In situ correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis

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Lehmann, Frank Serge, Luigi Terracciano, Ilaria Carena, Christian Baeriswyl, Jürgen Drew, Luigi Tornillo, Gennaro De Libero, and Christoph Beglinger. In situ correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis. Am J Physiol Gastrointest Liver Physiol 283: G481–G488, 2002. First published February 20, 2002; 10.1152/ajpgi.00422.2001.—Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are important for the pathogenesis of Helicobacter pylori-associated gastritis and peptic ulcer disease. Gastric biopsies from H. pylori-positive and -negative patients were used to examine the in situ correlation of TNF-α and IFN-γ with epithelial cell apoptosis, bacterial load, and histological parameters of gastritis. From the same patients, we isolated H. pylori-specific T cell lines and clones and examined their ex vivo release of proinflammatory cytokines. We found a highly significant correlation of TNF-α and IFN-γ production with activity and grade of gastritis (P < 0.01), H. pylori density (P = 0.01), epithelial cell apoptosis (P < 0.001), and Fas/Fas-ligand expression (P < 0.001). T cell lines and clones were all TCR-αδ+ and showed T helper 1 functional phenotype. With the use of serial histological sections, this study showed for the first time the in situ correlation of TNF-α and IFN-γ with epithelial cell apoptosis, bacterial load, and histological severity of disease and emphasizes the role of these cytokines in the pathophysiology of H. pylori-associated disease.

H. pylori infection generally persists for life with a widespread gastric inflammatory response, implying that the local mechanisms are ineffective in clearing the infection. Failure to resolve the infection probably results from induction of an inappropriate immune response and might be facilitated by an exaggerated activation of T cells.

In vitro studies suggest that in H. pylori infection, T cells infiltrating the gastric mucosa mainly produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). In previous studies, we examined the effect of TNF-α and IFN-γ on gastrin release (20) as well as on migration and proliferation of isolated gastric cells (18) and suggested that TNF-α could be one of the key cytokines in H. pylori-associated gastritis and peptic ulcer disease (20). Recently, several studies have been published about the origin of TNF-α- and IFN-γ-producing cells (1, 21). Fas and Fas-ligand (Fas-L) expression have been described in freshly isolated gastric epithelial cells, on mononuclear cells, and in gastric T cell lines, as well as in gastric adenocarcinoma (3). Furthermore, studies have shown the association among H. pylori gastritis, T cell infiltration, and class II major histocompatibility complex expression in gastric epithelial cells (29, 33).

Cell proliferation and apoptosis are essential events for normal cellular turnover of gastric tissue. In the healthy stomach, a balance exists between proliferation of new epithelial cells and death of senescent cells (15, 17). Induction of apoptosis by H. pylori has been observed in patients with gastritis and duodenal ulcers (15) and subsides after successful H. pylori eradication (15, 17, 23, 25). Sustained stimulation of apoptosis could ultimately result in excessive cell loss and ulcer development (in the acute phase) or atrophy (in the chronic phase) and promote the subsequent occurrence of gastric adenocarcinoma. The mechanism(s) by which H. pylori induces apoptosis is unknown. TNF-α and IFN-γ could be involved in the development of apoptosis as well as signaling pathways including stimulation...
of specific cell death receptors such as Fas/Fas-L. Fas expression has been described in gastric carcinoma cells as well as in tissue samples of \textit{H. pylori}-infected patients (26).

The aim of this study was to examine the in situ correlation of TNF-\( \alpha \) and IFN-\( \gamma \) with epithelial cell apoptosis, bacterial load, and histological parameters of gastritis by the use of serial histological sections from the same biopsies. From the same patients, we isolated \textit{H. pylori}-specific T cell lines and clones and investigated their ex vivo release of proinflammatory cytokines.

**MATERIALS AND METHODS**

**Patients and biopsies.** Twenty \textit{H. pylori}-positive patients and 12 \textit{H. pylori}-negative controls were included in the study. \textit{H. pylori}-positive patients had a median age of 57.5 (range 32–83) yr. Four patients had peptic ulcer disease, and 16 had dyspepsia. Patients treated with nonsteroidal anti-inflammatory drugs, antibiotics, proton pump inhibitors, H2 receptor antagonist, or bismuth at least 2 mo before study enrollment were excluded. \textit{H. pylori} status was determined in all patients by urease test, histology, and culture. Patients were considered positive if at least two of three tests were positive. All patients with positive culture or immunohistochemistry were \textit{H. pylori} positive. The \textit{H. pylori}-negative control group consisted of 12 dyspeptic patients with a median age of 54 (range 26–82) yr.

**Endoscopy.** Upper gastrointestinal endoscopy was performed after overnight fast. Six antrum and three body biopsies were taken for histology, urease test, and bacterial cultures. Two antrum and one body specimens were placed in formalin and embedded in paraffin for histopathological examination. Two antrum and one body biopsies were snap frozen for immunohistochemical characterization of cytokine production and dUTP nick-end labeling (TUNEL) assay. One antrum biopsy was used for the urease test; one antrum and one body specimen were put in cell culture medium.

**Histological and immunohistochemical assessment.** Paraffin sections were stained with hematoxylin-eosin and modified Giemsa. Each biopsy specimen was assessed for the presence, type, and density of the inflammatory infiltrate and the presence of \textit{H. pylori}. Gastritis was graded as absent, mild, moderate, and severe according to the updated Sydney system (10).

Characterization of inflammatory cells was performed by immunohistochemical staining with the ABC method according to the manufacturer’s instructions (Vectastain Elite, ABC kit). The following antibodies were used for immunohistochemical studies: anti-CD20 (clone L26), rabbit-anti-human CD3 (model A452), anti-CD68 (KP1) (all from DAKO, Carpinteria, CA), anti-CD14 (Coulter), anti-human leukocyte antigen D-related (HLA-DR) (model L243; Dade-Behring, Düdingen, Switzerland), anti-TNF-\( \alpha \) (model MAB11; Pharmingen, San Diego, CA), anti-IFN-\( \gamma \), (model 1598–00; Genzyme Diagnostics, Cambridge, MA), anti-Fas (APO/Fas, DAKO) and anti-Fas-L (model Q-20; Santa Cruz Biotechnology, Santa Cruz, CA). Streptavidin-biotinylated peroxidase (LAB-SA system, Zymed Laboratories, San Francisco, CA) or goat anti-mouse Ig-peroxidase (DAKO) were used to detect positive cells.

Four millimeter-thick frozen sections were obtained from each snap-frozen biopsy, postfixed in 4% paraformaldehyde, washed for 30 min in PBS, and again fixed for 2 min in ethanol. Slides were air-dried and stored at \(-20^\circ\text{C}\). Frozen sections were obtained, and a periodate-lysine-paraformaldehyde solution (4%) was used for postfixation. Identification of cytokine-secreting cells was accomplished by staining of adjacent serial sections, as previously described (31).

**Urease test.** The urease test (Delta West, Perth, Australia) was performed according to the manufacturer's instructions. Results were considered positive if a definite red color developed within 24 h.

**Bacteriologic assessment.** \textit{H. pylori} was cultured using two biopsies from the antral and body mucosa of each patient as previously described (11).

**TUNEL assay.** Apoptosis was examined by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) method. Slides were washed in PBS, then washed in \( \text{H}_2\text{O}_2 \), mounted with Gelvatol-Gallatin medium, and analyzed by fluorescence microscopy. At least 500 epithelial cells were counted in each section and the number of positive cells per 100 cells was expressed as the apoptotic index (in %).

**T cell isolation and culture.** T cells were cultured in RPMI 1640 medium supplemented with 2 mM Glutamax I, 10 \( \mu\text{g/ml} \) Kanamycin, 1 mM MEM nonessential amino acids, and 1 mM Na pyruvate (all from GIBCO-BRL, Life Technologies, Basel, Switzerland), 5% human serum (Blutspendezentrum, Basel, Switzerland), and 100 U of recombinant human interleukin-2 (IL-2). \textit{H. pylori}-specific T cell lines were established from peripheral blood mononuclear cells (PBMC) by incubating with 2 \( \times \) 10\(^5\) PBMC in IL-2-free RPMI medium and 100 \( \mu\text{g/ml} \) heat-inactivated \textit{H. pylori}. After 5 days of culture, 10 U/ml IL-2 were added and the cultures were further expanded. After 2 wk, growing cells were restimulated using autologous-irradiated PBMC and 100 \( \mu\text{g/ml} \) heat-inactivated \textit{H. pylori}. Specificity of T cells was assessed at days 28–32 of culture.

\textit{H. pylori}-specific T cell lines were also raised from gastric biopsies as previously described (8). Briefly, biopsies were collected in complete RPMI medium added to gentamycin (50 \( \mu\text{g/ml} \) (GIBCO-BRL), cyproflaxacin (10 \( \mu\text{g/ml} \) (Bayer, Zurich, Switzerland), and fungizone (2.5 \( \mu\text{g/ml} \) (GIBCO-BRL)), transferred in a petri dish and extensively washed to eliminate contaminating blood lymphocytes. Tissue was digested 5 h at 37\(^\circ\text{C}\) with collagenase type IV (200 U/ml; Sigma, Buchs, Switzerland) and deoxyribonuclease type I (2 mg/ml; Sigma). Cells were washed and seeded at 1–5 \times 10^6/ml together with the same amount of irradiated autologous PBMC as feeder cells and 100 \( \mu\text{g/ml} \) heat-inactivated \textit{H. pylori}.

T cell clones were established as previously described (8). Each clone was tested for reactivity against autologous Epstein-Barr virus (EBV)-transformed B cells in the presence and absence of heat-inactivated \textit{H. pylori} using ELISA for detection of cytokines. Ag-specific clones were expanded, restimulated, and used in further experiments. From each donor, autologous EBV-transformed B cells were established as previously described (8).

**Flow cytometry of cultured T cells.** The following monoclonal antibodies (MBs) were used: TR66 (anti-CD3), B1 (anti-pan TCR-\( \gamma \delta \)), anti-CD4, and anti-CD8 (Serotec, Oxford, UK) and FITC-labeled goat anti-mouse total Ig (Southern Biotechnology Associates, Birmingham, AL). Cells were stained as reported (9). After washing, cells were immediately analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Dead cells were excluded by propidium iodeid...
staining. Data analysis was performed using the CELLQuest program (Becton Dickinson).

Cytokine release assays. T cells (5 × 10⁴/well) were stimulated with heat-inactivated H. pylori (50 μg/ml) in triplicate cultures in flat-bottom 96-well plates in the presence of EBV-transformed autologous B cells as antigen-presenting cells (APCs; 5 × 10⁴/well). After 18 h of incubation, 150 μl of supernatant were removed and used to test the content of TNF-α, IFN-γ, and IL-4 by ELISA using commercial kits (R&D Systems, Wiesbaden, Germany). Recombinant humanTNF-α (0.1–100 ng/ml), IFN-γ (0.1–100 U/ml), and IL-4 by ELISA using commercial kits were used as standard. Recombinant human cytokines were used as standard.

Statistical analysis. A semiquantitative grading system (absent, minimal, moderate, and severe) was adopted for immunohistochemical evaluation. Results were further ranked into two grades: absent/minimal and moderate/severe. For comparison of incidence among patients, H. pylori-positive and H. pylori-negative patients, χ²-test or Fisher’s exact test was used, as appropriate. Furthermore, for correlation of semiquantitative parameters a Spearman rank correlation test was assessed as adequate. Calculations were performed running a SAS software package on an Apple G3 computer. P < 0.05 was considered significant.

RESULTS

Gastritis. In the H. pylori-positive group, four patients had mild, nine had moderate, and seven had severe chronic type B gastritis. In the H. pylori-negative control group, five had normal histology and seven had minimal lymphocytic infiltration of the tunica propria.

HLA-DR expression. HLA-DR expression was observed in foveolar epithelium, superficial stroma, and deep stroma. As suggested by Sarsfield et al. (28), HLA-DR expression was categorized as absent, scanty, moderate, or abundant. Results were further ranked into two grades: absent/scanty and moderate/abundant and a semiquantitative analysis was performed. In all compartments, HLA-DR expression was significantly more pronounced in H. pylori-infected samples (P < 0.001; Table 1). In the foveolar epithelium, HLA-DR expression was, in most cases, limited to the crypts and virtually never occurred in the superficial epithelium. HLA-DR expression was absent in intestinal metaplasia. In the stroma, the majority of mononuclear cells expressed HLA-DR (Fig. 1, A and B). HLA-DR expression was positively correlated with activity and grade of gastritis as well as density of H. pylori (P < 0.001; Table 2).

In situ characterization of TNF-α- and IFN-γ-secreting cells. Staining with anti-TNF-α MAb was exclusively detected in large mononuclear cells, which were also stained with anti-CD68 and CD14 MAbs (Fig. 1, C–E). In contrast, anti-IFN-γ MAb detected only T lymphocytes, identified by reactivity with anti-CD3 MAbs but not with anti-CD20 MAbs. The number of TNF-α- and IFN-γ-positive cells was significantly higher in H. pylori-positive biopsies (P < 0.01; Table 1). TNF-α- (Fig. 1C) and IFN-γ-positive cells showed a diffuse distribution in the stroma of patients with moderate and severe gastritis but were limited to the vicinity of gastric epithelium in mild gastritis. In H. pylori-positive biopsies, the number of TNF-α- and IFN-γ-expressing cells positively correlated with activity and grade of gastritis (P < 0.05; Table 2) and local density of H. pylori (P = 0.01; Table 2). Cells stained with anti-IFN-γ MAbs (i.e., recently activated T cells) could exclusively be detected in H. pylori-positive patients.

Positive correlation of apoptosis with H. pylori gastritis. Apoptotic epithelial cells were observed in the superficial gastric mucosa of all patients. In H. pylori-positive samples, apoptotic cells were detected also in deep glands (Fig. 1H). The number of apoptotic epithelial cells was significantly higher in H. pylori-positive (P < 0.01; Table 1) (apoptotic index of 24.8, median 11.2, range 1–56%) than in H. pylori-negative biopsies (apoptotic index of 3.6, median 2.4, range 0–10%). The correlation of apoptosis and grade of gastritis was highly significant (P < 0.001; Table 2).

Fas/Fas-L expression. Fas/Fas-L expression was mainly detected in the epithelial cells of H. pylori-positive patients and only occasionally in stromal inflammatory cells (Fig. 1, F and G). Germinal centers in lymphoid follicles also showed dull Fas expression. The number of Fas-expressing epithelial cells was significantly higher in H. pylori-positive biopsies and in severe rather than mild gastritis (P < 0.05; Table 1). Fas-L expression was also more pronounced in H. pylori-positive samples (P < 0.001; Table 1). The correlation of Fas-L and activity/grade of gastritis was highly significant (P < 0.001; Table 2).

Correlation of cytokine production, apoptosis, and Fas/Fas-L expression. Apoptosis of stromal cells was significantly correlated with the in situ detection of TNF-α (P < 0.001) and IFN-γ (P < 0.05; Table 2). Apoptosis of stromal cells was found in the vast majority of TNF-α-expressing samples and only in one TNF-α-negative patient. Furthermore, TNF-α and IFN-γ production was significantly correlated with Fas (P < 0.05) and Fas-L expression (P < 0.001 for TNF-α; P < 0.05 for IFN-γ).

Establishment of T cell lines and clones specific for H. pylori from gastric biopsies. To better characterize the nature of T lymphocytes infiltrating the gastric mucosa.

### Table 1. Correlation between Helicobacter pylori positivity, expression of different cytokines, and apoptotic index

<table>
<thead>
<tr>
<th>H. pylori positivity</th>
<th>Apoptotic index</th>
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<tr>
<td>Absent/minimal</td>
<td>3.6</td>
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<tr>
<td>Moderate/severe</td>
<td>24.8</td>
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P values were derived by the χ²-test with Fisher’s correction. TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; Fas-L, Fas-ligand, epit, epithelium; sup, superficial; HLA-DR, human leukocyte antigen D-related.
Fig. 1. Immunohistochemical detection of human leukocyte antigen D-related (HLA-DR), tumor necrosis factor-α (TNF-α) CD68, Fas, Fas ligand (Fas-L) and apoptosis in H. pylori-positive gastric biopsies. A: HLA-DR expression in infiltrating mononuclear cells. B: interferon-γ (IFN-γ) expression in mononuclear cells. C: distribution of TNF-α secreting cells in moderate chronic active gastritis (magnification ×100). D: serial section staining of large mononuclear cells with anti-TNF-α (magnification ×400) and E: with anti-CD68 monoclonal antibodies (MAbs) (magnification ×200). Fas (F) and Fas-L (G) expression in the epithelium. H: detection of apoptotic cells in superficial and in deep foveolar epithelial cells by dUTP nick-end labeling.
of *H. pylori*-infected patients, T cell lines were derived from antrum biopsies of 11 patients. Peripheral and mucosal T cell lines showed the same reactivity. As control, 11 lines were established from peripheral blood of the same donors. Ten lines were derived from *H. pylori*-positive patients and one from a negative donor. When the lines were tested in the presence of EBV-transformed B cells as APCs and heat-inactivated *H. pylori*, 6 of 11 lines derived from gastric tissue showed in vitro reactivity to *H. pylori*. The cell derived from the *H. pylori*-negative patient was nonreactive. Instead, 9 of 11 lines from peripheral blood were *H. pylori*-reactive (Fig. 2). These T cells specifically released both TNF-α and IFN-γ on stimulation with *H. pylori*.

Four bulk lines from two patients were further investigated by isolation of individual T cell clones. A total number of 279 T cell clones was isolated from two lines derived from gastric tissue and two lines from peripheral blood. Forty-eight clones showed *H. pylori*-specific release of TNF-α and IFN-γ. Twelve clones were isolated from one donor (1 from PBMC; 11 from gastric biopsy) and 36 from a second donor (23 from PBMC; 13 from gastric biopsy). All specific clones were TCR-αβ⁺, CD66a⁺, immunoglobulin-like transcript (ILT)-2, and ILT-3 negative. Two clones were CD8⁺, whereas all others were CD4⁺. The reactivity of one prototype T cell clone is shown in Fig. 3. Lymphokines released by these clones were assessed in parallel on the same supernatants. Most clones released IFN-γ and TNF-α but not IL-4, thus showing a T helper 1 (Th1) functional phenotype.

### DISCUSSION

*H. pylori* infection is histologically characterized by the presence of a large number of T cells in the lamina propria. A sizable fraction of these lymphocytes are specific for bacterial antigens and release proinflammatory lymphokines (32). Our data confirm that in the gastric mucosa, the host response to *H. pylori* is mainly characterized by T lymphocytic infiltration and local secretion of IFN-γ (21). In moderate and severe gastritis, IFN-γ-secreting T cells were diffuse in the stroma, likely because they were locally stimulated. In these forms of gastritis, the number of IFN-γ-secreting T cells correlated with the density of *H. pylori*. This finding suggests that in case of high bacterial density, a large amount of antigen is available to professional APCs that stimulate T cells in the stroma. In contrast, in mild gastritis, IFN-γ-secreting T cells were found in the proximity of epithelial cells, indicating that in this form of gastritis, the *H. pylori* stimulatory antigens remain mostly confined to epithelial cells, where the bacteria are located. Perhaps, the same epithelial cells present bacterial products to T cells, as shown in vitro with other types of antigens (24). The finding that a

<table>
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<tr>
<th>H. Pylori</th>
<th>Grade of Gastritis</th>
<th>Activity of Gastritis</th>
<th>TNF-α</th>
<th>IFN-γ</th>
</tr>
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<tbody>
<tr>
<td>HLA-DR epit.</td>
<td>0.6842 (P &lt; 0.001)</td>
<td>0.4748 (P &lt; 0.001)</td>
<td>0.5759 (P &lt; 0.001)</td>
<td>0.3647 (P = 0.04)</td>
</tr>
<tr>
<td>HLA-DR sup. stroma</td>
<td>0.7271 (P &lt; 0.001)</td>
<td>0.6328 (P &lt; 0.001)</td>
<td>0.6792 (P &lt; 0.001)</td>
<td>0.4538 (P &lt; 0.01)</td>
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<tr>
<td>HLA-DR deep stroma</td>
<td>0.6451 (P &lt; 0.001)</td>
<td>0.3277 (P &lt; 0.001)</td>
<td>0.6571 (P &lt; 0.001)</td>
<td>0.5920 (P &lt; 0.01)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.3543 (P &lt; 0.001)</td>
<td>0.5091 (P &lt; 0.002)</td>
<td>0.6462 (P &lt; 0.001)</td>
<td>0.6347 (P &lt; 0.01)</td>
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<td>Apoptosis</td>
<td>0.3855 (P &lt; 0.001)</td>
<td>0.4750 (P &lt; 0.05)</td>
<td>0.6554 (P = 0.004)</td>
<td>0.6347 (P &lt; 0.01)</td>
</tr>
<tr>
<td>Fas</td>
<td>0.4916 (P &lt; 0.001)</td>
<td>0.3839 (P &lt; 0.01)</td>
<td>0.4766 (P &lt; 0.001)</td>
<td>0.7801 (P &lt; 0.001)</td>
</tr>
<tr>
<td>Fas-L</td>
<td>0.4082 (P &lt; 0.01)</td>
<td>0.3695 (P &lt; 0.03)</td>
<td>0.5737 (P &lt; 0.001)</td>
<td>0.4027 (P &lt; 0.05)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.7874 (P &lt; 0.001)</td>
<td>0.6849 (P &lt; 0.001)</td>
<td>0.7338 (P &lt; 0.001)</td>
<td>0.7338 (P &lt; 0.001)</td>
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</table>

Values were derived by Spearman rank correlation test. P < 0.05 was considered significant.
strong epithelial and stromal expression of HLA-DR molecules is present during *H. pylori* infection supports this possibility. HLA-DR expression, which we also found positively correlated with *H. pylori* density as well as with activity and grade of gastritis, is not homogeneous in the gastric mucosal epithelium. Isthmic areas were brightly stained, whereas the surface epithelium was dull as previously reported (26). This is a paradoxical finding, considering that epithelial cells are in intimate contact with mononuclear cells secreting proinflammatory cytokines and that these soluble factors induce the expression of high levels of HLA-DR on epithelial cells in vitro (30). It is tempting to speculate that mature epithelial cells migrating upward in the gastric mucosa become less sensitive to these cytokines. The low expression of human leucocyte antigen (HLA) class II molecules has no relevant consequences for antigen presentation, because T cells are mainly in contact with the epithelial cell lining at the bottom of crypts or in the isthmic regions, which instead are HLA class II bright. The absence of HLA-DR on superficial epithelial cells might instead be important to reduce bacterial adhesion, because *H. pylori* uses HLA class II molecules as additional cellular receptors (13).

Our data show that TNF-α is released in the gastric mucosa during *H. pylori* infection. With the use of immunohistochemistry, CD68-positive cells were stained with anti-TNF-α MAbs, whereas T cells that appeared IFN-γ positive were TNF-α-negative. This result is different from what we observed in T cell clones. This difference might be attributed to the lower sensitivity of immunohistochemistry versus ELISA for the detection of this cytokine, or to the presence of high amounts of membrane-bound TNF-α on the surface of monocytes but not of T cells in vivo. However, it is also possible that indeed TNF-α is not produced by T cells when locally stimulated in the gastric mucosa, and it is thus conceivable that clones do not exactly reflect in vivo events. Further experiments are needed to determine whether presentation of bacterial antigens by monocytes, dendritic cells, and gastric epithelial cells stimulate secretion of different lymphokines by T cells. The finding that high numbers of locally activated T cells are present in patients with severe gastritis raises the question about the role of these cells in *H. pylori* infection. We agree with the current concept that *H. pylori*-specific T cells directly contribute to local inflammation.

*H. pylori*-specific T cell clones that we isolated from gastric tissue showed a Th1-like functional phenotype. They released large amounts of TNF-α and IFN-γ in vitro, but no IL-4. T cell clones specific for *H. pylori* isolated from infected gastric tissue have been previously studied, and they were predominantly Th1 as well (6, 7). In addition, other investigators reported prevalence of Th1 phenotype in mitogen-activated polyclonal T cells isolated from *H. pylori*-infected gastric mucosa (2).

In our study, a second important observation was the detection of a large number of apoptotic cells in *H. pylori*-positive biopsies. Apoptotic epithelial cells were present both on the superficial mucosa and deep glands. In both locations, epithelial cells may have been killed by interaction with soluble cytotoxic products released by bacterial cells. This possibility is supported by the highly significant correlation that we found between apoptosis and number of *H. pylori* cells. Apoptosis was also significantly correlated with activity and grade of gastritis, which is at odds with other reports (17, 25). One possible explanation for this discrepancy is that previously published studies were performed on paraffin-embedded and not on frozen material, as in our study. We have already previously found that detection of apoptotic cells is much more sensitive in frozen samples than in paraffin-embedded material.

It is likely that multiple mechanisms cause apoptosis of epithelial and stromal cells during *H. pylori* infection. Local release of TNF-α and IFN-γ interaction between Fas and Fas-L expressing cells and direct killing by activated T cells or by *H. pylori* cytotoxins may all contribute to the observed apoptosis. TNF-α may induce cell apoptosis directly (35) and can potentiate DNA-fragmentation induced by *H. pylori* in vitro (34). It is remarkable that among the parameters we studied, the presence of TNF-α-secreting cells and apoptosis were those showing the highest correlation (P < 0.001). Elevated and persistent TNF-α secretion may contribute to the establishment of tissue lesions reported in other inflammatory disease models (19).

Like TNF-α, IFN-γ may also facilitate the *H. pylori*-induced apoptosis of epithelial cells. In the literature, controversy exists whether local production of IFN-γ is increased in the gastric mucosa during *H. pylori* infection. In vitro data suggest that IFN-γ-producing T cells are present in large numbers in infected mucosa (7). In our study, IFN-γ-secreting T lymphocytes were exclusively found in *H. pylori*-infected biopsies. Furthermore, a positive correlation was detected between IFN-γ expression and TUNEL-positive cells. IFN-γ may modulate epithelial cell apoptosis by several mechanisms, for example, by increasing Fas surface expression, by sensitizing epithelial cells toward Fas-induced apoptosis, and by upregulating TNF-α receptors, thus facilitating TNF-α-mediated apoptosis (4, 16, 27).

Expression of Fas and Fas-L is a third mechanism that may directly contribute to apoptosis of epithelial cells. In contrast to another study (26), we found that epithelial cells and not stromal cells were Fas positive. Thus preferential expression of Fas by epithelial cells may explain why they were also the most frequent apoptotic cell type. We also found that epithelial cells express Fas-L.

Therefore, a cross talk among epithelial cells themselves might contribute to the development of the histological lesions. Similar findings have been reported in Hashimoto’s thyroiditis, in which Fas/Fas-L inter-
actions among thyrocytes lead to clinical hypothyroidism (14).

In conclusion, we found a positive correlation of TNF-α and IFN-γ with activity and grade of gastritis, H. pylori density, Fas/Fas-L expression, and apoptosis of epithelial cells. These findings support the important role of TNF-α and IFN-γ in H. pylori-associated gastritis and peptic ulcer disease.

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