Thalidomide Treatment Reduces Tumor Necrosis Factor α Production and Enhances Weight Gain in Patients with Pulmonary Tuberculosis

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ABSTRACT

Background: The monocyte-derived cytokine, tumor necrosis factor α (TNFα), is essential for host immunity, but overproduction of this cytokine may have serious pathologic consequences. Excess TNFα produced in pulmonary tuberculosis may cause fevers, weakness, night sweats, necrosis, and progressive weight loss. Thalidomide (α-N-phthalimidoglutarimide) has recently been shown to suppress TNFα production by human monocytes in vitro and to reduce serum TNFα in leprosy patients. We have therefore conducted a two-part placebo-controlled pilot study of thalidomide in patients with active tuberculosis to determine its effects on clinical response, immune reactivity, TNFα levels, and weight.

Materials and Methods: 30 male patients with active tuberculosis, either human immunodeficiency virus type 1 positive (HIV-1+) or HIV-1−, received thalidomide or placebo for single or multiple 14 day cycles. Toxicity of the study drug, delayed type hypersensitivity (DTH), cytokine production, and weight gain were evaluated.

Results: Thalidomide treatment was well tolerated, without serious adverse events. The drug did not adversely affect the DTH response to purified protein derivative (PPD), total leukocyte, or differential cell counts. TNFα production was significantly reduced during thalidomide treatment while interferon-γ (IFNγ) production was enhanced. Daily administration of thalidomide resulted in a significant enhancement of weight gain.

Conclusions: The results indicate that thalidomide is well tolerated by patients receiving anti-tuberculosis therapy. Thalidomide treatment reduces TNFα production both in vivo and in vitro and is associated with an accelerated weight gain during the study period.

INTRODUCTION

Tumor necrosis factor α (TNFα) is one of the important immunologic mediators generated by cells of the monocyte/macrophage lineage and has been shown to have significant effects on host immunity in bacterial and parasitic infections (1). Tuberculosis is a chronic mycobacterial infection (of macrophages and monocytes) in which TNFα is characteristically produced. TNFα is present in the pleural effusions of patients infected with Mycobacterium tuberculosis, and monocytes isolated from patients with active dis-

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ease and systemic symptoms release elevated amounts of TNFα into the culture supernatant in response to stimuli (2–4). In vitro, mycobacterial preparations have been shown to induce TNFα production by human monocytes (5). TNFα is essential for granuloma formation (6,7), which is intimately associated with resistance against mycobacteria (8).

While production of TNFα is essential for host immunity, overproduction of this cytokine may have serious pathologic consequences. In experimental animals, high levels of TNFα induce fever by direct action on the hypothalamus (1), and long-term administration of this cytokine causes marked losses of fat and muscle cells or cachexia (9). Elevated levels of TNFα produced in pulmonary tuberculosis may be responsible for many of the pathologic symptoms of the disease, including fevers, weakness, fatigue, night sweats, lung necrosis, and progressive weight loss (10).

The control of these pathologic effects of excess TNFα production has received increasing attention. In animal models of gram-negative sepsis, administration of either antibodies directed against TNFα or pharmaceutical agents that suppress TNFα production, leads to reduced fevers, less wasting, and significantly decreased mortality (11). One drug, thalidomide (α-N-phthalimidoglutarimide), has recently been shown to suppress TNFα production by human monocytes in vitro (12,13) and to normalize elevated levels of serum TNFα in vivo (12,14). The inhibitory activity of thalidomide is due to a selective destabilization of the TNFα mRNA (15); thalidomide selectively suppresses TNFα production in vitro without directly affecting the levels of other cytokines such as interleukin-1 (IL-1), IL-6, and granulocyte/macrophage-colony stimulating factor (GM-CSF) (13).

Since thalidomide inhibits but does not abolish TNFα production, thalidomide therapy might reduce the toxicities linked with overproduction of the cytokine, without interfering with its important role(s) in host immunity. Recent studies have shown that thalidomide has prompt and dramatic effects when given to leprosy patients with erythema nodosum leprosum (ENL), including the elimination of the lesions, a decrease in inflammation, and general enhancement of patient well-being. These studies suggest that thalidomide may play a therapeutic role in tuberculosis patients, where much of the pathology may be TNFα mediated, but where a normal immune response and granuloma formation is important in the control of the infection.

In order to determine the effect of thalidomide on TNFα levels and whether thalidomide treatment is associated with any untoward side effects, including drug toxicity or suppression of immune reactivity, we have conducted a two-part placebo-controlled pilot study of thalidomide in patients with active tuberculosis. The results of our study indicate that thalidomide reduces TNFα production by peripheral blood mononuclear cells (PBMCs) in patients receiving anti-tuberculosis therapy without any adverse effects. Furthermore, although all patients showed the normal expected response to anti-tuberculosis therapy, only patients treated with thalidomide demonstrated significant increased weight gain during the study period.

MATERIALS AND METHODS

Study Drug

Thalidomide (CG-217) was provided in 100-mg tablets (Lot 204 and 206) by Grunenthal GMBH (Aachen, Germany). Placebo tablets were also provided by Grunenthal GMBH (Aachen, Germany).

Study Design

A total of 30 male patients with active tuberculosis, either HIV-1$^+$ or HIV-1$^-$, were studied. Twenty of the patients were studied in Chiang Mai, Thailand (Group I), and 10 were studied in New York City, U.S.A. (Group II). In Thailand, patients were evaluated at the Tuberculosis Center in Chiang Mai and admitted to the Tuberculosis Sanitorium. In New York, patients were transferred from Bellevue Hospital to the Clinical Research Center (CRC) at the Rockefeller University Hospital.

All patients received 300 mg of thalidomide or placebo in tablet form (3 tablets) daily at bedtime. The Thailand patients received only a single 14-day cycle of thalidomide or placebo. In New York, after the first treatment cycle with thalidomide or placebo, Patients 25–30 were allowed a 7-day washout period and then treated with a second, third, and in some a fourth cycle of thalidomide or placebo. Of the 10 New York patients, one completed only 7 days of thalidomide during the initial cycle of treatment (Patient 21); the remaining nine completed the full initial cycle of 14 days of thalidomide or placebo.

INCLUSION CRITERIA. Table 1 summarizes the patient characteristics. Patients were included in the study if they had acid fast organisms on sputum examination with eventual growth of *M. tuberculosis* in culture and met one or more of the following criteria: fever greater than 38°C; weight loss greater than 5 kg; night sweats; and evidence of pulmonary disease on chest X-ray consistent with tuberculosis. Patients were excluded from the study if there was growth of mycobacteria other than tuberculosis or if patients refused human immunodeficiency virus type 1 (HIV-1) testing. Also patients with pre-existing peripheral neuropathy were excluded, since this is a well-described side effect of thalidomide.

CONCOMITANT THERAPIES. All patients received multidrug anti-tuberculosis regimens (MDT) as shown in Table 1. Anti-retroviral therapy was administered to the New York patients who were HIV-1$^+$ (Table 1) but was not available for the Thailand patients. The protocol was approved by the Institutional Review Board of the Rockefeller University Hospital and by the Ministry of Health in Thailand. Written consent was obtained for all patients.

Evaluation of Patients

All patients were evaluated by history and physical exam at baseline, at multiple time points during the study, and after cessation of the drug. Vital signs, including weight, were obtained twice daily. In Thailand, complete blood counts with differentials and biochemistry profiles were obtained before, on Day 7 of treatment and 5 days after the end of drug treatment. In New York, in addition to complete blood counts and biochemistry profile, laboratory evaluation included phenotyping of blood mononuclear leukocytes. Chest radiographs were obtained at baseline and after cessation of treatment with thalidomide or placebo. Delayed type hypersensitivity (DTH) was assessed by the response to intradermal injection of 0.1 ml of the tuberculin PPD (Mantoux, Connaught Laboratories, Inc., Swiftwater, PA, U.S.A.) and quantitated after 48 hr. DTH testing was performed at baseline, on Day 7 of treatment with drug or placebo, and 5 days after discontinuation of thalidomide or placebo. The size of the response is expressed as the mean of the two greatest diameters (in mm). Anergy testing was performed using the Multitest skin test reaction (Connaught). Patients 20, 23, 25, and 26 were not available for repeated

skin testing. Complete caloric intake was recorded for patients in New York (Group II) only.

PBMC PHENOTYPING. PBMCs were isolated as previously described (15). For indirect immunofluorescent staining, PBMCs were stained with a saturating amount of the following monoclonal antibodies: anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-CD19 (Leu-12) (Becton Dickinson, Mountain View, CA, U.S.A.), anti-CD56 (NKHI) (Coulter, Hialeah, FL, U.S.A.), and anti-CD14 (Ortho Diagnostics, Westwood, MA, U.S.A.). Cells were then analyzed with the Becton Dickinson FACScan flow cytometer.

Tissue Culture

PBMCs were isolated from blood obtained from all patients in the study and resuspended at a density of 2 × 10^6/ml in 2× culture medium (CM). CM consisted of RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% pooled human AB$^+$ serum (Biocell, Carson, CA, U.S.A.), 100 U penicillin/ml, 100 μg streptomycin/ml, and 2 mM L-glutamine (Gibco). Incomplete tissue culture results were obtained for Patients 21–23 because of tissue culture contamination; not all assays were available for Patients 20 and 30.

Cytokine Measurements

IN VITRO. Bacterial preparations: The Pasteur strain 1011 of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (Ataufo de Paiva Foundation, Rio de Janeiro, Brazil), the purified protein derivative (PPD) of *M. tuberculosis* for use in vitro (Statenseruminstitut, Copenhagen, Denmark), synthetic muramyl dipeptide (MDP) (Sigma, St. Louis, MO, U.S.A.), and gram-negative lipopolysaccharide isolated from *Salmonella typhimurium* (LPS) (List Biological Laboratories, Campbell, CA, U.S.A.) were obtained commercially.

For induction of cytokines, 0.1-ml aliquots of cell suspensions were added to each well of 96-well flat bottom tissue culture plates (Costar, Cambridge, MA, U.S.A.) containing 0.1-ml aliquots of RPMI 1640 alone, or bacterial preparations suspended in RPMI 1640, to give final concentrations of 10 μg PPD, 10 μg BCG, 0.5 μg MDP, or 10 ng LPS per ml.

Plates were incubated for 16 hr at 37°C, in 5% CO_2. Then enzyme-linked immunosorbent assays (ELISA) (Endogen, Boston, MA, U.S.A.), were performed according to the manufacturer's

TABLE 1. Patient characteristics, clinical status, and therapies

Patient Number	Study Drug[a]	HIV-1 Status	Age (years)	Baseline Weight (kg)	No. Lung Fields Involved[b]	Medication[c]
Thailand						
1	t	+	43	48.0	6	Rifater, E
2	t	+	51	49.0	4	Rifater, E
3	t	−	32	43.0	4	Rifater, E
4	t	−	65	40.5	4	Rifater, E
5	t	−	46	43.3	4	Rifater, E
6	t	+	60	41.3	4	RPE
7	t	−	31	52.8	1	RIP
8	t	+	38	48.3	2	Rifater, E
9	t	−	25	56.5	3	Rifater, E
10	t	+	25	49.5	1	Rifater, E
11	t	+	49	44.5	3	Rifater, E
12	t	−	38	50.5	2	Rifater, E
13	p	−	25	40.0	2	Rifater, E
14	p	−	53	50.0	4	Rifater, E
15	t	−	55	52.0	5	Rifater, E
16	p	−	64	49.0	5	Rifater, E
17	t	−	22	42.0	1	Rifater, E
18	p	−	46	63.3	1	Rifater, E
19	t	−	63	71.0	3	Rifater, E
20	p	−	42	58.0	NA	Rifater, E
New York						
21	t	+	44	63.3	1	RIPE + Anti retroviral
22	t	+	37	71.0	2	RIPE + Anti retroviral
23	p	+	33	77.7	1	RIPE + Anti retroviral
24	p	+	45	76.5	3	RIPE + Anti retroviral
25	t;t;p;t	−	32	53.7	3	RIPE
26	t;t	+	35	66.5	2	RIPE + Anti retroviral
27	t;t	+	30	63.3	3	RIPE + Anti retroviral
28	t;t;p;t	−	38	74.5	3	RIPE + CIP¶ + ETH¶
29	p;t;t	+	42	83.8	1	RIPE + Anti retroviral
30	t;p;t;p	−	46	89.6	2	RIPE

NA, not available; R, Rifampin; I, Isoniazid; P, Pyrazinamide; E, Ethambutol; CIP¶, Cipro for 16 days; ETH¶, Ethionamide for 16 days.

[a]Patients received either thalidomide (t), placebo (p), or multiple cycles of t or p in the sequence shown.

[b]Infiltration, granulomas, or cavities present on chest X-ray: a total of six lung fields were evaluated and results given for each patient.

[c]Rifater, Rifampin isoniazid pyrazinamide.

instructions on 0.1-ml aliquots of culture supernatants, or of 10-fold dilutions of the same supernatants, to determine TNFα, IL-1, and interferon-γ (IFNγ) levels. All data points were derived from triplicate cultures.

DIRECT ASSAY OF CYTOKINES IN PATIENT SERA. Samples of serum obtained from the patients at the same time points as PBMCs were assayed directly for TNFα, IL-1, and IFNγ by ELISA (Endogen). All assays were performed in triplicate. Serum samples were also assayed for total TNFα (receptor bound as well as free bioactive TNFα) by a commercial EIA (Medgenix, Fleurus, Belgium) (16).

Lymphocyte Transformation Tests

Culture conditions for lymphocyte transformation tests (LTTs) were identical to those described above except that U-bottom plates were used. After 5 days of incubation, 1 μCi of tritiated thymidine ([^{3}H])-TdR) (New England Nuclear, Boston, MA, U.S.A.) was added to U-bottom microwells. Following a 16-hr incubation, wells were harvested onto paper mats which were thoroughly washed, dried, and immersed in scintillation fluid for counting β emission. Data is also presented as stimulation index (SI) calculated as: (the cpm in the presence of stimulus)/ (cpm in the CM alone).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis of Cytokine mRNA Levels

Freshly isolated PBMCs obtained from all group II patients (New York) were used. RNA isolated from PBMCs obtained from Group I patients (Thailand) was partially degraded and not used for analyses. Reverse transcriptase-polymerase chain reaction (RT-PCR) for cytokine mRNA was carried out as described in detail elsewhere (17). Briefly, RNA was extracted from 4–6 $\times$ 10^6 PBMC immediately after isolation using RNAzol B (Cinna/Biotecx, Houston, TX, U.S.A.) according to the manufacturer's instructions. Absorption spectroscopy was used to measure purity and concentration of RNA with a $A_{260/280}$ ratio of 2.0 indicating highly purified RNA. RNA was reverse transcribed into cDNA which was then amplified using the Gene-Amp RNA-PCR kit (Perkin Elmer Cetus, Norwalk, CT, U.S.A.) with 30 cycles of 95°C for 1 min (denaturation) and 60°C for 1 min (primer annealing and extension). To normalize for the amount of input RNA, RT-PCR was performed on the constitutively expressed gene encoding glyceraldehyde phosphate dehydrogenase (GA3PD). PCR products were electrophoresed and transferred to nylon membranes (Zetabind, Cuno, Inc., Meriden, CT, U.S.A.). After prehybridization, membranes were hybridized with 5 $\times$ 10^5 cpm/ml of ^{32}P-labeled oligonucleotide probes for 8–12 hr at 40°C. After washing, membranes were exposed to X-ray film (Hyperfilm-MP, Amersham Corp., Arlington Heights, IL) at room temperature with an intensifier screen. Quantitative analysis by comparison with known standards, and primer and probe sequences have been detailed (17).

Statistical Analysis

Data obtained were evaluated by the Wilcoxon signed rank test (two-sided test), or by the Student's paired sample t test. p values of 0.05 or less were considered significant.

RESULTS

Drug Tolerance and Adverse Effects

At the inception of the study, 10 male patients (Table 1) were enrolled in Group I (Thailand). The patients had been recently diagnosed with active tuberculosis and all began anti-tuberculosis treatment with MDT simultaneously or within a few days of treatment with thalidomide. All patients received one 14-day cycle of thalidomide, as described in Materials and Methods. The thalidomide treatment was well tolerated. No peripheral neuropathy, excessive sedation, or other adverse events were observed. The only side effects noted were morning drowsiness, dry mouth, and constipation. In addition, all patients responded well to concomitant anti-tuberculosis therapy and showed clinical improvement.

Since there were no adverse effects, approval was granted for enrollment of an additional 10 patients in the study (in Thailand). These 10 additional male patients (Table 1) were randomized to receive either thalidomide or placebo. All patients completed the 14-day cycle of thalidomide or placebo treatment. Administration of either thalidomide or placebo did not result in any clinical or radiographic deterioration in any of the patients. Again, clinical improvement was noted after beginning anti-tuberculosis therapy. One patient (Patient 6, Table 1) developed a macular rash which resolved with discontinua-

tion of isoniazid and hence was not associated with thalidomide treatment.

This phase of the study established that a single 14-day cycle of thalidomide treatment was well tolerated and did not induce untoward side effects. We then received (FDA and IRB) approval to extend the studies for multiple cycles of thalidomide treatment. Therefore, an additional 10 male patients, ages 30 to 46, (Group II) were enrolled in the study in New York (Table 1). All had been diagnosed with active tuberculosis, and had received anti-tuberculosis therapy for 1–4 weeks prior to their entry into the study. During the study, 4 patients (Patients 21–24, Table 1) received a single cycle of thalidomide or placebo; the remaining 6 patients (Patients 25–30, Table 1) received multiple cycles of thalidomide and/or placebo (Table 1). All patients in Group II (New York) responded well to thalidomide treatment; there were no adverse responses in either the thalidomide- or placebo-treated patients, except for sedation, dry mouth, and constipation, as previously noted.

Effect of Thalidomide Treatment on Delayed Type Hypersensitivity

Delayed type hypersensitivity (DTH) was assessed in each patient by the skin test response to soluble antigen. The response to PPD was quantitated before, during, and after therapy to determine the effect of thalidomide on cell mediated immunity. At Day 0 (baseline) before the first cycle of thalidomide or placebo treatment, 21 patients were PPD$^+$ while 6 patients were PPD$^-$ (nonresponsive) (Table 2). Treatment with thalidomide did not alter the DTH response to PPD in patients who were already PPD$^+$ (Table 2). However, in the five patients who were nonresponsive to PPD at the beginning of the treatment cycle, thalidomide administration was associated with a significantly enhanced DTH reaction. This responsiveness returned to baseline after discontinuation of thalidomide in three of the patients. Both HIV-1$^-$ and HIV-1$^+$ showed similar DTH patterns in response to thalidomide treatment.

Reactivity to the Connaught panel of antigens was also tested. Responses to Old Tuberculin were similar to those observed with PPD (not shown). No patient became anergic to this or any other antigen during the course of the study.

TABLE 2. Delayed type hypersensitivity response to PPD during first cycle of study[a]

Patient Number	Skin Test Response to PPD (mm)[b]		
	Pre	Mid	Post
PPD$^+$			
1	26.5	20.5	24.0
3	14.0	25.0	18.5
4	19.0	16.0	20.5
5	16.5	18.0	24.5
6	14.0	12.0	16.5
7	23.5	65.0	ND[c]
9	18.5	14.5	21.5
11	14.5	18.0	13.0
12	19.5	26.0	17.0
13[d]	15.0	21.0	26.0
14[d]	26.5	22.0	31.0
15	16.0	18.0	10.0
16[d]	26.0	22.5	23.0
18[d]	20.0	20.5	17.0
19	15.5	18.0	16.0
20[d]	ND	ND	ND
21	11.0	15.5	12.0
22	22.0	19.0	17.0
23[d]	ND	ND	ND
25	ND	ND	ND
26	19.0	ND	ND
27	13.0	14.5	18.0
28	13.0	12.5	17.5
29[d]	18.0	14.0	10.0
PPD$^-$			
2	0	9.5	0
8	0	19.5	0
10	0	12.5	0
17	0	9.0	11.5
24[d]	0	0	0
30	0	19.0	ND

[a]Patients in Thailand and New York were skin tested 3× during the first cycle of study.
[b]Mean diameter of the indurated area at 48 hr after 5 u PPD administration. Diameter >10 mm is termed PPD$^+$.
[c]ND, not done.
[d]Patients received placebo during the first cycle of study.

TABLE 3. White blood cell counts and differentials

		Cell Count per mm^3 of Blood (mean ± SD)			
		Total WBCs	Lymphocyte	Monocyte	Eosinophil
Thalidomide					
HIV$^+$	pre	6765 ± 2286	1700 ± 927	359 ± 276	318 ± 247
	mid	6403 ± 2489	1438 ± 669	320 ± 237	463 ± 562
	post	6331 ± 1564	1784 ± 1165	427 ± 273	703 ± 562
HIV$^-$	pre	8435 ± 3519	1762 ± 876	579 ± 367	513 ± 492
	mid	7560 ± 2402	2402 ± 1294	393 ± 301	524 ± 613
	post	6941 ± 1735	2310 ± 801	454 ± 393	445 ± 451
Placebo					
HIV$^+$/1$^-$	pre	7573 ± 3289	1699 ± 440	616 ± 392	195 ± 151
	mid	6806 ± 3035	2140 ± 1019	336 ± 198	317 ± 321
	post	6588 ± 4012	2024 ± 806	282 ± 170	178 ± 167

Results are means ± one standard deviation (SD) evaluated before (pre), during (mid), and 5 days after (post) 14-day treatment cycles with 300 mg/day of thalidomide or placebo (see Table 1). No significant changes were observed. WBC, white blood cells.

Effect of Thalidomide Treatment on Blood Parameters

Complete blood counts and differential analyses were performed on all patients receiving thalidomide or placebo. Thalidomide treatment did not cause any significant changes in total leukocyte count or differential cell count (Table 3). Fluorescence-activated cell analysis (FACS) of leukocyte subsets was also carried out during the study in New York (Group II patients). No significant effect on subset distribution was observed either in thalidomide- or placebo-treated patients, regardless of whether patients were HIV-1$^-$ or HIV-1$^+$ (not shown). The percent of leukocyte subsets was similar in both HIV-1$^-$ or HIV-1$^+$ patients receiving either placebo or thalidomide treatment. There was, however, a slight increase in CD8$^+$ T cells in HIV-1$^+$ patients compared with HIV-1$^-$ patients.

Lymphocyte Function in Vitro

PBMCs were isolated from all patients prior to, on Day 7, and after thalidomide or placebo administration, and the proliferation in vitro in response to PPD and BCG was determined. Thalidomide did not significantly affect the ability of the cells to incorporate [^{3}H]-TdR in response to

the antigens tested (Table 4) although some fluctuations in the stimulation index were observed. HIV-1$^+$ patients were still responsive to PPD in vitro. This responsiveness was not significantly altered by treatment with thalidomide (Table 4).

Effect of Thalidomide Treatment on Cytokine Production

IFNγ PRODUCTION. To determine whether thalidomide treatment reduced the ability of the host to produce protective cytokines in response to infection, IFNγ production was evaluated both in vivo in the blood of thalidomide and placebo treated individuals and in vitro, in PBMCs.

In vivo. Patients treated with thalidomide had significantly enhanced serum IFNγ levels after 7 days of the first cycle of thalidomide treatment (Fig. 1). The serum IFNγ levels returned to baseline upon discontinuation of drug. In contrast, there was no increase in serum IFNγ levels in patients treated with placebo (Fig. 1).

In vitro. When PBMCs isolated from patients were stimulated with PPD, IFNγ was released into the culture medium (Fig. 1). Similar results

TABLE 4. Proliferative response of PBMC to PPD and BCG

	Lymphocyte Proliferation—counts per minute (stimulation index)		
	Thalidomide		Placebo
	HIV$^+$ CPM (SI)	HIV$^-$ CPM (SI)	HIV$^{+/-}$ CPM (SI)
Controls:			
pre	704 ± 337	1239 ± 1028	1037 ± 524
mid	2264 ± 4694	2620 ± 2949	1158 ± 664
post	1487 ± 1694	1140 ± 664	1632 ± 1376
PPD:			
pre	38253 ± 38987	57926 ± 41427	51878 ± 33764
	(60 ± 64)	(64 ± 46)	(63 ± 50)
mid	49341 ± 46031	44717 ± 23963	60007 ± 28475
	(52 ± 56)	(34 ± 31)	(80 ± 75)
post	44077 ± 43822	53078 ± 31411	52682 ± 43678
	(56 ± 61)	(59 ± 44)	(63 ± 52)
BCG:			
pre	32879 ± 36725	22096 ± 28567	26777 ± 18977
	(42 ± 37)	(28 ± 36)	(32 ± 26)
mid	44767 ± 54076	23788 ± 17519	34200 ± 29931
	(42 ± 58)	(23 ± 32)	(42 ± 53)
post	40172 ± 42663	23259 ± 17060	27891 ± 32902
	(43 ± 50)	(26 ± 19)	(33 ± 36)

Results are means ± one standard deviation (SD) of counts per minute (stimulation indexes) of proliferative responses to antigen stimulation of PBMC tested before (pre), during (mid), and 5 days after (post) treatment with thalidomide or placebo (see Table 1). No significant changes were observed.

were observed when PBMCs from patients were stimulated with BCG (Table 5), but not with LPS or MDP (not shown). Thalidomide treatment in vivo enhanced the release in vitro of IFNγ by PBMCs in response to mycobacterial antigens regardless of the patient's HIV status. The heightened response to both BCG and PPD was transient, and IFNγ levels returned to baseline levels after discontinuation of thalidomide treatment (Fig. 1 and Table 5). Thalidomide treatment did not appreciably alter levels of IFNγ mRNA expressed in freshly isolated PBMC (not shown). Placebo treatment had little effect on PPD- and BCG-stimulated release of IFNγ by PBMCs (Fig. 1 and Table 5).

TNFα PRODUCTION.   To determine whether treat-ment with thalidomide caused changes in production of inflammatory cytokines, PBMCs were stimulated with a variety of agonists in vitro. In one series of experiments, PBMCs isolated from all patient bloods during the first treatment cycle were treated with LPS in vitro, and TNFα released into the culture supernatant was quantitated. At the midpoint of thalidomide treatment (7 days), there was a reduction in TNFα levels released by LPS stimulated PBMCs when compared with the prestudy levels (Fig. 2). The inhibitory effect of thalidomide treatment on TNFα production was observed in HIV-1$^+$ as well as HIV-1$^-$ patients. Thalidomide treatment also reduced the amount of TNFα produced in vitro in response to BCG (Table 5) and PPD (not shown). The thalidomide-induced reduction of TNFα re-
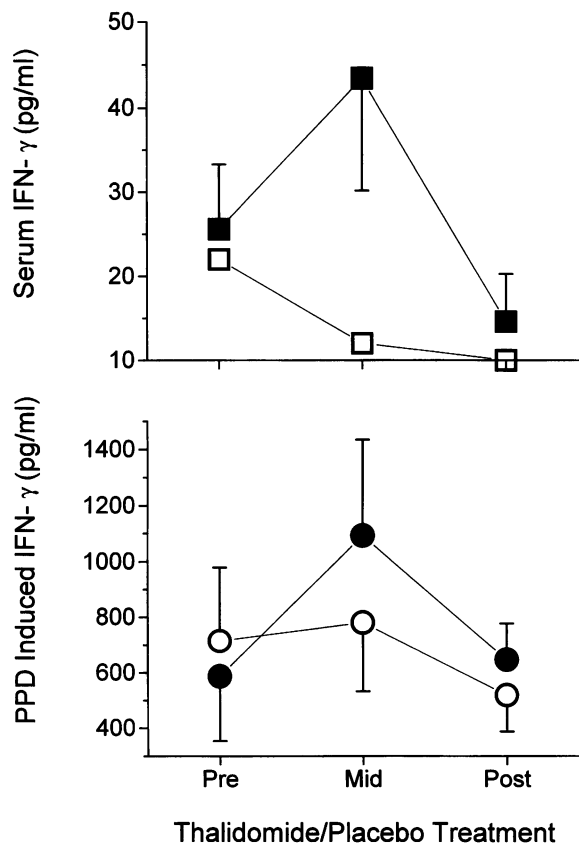
FIG. 1. The effect of thalidomide treatment on IFNγ production

IFNγ levels were evaluated by ELISA in the serum of patients (top) and in the supernatants of PBMCs obtained from patients and stimulated in vitro with PPD (bottom). (Top) 14 patients were treated for 14 days with 300 mg/day of thalidomide (■). Serum levels were significantly higher after 7 days on the drug (Mid) relative to the pretreatment time point ($p = 0.05$) and significantly lower after discontinuation of treatment (Post) ($p = 0.005$). Ten patients were treated with MDT and placebo (□). (Bottom) release of IFNγ into the culture supernatants by patient PBMCs stimulated in vitro with PPD before, during, and after thalidomide (●) or placebo treatment (○). Results are means ± one SEM.

FIG. 2. The effect of thalidomide treatment on TNFα production in vitro

TNFα levels were evaluated by ELISA in the supernatants of PBMCs obtained from 12 HIV-1⁻ and 10 HIV-1⁺ patients treated with MDT and 300 mg/day of thalidomide (left) and eight patients (5 HIV-1⁻) treated with MDT and placebo (right). PBMCs obtained before (Pre) on Day 7 of treatment (Mid) and 5 days after (Post) the first cycle of treatment were stimulated with LPS and culture supernatants were collected for assay. Results are means ± one SEM for samples obtained from all patients tested. TNFα levels were significantly lower during treatment with thalidomide relative to levels before ($p = 0.001$) or after ($p = 0.002$) thalidomide.

lease was transient, and, for most patients, levels of cytokine released by the cells returned to baseline or higher after thalidomide treatment was discontinued (Fig. 2 and Table 5). It is noteworthy that cells from patients receiving placebo failed to show any reduction in TNFα release at the midstudy time point, regardless of their HIV-1 status (Fig. 2 and Table 5).

Since the effect of thalidomide treatment on TNFα release by the cells in response to LPS was most pronounced at the midpoint of treatment

and waned when thalidomide treatment ended, a group of patients from Group II was treated with a second 14-day cycle of thalidomide after a 7-day washout period. When PBMCs were obtained from patients during the second cycle of thalidomide treatment and stimulated with LPS, the effect on TNFα levels was reproduced (Fig. 3).

To test whether the thalidomide-induced reduction in TNFα-producing capacity of PBMC was correlated with lower circulating TNFα levels in the patients, serum TNFα levels were measured during two consecutive 14-day cycles of thalidomide treatment (with washout periods between treatment cycles). When serum from patients was assayed for TNFα during repeated cycles of thalidomide treatment, a decrease in serum TNFα was observed at the midpoint of each thalidomide treatment (Fig. 3). In addition, mRNA was isolated from unstimulated PBMCs of patients receiving the sequential cycles of thalidomide treatment. Analysis of levels from unstimulated PBMC during thalidomide treatment, showed decreases in the number of copies of TNFα mRNA, mirroring the decrease in serum TNFα levels (Fig. 3).

TABLE 5. BCG induced cytokine production by PBMC

Cytokine	Thalidomide treatment $(n = 18)^a$			Placebo treatment $(n = 8)^a$		
	Pre	Mid	Post	Pre	Mid	Post
TNFα	1871 ± 601	1274 ± 565	2504 ± 793	1875 ± 797	2283 ± 775	1815 ± 790
IFNγ	218 ± 122	549 ± 330	218 ± 58	197 ± 69	236 ± 79	231 ± 55

TNFα and IFNγ levels were assayed by ELISA (as described in Materials and Methods) in the supernatants of PBMCs obtained from patients treated with either thalidomide or placebo. Results are expressed as pg/ml.
[a]The means for the number of patients shown in the parentheses ± SEM are given.

IL-1 PRODUCTION. TNFα and IL-1 are usually produced by PBMC in response to the same stimuli in vitro (e.g., LPS). Since thalidomide treatment of tuberculosis patients reduces the capacity of

PBMC to release TNFα, it was important to determine whether the production of other inflammatory cytokines, such as IL-1, was similarly affected. We therefore examined the plasma and PBMC for production and release of IL-1. IL-1 was not detected in either plasma or serum from patients receiving thalidomide. However, IL-1 release by LPS-stimulated PBMC in vitro was reduced by thalidomide treatment similar to the reduction in TNFα levels (Fig. 3). IL-1 levels rebounded after cessation of thalidomide treatment, but when thalidomide treatment was resumed, IL-1 levels again were reduced at the treatment midpoint (Fig. 3). No significant effect on IL-1 mRNA levels expressed by freshly tested PBMC was observed in response to multiple cycles of thalidomide treatment (Table 6).
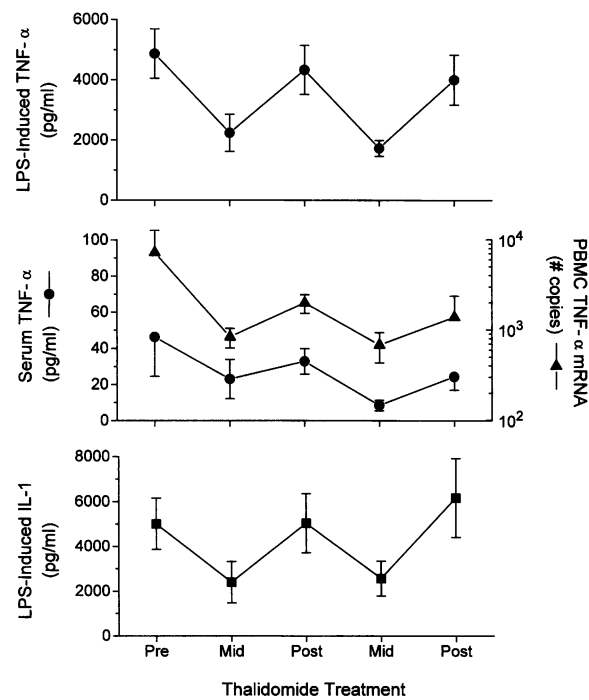
Effect of Thalidomide on Body Weight

Because thalidomide treatment causes a decrease in serum TNFα, and TNFα has been implicated in wasting, the effect of thalidomide on body weight was evaluated daily during the first cycle of treatment. Daily administration of thalidomide resulted in a significant enhancement of

FIG. 3. The effect of thalidomide treatment on TNFα and IL-1 production

In vitro LPS induced release of TNFα (top) and IL-1 (bottom) from PBMC. Serum TNFα levels and PBMC TNFα mRNA levels (middle). Results are means ± one SEM for samples obtained from five patients treated with two 14-day cycles of thalidomide (Material and Methods). TNFα mRNA levels and LPS induced TNFα were significantly lower as compared with the pretreatment levels ($p < 0.008$ and $p < 0.005$ respectively).

TABLE 6. IL-1β mRNA^a in PBMC from patients^b during thalidomide treatment

Pre	Mid	Post	Mid	Post
20 ± 9	14 ± 5	16 ± 6	16 ± 11	13 ± 7

[a]RNA copy number × 10^{-3} per 10 ng total RNA.
[b]$n = 5$ patients tested. Results are means ± SEM.

weight gain in tuberculosis patients (Fig. 4). Some of the patients receiving placebo also gained weight, but to a lesser extent than the thalidomide treated patients. Both HIV-1$^-$ and HIV-1$^+$ patients gained weight similarly (not shown). Upon withdrawal of thalidomide at 14 days, a reversal or cessation of weight gain was observed (Fig. 4).

In order to evaluate the reproducibility and persistance of the effect of thalidomide on weight gain, six of the Group II (New York only) patients were treated with sequential cycles of thalidomide or placebo in the following manner. Three patients were treated with a 14-day cycle of thalidomide, followed by a 7-day washout period, and then retreatment with thalidomide (Fig. 5). Three patients were treated with a cycle of thalidomide, followed by a washout period, followed by treatment with placebo (Fig. 5). Finally, two patients were treated with placebo during the first 14-day cycle, followed by a washout period, and then followed by thalidomide treatment (Fig. 5). The rate of weight gain was significantly higher while patients were receiving thalidomide, whether or not they had been treated with thalidomide in previous cycles. Moreover, the rate of weight gain was much lower while patients were on placebo or during the washout period. If patients were treated in the first cycle with thalidomide and then received placebo during the second treatment cycle, they stopped gaining weight at the accelerated rate, but these patients did not lose the weight gained during the thalidomide treatment. As a group, patients on placebo gained less weight than patients on thalidomide. This occurred despite maintaining a relatively constant caloric intake before, during, and after thalidomide and/or placebo treatment (Fig. 6). Thus, the weight gain during thalidomide treatment did not appear to be dependent on a change in caloric intake.

DISCUSSION

TNFα is an important regulatory cytokine of host response to bacterial and parasitic infections (1). A delicate balance must be maintained: enough TNFα must be produced to stimulate host immunity, but overproduction of this cytokine may have extremely deleterious effects. Increasing attention has therefore focussed on thalidomide, a therapeutic agent that appears to down-regulate TNFα overproduction without completely suppressing it (12–14). To determine whether tha-

lidomide might affect TNFα production and the subsequent disease course in tuberculosis, we carried out a two-part placebo-controlled pilot study of thalidomide treatment in patients with tuberculosis and wasting. Our observations showed that thalidomide at a dose of 300 mg per day for 14 days was well tolerated with minimal side effects. No adverse clinical manifestations were noted in either patients infected with *M. tuberculosis* alone or patients with dual infections of tuberculosis and HIV-1.

For thalidomide to be clinically useful in controlling the consequences of excessive TNFα production during a chronic infectious process, it should not adversely affect other parameters of host immunity. Therefore, the possible effects of thalidomide treatment on host immunity in tuberculosis were investigated in a number of ways. First, delayed type hypersensitivity was assessed in each patient during the study. Our results show that the response to PPD was not diminished during or after administration of thalidomide, as quantitated by skin test responsiveness (Table 2). On the contrary, there was evidence of enhanced reactivity. That is, HIV-1$^-$ and HIV-1$^+$ patients who were initially nonresponsive to mycobacterial antigen, developed skin test responsiveness during the administration of thalidomide. This was not observed in placebo-treated patients, suggesting that the observed reactivity was not due to a booster effect of repeated PPD administration.

Administration of thalidomide did not cause any significant changes in total leukocyte count or differential cell count (Table 3), nor was there any significant effect on lymphocyte subset distribution in either HIV-1$^+$ or HIV-1$^-$ patients. In addition, when PBMCs were isolated from patients during the course of thalidomide treatment, there was no change in the proliferative capacity of the PBMCs following in vitro stimulation with antigens (Table 4). Thalidomide did not suppress cell-mediated immunological responses in vitro during the treatment cycle.

The effect of thalidomide treatment on cytokine production was evaluated in all patients during the study. Thalidomide treatment resulted in increased levels of IFNγ secretion both in vivo, in the serum of patients, and in vitro by PBMCs stimulated with mycobacterial preparations (Fig. 1). This effect was observed in both HIV-1$^+$ and HIV-1$^-$ patients. The apparent immunostimulatory effect of thalidomide was limited to the treatment cycle, and when thalidomide therapy ceased, the effect waned (Fig. 1).
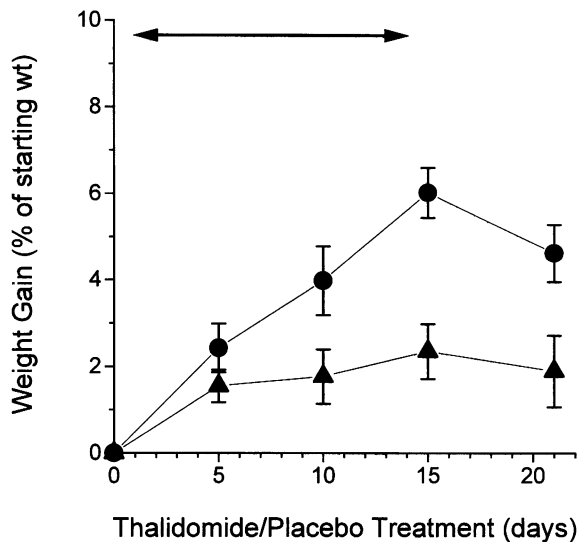
FIG. 4. The effect of thalidomide treatment on weight gain

Weight gain is expressed as the mean of the percent of body weight gained relative to starting weight ± one SEM for 21 patients treated for 14 days with MDT and 300 mg/day of thalidomide (●) and eight patients treated for 14 days with MDT and placebo (▲). The arrow above the figure denotes the duration of treatment with thalidomide or placebo. On Days 15 and 21, weight gain in all thalidomide-treated patients was significantly higher than that of placebo-treated patients ($p = 0.003$ and 0.04, respectively).

Thalidomide/Placebo Treatment (days)

FIG. 5. The effect of thalidomide on weight gain during two cycles of treatment

Weight gain was expressed as the percent of body weight gained relative to starting weight for each patient tested. Patients were evaluated during two cycles of treatment with thalidomide followed by a washout period and another cycle of thalidomide (three patients) (top), thalidomide followed by a washout and a placebo cycle (three patients) (middle), and placebo followed by a washout and a thalidomide cycle (two patients) (bottom). The arrows above the graphs denote thalidomide treatment (solid) and placebo treatment (broken). Statistical analyses of the differences in the slopes of each curve showed that weight gain was significantly different on thalidomide versus washout or placebo ($p = 0.0043$ and 0.031, respectively) and not significantly different between placebo and the washout period ($p = 0.36$).

The effect of thalidomide treatment on TNFα production, both in vitro and in vivo, was pronounced. PBMCs were isolated from patients during the study and then stimulated in vitro, with a variety of agonists. Thalidomide treatment of tuberculosis patients was clearly associated with a reduced production of TNFα in vitro by stimulated PBMCs (Fig. 2). The effect of thalidomide treatment on TNFα levels was transitory, and cytokine levels returned to baseline when thalidomide treatment ended. However, when patients were treated with a second 14-day cycle of thalidomide, following a 7-day washout period, the effect on TNFα was repeated and TNFα levels in vitro again declined (Fig. 3). These results showed that continuous thalidomide treatment was necessary to maintain the reduction in PBMC capacity to produce TNFα.

Simultaneously, we investigated the effect of thalidomide treatment on TNFα production in the patients. It had previously been noted that circulating TNFα levels are notoriously difficult to detect (16). In our study, serum or plasma TNFα was not detected by the Endogen ELISA kit

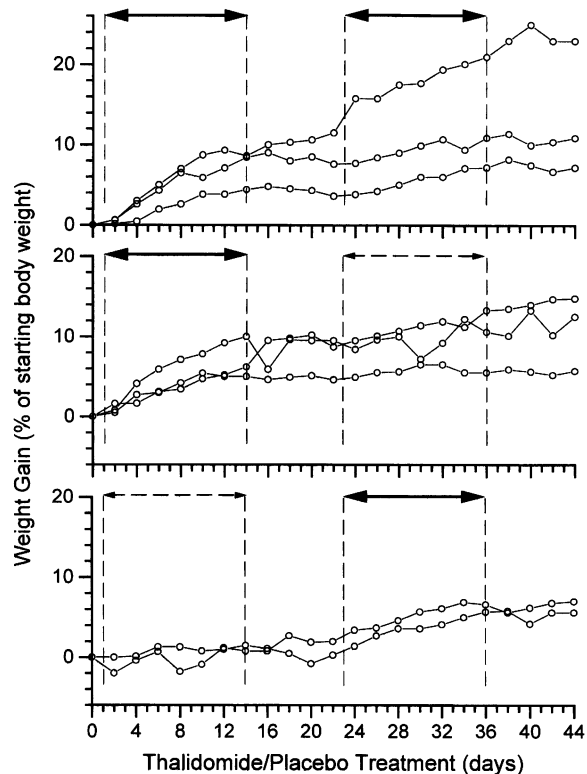(see Materials and Methods) which detects free, non-receptor-bound TNFα. However, TNFα was detected by the Medgenix kit (Materials and Methods) which detects total TNFα, whether or not it is bound to its receptor. When serum from patients was assayed with the latter kit, a reduction in levels of TNFα in the circulation of patients treated with thalidomide was noted. Again, the reduction was dependent on continuous treatment with thalidomide; when thalidomide was not given, TNFα levels returned to
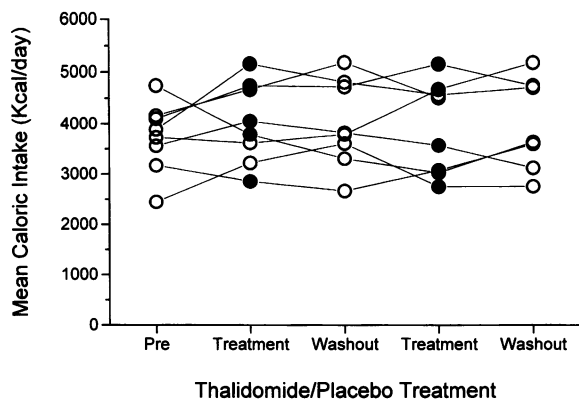
FIG. 6. Effect of thalidomide treatment on caloric intake in tuberculosis patients
Eight patients were treated as described in Fig. 5. Results are expressed as mean kcal/day for each patient. (O) mean caloric intake while on placebo or during the washout period. (●) mean caloric intake while on thalidomide.

baseline. TNFα mRNA levels in unstimulated circulating leukocytes, a sensitive assay for TNFα gene activation, were also decreased during thalidomide treatment (Fig. 3). This is a possible explanation for why cells from these patients release less of this cytokine in vitro in response to agonists.

We had previously shown that a short thalidomide pulse in vitro to PBMCs stimulated with LPS inhibited TNFα without affecting levels of IL-1 production (13). In the present study, the relatively long cycle of thalidomide administration in vivo had no significant effect on serum IL-1 or IL-1 mRNA levels. However, thalidomide treatment in vivo did cause a reduction in IL-1 release by LPS stimulated PBMCs in vitro (Fig. 3). These apparently contradictory effects may be due to the sensitivity of PBMC IL-1 production to TNFα levels, which are in turn, modulated by the duration of thalidomide treatment in vivo.

All patients in this study gained weight during treatment with thalidomide. While some of the patients treated with MDT and placebo also gained weight, the weight gain of patients treated with thalidomide was significantly greater than that of the placebo-treated group. After the cessation of thalidomide treatment, some patients lost some weight while other ceased to gain weight (Figs. 4 and 5). These observations suggest that thalidomide could be used clinically to accelerate weight gain during anti-tuberculosis therapy. In this study, weight gain was not a consequence of increased caloric intake, since there was no significant difference in this parameter between thalidomide and placebo treatment. Some of the thalidomide in-

duced weight gain persisted for at least 4 weeks after the end of thalidomide treatment (Fig. 5), suggesting that the weight gain was not due merely to transient fluid retention, nor was it due solely to MDT, since placebo-treated patients did not gain as much weight. However, measurement of total body and/or lean body mass are required to conclusively demonstrate the nature of the weight gain during thalidomide treatment. The results presented here suggest that thalidomide may exert a metabolic effect, perhaps mediated through TNFα levels. The nature of this potential metabolic change is being investigated in ongoing studies.

The results of our present study indicate strongly that thalidomide reduces TNFα production in both uncomplicated tuberculosis and tuberculosis associated with HIV-1 infection. The decrease in TNFα production was not associated with any clinical deterioration or negative effects on host immunity. How treatment with thalidomide impacts on the cytokine regulatory circuit and results in reduced TNFα production is as yet unknown. Our results suggest that cachexia may, at least in part, be mediated by chronic production of TNFα. Thalidomide therapy might thus be instrumental in reversing the debilitating symptoms of *M. tuberculosis* infection as well as in another syndrome with associated cachexia, the acquired immunodeficiency syndrome (17). Also, the reduction in TNFα induced by thalidomide may have profound effects on other physiologic pathways that are modulated by cytokine levels. For example, we have recently shown that thalidomide induced reduction in TNFα is associated with a reduction in activation of latent HIV-1 in vitro (18). The mechanism of this effect and its potential effect on clinical course is currently under investigation in our laboratory.

ACKNOWLEDGMENTS

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