Non-standard biological activities of lipopolysaccharide from *Helicobacter pylori*

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As assessed by the lipopolysaccharide (LPS)-specific chromogenic Limulus amoeboocyte lysate (LAL) assay, *Helicobacter pylori* LPS extracted by the phenol-water procedure showed full potency to coagulate LAL, as did LPS from *Salmonella minnesota* and *Escherichia coli*. However, pretreatment of *H. pylori* LPS with polymyxin B, which easily destroys the endotoxic activity of enterobacterial LPS/lipid A, had little effect on the LAL coagulation activity, although the same treatment of *E. coli* LPS markedly diminished its activity. The *H. pylori* LPS induced very weak production of nitric oxide (NO) or tumour necrosis factor (TNF) by murine macrophages and TNF by human peripheral whole blood *in vitro* in comparison with *S. minnesota* LPS. These findings indicate that *H. pylori* LPS has the unique endotoxic characteristic of retaining full LAL coagulation activity with polymyxin B resistance, despite losing its endotoxic potencies such as the ability to induce NO and TNF production.

**Introduction**

*Helicobacter pylori* is recognised as a primary cause of active chronic gastritis and is reported to be associated with peptic ulcer disease [1, 2], low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue and gastric adenocarcinoma [3, 4]. Like the cell envelope of all other gram-negative bacteria, that of *H. pylori* contains lipopolysaccharide (LPS). In general, LPS is composed of a lipid core (lipid A) and polysaccharide side chains of variable length. Lipid A represents the endotoxic principle of active LPS. The carbohydrate portion consists of a core region that usually is genus- or species-specific and an outermost chain that is strain-specific and forms the basis of the O-antigenic classification [5]. LPS from members of the family Enterobacteriaceae has been demonstrated to be highly pro-inflammatory and LPS released from the infecting organisms causes various inflammatory symptoms and pathophysiological disorders, including fever, disseminated intravascular coagulation (DIC), multiple organ failure (MOF) and septic shock [6, 7]. However, *H. pylori* LPS seems to lack strong endotoxicity [5, 8–11].

The most sensitive and rapid way to detect a trace amount of endotoxin/LPS or (1→3)-β-1-glucan in solution or serum is to use the amoeboocyte lysate of the horseshoe crab: the *Limulus* amoeboocyte lysate (LAL) test [12, 13]. To avoid the action of (1→3)-β-1-glucan-sensitive factor G in the LAL test [14–16], a highly sensitive and specific test for detecting endotoxin/LPS has been developed [17–21]. The improved LAL test is quite specific for LPS/lipid A and can detect picograms of endotoxin/LPS.

LAL coagulation activity of endotoxin/LPS has been thought to be closely correlated with its other biological activities, including pyrogenicity and macrophage (Mφ) activation. This study examined the biological activity of *H. pylori* LPS in comparison with its potency to coagulate LAL.

**Materials and methods**

**Reagents**

Four strains of *H. pylori* were used in this study: CG10 (isolated from a patient with chronic gastritis), GU2

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(isolated from a patient with gastric ulcer), DU8 (isolated from a patient with duodenal ulcer) and ATCC 43504. Each strain was grown on brain heart infusion agar plates supplemented with horse blood 10% at 37°C for 5 days under micro-aerophilic conditions (Gas Pack System without catalysis; BBL, Cockeysville, MD, USA) [22]. H. pylori LPS was extracted by the phenol-water method [23]. Briefly, phenol 90% was added to bacterial cells suspended in water and the mixture was heated at 65°C for 45 min. After cooling, the aqueous phase was separated by centrifugation, dialysed and lyophilised to give crude extract. The extract was treated with DNAase and RNAase at 37°C overnight and then treated with proteinase K at 37°C for 1 day. After dialysis, LPS was separated by ultracentrifugation, washed twice and lyophilised. LPS from Escherichia coli O55 and O111:B4 was purchased from Sigma and Difco, respectively. Re chromatope LPS (ReLPS) from Salmonella minnesota R595 was a gift from Dr K. Hisatsune, Josai University, Sakado, Japan. Rhodobacter sphaeroides diposphoryl lipid A (RsDPLA), which is known to be biologically inactive and a non-toxic LPS antagonist [24–29], was prepared from the LPSs of R. sphaeroides ATCC 17023 as described previously [30]. Polymyxin B was purchased from Sigma.

Mice

C3H/HeN mice (Nippon Clea, Tokyo, Japan) were bred and maintained in the animal facility of the Faculty of Medical School. Female mice were used at 10–15 weeks of age.

Cell preparations

Peritoneal Μφ were prepared from C3H/HeN mice according to the procedures described previously [31]. Briefly, murine peritoneal exudate cells (PEC) were isolated by peritoneal lavage 4 days after intraperitoneal injection of 1.5 ml of thioglycollate broth 3%. The PECs were plated at 2 × 10⁶ cells/well in serum-free RPMI 1640 medium in 96-well plates (Nunc, Roskilde, Denmark), cultured for 2 h and then washed to remove non-adherent cells. The remaining cells were used as Μφ. The viability of the cells was c. 99% as determined by trypan blue staining. TNF-sensitive L929 cells were provided by Dr M. J. Parmely, University of Kansas Medical Center and were grown at 37°C in air with CO₂ 5% in RPMI 1640 medium containing heat-inactivated fetal bovine serum (FBS; JHR Bioscience, Lenexa, KS, USA) 5%, 4 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 μg/ml.

NO and TNF production by Μφ stimulated by LPS

Μφ were incubated in the presence or absence of various doses of LPS in RPMI with FBS 2% at 37°C in CO₂ 5%. The supernates of the cultures were collected at 4 h for the TNF assay or at 48 h for the NO assay.

TNF activity was determined by a functional cytotoxic assay with the TNF-sensitive cell line L929, as described previously [32]. Briefly, L929 cells were plated in 96-well culture plates at 5 × 10⁴ cells per well in 100 μl of RPMI 1640 medium with FBS 5% and incubated for 20 h. The cells were then cultured for an additional 18 h in the presence of serial dilutions of test supernate and actinomycin D (Sigma) 5 μg/ml. The viability of the cells was determined by a quantitative colorimetric staining assay with a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), Sigma). The absorbance at 540 nm (A₅₄₀) was read with a model Biomec 1000 Spectrophotometer (Beckman Instruments, Palo Alto, CA, USA). TNF activity was expressed in units/ml, with 1 U being the amount of TNF causing 50% lysis of L929 cells.

NO formation was measured as its stable end product nitrite (NO₂⁻) in culture supernates with the Griess reagent [33]. Briefly, 100 μl of culture supernates was added to each well of 96-well plates and mixed with 100 μl of Griess reagent (1:1 v/v; N-[1-naphthyl]ethylenediamine dihydrochloride 0.1% in water and sulphanilamide 1% in H₃PO₄ 5%) and the A₅₄₀ was read with a model Biomec 1000 spectrophotometer.

LAL coagulation activity assay

The LAL coagulation activity was assessed with the LPS-specific chromogenic test kit (Endospecy; Seikagaku, Japan) The Endospecy kit reagent consists of coagulation factors (factor C, factor B and pro-clotting enzyme from horseshoe crab, Tachypleus tridentatus) and chromogenic substrate, t-butylxoyacarbonyl-Leu-Gly-Arg-p-nitroanilide (Boc-Leu-Gly-Arg-pNA). LPS and RsDPLA were dissolved in pyrogen-free water and serial dilutions were made. The Endospecy kit reagent was added to each dilution and the A₅₄₀ was measured every 15 s with a Well Reader SK601 (Seikagaku). The LAL coagulation activity was expressed as a maximum value of mAbsorbance/min in the assay. In the case of polymyxin B treatment, LPS samples were pre-incubated in the presence or absence of 15 μM polymyxin B for 30 min before the LAL assay.

Ex-vivo TNF production by human peripheral blood samples

Peripheral whole blood was obtained with a heparinised syringe from a healthy volunteer (39-year-old male, negative H. pylori infection was determined by the urease test.). The blood was diluted five times in serum-free RPMI 1640 medium. The diluted samples (270 μl/well) were pre-incubated in 96-well plates for 2 h at 37°C in CO₂ 5% and then incubated in the presence of various doses of LPS at 37°C in CO₂ 5%
[31]. The supernates of the cultures were collected at 20 h after the incubation and stored at –80°C until TNF levels of the supernates were assessed as described above.

Results

LAL coagulation activity

As shown in Fig. 1a, four preparations of \textit{H. pylori} LPS showed high levels of LAL coagulation activity that were similar to those of \textit{E. coli} and \textit{S. minnesota} LPS. In contrast, the activity of RdDPLA was 100 times less than those of \textit{H. pylori}, \textit{S. minnesota} and \textit{E. coli} LPS. However, RdDPLA did not inhibit the LAL activity of other LPS (data not shown). As polymyxin B has been documented to neutralise many of the biological activities of LPS by binding to lipid A [34], the study examined whether the activities of \textit{H. pylori} LPS were neutralised by polymyxin B. The LPS of \textit{H. pylori} GU2 and DU8 and LPS of \textit{E. coli} O55 were pre-incubated with 15 μM polymyxin B at 37°C for 30 min and then their LAL coagulation activities were assessed. As shown in Fig. 1b, the activities of these \textit{H. pylori} LPS preparations were not neutralised, although the activities were slightly decreased. In contrast, the activity of \textit{E. coli} LPS was quite sensitive to polymyxin B treatment. These findings indicate that \textit{H. pylori} LPS has enough potency to coagulate LAL, like other active LPS, but its potency is resistant to polymyxin B. The slight reduction of LAL activity in the absence of polymyxin B may be caused by the adsorption of LPS to the wall of the vessel.

TNF and NO production by murine peritoneal Mφ

Next, the potency of the preparations for induction of TNF and NO by murine peritoneal Mφ \textit{in vitro} was investigated. As shown in Fig. 2a, the addition of \textit{S. minnesota} LPS 10 ng/ml induced nitrite production of c. 35 μM, whereas the same dose of \textit{H. pylori} LPS produced no detectable nitrite. The addition of \textit{H. pylori} ATCC43504 or GC10 LPS 1000 ng/ml barely induced the production of a 30 μM level of nitrite. No \textit{H. pylori} LPS, except that of ATCC 43504, possessed TNF-inducing activity (Fig. 2b). These findings in-

![Fig. 1. LAL coagulation activities of LPS. (a) LPS of \textit{H. pylori} CG10 (○), GU2 (△), DU8 (□) and ATCC43504 (△) and \textit{E. coli} O55 (●) and O111:B4 (□); and \textit{S. minnesota} R595 (●) and RdDPLA (●) were analysed for their LAL coagulation activities. (b) LPS of \textit{H. pylori} GU2 (△) and DU8 (□) and \textit{E. coli} O55 (●) pre-incubated in the presence (---) or absence (——) of 15 μM polymyxin B for 30 min were analysed for their LAL coagulation activities.](image)

![Fig. 2. Induction of TNF and NO production by murine peritoneal Mφ in response to LPS. Peritoneal exudate Mφ of C3H/HeN mice were cultured at 2 × 10^5 cells/well in serum-free RPMI 1640 medium in 96-well plates in the presence or absence of various doses of LPS at 37°C in 5% CO2. The supernates of the cultures were collected at 4 h or 48 h, and the levels of TNF and NO were assessed in the 4- and 48-h samples, respectively. LPS of \textit{H. pylori} CG10 (○), GU2 (△), DU8 (□) and ATCC43504 (△) and of \textit{S. minnesota} R595 (●) were used for the assay.](image)
dicate that the endotoxic potency of *H. pylori* LPS to induce TNF and NO production by mouse Mφ is quite low when compared with the ability of *S. minnesota* LPS.

TNF production by human peripheral whole blood

*H. pylori* LPS only exhibited a low endotoxic activity as determined by its weak ability to induce TNF production by human peripheral whole blood cell cultures. As shown in Fig. 3, 1 ng of *S. minnesota* LPS/ml was enough to reach the plateau of TNF production, while 100 ng of *H. pylori* LPS/ml hardly induced any TNF production. The production was barely seen when ATCC 43504 LPS, strain CGI0 LPS or strain DU8 LPS 1000 ng/ml was added, but the same dose of strain GU2 LPS was not effective. These findings suggest that the ability of *H. pylori* LPS to induce TNF production by human peripheral monocytes is at least 1000-fold lower than that of *S. minnesota* LPS.

**Discussion**

It was reported that endotoxic activities of *H. pylori* LPS, such as mitogenicity in mouse spleen cells, pyrogenicity in rabbits and toxic lethality in galactosamine-sensitised mice, were 500–1000-fold lower than those of *Salmonella* LPS [8] and that the production of NO, TNF and prostaglandin E2 (PGE2) by murine or rat Mφ in response to *H. pylori* LPS was very low [5, 9–11]. It was also reported that the production of TNF, interleukin(IL)-1 and IL-6 by human peripheral mononuclear blood cells [35] and TNF, PGE2 and NO by human leukaemia cells [9] in response to *H. pylori* LPS were all apparently lower than those in response to *E. coli* LPS. Furthermore, the oxidative metabolism and enzyme release from human neutrophils in response to various preparations of *H. pylori* LPS were 10-fold lower than those induced by *Salmonella* LPS [36]. The findings in the present study that the biological activities of *H. pylori* LPS against murine Mφ and human monocytes were quite low coincided with the other reports described above, but the *H. pylori* LPS preparations in the former retained high LAL coagulation potency with resistance to polymyxin B treatment. To our knowledge, this is the first report of such an obvious dissociation of LAL coagulation potency from other biological activities of LPS. Matsushy–Balzter et al. reported that lipid A prepared from *H. pylori* LPS with acetic acid hydrolysis showed low solubility and less LAL activity in comparison with lipid A of *Campylobacter jejuni* or *S. typhimurium* [37]. Lipid A of *H. pylori* is known to have a unique structure that contains uncommonly long 3-hydroxy fatty acids and the backbone consisting of a D-glucosamine disaccharide with a 2-aminoethylphospho- plate group at position 1 [38, 39]. As neutral phospholipid is known to have no LAL activity [40], the neutral charge of lipid A moiety may be responsible for the low LAL activity of lipid A from *H. pylori*. Thus, removal of some structural moiety after acetic acid hydrolysis of *H. pylori* LPS, probably polysaccharide moiety, may affect its LAL coagulation potency: lipid A in combination with polysaccharide may activate the factor C enzyme. Moreover, polymyxin B did not interfere with the co-operative effect. As polymyxin B binds to negatively charged amphiphiles [41], polymyxin B may not interact with the lipid A moiety of *H. pylori* LPS. The structure of lipid A may be responsible for the polymyxin B resistance. Although endotoxic activity is not dependent on any single constituent of lipid A, a change in the lipid A structure can result in decreased endotoxic activity [10, 42]. Many lipid A analogues have been chemically synthesised. It has been found that structural alteration of the analogues may lower some activity without affecting other activities [43–45]. Although structural information about the LPS/lipid A in *H. pylori* responsible for the full retention of LAL coagulation potency without cytokine-inducing activity is not available at present, some novel chemical structure may exist in the LPS. There is a need for studies to determine structure–activity relationships in LAL coagulation and mechanisms involved in the stimulation of the LAL coagulation cascade.

![Fig. 3. Induction of TNF production by human whole peripheral blood in response to LPS. Human peripheral blood was obtained from a healthy male with a heparinised syringe and tube. The blood was diluted five-fold in RPMI 1640 medium and cultured in the presence of various doses of *H. pylori* LPS (0.01–100 ng/ml) or *S. minnesota* LPS (0.3 ng/ml), at 37°C in 5% CO2 for 4 h. At the end of culture, the blood samples were centrifuged (500 rpm for 3 min) and the supernatants were used for the TNF assay. LPS of *H. pylori* CGI0 (●), GU2 (△), DU8 (□) and ATCC43504 (○) and of *S. minnesota* R595 (■) were used for the assay.](image-url)