A Single Oral Dose of Thalidomide Enhances the Capacity of Lymphocytes to Secrete Gamma Interferon in Healthy Humans

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Thalidomide is increasingly being used as adjuvant therapy for patients with mycobacterial and human immunodeficiency virus (HIV) infections. The T-helper (Th1) cytokine–Th2 cytokine balance critically determines the outcomes of these diseases. To obtain insight into the effect of thalidomide on the capacity of lymphocytes to produce Th1 and Th2 cytokines, six healthy volunteers received an oral dose (400 mg) of thalidomide. Before and at 3, 6, and 24 h after ingestion of thalidomide, peripheral blood mononuclear cells (PBMCs) were isolated and stimulated for 24 h with the T-cell stimulant staphylococcal enterotoxin B (SEB) or anti-CD3/CD28. In all six volunteers ingestion of thalidomide was associated with enhanced SEB- and anti-CD3/CD28-induced production of the Th1 cytokine gamma interferon (IFN-γ) (P < 0.05) and a decrease in the level of anti-CD3/CD28-induced interleukin-5 (IL-5) production (P < 0.05). The levels of IL-2 (Th1) and IL-4 (Th2) released remained unchanged. These changes were accompanied by an increase in the amount of IL-12p40 released by the PBMCs 6 h after ingestion of thalidomide (P < 0.05). Thus, a single oral dose of thalidomide causes a Th1-type response in healthy humans. This finding offers a potential explanation for the positive effect of thalidomide in patients with mycobacterial and HIV infections.

Thalidomide is increasingly being used as adjuvant therapy for patients with mycobacterial and human immunodeficiency virus (HIV) infections. The clinical symptoms of patients with Mycobacterium avium infections refractory to conventional treatment improved dramatically with thalidomide treatment (3, 11). In patients with concomitant HIV and Mycobacterium tuberculosis infection, thalidomide increased the weights of patients significantly, while their HIV loads decreased (16). Thalidomide has also been found to be beneficial in a variety of other diseases, including aphthous ulcers, Behcet syndrome, erythema nodosum leprosum (ENL), and microsporidiosis (13, 15, 26, 28).

T-helper (Th) lymphocytes can be divided into subclasses depending on the patterns of the cytokines that they secrete upon stimulation. Th1 lymphocytes predominantly produce gamma interferon (IFN-γ) and interleukin-2 (IL-2), while Th2 lymphocytes mainly produce IL-4 and IL-5. IL-12 is a pivotal denominator of the balance between both lymphocyte subsets, as it drives naïve T cells into a Th1 direction (30). The Th1-Th2 balance critically determines the outcomes of mycobacterial and HIV infections. In mycobacterial infections a shift toward a Th1 response is protective, while a predominant Th2 response impairs host defenses. Indeed, mice deficient in IFN-γ or IL-12 are highly susceptible to infection with Mycobacterium tuberculosis (5, 9), and in patients with active tuberculosis the IL-4/IFN-γ ratio is increased (29, 32). Moreover, recurrent mycobacterial infections have been described in IL-12 receptor-deficient patients (8). Patients with progressive HIV disease show increased levels of Th2-type cytokines, such as IL-4 and IL-10, while long-term asymptomatic HIV-infected persons elicit a response with expression of Th1-type cytokines (4).

Evidence exists that thalidomide may influence the Th1-Th2 balance. In vitro experiments, thalidomide has been shown to suppress the production of IL-12 and IFN-γ and to enhance the production of IL-4 (19, 20). However, in vivo, determination of the effect of thalidomide on IFN-γ concentrations has yielded variable results, with increased as well as decreased levels of this cytokine found in patients with HIV infection or ENL (2, 16, 18, 26).

Knowledge of the effect of thalidomide on the Th1-Th2 balance may contribute to our understanding of the mechanisms underlying the previously reported beneficial effects of this compound during infection with HIV or mycobacteria. Since these diseases lead to complex cytokine activations that may obscure the effect of thalidomide, we sought to determine whether thalidomide can influence the pattern of lymphocyte-derived cytokine secretion upon activation of T cells in non-diseased individuals. For this purpose, six healthy males received a single oral, clinically relevant dose of thalidomide, and the capacity of their peripheral blood mononuclear cells (PBMCs), obtained before and at various time points after thalidomide ingestion, to produce Th1- and Th2-type cytokines after stimulation with T-cell stimuli was determined.

MATERIALS AND METHODS

Subjects and design. Six healthy male subjects (mean age, 38 years; age range, 33 to 44 years) were enrolled after documentation of normal liver and renal function and hematological parameters. The study was approved by the institutional research and ethics committees, and written informed consent was obtained from all subjects. All subjects took 400 mg of thalidomide (racemic mixture purchased from Grunenthal, GmbH, Stolberg, Germany) by mouth. Blood was obtained just before ingestion of thalidomide and at 3, 6, and 24 h thereafter. Venous blood samples were collected aseptically in sterile tubes prefilled with heparin (final concentration, 10 U/ml blood; Leo Pharmaceutical Products, Wiesbaden, Germany). PBMCs were isolated by Ficoll-Hypaque (Ficoll Paque; Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation and were diluted to 10⁶ cells/ml of RPMI 1640 (Bio Whittaker, Verviers,

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TABLE 1. Concentrations of thalidomide in serum after oral ingestion

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concn (µg/liter)*</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.5</td>
<td>252 ± 80</td>
</tr>
<tr>
<td>3</td>
<td>483 ± 86</td>
</tr>
<tr>
<td>6</td>
<td>619 ± 45</td>
</tr>
<tr>
<td>9</td>
<td>595 ± 51</td>
</tr>
<tr>
<td>24</td>
<td>353 ± 33</td>
</tr>
</tbody>
</table>

* Values are means ± standard errors of the means for six healthy volunteers. Thalidomide (400 mg) was taken orally at time zero.

The results from a study on the effects of thalidomide ingestion are presented in Table 1. The maximum concentration of thalidomide was found at 24 h after ingestion, with a concentration of 353 ± 33 µg/liter. The concentration decreased to 252 ± 80 µg/liter at 1.5 h after ingestion.

RESULTS

Serum thalidomide levels. The maximum concentration of thalidomide (619 ± 45 µg/liter) was measured 6 h after ingestion (Table 1). At 24 h after ingestion of the drug, thalidomide was still detectable.

Clinical response to thalidomide. Besides some drowsiness and euphoria, ingestion of thalidomide was not associated with any clinical effect.

Effect of thalidomide on leukocyte counts. No significant changes in leukocyte, lymphocyte, CD3⁺/CD4⁺, or CD3⁺/CD8⁺ lymphocyte counts were found after ingestion of thalidomide (Table 2). In control subjects not taking thalidomide, no significant circadian fluctuations in leukocyte, lymphocyte, CD3⁺/CD4⁺, or CD3⁺/CD8⁺ lymphocyte counts could be detected (data not shown). Cytokine concentrations measured after stimulation of PBMCs are expressed per milliliter of supernatant. Expression of the cytokine levels per 10⁶ CD3⁺/CD4⁺ or CD3⁺/CD8⁺ lymphocytes yielded similar results with respect to the effect of thalidomide (data not shown).

Th1 cytokines. The levels of IFN-γ and IL-2, which are prototypic Th1 cytokines, were measured. In the absence of a stimulus, incubation of PBMCs obtained before or at any time point after thalidomide ingestion did not result in detectable IFN-γ or IL-2 levels. The capacity of PBMCs to produce IFN-γ upon stimulation with SEB or anti-CD3/CD28 markedly increased after thalidomide ingestion (P < 0.05) (Fig. 1). The maximum effect of thalidomide was found at 24 h, when SEB-induced IFN-γ concentrations were increased 947% ± 284% and anti-CD3/CD28-induced IFN-γ concentrations were increased 697% ± 218% over the IFN-γ levels before the administration of thalidomide. The ability of PBMCs to release IL-2 did not change significantly after ingestion of thalidomide, although at 24 h a clear trend toward enhanced IL-2 secretion was found.

Th2 cytokines. The levels of IL-4 and IL-5, which are prototypic Th2 cytokines were measured. Without stimulus, incubation of PBMCs obtained before or at any time point after thalidomide ingestion did not result in detectable IL-4 or IL-5 levels. The level of IL-4 production by PBMCs was low after stimulation with SEB and did not change significantly over time. Anti-CD3/CD28-induced IL-4 levels were also low both before and after thalidomide ingestion, although a trend toward enhanced anti-CD3/CD28-induced IL-4 release was seen at 24 h (P = 0.22) (Fig. 1). The capacity of lymphocytes to produce IL-5 upon stimulation was severalfold greater than the ability to release IL-4. The level of SEB-induced IL-5 release by PBMCs did not change significantly over time (P = 0.29). However, the IL-5 levels measured after stimulation with anti-CD3/CD28 decreased significantly, reaching a nadir at 6 h after ingestion of thalidomide (relative decrease, 70% ± 15%; P < 0.05) (Fig. 1).

IL-12 and IL-10. Having established that thalidomide enhances the production of IFN-γ and decreases the level of IL-5 production, we wished to determine whether these effects were associated with altered levels of IL-12, a Th1-type cytokine, or IL-10, a Th2-type cytokine. After incubation without stimulus, the concentration of neither cytokines changed before or at any time point after the administration of thalidomide. The capacity of PBMCs to produce IL-12p40 was markedly increased at 6 h relative to the IL-12p40 concentration before thalidomide ingestion; SEB-induced IL-12p40 levels were increased 3,233% ± 1,750% (P < 0.05), and anti-CD3/CD28-induced IL-12p40 release was increased 348% ± 202% (P was not significant; Fig. 2). The level of neither SEB- nor anti-CD3/CD28-induced IL-12p70 production increased after ingestion of thalidomide.

TABLE 2. Effect of thalidomide administration on cell counts and differentials

<table>
<thead>
<tr>
<th>Time after thalidomide ingestion (h)</th>
<th>Leukocyte count (10⁹/liter)</th>
<th>Lymphocyte count (10⁹/liter)</th>
<th>CD3⁺/CD4⁺ lymphocyte count (10⁹/liter)</th>
<th>CD3⁺/CD8⁺ lymphocyte count (10⁹/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.25 ± 0.33</td>
<td>1.73 ± 0.21</td>
<td>0.91 ± 0.16</td>
<td>0.53 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>5.51 ± 0.32</td>
<td>1.82 ± 0.19</td>
<td>0.99 ± 0.17</td>
<td>0.59 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>5.84 ± 0.49</td>
<td>1.85 ± 0.16</td>
<td>1.01 ± 0.14</td>
<td>0.64 ± 0.08</td>
</tr>
<tr>
<td>24</td>
<td>5.10 ± 1.7</td>
<td>1.75 ± 0.21</td>
<td>0.91 ± 0.1</td>
<td>0.68 ± 0.13</td>
</tr>
</tbody>
</table>

* Values are means ± standard errors of the means for six healthy volunteers. Thalidomide (400 mg) was taken orally at time zero. No statistically significant differences in any of the cell counts were found at the four time points.
ingestion of thalidomide. Furthermore, IL-10 levels did not change over time (Fig. 2).

To determine the influence of thalidomide on monocyte-derived IL-12 levels, PBMCs were also stimulated with LPS. The capacity of PBMCs to produce IL-12p40 was increased at 6 h (69,881% ± 42,096%) and at 24 h (32,553% ± 9,631%) relative to that before thalidomide ingestion; however, this was not statistically significant (P = 0.11). No significant change in LPS-induced IL-12p70 levels was found after thalidomide ingestion (data not shown).

DISCUSSION

The immunomodulating capacity of thalidomide has led to its use for the treatment of different diseases, among which are mycobacterial and HIV infections. In the present study we sought to determine the effect of thalidomide on the Th1-Th2 balance in healthy volunteers by ex vivo stimulation of PBMCs with T-cell stimuli and subsequent determination of the level of cytokine release. We found that a single oral dose of thalidomide induced an increase in the capacity of PBMCs to secrete the Th1 cytokine IFN-γ upon stimulation, while their ability to release the Th2 cytokine IL-5 decreased. These changes were associated with a transient rise in the level of IL-12p40 production by PBMCs after administration of thalidomide. These data demonstrate for the first time that thalidomide may influence the Th1-Th2 balance in humans.

The increase in IFN-γ levels after stimulation of PBMCs with the specific T-cell stimuli SEB and anti-CD3/CD28 suggests a shift toward a Th1 response after oral ingestion of thalidomide. These findings are in accordance with previous reports on the in vitro effects of thalidomide. Indeed, thalidomide enhanced the level of IFN-γ production by T cells stimulated with immobilized anti-CD3 (6, 14). Others, however, found inhibition of IFN-γ release by PBMCs incubated with phytohemagglutinin (24). Our findings are in contrast to those of studies that have reported a shift toward Th2 after in vitro stimulation of PBMCs with phytohemagglutinin or streptokinase (19) and decreased levels of IFN-γ in serum in patients with ENL treated with thalidomide (26). Nonetheless, our ex vivo experiments are likely to be more representative of the human response to thalidomide and point toward a Th1-favoring effect of this drug.

Thalidomide ingestion was also associated with greater enhancement of the level of IFN-γ production and more inhibition of IL-5 release after stimulation with anti-CD3/CD28 than after incubation with SEB, while the enhancement of the level of IL-12p40 release was more profound after stimulation with SEB. It is conceivable that differences in the mechanisms by which anti-CD3/CD28 and SEB activate T cells contribute to
Thalidomide enhanced the level of IL-12p40 production but had no effect on the level of release of IL-12p70. A dissociation in IL-12p70 and IL-12p40 levels was significantly increased after ingestion of thalidomide, suggesting an agonistic role for the IL-12p40 dimer (10). Thus, it can be hypothesized that enhancement of IL-12p40 (dimer) production by thalidomide contributes to the release of increased levels of IFN-γ. In earlier in vitro studies, thalidomide suppressed both p70 and p40 production by human PBMCs stimulated with S. aureus or LPS (6, 20), while it enhanced the level of release of IL-12 (measured by an enzyme-linked immunosorbent assay that detects p70 and p40) by PBMCs incubated with immobilized anti-CD3 (6). Together, these data suggest that the effect of thalidomide on IL-12 release depends on the stimulus used, whereby primary T-cell stimulation results in the potentiation of IL-12 production.

The maximal serum thalidomide concentration was reached 6 h after drug administration, and the estimated elimination half-life was 11.1 h (27). The maximal measured effect of thalidomide was found 6 h (for IL-12p40 and IL-5) and 24 h (for IFN-γ and IL-2) after ingestion. Whether this discrepancy in serum thalidomide levels and the effect on cytokines must be attributed to an indirect effect of thalidomide or its metabolites or to other mediators remains to be established. Inhibition of TNF-α has been proposed as the most important biological effect of thalidomide. However, although thalidomide has been shown to inhibit monocyte TNF-α production in vitro (25), it does not seem to affect the level of TNF-α released by T cells (14), and several in vivo studies also showed no decrease in TNF-α levels (2, 15). In concordance with these results, we found neither an inhibition of TNF-α after stimulation of PBMCs with SEB or anti-CD3/CD28 nor a change in serum TNF-α levels after thalidomide ingestion (data not shown). Taken together, inhibition of TNF-α production does not seem to play a central role in the change in Th1 and Th2 cytokines induced by thalidomide.

A diurnal rhythm of antigen-induced Th1 and Th2 cytokine release, represented by IFN-γ and IL-10, respectively, has been described (22). We consider it unlikely that a circadian effect can explain our results for several reasons. The peak Th1 cytokine levels occurred 24 h after ingestion of thalidomide and fell in the described diurnal nadir of Th1 cytokine levels, while the lowest levels of IL-5 were in the expected period of peak Th2 cytokine levels (22). Furthermore, in our study IFN-γ and IL-2 release were especially enhanced at 24 h after thalidomide ingestion, ruling out a circadian effect.

Thalidomide has been found to exert beneficial effects in murine models of tuberculous meningitis (31) and pulmonary tuberculosis (21). In addition, thalidomide improved the clinical outcomes for patients with microsporidiosis (28) and HIV or mycobacterial infection (3, 11, 16). The common denominator in these pathological conditions in which thalidomide seems to be effective is the fact that a Th1 response is considered protective. Our present data therefore provide the first evidence of the possible mode of action of thalidomide in this discrepancy. Indeed, cross-linking of CD3 and CD28 results in direct T-cell activation, which is independent of the presence of antigen-presenting cells (APCs). SEB, a product of Staphylococcus aureus, is a superantigen which requires binding to both an APC and a T cell to induce T-cell stimulation; i.e., by binding to the major histocompatibility complex class II peptide of the APC, SEB can bind to the Vβ region of the T-cell receptor, resulting in polyclonal T-cell activation (17).

Thalidomide enhanced the level of IL-12p40 production but had no effect on the level of release of IL-12p70. A dissociation of LPS-induced production of IL-12p70 and p40 was found in endotoxin-tolerant mice (1), suggesting a different regulation of LPS-induced production of IL-12p70 and p40 was found in healthy volunteers. Black bars, cytokine levels after stimulation with SEB; open bars, cytokine levels after stimulation with anti-CD3/CD28. SEB-induced IL-12 was determined by an enzyme-linked immunosorbent assay (ELISA) that detects p70 and p40) by PBMCs incubated with immobilized anti-CD3 (6). Together, these data suggest that the effect of thalidomide on IL-12 release depends on the stimulus used, whereby primary T-cell stimulation results in the potentiation of IL-12 production.

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infections in humans. Further studies on the use of thalidomide as treatment for infections are warranted.

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REFERENCES