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Thermoregulatory responses of rats to conventional preparations of lipopolysaccharide are caused by lipopolysaccharide per se—not by lipoprotein contaminants

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Steiner, Alexandre A., Sumana Chakravarty, Jared R. Robbins, Alexander S. Dragic, Jennifer Pan, Miles Herkenham, and Andrej A. Romanovsky. Thermoregulatory responses of rats to conventional preparations of lipopolysaccharide are caused by lipopolysaccharide per se—not by lipoprotein contaminants. *Am J Physiol Regul Integr Comp Physiol* 289: R348–R352, 2005. First published April 28, 2005; doi:10.1152/ajpregu.00223.2005.—LPS preparations cause a variety of body temperature (T_b) responses: monophasic fever, different phases of polyphasic fever, and hypothermia. Conventional (c) LPS preparations contain highly active lipoprotein contaminants (endotoxin proteins). Whereas LPS signals predominantly via the Toll-like receptor (TLR) 4, endotoxin proteins signal via TLR2. Several TLR2-dependent responses of immunocytes to cLPS in vitro are triggered by endotoxin proteins and not by LPS itself. We tested whether any T_b response to cLPS from *Escherichia coli* 055:B5 is triggered by non-TLR4-signaling contaminants. A decontaminated (d) LPS preparation (free of endotoxin proteins) was produced by subjecting cLPS to phenol-water reextraction. The presence of non-TLR4-signaling contaminants in cLPS (and their absence in dLPS) was confirmed by showing that cLPS (but not dLPS) induced IL-1β expression in the spleen and increased serum levels of TNF-α and IL-1β of C3H/HeJ mice; these mice bear a nonfunctional TLR4. Yet, both cLPS and dLPS caused cytokine responses in C3H/HeOuJ mice; these mice bear a fully functional TLR4. We then studied the T_b responses to cLPS and dLPS in Wistar rats preimplanted with jugular catheters. At a neutral ambient temperature (30°C), a low (0.1 μg/kg iv) dose of cLPS caused a monophasic fever, whereas a moderate (10 μg/kg iv) dose produced a polyphasic fever. In the cold (20°C), a high (500 μg/kg iv) dose of cLPS caused hypothermia. All T_b responses to dLPS were identical to those of cLPS. We conclude that all known T_b responses to LPS preparations are triggered by LPS per se and not by non-TLR4-signaling contaminants of such preparations.

body temperature; fever; hypothermia; inflammation; Toll-like receptors; TLR2; TLR4; LPS

INTRAVENOUS ADMINISTRATION of bacterial LPS preparations to laboratory animals is widely used to induce thermoregulatory responses associated with systemic inflammation. These responses are dependent on both the dose of LPS preparation and ambient temperature (T_a); for a review, see Ref. 27. For example, rats respond to LPS in a thermoneutral or supraneutral environment with fever, either monophasic (a single rise in deep body temperature caused by low, near-threshold doses) or polyphasic (several sequential rises in body temperature caused by higher doses) (29, 30, 32, 33, 37). At a subneutral T_a, rats respond to LPS with either fever, hypothermia, or a combination of the two: a fever response is elicited by low doses; a mild hypothermic response followed by fever is elicited by intermediary doses; and pronounced hypothermia is elicited by high, shock-inducing doses (30, 31, 33, 37).

In vitro, conventional preparations of LPS activate immunocytes via signaling through both Toll-like receptor (TLR) 4 and TLR2 (5, 15, 43). However, these preparations contain highly active lipoprotein contaminants (so-called endotoxin proteins) that signal through TLR2 (16). Elimination of endotoxin proteins by phenol-water reextraction abolishes the ability of LPS preparations to produce TLR2-mediated effects (9, 40) or to activate cells with a nonfunctional TLR4 (4, 9, 18, 19, 38). These observations indicate that endotoxin protein-free LPS preparations signal largely through TLR4 and not through TLR2. They also show that many effects of conventional LPS preparations are caused, in part, by TLR2-activating endotoxin protein contaminants; these effects include the activation of nuclear factor-κB signaling and the production of TNF-α and IL-6 (9, 40). It is, therefore, important to ask whether any of the thermoregulatory responses to LPS preparations are caused, at least in part, by endotoxin proteins and not by LPS per se. To answer this question was the aim of the present study.

METHODS

Animals. The main study reported in this paper was conducted in 53 male Wistar rats (Harlan, Indianapolis, IN). Initially, the rats were housed three per standard "shoebox" cage; after surgery, they were housed individually. The cages were kept in a rack equipped with a Smart Bio-Pack ventilation system (model SB4100) and Thermo-Pak temperature control system (model TP2000; Allentown Caging Equipment, Allentown, NJ); the temperature of the incoming air was maintained at 28°C. Standard rat chow (Teklad Rodent Diet "W" 8604; Harlan Teklad, Madison, WI) and tap

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water were available ad libitum. The room was on a 12:12-h light-dark cycle (lights on at 7 AM). The cage space was enriched with artificial "rat holes" (cylindrical confiners made of stainless steel wire). In addition to spending time in the confiners voluntarily, the rats were systematically habituated to them (7 daily training sessions, 4 h each). The same confiners were used later in the experiments. Rodents are readily adaptable to restraint to an extent that habituated rodents respond to it with neither stress fever (32) nor other signs of stress (1, 7, 20, 36). The rats weighed 300–420 g at the time of the experiments. Each rat was used in an experiment once and euthanized with pentobarbital sodium (20 mg/kg iv) immediately thereafter. The rats were housed in the animal facility of St. Joseph's Hospital; all procedures in rats were conducted under the protocols approved by the St. Joseph's Hospital Animal Care and Use Committee.

To confirm successful removal of endotoxin proteins from LPS, a separate experiment in 18 mice was conducted. Male mice that express a nonfunctional TLR4, C3H/HeJ (25, 26), and their wild-type counterparts, C3H/HeOuJ, were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were housed in the animal facility of the National Institute of Mental Health; all procedures conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were conducted under Animal Care and Use Committee-approved protocols.

Removal of endotoxin proteins from LPS preparation. A conventional preparation of phenol-extracted *Escherichia coli* 055:B5 LPS (cat. no. L2880), here referred to as conventional (c) LPS, was purchased from Sigma (St. Louis, MO). Following the manufacturer's specifications, the cLPS we used contained up to 3% of protein. To remove endotoxin proteins, phenol-water reextraction was performed, as described by Manthey and Vogel (19). In brief, water-saturated phenol was added to an equal volume of cLPS suspension (5 mg/ml) in water containing 0.2% triethylamine and 0.5% deoxycholate. To separate the phenol and water phases, the mixture was cooled (4°C) and centrifuged (10,000 *g*; 2 min). Each phase was transferred to a separate tube and subjected to a second extraction. The water phase was subjected to extraction with water-saturated phenol; the phenol phase was subjected to extraction with water containing 0.2% triethylamine and 0.5% deoxycholate. After phase separation, the water phases (known to contain LPS) of the two tubes were pooled together. To precipitate LPS, ethanol and sodium acetate were added to the water phase to achieve final concentrations of 75% and 30 mM, respectively, and the system was allowed to rest for 1 h at −20°C. The precipitated LPS was separated by centrifugation (10,000 *g*; 10 min) at 4°C, washed with cold (4°C) ethanol, and air-dried. The LPS reextracted by this method is referred to as decontaminated (d) LPS. According to Manthey and Vogel (19), dLPS is essentially free of endotoxin proteins and all reagents used in the reextraction procedure, including deoxycholate, triethylamine, and sodium acetate.

Confirmation of endotoxin protein removal. Preparations of cLPS (contain endotoxin proteins) are known to stimulate immunocytes with either a functional or nonfunctional TLR4, whereas preparations of dLPS (free of endotoxin proteins) can stimulate immunocytes bearing a functional TLR4 but cannot stimulate cells with nonfunctional TLR4 (9–11, 18, 19, 21, 38). Such a difference between the effects of cLPS and dLPS was used in the present study to confirm the absence or presence of endotoxin proteins in the LPS preparations studied. Mice that express a nonfunctional TLR4, C3H/HeJ, and their wild-type counterparts, C3H/HeOuJ, were administered cLPS (1,000 μg/kg ip), the same dose of dLPS, or saline. Two hours after the injection, the mice were euthanized by cervical dislocation, and samples of their cardiac blood and spleen tissue were collected. The levels of the proinflammatory cytokines TNF-α and IL-1β in the blood serum were measured by immunoassay using R&D Systems (Minneapolis, MN) kits. The level of the IL-1β transcript in the spleen was visualized by in situ hybridization, as previously described (4).

In C3H/HeJ (TLR4 nonfunctional) mice, cLPS increased the levels of TNF-α and IL-1β in the serum and the level of the IL-1β transcript in the spleen, whereas dLPS elicited no cytokine responses (Fig. 1*A*). These results confirm that the cLPS preparation used contained non-TLR4-signaling contaminants (presumably endotoxin proteins), whereas dLPS was free of such contaminants. In C3H/HeOuJ (TLR4 functional) mice, both cLPS and dLPS produced increases in the serum levels of TNF-α and IL-1β and in the spleen level of the IL-1β transcript (Fig. 1*B*), thus confirming that dLPS retained the ability to induce immune responses in animals with a fully functional TLR4.

Surgical preparation and experimentation. Each rat was subjected to chronic catheterization of the jugular vein. Under ketamine-xylazine-acepromazine (55.6, 5.5, and 1.1 mg/kg ip, respectively) anesthesia and antibiotic (enrofloxacin 1.1 mg/kg sc) protection, the rat was placed on an operating board. A 1-cm longitudinal incision was made on the ventral surface of the neck, 1 cm left of the trachea. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (10 U/ml) pyrogen-free saline was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the 10-cm silicone catheter was knotted, tunneled under the skin, and exteriorized at the nape. The surgical wounds on the ventral and dorsal surfaces of the neck were sutured. The catheters were flushed with heparinized saline on *days 1* and *3* postsurgery.

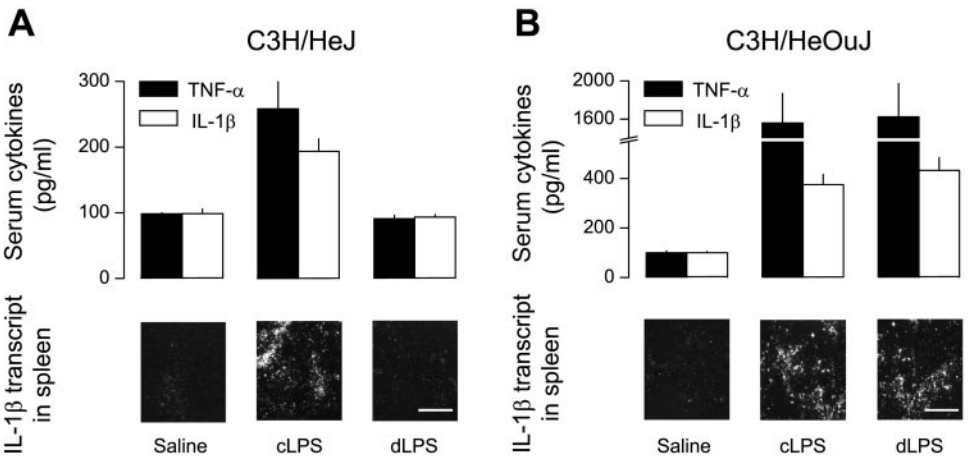

Fig. 1. Cytokine responses of C3H/HeJ [Toll-like receptor 4 (TLR4) nonfunctional; *A*] and C3H/HeOuJ (TLR4 functional; *B*) mice to conventional LPS (cLPS; contains endotoxin protein contaminants), decontaminated LPS (dLPS), or saline. The dose of cLPS or dLPS was 1,000 μg/kg ip. The levels of TNF-α and IL-1β in the blood serum and the level of the IL-1β transcript in the spleen at 2 h postinjection are shown. Serum levels of cytokines are presented as mean ($n = 3$) absolute values ± SE. Spleen levels of the IL-1β transcript can be visually evaluated as the amount of material labeled in white in the representative dark-field photomicrographs of emulsion-coated tissue sections (scale bar = 300 μm).

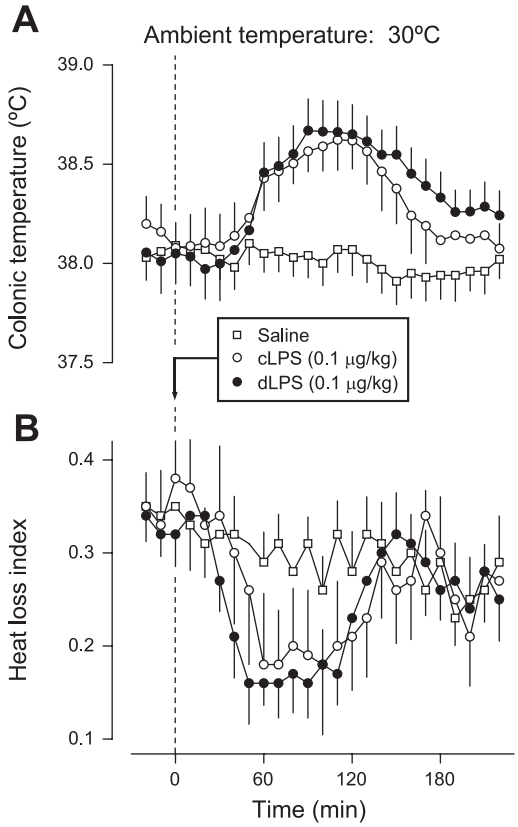

Fig. 2. Colonic temperature (*A*) and heat loss index (*B*) responses of Wistar rats to the intravenous injection (arrow) of the low (0.1 μg/kg) dose of cLPS ($n = 8$) or dLPS ($n = 7$) at a neutral ambient temperature (30°C); controls ($n = 6$) were injected with saline (1 ml/kg).

The experiments were performed on *day 5*. Each rat was placed in a confiner and equipped with two copper-constantan thermocouples: one for recording colonic temperature (T_c) and the other for recording tail skin temperature (T_{sk}). The colonic thermocouple was inserted 10 cm beyond the anal sphincter and fixed to the base of the tail with adhesive tape. The skin thermocouple was positioned on the lateral surface of the tail (at the boundary of the proximal and middle thirds of the tail) and insulated from the environment with tape. The thermocouples were plugged into a data logger (Dianachart, Rockaway, NJ), which was connected to a personal computer. The rat was transferred to a climatic chamber (Forma Scientific, Marietta, OH) set to either a neutral (30.0°C) or subneutral (20.0°C) T_a (28). The jugular catheter was extended with a length of PE-50 tubing filled with saline, and the extension was passed through a wall port and connected to a syringe filled with the drug of interest. This setup permitted intravenous drug administration without disturbing the rat. To induce fever, either cLPS or dLPS was injected at a neutral T_a of 30°C; a low dose (0.1 μg/kg iv) was used to evoke a monophasic fever; a moderate dose (10 μg/kg iv) was used to induce a polyphasic fever. To induce hypothermia, a high dose (500 μg/kg iv) of either cLPS or dLPS was injected at a subneutral T_a of 20°C. At either T_a, the controls were injected with saline (1 ml/kg iv).

Statistical analysis. The absolute value of T_c, rather than the change in T_c, was used to evaluate deep body temperature responses (for justification, see Ref. 34). The heat loss index (HLI) was used to evaluate thermoeffector responses of tail skin vasculature. As justified elsewhere (28), the HLI was calculated according to the formula: $HLI = (T_{sk} - T_a)/(T_c - T_a)$. The theoretical limits of the HLI are 0 (maximal skin vasoconstriction) and 1 (maximal vasodilation). In practice, however, the upper limit depends on the position of the tail skin thermocouple. When T_{sk} is measured at the boundary of the proximal and middle thirds of the tail, as in the present study, the HLI rarely exceeds 0.6. The T_c and HLI responses were compared across treatments and time points by a two-way ANOVA for repeated measures followed by the Tukey (honest significant difference) post hoc test. The analysis was performed using Statistica AX'99 (StatSoft, Tulsa, OK). The effects were considered significant when $P < 5.0 \times 10^{-2}$. The data are reported as means ± SE.

RESULTS

No marked changes in T_c or HLI occurred in response to saline administration at either a neutral (30°C) or subneutral (20°C) T_a, whereas administration of cLPS or dLPS evoked pronounced thermoregulatory responses (Figs. 2–4). At the neutral T_a, the low dose (0.1 μg/kg iv) of either cLPS or dLPS caused a monophasic rise in T_c ($P = 2.2 \times 10^{-5}$ for both cLPS and dLPS), with a peak at ~100 min postinjection (Fig. 2). A significant ($P = 2.5 \times 10^{-2}$ for cLPS; $P = 5.2 \times 10^{-4}$ for dLPS) decrease in the HLI (tail skin vasoconstriction) occurred immediately before the onset of the T_c response. At the same T_a, the moderate dose (10 μg/kg iv) of either LPS preparation elicited a high, polyphasic fever ($P = 2.2 \times 10^{-5}$ for both cLPS and dLPS) with three sequential peaks at ~40, 150, and 310 min postinjection (Fig. 3). Each febrile phase was preceded by a transient decrease in the HLI ($P = 1.3 \times 10^{-2}$ for cLPS; $P = 1.7 \times 10^{-4}$ for dLPS). At a subneutral T_a of 20°C, the high dose

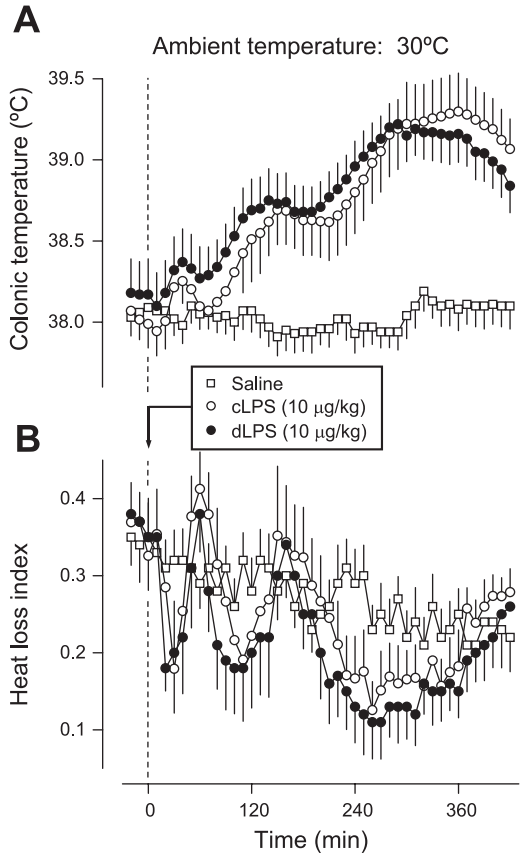

Fig. 3. Colonic temperature (*A*) and heat loss index (*B*) responses of Wistar rats to the intravenous injection (arrow) of the moderate (10 μg/kg) dose of cLPS ($n = 6$) or dLPS ($n = 7$) at a neutral ambient temperature; controls ($n = 6$) were injected with saline (1 ml/kg).

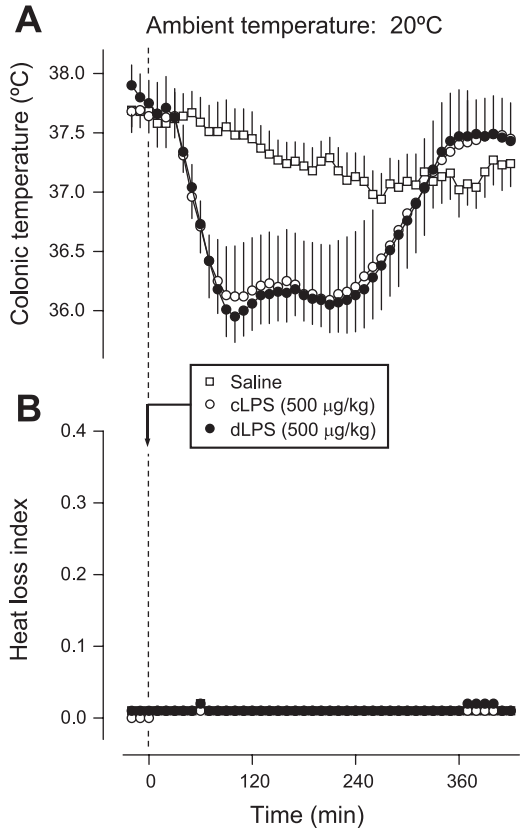

Fig. 4. Colonic temperature (*A*) and heat loss index (*B*) responses of Wistar rats to the intravenous injection (arrow) of the high (500 μg/kg) dose of cLPS ($n = 6$) or dLPS ($n = 6$) at a subneutral ambient temperature (20°C); controls ($n = 7$) were injected with saline (1 ml/kg).

(500 μg/kg iv) of either LPS preparation caused a pronounced hypothermia ($P = 2.2 \times 10^{-5}$ for both cLPS and dLPS) with a first nadir at ~100 min and second at ~200 min (Fig. 4). The HLI was nearly zero (maximal tail skin vasoconstriction) before the injection and remained unchanged throughout the response; the hypothermic response to LPS is brought about by inhibition of thermogenesis (31). For all thermoregulatory patterns studied (monophasic fever, three phases of polyphasic fever, and hypothermia), neither the T_c nor HLI response to dLPS differed from the corresponding response to cLPS, thus indicating that removal of endotoxin proteins from the LPS preparation did not change (and specifically did not reduce) any thermoregulatory response.

DISCUSSION

Recognition of LPS by TLRs has been a subject of vigorous debate in the last few years. Studies involving transfected cells suggested that LPS activates immunocytes by interacting with either TLR2 or TLR4 (5, 15, 43). However, other studies involving cells with a nonfunctional or absent TLR2 or TLR4 showed that only TLR4 is essential for LPS recognition (8, 12, 25, 26, 39). It was later shown that it is not LPS per se, but rather highly active lipoprotein contaminants of LPS preparations, the so-called endotoxin proteins, that activate TLR2 (9, 16, 40).

Because conventional LPS preparations cause multiple thermoregulatory responses (monophasic fever, at least three distinct phases of polyphasic fever, and hypothermia), and because some of these responses are caused only by high (hypothermia) or relatively high (third phase of polyphasic fever) doses of LPS preparations (for a review, see Ref. 27), it was necessary to investigate whether any of these responses are caused by contaminants. The present study provided a clear answer to this question. It showed that successful removal of non-TLR4-signaling contaminants from a conventional LPS preparation did not affect the ability of LPS to cause any of the thermoregulatory responses studied. Even those responses that are caused by higher doses of LPS (hypothermia and the third febrile phase) were found to be completely independent of the non-TLR4-signaling contaminants. It is, therefore, concluded that low amounts of endotoxin protein contaminants administered along with LPS do not produce thermoregulatory effects of their own and that all parts of the thermoregulatory responses studied are indeed triggered by LPS. This conclusion is in line with an early observation by Watson et al. (42), who found that LPS-induced hypothermia is absent in C3H/HeJ (TLR4 nonfunctional) mice. It is also in line with recent findings showing that some nonthermoregulatory responses to LPS in vivo, such as anorexia or production of proinflammatory cytokines, are mediated by TLR4 (4, 41) and do not require TLR2 (41).

The present study, however, does not rule out the possibility that TLR2-signaling endotoxin proteins at much higher doses (e.g., if such proteins were administered alone instead of as contaminants of LPS preparations) can have thermoregulatory effects of their own. At least some TLR2 agonists from Gram-positive bacteria are known to cause fever when injected in rats (6, 14), guinea pigs (17, 35), rabbits (3), and cats (2). Neither does the present study rule out the possibility that LPS causes some of its thermoregulatory effects by acting on receptors other than TLR4. LPS recognition can involve other cell-surface receptors, most notably CD11/CD18 β_2-integrin (24) and scavenger receptors (22, 23). Involvement of more than one receptor could provide a hypothetical explanation for how the same dose of LPS causes fever at a neutral T_a but hypothermia (at least transient) at a subneutral T_a. We speculated (13) that the dependence of the thermoregulatory effect of LPS on T_a reflects different distribution of the blood in the body at different T_as and, consequently, different distribution of LPS and its recognition by different cells possibly via different receptors. What the present study does show is that all known thermoregulatory responses to conventional LPS preparations (i.e., monophasic fever, different phases of polyphasic fever, and hypothermia) are triggered by LPS per se and not by common and highly active lipoprotein contaminants of such preparations.

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