Thalidomide does not modify the ability of cells in leprosy patients to incorporate $[^3]H$-thymidine when incubated with M. leprae antigens

AZEB TADESSE*,**, +, ENGEDA TAYE**, F. SANDOVAL* & E. J. SHANNON*
*Armauer Hansen Research Institute, AHRI. PO Box 1005, Addis Ababa, Ethiopia
**Department of Pathobiological Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA
+Gillis W. Long Hansen’s Disease Center, Lab Research Branch, Louisiana State University. Baton Rouge, LA, USA
++All Africa Leprosy Rehabilitation and Training Center, Addis Ababa, Ethiopia

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Summary The immune response in reversal reaction, (RR) and in erythema nodosum leprosum (ENL) is characterized in vitro by an enhancement in lymphocyte blast transformation against M. leprae. As thalidomide is an effective treatment for ENL, this study assessed the effect of this drug on these phenomena. Mononuclear cells from patients attending the clinic at ALERT and from healthy staff were cultured for 5 days with integral M. leprae (IM), or a modified Dharmendra antigen (Dhar), or PPD from M. tuberculosis. In one set of cultures, thalidomide was added once at the initiation of the culture; in the other set thalidomide was added a second time (2×), 18 h prior to harvesting the cells. The mononuclear cells, in the absence of thalidomide, from healthy staff, borderline tuberculoid patients (BT) and BT patients in RR (BT/RR) incorporated $[^3]H$-thymidine best when cultured with PPD>Dhar>M. leprae. The cells from patients with ENL did not respond well to the M. leprae antigens. Thalidomide (2×) enhanced proliferation to Dhar in the BTRR group (Wilcoxon signed rank test, $P < 0.05$). No significant changes occurred for the other groups. Comparing PPD-stimulated cells treated with thalidomide once to those treated with thalidomide twice, thalidomide (2×) suppressed incorporation of $[^3]H$-thymidine by the PPD-stimulated ($P < 0.05$) as well as IMI-stimulated ($P < 0.05$) cells in the healthy staff group. In the Dhar-stimulated cells from the healthy staff thalidomide significantly suppressed TNF-α ($P < 0.05$). A mixed effect was seen within and between the other groups, but there was a trend for thalidomide to suppress TNF-α induced by the M. leprae, Dhar and PPD antigens.
Introduction

Leprosy is a chronic skin disease characterized by episodes of reactions. The cause of these reactions is unknown. They may occur during the normal course of the disease, during treatment and even after treatment when the patient is bacteriologically negative. They are classified as type I, reversal reactions (RR), or type II, erythema nodosum leprosum (ENL). Although these reactions have different clinical manifestations, they share a similar immunological profile. The immune response in acute ENL\(^1\), as well as in acute RR,\(^2\) is characterized \textit{in vitro} by an enhancement in lymphocyte blast transformation to \textit{M. leprae} antigen(s). These changes indicate the presence as well as the emergence of antigen-reactive-T-cells in leprosy patients that are normally anergic to \textit{M. leprae}.

Among healthy individuals\(^3\) and HIV positive patients,\(^4\) thalidomide has a stimulating effect on the ability of mitogen and antigen simulated T-cells to synthesize IL-2. Since this cytokine is important in the evolution of events that culminate in the proliferation of lymphocytes, an objective of our study was to determine if thalidomide could modify lymphocyte blast transformation to \textit{M. leprae} antigens.

The mononuclear cells from patients attending the clinic at ALERT for treatment of their leprosy or reactional episodes were exposed to thalidomide and to IMI, or antigens prepared by sonication of IMI after depletion of lipids (a modified Dhar), or to PPD prepared from \textit{M. tuberculosis}. After 4 days in culture the cells were pulsed with \(^{3}H\)-thymidine and on day 5 the amount of tritiated thymidine incorporated by the cells was determined.

Materials and methods

STUDY SUBJECTS

Ethiopian healthy staff employed at AHRI \((n = 11)\), who are in frequent contact with leprosy and tuberculosis patients, and leprosy patients admitted to ALERT hospital for treatment of leprosy \((n = 33)\) were enrolled in the study. Fourteen of the patients were classified as having borderline tuberculoid leprosy (BT). 11 BT patients were experiencing reversal reaction BT/RR \((n = 11)\), and eight patients with ENL.

All of the ENL patients included in the study were being treated with steroids. This might have hindered the \textit{in vitro} immune responses of mononuclear cells isolated from these patients.

Signed written consent was obtained from each patient. All patients were evaluated by history and physical examination prior to preparation of mononuclear cell cultures. The clinical characteristics of the study group are presented in Table 1.

THALIDOMIDE

Thalidomide was kindly provided by Dr K. Zwingenberger (Grünenthal GmbH, Stolberg/ Rhineland, Germany). The drug was dissolved in dimethylsulfoxide DMSO (Sigma, USA) and, within 5–10 min after diluting it in RPMI, it was added into appropriate wells. Thalidomide was used at 4·0\(\mu\)g/ml in the cultures with a final concentration of DMSO of 0·25% v/v in thalidomide and control cultures.
Table 1. Characterization of study groups

<table>
<thead>
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<th>Age range</th>
<th>M</th>
<th>F</th>
<th>MDT</th>
<th>Anti-inflammatory</th>
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<tr>
<td>Healthy contacts n = 11</td>
<td>25–56</td>
<td>7</td>
<td>4</td>
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<td>Borderline tuberculoid patients n = 14</td>
<td>13–77</td>
<td>6</td>
<td>8</td>
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<tr>
<td>Borderline tuberculoid patients with reversal reactions n = 11</td>
<td>15–72</td>
<td>5</td>
<td>6</td>
<td>2/11</td>
<td>7/11</td>
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<tr>
<td>Patients with erythema nodosum leprosum n = 8</td>
<td>15–52</td>
<td>4</td>
<td>4</td>
<td>4/8</td>
<td>7/8</td>
</tr>
</tbody>
</table>

a WHO recommended multidrug regimen for leprosy.

b Prednisone.

ANTIGENS PREPARED FROM MYCOBACTERIA

Mycobacterium tuberculosis

PPD RT 23 was kindly provided by Statensersumitsitut, Copenhagen, Denmark. The preservative-free PPD was diluted in RPMI to contain 20 μg/ml and distributed in wells in a volume of 50 μl for a final concentration of 5.0 μg/ml.

Mycobacterium leprae

The antigens from M. leprae were prepared at the National Hansen’s Disease Programs, Laboratory Research Branch, Immunology Laboratory, Baton Rouge. IMI (acid-fast-bacteria, IMI) was isolated from the tissue of nude mice infected with M. leprae. The tissue was aseptically removed from footpads of infected mice, and homogenized in 7H12 medium. The homogenate was centrifuged at 30 g for 15 min. The pellet was discarded and the supernatant centrifuged at 2450 g for 45 min. The pellet was suspended in 20 ml of 0.1 N NaOH. After 10 min, the alkali treated material was centrifuged at 2100 g for 30 min. The pellet was washed twice with pyrogen free distilled water and suspended in pyrogen free distilled water. One half of this preparation was frozen and used as the IMI. The other half was prepared, as follows, for the Dhar. IMI was centrifuged at 450 g for 30 min. The pellet was suspended in 6 ml of chloroform and dried using nitrogen gas. The dried fraction was suspended in 6 ml of ether and centrifuged at 450 g for 30 min. The supernatant was discarded and the bacteria suspended in ether and transferred to an agate mortar and worked to dryness with a spatula. The dried bacilli were suspended in 5.0 ml of pyrogen free distilled water, and disrupted by sonic oscillation, on ice, for 15 min at 30% duty cycle (Untrasonic Homogenizer 4710, Cole-Palmer Inst. Co., Chicago, IL, USA). The IMI were enumerated and numbers adjusted to contain 100 AFB bacilli/50 μl/1 mononuclear cell. The Dhar was assayed for content of proteins (DC Protein Assay, Bio-Rad), and added to the cell cultures in a volume of 50 μl. The final concentration was 10 μg of protein/ml.

CELL CULTURES

Mononuclear cells were isolated from 30–40 ml of blood collected in Na-heparin-treated vacuum tubes. The blood was diluted to twice its volume with RPMI-1640 and centrifuged on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The mononuclear cells were harvested and washed three times in RPMI-1640 (Flow Labs, Irvine, UK). The cells were adjusted to
contain $2 \times 10^6$ cells per ml RPMI-1640 that had been supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml of streptomycin and 10% v/v fetal calf serum (Sigma) (RPMI-10%). A 100 µl aliquot of RPMI-10% containing $2 \times 10^5$ mononuclear cells was added into flat-bottom wells of a 96-well plate. The control cultures and cultures not treated with thalidomide received 50 µl of RPMI-10% with DMSO at 1% v/v. The cultures treated with thalidomide received 50 µl of RPMI-10% with DMSO at 1% v/v and thalidomide. The final concentration of unhydrolysed thalidomide was 4.0 µg/ml. The final concentration of DMSO in all cultures was 0.25% v/v. The control cultures not stimulated with antigen received 50 µl of RPMI-10%. The cultures stimulated with antigens received 50 µl of RPMI-10% containing Dhar, or IMI, or PPD (as described above).

**ASSAY FOR LYMPHOCYTE BLAST TRANSFORMATION**

The cells were incubated at 5% CO$_2$, high humidity, 37°C incubator. Culture systems were set up under two different conditions. One group, at the initiation of the 5-day culture period received 4.0 µg/ml of thalidomide (1×). Another set of cultures, to insure a source of unhydrolyzed thalidomide during the pulse with [³H]-thymidine, received 4.0 µg/ml of thalidomide for the second time on day 4 (2×). On day four in the 2× thalidomide treated cultures, the concentration of unhydrolyzed thalidomide was estimated to be 4.0009 µg/ml with 94 ng/ml of residual unhydrolyzed thalidomide remaining after the first 4 days in culture and the additional thalidomide added for the second time. This calculation is based on the estimated t½ of 8 h for thalidomide in tissue culture medium at 37°C.⁵

On day 4, the cultures were pulsed with 1 µCi per well of H³-thymidine (Boehringer Mannheim, Germany). After 18 h, cells were harvested on filters and the amount of radioactive thymidine incorporated into cellular DNA was determined using a Rack-β Liquid Scintillation Counter (LKB, Pharmacia, Uppsala, Sweden). The results were expressed as stimulation index (SI), which is the mean counts per minute (CPM) from three replicate cultures of the mycobacterial antigen alone or the mycobacterial antigen + thalidomide treated cells divided by the CPM of cultures not receiving antigen or thalidomide.

**DETERMINATION OF TNF-α**

On day 4, prior to the addition of thalidomide, 50 µl of supernatants was collected from each culture and frozen for later analysis of TNF-α. Commercial ELISA reagents were purchased (R&D) and used according to the manufacturer’s specifications. The amount of TNF-α in each sample was determined from the standard curve using a program called KCJunior Version 1.31-2 (Bio-Tek Instruments Inc, Vermont). The concentrations of TNF-α in cultures were expressed in ng/ml.

The percentage suppression of TNF-α was calculated from the following formula:

% Suppression = \left[ 1 - \left( \frac{\text{TNF-α in thalidomide treated wells}}{\text{TNF-α in untreated cultures}} \right) \right] \times 100

**Results**

**PROLIFERATIVE RESPONSES TO THE ANTIGEN PREPARATIONS IN THE ABSENCE OF THALIDOMIDE**

The modified Dhar and *M. leprae* antigen preparations were titrated for their ability to stimulate mononuclear cells to incorporate [³H]-thymidine as described previously.⁶ Using
mononuclear cells from two healthy lepromin and PPD positive individuals, the Dhar stimulated the cells at 10 µg/ml. After exposing the cells to a ratio of 1:1 or 10:1 or 100:1 *M. leprae* acid fast bacteria (AFB): mononuclear cells, the maximum stimulation with IMI was observed at a ratio of 100 AFB to 1 mononuclear cell (data not shown).

Figure 1, Effect of thalidomide on antigen treated cultures. Cells were incubated with PPD, Dhar or AFB. Thalidomide was added at 4 µg/ml. Culture supernatants were collected at day 4, thalidomide was replenished in the 2× cultures and wells were pulsed with tritiated thymidine. After 18 h of incubation, cultures were harvested and CPM determined. *P*-value derived from Wilcoxon signed rank test. A = PPD, no thalidomide; A' = PPD + thalidomide (1× or 2×); B = Dharmendra, no thalidomide; B' = Dharmendra + thalidomide (1× or 2×); C = integral *M. leprae*, no thalidomide; C' = integral *M. leprae* + thalidomide (1× or 2×).
The mononuclear cells from the healthy staff, BT and BT/RR, in the absence of thalidomide, responded to the three antigen preparations with the rank of response to PPD>Dhar>IMI. The patients in the ENL group were not stimulated as well to the *M. leprae* antigens.

**EFFECT OF THALIDOMIDE ON THE PROLIFERATIVE RESPONSE AGAINST THE ANTIGEN PREPARATIONS**

Thalidomide (1× and 2×) did not alter the lymphoproliferative response to the mycobacteria antigens in the BT, ENL and healthy groups.

Thalidomide (2×) empowered the mononuclear cells from the BTRR patient group to incorporate [3H]-thymidine when stimulated with Dhar (Wilcoxon signed rank test *P* = 0·01) compared to thalidomide untreated cultures (Figure 1).

Among the thalidomide 2× treated healthy cells, the addition of thalidomide for the second time resulted in a suppression of cell proliferation in response to PPD and AFB compared to thalidomide untreated cultures. This is showed by the statistically significant decrease in SI (*P* = 0·02 for PPD and *P* = 0·01 for AFB). For the rest of the groups, replenishment of thalidomide in cultures did not affect the SI (Figure 1).

![Figure 2. Effect of thalidomide on TNF-α production. At day 4 of incubation, supernatants were collected from the various cultures prior to pulsing with tritiated thymidine. ELISA was done to determine the amount of TNF-α produced in each sample. The level of TNF-α produced was calculated from the standard curves generated with KC Junior Version 1.31.2 ELISA program. Results are expressed as percentage of enhancement or suppression calculated from the formula:](image)

\[
\%
\text{Suppression} = \left[ 1 - \left( \frac{\text{TNF-α in thalidomide treated wells}}{\text{TNF-α in untreated cultures}} \right) \right] \times 100
\]
EFFECT OF THALIDOMIDE ON SYNTHESIS OF TNF-α

Thalidomide resulted in a significant suppression of TNF-α production in culture supernatants from cells from healthy staff exposed to the modified Dhar (P < 0.05) (Figure 2). A mixed effect was seen for the other groups with a trend of suppression in most cases.

It is interesting to note that among the BT/RR study group, four of seven patients cells were stimulated to produce TNF-α when incubated with IMI and Thalidomide (Figure 2).

Discussion

In diseases like leprosy, where cell mediated immunity (CMI) plays an important role in protection as well as in development of reactions and subsequent complications, the in vitro response of lymphocytes from patients against mycobacterial antigens is correlated well with the clinical manifestations. We studied the effect of thalidomide on the lymphocyte blast transformation of cells from leprosy patients with or without reactions in response to stimulation with mycobacterial antigens.

In vitro studies on the immunomodulatory properties of thalidomide have for the most part been based on mitogen stimulated cells from healthy individuals. Primed antigen-specific cells from patients may give a completely different picture. The direction of the differentiation of naive T-cells mainly depend on the dose and type of antigens and on the surrounding cytokines microenvironment. In this study we used mononuclear cells from patients harboring and sensitized to leprosy bacilli.

Type I reaction in leprosy is characterized by a sudden increase in cell mediated immune response to M. leprae antigens. Our results show proliferation of BT/RR patients’ cells in response to all three antigens compared to unstimulated controls. However, thalidomide failed to alter the in vitro proliferative response of the same patients against mycobacterial antigens.

ENL patients’ cells have been reported to incorporate [3H] thymidine in response to soluble and IMI antigens. Although significant, this response was relatively lower to that observed in tuberculoid leprosy patients. The ENL patients included in our study did not respond well to the M. leprae antigen preparations. These patients were being treated with prednisone (some as much as 30 mg per day) and were probably in a hyporeactivity or anergic phase usually observed in post-treatment of ENL.

The Dhar treatment of mycobacteria is thought to improve the antigen recognition and immune cell response without affecting the antigenic constituents of the bacilli. This is well confirmed in our study where the response to Dhar, be it in healthy staff or BT and BT/RR leprosy patients, is relatively better than that to integral bacilli.

Thalidomide has been previously shown to enhance the DTH response to a challenge dose of PPD in individuals with PPD skin test positivity. In vitro, the drug had been shown to increase synthesis of IL-2 in PPD positive individuals. In our study thalidomide facilitated the response against Dhar in BTTRR patients.

In various in vitro studies, the effect of thalidomide on the ability of antigen or mitogen stimulated cells to incorporate [3H] thymidine is controversial. Among healthy PPD positive individuals in Mexico, it was found that thalidomide or metabolites of thalidomide did not alter the ability of cells stimulated with PPD to incorporate [3H]-thymidine. Another study has shown that the drug enhances the proliferation of TB and HIV co-infected patient’s cells
stimulated with PPD. One possible explanation for such inconsistencies is the extremely quick hydrolysis of thalidomide. Even in a physiological environment like blood, thalidomide quickly hydrolyses. This characteristic of thalidomide is even faster at pH 7.0–7.5. The type of solvent used, incubation times and pH of media determine the extent of hydrolysis and the type of metabolite which will predominate in the assay. Our data might be the summation of the actions of different hydrolysis products of thalidomide.

Parallel to the proliferation assay the effect of thalidomide on the level of TNF-α was also assessed. Suppression of TNF-α was exhibited in healthy contacts in response to Dhar. Despite several studies conducted for over a quarter of a century, thalidomide’s mechanism of action in arresting ENL is still unknown. This lack of understanding is accentuated by the uncertainties that still remain today about the factors which cause ENL.

Treatment of a given clinical condition with thalidomide may be associated with a decrease in TNF-α as in ENL or an increase in TNF-α like in aphthous ulcers. In a trial involving the use of thalidomide to treat toxic epidermal necrolysis, the work was discontinued due to an elevated drug-related toxicity. TNF-α levels were elevated in the patients who received thalidomide compared to the placebo group. Comparison of prednisone, pentoxifylline and thalidomide in the treatment of ENL revealed that although thalidomide is the most effective drug to relieve this condition, prednisone suppresses TNF-α more than thalidomide. Besides, the level of TNF-α detected in ENL is so low that the authors suggested that this cytokine might not be the only factor responsible for the pathology of ENL. The most convincing evidence that TNF-α is not the sole cytokine targeted by thalidomide in ENL is the observation that this drug is not an effective treatment for reversal reaction. The pathology of RR, even more so than in ENL is associated with an increase in TNF-α protein and TNF-α mRNA in the skin and peripheral nerves.

With the exception of cells from ENL patients, we obtained a fairly good stimulation in the presence of all three mycobacterial antigens without thalidomide treatment. Thalidomide resulted in significant suppression in healthy controls in response to PPD and IMI antigens.

In order to adjust for drug hydrolysis, thalidomide was added in cell cultures a second time. This replenishment caused an enhanced response to Dhar in the BTRR group whereas no effect was seen in the other groups.

TNF-α production was suppressed only in the healthy control group in response to Dhar. In the BT/RR groups in four of the seven patients an enhancement of TNF-α production was observed in cells stimulated with IMI. Additional work is needed to confirm this interesting finding.

Overall, we were not able to obtain a clear picture of the immuno-modulatory effect of thalidomide in leprosy. ENL is one of the immune complications where thalidomide works best. With the presently growing number of indications for thalidomide, another approach, like phenotypic analysis of cell surface molecules, might result in a clearly demarcated effect to elucidate the mechanism of action of this drug in ENL. This will provide background information for the synthesis of the best analogue with minimal side effects.

Acknowledgements

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