Immunomodulatory Effects of Yersinia pestis Lipopolysaccharides on Human Macrophages

Motohiro Matsuura,1* Hideyuki Takahashi,2 Haruo Watanabe,2 Shinnji Saito,1,3 and Kazuyoshi Kawahara4

Department of Infection and Immunity, School of Medicine, Jichi Medical University, Tochigi 329-0498, Japan; Department of Bacteriology, National Institute of Infectious Disease, Tokyo 162-8640, Japan; Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan; and College of Engineering, Kanto Gakuin University, Yokohama 236-8501, Japan

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In the current study, we investigated the activity of lipopolysaccharide (LPS) purified from Yersinia pestis grown at either 27°C or 37°C (termed LPS-27 and LPS-37, respectively). LPS-27 containing hexa-acylated lipid A, similar to the LPS present in usual gram-negative bacteria, stimulated an inflammatory response in human U937 cells through Toll-like receptor 4 (TLR4). LPS-37, which did not contain hexa-acylated lipid A, exhibited strong antagonistic activity to the TLR4-mediated inflammatory response. The phagocytic activity in the cells was not affected by LPS-37. To estimate the activity of LPS in its bacterial binding form, formalin-killed bacteria (FKB) were prepared from Y. pestis cells grown at 27°C or 37°C (termed FKB-27 and FKB-37, respectively). FKB-27 strongly stimulated the inflammatory response. This activity was suppressed in the presence of an anti-TLR4 antibody but not an anti-TLR2 antibody. In addition, this activity was almost completely suppressed by LPS-37, indicating that the activity of FKB-27 is predominantly derived from the LPS-27 bacterial binding form. In contrast, FKB-37 showed no antagonistic activity. The results arising from the current study indicate that Y. pestis causes infection in humans without stimulating the TLR4-based defense system via bacterial binding of LPS-37, even when bacterial free LPS-37 is not released to suppress the defense system. This is in contrast to the findings for bacteria that possess agonistic LPS types, which are easily recognized by the defense system via the bacterial binding forms.

Yersinia pestis is the causative agent of bubonic, septicemic, and pneumatic plague in humans and is primarily a rodent pathogen that is transmitted intradermally to humans through the bite of an infected flea (24). Y. pestis must survive in two different temperature ranges. One temperature range represents that of a flea residing in rodent burrows or mammalian hair (21°C to 28°C), while the other represents the body temperature of the infected rodent or human (37°C to 41°C) (2, 4, 24). It has been demonstrated that the various cellular components of this bacterium are differentially expressed at these temperature ranges. The production of several virulent factors, such as the fraction 1 antigen (6), the pH 6 antigen (14), and Yop proteins (33), are known to be upregulated during growth of the bacterium at 37°C. In contrast, the production of murine toxin (12), which is required for the survival of Y. pestis in the midgut of fleas, is synthesized at approximately 27°C and is downregulated at 37°C.

Y. pestis is a gram-negative bacterium that contains bacterial lipopolysaccharide (LPS) as its major cell wall component. The lipid moiety in LPS that is responsible for immune system activation is lipid A and is composed of a glucosamine-disaccharide backbone carrying acyl chains and phosphate groups. The hexa-acylated type of lipid A was found to be the major component of the Escherichia coli LPS, which exhibits strong immunological activities (8, 13, 17). This particular type of lipid A is widely found among usual gram-negative bacteria, but it is not the sole type of lipid A species. The specific types of lipid A have been shown to differ among bacterial species, and heterogeneous types of lipid A often coexist in a single species (28). We have previously reported that the lipid A present in Y. pestis LPS is heterogeneous (from hexa-acylated to triacylated types) when the bacterium is grown at 27°C and shifts to the hypoacylated types (tetra- and triacylated types) when it is grown at 37°C (16). Such a temperature-dependent shift of lipid A types in the LPS of Y. pestis was further confirmed by another group (29), who demonstrated a similar shift in two additional pathogenic species of Yersinia. Hexa-acylated lipid A is able to strongly activate Toll-like receptor 4 (TLR4), while the tetra-acylated type is a poor activator and serves instead as an antagonist when the target cells are human in origin (10, 22). The alteration of lipid A to hypoacylated types is therefore favorable for the bacterium, as it allows the bacterium to evade the human innate immune system and avoid elimination. It has been indicated that lipid A alterations that result in the evasion of the bacteria from host TLR4 recognition play a more important pathogenic role than the additional virulence factors of Y. pestis (23).

In the current study, we purified LPS or prepared formalin-killed bacteria (FKB) from Y. pestis grown at either 27°C or 37°C and examined their influence on the immune system response in human U937 cells. Our findings suggested that the LPS isolated from Y. pestis grown at 37°C (LPS-37) strongly suppressed the human TLR4-dependent inflammatory response when it was free from the bacterial body. This

* Corresponding author. Mailing address: Department of Infection and Immunity, School of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. Phone: 81-285-58-7332. Fax: 81-285-44-1175. E-mail: mmatsuwr@jichi.ac.jp.

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suppressive activity was not observed when it was bound to the bacterial body. In contrast, the LPS isolated from Y. pestis grown at 27°C (LPS-27) was found to be agonistic toward TLR4 signaling in both the bacterium-free and -bound forms, as FKB grown at 27°C (FKB-27) showed agonistic activity similar to that of LPS-27.

MATERIALS AND METHODS

Bacterial culture, extraction of LPS, and preparation of FKB. Virulent Y. pestis strain Yreka (National Institute of Infectious Disease, Tokyo, Japan) was cultured on brain heart infusion agar (Difco Laboratories, Detroit, MI) at 27°C or 37°C for 48 h. The bacterial cells were suspended in saline to obtain heat-killed bacteria for the extraction of LPS by the method reported previously (16) and were purified via extraction with 45% phenol containing triethylamine and sodium deoxycholate (21). The LPS forms obtained following growth at 27°C and 37°C were termed LPS-27 and LPS-37, respectively. To prepare FKB, bacterial cells were suspended in 0.3% formalin solution at a dose of 30 mg (wet weight)/ml (9 cells were suspended in 0.3% formalin solution at a dose of 30 mg (wet weight)/ml) and were incubated at 37°C for 3 h with shaking for 5 days to kill the bacteria completely. These preparations, termed FKB-27 and FKB-37, respectively, were then washed and suspended in phosphate-buffered saline.

Cell culture. The murine macrophage cell line RAW264.7 and the human macrophage cell line U937 (both from the American Type Culture Collection, Manassas, VA) were used in the current study. To obtain mouse peritoneal exudate cells (PECs), 7- to 8-week-old C3H/HeN or C3H/HeJ mice (Japan Charles River, Tokyo, Japan) were injected intraperitoneally with 2 ml of thioglycollate broth (Difco Laboratories). PECs were then obtained 4 days later by peritoneal lavage. All animal experiments were carried out according to the guidelines of the Laboratory Animal Center, Jichi Medical University. The complete medium (CM) used for cell culture was composed of RPMI 1640 medium (ICN Biomedicals, Inc., Aurora, OH) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2% NaHCO3, and 10% heat-inactivated fetal bovine serum (FBS; Flow Laboratories Inc., Rockville, MD). The cells were cultured in a humidified chamber at 37°C with 5% CO2 and 95% air. RAW264.7 cells and PECs suspended in CM at 1 × 106 cells/ml were cultured overnight, and the adherent cells were used in all experiments. U937 cells were suspended in CM supplemented with 100 nM phorbol myristate acetate (PMA; Sigma-Aldrich Co., St. Louis, MO) at a cell density of 5 × 105/ml and were cultured for 3 days to induce differentiation into macrophage-like cells. Only the adherent cells were used in the current study.

Cytokine assay. RAW264.7 and U937 cells were cultured in 24-well culture plates (500 μl/well; Corning Inc., Corning, NY) and were stimulated with various LPSs, TLR ligands, and FKB. LPS purified from Salmonella enterica serovar Abortus-Equi (7) was a kind gift from C. Galanos (MPI für Immunobiologie, Freiburg, Germany) and was used as a reference for the TLR4 ligand. Pam3Cys-Ser-(Lys)4 hydrochloride (Pam3-Cys), purchased from Calbiochem, EMD Biosciences, Inc. (San Diego, CA), was used as a TLR2 ligand; and flagellin (Salmonella enterica S. Typhimurium), purchased from Invivogen (San Diego, CA), was used as a TLR5 ligand. The cytokine supernatant obtained 6 h after stimulation was assayed for mouse and human cytokines by a specific sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA was performed according to the instructions supplied by Endogen (Woburn, MA) and by using matched antibody pairs. Each cytokine was quantified (in ng/ml or pg/ml) on the basis of the standard curve obtained in each assay. In the suppression experiments, anti-human TLR4/CD284 (anti-TLR4 antibody; MBL Co., Ltd., Nagoya, Japan) (1), anti-human CD282 (TLR2) (anti-TLR2 antibody; BD Pharmingen, Franklin Lakes, NJ) (19), and a human TLR4 antagonist, compound 406 (Daiichi Kagaku Co., Tokyo, Japan) (10, 22), were used as suppressors. Cells were stimulated in the presence of suppressors (added 1 h prior to stimulation), and the culture supernatant was assayed 6 h after stimulation.

Detection of cytokine mRNA by RT-PCR. U937 cells were cultured in 60-mm culture dishes (Corning Inc.) containing 3 ml of medium and were stimulated with LPS or Pam3-Cys for 2 h in the presence or the absence of LPS-37. Total RNA was then extracted by using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). One microgram of total RNA was reverse transcribed by using SuperScript II reverse transcriptase (RT; Invitrogen Co., Carlsbad, CA). Aliquots of cDNA equivalent to 50 ng of RNA for the following genes were subsequently amplified by using the indicated primers (3, 5): for human tumor necrosis factor alpha (TNF-α), sense primer 5'-AGA GGG AAG AGT TTC CCA GGG AC-3' and antisense primer 5'-TGA GTC GTC GAC CCT TCT CCA G-3'; for human interleukin-1β (IL-1β), sense primer 5'-CCA GCT ACG AAT CTC GGA CCA CC-3' and antisense primer 5'-TTA GGA GAG ACA AAA TTG CAT GGT GAA GTC AGT-3'; for human IL-6, sense primer 5'-ATG AAC TCC TTT TCC ACA AGC GC-3' and antisense primer 5'-GAA GAG CCC TCA GGG TGC ACT G-3'; for human IL-8, sense primer 5'-ATG ACT TCC AAC CTG GCC GTG-3' and antisense primer 5'-TTA TGA ATT CTC AGC CCT TTA AAA ATT CTC-3'; for human IL-10, sense primer 5'-ATG CCC CAA GCT GAG AAC CAA GAC CCA-3' and antisense primer 5'-TCT CAA GGG GCT GGK TCA GAT CTC ACA-3'; for human IL-12p40, sense primer 5'-AGA GGC TCT TCT GAC CCC CAG-3' and antisense primer 5'-CCT TTG CTC TTG CCC TGG AGC TG-3'; and for human GAPDH, sense primer 5'-TGA AGG TCG GAG TAC GAT TTG GTT-3' and antisense primer 5'-CAT GTG GAC CAT GTC CAC CAC-3'. The PCR conditions included an initial denaturation at 94°C for 1 min, followed by 20 to 32 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Amplification was repeated for 32 cycles for IL-6, IL-10, and IL-12p40; 25 cycles for TNF-α and GAPDH; and 20 cycles for IL-1β and IL-8. The resulting PCR products were separated by electrophoresis on 1.2% agarose gels, and the gels were stained with ethidium bromide.

Phagocytic assay. U937 cells were cultured in 96-well culture plates (100 μl/well; Corning Inc.) and subjected to phagocytic assays, in accordance with the method described previously (36). Briefly, the cells were cultured for 1 h with cytochalasin D (a phagocytosis inhibitor; Wako Pure Chemical Industries, Ltd., Osaka, Japan), anti-TLR4 antibody, or LPS-37. After removal of the culture supernatant, the cells were cultured for 2 h in the presence of fluorescein-labeled Escherichia coli K-12 BioParticles (Molecular Probes, Inc., Eugene, OR). The culture supernatant containing the particles was then removed, and the remaining extracellular fluorescence was quenched by the addition of trypan blue solution (50 μg/ml, pH 4.4). After incubation at room temperature for 3 min, the dye was discarded and the fluorescence intensity (in relative fluorescence units) associated with the intracellular fluorescent particles was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm with a SPECTRMax M5 microplate fluorometer (Molecular Devices Corp., Sunnyvale, CA).

Estimation of LPS levels. The LPS levels present in the supernatants of the FKB were measured by using an Endospecy test chromogenic endotoxin-specific assay kit (Seikagaku Co., Tokyo, Japan). Briefly, the test sample solution was combined with the kit reagent in a 96-well microplate and the plate was incubated for 30 min at 37°C. The reaction was then stopped by adding 0.6 M acetic acid, and the color reaction was measured at 405 nm. LPS-27 and LPS-37 were used to construct the standard curves for the calculation of the LPS levels in the supernatants of FKB-27 and FKB-37, respectively.

Statistical analysis. The significance of any difference between the means for the experimental groups was determined by Student’s t test. The means were considered significantly different if P was <0.05.

RESULTS

Cytokine production by mouse and human cells stimulated with Y. pestis LPS-27 and LPS-37. The mouse macrophage cell line RAW264.7 was stimulated with LPS-27, LPS-37, or a reference Salmonella LPS; and the production of cytokines in the stimulated cells was assessed. The stimulation of cells with LPS-27 resulted in the production of both TNF-α and IL-6 when it was administered at a low dose of only 1 ng/ml. The stimulatory activity was similar to that achieved by the reference Salmonella LPS. LPS-37 also had stimulatory activity; however, the stimulation was significantly weaker than that of LPS-27. The activity of LPS-37 administered at 1,000 ng/ml was comparable to that of LPS-27 administered at 1 ng/ml (Fig. 1A). Similar results were also obtained when PECs isolated from C3H/HeN mice were used as target cells. However, the two LPS forms failed to induce cytokine production when PECs isolated from the TLR4 mutant mice (C3H/HeJ) were used as the target cells (data not shown). These results indicate that both LPS preparations function as specific mouse TLR4 agonists and are sufficiently free from detectable contaminants (i.e., they are pure enough) to stimulate the other TLRs.

We next examined the effects of these LPSs in human cells. As shown in Fig. 1B, LPS-27 exhibited strong stimu-
latory activity in human U937 cells comparable to that of the reference Salmonella LPS. In addition, the dose dependency of LPS-27 for this activity was similar to that for the activation of RAW264.7 cells. In contrast, the activity of LPS-37 in the human cells was remarkably reduced and was undetectable at doses as high as 10 μg/ml. This moderate agonistic activity observed in LPS-37-treated mouse cells and the antagonistic activity observed in treated human cells were similar to the

FIG. 1. Effects of LPS-27 and LPS-37 isolated from Y. pestis on the stimulation of mouse macrophage RAW264.7 cells and human macrophage U937 cells. (A) Mouse RAW264.7 cells were stimulated with LPS-27, LPS-37, and reference Salmonella LPS at the indicated doses. (B) Human U937 cells were stimulated as described for panel A. The cytokine levels in the culture supernatant at 6 h after stimulation were determined by ELISA. The data are presented as the means ± standard errors of triplicate samples, and a representative result from three independent experiments is shown. h, human; m, mouse.

FIG. 2. Effects of Y. pestis LPS-37 on cytokine production in U937 cells stimulated with TLR4 and TLR2 agonists. (A) U937 cells cultured in the presence of LPS-37 or compound 406 were stimulated with either Y. pestis LPS-27 (1 ng/ml) or Salmonella LPS (1 ng/ml). The cytokine levels in the culture supernatant were determined by ELISA. The data are presented as the means ± standard errors for triplicate samples. (B) U937 cells cultured in the presence of 10 ng/ml LPS-37 were stimulated with 1 ng/ml of LPS-27 or Salmonella LPS. Total RNA was isolated 2 h after stimulation and subjected to RT-PCR for determination of cytokine mRNA expression. PCR products were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide. (C) U937 cells cultured in the presence of LPS-37 were stimulated with Pam3-Cys (10 nM), and cytokine production was determined as described for panel A. (D) U937 cells cultured with LPS-37 (10 ng/ml) were stimulated with Pam3-Cys (10 nM), and cytokine mRNA expression was determined as described for panel B. Representative results of three (A and C) or two (B and D) experiments are presented.
Antagonistic activity of LPS-37 to stimulation of TLR4 in human cells. The suppressive effects of LPS-37 on the activation of human cells with various ligands to extracellular TLR were also examined. We used Y. pestis LPS-27 and the reference Salmonella LPS as TLR4 ligands, Pam3-Cys as the TLR2 ligand, and flagellin as the TLR5 ligand. Human U937 cells were stimulated with these agonists in the presence of LPS-37, or compound 406, and cytokine production was assessed. We found that the TLR4-dependent production of cytokines such as TNF-α and IL-6 was strongly suppressed by LPS-37. This result was similar to the response to compound 406 [Fig. 2A].

The strong antagonistic activity of LPS-37 against human TLR4 stimulation was confirmed by measuring the levels of expression of various cytokine mRNAs (Fig. 2B). LPS-37 was not observed to have suppressive effects against TLR2-stimulated cytokine production in terms of either protein production or mRNA expression (Fig. 2C and D). Similarly, LPS-37 was not observed to have antagonistic activity against TLR5 stimulation by flagellin (data not shown). These results indicate that LPS-37 acts as a strong antagonist against human TLR4 but not the additional human extracellular TLRs, such as TLR2 and TLR5.

Effects of LPS-37 on the phagocytic activity of U937 cells. Antiphagocytic factors play an important role in the evasion of bacteria from the host defense network by activating the innate immune system. As LPS-37 strongly suppressed cytokine production on the basis of the host (human) innate immune response, we next investigated the effects of LPS-37 on the phagocytosis of bacteria by U937 cells. The phagocytic activity of U937 cells was assayed with fluorescein-isothiocyanate-labeled E. coli K-12 BioParticles as the target bacteria, and the fluorescence of the engulfed intracellular bacteria was measured by quenching the fluorescence of the extracellular bacteria with trypan blue. Treatment with LPS-37 or anti-human TLR4 antibody failed to suppress the phagocytic activity in U937 cells, while the addition of cytochalasin D showed a significant suppressive effect [Fig. 3]. These results suggest that the suppression of TLR4 signaling by LPS-37 does not affect the role of Y. pestis in the enhancement of resistance to bacterial phagocytes by U937 cells.

Proinflammatory cytokines produce human cells stimulated with Y. pestis FKB. FKB-27 and FKB-37 were prepared by the method used in the production of formalin vaccine, and the ability of these FKB to stimulate cytokine production in U937 cells was investigated. Similar to treatment with LPS, the activity of FKB-27 was found to be strong, while the activity of FKB-37 was very weak, if it was present at all (Fig. 4A). The contribution of the free LPS released from the bacterial body to FKB-27 activity levels was found to be negligible. To estimate the amount of LPS released into the supernatant of the
FKB stock suspension, each of the supernatants isolated from the FKB-27- and FKB-37 suspensions was analyzed by the Endospecy test, and the levels were compared to the activity of the corresponding LPS (Fig. 4B). We found that 5 pg of LPS-27 was released from a suspension of $5 \times 10^4$ FKB-27 and that 2 pg of LPS-37 was released from a suspension of $1 \times 10^5$ FKB-37. These results indicate that the effect of the released LPS on FKB activity was negligible.

**Effects of TLR antibodies and LPS-37 on the activity of FKB-27.** To estimate the contribution of bacterium-bound LPS to the strong FKB-27 activity, we next investigated the effects of anti-TLR4 antibody, anti-TLR2 antibody, and LPS-37 on the activity of FKB-27 that stimulates human U937 cells. As presented in Fig. 5, we found that this activity was significantly suppressed in the presence of both anti-TLR4 antibody and LPS-37 but not in the presence of anti-TLR2 antibody. These findings were similar to the LPS-27 activity responses observed in the presence of antibody or suppressor. These results suggest that the bound form of LPS-27 significantly contributes to the activity of FKB-27.

**Effects of FKB-37 on cytokine response in U937 cells following TLR4 stimulation.** As the bacterium-free form of LPS-37 showed strong antagonistic activity to human TLR4 stimulation, we next examined whether FKB-37 also demonstrated a similar antagonistic activity. We found that FKB-37 did not exhibit antagonistic activity against the stimulation of human TLR4 in the presence of either LPS-27 or FKB-27. In addition, we also confirmed the strong antagonistic activity of LPS-37 (Fig. 6). In contrast to the bacterium-bound form of LPS-27, the bound form of LPS-37 did not exhibit the level of antagonistic activity observed in its bacterium-free form.

**DISCUSSION**

In the current study, we demonstrated that purified LPS-37 strongly suppresses proinflammatory cytokine production in human U937 cells stimulated with the TLR4 ligands *Salmonella* LPS and LPS-27. Activity was clearly observed when these ligands were administered at a dose of 1 ng/ml and reached a maximal level of activity (complete suppression) at approximately 10 ng/ml (Fig. 2). These doses were comparable to those required by *Salmonella* LPS and LPS-27 to exhibit agonistic activities, indicating that LPS-37 may serve as an antagonist in an infected host at a dose as low as that of an LPS that exhibits endotoxic activity. However, antagonistic activity was observed only when LPS-37 was in its bacterium-free form and not when it was bound to bacteria (FKB-37). Considering the low stimulatory activity of FKB-37 in U937 cells, the failure of *Y. pestis* LPS-37 to activate the host TLR4 defense system during the early stages of infection may significantly contribute to pathogenesis. Thus, suppression of the TLR4 function may contribute only to the later stages of infection, after bacterial lysis has already occurred.

We thought that it was interesting that the activity of FKB-27 was suppressed in the presence of the anti-TLR4 antibody but not in the presence of the anti-TLR2 antibody. Moreover, the activity was suppressed almost completely by LPS-37, which is a suppressor of TLR4 but not the additional extracellular TLRs, such as TLR2 and TLR5. Considering the relatively low levels of free LPS-27 released from FKB-27, together with the results mentioned above, the activity of FKB-27 may have been predominantly caused by LPS-27 in the form bound to the bacterial body. Similar LPS activity in its bacterium-bound form has not been reported previously. In contrast, FKB-37 did not exhibit antagonistic activity, suggesting that LPS-37 does not exhibit these activities in its bound form but does so in its free form. The human TLR4-based defense system may therefore remain unaffected during the early stages of *Y. pestis* infection.

*Francisella tularensis* is a gram-negative bacterium that causes the zoonotic infection tularemia. This bacterial species contains an LPS similar in structure and pathology to those of LPS-37 isolated from *Y. pestis*. One of the major lipid A mol-
ecules present in the LPS isolated from *F. tularensis* was reported to exhibit a tetra-acylated structure that contained three 3-OH C18 fatty acids, one C16 fatty acid, and one phosphate group (25, 34). We have previously reported that LPS-37 also exhibits a tetra-acylated structure but contains four 3-OH C18 fatty acids and two phosphate groups (16). In contrast to LPS-37, LPS isolated from *F. tularensis* was reported to be neither stimulatory nor antagonistic to human and murine cells via TLR4 (11). This difference was thought to be caused by the variations in their compositions, as monophosphoryl lipid A has been reported to have weaker activity than diphosphoryl lipid A (13) and longer acyl chains such as C16 have been reported to have weaker activity than C14 (22). These so-called silent characteristics of *F. tularensis* LPS are thought to contribute to its capacity to evade mammalian immune defense mechanisms and to promote survival in an infected host (9, 30). More recently, the effects of the preventive administration of a synthetic TLR4 agonist on the protection of mice from experimental pneumonic tularemia have been demonstrated (18). Similar prophylactic activation of TLR4 may also prove useful against *Y. pestis* infection.

The synthetic antagonist prototype termed compound 406 is a lipid IVa that is a biosynthetic precursor of *E. coli* lipid A (hexa-acylated) (26–27). This precursor structure, however, is not normally found in the outer membrane of *E. coli* or other usual members of the Enterobacteriaceae family. LPS is the major structural component of the cell wall outer membrane of gram-negative bacteria and plays an important role in the major structural component of the cell wall outer membrane of usual members of the *Enterobacteriaceae* family. LPS is the major structural component of the cell wall outer membrane of gram-negative bacteria and plays an important role in the stabilization of the bacterial body. A mutant strain of *E. coli* that synthesizes only the lipid IVa precursor and that contains only this type of lipid A species in its LPS has been reported to grow very slowly and to be unstable, as judged by its antibiotic hypersensitivity and easy lysis following centrifugation (35). However, *Y. pestis* grown at 37°C does not appear to be sensitive to oscillatory shock or susceptible to antibacterial factors in the serum of the infected host. It has been reported that the minimal LPS structure required for the viability of *E. coli* and *Salmonella enterica* serovar Typhimurium is lipid A glycosylated with 3-deoxy-D-manno-octulosonic acid (Kdo) residues but that *Y. pestis* can survive without Kdo residues in its LPS (32). In combination, these results suggest that *Y. pestis* differs from *E. coli* and other usual species of the Enterobacteriaceae, as it contains additional factors that may allow the construction of a strong outer membrane and the formation of a stable bacterial body, thus compensating for its insufficient LPS structure.

It has been reported that *Y. pestis* grown at a lower temperature (21°C) is readily phagocytosed by human neutrophils (15) but that *Y. pestis* grown at 37°C is resistant to phagocytosis (31). In the current study, we did not observe any antiphagocytic effects of LPS-37 on bacterial phagocytosis in U937 cells, even though LPS-37 strongly suppressed the TLR4-mediated inflammatory responses of these cells. Several antiphagocytic factors produced by *Y. pestis* are known to be upregulated during growth at 37°C. These factors include the type III secretion system (TTSS) and several effector Yop proteins expressed on plasmid pCD1 (33) and the F1 capsule protein expressed on plasmid pMT1 (6, 20). These factors have been shown to contribute to the extracellular survival of the bacterium following infection of humans. These results suggest that LPS-37 does not function as an antiphagocytic factor or that some additional factors expressed at 37°C are responsible for the antiphagocytic activity.

In conclusion, the current study reports on a possible role for the *Y. pestis* LPS during the infection of humans. LPS-37 appears to preserve the bacterium from elimination from the host via activation of the human TLR4 defense system during the early stages of infection when it is present in the bacterium-bound form and as a suppressor of the defense system when it is present in the bacterium-free form. We also found that LPS-27, an agonistic form of LPS expressed by numerous gram-negative bacterial species, was easily recognized by human TLR4 even in the bacterium-bound form.

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