EVALUATION OF THE ADJUVANTICITY OF ARTEMISININ WITH SOLUBLE LEISHMANIA MAJOR ANTIGENS IN BALB/c MICE

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN IMMUNOLOGY IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

OCTOBER 2009
DECLARATION

I, Albert Kimutai, do hereby declare that this thesis is my original work and has not been presented for a degree in any other University or for any other award

Signature _______________________________   Date_________________________

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DEDICATION

This thesis is dedicated to my beloved mother Susana Jemosop Sutter who passed on in the course of my studies, to my brother Edwin, wife Lydia and children Kiptoo and Kipkoech for their moral support and encouragement.
ACKNOWLEDGEMENTS

I would like to thank many individuals for their generous help and support that enabled me to complete this project and write this thesis.

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# TABLE OF CONTENTS

**TITLE PAGE**  
I  

**DECLARATION**  
II  

**DEDICATION**  
III  

**ACKNOWLEDGEMENTS**  
IV  

**TABLE OF CONTENTS**  
VII  

**LIST OF FIGURES**  
XII  

**LIST OF TABLES**  
XIII  

**LIST OF ACRONYMS AND ABBREVIATIONS**  
XIV  

**ABSTRACT**  
XVII  

## CHAPTER ONE: INTRODUCTION  
1  

1.1 Background information  
1  

1.2 Global distribution of leishmaniasis  
4  

1.3 Epidemiology of leishmaniasis in Kenya  
6  

1.4 Aetiology and clinical presentations of leishmaniasis  
7  

1.4.1 Visceral leishmaniasis  
7  

1.4.2 Cutaneous leishmaniasis  
8  

1.5 Research questions  
9  

1.6 Null hypothesis  
10  

1.7 Study objectives  
10  

1.7.1 General objective  
10  

1.7.2 Specific objectives  
10  

1.8 Significance of the study  
11  

1.9 Problem statement and justification of the study  
11
### CHAPTER TWO: LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 The biology of <em>Leishmania</em> parasites</td>
<td>13</td>
</tr>
<tr>
<td>2.1.1 Reservoir hosts</td>
<td>13</td>
</tr>
<tr>
<td>2.1.2 Sandfly vectors</td>
<td>13</td>
</tr>
<tr>
<td>2.1.3 The <em>Leishmania</em> life cycle</td>
<td>15</td>
</tr>
<tr>
<td>2.2 Immunity to <em>Leishmania major</em></td>
<td>16</td>
</tr>
<tr>
<td>2.3 Control of leishmaniasan</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Diagnosis of leishmaniasan</td>
<td>20</td>
</tr>
<tr>
<td>2.5 Chemotherapy of leishmaniasan</td>
<td>21</td>
</tr>
<tr>
<td>2.5.1 Treatment of cutaneous leishmaniasan</td>
<td>21</td>
</tr>
<tr>
<td>2.5.2 Treatment of visceral leishmaniasan</td>
<td>21</td>
</tr>
<tr>
<td>2.6 Vector and reservoir control</td>
<td>25</td>
</tr>
<tr>
<td>2.7 Vaccines against leishmaniasan</td>
<td>25</td>
</tr>
<tr>
<td>2.7.1 Leishmanization</td>
<td>25</td>
</tr>
<tr>
<td>2.7.2 Killed parasites</td>
<td>26</td>
</tr>
<tr>
<td>2.7.3 Live attenuated vaccines</td>
<td>27</td>
</tr>
<tr>
<td>2.7.4 Recombinant and synthetic vaccines</td>
<td>29</td>
</tr>
<tr>
<td>2.7.4.1 Expression of immunogens in bacteria and viruses</td>
<td>29</td>
</tr>
<tr>
<td>2.7.4.2 Synthetic peptides</td>
<td>32</td>
</tr>
<tr>
<td>2.7.4.3 Non protein antigens</td>
<td>32</td>
</tr>
<tr>
<td>2.7.4.4 Naked DNA vaccines</td>
<td>33</td>
</tr>
<tr>
<td>2.8 Soluble <em>Leishmania</em> antigens</td>
<td>33</td>
</tr>
<tr>
<td>2.9 Adjuvants in <em>Leishmania</em> vaccines</td>
<td>35</td>
</tr>
</tbody>
</table>
2.10 Artemisinin
   2.10.1 Introduction 38
   2.10.2 Metabolism of artemisinin drugs 41
   2.10.3 Mechanism of artemisinin drugs 42
   2.10.4 Antileishmanial activities of artemisinin and its derivatives 45
   2.10.5 Influences of artemisinin and its derivatives on immune function 45

CHAPTER 3: MATERIALS AND METHODS 48

3.1 Study site 48
3.2 Study design and experimental mice 48
3.3 *Leishmania major* parasites 48
3.4 Preparation of soluble *Leishmania* antigens 48
3.5 Artemisinin 49
3.6 Immunization of BALB/c mice and determination of lesion sizes 49
3.7 Infection of mice 50
3.8 Limiting dilution assay for quantification of *L. major* parasites 51
3.9 Cell cultures, proliferation of cytokine producing cells and measurement of cytokines 53
   3.9.1 Cell cultures 53
   3.9.2 Colorimetric assay for cell proliferation 53
   3.9.3 Cytokine assay 54
   3.9.1.0 Ethical and biosafety considerations 54
   3.9.1.2 Statistical analysis 55
REFERENCES

APPENDICES

Appendix I: Standard operating procedures

Appendix II: Cutaneous leishmaniasis lesion caused by *L. major*
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Map showing global distribution of leishmaniases</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>Life cycle of <em>Leishmania</em> parasites</td>
<td>15</td>
</tr>
<tr>
<td>2.2</td>
<td>Structure of artemisinin and its derivatives</td>
<td>40</td>
</tr>
<tr>
<td>2.3</td>
<td>Artemisia annua plant</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Subcutaneous infection of mice with <em>Leishmania major</em> parasites</td>
<td>51</td>
</tr>
<tr>
<td>3.2</td>
<td>Limiting dilution assay for quantification of <em>L. major</em> parasites</td>
<td>52</td>
</tr>
<tr>
<td>4.1</td>
<td>Proliferative responses of mouse splenocyte cultures before infection of mice with <em>Leishmania major</em></td>
<td>58</td>
</tr>
<tr>
<td>4.2</td>
<td>Proliferative responses of mouse splenocyte cultures after infection with <em>Leishmania major</em></td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Interferon gamma levels in supernatants of mouse splenocyte cultures and after infection with <em>Leishmania major</em></td>
<td>65</td>
</tr>
<tr>
<td>4.4</td>
<td>Interleukin-4 levels in supernatants of mouse splenocyte cultures before and after infection with <em>Leishmania major</em></td>
<td>68</td>
</tr>
<tr>
<td>4.5</td>
<td>Interleukin-5 levels in supernatants of mouse splenocyte cultures before and after infection with <em>Leishmania major</em></td>
<td>70</td>
</tr>
<tr>
<td>4.6</td>
<td>The course of <em>Leishmania major</em> infection in BALB/c mice</td>
<td>72</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Parasite burdens in footpads of <em>L. major</em> infected footpads of BALB/c</td>
<td>75</td>
</tr>
</tbody>
</table>
# Glossary of Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin Combination Therapy</td>
</tr>
<tr>
<td>ALM</td>
<td>Autoclaved <em>Leishmania major</em></td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette Guérin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>CBRD</td>
<td>Centre for Biotechnology Research and Development</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CP</td>
<td>Cysteine Proteinase</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine Phosphate Guanosine</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability Adjusted Year</td>
</tr>
<tr>
<td>DCL</td>
<td>Diffuse Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro diphenyl trichloroethane</td>
</tr>
<tr>
<td>DHA</td>
<td>Dihydroartemisinin</td>
</tr>
<tr>
<td>Dhf*ts</td>
<td>Dihydrofolate Reductase-thymidylate Synthetase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>gp 63</td>
<td>63 kilo Dalton Glycoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<td>-----------</td>
</tr>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
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</tr>
<tr>
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<td>Interleukin-4</td>
</tr>
<tr>
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<td>Interleukin-5</td>
</tr>
<tr>
<td>IL-10</td>
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</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ISCOMS</td>
<td>Immunostimulating Complexes</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>KO</td>
<td>Knock Out</td>
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<tr>
<td>LACK</td>
<td><em>Leishmania</em> Homologue of the mammalian Receptor for Activated C kinase</td>
</tr>
<tr>
<td>LeIF</td>
<td>Leishmanial Eukaryotic Ribosomal Protein</td>
</tr>
<tr>
<td>LCL</td>
<td>Localized Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>LPG</td>
<td>Lipophosphoglycan</td>
</tr>
<tr>
<td>LmSEAgS</td>
<td><em>Leishmania major</em> Soluble Exo-antigens</td>
</tr>
<tr>
<td>MCL</td>
<td>Mucocutaneous Leishmaniasis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2yl)-2, 5-Diphenyl Tetrazolium Bromide</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>OX40L-Fc</td>
<td>Chimeric Fusion Protein</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered Saline</td>
</tr>
<tr>
<td>pg/ml</td>
<td>Pico grams per millilitre</td>
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<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>PKDL</td>
<td>Post Kala-azar Dermal Leishmaniasis</td>
</tr>
<tr>
<td>PSA</td>
<td>Parasite Surface Antigen</td>
</tr>
<tr>
<td>rIL-12</td>
<td>Recombinant Interleukin-12</td>
</tr>
<tr>
<td>S-AcP</td>
<td>Secreted Acid Phosphatase</td>
</tr>
<tr>
<td>SLA</td>
<td>Soluble <em>Leishmania</em> Antigen</td>
</tr>
<tr>
<td>SOPs</td>
<td>Standard Operating Procedures</td>
</tr>
<tr>
<td>Th1</td>
<td>T Helper 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
</tr>
<tr>
<td>Th2</td>
<td>T Helper 2</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral Leishmaniasis</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
ABSTRACT

*Leishmania major* is an obligate intracellular protozoan parasite that causes chronic cutaneous lesions that often result in disfiguring scars. Artemisinin, a well known antimalarial drug, has been shown to be efficacious against *Leishmania* parasites both *in vivo* and *in vitro*. This study sought to determine the adjuvant potential of artemisinin when administered with a soluble leishmanial antigen. To test this hypothesis, seventy-two female BALB/c mice were randomly assigned into six treatment groups. The mice were vaccinated with soluble *Leishmania* antigens (SLA) alone, artemisinin co-administered with SLA, SLA and Bacille Calmette Guérin (BCG) vaccine and artemisinin alone on day 0 and boosted on day 13, then challenged with *Leishmania major* metacyclic promastigotes a week later. Unvaccinated mice formed the control group. The induction of cell-mediated immunity following vaccination was determined by measuring *ex vivo* lymphocyte proliferation and the production of interleukin (IL)-4, IL-5 and interferon gamma (IFN-γ) determined by flow cytometry. Mice receiving SLA plus artemisinin produced significantly high levels of IL-4 and IL-5 (*P*<0.05) and levels of IFN-γ level that did not differ significantly from those of the unvaccinated mice (*P*>0.05). Protection against *L. major* was determined by quantifying parasite burdens in *L. major* infected footpads using a limiting dilution assay and by measuring lesion sizes of the infected footpad as compared to the collateral uninfected footpad. Subcutaneous administration of SLA + artemisinin, artemisinin alone or SLA alone resulted in the development of large footpad swellings and high parasite loads that were comparable to those of the unvaccinated mice (*P*<0.05), resulting in exacerbated disease. These data suggest that artemisinin is not a suitable adjuvant for *Leishmania* vaccines. However, since artemisinin has been shown to be effective against *Leishmania* parasites *in vitro* and *in vivo*, further studies ought to be conducted to determine its immunochemotherapeutic potential when administered with *Leishmania* antigens.
CHAPTER ONE: INTRODUCTION

1.1 Background information

Leishmaniases are a group of diseases that are caused by obligate intracellular and kinetoplastid protozoa that belong to the *Leishmania* genus (family Trypanosomatidae) (Reithinger *et al*., 2007). The diseases are transmitted to humans by infected sandflies of the *Phlebotomous* genus in the Old World and *Lutzomyia* genus in the New World (Piscopo and Mallia, 2006). The leishmaniases have an important public health impact worldwide, with an estimated two million new cases per year and 350 million persons considered at risk of infection (World Health Organization, Leishmaniasis Control home page: [http://www.who.int/gb/ebwha/pdf_files/EB118/B118_4-en.pdf](http://www.who.int/gb/ebwha/pdf_files/EB118/B118_4-en.pdf)). With the advent of the Human Immunodeficiency Virus (HIV) epidemic, *Leishmania*/HIV co-infection is emerging as an extremely serious new disease with important clinical and epidemiological implications in many parts of the world (Alvar *et al*., 1997; Cruz *et al*., 2006).

In humans, infection with *Leishmania* parasites causes a wide spectrum of clinical manifestations ranging from localized cutaneous leishmaniasis (LCL) to mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL) (Garg and Dube, 2006; Akilov *et al*., 2007). Clinical outcomes depend on the parasite species and the host’s specific immune responses to *Leishmania* antigens (Goto and Lindoso, 2004; Roberts, 2006).

*Leishmania major* is the aetiological agent of Old World cutaneous leishmaniasis, a disease that is characterized by cutaneous lesions that can be self-resolving with life-long immunity or chronic when accompanied by defective cellular immune responses (Reed and Scott, 2000; Tonui *et al*., 2004a). Studies with the mouse model have demonstrated that protective
immunity against *L. major* in resistant mice strains such as C57BL/6, C3H and CBA is dependent on the ability to mount a CD4\(^+\) type 1 helper T-cell (Th1) response with interferon-gamma (IFN-\(\gamma\)) as the key effector cytokine able to activate macrophages to kill intracellular parasites (Reiner and Locksley, 1995; Noben-Trauth *et al*., 2003; Al-Wabel *et al*., 2007). In contrast, susceptibility to *L. major* as demonstrated by BALB/c mice has been attributed to a CD4\(^+\) type 2 helper T-cell (Th2) response associated with the production of interleukin(IL)-4 and IL-5 (Mahmoodi *et al*., 2005).

Control of leishmaniasis is currently based on chemotherapy to treat infected cases and on vector control to reduce transmission (Davies *et al*., 2003; Tonui *et al*., 2004a). To date, there are no proven vaccines against any form of leishmaniasis (Handman, 2001; Khamesipour *et al*., 2006). Treatment of the disease often involves the use of high doses of toxic pentavalent antimony compounds and various formulations of amphotericin B (Gicheru *et al*., 2001; Croft *et al*., 2006). However, the increasing prevalence of drug-resistant strains and the tendency for patients to relapse after an initially successful regimen of chemotherapy underscore the need for an effective prophylactic vaccine (Croft *et al*., 2006).

Abundant clinical and experimental evidence indicates that leishmaniasis should be preventable by vaccination (Handman, 2001; Requena *et al*., 2004; Murray *et al*., 2005). The only proven vaccine agent in human beings is live *L. major* (leishmanization), now discontinued because of unacceptable lesions in some recipients (Khamesipour *et al*., 2005). Current laboratory efforts are focused on novel antigens and adjuvants, live-attenuated vaccine, recombinant, purified and subunit proteins, naked deoxyribonucleic acid (DNA), bacteria expressing leishmanial antigens, and targeting dendritic cells (Coler and Reed, 2005; Ivens *et al*., 2005). Soluble *Leishmania* antigens with or without adjuvant have been tested as
potential vaccine candidates against leishmaniasis in both clinical and experimental studies with promising results (Scott et al., 1987; Ajdary et al., 2000; Rafati et al., 2000). These studies have further demonstrated that induction of immunity against leishmaniasis by vaccination with a Th1 promoting adjuvant and vaccine is a feasible strategy for control of the disease (Scott et al., 1987; Mahmoodi et al., 2003).

An adjuvant administered with an antigen influences the quantity and quality of the ensuing immune response (Tonui et al., 2004a). The adjuvants that have been tested in Leishmania vaccines such as Freund’s incomplete adjuvant and aluminium hydroxide are known to elicit mainly antibody and biased Th2 responses thus limiting their application in vaccines against Leishmania (Conacher et al., 2000). In addition, several other adjuvants such as recombinant interleukin-12 (rIL-12), BCG and Corynebacterium parvum studied in either experimental or clinical leishmaniasis, have been shown to elicit partially protective Th1 responses (Gicheru et al., 2001; Mahmoodi et al., 2003; Tonui et al., 2004a; Khamesipour et al., 2006). The search for effective Th1-promoting adjuvants is therefore an important priority.

Artemisinin, a natural component of the sweet wormwood plant Artemisia annua, has been used for over 2 000 years in traditional Chinese medicine for the treatment of fever (Woodrow et al., 2005). The drug is a sesquiterpene trioxane lactone, which contains an endoperoxide bridge essential for its biological activity (Klayman, 1985). Artemisinin has been shown conclusively to be effective in the treatment of drug resistant Plasmodium falciparum malaria (Abdin et al., 2003).

Artemisinin is hydrophobic and passes biological membranes easily (Woodrow et al., 2005). Another vital asset of artemisinin is its apparently excellent human safety and tolerability (Price et al., 1999). Artemisinin compounds have demonstrated efficacy against Leishmania
parasites achieving 50% killing at 750 nanomolar (nM) for *L. major* promastigotes, at 3 to 30 micromolar (µM) for intracellular amastigote stages in macrophages, and at 1.4 to 382.9 µM against *L. infantum* promastigotes (Yang et al., 1993; Avery et al., 2003). Artemisinin and its derivatives have also been shown to affect immune responses in a dose dependent manner (Golenser et al., 2006). Relatively low concentrations (up to 5 µM) of artemisinin, artesunate and Dihydroartemisinin (DHA) were shown to increase proliferation of concanavalin A (Con A) stimulated T cells and IL-2 production (Yang et al., 1993). In addition, cell-mediated immune functions were elevated in another study in mice with artesunate applied intramuscularly at a dose of 75 mg/Kg (Lin et al., 1995). On the other hand, concentrations above 50 µM, in the case of DHA, or 500 µM, in the case of artemisinin and artesunate, caused decreased proliferation, indicating a concentration-dependent effect (Yang et al., 1993). These studies suggest that artemisinin could be a valuable tool in the control of leishmaniasis. The purpose of this study was to determine the adjuvant potential of artemisinin when administered in combination with soluble *L. major* antigens in vaccinating BALB/c mice.

**1.2 Global distribution of leishmaniasis**

Leishmaniasis causes substantial clinical, public health and socioeconomic problems in endemic regions in more than 88 countries in the Indian sub continent, south western Asia, southern Europe, Africa, and central and south America (Desjeux, 2004; Fig. 1.1). There is a remarkable increase in risk factors for leishmaniases worldwide and the disease burden is increasing (Reithinger et al., 2007). Globally, there are an estimated 350 million people at risk of infection and disease, 14 million people are infected and 2 million new cases are reported each year (World Health Organization, Leishmaniasis Control home page: http://www.who.int/gb/ebwha/pdf_files/EB118/B118_4-en.pdf). The global burden of leishmaniasis has remained stable for some years, causing a morbidity and mortality loss of
2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Davies et al., 2003; Reithinger et al., 2007).

The global estimate for new cases of VL is 500,000 cases per year out of which 90% of the cases arise in just five countries-Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004). Each year, there are 1.5 million new cases of CL in more than 70 countries worldwide with 90% of the cases reported in Afghanistan, Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Syria (Ghalib and Modabber, 2007). On the other hand, 90% of all cases of MCL cases occur in Bolivia, Brazil and Peru (Desjeux, 2004).

In Africa, leishmaniasis is endemic to countries mostly in the north, central, east, the horn of Africa and West Africa (Boakye et al., 2005). Sudan is the most affected country, being one of the five countries that constitute 90% of all global cases of VL (Guerin et al., 2002). Algeria on the other hand is one of the eight countries that contribute 90% of worldwide cases of CL (Reithinger et al., 2007). The highest incidence of post kala-azar dermal leishmaniasis (PDKL) in the world is also found in Sudan (Ghalib and Modabber, 2007). Between 1984 and 1994, an epidemic in southern Sudan is thought to have had a mortality rate of 38-57%, killing 100,000 people (Seaman et al., 1996).
1.3 Epidemiology of leishmaniases in Kenya

In Kenya, both CL and VL are prevalent (Tonui, 2006). Leishmaniasis has been known to be endemic in the country from as far back as early in the 20th century (Fendall, 1961). An outbreak of VL was first reported among King’s African Rifles troops encamped north of Lake Turkana in the 1940s (Cole et al., 1942). Since then Turkana, Baringo, Kitui, West Pokot, Machakos, Meru, Keiyo and Marakwet districts have been considered to be endemic for VL with Baringo and West Pokot being considered as endemic foci (Tonui, 2006). The causative agent of VL in Kenya is *L. donovani* transmitted mainly by *Phlebotomous martini* (Wijers and Kiilu, 1984). In 2001, an outbreak of VL was reported in the previously non-endemic Wajir and Mandera districts of North Eastern Kenya where between May 2000 to August 2001, 904 patients were diagnosed with VL, with patients coming from as far as southern Somalia and southeast Ethiopia (Marlet et al., 2003).
In Kenya, CL is caused by *L. major*, *L. aethiopica* and *L. tropica* (Mebrahtu *et al.*, 1992). Cutaneous leishmaniasis due to *L. major* which is transmitted by *Phlebotomus duboscqi* is rare in humans, but underreporting is likely. Diffuse cutaneous leishmaniasis (DCL) was first reported in Kenya in 1969 in Bungoma district and the Mount Elgon area (Kungu *et al.*, 1972). In this region, *Leishmania aethiopica* has been identified as the aetiological agent, rodents as the animal reservoirs and *P. pedifer* as the vector of DCL (Mutinga, 1975; Sang and Chance, 1993; Ashford, 2000). Although various aspects of the transmission and control of leishmaniases have been studied in Kenya, the impact of the disease and particularly VL is still enormous (Tonui, 2006).

1.4 Aetiology and clinical presentations of leishmaniases

Approximately 21 out of 30 *Leishmania* species that infect mammals have been identified to be pathogenic to man (Singh, 2006).

1.4.1 Visceral leishmaniasis

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and is potentially fatal if left untreated (Garg and Dube, 2006). Parasites of the *L. donovani* complex are the typical aetiological agents of VL (Mukhopadhyay and Mandal, 2006). *Leishmania donovani* is the principal cause of VL in the Indian subcontinent and east Africa, *L. infantum* in the Mediterranean region and *L. chagasi* in the New World (Murray, 2001; Mukhopadhyay and Mandal, 2006). *Leishmania tropica* has been reported to produce visceral disease in immunocompromised persons while visceralization by *L. amazonensis* has also been reported (Magill *et al.*, 1993; Herwaldt, 1999). The cardinal symptoms of VL are prolonged fever, anemia, leukopenia, coughing, abdominal pain, diarrhea, epistaxis, splenomegaly, anemia, hepatomegaly, cachexia, pancytopenia and hypergammaglobulinaemia (Silva *et al.*, 2004;
Post-kala azar dermal leishmaniasis (PKDL) is a complication of VL (Zijlstra et al., 2003). The disease is characterized by macular, maculopapular or nodular skin lesions that usually spread from the peri-oral area to other areas of the body (Piscopo and Mallia, 2006). It is mainly seen in Sudan and India where it follows treated VL in 50% and 5–10% of cases, respectively (Zijlstra et al., 2003). Many PDKL patients heal spontaneously within 6 months but those who do not are difficult to treat, often requiring months of daily injections (Ghalib and Modabber, 2007). Post kala-azar dermal leishmaniasis patients harbour parasites in their skin and are believed to be an important reservoir of infection and possibly epidemics in endemic foci (Piscopo and Mallia, 2006).

### 1.4.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis (Desjeux, 2004). Multiple species produce CL in children and adults, primarily *L. major*, *L. tropica*, and *L. (L) aethiopica* (old world cutaneous leishmaniasis); *L. infantum* and *L. chagasi* (Mediterranean and Caspian Sea regions); and *L. mexicana*, *L. (L) amazonensis*, *L. braziliensis*, *L. Viannia panamensis*, *L. (V) peruviana*, and *L. (V) guyanensis* (New World Cutaneous Leishmaniasis) (Murray et al., 2005). The disease produces skin lesions mainly on the face, arms and legs (Akilov et al., 2007).

Localized cutaneous leishmaniasis (LCL) is the most common and the least drastic form of CL. The lesions are caused by *L. major*, *L. tropica*, *L. aethiopica*, and subspecies of *L. mexicana* (Murray et al., 2005). The first sign of an infection is typically a small erythema that develops after a variable prepatent period at the site where an infected sandfly has bitten
the host. The erythema develops into a papule, then a nodule that progressively ulcerates over a period of 2 weeks to 6 months to become the lesion that is characteristic of LCL (Akilov et al., 2007).

Diffuse cutaneous leishmaniasis (DCL) is a chronic, progressive, polyparasitic variant of CL that develops in context of leishmanial-specific anergy and is manifested by disseminated non-ulcerative skin lesions, which can resemble lesions of lepromatous leprosy (Aghaei et al., 2004). The lesions of DCL are very similar to those of LCL, except they are spread all over the body (Akilov et al., 2007). The body's immune system apparently fails to battle the protozoa, which are free to spread throughout the body (Handman, 2001).

Mucocutaneous leishmaniasis (MCL) or espundia as it is referred to in south America is a sequel of new world cutaneous leishmaniasis, where the initial skin lesion may cure but the parasites metastasize to mucous tissues by lymphatic or haematogenous dissemination (Reithinger et al., 2007). The disease can be caused by L. panamensis, L. guyanensis, L. amazonensis, L. major, L. tropica, and L. infantum but is most commonly associated with L. braziliensis thus, with some few exceptions; it is limited to south America (Piscopo and Mallia, 2006).

1.5 Problem statement and justification of the study
Infections caused by protozoa of the genus Leishmania are major worldwide problems with high endemicity in developing countries (Sharif et al., 2006). The situation has been aggravated by the fact that pentavalent antimonials, the drugs of choice for the treatment of leishmaniasis, are expensive, exhibit considerable toxicity, variable efficacy and recently, there is the emergence of antimony-resistant strains (Hadighi et al., 2006). The incidence of
leishmaniasis has also increased in the absence of an effective vaccine (Handman, 2001).

In leishmaniasis, the general consensus is that vaccines need to elicit Th1-type responses and adjuvants are likely important tools to help achieve this (Tonui et al., 2004a). Frequently used adjuvants like Freund’s incomplete adjuvant and aluminium hydroxide are known to induce mainly antibody and biased Th2 responses thus limiting their application in vaccines against *Leishmania* (Conacher et al., 2000). Several other adjuvants such as recombinant interleukin-12 (rIL-12), Bacille Calmette Guérin (BCG) and *Corynebacterium parvum* studied in either experimental or clinical leishmaniasis, have been shown to elicit partially protective Th1 responses (Gicheru et al., 2001; Mahmoodi et al., 2003; Tonui et al., 2004a; Khamesipour et al., 2006). The development of subunit vaccines for human vaccination against has also been hampered by the requirement for potentially toxic Th1-inducing adjuvants, which include BCG and IL-12 (Pinto et al., 2004). Other limitations associated with the use of BCG are the lack of batch-to-batch consistency, antigenic cross reactivity between *Mycobacteria* and *Leishmania* species (Smarskovski et al., 1977), and the suggestion that BCG immunotherapy may be a trigger mechanism for induction of autoimmune reactions (Convit et al., 1989; Sarples et al., 1994). The search for new, effective, safe and affordable Th1-promoting vaccine adjuvants against leishmaniasis therefore remains an important priority.

The therapeutic effects of artemisinin in *Leishmania* infected mice have been studied (Yang and Liew, 1993; Ying et al., 2004; Sharif et al., 2006). The immunostimulatory potential of artemisinin in normal mice has also been explored (Yang et al., 1993; Lin et al., 1995), and the drug has been shown to bias immunity at low concentrations towards cell-mediated immunity. This suggests that the drug could be of immunoprophylactic benefit against
leishmaniasis. The purpose of this study was to establish whether artemisinin potentiates the protective immunity of soluble *Leishmania* antigens and probably open new avenues for the development of new effective adjuvants for *Leishmania* vaccines.

### 1.6 Research questions

a) What are the levels of interleukin (IL)-4, IL-5 and interferon gamma (IFN-γ) in BALB/c mice following subcutaneous injections of artemisinin alone or in combination with soluble *Leishmania* antigens before and after challenge with *L. major* metacyclic promastigotes?

b) What is the effect of vaccination with either artemisinin alone or artemisinin co-administered with soluble *Leishmania* antigens on parasite burdens in *L. major* susceptible BALB/c mice?

c) What is the effect of vaccination with either artemisinin alone or artemisinin in combination with soluble *Leishmania* antigens on lesion sizes in *L. major* susceptible BALB/c mice?

### 1.7 Null hypothesis

Artemisinin does not enhance protective immune responses against *L. major* in susceptible BALB/c mice.

### 1.8 Study objectives

#### 1.8.1 General objective

To determine the adjuvant potential of artemisinin co-administered with soluble *Leishmania* antigens in susceptible BALB/c mice before and after challenge with *L. major*. 
1.8.2 Specific objectives

a) To determine levels of interleukin (IL)-4, IL-5 and interferon gamma (IFN-γ) following subcutaneous injections of artemisinin alone or in combination with soluble *Leishmania* antigens before and after challenge with *L. major* metacyclic promastigotes.

b) To determine parasite burdens in BALB/c mice that have been vaccinated with either artemisinin alone or artemisinin co-administered with soluble *Leishmania* antigens and then challenged with *L. major* metacyclic promastigotes.

c) To determine lesion sizes in BALB/c mice that had been vaccinated with either artemisinin alone or artemisinin co-administered with soluble *Leishmania* antigens and then challenged with *L. major* metacyclic promastigotes.

1.9 Significance of the study

The findings of this study will be useful in providing new insights on the potential of artemisinin as an adjuvant in *Leishmania* vaccines which could introduce new alternatives in the control of leishmaniasis.
CHAPTER TWO: LITERATURE REVIEW

2.1 The biology of Leishmania parasites

Leishmania parasites have a digenetic life cycle with an extracellular developmental stage in the female phlebotomine sandfly and a developmental stage in mammals, which is mostly intracellular (Roberts, 2006). In 1903, Leishman and Donovan separately described the protozoan now called Leishmania donovani in splenic tissue from VL patients in India (Ross, 1903). As a result, the amastigote stage seen in clinical samples is commonly known as Leishman-Donovan (LD) bodies (Singh, 2006).

2.1.1 Reservoir hosts

Leishmaniasis is primarily a zoonotic disease in which canines and rodents serve as reservoir hosts (Zijlstra and El-Hassan, 2001; Roberts, 2006). Domestic dogs by far play the most important role in harbouring and transmitting the disease to humans due to the close association between humans and dogs as pets (Arias et al., 1996). In anthroponotic VL due to L. donovani such as in India and Sudan, man is the principal reservoir host (Ghalib and Modabber, 2007). In these countries, asymptomatic carriers and PKDL patients are a particular source of infection for sandflies (Zijlstra et al., 2003).

2.1.2 Sandfly vectors

The only proven vector of the Leishmania parasite is the blood-sucking female of the genus Phlebotomous in the Old World and Lutzomyia in the New World (Murray et al., 2005). The insects are 2-3 mm long and are found throughout the tropical and temperate parts of the world. Only 30 or so of the over 500 species of Phlebotomine sandflies are known to transmit Leishmania parasites, these include P. argentipes on the Indian sub-continent, P. duboscqi, P. martini and P.orientalis in Africa and the Mediterranean basin, P. chinensis and P.
*alexandri* in China. In the new world *L. longipalpis* is the only known vector of *L. chagasi* (Murray *et al.*, 2005).

### 2.1.3 The *Leishmania* life cycle

In the vertebrate host, *Leishmania* parasites survive and multiply intracellularly in mononuclear phagocytes as tiny, ovoid to round, non-motile amastigotes about 3-5 µm in diameter (Singh, 2006). When a sandfly feeds on an infected host during a blood meal which is a requirement for oviposition; the fly ingests parasitized macrophages which are then released into the gut of the sandfly. The released amastigotes transform into the motile, elongated (10-20 µm), flagellate promastigote form (Roberts, 2006). The promastigotes then attach to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission and subsequently differentiate into the metacyclic promastigote stage that is infectious to the vertebrate host. By the time the sandfly takes a new blood meal, the metacyclics will have affected the feeding mechanism of the sandfly in such a way that they cause regurgitation of midgut content containing the parasites, into the feeding wound, thereby ensuring efficient transmission to the vertebrate host (Rogers *et al.*, 2002). A typical sandfly inoculum contains around $10^2$–$10^3$ metacyclic promastigotes (Roberts, 2006).

Although most promastigotes will be rapidly eliminated by the host via complement-mediated killing, some are opsonized and taken up by macrophages, neutrophils and dendritic cells where they rapidly revert to the amastigote form (Dominguez *et al.*, 2002). However, there’s evidence which suggests that cells other than mononuclear phagocytes, for example fibroblasts, may also harbour parasites (Solbach and Laskay, 2000). The *Leishmania* are able to resist the microbiocidal action of the acid hydrolases release from the lysozymes and so survive and multiply inside the macrophages, eventually leading to the lysis of the
macrophages (Olivier et al., 2005). The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow. The complete life cycle of *Leishmania* parasites is summarized below (Fig. 2.1).

Fig. 2.1: Life cycle of *Leishmania* spp (Adapted from Sacks and Noben-Trauth, 2002)
Occasionally, sandflies are not involved in transmission. Visceral leishmaniasis can be directly initiated by amastigotes via blood (shared needles, transfusion, and transplacental spread) or organ transplantation while CL can develop after inadvertent needlestick if the needle or syringe contains infected material (Singh et al., 1996; Alvar et al., 1997). Sexual transmission of leishmaniasis has also been implicated (Paredes et al., 2003).

2.2 Immunity to *Leishmania major*

Studies in mice and humans have demonstrated that acquired resistance to leishmaniasis is mediated by T cells (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). This view is supported by classical experiments which established that T-cell deficient mice rapidly succumb to inoculation with any one of several species of *Leishmania*, and that transfer of normal T cells confers resistance to the animals (Preston et al., 1972; Scott et al., 1988; Campos-Neto, 2005). Further evidence for the importance of cellular immune responses comes from the observation that in natural as well as experimental leishmaniasis, resistant individuals show low levels of specific antibodies and a high reactivity in delayed type hypersensitivity (DTH) reaction tests, whereas this is reversed in cases exhibiting clinical disease (Hale and Howard, 1981; Behforouz et al., 1983; Da-Cruz et al., 2002). Together, these have led to the view that cell-mediated but not humoral immunity is essential for protection against leishmaniasis.

Both CD4+ and CD8+ T cells have been shown to be important for protection in experimental leishmaniasis (Stern et al., 1988; Campos-Neto, 2005). Whereas the CD4+ subset of T cells is crucial for resistance, CD8+ T cells participate more in the memory events of the immune response than as effector cells involved in parasite elimination (Erb et al., 1996; Fowell et al., 1997). However, other studies have suggested that CD8+ T cells are also involved in the
clearance of primary infection (Belkaid et al., 2002a).

The most frequently studied model for leishmaniasis by far is that of *L. major* (Garg and Dube, 2006). Although not perfect, the spectrum of disease manifestations observed in human leishmaniasis can be mimicked in the laboratory by infection of inbred strains of mice with *L. major* (Handman, 2001). Studies to elucidate the immunologic pathways responsible for resistance or susceptibility to *L. major* in the murine model have given rise to the identification of the T-helper 1/T-helper 2 (Th1/Th2) dichotomy in the development of the immune response (Roberts, 2006). The subdivision of CD4⁺ cells into Th1 and Th2 is based on the pattern of cytokines the cells produce (Handman, 2001).

T helper 1 cells produce gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-12 and tumour necrosis factor alpha (TNF-α) which play a major role in protective immunity through macrophage activation while Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (De Krey et al., 1998; Roberts, 2006). More specifically, it is now a widely accepted fact that genetic predisposition for susceptibility or resistance to *L. major* infection in mice correlates with the dominance of an interleukin-4 (IL-4)-driven Th2 response that causes disease or an IL-12-driven interferon-γ (IFN-γ)-dominated Th1 response that promotes healing and parasite clearance, respectively (Sacks and Noben-Trauth, 2002). Evidence for the critical role for IFN-γ in the control of *Leishmania* infection comes from the demonstration that IFN-γ knockout (KO) mice fail to cure infection (Roberts, 2006). In addition, the apparent resolution of infection with *L. major* in BALB/c mice treated at the time of infection with an anti-IL-4 monoclonal antibody (Chatelain et al., 1992) or in IL-4-deficient BALB/c mice helped to establish the view that early production of IL-4 drives the polarized Th2 response that is responsible for suppressing Th1-cell development and inhibiting the high-level
secretion of IFN-γ that is required to activate infected macrophages for parasite killing (Kopf et al., 1996; Mohrs et al., 1999).

*Leishmania* amastigotes reside in phagolysosomes inside macrophages. For the host to be able to control *Leishmania* parasites, infected macrophages need to be activated to induce parasite killing through the production of reactive oxygen intermediates and nitric oxide (Serarslan and Atik, 2005). Several Th1 type cytokines (IFN-γ, TNF-α) are strong inducers of the macrophage enzyme inducible nitric oxide synthase (iNOS) that produce nitric oxide (Serarslan and Atik, 2005). Inbred strains of mice such as C57BL/6, C3H and CBA/N produce Th1 cytokine profiles and consequently are resistant to infection by *L. major* (Noben-Trauth et al., 2003). They develop small lesions which resolve in 10 to 12 weeks and are resistant to re-infection (Preston and Dumonde, 1976; Garg and Dube, 2006). On the other hand, BALB/c mice are highly susceptible; they mount a Th2 mediated immune response. Because macrophages are not activated to kill intracellular amastigotes, they develop severe and uncontrolled skin ulcers, which expand and metastasize; leading to death (De Krey et al., 1998). Most other strains of mice are intermediate in susceptibility (Preston and Dumonde, 1976).

Interferon-gamma is regulated by other cytokines such IL-10 and IL-12. Interleukin-10 inhibits the production of IFN-γ and thus IL-10 has been referred to as a cytokine that promotes the development of a Th2 response to *L. major* infection in mice (Noben-Trauth et al., 2003). Interleukin 12 has been shown to play a role in the optimal production of IFN-γ. Injection of recombinant IL-12 during the first week of infection with *L. major* in susceptible BALB/c mice resulted in the development of a Th1 response and allowed healing their lesions (Heinzel et al., 1993). Several observations have indicated that IL-13 is involved in
susceptibility to \emph{L. major} infection (Launois \emph{et al.}, 1998; Matthews \emph{et al.}, 2000). The use of IL-13 deficient mice and IL-13 transgenic mice indicates that IL-13 is important for the generation of the Th2 cells (Matthews \emph{et al.}, 2000).

Two other mechanisms of intracellular killing have been proposed involving destruction of infected macrophages by cytotoxic T lymphocytes (CTL) (Muller \emph{et al.}, 1991) and Fas Ligand (Fas-L)-mediated macrophage apoptosis (Huang \emph{et al.}, 1998). Other studies have suggested that susceptibility and resistance to \emph{Leishmania} infection is associated with the emergence of a unique subset of T cells, namely T regulatory cells (T reg) (Belkaid \emph{et al.}, 2002b).

\subsection*{2.3 Control of leishmaniasis}
Leishmaniasis is one of the most neglected tropical diseases in terms of the few available tools for control and the lack of criteria for methods of control (Davies \emph{et al.}, 2003). The diversity of epidemiological situations of leishmaniasis makes it virtually impossible for any single diagnosis, treatment or control measure to be suitable for all (Guerin \emph{et al.}, 2002). Control of leishmaniasis is currently based on chemotherapy to alleviate the disease through identification and treatment of infected cases and on vector control to reduce transmission (Handman, 2001). To date, there are no vaccines in routine use against the disease (Handman 2001; Khamesipour \emph{et al.}, 2006). However, there is consensus that in the longer term, vaccines ought to become a major tool in the control of this group of diseases (Handman, 2001; Requena \emph{et al.}, 2004).
2.4 Diagnosis of leishmaniasis

The clinical and epidemiological manifestations in various forms of leishmaniasis are nonpathognomonic and can mimic several other conditions; hence a laboratory diagnosis is necessary to confirm the clinical suspicions (Singh, 2006). The diagnostic tools used for each leishmanial syndrome may vary but the gold standard in each case remains to be the demonstration and isolation of the parasite from appropriate tissues (Singh and Sivakumar, 2003).

Parasitological diagnosis which includes microscopic examination of Giemsa-stained biopsy smears or aspirates, histopathological examination of fixed lesion biopsies, or culture of biopsy triturates or aspirates remains the gold standard in CL diagnosis because of its high specificity (Vega-Lopez, 2003). Culture and polymerase chain reaction (PCR) testing are technically difficult laboratory techniques that are not currently practical in developing countries (Reithinger et al., 2007). Serological diagnosis is rarely used in CL diagnosis because of variable sensitivity and specificity (Kar, 1995).

Diagnosis of VL is usually based on microscopic detection of amastigotes in smears of tissue aspirates or biopsy samples (Singh, 2006). An aspirate or biopsy of the bone marrow is frequently the tissue of choice because splenic aspirates may give rise to fatal bleeding in 2 out of 10,000 patients (Piscopo and Mallia, 2006; Singh, 2006). In other places, serum antileishmanial immunoglobulin G (IgG) in high titre is the diagnostic standard, primarily with direct agglutination test (DAT) (Zijlstra and El-Hassan, 2001). Freeze-dried antigen and rapid detection of anti-K39 antibody with finger stick blood in an immunochromatographic strip test have advanced field serodiagnosis (Murray et al., 2005). This test can safely substitute for invasive diagnostic procedures in Indian VL and is useful in PKDL (Sundar et al., 2002; Murray et al., 2005).
2.5 Chemotherapy of leishmaniasis

2.5.1 Treatment of cutaneous leishmaniasis

Old World CL is not a life-threatening disease and 90% of patients heal spontaneously within 3–18 months (Davies et al., 2003; Piscopo and Mallia, 2006). In New World CL, self-healing after 3 months is rapid in *L. mexicana* (75%) but slow in *L. braziliensis* (about 10%) and *L. panamensis* infections (about 35%) (Herwaldt et al., 1992; Navin et al., 1992; Soto et al., 2004).

Although non-fatal, CL is treated to accelerate cure to reduce scarring, especially in cosmetic sites, and to prevent parasite dissemination like the case of mucosal leishmaniasis or relapse (Reithinger, 2008). Treatment is commonly given for persistent (more than 6 months duration), multiple, or large lesions, and for lesions located on joints or on the face (Piscopo and Mallia, 2006).

Except for the immunotherapy policy in Venezuela (Convit, 1996), and the pentamidine treatment policies in French Guyana and Suriname (Lai et al., 2002; Roussel et al., 2006), WHO recommends treating CL with pentavalent antimonial drugs (sodium stibogluconate or meglumine antimoniate) at 20 mg/kg per day for 20–28 consecutive days (Reithinger et al., 2007). Barring one exception (Oliviera-Neto et al., 1997), the drug regimen has been shown to be more efficient than a daily dose of 10 mg/kg, 13 mg/kg or 15 mg/kg in treating LCL (Berman, 1997; Croft et al., 2006).

Treatment of MCL with antimonials is unsatisfactory, especially in severe disease (Franke et al., 1990). Amphotericin B and more recently liposomal amphotericin B have been used successfully in difficult cases while steroids may have to be used in patients in whom
respiratory compromise is possible (Sampaio et al., 1971; Amato et al., 2000).

2.5.2 Treatment of visceral leishmaniasis

Chemotherapy of VL relies on specific anti-leishmanial drugs and the aggressive management of any concomitant bacterial or parasitic infections, anemia, hypovolemia and malnutrition (Chappuis et al., 2007). The pentavalent antimonials sodium stibogluconate and meglumine antimoniate remain the therapeutic cornerstone of VL in all regions (Guerin et al., 2002; Murray, 2004) except two: Bihar State, India where the current approximate 35% cure response has ended the usefulness of antimony and southern Europe (Sundar et al., 2000; Murray et al., 2005).

Pentamidine proved to be an unsatisfactory substitute for antimony in India (Jha, 1983). However, conventional amphotericin B deoxycholate is highly effective, albeit an arduous treatment because of infusions, lengthy administration (20–30 days), and adverse reactions (Sundar et al., 2004; Thajur and Narayan, 2004). On the other hand, lipid formulations of amphotericin B, representing macrophage-targeted treatment, induce side effects much less frequently than the free drug and are very active in 5–10 day regimens (Murray et al., 2005). Despite short courses, the cost of these highly efficient agents restricts their use in developing countries (Murray, 2004).

Paromomycin, an aminoglycoside identical to aminosidine, has completed phase III testing in India and is being tested in east Africa (Croft et al., 2006). Once commercially available, paromomycin’s anticipated high-level efficacy, minimum toxicity, and low cost for the 21-day course is expected to provide an injectable alternative to amphotericin B in India and a potential substitute for antimony worldwide (Croft et al., 2006).
Miltefosine, the first effective oral treatment for VL, including for antimony-resistant infection, has opened the door to self-administered outpatient therapy; its rapid development in India heads the preceding list of tangible treatment advances (Berman, 2005). The drug, an alkylphospholipid which was approved in India 2002, Germany in 2004 and Colombia 2005 is administered in a 28-day course and has been shown to be active in adults and children, and common adverse gastrointestinal reactions are usually transient (Croft et al., 2006). Data on miltefosine use in east Africa are restricted to one study that was conducted in northern Ethiopia, in which it was found to be as safe and effective as sodium stibogluconate in HIV-negative patients and safer, but less effective, in HIV co-infected patients (Ritmeijer et al., 2006). Miltefosine is teratogenic in animals and should not be used in pregnant women; women of child-bearing age also need effective birth control during and for 2 months after treatment (Jha et al., 1999).

Other drugs that are currently under development include the 8-aminoquinoline derivative sitamaquine, currently in development with GlaxoSmithKline (Yeates, 2002). Limited Phase I/II clinical trials with varying levels of success in Brazil, Kenya and India (Dietze et al., 2001; Jha et al., 2005; Wasunna et al., 2005) have been reported. Imiquimod, an antiviral, has been shown to have an effect in experimental infections of CL (Buates and Matlashewski, 1999), and in conjunction with standard antimonial chemotherapy, has been used to successfully treat patients with CL which did not respond to antimonial therapy alone (Arevalo et al., 2001).

2.6 Vector and reservoir control

Control of transmission can be achieved by targeting the vector and/or the host reservoir. Sandfly control is now mostly dependent on pyrethroids, although the only reported
insecticide resistance in sandflies is for the organochlorine DDT (bis [4-chlorophenyl]-1,1,1-trichloroethane) in India (Davies et al., 2003). Indoor residual spraying with insecticide is the most widely used intervention for controlling sandflies that are endophilic and can considerably reduce CL cases (Davies et al., 2000; Reyburn et al., 2000). However, spraying programmes are often unsustainable (Murray et al., 2005). Where sandflies are endophagic and are active when people are asleep, insecticide bed nets impregnated with the synthetic pyrethroids permethrin, deltamethrin and Lambda-cyhalothrin provide considerable protection (Bern et al., 2000). The limitations associated with the use of bed nets include the discomfort generated by smaller mesh nets in warmer climates, the requirement for periodic re-impregnation of the nets and the high cost of long-lasting insecticide-treated bed nets (Murray et al., 2005).

The application of biolarvicides in the field condition is difficult due to diverse breeding habitat of sandfly and their practical application appears to be of limited use in the control of VL (Kishore et al., 2006). Satellite remote sensing for early prediction of disease by identifying the sandflygenic conditions and the use pheromones should be exploited in the control of leishmaniasis (Palit et al., 2002; Kishore et al., 2006).

In regions such as Latin America, then Mediterranean basin, central and south western Asia where VL is primarily zoonotic, reducing transmission to human beings by targeting the animal reservoir is a feasible strategy (Davies et al., 2003). However, culling infected domestic dogs in Brazil to reduce human VL was not effective because of incomplete coverage; delays between taking blood samples, diagnosis and culling; and the high dog population turnover rate (Courtenay et al., 2002). In view of the above shortcomings, dipping dogs in insecticide, applying topical insecticide lotions and the use of deltamethrin treated
collars are novel strategies that can substantially reduce sandfly bites on dogs and subsequent human infection (Davies et al., 2003).

2.7 Vaccines against leishmaniasis

Abundant clinical and experimental evidence indicates that leishmaniasis should be preventable by vaccination (Handman, 2001; Davies et al., 2003; Requena et al., 2004; Coler and Reed, 2005). A vaccine to prevent the disease has been a goal for nearly a century based on the knowledge that a cured infection protects the individual from re-infection (Selvapandinyan et al., 2006). However, prevention of leishmaniasis with an effective vaccine has, to date, not materialised (Piscopo and Mallia, 2006).

The completion of sequencing of \( L. \) major genome has added impetus to attempts to identify the genes that are responsible for resistance or susceptibility to leishmaniasis (Ivens et al., 2005). Many vaccine strategies have been pursued, including the use of whole lysate, killed, avirulent or irradiated parasites. Additionally, DNA vaccines and purified or recombinant parasite antigens have also been tested (Handman, 2001). Most of these strategies have shown some degree of effectiveness in animal models but little or no protection in humans (Requena et al., 2004). In general, the only successful immunization strategy in humans has been leishmanization, which is based on the development of durable immunity after recovery from infection at a chosen site, usually the arm, with viable non-attenuated parasites (Breton et al., 2005).

2.7.1 Leishmanization

The only proven vaccine agent in human beings is live \( L. \) major (leishmanization) (Ghalib and Modabber, 2007). In the Middle East, the deliberate infection with \( L. \) major was a
common and effective practice for immunization against subsequent infections, but a fraction of the vaccinated persons produced lesions that required medical treatment (Khamesipour et al., 2005). Leishmanization as a prophylactic vaccine was used on a large-scale in the Soviet Union and Israel with a high percentage of successful lesion development (Greenblatt, 1980; Kellina, 1981). Leishmanization was also employed in Iran in 1980s and then in a massive programme covering over 2 million people during the Iran-Iraq war of 1982-1985 (Nadim and Javaidan, 1988).

The concept of leishmanization has had many problems, including the development of large uncontrolled skin lesions, exacerbation of psoriasis and other skin diseases, and even immunosuppression as determined by low responses to the diphtheria, pertussis, and tetanus triple vaccine (Khamesipour et al., 2006). In addition, Leishmania parasites are believed to persist for a long time. As a result, leishmanization cannot be used on a large scale or in HIV endemic areas (Ghalib and Modabber, 2007). At present, there is only one prophylactic vaccine in use in Uzbekistan (Khamesipour et al., 2006).

### 2.7.2 Killed parasites

The earliest trials with killed Leishmania as a vaccine were conducted in Brazil in the 1940s. Later, from 1970s onwards Mayrink and colleagues developed a killed vaccine composed of five isolates of Leishmania containing four different species which was later simplified to a single L. amazonensis vaccine and tested for prophylactic potential in Columbia and Ecuador and as an adjunct to chemotherapy in Brazil (Modabber, 1995; Genaro et al., 1996).

In Venezuela, Convit et al. (1989) and his group used a combination of autoclaved L. mexicana or L. braziliensis promastigotes and M. bovis BCG either prophylactically or
immunochemotherapeutically against south American leishmaniasis (Castes et al., 1989). Cure was associated with the development of Th1-type immune responses in the recipients, with the production of IFN-\(\gamma\) and absence of IL-4 (Cabrera et al., 2000). In Venezuela, autoclaved killed \textit{L. mexicana} is being used to treat patients with CL (Convit et al., 2003), while in Ecuador, two doses of vaccine composed of \textit{L. amazonensis} and \textit{L. mexicana} mixed with BCG was shown to induce 73% protection (Armijos et al., 2004).

In the monkey model of CL, protective immunity was achieved using killed \textit{L. amazonensis} co-administered with recombinant IL-12 as adjuvant while another study using \textit{L. major} with recombinant human IL-12 resulted in a skewed Th1 immune response but did not protect the primates against challenge infection with the parasite (Kenney et al., 1999; Gicheru et al., 2001). In general, considering all trials, based on the immunogenicity of various killed \textit{Leishmania} preparations, it seems a better adjuvant than BCG would be required to produce a potent vaccine (Khamesipour et al., 2006).

### 2.7.3 Live attenuated vaccines

Recent advances in the ability to manipulate the \textit{Leishmania} genome by introducing or eliminating genes has the potential to make live-attenuated vaccines a reality. The idea of a live-attenuated vaccine is to expose the recipient to complex antigens in the right context over time without producing pathology (Selvapandiyan et al., 2006). Using gene-targeting tools, it is now possible to generate parasites lacking genes essential for long-term survival in the mammalian host, such as the gene encoding the enzyme dihydrofolate reductase-thymidylate synthetase (\textit{dhfr-ts}) (Titus et al., 1995). In a mouse model, \textit{L. major} parasites lacking \textit{dhfr-ts} induced protection against \textit{L. major} and \textit{L. amazonensis} but no protection was conferred to Rhesus monkeys (Titus et al., 1995; Veras et al., 1999; Amaral et al., 2002).
Other genetically engineered and tested mutant parasites include the null mutants for the glucose transporter gene family in *L. mexicana* that exhibited reduced infectivity to BALB/c mouse macrophages, demonstrating that a single gene deletion can render a parasite virulent (Burchmore *et al.*, 2003). Additionally, although *L. major* mutants deficient for leishmanolysin genes showed normal development in macrophages *in vitro*, they showed delayed lesion development in susceptible BALB/c mice (Joshi *et al.*, 1998; 2002). *Leishmania major* mutants that lack LPG1 (the gene encoding a galactofuranosyl transferase) showed attenuated virulence in mice whereas the parasites that lacked LPG2 (the gene encoding a golgi GDP-mannose transporter) persisted indefinitely at low level in mice without displaying disease and provided protection from virulent *L. major* challenge (Spath *et al.*, 2000; Uzonna *et al.*, 2004). Such protection from virulent challenges in mice has also been achieved after gene knockout for other genes: cysteine protease in *L. mexicana* and biopterin transporter in *L. donovani* (Alexander *et al.*, 1998; Papadopoulou *et al.*, 2002).

Attempts to develop avirulent parasites as vaccine candidates against leishmaniasis by irradiation, temperature sensitive mutations or random mutations induced by chemical agents has been hampered by reversion to virulence (Selvapandiyan *et al.*, 2006). These challenges can be met with the use of live attenuated strains which posses genetically defined mutations, can persist in the host without being virulent, have less chance of reversion to the virulent phenotype and can be produced in large quantities.

### 2.7.4 Recombinant and synthetic vaccines

The development of a defined vaccine against leishmaniasis has been accelerated by advances in the understanding of immunological mechanisms that mediate protection in animal models and to a lesser scale by supporting data from the characterization of immune
responses in *Leishmania* infected individuals (Khamesipour *et al.*, 2006).

Newer vaccines under consideration for leishmaniasis comprise recombinant DNA-derived antigens and peptides. Some of the target antigens are species and life cycle stage specific while others are shared by promastigotes and amastigotes. Since T cells usually recognize peptides derived from cytosolic proteins bound in the major histocompatibility complex (MHC) class I groove or peptides derived from the lysosomal compartment bound in the MHC class II groove on the antigen-presenting cell (APC) surface, all parasite proteins can function as antigens regardless of their location in the parasite (Handman, 2001).

Recombinant antigens can be delivered as purified proteins, as the naked DNA encoding them, or as bacteria manufacturing proteins *in situ*. Manipulations now allow targeting of the antigen to specific locations or to particular APCs, such as dendritic cells or langerhans cells, which are considered essential for the initiation of primary T-cell responses. Injection of bacteria or naked DNA may have the added advantage of providing adjuvant effect, which may “activate” or “licence” these APCs (Matzinger, 1998).

### 2.7.4.1 Expression of immunogens in bacteria and viruses

The encoding for the protein portion of the *Leishmania* surface glycoprotein (gp63) or leishmaniolysin was the first *Leishmania* vaccine delivered as a plasmid (Xu and Liew, 1994). Leishmaniolysin is a membrane protease present in promastigotes of all species and is one of the parasite receptors for host macrophages such that parasite mutants lacking the protein are avirulent (Chang *et al.*, 1990). The gene has been engineered in a number of delivery systems including BCG, vaccinia virus and *Salmonella typhimurium* with a view of inducing the appropriate Th1 immune response (Handman, 2001). The protective efficacy of
purified gp63 has been tested in several experimental models using different strains and adjuvants, giving rise to conflicting results (Olobo et al., 1995; Khamesipour et al., 2006). Both the recombinant and native proteins of gp63 seem to protect better against infection with *L. amazonensis* than against infection with *L. major*, suggesting species-specific epitopes in animal models (Russel and Alexander, 1988; Olobo et al., 1995). Overall, gp63 is still considered a vaccine candidate.

Another vaccine candidate tested in animal models is gp46/M2 or parasite surface antigen 2 (PSA-2), a membrane antigen of unknown function. Like gp63, PSA-2 belongs to a family of glycoinositol phospholipids anchored glycoproteins expressed in both promastigotes and amastigotes of all *Leishmania* species except *L. braziliensis* (Handman et al., 1995; 2001). It has been demonstrated that while vaccination with native PSA-2 with *C. parvum* as adjuvant protects mice against *Leishmania* through a Th1 mediated response, the recombinant PSA-2 purified from *Eschericia coli* and administered in immunostimulating complexes (ISCOMs) or mixed with *C. parvum* as adjuvant, does not induce protective immunity despite induction of Th1 responses (Sjölander et al., 1998; Khamesipour et al., 2006).

The *Leishmania* homologue of the mammalian receptor for activated c kinase (LACK) is another of the known antigen of *Leishmania* spp. that might be a candidate for vaccination (Mougneau et al., 1995). The antigen is expressed by both promastigotes and amastigotes and has been shown to protect mice from infection, especially when administered with IL-12 (Gurunathan et al., 1997). Very rapid IL-4 production in response to LACK has been documented in susceptible mice infected with *L. major*, which induced the typical Th2 cell response (Launois et al., 1997). In addition, immunization with LACK protein and IL-12 along with deoxyribonucleic acid (DNA) plasmids containing LACK gene and ‘altered’
LACK peptides made susceptible-mice resistant to infection with *L. major* (Mougneau *et al*., 1995; Guranathan *et al*., 1997). The presence of T cells that proliferate and produce cytokines such as IFN-γ and IL-10 in response to LACK has been demonstrated in PBMCs recovered from humans who have never been exposed to *Leishmania* and from patients during the early phase of the disease (Maasho *et al*., 2000). Thus, the role of LACK-specific cells in resistance and susceptibility to *Leishmania* infection (cutaneous leishmaniasis) is far from being established (Bourreau *et al*., 2003). The leishmanial eukaryotic ribosomal protein (LeIF), a homologue of the ribosomal of the ribosomal protein cIF4A, is being considered as a vaccine candidate based on its ability to induce Th1-type cytokines in humans (Skeiky *et al*., 1998).

Several other vaccine candidates that are amastigote specific include the A2, P4 and P8 of *L. mexicana pifanoi* (Soong *et al*., 2000). The A2 genes form part of a multigene family of at least 11 genes and are considered to be virulence factors that are required for the survival of *Leishmania* parasites in the mammalian host (Ghosh and Bandyopadhyay, 2003). Other antigens expressed in amastigotes are the cathepsin L-like cysteine proteinases (CPs), enzymes that belong to the papain superfamily (Mottram *et al*., 2004). These enzymes have been used in immunization experiments in the mouse model and it has been shown that the recombinant cysteine proteinases class B (CPB), in combination with adjuvant, induces long lasting immunity against *L. major* infection in BALB/c mice, while DNA vaccination is more efficient when a cocktail of plasmid DNAs encoding cysteine proteases class A (CPA) and CPB indicating the usefulness of a combination of these antigens (Rafati *et al*., 2000).

### 2.7.4.2 Synthetic peptides

Data obtained from experimental studies has provided evidence that a single subcutaneous
injection of a single synthetic T cell epitope is sufficient to provide long-lasting protection against two highly virulent strains (MRHP/SU/59 Neals and WHOM/IR/−/173) of *L. major* in BALB/c mice (Spitzer *et al*., 1999). In recent times however, the use of synthetic peptides and especially those with T-cell epitopes as vaccine candidates against leishmaniasis seems to have waned and focus has shifted to the use of recombinant DNA-produced polypeptides and to naked DNA (Khamesipour *et al*., 2006).

Several considerations make the peptide antigens less attractive. These include the magnitude of the T-cell memory induced, the inability of all individuals in the population to respond to the peptide and the economics of production. Some polypeptides such as PSA-2 need to be in their native conformation for antigen processing to take place, a requirement that may not be fulfilled by *Eschericia coli*-derived recombinant proteins (Sjölander *et al*., 1998). In addition, most synthetic peptides require delivery systems such as adjuvants but most effective adjuvants generally cause strong inflammation which although necessary for adjuvanticity, may preclude their use in humans owing to unacceptable side effects (Howard, 1993).

### 2.7.4.3 Nonprotein antigens

Early studies on vaccine development demonstrated that glycolipids such as the *Leishmania* lipophosphoglycan (LPG) provided excellent protection. However, protection was shown to rely on the use of adjuvants such as liposomes or *C. parvum* and on the integrity of the molecule (McConville *et al*., 1987; Russel and Alexander, 1988). In other studies, the water-soluble LPG lacking the glycophosphatidylinositol (GPI) anchor not only proved non protective, but it also exacerbated disease (Mitchell and Handman, 1986).
2.7.4.4 Naked DNA vaccines

DNA vaccines encoding gp63, LACK and PSA-2 have all been shown to protect both genetically resistant and susceptible mice from with *L. major* by inducing Th1 immune responses (Guranathan *et al.*, 1997; Sjölander *et al.*, 1998). Further improvements of the DNA vaccination approach are still needed to design a fully protective vaccine against leishmaniasis. Three directions of investigations are currently explored: DNA vaccines using a cocktail of antigens; prime boost approach; and association of immune modulators with the candidate antigens (Ahmed *et al.*, 2004).

The nucleoside hydrolase (NH36) DNA vaccine of *Leishmania donovani* has been shown to be cross-protective against murine VL caused by *L. chagasi* while vaccination with a multi-component DNA vaccine against VL in dogs produced Th1 cytokines and was not only shown to reduce incidences of canine VL, but also indirectly reduced the incidence of human VL (Gamboa-León *et al.*, 2006; Saldarriaga *et al.*, 2006).

2.8 Soluble *Leishmania* antigens

The soluble antigens of *Leishmania* promastigotes are primarily lipophosphoglycan, which comprise an albumin binding site, a hydrophilic lipophosphoglycan component, and a repeating phosphorylated saccharide (linked with secreted acid phosphatase [S-AcP]) (Ryan *et al.*, 2002). Using gelatin hydrolysis, Rafati *et al.* (1997), demonstrated that promastigote SLA preparation of *L. major* possesses proteinases with a molecular weight of 50 kD, as well another band of between 63-66 kD when promastigote cell lysates were used (Rafati *et al.*, 1997).

Soluble *Leishmania* antigens have been tested as potential vaccine candidates against leishmaniasis in both clinical and experimental studies with very promising results (Scott *et
al., 1987; Ajdary et al., 2000; Rafati et al., 2000). Scott et al. (1987) demonstrated that intraperitoneal immunization with a soluble, membrane-free parasite extract induced protection against *L. major* challenge equal to that obtained with whole organisms. Induction of immunity (89% protection in seven experiments) was most effective with 100 µg of SLA and required concomitant injection of the bacterial adjuvant, *C. parvum* (Scott et al., 1987). Other studies have shown that intranasal vaccination with a 10µg dose of SLA promoted increased resistance in BALB/c mice against *L. amazonensis* infection and promoted a balanced Th1-Th2 response as demonstrated by up regulation of both IFN-γ and IL-10 production in the popliteal lymph nodes at either 1 week or 4 months post vaccination (Pinto et al., 2004). In addition, oral vaccination of susceptible BALB/c mice and the more resistant C57BL/6 mouse strain with a total of 200 µg of *L. amazonensis* SLA rendered both mouse strains more resistant to *L. amazonensis* and *L. major* infections (Pinto et al., 2003).

In clinical studies, a comparison of the immune profile of nonhealing CL patients with those with active lesions and those who had recovered from infection, it was found that PBMCs from patients with active lesions and individuals who had recovered produced large amounts of IFN-γ and no or little IL-4 in response to SLA, whereas no or low IFN-γ and high IL-4 production were observed in nonhealing donors (Ajdary et al., 2000). In a related study, SLA was shown to induce proliferation of PBMC and several fold-higher frequency of IFN-γ and tumour necrosis factor alpha (TNF-α) producing cells with equivalent, or a tendency toward fewer IL-10 and IL-4 producing cells (Bottrel et al., 2001; Carrillo et al., 2007). All these studies have pointed to the induction of Th1 immune responses which are significant in the control of leishmaniasis. Soluble *Leishmania* antigens have been used to develop serological assays that demonstrated very high degrees of sensitivity and specificity (Ryan et al., 2002). A comparative evaluation of an enzyme-linked immunosorbent assay (ELISA) based on ten
recombinant or purified *Leishmania* antigens for the serodiagnosis of Mediterranean VL (MVL) demonstrated that the classic crude SLA represents a very good and less costly alternative to the more sophisticated recombinant proteins for development of serodiagnostic tests (Maalej *et al.*, 2003). These findings, supported by Western blot patient profiles, indicate that leishmanial soluble antigens are excellent diagnostic markers and are highly immunogenic in the infected host (Tonui *et al.*, 2004a).

### 2.9. Adjuvants in *Leishmania* vaccines

An adjuvant administered with an antigen influences the quantity and quality of the ensuing immune response (Tonui *et al.*, 2004a). The use of BCG as an adjuvant is regarded as an acceptable practice in man, and at present this adjuvant is routinely used in vaccination and immunotherapy trials against leishmaniasis (Frommel and Lagrange, 1989; Ghalib and Modabber, 2007).

In Iran, a series of double-blind randomized BCG-controlled trials with 1–3 injections of autoclaved *Leishmania major* (ALM) were conducted and no significant overall difference was seen between the vaccine group and the control (BCG alone) group (Momeni *et al.*, 1999). A second study showed that in the longer term, the vaccine combination provided better efficacy than BCG alone, suggesting that BCG may have had a transient effect (Sharifi *et al.*, 1998). However, those who had become leishmanin skin test (LST) positive were found to have significant protection (Momeni *et al.*, 1998; Sharifi *et al.*, 1998). Another study by Khalil *et al.* (2000) in Sudan found no evidence that two doses of ALM plus BCG offered significant protective immunity against visceral leishmaniasis compared with BCG alone (Khalil *et al.*, 2000). These studies concluded that there is a need to identify a suitable adjuvant that would help ALM to induce maximum protective immune response.
In experimental studies as in clinical studies, BCG has been only variably successful. Early studies suggested that intraperitoneal or subcutaneous inoculation of C3H mice with BCG did not alter lesion size in *Leishmania mexicana* infected animals (Grimaldi *et al*., 1980). Intravenous pretreatment of BALB/c mice with BCG was protective against systemic *L. major* disease but did little to protect these animals from cutaneous infection (Weintraub and Weinbaum, 1977). However, later studies to examine the protective effects of BCG on *Leishmania major* infections of BALB/c and P/J mice showed that BCG induced protection against chronic cutaneous disease, as well as lethal systemic infections (Fortier *et al*., 1987). Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant has been shown to be well tolerated when used in patients with VL and has been successful used with a defined antigen to treat MCL refractory to antimony (Badaro *et al*., 1994; 2001).

Bacille Calmette Guérin and *Salmonella typhimurium* vectors expressing gp63 have been successfully used to induce protection in *L. major* system (Handman, 2001). Other approaches that involve the use of immunostimulatory DNA (Oligodeoxynucleotides, ODN) which contain immunostimulatory cytosine-phosphate-guanosine (CpG) motifs (CpG ODN) as adjuvant to SLA and Montanide ISA 720 used with histone 1 (H1) recombinant vaccine (Masina *et al*., 2003) have been tested in BALB/c mice and proved to be partially protective (Stacey and Blackwell, 1999).

Studies with a primate model of cutaneous leishmaniasis (*Macaca mulatta*) have demonstrated that protective immunity against *L. amazonensis* can be achieved using recombinant human IL-12 and alum as adjuvants (Kenney *et al*., 1999). Similar results have been achieved with the vervet monkey model vaccinated with ALM and rIL-12, and
challenged with *L. major* (Gicheru *et al*., 2001). In both studies, high levels of IFN-γ, indicative of a Th1 immune response were detected in PBMCs. However, protection was only achieved in the former but not in the later study (Gicheru *et al*., 2001).

Soluble cytokines known to promote Th1 immune responses have been used to induce and maintain immune responses by *Leishmania* vaccines. Interleukin-12 has been shown to induce the development of CD4+ Th1 cytokines (Aebischer *et al*., 2000; Gicheru *et al*., 2001; Tonui *et al*., 2004a). Other types of particulate adjuvants and delivery systems that have been tested in animal models include liposomes, microparticles, immunostimulating complexes (ISCOMS) and micelles formed by intrinsically adjuvanted lipopeptides (Handman, 2001). In general, these substances induced partially protective immunity.

In Kenya, development of a vaccine against leishmaniasis is being spearheaded by the KEMRI and the IPR (Reviewed in Tonui, 2006). Vaccine research at KEMRI is still at the rodent model stage of development (Tonui, 2006). So far live-attenuated *L. major* parasites (Onyalo *et al*., 2005), *L. major* culture-derived soluble exogenous antigens (SEAgs) (Tonui *et al*., 2004b) and lipophosphoglycan (LPG) alone or LPG plus *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) (Tonui *et al*., 2003) have been tested. Transmission blocking vaccine studies in leishmaniasis have also been undertaken at KEMRI. These studies have shown that LPG is an excellent candidate as a transmission blocking vaccine against *L. major* infections (Tonui, 1999; Tonui *et al*., 2001a,b). In these studies, sand flies, which fed on BALB/c mice, immunized with *L. major*-derived LPG (Tonui *et al*., 2004b) or monoclonal antibodies raised against LPG showed that parasite development was inhibited at the log phase (procyclic) of the parasite. There was also a marked reduction in the numbers of metacyclic promastigotes developing, leading to reduced transmission of *L. major* to naïve
BALB/c mice (Tonui et al., 2001a). It has also been shown that *P. duboscqi* gut lysates and proteins present in *L. major*-derived LPG share two common proteins of molecular weights 105 kDa and 106 kDa (Tonui et al., 2004b). The main focus of these studies has been to develop a vaccine that can be utilized both to reduce transmissible infections within the sand fly and disease severity within the host.

At the Institute for Primate Research (IPR) in Nairobi, it has been shown that the vervet monkey (African Green monkey) is a good model for both human cutaneous and visceral leishmaniasis (Githure et al., 1987; Olobo et al., 2001). Using this model the safety and immunogenicity of the recombinant gp63 mixed with Baccille Calmette Guerin (BCG) vaccines (Olobo et al., 1995), and the adjuvant potential of two doses of IL-12 when used with a killed *L. major* vaccine (Gicheru et al., 2001) have been evaluated. It has also been demonstrated that high crossreactivity between *L. donovani* and *L. major*, and that *L. donovani* protects against *L. major* infections (Gicheru et al., 1997). These findings have relevance for vaccine development in leishmaniasis.

### 2.10 Artemisinin

#### 2.10.1 Introduction

Artemisinin (or *qinghaosu* in Chinese), is a natural component of the sweet wormwood plant *Artemisia annua* L., concoctions of which have been used for over 2000 years in traditional Chinese medicine for the treatment of fever (Woodrow et al., 2005; Fig. 2.3). The earliest reference to the plant also known to the Chinese as *qinghao* goes back to “‘52 descriptions”, found in the Mawangdui Tomb dating back to 168 BC (Klayman, 1985). Artemisinin was isolated in 1972 (Christen and Veuthey, 2001), and its structure determined in 1979 (Klayman, 1985).
Artemisinin is a sesquiterpene trioxane lactone, which contains an endoperoxide bridge essential for its biological activity (Klayman, 1985; Fig. 2.2). The presence of the endoperoxide bridge is essential for the drug's antimalarial activity since reduced forms of the compound such as deoxyartemisinin do not exhibit any antimalarial activity (Klayman, 1985; Woerdenbag et al., 1994). Artemisinin has low solubility in water or oil, and is usually administered orally (Woodrow et al., 2005). To resolve this problem, several semi-synthetic artemisinin derivatives were developed. These derivatives, chemically modified at the carbon 10 (C10) position, include the water soluble artesunate and artelinate and the oil-soluble derivatives artemether and arteether, dihydroartemisinin (DHA) and artelinic acid (Woodrow et al., 2005). Administration methods include the oral, parenteral, and intrarectal routes (Meshnick et al., 1996; Burk et al., 2005). New derivatives are currently being developed to improve solubility and pharmacokinetics (O’Neill, 2005).

The antimalarial activities of artemisinin and its derivatives have been studied and the drug has been shown conclusively to be effective in the treatment of drug resistant *Plasmodium falciparum* malaria (Abdin et al., 2003). The drug is a potent antimalarial with effective 50% inhibitory concentrations (IC$_{50}$ values) ranging from 4.2 to 16.2 nanomolar (nM) for different derivatives (Woodrow et al., 2005).
Fig. 2.2: Structure of artemisinin (Adapted from Woodrow *et al.*, 2005).

Fig. 2.3: *Artemisia annua* plant (Adapted from: [www.griffee.org/fieldnotes/artemisia-annua](http://www.griffee.org/fieldnotes/artemisia-annua))
Although artemisinin derivatives have been used to treat malaria cases around the world, their extensive usage has not been associated with any significant toxicity. These drugs have an apparently excellent safety and tolerability profile in humans (Price et al., 1999; Alkadi, 2007). In addition, no clinical resistance has been reported against the artemisinin class of drugs (Golenser et al., 2006). However, mutations in genes associated with multidrug resistance have been found to alter the sensitivity of some strains to artemisinin (Woodrow et al., 2005).

2.10.2 Metabolism of artemisinin drugs

Metabolism of artemisinin and the first-generation derivatives artemether and arteether is mediated by cytochrome P (CYP)-450 enzymes CYP2C19 and CYP2B6, which are associated with first pass drug metabolism (Burk et al., 2005). The drugs activate the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) in primary human hepatocytes, which cause induction of the CYP enzymes (Burk et al., 2005). Artemisinin metabolism in human liver microsomes is mediated primarily by CYP2B6, with probable secondary contribution from CYP3A4 and possibly CYP2A6 in individuals with low CYP2B6 expression (Svensson and Ashton, 1999).

Artemisinin has also been reported to induce CYP2C19 but not CYP3A4 (Svensson et al., 1998). While artemisinin is converted primarily to inactive metabolites, its derivatives artesunate, artelinate, artemether and arteether are metabolized to dihydroartemisinin (DHA), which has clinical efficacy at least equivalent to its parent compounds (Balint, 2001). However, it is not clear whether DHA activates PXR and thereby cytochrome P450 enzymes (Burk et al., 2005).
The apparent primary disadvantage of artemisinin drugs is that they are characterized by a short half life (Krishna et al., 2004). For parent compounds such as artesunate which have very short elimination half-times (<10 min), the antimalarial effect is less important than that of their metabolite, DHA, whose elimination half-time (Krishna et al., 2004) is somewhat longer (~1 h). Treatment with artemisinin drugs causes reduction of parasite burden below detectable levels without eliminating all parasites; this results in a higher risk of recrudescence (Krishna et al., 2004; Bjorkman and Bhattarai, 2005). In addition, a fraction of the parasites exposed to the drug are thought to become dormant and unsusceptible to further dosing until reactivation (Hoshen et al., 2000). In order to completely eliminate the parasites and prevent the emergence of resistant *P. falciparum*, combinations with other, longer acting drugs are necessary (Olliaro and Taylor, 2004; Menard et al., 2005). Artemisinin combination therapies (ACTs) are currently recommended by the World Health Organization (WHO) as the first-line antimalarial treatment for *P. falciparum* malaria (WHO, 2005). Several ACTs have been developed. These include Coartem®, the combination of artemether with lumefantrine, and the combination of artesunate with amodiaquine, mefloquine or sulfadoxine–pyrimethamine (Olliaro and Taylor, 2004; World Health Organization, 2005).

### 2.10.3 Mechanism of action of artemisinin drugs

Despite the growing reliance on artemisinin drugs, their exact mechanism of action is still unresolved: both parasite-specific and non-specific mechanisms of action have been proposed (Golenser et al., 2006).

Because artemisinin is structurally similar to thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), it has been hypothesized that
artemisinins specifically inhibit *P. falciparum* SERCA (Eckstein-Ludwig *et al*., 2003). Sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase is responsible for the maintenance of calcium ion concentrations, which is important for the generation of calcium-mediated signaling and the correct folding and post-translational processing of proteins (Golenser *et al*., 2006).

Three-dimensional modelling of the PfATP6 amino acid sequence and subsequent docking simulation demonstrated that artemisinins bind to the protein by hydrophobic interactions while leaving the peroxide bonds exposed (Jung *et al*., 2005). This allows cleavage of the peroxide bridge by iron to generate carbon-centred radicals leading to enzyme inactivation and parasite death. This theory is supported by the fact that a single amino acid mutation in the plasmodial SERCA modulates sensitivity to artemisinins *in vitro* (Uhlemann *et al*., 2005).

Artemisinins have been found to inhibit endocytosis by *P. falciparum* (Hoppe *et al*., 2004). In addition, artemisinins form covalent adducts with four major membrane-associated parasite proteins. None of these adducts has been functionally characterized, leaving the possibility that inhibition of additional proteins may also have a role in artemisinin-mediated parasite killing (Eckstein-Ludwig *et al*., 2003; Krishna *et al*., 2004). Li *et al*. (2005) suggested that artemisinin and its derivatives are in fact activated by, and interfere with, components of the electron transport chain of the parasite mitochondria. In the mechanism proposed, after activation, artemisinin causes local production of reactive oxygen species and depolarization of the mitochondrial membrane (Li *et al*., 2005). Artemisinin has been shown to inhibit the respiratory chain of both sexual and asexual stages of *P. falciparum* (Krungkrai *et al*., 1999). In *P. falciparum*, the loss of membrane potential affects pyrimidine biosynthesis (Krungkrai, 2004; Li *et al*., 2005), a key metabolic process for nucleic acid production, and
may thereby readily cause parasite death. Dihydroartemisinin (0.5–1.0 ng/ml) kills malaria parasites by generating free radicals, causing the reduction of red blood cell antioxidants and glutathione (Ittarat et al., 2003). The endoperoxide bridge, present in artemisinin and all its derivatives, is essential for antimalarial activity. This was demonstrated by the lack of activity of deoxyartemisinin, a reduced form of artemisinin in which a single oxygen replaces the endoperoxide bridge (Avery et al., 1993). Based on this finding, two models of artemisinin antimalarial mechanism of action were initially suggested, both of which suggest formation of free radicals, mediated by iron. In the reductive scission model, the peroxide bridge is cleaved by divalent Fe$^{2+}$ (hemin or ferrous heme), the by product of haemoglobin digestion. The origin of the iron is debatable. It was suggested that a cytosolic labile iron pool is used by the parasite for its metabolic purposes (Scholl et al., 2005). This iron is chelatable and is probably involved in free radical formation (Golenser et al., 2003); it might therefore activate artemisinin.

The second model, the open peroxide model, proposes that the peroxide ring is opened by protonation or by complex formation with Fe$^{2+}$, resulting in an open hydroxy- or metal-peroxide. The oxygen atom which is not in the ring stabilizes the positive charge of the open peroxide, lowering the energy needed to open the ring. In both models, the oxygen-centred radical formed attracts hydrogen ions, thereby causing the formation of carbon-centred radicals. The anticipated targets of the carbon-centered radicals included heme-binding proteins and proteinases involved in hemoglobin degradation (Olizaro et al., 2001; Haynes and Krishna, 2004). It is suggested that the parasite could be killed by a joint effect of stopping hemozoin formation while creating a more reducing milieu, which is not favorable for the formation of hemozoin (Kannan et al., 2005).
Artemisinin drugs have been shown to cause apoptosis in cancer cell lines (Singh and Lai, 2004). The apoptosis in cancer cells was found to be p53-independent (Disbrow et al., 2005) and mediated by the mitochondrial pathway (Wu et al., 2004; Disbrow et al., 2005), thus excluding a role for NF-κB in artemisinin-induced apoptosis. In contrast to the findings in cancer cell lines, artemisinin function has not been related to apoptosis of *Plasmodium*. The *in vitro* effect of artemether (5–10 µg/ml) on *Schistosoma* is also mediated by iron (12.5–100 µg/ml) or hemozoin. Xiao et al. (2003) reported that worm death is mediated by iron accumulation in the helminth, suggesting local reactive oxygen species (ROS) production. However, this does not necessarily contradict the evidence negating hemozoin activation of artemisinins in the malaria parasite: activation of artemisinins by iron may be more relevant in *Schistosoma* than in *P. falciparum* (Golenser et al., 2006).

### 2.10.4 Antileishmanial activities of artemisinin and its derivatives

Artemisinin compounds have been shown to be efficacious against *Leishmania* spp. of trypanosomatid parasites, achieving 50% killing at 750 nM for *L. major* promastigotes, at 3 to 30 µM for intracellular amastigote stages in macrophages, and at 1.4 to 382.9 µM against *L. infantum* promastigotes (Avery et al., 2003). Artemether treatment (50 mg/kg of body weight/day) of footpad lesions in mice, by oral, intralesional, intramuscular, or intravenous administration, significantly reduces lesion size and *L. major* parasite numbers (Yang and Liew, 1993). Oral DHA (25 or 50 mg/kg) also reduces parasite burdens by 75% in the spleens and livers of hamsters infected with *L. donovani* (Ma et al., 2004).

### 2.10.5 Influences of artemisinin and its derivatives on immune functions

Sesquiterpene lactones have been shown to exert in mice a potent stimulation of the *in vivo*
response (Valdès and Córdobora, 1975). Influences of artemisinin and its derivatives on immune functions have been reported but the results of these investigations have been rather complex and have not lend themselves to any easy interpretation. Artemisinins have been shown to affect immune responses in a dose dependent manner (Golenser et al., 2006). Relatively low concentrations (up to 5 μM) of artemisinin, artesunate and DHA were shown to increase proliferation of con A stimulated T cells and IL-2 production (Yang et al., 1993). On the other hand, concentrations above 50 μM, in the case of dihydroartemisinin, or 500 μM, in the case of artemisinin and artesunate, caused decreased proliferation, indicating a concentration-dependent effect (Yang et al., 1993).

Previous studies suggest that artemisinin appears to promote T-cell function selectively and these compounds have a potential application for the immune function (Yang et al., 1993). Artemisinin has been shown to synergize with antibiotics to protect mice against lethal live Escherichia coli challenge by decreasing proinflammatory cytokine release (Wang et al., 2006). Artesunate applied intramuscularly at a dose of 75 mg/kg twice a day for seven days was shown to suppress the humoral immune responses but to enhance the cell-mediated immunity in mice (Lin et al., 1995).

Other studies have demonstrated that artemisinin and its derivatives exhibit immunosuppressive activity in BALB/c mice. These include studies which indicated that artemisinin could suppress the delayed type hypersensitivity (DTH) against sheep blood capsule in BALB/c mice (Noori et al., 2004). In addition, a novel artemisinin derivative, 3-(12-b-artemisininoxo) phenoxy succinic acid (SM735), has also been shown to mediate immunosuppressive effects in vitro and in vivo (Zhou et al., 2005). Further studies to investigate the immunosuppressive activity of SM905, a new water-soluble artemisinin
derivative, on T lymphocytes both in vitro and in vivo, and explore its potential mode of action showed that SM905 had a high inhibitory activity in concanavalin A (con A)-induced splenocyte proliferation and mixed lymphocyte reaction, and a relatively low cytotoxicity in vitro. In ovalbumin-immunized mice, oral administration of SM905 dose-dependently suppressed T cell proliferative response to ovalbumin, and inhibited anti-ovalbumin interleukin-2 (IL-2) and interferon-γ (IFN-γ) production by T cells. Further studies showed that SM905 inhibited TCR (T cell receptor)/CD3 plus CD28-mediated primary T cell proliferation and cytokine production (IL-2 and IFN-γ), and exerted an inhibitory action on the phosphorylation of mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERK), p38 and Jun N-terminal kinase (JNK), and the activation of Ras (Wang et al., 2007).

In contrast to the iron-mediated formation of free radicals described, it has also been demonstrated that artemisinin suppresses nitric oxide (NO) synthesis (Aldieri et al., 2003). The inducible nitric oxide synthase (iNOS) gene in macrophages catalyzes the generation of NO, which is involved in inflammation and immune response. Human astrocytoma T67 cells, which contain iNOS, were incubated for 24 h with a mixture of 20 µg/ml lipopolysaccharide (LPS), 10 ng/ml IL-1β, 100 ng/ml IFN-γ and 50 ng/ml TNF-α. Addition of 10 µM artemisinin significantly reduced nitrite levels, suggesting inhibition of iNOS activity and consequently suppression of NO synthesis (Pahl, 1999).
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

The study was carried out at the Kenya Medical Research Institute’s (KEMRI), Centre for Biotechnology Research and Development (CBRD), in Nairobi, Kenya. The Institute has the requisite facilities that ensured that the studies were undertaken successfully.

3.2 Study design and experimental mice

The study design was a completely randomized block design (CRD) that consisted of six treatments. Seventy two female BALB/c mice that were used in the experiments were bred and maintained at KEMRI’s animal house facility following the laid down institutional guidelines. The mice aged between 6-8 weeks were then randomly assigned into six groups of twelve each. This allowed for four mice from each group to be followed for lesion size throughout the experiment and four subgroups of duplicate mice each to be used for assays to determine parasite burden and the nature of the immune response.

3.3 Leishmania major parasites

*Leishmania major* strain (NLB-144) was used. The parasite was originally isolated from *Phlebotomous duboscqi* in the Baringo District, Kenya, and maintained in BALB/c mice by serial subcutaneous passage (Anjili *et al.*, 1994). Parasites were maintained as previously described (Titus *et al.*, 1995). Briefly, *L. major* parasites were cultured in Schneider’s Drosophila insect medium supplemented with 20% fetal bovine serum (FBS), glutamine (2mM), penicillin G (100 U/ml), and streptomycin (100 µg/ml). Stationary-phase promastigotes were obtained from 5- to 7-day-old cultures. Metacyclics promastigotes for infection were isolated from stationary-phase cultures by negative selection using peanut agglutination (Sacks and Perkins, 1984; Tonui and Titus, 2006).
3.4 Preparation of soluble *Leishmania* antigens

Soluble *Leishmania* antigens (SLA) for vaccination were produced as previously described (Scott *et al*., 1987), with some modifications. Briefly, promastigotes were harvested from a population of mixed culture of *L. major* parasites, counted using a haemocytometer (10^9) then washed three times in cold phosphate-buffered saline (PBS) and resuspended in PBS. The parasites were then freeze thawed three times in liquid nitrogen then subjected to 3-5 sonication cycles of 20 seconds each on ice. The parasites were then viewed microscopically to ensure that all the parasites had disrupted. The resulting suspension was then centrifuged at 27 000 g for 20 min at 4 °C. Finally, the supernatant was collected and the protein concentration determined by the Bio-Rad protein assay and the final concentration was adjusted to 100µg/ml for immunization of mice and proliferation of cytokine producing cells (Lowry *et al*., 1951).

3.5 Artemisinin

Commercially available pure injectable liquid/soluble artemisinin for the study was purchased from Sigma Chemicals Sigma Chemical Laboratories, St. Louis-MO, USA. A concentration of 75mg/Kg was used (Lin *et al*., 1995).

3.6 Immunization of BALB/c mice and measurement of lesion sizes

The first group of mice was vaccinated with 100 µL of 75 mg/Kg of artemisinin alone administered in the rump subcutaneously and the second group with 100 µL artemisinin co-administered with 100µl of 100µg/ml of SLA. This level (100µg) of SLA was shown to be consistently protective in BALB/c mice infected with *L. major* (Scott *et al*., 1987). The third group of mice was vaccinated with 100 µg of SLA administered with 0.1 ml of a *Mycobacterium bovis* BCG suspension that contains 10^5 colony forming units (CFU) per
milliliter while the fourth group was vaccinated with 100µg/ml SLA in 100 µL PBS. The negative control group consisted of unvaccinated mice while BCG served as a control adjuvant. Thirteen days later, the immunizations were repeated.

3.7 Infection of mice
Seven days after the second immunization, all the mice were challenged with $1 \times 10^5$ metacyclic *L. major* in 50 µL of saline solution in their hind right footpad (Tonui *et al*., 2004a; Al-Wabel *et al*., 2007; Fig. 3.1). Lesion development was measured during the course of infection using a vernier calliper and expressed as the difference in thickness between the infected footpad and the contra-lateral uninfected footpad (Walker *et al*., 1999). At 2, 7 and 9 weeks post-infection, duplicate mice were sacrificed and a limiting dilution assay used to determine parasite numbers (Lima *et al*., 1997).
3.8 Limiting dilution assay for quantification of *L. major* parasite numbers

The number of living *L. major* parasites in infected footpads was quantified at 14, 35 and 63 days post infection using the parasite-limiting dilution assay (Lima *et al.*, 1997). Mice were sacrificed and the feet removed between the ankle joint and toes. Skin-peeled tissue from the footpad lesion was cut into several pieces then homogenized in a glass tissue homogenizer in complete Schneider’s insect medium (Fig. 3.2). Pooled samples were diluted in 5 ml complete Schneider’s medium per infected footpad. Serial dilutions of 200 µl of the footpad homogenate were then distributed in replicate wells of a 24-well plate. The plates were
sealed using a parafilm and incubated in the dark at 26°C. After 7 days, the assay was read microscopically, and the number of viable parasites in the tissue was determined as previously described (Titus et al., 1985). Plates were scored and the results evaluated using the ELIDA program (ELIDA software 1985–2005; Taswell, 1987).

Fig. 3.2: Limiting dilution assay for quantification of *Leishmania major* parasites (homogenizing *L. major* infected footpads in a laminar flow hood).
3.9 Cell cultures, proliferation of cytokine producing cells and measurement of cytokines

3.9.1 Cell cultures
Spleens from mice infected with *L. major* were harvested from duplicate mice in each experimental group of mice just prior to infection (day 0) and at day 35 post-infection and mononuclear cells purified with Ficoll-Paque ingredients (Bøyum, 1968). The viability of mononuclear cells was examined by trypan blue exclusion test. Splenocytes were then adjusted to $10^6$/ml in complete RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units of penicillin per ml, and 100 µg of streptomycin per ml. The splenocytes were then dispensed at the rate of 100 µL per well in 96 well flat bottomed tissue culture plate in triplicate for each of the experimental groups and either cultured alone or stimulated with Con A or 100 µg/ml of soluble *L. major* antigen. The cells were then incubated in a humidified atmosphere at 37 °C and 5% CO2 for 48 h. Colorimetric microassay was used to determine cell proliferation (Hay and Westwood, 2002).

3.9.2 Colorimetric assay for cell proliferation
Colorimetric microassay for cell proliferation was performed as previously described (Mosmann, 1983; Hay and Westwood, 2002) with some modifications. Forty eight hours post culture, 100 µL of thiazolyl blue tetrazolium bromide (MTT) was added to all wells of the plates after which the cells were incubated at 37 °C for 4 h then centrifuged again to remove medium. One hundred microlitres of culture supernatant was removed after centrifugation and 100 µL of dimethyl sulfoxide (DMSO) was added to all the wells followed by vigorous pipetting to dissolve the formazan crystals formed by metabolism of MTT by live cells. The plates were then read at 570 nm in an ELISA reader. Proliferative responses were expressed as stimulation indices (SI), which represents the ratio of mean
proliferation after stimulation with mitogen (concanavalin A) or antigen (soluble *Leishmania* antigen) to the mean proliferation of medium controls.

### 3.9.3 Cytokine assay

Culture supernatant fluids were collected at 48 hours post stimulation for both antigen-treated and con-A-treated wells and controls and stored at -20 °C till assayed. The cytometric bead array (CBA) kit was used to determine levels of IL-4, IL-5 and IFN-γ as previously described by Hodge *et al.*, 2004. Briefly, the Th1/Th2 cytokine standards were reconstituted and serially diluted using assay diluent. Ten microlitres of each mouse cytokine capture bead suspension was mixed and 50μl of mixed beads transferred to each assay tube. After the addition of 50 μl each of the standard dilutions and supernatant to the appropriate sample tubes, 50 μl of phycoerythrin (PE) detection reagent was added and the resulting mixture was incubated for 2 h in the dark at room temperature. The samples were washed and centrifuged at 500g for 5 min and the pellet was resuspended in 300 μL of wash buffer. The FACS Calibur flow cytometer (BD Pharmingen) was calibrated with setup beads and the samples acquired. Individual cytokine concentrations were computed using the standard reference curve of Cellquest and CBA software (BD Pharmingen).

### 3.10 Ethical and biosafety considerations

Approval for the study was sought from KEMRI ethical review committee and the Board of Postgraduate Studies of Kenyatta University. The experiments were done in compliance with KEMRI’s Animal Care and Use Committee (ACUC). Standard operating procedures (SOPs) available at the *Leishmania* laboratory at the CBRD included immunizing of animals using standard 21gauge needle, anesthetizing them using 6% sodium Pentobarbitone (Sagatal®) and killing them by using CO₂ asphyxiation. Biosafety issues were addressed by sterilizing
dead animals through dipping them into 70% ethanol and disposing into appropriate biohazard bags before transfer to the incinerator at KEMRI. Further precautionary measures involved putting on protective gear and carrying out the experiments in a laminar flow hood.

3.11 Statistical analysis

Statistical analysis was performed using SPSS version 13.0. Cytokine concentrations were expressed as means ± the standard deviation. The Student $t$ test was used to examine the differences in cytokine concentrations in culture supernatants as well as proliferation of stimulated and unstimulated splenocyte cultures. Data on lesion progression was analyzed for statistical significance using the analysis of variance (ANOVA). A $P$ value of $\leq 0.05$ was considered significant.
CHAPTER FOUR: RESULTS

4.1 Lymphoproliferative responses

Spleen cell proliferative responses in each of the six groups of mice were measured at 7 days after the booster injections and at 35 days post-infection with *L. major* parasites.

4.1.1 Lymphoproliferative responses of splenocyte cultures before infection of mice with *Leishmania major*

The first experiment, conducted at 7 days after the booster vaccinations sought to determine the effect of vaccination alone in the absence of challenge with *L. major* on the potential of the immune system to respond to stimulation *in vitro* with soluble *Leishmania* antigens. The *in vitro* lymphocyte proliferative responses of SLA and con-A stimulated splenocytes of mice which had received artemisinin + SLA, artemisinin alone, SLA alone, BCG alone, SLA + BCG and the control unvaccinated groups before infection with *L. major* are summarized in Fig. 4.1.

The results show that injection of 100µg of SLA co-administered with 75 mg/Kg of artemisinin is capable of increasing the proliferative response of splenocyte cultures against SLA compared to the control unvaccinated group (SI ± SD, 1.365 ± 0.093 vs SI ± S.E., 1.008 ± 0.062). However, using the Student’s *t*-test, no significant difference in proliferative responses was observed between this group and the control unvaccinated group (*P*=0.466). On the other hand, the splenocytes from the group of mice vaccinated with artemisinin alone virtually did not proliferate in response to SLA and had the least blastogenic response as compared to the control unvaccinated mice (SI ± S.E., 1.061 ± 0.062; *P*=0.378). The greatest lymphoproliferative response to SLA was realised from splenocytes of mice in the BCG + SLA group (SI ± S.E., 1.486 ± 0.036). However, this proliferative response did not reach statistical significance when compared with the control unvaccinated group (*P*= 0.139). On
the other hand, splenocytes from mice vaccinated with SLA alone proliferated modestly (SI ± S.E., 1.362 ± 0.093), but this response did not differ significantly from that of the control unvaccinated mice (P=0.4). Splenocytes from the BCG vaccinated group proliferated, though minimally in response to the antigen suggesting an effect on the immune system (SI ± S.E., 1.111 ± 0.046). Finally, spleen cells from the control group of mice that had not been vaccinated virtually did not proliferate when stimulated with SLA (SI ± S.E., 1.008 ± 0.118).

In general, there was no significant difference in the blastogenic response of spleen cells to SLA from all the vaccinated mice as compared to the unvaccinated mice (P > 0.05). As an internal control for cell viability, some cultures were also stimulated with a mitogen, concanavalin A. The cells from all the groups stimulated with con A showed high degrees of proliferative responses over the ones stimulated with SLA and medium (P<0.05). In addition, the results of these cultures demonstrated that the degree of proliferation of cells from all the groups stimulated with con A was almost equivalent (Fig. 4.1).
Fig. 4.1: Proliferative responses of splenocytes (2 x 10^5/well) from BALB/c mice before infection with *L. major*. The cells were cultured for 48 h in the presence of Con A (10 µg/well), SLA (100 µg/well) or medium. Results are expressed as arithmetic means of stimulation indexes (SI) of MTT adsorption at 570 nm (2 mice per group; test performed in triplicate).

Key: Art-artemisinin; SLA-soluble *Leishmania* antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline
4.1.2 Lymphoproliferative responses after infection of mice with *Leishmania major*

In the second cell proliferation assay carried out on the 35\textsuperscript{th} day after infection of mice with *L. major*, splenocytes from the mice showed mitogen and antigen specific proliferation. The *in vitro* lymphocyte proliferative responses of unstimulated cells, SLA and con-A stimulated splenocytes of mice which had received artemisinin + SLA, artemisinin alone, SLA alone, BCG alone, SLA + BCG and the control (unvaccinated groups) after infection with *L. major* are summarized in Fig. 4.2. Although the magnitude of the *Leishmania*-specific response varied, a strong splenocyte proliferative response to SLA was observed in all the groups compared to the unstimulated control wells (*P*<0.05). Splenocytes from the group of mice vaccinated with SLA co-administered with artemisinin proliferated modestly in response to SLA (SI ± S.E., 1.588 ± 0.096). However, the blastogenic response did not reach statistical significance in comparison with the controls (*P*= 0.117). On the other hand, the highest stimulation index (SI) to SLA was realised from the mice that had been vaccinated with BCG and SLA (SI ± S.E., 1.803 ± 0.026). This blastogenic response was 1.8 times higher than the controls and was highly significant (*P*= 0.001; Fig. 4.2).

Splenocytes from mice immunized with SLA proliferated strongly in response to SLA suggesting recall proliferative responses (SI ± S.E., 1.697 ± 0.060). In addition, this proliferative response reached statistical significance in comparison with splenocytes from the unvaccinated but *L. major* challenged mice (*P*= 0.02). Lymphoid cells from the BCG group similarly had high proliferative responses compared to the unvaccinated group (SI ± S.E., 1.598 ± 0.016), but lower than the SLA group (*P*=0.006). The lowest lymphocyte blastogenic response after the PBS control group was observed in the artemisinin group (SI ± S.E., 1.458 ± 0.053). Overall, the induction of *in vitro* proliferative responses to SLA was significantly higher after infection as compared to before infection with *L. major*.
promastigotes in the SLA + artemisinin, BCG, BCG + SLA and SLA vaccinated mice ($P<0.05$). No significant increase in blastogenic responses was observed in the artemisinin injected and unvaccinated group of mice ($P>0.05$). As in the first proliferation assay, results of the second assay also show that the degree of in vitro proliferation of spleen cells from all groups stimulated with con A was equivalent (Fig. 4.2).
Fig. 4.2: Proliferative responses of splenocytes (2 x 10^5/well) from *L. major* infected BALB/c mice. The cells were cultured for 48 h at 37 °C and 5% CO₂ in the presence of con A (10 µg/well), SLA (100 µg/well) or medium. Results are expressed as arithmetic means of optical densities and stimulation indices (SI) of MTT adsorption at 570 nm (2 mice per group; test performed in triplicate).

Key: Art-artemisinin; SLA-soluble *Leishmania* antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline

# 4.2 Cytokine production

In order to determine the nature of the immune response induced by immunization with SLA co-administered with artemisinin, SLA alone, BCG + SLA, BCG alone and artemisinin
alone, the cytokines (IFN-γ, IL-4 and IL-5) produced were determined. Seven days after the booster vaccination (but prior to challenge with *L. major*), duplicate vaccinated and control mice were sacrificed, their spleens were removed and mononuclear cell suspensions were prepared and cells stimulated *in vitro* with SLA and Con A. The process was repeated at 35 days post-infection with *L. major* parasites. Thirty five days of infection was deemed appropriate for these assays because by 35 days, clear, significant, and consistent differences had developed in the size of lesions of control mice compared with vaccinated mice. Therefore, the cytokines produced by these different groups of mice were expected to be distinguishable from each other. The culture supernatants were collected after 48 hours and stored at -20 °C till assayed for cytokines using the cytometric bead array (CBA) method on a FACSCalibur (Mahmoodi *et al.*, 2005). On the day of the assay, culture supernatant fluids were thawed at room temperature and cytokines levels were measured with commercial CBA kit for IFN-γ, IL-4 and IL-5 supplied by BD Biosciences according to the manufacturer's instructions.

### 4.2.1 Interferon gamma production of splenocyte cultures before infection with *Leishmania major*

Data on IFN-γ production by splenocytes after *in vitro* stimulation with SLA and concanavalin A prior to challenge with *L. major* are presented in Fig. 4.3. The mean production of IFN-γ production by splenocytes of mice vaccinated with SLA co-administered with artemisinin in response to *in vitro* stimulation with SLA at 7 days after the booster vaccination did not differ significantly from the corresponding values in the control unvaccinated group (mean ± S.E., 181.7 ± 27.178 pg/ml vs. 171.077 ± 10.414 pg/ml; *P*=0.734). On the other hand, the BCG + SLA group had the highest production of IFN-γ recording a value of 446.41 ± 8.458 pg/ml (mean ± S.E.), and differed significantly from that produced by the control unvaccinated group (*P* <0.001). The *in vitro* production of IFN-γ in
mice vaccinated with BCG was also high (mean ± S.E., 391.513 ± 14.752 pg/ml) and was significant when compared to the control group (P<0.001). Conversely, the lowest production of IFN-γ was realised from mice injected with artemisinin alone (mean ± S.E., 176.207 ± 7.727 pg/ml). In addition, this level of IFN-γ was not sufficient to reach statistical significance (P= 0.713). The group of mice vaccinated with SLA produced modest levels of IFN-γ (mean ± S.E., 218.640 ± 40.712 pg/ml), but this level did not differ from that produced by the control group (P=0.32; Fig. 4.3).

4.2.2 Interferon gamma production of splenocyte cultures after infection with Leishmania major

The levels of IFN-γ were then measured at 35 days post-infection with L. major parasites (Fig. 4.3). Thirty days of infection was chosen for these assays because by 35 days, clear, significant and consistent differences had developed in the size of lesions of control mice compared with vaccinated mice. Therefore, the cytokines produced by these different groups of mice should be distinguishable from each other. Splenocytes drawn from control (non-vaccinated) mice produced low levels of IFN-γ when restimulated with SLA in vitro (Fig. 4.3). There was a significant reduction in the level of IFN-γ produced by this group of mice as compared to the amount produced before infection (P= 0.007). On the other hand, there was a significant increase in the level of IFN-γ produced by spleen cells stimulated with SLA in vitro from the SLA+ BCG group compared to before infection with L. major (P= 0.02). A significant reduction of IFN-γ production was observed in supernatants of SLA-stimulated splenocytes culture of the SLA + artemisinin injected cases after infection with L. major (mean ± S.E., 127.215 ± 18.025, P=0.037).

The highest level of IFN-γ was realised from the BCG + SLA group (mean ± S.E., 543.338 ± 55.609). On the contrary, the level of IFN-γ produced by spleen cells from the artemisinin
alone group dropped from the previous value of $176.207 \pm 7.727$ (mean ± S.E.), to $161.249 \pm 18.7614$ (mean ± S.E.). However, this reduction was not statistically significant ($P > 0.05$).

Interferon-gamma produced by the SLA group was also lower than the previous level before infection (mean ± S.E., $163.823 \pm 7.826$), as was the level produced by the BCG alone group (mean ± S.E., $314.739 \pm 38.199$).

Overall, there was a reduction in the amount of IFN-γ detected in splenocyte culture supernatants of all the groups with the exception of the BCG + SLA group after infection as compared to before infection (Fig. 4.3).
Fig. 4.3: Interferon gamma production by spleen cells from vaccinated BALB/c mice before and after infection with *L. major*. The cells were stimulated *in vitro* with 100 µg SLA. After two days, the culture supernatants were harvested and assayed for the presence of IFN-γ by the cytometric bead assay (CBA) method using a FACSCalibur®.

Key: Art-artemisinin; SLA-soluble *Leishmania* antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline

### 4.2.3 *In vitro* interleukin-4 production before infection with *Leishmania major*

Data on IL-4 production by splenocytes culture of mice vaccinated with SLA + artemisinin, SLA alone, artemisinin alone, BCG + SLA and BCG alone after *in vitro* stimulation with SLA and mitogen is presented in Fig. 4.4. Splenocytes from mice vaccinated with SLA co-administered with artemisinin produced IL-4 when stimulated *in vitro* with SLA (mean ±
This cytokine level was significantly higher than the amount produced by the control unvaccinated group ($P=0.004$). The level of IL-4 detected from splenocytes culture supernatants of mice vaccinated with BCG + SLA was low (mean ± S.E., 10.984 ± 0.691), and did not differ significantly from that produced by the control unvaccinated mice ($P=0.137$).

The highest production of IL-4 from all the treatment groups in response to stimulation with SLA in vitro was observed in the group of mice that had been injected with SLA (mean ± S.E., 35.561 ± 2.502). There was a significant difference between this level of IL-4 compared to the controls ($P=0.003$). On the other hand, mice vaccinated with BCG alone produced similar amount of IL-4 as the control unvaccinated group (mean ± SE, 15.452 ± 2.45 vs 15.417 ± 2.266) while mice vaccinated with artemisinin produced significantly higher levels of IL-4 compared to the controls (mean ± SE, 32.325 ± 2.483, $P=0.007$).

Overall, the production of IL-4 detected from splenocyte culture supernatants of mice vaccinated with artemisinin alone, SLA alone and artemisinin co-administered with SLA after in vitro stimulation with SLA was significantly higher than that of the controls ($P<0.05$). The splenocytes cultures from all the groups that were stimulated with con A produced very high levels of IL-4, further underscoring con A as a non-specific inducer of cell proliferation and cytokine production. As expected, a low and almost undetectable level of IL-4 was observed from wells with unstimulated cultures (Fig. 4.4).

### 4.2.4 Interleukin-4 production after infection with *Leishmania major*

Data on IL-4 production after infection with *L. major* is presented in Fig. 4.4. At 35 days post infection with *L. major*, the level of IL-4 increased considerably in the control group of mice
that were unvaccinated. This increase was 5-fold as compared to before vaccination ($P=0.007$). On the other hand, there was modest increase in the IL-4 profile from mice vaccinated with artemisinin + SLA after infection (mean ± S.E., 57.143 ±1.938 pg/ml). This represented a 1.8-fold increase compared to before infection ($P= 0.002$).

The splenocytes from mice vaccinated with SLA produced higher levels of IL-4 as compared to before infection (mean ± S.E., 68.256 ± 6.372 pg/ml). The increase was significant ($P=0.015$), but did not differ from that of the controls ($P=0.818$). On the other hand, no IL-4 was detected in supernatants of SLA-stimulated splenocytes culture of SLA + BCG injected cases, while there was an significant increase in the profile of IL-4 in mice vaccinated with BCG alone (mean ± S.E., 28.763 ± 3.149 pg/ml, $P=0.026$). Unvaccinated but *L. major* challenged mice produced the highest amount of IL-4 compared to the treatment groups (mean ± S.E., 70.061 ± 3.730 pg/ml; Fig. 4.4).
Fig. 4.4: Interleukin-4 production by spleen cells from BALB/c mice immunized BALB/c mice before and after infection. Mononuclear cell suspensions adjusted to a concentration of $2 \times 10^5$/mL were prepared. The cells were stimulated in vitro with 100 µg SLA. After two days, the culture supernatants were harvested and assayed for the presence of IL-4 by the cytometric bead assay (CBA) method using a FACSCalibur®.

Key: Art-artemisinin; SLA-soluble *Leishmania* antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline

### 4.2.5 Interleukin-5 production before infection with *Leishmania major*

Data on IL-5 production by cell cultures of splenocytes after stimulation with soluble *Leishmania* antigen and concanavalin A are presented in Fig. 4.5. The mean production of IL-5 by cells of mice vaccinated with SLA + artemisinin in response to SLA was higher than
that of the corresponding control unvaccinated group (mean ± S.E., 13.215 ± 0.662 pg/ml vs 9.881 ± 0.837 pg/ml), and was statistically significant (P=0.035). On the other hand, the highest production of IL-5 was realised from the group of mice vaccinated with SLA (mean ± S.E., 19.514 ± 1.251 pg/ml). The mean production of IL-5 by cells of mice vaccinated with BCG alone and BCG + SLA was similar in both groups (mean ± S.E., 8.329 ± 0.628 pg/ml vs 8.547 ± 0.891 pg/ml, P=0.851; Fig. 4.5). The mean production of IL-5 by culture splenocytes of mice vaccinated with artemisinin was modest (mean ± S.E., 11.341 ± 0.962 pg/ml), and did not differ significantly from the controls (P=0.316). The splenocyte cultures from mice stimulated with con A produced relatively high but equivalent levels of IL-5 (P=0.109; Fig. 4.5).

4.2.6 Interleukin-5 production after infection with *Leishmania major*

Data on IL-5 production by cell cultures of splenocytes after stimulation with soluble *Leishmania* antigen and concanavalin A at 35 days post infection are presented in Fig. 4.5. The mean production of IL-5 by cells of mice vaccinated with SLA + artemisinin in response to SLA was significantly higher as compared to before infection with *L. major* (mean ± S.E., 29.296 ± 3.135 pg/ml, P=0.04). On the other hand, there was a two-fold increase in the mean IL-5 produced by splenocytes cultures of cells injected with artemisinin (mean ± S.E., 28.986 ± 2.520 pg/ml). This increase in IL-5 was statistically significant (P=0.03), and was 2.6 times higher than amount produced by the control mice (Fig. 4.5).

No IL-5 was detected from supernatants of splenocyte cultures stimulated *in vitro* with SLA from mice vaccinated with BCG + SLA whereas there was a significantly lower level of IL-5 produced by mice vaccinated with BCG as compared to the controls (P=0.006). Contrary to this observation, there was a slight increase in the mean production of IL-5 from splenocyte
culture supernatants of mice vaccinated with SLA (mean ± S.E., 32.182 ± 3.699 pg/ml). The highest production of IL-5 from splenocyte culture supernatants stimulated \textit{in vitro} with SLA was observed in the control group of mice (mean ± S.E., 38.320 ± 4.778 pg/ml).

Fig. 4.5: Interleukin-5 production by spleen cells from BALB/c mice immunized BALB/c mice before and after infection. Mononuclear cell suspensions adjusted to a concentration of $2 \times 10^5$/mL were prepared. The cells were stimulated \textit{in vitro} with 100 µg SLA. After two days, the culture supernatants were harvested and assayed for the presence of IL-4 by the cytometric bead assay (CBA) method using a FACSCalibur®.

Key: Art-artemisinin; SLA-soluble \textit{Leishmania} antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline
4.3 Results of lesion size measurements

In order to evaluate disease progression in mice vaccinated with soluble *Leishmania* antigens (SLA) co-administered with artemisinin, SLA alone, Bacille Calmette Guérin (BCG) alone, BCG + SLA, artemisinin alone and control non-vaccinated group, lesion development was followed by measuring the increase in the thickness of the infected footpad compared with the collateral uninfected footpad on a week-to-week basis. As shown in Fig. 4.6, lesion sizes had not developed in all the groups of mice between days zero and 7. To check the efficacy of artemisinin as an adjuvant with *L. major* SLA as antigen, footpad swelling in BALB/c mice vaccinated with SLA plus artemisinin and artemisinin alone was compared with that in unvaccinated controls (Fig. 4.6). In these two groups of mice, a significant difference with controls was observed during the first five weeks (*P*<0.05) post infection suggesting delay in infection but no protection was conferred as from 6 weeks (*P*>0.05) onwards till the time when the experiment was terminated. On the contrary, no significant difference with controls was observed in the footpad swelling of mice vaccinated with soluble *Leishmania* antigens alone during the same period (*P*>0.05) compared to the controls suggesting no protection.

On the overall, mice in the control unvaccinated but *L. major* challenged group developed large progressive footpad lesions (1.8 ± 0.04 mm; mean ± S.E., at week 13). Analysis of variance (ANOVA) further demonstrated that lesion sizes of mice injected with artemisinin, artemisinin plus SLA and SLA alone did not differ significantly from those of the control unvaccinated group (*P*>0.05). On the other hand, lesion sizes in the group of mice vaccinated with SLA + BCG and BCG alone were significantly smaller (*P*<0.001) compared with lesions that developed in control unvaccinated but *L. major* challenged mice. Remarkably, animals vaccinated with BCG + SLA had no measurable footpad lesions by week 13 post infection. Animals in the SLA + artemisinin, SLA alone, artemisinin alone and unvaccinated
control groups were euthanatized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain.

Fig. 4.5: The course of *L. major* infection as represented with footpad swelling in BALB/c mice. Data represent the mean ± S.E. of the difference between the infected and the contralateral uninfected footpad from the six experiments with 4 mice per group.

Key: Art-artemisinin; SLA-soluble *Leishmania* antigens, BCG- Bacille Calmette Guérin; Con A- concanavalin A; PBS- phosphate buffered saline.
4.4 Quantification of *Leishmania major* in infected footpads of BALB/c mice

The number of *L. major* parasites present in the infected footpads was quantified at 14, 35 and 63 days post infection using a published limiting dilution assay (Lima *et al.*, 1997) and the results analysed using the ELIDA statistical package (2001-2005) as described by Taswell *et al.* (1987). Results presented in Table 4.1 show that at 14 days post infection, *L. major* parasites were detected in both the vaccinated and unvaccinated groups of mice. Although the number of *L. major* in the footpads from mice vaccinated with SLA + artemisinin was lower than that of the controls at 14 days post infection, the overlapping of the 95% confidence limits indicates that the differences between the groups are not significant (*P* > 0.05). In addition, the parasite burdens in this group of mice did not differ significantly from those of the control unvaccinated mice as the disease progressed as quantified at days 35 and 63 post infection. Similarly, a persistent increase in parasite burdens was observed in footpad lesions from mice injected with SLA alone and artemisinin alone as the disease progressed.

Parasite recoveries from the footpads were significantly lower at all time points in BCG + SLA and BCG alone vaccinated mice than in unvaccinated mice (Table 4.1). The parasite loads in the BCG + SLA vaccinated BALB/c mice lymph nodes were 16- to 130 –fold less than their unvaccinated counterparts while those of the BCG vaccinated mice ranged between 4- to 8 -fold less than the control unvaccinated mice (Table 4.1). The data obtained for these two groups were statistically lower than those of the unvaccinated mice (*P* < 0.05). However, it is worth noting that although there was a reduction in parasite burdens in infected footpads of BCG vaccinated mice between days 14 and day 35 post infection, there was an increase, though insignificant, at day 63 post infection. On the other hand, parasite burdens in the unvaccinated control increased persistently as the disease progressed.
Overall, the parasite burdens in the footpads of mice vaccinated with SLA + artemisinin, SLA alone and artemisinin alone did not differ significantly from each other and from the unvaccinated control mice.
Table 4.1. Footpad parasite burden in BALB/c mice immunized subcutaneously twice at 0 and 13 days with artemisinin alone, soluble *Leishmania* antigen (SLA) plus artemisinin, SLA alone, BCG alone, SLA + BCG and PBS (control) then challenged with virulent *L. major* promastigotes at 7 days post vaccination. (*)$P < 0.005$ when the mice immunized are compared with mice that received PBS (control unvaccinated group).

<table>
<thead>
<tr>
<th>Days post infection</th>
<th>Artemisinin</th>
<th>Art + SLA</th>
<th>SLA</th>
<th>BCG</th>
<th>SLA + BCG</th>
<th>(PBS) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>14.4 (5.13-16.7)</td>
<td>10.1 (2.44-17.75)</td>
<td>13.7 (9.25-18.2)</td>
<td>4.97 (2.63-6.75)*</td>
<td>1.28 (0.493-3.64)*</td>
<td>20.55 (14.75-36.35)</td>
</tr>
<tr>
<td>35</td>
<td>19.25 (13.8-28.75)</td>
<td>20.99 (16.25-25.70)</td>
<td>22.2 (7.95-26.6)</td>
<td>3.44 (0.595-6.09)*</td>
<td>0.93 (0.25-1.32)*</td>
<td>30.85 (20.15-40.3)</td>
</tr>
<tr>
<td>63</td>
<td>56.41 (21.37-74.51)</td>
<td>38.13 (19.24-66.2)</td>
<td>48.83 (19.84-87.21)</td>
<td>8.18 (2.12-13.11)*</td>
<td>0.52 (0.13-1.12)*</td>
<td>67.34 (42.01-97.81)</td>
</tr>
</tbody>
</table>

$10^6$ parasites (95% confidence limits)
CHAPTER FIVE: DISCUSSION

5.1 Overview

*Leishmania major* is the causative agent of Old World Cutaneous Leishmaniasis, a disease that is characterised by cutaneous lesions that can be self healing or chronic when accompanied by defective cellular immune responses (Ajdary *et al*., 2000). For vaccination against CL in murine models, various adjuvants have been successfully tested with *Leishmania* antigens with varying results. These include complete Freund’s adjuvant (CFA), glucan, BCG, *Corynebacterium parvum*, IL-12 and CPG-ODNs among others (Afonso *et al*., 1994; Stacey and Blackwell, 1999; Handman, 2001). An essential role of adjuvants in vaccines is to direct CD4+ T cell subset differentiation (Afonso *et al*., 1994). Following the same pattern, the adjuvant potential of artemisinin a well known antimalarial was evaluated in this study. This is the first documented study that sought to determine the adjuvant potential of artemisinin co-administered with a soluble leishmanial antigen in BALB/c mice. Results presented here demonstrate that co-injection of artemisinin as adjuvant with soluble leishmanial antigen preparation does not modulate *Leishmania*-specific immunity towards a protective immune response.

5.2 Lymphoproliferative responses of mouse splenocytes to soluble *Leishmania* antigens

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses (Zhou *et al*., 2005). Proliferative responses of splenocytes to soluble leishmanial antigens have often been used as a measure of exposure to the parasite, as well as a measure of protection (Gicheru *et al*., 2001). In addition, it is now a widely accepted fact that cellular immune responses play a paramount role in the pathogenesis and healing of leishmaniasis (Garg *et al*., 2005). In the current study, splenocytes from mice injected with artemisinin alone virtually did not proliferate in response to *in vitro* stimulation with SLA compared to the control groups both before and after infection with *L. major* (*P* >
0.05). This observation is in tandem with previous results which suggest that artemisinin does not promote proliferation of cells in vitro and in vivo (Zhou et al., 2005; Wang et al., 2007). On the other hand, splenocytes from the group of mice vaccinated with soluble *Leishmania* antigens co-administered with artemisinin proliferated in response to SLA in vitro. This proliferative response could be attributed to SLA since SLA alone has been shown to stimulate proliferation of T-cells in both experimental and clinical CL systems (Ajdary et al., 2000; Gabaglia et al., 2004). On the other hand, the group of mice vaccinated with SLA + BCG had significantly high proliferative responses to SLA compared to unvaccinated mice both before and after infection (*P*<0.01). This is compatible with previous studies which demonstrated high proliferative responses in cells of mice vaccinated with SLA and a Th1 promoting adjuvant (Scott et al., 1987). Splenocyte blastogenic responses to SLA in vitro in mice vaccinated with SLA were modest, suggesting sensitization of mice. These results are in tandem with previous reports by Scott et al. (1988), which suggested that fractions of SLA separated by ion exchange chromatography stimulated proliferation of splenocytes (Scott et al., 1988). Absence of lymphoproliferative responses was also observed in control unvaccinated but *L. major* infected animals and was correlated with disease progression during the study. This is congruent with previous studies by Garg et al. (2005).

### 5.3 Cytokine production

One of the most predictive in vitro assessments of cellular immunity in murine and human CL is the ability of lymphoid cells and PBMCs respectively to secrete cytokines in response to antigenic stimulation (Coutinho et al., 1998). Extensive studies with experimental mouse models infected with *L. major* have shown that the outcome of infection with *Leishmania* parasites is critically dependent on the activation of one of the two subsets of CD4 T cells, Th1 or Th2 (Sacks and Noben-Trauth, 2002; Roberts, 2006). Gamma interferon (IFN-γ),
secreted by Th1 cells, is the most potent macrophage-activating cytokine leading to host resistance to infection with *Leishmania* parasites whereas IL-4 secreted by Th2 cells, is associated with down-modulation of IFN-γ-mediated macrophage activation (Sacks and Noben-Trauth, 2002). In addition, susceptibility to *L. major* as demonstrated by BALB/c mice has been attributed to the production of IL-5 (Sacks and Noben-Trauth, 2002). There is strong indirect and direct evidence of the importance of the Th1 and Th2 paradigm in the control of and resistance to human leishmaniasis respectively (Ajdary *et al*., 2000; Mahmoodi *et al*., 2005). This study sought to determine whether the co-administration of artemisinin with soluble *Leishmania* antigens will enhance the development of a Th1 or Th2 immune response as measured by IFN-γ production on one hand and IL-4 and IL-5 on the other, as correlates of resistance and susceptibility to *L. major* respectively in BALB/c mice.

The results presented here demonstrate that the mice vaccinated with SLA + artemisinin produced low levels IFN-γ levels that did not differ significantly from those of the controls both before and after infection (*P* < 0.05). Conversely, this group of mice produced significantly high levels of IL-4 and IL-5 compared to the controls suggesting that SLA co-administered with artemisinin induces a Th2 immune response and does not provide protection against cutaneous leishmaniasis. On the other hand, artemisinin alone produced IFN-γ levels that were comparable to the controls both before and after infection with *L. major*. On the other hand, the mice from this group produced significantly higher levels of IL-4 and IL-5 compared to the controls. The level of IL-4 and IL-5 increased significantly after infection (*P* < 0.05). The shift towards a Th2 immune response was responsible for the high parasite count and uncontrolled lesion sizes observed in these two groups. This is because interleukin-4 has been shown to suppress production of IFN-γ whose role is to activate macrophages to kill intracellular parasites (Noben-Trauth *et al*., 2003). The low
production of IFN-γ could also be attributed to artemisinin. Wang et al. (2007), recently demonstrated that SM905, a new water-soluble artemisinin derivative, inhibited TCR (T cell receptor)/CD3 plus CD28-mediated primary T cell proliferation as well as production of IL-2 and IFN-γ.

Previous studies by Afonso et al. (1994), have demonstrated that lymph node cells from BALB/c mice injected with SLA produce IL-4 but little IFN-γ (Afonso et al., 1994). In addition, studies conducted by other workers have shown that for SLA to protect BALB/c mice against challenge infection with *L. major*, co-administration with a Th1 promoting adjuvant such as *Corynebacterium parvum* and IL-12 among others is necessary (Scott et al., 1988; Afonso et al., 1994). To determine whether co-administration of artemisinin will alter the initial response to SLA in BALB/c mice, cytokine production by spleen cells taken a day before infection and 35 days post infection with *L. major* was assessed. Mice vaccinated with SLA produced a low level of IFN-γ and significantly high levels of IL-4 and IL-5 comparable to the unvaccinated control mice. Other studies have shown that separation of SLA yields fractions that secrete either Th1 or Th2 cytokines in *L. major* infected BALB/c (Scott et al., 1988). It is therefore not surprising that the SLA used in this study produced both Th1 and Th2 cytokine profiles. As a result, mice vaccinated with SLA developed uncontrolled lesions and had high parasite loads, comparable to those of the control unvaccinated mice. Exacerbation by SLA in the absence of a Th1-promoting adjuvant is not surprising in BALB/c mice, since they have an inherent tendency to develop Th2 responses (Stacey and Blackwell, 1999).

Vaccination of mice with SLA + BCG yielded significantly high levels of IFN-γ and significantly lower levels of IL-4 and IL-5 compared to the control unvaccinated mice.
experimental vaccine studies using killed *L. major* promastigotes (KLM) and BCG as adjuvant resulted in production of significantly lower levels of IL-4 and high levels of IFN-γ (Alimohammadian *et al.*, 2002). In this study, BCG was used as a control adjuvant. Interestingly, all the mice that received $10^5$ C.FU of BCG demonstrated an earlier healing response, as reported previously (Connel *et al.*, 1993; Alimohammadian *et al.*, 2002), because BCG promotes better antigen presentation and induces or accelerates expansion of T cells that secrete macrophage-activating cytokines (IL-2 and IFN-γ), leading to macrophage activation and intracellular parasite killing (Convit *et al.*, 1989). The use of BCG as an adjuvant has been well established for many years (Frommel and Lagrange, 1989). In addition, immunotherapy of the patients with leishmaniasis using killed parasites plus live BCG has been carried out successfully (Convit *et al.*, 1989). Furthermore, in vaccine trials using killed *L. major* candidate vaccine, BCG is commonly used as an adjuvant (Bahar *et al.*, 1996; Momeni *et al.*, 1999; Sharifi *et al.*, 1998; Khalil *et al.*, 2000).

**5.4 The course of *Leishmania major* infection in BALB/c mice**

Tracking lesion size (as a measure of the pathologic response to infection) is one method of monitoring the development of infection with *L. major* (Al-Wabel *et al.*, 2007). In the experiments performed here, mice vaccinated with SLA + artemisinin appear to have initially been protected at 2, 3 and 5 weeks post infection, while those vaccinated with BCG + SLA and BCG alone developed lesions that were comparable to the control unvaccinated mice early in infection. At these points in time, the parasite burdens in the SLA + artemisinin vaccinated mice were lower than those of artemisinin alone and SLA alone though not significantly different from those of the control unvaccinated group. Previous studies have shown that lesion pathology and parasite burden within lesions do not always correlate (Lima *et al.*, 1997). Since BALB/c resistance can be established with low doses of parasites (Bretscher *et al.*, 1992), one interpretation is that the parasites used here were insufficiently
infective. However, this seems unlikely since the parasite loads in the footpad lesions of all the groups was in the range of $10^5$-$10^6$/footpad.

BCG inoculation is known to lead to local inflammation followed by development of an immune response with cytokine production and induced macrophage activation for killing of intracellular and extracellular targets (Pappas et al., 1983; Fortier et al., 1987). It can therefore be concluded that the large lesion sizes initially observed in the BCG + SLA and BCG alone vaccinated mice was due to the inflammatory action of BCG before the induction of protective immune response.

To dissociate the nonspecific effect of artemisinin from *Leishmania*-specific adjuvant activity, it was necessary to wait for at least 1 week between the last vaccination and challenge infection. One week was deemed sufficient because artemisinin and its derivatives have a short half-life ranging between 10 min for parent compounds such as artesunate to slightly over 1 h for dihydroartemisinin (Krishna et al., 2004), and so it would be expected that by one week post infection, any effect on disease progression would only be attributed to the effect on the immune system and not direct killing of parasites. Since consistent differences had not developed in the size of lesions of control mice compared with vaccinated mice at between one and four weeks post infection, it was necessary to follow up the lesion sizes beyond this period. It is possible that some residual unexcreted artemisinin may have been responsible for the inhibition of parasite growth early in infection resulting in smaller lesions in the groups of BALB/c mice vaccinated with SLA co-administered with artemisinin and the artemisinin alone group in the first weeks of infection. This is because treatment with artemisinin drugs has been shown to cause reduction of parasite burden below detectable levels without eliminating all parasites; this results in a higher risk of
recrudescence (Krishna et al., 2004; Bjorkman and Bhattarai, 2005). In addition, a fraction of the parasites exposed to the drug are thought to become dormant and unsusceptible to further dosing until reactivation (Hoshen et al., 2000).

Overall, no protective effect of vaccination with SLA and artemisinin was observed since animals progressively developed ulcerating lesions and had to be killed if they reached a predetermined level of lesion severity. The response of unvaccinated BALB/c mice to infection in these experiments was consistent, with mice in this group progressively developing large uncontrolled lesions. In addition, mice vaccinated with BCG developed significantly smaller lesion sizes compared to the controls. However, in no case did vaccination make lesions ultimately resolve like those of the mice vaccinated with SLA + BCG.

5.5 Parasite burdens correlated with lesion development

To attempt to confirm the results obtained for lesion size measurements, and because lesion pathology and parasite burden within lesions do not always correlate (Lima et al., 1997), this study also determined the numbers of parasites in the lesions of infected BALB/c mice by using published techniques (Lima et al., 1997). Mice vaccinated with SLA + artemisinin had lower parasite burdens compared to the artemisinin alone and SLA alone vaccinated mice. However, these parasite loads did not differ significantly from those of the control unvaccinated groups or those in the footpad lesions of mice vaccinated with SLA alone or artemisinin alone throughout the experiment, with overlapping 95% confidence intervals. The relatively low parasite numbers could be attributed to the effect of SLA on the immune system since the parasite load was closer to those observed in the mice vaccinated with SLA alone than to those of the mice vaccinated with artemisinin alone. Previous studies have demonstrated that fractionation of SLA by ion-exchange chromatography identified only one
protective fraction out of nine and immunization of BALB/c mice with this fraction protected
the mice against a normally fatal challenge with *L. major*, and that this immunity was
associated with the induction of cell mediated immunity (Scott *et al*., 1987). It is possible that
this protective fraction of SLA was responsible for the lesser parasite burden observed in the
mice vaccinated with SLA alone and SLA + artemisinin group as compared to the control
unvaccinated mice.

Mice vaccinated with BCG + SLA and BCG alone had significantly reduced parasite
burdens. This is attributed to the production of significantly high production of IFN-γ and
low production of IL-4 and IL-5. Interferon gamma activates macrophages to kill
intramacrophage *Leishmania* parasites (Sacks and Noben-Trauth, 2002). Overall, in this
study, cutaneous lesion development directly correlated with lesion (footpad) parasite
burden.

The route of administration, which may have a different effect upon the host immune
response, has not been well defined either in humans or animal models is also another factor
that may have been responsible for exacerbation of the disease in mice vaccinated with SLA
+ artemisinin. Subcutaneous administration of a crude antigen (Liew *et al*., 1985) or even
candidate vaccine antigens selected by immune screening (Reiner *et al*., 1993) actually
expands Th2 cell populations and abrogates innate resistance or exacerbates protection in
susceptible mice. The same was found to be true in the mice group subcutaneously
vaccinated with SLA alone that produced low IFN-γ and relatively high levels of IL-4 and
IL-5 and went on to develop cutaneous lesions that did not differ from those of the
unvaccinated control mice. It also is possible that the concentration of 75 mg/Kg of
artemisinin that was used in this study was responsible for modulating the immune system
towards a non-protective response. Previous studies with artemisinin have shown that the drug affects the immune system in a concentration-dependent manner (Golenser et al., 2006).

Although artemisinin has been shown to be effective against *Leishmania* parasites both *in vitro* and *in vivo* (Avery et al., 2003; Ma et al., 2004), the experiments conducted in this study failed to show an adjuvant effect of artemisinin. Because the mechanism by which artemisinin was efficacious against these parasites has not been described and based on the fact that both *Leishmania* and *Plasmodium* are intracellular parasites, it is possible that the same mechanisms that the proposed mechanisms by which artemisinin kills *Plasmodium* parasites which include interference with plasmodial sarcoplasmic/endoplasmic calcium ATPase (SERCA) (Eckstein-Ludwig et al., 2003; Krishna et al., 2004), interference with mitochondrial electron transport (Jiang et al., 1985; Li et al., 2005), production of reactive species (Ittarat et al., 2003) were responsible for the reported efficacy against *Leishmania* parasites in the above documented studies.

Taken as a whole, these studies suggest that vaccination with artemisinin as adjuvant to soluble *Leishmania* antigens up-regulated a type 2 response to a greater extent than a type 1 response, since activation of IL-4 and IL-5 was more pronounced than that of IFN-γ. Previous studies have demonstrated that genetic predisposition for susceptibility to *L. major* infection in mice correlates with the dominance of an IL-4 driven Th2 response (Sacks and Noben-Trauth, 2002). It is therefore possible that lack of protection seen in vaccinated mice was due to down-regulation of IFN-γ production and up-regulation of IL-4 and IL-5.
6.1 Conclusions

a) These studies confirm the widely held belief which associates resistance and susceptibility to \textit{L. major} in BALB/c mice with the induction of the two phenotypically distinct subsets of CD4+ T cells, namely T helper cell type 1 (Th1) and type 2 (Th2) cells respectively, culminating in the production of different cytokine patterns during the disease process.

b) These results confirm previous studies implicating that soluble antigens derived from whole parasites can provide excellent protection only when it is administrated together with some adjuvant. Whereas artemisinin co-administered with SLA was non-protective, BCG a well established Th1 promoting adjuvant protected the mice from challenge infection with \textit{L. major}.

c) Immunization of BALB/c mice with SLA co-administered with 75 mg/Kg of artemisinin induces Th2 immune responses (IL-4 and IL-5) and does not protect BALB/c mice against challenge infection with \textit{L. major}. The lesion size results correlated with parasite burdens.

d) Antigen induced proliferation to soluble \textit{Leishmania} antigens was observed in response to vaccination with soluble leishmanial antigen and in response to challenge infection with \textit{L. major} parasites. The proliferative response was greater after infection than before infection.
Lesion sizes in this study correlated with parasite burdens in the infected footpads. Unvaccinated mice had a large number of parasites while mice vaccinated with BCG + SLA had the least parasite loads.

6.2 Recommendations

6.2.1 Future studies

a) Artemisinin affects the immune system in a concentration-dependent manner. More aspects of immune responses in mice vaccinated with SLA co-administered with different concentrations of artemisinin need to be explored, particularly with regard to the production of the regulatory cytokine IL-10 as well as immunoglobulins of the IgG2 subclass.

b) Artemisinin has been shown to be effective against *Leishmania* parasites both *in vitro* and *in vivo*. Since immunotherapies have been applied in the treatment of leishmaniasis in order to improve chemotherapy, it is important to investigate the immunochemotherapeutic potential of artemisinin with *Leishmania* antigens.

6.2.2 Applications of the findings

a) In the absence of a vaccine against *Leishmania*, BCG + SLA is a promising vaccine for CL.

b) In this study, lesion sizes correlated well with parasite burdens. For CL vaccine studies in the mouse model, lesion size is a good measure of protection.
6.2.3 Directions for future research

Since artemisinin has been shown to affect the immune system in a dose-dependent manner, further studies need to be conducted using different concentrations of artemisinin with soluble Leishmania antigens and other vaccines against leishmaniasis.
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APPENDICES

Appendix I

STANDARD OPERATING PROCEDURES

KENYA MEDICAL RESEARCH INSTITUTE (LEISHMANIASIS LABORATORY)

Research project title: Determination of the adjuvanticity of artemisinin with soluble Leishmania antigens

Protocol I

Preparation of soluble Leishmania antigens for vaccination

Responsible personnel:
Albert Kimutai
Dr. Willy Tonui

PROCEDURE

1. Harvest parasites by centrifugation at stationary phase
2. Wash 3x in sterile phosphate Buffered Saline
3. Count using a haemocytometer counting chamber
4. Resuspend the in a small volume of Phosphate Buffered Saline or Physiological saline
5. Freeze thaw in liquid nitrogen 3x
6. Subject to 3-5 sonication cycles of 20 seconds each on ice
7. View microscopically to ensure that all the parasites are disrupted
8. Centrifuge suspension at 27 000 g for 20 min at 4°C
9. Collect supernatant and quantify protein concentration
10. Aliquot and freeze in -70°C until when required

**Protocol II**

**Title: Cell cultures for proliferation and cytokine assays**

**Objective:** To determine cell proliferation and cytokine production by proliferated cells

**Materials and methods**

**Requirements:**
- Immunized mice (Two each from groups immunized with soluble *Leishmania major* antigen (SLA), artemisinin, artemisinin + SLA, *Mycobacterium bovis* Bacille Calmette Guerin (BCG), BCG + SLA and phosphate buffered saline (PBS).
- Sterile wire mesh
- Sterile RPMI wash media
- Sterile complete RPMI supplemented with 5% FBS
- Sterile Petri dishes
- Ficoll-paque
- Sterile syringe plunger
- Sterile 15 ml centrifuge tubes
- Microscope
- Haemocytometer
- Trypan blue
- 96 well plates
- Soluble *Leishmania* antigen 100 μg/ml
- Concanavalin A mitogen (10 μg/ml).

**Procedure**

**a) Preparation of splenocytes**

1. Sacrifice two BALB/c mice from each experimental group by CO₂ asphyxiation
2. Excise spleens aseptically through a fine wire mesh using a syringe plunger
3. Collect crushed spleen into sterile Petri dishes containing 5-7 mL RPMI wash media
4. Allow cells to stand in ice for 5-10 min for sedimenting of clumps
5. Transfer the upper portion of the medium containing the splenocytes using 1ml pipette, to a 15 mL sterile centrifuge tube.
6. Wet walls of a 15ml centrifuge tube with RPMI wash containing 5% FBS.
7. Remove RPMI and add 3 mL of thoroughly well mixed Ficoll-Paque™ PLUS to a 15 mL tube (mix Ficoll-Paque™ PLUS (Ficoll) thoroughly before use by inverting the bottle several times).
8. Carefully layer cell suspension (max of 500 x 10⁶ cells per gradient in 5mL of wash containing 5% FBS) over Ficoll being careful to minimize mixing with Ficoll.
9. Centrifuge the cell suspension at 400 X g for 30 min and wash cells once again in RPMI medium.
10. Remove and discard upper plasma layer without disturbing the Plasma-Ficoll Interface
11. Remove and retain lymphocytes at the plasma-Ficoll interface without disturbing the erythrocyte/granulocyte pellet.
12. Wash mononuclear cells once with medium at between 1500-2500 rpm.
13. Count cells using a small aliquot of cell suspension in Neubeur chamber and Trypan blue exclusion.

b) Dispensing and culturing of lymphocytes

1. Make cell suspension containing 2 x 10⁶ viable cells/mL in culture media containing 5% FBS
2. Dispense the cells at the rate of 100 μl per well in 96 well flat bottomed tissue culture plates in triplicate for each of the experimental groups
3. Culture cells from each experimental group with either 10 μg/ml of 100 μl of Con A, 100 μg/mL of SLA and medium alone.

4. Incubate cells in a humidified CO₂ incubator (5%) at 37 °C for 48h.

5. Harvest supernatants from each of the triplicate wells of Con A, SLA and medium alone for each of the experimental groups and pooled. They were then stored at --20 °C till assayed using a flow cytometer (FACSCalibur™).

c) Cell proliferation assay using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

1. Add 100 μl of 5 mg/ml of MTT solution to all wells of 48 h cultured lymphocytes and incubate for 4 h at 37 °C (formazan crystals will be formed at the bottom of each well).

2. Pipette out 100 μl of the spent medium along with suspension of cultured wells

3. Add 100 μl of dimethyl sulfoxide (DMSO) to all wells and pipette thoroughly to dissolve the dark blue crystals.

4. After a few minutes read the plates at room temperature using an ELISA reader at 570nm wavelength

5. Read plates normally within 1 hour of adding DMSO

6. Calculate cell proliferation as stimulation index

\[
\text{Stimulation Index} = \frac{A_{570 \text{ nm Stimulated lymphocytes}}}{A_{570 \text{ nm unstimulated lymphocytes}}}
\]

Where A570 = Absorbance at 570 nm

Appendix II

Cutaneous leishmaniasis lesion in man