Thalidomide Exerts Its Inhibitory Action on Tumor Necrosis Factor α by Enhancing mRNA Degradation

By Andre L. Moreira,* Elizabeth P. Sampaio,* Antonina Zmuidzinas,† Paula Frindt,* Kendall A. Smith,* and Gilla Kaplan*

From the *Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021; the †Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire 03755; and the ‡Leprosy Unit, Oswaldo Cruz Foundation, Rio de Janeiro 21045, Brazil

Summary

We have examined the mechanism of thalidomide inhibition of lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α) production and found that the drug enhances the degradation of TNF-α mRNA. Thus, the half-life of the molecule was reduced from ~30 to ~17 min in the presence of 50 μg/ml of thalidomide. Inhibition of TNF-α production was selective, as other LPS-induced monocyte cytokines were unaffected. Pentoxifylline and dexamethasone, two other inhibitors of TNF-α production, are known to exert their effects by means of different mechanisms, suggesting that the three agents inhibit TNF-α synthesis at distinct points of the cytokine biosynthetic pathway. These observations provide an explanation for the synergistic effects of these drugs. The selective inhibition of TNF-α production makes thalidomide an ideal candidate for the treatment of inflammatory conditions where TNF-α-induced toxicities are observed and where immunity must remain intact.

Thalidomide (α-N-phthalimidoglutarimide) is the drug of choice for the treatment of erythema nodosum leprosum (ENL), an acute inflammatory complication occurring in ~30% of lepromatous leprosy patients, usually in association with initiation of multidrug therapy (MDT). TNF-α seems to play a critical role in the pathogenesis of ENL. Serum levels of this cytokine have been reported to be elevated during ENL episodes, either induced by regular MDT (1), or in a combination of MDT and low-dose IFN-γ (1-3). Therapy with thalidomide promptly reduces the toxic symptoms associated with ENL, including fever, anorexia, and arthralgia, as well as the painful subcutaneous nodules. Thalidomide also decreases serum TNF-α levels in ENL patients, and inhibits the high levels of TNF-α produced in vitro by monocytes obtained from patients during ENL (2). Despite these obvious antiinflammatory actions of thalidomide, there have been no clinical indications that thalidomide reduces any cellular or humoral immune reaction in these patients.

In initial experiments to investigate the relationship between thalidomide and TNF-α, we found that thalidomide selectively inhibits the production of TNF-α by human monocytes stimulated in vitro with both LPS and mycobacterial products, without influencing the synthesis of other LPS-induced monocyte cytokines (3). In the present report, we examine the action of thalidomide as well as two other known TNF-α inhibitors. We found that thalidomide inhibits TNF-α production by enhancing the degradation of TNF-α mRNA. By comparison, pentoxifylline, a methylxanthine derivative, reduces TNF-α mRNA accumulation by inhibiting transcription of the TNF-α gene (4), while the glucocorticoid, dexamethasone, inhibits TNF-α production primarily posttranscriptionally by reducing translation (5). Our results provide an explanation for the selective effect of thalidomide on TNF-α production compared with the other monocyte cytokines.

Materials and Methods

Monocyte Isolation. PBMC from normal donors were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. Monocytes were enriched by incubation of PBMC with neuraminidase-treated (Vibrio cholerae neuraminidase; Calbiochem-Behring Corp., La Jolla, CA) sheep erythrocytes (Scott Laboratories, Friskvikki, RI) for 1 h on ice and separation by centrifugation over a Ficoll-Hypaque gradient from the rosetted population (E- cells). Cells were cultured at 37°C in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% AB* serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Adherent E- cells (monocytes) were used for the studies.

Cytokine Induction. LPS of Salmonella minnesota R595 (List Biological Laboratories, Campbell, CA) was diluted in PBS, pH 7.4, 1675 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/06/1675/06 $2.00
Volume 177 June 1993 1675-1680

1 Abbreviations used in this paper: ENL, erythema nodosum leprosum; MDT, multidrug therapy.
and used at 1 μg/ml. The concentration of the stimulating agent was determined in previous experiments to induce optimal TNF-α protein production by cultured monocytes. Monocytes were stimulated with LPS for 24 h unless otherwise specified. Culture supernatants were harvested, centrifuged to remove cells and debris, and kept frozen (−20°C) until TNF-α and other cytokine evaluations.

**Cytokine Inhibition.** Thalidomide (racemic mixture - D[+] and L[−] forms; lot no. 1055/8) was donated by Grunenthal, GBMH (Stolberg, Germany). Pentoxifylline and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were diluted in DMSO (Sigma Chemical Co.) and further dilutions were done in sterile RPMI. Final concentration of DMSO in all assays was 0.5%.

Percentage inhibition of cytokine was calculated as: 100 × [1−(cytokine experimental/cytokine control)]

where cytokine experimental represents cytokine secretion by stimulated monocytes that were cultured in the presence of thalidomide and 0.5% DMSO, and cytokine control represents cytokine secretion by stimulated monocytes that were cultured in 0.5% DMSO in the absence of the drug. Monocytes cultured in medium containing equivalent amounts of DMSO in the presence or absence of the stimulating agent were used as controls. Neither thalidomide nor DMSO had any effect on cell viability or function at the concentrations used (not shown).

**Cytokine Determination.** Commercial ELISA kits were used according to the manufacturer's specifications to determine the amount of TNF-α, GM-CSF (Endogen Inc., Boston, MA), IL-6 (Genzyme Corp., Cambridge, MA), and IL-1β (Cistron, Pine Brook, NJ) proteins in culture medium. Cytokine levels are expressed as picograms per milliliter.

**Northern Blot Hybridization.** Total cellular RNA from stimulated monocytes was isolated using the guanidine thiocyanate-phenol-chloroform method and quantified by absorbance at 260 nm. RNA was size fractionated by formaldehyde/agarose gel electrophoresis and transferred to nylon membranes (Bio-Rad Laboratories, Richmond, CA) in the presence of 10× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate). The membranes were crosslinked by UV radiation and then treated overnight at 42°C with prehybridization solution (50% formamide, 5× SSC, 5× Denhardt’s solution, 0.02 M NaHPO4, pH 6.5, 100 μg/ml of heat-denatured sheared salmon sperm DNA, and 10% dextran sulfate). This solution was then replaced with one containing 105 cpm/ml of heat-denatured 32P-labeled cDNA random-primer (U.S. Biochemical Corp., Cleveland, OH) probe for IL-1β (0.6-kb BamHI + SmaI fragment; American Type Culture Collection, Rockville, MD), TNF-α (1.3-kb PstI fragment; gift of Dr. A. Cerami), and β-actin (1.2-kb EcoRI-Xhol fragment) (6). The filters were hybridized at 42°C overnight. The membranes were washed twice for 15 min at room temperature with 2× SSC and 0.1% SDS, 30 min at room temperature with 0.1× SSC and 0.1% SDS, and 30 min at 60°C with 0.1× SSC and 0.1% SDS. After air drying, the blots were exposed to x-ray film for 12-24 h at −70°C and developed in a Kodak X-Omat processor stand. Densitometry was performed using a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA), and results are expressed as density units.

**mRNA Degradation Assay.** Monocytes were stimulated with LPS (1 μg/ml) for 2 h. Cells were washed twice in RPMI and further incubated in medium supplemented with actinomycin-D (5 μg/ml) (Calbiochem-Behring Corp.). After 30-60 min, cells were washed and lysed for RNA extraction. Thalidomide (50 μg/ml), pentoxifylline (30 μg/ml), and dexamethasone (100 μM/ml) were added together with actinomycin-D-supplemented medium. The degradation of TNF-α mRNA in the drug-treated groups was compared with RNA from cells treated only with actinomycin-D. In some experiments LPS-stimulated monocytes were cultured in the presence of cycloheximide (10 μg/ml) (Sigma Chemical Co.) or cycloheximide plus actinomycin-D for 1 h and then processed for RNA extraction. The decay of TNF-α mRNA was analyzed by Northern blot hybridization and quantitated as described above.

**Results**

**Comparison of the Effect of Thalidomide, Pentoxifylline, and Dexamethasone on Monocyte Cytokine Production.** Although pentoxifylline and dexamethasone are known to decrease TNF-α production, their effects on other monocyte cytokines have not been explored in detail. Therefore, thalidomide was first compared with these agents for suppressive effects on the secretion of several cytokines, including IL-1β, IL-6, and GM-CSF, in addition to TNF-α. As reported previously, thalidomide inhibited TNF-α release from LPS-stimulated monocytes in a concentration-dependent fashion without influencing the production of IL-1β and IL-6. GM-CSF appeared not to consistently be inhibited (Fig. 1). By comparison, pentoxifylline inhibited TNF-α and GM-CSF production, but had no effect on IL-1β or IL-6 release. Dexamethasone was broadly suppressive, inhibiting the production of all four cytokines tested.

Given that the three agents displayed different patterns of inhibition of monocyte cytokines, suggesting possible differences in mechanism, we next tested them for synergism of TNF-α suppression. Concentrations of pentoxifylline and dexamethasone that resulted in ~25% inhibition (IC50) were combined with varying concentrations of thalidomide. As shown in Fig. 2, the combination of thalidomide with pentoxifylline or dexamethasone resulted in synergistic inhibition of TNF-α production in both instances.

**TNF-α mRNA Expression.** In initial experiments thalidomide was found to suppress TNF-α mRNA expression, when assayed 6 h after stimulation with LPS. Since pentoxifylline was reported to inhibit TNF-α transcription, additional experiments were performed, comparing thalidomide and pentoxifylline. As shown in Fig. 3, thalidomide and pentoxifylline yielded comparable inhibition of TNF-α mRNA expression measured 6 h after LPS stimulation. As already reported by Han et al. (5), dexamethasone does not affect mRNA level for TNF-α.

Previous reports indicated that TNF-α mRNA expression peaks within 3 h of stimulation and declines thereafter (7). Therefore, a kinetic study of thalidomide inhibition of TNF-α mRNA levels was performed as shown in Fig. 4. There was little, if any, inhibition of mRNA expression at 3 h, but at later time intervals the suppressive effect of thalidomide became apparent. When the amount of mRNA present after 12 h of LPS stimulation was compared with the secreted TNF-α protein, the results were remarkably similar: thalidomide suppressed mRNA by 50% (Fig. 4) and TNF-α protein by 43% (not shown).

**The Effect of Thalidomide on TNF-α mRNA Degradation.** The lack of a discernible effect of thalidomide on TNF-α mRNA expression at early time intervals after LPS stimula-
Figure 1. Effect of thalidomide, pentoxifylline, and dexamethasone on LPS-induced cytokine production by human monocytes. Monocytes were stimulated with LPS (1 μg/ml) for 20 h. Drugs were added to the cells at the same time as the agonist. Cytokine release into the supernatant was assayed by ELISA. (Thal) thalidomide; (Pent) pentoxifylline; (Dex) dexamethasone.

tion, but clear effects at later time intervals, pointed towards a posttranscription level of control. Therefore, the effect of thalidomide on TNF-α mRNA stability was examined and compared with that of pentoxifylline and dexamethasone. As shown in Fig. 5, thalidomide accelerated the degradation of TNF-α mRNA twofold, such that the half-life (t½) decreased from 30 to 17 min. In contrast, pentoxifylline and dexamethasone had no effect on TNF-α mRNA stability. Degradation of TNF-α mRNA in LPS-stimulated monocytes was also evaluated in the presence of cycloheximide, a protein synthesis inhibitor. A superinduction of TNF-α mRNA was observed (8-10). In the presence of cyclohexi-
mide and thalidomide, there was less TNF-α mRNA, when compared with cycloheximide alone (not shown). When both cycloheximide and actinomycin-D were added to the cells, the presence of thalidomide still accelerated the decay of TNF-α mRNA, suggesting that thalidomide requires neither new mRNA synthesis nor protein synthesis to reduce TNF-α levels (Fig. 6).

Discussion
The results of these experiments indicate that thalidomide suppresses TNF-α production primarily by accelerating the degradation of TNF-α mRNA transcripts. Compared with two other known inhibitors of TNF-α production, the glucocorticoids dexamethasone and the methylxanthine derivative pentoxifylline, thalidomide is unique in its mechanism of action and its selectivity. Thus, our data suggest that the three TNF-α inhibitors affect TNF-α synthesis at distinct points of the cytokine biosynthesis pathways, providing a strong rationale for synergistic use of the drugs in patients.

For these experiments bacterial LPS was used as the agonist to promote monocyte TNF-α production. After stimulation by LPS, a marked increase in TNF-α gene transcription occurs, resulting in a rapid accumulation of mRNA (7). However, TNF-α production is also regulated at the level of mRNA, in that the transcripts are very short-lived, with a t1/2 of only 30 min (7). In addition, TNF-α translation has also been shown to be promoted by LPS stimulation, primarily by derepression of translational control (11). By accelerating the decay of TNF-α transcripts, thalidomide appears to have a marked regulatory influence on the total amount of TNF-α...
Figure 5. Effect of thalidomide on TNF-α mRNA degradation. Cells were stimulated with LPS for 2 h when actinomycin-D and thalidomide (50 μg/ml) were added to the culture. RNA extraction was performed 30 and 60 min after the addition of actinomycin-D. The decay of TNF-α mRNA was analyzed by Northern blot hybridization. The results are expressed as percentage of density units of TNF-α, normalized to β-actin for each lane evaluated by phosphorimaging exposed for 18 h for both mRNAs on the same blot. Cells were cultured with actinomycin-D only (○) or with actinomycin-D and thalidomide (●), pentoxifylline (▲), or dexamethasone (■). Results for actinomycin-D only and actinomycin-D and thalidomide are means ± SD of four and two experiments, respectively.

The selective inhibition of TNF-α, but not other cytokines, depends on different regulatory pathways for the different monocyte cytokines tested. The mechanisms by which LPS promotes the production of the other cytokines have been shown to differ from that of TNF-α, in minor but apparently important ways. For example, LPS stimulates IL-1 gene transcription but has no detectable effect on mRNA stability or rate of translation of IL-1 mRNA (12). By comparison, LPS promotes GM-CSF production by stabilizing the labile GM-CSF mRNA transcripts (13). Our results indicate that mRNA degradation of the other cytokines is also regulated differently from that of TNF-α and therefore not affected by thalidomide. Selective mRNA degradation or stabilization has been shown to be an important way for the cell to retain or downregulate the activity of a gene (14–16). Lindstein et al. (17) described that stimulation of the T cell by CD28 surface molecule leads specifically to stability of mRNA for IL-2, IFN-γ, TNF-α, and GM-CSF, while mRNA half-life for c-myc, c-fos, and IL-2 receptor is not affected.

The selective inhibition of TNF-α in the absence of general inhibition of other monocyte cytokines makes thalidomide an ideal candidate for the treatment of inflammatory conditions where immunity must remain intact. This selective inhibition of TNF-α does not mean that the drug does not impact on other inflammatory cytokines. Since TNF-α is known to modulate synthesis of other cytokines and other inflammatory molecules (18), the inhibition of TNF-α could ultimately lead to a reduction in other inflammatory mediators, including IL-1β, IL-6, IL-8, and GM-CSF. Thus, thalidomide could be used in acute infections such as meningitis or chronic infections such as tuberculosis, AIDS, and parasitic disease in order to overcome the toxic manifestations of TNF-α without compromising the host’s cellular immune response. Since pentoxifylline inhibits TNF-α production by suppressing transcription, one would anticipate that it would synergize with thalidomide, as well as synergizing with dexamethasone. Pentoxifylline is not immunosuppressive, suggesting that long-term treatment of patients with the combination of thalidomide and pentoxifylline may be better than long-term use of dexamethasone. This is especially true in diseases such tuberculosis and AIDS, where an intact cellular
immune response is crucial for disease control. Our results also suggest that a reduction in TNF-α production can be achieved by both isomers of thalidomide. It is not clear at this time whether racemization occurs between the two enantiomers. However, if racemization does not occur, this observation is specially important since the optical D(+) form of this drug has been reported not to be teratogenic (19).

We thank Judy Adams for help with the graphics and Susanne Dennehy for skillful technical help.

These studies were supported in part by U.S. Public Health Service grants AI-22616 and AI-33124, and by Celgene Corporation. A. L. Moreira and E. P. Sampaio are Villares Fellows.

Address correspondence to Gilla Kaplan, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. E. P. Sampaio’s present address is Oswaldo Cruz Foundation, Leprosy Unit, AV. Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil, 21045.

Received for publication 12 January 1993 and in revised form 10 March 1993.

References

1. Sarno, E.N., G.E. Grau, L.M.M. Vieira, and A.C. Nery. 1991. Serum levels of TNF-α and IL-1β during leprosy reactional states. Clin. Exp. Immunol. 84:103.
2. Sampaio, E.P., A.L. Moreira, E.N. Sarno, A.M. Malta, and G. Kaplan. 1992. Prolonged treatment with recombinant interferon γ induces erythema nodosum leprosum in lepromatous leprosy patients. J. Exp. Med. 175:1729.
3. Sampaio, E.P., E.N. Sarno, R. Galilly, Z.A. Cohn, and G. Kaplan. 1991. Thalidomide selectively inhibits tumor necrosis factor α production by stimulated human monocytes. J. Exp. Med. 173:699.
4. Doherty, G.M., J.C. Jensen, H.R. Alexander, C.M. Buresh, and J.A. Norton. 1991. Pentoxifylline suppression of tumor necrosis factor gene transcription. Surgery (St. Louis). 110:192.
5. Han, J., P. Thompson, and B. Beutler. 1990. Dexamethasone and pentoxifylline inhibit endotoxin induced cachectin/tumor necrosis factor synthesis at separate points in the signaling pathway. J. Exp. Med. 172:391.
6. Gunning, P., Ponte, H. Okayama, J. Engles, H. Blu, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α-β and γ- actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cystein that is subsequently removed. Mol. Cell. Biol. 3:787.
7. Beutler, B., and A. Cerami. 1988. The common mediator of shock, cachexia, and tumor necrosis. Adv. Immunol. 42:213.
8. Sen, R., and D. Baltimore. 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell. 47:921.
9. Sariban, E., K. Imamura, R. Luelbers, and D. Kufe. 1988. Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. J. Clin. Invest. 81:1506.
10. Zuckerman, S.H., G.F. Evans, and L. Guthrie. 1991. transcriptional and post-transcriptional mechanisms involved in the differential expression of LPS-induced IL-1 and TNF mRNA. Immunology 73:460.
11. Han, J., T. Brown, and B. Beutler. 1990. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J. Exp. Med. 171:465.
12. Sung, S.S., and J.A. Walters. 1991. Increased cyclic AMP levels enhance IL-1 alpha and IL-1 beta mRNA expression and protein production in human myelomonocytic cell lines and monocytes. J. Clin. Invest. 88:1915.
13. Thorens, B., J.J. Mermod, and P. Vassali. 1987. Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through posttranscriptional regulation. Cell. 48:671.
14. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell. 46:659.
15. Beulter, B., N. Krochin, I.W. Mitsark, C. Luedke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. Science (Wash. DC). 232:977.
16. Han, J., G. Huez, and B. Beutler. 1990. Interactive effects of the tumor necrosis factor promoter and 3'-untranslated region. J. Immunol. 146:1843.
17. Lindstein, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science (Wash. DC). 244:339.
18. Fong, Y., K.J. Tracey, and L.L. Maldawer et al. 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. 170:1627.
19. Blaschke, G. 1986. Chromatographic resolution of chiral drugs on polyamides and cellulose triacetate. J. Liq. Chromatogr. 9:341.