Thalidomide protects mice against LPS-induced shock

A.L. Moreira¹,², J. Wang¹, E.N. Sarno² and G. Kaplan¹

¹Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY, USA
²Setor de Hanseníase, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil

Abstract

Thalidomide has been shown to selectively inhibit TNF-α production in vitro by lipopolysaccharide (LPS)-stimulated monocytes. TNF-α has been shown to play a pivotal role in the pathophysiology of endotoxic shock. Using a mouse model of LPS-induced shock, we investigated the effects of thalidomide on the production of TNF-α and other cytokines and on animal survival. After injection of 100-350 µg LPS into mice, cytokines including TNF-α, IL-6, IL-10, IL-1β, GM-CSF and IFN-γ were measured in the serum. Administration of 200 mg/kg thalidomide to mice before LPS challenge modified the profile of LPS-induced cytokine secretion. Serum TNF-α levels were reduced by 93%, in a dose-dependent manner, and TNF-α mRNA expression in the spleens of mice was reduced by 70%. Serum IL-6 levels were also inhibited by 50%. Thalidomide induced a two-fold increase in serum IL-10 levels. Thalidomide treatment did not interfere with the production of GM-CSF, IL-1β or IFN-γ. The LD₅₀ of LPS in this model was increased by thalidomide pre-treatment from 150 µg to 300 µg in 72 h. Thus, at otherwise lethal doses of LPS, thalidomide treatment was found to protect animals from death.

Key words
- Endotoxic shock
- Thalidomide
- Cytokines
- Inflammation

Introduction

Injection of lipopolysaccharide (LPS) into experimental animals induces a massive production of tumor necrosis factor-α (TNF-α) and other endogenous inflammatory mediators which are associated with pathological manifestations such as fever, anorexia, malaise and shock (1,2). The role of TNF-α in the pathogenesis of endotoxic shock has been extensively investigated. Injection of recombinant TNF-α into experimental animals mimics the pathological alterations found in endotoxic shock (3). Monoclonal antibodies directed against TNF-α prevent lethality in the mouse (4) and in the primate models (5), when given prior to LPS challenge. Further evidence for the role of TNF-α in the pathogenesis of endotoxic shock has emerged from studies with TNF-α receptor knock-out mice, which are unresponsive to TNF-α, and are thereby protected from endotoxic shock (6). Other LPS-induced cytokines, such as interleukin-1 (IL-1) and interferon gamma (IFN-γ), have also been implicated in the pathogenesis of endotoxic shock (2). Administra-
tion of IL-1 receptor antagonist has been shown to abolish lethality in experimental models (7,8). In addition, IFN-γ receptor knock-out mice are resistant to lethal doses of LPS (9). This suggests an interactive cytokine network or cascade of effector molecules contributing to the pathophysiology of endotoxic shock syndrome.

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 pentoxifylline (10,11), hydrazine sulfate (12), and some metalloprotease inhibitors (13) have been shown to inhibit TNF-α production in vitro and in vivo; this inhibition is associated with protection from lethal doses of LPS injected into experimental animals.

Thalidomide, α-N-phthalimidoglutaramide, has been shown to selectively inhibit TNF-α production in vitro in human peripheral blood-derived monocytes stimulated with LPS or mycobacterial agonists (14,15). In the present study we utilized the murine model of endotoxic shock to investigate the effects of thalidomide on LPS-induced cytokine production in vivo and on animal survival after endotoxic shock.

Material and Methods

Mice

Female B6D2/F1 (C57/b6 x DBA2) mice (Jackson Laboratories, Bar Harbor, ME), 7-9 weeks old, were used in the experiments. Mice were housed in group cages at the Animal Research Center, Rockefeller University.

Reagents

Thalidomide was supplied by Celgene Corporation (Warren, NJ). The drug was dissolved in sterile dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) at 50 mg/ml (stock concentration), diluted in acidified pyrogen-free saline to the desired concentration and used immediately. The final concentration of DMSO was 2%. Lipopolysaccharide B from E. coli 026:B6 (Difco Laboratories, Detroit, MI) was reconstituted in saline to give a stock concentration of 1 mg/ml and stored at -20°C until use.

Experimental design

Thalidomide was administered intraperitoneally to the mice at two doses of 200 mg/kg in saline with 2% DMSO (unless otherwise specified) 18 h and 2 h prior to LPS challenge. This regimen of thalidomide administration induced sedation of the mice 30 min after the drug had been injected into the peritoneal cavity. Control animals received injections of saline with 2% DMSO. LPS at doses ranging from 100 to 350 µg per mouse was injected intravenously into a lateral tail vein at the beginning of the experiment to characterize the dose-response effect of LPS. For blood collection by cardiac puncture mice were anesthetized with a solution of ketamine (44 mg/kg) (Aveco Co., Inc., Fort Dodge, IO) and xylazine (5 mg/kg) (Rompum, Mobay Corp., Shawnee, KS). Blood was collected at regular intervals from 15 min to 12 h after LPS injection. Serum was separated and stored at -80°C until assay. Livers and spleens were obtained immediately after cardiac puncture and processed for RNA extraction (see below).

Serum TNF-α level determination

Serum TNF levels were measured by the cytotoxicity assay described by Fisch and Gifford (16). Briefly, the murine fibrosarcoma cell line L929B was used as the target for TNF-induced cytotoxicity. Cells were seeded at 5 x 10^4 cells/well in a 96-well flat bottom plate (Costar, Inc., Cambridge, MA) in RPMI 1640 (Gibco, Grand Island, NY)
supplemented with 5% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA) (culture medium) and incubated overnight at 37°C in a 5% CO₂ humidified incubator. Sequential two-fold dilutions of the sera were made in culture medium containing 2 µg/ml actinomycin-D (Calbiochem-Boehringer Corp., La Jolla, CA). One hundred µl of each dilution was added to wells containing a confluent monolayer of L929B cells. After 18-20 h of incubation, plates were fixed and stained with 0.2% crystal violet in 2% ethanol solution. Plates were washed and analyzed with an ELISA plate reader (Dynatech Laboratories, Chantilly, VA) at 570 nm. TNF titer (U/ml) was defined as the inverse of the dilution that caused 50% destruction of the monolayer. Serum TNF-α levels were also evaluated by ELISA (see below), and the correlation coefficient between the TNF-α levels measured by ELISA or bioassay was r = 0.93 and P = 0.0001.

Cytokine determination

TNF-α, IFN-γ, GM-CSF, IL-6, and IL-10 levels were measured with commercial ELISA kits for these cytokines (Endogen, Inc., Boston, MA). Kits were used according to manufacturer specification.

Northern blots

Total cellular RNA was obtained from 0.1 g each of liver or spleen of LPS-injected mice treated with thalidomide or DMSO. Tissues were homogenized in 2 ml of RNAzol B (Cinna/Biotecx Lab., Inc., Houston, TX) and RNA was extracted according to manufacturer instructions. RNA was size fractionated by agarose/formaldehyde gel electrophoresis and transferred onto a nylon membrane (Biorad, Hercules, CA). Membranes were hybridized as described elsewhere (15) with 32P-labeled random-primed probes specific for murine TNF-α (1.1-kb HindIII fragment), IL-10 (1.34-kb BamHI fragment), IFN-γ (0.7-kb PstI fragment) (ATCC, Rockville, MD) and ß-actin (1.2-kb EcoRI-XhoI fragment) (17). Densitometry was performed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and results are reported as density units.

Statistical analysis

The paired t-test and the Fisher exact p-test were used when appropriate.

Results

Effect of thalidomide on TNF-α production in vivo

LPS was administered intravenously to mice and TNF-α production was induced in a dose-dependent manner. Serum TNF-α levels peaked at 1.5 h after LPS injection and were no longer detectable after 5 h (Figure 1A). There was no subsequent peak of TNF-α, in accordance with previous observations.
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(18). The administration of 100 mg/kg thalidomide 2 h before LPS injection resulted in 60-70% inhibition of TNF-α production (19). When the animals were pre-loaded with thalidomide using two doses of 100 mg/kg thalidomide, 18 h and 2 h before the injection of 100 µg of LPS, the reduction in TNF-α levels reached 83 ± 7%. This effect was improved further by administration of two doses of 200 mg/kg thalidomide, now giving an inhibition of 95 ± 2.3% in TNF-α levels, thus demonstrating a dose-dependent effect (Figure 1A). Thalidomide given together or after the LPS challenge did not affect the production of TNF-α in vivo (data not shown). Regardless of the dose of LPS used there was a marked reduction in the production of TNF-α in mice treated with two doses of 200 mg/kg thalidomide (Figure 1B). However, thalidomide treatment did not inhibit TNF-α production completely.

At 30 min and 1 h after LPS injection, TNF-α mRNA production was detectable in spleens by Northern blot (Figure 2). TNF-α mRNA was also expressed at low levels in the livers (data not shown). No TNF-α mRNA expression was observed in organs of mice that had not been challenged with LPS (data not shown). Pre-treatment with two doses of 200 mg/kg thalidomide resulted in a 70% reduction in TNF-α mRNA levels in the spleens evaluated 30 min after LPS administration. A 65% reduction in TNF-α mRNA levels was observed 1 h after LPS challenge (Figure 2).

**Effect of thalidomide on LPS-induced cytokines in the mouse**

We also evaluated the effect of thalidomide on the production of other LPS-induced inflammatory cytokines. Serum GM-CSF levels peaked at 4 h and returned to baseline levels at 8 h after LPS injection; GM-CSF was not significantly affected by thalidomide treatment (Figure 3). Similarly, IFN-γ levels peaked in the serum at 8 h and were also not inhibited by thalidomide treatment. IL-10 peaked in the serum 1.5 h after the administration of LPS. In the presence of thalidomide, a significant increase in the level of this cytokine was observed (P = 0.02). Although thalidomide treatment did not modify the serum levels of IL-6 1.5 h after LPS injection, when TNF-α inhibition was maximal, a 50% reduction in serum IL-6 levels was observed 4 h after LPS injection in thalidomide-treated mice (Figure 3).

IL-1ß mRNA was detected in the spleens at 30 min after LPS injection, peaked at 1 h and was still detectable in the spleens 4 h after LPS injection. There was no significant inhibition in the expression of IL-1ß mRNA in thalidomide-treated mice (Figure 4). We could not detect mRNA expression for IL-10 and IFN-γ in the spleens of mice injected with LPS, with or without thalidomide treatment.

**LPS-mediated endotoxic shock**

Having demonstrated a significant inhi-
Thalidomide inhibits TNF-α in vivo

bition of TNF-α production by thalidomide, and the additional changes in cytokine production described above, we next evaluated the effects of the drug on the survival of animals following LPS-induced shock. DMSO-treated mice injected with LPS became lethargic, and developed pilo-erection and diarrhea. The severity of symptoms and the extent of survival of mice was dependent on the LPS dose used (Figure 5). Control mice began to die 24-48 h after LPS injection. After 72 h there were no further changes in the lethality curve. At higher doses of LPS, death was recorded as early as 24 h (Figure 5B). The LD₅₀ for LPS in these experiments ranged from 150 to 250 µg LPS per mouse. Mice treated with thalidomide had no diarrhea. Although these animals developed somnolence, they could be more easily aroused than the DMSO-treated control mice. Thalidomide (200 mg/kg) given 18 h and 2 h before the injection of 150 and 200 µg LPS protected mice from death (P = 0.03 for LPS injection of 150 µg/mouse and P = 0.04 for doses of 200 µg/mouse). The individual response of mice to LPS varied widely from experiment to experiment. At higher doses of LPS (250-300 µg/mouse) thalidomide treatment was not protective (P>0.05), but was associated with a delay in death. These results suggest that at the higher doses of LPS thalidomide may not be an
Discussion

Thalidomide was initially developed and used as a sedative without the knowledge of its mechanism of action or teratogenic effects. Recently, the drug has been shown to inhibit the production of TNF-α by reducing the half-life of the TNF-α mRNA in LPS-stimulated human monocytes (15) without directly affecting the production of other cytokines produced by monocytes. Thalidomide is insoluble in water and alcohol, and is very unstable in aqueous solutions. Studies on the pharmacokinetics of thalidomide are not available in the mouse; in a rabbit model, however, treatment of the animals with two or three doses of 150 mg/kg thalidomide improved absorption and accumulation of the drug in the serum (20). Peak serum levels obtained after three injections of 150 mg/kg thalidomide (20) are similar to those obtained in humans treated with thalidomide for graft-versus-host disease (21). In our experiments, the need to use two doses of 200 mg/kg thalidomide to achieve cytokine modulation may reflect the poor solubility and bioavailability of the drug (22). In fact, in our experiments thalidomide crystals were seen in the peritoneal cavity of the animals after injections of the drug. Based on these considerations, the animals were pre-loaded with thalidomide at doses adequate for sedation, thereby indicating effective pharmacological levels.

The present study demonstrates that administration of thalidomide to mice modified the cytokine response to the intravenous injection of LPS. The drug suppressed TNF-α and IL-6 production and enhanced the production of IL-10. However, thalidomide does not affect IL-6 production in LPS-stimulated monocytes in vitro (15,19). Indeed, following treatment with thalidomide, no inhibition of serum IL-6 was observed at 1.5 h, when TNF-α inhibition was maximal. We did observe a 50% reduction in serum IL-6 levels at 4 h after LPS injection. This temporal relationship suggests that the effect of thalidomide on IL-6 may be secondary to the inhibition of TNF-α production. Since it has been shown that TNF-α up-regulates IL-6 production (23), a reduction in the level of TNF-α may account for the lower levels of IL-6 observed at the later time point in our studies. Indeed, in animals treated with antibodies against TNF-α, a reduction in IL-6 and IL-1 production in response to bacteremia has also been observed (23).

Thalidomide-treated mice had a consistent elevation in serum IL-10 levels, although we could not detect IL-10 mRNA in the organs of LPS-injected mice whether or not mice were treated with thalidomide. The inhibition of TNF-α and IL-6 production...
observed in thalidomide-treated mice could be secondary to the enhancement of IL-10 production, since IL-10 has been shown to down-modulate the production of TNF-α and other inflammatory cytokines (24,25). However, in an in vitro system, thalidomide inhibited TNF-α production in the presence of neutralizing antibodies to IL-10, thus confirming that thalidomide has a direct effect on the inhibition of TNF-α production (19). This inverse relationship of TNF-α and IL-10 production in LPS-stimulated macrophages has been previously observed in cells treated with drugs that increase intracellular cAMP levels (26), as well as in mice injected with LPS and treated with cyclosporine in vivo (27). Therefore, the increase in IL-10 levels observed in our experiments may reflect an interference by thalidomide in a regulatory pathway between TNF-α and IL-10. The enhancement of IL-10 production by thalidomide may provide additional benefit in the treatment of inflammatory diseases (28,29).

The inhibition of TNF-α by thalidomide in LPS-challenged mice was also associated with protection from LPS-induced shock and death. This result confirms the importance of TNF-α in the pathophysiology of endotoxic shock. However, the protective effect of thalidomide in preventing death was dependent on the dose of LPS used. Mice could not be protected against very high doses of LPS, despite a measurable inhibition of TNF-α production (Figure 1). Endotoxic shock is multifactorial and the reduction in the levels of TNF-α achieved by the drug may not be sufficient for the prevention of death in every case. Cytokines other than TNF-α such as IL-1, IL-6 and IFN-γ (1,7,9,30,31), and inflammatory mediators such as nitric oxide (31-34) have been shown to contribute to the development of endotoxic shock and death. Higher doses of LPS might induce other pathways independent of TNF-α in the development of shock in this model.

The use of thalidomide as a TNF-α inhibitor is currently being tested in diseases that are associated with TNF-α toxicity such as wasting observed in AIDS (35). Whether thalidomide can be added to the current arsenal of drugs used for the therapy of endotoxic shock requires further investigation.

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