Effects of Thalidomide on Intracellular Mycobacterium leprae in Normal and Activated Macrophages

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Thalidomide is an effective drug for the treatment of erythema nodosum leprosum (ENL). ENL is an inflammatory reaction that may occur in multibacillary leprosy patients. Its cause(s) as well as the mechanism of thalidomide in arresting this condition are not fully understood. It has been suggested that ENL is an immune complex-mediated hypersensitivity precipitated by the release of Mycobacterium leprae from macrophages. The released antigen may complex with precipitating antibodies, initiating complement fixation and the production of inflammatory cytokines like tumor necrosis factor alpha (TNF-α). Thalidomide has been shown in vitro to reduce antigen- or mitogen-activated macrophage production of TNF-α. We investigated if thalidomide could also influence the viability of intracellular M. leprae. Mouse peritoneal macrophages were infected with M. leprae, activated with gamma interferon and endotoxin, or nonactivated, and treated with thalidomide. Intracellular bacilli were recovered, and metabolic activity was assessed by a radiorespirometric procedure. Thalidomide did not possess antimicrobial action against M. leprae in normal and activated host macrophages. This suggests that thalidomide does not retard the release of mycobacterial antigens, a possible prelude or precipitating factor for ENL. A distinct sequence of events explaining the mechanism of action for thalidomide’s successful treatment of ENL has yet to be established.

When administered following proper safety guidelines, thalidomide is the treatment of choice for erythema nodosum leprosum (ENL) (23). The sequence of events which precipitate ENL as well as the exact mechanism by which thalidomide arrests ENL have not yet been clearly delineated. The patients that may experience ENL during the course of their disease are those that are in the lepromatous end of the leprosy spectrum (lepromatous leprosy and borderline lepromatous leprosy) (7). These patients are characterized by the presence of precipitating antibodies in their sera and a tremendous load of mycobacterial antigen inside macrophages (16). As the likelihood of an episode of ENL increases after the initiation of treatment, it has been suggested that release of Mycobacterium leprae antigens from macrophages may be a factor which initiates ENL (7). The released antigen may then complex with antibodies, initiating complement fixation and production of inflammatory cytokines like tumor necrosis factor alpha (TNF-α).

Inhibition of TNF-α is one proposed mechanism by which thalidomide arrests ENL (18, 19). However, in a short open clinical study comparing the effects of thalidomide with other known inhibitors of TNF-α, like pentoxifylline and prednisone, the investigators concluded that thalidomide may have other targets in ENL (12).

Other evidence that TNF-α is not the sole cytokine targeted by thalidomide in ENL is the fact that thalidomide is not an effective treatment for reversal reaction (RR) (7). RR is another type of hypersensitivity reaction in leprosy, in which TNF-α is detected in skin and peripheral nerve lesions (9). Depending on experimental conditions and the type of cells stimulated, thalidomide may enhance or suppress the synthesis of TNF-α (20). We have recently shown that thalidomide enhances TNF-α in cells from RR patients stimulated with integral M. leprae (25).

The present report addresses a different tactic in exploring the efficacy of thalidomide in treating ENL reactions. Could thalidomide actually be inhibiting the killing and breakdown of M. leprae and thereby be inhibiting the release of antigens?

To study the effect of thalidomide on the fate of M. leprae in host macrophages, we assessed the viability of M. leprae residing in thalidomide-treated normal or gamma interferon (IFN-γ)–endotoxin–activated mouse macrophages.

MATERIALS AND METHODS

Bacterial source. Freshly isolated M. leprae from footpads of nude mice was used throughout the experiments (15). In each of the experiments the viability of 4 × 10⁸ acid-fast bacteria was assessed by inoculating the M. leprae suspension into vials containing BACTEC pyrazinamide (PZA) test medium (Becton Dickinson Diagnostic System, Sparks, Md.). The cumulative counts per minute (cpm) of ¹⁴CO₂ was measured daily for 7 days.

Mouse peritoneal macrophages. Macrophages were isolated from the peritoneal cavities of retired female Swiss Webster breeder mice (Simonsen Labs, Gilroy, Calif.). Briefly, 15 ml of cold heparinized phosphate-buffered saline was injected into the peritoneal cavity of each mouse. The peritoneal exudate was collected and washed. The cell pellet was resuspended in 10 ml of complete medium (RPMI 1640, 50 μg of ampicillin/ml, 2 mM glutamine, and 10% fetal bovine serum). Cells were counted and adjusted to 4 × 10⁶ per ml. The cell suspension was dispensed at 0.5 ml/well in 24-well plates containing LUX plastic coverslips (Miles Laboratory, Naperville, Ill.). The plates were incubated overnight in a humidified incubator at 37°C, 5% CO₂. Nonadherent cells were washed off by dipping coverslips in sterile phosphate-buffered saline and draining excess liquid on sterile gauze. The coverslips were placed in another 24-well plate containing 0.5 ml of complete medium/well.

Treatment conditions. Thalidomide was kindly provided by Celgene (Warren, N.J.). It was dissolved in a solution of 0.85% NaCl, pH 3.0 (acid saline), as described previously (21). Previous work had been done to monitor the degradation of thalidomide prepared as such. After a freeze-thaw cycle, thalidomide dissolved in acid saline and frozen at −70°C remains unhydrolyzed and stable for...
days at 4°C (unpublished observations). A stock solution of 50 μg/ml was aliquoted and kept in a −70°C freezer. Shortly before each experiment, thalidomide was diluted in acid saline to effect final concentrations of 0.625, 1.25, 2.5, and 5 μg/ml in the cell cultures. Based on the previously estimated half-life of 8.0 h for thalidomide in tissue culture medium at 37°C (6), cultures were replenished with 50 μl of acid saline containing thalidomide or 50 μl of acid saline daily to maintain an estimated concentration of ≥10 μg/ml of unhydrolyzed thalidomide in the 5-μg/ml-treated cultures.

**Activation of macrophages, infection with *M. leprae*, and treatment with thalidomide.** Monolayer cells on coverslips were incubated for 8 h with 200 U of mouse recombinant IFN-γ (IFN-γ; R&D Systems Inc., McKinley, Minn.)/ml and 2.0 ng of endotoxin (lipopolysaccharide [LPS]; Sigma, St. Louis, Mo.)/ml. Coverslips were washed as described previously and placed in fresh complete medium. Viable *M. leprae* organisms (4 × 10⁶) were added into the resting or activated macrophage monolayer at an estimated bacillus/macrophage ratio of 20:1. After an overnight incubation at 33°C, noningested bacilli were removed by washing the coverslips. The infected macrophages were incubated with the different concentrations of thalidomide and controls. Drug and media were replenished daily for 7 days in the nonactivated cultures and for 3 days in the activated cultures. A shorter exposure to thalidomide in the recombinant IFN-γ–LPS–activated macrophages was necessary, as the cells in the thalidomide- and acid saline-treated control wells were observed to progressively detach during the first 3 days of incubation. A confirmation of the microbialidal capacity of activated macrophages was based on their capacity to kill the ingested *M. leprae*.

**Assessment of *M. leprae* viability: radioisopotentometric assay.** A modified Bud- desmeyer radioisopotentometric assay was used to assess the viability of *M. leprae* released from macrophages as described previously (15). The coverslip containing the macrophage monolayer was placed in 400 μl of 0.1 M NaOH solution. Three hundred microliters of the lysate was gently mixed and transferred to a sterile glass vial containing 4.0 ml of PZA test medium with 1.0 μCi of [1-14C]palmitic acid and 50 μg of ampicillin/ml. The vials with loose caps were placed within PolyQ polyethylene vials containing a dried strip of Whatman DE42 filter paper (Whatman, Inc., Clifton, N.J.). The paper had been dipped in a mixture of Liquifluor PPO-POPOP (2,5 diphenyloxazole-1,4-bis(5-phenyloxazolyl) benzene) toluene concentrate (New England Nuclear, Boston, Mass.), Triton X-100, and 4.0 N NaOH-methanol. The strips were dried and kept at room temperature in a canister protected from light. The PolyQ vials with tightly closed caps were incubated at 33°C. The generation of radioactive 14CO₂ was determined daily with a Beckman model LS-6000IC liquid scintillation counter. Data are presented as cumulative cpm.

**Assessment of TNF-α in activated macrophage cultures.** Twenty-four hours after thalidomide treatment of activated and infected macrophages, an aliquot of the culture supernatant was removed and frozen at −70°C. TNF-α was measured by using a mouse TNF-α immunoassay kit according to the manufacturer’s specification (Quantikine; R&D Systems, Minneapolis, Minn.). The amount of TNF-α in each sample was determined from the standard curve using KCjunior version 1.31.2 (Bio-Tek Instruments Inc., Winooski, Vt.).

**Nitrile assay.** NO₃⁻ in culture supernatant was determined as described elsewhere (4). Briefly, Griess reagent was prepared and dilutions of a solution of NaNO₂ were used as standard. All samples were assayed in duplicate. Culture supernatant of activated macrophages treated with thalidomide for 24 h was collected as described for TNF-α. Fifty-microliter aliquots of standard and sample were dispensed into wells of a 96-well flat-bottom tissue culture plate. Duplicate blank wells with medium alone were included in each experiment. One hundred microliters of Griess reagent was added into each well, and the plate was incubated at room temperature for 20 min. Absorbance was read at 543 nm. The amount of NO₃⁻ in each sample was determined from the standard curve by using a program called KCjunior version 1.31.2 (Bio-Tek Instruments Inc.).

**RESULTS**

**Assessment of metabolic activity of *M. leprae* in resting and activated peritoneal macrophages.** Prior to infecting the macrophages, the viability of *M. leprae* was assessed by inoculation into PZA test medium. At day 7, the cumulative mean ± the standard deviation (SD) from four experiments was 36,344 ± 3,801 cpm.

During each experiment the metabolic activities of bacilli recovered from nonactivated as well as activated macrophages were assessed. Macrophages infected for 24 h were treated with alkali solution, the cell lysates were inoculated into PZA test medium, and the level of 14CO₂ released was measured daily for 1 week. Bacilli released from lysed macrophages were incubated in PZA test medium, and 14CO₂ was quantitated daily for 7 days. Shown are the mean ± SD cpm of three to eight replicates. *, P < 0.05; **, P < 0.01 (unpaired t-test).

**Effect of thalidomide on viability of *M. leprae* recovered from nonactivated and activated mouse peritoneal macrophages.** As *M. leprae* is an obligate intracellular pathogen, the focus of this work was on the effect of thalidomide on internalized bacilli. Over a 7-day period, a gradual daily increase in 14CO₂ was observed in the acid saline-treated control cultures and the thalidomide-treated wells at 0.625, 1.25, 2.5, and 5 μg/ml. There was no demonstrable titrated effect of thalidomide (data not shown). Figure 2 summarizes the cumulative mean cpm ± SD from three separate experiments on day 7 for thalidomide at 5.0 μg/ml. Comparison of the drug-free control group to the thalidomide-treated groups in nonactivated as well as activated macrophages revealed no significant difference (unpaired t-test).

**TNF-α and nitrite levels in activated macrophages.** Regardless of the duration of the cell cultures, nonactivated cells did not produce detectable amounts of TNF-α and/or NO₃⁻; therefore, TNF-α and NO₃⁻ were measured from IFN-γ–LPS–activated cells only.

After 24 h of exposure to thalidomide, no statistically significant differences in TNF-α and NO₃⁻ were observed compared to the medium- or acid saline-treated controls. Interestingly, although not statistically significant, the TNF-α levels were higher in thalidomide-treated cells (24.8 ± 3.47 pg/ml) than in acid saline-treated cells (18.12 ± 8.2 pg/ml) (Fig. 3).

**Morphological appearance of peritoneal macrophages.** Breakdown products of thalidomide have been described to inhibit the attachment of cells to plastic surfaces (3). For visual inspection of attachment of cells, coverslips from each treatment were stained with Quik-Diff (Dade Diagnostics, Aguada,
Puerto Rico), dried, and mounted on microscopic slides. The density of the cells on the coverslips appeared to be the same in all the treated groups.

Morphologically, the activated cells had a spindle-like shape and were clearly different than nonactivated cells. The thalidomide-treated activated macrophages were equally as dense on the coverslips as the acid saline-treated activated cells, and they were not morphologically different than acid saline-treated activated macrophages (Fig. 4).

DISCUSSION

In diseases like leprosy, a cell-mediated immune response plays a major role in the outcome of infection. The key components of this response are macrophages and T lymphocytes. Killing of *M. leprae* by activated macrophages is the main mechanism of elimination of the bacterium (24). ENL is characterized by the detection of a massive release of *M. leprae* from macrophages (16). Although thalidomide is an effective treatment for ENL, the mechanism by which it alleviates this reaction is not fully understood. It is possible that drugs like thalidomide, depending on their concentration, may enhance local production and release of TNF-α into the microenvironment of the ENL skin lesion and facilitate remodeling of inflamed tissue. When TNF-α is produced in lesser quantities and in a timely fashion, it mediates a variety of antiinflammatory events promoting wound healing and remodeling of injured tissue (27) and suppressing neutrophil migration (14).

The antiinflammatory and immunomodulatory properties ascribed to thalidomide offer multiple potential sites of action in arresting ENL. We have been trying to determine if thalidomide has any direct or indirect effect on the viability of *M. leprae*. Thalidomide in concentrations as high as 10 μg/ml did not directly alter the ability of *M. leprae* to oxidize [1-14C]palmitic acid (unpublished observations). In this work we studied if thalidomide could indirectly modulate the antimicrobial action of host macrophages against intracellular *M. leprae*.

Nonactivated mouse peritoneal macrophages infected with *M. leprae* were treated with 0.0625 to 5.0 μg of thalidomide/ml for a week. Thalidomide failed to show a dose-response relationship and failed to alter the metabolic activity of *M. leprae* in a significant manner. Higher concentrations of thalidomide may have shown a different result; however, the concentration of 5.0 μg/ml was, in our opinion, more relevant to that achieved in the treatment of ENL.

Our finding concurs with previous studies on the in vitro as well as in vivo antimicrobial action of thalidomide against other pathogens. Although thalidomide was effective in alleviating clinical symptoms associated with *Mycobacterium avium* complex infection in human immunodeficiency virus-infected patients (2), in vitro it was unable to kill laboratory-cultured strains of *M. avium* complex (26). In vivo, although thalidomide was effective in the treatment of microspordial diarrhea (22), when tested in vitro it failed to kill intracellular microsporida (17). In murine experimental tuberculosis (13) and BCG infection (5) models, thalidomide did not significantly alter bacterial load in organs.

For efficient activation of *M. leprae*-burdened macrophages, in addition to a primary stimulatory signal delivered by IFN-γ a secondary signal like endotoxin is required (10). We costimulated the peritoneal macrophages with IFN-γ and LPS. In the absence of thalidomide, the cumulative 14CO2 released from

![FIG. 2. Effect of thalidomide on viability of *M. leprae* recovered from nonactivated and activated mouse peritoneal macrophages. Infected mouse peritoneal macrophages were treated with 5 μg of thalidomide/ml (Thal 5) or acid saline (AS). Cells were lysed, and the viability of released bacilli was assessed by determining the cpm of cumulative 14CO2 released. Open bars, nonactivated macrophages; closed bars, activated macrophages. Shown are the mean ± SD cpm from three separate experiments.](http://cvi.asm.org/)

**FIG. 3.** Nitrite and TNF-α levels in *M. leprae*-infected LPS–IFN-γ–activated macrophages. Activated mouse peritoneal macrophages were infected with viable *M. leprae* cells and treated with thalidomide at 5 μg/ml for 24 h. Nitrite (A) and TNF-α (B) in culture supernatants were quantitated. Shown are the mean ± SD for four replicate wells. M, medium; AS, acid saline; T5, thalidomide (5 μg/ml).
oxidation of \(^{14}\text{C}\) palmitic acid in the PZA medium by \textit{M. leprae} recovered from nonactivated macrophages was higher than that from \textit{M. leprae} recovered from activated macrophages. This confirms a previous finding that activated mouse peritoneal macrophages kill or inhibit \textit{M. leprae} more effectively than nonactivated macrophages (1). The progressive detachment of IFN-\(\gamma\)/LPS–activated macrophages compared to nonactivated macrophages was to be expected (L. B. Adams, personal communication). When tested in this well-established activated macrophage system, thalidomide did not modify the viability of \textit{M. leprae} in a significant manner.

We have also examined the effect of thalidomide on TNF-\(\alpha\) produced in IFN-\(\gamma\)/LPS–activated \textit{M. leprae}-infected macrophages. Although statistical analyses revealed no significant difference between TNF-\(\alpha\) levels from thalidomide- and from acid saline-treated cells, a higher level of TNF-\(\alpha\) was observed in the thalidomide-treated group. This ability of thalidomide to enhance TNF-\(\alpha\) confirms an observation made in our laboratory (20). When human monocytes, isolated by a similar adherence technique, were stimulated with LPS and treated with 4.0 \(\mu\)g of thalidomide/ml, enhanced production of TNF-\(\alpha\) was observed. In an LPS-monocyte system where the human monocytes were isolated by depletion of lymphocytes by a sheep cell rosetting technique, thalidomide suppressed the production of TNF-\(\alpha\) (19). Depending on the type of cells stimulated and the nature of the stimulant, thalidomide tends to produce different effects on cytokines. In our previous study using peripheral blood mononuclear cells from healthy individuals occupationally exposed to \textit{M. leprae} and stimulated with a preparation of \textit{M. leprae} that had been depleted of lipids (Dharmendra antigen), TNF-\(\alpha\) was suppressed due to thalidomide treatment (25). In T-cell–anti-CD3 systems, thalidomide enhanced the production of TNF-\(\alpha\) in healthy individuals (11).

Reactive nitrogen intermediates, especially nitric oxide (NO), constitute a major antimicrobial effector armature of activated mononuclear phagocytes with activities against a broad spectrum of pathogens (8). A reduction in the viability of \textit{M. leprae} occurs when activated mouse peritoneal macrophages produce a high level of NO (1). In our study, although the level of NO produced was similar to that reported by others, thalidomide failed to influence the production of this molecule.

Using macrophages derived from the peritoneum of mice, we were not able to demonstrate a bactericidal effect of thalidomide on intracellular \textit{M. leprae} in normal or activated macrophages. Also, thalidomide did not alter the production of TNF-\(\alpha\) or NO in a significant manner. The use of a human monocyte cell line like THP-1 or adherent peripheral blood mononuclear cells (monocytes) may have given a different response. However, THP-1 cells require activation via phorbol 12-myristate 13-acetate or endotoxin before they differentiate into adherent cells, and adherent human peripheral blood mononuclear cells (monocytes) are poorly phagocytic to \textit{M. leprae} and require opsonins (unpublished data).

In this work, we were able to show that thalidomide does not possess a direct antimicrobial action against intracellular \textit{M. leprae} in normal or activated macrophages. Nor did it alter the production of TNF-\(\alpha\) and NO in a significant manner. A distinct sequence of events explaining the mechanism of action
for thalidomide to successfully treat ENL has yet to be established.

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REFERENCES