Helicobacter pylori lipopolysaccharide: Biological activities in vitro and in vivo, pathological correlation to human chronic gastritis and peptic ulcer

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AIM: To determine the biological activity of Helicobacter pylori (H pylori) lipopolysaccharide (H-LPS) and understand pathological correlation between H-LPS and human chronic gastritis and peptic ulcer.

METHODS: H-LPS of a clinical H pylori strain and LPS of Escherichia coli strain O55:B5 (E-LPS) were extracted by phenol-water method. Biological activities of H-LPS and E-LPS were detected by limulus lysate assay, pyrogen assay, blood pressure test and PBMC induction test in rabbits, cytotoxicity test in NIH 3T3 fibroblast cells and lethality test in NIH mice. By using self-prepared rabbit anti-H-LPS serum as the first antibody and commercial HRP-labeled sheep anti-rabbit sera as the second antibody, H-LPS in biopsy specimens from 126 patients with chronic gastritis (68 cases) or gastric ulcer (58 cases) were examined by immunohistochemistry.

RESULTS: Fibroblast cytotoxicity and mouse lethality of H-LPS were weaker than those of E-LPS. But the ability of coagulating limulus lysate of the two LPSs was similar (+0.5 ng/mL). At 0.5 h after H-LPS injection, the blood pressures of the 3 rabbits rapidly declined. At 1.0 h after H-LPS injection, the blood pressures in 2 of the 3 rabbits fell to zero causing death of the 2 animals. For the other one rabbit in the same group, its blood pressure gradually elevated. At 0.5 h after E-LPS injection, the blood pressures of the three rabbits also quickly declined and then maintained at low level for approximately 1.0 h. At 0.5 h after injection with H-LPS or E-LPS, PBMC numbers of the rabbits showed a remarkable increase. The total positivity rate of H-LPS from 126 biopsy specimens was 60.3% (76/126). H-LPS positivity rate in the biopsy specimens from chronic gastritis (50/86, 73.5%) was significantly higher than that from gastric ulcer (26/58, 44.8%) \((\chi^2=10.77, \ P<0.01)\). H-LPS positivity rates in biopsy specimens from chronic superficial gastritis (38/48, 79.2%) and chronic active gastritis (9/10, 90.0%) were significantly higher than that of the patients with atrophic gastritis (3/10, 30.0%) \((\chi^2=7.50-9.66, \ P<0.01)\).

CONCLUSION: The biological activities of H-LPS were weaker than those of E-LPS, the activities of H-LPS of lowering rabbit blood pressure and inducing rabbit PBMC were relatively stronger. H-LPS may play a critical role in inducing inflammatory reaction in human gastritis.

Luo YH, Yan J, Mao YF. Helicobacter pylori lipopolysaccharide: Biological activities in vitro and in vivo, pathological correlation to human chronic gastritis and peptic ulcer. World J Gastroenterol 2004; 10(14): 2055-2059

http://www.wjgnet.com/1007-9327/10/2055.asp

INTRODUCTION

Gastritis and peptic ulcer are the most prevalent gastric diseases. Gastric cancer is one of the malignant tumors with high morbidity in China\(^{11}\). Helicobacter pylori (H pylori) is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world’s populations\(^{21}\). Most infected individuals are asymptomatic. However, in some subjects, the infection causes acute, chronic gastritis or peptic ulceration, and plays an important role in the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma and primary gastric non-Hodgkin’s lymphoma\(^{3-7}\).

H pylori is a microaerophilic Gram-negative bacillus. It is well known that lipopolysaccharide (LPS) is a common and essential component in outer membrane of most Gram-negative bacteria responsible for the toxicity of endotoxin. Some literatures revealed H pylori possesses LPS (H-LPS) with a lower virulence compared to the typical bacterial endotoxins such as Escherichia coli LPS (E-LPS)\(^{8-12}\). However, some biological activities such as regulating blood pressure and inducing peripheral blood mononuclear cell (PBMC), which are clinically important in local tissue inflammation and injury, and pathological importance of H-LPS in human gastric diseases are still little understood. Besides, some previously published data demonstrated that different strains of the same bacterium and different extraction methods would significantly affect the biological activity of LPS\(^{13,14}\).

For measurement of the biological activities of H-LPS in vivo and in vitro compared to a typical LPS from E. coli, we used phenol-water method to extract LPS from a clinical isolated H pylori strain and applied routine assays for determining the endotoxin activity of H-LPS such as limulus lysate agglutination, rabbit pyrogenicity and mouse lethality. Furthermore, we also examined H-LPS activities on blood pressure regulation and PBMC inducement. To obtain direct evidence for the correlation of H-LPS and human chronic gastritis and peptic ulcer, we detected H-LPS in gastric biopsy specimens from patients with different gastric diseases.

MATERIALS AND METHODS

Bacterial strains and culture

A clinical H pylori strain named as Y06 was isolated from a • H pylori •
biopsy specimen of a male patient with chronic superficial gastritis and duodenal ulcer by using selected Columbia agar (bioMérieux) supplemented with 80 mL/L sheep blood, 5 g/L cyclolestin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and 2 500 U/L cefsludin. This strain was identified as _H pylori_ based on its typical Gram staining morphology, positivity for both urease and oxidase, and agglutination with a commercial rabbit antibody against whole cell of _H pylori_ (DAKO). _E. coli_ strain O55:B5 was offered by the National Institute for the Control of Pharmaceuticals and Biological Products of China (NICPBP) and cultured with BL agar.

**LPS extraction**

H-LPS from _H pylori_ strain Y06 and E-LPS from _E. coli_ strain O55:B5 were extracted by phenol-water method. Briefly, the two bacteria collected from Columbia agar and BL agar were ultrasonically broken, respectively. Each of the broken bacterial solutions was added with an equal volume of pre-warmed phenol-water (9:1, V:V) and then vibrated for 30 min at 68-70 °C. The aqueous phase was collected after centrifugation at 3 000 r/min for 30 min. This extraction step was repeated for 3 times. All the aqueous phases were combined and then dialyzed against distilled water for 48 h. This rough LPS extract was concentrated to 1/6 of the original volume and then digested with RNase H and DNase I (Sigma) to both the final concentration of 50 µg/mL at 37 °C for 4 h. The digested extract was bathed in boiling-water for 15 min and then placed at 4 °C overnight. The supernatant obtained after centrifugation at 3 000 r/min for 30 min was dialyzed against distilled water for 48 h and then precipitated with 6-fold volumes of anhydrous alcohol at 4 °C for 12 h. The precipitate was collected by centrifugation at 5 000 r/min for 30 min and then re-suspended with distilled water and dialyzed against distilled water for 24 h to remove residual alcohol. The LPS extract was ultra-centrifuged at 110 000 g for 3 h and the pellet was dialysed in distilled water and freeze-dried. The purified H-LPS and E-LPS were dissolved in pyrogen-free water or normal saline prepared with pyrogen-free water just before different uses.

**Limulus lysate assay**

Limulus lysate assay was applied by using E-TOXATE Reagent Kit (Sigma) to detect the H-LPS and E-LPS preparations according to the manufacturer’s instruction (Sensitivity = +, 1 ng/mL _E. coli_ O55:B5 LPS). In this assay, _E. coli_ O55:B5 LPS (Sigma) and pyrogen-free water were used as the positive and negative controls, respectively.

**Rabbit pyrogen assay**

Anal temperatures of normal New Zealand rabbits with 3.0±0.2 kg of body mass were detected for 3 times at an interval of 30 min before performing the test. A rabbit was suitable for the test if the fluctuant range of the three detected temperatures was ≤0.2 °C. The qualified rabbits were randomly divided into three groups and each group contained three animals. Each of the three rabbits in one group was injected with 0.5 mL normal saline containing H-LPS or E-LPS at the same dosage of 100 µg/kg through ear vein. Each of the three rabbits in the 3rd group was ear-intravenously injected with an equal volume of pyrogen-free saline as a negative control. The changes of blood pressure in the tested rabbits were observed.

**PBMC inducement test**

PBMC numbers in blood from ear vein of normal New Zealand rabbits with 3.0±0.2 kg of body mass were counted with hemacytometer twice at an interval of 30 min before performing the test. These rabbits were randomly divided into three groups and each contained 5 animals. Each of the five rabbits in one group was ear-intravenously injected with pyrogen-free saline containing H-LPS or E-LPS at the same dosage of 100 µg/kg and each of the five rabbits in the 3rd group were ear-intravenously injected with an equal volume of pyrogen-free saline as a negative control. The changes of PBMC numbers for each of the tested rabbits was counted at an interval of 30 min after injection.

**Cytotoxicity test**

Microtiter plates were inoculated with 1×10⁵ mouse 3T3 fibroblast cells in RPMI 1640 medium containing 100 mL/L bovine serum per well and then incubated at 37 °C overnight in 50 mL/L CO₂ atmosphere. The medium in the plates was discarded and then added with 200 µL of fresh medium containing H-LPS or E-LPS with different double dilutions. For each of the dilutions, 3 wells were repeated and then incubated for 48 h under the same conditions as mentioned above. In this test, the other 5 wells added with the same volume of LPS-free medium were set up as a negative control. H-TdR per well (37 kBq) was added and then continuously incubated for 24 h. CPM value for each of the wells was detected by using "H-TdR incorporation method to compare the cytotoxicity of H-LPS and E-LPS.

**Mouse lethality test**

NIH mice weighing 20±2 g were randomly divided into 7 groups and each contained 8 animals. For 6 of the 7 groups, each of the mice in one group was intraperitoneally injected with 0.2 mL pyrogen-free normal saline containing H-LPS or E-LPS at the different dosages of 0.25, 0.50 and 1.0 mg, respectively. Each of the mice in the last group was intraperitoneally injected with 0.2 mL pyrogen-free saline as a negative control. All the tested animals were observed for 7 d.

**Detection of H-LPS in biopsy specimens from patients with chronic gastritis and gastric ulcer**

Biopsy specimens with positive urease from 126 patients (86 male, 40 female, mean age: 40±18 years) with gastric diseases during January to September of 2003 were collected from three hospitals in Hangzhou. Among these patients, 68 suffered from chronic gastritis (48 cases with chronic superficial, 10 active and 1 atrophic gastritis) and 58 cases suffered from gastric ulcer (12 cases with gastric, 40 duodenal and 6 complex ulcer). The biopsy specimens were fixed with 50 g/L glutaraldehyde. Rabbit anti-H-LPS serum was prepared by using routine subcutaneous immunization. By using the
rabbit anti-H-LPS serum (1:200 dilution, self-prepared) as the first antibody and HRP-labeled sheep anti-rabbit serum (1:3 000 dilution, ImmunoResearch) as the second antibody. H-LPS in the biopsy specimens was detected by routine immunohistochemistry. Cells with at least one whole gastric gland in a biopsy section showing exactly brown color could be considered as positive.

RESULTS

**Limulus lysate coagulation**
The ability of coagulating limulus lysate of H-LPS was as low as 0.5 ng/mL, which was similar to E-LPS (Table 1).

| Group     | LPS (ng/mL) | PBMC induction |
|-----------|-------------|----------------|
| H-LPS     | 10.0        | +              |
| E-LPS     | 5.0         | +              |
| Pyrogen-free water | +   | +              |

**Cytotoxicity to mouse fibroblast**

At 0.5 h after injection with H-LPS or E-LPS, the PBMC numbers gradually decreased to low levels (Table 2).

| Group     | Dosage (µg/mL) | Death/Survival |
|-----------|----------------|----------------|
| H-LPS     | 0.25           | 0/8            |
| E-LPS     | 0.25           | 0/8            |

**Pyrogenic response**
The rabbits injected with H-LPS or E-LPS showed a similar biphasic fever but E-LPS could induce a stronger pyrogenic response. The animal temperature reached a peak at 1.5 h after injection and then showed a slight fall. At 3 h after injection, the second fever peak occurred (Figure 1).

**Regulation of rabbit blood pressure**

At 0.5 h after H-LPS injection, the blood pressures of the three rabbits rapidly declined from originally 11.331±0.152 kPa to 5.999±1.855 kPa. At 1.0 h after H-LPS injection, the blood pressures in two of the three rabbits fell to zero causing death of the two animals. For the other one rabbit in the same group, its blood pressure gradually elevated. At 0.5 h after E-LPS injection, the blood pressures of the three rabbits also quickly declined from originally 11.197±0.163 kPa to 8.531±2.424 kPa and then maintained the similar low blood pressure levels for approximately 1.0 h. At 2.5 h after injection, the blood pressures in all the three rabbits began to rise and returned to the original levels gradually.

**PBMC inducement**

At 0.5 h after injection with H-LPS or E-LPS, PBMC numbers of the rabbits showed a remarkable increase. From 1.0 h after injection with H-LPS or E-LPS, the PBMC numbers gradually and continuously decreased to low levels (Table 2).

**Cytotoxicity to mouse fibroblast**

At 0.5 h after injection with H-LPS or E-LPS, PBMC numbers gradually decreased to low levels (Table 2).

| Group     | Dosage (µg/mL) | Death/Survival |
|-----------|----------------|----------------|
| H-LPS     | 0.25           | 0/8            |
| E-LPS     | 0.25           | 0/8            |

**Mouse lethality**

H-LPS showed a significantly weaker virulence to mice than E-LPS. Although the injecting dosage was as high as 1 mg H-LPS per mouse, 2 of the 8 mice survived (Table 4).

**Positivity rate of H-LPS in biopsy specimens**

The total positivity rate of H-LPS in the 126 biopsy specimens was 60.3% (Table 5). Totally 73.5% of the biopsy specimens from chronic gastritis patients (50/68) were H-LPS positive, which was significantly higher than that (44.8%) from gastric ulcer patients (26/58) ($\chi^2=10.77, P<0.01$). H-LPS positivity rate in biopsy specimens of the patients with chronic superficial gastritis (38/48, 79.2%) was similar to that of the patients with chronic active gastritis (9/10, 90.0%) ($\chi^2=0.63, P>0.05$), but both positivity rates were much higher than that of the patients with atrophic gastritis (3/10, 30.0%) ($\chi^2=7.50-9.66, P<0.01$). Among the biopsy specimens from patients with any one of...
the three types of gastric ulcer, the H-LPS positive rates were similar to each other ($\chi^2=0.11-1.25, P>0.05$). A H-LPS positive biopsy specimen is shown in Figure 2.

**Figure 2** H-LPS positive biopsy specimen (Original magnification: x600).

**Table 5** H-LPS detection rates in the biopsy specimens from patients with chronic gastritis and gastric ulcer

| Group          | Number (n) | Positivity (n) | Positive rate (%) |
|----------------|------------|----------------|-------------------|
| Chronic gastritis |            |                |                   |
| Superficial     | 48         | 38             | 79.2              |
| Active          | 10         | 9              | 90.0              |
| Atrophic        | 10         | 3              | 30.0              |
| Gastric ulcer   |            |                |                   |
| Gastric         | 12         | 7              | 58.3              |
| Duodenal        | 40         | 16             | 40.0              |
| Complex         | 6          | 3              | 50.0              |
| Total           | 126        | 76             | 60.3              |

**DISCUSSION**

Bacterial endotoxin possesses broad biological activities and its toxicity is mainly dependent on lipid A\(^{[92-94]}\). Among the biological activities of LPS, limulus amoebocyte lysate coagulation, rabbit pyrogen and mouse lethality are most typical and important\(^{[6,7,14]}\). In some of the previously published data, H-LPS showed much lower activities of coagulating limulus lysate, pyrogenic response in rabbit and lethal potential in mice\(^{[8,9]}\). In our study, we found that the effect of H-LPS on causing fever in rabbits, death in mice and its cytotoxicity to NIH 3T3 fibroblast were less compared to the LPS from *E. coli*. However, the ability of coagulating limulus lysate of H-LPS (+/0.5 ng/mL) was similar to that of E-LPS based on several repeated results. As mentioned in Introduction, LPS preparations might have various biological activities if different extraction methods were used\(^{[13,14]}\). The result in limulus lysate assay of this study differed from the previously reported probably due to H-LPS from different strains and the distinction in LPS extraction methods.

Salgado et al. revealed that H-LPS from different strains could be divided into two types: One of low biological activity and one of high biological activity of inducing the mitogenicity and TNF-α synthesis of cells. And the strains with the high activity were demonstrated belonging to the low virulence genotypes with cagA and s1bm2 or s2m2 for vacA\(^{[18]}\). To our surprise, at the same injected dosage (100 µg/kg·b.w.) in our study, H-LPS caused death in two of the three tested rabbits in blood pressure regulation test but E-LPS did not. In addition, the ability of H-LPS of inducing PBMC at 0.5 h after injection (12.51±0.54×10⁵/L) was also stronger than that of E-LPS [(8.22±0.68)×10⁵/L]. These data, including the results reported by Salgado et al., indicated that the role of the LPS as a virulence factor from some *H pylori* strains should be re-evaluated.

It was reported that H-LPS acted as a modulator of host-dependent gastritis through inducing both gastric epithelial cells and macrophages to secrete IL-1, TNF and IL-8\(^{[19-21]}\). In this study, a high frequency of H-LPS in the biopsy specimens from chronic gastritis patients (73.5%) was found. However, the positivity rate of H-LPS in the biopsy specimens from gastric ulcer patients (44.8%) was relatively lower ($\chi^2=10.77, P<0.01$). Furthermore, the biopsy specimens from chronic superficial gastritis (79.2%) and chronic active gastritis (90.0%) showed significantly higher H-LPS positive rates compared to those from chronic atrophic gastritis (30.0%) ($\chi^2=7.50-9.66, P<0.01$). These data indicated that H-LPS might play a more important role in inducing human gastric inflammation than previously considered. It is well known that PBMC are a mixture of neutrophilic granulocytes, mononuclear macrophages and lymphocytes\(^{[22-24]}\). Mononuclear macrophages and lymphocytes are the major cells to produce IL-1, TNF and IL-8\(^{[19-21]}\). The result from our study showed that H-LPS had a stronger ability of inducing PBMC than E-LPS, suggesting the critical effect of H-LPS on inducing inflammatory reaction in human gastritis.

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Luo YH et al. Biological activities of Helicobacter pylori lipopolysaccharide 2059

Edited by Zhu LH and Chen WW Proofread by Xu FM