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Low Biological Activity of *Helicobacter pylori* Lipopolysaccharide

ANNA MUOTIALA,[1] ILKKA M. HELANDER,[1] LIISA PYHÄLÄ,[2] TIMO U. KOSUNEN,[3] AND ANTHONY P. MORAN[3]†*

Department of Bacteriology and Molecular Biology Program[1] and Laboratory Animal Unit,[2] National Public Health Institute, SF-00300 Helsinki, and Department of Bacteriology and Immunology, University of Helsinki, SF-00290 Helsinki,[3] Finland

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Lipopolysaccharide from the gastroduodenal pathogen *Helicobacter pylori* was tested for its ability to induce mitogenicity in mouse spleen cells, pyrogenicity in rabbits, and toxic lethality in galactosamine-sensitized mice. Compared with those for enterobacterial lipopolysaccharide, mitogenicity and pyrogenicity were a thousand-fold lower and lethal toxicity was 500-fold lower. We suggest that the phosphorylation pattern and acylation in lipid A are responsible for the low biological activity.

Extensive investigations worldwide have shown that *Helicobacter pylori* (formerly *Campylobacter pylori*) is the causal agent of active chronic gastritis and that a strong association between it and duodenal ulcers exists (16). Like the cell envelope of all other gram-negative bacteria, that of *H. pylori* contains lipopolysaccharide (LPS). In general, LPS is composed of a poly(oligo)saccharide and a lipid component termed lipid A (18, 20). It has been established that lipid A is essential for the endotoxic effects of gram-negative bacteria in vitro and in vivo (4, 7, 11, 20). Biologically active lipid A has a rather conservative structure which is characterized by a β(1→6)-linked (*gluco*-configured) D-hexosamine disaccharide backbone with phosphate groups at positions 1 and 4′ and carrying (*R*)-3-hydroxy- and (*R*)-3-acyloxyacyl residues at positions 2, 3, 2′, and 3′ (14, 18, 20); e.g., lipid A of *Escherichia coli* and *Salmonella enterica* serovar Minnesota has this structure. In order to enhance our knowledge of the biology of *H. pylori* and its surface structures, we chemically characterized the low-molecular-weight rough-form LPS of three *H. pylori* strains in a previous study (13). The results suggested that *H. pylori* lipid A, in addition to containing uncommonly long 3-hydroxy fatty acids (i.e., 3-hydroxyhexadecanoic and 3-hydroxyoctadecanoic), has as its backbone a D-glucosamine disaccharide which carries a phosphate group at position 1 but which is not phosphorylated at position 4′.

Studies with chemically defined synthetic analogs and partial structures of *E. coli* and *Salmonella* species lipid A have provided clear evidence that slight modifications to lipid A architecture (e.g., the loss of one constituent from the molecule or a different distribution of constituents) result in a significant reduction in biological activity (18, 20). Our chemical characterization of *H. pylori* LPS (13) suggested, therefore, that it would be less biologically active than enterobacterial LPS. To test this hypothesis, we investigated the ability of *H. pylori* LPS to induce a well-documented in vitro biological effect of endotoxin, B-lymphocyte mitogenicity, and two classical in vivo endotoxic effects, lethality in mice and pyrogenicity in rabbits.

H. pylori NCTC 11637, obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, England), was used throughout our investigations. NCTC 11637 was chosen because it is a well-characterized strain, and although it has been extensively passaged in vitro, changes that occur during passaging affect the O side chain (13) and would be unlikely to modify lipid A structure, as alterations in this conservative moiety of LPS would influence the viability of the organism (18, 20). The strain was grown on blood agar (Trypticase soy agar [Difco, Detroit, Mich.], 6% horse blood) supplemented with 10% IsoVitaleX (BBL, Cockeysville, Md.) which was incubated in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 at 37°C for 48 h. LPS was extracted from NCTC 11637 by the hot phenol-water technique (24) after pretreatment of bacteria with pronase (Calbiochem, Los Angeles, Calif.) and was purified by treatment with RNase A (Sigma Chemical Co., St. Louis, Mo.), DNase II (Sigma), and proteinase K (Sigma) and by subsequent ultracentrifugation as described previously (13). For comparison, LPS of *S. enterica* serovar Typhimurium SH2183 (22) and that of mutant strain *his-515* (15) (LPS chemotype Ra) were used. These strains were cultivated on L agar (21). Serovar Typhimurium SH2183 LPS was extracted as described for *H. pylori* LPS but without pronase pretreatment, whereas serovar Typhimurium *his-515* LPS was extracted by the phenol-chloroform-petroleum ether method (5).

The mitogenicity of LPS preparations was determined in (CBA × C57BL/6)F_1 mouse spleen cultures as described previously (6). Briefly, single-cell suspensions were cultured in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (GIBCO), and antibiotics (penicillin G, 50 U/ml; streptomycin, 50 μg/ml) in round-bottom microtiter plates (Nunc, Roskilde, Denmark) to a final concentration of 3×10^5 cells per well. Triplicate cultures were incubated without or with mitogens (concanavalin A [Sigma], 0.5 μg per well; LPS, 0.001 to 10 μg per well in tenfold dilutions) at 37°C for 48 h in a CO_2-enriched atmosphere (5% CO_2–95% air). After incubation, 0.5 μCi of [*methyl*-^{3}H]thymidine (Amersham International, Amersham, England; 25 Ci/mmol) in 20 μl of RPMI 1640 was added to each well and incubation was continued for a further 18 h. Cultures were harvested with a Nunc cell harvester, and incorporated ^{3}H was measured by conventional liquid scintillation counting.

In addition, the lethal toxicity of LPS was determined for D-galactosamine-sensitized mice (2). Groups of five female (CBA × C57BL/6)F_1 mice aged 10 to 11 weeks and weighing

* Corresponding author.
† Present address: Department of Microbiology, University College Galway, Galway, Ireland.

TABLE 1. Stimulation of the [*methyl*-^{3}H]thymidine uptake by mouse spleen cells by *H. pylori* and *S. enterica* serovar Typhimurium LPS[a]

Concn (μg/well) of LPS	Level of stimulation[b]		
	H. pylori NCTC 11637	*S. enterica* serovar Typhimurium	
		his-515	SH2183
0.001	544 ± 310	1,302 ± 88*	2,057 ± 785*
0.01	1,077 ± 180	10,736 ± 1,380***	11,099 ± 1,230***
0.1	1,792 ± 159	24,371 ± 505***	25,661 ± 3,648***
1	4,331 ± 315	30,187 ± 472***	25,833 ± 8,281***
10	14,149 ± 1,850	21,621 ± 2,004**	26,182 ± 1,726**

[a] Single-cell suspensions were cultured to a concentration of 3×10^5 spleen cells per well. Values for unstimulated cultures and cultures stimulated with concanavalin A (0.5 μg per well) were 320 ± 168 and 47,157 ± 1,192, respectively (mean count per minute ± standard deviation).

[b] Mean counts per minute ± standard deviation. Values include significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) of the difference between stimulation with *Salmonella* species LPS and that with *H. pylori* LPS as determined by Student's *t* test.

20 to 23 g were used in tests. *H. pylori* NCTC 11637 LPS was injected intravenously as indicated previously (2) for rough-form LPS. *S. enterica* serovar Typhimurium *his-515* LPS (chemotype Ra) was used as a positive control. Within 1 h of an intraperitoneal injection of galactosamine (Sigma; 12.5 mg per mouse) in 400 μl of endotoxin-free water, the mice received LPS (0.0001 to 10 μg per mouse in tenfold dilutions) in 200 μl of sterile saline intravenously. Control mice received only galactosamine. Deaths were recorded at 24 h, and values for the 50% lethal dose were calculated according to the method of Reed and Muench (17).

Finally, pyrogenicity was tested in chinchilla rabbits which were bred, reared, and maintained in a conventional environment in air-conditioned rooms at 21.0 ± 2°C with a relative humidity of 45 to 65% and a photoperiod of 14 h/day. Rabbits were housed individually, fed a commercial diet (K1; Ewos AB, Wadstena, Sweden) (120 g/day) and given water ad libitum. The pyrogen assay was performed according to the European Pharmacopoeia (1) with rabbits weighing 3.3 to 4 kg in groups of three to five. Rabbits were pretrained and tested with pyrogen-free saline in preliminary assays. Rectal temperature was monitored with indwelling thermistor probes (Ellab A/S, Copenhagen, Denmark) and a strip chart recorder. The minimal pyrogenic doses after 3 h from injection (MPD-3) (19) were titrated by using appropriate dilutions (0.05 to 5 μg per kg of body weight in 1 ml for *Helicobacter* species LPS; 0.005 to 0.5 μg per kg in 1 ml for *Salmonella* species LPS).

The mitogenicity of LPS of *H. pylori* NCTC 11637 and those of LPSs of *S. enterica* serovar Typhimurium *his-515* and SH2183 are shown in Table 1. Within the concentration range tested, the LPSs of both serovar Typhimurium strains brought about nearly a hundredfold stimulation compared with unstimulated cultures. For *H. pylori* LPS, the strongest stimulation was 44-fold, at a concentration of 10 μg of LPS per 0.2 ml of culture. This stimulatory effect was of a magnitude similar to those obtained with 0.01 μg of serovar Typhimurium LPS per 0.2 ml of culture. The LPS of *H. pylori* NCTC 11637, therefore, exhibited mitogenic activity about a thousand times lower than those of serovar Typhimurium strains.

In the lethal toxicity test with D-galactosamine-sensitized mice, *H. pylori* LPS also exhibited low activity. The 50% lethal doses were 2.1 and 0.0037 μg for LPS of NCTC 11637 and *his-515*, respectively, demonstrating a 500-fold difference in the lethalities of these LPSs. In addition, the pyrogenicity of *H. pylori* LPS in rabbits was low; its MPD-3 was 1.2 μg of LPS per kg. *S. enterica* serovar Typhimurium SH2183 and *his-515* gave MPD-3s on the order of 1 ng. Accordingly, the pyrogenicity of *H. pylori* LPS was a thousandfold lower than those of the serovar Typhimurium strains.

Our results show that *H. pylori* LPS possesses low mitogenic and pyrogenic activities and low lethal toxicity, suggesting that the primary role of lipid A should not be considered endotoxicity alone, but that it can be to provide a functional macromolecular matrix through which the bacterium interacts with its environment. It is interesting that *Bacteroides fragilis* LPS, which also expresses low pyrogenicity and toxicity, has a β(1→6)-linked D-glucosamine disaccharide backbone phosphorylated at position 1 only, with long 3-hydroxy fatty acids whose average chain length is 16 carbon atoms (23).

Studies on synthetic *Salmonella* species- and *E. coli*-like lipid A partial structures and analogs have shown that phosphorylation patterns (3, 7, 8, 10, 11), fatty acid composition (9, 12), and the presence of acyloxyacyl groups (19) are important for the full expression of a range of biological activities. As synthetic lipid A partial structures that are monophosphorylated and lipid A analogs that carry acyl chains of increased length have been shown to exhibit decreased biological activity (7, 11, 12), it is likely that the phosphorylation pattern and acylation in *H. pylori* lipid A (13), probably in a combined form, are the basis for the low biological activity of *H. pylori* LPS.

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