

Immunomodulating Effect of Lipopolysaccharide (LPS) Isolated From Virulent Strains of *H. Pylori*

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ABSTRACT

The mechanism by which *Helicobacter pylori*, which has little or no invasive activity, induces gastric-tissue inflammation and injury has not been well characterized. Extensive research during the past 2 decades has revealed the mechanism by which continued oxidative stress can lead to chronic inflammation, which in turn could mediate most chronic diseases including gastritis. The present study is aimed to analyze the effect of *H. pylori* Lipopolysaccharide LPS on immune system activation particularly oxidative stress. We have investigated the immunomodulatory effect of *H. pylori* LPS isolated from gastritis patients. LPS was challenged to neutrophils and macrophage, in response to which reactive oxygen species (ROS) and reactive nitrogen species (NO) were measured by chemiluminescence technique and spectrophotometry respectively. Additionally effect on T-cells proliferation was also studied by radioactive thymidine incorporation using scintillation counter. In this study, we have found that out of 10 different samples of *H. Pylori* LPS, 6 of the LPS samples resulted in 2-6 μ M of NO nitric oxide production in vitro. About 3 samples were aggravating reactive oxygen species production and almost all the samples moderately inhibited proliferation of T cell. All these events modulated by LPS clearly indicating the role of bacterial lipopolysaccharide in an increased proinflammatory activity which may lead to gastritis.

Keywords

Oxidative Stress, Inflammation, Lipopolysaccharide, *H. pylori*.

Abbreviation

NO: Nitric oxide, ROS: Reactive Oxygen Species, LPS: Lipopolysaccharide.

Introduction

There is growing evidence that oxidative stress, i.e. the imbalance between free-radical production and antioxidant defense and inflammation are involved in the pathogenesis of many chronic diseases like diabetes mellitus and gastritis [1]. Oxidative stress is likely to be involved in age-related development of cancer as well. The reactive species produced in oxidative stress can cause direct damage to the DNA and are therefore mutagenic, and it may also suppress apoptosis and promote proliferation, invasiveness and metastasis [2]. Infection by *Helicobacter pylori* which increases

the production of reactive oxygen and nitrogen species in human stomach is also thought to be important in the development of gastric cancer [3].

Since, LPS activated macrophages produce large amounts of nitric oxide and reactive oxygen species, which is considered as an essential mediator for host defence [4]. Basically, Nitric oxide and ROS secreted as an immune response act as free radicals and is toxic to bacteria [5]. However, this oxidative stress not only target bacteria but can damage the normal surrounding cells. Therefore ROS and RNS contribute to inflammation progression in excessive amount when release during inflammation [6]. In current studies we have examined effect of LPS on the production of reactive oxygen and nitrogen species on human peripheral blood phagocytes, and mouse macrophages respectively [7], which is responsible to promote inflammatory responses or cause tissue damage [8]. The diversity of LPS structures have been associated with several

bacterial diseases therefore, variability of bacterial ligands such as LPS and their innate immune receptors is an important factor in determining the outcome of infectious disease [9]. Much more about pylori has been explored regarding Bacterial virulence factors such as the cytotoxin-associated gene pathogenicity island-encoded protein CagA and the vacuolating cytotoxin VacA aid in this colonization of the gastric mucosa and subsequently seem to modulate the host's immune system.

It is widely accepted that LPS is primary factor to initiate innate immune response and well established that *E.coli* LPS is much stronger in activating the immune response to pathogen. However, Lipopolysaccharide from *H. Pylori* has not been studied as potential immunomodulating agent, our study focused on clinical isolates from Pakistani patients suffering from *H. Pylori* infection these isolates were subjected to isolation of LPS and then detailed immunomodulating studies were conducted to identify innate immune response in establishment of the active disease.

Experimental LPS preparations

The LPS from the four *H. pylori* strains was prepared by the hot-phenol–water method of [8] and subsequent purification steps were performed essentially as described by [10] In brief, bacterial cells from blood agar plates were scraped into saline, centrifuged at 5,000 g for 15 min, and resuspended in water with an equal volume of 90% phenol [Sigma, St. Louis, USA] at 6°C for 15 min. After the mixture was cooled to 10°C and centrifuged at 10,000 g for 20 min, the aqueous layer was removed. This extraction procedure was repeated twice, and the pooled water-extracted layers were dialyzed for 48 h against several changes of water and lyophilized.

Chemiluminescence Assay

Luminol-enhanced chemiluminescence assay was performed as described by [11] 25 µL of diluted whole blood (1:20 dilution in sterile Hanks Balanced Salt Solution containing Calcium Chloride and Magnesium Chloride (HBSS++)) [Sigma, St. Louis, USA] was incubated with 25 µL of LPS (10 µg/mL) each in triplicate. Control wells received HBSS++ and cells but no LPS. Test was performed in white half area 96 wells plates [Costar, NY, USA], which was incubated at 37°C for 15 minutes in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland]. After incubation a 25 µL of intracellular reactive oxygen detecting probe luminol [Research Organics, Cleveland, OH, USA] working solution (7 x 10⁻⁵ M), and 25 µL serum opsonized zymosan (SOZ) 2 mg/mL [Fluka, Buchs, Switzerland] was added into each well except blank wells (containing only HBSS++). The oxidative burst ROS production was monitored with the luminometer for 50 minutes in the repeated scan mode. The level of the ROS was recorded as total integral readings as relatively light units (RLU).

Nitrite concentration in Mouse Macrophage Culture Medium

The mouse macrophage cell line J774.2 (European Collection of Cell Cultures, UK) was cultured in 75 cc flasks (IWAKI Asahi Techno Glass, Tokyo, Japan) in DMEM (Sigma-Aldrich Steinheim, Germany) that contained 10% fetal bovine serum (GIBCO New

York U.S) supplemented with 1% streptomycin/penicillin. Flasks were kept at 37°C in humidified air containing 5% CO₂. Cells (106 cells/mL) were then transferred to a 24-well plate. The Nitric oxide synthase (NOS-2) in macrophages was induced by the addition 10 µg/mL *H. Pylori* (LPS). Soon after LPS stimulation the cells were again incubated at 37°C in 5% CO₂. The cell culture supernatant was collected after 48 hours for analysis. Nitrite accumulation in cell culture supernatant was measured using the griess method described previously [12]. In brief 50 µl of 1% sulphanilamide in 2.5% phosphoric acid, followed by 50 µl of 0.1% naphthyl-ethylene diamine dihydrochloride in 2.5% phosphoric acid was added to 50 µl of culture medium. After 10 minutes of incubation at room temperature the absorbance was read at 550 nm. Micro molar concentrations of nitrite were calculated from a standard curve generated using sodium nitrite which was used as reference compound.

T-cell proliferation assay

Cell proliferation was evaluated by standard thymidine incorporation assay following a method reported by [13] Briefly, cells were obtained from peripheral blood of healthy individuals as described by [13] and then cultured at a concentration of 5 × 10⁵/mL in a 96-well round bottom tissue culture plates (IWAKI Asahi Techq'1no Glass, Tokyo, Japan). Preliminary experiments were conducted to determine the optimum concentration of PHA on T-cell proliferation. PHA concentration 5 µg/mL was found to be optimum and used in our experiment. Cells were stimulated with 5 g/mL of PHA (Sigma Co. St. Louis, USA). LPS was added to obtain final concentrations of 10 µg/mL, each in triplicate. The plates were incubated for 72 h at 37°C in 5% CO₂ incubator.

After 72 hrs, cultures were pulsed with (0.5 µCi/well) tritiated thymidine (Amersham Pharmacia Biotech), and further incubated for 18 hrs. Cells were harvested onto a glass fiber filter (Cambridge Technology, USA) using cell harvester (Ionotech Dottikon, Switzerland). The tritiated thymidine incorporated into the cells was measured by a liquid scintillation counter (LS 6500, Beckman Coulter, USA). Results were expressed as mean count per minute (CPM). The inhibitory activity of compounds on T lymphocyte

$$\text{Inhibitory activity (\%)} = \frac{\text{control group (CPM)} - \text{experiment group (CPM)}}{\text{Control group (CPM)} \times 100}$$

proliferation was calculated using the following formula:

Statistical analysis

The data was expressed as the mean ± standard deviation. Graph was drawn using Microsoft Office excel, 2007 software.

Effect of H pylori LPS on Oxidative Burst

Reactive Oxygen Species play a very crucial role in inflammation and gastritis. In this study we observe the effect of LPS on ROS production in the whole blood phagocytes by using the luminol as probe, which react and detect extra cellular ROS and *H. pylori* LPS as an oxidative burst initiator. Our study elaborated that most of the LPS samples activated the phenomenon of oxidative burst by increasing the ROS production with% age activation between 20 to 30% (Table 1).

Effect of *H. pylori* LPS on Nitrite production

NO is produced in large quantities during host defense, inflammatory reaction and because of its cytotoxic properties to normal cells it is of great medical importance. Endotoxin of *H. pylori* is supposed to be inflammatory in nature and the extent of inflammatory phenomenon depend on virulence of strain we have observed that *h. pylori* LPS modulating the immune response via increasing NO concentration even some samples showed more strongly i.e 6.48 μM of NO that is significantly high as compare to untreated cells 0.43 μM of NO (Table 1).

<i>H. Pylori</i> LPS sample	NO (μM) at 10 $\mu\text{g}/\text{mL}$	% activation ROS at 10 $\mu\text{g}/\text{mL}$
1	4.63 \pm .002	20
2	0.73 \pm .00	12.1
3	1.90 \pm .01	13.3
4	2.97 \pm .04	20.6
5	2.29 \pm .07	13.7
6	1.56 \pm .00	14.2
7	4.55 \pm .08	21.1
8	1.65 \pm .00	12.6
9	5.16 \pm .005	34.2
10	6.48 \pm .06	35

Table 1: Effect of *H. pylori* LPS (10 $\mu\text{g}/\text{mL}$) on Nitrite and ROS production by mouse macrophages J774 and whole blood phagocytes was determined as described in Material and methods. Results are representative of Mean \pm SDV of three individual experiments.

Effect of compounds on T cell proliferation

In the present study, there was a marked proliferative response of PBMCs in cultures (105 cells/ well) stimulated 7.5 $\mu\text{g}/\text{mL}$ PHA alone which is known to stimulate the proliferation of T cells from healthy volunteers. However, when treated the cells with of *H. pylori* LPS 10 $\mu\text{g}/\text{mL}$ inhibition was seen in PHA - stimulated cultures containing *H. pylori* LPS within the range of (20 to 40) % (Table 2) which is a moderate inhibition of proliferation but still may contribute to pathogenicity (Figure 1).

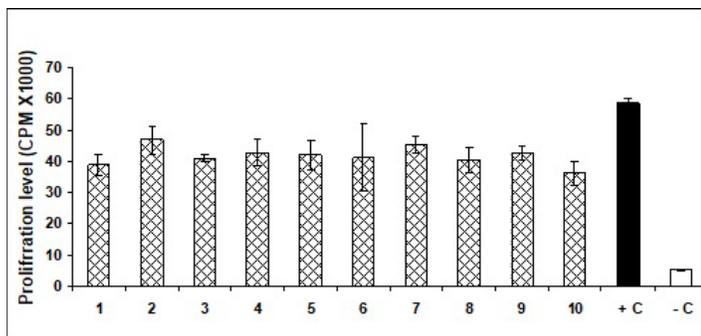


Figure 1: Effect of LPS on phytohemagglutinin (PHA) activated T-cell proliferation. The effect of LPS on the T-cell proliferation is compared with control. Each bar represents the mean value of triplicate reading \pm SD. + C represent control cells with PHA and -C represent only cells.

<i>H. pylori</i> LPS	% Inhibited at 10 $\mu\text{g}/\text{mL}$
1	34.0
2	20.5
3	30.3
4	27.2
5	28.6
6	29.9
7	22.8
8	31.5
9	37.2
10	38.3

Table 2: Percentage of T cell proliferation inhibited at 10 $\mu\text{g}/\text{mL}$ due to the effect of LPS isolated from *H. pylori* virulent strains.

Discussion

Chronic infection with the gastric bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to a high risk of developing gastric and duodenal ulcers, gastric cancer, and gastric lymphoma. We have investigated the role of LPS on inflammatory progression via reactive oxygen species (ROS) and reactive nitrogen species (RNS) and proliferation of T-cells. In order to look into effect of these LPS on the whole blood phagocytes ROS production, we have applied the luminol dependent CL assay which is a very sensitive system that can monitor the generation of the ROS in the phagocytes in internal and external environment [14,15]. Results showed that LPS of *H. pylori* is aggravating the ROS production of whole blood phagocytes, which can contribute to oxidative stress and eventually lead to chronic inflammation [16].

On the other hand, iNOS produces large amounts of NO as a defense mechanism, and is an important factor against parasites attack, bacterial infection, and tumor growth. In contrast, if over produced then react with superoxide and give rise to a very toxic radical peroxynitrite, which is one of the main cause of septic shock and may play a role in many diseases with an autoimmune etiology [17]. Eradication of *H. pylori* or identifying its existence in gut is really necessary to conceal the progression of disease. Because, our results identified the effect of LPS on increased NO release by inflammatory cells so, it's an important factor of pathogenicity as well.

Considering the diverse and important role, helper T cells play in the immune system; it is not surprising that these cells often influence the immune response against disease. In the worst case scenario, the helper T cell response could lead to a disaster and the fatality of the host [18]. Involvements of *H. Pylori* LPS in inhibiting T cell proliferation could be a major reason in establishing chronic gastritis. The immunomodulatory properties therefore allow the bacteria to persist for decades in infected individuals in the face of a vigorous innate and adaptive immune response.

Conclusion

In the present study, we examined the effect of *h. pylori* LPS on

cell based oxidative stress (ROS and RNS) and cell mediated immunity (T cell proliferation). Results indicated that out of 10 LPS samples, three of the samples showed more significant pathogenicity behavior via modulating immune system function by aggravating oxidative stress release by whole blood phagocytes. In addition, same LPS samples resulted in enhanced production of NO by mouse macrophages in cell culture medium as well as proliferation of T. cell was also moderately inhibited by almost all the samples. Modulating the immune system function in this way could contribute to pathogenicity of *H. Pylori* infection.

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