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Helicobacter pylori Urease Activity Is Toxic to Human Gastric Epithelial Cells

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A human gastric adenocarcinoma cell line was used to evaluate the contribution of urease from *Helicobacter* (formerly *Campylobacter*) *pylori* to its cytotoxicity. Gastric cells cultured in medium supplemented with 20 mM urea were exposed to 5×10^6 CFU of *H. pylori* per ml with or without the addition of a urease inhibitor, acetohydroxamic acid. Viabilities of cells exposed to *H. pylori* for 2, 24, and 48 h, assessed by incorporation of neutral red dye, were 60, 27, and 16%, respectively; however, the viabilities of cells exposed to both *H. pylori* and acetohydroxamic acid were 92, 46, and 20% after 2, 24, and 48 h, respectively, ($P < 0.001$). Therefore, the urease activity of *H. pylori* may play an important role in its pathogenicity, and inhibition of this enzyme activity may have therapeutic potential.

Helicobacter (formerly *Campylobacter*) *pylori* is strongly associated with active chronic gastritis and peptic ulcer disease (5, 6). Ingestion of *H. pylori* by volunteers has resulted in the development of acute gastritis, which was initially accompanied by an increase in gastric pH (7, 9). The cause of this increase in intragastric pH has been hypothesized to be due to hypochlorhydria, but it may also be a result of *H. pylori* urease activity. The urease produced by *H. pylori*, which hydrolyzes urea to ammonia and carbon dioxide, has an affinity for urea, such that the enzyme is likely saturated by levels of urea normally found in blood (8). Neutralization of the hydrochloric acid of the stomach with ammonia may allow colonization of the stomach by this acid-sensitive organism.

Ammonia, which has been found to be elevated in the gastric aspirates of some patients with *H. pylori*, has been postulated to have direct cytotoxic effects. However, there is little documented evidence that ammonia is toxic to human gastric epithelium (6). On the contrary, ammonium ion has been used in physiologic studies of gastric function without direct evidence of injury to gastric epithelium (10). In this study, we assessed cytotoxicity of *H. pylori* on gastric epithelial cells in vitro in the absence of hydrochloric acid and pepsin, examined the effect of urease inhibition on viability of gastric epithelial cells, and established a correlation between gastric epithelial cell injury and ammonia liberated by urea hydrolysis.

The human gastric adenocarcinoma cell line CRL 1739 (American Type Culture Collection, Rockville, Md.), which is a mucus-producing gastric epithelial cell line, was cultured in Ham F12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Inovar Chemicals, Inc., Gaithersburg, Md.), Skirrow selective antibiotic supplement (vancomycin, 10 μg/ml; polymyxin B sulfate, 2.5 IU/ml; trimethoprin lactate, 5 μg/ml) (Prolab Inc., Scarborough, Canada), and 0.25 μg of amphotericin B (Hazelton, Lenexa, Kans.) per ml (1). Cultures were incubated at 37°C in 5% carbon dioxide and humidified air. When used, urea was dissolved in sterile distilled water at a final concentration of 18 mg/ml (300 mOsm). Cells were grown in 24-well Falcon plastic tissue culture dishes (Becton-Dickinson & Co., Lincoln Park, N.J.) to approximately 80% confluency before each experiment. *H. pylori* cultured microaerophilically on sheep blood agar was added directly to 50 ml of medium at a final concentration of 5×10^6 CFU/ml. The suspension was then divided in half. Urea was added to one portion (25 ml) at a final concentration of 1.2 mg/ml (20 mM). The other portion (25 ml) received acetohydroxamic acid (AHA) at a final concentration of 500 μg/ml and urea (which was added 5 min after AHA) at a final concentration of 1.2 mg/ml (20 mM). In previous experiments by Mobley et al., AHA (500 μg/ml) was shown to completely inhibit urease activity without altering growth or viability of *H. pylori* (8). *H. pylori* in medium with and without AHA was then added to the gastric cells grown on 24-well plastic tissue culture dishes (2.0 ml per well). Control cells were cultured in medium supplemented with urea (1.2 mg/ml) alone and medium supplemented with both urea and AHA. The pH of the medium from controls and both experimental groups was measured at time zero and after 2, 24, and 48 h of culture. Control cells were cultured in medium at pH 7.4 and in medium with a pH matched to that of medium containing cells exposed to *H. pylori*. Medium osmolality was measured after 2, 24, and 48 h of culture. After 2, 24, and 48 h of culture, cell viability was determined by spectrophotometric measurement of the incorporation of neutral red dye by the method of Borenfreund and Puerner (2). Viability was also evaluated by cellular morphology under light microscopy (4). Cells were fixed in 100% ethanol and stained with Giemsa and Papanicolaou for morphological evaluation. Ammonia concentrations in medium were measured by enzymatic analysis by using glutamate dehydrogenase on a centrifugal analyzer after 2, 24, and 48 h of culture (3).

Ham F12 medium prepared as described above was supplemented with six different concentrations of urea (1, 5, 10, 15, 20, and 25 mM). Jack bean urease (Sigma Chemical Co., St. Louis, Mo.) with an activity of 50 U/mg (1 U liberates 1 μmol of ammonia per min from urea at 37°C) was added to culture medium containing urea at a final concentration of 0.1 mg/ml. This medium was then added to the gastric cells

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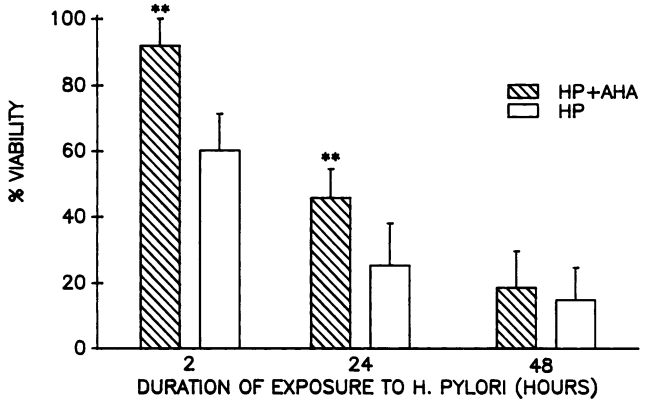

FIG. 1. Viability of gastric cells exposed to *H. pylori*. Monolayer cultures of a human gastric adenocarcinoma cell line (CRL 1739) were overlaid with suspensions of *H. pylori* (5×10^6 CFU/ml). Bacterial suspensions were either not preincubated or preincubated with 500 μg of AHA per ml. At 2, 24, and 48 h, the viability of monolayer cultures was assessed by cell incorporation of neutral red dye and expressed as a percentage of the control value. Cells exposed to *H. pylori* and AHA were significantly ($P < 0.0001$, by Student's *t* test) more viable than cells exposed to *H. pylori* alone after 2 and 24 h of culture (**). After 48 h of culture there was no significant difference in viability of cells in either group. Data are expressed as means ± standard deviations.

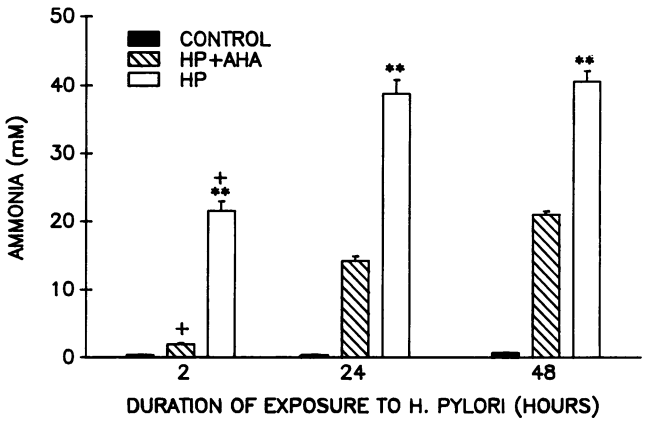

FIG. 2. Medium ammonia concentrations of cells exposed to *H. pylori*. Monolayer cultures of a human gastric adenocarcinoma cell line (CRL 1739) were overlaid with suspensions of *H. pylori* (5×10^6 CFU/ml). Bacterial suspensions were either not preincubated or preincubated with 500 μg of AHA per ml. Ammonia concentrations were measured after 2, 24, and 48 h of incubation with the bacterial suspension. After only 2 h in culture, the medium ammonia concentration of cells exposed to *H. pylori* with or without AHA was significantly higher ($P < 0.001$, by Student's *t*-test) than that of controls (+). Also, the medium ammonia concentration of cells exposed to *H. pylori* was significantly higher ($P < 0.0001$) at each time interval than that in cells exposed to *H. pylori* and AHA (**). Data are expressed as means ± standard deviations.

grown in 24-well plastic tissue culture dishes (2 ml per well). Control cells were cultured in medium without urea. After 24 h in culture, cell viability was determined by spectrophotometric measurement of neutral red dye incorporation and by cellular morphology (2, 4). Medium pH was measured at time zero and after 24 h of culture. The ammonia concentration in the medium was measured by enzymatic analysis after 24 h of culture (3). Cells were fixed in 100% ethanol and stained with Papanicolaou for morphological evaluation.

After 2, 24, and 48 h in culture, the viabilities of cultured gastric cells exposed to *H. pylori* without AHA in the culture medium (HP) were 60 ± 11, 27 ± 12 and 16 ± 9% (mean ± standard deviation), respectively (Fig. 1); viabilities of cells exposed to both *H. pylori* and AHA in the culture medium (HP+AHA) was 92 ± 9, 46 ± 8, and 20 ± 10% after 2, 24, and 48 h, respectively. Cell viabilities of cultures containing AHA in the medium were significantly higher ($P < 0.001$) than those of cells exposed to *H. pylori* alone after 2 and 24 h of culture. The addition of AHA to the medium of control cells did not alter cell viability. Gastric cells that were cultured in medium with either 1 mM (6 mg/dl) or 5 mM (30 mg/dl) urea showed no significant decrease in cell viability (99 and 96%, respectively) after 24 h of exposure to *H. pylori*.

The mean medium ammonia concentrations measured from the HP group, the HP+AHA group, and controls are shown in Fig. 2. AHA at 500 μg/ml only partially inhibited the urease activity of *H. pylori* in tissue culture medium. Ammonia concentrations of both the HP and HP+AHA treatment groups were significantly higher than those of controls after only 2 h of exposure of cultures to *H. pylori*. The pH of medium in the HP group measured 8.0, 8.2, and 8.2 after 2, 24, and 48 h in culture, respectively. The pH of medium in the HP+AHA group measured 7.5, 8.0, and 7.9 after 2, 24, and 48 h in culture, respectively. Control cells cultured at pH 8.2 showed no significant decrease in cell viability when compared with controls cultured at physiologic pH (7.4) for up to 48 h. Medium osmolalities (mean ± standard deviation) measured after 2, 24, and 48 h of exposure to *H. pylori* were 302 ± 3, 332 ± 9, and 330 ± 7 mOsm, respectively. Cells cultured in medium at 332 mOsm for 48 h were morphologically umchanged from control cells cultured at normal medium osmolality (300 mOsm) and maintained viability equivalent to that of cells cultured at normal medium osmolality.

The viability of cells exposed to jack bean urease for 24 h was inversely proportional to the resulting ammonia concen-

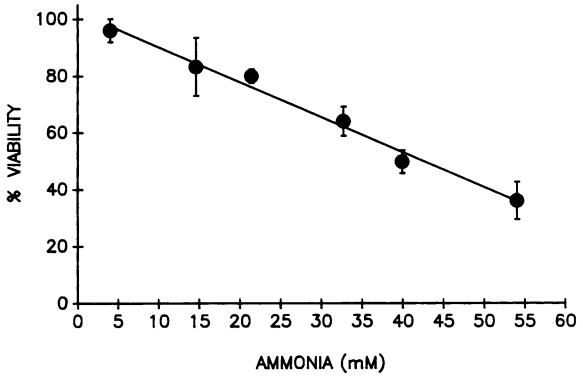

FIG. 3. Effect of jack bean urease on the viability of cultured gastric epithelial cells in medium containing urea. Viability was determined by spectrophotometric measurement of cell incorporation of neutral red dye and is expressed as percentages of control values. Control cells were cultured in medium containing urease but not supplemented with urea. There is a clear linear inverse relationship between cell viability and medium ammonia concentration ($r = 0.94$, by linear regression analysis). Cells exposed to ammonia at concentrations greater than 14 mM for 24 h were significantly ($P < 0.001$, by Student's *t* test) less viable than controls. Data are expressed as means ± standard deviations.

tration (Fig. 3). Cells exposed to >14 mM ammonia for 24 h were significantly less viable than controls. The viability of control cells cultured in medium with urease (without urea) was unchanged from that of control cells cultured in medium without urease. The pH of medium containing urea exposed to urease ranged from 7.8 to 9.0 after 2 h of culture and from 7.7 to 8.8 after 24 h of culture, depending on the initial urea concentration. The viabilities of control cells cultured at pH of 8.0, 8.5, and 9.0 for 24 h were 99, 95, and 92%, respectively, compared with control cells cultured at pH 7.4.

Histological specimens showed no notable changes morphologically between control cells and cells exposed to *H. pylori* for 2 h with or without AHA. However, after 24 h of exposure to *H. pylori* without AHA, more than two-thirds of the cells appeared injured morphologically with granular cytoplasm, pyknotic nuclei, cytoplasmic blebs, and decreased cell size and area. These same morphological changes were also seen in cells exposed to *H. pylori* with AHA for 24 h, but to a lesser degree (less than 50% of the cells). However, after 48 h of culture, these two groups could not be distinguished morphologically. Cells exposed to jack bean urease demonstrated similar morphological changes, which were directly proportional to the medium ammonia concentration.

These experiments demonstrate that ammonia liberated by *H. pylori* urease activity is directly cytotoxic to gastric epithelial cells in vitro. Inhibition of *H. pylori* urease significantly decreased this toxicity, suggesting that ammonia is at least partially responsible for the cytotoxicity found in association with this bacterium. In addition, ammonia produced by jack bean urease-mediated urea hydrolysis in the absence of *H. pylori* produced cytotoxicity similar to that seen after exposure of cultured gastric cells to *H. pylori*. Ammonia concentrations correlated directly with cell injury, further supporting the hypothesis that ammonia at high concentrations is cytotoxic to human gastric epithelium. Medium ammonia concentrations greater than 14 mM caused a significant decrease in cell viability. However, the viability of cells exposed to *H. pylori* was less than that of cells exposed to jack bean urease for any given medium ammonia concentration, suggesting that *H. pylori* may produce other toxins in addition to ammonia.

The cytotoxicity demonstrated by these experiments could not be explained by increases in pH or osmolality. Supplementing medium with 20 mM urea is equivalent to a blood urea nitrogen concentration of 120 mg/dl, which is much higher than that of normal patients. However, patients with chronic renal failure (without *H. pylori*) have urea concentrations averaging more than 18 mM and ammonia concentrations averaging 4 mM in gastric juice. Patients with chronic renal failure and with *H. pylori* infection have urea concentrations averaging 4 mM and ammonia concentrations averaging 19 mM in gastric juice, well within the range that produced significant cytotoxicity in this study (A. Triebling, M. Korsten, F. Paronetto, and S. Lieber, Gastroenterology **96**:A515, 1989).

In conclusion, this study demonstrates that *H. pylori* is directly cytotoxic to cultured gastric epithelial cells. This toxicity is at least in part secondary to ammonia produced by hydrolysis of urea. Therefore, the urease activity of *H. pylori* resulting in the production of ammonia appears likely to be partially responsible for the gastric mucosal injury found in association with *H. pylori* infection.

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