MICROBIAL PATHOGENICITY

Increased in-vitro and in-vivo biological activity of lipopolysaccharide extracted from clinical low virulence *vacA* genotype *Helicobacter pylori* strains

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Helicobacter pylori infection in man is associated with chronic gastritis and peptic ulcer disease. The virulence factors of the species are still under investigation. Among these, the lipopolysaccharide (LPS) is a potential pathogenic factor of the micro-organism, whose biological activity can be estimated by immunological parameters. The aim of this study was to determine the ability of pure LPS extracted from clinical isolates of *H. pylori* to induce mitogenicity, secretion of tumour necrosis factor- α (TNF- α), and spleen growth in a murine model. Rough and smooth LPS from *Salmonella typhimurium* were used as controls. The results showed that, like the control LPS, all extracts of LPS induced mitogenic activity, stimulated synthesis of TNF- α and induced spleen growth, although the effects produced by the majority of the *H. pylori* LPS samples analysed were less intensive than those produced by the *S. typhimurium* LPS. The immunological parameters analysed allowed the detection of two types of *H. pylori* LPS: one of low biological activity and one of high biological activity. The most active LPS was extracted from strains isolated from patients with increased mucous damage associated with epithelial regeneration. Surprisingly, these strains were *cagA* negative and belonged to a low virulence genotype according to *vacA* gene (s1bm2 and s2m2). The results suggest the need to re-evaluate the role of the LPS as a virulence factor for some strains of *H. pylori*.

Introduction

Helicobacter pylori is the main species involved in mucosal damage in the stomach [1]. The infection and persistence of *H. pylori* in the stomach have been associated with the appearance of more serious sequelae, such as MALT lymphoma and gastric carcinoma, pathologies that appear to develop within a specific subgroup of individuals [2, 3]. An interesting challenge in this field is to identify host and bacterial factors that might be involved in the remarkable variability of the diseases seen in infected patients.

Bacterial factors that favour pathogenicity of the species include CagA and VacA proteins [3, 4], urease [5], flagella [6], adhesins [6] and lipopolysaccharide (LPS)

[7, 8]. Of these, LPS has received less attention as a virulence factor in *H. pylori*, although variability in sugar and fatty acid composition as well as antigenicity have been observed [9–11]. Moreover, the role of LPS in ulcero-peptic disease has been questioned [12]. However, the increased evidence of the participation of LPS as an important virulence factor for gram-negative bacteria during infection, and the knowledge accumulated on their biological effects both *in vitro* and *in vivo*, suggest that this macromolecule might play a role in the inflammatory response and gastric epithelial damage observed *in vivo* during *H. pylori* infection [13, 14].

Keeping in mind the world-wide prevalence of *H. pylori* infection and the great impact of gastroduodenal diseases in health care, this laboratory has initiated an epidemiological surveillance of the *H. pylori* infection in Concepción, Chile, and a systematic study of the virulence factors of the species by investigating clinical isolates obtained from different diseases associated

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with *H. pylori*. Specifically, the effort has been concentrated on evaluating some immunological properties of *H. pylori* LPS, such as induction of mitogenicity, secretion of TNF- α and spleen growth, by the use of a mouse model.

This paper presents evidence of discrepancies between traditional genetic markers of virulence and immunological properties of *H. pylori* LPS that might contribute to the virulence and pathogenicity of particular strains.

Materials and methods

Mice

Female Rockefeller mice aged 7–8 weeks were used in the assays. Before the experiments, the animals were fed with extruded pellets and maintained under controlled environmental conditions (25°C and free access to water and food) for 1 week. The mice were handled according to the animal research guidelines of the Universidad de Concepción.

Bacterial strains and growth conditions

Eight strains of *H. pylori* were used in this study. The reference strain ATCC 43504 was a gift from Dr Arturo Yudelevich, Bios Chile Laboratory, Santiago, Chile. The other seven strains were isolated from gastric biopsies from patients with different gastric diseases (Table 1). The biopsies were macerated in 3–5 drops of saline solution, and the emulsions were inoculated on agar plates. All the cultures were performed on Columbia Agar (Merck Chemical Co., USA) supplemented with human defibrinated blood 5% and Dent inhibitors mix (Oxoid). The plates were incubated under micro-aerobic conditions [15] at 37°C for 96 h [16].

Identification of strains

Strains were identified by colony morphology, Gram's stain and determination of urease, oxidase and catalase activities [17].

Histopathological diagnosis

Biopsy specimens were fixed in buffered formalin 10%, embedded in paraffin, sectioned and stained as described previously for routine analysis [18]. Chronic gastritis, neutrophil infiltration and density of *H. pylori* was graded as suggested by the Updated Sydney System [18].

PCR-based *vacA* and *cagA* genotyping

Stock cultures of *H. pylori* were stored at –70°C under micro-aerophilic conditions as described above, and total DNA was obtained by suspending the cultures in 100 μ l of sterile distilled water, heated for 10 min in a boiling water bath and cooled on ice for an additional 10 min [19].

The strains were typed by PCR with the *vacA* primers described by Atherton *et al.* [4, 20] and the *cagA* primers described by Shimoyama *et al.* [21]. PCR amplification was performed in an automated thermal cycler (Perkin-Elmer 480 thermocycler) in 50 μ l of reaction mixture containing the following reagents: 50–100 ng of total DNA as template, 1.5 mM MgCl₂, 100 μ M NTPs, 500 μ M primers and Taq polymerase 1 U. The reaction mixtures were heated initially to 95°C for 120 s and then underwent 40 cycles of 60 s at 95°C, 60 s at 50°C and 60 s at 72°C. After cycling, the products were extended for a further 5 min at 72°C.

LPS extraction

LPS was extracted from bacterial cultures by the hot phenol-water technique described by Westphal and Jann [22]. The crude LPS preparations were further purified by treatment with lysozyme 25 mg/L, DNAase

Table 1. *H. pylori* strains isolated from patients with upper gastrointestinal symptoms used in this study: source, endoscopic and histopathological diagnosis, *cagA* status and *vacA* genotype of the strains

Strain no.	Patient		Endoscopic diagnosis	Histopathological diagnosis	<i>cagA</i>	Allelic <i>vacA</i> gene	
	age (years)	sex				s	m
839	38	F	NUD	ACG/ER	–	s1b	m2
128	48	F	PUD	ACG/ER	–	s2	m2
174	57	M	PUD	AMCG	–	s1b	m1
133	70	F	GC	ALCG	+	s2	m2
175	43	M	NUD	AMCG	–	s1b	m2
204	38	F	NUD	AMCG	+	s2	m2
630	54	F	NUD	ALCG	+	s1b	m2
43504	UN	UN	UN	AMCG	+	s1a	m1

M, male; F, female; s, *vacA* signal sequence; m, *vacA* middle region; PUD, peptic ulcer disease; GC, gastric cancer; NUD, non-ulcerous dyspepsia; UN, unknown; ACG/ER, active chronic gastritis associated with epithelial regeneration; AMCG, active moderate chronic gastritis; ALCG, active light chronic gastritis.

I 25 mg/L and RNAase 25 mg/L (all from Sigma) for 3 h at 37°C. Then, proteinase K (Sigma) 50 mg/L was added and the preparations were incubated for further 2 h at 60°C. Proteinase K was removed from the extracts by washing of LPS in pyrogen-free water followed by centrifugation at 105 000 *g* (Sorvall RC-2B, rotor SS34). Each pellet containing pure LPS fraction was lyophilised and kept at -20°C until use.

Limulus amoebocyte lysate (LAL) assay

The endotoxic activity was evaluated semi-quantitatively with a *Limulus* test (sensitivity 0.06 UE/ml) (Pyrotell) according to the manufacturer's directions.

B-cell proliferation

Assays were performed as described previously [23], in triplicate. Briefly, spleen cells were suspended in RPMI 1640 medium containing fetal calf serum 10%, penicillin 100 U/ml, streptomycin 100 mg/L and amphotericin B 0.25 mg/L (all from Sigma) and adjusted to 4×10^6 cells/ml. Equal 100- μ l portions of cell suspension and LPS preparation (containing 0.1, 1.0 and 10 μ g, respectively) were deposited in 96-well plates (Nunc, Denmark) and incubated for 72 h at 37°C, in air with CO₂ 5%. Then, cultures were pulse-labelled with 0.5 μ Ci of ³H-thymidine (50 Ci/mmol; Amersham, London) and incubation was continued for a further 18 h. The cells were harvested by filtration and the radioactivity was measured in a liquid scintillation counter (Analyzer Tri-Carb 1600TR, Packard Instruments, USA). Commercial LPS (Sigma) from a wild-type strain of *Salmonella typhimurium* and its isogenic Re mutant (strain SL 1181; Sigma) were used as positive controls; RPMI 1640 medium was the negative control. All assays were conducted in triplicate.

TNF- α determination

Spleen cell suspensions were obtained and challenged with 10 μ g of LPS as described above, but for a period of 24 h. The stimulated cells were concentrated by low speed centrifugation as described previously [24] and TNF- α was determined in the supernates of cell cultures by a Mouse TNF- α ELISA kit (BioSource International, Camarillo, USA), according to the manufacturer's instructions. Positive and negative controls were the same as those used for B-cell proliferation assays.

Splenic index

Blastogenic response was evaluated by the ability of LPS to induce spleen growth, expressed as a splenic index = [spleen weight (g)/mouse weight (g)] $\times$ 1000 [24]. Briefly, groups of four female mice were inoculated intraperitoneally with 10 μ g of LPS and killed after 4 days by cervical dislocation to allow extraction of the spleen. The weights of both the spleen and the

mouse were measured to calculate the splenic index. Positive controls were the same as those used for B-cell proliferation assays and isotonic saline solution was used as negative control. The results are presented as the arithmetic means for each group of mice.

Statistical analysis

All the results were submitted to statistical analysis by the Tukey test, with the Systat computing programme [25].

Results

In this study, pure LPS preparations obtained from clinical strains of *H. pylori* were used to challenge mouse spleen cells or mice to evaluate the ability of these preparations to elicit an immunological response both *in vitro* and *in vivo*, respectively.

The strains used in this study were isolated from patients with diverse underlying illnesses (Table 1), including one isolated from a patient with gastric carcinoma (strain 133). All the strains belonged to a low virulence genotype according to the type of middle region *vacA* allele (m2), except strain 174, which had an m1 genotype.

Pure LPS obtained from each strain, including the *H. pylori* ATCC 43504 control strain, was shown to be positive by the *Limulus* amoebocyte lysate test (≥ 0.06 endotoxin units/ml).

The immunological assays with pure LPS preparations were also shown to be mitogenic for mouse spleen cells (Table 2), although to a lesser extent than the commercial *S. typhimurium* smooth and rough LPS. Also, differences in the intensity of the biological activity among the *H. pylori* LPS were observed. Strains 128 and 839 showed high mitogenic activity (when cells were stimulated with 10 μ g of LPS) and highly increased ability to induce TNF- α secretion, comparable with the positive controls of *S. typhimurium* LPS (Fig. 1). The differences in mitogenicity observed between these LPS extracts and negative control were statistically significant ($p \leq 0.05$) and showed no significant differences from the positive control, as determined by the Tukey test. On the other hand, the LPS from *H. pylori* ATCC 43504 was the least active of all the LPS assayed, with biological activity only higher than the negative control.

The *in-vivo* assay confirmed the findings observed *in vitro*, as 100% of the *H. pylori* LPS induced spleen growth in mice (Fig. 2). Again, two groups of *H. pylori* LPS were observed, one of low activity (strains 133, 174, 175, 204, 630 and ATCC 43504) and one of high activity (strains 128 and 839), with splenic index values of 9.5 and 10.3, respectively. *S. typhimurium* LPS

Table 2. Mitogenicity induced by *H. pylori* LPS extracts

Strain no.	Mean (SD) ³ H-thymidine uptake (cpm) with LPS (μ g)		
	0.1	1	10
<i>H. pylori</i> 128	1072 (12.0)	3503 (6.5)	30 098 (141.4)
<i>H. pylori</i> 133	1810 (26.1)	2466 (34.2)	6856 (19.2)
<i>H. pylori</i> 174	1283 (29.2)	4366 (35.1)	13 854 (70.3)
<i>H. pylori</i> 175	1946 (7.4)	9802 (15.9)	10 444 (84.8)
<i>H. pylori</i> 204	946 (3.8)	2894 (10.2)	10 833 (101.8)
<i>H. pylori</i> 630	1507 (25.0)	3347 (38.8)	7714 (29.9)
<i>H. pylori</i> 839	1154 (4.5)	3400 (38.3)	27 030 (379.6)
<i>H. pylori</i> ATCC43504	2080 (18.7)	3913 (37.6)	6382 (60.8)
<i>S. typhimurium</i> (smooth)	5492 (23.9)	10 226 (72.1)	45 120 (141.0)
<i>S. typhimurium</i> (rough)	6938 (14.6)	12 242 (49.5)	56 645 (283.0)

Mouse spleen cells (4×10^6 cells/ml) were challenged for 72 h with various amounts of *H. pylori* LPS, pulse-labelled and counted to determine mitogenicity as described in *Materials and methods*. Each value represents the mean of three independent readings.

Fig. 1. Cytokine synthesis induced by *H. pylori* LPS extracts. A fraction of the induced cell mixture was centrifuged at 24 h after challenge with 10 μ g of LPS, and TNF- α was evaluated in the supernate with a mouse TNF- α ELISA kit. Each value represents the mean of three independent readings. 1, strain 128; 2, strain 839; 3, strain 174; 4, strain 175; 5, strain 630; 6, strain 204; 7, strain 133; 8, strain ATCC 43504; 9, rough LPS from *S. typhimurium*; 10, smooth LPS from *S. typhimurium*; 11, negative control (RPMI medium).

showed splenic index values of 13.5 for rough LPS and 10.8 for the smooth LPS.

Discussion

During the last two decades, *H. pylori* has caused great interest because of its high incidence as the aetiological agent of gastroduodenal diseases, including gastric adenocarcinoma [2]. However, the mechanisms involved in the pathogenesis of the species are still controversial, and the virulence factors are not well established. The results presented in this paper support the idea that LPS might play a crucial role as a pathogenic factor in *H. pylori* infection, particularly as it is known that several bacterial products – i.e., urease, cytotoxin or LPS – are released to the

epithelium during infection, thus inducing an inflammatory response in the host [26, 27]. Nonetheless, the importance of LPS as an inducer and maintenance factor in gastric disorders has been widely accepted only during the last few years, after the description of clinical strains with unusual LPS [9, 28].

Genetic approaches to detect more aggressive strains in clinical isolates of *H. pylori* have failed to explain the origin of several diseases, because genetically typed virulent strains have been isolated from asymptomatic individuals and low virulence genotypes have also been isolated from symptomatic patients [29]. These results indicate that new approaches are needed to understand the organic responses against *H. pylori* infection in man, and that strains with low virulence *vacA* alleles should not be disregarded and should be analysed for

Fig. 2. Spleen growth induced by *H. pylori* LPS. Four female mice were inoculated intraperitoneally with 10 μ g of LPS and killed after 4 days by cervical dislocation to extract the spleen, and the splenic indices were calculated (see *Materials and methods*). LPS extracts were the same as Fig. 1.

other attributes of virulence, such as biological activity of LPS, as it is known that this macromolecule is involved in events that induce epithelial disorganisation and mimicry in gastric epithelium [13, 14].

Interestingly, the two LPS extracts that have biological activity comparable to *S. typhimurium* LPS in all the assays were isolated from patients who had intestinal metaplasia. On the other hand, both strains were *cagA* negative and had low virulence *vacA* genotypes (middle region m2), which have been associated with production of vacuolating cytotoxin of poor vacuolating activity and low levels of interleukin (IL)-8 secretion in in-vitro assays [30]. The increased response against these LPS preparations that were observed in mice might be a consequence of the experimental quality of the lipid A and the better affinity of the endotoxin with the immunocompetent cells, resembling the behaviour of the gastric epithelium *in vivo* where free LPS interacts with epithelial cells [31], allowing the lipid A to reach the membrane and induce the release of pro-inflammatory cytokines such as TNF- α , IL-1 and IL-6 [13, 32, 33]. It is known that these related activities regulate secretion of other cytokines in the gastric mucosa [5, 27].

The LPS extracts of the other six clinical strains and the control ATCC *H. pylori* strain were not recognised as strong antigens, as they showed a smaller although homogeneous mitogenic activity and TNF- α induction capacity, similar to the results published elsewhere for this pathogen [12]. Also, the differences in the ability of LPS from various isolates to elicit mitogenic activity and TNF- α synthesis could be caused by variations in

the fatty acid content of lipid A, as has been demonstrated for the LPS of other pathogens [24].

In-vivo studies also showed the biological activity of LPS as an endotoxin (Fig. 2). This activity was positive for all isolates, although it is known that the murine model is more resistant to the endotoxic activity of LPS than man, so the in-vivo response against this macromolecule should be higher.

The results of the present study emphasise the need to re-evaluate the role of *H. pylori* LPS in the pathogenesis of the diseases caused by this species.

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