Helicobacter pylori-infected human antral primary cell cultures: effect on gastrin cell function

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Richter-Dahlfors, Agneta, Ursula Heczko, R. Mark Meloche, B. Brett Finlay, and Alison M. J. Buchan. Helicobacter pylori-infected human antral primary cell cultures: effect on gastrin cell function. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G393–G401, 1998.—Although Helicobacter pylori infection increases gastrin secretion, it is unknown whether this is a direct effect or requires activation of the immune system. We developed an H. pylori-infected human primary antral epithelial cell culture model to address this question. This culture protocol favors growth of H. pylori, and infected cultures could be maintained for up to 48 h. These cultures were enriched for gastrin (10–40%), somatostatin (2–5%), and gastric mucin (60–80%) cells but did not contain immunocytes. Bacterial attachment occurred in a random manner within 2 h of infection, although bacterial density was lower than in sections from infected patients. After 24 or 48 h, the bacterial microcolonies were similar in size to those seen in vivo, and at 24 h ultrastructural studies demonstrated well-developed pedestal formation underlying the bacteria. Coculture with H. pylori increased basal but not stimulated gastrin secretion at all time points >2 h. In conclusion, a newly developed cell culture model has been used to characterize the interactions between H. pylori and normal human antral epithelial cells.

Ultrastructure; bacterial pedestals; bacterial attachment; bombesin

The microaerophilic bacterium Helicobacter pylori is established as the leading cause of duodenal ulcers and has been indicated as a risk factor for gastric carcinoma (11, 14). The dose association between H. pylori infection, increased acid production, and duodenal ulcers is well established, and the mechanism underlying this association is thought to involve alterations in the release of the antral hormone gastrin (2, 9, 20).

Gastrin directly increases acid secretion from the parietal cell mass and potentiates the effect of both histamine and acetylcholine. Thus an increase in gastrin release will result in increased acid production with the attendant risk of mucosal ulceration. The regulation of gastrin release from antral G cells has been investigated in many species, with rat, dog, pig, and human being the most extensively studied (4). In humans, the primary regulators of G cell function are the neuropeptide gastrin releasing peptide (GRP) or its homologue bombesin (BN), epinephrine, luminal lumen contents (protein, calcium, and pH), and somatostatin (SS). Of these, SS and low gastric pH are the major inhibitory components.

H. pylori increases both basal and GRP- or BN-stimulated gastrin release and increases the total amount of gastrin present in antral biopsies (2, 7, 20). There is increasing evidence both in vivo and from isolated cell culture models that, although bacterial products such as lipopolysaccharide (LPS) can directly modify cell function, cytokines secreted from activated epithelial lymphocytes play a significant role in the end response to bacterial infection. The precise contribution of attached bacteria to the alterations observed in normal gastric epithelial cell function has yet to be determined because of the lack of a suitable model system.

The way in which H. pylori attaches to gastric epithelial cells remains controversial. Some studies indicate the bacterium causes rearrangement of the apical region of the cells to induce pedestal formation similar to that observed with the enteropathogenic form of Escherichia coli (6, 18). However, other groups have reported no evidence of pedestal formation or actin rearrangement (19). In addition, whether or not the bacterium becomes internalized into epithelial cells is debatable. In vivo, the vast majority of the bacteria remain at the apical region of the epithelial cells or in the mucin layer. In cultured cell models (AGS and HEP-2), significant internalization of the bacterium has been observed (6, 18). Whether this is representative of the response of normal gastric cells to bacterial attachment is unknown.

In most cases the models used to date to examine the interaction of H. pylori with epithelial cells have depended on human cell lines such as MKN 28, AGS, KATO III, or HEP-2 that differ significantly from normal gastric mucosa. An additional problem is generated by the fact that there is no antral cell line available that secretes gastrin. Studies of the effect of cytokines associated with H. pylori infection on endocrine secretion have relied on the use of primary cells isolated from canine or rabbit origin (1, 12, 21). The problem with this is that in vivo the organism does not colonize the stomach of either species; therefore, their use as models is not optimal.

The purpose of the present study was to develop a model of H. pylori-infected normal human antral epithelial cells enriched for gastrin-producing G cells. The model was used to examine the interaction between H. pylori and normal antral epithelial cells by light and electron microscopy and to investigate whether the bacterium alters gastrin cell function in the absence of macrophages or lymphocytes. Primary antral cell cul-

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turer were infected with the bacterium and maintained for up to 48 h. Infected cells at selected time points were fixed for light and electron microscopy. Control and infected cells were stimulated with either BN or calcium to determine whether H. pylori increased basal or stimulated gastrin release and whether this effect was stimulus specific.

MATERIALS AND METHODS

Growth Conditions of H. pylori

Wild-type H. pylori strain CCUG 17874 (8) was kept as 1 ml frozen aliquots at −80°C. Before experiments, the bacteria were thawed and used to inoculate 10 ml Brucella medium (DIFCO) supplemented with 10% FCS (GIBCO BRL), 1% IsoVitalex (Becton Dickinson, Cockeysville, MD), nalidixic acid (20 µg/ml, Sigma), vancomycin (10 µg/ml, Sigma), and polymyxin B sulfate (25 U/ml, Sigma). The cultures were incubated under shaking (150 rpm), microaerophilic conditions in a CampyPak jar (Becton Dickinson) at 37°C for 48 h. Bacteria were harvested (10,000 g, 20 min) and resuspended in fresh Brucella medium containing 10% FCS but no other additives, and the optical density at 600 nm was adjusted to 0.5. From this bacterial suspension, 10 µl per well were used as inoculum. To determine the exact number of bacteria in the inoculums, 0.1 ml of serial dilutions was plated on Brucella plates supplemented with 10% FCS, vancomycin (10 µg/ml), and polymyxin B (25 U/ml). After 3 days of incubation at 37°C under microaerophilic conditions, the colonies were counted. The size of the inoculum was 1 x 10^6 colony-forming units (cfu).

Cell Isolation

Human antrum was obtained from 11 multiple organ donors in association with the British Columbia Transplant Society. There were seven males and four females; the average age of the males was 26 yr and that of the females was 32 yr. Before digestion of the antral mucosa, a 0.25 cm strip was separated and fixed in 4% paraformaldehyde for 2 h to check for prior infection by H. pylori. A single-cell suspension of mucosal cells was prepared and separated by centrifugal elutriation as previously described (3). The F1 fraction containing the majority of the gastrin cells was used in subsequent experiments.

Cell Culture

Cells in the F1 fraction were resuspended at 0.5 x 10^6 cells/ml in growth medium comprising 50:50 DMEM/Ham's F-10, 1.0 mmol/l Ca^2+, 8 µg/ml insulin, 50 µg/ml hydrocortisone, 5% FCS, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 10 µg/ml gentamycin. The DMEM/Ham's F-10 was used to reduce the extracellular calcium concentration to 1 mmol/l rather than 1.8 mmol/l in DMEM alone. The cells were plated at 1 ml/well on 12-well Costar plates (Costar Data Packaging, Cambridge, MA), either untreated or coated with either rat tail collagen, laminin, fibronectin, or 3-aminopropyltriethoxysilane (APES) and maintained in a 5% CO_2 incubator at 37°C for 2 days before inoculation with the bacteria.

Before inoculation, the plates were washed in prewarmed (37°C) PBS (pH 7.4) to remove any nonadherent cells, and 1 ml of growth medium was added (same composition as the original medium with the addition of 10 µg/ml vancomycin rather than penicillin, streptomycin, and gentamycin). Approximately 1 x 10^8 cfu (10 µl) of H. pylori were added to each well, except for the control wells, to which the equivalent volume of Brucella medium (including 10% FCS) was added. From this point onward, the plates were maintained in 10% CO_2 at 37°C, to provide suitable growth conditions for H. pylori. At 2, 8, 24, or 48 h postinfection, plates were removed for release experiments or fixed for immunocytochemistry and electron microscopy.

Morphological Studies

Light microscopy. The cultured cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed twice in PBS, then incubated in 10% normal goat serum (NGS; GIBCO BRL) in PBS for 10 min before immunostaining. The composition of the revised cell cultures was determined by immunostaining with a panel of cell-specific antibodies (see Table 1). In all cases, the antibodies were incubated on the coverslips overnight at 4°C. For details of secondary antibodies and dilutions, see Table 1. The bound antibodies were localized using the avidin-biotin method (Vector ABC Elite kit) diluted 1:1,000 in PBS, and the peroxidase reaction was developed with diaminobenzidine as the chromogen with 0.1% nickel sulfate and 0.03% H_2O_2 in 0.5 M Tris buffer, pH 7.6, to give a black end product. After counterstaining with hematoxylin to stain the nuclei blue, the percentage of positive cells was calculated by counting the immunostained cells and the total number of cells (nuclei) in five random fields at x20 magnification using a Zeiss Axiovert microscope (n = 5).

To examine the location of bacteria in the cultures, an H. pylori antiserum (gift from Dr. T. J. Trust, Astra Pharmaceuticals, Cambridge, MA) was diluted to 1:250 in PBS with 0.2% saponin and 10% NGS and incubated on the cells overnight at 4°C. Bound antibodies were detected using biotin-conjugated goat-anti rabbit IgG at 1:1,000 with the avidin-biotin method (Vector ABC Elite kit), and the peroxidase reaction was developed with diaminobenzidine as the chromogen with 0.1% nickel sulfate and 0.03% H_2O_2 in 0.5 M Tris buffer, pH 7.6, to give a black end product. Once the staining for H. pylori was completed, the cells were double stained with monoclonal antibodies to gastrin (9303, provided by Center for Ulcer Research and Education/Gastroenteric Biology Center Antibody/RIA Core, National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-

All secondary antibodies were from Jackson Laboratories, West Grove, PA. PGP, protein gene product; UBC, University of British Columbia. *Serotec is in Kidlington, Oxford, UK.

Table 1. Details of the antibodies used to characterize the cell cultures

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Cell Type</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrin</td>
<td>Monoclonal Dr. J. Walsh</td>
<td>G</td>
<td>1,000</td>
<td>Goat anti-mouse</td>
<td>1,000</td>
</tr>
<tr>
<td>Gastric mucin</td>
<td>Monoclonal Sigma</td>
<td>Mucous</td>
<td>1,200</td>
<td>Goat anti-mouse</td>
<td>1,000</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Monoclonal Sigma</td>
<td>D</td>
<td>1,400</td>
<td>Goat anti-mouse</td>
<td>1,200</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Rabbit Sigma</td>
<td>Fibroblasts</td>
<td>1,100</td>
<td>Donkey anti-rabbit</td>
<td>1,100</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Monoclonal Serotec</td>
<td>Endothelial</td>
<td>1,500</td>
<td>Goat anti-mouse</td>
<td>1,100</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Rabbit</td>
<td>Neurons</td>
<td>1,200</td>
<td>Donkey anti-rabbit</td>
<td>1,200</td>
</tr>
</tbody>
</table>
The cells were incubated in the monoclonal antibody diluted 1:100 in PBS with 0.1% Triton X-100 (PBST) overnight at 4°C. After washing in PBST to remove unbound antibodies, the cells were incubated in peroxidase-conjugated donkey anti-mouse IgG, and the peroxidase reaction was developed with diaminobenzidine as the chromogen with 0.03% H2O2 in 0.5 M Tris buffer, pH 7.6, to give a brown end product.

To compare the density of H. pylori attached to the cultured cells with bacterial colonization in vivo, sections of antral biopsies from infected patients were obtained from the Pathology Department at the University of British Columbia. The sections were immunostained using the rabbit antiserum to H. pylori localized by the avidin-biotin method outlined above. At the same time, sections of the antrum taken before production of the primary cell cultures were stained to determine whether any of the individuals were infected with the bacterium.

Electron microscopy. The cultured cells were fixed in 2% glutaraldehyde for 30 min, dissociated from the wells using a plastic spatula, washed, and postfixed in 1% osmium tetroxide. The fixed cells were pelleted, dehydrated, and embedded in Epon at 60°C. Sections were cut at 80 nm, collected on 300-mesh copper grids, and counterstained with lead citrate and uranyl acetate before screening using a Zeiss 10 C/CR electron microscope.

Release Studies

Initial studies determined whether removing the rat tail collagen substrate or maintaining the cells for 4 days in culture altered basal or stimulated gastrin release. The wells were washed twice in release medium (Ham's F-10 with 5.5 mmol/l glucose, 0.1% bovine serum albumin, 1.0 mmol/l Ca2+) to remove any nonadherent cells and bacteria. After removal of nonadherent cells, duplicate wells were incubated in one of the following: control solution (975 µl release medium at 37°C plus 25 µl at 4°C), 10 nmol/l BN solution (975 µl release medium at 37°C plus 25 µl of 0.4 µmol/l BN at 4°C), or 1 ml release medium with 3.5 mmol/l Ca2+ at 37°C. To determine whether H. pylori infection altered either basal or stimulated gastrin cell secretion, plates were stimulated after 2, 6, 8, 24, or 48 h of infection using the same conditions. In all cases, after a 60-min incubation in 5% (initial studies without H. pylori) or 10% CO2 at 37°C, the medium was aspirated and centrifuged for 2 min to remove particulate matter. The supernatant was stored at −20°C until the gastrin content was determined by RIA.

The cells from control wells were extracted in boiling dH2O for 10 min and centrifuged to remove cell debris, and the supernatant was stored at −20°C. The data for the gastrin release were expressed as a percentage of the control gastrin release in the absence of bacteria for each cell preparation and presented as means ± SE. Statistical significance was determined using an ANOVA followed by unpaired Student's t-test; values of P < 0.05 were considered significant. The n values refer to the number of individual primary cell cultures used for each experiment.

RIA

The RIA for gastrin was performed using the CKG-2 polyclonal antibody as previously described (3). The assay detects human gastrin-17 and has a lower sensitivity limit of 5 fmol/l. Each sample was assayed in duplicate. The inter- and intra-assay variations were 10 and 5%, respectively.

RESULTS

Development of the Cell Culture Model

To provide a suitable growth environment for H. pylori, the culture conditions for the primary cells were modified. Two major changes were required. First, the usual rat tail collagen substrate was omitted because preliminary studies indicated that the bacteria attached to both the cultured cells and collagen. Substitution of alternative substrates, including fibronectin, laminin, and APES, also resulted in significant attachment of bacteria to the wells; therefore, the plates were left untreated. Second, because H. pylori is a microaerophilic bacterium, the CO2 concentration was increased from 5 to 10%. This resulted in a decrease in the pH of the medium from pH 7.2 to pH 6.9 that was maintained for the length of the culture period. To ensure that the plates were not contaminated by other bacteria, Brucella plates inoculated with samples taken after 6, 8, 24, and 48 h were screened and confirmed that H. pylori were the only bacteria in the cultures. If a contaminating bacterium was identified, the plates involved were discarded.

The modifications to the culture protocol did not alter the viability of the primary cell cultures. Trypan blue exclusion studies completed at each time point confirmed that >95% of the cells were viable. The attached cells reaggregated into clusters of flattened cells, and the G cell's repolarized, with the majority of the immunoreactive gastrin product concentrated to one pole of the cell. The initial studies were completed using cells plated at the normal density of 2 × 106/ml. However, this resulted in an almost confluent layer of cells and made distinguishing bacterial attachment sites difficult. In subsequent studies the plating density was reduced to 0.5 × 106/ml to provide smaller cell clusters. At the completion of the release studies, the viability of the attached cells was monitored by exclusion of trypan blue; both control (no added bacteria) and infected cultures maintained a >95% viability. The ultrastructural studies confirmed the viability of the cells, with very few necrotic (condensed nuclei) or apoptotic (fragmented nuclei) cells being observed.

Studies undertaken to characterize the newly developed culture preparation demonstrated that removal of the rat tail collagen substrate had no effect on total gastrin cell content after 2 days in culture: with collagen, 14,954 ± 689 pg/well; without collagen, 13,608 ± 459 pg/well (n = 3). In addition, there was no significant change in basal secretion or BN- and Ca2+-stimulated gastrin release after 2 days (Fig. 1). To ensure that increasing the length of the culture period from 2 to 5 days did not deplete gastrin cell content, the total gastrin cell content per well of the cultured cells was determined and compared between days 2, 3, 4, and 5 (Table 2). These data demonstrated that the amount of gastrin present in the G cells in the antral cell cultures was unaffected either by removing the collagen substrate or by increasing the length of the culture period during the H. pylori experiments.
Morphological Studies

The composition of the newly developed cell cultures was not significantly altered by reducing the cell seeding density or removing the collagen substrate. However, in the wells seeded with $0.5 \times 10^6$ cells/ml, the number of cells attaching to the plastic varied between preparations (see Table 3). The level of attachment did not correlate with age or sex of the organ donor and most probably resulted from the reduced cell density. The vast majority of cells attached to the plates were gastric mucous cells identified by immunostaining with the gastric mucin cell-specific antibody. The second most abundant cell type was gastrin immunoreactive, varying from 10 to 40% of the attached cells. SS-immunoreactive cells comprised <5% of the attached cells. Fibronectin-immunoreactive fibroblasts were present but at most comprised <0.02% of attached cells, and their number did not increase in the 4- or 5-day cultures. No immunoreactivity was detected with the antibodies to factor VIII or protein gene product (PGP) 9.5, indicating the absence of endothelial and nerve cells. This was not the result of lack of detection of the respective antigens in human material because both antisera stained cells in full-thickness sections of antral mucosa (data not shown). The percentages of mucin, gastrin, and SS cells in the six culture preparations used for the final H. pylori studies are shown in Table 3.

Cultures immunostained with the H. pylori antibody at different time points demonstrated an increase in bacterial numbers with time. The bacteria attached to the cells in the first 2 h and then increased in numbers around the initial attachment sites (Fig. 2). In many cases, the bacteria attached along the outside edge of the clustered cells, although cells in the center of the clusters also showed attached bacteria. At later time points, cells with bacteria attached were often obscured by the expanding microcolonies (Fig. 2D), indicating that active bacterial replication was occurring.

Double immunostaining of the infected cultures with the gastrin antibody demonstrated that while some gastrin cells were associated with attached bacteria, the majority of the cells with attached bacteria were not gastrin immunoreactive (Fig. 2). The bacteria attached to identified G cells were usually associated with the presumptive apical region of the cell and were not concentrated in the region with the strongest gastrin immunoreactivity (Fig. 2C). In the 48-h cultures, a small number of G cells were completely covered by bacteria (<2%).

In sections of antral mucosa from H. pylori-infected patients, although the density of immunoreactive bacteria was greatest at the luminal surface, significant numbers of bacteria were observed in the gastric pits in the region occupied by the gastric endocrine cells (Fig. 3). The number of bacteria attached in the gastric pit region was similar to that observed in the 24- and 48-h cultures. The immunostaining of sections from the antra used to provide the primary cell cultures with the antibody to H. pylori failed to demonstrate the presence of bacteria (data not shown). These results confirmed that the organ donors did not have a prior H. pylori infection. This was particularly relevant in the cell preparation obtained from donor 5, where the gastrin content was >100,000 pg/well. Sections of the antral mucosa from this individual showed a higher than normal number of intensely stained G cells, but no bacteria were observed nor was there evidence of infiltration of the mucosa with inflammatory cells.

The ultrastructural studies showed that the bacteria were directly attached to the cultured cells, with the attachment sites at 2 h resembling small pedestals (Fig. 4A). At 2 h, a single attachment site was observed on the majority of cells, with few cells demonstrating additional pedestals. After 24 h, although an increase in the number of cells with attachment sites was not

Table 2. Gastrin cell content in the cultures

<table>
<thead>
<tr>
<th>Donor</th>
<th>Day 2</th>
<th>6h</th>
<th>8h</th>
<th>24h</th>
<th>48h</th>
</tr>
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<tr>
<td>1</td>
<td>37,600</td>
<td>38,650</td>
<td>37,275</td>
<td>40,300</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>2,930</td>
<td>2,800</td>
<td>2,680</td>
<td>3,870</td>
<td>3,980</td>
</tr>
<tr>
<td>3</td>
<td>5,325</td>
<td>5,775</td>
<td>5,637</td>
<td>6,453</td>
<td>5,942</td>
</tr>
<tr>
<td>4</td>
<td>17,500</td>
<td>18,289</td>
<td>23,158</td>
<td>23,059</td>
<td>cont.</td>
</tr>
<tr>
<td>5</td>
<td>108,750</td>
<td>116,233</td>
<td>108,350</td>
<td>109,475</td>
<td>111,701</td>
</tr>
<tr>
<td>6</td>
<td>16,500</td>
<td>17,525</td>
<td>15,900</td>
<td>19,775</td>
<td>22,825</td>
</tr>
</tbody>
</table>

Values are in picograms per well. ND, not determined; cont., contaminated.

Table 3. Composition of the new cell preparation

<table>
<thead>
<tr>
<th>Donor</th>
<th>% Mucin Cells</th>
<th>% G Cells</th>
<th>% SS Cells</th>
<th>Total Cells/mm²</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>15</td>
<td>2.1</td>
<td>1,225</td>
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<tr>
<td>2</td>
<td>87</td>
<td>9.5</td>
<td>1.2</td>
<td>785</td>
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<tr>
<td>3</td>
<td>85</td>
<td>11</td>
<td>1.4</td>
<td>1,374</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>25</td>
<td>1.9</td>
<td>1,230</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>36</td>
<td>2.1</td>
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<tr>
<td>6</td>
<td>75</td>
<td>22</td>
<td>1.6</td>
<td>940</td>
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</table>

SS, somatostatin.
Gastrin Release Experiments

Initial studies demonstrated that no changes in basal or stimulated gastrin levels were observed with a 2-h incubation time in the presence of H. pylori (data not shown). Subsequent studies increased the shortest exposure time with H. pylori to 6 h. Due to significant variations in the total gastrin cell content between the six individual culture preparations (see Table 2), the data were expressed as a percentage of the gastrin released from the control cells for each experiment. The control was defined as the amount of gastrin released into the medium from the uninfected cells either unstimulated or in the presence of BN or Ca²⁺.

After 6 h, unstimulated gastrin release was 361 ± 261 pg/well (100%) in the absence of bacteria and 156 ± 14% in the presence of H. pylori (n = 6, P < 0.05). Although basal secretion was increased after 6 h, there was no significant effect on either BN- or Ca²⁺-stimulated gastrin release (n = 6, NS, Fig. 5). At the 8-h time point, gastrin released from unstimulated wells in the absence of H. pylori was 321 ± 245 pg/well (100%); the addition of the bacterium increased gastrin release, although to a lesser extent at 118 ± 10% (n = 6, P < 0.05). At this time point there was a significant reduction in BN-stimulated gastrin release in the presence of the bacteria. BN alone resulted in the release of 2,325 ± 989 pg/well (100%), which was reduced to 90 ± 4% in the presence of H. pylori (n = 6, P < 0.05). There was no effect on Ca²⁺-stimulated release (Fig. 5).

After 24 h, unstimulated gastrin release was 379 ± 205 pg/well (100%), and addition of H. pylori stimulated gastrin release to 149 ± 14% (n = 6, P < 0.05). At this time point there was no effect on BN-stimulated gastrin release, but Ca²⁺-stimulated release was reduced to 88 ± 6% (n = 6, P < 0.05). Of the four experiments in which plates were kept for 48 h with or without H. pylori, one had to be discarded because it was overgrown by contaminating bacteria. Unstimulated gastrin release in the absence of bacteria was 370 ± 221 pg/well (100%), and addition of H. pylori increased secretion to 122 ± 11% of control levels (n = 4, P < 0.05). No effect on either BN- or Ca²⁺-stimulated gastrin release was observed (Fig. 5).

DISCUSSION

We have developed a primary cell culture model of human antral epithelial cells infected with H. pylori for...
up to 48 h that can be used to determine the interaction of the bacteria with normal gastric cells. To develop these cultures, several parameters of the previously described antral cell culture model had to be altered. To avoid activation of the calcium-sensing receptor on the G cells (16), the concentration of calcium in the growth medium was reduced to 1.0 mmol/l. The culture conditions were altered to maintain viability of H. pylori and minimize attachment of the bacteria to noncellular elements in the cultures. Preliminary studies demonstrated that H. pylori attached to any substance used to coat the culture plates; therefore, the plates were left uncoated. This did not affect cell attachment and resulted in an increased viability of the culture preparation.

Fig. 3. Section of human antral mucosa immunostained using the H. pylori antibody and counterstained with hematoxylin. Note that although majority of bacteria are concentrated in the upper layer of the mucosa, gastric pits (arrow) contain a significant number of bacteria.

Fig. 4. A: typical attachment site following 2-h infection with H. pylori. Cocciid bacterium sits in a small cuplike structure on the cell surface. B: spherical bacteria were also attached to the cells at 2 h. Note that attachment site is at apical region of the cell close to the microvilli and the tight junction zone between 2 adjacent cells (arrow). C: at 24 h bacterial microcolonies were present, attached to apical region of the cells. Attachment pedestals formed clear protrusions above the cell surface, and no microvilli are present. D: microcolonies appear to favor the region around the apical microvilli, and at the attachment sites the normal microvilli (arrow) are lacking. E: in many cells, microcolonies were observed above large intracellular vacuoles.
antral sections. However, at 24 and 48 h, the microcolonies were either unstimulated or in the presence of either BN or Ca2+ is represented by line at 100%. Gastrin secretion in the infected wells was calculated as a percentage of the corresponding control level; n = 6 except for 48 h where n = 4, *P < 0.05.

Fig. 5. Effect of H. pylori infection on basal, BN- and Ca2+-stimulated gastrin release. Gastrin release from cells in wells uninfected by H. pylori either unstimulated or in the presence of either BN or Ca2+ is always close to the attachment site of H. pylori most probably reflects an insufficient exposure time to the bacterium. It has previously been reported that after attachment to a cell the spiral form of H. pylori converts to the coccoid form (18). In the present study, after 24 h, the majority of the bacteria attached to the gastric cells were in the coccoid form; the spiral bacteria were most often associated with the microvilli and not in direct contact with the underlying cells. These results would support the conversion of spiral to coccoid forms after cell attachment.

In cell lines, significant levels of internalization of the bacterium after attachment have been reported (6, 18). The attachment sites seen on AGS cells before internalization extend upwards, encircling the bacterium (18). Similar attachment sites were not observed in the present study, and no evidence of internalization of the bacteria was obtained. In vivo, internalized bacteria are rarely observed, suggesting that the extensive internalization observed with cell lines may not reflect the normal behavior of gastric epithelial cells.

Studies of primary human jejunal epithelial cells infected with H. pylori demonstrated significant vacuolation of the cytoplasm by bacteria expressing the vacuolating toxin VacA (10). We observed extensive cell vacuolation in a significant proportion of the cells with attached bacteria. After 24-h infection, the vacuoles were localized to the apical pole of the cells and were always close to the attachment site of H. pylori microcolonies. These results indicate that the VacA-positive wild-type H. pylori strain used in the present study secreted sufficient toxin to alter cell structure in the associated cells, although a similar level of vacuolation was not observed in surrounding cells lacking bacterial microcolonies.

Infection of antral epithelial cell cultures with H. pylori resulted in a small but significant increase in basal gastrin release at time points >2 h. The increased secretion did not reflect increased basal activity in the G cells cultured beyond the usual 2 days, because the gastrin levels secreted into the medium from the uninfected cultures did not increase with time. The absence of evidence indicating that H. pylori infection altered the integrity of the G cells in the trypan blue exclusion and ultrastructural studies would suggest that the increase in basal secretion does not reflect leakage from damaged cells. Furthermore, basal gastrin secretion from the H. pylori-infected cultures did not increase between 6 and 48 h postinfection, indicating that the large bacterial microcolonies had no further effect on G cell function. If decreased G cell integrity was the underlying cause for the elevated basal secretion, further increases should have been observed in the presence of additional bacteria. However, we cannot discount the possibility that the small increase in basal
gastrin release resulted from a nonspecific effect on the cultured epithelial cells. Due to the low seeding density used for these studies, we were unable to detect SS in the medium and could not monitor changes in secretion levels to establish whether the effects of H. pylori were specific to the G cells.

The lack of correlation between the increasing size of H. pylori microcolonies and basal gastrin release suggested that H. pylori was not directly stimulating the G cells. An alternative explanation would be that the increased basal secretion was the result of the interference of bacterial LPS with the binding of the inhibitory peptide, SS, to its cell surface receptor.

In studies of the effect of H. pylori LPS on rat gastric mucosal SS receptors, increasing LPS concentrations were correlated with a decrease in binding of radiolabeled SS to the receptors (15). In our cultures, the low level of SS secreted by the <5% cocultured D cells at pH 7.2 results in an inhibition of basal gastrin release (3). In previous experiments, the pH of the culture medium has been maintained at pH 7.6 to reduce SS levels and remove the inhibitory effect. In the present studies, the pH of the medium was 6.9; therefore, the levels of SS in the medium, although below the level of detection by the available radioimmunoassay, could be sufficient to decrease gastrin release. If H. pylori LPS blocks the ability of SS to bind to the SS receptors on the G cell membrane, this would remove the inhibitory effect of SS in the medium, resulting in a small increase in basal secretion. However, because the SS levels are low once this effect has been neutralized, no additional increase in basal secretion would be observed.

Interestingly, incubation of the G cell–enriched preparation with H. pylori for up to 48 h did not increase BN-stimulated gastrin release. These data suggest that the stimulatory effect seen in vivo is not generated by bacterial attachment. It has previously been demonstrated that cytokines such as tumor necrosis factor-α and interleukins increase gastrin secretion from isolated G cells after exposure for as little as 2 h (1, 12, 21). In the antral epithelial cell cultures, no lymphocytes were present; therefore, the secretion of cytokines will be minimal. Although H. pylori has been shown to initiate interleukin-8 secretion from gastric epithelial cells, this cytokine has not been linked to an increase in gastrin secretion at least in cultured rabbit G cells (21). Bacterial attachment alone does not appear to influence agonist-stimulated gastrin secretion; therefore it is probable that the effect of H. pylori on BN-stimulated gastrin secretion is secondary to activation of the immune system.

We have previously demonstrated that extracellular calcium levels stimulate gastrin release by activation of the calcium-sensing receptor (16). To determine whether the reported increase in BN-stimulated gastrin release was agonist specific, we examined the effect of bacterial infection on gastrin release in response to increasing extracellular calcium levels from 1.0 to 3.5 mmol/L. No further increase in calcium-stimulated gastrin release was observed during these studies.

In conclusion, we have developed an H. pylori-infected primary culture model of human antral epithelial cells that can be used to monitor the effect of the bacteria on epithelial cells without the confounding influence of factors secreted from immunocytes. The morphological studies indicated that the culture preparation appeared to closely duplicate the situation present in the epithelium of the gastric glands in infected patients. The ability to infect primary cultures with H. pylori and maintain these for up to 48 h without contamination by other microorganisms provides an ideal model to examine the interaction of H. pylori with normal gastric epithelial cells. Finally, although H. pylori infection modestly increased basal gastrin release, BN-stimulated release was not affected, suggesting that the latter is increased in vivo by other agents such as factors secreted by the activated immunocytes.

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