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A study of the anti-inflammatory, anti-microbial and immunomodulatory properties of thalidomide in leprosy

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A STUDY OF THE ANTI-INFLAMMATORY, ANTI-MICROBIAL AND IMMUNOMODULATORY PROPERTIES OF THALIDOMIDE IN LEPROSY

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ABSTRACT

During the course of their disease, leprosy patients may experience two types of inflammatory reactions- erythema nodosum leprosum (ENL) or reversal reaction (RR). Thalidomide is effective treatment for ENL, but not for RR. Using concentrations of thalidomide similar to that achieved in the treatment of ENL, we investigated thalidomide's effect on reactions, viability of *M. leprae*, and integrity of plasma membranes.

Cells from patients with and without RR were stimulated with *M. leprae* (AFB), a cytosol fraction of *M. leprae* (MLSA) or DHAR (DHAR) antigen, and the effect of thalidomide on lymphocyte proliferation, expression of TNF-α mRNA and synthesis of TNF-α was investigated. Thalidomide enhanced MLSA and DHAR induced proliferation of cells from patients with RR. The expression of TNF-α mRNA was variable, but thalidomide generally suppressed the synthesis of TNF-α. In a sub-set of RR patients, thalidomide enhanced AFB-induced cell proliferation, and the expression of TNF-α mRNA and TNF-α.

ENL has been described as a consequence of *M. leprae* antigens released from macrophages binding antibody and inducing inflammation. Thalidomide did not affect the viability of *M. leprae* residing in IFN-γ/LPS activated mouse macrophages, nor did it suppress TNF-α or nitrite.

Drugs may be anti-inflammatory by stabilizing cell membranes. Thalidomide failed to protect the plasma membrane of neutrophils and THP-1 cells from osmotic lysis. Thalidomide stabilized the membrane of erythrocytes from plasma free blood, but not from whole blood. *In*

vivo, the stability of erythrocytes membranes from subjects after ingestion of thalidomide was not affected.

In conclusion, thalidomide did not alter the viability of *M. leprae*, nor the integrity of the plasma membrane of inflammatory cells. It could enhance or suppress *M. leprae* antigen-induced synthesis of TNF-α. Interestingly, in 15 of 75 RR patients cells stimulated with AFB, thalidomide acted as a co-stimulant enhancing cell proliferation, synthesis of mRNA for TNF-α and TNF-α. Thalidomide's enhancing effect on TNF-α in RR appears to be dependent on the stimulant and IL-2 signaling. As the inflammation in RR is associated with the emergence of antigen-reactive T-cells and TNF-α, we speculate that the use of thalidomide in the treatment of RR may exacerbate the reaction.

CHAPTER 1 INTRODUCTION

1.1 History of Thalidomide

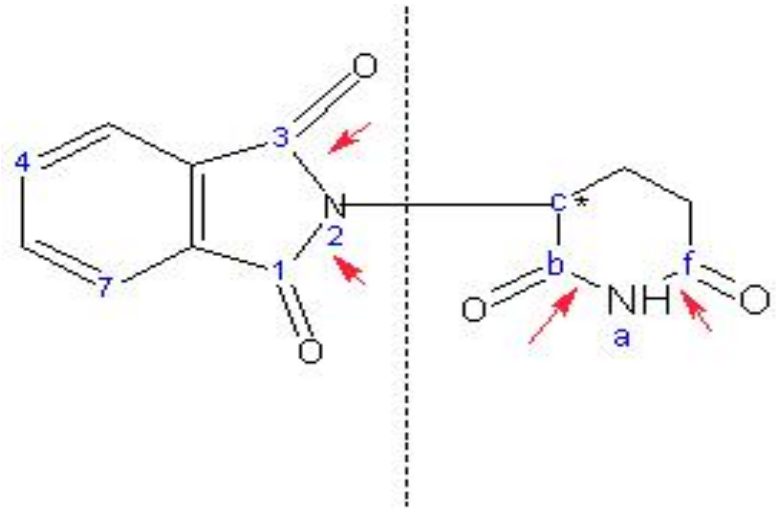
Chemie Grünenthal, a German pharmaceutical company, synthesized thalidomide in 1954. It was initially produced as a potential anti-histaminic drug but later discovered to possess marked sedative properties. Thalidomide's lack of toxicity in rodents at doses as high as 10g/kg facilitated its availability as an over-the-counter drug and by 1960, thalidomide was widely marketed in more than 20 countries. It quickly became a drug for the treatment of nausea associated with morning sickness during pregnancy. In 1961, suspicion regarding thalidomide's link with birth defects characterized by severe malformations started to appear. After more than 10,000 documented devastating cases of birth defects, thalidomide was withdrawn from the market in 1961 (Zwingerberger, K. and Wnendt, S., 1996).

In 1965, thalidomide made a major comeback after the accidental discovery of its dramatic effect in the treatment of an immunological reaction in leprosy known as erythema nodosum leprosum (ENL) (reviewed in Sheskin, J., 1980). Until 1998, thalidomide in the United States (US) was available only as an investigative new drug from the National Hansen Disease Program in Carville, Louisiana. In July 1998, the Food and Drug Administration (FDA) approved thalidomide for the treatment of ENL (FDA, 1998). Thalidomide is currently marketed in the US under the name Thalomid® (Thalidomide) by Celgene Company (Warren, New Jersey). In March 1999, the FDA in collaboration with Celgene implemented a distribution and training program known as System for Thalidomide Education and Prescribing Safety (S.T.E.P.S). In

order to guard against fetal exposure to thalidomide, this program requires physicians and pharmacists to register with the FDA before they can dispense thalidomide and all patients to complete an informed consent process and must agree to participate in a mandatory confidential surveillance registry (Zeldis, J.B. *et al*, 1999). In November 2001, Celgene expanded the availability of thalidomide worldwide by signing an agreement with Pharmion (Boulder, Colorado) a pharmaceutical company with European and Asian branches. In 2003, Thalomid® was granted approval by the Australian and New Zealand governments for the treatment of ENL as well as for the treatment of relapsed and refractory multiple myeloma (Celgene, website).

1.2 Chemical Properties

Thalidomide, α-phthalimidoglutarimide or 1,3-dioxo-2-(2',6'–dioxopiperidin-3'-yl) isoindoline is a white crystalline powder and has the following structural formula.



It has two ring systems, the phthalimide moiety on the left, and the glutaramide moiety on the right. Due to the asymmetry of the carbon atom (c*), on the glutaramide ring, thalidomide exists in optically active L and R isomers. *In vivo*, thalidomide inter-converts between isomeric forms to give an optically inactive racemic mixture. This inversion is very fast at 37 ^{0}C and is catalyzed by human serum albumin (Eriksson, T. *et al*, 1998). Thalidomide is insoluble in ether and benzene, sparingly soluble in water and ethanol and readily soluble in dimethyl sulfoxide (DMSO) and chloroform (The Merck Index, 1968).

At a pH of 6.0 and above, the 4-amide bonds of thalidomide (indicated by arrows) become susceptible to OH^- ions and undergo hydrolytic cleavage, yielding 4 major compounds, which undergo further breakdown to yield 8 compounds (Czejka, M.J. *et al*, 1987).

1.3 Pharmacokinetics

Thalidomide is absorbed slowly from the gastro-intestinal tract and is distributed throughout body fluids and tissues (Perri III, A.J. and Hsu, S., 2003). The pharmacokinetics of thalidomide has been studied in healthy as well as diseased individuals. With the exception of patients with Acquired Immuno-Deficiency Syndrome (AIDS), who may have gastro-intestinal absorption problems, thalidomide at a dose of 200 - 400 mg per os, achieves a peak plasma concentration of 1.15 – 3.44 mg/ml in 3 – 7 hours (hrs). The absorption half-life ($T^{1/2}$) is 1.5 – 1.7 hrs; whereas, the elimination $T^{1/2}$ is between 6.5 and 8.7 hrs (Eriksson, T. *et al*, 2001). Thalidomide is mainly degraded by spontaneous hydrolysis in the blood and tissues. A minute quantity of the drug is also metabolized by the hepatic cytochrome P450 system (Mujagic, H. *et*

al, 2002). Thalidomide is not excreted renally as indicated by the presence of less than 0.7% of intact drug in urine (Eriksson, T. *et al,* 1998).

1.4 Therapeutic Uses

Due to its anti-inflammatory, immuno-modulatory and anti-angiogenic properties, thalidomide is an effective treatment for a variety of clinical conditions. Currently, the only FDA approved indication for thalidomide is in the treatment of ENL (FDA, 1998). However, thalidomide has given promising therapeutic effects in various dermatological disorders like Behcet's disease (Hamuryudan, V. *et al*, 1998), chronic lupus erythematosus (Stevens, R.J. *et al*, 1997) actinic prurigo and prurigo nodularis (Perri III, A.J. and Hsu, S., 2003). It is also shown to be effective in Crohn's disease (Odeka, E.B. and Miller, V., 1997). Thalidomide has also been used for the treatment of graft-versus-host-disease (GVHD) both in a murine model and subsequently in a phase II trial (Voglesgang, G.B. *et al*, 1989; Voglesgang, G.B. *et al*, 1992; Cole, C.H. *et al*, 1994). But attempts to use thalidomide as a prophylactic agent in the prevention of GVHD resulted in a paradoxical outcome with thalidomide treated patients having a higher incidence in GVHD and a lower overall survival (Chao, N.J. *et al*, 1996). In complications related to HIV, thalidomide has been shown to treat wasting syndrome (Kaplan, G. *et al*, 2000), oropharyngeal, oesophageal and rectal apthous ulceration (Youle, M. *et al*, 1989; Jacobson, J. *et al* 1997), *Mycobacterium Avium* complex (MAC)–related infections (Bouza, E. *et al*, 1992) and microsporidial diarrhea (Sharpstone, D. *et al*, 1995).

1.5 Adverse Effects

The most severe side effect of thalidomide is teratogenicity. Thalidomide can easily cross the placenta and affect normal fetus formation. A single dose of 50 mg tablet in the first trimester of pregnancy has been reported to cause birth defects. The principal defect observed in a fetus is phocomelia where the hands and feet are attached to abbreviated arms and legs due to a poorly developed limb. In severe cases complete absence of limbs have been recorded. Additional abnormalities may include malformations of ears, bones, face, eyes, gastro-intestinal and genitourinary tracts. About 40% of exposed fetuses die at or shortly after birth (Perri III, A.J. and Hsu, S., 2003). The S.T.E.P.S. program for fetus protection ensures that thalidomide will not be given to pregnant women (Zeldis, J.B. *et al*, 1999).

Another potential adverse effect with prolonged use of thalidomide is peripheral neuropathy with risk of irreversible damage. This includes symmetrical, painful paresthesis of hand and feet often accompanied by numbness of lower limbs. Hypersensitivity reactions manifested by erythematous macular skin eruptions which if untreated could lead to fever, tachychardia and hypotension have also been reported. Somnolence and dizziness due to sedative properties of thalidomide have also been noted (Tseng, S. *et al*, 1996). Constipation can occur in some patients, which can lead to noncompliance if severe. Neutropenia is a rare side effect and is more common in HIV patients. In HIV-infected individuals, an increase in viral (median increase 0.42 $\log_{10}$ per milliliter) load has been reported after administration of thalidomide for the treatment of oral aphthous ulcers (Jacobson, J. *et al*, 1997).

1.6 Thalidomide and Erythema Nodosum Leprosum (ENL)

1.6.1 Historical Perspectives

The ability of thalidomide to effectively treat ENL was accidentally discovered in 1965, shortly after its ban from the world market. Professor Jacob Sheskin, an Israeli Dermatologist and Leprologist, was confronted with a patient suffering with ENL. In order to appease the patient's difficulty to sleep due to severe pain, Prof Sheskin gave him thalidomide as a sedative. Within 48 hours, he observed a significant improvement in the clinical manifestations of ENL. Prof. Sheskin reported his findings as an empirical study involving 6 ENL patients (Sheskin, J., 1980). Thereafter, various well-controlled clinical trials have shown the efficacy of thalidomide in the treatment of ENL. All of these studies, including a multi-centered worldwide survey organized by the World Health Organization (WHO) showed an efficacy of thalidomide in ENL as high as 90-99 % (Sheskin, J. 1980). The recommended dose of thalidomide in the treatment of ENL is initially 100 mg 3 to 4 times a day and tapered to 100 to 50 mg/day for maintenance dose (Hastings, R.C., 1985). Thalidomide alleviates symptoms of ENL 1-2 weeks after initiation of therapy. Systemic symptoms like fever, malaise, arthritic and neuritic pains dissipate within 24-48 hours after ingesting thalidomide. Disappearance of lesions takes relatively more time (Sampaio, E.P. *et al*, 1993)

1.6.2 ENL

ENL is an immunological reaction that occurs in 20 to 25 % of borderline leprosy and lepromatous leprosy patients. ENL occurs frequently after onset of treatment, but is also seen in untreated as well as treated patients (Petit, J.H.S. *et al*, 1967). ENL may be precipitated by

factors like pregnancy and parturition, protective immunization, stress, intercurrent infection and surgical operation (Jopling, W. H., 1988)

The major symptom of ENL is the appearance of crops of painful erythematous nodular lesions, which persist for days and then subside. These skin lesions, which are distinct from existing leprosy lesions, are round or oval and mainly appear on the face, trunk and extremities (Job, C.K. *et al*, 1964). Peripheral neuropathy especially polyneuritis with risks of disability, is another major complication of ENL. Patients also have severe symptoms of fever and general malaise. Other complications include arthralgia, polyarthritis, irritis and orchitis (Job, C.K. *et al*, 1964; Hastings, R.C., 1985).

1.6.3 Histopathology

ENL lesions are characterized with dense cellular infiltrates extending from lower dermis into sub-cutaneous fat (Job, C.K., *et al* 1964). At an early stage, lesions are characterized by a massive infiltration with polymorphonuclear neutrophils (PMN). As the inflammation subsides, the dominant cells in the lesion are lymphocytes and plasma cells. A slight increase in the number of histiocytes was also observed in chronic stages of ENL. The early stages of reaction also exhibit vasculitis due to swelling and edema of endothelium and to infiltration of blood vessel walls by PMN and eosinophils. The center of ENL lesions contain disintegrated histiocytes and fragmented and granulated acid-fast bacilli (Mabalay, M.C. *et al*, 1965; Ridley, M.J. and Ridley, D.S., 1983).

1.6.4 Immunology of Reactions in Leprosy

During the course of their disease, leprosy patients exhibit two types of immunological reactions. Dr W.H Jopling classified these reactions as Type I or reversal reaction (RR) and Type II reaction or ENL (Jopling, W.H., 1971). This classification is different from that of Gell and Coombs, who categorized reactions into four types. Type I hypersensitivity reactions are caused by IgE and allergens in complexes on mast cells. This results in mast cells degranulation and the release of inflammatory mediators like histamine. Type II hypersensitivity reactions are caused by covalent interactions of small molecules, like degradation products of penicillin, with cell surface components producing modified structures recognized as foreign by IgG antibodies. Type III hypersensitivity reactions are initiated by soluble immune complexes that get deposited on walls of blood vessels. These initiate complement fixation with marked neutrophilic infiltration resulting in tissue injury. Type IV hypersensitivity reactions are cell-mediated and occur due to an emergence of effector T-cells that react with antigens resulting in responses that may include macrophage activation, cytokine production and cytotoxicity (Williams, E.P., 2003).

In leprosy, reversal reaction (RR) mainly occurs in borderline leprosy patients and is caused by an increase in T-cell mediated immunity to *M. leprae* antigens expressed on dermal macrophages and Schwann cells. RR belongs to Type IV hypersensitivity group in the Gell and Coomb classification (Hastings, R.C., 1985). Clinical manifestations include a rapid change in the appearance of existing leprosy skin lesions, which become erythematous, more prominent, shiny and warm to touch. These lesions might sometimes break and ulcerate. A rapid swelling and inflammation of nerves with risks of motor disturbance is common. In RR, systemic

symptoms are very rare (Jopling, W.H., 1983). A transient influx of lymphocytes into lesions occurs at early stages. Edema around granulomatous lesions is very common. The cellular contents of granuloma changes progressively towards a more epitheloid form resulting in subsequent destruction of the bacilli within the granuloma (Ridley, D.S., 1969). RR is accompanied by an increase in the response and frequency of *M. leprae*-reactive T-cells in peripheral blood (Bjune, G. *et al*, 1976). Studies of skin lesions have demonstrated the infiltrating T-cells to be of $CD4^+$ phenotype. These T-cells show a ten-fold increase in IFN-γ production, a cytokine that causes activation of macrophages with subsequent elimination of bacilli and the induction of delayed-Type Hypersensitivity reaction (DTH) (Cooper, C.L. *et al*, 1989).

ENL is thought to be caused by antibody as well as cell-mediated immune processes. ENL was initially proposed to be a Type III hypersensitivity reaction due to the similarity of some of its symptoms like albuminuria and erythema to those encountered in serum sickness or in the experimental Arthus reactions (Ulrich, M. *et al*, 1971). The formation of immune complexes may be expected in lepromatous leprosy due to the concomitant presence of large amount of mycobacteria and their corresponding antibody (Shannon, E.J. *et al*, 1981). The presence of fixed complement and immunoglobulin deposits in ENL lesions has been demonstrated (Wemambu, S.N. *et al*, 1969). However demonstration of immune complexes and complement products in circulation has been an elusive task in ENL. Complement activation in ENL has also been shown by an increase in the complement split product C3d in serum of patients with active ENL. Determination of immune complexes in plasma of these same patients

by C1q-binding activity revealed a poor correlation between these complexes and the C3d level. This strongly indicates that immune complexes in ENL are extra-vascular (Bjorvatn, B. *et al*, 1976).

An additional and unique characteristic in the leprosy patients experiencing ENL is a transient improvement in T-cell functions indicating the involvement of a cell-mediated component as well. During the acute stage of ENL an emergence of reactive T-cells was demonstrated as shown by an enhancement in mitogen as well as *M. leprae*-antigen-induced lymphocyte proliferation. (Laal, S. *et al*, 1985). Immunohispathologic studies using *in situ* immunoperoxidase staining have demonstrated that ENL lesions have an increase in the percentage of cells with receptors for IL-2 and an increase in $CD4^+$ T-cells (Modlin, R.L. *et al*, 1986; Sampaio, E.P. *et al*, 1993). In ENL lesions enhanced expression of intercellular adhesion molecules (ICAM-1), leukocyte function antigen 1 (LFA-1) (Sullivan, L. *et al*, 1991) and expression of major histocompatibility complex class II (MHC II) molecules on immune cells and keratynocytes have also been described (Thangaraj, H. *et al*, 1988, Sampaio, E.P. *et al*, 1993).

1.6.5 Possible Mechanisms of Action of Thalidomide in the Treatment of ENL

Several studies have attempted to elucidate the mechanism of action of thalidomide in arresting ENL. Two main properties, anti-inflammatory and immunomodulatory represent the leading hypothesis regarding thalidomide's mechanisms.

1.6.5.1 Effect on Phagocytic cells

The influx of polymorphonuclear neutrophils (PMN) in early lesions and macrophages in ENL has prompted investigators to study the effect of thalidomide on chemotaxis, phagocytosis and effector mechanisms of phagocytic cells.

1.6.5.1.1 Expression of Cell Adhesion Molecules and Chemotaxis

In order to reach sites of inflammation, phagocytic cells in the blood must adhere and pass between the endothelial cells lining the walls of blood vessels. This migration process occurs in a step-wise fashion with the leukocyte rolling, tethering, adhering and then extravasation. These steps are mediated by interactions of cell surface molecules expressed on leukocytes and on vascular endothelial cells. Leukocyte rolling and tethering are mediated by selectins expressed on circulating leukocytes (L selectin) and on vascular endothelial cells (E and P selectins). Mucins, which are heavily glycocylated proteins, also interact with selectin in the initial phase of leukocyte adherence. A firm adhesion requires the interaction of integrins, such as LFA-1, Mac-1, and very late antigen-4 (VLA-4) expressed on leukocytes surfaces. Members of the immunoglobulin superfamily intercellular adhesion molecules (ICAMS) and vascular cell adhesion molecules (VCAM) pertain to distinct adhesion pathways. The ICAM-LFA-1 or Mac-1 pathway is mediated by constitutively expressed ligands whereas the VCAM-1/VLA-4 pathway bind to cytokine stimulated endothelial cells. (Williams, E.P., 2003).

Thalidomide's ability to modulate the adhesion cascade was investigated as a possible mechanism of action for this drug. Thalidomide is shown to enhance the shedding of L-selectin from PMN and to down regulate the expression of the inducible receptor of VCAM-1, which

might to some extent explain its anti-inflammatory mechanism. But contrary to expectations, thalidomide enhanced the TNF-α-mediated expression of ICAM-1 indicating a rather complex mechanism of interaction (Geitz, H. *et al*, 1996). Thalidomide is also shown to enhance the TNF-α-induced transmigration of PMN across a layer of human umbilical vein endothelial cells (HUVEC). It also synergized with TNF-α in the suppression of undirected as well as IL-8 and N-formyl-methionyl-leucyl-phenylalanine (fMLP)-mediated PMN chemotaxis (Dunzendorfer, S. *et al*, 1997).

1.6.5.1.2 Phagocytosis

The effect of thalidomide on phagocytosis is variable. In an experiment involving the phagocytosis of small latex beads thalidomide exhibited a bimodal activity with enhancement of the phagocytosis by monocytes at a 1.0 μg/ml and suppression at 10 μg/ml; whereas for PMN, it only suppressed the phagocytosis at 10 μg/ml and failed to affect it at the lower dose (Barnhill, R.L. *et al*, 1984). Thalidomide failed to affect the phagocytosis of larger zymosan particles by PMN (Hastings, R.C. *et al*, 1978).

1.6.5.1.3 Oxidative Effector Mechanism

Thalidomide failed to affect the respiratory burst of PMN as shown by chemiluminescence experiments (Dunzendorfer, S. *et al*, 1997) as well as Nitro Blue Tetrazolium reduction assay (NBT) (Golhman-Yahr, M. *et al*, 1978); whereas, chemiluminescence of monocytes was significantly depressed by thalidomide (Barnhill, R.L. *et al*, 1984).

1.6.5.1.4 Modulation of Cytokine Production by Thalidomide

- TNF-α

The presence of monocytes and lymphocytes in ENL lesions affords an opportunity for local production of inflammatory cytokines by these cells. TNF-α is one of the major cytokines produced by monocyte/macrophages. An enhanced level of TNF-α was detected in skin biopsies as well as in serum from ENL patients (Parida, S.K. *et al*, 1992; Moraes, M.O. *et al*, 1999). Thalidomide's mechanism in treating ENL is thought to be associated with its ability to partially inhibit the production of TNF-α. This reduction in TNF-α was first demonstrated *in vitro* in endotoxin or *M. leprae*-stimulated human monocytes (Sampaio, E.P. *et al*, 1991) and then *in vivo* in thalidomide-treated ENL patients (Sampaio, E.P. *et al*, 1993). So far, three mechanisms of TNF-α suppression by thalidomide have been described: (1) thalidomide was shown to reduce the half-life of TNF-α messenger RNA (mRNA) from 30 to 17 minutes (Moreira, A.L., *et al* 1993); (2) thalidomide is also shown to block NF-kB, a transcription factor involved in the regulation of several genes including TNF-α gene (Keifer, J.A. *et al*, 2001); (3) thalidomide is also shown to bind to α1-acid glycoprotein, a pro-inflammatory protein that belong to the acute phase proteins family (Turk, B.E. *et al*, 1996).

The suppression of TNF-α by thalidomide in conditions other than ENL was observed in tuberculosis (TB) patients with or without a co-infection with HIV (Tramontana, J.M. *et al*, 1985).

The inhibitory effect of thalidomide on TNF-α became controversial when studies indicating an enhanced production of TNF-α by thalidomide, started to appear. In 1996, Shannon

et al showed thalidomide to enhance TNF-α production by LPS-stimulated human monocytes as well as in the LPS-stimulated THP-1-monocyte-like cell line (Shannon, E.J. and Sandoval, F., 1996). This work supported subsequent findings of thalidomide-induced enhancement of TNF-α in the serum of apthous ulcers in HIV patients (Jacobson, J. *et al*, 1997) and in toxic epidermal necrolysis (Wolkenstein, P. *et al*, 1998). A recent study by Marriott *et al* has explained the varying effect of thalidomide on TNF-α as being conditional on the stimulant and the cell stimulated. In T-cell-independent systems like LPS-stimulated monocytes, thalidomide suppresses TNF-α; whereas, in a T-cell-dependent system such as PBMC stimulated by cross-linking of T-cell receptor (TCR), thalidomide can enhance the production of TNF-α. This enhancement is seen at late stages of incubation (48-72 hours) and is thought to be dependent on IL-2 signaling (Marriott, J.B. *et al*, 2002).

- Cytokines Other Than TNF-α

The effect of thalidomide on the monocyte production of the pro-inflammatory cytokines IL-1 and IL-6 is not very clear. Thalidomide (0.1-10 μg/ml) was shown to have no effect on the production of IL-1β, IL-6 or granulocyte macrophage colony stimulating factor (GM-CSF) by monocytes from healthy humans stimulated with LPS (Sampaio, E.P. *et al*, 1991). On the other hand, the release of IL-1 by LPS-stimulated PBMC from TB patients was suppressed in thalidomide-treated patients. In this same study the *in vivo* treatment of TB patients with thalidomide failed to affect the plasma IL-1 level (Tramontana, J.M. *et al*, 1985).

Thalidomide affects the production of IL-12 in a selective manner similar to its effect on TNF-α. In LPS stimulated monocytes, it suppressed the production of IL-12 (Moller, D.R. *et al*,

1997) whereas in T-cells stimulated via the TCR, it enhanced the production of IL-12 (Corral, L.G. *et al*, 1999).

1.6.5.2 Modulation of Lymphocyte Activity by Thalidomide

1.6.5.2.1 Lymphocyte Proliferation

The effect of thalidomide on primary T-cell responses has been difficult to evaluate using lymphocyte proliferation assay. Treatment of antigen or mitogen stimulated peripheral blood mononuclear cells (PBMC) from healthy as well as HIV and/or TB patients has given inconsistent results. Thalidomide given *in vivo* as well as *in vitro* in PBMC cultures from healthy individuals failed to affect lymphocyte proliferation in response to Concanavalin A (ConA) or purified protein derivatives (PPD) (Santos-Mendoza, T. *et al*, 1996). Günzler *et al* obtained a similar result in PBMC from healthy individuals stimulated with ConA and treated *in vitro* with 5 or 40 μMol/l (1.29-10.3 μg/ml) of thalidomide (Günzler, V. *et al*, 1986). In a study of tuberculosis (TB) patients with or without HIV co-infection, thalidomide treatment *in vivo* failed to significantly affect the ability of PBMC to proliferate in response to mycobacterial antigens (Tramontana, J.M. *et al*, 1985). Thalidomide at 3-30 ug/ml, failed to affect the ability of PBMC from a healthy individual to incorporate ^{3}H-thymidine in response to phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) (Fernandez, L.P. *et al*, 1995).

Some studies have demonstrated thalidomide's ability to suppress or stimulate the response of PBMC to various antigens and mitogens. Thalidomide suppressed the mitogenic and allogeneic stimulations of lymphocytes from healthy donors (Keenan, R.J. *et al*, 1991). *In vitro* treatment of thalidomide resulted in an enhanced proliferative response of PBMC from healthy

volunteers stimulated by cross-linking of the T-cell receptor (TCR) by immobilized monoclonal mouse anti-human CD3 antibody (Corral, L.G. *et al*, 1999). Similarly, in a study of whole PBMC as well as purified $CD4^+$ and $CD8^+$ T-cells from HIV-infected individuals, treatment with thalidomide at 1 and 10 μg/ml enhanced proliferative responses of these cells to anti-CD3 antibodies (Haslett, P.A.J. *et al*, 1998). The varying effect of thalidomide on lymphocyte proliferation assay was recently explained to be dependent on the types of dominant cell population and the signaling pathway involved. In T-dependent systems, thalidomide act as a secondary co-stimulator required for optimal T-cell activation resulting in enhanced proliferation. This was demonstrated in PBMC stimulated with the T-cell receptor cross-linker anti-CD3 antibody, where thalidomide enhanced the proliferation of PBMC in a concentration dependent fashion (Marriott, J.B. *et al*, 2002; Corral, L.G. *et al*, 1999).

1.6.5.2.2 T-cell Cytokine Production

Thalidomide is shown to enhance the production of TNF-α in T-cell dependent systems. CC-4047 is an analogue of thalidomide classified as an ImiDs (immunomodulatory drugs). CC-4047 is functionally similar to but more potent than thalidomide. A study involving CC-4047 has shown that the sources of elevated TNF-α are activated lymphocytes. This thalidomide-induced production of TNF-α occurs as a late event and is dependent on IL-2–mediated signalling. Abrogation of the IL-2 signal by anti-IL-2 antibody disrupted the enhancement of TNF-α by CC-4047 (Marriot, J.B. *et al*, 2002).

Several works have shown that thalidomide enhances the production of IL-2 by healthy as well as HIV-infected PBMC stimulated with mitogen and antigens (Shannon, E.J. and

Sandoval, F., 1995; Santos-Mendoza, T. *et al*, 1996; Marriott, J.B. *et al*, 2002; Haslett, P.A.J. *et al*, 1998). One particular group, which studied the expression of IL-2 by Jurkat cells, in response to PHA/PMA-induced stimulation, failed to demonstrate an effect of thalidomide on IL-2 (Fernandez, L.P. *et al*, 1995).

Thalidomide also enhanced the production of IFN-γ by mycobacterial antigen stimulated PBMC from thalidomide-treated TB patients (Tramontana, J.M. *et al*, 1985). A similar result was obtained in healthy individuals after ingestion of 400 mg thalidomide tablets where *in vitro* stimulation of PBMC with staphylococcal enterotoxin B (SEB) or anti-CD3 antibody resulted in a significant enhancement of IFN-γ (Verbon, A. *et al*, 2000).

Thalidomide is also shown to slightly enhance the LPS-induced production of IL-10 by PBMC from healthy volunteers (Corral, L.G. *et al*, 1999).

1.6.5.2.3 Effect on Plasma Cells and Antibody Production

Although formation of complexes between *M. leprae* antigens and their corresponding antibody is described as an initial event in ENL, not much work has been done regarding the effect of thalidomide on humoral immune responses. Production of primary IgM response to sheep red blood cells (sRBC) was suppressed in Swiss Webster mice fed with a thalidomide containing diet for 5 to 7 days prior to sensitization with sRBC; whereas, assessment of secondary humoral immune response revealed no effect of thalidomide on anti-sRBC IgG. In this same study, a marked reduction in serum IgM was also demonstrated in LL patients treated with both thalidomide and dapsone compared to healthy individuals, untreated active LL patients as well as dapsone treated patients (Shannon, E.J. *et al*, 1981). An opposite finding was reported in

another study where B6C3F1 mice were treated with thalidomide and sensitized with sRBC intravenously (i.v.). Evaluation of direct anti-sRBC plaque forming cells indicated significant enhancement in spleen IgM level due to thalidomide treatment (Karrow, N.A. *et al*, 2000). The difference of these two works was the method of thalidomide preparation, administration and dose used. In the study by Shannon *et al* 1981, thalidomide was incorporated into a powdered diet at 0.03% w/w which is the concentration required to achieve 0.84 μg/ml, an amount equivalent to plasma thalidomide level obtained after intake of a100 mg tablet in humans. While Karrow *et al* mixed thalidomide with water and injected mice by the i.p route at 30-150 mg/kg.

1.6.5.2.4 Effect on Lymphocyte Surface Expression Molecules

Patients experiencing ENL have an increase in $CD4^+$ cells in blood as well as in skin lesions (Modlin, R.L. *et al*, 1986). Thalidomide administration *in vivo* resulted in alteration of the ratio of $CD4^+$: $CD8^+$ cells in blood of healthy individuals (Gad, S.M. *et al*, 1985). A reduction in the total $CD4^+$ cells and an increase in $CD8^+$ cells were observed in thalidomide treated ENL patients (Shannon, E.J. *et al*, 1992). Treatment of ENL patients with thalidomide resulted in a prompt reduction in $CD4^+$ T-cells in lesions. A reduction in the expression of MHC Class II antigen and ICAM-1 by epidermal keratinocytes was also reported (Sampaio, E.P. *et al*, 1993). *In vitro*, treatment of PBMC with thalidomide and analysis of the expression of cell surface markers like CD2, CD4, CD5, CD8, HLA-A, B, C and HLA-DR molecules revealed no significant change (Shannon, E.J. *et al*, 1994). In HIV-infected individuals, treatment with thalidomide resulted in marked increase in soluble CD8 antigen in plasma while no change was

observed in soluble CD4 antigen. No significant difference was detected in $CD4^+$ as well as $CD8^+$ T-cell subsets between thalidomide treated group and placebo (Haslett, P.A.J. *et al*, 1998).

1.7 Thalidomide in Cancer

The immunosuppressive properties of thalidomide have led investigators to test this drug in the treatment of cancers as early as in the 1960's. The interest in the therapeutic potential of thalidomide in oncology quickly faded away as no dramatic effect was seen. Recently this interest was rekindled when thalidomide's anti-angiogenic activity was demonstrated (D'Amato J.R. *et al*, 1994). Angiogenesis or the formation of new blood vessels from already existing ones is crucial for the growth of tumors and for metastasis (Gupta, M.K. and Qin, R.Y., 2003).

Several studies of thalidomide in the treatment of various cancers have been carried out. Multiple myeloma (MM) is one example with a dramatic response in patients that are refractory to conventional therapy (Singhal, S. *et al*, 1999; Tosi, P. *et al*, 2002). This drug recently received approval from the governments of Australia and New Zealand for the treatment of refractory multiple myeloma (www.Celgene.com).

In MM, increase in bone marrow (BM) angiogenesis and an elevated BM plasma cell labeling index are associated with disease progression and poor prognosis. Conditioned medium of plasma cells isolated from MM patients showed a highly pronounced angiogenic activity in chick embryo chorioallantoic membrane, an enhanced chemotaxis and proliferation of endothelial cells (HUVEC) (Vacca, A. *et al*, 1999).

Malignant cells in MM are shown to secrete angiogenic cytokines known as endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF is a potent inducer of

vascular permeability and a potent mitogenic activator of endothelial cells (Bellamy, W.T. *et al*, 1999). Stimulation of bone marrow stromal cells from multiple myeloma patients with VEGF is shown to enhance the secretion of IL-6 a potent growth factor of myeloma cells. IL-6 is also an inhibitor of plasma cells apoptosis (Dankbar, B. *et al*, 2000).

Thalidomide inhibited bFGF-induced angiogenesis in a rabbit cornea micropocket assay (D'Amato, J.R. *et al*, 1994). Successful treatment of refractory MM patients with thalidomide was demonstrated to be associated with a reduction in plasma VEGF (Kakimoto, T. *et al*, 2002). Thalidomide also suppressed the proliferation of HUVEC and inhibited the production of TNF-α by these cells (Moreira, A. *et al*, 1999).

Interaction of integrins with the extra-cellular matrix of endothelium is important in endothelial cells survival, proliferation and migration during angiogenesis. The ability of thalidomide to suppress the β3 subunit of the αvβ3 integrin and to inhibit the release of FGF, which can stimulate the production of this integrin, suggests that thalidomide might modulate the expression of this integrin on endothelial cell surfaces (reviewed by Stephen, T.D. *et al*, 2000).

But the inconsistent decrease in BM microvessel density (MVD) observed after treatment in MM patients (Kumar, S. *et al*, 2002a) has led to uncertainties regarding the importance of angiogenesis in MM. This has led to investigations of other possible anti-tumor mechanism of thalidomide beside its anti-angiogenic effect (Kumar, S. *et al*, 2002b). Alternative anti-MM mechanisms such as the induction of plasma cell apoptosis, inhibition of TNF-α secretion by bone marrow stromal cells, modulation of T-cell immune response and induction of NK-cell

mediated lysis of malignant cells have all been investigated (Reviewed by Tosi, P. and Cavo, M., 2002).

In other hematological malignancies like acute myelogenous leukemia and non-Hodgkins lymphoma, thalidomide resulted in complete or partial response rates ranging from 20%-40%. In solid tumors like prostate, renal, brain cancers and Kaposi's sarcoma, treatment with thalidomide resulted in a response rate ranging from 6 % to 47% (Kumar, S. *et al*, 2002a).

1.8 Aim

It has been almost 40 years since Professor Sheskin discovered thalidomide to be an effective drug for the treatment of ENL. Since that discovery, thalidomide has been used with successes and failures in the treatment of a variety of inflammatory condition especially those refractory to standard treatment with anti-inflammatory drugs.

A precise mechanism of action for thalidomide is still lacking. Research efforts have pointed towards various possibilities but it has mostly been very hard to reproduce reported findings. The major sources of variation are the methods of drug preparation employed by each lab and the *in vitro* concentrations utilized. Thalidomide is poorly soluble in water and once in solution it quickly hydrolyses to different metabolic products.

The objective of this study is to assess possible mechanisms of action of thalidomide by using a method of drug preparation that ensures minimum hydrolysis. The concentration of thalidomide used was within the range of physiological level achieved after ingestion of a 400 mg tablet.

The controversial effect of thalidomide on primary T-cell response is rarely investigated in leprosy patients. In the first part of this work (part I), the effect of thalidomide on the immune response of PBMC from leprosy patients with or without reaction was investigated. The second part of this study (part II) assessed the effect of thalidomide on TNF-α production in patients with reversal reactions (RR). Although TNF-α level is elevated in RR patients, thalidomide has no therapeutic effect on this condition. This work was based on a finding from part I where thalidomide enhanced the production of TNF-α by PBMC from patients with borderline tuberculoid leprosy experiencing RR (BTRR) stimulated with integral *M. leprae*. This finding was further investigated by using a statistically determined sample size of untreated RR patients. The production of TNF-α by PBMC stimulated with mycobacterial antigens and treated with thalidomide *in vitro* was quantitated at protein as well as mRNA levels.

Thalidomide is shown to work in various inflammatory conditions while a convincing mechanism for its anti-inflammatory mechanism is still lacking. As the third chapter of this study (part III), the effect of thalidomide on the integrity of erythrocytes, PMNs and THP-1 cells membranes was studied.

The last part of this study (part IV) assessed the anti-microbial action of thalidomide on intracellular *M. leprae*. Although some speculations exist regarding the direct effect of thalidomide on *M. leprae*, no work has been done so far to prove or disprove this. Intracellular bacilli in resting as well as activated mouse peritoneal macrophages were treated with thalidomide and the viability of *M. leprae* was investigated by radio-respirometric assay.

CHAPTER 2 THE EFFECT OF THALIDOMIDE ON THE IMMUNE RESPONSE OF CELLS FROM LEPROSY PATIENTS

2.1 Introduction

Leprosy is a chronic skin disease characterized by episodes of reactions. The cause of these reactions is unknown. They may occur during the normal course of the disease, during treatment and even after treatment when the patient is bacteriologically negative. They are classified as type I /reversal reactions (RR) or type II/ erythema nodosum leprosum (ENL). Although these reactions have different clinical manifestations, they share a similar immunological profile. The immune response in acute ENL (Laal, S.S. *et al*, 1985), as well as in acute RR (Bjune, G. *et al*, 1976), is characterized *in vitro* by an enhancement in lymphocyte blast transformation to *M. leprae* antigen (s). These changes indicate the presence as well as the emergence of antigen-reactive-T-cells in leprosy patients that are normally anergic to *M. leprae*.

Among healthy individuals (Haslett, A.J.P. *et al*, 1998) and HIV positive patients (Shannon, E.J. *et al*, 2000), thalidomide has a stimulating effect on the ability of mitogen and antigen simulated T-cells to synthesize IL-2. Since this cytokine is important in the evolution of events that culminate in the proliferation of lymphocytes, an objective of our study was to determine if thalidomide could modify lymphocyte blast transformation to *M. leprae* antigens.

The mononuclear cells from patients attending the clinic at All Africa Leprosy, Tuberculosis and Rehabilitation Training Center (ALERT) for treatment of their leprosy or reactional episodes were exposed to thalidomide and to integral *M. leprae*, or antigens prepared

by sonication of integral *M. leprae* after depletion of lipids (a modified DHAR antigen), or to PPD prepared from *M. tuberculosis*. After 4 days in culture, the cells were pulsed with ^{3}H-thymidine and on day 5 the amount of tritiated thymidine incorporated by the cells was determined.

2.2 Materials and Methods

2.2.1 Study Subjects

Ethiopian healthy staff employed at Armauer Hansen Research Institute (AHRI) (N=11), who are in frequent contact with leprosy and tuberculosis patients, and leprosy patients admitted to ALERT hospital for treatment of leprosy (N=33) were enrolled in the study. Fourteen of the patients were classified as having borderline tuberculoid leprosy (BT), eleven BT patients were experiencing reversal reaction BT/RR, and 8 patients with ENL. All of the ENL patients included in the study were being treated with steroids. This might have hindered the *in vitro* immune responses of mononuclear cells isolated from these patients.A signed written consent was obtained from each patient. All patients were evaluated by history and physical examination prior to preparation of mononuclear cell cultures.

Table 2.1. Characterization of study groups

	Age Range	**Sex**		**MDT***	**Anti-inflammatory^{+}**
		Male	**Female**		
Healthy contacts N=11	25-56	7	4	0/11	0/11
BT N=14	13-77	6	8	1/14	1/14
BT/RR N=11	15-72	5	6	2/11	7/11
ENL N=8	15-52	4	4	4/8	7/8

* World Health Organization recommended multi-drug regimen, $^{+}$ Prednisone

2.2.2 Thalidomide

Thalidomide was kindly provided by Dr. K. Zwingenberger (Grünenthal GmbH, Stolberg/Rhineland, Germany). The drug was dissolved in dimethylsulfoxide DMSO (Sigma, USA) and, within 5-10 min after diluting it in RPMI, it was added into appropriate wells. Thalidomide was used at 4.0 µg/ml in the cultures with a final concentration of DMSO of 0.25% v/v in thalidomide and control cultures.

2.2.3 Antigens Prepared from Mycobacteria

2.2.3.1 Mycobacterium Tuberculosis

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

2.2.3.2 Mycobacterium Leprae

The antigens from *M. leprae* were prepared at the National Hansen's Disease Programs, Laboratory Research Branch, Immunology Lab, Baton Rouge La. Integral *M. leprae* (acid-fast-bacteria, AFB) was isolated from the tissue of nude mice infected with *M. leprae*. The tissue was aseptically removed from footpads of infected mice, and homogenized in 7H12 medium. The homogenate was centrifuged at 30 x g for 15 minutes (min). The pellet was discarded and the supernatant centrifuged at 2450 x g for 45 min. The pellet was suspended in 20 ml of 0.1N NaOH. After 10 min, the alkali treated material was centrifuged at 2100 x g for 30 min. The pellet was washed twice with pyrogen free distilled water and suspended in pyrogen free distilled water. One half of this preparation was frozen and used as the integral *M. leprae* (AFB). The

other half was prepared, as follows, for the DHAR antigen. Integral *M. leprae* was centrifuged at 450 x g for 30 min. The pellet was suspended in 6 ml of chloroform and dried using nitrogen gas. The dried fraction was suspended in 6 ml of ether and centrifuged at 450 x g for 30 min. The supernatant was discarded and the bacteria suspended in ether and transferred to an agate mortar and worked to dryness with a spatula. The dried bacilli were suspended in 5.0 ml of pyrogen free distilled water, and disrupted by sonic oscillation, on ice, for 15 min at 30% duty cycle (Untrasonic Homogenizer 4710, Cole-Palmer Inst. Co., Chicago). The integral *M. leprae* were enumerated and numbers adjusted to contain 100 AFB bacilli/ 50 µl / 1 mononuclear cell. The DHAR preparation was assayed for content of proteins (DC Protein Assay, Bio-Rad), and added to the cell cultures in a volume of 50 µl. The final concentration was10 µg of protein /ml.

2.2.4 Cell Cultures

Mononuclear cells were isolated from 30-40 ml of blood collected in Na-heparin-treated vacuum tubes. The blood was diluted to twice its volume with RPMI-1640 and centrifuged on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The mononuclear cells were harvested and washed three times in RPMI-1640 (Flow Labs, Irvine, UK). The cells were adjusted to contain 2 x 10^6 cells per ml RPMI-1640 that had been supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml of streptomycin and 10% v/v fetal calf serum (Sigma) (RPMI-10%). One hundred µl of RPMI-10% containing 2 x 10^5 mononuclear cells was added into flat-bottom wells of a 96-well plate. The control cultures and cultures not treated with thalidomide received 50 µl of RPMI-10% with DMSO at 1% v/v. The cultures treated with thalidomide received 50 µl of RPMI-10% with DMSO at 1% v/v and thalidomide. The final concentration of unhydrolyzed

thalidomide was 4.0 µg/ml. The final concentration of DMSO in all cultures was 0.25% v/v. The Control cultures, not stimulated with antigen, received 50 µl of RPMI-10%. The cultures stimulated with antigens received 50 µl of RPMI-10% containing DHAR antigen, or integral *M. leprae,* or PPD (as described above).

2.2.5 Assay for Lymphocyte Blast Transformation

The cells were incubated at 5% CO_2 in a high humidity, 37^0C incubator. Culture systems were set up under two different conditions. One group, at the initiation of the 5-day culture period, received 4.0 µg/ml of thalidomide (1X). Another set of cultures, to insure a source of unhydrolyzed thalidomide during the pulse with ^{3}H-thymidine, received 4.0 µg/ml of thalidomide for the second time on day 4 (2X). On day four in the 2X thalidomide treated cultures, the concentration of unhydrolyzed thalidomide was estimated to be 4.0009 µg/ml with 94 ng/ml of residual unhydrolyzed thalidomide remaining after the first 4 days in culture and the additional thalidomide added for the second time. This calculation was based on the estimated t½ of 8.0 hrs for thalidomide in tissue culture medium at 37^0C (Gunzler, V. *et al*, 1986).

On day 4, the cultures were pulsed with 1µCi per well of H^3-thymidine (Boehringer Mannheim, Germany). After 18 hours, cells were harvested on filters and the amount of radioactive thymidine incorporated into cellular DNA was determined using a Rack-β Liquid Scintillation Counter (LKB, Pharmacia, Uppsala, Sweden). The results were expressed as stimulation index (SI), which is the mean counts per minute (CPM) from three replicate cultures of the mycobacterial antigen alone or the mycobacterial antigen + thalidomide treated cells divided by the CPM of cultures not receiving antigen or thalidomide.

2.2.6 Determination of TNF-α

On day 4, prior to the addition of thalidomide, 50 µl of supernatants was collected from each culture and frozen for later analysis of TNF-α. Commercial ELISA reagents were purchased (R&D Systems, Minneapolis) and used according to the manufacturer's specifications. The amount of TNF-α in each sample was determined from the standard curve using the program KCjunior Version 1.31.2 (Bio-Tek Instruments Inc, Vermont). The concentrations of TNF-α in cultures were expressed in ng/ml.

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

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

2.3 Results

2.3.1 Proliferative Responses in the Absence of Thalidomide

The modified DHAR and *M. leprae* antigen preparations were titrated for their ability to stimulate mononuclear cells to incorporate ^{3}H-thymidine as described previously (Shannon, E.J, *et al*, 1984). Using mononuclear cells from two healthy lepromin and PPD positive individuals, the DHAR antigen preparation stimulated the cells at 10 µg/ml. After exposing the cells to a ratio of 1:1 or 10:1 or 100:1 *M. leprae* acid fast bacteria (AFB): mononuclear cells, the maximum stimulation with integral *M. leprae* was observed at a ratio of 100 AFB to 1 mononuclear cell (data not shown).

The mononuclear cells from the healthy staff, BT and BT/RR, in the absence of thalidomide, responded to the three antigen preparations with the rank of response to PPD >

DHAR> AFB. The patients in the ENL group were not stimulated as well to the *M. leprae* antigens.

2.3.2 Effect of thalidomide on Proliferative Responses

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

Thalidomide (2X) empowered the mononuclear cells from the BTRR patient group to incorporate [H3]-thymidine when stimulated with DHAR (Wilcoxon signed rank test p= 0.01) compared to thalidomide untreated cultures (Figure 2.1)

Among the thalidomide 2X treated healthy cells, the addition of thalidomide for the second time resulted in a suppression of cell proliferation in response to PPD and AFB compared to thalidomide untreated cultures. This was shown by the significant decrease in SI (p=0.02 for PPD and p=0.01 for AFB). For the rest of the groups, replenishment of thalidomide in cultures did not affect the SI (Figure2.1).

2.3.3 Effect of Thalidomide on Synthesis of TNF-α

Thalidomide resulted in a significant suppression of TNF-α production in culture supernatants from cells from healthy staff exposed to the modified DHAR antigen (p=0.0312) (Figure 2.2). A mixed effect was seen for the other groups with a trend of suppression in most cases. It is interesting to note that among the BT/RR study group, four of seven patients cells were stimulated to produce TNF-α when incubated with integral AFB and Thalidomide (Figure 2.2).

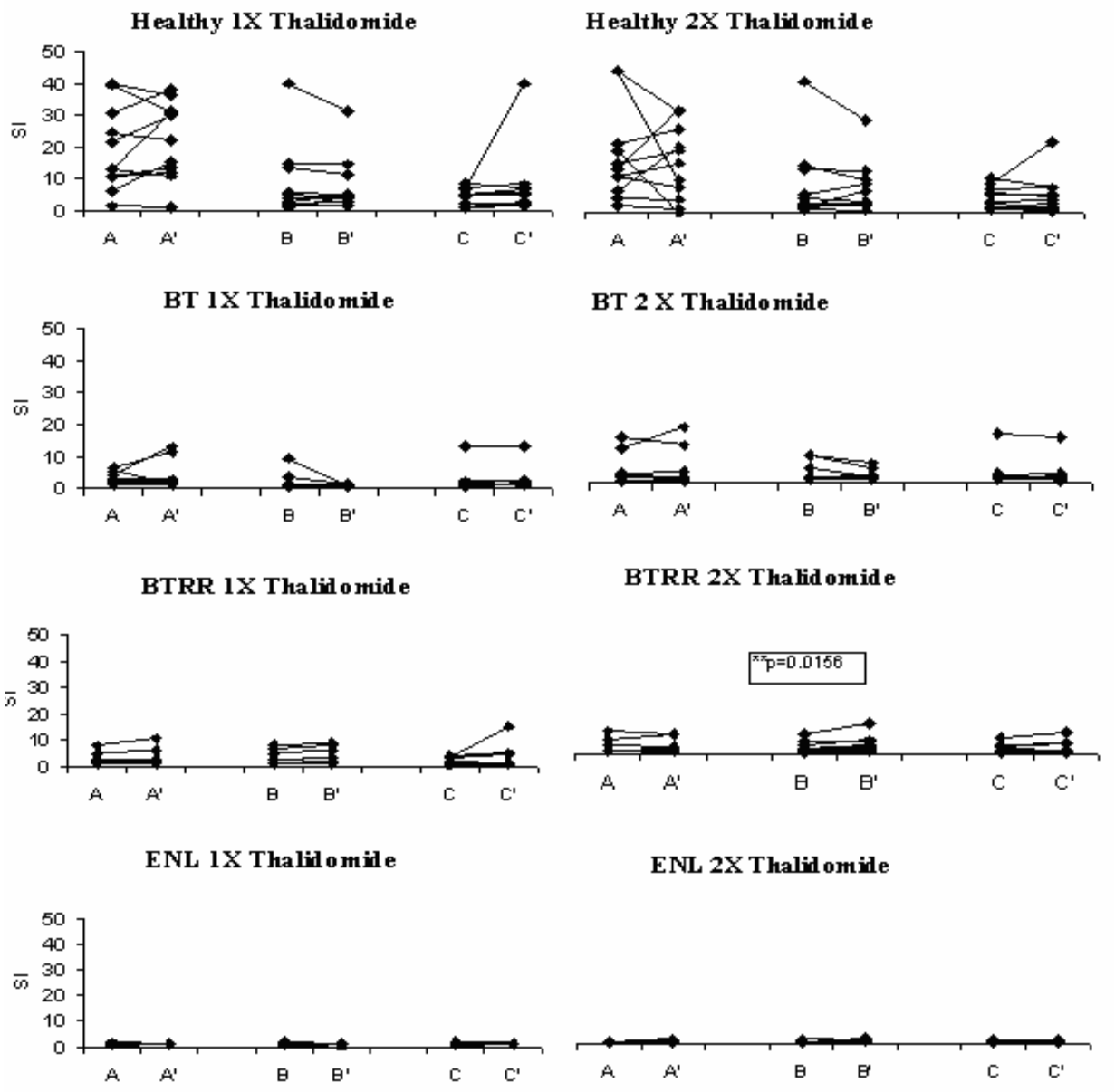


Figure 2.1. The Effect of Thalidomide Added at the Initiation of Incubation Period (1x) and Thalidomide Added a Second Time 24 hours Prior to Termination of 5 days Cultures (2x). P value derived from Wilcoxon signed rank test. A=PPD, No Thalidomide; A'=PPD + Thalidomide (1x or 2x); B=Dharmendra, No Thalidomide; B'=Dharmendra + Thalidomide (1x or 2x); C=Integral *M. leprae, No* Thalidomide;C'=Integral *M. leprae* + Thalidomide (1x or 2x)

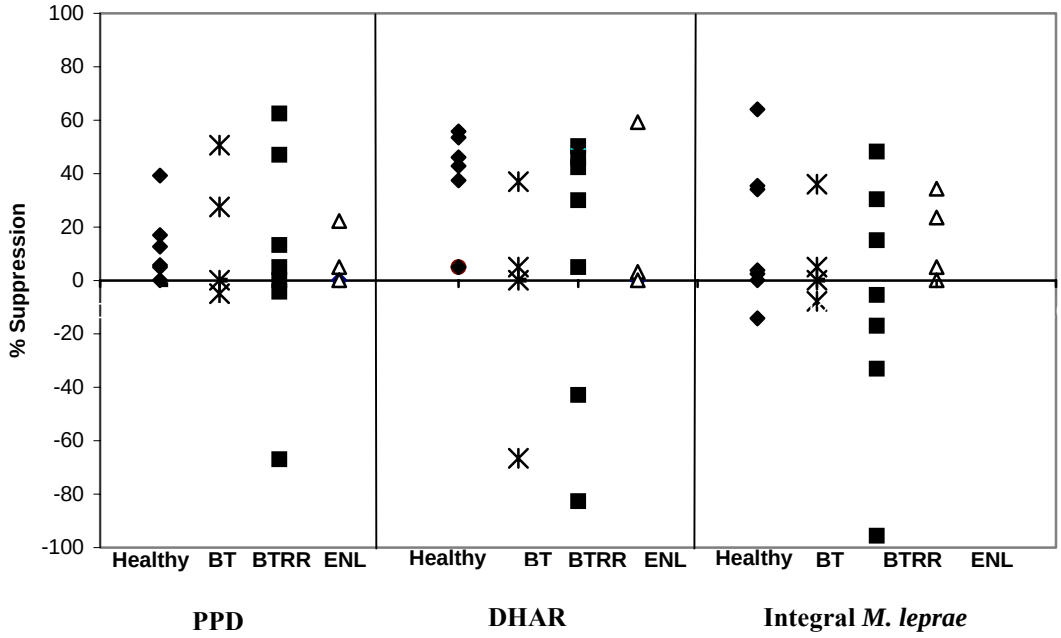


Figure 2.2 Effect of Thalidomide on TNF-α Production. Culture supernatants, from cells treated with thalidomide and antigen or DMSO and antigen, were used in ELISA to measure TNF-α levels. Values in pg/ml were used in the following formula to calculate percent suppression.

2.4 Discussion

In diseases like leprosy, where cell mediated immunity (CMI) plays an important role in protection as well as in development of reactions and subsequent complications, the *in vitro* response of lymphocytes from patients against mycobacterial antigens is correlated well with the clinical manifestations (Myrvang, B. *et al*, 1973). We studied the effect of thalidomide on the lymphocyte blast transformation of cells from leprosy patients with or without reactions in response to stimulation with mycobacterial antigens.

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

Type I reaction in leprosy is characterized by a sudden increase in cell mediated immune response to *M. leprae* antigens (Bjune, G. *et al*, 1976). Our results show proliferation of BT/RR patients' cells in response to all three antigens compared to unstimulated controls. However thalidomide failed to alter the *in vitro* proliferative response of the same patients against mycobacterial antigens.

ENL patients' cells have been reported to incorporate ^{3}[H] thymidine in response to soluble and integral *M. leprae* antigens (Laal, S.S. *et al*, 1985). Although significant, this

response was relatively lower to that observed in tuberculoid leprosy patients. The ENL patients included in our study did not respond well to the *M. leprae* antigen preparations. These patients were being treated with prednisone (some as much as 30 mg per day) and were probably in a hyporeactivity or anergic phase usually observed in post-treatment of ENL.

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

Thalidomide has been previously shown to enhance the DTH response to a challenge dose of PPD in individuals with PPD skin test positivity (Tramontana, J.M. *et al*, 1995). *In vitro*, the drug had been shown to increase synthesis of IL-2 in PPD positive individuals (Shannon, E.J. *et al*, 1995). In our study, thalidomide facilitated the response against DHAR in BTRR patients.

In various *in vitro* studies, the effect of thalidomide on the ability of antigen or mitogen stimulated cells to incorporate ^{3}[H] thymidine is controversial. Among healthy PPD positive individuals in Mexico, it was found that thalidomide or metabolites of thalidomide did not alter the ability of cells stimulated with PPD to incorporate ^{3}H- thymidine (Santos-Mendoza, T. *et al*, 1996). Whereas, another study showed that the drug enhances the proliferation of TB and HIV co-infected patient's cells stimulated with PPD (Bekker, L.G. *et al*, 2000). One possible explanation for such inconsistencies is the extremely quick hydrolysis of thalidomide. Even in a physiological environment like blood, thalidomide quickly hydrolyses. This characteristic of thalidomide is even faster at pH 7.0 to 7.5 (Ericsson, T. *et al*, 1998). The type of solvent used,

incubation times and pH of media determine the extent of hydrolysis and the type of metabolite, which will predominate in the assay (Shannon, E.J. *et al*, 1997). Our data might be the summation of the actions of different hydrolysis products of thalidomide.

Parallel to the proliferation assay, the effect of thalidomide on the level of TNF-α was also assessed. Suppression of TNF-α was exhibited in healthy contacts in response to DHAR antigens.

Despite several studies conducted for over a quarter of a century, thalidomide's mechanism of action in arresting ENL is still unknown. This lack of understanding is accentuated by the uncertainties that still remain today about the factors which cause ENL.

Treatment of a given clinical condition with thalidomide may be associated with a decrease in TNF-α like in ENL (Sampaio, E.P. *et al*, 1992) or an increase in TNF-α like in aphthous ulcers (Jacobson, J. *et al*, 1997). In a trial involving the use of thalidomide to treat toxic epidermal necrolysis, the work was discontinued due to an elevated drug-related toxicity. TNF-α levels were elevated in the patients who received thalidomide compared to the placebo group (Wolkenstein, P. *et al*, 1998). Comparison of prednisone, pentoxifylline and thalidomide in the treatment of ENL revealed that although thalidomide is the most effective drug to relieve this condition, prednisone suppresses TNF-α more than thalidomide. The level of TNF-α detected in ENL is so low that the authors suggested that this cytokine might not be the only factor responsible for the pathology of ENL (Moreira, A. *et al*, 1998). The most convincing evidence that TNF-α is not the sole cytokine targeted by thalidomide in ENL is the observation that this drug is not an effective treatment for reversal reaction (Hastings, R.C. *et al*, 1985). The

pathology of RR, even more so than in ENL is associated with an increase in TNF-α protein and TNF-α mRNA in the skin and peripheral nerves (Naffs, B., 1994; Khanolkar-Young, S. *et al*, 1995).

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

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

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

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

CHAPTER 3 THE EFFECT OF THALIDOMIDE ON THE EXPRESSION OF TNF-α MRNA AND THE SYNTHESIS OF TNF-α IN CELLS FROM LEPROSY PATIENTS WITH REVERSAL REACTION

3.1 Introduction

Leprosy is a spectral disease characterized by the severe form of lepromatous leprosy (LL) at one end of the spectrum and the milder tuberculoid leprosy (TT) at the other. Borderline leprosy patients lie between LL and TT and are immunologically unstable (Hastings, R.C., 1985). This group of patients has a tendency to downgrade or upgrade their immune response to *M. leprae* with a consequence of adverse reactions.

Reactions associated with an upgrading in the immune response to *M. leprae* are known as reversal reactions (RR). Symptoms of RR include inflammation of pre-existing lesions and acute neuritis with risks of permanent disability. The other type of immunological reaction in leprosy, known as erythema nodosum leprosum (ENL), is mainly confined to LL patients. ENL is manifested by the appearance of crops of tender erythematous nodules, neuritis and systemic symptoms like fever, myalgia and general malaise (Ridley, D.S., 1969).

Common to both RR and ENL is a transient enhancement in cell-mediated immunity towards *M. leprae* (Bjune, G. *et al*, 1976; Laal, S. *et al*, 1985); an elevated production of TNF-α (Sarno, E.N. *et al*, 1991; Khanolkar-Young, S. *et al*, 1995; El-din, A. *et al*, 1998), with TNF-α reported to be even more elevated in RR (Gru, G. *et al*, 1992).

Thalidomide is the drug of choice for the treatment of ENL, but it lacks any effect in the treatment of RR (Hastings, R.C., 1985). Thus the beneficial effect of thalidomide in ENL, which has been mainly attributed to an inhibition of TNF-α, does not explain why the drug is not

equally successful in the treatment of RR. We observed, among 4 of 7 steroid-treated RR patients, that thalidomide enhanced TNF-α when PBMC cultures were stimulated with integral *M. leprae* antigen (AFB) (Tadesse, A. *et al*, 2003). This study is an extension encompassing a larger non-steroid treated population of patients and also assesses mRNA expression for TNF-α in the cultured cells.

PBMC from a group of 68 RR patients and two control groups were treated with thalidomide and stimulated with integral γ-irradiated *M. leprae* (AFB), a cytosolic preparation from *M. leprae* (MLSA) and DHAR antigen**.** Lymphocyte proliferation was determined by incorporation of ^{3}H-thymidine; TNF-α was measured by ELISA, and TNF-α mRNA was semi-quantitated using reverse transcription polymerase chain reaction (RT-PCR).

3.2 Materials and Methods

3.2.1 Study Subjects

Based on previous observations with 4 of 7 RR patients having an enhanced TNF-α production due to thalidomide (Tadesse, A. *et al*, 2003), The sample size was calculated with a 10% margin of error and a 90 % confidence level to be 68. The two control groups used were: 12 borderline leprosy patients without reversal reactions (BT, BB and BL), and 15 healthy individuals with frequent contact with leprosy and tuberculosis patients. Patients were attending the clinic at All Africa Leprosy Rehabilitation and Training Center (ALERT). Those experiencing RR had blood drawn immediately upon admission and prior to treatment with anti-inflammatory drugs. The subjects were informed about the purpose, possible risks and benefits of the study and a written signed consent was obtained from each subject. Only adults of 18

years and above were enrolled in the study. All patients were evaluated by history and physical examination and the clinical characteristics of each patient were recorded in table 3.1.

Table 3.1. Characterization of Study Groups

	Age Range	Sex		Total
		Male	Female	
Patients with RR				
BT/RR	18-65	15	17	22
Borderline lepromatous (BL/RR)	18-70	26	17	43
Lepromatous (LL/RR)	18-65	3	0	3
Patients without RR (Control 1)				
Mid-borderline (BB)	55	1	0	1
BT	21-73	3	0	3
BL	19-55	5	2	7
Neural leprosy	60	1	0	1
Healthy Individuals (Control 2)				
	24-45	9	6	15

3.2.2 Thalidomide

Thalidomide (kindly provided by Celgene, Warren, New Jersey) was dissolved in dimethylsulfoxide (DMSO). Within 5-10 min after preparation, dilutions were made in complete RPMI. As previously described, a concentration of 4.0 μg/ml of thalidomide was used (Tadesse, A. *et al*, 2003). [This concentration of thalidomide best approximates the area under the curve where thalidomide has been used effectively in the treatment of a variety of inflammatory conditions (Erikson, T., 1997)]. A concentration of 0.25% v/v of DMSO was maintained in all drug-treated and control wells.

3.2.3 Antigens

γ-irradiated integral *M. leprae* (AFB) and a cytosol fraction of *M. leprae* (MLSA) were kindly provided by an NIH awarded contract to Colorado State University (Department of Microbiology, CSU). The γ-irradiated bacteria were used at a final concentration of 25 µg/ml and the cytosol fraction of *M. leprae* (MLSA) was used at a final concentration of 25 µg/ml.

The DHAR (DHAR) antigen was prepared from *M. leprae* at the Laboratory of the National Hansen's Disease Programs in Baton Rouge, Louisiana as described previously (Tadesse, A. *et al*, 2003). Based on a determination of protein content, a final concentration of 5µg/ml was used in cultures.

Purified protein derivative PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) contained a final concentration at 10µg/ml in cultures. Phytohemagglutinin (PHA) (Sigma, Saint Louis MI) was used at 10 µg/ml.

3.2.4 Cell Cultures

Thirty ml of blood was collected from the leprosy patients and controls. PBMC were isolated by gradient density centrifugation. Briefly, the blood was diluted with an equal volume of RPMI-1640 (Flow Labs, Irvine, UK) and centrifuged on Ficoll-Paque^{TM} (Amersham Biosciences AB, Uppsala, Sweden). Mononuclear cells were harvested and washed twice with RPMI-1640. The cell pellet was resuspended in complete medium (RPMI-1640 supplemented with 10 % fetal calf serum, 2mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin) and cells were adjusted to contain 2 x 10^6/ml. One hundred µl of cells was added per well into tissue culture plates. The cultures treated with thalidomide received 50 µl thalidomide dissolved

in DMSO and further diluted with complete media. The control cultures, not treated with thalidomide, received 50 µl of complete RPMI containing DMSO. The final concentration of thalidomide was 4.0 µg/ml and the final concentration of DMSO in all cultures was 0.25% v/v. Wells stimulated with antigens received 50 µl of the above-described antigens while control wells received 50 µl of complete RPMI.

3.2.5 Assay for Lymphocyte Blast Transformation

The cell cultures were incubated at 5% CO_2, high humidity and 37^0C. For cells stimulated with mycobacterial antigens the cultures were incubated for 5 days; whereas, the cultures stimulated with PHA were incubated for 3 days.

On day four (day two for PHA treated cultures), cells were pulsed with 2µCi per well of H^3-thymidine (Amersham, Sweden). After 18 hours, the cells were harvested on filters and the amount of radioactivity was determined using a Rack-β Liquid Scintillation Counter (LKB, Pharmacia, Uppsala, Sweden). A stimulation index (SI) was calculated. The SI is the mean counts per minute (CPM) from three replicate wells of antigen or antigen + thalidomide treated wells divided by the CPM of DMSO treated wells. The following formula was used to determine the percent suppression (% S) by thalidomide.

$$\% S = [1- (\underline{\text{SI of thalidomide + antigen-treated wells}})] \times 100$$
$$\text{SI of DMSO+ antigen-treated wells}$$

[When the % S is a positive number suppression occurred; whereas, a negative number indicates enhancement].

3.2.6 Determination of TNF-α

Separate cultures were set in parallel for TNF-α analysis. On day 4 of incubation, 50 µl

of culture supernatants were pooled from each of 3 wells and frozen at -70^0C for later TNF-α protein quantitation. The remaining cell suspension was collected in Nunc tubes (Nalgen Nunc International, Denmark) and snap-frozen in liquid nitrogen for determination of TNF-α mRNA by RT-PCR.

3.2.6.1 TNF-α Protein Determination

A human TNF-α commercial ELISA kit (Pierce Endogen, Woburn, MA) was used to quantitate TNF-α in culture supernatants from AFB, DHAR and MLSA treated cultures. The provided manufacturer's specifications were strictly followed. The amount of TNF-α in each sample was determined from the standard curve using Genesis Lite Version 3.03 (Life Sciences UK, Ltd). TNF-α concentration, expressed in pg/ml, was used to calculate the percent suppression using the following formula:

$$\% S = \left[1 - \frac{\text{TNF-}\alpha \text{ in thalidomide and antigen treated wells)}}{\text{TNF-}\alpha \text{ in drug free and antigen treated wells}}\right] \times 100$$

3.2.6.2 Determination of TNF-α MRNA

Frozen cell pellets were thawed and total RNA was extracted by using the RNeasy RNA isolation kit (Qiagen, gmbH, Germany). The RNA was resuspended in RNAse free water and the concentration was determined by spectrophotometer at 260 nm. The total RNA per each sample was used to synthesize complementary DNA (cDNA) using the Omniscript Reverse transcription kit (Qiagen, gmbH, Germany). The Reverse transcription (RT) mix consisted of 2 µl 10x buffer, 2 µl dNTP mix, 2 µl oligodT primer, 1 µl RNAse inhibitor and 1 µl Omniscript reverse

transcriptase. The reaction was allowed to proceed in a Multi-block systems thermal cycler (Hybaid, Germany) at 37^0C for 1 hour, 93 ^{0}C for 5 minutes and kept at 4^0C.

The newly synthesized c-DNA was transferred to a sterile tube. A 1:10 dilution of the c-DNA was used to measure the concentration of nucleic acids at 260 nm and to check for protein contamination by 260/280 ratios. The c-DNA was adjusted to 0.1 µg/µl with DNAse free sterile water. Ten µl of cDNA (1 µg) was used as template in each PCR work (Multi-block systems software, Hybaid Germany). The TNF-α primer pair sequence (Johnson B.J. and McMurray D.N., 1994) 5′-CCTTGGTCTGGTAGGAGACG-3′ and 5′-CAGAGGGAAGAGTTCCCCAG was used for amplification. Normalization was done with the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GA3PD) (Villiger P.M *et al* 1990) primers 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′and 5′-TGGTATCGTGGAAGGACTCATGAC-3′. Both TNF-α and GA3PD primers were synthesized at the Gene Lab of BioMMED, Division of Biotechnology and Molecular Medicine (Louisiana State University, Baton Rouge, LA). The PCR milieu for each tube consisted of 25 µl master mix (HotStatTaq^{TM} PCR, Qiagen, gmbH, Germany) 1.5 µl of GA3PD or 2.5 µl of TNF-α primers, 10 µl cDNA and 13 µl of sterile distilled H_2O. For TNF-α, the PCR program used was 95^0C 15 min 1 cycle; 94^0C 1 min, 62^0C 1 min, 72^0C 1 min 45 cycles; 72^0C 10 min final extension. For GAP3D the PCR program used was 95^0C 15 min 1 cycle; 94^0C 1 min, 62^0C 1 min, 72^0C 1 min 35 cycles; 72^0C 10 min final extension. A mixture of 8 µl of each PCR product and 2 µl of loading buffer was resolved on ethidium bromide stained 1% agarose gel. Bands were visualized by a UV transilluminator and the gel pictures were analyzed by computer software (LabWork ^{TM} Version 3.00, Upland,

California). The maximum optical density (OD Max) was used to score the relative intensity of the TNF-α-specific bands against the glyceraldehype-3-phosphate dehydrogenase (GA3PD), which was used as a control to normalize the cytokine signals within and between samples.

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

$$\% \text{ TNF mRNA} = \frac{\text{OD Max of TNF-}\alpha\text{ specific band of the sample}}{\text{OD Max of GA3PD band for the same sample}} \times 100$$

3.2.7 Analysis of Data

GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA) was used to analyze the data. Wilcoxon signed rank test was used to compare thalidomide treated to untreated groups. Differences were considered to be significant when the $p \leq 0.05$, 2-tail test.

3.3 Results

3.3.1 Lymphocyte Proliferation

Cell viability was assessed by 3-day stimulation with PHA. The CPM in the PHA stimulated cultures ranged from 493 to 103,077 in the RR patients, from 372 to 37,216 in the BL patients, and from 17,405 to 117,576 in the healthy individuals. Proliferation to the mycobacterial antigens was assessed after 5 days in culture. Fifty three percent of healthy individuals had a response with a SI above 2.0 for AFB, 93% for DHAR and 33% for MLSA. In RR patients the proportion of responders was 13% for AFB, 47% for DHAR and 13% for MLSA. Overall, the proliferative responses in the RR group were lower than the healthy control group, but much higher than leprosy patients without reaction (Table 3.2.).

Table 3.2. Proportion of Subjects with a Stimulation Index Above the Cut-off Value of 2.0

Antigens	RR (n=68)	No RR (n=15)	Healthy (n=12)
AFB	9 (13.2*)	2 (16.6)	8 (53)
DHAR	32 (47)	0	14 (93.3)
MLSA	9 (13.2)	1 (8.3)	5 (33.3)

*Values in bracket represent percentages

3.3.2 Effect of Thalidomide on Lymphocyte Proliferation

The SI in drug free + antigen-treated wells with the SI in thalidomide + antigen-treated wells, inn the RR patient group thalidomide significantly enhanced proliferation in response to DHAR (p= 0.004) and MLSA (p= 0.013); it failed to significantly stimulate proliferation in response to AFB (p= 0.0629). Thalidomide also suppressed AFB induced proliferation among healthy controls (P= 0.0084) (Figure 3.1). In all of the groups, thalidomide did not significantly modify the response of PBMC to PPD (data not shown).

3.3.3 Effect of Thalidomide on the Synthesis of TNF-α

The TNF-α levels in drug free + antigen-treated wells with TNF-α in thalidomide + antigen-treated wells, in all the groups, thalidomide: suppressed the synthesis of TNF-α, in response to the antigen preparations from AFB, DHAR and MLSA ($p < 0.0001$) (Figure 3.2A). A similar suppression of TNF-α due to treatment with thalidomide was seen in healthy individuals (AFB p=0.0017, DHAR p= 0.0134 and MLSA p= 0.0266) (Figure 3.2B). For borderline leprosy patients without RR, the suppression was significant in the AFB (p=0.031) and MLSA (p=0.0156) stimulated groups (Figure 3.2C).

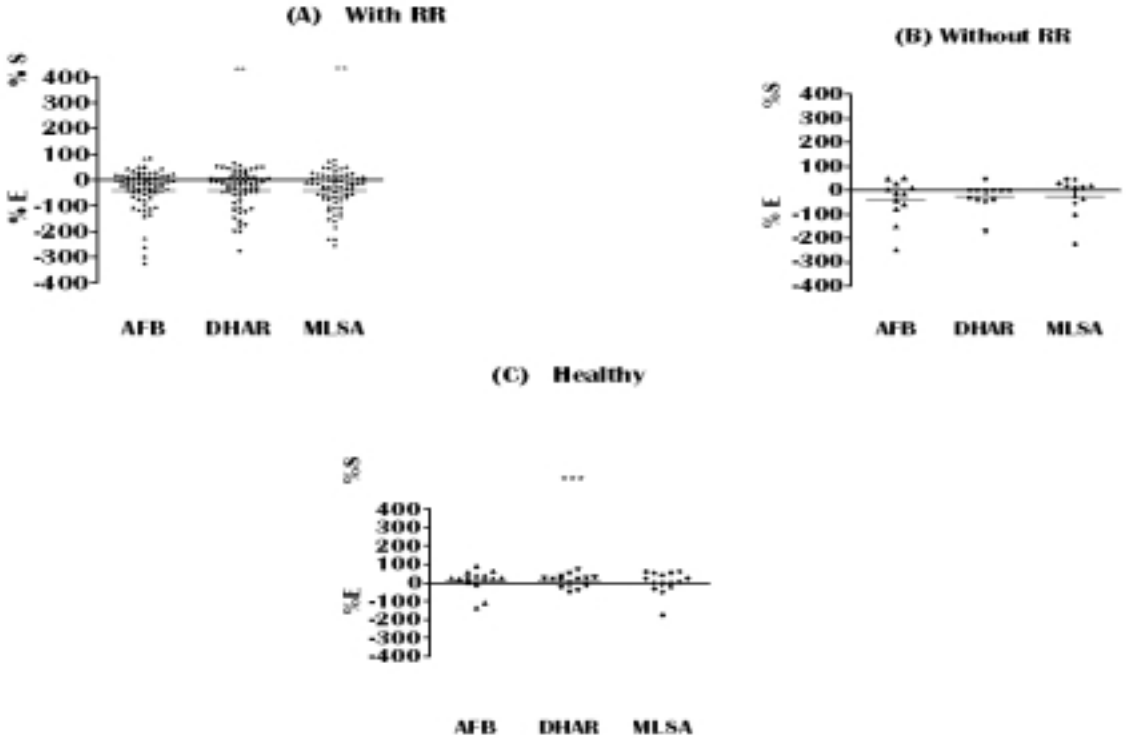


Figure 3.1. Effect of Thalidomide on Lymphocyte Proliferation to *M. Leprae* Antigens. PBMCs from leprosy patients with RR (A), patients without RR (B) and healthy individuals (C) were incubated with mycobaterial antigens and thalidomide for 5 days. At day 4, cultures were pulsed with tritiated thymidine. Counts per minutes were used to calculate SI, which are then used to determine the percentage enhancement (%E) or suppression (%S) by thalidomide.

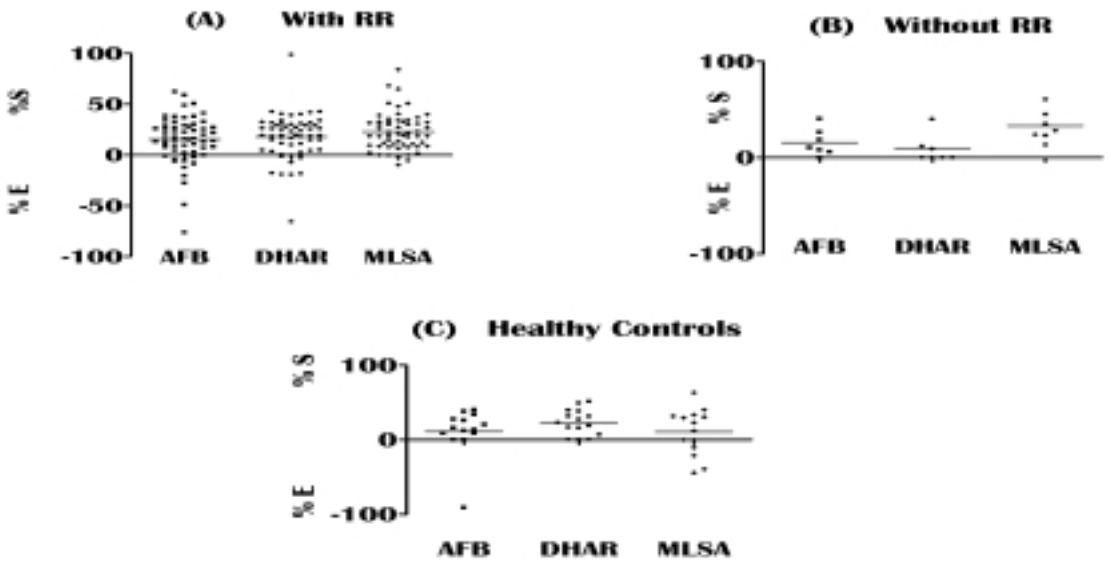


Figure 3.2. The Effect of Thalidomide on TNF-α Production. PBMCs from leprosy patients with RR (A), with leprosy patients without RR (B) and healthy individuals (C) were stimulated with AFB, DHAR and MLSA antigens for 4 days. Pools of 3 wells culture supernatant were used in ELISA to quantitate the level of TNF-α. The percentage suppression (%S) and enhancement (%E) were used as y intercepts.

3.3.4 Effect of Thalidomide on TNF-α MRNA Level

The TNF-α primers used were designed to span the junctions of 2 exons, making them specific for cDNA and not capable of recognizing contaminating genomic DNA (Johnson, B.J. and McMurray, D.N., 1994). As shown in Figure 3.3, the presence of TNF-α mRNA was validated by the presence of a 325 bp band in the agarose gels (Wang, A.M. *et al*, 1989). Although a fairly consistent cDNA band for TNF-α was detectable in almost all PBMCs in response to all three antigens (Figure 3.4), thalidomide did not significantly influence the TNF-α mRNA expression pattern in antigen stimulated cells (Wilcoxon signed rank test). The spread

was more pronounced among RR patients. GA3PD gave a fairly consistent band intensity of 190 bp.

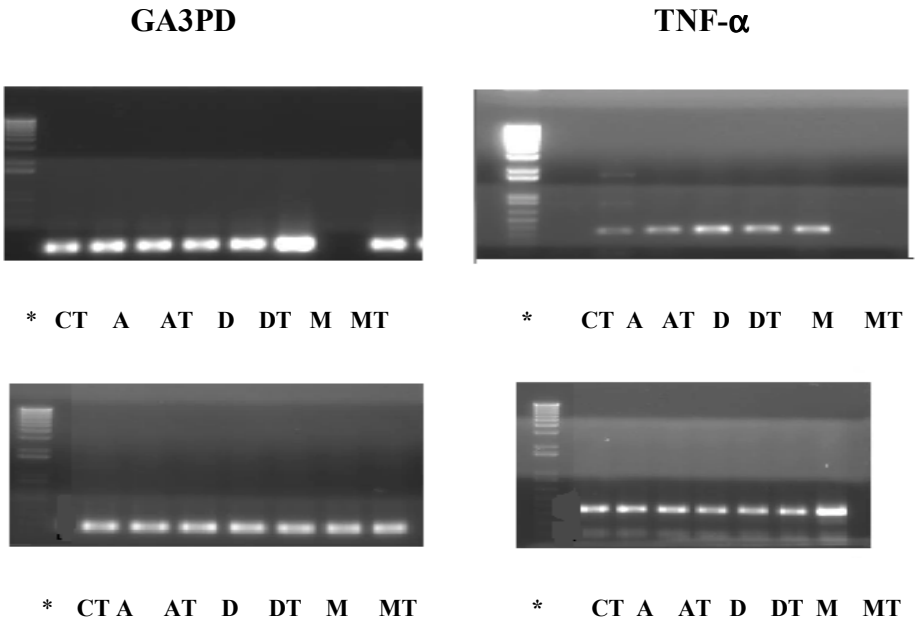


Figure 3.3. Effect of Thalidomide on TNF-α MRNA Expression. PBMC were stimulated with antigens for 5 days. Total mRNA was extracted; c-DNA synthesized and TNF-α mRNA expression was assessed by RT-PCR using specific primers. The housekeeping gene GA3PD was used for normalization. Lane legend: (*) molecular mass marker 123 bp DNA ladder, (CT) unstimulated cells + thalidomide, (A) AFB, (AT) AFB + thalidomide, (D) DHAR, (DT) DHAR + thalidomide, (M) MLSA and (MT) MLSA + thalidomide.

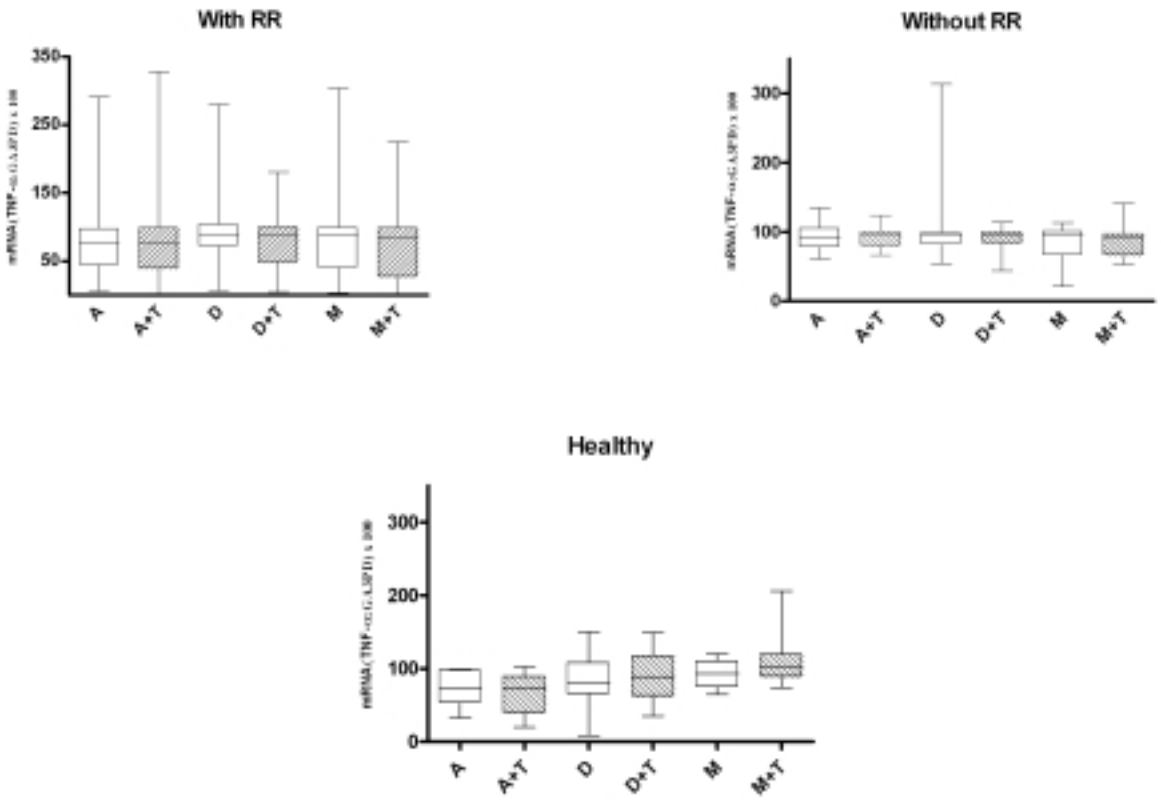


Figure 3.4. The Effect of Thalidomide on the Expression of TNF-α MRNA in Antigen Stimulated Cells. Total RNA was extracted and used to synthesize cDNA. PCR was done using TNF-α specific primers. Values are normalized with the housekeeping gene GA3PD. A: AFB, A+T: AFB + thalidomide(T), D: DHAR, D+T: DHAR+T, M: MLSA, M+T: MLSA+T.

3.3.5 Thalidomide-Induced Enhancement of TNF-α and TNF-α MRNA

In 11 of the 68 RR patients, stimulation of PBMC with AFB antigen resulted in enhanced production of TNF-α (Table 3.4). These patients were of the BL/RR type with only one BT/RR patient. The SI was also enhanced due to treatment with thalidomide except for patients 1398/02 and 360/03. Six out of 8 patients had a correspondingly enhanced expression of TNF-α mRNA.

Table 3.4. Characteristics of the Sub-Group of RR Patients with Enhanced TNF-α

ID	Age	Sex	RR Type	* % E or S, SI	* % E or S, TNF-α	* % E or S TNF-α mRNA
1329/02	60	F	BL/RR	-12	-12	-27
1398/02	45	M	BL/RR	26	-6	Not available
049/03	18	F	BL/RR	-69	-4	-88
165/03	60	F	BL/RR	-297	-6	Not available
318/03	18	M	BL/RR	-18	-1	24
347/03	40	F	BL/RR	-76	-28	Not available
360/03	20	F	BL/RR	8	-49	-47
383/03	55	M	BL/RR	-109	-76	-39
385/03	18	F	BL/RR	-109	-9	-13
405/03	70	M	BL/RR	22	-21	Not available
422/03	32	M	BT/RR	-118	-2	-167

% E: percent enhancement, % S: percent suppression

3.4 Discussion

Thalidomide has been shown to be effective in the treatment of pathologies associated with elevated TNF-α such as ENL (Sampaio, E.P. *et al*, 1993), and wasting syndrome in tuberculosis and HIV infections (Klausner, J.D. *et al*, 1996). This has been attributed mainly to the ability of thalidomide to suppress TNF-α (Sampaio, E.P. *et al*, 1993). There are, however, conditions such as toxic epidermal necrolysis where the rationale for using thalidomide to suppress TNF-α failed and an enhanced production of TNF-α was reported (Wolkenstein, P. *et al*, 1998).

In RR, TNF-α was elevated even more so than in ENL (Gru, G. *et al*, 1992) in lesions as well as in the serum, yet thalidomide lacked any effect against this condition**.** Previously it was observed among steroid treated patients with RR that thalidomide induced an enhancement of TNF-α when mononuclear cells were stimulated with integral *M. leprae* (Tadesse, A. *et al*, 2003). Non-steroid treated RR patients were studied to corroborate this observation and the influence of thalidomide on TNF-α in RR patients cells stimulated with mycobacterial antigens was assessed by measuring the expression of TNF-α mRNA in parallel with the synthesis of TNF-α.

Cells stimulated with mycobacterial antigens, in the absence of thalidomide, gave an enhanced proliferative response in RR patients compared to patients without reaction. This confirmed the frequently described transient augmentation of cell-mediated immunity or the enhanced delayed type hypersensitivity (DTH) response to *M. leprae* antigens in RR (Bjune, G. *et al*, 1976; Cooper, C.L *et al*, 1989). Thalidomide co-stimulated the proliferative responses in

RR patients to the MLSA and DHAR antigens, but not to the AFB. The immuno-modulatory action of thalidomide has been shown to vary depending on the stimulant, the cells stimulated and the signalling pathway involved. The thalidomide-induced enhancement of proliferation of antigen specific T-cells in this study was similar to studies where PBMC were stimulated by cross-linking of T-cell receptor with anti-CD3. In these studies, thalidomide acts as a co-stimulator resulting in enhanced proliferation of lymphocytes (Haslett, P.A.J. *et al*, 1998; Corral, L.G. *et al*, 1999; Marriot, J.B. *et al*, 2002).

Among the RR group in response to DHAR and MLSA antigens and when comparing antigen stimulated cultures to antigen and thalidomide treated cultures, thalidomide enhanced lymphocyte proliferation but significantly suppressed TNF-α production. This is in contrast to a previous report by Marriot JB *et al* 2002 who showed that enhanced production of TNF-α is correlated with enhanced lymphocyte proliferation. In their study T-cells were stimulated directly by using anti-CD3 antibody; whereas, in this study, antigens derived from *M. leprae* that are likely to require processing and presentation to T-cell were.

The effect of thalidomide on the expression of TNF-α mRNA in PBMC stimulated with the mycobacterial antigens was also assessed. Samples for the assessment of TNF-α and TNF-α mRNA were harvested at the same time but no correlation with a correspondingly consistent reduction of TNF-α mRNA with TNF-α was observed due to thalidomide treatment. When purified monocytes were stimulated with LPS, Moreira A., *et al* 1993 showed thalidomide to suppress TNF-α by reducing the half-life of TNF-α mRNA from 30 to 17 minutes. A likely explanation for this discrepancy is the varying effects of thalidomide related to the dominant cell

type and antigen used for stimulation (Marriot, J.B. *et al*, 2002). Alternative mechanisms of TNF-α suppression by thalidomide are blocking of NF-kB, a transcription factor involved in the regulation of several genes including TNF-α gene (Keifer, J.A. *et al*, 2001) and binding of thalidomide to α1-acid glycoprotein, a pro-inflammatory protein that belong to the acute phase proteins family (Turk, *et al*, 1996).

Previous observation made among 4 of 7 RR patients of a thalidomide-induced enhanced production of TNF-α in response to stimulation with AFB was again observed in 11 out of 68. When comparing the effect of thalidomide in AFB stimulated PBMCs from all 68 RR patients, thalidomide failed to act as a co-stimulant. But, it did act as a co-stimulant in the subgroup of 11 RR patients. In systems where thalidomide acts as a co-stimulator of T-cell activation, a subsequent induction of TNF-α was demonstrated (Marriot, J.B. *et al*, 2002). This co-stimulatory property of thalidomide is dependent on IL-2-mediated signalling indicating that T-cell activation rather than the direct effect of thalidomide resulted in increased production of TNF-α. One proof of IL-2-mediated signalling is an enhanced stimulation index in response to AFB in 8 of these 11 RR patients. Although the level of IL-2 was not measured in this study, an augmentation of IL-2 by thalidomide was demonstrated by Shannon *et al*, (Shannon, E.J. and Sandoval, F., 1995) and confirmed by Haslett *et al*, (Haslett, P.A.J. *et al*, 1998).

Cytokine secretion by T-cells is a transient process and requires activation of T-cells. Usually in T-cells, cytokine proteins and mRNA are not stored intra-cellularly but rather synthesized *de novo*. The transcription of these cytokines genes is highly regulated with most of their mRNAs having a short $T^{1/2}$ (Lohning, M. *et al*, 2002). An exception to this rule is TNF-α,

whose mRNA is stored in resting cells in an unspliced form called pre-TNF-α. T-cell stimulant like anti-CD3 and PMA result in massive release of TNF-α, which is due to an induction of transcription of TNF-α gene as well as enhanced splicing of accumulated pre-TNF-α mRNA (Yang, Y. *et al*, 1998). Thalidomide might alter TNF-α at a post-transcriptional level by influencing the splicing of existing pre-TNF-α mRNA. Makonkawkeyoon *et al*, (1993) showed that the addition of thalidomide increased the amount of PMA-induced TNF-α mRNA in ACH-2 cells- a T-cell line. If thalidomide acts as a co-stimulator as previously reported (Mariott, J.B. *et al*, 2002) it might also stabilize the mature TNF-α mRNA leading to increased production of TNF-α.

The thalidomide-induced augmentation of TNF-α mRNA and TNF-α that was observed in cultures was limited to stimulation with integral *M. leprae* (AFB). Processing of the integral *M. leprae* antigen (AFB) by macrophages and Schwann cells and presentation of certain specific peptides might be responsible for activation of T-cells and enhancement of TNF-α production in RR.

No striking *in vitro* parameter that can explain the absence of an effect of thalidomide in the treatment of RR was detected in this work. The pathogenesis of RR is the outcome of a complex interaction of antigen specific cell mediated immune responses and non-specific anti-inflammatory events. The differential expression of the *M. leprae* antigens lipoarabinomanan (LAM) and phenolic glycolipd-1 (PGL-1) in lesions of RR patients (Verhagen, C. *et al*, 1999) and the concordant association of lymphocyte proliferation to *M. leprae* with the degree of inflammation among RR patients (Bjune, G. *et al*, 1976) are two examples of this complexity.

Unique to leprosy is the homing of the bacilli into Schwann cells. These cells have been shown to process and present *M. leprae* to MHC-II restricted $CD4^+$ (Ottenhoff) and MHC-I restricted $CD8^+$ T-cells (Steinhoff, U. *et al*, 1988). This dual property of the Schwann cell makes it a candidate for direct lysis by cytotoxic T-cell and probably explains the intense neuritis characteristic of RR. In an experimental autoimmune encephalitis (EAE) model using rats, thalidomide prolonged the duration of EAE and enhanced the inflammation of sciatic nerve (Zhu, J. *et al*, 1998). Similar to this model of T-cell-induced neuritis, the use of thalidomide in the treatment of RR may result in an exacerbation of the reaction.

CHAPTER 4 THE EFFECT OF THALIDOMIDE ON THE VIABILITY OF INTRACELLULAR M. LEPRAE IN ACTIVATED MACROPHAGES

4.1 Introduction

Thalidomide is the treatment of choice for erythema nodosum leprosum (ENL) (Sheskin, J., 1980). The sequence of events, which precipitate ENL as well as the exact mechanism by which thalidomide arrest ENL has not yet been clearly delineated. The patients that may experience ENL during the course of their disease are those in the lepromatous end of the leprosy spectrum (lepromatous leprosy, LL and borderline lepromatous leprosy, BL) (Hastings, R.C., 1985). These patients are characterized by the presence of precipitating antibodies in their sera and a tremendous load of mycobacterial antigen inside macrophages (Ridley, M.J. and Ridley, D.S., 1983). As the likelihood of an episode of ENL increases after the initiation of treatment, it has been suggested that release of *M. leprae* antigens from macrophages may be a factor, which initiates ENL (Hastings, R.C., 1985). The released antigen may then complex with antibodies, initiating complement fixation and production of inflammatory cytokines like TNF-α.

Inhibition of TNF-α is one proposed mechanism by which thalidomide arrests ENL (Sampaio, E.P. *et. al*, 1991). However in a short open clinical study comparing the effects of thalidomide with other known inhibitors of TNF-α like pentoxifylline and prednisone, the authors concluded that thalidomide might have other targets in ENL (Moreira, A. *et. al*, 1998). 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) (Hastings, R.C., 1985).

RR is another type of hypersensitivity reaction in leprosy where TNF-α is detected in skin and peripheral nerve lesions (Khanolkar-young, S. *et. al*, 1995). Depending on experimental conditions, and the type of cells stimulated, thalidomide may enhance or suppress the synthesis of TNF-α (Shannon, E.J. and Sandoval, F., 1996). Previous work has recently shown that thalidomide enhanced TNF-α in cells from RR patients stimulated with integral *M. leprae* (Tadesse, A. *et. al*, 2003).

This report addresses a different tact in exploring the efficacy of thalidomide in treating ENL reactions. Could thalidomide actually be inhibiting the killing and breakdown of *M. leprae* and thereby inhibit the release of antigens? To study the effect of thalidomide on the fate of *M. leprae* in host macrophages, the viability of *M. leprae* residing in thalidomide-treated normal or IFN-γ-endotoxin activated mouse macrophages was assessed.

4.2 Materials and Methods

4.2.1 Source of M. Leprae

Freshly isolated *M. leprae* from footpads of nude mice was used throughout the experiments (Ramesh, N. *et al*, 1991). In each of the experiments, the viability of 4 x 10^7 acid-fast bacteria (AFB) was assessed by inoculating the *M. leprae* suspension into vials containing BACTEC™ PZA Test Medium (Becton Dickinson Diagnostic System, Sparks, MD). The cumulative $^{14}CO_2$ was measured daily for 7 days.

4.2.2 Mouse peritoneal macrophages

Macrophages were isolated from the peritoneal cavities of retired female Swiss Webster breeder mice (Simonsen Labs, Gilroy, CA). Briefly, 15 ml of cold heparinized phosphate

buffered saline (PBS) was injected into the peritoneal cavity of each mouse. The peritoneal exudates was collected and washed. The cell pellet was resuspended in 10 ml of Complete Medium (RPMI 1640, 50 μg/ml ampicillin, 2 mM glutamine, 10% fetal bovine serum). Cells were counted and adjusted to 4 x 10^6 per ml. The cell suspension was dispensed at 0.5 ml/well in 24-well plates containing LUX ® plastic cover slips (Miles Laboratory, Naperville, IL). The plates were incubated overnight in a humidified incubator at 37 ^{0}C, 5% CO_2. Non-adherent cells were washed off by dipping coverslips in sterile PBS and draining excess liquid on sterile gauzes. The coverslips were placed in another 24-well plate containing 0.5ml/well Complete Medium.

4.2.3 Treatment Conditions

Thalidomide was provided by Celgene (Warren, NJ) and was dissolved in a solution of 0.85% NaCl, pH 3.0 (Acid Saline, AS) as described previously (17). A stock solution of 50 μg/ml was aliquoted and kept in –70 ^{0}C freezer. Shortly before each experiment, dilutions of thalidomide at concentrations of 0.625 μg/ml, 1.25 μg/ml, 2.5 μg/ml and 5 μg/ml were done in AS. Based on the previously estimated half-life of 8.0 hrs for thalidomide in tissue culture medium at 37^{0}C (Gunzler, V. *et al*, 1986), cultures were replenished with 50 μl of AS containing thalidomide or 50 μl of AS daily to maintain an estimated concentration of ≥ 1.0 μg/ml of un-hydrolyzed thalidomide in the 5 μg/ml treated cultures.

4.2.4 Activation of Macrophages - Infection - Treatment with Thalidomide

Monolayer cells on coverslips were incubated for 8 hours with 200 Units/ml of mouse recombinant IFN-γ (R and D Systems Inc., McKinley, MN) and 2.0 ng/ml of endotoxin (Sigma,

St Louis, MO). Coverslips were washed as described previously and placed in fresh Complete Medium. Viable *M. leprae* (4×10^7) was added into the resting or activated macrophage monolayer at an estimated 20:1 bacilli: macrophage. After an overnight incubation at 33^0C, non-phagocytosed bacilli were removed by washing the coverslips. The infected macrophages were incubated with the different concentrations of thalidomide and controls. Drug and media was replenished daily for 7 days in the non-activated cultures and 3 days in the activated cultures. A shorter exposure to thalidomide in the activated macrophages was necessary as the cells were observed to detach after 3 days.

4.2.5 Assessment of M. Leprae Viability: Radiorespirometric Assay

A modified Buddemeyer radiorespirometric assay was used to assess the viability of *M. leprae* released from macrophage as described previously (Ramesh N. et. al., 1991). The coverslip containing the macrophage monolayer was placed in 400 µl of 0.1 N NaOH solution. Three hundred µl 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-^{14}C] palmitic acid and 50 µg/ml ampicillin. The vials with loosen cap were placed within PolyQ polyethylene vials containing a dried strip of Whatman DE42 filter paper (Whatman, Inc., Clifton, NJ). The paper had been dipped in a mixture of Liquifluor PPO-POPOP [2,5 diphenyloxazole-1, 4-bis (5-phenyloxazoly) benzene] toluene concentrate (New England Nuclear, Boston, MA) 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 cap were incubated at 33^0C. The generation of radioactive

$^{14}CO_2$ was determined daily by reading in a Beckman model LS-6000IC liquid scintillation counter. Data are presented as cumulative counts per minutes (CPM).

4.2.6 Assessment of TNF-α in Activated Macrophages

Twenty-four hours after thalidomide treatment of activated and infected macrophages, an aliquot of the culture supernatant was removed and frozen at -70 ^{0}C. TNF-α was measured by using a mouse TNF-α immunoassay kit according to the manufacturer's specification (Quantikine, R&D Systems, Minneapolis, MN). The amount of TNF-α in each sample was determined from the standard curve using KCjunior Version 1.31.2 (Bio-Tek Instruments Inc, Vermont).

4.2.7 Nitrite Assay

NO_2^- levels in culture supernatants were determined as described previously (Drapier, J.C. *et al*, 1988). Briefly, Griess reagent was prepared and dilutions of $NaNO_2$ solution were used as a standard. All samples were assayed in duplicate. Samples were culture supernatants of activated macrophages treated with thalidomide for 24 hours and collected as described for TNF-α. 50 µl of standard and samples were dispensed into wells of a 96 well flat bottom tissue culture plate. Duplicate blank wells with media alone were also included in the experiment. 100 µl of Griess reagent was added into each well. The plate was mixed by gently tapping and incubated at room temperature for 20 minutes. Absorbance was read at 543 nm. The amount of NO in each sample was determined from the standard curve using KCjunior Version 1.31.2 (Bio-Tek Instruments Inc, Vermont).

4.3 Results

4.3.1 Assessment of Metabolic Activity of Intracellular M. Leprae

Prior to infecting the macrophages the viability of *M. leprae* used for each experiment was tested by inoculating into Pyrazinamide (PZA) test medium. At day 7, the cumulative mean +/- SD from 4 experiments was 36,344 +/- 3,801.

Bacilli recovered from non-activated macrophages had a higher $^{14}CO_2$ level (CPM range day 1- day 7: 2989 +/- 1391 to 7582 +/- 5481) compared to IFN-γ and LPS activated macrophages (CPM range day 1- day 7: 1488 +/- 324 to 3318 +/- 1129) (Figure 4.1). The difference between activated and resting macrophages was significant for all days except day 7 (unpaired T test, GraphPad Prism V 4.0).

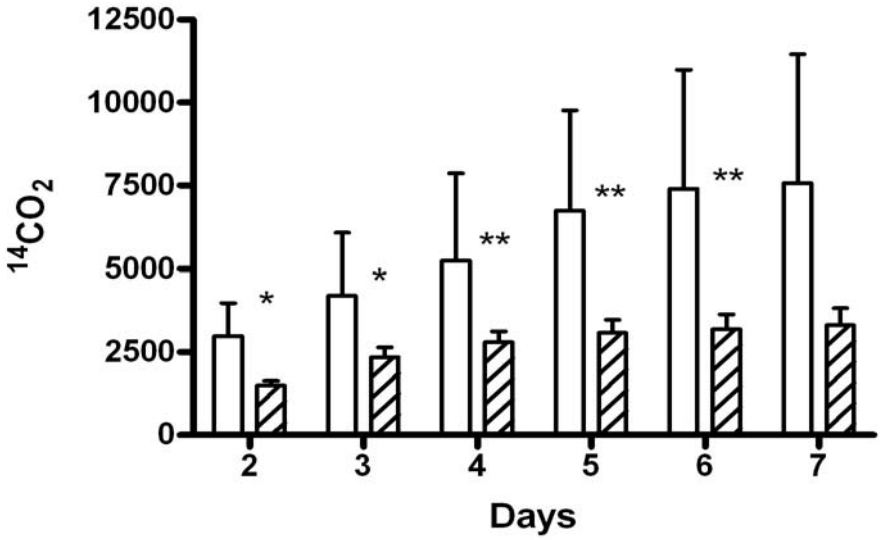


Figure 2. Viability of M. Leprae in Activated Versus Resting Macrophages. Resting (open bar) and IFN-g -LPS-activated (hatched bar) mouse peritoneal macrophages were infected with M.leprae for 24 hours. Bacilli released from lysed macrophages were incubated in PZA test medium and14CO2 was quantitated daily for 7 days. Mean CPM+/- SD of 3 to 8 replicates. Unpaired t test,* $p< 0.05$,** $p< 0.01$.

4.3.2 Effect of Thalidomide on Viability of M. Leprae

Over a 7-day period, a gradual daily increase in $^{14}CO_2$ was observed in the AS-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 4.2 summarizes the cumulative mean CPM +/- S.D from 3 separate experiments on day 7 for thalidomide at 5 μg/ml. Comparison of the drug-free control group to the thalidomide treated groups in non activated as well as activated macrophages revealed no significant difference (unpaired T test).

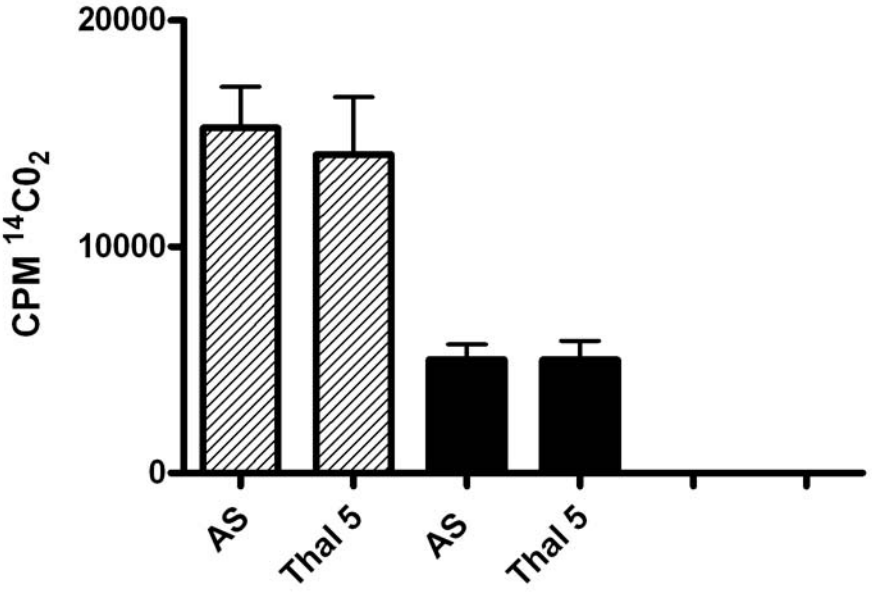


Figure 4.2. Effect of Thalidomide on Viability of M. Leprae Recovered From Non-Activated and Activated Mouse Peritoneal Macrophages. Infected mouse peritoneal macrophages were treated with 5 μg/ml of thalidomide (Thal 5) and acid saline (AS). Cells were lysed and the viability of released bacilli was assessed by determining counts per minute (CPM) of cummulative $^{14}C0_2$ released. Open bars represent non-activated macrophages, closed bars represent activated macrophages. Means +/- SD from 3 separate experiments.

4.3.3 TNF-α and Nitrite Levels in Activated Macrophages

After 24 hours exposure to thalidomide, no significant changes in TNF-α and NO_2^- was observed compared to media or AS controls. Although TNF-α levels were higher in thalidomide treated cells (24.8 pg/ml +/- 3.47) compared to AS-treated ones (18.12 pg/ml +/- 8.2), it was not statistically significant (Figure 4.3).

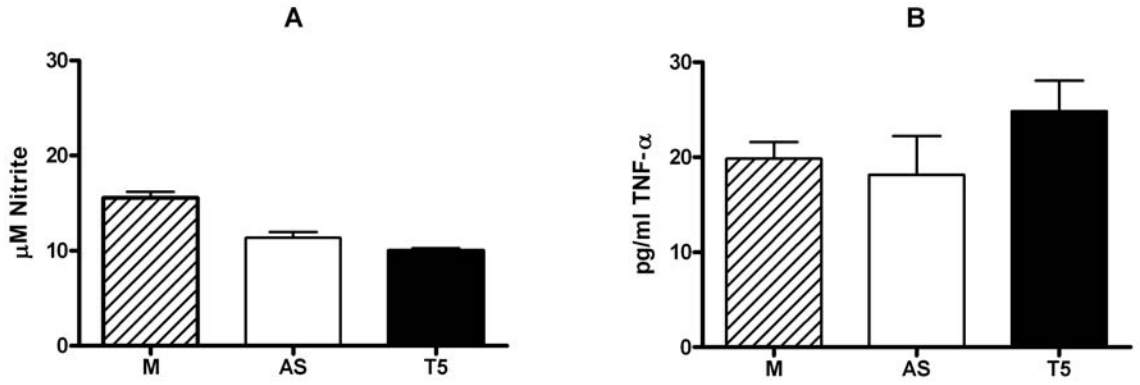


Figure 4.3. Nitrite and TNF-α Levels in M. Leprae-Infected LPS-IFN-γ-Activated Macrophages. Activated mouse peritoneal macrophages were infected with viable *M.leprae* and treated with thalidomide at 5 µg/ml for 24 hours. Nitrite (A) and TNF-α (B) in culture supernatants were quantitated. Mean and SD for 4 replicate wells controls; M= media, AS= acid saline and T5= thalidomide.

4.3.4 Morphological Appearance of Peritoneal Macrophages

Breakdown products of thalidomide have been described to inhibit the attachment of cells to plastic surfaces (Braun, A.G. and Weinreb, S.L., 1984). For visual inspection of attachment of cells, coverslips from each treatment were stained with quik-Diff® (Dade Diagnostics, Aguada, PR), dried and mounted on microscopic slides.

The density of the cells appears to be the same in all the treated groups. Activated cells had a spindle-like shape and are clearly different than non-activated cells. Thalidomide treated activated macrophages were not morphologically different than AS treated activated macrophages (Figure 4.4).

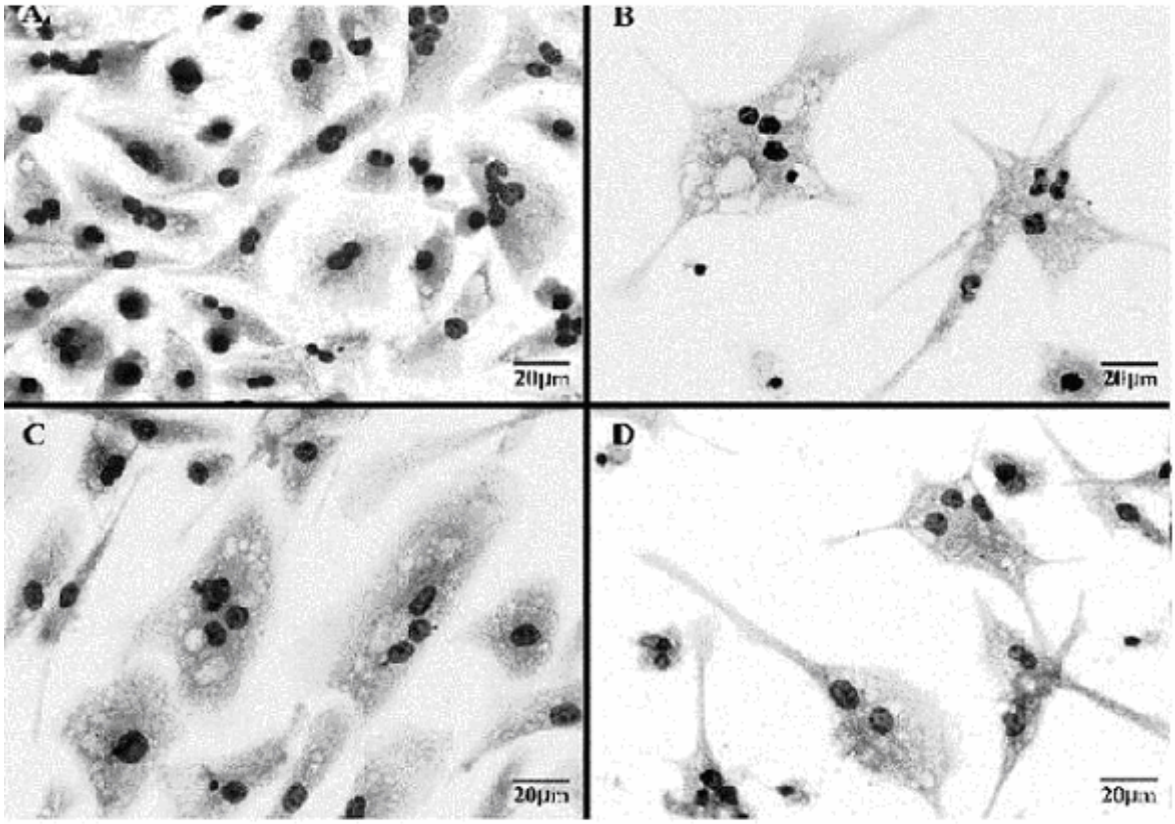


Figure 4.4. Monolayers of Mouse Peritoneal Macrophages.
(A) Media control, non-activated
(B) LPS + IFN-γ-activated and M. leprae Infected
(C) LPS + IFN-γ-activated, *M. leprae* infected and thalidomide-treated
(D) LPS + IFN-γ-activated, *M. leprae* Infected and acid saline-treated

4.4 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 bacteria (Sibley, D. *et al*, 1987). ENL is characterized by the detection of granulated beaded bacteria indicating a massive release of *M. leprae* by macrophages (Ridley, M.J. and Ridley, D.S, 1983). 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 facilitates remodeling of inflamed tissue. When TNF-α is produced in lesser quantities and in a timely fashion, it mediates a variety of anti-inflammatory events promoting wound healing and remodeling of injured tissue (Vlassara, H. *et al*, 1988) and suppressing neutrophil migration (Otsuka, Y. *et al*, 1990).

The anti-inflammatory and immunomodulatory properties ascribed to thalidomide offer multiple potential sites of action in arresting ENL. In this work we have tried to study whether thalidomide modulates the anti-microbial action of host macrophages against intracellular *M. leprae* as a possible prelude to inhibiting the release of mycobacterial antigen.

Non-activated mouse peritoneal macrophages infected with *M. leprae* were treated with 0.625-5.0 μg/ml of thalidomide 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, considered to be, more relevant to that achieved in the treatment of ENL.

These findings concur with previous studies on the *in vitro* as well as *in vivo* anti-microbial action of thalidomide against other pathogens. Although thalidomide was effective in alleviating clinical symptoms associated with *Mycobacterium Avium Complex* (MAC) in HIV patients (Bouza, E.R. *et al*, 1992), *in vitro* it was unable to kill laboratory-cultured strains of MAC (Vicente, T. *et al*, 1993). Thalidomide is also effective against microsporidial diarrhea, but when tested *in vitro*, it failed to kill intracellular microsporidia (Ridoux, O. *et al*, 1999). In murine experimental tuberculosis (Moreira, A.L. *et al*, 1997) and BCG infection (Fazal, N.L. *et al*, 1988) 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 (Krahenbuhl, J.L. *et al*, 1990). The peritoneal macrophages were co-stimulated with IFN-γ and LPS. In the absence of thalidomide, the cumulative $^{14}CO_2$ from activated macrophages was higher than that of non-activated macrophages confirming previous findings where activated mouse peritoneal macrophages killed or inhibited *M. leprae* more effectively than non-activated macrophages (Adams, L.B. *et al*, 2000). When tested in this activated macrophage system, thalidomide did not modify the viability of *M. leprae* in a significant manner.

The effect of thalidomide on TNF-α produced in IFN-γ- LPS- activated-*M. Leprae*-infected macrophages were also examined. Although statistical analyses revealed no significant

difference between TNF–α level from thalidomide and from AS treated cells, a higher level of TNF-α was observed in the thalidomide treated group.

Reactive nitrogen intermediates, especially nitric oxide (NO) constitute a major anti-microbial effector armature of activated mononuclear phagocytes with activities against a broad spectrum of pathogens (Hibbs, J.B. *et al*, 1987). A reduction in the viability of *M. leprae* occurs when activated mouse peritoneal macrophages produce a high level of NO (Adams, L.B. *et al*, 2000). In this study, although the level of NO_2^- produced was similar to that reported by others, thalidomide failed to influence the production of this molecule.

This work showed that thalidomide does not possess a direct anti-microbial action against intracellular *M. leprae* in normal or activated macrophages. Nor did it alter the production of TNF-α 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.

CHAPTER 5 THE EFFECT OF THALIDOMIDE ON THE INTEGRITY OF PLASMA CELL MEMBRANES

5.1 Introduction

The anti-inflammatory properties of thalidomide are demonstrated by its beneficial effects in the treatment of inflammatory conditions like erythema nodosum leprosum (ENL), which may occur in leprosy patients, and apthous ulcers which may occur in AIDS patients (Zwingenberger, K. and Wendt, S., 1996; Tseng, S.P. *et al*, 1996). The sequences of events that precipitate these inflammatory conditions as well as the molecular and biochemical basis for the anti-inflammatory properties of thalidomide are not understood. A common finding in the lesions of patients experiencing ENL or apthous ulcers is an influx of polymorphonuclear neutrophils (PMN) (Mabalay, M.C. *et al*, 1964; Burns, R.A. *et al*, 1985). Reactive oxygen species (ROI) produced by the PMN are important in intracellular killing of pathogens; however these ROI can also combine with enzymes released from lysosomes causing tissue damage and inflammation of the surrounding extra-cellular environment (Parham, P., 2000).

Stabilization of lysosomal membranes by thalidomide could be a mechanism for its anti-inflammatory action in ENL. Hastings showed that *in vitro* thalidomide prevented the release of B-glycerophosphatase from lysosomes isolated from rat livers. The drug was effective at concentrations similar to those achieved in the blood of leprosy patients effectively treated for their ENL (Hastings, R.C., 1971). Based on this interesting finding, the effect of thalidomide on cell membrane integrity was studied.

For stabilization of erythrocyte membrane, we used a modification of Dacie's osmotic fragility test (Dacie, J.V., 1975). This test measures hemoglobin released from lysed RBCs after

their exposure to hypotonic solutions of sodium chloride (NaCl). The *in vitro* as well as *in vivo* effects of thalidomide on hemolysis of RBCs from healthy humans were investigated. To probe the effect of thalidomide on the stability of membranes of inflammatory cells, human PMNs and the human-monocytic cell line THP-1 were employed. The detection of lactic acid dehydrogenase (LDH) released in culture supernatants as a result of leakage was used as an indicator of membrane dysfunction.

5.2 Materials and Methods

5.2.1 Preparation of Thalidomide

The drug for *in vitro* and *ex vivo* use was provided by Celgene and dissolved in acid saline (AS) as previously described (Shannon, E.J., et Al, 1997). AS was chosen as the solvent for thalidomide rather than the commonly used DMSO. This is due to reported interference of DMSO with cell metabolism and membrane integrity resulting in cellular damage (Penninck, F. *et al*, 1983).

5.2.2 In Vitro Thalidomide Treatment

Ten ml of peripheral blood was collected in a Na-heparin vacuum tube from 12 healthy adult volunteers by venipuncture. The blood sample was equally divided into 2 tubes. One tube of blood was centrifuged at 913 x g, at room temperature for 10 minutes. The plasma was removed and replaced with an equal volume of AS (normal saline, pH 3.0). The tube was labeled Washed RBC. The second tube containing the plasma was labeled Not Washed RBC.

5.2.3 Ex Vivo Thalidomide Treatment

Sixteen healthy male volunteers were included in this part of the study. Twelve received drug and 4 received placebos. A blood sample was taken prior to ingestion of a 100 mg tablet and after the subject has consumed a cumulative dose of 600 mg of thalidomide given as one 100 mg tablet every 4 hours. The blood was directly used (at 10 µl/well) in osmotic fragility experiments.

5.2.4 Osmotic Fragility

For convenience, Dacie's method (Dacie, J.V., 1975) of osmotic fragility was modified to a micro-scale assay. Briefly, a 100 ml of a stock buffered NaCl solution containing 9 g of NaCl; 1.365 g of Na_2HPO_4 and 0.215 g of $NaH_2PO_4.H_2O$ was prepared. A 1% solution of buffered NaCl was prepared by diluting the stock NaCl in deionized water. In a 96-well V-bottom microtiter plate, the dilution scheme in Table 5.1 was used to prepare hypotonic solutions.

Table 5.1. Dilution Scheme of NaCl and Water to Give the Various % Salt Concentrations

Dilution	1% buffered NaCl (µl)	Deionized water (µl)	Final NaCl Concentration %
1	200	0	1.00
2	170	30	0.85
3	150	50	0.75
4	130	70	0.65
5	120	80	0.60
6	110	90	0.55
7	100	100	0.50
8	90	110	0.45
9	80	120	0.40
10	70	130	0.35
11	60	140	0.30
12	40	160	0.20
13	20	180	0.10
14	0	200	0.00

The final volume in each well was 200 µl, 3 to 6 replicate wells were used for each concentration of salt. Ten µl of ThalW or ASW or Thal or AS treated blood was added into each well and the plate was incubated for 30 minutes at room temperature. At the end of incubation, the plates were spun at 913 x g, at room temperature (RT) for 5 minutes. 100 µl of supernatant was carefully transferred from each well into wells of a clean flat bottom microtiter plate. The extent of hemolysis was determined by reading the plate in an ELISA reader (Synergy HT Multi-detection microplate reader, Biotek) at 550 nm. Optical density (OD) readings were analyzed by using the software KC4 Version 3.0 ELISA Program (Biotek, Vermont).

The percentage of hemolysis was calculated from the following formula:

$$\% \text{ Hemolysis} = \frac{\text{OD of the specific NaCl concentration}}{\text{OD of 100\% hemolysis (0\% NaCl)}}$$

For each drug treatment, osmotic fragility curves were prepared by plotting % hemolysis versus % NaCl in GraphPad Prism4 (GraphPad Software Inc.San Diego, CA). Statistical analysis (non parametric Mann Whitney test) was done in Prism 4.

5.2.5 THP-1 Cell Culture

The acute monocyte-like cells THP-1 were purchased from American Type Culture Collection (ATCC TIB 202, Rockville MD). The cells were maintained in complete media composed of RPMI-1640 containing 10 mM Hepes buffer, 1 mM sodium pyruvate, 1 mM L-glutamine, 4.5 g/l D-glucose, 0.05 µM 2-mercaptoethanol and 10% fetal bovine serum. Cells were kept in tissue culture flasks in a humidified incubator at 37^0C, 5% CO_2.

5.2.6 THP-1 Membrane Fragility Experiments

Cells were pelleted by centrifugation at 250 x g, 4^0C, for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1.0 ml AS. Viability, as checked by trypan blue, ranged between 95 – 99 %. Cells were enumerated and split into two tubes. In one tube labeled as Thal-THP-1, thalidomide was added at 4 μg/ml; whereas, in another tube, an equivalent volume of AS was added and the tube was labeled as A/S-THP-1. The tubes were placed on a rocker for 30 minutes at room temperature (RT). The cell suspensions were then centrifuged at 250 x g for 10 minutes, 4^0C. Supernatants were discarded and the pellet of each tube was resuspended in 1.0 ml AS. Cells were added in each well of plates containing dilutions of salt as described in Table 1. The plates were incubated at RT for 30 minutes. After centrifugation 230 x g for 15 minutes at RT, supernatants were harvested and stored at – 80^0C.

5.2.7 Isolation of Neutrophils and Fragility Experiments

The dextran sedimentation technique, as described in the Current Protocols in Immunology, was used (Wiley and Sons., 2003). Briefly, whole blood was mixed with an equal volume of 3% dextran solution. After 20 minutes of incubation at RT the upper layer was collected in a clean tube. The suspension was pelleted at 250 x g, 5^0C for 10 minutes. The pellet was resuspended in normal saline and mononuclear cells were removed by Ficoll-Hypaque gradient centrifugation. The RBC/neutrophil pellet was subjected to hypotonic lysis to remove the RBC. After the final wash the neutrophils were resuspended in PBS containing 10 mM D-glucose solution. The cells were pre-treated for 30 minutes with either thalidomide at 4.0 μg/ml or acid saline control. Cells were then exposed to various salt solutions as mentioned in Table 1.

After 30 minutes of incubation at RT, plates were centrifuged at 250 x g at RT for 10 minutes; 100 µl of supernatant was transferred from each well in a plate and frozen at –70 ^{0}C.

5.2.8 LDH Assay

Supernatants were thawed and dispensed at 50 µl/well in microtiter plates. The Cyto Tox 96 ® non-radioactive cytotoxicity assay (Promega, WI, USA) was used to assess the LDH presence in the supernatants. The substrate was reconstituted in assay buffer and 50 µl of this substrate mix was added into wells containing the supernatants. The plate was covered and incubated in the dark, at RT for 30 minutes. The reaction was stopped with 50 µl /well of stop solution and absorbance was determined at 490 nm. . The intensity of color produced is directly proportional to the concentration of LDH in the culture supernatants. The OD obtained was used to calculate the % LDH from the following formula:

$$\% \text{ LDH} = \frac{\text{OD of experimental wells.}}{\text{OD at maximum lysis (i.e. 0\% NaCl)}} \times 100$$

5.3 Results

5.3.1 Thalidomide Stabilized the Erythrocyte Membrane

In blood that was washed and at most of the salt concentrations tested, a significant reduction in % lysis was observed in thalidomide treated cells compared to AS treated controls. In blood that was not washed, no significant difference was obtained between drug and AS treated blood (Figure 5.1).

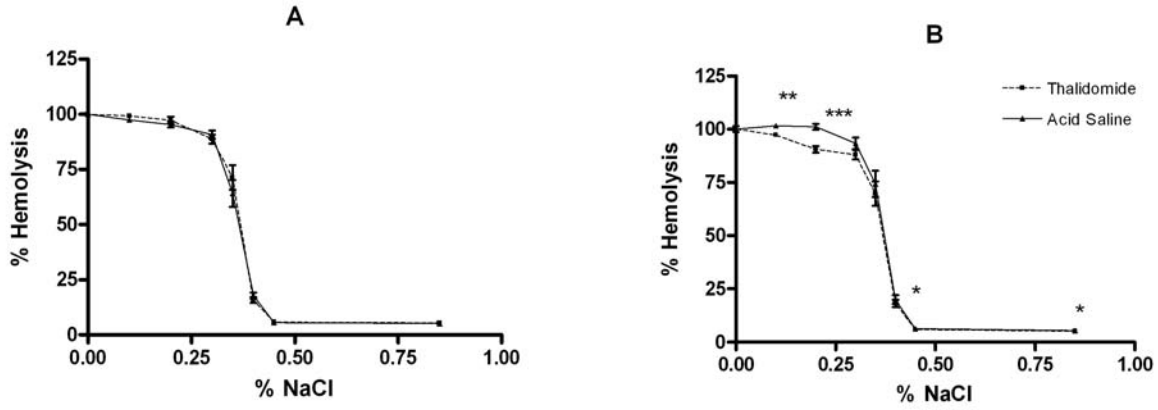


Figure 5.1. Osmotic Fragility Experiment of Washed Versus Not Washed RBC. Not washed blood (A) or washed blood (B) was incubated for 30 minutes with thalidomide or acid saline. Osmotic fragility experiment was done. The % hemolysis of RBC was plotted against the different hypotonic NaCl solution Values represent average +/-standard deviation of 5 experiments containing 4 replicate wells per treatment. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Mann-Whitney U test).

5.3.2 Effect of Thalidomide on LDH Release by Neutrophils

At 0.05 % NaCl, the median % LDH of thalidomide treated cells was markedly lower than that of acid saline treated; however, it was not significant ($p = 0.1359$). At all salt concentrations, no significant difference in LDH release was obtained between drug and solvent treated PMN (Figure 5.2).

5.3.3 Effect of Thalidomide on LDH Release by THP-1 cells

Although the median % LDH from thalidomide-treated cells was slightly lower than that of acid saline treated cells, especially at 0.2 % salt concentration, no significant difference was obtained between drug and control groups (Figure 5.3).

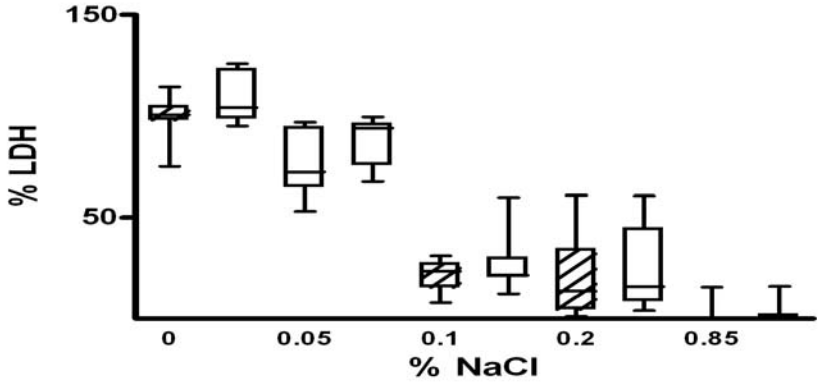


Figure 5.2.**Lactate Dehydrogenase (LDH) Release Assay of Thalidomide-Treated Neutrophils (PMN).** PMNs, isolated from 3 healthy subjects, were pre incubated with thalidomide (hatched box) or acid saline (open box) and exposed to different concentrations of buffered NaCl

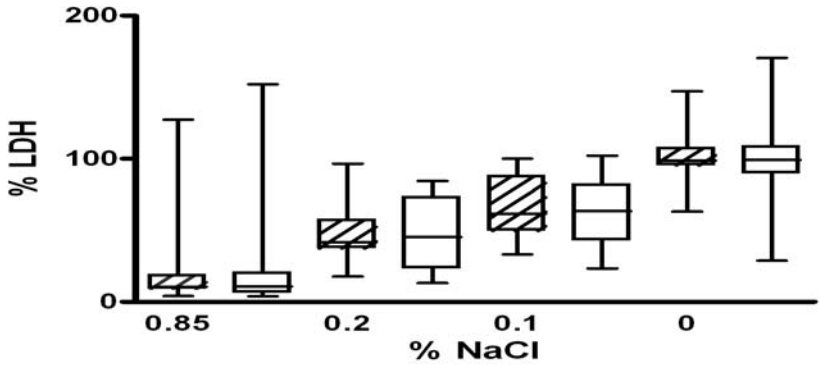


Figure 5.3. Lactate Dehydrogenase Release Assay of Thalidomide-Treated THP-1 Cells. THP-1 monocyte-like cells were incubated with thalidomide (hatched bar) or acid saline (open bar) and subjected to solutions with different salt concentration. Cytotoxicity was measured by quantifying the amount of LDH released. .

5.3.4 Influence of Ingested Thalidomide on Osmotic Fragility of RBC

The *ex vivo* osmotic fragility experiments showed no significant difference between pre-thalidomide and post-thalidomide. The usual pattern with increased lysis at low salt concentration and gradual decrease in lysis as isotonic normal saline was approached in all experiments (Figure 5.4).

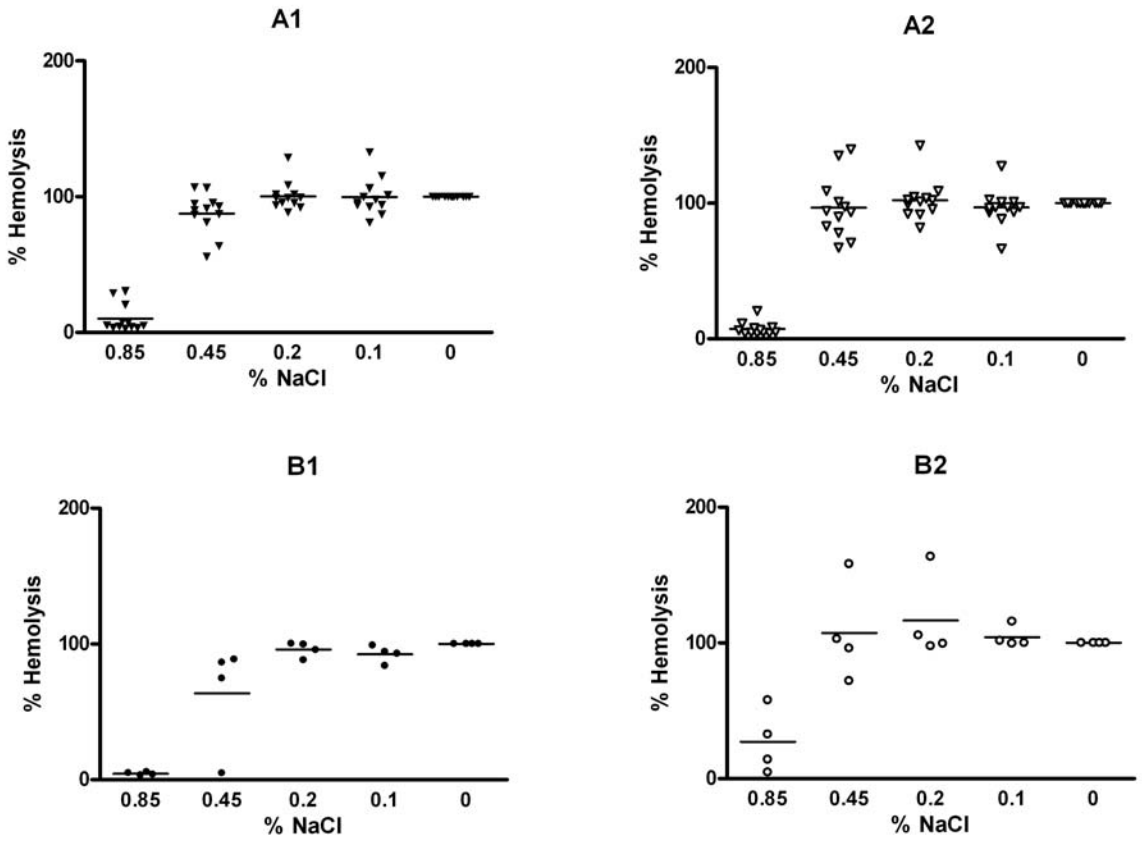


Figure 5.4. Osmotic Fragility of RBC from Subjects Treated with Thalidomide In Vivo. Blood was drawn from 12 subjects before (A1) and after (A2) intake of thalidomide. The placebo group of 4 subjects is illustrated as (B1) and (B2).

5.4 Discussion

The beneficial effect of thalidomide in the treatment of inflammatory conditions has been well established. But no definitive mechanism for this anti-inflammatory property has been obtained so far. A characteristic common to such conditions treated by thalidomide is an influx of PMNs at the site of inflammation, resulting in tissue damage (Mabalay, M.C. *et al*, 1964; Burns, R.A. *et al*, 1985).

A straightforward hypothesis for thalidomide's anti-inflammatory mechanism may be a perturbation of membranes on cells with immune potential. It has been shown that thalidomide did not alter mononuclear cell surface molecules like CD4, CD5, CD2, CD8, HLA-DR, HLA-A, B and C (Shannon, E.J. *et al*, 1994). Inhibition of functions of inflammatory cells like PMN phagocytosis and/or perturbation of membranes inhibiting the oxidative burst may also be another possibility. Thalidomide has failed to influence the oxidative burst of neutrophils as shown in chemiluminescence as well as in NBT reduction assays (Barnhill, R.L. *et al*, 1984; Golhman-Yahr, M. *et al*, 1978). This drug showed a bimodal activity with suppression of phagocytosis of latex beads by PMNs at a higher concentration and enhancement at a lower concentration (Barnhill, R.L. *et al*, 1984). But, it failed to affect the phagocytosis of larger particles like zymosan (Hastings, R.C. *et al*, 1978).

The initial step in the oxidative burst event is the reduction of oxygen to O_2^- at the expense of NADPH. This reaction is catalyzed by neutrophil membrane-associated enzymes known as NADPH oxidase. Agents that perturb the integrity of the PMN and/or the lysosome membranes

have been demonstrated to significantly reduce the production of reactive oxygen species (Wiles, M.E *et al*, 1994; Hastings, R.C., 1971). Membrane stabilization of neutrophils as well as other eukaryotic cells as a possible mechanism of anti-inflammatory role exerted by thalidomide was investigated.

Certain drugs like the macrolide antibiotics erythromycin and clarithromycin are shown to exert their anti-inflammatory action by stabilizing PMN membranes (Eriksson, T. *et al*, 1997). Thalidomide stabilized the RBC membrane as seen by the reduction in the percentage hemolysis of RBC exposed to low salt concentrations. This stabilization was confined to washed blood only. Thalidomide failed to affect the integrity of RBCs from blood that was not washed. This could be due to binding of thalidomide to proteins like human serum albumin and effectively depleting the concentration of thalidomide in the plasma (Eriksson, T. *et al*, Al., 1997). However, concentrations of thalidomide as high as 16 μg/ml in not washed RBC did not alter the integrity of the membranes (data not shown). Washing would remove these proteins increasing the interaction of the drug with RBC membranes.

The ex *vivo* study of osmotic fragility on blood samples from human subjects before and after ingestion of 100 mg/ml thalidomide tablets, revealed no significant difference in the resilience of the RBC to the hypotonic solutions before and after intake of thalidomide. The concentrations of unhydrolysed thalidomide under this steady state condition are likely to be between 2 and 4 μg/ml (Eriksson, T. *et al*, 1997). Partial interaction of thalidomide with soluble proteins might have hindered the stabilization effect of thalidomide.

Lactate dehydrogenase, a stable cytosolic enzyme released during cell lysis, was used to assess the effect of thalidomide on neutrophils and THP-1 cell membranes. There was no significant difference between thalidomide-treated neutrophils compared to AS treated-ones. The membrane stabilizing property of macrolide antibiotics is strongly associated with significant reduction in the oxidative burst by PMN (Theron, A.J. *et al*, 2000). The lack of stabilizing effect on PMN by thalidomide in this study is in concordance with previous findings where thalidomide failed to affect the production of reactive oxygen intermediates by neutrophils (Barnhill, R.L. *et al*, 1984; Golhman-Yahr, M. *et al*, 1978).

To assess the effect of thalidomide on monocyte/macrophage membranes the monocyte like cell THP-1 was used. Although thalidomide was previously reported to suppress the production of ROI by activated human monocyte in a chemiluminescence assay (Barnhill, R.L. *et al*, 1984), a difference between drug treated and solvent treated THP-1 cells was not seen. This confirms other studies that the anti-inflammatory mechanism of thalidomide is not explained by events associated with the modulation of superoxide anions.

CHAPTER 6 CONCLUSION

The objectives of this work were to study the immunomodulatory, anti-inflammatory and anti-microbial properties of thalidomide in relation to leprosy. Elucidating the mechanism behind the dramatic effect of thalidomide in the treatment of ENL would facilitate an understanding of the cause(s) of ENL and the development of non-teratogenic analogues that can be used in the treatment of ENL.

The influence of thalidomide on the immune response of leprosy patients' cells, with ENL and RR or without reactions, to *M. leprae*-specific antigens was assessed. In the main phase of the study lymphocyte proliferation assays and the assessment of the synthesis of TNF-α were performed. The effect of thalidomide on these two parameters was controversial. Thalidomide has been previously reported to suppress TNF-α (Sampaio, E.P., *et al* 1991) or enhance TNF-α (Shannon, E.J. and Sandoval, F., 1996). In lymphocyte proliferation studies, thalidomide enhanced (Marriott, J.B. *et al*, 2002; Corral, L.G. *et al*, 1999 Haslett, P.A.J. *et al*, 1999), suppressed (Keenan, R.J. *et al*, 1991) or had no effect (Günzler, V. *et al*, 1986 Santos-Mendoza, T. *et al*, 1996; Shannon, E.J. and Sandoval, F., 1995). In some of these studies, the method of thalidomide preparation was not described or used procedures that indicate probable degradation of thalidomide. In other studies, concentrations as high as 50 μg/ml were used. In this study, thalidomide was prepared with an awareness of its susceptibility to hydrolysis and used in concentrations equivalent (i.e. 4.0 μg/ml) to that achieved in the plasma of patients treated for ENL.

In the first phase of the study, with the exception of a significant enhancement of proliferative responses to DHAR antigen in RR patients, thalidomide failed to modify the ability of cells to incorporate ^{3}H-Thymidine. Thalidomide suppressed TNF-α in response to DHAR in healthy controls. Patients with ENL did not respond well in the assays to the AFB, DHAR or PPD antigen preparations. The majority of these patients were being treated with prednisone and their cells were probably in a hyporeactive or anergic state. Interestingly, in 4 of 7 RR patients, thalidomide enhanced the production of TNF-α in response to AFB. Some of these RR patients were under steroid treatment. This prompted further investigation of this observation among non-steroid-treated RR patients.

In a second phase of the study, a significant enhancement in proliferation in response to DHAR and the cytosolic antigen MLSA was observed in non-treated RR patients. In the RR group as well as the non-reactional leprosy controls and the healthy controls, TNF-α was significantly suppressed due to thalidomide treatment. This confirmed a recently reported co-stimulatory property of thalidomide (Marriott, J.B. *et al*, 2002), which might explain the absence of effect against RR. As in the first phase of the study, 11 of 68 RR patients responded to treatment with thalidomide and AFB, with enhanced lymphocyte proliferation, enhanced expression of TNF-α mRNA and enhanced the synthesis of TNF-α.

In a third phase of this work, the effect of thalidomide on the viability of *M. leprae* was assessed. Histopathologic studies of ENL have indicated the abundant presence of granulated *M. leprae* in lesions. The mechanism behind the release of *M. leprae* antigen from macrophage from certain lepromatous leprosy patients is not understood. Whether thalidomide interferes with the

viability of intracellular *M. leprae* and thereby may retard the release of *M. leprae* antigens was investigated. Mouse peritoneal macrophages non-activated or IFN-γ-LPS-activated and infected with *M. leprae* were treated with thalidomide in culture. This drug failed to affect the viability of intracellular *M. leprae* in a significant manner in the non-activated or activated macrophages. Thalidomide did not influence the production of neither TNF-α nor NO_2^- by these activated and infected macrophages.

Thalidomide had been shown to stabilize lysosomal membranes derived from rat liver homogenates. This property of thalidomide was proposed as its possible anti-inflammatory mechanism in the treatment of ENL (Hastings, R.C. 1971). In the last phase of this study the effect of thalidomide on the plasma membranes of neutrophils, THP-1 cells and red blood cells was studied. Thalidomide failed to protect the plasma membrane of neutrophils and THP-1 cells from osmotic lysis. *In vitro*, thalidomide stabilized RBC in blood depleted of plasma but it failed to do so in whole blood and in the blood of normal males ingesting thalidomide. Overall thalidomide did not alter the viability of *M. leprae*, nor the integrity of the plasma membrane of inflammatory cells. It could enhance or suppress *M. leprae* antigen-induced synthesis of TNF-α.

Interestingly, in 15 of 75 RR patients cells stimulated with AFB, thalidomide acted as a co-stimulant enhancing cell proliferation, synthesis of mRNA for TNF-α and TNF-α. Thalidomide's enhancing effect on TNF-α in RR appears to be dependent on the stimulant and IL-2 signaling. As the inflammation in RR is associated with the emergence of antigen-reactive T-cells and TNF-α, we speculate that the use of thalidomide in the treatment of RR may exacerbate the reaction.

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APPENDIX - RELEVANT EXTRA MATERIAL

DEVELOPMENT AND VALIDATION OF A GEAOGRAPHIC INFORMATION RISK ASSESSEMENT MODEL FOR THE CONTROL OF LEPROSY IN ETHIOPIA

Introduction

The multi-drug treatment approach of the WHO Global Leprosy Elimination Campaign has reduced the prevalence of leprosy in the world. According to WHO, since 1985, the global prevalence has been reduced by 90 % with a current prevalence/10,000 of 1.4 (*Int. J. Lepr. Other Mycobact. Dis* S21-S22, 2002). However, this reduction in prevalence does not appear to be successful in interrupting the transmission of leprosy. For the past 10-15 years, stable numbers of new cases have been recorded yearly, and in the 6 countries that account for approximately 90 % of new cases the incidence is even rising (Durheim, D.N. and Speare, R., 2003; Lockwood D.N.J., 2002). This increase in incidence is explained by some agencies to be due to increased detection of hidden cases through improved case detection services and special education campaigns. Although this might be true for the incidence among patients with advanced disease or disability, it does not explain why children make up 13 % of the new cases (Lockwood, D.N.J. 2002).

The relationship among *Mycobacterium leprae*, the human host and the chain of infection, which may lead to development of leprosy, are not clear. Leprosy is believed to be transmitted by inhalation of nasal droplets containing *M. leprae*. Lepromatous leprosy (LL) patients, who harbor viable bacilli in nasal secretions, are thought to be the main source of infection (Hastings, R.C., 1993). Yet little correlation exists between prevalence of LL and the

total new cases registered. The incidences of conjugal leprosy and of individuals with frequent contact with cases are also insignificant (Jopling, W.H., 1988).

The rate of infection by *M. leprae* is much greater than the rate of disease development indicating the presence of sources of infection other than the leprosy patients (Fine, P.E. *et al*, 1997). Alternative sources of *M. leprae* infection could be a sub-clinically infected individual, an animal or insect vector or a contamination in the environment.

According to the WHO, Ethiopia in 1999 reached the leprosy elimination target of 1case /10,000 populations; however, as with other countries, the incidence has not changed appreciably with about 5,000 new cases detected yearly. In 2002, clusters of endemicity with prevalence rates higher than the elimination target was recorded in 4 of the 14 administrative divisions namely the Oromia, Amhara, the Southern Nations and Nationalities (SNNP) and Tigray (Tuberculosis and Leprosy Control of Ethiopia10th Annual Review Meeting, 2002).

Environmental sources have long been suspected to play a role in the spread of leprosy especially due to the geographical differences in leprosy distribution and local clustering. Previous beliefs that *M. leprae* cannot survive once outside the human body are now changing. Under favorable conditions such as hot humid climate, *M. leprae* is capable of surviving for months (Dessikan, K.V. and Sreevatsa, 1995).

An objective of this work was to use geographic information systems (GIS) to assess the influence of environmental factors on the prevalence of leprosy in Ethiopia. The application of GIS in epidemiology has facilitated our understanding of spatial variations of diseases and their relationship to the environment. The environmental attributes selected for analysis were

normalized difference in vegetation index (NDVI), earth surface temperature (TMax), and a climate data including point potential evapo-transpiration (PPE), average maximum and minimum temperatures. These attributes were analyzed for their suitability for leprosy prevalence in the Oromia region in Ethiopia. Arc View GIS 3.3 and Genetic Algorithm for Rule-set Prediction (GARP) were used to build models and to create maps showing the distribution of leprosy in the region. The independent leprosy data set from the Amhara region was then used to validate the leprosy prediction model.

Material and Methods

Baseline Data. The data was prepared by the Amhara and Oromia Regional Bureaus of Health and the Ethiopian Ministry of Health. Cases include both the severe forms of multibacillary leprosy (MB) as well as the milder form known as paucibacillary leprosy (PB). As of June 2001, the total number of PB (N=1367) and PB (318) cases recorded in 285 zonal health institutions in the Amhara region was used in this study. The total PB (N=136) and MB (N=2402) cases for the Oromia region collected as of August 2003, comprised data from 124 health institutions.

Determination of geographical coordinates. The latitude and longitude of each clinic was obtained in geographic decimal degree format from the website of the National Imagery and Mapping Agency NIMA)(http://gnps.www. nima). Coordinates not found were obtained from Encarta (Microsoft Encarta, 2002). Digital mapping of the point coordinates for each clinic was prepared in Arc View GIS 3.3.

The Minimum Medical Database (MMDb). This database was prepared by collaborating health workers and earth scientists interested in the application of geospatial sciences to control

program management and research for schistosomiasis and other snail-borne diseases. The database contains information for the Intergovernmental Authority of Development/Nile Basin (IGAD/Nile) region of East Africa, which includes Ethiopia. The MMDb available in CDROM (http://www.gnosis.org) contains useful maps and compiled data on environmental attributes such as normalized difference in vegetation index (NDVI), earth surface maximum temperature (TMax), elevation, soil types; infrastructures like roads, rail roads, utility lines; political boundaries and population data for the region of East Africa (Malone, J.B. *et al*, 2001). All parameters tested in this study were taken from the MMDb .

Table.1. Data Used for Analysis

Parameter	**Feature Type**	**Source**
NDVI	8-bit Image	United States Geological Survey (USGS)
TMax	16-bit Image	USGS
ACT Climate	Grid	Blackland Research and Extension Center, Texas Agricultural experiment station
NDVI, TMax for GARP	Image	Clipped and modified with GARP Dataset Manager

Prevalence Calculation and Normalization of Data. To avoid confounding factors like clinic size and number of people living around each clinic and to obtain comparable data, we calculated the prevalence of leprosy per 10,000 populations for both the Amhara and Oromia regions.

Oromia Region: the data for this region contains number of inhabitants in each zone. We used these data to calculate the prevalence of leprosy/10,000 for each clinic.

Amhara Region: the total population per each zone was estimated by creating a 10-km diameter buffer zone surrounding each leprosy clinic and extracting the density; the total

population was calculated from the population density and used for the determination of prevalence/10,000 for each clinic.

Extraction of Environmental Data. All extractions of environmental feature data, including Advanced Very High Resolution Radiometer (AVHRR) satellite sensor data, were based on the 10-km diameter buffer zone around each clinic. We used the Oromia data for creation of models and the Amhara data for validation of created models.

Normalized Difference in Vegetation Index NDVI (1992-1995)

AVHRR data for daytime land surface temperature (TMax) and Normalized Difference Vegetation Index (NDVI) for 1992-1993 and 1995-1996 was obtained from the United States Geological Survey (USGS) global 1km^2 website (Huh, O.K. *et al*, 2001). NDVI is an index that ranges from 0 to 200 with increasing green vegetation. It is an indicator of soil moisture based on vegetation. The AVHRR data, which consists of decadal (10-day) composites of daytime imagery, was processed by the USGS to minimize cloud cover and atmospheric attenuation of sensor signal. All downloaded images were calibrated and geo-referenced to a geographic decimal degree latitude and longitude coordinate system using ERDAS imagine 8.6 image processing software. The decadal images were averaged to create monthly composites, which were averaged together to create maps for dry (October-March) and wet (April-September) seasons and an annual composite map, which included both seasons.

The NDVI image files were converted into a grid file and used for extraction of mean NDVI value for each buffer zone. The extracted data were exported as Dbase files into Microsoft excel datasheets. Scatter diagrams of Cases/10,000 versus mean NDVI were plotted in Microsoft

Excel. Outliers such as buffer zones falling fully or partially in water were excluded. A range of NDVI that comprises all prevalence data points was determined from the plot and used to perform queries in Arc View 3.3 GIS to create map overlays showing areas suitable for leprosy prevalence.

TMax, (1992-1995)

TMax is the earth surface maximum temperature from AVHRR channel 4, which is used as an indicator of temperature (Huh, O.K. *et al*, 2001). Similar to the NDVI data, the image files for TMax were prepared for the dry, wet and annual seasons. The same extraction procedures were applied to obtain a range of TMax suitable for the prevalence of leprosy in the regions. Scatter plots were prepared in Microsoft excel and the range was then used as the criteria for queries in Arc view 3.3 GIS to create map overlays. The Amhara prevalence data points were overlaid on the model and points falling inside and outside the model were determined for calculation of positive and negative predictive values.

Almanac Characterization Tool (ACT) Climate Data. This is a climate grid file (5 x 5 km^2 cell size), which contains 30 years average data for precipitation (pre), potential evapo-transpiration (pet), point potential evapo-transpiration (ppe= pre/pet) and the minimum (It) and maximum temperature (xt) for January up to December. Ppe is an indicator of water budget of the soil. The leprosy prevalence point table was joined to the climate data table based on the grid cell. The linked tables was exported as a DBase file and mean temperature ([It +xt]/2) and mean ppe were calculated for dry season (October to March) and wet season (April to September) to give dry T, wet T, dry PPE and wet PPE values. Scatter plots of these values versus the stratified

prevalence/10,000 (0-0.1, 0.1-0.7 and > 0.7) were used to define ranges that included 95% of all points (Table 2) for creation of map overlays in Arc view 3.3 GIS**.**

Ecologic Niche Modeling GARP. The annual composites data for TMax and NDVI were used as environmental dimensions for the ecologic niche modeling. Environmental layers for the analysis were prepared by converting the image files to grid and processing of these files using the GARP module in Arc View GIS 3.3. These files were transformed to GARP compatible format with the GARP Dataset Manager. The Amhara and Oromia leprosy prevalence data were combined to generate a total of 317 occurrence points. These distributional points were loaded into Desktop GARP and used to model ecologic niches and potential geographic distribution of *M. leprae*. GARP equally divides the loaded prevalence data into training and test data sets. Multiple iterations based on rule selection, evaluation, testing and incorporation or rejection is performed. The training data sets are subjected to randomly selected statistical methods like logistic regression, bioclimatic rules to develop a procedure to be used to evaluate the predictive accuracy based on test data sets and points randomly sampled from the whole region as a whole (Towsend, A.P. *et al*, 2001). For each set of data used GARP generates 20 models. Three criteria were used for the selection of the best model; (1) elimination of models with high number of training points omitted and when possible including only those models with 0 omission; (2) the average of total area predicted for species to be present was calculated and models falling within 5% range below or above this average were selected and (3) models that passed the two criteria were compared based on the p value for Chi-square analysis of the probability of a random sample predictions being similar to the one generated by GARP.

Results

Point locations of the 123 health institutions in the Oromia region and the 285 health institutions in the Amhara region were mapped in Arc View 3.3.

Scatter plots of Oromia prevalence data versus NDVI or TMax, prepared in Microsoft Excel, were determined to define ranges that included 95 % of all leprosy prevalence points. The NDVI value ranges were 116-154.5 for the annual composite, 116-161 for the dry season map and 115-155 for the wet season map. TMax ranges were 9.4-28.8^{0}C for the annual composite, 11.7-32.5^{0}C for the dry season map, 7.7-25.8^{0}C for the wet season map. These ranges were used to prepare query-based map overlays showing all areas that met both the NDVI and TMax criteria ranges in the country. We selected the annual composite model that covers all seasons throughout the year. This model showed except for areas in the South East (Somali) and North East (Afar), that most places in the country were found to be suitable for leprosy (Figure 1). The positive predictive value calculated using the Amhara data was 90.5 %. All of the prevalence points that fell outside the predicted area were in the lowest ranges of prevalence/10,000 (0 – 0.1).

The ACT climate grid was used to determine the influence of thermal-hydrologic regime (PPE and PET) in relation to leprosy distribution. Table.2 shows the defined ranges of potential evapo-transpiration and minimum and maximum temperatures for the dry and wet seasons The climate model predicted most of the Amhara and the Oromia region except the southern part, most of the Southern Nation and Nationality (SNNP), Harari and DireDawa (Figure 2.). The model excluded Somali, most parts of Afar especially the Eastern part, Northern Tigray and Western Gambela.

The positive predictive value generated using the Amhara data was 87.8%. Except for Chagnie (prevalence/10,000 = 1.32), all the prevalence points not predicted by the model had a prevalences/10,000 in the 0-0.1 ranges.

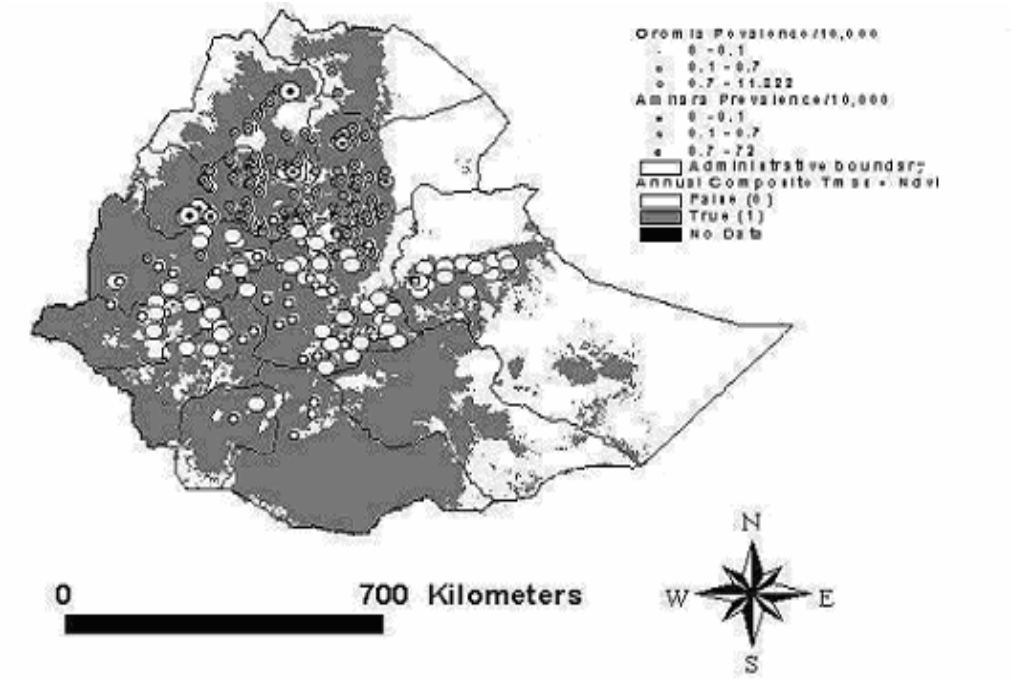

Figure 1. GIS Annual Composite Model for leprosy in Ethiopia. Query that met the ranges of NDVI (116.2 - 154.46) and Tmax (9.4 - 28.82) in relation to leprosy prevalence in the Orormia region was overlaid on the polytical boundary map of Ethiopia. Area in grey represent the 'best fit' model. the positive predictive value calcaulated by usong data from the Amhara region was 90.476%

.

Table 2. Temperature and PPE Ranges Used in ACT Climate Query

Prevalence Class	Dry season T in ^{0}C	Wet season T in ^{0}C	Dry season PPE	Wet season PPE
0 - 0.1	11.6 - 17.9	11.6 - 21.0	0.12 - 0.42	1.03 - 2.61
0.1 - 0.7	9.8 - 24.3	9.6 - 25.3	0.12 - 0.6	0.59 - 2.9
> 0.7	8.9 - 21.8	9.7 - 24.5	0.13 - 0.76	0.6 - 3.05

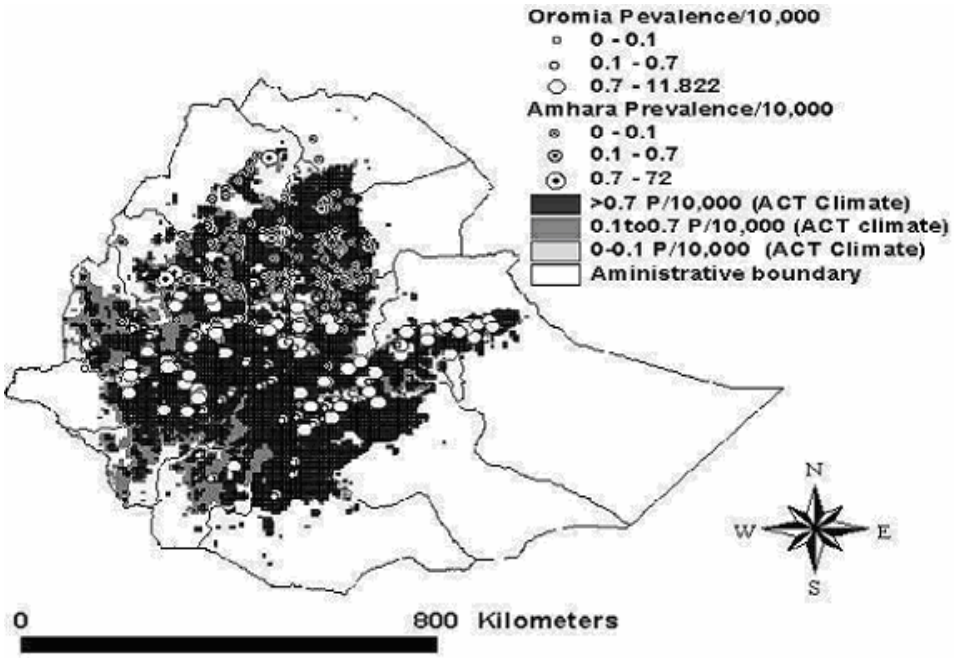

Figure .2 Climate Prediction Model for leprosy in Ethiopia. Potential evapotranspiration (PPE), average maximum and minimum temperatures for dry and wet seasons were used to study the influene of thermal-hydrologic regime on the prevalene of leprosy in Ethiopia. The ACT 5 x 5 climate grid was used for extraction. The Oromia prevalence table was joined to the ACT data and exported as a DBase file. Scatter plots

The GARP *M. leprae* distribution model is the most powerful of all models due to restrictions and statistical methods employed for its generation. This model is inclusive of all of the Amhara and Oromia points while the other two models were based on the Oromia data only. The prediction pattern of this ecologic niche model resembles the annual composite prediction model. It included most of the Amhara, Oromia, SNNP, Tigray and Gambela regions and excluded the Afar and Somali regions (Figure.3).

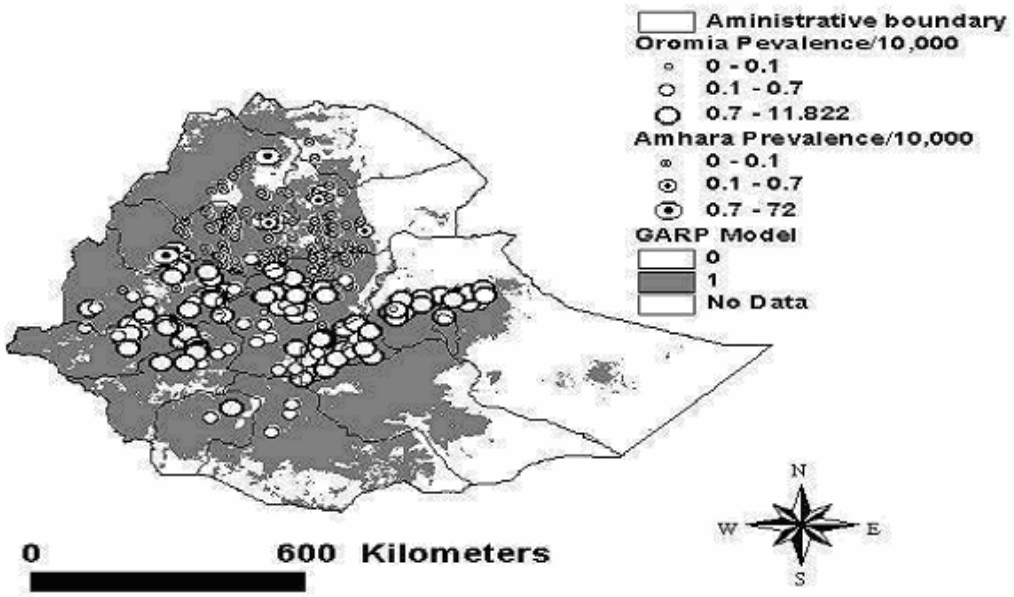

Figure .3 GARP predictive model for the distribution of M. leprae in Ethiopia. The environmental layers used are the annual TMax and NDVI. The model with minimum percentage of omission and within 5% range of the average presence area was selected.

Discussion

Understanding of the natural history of a disease, its ecologic distribution and the essential host-pathogen interactions are necessary in order to establish a successful control program. The WHO leprosy elimination campaign, mainly based on case finding, treatment and follow-up of household contacts, has not reduced the incidence of leprosy. Enhanced detection of hidden cases cannot explain the new occurrence of leprosy among young children, an indicator of ongoing active transmission (*Int. J. Lepr. Other Mycobact. Dis* S21-S22, 2002).

Several countries that reached the elimination target have localities with clusters of leprosy. These high endemicity regions must be eliminated in order to achieve sustained low transmissions and reduced incidence rates. Ethiopia is one example where leprosy elimination is achieved but there are districts with leprosy prevalence above the 1 case/10,000 cut-off defined by WHO to be an endemic area. The transmission rate in children below 15 years of age is about 6%. Out of the total leprosy cases recorded in Ethiopia in 2002, 46% occurred in Oromia, the largest administrative region in the country, followed by the Amhara region with 31.5 % of the total new cases (Tuberculosis and Leprosy Control of Ethiopia10th Annual Review Meeting, 2002).

GIS techniques were used in the current study to develop leprosy prediction models for Ethiopia. Thermal-hydrologic regime, as defined by AVHRR satellite sensor data on moisture or humidity, land surface temperature plus conventional climate data were included as potential environmental factors that might determine the distribution and abundance of the disease. Annual composite image files for NDVI, an indicator of moisture regime and vegetation vigor,

and TMax an indicator of temperature regime (Huh, O.K. and Malone, J.B., 2001) were used to create the AVHRR satellite sensor based model. This TMax/NDVI combined model showed a major part of the country was suitable for occurrence of leprosy. A second model based on climate station data on precipitation (PRE), Potential evapo-transpiration (PET), the PRE/PET ratio also known as point potential evapo-transpiration (PPE) and maximal and minimal temperatures for each month was also created. PPE indicates the moisture content of soil (water balance) was included to validate the previously reported association of *M. leprae* with moisture (10). The climate-based model with stratified prevalences (0-0.07, 0.07 – 0.1 and 0.1 to 71.3 cases/10,000 population) predicted the highest risk in Amhara and Oromia, the two regions with highest leprosy incidence (Tuberculosis and Leprosy Control of Ethiopia10th Annual Review Meeting, 2002).

The ecologic niche of a species is the suitable ecologic space within which a species is potentially able to maintain population without immigration. GARP is a high precision computer based genetic algorithm system used in ecologic niche modeling. It has a high predictive ability of species potential distributions based on user selected environmental layers. GARP incorporates different procedures that involve powerful rules including atomic rules, logistic regression analysis and the frequency distribution-based bioclimatic rules. The GARP model has minimum errors of omissions and commissions making it an important tool in understanding the geographical distribution of the species of interest (Townsend, A.P. *et al*, 2001; Costa, J. *et al*, 2002).

The third model GARP, based on input variable of NDVI and TMax annual composite value confirmed what we saw in the annual TMax/NDVI model. It predicted most of Amhara and Oromia regions as the two major leprosy areas in Ethiopia.

All three models excluded the dry and hot Afar and Somali area, the 2 regions with the least number of leprosy cases in Ethiopia in 2002 (Tuberculosis and Leprosy Control of Ethiopia10th Annual Review Meeting, 2002).

M. leprae is capable of withstanding severe adverse environmental conditions while to remain viable. Hot and humid weather, wet soils, water and low temperatures, are all demonstrated to favor survival of the bacilli for a few months (Desikan, K.V. and Sreevatsa, 1995; Desikan, K.V., 1977). *M. leprae* DNA has been detected in water in Indonesia at locations where a high proportion of individuals utilizing this water were affected by leprosy (Matsuoka, M. *et al*, 1999). A well-controlled study in Karonga district in Malawi has shown water and soil moisture to be associated with high prevalence of leprosy (Sterne, J.A.C. *et al*, 1995). Indirect transmission of leprosy is further supported by the natural occurrence of leprosy in animals like armadillos (Job, C.K. *et al*, 1986) and reports on the potential of insects to transmit leprosy (Geater, J.G., 1975; Banerjee, R. *et al*, 1990).

Our study indicates that leprosy may be associated with specific environmental features. The models we developed seem to be in concordance with leprosy distribution in Ethiopia and support the conclusion that: (1) certain thermal-hydrological regimes favor survival of leprosy in the environment and that (2) both NDVI and TMax may be incorporated in the predictive model

of leprosy. This can be measured by conventional climate station data or by satellite-sensor data on NDVI and TMax as surrogates of moisture and temperature respectively.

Various parameters have been identified as risk factors in acquiring leprosy disease. These include demographics like age and sex and immunological factors like the absence of BCG vaccination (Ponnighaus, J.M. *et al*, 1993). A significant negative correlation between the incidence of leprosy and higher economic indices has been reported (Di, Z. *et al*, 1993). Rural endemic settings with poor housing and sanitation conditions have been described as important factors in the transmission of leprosy (Mani, M.Z., 1996). For any of these factors to have a role in the development of leprosy, the sustained presence of *M. leprae* in the environment is necessary. We propose that leprosy occurs most frequently when a suitable microenvironment like moist soil coexists with other known or unknown predisposing factors. This study provides evidence that environmental factors are of importance in the prevalence of leprosy. Such factors must be taken into consideration when planning a control program. Further studies that incorporate field-collected validation data (ground truth) may shed more light into the precise thermal-hydrological regime factors or other risk factors associated with the environmental risk of leprosy.

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