

Helicobacter pylori L-form and patients with chronic gastritis

Ke-Xia Wang, Lin Chen

Ke-Xia Wang, Lin Chen, School of Medicine, Anhui University of Science & Technology Huainan 232001, Anhui Province, China
Supported by The Education Department of Anhui Province, China No.2003kj111

Correspondence to: Dr. Ke-Xia Wang, School of Medicine, Anhui University of Science & Technology, Huainan, 232001, Anhui Province, China. kexiawang2003@yahoo.com.cn

Telephone: +86-554-6658770 **Fax:** +86-554-6662469

Received: 2003-10-20 **Accepted:** 2003-12-08

Abstract

AIM: To study the relationship between infection with *Helicobacter pylori* L-forms and chronic gastritis and its association with possible changes of cellular immune function.

METHODS: Gastric mucosal biopsies were taken from 428 patients with chronic gastritis to detect *H pylori* L-form by Gram staining and immunohistochemistry staining. Peripheral venous blood samples of patients were taken to detect the percentage of CD3+, CD4+ and CD8+ by the biotin-streptavidin (BSA) assay and the levels of IL-2, IL-6 and IL-8 by ELISA.

RESULTS: The rate of infection with *H pylori* L-forms was 48.83% (209/428). The rate was 50.47% (216/428) and 52.80% (226/428), respectively, as detected by immunohistochemistry staining and Gram staining ($P>0.05$). The rate of *H pylori* L-forms in males and females was 57.8% (136/235) and 37.28% (73/193), respectively, ($\chi^2=17.05$, $P<0.01$). Furthermore, the rate increased with age, with the rate being significantly greater in patients ≥ 40 years old than in those <40 years old ($P<0.01$). The percentage of CD3+, CD4+, CD8+, the ratio of CD4+ / CD8+, and the levels of IL-2, IL-6, IL-8 in *H pylori*-positive patients were 47.58 \pm 4.44%, 25.51 \pm 4.74%, 22.77 \pm 7.46%, 1.44 \pm 0.51%, 1.56 \pm 0.47 mg/L, 103.62 \pm 5.85 ng/L, and 109.79 \pm 7.18 ng/L, respectively. Compared with *H pylori*-negative patients, the percentage of CD3+, CD4+ and the ratio of CD4+ / CD8+ and the IL-2 level decreased, but the levels of IL-6, IL-8 increased ($P<0.001$ - $P<0.01$). Moreover, the percentage of CD3+, CD4+, CD8+, the ratio of CD4+ / CD8+, and the levels of IL-2, IL-6 and IL-8 in patients infected with both *H pylori* L-forms and vegetative forms were 46.67 \pm 5.21%, 30.75 \pm 4.89%, 22.15 \pm 6.45%, 1.32 \pm 0.47%, 1.16 \pm 0.38 mg/L, 116.45 \pm 5.44 ng/L, and 118.64 \pm 6.24 ng/L, respectively. Compared with patients infected with only vegetative forms, the percentage of CD4+, the ratio of CD4+ / CD8+ and IL-2 level decreased, but the levels of IL-6 and IL-8 increased ($P<0.001$ or $P<0.01$).

CONCLUSION: L-form variation often occurs in patients with chronic gastritis and is commonly found in male patients and associates with ages. The L-form variation may be an important factor causing disorder of cellular immune function in the patients with *H pylori*-induced chronic gastritis.

Wang KX, Chen L. *Helicobacter pylori* L-form and patients

with chronic gastritis. *World J Gastroenterol* 2004; 10(9): 1306-1309

<http://www.wjgnet.com/1007-9327/10/1306.asp>

INTRODUCTION

Helicobacter pylori infection of the gastric mucosa can be found in approximately 50% of the world's population, and is associated with a range of pathologies, including chronic gastritis, peptic ulcer, atrophic gastritis, and gastric cancer^[1-6]. Infection of *H pylori* is life-long that elicits a marked host inflammatory response^[7-9]. However, natural infection fails to yield protective immunity^[10,11]. *H pylori* is a gram-negative, spiral and microaerophilic bacterium that colonizes the gastric epithelium of humans^[12-15]. Under some hostile conditions, *H pylori* changes from vegetative forms into L-forms by which *H pylori* can escape the body's immune response and live in the body for a long time. Once conditions return to normal *H pylori* reverts into vegetative forms, which further deteriorates the pathological changes. Therefore, *H pylori* L-forms appear to be more pathogenic and virulent than vegetative forms^[16-18]. Animal experiments and clinical studies have demonstrated that damages induced by *H pylori* are associated with Th1 cell-mediated immune response^[19-23]. The aim of this study was to confirm the occurrence of L-forms variation of *H pylori* vegetative forms, and to determine the relationship between *H pylori* L-form infection and chronic gastritis, and its association with possible changes of cellular immune function in the patients. In the study, gastric mucosal biopsies were taken from 428 patients with chronic gastritis to detect *H pylori* L-form by Gram staining and immunohistochemistry staining, and T lymphocyte subsets and serum levels of IL-2, IL-6 and IL-8 of patients were also detected.

MATERIALS AND METHODS

Patients

A total of 428 patients (aged from 14 to 67 years, 235 males and 193 females) with chronic gastritis diagnosed in our affiliated hospital from September 2000 to December 2002 were included in this study. Patients with other diseases were excluded.

Reagents

Gram staining and immunohistochemistry staining were used in this study. The biotin-streptavidin (BSA) reagents for T lymphocyte subset classification were provided by Jin' an Medical Laboratory Institute in Shanghai. Separating medium for lymphocyte was supplied by The Second Biochemical Reagent Factory in Shanghai (batch No.011215), and the test kits for IL-2 (batch No.1002-21), IL-6 (batch No.1006-32) and IL-8 (batch No.1008-25), were offered by Besancon Company (France).

Detection of *H pylori* L-forms

Biopsies of gastric antrum and gastric corpus were taken from 428 patients during upper endoscopy, fixed in 40 g/L formaldehyde, then embedded in paraffin and cut into section in 4 μ m thickness. The sections were used for Gram staining and immunohistochemistry staining separately. Gram stained slide was observed under oil lens (10 \times 100), and a total of 10-15

fields were randomly counted for *H pylori* L-forms. We regarded it as positive only when the average number was greater than 20. The antigen and antibody used in immunohistochemistry staining for *H pylori* L-forms were made in our laboratory, and the concentration of first antibody is 1:80. Other steps were performed according to the instruction of manufacturer (Dako company), using the known positive slide as a positive control and using PBS instead of first antibody as a negative control. A specimen was defined to be infected with *H pylori* L-forms when both staining methods produced a positive result.

Detection of cellular immune function

To investigate the possible changes of cellular immune function in *H pylori*-infected individuals, including the patients infected by *H pylori* L-forms and vegetative forms, the percentage of CD3+, CD4+, CD8+, ratio of CD4+/CD8+, and the levels of IL-2, IL-6, IL-8 in peripheral blood of *H pylori*-positive individuals were tested with the biotin-streptavidin (BSA) method. The peripheral venous blood of the subjects was taken, anticoagulated with heparin, and diluted with a solution free of Ca²⁺, Mg²⁺. Then, peripheral blood mononuclear cells were separated with lymphocytes separating medium and cleaned, and the number of cells was adjusted to (1-3)×10⁹/L of which 10 μL was taken and smeared in an acidproof varnish circle on the surface of a slide. When it dried naturally, McAb of anti-CD3+, anti-CD4+ and anti-CD8+, and sheep anti-guinea pig IgG, and SA-HRP. Cells were regarded as positive when the membrane was stained in brown color. A total of 200 cells were counted, and the positive percentages of cells were calculated. In addition, the serum levels of IL-2, IL-6 and IL-8 were detected by ELISA following the procedures detailed in the product instructions.

Statistical analysis

Data were expressed as mean±SD. Multiple comparison tests were performed with the χ^2 test and *t*-test.

RESULTS

Examination of *H pylori* L-forms with gram staining

Of the 428 patients studied, 226 (52.80%) were positive for both *H pylori* vegetative forms and L-forms, and other 17 (3.97%) were positive for vegetative forms only by Gram staining. The morphology of *H pylori* L-forms was highly variable, such as spheroid, coccoid form, big body, elementary body, long filament body, as seen on the smears under microscope.

Comparison between gram staining and immunohistochemistry staining

H pylori L-forms was detected in 216 (50.47%) patients by immunohistochemistry staining, of whom 209 (48.83%) were also detected *H pylori* L-forms by Gram staining. There was no significant difference in detection rate of *H pylori* L-forms between the 2 methods ($P>0.05$) (Table 1).

Relationship between infection of *H pylori* L-forms and gender as well as age of patients with chronic gastritis

H pylori L-forms were present in 57.87% (136/235) of males and 37.82% (73/193) of females ($\chi^2=17.05, P<0.01$). Furthermore, the rate of *H pylori* L-forms was significant difference between patients <40 years old and those ≥ 40 years old ($P<0.01$) (Table 2).

Detection of cellular immune function

The percentages of CD3+, CD4+ and the ratio of CD4+/CD8+ and IL-2 decreased, but the levels of IL-6 and IL-8 increased in *H pylori*-positive patients, compared with *H pylori*-negative patients, (Tables 3 and 4). In addition, the percentage of CD4+, the ratio of CD4+/CD8+ and the levels of IL-2 decreased, but

the levels of IL-6 and IL-8 increased in the patients infected by both L-forms and vegetative forms of *H pylori*, compared with those only infected by vegetative forms (Tables 3, 4).

Table 1 Comparison of Gram staining and immunohistochemistry staining in detection of *H pylori* L-forms

Gram staining (n)	Immunohistochemistry staining (n)		Total (n)
	Positive	Negative	
Positive	209	17	226
Negative	7	195	202
Total	216	212	428

$\chi^2=0.46779, P>0.05$ between the 2 methods.

Table 2 Relationship of infection of *H pylori* L-forms with gender and age of patients with chronic gastritis

Age(yr)	n	Male		Female		Total	
		n	Detection rate (%)	n	Detection rate (%)	n	Detection rate (%)
14-	62	31 (50.00)	75	17 (22.67)	137	48 (35.04) ^b	
30-	37	14 (37.84)	48	18 (37.50)	85	32 (37.65) ^b	
40-	55	35 (63.64)	23	12 (52.17)	78	47 (60.26) ^b	
50-	81	56 (69.14)	47	26 (55.32)	128	82 (64.06) ^b	
Total	235	136 (57.87) ^d	193	73 (37.82) ^d	428	209 (48.83)	

^d $\chi^2=17.05, P<0.01$; ^b $\chi^2=33.74, P<0.01$.

Table 3 Detection of T lymphocyte subsets in patients with chronic gastritis in relation to infection with *H pylori* (Hp) and its L-forms

Group	n	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD4+/CD8+
Hp (-)	202	60.72±8.38 ^b	36.33±3.68 ^d	25.83±6.93	1.55±0.34 ^j
Hp (+)	226	47.58±4.44 ^b	25.51±4.74 ^d	22.77±7.46	1.44±0.51 ^j
Hp-L (-)	17	50.81±6.20	33.74±5.34 ^f	25.04±10.48	1.68±0.58 ^h
Hp-L (+)	209	46.67±5.21	30.75±4.89 ^f	22.15±6.45	1.32±0.47 ^h
Total	428	52.20±6.21	34.31±4.18	23.49±6.86	1.16±0.466

^b $P<0.001, t=20.61$; ^d $P<0.001, t=26.21$; ^j $P<0.001, t=4.36$; ^f $P<0.001, t=7.78$; ^h $P<0.01, t=2.99$.

Table 4 Detection of serum IL-2, IL-6 and IL-8 in patients with chronic gastritis in relation to infection with *H pylori* (Hp) and its L-forms

Group	n	IL-2 (mg/L)	IL-6 (ng/L)	IL-8 (ng/L)
Hp (-)	202	6.14±0.65 ^b	36.82±3.15 ^d	48.35±3.63 ^f
Hp (+)	226	1.56±0.47 ^b	103.62±5.85 ^d	109.79±7.18 ^f
Hp-L (-)	17	3.39±0.57 ^h	63.35±6.51 ^j	82.33±8.45 ^j
Hp-L (+)	209	1.16±0.38 ^h	116.45±5.44 ^j	118.64±6.24 ^j
Total	428	3.27±0.52	81.83±4.73	87.53±5.68

^b $P<0.001, t=83.06$; ^d $P<0.001, t=145.3$; ^f $P<0.001, t=110.3$; ^h $P<0.001, t=21.08$; ^j $P<0.001, t=38.45$; ⁱ $P<0.001, t=22.61$.

DISCUSSION

Helicobacter pylori was first isolated by Warren and Marshall in 1983 from gastric mucosa of patients with gastritis. It is now accepted to be an etiological agent of chronic gastritis, peptic ulcer and gastric cancer. It is a Gram-negative, spiral-shaped, microaerophilic bacterium that colonizes human gastric epithelium, with a curved, S or arc-like appearance in the stomach. When exposed to factors such as gastric juice, bile,

antibiotics and other hostile conditions, some bacterial cells turn into pleomorphic variations^[24-26] of which L-forms (spheroid) is the most common one^[27-30]. In this study, gastric mucosal biopsy specimens from 428 patients with chronic gastritis were taken for the detection of *H pylori* L-forms. Gram staining showed that 226 patients were infected with both *H pylori* L-forms and vegetative forms, and 17 patients were infected with *H pylori* vegetative forms only. This indicates that L-forms variation of *H pylori* is common in patients with chronic gastritis. Due to the loss of cell walls, or certain components and antigens of cell walls, *H pylori* L-forms differ from the vegetative forms in antigenicity, which may affect the result of serology diagnoses and, more importantly, enables the bacteria to live in the stomach for a long time by escaping the body's immunity. Moreover, when conditions improve, L-form can revert to typical vegetative forms, which may be an important factor leading to deterioration and relapse of infection. Therefore, *H pylori* L-forms are more adhesive, invasive and pathogenic, and the variation of *H pylori* results in the deferment and recurrence of chronic gastritis^[9,31].

In this study, the detection rate of *H pylori* L-forms was significantly greater in males than in female (57.87% vs 37.82%). This may be related to some male habits, such as smoking, drinking, irregular diets that might damage gastric mucosa and change the gastric internal environment^[9,30-35]. In addition, the presence of *H pylori* L-forms seems to be related to patients' ages, as the detection rate of *H pylori* L-forms increases with age.

Cellular immune function of patients with *H pylori* has been described in recent years. The chronic inflammatory responses associated with natural infection do not provide protection, but contribute to tissue damages and pathogenesis of gastroduodenal diseases, including atrophic gastritis, peptic ulcer, and gastric cancer. These immune responses are likely to attribute to a subject of T helper lymphocytes, so-called Th1 cells, which enhance cell-mediated immunity and induce damage to the gastric epithelium. To investigate the mechanisms for Th1 immune response caused by *H pylori* based on the variation of L forms, T lymphocyte subsets and the levels of IL-2, IL-6, and IL-8 in peripheral blood of the patients were detected. The results showed that in *H pylori*-positive patients, CD3+, CD4+, CD4+/CD8 and IL-2 decreased, but IL-6 and IL-8 increased, compared with those in *H pylori*-negative, indicating that *H pylori* infection may weaken the immune function of the host and cause a predominant Th1 cellular response. Moreover, the percentage of CD4+, the ratio of CD4+/CD8 and the level of IL-2 were lower but the levels of IL-6 and IL-8 were higher in the patients infected with both L-forms and vegetative forms, compared with those infected with vegetative forms only. Thus, *H pylori* L-forms infection may be closely related to disorder of the immune function, and may be one of the crucial factors causing Th1 immune response. We postulate that *H pylori* L-forms may invade into the host cells where they may serve as a pronounced inducer for Th1-type CD4 (+) T cell response, leading to the decrease in the percentage of CD4+ and the ratio of CD4+/CD8. Active CD4+-T cell may also inhibit the activation of Th1 cells cytokines, and the outcome of IL-2 is a risk factor of cellular immune response.

In addition, in this study, the levels of IL-6 and IL-8 in peripheral blood of the patients increased significantly, which is likely to be associated with ulceration inflammation, blood macrophage stimulation and active secretion by the neutrophils and vascular endothelial cells. Once attached to the gastric epithelial cells, *H pylori* incites an immune response characterized by the increased pro-inflammatory cytokine of IL-8, IL-12 and TNF-alpha. Activated inflammatory and immunologically competent cells such as neutrophils, lymphocytes and monocytes release cytokines such as IL-6, IL-8 and IFN-gamma. As a result, the serum levels of IL-6 and IL-8 increase^[18].

In conclusion, Co-infection with both *H pylori* vegetative forms and L-forms is common in patients with chronic gastritis. The rate of infection with *H pylori* L-forms in males is higher than in females, and the rate increases with age. Once *H pylori* L-forms occurs, the morphology and microstructure of the organisms change, *i.e.*, the cell walls of the L-forms are partly or completely lost, the charge of the bacterial surface increases, and the adherence and invasiveness of the bacteria become more powerful. All of these changes may play an important role in the deferment and relapse of chronic gastritis and in the disordered cellular immune function in patients with *H pylori* infection.

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Edited by Xia HHX and Xu FM