Thalidomide Selectively Inhibits Tumor Necrosis Factor α Production by Stimulated Human Monocytes

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Summary

Thalidomide selectively inhibits the production of human monocyte tumor necrosis factor α (TNF-α) when these cells are triggered with lipopolysaccharide and other agonists in culture. 40% inhibition occurs at the clinically achievable dose of the drug of 1 μg/ml. In contrast, the amount of total protein and individual proteins labeled with [^{35}S]methionine and expressed on SDS-PAGE are not influenced. The amounts of interleukin 1β (IL-1β), IL-6, and granulocyte/macrophage colony-stimulating factor produced by monocytes remain unaltered. The selectivity of this drug may be useful in determining the role of TNF-α in vivo and modulating its toxic effects in a clinical setting.

Thalidomide (α-*N*-pthalimidoglutarimide) has a long pharmacological history, having been used as a sedative, an antiinflammatory, and an immunosuppressive agent (1–3). Currently, it is used for the therapy of erythema nodosum leprosum (ENL),[1] or type II reaction, an acute inflammatory state occurring in lepromatous leprosy characterized by severe systemic symptoms, including fever, painful cutaneous lesions, arthritis, glomerulonephritis, and the presence of circulating immune complexes (4). In this serious complication of leprosy, thalidomide has a prompt and dramatic effect, decreasing inflammation and enhancing patient well-being so that it remains the drug of choice for the therapy of ENL (5). The fever, weight loss, and general debility of ENL, as in other forms of acute and chronic disease, including sepsis and cancer, may be associated with the production of macrophage-derived cytokines (6, 7). Recently, serum levels of TNF-α and IL-1β released mainly by mononuclear phagocytes were found to be markedly elevated in ENL (8). In addition, it was noted that TNF-α levels were reduced after treatment of patients in ENL. This prompted a detailed examination of the effects of thalidomide on cytokine production by monocytes.

[1] *Abbreviations used in this paper:* CWP-ML, cell wall protein of *Mycobacterium leprae*; ENL, erythema nodosum leprosum; GM-CSF, granulocyte/macrophage colony-stimulating factor; PPD, purified protein derivative of tuberculin.

Materials and Methods

Monocyte Isolation. PBMC obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation were rosetted with neuraminidase-treated (*Vibrio cholerae* neuraminidase; Calbiochem-Behring Corp., La Jolla, CA) sheep erythrocytes (Scott Laboratories, Friskville, RI) (SRBC rosetting), and the nonrosetted cells were counted (E^- population monocytes enriched). 10^6 cells were cultured at 37°C in 24-well plates (Corning Glass Works, Corning, NY) in 1 ml of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% AB^+ serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Adherent E^- cells were used for the studies.

Cytokine Agonists. LPS of *Salmonella minnesota* R595 (List Biological Laboratories, Campbell, CA) was diluted in PBS, pH 7.4, and used at 1 μg/ml; Purified protein derivative of tuberculin (PPD) was purchased from Statens Seruminstitut, Copenhagen, Denmark; cell wall protein of *Mycobacterium leprae* (CWP-ML) was prepared through the National Institute of Allergy and Infectious Diseases, and provided by Dr. Patrick Brennan (Department of Microbiology, Colorado State University, Fort Collins, CO). Concentration of the stimulating agents were determined in previous experiments to induce optimal TNF-α protein production by cultured monocytes. The endotoxin content of solutions and mycobacterial preparations was estimated by the *Limulus* amebocyte lysate assay (LAL; Whittaker M.A. Bioproducts, Walkersville, MD). All solutions used contained <10 pg/ml of endotoxin.

Cytokine Induction. Adherent E^- cells were stimulated with 1 μg/ml of LPS, 10 μg/ml of PPD, or 10 μg/ml of CWP-ML for up to 18–20 h. At various times, supernatants were harvested, centrifuged to remove cells and debris, and kept frozen until use (−20°C).

TNF-α Assay. TNF-α concentration in the supernatants was determined with a TNF-α-specific ELISA, specific for the biologically active molecule. The reagents were a donation from Dr. Baron R. Reed, Genentech, Inc., South San Francisco, CA. Assays were performed in 96-well plates (Nunc Immunoplates, Roskilde, Denmark) coated with the affinity-purified rabbit anti-TNF-α antibody (0.5 μg/ml; 12–16 h; 4°C) and blocked for 2 h at room temperature with PBS/0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) containing 5 mg/ml BSA. After washing, 100 μl of TNF-α standards, samples, and controls were applied to the wells, and the plates were incubated for 12–24 h at 4°C. After the incubation, plates were washed and a second antibody, horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-TNF-α, diluted 1:2,000 in PBS/BSA/Tween, was applied to the wells and incubated for 2 h at room temperature. The color reaction was developed with the OPD substrate (0.4 mg/ml *o*-phenylenediamine [Sigma Chemical Co.] in 24 mM citric acid, 51 mM sodium phosphate, pH 5.0 [phosphate-citrate buffer; Sigma Chemical Co.], containing 0.012% hydrogen peroxide [H_2O_2; Fisher Scientific Co., Pittsburgh, PA]) and absorbance read at 492 nm in an automated ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA).

IL-1β Assays. IL-1β levels were determined using a commercial ELISA kit (Cistron Biotechnology, Pine Brook, NJ) according to the manufacturer's specifications. IL-1β levels are expressed as picograms per milliliter of protein.

IL-6 Assay. IL-6 levels were determined using a biological assay as described (9). Proliferation of 7TD1 hybridoma cell line specifically sensitive to IL-6 was measured by colorimetric determination of hexosaminidase levels (10), and values for IL-6 in the samples were obtained by interpolation from a standard curve. 1 U/ml of IL-6 corresponds to the concentration that yields half-maximal growth.

Granulocyte/Macrophage CSF (GM-CSF) Assay. GM-CSF levels were determined using a commercial ELISA kit (Genzyme, Boston, MA) according to the manufacturer's specifications, and were expressed as picograms per milliliter of protein.

Thalidomide Inhibition. The thalidomide used in this study was the purified drug (racemic mixture of D [+] and L [−] forms) (lot No. JB-I-114; Andrulis Research Corporation, Beltsville, MD). The compound was shown to be at least 99% pure, melting sharply at 270°C (published melting range, 269–271°C) (11). It was then diluted in DMSO (Sigma Chemical Co.); further dilutions were done in sterile PBS.

Percentage inhibition of TNFα secretion was calculated as: 100× [1-(TNF-α experimental/TNF-α control)]; where TNF-α experimental represents TNF-α secretion by stimulated monocytes that were cultured in the presence of thalidomide, and TNF-α control represents TNF-α secretion by stimulated monocytes that were cultured in the absence of the drug. Monocytes cultured in medium containing equivalent amounts of DMSO in the presence or absence of the stimulating agent were used as controls for thalidomide-treated cells. Neither thalidomide nor DMSO had any effect on cell viability or function at the concentrations used.

Protein Synthesis. Human monocytes were cultured in teflon beakers in methionine-free RPMI with 10% AB^+ serum at 37°C for 1 h, when 200 μCi/ml [^{35}S]methionine (1,153 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) was added to the cultures for the next 3 h with or without the stimulating and the suppressive agent. At the end of the labeling period, ^{35}S-labeled cells were washed twice in ice-cold PBS and lysed directly in 500 μl lysis solution (10 mM Tris-HCl buffer, pH 7.4, 150 NaCl, 1 mM EDTA, and 1% SDS). Resolving 8% SDS-PAGE was performed overnight. The gel was washed, dried, and analyzed by autoradiography at −70°C using XAR-5 radiographic film (Kodak, Rochester, NY) with an intensifying screen.

Results

Monocytes were enriched from PBMC of normal donors and stimulated in vitro for 18–20 h with bacterial LPS and mycobacterial products, known agonists of monocyte TNF-α synthesis and secretion (12, 13). Thalidomide suppressed LPS-stimulated TNF-α production (Fig. 1 *A*) with a 50% inhibitory concentration (IC_{50}) of 1–4 μg/ml, and ~90% inhibition observed at 10 μg/ml (18–20-h assay). Similar results were obtained when PPD and CWP-ML were used as stimulants (Fig. 1, *B* and *C*, respectively).

The inhibition of TNF-α secretion by thalidomide was dependent upon the state of monocyte stimulation (Table 1). Preincubation of unstimulated monocytes with thalidomide, followed by removal of the drug before LPS stimulation, did not lead to suppression. By comparison, when LPS and thalidomide were added simultaneously to the cultures, irreversible suppression occurred, even when the drug was re-

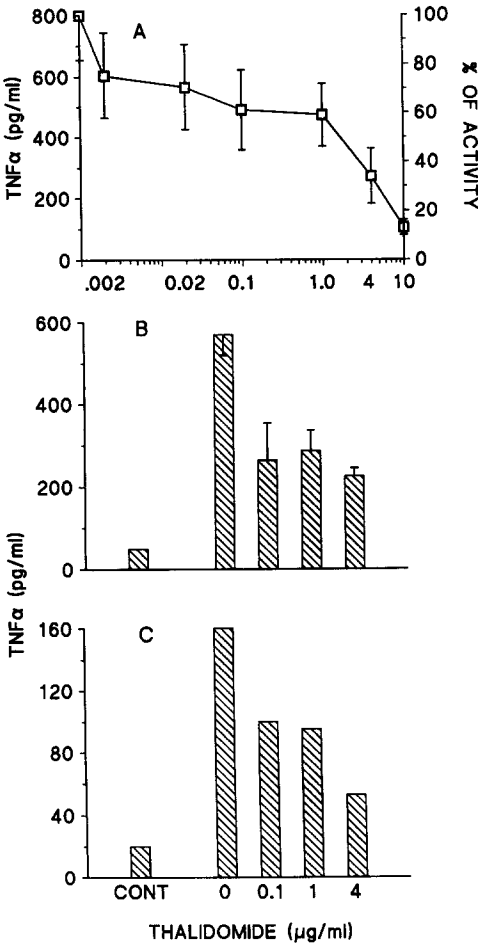

Figure 1. Effect of thalidomide on (*A*) bacterial endotoxin (LPS, 1 μg/ml), (*B*) PPD, (10 μg/ml), and (*C*) CWP-ML (10 μg/ml)-induced TNF-α production. Monocytes were simultaneously incubated with 2 ng/ml to 10 μg/ml of thalidomide in the culture medium. Control cells were cultured in medium alone. A dose-dependent inhibition of TNF-α secretion by thalidomide was noted. No detectable production of TNF-α protein was observed in supernatants of unstimulated monocytes. Data represent mean ± SD of 15 (*A*), two (*B*), and one (*C*) different experiments, respectively.

Table 1. *Effect of Preincubation of Monocytes with Thalidomide*

	Preincubation			Incubation			
	Time	Thalidomide (4 μg/ml)	LPS (1 μg/ml)	Time	Thalidomide (4 μg/ml)	LPS (1 μg/ml)	Percent activity
	h			*h*			
A	0–4	0	0	4–20	0	+	100
B	0–4	+	0	4–20	0	+	90 ± 4.6
C	None	0	0	0–4	+	+	48 ± 15
D	0–4	+	+	4–20	0	+	56 ± 0.5
E	None	0	0	0–20	+	+	52 ± 9.3

Human monocytes cultured in 24-well plates were preincubated with the inhibitory drug with or without the stimulating agent. After 4 h, the cultures were washed, medium was replaced, and LPS was added again for the next 16 h. Culture supernatants were recovered at the different periods and TNF-α levels determined as described. LPS-induced release of TNF-α by monocytes cultured for 20 h in the absence of thalidomide (*A*). No inhibitory action of thalidomide was detected when the drug was washed away before the addition of the stimulating agent (*B*). Thalidomide-induced inhibition of TNF-α production in the presence of LPS after 4 h of stimulation (*C*), which perisisted even after the drug was washed away (*D*). Control experiment in which thalidomide was kept in the cultures with the stimulating agent during the whole assay (*E*). Data represent mean ± SD of two different experiments.

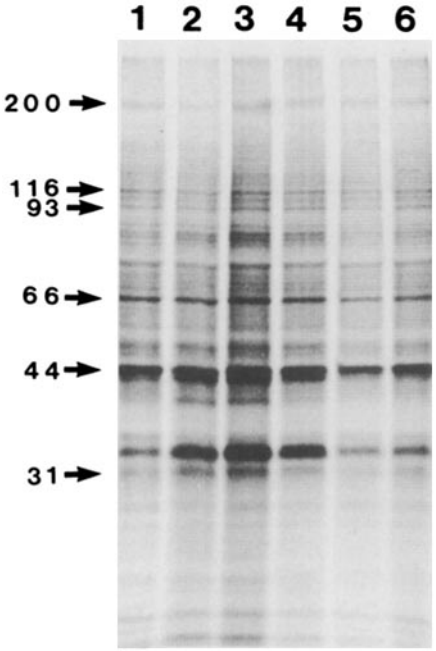

Figure 2. Effect of thalidomide on protein synthesis by human peripheral blood monocytes. Electrophoretic analysis of lysates from monocytes incubated with [^{35}S]methionine was performed. Cells were stimulated in vitro with and without LPS in the presence or absence of thalodomide at 1 and 4 μg/ml. TCA-precipitable radioactivity (10% TCA precipitation) was measured by liquid scintillation counting. The amount of radioactivity in the pellets is expressed as cpm × 10^{-3} and represents the mean of three precipitates with a SD of 10%. Neither total radioactivity nor the pattern of most of the protein bands in the gel was affected by thalidomide. (Lane *1*) Unstimulated cells, 3.3 × 10^{-2} cpm in TCA precipitates; (lane *2*) cells stimulated with 1 μg/ml LPS, 4.2 × 10^{-2} cpm in TCA precipitate; (lane *3*) cells stimulated with LPS in the presence of 1 μg/ml thalidomide, 4.2 × 10^{-2} cpm in TCA precipitate; (lane *4*) cells stimulated with LPS in the presence of 4 μg/ml thalidomide, 4.1 × 10^{-2} cpm in TCA precipitate; (lanes *5* and *6*) cells incubated only with thalidomide at 1 or 4 μg/ml, respectively, 3.2 × 10^{-2} and 2.8 × 10^{-2} cpm in TCA precipitates, respectively.

moved after a few hours (Table 1). Therefore, the thalidomide-sensitive reaction(s) occurs only after the LPS induction of TNF-α production.

The inhibition of LPS-stimulated TNF-α secretion by thalidomide occurs in a setting in which many other proteins are being synthesized by both constitutive and induced mechanisms (14). Thus, a simple explanation for the effect of the drug on TNF-α production could be a suppression of overall protein synthesis. Fig. 2 illustrates the effect of thalidomide on the pattern and quantity of proteins synthe-

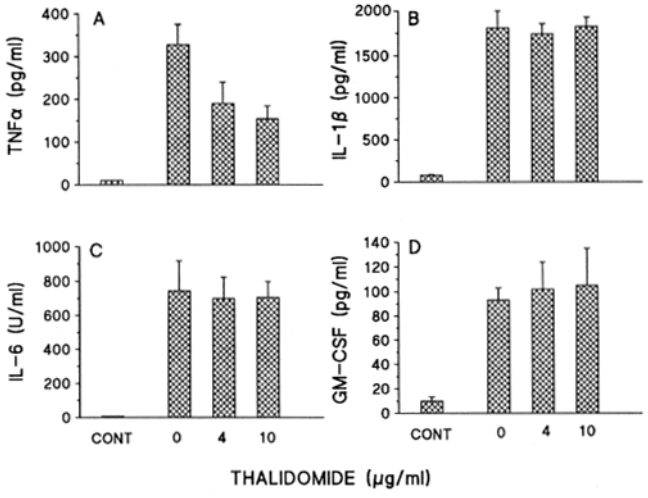

Figure 3. Levels of different cytokines tested in culture supernatants of human monocytes stimulated with LPS for 6 h (*A–C*) or 20 h (*D*) in the presence or absence of 4 or 10 μg/ml of thalidomide. Data represent mean ± SD of six different experiments for TNF-α and IL-1β determinations and three experiments for IL-6 and GM-CSF measurements. About 41.9 ± 14.6% and 52.8 ± 14.7% inhibition of TNF-α secretion was found in the presence of 4 and 10 μg/ml of thalidomide, respectively. (Cont) Unstimulated cells cultured in medium. No effect on IL-1β, IL-6, or GM-CSF secretion was detected in these cultures.

sized after a 3-h pulse of [^{35}S]methionine (legend to Fig. 2). The total incorporation of isotope into TCA-precipitable proteins as well as the intensity of most of the individual bands on SDS-PAGE of LPS-triggered monocytes remained unchanged after thalidomide treatment.

Several cytokines are produced by monocytes in response to LPS in addition to TNF-α, including IL-1β and IL-6 (15–16). Fig. 3 shows that thalidomide exerts a selective effect by suppressing only TNF-α secretion by LPS-stimulated monocytes. Whereas 4 μg/ml thalidomide suppressed TNF-α production (41.9% inhibition) (Fig. 3 *A*), neither IL-1β (Fig. 3 *B*), IL-6 (Fig. 3 *C*), nor GM-CSF production (Fig. 3 *D*) was influenced by the drug. Similar but more extensive selective suppression was observed with much higher (up to 20 μg/ml) concentrations of thalidomide (not shown).

Discussion

These experiments demonstrate that thalidomide inhibits TNF-α production by human blood monocytes, without influencing either general protein synthesis or the expression of three other monocyte-derived cytokines. Although cells of the mononuclear phagocyte series appear to be the major producers of TNF-α, other cells, in particular, T lymphocytes and NK cells, also synthesize this molecule. Therefore, additional experiments clarifying the spectrum of cell types sensitive to thalidomide action are now warranted. Experiments in animals and clinical observations in various inflammatory diseases (17–19) suggest that the production of excess TNF-α is related to a number of toxic manifestations of infection. Fever, cachexia, and general debilitation are usually associated with elevated TNF-α levels, found in cancer (20) and a variety of chronic infectious and parasitic diseases, including tuberculosis and the opportunistic infections of the acquired immunodeficiency syndrome (21, 22). Therefore, it is possible that the use of thalidomide may significantly improve the quality of life for these patients. In this regard, it is important to note that the thalidomide concentrations found to be effective in vitro (IC_{50} at 1–4 μg/ml) are similar to the plasma concentration obtained in man (i.e., up to 1.5 μg/ml) after the administration of a single oral dose of 150 mg thalidomide (23). As mentioned, this dose is strikingly effective at alleviating the acute symptoms of ENL in leprosy patients.

We must, however, be aware that TNF-α may play a positive role in host resistance, in infections (24) as well as against malignancies. Therefore, its suppression could have deleterious effects on host immunity. In this regard, since total inhibition of TNF-α secretion in vitro does not occur at 1–4 μg/ml, careful dosing of the thalidomide during therapy would lead to the persistence of some TNF-α production. Finally, the potent teratogenicity of the drug is well established and its use in females of child-bearing age must be carefully controlled. In this respect, the observation that only one of the enantiomers of thalidomide (the S or optical L [−] form) displays teratogenic properties (25) is of interest. Preliminary results obtained in this laboratory suggest that the purified nonteratogenic enantiomer R or D (+) isomeric form alone is capable of suppressing TNF-α production, similar to the racemic mixture.

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References

1. Mellin, G.W., and M. Katzenstein. 1962. The saga of thalidomide. *N. Engl. J. Med.* 267, 1184.
2. Moncada, B., M.L. Baranda, R. Gonzalez-Amaro, R. Urbina, and C.E. Loredo. 1985. Thalidomide-effect on T cell subsets as a possible mechanism of action. *Int. J. Lepr.* 53:201.
3. Vogelsang, G.B., S. Taylor, G. Gordon, and A.D. Hess. 1986. Thalidomide, a potent agent for the treatment of graft-versus-host disease. *Transplant. Proc.* 23:904.
4. Ridley, D.S. 1969. Reactions in leprosy. *Int. J. Lepr.* 40:77.
5. Barnhill, R.L., and C. McDougall. 1982. Thalidomide: Use and possible mode of action in reactional lepromatous leprosy and in various other conditions. *J. Am. Acad. Dermatol.* 7:317.
6. Tracey, K.J., H. Wei, K.R. Manogue, Y. Fong, D.G. Hesse, H.T. Nguyen, G.C. Kuo, B. Beutler, S. Cotran, A. Cerami, and S.F. Lowry. 1988. Cachetin/tumor necrosis factor induces cachexia, anemia, and inflammation. *J. Exp. Med.* 167:1211.
7. Girardin, E., G.E. Grau, J.M. Dayer, P. Roux-Lombard, The J5-Study Group, and P.-H. Lambert: 1988. Tumor necrosis

factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med.* 319:397.
8. Sarno, E.N., G.E. Grau, L.M.M. Vieira, and A.C. Nery. 1990. Serum levels of TNF alpha and IL-1 beta during leprosy reactional states. *Clin. Exp. Immunol.* In press.
9. Finkelman, F.D., I.M. Katona, J.F. Urban, Jr., C.M. Snapper, J. Ohara, and W.E. Paul. 1986. Suppression of in vivo polyclonal IgE response by monoclonal antibody to the lymphokine B cell stimulatory factor 1. *Proc. Natl. Acad. Sci. USA.* 83:9675.
10. Landegren, J. 1984. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokine and cell surface antigens. *J. Immunol. Methods.* 67:379.
11. Budavari, S., Editor. 1989. The Merck Index. Merck and Co., Inc., Rahway, NJ. Compound no. 9182.
12. Beutler, B., N. Krochin, I.W. Milsark, C. Lvedke, and A. Cerami. 1986. Control of cachetin (tumor necrosis factor) synthesis: mechanism of endotoxin resistance. *Science (Wash. DC).* 232:977.
13. Wallis, R.S., M. Amir-Tahmasseb, and J.J. Ellner. 1990. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte western blot. *Proc. Natl. Acad. Sci. USA.* 87:3348.
14. Bursten, S.L., R.M. Locksley, J.L. Ryan, and D.H. Lovett. 1988. Acetylation of monocyte and glomerular mesangial cell proteins. Myristylacylation of the interleukin 1 precursors. *J. Clin. Invest.* 82:1479.
15. Wallis, R.S., H. Fujiwara, and J.J. Ellner. 1986. Direct stimulation of monocyte release of interleukin 1 by mycobacterial protein antigens. *J. Immunol.* 136:193.
16. Navarro, S., N. Debili, J.F. Bernaudin, W. Vainchenker, and J. Doly. 1989. Regulation of the expression of IL-6 in human monocytes. *J. Immunol.* 142:4339.
17. Oliff, A., D. Defeo-Jones, M. Boyer, D. Martinez, D. Kiefer, G. Vocolo, A. Wolfe, and S.H. Socher. 1987. Tumours secreting human TNF/cachetin induce cachexia in mice. *Cell.* 50:555.
18. Scuderi, P., K.E. Sterling, K.S. Lam, K.J. Ryan, E. Petersen, P.R. Finley, R.G. Ray, D.J. Slymen, and S.E. Salmon. 1986. Raised serum levels of tumor necrosis factor in parasitic infections. *Lancet.* ii:1364.
19. Waage, A., P. Brandtzalg, A. Halstensen, P. Kierlulf, and T. Espevibe. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. *J. Exp. Med.* 169:333.
20. Balkwill, F., F. Burke, D. Talbot, J. Taveinier, R. Osborne, S. Naylor, H. Durbin, and W. Fliers. 1987. Evidence for tumour necrosis factor/cachetin production in cancer. *Lancet.* ii:1229.
21. Barnes, P.F., S.J. Fong, P.J. Brennan, P.E. Twomey, A. Mazumder, and R.L. Modlin. 1990. Local production of tumor necrosis factor and IFN-γ in tuberculous pleuritis. *J. Immunol.* 145:149.
22. Lahdevirta, J., C.P.J. Maury, A.-M. Teppon, and H. Repo. 1988. Elevated levels of circulating cachetin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am. J. Med.* 85:289.
23. Green, J.N., and B.C. Benson. 1961. The spectrophotometric determination of thalidomide in body fluids. *J. Pharm. Pharmacol.* 13(Suppl.):117.
24. Havell, E.A. 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J. Immunol.* 143:2894.
25. Blaschke, G. 1986. Chromatographic resolution of chiral drugs on polyamides and cellulose triacetate. *J. Liq. Chromatogr.* 9:341.