig. 4 A) and an AR immunoreactive peptide of ~ 10 kD (Fig. 4 B) were secreted in the conditioned medium from MKN 28 cells after 24 h of incubation with *H. pylori* 60190 broth culture filtrate, but were not detected in conditioned medium from MKN 28 cells treated with uninoculated broth filtrates. No HB-EGF or AR immunoreactive peptides were detected in *H. pylori* 60190 broth culture filtrate alone (data not shown). As expected, anti-human HB-EGF antibody and anti-human AR antibody also recognized recombinant human HB-EGF and AR peptides of 10 and 11 kD, respectively (Fig. 4). The differences in molecular mass between rhHB-EGF and the rhAR and HB-EGF and AR secreted by MKN 28 cells might be due to differential processing of the prepropeptides in MKN 28 cells. In fact, it has been demonstrated that human keratinocytes secrete different molecular mass forms of HB-EGF ranging from 14.5 to 30 kD (45). Similarly, low (9.5 kD) and high (16.5 kD) molecular mass forms of AR have been described in human breast carcinoma cells (46).

**Effect of *H. pylori* broth culture filtrates on HB-EGF- or AR-induced MKN 28 cell proliferation.** To determine whether HB-EGF or AR stimulates mitogenesis of MKN 28 cells, we studied the effect of adding rhHB-EGF (0.1–50 nM) or rhAR (0.1–50 nM) on MKN 28 cell growth, as assessed by [<sup>3</sup>H]thymidine incorporation. Each peptide caused significant and dose-dependent increases in DNA replication (Fig. 5). In particular, at a concentration  $\geq 10$  nM, HB-EGF and AR caused a 2- and



**Figure 3.** Comparative effect of undialyzed or dialyzed *H. pylori* broth culture filtrates on HB-EGF and AR mRNA expression in MKN 28 cells. Approximately  $2 \times 10^6$  cells were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in culture medium, i.e., DMEM supplemented with 10% FCS) from *H. pylori* 60190 strain (wild-type) or its isogenic vacA<sup>-</sup> mutant. (A) Undialyzed filtrates; (B) dialyzed filtrates. Isolation of total RNA, Northern blotting, and filter hybridization were as described in Fig. 1. A representative autoradiograph of three separate experiments is shown.

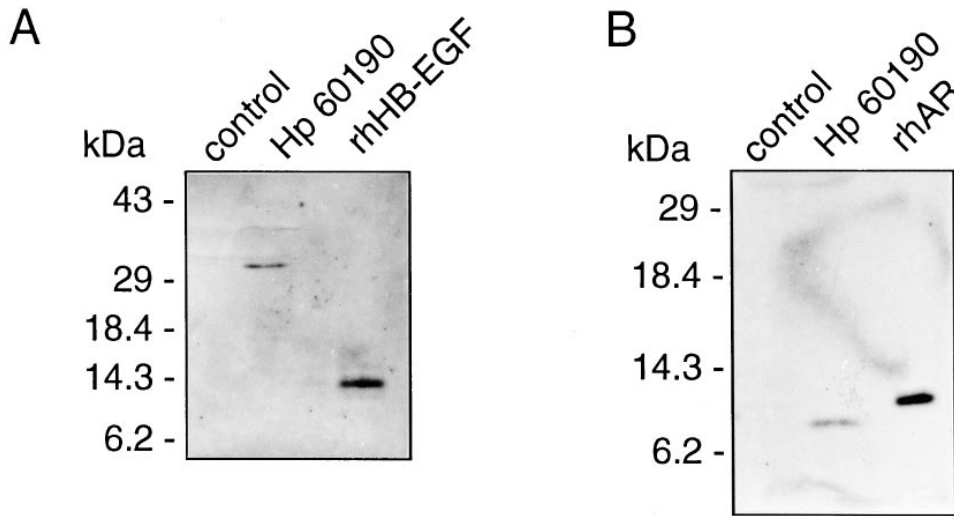


Figure 4. Effect of *H. pylori* broth culture filtrates on HB-EGF and AR protein levels in the conditioned medium of MKN 28 cells. Approximately  $2 \times 10^6$  cells were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrate from *H. pylori* 60190 strain (wild-type; each diluted 1:3 in serum-free medium). 50  $\mu$ l of concentrated conditioned medium was electrophoresed through 12% (A) or 15% (B) SDS-PAGE under non-reducing conditions and blotted to nitrocellulose filters. Immunodetection was performed using anti-human HB-EGF (A) or anti-human AR (B) antibody. The amount of samples loaded was standardized according to protein concentration. 10 ng of rhHB-EGF or rhAR was loaded in the third lanes as controls. Migration of molecular weight markers is indicated on the left of each panel.

a 2.5-fold increase in [ $^3$ H]thymidine uptake, respectively ( $P < 0.05$  vs. control).

We previously have shown that *H. pylori* broth culture filtrates cause a significant decrease in the proliferative activity of MKN 28 cells (15). This finding seemed to contradict our current data, which show that *H. pylori* induces HB-EGF and AR expression in MKN 28 cells, and that these cells proliferate

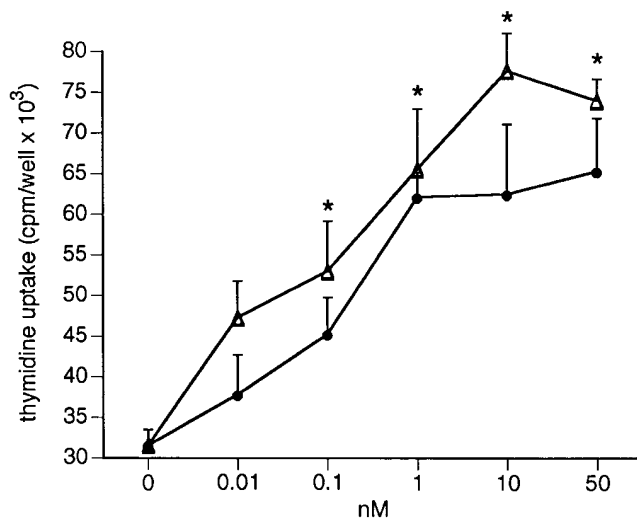
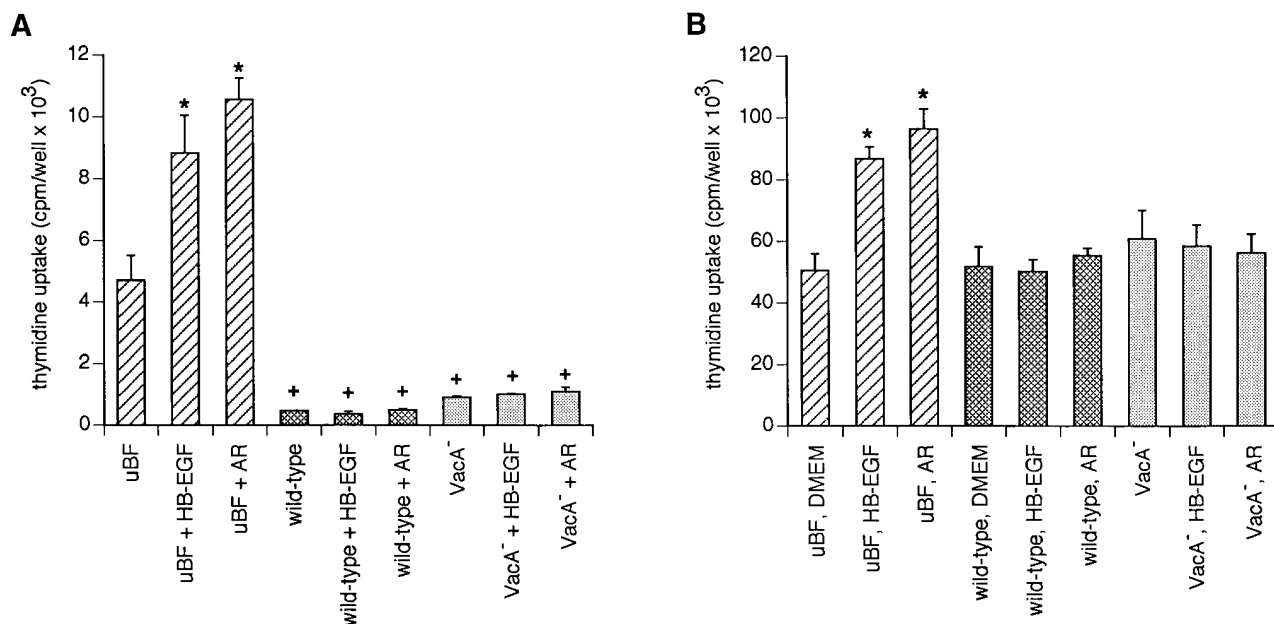


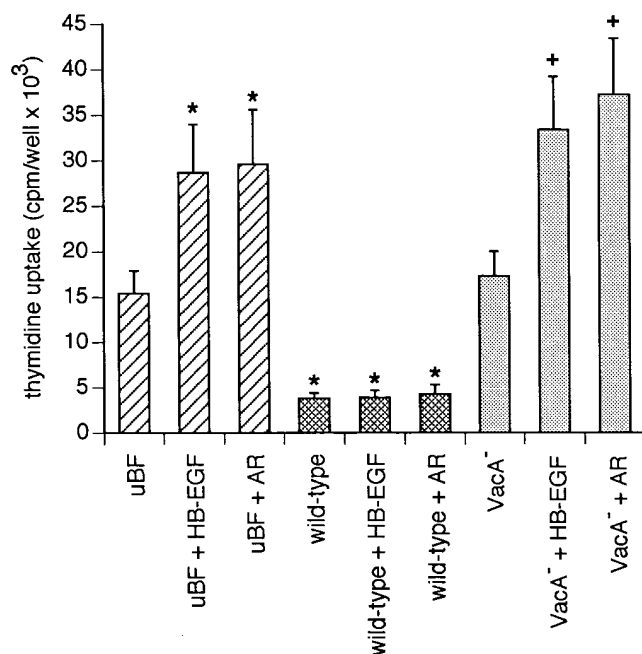
Figure 5. Effect of rhHB-EGF or rhAR on DNA replication in MKN 28 cells.  $5 \times 10^4$  cells/well were seeded onto 24-well dishes. 12 h after seeding, medium was removed and replaced with serum-free medium so as to synchronize cell cycles. 12 h later, cells were incubated with serum-free medium (control), rhHB-EGF (●; 0.01–50 nM), or rhAR (△; 0.01–50 nM). After 21 h, 1  $\mu$ Ci/ml [ $^3$ H]thymidine was added, and incubation was continued for an additional 3 h. Mean  $\pm$  SEM of three experiments run in quadruplicate. \* $P < 0.05$  vs. control (0 nM). The basal [ $^3$ H]thymidine uptake by MKN 28 cells in the absence of ligand (control) was  $\sim 30,000$  cpm/well.

in response to these two factors. Therefore, we studied whether *H. pylori* broth culture filtrates interfered with HB-EGF- or AR-induced proliferation of MKN 28 cells. We confirmed that broth culture filtrates from *H. pylori* wild-type strain 60190 or its isogenic *vacA*<sup>−</sup> mutant nearly abolished the proliferative activity of cultured cells, and now found (Fig. 6 A) that this effect was not counteracted by coadministration of rhHB-EGF or rhAR at a dose (10 nM) that, when given alone, caused an approximately twofold increase in [ $^3$ H]thymidine uptake. After pretreatment of cells with uninoculated broth filtrate, administration of rhHB-EGF (10 nM) or rhAR (10 nM) caused a significant increase ( $P < 0.05$  vs. control) in [ $^3$ H]thymidine uptake of MKN 28 cells (Fig. 6 B). In contrast, pretreatment with *H. pylori* broth culture filtrates abrogated growth factor-induced mitogenesis (Fig. 6 B). We also evaluated whether dialyzed broth culture filtrates from cytotoxic (wild-type) or noncytotoxic (*VacA*<sup>−</sup>) *H. pylori* strains retained their ability to counteract EGF ligand-induced cell proliferation. We confirmed that dialyzed broth culture filtrate from the *H. pylori* wild-type strain retained its antiproliferative effect, and showed that this effect was not counteracted by coadministration of rhHB-EGF (10 nM) or rhAR (10 nM; Fig. 7). On the other hand, dialyzed broth culture filtrate from the isogenic *vacA*<sup>−</sup> mutant lost its ability to inhibit basal as well as EGF-related peptide-stimulated cell growth (Fig. 7). These observations confirm that the vacuolating cytotoxin and a low (< 12 kD) molecular mass component play a role in inhibiting gastric cell proliferation, and suggest that both factors are important in counteracting EGF-related peptide-induced cell growth stimulation.

**Effect of *H. pylori* broth culture filtrate on EGF binding affinity and on number of specific binding sites for EGF.** Since the proliferative effects of HB-EGF and AR are mediated through the EGFr (16, 27), we hypothesized that *H. pylori*-induced inhibition of EGF-related peptide-dependent cell growth might be mediated by EGFr downregulation. To address this issue, we performed experiments designed to quan-



**Figure 6.** Effect of *H. pylori* broth culture filtrates on HB-EGF- or AR-induced stimulation of MKN 28 cell proliferation.  $5 \times 10^4$  cells/well were seeded onto 24-well dishes. 12 h after seeding, medium was removed and replaced with serum-free medium for an additional 12 h. (A) cells were incubated for 24 h with control (uninoculated) broth filtrate (uBF) or broth culture filtrate from *H. pylori* 60190 (wild-type) or its isogenic *vacA*<sup>-</sup> mutant (VacA<sup>-</sup>) strain without or with simultaneous exposure to rhHB-EGF (10 nM) or rhAR (10 nM). (B) Cells were incubated for 24 h with uninoculated broth filtrate (uBF) or broth culture filtrate from *H. pylori* 60190 (wild-type) or its isogenic *vacA*<sup>-</sup> mutant (VacA<sup>-</sup>) strains. At the end of the incubation period, supernatant medium was removed, and cells were incubated for an additional 24 h with serum-free medium (DMEM) without or with rhHB-EGF (10 nM) or rhAR (10 nM). In both experiments, 1  $\mu$ Ci/ml [<sup>3</sup>H]thymidine was added 3 h before the end of the last incubation period. Mean  $\pm$  SEM of three experiments run in quadruplicate. \* $P < 0.05$  vs. uBF (A) and uBF, DMEM (B); + $P < 0.05$  vs. uBF (A).



**Figure 7.** Effect of dialyzed *H. pylori* broth culture filtrates on HB-EGF- or AR-induced stimulation of MKN 28 cell proliferation.  $5 \times 10^4$  cells/well were seeded onto 24-well dishes. 12 h after seeding, medium was removed and replaced with serum-free medium for an additional 12 h. Cells were incubated for 24 h with dialyzed control (uninoculated) broth filtrate (uBF) or dialyzed broth culture filtrate from

tify binding of [<sup>125</sup>I]EGF to MKN 28 cells after incubation with *H. pylori* 60190 (wild-type) broth culture filtrates or with control (uninoculated) broth filtrate. A competitive binding assay showed that [<sup>125</sup>I]EGF binding was displaced in a dose-dependent manner by EGF, HB-EGF, or AR cold ligands (Fig. 8A). When the specific binding was evaluated by Scatchard plot analysis, a single class of high-affinity binding sites for EGF, HB-EGF, and AR was identified (Fig. 8B and data not shown). The estimated  $K_d$  values for EGF, HB-EGF, and AR binding to MKN 28 cells incubated with uninoculated broth filtrate were  $6.3 \times 10^{-10}$ ,  $1.1 \times 10^{-9}$ , and  $7.2 \times 10^{-9}$  M, respectively. The  $K_d$  value and total number of specific binding sites ( $B_{max}$ ) for EGF was not significantly altered in *H. pylori*-treated cells compared with controls (Table I). *H. pylori*-treated cells possessed a high number of surface EGFr ( $\sim 240,000$  binding sites/cell), which was similar to findings for control cells.

**Effect of *H. pylori* broth culture filtrates on tyrosine phosphorylation of the EGFr.** As another approach to explain the inhibitory effect of *H. pylori* on HB-EGF- or AR-induced cell

*H. pylori* 60190 (wild-type) or its isogenic *vacA*<sup>-</sup> mutant (VacA<sup>-</sup>) strain with or without simultaneous exposure to rhHB-EGF (10 nM) or rhAR (10 nM). After 21 h, 1  $\mu$ Ci/ml [<sup>3</sup>H]thymidine was added, and incubation was continued for an additional 3 h. Mean  $\pm$  SEM of three experiments run in quadruplicate. \* $P < 0.05$  vs. uBF; + $P < 0.05$  vs. VacA<sup>-</sup>.

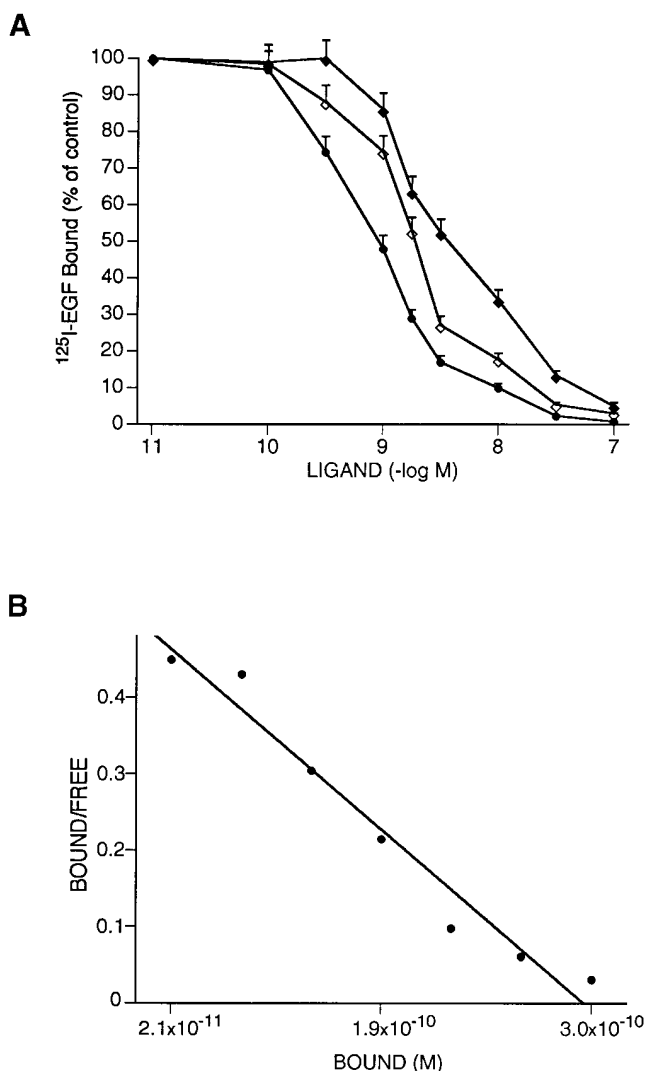


Figure 8. Effect of *H. pylori* on [ $^{125}\text{I}$ ]EGF binding to MKN 28 cell monolayers. (A) Competitive inhibition of [ $^{125}\text{I}$ ]EGF binding to cultures of MKN 28 cells after incubation with broth culture filtrate from *H. pylori* 60190 strain in the presence of different concentrations of unlabeled EGF (●), HB-EGF (◇), or AR (◆). All values of [ $^{125}\text{I}$ ]EGF with competitors were expressed relative to specific binding of [ $^{125}\text{I}$ ]EGF without competitor (100% control). Each point represents mean  $\pm$  SEM of quadruplicate measurements from three separate cell preparations. Divergence between quadruplicate values was  $< 15\%$ . (B) Representative Scatchard plot of [ $^{125}\text{I}$ ]EGF binding to MKN 28 cell monolayers in the presence of increasing concentrations of unlabeled EGF after treatment with broth culture filtrate from *H. pylori* 60190 strain. Each point is the mean of triplicate determinations from a representative experiment.

growth, we hypothesized that *H. pylori* broth culture filtrate might interfere with HB-EGF or AR-dependent EGFr activation. Treatment of MKN 28 cells with HB-EGF (10 nM) or AR (10 nM) in the presence of either uninoculated broth filtrate or *H. pylori* 60190 broth culture filtrate induced tyrosine phosphorylation in proteins of  $\sim 170$  and 70 kD (Fig. 9 A, top). Also, the EGFr total protein levels were similar in cells treated with supernatants from *H. pylori* cultures or from uninoculated broth filtrate (Fig. 9 A, bottom). Immunoprecipitation of protein extracts with an anti-EGFr antibody followed by

Western blot analysis with an antiphosphotyrosine antibody showed that control (untreated) cells had undetectable levels of tyrosine-phosphorylated EGFr, whereas *H. pylori* treatment caused detectable tyrosine phosphorylation of the EGFr (Fig. 9 B). Also, *H. pylori* treatment did not alter HB-EGF- or AR-dependent EGFr tyrosine phosphorylation (Fig. 9 B). In particular, densitometric analysis of the experiment shown in Fig. 9 B demonstrated that the antiphosphotyrosine/anti-EGFr ratio was close to 0 in control untreated cells, 0.6 in *H. pylori*-treated cells, and 3 and 2.5 vs. 4 and 3.5 after administration of rhHB-EGF (10 nM) or rhAR (10 nM) to control untreated cells and *H. pylori*-treated cells, respectively. Thus, there was no evidence that *H. pylori* filtrate inhibited activation of the EGFr.

## Discussion

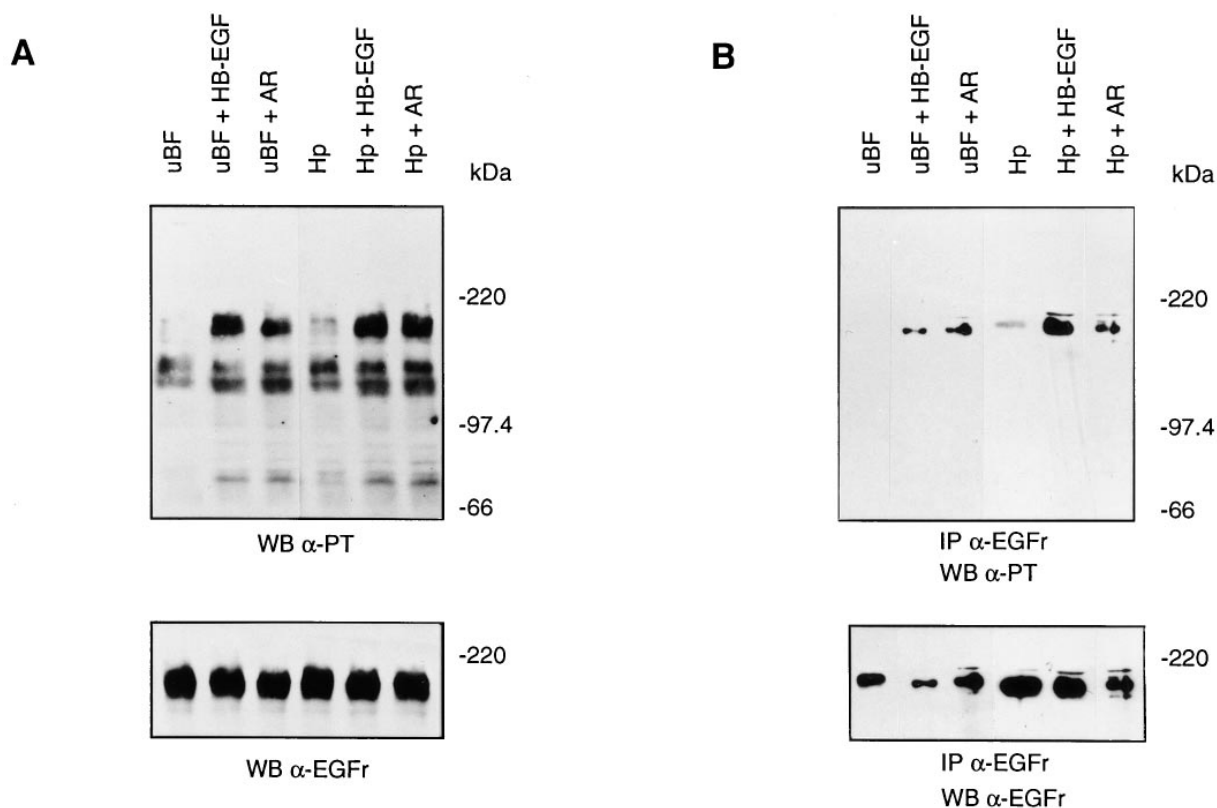
*H. pylori* infection is associated with development of gastroduodenal mucosal ulceration (1, 2), and with an increased risk of gastric adenocarcinoma in humans (3, 4). Host response and environmental factors are important in the pathogenesis of *H. pylori*-related gastroduodenal disease (5, 6). However, molecules expressed by  $\sim 60\%$  of wild-type isolates of *H. pylori* have been identified that may participate in the pathogenesis of *H. pylori*-induced disease (7–11). In particular, *H. pylori* strains preferentially associated with development of peptic ulcer or gastric adenocarcinoma express vacuolating cytotoxin activity (i.e., Tox $^{+}$ ; 7–9) and an immunodominant antigen associated with the cytotoxin (i.e., CagA; references 10 and 11). Multiple genes in the *cag* island have recently been described, whose expression are necessary for cytokine production by gastric epithelial cells in vitro (33, 47).

EGF-related peptides have been shown to prevent drug-induced damage to the stomach, and to accelerate healing of gastric mucosal ulcerations (17, 18), suggesting that EGF ligands play a major role in maintaining gastric mucosal integrity (26, 27, 48, 49). In particular, HB-EGF recently has been demonstrated to be expressed in the normal human gastric mucosa (50), and to be upregulated after acute injury to the rat stomach (51) or kidney (52). Therefore, we studied whether *H. pylori* might affect EGF-related peptide expression in gastric mucosal cells in vitro. Our data show that *H. pylori* upregulates expression of AR and HB-EGF at the mRNA and protein levels, effects not only observed with *H. pylori* suspensions, but also with broth culture filtrates, suggesting that it might be mediated by a soluble product released from the bacterium.

Table I. Effect of *H. pylori* 60190 (Wild-Type) Broth Culture Filtrate on EGF Binding Affinity ( $K_d$ ) and Number of Specific Binding Sites ( $B_{\text{max}}$ ) in MKN 28 Cells

	$K_d$	$B_{\text{max}}$
	$\text{M} \times 10^{-10}$	$\text{fM}/10^4 \text{ cells}$
DMEM	$6.6 \pm 1.2$	$3.3 \pm 0.6$
Uninoculated broth filtrate	$6.3 \pm 1.5$	$3.5 \pm 0.7$
<i>Hp</i> 60190 BCF*	$6.6 \pm 0.9$	$4.2 \pm 0.8$

\*Broth culture filtrate. Mean  $\pm$  SEM of four independent experiments.  $K_d$  and  $B_{\text{max}}$  values were obtained using the EBDA/LIGAND program (44).



**Figure 9.** Effect of *H. pylori* broth culture filtrates on tyrosine phosphorylation of EGFR. Approximately  $2 \times 10^6$  cells were incubated for 24 h with uninoculated broth filtrate (uBF), or *H. pylori* 60190 broth culture filtrate (Hp). Cells were then incubated for 10 min with uBF or *H. pylori* broth culture filtrate in the absence or presence of rhHB-EGF (10 nM) or rhAR (10 nM). (A) Western blot analysis (WB) of cell extracts (50 µg/sample) using an antiphosphotyrosine antibody ( $\alpha$ -PT; top) or an antihuman EGFR antibody ( $\alpha$ -EGFR; bottom). (B) Immunoprecipitation of cell extracts (1 mg/sample) with an antihuman EGFR antibody followed by Western blot analysis using an antiphosphotyrosine antibody (top) or an antihuman EGFR (bottom). Protein samples were electrophoresed through a 7.5% SDS-PAGE under reducing conditions. The migration of molecular weight markers is indicated on the right of each panel.

Our data indicate that the *H. pylori* factor involved in upregulation of EGF-related peptide expression is  $> 12$  kD, and that none of several known virulence factors, including *vacA*, *cagA*, *pilA*, or *pilB*, mediates this effect. Moreover, urease-generated ammonia does not seem to play a role in this effect since  $\text{NH}_4\text{Cl}$  did not increase AR or HB-EGF mRNA expression, and a urease-negative *H. pylori* strain was as effective as its isogenic wild-type (urease positive) strain in upregulating AR and HB-EGF mRNA transcripts. The possibility that overexpression of AR and HB-EGF is a non specific phenomenon due to a component common to all Gram-negative bacteria is ruled out by the finding that *E. coli* did not affect AR or HB-EGF mRNA levels.

We also found that *H. pylori* treatment induced release of HB-EGF and AR protein in the conditioned media of cultured cells. An HB-EGF immunoreactive peptide of 30 kD and an AR immunoreactive peptide of 10 kD were identified. These different molecular weight forms of HB-EGF and AR have been found to be biologically active (45, 46). Secretion of HB-EGF and AR in the conditioned medium together with the finding that either peptide dose-dependently stimulated gastric cell proliferation as assessed by [ $^3\text{H}$ ]thymidine incorporation suggests that these peptides may play an autocrine role in MKN 28 cell proliferation. Recent epidemiological studies in-

dicate that *H. pylori* is involved in the development of adenocarcinoma of the distal stomach (3,4). The pathogenetic mechanisms are not known, but increased proliferative activity of epithelial cells in gastric mucosa colonized by *H. pylori* might contribute (53, 54). Since increased expression of HB-EGF and AR has been described in gastrointestinal malignancies in humans (55, 56), we speculate that sustained overexpression of EGF-related peptides in persons colonized with *H. pylori* might overcome the acute inhibitory effect on cell growth exerted by *H. pylori* (15), thus leading to increased proliferation of gastric mucosal cells and, in turn, to development of gastric cancer. However, in vivo studies in *H. pylori*-colonized gastric mucosa are needed to verify this hypothesis.

We have previously shown in the same experimental model that *H. pylori* broth culture filtrates inhibited gastric mucosal cell proliferation (15). That *H. pylori* broth culture filtrates given either as a pretreatment or together with growth factors, completely abolished the growth stimulatory effect of AR or HB-EGF, suggests that increased production of these peptides after acute exposure to *H. pylori* is insufficient to counteract the marked inhibitory effect on cell growth exerted by *H. pylori*. We previously described that after dialysis with a 12-kD cutoff, broth culture filtrate from *H. pylori* wild-type strain 60190 retained its ability to inhibit cell proliferation, whereas



broth culture filtrate from its isogenic noncytotoxic strain did not. Moreover, inhibition of cell proliferation induced by dialyzed broth culture filtrate from the cytotoxic *H. pylori* strain was specifically counteracted by pretreatment with a neutralizing polyclonal antiserum raised against purified VacA (15). These observations suggest that *H. pylori*-induced inhibition of cell proliferation is due in part to the vacuolating cytotoxin, and in part to a low (< 12 kD) molecular mass component. Herein we have shown that after dialysis, the *H. pylori* wild-type strain retained its ability to counteract HB-EGF- or AR-induced cell growth, whereas the *H. pylori* noncytotoxic strain did not. This observation, together with the demonstration that neither the vacuolating cytotoxin nor a low molecular mass bacterial product are involved in upregulating HB-EGF or AR mRNA expression (Fig. 3), suggests that separate factors are involved in inducing EGF-related peptide expression, and in inhibiting their proliferative activity. In particular, upregulation of HB-EGF and AR expression appears to be due to a high (> 12 kD) molecular mass component other than the vacuolating cytotoxin, since this effect is observed with both cytotoxic and noncytotoxic strains after dialysis. On the contrary, inhibition of basal as well as EGFr ligand-stimulated cell growth depends on the vacuolating cytotoxin, and is also contributed to by a low (< 12 kD) molecular mass bacterial product. That the cytotoxic strain retains its antiproliferative activity after dialysis, whereas the noncytotoxic strain does not, suggests that these separate factors might ultimately be the targets of different therapeutic interventions.

The biological effects of EGF-related peptides are mediated through interaction with a common cell membrane receptor (i.e., the EGFr; references 16 and 27). EGFr expression is upregulated after injury to the mucosa of the gastrointestinal tract (25). Inhibition of the tyrosine kinase specific for the EGFr impairs the protective effect of EGF against experimentally induced colitis in the rat (57). We found that either HB-EGF or AR competed at high affinity for [<sup>125</sup>I]EGF binding to MKN 28 cells, and that MKN 28 cells expressed a single class of binding sites for EGF with a  $K_d$  of  $6.3 \times 10^{-10}$  M and a  $B_{max}$  of  $\sim 210,000$  receptors/cell. This result is in partial agreement with Rutten et al., who found in primary culture of guinea pig gastric mucosal cells a single class of binding sites with a  $K_d$  of  $2.6 \times 10^{-10}$  M and a  $B_{max}$  of  $\sim 12,000$  receptors/cell (58). The higher EGFr expression in MKN 28 cells is similar to that reported for other tumor cell lines (59). Our data indicate that EGF binding affinity and the total number of specific binding sites for EGF are not significantly altered by incubation of MKN 28 cells with *H. pylori* broth culture filtrates, making it unlikely that downregulation of the EGFr plays a role in inhibiting EGF-related peptide-induced stimulation of cell growth by *H. pylori*.

Another mechanism of *H. pylori*-induced inhibition of growth factor-stimulated cell growth might be through inhibition of HB-EGF or AR-dependent activation of the EGFr (60). We therefore studied the effect of *H. pylori* on tyrosine phosphorylation of the EGFr induced by exogenous administration of HB-EGF or AR. Because of the comparable levels of either HB-EGF or AR-dependent tyrosine phosphorylation of the EGFr observed in monolayers treated with *H. pylori* broth culture filtrates and in control (untreated) monolayers, we speculate that *H. pylori* may act by altering the EGFr signal transduction pathway downstream to the activation of the receptor directly, or through activation of other signal transduc-

tion pathways, such as the JNK/SAPK signalling pathway, that leads to inhibition of cell growth (61). Studies are underway in our laboratory to address this issue.

In conclusion, our study suggests that exposure of gastric mucosal cells to *H. pylori* leads to increased production of EGF-related growth factors. Induction of these growth factors is not sufficient to counteract the growth inhibitory effect exerted by *H. pylori*, at least in this acute experimental setting. Inhibition of EGF-related peptide-dependent cell growth does not seem to be due to downregulation of the EGFr, or to inhibition of EGFr activation. Finally, upregulation of EGF-related growth factors and inhibition of their stimulatory effect on cell growth appear to be mediated by separate *H. pylori* factors. We speculate that inhibition of reparative events mediated by EGF-related peptides might play a role in the pathogenesis of *H. pylori*-induced acute damage to the gastroduodenal mucosa in vivo.

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