Effective Treatment of Erythema Nodosum Leprosum with Thalidomide Is Associated with Immune Stimulation

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The immunomodulatory drug thalidomide is the treatment of choice for erythema nodosum leprosum (ENL), an inflammatory cutaneous and systemic complication of multibacillary leprosy. To elucidate the mechanism of action of thalidomide in this syndrome, we prospectively investigated 20 patients with ENL who were treated with thalidomide for 21 days. All patients responded to treatment, with the majority of them having complete resolution of cutaneous lesions within 7 days. This response was associated with a marked but transient increase in ex vivo mitogen-induced expression of interleukin (IL)–2 and interferon-γ by CD4+ and CD8+ T cells that was observed on treatment day 7, but these returned to pretreatment levels by day 21. Plasma tumor necrosis factor–α levels were not high at baseline, and they increased modestly during treatment. Plasma levels of IL-12 increased steadily during thalidomide treatment. Hence, the therapeutic effect of thalidomide in ENL appears to be associated with transient immune stimulation, which suggests that the drug may promote an active immunoregulatory response.

Erythema nodosum leprosum (ENL) is a debilitating inflammatory complication of leprosy, an infectious disease caused by *Mycobacterium leprae*. Leprosy presents as a spectrum of disease affecting peripheral nerves and skin, with 2 defined poles [1]: tuberculoid leprosy, in which *M. leprae*–specific Th1-type immunity is robust, clinical disease is limited, and numbers are bacilli are few; and lepromatous or multibacillary leprosy, which is associated with absent or weak *M. leprae*–specific Th1-type immunity, widespread disease, and a huge bacillary burden.

Much of the morbidity associated with leprosy results from episodic lepra reactions, which are caused by shifts in unstable *M. leprae*–specific host responses. Two types of lepra reaction are recognized: type I reactions, which are caused by transient increases in Th1-type immunity, and type II lepra reactions, or ENL, which occur by an unknown immunopathologic process in patients with multibacillary disease. ENL is characterized by the episodic development of clusters of localized or generalized inflammatory skin nodules. In severe cases, nodules may ulcerate, and there may be systemic symptoms, such as fever and anorexia, and organ-specific manifestations, such as neuritis, iritis, and nephritis. Histologically, ENL nodules are characterized by a mixed infiltrate of neutrophils and mononuclear cells. An in-
triguing feature of ENL is its exquisite sensitivity to treatment with the immunomodulatory drug thalidomide, which was discovered serendipitously in the early 1960s [2]. Thalidomide is now the treatment of choice for ENL, although its mechanism of action is still unclear.

Several immunologic and potentially anti-inflammatory activities of thalidomide have been described, including the inhibition of the production of the proinflammatory cytokine tumor necrosis factor (TNF)–α with angiogenesis and costimulation of interleukin (IL)–2 production by T cells [3–7]. ENL has been associated with increased plasma TNF-α levels [4, 8], which were also reported to normalize after thalidomide treatment [4, 8]. However, thalidomide treatment can increase plasma TNF-α levels in other diseases [9–13]. It is therefore uncertain whether the efficacy of thalidomide in ENL is exclusively mediated by the inhibition of TNF-α.

Could the costimulation of IL-2 production by thalidomide contribute to the anti-inflammatory properties of this drug? Mice deficient in IL-2 have uncontrolled T cell proliferation and inflammatory bowel disease [14] that is reversed by the passive transfer of regulatory T cells that express CD25, the high-affinity IL-2 receptor (IL2R) [15]. In mice, the generation of regulatory T cells is the only nonredundant function of IL-2 [16–18]. Moreover, physiologic T cell costimulation via CD28 is necessary for the maintenance of regulatory T cell function and immune homeostasis [19]. Thus, it is conceivable that the T cell costimulatory effect of thalidomide treatment may augment the IL-2–dependent number and/or function of regulatory T cells.

To further delineate immune changes in response to thalidomide in the presence of ENL, we performed a prospective, open-label investigation of thalidomide treatment in 20 patients with ENL. The focus of the study was to monitor changes in proinflammatory cytokines and in the functional status of T cells. In a subset of patients, we also measured levels of expression of the transcription factor foxp3, because this gene is uniquely expressed in regulatory T cells and may therefore provide a measurement of T cell–mediated immune regulation.

**PATIENTS, MATERIALS, AND METHODS.**

**Patients and clinical evaluations.** Patients with a histologically confirmed clinical diagnosis of ENL were recruited from the leprosy clinic of Anandaban Hospital (Anandaban, Nepal). Female patients of child-bearing potential were excluded from the study because of the teratogenicity of thalidomide. Patients with moderate or severe ENL-associated neuritis (defined as nerve swelling, tenderness, or sensorimotor loss since the onset of ENL) were excluded, because this complication does not improve with thalidomide treatment. Patients who had received immunomodulating therapy within the preceding month were excluded from the study.

We recruited patients with multibacillary (lepromatous) leprosy but without ENL or type 1 lepra reaction and who had no history of ENL within the preceding 30 days to serve as control subjects. All patients provided written, informed consent for participation in the study. Because thalidomide is the treatment of choice for ENL, the study was not placebo controlled. The Nepal Health Research Council and the institutional review board of Rockefeller University (New York, NY) approved the research protocol. Patient recruitment took place during 1999–2002.

The severity of the cutaneous manifestations of ENL was scored according to a scheme devised for the study. The extent of lesions in each of 7 anatomic areas (each limb, the head, and the front and back of the torso) was scored from 0 to 3 as follows: 0, no lesions; 1, lesions present on <25% of the region; 2, lesions present on 25%–75% of the region; and 3, lesions present on >75% of the region. An additional 10 points were added to the total if any ENL lesions were blistered, pustular, or ulcerated. Hence, the maximum possible score was 31. All study subjects were treated with a supervised thalidomide regimen, administered by mouth once daily, of 300 mg on days 0–6, followed by 200 mg on days 7–13, then 100 mg on days 14–20. Clinical evaluations, including repeated cutaneous ENL scoring and monitoring for neurotoxicity, were performed on days 0, 3, 7, 14, 21, and 28. Control subjects were evaluated only once, at baseline. Thalidomide was purchased from Penn Pharmaceuticals.

**Immunologic assessments.** Heparin-anticoagulated blood was obtained on days 0, 3, 7, 14, 21, and 28 and was immediately processed to separate plasma and isolate peripheral blood mononuclear cells (PBMCs) by density centrifugation.

**ELISA.** Plasma was stored in aliquots at −80°C. Subsequently, ELISA was performed using paired antibodies (obtained from R&D Systems) to determine levels of interferon (IFN)–γ, as described elsewhere [20], and using kits to determine levels of TNF-α (BioSource International), soluble (s) IL2R (Pierce Biotechnology), and total levels of IL-12 (i.e., both IL-12 p40 and IL-12 p70; Pierce Biotechnology).

**Enzyme-linked immunospot (ELISPOT) assay.** Single-cell IFN-γ production by PBMCs was assessed on days 0, 7, 21, and 28 by ELISPOT, performed as described elsewhere [21]. Briefly, 1 × 10⁵ freshly isolated PBMCs suspended in 200 μL of culture medium (RPMI 1640 supplemented with 10% human AB serum, penicillin, and streptomycin) were placed in wells of a 96-well ELISPOT plate (Millipore) coated with anti–IFN-γ antibody (clone 1-DIK; MabTech). Duplicate cultures were left unstimulated or were stimulated with a cytosolic extract of *M. leprae* (provided by Dr. Patrick Brennan, Colorado State University, Fort Collins), at a final concentration of 10 μg/mL. Phytohemagglutinin (PHA; Sigma), at a concentration of 5 μg/mL, was used as a positive control stimulus. Cultures...
were incubated for 16 h at 37°C; then cells were lysed, and spots of captured IFN-γ were revealed with a secondary peroxidase-conjugated IFN-γ antibody (clone 7-B6-1; MabTech). Spots, representing IFN-γ production by a single cell, were counted with a dissecting microscope.

**Cytokine flow-cytometric assay.** Cytokine production by individual T cells was evaluated on study days 0, 7, and 21 by stimulation of freshly isolated PBMCs with PMA and ionomycin (Sigma) at concentrations of 10 ng/mL and 1 μmol/L, respectively. Cultures were set up in culture medium for 5 h at 37°C in the presence of 10 μg/mL brefeldin A (Sigma). PBMCs were then fixed with 4% paraformaldehyde and cryopreserved. Subsequently, sequential samples were simultaneously thawed, permeabilized in 0.01% saponin buffer, and stained with combinations of fluorescence-conjugated antibodies (Becton Dickinson Immunocytochemistry): CD3 peridinin-chlorophyll-protein complex; CD8 fluorescein isothiocyanate; and either IFN-γ phycoerythrin (PE), IL-2 PE, or IL-4 PE, as described elsewhere [22]. The flow-cytometric assay was performed with a FACScan instrument (Becton Dickinson), and the cytokine expression of CD3+ cells was analyzed. PMA causes a degradation of CD4 [23], so CD3+CD8− cells were assumed to be CD4+ cells. Data were analyzed with FlowJo software (version 4.6.2; Treestar).

**Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).** For some patients, PBMCs collected on days 0, 7, and 21 were immediately placed in a cell lysis/RNA-stabilizing solution (RNA-STAT-50; Tel-Test) and stored in liquid nitrogen. A portion of the baseline diagnostic skin-biopsy sample was stored at −70°C in RNAlater (Ambion). RNA was isolated from thawed sets of specimens by use of RNA isolation kits, in accordance with the manufacturer’s instructions (Qiagen). RT with random primers generated cDNA (Qiagen). IL-2 and β-actin primers and Taqman probes with sequences described elsewhere [24, 25] were obtained from Biosource International, and foxp3 primers and Taqman probes were obtained from Applied Biosystems (ABI assay Hs00203958 m1). qRT-PCR was performed on an ABI PRISM 7700 instrument for the amplification of IL-2 and foxp3 genes, and β-actin was amplified as the endogenous control. Test-gene expression was calculated as a fraction of that of β-actin by finding the difference in the number of PCR cycles needed to achieve threshold fluorescence for each gene (ΔCt). Under the assumption that a doubling of fluorescence occurs with each cycle, relative gene expression was calculated as 2−ΔCt. Equal efficiencies of amplification of the 3 gene products were indicated by equal and parallel linearity of standard curves of serially diluted cDNA derived from PHA-activated PBMCs.

**Statistical analysis.** Data from sequentially collected samples from patients with ENL were tested for normality of distribution and analyzed by repeated-measures analysis of variance (ANOVA), followed by a posttest Dunnett’s multiple comparison when ANOVA revealed a significant difference between time points. An unpaired t test was used to compare baseline data between patients with ENL and control subjects. Statistical significance was set at P < .05. Nonparametric analyses were performed to test for differences between subgroups of patients with ENL, categorized by disease duration or severity.

**RESULTS**

**Study subjects.** Twenty consecutive male patients with ENL and 20 male control subjects were enrolled in the study. The demographic and clinical details of patients with ENL are presented in table 1. Five subjects had had ENL for ≥30 days, because of mild disease treated intermittently with aspirin (patients 3 and 14); late presentation to the clinic (patient 5); a new diagnosis of leprosy with long-standing ENL (patient 10), or transfer from another area and undiagnosed ENL (patient 17).

**Clinical responses.** After thalidomide treatment, all patients showed prompt improvement in ENL, as reflected by improved skin scores (figure 1). Eighteen of 20 patients had achieved a skin score of 0 on at least one occasion by day 21. However, 9 subjects exhibited persistent ENL (n = 2) or minor recurrences (n = 7) while receiving the reducing schedule of thalidomide treatment. Interestingly, the latter group of incomplete responders had significantly lower ENL skin scores at baseline than did the patients in whom ENL resolved completely (figure 1, inset). There were no differences between the groups in baseline immunologic indices or changes in immune parameters during thalidomide treatment (data not shown). Additionally, 6 subjects exhibited flare-ups in ENL activity on day 28, after the discontinuation of thalidomide treatment (figure 1). All patients tolerated thalidomide well; no toxicities attributable to thalidomide were observed.

**Plasma cytokines.** Serially collected plasma samples were assayed by ELISA for TNF-α, IFN-γ, IL-12, and sIL2R. Levels of circulating TNF-α were low at the time of ENL diagnosis and were significantly lower than those in control subjects (P < .01) (figure 2A). An upward trend in plasma TNF-α levels was observed in the patients with ENL during thalidomide treatment (P = .0016, repeated-measures ANOVA); this trend achieved statistical significance on days 7 and 14 of thalidomide treatment. Plasma levels of this cytokine returned to baseline levels on study day 28, after the discontinuation of thalidomide treatment (figure 2A).

In patients with ENL, baseline plasma levels of IL-12 were also significantly lower than levels in control subjects (P < .01) (figure 2B). Thalidomide treatment was associated with an upward trend in levels of this cytokine (P = .044, repeated-measures ANOVA); this trend reached statistical significance on days 14 and 21 of treatment. Levels of IL-12 approached those
Table 1. Demographic and clinical details of patients with erythema nodosum leprosum (ENL) and control subjects with non-ENL lepromatous leprosy (LL).

<table>
<thead>
<tr>
<th>Patients with ENL</th>
<th>Age, years</th>
<th>Sex</th>
<th>Duration of leprosy treatment, months</th>
<th>Status at diagnosis of leprosy Ridley-Jopling classification</th>
<th>Bacillary index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Duration of present ENL episode, days</th>
<th>Previous episodes of type II ENL reactions, no.</th>
<th>Previous episodes of type I lepra reaction, no.</th>
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Patients with ENL, median (range) 40 (17–54) ... 10 (0–34) ... 4.3 (1–5.25) 10 (2–240) 1 (0–3) 0 (0–1)

Control subjects (n = 20), median (range) 34 (11–61) ... 6 (0–50) ... 3.3 (1–4.75) NA 0 (0–7) 0 (0–1)

**NOTE.** BL, borderline lepromatous; NA, not applicable.

<sup>a</sup> Logarithmic expression of bacillary density in skin-biopsy specimens (1, >1 bacillus/100 high-power fields; 2, >1 bacillus/10 high-power fields; 3, >1 bacillus/1 high-power field; 4, >10 bacilli/1 high-power field; 5, >100 bacilli/1 high-power field).
Thalidomide in Erythema Nodosum Leprosum

In contrast to TNF-α and IL-12, baseline levels of sIL2R were significantly higher in patients with ENL than in control subjects (P < .01) (figure 2C), and levels showed a downward trend during thalidomide treatment (P = .025, repeated-measures ANOVA), reaching statistical significance on day 21 of treatment, at which time sIL2R levels were not significantly different from those in the control subjects.

IFN-γ was not detected in the majority of plasma samples (data not shown). Subgroup analysis to compare plasma TNF-α, IL-12, and sIL2R levels in subjects with longstanding (≥30 days, n = 5) versus recent-onset (<30 days, n = 15) ENL revealed no statistically significant differences at baseline or during thalidomide treatment (data not shown).

In summary, analysis of these immunologic soluble factors in plasma showed an association between thalidomide treatment and increases in TNF-α and IL-12 levels and reductions in sIL2R levels, which suggests both an immune activating and an anti-inflammatory effect of the drug. Thus, a trend toward “normalization,” as was seen in the values obtained for the untreated control subjects, was noted.

Intracellular T cell cytokines. To assess the contribution of the modulation of T cell function to the response to thalid-
omide treatment, we next examined intracellular cytokine expression in circulating T cells. Representative results from a patient with ENL are shown in figure 3. At baseline, PMA and ionomycin–stimulated IL-2 expression predominated in the CD8^- (CD4+) subset, but IFN-γ expression was more evenly distributed between the CD8^- and CD8^+ subsets. Thalidomide treatment was associated with a transient increase in the proportion of T cell subsets that expressed both IL-2 and IFN-γ that was clear on day 7 but was no longer evident on day 21. IL-4 expression was weak at baseline and was not detectably affected by thalidomide treatment. This pattern of cytokine responses after thalidomide treatment was evident in the entire group of patients with ENL (figure 4). Overall, a statistically significant change in IL-2 expression in CD4^+ and CD8^+ T cells was demonstrated by repeated-measures ANOVA, whereas changes in IFN-γ achieved statistical significance only in CD4^+ cells (figure 4). Dunnett’s posttest analysis revealed that all significant changes occurred on day 7, with uniform increases in IL-2 and IFN-γ levels (figure 4). These results demonstrate a transient thalidomide treatment–associated increase in PMA and ionomycin–induced Th1 T cell cytokine expression.
M. leprae–specific IFN-γ responses. M. leprae–specific IFN-γ responses were assessed by ELISPOT analysis of M. leprae–stimulated PBMCs on study days 0, 7, 21, and 28. A broad range of initial responsiveness to this antigen was observed; 13 of 20 patients with ENL exhibited positive responses, with a mean ± SE of 34 ± 15 IFN-γ–producing sfc/10^6 PBMCs. Repeated-measures ANOVA revealed no significant change in this response during thalidomide treatment (data not shown).

Expression of foxp3 and IL-2 genes in PBMCs and skin. RNA was available from sequentially collected PBMCs (days 0, 7, and 21) and from skin-biopsy samples obtained at baseline from 5 patients with ENL (patients 8, 10, 11, 12, and 13). β-actin was readily detected in all specimens after a mean of 17.6 cycles of PCR (range, 15.6–22.8 cycles of PCR). No IL-2 mRNA was detected in any samples (data not shown), which is consistent with the lack of IL-2 protein in unstimulated T cells (figures 3 and 4). By contrast, the regulatory T cell–specific gene foxp3 was detected in all skin-biopsy samples and PBMCs, and an upward trend was observed in PBMCs during the 21 days of thalidomide treatment (figure 5). However, the changes in foxp3 expression did not reach statistical significance (figure 5).

DISCUSSION

The proven efficacy of thalidomide in the treatment of ENL [26, 27] presents an opportunity to correlate the clinical and immunologic effects of this drug. The present study was not placebo controlled, so we cannot distinguish with absolute confidence the immunologic effects of thalidomide from those secondary to disease resolution. However, the dynamic patterns of immunologic changes that we observed, when considered together with the effects of thalidomide reported in previous studies, suggest some new ideas about the possible mechanism of action of the drug against ENL.

In previous studies, the finding in patients with ENL of increased plasma levels and ex vivo production of TNF-α suggested a role for this molecule in the pathogenesis of ENL [4, 28–31]. The first clinical clue to the capacity of thalidomide to inhibit TNF-α production was obtained in a landmark investigation by Sampaio et al. [4]. In that study, high levels of circulating TNF-α in Brazilian patients with severe systemic ENL decreased precipitously after thalidomide treatment [4]. By contrast, in the present study of 20 consecutive patients with ENL of varying degrees of severity, levels of TNF-α were, in some cases, undetectable; in the group as a whole, levels were lower than those in the control subjects. Moreover, thalidomide treatment for 21 days was associated with a modest increase in plasma TNF-α levels. TNF-α may be particularly important in the pathogenesis of ENL with systemic complications [28], so the variable severity of disease in patients reported here may account for the modest and variable TNF-α levels observed. Also, regional differences in the severity of ENL may be reflect-
ment. However, variability in these responses may have obscured any effect of thalidomide on M. leprae–specific immunity.

Although the costimulatory effect of thalidomide on T cells provides a ready explanation for the observed increases in mitogen-induced IL-2 and IFN-γ production (figures 3 and 4), the mechanism for increased IL-12 production, which is secreted by antigen-presenting cells, is less obvious. In vitro, thalidomide inhibits IL-12 secretion by lipopolysaccharide-stimulated monocytes [38]. However, we have reported that the drug induces the up-regulation of CD40L on activated CD4+ cells, mediating increased IL-12 production by CD40+ antigen-presenting cells [37, 39]. Thus, the effect of thalidomide on IL-12 depends on whether T cells are activated and whether IL-12 induction is T cell dependent or independent. This interpretation would suggest that the increases in plasma IL-12 production that we observed were T cell dependent.

The present investigation did not show a purely immunostimulatory effect of thalidomide: levels of plasma sIL2R, an inflammatory marker that is increased in ENL [29, 40, 41], decreased during thalidomide treatment, as has been reported elsewhere [41]. However, Tung et al. [40] observed no effect of corticosteroid treatment on plasma sIL2R levels in ENL. These contrasting results may reflect differences in the mechanisms of action of these immune modulatory agents or differences in the severity of ENL in different study populations.

Together, our results suggest a complex effect of thalidomide that is anti-inflammatory (as demonstrated by the resolution of ENL and reductions in plasma sIL2R levels) but also involves a transient boosting of T cell function, with secondary IL-12 secretion by antigen-presenting cells. Oliver et al. [12] reported a similar association of T cell activation with the resolution of dermatologic sarcoidosis after thalidomide treatment. In that study, serial skin-biopsy samples revealed a treatment-associated influx of T cells, together with the maturation and subsequent resolution of sarcoid granulomata. We suggest that thalidomide may actively promote T cell regulation in these diseases. foxp3-expressing CD25+ regulatory T cells have been described in human inflammatory lesions [42]. The expression of this gene in the skin of 5 subjects in the present study is consistent with regulatory T cell activity in ENL lesions. The propensity of thalidomide to augment the production of IL-2 by T cells [6, 7], together with the exquisite and unique dependence of regulatory T cells on this cytokine [16–18], suggest that the boosting of regulatory T cells is a potential mechanism of action of thalidomide. Our finding of a trend toward increased foxp3 gene expression in PBMCs during thalidomide treatment is consistent with this notion. However, RNA was available from only 5 subjects, so the changes in sequential data fell short of statistical significance. We therefore consider the present findings to be provocative but preliminary and have reported them because they offer a potential explanation for the association of T cell stimulation with the resolution of inflammation in ENL. Further exploration of the effects of thalidomide on regulatory T cell function might be a worthwhile avenue of future enquiry in ENL and other diseases.