Infection with the gastric pathogen *Helicobacter pylori* causes chronic active gastritis and peptic ulcer disease (35). In addition, *H. pylori* infection has been associated epidemiologically with the development of gastric cancers, including adenocarcinoma (14) and lymphoma (31). The mechanisms by which *H. pylori* mediates these host responses, however, remain unknown.

Aptosis is a genetically programmed form of cell death characterized by distinct morphologic and molecular features (32). Programmed cell death plays an important role in the regulation of epithelial cell numbers in the gastrointestinal tract (15). In addition, deregulation of the apoptotic pathway is characterized by distinct morphologic and molecular features (28). Under physiologic conditions, the Fas system is involved in regulating the immune response by eliminating activated lymphocytes (23). Virus-infected cells are also eliminated through Fas-Fas-ligand interactions (1). In addition, present evidence links excessive activity of the Fas system with the pathogenic effects associated with infection by certain microbes. For example, the lymphocyte depletion observed in patients infected with the human immunodeficiency virus appears to be Fas mediated (3, 19).

Alterations in the gastric epithelial cell cycle, including both enhanced proliferation and increased apoptosis of gastric cells, are identified during infection with *H. pylori* (4, 27). These changes in cell turnover are present in both *H. pylori*-infected children (16) and adults (25). Investigations of the molecular determinants mediating apoptosis have identified both enhanced expression of the tumor suppressor p53 (16) and increased expression of the proapoptotic protein Bak in response to *H. pylori* infection (5). Among *H. pylori*-infected children, gastric epithelial cell apoptosis returns to baseline levels only following both eradication of the bacterium and resolution of the accompanying gastritis (16). These findings suggest a role for immune-mediated apoptosis of gastric epithelial cells during *H. pylori* infection. Therefore, the aims of this study were to determine if *H. pylori* can directly stimulate programmed cell death of gastric epithelial cells and to characterize the role of Fas-Fas ligand signaling in this cell death cascade.

Infection with *H. pylori* directly induces apoptosis of gastric epithelial cells in vitro and to define the role of the Fas-Fas ligand signal transducing cascade, human gastric epithelial cells were infected with *H. pylori* for up to 72 h under microaerophilic conditions. As assessed by both transmission electron microscopy and fluorescence microscopy, incubation with a cagA-positive, cagE-positive, VacA-positive clinical *H. pylori* isolate stimulated an increase in apoptosis compared to the apoptosis of untreated AGS cells (16.0% ± 2.8% versus 5.9% ± 1.4%, *P* < 0.05) after 72 h. In contrast, apoptosis was not detected following infection with cagA-negative, cagE-negative, VacA-negative clinical isolates or a *Campylobacter jejuni* strain. In addition to stimulating apoptosis, infection with *H. pylori* enhanced Fas receptor expression in AGS cells to a degree comparable to that of treatment with a positive control, gamma interferon (12.5 ng/ml) (145% ± 2.4% and 167% ± 24% of control, respectively). The enhanced Fas receptor expression was associated with increased sensitivity to Fas-mediated cell death. Ligation of the Fas receptor with an agonistic monoclonal antibody resulted in an increase in apoptosis compared to the apoptosis of cells infected with the bacterium alone (38.5% ± 7.1% versus 16.0% ± 2.8%, *P* < 0.05). Incubation with neutralizing anti-Fas antibody did not prevent apoptosis of *H. pylori*-infected cells. Taken together, these findings demonstrate that the gastric pathogen *H. pylori* stimulates apoptosis of gastric epithelial cells in vitro in association with the enhanced expression of the Fas receptor. These data indicate a role for Fas-mediated signaling in the programmed cell death that occurs in response to *H. pylori* infection.

*H. pylori* Induces Gastric Epithelial Cell Apoptosis in Association with Increased Fas Receptor Expression

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Bacteria and growth conditions. *H. pylori* LC 11, a cagA-positive, cagE-positive, vacuolating cytotoxin (VacA)-producing *H. pylori* strain originally isolated from the stomach of a patient with peptic ulcer disease, and two cagE-negative, cagA-negative *H. pylori* strains, LC 3 and LC 20, were employed for these studies (13). The presence of cagE and cagE was determined by isolation of genomic DNA and PCR (24) with the primer pair GCTTACTGGTTGGGATTTG and GCTT GACTTTGCTTTC for cagE and both primer pair AGACATGCAAAG TAT and CACCTGTGGGTTGTA and primer pair TCTGCTAGCAT TAGAGA and TAGTCCTTATGTAGATG (kindly provided by Robin Beech, McGill University, Montreal, Quebec, Canada) for cagA. The presence of the vacuolating cytotoxin was determined by the method of Cover et al. (7). Concentrated broth supernatants were incubated with HEp-2 cells for 24 h at 37°C, and vacuolation was assessed by bright-field microscopy (24).

Results: Evaluation of apoptosis in *H. pylori*-infected gastric epithelial cells. To determine if infection with *H. pylori* alone could stimulate apoptosis of gastric epithelial cells in vitro, AGS and KATO III cells were incubated with the bacterium for up to 72 h. AGS cells infected with a cagA-positive, cagE-positive, VacA-positive isolate, strain LC 11, underwent apoptosis as assessed by fluorescence microscopy. As shown in Fig. 1, apoptotic cells displayed the characteristic features of reduced size, cytoplasmic vacuolization, and enhanced fluorescence of condensed and margined nuclear chromatin. These results were confirmed by transmission electron microscopy. Unlike untreated cells, AGS cells infected with strain LC 11 demonstrated the ultrastructural features which characterize the process of programmed cell death, including cytoplasmic vacuolation, condensed nuclear chromatin, and formation of apoptotic bodies (Fig. 2).

Quantitation of apoptotic AGS cells by fluorescence microscopy demonstrated that *H. pylori* LC 11-mediated cell death was time dependent. An increase in the death of gastric epithelial cells was observed following 72 h of infection with the bacterium (16.0% ± 2.8% versus 5.9% ± 1.4%, *P < 0.05*) (Fig. 3). When AGS cells were infected for 72 h with two clinical isolates which lack the putative virulence genes cagE and cagA and vacuolating cytotoxin activity, apoptosis of gastric epithelial cells was not detected (6.5% ± 1.3% versus 7% ± 2.1%). Similarly, infection with the related enteric pathogen *C. jejuni* did not induce apoptosis of gastric cells (5.9% ± 2.7% versus 6.4% ± 2.5%). In contrast to AGS cells, KATO III cells more readily underwent necrosis in response to infection with *H. pylori* strain LC 11 for 72 h; therefore, the remaining studies were performed with AGS cells.

Expression of the Fas receptor during *H. pylori* infection. To determine if AGS cells had a basal expression of Fas, which could be enhanced by IFN-γ, which is known to upregulate expression of the receptor in other cell lines (12), fluorescence microscopy with a monoclonal antibody to the Fas receptor was employed. As shown in Fig. 4, AGS cells had a low level of Fas expression which was enhanced following stimulation by IFN-γ (12.5 ng/ml).

Compared to uninfected (control) cells, infection with *H. pylori* LC 11 also enhanced expression of Fas as determined by enzyme-linked immunosorbent assay (Fig. 5). *H. pylori*-stimulated Fas receptor expression was comparable to that mediated by IFN-γ (148% ± 24% and 167% ± 24% of the control, respectively; *n = 3*). The *H. pylori*-mediated Fas expression was not a result of cross-reactivity with a bacterial product, since assessment of bacterial extracts alone showed no detectable Fas expression.

Sensitivity of *H. pylori*-infected cells to Fas-stimulated cell death. An agonistic monoclonal antibody to the Fas receptor, which stimulates Fas-sensitive cells to undergo apoptosis (12), was then employed to determine if the enhanced Fas expression was functional. As shown in Fig. 6, incubation of AGS cells with the anti-Fas antibody mediated an increase in apoptosis compared to the apoptosis of untreated cells (21.1% ± 1.8% versus 5.9% ± 1.4%). Furthermore, incubation of LC
11-infected gastric cells with the anti-Fas antibody resulted in a marked increase in programmed cell death compared to the death of cells infected with the bacterium alone (38.5% ± 7.1% versus 16.0% ± 5.5%, \( P < 0.05 \) [ANOVA]). Incubation of LC 11-infected AGS cells with a neutralizing anti-Fas receptor antibody did not prevent cell death (7.7% versus 9.9%), indicating that the induction of apoptosis observed following infection with the bacterium alone was not mediated by the enhanced Fas receptor expression.

**DISCUSSION**

In vivo studies demonstrate that infection with *H. pylori* triggers apoptosis of gastric epithelial cells (34). However, in this setting it is unclear whether immune factors or bacterial factors contribute to cell death. This study supports and extends recent evidence indicating that several mechanisms are involved in stimulating apoptosis of gastric epithelial cells during *H. pylori* infection (2, 33, 36).

These data demonstrate that *H. pylori* is capable of directly inducing the death of gastric epithelial cells in vitro in the absence of immune cells. The mechanism of cell death differed between the two gastric cell lines. KATO III cells underwent necrosis in response to prolonged infection with the bacterium, while AGS cells underwent apoptosis. The response of AGS cells to infection with the bacterium mimics the in vivo setting, indicating that the AGS cell line serves as a better model system for investigating these apoptotic pathways than the KATO III cell line.

**FIG. 1.** Identification of apoptotic cells in untreated AGS cells (A) and *H. pylori*-infected cells (B) after 72 h by acridine orange-ethidium bromide staining and fluorescence microscopy. (A) AGS cells demonstrate normal morphology. (B) *H. pylori*-infected AGS cells show morphologic features of apoptosis (arrow), including condensed and margined chromatin with apoptotic body formation. Approximate magnifications, ×1,000.

**FIG. 2.** Transmission electron photomicrographs of uninfected (A) and *H. pylori*-infected (B) AGS cells. (A) Control cells show normal cellular morphology. (B) *H. pylori*-infected AGS cells demonstrate the characteristic features of programmed cell death, including cytoplasmic vacuolation (arrowhead) and apoptotic body formation (arrow). Approximate magnifications, ×7,800.
The exact bacterial factors which directly mediate the death signal are not known. Fan et al. (10) recently provided evidence that binding of H. pylori to the class II major histocompatibility complex expressed on gastric epithelial cells can transduce the cell death signal in vitro. In this study, the presence of factors considered to be associated with virulence, including cagE and cagA, two genes found on the pathogenicity island, as well as vacuolating cytotoxin activity, was associated with apoptosis. In contrast, apoptosis was not detected following infection with clinical isolates lacking these virulence factors. These results are in agreement with the recent findings of Rudi et al. (33), who detected apoptosis of gastric epithelial cells following incubation with culture supernatants from a cagA-positive H. pylori isolate with cytotoxic activity, but not with supernatants from a cagA-negative, noncytotoxic strain. In contrast, another study detected apoptosis during infection with both cagA-positive, VacA-producing strains and cagA-negative, VacA-negative H. pylori strains (36). However, the cagE status of the strains utilized in both of these studies was not determined. Taken together, these findings suggest that the induction of programmed cell death could play a role in mediating disease outcome. Of interest, a preliminary study demonstrated that infection with cagE-positive strains is associated with peptic ulcer disease in children (8).

In addition to cell death triggered directly by infection with H. pylori, upregulation of the Fas receptor is observed in association with increased sensitivity to apoptosis upon ligation of the receptor. Wagner and colleagues (36) also identified enhanced apoptosis in the gastric epithelial cell line HM02 following infection with H. pylori and Fas ligation. However, the mechanism for the enhanced sensitivity to Fas signaling was not determined. This study suggests that H. pylori infection enhances expression of the Fas receptor in gastric epithelial cells, thereby resulting in an increased sensitivity to Fas-triggered cell death. H. pylori-mediated enhanced Fas expression does not directly stimulate apoptosis since a neutralizing anti-

FIG. 3. Quantitation of apoptotic AGS cells infected with H. pylori for various lengths of time. Incubation with the bacterium resulted in an increase in apoptosis compared to the apoptosis of untreated cells at 72 h (P < 0.05 [ANOVA]). Results are expressed as the mean percentages of apoptotic cells per 500 cells enumerated. Variations are represented as the SE.

FIG. 4. Fluorescence micrograph demonstrating Fas expression. (A) A low level of Fas expression is detected in untreated AGS cells. (B) Fas expression is enhanced following incubation of AGS with IFN-γ (12.5 ng/ml).

FIG. 5. Effect of H. pylori on Fas receptor expression in AGS cells. Incubation with H. pylori enhanced Fas receptor expression in AGS cells to a degree comparable to that obtained by treatment with IFN-γ (12.5 ng/ml). Results are expressed as the percent increase in Fas receptor expression compared to the expression in untreated (control) cells (+ SE).
Results are expressed as the percentages of apoptotic cells per 500 cells presence or absence of an agonistic monoclonal antibody (mab) to the Fas receptor, and apoptosis was assessed. Incubation with the Fas agonist enhanced programmed cell death in control cells (21.1% ± 1.9% versus 5.9% ± 1.4%, P < 0.05 [ANOVA]). The apoptotic index of H. pylori-infected cells incubated with an anti-Fas antibody increased compared to that of AGS cells incubated with the bacterium alone (38.5% ± 7.1% versus 16.0% ± 2.8%, P < 0.05 [ANOVA]). Results are expressed as the percentages of apoptotic cells per 500 cells enumerated (+ SE).

A number of studies have indicated that immune system-mediated cell death through the Fas-Fas ligand pathway likely also contributes to the apoptosis that is observed during infection in vivo. The factors mediating enhanced Fas receptor expression and Fas-mediated cell death during H. pylori infection are not known. A recent study demonstrated that IFN-γ, which is increased in the gastric mucosa during H. pylori infection (18), augments apoptosis. IFN-γ also upregulates expression of the Fas death receptor (30). Cytokines produced by inflammatory cells in the lamina propria in response to H. pylori infection could also modulate cell death. Further studies are required to determine the factors which increase the susceptibility of gastric cells to the Fas death cascade.

Our findings indicate that Fas-stimulated cell death could play a role in H. pylori-mediated pathogenesis in vivo. During infection with the bacterium, gastric epithelial cells exhibiting enhanced Fas receptor expression could be eliminated by infiltrating lymphocytes that express the Fas ligand. In support of this contention, a recent study identified an increase in Fas ligand mRNA expression in lymphocytes within the lamina propria and enhanced Fas receptor expression in both gastric epithelial cells and cells within the superficial lamina propria of H. pylori-infected gastric biopsy tissue (33). Furthermore, the region of the gastric mucosa with enhanced Fas ligand mRNA corresponded to areas of enhanced apoptosis.

Also, animal models suggest that Fas signaling plays a role in gastric injury. In a murine model of autoimmune gastritis generated by either thymectomy or adoptive transfer of a Th1 cell clone recognizing an epitope of the H⁺, K⁺-ATPase, enhanced Fas expression was detected on gastric parietal cells (29). In contrast, gastric tissue from normal controls lacked detectable Fas. The topographic expression of this death receptor in parietal cells correlated with the induction of apoptosis as assessed by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling method (29). This indicates that one mechanism by which autoimmune-mediated target cell destruction may be effected is Fas-Fas ligand interactions. This is of particular interest since autoantibodies directed against gastric parietal H⁺, K⁺-ATPase are detected in sera from H. pylori-infected subjects and correlate with the presence of gastric atrophy (6).

In summary, the present study shows that H. pylori infection is capable of activating the apoptotic cell death cascade in gastric epithelial cells by more than one mechanism. The bacterium can directly stimulate programmed cell death and also enhances both expression of the cell death receptor Fas and sensitivity to Fas-mediated apoptosis. In vivo studies, including those with H. pylori-infected animal models of human disease (22), should now be undertaken to further delineate the role of Fas signaling in the pathogenesis of H. pylori-mediated disease.

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