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Production of Reactive Oxygen Species by Gastric Cells in Association with *Helicobacter Pylori*

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Reactive oxygen species (ROS) and *Helicobacter pylori* have been identified as pathogenic factors in several gastrointestinal disorders. Since little information is available regarding the mechanistic pathways of *H. pylori*-induced gastric injury, the potential role of ROS was investigated. The induction of ROS in gastric cells (GC) by *H. pylori* was assessed using chemiluminescence, cytochrome *c* reduction, nitrobluetetrazolium (NBT) reduction and lactate dehydrogenase (LDH) leakage. The dose-dependent protective abilities of selected ROS scavengers on LDH leakage were determined. Following incubation of GC with three strains of *H. pylori* (1:1), approximately 5.7–8.0 and 3.8–4.3 fold increases were observed in cytochrome *c* and NBT reduction, respectively, demonstrating production of ROS. Enhanced chemiluminescence responses of 2.1- and 3.7-fold were observed following incubation of GC with *H. pylori* (ATCC 43504) at ratios of 1:1 and 1:10, respectively. Approximately 2.2- and 3.5-fold increases in LDH leakage were observed at GC:*H. pylori* (ATCC 43504) ratios of 1:1 and 1:10, respectively. Substantial inhibition of LDH leakage from GC in the presence of *H. pylori* was observed following co-incubations with selected ROS scavengers with cimetidine serving as the best chemoprotectant. The antioxidants and H_2-receptor antagonists had no effect on growth of *H. pylori* cells. This study demonstrates that *H. pylori* induces enhanced production of ROS in GC, and enhances membrane damage.

Key words: *H. pylori*, gastric cells, chemiluminescence, cytochrome *c* reduction, nitrobluetetrazolium reduction, lactate dehydrogenase, catalase, superoxide dismutase, mannitol, allopurinol, vitamin E, cimetidine, famotidine, ranitidine

INTRODUCTION

Helicobacter pylori, a gram negative spiral bacterium, was first reported to be associated with human gastric mucosa as early as 1896,[1] but was largely ignored until 1982.[2] In the duodenum *H. pylori* is restricted to areas of gastric metaplasia,[3,4] usually accompanied by an inflammatory response. Furthermore, prevailing evidence indicates that *H. pylori* plays an important role in producing an imbalance between aggressive and defensive factors which lead to mucosal damage.[5,6] Extensive data exists showing that *H. pylori* is associated with duodenitis, duodenal ulcer, gastric ulcer, non-ulcer dyspepsia,[5,7–9] chronic gastritis,[5] 90% of cases with chronic type B gastritis,[10] and gastric carcinoma.[11] Furthermore, duodenal

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and gastric ulcer recurrence rates are significantly reduced by erradicating *H. pylori*, implying that this organism is etiologically very important in these conditions.[8,12]

H. pylori is an ubiquitous organism, and its prevalence seems to rise with age, being present in more than 60% of Americans in their seventh decade.[13] This bacterium is found not only adjacent to mucus cells, but has been observed in the canaliculi of parietal cells, with observed degenerative changes in the latter.[14] Cell membranes are disrupted and cellular organelles disappear in the vicinity of *H. pylori*.[14]

By using chemiluminescence, Davies *et al.*[15] have demonstrated elevated levels of oxygen free radicals in duodenal biopsies taken from patients with active duodenal ulcers. They also demonstrated that *H. pylori* infected antral mucosa generates more reactive oxygen species than non-*H. pylori* infected tissue, and furthermore, the oxygen free radical production was not related to the intensity of leucocytic infiltrate.[16]

The present study investigated the effects of *H. pylori* on the release of lactate dehydrogenase (LDH) by cultured gastric cells as an index of cellular damage and cytotoxicity. The generation of reactive oxygen species in gastric cells and in *H. pylori* cells as well as in gastric cells in association with *H. pylori* was also determined by enhanced chemiluminescence, and cytochrome *c* and NBT reduction assays, and the relationship between oxidative stress and the relative scavenging abilities of selected oxygen free radical scavengers, antioxidants and histamine H_2-receptor antagonists was assessed.

MATERIALS AND METHODS

The ATCC human gastric CRL 1739 adenocarcinoma cell line and *H. pylori* strains ATCC 43504 and ATCC 43629 were obtained from American Type Culture Collection (Rockville, MD). Cytotoxin producing *H. pylori* strain 60190 was obtained from Dr. T.L. Cover, Vanderbilt University, Nashville, TN. Ham's F-12 HEPES modification, NADH (disodium salt), pyruvic acid (disodium salt), monobasic sodium phosphate, dibasic sodium phosphate, and tris(hydroxymethylamino-methionine) chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin-streptomycin, gentamycin, and trypsin were obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). *Brucella albimi* broth (BBL 99125), Trypticase soy agar (BBL 11043) and defibrinated sheep blood (BBL 11945) were purchased from Baxter (Kansas City, MO), a distributor of Baltimore Biological Laboratories. All other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or of the highest grade available. CLOtest strips for detecting *Helicobacter pylori* were obtained from Tri-Med Specialties Inc. (Lenexa, KS). Detailed investigations were performed on *H. pylori* strain ATCC 43504, while selected experiments were conducted with *H. pylori* strains ATCC 43629 and 60190 to determine whether the two later strains produced similar results to the bacterial strain ATCC 43504.

Gastric Cell Line

The human gastric adenocarcinoma cell line (ATCC, CRL 1739) was maintained and grown in culture flasks in Ham's F12 medium containing 10% fetal bovine serum, 0.2% gentamycin and 0.25% penicillin-streptomycin solution. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Trypsin solution was used to split cultures whenever they were grown to confluence. The number of cells was determined using a Coulter counter. Viability was checked by the Trypan blue exclusion method. Bull-Henry *et al.*[17] have previously used this human gastric adenocarcinoma cell line and demonstrated *in vitro* invasion and injury by *H. pylori*.

For the determination of lactate dehydrogenase (LDH) leakage as a parameter of *H. pylori*-induced cytotoxicity, cultured gastric CRL/1739

cells were plated at 25×10^4 cells/35 mm petri dish (Corning, NY) in 2 ml of Ham's F12 media having the same composition as described above. After incubation for 2 hrs to allow cell adherence, various concentrations of *H. pylori* were added to different petri dishes, and the incubations were continued for 0, 6, 12, 18, 24 and 30 hrs. To determine total LDH in gastric cells in the absence of *H. pylori*, the cells were lysed using 0.01% triton X-100 followed by freeze-thaw cycles at 6, 18 and 30 hrs of incubation. For studies involving selected oxygen free radical scavengers including superoxide dismutase (SOD), catalase, allopurinol and mannitol, the antioxidants vitamin E and vitamin E succinate, and the histamine H_2-receptor antagonists cimetidine, ranitidine and famotidine, various concentrations were added alone or in combination with *H. pylori* to different gastric cell cultures, and incubations were continued for 0, 12 or 24 hr. SOD, catalase and mannitol were individually dissolved in the media, and added to the incubation mixture in a volume of 10 µl, while allopurinol was dissolved in slightly alkaline solution and added in a volume of 10 µl. Cimetidine and ranitidine were dissolved in a 1:1 mixture of media and ethanol and added to the incubation mixture in a volume of 10–30 µl. Vitamin E, vitamin E succinate and famotidine were individually dissolved in ethanol, and added to the incubation mixture between 10–30 µl. Vehicles without these drugs were added to the control petri dishes. An additional volume of the vehicle was added to all petri dishes to maintain a total volume of 2.04 ml in each petri dish. The media from the incubated petri dishes were collected for LDH assay and stored at –70°C. Enzyme assays were completed within 1–3 days of media collection.

For the determination of chemiluminescence, cytochrome *c* and NBT reduction, samples containing 3×10^6 gastric cells/ml were precubated at 37°C for 30 min. After preincubation, various concentrations of *H. pylori* and/or selected oxygen free radical scavengers including SOD and catalase were added and the mixtures were incubated at 37°C for 15 min for the determination of cytochrome *c* and NBT reduction. Chemiluminescence was monitored for 6 min at continuous 30 sec intervals.

Helicobacter pylori Cell Lines

The *H. pylori* strain 60190, a cytotoxin producing strain, and the *H. pylori* strains 43504 and 43629 obtained from ATCC, were rehydrated with 0.3 ml *Brucella albimi* broth, and the entire suspension was transferred to a fresh blood agar plate (Trypticase soy agar with 5% defibrinated sheep blood) and grown in a microaerophillic environment (5% O_2, 10% CO_2 and 85% N_2) at 37°C. Bacteria counts were performed using the serial dilution technique. Viable cells were determined by spreading measured aliquots of diluted bacterial cultures onto fresh blood agar plates (Trypticase soy agar with 5% defibrinated sheep blood) and counting the resulting colonies after 12 hr and 24 hr of incubation.[18] The bacteria was characterized by developing red color in Christensen's urea agar plate (composition: peptone 0.1% w/v, NaCl 0.5% w/v, glucose 0.1% w/v, monopotassium phosphate 0.2% w/v, phenol red 0.012% w/v, urea 2%, and agar 1.5% w/v in sterile deionized water), and also using a CLOtest strip (Tri-Med Specialities, Inc.).

Chemiluminescence Measurement

Chemiluminescence was measured[19] in a Chronolog Lumivette luminometer (Chronolog Corp., Philadelphia, PA). The assay was conducted in 3 ml glass minivials. The vials were incubated at 37°C prior to measurement and the background chemiluminescence of each vial was checked before use. Samples containing 3×10^6 cells/ml were pre-incubated at 37°C for 30 min. After incubation of the gastric and/or *H. pylori* cells, 4 µM luminol was added to enhance chemiluminescence. All additions to the vials as well as chemiluminescence counting procedures were performed under dim lighting conditions. Cell suspensions were maintained at pH 7.4 during incubations. Results

were examined as counts/unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30 second intervals.

Measurement of Lactate Dehydrogenase (LDH) Leakage

Leakage of LDH from cells into the media was determined as described by Moss *et al.*,[20] by dilution of 10 µl of media to 2.6 ml with phosphate buffer (0.1 M, pH 7.4) and warming the mixture to 37°C. The reaction was then initiated in a cuvette by adding 100 µl of NADH (125 µM, final concentration) and 100 µl of sodium pyruvate (300 µM, final concentration) to the diluted media. Changes in absorbance of the reaction solution were measured at 340 nm for 3 minutes in a Perkin-Elmer Lambda 6 spectrophotometer. The concentrations of LDH in the media were determined by direct calculation based upon the decrease in absorbance.

Nitrobluetetrazolium (NBT) Dye Reduction

Reduction of NBT is a convenient system for assessing the formation of reactive oxygen species.[21] The 1.0 ml assay mixture contained 3×10^6 cells and 50 µM NBT in the assay buffer. The mixture was incubated at 37°C for 15 min and spectrophotometric determination was carried out at 510 nm to assess the amount of formagan produced. The amount of reactive oxygen species produced was expressed as nmoles NBT reduced/3×10^6 cells/15 min.

Cytochrome *c* reduction

Superoxide anion production was measured by the cytochrome *c* reduction assay of Babior *et al.*[22] Each 1.0 ml reaction mixture contained 3×10^6 cells and 0.05 mM cytochrome *c*. The reaction mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures on ice, and spectrophotometric determination was carried out at 550 nm. Absorbance values were converted to nmoles of cytochrome *c* reduced using the extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$/15 min, and the data were expressed as nmol cytochrome *c* reduced/3×10^6 cells/15 min.

Statistical Methods

The presence of significant differences between mean values was determined using Student's *t* test or by Analysis of Variance (ANOVA) followed by Scheffe's S method as the post hoc test. Each value is the mean ± S.D. from at least 4–6 experiments. The level of statistical significance employed in all cases was $P<0.05$.

RESULTS

The release of the enzyme lactate dehydrogenase (LDH) into the media of incubated gastric CRL/1739 cultured cells is indicative of cellular and membrane damage. Therefore, cultured gastric cells were incubated in the presence of various concentrations of *H. pylori* (ATCC 43504), and the release of LDH by the cells was measured as an index of cytotoxicity. The amount of LDH released is presented in Figure 1 as a function of *H. pylori* (ATCC 43504) concentration and incubation time. The total determination of LDH following complete cell lysis of gastric cells in the absence of *H. pylori* at 6, 18 and 30 hrs resulted in the release of 652 ± 99, 811 ± 140 and 786 ± 102 U LDH/L, respectively. In most cases, the LDH leakage was maximal following 12 hrs of incubation, and enhanced LDH leakage was observed at each concentration of *H. pylori*. For example, at the 12 and 24 hr time points, approximately 2.0-fold and 2.2-fold enhanced LDH leakage ($P<0.05$) was observed, respectively, at a 1:1 ratio of gastric cells:*H. pylori* ATCC 43504 cells. Approximately 4.5-and 4.7-fold increases in LDH leakage ($P<0.05$) were observed at the 12 and 24 hr incubation times, respectively, following incubation of gastric and *H. pylori* ATCC 43504 cells at a 1:100 ratio. At the 24 hr time point with the 1:100 ratio of gastric cells to *H. pylori* cells, the released LDH

TABLE 1 Effects of Free Radical Scavengers on the Release of Lactate Dehydrogenase (LDH) from Human Gastric CRL/1739 Cells in Association with Helicobacter pylori

Gastric cell: *H. pylori* cell ratio	Oxygen radical scavengers	Units LDH/liter		
		0 hr	12 hr	24 hr
Medium (Ham's F12 and additives)	–	28.8 ± 4.0	24.7 ± 3.3	31.2 ± 2.6
1:0 (gastric cells alone)	–	71.6 ± 8.1	73.0 ± 6.4	75.4 ± 8.5
0:1 (ATCC 43504)	–	49.6 ± 5.6	57.6 ± 8.2	51.8 ± 6.7
0:10 (ATCC 43504	–	61.7 ± 10.2	59.8 ± 8.4	63.0 ± 7.2
0:100 (ATCC 43504)	–	82.7 ± 9.3	85.8 ± 7.7	84.4 ± 10.6
1:1 (ATCC 43504	–	92.4 ± 10.1	184.1 ± 16.4*	203.8 ± 12.5*
1:1 (ATCC 43629)	–	94.1 ± 10.5	178.2 ± 22.3*	205.4 ± 21.7*
1:1 (strain 60190)	–	95.0 ± 8.8	247.2 ± 22.6*	284.8 ± 30.3*
1:10 (ATCC 43504)	–	95.6 ± 8.7	276.3 ± 24.3*	319.5 ± 25.4*
1:10 (ATCC 43629)	–	–	261.8 ± 28.4*	311.7 ± 33.3*
1:10 (strain 60190)	–	–	299.6 ± 40.2*	349.3 ± 45.4*
1:0	SOD (200 μg/ml)	–	–	67.5 ± 7.3
1:1 (ATCC 43504)	SOD (200 μg/ml)	88.7 ± 9.5	181.4 ± 15.4*	199.8 ± 11.4*
1:0	Catalase (200 μg/ml)	–	–	68.4 ± 7.0
1:1 (ATCC 43504)	Catalase (200 μg/ml)	81.4 ± 7.4	176.2 ± 11.2*	191.6 ± 11.4*
1:0	Mannitol (1.25 μM)	–	–	62.5 ± 7.7
1:1 (ATCC 43504)	Mannitol (1.25 μM)	90.5 ± 6.9	167.4 ± 11.5*,**	184.8 ± 4.6*,**
1:0	Allopurinol (1.47 mM)	–	–	73.6 ± 10.1
1:1 (ATCC 43504)	Allopurinol (1.47 mM)	88.8 ± 8.8	181.0 ± 14.5*	190.2 ± 16.3*
1:1	SOD + Catalase (200 μg/ml each)	94.7 ± 10.4	144.5 ± 8.7*,**	165.4 ± 9.5*,**
1:1 (ATCC 43504)	SOD + Catalase + Mannitol + Allopurinol	86.4 ± 4.8	131.8 ± 10.3*,**	147.2 ± 10.4*,**
1:10 (ATCC 43504)	SOD + Catalase (200 μg/ml each)	87.3 ± 7.9	230.5 ± 14.8*	251.1 ± 22.5*
1:10 (ATCC 43504)	SOD + Catalase + Mannitol + Allopurinol	82.5 ± 6.7	151.7 ± 8.5*,**	169.5 ± 11.7*,**

Human gastric CRL1739 cells (25×10^4 cells/35 mm petri dish) in 2 ml of Ham's F12 were incubated for 2 hrs to allow cell adherence and *H. pylori* and/or various concentrations of selected oxygen free radical scavengers, singly and in combination, were added to the cultures. The incubation was continued at 37°C in an atmosphere of 5% CO_2 for 24 hrs. Media was collected from the cultures and assayed for lactate dehydrogenase activity. Data are expressed as the mean value of 6 experiments ± S.D. $P<0.05$ with respect to the control gastric cells in the absence of *H. pylori* cells. **$P<0.05$ with respect to the gastric cells treated with *H. pylori* ATCC 43504 cells at 1:1 ratio.

represents approximately 50% of the total LDH present in the gastric cells.

In order to determine whether the increase in LDH leakage associated with the increasing ratio of *H. pylori* ATCC 43504 cells in the presence of the gastric cells was due to loss of the enzyme from the *H. pylori* ATCC 43504 cells, various concentrations (0:1, 0:10, and 0:100) were incubated for up to 24 hrs in the absence of gastric cells. These results are presented in Table 1. The results demonstrate that a small increase in LDH release was observed with increasing concentration of *H. pylori* ATCC 43504 cells in the absence of gastric cells. However, in the presence of gastric cells the *H. pylori* ATCC 43504 cells resulted in approximately a 4–5-fold greater release of LDH into the medium. Therefore, these results suggest that *H. pylori* ATCC 43504 cells induce concentration-dependent cytotoxic effects on gastric cells as shown by release of LDH into the medium from the cultured gastric cells.

In order to determine whether other *H. pylori* strains produce similar results, the abilities of *H. pylori* strains ATCC 43629 and 60190 to induce the leakage of LDH from cultured gastric cells following coincubation were examined. The *H. pylori* strain 60190 is known to be a cytotoxin producing strain. The results clearly indicate that at the 12 and 24 hr incubation times with gastric cell to *H. pylori* cell ratios of 1:1 and 1:10 significant increases in LDH leakage were observed. Similar results were observed for *H. pylori* strains ATCC 43629 and 43504 (1.9–3.3 fold increases), while the highly cytotoxic strain 60190 produced greater leakage of LDH (2.6–3.7 fold increases) than the other two strains.

The effects of chemoprotectants including selected oxygen free radical scavengers, antioxidants and histamine H_2-receptor antagonists were assessed on the release of LDH into the medium following incubation of gastric cells with two different ratios of *H. pylori* ATCC 43504 (1:1 and 1:10). The dose-dependent effects of SOD, catalase, allopurinol and mannitol, singly and in combination, on LDH leakage are presented in Table 1. The individual use of oxygen free radical scavengers did not attenuate the *H. pylori*-induced LDH leakage from gastric cells. However, following incubation of gastric cells with *H. pylori* in combination with SOD (200 μg/ml) and catalase (200 μg/ml) decreases in LDH leakage of approximately 20% ($P<0.05$) were observed at the 12 and 24 hr incubation times at both cell ratios (1:1 and 1:10). Incubation of gastric cells with *H. pylori* at a 1:1 ratio in combination with SOD (200 μg/ml), catalase (200 μg/ml), mannitol (1.25 μM) and allopurinol (1.47 mM) decreased LDH leakage by approximately 28% ($P<0.05$) at both 12 and 24 hr time points. At a cell ratio of 1:10 in the presence of the four free radical scavengers LDH leakage decreased by approximately 46% at the two time points relative to the combined cells in the absence of the free radical scavengers.

Table 2 demonstrates the dose-dependent inhibition of LDH release by vitamin E, vitamin E succinate and the histamine H_2-receptor antagonists cimetidine, ranitidine and famotidine on gastric cells:*H. pylori* ATCC 43504 cells (1:1) at the 12 and 24 hr incubation times. Inhibition was observed at every concentration and time point. Approximately 26% and 25% ($P<0.05$) inhibitions were observed at the 12 hr and 24 hr incubation times, respectively, with 75 μM vitamin E. Vitamin E succinate provided concentration-dependent protection against *H. pylori*-induced LDH release from gastric cells. Approximately 12% and 19% inhibitions in LDH release were observed at the 12 and 24 hr incubation times, respectively, with 75 μM vitamin E succinate. Inhibition was significant only at the 24-hour time point. In the case of histamine H_2-receptor antagonists, cimetidine was the most effective agent in attenuating LDH leakage. Following incubation of gastric cells with *H. pylori* cells (1:1) in combination with 100 μM concentrations of cimetidine, famotidine or ranitidine for 12 hr, approximately 42%, 37% and 32% decreases in LDH leakage ($P<0.05$) were observed, respectively, with similar results being observed after 24 hrs. Vitamin E succinate and histamine H_2-receptor antagonists

TABLE 2 Effects of antioxidants and histamine H_2-receptor antagonists on the release of lactate dehydrogenase (LDH) from human gastric CRL/1739 cells in association with *Helicobacter pylori* ATCC 43504

Gastric cell: *H. pylori* cell ratio	Antioxidants/histamine H_2-receptor antagonists	Units LDH/liter		
		0 hr	12 hr	24 hr
1:1	–	92.4 ± 10.1	184.1 ± 16.4*	203.8 ± 12.5*
1:1	Vitamin E (25 μM)	–	171.0 ± 11.2*	184.5 ± 16.2*
1:1	Vitamin E (50 μM)	–	144.5 ± 10.8*,**	167.1 ± 11.2*,**
1:1	Vitamin E (75 μM)	–	137.0 ± 8.8*,**	154.2 ± 13.4*,**
1:0	Vitamin E (75 μM)	–	–	70.5 ± 8.2
1:0	Vitamin E Succinate (75 μM)	–	–	121.3 ± 11.8*
1:1	Vitamin E Succinate (25 μM)	–	185.3 ± 11.5*	189.4 ± 14.1*
1:1	Vitamin E Succinate (50 μM)	–	167.7 ± 12.4*	169.2 ± 16.3*,**
1:1	Vitamin E Succinate (75 μM)	–	163.5 ± 15.4*	164.1 ± 8.3*,**
1:0	Cimetidine (100 μM)	–	–	72.2 ± 10.5
1:0	Famotidine (100 μM)	–	–	79.5 ± 6.3
1:0	Ranitidine (100 μM)	–	–	74.7 ± 7.6
1:1	Cimetidine (25 μM)	–	140.3 ± 11.7*,**	162.2 ± 13.1*,**
1:1	Cimetidine (50 μM)	–	121.4 ± 8.7*,**	139.3 ± 6.6*,**
1:1	Cimetidine (100 μM)	–	107.5 ± 12.2*,**	119.5 ± 13.3*,**
1:1	Famotidine (25 μM)	–	152.6 ± 14.5*,**	164.4 ± 18.6*,**
1:1	Famotidine (50 μM)	–	124.8 ± 11.6*,**	133.8 ± 11.6*,**
1:1	Famotidine (100 μM)	–	116.8 ± 10.8*,**	130.1 ± 14.5*,**
1:1	Ranitidine (25 μM)	–	161.6 ± 8.3*,**	168.8 ± 8.4*,**
1:1	Ranitidine (50 μM)	–	140.7 ± 18.4*,**	154.5 ± 9.6*,**
1:1	Ranitidine (100 μM)	–	126.8 ± 11.2*,**	142.7 ± 15.3*,**

Human gastric CRL1739 cells (25×10^4 cells/35 mm petridish) in 2 ml of Ham's F12 were incubated for 2 hrs to allow cell adherence and *H. pylori* and/or various concentrations of antioxidants/histamine H_2-receptor antagonists were added to cultures. The incubation was continued at 37°C in an atmosphere of 5% CO_2 for 24 hrs. Media were collected from the cultures and assayed for LDH activity. *$P<0.05$ with respect to the control gastric cell: *H. pylori* cell mixture at zero time. **$P<0.05$ with respect to the gastric cells treated with *H. pylori* ATCC 43504 cells at 1:1 ratio.

had no growth inhibitory effects on cultured *H. pylori* cells (data not shown).

The production of superoxide anion based on cytochrome *c* and NBT reduction, by human gastric CRL/1739 cells in association with *H. pylori* (ATCC 43504), is shown in Table 3. Cell ratio dependent increased cytochrome *c* and NBT reduction was observed. At the highest concentration of *H. pylori* with a gastric to *H. pylori* cell ratio of 1:10, 12.3-and 5.7-fold increases in cytochrome *c* and NBT reductions, respectively, were observed as compared to gastric cells alone.

The *H. pylori* strains ATCC 43629 and 60190 were similarly assessed to determine the production of superoxide anion (Table 3). At a 1:10 ratio of gastric and *H. pylori* (ATCC 43629) cells approximately 10.8-fold and 5.2-fold increases ($P<0.05$) were observed in cytochrome *c* and NBT reduction, respectively, while at this same ratio of gastric and *H. pylori* (strain 60190) cells approximately 13.9-fold and 6.4-fold increases ($P<0.05$) were observed in cytochrome *c* and NBT reduction, respectively, relative to control gastric cells.

The fraction of cytochrome *c* and NBT reduction that is inhibitable by SOD or SOD plus catalase can be considered to be due to superoxide. Therefore, the effects of these two enzymes on cytochrome *c* and NBT reduction by gastric cells in the presence of various ratios of *H. pylori* (ATCC 43504) are presented in Table 3. In the absence of *H. pylori*, SOD plus catalase had little effect on cytochrome *c* and NBT reduction. However, in the presence of various ratios of gastric to *H. pylori* cells, the addition of SOD or SOD plus catalase

TABLE 3 *In Vitro* Production of Superoxide Anion (Cytochrome *c* and NBT reduction) Following Incubation of human gastric CRL/1739 cells with *Helicobacter pylori* and the effects of specific oxygen free radical scavengers

Gastric cell:*H. pylori* cell ratio	Oxygen free radical scavengers	Cytochrome *c* reduction nmoles/15 min/3 × 10^6 gastric cells	NBT reduction nmoles/15 min/3 × 10^6 gastric cells
1:0	–	3.2 ± 0.1^a	9.4 ± 1.6^a
0:1 (ATCC 43504)	–	5.0 ± 0.7^b	13.3 ± 1.4^b
1:1 (ATCC 43504)	–	22.0 ± 3.4^c	40.0 ± 5.2^c
1:3 (ATCC 43504)	–	34.6 ± 5.7^d	48.5 ± 6.6^d
1:5 (ATCC 43504)	–	37.0 ± 6.2^d	51.2 ± 7.3^d
1:10 (ATCC 43504)	–	39.4 ± 5.2^d	53.1 ± 6.2^d
1:1 (ATCC 43629)	–	18.3 ± 3.7^c	36.6 ± 4.0^c
1:10 (ATCC 43629)	–	34.6 ± 5.2^d	48.4 ± 5.7^d
1:1 (strain 60190)	–	30.4 ± 3.9^c	47.3 ± 6.5^c
1:10 (strain 60190)	–	44.4 ± 6.6^d	59.7 ± 10.2^d
1:0	SOD (100 μg/ml) + Catalase (100 μg/ml)	3.0 ± 0.4^a	9.3 ± 0.8^a
1:1 (ATCC 43504)	SOD (100 μg/ml)	10.0 ± 1.2^e	20.3 ± 2.7^e
1:1 (ATCC 43504)	SOD (100 μg/ml) + Catalase (100 μg/ml)	7.8 ± 1.4^e	18.5 ± 2.0^e
1:3 (ATCC 43504)	SOD (100 μg/ml)	22.6 ± 6.7^c	31.3 ± 5.0^d
1:3 (ATCC 43504)	SOD (100 μg/ml) + Catalase (100 μg/ml)	20.3 ± 3.4^c	27.6 ± 3.3^f
1:5 (ATCC 43504)	SOD (100 μg/ml)	23.5 ± 7.4^c	$30.9 \pm 8.4^{c,d}$
1:5 (ATCC 43504)	SOD (100 μg/ml) + catalase (100 μg/ml)	19.8 ± 5.5^c	27.7 ± 4.9^c
1:10 (ATCC 43504)	SOD (100 μg/ml)	$27.2 \pm 10.1^{c,d}$	$30.2 \pm 10.7^{c,d}$
1:10 (ATCC 43504)	SOD (100 μg/ml) + catalase (100 μg/ml)	24.6 ± 7.2^c	26.1 ± 8.8^c

Each value is the mean ± S.D. of 4–6 experiments. NBT: nitrobluetetrazolium. The incubation time was 15 min at 37°C. Values with non-identical superscripts are significantly different. ($P < 0.05$)

resulted in 31–55% and 38–65% decreases, respectively, in cytochrome *c* reduction. SOD and SOD plus catalase produced similar decreases in NBT reduction at the various cell ratios.

The dose-dependent production of enhanced chemiluminescence as an index of reactive oxygen species production by human gastric CRL/1739 cells in association with three different ratios of *H. pylori* ATCC 43504 (1:1, 1:5 and 1:10) is presented in Figure 2. The chemiluminescence response rapidly rises, reaching a maximum at approximately 4 min of incubation. No further increases were observed thereafter. Cell ratio-dependent enhanced chemiluminescence was observed. Approximately 2.1-, 2.9- and 3.7-fold increases ($P<0.05$) in chemiluminescence were observed at gastric cell:*H. pylori* (ATCC 43504) cell ratios of 1:1, 1:5 and 1:10, respectively, relative to gastric cells alone after 6 min of incubation.

DISCUSSION

Extensive data demonstrates that *H. pylori* is associated with various gastrointestinal disorders including gastric carcinoma.[7–11] The involvement of reactive oxygen metabolites has been postulated in several gastrointestinal pathologies.[23] Reactive oxygen species appear to be of great importance in the development of gastrointestinal injury after intestinal ischemia and reperfusion,[24] and after hemorrhagic shock.[23] Hepatic injury after ischemia and reperfusion is attributed to the enhanced production of reactive oxygen species.[25] Oxygen free radicals are also proposed to be involved in

gastrointestinal inflammatory diseases. Production of reactive oxygen species has been evidenced during acute pancreatitis[26] and inflammatory bowel diseases.[27] Furthermore, oxygen free radicals are produced during the gastrointestinal metabolism of xenobiotics, which may lead to intestinal disorders.[28]

Previous studies by Davies *et al.*[29] have demonstrated elevated levels of oxygen free radicals in duodenal biopsies from patients with active duodenal ulcers. Chemiluminescence was greater in *H. pylori* positive as compared with negative tissue when samples were grouped by equivalent macroscopic or microscopic damage. This difference was in part accounted for by a greater neutrophil infiltration in the *H. pylori* positive mucosa, but when biopsy specimens with equivalent neutrophil infiltration could be compared directly, positive specimens gave greater chemiluminescence than negative tissue specimens. There was no evidence for reactive oxygen species in the pathogenesis of gastric mucosal injury in cases unrelated to *H. pylori* infection.[29] Thus, these studies indicate that excess reactive oxygen species production is associated with *H. pylori* positive antral infection and may be an important pathogenic mechanism.

The involvement of reactive oxygen species has also been demonstrated by Salim[30] in the relapse of duodenal ulceration in patients infected with *H. pylori*. Various oxygen free radical scavengers including allopurinol, dimethyl sulfoxide (DMSO) and cimetidine were individually administered to patients to investigate the possibility that duodenal ulcer relapse associated with *H. pylori* infection was mediated by reactive oxygen species. Cimetidine was effective in preventing the relapse. However, allopurinol and DMSO were superior to cimetidine in this respect.[30] Ching *et al.*[31] have demonstrated that cimetidine is a powerful hydroxyl radical scavenger with a much higher rate constant for reaction with hydroxyl radicals than the rate constant found for the well-known hydroxyl radical scavenger mannitol.

The present study examined the effects of *H. pylori* on LDH leakage from cultured gastric cells as an index of membrane damage and assessed the production of reactive oxygen species based on chemiluminescence as well as cytochrome *c* and NBT reduction. The use of a cell culture system was selected in order to avoid confounding factors that might alter free radical production and the interaction between gastric and *H. pylori* cells, such as the immune and hormonal systems. Most of the studies were conducted with the *H. pylori* strain ATCC 43504. The two *H. pylori* strains ATCC 43629 and the cytotoxin producing strain 60190 exhibited similar performances to ATCC 43504 in terms of enhanced LDH leakage and production of superoxide anion, suggesting that the results are not strain specific. Greatest LDH leakage and superoxide anion production was caused by strain 60190.

The abilities of selected free radical scavengers, antioxidants and histamine H_2-receptor antagonists to provide partial inhibition of the membrane damage and subsequent leakage of LDH provides additional evidence for the role of reactive oxygen species in the cell damage induced by *H. pylori*. Similar results were observed in chemiluminescence, and cytochrome *c* and NBT reduction experiments. When vitamin E succinate and the histamine H_2 receptor antagonists were incubated alone with *H. pylori*, the growth of *H. pylori* was not inhibited (data not shown). Therefore, the protective effects of these antioxidants and H_2-receptor antagonists were not due to the inhibition of *H. pylori*.

The results in Figure 1 and Table 1 demonstrate that the incubation of gastric cells with *H. pylori* cells results in enhanced leakage of LDH in the incubation medium. The leakage of LDH was dependent upon the concentration of *H. pylori* cells, and the large increase in LDH in the medium could not be attributed to LDH associated with the *H. pylori* cells. Leakage of LDH did not occur from gastric cells in the absence of *H. pylori*. Thus, the results indicate that *H. pylori* produces membrane damage in gastric cells with loss of LDH into the medium.

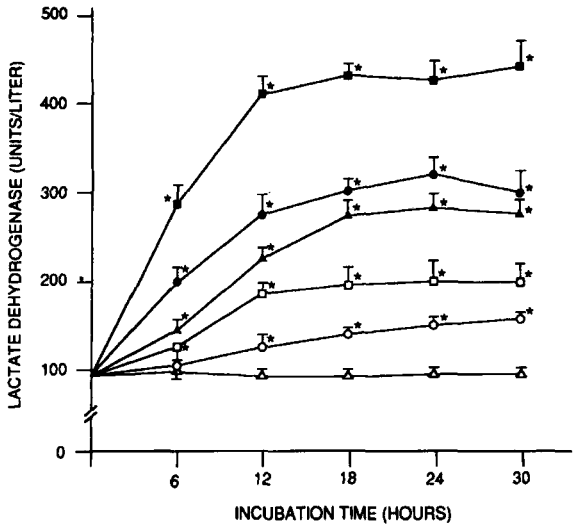

FIGURE 1 Concentration- and time-dependent effects of *H. pylori* (ATCC 43504) on gastric cells. Human gastric CRL 1739 cells (25×10^4 cells/35 mm petri dish) in 2 ml of Ham's F12 medium were incubated for 2 hrs to allow cell adherence, and individually incubated with the following ratios of *H. pylori*: 1:0 (△—△), 1:0.1(○—○), 1:1(□—□), 1:5 (▲—▲), 1:10 (●—●) or 1:100 (■—■). The cells were incubated at 37°C in an atmosphere of 5% CO_2. Media were collected from the cultures after 0, 6, 12, 18, 24 or 30 hrs of incubation, and assayed for lactate dehydrogenase (LDH) activity. Each value represents the mean ± S.D. of six experiments *P<0.05 with respect to the corresponding control group.

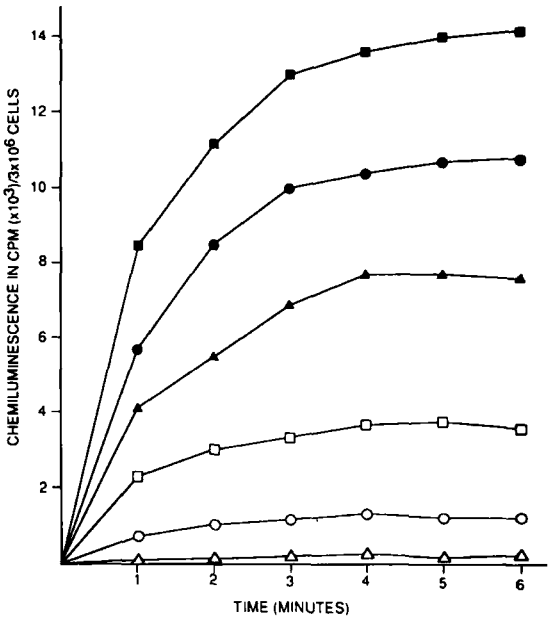

FIGURE 2 Production of chemiluminescence by gastric CRL 1739 cells (3×10^6 cells/ml) incubated *in vitro* at 37°C with three different ratios of *H. pylori* (ATCC 43504), 1:1, 1:5 and 1:10. Luminol (4 μM) was added to each sample to enhance chemiluminescence. Buffer (△—△); buffer + Ham's F12 medium (○—○); buffer + Ham's F12 medium + gastric cells (□—□) buffer + Ham's F12 medium + gastric cells + *H. Pylori* (1:1) (▲—▲); buffer + Ham's F12 medium + gastric cells + *H. pylori* (1:5) (●—●); and buffer + Ham's F12 medium + gastric cells + *H. pylori* (1:10 (■—■). The data are representative of 4–6 different experiments.

The results in Table 1 clearly demonstrate that selected oxygen free radical scavengers can ameliorate *H. pylori*-induced cytotoxicity in gastric cells. Combination of SOD and catalase partially attenuated *H. pylori*-induced LDH leakage from gastric cells, which indicates that superoxide anion and hydrogen peroxide may be involved. Furthermore, the use of the free radical scavengers mannitol and allopurinol suggest that hydroxyl radical and other oxygen free radicals may be involved in *H. pylori*-induced cytotoxicity in gastric cells.

These data provide a rational explanation for the *H. pylori*-induced cell damage to gastric cells. Histamine H_2-receptor antagonists served as better chemoprotectants based on LDH leakage from gastric cells in association with *H. pylori* (Table 2) as compared to the use of combined oxygen free radical scavengers. Cimetidine and other H_2-receptor antagonists have been identified as potent hydroxyl radical scavengers,[31] which can account for the chemoprotective activity. As noted above, H_2-receptor antagonists do not reduce LDH leakage from gastric cells by a direct growth inhibitory effect on *H. pylori*. The results suggest that *H. pylori* induced oxidative stress in gastric cells may act as a major pathway in gastric injury, although other pathways may also be involved. However, based on these and other studies, it is not clear whether reactive oxygen species play a primary or secondary role in gastric injury. The ability of free radical scavengers to partially inhibit cell death does not confirm a role for free radicals in the process, but does provide supporting evidence.[32,33]

The production of reactive oxygen species by

gastric cells in association with *H. pylori* was assessed by measuring enhanced chemiluminescence (Figure 2) and the reduction of cytochrome *c* and NBT (Table 3). Cytochrome *c* and NBT reduction are relatively specific tests for superoxide anion production,[22,34] while chemiluminescence is a general assay for the production of reactive oxygen species.[35] The three assays clearly indicate that reactive oxygen species are produced by the association of *H. pylori* cells with gastric cells. The abilities of oxygen free radical scavengers including SOD and catalase to inhibit *H. pylori*-induced cytotoxicity in gastric cells strongly suggests a role for reactive oxygen species and free radicals in this response, and provides information regarding the mechanism of *H. pylori*-induced cellular injury.

In summary, an *in vitro* cell culture system was used to assess production of reactive oxygen species and cell damage of gastric cells by *H. pylori*. Recognizing that the results of this *in vitro* system can not be directly extrapolated to the interaction of the same cells *in vivo*, the findings suggest that *H. pylori*-induced oxidative stress in gastric cells may act as a major pathway in gastric injury, although other pathways may also be involved.

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