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L-forms of *H. pylori*

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Abstract

AIM: To study the occurrence of L-forms of *H. pylori* infection in patients with peptic ulcers and its association with possible changes of cellular immune function in the patients.

METHODS: Endoscopic biopsy specimens of gastric antrum and gastric corpus were taken from 228 patients with peptic ulcers and inoculated into Skirrow selective medium for *H. pylori* vegetative forms and special medium for *H. pylori* L-forms, followed by bacterial isolation and identification. And peripheral venous blood of the patients was taken to detect the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ with biotin-streptavidin (BSA) and the level of IL-2, IL-6 and IL-8 with ELISA.

RESULTS: (1) The detection rates of *H. pylori* L-forms and vegetative forms in the patients were 50.88 % (116/228) and 64.91 % (148/228) respectively, and the co-infection rate of *H. pylori* L-forms and vegetative forms was 78.38 % (116/148). To be more exact, the detection rates of *H. pylori* L-forms in male and female patients were 57.04 % (77/135) and 41.94 % (39/93) respectively, and statistics found significant difference between them ($P<0.05$). Furthermore, the detection rates of *H. pylori* L-forms in patients aged 14 years-, 30 years-, 40 years- and 50 years- were 31.91 % (15/47), 42.86 % (24/56), 56.94 % (41/72) and 67.92 % (36/53) respectively, and there was significant difference between them ($P<0.01$). (2) The percentages of $CD3^+$, $CD4^+$, $CD8^+$, the ratio of $CD4^+/CD8^+$, and the level of IL-2, IL-6, IL-8 in *H. pylori*-positive patients were (52.59±5.44) %, (35.51±5.74) %, (27.77±8.64) %, (1.56±0.51), (2.66±0.47) mg/L, (108.62±5.85) ng/L and (115.79±7.18) ng/L respectively, compared with those in *H. pylori*-negative patients, the percentages of $CD3^+$, $CD4^+$ and the ratio of $CD4^+/CD8^+$ decreased, but the level of IL-2, IL-6 increased, and the difference was significant ($P<0.001$-$P<0.01$). Moreover, the percentages of $CD3^+$, $CD4^+$, $CD8^+$, the ratio of $CD4^+/CD8^+$, and the level of IL-2, IL-6, IL-8 in the patients with mixed infection of both *H. pylori* L-forms and vegetative forms were (51.69±5.28) %, (34.75±5.89) %, (27.15±7.45) %, (1.48±0.47), (2.16±0.38) mg/L, (119.45±5.44) ng/L and (123.64±6.24) ng/L respectively, compared with those in patients with simple infection of *H. pylori* vegetative forms, the percentage of $CD4^+$, the ratio of $CD4^+/CD8^+$ and the level of IL-2 increased, but the level of IL-6 and IL-8 decreased, statistical difference was found between them ($P<0.001$-$P<0.05$).

CONCLUSION: L-forms variation often occurs in patients with peptic ulcers who are infected by *H. pylori*, which is commonly found in male patients and related to ages. The L-forms variation of *H. pylori* can be an important factor causing disorder of cellular immune function in the patients with peptic ulcers who are infected by *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the commonest bacteria causing chronic infection, which infects more than 50 % of the human population, and is associated with a range of pathology, such as chronic gastritis, peptic ulcer and gastric cancer[1-5]. And infection of *Helicobacter pylori* is life-long that elicits a marked host inflammatory response. However, natural infection fails to yield protective immunity[6].

H. pylori is a gram-negative, spiral and microaerophylic bacterium that colonizes the gastric epithelium of humans[7]. It causes chronic gastritis and, together with non-steroidal anti-inflammatory drugs, is considered as the most frequent etiologic agent of peptic ulcer[8-10]. After long-term treatment with antibiotic, cell walls deficiency of *H. pylori* vegetative forms occurs and *H. pylori* L-forms comes into being[11,12]. Whether in animal experiments or clinical studies, damages induced by *H. pylori* were reported to be associated with Th1 cell-mediated immune response[13-15].

In order to confirm the occurrence of L-forms variation of *H. pylori* vegetative forms and its association with possible changes of cellular immune function in the patients, gastric mucosa biopsy specimens were taken from 228 patients with peptic ulcers randomly enrolled in this study to isolate and identify *H. pylori* vegetative forms and L-forms, and T lymphocyte subsets and the level of IL-2, IL-6 and IL-8 of the patients were detected at the same time.

MATERIALS AND METHODS

Materials

Patients A total of 228 patients with peptic ulcer (gastric ulcers 66, duodenal ulcers 132, and complex ulcers of gastric and duodenal 30) diagnosed in our affiliated hospital from August, 2000 to April 2002 were involved in this study. All of them were except aged from 14 to 67 years, 135 male and 93 female, other were eliminated for presence of other diseases.

Reagents *H. pylori* L- forms solid medium designed by Jia JH was served in this study. Based on broth culture medium, the medium consisted of peptone 1 %, tryptone1 %, glucose 0.1 %, yeast powder 0.2 %, D-methionine 0.02 %, NaCl 1.5 %, $MgSO_4 \cdot 7H_2O$, 15mM, agar 0.8 % and Caprine plasma 15 % with the inducer of carbenicillin and made into pour plate. BSA reagent for T lymphocyte subsets was 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 kit for IL-6 (batch No. 1006-32), IL8 (batch No.1008-25), IL-2 (batch No.1002-21) was offered by Besancon Company in France.

Methods

We used gastric antral and corporal biopsies for bacterial culture, blood samples for detection of T lymphocyte subsets and IL-2, IL-6, IL-8.

Bacteriological examination Endoscopic biopsies of gastric antrum and gastric corpus were taken from 228 patients with peptic ulcers and inoculated into Skirrow selective medium for *H. pylori* vegetative forms and special medium for *H. pylori* L-forms. Then the plates were incubated at 37 ℃ under microaerobic conditions (5 % O_2, 8 % H_2, 7 % CO_2 and 80 % N_2) for 72 hours. Based on the results of Gram staining, cell morphology and positive reaction for urease, oxidase and immunoenzyme staining,, the identification was carried out.

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 level of CD3+, CD4+, CD8+, CD4+/CD8+ and IL-2, IL-6, IL-8 in peripheral blood of *H. pylori*-positive individuals were tested with biotin-streptavidin (BSA) method. Firstly, the peripheral venous blood of the subjects was taken, anticoagulated with heparin, and diluted with fluid free of Ca^{2+}, Mg^{2+}. Secondly, peripheral blood mononuclear cells were separated with lymphocytes separating medium, cleaned, and the number of cells was adjusted to (1-3)×10^9/L of which 10 μl was taken and smeared in an acid-proof varnish circle on the surface of the slides. When it dried naturally, McAb of anti-CD3+, anti-CD4+ and anti-CD8+ and sheep anti-guineapig IgG, SA-HRP was added into the circle. After development with DAB, the slides were observed under microscope. Only brown cytomembrane staining was regarded as positive, otherwise, as negative specimen. A total of 200 cells were counted, and the positive percentages of cells were analyzed respectively. In addition, the level of IL-2, IL-6 and IL-8 were detected by ELLISA following the procedure detailed in the product description.

Statistical analysis

Data were expressed as mean ±standard deviation. And multiple comparison tests were performed with χ^2 test and *t*-test.

RESULTS

Bacteriological examination By endoscopic biopsy of gastric mucosa, 116 out of all the 228 patients were detected to be positive for both *H. pylori* vegetative forms and L-forms, and 32 (14.04 %) to be positive for vegetative forms only. The detection rates of *H. pylori* L-forms and vegetative forms were 50.88 % (116/228) and 64.91 % (148/228) respectively. "Fried Egg" colonies grew in the special medium for *H. pylori* L-forms after induction with carbenicillin. And the morphology of *H. pylori* L-forms was highly variably seen on the smears under microscopy, such as spheroid, coccoid form, big body, elementary body, long filament body.

Relationship between infection of *H. pylori* L-forms and gender as well as age of the patients with peptic ulcer The detection rates of *H. pylori* L-forms and vegetative forms in the patients were 50.88 % (116/228) and 64.91 % (148/228) respectively, and among the vegetative forms of *H. pylori*-positive patients, it was 78.38 % (116/148) which was the co-infection rate of L-forms of *H. pylori*. To be more exact, the detection rates of *H. pylori* L-forms in male and female patients were 57.04 % (77/135) and 41.94 % (39/93) respectively, and with statistically significant difference between them (P<0.05). In addition, the detection rate of *H. pylori* L-forms was associated with age. The detailed results were shown in Table 1.

Detection of cellular immune function Compared with the percentages of CD3+, CD4+, CD8+, the ratio of CD4+/CD8+, and the level of IL-2, IL-6 , IL-8 in *H. pylori*-negative patients, the percentages of CD3+, CD4+ and the ratio of CD4+/CD8+ decreased in *H. pylori*-positive patients, but the level of IL-2, IL-6 increased. And compared with the percentages of CD3+, CD4+, CD8+, the ratio of CD4+/CD8+, and the level of IL-2, IL-6 , IL-8 in the patients only infected by vegetative forms of *H. pylori*, the percentage of CD4+, the ratio of CD4+/CD8+ and the level of IL-2 increased in the patients not only infected by L-forms of *H. pylori* but also the vegetative forms, but the level of IL-6 and IL-8 decreased, statistical difference was found between them (P<0.001-P<0.05). All of these were shown in Table 2 and Table 3.

Table 1 Relationship between *H. pylori* L-forms and gender as well as ages of the patients with peptic ulcer (*n*, %)

Age	Male		Female		Total	
	n	Detection Rate (%)	*n*	Detection Rate (%)	*n*	Detection Rate (%)
14~	28	9 (32.14)	19	6 (31.58)	47	15 (31.91)[b]
30 ~	36	13 (36.11)	20	11 (55.00)	56	24 (42.86)[b]
40~	42	28 (66.67)	30	13 (43.33)	72	41 (56.94)[b]
50~	29	27 (93.10)	24	9 (37.50)	53	36 (67.92)[b]
Total	135	77 (57.04)[a]	93	39 (41.94)[a]	228	116 (50.88)

[a]P<0.05, χ^2=5.02; [b]P<0.01, χ^2=15.43.

Table 2 Detection of the T lymphocyte subsets in 228 patients with peptic ulcers

Group	*n*	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4^+/CD8^+$ (%)
Hp(-)	80	65.72±9.38[c]	46.33±4.86[d]	29.83±7.39	1.74±0.34[e]
Hp(+)	148	52.59±5.44[c]	35.51±5.74[d]	27.77±8.64	1.56±0.51[e]
Hp(+)& Hp-L(-)	32	55.87±6.23	38.27±6.43[f]	30.04±11.84	1.86±0.58
Hp(+)& Hp-L(+)	116	51.69±5.28	34.75±5.89[f]	27.15±7.45	1.48±0.47★
Total	228	57.20±6.43	39.31±5.18	28.49±7.86	1.61±0.466

[c]P<0.001, t=13.38; [d]P<0.001, t=14.31; [e]P<0.01, t=2.82; [f]P<0.01, t=2.93; [g]P<0.05, t=2.65.

Table 3 Detection of IL-2, IL-6, IL-8 in patients with peptic ulcers

Group	*n*	IL-2 (mg/L)	IL-6 (ng/L)	IL-8 (ng/L)
Hp(-)	80	7.24±0.65[h]	40.82±3.15[i]	52.35±3.63[j]
Hp(+)	148	2.66±0.47[h]	108.62±5.85[i]	115.79±7.18[j]
Hp(+)&Hp-L(-)	32	4.49±0.57[k]	69.35±6.51[l]	87.33±8.45[m]
Hp(+)&Hp-L(+)	116	2.16±0.38[k]	119.45±5.44[l]	123.64±6.24[m]
Total	228	4.27±0.52	84.83±4.73	93.53±5.68

[h]P<0.001, t=61.14; [i]P<0.001, t=96.32; [j]P<0.001, t=74.03; [k]P<0.001, t =27.30; [l]P<0.001, t =43.95; [m]P<0.001, t=26.86.

DISCUSSION

Helicobacter pylori is a gram-negative, spiral-shaped, microaerophylic bacterium that colonizes the human gastric epithelium but not cleared by the host immune response, which can be found in approximately 50 % of the world' s population, and which plays a causative role in peptic ulcer and perhaps gastric cancer. As a matter of fact, it results in a release of various bacterial and host dependent cytotoxic substances including ammonia, platelet activating factor, cytotoxins and lipopolysaccharides as well as cytokines such as interleukins (IL)-1-12, tumor necrosis factor alpha (TNF-alpha), interferon

gamma (INF-gamma) and reactive oxygen species[16-22]. Although the mucus layer is the major reservoir of *H. pylori in vivo*, a growing body of evidence suggests that *H. pylori* can persist in multiple intracellular sites[23]. And primary gastritis, duodenitis, peptic ulcer are no longer considered to be disorders of the balance of secretion of acid and immune responses of the gastric mucosa, but it is thought to be caused by *Helicobacter pylori* infection. Moreover, the chronic inflammatory response associated with natural infection can contribute to tissue damage and the pathogenesis of gastroduodenal disease[24-27]. In 1994, the connection of *H. pylori* to stomach cancer became so certain that the World Health Organization International Agency for Research in Cancer (IARC) classified it as a class I carcinogen. So *H. pylori* became the first bacteria to be connected with carcinogenesis[28-30].

Recurrence of *H. pylori* infection after successful dual or triple therapy is fairly common, and gastroduodenal disease, gender, and gastritis activity seem to affect relapse of infection[31,32]. Many scholars believed that pleomorphic variation of the *H. pylori* is a crucial factor to the occurrence and development of gastric malignant tumor, and some detailed it to L-forms variation of *H. pylori* that played an important role in prolongation and relapse of peptic ulcer as well as chronic gastritis.

In this study, gastric mucosal biopsy specimens from 228 patients with peptic ulcers were inoculated into Skirrow selective blood plate for *H. pylori* vegetative forms and special plate for *H. pylori* L-forms. The experimental results showed that 116 patients were infected with both *H. pylori* L-forms and vegetative forms, but 32 patients were infected with *H. pylori* only. This indicated that L-form variation of *H. pylori* is common in patients with peptic ulcers related to *H. pylori*. Under circumstances of gastric juice, bile, antibiotic and other factors either *in vitro* or *in vivo*, many bacteria could come into pleomorphic variation[33-36]. For example, in penicillin-susceptible bacteria, penicillin causes growth of a small fraction of cells as wall-deficient forms if an appropriate osmo protection is provided (unstable L-forms). According to some scholar' s reports, *H. pylori* vegetative forms could turn into *H. pylori* L-forms in the treatment of peptic ulcer with antibacterial, so co-infection of *H. pylori* L-forms and vegetative forms yields in some patients with *H. pylori*. Following the loss of cell walls, the L-forms lose certain components of antigen and become less antigenic, and with increase of charge of the bacteria surface, which make it more adhesive when cultivating in a liquid L-form medium. Moreover, when condition is available, L-forms can revert to typical vegetative forms, which may be an important factor leading to deterioration and relapse of infection[33]. That is to say *H. pylori* L-forms possess more adhesiveness, invasiveness and risky. Therefore, co-infection of *H. pylori* vegetative forms in extracellular and L-forms in intracellular can result in chronic damage to the host cells.

In this study, the detection rates of *H. pylori* L-forms in male and female were 57.04 % (77/135) and 41.94 % (39/93), which were significantly different. This may be related to some male habits, such as smoking, drinking, irregular diet, which might damage gastric mucosa and change the gastric internal environment[37-39]. In addition, *H. pylori* L-forms infection could occur in different age groups and the positive rate of *H. pylori* L-forms seemed to be related to the patients' ages, as opportunities of infection increase with time due to age, smoking, exposure to antibacterial agents, eating habit, etc.

In recent years, cellular immune function of the patients with *H. pylori* has been discussed very frequently. Rather than providing protection, the chronic inflammatory response associated with natural infection can contribute to tissue damages and pathogenesis of gastroduodenal disease, including atrophic gastritis, peptic ulcer, and gastric cancer. These immune responses are likely to attribute to a subset of T helper lymphocyte, so-called Th1 cells, that enhance cell-mediated immunity and induce damage to the gastric epithelium[13-15]. To investigate the reason for Th1 immune response caused by *H. pylori* based on the variation of L forms, detection of T lymphocyte subsets and the level of IL-2, IL-6, IL-8 in peripheral blood of the patients were performed. The results showed that in 148 *H. pylori*-positive patients, CD3+, CD4+, CD4+/CD8 and IL-2 decreased, IL-6 and IL-8 increased, which were significantly different from those in *H. pylori*-negative ($P<0.001$, $P<0.01$). It indicated that *H. pylori* infection might weaken the immune function of the host and cause a predominant Th1 cellular response.

The results showed that among the 148 patients with *H. pylori* vegetative forms, 116 were co-infected with *H. pylori* L-forms. That is to say, only 32 patients were found with *H. pylori* vegetative forms only. Therefore, the roles of *H. pylori* L-forms in the change of immune function of the patients with peptic ulcers are worthy of great awareness. Compared the T lymphocyte subsets and the levels of IL-2, IL-6, IL-8 in patients with *H. pylori* vegetative forms only and co-infection of *H. pylori* vegetative forms and L-forms, the percentage of CD4+, CD4+/CD8 and the level of IL-2 in the patients with co-infection were lower than those with vegetative forms infection only, but the level of IL-6 and IL-8 was higher. As a conclusion, *H. pylori* L-forms infection is related to disorder of the immune function of the host, and may be one of the crucial factors causing Th1 immune response. When infesting in the host cell, *H. pylori* L-forms may be a pronounced inducer for Th1-type CD4 (+) T cell response and decrease the percentage of CD4+ and the ratio of CD4+/CD8. Active CD4+-T cell may also inhibit the activation of Th1 cells cytokine, 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 as neutrophils, lymphocytes, monocytes, could release cytokine as IL-6, IL-8 and IFN-gamma. As a result, the level of IL-6 and IL-8 in serum of the patients increased[40].

To sum up, vegetative forms of *H. pylori*' s susceptibly turning into L-forms is because of antibacterial agent and alteration of gastric environment in patients with peptic ulcers, and it is common for patients to be co-infected by both *H. pylori* vegetative forms and L-forms. Once *H. pylori* L-forms occurs, the morphology and microstructure of the organisms change, to be exact, the cell walls of the L- forms are partly or completely lost, the charge of the bacteria surface increases, and the adherence and invasiveness of the bacteria become more powerful. All of these make the pathogen invading into intracellular compartment Mac easily, which can be an important factor causing disordered cellular immune function in the patients with peptic ulcers infected by *H. pylori*.

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