Selection of Novel Analogs of Thalidomide with Enhanced Tumor Necrosis Factor α Inhibitory Activity

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ABSTRACT

Background: Tumor necrosis factor α (TNFα) is thought to mediate both protective and detrimental manifestations of the inflammatory response. Recently, thalidomide (α-N-phthalimidoglutarimide) was shown to partially inhibit monocyte TNFα production (by 50–70%) both in vivo and in vitro. More efficient inhibition of TNFα may, however, be necessary to rescue the host from more acute and extensive toxicities of TNFα-mediated inflammation.

Materials and Methods: Three structural analogues of thalidomide were selected for study based on increased activity against TNFα production. The parent drug and the analogs were tested in vitro in human peripheral blood mononuclear cell cultures for their effects on lipopolysaccharide (LPS) induced cytokine protein and mRNA production using ELISAs and Northern blot hybridization. The in vitro effects of the drugs were then confirmed in vivo in a mouse model of LPS induced lethality.

Results: The new compounds (two esters and one amide) showed increased inhibition of TNFα production by LPS-stimulated human monocytes, relative to the parent drug thalidomide. The analogs and the parent drug enhanced the production of interleukin 10 (IL-10), but had little effect on IL-6 and IL-1β protein and mRNA production. When tested in vivo, the amide analog protected 80% of LPS-treated mice against death from endotoxin induced shock.

Conclusions: Analogs of thalidomide designed to better inhibit TNFα production in vitro have correspondingly greater efficacy in vivo. These finding may have therapeutic implication for the treatment of human diseases characterized by acute and extensive TNFα production such as tuberculous meningitis or toxic shock.

INTRODUCTION

Thalidomide was initially used as a sedative without knowledge of its mechanism of action, its immunoregulatory properties, or its teratogenic activity (1). Recent studies have shown that, in addition to its other known effects, thalidomide is an inhibitor of tumor necrosis factor α (TNFα) production by monocytes in vitro (2,3). When leprosy patients with erythema nodosum leprosum (ENL) are treated with thalidomide, there is an inhibition of TNFα production in vivo as well as a delayed reduction in interferon-γ (IFNγ) serum levels (4). In patients with tuberculosis, thalidomide treatment lowers TNFα protein and TNFα mRNA production and also reduces the levels of interleukin 1β (IL-1β) mRNA in peripheral blood mononuclear cells (5). Thalidomide appears to inhibit TNFα production by enhancing the degradation of TNFα mRNA (3). However, thalidomide inhibits only 50–70% of the TNFα mRNA and TNFα protein produced by monocytes. This may be in part attributed to the instability of the drug in aqueous solution. Thalidomide undergoes rapid degradation at physiological pH (6) through the hydrolysis of the glutarimide ring to generate either a N-phthaloyl substituted glycinamide or a N-phthaloyl substituted γ-aminobutamide (7).

Although TNFα production is important in the host defense against infection, the accompa-

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nying toxicities associated with increasing levels of TNFα and other cytokines may lead to serious pathology in the host. For example in experimental models of endotoxin induced shock, TNFα production as well as the generation of other pro-inflammatory cytokines has been shown to be associated with the rapid onset of multiorgan failure and death. Reversal of shock is dependent on, among other interventions, complete inhibition of production of cytokines such as TNFα (8,9). Selective inhibitors of TNFα would therefore be useful in this situation. Since the efficiency of thalidomide in inhibition of TNFα production may depend on its stability in aqueous solution, we designed and synthesized a series of thalidomide analogs with improved activity and stability (10). We then selected three of the structural analogs which had superior TNFα inhibitory activity in preliminary experiments. These compounds contained the phthaloyl ring in which the glutarimide moiety was replaced. The resulting analogs, one amide and two esters, were evaluated for their ability to inhibit and or increase monocyte cytokine production. The analogs were also compared with thalidomide in respect to cytokine mRNA accumulation in lipopolysaccharide (LPS)–stimulated human monocytes in vitro and evaluated in vivo for efficacy in a mouse model of LPS-induced death.

MATERIALS AND METHODS

Monocyte Isolation

Peripheral blood mononuclear cells (PBMC) from normal donors were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) density centrifugation (2). Monocytes were enriched by incubation of PBMC with neuraminidase-treated (*Vibrio cholera* neuraminidase; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) sheep erythrocytes (Cocalico Biologicals, Reamstown, PA, U.S.A.) for 1 hr at 4°C and separated by Ficoll gradient centrifugation. Cells (10^6 cells/ml) were cultured in RPMI (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10 AB^+ serum (Biocell, Rancho Dominguez, CA, U.S.A.), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco).

In Vitro Cytokine Induction

Monocytes were stimulated with 1 μg/ml of LPS prepared from *Salmonella minnesota* R595 as previously described (2) (List Biological Labs, Campbell, CA, U.S.A.). Triplicate cultures were incubated with LPS for 18–20 hr at 37°C in 5% CO_2. Supernatants were then harvested and assayed for cytokines levels. In some experiments, supernatants were kept frozen at −70°C until assay.

In Vitro Cytokine Inhibition

Thalidomide and analogs were synthesized (Celgene Corporation, Warren, NJ, U.S.A.) as described (10). The drugs were dissolved in DMSO (Sigma Chemical, St Louis, MO, U.S.A.); further dilutions were done in culture medium as previously described (3). The final DMSO concentration in all assays was 0.25%. Drugs were added to cell 1 hr before LPS stimulation. The toxicity of the compounds for the cells was assayed by trypan blue exclusion dye method, immediately after harvesting of the culture supernatant.

Cytokine Determination

The concentration of the cytokines TNFα, IL-1β, IL-6, granulocyte macrophage-colony–stimulating factor (GM-CSF), and IL-10 in culture supernatants was determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, MA, U.S.A.) according to the manufacturer's directions. The concentration of the cytokines TNFα and IL-10 in mouse serum was determined similarly by commercial ELISA kits (Endogen). Cytokine levels are expressed as ng/ml. Percentage inhibition was determined as $100 \times [1 - (\text{cytokine experimental/cytokine control})]$.

Northern Blot Hybridization

Cells were treated with thalidomide or analogs and stimulated with LPS for 6 hr, and total cellular RNA was extracted using RNAzol (Cina/Biotecx Lab. Inc., Houston, TX, U.S.A.). RNA was size fractionated by electrophoresis in 1% formaldehyde/agarose gel. RNA was transferred overnight onto nylon membranes (BioRad Labs., Richmond, CA, U.S.A.). Membranes were hybridized for TNFα (1.1-kb *Pst*I fragment), IL-1β (0.6-kb *Bam*HI + *Sma*I fragment), IL-6 (1.0-kb

EcoRI fragment), and β-actin (1.4-kb *Eco*RI/*Xho*I fragment) (ATCC, Rockville, MD, U.S.A.). Filters were washed as previously described (3). Blots were exposed to X-ray film for 2 to 24 hr at −70°C. Densitometry of the Northern blots were done using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The density units for the cytokines mRNA were normalized to the density for β-actin mRNA.

Anti–IL-10 Treatment

Human monocytes were stimulated with LPS (1 μg/ml) for 18–20 hr in culture in the presence of anti–IL-10 neutralizing antibody (10 μg/ml) or control IgG (10 μg/ml) (Endogen). Thalidomide or thalidomide analogs were added at varying concentrations 1 hr prior to LPS stimulation of the cells. Supernatants were harvested and assayed for TNFα levels by ELISA as described above.

Mice

Female BALB/c mice, 7–9 weeks old, weighing 18–20 g, were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.) and housed under pathogen-free conditions.

Drug Stability in Human Plasma

Human plasma (Sigma) was spiked with thalidomide or analogs and kept at 37°C. Samples were taken every 30 min, and the levels of the drugs were determined by HPLC as described (11).

In Vivo Cytokine Induction

Cytokines production in the circulation of mice was induced by administration of *Salmonella abortus equi*–derived LPS (Sigma) at 5 mg/kg. LPS was injected intraperitoneally (i.p.) in a volume of 200 μl of PBS/mouse. Blood was collected by cardiac puncture under anesthesia 1.5 hr after LPS injection. Blood samples were allowed to clot at room temperature for 2 hr and centrifuged. Sera were collected, immediately frozen, and kept at −70°C. Cytokine levels in serum were determined by ELISA as described above.

In Vivo Cytokine Inhibition

Thalidomide and analogs were suspended in sterile saline and administered by i.p. injection (0.5 ml/mouse) 2 hours before LPS challenge. Control mice were injected with the same volume of vehicle.

LPS-Induced Death and Protection Studies

For the lethality experiments, an LD_{100} dose of LPS (7.5 mg/kg) was injected i.p. in a volume of 200 μl PBS. For the protection studies, drugs were given i.p. at a single dose of 100 mg/kg in 0.5 ml of saline, 2 hr before LPS injection. Survival of treated mice was assessed two to three times a day. A minimum of 10 animals per group was used in each experiment.

Statistical Method

Student's *t* test was used to determine statistical significance and *p* values of <0.05 were considered significant.

RESULTS

Selection of Thalidomide Analogs with Enhanced Capacity to Inhibit TNFα Production in Vitro

Thalidomide has been shown in previous studies to partially inhibit TNFα production by peripheral blood monocytes stimulated in vitro with LPS. In order to generate thalidomide analogs with greater inhibitory activity, we introduced structural modifications on different moieties of the parent thalidomide molecule. Initial experiments indicated that neither the phthalimide nor the glutarimide rings of the thalidomide molecule were essential for inhibition of TNFα production, but that the presence of the phthalimide ring greatly improved inhibitory activity (10). For this reason, we initially chose to focus on modifying the glutarimide ring. Simple hydrolysis products with an open glutarimide ring were tested (Fig. 1), and little or no inhibition of TNFα production was observed (not shown). Straight chain N-phthaloyl amino alkyl amides which could mimic the glutarimide degradation products were also prepared and evaluated. The derivative (CC-1003) prepared from β-alanine showed low activity in inhibiting TNFα production. The addition of a phenyl group to this chain (CC-1018) (Fig. 1) rendered the compound capable of inhibiting TNFα production at a level similar to that observed with thalidomide (data not shown).

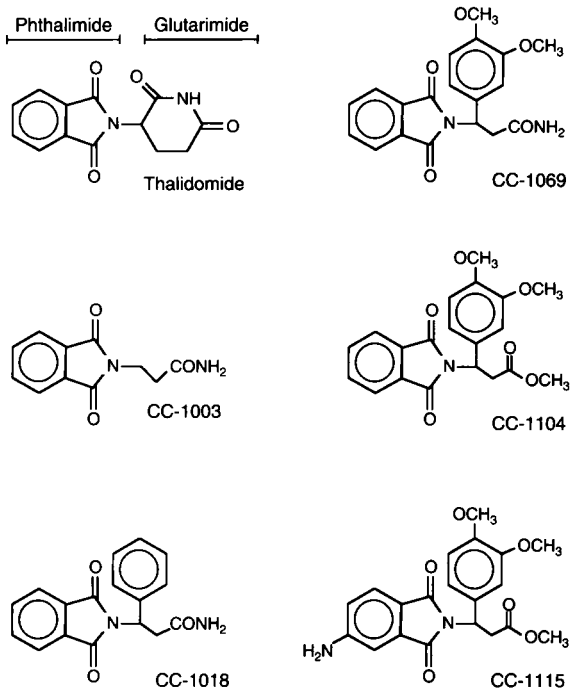

FIG. 1. Chemical structures of thalidomide and analogs

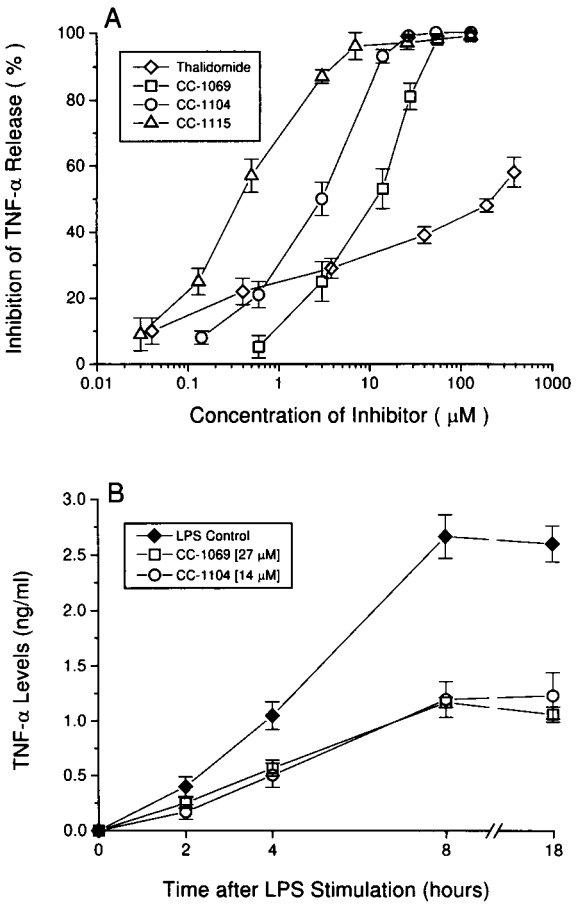

FIG. 2. (A) Dose-dependent inhibition of TNFα produced by LPS-stimulated human PBMC treated with thalidomide (◇), CC-1069 (□), CC-1104 (○), and CC-1115 (△); (B) kinetics of inhibition of TNFα release by LPS-stimulated human monocytes following treatment with none (◆), CC-1069 (□), CC-1104 (○)

(A) Cells were cultured with 1 μg/ml of LPS in the absence or presence of the drugs. Culture supernatants were collected after 20 hr and assayed for TNFα by ELISA. Results are means ± SEM of five experiments done in triplicate. (B) Results are means ± SD of three experiments done in triplicate.

Modification in the position and type of chemical group around the phenyl ring had a great effect on the ability of these compounds to inhibit TNFα production. We found that the addition of two methoxy groups at positions 3 and 4 of the phenyl group (Fig. 1) resulted in the more active analogs (e.g., the compound CC-1069). This compound inhibited 50% of TNFα production (IC_{50}) at the concentration of 12.5 μM (Fig. 2A). This analog is thus more active than thalidomide, which has an IC_{50} of 194 μM (Fig. 2A). We next replaced the primary amide of analog CC-1069 with a carboxy-methyl group yielding compound CC-1104 (Fig. 1). This compound had a TNFα inhibitory IC_{50} of 2.7 μM (Fig. 2A). The substitution of an amino group in the phthalimide ring of the analog CC-1104 resulted in the analog CC-1115 (Fig. 1), which had a TNFα inhibitory IC_{50} of 0.5 μM (Fig. 2A).

Dose-response curves for CC-1069, CC-1104, and CC-1115 are the standard sigmoidal curves seen for classical pharmacological antagonists compared with the dose-response curve for thalidomide, which shows a partial antagonist type dose-dependent inhibition (Fig. 2A). Although thalidomide inhibition of TNFα production never reaches 100% (even at concentrations up to 780 μM; not shown), all three analogs completely inhibited TNFα production (Fig. 2A). Treatment of the cells at this range of concentrations with the parent drug or with any of the analogs did not affect cell viability as assayed by trypan blue exclusion.

In our experiments, TNFα released by LPS-stimulated cells was usually monitored after about 20 hr of incubation with the drugs. However, inhibition of TNFα production by thalidomide (2) and its analogs could be documented as early as 2 hr after LPS stimulation throughout the 20 hr incubation period (Fig. 2B).

TABLE 1. Effect of thalidomide and analogs on cytokine production by LPS-stimulated human monocytes

Compound (conc)	*Cytokine Levels*				
	TNFα	**GM-CSF**	**IL-1β**	**IL-6**	**IL-10**
LPS (1 μg/ml) +:					
0.25% DMSO (control)	6.1 ± 2.0 ng/ml	0.4 ± 0.4 ng/ml	23.8 ± 7.0 ng/ml	540 ± 11 ng/ml	1.9 ± 1.1 ng/ml
Thalidomide (194 μM)	49 ± 4.0%[a,b]	86 ± 5.50%	86 ± 20.3%	100 ± 13.5%	149 ± 26.0%[b]
CC-1069 (12.5 μM)	40 ± 5.9%[b]	56 ± 1.50%[b]	77 ± 4.60%[b]	97 ± 5.60%	130 ± 16.4%
CC-1104 (2.7 μM)	50 ± 8.6%[b]	62 ± 13.9%	91 ± 13.9%	88 ± 7.00%	140 ± 21.8%
CC-1115 (0.5 μM)	44 ± 8.9%[b]	25 ± 0.90%[b]	99 ± 10.9%	102 ± 8.60%	160 ± 14.9%[b]

Cytokine levels in culture medium were measured by ELISA. Drug concentrations are the IC_{50} for TNFα for each drug. Results are expressed as absolute level (ng/ml) and as percentages of cytokine activity and represent the mean ± SEM of five experiments done in triplicate.

[a]Percentage activity.

[b]Significant ($p < 0.05$) difference compared with control values.

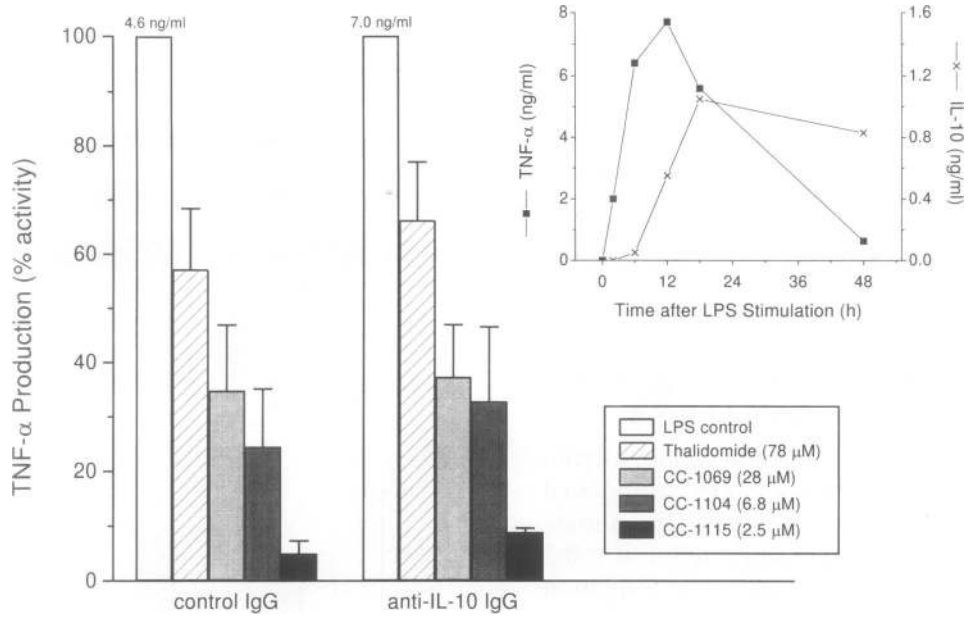

FIG. 3. The effects of anti–human IL-10 monoclonal antibody (anti–hu IL-10) on the inhibition of TNFα by thalidomide and the analogs in LPS-stimulated monocytes.

Cells were activated with LPS (1 μg/ml) and cultured with drugs in the presence of control IgG or neutralizing anti–huIL-10 (10 μg/ml) for 20 hr. Production of TNFα was determined by ELISA. The ability of anti–huIL-10 to completely neutralize secreted IL-10 was confirmed by ELISA. Results are means ± SD of three experiments done in triplicate. (insert) Kinetics of TNFα and IL-10 release into the culture supernatant after LPS stimulation of human monocytes. Cytokine levels were determined by ELISA.

Effect of Thalidomide and the Analogs on LPS-Induced Cytokine Production in Vitro

The effects of thalidomide and its analogs on induction of cytokines following LPS stimulation of monocytes was investigated. TNFα, GM-CSF, IL-6, IL-1β, and IL-10 were evaluated. The levels of these cytokines released into the culture medium in response to LPS stimulation are shown in Table 1. At concentrations near the IC_{50} for TNFα production for thalidomide and the analogs, the levels of IL-6 and IL-1β produced were not significantly reduced except for a slight reduction in IL-1β levels by CC-1069 (Table 1). Two of the analogs (CC-1069 and CC-1115) induced significant inhibition of GM-CSF production at the TNFα IC_{50}. This inhibition of GM-CSF production was not observed following treatment with thalidomide (Table 1).

In addition, when LPS stimulated monocytes were treated with thalidomide and its analogs, there was an increase in IL-10 release compared with the DMSO control (Table 1). Thalidomide and the analogs did not induce IL-10 production in unstimulated cells (data not shown). Since IL-10 has been reported to inhibit TNFα production by monocytes in vitro (12), we investigated the relationship between release of IL-10 and the inhibition of TNFα mediated by thalidomide and the analogs. The inhibitory effect of thalidomide and analogs was therefore studied in the presence of neutralizing antibodies to IL-10. Control IgG had no effect on TNFα production by LPS-stimulated monocytes (not shown). However, total TNFα production (in ng/ml) was higher (7.0 ng/ml) in the LPS-treated monocyte culture supernatants containing anti–IL-10 IgG compared with cultures containing control IgG (4.6 ng/ml) (Fig. 3). In the presence of neutralizing antibodies to IL-10, thalidomide and its analogs still induced a significant reduction in TNFα levels released into the culture supernatant (Fig. 3). The results show that even when IL-10 was completely neutralized by anti–IL-10 antibodies, the percentage inhibition of TNFα production by thalidomide and the analogs was similar to that observed without neutralization of IL-10.

We next studied the kinetics of IL-10 and TNFα production in human monocytes in vitro. Production of the two cytokines in response to LPS stimulation of human monocytes followed different kinetics (Fig. 3, insert) confirming previously reported results (12). TNFα was measur-

able in the culture supernatant as early as 2 hr after LPS stimulation and continued to accumulate in the supernatant for up to 12 hr and then the concentration waned. In contrast, IL-10 first appeared in the culture supernatant at 6 hr after LPS stimulation and peaked at 18 hr (Fig. 3, insert). These observations taken together suggest that thalidomide and its analogs directly inhibit TNFα production, rather than acting via the induction of IL-10.

Effect of Thalidomide and Its Analogs on Cytokine mRNA Expression in Vitro

Thalidomide has been reported to suppress TNFα mRNA expression levels in LPS stimulated monocytes (3). In our experiments, thalidomide and all three analogs significantly reduced TNFα mRNA accumulation in the cells. The inhibition of TNFα mRNA expression by the drugs was dose dependent (Fig. 4A). The inhibition of TNFα mRNA by the analogs ranged from 55 to 80%, at concentrations lower than those required for a 40% reduction of TNFα mRNA levels by thalidomide (Fig. 4B). In addition, while TNFα mRNA expression was significantly affected by all four drugs, levels of IL-6 mRNA and IL-1β mRNA were not significantly affected (Fig. 4).

Effect of Thalidomide and Thalidomide Analogs on TNFα and IL-10 Production in Vivo

The drugs were tested for their ability to inhibit TNFα production in vivo in LPS-treated mice. When mice challenged with LPS were treated with either thalidomide or any of the analogs, TNFα levels in sera were reduced. Analog CC-1069 inhibited TNF-α in a dose dependent manner with a reduction of almost 90% of circulating TNFα at 100 mg/kg (Table 2). The other drugs produced more modest inhibitions. Treatment with thalidomide administrated at a single dose of 100 mg/kg, never resulted in more than 62% inhibition of TNFα production. Treatment with thalidomide or the analogs which resulted in inhibition of TNFα was also associated with enhancement of IL-10 levels in the sera of LPS-treated mice (Table 3). Analog CC-1069, which was the most active analog in vivo against TNFα, was also the analog that produced the greatest enhancement of IL-10 levels. Interestingly, in vivo, both IL-10 and TNFα levels peak at about 1.5 hr after administration of LPS. These results demonstrated that, while analog CC-1115 was the most

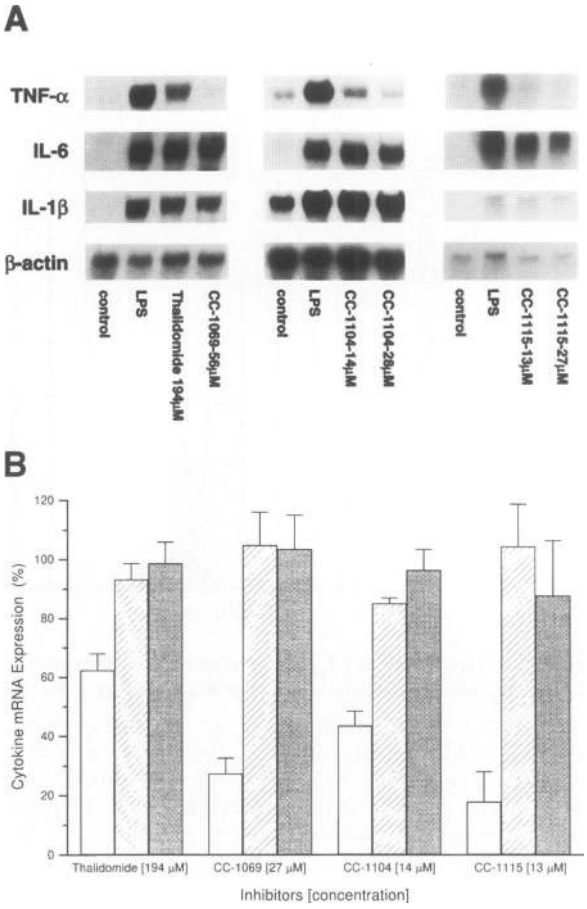

FIG. 4. Northern blot of cytokine mRNA expression by LPS stimulated human monocytes treated with thalidomide or the analogs (A) and cytokine mRNA quantified by phosphorimaging and normalized to the density of β-actin on the same blot (B)

(A) RNA was extracted from cells after 6 hr of incubation at 37°C and 20 μg of RNA were loaded per lane. (B) □, TNFα mRNA; ▨, IL-6 mRNA; ■, IL-1β mRNA. Results represent mean ± SEM of three to seven experiments. TNFα mRNA inhibition was statistically significant ($p < 0.05$) for all drugs tested.

active in vitro, analog CC-1069 was the most effective in vivo. This may be due to one or more factors, including bioavailability, other pharmacokinetic parameters and inherent differences between human and murine systems.

To determine the relative stability of thalidomide and the three analogs in vivo, the drugs were evaluated for half-life in human plasma. The three thalidomide analogs showed improved stability in human plasma over the parent drug (Table 3). While thalidomide had a half-life ($t_{1/2}$) of less than 2 hr under these assay conditions, the analogs had longer $t_{1/2}$. Analog CC-1069 was the most stable with a $t_{1/2}$ of 8 hr in human

TABLE 2. Effect of thalidomide and analogs on serum cytokine levels in LPS treated mice

Compound	Amount (mg/kg)	Cytokine Level (ng/ml)	
		TNFα	IL-10
LPS (5 mg/kg) +:			
None (control)	—	4.5 ± 1.5	19.9 ± 5.1
Thalidomide	1	2.2 ± 0.7[a]	25.6 ± 5.6
	10	2.1 ± 0.8[a]	25.3 ± 5.6
	100	1.7 ± 0.5[a]	32.4 ± 4.8[a]
CC-1069	1	3.1 ± 1.0	25.7 ± 2.1
	10	1.5 ± 0.2[a]	28.7 ± 4.1[a]
	100	0.6 ± 0.3[a]	44.8 ± 5.6[a]
CC-1104	1	4.9 ± 0.8	13.0 ± 0.3
	10	3.2 ± 0.3	16.7 ± 3.8
	100	1.9 ± 0.4[a]	17.9 ± 2.3
CC-1115	1	2.7 ± 0.9	26.6 ± 2.8
	10	2.7 ± 0.4	29.9 ± 2.5[a]
	100	1.6 ± 0.4[a]	32.4 ± 2.4[a]

Concentration of cytokines in serum after LPS injection was evaluated by ELISA. Drugs were given i.p. 2 hr before i.p. administration of LPS. Blood samples were collected 1.5 hr later. Results are expressed as the mean ± SD of three to five experiments (four mice per group in each experiment).

[a]Denotes significant ($p < 0.05$) difference compared with control values.

plasma (Table 3). This improved stability of CC-1069 in human plasma could be due to the fact that CC-1069 is an amide while the other analogs are esters and may therefore be more readily degraded by plasma esterases.

Effect of Thalidomide and CC-1069 on the Survival of Mice Treated with a Lethal Dose of LPS

Since CC-1069 was both most active at modulating cytokine levels in vivo and more stable in plasma, we tested its efficacy in protecting mice from death due to LPS-induced shock.

A single dose of either thalidomide or CC-1069 protected mice treated with a lethal dose of LPS against death. However, the analog was more effective than thalidomide in this regard. Treatment with CC-1069 resulted in the survival of 80% of LPS-injected mice, compared with survival of 60% of LPS-injected mice treated with thalidomide (Fig. 5).

DISCUSSION

It has long been known that reducing the levels of TNFα and other cytokines during an inflammatory episode can improve clinical outcome by

TABLE 3. Stability of thalidomide and analogs in human plasma

Half-Lives ($t_{1/2}$) of Compounds (hr)			
Thalidomide	CC-1069	CC-1104	CC-1115
1.52 ± 0.11	7.80 ± 0.66	2.57 ± 0.53	3.77 ± 0.32

Human plasma (Sigma) was spiked with thalidomide or analogs and incubated at 37°C. Drug levels were determined by HPLC.

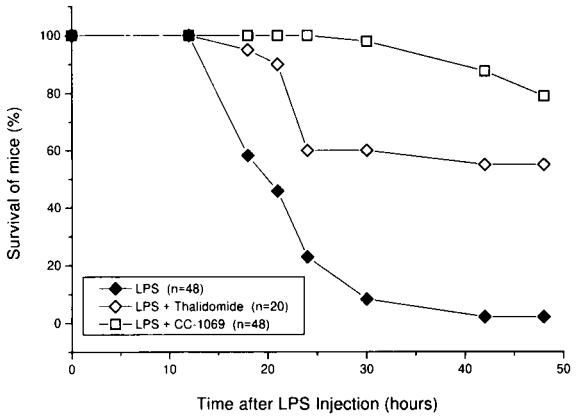

FIG. 5. Effect of thalidomide and analog CC-1069 on LPS-induced lethality in mice

Mice were treated with one dose of thalidomide (100 mg/kg) or CC-1069 (100 mg/kg) 2 hr before the administration of LPS (7.5 mg/kg). Results are expressed as percentage of mice surviving at 48 hr after LPS administration. Results are from four independent experiments. LPS + DMSO (control) (◆), 48 mice; LPS + thalidomide (◇), 20 mice; LPS + CC-1069 (□), 48 mice.

ameliorating the toxic effects of the inflammatory cascade (13). Strategies for the design of optimal TNFα inhibitors aim at the synthesis of drugs which are effective at low doses, nontoxic, and specific enough to modulate the inflammatory response without shutting down the protective immune response. Recently one drug, thalidomide, has been shown to selectively, albeit partially, inhibit TNFα production in vitro. Following treatment with thalidomide, we have observed a decrease in TNFα and other cytokines, and a clinical improvement in leprosy (ENL) patients (4) and tuberculosis patients (5), in the absence of significant toxicities. We have also observed partial inhibition of TNFα release as well as only partial reduction in inflammatory manifestations in the central nervous system following administration of thalidomide in rabbits with bacterial meningitis (14). To obtain drugs which are more efficient than thalidomide and retain the specificity of the parent drug and will therefore not be immune suppressive, we have modified the parent drug and examined some of the resulting compounds for the desired profile of activities.

In this report, we describe three new drugs with significantly improved (up to 400-fold) capacity to inhibit TNFα production in vitro. In addition to the effect on TNFα production, the new compounds along with thalidomide produced an enhancement in the production of IL-10 in LPS-stimulated cells. IL-10 is known to suppress the production of TNFα by human and mouse cells (15,16). TNFα inhibition by these compounds, however, was not mediated by IL-10 since comparable inhibition was found in the presence of neutralizing IL-10 antibodies. The enhancement of IL-10 production by LPS-stimulated cells in association with TNFα inhibition has been reported previously in LPS-stimulated macrophages in vitro (16,17). TNFα inhibition and IL-10 enhancement by thalidomide and analogs was also observed in vivo. The increased production of IL-10 may be partially responsible for the protection from LPS lethality achieved by these drugs since IL-10 neutralization is associated with increased lethality in endotoxemia (18,19). The ability of CC-1069 to protect mice from death due to LPS-induced shock suggests that this class of compounds might be useful in similar clinical situations in humans. Since the potent teratogenic effect of thalidomide is well documented, the analogs are now being tested for their teratogenic potential. Preliminary results are exciting because several analogs appear to be less teratogenic than the parent compound, thalidomide.

Although the focus of the present study was on identifying molecules that inhibit TNFα production more efficiently than thalidomide, it is obvious from these studies, as well as from other published studies (20), that thalidomide and possibly some of its analogs are pleiotropic in their effects. Thalidomide has been shown to act as a teratogen and as a sedative, and also is capable of inhibiting angiogenesis (1,21). It is not clear that these varied physiologic effects are mediated by a common mechanism. The separation of the different effects of thalidomide in the different physiologic situations is a central focus of the ongoing rational drug design program. This should enable us to design drugs that retain the positive effects and eliminate or minimize toxicities and side effects.

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