Decreased Epithelial Cytokine Responses in the Duodenal Mucosa of Helicobacter pylori-Infected Duodenal Ulcer Patients

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Helicobacter pylori colonization is the most common gastrointestinal bacterial infection worldwide. However, even though half of the world's population is infected with H. pylori, only 10 to 15% of those infected develop peptic ulcer disease (29). The different clinical outcomes may be explained by variations in bacterial virulence factors, as well as differences in the host immune responses. H. pylori induces a strong immune response in the stomach, which nevertheless usually fails to resolve the infection. In the gastric mucosa, H. pylori antigens, together with cytokines induced by the infection, attract and activate leukocytes. During the acute phase, polymorphonuclear neutrophils (PMNs) and monocytes accumulate in the gastric mucosa (36), which leads to increased epithelial permeability and acute mucosal damage (49). After the initial acute phase the inflammation is maintained and is characterized by infiltration of lymphocytes into the mucosa. It has been suggested that the H. pylori-induced T-cell response is predominantly a Th1-type response since increased numbers of gamma interferon (IFN-γ)-producing T cells can be detected in the infected gastric mucosa compared to the numbers of interleukin-4 (IL-4) and IL-5-producing cells (Th2-type cytokines), which remain unchanged (3, 18, 28). Furthermore, D'Ellios et al. (11) have shown that T-cell clones generated from antral biopsy specimens of H. pylori-infected peptic ulcer patients produce IFN-γ and IL-12 but usually not IL-4 or IL-5 in response to H. pylori antigen stimulation. The levels of several cytokines, both proinflammatory and immunoregulatory, have been shown to be elevated in the H. pylori-infected gastric mucosa. IL-8, a neutrophil-attracting cytokine, has been suggested to be important in H. pylori-associated disease (2, 13, 31, 39). Furthermore, the levels of expression of IL-1β, IL-6, tumor necrosis factor alpha, and transforming growth factor β (TGF-β), and IFN-γ-positive mononuclear cells were observed in the duodenal lamina propria of both DU patients and AS carriers than in that of the uninfected controls. Our finding that a number of cytokines that may be important for the mucosal host defense against H. pylori are strongly decreased in the duodenal epithelium of ulcer patients suggests that a down-regulated immune response plays a role in the development of duodenal ulcers.

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differences in the local immune response. The cytokine levels in both the epithelium and the lamina propria of normal mucosa as well as metaphasic duodenal mucosa of *H. pylori*-infected AS carriers and DU patients were analyzed by immunochemistry.

**MATERIALS AND METHODS**

**Subjects.** This study was approved by the Human Research Ethics Committee of the Medical Faculty, Göteborg University, Göteborg, Sweden. Six DU patients (mean age, 45.3 years; age range, 24 to 50 years; five men and one woman), six *H. pylori*-infected AS carriers (mean age, 53.7 years; age range, 25 to 78 years; five men and one woman), and six uninfected, healthy Swedish volunteers (mean age, 46.5 years; age range, 25 to 61 years; three men and three women) were included in the study. Informed consent was obtained from all participants. *H. pylori* infection was confirmed by serology and culture. The healthy individuals and the AS *H. pylori*-infected subjects had no history of gastrointestinal disease or symptoms. None of the *H. pylori*-infected subjects had taken any antibiotic medication prior to sampling of duodenal biopsy specimens and had not taken any antisecretory medication for at least 10 days prior to endoscopy.

**Specimen collection and staining technique for identification of metaplastic areas in the duodenum.** The study subjects were given an antifoaming agent (Minifoam), and then gastroduodenal endoscopies were performed while the subjects were under local anesthesia with lidocain (Xylocain). A previously described method (14, 34), with some modifications (A. Edebo et al., unpublished data), was used to identify areas of DGM. The endoscope and the duodenal bulb were flushed with water, and 7 to 10 ml of methylene blue (1 mg/ml) was instilled. Two adjacent biopsy samples were taken from different areas of DGM and normal mucosa, respectively, and all individual biopsy specimens were cut in half. To avoid contamination, the biopsy forceps were flame sterilized and the individual biopsy specimens were stored at −20°C until they were assayed for *H. pylori*-specific antibodies.

**Histological examination of biopsy specimens.** Histological examination was done by one experienced pathologist, who was blinded to the patients’ clinical diagnosis. The extent of DGM in each biopsy specimen was determined as the percentage of the total epithelial surface and was graded 0, 1, 20, 20, or more than 50%. Only biopsy specimens with DGM in more than 20% of the epithelium were included in the study, and biopsy specimens regarded as normal lacked DGM completely. The chronic inflammation score, i.e., the density of mononuclear cells (MNCs), was determined separately from the active inflammation score, i.e., PMN infiltration; and both were graded from 0 to 3 (for none, mild, moderate, and severe, respectively) according to the Sydney system (43).

**Diagnosis of *H. pylori* infection.** One duodenal biopsy specimen from each sampling area and the antral biopsy specimen were used for culture of *H. pylori*.

**Cytokines in *H. pylori*-infected duodenum**

Collection of biopsy specimens from normal and metaphasic duodenal mucosa. *H. pylori* colonizes the duodenum only in areas of DGM (48, 53). In this study sections from one normal biopsy specimen and one metaphasic duodenal biopsy specimen from the same individual were analyzed in parallel. To discriminate between normal duodenal mucosa and the patchy islands of DGM, the duodenal bulb was stained with methylene blue, which stained the normal mucosa dark blue, while the areas of DGM remained unstained. The biopsy specimens were histologically evaluated for the degree of metaplasia, and the in vivo detection of gastric metaplasia in the duodenal bulb was found to agree well with the histological findings of DGM.

**MABs.** MABs were as follows: anti-IL-8 (NAP-1), Skafte-Claesson, Malmö, Sweden; anti-IFN-γ (1-D1K), MABTECH AB, Nacka, Sweden; anti-IL-6 and anti-TGF-β, Genzyme Diagnostics, Cambridge, Mass.; anti-IL-4 (clone SF12). Immunohistochemistry was performed with the corresponding recombinant cytokine for 3 h at 4°C, which abolished the immunostaining.

**Immunohistochemistry.** (i) Detection of cytokines. Immunohistochemical staining of duodenal biopsy specimens was performed on 8-μm frozen sections mounted on glass slides (SuperFrost/Plus; Menzel-Glaser, Braunschweig, Germany), as described previously (1, 5, 31). Briefly, sections were fixed in 2% paraformaldehyde, air dried, and frozen at −20°C for at least an hour. After permeabilization in phosphate-buffered saline (PBS)−0.1% saponin (Sigma, St. Louis, Mo), endogenous peroxidase activity was blocked by treating the tissue sections with 1% H2O2−0.02% NaN3 in PBS, and endogenous biotin was blocked with an Avidin-Biotin-Blocking kit (Vector Laboratories Inc., Burlingame, Calif.,) according to the instructions of the manufacturer. The tissue sections were incubated with the cytokine-specific MABs at 4°C overnight. After the sections were washed in PBS−0.1% saponin, they were treated with 1% normal goat serum and subsequently incubated with biotinylated goat anti-mouse IgG1 (Callig Laboratories, San Francisco, Calif.) diluted 1:300. Avidin-biotin-horseradish peroxidase complex (Vectastain ABC-HRP kit; Vector Laboratories Inc.) was added, and the sections were developed with the chromogen substrate 3,3-diaminobenzidine (Vector Laboratories Inc.) was added, and the sections were developed with the chromogen substrate

**RESULTS**

Collection of biopsy specimens from normal and metaphasic duodenal mucosa. *H. pylori* colonizes the duodenum only in areas of DGM (48, 53). In this study sections from one normal biopsy specimen and one metaphasic duodenal biopsy specimen from the same individual were analyzed in parallel. To discriminate between normal duodenal mucosa and the patchy islands of DGM, the duodenal bulb was stained with methylene blue, which stained the normal mucosa dark blue, while the areas of DGM remained unstained. The biopsy specimens were histologically evaluated for the degree of metaplasia, and the in vivo detection of gastric metaplasia in the duodenal bulb was found to agree well with the histological findings of DGM.

**Cytokine-specific MABs.** All cytokine-specific MABs used were mouse anti-human antibodies of the immunoglobulin G1 (IgG1) isotype. The sources of the MABs were as follows: anti-IL-8 (NAP-1), Skafte-Claesson, Malmö, Sweden; anti-IFN-γ (1-D1K), MABTECH AB, Nacka, Sweden; anti-IL-6 and anti-TGF-β, Genzyme Diagnostics, Cambridge, Mass.; anti-IL-4 (clone SF12). Immunohistochemistry was performed with the corresponding recombinant cytokine for 3 h at 4°C, which abolished the immunostaining.
In the duodenum of DU patients, with significantly higher levels of TGF-β (P = 0.0022), IL-1β (P = 0.0087), IFN-γ (P = 0.0043) in the metaplastic biopsy specimens and significantly lower levels of TGF-β (P = 0.0095), IL-6 (P = 0.0173), and IFN-γ (P = 0.0303) in normal duodenal biopsy specimens. Also, when the epithelial cytokine levels in DU patients and uninfected controls were compared, significantly lower levels of IL-8 (P = 0.03), IL-6 (P = 0.03), TGF-β (P = 0.0043), IFN-γ (P = 0.0043), and IL-4 (P = 0.0043) were found in the DU patients (Fig. 1).

To test whether the lower cytokine levels in the epithelium of DU patients were due to apoptosis of epithelial cells, biopsy specimens were also analyzed for DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labeling staining technique. However, the numbers of apoptotic cells in biopsy specimens from DU patients, AS carriers, and uninfected individuals were found to be similar.

Since we observed staining of the epithelium for IFN-γ, a cytokine not produced by epithelial cells, sections were also analyzed for the expression of the IFN-γ receptor. The epithelial cells were positively stained for the IFN-γ receptor (Fig. 2), and areas with strong IFN-γ staining were found to be associated with higher levels of IFN-γ receptor staining, and vice versa. No differences in the intensities of epithelial staining for any of the cytokines were observed between AS carriers, DU patients, and uninfected individuals.

**H. pylori infection induces cytokine production by MNCs in the lamina propria.** Tissue sections from normal and metaplastic biopsy specimens from AS carriers, DU patients, and uninfected controls were also examined for the presence of cytokine-specific MNCs in the lamina propria. Since the frequencies of cytokine-specific cells in the lamina propria were very similar in biopsy specimens from AS carriers and DU patients, the results for these two groups were combined and compared with those for biopsy specimens from uninfected individuals (Table 1). When normal and metaplastic biopsy specimens from the same H. pylori-infected individual were compared, significantly higher numbers of TGF-β-specific MNCs (P = 0.0078) and slightly increased numbers of IL-6-, IFN-γ-, and IL-1β-positive MNCs were found in metaplastic biopsy specimens than in normal biopsy specimens from DU patients (Table 1). Furthermore, significantly higher numbers of MNCs specific for IL-8 (P = 0.0026), IL-6 (P = 0.0186), TGF-β (P = 0.0057), and IFN-γ (P = 0.0006) were found in the H. pylori-infected subjects than in the uninfected individuals (Table 1).

The intensity of cytokine staining, i.e., the amount of cytokine produced, was not calculated. However, when the intensity was subjectively scored, a considerably more intense staining of MNCs was observed for all cytokines in biopsy specimens from DU patients than from the other subject groups (exemplified in Fig. 2B, D, and F) and was observed most markedly for TGF-β.

**The degree of inflammation does not influence cytokine levels.** In order to determine whether the degree of inflammation was related to cytokine production, the extent of active inflammation (i.e., PMN infiltration) and signs of chronic inflammation (i.e., the density of MNCs in the duodenal biopsy specimens) were evaluated and scored. Five of the six DU patients but only one of the six AS carriers had detectable active inflammation in the duodenum. Furthermore, the active inflammatory score was higher in the DU patients than in either the AS carriers or the uninfected controls (Table 2). It was also found to be higher in metaplastic biopsy specimens than in biopsy specimens from normal duodenal mucosa. All H. pylori-infected individuals showed histological signs of chronic inflammation in the duodenum of at least grade 1 (Table 2), according to the Sydney system (43). No relation between the degree of active or chronic inflammation and the staining of the epithelium or lamina propria was observed for any of the cytokines studied.

**DISCUSSION**

The fact that only 10 to 15% of those who are *H. pylori* infected develop peptic ulcers and that most remain asymptomatic throughout their lives (29) might be explained by differences in bacterial virulence factors, as well as differences in the host immune response. Previous studies have shown that *H. pylori* induces strong B-cell responses (33), as well as activation of T cells (47, 50), locally in the gastric mucosa. Studies comparing the mucosal immune responses in AS carriers and DU patients have all been limited to the immune responses in the stomach, and differences that may explain the development of DU have not been identified (23, 31, 56). However, we have previously shown that at the site of most ulcers, i.e., the duodenum, the levels of gastric metaplasia and the densities of *H. pylori* are considerably higher in DU patients than in AS carriers (20). Furthermore, some differences in the levels of expression of virulence factors between *H. pylori* strains isolated from the duodenum of DU patients and AS carriers have been identified (51). To investigate whether the observed differences in bacterial colonization can be explained, at least in part, by
FIG. 1. Epithelial cytokine staining in normal (N) and metaplastic (M) biopsy specimens from AS carriers, DU patients, and uninfected individuals (H. pylori−). Staining of both superficial epithelium and epithelial cells of the deeper regions of the duodenal crypts was determined and expressed as a percentage of the total epithelial area in the section. Each circle represents a biopsy specimen from one individual, and normal and metaplastic biopsy specimens from the same individual are connected with a dotted line. Horizontal lines represent the median for each group. **, P < 0.01; *, P < 0.05.
FIG. 2. Light microphotographs showing immunohistochemical detection of cytokines in duodenal tissue sections. (A and B) IL-8-specific staining (arrows indicate positive MNCs, and the arrowhead indicates a positive neutrophil) of biopsy specimens from the metaplastic mucosa of an AS carrier (A) and a DU patient (B); (C and D) TGF-β staining (the arrow indicates a positive MNC) of biopsy specimens from the metaplastic mucosa of an AS carrier (C) and a DU patient (D); (D and E) IFN-γ receptor staining (the arrow indicates positively stained epithelia, and arrowheads indicates a positive MNC) of biopsy specimens from the normal mucosa of an AS carrier (E) and a DU patient (F). Magnification ×97 (original magnification, ×100).
differences in the local immune responses, we compared the cytokine responses in the duodenum of \textit{H. pylori}-infected DU patients and AS carriers, and for comparison, we also evaluated the cytokine responses in uninfected controls.

Since \textit{H. pylori} colonizes the duodenum only in areas of gastric metaplasia (48, 53), we studied the cytokine responses in both normal and metaplastic duodenal mucosa. Even though \textit{H. pylori} colonization was restricted to the metaplastic areas, the levels of all cytokines analyzed were found to be comparable in the normal and metaplastic mucosa. This might be explained by the bacterial factors that are released, e.g., urease and other enzymes or toxins, which may also affect the surrounding epithelium. In contrast to our results, Noshiro et al. (40) have reported that cultured metaplastic duodenal biopsy specimens from \textit{H. pylori}-infected DU patients produce higher levels of IL-8 than normal biopsy specimens from the same individuals. However, in the study by Noshiro and coworkers (40), the IL-8 levels in culture supernatants were measured by enzyme-linked immunosorbent assay, which reflects the accumulated IL-8 produced over a period of time and by several different cell types, i.e., also by neutrophils, which have been shown to be present in increased numbers in the metaplastic areas (20). Furthermore, we have previously found that culturing of mucosal biopsy specimens itself induces the spontaneous production of IL-8 (32).

\textit{H. pylori} has been shown to induce a strong cytokine response in both human gastric epithelial cells (31) and gastric epithelial cell lines (10, 25, 26). Whether this is also true for the duodenal epithelium of \textit{H. pylori}-infected subjects has not previously been analyzed. Here we demonstrate a substantial down-regulation of several cytokines, especially IL-8, TGF-\(\beta\), and IFN-\(\gamma\), in the duodenal epithelium of DU patients compared to the levels in AS carriers and uninfected individuals.

There are several possible explanations for why DU patients may have decreased epithelial cytokine responses, e.g., apoptosis of the epithelium, bacterial factors, genetic host factors, or possibly, down-regulation by other immune cells. Since apoptosis of epithelial cells has previously been demonstrated in the \textit{H. pylori}-infected stomach (37, 52), we evaluated whether the lower cytokine levels observed in DU patients simply could be explained by an inability of the epithelial cells to produce cytokines due to apoptosis. However, the epithelium did not show any signs of disruption microscopically, and when epithelial cells were analyzed for DNA fragmentation, the numbers of apoptotic cells were shown to be similar in AS carriers and DU patients. Apoptosis is therefore not a likely explanation for the marked differences in cytokine staining. The considerably lower level of epithelial cytokine staining observed in DU patients could also be explained by differences in the infecting bacterial strains (51), i.e., some bacterial factors that may interfere with cytokine production. \textit{cagA} is one of the most

| Cytokine | Mean no. of cytokine-specific MNCs/mm\(^2\) in metaplastic and normal biopsy specimens\(^\text{a}\) | \(P\) value\(^b\) |
|----------|--------------------------------------------------------------------------------|------------------|
|          | \(H. pylori\) positive | \(H. pylori\) negative | Normal vs metaplastic\(^c\) | Normal vs \(H. pylori\) negative\(^d\) |
| **IL-8** | normal vs metaplastic\(^c\) | Normal vs \(H. pylori\) negative\(^d\) |
| AS carriers \((n = 6)\) | Metaplastic specimen | Normal specimen | Metaplastic specimen | Normal specimen |
| 9.8 (3.8–16) | 14.7 (7.5–37) | 10.2 (2.5–22) | 5.7 (3.3–20) | 3.2 (3.0–4.0) |
| 6.7 (4.0–38) | 9.5 (1.5–31) | 11.6 (5.0–54) | 10.0 (4.8–15) | 4.3 (2.5–8.3) |
| 9.7 (2.5–16) | 7.5 (1.0–14) | 20.6 (3.3–56) | 8.0 (3.3–27) | 4.3 (3.2–11) |
| 19.9 (4.0–29) | 11.5 (4.0–24) | 18.3 (8.0–39) | 8.8 (3.0–28) | 3.5 (2.0–6.7) |
| 7.6 (3.0–8.6) | 8.3 (5.5–20) | 14.0 (10–42) | 6.3 (5.0–29) | 2.8 (1.5–4.0) |
| 3.6 (0–16.0) | 2.6 (1.0–14) | 4.8 (2.0–10) | 5.0 (0.7–6.9) | 2.5 (0.5–10) |

\(^a\) Values are given as medians (interquartile ranges).

\(^b\) \(P < 0.001; \star, P < 0.01; \dagger, P < 0.05; \text{ns, not significant}\).

\(^c\) AS carriers and DU patients combined.

\(^d\) Determined by Wilcoxon matched pairs test.

\(^e\) Determined by Mann-Whitney test.
extensively studied virulence factors of *H. pylori* and has been associated with cytokine expression (42, 54) and has also been linked to DU disease (20, 51). However, the differences in epithelial cytokine levels that we observed could not be explained by differences in *cagA* expression since all strains, i.e., those collected from both AS carriers and DU patients, were *cagA* positive. Various genetic host factors have been suggested to be of importance for the development of DUs (15, 30). It is possible that the down-regulated epithelial cytokine levels that we observed in the DU patients in this study may be due to differences in genetic host factors. However, this needs to be further investigated. Another possible explanation for the deceased level of cytokine staining of the epithelium in DU patients is down-regulation by other immune cells. Indeed, we have recently observed higher numbers of down-regulatory T cells in the lamina propria of the duodenal mucosa of DU patients compared to the numbers in AS carriers (48a). Furthermore, regulatory T cells have been shown to suppress *H. pylori*-specific immune responses in humans (A. Lundgren et al., submitted for publication), as well as in an experimental mouse model of *H. pylori* infection (S. Raghavan et al., submitted for publication). However, whether these T cells may also have a down-regulatory effect on the cytokines produced by or bound to epithelial cells remains to be elucidated.

IFN-γ is a cytokine that has been shown to be involved in intestinal epithelial cell growth (4) and barrier function (27), as well as in the induction of major histocompatibility complex class II expression on human intestinal epithelial cells (7, 24). In mice, IFN-γ has been shown to play a major role in *H. pylori* infection by increasing the level of gastric inflammation and reducing the level of bacterial colonization (35, 46). Epithelial cells are not believed to produce IFN-γ but can express the IFN-γ receptor and thereby respond to this cytokine. The substantial epithelial IFN-γ staining of biopsy specimens from AS carriers and uninfected subjects was found to reflect receptor-bound IFN-γ, produced by MNCs. Accordingly, the decreased level of epithelial IFN-γ staining in DU patients corresponded to lower levels of IFN-γ receptor staining, as previously described for the numbers of IFN-γ and TGF-β receptors in *Shigella*-infected rectal epithelium (44).

We believe that the down-regulated epithelial cytokine responses in the duodenum may be indirectly involved in the development of DUs. The decreased cytokine responses in the epithelium of DU patients may allow a higher degree of *H. pylori* colonization, which is indeed observed in the duodenum of DU patients compared to the degree of colonization in AS carriers (20). A higher bacterial load may lead to increased levels of production of enzymes, toxins, and other bacterial factors that may be harmful, which in turn may cause the formation of DUs.

Another consequence of a decreased epithelial cytokine response might be defective signaling from the epithelium to the lamina propria. Thus, the epithelial levels of IL-8 have been demonstrated to be of major importance for neutrophil transport over the epithelium (16). Also, mice deficient in the IL-8 receptor have been shown to have impaired transepithelial neutrophil transport, leading to neutrophil entrapment within the lamina propria and subsequent tissue destruction (22). It is possible that the lower IL-8 levels in the epithelium of DU patients may in part explain the increased numbers of neutrophils that are observed in the lamina propria of DU patients (20).

As previously shown for the antrum (2, 13, 31, 39), *H. pylori* infection was found to significantly induce production of the proinflammatory cytokines IL-8, IL-6, and IFN-γ and the anti-inflammatory cytokine TGF-β by MNCs in the lamina propria of the duodenum of both AS carriers and DU patients. The higher levels of IL-8 in the lamina propria of the duodenum of *H. pylori*-infected individuals compared to those in uninfected controls that were observed in this study are consistent with previous findings (9, 40). The numbers of cytokine-positive MNCs in the lamina propria were similar in AS carriers and DU patients. However, substantially more intense staining was observed in the ulcer patients, most markedly for TGF-β. Considering that we have also observed an increased number of cytotoxic T-lymphocyte-associated antigen 4-positive cells, i.e., possibly regulatory T cells, in the duodenal lamina propria of DU patients (48a), we believe that the TGF-β-positive cells are at least partly regulatory T cells (6, 38, 45). This further supports the hypothesis of a down-regulated duodenal immune response in DU patients.

The mucosal epithelium plays an important role as a first line of defense against gastroduodenal infections, and an impaired epithelial barrier may play a role in the development of *H. pylori*-induced ulcers. In this study we have shown that the epithelial cytokine responses in the duodenum of DU patients differ from those in AS carriers and uninfected subjects, with significantly lower levels of IL-8, IFN-γ, and TGF-β in the ulcer patients. Together with our previous observations of higher numbers of down-regulating T cells and, as shown here, more intense staining of the anti-inflammatory cytokine TGF-β in the lamina propria, these results indicate that DU patients have a down-regulated duodenal immune response. The lower epithelial cytokine response in the duodenum may explain the higher bacterial load observed in ulcer patients and is therefore likely to be of importance for the pathogenesis of *H. pylori*-induced DUs.

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