To the Graduate Council:

I am submitting herewith a dissertation written by Sarojini Adusumilli entitled "Understanding Immune Response in *Mycobacterium ulcerans* Infection." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Pamela Small, Major Professor

We have read this dissertation
and recommend its acceptance:

Robert N. Moore

Stephen P. Oliver

David A. Bemis

Accepted for the Council:

Vice Chancellor and Dean of
Graduate Studies

UNDERSTANDING
IMMUNE RESPONSE IN *MYCOBACTERIUM ULCERANS* INFECTION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

SAROJINI ADUSUMILLI

December, 2005

DEDICATIONS

This work is dedicated to my loving grandfather (Sri Satyanarayana Rao), parents (Smt. Raja Kumari and Sri. Venkat Rao) and my aunt and uncle (Smt. Lakshmi Kumari and Sri. Rama Mohan Rao) for imparting the importance of higher education, guiding me, believing in me and helping me come this far. This is also dedicated to all the people who are passionate enough to reach their goals and make a difference in other people's lives.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my major professor, Dr. Pam Small, for giving me the opportunity to work in her lab, guiding me in the process of evolving into an independent thinker and for being a wonderful mentor. Her constant zest for the fun things in life (including science) are constant energy boosters to the people around her.

I would also like to thank my committee members Dr. Steve Oliver, Dr. Robert N. Moore and Dr. David Bemis, for their invaluable critical comments and suggestions.

A super duper thank you to all the "stupid guys" in my lab (Brian Ranger, Heather Williams, Lydia Mosi) who have now become my family here, especially Brian Ranger for his constant help and advice and more than all for putting up with me.

Also I would like to thank Nirisha, Shalini, Kiran and Deepthi for their friendship and all the fun things we did together.

Finally, I would like to thank my family, friends and all the other people who have helped me reach this stage of life.

ABSTRACT

Buruli ulcer is a necrotizing skin infection and is the third most important mycobacterial disease in immune competent individuals after tuberculosis and leprosy in humid tropical countries. The causative agent, *Mycobacterium ulcerans* is unlike other mycobacterial pathogens in that it appears to maintain an extracellular location during infection. Another unusual feature of the bacterium is that it is the only mycobacterium known to produce a dermo-necrotic polyketide toxin called mycolactone. A single Buruli ulcer, which can cover 15% of a person's skin surface, contains huge numbers of extracellular bacteria. The infection is characterized by massive necrosis at the site of infection followed frequently by debilitating disfiguration. Despite their abundance and extensive tissue damage, there is a remarkable absence of acute inflammatory response to the bacteria, and lesions are often painless. Though there is extensive literature on interaction of other mycobacterial species with innate immune cells, information concerning interaction of *M. ulcerans* with macrophages and neutrophils is scarce and requires further investigation. Research in this dissertation was geared towards understanding the poor innate immune response generated following *M. ulcerans* infection.

One hallmark of most diseases caused by mycobacteria including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. marinum*, *M. haemophilum* is the ability of the bacilli to grow within host cells and cause granulomas. In contrast, *M. ulcerans* primarily forms extracellular microcolonies within necrotic tissues and is rarely found within host cells. The role of mycolactone in the extracellular location of the bacteria was investigated using a macrophage infection model. Experiments using a panel of mycolactone negative

(*myc*-) and wild type (WT) *M. ulcerans* strains showed that presence or absence of mycolactone determines whether the bacteria are extracellular or intracellular. Exposure of macrophages to high concentrations of mycolactone interfered with their phagocytic ability. These observations that a mycolactone mutant is better phagocytized than the wild type strain is consistent with the presence of almost exclusively extracellular bacteria in Buruli ulcer patients.

Experiments studying the effect of mycolactone concentrations on fibroblast cell lines showed that mycolactone-mediated apoptosis and necrosis was concentration dependent. Mycolactone caused necrosis at high concentrations and apoptosis at low concentrations. Chemotaxis assays using human neutrophils showed that neutrophils do not respond to *M. ulcerans* (WT or *myc*-) or mycolactone. Mycolactone treatment resulted in rapid necrosis of human neutrophils in a dose dependent manner *in vitro*. These data could be relevant *in vivo* in human infections where toxin gradients produced by a pool of extracellular *M. ulcerans* may cause apoptosis or necrosis of inflammatory cells trying to move into the focus of infection and clear the bacteria.

Lack of an inflammatory reaction during the necrotizing stage of Buruli ulcer could be due to abrogation of production of inflammatory cytokines (e.g. TNF-α, IL-1, IFN-γ), chemokines (IL-8) and lowered expression of cell-adhesion molecules (e.g. ICAM-1, selectins, VCAM) which help inflammatory cells reach the site of infection. TNF-α and IL-8 are key players in immuno-inflammatory responses. Studies regarding TNF-α response to bacterial infection and mycolactone treatment *in vitro* showed that WT *M. ulcerans* and mycolactone did not induce TNF-α production while *myc- M.*

ulcerans did. Interestingly, LPS mediated TNF-α production was inhibited by WT *M. ulcerans* and mycolactone. Both WT and *myc- M. ulcerans* as well as mycolactone did not induce IL-8 production. WT *M. ulcerans* and mycolactone induced expression of the cell adhesion molecule ICAM-1 was less than that induced by *myc- M. ulcerans* or LPS.

Microarray analysis of genes modulated by mycolactone yielded interesting information. Genes that were significantly upregulated by mycolactone included those related to transcriptional repressors, cytoskeletal rearrangement, cell cycle control/proliferation, apoptosis, G-protein receptors, tumor supression and immune response. Genes downregulated by mycolactone included those related to DNA repair, inactivation of complement and metalloproteinases, immune response, leukotrienes production, receptors for collagen and laminin. Data from the present study provide new insight into the effect of mycolactone on macrophages, fibroblasts, neutrophils and host gene-expression pathways induced or repressed by mycolactone. Knowledge obtained from the present study can be expected to contribute to a better understanding of the role of mycolactone in host-pathogen interactions as well as pathophysiology of the disease.

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)
AFB	acid fast bacilli
AP	activator protein
ASLs	acetone soluble lipids
ATCC	American Type Culture Collection
BCG	bacillus Calmette-Guerin
BSA	Bovine serum albumin
BU	Buruli ulcer
BUD	Buruli ulcer disease
^{O}C	degrees celcius
cDNA	complimentary DNA
CFUs	colony forming units
CO_2	carbon dioxide
CPA	cytopathic affect
CR	complement receptor
CsA	cyclosporine
d	distilled
dd	double distilled
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide

DSCR1L1	Down syndrome critical region gene 1-like 1
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate disodium salt
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
FCS	fetal calf serum
g	gram
h	hour
H_2O_2	water
HDL	high density lipid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
Ig	immunoglobulin
I-κB	inhibitory-κB
IL	interleukin
iNOS	inducible (calcium-independent) nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactoside
IS	insertion sequences
kDa	kilodalton
LB	Luria-Bertani medium

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	molar
M7H9	Middlebrook 7H9 broth
M7H10	Middlebrook 7H10 agar
MAP	mitogen activated protein
MCP-1	monocyte chemotactic protein-1
MOI	multiplicity of infection
MU	*M. ulcerans*
MUCF	*M. ulcerans* culture filtrate
mg	milligram
min	minutes
mM	millimolar
ml	milliliter
mol	moles
mRNA	messenger ribonucleic acid
NAD/NADH	nicotinamide adenine dinucleotide
NFAT	nuclear factor of activated T cells
NFDM	nonfat dry milk
NF-κB	nuclear factor-κB
NK	natural killer
ng	nanogram
nmol	nanomoles

OADC	oleic acid-albumin-dextrose-complex
OD	optical density
PBS	phosphate buffered saline
PBS-Tween	phosphate buffered saline with 0.05% Tween 20
PCR	polymerase chain reaction
PKS	polyketide synthase
PMA	phorbal 12-myristate-13 acetate
PNG	polymorphonuclear granulocytes
PVDF	polyvinylchloride
pmol	picomol
Rf	ratio to front
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
TAE	tris, acetic acid, EDTA
Taq	*Thermus aquaticus* DNA polymerase
TB	tuberculosis
TBE	tris, borate, EDTA
TBS	tris buffered saline
Th	helper T cells

TLC	thin-layer chromatography
TLR	Toll-like receptor
TMC	Trudeau Mycobacterial Culture Collection
TNF-α	tumor necrosis factor alpha
U	unit
UV	ultraviolet
V	volts
WHO	World Health Organization
WT	wild type
YEPD	yeast extract potato dextrose
µF	microfaraday, unit of capacitance
µg	microgram
µl	microliter
µm	micrometer
µmol	micromoles
m/z	mass/charge
myc-	mycolactone negative
x g	times gravity

Chapter 1

BACKGROUND AND RESEARCH OBJECTIVES

Introduction

The genus Mycobacterium is highly diverse, with 85 different species identified since the identification of *Mycobacterium leprae* in 1873. The vast majority of *Mycobacterial* species are environmental free-living saprophytes (1). Of all the known species of the genus Mycobacterium, only a few are known to cause disease in man [9].

Mycobacterial diseases cause substantial illness and death thoughout the world, despite years of public health efforts. *Mycobacterium ulcerans* (*M. ulcerans*) is the etiological agent of Buruli ulcer (BU), the third most common mycobacterial infection of immunocompetent humans after tuberculosis and leprosy (2). Buruli ulcer is the only mycobacterial disease known in which a bacterial toxin, rather than a host response, causes tissue destruction. The first cases of Buruli ulcer were described from central Africa in 1897 although the first African isolates were not cultured until 1964 (3). The disease has since been recorded in many, mostly tropical countries in Africa, Central and South America, Australia and Southeast Asia (4) (Fig. 1.1). In some West African communities, BU has replaced tuberculosis (TB) and leprosy as the most prevalent mycobacterial disease, affecting up to 22% of the population. However, the true global impact of the disease is unknown because reliable tools for its rapid diagnosis and surveillance are lacking (5). BU was recently recognized by the World Health Organization as an important emerging disease (6).

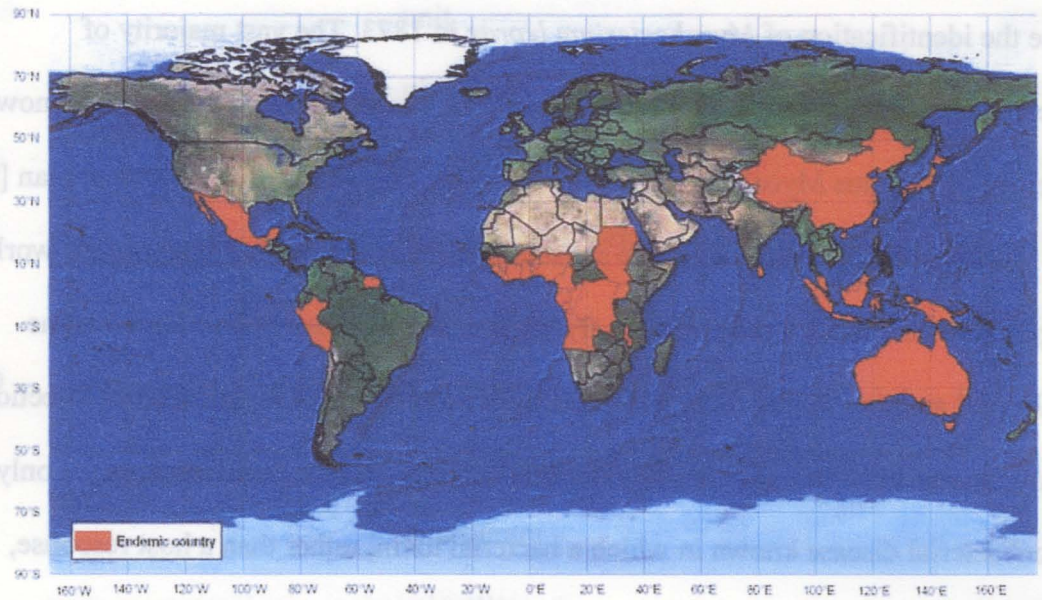


Fig. 1.1. Countries where Buruli ulcer has been reported. Countries shown in red are endemic regions. (Taken from WHO Buruli ulcer website: http://www.who.int/gtb-buruli/global__situation/index.html.)

Disease manifestation, transmission and therapy

BU is characterized by extensive tissue damage, which occurs in the absence of an acute inflammatory response. There is considerable variation in the clinical presentations of BU patients, and evidence suggests that there are geographical differences in the severity and forms of the disease. Most researchers believe that *M. ulcerans* infection is acquired from the natural environment, and that small penetrating injuries transmit the bacterium to the subcutaneous fat of exposed parts of the body. The first sign of *M. ulcerans* is a papule or pimple (like a spider bite) in the skin, with no apparent involvement of subcutaneous fat (2) (Fig. 1.2). A characteristic BU has scalloped edges and a base covered with necrotic slough. Although the mycobacteria are concentrated in the center of the lesion, the ulcer is widely undermined, with vast extension of necrosis into the subcutaneous fat.

Histopathologic examination reveals little acute or chronic inflammation in the lesion, and *M. ulcerans* is seldom seen in macrophages. Further, patients with BU experience neither fever, malaise, nor regional lymphadenopathy. These features suggest that toxin from *M. ulcerans* exerts local immunosuppressive and/or antiphagocytic effects (7). Histological specimens typically show large clumps of extracellular acid-fast organisms surrounded by areas of necrosis and a poor or absent inflammatory response. Later in the natural history of the disease, the immunosuppressive effect of the toxin is somehow overcome by the host, immunity develops, and healing commences (8). An intense necrosis is seen in the lesion but previously it was not clear whether this is induced by the toxin or has an immunobiological basis (9).

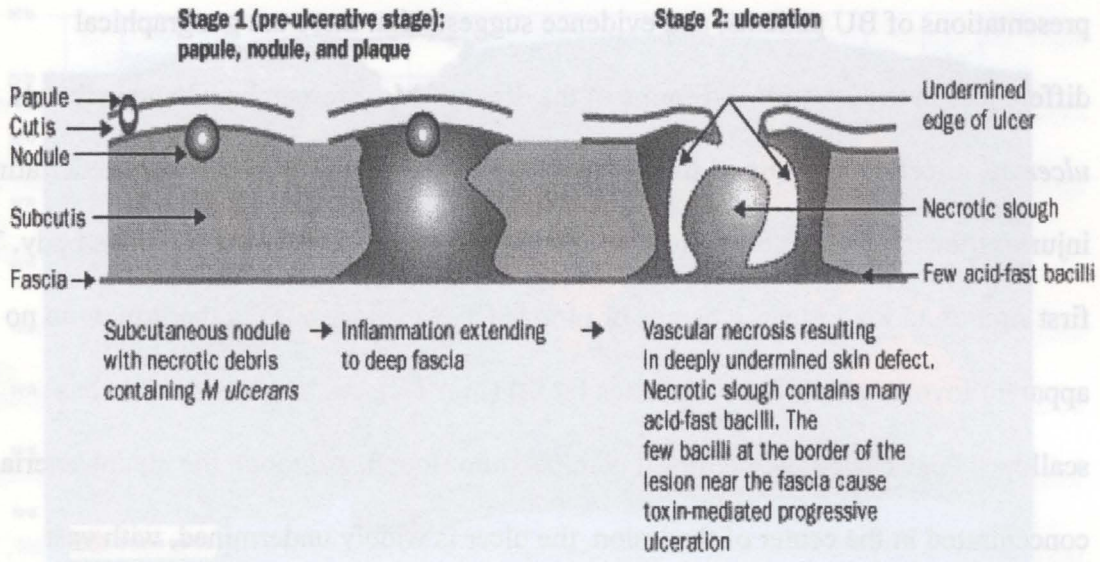


Fig. 1.2. Progression of Buruli ulcer lesion to the ulcerative stage, (Taken from van der Werff *et al.*, 1999)

Recent studies have shown that the tissue necrosis is, at least in part, due to the bacterial toxin mycolactone. Widely used animal models to study the pathogenesis of *M. ulcerans* are mice and guinea pigs (10-13).

Lesions may occur on any part of the body though they are more common on exposed parts, especially the limbs. Lesions are single, or less frequently multiple. Most ulcers occur on the extremities and there is frequently a history of minor injury or insect bite which fails to heal, or the spontaneous appearance of a small boil or papule which becomes indurated, crusted, and eventually ulcerated (Fig. 1.3). Over a period of weeks the ulcer enlarges, with widespread thickening of subcutaneous tissue. Subsequent necrosis produces "undermining" of the ulcer margins, and a sharply defined ulcer edge develops. The base of the lesion appears mucoid and there is frequently a clear serous discharge. Where fat is exposed, it may appear white and lusterless. In large lesions there is extensive induration adjacent to the ulcer. Beyond this area of thickening, however, the skin appears normal with no cellulites, lymphangitis or lymphadenopathy. Without therapy there is progressive enlargement, formation of satellite lesions, and necrosis extending between lesions in subcutaneous tissue beneath apparently healthy surface epithelium. Necrosis may extend down to muscle fascia and even to bone, but there is seldom actual bone involvement. Irregular healing occurs with production of fibrous scar tissue and calcification (14).

All the forms of disease-nodular, oedematous, plaque, ulcerative and osteomyelitis are found among African patients. However, there also appears to be geographical variation in the pathology of the disease. For example, the plaque form of Buruli ulcer, though common in Benin, appears to be entirely absent in Australia.





Fig. 1.3. Buruli ulcer (A) Nodule (B) Ulcerative lesion. (Taken from Buruli ulcer: Diagnosis of *Mycobacterium ulcerans* disease, WHO 2001)

Osteomyelitis, which is fairly common in Benin and Ghana, has not been reported in Australia or Mexico suggesting that *M. ulcerans* isolates from outside West Africa may be less virulent. Clinical data suggests that infections with Asian isolates may also be less virulent than the African strains (3).

Neither the mode of transmission nor the environmental reservoir for *M. ulcerans* is known, although observational studies suggest that *M. ulcerans* infection is transmitted through the skin after contact with contaminated water, vegetation, or insects (5). The organism has only been recovered from lesions of humans or, in one case, a koala, and there has been only one report of person-to-person spread (15-16). Thus, environmental exposure, either by direct inoculation or an insect vector, is the likely route of infection. Epidemiologic studies suggest the proximity to water sources, such as freshwater lakes or rivers, predisposes to disease, but specific contact with water that might lead to transmission of the bacteria has not been identified. Cultures of water near BU-endemic areas have not yielded *M. ulcerans*, though testing of water samples by polymerase chain reaction (PCR) detected *M. ulcerans* in one Australian study. Soil also has been considered a possible reservoir, but the organism has not been isolated or detected in soil samples (17).

The most recent data regarding the environmental source of *M. ulcerans* is the detection and culture of *M. ulcerans* from aquatic insects collected from the environment in Benin by Portaels *et al.* (18). In another study, also in areas of Benin where BU is endemic, it was shown that small fish were positive for *M. ulcerans* by PCR (19). Marsollier *et al.* (20) demonstrated that *M. ulcerans* may multiply in the salivary glands

of aquatic insects following experimental infection and that bites of such insects transmitted *M. ulcerans* to laboratory mice.

BU is primarily a disease of children, with the highest rates found in children ages 2 to 14 years. Although reported in persons with human immunodeficiency virus (HIV), no predilection for BU has been noted in HIV-infected persons or other immunodeficient patients (21, 22).

Culture and detection

M. ulcerans is a slow-growing environmental mycobacterium that can be cultured from human lesions on mycobacterial medium at 30-32°C (23). *M. ulcerans* is phylogenetically related to the ubiquitous waterborne *M. marinum*, and can be differentiated on the basis of phenotypic characteristics, slight nucleotide variations in targeted genes, and the presence of two insertion sequences, IS 2404 and IS 2606 (24). Confirmation of BU disease can be performed with tissues obtained directly from the excised skin or ulcer by combined laboratory methods such as Ziehl-Neelsen staining for acid fast bacilli (AFB), bacterial culture, histopathology, and/or PCR (25).

Treatment

The treatment of BU depends on the stage of the disease. Chemotherapy has little impact on the course of the disease and surgery is often required. Early lesions respond to simple excision but ulcerative lesions require very extensive excision and skin grafting (9). Pathologic changes that characterize early *M. ulcerans* infections depend in large measure on two distinct properties of the etiologic agent: (i) *M. ulcerans* grows optimally

at temperatures lower than central body temperatures, i.e., at 30 to 33°C, and (ii) *M. ulcerans* produces a toxin. This temperature growth requirement largely limits the infection to body surfaces, and the toxin destroys tissue and suppresses local immune responses (26).

Because lesions are painless, BU is rarely detected in an early and readily curable stage. Most lesions are widely ulcerated when they are detected and require extensive surgical excision and skin grafting. Primary culture of *M. ulcerans* under optimal conditions may take several months (27). Traditionally, drug therapy has been considered ineffective, but recent data suggest that combinations of antimycobacterial antibiotics that include rifampicin and either streptomycin or amikacin are able to kill *M. ulcerans* in human lesions. The persistence of *M. ulcerans* infection and ineffectiveness of antibiotics are thought, at least in part, to relate to a local immunosuppression at the site of infection (28). Untreated Buruli ulcer will eventually subside with the gradual development of host immunity in most cases. However, by this time, tissue damage may be very extensive and healing by scar can lead to permanent functional and cosmetic deformity (8). Recent studies by Phillips *et al.* have shown that topical application of acidified nitrite increases the rate of healing (29, 30).

Mycolactone-a major virulence determinant

Even though the better known pathogenic members of the genus Mycobacterium, such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium leprae* (*M. leprae*), are not associated with toxins, the presence of a toxin in *M. ulcerans*, which is an extracellular pathogen, has been hypothesized for almost 40 years (31). Early studies by

Meyer *et al.* (32) have shown that *M. ulcerans* secretes an exotoxin into culture filtrate. The inoculation of concentrated culture filtrate into guinea pig skin, causes inflammation, necrosis, edema, and other histopathological changes which resemble infections of humans (32-33). The resulting pathogenesis and histological appearance was explained by a recently identified diffusible lipid toxin, mycolactone (3, 8).

Mycolactone was originally isolated, purified and characterized from *M. ulcerans* 1615, a Malaysian isolate, as a mixture of cis/trans isomers designated mycolactone A and mycolactone B (Fig. 1.4) (3). The discovery of mycolactone in *M. ulcerans* is the first report of macrolides from *Mycobacteria* species as well as the first identification of a macrolide from any bacterial pathogen (34). Macrolides are an important class of complex polyketides produced as secondary metabolites primarily by soil bacteria in the order Actinomycetales (34). Mycolactone is a 12- membered ring macrolide which is the group of macrolides containing the smallest lactone ring size. Well-known polyketides include antibiotics (erythromycin), immunosuppressants (rapamycin, FK506), antifungal agents (amphotericin B), antihelmetic agents (avermectin), and cytostatins (bafilomycin). Other macrolides that induce apoptosis in tissue culture cells include erythromycin, bafilomycin A1, antimycin A, and ionomycin (13).

The activity of mycolactone *in vitro* on cultured fibroblasts and macrophage cell lines produces a distinct cytopathic phenotype. The earliest effect is cell rounding caused by radical alterations in the cytoskeleton of cells, which occurs within 10 h after addition of mycolactone to cultured cells. At 36 h, treated cells are arrested in G_0/G_1 of the cell cycle, and by 72 h, cells begin to die via apoptosis (13). Further experimentation revealed

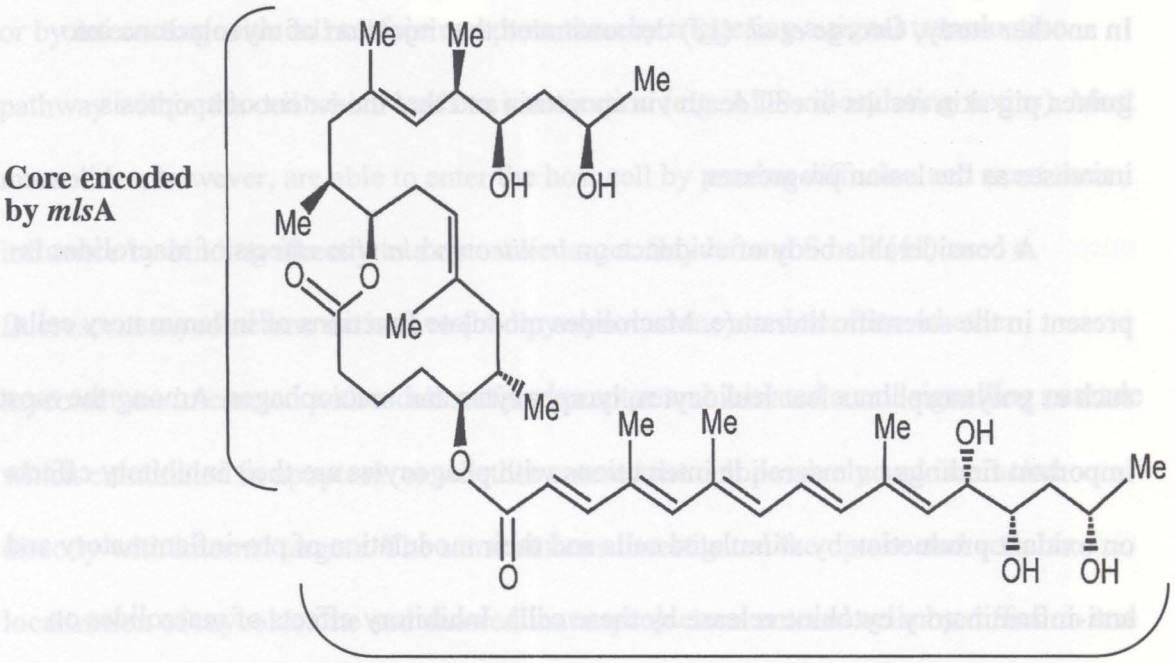


Fig. 1.4. Structure of mycolactoneA/B. (Taken from Gunawardanna, *et al.* 1999 and Song, B., and Kishi, Y., 2001)

a general inhibition of protein synthesis in response to mycolactone (35). *In vivo* studies using a guinea pig model of infection suggest that mycolactone is responsible for both the extensive tissue damage and immunosuppression which accompanies Buruli ulcer (36). In another study, George *et al.* (13) demonstrated that injection of mycolactone into guinea pig skin results in cell death via apoptosis and that the extent of apoptosis increases as the lesion progresses.

A considerable body of evidence on *in vitro* and *in vivo* effects of macrolides is present in the scientific literature. Macrolides modulate functions of inflammatory cells, such as polymorphonuclear leukocytes, lymphocytes and macrophages. Among the most important findings on macrolide interactions with phagocytes are their inhibitory effects on oxidant production by stimulated cells and their modulation of pro-inflammatory and anti-inflammatory cytokine release by these cells. Inhibitory effects of macrolides on leukocyte chemotaxis have also been documented (37, 38). Macrolides inhibit production of proinflammatory cytokines including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α, and inhibit expression of transcription factors, including nuclear factor (NF)-κB and activator protein (AP)-1. Macrolides also inhibit neutrophil elastase and reactive oxygen radicals; suppress expression of neutrophil adhesion molecules and selectins, thus decreasing infiltration of inflammatory cells (39). Mycolactone could play an important role not only in the immunosuppression and tissue destruction which occurs in the disease but also in the ability of the organism to survive in an intracellular environment (34).

Mycolactone appears to play a role in inhibiting recruitment of inflammatory cells to the site of infection (40). The specific cellular effects of mycolactone suggest that

mycolactone might have therapeutic potential as an antineoplastic, anti-inflammatory and/or immunosuppressive agent. Many bacterial toxins are proteins. These molecules generally act either by forming pores in the host cell (hemolysins) and inducing cell lysis, or by interacting with cell surface receptors thereby triggering a signal transduction pathway within the cell which leads to intoxication (e.g. ADP-ribosylating toxins). Most macrolides, however, are able to enter the host cell by passive diffusion and exert their influence by binding directly to a cytosolic target. Snyder and Small (41) used a fluorescent mycolactone derivative (bodipymycolactone) to determine whether mycolactone interacted with a cell surface receptor and initiated a cell signalling cascade which culminated in apoptosis or whether entered the cell passively and interacted directly with internal targets. The same authors investigated the uptake and cellular localization of mycolactone and showed that mycolactone entered cells by diffusion and accumulateed in the cytosol (41).

Biosynthesis of mycolactone requires three polyketide synthase enzymes and at least two accessory enzymes, all of which are located within a contiguous 110-kb cluster on a large plasmid (8). The lactone core is encoded by two polyketide synthase genes, *mls*A1 and *mls*A2, and a third polyketide synthase gene, *mls*B, encodes the fatty acid side chain (42).

Immune response

Early immune response

In contrast to our knowledge of tuberculosis and leprosy, and despite the frequency of *M. ulcerans* infection, there is a paucity of knowledge about the human immune response to *M. ulcerans*. *M. ulcerans* occurs in lesions primarily as extracellular microcolonies (36). Unlike most other mycobacterial pathogens like *M. tuberculosis* and *M. leprae* which are intracellular, *M. ulcerans* infected tissues primarily contain extracellular bacilli (AFB) in the center of lesions (17). Although lesions may be quite extensive, covering up to 15% of a patient's skin surface, they are relatively painless (13). *M. ulcerans* differs from *M. tuberculosis* and *M. marinum* in terms of tissue tropism and phenotype of the host immune response. Whereas *M. tuberculosis* and *M. marinum* disseminate in the host and proliferate within macrophages, *M. ulcerans* is found almost exclusively extracellularly in cutaneous and subcutaneous tissues (43) (Fig. 1.5).

Ashford *et al.* (44) and others recently reported that in Buruli ulcer disease inflammation appears to be minor for the amount of necrosis. Connor and Lunn hypothesized that the toxin may induce necrosis of the inflammatory infiltrate (31, 45). Similarly, Pahlevan *et al.* have postulated that local secretion of mycolactone reduces the cutaneous inflammatory response and may contribute to the development of the ulcer (28) (7). These features suggest that the toxin from *M. ulcerans* exerts local immunosuppressive and/or antiphagocytic effects. Gooding *et al.* (46) showed that patients with *M. ulcerans* infection mount an immune response to *M. ulcerans* as

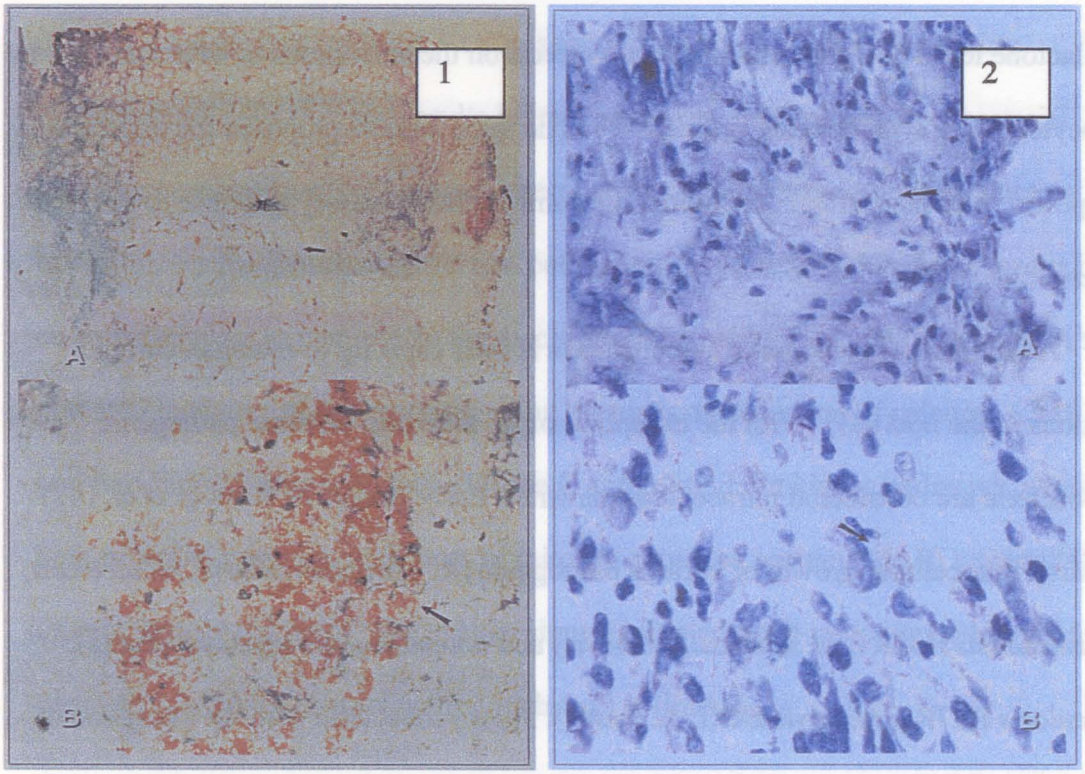


Fig. 1.5. Cellular location of *M. ulcerans* (1) and *M. marinum* (2).

1. Early-(**A**) and late- stage (**B**) disease histopatho- logic sections of the dermis stained for acid-fast bacilli (AFB) from a patient with a *Mycobacterium ulcerans* infection. In A, arrows indicate necrosis of adipose tissue distant from the location of AFB, and in B, the arrow indicates predominance of extracellular bacilli and microcolonies. Patients' samples were obtained from the study conducted in Côte d'Ivoire.

2. A and **B.** Active disease histopathologic sections of soft tissue stained for acid-fast bacilli from a patient with a *Mycobacterium marinum* infection. In **A**, the arrow indicates localized necrosis, and in **B**, the arrow indicates predominance of intracellular bacilli.

(Taken from Dobos, K. M., *et al.* 1999).

evidenced by antibody production, but they demonstrate profound systemic T-cell anergy to mycobacterial antigens (2). Pahlevan *et al.* (28) reported that the partially purified mycolactone toxin had profound inhibitory effects on the elaboration of TNF and IL-2 by monocytes and T lymphocytes, respectively. The same authors proposed the toxin would have potent inhibitory effect on a mounting immune-inflammatory response to *M. ulcerans* (28).

A hallmark of pathogenic mycobacteria is their capacity to establish chronic infections in the host (43). With the exception of *M. ulcerans*, all other pathogenic mycobacteria are considered intracellular parasites. Early studies by Rastogi *et al.* (47) compared intracellular growth of various pathogenic (*M. paratuberculosis*, *M. ulcerans*, *M. tuberculosis* H37Rv, *M. kansasii*, *M. bovis*) and nonpathogenic mycobacteria (*M. tuberculosis* H37Ra, *M. bovis* BCG, *M. gastri*). All mycobacteria with the exception of *M. ulcerans* and *M. gastri* effectively multiplied inside J774-macrophages. Inability to propagate *M. ulcerans* inside J-774 macrophages was hypothesized to be due to a toxin secreted by the bacteria inside macrophages leading to massive lysis after infection. J-774 model has been used extensively to study in vitro host-mycobacteria interactions (47). The pathogenesis of *M. ulcerans* is closely associated with expression of mycolactone, as a natural mycolactone deficient mutant failed to induce ulcers in guinea pigs (36).

Most pathogenic mycobacteria are primarily intracellular when residing in vertebrate hosts. One of the oldest and most well-supported observations regarding the interaction between pathogenic mycobacteria and the macrophage is the intracellular location of mycobacteria. Macrophages produce various chemokines (e.g. RANTES and MCP-1) and cytokines (e.g. TNF-α and IL-1β) following a mycobacterial infection and

these immune effector molecules are required for recruitment and activation of leucocytes and subsequent control of the mycobacterial infection. In particular, activation of macrophages by stimulated T cells is thought to play a critical role during mycobacterial infection and results in increased expression of antimycobacterial mediators such as reactive nitrogen intermediates. The ability to limit production of inflammatory mediators suggests that pathogenic mycobacteria like *M. ulcerans* initiate an active process to manipulate the macrophage signal transduction pathways. A major potential mediator of upstream signaling events is mobilization of calcium. Ca2+ is required for activation of protein kinase C and calmodulin kinase pathways, which are both potential upstream activators of mitogen activated protein (MAP) kinases (48). The current understanding of macrophage signals initiated upon infection and how *M. ulcerans* modulates these signaling pathways is limited.

Adaptive immune response

During the necrotic phase of the disease, there is virtually no cellular response to the infection. No skin-test reactivity to burulin (a skin-test reagent prepared from *M. ulcerans* organisms) develops in patients during this phase of the disease. Skin-test reactivity is observed during healing and is accompanied by granuloma formation in the ulcer bed and surrounding tissue. Pimsler *et al.* (49) speculated that healing begins to accelerate when *M. ulcerans* no longer produces toxin, thus permitting development of the host response to other antigenic components of the etiologic agent (e.g., cell wall). Suppression of immune responses is an important factor in the pathogenesis of several mycobacterial diseases of humans. In susceptible individuals, infection by *M. leprae* is

accompanied by development of suppressor T cells that are *M. leprae* specific. *Mycobacterium avium* and *Mycobacterium intracellulare* infections are infrequent in healthy individuals but are common and life threatening in immunodeficient patients with AIDS. Pimsler *et al.* (49) reported that *M. ulcerans* produced a toxin that prevented T cell activation and suppressed phagocytosis by macrophage. Pahlevan *et al.* (28) showed that high density lipid fractions from culture filtrates of *M. ulcerans* inhibited LPS-induced TNF and IL-10 production. These fractions also blocked production of IL-2 from activated T lymphocytes. Interestingly, TNF induced NF-κB activation was inhibited whereas a synergy was observed between the factor and phorbol ester-directed NF-κB activation (28).

Gooding *et al.* (7) postulated that T cell anergy to mycobacteria may contribute to the pathogenesis of Buruli ulcer. Absence of a positive skin test response in most patients may indicate that cellular immune responses are not or are only weakly induced. Interestingly, spontaneous healing is often accompanied by skin test conversion, again suggesting a pivotal role of the immune system in the control of the disease (50). BCG vaccination had an incomplete but significant protective effect against Buruli ulcer in two controlled trials in Uganda (2). No study has addressed whether cytotoxic or immunosuppressive factors are released from *M. ulcerans* during the early, active, or late stages of infection; mechanisms by which such factors might act on host tissues are also not known (17). In another study, Gooding *et al.* (7) reported marked anergy to *M. ulcerans* in patients with current or past *M. ulcerans*-induced disease, comparable to that to *M. bovis* BCG observed in Mantoux test-negative subjects. They showed that the anergy is not generalized, as evidenced by a normal proliferative response and IFN-γ

production following stimulation with PHA. This anergy was due to failure to recognize *M. ulcerans*, as antibodies to these bacteria were demonstrated in patients with unreactive peripheral blood mononuclear cells (PBMC) but in none of the control subjects. The same authors suggested that in individuals who develop overt disease, the bacteria are ineffectively phagocytosed or escape from phagocytes before being killed, processed, and presented to T lymphocytes (7).

Cytokine response

Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of costimulatory signals and cytokines (51). IFN-γ is a major factor in macrophage activation and plays a critical role in protection against infection with mycobacteria. T-cell anergy and lack of IFN-γ production in patients with *M. ulcerans* disease may account for the persistence of extracellular mycobacteria, the indolent nature of the disease and its failure to respond to conventional antimycobacterial chemotherapy (7, 52). In addition, inappropriate cytokine production may divert immune systems of patients toward a predominantly Th2-type response, thus, accounting for enhanced antibody responses of patients (46). Mycolactone inhibits IL-2 production of activated lymphocytes, and TNF-α expression of LPS-stimulated monocytes (28). A comparative analysis of systemic and intralesional cytokine production in patients suffering from the nodular and ulcerative form of the disease showed that patients with nodular forms demonstrated higher IFN-γ and lower IL-10 production against *M. ulcerans* than patients with ulcerative forms (53). IL-10 is a well known powerful downregulator of IFN-γ production (54). Other Th2 type cytokines that

may be responsible for the low *M. ulcerans* specific IFN-γ response are IL-4, 5, 6 and 13. Huygen *et al.* (55) hypothesized that mycolactone could stimulate production of IL-10 in the skin, which, in turn, would have a direct anti-inflammatory effect and downregulate production of IFN-γ by Th1 T-lymphocytes. Regulatory T-cells are possible candidates responsible for elevated production of IL-10, since another cytokine produced by regulatory T-cells, i.e., transforming growth factor (TGF)-β, is also produced by PBMC cultures from BU patients stimulated with *M. ulcerans* bacilli (55). Recent studies by Westenbrink *et al.* (56) showed that early-stage BUD patients produced significantly lower levels of IFN-γ and IFN-γ/IL-4 ratios compared to late-stage BUD patients after PHA stimulation. Compared to that of controls, IFN-γ production after tuberculin stimulation was significantly higher in late-stage but not in early-stage BUD patients. The same authors concluded that differences in Th-1-type cytokine production between early- and late-stage BUD might reflect an improved immune defense over time (56).

Humoral response

Significant immunoglobulin (Ig)G antibody production against *M. ulcerans* can be demonstrated in both patients and in unaffected household contacts, as tested by immunoblot analysis with extracts of *M. ulcerans*. This antibody production in unaffected contacts may be related to the extracellular localization of *M. ulcerans*, even in the early forms of infection (25). It forms a marked contrast with the serological findings for tuberculosis and leprosy, in which only patients with advanced disease display significant antibody production against the pathogen that is initially confined to an exclusive intracellular localization (57-58). IgG responses against *M. ulcerans* culture filtrate

(MUCF) antigens cannot distinguish between diseased, healed or healthy individuals in endemic regions. On the other hand, primary IgM antibody responses against these MUCF proteins can be detected in sera from 85% of confirmed BU patients and only in 4.5% sera from healthy family controls. From a group of 56 patients who were IgM-positive to MUCF antigens, 21 patients were at the early nodular stage, while 35 were at the later ulcerative stage (25).

Vaccine

Currently, there is no specific vaccine against *M. ulcerans* but evidence in the literature suggests a cross-reactive protective role of the *M. bovis* BCG vaccine used against tuberculosis. Although BCG vaccination seems to offer short-term protection against the development of BU, it does not seem to influence actual incidence of BU in the long run (59, 60). Mycolactone-negative isolates of *M. ulcerans* have been identified, both as spontaneous mutants that lack yellow pigmentation and more recently by screening of kanamycin resistance in isolates generated by transposon mutagenesis. These mycolactone-negative mutants may offer a better and more specific protection than BCG. Mycolactone is an obvious candidate for a vaccine but by virtue of its chemical structure, a polyketide is not in itself immunogenic (55). So far, antibodies against mycolactone have not been detected either in healthy contacts or in BU patients. A key question which may be essential for the development of an effective vaccine: is *M. ulcerans* intracellular or extracellular during the early stages of infection?

A number of mycobacterial lipids have been postulated to play a role in pathogenesis and have multiple effects on host cells, particularly immune cells. The lack

of an acute inflammatory response to infection with *M. ulcerans*, the cytopathology of the toxin on tissue culture cells, and the lipid nature of the toxin suggest that the *M. ulcerans* toxin may function in a unique, as yet uncharacterized mechanism (35). The most distinctive feature of *M. ulcerans* pathology is the presence of cell damage in the absence of an acute inflammatory response. Apoptosis as a phenomenon is associated with a lack of inflammatory response. If apoptosis is a major cause of pathology in Buruli ulcer, this could explain why an acute inflammatory reaction does not develop in Buruli ulcer lesions despite extensive cell damage. Mycolactone as a single molecule might lead to this property (13).

In *M. ulcerans* infection, as in other mycobacterial infections (eg, *M. tuberculosis*), the immune system may fail to recognize common mycobacterial antigens; this failure facilitates disease progression (2). Loss of protective immunity could be innate, acquired from environmental pressure, or induced by *M. ulcerans* infection itself (40). The immune mechanisms involved in protection against Buruli ulcer are largely unknown at present. A better definition of cellular immune responses to *M. ulcerans* may have an important impact on vaccine development (7, 46, 52).

Research objectives

Though there is extensive literature on interaction of other Mycobacterial species with innate immune cells, information concerning interaction of *M. ulcerans* with macrophages and neutrophils is scarce and requires further investigation. Bacterial resistance to phagocytosis may represent a pathogenic strategy for successfully establishing disease. This characteristic of *M. ulcerans* may be relevant *in vivo* for

maintenance of skin lesions and bacterial persistence in tissues or ulcers. Specific virulence factors that might be responsible for inhibition of phagocytosis and subsequently extracellular location of the bacilli have not been identified. Understanding the response of innate immune cells to *M. ulcerans* may provide insights to host defenses and tactics used by the pathogen to circumvent these defenses.

Phagocytosis constitutes the primary line of host innate defense against incoming microbial pathogens, providing an efficient means for their removal and destruction. Phagocytic cells such as granulocytes, monocytes, and macrophages, defend against different pathogens by ingesting and killing invaders. However, several virulent bacteria have evolved mechanisms to prevent their phagocytosis as part of their pathogenic profile. Such mechanisms allow the organism to block phagocytic responses and in some instances to avoid destruction via degradative endocytic pathways, thereby impairing development of cellular immunity and enhancing extracellular survival (61). How microorganisms are phagocytosed and killed, and why certain pathogens resist these mechanisms, are crucial questions for an understanding of the pathogenesis of infectious diseases and development of innovative treatment approaches.

In order to better understand the poor immune response generated following *M. ulcerans* infection, the following hypotheses were tested:

- The diminished acute inflammation in early infection with *M. ulcerans* may be partially because the bacteria are ineffectively phagocytosed. Mycolactone, the *M. ulcerans* toxin, may paralyze macrophages rendering them incapable of

phagocytosis. Cellular response to mycolactone may be dose dependent.

- Lack of an inflammatory reaction and recruitment of immune cells during the necrotizing stage of Buruli ulcer could be due to abrogation of production of inflammatory cytokines and lowered expression of cell adhesion molecules.

- Gene transcription profiling of cells treated with mycolactone may yield valuable information that may give an insight into the pathway/s of action of mycolactone.

To test these hypotheses, our objectives were as follows:

1. Elucidate the kinetics and efficiency *in vitro* of phagocytosis of *M. ulcerans* by macrophages and the capacity of the bacteria to survive within phagocytic cells.
2. Examine the effect of mycolactone on the cellular and immunological responses to *M. ulcerans* by comparing wild type (WT) *M. ulcerans* with an isogenic mycolactone negative mutant.
3. Identify signaling pathways involved in the cellular response to mycolactone using microarrays.

Chapter 2

MATERIALS AND METHODS

Bacterial and yeast cultures

A well characterized clinical isolate, *Mycobacterium ulcerans* 1615 (ATCC 35840; ATCC, Manassas, VA) (62) (36) isolated originally from a Malaysian patient, was obtained from the Trudeau Collection (Trudeau Institute, Saranac lake, NY) and used as wild type (WT) strain in all studies. MU 1615A was isolated as a nonpigmented, spontaneous mutant from MU 1615 and characterized by mass spectroscopy, PCR analysis and cytopathicity assays (36). PCR analysis using probes to the mycolactone plasmid and mycolactone genes (63) showed that loss of mycolactone production in this isolate occurred due to plasmid deletions in which partial deletion of *mls*A and absence of *mls*B resulted in loss of mycolactone production (Fig. 1.1). Deletions in this portion of the plasmid have been reported recently in other isolates (64).

The mycolactone core lactone is encoded by *mls*A and the fatty acid side chain by *mls*B (42). Transposon mutagenesis was used to generate strains with insertions in polyketide synthase genes, *mls*A and *mls*. Because of the extreme redundancy in the mycolactone gene sequence, mass spectroscopic analysis was used to determine whether a particular transposon insertion had occurred in *mls*A or *mls*B (64). Although the loss of *mls*A results in total absence of mycolactone, core lactone lipid was detected in strains MU1615::Tn84, MU1615::Tn94 and MU1615::Tn100 confirming that these isolates contain insertions in *mls*B. Mass spectroscopy analysis and cytopathicity assays were used to confirm the absence of mycolactone in all mycolactone negative mutants (*myc*-). Mycobacterial strains were grown in Middlebrook 7H9 medium (Difco, Detroit, MI)

supplemented with 10% oleic acid-albumin-dextrose enrichment (OADC) and Middlebrook 7H10 agar (Difco, Detroit, MI) and incubated at 32^0C. OADC was prepared with bovine serum albumin (BSA; Sigma, St. Louis, MO) and all other reagents (Fischer Scientific, Pittsburg, PA). *Candida albicans* SC 5364 was grown overnight in yeast extract potato dextrose (YEPD; Difco, Detroit, MI) medium at 30°C.

Cell culture

J774A.1 mouse macrophage cells (ATCC TIB 67), L929 mouse fibroblasts (ATCC CCL1) and THP-1 human monocytes (ATCC TIB-202) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco modified Eagle medium (DMEM, Mediatech Inc.) supplemented with 2mM L-glutamine (Bio Whittaker) and 10% (macrophages, monocytes) or 5% (fibroblasts) heat inactivated fetal calf serum (FCS, Mediatech Inc.) at 37 ^{0}C in a humidified atmosphere containing 5% CO_2.

Bacterial uptake assay

Mouse macrophages (J774, 10^5cells/ml) were grown overnight in a 24-well plate and infected with WT and mycolactone mutant strains of *M. ulcerans*. Mycobacteria were passed through a 25-gauge needle 10 times to break up clumps, and 10µl of the suspension were added to each well to give a multiplicity of infection (MOI) of approximately 10 bacteria: 1 macrophage (quantified by plating dilutions of inoculum). Plates were centrifuged at 1500rpm for 5 min to facilitate contact between bacteria and adherent macrophages. Phagocytosis was allowed to occur at 37°C, 5%CO_2. After 24 h,

infected monolayers were washed with phosphate-buffered saline (PBS), lysed with 0.01% sodium dodecyl sulfate (SDS; Fischer Scientific) and plated. Plates were incubated at 32^0Cfor 6 weeks and percentage of inoculum internalized was determined. Samples were tested in triplicate and repeated at least twice.

Intracellular survival assay

Cells were infected as described in the bacterial uptake assay. After 8 h, the supernatant was removed, cells were washed to remove non-adherent bacterial cells and fresh tissue culture medium with 100 μg/ml of amikacin was added. Cells were washed every two days and tissue culture media containing 20 μg/ml of amikacin was added. At 1, 3, and 6 days following infection, macrophages were lysed with 0.01%SDS, the lysate was diluted ten-fold and plated on M7H10 agar supplemented with 10% OADC. Plates were incubated at 32^0C for 6 weeks and colony forming units (CFUs) were determined. Samples were run in triplicate and repeated at least twice.

Inhibition of phagocytosis

Murine macrophages (J774) were treated with mycolactone, 10ng/ml, 400ng/ml or 7.9μg/ml, for 12 h. *C. albicans* was then added at a MOI of 10:1. After 3 h, the supernatant was removed and cells were washed with PBS. After lysis of cells with 0.01% SDS, lysate was diluted ten-fold and plated on YEPD agar to determine the percentage of inoculum internalized. Plates were incubated at 30^0C for 24 h and CFU were determined. Results are from triplicate samples from a typical experiment.

Neutrophil chemotaxis asays

Peripheral blood neutrophils were isolated from 30 ml of human blood sample collected from the antecubital vein of a healthy individual using sodium citrate as anticoagulant. The protocol for drawing blood from healthy volunteers was approved by the institutional review board of the University of Tennessee (IRB # 6476B). Blood was mixed with equal volume of saline and centrifuged for 30 minutes at room temperature in a Ficoll-Histopaque discontinuous gradient (Mediatech, Herndon, VA). The upper layer was discarded and the pellet was mixed with 20 ml of 3% dextran, and centrifuged at 400 x g for 5 minutes. Neutrophils were recovered and contaminating erythrocytes were lysed by hypotonic shock. Cells were washed and the pellet was resuspended at a concentration of 5×10^6 cells/ml in fresh chemotaxis buffer (prepared from Hank's balanced salt solution, Gibco BRL, Grand Island, NY; 0.1% BSA, Roche, Indianapolis, IN; and 10mM HEPES, Gibco BRL). Neutrophils suspended in chemotaxis buffer were added to the upper compartment of a 96-well ChemoTx System chemotaxis chamber (NeuroProbe, Inc., Gaithersburg, MD.).

For the study of chemotaxis, bacterial samples were added in 30 µl chemotaxis buffer to the lower compartment of the chemotaxis chamber. IL-8 served as positive control. Plates were incubated for 1 h at 37°C under a 5% CO_2 atmosphere. The number of cells that migrated to the lower wells was calculated by counting 5 fields of triplicate 10 µl aliquots on a microscope using a hemocytometer. Each experiment was repeated at least three times and samples were run in triplicate.

LDH release and apoptosis

L929 fibroblasts and peripheral blood human neutrophils were assayed for cell death via apoptosis and necrosis. Cytotoxicity/necrosis was measured using a colorimetric kit from Promega (Madison, WI) as described previously (65). Briefly, cells were suspended in DMEM supplemented with 5% (fibroblasts) or 10% (neutrophils) FCS and L-glutamine and were seeded at a concentration of 6×10^3 cells/well in a 96 well plate. The release of the cytoplasmic enzyme, lactate dehydrogenase (LDH), from mycolactone treated, permeabilized cells was measured 4 and 24 h post infection by using the colorimetric kit from Promega and following manufacturer's instructions. Background release of LDH was obtained by measurement of ethanol treated cells, and maximum release of LDH was obtained by lysis of untreated cells according to manufacturer's protocol (Fig. 2.1). The percent LDH release was then determined using the following calculation: [(release of LDH from mycolactone treated cells-background release from untreated cells)/(Maximum release of LDH by cell lysis-background release)] x 100.

Apoptosis was measured from mycolactone treated neutrophils and fibroblasts at 4 h and 24 h post treatment using the Cell Death Detection Plus enzyme-linked immunosorbent assay (Roche) as described previously (Fig. 2.2) (65). Apoptosis was then determined as fold enrichment of nucleosomes using the following calculation: [(measurement of DNA-histone complex from treated cells / background measurement of untreated cells)]. Samples were run in triplicate and repeated at least twice.

The general chemical reactions of the CytoTox 96® Assay are as follows:

$$\text{NAD}^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\text{Diaphorase}} \text{NAD}^+ + \text{formazan (red)}$$

Fig. 2.1. Schematic showing the cytotoxicity assay chemical reaction. Taken from Promega catalogue 2003

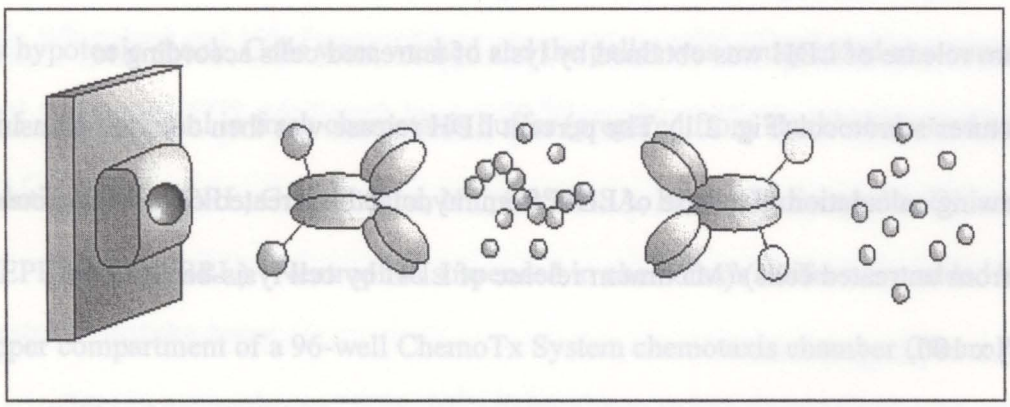


Fig. 2.2. Schematic illustrating the ELISA assay used to detect cell death. Taken from Roche catalogue 2003.

Mycolactone isolation

Mycolactone was isolated from MU 1615 in late exponential growth phase as described previously (3, 36, 66). Briefly, lipids were extracted from WT 1615 using chloroform:methanol 2:1 (vol/vol) and phospholipids were removed by precipitation with ice cold acetone. Purified mycolactone was obtained using a chromatotron (Harrison, CA) as described previously and analyzed by running on a silica thin layer chromatography (TLC) plate (Alltech, Deerfield, IL) in a solvent system of chloroform:methanol:water (90:10:1). Lipids were visualized with ceric sulfate-ammonium molybdate in 2 M sulfuric acid stain. Mycolactone was then tested in triplicate on L929 murine fibroblasts for cytopathicity (CPA). This CPA phenotype has been established previously as the minimal concentration of toxin per milliliter that causes inhibition of cell growth by 24 h and 90% cell rounding with loss of the monolayer by 48 h (13, 35-36).

Enzyme linked immunosorbent assays (TNF-α, IL-8)

Human THP-1 cells seeded at 1×10^5 cells/well were grown in a 24-well plastic tissue culture plate and differentiated using phorbal 12-myristate-13 acetate (Sigma) (100 ng/ml). After 24 h, the supernatant was removed and fresh tissue culture medium supplemented with 10% FCS and L-glutamine was added. Cells were stimulated with wild type *M. ulcerans* (1615), mycolactone negative mutants of *M. ulcerans* (MU 1615A, MU 1615::Tn119), *M. fortuitum* (MOI of 10:1), mycolactone (50 ng/ml, 400 ng/ml or 50 μg/ml), LPS (10ng/ml) or wild type *M. ulcerans* and LPS (Sigma) (10ng/ml). Untreated and ethanol treated cells served as negative controls. Culture supernatants of triplicates

were collected 24 h post infection and used for quantification of secreted TNF-α and IL-8 by sandwich ELISA using commercially available antibody pairs according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Briefly, culture supernatants were added to TNF-α or IL-8 specific monoclonal antibody-coated wells followed by biotinylated specific monoclonal secondary antibody. After incubation and washing with PBS tween, strepavidin peroxidase was added. After incubation and washing to remove unbound enzyme, a substrate solution (ABTS, 2,2'-Azino-di-(3-ethylbenz-thiazoline Sulfonic Acid, Zymed, San Fransisco, CA) was added to induce a colored reaction product. The intensity of the color reaction was measured at 405nm to estimate the concentration of TNF-α or IL-8 present in the culture supernatant. Sample concentrations were calculated using a standard curve. Results were expressed as amount of cytokine or chemokine in pg/ml. Data are expressed as means ± S.D. (n=3).

Intracellular adhesion molecule-1 (ICAM-1) expression

Human THP-1 cells seeded at 1×10^5 cells/ml were grown in a 12-well plastic tissue culture plate and differentiated using phorbal 12-myristate-13 acetate (100 ng/ml). After 24 h, the supernatant was removed and fresh tissue culture medium supplemented with 10% FCS and L-glutamine was added. Cells were infected with WT *M. ulcerans*, *myc*- (mycolactone negative) *M. ulcerans* (119, 1615A), mycolactone (100 ng/ml, 1000 ng/ml), LPS (10 ng/ml, 100 ng/ml), or mycolactone (100 ng/ml, 1000 ng/ml) and LPS (10ng/ml). Uninfected and ethanol treated cells served as negative controls. Supernatant was removed 24 h post infection and cells were washed 3 times with PBS. Cells were

then incubated with diluted (1:200) fluorescein labeled anti-human ICAM-1 antibody (Leinco Technologies, St. Louis, MO) for 45 min at room temperature according to manufacturer's instructions. Cells were washed 3 times with PBS tween to remove any unbound antibody and percentage of ICAM-1 positive-cells was determined using a fluorescent activated cell sorter (FACS, BD FACScan, San Jose, CA) and analyzed using CellQuest software.

Western immunoblot analysis of I-kB degradation/inhibition

J774 cells (1×10^5) were grown in a 6-well tissue culture plate overnight. Cells were exposed to LPS (20 ng/ml) for 15 or 30 min, mycolactone (100 ng/ml) for 1 h mycolactone 1 h and LPS for 15 min or 30 min. Untreated and ethanol treated cells served as controls. After the indicated time points, the supernatant was removed and cells were washed 3 times with ice cold PBS. Cells were lysed with 100 µl of sample loading and lysis buffer, boiled at 95°C for 5-10 min and stored at -20°C for future use. Cell extracts (50 µl) were then analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis using a Mini Protean II gel apparatus (BioRad, Hercules, CA) according to the method described by Laemmli (67). Stacking gels of 4% and resolving gels of 10%(acrylamide/bisacrylamide 30:0.8, National Diagnostics, Atlanta, GA) were used. Protein samples in 3x sample buffer (prepared with 1M Tris-cl pH 6.8 2.4ml, 20% SDS 3ml, Glycerol (100%) 3ml, β-Mercaptoethanol 1.6ml, Bromophenol blue 0.006g, total volume of 10ml stored at 4°C) were electrophoresed at 100V for 1-1.5 h.

Gels were subsequently soaked in protein transfer buffer (9g Tris base, 43.2g glycine suspended in 2.4L ddH2O and 0.6L methanol) for 15 minutes. Proteins were then transferred onto Immobilon-P polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA), prewetted with methanol and soaked in protein transfer buffer, using a semi-dry apparatus (Bio-Rad, Hercules, CA) set at 100V for 1.5 h. For western blot, unbound protein sites on the membrane were first blocked for 1 h in 5% NFDM in TBS-Tween 20 (8g Nacl, 20ml 1M Tris Hcl, 1L ddH2O, pH 7.6, 0.1% tween 20) with 0.1% sodium azide. IκBα was detected by incubating the membrane with primary antibody (Santa-Cruz Biotechnology Inc., CA) 1: 1000 dilution in 5% NFDM in TBS- tween 20 overnight at 4°C with gentle agitation, washed with TBS-Tween 20 three times for 10 min each time and incubated with secondary horse radish peroxidase-antibody (Santa-Cruz Biotechnology Inc., Santa Cruz, CA) 1:1000 dilution in 5% NFDM in TBS-tween 20. Immunoblots were developed by addition of 15 mg 4-chloro-1-napthol (Bio-Rad) dissolved in 5ml ice cold methanol and 15μl of ice cold 30% H_2O_2 in 25 ml Tris buffered saline (TBS, 20nM Tris, 0.5M NaCl, pH 7.2). Development was at room temperature for 30 min and was terminated by washing the membrane extensively with water. Protein sizes were determined by comparison to a set of standards (Rainbow markers, Bio-Rad).

Coomassie blue visualization of proteins, SDS/PAGE was performed as described previously. Gels were placed in Coomassie staining solution (0.5% Coomassie brilliant blue R (Bio-Rad) in 50% dH2O, 40% methanol, and 9.5% glacial acetic acid) and incubated for 1 h. All unbound dye was removed by 3 to 5 washes with destaining solution (50% dH_2O_2, 40% methanol and 10% glacial acetic acid).

Statistical analysis

Data were analyzed by using Students t-test. Bars represent the mean ± standard deviation of experiments performed in triplicate.

Chapter 3

INTERACTION OF *MYCOBACTERIUM ULCERANS* WITH MACROHAGES, FIBROBLASTS AND NEUTROPHILS

Introduction

Monocytes/macrophages and neutrophils represent two arms of the innate immune response. Neutrophils are found in blood; however their site of action is the extravascular space. Neutrophils actively migrate to the site of infection through a process involving adhesion to vascular endothelium and chemotaxis towards the site of infection. Macrophages are resident phagocytes found in many different organs (13, 51, 68). Monocytes, which are macrophage precursor cells, migrate from the blood towards the respective tissues, where they differentiate into macrophages. Macrophages play key roles in host defense by recognizing, engulfing, and killing microorganisms. A key element in the cellular machinery that allows phagocytosis is the actin cytoskeleton. It has been known for a long time that disruption of the actin cytoskeleton by certain toxins inhibits phagocytosis (69).

Pathogenic mycobacteria such as *M. tuberculosis* and *M. leprae* can survive and grow within host cells, particularly macrophages. The initial response to macrophage at the site of invasion is likely to play a key role in determining the outcome of the interaction and the overall regulation of the ensuing, adaptive, response (70). Unlike most other mycobacterial pathogens, such as *M. tuberculosis* and *M. leprae* which are intracellular, *M. ulcerans* infected tissues primarily contain extracellular bacilli, with microcolonies containing large number of extracellular acid-fast bacilli (AFB) in the

center of the lesions (17). Infection with mycobacterial pathogens elicits a strong inflammatory response. In contrast, *M. ulcerans*, the causative agent of Buruli ulcer, is unique among human mycobacterial pathogens in that the bacteria are primarily extracellular and there is a limited inflammatory response to the infection (4, 35, 71). Here I show that mycolactone is the major virulence factor that influences phagocytosis of *M. ulcerans* by macrophages.

A wide variety of pathogenic microorganisms have been demonstrated to cause eukaryotic cell death, either as a consequence of infecting host cells or by producing toxic products (72). Host cell death may impair normal organ function and lead to associated signs and symptoms of disease. Microbial pathogens may improve their ability to persist in infected hosts by causing death of cells required for host defense (73). Pathogen-induced cell death may occur by a variety of complex mechanisms. Elucidating the factors required by a pathogen to kill host cells is, therefore, critical to uncovering mechanisms of pathogenesis. Understanding the process of dying may reveal why certain cells may be more or less susceptible to pathogen-induced cell death and reveal novel therapeutic targets. Furthermore, the mechanism of cell death may have significant consequences in terms of the ensuing response to the dead cell by modulating inflammation or influencing the immune response (74). Cell death is typically discussed dichotomously as either apoptosis or necrosis. Apoptosis is described as an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation. Necrosis has been characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents. Multiple types of death may be observed simultaneously in tissues or cell

cultures exposed to the same stimulus. The local intensity of a particular initial insult may influence the mechanism of demise taken by individual cells in a population (72).

Preliminary data indicated that WT *M. ulcerans* are taken up less efficiently by macrophages in comparison to *myc*- spontaneous mutants of *M. ulcerans*. The purpose of this study was therefore, first to investigate whether *M. ulcerans* can be taken up by macrophages and second to further elucidate the role of mycolactone in the interaction of *M. ulcerans* with macrophages and neutrophils by using well characterized transposon insertion *myc*- mutants. *M. ulcerans* strains tested are listed in Table 1. The results of this study demonstrate that mycolactone inhibits the uptake of *M. ulcerans* by macrophages. Also described, for the first time is the effect of mycolactone concentration on whether the cell undergoes apoptosis or necrosis.

Results

Mycolactone inhibits macrophage uptake of *M. ulcerans*

A partial explanation for the absence of detectable intracellular *M. ulcerans* in macrophages from tissue from Buruli ulcer patients might be that mycolactone is antiphagocytic. To test this hypothesis, J774 murine macrophages were assayed for the ability to internalize *M. ulcerans* and three Mu*myc*-, Mu1615::Tn84, Mu1615::Tn94, and Mu1615::Tn119. Mu*myc*- were internalized in significantly greater numbers than the parental *M. ulcerans* 1615 (Figs. 3.1, 3.2). The percentage of inoculum internalized for the mutants was in the range of 10.0 - 90.0% compared with 0.7% for isogenic WT *M.*

Table 1. *M. ulcerans* strain list.

Name of the strain	Characteristics	Source
M. ulcerans 1615	Virulent wild type strain	Trudeau Collection
M. ulcerans 1615::Tn84, *M. ulcerans* 1615::Tn100	Mycolactone negative; insertion in polyketide synthase gene *mls*B	Small lab
M. ulcerans 1615::Tn89	Mycolactone negative; insertion in protoporphyrin IX magnesium chelatase gene	Small lab
M. ulcerans 1615::Tn90	Mycolactone negative	Small lab
M. ulcerans 1615::Tn94, *M. ulcerans* 1615::Tn119	Mycolactone negative; insertion in polyketide synthase gene *mls*B	Small lab
M. ulcerans 1615::Tn96	Mycolactone negative	Small lab
M. ulcerans 1615::Tn97	Mycolactone negative; insertion in FurA gene	Small lab
M. ulcerans 1615::Tn107	Mycolactone negative; insertion element (IS2606)	Small lab
M. ulcerans 1615::Tn114	Mycolactone negative; insertion in succinate dehydrogenase gene	Small lab
M. ulcerans 1615::Tn120	Mycolactone negative; insertion in hypothetical Rv2079 gene	Small lab

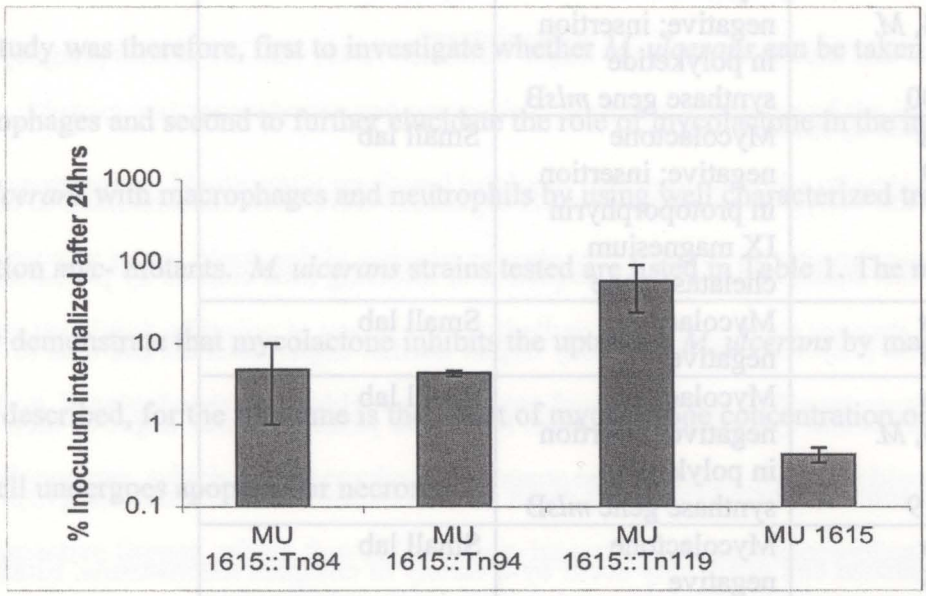


Fig. 3.1. Uptake of wild and mycolactone mutant strains of *M. ulcerans*. J774 cells were infected with wild and mycolactone mutant strains of *M. ulcerans* (MOI 10:1). Data are expressed as percentage of total inoculum internalized after 24 h. MU1615 versus MU1615::Tn84, MU1615::Tn94 and MU1615::Tn119, $P<0.22$, 0.001 and 0.025 respectively.

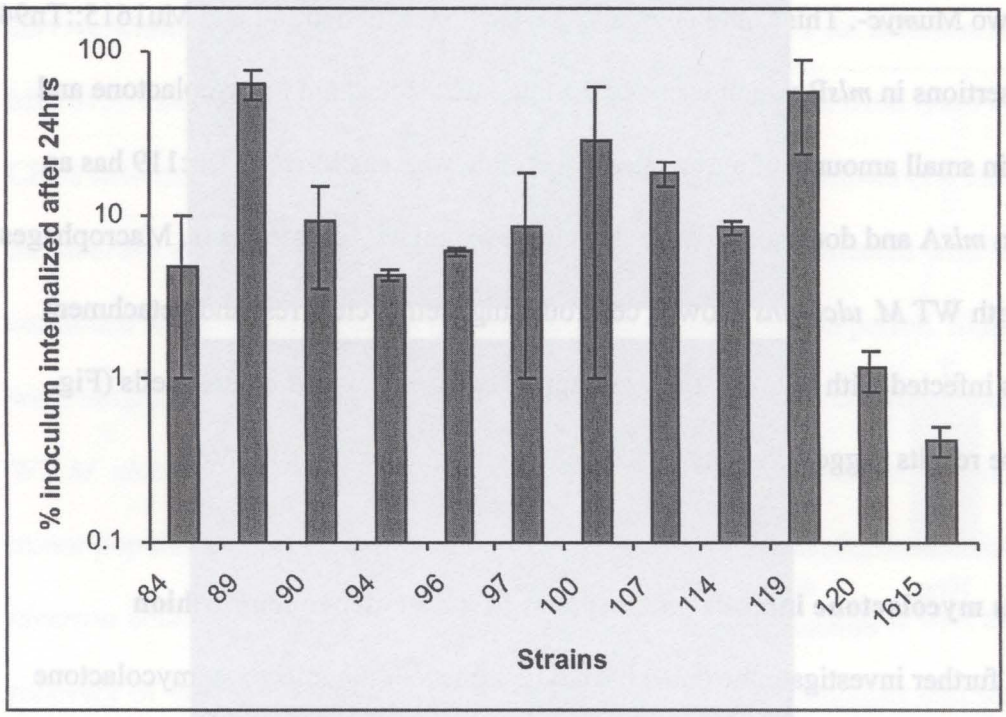


Fig. 3.2. Uptake of wild and several mycolactone mutant strains of *M. ulcerans*. J774 cells were infected with wild and mycolactone mutant strains of *M. ulcerans* (MOI 10:1). Data are expressed as percentage of total inoculum internalized after 24 h.

ulcerans. There were also significant differences between the macrophage uptake of different Mu*myc*-. Mu1615::Tn119 was phagocytosed significantly ($P<0.05$) better than the other two Mu*myc*-. This could be due to the fact that Mu1615::84 and Mu1615::Tn94 contain insertions in *mls*B which encodes the fatty acid side chain of mycolactone and may contain small amounts of a truncated polyketide whereas Mu1615Tn::119 has an insertion in *mls*A and does not produce detectable mycolactone precursors. Macrophages infected with WT *M. ulcerans* showed cell rounding, cell cycle arrest and detachment while cells infected with myc- *M. ulcerans* appeared normal as did control cells (Fig. 3.3). These results suggest that mycolactone has anti-phagocytic activities.

Exogenous mycolactone inhibits phagocytosis in a dose-dependent fashion

To further investigate the anti-phagocytic effects of mycolactone, mycolactone treated macrophages were assayed for their ability to take up *Candida albicans*. In these studies, *C. albicans* cells were added to macrophages which had been treated with 10 ng, 400 ng, and 7.9 μg mycolactone for 12 h and the percent inoculum internalized was determined by CFUs. Despite the fact that both 10 ng and 400 ng of mycolactone drastically altered the morphology of macrophages, this did not alter their phagocytic ability (Fig. 3.4). However, mycolactone did inhibit phagocytosis of *C. albicans* at a concentration of 7.9 μg. These results show that the antiphagocytic activity of mycolactone is a characteristic of cell-free, as well as bacterial bound mycolactone but only at high concentrations.

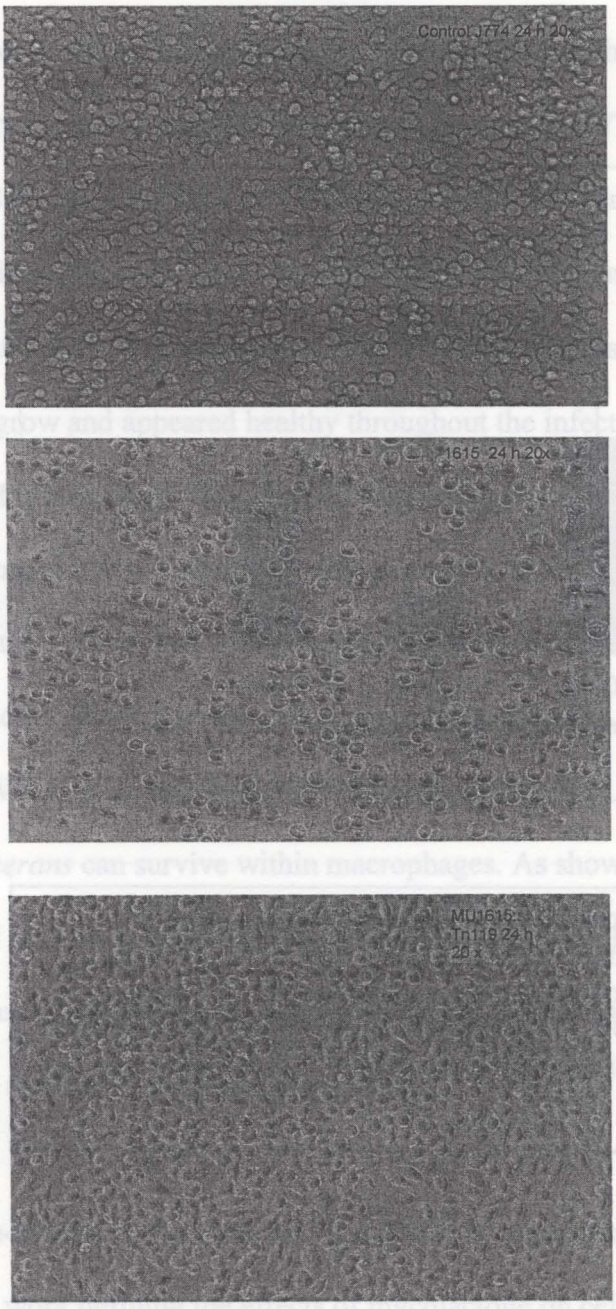


Fig. 3.3. Effect of WT *M. ulcerans* and *myc*- MU 1615::Tn119 on J774 murine macrophage cell line.

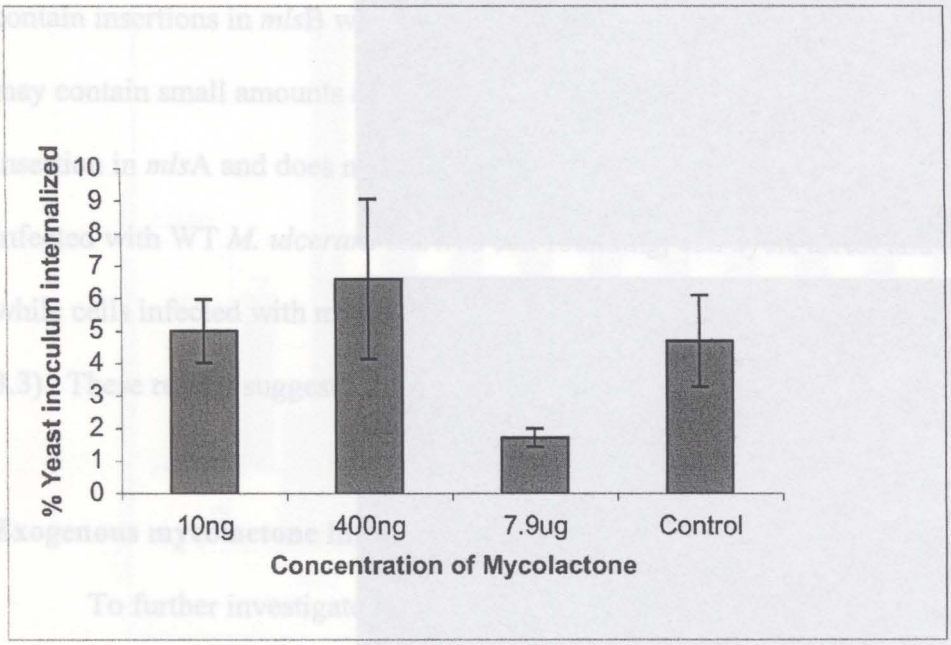


Fig. 3.4. Effect of mycolactone pretreatment on phagocytosis of *C. albicans* by murine macrophage cell line (J774). Macrophages were treated with different concentrations of mycolactone for 12 h prior to addition of *C. albicans* (MOI 10:1). After 3 h cells were lysed and plated to determine percentage of inoculum internalized. Control versus 7.9 μg treated cells, $P \leq 0.02$.

M. ulcerans mycolactone negative mutant survives within macrophages

In order to determine whether mycolactone affected the ability of *M. ulcerans* to survive within macrophages, J774 cells were infected with WT *M. ulcerans* and Mu1615::Tn84 for 6 d. At 2 d intervals, bacteria were recovered from lysed cells and CFUs determined. Mu1615::Tn84 were recovered in greater numbers ($P \leq 0.01$) than WT *M. ulcerans* at 3 and 6 d post-infection (Fig. 3.5). Monolayers infected with MU *myc*- continued to grow and appeared healthy throughout the infection period. Similar results were obtained with Mu*myc*- strains 1615A (data not shown). In contrast, infection with WT *M. ulcerans* resulted in destruction of over 90% of the monolayer by 2 d. However, a minor population of attached cells was present throughout the infection. A few (< 10) *M. ulcerans* could be detected in these cells by acid fast staining. This, as well as the recovery of WT *M. ulcerans* throughout the infection period, suggests that small numbers of WT *M. ulcerans* can survive within macrophages. As shown in Fig. 3.6, no difference was observed in *in vitro* growth of MU 1615 and MU 1615::Tn84 in M7H9 liquid broth up to 15 wk as determined by the number of CFUs. These studies show that Mu*myc*- are not cytotoxic and replicate within macrophages.

Mycolactone-mediated necrosis and apoptosis is concentration dependent

Early work defining the effects of mycolactone on cells showed that the molecule caused apoptosis at concentrations as low as 2 pg/ml (13, 36). However, the limited availability of chemically pure mycolactone restricted investigations to the use of small concentrations of mycolactone. Preliminary evidence suggested that *M. ulcerans* might

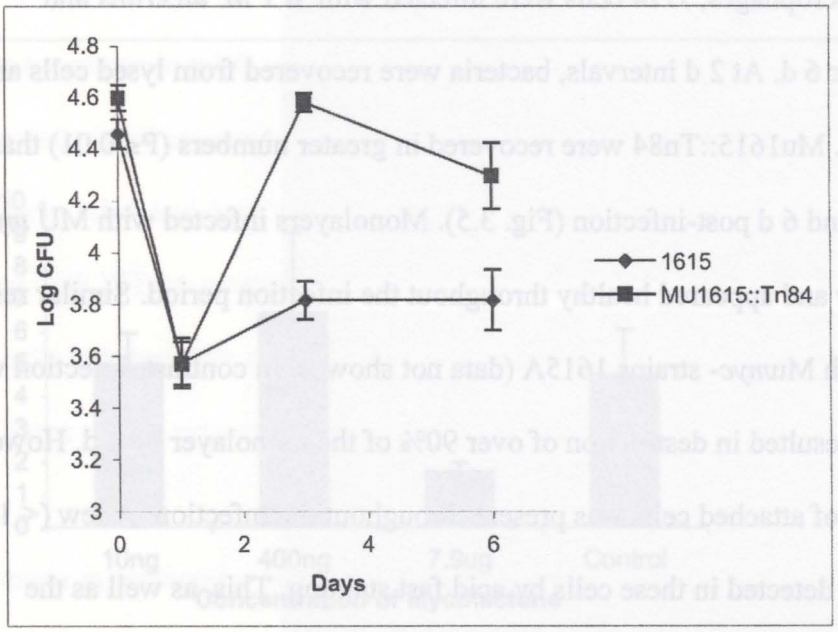


Fig. 3.5. Intracellular survival of wild and mycolactone mutant strains of *M. ulcerans* in J774 cells. The intracellular survival of WT *M. ulcerans* 1615 and Mu1615::Tn84 (MOI 10:1) after 1, 3 ($P < 0.01$) and 6 ($P < 0.01$) days of infection was measured by lysing cells and plating lysate.

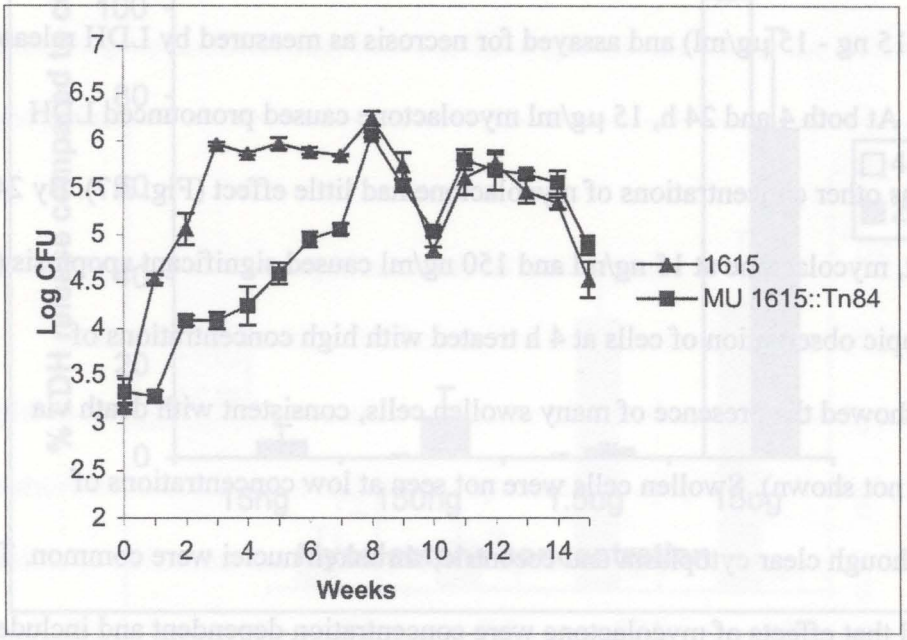


Fig. 3.6. Growth curve of WT *M. ulcerans* and MU 1615::Tn84 in M7H9 broth. Growth was determined by diluting and plating the culture on M7H10 agar plates every 5 d for a duration of 15 wk.

secrete a protein toxin which caused necrosis but results from these studies were complicated by the fact the protein fractions were contaminated with mycolactone (65). In order to investigate the concentration-dependent activity of mycolactone more thoroughly, fibroblasts were exposed to increasing concentrations of mycolactone (15 ng - 15 μg/ml) and assayed for necrosis as measured by LDH release, and apoptosis. At both 4 and 24 h, 15 μg/ml mycolactone caused pronounced LDH release whereas other concentrations of mycolactone had little effect (Fig. 3.7). By 24 h post-treatment, mycolactone at 15 ng/ml and 150 ng/ml caused significant apoptosis (Fig. 3.8). Microscopic observation of cells at 4 h treated with high concentrations of mycolactone showed the presence of many swollen cells, consistent with death via necrosis (data not shown). Swollen cells were not seen at low concentrations of mycolactone though clear cytoplasm and eccentric, shrunken nuclei were common. These results showed that effects of mycolactone were concentration dependent and included both rapid changes in cell permeability, as measured by LDH release, and apoptosis.

M. ulcerans is defective in neutrophil chemotaxis

The relative paucity of neutrophils in human and guinea pig *M. ulcerans* lesions compared with *M. marinum* lesions could be due to a defect in neutrophil recruitment and/or because neutrophils that are attracted to the site of infection are rapidly killed. To explore the possibility of a defect in neutrophil chemotaxis, *M. ulcerans* 1615, and MU 1615::Tn119, were tested for their ability to recruit human neutrophils in an *in vitro* chemotaxis assay. Neither WT *M. ulcerans* nor MU 1615::Tn119 induced a chemotactic

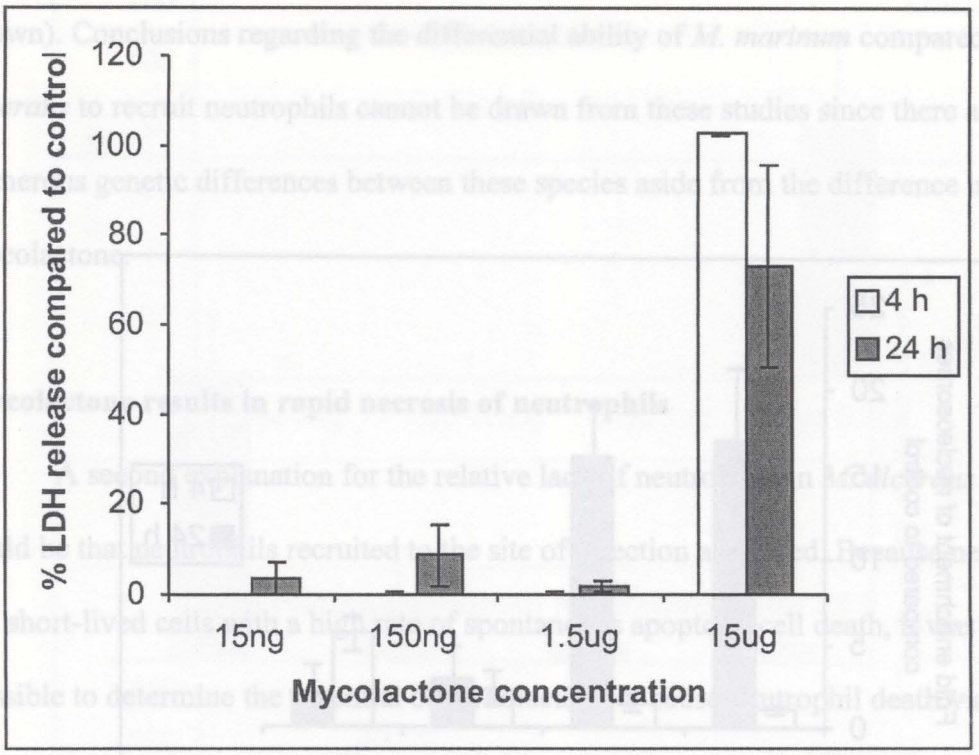


Fig. 3.7. Mycolactone-mediated necrosis of L929 murine fibroblasts. Cytotoxicity measured by LDH release. Culture supernatants were collected from wells containing L929 cells 4 and 24 h after mycolactone treatment, and the amount of LDH was measured using a CytoTox 96 assay kit (Promega). 15 μg versus all other treatments, $P < 0.005$.

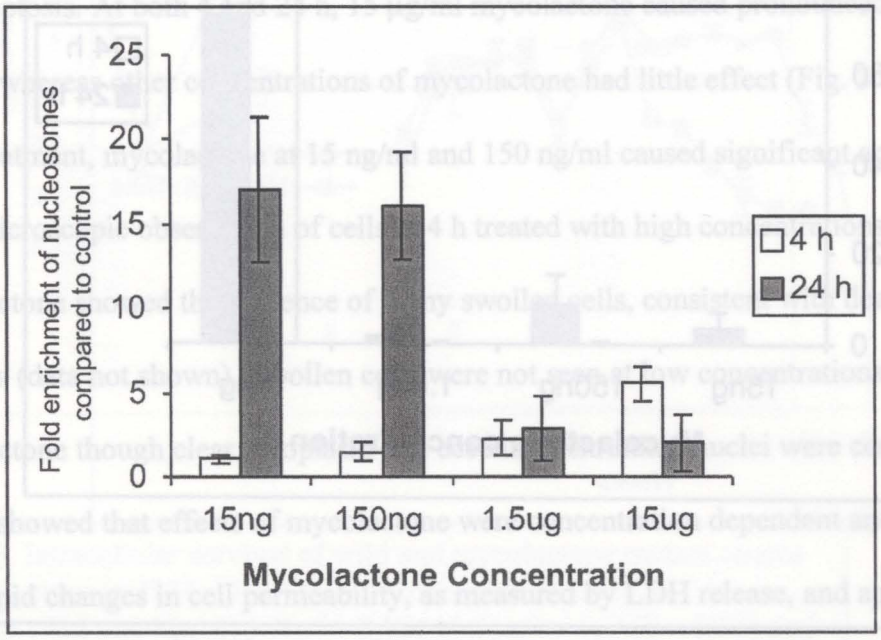


Fig. 3.8. Mycolactone-mediated apoptosis of L929 murine fibroblasts. Apoptosis was assessed with the cell death detection enzyme-linked immunosorbent assay (ELISA) kit and expressed as fold enrichment of nucleosomes. Data are mean and standard deviation of the values obtained from triplicate samples. 15ng, 150ng versus all other treatments, $P < 0.005$.

response in human neutrophils (Fig. 3.9). In contrast, *M. marinum*, a mycobacterial control, elicited as great a chemotactic response as the positive IL-8 control (data not shown). Conclusions regarding the differential ability of *M. marinum* compared with *M. ulcerans* to recruit neutrophils cannot be drawn from these studies since there are numerous genetic differences between these species aside from the difference in mycolactone.

Mycolactone results in rapid necrosis of neutrophils

A second explanation for the relative lack of neutrophils in *M. ulcerans* lesions could be that neutrophils recruited to the site of infection are killed. Because neutrophils are short-lived cells with a high rate of spontaneous apoptotic cell death, it was only possible to determine the potential of mycolactone to cause neutrophil death via necrosis. Results from these studies show that mycolactone causes dose-dependent necrosis of human neutrophils within 24 h (Fig. 3.10 A, B). Neutrophils treated with high concentrations of mycolactone showed severe loss of membrane permeability as shown by cytotoxic effects seen in Fig. 3.11. Taken together, these results suggest that the paucity of neutrophils in *M. ulcerans* infection focus may be due to a lack of chemotactic stimuli as well as to killing of cells which are attracted to the site of infection.

Discussion

M. ulcerans is unique among mycobacterial pathogens in three respects: 1) the organism produces an extracellular infection and 2) virulence is toxin-mediated and 3)

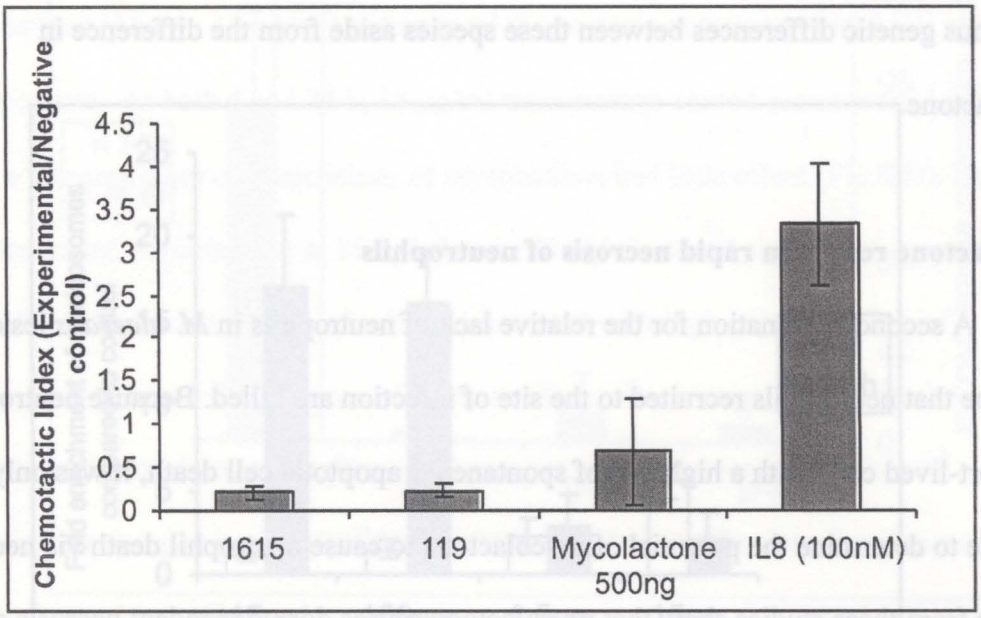


Fig. 3.9. Migration of human neutrophils in response to wild and mutant strains of *M. ulcerans* or purified mycolactone. Chemotaxis assays were performed using a double-chamber system. Wild and mycolactone mutant strains of *M. ulcerans* were used as chemoattractants in the lower chamber and human neutrophils purified from peripheral blood were seeded in the upper chamber. Interleukin 8 (IL-8) (100nM) served as positive control and buffer alone as a negative control. After 1 h transmigrated cells were recovered from the lower chamber and counted using a hemocytometer. Data are expressed as chemotactic index (ratio of chemotactic movement to random migration) ±standard deviation. IL-8 versus WT *M. ulcerans* 1615 and Mu1615::Tn119, $P \leq 0.01$.

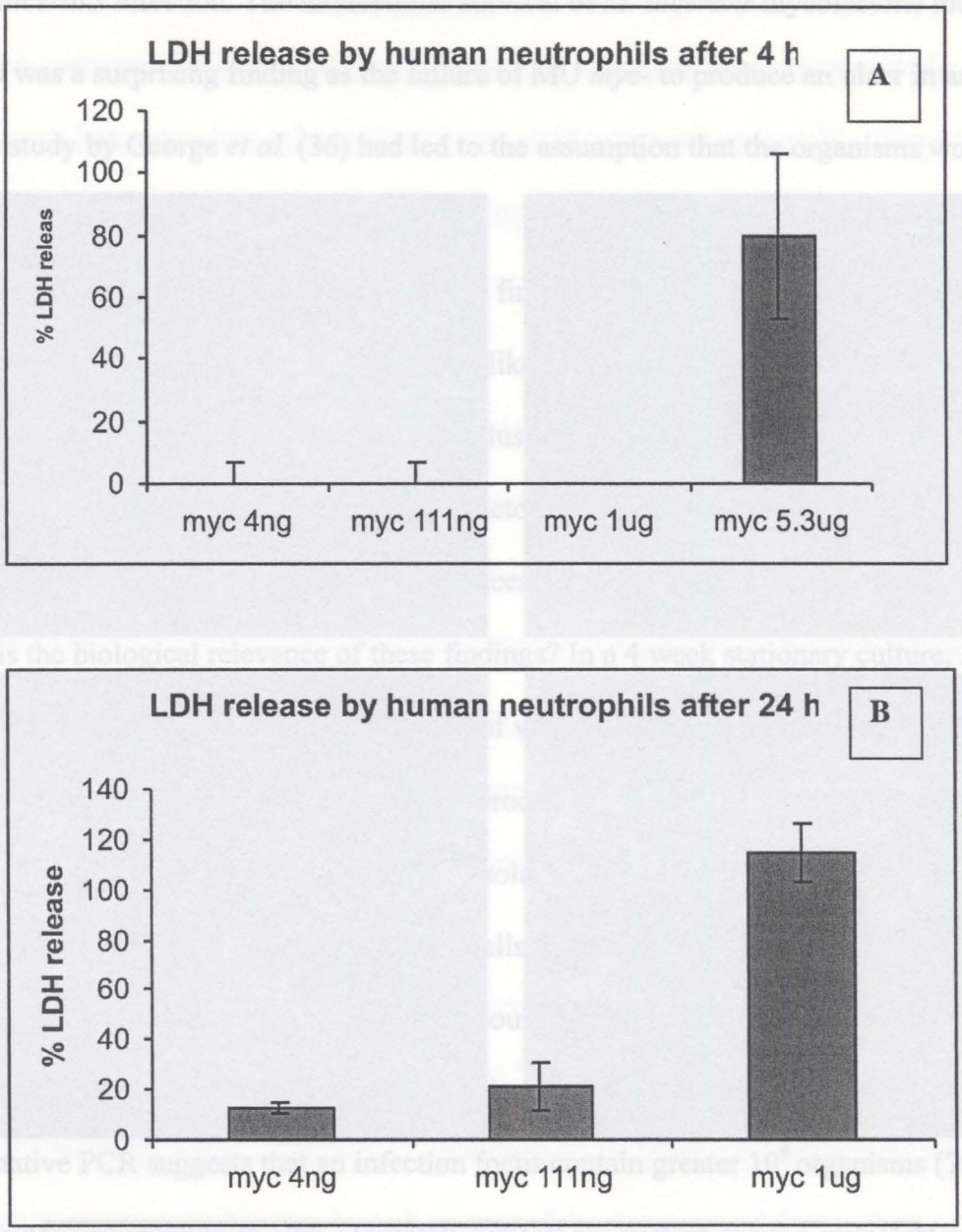


Fig. 3.10. Necrosis of human neutrophils treated with different concentrations of mycolactone for 4 **(A)** and 24 **(B)** h. Values obtained were normalized by subtraction of background levels of necrosis of untreated cells at each time point. All experiments were performed in triplicate. Data are means ± standard deviations (n=3) from one representative experiment of two. Highest concentration (5.3 μg, 1 μg) versus all other treatments, $P \leq 0.005$.

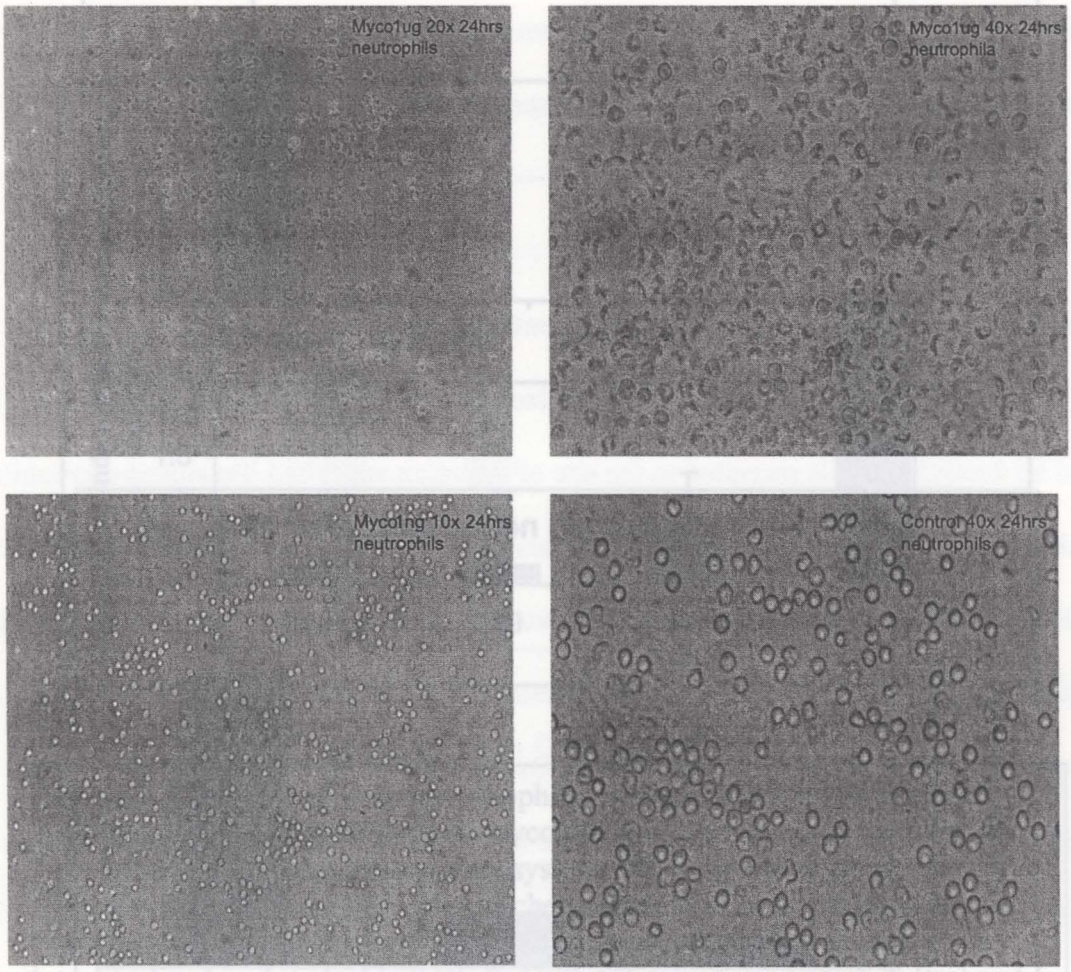


Fig. 3.11. Cytotoxic effect of mycolactone on human neutrophils.

of *M. ulcerans* infection. The intracellular survival of *M. ulcerans* mycolactone mutants *in vivo* was a surprising finding as the failure of MU *myc-* to produce an ulcer in an earlier study by George *et al.* (36) had led to the assumption that the organisms would be cleared.

An interesting implication from these findings is that if mycolactone production was turned off during infection, *M. ulcerans* like other pathogenic *Mycobacteria* species might persist within macrophages. This conclusion is also supported by *in vitro* macrophage studies. The finding that mycolactone causes a rapid change in cell permeability resulting in necrosis at high concentrations is a newly reported phenotype. What is the biological relevance of these findings? In a 4 week stationary culture, *M. ulcerans* produces 4 - 6 μg/ml mycolactone of which 75% (3 - 4.5 μg) is cell associated (75). By extrapolation from *in vitro* growth production, it is estimated that injection of 10^7 *M. ulcerans* introduces 0.3 - 0.45 μg mycolactone into the dermis. Mycobacteria are nonmotile. In the absence of inflammatory cells, localized replication of mycobacteria leads to formation of micro-colonies which could result in extremely high local mycolactone concentrations. Analysis of the bacterial load in human tissue by quantitative PCR suggests that an infection focus contain greater 10^9 organisms (76). If mycolactone production *in vitro* accurately reflects production *in vivo*, mycolactone concentration used in our studies is well within the range likely to be present in natural infections. This suggests that both necrosis and apoptosis are relevant mycolactone effects.

Based on these studies the following role for mycolactone in the virulence of *M. ulcerans* is proposed. As organism replicates and mycolactone is secreted, a toxin

gradient forms. The death of adipocytes leads to release of fatty acids which creates a hydrophobic environment facilitating diffusion of mycolactone. Inflammatory cells are killed rapidly when they encounter high concentrations of mycolactone and cannot disperse bacteria. Inflammatory cells more distant from the necrotic center are killed via apoptosis. This sequence of events creates and maintains locally high mycolactone concentrations which results in death of inflammatory cells, and prevents amplification of the initial immune response. Unfortunately, the route of transmission, the infective dose of *M. ulcerans*, the regulation of toxin production *in vivo*, the amount of mycolactone in a human lesion and the stability of the molecule *in vivo* are unknown. Studies with MU *myc*- suggest that if mycolactone production was turned off at some point in infection, *M. ulcerans* might indeed have an intracellular phase. This is consistent with the finding that *M. ulcerans* patients mount a Th1 immune response to infection leading to the widely held conviction that *M. ulcerans* has an intracellular phase which has not been detected (7, 52, 56, 77).

In vivo, the relative paucity of neutrophils in *M. ulcerans* infection as well as the differential neutrophilic response to WT *M. ulcerans* and MU *myc*- suggest that mycolactone might have an effect on neutrophil recruitment. However, results from our *in vitro* studies with human neutrophils suggest that whereas *M. ulcerans* promotes little chemotaxis, this is not likely to be due to the presence of mycolactone. The immunomodulatory effects of mycolactone are not surprising considering that many antibiotic macrolides such as erythromycin have anti-inflammatory properties (37).

In the case of pathogenic mycobacteria such as *M. tuberculosis*, *M. bovis* and *M. leprae*, phagocytic uptake has been shown to occur by binding to receptors on the plasma

membrane of phagocytic cells. These receptors include complement receptors (CR1, CR3, CR4), for opsonized and non-opsonized entry, mannose receptors (MR) that bind mannosylated structures on the bacterial surface, Fc_{γ} receptors that internalize IgG-opsonized bacteria and scavenger receptors (43, 48, 51, 78). In addition to binding one or more receptor molecules, *M. tuberculosis* has been shown to interact with the plasma membrane sterol cholesterol. It has been hypothesized that the extremely glycolipid-rich mycobacterial cell wall may contain components that interact directly with cholesterol (78, 79).

Toll-like receptors are also involved in cellular recognition of mycobacteria (51). TLR2, TLR4 and, more recently, TLR1/TLR6 that heterodimerize with TLR2, have been implicated in the recognition of mycobacterial antigens. The mycobacterial cell wall is composed of different glycolipids such as lipoarabinomannan (LAM-AraLAM, ManLAM, PILAM), mycolic acid, lipopeptides, and phosphoinositol. PILAMs are usually considered as pro-inflammatory molecules while ManLAMs are anti-inflammatory molecules. TLR-2 mediated cell activation has been described for LAM, LM (lipomannan), phosphatidyl-myo-inositol mannoside (PIM), and the 19-kDa mycobacterial lipoprotein which is a potent inducer of T-cell responses. It is thought that a balance between PIM, LM and LAM synthesis by pathogenic mycobacteria might provide pro- or anti-inflammatory immunomodulatory signals during infection (80, 81). From several lines of evidence, it has become clear that phagocytosis does not lead to immune activation in the absence of functional TLRs (51). Comparatively, little is known regarding the cell wall of *M. ulcerans* and its interaction with host cells. Determination of all the cell wall components of *M. ulcerans* and comparison with that of other

intracellular pathogenic mycobacteria may help in understanding what role the cell wall plays in the difference in initial recognition and interaction with resident immune cells, phagocytosis and further amplification of the immune response.

Chapter 4

CYTOKINE AND CELL-ADHESION MOLECULE RESPONSE TO *MYCOBACTERIUM ULCERANS*

Introduction

M. ulcerans infection is characterized pathologically by a predominance of extracellular bacteria, massive necrosis, little acute or chronic inflammation in the lesion and a paucity of mononuclear cell inflammatory response (2, 46, 49). Tissue necrosis has been attributed to mycolactone, the toxin produced by *M. ulcerans* (36). After infection, a critical role played by the innate immune system is to emit 'alarm signals' that warn of pathogens in the vicinity. These signals (cytokine, chemokine, adhesion molecule responses) result in rapid recruitment and activation of antigen presenting cells, which are critical early events in launching a productive immune response (82). Virtually all pathogenic mycobacteria except *M. ulcerans* produce granulomas in their vertebrate hosts. Granuloma formation typically starts from aggregates of mononuclear phagocytes surrounding individual infected macrophages and include giant cells and T cells (43). Production of inflammatory mediators by infected phagocytes, such as TNF-α, has been shown to be an essential requirement to initiate timely chemokine-induced cell recruitment during infection with *M. tuberculosis* (83).

Information regarding the pro-inflammatory cytokine, chemokine and cellular adhesion molecule response to *M. ulcerans* is limited. Table 2 includes a list of cytokine responses to *M. ulcerans in vitro* and *ex vivo* that have been studied to date. Previous studies have shown that a high density lipid fraction from *M. ulcerans* culture filtrate

Table 2. Cytokine response to *M. ulcerans*.

Cytokine Response	Cells used	Stimulant	Response	Reference
Interleukin-2	Human T lymphocytes (T cell line 3895, Jurkat T cells)	High density lipid fraction from *M. ulcerans* culture filtrate	Inhibited	Pahlevan *et al.*, 1999
Tumor necrosis factor-alpha	Human PBMC from healthy subjects	High density lipid fraction from *M. ulcerans* culture filtrate	Inhibited	Pahlevan *et al.*, 1999
Interleukin-10	Human PBMC from healthy subjects	High density lipid fraction from *M. ulcerans* culture filtrate	Inhibited	Pahlevan *et al.*, 1999
Interferon-gamma	Human PBMC from early stage Buruli ulcer disease patients	PPD	Inhibited	Westenbrink *et al.*, 2005
	Human PBMC from patients with late stage or healing Buruli ulcer disease	PPD	Increased	Gooding *et al.*, 2001
	Human PBMC from subjects with current or past Buruli ulcer disease	Live *M. ulcerans*	Inhibited	Gooding *et al.*, 2001, 2002
Interleukin-4, 5, 6, 10	Human PBMC from Buruli ulcer patients	Live *M. ulcerans*, PCR analysis,	Increased	Gooding *et al.*, 2003

inhibits IL-2 production of activated lymphocytes, and TNF-α expression of LPS-stimulated monocytes. Immunomodulatory effects of *M. ulcerans* and purified mycolactone toxin on TNF-∝ production, secretion of IL-8, an important neutrophil chemotactic factor and cell-adhesion molecules have not been addressed so far.

Important effector mechanisms of host defense include secretion of chemokines and cytokines (83-86). Regulation of these host effector mechanisms depends on pathways initiated by ligand-receptor interactions on the surface of cells in the area of infection. Central roles in these intracellular pathways have been assigned to the nuclear factor kappa B (NF-κB) system and to mitogen-activated protein kinase (MAPK) pathways, such as the extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38. Disruption of these pathways leads to impaired secretion of antibacterial effectors like TNF-α, IL-1, and IL-8 (87) (88, 89).

Early inflammatory responses are essential for generating antigen-specific effector cells of adaptive immunity. Primary cells that produce TNF-α are monocytes and macrophages (90). Cytokines produced immediately after infection orchestrate the next wave of the innate immune response, cell recruitment and activation (68). Protective innate immunity against mycobacteria depends on TNF-α and IL-12 production to regulate the activation of T-lymphocytes and to stimulate the antimycobacterial capacity of infected macrophages (84). Polymorphonuclear granulocytes (PNG) are the first cells which, in response to microbial invasion, migrate from the blood into tissue sites, where they participate in the early inflammatory response. Chemokines like IL-8 are potent

leukocyte attractants. IL-8 is expressed by macrophages and PNG upon appropriate stimulation. Receptors for IL-8 have been identified on PNG and natural killer (NK) cells. IL-8 has many biological activities including induction of chemotaxis, exocytosis, respiratory burst, and up regulation of complement receptors 1 and 3 (85).

The NF-κB family of transcription factors is a group of evolutionarily conserved proteins which are important in regulation of the immune system. These transcription factors are involved in the development of accessory cell and lymphocyte populations and expression of numerous proteins involved in innate and adaptive immunity. Invasion of a host by a pathogen is frequently associated with activation of NF-κB, which coordinates various aspects of immune function required for resistance to infection (91). NF-κB activation can be inhibited by blocking various points in the NF-κB/IκB pathway. Interfering with degradation of I-κB is a strategy commonly employed by some pathogens to inhibit activation of NF-κB (86).

Adhesion molecules expressed on immunocompetent cells play important roles in cellular interactions for the development of immunological responses. Intercellular adhesion molecule-1 (ICAM-1) plays a role in lymphocyte trafficking by allowing ICAM-1 bearing endothelial cells to interact with leukocyte function-associated antigen-1 (LFA-1) on lymphocytes, and thereby facilitate migration of lymphocytes to sites of inflammation along endothelial cells (92, 93). Moreover, the interaction of LFA-1 with ICAM-1 is critical for effective cellular interactions of T cells with antigen presenting cells, including macrophages, and the consequent activation of resting T cells. ICAM-1 augments T-cell proliferation and is pivotal in recall antigen responses of T cells to purified protein derivative of *M. tuberculosis* (93). A recent study by Lopez Ramirez *et*

al. (94) showed that ICAM-1 expression on THP-1 human monocyte- derived cell line was potently increased by stimulation with *M. tuberculosis*. In addition, the *in vivo* study by Sullivan *et al.* (95) concerning *M. leprae* infection revealed that granulomas from tuberculoid leprosy displayed ICAM-1 expression. This implies that ICAM-1 plays important roles in enhancing the immune and inflammatory responses to mycobacteria (96). Therefore, it is of interest to know the profiles of ICAM-1 expression by macrophages after stimulation with *M. ulcerans*.

This study demonstrates that *M. ulcerans* and mycolactone do not induce TNF-α and IL-8 production by macrophages at the protein level. Iκ-B degradation is not affected by mycolactone. Decreased expression of cell-adhesion molecules like ICAM-1 may contribute to poor inflammatory response-lack of cellular interactions which prevents release of inflammatory cytokines as well as the influx of macrophages and neutrophils seen in Buruli ulcer lesions.

Results

TNF-α secretion by THP1 cells in response to wild and mycolactone mutant strains of *M. ulcerans*

Levels of TNF-α produced by THP-1 cells following infection with *M. ulcerans*, mycolactone mutant strain of *M. ulcerans* or mycolactone are shown in Fig. 4.1. In response to infection with WT *M. ulcerans* or mycolactone, very low levels of TNF-α (almost equivalent to background levels of TNF-α secreted by control untreated cells) were detected at 24 h. In contrast, a mycolactone mutant strain MU 1615A induced

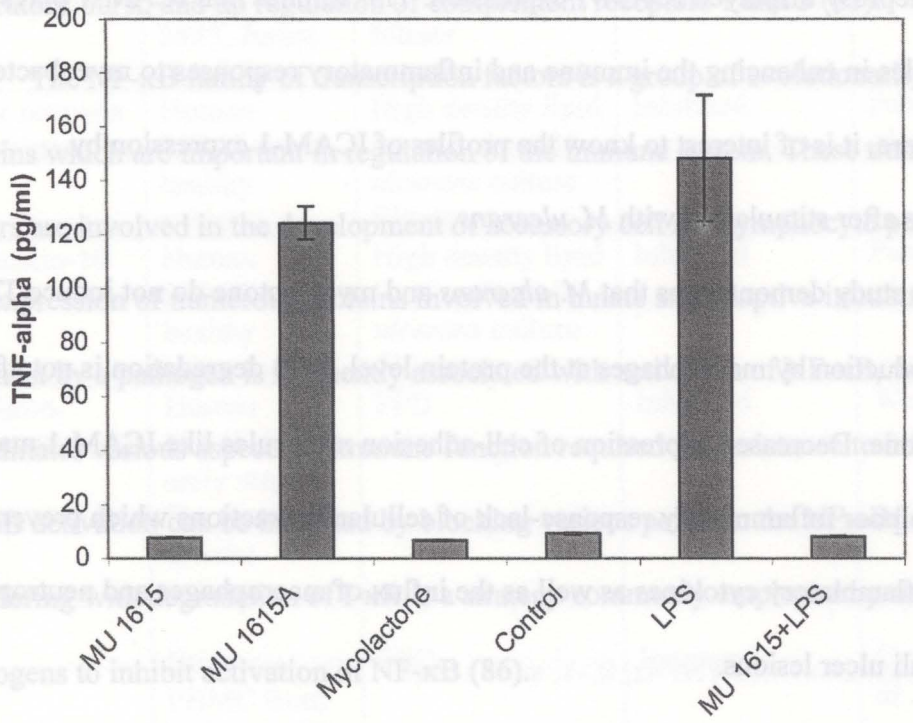


Fig. 4.1. TNF-α production by differentiated THP-1 cells. Cells were treated with wild type *M. ulcerans* (1615), a mycolactone mutant of *M. ulcerans* (MU 1615A), mycolactone (400 ng/ml), LPS (10 ng/ml) or wild type *M. ulcerans* and LPS (10 ng/ml). Untreated cells served as controls. Supernatants were harvested 24 h post infection and secreted TNF- α levels were determined by sandwich ELISA. Data are expressed as mean ± S.D (n=3).

significant levels of TNF-α. Interestingly, WT MU 1615 inhibited LPS induced TNF-α release by THP-1 cells.

IL-8 secretion by THP-1 cells following infection with *M. ulcerans* strains

Differentiated human monocytes (THP-1 cells) were treated with WT *M. ulcerans*, myc- *M. ulcerans* (119), *M. marinum*, *M. fortuitum*, or mycolactone for 24 h. Culture supernatants were collected, and IL-8 concentrations were determined by ELISA. The levels of IL-8 produced by THP-1 cells following infection with WT *M. ulcerans* or *myc- M. ulcerans* are shown in Fig. 4.2. WT *M. ulcerans, myc- M. ulcerans* or mycolactone fail to stimulate IL-8 secretion beyond baseline level secreted by untreated control cells. In contrast, other pathogenic mycobacteria like *M. marinum* and *M. fortuitum* stimulate IL-8 production. LPS treatment also stimulated IL-8 production (data not shown).

Macrophage surface expression of ICAM-1 following infection with *M. ulcerans* or treatment with mycolactone

In this the level of macrophage surface expression of ICAM-1, a key protein known to play a major role in cell-cell adhesion was also examined. As shown in Fig. 4.3, flow cytometric analysis revealed an increase in ICAM-1-positive macrophages in WT *M. ulcerans* infected cells relative to uninfected cells at 24 h. However, infection of cells with the mycolactone negative mutant MU 1615::Tn119 resulted in a significant increase

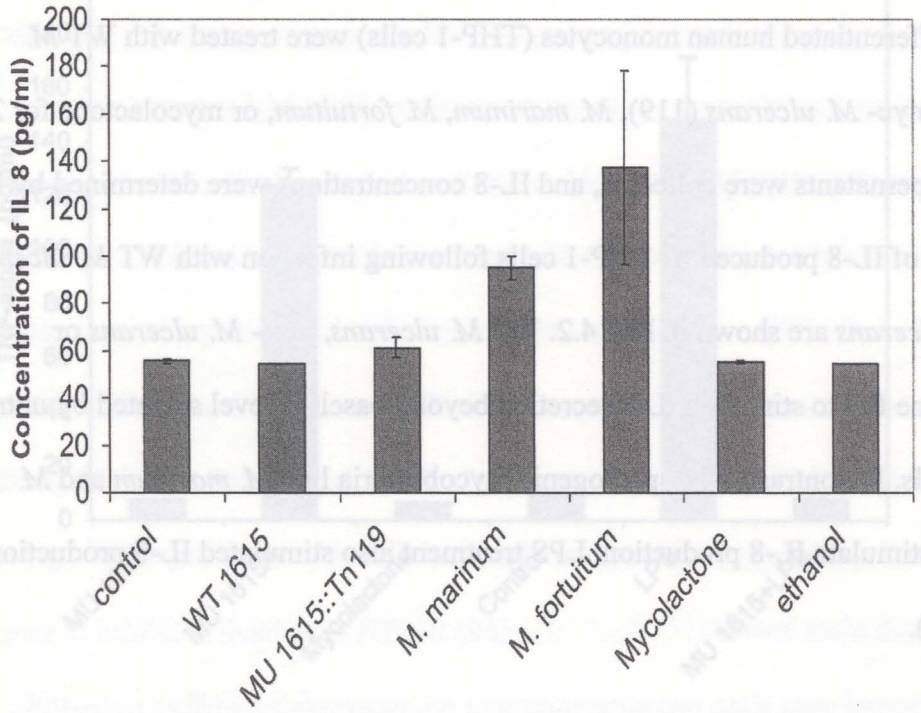


Fig. 4.2. IL-8 production by differentiated THP-1 cells. Cells were treated with wild type *M. ulcerans* (1615), a mycolactone mutant of *M. ulcerans* (MU 1615::Tn119), mycolactone (500 ng/ml), *M. marinum* or *M. fortuitum*. Untreated and ethanol treated cells served as controls. Supernatants were harvested 24hrs post infection and secreted IL-8 levels were determined by sandwich ELISA. Data are expressed as mean ± S.D (n=3).

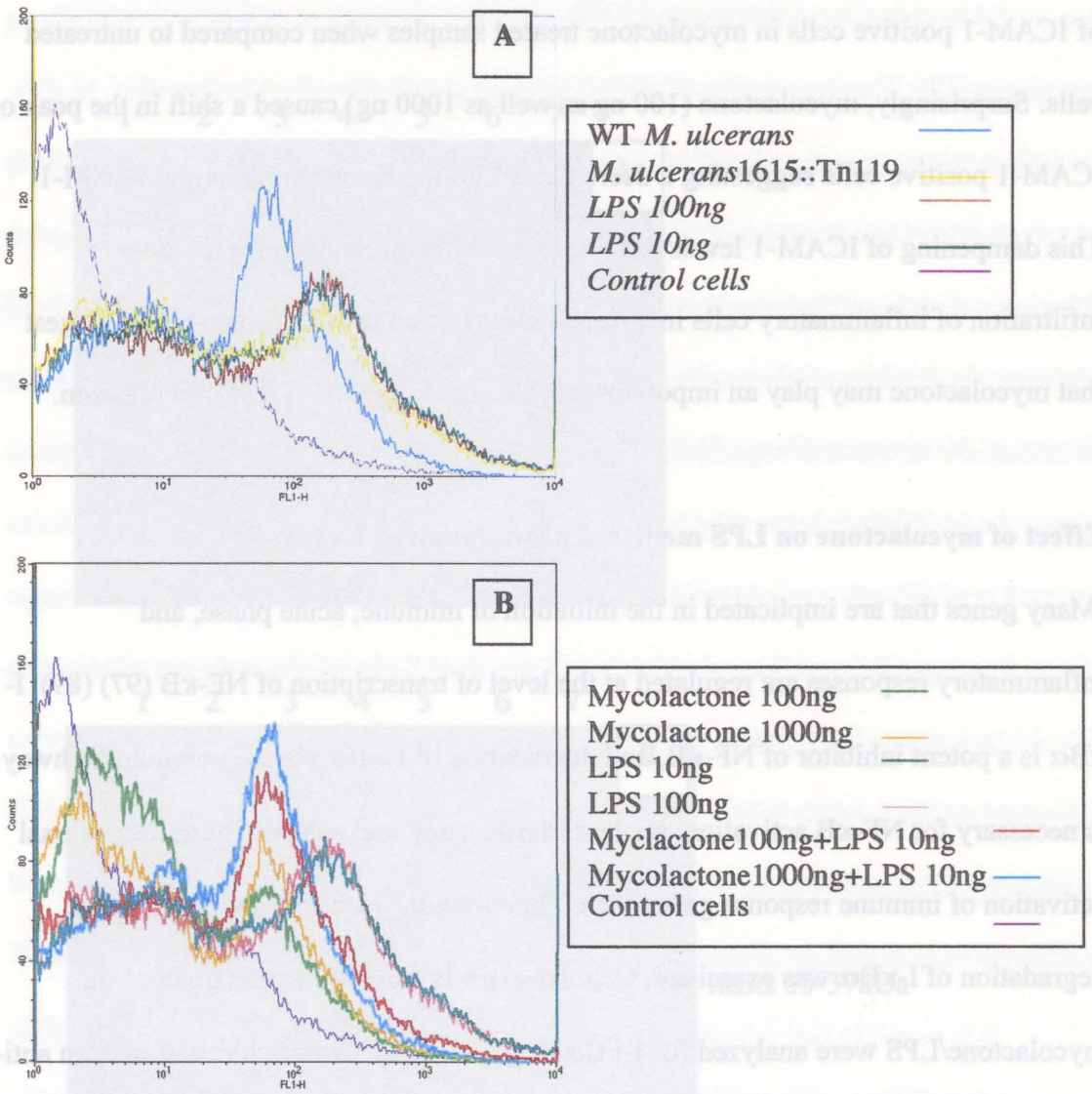
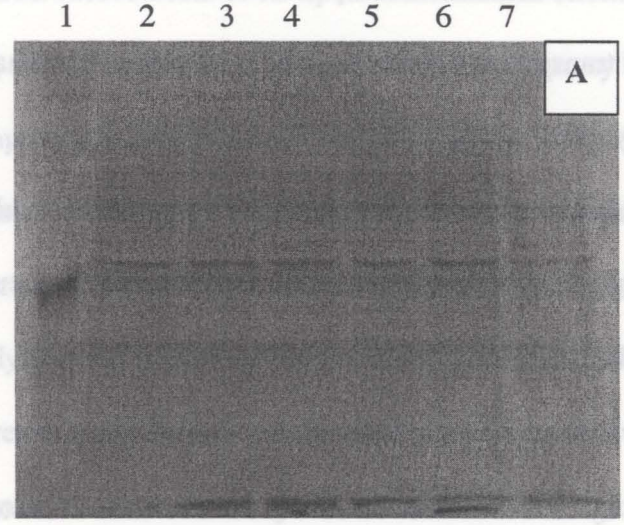


Fig. 4.3. Flow cytometry analysis of ICAM-1 expression by THP-1 cells. **(A)** in response to 24 h infection with WT MU1615 (blue line), *myc*- MU 1615::Tn119 (yellow line), LPS (brown-100ng or green-10ng line) or control cells (purple line). **(B)** ICAM-1 expression by THP-1 cells in response to 24 h treatment with mycolactone (100 ng/ml, fluorescent green line, 1000 ng/ml, orange line), LPS (10 ng/ml, dark green line, 100ng, pink line) or mycolactone (1000 ng/ml, blue line, 100 ng/ml, red line) and LPS (10 ng/ml) or control cells (purple line).

in expression of ICAM-1 as did LPS treated cells. There was an increase in the number of ICAM-1 positive cells in mycolactone treated samples when compared to untreated cells. Surprisingly, mycolactone (100 ng as well as 1000 ng) caused a shift in the peak of ICAM-1 positive cells suggesting a decrease in LPS mediated expression of ICAM-1. This dampening of ICAM-1 levels provides a possible mechanism for the poor infiltration of inflammatory cells in a Buruli ulcer human lesion. These results suggest that mycolactone may play an important role as a modulator of ICAM-1 expression.

Effect of mycolactone on LPS mediated degradation of I-κBα

Many genes that are implicated in the initiation of immune, acute phase, and inflammatory responses are regulated at the level of transcription of NF-κB (97) (89). I-κBα is a potent inhibitor of NF-κB, and degradation of I-κBα via the ubiquitin pathway is necessary for NF-κB activation, nuclear translocation and subsequent transcriptional activation of immune response genes (88). Therefore, the effect of mycolactone on degradation of I-κBα was examined. Cellular extracts from J774 cells treated with mycolactone/LPS were analyzed for I-κBα degradation by western blotting with an anti-I-κBα polyclonal antibody. As shown in Fig. 4.4, treatment with LPS led to the rapid disappearance of the immunoreactive I-κBα band within 15 min; this band returned to basal levels within 30 min. LPS induced degradation of I-κBα was not inhibited by mycolactone at the concentration tested. Taken together, these results suggest that the molecular target for mycolactone may not be dissociation of NF-κB from I-κB and mycolactone may act on some other targets in the signaling pathway.



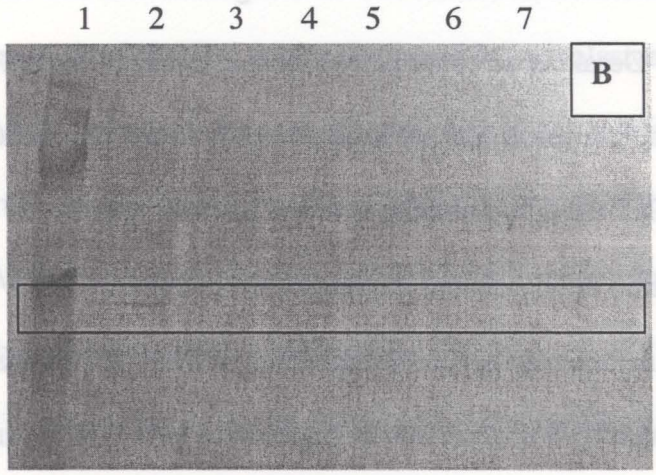


Fig. 4.4. (A) Commassie stain of proteins from J774 cell extract **(B)** Western blot analysis of IkBα degradation in J774 cell line. Lanes: 1, protein standard markers; 2, control ; 3, LPS 20 ng/ml 15 min; 3, LPS 20 ng/ml 30 min; 4, mycolactone 100 ng/ml 1 h; 5, mycolactone 100 ng/ml 1 h and LPS 15min; 6, mycolactone 100 ng/ml 1 h and LPS 30 min.

Discussion

Monocytes/macrophages and TNF-α are key elements in anti-mycobacterial responses (28). The importance of NF-κB in the transcription of a number of proinflammatory genes makes this transcription factor key to the immune inflammatory response (98). Data from this study using whole bacterial cells of WT *M. ulcerans*, *myc-M. ulcerans* and purified mycolactone confirms studies published by Pahlevan *et al.* (28) showing the inhibitory effect of high density lipid (HDL) fraction extracted from culture filtrate of *M. ulcerans* (Strain 7634) on TNF-α production by RAW 264.7 murine macrophage cell line. Interestingly, the same group showed that TNF-α-induced activation of NF-κB was inhibited by the HDL fraction of WT *M. ulcerans*. This study shows that mycolactone does not inhibit LPS induced I-κBα degradation in J774 mouse macrophages. Recent studies by Desaki *et al.* (88) showed similar results in human bronchial epithelial cells with another macrolide erythromycin. Erythromycin did not inhibit degradation of I-κBα or NF-κB DNA-binding activity. Interestingly, erythromycin suppressed activation of NF-κB and production of IL-8 (88).

It is possible that mycolactone may act on the signaling pathway via a different downstream target. It is known that IL-8 is expressed in response to LPS, TNF-α and aggregated immune complexes (99). The inability of *M. ulcerans* to stimulate TNF-α production and the possible inability to form immune complexes due to the presence of large clumps/masses of extracellular bacteria may explain the inability of cells to secrete IL-8.

Lymphocyte adhesion is a necessary event for cell to cell interactions required to produce an immune response (92). Recently, it was found that stimulation of THP1 cells with *M. tuberculosis* or its lipoarabinomannan elicited an increase in ICAM-1 expression (93, 94). In addition, in cases of tuberculoid leprosy lesions characterized by strong delayed-type hypersensitivity (DTH) against *M. leprae*, keratinocytes exhibited pronounced ICAM-1 expression. Conversely, in lepromatous leprosy patients who show a serious defect in DTH, keratinocytes ICAM-1 expression was low (95, 100). Another study showed that infection of murine peritoneal macrophages with *M. avium* lead to an increase in ICAM-1 expression (101). In the present study macrophage ICAM-1 expression is decreased by WT *M. ulcerans* and mycolactone in comparison to the mycolactone mutant MU1615::Tn119 stimulated cells. In addition, LPS induced expression of ICAM-1 is also decreased by mycolactone. Decreased expression of ICAM-1 on cells in the area of lesion may play a role in the immune response seen in Buruli ulcer patients. It would be interesting to see if there is a difference in expression of ICAM-1 and other cell adhesion molecules in skin samples from Buruli patients with early, ulcerative and healed lesions.

A number of studies have shown the inhibitory effect of macrolides on inflammatory immune responses (37-39, 88, 99). Data from studies *in vitro* and in animal models support the inhibitory effects of macrolides on neutrophil influx and chemotactic activity (37, 99). Long-term treatment with macrolide antibiotics has been shown to decrease levels of IL-8 in the bronchial alveolar lavage (BAL) fluid of patients with diffuse parabronchiolitis, and to decrease concentrations of IL-8 released by eosinophils

from atopic subjects. Cultured human bronchial epithelial cells treated with erythromycin showed reduced levels of IL-6, IL-8 and ICAM-1 (88).

Results from the present study suggest that *M. ulcerans* and mycolactone inhibit pro-inflammatory cytokine response and decreased ICAM-1 expression which may contribute to the paucity of inflammatory cells seen in the area of infection. Further studies to understand how mycolactone may either disrupt or redirect signaling pathways that lead to immense tissue damage and a lack of immune response may help in developing new strategies to treat Buruli ulcer and ameliorate symptoms of the disease.

Chapter 5

MICROARRAY ANALYSIS TO INVESTIGATE MODE OF ACTION OF MYCOLACTONE

Introduction

The unique pathology seen in Buruli ulcer is attributed to mycolactone (13, 36, 40, 102). Its mode of action is unclear, however, in a guinea pig model of the disease, purified mycolactone injected subcutaneously reproduces the natural pathology, and mycolactone-negative variants were avirulent, implying a key role for the toxin in pathogenesis. Mycolactone was also shown to have cytotoxic, anti-inflammatory and immunosuppressive properties (36). On cultured fibroblasts and macrophage cell lines, mycolactone induced cell rounding, inhibited protein synthesis, arrested cells in G0/G1 stage, and eventually cell death (13, 35, 36). *In vitro* experiments showed that the major mycolactones, a mixture of cis-trans isomers (mycolactone A and B), produced apoptosis and necrosis in many human cell types (13, 65, 103). Mycolactone has also been shown to induce cell-cycle arrest in cultured lymphocytes, followed by apoptotic cell death (28). There is a general inhibition for protein synthesis *in vitro* in response to mycolactone (35). It has been postulated that local secretion of mycolactone reduced the cutaneous inflammatory response and may contribute to the development of the Buruli ulcer (40). However, it is still not clear if mycolactone causes global immunosuppression in the host.

The mode of action of mycolactone is still unknown. Studies using a biologically active fluorescent mycolactone derivative, bodipy mycolactone, have suggested that mycolactone enters cells by diffusion and accumulates in the cytosol (41). The same researchers showed that uptake of mycolactone is both nonsaturable and noncompetitive

with excess mycolactone, consistent with passive diffusion of this toxin through the cell membrane. These facts, combined with the inability of signal transduction inhibitors to inhibit mycolactone cytopathicity point towards the presence of a cytosolic target for mycolactone (41). The molecular mechanism/s underlying the cytotoxic effect caused by mycolactone have not yet been uncovered in detail. In view of the complex factors that may contribute to mycolactone activity, gene expression profiling may make it possible to identify various genes involved in mycolactone-mediated cytotoxicity.

In the last few years, microarray technology has emerged as the method of choice for large-scale gene expression studies. It provides a rapid and efficient method to investigate the entire transcriptome of a cell (104-108). Microarrays have become an important tool for analyzing genomic transcriptional programs in diverse biological processes. Microarray analyses are utilized in a variety of research areas such as investigation of gene networks in physiological pathways, analysis of genetic variation, host-pathogen interactions or for studying host gene expression programs modulated by different drugs and to identify the pathway of action of the compounds new therapeutic drug targets. The term toxicogenomics describes another exciting field of microarray application, which offers the possibility to characterize transcriptional responses towards toxic compounds (109-110). Toxicogenomic approaches comprise the assessment of gene expression profiles of animal tissues or cell cultures after administration of toxicants, which may lead to the establishment of toxicant-specific "fingerprints." Another challenge provided by toxicogenomic-based investigations is the possibility to assess the toxic mode of action of a compound. Interpretation of transcriptional alterations and assignment of differentially expressed genes to biological pathways might culminate in

the generation of hypotheses about toxicological mechanisms of compounds (111). The simplest way to identify genes of potential interest through several related experiments is to search for those that are consistently either up- or downregulated (112). The principle of a microarray experiment is that mRNA from a given cell line or tissue is used to generate a labelled sample, sometimes termed the 'target', which is hybridized in parallel to a large number of DNA sequences, immobilized on a solid surface in an ordered array (Fig. 5.1). Using this method, tens of thousands of transcript species can be detected and quantified simultaneously (113).

In this study, microarray technology was used to gain a global and molecular view of cellular transcriptional responses to mycolactone and to identify important genes modulated by the toxin. To investigate cellular responses to mycolactone, changes in gene expression caused by treatment of human monocytes with different concentrations of mycolactone were analyzed using DNA microarrays. Analysis of genes induced by mycolactone revealed up-regulation and down-regulation of several interesting genes. Here the significance of modulation of some of these genes by mycolactone in *M. ulcerans* pathogenicity, mycolactone-mediated cytotoxicity and disease outcome is discussed.

RNA preparation and microarray analysis

THP-1 human monocyte cells were plated at 5×10^5 cells/ml in 35mm tissue culture flasks and differentiated using phorbal 12-myristate-13 acetate (100 ng/ml). After 24 h, the

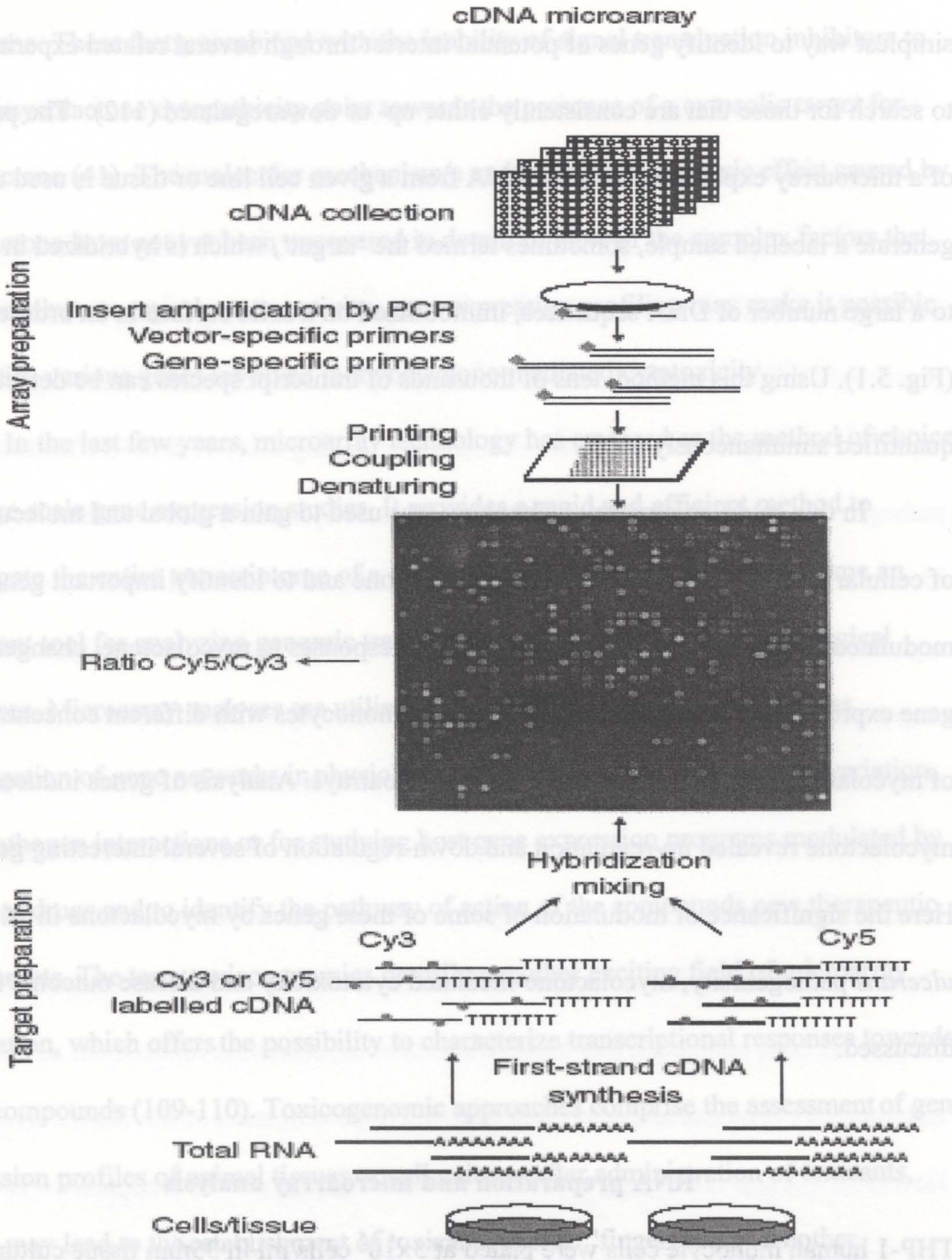


Fig. 5.1. Schematic overview of probe array and target preparation for spotted cDNA microarrays. Array preparation: inserts from cDNA collections or libraries (such as IMAGE libraries) are amplified using either vector-specific or gene-specific primers. (Taken from Schulze, A., *et al.*, 2001).

supernatant was removed and fresh tissue culture medium supplemented with 10% FCS and L-glutamine was added. Cells were treated with two different concentrations of mycolactone (15 ng/ml or 100 ng/ml) for 0.5, 2 or 8 h. Control cells were treated with an equal volume of ethanol. Total RNA was isolated at the indicated time points from mycolactone-treated and control macrophages using Trizol™ (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA was further purified using the Rneasy kit (Qiagen, Valencia, CA). Quality and quantity of RNA was assessed by measuring the A260/A280 ratio and by analysis on ethidium bromide stained 1% agar gels.

Probe synthesis and DNA microarray hybridization were performed by Drs. Anne Mueller and Karen Guillemin in Dr. Stanley Falkow's laboratory at Stanford University School of Medicine. Detailed protocols for RNA labeling and hybridization can be found at http://cmgm.stanford.edu/pbrown/protocols/index.html. Briefly, total RNA was used for single stranded cDNA probe synthesis incorporating aminoallyl-dUTP, which was subsequently coupled with Cy3 (for the control sample) or Cy5 (for the experimental sample). The fluorescently labeled experimental and control samples were combined and hybridized to a 40,000-element human cDNA microarray.

Arrays were scanned using a GenePix 4000A scanner and images were analyzed with GENEPIX PRO software (Axon Instruments, Foster City, CA). Data were filtered by omission of spots, with regression correlations < 0.6, and by selection of genes whose $\log_2$ of red:green normalized ratio was > 2 (i.e., normalized ratio is more than two standard deviations away from mean). Only genes with > 70% good data for all arrays

were included for $\log_2$ transformation and analysis. These criteria were met by 1596 genes. Data were then filtered so that the absolute values of the fold change between experimental and reference sample was ≥ 4. Annotations about individual genes were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.goc) and SOURCE (http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch) websites.

Results

Data were used to identify genes that might be critical in the cytotoxic effects caused by mycolactone. To identify transcriptional events regulated by mycolactone, data obtained from THP-1 cells treated with two different concentrations (low-15 ng/ml, high-100 ng/ml) of mycolactone for 0.5 (early), 2 (intermediate) and 8 (late) h were compared. Raw microarray data (shown in appendix figures A 1.1- A 1.4) represent genes whose absolute values of the fold change between experimental and reference sample was ≥ 4 in at least two sets of microarray experiments for each condition. Genes that were significantly up-regulated included those related to transcriptional repressors, cytoskeletal rearrangement, cell cycle control/proliferation, apoptosis, G-protein receptors, tumor supression and immune response. Genes down-regulated throughout the time course included those related to DNA repair, inactivation of complement and metalloproteinases, immune response, leukotrienes production, receptors for collagen and laminin.

Genes up-regulated by mycolactone treatment

Treatment of THP-1 cells with mycolactone for 0.5 h (Table 3) caused an increase in expression of genes related to transcription (TLE4, DSCR1L1, RING1, several zinc finger proteins), immune response (HLA-DPB1, DEFA4, CCL7, ITIH4, CD28, STAT4), sulfotransferase enzymes (involved in modification of drugs, hormones, xenobiotic compounds), carbohydrate metabolism (PFKP-key control step of glycolysis), apolipoprotein receptor (LRP8), transmembrane signaling (PLCG2), tumor suppressors (LLGL1), calcium transport (PLN- regulates activaty of calcium pump) and apoptosis (CASP3).

Mycolactone treatment for 2 h (Table 4) resulted in an induction of genes related to transcriptional repressors/inhibitors/regulators (SIRT4, T, HOXB2, FLJ36991), cell cycle and maintenance (PKYMT1, AK2), immune response (IL1R2, AZU1, COLEC10), fatty acid, steroid metabolism (RODH, CYP11B1, RGRX), receptors (GPR105, AVPR1A, PTGER3, ROR2, GC, OXTR), signaling (PRKCM), tumor (BAI1, TSSC1, PRKCG, KLK6) and voltage activated potassium channel (KCNH2). Genes modulated by 8 h (Table 5) treatment of mycolactone are related to apoptosis (BAX), immune response (CD86, FREB), cytochrome P450 (CYP2S1), transport/trafficking (STARD4, DDHD1, SLC27A4, ARFGAP1, APP) and pH regulation (SCL9A2).

Genes down-regulated by mycolactone treatment

Genes down-regulated throughout the time course (0.5, 2 and 8 h) as shown in Table 6 were those related to transcription (JUN, MATR3, POGZ), cell cycle control

Table 3. Genes up-regulated by mycolactone ≥ 4 times after 0.5 h.

Gene name	Biological process/molecular function
TLE4	Transcriptional repressor
DSCR1L1	Calcineurin inhibitor
RING1	Transcriptional repressor
ZNF274	Chromatin reorganization
CASP3	Apoptosis
Map4	Microtubule assembly
SGCB	Cytoskeletal reorganization
EPB41	Membrane mechanical stability
HLA-DPB1	MHC Class II
DEF4	Defensins
CCL7	Chemotaxis of macrophages, eosinophils
ITIH4	Acute phase reactions
STAT4	Transcriptional activator of IL12
SEPT6	Cytokinesis
SULT1A3	Sulfotransferase
LSS	Sterol biosynthesis
PFKP	Glycolysis
LRP8	Apolipoprotein E receptor
PLCG2	Signal transduction (phospholipase)
SPR1B	Tissue damage
PLGL	Extracellular matrix destruction
LOH11CR2A	Tumor Suppressor
LLGL1	Tumor suppressor
PLN	Calcium pump regulation
NDUFS1	NADH dehydrogenase and oxidoreductase
FBXL3A	Ubiquination (phosphorylation dependant)

Table 4. Genes up-regulated by mycolactone ≥ 4 times after 2 h.

Gene name	Biological process/molecular function
SIRT4	Gene Silencing
T	Transcriptional regulation
PKYMT1	Negative regulator of cell cycle (mitosis)
AK2	Apoptosis, Cell maintenance
ZNF3	Cell differentiation, proliferation
KIFAP3	Acts on G proteins (Rho,Rap1,Ki-Ras)
PAFAH1B1	Dynein mediated microtubule sliding inactivates PAF
MST1R	Receptor for Φμ stimulating factor
MRC1	Receptor (endocytosis of glycoproteins by Φμ)
IL1R2	Decoy receptor of IL1
AZU1	Azurophil granules in neutrophils
MYH10	Cytokinesis
COLEC	C-lectin family
CD86	T cell immunity/anergy
RODH	epimerase, oxidoreductase (retinoic acid biosyn)
CYP11B1	Cyt P450
AGL	Glycogen degradation
GPR105	Receptor for UDP-glucose
PRKCM	Signal transduction
AVPR1A	G-protein receptor
PTGER3	G-protein receptor (prostaglandin E2)
ROR2	Receptor protein tyrosine kinase
GC	Transport of Vit D sterols to target tissues
SLC22A1	Glucose transporter
OXTR	G-protein receptor (oxytocin)
AQP2	Water channel
ACE	Angiotensin catalyzing enzyme (bradylinin inhibitor)
BAI1	Angiogenesis inhibitor, suppressor of glioblastoma
PRKCG	Serine-threonine enzyme
GALGT	Synthesis of glycosphingolipids

Table 5. Genes up-regulated by mycolactone≥ 4 times after 8 h.

Gene name	Biological process/molecular function
EPO	Erythrocyte differentiation
BAC	Apoptosis
DRIL2	Cell cycle control
CAPN1	Cytoskeletal remodelling, signal transduction
TNC	Inhibit cell migration, ligand for integrins
FREB	Mediate phagocytosis of IgG coated pathogens
IL6R	Immune response, acute phase reactions
CYP2S1	Cyt p450
UGDH	UDP-glucose dehydrogenase
SLC27A4	Translocation of long chain FA
ARFGAP1	Membrane traficking
APP2	Cell surface receptor
GIT2	GTPases activating factor
RANGAP1	GTPase activity of RAN
CTSZ	Carboxypeptidase (tumorigenesis)
SCL9A2	Proton extruding system (pH balance)
TMEM8	DNA-dependent RNA polymerase
WWP1	Protein degradation,transcription, RNA splicing
TGM3	Transglutaminase
FBX09	Ubiquination (phosphorylation dependant)
OAZIN	Ornithine decarboxylase

Table 6. Genes down-regulated by mycolactone throughout time course (0.5, 2, 8 h).

Gene name	Biological process/molecular function
THIL	Repress transcriptional elongation by RNA pol II
POGZ	Transcription regulator
ARNT	Transcriptional regulator of adaptive response to hypoxia
JUN	Transcription regulator
CDK2AP1	Cell cycle (S phase)
CAPZA1	Actin capping
MACF1	Cytoskeletal rearrangement
FCER1G	IgE receptor
RGS1	Inhibits signal transduction (B cell proliferation regulation)
PTMA	Immunity to opportunistic infection
LTC4S	Production of leukotrienes
PRDX4	Antioxidant enzyme
ITGA1	Integrin (cell surface receptor for collagen, laminin)
ACADM	Acyl coA dehydrogenase
AKR1B1	Aldo/keto reductase
HAGL	Thiolesterase
THRA	Receptor for triiodothyronine
YWHAZ	Signal transduction
TFRC	Transferrin receptor (iron)
CD36	Receptor for collagen, thrombospondin
ARHA	Signal transduction
CEPT1	Cholinephosphotransferase
TIMP2	Inhibitors of metalloproteinases
MCP	Inactivation of C3b, C4b
Clic4	Chloride channel
GABARAP	Receptor
RAD23B	DNA repair
RISC	Needed for neuraminidase, galactosidase activity
UBE1	Ubiquination
UBQLN2	Ubiquination

(PPP2R1B, RCL, CDK2AP1, MAGED1), cytoskeletal rearrangement (MACF1, CAPZA1), inflammation (FCGRT, SCYE1, ITGA1, PRDX4, LTC4), immune response (IL6R, FCER1G, RGS1, PTMA), signal transduction (SPRY2, RANBP7, ARHA, YWHAZ), tissue repair/damage (MCP, TIMP2) and DNA repair (RAD23B)

Discussion

Taken together, the array data provided for the first time a global view of mycolactone-mediated signal transduction in host cells at the molecular level. The cytotoxic response to mycolactone involved several genes modulated significantly and consistently. The most striking changes were in genes required for cell cycle progress and transcription factors (regulators, repressors). Others included genes related to cell cycle arrest, apoptosis, tumor suppression and immune response. Additionally, several signal-transduction, cell growth/division, transcription regulation and inflammation related genes that were significantly down-regulated in mycolactone treated macrophages were identified. Data from this study provide new insight into the host gene-expression pathways induced by mycolactone.

Pimsler *et al.* (49) showed that a crude culture filtrate of *M. ulcerans* inhibited T cell activation. Another group, Pahlevan *et al.* (28) also showed that a high density lipid fraction of *M. ulcerans* inhibited T cell activation. The same group also showed that the factor abrogated TNF-induced NF-κB activation but did not inhibit degradation of IκBα induced by TNF, indicating that the target for its activity lies within an undefined part of the TNF signaling mechanism. The factor had no effect on IL-1 or LPS-induced NF-κB activity (28).

Calcium is used by cells to modulate gene expression programs by various means. The best known example of calcium regulation through phosphatases is the NF-AT transcription factor (114, 115). Calcineurin becomes activated on binding calcium and calmodulin when cytosolic calcium rises, and is inhibited on binding the immunosuppressants cyclosporin A and FK506 in complexes with their respective cellular receptors (116).

Calcineurin is a serine/threonine protein phosphatase under the control of Ca2+/calmodulin and plays a pivotal role in developmental and homeostatic regulation of a wide variety of cell types (116). It functions as a heterodimer composed of a catalytic A subunit (CaNA) and a calcium binding regulatory B subunit (CaNB). CaNA displays a multidomain structure with a catalytic domain at its N-terminus, followed by the binding regions for the B subunit and calmodulin (CaM). An autoinhibitory domain is located near the C-terminus, which is thought to be displaced upon CaM binding (117).

The interaction of calcineurin with transcription factors of the NFAT family following activation of the T cell receptor in leukocytes provides the best characterized example of how calcineurin regulates gene expression (114, 115, 118, 119). Changes in intracellular calcium promote binding of Ca^{2+}/calmodulin to the catalytic subunit of calcineurin (CnA), thereby displacing an autoinhibitory region and allowing access of protein substrates to the catalytic domain. Dephosphorylation of NFAT by activated calcineurin promotes its translocation from the cytoplasm to the nucleus, where NFAT binds DNA cooperatively with an AP1 heterodimer to activate transcription of genes encoding cytokines such as IL-2. This basic model of NFAT activation has been shown to transduce Ca^{2+} signals via calcineurin in many cell types and to control transcription of

diverse sets of target genes unique to each cellular environment (115). In each case, NFAT acts cooperatively with other transcription factors that include proteins of the AP1, cMAF, GATA, or MEF2 families (118). In addition to T cell activation, cellular responses controlled by calcineurin/NFAT signaling include gene transcription factors, signaling proteins, cell surface receptors, other effector proteins (115, 118).

Down syndrome critical region gene 1-like 1 (DSCR1L1), also known as calcipressin 2/ZAKI4, was identified to be up-regulated early (0.5 h) by microarray analysis. Previous studies showed that different isoforms of DSCR1L1 were expressed in the brain, heart, skeletal muscle, kidney, skin and other organs (120-122). DSCR1L1 is a feedback inhibitor of calcineurin. It binds to CaNA subunit of calcineurin and inhibits its phosphatase activity, preventing dephosphorylation of cytosolic NF-AT and its subsequent nuclear import (117). One possible mechanism by which mycolactone inhibits the signaling pathway through calcineurin/NF-κB activation could be by up-regulating DSCR1L1, thus preventing translocation of NFAT to the nucleus.

Further more, down-regulation of transcription factors such as JUN which is a member of the activator protein-1 (AP-1) complex could inhibit transcription of downstream immune response gene. Fos and Jun proteins, which are components of the AP-1 complex of nuclear transcription factors, have been implicated in the regulation of inflammatory responses (88). This could explain the inhibition of production of several pro-inflammatory cytokines observed in *M. ulcerans* infection *in vitro* and *in vivo* by several researchers (5, 25, 28, 46, 49, 52, 56). It would be interesting to see if expression of DSCR1L1 is a direct effect of mycolactone and to look at the effect of mycolactone on DNA-binding activity of the AP-1 complex.

Other macrolides like cyclosporin, FK-506 and rapamycin have similar but distinct modes of interaction with cyclophilins, calcineurin and transcription factors (116, 119). These immunosuppressive drugs have also been shown to inhibit inflammatory responses in macrophages (123). FK506 and cyclosporin, when complexed to specific immunophilins (FKBP12 and cyclophilin A, respectively), bind calcineurin at multiple sites, inhibiting calcineurin activity (114, 115, 124). By inhibiting calcineurin activity, cyclosporin and FK 506 prevent the nuclear translocation of NF-AT secondary to dephosphorylation, thereby suppressing T-cell activation (122) (Fig. 5.2). In a similar manner, the inhibitory effect of mycolactone on the activation of transcription factors can play a pivotal role in anti-inflammatory action of mycolactone.

Up-regulation of several apoptosis related genes like BAX and CASP3 is consistent with data reported by other groups showing induction of apoptosis *in vitro* as well as *in vivo* (13) (65) . Other genes of interest modulated by mycolactone include LTC4S, ITGA1, TIMP2 and RAD23B. TIMP2, which is an inhibitor of metalloproteinases (MMPs), was downregulated throughout the time course of the microarray study. During healthy states, the integrity of the extracellular matrix requires a correct balance between the amount and activity of matrix-degrading proteolytic enzymes and their associated inhibitors. It has been demonstrated that MMPs cause tissue destruction during disease (125). Inhibition of TIMP2 could contribute to the extensive tissue destruction seen in Buruli ulcer.

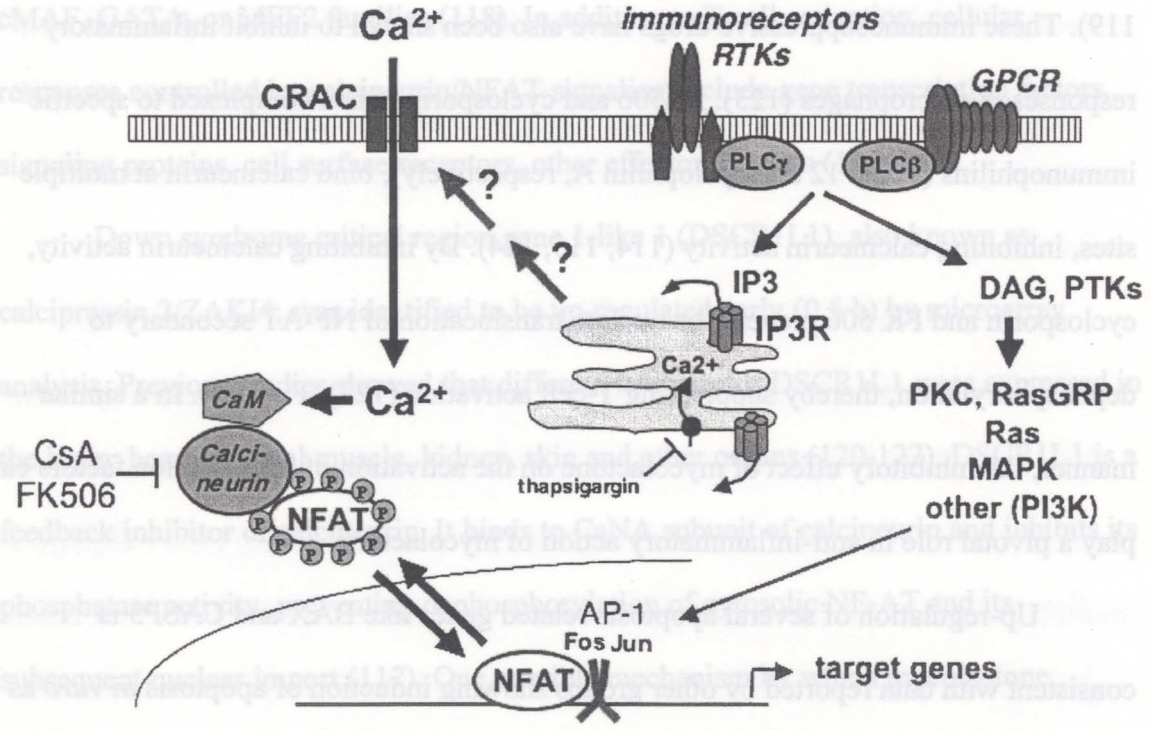


Fig. 5.2. Schematic view of the NFAT activation cycle. NFAT is activated by cell-surface receptors coupled to Ca2+ mobilization: Immunoreceptors and receptor tyrosine kinases (RTKs) activate phosphatidylinositol-specific phospholipase C (PLC)γ , while G-protein-coupled receptors (GPCR) activate PLCβ . PLC activation raises intracellular free Ca2+ levels ([Ca2+]I) in several sequential steps. [Ca2+] increases result in activation of many calmodulin (CaM)-dependent enzymes, including the phosphatase calcineurin and the CaM-dependent kinases CaMKII and CaMKIV. Calcineurin dephosphorylates multiple phosphoserines on NFAT, leading to its nuclear translocation and activation. Calcineurin activity is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506, which act as complexes with their intracellular immunophilin receptors cyclophilin and FKBP12, respectively. In parallel, hydrolysis of PIP2 by PLC results in production of diacylglycerols (DAG), which activate RasGRP and protein kinase C (PKC). Receptor activation is coupled to activation of protein tyrosine kinases (PTKs), Ras, MAP kinases (MAPK), and PI-3 kinase (PI3K). MAP kinase activation leads to synthesis and activation of Fos and Jun, the components of the heterodimeric transcription factor AP-1, which then binds cooperatively with NFAT to composite NFAT:AP-1 sites found in the regulatory regions of many NFAT target genes. (Taken from Hogan, P.G., *et al.*, 2003).

Arachidonic acid is a precursor of prostaglandins, leukotrienes (LTs) and related compounds that have important roles as mediators and regulators of inflammation (126). Formation of LTC_4 by the LTC_4 synthase enzyme represents the first committed step in the synthesis of LTs (127). LTs participate in a wide array of pathologic inflammatory, innate and acquired immune response processes (128). This could be interesting in the context of Buruli ulcer where there is minimal inflammation during infection. ITGA1, a gene that encodes for an intergrin receptor is involved in cell-cell adhesion and plays a role in inflammation and fibrosis is down-regulated by mycolactone. Another interesting gene that is down-regulated is RAD23B which is a DNA repair enzyme (109). Further experiments need to be performed in future to validate expression of the above genes by real-time PCR, northern or western blot analysis. Expression data described here can be expected to provide a foundation for further analysis of the role of interesting genes in mycolactone mediated activity.

Chapter 6

CONCLUSIONS

Buruli ulcer causes great morbidity and is a major health problem in many tropical countries (133, 134). A single Buruli ulcer, which can cover 15% of a person's skin surface, contains huge numbers of extracellular bacteria. The infection is characterized by massive necrosis at the site of infection followed frequently by debilitating disfiguration. Despite their abundance and extensive tissue damage, there is a remarkable absence of acute inflammatory response to the bacteria, and lesions are often painless (4, 71). A major breakthrough in the field came when the toxin made by the bacteria was identified to be a macrolide named mycolactone and was shown to be required for virulence (36). The persistence of *M. ulcerans* infection and the ineffectiveness of antibiotics are thought, at least in part, to relate to a local immunosuppression caused by the toxin at the site of infection (28, 49). Since the first documentation of the disease in 1898, not much progress has been made in understanding the immune response to the bacterium.

One hallmark of most diseases caused by mycobacteria including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. marinum*, *M. haemophilum* is the ability of the bacilli to grow within host cells and cause granulomas. In contrast, *M. ulcerans* primarily forms extracellular microcolonies within necrotic tissues and is rarely found within host cells (47). Using a macrophage infection model, the role of mycolactone in the extracellular location of the bacteria was investigated. Data obtained from the present study showed that wild type *M. ulcerans* is significantly less efficiently phagocytosed by macrophages

compared to isogenic mycolactone-negative *M. ulcerans* mutants. Experiments using a wide panel of *myc- M. ulcerans* strains have shown that presence or absence of mycolactone determines whether the bacteria are extracellular or intracellular. Exposure of macrophages to high concentrations of mycolactone interferes with their phagocytic ability. These observations that a mycolactone mutant is better phagocytized than the wild type strain is consistent with the presence of almost exclusively extracellular bacteria in Buruli ulcer patients. Surprisingly *myc- M. ulcerans* were not cleared by macrophages and survived within macrophages. Further studies to determine the intracellular location and compartmentalization of mutants within the macrophage may yield insight into strategies used by *myc- M. ulcerans* strains for survival within cells.

One of the intriguing findings is that although few WT *M. ulcerans* could be found inside cells *in vitro*, intracellular bacteria are rarely found in skin lesions from patients with clinical disease. This may be a limitation of the *in vitro* model used which may not reflect collective events that take place *in vivo*. Also, the disease is usually detected in patients at a much later stage where the lesion has already developed. These patients may be representative of a scenario where progression to the disease stage is due to a defect in the immune response leading to unrestricted multiplication of bacteria, subsequently leading to production of toxin thereby causing tissue damage, necrosis, cell death and extracellular location of the bacteria.

Though studies showed that uptake of WT *M. ulcerans* is inhibited, specific events that may lead to this have not been addressed. Recognition of *M. ulcerans* and binding by the phagocyte may be affected by mycolactone and other cell wall components of *M. ulcerans*. Also molecular events involved in internalization of the

bacteria may be interrupted. It is still not clear whether antiphagocytic activity of mycolactone is specific or is a more general cytotoxic response with lack of phagocytosis being a consequence of cytoskeletal disruption and paralysis of the cell. Future studies to understand the initial events leading to disruption of phagocytosis will help in understanding the extracellular location of *M. ulcerans* and the specific effect if any of mycolactone and other cell wall components of *M. ulcerans* on phagocytic receptors and uptake. Determining the cell wall constituents of *M. ulcerans* and comparison to that of other pathogenic intracellular mycobacteria may aid in understanding the contrast in immune response and cellular location of the bacteria.

TLR activation has been shown to be necessary for innate immunity by inducing pro-inflammatory cytokines and antimicrobial activity (80, 81). *M. tuberculosis* and its cell wall components have been show to activate TLR2 and TLR4 pathways leading to an activation of several signal transduction pathways important in the immune response. In this context, studying the effect of *M. ulcerans* and mycolactone on TLR signaling may be relevant. The complement system plays an important role in clearance of bacterial pathogens by participating in their opsonization through complement deposition following its activation via the classical or alternative pathways (135). Complement-opsonized organisms are then recognized by phagocytes through complement receptors and phagocytosed. Potentially, any pathogen able to interefere with complement deposition is likely to impair phagocytosis by preventing its recognition as a particle to be destroyed (61). Pathogenic mycobacteria are known to activate the alternative complement pathway. Complement activation by *M. ulcerans* has not been studied thus far and may yield useful information about the role if any, of complement in the disease.

Experiments studying the effect of mycolactone concentrations on fibroblast cell lines showed that mycolactone-mediated apoptosis and necrosis was concentration dependent. Mycolactone caused necrosis at high concentrations and apoptosis at low concentrations. Chemotaxis assays using human neutrophils showed that neutrophils do not respond to *M. ulcerans* (WT or *myc*-) or mycolactone. Mycolactone treatment resulted in rapid necrosis of human neutrophils in a dose dependent manner *in vitro*. These data could be relevant in vivo in human infections where toxin gradients produced by a pool of extracellular *M. ulcerans* may cause apoptosis or necrosis of inflammatory cells trying to move into the focus of infection and clear the bacteria.

Lack of an inflammatory reaction during the necrotizing stage of Buruli ulcer could be due to abrogation of production of inflammatory cytokines (e.g. TNF-α, IL-1, IFN-γ), chemokines (IL-8) and lowered expression of cell-adhesion molecules (e.g. ICAM-1, selectins, VCAM) which help inflammatory cells reach the site of infection. TNF-α and IL-8 are key players in immuno-inflammatory responses. Studies regarding TNF-α response to bacterial infection and mycolactone treatment *in vitro* showed that WT *M. ulcerans* and mycolactone did not induce TNF-α production while *myc- M. ulcerans* did. Interestingly, LPS mediated TNF-α production was inhibited by WT *M. ulcerans* and mycolactone. Both WT and *myc- M. ulcerans* as well as mycolactone did not induce IL-8 production. WT *M. ulcerans* and mycolactone induced expression of the cell adhesion molecule ICAM-1 was less than that induced by *myc- M. ulcerans* or LPS. It will be of interest to look at expression of anti-inflammatory cytokines (IL-4, IL-6, IL-

10, TGF-β) and their effect if any, in decreasing the inflammatory response during infection.

Finally, microarray analysis of genes modulated by mycolactone has yielded interesting information. Genes that are significantly upregulated by mycolactone included those related to transcriptional repressors, cytoskeletal rearrangement, cell cycle control/proliferation, apoptosis, G-protein receptors, tumor supression and immune response. Genes downregulated by mycolactone included those related to DNA repair, inactivation of complement and metalloproteinases, immune response, leukotrienes production, receptors for collagen and laminin.

To validate the microarray data, real time PCR, northern or western blot analysis of genes of interest need to be performed. Interest in the calicneurin inhibitor DSCR1L1 resulted from previous research on other macrolide immunosuppressants like FK506 and rapamycin which are calcineurin inhibitors. It is tempting to speculate that mycolactone may also act via a similar pathway. Upregulation of DSCR1L1 by mycolactone, if true, could be very important. Further studies to address the modulation of DSCR1L1 may yield valuable information regarding the mode of action of mycolactone. Since the major role of calcineurin is to dephosphorylate NFAT and regulate the location and activity of NFAT, the effect of mycolactone on NFAT/calcineurin pathway and subsequent expression of NFAT dependent genes needs to be studied in detail.

Knowledge obtained from the present study will contribute to a better understanding of the role of mycolactone in host-pathogen interactions as well as pathophysiology of the disease and can be expected to have a significant impact on immuno-therapeutic (vaccine) interventions for Buruli ulcer.

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APPENDICES

Appendix 1

MICROARRAY DATA

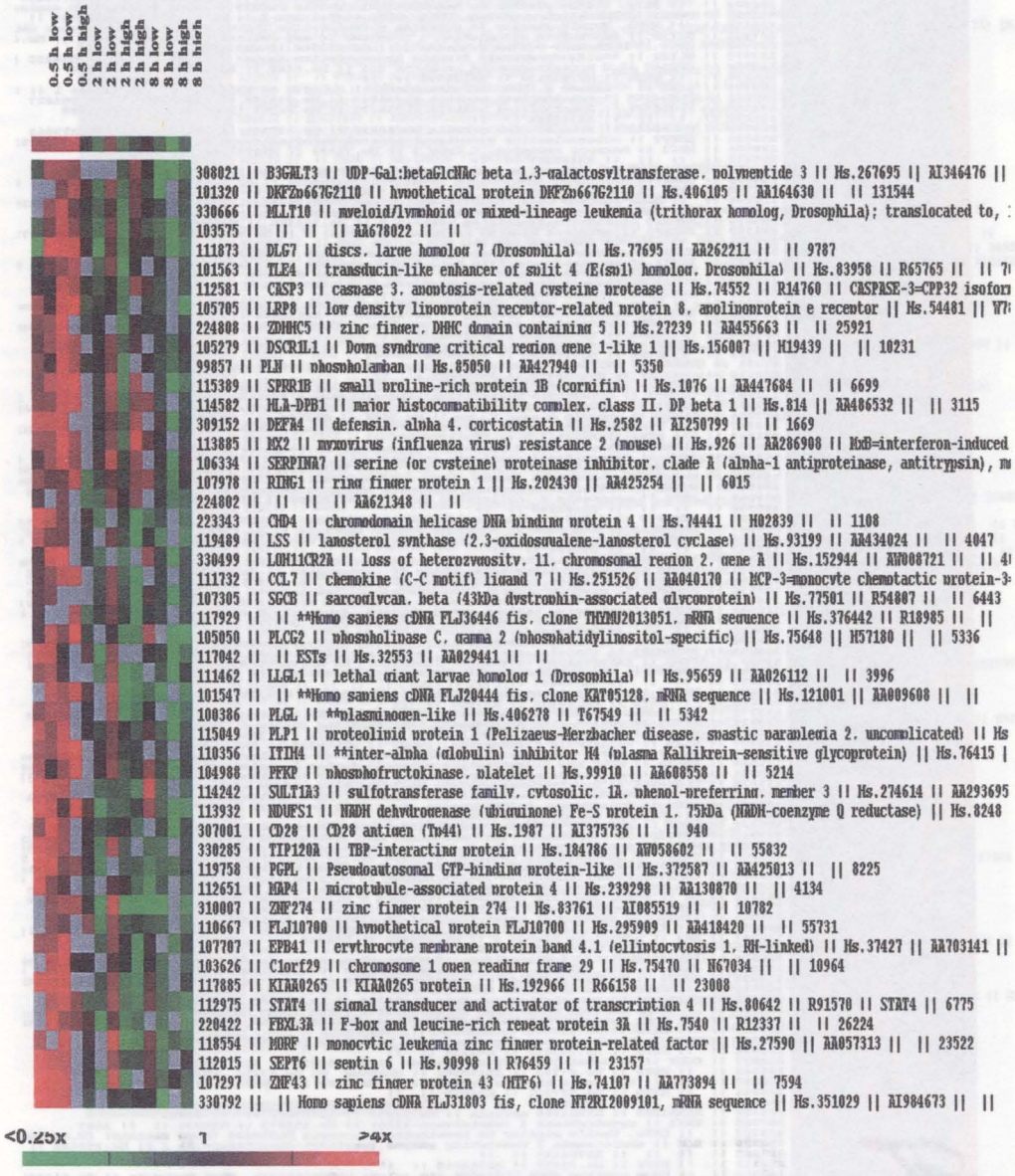


Fig. A 1.1. Genes up-regulated 0.5 h post mycolactone treatment.



Fig. A 2.2. Genes up-regulated 2 h post mycolactone treatment.

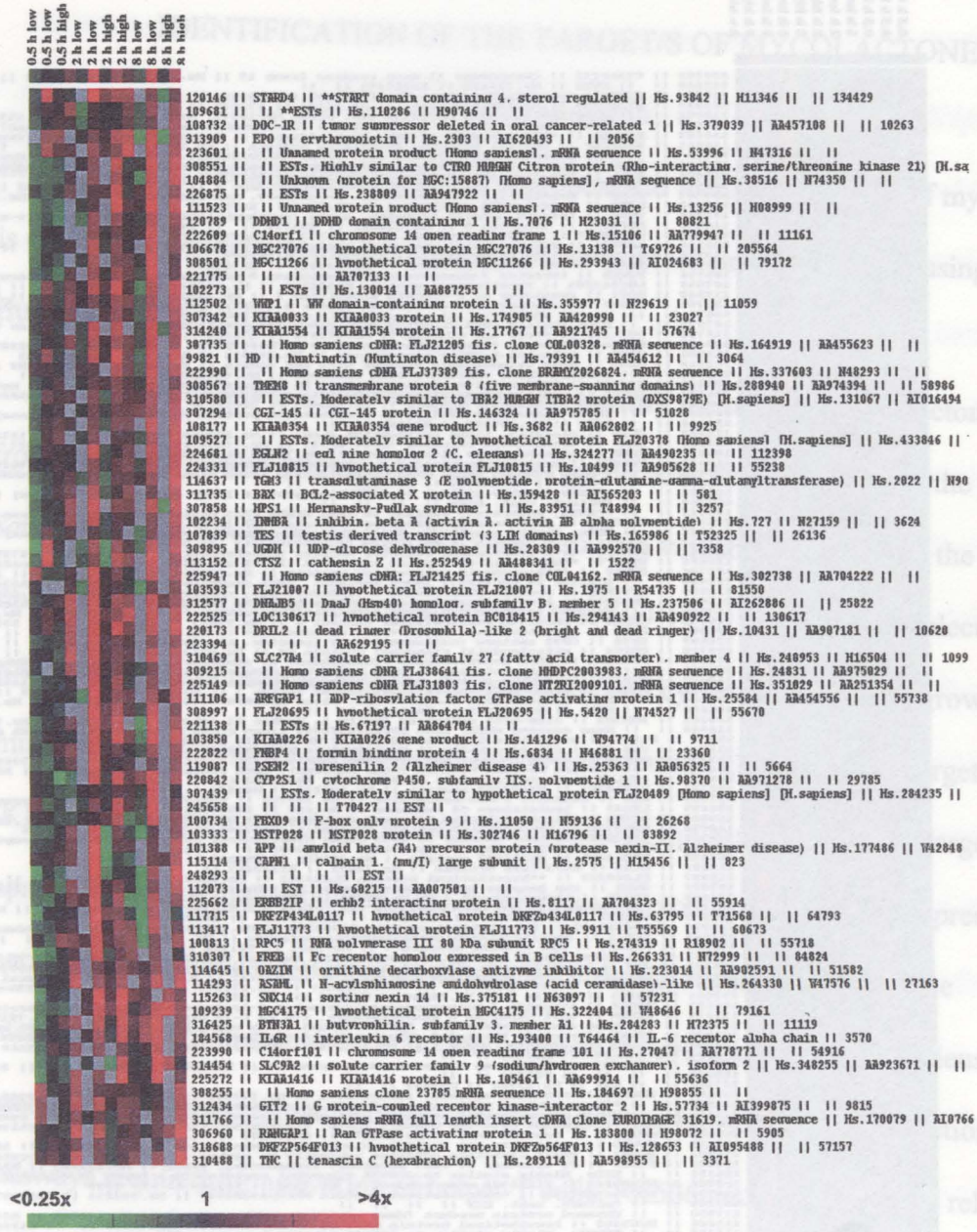


Fig. A 1.3. Genes up-regulated 8 h post mycolactone treatment.



Fig. A 1.4. Genes down-regulated throughout time course (0.5, 2, 8 h) post mycolactone treatment.

Appendix 2

IDENTIFICATION OF THE TARGET/S OF MYCOLACTONE

Introduction

In this chapter, the use of two different approaches to identify the target of mycolactone is described: a genetic approach using yeast, and a biochemical approach using fluorescently tagged mycolactone.

Interest in using a genetic approach to identify the target of mycolactone resulted from extensive studies published in the past two decades describing use of the yeast *Saccharomyces cerevisiae* as a model system to gain valuable insights into the targets and modes of action of a diverse array of drugs (119, 129-131). Genetic and molecular studies in yeast have demonstrated that the drugs FK506 and cyclosporine inhibit growth via immunophilin dependent inhibition of fungal calcineurin (116, 129). The target of the FKBP12/rapamycin complex was also first identified in yeast as the TOR (target of rapamycin) kinase homologues (130). In higher eukaryotes, the immunosuppressive macrolide compounds cyclosporine (CsA), FK506, and rapamycin inhibit the intermediate steps in signal transduction between the cytoplasm and the nucleus. These three compounds are immunosuppressive because they block signal transduction pathways required for activation of helper T cells. FK506 and rapamycin are related macrolides and share no chemical similarity with CsA, a cyclic undecapeptide (119, 131). Recent studies revealed that the molecular mechanisms of the immunosuppressive and antimicrobial effects of FK506 and rapamycin were remarkably similar and involved inhibition of highly conserved target proteins (37, 39). Both drugs are hydrophobic and enter the cell by diffusion. They then bind to highly conserved intracellular protein,

FKBP12, which is conserved from unicellular eukaryotes such as *S. cerevisiae* to man. FKBP12 is an enzyme that catalyses cis-trans peptidyl prolyl isomerization, a rate-limiting step during protein refolding *in vitro* (130). Both FK506 and rapamycin bind to the FKBP12 active site and inhibit activity, but this is not the mechanism by which these drugs act. Instead, the protein/drug complexes are the active *in vivo* agents (37, 39). It was observed that yeast mutants lacking FKBP12 are viable and resistant to FK506 and rapamycin and increasing FKBP12 protein levels in mammalian cells by transfection results in increased drug sensitivity. The target of FKBP12/FK506 complex is calcineurin (119, 131). Calcineurin is a calcium/calmodulin activated protein phosphatase that plays a central role in signal transduction from the T-cell receptor to regulate T-cell activation and plays diverse roles in yeast involving gene regulation and cation homeostasis (115, 123). The target of the FKBP12/rapamycin compex is the TOR (target of rapamycin) kinase homologues (119, 124, 130-131). The success met by researchers in identifying the targets of the macrolides by using yeast as a model organism led us to use a similar approach to investigate the target of mycolactone.

Use of yeast as a model system to identify the target of mycolactone

To detect the effect (cytopathic/cytostatic) of mycolactone on yeast, a variety of strains were used. Strains used and their sources are listed in Table 7. The strains tested included 4 strains of *S. cereviceae* and 2 strains of *Cryptococcus neoformans* kindly provided by Dr. Joseph Heitman (Duke University Medical center, Durham, NC). SMY47-9.1d and SMY76-13b are SMY44-1 derivatives. MLY61a/α is a diploid strain with ∑1278b background. JEC21 is of serotype D and is avirulent whereas H99 is of

serotype A and is virulent. The *C. neoformans* strains were sensitive to rapamycin, FK506 and CsA while *S. cereviceae* strains are sensitive to rapamycin (116). In addition to the strains mentioned above, *C. albicans* SC 5364 and *S. cereviceae* BY4742 provided by Dr. Jeffrey Becker's lab at the University of Tennessee, Knoxville were also tested for sensitivity to mycolactone. Sensitivity to mycolactone was tested by disc diffusion method as well as broth dilution method.

Materials and methods

Disc diffusion method: *S. cereviceae*, *C. neoformans* and *C. albicans* were cultured in YEPD (Difco) liquid medium overnight at 30°C (*S. cereviceae*, *C. albicans*) or 37°C (*C. neoformans*). Cells were pelleted and resuspended in fresh culture medium at 5×10^6 cells/ml. Yeast cells (1ml) were added to 2 ml of 0.2% noble agar which was poured onto the surface of YEPD or minimal agar (with amino acid supplements and uracil) medium and allowed to solidify to allow even distribution of inoculum. Paper discs (6.35mm, Becton and Dickinson, Cockeysville, MD) were placed on the agar surface and different concentrations of mycolactone (50 μg, 5 μg, 1 μg, 500 ng) were added to discs, ethanol served as negative control. Plates were incubated at 30°C or 37°C for 24-72 h. Growth inhibition was quantified by measuring the distance from the edge of the disc to the zone of growth (the radius of growth inhibition).

A similar assay was repeated by making YEPD plates with the above concentrations of mycolactone added to the agar before pouring the plates. Yeast strains were streaked onto plates and observed after 24, 48 and 72 h for growth inhibition.

Table 7. Yeast strain list.

Strain used	Description	Source
S. cerevisiae	SMY44-1 (Matα trp1 his3 ura3 60pS-LEU2)	Joseph Heitman
S. cerevisiae	SMY47-9.1d (Matα trp1 his3 ura3 60pS-LEU2 erg6)	Joseph Heitman
S. cerevisiae	SMY76-13b (Matα trp1 his3 ura3 60pS-LEU2 erg6 pdr5 snq2)	Joseph Heitman
S. cerevisiae	MLY61a/α (Mata/α ura3-52/ura3-52)	Joseph Heitman
S. cerevisiae	BY4742 (Matα HisΔ LeuΔ LysΔ Ura3)	Jeff Becker
C. neoformans	JEC21	Joseph Heitman
C. neoformans	H99	Joseph Heitman
C. albicans	SC5364	Jeff Becker

Broth dilution method: Broth dilution method was set up according to the NCCLS reference method for antifungal susceptibility testing for yeasts (132). Briefly, 0.1 ml yeast strains was added to 0.1ml YEPD or RPMI medium with different concentrations of mycolactone (10 ng, 100 ng, 500 ng, 1000 ng, 7.9 μg) in a 96-well plate. Ethanol was used as a negative control. Inoculum was prepared from 24 h (*S. cerevisiae*, *C. albicans*) or 48 h (*C. neoformans*) cultures at a concentration of 5×10^2 to 2.5×10^3 cells/ml. Plates were incubated at 35°C for 46-50 h (*C. albicans*, *S. cerevisiae*) or 70-74 h (*C. neoformans*). Growth in each well was compared to growth in ethanol treated and control wells. Plating samples showed that there was no inhibition of growth by mycolactone as the number of viable yeast was the same between the test and control groups.

Results and Discussion

A sensitive phenotype was not found in the yeast strains used as there was no inhibition of growth of all strains tested by either the disc diffusion method or broth dilution method. The possible reasons as to why we did not see a sensitive phenotype in yeast that were tested could be because the target for mycolactone may not be present in yeast. Another possibility is that mycolactone may not be able to enter the yeast cell in the same way as other macrolides like rapamycin and FK506 do. In the future, one way to test this would be to use fluorescently tagged mycolactone to visualize the permeability of yeast cell wall to mycolactone.

Biochemical approach to identify the target of mycolactone using fluorescent mycolactone analogs

An alternative approach taken to identify the target of mycolactone was analysis of the interaction of L929 fibroblast cell proteins (cytosolic and cell membrane proteins) with fluorescent mycolactone analogs. L929 fibroblast cells were used because they are sentitive to mycolactone and the cytopathic effect of mycolactone on these cells has been well studied (35, 36). Structures of the fluorescent mycolactone analogues are as shown in Fig. A 2.1. The fluorescent analogues were kindly provided by Dr. Michael Burkart at the University of San Diego, CA. Both the analogs were cytopathic to L929 cells though the cytopathicity was roughly 100-fold less than that of the native myctoalctone.

Materials and methods

Cytoplasmic and cell membrane proteins were isolated from L929 cells as follows. After growing confluently for 48 h, cells were washed three times with 10ml of sterile PBS (pH 7.4), and centrifuged at 5000 rpm (10 min, 4°C) between each wash. Cell pellets were then frozen at –20°C for 3 h after which they were resuspended in 10ml of ice cold HB-1 buffer (250mM sucrose and 3mM imidazole, pH 7.4) and pelleted at 5000 rpm (5 min, 4°C). The resulting pellet was suspended in 5ml HB-2 buffer (250mM sucrose, 3mM imidazole, 0.5mM EDTA, 0.5mM PMSF, pH 7.4). The pellet was passed 5 times through a 20 g needle followed by 10 times through a 25 g needle and centrifuged

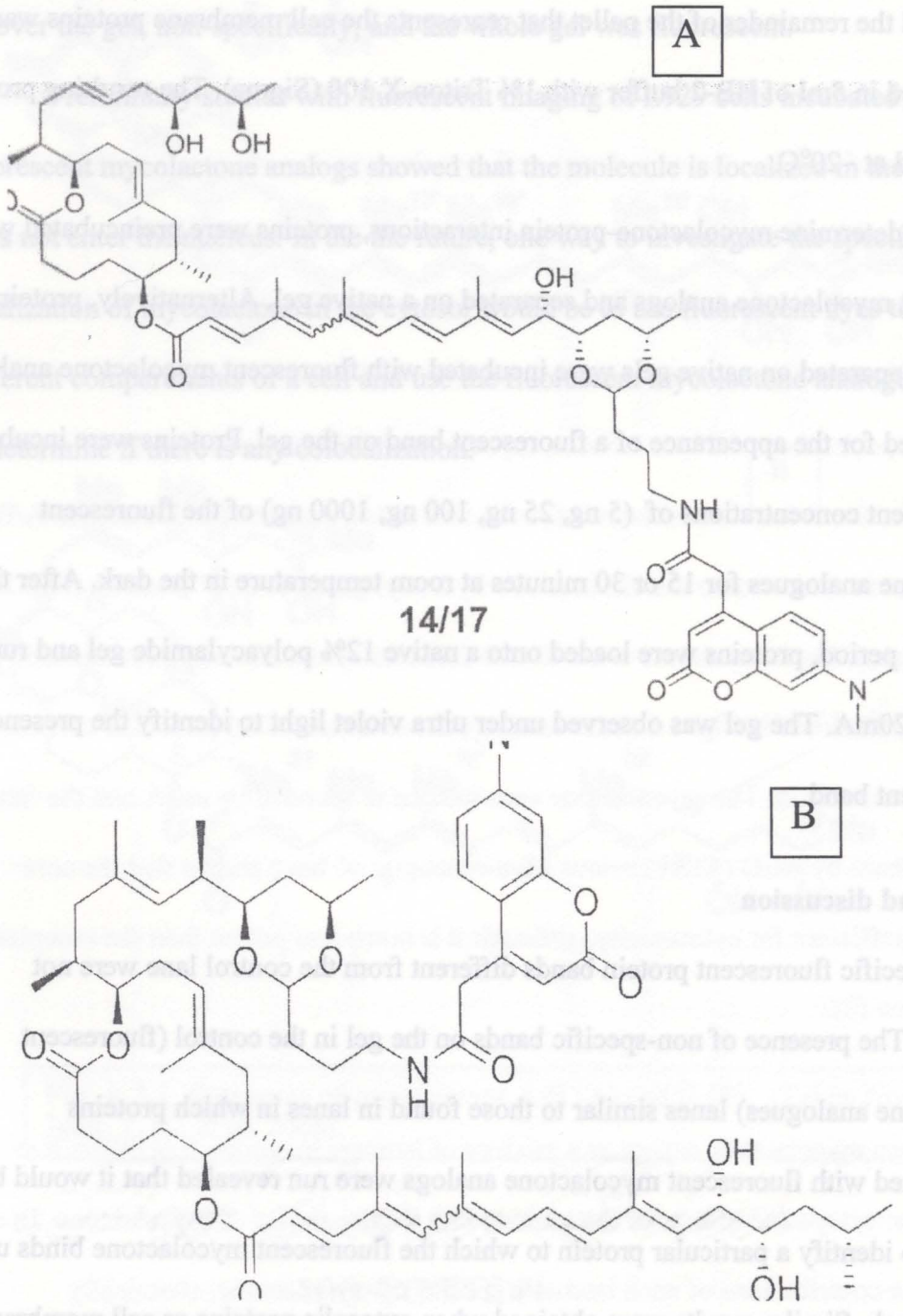


Fig. A 2.1. Structure of fluorescent mycolactone analogues 14 **(A)** and 17 **(B)**.

at 1500 rpm (5 min, 4°C). Supernatant was removed and recentrifuged at 68,000 g (30 min, 4°C). After centrifugation, the supernatant that represents cell cytosol proteins was pooled and the remainder of the pellet that represents the cell membrane proteins was resuspended in 5ml of HB-2 buffer with 1% Triton-X 100 (Sigma). The resulting proteins were stored at –20°C.

To determine mycolactone-protein interactions, proteins were preincubated with fluorescent mycolactone analogs and separated on a native gel. Alternatively, proteins that were separated on native gels were incubated with fluorescent mycolactone analogs and checked for the appearance of a fluorescent band on the gel. Proteins were incubated with different concentrations of (5 ng, 25 ng, 100 ng, 1000 ng) of the fluorescent mycolactone analogues for 15 or 30 minutes at room temperature in the dark. After the incubation period, proteins were loaded onto a native 12% polyacylamide gel and run for 1-1.5 h at 20mA. The gel was observed under ultra violet light to identify the presence of a fluorescent band.

Results and discussion

Specific fluorescent protein bands different from the control lane were not observed. The presence of non-specific bands on the gel in the control (fluorescent mycolactone analogues) lanes similar to those found in lanes in which proteins preincubated with fluorescent mycolactone analogs were run revealed that it would be difficult to identify a particular protein to which the fluorescent mycolactone binds using this approach. Similar results were obtained when cytosolic proteins or cell membrane proteins were used. When proteins separated on a gel were incubated with different

concentrations of the fluorescent mycolactone analogues for 30 min, 1 h or overnight, specific bands could not be found. A major pitfall was that mycolactone seemed to bind all over the gel, non-specifically, and the whole gel was fluorescent.

Preliminary studies with fluorescent imaging of L929 cells incubated with fluorescent mycolactone analogs showed that the molecule is localized in the cytosol and does not enter the nucleus. In the the future, one way to investigate the specific localization of mycolactone in the cytosol would be to use fluorescent dyes to stain the different compartments of a cell and use the fluorescent mycolactone analogues as tools to determine if there is any colocalization.

Appendix 3

ACTIVITY OF MYCOLACTONE

Introduction

Mycolactone was isolated originally from *M. ulcerans* 1615, a Malaysian isolate, as a mixture of cis/trans isomers designated mycolactone A and mycolactone B (34). Cadapan *et al.* have recently shown that *M. ulcerans* 1615 produces several minor species of mycolactone (congeners) in addition to mycolactone A/B which differ primarily in the number of hydroxyl groups and double bonds (75). While mycolactone A/B is the dominant species from African isolates, mycolactone C is the dominant mycolactone species from the Australian isolates. Mycolactone C is a variant of mycolactone A/B with an altered side chain due to the absence of a hydroxyl group at C12' position (Fig. 7.1). Mycolactone C has been shown to be 10,000-fold less cytopathic than mycolactone A and B (3). As shown in Fig. 1.1 (introduction), the structure of mycolactone consists of a core and a side chain. The mycolactone core lactone is encoded by *mls*A and the fatty acid side chain by *mls*B (42). Previous Mve-obiang *et al.* have shown that the core lactone is sufficient for cytotoxicity, although it is much less potent than the complete mycolactone (3).

Since mycolactone occurs as a mixture of isomers in nature, it is difficult to attribute the cytopathic effects of the toxin to any single species of mycolactone. In order to study the contributions of each isomeric species of mycolactone, chemically synthesized mycolactones A, B, C, core and side chain were used. These chemical

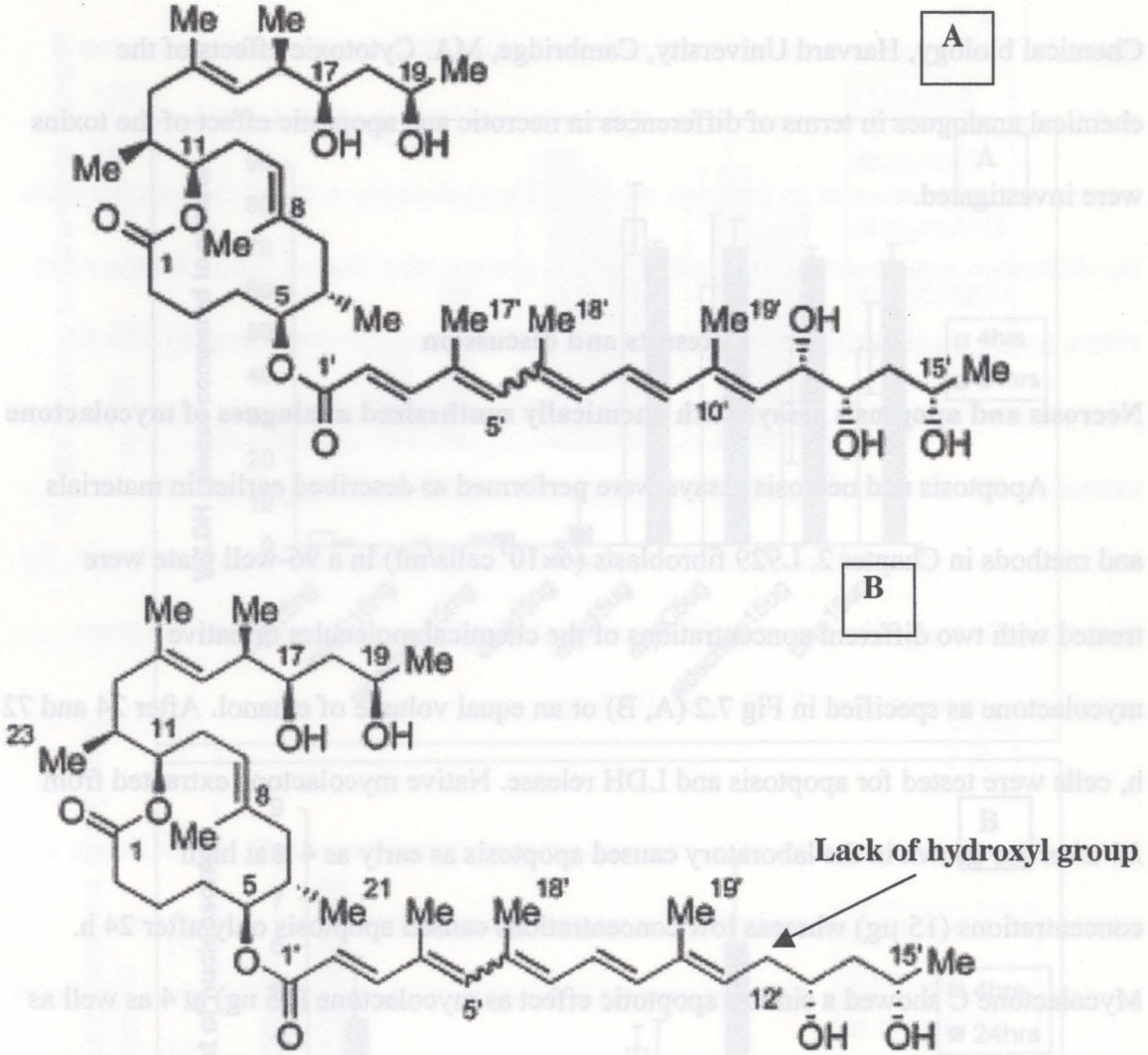


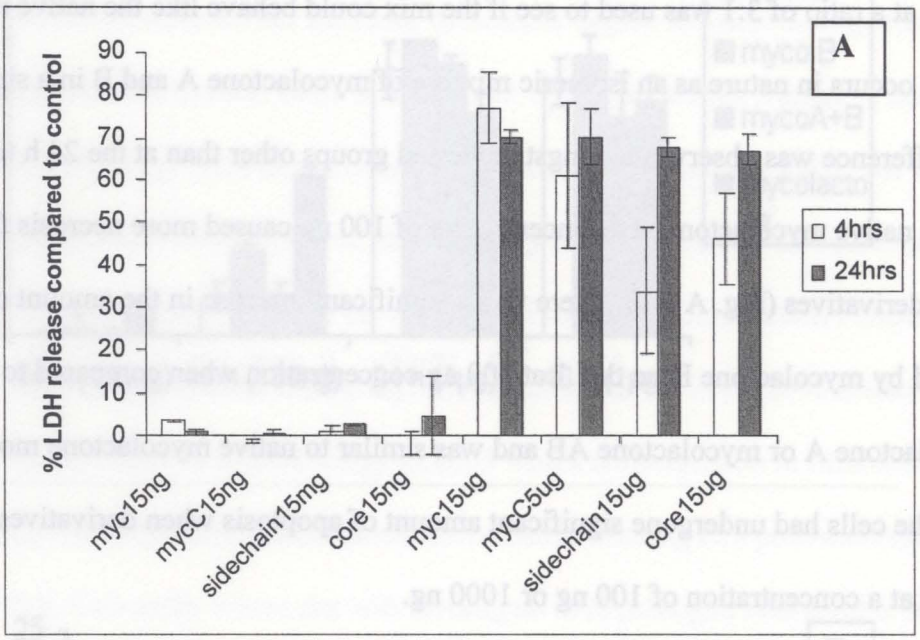
Fig. A 3.1. Structures of mycolactone A/B **(A)** and C **(B)**. (Taken from Judd, T. C., *et al.* 2004)

analogues were provided by Dr. Yoshito Kishi at the Department of Chemistry and Chemical biology, Harvard University, Cambridge, MA. Cytotoxic effects of the chemical analogues in terms of differences in necrotic and apoptotic effect of the toxins were investigated.

Results and discussion

Necrosis and apoptosis assays with chemically synthesized analogues of mycolactone

Apoptosis and necrosis assays were performed as described earlier in materials and methods in Chapter 2. L929 fibroblasts (6×10^3 cells/ml) in a 96-well plate were treated with two different concentrations of the chemical molecules or native mycolactone as specified in Fig 7.2 (A, B) or an equal volume of ethanol. After 24 and 72 h, cells were tested for apoptosis and LDH release. Native mycolactone extracted from *M. ulcerans* grown in the laboratory caused apoptosis as early as 4 h at high concentrations (15 μg) whereas low concentrations caused apoptosis only after 24 h. Mycolactone C showed a similar apoptotic effect as mycolactone (15 ng) at 4 as well as 24 h time points. Side chain and core showed very little apoptotic activity after 4 and 24 h. The cytotoxicity profile as measured by LDH release was the same for all the chemical derivatives and native mycolactone added at a concentration of 15 ng. Similar results were obtained when higher concentrations were added with all the analogues exhibiting an increase in cell wall permeability at 4 h as well as 24 h. Mycolactone C was added at a lower concentration than the other derivatives due to limited availability.



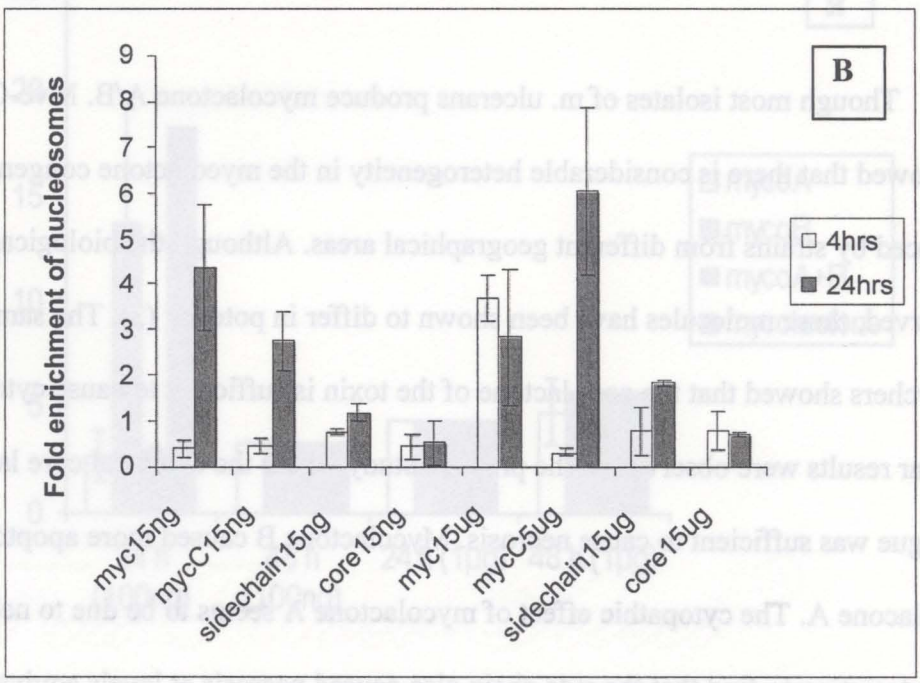


Fig A 3.2. Necrosis (**A**) and Apoptosis (**B**) of L929 fibroblasts 4 and 24 h post treatment with chemically synthesized mycolactone core, side chain, mycolactone C and native mycolactone.

In another set of experiments, chemically synthesized mycolactone A and B were also tested for ability to cause necrosis and cell death. In addition, a mix of mycolactone A and B at a ratio of 3:1 was used to see if the mix could behave like the native molecule which occurs in nature as an isomeric mixture of mycolactone A and B in a similar ratio. No difference was observed amongst the tested groups other than at the 24 h time point where native mycolactone at a concentration of 100 ng caused more necrosis than the other derivatives (Fig. A 3.3). There was a significant increase in the amount of apoptosis caused by mycolactone B on day 1 at 100 ng concentration when compared to mycolactone A or mycolactone AB and was similar to native mycolactone molecule. By 48 h the cells had undergone significant amount of apoptosis when derivatives were added at a concentration of 100 ng or 1000 ng.

Though most isolates of m. ulcerans produce mycolactone A/B. Mve-Obiang *et al.* showed that there is considerable heterogeneity in the mycolactone congeners produced by strains from different geographical areas. Although the biological activity is conserved, these molecules have been shown to differ in potency (3). The same researchers showed that the core lactone of the toxin is sufficient to cause cytopathicity. Similar results were observed in the presernt study where the chemical core lactone analogue was sufficient to cause necrosis. Mycolactone B caused more apoptosis than mycolacone A. The cytopathic effect of mycolactone A seems to be due to necrosis. It was interesting to find that the side chain also caused necrosis at levels equivalent to the core and native mycolactone. Though mycolactone C was added at a lower concentration than the other analogues, its activity was similar to native mycolactone. Further

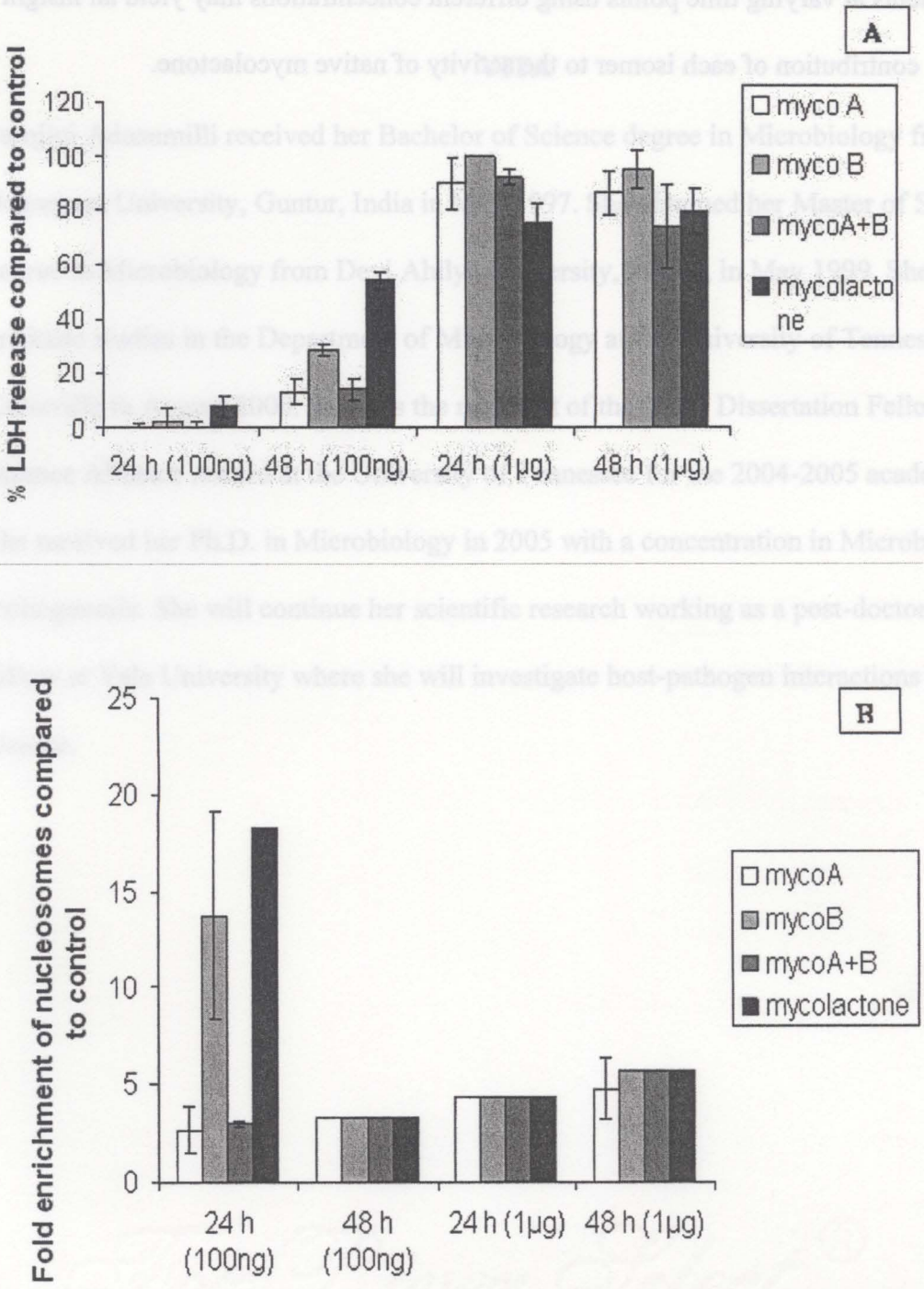


Fig. A 3.3. Necrosis (**A**) and Apoptosis (**B**) of L929 fibroblasts 1 and 2 d post treatment with chemically synthesized mycolactone A, B and native mycolactone.

experiments at varying time points using different concentrations may yield an insight into the contribution of each isomer to the activity of native mycolactone.

VITA

Sarojini Adusumilli received her Bachelor of Science degree in Microbiology from Nagarjuna University, Guntur, India in May 1997. She obtained her Master of Science degree in Microbiology from Devi Ahilya University, Indore, in May 1999. She began graduate studies in the Department of Microbiology at the University of Tennessee at Knoxville in August 2000. She was the recipient of the Yates Dissertation Fellowship and Science Alliance Award at the University of Tennessee for the 2004-2005 academic year. She received her Ph.D. in Microbiology in 2005 with a concentration in Microbial Pathogenesis. She will continue her scientific research working as a post-doctoral fellow at Yale University where she will investigate host-pathogen interactions in Lyme disease.