

# Prolonged Treatment with Recombinant Interferon $\gamma$ Induces Erythema Nodosum Leprosum in Lepromatous Leprosy Patients

By Elizabeth P. Sampaio,\* Andre L. Moreira,† Euzenir N. Sarno,† Ana M. Malta,† and Gilla Kaplan\*

From the \*Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021; and the †Leprosy Unit, Oswaldo Cruz Foundation, Manguinhos, Rio de Janeiro, Brazil, 21045

## Summary

10 patients with borderline and lepromatous leprosy were selected for a prolonged trial with recombinant interferon  $\gamma$  (rIFN- $\gamma$ ). Patients received 30  $\mu\text{g}$  intradermally for six injections over a 9-d period, and then either 100  $\mu\text{g}$  intradermally every 1 mo for 10 mo or every 2 wk for 5 mo (total, 1.2 mg). Erythema nodosum leprosum (ENL) was induced in 60% of the patients within 6–7 mo, as compared with an incidence of 15% per year with multiple drug therapy alone. The mean whole-body reduction in bacterial index over the first 6 mo was 0.9 log units. Cutaneous induration at the intradermal injection sites of  $\geq 15$  mm predicted the development of a subsequent reactional state. Monocytes obtained from patients receiving the lymphokine demonstrated an increased respiratory burst and a 2.5–5.1-fold increase in tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) secretion in response to agonists. Patients in ENL had an even higher release of TNF- $\alpha$  from monocytes as well as high levels of TNF- $\alpha$  in the plasma (mean, 2,000 pg/ml). Thalidomide therapy was required to treat the systemic manifestations of ENL. Control of toxic symptoms with thalidomide was associated with a 50–80% reduction in agonist-stimulated monocyte TNF- $\alpha$  secretion. IFN- $\gamma$  enhanced the monocyte release of TNF- $\alpha$  by 3–7.5-fold (agonist dependent) when added to patient's cells in vitro, and this could be suppressed by the in vitro addition of 10  $\mu\text{g}/\text{ml}$  of thalidomide.

Our previous studies have described the response of patients with lepromatous leprosy to the administration of rIFN- $\gamma$  (1–3). This T cell- and NK cell-derived lymphokine is a major activator of monocyte/macrophage antimicrobial activity, and activation is attributed to the enhancement of oxygen radical formation (4). The anergy of lepromatous leprosy patients to antigens of *Mycobacterium leprae* is reflected in their inability to produce IFN- $\gamma$  (5). We therefore considered that replacement therapy with rIFN- $\gamma$  might be useful in these patients. Our studies, initiated in 1985 with the intradermal injection of rIFN- $\gamma$  to multibacillary patients, revealed the local accumulation of T cells and monocytes, the destruction of parasitized macrophages, and a reduction in the number of acid-fast bacilli (AFB)<sup>1</sup> at the site of injection (1–3). More recently, 10 daily intramuscular injections

of 10–30  $\mu\text{g}$  rIFN- $\gamma/\text{m}^2$  led to a systemic, cutaneous T cell infiltrate, although a reduction in AFB was not observed (6).

We now report our experience with the administration of rIFN- $\gamma$  to lepromatous leprosy for 6–10 mo. Lymphokine injections were associated with the development of erythema nodosum leprosum (ENL) in 6 of 10 patients. In addition, an enhanced reduction of whole body bacterial load was noted. Monocytes obtained from ENL patients secreted high levels of TNF- $\alpha$ . Thalidomide therapy of systemic ENL promptly led to a cessation of symptoms associated with a reduction in TNF- $\alpha$  production. The effects of IFN- $\gamma$  and thalidomide on TNF- $\alpha$  production could be reproduced in vitro.

## Materials and Methods

**Patient Population.** 10 lepromatous leprosy patients diagnosed according to the Ridley-Jopling classification (7) (three borderline lepromatous [BL], two subpolar lepromatous [LLs], and five polar lepromatous [LL]) from the Leprosy Out-Patient Unit, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were selected for the in vivo rIFN- $\gamma$  injections (Table 1). All patients had been treated for 3–8 mo (mean, 3.9 mo) with multidrug therapy (MDT) (600 mg Rifampin and 300 mg clofazimine, monthly supervised, and 100

<sup>1</sup> Abbreviations used in this paper: AFB, acid-fast bacilli; BCG, Bacillus Calmette-Guerin; B.I., bacterial index; BL, borderline lepromatous leprosy; CWP-ML, cell wall protein of *M. leprae*; ENL, erythema nodosum leprosum; LL, lepromatous leprosy; LAM-ML, lipoarabinomannan of *M. leprae*; ML, *Mycobacterium leprae*; MDT, multidrug therapy; PGL-I, phenolic glycolipid I; PPD, purified protein derivative of tuberculin.

mg dapsone, daily, and 50 mg clofazimine, daily unsupervised). The mean  $\pm$  SEM bacterial index (B.I.) at diagnosis for all 10 patients was  $3.0 \pm 0.25$ , and at initiation of the study  $2.49 \pm 0.36$ . Medication was continued during the study. B.I.s reported here for each subject were determined from slit smears collected from six sites on the body. None of the patients had presented with any reactional manifestations before the beginning of the study. For in vitro TNF- $\alpha$  measurements, blood cells from nine normal donors (laboratory personnel), 15 lepromatous leprosy patients (seven BL, five LLs, and three LL), and 13 lepromatous patients (four BL, three LLs, and six LL) with ENL were tested. These patients had been treated for 0–2 yr with MDT. Seven ENL patient blood samples were taken before starting treatment with thalidomide. The other six patients had been treated with thalidomide (300 mg/d) for 1–2 wk before blood samples were collected.

89 LL/BL patients diagnosed and treated with MDT at the same clinic from 1 yr before and during the study were evaluated for their rate of reduction in B.I. during 18 mo of therapy. The patients were selected for compliance and for clinical matching (LL/BL) with the study population and were used as controls. The mean  $\pm$  SEM B.I. at diagnosis for all 89 patients was  $2.92 \pm 0.06$ , and at 3 mo after initiation of therapy (about the mean time the test group entered into the study) was  $2.35 \pm 0.13$ .

**rIFN- $\gamma$  Injections.** rIFN- $\gamma$  (6507; Boehringer Ingelheim, Ingelheim am Rhein, Germany) was purified from *Escherichia coli* transfected with cDNA from human rIFN- $\gamma$ . The lyophilized product was reconstituted in pyrogen-free sterile distilled water as described (1) and had a sp act of  $2 \times 10^7$  U/mg of protein. After written consent, lepromatous patients were injected into a leprosy lesion with six intradermal 100- $\mu$ l injections of 30  $\mu$ g rIFN- $\gamma$  (three daily injections, 3 d rest, and another three daily injections). 1 mo after the last 30- $\mu$ g injection, patients received the next intradermal injection (100  $\mu$ l) of 100  $\mu$ g rIFN- $\gamma$  in the back. A total of 10 100- $\mu$ g doses were administered: six patients were injected on a monthly basis and four patients were injected every 2 wk. Patients were evaluated before the injections, after the sixth 30- $\mu$ g injection, and after 1, 6, and 12 mo of study. Induration at the site of lymphokine administration was recorded 24 h after each 30- $\mu$ g injection.

**Cell Cultures.** Heparinized venous blood was collected for in vitro tests, and PBMC were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. For monocyte enrichment, PBMC were rosetted with neuraminidase-treated (*Vibrio cholera* neuraminidase; Calbiochem-Behring Corp., La Jolla, CA) sheep erythrocytes (Calico Biologicals, Inc., Reamstown, PA) (8). A total of  $10^6$  nonrosetted cells ( $E^-$  population) were cultured in 24-well plates (Corning Glass Works, Corning, NY) in 1 ml RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 10% pooled AB $^+$  serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (complete medium). Nonadherent cells were washed away and the adherent cells were stimulated with TNF- $\alpha$ -inducing agonists for 18–20 h, when supernatants were harvested and kept frozen until use ( $-20^\circ\text{C}$ ). Blood was also used for total leukocyte and differential leukocyte counts.

**Culture Stimulation.** LPS of *Salmonella minnesota* R595 (List Biological Laboratories, Campbell, CA) was diluted in PBS, pH 7.4, and used at 1  $\mu$ g/ml; Bacillus Calmette-Guerin (BCG) was obtained from Ataufo de Paiva Foundation (Rio de Janeiro, Brazil), heat killed, and used at 10  $\mu$ g/ml. Armadillo-derived *M. leprae* (ML) antigen was provided by Dr. R. J. W. Rees (IMMLEP Bank, The National Institute of Medical Research, Mill Hill, England). Cell wall protein of ML (CWP-ML), lipoarabinomannan of ML (LAM-ML), and deacylated phenolic glycolipid I of ML (dPGL-I) (produced through the National Institute of Allergy and Infectious

Disease) were provided by Dr. Patrick Brennan (Department of Microbiology, Colorado State University, Fort Collins, CO). Unless otherwise stated, all products were used at 10  $\mu$ g/ml. Purified protein derivative of tuberculin (PPD; Statens Serum Institut, Copenhagen, Denmark) was used at 10  $\mu$ g/ml. Muramyl dipeptide (MDP; Sigma Chemical Co., St. Louis, MO) was used at 5  $\mu$ g/ml. The concentration of contaminating endotoxin in stock solutions and antigens was estimated by the *Limulus* amoebocyte lysate assay (LAL; Whittaker M. A. Bioproducts, Walkersville, MD). All final cultures contained  $<3$  pg/ml LPS, suggesting that the TNF- $\alpha$  levels were induced by the ML products themselves and not by contaminating LPS (9).

**Thalidomide Preparation.** Thalidomide (the racemic mixture: D [+] and L [-] forms; lot no. JB-I-114) was donated by Andrus Research Corporation (Beltsville, MD) and by Grunenthal GMBH (Stolberg, Germany) (lot no. 1055/8). The compound was dissolved in DMSO (Sigma Chemical Co.) at 10 mg/ml, and further dilutions were made in sterile RPMI, pH 4.0. The compound from both companies gave similar results. The effect of thalidomide on in vitro TNF- $\alpha$  release is expressed as the mean percent of inhibition  $\pm$  SEM (10).

**TNF- $\alpha$  ELISA.** TNF- $\alpha$  concentration in serum samples (collected before and at 4 and 24 h after rIFN- $\gamma$  injections) and in cell culture supernatants was determined using a TNF- $\alpha$ -specific ELISA (Endogen Inc., Boston, MA) as recommended by the manufacturer (10). TNF- $\alpha$  levels are expressed as nanograms per milliliter of protein.

**Lymphocyte Transformation Test (LTT).** PBMC ( $2 \times 10^5$  cells) were cultured in 96-well plates in 200  $\mu$ l of complete medium. Mycobacterial antigens (ML, 25  $\mu$ g/ml; BCG, 20  $\mu$ g/ml, or PPD, 10  $\mu$ g/ml) or the mitogen PHA (1%; Gibco Laboratories) were added to the cultures, and the assay was performed as described (11). Results (cpm) are expressed as  $\Delta$ cpm (cpm obtained in stimulated cultures minus cpm obtained in control cultures).

**Chemiluminescence Assay.** PBMC ( $10^6$  cells) were cultured on glass coverslips in 24-well plates for 2–20 h and washed three times with warm RPMI. Coverslips with the adherent cells were then transferred to scintillation vials containing 1 ml RPMI with 5% FCS. Luminol ( $5 \times 10^{-5}$  M; Sigma Chemical Co.) and PMA (0.5  $\mu$ g/ml) (Sigma Chemical Co.) were added to the vials in the dark, and respiratory burst activity of monocytes was measured on a  $\beta$  scintillation counter. Control cells received only luminol. Results are expressed as  $\Delta$ cpm (cpm obtained from cells stimulated with PMA minus cpm obtained in control cells).

**HLA Class II Expression.** Cytospins were prepared by centrifugation of  $10^5$  PBMC onto glass slides for 3 min at 800 rpm. After air drying, cells were fixed in 3% paraformaldehyde, 75 mM lysine, and 10 mM sodium-m-periodate in PBS (PLP) for 10 min and rinsed in PBS. The slides were stored desiccated at  $-20^\circ\text{C}$  until use. Immunocytochemical staining was performed as described (2) using a mouse mAb, 9.3F10 (anti-MHC class II antigen), produced in this laboratory (12).

**B.I. Estimation.** Slit smears taken from six sites, including a lesion in each patient, were obtained for whole-body B.I. evaluation at the time of diagnosis. Patients were given MDT for 3–8 mo (mean, 3.9 mo) before entering into the study, at which time a new B.I. evaluation was carried out. In addition to the six sites, a sample was taken from the back of the patients before rIFN- $\gamma$  injection (seventh site). Patients were retested at the same sites at intervals thereafter. In addition, 89 LL/BL patients were evaluated for their rate of reduction in B.I. during 18 mo of MDT alone.

**Histological Analysis.** Biopsies (6-mm punch) of matched, distal lepromatous lesions not injected with rIFN- $\gamma$  and not in areas within

the lymphatic drainage of injected sites were taken before, and during, the rIFN- $\gamma$  injections. In addition, biopsies were taken of ENL lesions that developed in response to rIFN- $\gamma$  treatment. A part of each biopsy was fixed in 10% neutral buffered formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological diagnosis.

**Immunohistology.** A part of each biopsy specimen was fixed in PLP for 3 h, as described (2). This fixative preserves structural details without inhibiting the binding of mAbs to their antigen. Biopsies were washed in PBS containing sucrose (10%) and digitonin ( $5 \times 10^{-5}$  M), and then serially suspended in graded solutions of sucrose (15–25%). The tissue was stored at 4°C in PBS with sucrose/glycerol (25% and 5%, respectively), embedded in OCT compound, and frozen at -20°C. Biopsies were sectioned and stained as described (13).

**mAbs.** Mouse mAbs were used for the identification of specific cell types in frozen tissue sections and on cytopins. Leu-1, Leu-2a, and Leu-3a (CD5, CD4, and CD8 anti-T cells) and Leu-M5 (CD11c anti-monocyte/macrophage) (13) were obtained from Becton Dickinson & Co. (Mountain View, CA). Antibody 9.3F10 was produced in this laboratory (12).

## Results

### Clinical Responses to rIFN- $\gamma$

The dose, schedule of administration, and clinical status of the patients receiving rIFN- $\gamma$  are shown in Table 1. All patients received 30  $\mu$ g i.d. daily for three doses, and a respite period of 3 d was followed by another three injections for a total of 180  $\mu$ g rIFN- $\gamma$ . Thereafter, six patients (nos. 1, 2, 6, 7, 8, and 9) received 100  $\mu$ g i.d. every 1 mo for 10 mo, and four patients (nos. 3, 4, 5, and 10) 100  $\mu$ g i.d. every 2 wk for 5 mo.

The local response to intradermal rIFN- $\gamma$  of erythema and

induration varied widely from patient to patient as noted previously (1–3) (Table 1). Reinjections into the same site resulted in smaller reactions. Almost all patients exhibited mild systemic symptoms. These included malaise and fatigue (10/10), low grade fever enhancement (1°C) (5/10), headache and myalgia (3/10), nausea, and weight loss (1/10).

Biopsies of distal skin sites taken 1 mo after initiation of rIFN- $\gamma$  injections revealed greater numbers of CD3<sup>+</sup> T cells in the dermis as compared with preinjection biopsies (not shown). However of the 10 patients biopsied, only 4 had extensive CD3<sup>+</sup> cell counts. In addition, keratinocytes did not express HLA class II antigens on their surface in 8 of 10 biopsies of the patients studied.

### The Induction of ENL and Its Therapy

6 of the 10 patients developed ENL during the course of their rIFN- $\gamma$  treatment, as outlined in Table 2. In the early phase of low dose intradermal injections, these were characterized by single, painful, subcutaneous nodules that were transitory in nature and subsided within 8 d. All of these patients developed disseminated, multiple, painful subcutaneous nodules during the high-dose, bolus injections. There was a correlation between areas of induration ( $\geq 15$  mm) (Table 1) occurring with intradermal injections and the subsequent development of ENL (5/6 patients). The typical microscopic appearance of biopsied nodules is illustrated in Fig. 1. These were characterized by dense cellular infiltrates extending from the lower dermis into the subcutaneous fat, comprised predominantly of mononuclear cells and granulocytes. The mononuclear cells (evaluated by immunohistological staining) were largely CD3<sup>+</sup> T cells (many CD4<sup>+</sup>) and monocytes, whereas the granulocytes (evaluated by H&E

**Table 1.** Clinical Status and Response of LL/BL Patients before and after Intradermal rIFN- $\gamma$  Injection

Patient	Diagnosis	MDT*	B.I. (Pre)	Duration of treatment	Local induration		B.I. (first month)	B.I. (sixth month)
					Day 3	Day 6		
		<i>mo</i>		<i>mo</i>	<i>mm</i>			
1	BL	4	4.0	12 <sup>†</sup>	16	7	1.6	1.0
2	LL	4	3.3	12	15	19	3.0	2.1
3	LL	3	2.5	6 <sup>§</sup>	35	27	2.1	1.3
4	BL	3	2.3	6	4	8	0.5	1.3
5	LL	3	2.6	6	32	17	1.6	2.6
6	BL	3	4.0	12	0	0	2.8	0.3
7	LL	3	2.9	12	35	25	3.3	2.6
8	LLs	8	0.5	12	5	0	2.1	2.0
9	LLs	5	1.5	12	6	7	2.1	1.1
10	LL	3	1.3	6	18	14	2.0	ND

\* See Materials and Methods.

† Six injections of 30  $\mu$ g and one monthly injection of 100  $\mu$ g each for a total of 10 doses.

§ Six injections of 30  $\mu$ g and one injection every 2 wk of 100  $\mu$ g each for a total of 10 doses.

**Table 2.** Induction of ENL in Patients Receiving rIFN- $\gamma$ 

Patient	rIFN- $\gamma$ dose schedule			
	30 $\mu$ g (six doses)		100 $\mu$ g (10 doses)	
	Time of appearance	Clinical status	Time of appearance	Clinical status
2	None	None	14 d after sixth dose	Multiple painful nodules
3	1 d after fifth dose	One painful nodule*	3 d after second dose	Systemic ENL
5	13 d after sixth dose	One painful nodule	14 d after second dose	Multiple painful nodules
7	1 d after second dose	Two painful nodules	6 d after first dose	Systemic ENL
8	None	None	1 d after fourth dose	Multiple painful nodules
10	1 d after second dose	Three painful nodules	14 d after third dose	Multiple painful nodules

Course of intradermal rIFN- $\gamma$  injections included three 30- $\mu$ g doses daily, a 3-d respite, and another three 30- $\mu$ g injections daily. This was followed in 30 d by a 100- $\mu$ g dose every 2 wk for 5 mo, or every 1 mo for 10 mo.

\* Painful nodules in subcutaneous tissue.

staining of histological sections) included mostly neutrophils and a few eosinophils. Therefore, these nodules resembled the ENL lesions that occur spontaneously in patients receiving MDT (14).

Two of the patients developed severe systemic symptoms including multiple subcutaneous nodules, fever, malaise, polyneuritis, arthritis, and weight loss, and required therapy with thalidomide. This symptom complex was also similar to the reactional states occurring during chemotherapy. These two patients responded promptly to an initial dose of 300 mg/d of thalidomide, which was then reduced weekly by 100 mg for a total course of 21 d. Within 48 h, fever, malaise, and the arthritic and neuritic pain were absent. The tenderness of subcutaneous nodules responded just as promptly, and by 10–15 d the nodules themselves were gone. One of these patients (Table 2, no. 3) was treated for 1 wk with thalidomide together with all subsequent rIFN- $\gamma$  injections and did not develop further episodes of ENL. The other patient continued to express symptoms of systemic ENL after the next three rIFN- $\gamma$  injections. Injections of rIFN- $\gamma$  were discontinued after 4 mo (Table 2, no. 7).

#### *The Influence of rIFN- $\gamma$ Injections on the B.I.*

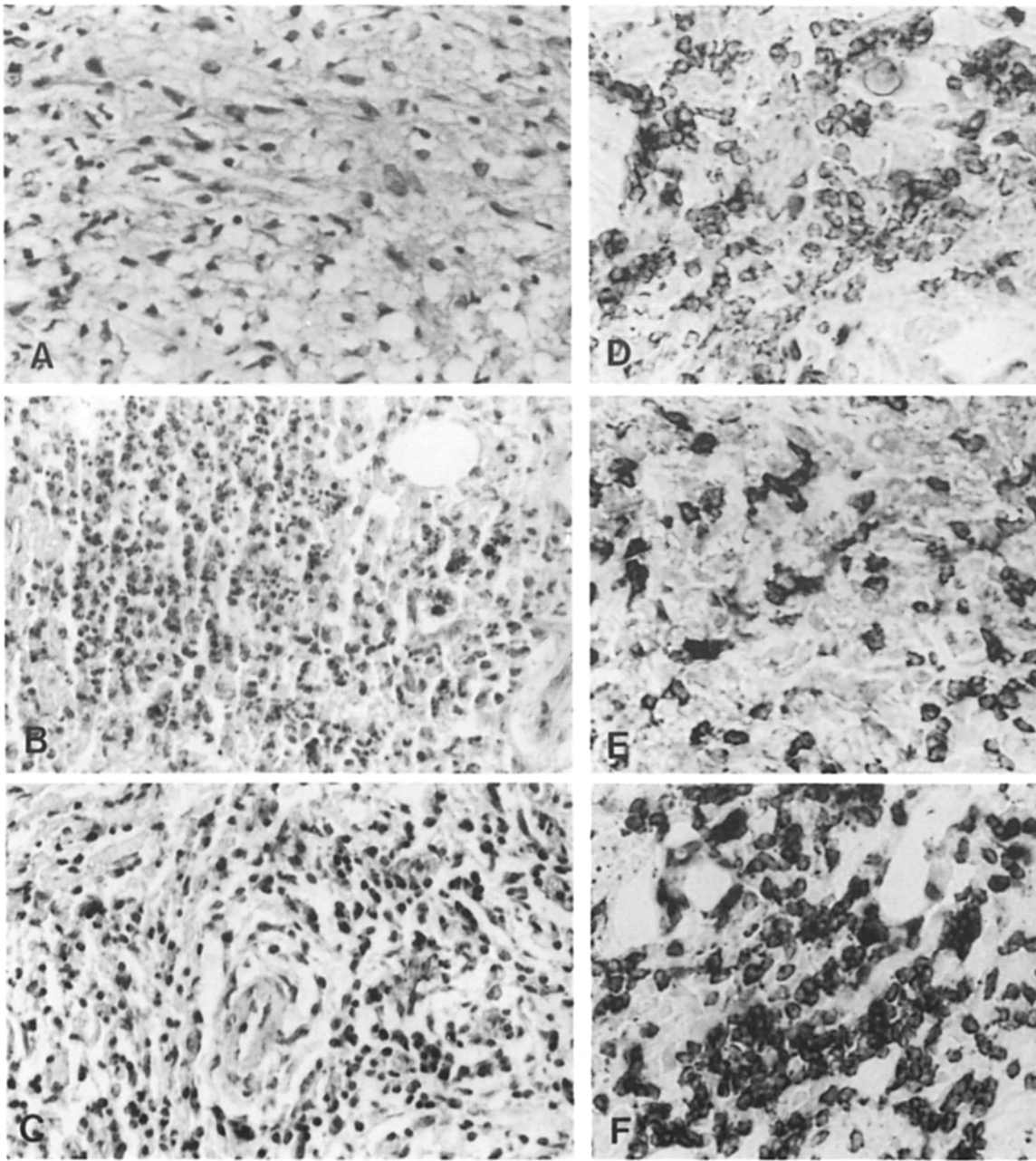
Patients receiving MDT demonstrated a gradual reduction in the number of acid-fast bacilli in their tissues. The effect of chemotherapy alone on the total-body B.I. of 89 LL/BL patients was evaluated over a 12-mo period of therapy. A mean  $\pm$  SEM of  $0.6 \pm 0.12$  log reduction in B.I. was observed using slit smear specimens obtained from six sites. The B.I. of all 10 patients receiving rIFN- $\gamma$  is shown in Table 1. A mean  $\pm$  SEM log B.I. reduction during 1 and 6 mo of IFN- $\gamma$  injections was  $0.4 \pm 0.2$  and  $0.9 \pm 0.3$ , respectively. The values indicated a possible accelerated reduction in systemic

B.I. in 6 of 10 patients during the first month of therapy, which continued at a slower rate for up to 6 mo.

#### *The Influence of rIFN- $\gamma$ on the Function of Circulating Cells of Injected LL and BL Patients*

**Leukocyte Numbers and MHC Class II Antigen Expression.** Total leukocyte counts performed on patient bloods collected before and 1 h after the sixth 30- $\mu$ g IFN- $\gamma$  as well as 1 h after the first 100- $\mu$ g injection, showed no significant changes (mean  $\pm$  SEM of white blood cells per mm<sup>3</sup> of  $7,575 \pm 890$  for pre-IFN- $\gamma$  bloods and  $8,785 \pm 1,560$  for post-IFN- $\gamma$  bloods). Differential counts for the percent of T cells and monocytes also showed no significant changes (mean percent of PBMC for monocytes: pre-IFN- $\gamma$ , 4.8%; and post-IFN- $\gamma$ , 5.8%). However, some (25%) patients in ENL had enhanced total leukocyte counts (mean,  $10,450 \pm 3,088$ ), which were accounted for by enhanced numbers of granulocytes, mostly PMN leukocytes (ENL,  $6,685 \pm 2,847$  vs. LL/BL,  $3,949 \pm 627$  per mm<sup>3</sup> blood). Immunostaining of PBMC showed that the percent of PBMC-expressing MHC class II antigens was not significantly affected after lymphokine injections (mean  $\pm$  SEM:  $8.7 \pm 1.3\%$  positive cells before vs.  $12.5 \pm 2.0$  after six 30- $\mu$ g doses). PBMC isolated from patient bloods contained a maximum of 5–8% PMN leukocytes.

**Monocyte Chemiluminescence.** The luminol assay was used as an index of the respiratory burst of monocytes obtained from patients before and 4 h after an injection of 30  $\mu$ g of rIFN- $\gamma$  (Table 3). Both the spontaneous and PMA-stimulated activity were increased after rIFN- $\gamma$  administration, as reported previously (1). In addition, monocytes obtained from patients who had developed ENL exhibited higher activity in the luminol assay (threefold higher than LL/BL patients). Thalido-



**Figure 1.** Light micrographs demonstrating the cellular infiltration in lepromatous and in rIFN- $\gamma$ -induced ENL lesions. H&E stained section of LL pre-IFN- $\gamma$  lesion (A) with mostly foam macrophages, and post-IFN- $\gamma$  ENL lesion (B and C) with an increase in cellularity mostly PMN leukocytes in B and lymphoid cells in C. Immunohistologic staining for CD3<sup>+</sup> T cells (D) and CD4<sup>+</sup> T cells (E) of serial sections of ENL lesion (same biopsy as B, patient no. 3) and CD3<sup>+</sup> T cells (F) of another ENL lesion (same as C, patient no. 7).  $\times 300$ .

mide (10–20  $\mu\text{g/ml}$ ) added to the cultures during the assay did not affect the activity measured.

**Mitogenic Response of PBMC.** The thymidine incorporation of PBMC obtained before and after the sixth intradermal dose of rIFN- $\gamma$  was examined. No difference was noted in the proliferative response to either mitogens (mean cpm  $\pm$  SEM: 72,713  $\pm$  9,006 vs. 80,917  $\pm$  14,456) or the ML antigen (mean cpm  $\pm$  SEM: 927  $\pm$  408 vs. 1,682  $\pm$  666). Therefore, there was no evidence that rIFN- $\gamma$  had appreciably

modified the T cell anergy of lepromatous leprosy patients to the antigens of ML.

**Synthesis of TNF- $\alpha$  by Peripheral Blood Monocytes.** The relative efficacy of mycobacterial antigens for the induction of TNF- $\alpha$  secretion in the absence of IFN- $\gamma$  was of interest. ML and CWP-ML antigens from ML as well as MDP, BCG, and PPD gave similar elevations that were  $\sim 100$ -fold above unstimulated cultures (Table 4). In contrast, dPGL-I ML failed to enhance secretion. It appears that there may be common

**Table 3.** Effect of rIFN- $\gamma$  Injections and ENL Development on the Respiratory Burst Activity of Monocytes from Lepromatous Leprosy Patients

Leprosy patients	Luminol assay* (no. of patients)
Pre-rIFN- $\gamma$ injections	1.4 $\pm$ 0.7 (5)
Post-rIFN- $\gamma$ injections <sup>†</sup>	3.4 $\pm$ 1.2 (5)
ENL	4.0 $\pm$ 1.3 (6)

\* Results are expressed as cpm  $\times 10^{-3} \pm$  SEM for each group of patients tested.

<sup>†</sup> Cells were obtained 4 h after one 30- $\mu$ g rIFN- $\gamma$  injection.

components of mycobacteria, e.g., MDP, but not the specific ML molecule PGL-1, which stimulates secretion of TNF- $\alpha$  from monocytes of lepromatous patients, even in the presence of selective ML T cell anergy.

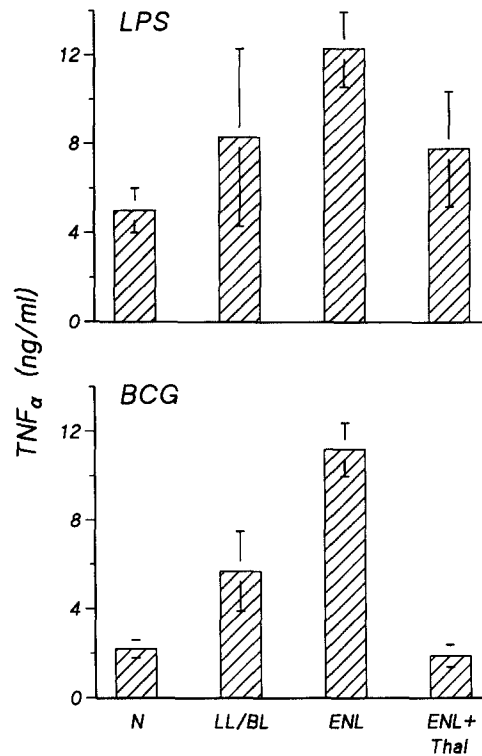
Monocyte-enriched cultures were examined in LL/BL patients for their capacity to produce TNF- $\alpha$  before and 4 h after the injection of a 30- $\mu$ g dose of rIFN- $\gamma$ . Monocyte-enriched cells explanted in vitro were exposed to a number of antigenic agonists. In the absence of exposure to agonists in vitro, and 4 h after the intradermal injection of rIFN- $\gamma$ , only low TNF- $\alpha$  concentrations were released (0.06  $\pm$  0.01 and 0.08  $\pm$  0.02 ng/ml of TNF- $\alpha$ , respectively). The addition of the agonists ML, BCG, and LPS to the monocytes in vitro induced levels of secretion of TNF- $\alpha$  similar to those observed in Table 4 and Fig. 2. Of interest, however, was the higher secretion obtained from monocytes after a single intradermal injection of 30  $\mu$ g rIFN- $\gamma$  (4 h post-IFN- $\gamma$ ). For the ML antigen, this was 2.5-fold higher than uninjected BL/LL patients, 4.0-fold higher for BCG, and 5.1-fold higher for LPS (mean results for three patients tested). This enhanced TNF- $\alpha$  release had disappeared by 24 h after rIFN- $\gamma$  administration. It appears that the lymphokine primes monocytes

**Table 4.** Induction of Monocyte TNF- $\alpha$  Production by ML Components

Agonist	TNF- $\alpha$ ng/ml
Control	0.04 $\pm$ 0.01*
ML	5.5 $\pm$ 0.7
CWP ML	4.5 $\pm$ 0.4
LAM ML	2.0 $\pm$ 0.4
dPGL-I ML	0.04 $\pm$ 0.01
MDP	4.7 $\pm$ 0.8

TNF- $\alpha$  release from lepromatous leprosy patient monocytes induced by optimal concentrations of ML and other mycobacterial products.

\* Values represent the mean  $\pm$  SEM of 12 different experiments.



**Figure 2.** Levels of TNF- $\alpha$  released in vitro from monocyte-enriched cultures from lepromatous patients. Cells were stimulated with LPS (1  $\mu$ g/ml) (A) or BCG (10  $\mu$ g/ml) (B) for 20 h. Cells from normal individuals (N, n = 8), lepromatous patients without ENL (LL/BL, n = 12), untreated ENL patients (ENL, n = 7), and thalidomide-treated (300  $\mu$ g 1 d for 1 wk) ENL patients (ENL + Thal, n = 6) were tested. Results represent means  $\pm$  SEM.

in vivo for transient enhanced antigen-induced TNF- $\alpha$  secretion. No TNF- $\alpha$  was observed in the serum of patients 4 h or 24 h after rIFN- $\gamma$  administration.

#### TNF- $\alpha$ Production during ENL and Its Suppression by Thalidomide Therapy

Patients developing generalized ENL were evaluated for the levels of TNF- $\alpha$  in the plasma, in PBMC and isolated monocytes. Of 14 patients studied, nine had appreciable levels of plasma TNF- $\alpha$  (mean of 2,201  $\pm$  1,178 pg/ml) and the other five had no detectable cytokine. 46 patients with LL/BL leprosy (not in ENL), 24% had TNF- $\alpha$  levels in their plasma (1,339  $\pm$  550 pg/ml). TNF- $\alpha$  was not detected in the plasma of normal controls. Monocyte-enriched cultures obtained from all patients in ENL exhibited elevated levels after exposure to a variety of agonists (Fig. 2). The release of TNF- $\alpha$  in response to LPS and BCG from monocyte-enriched cultures of patients in ENL was approximately twofold above that seen with uncomplicated LL/BL patients and three- to sixfold above normal controls. Similar results were obtained with other agonists (not shown).

Patients with ENL who received thalidomide therapy were also examined for the production of TNF- $\alpha$  by their monocytes. As seen in Fig. 2, oral doses of 300 mg/d thalidomide resulted in a 50–80% reduction in the ability of monocyte-

**Table 5.** Effect of *In Vitro* IFN- $\gamma$  on TNF- $\alpha$  Release by Patient Monocytes

IFN- $\gamma$	TNF- $\alpha$ release (percent of control)		
	ML (10 $\mu$ g/ml)	MDP (5 $\mu$ g/ml)	LPS (1 $\mu$ g/ml)
None	100*	100*	100†
1 U/ml	205 $\pm$ 32	349 $\pm$ 93	491 $\pm$ 188
10 U/ml	291 $\pm$ 86	549 $\pm$ 95	684 $\pm$ 225
100 U/ml	315 $\pm$ 23	628 $\pm$ 99	748 $\pm$ 236

Results are the mean  $\pm$  SEM of three different experiments.

\* For range of TNF- $\alpha$  release see Table 4.

† For range of TNF- $\alpha$  release see Fig. 2.

enriched cultures to secrete TNF- $\alpha$  after stimulation with LPS and BCG.

#### *The Influence of rIFN- $\gamma$ and Thalidomide When Added to Patient's Monocytes In Vitro*

The previous information suggested that the administration of rIFN- $\gamma$  to patients resulted in a high incidence of ENL and enhanced the production of TNF- $\alpha$  by circulating monocytes. Both the inflammatory components of ENL and the secretion of monocyte-derived TNF- $\alpha$  could be inhibited by the *in vivo* administration of thalidomide. In this secretion we evaluate the effects of IFN- $\gamma$  and thalidomide when added *in vitro*.

Monocytes from LL/BL patients were incubated in the presence or absence of rIFN- $\gamma$  for 2–24 h and then assayed with and without agonists. Table 5 shows the effects of three concentrations of rIFN- $\gamma$  on TNF- $\alpha$  secretion. Maximum release occurred with 100 U/ml of rIFN- $\gamma$  incubated with the cells and agonists for 20 h. Elevated secretion of TNF- $\alpha$  after rIFN- $\gamma$  addition failed to occur in the absence of an agonist (not shown). In addition, the exposure of monocytes to rIFN- $\gamma$  for as short a period as 2 h was sufficient to prime the cells for maximum agonist-induced secretion (not shown).

The *in vitro* addition of thalidomide (10  $\mu$ g/ml) to LL/BL patient's monocytes primed and not primed with rIFN- $\gamma$  inhibited agonist-induced TNF- $\alpha$  production to the same extent (not shown). The mean percent inhibition  $\pm$  SEM for three different mycobacterial agonists tested is as follows: ML, 66  $\pm$  4%; CWP-ML, 60  $\pm$  16%; MDP, 60  $\pm$  9%. It therefore appeared that the effects of rIFN- $\gamma$  and thalidomide on TNF- $\alpha$  production as exhibited in LL/BL patients *in vivo* could be mimicked by their addition to isolated monocytes *in vitro*.

#### **Discussion**

After multiple low-dose intradermal rIFN- $\gamma$  injections administered over a 6- or 12-mo period, ENL was induced in a much higher number of patients (60% in 6–7 mo) than would have been expected during regular MDT in this pa-

tient population (15% in 12 mo). The severity of the ENL symptoms was dependent on the dose of rIFN- $\gamma$  administered. After the 30- $\mu$ g-dose injections, only single subcutaneous painful nodules were induced, while 100- $\mu$ g doses induced multiple nodules as well as other systemic effects. In most patients ENL was easily controlled. The short half-life and prompt clearance of the cytokine from the circulation probably accounted for the fact that the nodules cleared spontaneously without thalidomide therapy.

In addition to the IFN- $\gamma$ -induced ENL, the whole-body B.I. of all 10 patients treated with IFN- $\gamma$  was reduced by a mean log of 0.4  $\pm$  0.2 and 0.9  $\pm$  0.3 during the first month and 6 mo of treatment, respectively. In comparison, MDT alone gave a 0.6  $\pm$  0.12 log reduction in whole body B.I. load during 12 mo. The reduction in the systemic ML load occurred in the absence of enhanced T cell responsiveness to mycobacterial antigens, and thus patient energy to the bacilli remained unaffected. The mechanism underlying the B.I. reduction can be explained by our recent observations. We have previously shown that the local administration of rIFN- $\gamma$  into lepromatous lesions induces accumulation of T cells and monocytes, destruction of parasitized macrophages, and a local reduction of ML at the site of cytokine injection (1–3). Multiple intramuscular (6) as well as intradermal injections of the cytokine have given a generalized hypercellularity in the skin with a selective enrichment in T cells and monocytes in the lesions. We believe that the accumulation of lymphoid cells in the dermis led to selective killing of parasitized macrophages and release of the bacilli into the extracellular space, as observed by electron microscopy (2, 3). Destruction of the host cells could be mediated by a population of cytotoxic T cells and/or NK cells recruited to the site and activated locally. Rephagocytosis of the extracellular ML by oxidatively competent monocytes (15), activated by IFN- $\gamma$  locally, might lead to killing of the bacilli and their clearance (16).

These effects of rIFN- $\gamma$  administration were accompanied by monocyte activation for enhanced oxygen radical formation as well as an increased release of TNF- $\alpha$  relative to the precytokine levels. Thalidomide therapy, which rapidly reduced systemic toxicity and the painful subcutaneous nodules of ENL, also inhibited the high levels of TNF- $\alpha$  produced by monocytes during ENL. The enhanced secretion of TNF- $\alpha$  could be reproduced by *in vitro* treatment of monocytes with rIFN- $\gamma$  and was readily reversed *in vitro* with thalidomide.

A scenario that could explain these results involves rIFN- $\gamma$  priming of the patient's monocytes for enhanced TNF- $\alpha$  release (17–19). Primed monocytes were then triggered by mycobacterial agonists found in high concentrations in the circulation as a result of enhanced bacterial killing. Thus, abnormally high levels of TNF- $\alpha$ , and possibly other cytokines, would have been released into the circulation bringing about the clinical symptoms and toxicities of ENL. Thalidomide would be so effective at controlling ENL because it reduces TNF- $\alpha$  levels released by monocytes.

It is not clear whether a similar scenario is involved in the generation of ENL in patients treated with MDT alone. The initiation of therapy would give rise to bacterial killing, releasing into the circulation ML components capable of trig-

gering TNF- $\alpha$  secretion from monocytes and macrophages (20–22). We have demonstrated that ENL patient's monocytes release higher levels of TNF- $\alpha$  than do cells from normal controls or BL/LL patients not in reaction. The ENL patients also have high TNF- $\alpha$  plasma levels in the circulation (23). Whether IFN- $\gamma$  is involved in this process is unknown. IFN- $\gamma$ -producing cells were shown to be increased in the lesions of lepromatous patients during ENL (24). The emergence of T cells reactive to ML during ENL has also been suggested (25, 26), but this response appears transient.

The efficacy of thalidomide in ENL and its inability to modify the course of reversal reaction suggest differences in the underlying pathophysiology of these two reactional states (27–29). Enhanced T cell responsiveness is a prominent fea-

ture of the reversal reaction, and lesions of skin and nerve are characterized by mononuclear infiltration. In contrast, ENL has little or no enhancement of T cell reactivity and exhibits mixed cellular infiltrates containing both granulocytes and mononuclear cells. ENL exhibits extensive vasculitis probably as a result of the high TNF- $\alpha$  levels and the activation of the complement cascade. It is the systemic, toxic manifestations of this state and others (30, 31) that are so responsive to thalidomide. Two possibilities are suggested by these considerations. The first is that mycobacterial products serve as particulate agonists of monocyte cytokine synthesis, including TNF- $\alpha$ , and the second is that the large number of tissue granulocytes make appreciable amounts of TNF- $\alpha$  (32–34). These points are now being explored in our laboratory.

---

We thank Dr. Z. A. Cohn for helpful advice with the project and the manuscript; Drs. M. E. N. Gallo and J. A. Nery for their support throughout the study; T. S. Guedes for performing the B. I. analysis; and C. F. Miranda and P. Frindt for technical assistance. Thanks are due to A. R. de Moura for the graphic work and to M. Nulty and M. Garcia for efficient secretarial help.

This study was supported in part by U.S. Public Health Service grant AI-22616 and by Boehringer Ingelheim. E. P. Sampaio is a Villares Fellow.

Address correspondence to Gilla Kaplan, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

Received for publication 24 September 1991 and in revised form 3 March 1992.

## References

1. Nathan, C.F., G. Kaplan, W.R. Levis, A. Nusrat, M.D. Witterm, S.A. Sherwin, C.K. Job, C.R. Horowitz, R.M. Steinman, and Z.A. Cohn. 1986. Local and systemic effects of intradermal recombinant interferon- $\gamma$  in patients with lepromatous leprosy. *N. Engl. J. Med.* 315:6.
2. Kaplan, G., A. Nusrat, E.N. Sarno, C.K. Job, J. McElrath, J.A. Porto, C.F. Nathan, and Z.A. Cohn. 1987. Cellular responses to the intradermal injection of recombinant human  $\gamma$ -interferon in lepromatous leprosy patients. *Am. J. Pathol.* 128:345.
3. Kaplan, G., N.K. Mathur, C.K. Job, I. Nath, and Z.A. Cohn. 1989. Effect of multiple interferon  $\gamma$  injections on the disposal of *Mycobacterium leprae*. *Proc. Natl. Acad. Sci. USA.* 86:8073.
4. Nathan, C.F., and R. Yoshida. 1988. Interferon- $\gamma$ . In *Inflammation: Basic Principles and Clinical Correlates*. J. Gallin, I. Goldstein, and R. Snyderman, editors. Raven Press, Ltd., New York. 229–251.
5. Kaplan, G., and Z.A. Cohn. 1986. The immunobiology of leprosy. *Int. Rev. Exp. Pathol.* 28:45.
6. Nathan, C., K. Squires, W. Griffo, W. Levis, M. Varghese, C.K. Job, A.R. Nusrat, S. Sherwin, S. Rappoport, E. Sanchez, R.A. Burkhardt, and G. Kaplan. 1990. Widespread intradermal accumulation of mononuclear leukocytes in lepromatous leprosy patients treated systemically with recombinant interferon  $\gamma$ . *J. Exp. Med.* 172:1509.
7. Ridley, D.S., and W.H. Jopling. 1966. Classification of leprosy according to immunity. A five-group system. *Int. J. Lepr.* 34:255.
8. Young, J.W., and R.M. Steinman. 1988. Accessory cell requirements for the mixed-leukocyte reaction and polyclonal mitogens, as studied with a new technique for enriching blood dendritic cells. *Cell. Immunol.* 111:167.
9. Molloy, A., G. Gaudernack, W.R. Levis, Z.A. Cohn, and G. Kaplan. 1990. Suppression of T-cell proliferation by *Mycobacterium leprae* and its products: the role of lipopolysaccharide. *Proc. Natl. Acad. Sci. USA.* 87:973.
10. Sampaio, E.P., E.N. Sarno, R. Galilly, Z.A. Cohn, and G. Kaplan. 1991. Thalidomide selectively inhibits tumor necrosis factor  $\alpha$  production by stimulated human monocytes. *J. Exp. Med.* 173:699.
11. Kaplan, G., D.E. Weinstein, R.M. Steinman, W.R. Levis, V. Elvers, M.E. Patarroyo, and Z.A. Cohn. 1985. An analysis of *in vitro* T cell responsiveness in lepromatous leprosy. *J. Exp. Med.* 159:917.
12. Van Voorhis, W., R. Steinman, L. Hair, J. Luban, M. Witmer, and Z.A. Cohn. 1983. Specific mononuclear phagocyte monoclonal antibodies: Application to the purification of dendritic cells and the tissue localization of macrophages. *J. Exp. Med.* 158:126.
13. Kaplan, G., W.J. Britton, G.E. Hancock, W.J. Theuvenet, K.A. Smith, C.K. Job, P.W. Roche, A. Molloy, R. Burkhardt, J. Barker, H.M. Pradhan, and Z.A. Cohn. 1991. The systemic influence of recombinant interleukin 2 on the manifestation of lepromatous leprosy. *J. Exp. Med.* 173:993.
14. Modlin, R.L., J.F. Gebhard, C.R. Taylor, and T.H. Rea. 1983.



- In situ characterization of T lymphocyte subsets in the reactional states of leprosy. *Clin. Exp. Immunol.* 53:17.
15. Lepay, D.A., R.M. Steinman, C.F. Nathan, H.W. Murray, and Z.A. Cohn. 1985. Liver macrophages in murine listeriosis. Cell-mediated immunity is correlated with an influx of macrophages capable of generating reactive oxygen intermediates. *J. Exp. Med.* 161:1503.
  16. Klebanoff, S.J., and C.C. Shepard. 1984. Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on mycobacterium leprae. *Infect. Immun.* 44:534.
  17. Rook, G.A.W., J. Taverne, C. Leveton, and J. Steele. 1987. The role of gamma-interferon, vitamin D<sub>3</sub> metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology.* 62:229.
  18. Debets, J.M.H., C.J. Van Der Linden, I.E.M. Spronken, and W.A. Buurman. 1988. T cell-mediated production of tumour necrosis factor- $\alpha$  by monocytes. *Scand. J. Immunol.* 27:601.
  19. Hart, P.H., G.A. Whitty, D.S. Piccoli, and J.A. Hamilton. 1989. Control by IFN- $\gamma$  and PGE<sub>2</sub> of TNF $\alpha$  and IL-1 production by human monocytes. *Immunology.* 66:376.
  20. Valone, S.E., E.A. Rich, R.S. Wallis, and J.J. Ellner. 1988. Expression of tumor necrosis factor *in vitro* by human mononuclear phagocytes stimulated with whole *Mycobacterium bovis* BCG and mycobacterial antigens. *Infect. Immun.* 56:3313.
  21. Moreno, C., J. Taverne, A. Mehlert, C.A.W. Bate, R.J. Brealey, A. Meager, G.A.W. Rook and J.H.L. Playfair. 1989. Liparabinomannan from *Mycobacterium tuberculosis* induces the production of tumour necrosis factor from human and murine macrophages. *Clin. Exp. Immunol.* 76:240.
  22. 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- $\gamma$  in tuberculosis pleuritis. *J. Immunol.* 145:149.
  23. Sarno, E.N., G.E. Grau, L.M.M. Vieira, and J.A.C. Nery. 1991. Serum levels of tumour necrosis factor-alpha and interleukin-1 $\beta$  during leprosy reactional states. *Clin. Exp. Immunol.* 84:103.
  24. Cooper, C.L., C. Mueller, T.-A. Sinchaisri, C. Pirmez, J. Chan, G. Kaplan, S.M.M. Young, I.L. Weissman, B.R. Bloom, T.H. Rea, and R.L. Modlin. 1989. Analysis of naturally occurring delayed-type hypersensitivity reactions in leprosy by in situ hybridization. *J. Exp. Med.* 169:1565.
  25. Rao, R.D., and P.R. Rao. 1987. Enhanced cell-mediated immune response in erythema nodosum leprosum reactions of leprosy. *Int. J. Lepr.* 55:36.
  26. Laal, S., L.K. Bhutani, and I. Nath. 1985. Natural emergence of antigen-reactive T cells in lepromatous leprosy patients during erythema nodosum leprosum. *Infect. Immun.* 50:887.
  27. Sehgal, V.N. 1987. Reaction in leprosy. Clinical aspects. *Int. J. Dermatol.* 26:278.
  28. Ridley, D.S. 1969. Reactions in leprosy. *Int. J. Lepr.* 40:77.
  29. 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.
  30. 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.
  31. Gorin, I., B. Vilette, P. Gehanno, and J.P. Escande. 1990. Thalidomide in hyperalgalic pharyngeal ulceration of AIDS. *Lancet.* 335:1343.
  32. Djeu, J.Y., Serbousek, D., and Blanchard, D.K. 1990. Release of tumor necrosis factor by human polymorphonuclear leukocytes. *Blood.* 76:1405.
  33. Dubravec, D.B., Spriggs, D.R., Mannick, J.A., and Rodrick, M.L. 1990. Circulating human peripheral blood granulocyte synthesize and secrete tumor necrosis factor  $\alpha$ . *Proc. Soc. Natl. Sci. Acad. USA.* 87:6758.
  34. Bazzoni, F., M.A. Cassatella, C. Laudanna, and F. Rossi. 1991. Phagocytosis of opsonized yeast induces tumor necrosis factor- $\alpha$  mRNA accumulation and protein release by human polymorphonuclear leukocytes. *J. Leukocyte Biol.* 50:223.