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Investigating the immunological effects of *H.pylori* infection in peptic ulcer patients.

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ABSTRACT

H. pylori colonization of the stomach elicits humoral and cellular immune responses, which is associated mostly with an increased risk for development of peptic ulcer disease. So different immunological markers evaluate in the sera of H. pylori peptic ulcer patients. inflammatory cytokines (IL-1α and IL-8) showed a significant increase in sera of patients as compared to control subjects. The results of lymphocyte immunophenotypes percentage of CD3+, CD4+, CD8+ and CD56+cells showed a significant increase in patients when compared with those of normal subjects (37, 51, 40, and 33% respectively in patients vs. 32, 30, 35, and 21%, respectively in control). Phagocytes using heat-killed yeast and NBT reaction showed significant ($P \leq 0.001$) increase activity of phagocytosis (Phagocytic index; PI) in total patients as compared to control subjects. We conclude that non specific immune responses (inflammatory cytokines, different immune cells such as phagocytic cells) formed against H. pylori participate in inflammatory and damage process in gastric tissue and its progression to peptic ulcer disease.

Introduction

A peptic ulcer, also known as ulcus pepticum, peptic ulcer disease or PUD, is an ulcer (defined as mucosal erosions equal to or greater than 0.5 cm) of an area of the gastrointestinal tract that is usually acidic and thus extremely painful. *Helicobacter pylori (H. pylori)* is a spiral-shaped bacterium that is found in the gastric mucous layer or adherent to the epithelial lining of the stomach. *H. pylori* causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers. Contrary to general belief, more peptic ulcers arise in the duodenum than in the stomach (1,2,3,4).

A gastric peptic ulcer is a mucosal defect which penetrates the muscularis mucosae and muscularis propria, produced by acid-pepsin aggression. Ulcer margins are perpendicular and present chronic gastritis. During the active phase, the base of the ulcer shows 4 zones: inflammatory exudate, fibrinoid necrosis, granulation tissue and fibrous tissue. The fibrous base of the ulcer may contain vessels with thickened wall or with thrombosis (3,5).

H. pylori weakens the protective mucous coating of the stomach and duodenum, which allows acid to get through the sensitive lining beneath. Both acid and bacteria irritate the lining and cause a sore, or ulcer.Contributing to the protection of *H. pylori* is the fact that the body's natural defenses cannot reach the bacterium in the mucus lining of the stomach. The immune system will respond to an *H. pylori* infection by sending white cells, killer T cells, and other infection fighting agents. However, these potential *H. pylori* eradicators cannot reach the infection, because they cannot easily get through the stomach lining. They do not, however, go away - the immune response just grows and grows. White cells die and spill their destructive compounds (superoxide radicals) on stomach lining cells. Extra nutrients are sent to reinforce the white cells, and *H. pylori* can feed on this. Within a few days, gastritis and perhaps eventually a peptic ulcer results. It may not be *H. pylori* itself which causes peptic ulcer, but the body's response (inflammation of the stomach lining) (6,7,8).

Because of its low potency, *H. pylori* LPS had been considered to play a minor role in disease pathogenesis. However, antigenic mimicry by *H. pylori* LPS may also incite an autoimmune response, resulting in gastric mucosal injury (9). *H. pylori* LPS causes an acute gastritis and results in the induction of gastric epithelial cell apoptosis (10). Studies examined peripheral blood monocytes and may not be directly relevant to gastric lamina propria macrophages. However, H. pylori LPS is an important virulence factor that plays a key role in CD14-mediated monocyte activation and inflammatory cell recruitment in *H. pylori* gastritis (11).

Helicobacter pylori is a gram-negative bacterium that persistently colonizes more than half of the global human population. In order to successfully colonize the human stomach, *H. pylori* must initially overcome multiple innate host defenses. Remarkably, *H. pylori* can persistently colonize the stomach for decades or an entire lifetime despite development of an acquired immune response (12,13,14). This review focuses on the immune response to *H. pylori* and the role of immune reactions such as inflammatory responses in progression of infection to ulcer. The topics addressed in this review are important for understanding how *H. pylori* resists

immune clearance and also are relevant for understanding the pathogenesis of diseases caused by *H. pylori.*

The goals of this study were (i) to determine whether *H. pylori* affectinng the release of proinflammatory chemokines, including IL-8, IL-1 in sera of patients with peptic ulcer; (ii) The peripheral lymphocytes of patients with peptic ulcer and controls were immunophenotyped for four CD markers, which were CD3, CD4, CD8 and CD56; and (iii) to determine whether H. pylori could stimulate phagocytic activity.

MATERIALS AND METHODS

Subjects

Fifty patients with *H.pylori* peptic ulcer (age range: 25 – 50 years) were investigated. The patients were referred to the hospital. The diagnosis was made by the consultant medical staff, which was based on a history inspection, clinical examination, endoscope and other laboratory tests. A control sample of 50 was included in the study

Blood Samples

From each subject, 10 ml of blood were obtained by veinepuncture, a disposable syringe. The blood sample was divided into two aliquots, each of 5ml. The first was dispensed in a plain tube, and left for 15 minutes at 4ºC to clot. Then, centrifuged at 3000 rpm for 10 minutes to collect serum. The serum was divided into aliquots (0.5 ml) and stored in freezer (-20ºC) until use. The second aliquot was dispensed in a tube containing heparin (5000 unit/ml) to prevent coagulation. This blood sample was processed in less than three hours and was used for lymphocyte immunophenotyping and phagocytosis**.**

Laboratory Methods

Serum Level of Cytokines

Serum levels of eight cytokines (IL-1α, and IL-8) were quantitatively determined in patients and control subjects by means of indirect sandwich ELISA test using ready kits manufactured by the French company Immunotech(IL-1α, and IL-8).

Calculations: The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample, using a curve fit equation.

Isolation of lymphocytes

By means of a density gradient centrifugation (modified by Ad'hiah, 1990)(32), the lymphocytes were isolated from the whole blood. The following steps were followed:

i. The blood (5 ml) was centrifuged (1000 rpm) for 15 minutes.

ii. The plasma, Buffy coat and the most upper layer of erythrocytes were collected in a 5 ml centrifuge tube, and the cell-suspension was diluted with physiological saline up to 5 ml.

iii. The diluted cell suspension (2.5 ml) was layered on 2 ml of Ficoll-isopaque separation fluid (lymphoprep; specific gravity = 1.077).

iv. The tubes were centrifuged (2100 rpm) for 30 minutes in a cooled centrifuge.

v. After centrifugation, the lymphocytes were visible as cloudy band between the plasma and lymphoprep layers.

vi. The band was collected in a 5 ml test tube, and the cells were suspended in washing medium (2 ml).

vii. The tube was centrifuged (2000 rpm) for 5 minutes (first wash), then the supernatant was discarded,

and the cells were re-suspended in 3 ml washing medium. This step was repeated (second wash).

viii. A third wash was also done but at a lower speed (1000 rpm) and for 10 minutes. This step is very necessary, because it helps to maintain most of the platelets in the supernatant.

ix. The obtained cells were suspended in 1 ml Terasaki medium supplemented with 5 % heat inactivated (56ºC for 30 minutes) foetal calf serum.

:Lymphocyte Immunophenotyping for CD markers

: Principles

Lymphocytes express a large number of different molecules on their surfaces, which can be used to distinguish cell subsets. Many of these cell markers can be identified by specific monoclonal antibodies. A systematic nomenclature has been developed in which the term CD (cluster designation) refers to groups of monoclonal antibodies that bind specifically to particular markers (33). In the present study, four CD markers were investigated: CD3 (Pan T-lymphocytes), CD4 (T-helper lymphocytes), CD8 (T-cytotoxic lymphocytes) and CD56 (natural killer cells).

: Procedure

The isolated lymphocytes prepared were adjusted to a cell count of 1 x 10^6 cell /ml, and 10 µl of the cell suspension were dispensed in the well of a CD marker specific slide. Then, the slide was left for air-drying at room temperature for 30 minutes. After drying, 10 µl of the fixative solution were added to each well on the slides and left for air-drying (about 2 minutes) at room temperature. Then, the slide was covered with Aluminium foil and stored in the freezer (-20ºC).

Before carrying out the procedure of CD typing, the slide was taken out from the freezer and left for thawing at room temperature for 15 minutes. Then, 10 µl of a specific anti-CD marker antibody (Fluorescin Isothiocyanate labeled monoclonal antibody) were added to the well, and the slide was incubated at room temperature in a humid chamber for 60 minutes. After incubation, the well was washed with phosphate buffer saline to remove any unreacted antibody. The slide was air-dried and examined under fluorescent microscope to score the percentage of fluorescent cells (Serotec Data Sheet, 2005).

: Phagocytosis

The method of Metcalf *et al*. (1986) (34) was adapted to carry out the procedure of assessing phagocytosis in the peripheral blood. The method is based on assessing the percentage of polymorphonuclear cells that phagocytose heat-killed yeast.

Preparation of Heat-Killed Yeast

Ten grams of yeast (*Saccharomyces cervisiae*) were suspended in a warm (37ºC) physiological saline (150 ml). The cell suspension was heated in boiling water bath for 60 minutes. After heating, the cells suspension was cooled to 37ºC, and filtered using sterile double layers of gauze. The filtered cell suspension was assessed for yeast cell viability by dye exclusion test (trypan blue) to assure that all cells were dead. Then, the cell suspension was divided into aliquots (5 ml) and stored at -20ºC until use.

Phagocytic Index

To determine the phagocytic index (PI), an aliquot of the heat killed yeast suspension was obtained from the freezer and left in a water bath (37ºC) for

thawing. Then, the cell suspension was washed twice with physiological saline and the yeast cells were adjusted to a concentration of 1 x 10^7 cells/ml. Then, 0.25 ml of heparinized blood was mixed with 0.15 ml of yeast suspension together with 0.20 ml of Hank's balanced salt solution, and the mixture was incubated for 30 minutes at 37ºC, with gentle shaking every 5 minutes. After incubation, the mixture was mixed thoroughly, and a blood film was made and stained with Wright stain for 3 minutes. Then, the slide was washed with tap water and air-dried. The slide was inspected for yeast-phagocytic cells using light microscope, and for each samples at least 100 polymorphonuclear cells (phagocytic and non-phagocytic cells) were randomly counted, and the percentage of yeast-phagocytic cells was scored according to the following formula:

$$\text{Phagocytic Index (\%)} = \left(\frac{\text{Number of Phagocytic cells}}{\text{Total Count}}\right) \text{x } 100$$

:Nitroblue Tetrazolium (NBT) Index

The procedure of Metcalf *et al*. (1986) (33) was followed to assess the nitroblue tetrazolium index.

The following materials were mixed in a test tube and incubated at 37ºC for 25 minutes:

- Heparinized blood: 0.25 ml
- Hank's balanced salt solution: 0.20 ml
- NBT solution: 0.10 ml
- Yeast suspension: 0.05 ml

After that, a blood smear was prepared on a slide and left for air-drying. The slide was stained with Wright stain (3 minutes), washed with tap water and air-dryed. Then, the slide was examined under light microscope to determine the polymorphonuclear cells that reduced the NBT substance, and their percentage was calculated (NBT index; %) using the equation of phagocytic index.

Differences between Means

The data of other parameters were tabulated in a data sheet, and the data were analyzed using the computer programme SPSS (Statistical Package for Social Sciences) version 11.5. The investigated parameters were presented in terms of means ± standard errors (S.E.), and differences between means of patients and controls were assessed by ANOVA test and the Least Significant Difference (LSD). The difference was considered significant when the probability (P) value was ≤ 0.001. Other data was analyzed using Chi-square test.

Results

Serum Cytokine Levels

The serum levels of interlukin-1α (IL-1α), and IL-8, were assessed in patients and controls.

Interlukin-1α (IL-1α)

A highly significant (P ≤ 0.001) increased mean serum level of IL-1α was observed in the patients as compared to control subjects (210.61 vs. 33.20 pg/ml), (Table 1).

Table 1: Serum level (mean ± S.E.) of IL-1α in peptic ulcer patients and controls.

GROUPS	NUMBER	SERUM LEVEL OF IL-1A (PG/ML)			PROBABILITY
		Mean ± S.E.	Minimum	Maximum	≤
Controls	50	33.20 ± 0.04	19.50	43.50	
patients	50	210.61 ± 1.89	114.40	264.80	0.001

Interlukin-8 (IL-8)

A highly significant ($P \leq 0.001$) increased mean serum level of IL-8 was observed in the total patients as compared to control subjects (270.58 vs. 44.86 pg/ml), (Table 2).

Table 2: Serum level (mean ± S.E.) of IL-8 in peptic ulcer patients and controls.

GROUPS	NUMBER	SERUM LEVEL OF IL-8 (PG/ML)			PROBABILITY ≤
		Mean ± S.E.	Minimum	Maximum	
Controls	50	44.86 ± 1.84	23.90	51.10	
patients	50	270.58 ± 1.20	96.87	320.00	0.001

Lymphocyte Immunophenotypes

The peripheral lymphocytes of patients and controls were immunophenotyped for four CD markers, which were CD3, CD4, CD8 and CD56.

The total patients showed a significant ($P \leq 0.05$) increased percentage mean of CD3, CD4, CD8 and CD56 lymphocytes when a comparison was made with the controls (37, 51, 40, and 33% respectively in patients vs. 32, 30, 35, and 21%, respectively in control) as showed in (fig.1)

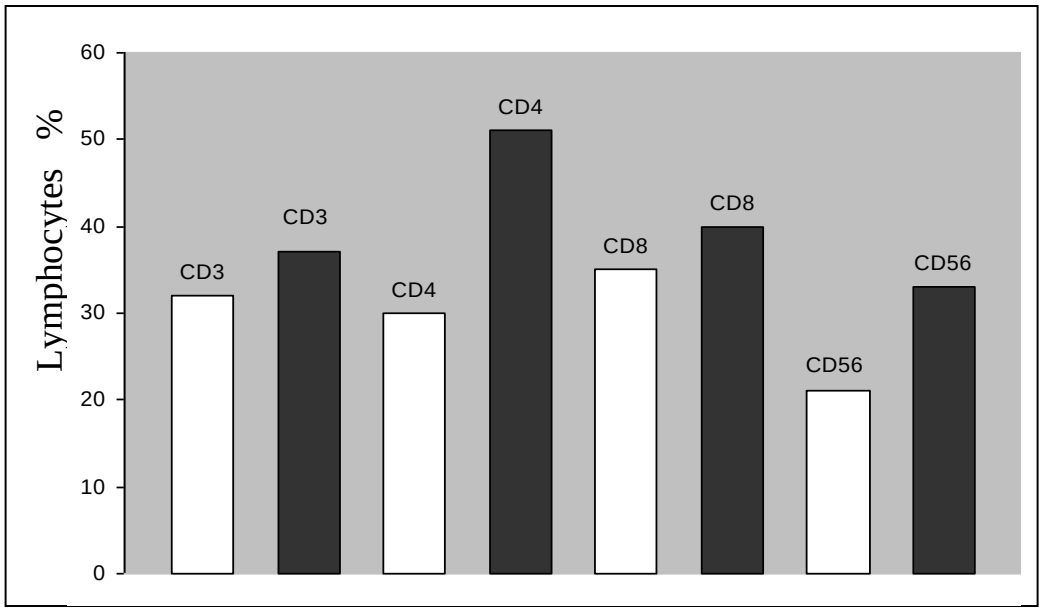

Figure 1: Percentage means of Lymphocytes in peripheral blood of controls (white bars) and patients (black bars).

Phagocytosis

A highly significant ($P \leq 0.001$) increased activity of phagocytosis (Phagocytic index; PI) was observed in total patients (73.68 %) as compared to control subjects (21.30 %) (Table 3). Similarly, the nitroblue-tetrazolium (NBT) index showed a significant increased percentage in patients (121.0%) as compared to controls (30.10) (Table 4).

Table 3: Phagocytic index (mean ± S.E.) of peripheral phagocytes in patients and controls.

GROUPS	NUMBER	PHAGOCYTIC INDEX (%)			PROBABILITY ≤
		Mean ± S.E.	Minimum	Maximum	
Controls	50	21.30 ± 1.84	8.90	30.50	
patients	50	73.68 ± 1.67	27.60	86.84	0.001

Table 4: Nitroblue-tetrazolium index (mean ± S.E.) of peripheral phagocytes in patients and controls.

GROUPS	NUMBER	NITROBLUE-TETRAZOLIUM INDEX (%)			PROBABILITY ≤
		Mean ± S.E.	Minimum	Maximum	
Controls	50	30.10 ± 0.44	19.00	55.80	
patients	50	121.00 ± 1.14	70.60	155.84	0.001

Disscution

Helicobacter bacteria colonize in the stomach and induce strong, specific local and systemic humoral and cell-mediated immunity. Helicobacter binds to the host epithelial cells, directly triggering the recruitment of neutrophils. Local inflammatory processes in the gastric mucosa are followed by extensive immune cell infiltration, resulting in chronic active gastritis (1,15,16).

Different immunological parameters (lymphocyte immunophenotypes, cytokines, and phagocytosis,) were evaluated in the patients. The results of lymphocyte immunophenotypes defined by CD markers, serum level of cytokines, and phagocytosis were in favour of such generalization, and the *H. pylori* may have either a direct or indirect effect in these evaluations. The results of lymphocyte immunophenotypes support such suggestion, and percentages of CD3+, CD4+, CD8+ and CD56+ showed significant deviations from the normal values in controls (Fig.1).

The percentage of CD3+ cells (pan T lymphocytes) in control group was lower than that detected in group of patients.

These findings may indicate that T lymphocytes were activated numerically and functionally The CD4+ lymphocytes, which are known as helper/inducer lymphocytes, showed a significant increase in group patients when compared with CD4+ cells level in normal subjects.

The percentage of CD8+ cells showed a significant increase in group patients when compared with CD8+ cells level in normal subjects.

A further important cell of lymphocytes, which showed a significant increased percentage in patients, is CD56+ cells. Previous experimental studies showed that the gastric mucosal inflammation that develops in wild-type mice infected with *H. pylori* consists primarily of lymphocytes and other mononuclear cells. Most of the infiltrating cells are CD4+ T cells, but CD8+ T cells, B cells, dendritic cells, and monocytes are also present (17, 18). The intensity of inflammation that develops in *H. pylori*-infected mice is relatively mild compared to that which develops in *H. pylori*-infected humans and is also relatively mild compared to that which develops in *H. pylori*-infected Mongolian gerbils (19)

In the present study, the investigated proinflammatory cytokines (IL-8, IL-1α) were significantly increased in the sera of patients as compared to health subjects (table 1, 2). These result agreed with other studies demonstrate that *H. pylori* stimulates the release of neutrophil-activating, IL-8 from human monocytes (20). This chemokine is potent leukocyte chemoattractants and may play an important role in regulating inflammatory cell infiltration of *H. pylori*-infected gastric mucosa (21). The mean serum level of IL-1α in healthy group was very low (below 33.20 pg/ml), and this is expected since, it is well known that most IL-1 α remains in the cytosol of the cell in its precursor form, where it may function as an autocrine messenger. There is also evidence that the precursor is transported to the cell surface that is associated with the membrane. This membrane-bound precursor is biologically active, perhaps serving as a paracrine messenger to adjacent cells (22, 23). The IL-1α showed a highly significant difference between patients and health control, and this result agrees with findings of Innocenti et al. (2001) who reported that *H. pylori* infection is characterized by a local increase in the expression of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α), as well as high levels of the neutrophil-recruiting chemokine IL-8, in the H. pylori-infected stomach has also been documented. *H. pylori* has been demonstrated to have a possible pro-inflammatory role in chronic inflammatory diseases, and consequently, it stimulates T and B-lymphocytes which lead to a more pro-inflammatory situation with elevated serum levels of inflammatory cytokines such as IL-8, which showed a significant increased in patients (24).Chemokines are a superfamily of closely related chemoattractant cytokines which specialize in mobilizing leukocytes to areas of immune challenge (25). These inducible proinflammatory peptides potently stimulate leukocyte migration along a chemotactic gradient. They also modulate leukocyte adhesion molecule expression and other leukocyte functions that are necessary for leukocytes to leave the circulation and infiltrate tissues. Thus, increased chemokine production and release is an important mechanism

for leukocyte recruitment in response to injury or infection (25, 26).

The scope of peptic ulcer and immunity was further explored but this time taking a non-specific measure of immune response; it was phagocytosis. A highly significant increased activity of phagocytosis in terms of phagocytosing heat-killed yeast and NBT reaction was observed in total patients as compared to control subjects (Table 3, 4). One way in which *H. pylori* might resist being killed by macrophages is by blocking the production of nitric oxide. This effect is mediated by *H. pylori* arginase, which competes with nitric oxide synthase (NOS) for Arginine (27, 28). Kaparakis et al. (2008), and their results demonstrated that macrophages have a central role in Helicobacter infection-induced gastritis but do not affect *H. pylori*-specific antibody responses," say the researchers. "In identifying a role for macrophages in the initiation of gastritis during *H. pylori* infection, this study may assist in future studies targeting the inhibition of gastritis in the host and provide a stimulus to study the capacity of macrophage-modifying drugs to reduce the gastritis associated with Helicobacter disease(29).

The pathophysiologic mechanisms leading to neutrophil infiltration in *H. pylori* gastritis have been the subject of intense investigation (18, 23). H. pylori is minimally invasive; for this reason most investigators have focused on soluble factors, of either host or bacterial origin, which may mediate neutrophil recruitment. Host factors include proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-8, all of which are increased in the antral mucosae of individuals with H. pylori gastritis (29). Chemokines provide a chemoattractant signal to direct neutrophil migration toward an inflammatory focus. However, endothelial cell activation is required also for neutrophils to adhere to the vascular endothelium, migrate between endothelial cell tight junctions, and infiltrate tissues (25,26,30).

Our results showed that H. pylori infection is characterized by an active chronic gastric inflammation with invasion of polymorphonuclear as well as mononuclear cells. This concludes agreed with previous study showed that Helicobacter pylori infection of the stomach epithelium is characterized by an infiltration of polymorphonuclear and mononuclear cells (26, 30). These immune cells contribute to mucosal damage which may eventually lead to gastritis, peptic ulcer, gastric cancer, and/or MALT-associated gastric lymphoma (31).

In this review, we conclude that immune responses (inflammatory cytokines, different immune cells such as phagocytic cells) formed against *H. pylori* participate in inflammatory and damage process occur in gastric and its progression to peptic ulcer disease.

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دراسة لبعض ألعوامل ألمناعية لدى ألمصابين بقرحة ألمعده و ألأمعاء ألناتجه عن بكتريا *Helicobacter pylori* .

منال بادي صالح التميمي

فرع الأحياء ألمجهرية ألطبية - كلية الطب - جامعة ذي قار

ألخلاصة

تحفز بكتريا *Helicobacter pylori* ألمستعمره للمعدة و المتسببة بالتهاب المعدة المزمن أو التهاب أغشيتها المخاطية ألمناعة ألخلوية وألخلطيه في كل ألجسم وتتركز في موقع ألالتهاب. أن هذه ألاستجابة لا تؤدي إلى ألتخلص من هذه ألبكتريا بل تؤدي إلى تفاقم ألالتهاب مكون قرحة ألأمعاء.

لذ اقيمت في المرضى عوامل مناعية مختلفة شملت أنواع الخلايا اللمفية والحركيات الخلوية والفعالية البلعمية.

أظهرت ألحركيات ألخلويه (ألسايتوكينات) ألمدروسة (IL-1α, and IL-8) ارتفاعا معنويا في المرضى عند المقارنة مع السيطرة. وصفت الخلايا اللمفية في المرضى و السيطرة في ضوء واسماتها السطحية و التي شملت CD3، CD4، CD8،وأخيرا CD56.اظهر العدد الكلي للمرضى ارتفاعا معنويا بمعدل النسبة المئوية للخلايا (37, 51, 40, and 33%) في المرضى مقابل (32, 30, 35, and 21%) عند المقارنة مع السيطرة. أزدادت أيضا ألفعاليه ألبلعميه (معاملة بلعمة خلايا الخميرة المقتولة بالحرارة و فعالية NBT) في العدد الكلي للمرضى زيادة معنويه.

نستنتج من هذه الدراسة أن استجابة ألجسم ألمناعية ألمتمثلة بالعوامل ألمناعية أعلاه نتيجة الإصابة ببكتريا *Helicobacter pylori* لها دور كبير في تفاقم ألمرض إلى تكون قرحة ألأمعاء.

أن ألهدف من هذه ألدراسة هو لتأكيد ألمقترح ألذي يشير إلى أن هذه ألبكتريا ليس هي ألمسبب للتقرح ألحاصل بالأمعاء نتيجة الإصابة بها بل أن ألسبب يعود إلى استجابة ألجسم ألمناعية تجاه هذه ألإصابة والعوامل ألالتهابيه ألمتجمعة في موقع ألالتهاب.