Bovine dialyzable leukocyte extract protects against LPS-induced, murine endotoxic shock

Moisés A. Franco-Molina, Edgar Mendoza-Gamboa, Leonardo Castillo-León, Reyes S. Tamez-Guerra, Cristina Rodríguez-Padilla*

Laboratorio de Inmunología y Virología, Departamento de Microbiología e Inmunología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Apartado Postal 46 “F”, San Nicolás de los Garza, N.L., México

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Abstract

The pathophysiology of endotoxic shock is characterized by the activation of multiple pro-inflammatory genes and their products which initiate the inflammatory process. Endotoxic shock is a serious condition with high mortality. Bovine dialyzable leukocyte extract (bDLE) is a dialyzate of a heterogeneous mixture of low molecular weight substances released from disintegrated leukocytes of the blood or lymphoid tissue obtained from homogenized bovine spleen. bDLE is clinically effective for a broad spectrum of diseases. To determine whether bDLE improves survival and modulates the expression of pro-inflammatory cytokine genes in LPS-induced, murine endotoxic shock, Balb/C mice were treated with bDLE (1 U) after pretreatment with LPS (17 mg/kg). The bDLE improved survival (90%), suppressed IL-10 and IL-6, and decreased IL-1β, TNF-α, and IL-12p40 mRNA expression; and decreased the production of IL-10 (P<0.01), TNF-α (P<0.01), and IL-6 (P<0.01) in LPS-induced, murine endotoxic shock. Our results demonstrate that bDLE leads to improved survival in LPS-induced endotoxic shock in mice, modulating the pro-inflammatory cytokine gene expression, suggesting that bDLE is an effective therapeutic agent for inflammatory illnesses associated with an unbalanced expression of pro-inflammatory cytokine genes such as in endotoxic shock, rheumatic arthritis and other diseases.

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1. Introduction

Sepsis due to Gram-negative bacterial infection remains a major cause of mortality [14,16]. Invasion of the host with Gram-negative bacteria may lead to a systemic inflammatory syndrome characterized by hypotension, disseminated intravascular coagulation, and renal, hepatic, and cerebral damage [19]. Most
of these deleterious effects can be mimicked by an infusion of endotoxin, the lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria [16,17]. Lethal endotoxemia has been extensively used as an experimental model of Gram-negative septic shock [19]. LPS exerts its profound effect on the host by activating LPS-sensitive cells such as monocytes and endothelial cells to release various cytokines, lipid mediators, and free radicals [14]. The pro-inflammatory cytokines include tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12). These pro-inflammatory cytokines are known to be important mediators in the pathogenesis of lethal septic shock, systemic inflammatory response syndrome, tissue injury, cachexia, hemorrhagic shock, vascular leakage syndrome, transplant rejection and various inflammatory diseases. The anti-inflammatory cytokine, interleukin-10, inhibits pro-inflammatory cytokine production and suppresses their inflammatory reactions [8,9]. The development of therapies directed towards the inhibition of TNF-α production has been an important goal in improving the management of endotoxic shock and TNF-α-induced pathology. Drugs such as JTE-607 [8], pentoxifyline [11], 21-aminosteroids [7], thalidomide [18], and dexamethasone [2] have been shown to inhibit TNF-α production in vivo and in vitro; this inhibition is associated with protection from lethal doses of LPS injected into experimental animals. However, inhibitors of cytokines must be administered with caution because cytokine effects are complex. Removal of cytokines might be more harmful than beneficial because they often permit a compensatory response of the host, with salutary effects. Sublethal injections of TNF-α induce a kind of endotoxin tolerance and protect animals against subsequent administration of LPS. Furthermore, removal of TNF-α and IL-1 leaves the host immunocompromised. This might explain why clinical therapies directed against inflammatory cytokines have been so disappointing [24]. Dialyzable leukocyte extract (DLE) is a dialyze of a heterogeneous mixture of low molecular weight substances released from disintegrated leukocytes of the blood or lymphoid tissue [25]. DLE has ability to transfer specific cellular immunity from an immune donor to a nonimmune recipient [5,10]. Since the discovery of transfer factor by Lawrence over 40 years ago, many clinical reports have established its usefulness as an immuno-modulator in illnesses. Transfer factor contains many molecules; some act in an antigen-specific manner (>5000 Da), whereas smaller molecules (<3500 Da) have been shown to have immune modulating activities. DLE contains molecules with nonspecific adjuvant-like activities such as serotonin, histamine, bradykinin, ascorbate, nicotinamide, cyclic nucleotides, and thymosin α1 [13]. DLE is used clinically as a preventive or therapeutic agent in a variety of cases: immunity disorders, immunosuppressive pharmacological therapies and chronic infections [10,21,25,27].

In this paper, we determine whether or not bDLE improves survival and modulates the expression of pro-inflammatory cytokine genes in LPS-induced endotoxic shock in mice.

2. Materials and methods

2.1. Animals

Male Balb/C mice, 6 weeks old, were obtained from the animal production facility of the Laboratorio de Inmunología y Virología of the Facultad de Ciencias Biológicas de la UANL. The mice were maintained on pelleted food and water ad libitum.

2.2. Bovine dialyzable leukocyte extract

Bovine dialyzable leukocyte extract (bDLE) produced by Laboratorio de Inmunología y Virología of the Facultad de Ciencias Biológicas de la UANL (San Nicolás de los Garza, Nuevo León, México) is a low molecular weight product (10-12 kDa) from bovine spleen, dialyzed, lyophilized, and endogenous pyrogen-free as determined by a Limulus of amoebocyte lysate assay (Endotoxin-Kit-Timed Gel Formation; Sigma, St. Louis, MO, USA), and free of bacteria as determined by the culture of bDLE in different culture media and in vivo inoculation of mice. The bDLE obtained from 1×10^8 leukocytes is defined as one unit (1U) and was dissolved in 0.2 ml of pyrogen-free water and administered via intramuscular injection for each treatment.
2.3. Treatments

Lipopolysaccharide B from E. coli 026:B6 (Sigma) was reconstituted in pyrogen-free water to give a stock concentration of 1 mg/ml and stored at −20 °C until use. LPS at doses from 1 to 17 mg/kg weight per mouse was injected intraperitoneally to demonstrate the dose-response effect of LPS during a 72-h period. Mice in the control group received intramuscular injections of pyrogen-free water. Balb/C mice each received intramuscular injections of bDL (1 U) at the following times: 30 min, and 4, 8, 12, 24, 48, and 72 h (n=10 each group); they were then injected intraperitoneally with LPS (17 mg/kg) and monitored for 72 h. The mice in the LPS group received a pretreatment with LPS (17 mg/kg); thereafter (at 30 min, and at 4, 8, and 12 h), they received intramuscular injections of bDL (1 U) dissolved in 0.2-ml pyrogen-free water and were then monitored for 72 h. After the monitoring period, the animals were killed by cervical dislocation. The spleen and blood were collected. Total RNA was isolated from spleens for RT-PCR of pro-inflammatory cytokines mRNA analysis, and serum was kept at −70 °C for cytokine determination by ELISA.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from mice spleen was prepared using TRIzol reagent (Gibco, Grand Island, NY). Concentration and integrity of RNA were determined by measuring absorbance at 260 nm and analyzed by electrophoresis on 1% agarose gels. For RT-PCR, 1 μg of total RNA was reverse-transcribed using SUPER-SCRIPT™ II RT (Gibco) and oligo (dT)12−18 primer (Gibco). The cDNA (1 μl) obtained was amplified using Taq DNA polymerase (Gibco), and specific primers were used (MPCR: Multiplex PCR Kit for Mouse Sepsis Cytokines Set-2, with GAPDH as internal control, cat. no. MP-70132, Maxim Biotech, San Francisco, CA). These procedures—including 4 min of 94 °C denaturation period, thereafter, 30 cycles of 94 °C for 1 min, 57 °C for 2 min and 72 °C for 2 min followed by final extension at 72 °C for 7 min—were performed using PT-C-200, Peltier Thermal Cycler (MJ Research, Massachusetts, USA) according to the manufacturer’s specifications. The amplified DNA size was 658 bp for GAPDH, 538 bp for IL-10, 453 bp for IL-6, 351 bp for TNF-α, 294 bp for IL-1β, 237 bp for IL-12 p40. The size and amount of PCR products were analyzed by electrophoresis in 2% agarose gels, visualized by ethidium bromide staining with UV light, then photographed and OD quantified using a scanning densitometer. All values were normalized to the constitutive expression of the control gene (GAPDH).

2.5. Cytokine determination

To determine whether bDL modulates the production of pro-inflammatory cytokines in LPS-induced, murine endotoxic shock, Balb/C mice (n=10) were injected intramuscularly with bDL (1 U) at 8 h after pretreatment with the LPS (17 mg/kg, injected intraperitoneally). Twenty hours later, the animals were killed by cervical dislocation, the blood was collected, and the serum was kept at −70 °C for cytokine determination by ELISA. The TNF-α, IL-6, and IL-10 levels were measured with commercial ELISA kits for these cytokines (Biosource International, Camarillo, CA, USA). Kits were used according to manufacturer’s specifications.

2.6. Statistical analysis

The values are presented as mean±standard error (S.D.). All experiments were done three times. Statistical significance was evaluated by ANOVA.

3. Results

3.1. Dose-response effect of LPS on survival

LPS induced lethargy, piloerection, and diarrhea in all the doses tested. The severity of the symptoms and the survival rate were LPS dose-dependent (Fig. 1). LPS at 17 mg/kg induced 100% mortality within 22 h, and it was used to determine the effect of bDL on the survival and expression of pro-inflammatory cytokine genes in LPS-induced, murine endotoxic shock.

The LPS (17 mg/kg) test resulted in 20% morbidity beginning 2 h after its administration, reaching its maximum effect between 4 and 12 h. Mortality was 20% by 16 h and reached 100% by 20–22 h after LPS
administration (Fig. 2). The LPS treatment significantly (P< 0.05) induced morbidity and mortality, respectively (Table 1).

### 3.3. Effect of bDLE on the expression of pro-inflammatory cytokine genes in LPS-induced, murine endotoxic shock

The bDLE modulated the mRNA expression of pro-inflammatory cytokine genes in LPS-induced endotoxic shock murine. The LPS treatment significantly (P<0.05) induced the mRNA expression of the cytokine genes: IL-10 [30 min (8.2-fold), 4 h (8.1-fold), 8 h (8.1-fold), 12 h (8.1-fold), and 20 h (8.2 h)], IL-6 [30 min (11-fold), 4 h (10-fold), 8 h (9.5-fold), 12 h (10-fold)], and increased the mRNA expression of TNF-α [30 min (17.4-fold), 4 h (18.2-fold), 8 h (16.8-fold), and 12 h (9.5-fold)], IL-1β [30 min (18-
fold), 4 h (17.7-fold), 8 h (17.7-fold), 12 h (17.2-fold),
and 20 h (8.2-fold)], and IL-12p40 [30 min (15-fold),
4 h (15.1-fold), 8 h (16.3-fold), 12 h (17-fold), and 20
h (2.2-fold)] after the LPS challenge (Fig. 3). The
bDLE treatment alone induced (P<0.05) the mRNA
expression of IL-10 [30 min (2.2-fold), 4 h (2.8-fold),
8 h (2.3-fold), 12 h (2.3-fold), 24 h (2.4-fold), and 48
h (1.3-fold)] and increased (P< 0.05) the mRNA
expression of TNF-α [4 h (3.7-fold), 8 h (3.2-fold), 12
h (3.2-fold), 24 h (3.2-fold), and 48 h (3.1-fold)] and
IL-12p40 [4 h (3.7-fold), 8 h (3.2-fold), 12 h (3.3-
fold), 24 h (3.3-fold), and 48 h (3.3-fold)]; IL-1β
mRNA expression was not affected (P>0.05) and the
IL-6 mRNA expression was not detected (P<0.05)
(Fig. 4). The bDLE treatment at 8 h after the
pretreatment with LPS modulated the mRNA expres-
sion of the pro-inflammatory cytokine genes; sup-
pressed (P<0.05) IL-10 and IL-6 mRNA expression
and decreased (P<0.05) TNF-α [30 min (2.4-fold),
1.5 h (2.1-fold), 4 h (2.2-fold), 8 h (0.2-fold), 12 h
(0.2-fold), 24 h (0.2-fold), 48 h (0.3-fold), and 72 h
(0.3-fold)], IL-1β [30 min (3.8-fold), 1.5 h (3.9-fold),
4 h (3.5-fold), 8 h (3.3-fold), 12 h (3.3-fold), 24 h
(2.4-fold), 48 h (2.6-fold), and 72 h (0.7-fold)], and
IL-12p40 [30 min (0.7-fold), 1.5 h (3.2-fold), 4 h (3.1-
fold), 8 h (3.5-fold), 12 h (3.2-fold), 24 h (0.8-fold),
48 h (0.7-fold), and 72 h (0.7-fold)] mRNA expres-
sion in all the times evaluated (Fig. 5).

3.4. Effect of bDLE on the production of TNF-α, IL-6,
and IL-10 in LPS-induced endotoxic shock in mice

bDLE modulated the production of pro-inflamm-
atory cytokines in LPS-induced, murine endotoxic

![Image](305x412)

**Fig. 3.** Cytokine gene expression in LPS-induced endotoxic shock. Total RNA was extracted from mouse spleens 30 min, and 4, 8, 12, and 20 h
after LPS treatment (17 mg/kg). RNA was analyzed for the expression of cytokine mRNA (IL-10, IL-6, TNF-α, IL-1β, and IL-12p40) by RT-PCR, as described in Materials and methods. (A) The size and amount of PCR products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining with UV light, and photographed. C=untreated mice. The data represent one of 10 independent experiments (spleens); the 10 experiments having shown similar results. (B) The level of mRNA in the cytokines was quantified by densitometry and normalized to the constitutive expression of the control gene (GAPDH). (C) Mean values of 10 independent experiments (spleens)± S.D. **P<0.01, *P<0.05 compared with untreated control mice.
The cytokine production of TNF-α, IL-6, and IL-10 (P<0.01) was not detected in the control and the bDLE group. The LPS treatment significantly increased the production of IL-6, TNF-α, and IL-10 (P<0.01). The bDLE treatment at 8 h after pretreatment with LPS significantly decreased the cytokine production of IL-6, TNF-α, and IL-10 (P<0.01) (Figs. 6–8).

4. Discussion

The injection of high doses of LPS induces a septic-shock-like state. Sepsis is a complex condition involving a wide variety of local and systemic inflammatory responses [6]. All the LPS test dosages induced morbidity 2 h after being administered to the mice. The severity of the symptoms and rate of mortality were LPS dose-dependent. LPS at 17 mg/kg induced 100% mortality within 22 h (Fig. 1, Table 1); it induced 20% morbidity beginning at 2 h, reaching its maximum effect between 4 and 12 h after its administration (Fig. 2). Victor [26] and Moreira et al. [18] similarly demonstrated that morbidity and mortality in mice were LPS dose-dependent. Pretreatment with bDLE did not affect the morbidity and mortality of the LPS tests, suggesting that bDLE does not prevent the induction of endotoxic shock (Table 1). Lokaj et al. [15] reported similar effects in endotoxic shock patients and other related diseases treated with dialyzable leukocyte extract. The pathophysiological effects of severe sepsis, endotoxic shock and other related syndromes are a consequence of uncontrolled production of inflammatory cytokines. Potential roles for TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p40 have been suggested by the results of in vivo studies.
of bacteremia and septic shock caused by both Gram-positive and Gram-negative bacteria [1,23]. We demonstrated that LPS administration in animal models induced the overexpression of inflammatory cytokines genes IL-10, IL-6, TNF-α, IL-1β, and IL-12p40 (Figs. 7 and 8). Similar results have demonstrated that the primary target cells for LPS are monocytes and macrophages; picogram amounts of endotoxin are sufficient to trigger a massive production of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-8, IL-12, reactive oxygen and nitrogen intermediates, procoagulants, and cell adhesion molecules. In turn, expression of these LPS-responsive factors contributes to collapse of the circulatory system, to disseminated intravascular coagulation, and to a 30% mortality rate. The ability to duplicate many of the pathophysiologic changes associated with septic shock by administration of IL-1β or TNF-α in mice, rabbits, and baboons suggests a central role for these mediators in endotoxin shock [1,8]. Numerous laboratory studies with animal models demonstrate that inhibition of TNF-α or IL-1β protects against the deleterious effects of excessive cytokine activity and significantly improves the survival rate in cases of experimental endotoxic shock [17]. Our results showed that although the posttreatment with bDLE increased the survival of mice with LPS-induced endotoxic shock, the treatments did not affect the morbidity; however, 72 h after the treatment with bDLE, the signs and symptoms of endotoxic shock disappeared. Because the bDLE posttreatment 8 h after injection with LPS induced the highest survival rate (90%), this treatment was chosen for bDLE studies. These results suggest that bDLE decreases mortality induced by LPS (Table 1).
and TNF-α) and decreased the serum cytokine production of IL-6, IL-10, and TNF-α. These results suggest that bDLE modulated the expression and production of pro-inflammatory cytokine genes associated with murine endotoxic shock. These results correlate with the studies reported by Ojeda et al., 1996, where bDLE treatment decreased the TNF-α production in LPS-stimulated human leukocytes in vitro [20]. Various cytokines have been found to inhibit LPS-induced TNF-α production. For instance, IL-10 inhibited TNF-α production both peripherally (when injected systemically) and in the brain (when injected locally) [3]. IL-6 is known to inhibit the synthesis of TNF-α in vivo and in vitro; furthermore, IL-6-deficient mice produced higher serum TNF-α levels when injected with LPS, suggesting that endogenous IL-6 may play a role in down-regulating TNF-α production [4]. The increased morbidity and mortality of neonatal sepsis does not seem to be due to an inadequate IL-10 response, because newborns can produce IL-10 in high quantities during sepsis [22]. IL-10 has many anti-inflammatory and immunosuppressive activities. In mice, IL-10 protects against death during endotoxemia and staphylococcal enterotoxin B (SEB)3-induced shock [12]. However, other studies suggest that nontoxic agents such as AS101, with the capacity to inhibit IL-10 and stimulate macrophage functions, may have clinical potential in

![Fig. 6. Effect of bDLE on the production of TNF-α in LPS-induced endotoxic shock in mice. Animals in control, bDLE, and LPS groups (n=10) were injected intramuscularly or intraperitoneally with either pyrogen-free water, bDLE (1 U), or LPS (17 mg/kg), respectively. Balb/C mice (n=10) were injected intramuscularly with bDLE (1 U) at 8 h after pretreatment with LPS (17 mg/kg) injected intraperitoneally; 20 h later, the animals were killed by cervical dislocation, the blood was collected, and the serum was kept at −70 °C for quantification of cytokines by ELISA. The TNF-α levels were measured with commercial ELISA kits for this cytokine (Biosource International). Kits were used according to manufacturer’s specifications. (A) Mean values of 10 independent experiments (serum) ± S.D. * * P < 0.01, compared with untreated control mice.](image1)

![Fig. 7. Effect of bDLE on the production of IL-6 in LPS-induced endotoxic shock in mice. Animals in control, bDLE, and LPS groups (n=10) were injected intramuscularly or intraperitoneally with either pyrogen-free water, bDLE (1 U), or LPS (17 mg/kg), respectively. Balb/C mice (n=10) were injected intramuscularly with bDLE (1 U) at 8 h after pretreatment with LPS (17 mg/kg) injected intraperitoneally; 20 h later, the animals were killed by cervical dislocation, the blood was collected, and the serum was kept at −70 °C for quantification of cytokines by ELISA. The IL-6 levels were measured with commercial ELISA kits for this cytokine (Biosource International). Kits were used according to manufacturer’s specifications. (A) Mean values of 10 independent experiments (spleens) ± S.D. * * P < 0.01, compared with untreated control mice.](image2)

![Fig. 8. Effect of bDLE on the production of IL-10 in LPS-induced endotoxic shock in mice. Animals in control, bDLE, and LPS groups (n=10) were injected intramuscularly or intraperitoneally with either pyrogen-free water, bDLE (1 U), or LPS (17 mg/kg), respectively. Balb/C mice (n=10) were injected intramuscularly with bDLE (1 U) at 8 h after pretreatment with LPS (17 mg/kg) injected intraperitoneally; 20 h later, the animals were killed by cervical dislocation, the blood was collected, and the serum was kept at −70 °C for quantification of cytokines by ELISA. The IL-10 levels were measured with commercial ELISA kits for this cytokine (Biosource International). Kits were used according to manufacturer’s specifications. (A) Mean values of 10 independent experiments (spleens) ± S.D. * * P < 0.01, compared with untreated control mice.](image3)
the treatment of sepsis, provided they are administered during the phase of sepsis characterized by immune suppression. Septic patients have shown immunosuppression by steroid administration, neutropenia, and genetic and acquired immunologic deficiencies with an inadequate inflammatory response to infection. Regulation of host defense mechanisms may accelerate the elimination of the bacteria and their toxins, decreasing tissue injury. Although we found that the treatment with only bDLE was able to induce the expression of IL-10 and increase the expression of TNF-α and IL-12p40 cytokine, this did not induce any symptomatology associated with disease in the treated mice. Our results demonstrate that bDLE leads to improved survival in LPS-induced endotoxic shock in mice, modulating the expression of pro-inflammatory cytokine genes, and suggesting that bDLE is an effective therapeutic agent for inflammatory illnesses associated with an unbalanced expression of pro-inflammatory cytokine genes such as endotoxic shock, rheumatic arthritis and other diseases.

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