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• *Helicobacter pylori* •

Interleukin-17 levels in *Helicobacter pylori*-infected gastric mucosa and pathologic sequelae of colonization

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Abstract

AIM: To determine the role of interleukin (IL)-17 in gastric ulcerogenesis.

METHODS: Thirty-six gastric ulcer (GU) patients and 29 non-ulcer (NU) patients were enrolled in this study. Mucosal biopsy samples were obtained from the gastric antrum and GU site during endoscopy. Samples were used in *in situ* stimulation for 48 h in the presence of 10 μg/mL phytohemagglutinin-P (PHA), histological examination, and *Helicobacter pylori* (*H pylori*) culture. IL-17 and IL-8 protein levels in culture supernatants were assayed by ELISA. IL-17 mRNA expression was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). *H pylori cagA* and *vacA* status was assessed by reverse hybridization using a line probe assay (LiPA). IL-8 levels in culture supernatants were assayed after AGS cells were co-cultured with *H pylori* strain 26 695 or recombinant human (rh) IL-17.

RESULTS: All 36 GU patients and 15 of 29 NU patients were found to be *H pylori*-positive, while 14 NU patients were *H pylori*-negative. All 51 *H pylori* strains from both GU and NU patients were *cagA*- and *vacA*s1/m1-positive. Antral mucosal tissues from *H pylori*-positive patients contained significantly (*H pylori*-positive NU patients: median 467 pg/mg/protein, range 53-2 499; *H pylori*-negative NU patients: median 104 pg/mg/protein, range 16-312, $P<0.0005$) higher levels of IL-17 than those from uninfected patients. IL-17 levels at the ulcer site were significantly (ulcer site: median 1 356 pg/mg/protein, range 121-1 3730; antrum: median 761 pg/mg/protein, range 24-7 620, $P<0.005$) higher than those at distant sites in the antrum. Biopsies from *H pylori*-positive GU and NU patients showed IL-17 mRNA expression in all samples whereas those from the antrum of the *H pylori*-negative controls showed no detectable expression. A significant correlation was seen between IL-17 and IL-8 levels at each biopsy site (ulcer: $r = 0.62$, $P<0.0001$; antrum: $r = 0.61$, $P<0.0001$) in GU patients. RhIL-17 and *H pylori* strain 26 695 each stimulated IL-8 production from AGS cells.

CONCLUSION: IL-17 may play an important role in the inflammatory response to *H pylori* colonization, and may ultimately influence the outcome of *H pylori*-associated diseases that arise within the context of gastritis.

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Key words: *Helicobacter pylori*; Gastric ulcer; Histological gastritis; Interleukin-17; Interleukin-8

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INTRODUCTION

Helicobacter pylori (*H pylori*), a microaerophilic, Gram-negative bacterium that selectively colonizes the human stomach, is the major cause of chronic active gastritis and peptic ulcer disease[1-3]. In *H pylori* infection, neutrophils are present within epithelial glands and the underlying lamina propria, and an increase in chronic inflammatory cells such as lymphocytes, macrophages, eosinophils, and plasma cells is also found in the lamina propria[4-8]. Recent studies have shown that *H pylori* produces various cytokines that are related to neutrophil or mononuclear cell accumulation, such as interleukin-β (IL-1β), interferon (IFN)-γ, IL-6, IL-8, tumor necrosis factor (TNF)-α and MIP-1α[7-10]. Among these cytokines, IL-8, the major human PMN chemoattractant, plays an important role in *H pylori*-associated acute inflammatory responses[7,8,11]. IL-8 mRNA has been detected in the vast majority of antral biopsy tissues from *H pylori*-positive patients[8], indicating that *H pylori*-infected gastric mucosa is an active site of IL-8 synthesis. It was reported that IL-8 activity increases at ulcer sites[12], a finding which may be important to gastric ulcerogenesis.

IL-17, originally identified as cytotoxic T lymphocyte-associated antigen 8 (CTLA-8)[13], is a proinflammatory cytokine derived mainly from activated T cells[14-16]. A number of recently discovered related molecules form a family of

cytokines, the IL-17 family, and have been designated as IL-17A-F, with the prototype member being IL-17A[13,17-22]. IL-17A is produced exclusively by CD4$^+$ activated T cells[13,23], and more specifically by CD4$^+$CD45RO$^+$ memory T cells[18,24]. The human IL-17A gene product is a protein of 155 amino acids with a molecular weight of 20-30 ku which is secreted as a disulfide-linked homodimer of a 30-35-ku glycoprotein[25,26]. IL-17 stimulates IL-8 release by gastric epithelial cells and facilitates the chemotaxis of neutrophils through an IL-8-dependent mechanism, and contributes to the enhancement of IL-8 levels in *H pylori*-colonized gastric mucosa[27].

Recently, several *H pylori* genes related to an increased risk of disease have been identified[28]. The cytotoxin-associated gene (*cagA*) is a marker for the *cag* island, the presence of which is associated with a more severe clinical outcome[29-32]. *vacA* encodes a protein that induces vacuoles in epithelial cells[33]. *vacA* is present in all *H pylori* strains and contains at least two variable regions[34]. The s-region (encoding the signal peptide) exists as a s1 (including s1a, s1b, and s1c) or a s2 allelic type[35], while the m-region (middle) occurs as a m1 or a m2 allelic type. It was reported that *vacA*s1/m1 *H pylori* strains are more strongly associated with disease than *cagA*-negative *vacA*s2/m2 strains[36,37]. The evaluation of these genes is therefore an essential part of any investigation into the association between *H pylori* infection and pathogenesis.

In view of evidence that the eradication of *H pylori* reduces the risk of peptic ulcer recurrence[38] and ameliorates associated gastric inflammatory changes and chemokine production in the background gastric mucosa[7,8], clarification of the pathogenesis of *H pylori*-positive peptic ulcer disease requires an understanding of the host inflammatory response to this pathogen. We hypothesized that IL-17 secreted at the ulcer site might play a role in ulcerogenesis through the mucosal damage caused by the emigration and activation of various inflammatory effector cells together with IL-8. In the present study, we examined the secretion of IL-17 and expression of IL-17 mRNA using biopsy specimens from the ulcer site in 36 gastric ulcer (GU) patients with *H pylori* infection, and compared results to the severity of gastritis and IL-8 levels.

MATERIALS AND METHODS

Study groups and mucosal biopsy samples

Mucosal biopsy samples were obtained from the gastric antrum and ulcer site in 36 patients with GU, 15 with *H pylori* infection but without ulcers and 14 without either *H pylori* infection or ulcers whose conditions were diagnosed during upper gastroduodenal endoscopic examination in Nagoya University Hospital from October 2001 to January 2002 (Table 1). Thirteen patients had an active open ulcer and 23 had ulcer scaring. None of the patients took non-steroidal anti-inflammatory drugs, antibiotics or bismuth compounds during the preceding 3 mo. Six antral specimens were taken from adjacent areas of endoscopically intact mucosa in the GU and control patients, with one for bacterial culture, and rapid urease test (CLO test; Delta West, Bentley, Australia) and histological examination, and three for *in vitro* organ cultures for IL-17 and IL-8 measurement. Four additional specimens were taken from the margin of the open ulcer or the center of the ulcer scar in GU patients, one for histological examination and three for IL-17 and IL-8 measurement. All samples were obtained under informed consent in accordance with the Helsinki Declaration. *H pylori* infection was confirmed by positive results in at least two of four diagnostic methods, namely bacterial culture, rapid urease test, [^{13}C]-urea breath test and identification of the organism on tissue sections (Giemsa stain). The absence of infection was defined by a negative result in all the four tests.

Table 1 Characteristics of study groups (mean±SD)

	Patients (*n*)	Sex (male/female)	Age (yr)
GU	36	27/9	57.8±8.4
H pylori-positive			
Open ulcer	13	12/1	56.5±9.5
Ulcer scar	23	15/8	58.5±7.8
NU	29	17/12	47.1±15.6
H pylori-positive	15	10/5	49.8±18.9
H pylori-negative	14	7/7	44.1±11.0
Total	65	44/21	53.0±13.2

Organ culture

Mucosal biopsy tissues were weighed and cultured in a 50 mL/L CO_2 incubator for 48 h on a culture insert (Falcon, Oxnard, CA, USA) placed over polystyrene plates (Falcon) containing RPMI 1640 (Life Technologies, Inc., Rockville, MD, USA) medium with 5% heat-inactivated fetal calf serum, 15 mmol/L HEPES buffer, 100 U/mL of penicillin-G, and 100 μg/mL of streptomycin (culture medium) under the conditions of 10 μg/mL of phytohemagglutinin-P (PHA; Sigma Chemical Co., St. Louis, MO, USA). At the end of the culture period the supernatants were collected and kept at -70 ℃ until assayed for IL-17 and IL-8 levels. Total protein in biopsy homogenates was assayed by a modification of the Lowry method.

Histology

In accordance with the updated Sydney system[6], activity (neutrophil infiltration), inflammation (mononuclear cell infiltration), glandular atrophy and intestinal metaplasia and *H pylori* density were assessed on a four-grade scale of 0, 1, 2, and 3 (corresponding to none, mild, moderate, and severe in the Sydney system) using tissue sections stained with hematoxylin-eosin and those stained with Giemsa. In addition, the numbers of neutrophils and mononuclear cells infiltrating the lamina propria were counted in five high-power fields (×200 magnification). Respective numbers were classified into four grades of 0, 1, 2, and 3. All histologic evaluations were conducted without the knowledge of *H pylori* status or experimental results.

Cell cultures

AGS human gastric epithelial cells (American Type Culture Collection CRL 1739) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and

gentamicin 20 μg/mL in an atmosphere of 50 mL/L CO_2 at 37 ℃. For coculture experiments, *H pylori* was grown in brucella broth with 5% FBS for 48 h. Cells were harvested by centrifugation (2 000 r/min) and resuspended in antibiotic-free RPMI 1640 with 10% FBS to yield a final concentration of 10^9 CFU/mL. *H pylori* was added to AGS cells at a bacteria/cell ratio of 1 000:1 for the IL-8 assay. Experiments were performed in antibiotic-free medium with 10% FBS using T-150 flasks (Corning Costar, Cambridge, MS, USA) or 6-well polypropylene tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA).

IL-17 and IL-8 assay

IL-17 and IL-8 protein levels in culture supernatants were assayed in duplicate using ELISA kits specific for IL-17 (R&D Systems, Minneapolis, MN, USA) and IL-8 (Biosource International, Camarillo, CA, USA) according to the manufacturer's instructions. In these assays, the lower limits of detection were 15 pg/mL for IL-17 and <5.0 pg/mL for IL-8. The amount of IL-17 and IL-8 in the organ cultures was expressed relative to protein content in the homogenate of biopsy tissues (IL-17, pg/mg protein; IL-8, ng/mg protein).

Extraction of RNA and RT-PCR amplification for human IL-17 mRNA

RNA was extracted from freshly obtained biopsy tissue by the acid guanidinium thiocyanate-phenol-chloroform method[41]. As a positive control, RNA was also extracted from peripheral blood mononuclear cells (PBMC) stimulated with PHA for 3 h. Two micrograms of extracted RNA was reverse-transcribed into first-stand complementary DNA (cDNA) at 42 ℃ for 1 h using 0.5 μL of Rous-associated virus 2 reverse-transcriptase (Takara Biomedicals, Ohtsu, Japan) and 0.1 μmol/L of oligo d(T) (Amersham Pharmacia Biotech, UK) in a 50-μL reaction mixture. Twenty-five microliters of cDNA from samples was amplified with 0.2 μmol/L of the sense and anti-sense primers for β-actin or IL-17 in a 100 μL reaction mixture containing 0.5 μL of Taq polymerase (Promega Corporation, Madison, WI, USA) for 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 2 min in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CO, USA). PCR primers were as follows: β-actin, 5'-GTG GGG CGC CCC AGG CAC CA-3' and 5'-CTC CTT AAT GTC ACG CAG GAT TTC-3'[42], and IL-17, 5'-AGA GAT ATC CCT CTG TG ATC-3' and 5'-TAC CCC AAA GTT ATC TCA -3'[43]. Negative controls for PCR amplification were prepared by omitting the cDNA sample from the reaction mixture. The amplified products were subjected to electrophoresis in an 1.5% agarose gel containing ethidium bromide and visualized by UV transillumination.

Assessment of H pylori cagA and vacA status

A chloroform-phenol extraction method was used to obtain DNA from the *H pylori* isolates as previously described[44]. *cagA* and *vacA* status was assessed by reverse hybridization using a line probe assay (LiPA)[45].

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* and paired tests and linear regression analysis. The results were expressed as mean±SD. $P<0.05$ was considered statistically significant.

RESULTS

Mucosal IL-17 and IL-8 levels

Mucosal IL-17 (Figure 1A) and IL-8 (Figure 1B) levels were determined using *in vitro* organ cultures of mucosal tissues taken from the gastric antrum and ulcer site of 36 patients with GU and from the gastric antrum of 29 patients with non-ulcer (NU) under PHA stimulation. Among the 36 GU patients, the ulcer was located in the corpus in 27 and in the antrum in 9. Culture supernatants contained measurable levels of IL-17 and IL-8 in both *H pylori*-positive and -negative subjects. IL-17 levels were significantly higher in antral mucosal tissues from GU (median 761 pg/mg protein, range 24-7 620: $P<0.0001$) and *H pylori*-positive NU patients (median 467 pg/mg protein, range 53-2 499: $P<0.0005$) than in those from *H pylori*-negative NU patients (median 104 pg/mg protein, range 16-312). There was no significant difference in IL-17 levels in the supernatants of antral gastric tissue between GU and *H pylori*-positive NU patients. Levels were significantly higher in mucosal tissues from the ulcer site of GU patients (median 1 356, range 121-13 730) than in those from the antrum of any patient group, including GU patients themselves (median 466, range 24-7620: $P<0.05$, Figure 1A). IL-8 levels were significantly higher in mucosal tissues of the antrum from GU (median 30 ng/mg protein, range 3-261: $P<0.0001$) and *H pylori*-positive NU patients (median 38 ng/mg protein, range 7-107: $P<0.0005$)

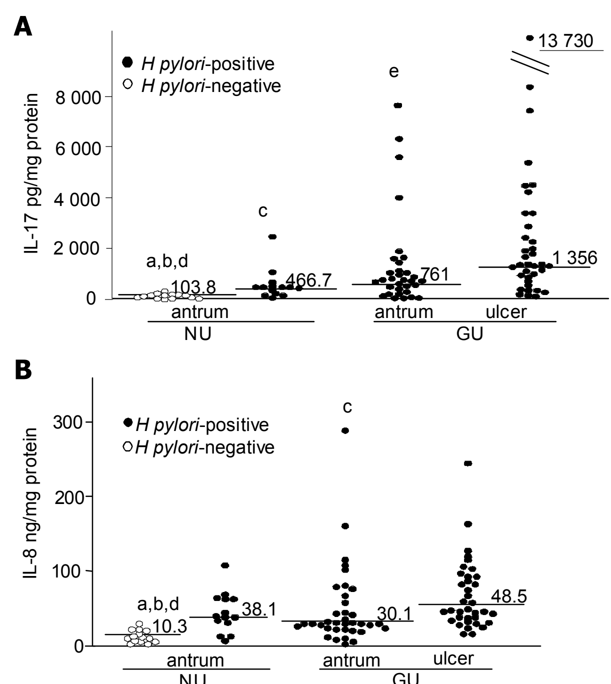

Figure 1 Levels of IL-17 (**A**) and IL-8 (**B**) secreted in organ cultures of biopsies from *H pylori*-positive (closed circle) and -negative (open circle) patients. The bar indicates the median value for each group. **A**: [a]$P<0.0005$ a *vs* b, [b]$P<0.0001$ a *vs* c, [c]$P<0.0005$ b *vs* c, [d]$P<0.0001$ a *vs* d, [e]$P<0.0005$ c *vs* d; **B**: [a]$P<0.0005$ a *vs* b, [b]$P<0.0001$ a *vs* c, [c]$P<0.0005$ a *vs* d, [d]$P<0.0001$ a *vs* d (NS:not significant).

than in those from *H pylori*-negative NU patients (median 10 ng/mg protein, range 1-22). There was no significant difference in IL-8 levels in the supernatant of gastric tissue from the antrum between GU and *H pylori*-positive NU patients. Levels were also significantly higher in mucosal tissues of the ulcer site of GU patients (median 49, range 15-164) than in those from the antrum of any patient group, including GU patients themselves (median 30, range 1-161: $P<0.05$, Figure 1B). There was a significant relationship between IL-17 and IL-8 levels in both antrum ($r = 0.61$, $P<0.0001$, Figure 2A) and ulcer site ($r = 0.62$, $P<0.001$, Figure 2B). With regard to ulcer stage in GU patients, there was no significant difference in IL-17 and IL-8 levels between samples from open ulcers (Figure 3A) and ulcer scars (Figure 3B).

Cytokine levels and histological findings

The association between cytokine levels and histological findings was investigated by comparing levels of IL-17 and IL-8 secretion *in vitro* with the severity of gastritis (activity, inflammation, atrophy, and metaplasia). A significant association was identified between IL-17 and IL-8 levels and the histological degree of activity and inflammation, but IL-17 and IL-8 levels were not associated with the degree of glandular atrophy or intestinal metaplasia (data not shown). The association was further investigated by quantifying the number of infiltrating neutrophils and mononuclear cells. In the antrum, IL-17 showed a stronger association ($r = 0.55$, $P<0.0001$) with the number of infiltrating mononuclear cells than IL-8 ($r = 0.45$, $P<0.0005$), whereas IL-17 ($r = 0.51$, $P<0.0001$) and IL-8 ($r = 0.54$, $P<0.0001$) showed a similarly strong association with the number of neutrophils. At the ulcer site, IL-17 had a stronger correlation with mononuclear cells (IL-17: $r = 0.64$, $P<0.0001$; IL-8: $r = 0.38$, $P = 0.012$, Figures 4A and B) and neutrophils (IL-17: $r = 0.74$, $P<0.0001$; IL-8: $r = 0.44$, $P = 0.078$) than IL-8 (Figures 4C and D).

IL-17 mRNA expression

The potential of gastric mucosa to synthesize IL-17 was studied by RT-PCR analysis using biopsy specimens from the ulcer sites and antrum of 5 *H pylori*-positive GU patients, and from the antrum of 5 *H pylori*-positive and 5 *H pylori*-negative NU patients. Consistent with the enhanced activity of IL-17 identified in organ cultures, all fresh biopsy specimens from the ulcer sites and antrum of *H pylori*-positive GU or NU patients had detectable IL-17 mRNA expression (IL-17: GU patients; ulcer sites 5/5, antrum 5/5, NU patients; antrum 5/5) (Figure 5). In contrast, IL-17 transcripts were not detected in any of the 5 *H pylori*-negative NU patients.

Effect of IL-17 on IL-8 production in AGS cells

To further investigate the role of IL-17 in the induction of IL-8 in gastric mucosa, we tested the effect of recombinant human (rh) IL-17 on the secretion of IL-8 by AGS cells (Figure 6). Stimulation of AGS cells cultured with graded doses of rhIL-17 at 1, 10 or 100 ng/mL for 24 h was followed by a dose-dependent increase in IL-8 secretion as measured by ELISA compared to unstimulated cell culture

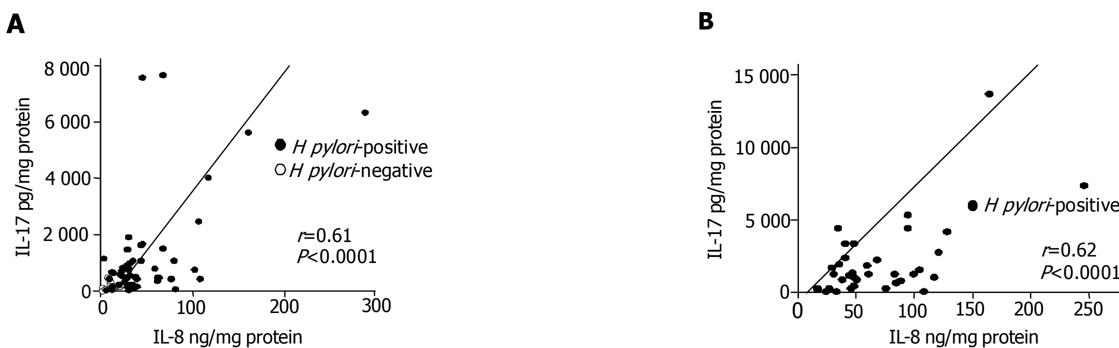

Figure 2 Relationship between IL-17 and IL-8 levels in biopsy culture supernatants from antrum (**A**) and ulcer site (**B**). The bar indicates the median value for each group.

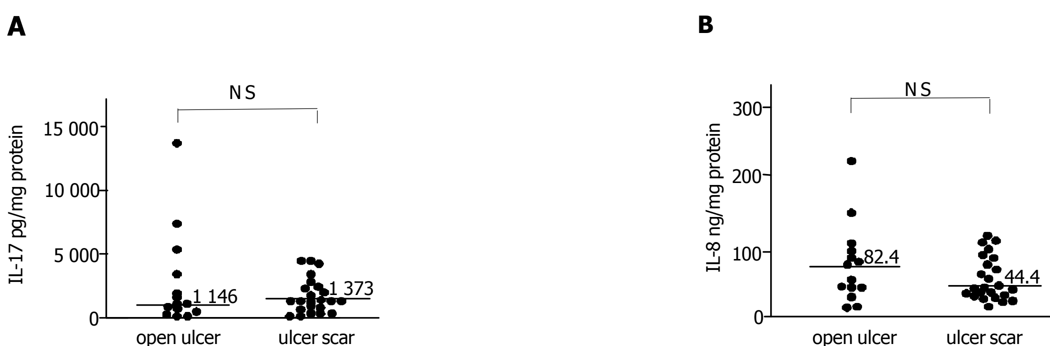

Figure 3 IL-17 (**A**) and IL-8 (**B**) levels in biopsy culture supernatants of GU patients according to ulcer stage.

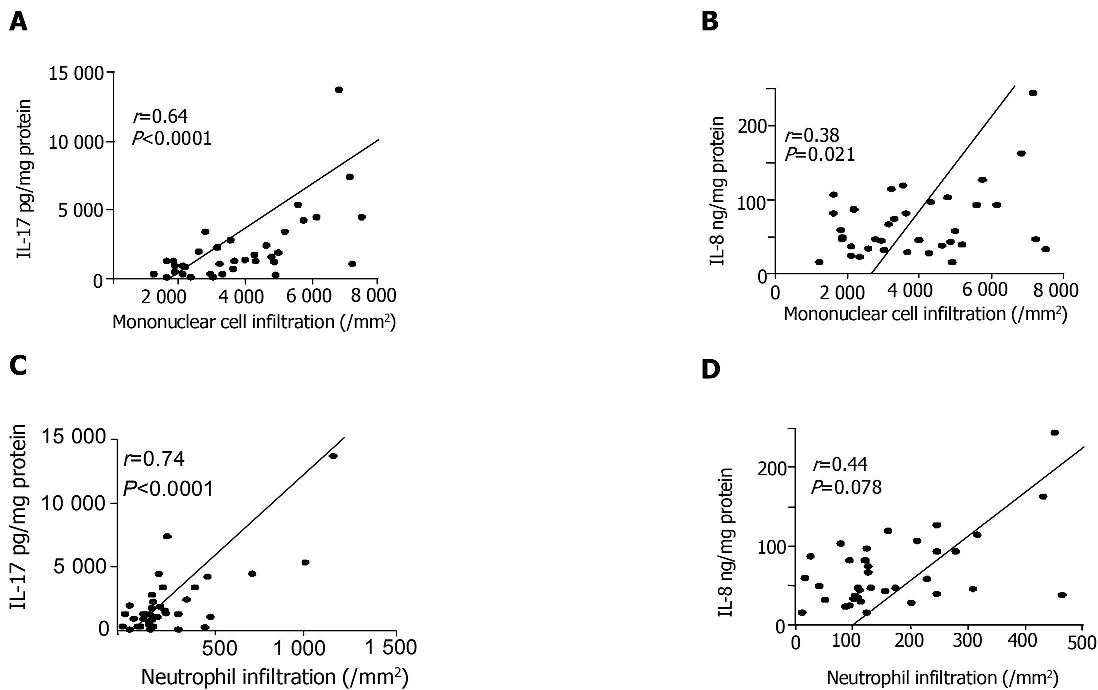

Figure 4 Relationship between IL-17 (**A** and **C**) or IL-8 (**B** and **D**) levels and mononuclear cell infiltration or neutrophil infiltration at ulcer sites.

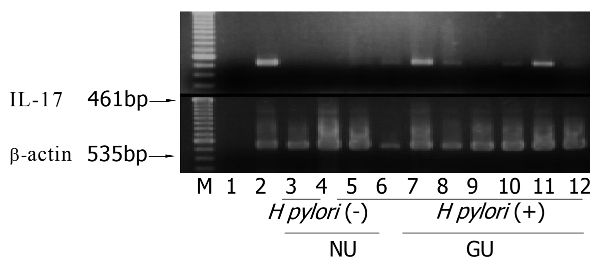

Figure 5 RT-PCR analysis of IL-17 and β-actin mRNA. M: 100-bp ladder marker; lane 1: negative (no cDNA sample); lane 2: positive control (PBMC stimulated by PHA for 3 h); lanes 3 and 4: antral biopsies from *H pylori*-negative NU patients with endoscopically normal mucosa; lanes 5 and 6: antral biopsies from *H pylori*-positive NU patients with gastritis; lanes 7, 9, and 11: antral biopsies from *H pylori*-positive GU patients; lanes 8, 10, and 12: mucosal biopsies from the ulcer site of *H pylori*-positive GU patients.

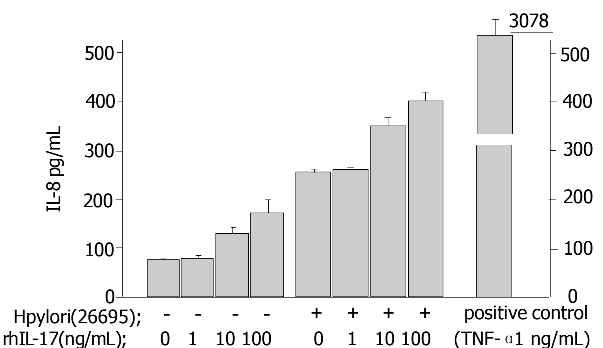

Figure 6 IL-8 levels in culture supernatants of AGS cells co-cultured with graded doses of rhIL-17, *H pylori* strain 26 695, or both.

supernatants. The addition of *H pylori* strain 26 695 to the cell culture at any concentration of rhIL-17 produced a further increase in IL-8 production.

Assessment of H pylori cagA and vacA status

Determination of genotype of *H pylori* strains isolated from GU and NU patients by LiPA confirmed that all 51 strains (GU, *n* = 36; NU, *n* = 15) were *cagA*-positive *vacA*s1/m1 (Table 2). Most strains were *vacA* subtype s1c, while the rest were *vacA* s1a. Thus, GU and NU patients had a similar *cagA* and *vacA*s1/m1 status.

DISCUSSION

On the basis of previous findings that IL-8 production increases at the ulcer site in patients with GU[12] and that IL-17 has the ability to stimulate IL-8 production in both epithelial cells[27] and fibroblasts[22], we investigated the production of IL-17 at ulcer and exogenous NU sites in GU patients. Among 36 GU patients, the ulcer was located in the corpus in 27 and in the antrum in 9. In preliminary studies to determine the site of biopsy for IL-17 measurement, IL-17 production in the antrum, corpus, and ulcer site was compared in 10 patients (5 GU and 5 gastritis). Because specimens from the corpus and the antrum showed no significant difference in IL-17 production (data not shown), specimens for comparison to the ulcer site were limited to the antrum to reduce the number of gastric biopsies. Our results show that IL-17 and IL-8 activities in organ cultures of biopsy specimens from the antrum are substantially higher in *H pylori*-positive patients than in *H pylori* -negative patients, and that among *H pylori*-infected GU patients, the gastric mucosa at the ulcer sites contains significantly more IL-17 and IL-8 than the mucosa of the antrum.

Further, IL-17 and IL-8 levels were significantly correlated with the number of infiltrating mononuclear cells

Table 2 *H pylori cagA* and *vacA* gene status

		cagA status		*vacA* status					
				Signal region				Middle region	
	n	Positive	Negative	s1a	s1b	s1c	s2	m1	m2
GU	36	36	0	5	0	31	0	36	0
NU	15	15	0	2	0	13	0	15	0
Total	51	51	0	7	0	44	0	51	0

and neutrophils at both antral and ulcer sites, suggesting that IL-17 and IL-8 generated locally within the gastric mucosa, may be relevant to the background histological gastritis caused by *H pylori* infection. IL-17 and IL-8 levels in the antrum showed significant associations with the number of infiltrating mononuclear cells and neutrophils. At the ulcer site, however, IL-17 had a stronger correlation with mononuclear cells and neutrophils than IL-8, and a stronger correlation with neutrophils than with mononuclear cells. IL-17 and IL-8 have been reported to cause neutrophil recruitment[44], and neutrophils are known to cause damage to tissues they infiltrate[27]. On the basis of these results, we hypothesize that, together with IL-8, IL-17 might contribute to ulcerogenesis in GU patients as a result of its induction of neutrophil recruitment at the ulcer site.

When IL-17 and IL-8 levels in gastric mucosa at the ulcer site were compared between GU patients in the active stage and those in the healed stage, no significant difference was seen. Moreover, comparison of infiltrating neutrophils and mononuclear cells at ulcer site between ulcer stages showed no significant difference in IL-17 and IL-8 levels (data not shown). These results suggest that immunological background might not differ by GU stage, but might rather be fundamentally the same, regardless of stage.

Another important finding is the significant correlation between IL-17 and IL-8 levels at both the antrum and ulcer site. As this indicates a likely association between IL-17 and IL-8, we subsequently examined the effect of IL-17 as well as *H pylori* on IL-8 production by AGS cells. Co-culture of AGS cells with *H pylori* strain 26 695, in the presence or absence of rhIL-17, showed that both *H pylori* strain 26 695 and rhIL-17 stimulated IL-8 production from AGS cells.

Consistent with the increased secretion of IL-17 protein in organ cultures, biopsies from *H pylori*-positive GU and NU patients contained mRNA for this cytokine in all samples tested, but no expression in samples from the antrum of *H pylori*-negative controls. We previously reported that IL-8 mRNA expression is often detected in gastric biopsy tissues from *H pylori*-positive patients, but rarely in those from *H pylori*-negative controls[7], indicating that gastric mucosa is an active site for the synthesis of both IL-17 and IL-8 in *H pylori*-infected GU patients. Considering that IL-17 and IL-8 induce exocytosis and a respiratory burst in neutrophils[46], we hypothesize that locally secreted IL-17 and IL-8 are biologically active key chemotactic factors for the recruitment and activation of neutrophils, particularly at ulcer sites in *H pylori*-infected GU patients. We additionally speculate that enhanced synthesis and secretion of IL-17 and IL-8 may contribute to ulcerogenesis in *H pylori*-infected patients.

In conclusion, IL-17 and IL-8 may play an important role in the inflammatory response to *H pylori* colonization, and may ultimately influence the outcome of *H pylori*-associated diseases arising within the context of gastritis. Further studies are necessary to determine the mechanisms of this elevation in mucosal IL-17 and IL-8 activity in *H pylori* infection.

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