Immunobiological activities of a new nontoxic lipopolysaccharide from *Acidiphilium* GS18h/ATCC55963, a soil isolate from an Indian copper mine

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**Abstract**

A novel nontoxic lipopolysaccharide (LPS) was purified from *Acidiphilium* strain GS18h/ATCC55963. The chemical composition of the lipid A part of this LPS is distinctly different from that of known lipid A molecules. The LPS was investigated to determine its capacity to provide protection against toxic LPS or endotoxic shock, as has been reported for other nontoxic LPSs (*Rhodobacter sphaeroides* and *Rhodobacter capsulatus*), and also the extent and type of immunomodulatory response in terms of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6 release as well as NO secretion by stimulated monocyte–macrophage systems. This study demonstrates clearly that mice immunized or primed with this LPS are fully protected against challenge with toxic *Escherichia coli* LPS. Unlike most of the extensively studied nontoxic LPSs, this LPS induced reactive nitrogen intermediates and released TNF-α, IL-1β and IL-6 in both mouse and human monocyte–macrophage systems. However, the extent of the cytokine and lymphokine releasing response was well below the range of the toxic LPS, for example that of *E. coli*. Owing to its capacity to provide immunostimulation of the host without causing any lethality to ensure protection against endotoxic shock, this LPS appears to have potential therapeutic value.

**Introduction**

Bacterial lipopolysaccharides (LPSs), also known as endotoxin, are capable of overstimulating the monocyte–macrophage system and are an integral part of the Gram-negative bacterial cell wall. They activate cells of the immune system that produce inflammatory cytokines, proteases, eicosanoids, and reactive oxygen and nitrogen intermediates (West & Heagy, 2002). The relationship between the chemical structure/composition and biological activity of LPS is of paramount importance to the outcome of proinflammatory cytokine production and its action. The proinflammatory cytokines produced by LPS activity are indispensable for countering the growth and dissemination of Gram-negative bacteria, while overproduction manifests as sepsis syndrome and endotoxic shock. The manifestation of LPS activity in biological systems is therefore under numerous controls. It depends on the species, cell type, and the structural architecture and/or compositional make up of the LPS, and may be host-protective or host-deleterious, for example by inducing hyper-toxic or shock syndrome.

Biologically less active or weakly toxic lipopolysaccharides are distributed in bacteria phylogenetically not related closely to or existing distinctly from *Enterobacteriaceae*. The lipid A moieties of such bacterial LPSs are structurally distinct from those of the toxic lipid A owing to their sugar backbone and the fatty acid spectrum (Johnson, 1994; Rietschel et al., 1994; Zahringer et al., 1995). These types of lipid A are of particular biological interest as they lack endotoxic activity, and are capable of inducing cytokines having an entirely different spectrum and of modifying the response of toxic LPS. The LPSs originating from other sources, such as the soil bacteria *Acidiphilium* (Urkami et al., 1989; Kishimoto et al., 1991; Basu et al., 1994), are closely related to the *Thiobacillus* LPS, which is reported to be nontoxic or weakly toxic (Mayer et al., 1989). We have isolated and purified the LPS from the new *Acidiphilium* strain ATCC55963 (deposited in the ATCC patent
depository as a new *Acidiphilium* strain), which has been placed phylogenetically along with *Acidiphilium cryptum* and *Acidiphilium symbioticum* (Banerjee et al., 1996). We found that the type, nature, linkage and chain length of fatty acids of lipid A in this LPS are very different from those of the toxic LPSs of *E. coli, Salmonella* and other enterobacterial types, but partially similar to those of the LPSs of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. The *Acidiphilium* ATCC55963 LPS was found to contain mostly long-chain fatty acids that are either β-hydroxylated or unsaturated (Bera et al., 2005). The isolation, chemical characterization and lethal toxicity of this LPS in galactosamine-sensitized mice have been described in an earlier report (Bera et al., 2005). However, the profile of cytokines/lymphokine and the reactive nitrogen intermediates released remained to be elucidated in respect of evaluating the extent of its toxicity and relevance to all these biological responses in different cell systems, namely mouse, human and a permanent macrophage cell line. Septic shock is associated with all these responses, which are mediated by the release of multiple mediators from phagocyte and endothelial cells. In particular, interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) are the most important mediators in response to toxic LPS inducing septic shock syndrome.

For the maximum therapeutic effect, it may be necessary to control the action of the endotoxin at the primary level of receptor stimulation in order to block the LPS-induced cellular response over the activation and subsequent release of the broad spectrum of potentially deleterious cytokines and mediators responsible for septic shock. It has been reported that the LPSs from the nonenteric, nonpathogenic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are poor agonists, and that the lipid A derived from these LPSs can antagonize more pathogenic LPSs (Kobayashi et al., 1998). These findings imply that certain forms of lipid A derivatives can inhibit the acute inflammatory response to LPS and may be useful for the treatment of LPS-induced shock or mortality. In this study, we have compared the effect of LPS from *Acidiphilium* ATCC55863 with that of LPS from *E. coli* (serotype 0111:B4) in various systems to assess whether a new nontoxic LPS would emerge to act as a preventive for endotoxic shock in humans and whether it could be proposed for use therapeutically.

**Materials and methods**

**Animals**

Female BALB/c mice were obtained from the animal house of our institute. All mice were 10–12 weeks old and weighed 20 ± 2 g. Mice were housed in groups of 10 at a constant temperature of 23 ± 1 °C in 55 ± 5% humidity and were provided with pellet food and water ad libitum. Indian domestic white male rabbits each weighing 2–2.5 kg were also obtained from the animal house of our institute.

**LPS preparation**

The preparation of LPS from *Acidiphilium* ATCC55963 is described in an earlier paper (Bera et al., 2005). Briefly, the LPS was extracted from wet cells by a conventional hot phenol-water method (Westphal & Jann, 1965), and further purified by repeated ultracentrifugation and a ‘phenol re-extracted LPS’ procedure by treatment with triethylamine, phenol, and deoxycholate (DOC) as per a previously reported method (Hirschfeld et al., 2000). Purified LPS was free from protein which is usually associated with endotoxin, nucleic acid and phospholipids (Bera et al., 2005). LPS from *E. coli* (a phenol extract of serotype 0111:B4) was obtained from Sigma Chemical Co. (St Louis, MO).

**Preparation of mouse peritoneal macrophages**

Mice were injected intraperitoneally with 1.5 mL of 3% sterile thioglycolate broth (Difco, Detroit, MI). Three days after injection, the mice were killed by cervical dislocation, and 10 mL of cold RPMI-1640 medium (Life Technologies, GibcoBRL, MD) was used to wash the peritoneal cavity. Macrophages were collected by centrifugation and again washed with 10 mL RPMI-1640 medium. Cell viability was verified by the trypan blue exclusion technique. Cells were resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotic–antimycotic (Life Technologies, GibcoBRL, MD). Cells were plated at 1 × 10⁶ density per well in a 24-well tissue-culture plate and incubated for 6 h at 37 °C in a 5% CO₂ humidified incubator to allow the macrophages to adhere. After 6 h, nonadherent cells were aspirated and the cells were washed with phosphate-buffered saline (PBS). Finally, 1 mL of complete culture medium was added to each well. The cells were then exposed to LPS in the same medium for stimulation. At the end of the stimulation period, the culture supernatants were collected and kept at −70 °C until analysed.

**Mouse macrophage cell-line RAW 264.7 culture**

For RAW 264.7 cell culture, Dulbecco’s Modified Eagle’s Medium (DMEM) medium supplemented with 10% FCS and antibiotic–antimycotic was used. Cells from the murine macrophage cell line RAW 264.7 were procured from the National Cell Center (Pune, India) and maintained in the above culture medium in a humidified chamber with 5% CO₂ at 37 °C. A culture medium supplemented with 2% FCS was used for various stimulation experiments with such a macrophage cell line. The cells were plated at 1 × 10⁶ density per well in a 24-well tissue-culture plate and incubated for 2 h in a cell-culture incubator to allow them
to adhere to the plates. After being washed three times with PBS, the cells were cultured in culture media containing different stimulants and/or inhibitors.

**Isolation of human peripheral blood monocytes**

Peripheral venous blood was obtained from healthy volunteers, with heparin (10 U mL\(^{-1}\)) as the anticoagulant. Mononuclear cells (MNC) were isolated by density gradient on Histopaque (density 1.077 ± 0.001, Sigma Chemicals Co.). The mononuclear cell fraction was collected, and the cells were washed twice in PBS. Cells were then resuspended in RPMI 1640 medium supplemented with 10% FCS and antibiotic–antimycotic. Viability of the cells was assessed by the trypan blue exclusion method. Cells were seeded into 24-well culture plates at a concentration of 1 × 10\(^6\) per well and incubated for 2 h at 37°C with 5% CO\(_2\). After 2 h, nonadherent cells were aspirated, and the adhering cells were washed three times with PBS. Finally, 1 mL of culture medium (RPMI 1640) was added to each well. Stimulation of the cells by LPS was performed in the same medium for 24 h. At the end of the stimulation period, the culture supernatants were collected and kept at −70°C until analysed.

**NO assay**

NO released by the macrophages was measured as a stable form of nitrite (NO\(_2\)) in the culture supernatants using Griess reagent (Green et al., 1982). Briefly, 100 μL of test samples were mixed with the same volume of Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in H\(_2\)O, 1% (w/v) sulfanilamide in 5% (v/v) H\(_3\)PO\(_4\); 1:1 (v/v)] in a 96-well plate. The colorimetric reaction was allowed to proceed for 10 min at 30°C, and the optical density (OD) was measured at 550 nm in an Emax microplate reader (Molecular Devices). In a similar way, a standard curve was established from serial dilution (1–100 μM) of NaNO\(_2\) in culture medium, and the measured OD values of experimental samples were converted to concentrations. All samples were assayed in triplicate.

**Cytokine assay**

The release of TNF-α, IL-1β and IL-6 by human monocytes was quantified in culture supernatants of the in vitro experiment using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (Quantikine, R & D Systems, Minneapolis, MN). Assays of TNF-α and IL-1β released by the murine macrophage cell line RAW 264.7 and mouse peritoneal macrophages were performed by an indirect ELISA method previously described in Garde et al. (1995). Briefly, 96-well ELISA plates were coated with 100 mL of supernatant containing TNF-α in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) by overnight incubation at 4°C in triplicate. The same volume of RPMI or DMEM medium was added to three wells as a control. Serially diluted murine TNF-α (Sigma) ranging from 0.1 to 100 ng mL\(^{-1}\) was also included as standard. The coated plates were then washed five times with washing buffer (PBS containing 0.05% Tween 20) and blocked for 12 h with 200 μL of blocking solution (2% nonfat dry milk solution in coating buffer) at 4°C. The plates were then emptied and washed six times with washing buffer. Then goat antimouse TNF-α monoclonal antibody (Sigma, 100 ng 100 μL\(^{-1}\) per well) was added to each well and incubated at 37°C for 2 h. After flicking out the medium, the wells were washed six times with the washing buffer. Next, rabbit anti-antibody alkaline phosphatase conjugates were added as the detecting antibody and incubated at 37°C for 2 h. The wells were washed, the substrate pNPP (1 mg mL\(^{-1}\) in diethanolamine buffer, pH 9.3) was added, and the wells kept in the dark for 40 min. The OD was then measured at 405 nm using an Emax microplate reader. Samples with OD values outside the standard range were assayed again at an appropriate dilution.

The amount of IL-1β was determined by ELISA using goat antimouse IL-1β monoclonal antibody (Sigma) as the first antibody, and rabbit anti-antibody conjugated with horseradish peroxidase (Sigma) as the detection antibody. O-phenylenediamine (OPD) was used as the substrate. The reaction was stopped by the addition of 4 N sulfuric acid, and the OD was measured at 495 nm using an Emax microplate reader.

**In vivo study of the antagonistic activity of purified nontoxic Acidiphilium ATCC55963 LPS against toxic E. coli LPS**

In order to study the antagonistic effect of Acidiphilium ATCC55963 LPS, fresh noninfected BALB/c mice were used in all studies. All LPS preparations were made in fresh pyrogen-free saline and administered intravenously via a tail vein. The naïve mice were treated with ATCC55963 LPS before or after the challenge of E. coli toxic LPS, and mortality was recorded for the next 72 h. The effect of administration of ATCC55963 and E. coli LPS in mice using various doses and at various times of administration ranging from 0 to 12 h was observed to determine the mortality rate.

**Rabbit pyrogenicity**

The pyrogenic property of Acidiphilium nontoxic LPS was determined by means of the rabbit pyrogenicity test method as described previously (Ogawa et al., 2000), with a slight modification. The test solution in 0.5 mL of pyrogen-free saline was injected in rabbits via an ear vein. The thermister probe, connected to a temperature-recording device,
measured the rectal temperature of each rabbit continuously. The measurement in this study was simplified to determine the rectal temperature of each rabbit every 30 min between 1 and 1.5 h and between 2.5 and 4 h after the injection of test specimens for the first and second-phase temperatures, respectively. The least amount of LPS leading to an increased rectal temperature of more than 0.6 °C was taken as a positive minimal pyrogenic dose.

Statistical analysis

Results are expressed as the mean ± SD of the mean. Differences between results were assessed for significance using Student’s t-test. Differences with P value of less than 0.05 were considered statistically significant.

Results

Induction of NO production by mouse peritoneal macrophages and RAW 264.7 cells upon stimulation with Acidiphilium ATCC55963 and E. coli LPS

The role of NO in the pathogenesis of various inflammatory diseases is well known. LPS stimulates macrophages to release NO, which plays an important role in inflammation. Both the murine macrophage-like cell line RAW 264.7 and mouse peritoneal macrophages are useful models with which to study the macrophage synthesis of NO. In this study, mouse peritoneal macrophages and RAW 264.7 cells were used to compare the production of NO by the LPS of Acidiphilium ATCC55963 and that of E. coli. Stimulation with 1000 ng mL⁻¹ of either type of LPS caused a marked elevation in NO secretion. In the time kinetics studies of NO release by RAW 264.7 cells upon stimulation with LPS, it was observed that the production of NO was initiated by 3 h, peaked at 48 h, and declined over 72 h (data not shown).

In a dose-dependent study, the production of NO in the culture supernatant was measured 48 h after treatment of LPS. Unstimulated macrophages were not observed to secrete NO into the culture medium during 48 h of incubation. As shown in Fig. 1a and b, the level of NO production induced by Acidiphilium ATCC55963 LPS was always considerably lower than that observed with E. coli LPS. It was also observed that the use of thioglycolate-elicited mouse peritoneal macrophages instead of RAW 264.7 cells produced similar results, but that the maximum production of NO upon stimulation with either Acidiphilium LPS or E. coli LPS was smaller than that with RAW 264.7 cells. A representative result obtained from three to five independent experiments is shown (Fig. 1b).

Potency of Acidiphilium ATCC55963 LPS to activate murine macrophages and RAW 264.7 cells for the induction of cytokines

The potency of Acidiphilium LPS preparations to activate RAW 264.7, a murine macrophage cell line, and thioglycolate-elicited peritoneal macrophages of BALB/c mice for the induction of TNF-α and IL-1β was investigated. LPS from both Acidiphilium ATCC55963 and E. coli triggered TNF-α and IL-1β release in a dose-dependent manner. Significant amounts of TNF-α were produced by murine peritoneal macrophages upon stimulation with ATCC55963 LPS at concentrations over 1 ng mL⁻¹, and the trend was similar to that noted with E. coli LPS, although the amount of TNF-α produced by the former was very much lower than that produced by the reference E. coli LPS (Fig. 2a and b). Both types of LPSs released the maximum amount of TNF-α at a concentration of 1 μg mL⁻¹; the concentration of released TNF-α was 19.8 ng mL⁻¹ for E. coli LPS but 8.4 ng mL⁻¹ for ATCC55963 LPS. Similarly, ATCC55963 LPS exhibited weak potency in the induction of IL-1β compared with toxic E. coli LPS. The maximum release of IL-1β, 5.2 ng mL⁻¹, occurred with E. coli LPS at a concentration of 1 μg mL⁻¹. In contrast, the maximum concentration of IL-1β in response to Acidiphilium ATCC55963 LPS, 2.4 ng mL⁻¹, was reached with the highest dose tested, namely 10 μg mL⁻¹ (Fig. 2b).

A murine macrophage cell line, RAW 264.7, was then used to investigate the activity of ATCC55963 LPS to induce
TNF-α and IL-1β. As shown in Fig. 3a and b, large amounts of TNF-α and IL-1β were produced by RAW 264.7 compared with peritoneal mouse macrophages upon stimulation with ATCC55963 LPS and the reference E. coli LPS. The results clearly indicate that induction of TNF-α and IL-1β production was much higher with E. coli LPS than with Acidiphilium ATCC55963 LPS.

Potency of Acidiphilium ATCC55963 LPS to activate human monocytes for the induction of cytokines

In the past decade, a number of studies have strongly implicated the proinflammatory cytokines TNF-α and IL-1β as potentially important mediators of septic responses during Gram-negative infections. Moreover, IL-6 is known to be an important pro- and anti-inflammatory cytokine, which is upregulated during sepsis. We therefore carried out detailed quantitative assessments of LPS-induced TNF-α, IL-1β, and IL-6 secretion by human monocytes using ELISA methods. Because IL-1β and TNF-α have been implicated in the pathogenesis of endotoxic shock (Dinarello, 1988; Riedemann et al., 2003), it was important to determine whether ATCC55963 LPS had any effect on their release from monocytes. LPSs from both Acidiphilium ATCC55963 and E. coli triggered TNF-α, IL-1β and IL-6 in a dose-dependent manner. The maximum amount of TNF-α produced was 4.87 ± 0.15 and 1.20 ± 0.09 ng mL⁻¹ at a concentration of 1 mg mL⁻¹ of E. coli and ATCC55963 LPS, respectively (Fig. 4a), and the release of this cytokine was decreased by both the LPSs at higher doses. Similarly, Acidiphilium ATCC55963 LPS exhibited weak potency in the induction of IL-1β compared with the reference E. coli LPS (Fig. 4b). The maximum release of IL-1β, 2.85 ± 0.15 ng mL⁻¹, occurred with E. coli LPS at a concentration of 1 μg mL⁻¹. In contrast, the maximum concentration of IL-1β released in response to ATCC55963 LPS was 1.25 ± 0.05 ng mL⁻¹ with the highest dose tested, 10 μg mL⁻¹. In view of the release of TNF-α and IL-1β, endotoxic shock mediators, the secretion of monokines induced in human monocytes in the in vitro system by the ATCC55963 LPS was far lower than that by E. coli LPS. In addition, the level of IL-6 released by E. coli LPS was also much higher than that released by ATCC55963 LPS (Fig. 4c). It is interesting that IL-6 has been known since 1989 to be greatly upregulated in the serum of patients with a bacterial infection or sepsis (Wagge et al., 1989; Riedemann et al., 2003), and a positive correlation exists between IL-6 serum levels and severity in septic patients (Calandra et al., 1991; Gardlund et al., 1995).

Pyrogenicity

Previously we observed in the Limulus amoebocyte lysate assay that the lowest concentration of LPS that produced a
positive test was 0.1 ng mL\(^{-1}\) for \(E.\ coli\) and \(>10\) ng mL\(^{-1}\) for \(Acidiphilium\) ATCC55963. Similarly, ATCC55963 LPS induced no febrile response in rabbits up to a dose of \(10\) µg kg\(^{-1}\), whilst rabbits injected with \(0.001\) µg kg\(^{-1}\) of \(E.\ coli\) LPS showed marked febrile responses of both first and second phase.

### Protective antagonistic effect of \(Acidiphilium\) ATCC55963 LPS against toxic \(E.\ coli\) LPS

The structure–activity relationship of LPS or lipid A has revealed basic principles for manifesting the endotoxic properties of LPS. In previous studies, it was found that the new nontoxic ATCC55963 LPS possessed various key unique structures, which implied its low or no toxicity compared with toxic \(E.\ coli\) LPS (Bera \textit{et al.}, 2005). We therefore investigated its possible role as a preventive agent in a mouse model for endotoxemia. From Table 1, it is evident that priming/treating mice with ATCC55963 LPS for \(72\) h assured full protection to the lethal dose (LD\(_{100}\)) of toxic \(E.\ coli\) LPS. Even at a dose of \(300\) µg mouse\(^{-1}\) (1.5 times the lethal dose), \(E.\ coli\) LPS was antagonized when the mouse was primed with \(300\) µg of ATCC55963 LPS. It is also evident that cent percent protection was achieved when ATCC55963 LPS and \(E.\ coli\) LPS were coadministered, provided that the toxic LPS dose was either equal to or less than the nontoxic ATCC55963 LPS dose (Table 2). Protective antagonism was observed throughout the time course followed, except at \(3–6\) h, when ATCC55963 LPS was administered either before or after the challenge dose of toxic \(E.\ coli\) LPS (Table 2).

### Discussion

Previously we reported on the isolation, purification and chemical characterization of a novel LPS from the new \(Acidiphilium\) strain GS18h/ATCC55963; unlike other Gram-negative bacteria, it contains mostly long-chain fatty acids that are either β-hydroxylated or unsaturated (Bera \textit{et al.}, 2005). In galactosamine-sensitized mice, this LPS was nonlethal up to a dose of \(500\) µg mouse\(^{-1}\), which is \(450\) times higher than the lethal dose reported for the extensively studied nontoxic LPS of \(R.\ sphaeroides\) (Strittmatter \textit{et al.}, 1983).

In this study, we have shown that the new \(Acidiphilium\) ATCC55963 LPS activated mouse macrophages RAW 264.7 (a murine macrophage-like cell) and human peripheral blood monocytes with the release of NO, TNF-\(\alpha\), IL-1β and IL-6. Compared with the \(E.\ coli\) LPS, the extent of the release was about four to six times lower, although it was dose-dependent. TNF-\(\alpha\) and IL-1β, potentially important

### Table 1. Protective antagonism of \(Acidiphilium\) ATCC55963 LPS against toxic \(Escherichia\) coli LPS

<table>
<thead>
<tr>
<th>Acidiphilium ATCC55963 LPS (µg/mouse)</th>
<th>(E.\ coli) LPS (µg/mouse)</th>
<th>No. of dead mice/no. of tested mice</th>
<th>Percentage of mice surviving after 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0</td>
<td>0/6</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
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</tr>
<tr>
<td>200</td>
<td>300</td>
<td>6/6</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mice were treated with \(Acidiphilium\) ATCC55963 LPS \(72\) h before toxic \(E.\ coli\) LPS challenge.*
mediators of the septic responses during Gram-negative infections, and IL-6, a pro- and anti-inflammatory cytokine, are highly upregulated during sepsis by toxic LPS. However, Acidiphilium ATCC55963 LPS was effectively less potent in eliciting TNF-α, IL-1β and IL-6 secretion and did not induce septic shock or lethality, as we have observed in galactosamine-sensitized mice (Cerami & Beutler, 1988; Dinarello, 1988).

Acidiphilium ATCC55963 LPS was 1000–10 000 times less potent in lethality or pyrogenicity (Bera et al., 2005), but only four to six times less potent in respect of cytokine release in murine macrophage-like cells and human monocytes when compared with E. coli LPS. We therefore could not identify a strong relationship between the activities of this LPS in terms of Limulus Amebocyte Lysate (LAL) assay and in vitro TNF-α production, unlike the observations made with E. coli LPS. Indeed, our findings are not consistent with the results recently published by Amura et al. (1998), who reported a lack of correlation between in vivo lethality of LPS and in vitro ability to induce TNF-α production. A number of laboratories investigating the events leading to endotoxic shock have pointed to TNF-α as one of the main mediators of the sepsis syndrome (Hesse et al., 1988; Cannon, 1992), although there are a number of studies that suggest strongly that the serum level of TNF-α alone cannot fully explain the lethal effect of LPS (Sanchez-Cantu et al., 1991; Rigato et al., 1996). In this regard, it has been reported that TNF-α serum levels do not correlate with mortality (Amura et al., 1998).

The activity of Acidiphilium LPS was evaluated by studying its protective nature against E. coli LPS in a mouse model. The observation of excellent protection provides strong arguments for its use as an antagonist in the mouse model. Even when the mice were challenged with E. coli LPS at a dose much higher than lethal, they were fully protected if primed earlier or treated within 2 h of challenge with ATCC55963 LPS at a dose either equal to or higher than that of toxic E. coli LPS. The results apparently suggest that ATCC55963 LPS antagonized or interfered in such a way that its antagonistic action was fully expressed if present at a concentration higher than or equal to that of E. coli LPS within the stipulated time period. If priming was carried out 3–6 h before or 2 h after the challenge, there was drastic change in the antagonistic activity. In the case of lipid X (2,3-diacylglucoamine 1-phosphate), a novel monosaccharide precursor of lipid A, the protective action was reported when the mouse was primed 0.5–0 h before toxic challenge (Proctor et al., 1986). The action of LPS expressed on the cell-surface molecule by unloading LPS from the LPS-LPS binding protein (LBP) complex has been proved, so the membrane-bound CD14 (mCD14) has been described as a key cell-surface component for the biological response to toxic LPS (Landmann et al., 1996). Only after 24 h and between 3 and 6 h after administration of toxic E. coli LPS, does the mCD14 level show a decreasing trend (Landmann et al., 1996). In this study, E. coli LPS challenge was counteracted for up to 2 h after the mouse was primed with ATCC55963 LPS. Beyond 3 h, when mCD14 was reported to be downregulated (Landmann et al., 1996), the protection was reduced, as shown in Table 2. Thus the results suggest that mCD14 is possibly involved in the protective/binding interaction of nontoxic LPS during its expression of antagonism, and this indicates the involvement of CD14. The interaction of the LPS CD14 complex with other membrane complex component(s) serves as the transducer of a signal pathway (Lee et al., 1993). All this proves that the macrophage, the well-documented effector cell having mCD14, is possibly the target for interaction with nontoxic LPS. The blockade of the inflammatory response by nontoxic LPS/lipid A has been demonstrated in other in vivo mouse infection models (Kobayashi et al., 1998; Nayak, 1999). In order to study the actual clinical sepsis situation, we studied

<table>
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<tr>
<th>Time of administration</th>
<th>16 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>Percentage of mice surviving</th>
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<td>0/10</td>
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<td>100</td>
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<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>100</td>
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<td>0/10</td>
<td>0/10</td>
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<td>100</td>
</tr>
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<td>2/10</td>
<td>2/10</td>
<td>80</td>
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<td>5/10</td>
<td>8/10</td>
<td>9/10</td>
<td>10</td>
</tr>
<tr>
<td>ATCC55963 LPS treated 12 h before E. coli LPS</td>
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<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>100</td>
</tr>
<tr>
<td>E. coli LPS treated 1 h before ATCC55963 LPS</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
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<td>100</td>
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<tr>
<td>E. coli LPS treated 2 h before ATCC55963 LPS</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
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<tr>
<td>E. coli LPS treated 3 h before ATCC55963 LPS</td>
<td>0/10</td>
<td>0/10</td>
<td>3/10</td>
<td>4/10</td>
<td>60</td>
</tr>
<tr>
<td>E. coli LPS treated 6 h before ATCC55963 LPS</td>
<td>0/10</td>
<td>5/10</td>
<td>8/10</td>
<td>10/10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mice were administered equal amounts (200 μg/mouse) of Acidiphilium ATCC55963 and E. coli LPS. The dose of 200 μg/mouse of E. coli LPS was completely lethal in BALB/c mice (Bera et al., 2005).*
the live *E. coli* infection model. *Escherichia coli* administration with ceftazidime (CAZ), either alone or combined with ATCC55963 LPS, was included to examine the antagonism of this LPS. Although CAZ released endotoxin at a faster rate from bacteria into circulation, much better protection was observed with the combination of CAZ and ATCC55963 LPS (Nayak, 1999).

It has been reported that the LPS from the Gram-negative bacterium *Pantoaea agglomerans* shows a higher macrophage-inducing activity in terms of the production of TNF than does *E. coli* LPS (Tsukioka *et al*., 1997). However, this LPS showed preventive and curative effects for various intractable diseases (ulcers, diabetes, hyperlipidemia), and actually showed antitumor effects without severe side effects when administered intradermally in a clinical study of human patients (Goto *et al*., 1996). It has been proposed that it must be produced in the right place, at the right time, and in an appropriate concentration for beneficial host response (Grivennikov *et al*., 2005), because overproduction results in sepsis syndrome and endotoxic shock. We found that *Acidiphilium* ATCC55963 LPS produced a significant amount of TNF-α but did not exhibit harmful effects under *in vivo* and *in vitro* conditions. The level of serum TNF-α released without harming the host is a good indication of the therapeutic usefulness of *Acidiphilium* ATCC55963 LPS.

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References


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