Thalidomide Costimulates Primary Human T Lymphocytes, Preferentially Inducing Proliferation, Cytokine Production, and Cytotoxic Responses in the CD8+ Subset

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Summary

The efficacy of thalidomide (α-phthalimido-glutarimide) therapy in leprosy patients with erythema nodosum leprosum is thought to be due to inhibition of tumor necrosis factor α. In other diseases reported to respond to thalidomide, the mechanism of action of the drug is unclear. We show that thalidomide is a potent costimulator of primary human T cells in vitro, synergizing with stimulation via the T cell receptor complex to increase interleukin 2–mediated T cell proliferation and interferon γ production. The costimulatory effect is greater on the CD8+ than the CD4+ T cell subset. The drug also increases the primary CD8+ cytotoxic T cell response induced by allogeneic dendritic cells in the absence of CD4+ T cells. Therefore, human T cell costimulation can be achieved pharmacologically with thalidomide, and preferentially in the CD8+ T cell subset.

Key words: thalidomide • costimulation • T cells • CD8+ • pharmacologic immune modulation

Thalidomide’s immune-modulating effects were discovered serendipitously over 30 years ago. Administration of the drug, when prescribed as a sedative (its original indication) to leprosy patients with erythema nodosum leprosum (ENL), was found to heal the inflammatory skin lesions and eliminate the systemic symptoms that characterize that condition (1). We showed subsequently that the efficacy of thalidomide in ENL is associated with a rapid reduction in the circulating level of the proinflammatory cytokine TNF-α (2). In further studies, we demonstrated that thalidomide exerts a specific inhibitory effect on the production of TNF-α by lipopolysaccharide-stimulated monocytes in vitro (3). These results suggested that inhibition of this cytokine is a possible mechanism for the antinflammatory effects of thalidomide therapy.

Recently, studies in animal models and human diseases have been initiated to explore the potential application of this agent in a range of inflammatory and immunologic disorders. Compelling data have now accumulated confirming the efficacy of thalidomide in ENL (4) and in aphthous ulceration complicating HIV disease (5, 6). Other studies have suggested a potential application in chronic GVHD (7, 8). The observation that thalidomide appears to be effective in these conditions when conventional immunosuppressive therapy (usually with corticosteroids) has failed continues to stimulate interest in identifying the possible mechanism(s) of action of this controversial agent.

There has been much interest in the possibility that thalidomide might not only affect monocyte cytokine production, but also influence the functions of T cells, since the latter have central effector and regulatory roles in immune responses. However, the results of investigations by several different groups have revealed no clear or consistent effect of thalidomide on primary T cell responses. Taken together, these observations have suggested either that any effect on T cell function is indirect, or that such an effect is elusive in conventional T cell assays.

Recent studies have shown that optimal activation of T cells requires the delivery of a second, costimulatory signal in addition to antigenic stimulation via the TCR (9). Physiologically, these costimulatory signals are effected by interactions between molecules on the surface of specialized APCs and their ligands on T cells. Thus, modifying T cell costimulation may have a profound impact on the integrity of the immune response. This is exemplified by the immunosuppressive effect of specifically blocking the interaction of one such ligand pair, B7 (on the APC) and CD28 (on the T cell) (10). Therefore, it is important to consider such accessory pathways when investigating T cell function.

Abbreviations used in this paper: DC, dendritic cell; ENL, erythema nodosum leprosum; ER, erythrocyte-rosetting; GAF, glutaraldehyde-fixed; r-hu, recombinant human; SEA and SEB, staphylococcal enterotoxin A and B.
We set out to examine the effects of thalidomide on primary human T cell responses in vitro. Initially, we were interested in studying the effects of thalidomide on TNF-α production by these cells, but found no effect of the drug on this response. Unexpectedly, when purified T cells were stimulated by antibodies to CD3, a component of the TCR complex, thalidomide provided an essential costimulatory signal for T cell proliferation and lymphokine production. The costimulatory effect of thalidomide was more pronounced in the CD8+ subset of T cells. This is the first example of a pharmacologic agent used in the treatment of human disease that acts as a costimulator for T cells. Moreover, thalidomide preferentially stimulates CD8+ T cells.

Materials and Methods

Preparation of Cells

PBMCs and T Cells. PBMCs were isolated from the blood of healthy volunteers by Ficoll density gradient separation. T lymphocytes were purified by rosetting with neuraminidase-treated sheep erythrocytes and subsequent incubation of erythrocyte-rosetting (ER+) cells on a nylon wool column. Nonadherent cells were eluted from the column and were ≥97% CD3 antigen-positive by flow cytometry (FACStar; Becton Dickinson, San Jose, CA). The latter population we refer to herein as “bulk” T cells. Primary CD4+ and CD8+ T cells were purified from PBMCs by positive selection with magnetic beads coated with mouse anti-human CD4 and anti-CD8 antibodies, respectively (Dynal A.S., Oslo, Norway), at a bead/cell ratio of 2–3:1. Positively selected cells were then dissociated from the beads by incubation with a second antibody competing for the antigen binding site on the primary anti-human antibody (Detachabead; Dynal A.S.). These cells were ≥98% CD4+CD3+ or CD8+CD3+ by flow cytometry.

APCs. PBMCs were enriched for APCs in experiments with staphylococcal enterotoxin superantigens or in one-way alloimmune reactions by depletion of ER+ populations. For the latter experiments, these non-erythrocyte-rosetting (ER−) populations were irradiated with 2,000 rads from a cesium source. In experiments with staphylococcal superantigens, ER− populations were rendered costimulator-deficient by 0.05% glutaraldehyde fixation for 30 s, followed by the addition of 0.2 M MOPS to stop the reaction and four washes in PBS.

These cell cultures were set up in RPMI medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS and penicillin/streptomycin.

Dendritic Cells. Dendritic cells (DCs) were derived from peripheral blood progenitors as described by Bender et al. (11). In brief, ER− cells were incubated for 7 d with recombinant human (r-hu) IL-4 (Genzyme Corp., Cambridge, MA) and r-hu GM-CSF (Immunex, Seattle, WA), each at 1,000 u/ml, followed by a 4-d incubation with macrophage-conditioned medium. At the end of this period, the cells exhibited stellate morphology characteristic of DCs, were CD83+, HLA-DR+, and CD14− by flow cytometry, and were highly efficient inducers of proliferation and cytotoxicity in purified alloreactive T cells.

Target Cells for CTL Assays. To obtain target cells for the CTL 51Cr-release assays, polystyrene-adherent ER− cells from either the same donor as the DCs (alloimmune targets) or the same donor as the T cells (autologous control targets) were cultured in Teflon tissue culture dishes in RPMI containing 5% human serum at 37°C until required for the CTL assay (up to 1 wk).

Thalidomide

Thalidomide (Celgene Corp., Warren, NJ) was dissolved in DMSO to give a stock solution of 20 mg/ml, which was kept for up to 1 wk. Before stimulation, T cells were preincubated for 1–2 h at 37°C with thalidomide or DMSO (control) at the concentrations indicated in Results. Cell cultures were treated daily with the appropriate dilutions of thalidomide stock or DMSO.

T Cell Stimulation

Purified T cells were stimulated in three experimental systems: cross-linking of the TCR by immobilized monoclonal anti-human CD3. The anti-CD3 antibody (OKT3; a gift of Dr. Robert Z. Lieh, OrthoBiotec Inc., Raritan, NJ) was diluted in PBS and coated onto flat-bottomed polystyrene tissue culture plates by overnight incubation at 4°C, at concentrations indicated in Results. In some experiments, exogenous r-hu IL-2 (Chiron Corp., Emeryville, CA) was added at a final concentration of 10 U/ml. Thalidomide- or DMSO-treated T cells were added to 96-well plates at 105 cells per well for triplicate proliferation assays, and to 24-well plates at 106 cells per ml for the measurement of cytokine production in culture supernatant fluid. In preliminary experiments, unsorted PBMCs were also stimulated in this system. In cytokine blocking experiments, cultures were treated with polyclonal rabbit anti-human IL-2 or polyclonal sheep anti-human IL-4 antibodies (Endogen, Inc., Cambridge, MA) at final concentrations of 40 and 10 μg/ml, respectively.

Presentation of the Superantigens Staphylococcal Enterotoxin A and B by Costimulator-deficient APCs. Glutaraldehyde-fixed (GAF) APCs were incubated with purified CD4+ or CD8+ T cells and both staphylococcal enterotoxin A and B (SEA and SEB; Toxin Technology Inc., Sarasota, FL) so that the final concentration of each superantigen was 10 ng/ml and the APC:T cell ratio was 1:1. Cultures were set up in triplicate in 96-well round-bottomed tissue culture plates with 106 of each cell type per well. Stimulation of T Cells by Allogeneic DCs. DCs/T cell cocultures were set up in round-bottomed 96-well plates. T cells were purified CD4+, CD8+, or recombined CD4+ and CD8+ (ratio of 2:1), and were treated with thalidomide or DMSO as above. Each well contained 105 T cells in a final volume of 0.2 ml. The ratio of DC to T cells was 1:30.

T Cell Proliferation Assays

T cell proliferative responses were assayed by measuring [3H]thymidine incorporation during the last 12 h of 72-h cultures. DNA was harvested onto filter mats with an automatic cell harvester (Skatron Instruments Inc.) after a 6-h incubation at 37°C. Incorporation of [3H]thymidine into T cells was measured with a beta plate liquid scintillation counter (model LKB 1205; Wallac, Gaithersburg, MD).

CTL Assays

Standard 6-h 51Cr-release assays were set up as described by Young and Steinman (12). On the day of assay, 5 × 104 51Cr-labeled mononuclear target cells were added to the wells in the 96-well plates, and supernatants were collected with a multichannel harvesting frame (Skatron Instruments Inc.) after a 6-h incubation. Released radioactivity was measured with an automated γ counter (Wallac). Identical 96-well plates set up in parallel were...
Results

Effect of Thalidomide on T Cell Proliferative Responses after Stimulation by Immobilized Anti-CD3. Preliminary experiments carried out on PBM C s obtained from two healthy tuberculin-sensitive donors showed no effect of thalidomide on lymphocyte proliferative responses to the mitogens PHA and PMA, the superantigens SEA and SEB, or purified protein derivative (data not shown). However, when PBMCs from three individual donors were stimulated by cross-linking the TCR with immobilized anti-CD3 mAbs alone, thalidomide induced a concentration-dependent increase in proliferative responses (Fig. 1).

In subsequent studies, the effect of thalidomide on purified peripheral blood T cells (“bulk” T cells) was evaluated. Exposure to a constant concentration of thalidomide (10 μg/ml) caused a marked enhancement of proliferative responses to increasing concentrations of anti-CD3 (Fig. 2 A). At low concentrations of anti-CD3, the T cells failed to respond unless thalidomide was added. This effect was also evident in purified CD4+ and CD8+ T cell subsets (Fig. 2 B). Again, there was no proliferative response to thalidomide in the absence of anti-CD3, indicating that thalidomide was not mitogenic per se, but acted as a costimulator. We observed that significant augmentation of the proliferative response by thalidomide occurred at lower concentrations of anti-CD3 for bulk and CD8+ T cells than for CD4+ T cells (Fig. 2, A and B). Fig. 2 C shows that when the concentration of anti-CD3 was constant, there was a thalidomide concentration–dependent increase in proliferative responses in all three lymphocyte populations. This effect was relatively modest in the CD4+ T cell subset compared with bulk or CD8+ T cells within individual donors, although large thalidomide-induced increases in the CD4+ T cell response were seen in some individuals. To confirm that the differential effect of thalidomide on the two T cell subsets was not due to an incapacity of the CD4+ population to respond fully, experiments were carried out using PMA, a well-described chemical costimulator of T cells. Addition of PMA stimulated CD4+ and CD8+ T cell proliferation similar to thalidomide (data not shown).

In further experiments, when the TCR was cross-linked by anti-CD3 in the presence of exogenous IL-2 (10 U/ml), no effect of thalidomide on T cell proliferation was apparent (Fig. 2 D). However, when the cytokine was not added, thalidomide substituted almost completely for exogenous IL-2 in stimulating the proliferative responses of CD8+ T cells, while restoring ~50% of the CD4+ T cell response under the same conditions (Fig. 2 D).

Effect of Thalidomide on T Cell Production of Cytokines. We next examined the effect of thalidomide on cytokine production by the three T cell populations when stimulated by anti-CD3 in the absence of exogenous IL-2. Thalidomide induced a consistent concentration-dependent increase in IL-2 production at 12 h (time of peak production determined in preliminary kinetic experiments), in CD8+ and in bulk T cells (Fig. 3 A). Although a trend towards a thalidomide dose response in IL-2 production by purified CD4+ T cells was apparent, production of this cytokine was modest in comparison to that of bulk and CD8+ populations. Exposure to thalidomide consistently increased IFN-γ production by all three T cell populations. Since there was no clear peak in production of the latter cytokine, the results of a kinetic experiment are shown for CD4+ and CD8+ T cells (Fig. 3 B) using a single concentration of thalidomide (10 μg/ml). Again, the drug induced relatively greater production of IFN-γ by purified CD8+ T cells. However, the thalidomide-induced augmentation of IFN-γ production by CD4+ T cells was marked in comparison to the drug’s weak effect on IL-2 production.

Cytokines

Tissue culture supernatants were harvested and frozen immediately at −70°C until assay in duplicate for IL-2, IFN-γ, IL-4, or TNF-α by enzyme-linked immunoassay (Endogen, Inc.).

Donor Blood and Experimental Design

Blood for these experiments was drawn from a total of nine healthy adult donors. Each experiment was repeated at least three times with different donors. The observations reported here were consistently present in experiments from all donors. Findings from individual representative experiments are shown to illustrate each point.
by this subset. Bulk T cells displayed a similar IFN-γ profile to CD8+ T cells (data not shown). All three lymphocyte populations showed a concentration-dependent increase in IFN-γ production in response to thalidomide (data not shown). In contrast, thalidomide did not affect significantly the production of TNF-α by either bulk or CD8+ T cells, and the production of TNF-α by CD4+ cells was minimal in this system (Fig. 3C). A critical consequence of T cell costimulation is the production of IL-2. Thus, experiments to evaluate the effect of the drug on TNF-α production were also carried out in which exogenous IL-2 was added to eliminate the costimulatory effect.
of thalidomide. Again, no consistent inhibitory effect of thalidomide on TNF-α production by T cells was found (data not shown). IL-4 was not detected in the supernatants of T cells stimulated with anti-CD3 in the presence or absence of thalidomide (data not shown).

Taken together, these observations suggested that thalidomide-induced T cell proliferation was dependent on the autocrine production of IL-2 but not IL-4. To confirm these findings, neutralization of IL-2 by a polyclonal antibody was carried out. Anti-IL-2 abrogated the proliferative effect of thalidomide, whereas the addition of anti-IL-4 antibody had no effect. This observation was consistent in the three T cell populations under study (Fig. 3 D), and confirmed that the stimulatory effect of thalidomide was dependent on the induction of endogenous IL-2 production. Together, these experiments suggest that thalidomide is acting as a costimulus to T cells, which have received a primary signal through the cross-linked TCR.

Effect of Thalidomide on Lymphocyte Proliferative Responses to Staphylococcal Superantigens Presented by Autologous GAF APCs. Next, we sought to determine whether thalidomide could costimulate T cell responses after superantigen presentation by APCs. PBMCs enriched for APCs were fixed in 0.05% glutaraldehyde. This chemical modification rendered the APCs deficient in costimulatory function (13, 14) while preserving their capacity to present staphylococcal superantigens to purified autologous CD4+ and CD8+ T cells. When T cells were exposed to thalidomide in this system, there was a marked increase in CD4+ and CD8+ T cell proliferative responses (Fig. 4). The absolute response of CD8+ T cells was modest in comparison with that of CD4+ T cells, since characteristically, CD4+ T cells respond more vigorously to superantigens. However, the fold increase in response to thalidomide relative to cells treated with DMSO was consistently greater in CD8+ than in CD4+ T cells if expressed as a “stimulation ratio” (thalidomide cpm ÷ DMSO cpm). Accordingly, the mean (±SD) stimulation ratio was 7.14 (±3.55) for CD8+ T cells in three experiments versus 3.88 (±1.04) for CD4+ T cells in six experiments.

Effect of Thalidomide on the Proliferative and Cytotoxic Responses of Purified CD8+ T Cells in a DC/T Cell Allogeneic Reaction. To evaluate the effect of thalidomide on T cells receiving a more physiologic stimulus, we set up cocultures of purified T cells with blood-derived HLA-mismatched DCs and studied primary CTL killing of allogeneic targets. A standard 6-h microassay was used. Monocytes (from the same donor as the DCs) labeled with 51Cr were used as targets. No significant cytotoxic activity was observed before day 4 of DC/T cell coculture. Maximum CTL activity by purified CD8+ T cells was observed on days 4–5 (Fig. 5 D), whereas recombined CD4+ and CD8+ T cells showed peak CTL activity on days 5–6 (Fig. 5 F). Purified CD4+ T cells stimulated with allogeneic DCs were not cytotoxic (Fig. 5 E). Thalidomide increased both proliferative and cytotoxic responses of purified CD8+ T cells in the absence of CD4+ T cells (Fig. 5, A and D). However, when a mixed CD4+/CD8+ T cell population was reconstituted at a physiologic ratio of 2:1, no significant enhancing effect of thalidomide was evident (Fig. 5, C and F). Similarly, thalidomide did not enhance the proliferative response of a purified CD4+ T cell population (Fig. 5 B). Thus, CD8+ T cell functional responses to allogeneic DCs can be further enhanced by thalidomide, but only in the absence of CD4+ T cell “help.”

Discussion

Several different immunomodulating and/or antiinflammatory mechanisms of action have been suggested for thalidomide, including the specific inhibition of TNF-α both in vitro and in vivo (2, 15). In addition, in vitro investigations have shown that thalidomide can modify surface adhesion molecules on leucocytes (16), cause a shift from Th1 to Th2 type T cell responses (17), and inhibit production of monocyte IL-12 (18), as well as inhibit angiogenesis (in an animal model of corneal neovascularization [19]). Reports of thalidomide’s efficacy in GVHD in animal models (20–22) and in clinical studies (7, 8) suggested that the drug might modify T cell responses. However, several investigations seeking to demonstrate an inhibitory action of thalidomide on T cell responses have resulted in inconsistent findings (23–25).

In this study, we report that thalidomide can consistently boost primary human T cell responses in vitro by acting as a costimulatory molecule. The optimal demonstration of this effect required that the T cells be highly purified, and that a single stimulus be delivered to the T cell via the TCR. Experiments with sorted cells revealed that for every
Thalidomide Costimulation of T Cells

donor, the relative response to thalidomide was more pronounced in the CD8+ than the CD4+ T cell subset, although substantial costimulation of CD4+ T cells was observed occasionally. This differential effect contrasted with the effect of PMA, a reagent used in vitro to costimulate T cells, which exerted a similar effect on both CD4+ and CD8+ T cells. Pharmacokinetic data indicate that the costimulatory effects of thalidomide could be achieved at concentrations as low as the 1-2 µg/ml attained in the plasma of human subjects after thalidomide treatment (26).

In contrast to systems in which we tested for costimulation of T cells stimulated with anti-CD3, no effect of the drug on proliferative responses was seen when unsorted PBMCs were stimulated by recall antigens or mitogens. Therefore, the costimulatory effect of thalidomide is apt to be overlooked in in vitro systems where the dominant response is by CD4+ T cells, and in experiments designed to reveal inhibition rather than stimulation. Interestingly, two recent studies showed a stimulatory effect of thalidomide (27) and thalidomide metabolites (28) on IL-2 production by mitogen-stimulated PBMCs, but found no effect on T cell proliferation.

The differential costimulatory effect of thalidomide on CD8+ T cell proliferation is intriguing. To examine the effect of thalidomide on a well-characterized functional CD8+ response in a more physiologically relevant system, we induced primary cytotoxic T cell responses by stimulation with allogeneic DCs. The latter experiments confirmed the differential effect of thalidomide in stimulating proliferative responses in CD8+ but not CD4+ T cells, and in increasing CD8+ CTL responses. Moreover, the costimulatory effect on CD8+ T cells was not observed in the presence of CD4+ T cells, suggesting that in the presence of physiologic CD4+ T cells, no further help is required, and that the effects of thalidomide on CD8+ T cells are superfluous. Similarly, any costimulatory effect of the drug on CD4+ T cells is inapparent in the presence of a full complement of physiologic costimulatory signals, as delivered by DCs. Thus, the costimulatory effects of thalidomide on CD8+ T cell function may have particular relevance in clinical settings where CD4+ T cell function is defective, such as in HIV disease, or in situations where there is a defect in T cell costimulation.

These in vitro observations of differential costimulation of T cells by thalidomide may have an in vivo counterpart. We reported recently a differential increase in circulating CD8+ over CD4+ T cell numbers in 13 patients with HIV infection treated with thalidomide, resulting in a drop in the CD4+/CD8+ ratio (29). A consistent drug-induced increase in plasma levels of soluble IL-2 receptor, a marker of T cell activation, was also seen in this cohort. Furthermore, we have observed the restoration of delayed-type cutaneous hypersensitivity to purified protein derivative of tuberculin during thalidomide treatment in five previously anergic patients with HIV infection (reference 15, and our unpublished findings), which was lost after stopping the drug. These findings are consistent with T cell stimulation by thalidomide in patients in vivo, and raise the interesting possibility that this agent may have a novel application as an immunologic adjuvant in HIV disease.

Although thalidomide is the drug of choice in ENL (4), corticosteroids are often used in the management of this condition when thalidomide is unavailable. A recent controlled trial has established the efficacy of thalidomide in the treatment of refractory HIV-associated oral aphthous ulceration (5). This agent has also been used in the management of chronic GVHD when alternatives, notably corticosteroids, have failed. The ability of thalidomide to act in
T cell responses, without inhibiting normal immunity. Inhibition of TNF-
immune modulation by thalidomide may occur through the
Together with these observations, our data suggest that im-
findings presented here to note anecdotal reports of the ef-
Thalidomide may also play an important role in this setting
In conclusion, we have shown a novel and unique prop-
property of thalidomide in differentially costimulating CD8+ T cells.
Further clinical and laboratory-based studies are neces-
response contribute to its efficacy in a wide range of clinical disorders.

The authors wish to thank Judy Adams for help in preparing the
ting, and Drs. Ralph Steinman, Victoria H. Freedman, and Chau-ching Liu for critical discussions during the
preparation of the manuscript.

This work was supported by Public Health Service grants AI-22616 and AI-33124. We also acknowledge
from Celgene Corp. (Warren, NJ) and the "Direct Effect" HIV research support organization.

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Received for publication 28 January 1998.

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1891 Haslett et al.