DISSERTATION

# DEVELOPMENT AND EVALUATION OF NEW LEPROSY SKIN TEST ANTIGENS AS DIAGNOSTIC TOOLS

Submitted by

Becky Louise Rivoire

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2012

Doctoral Committee:

Advisor: Patrick Brennan

Barry Beaty
Mercedes Gonzalez-Juarrero
Olve Peersen

# ABSTRACT

## DEVELOPMENT AND EVALUATION OF NEW LEPROSY SKIN TEST ANTIGENS AS DIAGNOSTIC TOOLS

An early diagnostic test for leprosy that is adequately sensitive and specific to identify infected individuals before the onset of clinical symptoms continues to be one of the greatest needs in the field. Preclinical diagnosis would expedite the delivery of chemotherapy to patients, prevent disabilities, decrease stigma, intercept transmission, and measure the true incidence of disease. To address this pressing need, three new leprosy skin test antigens were investigated: MLSA-LAM [*M. leprae* soluble antigens devoid of lipoglycans, primarily lipoarabinomannan (LAM)], MLCwA (*M. leprae* cell wall associated antigens), and MLMA-LAM (*M. leprae* membrane antigens devoid of lipoglycans, primarily LAM). Two of these antigens, MLSA-LAM and MLCwA, were developed for manufacturing and testing for safety and efficacy in phase I and phase II human clinical trials.

Skin test antigens were derived from *M. leprae* purified from experimentally infected armadillo tissues under current good manufacturing practice conditions. A skin test pilot plant was created at Colorado State University for this purpose. Quality control testing of skin test antigens included potency and stability testing in guinea pigs, safety testing in guinea pigs and mice, integrity testing by gel electrophoresis and immunoblotting, and purity testing for residual dextran, collagenase, detergent, and endotoxin. An investigational new drug (IND) application was submitted to the Food and Drug Administration (FDA) and clinical protocols with respective informed consent forms were generated. Training in good laboratory, manufacturing, and clinical practice (GLP, GMP, and GCP) was a prerequisite for these studies.

The phase I clinical trial was conducted at a non-endemic region for leprosy with both antigens at 2.5, 1.0, and 0.1 µg dosages. A randomized double blind phase II clinical trial (stages A, B, and C) followed in an endemic region for leprosy with both antigens at 1.0, and 0.1 µg dosages. Antigens were tested in the phase I and phase II, stage A/B trials using the intradermal delayed type hypersensitivity (DTH) skin test in healthy subjects without known exposure to leprosy, while the phase II, stage C trial compared the DTH skin test to the IFN-γ test and the *M. leprae* specific phenolic glycolipid I antibody test in target populations, including: leprosy patients, household contacts of leprosy patients, and tuberculosis patients.

Both skin test antigens, MLSA-LAM and MLCwA, were found to be safe at each dose tested in the phase I and II clinical trials. The phase II, stage A/B clinical trials showed the baseline in healthy endemic controls for both leprosy antigens at the low dose of 0.1 µg was negligible, while slightly elevated with the high dose of 1.0 µg. Efficacy findings from the phase II, stage C clinical trial showed that the antigens were immunologically potent; highly specific, but lacked sensitivity at the low dose. The response to PPD did not correlate with either leprosy antigen at either dose. The IFN-γ release test provided the best diagnostic accuracy at the high dose with both antigens. Household contacts with the highest risk of infection reacted in each test.

MLSA-LAM and MLCwA are the first skin test antigens to show specificity for leprosy in the field. The interferon gamma release assay with MLSA-LAM at the high dose provides the best diagnostic accuracy for tuberculoid leprosy patients. The PGL-I antibody assay provides the best diagnostic accuracy for lepromatous leprosy patients. Optimization of the antigen dosage or use of these tests in parallel or combination could lead to enhanced sensitivity, resulting in a good early diagnostic test for leprosy. Results from these research studies prove that a product can be translated from the bench to the clinic in an academic setting.

# ACKNOWLEDGMENTS

I would like to thank Ms. Angela Marques, Mr. Nathan Groathouse, Ms. Emily Stump, and Ms. Mary Sanders for their assistance on this research project, and Ms. Heejin Kim for her constant moral support and valued friendship. I am also thankful to Mr. Kapil Neupane, Dr. Murdo Macdonald, Dr. Rachel Hawksworth, and Dr. Deanna Hagge at Anandaban Hospital, Kathmandu Nepal for implementing the phase II clinical study; Ms. Melinda Tibbals, Dr. Mark Wolfe, and Ms. Carol Smith at The Emmes Corporation, Rockville, MD for statistical evaluation; Ms. Robin Mason and Dr. Christine Sizemore at the Division of Microbiology of Infectious Diseases, National Institute of Allergy and Infectious Diseases for their tremendous regulatory and advisory role on the phase II study; and, the human subjects in Colorado, USA and Kathmandu, Nepal who courageously volunteered for the clinical trials.

I owe sincere and earnest thankfulness to my graduate committee members, Dr. Patrick Brennan, Dr. Barry Beaty, Dr. Mercedes Gonzales Juarrero, and Dr. Olve Peersen for instruction, examination, and counsel. Particularly, I am indebted to my primary advisor, Dr. Patrick Brennan, who provided this incredible opportunity and supported me without hesitation through many challenges over the years. Words cannot express my deep gratitude and appreciation.

Finally, this dissertation would not have been possible without the constant and loving support of my sisters, Linda Blish, Beverly Wicker, Deni Stubbs, and my mother, Janet Peters; unconditional love and patience of my children, Michael and Christopher Rivoire; full and unwavering emotional and physical support from my husband, my friend, and my rock, Stephen Rivoire; and, innumerable answered prayers by The Lord. This endeavor was fortified by a tremendous team for which I am eternally grateful. Becky Rivoire

# PREFACE

As a young member of the Brennan Laboratory in the late 1980's, elucidation of the major proteins of *Mycobacterium leprae* was underway. My involvement was multifaceted, including harvesting bacteria from armadillo tissues; disrupting bacteria by sonication; antigen fractionation by centrifugation and high pressure liquid chromotagraphy; analysis by gel electrophoresis and immunoblotting, ELISA, and gas chromatography; and, delayed type hypersensitivity potency testing in guinea pigs.

Working together with colleagues in the early days of the Mycobacterial Research Laboratories (MRL) to remove immunosuppressive lipoglycan components from subcellular fractions resulted in two new leprosy antigens that were potent in guinea pigs. At the time, numerous articles and recommendations by the World Health Organization were being published on the urgent need for a diagnostic tool for the detection of asymptomatic leprosy to aid in the elimination and understanding of the disease. Skin testing had already been discussed as being the most feasible method and with the excitement of the two new antigens, efforts towards an investigational new drug (IND) application was initiated. I remember the day, when I naively went into Dr. Brennan's office and suggested that we move these antigens from the bench to the clinic. With enthusiasm about developing the first early diagnostic, yet unaware of the full challenges that lay ahead, he agreed.

Contract manufacturing organizations were not easily identified, due to the cost and fear of working with *M. leprae*, therefore a decision was made to renovate a BSL-3 laboratory in the Microbiology building into a pilot manufacturing plant to prepare skin test antigens for phase I and phase II clinical trials. With support from our contract sponsor we pressed

Forward through a maze of regulations, facility renovations, documentation, documentation, and more documentation. Work through the IND process, manufacturing, phase I and phase II, stage A/B clinical trials was performed between 1992 and 2005.

In 2004, an opportunity to expand cGMP manufacturing capabilities at Colorado State University under the Regional Center of Excellence Program prompted a grant proposal for a unique academic-based product translational core. An RCE award was received under the direction of Dr. Barry Beaty to support many research investigators and three Core facilities, one of which was The Product Development and Manufacturing (PDM) Core. I transitioned into full time work with the RMRCE and part-time work as a graduate student in 2005 and continued through April, 2012. The phase II, stage C clinical study was completed in 2011 and the final clinical study report was submitted in February 2012.

Following the completion of this dissertation, my hope is that these new antigens will be tested further for diagnostic-epidemiological capabilities of screening individuals infected with asymptomatic leprosy. To have played a small part of such a success story will make every ounce of energy expended on this project and my limited life outside of work and school over these past 7 years worthy. Although my "bubbly enthusiasm" as described by one of my mentors has waned with work and personal circumstances over this last year, I am confident that it will return, and with that surge of effervescent energy, I plan to apply my higher education in areas of applied research, where I passionately believe in the need to move product ideas from the bench to the clinic.

# AUTOBIOGRAPHY

I will never forget my father's words, "when you do something, do it right." That has been my mantra, my fervor, my dedication throughout my life. My father was the apple of my eye and I wanted to be just like him; hence I was all tomboy and proud of it. My mother accepted the fact, as she had a bit of "tom" in her too. I always took the dirty jobs, the hard jobs, the thankless jobs as a kid. No reason, other than to say I could do the job and do it well. My love for horses was unlocked when I trotted and galloped in circles for 2 hours, while my youth group went on a trail ride. I didn't have enough money to go with them, but I had enough coins for 3 rides in a round pen. The owner felt sorry for me and let me ride for 2 hours (poor horse).

School always came easy, and I excelled at math and science. Nobody in our family had ever been to college and therefore the prospect never entered my mind, until I began working on my Aunt and Uncle's farm in Kansas. I learned that not only did I have a passion for horses, but I loved to be around and care for all kinds of animals. It was clear at that time that I was meant to be a veterinarian. Although my father told me that I shouldn't go to college, I had already decided it was my destiny.

After four quick years at CSU, I received my Bachelor of Science degree in animal science and a minor in biochemistry. Veterinary Medicine, as it turns out, was not my destiny. After graduating in 1981, I started working at Elars cleaning animal cages and quickly advanced up the ranks to making monoclonal antibodies, followed by assisting and then running good laboratory practice (GLP) animal studies on small and then large animals.

I met my husband, the new "apple of my eye" in 1980 and married in 1982. We had two wonderful boys in 1984 and -85 that filled our lives with immense joy and agreeably, a new

lifestyle. My husband completed his undergraduate and master's degree in physical therapy, as I worked to support the family and advance my career. Five years later, with Elars soon to be absorbed back into the Tech America parent company, my supervisor, Dr. Yoshio Teramoto asked me to visit Dr. Brennan at CSU, who was looking for a technician. He told me that I was the only one at Elars that he recommended, because Dr. Brennan was quite demanding and challenging of his staff. I began working with Dr. Brennan in 1986 on many different projects and flourished with the added responsibilities and challenges.

My hours at work were long, partially because of my mantra and multiple responsibilities. In 1996, I took a hiatus from mycobacteria and the skin test antigen project to work at Heska Veterinary Pharmaceuticals and then Mycos, LLC, with plans to spend more time for my boys. Unfortunately, "cookies and milk" were not on their agenda, so I returned to CSU in 2000, when given a chance to finish the skin test antigen project. It was during this time that Dr. Brennan suggested that we propose a product development core facility as a unique core to support the Regional Center for Excellence proposal. As my interest in product development had begun to blossom, I was very excited about this opportunity. With painful effort, I wrote my first grant proposal and by the grace of Dr. Beaty and Dr. Brennan, ran the Product Development and Manufacturing Core. My heart and soul went into making the Core a nationally recognized program, but it was clear that with this vision came the need to further my education. For that reason, I embarked on the long and arduous journey of a higher education.

# DEDICATION

I dedicate this dissertation to my younger sister, Beverly Billings Wicker. She always told me that I was her hero for going back to school. In truth, she was my hero for her strength and courage exemplified during her short battle with glioblastoma. Her jovial and loving spirit will live in my heart forever.

Additionally, I dedicate this work to the sisters, brothers, fathers, mothers, sons, and daughters afflicted with the terrible disease of leprosy. Their physical, social, and psychological suffering is incomprehensible. It is my greatest desire that the results of this research will in some way lead to the early diagnosis and eventual eradication of this age old, and highly feared disease.

TABLE OF CONTENTS

CHAPTER THREE: SAFETY ASSESSMENT OF TWO NEW LEPROSY SKIN TEST ANTIGENS IN HEALTHY SUBJECTS WITHOUT KNOWN EXPOSURE TO LEPROSY: RANDOMIZED DOUBLE BLIND CLINICAL STUDY

# LIST OF KEY WORDS

Leprosy

Diagnosis

MLSA-LAM

MLCwA

Skin Test

Delayed Type Hypersensitivity

Interferon Gamma Release Assay

PGL-I

CHAPTER ONE: LITERATURE REVIEW

## TAXONOMY AND GENERAL CHARACTERISTICS OF *MYCOBACTERIUM LEPRAE*

The etiological agent of leprosy is of the suborder Corynebacterineae, family Mycobacteriaceae, genus *Mycobacterium,* and species *leprae*. [1] *M. leprae* was identified by Gerhard Armauer Hansen in 1873 as the first bacterium to cause human disease. The bacteria are straight or slightly curved rod-shaped organisms. They are 1-8um long and 0.3um in diameter, and divide by binary fission. *M. leprae* is gram-positive, but also acid fast due to the presence of mycolic acids in the waxy coating on the surface of the organism. The pathogen is an obligate intracellular parasite, predominantly found in macrophages and Schwann cells. *M. leprae* cannot be grown axenically, but can be grown to concentrated levels in the nine banded armadillo and nude mouse foot pad. [2,3] The preferred growth temperature is between 30-34°C, supported by the low body temperature of the armadillo and mouse foot pad. This temperature preference is also reflected clinically, as the cooler areas of the body such as the skin, nasal mucosa, and peripheral nerves are predominant sites of infection.

## LEPROSY DISEASE

Leprosy was referred to as the "aristocrat of diseases" in colonial and imperial medical exchanges in the 19th century, because it was the oldest and most mysterious disease. [4] The first evidence of leprosy disease is 2000 BC, as found in ancient skeletal remains from India.[5] Single nucleotide polymorphism analysis suggests that Eastern Africa or the Near East was the origin of leprosy, and that it spread with human migration.[6] Leprosy disease, also called Hansen's disease, presents with different clinical manifestations, depending on the host response

to the organism.[7] Mycobacteria survive and multiply in phagocytes. They evade microbicidal host defense systems and interfere with the antigen presentation by macrophages, leaving the immune system defective and unable to mount a concerted protective immune response.[8,9] The clinical spectrum of leprosy disease is directly related to the strength of activation of the immune system, which is critical to protection from leprosy.[10] The innate immune response is directly correlated with development of leprosy disease and disease type. [11]

Leprosy affects the skin, nerves, limbs, and eyes. Early clinical symptoms include loss of feeling for heat followed by touch and pain, beginning at the extremities. Skin lesions appear later during the course of the disease. Hansen's disease of PB type is associated by one or more hypopigmented skin macules, while MB type is associated with symmetric skin lesions, nodules, plaques, thickened dermis, and frequent involvement of the nasal mucosa resulting in nasal congestion and epistaxis.[12] Advanced leprosy leads to paralytic or other neurologic disabilities, observed as physical deformities of the extremities or ocular degeneration.[13] Noticeable disabilities lead to emotional and social effects and shunning by the community.[14-17]

**CLINICAL DIAGNOSIS AND DISEASE CLASSIFICATION**

Leprosy diagnosis is currently based on clinical symptoms. An individual in an endemic area is diagnosed with leprosy based on one of two cardinal signs, 1) a skin lesion consistent with leprosy and with definite sensory loss, with or without thickened nerves, or 2) positive skin smears. **[18]** Clinical leprosy is a polar disease with limited pathogenesis on one pole and severe pathogenesis on the other pole. Classification of the disease spectrum was first described by Ridley and Jopling. [19] Britton and Lockwood have summarized the clinical

immunopathological range of leprosy in a diagram, shown as **Figure 1.** [18] Assignment of disease type was based on the number of lesions, lepromin response, and number of bacteria in a

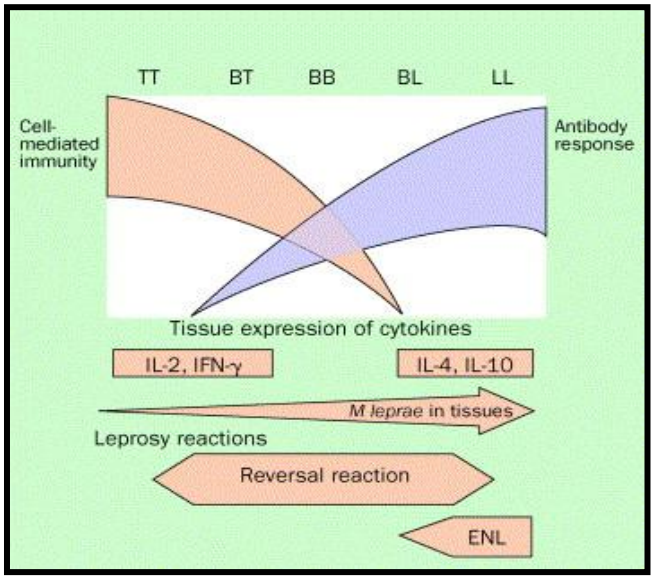



**Figure 1: Clinical Immunopathological Range of Leprosy**
The full spectrum of leprosy disease and clinical manifestation of disease; ENL: erythema nodosum leprosum leprosy reaction.

slit skin smear. Tuberculoid leprosy (TT) and lepromatous leprosy (LL) where the extreme types at each pole and borderline tuberculoid (BT), borderline leprosy (BB), and borderline lepromatous leprosy (BL) were between the polar ends. Polar TT patients have a high degree of cell-mediated immunity (CMI), usually with a single lesion that is well demarcated with a central hypopigmentation and hypoesthesia. Biopsies contain developed granulomatous inflammation and rarely contain acid fast bacteria. On the opposite end of the spectrum are Polar LL patients who do not have resistance to *M. leprae* and as a result develop multiple poorly demarcated, raised or nodular lesions on all parts of the body. Biopsies contain sheets of foamy macrophages in the dermis and a large number of acid fast bacilli. LL patients are anergic to *M. leprae* antigens, but do retain the immunological capacity to mount a humoral response to *M. leprae* antigens. This is the highly infectious form of the disease. [7]

## TREATMENT

Disease classification was simplified by the WHO for implementation of multi-drug therapy (MDT: clofazimine, rifampicin, and dapsone). Paucibacillary (PB) leprosy patients presented with 1-5 skin lesions and multibacillary (MB) presented with > 5 skin lesions. The PB subtype is reflective of a Th1 (cell-mediated) immune response and MB subtype is reflective of a Th2 (humoral) immune response. [7] Adult leprosy patients with MB leprosy receive a 12 month treatment regimen of 600 mg rifampicin once per month, 100 mg dapsone daily, 300 mg clofazimine once per month and 50 mg daily. Leprosy patients with PB leprosy receive a 6 month regimen of 600 mg rifampicin once per month and 100 mg dapsone daily. MDT is provided in pre-dose blister packs, free of charge, by the WHO.[20]

## LEPROSY REACTIONS

Leprosy patients can also develop painful immune-mediated reactions that may lead to added neurological disabilities. There are two primary types of leprosy reactions, Type 1 (Reversal Reaction) and Type 2 (Erythema Nodosum Leprosum). Type I reactions can occur in any type of leprosy, with a sudden increase in cell mediated immunity against *M. leprae* antigens and an increase in Th1 cytokines including IL-1 beta, TNF alpha, IL-2 and INF-γ, and a decrease in Th2 cytokines IL4, IL5, and IL10. Inflammatory reactions result in pressure on the nerve axon, resulting in nerve cell death. Type 2 reactions only occur in BL/LL leprosy and are thought to be related to an Arthus reaction involving the complement system. Research in this area is limited, although it is known that physical stress such as viral infections and pregnancy[21] or psychological stress can evoke a reactional episode. [22,23]

## GENETIC SUSCEPTIBILITY

Host genetics appear to play a two stage role. The PARK2, LTA, 13q22.1, or 20p12.3 genes confer susceptibility or resistance. For the approximate 5-10% of infected individuals who develop leprosy, HLA-DRB1*15 or 10p13 genes determine the type of host immune response elicited and subsequent clinical manifestation.[24] Polymorphism have been found in some of these same genes and others, such as RIPK2, TNFSF15, LRRK2, C13orf31/CCDC122, NOD2;[25] TLR1, C13orf31 and, CDDC1222.[26] Genetic makeup is one risk factor for the development of leprosy.

## TRANSMISSION

*M. leprae* is not highly infectious, but is pathogenic depending on the form of disease. Evidence suggests that bacteria are carried through nasal droplets and infect the upper respiratory tract. Infection requires more than just casual contact with infected patients. Living in a household with a patient that has high bacillary loads over long periods presents 8-fold higher risk of contracting the disease.[27,28]

The incubation period from infection to clinical manifestation of disease varies from several months to 30 years, depending on the type of leprosy. [29] On average, TT patients develop clinical symptoms within 4 years and LL patients develop clinical symptoms within 10 years. With the long incubation period from infection to manifestation of disease, a low rate of transmission can continue for many years. Risk factors include genetic susceptibility, age under 14 years or older adults, male gender, antibody response to the M. leprae specific antigen, phenolic glycolipid I (PGL-I), and lepromin negative response. [30] As a result, contacts of patients, especially MB patients, are at an 8-fold higher risk of contracting leprosy compared to non-contacts. Contacts of PB patients are only slightly more at risk than non-contacts.

Intercepting human to human transmission of infectious bacilli is critical for preventing leprosy disease, nerve damage, stigmatization by local communities, and disabilities. [29]

## VACCINES

With issues complicating the development of early diagnostic tests for leprosy, a protective vaccine with higher efficacy than that seen with BCG would be useful. Alternatively, identifying protective correlates of immunity to identify those individuals who are/are not infected, but self-cured, would be beneficial. The vaccine trial in Karong District, Northern Malawi showed that a single dose of BCG provided greater than 50% protection against leprosy, but not tuberculosis. These results were consistent with the study in Venezuela.[31,32] The role of the BCG vaccination was verified with an analysis of 19 observational and 7 experimental papers. Age is not a predictor of protective effect, but an additional dose was more protective in prevention compared to one dose.[33] The hope for the field of leprosy is that the intensive efforts to find a better vaccine for tuberculosis will also have cross-protective characteristics and protect individuals, especially children who have an increased incidence, from leprosy. A concern is that as the tuberculosis vaccines become more specific, cross-reactive epitopes to protect against leprosy may be lost.[34]

## EPIDEMIOLOGY

Detection of asymptomatic leprosy has been identified by the World Health Organization (WHO) as an important need.[35] Emphasizing this point, the need for a diagnostic test for early leprosy infection has been reported by the scientific community for more than 90 years, since Mitsuda tested the value of a skin reaction using a suspension of leprosy nodules termed Lepromin-H.[36] With the introduction of multiple drug therapy (MDT) by the WHO in 1982 to prevent Mycobacterium leprae resistance to dapsone monotherapy, leprosy prevalence began a

dramatic decline. [37] Over the past 30 years, the prevalence of leprosy has dropped 98.3% from an estimated 11.5 million cases, of which only 5.3 million cases were registered in 1983, to the current figure in 2010 of 192,246.[35,38] Contrary to this remarkable achievement, leprosy incidence or new case detection remained relatively constant or increased slightly from 1985 at 555,188 new cases in the top 33 endemic countries [39] to 571,792 new cases in 1990 [40] and 620,672 new cases in 2002.[41] A significant decline of 51.4% in leprosy incidence was observed between 2002 to 2005 at 299,036 new cases, followed by another decline of 23.6% to the current 2010 figure of 228,474. [35] A total decline of 58.8% in detection of new cases from 1985 to 2010 has been observed. Although many investigators have questioned the value of these numbers based on confounding operational factors, one basic fact remains. Incidence has exceeded prevalence, and therefore transmission of M. leprae from infected individuals to susceptible individuals remains a serious concern. [42]

## DIAGNOSTIC METHODS

Multiple test methods are being investigated in the field for the diagnosis of asymptomatic leprosy including: serology, IFN-γ assays, polymerase chain reaction (PCR) with *M. leprae* specific gene primers, and intradermal DTH skin test. [43-47] All of these methods have shown some level of diagnostic potential, however, none to date perform at a sensitive and specific level across the complicated spectrum of leprosy disease.

### Serology

The most notable contribution in serological detection of *M. leprae* was the discovery of phenolic glycolipid I (PGL-I) as a specific cell surface marker of *M. leprae*. [48] Translation of this IgM antibody detection method into a lateral flow immunodiffusion test was accomplished for ease of testing in the field. Numerous studies have been performed with PGL-I enzyme

linked immunoadsorbant assays in the native form and synthetic ND-O-BSA/HSA forms. [30,49] The test does not detect early infection, but has been shown to classify BL/LL from BT/TT leprosy disease.

Serology has been attempted on a vast number of native proteins, recombinant proteins, and synthetic peptides such as the serine rich 45 kDa protein, ESAT-6, CFP-10, 35 kDa major membrane protein, antigen 85 proteins, hypothetical proteins, etc. [43,50-52] derived by conventional identification, and *in silico* analysis of the *M. leprae* genome, and/or use of specialized software programs to detect T- and B- cell epitopes, secretory sequences, lipoprotein sequences, sequences specific to *M. leprae*, etc. More recently combinations of peptides and expression of PGL-I/peptide conjugates have proven useful [example: LID-1 (ML0405 and ML23310). [43]

**T-cell Assays**

Early studies to identify a diagnostic test for asymptomatic leprosy included T-cell proliferation assays [53,54], while later studies used T cell cytokine detection assays, specifically interferon gamma.[42] Cell mediated immunity against *M. leprae* was measured by the level of IFN-γ present following *in vitro* T-cell stimulation. [55,56] Gene's specific to *M. leprae* have been identified and respective proteins expressed or peptides synthesized for testing in whole blood or peripheral blood monocyte assays.[57,58] Present day focus on interferon gamma release assays (IGRA) providing some level of early diagnostic capabilities is actively progressing.[58-62] Equally active are IGRA studies for the detection of early or latent *M. tuberculosis.*[63-65]

**PCR**

PCR assays have shown promise in detecting *M. leprae* DNA from nasal swabs. This method holds promise for detecting both forms of disease at an early stage, because it is sensitive and specific to the organism. The concern is that it might be too sensitive, as it detects *M. leprae* in nasal carriages of healthy controls. It is also not able to distinguish between viable and non-viable bacilli, and a test for mRNA is not yet developed. Moreover, this test requires an even higher skill set, expensive equipment, and costly reagents. The likelihood that a diagnostic test would ever be commercialized for leprosy is very low, because the people and governments living in leprosy endemic regions would not be able to afford an expensive test. [66,67]

**Intradermal DTH Skin Test**

The intradermal DTH test measures cell-mediated immunity (CMI). [68] Based on experience with the Tuberculin PPD skin test for tuberculosis, subjects must be exposed to the infectious agent for at least 4-6 weeks prior to testing for antigen recall. [69] A DTH type IV reaction is initiated when antigen is injected into subcutaneous tissue and processed by antigen presenting cells. A $TH_1$ effector cell recognizes the antigen and releases cytokines IL-2, IFN-$\gamma$, and TNF, which act on vascular endothelium causing erythema and recruitment of T-cells, phagocytes, fluid, and protein which causes a measurable induration response within 48-72 hours in humans. A lack of DTH response to recall antigen is evidence of anergy.

Sensitivity of the DTH test method has been proven with Purified Protein Derivative (PPD) prepared from secreted antigens of the culture filtrate of *M. tuberculosis* and employed as a DTH diagnostic test for Tuberculosis. [70] The Tuberculin Mantoux response is highly sensitive in detecting exposure or infection with *M. tuberculosis*, however, the test is not very specific in endemic regions for tuberculosis or in populations that have been vaccinated against Bacillus

Calmette Guerin (BCG) and exposed to related mycobacteria. On the contrary, it is specific for tuberculosis in non-endemic regions where subjects have not been exposed or vaccinated. [71] Tuberculin PPD has been used across the world with an exceptional safety record.[72,73]

**HISTORY OF LEPROSY SKIN TEST ANTIGENS**

The earliest published information on leprosy skin test antigens was by Mitsuda in 1919 [36] where an autoclaved suspension of macerated nodules from untreated leprosy patients termed Lepromin-H was administered to volunteers. Lepromatous leprosy patients did not respond, but tuberculoid leprosy patients and a certain percentage of indeterminate and borderline patients produced a nodule varying in size from 3 to 4 mm on day 21 - 30. This nodule was termed the Mitsuda reaction. This reaction is unique and considered to be a measure of response capability to *M. leprae* antigens; however, because about 90% of normal subjects in endemic areas produce a positive response the test is not considered useful for diagnosing leprosy. In 1940, Fernandez[74] described another response produced by this antigen, which appeared 48 hours after injection in those individuals with tuberculoid leprosy and in a certain number of normal people who had been in contact with leprosy patients. The presence of a Fernandez reaction was considered indicative of CMI against *M. leprae.* A derivative of Lepromin-H, consisting of a chloroform ether extracted suspension of *M. leprae*, named Dharmendra Lepromin, produces only the 48 hour reaction.[75] The protocol for this leprosy skin test antigen is on file with the FDA under Investigation New Drug (IND) number: BB-IND-2399.

As leprosy declined and MDT was widely implemented, the number of patients with fulminating leprosy declined and the availability of human lepra nodules was reduced dramatically. As a result, it became important to find another source of antigen. In 1975, investigators demonstrated that armadillo-derived Lepromin-A elicited a DTH and Mitsuda

reaction equal to or better than Lepromin-H in human clinical studies. [76-78] These results led to the submission of an IND for Lepromin-A (IND number: BB-IND 2401) in 1981 by Dr. W. A. Krotoski and Dr. R. C. Hastings at the Gillis W. Long Hansen's Disease Center (GWLHDC), Louisiana State University, Baton Rouge, LA.[79,80] Lepromin-A, like previous Lepromins, has prognostic value for classifying disease. By January 1993, under a contract with the WHO, approximately one million doses of Lepromin-A had been distributed to physicians and institutions in leprosy endemic areas. Its general safety and effectiveness are well established, however, two unfavorable results are apparent. First is the appearance of a nodule and/or necrosis, 3-4 weeks following injection, a.k.a. Mitsuda reaction, and second, the material itself can act as a weak vaccine and immunologically disturb any population receiving this reagent.[81]

By 1984, it became apparent that a skin test reagent capable of specifically diagnosing leprosy without undesirable affects would have to come from fractionated *M. leprae*. To mimic the classical Tuberculin PPD in which activity and specificity are related to low molecular weight secretory proteins in the culture filtrate,[82] efforts were directed to the soluble fraction of the leprosy bacilli, since *M. leprae* is not cultivable. Two laboratories formulated different subcellular skin test antigens at the same time. Convit's antigen was designated Soluble Protein Antigen (SPA) or Soluble Antigen (SA), while the Rees Antigen was designated *M. leprae* soluble antigen (MLSA) or Leprosin.[83] Convit's SPA was prepared from bacilli purified from *M. leprae* infected armadillo tissues using the 1/79 Draper protocol.[84] Purified bacilli were disrupted by French Press and centrifuged at 48,000 x g for 1 hour. The supernatant containing cytosolic and membrane components were filtered through a 0.45μm filter, diluted in borate buffer, and bottled in vials. Vials were then autoclaved, cooled, and stored at -20 °C. The Rees

antigen was prepared from bacilli purified from gamma-irradiated *M. leprae* infected armadillo tissues using the 3/77 Draper protocol.[85] Purified bacilli were disrupted by sonication and centrifuged at 27,000 x g to remove the cell wall and a 105,000 x g to remove the membrane. The supernatant containing cytosolic components was filtered through a 0.8µm and 0.2µm filter, diluted in borate/Tween buffer, and bottled in vials. Vials were stored at 4°C.

Extensive testing of human subjects with both the Rees and Convit antigens was undertaken in Malaysia, Malawi, Venezuela, and India with no adverse effects. [31,83,86-88] Reactions observed in these studies ranged from soft, meaning that the reaction merged almost imperceptibly with the surrounding skin to hard, meaning that the reaction was quite distinct in the margins. Reactions to Tuberculin are hard in consistency. The reason for the soft reaction is unknown, but obviously makes the test extremely difficult to read consistently.[86] Results from these studies indicated that the Rees and Convit antigens are not useful in the identification of *M. leprae* infection or in the confirmation of leprosy diagnosis in a leprosy endemic population with a high prevalence of non-specific sensitivity.[87] Promising features of both antigens showed that neither exhibited sensitizing potential, both were very potent immunologically, and could classify leprosy in human vaccine trials in Venezuela, Malawi, and India.[31,83]

Both antigens were found to be safe, and in a limited sense, useful. Samuel, et al. documented that the Rees antigen reactions were positive in highly resistant forms of leprosy and negative in low-resistant lepromatous forms of leprosy in India, Uganda, Kenya, Nepal, and Bhutan. Wide variations in response to both of these antigens was well documented by Gupte, et. al.[87] Reasons for these differences could be the use of different batches of antigens with suspected variability of protein content of the earlier preparations, prevalence in different areas, non-specific desensitization, and geographical differences. In general, the results of these studies

indicate that the Convit and Rees antigen skin test response are variable due to product or population variation, may not be sensitive enough to detect leprosy, and do not appear to be specific enough to confirm clinical diagnosis of leprosy.[47] Both antigens meet the ideal for potency while falling short in terms of sensitivity and specificity.

**NEW LEPROSY SKIN TEST ANTIGENS**

Criteria for a useful early diagnostic test for leprosy had been established. To have any chance of successful implementation, the test must be able to detect an early and specific immunological response, be simple to implement in the field, and be inexpensive. Information gained from preparation and testing of the Rees and Convit skin test antigens provided a frame-work in the early 1990's for the development of two new leprosy skin test antigens.[47,89] Elucidation of the major proteins of the *M. leprae* bacillus was underway. Subcellular fractions were being prepared by ultrasonication and centrifugation and lipoglycans were extracted with detergent to visualize and enable N-terminal sequencing of major proteins resolved by reduced acrylamide gel electrophoresis. [90-92] Subcellular fractions and purified proteins were tested in the delayed type hypersensitivity (DTH) guinea pig animal model to assess their ability to stimulate a cell mediated immune response in *M. leprae* sensitized compared to *M. tuberculosis* infected or naïve animals. [93]

The first new antigen candidate was modified from the Rees MLSA by removing immunosuppressive and cross-reactive components (LAM, LM, PIMS, and other lipids) to produce MLSA-LAM. [94,95] The second new antigen consisted of cell wall associated antigens, which were found to be powerful immunogens in extensive immunological studies and skin testing in guinea pigs.[96,97] Both MLSA-LAM and MLCwA were found to strongly induce proliferation of lymphocytes and stimulate secretion of IFN-γ from immune cells.[98,99]

An equivalent *M. leprae* membrane antigen (MLMA-LAM) was also developed, but was not moved forward due to low yields.

In 1992, a decision was made to take the two new leprosy antigens forward as skin test antigens in human clinical trials. Multiple preclinical batches were prepared to optimize and refine the production process, test stability, and to provide ample material for developing assays for assessing quality. Preparation of early batches also showed that the process was reproducible and potency between batches was consistent and that the antigens were active.

A rational approach to moving these two antigens from the bench to the clinic involved intense and frequent discussions with our National Institutes of Health (NIH) sponsor who funded the project under Dr. Brennan's Leprosy Support Contract. Of importance was the need to produce these new skin test antigens under the rigor of cGMP for consistency and reproducibility between batches, based on the variability of protein content seen in earlier Rees and Convit preparations. The regulatory scenario had advanced dramatically contributing to a higher quality product for first in human phase I clinical trial. Considerable effort to identify a contract manufacturing organization (CMO) was initiated, but exorbitant cost and the fear of working with *M. leprae* prohibited use. As a result, the laboratory embarked on the monumental task of creating a current good manufacturing practices (cGMP) skin test pilot plant in the microbiology department building at Colorado State University to prepare material for phase I and phase II human clinical trials.

Applied research efforts progressed with the anticipation of developing an early diagnostic test for leprosy. Between 1992 and 2005, the following achievements occurred: 1) candidate antigens were identified; 2) concerted decision to move forward with product translation of MLSA-LAM and MLCwA; 3) Renovation of a cGMP Pilot Plant Facility dedicated to the

production of new leprosy skin test antigens; 4) Creation of a Quality System, batch records, and supporting standard operating procedures (SOPs); 5) pre-IND meetings; 6) manufacturing; 7) submission of the IND; 8) completion of a phase I clinical trial at Colorado State University, Fort Collins, Colorado, USA, a nonendemic region for leprosy; and, 8) completion of a phase II, stage A/B clinical trial on healthy subjects without known exposure to leprosy at Anandaban Hospital, Kathmandu, Nepal, an endemic region for leprosy. A visit to the phase II clinical site took place on November 17-19, 2004 by Dr. Brennan, Research Principal Investigator, Ms. Rivoire, Research Study Coordinator, and Dr. Robert Gelber, Safety Monitoring Committee Chair, to meet regulatory and clinical staff, discuss proposed protocol changes, and to tour the hospital. With permission, photographs of staff and patients were taken to document participants in the clinical trial (**Appendix 1**).

## RESEARCH OBJECTIVES

### Personal Statement

Product translation from discovery to first-in-human testing is a challenging and difficult road for academicians working on a product idea without commercial interest. Such was the situation with the two new leprosy skin test antigens, as leprosy was a disease of mostly poor, developing countries, also experiencing a rapid decline in prevalence (registered cases) following the implementation of MDT. Incidence (new case detection) however; remained constant suggesting that transmission was still occurring. The desperate need for an early diagnostic test for leprosy to allow chemotherapeutic intervention prior to debilitating clinical manifestation of disease and to prevent transmission remained a concern then, as it does today.

The Leprosy Skin Test Initiative was launched following a passionate plea to advance two scientifically sound diagnostic skin test antigen candidates from the bench to the clinic. Without

a pharmaceutical partner and failed attempts at finding a CMO, product translation had to come from within the university or be abandoned. The potential prospect of these two skin test antigens being efficacious as early diagnostic tools for leprosy was a strong motivator.

I embarked on this very unique project with a focus on translational research, rather than pure basic research to apply the knowledge we had at the time. This special dissertation was agreed upon and supported by my graduate committee members and represents the first product translational project within the department. The work presented here validates the appropriateness of product translation in an academic environment for medical interventions that have potential use, but lack commercial sponsorship. Prospective products should not be left on the shelf, when people are suffering or are at risk of contracting or already suffering from a debilitating disease.

**Hypothesis and Aims**

This graduate program formally began in the Spring of 2005, following the Phase II, Stage B clinical trial study and continued through February, 2012, the end of the phase II, stage C-1 clinical trial. The hypothesis of this research was that the new leprosy skin test antigens, MLSA-LAM and MLCwA, at doses of 1.0μg and/or 0.1μg, would be safe and efficacious as diagnostic-tools to detect leprosy and allow treatment of patients earlier and to measure the extent of leprosy infection in human subjects living in a leprosy endemic area. There were three specific aims: 1) assess both antigens and antigen doses for safety and efficacy in leprosy patients, household contacts of leprosy patients, and tuberculosis patients in an endemic region for leprosy; 2) compare the skin test method against two *in vitro* test methods, IFN-γ assay and PGL-I antibody assay; and, 3) successfully complete product translation within an academic environment under government support.

My involvement with this project started in 1992, upon the launch of the Leprosy Skin Test Initiative, providing laboratory research and management skills to move this project forward. Under this graduate program, my role was the primary study coordinator, responsible for creating, updating, and reviewing regulatory documents, reporting to institutional review boards (IRB), *in vitro* laboratory assay procedures, communications, data analysis, and publications. This dissertation is the culmination of translational research of MLSA-LAM and MLCwA as potential asymptomatic leprosy diagnostic tests.

**Research Responsibilities**

A timeline of the Leprosy Skin Test Initiative can be seen in **Figure 2**. A significant amount of the work was performed by me and coworkers from 1992 through the inception of this program in 2005. I left the project from 1996 through 2000 for personal reasons, and Dr. Stephen Terlow performed the manufacturing of new skin test antigens, Dr. Patrick Brennan submitted the IND, and Dr. Terlow served as research coordinator of the phase I study. Upon Dr. Terlow's exit in 2001, I resumed the coordinator position on the Leprosy Skin Test Initiative project. Formulation of antigens was performed from stock vials to increase our clinical supplies, prior to the phase II, stage B studies and verification of stability at 2 and 6 years post-manufacturing was performed in guinea pig potency assays.

In 2005, the phase II protocol was rewritten using the NIH Clinical Trial Protocol Template. [100] Development and preparation of reagents for the IFN-γ assay and PGL-I Antibody assay were performed, and related standard operating procedures were created for approval by the team, prior to implementation in the Stage C study. Document control, ethical review board notification, and team correspondence was managed, protocols were amended, and safety reports

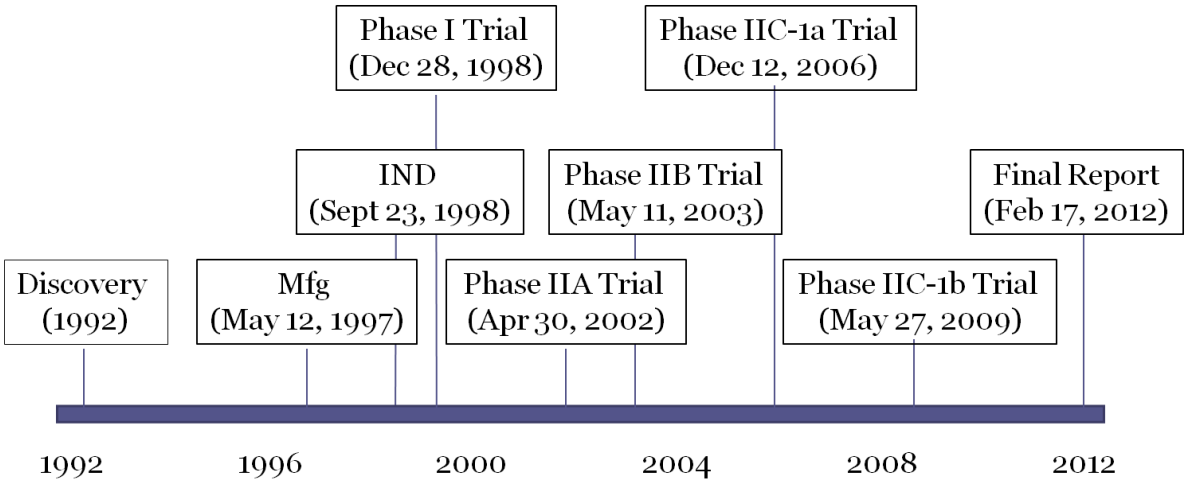



**Figure 2: Leprosy Skin Test Initiative Timeline**
Product translation of MLSA-LAM and MLCwA from discovery to pilot scale clinical trials spanned 20 years.

and the final comprehensive report were reviewed. Statistical data analysis and drafting of manuscripts was the final momentous task of the first part of this research study. As described in the background of new leprosy skin test antigens, discovery, manufacturing, IND, phase I, and phase II, stage A/B clinical trials were completed prior to the initiation of this research program. Studies covered under this program include the phase II, stage C-1a/b clinical trials, final report review, data analysis, and publication of the Leprosy Skin Test Initiative.

**Dissertation Organization**

Product translation of the two products under investigation could not be reported until the closure of the phase II protocol, to prevent compromising on-going studies. Consequently, this dissertation includes full reporting of the translational process from discovery through phase I and phase II clinical trials. Chapter 1 provides a literature review and background work supporting this dissertation. Details of key personnel responsible for background work have been provided under research responsibilities. Chapter 2 provides a concise report of developing products suitable for human testing with minimal resources. This work occurred between 1992

and 1998, primarily by me, Dr. Steve Terlow and Dr. Patrick Brennan. Chapter 3 covers the phase I and phase II, stage A and B clinical trials to assess safety of these skin test antigens in healthy contacts living in a nonendemic region or endemic region for leprosy. This work occurred between 1999 and 2004, primarily by Dr. Terlow, Dr. Brennan, CSU Hartshorn Health Center staff, and me. Chapter 4 describes the phase II, stage C-1a and C-1b clinical trials to assess both safety and efficacy in target populations, including leprosy patients, household contacts of leprosy patients, and tuberculosis patients. The work occurred between 2005 and 2012, primarily by me, Dr. Brennan, and the Anandaban Hospital clinical team. Chapter 5 summarizes the basic and applied research presented in this dissertation, lessons learned, and future directions.

REFERENCES


1. Katoch VM (2002) Advances in the diagnosis and treatment of leprosy. Expert Rev Mol Med 4: 1-14.

2. Storrs EE (1971) The nine-banded armadillo: a model for leprosy and other biomedical research. Int J Lepr Other Mycobact Dis 39: 703-714.

3. McDermott-Lancaster RD, Ito T, Kohsaka K, Guelpa-Lauras CC, Grosset JH (1987) Multiplication of Mycobacterium leprae in the nude mouse, and some applications of nude mice to experimental leprosy. Int J Lepr Other Mycobact Dis 55: 889-895.

4. Robertson J (2003) Leprosy and the elusive M. leprae: colonial and Imperial medical exchanges in the nineteenth century. Hist Cienc Saude Manguinhos 10: 13-40.

5. Robbins G, Tripathy VM, Misra VN, Mohanty RK, Shinde VS, et al. (2009) Ancient skeletal evidence for leprosy in India (2000 B.C.). PLoS One 4: e5669.

6. Monot M, Honore N, Garnier T, Araoz R, Coppee JY, et al. (2005) On the origin of leprosy. Science 308: 1040-1042.

7. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, et al. (2006) The continuing challenges of leprosy. Clin Microbiol Rev 19: 338-381.

8. Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM (1989) Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J Exp Med 169: 59-72.

9. Myrvang B, Godal T, Ridley DS, Froland SS, Song YK (1973) Immune responsiveness to Mycobacterium leprae and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clin Exp Immunol 14: 541-553.

10. Hussain R, Kifayet A, Chiang TJ (1995) Immunoglobulin G1 (IgG1) and IgG3 antibodies are markers of progressive disease in leprosy. Infect Immun 63: 410-415.

11. Modlin RL (2010) The innate immune response in leprosy. Curr Opin Immunol 22: 48-54.

12. Centers for Disease Control and Prevention Hansen's Disease (Leprosy): Clinical Features. http://wwwcdcgov/nczved/divisions/dfbmd/diseases/hansens_disease/technicalhtml/#clinical.

13. Boku N, Lockwood DN, Balagon MV, Pardillo FE, Maghanoy AA, et al. (2010) Impacts of the diagnosis of leprosy and of visible impairments amongst people affected by leprosy in Cebu, the Philippines. Lepr Rev 81: 111-120.

14. Kumar A, Girdhar A, Girdhar BK (2012) Risk of developing disability in pre and post-multidrug therapy treatment among multibacillary leprosy: Agra MB Cohort study. BMJ Open 2: e000361.

15. Smith WC, Saunderson P (2010) Leprosy. Clin Evid (Online) 2010.

16. van Brakel WH, Nicholls PG, Wilder-Smith EP, Das L, Barkataki P, et al. (2008) Early diagnosis of neuropathy in leprosy--comparing diagnostic tests in a large prospective study (the INFIR cohort study). PLoS Negl Trop Dis 2: e212.

17. Teles RM, Krutzik SR, Ochoa MT, Oliveira RB, Sarno EN, et al. (2010) Interleukin-4 regulates the expression of CD209 and subsequent uptake of Mycobacterium leprae by Schwann cells in human leprosy. Infect Immun 78: 4634-4643.

18. Britton WJ, Lockwood DN (2004) Leprosy. Lancet 363: 1209-1219.

19. Ridley DS, Jopling WH (1966) Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis 34: 255-273.

20. World Health Organization Leprosy Elimination: WHO Recommended MDT Treatment. http://wwwwhoint/lep/mdt/regimens/en/indexhtml.

21. Burki T (2009) Old problems still mar fight against ancient disease. Lancet 373: 287-288.

22. Van Veen NH, Lockwood DN, Van Brakel WH, Ramirez J, Jr., Richardus JH (2009) Interventions for erythema nodosum leprosum. A Cochrane review. Lepr Rev 80: 355-372.

23. Naafs B (1994) Leprosy reactions. New knowledge. Trop Geogr Med 46: 80-84.

24. Alter A, Grant A, Abel L, Alcais A, Schurr E (2011) Leprosy as a genetic disease. Mamm Genome 22: 19-31.

25. Zhang FR, Huang W, Chen SM, Sun LD, Liu H, et al. (2009) Genomewide association study of leprosy. N Engl J Med 361: 2609-2618.

26. Vannberg FO, Chapman SJ, Hill AV (2011) Human genetic susceptibility to intracellular pathogens. Immunol Rev 240: 105-116.

27. Curtiss R, 3rd, Blower S, Cooper K, Russell D, Silverstein S, et al. (2001) Leprosy research in the post-genome era. Lepr Rev 72: 8-22.

28. Rodrigues LC, Lockwood DNJ (2011) Leprosy now: epidemiology, progress, challenges, and research gaps. The Lancet Infectious Diseases 11: 464-470.

29. van Brakel W, Cross H, Declercq E, Deepak S, Lockwood D, et al. (2010) Review of leprosy research evidence (2002-2009) and implications for current policy and practice. Lepr Rev 81: 228-275.

30. Douglas JT, Cellona RV, Fajardo TT, Jr., Abalos RM, Balagon MV, et al. (2004) Prospective study of serological conversion as a risk factor for development of leprosy among household contacts. Clin Diagn Lab Immunol 11: 897-900.

31. Convit J, Sampson C, Zuniga M, Smith PG, Plata J, et al. (1992) Immunoprophylactic trial with combined Mycobacterium leprae/BCG vaccine against leprosy: preliminary results. Lancet 339: 446-450.

32. (1996) Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed Mycobacterium leprae vaccine for prevention of leprosy and tuberculosis in Malawi. Karonga Prevention Trial Group. Lancet 348: 17-24.

33. Setia MS, Steinmaus C, Ho CS, Rutherford GW (2006) The role of BCG in prevention of leprosy: a meta-analysis. Lancet Infect Dis 6: 162-170.

34. Duthie MS, Gillis TP, Reed SG (2011) Advances and hurdles on the way toward a leprosy vaccine. Hum Vaccin 7: 1172-1183.

35. Anonomyous (2011) Leprosy update. Weekly Epidemiological Record 86: 389-400.

36. Mitsuda K. (1919) On the value of a skin reaction to a suspension of leprosy nodules. Jap J Derm Urol 19: 697-708.

37. World Health Organization (1982) Report of the Eleventh and Twelfth Meetings of the Steering Committee of the Scientific Working Group on the Chemotherapy of Leprosy. Geneva. March 30-31 and Oct 10. TDR/THELEP-SC (11-12) 82: 1-6.

38. Sansarricq H (1983) Recent changes in leprosy control. Lepr Rev Spec No: 7-16.

39. Meima A, Richardus JH, Habbema JD (2004) Trends in leprosy case detection worldwide since 1985. Lepr Rev 75: 19-33.

40. Anonomyous (1998) Elimination of leprosy as a public health problem (update) Weekly Epidemiological Record 73: 308-312.

41. World Health Organization (2004) Leprosy Elimination Project: Status Report 2003. Geneva.

42. Dockrell HM, Geluk A, Brennan P, Saunderson PR, Oskam L, et al. (2011) Report on the sixth meeting of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium held in Beijing, China on 23-25 August 2010. Lepr Rev 82: 80-85.

43. Geluk A, Duthie MS, Spencer JS (2011) Postgenomic Mycobacterium leprae antigens for cellular and serological diagnosis of M. leprae exposure, infection and leprosy disease. Lepr Rev 82: 402-421.

44. Dockrell HM (2011) Keep the faith--leprosy still needs new diagnostic tools and laboratory research. Lepr Rev 82: 340-343.

45. Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon MV, et al. (2011) Analysis of antibody responses to Mycobacterium leprae phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. Clin Vaccine Immunol 18: 260-267.

46. Banerjee S, Biswas N, Kanti Das N, Sil A, Ghosh P, et al. (2011) Diagnosing leprosy: revisiting the role of the slit-skin smear with critical analysis of the applicability of polymerase chain reaction in diagnosis. Int J Dermatol 50: 1522-1527.

47. Brennan PJ (2000) Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. Lepr Rev 71 Suppl: S50-54.

48. Cho SN, Cellona RV, Villahermosa LG, Fajardo TT, Jr., Balagon MV, et al. (2001) Detection of phenolic glycolipid I of Mycobacterium leprae in sera from leprosy patients before and after start of multidrug therapy. Clin Diagn Lab Immunol 8: 138-142.

49. Buhrer-Sekula S, Smits HL, Gussenhoven GC, van Leeuwen J, Amador S, et al. (2003) Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. J Clin Microbiol 41: 1991-1995.

50. Parkash O (2011) Serological detection of leprosy employing Mycobacterium leprae derived serine-rich 45 kDa, ESAT-6, CFP-10 and PGL-I: a compilation of data from studies in Indian populations. Lepr Rev 82: 383-388.

51. Roche PW, Failbus SS, Britton WJ, Cole R (1999) Rapid method for diagnosis of leprosy by measurements of antibodies to the M. leprae 35-kDa protein: comparison with PGL-I antibodies detected by ELISA and "dipstick" methods. Int J Lepr Other Mycobact Dis 67: 279-286.

52. Kumar G, Dagur PK, Singh PK, Shankar H, Yadav VS, et al. (2010) Serodiagnostic efficacy of Mycobacterium tuberculosis 30/32-kDa mycolyl transferase complex, ESAT-6, and CFP-10 in patients with active tuberculosis. Arch Immunol Ther Exp (Warsz) 58: 57-65.

53. Mutis T, Kraakman EM, Cornelisse YE, Haanen JB, Spits H, et al. (1993) Analysis of cytokine production by Mycobacterium-reactive T cells. Failure to explain Mycobacterium leprae-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. J Immunol 150: 4641-4651.

54. Shankar P, Agis F, Wallach D, Flageul B, Cottenot F, et al. (1986) M. leprae and PPD-triggered T cell lines in tuberculoid and lepromatous leprosy. J Immunol 136: 4255-4263.

55. Kaplan G, Weinstein DE, Steinman RM, Levis WR, Elvers U, et al. (1985) An analysis of in vitro T cell responsiveness in lepromatous leprosy. J Exp Med 162: 917-929.

56. Lindh J, Anderson U, Britton S, De Ley M (1987) A single cell assay for the study of gamma-interferon formation in leprosy patients. Clin Exp Immunol 67: 51-54.

57. Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, et al. (2005) Identification of specific proteins and peptides in Mycobacterium leprae suitable for the selective diagnosis of leprosy. J Immunol 175: 7930-7938.

58. Sampaio LH, Stefani MM, Oliveira RM, Sousa AL, Ireton GC, et al. (2011) Immunologically reactive M. leprae antigens with relevance to diagnosis and vaccine development. BMC Infect Dis 11: 26.

59. Geluk A, van der Ploeg-van Schip JJ, van Meijgaarden KE, Commandeur S, Drijfhout JW, et al. (2010) Enhancing sensitivity of detection of immune responses to Mycobacterium leprae peptides in whole-blood assays. Clin Vaccine Immunol 17: 993-1004.

60. Martins MV, Guimaraes MM, Spencer JS, Hacker MA, Costa LS, et al. (2012) Pathogen-specific epitopes as epidemiological tools for defining the magnitude of Mycobacterium leprae transmission in areas endemic for leprosy. PLoS Negl Trop Dis 6: e1616.

61. Lobato J, Costa MP, Reis Ede M, Goncalves MA, Spencer JS, et al. (2011) Comparison of three immunological tests for leprosy diagnosis and detection of subclinical infection. Lepr Rev 82: 389-401.

62. Venturini J, Soares CT, Belone Ade F, Barreto JA, Ura S, et al. (2011) In vitro and skin lesion cytokine profile in Brazilian patients with borderline tuberculoid and borderline lepromatous leprosy. Lepr Rev 82: 25-35.

63. Bergstedt W, Tingskov PN, Thierry-Carstensen B, Hoff ST, Aggerbeck H, et al. (2010) First-in-man open clinical trial of a combined rdESAT-6 and rCFP-10 tuberculosis specific skin test reagent. PLoS One 5: e11277.

64. Chiappini E, Accetta G, Bonsignori F, Boddi V, Galli L, et al. (2012) Interferon-gamma release assays for the diagnosis of Mycobacterium tuberculosis infection in children: a systematic review and meta-analysis. Int J Immunopathol Pharmacol 25: 557-564.

65. Simpson T, Fox J, Crouse K, Field K (2012) Quantitative and qualitative QuantiFERON((R))-TB Gold In-Tube results among groups with varying risks of exposure to tuberculosis. Heart Lung.

66. Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, et al. (2010) Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts--a pilot study from India. BMC Infect Dis 10: 252.

67. Ottenhoff TH (1994) Immunology of Leprosy: lessons from and for leprosy. Int J Lepr Other Mycobact Dis 62: 108-121.

68. Black CA (1999) Delayed type hypersensitivity: current theories with an historic perspective. Dermatol Online J 5: 7.

69. Ernst JD (2012) The immunological life cycle of tuberculosis. Nat Rev Immunol 12: 581-591.

70. Rieder HL, Chadha VK, Nagelkerke NJ, van Leth F, van der Werf MJ (2011) Guidelines for conducting tuberculin skin test surveys in high-prevalence countries. Int J Tuberc Lung Dis 15 Suppl 1: S1-25.

71. Arnadottir T, Rieder HL, Trebucq A, Waaler HT (1996) Guidelines for conducting tuberculin skin test surveys in high prevalence countries. Tuber Lung Dis 77 Suppl 1: 1-19.

72. Snider DE, Jr. (1982) The tuberculin skin test. Am Rev Respir Dis 125: 108-118.

73. Huebner RE, Schein MF, Bass JB, Jr. (1993) The tuberculin skin test. Clin Infect Dis 17: 968-975.

74. Fernandez JMM. (1940) The early reaction induced by lepromin. Int J Lepr 8: 1-14.

75. Dharmendra D (1942) The immunological skin tests in leprosy: the isolation of a protein antigen of *Mycobacterium leprae*. Ind J Med Res 30: 1-7.

76. Convit J, Pinardi ME, Avila JL, Aranzazu N (1975) Specificity of the 48-hour reaction to Mitsuda antigen. Use of a soluble antigen from human and armadillo lepromin. Bull World Health Organ 52: 187-191.

77. Meyers WM, Kvernes S, Binford CH (1975) Comparison of reactions to human and armadillo lepromins in leprosy. Int J Lepr Other Mycobact Dis 43: 218-225.

78. Millar JW, Gannon C, Chan CS (1975) Comparison in leprosy patients of Fernandex and Mitsuda reactions using human and armadillo antigens. A double-blind study. Int J Lepr Other Mycobact Dis 43: 226-233.

79. Krotoski WA, Mroczkowski TF, Rea TH, Almodovar PI, Clements BC, et al. (1993) Lepromin skin testing in the classification of Hansen's disease in the United States. Am J Med Sci 305: 18-24.

80. Krotoski WA, Mroczkowski TF, Shannon EJ, Millikan LE, Sanchez RM, et al. (1993) Lepromin responses in recipients of a candidate antileprosy bacterin vaccine (WHO-IMMLEP Mycobacterium leprae killed preparation) in the USA. Int J Dermatol 32: 191-193.

81. WHO IMMLEP. (1982) Testing of purified armadillo-derived M. leprae in man. Document finalized by the IMMLEP Steering Committee at its meeting, 10-12 June 1981. WHO Document TDR/IMMLEP/SC/TEST 81: 1.

82. Seibert FB. (1934) The isolation and properties of the purified protein derivative of tuberculin. Am Rev Tuberc 30: 713-720.

83. WHO IMMLEP. (1982) Vaccination trials against leprosy: a meeting of the epidemiology subgroup of Scientific Working Group on the Immunology of Leprosy, Geneva, 11-13 February, 1985. WHO Document TDR/IMMLEP/EDP 85: 7-8.

84. WHO IMMLEP. (1979) Draper, P. Protocol 1/79: Purification of M. leprae. Annex 1 of the Enlarged Steering Committee for Research on the Immunology of Leprosy (IMMLEP) Meeting of 7-8 February, 1979. Geneva. World Health Organization: 4.

85. WHO IMMLEP. (1977) Report of the third IMMLEP scientific working group on leprosy protocol 3/77. WHO Document TDR/SWG/IMMLEP: 20.

86. Gupte MD, Anantharaman DS (1988) Use of soluble antigens in leprosy epidemiology. Lepr Rev 59: 329-335.

87. Gupte MD, Anantharaman DS, Nagaraju B, Kannan S, Vallishayee RS (1990) Experiences with Mycobacterium leprae soluble antigens in a leprosy endemic population. Lepr Rev 61: 132-144.

88. Samuel NM, Stanford JL, Rees RJ, Fairbairn T, Adiga RB (1984) Human vaccination studies in normal and contacts of leprosy patients. Indian J Lepr 56: 36-47.

89. Brennan PJ, Cho SN, Klatser PR (1996) Bangkok Workshop on Leprosy Research. Immunodiagnostics, including skin tests. Int J Lepr Other Mycobact Dis 64: S58-62.

90. Hunter SW, Rivoire B, Mehra V, Bloom BR, Brennan PJ (1990) The major native proteins of the leprosy bacillus. J Biol Chem 265: 14065-14068.

91. Young DB, Kaufmann SH, Hermans PW, Thole JE (1992) Mycobacterial protein antigens: a compilation. Mol Microbiol 6: 133-145.

92. Rivoire B, Pessolani MC, Bozic CM, Hunter SW, Hefta SA, et al. (1994) Chemical definition, cloning, and expression of the major protein of the leprosy bacillus. Infect Immun 62: 2417-2425.

93. Collins FM, Morrison NE, Watson SR (1983) Fernandez and Mitsuda reactivity in guinea pigs sensitized with heat-killed Mycobacterium leprae: persistence and specificity of skin reactivity to soluble and particulate antigens. Int J Lepr Other Mycobact Dis 51: 481-489.

94. Chatterjee D, Khoo KH (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 8: 113-120.

95. Barnes PF, Chatterjee D, Abrams JS, Lu S, Wang E, et al. (1992) Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. J Immunol 149: 541-547.

96. Hunter SW, McNeil M, Modlin RL, Mehra V, Bloom BR, et al. (1989) Isolation and characterization of the highly immunogenic cell wall-associated protein of Mycobacterium leprae. J Immunol 142: 2864-2872.

97. Melancon-Kaplan J, Hunter SW, McNeil M, Stewart C, Modlin RL, et al. (1988) Immunological significance of Mycobacterium leprae cell walls. Proc Natl Acad Sci U S A 85: 1917-1921.

98. Weir RE, Brennan PJ, Butlin CR, Dockrell HM (1999) Use of a whole blood assay to evaluate in vitro T cell responses to new leprosy skin test antigens in leprosy patients and healthy subjects. Clin Exp Immunol 116: 263-269.
99. Manandhar R, LeMaster JW, Butlin CR, Brennan PJ, Roche PW (2000) Interferon-gamma responses to candidate leprosy skin-test reagents detect exposure to leprosy in an endemic population. Int J Lepr Other Mycobact Dis 68: 40-48.
100. NIH NIAID (2008) Clinical Trial Protocol Template. http://wwwniaidnihgov/labsandresources/resources/toolkit/protocol/Pages/protocolaspx.

# CHAPTER TWO: THE CHALLENGE OF PRODUCING SKIN TEST ANTIGENS WITH MINIMAL RESOURCES SUITABLE FOR HUMAN APPLICATION AGAINST A NEGLECTED TROPICAL DISEASE; LEPROSY

## SYNOPSIS

True incidence of leprosy and its impact on transmission will not be understood until a tool is available to measure subclinical infection. Diagnosis is currently based on clinical symptoms, which on the average take 3-10 years to manifest. The fact that incidence as judged by new case detection equates with prevalence i.e., registered cases, suggests that the cycle of transmission has not been fully intercepted by implementation of multiple drug therapy. This is supported by high incidence of childhood leprosy. Epidemiological evaluation of asymptomatic leprosy in large endemic populations is required to facilitate targeted chemoprophylactic interventions. Such a test must be sensitive, specific, simple to administer, cost-effective, and easy to interpret. The intradermal skin test method that measures cell mediated immunity was deemed the best option. Prior knowledge on skin testing of healthy subjects and leprosy patients with whole or partially fractionated Mycobacterium leprae bacilli, such as Lepromin or the Rees' or Convit' antigens has established an acceptable safety and potency profile. These data, along with immunoreactivity data, laid the foundation for two new leprosy skin test antigens, MLSA-LAM (M. leprae soluble antigen devoid of mycobacterial lipoglycans, primarily lipoarabinomannan) and MLCwA (M. leprae cell wall antigens). In the absence of commercial interest, the challenge was to develop these antigens under current good manufacturing practices in an acceptable local pilot facility and submit an Investigational New Drug Application to the Food and Drug Administration to allow a first-in-human phase I clinical trial.

**Funding**

Leprosy Research Support, Contract NO1 AI-25469; National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH)

**AUTHOR SUMMARY**

Despite reaching the global elimination target for leprosy, the need for a diagnostic tool to detect asymptomatic disease remains. Transmission has not been completely intercepted despite over 30 years of extensive curative treatment. With limited resources, two new leprosy skin test antigens MLSA-LAM and MLCwA, suitable for human application were developed and manufactured in a local pilot plant. Requirements for manufacturing and clinical testing were met and an Investigational New Drug Application was established with the Food and Drug Administration to test both antigens in a phase I clinical trial for safety in a non-endemic region for leprosy and a phase II clinical trial for safety and efficacy in an endemic region for leprosy.

**INTRODUCTION**

Detection of subclinical leprosy continues to be identified by the World Health Organization (WHO) as a priority. [1] With the introduction of multiple drug therapy (MDT) by the WHO in 1982 to prevent M. leprae resistance to dapsone monotherapy, the prevalence of leprosy began a dramatic decline. [2] Over the past 30 years, prevalence has dropped by about 98% from an estimated historical high of 11.5 million cases in 1983 [3] to the current figure of 192,246 registered cases. [1] Contrary to this remarkable achievement, leprosy incidence or new case detection remained relatively constant or increased slightly from 1985 at 555,188 new cases in the top 33 endemic countries [4] to 571,792 in 1990 [5] and 620,672 in 2002. [6] A significant decline of 51.4% in new cases was then observed between 2002 and 2005 to 299,036, followed by another decline of 23.6% to the current figure of 228,474. [1] A total decline of 58.8% in

detection of new cases from 1985 to 2010 has been observed. Although many investigators have questioned the value of these numbers based on confounding operational factors [7], one fact remains; incidence has generally exceeded prevalence. Of concern is the increased number of childhood leprosy observed in India signifying active and recent transmission of disease. [8,9] These findings provide evidence that transmission of M. leprae from infected to susceptible individuals remains a problem.

Little is known of the extent of leprosy in the endemic regions of the world, or reservoirs of infection, or bacterial or immunological basis of the distinctive pathogenesis of leprosy, notably nerve damage; [10,11] however, we do know that early detection and treatment does reduce transmission [12] and disease sequelae [13-15]. MDT is curative for leprosy disease, and if administered early during infection can prevent transmission from individuals harboring *M. leprae* bacilli. [16,17]

Classification of leprosy was first described by Ridley and Jopling based on the number of lesions, lepromin skin test response, and number of bacteria in a slit skin smear. [18] Clinical leprosy is an immunologically polar disease with limited pathogenesis on the tuberculoid (TT) pole and severe pathogenesis on the lepromatous (LL) pole; borderline tuberculoid (BT), borderline (BB), and borderline lepromatous (BL) leprosy fall between the polar ends. [10] BT/TT leprosy patients typically exhibit few lesions (five or less), high cell-mediated immunity (CMI), and few if any detectable bacteria (paucibacillary), whereas BL/LL leprosy patients exhibit multiple lesions (greater than five), specific T cell anergy, and large numbers of bacteria (multibacillary). [2] This spectrum of disease is determined by the immunological status of the host, [19] wherein the effectiveness of the adaptive T-cell response is dependent on instruction from the innate immune response. [20]

The incubation time of infection is difficult to assess due to the slow and variable onset of disease and the lack of adequate immunological tools.[11] Likewise, details of the early CMI events are unknown. Patients with borderline forms of leprosy, who are considered immunologically unstable, downgrade toward the lepromatous pole when immunocompromised. [19] Also, T cell anergy/hyporesponsiveness has been shown to result from immunological deregulation during leprosy progression. [21] These changes in the immune response along the continuum of disease suggest that a CMI test may be adequate to detect subclinical leprosy.

Our approach has been focused on the CMI delayed hypersensitivity (DTH) immune response. A DTH type IV reaction is initiated when antigen is injected into subcutaneous tissue and processed by antigen presenting cells. A $Th_1$ effector cell recognizes the antigen and releases cytokines IL-2, IFN-γ, and TNF, which act on vascular endothelium causing erythema and recruitment of T-cells, phagocytes, fluid, and protein which causes a measurable induration response within 48-72 hours in humans. A lack of DTH response to recall antigen is evidence of anergy. [22]

Early skin test studies with whole bacilli preparations such as Lepromin-H (Mitsuda) [23] and Lepromin A (Krotoski, Hastings) [24,25] had proven utility in classification of disease with the 21 day Mitsuda reaction, but the Lepromin antigen immunologically primed the immune response and was not specific for leprosy. Lepromin Dharmendra (Dharmendra) [26], Soluble Protein Antigen (SPA) or Leprosin (Convit) and *Mycobacterium leprae* soluble antigens (MLSA) (Rees) [27] measured the Fernandez 48 hour DTH reaction and have shown potency, but fall short of sensitivity and specificity. [28-31] Leprosy patients classified as tuberculoid leprosy (TT) have a characteristic DTH response to leprosy skin test antigens Lepromin A, SPA or Leprosin, and MLSA, whereas lepromatous leprosy (LL) patients are anergic to these

antigens, but not other mycobacterial antigens [32]. The DTH response of borderline patients typically fall within the spectrum of their disease classification, however, there are a few, albeit low number of outliers. [29,31]

Two refined leprosy skin test antigens: MLSA-LAM (*M. leprae* soluble antigen devoid of lipoglycans, primarily the immunosuppressive and cross-reactive lipoarabinomannan (LAM), and also lipomannan (LM), and phosphatidylinositol mannoside (PIM) and other lipids [33-35], and MLCwA (*M. leprae* cell wall antigen consisting of the powerful immunogens of the cell wall) [36,37] were identified [38]. Both antigens strongly induce proliferation of lymphocytes and stimulate secretion of IFN-γ from immune cells [39,40]. These early research studies led to the development and manufacturing of these antigens suitable for first-in-human clinical trials. [38]

## METHODS

### Propagation of M. leprae in Armadillos

*M. leprae* cannot grow axenically, but can be propagated in the nine-banded armadillo, *Dasypus novemcinctus*, [41] which are numerous and highly inbred in Florida. At the Florida Institute of Technology (FIT), Melbourne, Florida, Eleanor. E. Storrs and subsequently Arvind Dhople, et al. under National Institutes of Allergy and Infectious Diseases (NIAID) captured armadillos from state or nationally managed land areas in Central Florida. The following information was supplied by FIT. Upon arrival at the FIT facility, armadillos were placed in quarantine and dipped in 50% malathion initially and every 6 months to kill external parasites. Equizole A liquid was administered initially and every 6 months to treat for internal parasites. The following tests were performed before releasing armadillos from quarantine: 1) acid fast staining of ear snips, nasal swabs, and blood for evidence of acid-fast bacilli, 2) culturing of a

blood sample for sterility in Trypticase Soy Broth and thioglycollate broth, 3) hematology, 4) serodiagnosis for IgM antibodies to phenolic glycolipid-I, and 5) Lepromin test to determine susceptibility to *M. leprae.* [41,42] Armadillo quarantine ranged from 3 to 6 months.

The source of *M. leprae* was an untreated individual from Guyana with large numbers of highly bacilliferous subcutaneous nodules and lepromas. Genetic evidence has since indicated that *M. leprae* isolates were antigenically homogeneous. [43,44] Infected armadillos were sacrificed and the livers and spleens were homogenized and fractionated to separate *M. leprae* bacilli to serve as the Master Seed Stock in 2 ml volumes ($3 \times 10^8$ bacilli/ml) frozen at -70 °C. Master Seed Stock (1 ml) was inoculated into each armadillo. Infected armadillos with disseminated leprosy were sacrificed and the tissues (liver and spleen) aseptically removed. Samples from each tissue were tested for bacterial quantitation and sterility (Lowenstein-Jensen agar, Middlebrook 7H11 agar, nutrient broth, Trypticase Soy Broth, and thioglycollate broth at 32 °C and 37 °C for eight weeks). The infected armadillo tissues were shipped to the Pilot Plant Skin Test Antigen Facility at Colorado State University (CSU). The FIT armadillo facility was in-compliance with United States Department of Agriculture-American Public Health Association, United States Public Health Service-Office for Protection from Research Risks, and Institutional Animal Care and Use Committee standards.

**Tissue fractionation**

A total of 242 g of *M. leprae* infected tissue (spleen, 19 g; liver, 223 g) from three infected armadillos [animal nos. A563 (19 g spleen, one preparation), A572 (109 g liver, divided into three preparations), and A581 (114 g liver, divided into three preparations)] were fractionated using a modified 3/77 Draper protocol, [45] (**Figure 3**) except for omission of the step involving protease digestion with chymotrypsin and trypsin and alterations in buffer composition. Protease

digestion of homogenate was removed since no difference was seen between treated and untreated tissue preparations in terms of purity, protein content, and immunological potency of the recovered *M. leprae*.

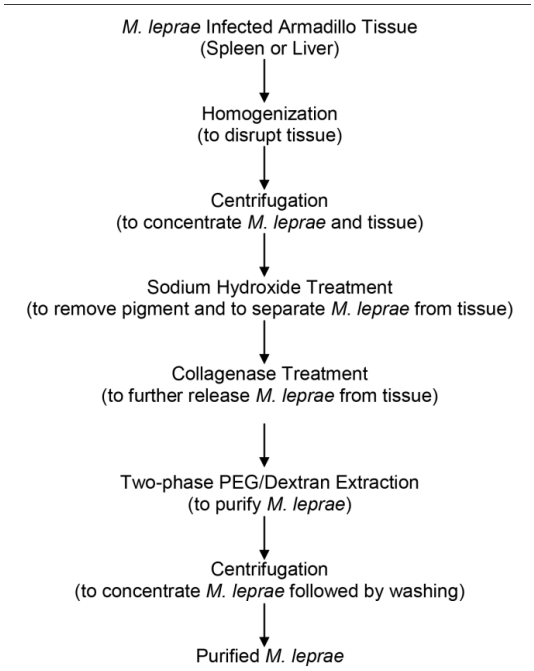



**Figure 3: Tissue Fractionation Flow Chart**
*M. leprae* was purified from the tissues of experimentally infected armadillos. A total of seven batches were prepared to generate an adequate quantity of bacteria (128.4 mg) for bacterial fractionation.

In brief, tissue sections ranging from 19 g to 36.5 g were homogenized with 10 mM disodium ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, Mo.), pH 8.0 at 3 ml/g of tissue; homogenates were tested for sterility on brain heart infusion agar, blood agar, and Lowenstein-Jensen agar (BD, Franklin Lakes, NJ). Tissue fragments were pelleted and washed twice with 10 mM EDTA by centrifugation (Sorvall RC5, Thermo Fisher Scientific, Inc., Rockford, IL) at 15,000 x g for 10 min at 4 °C in 50 ml Teflon Oakridge tubes, followed by extraction with 0.1M sodium hydroxide (Mallinckrodt Baker Inc., Phillipsburg, NJ) in 10 mM EDTA while stirring at

room temperature for 2 h to remove pigment and to separate *M. leprae* from tissue. The suspension was pelleted and washed twice with 0.1mM sodium phosphate/0.1% Tween 80 (Mallinckrodt/Fisher) designated buffered water followed by digestion with 20 mg collagenase (Sigma, St. Louis, Mo.) and 0.23 mM calcium chloride (Sigma) in 200 ml buffered water while stirring overnight at 37 °C.

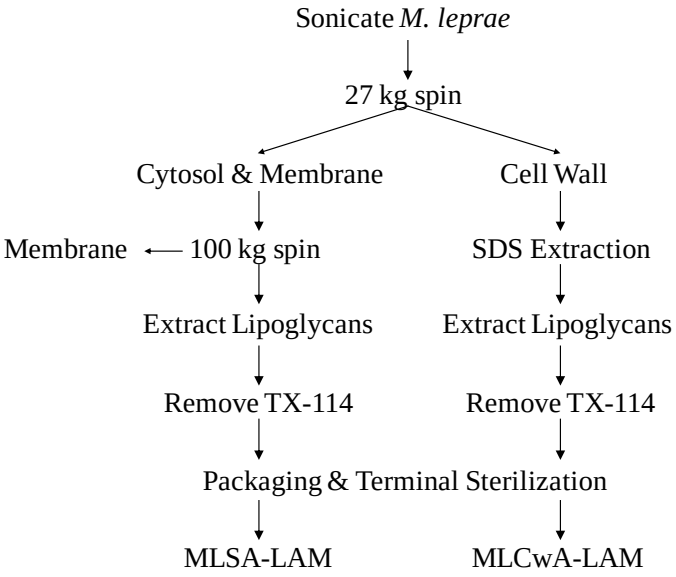



**Figure 4**. **Bacterial Fractionation Flow Chart**
Bacteria were sonicated and fractionated into subcellular components: cell wall, cytosol, and membrane. The membrane antigen was a by-product saved for next generation skin test antigen studies. Cell wall associated proteins were extracted with SDS and both the cytosol and cell wall fractions were then extracted with TX-114 to remove immunosuppressive lipoglycans. Residual detergent was removed by affinity chromatography. Antigens were diluted to prescribed concentrations, vialed, labeled, and autoclaved.

The digest was again pelleted and washed prior to two-phase extraction with 6% polyethylene glycol 6,000 and 8% Dextran T-500 (Sigma) in 0.1M sodium phosphate/150 mM sodium chloride at 10 ml/g of tissue in a separatory funnel. The upper phase containing bacteria was removed and an equal volume of 0.2% Tween 80 added prior to centrifugation at 27,000 x g for 30 min at 4°C. Purified *M. leprae* was then washed twice at 15,000 x g with buffered water

and the concentration of bacilli estimated with a 1:100 and 1:200 dilution by optical density at $A_{540}$ using an empirically determined conversion factor of 0.362 based on dry weight, i.e., $A_{540}$ of 1.0 = 0.362 mg *M. leprae*/ml multiplied by the dilution factor. Samples of the bacilli were tested for sterility by culturing on brain heart infusion agar, blood agar, and Lowenstein-Jensen agar. Purity was subjectively determined by acid fast staining using methlyene blue as a counterstain for residual tissue, with acceptance criteria of $\geq$ 90%. [46,47]

**Bacterial fractionation**

*M. leprae* (128.43 mg) from seven such preparations were pooled and washed twice with 25 ml phosphate buffered saline (PBS) by centrifugation at 27,000 x g for 15 min at 4 °C (**Figure 4**). Bacteria were suspended in 5 ml PBS and disrupted by sonication on cold packs with an ultrasonic processor (Sanyo Soniprep 150, MSE Ltd., Lower Sydenham, London) at 1.5 MHz, 50% duty, and 1 second pulse intervals over six 5 min cycles with 5 min cooling between each cycle. Pre and post-sonicated bacteria were stained using the TB Acid Fast Stain Kit (Thermo Fisher Scientific Inc.) for counting to verify greater than 80% breakage.

Disrupted bacteria were centrifuged at 27,000 x g for 30 min. Supernatant consisting of cytosol and membrane was transferred to a fresh tube and centrifugation repeated. The pellet consisting of *M. leprae* cell wall was washed three times with 10 ml PBS. The cytosol/membrane containing supernatant was transferred to an Ultra Clear 5 ml (13 x 51 mm) tube and ultracentrifuged (Optima TLX 120, Beckman Coulter Inc., Brea, CA) at 100,000 x g for 2 h at 4 °C to pellet the membrane. To remove lipoglycans [48] cold 20% condensed Triton X-114 (Baxter, Deerfield, IL) was added to the supernatant (cytosol) to a final concentration of 4%, followed by rocking at 4 °C overnight. The tube was placed in a beaker of water at 37 °C for 10 minutes to condense the TX-114 followed by centrifugation for 15 minutes at 3,900 x g at 22 °C

to separate detergent and aqueous layers. The top layer was transferred onto tandem 1 ml Extracti-gel D (Fisher) columns to remove residual detergent. Extraction and removal of residual detergent was then repeated.

Cell wall pellet was resuspended with 2 ml of 2% sodium dodecyl sulfate (SDS, Fisher)/PBS and stirred while heating at 56 °C for 1 h followed by centrifugation for 15 min at 27,000 x g at 22 °C to remove the SDS solubilized *M. leprae* cell wall antigens; the residual *M. leprae* cell walls consisting of the mycolylarabinogalactan has been the subject of much research. [49,50] The supernatant was transferred to a fresh tube and the extraction was repeated. MLCwA preparation was passed over two 1 ml Extracti-gel D columns to remove residual SDS and finally subjected to two rounds of TX-114 extraction followed by removal of residual detergent as described above.

The protein concentration of each of the antigen preparations was assessed by the Bicinchoninic Acid assay (Fisher). The process yielded 4.6 mg of MLSA-LAM and 5.0 mg MLCwA. Antigens were diluted with phosphate buffered saline containing 0.0005% Tween 80 to a final dosage of 10.0, 5.0, 2.5, 1.0, and 0.1 μg protein per 0.1 ml followed by 0.2 μm filtration to remove residual particulates. A total of 1 ml of each of the antigen doses was aliquoted into prewashed and sterilized 2 ml borosilicate vials with 13 mm silicon rubber stoppers and aluminum caps (Wheaton, Millville, NJ). Vials were labeled in accordance with FDA labeling requirements, including the statement, "Caution: New Drug-Limited by Federal Law to Investigational Use"[51], autoclaved for 20 min at 121 °C; cooled at room temperature, and placed at -70 °C for storage as MLSA-LAM and MLCwA batch no. 23 and lot no. 051297. Vials used in the phase I clinical trial remained at CSU, while those used in the phase II clinical

trial were sent to Fisher Bioservices Repository (Rockville, MD) for relabeling with randomly assigned codes and shipment to the phase II clinical site (**Figure 5**).

**Figure 5: Packaged and Labeled Leprosy Skin Test Antigens**
Product interventions, MLSA-LAM and MLCwA, each at 1.0 µg and 0.1 µg, and control antigens saline and Tuberculin 5 TU were coded by Fisher Bioservices Repository prior to shipment to the phase II clinical site for blinding blinded applications.

**Residual Collagenase Assay**

A collagenase enzymatic assay adapted from Sigma was used to test for residual collagenase in skin test antigen preparations. [52,53] A single unit of collagenase liberates 1 µmole of 4-phenylazobenzyloxycarbonyl (Pz)-Pro-Leu from the substrate Pz-Pro-Leu-Gly-Pro-dArg in 15 minutes at pH 7.1 at 37 °C. [54] Collagenase was not detectable in the final products within the assay sensitivity limit of 2.0 µg/ml.

**Residual SDS Assay**

Residual SDS was measured by the Anionic Detergent Assay using methylene blue and chloroform. [55,56] Both skin test antigens contained less than 5 ng/ml SDS.

**Residual Triton X-114 Assay**

Residual Triton X-114 was measured by the Nonionic Detergent Assay using dichloromethane and cobaltothiocyanate reagent. [57] Triton X-114 was not detectable in either skin test antigen preparation within the assay sensitivity limit of 4µg/ml.

**Evaluation of Protein and Soluble Carbohydrate Identity**

Following filtration and prior to dose formulation, a sample was removed from each antigen to evaluate the protein profile by separation of proteins on reduced 15% polyacrylamide gel electrophoresis gels [58] and staining with silver nitrate to detect proteins [59] or sliver nitrate with periodate to detect glycans [60]. Antigens were loaded onto gels at 1.0, 0.5, 0.25, 0.1, and 0.01 µg/lane. M. leprae whole sonicate (2 µg) was used as a reference standard. Antigens were transferred to nitrocellulose in Tris, glycine, methanol transfer buffer for 1 h at 50 V. [61] Nitrocellulose panels were blocked with 1% Bovine Serum Albumin (Sigma) in Tris buffered Saline (TBS)/0.05% Tween 80 as diluent for 1 h at room temperature and then incubated in one of the following primary antibodies for 1 h at room temperature: mouse monoclonal antibody (mab) anti-LAM (CS-35), mab anti-GroES (CS-01), mab anti-superoxide dismutase (SOD), (CS-18), mab anti-major membrane protein-I (MMP-I), (CS-38), mab anti-GroEL (CS-43), and rabbit polyclonal antibody against non-infected armadillo liver. All antibodies were prepared in-house. After washing three times in TBS/0.05% Tween 80, a dilution of goat anti-mouse IgG or goat anti-rabbit IgG conjugated to alkaline phosphatase were added to each panel following incubation for 1 h at room temperature. Panels were washed 3 times with TBS and once with water prior to developing in NBT-BCIP substrate (Sigma) for approximately 3 min before stopping the reaction with water.

**General Sterility Test**

The General Sterility Test procedure specified in Title 21 of the Code of Federal Regulations (CFR) Part 610.12 was performed. [62] A total of 10 vials of each dosage to be tested in humans (2.5, 1.0, and 0.1 µg/ml) was tested with fluid thioglycollate medium at 30 – 35 °C and soybean casein digest medium at 20 – 25 °C, each for a total of 14 days. All controls were positive for

growth within 2-4 days and all vials tested in both medium conditions were negative for growth at each observation point.

**General Safety Test**

The General Safety Test procedure specified in 21 CFR 610.11 was performed in mice and guinea pigs with the 5.0 µg/0.1 ml dosage of each antigen preparation. [63] No adverse reactions were observed and all animals increased in weight by the end of the study. All animal studies were conducted at CSU, adhering to Institutional Animal Care and Use Committee guidelines for animal husbandry.

**Assay for Endotoxin Content**

The Limulus Amebocyte Lysate third generation pyrogen test from BioWhittaker, License No. 709 was used for this test. [64] The standard concentration range was 0.1 – 1.0 EU/ml. All vials of antigen preparations had endotoxin concentrations less than the limit of detection.

**DTH Guinea Pig Potency Assay**

Guinea pigs of the outbred Hartley strain were sensitized by subcutaneous injection in the base of the neck with M. leprae inactivated at 80 °C and suspended in Freund's Incomplete Adjuvant. After 4-8 weeks, 0.1 ml skin test antigens were administered intradermally on the freshly shaven back of each animal. Induration was measured at 24 and 48 hours post-injection. A measurement over 5 mm was considered positive. [65]

**Stability Testing**

Skin test antigen batch no. 9, prepared in the general research laboratory was used for preliminary stability testing. Each antigen was diluted with PBS or borate buffer to a dosage of 10.0 µg/0.1 ml and either filtered or filtered and terminally sterilized. Immediately after packaging, each sample was placed at -70 °C, 4 °C, 37 °C, or 56 °C. Samples were analyzed for

stability in the DTH Guinea Pig Potency Assay on days 45, 90, 120, and 360 at 1.0 µg and 0.1 µg doses. Abbreviated stability testing was performed on the cGMP batch no. 23, lot no. 051297. Antigens vialed at doses of 1.0 µg and 0.1 µg were tested at 4 °C and 20 °C against equivalent antigens stored at -70 °C for 90 d, 120 d, 360 d, 2 y and 4 y.

### Adventitious Agent (Virus) Testing

Liver homogenates from each tissue fractionation and MLSA-LAM and MLCwA final product at 10.0 and 5.0 µg/0.1 ml were tested for human viral pathogens using cell based assays and polymerase chain reaction (PCR). Viral identification by cytopathic effect for Adenovirus; Parainfluenza 1, 2, and 3 Viruses; Influenza Virus; Poliovirus; Cytomegalovirus; Herpes Simplex 1, and 2 Viruses; and Respiratory Syncytial Virus was conducted at the University of Colorado Diagnostic Virology Laboratory (Boulder, Colorado). Positive and negative controls were included during testing. PCR for Hepatitis B Virus and Human Immunodeficiency Virus was performed by Specialty Laboratories, Inc.[66].

## RESULTS

### Choice and Characterization of Antigen Preparations

Similar antigen preparations, forerunners of the present ones were tested in guinea pig DTH potency studies to evaluate their skin test potential and an in vitro whole blood T cell assay to evaluate their ability to stimulate a response from healthy subjects and leprosy patients. [39] The M. leprae membrane antigen (MLMA) was immunoreactive, but contained too little protein content after removal of lipoglycans to proceed further. Although use of subcellular fractions was the only viable choice at the onset of this project, it was not without the realization that use of complex antigen mixtures would likely impact specificity. Nonetheless, on a par with

Tuberculin PPD, a decision was made to move forward with development of MLSA-LAM and MLCwA to establish a prototype for next generation skin test antigen candidates.

The active ingredients of these two intradermal skin test antigens are protein antigens of M. leprae. MLSA-LAM contains the soluble protein antigens of M. leprae; over 100 individual proteins were initially recognized on two-dimensional gels, and about 30 of these had been sequenced and the immunological responses studied in part. [67,68] Foremost among these antigens are the 70 kDa (DnaK), 65 kDa (GroEL), 45 kDa, 38 kDa, 35 kDa (MMP-I), 23 kDa (SOD), 18 kDa small heat shock protein (SmHSP), 18 kDa bacterioferritin (Bfr), 10 kDa (GroES), and the ribosomal proteins S7/S12. [69-74] More recently, the full spectrum of proteins in soluble and insoluble subcellular fractions of M. leprae have been demonstrated and many more identified through the modern-day "proteomics" approach. [75-77] MLCwA contains many of the same proteins as MLSA-LAM, particularly the 70 kDa and 65 kDa kDa and degradation products of these, the export/secretory proteins (notably the 30/31 kDa, multigene antigen 85 complex), and also some larger, uncharacterized proteins. [76] Details of the full spectrum of MLCwA constituent proteins have since been published. [77]

**Leprosy Skin Test Antigen Pilot Plant**

Options for manufacturing the two new leprosy skin test antigens under current good manufacturing practices (cGMP), suitable for human application were limited. Costs for using a contract manufacturing organization (CMO) was prohibitive; it was difficult to find any with an open schedule, and few had biosafety level 2 (BSL-2)/cGMP clean rooms required for safe manufacturing of these antigens. In addition, service providers acknowledged that they were fearful of working with *M. leprae*. Consequently, a retired BSL-3 research laboratory was converted to a cGMP Pilot Facility (**Figure 6**) at CSU for the sole purpose of manufacturing

these leprosy skin test antigens. To this end, the manufacturing and testing process for MLSA-LAM and MLCwA was developed to meet 21 CFR part 210, 211 for current Good Manufacturing Practices. [78,79]

The Pilot Facility consisted of a suite of five rooms, 1) Gowning and Material Transfer Room, 2) Manufacturing Suite A, 3) Manufacturing Suite B, 4) Quarantine/Released Goods Room, and 5) Quality Control Laboratory. Both the manufacturing and quality control rooms were under positive pressure cascading from the innermost room to the entry foyer. Air was supplied by a dedicated heating ventilation air conditioning system with single pass air flow monitored with gauges in the entry room and with an anemometer prior to entry of the manufacturing suite. High efficiency particulate air filters were positioned on both the supply and exhaust air streams to purify air entering and exiting the clean rooms. The manufacturing rooms were classified [80] as international standard organization (ISO) 7 clean rooms. The innermost manufacturing room was used for downstream processing (antigen purification, formulation, and vialing), while the outermost manufacturing room was used for upstream processing (tissue fractionation and bacteria sonication). The gowning and material handling room was classified as an ISO8 clean room for personnel aseptic tyvek gowning, wipe down and transfer of materials and equipment into the manufacturing area, and entering and exiting of personnel. The innermost quality control room, an ISO8 clean room was used for testing raw materials, intermediate product, and final product, while the quarantine/released goods room was a clean, non-classified clean room used for quarantine and release of raw materials.

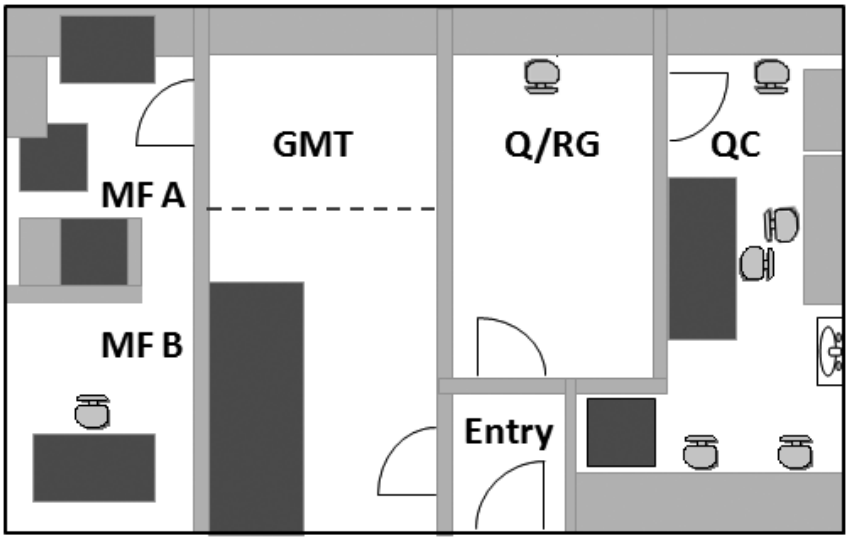



**Figure 6. Leprosy Skin Test Antigen Pilot Facility**
Five room cGMP suite consisting of 1) Gowning and Material Transfer (GMT) Room for entry and exit of personnel, materials, equipment, product, and product into manufacturing suites - ISO8 clean room; 2) Manufacturing Suite A (MF A) for tissue fractionation – ISO7 clean room; 3) Manufacturing Suite B (MF B) for bacterial fractionation, purification, formulation, and vialing – ISO7 clean room; 4) Quarantine/Release Goods Room (Q/RG) for quarantine and release of raw materials, buffers, and media – clean/non-classified clean room; and, 5) Quality Control Laboratory (QC) for laboratory testing of raw materials, intermediate product, and final product – ISO8 clean room.

Renovation of the Pilot Plant Facility to meet cGMP regulations involved firstly, replacement of the heating, ventilation, air conditioning system with a new unit for dependability; due to cost restraints and limited use, a redundant system was not installed. High efficiency particulate air filter casings were resealed and new filters installed. The air flow direction was switched to positive pressure and balanced to supply adequate air exchange and a pressure differential cascading from the most inner room to the entry room on each side of the suite to reduce the potential for product contamination during processing. Ceilings and wall surfaces were already non-porous and therefore only required cleaning and a fresh coat of paint. The floor was cleaned and resealed with an epoxy floor coating. A biosafety cabinet (Forma Scientific, class II/AB3) that met ISO 5 classification for clean rooms was installed in the two most inner production

rooms and the inner most laboratory for handling open product or quality control testing, respectively. All processing equipment was dedicated during the manufacturing campaign.

**Quality Management System**

A quality system [81] was created for processing and testing leprosy skin test antigens in the renovated pilot plant. [82] The system covered six parts: facility and equipment, materials, production, product labeling, laboratory control, and quality. [83] Two batch records were written, one for tissue fractionation and one for bacteria fractionation. A total of 255 supporting standard operating procedures (SOPs) were written to cover the quality system and manufacture of antigens. Facility and equipment SOPs were written for operation, maintenance, and calibration of dedicated equipment. SOPs for directing and tracking the chain of custody for raw materials transferred through purchasing, receiving, quarantine, release, and storage were created. Process directives supporting environmental monitoring, gowning, transferring material, manufacturing, in-process testing, and release testing were written into SOP format with data forms to collect relevant information. Explicit details for product labeling were captured in the batch record. All levels of training, including equipment use, biosafety, good laboratory practice, cGMP, and good clinical practice were directed through SOPs. Logs were created to track part numbers, documents, raw materials, sample submission, equipment usage and room usage. Documents were subjected to the mandated review and approval process prior to implementation. [84]

Finally, commissioning of the cGMP Pilot Plant for manufacturing skin test antigens was performed. Rooms were decontaminated with para-formaldehyde.The Pilot Plant was cleaned and the environment was monitored on three consecutive days and three consecutive weeks following directive documents to assess the cleanliness of the facility. Monitoring viable

airborne organisms was performed with the Rotary Centrifugal Air Sampler (Biotest Diagnostics, Brooklyn Park, MN) and settling plates, both using Trypticase Soy Agar strips/plates. Monitoring viable surface organisms was performed with Rodac plates containing Trypticase Soy Agar and neutralizer for cleaning agents. Isolates were identified to the genius and species level using API Test Kits (Biomerieux, Etolile, France; distributed by VWR). Total particle counts in each clean room were measured using a Particle Counter (Metone Instruments, Grants Pass, Oregon). Acceptance criteria were met with each test enabling release of the Pilot Plant for cGMP manufacturing. Data was filed reported and then filed at CSU with original records.

**Pre-IND**

Prior to manufacturing skin test antigens, a draft Investigational New Drug (IND) application [85] and specific questions related to IND enabling studies, manufacturing, and phase I clinical trial design was sent to our NIH, NIAID, Division of Microbiology and Infectious Diseases (DMID) program officer at the time (the late Dr. Darryl Gwinn) and Regulatory Affairs Specialist (Ms. Carol Manning) for a preliminary review prior to submission to the FDA Center for Biologics and Evaluation Research (CBER) for review and comment. A comprehensive list of queries were received and addressed prior to submission of the IND application. The first topic of focus was the armadillo infected tissue and included questions on the following subject matters: 1) the origin, isolation, and characterization of the *M. leprae* strain; 2) creation, storage, maintenance, and viability testing of the master seed stock; 3) armadillo quarantine, test for human pathogens, and general health status; 4) potential human infectivity of indigenous armadillo microorganisms; 5) armadillo inoculation procedures and biosafety procedures for staff; and 6) test for viral adventitious agents. The second topic of focus was the manufacturing and characterization process, including questions on: 1) procedural flow charts; 2) potential or

known human toxicities and quantitative tests for reagents used in the manufacturing process; 3) qualitative compositional analyses for each skin test antigen; 4) presence of cross-reactive antigens; 5) level of host contamination, endotoxin, and sterility; 6) i*n-vitro* and *in-vivo* potency assays conforming to intended clinical use in humans; 7) stability testing prior to clinical studies; and 8) preclinical testing of clinical lots for safety, activity, and skin test conversion in a dose ranging study. The last topic of focus was the clinical phase I study design, including the following issues: 1) clinical study details; 2) potential impact of anergy regarding leprosy and HIV patients; 3) consent form and Institutional Review Board for each study site; 4) Case Report Forms for data collection; 5) references supporting related antigens and clinical studies; and 6) distinguishing subjects that are infected or harboring live bacilli from those who are infected and cured. A response to the FDA Response Letter was satisfactory and a Pre-IND Meeting followed. The following is an outcome of that meeting.

**Manufacturing of Antigens**

The manufacture of antigens was a two step process beginning with receipt, tracking, and release of raw materials. The primary raw material was spleen and liver tissues laden with *M. leprae* propagated in armadillos at FIT. Upon aseptic harvest, tissues were tested for the presence of contaminating bacteria using microbiological medium and then sent to the Pilot Plant, where they were frozen at -70 °C in a qualified freezer until used. SOPs covering each step of the propagation process were provided by FIT. All other reagents were United States Pharmacopeia grade or equivalent, if available. Otherwise the highest purity was specified. Each reagent was released for use based on a certificate of analysis provided by the vendor, per an approved in-house specification sheet. Materials were tracked using a receiving code and part number system.

Tissue fractionation under the respective Master Production and Control Record (MPCR) was performed to release and purify *M. leprae* from the armadillo tissue as the intermediate product. A total of 7 tissue runs were performed to accumulate 100 – 150 mg bacteria. Tissue weights ranged from 19 – 36.5 g for manageability and to maximize yields. A total of 128.4 mg *M. leprae* was purified from 242 g tissue, resulting in a yield of 0.05% (**Table 1**). Sterility testing was performed on each bacterial lot and material was stored at -70 °C until use. Bacterial fractionation under the respective MPCR was performed using the pooled intermediate product. A total of 4.6 mg of MLSA-LAM and 5.0 mg of MLCwA was obtained representing a yield of 3.57% and 3.88% from intact bacteria, respectively. Quality control was performed on the final product.

**Quality Control of Antigens**

Assays to assess MLSA-LAM and MLCwA critical quality attributes of identity, purity, sterility, potency, and safety were performed. [86] Ten vials of each antigen dose (2.5, 1.0, and 0.1 µg/0.1 ml) planned for clinical studies were tested on all assays with two exceptions. Identity testing by gel electrophoresis and immunoblotting was performed on samples taken prior to

**Table 1. Leprosy Skin Test Antigen Purification Yields**

| Step | Starting Material | Tissue | Animal No. (tissue wt) | Total Yield | Percent of prior step |
|---|---|---|---|---|---|
| 1 | Tissue | Spleen | A563 (19 g) | --- | --- |
| | Tissue | Liver | A572 (109 g)[a] | --- | --- |
| | Tissue | Liver | A581 (114 g)[a] | 242.0 g | --- |
| 2 | *M. leprae* | --- | --- | 128.4 mg | 0.05% |
| 3 | MLSA-LAM | --- | --- | 4.6 mg | 3.57% |
| | MLCwA | --- | --- | 5.0 mg | 3.88% |

[a] Liver tissues were divided into three sections with an average weight of 32 g $\pm$ 0.9 g/run.

autoclaving, which degrades proteins resulting in smearing of bands on gels and immunoblots. A representative silver stained gel of both antigen preparations is shown in **Figure 7**.

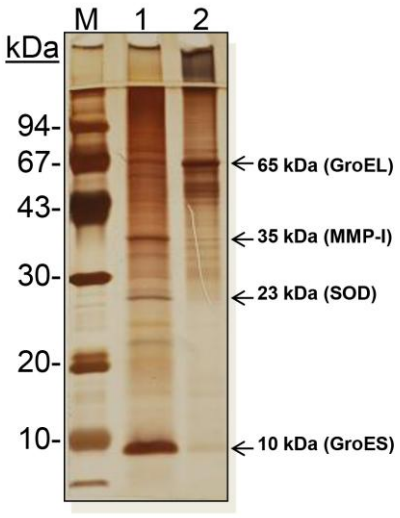



**Figure 7. Protein Profile of MLSA-LAM and MLCwA**
Leprosy skin test antigens MLSA-LAM and MLCwA (pre-autoclaved; 2 µg load) were separated on a 15% reduced polyacrylamide gel and visualized by staining with silver nitrate. Proteins which reacted by immunoblot are depicted. The SOD protein is a 23 kDa protein based on amino acid sequence, but resolves at 28 kDa under reduced gel electrophoresis conditions.

Immunoblotting results showed that neither antigen preparation had detectable armadillo tissue or LAM present, both contained MMP-I, and only MLSA-LAM contained GroES and SOD, while only MLCwA contained GroEL proteins. Purity testing for adventitious agents was performed on tissue homogenates and concentrated final product (10.0 µg and 5.0 µg/0.1 ml); both were free of detectable human viral pathogens.

Purity was also assessed by measuring endotoxin concentration and running specific assays for residual excipient, unless justified otherwise. Collagenase, Triton X-114, and SDS were tested and found to be lower than the limit of detection for each assay. Extracti gel D ligand was not tested, because if detached, would be removed by filtration prior to vialing. Calcium chloride (0.23mM) was not tested, because following multiple washes, the calculated residual

concentration in the purified bacteria suspension had decreased by 46-fold to 5 µM and was found to be harmless as demonstrated in animal safety studies. This was equally the case for polyethylene glycol, Dextran T-500, and sodium hydroxide excipients.

Antigen preparations were found to be sterile under aerobic and anaerobic conditions and potent when assessed for a DTH response in guinea pigs sensitized with *M. leprae* or infected with *M. tuberculosis*. Stability, although not a critical quality attribute was assessed during product development using a research batch and prior to and during clinical testing, resulting in 4 years of satisfactory results. The Lot Release Summary and stability results for both MLSA-LAM and MLCwA can be found in **Table 2.**

**IND Application**

The IND chemistry, manufacturing, and control section was updated following antigen manufacturing and testing. Our DMID Study Sponsor submitted the IND Application to CBER for review and allowance of the clinical investigation of two new drugs, MLSA-LAM and MLCwA, each at 3 doses (2.5, 1.0, and 0.1 µg) initially in a phase I clinical trial with ten healthy subject residing in a non-endemic region for leprosy, and subsequently in a phase II clinical trial with healthy subjects, leprosy patients, leprosy patient contacts, and tuberculosis patients residing in an endemic region for leprosy. This IND is filed with the FDA (IND number: BB IND 7938).

**Table 2. Lot Release and Stability Summary: MLSA-LAM and MLCwA Batch No. 23, Lot No. 051297**

| Quality Attribute | Test Method | Specification | Results | |
|---|---|---|---|---|
| | | | MLSA-LAM | MLCwA |
| Identity | Protein Concentration | Diluted to concentration | Pass | Pass |
| | Reduced Silver Stain Gel | Expected profile | Pass | Pass |
| | Immunoblots | Expected profile | Pass | Pass |
| Purity | Viruses: Culture for CPE, PCR | Not detected | Pass | Pass |
| | Endotoxin Concentration | $\leq$ 0.5 EU/mL | Pass | Pass |
| | Collagenase | Not detected | Pass | Pass |
| | Residual SDS | $\leq$ 0.001% | Pass | Pass |
| | Residual Triton X-114 | Not detected | Pass | Pass |
| Potency | DTH in Guinea Pigs | Induration at 1µg/0.1mL | Pass | Pass |
| Sterility | 21 CFR 610.12 | No growth | Pass | Pass |
| Safety | 21 CFR 610.11, guinea pigs & mice | All survive, no weight loss at 7 days, no AE | Pass | Pass |
| Stability | DTH in Guinea Pigs Temp: 4 °C and 20 °C Time: d90, d120, d360, y2 and y4 | Induration at 1µg/0.1mL | Pass | Pass |

## DISCUSSION

Development of two new leprosy skin test antigens, MLSA-LAM and MLCwA, began in 1992. A tool for detection of subclinical leprosy was then, as it is now, an urgent need. [87,88] Serological and gene approaches had not proven satisfactory for this purpose. [89] Even so, these and other test methods are continually being refined and evaluated today, including: antibodies [90,91], *M. leprae specific* DNA polymerase chain reaction [92-94], and cell mediated immune response primarily based on IFN- γ release assays [95,96]. While tests for PGL-I IgM antibodies

have found favor for certain applications, most are not suitable for mass epidemiological application. [97]

With our focus on the cell mediated immune response for detection of asymptomatic leprosy, historical data from early leprosy skin test antigens [98] established precedence for these studies. Promising features of the Rees MLSA and Convit SPA included: neither had sensitizing potential like Lepromin A [99]; both were potent immunologically; and, both were found to be safe in human vaccine trials in Venezuela, Malawi, and India. [30,100] Shortcomings included inconsistent readings due to soft rather than hard DTH reaction in some individuals; variations in potency between batches due to quality control issues; and, lack of adequate sensitivity and specificity. A more refined skin test antigen was needed.

Sensitivity of the skin test method for detecting infection has been proven with Tuberculin PPD skin test antigen for tuberculosis. [101] Tuberculin PPD has been used across the world with an exceptional safety record. Specificity, however, depends on age, genetic factors, certain medical conditions, climate, geography and other factors such as vaccination with bacilli Calmette Guerin (BCG). [102] Depending on the location, exposure to local environmental mycobacteria may cross react with the antigen and obscure test results. The antigen consists of a mixture of soluble, low molecular weight, secreted proteins of *M. tuberculosis*, precipitated from culture broth filtrate. [103]

Our goal was to produce a leprosy skin test antigen equal to the sensitivity and better than the specificity of Tuberculin PPD. Two leprosy skin test antigens were chosen as candidates for development based on prior knowledge, adequate yield, a robust DTH response in *M. leprae* sensitized compared to *M. tuberculosis* infected guinea pigs, and *in vitro* stimulation of TT leprosy patient T-cells to release IFN-γ when compared to controls.[38] The first antigen,

MLSA-LAM was modified from Rees MLSA by removing immunosuppressive and cross-reactive components (LAM, LM, and PIMs) and other lipids. The second antigen, MLCwA contained powerful cell wall derived immunogens. Since these antigens were identified, further characterization has shown that each are made up of complex protein mixtures. [76,77]

Developing and manufacturing MLSA-LAM and MLCwA investigative products suitable for human application for leprosy was challenging without industry expertise and funding. Attempts to identify a qualified, willing, and reasonably priced CMO failed, leading to the decision to manufacture the skin test antigens within an academic setting.

Deducing the product development process and related regulatory requirements was daunting in the early days. A product development roadmap [104] or FDA Translational Critical Path [105] was not available. There were FDA guidelines on some topics and a consultant was used to jump start the skin test initiative. Our NIH, NIAID, DMID study sponsor provided guidance and regulatory assistance and served as a conduit to the Food and Drug Administration (FDA) for cGMP questions covering construction, equipment, raw materials, production, testing, and quality; IND questions covering chemistry, manufacturing, and control; and, GCP questions covering clinical studies and required documentation.

Keeping the project on-schedule during edification and formulation of systems supporting facilities and equipment, raw materials, the manufacturing process, quality control, documentation, and quality assurance in-compliance with cGMP regulations [78,79] was challenging. With limited resources, renovating and commissioning a BSL-2/cGMP pilot plant, establishing batch records and supporting SOPs, performing IND enabling studies as an outcome of sponsor and FDA review of the draft IND, and managing multiple rounds of document review

and approval were time consuming and onerous. The immensity of the project led to prolonged timelines.

As challenges were overcome, the draft IND was submitted by our study sponsor to the FDA for comment in April, 1994; antigens were successfully manufactured in May, 1997; and the IND Application was submitted to the FDA in September, 1998 for allowance to move clinical trials forward to assess the safety and efficacy of MLSA-LAM and MLCwA as early diagnostic tools for leprosy. This work also provides a generalized template supporting product translation for a diagnostic, therapeutic, or vaccine for other neglected tropical diseases.

REFERENCES


1. Anonomyous (2011) Leprosy update. Weekly Epidemiological Record 86: 389-400.

2. World Health Organization (1982) Report of the Eleventh and Twelfth Meetings of the Steering Committee of the Scientific Working Group on the Chemotherapy of Leprosy. Geneva. March 30-31 and Oct 10. TDR/THELEP-SC (11-12) 82: 1-6.

3. Sansarricq H (1983) Recent changes in leprosy control. Lepr Rev Spec No: 7-16.

4. Meima A, Richardus JH, Habbema JD (2004) Trends in leprosy case detection worldwide since 1985. Lepr Rev 75: 19-33.

5. Anonomyous (1998) Elimination of leprosy as a public health problem (update) Weekly Epidemiological Record 73: 308-312.

6. World Health Organization (2004) Leprosy Elimination Project: Status Report 2003. Geneva.

7. Sachdeva S, Khan Z, Ansari MA, Amin SS (2011) Leprosy: down but not out. Trop Doct 41: 28-30.

8. Singal A, Sonthalia S, Pandhi D. (2011 ) Childhood leprosy in a tertiary-care hospital in Delhi, India: a reappraisal in the post-elimination era. Lepr Rev 82: 259-269.

9. Sachdeva S, Amin SS, Khan Z, Sharma PK, Bansal S (2011) Childhood leprosy: lest we forget. Trop Doct 41: 163-165.

10. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, et al. (2006) The continuing challenges of leprosy. Clin Microbiol Rev 19: 338-381.

11. Bhat RM, Prakash C. (2012) Leprosy: an overview of pathophysiology. Interdiscip Perspect Infect Dis 2012: 181089.

12. Richardus JH, Habbema JD (2007) The impact of leprosy control on the transmission of M. leprae: is elimination being attained? . Lepr Rev 78: 330-337.

13. Rodrigues LC, Lockwood DNJ (2011) Leprosy now: epidemiology, progress, challenges, and research gaps. The Lancet Infectious Diseases 11: 464-470.

14. Saunderson P. (2000) The epidemiology of reactions and nerve damage. Lepr Rev Suppl 71: 106-110.

15. Van Brakel WH, Nicholls PG, Das L, Barkataki P, Maddali P, et al. (2005) The INFIR Cohort Study: assessment of sensory and motor neuropathy in leprosy at baseline. Lepr Rev 76: 277-295.

16. Feenstra SG, Nahar Q, Pahan D, Oskam L, Richardus JH (2011) Acceptability of chemoprophylaxis for household contacts of leprosy patients in Bangladesh: a qualitative study. Lepr Rev 82: 178-187.

17. Moet FJ, Pahan D, Oskam L, Richardus JH (2008) Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ 336: 761-764.

18. Ridley DS, Jopling WH (1966) Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis 34: 255-273.

19. George M, Rajan U, George S, Pakran J, Thomas S (2010) Sub-polar lepromatous leprosy localized to the face. Dermatol Online J 16: 8.

20. Modlin RL (2010) The innate immune response in leprosy. Curr Opin Immunol 22: 48-54.

21. Kumar S, Naqvi RA, Khanna N, Rao DN (2011) Disruption of HLA-DR raft, deregulations of Lck-ZAP-70-Cbl-b cross-talk and miR181a towards T cell hyporesponsiveness in leprosy. Mol Immunol 48: 1178-1190.

22. Black CA (1999) Delayed type hypersensitivity: current theories with an historic perspective. Dermatol Online J 5: 7.

23. Mitsuda K. (1919) On the value of a skin reaction to a suspension of leprosy nodules. Jap J Derm Urol 19: 697-708.

24. Krotoski WA, Mroczkowski TF, Rea TH, Almodovar PI, Clements BC, et al. (1993) Lepromin skin testing in the classification of Hansen's disease in the United States. Am J Med Sci 305: 18-24.

25. Krotoski WA, Mroczkowski TF, Shannon EJ, Millikan LE, Sanchez RM, et al. (1993) Lepromin responses in recipients of a candidate antileprosy bacterin vaccine (WHO-IMMLEP Mycobacterium leprae killed preparation) in the USA. Int J Dermatol 32: 191-193.

26. Dharmendra D (1942) The immunological skin tests in leprosy: the isolation of a protein antigen of Mycobacterium leprae. Ind J Med Res 30: 1-7.

27. Special Programme for Research and Training in Tropical Diseases World Health Organization Scientifice Working Group on the Immunology of Leprosy Epidemiology Subgroup (1985) Vaccination Trials Against Leprosy: A Meeting of the Epidemiology Subgroup of the Scientific Working Group on the Immunology of Leprosy : Geneva, 11-13 February 1985. World Health Organization 85: 7-8.

28. Gupte MD, Anantharaman DS (1988) Use of soluble antigens in leprosy epidemiology. Lepr Rev 59: 329-335.

29. Gupte MD, Anantharaman DS, Nagaraju B, Kannan S, Vallishayee RS (1990) Experiences with Mycobacterium leprae soluble antigens in a leprosy endemic population. Lepr Rev 61: 132-144.

30. Convit J, Sampson C, Zuniga M, Smith PG, Plata J, et al. (1992) Immunoprophylactic trial with combined Mycobacterium leprae/BCG vaccine against leprosy: preliminary results. Lancet 339: 446-450.

31. Samuel NM, Stanford JL, Rees RJ, Fairbairn T, Adiga RB (1984) Human vaccination studies in normal and contacts of leprosy patients. Indian J Lepr 56: 36-47.

32. Bloom BR, Mehra V (1984) Immunological unresponsiveness in leprosy. Immunol Rev 80: 5-28.

33. Chatterjee D, Khoo KH (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 8: 113-120.

34. Torrelles JB, Sieling PA, Zhang N, Keen MA, McNeil MR, et al. (2012) Isolation of a distinct Mycobacterium tuberculosis mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. Glycobiology 22: 1118-1127.

35. Barnes PF, Chatterjee D, Abrams JS, Lu S, Wang E, et al. (1992) Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. J Immunol 149: 541-547.

36. Hunter SW, McNeil M, Modlin RL, Mehra V, Bloom BR, et al. (1989) Isolation and characterization of the highly immunogenic cell wall-associated protein of Mycobacterium leprae. J Immunol 142: 2864-2872.

37. Melancon-Kaplan J, Hunter SW, McNeil M, Stewart C, Modlin RL, et al. (1988) Immunological significance of Mycobacterium leprae cell walls. Proc Natl Acad Sci U S A 85: 1917-1921.

38. Brennan PJ, Cho SN, Klatser PR (1996) Bangkok Workshop on Leprosy Research. Immunodiagnostics, including skin tests. Int J Lepr Other Mycobact Dis 64: S58-62.

39. Weir RE, Brennan PJ, Butlin CR, Dockrell HM (1999) Use of a whole blood assay to evaluate in vitro T cell responses to new leprosy skin test antigens in leprosy patients and healthy subjects. Clin Exp Immunol 116: 263-269.

40. Manandhar R, LeMaster JW, Butlin CR, Brennan PJ, Roche PW (2000) Interferon-gamma responses to candidate leprosy skin-test reagents detect exposure to leprosy in an endemic population. Int J Lepr Other Mycobact Dis 68: 40-48.

41. Storrs EE (1971) The nine-banded armadillo: a model for leprosy and other biomedical research. Int J Lepr Other Mycobact Dis 39: 703-714.

42. Kirchheimer WF, Storrs EE (1971) Attempts to establish the armadillo (Dasypus novemcinctus Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int J Lepr Other Mycobact Dis 39: 693-702.

43. Monot M, Honore N, Garnier T, Araoz R, Coppee JY, et al. (2005) On the origin of leprosy. Science 308: 1040-1042.

44. Williams DL, Gillis TP, Portaels F (1990) Geographically distinct isolates of Mycobacterium leprae exhibit no genotypic diversity by restriction fragment-length polymorphism analysis. Mol Microbiol 4: 1653-1659.

45. WHO IMMLEP. (1977) Report of the third IMMLEP scientific working group on leprosy protocol 3/77. WHO Document TDR/SWG/IMMLEP: 20.

46. Shepard CC, McRae DH (1968) A method for counting acid-fast bacteria. Int J Lepr Other Mycobact Dis 36: 78-82.

47. Dyachina MN, Pynchuck LM, Lazovskaya AL, Juscenko AA (1994) Assessment of the purity of M. leprae preparations from tissues of leprosy-infected laboratory animals. Int J Lepr Other Mycobact Dis 62: 299-301.

48. Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256: 1604-1607.

49. Brennan PJ (2009) IDEAL: in the footsteps of IMMLEP and THELEP. Lepr Rev 80: 236-245.

50. Mahapatra S, Crick DC, McNeil MR, Brennan PJ (2008) Unique structural features of the peptidoglycan of Mycobacterium leprae. J Bacteriol 190: 655-661.

51. FDA (2011) Investigational New Drug Application; Labeling of an Investigational New Drug. 21 CFR 312.6

52. Wunsch E, Heidrich HG. (1963) Zur Quantitativen Bestimmung der Kollagenase. Zeitschrift fur Physiologische Chemie 333: 149-151.

53. Sigma Aldrich Enzymatic Assays (2008) Enzymatic assay of collagenase using N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) as the substrate. http://wwwsigmaaldrichcom/sigma/enzyme%20assay/collagenfalgpasubpdf.

54. Morales TI, Woessner JF, Jr. (1977) PZ-peptidase from chick embryos. Purification, properties, and action on collagen peptides. J Biol Chem 252: 4855-4860.

55. Milwidsky BM, Gabriel DM. (1982) Calibration curves for anionic detergents. Detergent Analysis: a Handbook of Cost-Effective Quality Control: 73.

56. Mukerjee P (1956) Use of Ionic Dyes in Analysis of Ionic Surfactants and Other Ionic Organic Compounds. Analytical Chemistry 28: 870-873.

57. Milwidsky BM, Gabriel DM (1982) Calibration curves for nonionic detergents. Detergent Analysis: A Handbook of Cost-Effective Quality Control: 67.

58. Laemmli UK, Johnson RA (1973) Maturation of the head of bacteriophage T4. II. Head-related, aberrant tau-particles. J Mol Biol 80: 601-611.

59. Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307-310.

60. Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119: 115-119.

61. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4354.

62. FDA (2011) General Biological Products Standards; Sterility. 21 CFR 610.12.

63. FDA (2011) General Biological Products Standards; General Safety. 21 CFR 610.11

64. USP (2011) Chapter <85>, Bacterial Endotoxins Test

65. Collins FM, Morrison NE, Watson SR (1983) Fernandez and Mitsuda reactivity in guinea pigs sensitized with heat-killed Mycobacterium leprae: persistence and specificity of skin reactivity to soluble and particulate antigens. Int J Lepr Other Mycobact Dis 51: 481-489.

66. Sevall JS, Prince H, Garratty G, O'Brien WA, Zack JA (1993) Rapid enzymatic analysis for human immunodeficiency virus type 1 DNA in clinical specimens. Clin Chem 39: 433-439.

67. Thole JE, Wieles B, Clark-Curtiss JE, Ottenhoff TH, Rinke de Wit TF (1995) Immunological and functional characterization of Mycobacterium leprae protein antigens: an overview. Mol Microbiol 18: 791-800.

68. Young DB, Kaufmann SH, Hermans PW, Thole JE (1992) Mycobacterial protein antigens: a compilation. Mol Microbiol 6: 133-145.

69. Pessolani MC, Brennan PJ (1992) Mycobacterium leprae produces extracellular homologs of the antigen 85 complex. Infect Immun 60: 4452-4459.

70. Rivoire B, Pessolani MC, Bozic CM, Hunter SW, Hefta SA, et al. (1994) Chemical definition, cloning, and expression of the major protein of the leprosy bacillus. Infect Immun 62: 2417-2425.

71. Mehra V, Bloom BR, Bajardi AC, Grisso CL, Sieling PA, et al. (1992) A major T cell antigen of Mycobacterium leprae is a 10-kD heat-shock cognate protein. J Exp Med 175: 275-284.

72. Pessolani MC, Smith DR, Rivoire B, McCormick J, Hefta SA, et al. (1994) Purification, characterization, gene sequence, and significance of a bacterioferritin from Mycobacterium leprae. J Exp Med 180: 319-327.

73. Hunter SW, Rivoire B, Mehra V, Bloom BR, Brennan PJ (1990) The major native proteins of the leprosy bacillus. J Biol Chem 265: 14065-14068.

74. Pessolani MC, Brennan PJ (1996) Molecular definition and identification of new proteins of Mycobacterium leprae. Infect Immun 64: 5425-5427.

75. Marques MA, Chitale S, Brennan PJ, Pessolani MC (1998) Mapping and identification of the major cell wall-associated components of Mycobacterium leprae. Infect Immun 66: 2625-2631.

76. Marques MA, Espinosa BJ, Xavier da Silveira EK, Pessolani MC, Chapeaurouge A, et al. (2004) Continued proteomic analysis of Mycobacterium leprae subcellular fractions. Proteomics 4: 2942-2953.

77. Marques MA, Neves-Ferreira AG, da Silveira EK, Valente RH, Chapeaurouge A, et al. (2008) Deciphering the proteomic profile of Mycobacterium leprae cell envelope. Proteomics 8: 2477-2491.

78. FDA (2011) Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General. 21 CFR 210

79. FDA (2011) Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211.

80. ISO (2004) Cleanrooms and associated controlled environments. 14644-7.

81. ICH (2009) Guidance for Industry: Pharmaceutical Quality System. Q10.

82. Desain C. (1993) Documentation Basics: That Support Good Documentation Practices. Aster Pub Corp.

83. FDA (2006) Quality Systems Approach to Pharmaceutical cGMP Regulations. Guidance for Industry.

84. FDA (2012) Records and Reports, Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211 Subpart J

85. FDA (2011) Investigational New Drug Application. 21 CFR 312

86. FDA (2011) General Biological Products Standards: General Provisions. 21 CFR 610, subpart B

87. Noordeen SK (1994) Elimination of leprosy as a public health problem. Int J Lepr Other Mycobact Dis 62: 278-283.

88. Anonomyous (1992) Leprosy situation in the world and multidrug therapy coverage. Weekly Epidemiological Record 67: 153-160.

89. Brennan PJ (1995) Prospects for Contributions from Basic Biological Research to Leprosy Control. Int J Lepr 63: 285-286.

90. Parkash O (2011) Serological detection of leprosy employing Mycobacterium leprae derived serine-rich 45 kDa, ESAT-6, CFP-10 and PGL-I: a compilation of data from studies in Indian populations. Lepr Rev 82: 383-388.

91. Spencer JS, Brennan PJ (2011) The role of Mycobacterium leprae phenolic glycolipid I (PGL-I) in serodiagnosis and in the pathogenesis of leprosy. Lepr Rev 82: 344-357.

92. Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, et al. (2010) Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts--a pilot study from India. BMC Infect Dis 10: 252.

93. Banerjee S, Biswas N, Kanti Das N, Sil A, Ghosh P, et al. (2011) Diagnosing leprosy: revisiting the role of the slit-skin smear with critical analysis of the applicability of polymerase chain reaction in diagnosis. Int J Dermatol 50: 1522-1527.

94. Martinez AN, Ribeiro-Alves M, Sarno EN, Moraes MO (2011) Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens. PLoS Negl Trop Dis 5: e1354.

95. Geluk A, Duthie MS, Spencer JS (2011) Postgenomic Mycobacterium leprae antigens for cellular and serological diagnosis of M. leprae exposure, infection and leprosy disease. Lepr Rev 82: 402-421.

96. Geluk A, Bobosha K, van der Ploeg-van Schip JJ, Spencer JS, Banu S, et al. (2012) New biomarkers with relevance to leprosy diagnosis applicable in areas hyperendemic for leprosy. J Immunol 188: 4782-4791.

97. Lobato J, Costa MP, Reis Ede M, Goncalves MA, Spencer JS, et al. (2011) Comparison of three immunological tests for leprosy diagnosis and detection of subclinical infection. Lepr Rev 82: 389-401.

98. Brennan PJ (2000) Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. Lepr Rev 71 Suppl: S50-54.

99. WHO IMMLEP. (1982) Testing of purified armadillo-derived M. leprae in man. Document finalized by the IMMLEP Steering Committee at its meeting, 10-12 June 1981. WHO Document TDR/IMMLEP/SC/TEST 81: 1.

100. WHO IMMLEP. (1982) Vaccination trials against leprosy: a meeting of the epidemiology subgroup of Scientific Working Group on the Immunology of Leprosy, Geneva, 11-13 February, 1985. WHO Document TDR/IMMLEP/EDP 85: 7-8.

101. Rieder HL, Chadha VK, Nagelkerke NJ, van Leth F, van der Werf MJ (2011) Guidelines for conducting tuberculin skin test surveys in high-prevalence countries. Int J Tuberc Lung Dis 15 Suppl 1: S1-25.

102. Arnadottir T, Rieder HL, Trebucq A, Waaler HT (1996) Guidelines for conducting tuberculin skin test surveys in high prevalence countries. Tuber Lung Dis 77 Suppl 1: 1-19.

103. Seibert FB. (1934) The isolation and properties of the purified protein derivative of tuberculin. Am Rev Tuberc 30: 713-720.

104. Martin-Moe S, Lim FJ, Wong RL, Sreedhara A, Sundaram J, et al. (2011) A new roadmap for biopharmaceutical drug product development: Integrating development, validation, and quality by design. J Pharm Sci 100: 3031-3043.

105. Barratt RA, Bowens SL, McCune SK, Johannessen JN, Buckman SY (2012) The critical path initiative: leveraging collaborations to enhance regulatory science. Clin Pharmacol Ther 91: 380-383.

# CHAPTER THREE: SAFETY ASSESSMENT OF TWO NEW LEPROSY SKIN TEST ANTIGENS IN HEALTHY SUBJECTS WITHOUT KNOWN EXPOSURE TO LEPROSY: RANDOMIZED DOUBLE BLIND CLINICAL STUDY

## SYNOPSIS

**Background:** A diagnostic tool for asymptomatic leprosy is needed to treat patients earlier during infection and to measure the extent of leprosy in endemic regions of the world. To address this need, two new skin test antigens, MLSA-LAM and MLCwA, were developed and manufactured for clinical testing; initially to assess safety in healthy subjects without known exposure to leprosy in endemic and non-endemic regions.

**Methods:** A phase I clinical trial was first conducted in a non-endemic region for leprosy followed by a randomized double blind phase II, stage A and B clinical trial in an endemic region for leprosy. The phase I study consisted of two groups, whereby 10 healthy non-exposed subjects received three titrated doses (2.5 µg, 1.0 µg and 0.1 µg) of MLSA-LAM (n=5) or MLCwA (n=5) and control antigens [Rees MLSA Antigen (1.0 µg) and saline]. The phase II, stage A (n=10) and stage B (n=90) studies were an expansion of the phase I study, except only the 1.0 µg (high dose) and 0.1 µg (low dose) of each antigen, and 5TU dose of Tuberculin PPD were tested. The primary outcome measure was induration induced by a delayed type hypersensitivity reaction.

**Findings:** In the phase I trial, reactogenicity was primarily against the 2.5 µg dose of both antigens and Rees control antigen, which consequently were not tested further; seven adverse events were deemed unrelated to the study products. In the phase II study, 20% of subjects (10/50) showed induration or erythema against the high dose of each antigen, and 4% (2/50)

reacted to the low dose of MLSA-LAM with minimal pain or itching; one adverse event was possibly related to MLCwA, while fourteen adverse events and two serious adverse events were deemed unrelated to the study products.

**Interpretation:** MLSA-LAM and MLCwA at both the 1.0 µg and 0.1 µg doses were found to be safe for use in humans without known exposure to leprosy. Assessment of safety and efficacy in target populations was subsequently undertaken.

**Funding:** Leprosy Research Support, Contract NO1 AI-25469; National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH)

## REGISTRATION

Phase I Clinical Trial:

Registry: none

Phase II Clinical Trial, Stages A/B:

Registry name: ClinicalTrials.gov

Registry number: NCT00128193

URL: http://clinicaltrials.gov/ct2/show/NCT00128193?term=leprosy&rank=3

## AUTHOR SUMMARY

Given the pressing need for a diagnostic tool to detect asymptomatic leprosy, two new skin test antigens, MLSA-LAM and MLCwA, were assessed for safety in healthy subjects (n=10) without known exposure to leprosy in a phase I trial performed at the leprosy non-endemic site of manufacture (Fort Collins, Colorado, USA), followed by a phase II, two-part trial, stages A (n=10) and B (n=90) performed in an endemic region for leprosy (Kathmandu, Nepal). Both

antigens and antigen doses, 1.0 µg and 0.1 µg, were considered to be safe for use in humans without known exposure to leprosy, allowing further testing for safety and efficacy in patient populations.

## INTRODUCTION

Diagnosis of subclinical leprosy remains difficult, despite being recognized as one of the missing elements in eliminating this debilitating disease. [1,2]' Early diagnosis would enable earlier treatment and prevent disabilities, reduce social stigma, and intercept transmission of disease; [3] breaking the incessant pattern of incidence exceeding prevalence. [4,5] Until there is a shift in the incidence gridlock, nearly a quarter of a million new patients will continue to be diagnosed annually with leprosy.

With a focus on the attributes of a functional early diagnostic test for leprosy in countries with limited resources, we surmised that such a test must be sensitive to an early response to infection, inexpensive, and simple to implement in large epidemiological studies. Historically, mycobacterial skin test antigens for both leprosy (Lepromin A, Rees Antigen, Convit Antigen) [6] and tuberculosis (Tuberculin PPD) [7,8] were safe when used in humans. The immensity of data on their use over 37 years for Lepromin A[9,10]; 6 years for the Rees and Convit antigens [11-14]; and 73 years for a purer and more standardized preparation of PPD in the context of Tuberculin [15] provides a solid foundation of safety for refined leprosy skin test antigens. Two partially fractionated antigens, *Mycobacterium leprae* soluble antigens (MLSA) devoid of lipoglycans, particularly lipoarabinomannan (LAM) called MLSA-LAM and *M. leprae* cell wall associated antigens called MLCwA were developed and then manufactured at Colorado State University (CSU) in Fort Collins (Chapter 2) [16], Colorado, under current good manufacturing

practices (GMP) for first-in-human clinical studies. [17,18] Commercial development of products supporting early diagnosis of leprosy, a neglected tropical disease, was not feasible.

Clinical safety assessment of investigational products, MLSA-LAM and MLCwA, was required by law under the United States Federal Food, Drug, and Cosmetic Act codified in Title 21 Code of Federal Regulations (CFR) part 312.21. [19] Data and historical information were supplied to the Food and Drug Administration (FDA) in an Investigational New Drug (IND) Application [20] supporting the premise that the product will not expose humans to unreasonable risks when used in limited early stage clinical studies. IND permission allowed the transfer of experimental products to the clinical investigator for initial testing of small numbers of healthy subjects without known exposure to leprosy, first in a nonendemic region for leprosy under a phase I clinical trial, and subsequently in an endemic region for leprosy under a phase II, stage A and then stage B clinical trial. The primary goal was to determine if the products were reasonably safe for humans and if they exhibited any pharmacological activity that justifies further development.

## METHODS

### Interventions and Control Products

Two medical interventions were tested, MLSA-LAM and MLCwA. A detailed description of each antigen can be found in chapter 2.[16] Three control products were used in these studies: 1) 0.9% sterile saline, approved for human use (Abbott Laboratories Inc., Abbott Park, IL) for use in the phase I and phase II, stage A trials, 2) Rees MLSA antigen [21], 20 x 1.0 ml vials at 1.0 µg/0.1 ml dose were received as a gift from Philip Draper and the late Joseph Colston (National Institute Medical Research, Mill Hill, UK) for use in the phase I trial, and, 3) Tubersol®, Tuberculin PPD, 5TU dose (Aventis Pasteur Inc., Swiftwater, PA) for use in the phase II stage A

and B trials. Phosphate buffered saline containing 0.0005% Tween 80 was the diluent used to manufacture both new leprosy skin test antigens. At the time of manufacturing, diluent was not vialed; therefore, 0.9% saline for use in humans was used as a control.

**Human Subjects Recruitment**

Phase I study participants were recruited from the Mycobacterial Research Laboratories, Department of Microbiology, CSU, by posting notices in the department building. Phase II study participants were recruited from the Lalitpur Nursing Campus, Sanepa, Kathmandu, following delivery of a recruitment talk by a senior member of the research team from Anandaban Hospital, Kathmandu, Nepal, using the local Nepali language or English with immediate translation to Nepali. Attendees were contacted directly and invited to participate in the study one week prior to antigen administration.

**Sample Size**

In the phase I clinical trial, the total sample size was 10 subjects divided between two antigen groups; 5 subjects received titrated doses of MLSA-LAM or MLCwA, plus control antigens. The phase II, stage A/B clinical trial was identical to the phase I clinical trial, except that the sample size was 100 subjects divided between two antigen groups; 50 subjects received titrated doses of MLSA-LAM and MLCwA, plus control antigens. Stage A was a preliminary safety screen with 10 subjects, while stage B completed the study with 90 subjects. In the Kathmandu region of Nepal, experience in clinical trials suggested that no more than 10% of subjects were expected to be lost to follow-up. Sample size consideration analysis indicated that the study would be able to meet the primary statistical objectives, should up to 10% of the subjects be lost to follow-up.

**Antigen Administration**

In the phase I clinical trial, each participant received five 100 µl intradermal injections of titrated doses (2.5 µg, 1.0 µg, and 0.1 µg) of one of the two skin test antigens, one injection of 0.9% sodium chloride, and one injection of Rees MLSA control leprosy skin test antigen at a 1.0 µg dose, between both forearms. Injections were administered on the flexor surface of both forearms, about 2 inches, 3 inches, and 6 inches below the bend of the elbow on one forearm, and about 3 inches and 6 inches below the bend of the elbow on the other forearm. The skin of the forearm was cleansed with alcohol and allowed to dry and the test dose was administered with a sterile 1 ml syringe calibrated in tenths and fitted with a sterile, one-half inch, 26 or 27 gauge needle. The point of the needed was inserted into the most superficial layers of the skin with the needle bevel pointing upward. A definitive raised bump or bleb was observed at the needle point about 10mm in diameter, which disappeared within minutes.

In the phase II, stage A/B clinical trial, each participant received four 100 µl intradermal injections of titrated doses (1.0 µg and 0.1 µg) of one of the two skin test antigens, one injection of 0.9% sterile saline (stage A only), and one injection of Tuberculin/PPD Tubersol® 5TU, between both forearms. Injections and readings were performed as described for the phase I trial, except that the test dose was administered with a sterile 1 ml Tuberculin syringe calibrated in tenths and fitted with a sterile, one-quarter inch, 30 gauge needle

**Read-Out Measurements**

Each antigen site was evaluated for reactogenicity, defined as a reaction at the site of injection that is common and reasonably expected for the intervention being studied. Specifically, the maximal diameter of induration and erythema, and presence of pain, pruritis (itching), bleeding, urticaria (hives), infection, or blistering were possible reactions based on

Tuberculin skin testing.[22,23] The method for measuring induration was adapted from "Guidelines for Conducting Skin Test Surveys in High Prevalence Countries," issued by the International Union Against Tuberculosis and Lung Disease.[22] In brief, skin test sites were palpated, the limits of the induration determined with the fingers, and the largest transverse diameter measured with calipers or a soft, flexible, transparent ruler.

Measurements were taken by one reader in the phase I clinical trial and two independent readers in the phase II clinical trial. When two readers were used, readings from both readers were analyzed for correlation (the most experienced administrator/reader was used as the 'gold standard'). In case of systematic reading errors or terminal digit preference on the part of the second reader, the reader was given more supervision or further training without explaining the reason, in order to avoid correcting one bias by potentially introducing another. Training of readers occurred prior to study initiation.

DTH responses were read at ~15 min, 48 $\pm$ 3 h and 72$\pm$ 3 h in the phase I and phase II, stage A trials and ~15 min, 72$\pm$ 3 h, and 7 $\pm$ 1 d in the phase II, stage B trial. The 15 min observation was primarily a safety measure to watch for immediate adverse events, such as anaphylaxis. If a subject was observed to have an induration greater than 10 mm at any injection site and either study visit, they were asked to return at 28 $\pm$ 3 d for a final induration measurement. Any persistent reaction was followed-up until resolved or stabilized.

The protocol was amended before starting stage B to delete the 48 h reading and to add a 7 d reading. This change was recommended by the clinical staff for two reasons: 1) during stage A, maximal induration was typically observed at 72 h, with 48 h readings being nearly equivalent, and 2) there was a concern about the prolonged time between the last reading at 72 h and day 28 d, if a problem arose.

**Institutional Review Boards**

The phase I clinical trial was locally monitored by the CSU Institutional Review Board (IRB) [24] under the U.S. Department of Health and Human Services/U.S. Public Health Service/National Institutes of Health (DHHS/PHS/NIH) assurance identification no. M-1153 and CSU IRB no. 01. The phase II clinical trial was locally monitored by both the CSU IRB and the Nepal Health Research Council (NHRC) under the Office for Human Research Protections (OHRP) single project assurance no. S-017469-02 issued at the beginning of the study, followed by a federal wide assurance no. FWA 00000647 and CSU IRB no. 00000202. A letter stating the equivalent assurance of protection for human subjects in international research was also obtained from the NHRC. IRBs provided protocol and informed consent form approval, protocol amendment approvals, safety monitoring report reviews, and annual approval to perform respective trials.

**Safety Monitoring Committee**

The Safety Monitoring Committee (SMC) [25] for the phase I study consisted of two off-site physicians. The phase II, stage A/B study SMC consisted of 4 physicians: three off-site, and one on-site who served as the independent safety monitor (ISM). The ISM reviewed all adverse events (AEs) and serious adverse events (SAEs) and reported the findings to the clinical principal investigator, who then forwarded reports to the study sponsor, Data Control Center (DCC); The EMMES Corporation, Rockville, MD), SMC, and pharmacovigilance contractor (PPD Development, Inc., Wilmington, NC). The SMC reviewed all SAE and Safety Monitoring Reports and made recommendations to the study sponsor and both IRBs, as to whether the study should proceed, be stopped or the protocol altered before proceeding.

**Documentation**

Regulatory documentation for the Phase I clinical trial included an FDA IND Application Form 1571, IND Application [20], an Investigator's Brochure [26], an FDA Statement of Investigator Form 1572, Phase I Study Protocol [27], and Informed Consent Form. [28] All documentation was submitted to our study sponsor for review and submission to the FDA. Likewise, a Study Protocol for the Phase II clinical trial (stages A, B, and C) [29] prefaced with the Principal Investigator FDA Form 1572 with associated Informed Consent Forms were submitted to our study sponsor. The current phase II protocol titled, Two New Leprosy Skin Test Antigens: MLSA-LAM and MLCwA in a Leprosy-Endemic Region; version 9.0, dated March 2, 2009 has been attached as a supplement.

**Data Capture, Analysis and Reporting**

Case report forms (CRFs) for the phase I trial consisted of an Eligibility Checklist, Demographic Information Form, Clinical Evaluation Form, Adverse Events Form, Study Termination Form, and Volunteer Symptom Diary. The Clinical Evaluation Form was used by medical staff to record antigen administration, induration and erythema measurements, side effects, and other observations. The Adverse Event Form was completed by the clinical principal investigator and covered the event, outcome, severity, seriousness, causality, and action taken for adverse events, including serious adverse events. The Volunteer Symptom Diary was used by volunteers to record the site, reaction, and severity of a response for each day of the study. Similarly, the phase II, stage A/B study CRFs included an Eligibility Checklist, Demographics and Medical History Form, Antigen Administration Form, Follow-up Examination and Reaction Form, Skin Test Reading Forms for each time point, Study Termination Form, Adverse Event Form, and Serious Adverse Event Form. In addition, a Manual of Operations (MOO) including

study personnel qualifications, roles and responsibilities; approved standard operating procedures (SOPs), CRF guidelines, and, CRF templates were developed to provide additional quality control for the phase II clinical trial.

The phase I clinical trial data was captured on CRF source documents from which Zerox copies were made for analysis by both the clinical and scientific investigators. An Adverse Events Report and a Phase I Clinical Trial Report was sent to the study sponsor and CSU IRB for review. The study sponsor submitted a final Study Report to the FDA. The phase II, stage A/B clinical trial data was captured on CRF source documents printed on two-ply-no-carbon required (NCR) paper. Data entry was verified by the study coordinator and a copy of each CRF was sent to the DCC. The DCC analyzed blinded data from stage A and submitted a Safety Monitoring Report to the study sponsor, SMC, and both IRBs. Following the completion of stage B, data from both stages A and B were unblinded and a final Safety Monitoring Report for stages A and B was submitted.

Classification of reactogenicity by grade was outlined in the Clinical Study Reactogenicity Assessment Table (**Table 3**), present in the phase II clinical protocol. Reactions were graded as mild (1), moderate (2), severe (3), or life-threatening (4). Severe reactions were recorded as adverse events, while life-threatening reactions were recorded as severe adverse events. Adverse events (AEs) were coded by the Medical Dictionary for Regulatory Activities (MedDRA®) [30] for preferred term and system organ class (SOC). In addition, AEs were tabulated by possible relationship to antigen and greatest reported intensity, and listed by subject identification code, antigen injection date, event description, MedDRA® preferred term, onset date and time, end date, maximal intensity, antigen association, action taken, outcome, resolution and brief notes.

**Table 3. Reactogenicity Classification**

| SKIN | | | | |
|---|---|---|---|---|
| Reactogenicity<br><br>Form(s) used | Grade 1<br>Reaction Form | Grade 2<br>Reaction Form | Grade 3<br>Adverse Events and Reaction Forms | Grade 4<br>SAE, Adverse Events and Reaction Forms |
| Mucocutaneous | erythema;<br>pruritus | diffuse, maculo papular rash, or dry desquamation | vesiculation or moist desquamation or ulceration<br><br>infection at injection site | exfoliative dermatitis, mucous membrane involvement or erythema, multiforme or suspected Stevens-Johnson, or necrosis requiring surgery<br><br>anaphylaxis<br><br>systemic infection |
| Induration | < 15mm | 15-30 mm | >30mm | N/A |
| Pain at Injection Site | Barely noticeable at injection site | Slightly uncomfortable at injection site` | Moderately uncomfortable at injection site | Prevents minimal activity |
| Erythema | < 15mm | 15-30 mm | >30mm | N/A |
| Edema | < 15mm | 15-30 mm | >30mm | N/A |
| Rash at Injection Site Other than Erythema | < 15mm | 15-30 mm | >30mm | N/A |
| Pruritus | slight itching at injection site | moderate itching at injection extremity | itching over entire body | N/A |

**Participants**

A total of ten participants between the ages of 18 and 40 years and with a weight greater than 100 lbs (45 Kg) for females and 140 lbs (64 Kg) for males were enrolled in the phase I clinical trial. All were U.S. citizens, healthy, and free of evidence of leprosy or tuberculosis by clinical examination. Females were certified as not being pregnant, by urine pregnancy testing within 7 days of skin test administration. All were Tuberculin skin test negative when tested 3 weeks prior to study initiation and did not have any known hypersensitivities or allergies. Females who were lactating, or any individual who was currently on oral corticosterioid treatment, had a chronic illness or immunosuppressive condition, or had extensive travel (2-3 trips/year) in a leprosy and/or tuberculosis endemic region were excluded from the study.

A total of 100 participants were enrolled in the phase II, stage A (n=10) and B (n=90) clinical trial. All were healthy Nepali residents, including expatriates from India, without any known contact with tuberculosis or leprosy patients. To assess eligibility, volunteers were asked a series of health related questions, given a general physical exam (vital signs and body weight) and standard examination for signs of leprosy, including skin lesions associated with leprosy and palpation of the greater auricular nerve, ulnar nerve at elbows, radial nerve at wrists, lateral popliteal nerve at the back of the knees, and posterial tibial nerve at the heel. [31,32]

Overall, 70% were males and 30% females, between the ages of 18 – 54 years, with the average age of 29 years. All participants had a weight greater than 30 Kg for females and 38 Kg for males. Females who were pregnant as determined by a urine pregnancy test or lactating on day 0 of the study, or individuals who were on corticosterioid or other immunosuppressive treatment, had cancer, diabetes, extra-pulmonary tuberculosis, or other chronic illness, or had a history of being treated for tuberculosis or leprosy, known hypersensitivities or allergies,

expatriates other than those from India, had participated in an earlier stage of this study, or was concurrently participating in another clinical trial were excluded from this study.

A Tuberculin test was not part of the screening process, because it was used in the clinical trial as a comparator to the medical interventions. Females were tested for pregnancy. Demographic information was collected, and BCG scar measured across the diameter (if present). A medical officer, nurse, or paramedic reviewed the screening data, demographics, and medical history forms against a checklist to determine eligibility.

**Ethics**

The phase I and phase II, stage A/B clinical trials were conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research [33] and codified in 45 CFR Part 46, [28] the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Good Clinical Practice ICH E6 regulations and guidelines, [34] and the Declaration of Helsinki. [35] The present studies included women, but excluded special minority populations, including children. Minors were excluded, because the research involved greater than minimal risk and there was no direct benefit to the participants at this stage of the study, thus not satisfying federal regulations for inclusion of children. [36]

For the phase I study, the informed consent form was written in English and approved by the study sponsor and CSU IRB prior to subject recruitment and enrollment. For the phase II, stage A/B study, informed consent forms were written in English, translated to Nepali, and back-translated to English. All versions were submitted to the study sponsor and each IRB for approval prior to subject recruitment and enrollment. .The informed consent form for the phase II, stage A/B trial, version 4.6, dated November 07, 2003 has been attached as a supplement.

Subject confidentiality was strictly held in trust by the investigators, their staff, and the study sponsor. Confidentiality was described to potential subjects during the recruitment talk, and mentioned again in the informed consent document. In summary, the subject was informed that results from these research studies may be published, but their name or identity would not be revealed. To maintain subject confidentiality, each volunteer who agreed to enter the study and signed a consent document, was assigned a study number. The number and the name of volunteers were recorded in a log, which was kept in a locked file by the study coordinator. Files will be held for a minimum of 5 years following the end of this study.

**Risks and Benefits**

Anticipated risks were the same as those encountered with other intradermal skin test antigens such as Tuberculin/PPD. [22,23] Localized areas of erythema, induration, itching, and pain were expected to occur in those responding to the antigens, but were not expected to cause significant discomfort. For strongly reactive individuals, blistering and ulceration was a possibility at the injection site. Individuals sensitive to Tween 80 were expected to exhibit additional reactions and discomfort at the injection site for approximately 24 h after administration. Medical personnel and equipment were available for those subjects who might experience adverse reactions caused by the procedures. Participation in this study did not directly benefit the volunteers; however, the information gained about the early detection of individuals infected with leprosy is hoped to benefit others with this affliction.

**Randomization and Blinding**

Phase I subjects were assigned to either the MLSA-LAM or MLCwA antigen group based on a random sequence of integers. Phase II, stage A/B subjects, were assigned an antigen and administration template based on a fixed block randomization sequence provided by the DCC.

.Phase II study antigens were concealed by antigen codes randomized for each antigen and antigen dose by the DCC. The phase II study was a double blind study; both randomization schemes were sent to the clinical study principal investigator in the event that unblinding was necessary. Antigen codes were provided in separate envelopes, such that if only one antigen required unblinding, the others were not compromised. Unblinding did not occur until stage B data were analyzed.

### Statistical Considerations

For the phase I clinical study, both antigens at each dose were not expected to elicit a DTH skin test response, therefore a sample size of 10 subjects (5 per group) was expected to be satisfactory as a preliminary safety screen in a non-endemic region for leprosy. For the phase II, stage A clinical study, both antigens and antigen doses were expected to show minimal reactions, if any, and therefore a sample size of 10 subjects was expected to uncover any major safety concerns. For the phase II, stage B clinical study, the sample size was increased by 40 subjects for each antigen, to generate statistically significant data. A power analysis was not required for these pilot scale studies.

## RESULTS/DISCUSSION

### Study Design

Phase I Trial

The phase I clinical trial was performed at Hartshorn Health Center at CSU. Eleven volunteers were recruited from the Mycobacterial Research Laboratories on campus as depicted in the Phase I Consort Flow Diagram (**Figure 8**). Ten volunteers met the inclusion criteria and were enrolled in the study; one volunteer was unable to participate. Study objectives were two-fold: 1) to determine that MLSA-LAM and MLCwA were safe for use in humans as a skin test

antigen, and 2) to determine that the range of concentrations chosen for skin testing did not elicit a reactive response in a negative control group of human subjects living in a non-endemic region for leprosy.

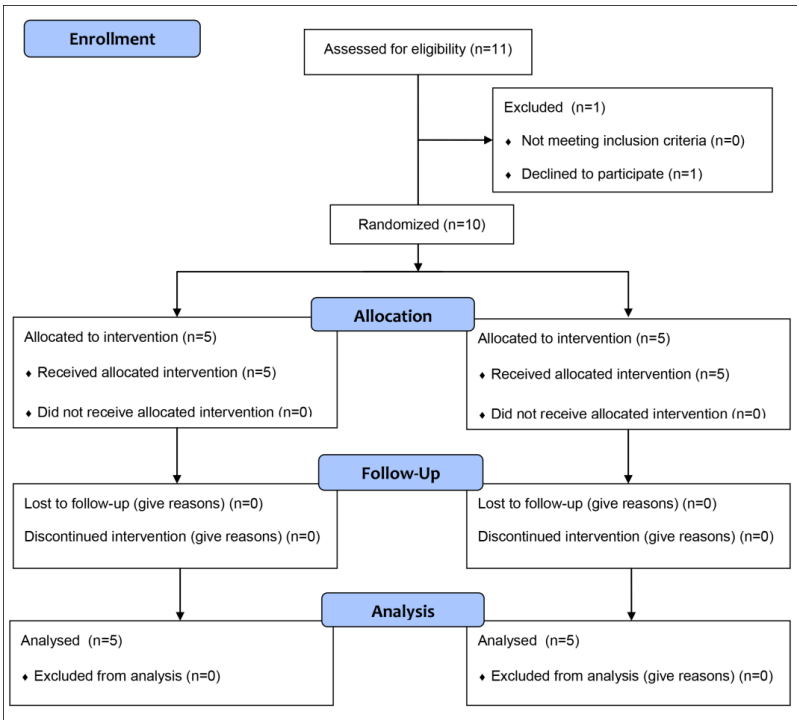



**Figure 8. Phase I Clinical Trial Consort Flow Diagram**

The expected outcome for the phase I clinical trial was that all three concentrations of the two leprosy skin test antigens, saline, and control Rees MLSA leprosy antigen would not evoke a skin test antigen response. Any untoward local reaction such as severe erythema or necrosis would result in those antigen doses being dropped from further testing.

Phase II, Stage A/B Trial

Phase II, stage A and B clinical trials were performed by staff from Anandaban Hospital. To recruit adequate numbers of healthy subjects without exposure to leprosy, the trials were performed at Lalitpur Nursing Campus, Sanepa, Kathmandu, Nepal. One hundred and one volunteers were recruited for stage A and B, as depicted in the Phase II, stage A/B Consort Flow

Diagram (**Figure 9**) and one declined participation. Phase II, stage A/B study objectives were to evaluate the safety and to select a dose of MLSA-LAM and MLCwA causing minimal induration in healthy subjects without known exposure to clinical leprosy or tuberculosis, living in a region endemic for leprosy.

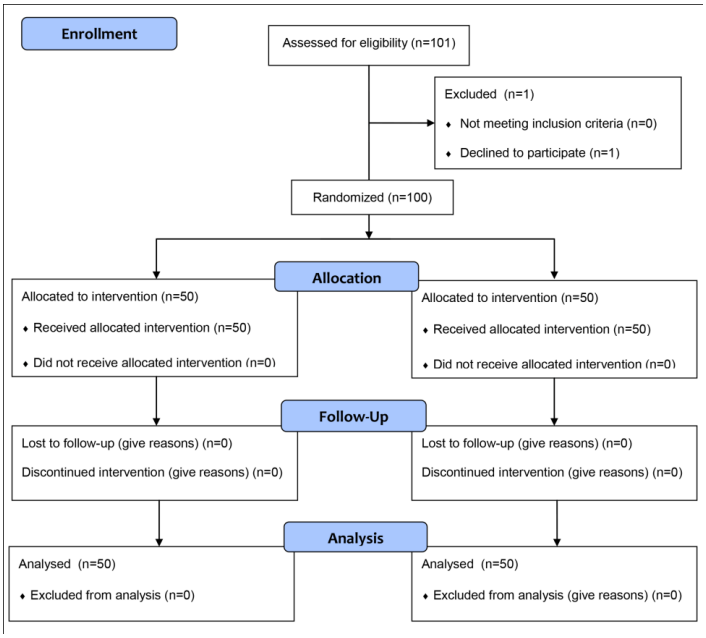



**Figure 9. Phase II, Stage A/B Consort Flow Diagram**

The expected outcome for the phase II, stage A/B clinical trial was that subjects would have a small (less than 10mm) induration reaction to the leprosy skin test antigens, due to cross-reactivity with M. tuberculosis, BCG vaccination, and/or environmental mycobacteria [37-39] Known environmental mycobacteria present in soil and water include: M. avium-intracellulare, M. kansasii, M. xenopi, M. ulcerans, M. marinum, M. malmoense, M. fortuitum, and M. chelonei. Since tuberculosis is prevalent in Nepal and many individuals were vaccinated with BCG, most subjects were expected to respond to Tuberculin PPD; hence, Tuberculin PPD testing was a measure of not only exposure to tuberculosis, but also BCG vaccination, and non-pathogenic environmental mycobacteria, to some extent.

**Study Outcome**

Phase I Trial

Safety was analyzed by reactogenicity, i.e., the proportion of study participants with erythema, itching, pain/tenderness, bleeding, urticaria, infection and blistering for each time point and antigen concentration; and frequency, severity, and relationship of adverse events to the products under investigation. Reactogenicity observations were tabulated in **Table 4** by maximum number of events across all readings by antigen, except the 15 minute reading post injection, which was merely the raised bleb on the skin from product administration. The 48 and 72 hour induration measurements were very similar; hence, since the 48 hour reading was dropped from the phase II, stage B study, only the 72 hour values have been provided as supplementary data in **Appendix 2**.

**Table 4. Phase I – Maximum Reactogenicity**

| **Reaction** | **No. Subjects with Reactions Across All Study Visits** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **MLSA-LAM** | | | **MLCwA** | | | **Rees** | **Saline** |
| | **2.5 µg** | **1.0 µg** | **0.1 µg** | **2.5 µg** | **1.0 µg** | **0.1 µg** | **1.0 µg** | **N/A** |
| Induration | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| Erythema | 5 | 3 | 0 | 3 | 2 | 2 | 8 | 1 |
| Pruritis (itching) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total No. Events** | **6** | **3** | **0** | **4** | **2** | **2** | **9** | **1** |
| **Total No. that were AE** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |

Of the ten participants tested with titrated doses of MLSA-LAM or MLCwA, only one subject elicited a DTH skin test response of induration against the 2.5 µg dose of MLCwA at 48 h and 72 h readings. This particular individual worked with Mycobacterium species on a daily basis and may have been exposed to mycobacterial antigens. This is further justified by the fact

that this individual was the only one of ten subjects to also elicit a positive induration reaction to the Rees control antigen. Adverse events from the phase I study are listed by the MedDRA® SOC in **Table 5**. A total of 7 adverse events were recorded, all of which were deemed not related to the investigational products.

Both skin test antigens were found to be safe at all doses tested, but the 2.5ug dose of both MLSA-LAM and MLCwA were responsible for 100% (1//1) of induration events, 53% (8/15) of erythema events and 100% (1/1) of itching events noted with investigative products. Although these events were expected skin test reactions, they were not expected in healthy controls; therefore, as a precaution, the 2.5 µg dose was dropped from further testing. It should be noted that the Rees antigen at the 1.0 µg dose produced 8 erythema events; yet historically has been shown to be safe for use in humans across multiple vaccine and skin test clinical trials. [40] A final study report was submitted to the study sponsor and CSU IRB with recommendations to test the new leprosy skin test antigens in an endemic region for leprosy at 1.0 µg and 0.1 µg doses. Following FDA review of the study report, the next stage was initiated.

**Table 5. Phase I – Adverse Events Classified by MedDRA®**

| MedDRA® System Organ Class | Preferred Term | Severity Grade | Number of AEs | | Relationship to Study Product |
|---|---|---|---|---|---|
| | | | MLSA-LAM | MLCwA | |
| Infections and infestations | Common cold | 2 | 1 | 1 | Probably not |
| Nervous system disorders | Headache | 1 | 1 | 1 | Probably not |
| Skin and subcutaneous tissue disorders | Mild Rash – both forearms | 1 | 1 | --- | Probably not |
| | Redness, itching under wristwatch | 1 | --- | 1 | Probably not |
| Vascular disorders | Tender occipital node | 2 | --- | 1 | Probably not |
| **Total No. AEs** | | | **3** | **4** | |

Phase II, Stage A/B Trial

Phase II, stage A maximum reactogenicity observations are tabulated in **Table 6**. The 72 hour induration measurements are tabulated as a supplement in **Appendix 2**. Of participants tested in the phase II, stage A study, only one subject in each group elicited a DTH skin test response of induration against the high (1.0 µg) dose of MLSA-LAM and MLCwA. Three subjects exhibited erythema against both antigens at the high dose and 1 subject exhibited erythema against the low dose of MLCwA only. Itching was observed in one subject from the low dose of MLSA-LAM. Nearly all subjects responded to Tuberculin PPD by induration and erythema, with some exhibiting pain, urticaria, and one subject exhibiting blistering at the skin test site.

**Table 6. Phase II, Stage A - Maximum Reactogenicity**

| **Reaction** | **No. Subjects with Reactions Across All Study Visits** | | | | | |
|---|---|---|---|---|---|---|
| | **MLSA-LAM** | | **MLCwA** | | **Tuberculin** | **Saline** |
| | **1.0 µg** | **0.1 µg** | **1.0 µg** | **0.1 µg** | **5TU** | **N/A** |
| Induration | 1 | 0 | 1 | 0 | 8 (3 AE) | 0 |
| Erythema | 3 | 0 | 3 | 1 | 9 | 0 |
| Pruritis (itching) | 1 | 0 | 1 | 0 | 5 | 0 |
| Pain | 0 | 1 | 0 | 0 | 4 | 1 |
| Bleeding | 0 | 0 | 0 | 0 | 0 | 0 |
| Urticaria (hives) | 0 | 0 | 0 | 0 | 2 | 0 |
| Infection | 0 | 0 | 0 | 0 | 0 | 0 |
| Blistering | 0 | 0 | 0 | 0 | 1 | 0 |
| Other | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total No. Events** | **5** | **1** | **5** | **0** | **29** | **1** |
| **Total No. AE** | **0** | **0** | **0** | **0** | **3** | **0** |

Potential adverse events are listed by the MedDRA® SOC in **Table 7**. Three adverse events were related to the Tuberculin PPD antigen. A safety report was created by the DCC maintaining

the study blind for submission to the SMC, study sponsor, FDA, and both IRBs. Both antigens and both the high and low doses were found to be safe for use in humans and phase II, stage B clinical trial was allowed to proceed.

**Table 7. Phase II, Stage A/B - Adverse Events Classified by MedDRA®**

| Stage | MedDRA® System Organ Class | Preferred Term | Severity Grade | Number of AEs | | | Relation-ship to Study Product |
|---|---|---|---|---|---|---|---|
| | | | | MLSA-LAM | MLCwA | PPD | |
| A | Skin and subcutaneous tissue disorders | Induration > 30 mm | 3 | --- | --- | 3 | Probably |
| | | | | | | | |
| B | Skin and subcutaneous tissue disorders | Induration > 30 mm | 3 | --- | --- | 1 | Probably |
| | | Erythema > 30 mm | 3 | --- | --- | 5 | Probably |
| B | Infections and infestations | Herpes zoster | 2 | --- | 1 | --- | Probably not |
| | | Appendicitis Hospitalization | 4 | 1 | --- | --- | Probably not |
| B | Injury, poisoning and procedural complications | Blister | 1 | --- | 1 | --- | Probably not |
| B | Nervous system disorders | Syncope vasovagal | 1 | 1 | --- | --- | Probably not |
| | | Death, Cerebral Hemorrhage | 5 | 1 | --- | --- | Probably not |
| B | Vascular disorders | Lymphangitis | 1 | --- | 1 | --- | Possibly |
| **Total No. of AE** | | | | 3 | 3 | 9 | |

The phase II, stage B maximum reactogenicity observations are tabulated in **Table 8**. The 72 hour induration measurements are tabulated as a supplement in **Appendix 2**. Of the ninety

participants tested in the phase II, stage B study, 10 and 8, respectively, elicited induration and 10 and 11, respectively, showed erythema for the high dose of MLSA-LAM and

**Table 8. Phase II, Stage B - Maximum Reactogenicity**

| Reaction | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | MLSA-LAM | | MLCwA | | Tuberculin | Saline |
| | 1.0 µg | 0.1 µg | 1.0 µg | 0.1 µg | 5TU | N/A |
| Induration | 10 | 2 | 8 | **0** | 67 (1 AE) | 0 |
| Erythema | 10 | 3 | 11 | 1 | 67 (5 AE) | 0 |
| Pruritis (itching) | 0 | 0 | 1 | 0 | 34 | 0 |
| Pain | 3 | 1 | 2 | 0 | 25 | 2 |
| Bleeding | 0 | 0 | 0 | 0 | 0 | 0 |
| Urticaria (hives) | 0 | 0 | 0 | 0 | 1 | 0 |
| Infection | 0 | 0 | 0 | 0 | 0 | 0 |
| Blistering | 0 | 0 | 0 | 0 | 5 | 0 |
| **Total No. Events** | **23** | **6** | **22** | **1** | **199** | **2** |
| **Total No. AE** | **0** | **0** | **0** | **0** | **6** | **0** |

MLCwA. Only 2 subjects showed induration at the high dose of both antigens and 3 and 1 subjects respectively showed erythema at the low dose of both antigens. Itching was only observed in one subject at the high dose of MLCwA and pain was observed in 3 and 2 or 1 and 0 subjects in the high and low dose of MLSA-LAM and MLCwA, respectively. Again, significant reactions were observed with the Tuberculin PPD control, of which 6 were recorded as AEs. At the end of this stage of the study, the clinical team requested that a lower dose of Tuberculin PPD control antigen be used in stage C to save the participants from unnecessary pain and suffering. Phase II, stage A/B adverse events, including serious adverse events, are listed by the MedDRA® SOC in Table 7. All adverse events resulting from reactogenicity were related to the Tuberculin control antigen and not the study products. Only one mild adverse event recorded as

lymphangitis was listed as possibly being related to the study product. The subject who experienced this adverse event was administered MLCwA and Tuberculin PPD. With the strong reactions seen with Tuberculin, it is possible that this event could be related to either or both the study product or control antigen.

Two SAEs were observed and procedures for treating, monitoring, recording, and notification of regulatory officials were followed. Although the appendicitis hospitalization was after the 28 day study period, the subject's medical condition was still monitored by the clinical staff.The subject had no past medical, surgical, and allergy histories and was not on any concomitant medications prior to this event. He underwent an appendectomy on day 34, with surgical findings consistent with uncomplicated appendicitis. The event was considered resolved without sequelae on day 38. The clinical investigator and medical monitor assessed the event as not associated to the study product. The second SAE involved a death due to cerebral hemorrhaging possibly secondary to an A-V anerurymsm. The subject was hospitalized two days prior with seizures, headache, blurred vision and chest pain and a CT scan confirmed the diagnosis of intracranial bleeding secondary to an arterio-venous aneurysm. This individual was on concomitant medications. The subject's condition deteriorated and he died on day 25 of the study. Both the investigator and medical monitor assessed the event as serious and not associated with the study drug.

Data from both stage A and B were unblinded and a safety report was created by the DCC for submission to the SMC, study sponsor, FDA, and both IRBs. Results showed that Tuberculin PPD, as opposed to one of the study products was responsible for the high degree of reactogenicity and adverse events. Both study antigens and doses were found to be safe for use in humans without known exposure to leprosy.

Baseline Data

A baseline can be derived for each antigen at each antigen dose based on phase II, stage A/B data from healthy controls in an endemic region without known exposure to leprosy. Induration measurements (mm) are graphed for each subject; **Figure 10** depicts data for MLCwA (low dose and high dose), while **Figure 11** depicts data for MLSA-LAM (low dose and high dose). Tuberculin results are graphed in **Figure 12** as a comparator. MLCwA low dose did not elicit an induration response in any of the 50 subjects, whereas the high dose caused induration in 8/50 subjects, with one falling below 5 mm. MLSA-LAM low dose elicited an induration response in 2/50 subjects, with one subject below 5 mm, whereas the high dose caused an induration response in 10/50 subjects, with two subjects below 5 mm. Tuberculin PPD elicited a reaction in 67/100 subjects, with 33 subjects below 5 mm. Both leprosy skin test antigens are showing potency at the high dose and a level of specificity at the low dose, when compared to Tuberculin PPD.

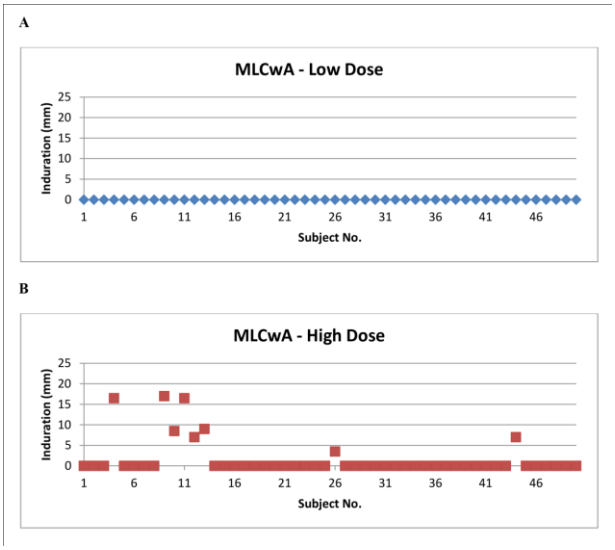



**Figure10. Phase II, Stage A/B - MLCwA Induration by Subject**
Phase II, stage A/B graph depicting DTH induration elicited by MLCwA. A) The low dose antigen did not elicit an induration response in any of the 50 subjects. .B) The high dose antigen caused an induration response in 8/50 subjects, with one falling below 5 mm.

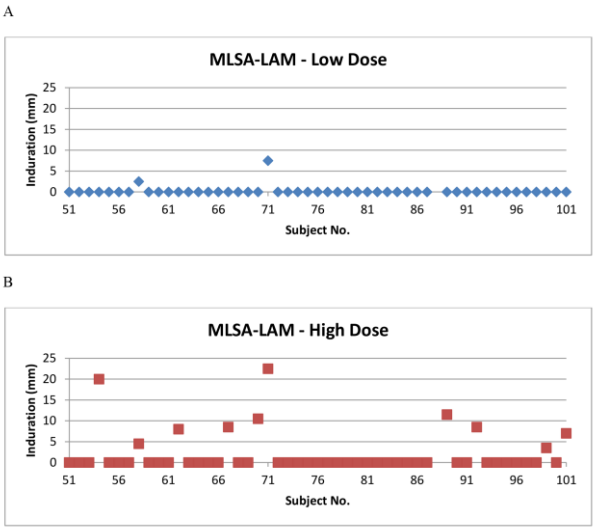



**Figure 11. Phase II, Stage A/B - MLSA-LAM Induration by Subject**
Phase II, stage A/B graph depicting DTH induration elicited by MLSA-LAM. A) The low dose antigen elicited an induration response in 2/50 subjects, with one subject below 5 mm. B) The high dose antigen elicited an induration response in 10/50 subjects, with two subjects below 5 mm.

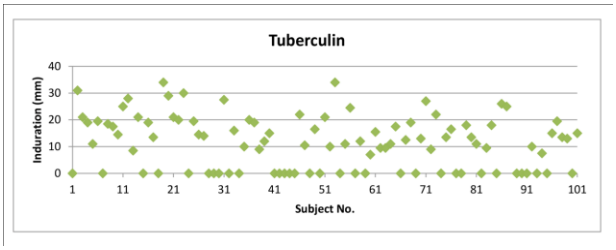



**Figure 12. Phase II, Stage A/B - Tuberculin**
Phase II, stage A/B graph depicting DTH induration elicited against Tuberculin PPD by subject number.

The frequency of distribution for induration is graphed for both antigens and antigen doses, and Tuberculin PPD as a comparator in **Figure 13** to discern a cut off value based on biomodal distributions. Results suggest that a cut off value for MLCwA low dose would be any reaction greater than 0 mm with 100% confidence; MLCwA high dose would be 5 mm with 86% confidence; MLSA-LAM low dose would be 5 mm with 98% confidence; and, MLSA-LAM high dose would be 5mm with 84% confidence. By comparison, Tuberculin PPD at a 5 TU dose

would be 5 mm with 34% confidence or 10 mm with 37% confidence. These results require further analysis following collection of data from testing leprosy and tuberculosis patients.

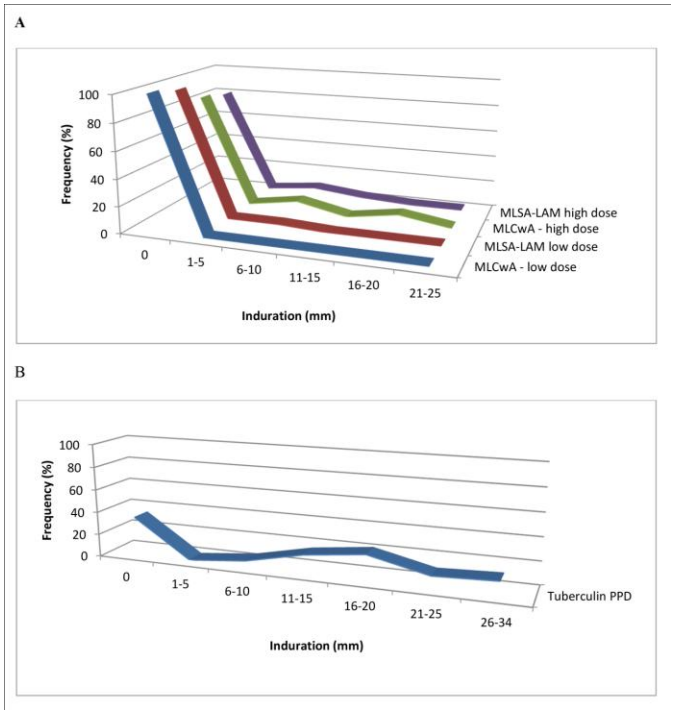



**Figure 13. Phase II, Stage A/B - Distribution Frequency of Induration**
The distribution curve: A) comparing frequency of induration between antigen preparations and dosage suggests that the cut off value for MLCwA and MLSA-LAM low dose is 0 mm with 100% of subjects (50/50) and 96% of subjects (48/50) falling at or below the baseline, respectively. The high dose of each antigen have an estimated cut off value of 5 mm with 86% of subjects (43/50), and 84% of subjects (42/50) falling below the baseline, respectively. B) Distribution frequency of induration response against Tuberculin PPD.

## Conclusion

The phase I clinical trial commenced in December 1998 and was completed in February 1999. It was the first-in-human study with MLSA-LAM and MLCwA at three concentrations. Both antigens at each dose were found to be safe, but the highest dosage caused reactogenicity and was therefore not tested in the phase II clinical trial. The Rees MLSA leprosy control antigen was a good comparator, as shown when the only individual who responded by induration was the same individual who responded to the 2.5 µg dose of MLCwA. Even so, this antigen was not

commercially available or registered under a U.S. IND, therefore it was not allowed to be used as a control antigen in the phase II clinical study. Tuberculin PPD, the skin test antigen for tuberculosis, was therefore used as a control for the phase II clinical trials.

The phase II, stage A clinical trial commenced in April 2002 and was completed in July 2002, and the stage B trial commenced in May 2003 and was completed in January 2004. These trials reflect the first testing of MLSA-LAM and MLCwA in an endemic region for leprosy. As summarized in Figure 13, thirty individuals did not respond to either intervention or Tuberculin PPD, three responded to one or the other interventions, but not Tuberculin PPD, 52 responded to Tuberculin PPD only, and 15 responded to both the intervention and Tuberculin PPD. These results suggest that the leprosy antigens may provide specificity, which was missing from the Rees MLSA or Convit antigens.

All the objectives of the phase I and phase II, stage A/B clinical trials were met. The antigens have been shown to be safe for use in healthy individuals, without known exposure to leprosy. In addition these studies show that the new leprosy skin test antigens are immunologically potent in humans and may provide a level of specificity in subsequent studies to establish safety and efficacy in target populations.

# REFERENCES


1. Nsagha DS, Bamgboye EA, Assob JC, Njunda AL, Kamga HL, et al. (2011) Elimination of leprosy as a public health problem by 2000 AD: an epidemiological perspective. Pan Afr Med J 9: 4.

2. Pinheiro RO, de Souza Salles J, Sarno EN, Sampaio EP (2011) Mycobacterium leprae-host-cell interactions and genetic determinants in leprosy: an overview. Future Microbiol 6: 217-230.

3. Rodrigues LC, Lockwood DNJ (2011) Leprosy now: epidemiology, progress, challenges, and research gaps. The Lancet Infectious Diseases 11: 464-470.

4. Anonomyous (2011) Leprosy update. Weekly Epidemiological Record 86: 389-400.

5. Dockrell HM, Geluk A, Brennan P, Saunderson PR, Oskam L, et al. (2011) Report on the sixth meeting of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium held in Beijing, China on 23-25 August 2010. Lepr Rev 82: 80-85.

6. Brennan PJ (2000) Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. Lepr Rev 71 Suppl: S50-54.

7. Snider DE, Jr. (1982) The tuberculin skin test. Am Rev Respir Dis 125: 108-118.

8. Huebner RE, Schein MF, Bass JB, Jr. (1993) The tuberculin skin test. Clin Infect Dis 17: 968-975.

9. Meyers WM, Kvernes S, Binford CH (1975) Comparison of reactions to human and armadillo lepromins in leprosy. Int J Lepr Other Mycobact Dis 43: 218-225.

10. Millar JW, Gannon C, Chan CS (1975) Comparison in leprosy patients of Fernandex and Mitsuda reactions using human and armadillo antigens. A double-blind study. Int J Lepr Other Mycobact Dis 43: 226-233.

11. Gupte MD, Anantharaman DS, Nagaraju B, Kannan S, Vallishayee RS (1990) Experiences with Mycobacterium leprae soluble antigens in a leprosy endemic population. Lepr Rev 61: 132-144.

12. Gupte MD, Anantharaman DS (1988) Use of soluble antigens in leprosy epidemiology. Lepr Rev 59: 329-335.

13. Convit J, Sampson C, Zuniga M, Smith PG, Plata J, et al. (1992) Immunoprophylactic trial with combined Mycobacterium leprae/BCG vaccine against leprosy: preliminary results. Lancet 339: 446-450.

14. Samuel NM, Stanford JL, Rees RJ, Fairbairn T, Adiga RB (1984) Human vaccination studies in normal and contacts of leprosy patients. Indian J Lepr 56: 36-47.

15. Seibert FB. GJ (1941) Tuberculin purified protein derivative: prepartion and analyses of a large quantity for standard. Am Rev Tuberc 44: 9-24.

16. Rivoire BL, Terlow S, Brennan PJ (Submitted) The Challenge of Producing Skin Test Antigens with Minimal Resources Suitable for Human Application against a Neglected Tropical Disease; Leprosy. PLoS Negl Trop Dis.

17. FDA (2011) Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs; General. 21 CFR 210

18. FDA (2011) Current Good Manufacturing Practice for Finished Pharmaceuticals. 21 CFR 211.

19. FDA (2011) Investigational New Drug Application: Phases of an Investigation. 21 CFR 312.21.

20. FDA (2011) Investigational New Drug Application. 21 CFR 312

21. Smelt AH, Rees RJ, Liew FY (1981) Induction of delayed-type hypersensitivity to Mycobacterium leprae in healthy individuals. Clin Exp Immunol 44: 501-506.

22. Arnadottir T, Rieder HL, Trebucq A, Waaler HT (1996) Guidelines for conducting tuberculin skin test surveys in high prevalence countries. Tuber Lung Dis 77 Suppl 1: 1-19.

23. Sanofi Pasteur Limited Toronto Ontario Canada (2009) Tubersol, Tuberculin Purified Protein Derivative (Mantoux) Package Insert R19-0905 Canada.

24. FDA (2011) Institutional Review Boards. 21 CFR 56

25. FDA (2011) Investigational New Drug Application; Responsibilities of Sponsors and Investigators. 21 CFR 312 Subpart D.

26. FDA (2011) Investigational New Drug Application; IND Content and Format. 21 CFR 312.23.

27. FDA (2011) Investigational New Drug Application; Protocol Amendments. 21 CFR 312.30

28. FDA (2011) Protection of Human Subjects. 21 CFR 56

29. NIH NIAID (2008) Clinical Trial Protocol Template. http://wwwniaidnihgov/labsandresources/resources/toolkit/protocol/Pages/protocolaspx.

30. ICH (2012) MeDRA Term Selection. Technical Requirements for Registration of Pharmaceuticals for Human Use. Points to Consider 4.3

31. Britton WJ, Lockwood DN (2004) Leprosy. Lancet 363: 1209-1219.

32. (2006) Global strategy for further reducing the leprosy burden and sustaining leprosy control activities 2006-2010. Operational guidelines. Lepr Rev 77: IX, X, 1-50.

33. (1979) Protection of human subjects; Belmont Report: notice of report for public comment. Fed Regist 44: 23191-23197.

34. ICH (1996) Guideline for Good Clinical Practice. Points to Consider E6(R1).

35. (2008) Ethical Principles for Medical Research Involving Human Subjects; Declaration of Helsinki. World Medical Association.

36. FDA (1998) Protection of Human Subjects; NIH Policy and guidelines on the inclusion of children as participants in research involving human subjects. 45 CFR 46 subpart 401-409

37. Holland SM (2001) Nontuberculous mycobacteria. Am J Med Sci 321: 49-55.

38. Parashar D, Das R, Chauhan DS, Sharma VD, Lavania M, et al. (2009) Identification of environmental mycobacteria isolated from Agra, north India by conventional & molecular approaches. Indian J Med Res 129: 424-431.

39. Fine PE, Floyd S, Stanford JL, Nkhosa P, Kasunga A, et al. (2001) Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. Epidemiol Infect 126: 379-387.

40. WHO IMMLEP. (1982) Vaccination trials against leprosy: a meeting of the epidemiology subgroup of Scientific Working Group on the Immunology of Leprosy, Geneva, 11-13 February, 1985. WHO Document TDR/IMMLEP/EDP 85: 7-8.

# CHAPTER FOUR: SAFETY AND EFFICACY ASSESSMENT OF TWO NEW LEPROSY SKIN TEST ANTIGENS: RANDOMIZED DOUBLE BLIND CLINICAL STUDIES

## SYNOPSIS

**Background:** The extent of true leprosy infection will not be revealed until a sensitive and specific test is identified and developed for field use. Detection of leprosy prior to the onset of clinical symptoms would prevent disabilities by earlier implementation of chemotherapy and reduce the number of new cases by intercepting transmission Continuation of the phase II clinical trial of the leprosy skin test antigens MLSA-LAM and MLCwA was implemented to assess both the safety and efficacy in target populations.

**Methods:** A randomized double blind phase II, stage C-1 clinical trial was conducted in Kathmandu, Nepal, following a phase II, stage A and B safety study in healthy subjects without known exposure to leprosy. Stage C-1 was divided into two parts to test the high dose (1.0µg) and low dose (0.1 µg) of each leprosy skin test antigen; Tuberculin PPD (2TU) served as a control. Each study enrolled 80 participants, including 20 BL/LL leprosy patients, 20 BT/TT leprosy patients, 20 BL/LL leprosy patient household contacts, and 20 tuberculosis patients. A whole blood interferon gamma release assay (IGRA) and phenolic glycolipid-I (PGL) antibody assay were directly compared to the skin test. The primary outcome measure for the skin test was delayed type hypersensitivity induration, the IGRA test was IFN-γ concentration, and the PGL-I test was color intensity. Gold standard

**Findings:** Diagnostic test performance of MLSA-LAM and MLCwA at the low dose exhibited high specificity at 100% and 95%, but low sensitivity at 20% and 25% in tuberculoid leprosy patients compared to tuberculosis patients. The positive predictive value was 100% and

83%, while the negative predictive value was 81% and 82%, respectively. The high dose of both antigens showed lower specificity (70% and 60%) and sensitivity (10% and 15%). Lepromatous patients were completely anergic to the leprosy antigens, with one exception. In contrast, the IGRA showed the low dose of both antigens to be moderately sensitive at 83% and 72% with a specificity of 53% at a cut off value of 1.3 and 1.2 IU/mL. Surprisingly, the high dose of both antigens displayed 95% specificity at a cut off value of 0.14 and 0.22, respectively. The PGL antibody assay exhibited moderate specificity and sensitivity (77% and 80%) in BL/LL leprosy patients.

**Interpretation:** In small scale sample sizes, MLSA-LAM and MLCwA at 1.0 µg and 0.1 µg dosages were found to be safe for human use in target populations. Efficacy of both antigens in terms of sensitivity was poor in the skin test and IGRA; however, specificity was high for tuberculoid leprosy patients. These native antigens represent a step forward in the critical search for an early diagnostic tool for leprosy.

**Funding:** Leprosy Research Support, Contract NO1 AI-25469; National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH)

**REGISTRATION:**

Phase I Clinical Trial: Not registered

Phase II Clinical Trial, Stages A/B:

Registry name: ClinicalTrials.gov

Registry number: NCT00128193

URL: http://clinicaltrials.gov/ct2/show/NCT00128193?term=leprosy&rank=3

## AUTHOR SUMMARY

The aim of this work was to assess the safety and efficacy of two new leprosy antigens, MLSA-LAM and MLCwA, as diagnostic skin tests for the detection of asymptomatic leprosy. The resulting test must be sensitive enough to detect infection prior to manifestation of clinical symptoms and specific enough to differentiate between leprosy, tuberculosis, vaccination with BCG, and exposure to environmental mycobacterium. Phase I and phase II, stage A/B clinical trials showed that these two new skin test antigens were safe for use in humans in a nonendemic and endemic area for leprosy. The phase II, stage C clinical trial followed to provide preliminary data on safety and efficacy of these two leprosy antigens at high and low doses in small groups (n = 20) of leprosy patients and their contacts and tuberculosis patients.

## INTRODUCTION

Leprosy transmission continues today with new cases occurring in high pockets of burden against low burden background in nearly all endemic countries, further emphasizing the importance of early detection. [1] Without a prophylactic vaccine better than the incomplete protection provided by the BCG vaccine for tuberculosis, [2] a tool for detecting asymptomatic leprosy is paramount. A sensitive and specific test would facilitate early detection allowing earlier treatment to interrupt disease pathogenesis; thus, a reduction of patient disabilities, reservoir of infection, and level of transmission. [3] Likewise, understanding the true incidence of leprosy may reveal underlying factors related to disease persistence; shifting public health resources back to this neglected tropical disease for full elimination. [4]

To address detection of subclinical leprosy, the cell mediated immune response (CMI) against *Mycobacterium leprae* was targeted for measurement by a delayed type hypersensitivity (DTH) type IV response from intradermal skin testing. Two new leprosy skin test antigens,

MLSA-LAM (*M. leprae* soluble antigens devoid of lipoglycans, primarily lipoarabinomannan; LAM) and MLCwA (*M. leprae* cell wall proteins) were developed for testing in humans. (Chapter 2) [5] Phase I and phase II, stage A/B safety studies were successfully conducted on both antigens at the high (1.0 μg) and low (0.1 μg) dosages in healthy subjects without known exposure to leprosy. (Chapter 3) [6]

Continuation of the phase II clinical trial included compulsory testing for safety and efficacy of MLSA-LAM and MLCwA in target populations: borderline tuberculoid/tuberculoid (BT/TT) and borderline lepromatous/lepromatous (BL/LL) leprosy patients, household contacts of BL/LL leprosy patients (HC), and tuberculosis patients (TB). Prior to initiation, the protocol was amended: 1) to allow testing in smaller group sizes (n = 20) to assure safety before ramping and to improve the likelihood of recruiting the requisite number of subjects; 2) to decrease the number of injections for subject comfort and to aid recruitment; and, 3) to add comparative *in vitro* assays to maximize the potential of this study. Stage C was divided into stage C-1 for small scale studies and stage C-2 for ramping to achieve statistical significance. Stage C-2 was not feasible, due to cessation of dedicated funding. Stage C-1 was divided into two parts, a and b, to test the high dose and low dose of each antigen subsequently. This protocol change enabled both the reduction of sample size and number of injections per subject, while remaining within the scope of the original protocol. The phase II, stage C-1a/b clinical trial was performed at Anandaban Leprosy Hospital and Patan Hospital Tuberculosis Clinic, Kathmandu, Nepal.

## METHODS

### Skin Test Antigens and Control Products

Two skin test antigens were tested, MLSA-LAM and MLCwA. A detailed description of each antigen can be found in chapter 2. [5] Tubersol®, Tuberculin PPD, 5TU dose (Aventis

Pasteur Inc., Swiftwater, PA) served as the control antigen for preliminary testing of stage C-1a. Strong reactogenicity readings prompted unblinding and SMC review for this one product. The outcome was a recommendation to use a lower dose for the remainder of stage C-1 a/b studies; this change was not expected to significantly impact the study results. Following a protocol amendment and proper approvals, the proposed change was authorized. The lower dose was not available from the same vendor; therefore, Tuberculin PPD RT 23, 2 TU dose, solution for injection, Statens Serum Institute (SSI), Copenhagen, Denmark was used.

**Human Subjects Recruitment**

Leprosy patients and their household contacts were recruited at Anandaban Hospital, Kathmandu and tuberculosis patients were recruited at Patan Hospital, Lagankhel, Lalitpur, Kathmandu. Recruitment was informal between the study investigator and interested individuals. Explanation of the study was guided by use of a flip chart and the appropriate consent form translated into the native language, either Nepali or Hindi. In the case of illiterate subjects, information was read to them by a staff member. Recruits were encouraged to ask questions prior to signing the informed consent form.

A total of 160 participants were enrolled in the phase II, stage C-1a/b clinical trials. All were healthy Nepali residents, including expatriates from India. Details, including exclusion criteria were described in chapter 3. [6] The Kruskal-Wallis test was used to compare age by study group; age did not vary significantly across study groups for stages A, B, or C.

Leprosy patients had one of the hallmark symptoms of leprosy including: hypopigmented or erythematous skin lesion(s) with definite loss of sensation; damage to the peripheral nerves as demonstrated by palpable thickening with or without impairment of sensation and/or weakness of the muscles of hands, feet or face; presence of acid-fast bacilli in slit skin smears, histological

changes diagnostic of leprosy in a skin biopsy, receiving standard multi-drug therapy (MDT) treatment for leprosy, or completed treatment for leprosy no more than 4 years prior to study enrollment. [7,8] Household contacts were determined to be healthy by history and physical examination and have resided in the same residence as the BL/LL leprosy index case for at least 6 months duration and within 6 months of this study, or a person professionally exposed to BL/LL leprosy for at least 5 years duration, and within 6 months of this study.

Tuberculosis patients had active tuberculosis defined by a culture positive test; a productive cough of more than 3 weeks duration accompanied by night sweats, loss of appetite, haemoptysis, weight loss, chest pain, shortness of breath and sputum smear positive, or sputum smear negative with x-ray evidence consistent with pulmonary tuberculosis that does not clear with non-tuberculous antibiotics; or, culture positive for M. tuberculosis. All patients had completed the intensive phase of chemotherapy for tuberculosis, but were still undergoing the continuation phase of therapy.

**Sample Size**

A total of 160 participants were enrolled in the phase II, stage C-1clinical trial. Stage C-1a (n=80) and stage C-1b (n=80) each included 20 BT/TT leprosy patients, 20 BL/LL leprosy patients , 20 HC, and 20 TB patients. Based on prior clinical trial experience in the Kathmandu region, subject loss to follow-up was not expected to exceed 10%, which was within sample size consideration to meet study objectives.

**Antigen Administration and Read-out Measurements**

Antigen administration and read-out measurements were identical to those described for the phase II, stage B clinical trial. (Chapter 3) [6] Briefly, stage C-1a subjects received 100 µl intradermal injections of the high dose (1.0 µg) of each intervention and control antigen using a 1

ml Tuberculin syringe calibrated in tenths and fitted with a sterile, one-quarter inch, 30 gauge needle.

Each antigen site was evaluated for reactogenicity, defined as a reaction at the site of injection that is common and reasonably expected for the intervention being studied. Specifically, the maximal diameter of induration and erythema, and presence of pain, pruritis (itching), bleeding, urticaria (hives), infection, or blistering were possible reactions based on Tuberculin skin testing. [9,10] The method for measuring induration was adapted from "Guidelines for Conducting Skin Test Surveys in High Prevalence Countries," issued by the International Union Against Tuberculosis and Lung Disease.[9]

DTH responses were read at ~30 min, 72 $\pm$ 3 h, and 7 $\pm$ 1 d. The 30 min observation was changed from the 15 min observation in the phase II, stage B study to assure subjects were not adversely affected from the added blood draw for *in vitro* testing. If a subject was observed to have an induration greater than 10 mm at any injection site, they were asked to return at 28 $\pm$ 3 d for a final induration measurement. Any persistent reaction was followed-up until resolved or stabilized.

**Regulatory Boards, Documentation, and Reporting**

Details were identical to those provided for the phase II, stage A/B trial, with two exceptions. (Chapter 3) [6] One member of the Safety Monitoring Committee (SMC) could not participate and was therefore replaced. The new committee consisted of three off-site and one on-site physician who served as the independent safety monitor (ISM). Subjects were not asked to complete a Volunteer Symptom Diary, because entries did not add value when used in the phase II, stage A/B study.

**Ethics**

Compliance with regulatory requirements for the protection of human subjects enrolled in the phase II, stage C-1a/b study was described. (Chapter 3) [6] Three flip charts and three informed consent forms were written in English, translated to Nepali and Hindi, and back-translated to English. Each supported recruitment of leprosy patients; TB patients and HC; or, access to medical records of the HC index case. All versions were submitted to the study sponsor and each IRB for approval prior to subject recruitment and enrollment. Informed consent forms, version 5.0, dated December 22, 2005 and flip charts, dated April 20, 2005 have been attached as supplements.

**Risks and Benefits**

Details were identical to those provided for the phase II, stage A/B trial. (Chapter 3) [6]

**Randomization, Blinding, and Statistical Considerations**

The phase II, stage C-1a/b trial was a randomized double blind study, as described. (Chapter 3) [6]. The study was designed to assess the safety and primary response measure of induration resulting from skin test antigen DTH responses in small numbers (n=20) of participants within each of four different groups that theoretically may be at higher risk of serious adverse responses to novel antigens. A power analysis was not required for this pilot scale study. The probability of observing one or more serious adverse event related to antigen administration was calculated. If the true serious adverse event rate is 10% then there is an 85% chance of observing one or more serious adverse events in any one of the four groups with loss during follow-up of 10% of the subjects, or 88% if there is no loss during follow-up.

Efficacy analyses were performed using the receiver operating characteristic (ROC) curve. [11] A hypothesis concerning the AUC was not made, since this study was the first using these

new leprosy skin test antigens. The Kruskal-Wallis test was used to compare ages between phase II, stages A, B, and C. [12] Graph Pad, Prism for Windows, version 5.04 (La Jolla, CA) was used for graphing and analyzing ROC curves.

**Laboratory Assays**

The QuantiFERON-CMI kit (Cellestis Limited, Valentia, California) was the IGRA of choice used to quantify IFN-γ following stimulation with each of the skin test antigens and antigen dosages or PPD. Mitogen was included with the test kit as a positive control and Aims V media (Life Technologies, Grand Island, NY) was used to prepare antigen dilutions at 10x concentration and as nil control. Antigens were aliquoted and lyophilized for shipment in a one batch and single use vials. An SOP was written and approved prior to use.

Due to limited blood collected, a single test well was run for each sample. Approximately 10-12 ml blood was collected and 3 ml was aliquoted for the PGL assay, while the remaining was added to a BD heparinized tube to prevent clotting. Blood was then transferred at RT to the laboratory for aliquoting 0.5 mL/well into 48 well plates within 6-12 hours of collection and any extra blood was sterilized and disposed per request by the NHRC. Antigen, mitogen, or Aims V media were added (50 µl/well) and wells were mixed by consistent pipetting. Plates were incubated at 37 °C overnight and approximately 200 µl/well plasma was collected in a 48 well plate. The ELISA was performed per the test kit instructions: 1) conjugate was added to ELISA wells at 50µl/well, 2) Plasma or IFN-γ standards were then added to appropriate wells at 50 µl/well, 3) Plate was incubated for 120 minutes at 20-25 °C, 4) wells were aspirated and washed 6 times with diluted buffer provided with the kit, 5) Kit substrate was added at 100 µl/well and incubated for 30 min at 20-25 °C, 6) stop solution was added at 50 µl/well, and 7) plate was read at 450nm. The IGRA read out was concentration of IFN-γ extrapolated from a standard curve.

The lateral flow immunodiffusion PGL-I Rapid Antibody Test Kits were provided by Dr. Sang Nae Cho, Seoul, South Korea. An SOP was written and approved prior to use. A total of 3 ml of blood was allowed to clot and serum was removed following centrifugation. Serum was diluted 1:10 in phosphate buffered saline and apply 100 µl to the sample well of the test cassette. A control line verified the test ran properly. Results were read at 10 minutes $\pm$ 1 minute as negative, weak positive, moderate positive, or strong positive. Remaining serum was aliquoted and stored frozen until the end of the study, at which time it was proper discarded at the request of the NHRC.

## RESULTS

### Study Design

Stage C-1 was designed as a preliminary safety and efficacy study for evaluating the high dose (1.0 µg) and low dose (0.1µg) of MLSA-LAM and MLCwA in small scale sized groups of subjects who were expected to respond to the products under investigation. Although not statistically relevant with 20 subjects per group, these ramping studies were justified as being satisfactory for initial evaluation of safety and for identifying trends for efficacy. Both studies were performed by staff from Anandaban Hospital. Leprosy patients and their contacts were mostly recruited at Anandaban Hospital and tuberculosis patients from Patan Hospital. The Phase II, stage C-1a/b Consort Flow Diagram is shown in **Figure 14**. Over two subsequent studies, one-hundred sixty-one subjects (81 for stage C-1a and 80 for stage C-1b) were recruited; one household contact in stage C-1a declined participation.

Eighty subjects (20 in each of four groups: BT/TT, BL/LL, HC, and TB) for each study were randomized to receive antigens in different patterns, according to different templates. Participants, antigen administrators, and readers were blinded to the antigen (labeled in code)

and the pattern of antigen delivery. Volunteers' participation in each study lasted for 7 ± 1 d, unless induration greater than 10 mm was observed, an additional study visit occurred on day 28. Total time involvement for each participant for all visits was approximately 5 h.

The primary objective of the phase II, stage C-1 study was safety assessment of the two skin test antigens by measuring reactogenicity and adverse events. The secondary objective was efficacy assessment of the two skin test antigens by comparing induration measured at 72 ± 3 h following skin test administration. Results from leprosy patients and HC were compared to those of EC (phase II, stage A/B) and TB patients to: 1) quantify the number of positive reactors and mean induration between groups; 2) to identify an induration size that defines a positive skin test reaction in leprosy patients; and, 3) to assess sensitivity and specificity of both antigens and antigen doses.

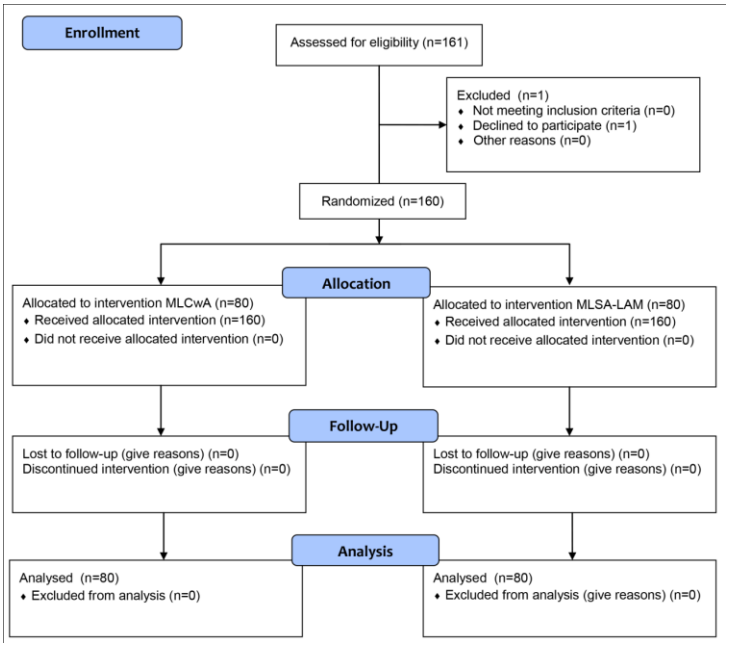



**Figure 14. Phase II, Stage C-1 a/b Consort Flow Diagram**

The expected safety outcomes were that both antigens and antigen dosages would be deemed safe for use in leprosy patients, HC, and TB patients. The expected efficacy outcomes were that

the skin test reactions would vary among the groups participating. It was expected that BT/TT leprosy patients and some healthy contacts of leprosy patients would have large indurations at *M. leprae*-derived antigen sites and a variable reaction at the Tuberculin PPD site due to prior BCG vaccination or exposure to related environmental mycobacteria, including *M. tuberculosis*. [13-15]. BL/LL leprosy patients would have negative indurations at all leprosy skin test sites due to *M. leprae* specific T-cell anergy with a variable reaction at the Tuberculin PPD site. Tuberculosis patients were expected to react with a large induration at the Tuberculin PPD site and may react with an induration less than 10 mm at the other sites.

**Study Outcome**

*Safety*

Product safety was assessed by the number and severity of reactogenic events and adverse events, as described for the phase II, stage A/B trials. (Chapter 3) [6] Reactogenic events were tabulated as the maximum number of events by subject, antigen, and antigen dose. Stage C-1a and C-1b reactogenic events are listed as supplements in **Appendix 3**; a summary of this data is provided in **Table 9**. Most reactions consisted of mild to moderate induration and erythema with only a few cases of mild pruritis and pain and one case of urticaria, infection, and blistering with MLSA-LAM at both the high and low dose. One case of bleeding was seen with MLSA-LAM at the low dose. The HC and TB groups had the highest number of reactions in the high dose study, whereas the BT/TT and HC groups had more reactions in the low dose study. The BL/LL group had the lowest number of reactions across both the high and low dose of each leprosy antigen. A total of 55 and 68 reactogenic events were observed with MLSA-LAM and MLCwA at the high dose, respectively; and, 28 and 38 reactogenic events were observed at the low dose,

respectively. No adverse events (AE) were observed based on reactogenicity at the site of injection for either intervention.

A significantly greater number of reactogenic events were observed with Tuberculin PPD over prescribed study visits. A total of 182 and 152 reactogenic events were observed in stage C-1a and C-1b, respectively. Most were mild and moderate induration and erythema; however, 14 events were classified as severe reactions and therefore recorded as mild AEs. The number of

**Table 9. Maximum Reactogenicity[a] by Subject Across all Visits (Summary)**

| Summary [b] | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | Stage C-1a | | | Stage C-1b | | |
| Reactions | MLSA-LAM | MLCwA | PPD | MLSA-LAM | MLCwA | PPD |
| | 1.0 µg | 1.0 µg | 5 TU | 0.1 µg | 0.1 µg | 5 TU |
| Induration | 16 | 22 | 61 | 6 | 12 | 55 |
| Erythema | 29 | 29 | 62 | 15 | 23 | 61 |
| Pruritis (itching) | 5 | 12 | 29 | 2 | 3 | 25 |
| Pain | 2 | 5 | 27 | 1 | 0 | 10 |
| Bleeding | 0 | 0 | 0 | 1 | 0 | 0 |
| Urticaria (hives) | 1 | 0 | 0 | 1 | 0 | 0 |
| Infection | 1 | 0 | 0 | 1 | 0 | 0 |
| Blistering | 1 | 0 | 3 | 1 | 0 | 1 |
| ***Total No. Sites*** | ***80*** | ***80*** | ***80*** | ***80*** | ***80*** | ***80*** |
| **Total No. Events** | **55** | **68** | **182** | **28** | **38** | **152** |
| **Total No. AE** | **0** | **0** | **10** | **0** | **0** | **4** |

[a] Maximum reactogenicity is the no. of subjects exhibiting specific reactions across all visits.
[b] Summary includes all target groups: BT/TT, BL/LL, HC, and TB; tabulated as supplements

reactogenic events caused from Tuberculin PPD was nearly three times the number observed for the products under investigation at the high dose and four times that observed at the low dose.

Each AE was graded and coded by the MedDRA® SOC (Chapter 2) [16]; presented as a supplement in **Appendix 3**, because none were found to be associated with the investigative

products. A total of 14 AEs were observed as a result of severe reactogenicity reactions; 10 AEs in Stage C-1a and 4

AEs in Stage C-1b. All were assigned an AE maximum severity level of mild and were found to have an antigen association with Tuberculin PPD. Two subjects in the BL/LL group and one subject in the HC group experienced blistering and received treatment with resolution, except for a dark or hypopigmented spot by day 28. One unexpected AE described as a type I hypersensitivity reaction was observed in stage C-1b in a BT/TT subject on day 12. The event was assigned an AE maximum severity level of mild and was found to probably not be related to the study treatments. The subject was given prednisolone and the event was ongoing upon termination.

*Safety Analysis*

Maximum reactogenicity measured across all study visits (day 3, 7, and 28) was greater with MLCwA compared to MLSA-LAM and the high dose compared to the low dose. Reactions were most prevalent in the TB group at the high dose of both antigens. There were no AEs observed for either study intervention. Tuberculin PPD control antigen elicited considerably more reactions of greater severity in subjects across all groups, resulting in a total of 14 AEs. Dropping the Tuberculin PPD dose from 5TU to 2TU did help decrease the number of reactions and AEs in the low dose study. Following review of unblinded data presented in the Safety Monitoring Reports, the SMC found the two new antigens at both doses to be safe for use in humans.

*Efficacy*

Stage C-1a (high dose) and C-1b (low dose) 72 $\pm$ 3h induration measurements are listed as a supplement in **Appendix 3**. A dot plot of these induration measurements across cohorts tested with MLSA-LAM and MLCwA can be found in **Figure 15**. The most dramatic and decisive

result of these analyses was the near total lack of response of the BL/LL subjects to the skin test antigens, yet a vigorous response to Tuberculin PPD. The leprosy antigens were behaving according to precedent. [17] The low number of positive responders in the BT/TT group could be related to the choice of antigen, immune status of the patient since treatment, or lack of sensitivity. Leprosy patients were allowed in the study after receiving MDT treatment up to 4 years.

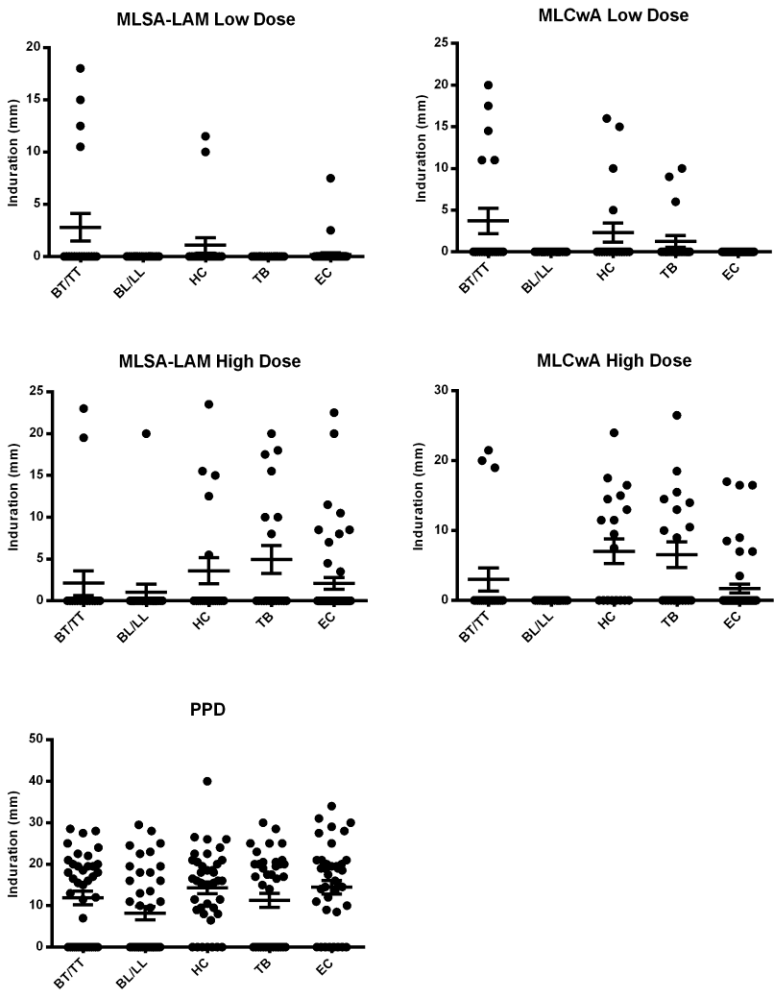



**Figure 15. Dot Plot of Induration Measurements**
Induration results are provided across five subject groups, including ECs ( *n* = 50), BT/TT leprosy patients ( *n* = 20), BL/LL leprosy patients ( *n* = 20), HC (*n* = 20), and TB (*n* = 20). EC data was presented in a parallel article describing the phase II, stage A/B safety study. Mean and standard deviation are shown. .

Proportion of Positive Reactors.

A direct comparison of the proportion of positive reactors and mean induration for each study group was compared to the EC group. Results are shown as a supplement in **Appendix 3**. Results show that both skin test antigens are immunologically active in target populations. MLSA-LAM low dose elicits a response in twice the number of BT/TT leprosy patients as HC and does not react with BL/LL leprosy or TB patients. MLCwA low dose stimulates BT/TT leprosy patients and HC nearly equally, but also some TB patients. Interestingly, this was the only antigen dose that did not elicit a response in EC, despite 67% reacting with Tuberculin PPD.

The high doses of both leprosy skin test antigens elicited a response in HC and TB patients greater than BT/TT leprosy patients. The reason is unknown, but suggests that either BT/TT leprosy patients are cured and their CMI against *M. leprae* has waned, or they have some degree of specific T-cell anergy. One BL/LL subject in the MLSA-LAM high dose group reacted with an induration of 20 mm. This subject was a 46 year old male with LL leprosy, who had been treated with MDT for a period of 1 month before enrolling in the high dose study. This participant was smear positive, had a bacterial index of 4.0, did not have a history of Type I or ENL reactions, had a single BCG scar, and, was taking concomitant hormones/steroids for the treatment of eczema.

Comparison of mean induration measurements across all subjects showed a higher response in the BT/TT leprosy patients compared to HC with both antigens at the low dose. Antigens at the high dose showed a lower response in BT/TT leprosy patients compared to HC and TB patients. The mean induration of only positive responders has been graphed in box plots in **Figure 16**. Either by antigen or antigen dosage, BT/TT leprosy patients respond with the highest induration. HC subjects react higher than TB or EC with lose dose antigens, but equal with TB

patients with high dose antigens. No difference in response across groups was seen with Tuberculin PPD.

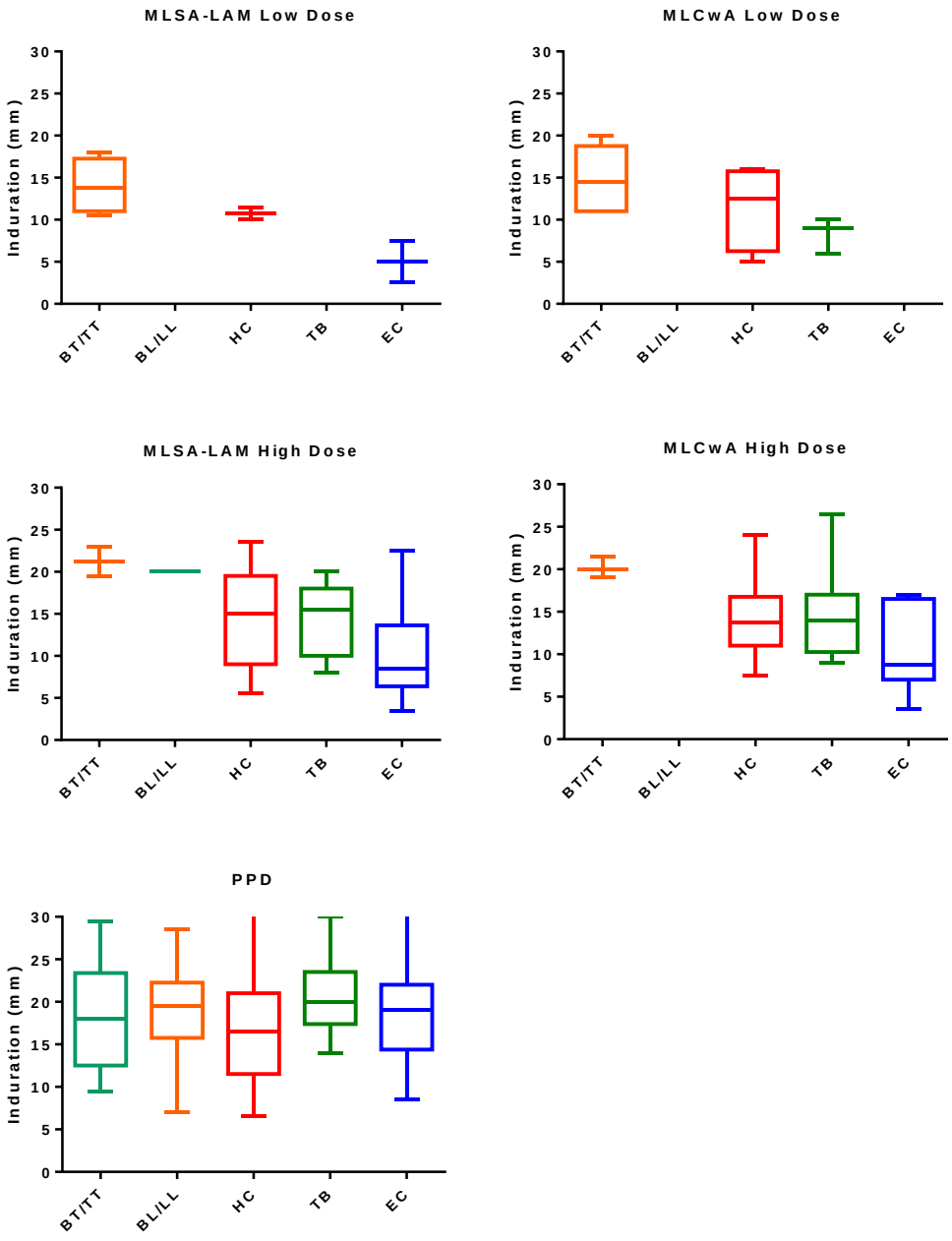



**Figure 16**. Mean Induration of Positive Responders
Mean induration of the positive responders in each subject group represented in box plot format.

With different responses being recognized between the two skin test antigens and dosages, results were compared by linear regression in **Figure 17** to look for correlations using the BT/TT group, albeit with few responders. The highest correlation was found between the two leprosy antigens at the low dose with a covariance ($r^2$) value of 0.81, followed by the high dose with a covariance of 0.67. There was not a correlation between MLSA-LAM high and low dose or either antigen at either dose against Tuberculin PPD (results shown for MLSA-LAM low dose).

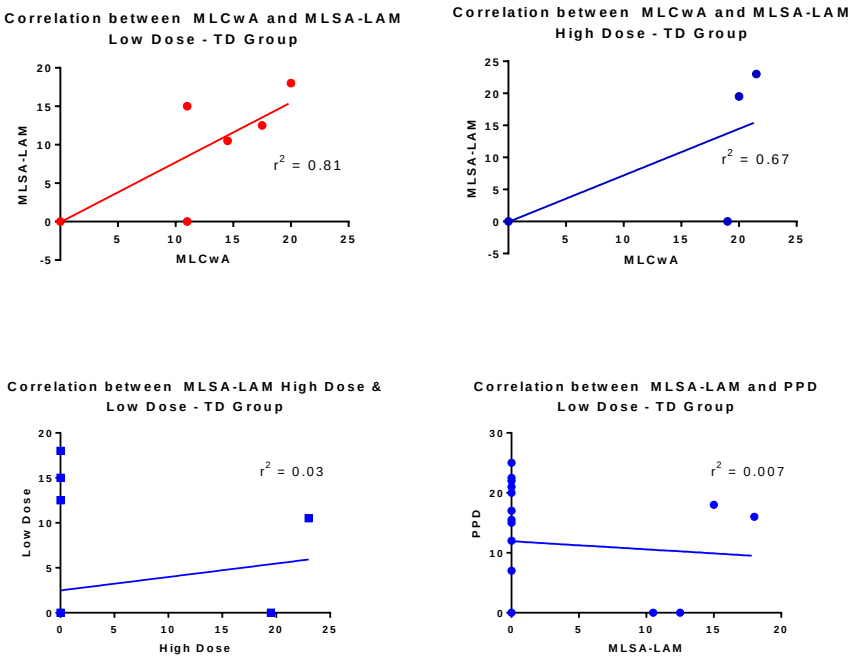



**Figure 17. Antigen and Antigen Dose Correlations**
Antigens were compared by unilateral linear regression to assess correlation. Covariation was calculated by the Pearson correlation coefficient ($r^2$) and did not improve with transformation of data. Analysis was performed on reactions from the BT/TT group with A) both antigens at the low dose, B) both antigens at the high dose, C) MLSA-LAM high dose vs low dose, and D) Tuberculin PPD at the low dose. Covariation is reported on each graph.

Definition of a Positive Skin Test.

A frequency distribution of induration size was used to compare BT/TT to EC and TB groups to identify a cutoff point, if possible. The distribution curve shown in **Figure 18** was difficult to interpret due to limited sample size and few reactors in the BT/TT groups. The EC response served as the baseline, while the TB response provided the worst case scenario with individuals infected with a related mycobacterial species. The projected cut off point is at the anti-mode, or the point at which the control groups no longer respond and the patient groups begin responding. MLSA-LAM and MLCwA low dose presented an anti-mode at 8mm and 10 mm, respectively. The curves for the high dose antigens did not present a biomodal distribution; therefore, a cutoff point could not be determined. ROC curve analysis calculated the cut off point for MLSA-LAM and MLCwA low dose to be greater than 5.2 mm and 9.5 mm, respectively. The likelihood ratios were high; however, p-values were not significant ($p = 0.28$ and 0.46, respectively) due to limited BT/TT group responses. A larger sample size is needed to properly evaluate this parameter.

Specificity and Sensitivity.

Diagnostic statistics provide a measurable assessment of the leprosy skin test antigens. [18] Four statistics provide the foundation for assessing a diagnostic test: 1) sensitivity; 2) specificity; 3) positive predictive value; and, 4) negative predictive value. Sensitivity (Se) is the likelihood to detect the presence of disease; specificity (Sp) is the likelihood to detect absence of disease; positive predictive value (PPV) is the likelihood that if a subject tests positive, they will have disease; and, negative predictive value (NPV) is the likelihood that if a subject tests negative, they will not have disease. Generally, a good diagnostic test is both sensitive and specific.[19]

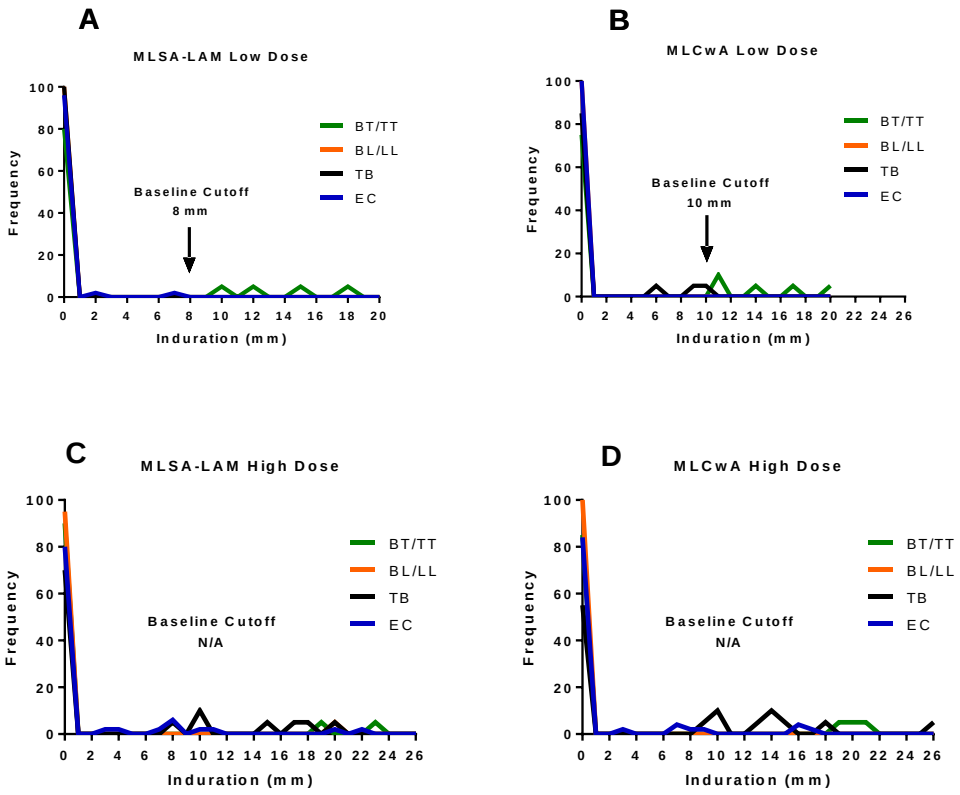



**Figure 18. Biomodal Distribution of Induration**
Frequency distribution graphs were used to establish cut off points for each skin test antigen at each dosage tested. Frequency of induration reaction (mm) of EC and TB groups were graphed against BT/TT and BL/LL leprosy groups. The anti-mode between the control and leprosy patient group represents the cut off for each antigen and antigen.

Diagnostics statistics have been calculated for the two new antigens and antigen doses in **Table 10**. Caution was taken when interpreting these values, because of the small sample sizes and limited BT/TT responders. Results showed that MLSA-LAM and MLCwA at the low dose were highly specific (100% and 95%), but lacked sensitivity (20% and 25%). Worst-case PPV was 100% and NPV was 56% with the low dose of MLSA-LAM. NPV is related to sensitivity, which must be enhanced to develop a viable diagnostic test. Tuberculin PPD as a diagnostic for tuberculosis was sensitive (90%), but not specific (41%).

**Table 10. Diagnostic Test Statistics – Skin Test Method**

| Antigen | Sensitivity (BT/TT) | Sensitivity (BL/LL) | Specificity (EC) | Specificity (TB) | PPV | NPV | NPV |
|---|---|---|---|---|---|---|---|
| | | | | | EC + TB (worst case) | EC + TB | TB (worst case) |
| MLSA-LAM Low | (4/20) 20% | (0/20) 0% | (50/50) 100% | (20/20) 100% | (4/4) 100% | (70/86) 81% | (20/36) 56% |
| MLCwA Low | (5/20) 25% | (0/20) 0% | (50/50) 100% | (19/20) 95% | (5/6) 83% | (69/84) 82% | (19/34) 56% |
| MLSA-LAM High | (2/20) 10% | (1/20) 5% | (43/50) 86% | (14/20) 70% | (2/15) 13% | (57/75) 76% | (14/32) 44% |
| MLCwA High | (3/20) 15% | (0/20) 0% | (47/50) 94% | (12/20) 60% | (3/14) 21% | (59/76) 78% | (12/29) 41% |

Diagnostic test statistics were calculated for each test method. Sensitivity (Se) is the likelihood to detect the presence of disease (TP/TP+FN). Specificity (Sp) is the likelihood to detect absence of disease (TN/TN+FP). Positive Predictive Value (PPV) is the likelihood that if a subject tests positive, they will have disease (TP/TP+FP). Negative Predictive Value (NPV) is the likelihood that if a subject tests negative, they will not have disease (TN/ TN + FN). Tuberculin PPD served as an antigen control. Statistics for detecting tuberculosis: Sensitivity is (36/40) 90%, specificity is (41/100) 41%, PPV is (36/95) 38%, and NPV is (41/45) 91%.

IGRA Results. Stage C-1a (high dose) and C-1b (low dose) IGRA data are listed as a supplement in **Appendix 3**. A dot plot of IGRA results is shown in **Figure 19**. The low dose antigens show a slightly higher background with the TB group, but overall the number of responders is higher. The mean induration of positive responders above a concentration of 0.1 IU/mL IFN-γ has been graphed in **Figure 20**. The cut off value was chosen based on the ROC Curve Analysis. The assay was not optimized for antigen concentration, because this assay was a direct comparison to the skin test antigen method, where doses were chosen based on prior knowledge with Rees MLSA skin test antigen in humans and new skin test antigen reactivity in guinea pigs.

PGL-I Antibody Assay Results. Stage C-1a (high dose) and C-1b (low dose) PGL Antibody data are listed as a supplement in **Appendix 3**. The lateral flow immunodiffusion cassettes were prepared with Neo Disaccharide O-linked Human Serum Albumin (NDO-BSA) prepared by Dr. Delphi Chatterjee, Colorado State University, Fort Collins, Colorado, USA for the detection of antibodies against this *M. leprae* specific surface antigen. The intensity of the positive reaction was visually observed and assigned a number, 1 was a weak response, 2 was a moderate response, and 3 was a strong response. Results were then graphed in a dot plot matrix in **Figure 21**. The LP group has a significant number of strong reactors, compared to multiple mild and weak responders in other groups. Stage C-1b (high dose group) had more responders in the TB group than was seen in the stage C-1a (low dose group). This suggested that some of the TB subjects were possibly co-infected with *M. leprae*.

Comparison of *In vivo* and *In vitro* Test Methods. The diagnostic performance of a particular test to differentiate individuals with disease from individuals without disease was evaluated using the ROC curve analysis method.[19] ROC plots the true positive rate (Se) against the false

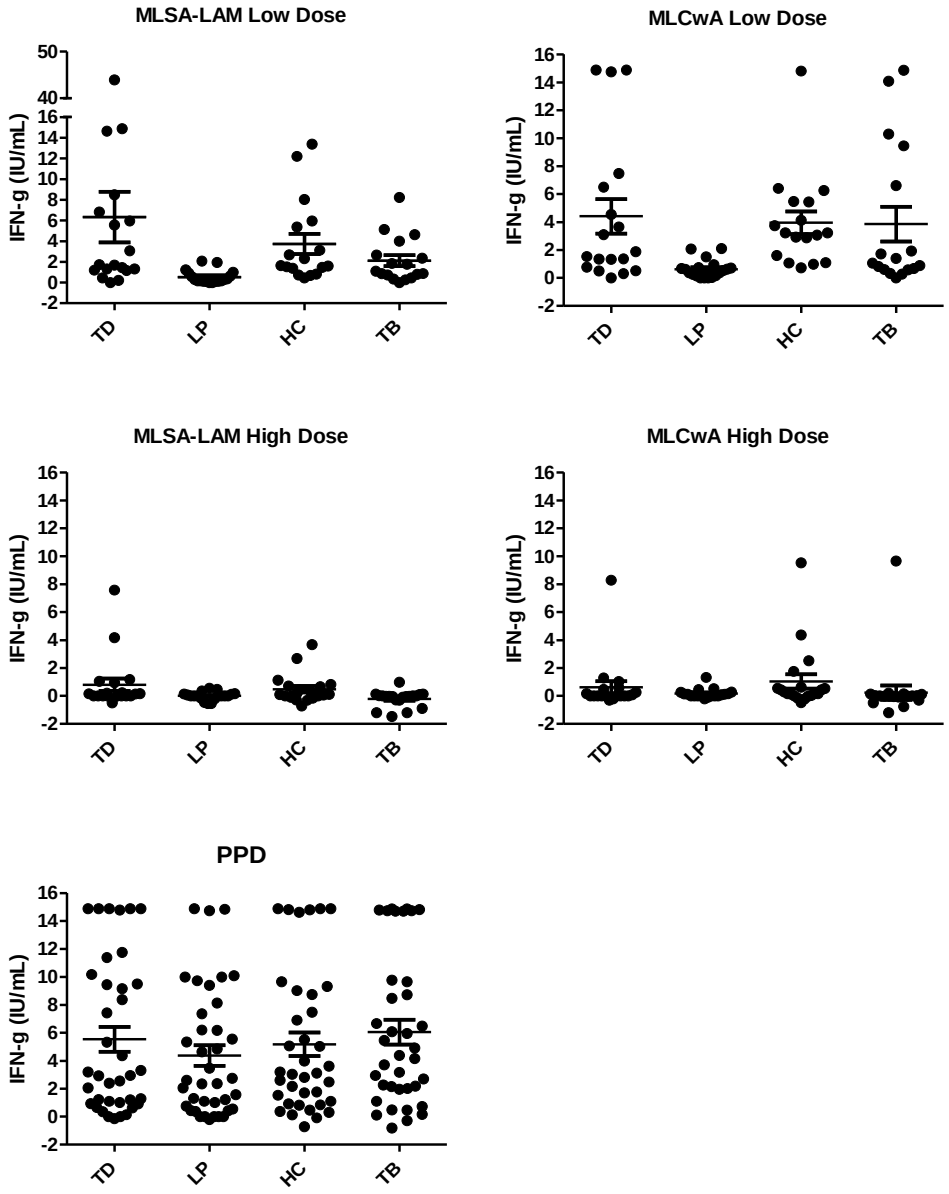



**Figure 19. IGRA Test Method Dot Plot Results**
IFN-γ production was measured by the QuantiFERON CMI Test kit (Cellestis, Valentia, CA) after culturing undiluted whole blood with skin test antigen MLSA-LAM or MLCwA at the high (1.0 µg) or low (0.1 µg) dose, PPD 2TU, or mitogen for 24 h at 37°C. Tests were performed in a single replicate. Reported values were extrapolated from an IFN-γ standard curve and subtracted from the medium control. Subject groups included TD, LP, HC, and TB. Single replicate Graph against medium control.

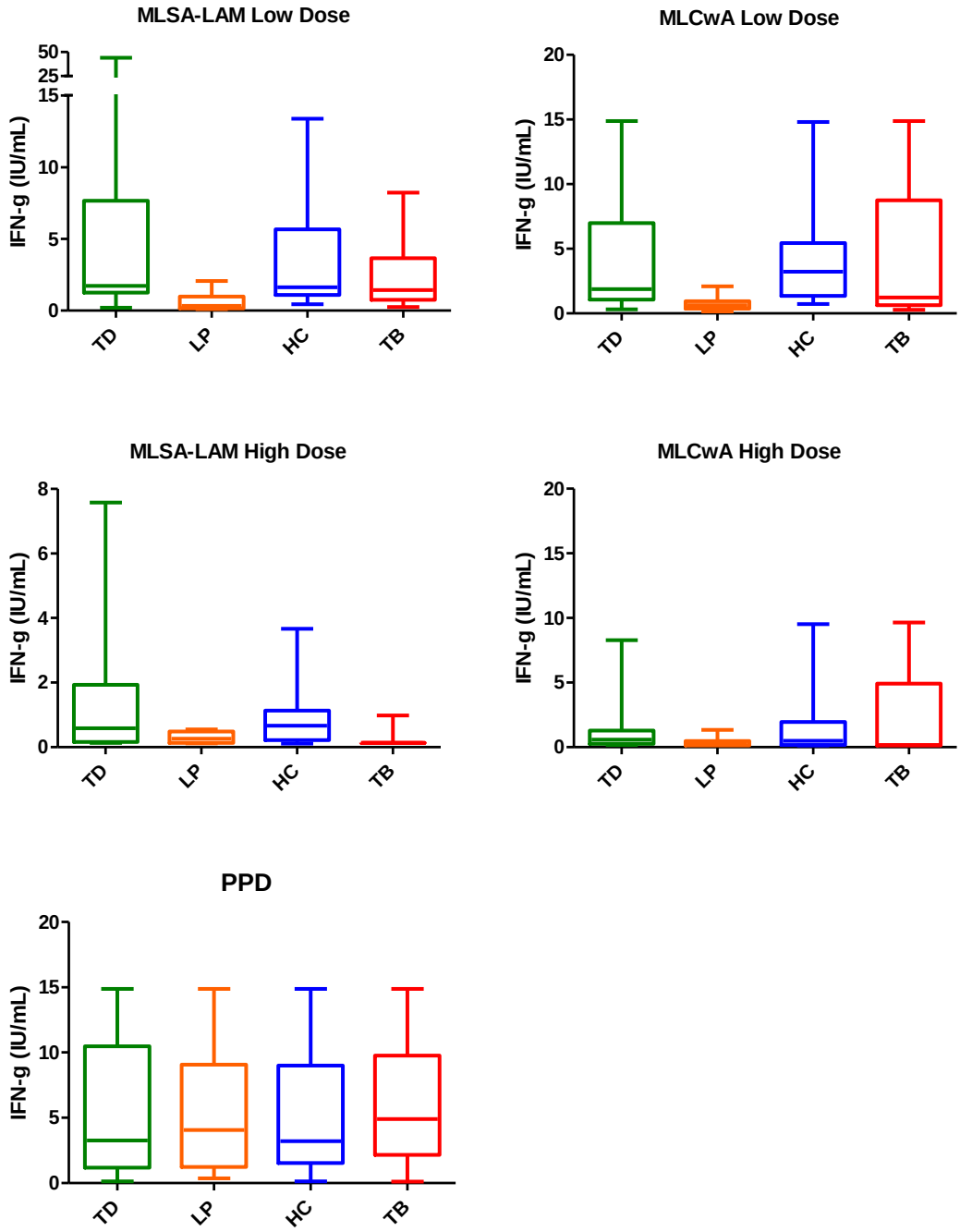



**Figure 20. IGRA positive reactors only**
Box plot of the positive responders for each antigen and antigen dose.

positive rate (Sp) for different cut-off points. The output is a Se/Sp prediction for each cut-off point. Accuracy of the test increases as Se and Sp approach 100%. The positive likelihood ratio is the probability of a positive test result given the presence of the disease over the probability of a positive test result given the absence of the disease. A p value <0.05 suggests that the area under the curve (AUC) is significantly different from a true population AUC of 0.5, and therefore there is evidence that the test has the ability to distinguish between the two groups. The AUC for ROC curve analysis is equal to the probability that an individual randomly chosen from the positive group will test positive compared to a randomly chosen individual from the negative group. When there is no difference between the distributions, the area will be equal to 0.5. With this planned analysis in-hand, test methods were compared individually and combinatorial to look for synergistic effects. The TD group was compared to the TB group across antigens and antigen doses, except for the PGL antibody assay, which included a comparison of the LP group compared to the TB group across stage C-1a (high dose) and stage C-1b (low dose) groups. The results of the ROC analysis can be found in **Table 11**.

The skin test high dose group results showed high sensitivity (90% and 85%) for MLSA-LAM and MLCwA, respectively; however, the cut off values derived from the curve were not realistic induration values at < 4.0 mm and < 9.5 mm. The low likelihood ratios, high p values, and low AUC values were indicators that something was flawed. On the contrary, the low dose group results showed high specificity with a cut off at > 5.2 mm and > 9.5 mm, with a strong likelihood ratio, but low p value and AUC. Specificities were similar to what had been determined using the biomodal distribution to identify the cut off values and calculations by hand. Unfortunately, a good diagnostic test has both good specificity and sensitivity.

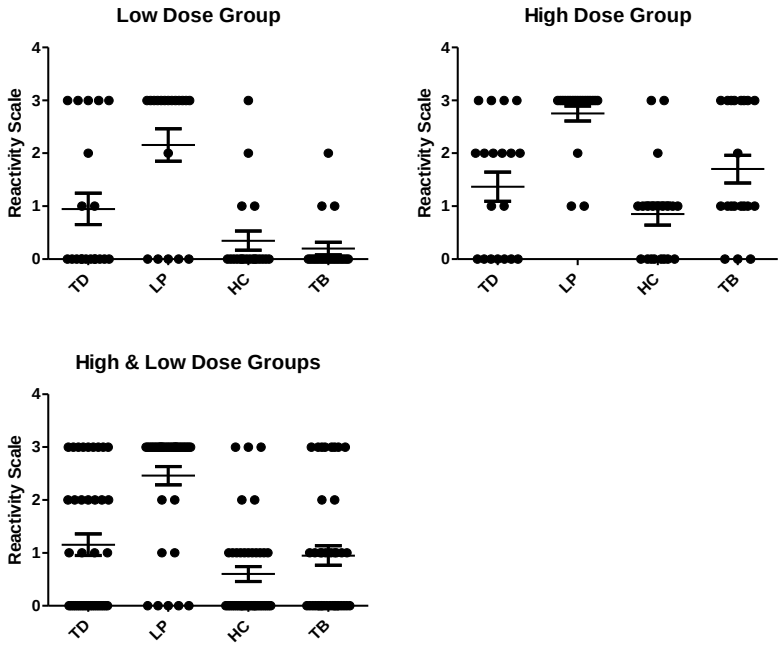



**Figure 21. PGL-I Antibody Test Method Dot Plot Results**
Presence of the *M. leprae* specific PGL-I antibody was measured with serum by lateral flow immunodiffusion using the Rapid PGL-I Antibody Test kit (Dr. Sang Nae Cho, Yonsei University, Seoul, South Korea). Results were stratified to allow graphing (0 – negative, 1 – weak positive, 2 – moderate positive, and 3 – strong positive).

**Table 11. ROC Curve, Diagnostic Test Statistics**

| Test Method | Antigen | Dose | Cut off* | Se | Sp | Likelihood Ratio | P value | AUC |
|---|---|---|---|---|---|---|---|---|
| Skin Test | MLCwA | Low | >9.5 mm | 25% | 95% | 5.00 | 0.46 | 0.57 |
| | MLSA-LAM | Low | > 5.2 mm | 20% | 100% | none | 0.28 | 0.60 |
| | MLCwA | High | < 9.5 mm | 85% | 40% | 1.42 | 0.57 | 0.55 |
| | MLSA-LAM | High | < 4.0 mm | 90% | 35% | 1.38 | 0.23 | 0.61 |
| IGRA | MLCwA | Low | >1.20 | 72% | 53% | 1.00 | 0.50 | 0.57 |
| | MLSA-LAM | Low | >1.30 | 83% | 53% | 1.77 | 0.15 | 0.64 |
| | MLCwA | High | >0.22 | 32% | 95% | 6.32 | 0.03 | 0.70 |
| | MLSA-LAM | High | >0.14 | 47% | 95% | 9.47 | 0.001 | 0.80 |
| PGl-I Antibody | LP | High/Lo | ➢2 | 77% | 80% | 3.85 | <0.0001 | 0.80 |
| | TD | High/Lo | > 1 | 41% | 75% | 1.64 | 0.62 | 0.53 |

The results seen with the IGRA compared to the skin test assay were further elucidated with the ROC analysis. Both high dose antigens showed excellent specificity (95% each), albeit poor sensitivity (47% and 32%) with very high likelihood ratios (9.47 and 6.32), significant p values (0.001 and 0.03), and excellent AUC values (0.80 and 0.70) at low cut off values (> 0.14 and > 0.22). The sensitivity was however, 27% better than the skin test method. When considering the low dose of each antigen, sensitivity improved dramatically, especially with MLSA-LAM (83%) at a higher cut off value (> 1.30), while specificity dropped to 53%. The likelihood ratio was low (1.77), while the p value was good, it was not significant (0.15) and the AUC was poor (0.64). The reason for this inverted data compared to the skin test method is unknown. This phenomenon will need to be verified. Dose optimization may identify a dose that provides both good specificity and sensitivity.

Comparing the LP group to the TB group across both the high and low dose studies, the PGL antibody assay shows the best diagnostic statistics. With a cut off greater than moderate (3 intensity level), sensitivity is moderate (77%) and specificity is also moderate (80%) with a high likelihood ratio (3.85), highly significant p value (< 0.0001) and excellent AUC (0.80). For diagnostic accuracy of TD subjects, the best cut off value was greater than mild (2 or 3 intensity level), with moderate specificity (75%), but poor sensitivity (41%) and poor diagnostic statistics. These results were as expected, since the PGL antibody test is excellent at classifying clinical disease. The question is the utility for early diagnosis. From the HC low dose and high dose groups, there were 3 individuals that had a PGL test result of "3" and 2 individuals that had a test result of "2". Verification that these 5 individuals develop leprosy would be accomplished by 6 month follow-up. Past studies that showed PGL was not sensitive maybe required a tighter cut-off value.

Based on the ROC curve analysis results, the three test methods were compared individually and together to determine if one was adequately useful or if more than one was better in diagnosing clinical leprosy. The best antigen and dose were used and the cut off was based on the best likelihood ratio. The number of positive subjects based on cut off values was tallied and the percent positive was graphed. MLSA-LAM low dose at > 5.2 mm cut off was used for the skin test method, MLSA-LAM low dose at > 1.30 IU/mL was used for the IGRA method, and a cut off of 3 was used for the PGL method. Adding MLSA-LAM high dose at > 0.14 cut off was evaluated and found to probably add no additional value. The TD group would have had 3 additional positive subjects, one of which was positive by PGL. The LP group would have had 13 additional positives, ten of which were positive by PGL. The HC group would have had 4 additional positives, none of which were positive by either skin test or PGL assay. The TB group would have had 7 additional positives, none of which were positive by either the skin test or PGL assay. Results are shown in **Figure 22**. The best scenario is the combination of the IGRA and PGL assays, which is better than either assay alone. Addition of the skin test did not improve the number of positive individuals detected. On the other hand, the combination of the skin test and PGL antibody test resulted in the least background reactors from the TB group. In the comparison with antigens at the high dose, all have background reactors, except IGRA with MLSA-LAM high dose.

Efficacy Analysis.

Results from the stage C-1a/b clinical trial showed that both new leprosy skin test antigens are immunologically potent as skin test antigens when tested in leprosy patients and their household contacts. The low doses of both antigens were found to cause minimal induration in EC and TB groups and a baseline was able to be determined for the low dose antigens only. MLSA-LAM low dose antigen cut off value was 5.2 mm and MLCwA low dose antigen cut off

value was 9.5 mm, when compared to TB subjects. Both skin test antigens at the low dose were found to be highly specific, but poorly sensitive.

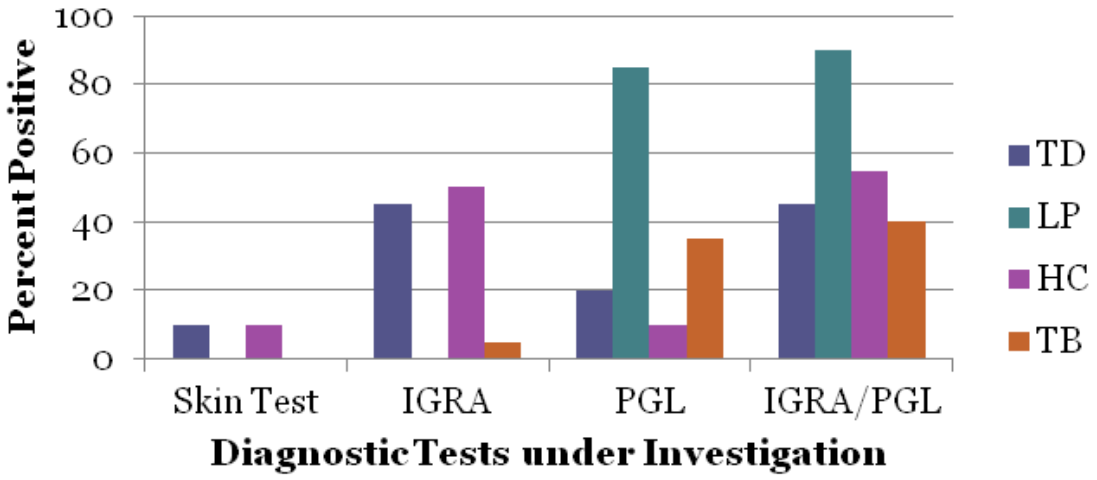



**Figure 22. Comparison of Test Methods**
Percent of positive reactors within each test group were graphed by test method. Individual test method was compared to combined test methods to assess the value of one or more test methods.

The IGRA test was found to be highly specific at the high dose and moderately specific at the low dose with both antigens. PGL antibody test once again proves to be both specific and sensitive in diagnosing BL/LL patients with clinical leprosy, rarely diagnosed by skin testing. Under these conditions, some household contacts were also identified as being positive to the PGL assay, suggesting the test may be useful for early diagnosis with a higher cut off. Combinatorial analysis of the three test methods showed that the best candidates for epidemiological screening may be the IGRA combined with the PGL assay, as the skin test method did not seem to add value to the total true positive subjects identified.

**DISCUSSION**

The phase II, stage C-1a clinical trial commenced in December 2006 and was completed in March 2008. The stage C-1b trial commenced in May 2009 and was completed in September

2009. Two protocol amendments were filed, one in May 2007 to decrease the control antigen dose; and, one in March 2009 to reduce the study size and to add comparative *in vitro* tests. These trials reflect the first testing of MLSA-LAM and MLCwA in leprosy patients, household contacts of leprosy patients and tuberculosis patients in an endemic region for leprosy. A final Clinical Study Report was submitted to the study sponsor by the data control center (The Emmes Corporation, Rockville, MD) on February 2012.

Study results were presented as trends, due to small scale sample sizes. Both antigens and antigen dosages have been shown to be safe for use in BT/TT and BL/LL leprosy patients, HC of lepromatous leprosy patients, and TB patients. The diagnostic accuracy of both skin test antigens at the low dose (0.1 μg) was found to be inadequate in terms of sensitivity, but excellent in terms of specificity. MLSA-LAM was shown to have slightly higher specificity than MLCwA at the low dose when comparing BT/TT leprosy patients against individuals infected with *M. tuberculosis*. At the high dose (1.0 μg) both antigens were limited in both sensitivity and specificity. Leprosy skin test antigens were found to be unresponsive in BL/LL leprosy patients confirming *M. leprae* specific anergy, yet capable of eliciting a response in some HC of BL/LL leprosy patients advocating promise for these antigens as early diagnostic tools for leprosy. A cut-off point for each antigen and antigen dose could not be determined with significance, due to limited positive responders in the BT/TT leprosy group.

Sensitivity and specificity were lacking with the Rees and Convit soluble antigens when tested in 2,602 Indian subjects. [20] A biomodal distribution of induration was seen with both antigens, but newly diagnosed leprosy patients, contacts, and non-contacts responded equally. In Northern Malawi, 15,630 subjects were tested with 5 batches of the Rees MLSA antigen prepared from two protocols. [21] With first and second generation antigens, a response from

paucibacillary (similar to BT/TT) [22] leprosy patients was seen in 76% and 38% of the subjects, respectively; however, endemic controls responded in 42% and 32% of the subjects, resulting in a difference of 34% and 6% detection rates, respectively. These percentages represent responders over baseline and are close to the 10-20% detection rates seen with low dose MLSA-LAM and MLCwA antigens.

The low detection rate of known BT/TT leprosy patients with these antigens suggests that they are not suitable for detection of clinical leprosy. They do however; elicit a response in 10-20% of HC suggesting that they might be suitable for detection of subclinical leprosy. The proportion of positive HC responding in these studies was consistent with documented risk of infection from a high bacillary index case at one in seven (14%) of 178 households studied. [23] Whole blood IFN-γ assay studies with MLSA-LAM and MLCwA showed nearly identical results to these skin test studies, except that HC responded with a higher geometric mean than BT/TT leprosy patients; EC and TB patients did not respond. [24] The authors also found that recent exposure resulted in substantially stronger responses.

At the high dose compared to the low dose, both antigens elicited a response in a higher number of HC, TB, and EC subjects, but the number of BT/TT responders remained the same. These phenomena maybe related to that observed when Leprosin A (Rees antigen) was shown to immunologically suppress the skin test response to Tuberculin PPD in both BT/TT and BL/LL leprosy patients. [25] This data supports the idea that there may be a difference in the antigenic profile that stimulates a response in subclinical, but not clinical leprosy.

The immunological environment of subclinical leprosy is unknown; however, advances have been made in understanding the innate and adaptive immune mediated pathways that promote and control disease pathology. [26,27] In tuberculosis, the infection delays onset of adaptive

immunity, which provides a window to establish a successful infection. Disease progression in tuberculosis, like leprosy, is then dependent on the immunological status of the host. [28]. Striking similarities of the immunology and pathology between these two diseases suggest that TT leprosy could be a latent form of disease, under the control of the immune system, whereas LL leprosy is known to be the active form of disease with T-cell hyporesponsiveness. [29] Borderline forms are immunologically unstable and can downgrade depending on the immunologically position of the host. [17] This continuum of immunological events probably occur prior to and during manifestation of clinical symptoms, providing opportunities for a subclinical diagnostic tool.

Antigen specificity at the low dose was thought to be related to the removal of lipoglycans, including the immunosuppressive and cross-reactive LAM, LM, PIM, and other lipids and lipoproteins. [30-32] Remaining proteins were numerous, but many shared sequence homology with *M. tuberculosis*. [33] Nonetheless, of the 100 EC tested, 77% had been vaccinated with BCG and 67% reacted with Tuberculin PPD, while only 2% reacted to the low dose leprosy antigens (2 with MLSA-LAM and 0 with MLCwA). Of the 20 TB subjects tested in the phase II, stage C-1b study, 95% (n=19) reacted to Tuberculin PPD, but only 10% (n=2) reacted to MLCwA and none reacted to MLSA-LAM. Another possibility is that the dose alone, or in combination with the removal of lipoglycans resulted in high specificity. At the low dose, *M. leprae* specific proteins may be available for recognition; whereas at the high dose those same proteins may be overpowered by ubiquitous mycobacterial proteins lending to cross-reactive responses with TB patients and EC exposed to environmental mycobacteria or vaccinated with BCG. [15]

The IGRA test and PGL antibody test did exhibit both sensitivity and specificity and when combined demonstrated enhanced sensitivity for detecting both tuberculoid and lepromatous leprosy patients in both known patients and household contacts. The skin test assay was compared to an interferon-gamma release assay and PGL antibody assay to see if either or a combination of *in vitro* tests could provide an alternative to the *in vivo* skin test. The interferon test at the high dose was found to be highly specific for tuberculoid leprosy patients and the low dose was found to be reasonably sensitive. A dose optimization between the high and low dose may lead to a single dose that is both sensitive and specific for detecting tuberculoid leprosy. The PGL antibody test was found to be both sensitive and specific for lepromatous leprosy at a particular cut off value. The combination of the interferon and PGL antibody test was found to be synergistic in detecting known leprosy patients.

The strength of these studies was in the verification that new refined leprosy skin test antigens were immunologically potent in BT/TT leprosy patients, anergic in BL/LL leprosy patients, and highly specific in BT/TT leprosy patients. The skin test method was simple, easy for field use, and minimally invasive, affording a feasible early diagnostic test tool. Limitations of these studies were difficulties shipping materials through customs, lengthy document review and approvals, multiple stages in the phase II protocol; prolonged duration to complete the study; political turmoil in the endemic country; and, intermittent communication services. Likewise, clues regarding efficacy were somewhat stymied by lack of subject numbers. Although the decrease in subject numbers was decided based on pragmatic reasons, increased numbers as established for the original study for statistical significance could have been achieved and would have strengthened this work.

As a result of these studies, the MLSA-LAM antigen at the 0.1 µg dose was found to be the best skin test antigen candidate and although the test configuration resulted in exceptional specificity it was paired with extremely poor sensitivity. Since sensitivity did not increase with the higher dose, optimizing the dose will probably not help improve the accuracy of the skin test diagnostic. The interferon release assay also favored the MLSA-LAM antigen at the 0.1 µg dose for sensitivity, but the 1.0 µg dose for specificity. Antigen concentration optimization should result in an improved diagnostic test with one antigen concentration. Finally, this study confirmed the sensitivity and specificity of PGL antibody assay in detecting clinical lepromatous leprosy; however, it also provides information of cut off values that may aid in enhancing specificity for use as an early diagnostic tool to complement a cell mediated diagnostic for detecting tuberculoid subjects. Albeit monumental, the definitive goal of developing an early diagnostic test for leprosy is to detect lepromatous leprosy patients early, because they are at greater risk of developing disabilities and spreading disease. This study suggests that combining an optimized interferon assay with the PGL assay provides an alternative to an *in vivo* test with the most potential for detecting early infection.

Further testing in a large scale randomized study of HC with follow-up is needed to reveal whether these skin test antigens could tip the balance toward intercepting transmission. Inclusion of a dose optimization study may improve sensitivity, but compromise specificity. Combinatorial testing of the skin test method against the *in vitro* IFN-γ test and phenolic glycolipid-I (PGL-I) antibody test may provide options to enhance sensitivity. [34] Recent testing of multiple proteins and peptides using the IFN-γ assay has shown promising results. [35-37] Finally, use of these skin test antigens may help in elucidation of the early immunological response following infection with *M. leprae.*

# REFERENCES


1. (2012) WHO Expert Committee on Leprosy. World Health Organ Tech Rep Ser: 1-61, 61 p following 61.

2. Duthie MS, Gillis TP, Reed SG (2011) Advances and hurdles on the way toward a leprosy vaccine. Hum Vaccin 7: 1172-1183.

3. Suzuki K, Akama T, Kawashima A, Yoshihara A, Yotsu RR, et al. (2012) Current status of leprosy: epidemiology, basic science and clinical perspectives. J Dermatol 39: 121-129.

4. Hotez P (2011) Enlarging the "Audacious Goal": elimination of the world's high prevalence neglected tropical diseases. Vaccine 29 Suppl 4: D104-110.

5. Rivoire BL, Terlow S, Brennan PJ (Submitted) The Challenge of Producing Skin Test Antigens with Minimal Resources Suitable for Human Application against a Neglected Tropical Disease; Leprosy. PLoS Negl Trop Dis.

6. Rivoire BL, McDonald M, Hawksworth RA, Neupane KD, Hagge D., et al. (Submitted) Safety Assessment of Two New Leprosy Skin Test Antigens in Healthy Subjects without known Exposure to Leprosy: Randomized Double Blind Clnical Study. PLoS Negl Trop Dis.

7. Britton WJ, Lockwood DN (2004) Leprosy. Lancet 363: 1209-1219.

8. (2006) Global strategy for further reducing the leprosy burden and sustaining leprosy control activities 2006-2010. Operational guidelines. Lepr Rev 77: IX, X, 1-50.

9. Arnadottir T, Rieder HL, Trebucq A, Waaler HT (1996) Guidelines for conducting tuberculin skin test surveys in high prevalence countries. Tuber Lung Dis 77 Suppl 1: 1-19.

10. Sanofi Pasteur Limited Toronto Ontario Canada (2009) Tubersol, Tuberculin Purified Protein Derivative (Mantoux) Package Insert R19-0905 Canada.

11. Zhou, Obuchowski, McClish (2002) Statistical Methods in Diagnostic Medicine. Wiley.

12. Kruskal W.H., W. A. Wallis (1952) Use of ranks in one-criterion variance analysis. Journal of the American Statistical Association 47 583–621.

13. Holland SM (2001) Nontuberculous mycobacteria. Am J Med Sci 321: 49-55.

14. Parashar D, Das R, Chauhan DS, Sharma VD, Lavania M, et al. (2009) Identification of environmental mycobacteria isolated from Agra, north India by conventional & molecular approaches. Indian J Med Res 129: 424-431.

15. Fine PE, Floyd S, Stanford JL, Nkhosa P, Kasunga A, et al. (2001) Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. Epidemiol Infect 126: 379-387.

16. ICH (2012) MeDRA Term Selection. Technical Requirements for Registration of Pharmaceuticals for Human Use. Points to Consider 4.3

17. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, et al. (2006) The continuing challenges of leprosy. Clin Microbiol Rev 19: 338-381.

18. Leeflang MM, Deeks JJ, Gatsonis C, Bossuyt PM (2008) Systematic reviews of diagnostic test accuracy. Ann Intern Med 149: 889-897.

19. Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39: 561-577.

20. Gupte MD, Anantharaman DS, Nagaraju B, Kannan S, Vallishayee RS (1990) Experiences with Mycobacterium leprae soluble antigens in a leprosy endemic population. Lepr Rev 61: 132-144.

21. Sterne JA, Fine PE, Ponnighaus JM, Rees RJ, Chavula D (1998) Delayed-type hypersensitivity to Mycobacterium leprae soluble antigens as a test for infection with the leprosy bacillus. Int J Epidemiol 27: 713-721.

22. World Health Organization (1982) Report of the Eleventh and Twelfth Meetings of the Steering Committee of the Scientific Working Group on the Chemotherapy of Leprosy. Geneva. March 30-31 and Oct 10. TDR/THELEP-SC (11-12) 82: 1-6.

23. Douglas JT, Cellona RV, Fajardo TT, Jr., Abalos RM, Balagon MV, et al. (2004) Prospective study of serological conversion as a risk factor for development of leprosy among household contacts. Clin Diagn Lab Immunol 11: 897-900.

24. Manandhar R, LeMaster JW, Butlin CR, Brennan PJ, Roche PW (2000) Interferon-gamma responses to candidate leprosy skin-test reagents detect exposure to leprosy in an endemic population. Int J Lepr Other Mycobact Dis 68: 40-48.

25. Sengupta U, Sinha S, Ramu G, Lamb J, Ivanyi J (1987) Suppression of delayed hypersensitivity skin reactions to tuberculin by M. leprae antigens in patients with lepromatous and tuberculoid leprosy. Clin Exp Immunol 68: 58-64.

26. Ottenhoff TH (2012) New pathways of protective and pathological host defense to mycobacteria. Trends Microbiol 20: 419-428.

27. Modlin RL (2010) The innate immune response in leprosy. Curr Opin Immunol 22: 48-54.

28. Bhat RM, Prakash C. (2012) Leprosy: an overview of pathophysiology. Interdiscip Perspect Infect Dis 2012: 181089.

29. Kumar S, Naqvi RA, Khanna N, Rao DN (2011) Disruption of HLA-DR raft, deregulations of Lck-ZAP-70-Cbl-b cross-talk and miR181a towards T cell hyporesponsiveness in leprosy. Mol Immunol 48: 1178-1190.

30. Chatterjee D, Khoo KH (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 8: 113-120.

31. Torrelles JB, Sieling PA, Zhang N, Keen MA, McNeil MR, et al. (2012) Isolation of a distinct Mycobacterium tuberculosis mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. Glycobiology 22: 1118-1127.

32. Barnes PF, Chatterjee D, Abrams JS, Lu S, Wang E, et al. (1992) Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. J Immunol 149: 541-547.

33. Marques MA, Neves-Ferreira AG, da Silveira EK, Valente RH, Chapeaurouge A, et al. (2008) Deciphering the proteomic profile of Mycobacterium leprae cell envelope. Proteomics 8: 2477-2491.

34. Spencer JS, Brennan PJ (2011) The role of Mycobacterium leprae phenolic glycolipid I (PGL-I) in serodiagnosis and in the pathogenesis of leprosy. Lepr Rev 82: 344-357.

35. Geluk A, Bobosha K, van der Ploeg-van Schip JJ, Spencer JS, Banu S, et al. (2012) New biomarkers with relevance to leprosy diagnosis applicable in areas hyperendemic for leprosy. J Immunol 188: 4782-4791.

36. Dockrell HM (2011) Keep the faith--leprosy still needs new diagnostic tools and laboratory research. Lepr Rev 82: 340-343.

37. Sampaio LH, Stefani MM, Oliveira RM, Sousa AL, Ireton GC, et al. (2011) Immunologically reactive M. leprae antigens with relevance to diagnosis and vaccine development. BMC Infect Dis 11: 26.

# CHAPTER FIVE: CONCLUSIONS

## SUMMARY

Translation of two new leprosy skin test antigens, MLSA-LAM and MLCwA, under the Leprosy Skin Test Initiative has been completed. Achievements are outlined in **Table 12.** Both antigens and antigen dosages (0.1 µg and 1.0 µg) were found to be safe for use in humans and were highly specific for tuberculoid leprosy, but lacked sensitivity for tuberculoid and lepromatous leprosy. Due to small sample sizes and limited response by tuberculoid leprosy patients to the skin test antigens, efficacy results were not statistically significant and therefore are presented only as promising trends.

Several other important findings were identified. Firstly, lepromatous leprosy patients were anergic to these *M. leprae* specific antigens, as expected. Secondly, the response to both skin test antigens correlated reasonably well at the low dose, but not at the high dose; and, the response to PPD did not correlate with either leprosy antigen at either dose, even though most subjects across all groups recognized PPD. Thirdly, the interferon gamma release method using both antigens at the high dose provided the best diagnostic accuracy. Finally, the PGL Antibody Assay was a very good diagnostic test for lepromatous leprosy patients, which were not detected with the skin test or interferon test.

In summary, both new leprosy antigens were found to be specific to tuberculoid leprosy patients at the low dose. This is the first documented report of a specific leprosy skin test antigen. Recognition of high specificity was likely the result of using a low dose and the removal of cross-reactive lipoglycans. Although specific, they were not adequately sensitive. This could be a function of inadequate diversity of the antigen repertoire, reduced concentration

of diagnostic antigens in these preparations, or poor choice of test method; however, household contacts did respond at a rate which aligns with infectivity noted in the literature. Overall, MLSA-LAM low dose showed better specificity in the skin test, but the MLSA-LAM high dose provided the best diagnostic accuracy in the interferon assay. The PGL-I Antibody assay showed good diagnostic accuracy for lepromatous patients, but the cut off value is qualitative using the lateral flow immunodiagnostic kit.

**EVALUATION**

The results of this research partially prove the hypothesis that the new leprosy skin test antigens, MLSA-LAM and MLCwA, at doses of 1.0μg and/or 0.1μg, would be safe and efficacious as diagnostic- tools to detect leprosy and allow treatment of patients earlier and to measure the extent of leprosy infection in human subjects living in a leprosy endemic area.

Specific aim 1 findings supported the safe use of these antigens and antigen dosages in leprosy patients, household contacts of leprosy patients, and tuberculosis patients living in an endemic region for leprosy. Moreover, in the small sample size studies, both antigens were found to be specific for tuberculoid leprosy, but not specific as skin test antigens when evaluating clinical leprosy patients who were either being treated concurrently or had completed treatment for leprosy. Both antigens at both dosages did elicit a response in some household contacts, suggesting that these antigens might be possible candidates for early diagnostic tools for leprosy. Specific aim 2 was successfully completed the comparison of the skin test method against the IGRA and PGL Antibody Assay. These studies suggested that the IGRA with MLSA-LAM low dose was the best antigen, dose, and test method provided the best diagnostic accuracy for detection of tuberculoid leprosy patients. Lepromatous leprosy patients were not detected with

**Table 12. Leprosy Skin Test Initiative Achievements**

| Stage | Date | Task |
|---|---|---|
| Discovery | 1992 | Target antigens identified |
| Draft IND | January, 1994 | Submitted to Sponsor |
| | April 20, 1994 | FDA comments received |
| Manufacturing | May 12, 1997 | Clinical grade MLSA-LAM and MLCwA |
| Investigator's Brochure | September 18, 1998 | Submitted to Sponsor |
| Final IND/Phase I Protocol | September 23, 1998 | Submitted to Sponsor |
| Phase I | December 28, 1998 | Trial commenced |
| | February 16, 1999 | Trial completed |
| | June 7, 1999 | Final Report |
| Phase II Protocol | June 25, 2001 | Submitted to Sponsor (version 6.1) |
| Phase II, Stage A | April 30, 2002 | Trial commenced |
| | July 10, 2002 | Trial completed |
| | September 10, 2002 | Safety Monitoring Report |
| Phase II Protocol | October 29, 2002 | Amendment (version 6.2) |
| Phase II, Stage B | May 11, 2003 | Trial commenced |
| | January 6, 2004 | Trial completed |
| | August 31, 2004 | Safety Monitoring Report |
| Phase II Protocol | March 8, 2006 | Amendment (version 7.0) |
| Phase II, Stage C-1a | December 12, 2006 | Trial commenced |
| Phase II Protocol | May 25, 2007 | Amendment (version 8.0) |
| Phase II, Stage C-1a | March 30, 2008 | Trial completed |
| | September 25, 2008 | Safety Monitoring Report |
| Phase II Protocol | March 2, 2009 | Amendment (version 9.0) |
| Phase II, Stage C-1b | May 27, 2009 | Trial commenced |
| | September 13, 2009 | Trial completed |
| Final Clinical Study Report | February 17, 2012 | Submitted to Study Sponsor by the Data Coordinating Center |

the IGRA method; however, the PGL Antibody Assay showed promise using a high cut off value. A parallel diagnostic approach using MLSA-LAM high dose in the IGRA and PGL

Antibody Assay as a primary epidemiological screen, following up with MLSA-LAM low dose skin test for confirmation of tuberculoid leprosy patients provides an option. Moreover, optimization of MLSA-LAM concentration in the IGRA may improve diagnostic accuracy in both areas of sensitivity and specificity.

Specific aim 3 was successfully completed by translating these two skin test antigens in an academic environment; moving the antigens from the bench to clinical trials to assess their utility as safe and efficacious diagnostic tools. Although the early steps of product translation were not covered under the auspice of his dissertation, the reporting of the translational process was a culmination of this research. This research proves that the translation of a product from discovery, through manufacturing under cGMP, submission of an IND, and testing products in human clinical trials can be done under government sponsorship in a university setting. Likewise, the teaching potential for such an endeavor is tremendous for undergraduate and graduate students leaving the university with an interest in pharmaceutical drug discovery.

**FUTURE DIRECTIONS**

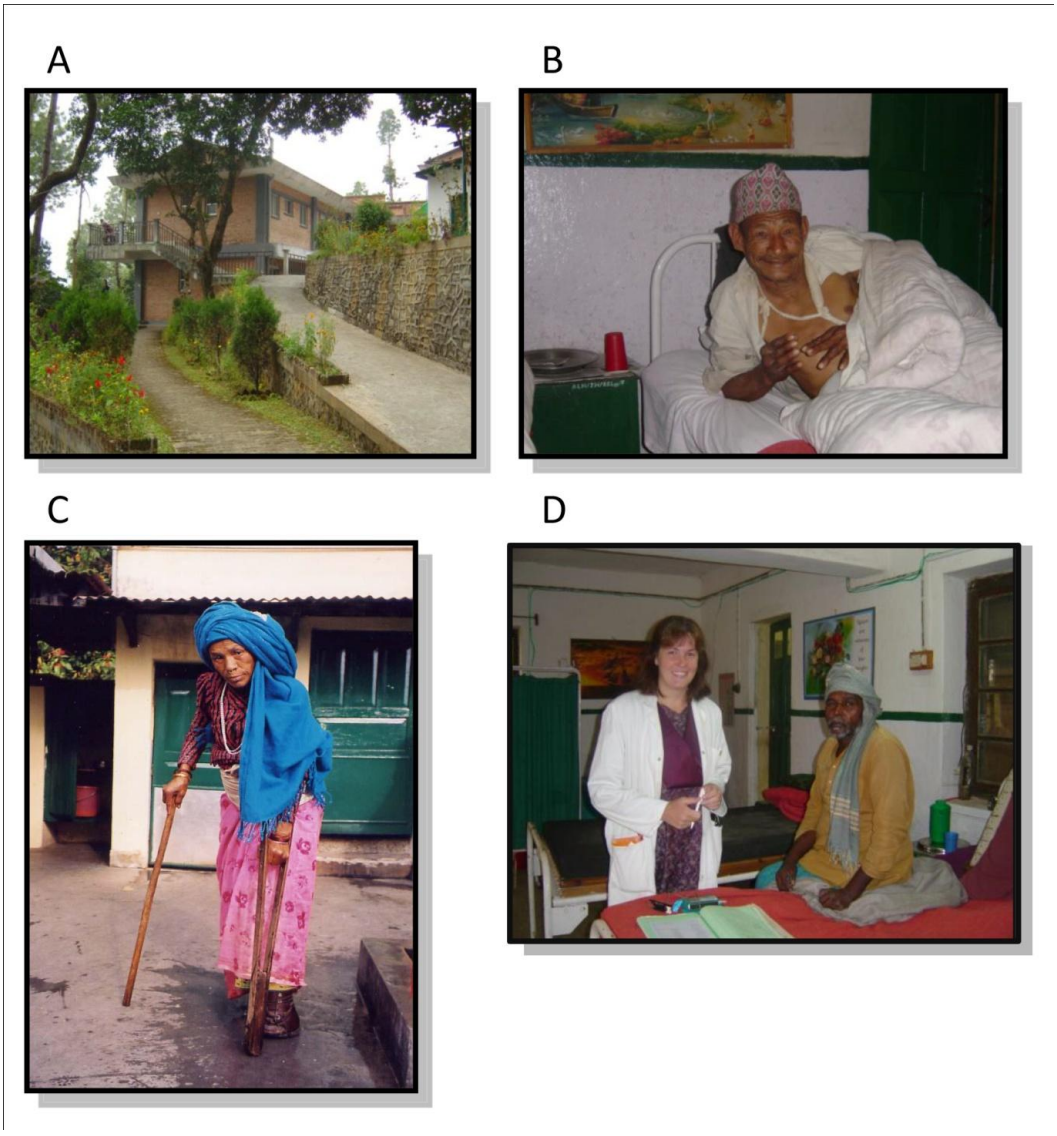
Results from these research studies provide new translational knowledge and opportunities for further investigation and combinatorial approaches to reach the unmet need of developing and implementing an early diagnosis test for leprosy. Further testing is required to optimize the IGRA dose to increase sensitivity, while maintaining reasonable specificity. Once optimized and proof of concept has been appropriately tested in clinical studies, the combination of the IGRA and PGL-I Antibody Assay may provide epidemiological value to assess the true incidence of leprosy and to identify asymptomatic patients for prophylactic chemotherapy.

APPENDICES

## APPENDIX 1: PHASE II CLINICAL SITE, STAFF, AND LEPROSY PATIENTS

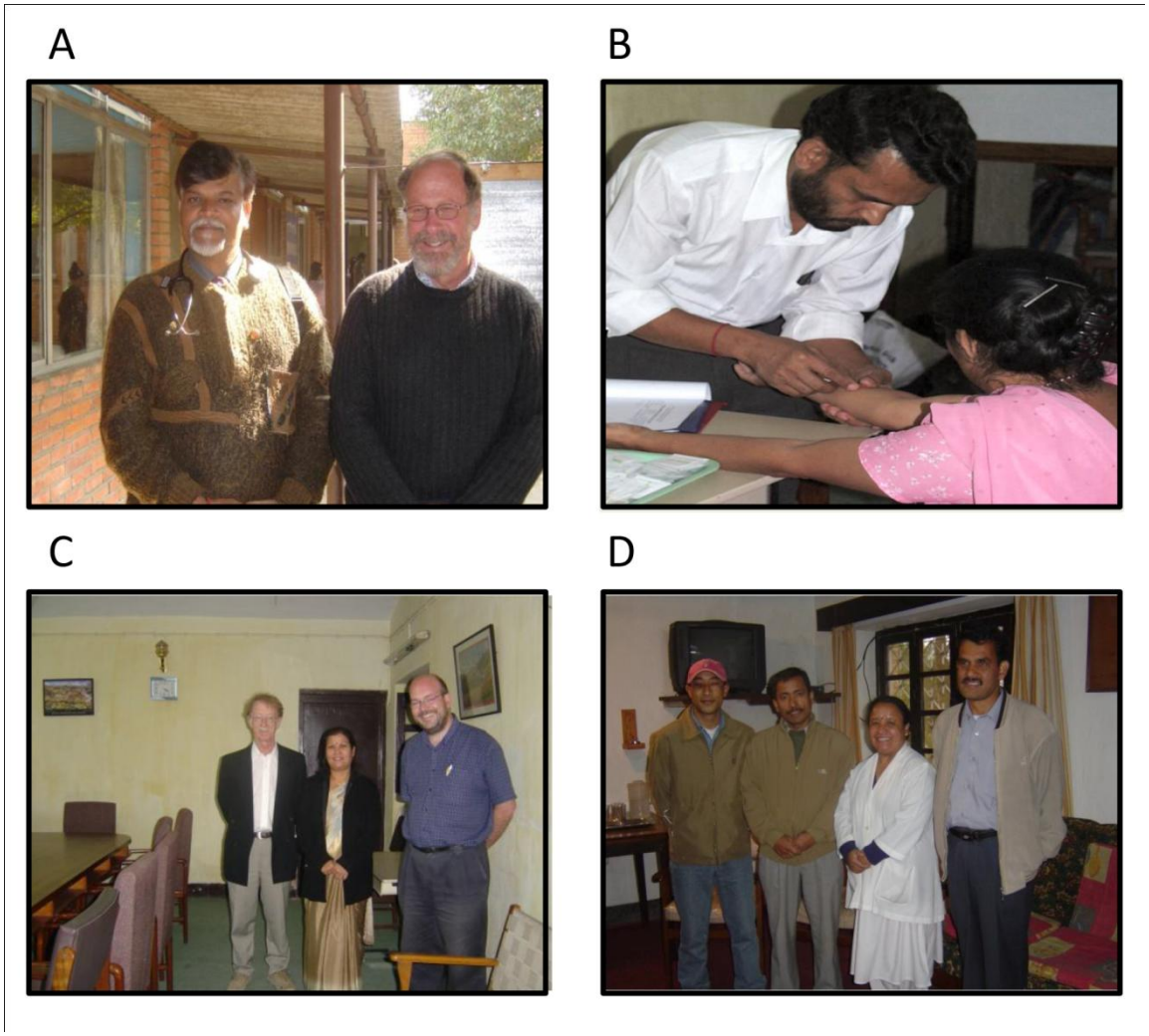
## PHOTOGRAPHS

A) Anandaban Hospital, Kathmandu, Nepal. Site of Phase II, Stage C Clinical Trial. Founded in 1957 by The Leprosy Mission International (TLMI) as the main referral hospital for leprosy in the central region of Nepal. B) Leprosy patient with hand neuropathy and bone resorption, which may require surgery and physical therapy, C) Leprosy patient with foot support to assist with movement and prevent further disabilities, D) Dr. Rachel Hawksworth, Clinical Principal Investigator, Stage C, with patient,



CONTINUED APPENDIX 1

A) Dr. Yadav (Internal Safety Monitor) and Dr. Gelber (Safety Monitoring Committee, Chair) at Patan Hospital, where tuberculosis patients were recruited, B) Mr. Kapil Neupane, Administrator and Gold Standard reader, C) Mrs. Bangdel (Radha Bangdel, Assistant, Lalitpur Nursing College, Sanepa, Kathmandu), Dr. Murdo Macdonald (Clinical Study Coordinator, Anandaban Hospital), and Dr. Patrick Brennan (Research Principal Investigator) standing in the injection/reading room, which is adjacent to an exit into the courtyard where an ambulance was stationed during the study, and E) Skin test injectors and readers from left to right: HA Krishnaman Shrestha, LT Subash C. Silwal (not shown), LT Ishwor Raj Shrestha, Sr. Niru Shrestha, and Kapil Neupane.



## APPENDIX 2: CHAPTER THREE SUPPLEMENTARY DATA

### Phase I - Induration Measurements at 72 h

| Subject No. | Assigned Sequential No. | Intervention | Induration (mm) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Intervention | | | Rees | Saline |
| | | | 0.1 µg | 1.0 µg | 2.5 µg | 1.0 µg | N/A |
| 2 | 1 | MLCwA | 0 | 0 | 0 | 0 | 0 |
| 3 | 2 | MLCwA | 0 | 0 | 0 | 0 | 0 |
| 6 | 3 | MLCwA | 0 | 0 | 9 | 7 | 0 |
| 8 | 4 | MLCwA | 0 | 0 | 0 | 0 | 0 |
| 10 | 5 | MLCwA | 0 | 0 | 0 | 0 | 0 |
| 1 | 6 | MLSA-LAM | 0 | 0 | 0 | 0 | 0 |
| 4 | 7 | MLSA-LAM | 0 | 0 | 0 | 0 | 0 |
| 7 | 8 | MLSA-LAM | 0 | 0 | 0 | 0 | 0 |
| 9 | 9 | MLSA-LAM | 0 | 0 | 0 | 0 | 0 |
| 11 | 10 | MLSA-LAM | 0 | 0 | 0 | 0 | 0 |

The 48 and 72 hour induration measurements were very similar and since the 48 hour response was dropped from phase II, stage B study, only the 72 hour values are provided for comparison.

CONTINUED APPENDIX 2

**Phase II, Stage A - Induration Measurements at 72 h**

| Subject No. | Assigned Sequential No. | Intervention | Induration (mm) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Intervention | | Tuberculin | Saline | |
| | | | 0.1 µg | 1.0 µg | 5TU | N/A | |
| 2 | 1 | MLCwA | 0 | 0 | 0 | 0 | * |
| 3 | 2 | MLCwA | 0 | 0 | 31 | 0 | *** |
| 5 | 3 | MLCwA | 0 | 0 | 21 | 0 | *** |
| 6 | 4 | MLCwA | 0 | 16.5 | 19 | 0 | **** |
| 9 | 5 | MLCwA | 0 | 0 | 11 | 0 | *** |
| 1 | 6 | MLSA-LAM | 0 | 0 | 21 | 0 | *** |
| 4 | 7 | MLSA-LAM | 0 | 0 | 10 | 0 | *** |
| 7 | 8 | MLSA-LAM | 0 | 0 | 34 | 0 | *** |
| 8 | 9 | MLSA-LAM | 0 | 20 | 0 | 0 | ** |
| 10 | 10 | MLSA-LAM | 0 | 0 | 11 | 0 | *** |

CONTINUED APPENDIX 2

**Phase II, Stage B - Induration Measurements at 72 h**

| Subject No. | Assigned Sequential No. | Intervention | Induration (mm) | | | |
|---|---|---|---|---|---|---|
| | | | Intervention | | Tuberculin | |
| | | | 0.1 µg | 1.0 µg | 5TU | |
| 12 | 6 | MLCwA | 0 | 0 | 19.5 | *** |
| 16 | 7 | MLCwA | 0 | 0 | 0 | * |
| 18 | 8 | MLCwA | 0 | 0 | 18.5 | *** |
| 19 | 9 | MLCwA | 0 | 17 | 17.5 | **** |
| 20 | 10 | MLCwA | 0 | 8.5 | 14.5 | **** |
| 22 | 11 | MLCwA | 0 | 16.5 | 25 | **** |
| 24 | 12 | MLCwA | 0 | 7 | 28 | **** |
| 25 | 13 | MLCwA | 0 | 9 | 8.5 | **** |
| 26 | 14 | MLCwA | 0 | 0 | 21 | *** |
| 29 | 15 | MLCwA | 0 | 0 | 0 | * |
| 33 | 16 | MLCwA | 0 | 0 | 19 | *** |
| 34 | 17 | MLCwA | 0 | 0 | 13.5 | *** |
| 35 | 18 | MLCwA | 0 | 0 | 0 | * |
| 36 | 19 | MLCwA | 0 | 0 | 34 | *** |
| 40 | 20 | MLCwA | 0 | 0 | 29 | *** |
| 41 | 21 | MLCwA | 0 | 0 | 21 | *** |
| 42 | 22 | MLCwA | 0 | 0 | 20 | *** |
| 44 | 23 | MLCwA | 0 | 0 | 30 | *** |
| 47 | 24 | MLCwA | 0 | 0 | 0 | * |
| 50 | 25 | MLCwA | 0 | 0 | 19.5 | *** |
| 53 | 26 | MLCwA | 0 | 3.5 | 14.5 | **** |
| 54 | 27 | MLCwA | 0 | 0 | 14 | *** |
| 56 | 28 | MLCwA | 0 | 0 | 0 | * |
| 57 | 29 | MLCwA | 0 | 0 | 0 | * |
| 59 | 30 | MLCwA | 0 | 0 | 0 | * |
| 64 | 31 | MLCwA | 0 | 0 | 27.5 | *** |
| 65 | 32 | MLCwA | 0 | 0 | 0 | * |
| 66 | 33 | MLCwA | 0 | 0 | 16 | *** |
| 68 | 34 | MLCwA | 0 | 0 | 0 | * |
| 70 | 35 | MLCwA | 0 | 0 | 10 | *** |
| 71 | 36 | MLCwA | 0 | 0 | 20 | *** |
| 72 | 37 | MLCwA | 0 | 0 | 19 | *** |
| 75 | 38 | MLCwA | 0 | 0 | 9 | *** |
| 79 | 39 | MLCwA | 0 | 0 | 12 | *** |
| 80 | 40 | MLCwA | 0 | 0 | 15 | *** |

| Subject No. | Assigned Sequential No. | Intervention | Induration (mm) | | | |
|---|---|---|---|---|---|---|
| | | | Intervention | | Tuberculin | |
| | | | 0.1 µg | 1.0 µg | 5TU | |
| 81 | 41 | MLCwA | 0 | 0 | 0 | * |
| 82 | 42 | MLCwA | 0 | 0 | 0 | * |
| 85 | 43 | MLCwA | 0 | 0 | 0 | * |
| 86 | 44 | MLCwA | 0 | 7 | 0 | ** |
| 90 | 45 | MLCwA | 0 | 0 | 0 | * |
| 91 | 46 | MLCwA | 0 | 0 | 22 | *** |
| 94 | 47 | MLCwA | 0 | 0 | 10.5 | *** |
| 97 | 48 | MLCwA | 0 | 0 | 0 | * |
| 99 | 49 | MLCwA | 0 | 0 | 16.5 | *** |
| 100 | 50 | MLCwA | 0 | 0 | 0 | * |
| 11 | 56 | MLSA-LAM | 0 | 0 | 24.5 | *** |
| 13 | 57 | MLSA-LAM | 0 | 0 | 0 | * |
| 14 | 58 | MLSA-LAM | 2.5 | 4.5 | 12 | **** |
| 15 | 59 | MLSA-LAM | 0 | 0 | 0 | * |
| 17 | 60 | MLSA-LAM | 0 | 0 | 7 | *** |
| 21 | 61 | MLSA-LAM | 0 | 0 | 15.5 | *** |
| 23 | 62 | MLSA-LAM | 0 | 8 | 9.5 | **** |
| 27 | 63 | MLSA-LAM | 0 | 0 | 9.5 | *** |
| 28 | 64 | MLSA-LAM | 0 | 0 | 11 | *** |
| 30 | 65 | MLSA-LAM | 0 | 0 | 17.5 | *** |
| 31 | 66 | MLSA-LAM | 0 | 0 | 0 | * |
| 32 | 67 | MLSA-LAM | 0 | 8.5 | 12.5 | **** |
| 37 | 68 | MLSA-LAM | 0 | 0 | 19 | *** |
| 38 | 69 | MLSA-LAM | 0 | 0 | 0 | * |
| 39 | 70 | MLSA-LAM | 0 | 10.5 | 13 | **** |
| 43 | 71 | MLSA-LAM | 7.5 | 22.5 | 27 | **** |
| 45 | 72 | MLSA-LAM | 0 | 0 | 9 | *** |
| 46 | 73 | MLSA-LAM | 0 | 0 | 22 | *** |
| 48 | 74 | MLSA-LAM | 0 | 0 | 0 | * |
| 49 | 75 | MLSA-LAM | 0 | 0 | 13.5 | *** |
| 51 | 76 | MLSA-LAM | 0 | 0 | 16.5 | *** |
| 52 | 77 | MLSA-LAM | 0 | 0 | 0 | * |
| 55 | 78 | MLSA-LAM | 0 | 0 | 0 | * |
| 58 | 79 | MLSA-LAM | 0 | 0 | 18 | *** |
| 60 | 80 | MLSA-LAM | 0 | 0 | 13.5 | *** |
| 61 | 81 | MLSA-LAM | 0 | 0 | 11 | *** |
| 62 | 82 | MLSA-LAM | 0 | 0 | 0 | * |
| 63 | 83 | MLSA-LAM | 0 | 0 | 9.5 | *** |

| Subject No. | Assigned Sequential No. | Intervention | Induration (mm) | | | |
|---|---|---|---|---|---|---|
| | | | Intervention | | Tuberculin | |
| | | | 0.1 µg | 1.0 µg | 5TU | |
| 67 | 84 | MLSA-LAM | 0 | 0 | 18 | *** |
| 69 | 85 | MLSA-LAM | 0 | 0 | 0 | * |
| 73 | 86 | MLSA-LAM | 0 | 0 | 26 | *** |
| 74 | 87 | MLSA-LAM | 0 | 0 | 25 | *** |
| 76 | 89 | MLSA-LAM | 0 | 11.5 | 0 | ** |
| 77 | 90 | MLSA-LAM | 0 | 0 | 0 | * |
| 78 | 91 | MLSA-LAM | 0 | 0 | 0 | * |
| 83 | 92 | MLSA-LAM | 0 | 8.5 | 10 | **** |
| 84 | 93 | MLSA-LAM | 0 | 0 | 0 | * |
| 87 | 94 | MLSA-LAM | 0 | 0 | 7.5 | *** |
| 89 | 95 | MLSA-LAM | 0 | 0 | 0 | * |
| 92 | 96 | MLSA-LAM | 0 | 0 | 15 | *** |
| 93 | 97 | MLSA-LAM | 0 | 0 | 19.5 | *** |
| 95 | 98 | MLSA-LAM | 0 | 0 | 13.5 | *** |
| 96 | 99 | MLSA-LAM | 0 | 3.5 | 13 | **** |
| 98 | 100 | MLSA-LAM | 0 | 0 | 0 | * |
| 101 | 101 | MLSA-LAM | 0 | 7 | 15 | **** |

In the phase II, stage A study, the 48 and 72 hour induration measurements were very similar, and since the 48 hour response was dropped from stage B, only the 72 hour values are provided for comparison. (*) a total of 30 individuals did not respond to either the intervention or to Tuberculin PPD, (**) a total of 3 individuals responded to one or the other antigens, but not Tuberculin, (***) a total of 52 individuals responded to Tuberculin PPD only, and (****) a total of 15 individuals responded to both the intervention and Tuberculin PPD.

## APPENDIX 3: CHAPTER FOUR SUPPLEMENTARY DATA

### Maximum Reactogenicity[a] by Subject Across all Visits (BT/TT)

| BT/TT | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | Stage C-1a | | | Stage C-1b | | |
| Reactions | MLSA-LAM | MLCwA | PPD | MLSA-LAM | MLCwA | PPD |
| | 1.0 µg | 1.0 µg | 5 TU | 0.1 µg | 0.1 µg | 2 TU |
| Induration | 2 | 3 | 12 (1-AE) | 4 | 5 | 13 |
| Erythema | 4 | 5 | 13 (2-AE) | 5 | 8 | 14 |
| Pruritis (itching) | 0 | 1 | 5 | 1 | 2 | 8 |
| Pain | 0 | 1 | 7 | 0 | 0 | 1 |
| Bleeding | 0 | 0 | 0 | 0 | 0 | 0 |
| Urticaria (hives) | 0 | 0 | 0 | 0 | 0 | 0 |
| Infection | 0 | 0 | 0 | 0 | 0 | 0 |
| Blistering | 0 | 0 | 0 | 0 | 0 | 0 |
| ***Total No. Sites*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** |
| **Total No. Events** | **6** | **10** | **37** | **10** | **15** | **36** |
| **Total No. AE** | **0** | **0** | **3** | **0** | **0** | **0** |

[a] Maximum reactogenicity is the no. of subjects exhibiting specific reactions across all visits.

CONTINUED APPENDIX 3

**Maximum Reactogenicity[a] by Subject Across all Visits (BL/LL)**

| BL/LL | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | Stage C-1a | | | Stage C-1b | | |
| Reactions | MLSA-LAM | MLCwA | PPD | MLSA-LAM | MLCwA | PPD |
| | 1.0 µg | 1.0 µg | 5 TU | 0.1 µg | 0.1 µg | 2 TU |
| Induration | 1 | 0 | 11 | 0 | 0 | 7 |
| Erythema | 2 | 2 | 12 (2-AE) | 4 | 4 | 10 |
| Pruritis (itching) | 0 | 0 | 5 | 0 | 0 | 0 |
| Pain | 1 | 1 | 6 | 0 | 0 | 3 |
| Bleeding | 0 | 0 | 0 | 0 | 0 | 0 |
| Urticaria (hives) | 0 | 0 | 0 | 0 | 0 | 0 |
| Infection | 0 | 0 | 0 | 0 | 0 | 0 |
| Blistering | 0 | 0 | 2 (2-AE) | 0 | 0 | 0 |
| ***Total No. Sites*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** |
| **Total No. Events** | **4** | **3** | **36** | **4** | **4** | **20** |
| **Total No. AE** | **0** | **0** | **4** | **0** | **0** | **0** |

[a] Maximum reactogenicity is the no. of subjects exhibiting specific reactions across all visits.

**Maximum Reactogenicity[a] by Subject Across all Visits (HC)**

| HC | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | Stage C-1a | | | Stage C-1b | | |
| Reactions | MLSA-LAM | MLCwA | PPD | MLSA-LAM | MLCwA | PPD |
| | 1.0 µg | 1.0 µg | 5 TU | 0.1 µg | 0.1 µg | 2 TU |
| Induration | 5 | 10 | 16 | 2 | 4 | 16 (1-AE) |
| Erythema | 11 | 8 | 17 (1-AE) | 4 | 6 | 17 (1-AE) |
| Pruritis (itching) | 4 | 5 | 9 | 1 | 1 | 5 |
| Pain | 1 | 1 | 5 | 1 | 0 | 3 |
| Bleeding | 0 | 0 | 0 | 1 | 0 | 0 |
| Urticaria (hives) | 0 | 0 | 0 | 1 | 0 | 0 |
| Infection | 0 | 0 | 0 | 1 | 0 | 0 |
| Blistering | 0 | 0 | 1 | 1 | 0 | 1 (1-AE) |
| ***Total No. Sites*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** |
| **Total No. Events** | **21** | **24** | **48** | **12** | **11** | **42** |
| **Total No. AE** | **0** | **0** | **1** | **0** | **0** | **3** |

[a] Maximum reactogenicity is the no. of subjects exhibiting specific reactions across all visits.

**Maximum Reactogenicity[a] by Subject Across all Visits (TB)**

| TB | No. Subjects with Reactions Across All Study Visits | | | | | |
|---|---|---|---|---|---|---|
| | Stage C-1a | | | Stage C-1b | | |
| Reactions | MLSA-LAM | MLCwA | PPD | MLSA-LAM | MLCwA | PPD |
| | 1.0 µg | 1.0 µg | 5 TU | 0.1 µg | 0.1 µg | 2 TU |
| Induration | 8 | 9 | 18 | 0 | 3 | 19 |
| Erythema | 12 | 14 | 20 (2-AE) | 2 | 5 | 20 (1-AE) |
| Pruritis (itching) | 1 | 6 | 14 | 0 | 0 | 12 |
| Pain | 0 | 2 | 9 | 0 | 0 | 3 |
| Bleeding | 0 | 0 | 0 | 0 | 0 | 0 |
| Urticaria (hives) | 1 | 0 | 0 | 0 | 0 | 0 |
| Infection | 1 | 0 | 0 | 0 | 0 | 0 |
| Blistering | 1 | 0 | 0 | 0 | 0 | 0 |
| ***Total No. Sites*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** | ***20*** |
| **Total No. Events** | **24** | **31** | **61** | **2** | **8** | **54** |
| **Total No. AE** | **0** | **0** | **2** | **0** | **0** | **1** |

[a] Maximum reactogenicity is the no. of subjects exhibiting specific reactions across all visits.

**Adverse Events Classified by MedDRA®**

| Stage | MedDRA® System Organ Class | Preferred Term | Number of AEs | Maximum Severity | Antigen Association | Relationship to Treatment |
|---|---|---|---|---|---|---|
| C-1a | Skin and subcutaneous tissue disorders | Induration > 30 mm | 1 | 1 | PPD | Associated |
| | | Erythema > 30 mm | 7 | 1 | PPD | Associated |
| | Injury, poisoning and procedural complications | Blistering | 2 | 1 | PPD | Associated |
| C-1b | Skin and subcutaneous tissue disorders | Induration > 30 mm | 1 | 1 | PPD | Associated |
| | | Erythema > 30 mm | 2 | 1 | PPD | Associated |
| | Injury, poisoning and procedural complications | Blistering | 1 | 1 | PPD | Associated |
| | Immune system disorders | Type I hypersensitivity reaction | 1 | 1 | None | Probably not associated |
| | **Total No. of AE** | | 15 | | | |

CONTINUED APPENDIX 3

**Stage C-1a (Antigen High Dose) Induration at 72 h**

| Stage C-1 a | BT/TT | | | | Stage C-1 a | BL/LL | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subject No. | MLCwA | MLSA-LAM | PPD | Rxn | Subject No. | MLCwA | MLSA-LAM | PPD | Rxn |
| 1 | 0 | 0 | 18 | *** | 1 | 0 | 20 | 0 | ** |
| 2 | 0 | 0 | 19.5 | *** | 2 | 0 | 0 | 0 | * |
| 3 | 0 | 0 | 0 | * | 3 | 0 | 0 | 29.5 | *** |
| 4 | 0 | 0 | 28.5 | *** | 4 | 0 | 0 | 28 | *** |
| 5 | 0 | 0 | 0 | * | 5 | 0 | 0 | 0 | * |
| 6 | 21.5 | 23 | 27.5 | **** | 6 | 0 | 0 | 11 | *** |
| 7 | 0 | 0 | 11.5 | *** | 7 | 0 | 0 | 0 | * |
| 8 | 0 | 0 | 28 | *** | 8 | 0 | 0 | 18 | *** |
| 9 | 0 | 0 | 0 | * | 9 | 0 | 0 | 19.5 | *** |
| 10 | 0 | 0 | 24 | *** | 10 | 0 | 0 | 16 | *** |
| 11 | 0 | 0 | 18.5 | *** | 11 | 0 | 0 | 11 | *** |
| 12 | 0 | 0 | 19.5 | *** | 12 | 0 | 0 | 22.5 | *** |
| 13 | 0 | 0 | 19.5 | *** | 13 | 0 | 0 | 0 | * |
| 14 | 0 | 0 | 16.5 | *** | 14 | 0 | 0 | 0 | * |
| 15 | 0 | 0 | 0 | * | 15 | 0 | 0 | 0 | * |
| 16 | 0 | 0 | 0 | * | 16 | 0 | 0 | 13.5 | *** |
| 17 | 0 | 0 | 13 | *** | 17 | 0 | 0 | 0 | * |
| 18 | 0 | 0 | 0 | * | 18 | 0 | 0 | 13 | *** |
| 19 | 20 | 19.5 | 0 | ** | 19 | 0 | 0 | 0 | * |
| 20 | 19 | 0 | 0 | ** | 20 | 0 | 0 | 16 | *** |
| Mean | 3.03 | 2.13 | 12.20 | | Mean | 0.00 | 1.00 | 9.90 | |
| Median | 0.00 | 0.00 | 14.75 | | Median | 0.00 | 0.00 | 11.00 | |
| Std Error | ± 1.66 | ± 1.47 | ± 2.48 | | Std Error | ± 0.00 | ± 1.00 | ± 2.30 | |
| 95% CI | ± 3.24 | ± 2.88 | ± 4.86 | | 95% CI | ± 0.00 | ± 1.96 | ± 4.51 | |

CONTINUED APPENDIX 3

| Stage C-1 a | HC | | | | Stage C-1 a | TB | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subject No. | MLCwA | MLSA-LAM | PPD | Rxn | Subject No. | MLCwA | MLSA-LAM | PPD | Rxn |
| 1 | 0 | 0 | 15.5 | *** | 1 | 14 | 8 | 20 | **** |
| 2 | 13 | 0 | 15.5 | **** | 2 | 14.5 | 0 | 15 | **** |
| **4** | 11.5 | 15.5 | 22.5 | **** | 3 | 0 | 0 | 22 | *** |
| 5 | 0 | 0 | 0 | * | 4 | 0 | 0 | 21 | *** |
| 6 | 0 | 0 | 18 | *** | 5 | 0 | 0 | 22 | *** |
| 7 | 0 | 0 | 16.5 | *** | 6 | 13 | 10 | 17 | **** |
| 8 | 11.5 | 0 | 8 | **** | 7 | 15.5 | 0 | 7.5 | **** |
| 9 | 0 | 5.5 | 11.5 | **** | 8 | 0 | 20 | 25 | **** |
| 10 | 15 | 0 | 26.5 | **** | 9 | 0 | 0 | 18.5 | *** |
| 11 | 0 | 0 | 11.5 | *** | 10 | 26.5 | 18 | 28 | **** |
| 12 | 16.5 | 15 | 16 | **** | 11 | 18.5 | 17.5 | 23 | **** |
| 13 | 17.5 | 0 | 9.5 | **** | 12 | 0 | 15.5 | 25 | **** |
| 14 | 0 | 0 | 21 | *** | 13 | 0 | 0 | 0 | * |
| 15 | 7.5 | 0 | 10.5 | **** | 14 | 0 | 0 | 17.5 | *** |
| 16 | 9.5 | 0 | 0 | ** | 15 | 10 | 0 | 18.5 | **** |
| 17 | 24 | 23.5 | 26 | **** | 16 | 10.5 | 10 | 18.5 | **** |
| 18 | 0 | 0 | 22.5 | *** | 17 | 0 | 0 | 0 | * |
| 19 | 0 | 0 | 0 | * | 18 | 9 | 0 | 24 | **** |
| 20 | 14.5 | 12.5 | 16 | **** | 19 | 0 | 0 | 16.5 | *** |
| 21 | 0 | 0 | 24 | *** | 20 | 0 | 0 | 22 | *** |
| Mean | 7.03 | 3.60 | 14.55 | | Mean | 6.58 | 4.95 | 18.05 | |
| Median | 3.75 | 0.00 | 15.75 | | Median | 0.00 | 0.00 | 19.25 | |
| Std Error | $\pm$ 1.76 | $\pm$ 1.58 | $\pm$ 1.84 | | Std Error | $\pm$ 1.84 | $\pm$ 1.66 | $\pm$ 1.70 | |
| 95% CI | $\pm$ 3.46 | $\pm$ 3.09 | $\pm$ 3.61 | | 95% CI | $\pm$ 3.61 | $\pm$ 3.25 | $\pm$ 3.32 | |

CONTINUED APPENDIX 3

**Stage C-1b (Antigen Low Dose) Induration at 72 h**

| Stage C-1 b | BT/TT | | | | Stage C-1 b | BL/LL | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subject No. | MLCwA | MLSA-LAM | PPD | Rxn | Subject No. | MLCwA | MLSA-LAM | PPD | Rxn |
| 21 | 0 | 0 | 0 | * | 21 | 0 | 0 | 9.5 | *** |
| 22 | 0 | 0 | 12 | *** | 22 | 0 | 0 | 24.5 | *** |
| 23 | 0 | 0 | 15 | *** | 23 | 0 | 0 | 18 | *** |
| 24 | 0 | 0 | 17 | *** | 24 | 0 | 0 | 23 | *** |
| 25 | 0 | 0 | 0 | * | 25 | 0 | 0 | 0 | * |
| 26 | 14.5 | 10.5 | 0 | ** | 26 | 0 | 0 | 0 | * |
| 27 | 0 | 0 | 20 | *** | 27 | 0 | 0 | 19.5 | *** |
| 28 | 0 | 0 | 22.5 | *** | 28 | 0 | 0 | 10 | *** |
| 29 | 0 | 0 | 25 | *** | 29 | 0 | 0 | 0 | * |
| 30 | 0 | 0 | 7 | *** | 30 | 0 | 0 | 0 | * |
| 31 | 20 | 18 | 16 | *** | 31 | 0 | 0 | 0 | * |
| 32 | 0 | 0 | 20 | *** | 32 | 0 | 0 | 0 | * |
| 33 | 0 | 0 | 21 | *** | 33 | 0 | 0 | 0 | * |
| 34 | 17.5 | 12.5 | 0 | ** | 34 | 0 | 0 | 0 | * |
| 35 | 0 | 0 | 22 | *** | 35 | 0 | 0 | 0 | * |
| 36 | 11 | 15 | 18 | **** | 36 | 0 | 0 | 0 | * |
| 37 | 0 | 0 | 0 | * | 37 | 0 | 0 | 25 | *** |
| 38 | 0 | 0 | 0 | * | 38 | 0 | 0 | 0 | * |
| 39 | 0 | 0 | 0 | * | 39 | 0 | 0 | 0 | * |
| 40 | 11 | 0 | 15.5 | **** | 40 | 0 | 0 | 0 | * |
| Mean | 3.70 | 2.80 | 11.55 | | Mean | 0.00 | 0.00 | 6.48 | |
| Median | 0.00 | 0.00 | 15.25 | | Median | 0.00 | 0.00 | 0.00 | |
| Std Error | 1.53 | 1.32 | 2.13 | | Std Error | 0.00 | 0.00 | 2.18 | |
| 95% CI | $\pm$ 2.99 | $\pm$ 2.58 | $\pm$ 4.16 | | 95% CI | $\pm$ 0.00 | $\pm$ 0.00 | $\pm$ 4.28 | |

CONTINUED APPENDIX 3

| Stage C-1 b | HC | | | | Stage C-1 b | TB | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subject No. | MLCwA | MLSA-LAM | PPD | Rxn | Subject No. | MLCwA | MLSA-LAM | PPD | Rxn |
| 22 | 0 | 0 | 21 | *** | 21 | 0 | 0 | 20 | *** |
| 23 | 0 | 0 | 0 | * | 22 | 0 | 0 | 17 | *** |
| 24 | 0 | 0 | 6.5 | *** | 23 | 0 | 0 | 17 | *** |
| 25 | 0 | 0 | 26 | *** | 24 | 6 | 0 | 20.5 | **** |
| 26 | 0 | 0 | 8 | *** | 25 | 0 | 0 | 19.5 | *** |
| 27 | 0 | 0 | 0 | * | 26 | 0 | 0 | 20.5 | *** |
| 28 | 0 | 0 | 16 | *** | 27 | 0 | 0 | 19 | *** |
| 29 | 0 | 0 | 9 | *** | 28 | 0 | 0 | 20 | *** |
| 30 | 0 | 0 | 19.5 | *** | 29 | 0 | 0 | 0 | * |
| 31 | 0 | 0 | 18 | *** | 30 | 0 | 0 | 17.5 | *** |
| 32 | 0 | 0 | 9.5 | *** | 31 | 0 | 0 | 25 | *** |
| 33 | 0 | 0 | 40 | *** | 32 | 0 | 0 | 28.5 | *** |
| 34 | 10 | 11.5 | 20 | **** | 33 | 0 | 0 | 17.5 | *** |
| 35 | 0 | 0 | 15 | *** | 34 | 0 | 0 | 16.5 | *** |
| 36 | 5 | 0 | 15 | *** | 35 | 0 | 0 | 14 | *** |
| 37 | 0 | 0 | 20.5 | *** | 36 | 9 | 0 | 20 | **** |
| 38 | 0 | 0 | 0 | * | 37 | 0 | 0 | 21 | *** |
| 39 | 0 | 0 | 0 | * | 38 | 0 | 0 | 25 | *** |
| 40 | 15 | 10 | 18.5 | **** | 39 | 0 | 0 | 25 | *** |
| 41 | 16 | 0 | 18.5 | **** | 40 | 10 | 0 | 30 | **** |
| Mean | 2.30 | 1.08 | 14.05 | | Mean | 1.25 | 0.00 | 19.68 | |
| Median | 0.00 | 0.00 | 15.50 | | Median | 0.00 | 0.00 | 20.00 | |
| Std Error | $\pm$ 1.15 | $\pm$ 0.75 | $\pm$ 2.27 | | Std Error | $\pm$ 0.70 | $\pm$ 0.00 | $\pm$ 1.38 | |
| 95% CI | $\pm$ 2.25 | $\pm$ 1.45 | $\pm$ 4.45 | | 95% CI | $\pm$ 1.37 | $\pm$ 0.00 | $\pm$ 2.71 | |

Response patterns for individual subjects have been marked (*) no reaction to either intervention or PPD, (**) reaction to one or both interventions, but not to PPD, (***) reaction to PPD only, and (****) reaction to one or both intervention and PPD.

CONTINUED APPENDIX 3

**Number of Positive Responders and Mean Induration Compared to Endemic Controls**

| Antigen Dose | Test Group | Number of Subjects | | | | Mean Induration | | |
|---|---|---|---|---|---|---|---|---|
| | | No. | (%) | Ratio | Proportion | Mean | Ratio | Proportion |
| MLSA-LAM Low Dose | EC | 2/50 | 4.0 | na | na | 0.2 | na | na |
| | BT/TT | 4/20 | 20.0 | 20.0/4.0 | 5.0 | 2.8 | 2.8/0.2 | 14.0 |
| | BL/LL | 0/20 | 0.0 | 0.0/4.0 | 0.0 | 0.0 | 0.0/0.2 | 0.0 |
| | HC | 2/20 | 10.0 | 10.0/4.0 | 2.5 | 1.1 | 1.1/0.2 | 5.5 |
| | TB | 0/20 | 0.0 | 0.0/4.0 | 0.0 | 0.0 | 0.0/0.2 | 0.0 |
| MLCwA Low Dose | EC | 0/50 | 1.0[a] | na | na | 0.1 [a] | na | na |
| | BT/TT | 5/20 | 25.0 | 25.0/1.0 | 25.0 | 3.7 | 3.7/0.1 | 37.0 |
| | BL/LL | 0/20 | 0.0 | 0.0/1.0 | 0.0 | 0.0 | 0.0/0.1 | 0.0 |
| | HC | 4/20 | 20.0 | 20.0/1.0 | 20.0 | 2.3 | 2.3/0.1 | 23.0 |
| | TB | 3/20 | 15.0 | 15.0/1.0 | 15.0 | 1.3 | 1.3/0.1 | 13.0 |
| MLSA-LAM High Dose | EC | 10/50 | 20.0 | na | na | 2.1 | na | na |
| | BT/TT | 2/20 | 10.0 | 10.0/20.0 | 0.5 | 2.1 | 2.1/2.1 | 1.0 |
| | BL/LL | 1/20 | 5.0 | 5.0/20.0 | 0.3 | 1.0 | 1.0/2.1 | 0.48 |
| | HC | 5/20 | 25.0 | 25.0 /20.0 | 1.3 | 3.6 | 3.6/2.1 | 1.7 |
| | TB | 7/20 | 35.0 | 35.0 /20.0 | 1.8 | 5.0 | 5.0/2.1 | 2.4 |
| MLCwA High Dose | EC | 8/50 | 16.0 | na | na | 1.7 | na | na |
| | BT/TT | 3/20 | 15.0 | 15.0/16.0 | 0.9 | 3.0 | 3.0/1.7 | 1.8 |
| | BL/LL | 0/20 | 0.0 | 0.0/16.0 | 0.0 | 0.0 | 0.0/1.7 | 0.0 |
| | HC | 10/20 | 50.0 | 50.0/16.0 | 3.1 | 7.0 | 7.0/1.7 | 4.1 |
| | TB | 9/20 | 45.0 | 45.0/16.0 | 2.8 | 6.6 | 6.6/1.7 | 3.9 |

[a] To allow calculations, the EC percent positive has been changed to 1.0.

CONTINUED APPENDIX 3

**Whole Blood IGRA Data**

**Stage C-1a (Antigen High Dose)**

| **Stage C-1a** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TD** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 1 | 0 | 0 | 1.22 | 0 | 0 | 0 | 1.22 |
| 2 | 0 | 0 | 5.32 | 0 | 0 | 0 | 5.32 |
| 3 | 0 | 0 | 0.66 | 0 | 0 | 0 | 0.66 |
| 4 | 0 | 0.17 | 8.37 | 0 | 0 | 0.17 | 8.37 |
| 5 | 1.28 | 1.18 | 2.05 | 0 | 1.28 | 1.18 | 2.05 |
| 6 | 9.11 | 8.41 | 9.99 | 0.83 | 8.28 | 7.58 | 9.16 |
| 7 | 1.52 | 1.53 | 9.99 | 0.49 | 1.03 | 1.04 | 9.5 |
| 8 | 9.99 | 9.99 | 9.99 | 9.999 | 0 | 0 | 0 |
| 9 | 0.31 | 0.49 | 1.44 | 0.51 | -0.2 | -0.02 | 0.93 |
| 10 | 0.25 | 0.13 | 7.43 | 0 | 0.25 | 0.13 | 7.43 |
| 11 | 0.48 | 0.69 | 3.84 | 0.54 | 0 | 0.15 | 3.3 |
| 12 | 0.63 | 0.78 | 9.99 | 0.55 | 0.08 | 0.23 | 9.44 |
| 13 | 0.52 | 0.33 | 1.17 | 0.82 | -0.3 | -0.49 | 0.35 |
| 14 | 0.48 | 0.55 | 1.38 | 0.47 | 0.01 | 0 | 0.91 |
| 15 | 0.31 | 0.35 | 0 | 0.15 | 0.16 | 0.2 | -0.15 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 1.09 | 0 | 0 | 0 | 1.09 |
| 18 | 1.99 | 5.69 | 2.72 | 1.52 | 0.47 | 4.17 | 1.2 |
| 19 | 0.58 | 0.94 | 1.28 | 0 | 0.58 | 0.94 | 1.28 |
| 20 | | | | | | | |
| Mean | | | | | 0.61 | 0.80 | 3.3 |
| Median | | | | | 0.00 | 0.13 | 1.20 |
| Std Error | | | | | ± 0.44 | ± 0.44 | ± 0.83 |
| 95% CI | | | | | ± 0.90 | ± 0.91 | ± 1.75 |

CONTINUED APPENDIX 3

| Stage C-1a | | | | | | | |
|---|---|---|---|---|---|---|---|
| **LP** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 1 | 0 | 0 | 9.99 | 0 | 0 | 0 | 9.99 |
| 2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 3 | 0.67 | 0.13 | 9.99 | 0.59 | 0 | -0.46 | 9.4 |
| 4 | 0.15 | 0 | 9.99 | 0 | 0.15 | 0 | 9.99 |
| 5 | 1.52 | 0.75 | 5.54 | 0.2 | 1.32 | 0.55 | 5.34 |
| 6 | 3.09 | 2.1 | 9.99 | 2.64 | 0.45 | -0.54 | 7.35 |
| 7 | 0.66 | 1 | 0.98 | 0.54 | 0.12 | 0.46 | 0.44 |
| 8 | 0.52 | 0.4 | 3.74 | 0.27 | 0.25 | 0.13 | 3.47 |
| 9 | 0.12 | 0 | 0.76 | 0 | 0.12 | 0 | 0.76 |
| 10 | 0.44 | 0.28 | 0.58 | 0.16 | 0.28 | 0.12 | 0.42 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 1.18 | 1.04 | 5.3 | 0.67 | 0.51 | 0.37 | 4.63 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0.25 | 0.25 | 0.24 | 0.45 | -0.2 | -0.2 | -0.21 |
| 16 | 1.21 | 0.65 | 2.41 | 1.2 | 0.01 | -0.55 | 1.21 |
| 17 | 0.14 | 0.16 | 0.55 | 0 | 0.14 | 0.16 | 0.55 |
| 18 | 0.29 | 0.27 | 6.38 | 0.21 | 0.08 | 0.06 | 6.17 |
| 19 | | | | | | | |
| 20 | 0.42 | 0.36 | 6.72 | 0.52 | -0.1 | -0.16 | 6.2 |
| Mean | | | | | 0.16 | 0.00 | 3.50 |
| Median | | | | | 0.08 | 0.00 | 1.20 |
| Std Error | | | | | ± 0.08 | ± 0.07 | ± 0.86 |
| 95% CI | | | | | ± 0.16 | ± 0.15 | ± 1.80 |

CONTINUED APPENDIX 3

| Stage C-1a | | | | | | | |
|---|---|---|---|---|---|---|---|
| **HC** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 1 | 5.62 | 4.93 | 9.99 | 1.26 | 4.36 | 3.67 | 8.73 |
| 2 | 0.64 | 0.7 | 3.04 | 0 | 0.64 | 0.7 | 3.04 |
| 4 | 2.42 | 1.48 | 9.99 | 0.67 | 1.75 | 0.81 | 9.32 |
| 5 | 0 | 0 | 0.53 | 0.16 | -0.16 | -0.16 | 0.37 |
| 6 | 0.12 | 0 | 3.98 | 0 | 0.12 | 0 | 3.98 |
| 7 | 0.57 | 0 | 0.4 | 0.28 | 0.29 | -0.28 | 0.12 |
| 8 | 1.47 | 2.06 | 3.09 | 0.93 | 0.54 | 1.13 | 2.16 |
| 9 | 0 | 0 | 0.3 | 0 | 0 | 0 | 0.3 |
| 10 | 0.24 | 0 | 0 | 0.71 | -0.47 | -0.71 | -0.71 |
| 11 | 0.32 | 0.54 | 0.97 | 0.16 | 0.16 | 0.38 | 0.81 |
| 12 | 0.56 | 0.35 | 1.23 | 0.13 | 0.43 | 0.22 | 1.1 |
| 13 | 0.67 | 0.58 | 2.61 | 0.14 | 0.53 | 0.44 | 2.47 |
| 14 | 0.16 | 0.19 | 2.6 | 0 | 0.16 | 0.19 | 2.6 |
| 15 | 0.89 | 1.11 | 5.48 | 0.45 | 0.44 | 0.66 | 5.03 |
| 16 | 9.99 | 0.55 | 1.31 | 0.46 | 9.53 | 0.09 | 0.85 |
| 17 | 2.87 | 3.02 | 9.99 | 0.35 | 2.52 | 2.67 | 9.64 |
| 18 | 0.41 | 0.43 | 3.48 | 0.37 | 0.04 | 0.06 | 3.11 |
| 19 | 0.64 | 0.35 | 1.13 | 0.67 | -0.03 | -0.32 | 0.46 |
| 20 | 0 | 0.12 | 0.13 | 0.22 | -0.22 | -0.1 | -0.09 |
| 21 | 0.28 | 0.24 | 1.04 | 0.13 | 0.15 | 0.11 | 0.91 |
| Mean | | | | | 1.00 | 0.48 | 2.70 |
| Median | | | | | 0.23 | 0.15 | 1.60 |
| Std Error | | | | | ± 0.51 | ± 0.23 | ± 0.71 |
| 95% CI | | | | | ± 1.07 | ± 0.48 | ± 1.50 |

CONTINUED APPENDIX 3

| Stage C-1a | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TB** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 1 | 0 | 0 | 3.36 | 1.19 | -1.19 | -1.19 | 2.17 |
| 2 | 0.15 | 0.13 | 0.64 | 0.16 | -0.01 | -0.03 | 0.48 |
| 3 | 0.23 | 0.4 | 8.99 | 0.27 | -0.04 | 0.13 | 8.72 |
| 4 | 0.17 | 0.14 | 5.64 | 0.19 | -0.02 | -0.05 | 5.45 |
| 5 | 9.99 | 0.31 | 9.99 | 0.35 | 9.64 | -0.04 | 9.64 |
| 6 | 0.5 | 0.5 | 5.19 | 0.81 | -0.31 | -0.31 | 4.38 |
| 7 | 0.18 | 0.2 | 0.68 | 0.21 | -0.03 | 0 | 0.47 |
| 8 | 0.28 | 0.28 | 2.94 | 0.26 | 0.02 | 0.02 | 2.68 |
| 9 | 2.4 | 3.94 | 4.84 | 5.13 | -2.73 | -1.19 | -0.29 |
| 10 | 0.34 | 0.41 | 2.56 | 0.41 | -0.07 | 0 | 2.15 |
| 11 | 1.72 | 2.51 | 9.99 | 1.53 | 0.19 | 0.98 | 8.46 |
| 12 | 0.4 | 0.33 | 1.13 | 0.41 | 0.00 | -0.08 | 0.72 |
| 13 | 0.49 | 0.35 | 0.42 | 1.25 | -0.76 | -0.9 | -0.83 |
| 14 | 0.21 | 0.2 | 0.34 | 0.23 | -0.02 | -0.03 | 0.11 |
| 15 | 0.4 | 0.36 | 3.22 | 0.27 | 0.13 | 0.09 | 2.95 |
| 16 | 0.41 | 0.42 | 3.46 | 0.29 | 0.12 | 0.13 | 3.17 |
| 17 | 3.89 | 2.28 | 9.69 | 3.74 | 0.15 | -1.46 | 5.95 |
| 18 | 0.22 | 0.22 | 2.49 | 0.22 | 0 | 0 | 2.27 |
| 19 | 0.27 | 0.48 | 0.92 | 0.76 | -0.49 | -0.28 | 0.16 |
| 20 | 0.22 | 0.17 | 2.15 | 0.2 | 0.02 | -0.03 | 1.95 |
| Mean | | | | | 0.23 | -0.21 | 3.0 |
| Median | | | | | -0.15 | -0.03 | 2.2 |
| Std Error | | | | | ± 0.52 | ± 0.13 | ± 0.70 |
| 95% CI | | | | | ± 1.08 | ± 0.27 | ± 1.45 |

**Stage C-1b (Antigen Low Dose)**

| Stage C-1b | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TD** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 21 | | | | | | | |
| 22 | | | | | | | |
| 23 | 1.76 | 1.41 | 15 | 0.22 | 1.54 | 1.19 | 14.78 |
| 24 | 0.54 | 0.43 | 3.14 | 0.23 | 0.31 | 0.2 | 2.91 |
| 25 | 1.47 | 1.44 | 3.06 | 0.12 | 1.35 | 1.32 | 2.94 |
| 26 | 15 | 6.21 | 12 | 0.25 | 14.75 | 5.96 | 11.75 |
| 27 | 1.46 | 1.84 | 15 | 0.12 | 1.34 | 1.72 | 14.88 |
| 28 | 7.6 | 6.92 | 15 | 0.12 | 7.48 | 6.8 | 14.88 |
| 29 | 6.62 | 8.61 | 15 | 0.12 | 6.5 | 8.49 | 14.88 |
| 30 | 1.48 | 1.44 | 2.51 | 0.12 | 1.36 | 1.32 | 2.39 |
| 31 | 15 | 15 | 3.32 | 0.12 | 14.88 | 14.88 | 3.2 |
| 32 | 0.12 | 0.12 | 11.51 | 0.12 | 0 | 0 | 11.39 |
| 33 | 0.98 | 1.65 | 10.38 | 0.21 | 0.77 | 1.44 | 10.17 |
| 34 | 3.21 | 44.04 | 0.25 | 0.12 | 3.09 | 43.92 | 0.13 |
| 35 | 0.61 | 1.79 | 15 | 0.12 | 0.49 | 1.67 | 14.88 |
| 36 | 15 | 14.75 | 15 | 0.12 | 14.88 | 14.63 | 14.88 |
| 37 | 3.76 | 5.68 | 4.48 | 0.12 | 3.64 | 5.56 | 4.36 |
| 38 | 4.72 | 3.24 | 1.18 | 0.18 | 4.54 | 3.06 | 1 |
| 39 | 2.03 | 1.31 | 0.77 | 0.15 | 1.88 | 1.16 | 0.62 |
| 40 | 0.67 | 0.6 | 2.71 | 0.16 | 0.51 | 0.44 | 2.55 |
| Mean | | | | | 4.41 | 6.32 | 7.92 |
| Median | | | | | 1.71 | 1.70 | 7.27 |
| Std Error | | | | | ± 1.23 | ± 2.46 | ± 1.43 |
| 95% CI | | | | | ± 2.60 | ± 5.19 | ± 3.02 |

CONTINUED APPENDIX 3

| Stage C-1b | | | | | | | |
|---|---|---|---|---|---|---|---|
| **LP** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 21 | 0.39 | 0.5 | 8.27 | 0.14 | 0.25 | 0.36 | 8.13 |
| 22 | 0.16 | 0.22 | 2.48 | 0.12 | 0.04 | 0.1 | 2.36 |
| 23 | 1.06 | 0.28 | 9.84 | 0.12 | 0.94 | 0.16 | 9.72 |
| 24 | 0.65 | 0.23 | 15 | 0.12 | 0.53 | 0.11 | 14.88 |
| 25 | 0.82 | 0.36 | 2.8 | 0.21 | 0.61 | 0.15 | 2.59 |
| 26 | 0.28 | 0.28 | 1.7 | 0.13 | 0.15 | 0.15 | 1.57 |
| 27 | 0.53 | 0.44 | 15 | 0.16 | 0.37 | 0.28 | 14.84 |
| 28 | 0.26 | 0.23 | 1.22 | 0.12 | 0.14 | 0.11 | 1.1 |
| 29 | 0.84 | 1.13 | 2.49 | 0.14 | 0.7 | 0.99 | 2.35 |
| 30 | | | | | | | |
| 31 | 2.24 | 2.21 | 5.7 | 0.14 | 2.1 | 2.07 | 5.56 |
| 32 | 1.63 | 2.06 | 4.96 | 0.12 | 1.51 | 1.94 | 4.84 |
| 33 | 0.48 | 0.68 | 0.48 | 0.12 | 0.36 | 0.56 | 0.36 |
| 34 | 0.14 | 0.23 | 1.45 | 0.14 | 0 | 0.09 | 1.31 |
| 35 | 0.92 | 0.49 | 2.24 | 0.19 | 0.73 | 0.3 | 2.05 |
| 36 | 0.12 | 0.12 | 0.12 | 0.12 | 0 | 0 | 0 |
| 37 | 0.9 | 0.9 | 15 | 0.27 | 0.63 | 0.63 | 14.73 |
| 38 | 2.26 | 1.43 | 10.27 | 0.19 | 2.07 | 1.24 | 10.08 |
| 39 | 0.98 | 1.21 | 3.06 | 0.31 | 0.67 | 0.9 | 2.75 |
| 40 | 0.12 | 0.12 | 0.12 | 0.12 | 0 | 0 | 0 |
| Mean | | | | | 0.62 | 0.53 | 5.22 |
| Median | | | | | 0.53 | 0.28 | 2.59 |
| Std Error | | | | | ± 0.15 | ± 0.14 | ± 1.20 |
| 95% CI | | | | | ± 0.31 | ± 0.30 | ± 2.52 |

CONTINUED APPENDIX 3

| Stage C-1b | | | | | | | |
|---|---|---|---|---|---|---|---|
| **HC** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 22 | 15 | 13.56 | 15 | 0.19 | 14.81 | 13.37 | 14.81 |
| 23 | 4.32 | 1.57 | 5.69 | 0.19 | 4.13 | 1.38 | 5.5 |
| 24 | | | | | | | |
| 25 | | | | | | | |
| 26 | | | | | | | |
| 27 | 3.03 | 1.75 | 5.18 | 0.12 | 2.91 | 1.63 | 5.06 |
| 28 | 6.61 | 6.15 | 15 | 0.2 | 6.41 | 5.95 | 14.8 |
| 29 | 1.22 | 0.85 | 1.66 | 0.12 | 1.1 | 0.73 | 1.54 |
| 30 | 1.36 | 1.9 | 7.85 | 0.38 | 0.98 | 1.52 | 7.47 |
| 31 | 3.35 | 2.79 | 15 | 0.12 | 3.23 | 2.67 | 14.88 |
| 32 | 1.19 | 0.81 | 1.88 | 0.12 | 1.07 | 0.69 | 1.76 |
| 33 | 3.85 | 8.14 | 15 | 0.12 | 3.73 | 8.02 | 14.88 |
| 34 | 2.99 | 3.23 | 15 | 0.12 | 2.87 | 3.11 | 14.88 |
| 35 | 3.36 | 2.43 | 9.14 | 0.13 | 3.23 | 2.3 | 9.01 |
| 36 | 6.38 | 0.92 | 3.32 | 0.12 | 6.26 | 0.8 | 3.2 |
| 37 | 0.84 | 0.56 | 3.72 | 0.12 | 0.72 | 0.44 | 3.6 |
| 38 | 3.5 | 1.9 | 3.25 | 0.44 | 3.06 | 1.46 | 2.81 |
| 39 | 1.73 | 1.71 | 1.81 | 0.12 | 1.61 | 1.59 | 1.69 |
| 40 | 5.83 | 5.74 | 15 | 0.37 | 5.46 | 5.37 | 14.63 |
| 41 | 5.68 | 12.45 | 7.16 | 0.25 | 5.43 | 12.2 | 6.91 |
| Mean | | | | | 3.94 | 3.72 | 8.08 |
| Median | | | | | 3.23 | 1.63 | 6.91 |
| Std Error | | | | | ± 0.81 | ± 0.97 | ± 1.34 |
| 95% CI | | | | | ± 1.72 | ± 2.06 | ± 2.83 |

CONTINUED APPENDIX 3

| Stage C-1b | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TB** | **Raw Data (IFN-γ IU/ml)** | | | | **Media Subtracted (IFN-γ IU/ml)** | | |
| **Subject No.** | **MLCwA** | **MLSA-LAM** | **PPD** | **Aim V** | **MLCwA** | **MLSA-LAM** | **PPD** |
| 21 | | | | | | | |
| 22 | | | | | | | |
| 23 | | | | | | | |
| 24 | 10.58 | 4.27 | 15 | 0.29 | 10.29 | 3.98 | 14.71 |
| 25 | 1.6 | 1.06 | 6.28 | 0.2 | 1.4 | 0.86 | 6.08 |
| 26 | 1.08 | 2.02 | 15 | 0.26 | 0.82 | 1.76 | 14.74 |
| 27 | 6.74 | 2.8 | 15 | 0.14 | 6.6 | 2.66 | 14.86 |
| 28 | 1.3 | 2.09 | 6.9 | 0.24 | 1.06 | 1.85 | 6.66 |
| 29 | 0.69 | 0.57 | 4.28 | 0.12 | 0.57 | 0.45 | 4.16 |
| 30 | 0.44 | 0.46 | 2.14 | 0.12 | 0.32 | 0.34 | 2.02 |
| 31 | 15 | 4.74 | 15 | 0.13 | 14.87 | 4.61 | 14.87 |
| 32 | 14.38 | 5.43 | 15 | 0.3 | 14.08 | 5.13 | 14.7 |
| 33 | 0.83 | 1 | 9.91 | 0.15 | 0.68 | 0.85 | 9.76 |
| 34 | 1.07 | 0.92 | 5.1 | 0.19 | 0.88 | 0.73 | 4.91 |
| 35 | 0.75 | 0.94 | 6.63 | 0.15 | 0.6 | 0.79 | 6.48 |
| 36 | 2.14 | 2.57 | 15 | 0.22 | 1.92 | 2.35 | 14.78 |
| 37 | 1.98 | 1.37 | 15 | 0.27 | 1.71 | 1.1 | 14.73 |
| 38 | 0.4 | 0.37 | 3.83 | 0.12 | 0.28 | 0.25 | 3.71 |
| 39 | 0.12 | 0.12 | 1.22 | 0.12 | 0 | 0 | 1.1 |
| 40 | 9.64 | 8.41 | 15 | 0.19 | 9.45 | 8.22 | 14.81 |
| Mean | | | | | 3.86 | 2.11 | 9.59 |
| Median | | | | | 1.06 | 1.10 | 9.76 |
| Std Error | | | | | ± 1.24 | ± 0.54 | ± 1.30 |
| 95% CI | | | | | ± 2.63 | ± 1.14 | ± 2.76 |

CONTINUED APPENDIX 3

**PGL-I Antibody Assay Data**

| Reactivity Scale | |
|---|---|
| 0 | negative |
| 1 | weak positive |
| 2 | moderate positive |
| 3 | strong positive |

**Stage C-1a (Antigen High Dose)**

| Stage C-1a – PGL Antibody Results | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TD** | | **LP** | | **HC** | | **TB** | |
| **Subject** | **PGL-I** | **Subject** | **PGL-I** | **Subject** | **PGL-I** | **Subject** | **PGL-I** |
| 1 | 2 | 1 | 1 | 1 | 3 | 1 | 1 |
| 2 | 2 | 2 | 3 | 2 | 0 | 2 | 3 |
| 3 | 2 | 3 | 1 | 4 | 1 | 3 | 3 |
| 4 | 2 | 4 | 3 | 5 | 0 | 4 | 1 |
| 5 | 0 | 5 | 3 | 6 | 1 | 5 | 3 |
| 6 | 3 | 6 | 3 | 7 | 1 | 6 | 1 |
| 7 | 1 | 7 | 3 | 8 | 1 | 7 | 1 |
| 8 | 3 | 8 | 2 | 9 | 3 | 8 | 1 |
| 9 | 3 | 9 | 3 | 10 | 2 | 9 | 3 |
| 10 | 0 | 10 | 3 | 11 | 1 | 10 | 3 |
| 11 | 0 | 11 | 3 | 12 | 1 | 11 | 1 |
| 12 | 3 | 12 | 3 | 13 | 0 | 12 | 0 |
| 13 | 0 | 13 | 3 | 14 | 1 | 13 | 3 |
| 14 | 0 | 14 | 3 | 15 | 0 | 14 | 3 |
| 15 | 0 | 15 | 3 | 16 | 0 | 15 | 0 |
| 16 | 0 | 16 | 3 | 17 | 0 | 16 | 1 |
| 17 | 2 | 17 | 3 | 18 | 0 | 17 | 3 |
| 18 | 1 | 18 | 3 | 19 | 0 | 18 | 0 |
| 19 | 2 | 19 | 3 | 20 | 1 | 19 | 1 |
| 20 | | 20 | 3 | 21 | 1 | 20 | 2 |
| Mean | 1.37 | | 2.75 | | 0.85 | | 1.7 |
| Median | 2 | | 3 | | 1 | | 1 |
| Std | 0.28 | | 0.14 | | 0.21 | | 0.26 |
| 95% CI | 0.58 | | 0.30 | | 0.44 | | 0.55 |

**Stage C-1b (Antigen Low Dose)**

| **Stage C-1b– PGL Antibody Results** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **TD** | | **LP** | | **HC** | | **TB** | |
| **Subject No.** | **PGL-I** | **Subject No.** | **PGL-I** | **Subject No.** | **PGL-I** | **Subject No.** | **PGL-I** |
| 21 | 0 | 21 | 0 | 22 | 3 | 21 | 0 |
| 22 | 0 | 22 | 3 | 23 | 0 | 22 | 0 |
| 23 | 2 | 23 | 3 | 24 | 0 | 23 | 0 |
| 24 | 3 | 24 | 0 | 25 | 0 | 24 | 2 |
| 25 | 0 | 25 | 3 | 26 | 1 | 25 | 0 |
| 26 | 0 | 26 | 3 | 27 | 0 | 26 | 1 |
| 27 | 3 | 27 | 3 | 28 | 0 | 27 | 0 |
| 28 | 0 | 28 | 3 | 29 | 0 | 28 | 0 |
| 29 | 3 | 29 | 3 | 30 | 1 | 29 | 0 |
| 30 | 0 | 30 | | 31 | 0 | 30 | 0 |
| 31 | 3 | 31 | 0 | 32 | 0 | 31 | 1 |
| 32 | 0 | 32 | 3 | 33 | 0 | 32 | 0 |
| 33 | 0 | 33 | 3 | 34 | 0 | 33 | 0 |
| 34 | 3 | 34 | 3 | 35 | 0 | 34 | 0 |
| 35 | 1 | 35 | 0 | 36 | 0 | 35 | 0 |
| 36 | 0 | 36 | 3 | 37 | 0 | 36 | 0 |
| 37 | 1 | 37 | 3 | 38 | 0 | 37 | 0 |
| 38 | 0 | 38 | 3 | 39 | 2 | 38 | 0 |
| 39 | 0 | 39 | 2 | 40 | 0 | 39 | 0 |
| 40 | 0 | 40 | 0 | 41 | 0 | 40 | 0 |
| Mean | 0.95 | | 2.16 | | 0.35 | | 0.20 |
| Median | 0.00 | | 3.00 | | 0.00 | | 0.00 |
| Std Error | 0.29 | | 0.31 | | 0.18 | | 0.12 |
| 95% CI | 0.62 | | 0.65 | | 0.38 | | 0.24 |

GLOSSARY

| Term | Definitions |
|---|---|
| CBER | One of six centers of the U.S. FDA under the U.S. DHHS. CBER is responsible for assuring the safety, purity, potency, and effectiveness of biologics (such as vaccines, blood products, and monoclonal antibodies) and related products. |
| CRF | Formal document created to record specific data during a clinical trial. CRFs are tools used to collect, verify, and compile data supporting a clinical study. |
| DTH Reaction | A delayed inflammatory reaction observed 48-72 hours after antigen exposure. $TH_1$ effector cells recognizes the antigen and release cytokines IL-2, IFN-γ, and TNF, which act on vascular endothelium causing erythema and recruitment of T-cells, phagocytes, fluid, and protein which causes a measurable Type IV DTH induration response. A lack of DTH response to recall antigen is evidence of anergy. |
| ISO | The international standards for organization provide recommendations and guidelines to ensure that products and services are safe, reliable and of good quality. They also provide strategic tools for business. |
| Lipoglycans | Macromolecules consisting of lipids and polysaccharides, derived from the cell envelope of mycobacteria or cell wall of gram-negative bacteria (LPS). Lipoglycans contain pathogen-associated molecular patterns (PAMPS) and are shown to be TLR2 agonists in mycobacteria and other related genera within the actinomycetales phylogenetic order. In mycobacteria, LAM, LM, and PIM are common lipoglycans extracted with TX-114 detergent. |
| Medra® | Medical dictionary consisting of terms used to classify adverse events resulting from the intervention of biopharmaceuticals and other medical products. Adverse events are coded to a standard set of Medra® system organ classification (SOC) terms for sharing and analysis of safety data among the biopharmaceutical industry. Medra® was created by the ICH and sustained by the Maintenance and Support Services Organization (MSSO). |

| | |
|---|---|
| Mitsuda Reaction | A granulomatous reaction observed 21 days after an intradermal injection of Lepromin (inactivated *M. leprae* suspension), first described by Kensuke Mitsuda in 1919. The Mitsuda Reaction is elicited in BT or TT leprosy patients, but not BL or LL leprosy patients. It is useful in classification, but not diagnosis of leprosy disease. |
| MOO | A document created for the clinical site that covers policies and procedures for execution of the clinical study. The manual contains descriptions of responsibilities, staff qualifications, list of authorities and resources, guidelines, and procedures for the safe and efficient conduct of the clinical research. |
| Sensitivity | Likelihood of detecting the presence of disease (number of true positives/number of true positives plus the number of false negatives), or the probability of a positive test when the subject has disease. Calculations are based on binomial classification using statistics. |
| Specificity | Likelihood to detect the absence of disease (number of true negatives/number of true negatives plus the number of false positives), or the probability of a negative test when the subject does not have disease. Calculations are based on binomial classification using statistics. |

LIST OF ABBREVIATIONS

| Acronymn | Full Name |
|---|---|
| AE | Adverse Event |
| ATCC | American Type Culture Collection |
| AUC | Area Under the Curve |
| BB | Borderline (Ridley Jopling Classification) |
| BCA | Bicinchoninic Acid |
| BCG | *Mycobacterium bovis*, strain Bacillus Calmette Guerin |
| BL | Borderline Lepromatous (Ridley Jopling Classification) |
| BT | Borderline Tuberculoid (Ridley Jopling Classification) |
| CBER | Center for Biological Evaluation and Research |
| CFR | Code of Federal Regulations |
| cGMP | current Good Manufacturing Practice |
| CMC | Chemistry Manufacturing Control |
| CMI | Cell Mediated Immunity |
| CMO | Contract Manufacturing Organization |
| CPE | Cytopathic Effect |
| CRF | Case Report Form |
| CSU | Colorado State University |
| DCC | Data Coordinating Center |
| DHHS | Department of Human Health Services |
| DTH | Delayed Type Hypersensitivity |
| EC | Endemic Control (Study Acronymn) |
| EPI | Electronic Password Information |
| FALGPA | N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala |
| FDA | Food and Drug Administration |
| FIT | Florida Institute of Technology |
| FPR | False Positive Rate |
| FWA | Federal Wide Assurance |
| GCP | Good Clinical Practice |
| GDP | Good Documentation Practice |
| GLP | Good Laboratory Practice |
| GMP | Good Manufacturing Practice |
| GroEL | Large Chaparone Protein from the GroE Operon |
| GroES | Small Chaparone Protein from the GroE Operon |
| GWLHDC | Gillis W. Long Hansen's Disease Center |
| HBV | Hepatitis B Virus |
| HC | Household Contact of BL/LL Leprosy Patient (Study Acronymn) |
| HHS | Health and Human Services |

| | |
|---|---|
| HIV | Human Immunodeficiency Virus |
| HVAC | Heating, Ventilation, and Air Conditioning |
| IAUCU | Institutional Animal Care & Use Committee |
| IFN-γ | Interferon gamma |
| IGRA | Interferon Gamma Release Assay |
| IND | Investigational New Drug |
| IRB | Institutional Review Board |
| ISM | Internal Safety Monitor |
| ISO | International Standards Organization |
| LAM | Lipoarabinomannan |
| LL | Lepromatous Leprosy (Ridley Jopling Classification) |
| LM | Lipomannan |
| LP | Lepromatous Leprosy Patient (Study Acronymn) |
| LSTA | Leprosy Skin Test Antigens |
| MAB | Monoclonal Antibody |
| MB | Multibacillary (WHO Leprosy Classification) |
| MDT | Multiple Drug Therapy |
| Medra® | Medical Dictionary for Regulatory Activities |
| MI | Morphological Index |
| MLCwA | *M. leprae* Cell Wall Associated Antigens devoid of lipoglycans |
| MLMA | *M. leprae* Membrane Antigens |
| MLMA-LAM | *M. leprae* Membrane Antigens devoid of lipoglycans |
| MLSA | *M. leprae* Soluble Antigen |
| MLSA-LAM | *M. leprae* Cytosolic Antigens devoid of lipoglycans |
| mm | millimeters |
| MMP-I | Major Membrane Protein - I |
| MOO | Manual of Operation |
| MRL | Mycobacterial Research Laboratories |
| NBT-BCIP | nitro blue tetrazolium - (5-bromo-4-chloro-3-indolyl-phosphate) |
| NCR | No Carbon Required |
| ND-O-HSA | Natural Disaccharide Octyl Bovine Serum Albumin |
| NHRC | Nepal Health Research Council |
| NIAID | National Institute of Allergy and Infectious Diseases |
| NIH | National Institutes of Health |
| NPV | Negative Predictive Value |
| OHRP | Office of Human Research Protections |
| PB | Paucibacillary (WHO Leprosy Classification) |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDM | Product Development and Manufacturing |
| PEG | Polyethylene Glycol |

| | |
|---|---|
| PGL-I | Phenolic Glycolipid-I |
| PHS | Public Health Services |
| PIM | Phosphatidylinositol Mannoside |
| PPD | Purified Protein Derivative |
| PPV | Positive Predictive Value |
| RMRCE | Rocky Mountain Regional Center of Excellence |
| ROC | Receiver Operating Characteristics |
| SA | Soluble Antigen |
| SAE | Serious Adverse Event |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| Se | Sensitivity |
| SMC | Safety Monitoring Committee |
| SOC | System Organ Class |
| SOD | Superoxide Dismutase |
| SOP | Standard Operating Procedure |
| Sp | Specificity |
| SPA | Soluble Protein Antigen |
| TB | Tuberculosis Patient (Study Acronymn) |
| TD | Tuberculoid Leprosy Patient (Study Acronymn) |
| TPR | True Positive Rate |
| TSA | Trypticase Soy Agar |
| TSB | Trypticase Soy Broth |
| TT | Tuberculoid Leprosy (Ridley Jopling Classification) |
| TU | Tuberculin Units |
| TX-114 | Triton X-114 |
| UCSF | University of California, San Francisco |
| WHO | World Health Organization |