Changes in Cytokine levels related to the Immunopathogenesis of Helicobacter pylori disease. Immunological and histological effects of triple treatment (omeprazol, azitromycin, and amoxycillin)

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RESUMEN
Las enfermedades gastroduodenales causadas por H. pylori son consecuencia de una inapropiada respuesta inmunitaria del huésped. Los objetivos del presente estudio han sido evaluar: 1) niveles séricos de citocinas en pacientes infectados o no con H. pylori y la relación de los primeros con la inmunopatogénesis de las lesiones en la mucosa gástrica y duodenal causada por este agente; 2) cambios en los niveles de citocinas posteriores al tratamiento y relacionados con algunas alteraciones histológicas. Se estudiaron 40 pacientes ambulatorios (edad media 68.7 años; 59-80 años), de ambos sexos, con diagnóstico clínico y endoscópico de úlcera gástrica, úlcera duodenal y gastritis erosiva y/o lesiones duodenales. Se diagnosticó H. pylori en muestras de tejido gástricas y duodenales por el Test de la Ureasa rápido, exámenes histológicos y cultivo de tejido. La sensibilidad antibiótica se determinó por la Técnica Elipsométrica, para evaluar la concentración inhibitoria mínima (CIM) en g.ml⁻¹ de azitromicina y amoxicilina. Se evaluaron los niveles séricos de IL-2, IL-4, IL-10, IFN-γ y TGF-β antes y después del tratamiento. Los pacientes con diagnóstico confirmado de infección por H. pylori fueron tratados con omeprazol, amoxicilina y azitromicina. Aquellos no infectados con H. pylori sólo recibieron omeprazol. Se detectó una diferencia significativa en los niveles de todas las citocinas entre los pacientes H. pylori (+) y H. pylori (-) (p<0.01). Se determinaron niveles significativamente menores de IL-2 (p<0.05) e IFN-γ y mayores de IL-4, IL-10 y TGF-β en pacientes H. pylori (+) comparados con los de pacientes H. pylori (-) (p<0.01). También las diferencias fueron significativas entre las determinaciones pre y post tratamiento de los pacientes H. pylori (+) (p<0.01). La eficacia del tratamiento empleado se evidenció tanto por los valores de las citocinas evaluadas así como en la reversión las alteraciones histológicas observadas antes de la terapia.

PALABRAS CLAVE: Helicobacter pylori / respuesta inmune / IL-2 / IL-4 / IL-10 / IFN-γ / TGF-β.

ABSTRACT
Gastroduodenal diseases caused by Helicobacter pylori are a consequence of an inappropriate host’s immune response. This study aims to evaluate: 1) cytokine serum levels in H. pylori infected patients and its relationship with the immunopathogenesis of the gastric and duodenal mucosa lesions caused by this agent; 2) the immunological changes that could be detected after treatment and linked to some histological alterations. Forty ambulatory patients (mean age 68.7 years; 59-80 years), both sexes with clinical and endoscopical diagnosis of gastric ulcer, duodenal ulcer or erosive gastric and/or duodenal lesions, were studied. H. pylori was diagnosed in gastric and duodenal tissue samples by urease rapid test, histological examination and tissue culture. Antibiotic sensitivity tests by the ellipsometric technique was performed to determine the minimal inhibitory concentration (MIC) in g.ml⁻¹ of azitromycin, and amoxicillin. The serum levels of IL-2, IL-4, IL-10, IFN-γ, and TGF-β were assessed before and after treatment. Patients with confirmed diagnosis of H. pylori infection were treated with omeprazol, amoxicillin and azitromycin. H. pylori non infected patients only received omeprazol. A significant difference was detected in cytokine levels between H. pylori (+) (n=24) and H. pylori (-) (n=16) patients (p<0.01). In H. pylori (+) patients IL-2 (p<0.05) and IFN levels were lower and IL-4, IL-10 and TGF-β were higher when compared with H. pylori (-) subjects (p<0.01). Pre and post treatment assessments were also significantly different (p<0.01). The employed treatment efficiency was expressed not only on cytokine values but also on the reversion of the histological changes.

KEY WORDS: Helicobacter pylori / Immune response / IL-2 / IL-4 / IL-10 / IFN-γ / TGF-β.
INTRODUCTION
Infection with Helicobacter pylori leads to different clinical and pathological outcomes in humans. Several authors have reported the relationship between this agent and the pathogenesis of chronic gastritis, peptic ulcer, gastric cancer and MALT lymphomas. Epidemiological studies demonstrating that these severe clinical manifestations do not frequently occur in most people infected with H. pylori, led us to think that the different clinical disorders caused by this agent could be a consequence of microorganism characteristics, environmental influences and the host response against H. pylori. There are bacterial determinants associated with its pathogenicity, which enable some bacteria species to synthesize products that directly or indirectly affect the gastric mucosal, cause an inflammatory reaction and alter mucosa secretion. These risk factors associated with the host’s disregulated immune response, genetic influences and environmental conditions are responsible for the mucosa alterations and subsequent development of a disease.

The local acute inflammatory reaction and the systemic immune response developed against the H. pylori organisms are neither gastric nor intestinal mucosa protectors. It is generally accepted that they produce mucosal injury. Several cellular and humoral immune mechanisms are involved in the control of H. pylori infection. These different humoral, cellular and cytokine responses may reflect the presence of different H. pylori strains eliciting different host responses.

The purpose of the present study was to investigate the serum levels of cytokines and their regulatory action on the immunopathogenesis of H. pylori infection, as well as to provide information regarding the immunological changes linked to some histological alterations that could be detected after treatment. With these aims, a group of patients with gastroduodenal disease, infected or not with H. pylori were studied. Different diagnostic methods were used to detect H. pylori infection, and IL-2, IL-4, IL-10, IFN-γ and TGF-β serum levels were evaluated before and after the treatment.

MATERIALS AND METHODS
Patients
Forty ambulatory patients (mean age 68.7 years; 59-80 years), with clinical and endoscopic diagnosis of gastric ulcer, duodenal ulcer or erosive gastric and/or duodenal lesions, were studied. H. pylori (+) patients were assigned to the eradication therapy, while H. pylori (-) patients were included as a control group. According to the endoscopic studies and considering the evaluated parameters, the examined population was classified as follows: a) patients with gastric ulcer (4 cases); 2) gastric ulcer plus erosive lesion (2 cases); 3) erosive lesions (24 cases); 4) erosive gastric and duodenal lesions (2 cases); 5) erosive duodenal lesions (1 case); 6) chronic inflammatory reaction (12 cases); 7) acute inflammatory reaction (12 cases); 8) subacute inflammatory reaction (12 cases); 9) atrophy (10 cases). Some patients showed two or more lesions concomitantly.

Clinical examination
The patients were subjected to a complete clinical examination and routine complementary laboratory tests were performed. Five samples from the antrum and five from the gastric cavity were obtained by endoscopy and the characteristics of these tissues were observed.

Diagnosis
Presence of H. pylori was assessed in gastric tissue samples. Several methods were utilized, namely, rapid urease test (RUT) in gastric tissue, tissue culture in enriched and/or selective media and histological examination. It was considered H. pylori positive (Hp +) those cases with confirmed diagnosis by anatomopathological examination (100% of cases). Patients assigned as H. pylori (+) and H. pylori (-) subjected to the diagnostic tests before and after treatment were examined.

Bacteriological diagnosis
Five antrum and five body samples were obtained during the endoscopy. In one section of each of the three samples used for bacteriological diagnosis the RUT was performed. Smears for direct Gram Nicolle stained bacteria examination were prepared from the well-preserved gastric sample biopsy. Cultures with selective and non-selective specific media, plus the nutritional components conditioned with blood and antibiotics, were also performed. The bacterial growth was evaluated by semi-quantitative seeding in non-selective media, typifying colonies through morphology, biochemical and protein profile, preformed enzymes as urease, catalase and oxidase.

Antibiotic sensitivity tests were performed by ellipsometric technique (E-test), to determine the Minimal Inhibitory Concentration (MIC) in g.ml⁻¹ of azitromycin and amoxycillin.

Histopathology
Four samples (2 of each region) were subjected to histological examination to characterize H. pylori, for
evaluating the cell alterations the organism may produce and the accompanying inflammatory reaction, linked to some cytokines changes. Two samples from the antrum and 2 from the stomach body, were fixed in formaldehyde, embedded in paraffin and stained with hematoxin-eosin (HE) and modified Giemsa (MG). It was studied the localization of inflammatory and other active lesions of the mucosa, and the parameters analyzed were: a) presence of acute, subacute or chronic inflammatory reaction; b) erosion; c) metaplasia, displasia or intestinal atrophy; and d) ulcerative lesions.

Both H. E. and Giemsa confirmed diagnosis by the identification of a curved bacillus in the luminal surface of the gastric epithelial cells. Satoh’s parameters as well as Sidney’s modified histopathological classification were applied\(^{(21,22)}\). Diagnosis was also confirmed by the histological alterations such as, chronic active gastritis, focal necrosis, absence of mucus, PMN infiltrate related to inflammation, association with gastric metaplasia in duodenum and absence of \(H.\) \textit{pylori} in case of intestinal metaplasia presence.

**Cytokine assays**

Peripheral blood levels of interleukins IL-2, IL-4, IL-10, Interferon gamma (IFN-\(\gamma\)) and Transforming Growth Factor beta (TGF-\(\beta\)) were analyzed by commercially available ELISA methods (R&D Systems). Pre and post treatment sera were stored at -70°C until used. Assays were performed in duplicate according to the manufacturer’s instructions.

**Treatment**

Patients with confirmed diagnosis of \(H.\) \textit{pylori} infection were treated with 20 mg omeprazol daily for 30 days, 1.5 gr amoxycillin three times/day for 10 days, and azitromycin 500 mg daily during 6 days. On day 60, all the diagnostic tests were repeated to determine \(H.\) \textit{pylori} presence. Treatment efficacy was investigated by repeating the histopathological, bacteriological and immunological tests. The \(H.\) \textit{pylori} (+) patients were assigned to the control group and received only omeprazol (20 mg/day for 30 days). All participants received proper information and signed a written consent form. This investigation conforms to the principles outlined in the Declaration of Helsinki (Br. Medical J. 1964; 11:177).

**Statistical Analysis**

Data regarding cytokine levels were analyzed by Kruskall-Wallis and Mann-Whitney U Tests. Wilcoxon test was used to compare the cytokine levels after and before treatment. A probability value of 0.05 was set as the limit of statistical significance. To analyze the relationship between cytokines and successful therapy Spearman rank correlation was employed.

**RESULTS**

Twenty four out of 40 patients were tested positive for \(H.\) \textit{pylori}. Therefore the infection prevalence in the studied group of patients was 60%. The \(H.\) \textit{pylori} infection was considered eradicated by day 60 if the assessed bacteriological and histopathological tests were negative. Following these criteria 18/24 (75%) of patients eradicated the \(H.\) \textit{pylori} infection after 60 days of treatment.

The immunological test results evidenced significant modifications in cytokine levels mainly in \(H.\) \textit{pylori} (+) patients. Levels of different cytokines were compared in \(H.\) \textit{pylori} (-) patients (n=16) and \(H.\) \textit{pylori} (+) patients (n=24). In \(H.\) \textit{pylori} (+) patients IL-2 (p<0.05) and IFN-\(\gamma\) levels were lower and IL-4, IL-10 and TGF-\(\beta\) were higher when compared with \(H.\) \textit{pylori} (-) subjects (p<0.01; Fig. 1). The effect of treatment on the serum levels of cytokines in Hp(-) and Hp(+) patients was also analysed. The treatment did not modify the serum cytokine levels in \(H.\) \textit{pylori} (-) patients (Fig. 2a). In contrast, it could be observed that in \(H.\) \textit{pylori} (+) patients the levels of IL-2 and IFN-\(\gamma\) significantly increased and those of IL-4, IL-10 and TGF-\(\beta\) significantly decreased compared to before treatment (p<0.01; Fig. 2b). Interestingly, the serum concentrations of cytokines in post treated Hp(+) patients (Fig. 2b) did not differ from those of non infected patients shown in Figure 1. The analysis of the relationship...
between cytokines changes and successful therapy showed a strong association among the variables under study (Spearman rank correlation; rs=0.52; p<0.04).

In patients with confirmed \textit{H. pylori} (+), a superficial chronic gastritis inflammatory reaction of variable degree was detected, with lymphocytes and plasma cells being identified in chronic processes and PMN and eosinophils in the acute ones. There were no bacteria neither in glands nor in intestinal metaplasia in \textit{H. pylori} (+).

Histopathological studies evidenced several concluding remarks (Table I). Twenty four out of the 40 studied patients were (\textit{Hp} +). Eighteen of the 24 \textit{H. pylori} (+) diagnosed patients were negative after treatment (75%). A decrease of positiveness was observed in 3 cases (12.5%) and persistence in 1 case (4.2%). The data analysis showed a significant difference (p<0.01).

Chronic inflammation detected before treatment disappeared in some cases, and decreased remarkably in the rest after the combined treatment. Chronic (12/24) and acute inflammatory reaction (12/24) detected before the treatment disappeared or decreased in several cases (8/24). Subacute infection also decreased after treatment (6/24). Erosion was absent at all in 10 cases. Neither intestinal metaplasia nor slight focal displasia showed post-treatment modifications. Pre-treatment atrophy (n=10) disappeared in 6 patients and decreased in 1 case (Table I).

**DISCUSSION**

Our study indicates a high prevalence of infection with \textit{H. pylori} (60%) among patients with digestive disorders. The fact that several subjects also had erosive lesions and gastric and/or duodenal ulcers reinforces the view of \textit{H. pylori} as a being involved in such pathologies. Accompanying these results there were significant differences in the serum levels of cytokines when comparing \textit{H. pylori} (+) and \textit{H. pylori} (-) patients. Data represent mean ± standard deviation. *Comparisons between pre- and post-treatment values were significant in all cases (p<0.01).

**TABLE I.** Pre- and post-treatment findings from histological studies

<table>
<thead>
<tr>
<th>H. pylori</th>
<th>Infection with H. pylori</th>
<th>Chronic</th>
<th>Subacute</th>
<th>Persistent</th>
<th>Erosion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>16/40</td>
<td>Decreased</td>
<td>12/24</td>
<td>50%</td>
<td>41.6%</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>18/24</td>
<td>Decreased</td>
<td>8/24</td>
<td>8/24</td>
<td>6/24</td>
</tr>
</tbody>
</table>

*Significantly different from Pre-treatment H. pylori (+) patients p<0.0001.

![Figure 2.](image-url)
Asymptomatic ones(25). i.e., IL-8, and reactive oxygen and nitrogen species may be increased mucosal production of inflammatory cytokines, reported that in addition to the bacterial virulence factor, mucosal inflammation by cagA gene positive induction of distinct cytokines and development of severe pylori exposure may reverse such strategy by driving a predominant mucosal inflammation along with a persistent antigen recovery. It has been proposed that some infected individuals develop clinical manifestations, such as gastric or duodenal ulcers, and even gastric cancer while others remain symptomless or unaffected. This is probably due to differences in H. pylori strains, the existence of a toxin encoded in the cagA gene, or several host and environmental risk factors(7,12-25). Yamaoka et al. reinforced this idea demonstrating the magnitude and type of effector immunological mechanisms are largely determined by cytokines(11,23,24). Klausz and col. reported that in addition to the bacterial virulence factor, increased mucosal production of inflammatory cytokines, i.e., IL-8, and reactive oxygen and nitrogen species may be relevant to the pathophysiology of H. pylori-induced duodenal ulcer(26).

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At the microscopic level, H. pylori (+) cases developed chronic superficial gastritis, mostly in the antrum, with a variable degree of inflammatory reaction composed of lymphocytes and plasma cells, whereas patients with acute or subacute inflammation showed a PMN and eosinophil predominance in their gastric infiltrates. All H. pylori (+) patients were subjected to combined therapy (azitromycin, amoxicillin and omeprazol) showing a remarkable improvement either in the histopathological analysis or endoscopic examination, no matter the length of the inflammatory process, even in one case of H. pylori persistence. In general terms, there was no inflammation or the degree of the reaction was minimal after treatment. This histological reparation may be linked to cytokines changes. It can be concluded that the cytokine response may be a substantial determinant for disease outcome.

REFERENCES