Thalidomide Promotes the Release of Tumor Necrosis Factor- α (TNF- α) and Lethality by Lipopolysaccharide in Mice

Masaaki ISHIKAWA,* Shu-ichi KANNO, Motoaki TAKAYANAGI, Yoshio TAKAYANAGI, and Ken-ichi SASAKI

Department of Pharmacology and Toxicology, Institute of Cancer Research, Tohoku College of Pharmacy, 4-4-1 Komatsu-shima, Aoba-ku, Sendai 981-8558, Japan. Received December 8, 1997; accepted February 7, 1998

We investigated the *in vivo* effects of thalidomide on the production of tumor necrosis factor- α (TNF- α). An *in vivo* systemic release of TNF- α occurred after the injection of lipopolysaccharide (LPS) in male ddY mice, and the TNF- α serum levels reached 652.2 ± 75.7 pg/ml 90 min after the injection of LPS (0.3 mg/kg, i. p.). When thalidomide (1, 3, or 6 mg/kg) was administered intraperitoneally 3 h before the injection of LPS (0.3 mg/kg, i. p.), thalidomide markedly enhanced LPS-induced TNF- α release in a dose-dependent manner. The TNF- α serum levels at 90 min were 640 ± 58.6 , 1985 ± 132.6 , and 2795 ± 203.5 pg/ml, respectively, compared to 628.6 ± 64.4 pg/ml in mice treated with LPS-alone. Pretreatment with a single injection of thalidomide (1, 3, or 6 mg/kg, i. p.) dose-dependently increased the subsequent mortality caused by a challenge with LPS (15 mg/kg, i. p.), a dose that caused death in 10% of the control mice. We conclude that thalidomide enhances *in vivo* TNF- α secretion and the lethality of LPS in mice.

Key words thalidomide; tumor necrosis factor- α (TNF- α); lipopolysaccharide; mouse

Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria, and it contributes to the initiation of the wide variety of pathophysiological responses when injected into experimental animals.¹⁾

Recent studies on the mechanism of LPS action have focused on cytokines such as tumor necrosis factor (TNF)- α . Endotoxin shock is mediated by macrophages and monocytes and contributes to the overproduction of cytokines by LPS. LPS stimulates immune cells to produce proinflammatory cytokines, such as TNF- α , interleukin 1 (IL-1), IL-6, and interferon- γ . The production of these cytokines is necessary for the development endotoxin shock because the administration of TNF- α can induce IL-1 and IL-6, which synergistically induce shock and possibly death.^{2,3)} Neutralizing antibodies to murine TNF protect mice against endotoxin shock.⁴⁾ Inhibitors of TNF- α production might block endotoxin shock.

Thalidomide (*N*-phthalylglutamic acid imide) was used clinically for its central nervous system effects, but its teratogenicity caused its removal from the market in many countries. Thalidomide was subsequently found to have a variety of antiinflammatory and immunosuppressive effects. Thalidomide can inhibit TNF- α production by LPS-induced stimulated human monocyte cultures.⁵⁾ Thalidomide might therefore reduce LPS toxicity *in vivo*. To test this hypothesis, we analyzed the effects of thalidomide administration before an LPS challenge in mice, focusing on the systemic release of TNF- α and lethality.

We demonstrated that thalidomide enhances in a dose-dependent manner the production of TNF- α in mice stimulated with LPS.

MATERIALS AND METHODS

Animals Male ddY mice (Japan SLC, Hamamatsu), 4 weeks old and weighing 20–22 g, were acclimated for 3 d in animal facilities with a 12 h-light/12 h-dark cycle. Mice were

administered LPS from *Escherichia coli* 0127:B8 (Difco Laboratories, Detroit, Michigan, U.S.A.) dissolved in pyrogen-free saline after the administration of thalidomide (Nacalai Tesque, Kyoto, Japan) dissolved in 20% Tween 80 in saline. Serum TNF- α levels were determined at 0.5, 1, 1.5, 2, and 2.5 h after LPS injection. In separate experiments, we evaluated the effects of thalidomide on the lethality induced by a single administration of LPS.

Animals were housed in facilities approved by the Japan Association of Laboratory Animals Care, and the research protocols were approved by the Institutional Animal Care and Use Committee of the Tohoku College of Pharmacy.

TNF- α Assay After decapitation, blood samples were collected at the indicated times after the LPS administration, and were allowed to clot at 4 °C for 2 h; they were then centrifuged at $2500 \times g$ for 15 min at 4 °C. The sample was frozen at –80 °C until being assayed. Serum TNF- α was assayed using a cytokine-specific ELISA (Amersham Life Science) according to the manufacturer's instructions.

Mortality Lethality was determined for 72 h after the intraperitoneal injection of LPS. Control animals were administered the vehicle for thalidomide.

Statistics For the statistical analysis of the results for the time points, Student's *t* test was used, and a *p* value of 0.05 was regarded as significant.

RESULTS AND DISCUSSION

A preliminary study showed that LPS-induced TNF- α production was significantly increased in mice administered thalidomide (6 mg/kg, intraperitoneally 30 min before) compared to those which received saline. Therefore, the time- and dose-dependence of this increase was examined. Mice were challenged with LPS (0.3 mg/kg, i. p.), and serum TNF- α levels were measured at various times by ELISA. The serum from the control ddY mice did not contain detectable TNF- α as determined by ELISA, in which the lower limit of detection was 10 pg/ml, and the injection of LPS caused cir-

* To whom correspondence should be addressed.

Fig. 1. Time-Course of LPS-Induced TNF- α in Mice

Groups ($N=4-6$ in each group) of ddY mice received LPS (0.3 mg/kg, i. p.) 3 h after the injection of thalidomide (6 mg/kg, i. p., ●) or saline (○), and then blood samples were collected at the indicated times. TNF- α levels are the mean $\pm$ S.E. of duplicate results in 4–6 mice. Similar results were obtained in three separate experiments. * $p<0.05$ compared with the amount determined in the LPS alone group.

Fig. 2. Time-Course of Enhanced LPS-Induced Production of TNF- α by Thalidomide in Mice

Groups ($N=4-6$ in each group) of mice received LPS (0.3 mg/kg, i. p.) at 1, 2, 3, 4, or 5 h after the injection of thalidomide (6 mg/kg, i. p.), then blood samples were collected 90 min after LPS. TNF- α levels are the mean $\pm$ S.E. of duplicate results in 4–6 mice. * $p<0.05$ compared with levels found in the LPS alone group.

culating TNF- α . Serum TNF- α was detected at 30 min in mice (mean $\pm$ S.E.: 214.5 ± 23.5 pg/ml), peaked at 90 min (mean $\pm$ S.E.: 759.5 ± 70.3 pg/ml), and returned to baseline background levels at 150 min (Fig. 1). These results are similar to the TNF- α levels that peaked at 90 min after the LPS challenge in mice.^{6,7)}

Thalidomide (6 mg/kg, i. p.) was shown to markedly increase serum TNF- α production 3 h after injection (Fig. 2). The *in vivo* effect of thalidomide on the systemic release of TNF- α was determined in mice pretreated with thalidomide 3 h before the challenge with LPS (0.3 mg/kg, i. p.).

Serum levels of TNF- α increased after the administration of LPS (0.3 mg/kg, i. p.) and peaked at 90 min in the control and thalidomide (6 mg/kg, i. p.)-treated mice (Fig. 1). However, the peak level of TNF- α was significantly higher in the thalidomide-treated mice than in the control mice ($p<0.005$). In addition, the level of TNF- α in the thalidomide-treated mice, which was significantly different from that in control mice, remained for 150 min. TNF- α levels in thalidomide-treated mice were higher than those of the control mice

Fig. 3. Dose-Response of Enhancement of LPS-Induced Production of TNF- α by Thalidomide in Mice

Groups ($N=4-6$ in each group) of mice received LPS (0.3 mg/kg, i. p.) 3 h after the injection of thalidomide (1, 3 and 6 mg/kg, i. p.), then blood samples were collected 90 min after LPS. TNF- α levels are the mean $\pm$ S.E. of duplicate results in 4–6 mice. * $p<0.05$ compared with levels found in the LPS alone group.

Fig. 4. Effect of Thalidomide on LPS-Induced Lethality in Mice

Mice ($N=10$ in each treatment group) were treated as described in the Materials and Methods section. Mouse death was monitored twice daily for 72 h. No lethality was observed between day 3 and 21 after injection.

during the 150 min after injection.

To assess the *in vivo* effects of thalidomide administration on LPS-induced TNF- α release, three doses of thalidomide (1, 3, or 6 mg/kg) were administered intraperitoneally 3 h before the intraperitoneal injection of LPS (0.3 mg/kg) (Fig. 3). Because previous studies showed that TNF- α levels peaked 90 min after the LPS challenge, blood samples were taken at that time. Thalidomide pretreatment increased the amount of TNF- α released in the circulation after LPS challenge, as shown in Fig. 3. To evaluate the *in vivo* effect of thalidomide on LPS-induced toxicity, we monitored acute toxicity for 72 h after the challenge with LPS (15 mg/kg, i. p.), a dose which is lethal within 72 h in 10% of the animals (Fig. 4). As has been previously reported, LPS was markedly lethal, which might have been dependent on TNF- α release.⁶⁻⁸⁾ Mice pretreated with thalidomide (1, 3 or 6 mg/kg, i. p.) died after LPS injection in a dose-dependent manner (Fig. 4). These results indicate that pretreatment with thalidomide augments the toxicity of LPS in mice. The increasing adverse effect of LPS by thalidomide could be caused by TNF- α re-

lease. *In vitro* results suggest that thalidomide might enhance the release of monocyte/macrophage-derived cytokines involved in the pathogenesis of septic shock, especially TNF- α .^{5,7,8)}

We found that thalidomide enhances LPS-induced TNF- α production in a dose-dependent manner in mice, although LPS inhibits TNF- α production by human monocytes and the U1 cell line (human leukemia cell line U937 infected with human immunodeficiency virus type I (HIV-1), which are stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or cytokine agonists such as interleukins and LPS.⁹⁻¹¹⁾ Furthermore, thalidomide had been reported to be an inhibitor of TNF- α production in rats by Schmidt *et al.*¹²⁾ The difference between our results and those of Kaplan *et al.*⁹⁾ and Schmidt *et al.*¹²⁾ remain unknown. These discrepancies in the results may be due to the use of different animals or cell type-specificity of the action of thalidomide. Studies on the molecular mechanisms of the effects of thalidomide, TPA, and cytokine agonists are needed, and would contribute to the development of inhibitors of TNF- α production. Our

present results do not support the hypothesis that thalidomide inhibits TNF- α production.

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