Local and peripheral cytokine response and CagA status of *Helicobacter pylori*-positive patients with duodenal ulcer

Gergely Klausz[1], Andrea Tiszai[2], László Tiszlavicz[3], Zsófia Gyulai[1], Zsuzsa Lénárt[2], János Lonovics[2], Yvette Mándi[1]

[1] Department of Medical Microbiology and Immunobiology, [2] 1st Department of Internal Medicine, [3] Department of Pathology, Faculty of Medicine, University of Szeged, Szeged, Hungary

Correspondence: Prof. Dr. Yvette Mándi, Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dóm tér 10., H-6720 Szeged, Hungary. Phone: + 36-62-545 115, Fax: + 36- 62-545 113

ABSTRACT. The mucosal production of TNF-α, IL-6, IL-8, IL-10 and nitrotyrosine was investigated in *H. pylori*-positive patients with duodenal ulcer (DU). The concentrations of these cytokines in gastric antrum mucosal specimens from patients infected with *H. pylori* (n = 40) were determined by ELISA and compared with data on mucosal specimens from *H. pylori*-negative patients (n = 12). Nitrotyrosine was determined by ECL Western blotting. It was additionally investigated whether the tissue levels of the cytokines correlated with the peripheral cytokine levels, and the CagA status of the patients.

The local TNF-α, IL-6 and IL-8 concentrations in the antral biopsy samples were significantly higher ($p < 0.001$) in the patients infected with *H. pylori* than in the samples from the *H. pylori*-negative subjects. There was a negative correlation between the TNF-α and IL-10 concentrations. Further more, in 23 of the 40 biopsy specimens, considerable nitrotyrosine production was detected by ECL Western blotting. There was no significant difference in peripheral TNF-α and IL-6 production between the DU patients and healthy blood donors (n = 100; 58% of whom were also *H. pylori*-positive). Only the *in vitro* IL-8-producing capacity was higher in the peripheral blood of the DU group after *ex vivo* induction with *H. pylori*.

CagA positivity was demonstrated in 39 (97.5%) of the 40 patients with DU, and in 41 (70.7%) of the 58 *H. pylori*-positive, healthy blood donors.

This study suggests that besides the bacterial virulence factor, the host response, with an increased mucosal production of inflammatory cytokines and reactive oxygen and nitrogen species could be relevant to the gastric pathophysiology in *H. pylori*-induced DU. There is no generalized cytokine overproduction in these DU patients, but the moderate increase in *in vitro* IL-8 production might be of pathophysiological importance.

Keywords: *H. pylori*, cytokine, nitrotyrosine, CagA, ELISA, Western blot

INTRODUCTION

Helicobacter pylori infection almost invariably causes chronic gastritis, but only a proportion of the infected subjects develop peptic ulcers [1]. The host immune response might be of importance as regards the outcome of the infection [2]. The local inflammation in *H. pylori* infection is characterized by the infiltration of neutrophils and lymphocytes into the gastric mucosa and by an increased production of the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α [2, 3]. In particular, IL-8 as a neutrophil chemotactic and activating factor has been suspected of playing an important role in *H. pylori*-associated disease. *H. pylori* strains carrying the pathogenicity island including the gene encoding the cytotoxicity-associated protein (CagA) are more commonly isolated from duodenal ulcer (DU) patients [4], and these CagA positive strains display an increased IL-8 – inducing capacity [5]. The effects of proinflammatory cytokines however, might be counteracted by locally produced IL-10, a TH2 cytokine which has anti-inflammatory effects [6, 7].

H. pylori infection could lead to increased nitric oxide (NO) production by activated macrophages and neutrophils in the gastric mucosa. INOS is increased in symptomatic and asymptomatic humans infected with *H. pylori*, and a decrease in staining for iNOS and catalase was observed after *H. pylori* eradication [8] In the presence of oxygen free radicals, NO can form a genotoxic peroxynitrite, which can contribute to further destruction of the mucosa. Nitrotyrosine as a stable end product of peroxynitrite, can be used for the detection of NO and peroxynitrite formation [9, 10].

The aim of this study was an investigation of the mucosal production of TNF-α, IL-6, IL-8, IL-10 and nitrotyrosine in *H. pylori*-infected patients. We determined the concentrations of these cytokines in gastric antral mucosal specimens from *H. pylori*-positive DU patients and compared them with those in mucosal specimens from *H. pylori*-negative subjects. In addition, we investigated whether the tissue levels of the cytokines correlated with the peripheral cytokine levels, and the CagA status of patients.

METHODS

Patients

Forty *H. pylori*-positive patients with DU (mean age 47.6 years, male/female 10/30) were studied. Twelve *H. pylori*-negative subjects (mean age 36.8 years, male/female 8/4) from whom biopsies were taken for different reasons served as control. The project was approved by the Clinical Ethical Committee of the Medical Faculty of Szeged University and informed consent was obtained all from of the patients. Multiple biopsy specimens were taken during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum and corpus for histology, for rapid urease test (Controloc) and for PCR. The presence of *H. pylori* was confirmed, and the severity of gastritis was graded with the Sydney Classification System [11]. Only individuals who gave positive histology and urease tests were regarded as infected with *H. pylori*. Additionally, the PCR assays with oligonucleotide primers homologous to a portion of the urease C gene of *H. pylori* [12] were performed with DNA extracted from biopsy materials [13], and results demonstrated 100% specificity. Additionally, two biopsy specimens were taken for cytokine ELISA and nitrotyrosine Western blotting.

For serological investigations, the sera of the 40 DU patients patients were compared with those of 100 apparently healthy blood donors.

Cytokine assays

Biopsy specimens were immediately placed in RPMI 1640 medium and transported to the laboratory. Specimens were homogenized in 0.5 ml of 3.3 mM $CaCl_2$. Aliquots of the homogenate were assayed for total proteins by the Lowry method [14], specimens were stored at – 70°C until assayed.

Concentrations of cytokines in homogenates were determined by ELISA assays, with the following ELISA kits: TNF-α BIOSOURCE, IL-6 BIOSOURCE, IL-8 BIOSOURCE, and IL-10 BIOSOURCE. The procedures were performed in full accordance with the instructions of the manufacturer. The mucosal levels of cytokines were expressed as pg/mg biopsy protein.

Nitrotyrosine detection

Nitrotyrosine was detected by an ECL Western blot technique with the application of anti-nitrotyrosine monoclonal antibody (HM 11, HyCult Biotechnology). This highly specific monoclonal antibody reacts with proteins containing nitrotyrosine.

Total protein from biopsy samples was solubilized after ultrasonication, with subsequent processing in Laemmli buffer [15], and individual protein samples (10 μg/lane) were fractionated on 15% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose blotting membrane (Trans Blot BioRad) using a semi-dry HOEFER transfer system. Membranes were blocked in 5% nonfat dried milk. Immunoblotting was performed with anti-human nitrotyrosine MoAb HM 11 at a dilution of 1:500. A goat anti-mouse immunoglobulin G antibody conjugated to horse radish peroxidase (Bio-Rad) was used to form antigen-antibody complexes, which were detected via a chemiluminescent reaction (ECL Amersham), followed by exposure to X-ray film (KODAK BIOMAX).

The presence of nitrotyrosine in tissue homogenate was regarded as positive in the event of the detection of at least two or three positive bands, indicating the nitrosylation of tyrosine residues. For positive and negative controls, peroxynitrite-treated and control AGS (human gastric epithelial cells #CRL –1739; American Type Culture Collection) cell lysates were processed in the same way. To confirm the specificity of the signal for Nytr the following procedures were included: a) omission of the primary antibody, b) chemical reduction of Nytr with 100 mM dithionite before the addition of primary antibody.

Serology

For the simultaneous evaluation of *H. pylori* positivity and CagA status, sera from the 40 patients with DU and sera from the 100 blood donors were processed with a Western blot assay elaborated by MICROGEN GmbH Germany. In this test, the strips are incubated with 1:100 dilution of human serum, and at the end of Western assay individual sharp bands demonstrate the presence of antigen-antibody complexes. This test allows the detection of different antibodies against bacterial antigens (UreB, HspA, HspB, UreA, and Fla), VacA, and CagA. The molecular weights and names of the antigens are provided by the manufacturer.

In vitro *cytokine production in peripheral blood samples*

For the *in vitro* cytokine induction experiments, blood was drawn with anticoagulant (EDTA), For whole blood cultures, 1 ml blood was mixed with 1 ml RPMI 1640 supplemented with 10% fetal calf serum (FCS), and incubated with 10^9 *H. pylori* 26695 for 18 h at 37°C. Supernatants of whole blood cultures were thereafter tested for the presence of TNF-α, IL-6 and IL-8 by ELISA. For the coculture experiments Cag A positive *H. pylori* 26695 strain was used, which was a kind gift from D.E. Berg (Department of Molecular Biology and Genetics, Washington University Medical School, St. Louis, USA).

Bacteria were maintained on Brain Heart infusion agar containing 10% horse blood, and incubated in a microaerophilic atmosphere [16]. Inocula for coculturing were diluted from suspensions that had been prepared from 72 h subcultures and adjusted by comparison of the absorbance with standards.

Statistics

All statistical calculations were performed with the GraphPad Prism statistical program. Differences between levels of cytokine production were computed with the Mann-Whitney, and Student tests.

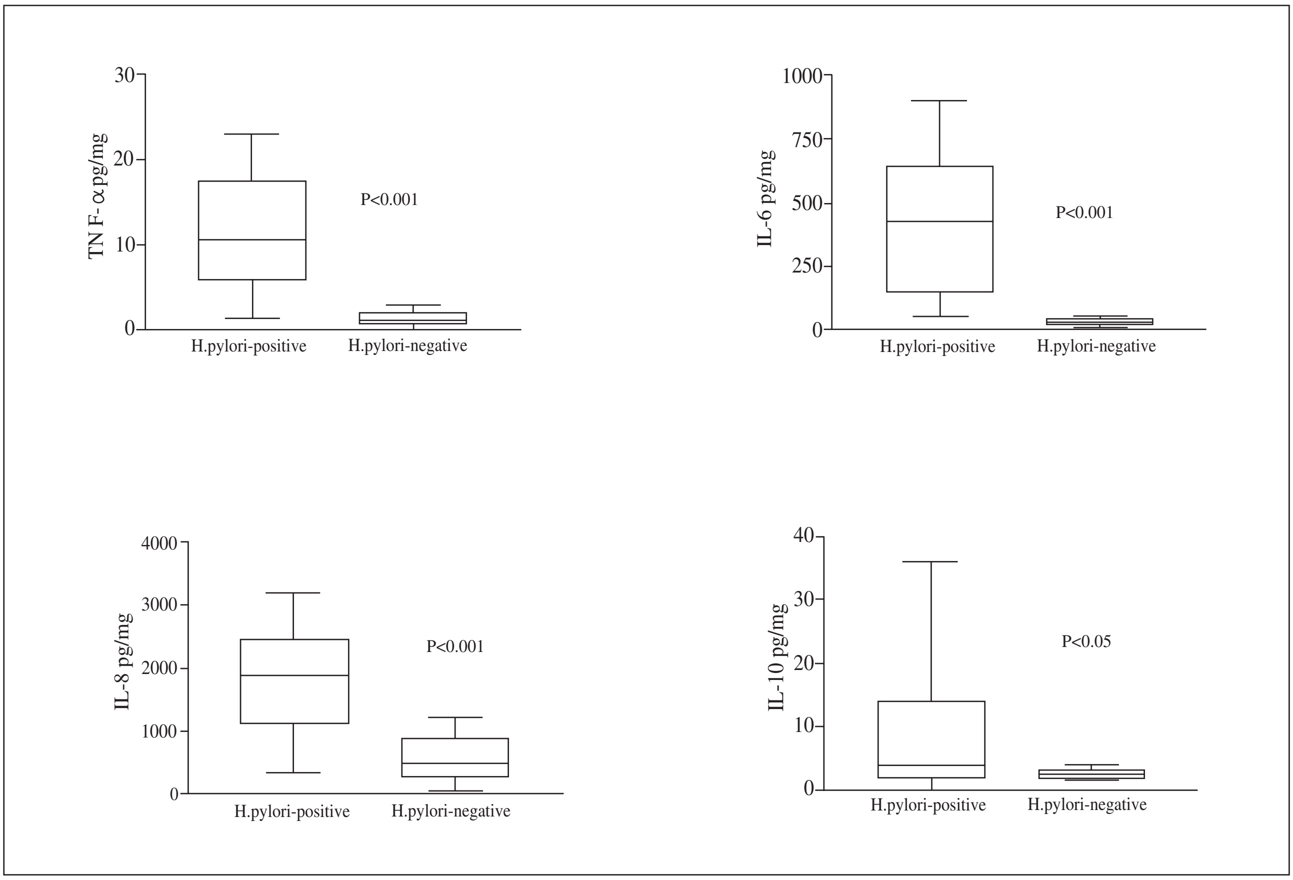

Figure 1
Mucosal cytokine production from biopsy specimens. Cytokine concentrations are expressed as pg/mg biopsy protein. The data are depicted as box-and whiskers plots (the box shows the median and the upper 75% and lower 25%, and the whiskers show the 95 percentile). P values were calculated by the Mann-Whitney U test.

RESULTS

Mucosal TNF-α, IL-6, IL-8 and IL-10 production from biopsy specimens

DU patients infected with *H. pylori* exhibited a higher antral cytokine production than did *H. pylori*-negative patients. Results are summarized in Figure 1: TNF-α: median = 10.55 (range 1.2-23) pg/mg protein, versus 0.85 (range 0.0-2.7) pg/mg protein, $p < 0.001$; IL-6: median = 425 (range 50.0-900.0) pg/mg protein, versus 25 (range 6.0-55) pg/mg protein, $p < 0.001$; IL-8: median = 1890 (range 350-3200) pg/mg protein, versus 485 (range 50-1200) pg/mg protein, $p < 0.001$; and IL-10: median = 3.95 (range 0.9-36.0) pg/mg protein, versus 2.4 (range 1.5-3.8) pg/mg protein, $p < 0.05$.

There was an inverse correlation between the levels of TNF-α and the anti-inflammatory cytokine IL-10 (Figure 2), which clearly demonstrates the imbalance between TH1 and TH2 cytokines in DU patients [17]. The highest TNF-α content was measured in those specimens, in which the lowest IL-10 concentrations were detected.

Mucosal nitrotyrosine formation

Nitrotyrosine (Nytr) is formed in tissues in the presence of the active metabolite NO Nitrotyrosine is a stable end-product of nitrosylation of tyrosine and can be detected by histology on paraffin sections and by Western blot [9, 10]. ECL Western blotting was applied for the immunodetection of nitrated proteins in protein lysates of biopsy samples. Figure 3 reveals a representative result of immune detection of nitrotyrosine, showing several distinct bands, especially in the lower molecular range between 20 and 47.5 kDa. (Lanes 2 and 3). By comparison, no specific bands were obtained in biopsy specimens of *H. pylori*-negative subjects (Lane 1). Lane 4 demonstrates a negative result obtained with a sample from one of the *H. pylori*-positive DU patients. AGS cells (gastric epithelial cells)

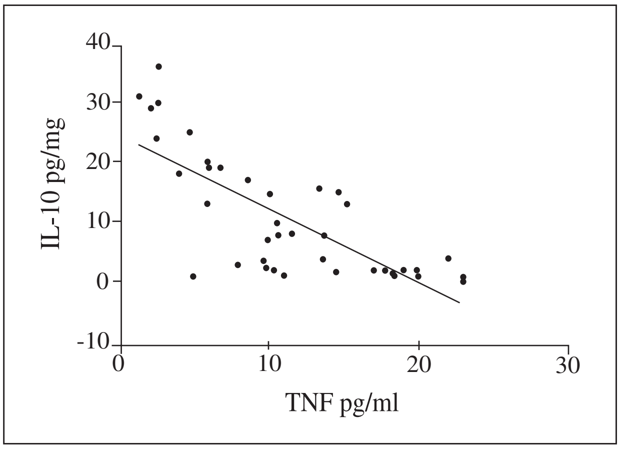

Figure 2
Relationship between mucosal TNF-α and IL-10 production in DU patients. Cytokine concentrations are expressed as pg/mg biopsy protein. ($p < 0.01$, $r^2 = 0.58$).

treated with peroxynitrite (100-200 µM) served as a positive control. Western blotting gave positive results only with the peroxynitrite-treated cells (data not shown). In 23 of the 40 biopsy samples, positive results were obtained with Western blotting, as in the representative experiment illustrated in Figure 3. None of the biopsy specimens originating from the *H. pylori*-negative controls was positive in this test. In a comparison of the nitrotyrosine assay results with the Sydney scores, all of these 23 samples were evaluated at grade 3, and their mean concentrations of TNF-α and IL-8 were 17.5 ± 2.1 pg/mg and 2540 ± 650 pg/mg, respectively.

Comparison of serum cytokine levels and ex vivo inducible cytokine release in whole blood from DU patients and healthy blood donors

We investigated whether the high cytokine production in *H. pylori*-infected patients was characteristic only locally, or whether the gastric infection could lead to an elevated cytokine production at the periphery too. For this purpose, the cytokine concentrations in the sera and the *in vitro* cytokine production of peripheral white blood cells were determined. Table 1 summarizes the results on such patients and on healthy blood donors attending the the South Hungarian Regional Blood Bank of National Transfusion Service, Szeged. These subjects were age- and sex-matched (mean age 51.2 years, male/female 25/75), with no gastrointestinal disorders or symptoms, and without a history of gastrointestinal disease or any other relevant illness. TNF-α is rarely measurable in healthy humans, and was almost undetectable in DU patients. There was no difference in mean IL-6 and IL-8 concentrations in the sera of the DU patients and the blood donors. *H. pylori* 26695 induced moderate TNF-α and IL-6 production when whole blood cultures were coincubated with *H. pylori* for 18 h. There was no significant difference between the amounts of these cytokines in supernatants of cultures of whole blood from the DU patients and healthy blood donors. The *in vitro H. pylori* infection caused a greater IL-8 production in the DU patients than in the controls (mean ± SD = 46954 ± 26100 pg/ml *versus* 36484 ± 22411 pg/ml, $p < 0.01$) (Table 1). More details concerning *in vitro* IL-8 production in individual whole blood cultures (WBC) are presented in Figure 4. Despite the great variation in IL-8 production, the DU patients exhibited significantly greater IL-8 production at the periphery than that of the healthy blood donors. There was no further difference in the *in vitro* cytokine-producing capacity between the *H. pylori*-positive Cag A+ and CagA– healthy blood donors, or between the *H. pylori*-positive and negative blood donors. It is interesting, that in DU patients not only in the *H. pylori*-stimulated, but also in the basal, nonstimulated WBC culture supernatants, relatively higher IL-8 concentrations were measured, but statistically the differences were not significant (3.024 ± 1.933 ng/ml *versus* 2.45 ± 1.257 ng/ml). (Figure 4).

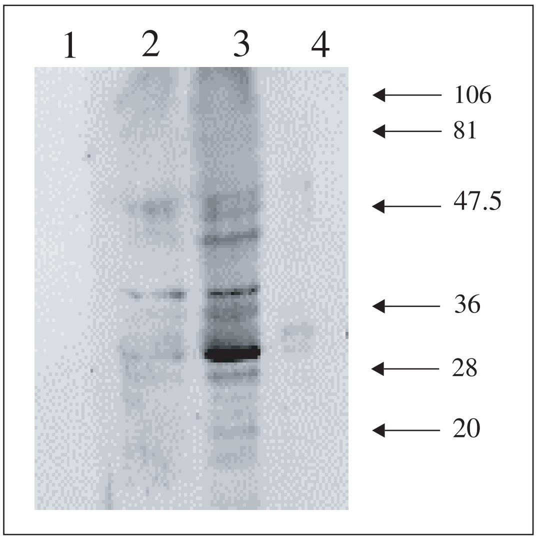

Figure 3
Western blot analysis for nitrotyrosine of biopsy homogenates of *H. pylori*-positive DU patients (Lanes 2, 3, 4) and *H. pylori*-negative patient (Lane 1) Equal amounts of protein (10 ug/well) were loaded onto the gel (15% SDS-PAGE), transferred to nitrocellulose, and reacted with MoAb against nitrotyrosine HM1 1:500. For visualization, ECL reaction was used, using KODAK-BIOMAX film. (Corresponding blot treated with dithione gave no signal for nitrotyrosine; data not shown).

Cag A status

The MICROGEN Western blot kit revealed that 39 (97.5%) of the 40 DU patients were CagA-positive. Among the 100 healthy blood donors, 58 were *H. pylori*-positive: serum samples from the latter, in a dilution of 1:100, reacted with at least 4 different *H. pylori* antigens, and 41 of them (70.7%) had antibodies against CagA antigen too. Our data are consistent with earlier observation, that CagA-positive bacteria are more frequently able to cause ulcerative diseases [4, 18]. However, the frequency of CagA positivity even in the healthy control group is high, and we therefore suggest that CagA positivity alone cannot explain ulcer development in the *H. pylori*-positive DU group.

Table 1
Serum cytokine levels and *ex vivo* cytokine release capacity* in whole blood from DU patients and healthy donors

	TNF-α pg/ml		IL-6 pg/ml		IL-8 pg/ml	
	in sera	in WBC**	in sera	in WBC**	in sera	in WBC**
DU patients	3.5 ± 0.2	$5 \pm 1.25 \times 10^2$	20 ± 5	$5 \pm 0.5 \times 10^3$	75 ± 58	$4.6 \pm 2.61 \times 10^4$***
Controls	3.1 ± 0.3	$4.8 \pm 1.8 \times 10^2$	15 ± 10	$4.9 \pm 0.45 \times 10^3$	68 ± 65	$3.6 \pm 2.24 \times 10^4$

* Whole blood samples from DU patients (n = 40) and healthy donors (n = 100) was diluted 1:1 with RPMI 1640 + 10% FCS (whole blood cultures**) and was incubated in the presence of 10^9 *H. pylori* 26695 for 18 h at 37⁰C. Cytokine levels in cell-free supernatants were measured by ELISA. Results are expressed as means ± SD statistical significance was detected by Student's t test.*** $p < 0.01$.

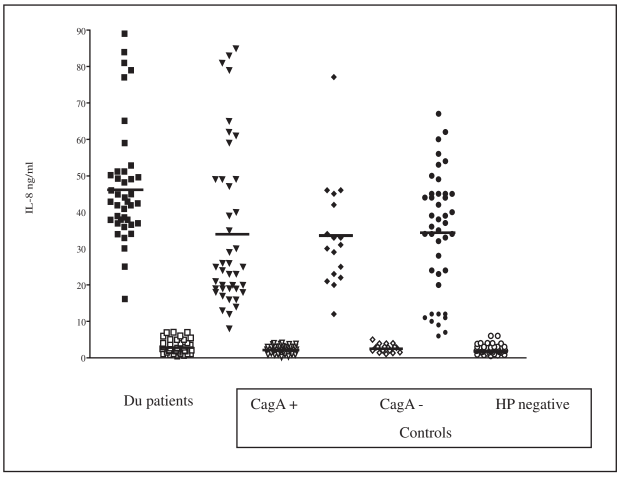

Figure 4
Individual titration values of IL-8 in culture supernatants of whole blood cultures (WBC). WBC of DU patients, CagA+ and CagA- controls, and *H. pylori*-negative controls were incubated with (stimulated level; filled symbols) and without (basal level; open symbols) *H. pylori* 26695 for 18 h at 37°C. Each dot represents the individual value for one subject, and the horizontal lines denote the means for individual groups.

DISCUSSION

In *H. pylori* infection both bacterial virulence factors and the inflammatory response of the host contribute to mucosal damage [2, 19, 23]. The role of cytokines in the initiation and modulation of gastrointestinal inflammatory responses is crucial [3]. We set out to investigate the local cytokine activation and the concomitant production of nitrating agents in the mucosa, comparing the results with the peripheral cytokine pattern in *H. pylori*-positive patients with duodenal ulcer.

In antral biopsy specimens from the DU patients, significantly higher levels of TNF-α, IL-6 and IL-8 were observed than in the *H. pylori*-negative subjects. *H. pylori* infection is characterized by a marked neutrophil infiltration. The activation and recruitment of neutrophils by IL-8 at the site of inflammation can lead to an increased formation of oxygen free radicals. At the same time, *H. pylori* infection can lead to the expression of iNOS and the sustained production of NO by host macrophages. Oxygen free radicals and NO can form potentially genotoxic nitrating species such as peroxynitrite [20, 21]. We examined the presence of nitrotyrosine as a marker of peroxynitrite formation in biopsy specimens. Nitrotyrosine was detected in those samples, in which the highest TNF-α and IL-8 cytokine concentrations were measured. Accordingly, we concluded that these cytokines, which activate inflammatory cells, will result in the generation of nitrating and oxidizing agents. This can lead to increased rates of DNA damage in the gastric mucosa, inducing NO production and the production of oxygen free radicals, and resulting overall in peoxynitrite formation. Considerable evidence indicates that tyrosine residues in proteins become nitrated preferentially at sites of inflammation-induced tissue injury, leading to the suggestion that nitrotyrosine may be deleterious or serve as a biomarker for the effects of reactive nitrogen oxides [21]. These free radicals are of pathophysiological importance in the destruction of the gastric mucosa. The apoptotic effect of peroxynitrite is extremely important in this process.

IL-10 inhibits the secretion of proinflammatory cytokines and chemokines from macrophages and polymorphs, thereby potentially reducing neutrophil activation and the generation of damaging oxygen metabolites [6, 7]. Hence, IL-10 is an important counter-regulatory cytokine, and could damp down local inflammation in some *H. pylori*-positive DU patients. Despite the individual variation in the levels of IL-10, the gastric tissue of the DU patients displayed relatively low levels of IL-10 (Figure 1), and the diference was only marginally significant ($p < 0.05$) as compared with samples from *H. pylori*-negative patients. Our study revealed a negative correlation between local IL-10 and TNF-α production, indicating an imbalance between the induction of the proinflammatory cascade and inhibitory cytokines, disturbing the mucosal homeostasis [17].

The circulating TNF-α level was almost undetectable not only in the healthy controls, but also in the DU patients. The mucosal production of TNF-α and proinflammatory cytokines at the site of challenge can therefore be considered as occurring separately from the systemic circulation with respect to cytokine production. In our *in vitro* experiments, the TNF-α and IL-6-inducing ability of the *H. pylori* 26695 CagA + strain was similar, regardless of whether the white blood cells originated from the patients with DU or from the apparently healthy blood donors, irrespectively of the *H. pylori* status. It is noteworthy, however, that the peripheral blood cells from the DU patients produced a moderately higher level of IL-8 (Table 1, Figure 4). IL-8 is one of the most important cytokines in the pathogenesis of local inflammation at the site of *H. pylori* multiplication [5, 18], where not only inflammatory cells, but also gastric epithelial cells themselves can be the source at least of IL-8 production [22]. It is not known, why the IL-8 production capacity is higher in *H. pylori*-positive DU patients, whereas there is no significant difference in TNF-α and IL-6 production. The secretion of IL-8 is unlikely to be exclusively dependent on TNF-α, and the relation between these cytokines is complex.

A considerable number of data indicate that CagA-positive strains are more virulent, and are able to induce more IL-8 [5, 19]. In our study, 39 of the 40 DU patients had a CagA-positive status. In the non-ulcer group, the CagA positivity among the *H. pylori*-positive subjects was as high as 70.7%. This frequency of CagA positivity in a healthy control group is relatively high. We therefore suggest that CagA positivity alone can not explain the development of ulcer in the *H. pylori*-positive DU group. Thus, besides the bacterial virulence, host factors appear to be important in the outcome of the infection [23]. Among them, the intensity of the local cytokine response, with a consequent elevation of nitrating agents, can contribute to the development of mucosal destruction. Further studies are required to elucidate whether the higher IL-8-producing ability of the peripheral white blood cells in duodenal ulcer patients and the intensity of the local IL-8 response are connected. IL-8 is a chemokine, that is mainly secreted in digestive epithelial cells after bacterial interaction. Accordingly, its role in DU patients is obviously local. It is therefore tempting to speculate that there may be a difference in *in vitro* IL-8 production by the peripheral white blood cells in DU patients and *H. pylori*-positive healthy blood donors. The *H. pylori*-positive and CagA-positive normal blood donors (with no history of ulcerative disease) exhibited the same basal *in vitro*, inducible IL-8-producing capacity as that of the *H. pylori*-negative controls (Figure 4). It may be that a predisposition to a higher IL-8 response to a given bacterial stimulus might also be a predisposing factor for ulcerative processes. It is obvious, however, that merely this one factor cannot determine the final outcome of *H. pylori* infection. Further investigations of the connections between the genetic determination of an IL-8-producing capacity and the frequency of ulcer diseases could be informative, and are therefore mandatory.

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