Thalidomide-Induced Antigen-Specific Immune Stimulation in Patients with Human Immunodeficiency Virus Type 1 and Tuberculosis

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Thalidomide, which inhibits monocyte tumor necrosis factor (TNF-α) production and co-stimulates T cells, was tested for immune modulation in patients with human immunodeficiency virus (HIV) infection and tuberculosis (TB) in a placebo-controlled study. Thalidomide therapy resulted in increased levels of plasma interleukin (IL)-2 receptor, soluble CD8, interferon-γ, and IL-12, indicating immune stimulation. TNF-α levels were not reduced. Thalidomide treatment increased CD4+ and CD8+ T cell counts and lymphocyte proliferation to purified protein derivative. Immune stimulation was not associated with an increase in plasma HIV levels. In vivo, a thalidomide dose-dependent costimulatory effect on T cell proliferation and HIV replication was observed after stimulation with antigens or anti-CD3, respectively. Thalidomide-induced increased viral replication in CD4+ T cells was abrogated by adding back autologous CD8+ T cells. Thus, in the presence of thalidomide, antigen-specific immune responses in vitro and in patients with HIV/TB were enhanced.

Tuberculosis (TB) is highly prevalent in South Africa, with a maximal incidence of up to 800–1200 per 100,000 in parts of the Western Cape [1]. In addition, TB is the most common coinfection in patients with human immunodeficiency virus type 1 (HIV-1) infection in South Africa, causing severe morbidity and accelerated mortality [2]. This is probably the result of the immune suppression of HIV infection, which appears to lead to a greater susceptibility to development of active disease after exposure to Mycobacterium tuberculosis [3], as well as an increased rate of reinfection and/or a higher incidence of disease reactivation after anti-TB therapy [4, 5].

TB is a potent inducer of HIV replication and results in up to a 160-fold increase in virus particles in plasma [6], probably because virus replication is stimulated by the immune activation triggered by concomitant infection [7]. Immune activation after administration of recombinant interleukin (IL)-2 [8] or immunization [9] also results in increased virus load. In vitro, antigenic or mitogenic stimulation of blood cells from HIV-infected individuals results in increased viral replication and increased cell infection [10, 11].

Tumor necrosis factor (TNF-α) is a proinflammatory molecule produced during the course of immune activation [12]. Plasma levels of this cytokine are higher in patients with concomitant HIV and TB infections (HIV/TB) than in patients infected with HIV or M. tuberculosis alone [13]. TNF-α induces HIV replication via a shared NFκB-dependent transcription control mechanism. Increases in the levels of this proinflammatory cytokine may, therefore, contribute to enhanced viremia, as well as to the wasting observed in HIV/TB coinfection [14, 15]. Recently, we observed that initiation of anti-TB therapy in TB patients often results in clinical worsening, including weight loss associated with a transient increase in plasma levels of TNF-α [16].

Thalidomide is an immunomodulatory drug that inhibits monocyte TNF-α production in vivo and in vitro [17, 18] but does not affect production of TNF-α by T cells [19]. The drug also acts as a costimulator of human T cells in vitro, resulting in increased production of Th1 cytokines (interferon [IFN]-γ and IL-12) [19, 20]. In HIV-infected patients, thalidomide stimulates T cell responses and IL-12 production [21]. To test whether thalidomide would affect the production of TNF-α and of Th1-type cytokines (IFN-γ and IL-12) in HIV-infected patients with TB, we performed a double-blind, placebo-controlled study. We studied the ability of the drug to stimulate antigen-specific T cell responses in vivo and in vitro, as well as the effect of the drug on HIV levels in vivo and on HIV replication in vitro.

Received 3 August 1999; revised 2 September 1999; electronically published 8 March 2000.


Informed consent was obtained from all patients. Human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of this clinical research. The study was approved by the Ethics Committee at the University of Cape Town and by the Institutional Review Board of the Rockefeller University, New York.

Financial support: World Health Organization Global Program on AIDS (WHO AIDS; Geneva, Switzerland); research grant from Direct Effect (New York.

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The Journal of Infectious Diseases 2000;181:954-65
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0022-1899/2000/18103-0020$02.00

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Subjects and Methods

Study patients. Thirty patients seropositive for HIV-1 infection who had never received antiretroviral therapy and had been hospitalized with a recent diagnosis of sputum-smear–positive pulmonary TB were enrolled in the study. Exclusion criteria included patients in whom multidrug-resistant M. tuberculosis was subsequently identified, patients in whom treatment with immunomodulatory or antiretroviral agents had occurred, and patients in whom an additional opportunistic infection had occurred within 4 weeks of the recruitment date. The patients were recruited over a period of 8 months from 3 acute inpatient hospitals in Cape Town. Patients were hospitalized for the duration of the study in a TB hospital in the Cape Town area.

Female patients with childbearing potential underwent pregnancy tests within 72 h before joining the study and weekly thereafter. Female patients were required to practice 2 methods of contraception initiated before taking the study drug and to continue contraception use for the duration of treatment and for at least 4 weeks thereafter. In most cases, long-acting, intramuscular progesterone and condoms were the methods of choice.

In addition, blood was obtained from 8 asymptomatic HIV-infected patients for in vitro studies (as discussed later). The median age of the patients was 42 years (range, 27–52 years), and the median CD4 cell count was 521/mm^3 (range, 380–657/mm^3); 4 patients were women and 4 were men, and 6 of the 8 patients were receiving highly active antiretroviral therapy. The patients in this group did not receive treatment with thalidomide.

Treatment regimen. HIV/TB patients were enrolled into the study within 7 days of the initiation of a standard 4-drug regimen (rifampicin, 10 mg/kg; isoniazid, 5 mg/kg; pyrazinamide, 30 mg/kg; and ethambutol, 25 mg/kg) of anti-TB therapy. All patients were also treated daily with 25 mg of oral pyridoxine. Patients were randomized in a double-blind manner to receive either thalidomide (200 mg/day) or an identical placebo provided by Celgene Corporation (Warren, NJ). The drug was given orally at night. Pharmacokinetic studies in HIV-infected patients have shown that a single oral dose of 200 mg of thalidomide resulted in plasma levels of 1.9 μg/mL [22]. Randomization was done by a research pharmacist at a ratio of 2 : 3 (placebo: thalidomide), and the code was deciphered at the end of the study. This ratio was chosen to accommodate an anticipated higher dropout rate in the thalidomide-treated group owing to the risk of drug-induced rashes, as reported elsewhere [23]. The study drug was administered for 42 days, followed by a 2-week observation period. If a drug rash developed, all drugs were stopped, and patients were treated symptomatically with antihistamines and antipyretics until the rash disappeared. Subsequently, medications were reintroduced sequentially at half-dose for 2 days, then at full dose if tolerated. Rechallenge was not attempted if there was any mucosal or ocular involvement. Standard anti-TB therapy was continued throughout and after the completion of the study. Antiretroviral therapy is limited in Africa; these patients did not receive antiretroviral therapy and are thus representative of the overwhelming majority of HIV-infected individuals in the third world.

Clinical evaluation. Initial baseline assessment of each patient was done, including full history and physical examination. A careful assessment was made for peripheral neuropathy. A Karnofsky score was assigned for each patient. Patients were weighed, their body mass index was calculated, their right midarm circumference was measured, and their triceps skin-fold thickness was determined by use of a Lange skinfold caliper (Beta Technology, Cambridge, MD). We did not record caloric intake, but all patients received a standard hospital diet. In many cases, standard hospital diet constituted an improvement over the patient’s typical diet.

The assessments were repeated at weekly intervals for the duration of the study. Signs of drug toxicity were regularly (at least weekly) sought throughout the study, with particular attention paid to signs of peripheral neuropathy, rash, or excessive sedation.

Clinical laboratory measurements. Full blood count, serum chemistry, and urinalysis were performed on samples obtained from all patients at study day 0 and every 3 weeks thereafter (days 0, 21, and 42). A final blood sample was also collected at 2 weeks after treatment (day 56).

Bacteriology. Sputum was collected on day 0 and sent for M. tuberculosis smear, culture (Bactec; Becton Dickinson, San Jose, CA), and sensitivity to isoniazid and rifampicin. Sputum was collected and reexamined weekly if still available.

Virologic measurements. EDTA-anticoagulated plasma samples were collected on days 0, 21, 42, and 56 and were frozen at −70°C until assayed. HIV-1 titers were measured by nucleic acid sequence–based amplification (Organon Teknika, Oss, the Netherlands). Virus titers in equivalents per milliliter were expressed as log_{10} RNA units per milliliter.

Plasma cytokines and markers of T cell activation. Blood samples collected in EDTA were used for these studies. The following cytokines and soluble markers of T cell activation were measured in plasma by ELISA, in accordance with each manufacturer’s instructions: total TNF-α (unbound and bound to soluble TNF-α receptor), TNF-α receptor (TNF-αR), IFN-γ, IL-6, and IL-10 (all from Medgenix, Fleuris, Belgium); soluble IL-2R (sIL-2R; Genzyme, Cambridge, MA); and IL-12 (this assay measures both p40 and p70 subunits of IL-12) and soluble CD8 (sCD8) antigen (Endogen, Woburn, MA). For each cytokine kit, the range of expected values for normal control is provided by the manufacturer and was confirmed in our laboratory for each batch of kits.

T cell subsets. T lymphocyte subsets (CD3⁺ CD4⁺ and CD3⁺ CD8⁺) in peripheral blood were enumerated by flow cytometry (FACSCOUNT; Becton Dickinson) at days 0, 21, 42, and 56.

M. tuberculosis–specific immune responses. Skin tests for delayed-type hypersensitivity were conducted at baseline and on day 42. Five units of purified protein derivative (PPD) of tuberculin (Connaught Laboratories, Willowdale, Ontario, Canada) was injected intradermally into the volar aspect of the left forearm, and induration in 2 perpendicular diameters was measured 48 h later. An induration ≥5 mm in diameter was considered positive [24].

Peripheral blood mononuclear cells (PBMC) and lymphocyte proliferation assay. PBMC were isolated by Ficoll-Hypaque gradient centrifugation, as described elsewhere [25], and resuspended at a density of 2 × 10⁶/mL in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% human AB+ serum (Biocell, Carson, CA), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (R10) (Gibco). Aliquots of 100 μL of cell suspensions in R10 were added to wells of 96 round-bottom tissue culture plates
in triplicates (Costar, Corning, NY). R10 medium (unstimulated control) or R10 medium containing PPD (Statens Seruminstitut, Copenhagen, Denmark) (stimulus) was added to obtain a final concentration of 20 μg/mL of PPD per well. Plates were incubated for 5 days at 37°C in 5% CO₂. On day 5, 1 μCi of tritiated thymidine ([³H]TdR; New England Nuclear, Boston) was added to each microwell for the last 18 h of culture. DNA was then harvested onto fiber mats, which were dried and immersed in scintillation fluid for counting β emission. Data are presented as a stimulation index (SI), calculated as counts per minute (cpm) in the presence of PPD per cpm in the culture medium alone.

In vitro analysis of thalidomide effect on antigen-specific immune responses. Blood was obtained from HIV-infected individuals, and PBMC were isolated as described earlier.

Monocyte antigen-presenting cells. PBMC (3 × 10⁶) were incubated in 1 mL of RPMI 1640 containing 2% pooled human serum and penicillin/streptomycin (R2) in 12-well polystyrene tissue culture plates (Costar) for 1 h at 37°C. Subsequently, nonadherent cells were washed off with warmed RPMI. The remaining adherent cells (monocyte antigen-presenting cells [M-APCs]) were reincubated in R2 overnight with or without antigens (as discussed later). M-APCs were then detached by incubation on ice for 20 min and vigorous pipetting, washed 3 times, and resuspended.

Dendritic cells (DCs). DCs were prepared from progenitor cells derived from PBMC by an adaptation of the method of Bender et al [26]. Briefly, PBMC were incubated with neuraminidase-treated sheep red blood cells (SRBCs) on melting ice for 40 min to remove T cells. Non-SRBC-rosetting cells were subsequently isolated by density centrifugation. The latter cells were then plated at 10⁷ cells in 1 mL/well in 12-well tissue culture plates in RPMI containing 1% autologous plasma, 10 mM HEPES buffer, and penicillin/streptomycin. Recombinant human IL-4 (Genzyme) and granulocyte-macrophage colony-stimulating factor (Immunex, Seattle) at 1000 U/mL each were added on days 0, 2, and 4, and the cells were incubated for 7 days. The cells thus derived exhibited the characteristic stellate morphology of immature DCs and were strongly HLA-DR⁺ and CD14⁺ by flow cytometry. The DCs were “pulsed” with antigens for 4 h while being maintained in the cytokine-supplemented media. Subsequently, DCs were harvested and washed 3 times. Each preparation of DCs was divided into 2 aliquots: 1 was added directly (live DCs) to autologous purified CD4⁺ T cells, and the other was fixed for 30 s in 0.05% glutaraldehyde, as described elsewhere [19], followed by 3 additional washing steps before T cell coculture.

CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells were purified from PBMC by positive selection by use of antibody-coated magnetic beads (Dynal, Oslo, Norway), in accordance with the manufacturer’s instructions. Positively selected cells were separated from the beads by a further incubation with a secondary antibody that competes for the binding site of the primary antibody (Detachabead; Dynal). Flow cytometric analysis revealed that cells isolated in this way were 95.3%–99.4% CD3⁺ CD4⁺ and 97.0%–99.0% CD3⁺ CD8⁺.

Incubation with antigens. M-APCs and DCs were incubated with the following antigens: PPD (Statens Seruminstitut), 10 μg/mL; recombinant baculovirus-derived HIV-1 p24 gag antigen or a control baculovirus protein (Protein Sciences, Meriden, CT), 10 μg/mL; tetanus toxoid (Statens Seruminstitut), 10 μg/mL; cytomegalovirus (CMV) antigen (1 : 100 dilution of CMV-infected cell line culture supernatant (Biowhittaker, Walkersville, MD); control antigen (diluted supernatant of uninfected culture); or no antigen (negative control).

Antigen-presenting cells were added to purified autologous CD4⁺ T cells in triplicate cocultures in 96-well plates, so that each well contained 10⁵ T cells and M-APCs (ratio of T : M-APC, 3 : 1) or DCs (T : DC, 10 : 1) in a total volume of 200 μL of R10 culture medium. Thalidomide (Celgene) was dissolved in dimethyl sulfoxide (DMSO), to give a stock solution of 20 mg/mL, and immediately added to the cultures in serial dilutions to achieve the final concentrations indicated in the Results section. Dilutions were made so that all culture conditions contained the same concentration of DMSO. Cultures were treated daily with fresh DMSO/thalidomide in 50% volume exchanges.

On the 6th day of culture, 1 μCi of [³H]TdR was added to each well for 6 h, and DNA was harvested as described earlier. Data are presented as cpm (mean of triplicate cultures).

In vitro analysis of the effect of thalidomide on HIV activation in T cells. PBMC or T cells were stimulated by cross-linking CD3, a component of the T cell receptor complex. Forty-eight-well flat-bottomed tissue culture plates were coated with mouse anti-human CD3 monoclonal antibody (a kind gift of Dr. Robert Zivin, Orthobiotech, Raritan, NJ) at a coating concentration of 1–10 μg/mL, as described elsewhere [19]. For each experimental condition, duplicate cultures were set up at 0.5–1.0 × 10⁶ cells per well (either purified T cells or PBMC) in 48-well plates. Cultures were treated daily with thalidomide at various concentrations, as described earlier. At different time points, culture supernatants were collected and stored at −70°C for assay of HIV p24 antigen by ELISA (Immunotech, West Rook, ME) in accordance with the manufacturer’s instructions.

Statistical analysis. Student’s paired t test was used for continuous variables when follow-up results were compared with baseline results within a treatment group. To analyze differences between the 2 treatment groups, Student’s unpaired t test was used. All calculations (Microsoft, Richmond, WA).

Results

Clinical outcome and adverse events. Thirty HIV-1/TB-infected patients were randomized to receive thalidomide (n = 18) or placebo (n = 12). There were no significant differences in clinical characteristics and immunologic markers between the 2 groups at baseline (table 1). Three patients were excluded early in the study. One (assigned to thalidomide) was infected with multidrug-resistant M. tuberculosis, as noted when mycobacterial sensitivities became available. An excluded 2d patient (assigned to thalidomide) had a complicating infection (bacterial empyema) at the time of enrollment. The 3d excluded patient (assigned to placebo) developed evidence of early HIV-1–associated dementia within a week of study commencement and withdrew consent. Of the 27 remaining patients, 11/11 in
the placebo group and 14/16 in the thalidomide group completed the study. Two patients (patients 2 and 21, both assigned to thalidomide) died. Postmortem examinations were performed, confirming the causes of death as hepatitis (probably induced by the anti-TB drugs) and Pneumocystis carinii pneumonia (considered unrelated to the study drug), respectively (table 2).

In the 14 patients assigned to thalidomide who completed the study, the dose of 200 mg/day of thalidomide appeared to be well tolerated, with no dropouts related directly to therapy (table 2). Two patients complained of increased tingling of their feet, but there were no objective clinical changes to suggest progressive peripheral neuropathy. Doubling of the dose of pyridoxine (to 50 mg) and addition of amitriptyline (25 mg) at night led to resolution of all symptoms. Three patients developed maculopapular rash, with fever and erythema, typical of the rash seen previously in thalidomide-sensitive patients [23] (table 2). No mucocutaneous or ocular involvement was present in any of these patients, and thalidomide was successfully rechallenged and completed a full course of anti-TB drugs (table 2).

The patients included in the study were severely immunosuppressed individuals who required hospitalization for TB. Despite the severity of disease, patient responses to antibiotic treatment of TB with or without thalidomide were similarly good in both study groups. After the commencement of anti-TB therapy, Karnofsky scores (used as a subjective measure of patient well-being), shown as mean ± SD, showed a significant improvement, from 49.4 ± 11.97 and 50.83 ± 10.84 to 91.53 ± 16.75 and 92.7 ± 12.72 for thalidomide and placebo, respectively.

The mean baseline weight in both groups was similar (50.3 kg in the thalidomide and 51.3 kg in the placebo group). Patients treated with anti-TB drugs and either thalidomide or placebo experienced significant (P < .004 for both groups) weight gain beginning at day 21 (figure 1). A similar steady increase in weight was observed in both groups.

The mean body mass index for all patients studied at baseline (range, 15–30; mean, 18.6) was below the normal values expected for this adult population (normal range, 20–25). In both study groups, there was a similar significant increase (P < .02 for both groups) in body mass index beginning at day 21 after hospitalization and commencement of anti-TB therapy (figure 1). No significant difference in increase in body mass index was seen between the 2 groups.

An increase in triceps skin thickness and midarm circumference was measured in both groups of patients. The skin-fold thickness increased in both groups, but the increase from baseline was statistically significant only in the thalidomide-treated patients (P < .05; figure 1). Although there was a clear trend toward a larger increase in skin-fold thickness in response to thalidomide, the difference between the thalidomide- and placebo-treated patients was not statistically significant.

**Sputum collection and culture of M. tuberculosis.** Sputum collected weekly was evaluated for smear positivity and growth of M. tuberculosis. The median time to negative culture or the time to inability to produce sputum was found to be 7 days (range, 3–14 days) in the thalidomide-treated and 14 days (range, 7–42 days) in the placebo-treated patients. The accelerated clearance of acid-fast bacilli from the sputum of the thalidomide-treated patients compared with that of the placebo-treated patients was not statistically significant.

**Virus load.** The effect of anti-TB therapy and either thalidomide or placebo treatment on plasma HIV-1 levels was evaluated. At baseline, the mean ± SE plasma HIV-1 titer in

### Table 1. Patient characteristics at baseline.

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Treatment group</th>
<th>Placebo (n = 12)</th>
<th>Thalidomide (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td>32.4 (8.6)</td>
<td>34.1 (11.6)</td>
</tr>
<tr>
<td>Sex, male : female</td>
<td></td>
<td>4 : 8</td>
<td>9 : 7</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
<td>18.8 (2.9)</td>
<td>18.7 (3.4)</td>
</tr>
<tr>
<td>CD4⁺ cells/mm³</td>
<td></td>
<td>94 (46)</td>
<td>97.4 (23)</td>
</tr>
<tr>
<td>Virus load, RNA U/mL</td>
<td></td>
<td>Log₁₀ 5.25 (0.18)</td>
<td>Log₁₀ 5.57 (0.22)</td>
</tr>
<tr>
<td>Karnofsky score</td>
<td></td>
<td>50 (10.8)</td>
<td>49 (11.9)</td>
</tr>
</tbody>
</table>

**NOTE.** All parameters are calculated as means. SDs are given in parentheses.

BMI, body mass index.
Figure 1. Effect of thalidomide treatment (horizontal arrows) on patient weight, body mass index, and triceps skin-fold thickness. Patients receiving thalidomide (●); patients receiving placebo (○). Results are expressed as mean ± SE. In thalidomide-treated patients, double asterisk (**) denotes significant difference at day 56 compared with baseline.

The 2 treatment groups was $5.25 ± 0.18 \log_{10}$ RNA equivalents/mL (placebo) and $5.57 ± 0.22 \log_{10}$ RNA equivalents/mL (thalidomide). No change in plasma HIV-1 levels was observed during the study in the placebo-treated patients (figure 2). By comparison, a trend ($P = .1$) toward a reduction in virus load compared with baseline was noted in the thalidomide-treated patients. However, the differences in virus load between the 2 treatment groups were not significant.

Total lymphocyte counts and T cell subsets. Total lymphocyte counts were similar at baseline in the 2 treatment groups ($973 ± 177$ and $832 ± 236$ for the thalidomide and placebo groups, respectively) and were not significantly different over the study period ($1131 ± 171$ and $1223 ± 245$, respectively). The CD4$^+$ T cell counts increased in both groups. The increase from baseline in the thalidomide-treated patients occurred earlier than (day 21) and was significantly different ($P = .05$) from that in the placebo-treated patients (figure 3). Similarly, the CD8$^+$ T cell counts increased in both groups. The increase compared with the baseline in the thalidomide-treated patients at day 21 was more pronounced and significantly higher ($P = .007$) than that in the placebo-treated patients (figure 3). Also, a significant ($P = .05$) increase in sCD8 was observed on day 21 in the thalidomide-treated patients compared with the placebo-treated patients, in whom sCD8 levels remained unchanged (figure 3).

Effect of thalidomide on plasma cytokine and cytokine receptor levels. At baseline, TNF-α levels were higher than normal in all patients (mean normal, 6 pg/mL). During the study there was a transient increase in TNF-α levels observed on day 21 in the thalidomide ($P = .05$) and the placebo (not statistically significant) treatment groups (table 3). By the end of the study, the levels of TNF-α had decreased to just below baseline levels in both groups. Nevertheless, levels at 56 days remained elevated compared with normal levels (uninfected controls). At baseline, IFN-γ levels in plasma were also above normal (mean normal, 0.2 IU/mL). An early increase in plasma IFN-γ levels was noted in the thalidomide-treated patients but not in the placebo-treated group. Thereafter, in both treatment groups there was a reduction in plasma IFN-γ levels by day 56, which was statistically significant ($P = .01$) only in the patients treated with placebo (table 3). However, at day 56, plasma IFN-γ levels were still higher than normal. IL-6 plasma levels, which were elevated at baseline in all patients, were also reduced during treatment. The reduction in plasma IL-6 levels was statistically significant ($P = .005$) in the placebo-treated but not in the thalidomide-treated patients (table 3; mean normal, 8.5 pg/mL).

Figure 2. Effect of thalidomide treatment (horizontal arrows) on plasma human immunodeficiency virus type 1 (HIV-1) virus load. Results are expressed as mean ± SE $\log_{10}$ RNA U/mL for thalidomide-treated group (●) and placebo-treated group (○).
Figure 3. Effect of thalidomide treatment (horizontal arrows) on T cells. CD4*T cell counts at baseline (mean ± SE) were 103 ± 23 and 108 ± 46, and mean ± SE CD8*T cell counts at baseline were 590 ± 125 and 434 ± 130 for thalidomide and placebo, respectively. Results are expressed as mean percentage of baseline ± SE. Mean plasma soluble CD8 is expressed in U/mL ± SE. Thalidomide-treated patients (○); placebo-treated patients (●). Asterisk (*) denotes significant difference noted between the 2 treatment groups.

IL-12 plasma levels were elevated in both treatment groups at baseline (figure 4; mean normal, 81 pg/mL). Thereafter, a significant (P = .022) difference in the plasma IL-12 levels between the 2 treatment groups was noted. Plasma IL-12 levels increased significantly (P < .001) in response to thalidomide treatment (figure 4). In contrast, the IL-12 level in the placebo-treated patients remained constant throughout the study.

In keeping with the thalidomide-induced immune activation reflected by CD8*T cell increases and indicated by the enhanced IL-12 and IFN-γ production, nonspecific markers of immune activation were also affected. The sIL-2R levels, which were elevated in all patients at baseline (mean normal, 1250 pg/mL), had significantly increased (P = .03) at days 21 and 42 in the thalidomide-treated patients, as compared with the placebo-treated group. The sIL-2R levels remained elevated during thalidomide treatment and were reduced only after discontinuation of the drug. In the placebo-treated patients, plasma levels of sIL-2R were reduced to normal levels by day 21 (figure 4).

At baseline, TNF-αR levels in plasma were elevated similarly in both groups of patients (figure 4; mean normal, 1.2 ng/mL). Here too there was a significant delay (day 21, P = .008; day 42, P = .002) in the decrease in plasma levels of TNF-α relative to normal in the thalidomide-treated patients compared with the placebo-treated patients. However, the levels of the soluble receptor were reduced significantly in both treatment groups by the end of the study (figure 4). Taken together, the changes in cytokine and cytokine receptor levels suggest that thalidomide treatment of HIV/TB patients induced an activation of the host immune cells.

The effect of thalidomide on delayed-type hypersensitivity responses and antigen-specific lymphocyte proliferation. The skin test response in situ to PPD was compared between the 2 treatment groups. At baseline, intradermal PPD–induced induration was observed in 3 (21%) of 14 thalidomide and 3 (27%) of 11 placebo patients. At 4 weeks of treatment, 10 (71%) of 14 thalidomide and 6 (54%) of 11 placebo patients had positive responses. Although these results suggested that thalidomide treatment may have enhanced the antigen-specific T cell response in these patients, statistical significance was not attained (P = .2).

To further evaluate the T cell response to PPD ex vivo and to monitor any effect that thalidomide may have had on this response, lymphocyte proliferation assays were done before and during treatment. In response to thalidomide treatment, a significant increase in mean SI was observed compared with baseline (P = .0006 and P = .0001 at days 21 and 42, respectively; figure 5). After discontinuation of thalidomide therapy, lym-

Table 3. Plasma levels of TNF-α, IFN-γ, and IL-6

<table>
<thead>
<tr>
<th>Cytokine, treatment group</th>
<th>Day 0 (baseline)*</th>
<th>Day 21b</th>
<th>Day 42b</th>
<th>Day 56b</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, pg/mL</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Thalidomide</td>
<td>95.6 (17.2)</td>
<td>162 (33)</td>
<td>132 (28)</td>
<td>92 (22)</td>
</tr>
<tr>
<td>Placebo</td>
<td>94.1 (23.5)</td>
<td>125 (26)</td>
<td>86 (14)</td>
<td>84 (13)</td>
</tr>
<tr>
<td>IFN-γ, IU/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalidomide</td>
<td>1.2 (0.3)</td>
<td>130 (26)</td>
<td>58 (13)</td>
<td>60 (13)</td>
</tr>
<tr>
<td>Placebo</td>
<td>2.3 (0.4)</td>
<td>91 (21)</td>
<td>52 (10)</td>
<td>48 (12)</td>
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<tr>
<td>IL-6, pg/mL</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Thalidomide</td>
<td>86.9 (38.7)</td>
<td>82 (31)</td>
<td>71 (36)</td>
<td>56 (19)</td>
</tr>
<tr>
<td>Placebo</td>
<td>62.2 (25.6)</td>
<td>54 (28)</td>
<td>32 (30)</td>
<td>20 (14)</td>
</tr>
</tbody>
</table>

NOTE. TNF, tumor necrosis factor; IFN, interferon; IL, interleukin.

* Absolute values.

* Values are mean % of baseline ± SE.

* P < .05 (day of evaluation compared with baseline).
phoproliferative responses returned to baseline. In contrast, in the placebo-treated patients, lymphocyte proliferation did not change throughout the study period (figure 5). Thus, in patients treated with thalidomide there was an activation of antigen-specific T cell responses.

**In vitro thalidomide stimulation of CD4+ T cell proliferative responses to antigen.** To determine whether thalidomide could directly stimulate T cells obtained from HIV-1-infected individuals, the effect of increasing doses of thalidomide on the in vitro proliferative response of CD4+ T cells to different recall antigens was evaluated. When antigen-pulsed monocytes were incubated with purified CD4+ T cells, we observed a thalidomide concentration-dependent increase in CD4+ T cell proliferation in response to CMV, PPD, and HIV-1 p24 antigen (figure 6A).

To identify the cellular target of the thalidomide-induced increase in antigen-specific proliferation of T cells, the assays were done with antigen-pulsed DCs that were either live or fixed with glutaraldehyde. Glutaraldehyde fixation renders the DCs deficient in costimulatory function, while preserving their capacity to present antigens to T cells [28]. When live antigen-pulsed DCs were incubated together with purified CD4+ T cells in the presence of thalidomide, a variable enhancing effect of the drug on CD4+ proliferative responses was observed (figure 6B). However, when the antigen-pulsed DCs were fixed, thalidomide induced a consistent, concentration-dependent increase in CD4+ proliferative responses (figure 6C). These results show that thalidomide can stimulate an increase in antigen-specific CD4+ T cell responses in vitro, which is most readily apparent in the absence of additional costimulatory activity. The drug appears to have a costimulatory effect directly on the CD4+ T cell.

**Effect of CD8+ T cells on augmented HIV replication occurring in response to thalidomide costimulation of patients’ CD4+ T cells.** It has been reported that thalidomide treatment of patients with advanced HIV disease resulted in an increase in plasma levels of HIV [29]. However, in the present clinical study we observed that, despite clear evidence of immune stimulation by thalidomide, no stimulatory effect of the drug on HIV replication was observed. To address this question in vitro, we examined the effect of thalidomide on PBMC and CD4+ T cells obtained from HIV-infected individuals. Figure 7 shows the results of a representative experiment. When PBMC were stimulated with immobilized anti-CD3 antibody, very low levels of HIV p24 antigen were detectable in the culture supernatant, with no effect of thalidomide (figure 7A). When CD8+ T cells were isolated and similarly stimulated, as expected, HIV was not detected (figure 7B). When the CD8+ T cell-depleted PBMC were stimulated with anti-CD3, HIV p24 antigen was readily detected, and p24 production was modestly increased by thalidomide at 10 μg/mL (figure 7C). However, when purified CD4+ T cells were similarly stimulated, thalidomide treatment at either 1 or 10 μg/mL caused a marked increase in HIV p24 antigen secretion (figure 7D). When these cultures were reconstituted with CD8+ T cells at CD4 : CD8 ratios of 10 : 1 or 1 : 1, the enhancing effect of thalidomide on HIV replication was abrogated (figure 7E and 7F, respectively). These results suggest that, although thalidomide has the potential to stimulate increased HIV replication in activated CD4+ T cells, this effect is held in check in the presence of CD8+ T cells.

**Discussion**

In this study, thalidomide stimulated antigen-specific T cell immunity in patients with advanced HIV/TB coinfection, as
indicated by enhanced T cell proliferation in response to mycobacterial antigens (PPD). The immunostimulatory effect of thalidomide was also indicated by increases in plasma levels of some of the Th1-type cytokines and cytokine receptors, including IFN-γ, IL-12, IL-2R, and TNF-αR. In addition, increases in the numbers of CD4⁺ and CD8⁺ T cells and plasma sCD8 levels were noted. In the in vitro studies of CD4⁺ T cells obtained from HIV-infected donors, thalidomide induced increased cell proliferation that was also antigen specific.

Because all the HIV-infected patients also had documented TB and were therefore exposed to M. tuberculosis antigens, we had a unique opportunity to evaluate the potential of thalidomide to stimulate antigen-specific T cell responses. Optimal T cell activation, resulting in T cell proliferation and cytokine production, requires 2 signals: primary T cell receptor stimulus delivered by antigen-loaded APCs and a secondary costimulatory signal [30]. We have previously shown that thalidomide has an in vitro costimulatory effect on purified primary human T cells that are receiving simultaneous stimulation via the T cell receptor by means of a monoclonal antibody to CD3 [19]. Exposure of the cells to thalidomide in vitro resulted in increased IL-2–mediated T cell proliferation and production of IFN-γ, a Th1-type cytokine. Here we show that the in vivo immune stimulatory effects of the drug in HIV-infected patients can enhance antigen (PPD)–specific responses (figure 5). This is consistent with our observation that more patients converted to PPD skin test positivity (>5 mm of induration) in the thalidomide-treated group than in the placebo-treated group.

Figure 5. Effect of thalidomide treatment on lymphocyte proliferation response to 20 μg/mL of purified protein derivative. Top panels show individual stimulation indices at days 0, 21, and 42 for placebo-treated patients (○) and thalidomide-treated patients (●). Lower panel indicates mean stimulation indices (±SE) for each group over the whole study period. Double asterisk (**) denotes significant difference from baseline at specified time points for thalidomide-treated patients.
Figure 6. Effect of thalidomide on antigen-specific proliferation of purified CD4+ T cells from individuals infected with human immunodeficiency virus (HIV). Purified CD4+ T cells were incubated in the presence of varying concentrations of thalidomide with monocyte antigen-presenting cells (A), live dendritic cells (DCs) (B), or glutaraldehyde-fixed DCs (C) that had been pulsed with the following antigens: cytomegalovirus (CMV) (●), purified protein derivative (△), tetanus toxoid (●), HIV p24 antigen (○), control protein for CMV (□), control protein for HIV p24 antigen (□), or no antigen (△). Results are for individual patients (means of triplicate samples). [3H]TdR, tritiated thymidine.

in vitro experiments reported here indicate that the drug can act directly on CD4+ T cells from HIV-infected individuals to costimulate antigen-specific T cell responses (figure 6).

Studies in vitro have shown that thalidomide treatment inhibits IL-12 production by lipopolysaccharide (LPS)–stimulated monocytes (T cell independent) [31] but that the drug stimulates the production of IL-12 in T cell–dependent systems [20, 21]. Thus, the effect of the drug on production of IL-12 varies according to the stimulus and the target cell type. IL-12 is a central regulatory cytokine in the cellular immune response. The cytokine stimulates Th1-type T cell activation [32], and T cell activation, in turn, stimulates the production of monocyte IL-12, completing a positive feedback loop. T cell–dependent stimulation of IL-12 production requires the interaction of CD40 on antigen-presenting cells (monocytes and DCs) with CD40L, expressed on the surface of activated T cells. In the present study, levels of T cell activation markers were elevated before increases in plasma IL-12 levels were noted, suggesting that IL-12 production occurred as a consequence of thalidomide-induced T cell activation. In previous studies, we observed that thalidomide costimulation induced increases in CD40L expression on T cells isolated from HIV-infected [21] and uninfected [20] donors. These observations, taken together, suggest that, in the patients studied here, thalidomide treatment may have activated T cells to upregulate CD40L, which in turn stimulated increased APC (DCs and monocyte) IL-12 production (T cell dependent). However, because CD40L expression on peripheral blood T cells was not measured in this study, a
conclusive understanding of the mechanism of in vivo augmentation of IL-12 production must await further patient studies.

The thalidomide-induced increase in endogenous IL-12 production may be important in controlling both TB and HIV infection. In vivo, an intact IL-12 signaling pathway is required to generate protective immunity to TB. Indeed, disseminated TB has been reported in a patient with a genetic deficiency in the IL-12 receptor [33]. In addition, HIV infection is accompanied by deficiencies in the production of IL-12 [34]. Interestingly, in vitro IL-12 production is restored in cells from HIV patients by IFN-γ together with CD40 ligand [35], and in vitro IL-12 restores HIV-specific cellular immunity [36] and enhances HIV-specific CTL responses [37]. Finally, local mucosal delivery of rIL-12, together with a vaccine, increases CD8+ CTL responses in a murine vaccine model [38]. The use of recombinant IL-12 therapy in HIV infection has been advocated as an immunostimulatory intervention but is limited by the toxic effects after the administration of the recombinant cytokine [39]. Thus, an intervention such as thalidomide, which stimulates endogenous IL-12 production, may be a less toxic therapeutic approach. Indeed, there is an anecdotal report of thalidomide treatment–induced clinical improvement of drug-resistant M. avium infection in a patient with advanced HIV disease [40]. However, the benefits or potential adverse consequences of immune stimulation in infectious diseases remain to be elucidated.

In association with the observed thalidomide-induced immune stimulation in these patients, we would have expected an increase in virus load. Thalidomide treatment of HIV-infected patients has been reported in the past to induce an increase in HIV levels [29]. However, this was not observed here. In HIV infection, CD8+ T cells have been reported to reduce replication of virus in CD4+ cells by a variety of mechanisms, including CTL activity and chemokine production [41]. We suggest that the increased number and/or activation of CD8+ T cells noted in our thalidomide-treated patients may have down-regulated any viral production by thalidomide-stimulated CD4+ T cells by an as yet undefined mechanism. Our in vitro studies (figure 7) indicate that this may indeed be the case. The increase in CD8+ T cells in the thalidomide-treated patients may be important also in combating the concomitant TB infection, since CD8+ T cells have been shown to contribute to the immune response to TB infection [42].

In these studies, as well as in one of our previous studies [21], we noted that thalidomide treatment of HIV-infected patients did not reduce plasma TNF-α levels. These results con-
trast with those of some previous studies, in which thalidomide treatment induced a reduction in plasma TNF-α levels in patients with leprosy or with TB [17, 43]. In another study of HIV/TB patients, a decrease in plasma TNF-α levels was observed after 21 days of thalidomide treatment only in those individuals with the highest levels of TNF-α [13]. Our present observations may be explained by the differential effects of thalidomide on monocyte and T cell TNF-α production [19, 20]. As already mentioned, thalidomide has been shown to inhibit TNF-α production by LPS-stimulated monocytes. However, it is important to note that the drug fails to inhibit TNF-α production by activated T cells [19]. These findings suggest that, in the present study, at least some of the TNF-α production may be T-cell derived and therefore not inhibited by thalidomide. The transient increase in TNF-α levels seen at day 21 of treatment may be attributed to the anti-TB therapy [16]. In the thalidomide-treated group, the drug may have augmented this increase, because thalidomide activates T cells.

In conclusion, our study has provided in vivo data showing that thalidomide treatment can enhance antigen-specific immunity. This finding suggests that thalidomide may have a hitherto unappreciated potential as an immunologic adjuvant. However, because the effect wanes after removal of the drug, long-term treatment may be required. For example, to boost an anti-TB effect, the drug may need to be used for the duration of TB therapy.

Acknowledgments

We thank Dr. Peter Morris and the staff of Brooklyn Chest Hospital, Cape Town, South Africa; Dr. Victoria Freedman for help with the manuscript; Zhu Shen for her help with the in vitro studies; Judy Adams for preparation of the figures; and Marguerite Nulty for secretarial assistance.

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