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Helicobacter pylori Modulates Lymphoepithelial Cell Interactions Leading to Epithelial Cell Damage through Fas/Fas Ligand Interactions

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Helicobacter pylori **causes a common chronic infection of humans that leads to epithelial cell damage. Studies have shown that apoptosis of the gastric epithelium is increased during infection and this response is associated with an expansion of gastric T-helper type 1 (Th1) cells. We report that gastric T cells contribute to apoptosis of the epithelium by a Fas/Fas ligand (FasL) interaction. Fas receptor expression was detected on freshly isolated gastric epithelial cells by flow cytometry and immunohistochemistry, and this level of expression was increased during infection with *H. pylori*. The expression of Fas receptor on three gastric epithelial cell lines was increased by *H. pylori*, either alone or in combination with gamma interferon or tumor necrosis factor alpha. The role of Fas in apoptosis of gastric epithelial cell lines was evidenced by DNA fragmentation after cross-linking of Fas with specific antibodies. FasL expression was detected by immunohistochemistry on mononuclear cells in gastric biopsy specimens of infected but not uninfected subjects. Gastric T-cell lines were also shown to express FasL, as evidenced by reverse transcription-PCR and killing of target cells expressing Fas receptor. Moreover, these T-cell lines were capable of killing cultured gastric epithelial target cells and antibodies that block the interaction between Fas receptor and FasL inhibited this cytotoxic activity. These observations demonstrate that local Th1 cells may contribute to the pathogenesis of gastric disease during *H. pylori* infection by increasing the expression of Fas on gastric epithelial cells and inducing apoptosis through Fas/FasL interactions.**

Helicobacter pylori causes a lifelong infection of the gastric antrum that affects more than 50% of humanity. Infection is associated with chronic antral gastritis, characterized by a mucosal infiltration of polymorphonuclear and mononuclear leukocytes (10, 14). Evidence for a pathogenic role of *H. pylori* infection in peptic ulcer is derived from clinical investigations showing that cure of *H. pylori* infection accelerates ulcer healing and prevents ulcer relapse (20). Besides ulcer disease, *H. pylori* infection has also been implicated as a cause of gastric lymphoma or carcinoma in some patients (7).

There are many reports that *H. pylori* infection interferes with the equilibrium between proliferation and apoptosis of the gastric epithelium (4, 7, 25, 32, 35, 49). Most of the in situ studies have shown that the number of apoptotic epithelial cells increases during *H. pylori* infection (25, 32, 35, 41). Numerous mechanisms could account for this, including the direct effects of the bacteria, as well as the inflammatory response elicited by the infection (13, 24, 41, 49).

Several independent approaches have suggested that T-helper type 1 (Th1) cells are selectively increased during infection (3, 9, 18, 26, 31). Th1 cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), can increase the release of proinflammatory cytokines, such as interleukin-8, from the epithelium (52), as well as Fas and Fas ligand (FasL) (6). Furthermore, these cytokines can also increase the expression of major histocompatibility complex class II molecules by gastric epithelial cells, thereby increasing the binding of *H. pylori* to the gastric epithelium (13). As Th1 cells are associated with cell-mediated immune responses, they may also play a role in damaging gastric tissues directly by triggering apoptosis in gastric epithelial cells.

Th1 cells can also express higher levels of FasL than Th2 cells (40, 46). This molecule belongs to the TNF family of proteins. The receptor of FasL, Fas (CD95), is a 45-kDa protein belonging to the TNF receptor family. Cross-linking of Fas with an agonistic immunoglobulin M (IgM) antibody or FasL will transduce the death signal to the cells, resulting in the induction of apoptosis. However, studies to date have not shown that gastric T cells express FasL and induce epithelial cell death by Fas/FasL interactions. The purpose of this study was to investigate the role of *H. pylori* in the modulation of lymphoepithelial interactions in the stomach that are mediated through Fas/FasL.

MATERIALS AND METHODS

Subjects. Material from human tissue was obtained from consenting adults aged 20 to 55 years as approved by the respective institutional review boards at the National University of Ireland, the University of Göteborg, and the University of Texas Medical Branch. Individuals regularly using nonsteroidal anti-inflammatory drugs or antisecretory drugs were excluded from the study population. Biopsy specimens of the gastric antrum were obtained from consenting subjects undergoing gastroesophageal duodenoscopy for various clinical indications. Subjects were considered infected if *H. pylori* was detected either by a rapid urease test or by histopathology on biopsy specimens.

Preparation of freshly isolated gastric epithelium and T cells. (i) Gastric epithelium. To evaluate the expression of Fas receptor, gastric epithelial cells were partially enriched using a modification of previously described techniques (13, 53). Briefly, biopsy specimens from subjects infected with *H. pylori* were collected into sterile collection medium (calcium- and magnesium-free Hanks balanced salt solution [HBSS] with 5% fetal calf serum [FCS] and penicillin plus streptomycin). Biopsy specimens were rinsed with HBSS medium containing 1

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mM dithiothreitol and 1 mM EDTA (Sigma Chemical Co., St. Louis, Mo.). The specimens were agitated for 1 h at 37°C to obtain epithelial cells. The resulting cell suspensions were washed, and the viability of the gastric epithelial cells was determined by trypan blue exclusion. Cells were not used if viability did not exceed 80%. The yield from a single subject varied from 5×10^5 to 1×10^6, so further purification by density gradients was not possible. To determine purity, freshly isolated cells were stained for an epithelium-specific antigen using fluorescein isothiocyanate-conjugated monoclonal antibody (clone Ber-EP4; Dako, Flostrup, Denmark) as previously described (3, 17). Purity ranged from 50 to 80% with epithelial lymphocytes being the major contaminating cell type (3). Cells stained by the epithelial cell antigen were selected by electronic gating, and this population was examined by flow cytometry for the expression of Fas receptor.

(ii) Gastric T cells. Gastric T cells were isolated using a modification of previously described techniques (3, 12). Briefly, biopsy specimens were collected into collection medium (calcium- and magnesium-free HBSS with 5% FCS and penicillin plus streptomycin). In some cases, biopsy specimens were stored at 4°C for up to 18 h prior to processing, this having previously been shown not to alter T-cell function. All manipulations were carried out using aseptic techniques. Biopsy specimens were rinsed with aqueous Betadine and immediately rinsed four times in collection medium before being placed in collection medium containing 1 mM dithiothreitol and 1 mM EDTA. The specimens were agitated for 1 h at 37°C to remove intraepithelial lymphocytes and epithelial cells before being placed in complete RPMI medium (RPMI 1640 medium with 10% FCS, penicillin, and streptomycin) and washed three times. Subsequently, lamina propria T cells were liberated by treatment with collagenase (30 U/ml; Sigma Chemical Co.) in complete RPMI medium for 3 h. Undigested biopsy material was removed, and the cells were washed three times with complete RPMI medium. The resulting cell suspensions were washed, and the viability of the mononuclear cells was determined by trypan blue exclusion. Cells were not used if viability did not exceed 90%.

Gastric T cells were cultured in RPMI 1640 medium containing 15% FCS, 10 mM HEPES, penicillin plus streptomycin, 10 U of recombinant interleukin-2 (ICN Pharmaceuticals Inc., Irvine, Calif.) per ml, and 10% phytohemagglutinin-stimulated lymphocyte supernatant. T cells were fed with 5 mg of phytohemagglutinin (Difco Laboratories, Detroit, Mich.) per ml and an irradiated (3,000 rads) allogeneic peripheral B-cell line every 2 weeks.

Immunohistochemistry. (i) Cytokines. For cytokine detection studies, frozen sections were prepared from two biopsy specimens of the gastric antrum from infected and uninfected subjects. Two sections were examined from each biopsy. The slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 10 min, washed in PBS, air dried, and kept at −20°C until used. After rehydration, the slides were treated with 0.1% saponin (Sigma Chemical Co.) in PBS to permeabilize the cell membranes. Endogenous peroxidase and biotin activities were blocked with 1% H_2O_2 and 0.02% NaN_3 in PBS and by use of an avidin-biotin blocking kit (Vector Laboratories, Burlingame, Calif.), respectively. The slides were then incubated overnight at 4°C with anti-TNF-α (MAB11; PharMingen, San Diego, Calif.) or anti-IFN-γ (DIK-1; Chromogenix, Molndal, Sweden) monoclonal antibodies at a concentration of 5 μg/ml. An irrelevant isotype-matched monoclonal antibody (Dako) was used as a control for nonspecific staining. After blocking with 1% normal goat serum, biotinylated goat anti-mouse IgG, absorbed against human IgG (Caltag Laboratories, San Francisco, Calif.), was added as a secondary antibody, and the mixture was incubated for 30 min at room temperature. The sections were then overlaid with an avidin-biotin-horseradish peroxidase complex (Vectastain ABC-HP kit; Vector Laboratories), followed by the substrate chromagen diaminobenzidine (Vector Laboratories), and finally counterstained with Mayer's hematoxylin.

(ii) FasL and FasR. Paraffin-embedded sections of normal gastric tissues were deparaffinized in xylene and rehydrated prior to analysis. Slides were washed twice for 5 min each time in a wash buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 5 min. Slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum, and then blocked for 1 h in a wash buffer containing 5% normal goat serum. After washing and incubation overnight at 4°C with affinity-purified rabbit polyclonal anti-human FasL- or FasR-specific IgG (Santa Cruz Biotechnology, Santa Cruz, Calif.) at 0.1 μg/ml in wash buffer, antibody binding was localized using a biotinylated secondary antibody, avidin-conjugated horseradish peroxidase, and diaminobenzidine substrate (contained within the Vectastain ABC-HP kit [Vector Laboratories]). The appropriate immunizing peptide to which the antibody was raised (FasL amino acids 260 to 279 or FasR amino acids 316 to 335; Santa Cruz Biotechnology) was included at 1 μg/ml during primary antibody incubation as a direct internal competitive control for antibody specificity. Slides were counterstained with hematoxylin.

Bacteria and human cell lines. *H. pylori* LC-11 is a *cagA*-bearing strain that was isolated from a child with duodenal ulceration as described previously (8, 11). To investigate the impact of the *cag* pathogenicity island (PAI) on the induction of Fas, strain 26695 and an isogenic mutant lacking the entire *cag* PAI, 8-1 (2), were used. Briefly, bacteria were grown on blood agar base (Becton Dickinson, San Jose, Calif.) at 37°C under microaerobic conditions and harvested on day 3 into PBS. After centrifugation at 2,500 × *g* for 15 min, bacteria were resuspended in sterile PBS (pH 7.4) to a concentration of 2×10^8/ml. Bacterial numbers were estimated by measuring the A_{530} using a DU-65 spectrophotometer (Beckman, Fullerton, Calif.). One unit of optical density at 530 nm (OD_{530}) was equivalent to 2×10^8 bacteria per ml as determined by comparing the measured value to a standard curve generated by quantifying viable organisms from aliquots of bacteria at various concentrations that were also assessed for absorbance. Motility was confirmed by phase-contrast microscopy prior to experimental use.

Kato-III, N87, and AGS-NY2 are gastric epithelial cell lines, while Jurkat E6-1 is a leukemia T-cell line. Kato-III, N87, and Jurkat E6-1 cells were purchased from the American Type Culture Collection, Manassas, Va., while AGS-NY2 cells were generously provided by S. Moss. All cells were maintained in RPMI 1640 medium supplemented with 10% FCS as previously described (13).

Examination of Fas receptor expression by flow cytometry. To measure Fas receptor expression, freshly isolated gastric epithelial cells were harvested, washed, and stained with an optimal amount of mouse anti-human Fas receptor (CD95) (DX2 at 1 μg/ml; PharMingen) or an appropriate isotype control. Subsequently, cells were washed with PBS with 0.1% bovine serum albumin and fixed in 1% paraformaldehyde in PBS before specific fluorescence was measured using a FACScan (Becton Dickinson) after correction for nonspecific fluorescence using the appropriate isotype controls. The results are presented as the mean fluorescence intensity (MFI) and percentage of positive cells.

In other experiments, resting or stimulated gastric epithelial cell lines were stained in an identical fashion for the evaluation of CD95 expression. Briefly, cells were exposed to the various strains of *H. pylori* (300 bacteria/epithelial cell), TNF-α (40 ng/ml; R&D Systems, Minneapolis, Minn.), or IFN-γ (100 U/ml; R&D Systems) either alone or in combination. These cytokine concentrations were based on a previous determination of optimal responses as reported by several laboratories (8, 13, 21, 24, 49). After incubation for 48 h, the cells were harvested and Fas expression was detected by flow cytometry. The results are presented as the relative MFI, which equals (MFI of Fas/MFI of isotype) × 100, to compare the changes in Fas expression.

To determine if functional Fas was expressed on gastric epithelial cells, Kato-III, AGS-NY2, and N87 cells were incubated with medium or various stimuli as described above for 6 h, followed by incubation with medium or 30 to 100 ng of IgM anti-Fas antibody CH11 (Kamiya Biomedical Company, Thousand Oaks, Calif.) per ml for 12 h. DNA fragmentation was evaluated by enzyme-linked immunosorbent assay (ELISA) and a DNA ladder assay as described below.

Detection of FasL mRNA. (i) Extraction of total RNA and reverse transcription (RT). Total RNA was extracted from gastric biopsy specimens or cell lines using Trizol reagent (Life Technologies, Houston, Tex.) in accordance with the protocol provided by the manufacturer (8). Briefly, cells or tissues were lysed using Trizol reagent, followed by extraction with chloroform and precipitation with isopropanol, washed one time using 70% ethanol, and then diluted into diethylpyrocarbonate-treated distilled water. The purity and amount of the RNA were determined by measuring OD_{260} and OD_{280} using a DU-65 spectrophotometer (Beckman). cDNA was synthesized using Superscript II reverse transcriptase and oligo(dT)s primer (Life Technologies, Gaithersburg, Md.) at 42°C in accordance with the protocol provided by the manufacturer.

Amplification of FasL. Primers for FasL were designed in accordance with the human gene sequences as described elsewhere (37). The length of the product is 344 bp. β-Actin primers were purchased from Clontech Laboratory (Palo Alto, Calif.); the length of the product is 838 bp. The sequences of FasL primers were as follows: sense primer, 5′-CAGCTCTTCCACCTACAGAAGG-3′; antisense primer, 5′-GAGAGACCAGTTAAAACTCCTTAGA-3′. Thermal cycling was done as follows: denaturation at 96°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 150 s; 40 cycles were performed. Primers were used at a final concentration of 0.1 μM each in a 50-μl volume, and the concentration of Mg^{2+} was 1.0 mM. PCR products were identified after separation by electrophoresis using 1.2% agarose gels, followed by staining with ethidium bromide.

Detection of DNA fragmentation. (i) JAM test. Induction of apoptosis was detected using a cytotoxicity assay termed the JAM test (33). Briefly, target cells (T) were labeled with [^{3}H]thymidine (ICN Pharmaceuticals Inc., Irvine, Calif.) at 10 μCi/ml for 12 h and seeded into 96 wells at a concentration of 2×10^4 cells per well. Effector cells (E) were cocultured with targets at various E/T ratios for 12 h. Subsequently, cells were collected with a cell harvester (Skatron Instruments Inc., Sterling, Va.) and radioactivity was determined using a liquid scintillation counter (Beckman). The degraded DNA was washed through the membrane, and the undegraded, high-molecular-weight DNA was captured on the membrane. Thus, killing (expressed as the percentage of killed cells) can be calculated as $(S - E)/S \times 100$, where S is counts per minute in the medium control and E is the counts per minute of treated cells.

To study Fas/FasL interactions, gastric T cells expressing FasL were incubated with target cells expressing Fas (Jurkat T cells or gastric epithelial cells) in the presence or absence of 1 μg of anti-Fas IgG antibody (ZB4; Kamiya Biomedical Company) or the appropriate isotype control (37) per ml.

(ii) ELISA. DNA fragmentation was also measured using a commercially available ELISA (Boehringer, Mannheim, Germany). This assay detects low-molecular-weight nucleosome fragments in the cytoplasmic fractions of affected cells that arise during apoptosis but not as a result of necrosis. A_{405} was measured by Titertek Multiskan MCC/340 (ICN Pharmaceuticals Inc.) and compared to that of the substrate solution used as a blank. The apoptotic index was calculated

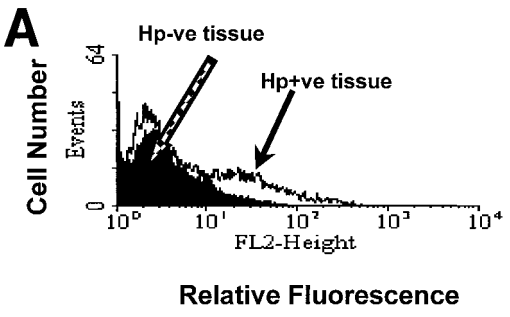
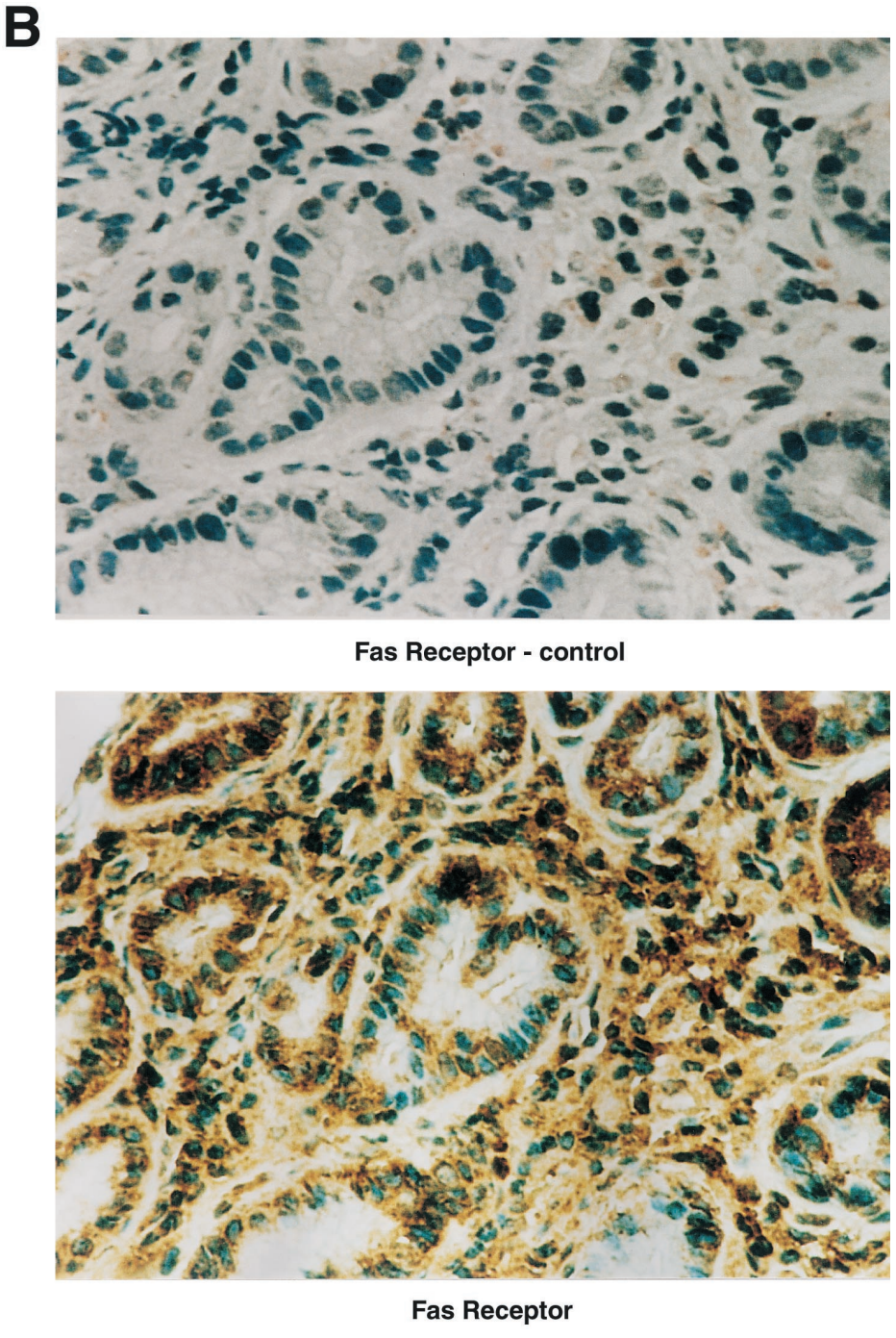


FIG. 1. Fas receptor expression is increased in vivo during infection with *H. pylori*. (A) Epithelial cells were isolated from multiple biopsy specimens obtained from *H. pylori*-positive or -negative subjects, and Fas expression was detected by flow cytometry. Shown is a representative histogram in which Fas expression on epithelial cells from one *H. pylori*-infected subject (solid arrow) was higher than that on cells from an uninfected subject (open arrow). The mean percentage of positive cells expressing Fas ± SEM was 11.3% ± 5.6% for infected subjects versus 1.4% ± 0.3% for uninfected subjects ($n = 8$). Hp−ve, *H. pylori* negative; Hp+ve, *H. pylori* positive. (B) Fas expression on gastric epithelium was detected in paraffin-embedded gastric specimens from *H. pylori*-infected subjects using immunohistochemistry as described in Materials and Methods. This image shows that Fas was expressed on the gastric epithelium of infected subjects ($n = 5$) and the detection was blocked when Fas peptide at 1 μg/ml was added with the primary antibody (control). Expression in uninfected tissues was almost undetectable ($n = 5$; data not shown).



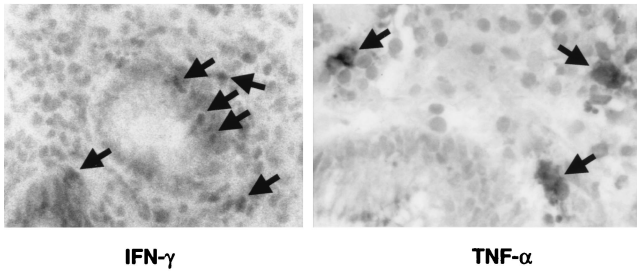


FIG. 2. Detection of Th1 cytokines adjacent to gastric epithelial cells during infection with *H. pylori*. Biopsy specimens from infected and uninfected subjects were stained with antibodies recognizing human IFN-γ or TNF-α as described in Materials and Methods. Whereas few positive cells were detected in uninfected subjects, cytokine-positive cells were observed within or adjacent to the gastric epithelium (arrows). These images are representative of eight different subjects.

in accordance with the manufacturer's instructions by dividing the absorbance of stimulated cells by the absorbance of control cells (13).

Gel electrophoresis. Agarose gel electrophoresis to detect DNA fragmentation was conducted in accordance with a standard procedure for assaying fragmentation in total genomic DNA (48). Briefly, cells under different culture conditions were harvested and pelleted by centrifugation at 400 × *g* for 5 min. Subsequently, 10^6 epithelial cells were washed with PBS, pelleted, and resuspended in sample buffer composed of Tris-buffered glycerol with $ZnSO_4$ at 1.4 mg/ml, bromophenol blue, and RNase (Sigma) at 10 mg/ml. Nucleosomal ladders were visualized in 2% agarose gels in TBE buffer (0.09 M Tris, 0.09 mM boric acid, 0.24 M EDTA, pH 8.0). Cells were lysed by 2% sodium dodecyl sulfate and proteinase K (Sigma) at 53 μg/ml contained within a 1% agarose gel slice above the wells. The gel was run for 10 to 14 h at 30 V, stained with ethidium bromide at 2 μg/ml for 1 h, and washed overnight with a large volume of distilled water to remove excess stain. UV light was used to visualize DNA fragments in the gel that were photographed, and their migration was compared to that of the *Hae*III-digested fX174 standard (Promega, Madison, Wis.).

Statistical analyses. Results are expressed as the mean ± the standard error of the mean (SEM). Fas/FasL expression and killing rates were compared using a two-tailed Student *t* test, and differences were considered significant if *P* values were <0.05.

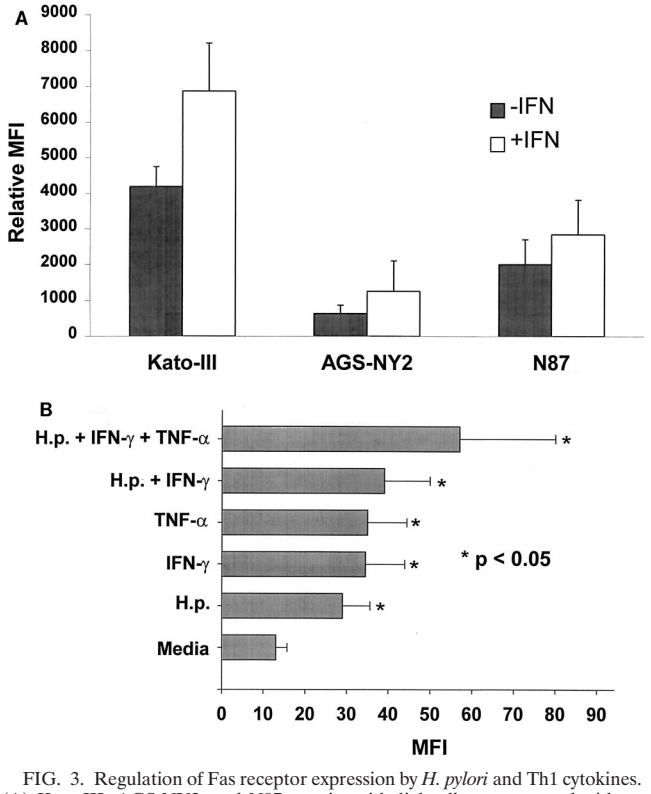


FIG. 3. Regulation of Fas receptor expression by *H. pylori* and Th1 cytokines. (A) Kato-III, AGS-NY2, and N87 gastric epithelial cells were treated with or without IFN-γ (100 U/ml) for 24 h, and Fas expression was detected by flow cytometry. It was found that IFN-γ was capable of increasing Fas expression in all of these epithelial cell lines ($n = 3$, $P < 0.05$), with Kato-III being the most sensitive cell line. (B) Gastric epithelial cells (Kato-III) were treated for 48 h with medium, *H. pylori* (H.p.), IFN-γ (100 U/ml), TNF-α (40 ng/ml), *H. pylori* and IFN-γ, or *H. pylori*, IFN-γ, and TNF-α. Subsequently, Fas expression was detected by flow cytometry. This graph shows that all of these stimuli, either alone or in combination, increased the expression of Fas compared to medium (*, $P < 0.05$). These results were obtained from three separate experiments.

TABLE 1. Relative expression of Fas by gastric epithelial cells after stimulation with strains of *H. pylori* that vary in the presence of the *cag* PAI

Cell line	% of control MFI in *H. pylori* strain:	
	26695	8-1
Kato-III	248	267
AGS-NY2	322	233
N87	545	339

[a] Data are reported as averages of two assays detecting the MFI (expressed as a percentage relative to the control) for Fas expression as measured by flow cytometry in cell lines exposed to medium or strains of *H. pylori* with (26695) or without (8-1) the *cag* PAI.

RESULTS

Gastric epithelial cells express Fas receptor in vivo. In order for gastric T cells to mediate epithelial cell death through Fas/FasL interactions, the target cell must express Fas. To confirm the expression of Fas by gastric epithelium and to compare the expression on gastric epithelial cells in the presence or absence of *H. pylori* infection, gastric biopsy specimens were collected from infected and uninfected subjects and isolated epithelial cells were examined by flow cytometry (Fig. 1A). After staining to detect surface Fas, the MFI was 19.0 in eight infected subjects versus 7.8 in eight uninfected subjects, while the mean percentage of Fas-positive cells was 11.3% versus 1.4%, respectively ($P < 0.05$). Since the expression of Fas was restricted to a small subset of the isolated epithelial cells from infected subjects, the distribution of Fas expression was examined by immunohistochemistry. Consistent with the findings obtained using flow cytometry, Fas expression was limited to an extremely small percentage of the gastric epithelial cells in normal, uninfected specimens (data not shown) whereas Fas expression by epithelial cells from infected tissues was much greater (Fig. 1B). Thus, Fas expression by gastric epithelial cells is increased during infection with *H. pylori*.

Th1 cytokine production adjacent to the gastric epithelium. Since Fas expression on epithelial cells was increased and this can be achieved by cytokines from Th1 cells, the expression of IFN-γ and TNF-α adjacent to gastric epithelial cells was examined by immunohistochemistry. IFN-γ-positive cells were found in all but one of the *H. pylori*-infected subjects, whereas TNF-α was found in five of eight infected biopsy specimens. IFN-γ or TNF-α was never found in biopsy specimens from healthy uninfected subjects using this approach. As shown in Fig. 2, both cytokines were detected in mononuclear cells within, or adjacent to, the gastric epithelium. The median number of IFN-γ-stained intraepithelial lymphocytes was 1.7/mm^2 of tissue area (interquartile range, 0.8 to 4), and the median number of positively stained lamina propria was 4.8 (interquartile range, 1.8 to 11). TNF-α-positive cells, median number, 1.2 (interquartile range, 0 to 2.9), were often located in the lamina propria surrounding the neck region of the gastric glands, but no intraepithelial lymphocytes were specifically

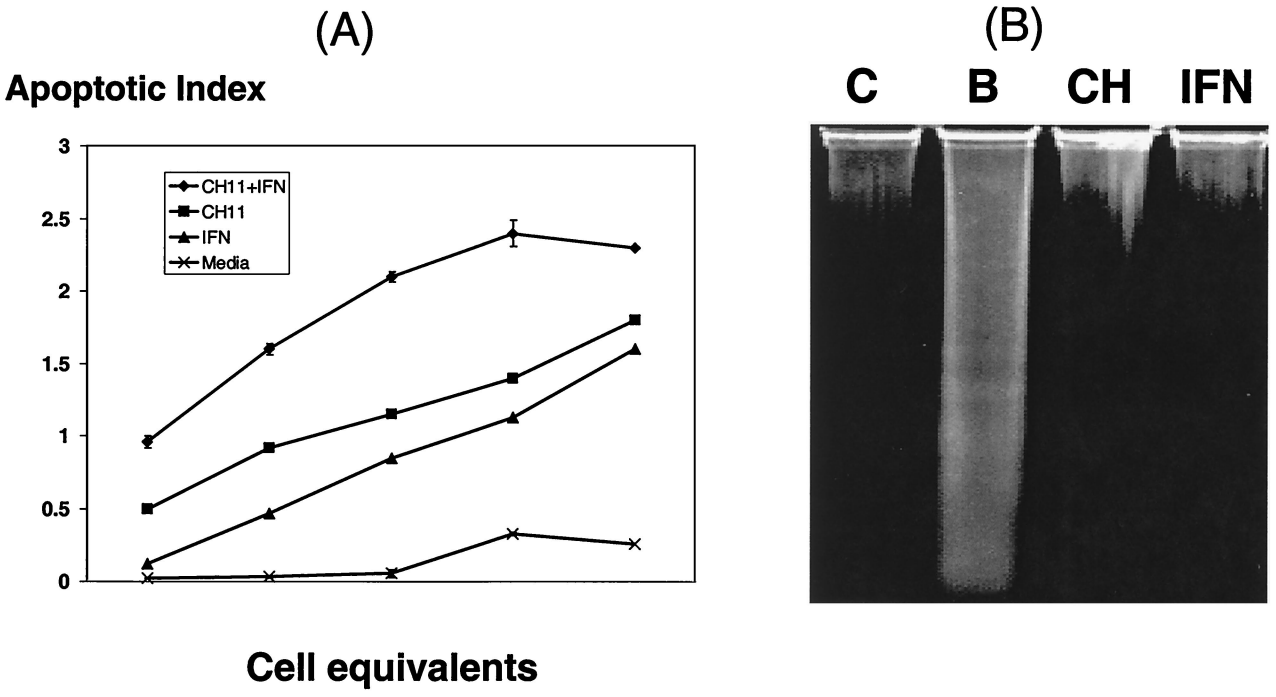


FIG. 4. Induction of Fas-mediated apoptosis in gastric epithelial cells. Kato-III cells treated with medium or IFN-γ (100 U/ml, 48 h) were incubated with anti-Fas IgM antibody (CH11; 100 ng/ml) for 12 h. Subsequently, apoptosis was evaluated by measuring DNA degradation using the ELISA and the DNA ladder assay as described in Materials and Methods. (A) The apoptotic index of Kato-III cells after pretreatment with IFN-γ and CH11 was higher than that detected after exposure to IFN-γ or CH11 alone, although all of the stimuli induced greater DNA fragmentation than the medium control. (B) The sensitivity for the detection of DNA degradation using agarose gel electrophoresis was less than that of the ELISA, as evidence for apoptosis was only found in Kato-III cells treated with IFN-γ and CH11. Lanes: C, untreated control; B, both IFN-γ and CH11; CH, CH11; IFN, IFN-γ.

stained for TNF-α. Interestingly, positive staining for both cytokines was occasionally also found in the epithelial cells themselves. The finding of IFN-γ and TNF-α-positive cells was consistent with the reports describing the cytokine production in gastric tissue during infection (3, 9, 26, 31) and suggested a potential role for Th1 cells in the regulation of Fas/FasL interactions.

Regulation of Fas expression on gastric epithelial cells by *H. pylori* and Th1 cytokines. Since Th1 cytokines are present adjacent to the gastric epithelium in *H. pylori*-infected tissues, we determined if these factors might account for the observed increase in Fas expression by gastric epithelial cells. Gastric epithelial cell lines were stimulated with *H. pylori* in the presence or absence of various cytokines and examined by flow cytometry. Figure 3A shows that Fas expression on three separate gastric epithelial cell lines was increased by IFN-γ, with Kato-III being the most sensitive cell line. In another experiment, Fas expression on Kato-III cells was detected after incubation with *H. pylori*, IFN-γ, and TNF-α, either alone or in various combinations. Figure 3B shows that Fas expression on Kato-III cells was increased consistently by all of these stimuli alone while the combination of *H. pylori*, IFN-γ, and TNF-α yielded the greatest induction of Fas. To investigate the role of the *cag* PAI in the induction of Fas, Kato-III, AGS-NY2, and N87 cells were incubated with strain 26695 or the *cag* PAI-deficient isogenic mutant 8-1. As shown in Table 1, both strains induced Fas expression although the level of induction was greater with *cag*-bearing strain 26695.

TABLE 2. Relative sensitivities of different gastric epithelial cell lines to Fas-mediated apoptosis after induction of Fas[a]

Cell line	Mean % specific killing ± SEM			
	Medium	*H. pylori* 26695	*H. pylori* 8-1	IFN-γ
Kato-III	9 ± 3	15 ± 1	12 ± 1	35 ± 2
AGS-NY2	16 ± 3	32 ± 1	18 ± 2	28 ± 2
N87	31 ± 8	55 ± 4	36 ± 4	61 ± 3

[a] Fas expression was induced as described in Table 1, footnote *a*; the legend to Fig. 3; and Materials and Methods. Subsequently, Fas-mediated killing was detected using anti-Fas antibody CH11 at 50 ng/ml and assayed by the JAM test. Data represent the mean ± SEM of three observations measuring percent specific killing. Significant killing was detected under all of the conditions tested ($P < 0.05$), confirming the functional capacity of epithelial Fas.

Fas expressed by gastric epithelium is functional. The function of Fas expressed on three separate gastric epithelial cell lines was examined after control or IFN-γ-treated cells were incubated with anti-Fas IgM antibody. DNA degradation products isolated from the cytosol of stimulated and control cells were evaluated by an ELISA to detect DNA fragments released from the nuclei of cells undergoing apoptosis. Figure 4A shows that the ELISA used was able to detect DNA fragmentation induced during apoptosis in as few as 10 cells. IFN-γ and anti-Fas antibody alone killed gastric epithelial cells; however, the combination of both markedly increased the apoptosis of gastric epithelial cells. Figure 4B shows the low-molecular-weight DNA isolated from Kato-III cells (10^6/ml) resolved on a 2% agarose gel after control or IFN-γ-treated cells were incubated with anti-Fas IgM antibody and not an isotype control antibody. Moreover, Fas was shown to be functional on other cell lines, including AGS-NY2 and N87 (Table 2). Significant augmentation of killing ($P < 0.05$) was observed in all

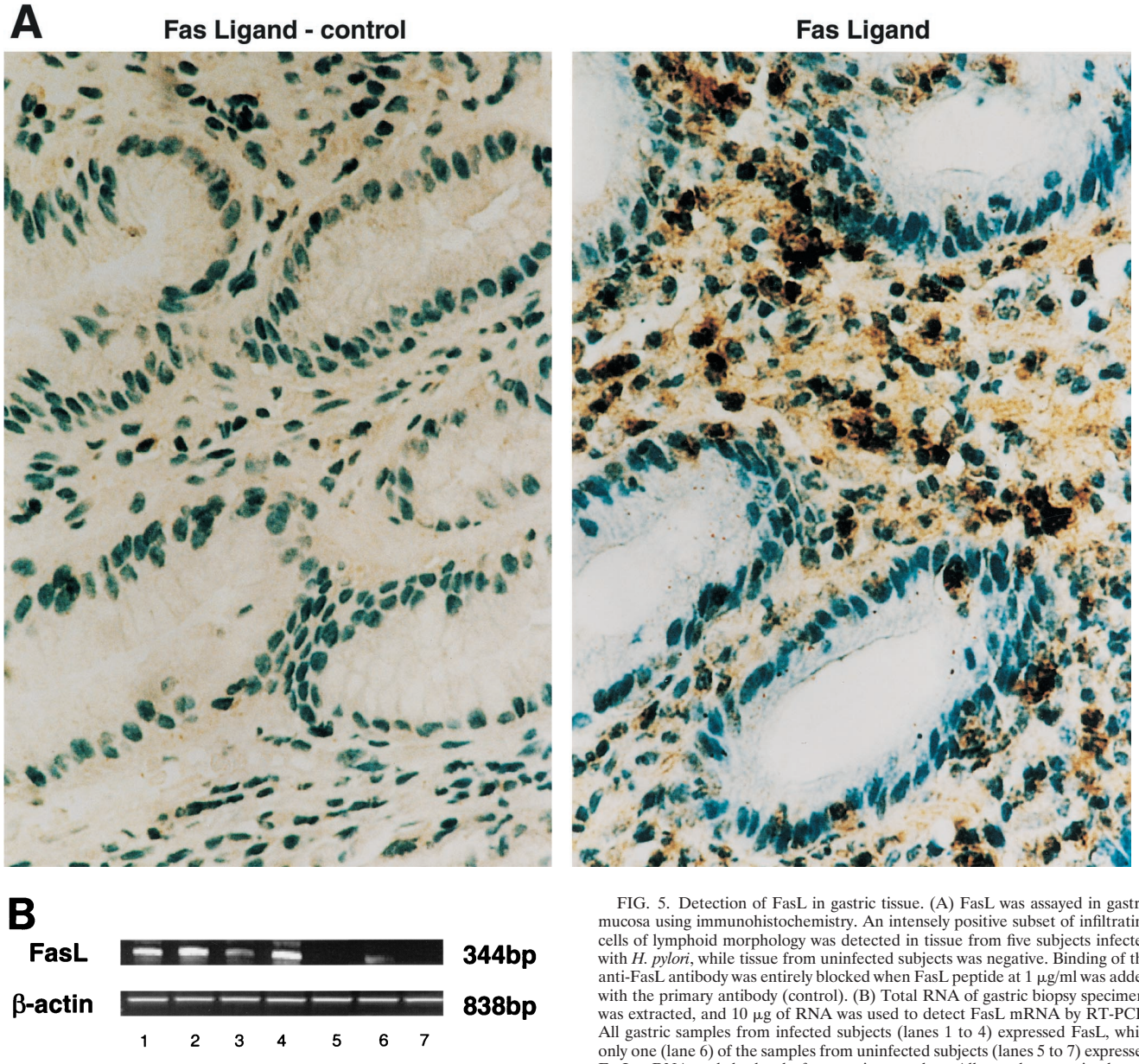


FIG. 5. Detection of FasL in gastric tissue. (A) FasL was assayed in gastric mucosa using immunohistochemistry. An intensely positive subset of infiltrating cells of lymphoid morphology was detected in tissue from five subjects infected with *H. pylori*, while tissue from uninfected subjects was negative. Binding of the anti-FasL antibody was entirely blocked when FasL peptide at 1 μg/ml was added with the primary antibody (control). (B) Total RNA of gastric biopsy specimens was extracted, and 10 μg of RNA was used to detect FasL mRNA by RT-PCR. All gastric samples from infected subjects (lanes 1 to 4) expressed FasL, while only one (lane 6) of the samples from uninfected subjects (lanes 5 to 7) expressed FasL mRNA and the level of expression was low. All samples contained comparable amounts of β-actin mRNA.

lines treated with IFN-γ and in AGS-NY2 or N87 cells exposed to *H. pylori* 26695 but not under the other conditions tested. These findings indicate that innately expressed Fas and IFN-γ-induced Fas are functional in the transduction of the apoptotic signal.

FasL is expressed in gastric tissues. To determine if Fas-bearing epithelial cells would be exposed to effector cells expressing FasL, gastric biopsy specimens from infected or uninfected subjects were examined for FasL protein by immunohistochemistry. As shown in Fig. 5A, FasL protein was present on mononuclear cells in the gastric mucosa of infected individuals, whereas the expression in normal uninfected tissues was undetectable (M. Bennett et al., unpublished observations). To detect FasL mRNA, total RNA of gastric biopsy specimens with or without *H. pylori* infection was extracted and assayed using RT-PCR. As shown in Fig. 5B, all four infected gastric tissues contained mRNA for FasL while only one weakly positive band was detected in the three uninfected biopsy specimens.

Functional FasL is expressed by gastric T cells. Gastric T cells were isolated from biopsy specimens and expanded in vitro in order to evaluate the expression and function of FasL. As shown in Fig. 6A, FasL mRNA was detected by RT-PCR in gastric T cells isolated from *H. pylori*-infected individuals. To evaluate the function of FasL, the T-cell lines were assayed using the JAM test to determine if they induced apoptosis in Jurkat T-cell line E6-1. The Jurkat E6.1 clone expressed a high level of Fas and was susceptible to IgM anti-Fas (CH11) antibody-mediated killing (data not shown). As shown in Fig. 6B, gastric T-cell lines could kill these target cells and this cytotoxicity was inhibited by antibody ZB4, which blocks Fas/FasL interactions while an isotype control, mouse IgG, did not ($P < 0.05$). These results imply that FasL expressed by T cells in the

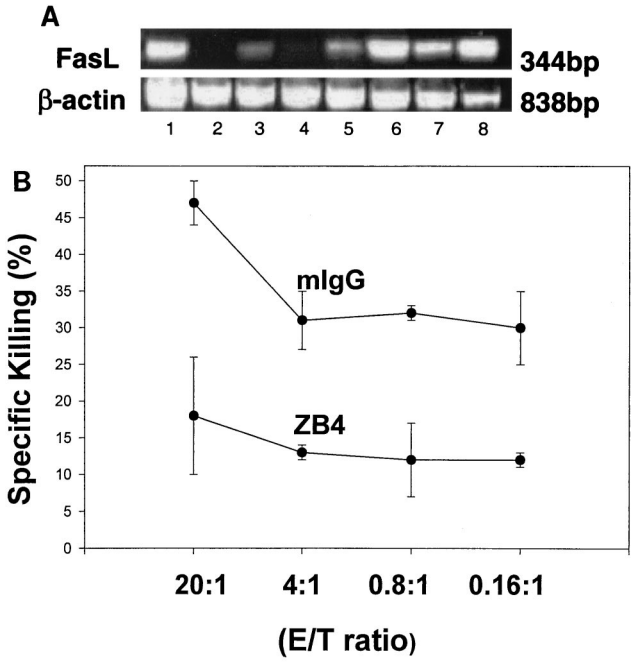


FIG. 6. Gastric T cells express functional FasL. T cells were isolated from gastric biopsy specimens and expanded in vitro. (A) Total RNA was extracted from gastric T-cell lines, and RT-PCR was used to detect mRNAs for FasL and β-actin. The mRNA for FasL was detected in T-cell lines regardless of whether they were obtained from infected or uninfected subjects, although products were more easily detected in T-cell lines from infected subjects. Lanes: 1, positive control; 2 to 4, gastric T-cell lines from uninfected subjects; 5 to 8, gastric T-cell lines from infected subjects. (B) Functional evidence for FasL expression on gastric T cells was obtained using the JAM test to detect DNA fragmentation of Fas-expressing target cells after exposure to T cells. Cytotoxicity was calculated by comparing the levels of radioactivity in cells that had or had not been exposed to the T cells. Murine anti-Fas IgG (ZB4; 500 ng/ml), but not isotype control antibody (mouse IgG [mIgG]), blocked the killing after being preincubated with target cells for 1 h ($P < 0.05$). These data are representative of three separate experiments.

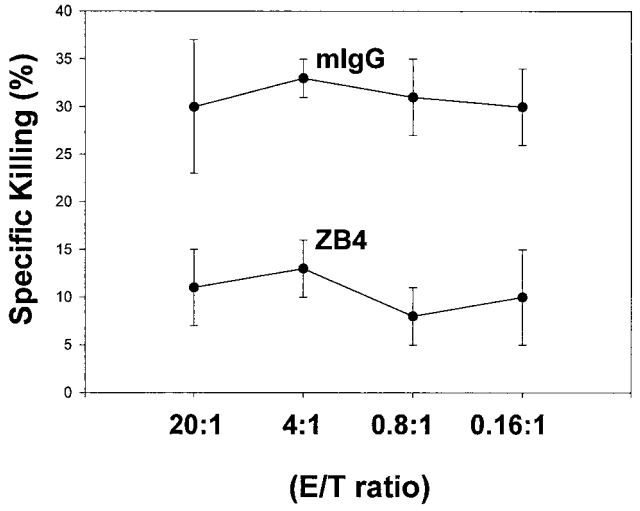


FIG. 7. Killing of gastric epithelium by T cells through Fas/FasL interactions. Expanded gastric T cells were harvested and incubated with [^{3}H]thymidine-labeled gastric epithelial (Kato-III) cells for 12 h at the indicated E/T ratios. Specific killing was calculated by comparing the levels of radioactivity of cells with or without exposure to T cells. Gastric T cells could kill Kato-III cells, and this cytotoxicity was blocked effectively by anti-Fas IgG antibody (ZB4; 500 ng/ml) but not by an isotype control (mouse IgG [mIgG]) ($P < 0.05$). These data are representative of three separate experiments.

stomach during *H. pylori* infection was capable of mediating apoptosis in Fas-bearing cells.

To confirm the Fas/FasL interaction between T cells and epithelial cells, Kato-III cells were incubated with gastric T-cell lines and assayed for apoptosis using the JAM test. As shown in Fig. 7, gastric T cells killed gastric epithelial cells and this interaction was blocked by anti-Fas antibody ZB4 ($P < 0.05$ compared to the isotype control, mouse IgG). This observation further supports the hypothesis that Fas/FasL interactions are involved in the lymphoepithelial cell interactions that lead to epithelial cell damage during *H. pylori* infection.

DISCUSSION

Diseased tissue is often associated with cell death due to various processes, including necrosis and apoptosis. Apoptosis is a normal mechanism that controls epithelial cell turnover; however, the rate of apoptosis can be altered in response to infection and inflammation. Induction of apoptosis by *H. pylori* has been reported in subjects with duodenal ulcer and gastritis (25). Jones et al. found that the apoptotic index increased threefold in persons with *H. pylori* gastritis compared to non-inflamed controls, and this change decreased following the eradication of *H. pylori* and resolution of gastritis (25). Other authors have described an increase in both the proliferation rate and the apoptotic index in *H. pylori*-infected stomachs (4, 29, 39). These observations imply that during infection with *H. pylori*, gastric epithelial apoptosis is induced and followed by an increase in the repair process. However, the mechanisms regulating cell death have only begun to be defined. Recently, it has been shown that *H. pylori* can induce apoptosis directly (13, 24, 41, 49). In addition, some cytokines, such as TNF-α or IFN-γ, can induce apoptosis directly, as well as augment the effects of the bacteria alone (13, 49). The results presented here provide evidence that T cells can contribute to epithelial cell death through Fas/FasL interactions.

IFN-γ-producing T cells, both $CD4^+$ and $CD8^+$, have been reported to be increased in the gastric mucosa of infected subjects (3, 17). Importantly, D'Elios and colleagues have shown that some gastric Th1 cell lines specifically recognize *H. pylori* antigens and produce both TNF-α and IFN-γ (9). More recent immunohistochemical evidence suggests that cytokine-producing Th1 cells predominate in the gastric antral mucosa during infection (31, 44). In addition, Th1 cells also dominate in the healthy uninfected gastric mucosa (17) or in a state of gastritis for other reasons (26). Thus, the predominance of Th1 cells suggests that local T cells contribute significantly to cell-mediated immunity targeted toward the epithelium during gastritis for various reasons.

One mechanism by which T cells kill their targets is expression of FasL and its binding to the Fas receptor. This mechanism of killing can be achieved by both $CD8^+$ cytotoxic T lymphocytes, as well as $CD4^+$ Th1 cells (18, 46, 51). T cells isolated from gastric tissues and expanded in vitro expressed FasL mRNA and were capable of inducing apoptosis in target cells bearing Fas. Unfortunately, too few T cells were isolated from gastric biopsy specimens to permit direct evaluation of FasL expression and function, therefore requiring the activation in vitro that may have induced FasL expression artificially. However, RT-PCR showed that FasL expression was increased in gastric tissue and immunohistochemistry confirmed the increase in FasL protein, including its expression on mononuclear cells adjacent to the epithelium. These data provide a

human correlate of Fas/FasL-mediated epithelial cell damage that occurs in response to lymphoepithelial interactions in mice (16, 23, 30, 42, 45). Thus, epithelial cell damage may play a role in the pathogenesis of gastrointestinal disease in humans, as well as in the animal models studied to date.

In order for T cells bearing FasL to mediate apoptosis in gastric epithelial cells, the target must express the Fas receptor. Fas is expressed on T cells and is a key factor in the differentiation of T cells and prevention of autoimmune diseases. However, Fas can be expressed by a variety of cells that can be damaged by T cells expressing FasL and has therefore been associated with the development of some autoimmune diseases (5). For example, it has been found that Fas is involved in the development of murine autoimmune gastritis (36).

Previous reports have described the expression of Fas on gastric epithelial cell lines (19, 21, 24, 28). Moreover, Fas molecules on the surface of gastric epithelial cells can be stimulated to induce apoptosis (19, 22, 24). Rudi et al. recently reported that *cagA*$^+$ *H. pylori* strains can upregulate the expression of Fas and FasL on gastric tissues, and this may contribute to the apoptosis during infection (41). In our study, gastric epithelial cells expressed Fas both in vivo and in vitro, verifying that they can serve as target cells in Fas-mediated cell death. Approximately 20% of the freshly isolated cells expressed Fas, as detected by fluorescence-activated cell sorter. Immunohistochemistry supported the fluorescence-activated cell sorter findings by demonstrating that Fas expression on gastric epithelial cells in gastric biopsy specimens from infected subjects was higher than that in uninfected tissues. Furthermore, the high levels of Fas expression were unevenly distributed, preferentially in the neck of the stomach. This is the region in which cytokine-producing T cells are enriched (3), suggesting that induction of Fas is greatest adjacent to cytokine-producing cells.

Th1 cytokines (52), including IFN-γ and TNF-α, and *H. pylori* infection (15, 27, 34, 43, 47) activate transcription factors in gastric epithelial cells that bind to the NF-κB and AP-1 regulatory sites. Since these regulatory sites are located in the 5′ region of the *fas* gene, it is possible that both can contribute to the induction of Fas expression. However, our results showed that *H. pylori* strain 8-1, which lacks the *cag* PAI, induced Fas expression, although to a lesser degree. Since the activation of NF-κB by *H. pylori* is dependent on the presence of the *cag* PAI (15, 52), AP-1 likely plays a role in the induction of Fas. Possibly, other transcription factors play a role but remain to be defined.

Three different cell lines expressing Fas were susceptible to apoptosis after being exposed to anti-Fas antibodies and/or T cells expressing FasL. Interestingly, the ability to detect apoptosis was dependent on the assay being used. Based on the detection of DNA fragmentation using electrophoresis, apoptosis could only be detected after pretreatment with IFN-γ to increase the expression of Fas. This is similar to another observation using intestinal epithelial cell lines (1). However, using the ELISA or the JAM test, apoptosis could be detected without boosting the expression of Fas. Therefore, cytokines can not only induce apoptosis directly but also increase the expression of Fas and enhance cell death by Fas/FasL interactions. It remains to be determined if some of the synergism is due to the effect of cytokines on signaling pathways that "arm" the cell to respond more efficiently subsequent to the engagement of the Fas receptor.

Further evidence in support of the notion that gastric T-cell cytokines collaborate in the regulation of epithelial cell death is found in the presence of IFN-γ- and TNF-α-producing cells within the gastric mucosa. IFN-γ and TNF-α are known to increase Fas expression on several types of cells, including renal endothelium (38), intestinal epithelium (50), and astrocytes (6). We found that these Th1 cytokines could act synergistically with *H. pylori* to increase Fas expression on all three of the gastric epithelial cell lines tested (AGS-NY2, Kato-III, and N87), which extends previous observations describing the regulation of Fas on gastric epithelial cells (24). Thus, Th1 cytokines can not only injure the gastric epithelium directly (13, 49) but also accelerate epithelial damage by modulating Fas/FasL interactions.

In conclusion, *H. pylori* infection induces the expansion of Th1-like cells that have the potential to express FasL. T cells with this phenotype can increase the expression of Fas on gastric epithelial cells and induce cell death through Fas/FasL interactions. Thus, apoptosis of the gastric epithelium can occur due to multiple mechanisms and thereby lead to a breach in the epithelial barrier that facilitates tissue damage due to luminal acid and pepsin.

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