EVALUATION OF BACILLE CALMETTE GUÉRIN, MONTANIDE ISA 720
AND ALUMINIUM HYDROXIDE AS ADJUVANTS FOR LEISHMANIA
VACCINE IN BALB/c MICE

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Evaluation of Bacille calmette guerine
DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other university

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Supervisors’ approval

We confirm that, the work reported in this thesis was carried out by the candidate under our supervision

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DEDICATION

A thesis dedicated to all those involved in the fight against leishmaniasis
ACKNOWLEDGEMENTS

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VL  Visceral Leishmaniasis

WHO  World Health Organisation

wk  Weeks

μCi  Microcurie

μg  Microgram

μl  Microliters
Abstract

Leishmaniases are parasitic diseases caused by protozoan flagellates of the genus *Leishmania*. These parasites infect numerous mammalian species, including humans, and are transmitted through the infective bite of an insect vector, the female *Phlebotomus* sandfly. Leishmaniasis is currently endemic in 88 countries, and is a threat to 350 million people with a worldwide prevalence of 14 million clinical cases and 12 million new cases each year. Drugs for leishmaniasis are generally toxic and their cost is prohibitive. Vector control measures are poorly implemented and inaccessible to many people in developing countries. In leishmaniasis, protection requires leishmanial-specific CD4+ T helper-I (Th1) cells. Effective vaccination against leishmaniasis would be cheaper and accessible. Unfortunately, so far there is no vaccine against leishmaniasis for routine use. Immunity to cutaneous leishmaniasis, a chronic skin-disfiguring lesion affecting millions, has been historically achieved by inoculation with live virulent preparations of the parasite. Killed antigens that could be safer as vaccines generally require an adjuvant for the induction of strong Th1 response in murine models. The objective of this study was to assess and compare the immune responses and efficacy of a vaccine containing Bacille calmette guerin (BCG), aluminium hydroxide (alum) and Montanide ISA 720 (MISA 720) as adjuvants combined with killed *Leishmania major* vaccine (KLM) in BALB/c mice. Sixty mice were immunized three times at weeks 0, 4 and 6 and two weeks later, either sacrificed for comparative immunogenicity analysis or challenged with virulent *L. major* for efficacy monitoring. Splenic blood lymphocytes were stimulated in culture with KLM antigens or concanavalin A and their proliferation quantified. Sera immunoglobulin gamma (IgG) and *in vitro* interferon gamma (IFN-γ) production were measured by enzyme linked immunosorbent assay (ELISA) using *Leishmania* antigen and cross-reactive monoclonal antibody. Lesion development in infected mice footpads were monitored for 8 weeks and parasite loads determined there after. The results indicated higher IgG responses in the BCG-KLM and alum-KLM vaccinated mice as compared to the MISA-KLM mice. Antigen-specific lymphoproliferative *in vitro* response showed significantly higher (*p<0.01*) responses in the MISA-KLM as compared to alum-KLM and also BCG-KLM groups. The BCG-KLM group recorded significantly higher (p<0.001) IFN-γ production as compared to both alum-KLM and MISA-KLM vaccinated groups. Efficacy evaluations showed significantly reduced lesion sizes in the MISA-KLM than in both alum-KLM and BCG-KLM vaccinated groups. There were significantly reduced (p<0.001) parasite loads in both the MISA-KLM and BCG-KLM groups as compared to the alum-KLM vaccinated groups. It was however, noted that, BCG vaccination caused inflammatory reaction that led to highest lesion sizes observed in the mice vaccinated with BCG as compared to both the alum and Montanide ISA 720 vaccinated mice groups. The results from this study conclude that Montanide ISA 720 adjuvant is safe and could be used to induce protection against cutaneous leishmaniasis caused by *L. major* in susceptible BALB/c mice. The study has contributed valuable data to be used in further studies and the development of a potential adjuvant for human *Leishmania* vaccine.
CHAPTER 1: INTRODUCTION

1.1 General introduction

Leishmaniases are parasitic diseases caused by protozoan flagellates of the genus *Leishmania* which multiplies in certain vertebrates that act as reservoirs of the disease. The parasite is transmitted to humans through the bite of female sandflies that have previously fed on an infected reservoir (WHO, 2007). The leishmaniases are currently threatening 350 million people in 88 countries of four continents. The disease is endemic throughout parts of Africa, India, the Middle East, southern Europe, and Central and South America (Figure 1.1) and epidemics are also well recognized (Handman, 2001). An estimated 14 million people are infected, and each year about two million new cases occur (WHO, 2007; Desjeux, 1999). The disease contributes significantly to the propagation of poverty, because treatment is expensive and hence either unaffordable or it imposes a substantial economic burden, including loss of wages (WHO, 2007).

About 22 species and subspecies are known to cause disease in humans and 30 Phlebotomine species are confirmed vectors (Herwaldt, 1999; Desjeux, 1996). Both parasites and vectors can be classified regionally into Old and New world species depending on the geographical region where they are found. The species found in America are considered New World species while those occurring in the rest of the world are Old World species. The overlapping geographical distribution of human immunodeficient virus (HIV) infection and leishmaniasis is increasing due to the spread of leishmaniasis from rural into urban areas (Alvar *et al.*, 1997).
Figure 1.1. World map highlighting areas where cutaneous, visceral, and mucocutaneous leishmaniasis are endemic (Adopted and modified from Handman, 2001).
Leishmania-HIV coinfection is regarded as an emerging infectious disease, for in certain countries up to 70% of adult cases of leishmaniasis are related to HIV and AIDS infection (Alvar et al., 1997). Two forms of leishmaniasis are found in Africa, visceral disease or kala-azar, caused by *L. donovani* and cutaneous leishmaniasis caused by *L. major*.

*Leishmania donovani* causes destructive infections of the liver, spleen, lymph nodes and bone marrow while *L. major* is usually restricted to the skin where it causes disfiguring large ulcers. Leishmaniasis has been known to be endemic in many parts of Kenya from as back as early 19\textsuperscript{th} century. Nine sporadic cases of leishmaniasis were reported between 1911 and 1939 (Findall, 1961). The main endemic foci of visceral leishmaniasis in Kenya are Eastern and the Rift valley provinces (Muigai et al., 1987). Cutaneous leishmaniasis occurs on the eastern slopes of Mount Elgon in Western Kenya, parts of the Rift valley and some parts of Central Kenya among other areas (Mutinga et al., 1975b). Some of the districts affected by leishmaniasis include: Baringo, Isiolo, Meru, Turkana, Laikipia, Kajiado, Machakos, Kitui and Wajir districts (Figure 1.2).

Vector control measures, such as residual insecticide spraying and the use of insecticide-treated bed nets or curtains, offer effective protection (Desjeux, 2004; Reyburn, 2000). However, treated bed-net programs are poorly implemented in many endemic countries (Thakur, 2000) and are beyond the means of many families in endemic villages (Desjeux, 2004).
Figure 1.2. Geographical distribution of leishmaniases in Kenya (Adopted and modified from Killick-Kendrick, 1999).

Key:  CL- Cutaneous leishmaniasis,

VL- Visceral leishmaniasis,

DCL- Diffuse cutaneous leishmaniasis
Treatment for leishmaniasis often involves the use of pentavalent antimony compounds or various formulations of amphotericin B. However, the increasing prevalence of drug-resistant organisms and the tendency for patients to relapse after an initially successful regimen of chemotherapy underscore the need for an effective prophylactic vaccine (Reed and Scott, 2000).

To date, there are no vaccines against leishmaniasis. However, there is consensus that in the longer term, vaccines ought to become a major tool for the control of Leishmaniasis (Handman, 2001). Protection against leishmaniasis depends on induction of cell-mediated immunity of Th 1 phenotype. Efforts to develop an effective Leishmania vaccine so far have been limited due to lack of an appropriate adjuvant (Sohrabi et al., 2005). The addition of adjuvant becomes necessary to enhance immune responses and improve vaccine potency (Aucouturier et al., 2002).

The goal of this study was to test the efficacy and immune responses induced by Bacille Calmette Guérin, Montanide incomplete seppic and aluminium hydroxide adjuvants co-administered with killed Leishmania parasites in susceptible BALB/c mice with the aim of identifying the most appropriate adjuvant for Leishmania vaccines.

1.2 Problem statement
Leishmaniasis is one of the most neglected diseases targeted by the World Health Organization (Amaral et al., 2002). Presently, 88 countries in four continents suffer huge economic losses from infections of Leishmania that currently has a prevalence of 14
million clinical cases worldwide, 2 million new infections annually and 350 million people at risk of infection (WHO, 2007; Alvar et al., 2006). The overlapping geographical distribution of human immunodeficiency virus (HIV) infections and leishmaniasis is increasing due to the spread of the disease from rural into urban areas causing a threatening emergency of leishmaniasis/HIV co-infections. The disease is mainly prevalent in areas where the population is scattered and nomadic, making the logistic of regular drug distribution for chemotherapy problematic.

Vector control measures are poorly implemented in many endemic countries and are beyond the means of many families in endemic villages (Alvar et al., 2006). Moreover, chemotherapy is very expensive and therefore inaccessible to many people and the drugs are highly toxic, causing severe reactions (Sharifi et al., 1998), including death in 10% of those who are treated. There is also increasing prevalence of drug-resistant organisms and tendency for patients to relapse after an initially successful regimen of chemotherapy.

Developing an effective vaccine is a better option in the control of leishmaniasis (Reed and Scott, 2000). Leishmanization, an ancient technique which traditionally involved the exposure of babies’ bottoms to sandfly bites in order to protect them from facial lesions or the use of a thorn to transfer infectious material from lesions to uninfected individuals was shown to induce long lasting protection (Handman, 2001). Unfortunately, the practice is no longer acceptable as it was found to cause the development of large uncontrolled skin lesions, immunosuppression and other diseases (Modabber, 1995). There is need to develop a safer vaccine based on killed parasites.
Killed *Leishmania* parasites are poorly immunogenic and generally need to be administered together with an adjuvant to improve immunogenicity (Kenney *et al.*, 1999). Only few adjuvants are approved for human use. It was therefore important to test approved human adjuvants with potential for *leishmania* vaccines to identify the most appropriate adjuvant for *Leishmania* vaccines.

1.3 Research questions

a) What are the immune responses induced by Bacille calmette guérin, Montanide incomplete seppic and Aluminium hydroxide adjuvants co-administered with *L. major* killed promastigotes in BALB/c mice?

b) What is the efficacy of the three different adjuvants co-administered separately with *Leishmania major* killed vaccine in mice model for Leishmaniasis?

1.4 Null hypothesis

Bacille Calmette Guérin, Montanide Incomplete Seppic and Aluminium hydroxide adjuvants co-administered with killed *Leishmania* major do not induce protective immune responses in mice following vaccination, thus are not efficacious and appropriate for *Leishmania* vaccines.
1.5 Objectives

1.5.1 General objective
To evaluate Bacille calmette guérin (BCG), Montanide incomplete seppic (MISA) and Aluminium hydroxide as adjuvants for killed Leishmania vaccine in BALB/c mice.

1.5.2 Specific objectives

a) To determine the immune responses induced by Bacille Calmette Guérin (BCG), Montanide Incomplete Seppic (MISA) and Aluminium hydroxide (Alum) adjuvants co-administered with *L. major* killed promastigotes in BALB/c mice.

b) To determine the efficacy of Bacille Calmette Guérin, Montanide Incomplete Seppic Adjuvant and Aluminium hydroxide adjuvants when used independently with *L. major* killed antigens in mice following vaccination and challenge.

1.6 Significance of the study
The lack of cheaper and safe antileishmanial drugs coupled with the poorly implemented and inaccessible vector control measures and the fact that an effective vaccine against leishmaniasis is feasible prompted this study. The study was relevant as it tried to utilize readily available and cheap tools in the search for an effective *Leishmania* vaccine. The identification of an adjuvant with potential for *Leishmania* vaccine can bring closer the realization of a cheaper universal control method for this disease.
CHAPTER 2: LITERATURE REVIEW

2.1 Life cycle of *Leishmania*

In nature, *Leishmaniae* are alternately hosted by the insect (flagellated promastigote) and by mammals (intracellular amastigote stage). When a female sandfly takes a blood meal from a *Leishmania* infected mammal, intracellular (and may be also extra-cellular) amastigotes are ingested by the insect (Figure 2.1). Inside the blood meal, amastigotes transform into motile promastigotes, which escape through the peritrophic membrane enveloping the blood meal. The promastigotes multiply intensively inside the intestinal tract of the sandfly, successively as free elongated promastigotes (nectomonads) or as attached pro- and paramastigotes (haptomonads; Walters, 1993). This intraluminal development occurs in the midgut. Whatever the multiplication site, the parasites subsequently migrate to the anterior part of the sandfly midgut, where they change into free swimming metacyclic promastigotes, the stage infective for the vertebrate host (http://www.stanford.edu/class/humbio103/paraSites2006/leishmaniasis/LandR.htm).

The bite of an infected sandfly deposits infective metacyclic promastigotes in the mammalian skin (Figure 2.1), which are rapidly phagocytosed by cells of the mononuclear phagocyte system. The intracellular parasites change into amastigotes, which multiply by simple mitosis.
Figure 2.1. Schematic diagram of the *Leishmania* digenetic life cycle (Adopted from Handman, 2001).
2.2 Transmission of *Leishmania*

The inoculation of metacyclic promastigotes through the sandfly bite is the usual method of leishmaniasis transmission. Other routes remain exceptional. In visceral leishmaniasis (VL), a few cases of congenital and of blood transfusion transmission have been reported. A case of direct transmission by sexual contact has been reported (Symmers, 1960). Exchange of syringes has been incriminated to explain the high prevalence of *L. infantum/HIV* co-infection in intravenous drug-users in southern Europe (Dye and Wolpert, 1988). In cutaneous leishmaniasis (CL), contact with the active lesion is innocuous; infection should require inoculation of material from active sores, as was carried out in ancient times by various populations of endemic areas as a crude form of vaccination.

2.3 Clinical manifestations of *Leishmaniasis*

*Leishmaniasis* is a disease attributed to any of a number of protozoan species of the genus *Leishmania* (Table 2.1). It is transmitted by the bite of an infected female *Phlebotomus* sandfly. There are four major clinical types of this infection: cutaneous, diffuse cutaneous, mucocutaneous, and visceral leishmaniasis (Enrique *et al.*, 2005). Visceral leishmaniasis (VL), commonly known as kala-azar from the Hindu vernacular, is a systemic disease. An estimated 500,000 new cases of VL occur each year, and a tenth of these patients will die (http://www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html).

The disease causes splenomegaly, recurring and irregular fever, anaemia, pancytopenia, weight loss, and general weakness. Visceral leishmaniasis is a silent killer, invariably
killing almost all untreated patients (Boelaert et al., 2000). Mucocutaneous leishmaniasis (ML) is a severe disfiguring disease that usually evolves chronically and is extremely difficult to treat (Marsden, 1986). Cutaneous leishmaniasis is caused by different species of the hemoflagellatic protozoan *Leishmania* and affects approximately 1.5 million individuals per year. Old World CL is separated into four distinct types (Table 2.1): *L. aethiopica*, *L. major*, *L. tropica* and *L. infantum* (Enrique et al., 2005). *Leishmania aethiopica* in eastern Africa and the *L. mexicana* species complex in the Americans are also the aetiological agents of DCL. Unlike CL, DCL is difficult to treat due to disseminated cutaneous lesions that resemble leprosy and which do not heal spontaneously. It is associated with a defective immune system, with most patients failing to display *Leishmania* specific cell-mediated immunity (Castes et al., 1984).

Table 2.1. Spectrum of leishmaniases, aetiological agents and worldwide distribution.

<table>
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<tr>
<th>Type of leishmaniasis</th>
<th>Causative organism</th>
<th>Worldwide distribution</th>
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<tbody>
<tr>
<td>Visceral leishmaniasis</td>
<td><em>L. donavani</em></td>
<td>China, India, Iran, Sudan, Kenya, Ethiopia, Mediterranean basin, Brazil, Colombia, Venezuela, Argentina</td>
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<tr>
<td>Post kala-azar dermal leishmaniasis</td>
<td><em>L. infantum</em></td>
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<td><em>L. chagasi</em></td>
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<tr>
<td>Cutaneous leishmaniasis</td>
<td><em>L. tropica</em></td>
<td>Mediterranean basin, Afghanistan, Middle East, W. and N. Africa, Kenya, Ethiopia, Central America and Amazon basin</td>
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<td><em>L. major</em></td>
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<td><em>L. aethiopica</em></td>
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<td><em>L. mexicana</em></td>
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<td>Mucocutaneous leishmaniasis</td>
<td><em>L. braziliensis</em></td>
<td>Brazil, Peru, Ecuador, Columbia, Venezuela</td>
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Cutaneous leishmaniasis or oriental sores, produces disfiguring skin lesions on the face, arms and legs, and is often self-healing (Ashford and Bates, 1998). The initial papule rapidly gives rise to an ulcer. Most patients have one or two lesions but the lesions may be multiple and occur in groups. Lesions are usually present on the exposed sites. Their sizes vary from 0.5 cm to 3 cm in diameter (Shiraz and Syed, 2007). Some lesions do not ulcerate at all and remain as bluish papules; others develop sporotrichoid nodular lymphangitis. Secondary bacterial infection is common and must always be suspected if the painless lesion becomes painful. Most lesions heal over months or years, leaving an atrophic scar. In general 50 percent of those lesions associated with *Leishmania major* will have healed in 3 months; those associated with *Leishmania tropica* take about a year, and those associated with *Leishmania braziliensis* persist much longer (Shiraz and Syed, 2007). Cutaneous leishmaniasis infection induces immunity to re-infection by the particular species that caused the disease, upon recovery or successful treatment (Ashford and Bates, 1998).

2.4 Immunology of leishmaniasis

The correlation between the development of protective immunity/resistance and the development of CD4+ Th 1 cell response has long been established in murine models, whereas susceptibility or development of chronic leishmaniasis is associated with the development of a CD4+ Th 2 response (Locksley *et al.*, 1987; Scott *et al.*, 1988; Heinzel *et al.*, 1989). The two functionally different CD4+ T cells originate from common naïve CD4+ T cell precursors and can only be distinguished by the pattern of cytokines that they produce upon stimulation (Rocken *et al.*, 1992; Mosmann and Coffman, 1989). Th 1
cells are distinguished by secretion of IFN-γ and lymphotoxin that mobilize the host’s defenses against intracellular pathogens as described above, whereas Th 2 cells secrete IL-4, IL-5 and IL-13 that favor the development of humoral immune responses against extracellular pathogens. The two subsets often work antagonistically to each other so that one gains predominance while down regulating the other (Liew et al., 1989). Early cytokine environment has been shown to play a critical role in the polarising of Th 1 and Th 2 immune responses in vivo (Sacks and Noben-Trauth, 2002).

Whereas both resistant and susceptible mice produce a wave of Th 2 cytokines, the important difference between them is the ability of the resistant types to switch to a Th 1 profile thus checking the disease (Skeiky et al., 1998; Morris et al., 1993; Morris et al., 1992). The possible events that could lead to the development of Th 1 include the early production of IL-2, IL-12 and IL-27 by infected antigen presenting cells leading to the differentiation by naïve T cells into Th 1 cells. Down regulation of IL-4 production and possible release of other cytokines by Leishmania homologue of the receptor for activated C kinase (LACK) reactive CD4+ T cells, may then serve to curtail Th2 cell development.

Maintenance of IL-12 receptor expression by T-beta, IL-18 and IL-23 is then able to strengthen Th1 maturation and commitment, leading to the subsequent production of interferon gamma (IFN-γ) by the Th1 cells and natural killer (NK) cells (Gorak et al., 1998; Scharton-Kerstem, 1995; Afonso et al., 1994). The IFN-γ together with tumor necrosis factor-alpha (TNF-α), produced by the infected macrophages, activates the
inducible nitric oxide synthetase (iNOS) gene, resulting in the production of nitric oxide (NO), which is fatal to the parasite (Bogdan, 1997). Inducing the expansion of IFN-γ secreting Th1 clones is therefore the key to eradicating the parasite through iNOS activation. The *Leishmania* parasite has therefore developed many strategies in order to subterfuge the host's mechanisms that mount the Th1 immune response.

**Primary** to the survival of *Leishmania* is by inducing transforming growth factor-beta (TGF-β), which is a macrophage deactivator, within the first 3 days of infection (Bogdan *et al.*, 1996; Stenger *et al.*, 1994). TGF-β suppresses the production of nitric oxide by macrophages and also inhibits the expression of T-beta, a transcription factor central to Th1 differentiation (Gorelik *et al.*, 2002; Li *et al.*, 1999). Early IL-4 production by LACK reactive CD4+ further instructs Th2 cell maturation, while inhibiting IL-12 signaling, which is pivotal in the maturation of Th1 cells (Gumy *et al.*, 2003).

The *Leishmania* parasite also induces IL-10, which has recently been shown to play a role in mice susceptibility to *L. major*. IL-10 has also been implicated in the persistence of parasite, which remain in the host indefinitely even after overcoming of the disease (Belkaid *et al.*, 2001). In addition to Th1 induced iNOS, the *Leishmania* parasite also faces many other defensive mechanisms such as hydrolytic enzymes, acidic pH, calcium chelation and host cell apoptosis. *Leishmania* has been demonstrated to surmount these challenges via several strategies including: the evasion of oxidative burst upon phagocytosis by macrophages; cytoplasmic localization; inhibition of phagosome-lysosome fusion; inhibition of hydrolytic enzymes; inhibition of calcium chelation;
inhibition of host signaling pathways; inhibition of nitric oxide production; modulation of macrophage apoptosis; modulation of macrophage cytokine production; inhibition of antigen processing, presentation and T-cell stimulation; and activation-induced T-cell death (Cunningham, 2002; Bogdan and Rollinghoff, 1999).

2.5 Experimental leishmaniasis

Many experimental models of leishmaniasis have been developed. These models have the major attraction of allowing control over the genetics of both the parasite and the host, but none entirely reproduces the disease in humans (Handman, 2001). One of the factors contributing to differences between humans and animal models is the size and nature of the parasite inoculum. In natural infections, the sandfly introduces into the skin a very small number (possibly as few as 100 to 1,000) metacyclic promastigotes together with strongly bioactive saliva, whereas in laboratory infections thousands to millions of culture-derived promastigotes or tissue-derived amastigotes are injected (Handman, 2001).

The sandfly is a blood pool feeder, using its mandibles to cut a wound in the skin and sucking up the blood that accumulates. It is in this superficial pool that the infective parasite inoculum is deposited, most probably in a very small volume. In contrast, the laboratory infection is commonly done in relatively large volumes of 50 μl or more. In addition, in the laboratory the syringe-delivered parasites are deposited mostly subcutaneously or, in visceral leishmaniasis models, intravenously. A better understanding of the molecular mechanisms involved in parasite maturation in the
sandfly and the ability to mimic some of these in the laboratory are leading to much improved protocols for infection (Dekrey and Titus, 1999). Investigators are now using small numbers of *in vitro*-derived metacyclic promastigotes and intradermal rather than subcutaneous infection into the ears of mice (Dekrey and Titus, 1999).

2.5.1 Experimental models of cutaneous leishmaniasis

*Leishmania enriettii* infection of guinea pigs was the first model to be well characterized. It established the requirement for cell-mediated immunity for recovery from cutaneous disease. Guinea pigs develop T-cell responses to parasite antigens within 2 weeks of infection, and the lesions heal within about 10 weeks (Mann *et al.*, 1998). A major attraction of this animal model is the fact that the host-parasite combination is a natural one and that the disease pattern is similar to that observed in human cutaneous leishmaniasis caused by *L. major*. The *L. enriettii* guinea pig model has now been superseded by infection of inbred mice with *Leishmania* species pathogenic for humans. Although not perfect, the spectrum of disease manifestations observed in human leishmaniasis can be mimicked in the laboratory by infection of different inbred strains of mice with *L. major*.

The mouse model reproduces many aspects of the human disease, including a range of susceptibility states depending on the strain of mouse used. In this animal model, the use of a clonal parasite population eliminates the contribution of the genetic diversity of the parasites and allows analysis of the host factors, which determine disease manifestations. BALB/c mice are highly susceptible; upon infection they develop large skin ulcers,
which expand and metastasize, leading to death. On the other hand, C57BL/6 and CBA/N mice are resistant, develop small lesions which cure in 10 to 12 weeks, and are resistant to reinfection. Most other strains of mice are intermediate in susceptibility (Preston and Dumode, 1976).

In mice, the outcome of infection depends on the polarized activation of one of two subsets of CD4+ T cells, Th1 or Th2. The subdivision into Th1 and Th2 cells is based on the pattern of cytokines that they produce. Th1 cells produce gamma interferon (IFN-γ) and interleukin-2 (IL-2), whereas Th2 cells produce IL-4, IL-5, and IL-10. Protective immunity depends on the induction of T cells producing Th1 cytokines, which activate macrophages to kill the intracellular organisms primarily through a nitric oxide-mediated mechanism (Liew et al., 1990).

BALB/c mice produce mainly Th2 cytokines, in particular IL-4, and this pattern is established within hours of infection (Bogdan, 1998; Solbach and Laskay, 2000). However, during the period of active lesion development, both susceptible and resistant mice produce a wave of Th2 cytokines (Morris et al., 1993; Morris et al., 1992). An important difference between susceptible and resistant mice is that the resistant mice are able to switch to a Th1 profile and control the disease (Solbach and Laskay, 2000; Heinzel et al., 1991). An important factor in the “decision” to form a Th1 or Th2 phenotype is the early cytokine environment, and IL-12 is one of the cytokines that contributes significantly to the establishment of the Th1 phenotype (Solbach and Laskay, 2000). While it is useful in many ways, one must remember that the mouse model for
Leishmaniasis is just a model and that the mechanisms of pathogenesis and immunity may be a little different in humans. Extrapolation from mouse to human requires much care (Kelso, 1998; Kelso, 1995).

Promising future animal models of leishmaniasis include the use of dogs and non-human primates such as the vervet monkey (Cercopithecus aethiops). Dogs are natural reservoir of *L. donovani* strains in endemic zoonotic VL foci such as Brazil. Disease symptoms and pattern in dogs are very similar to that of humans with the exception of additional cutaneous lesion in dogs, making them very ideal models for VL (Moody et al., 2000; Mendonca et al., 1995; Pinelli et al., 1995). The vervet monkey (Cercopithecus aethiops) has also been demonstrated to be a natural host for *L. major* in Kenya (Binhazim et al., 1987). Vervet monkey has very similar symptoms and disease patterns to that of humans and has been characterized as a model for both CL and VL that is very ideal for use in vaccine and drug development (Gicheru et al., 1995; Githure et al., 1987).

### 2.6 Control of Leishmaniasis

Improved control reduces both mortality and morbidity. It also reduces the role of humans as a reservoir in anthroponotic cycles and makes it possible to avert progression of the disease to complicated forms (WHO, 2007). Proper species and strain identification is important before the correct medication can be instituted. This diagnosis may involve direct sample observation from patients or samples may be cultured for identification of isolate. Control measures aims at eradicating the parasite reservoir in
anthroponotic transmission cycles and using control strategies against the vector and zoonotic reservoir in order to break transmission in zoonotic cycles (Lacerda, 1994).

2.6.1 Diagnosis of leishmaniasis

2.6.1.1 Visceral leishmaniasis

The gold standard for diagnosing visceral leishmaniasis is parasite identification in tissue smears, with splenic aspirate being more sensitive than bone marrow or lymph node aspirates. However, difficulties in obtaining and examining tissues mean that serological methods are increasingly being used. The direct agglutination test, in which stained parasites are agglutinated by serum antibodies, is popular in Iran and Africa, but variation between batches and the high cost of commercially available antigen are limiting factors. In the Indian subcontinent (Sundar et al., 1998) but less so in Europe and Africa (Zijlstra et al., 2001) a rapid strip test is used to detect antibody to rK39 (a conserved antigen of *L infantum*) and is both sensitive (67-100%) and specific (93-100%).

Weak responses in some patients, persistence of antibodies after cure, and presence of antibodies in some healthy individuals are inherent limitations with antibody based diagnostics. Detection of leishmanial antigen in urine through a latex agglutination test (Katex) seems to be promising for both diagnosis and prognosis (Attar et al., 2001). Techniques based on polymerase chain reaction are potentially highly sensitive and specific (Martin-Sanchez et al., 2001) but they need to be made more suitable for field
use in terms of cost and user skills required. In patients co-infected with HIV and visceral leishmaniasis, blood smears and culture might yield good results.

2.6.1.2 Cutaneous and mucocutaneous leishmaniasis

Touch smears or culture of exudates or scrapings yield good results in the diagnosis of cutaneous leishmaniasis. From a nodule, slit skin smears are often rewarding. Tissue biopsy can be used for impression smears, culture, or animal inoculation, especially for mucocutaneous leishmaniasis (Davies et al., 2003). Although multiple *Leishmania* species sometimes coexist, species identification is unlikely to be cost effective in the field unless major treatment decisions for cutaneous leishmaniasis become species specific (Davies et al., 2003).

2.6.2 Vector and reservoir control

Vector control using indoor spraying of insecticides is always determined by the behaviour of the species of sandfly present in each area: whether it is endophilic or exophilic and endophagous or exophagous (WHO, 2007). Spraying houses with insecticide is the most widely used intervention for controlling sandflies that are endophilic. Where sandflies are endophagic (mainly feed indoors) and most active when people are asleep, bed nets provide considerable protection (Davies et al., 2003). Whatever the case, logistics and costs limit the sustainability of periodic spraying of walls. Sustained vector control utilizing large-scale insecticide spraying (Dye, 1996) in developing countries is costly and not feasible. In Brazil, about 200,000 houses are sprayed and 20,000 dogs are culled each year to prevent zoonotic visceral leishmaniasis.
After annual surveys in endemic regions, dogs are culled if their blood samples are diagnosed positive by immunofluorescence (Davies et al., 2003). Combined campaigns targeting *Anopheles* mosquitoes and sandflies, however, are more cost effective. A suitable alternative, at an estimated cost of US$ 5 per unit, is the use of bednets impregnated with long-lasting insecticide; on average, the nets last for five years (WHO, 2007).

2.6.3 Chemotherapy
The first line approach for the treatment of leishmaniasis is administration of pentavalent antimony compounds (Herwaldt, 1999). Often, serious adverse reactions occur such as cardiac arrhythmias, severe arthritis, liver dysfunction, lethargy and, eventually, sudden death (Pearson et al., 1999). Historically, thousands of leishmaniasis patients treated with antimonials are successfully cured, but always with the danger of well-documented side effects of heavy metal poisoning (WHO, 1984). In addition, emergence of leishmanial resistant organisms to the pentavalent antimony is well documented and, in some endemic areas of the world, treatment failure has reached a level of 60% to 80% (Grogl et al., 1992). Unfortunately, second line alternative drugs are more toxic than antimonial compounds (Sampaio et al., 1971).

Amphotericin B and Pentamidine have shown reasonably good efficacy results in a series of cases reported, but both have been associated with severe, life threatening organ dysfunction and death (Herwaldt, 1999; Pearson et al., 1999). During the last decade, new formulations of Amphotericin B in a liposome or other lipid-complex drug delivery
system have significantly decreased the side effects of Amphotericin based therapy (Thakur et al., 1996). However, the price of the liposome-Amphotericin B preparation is prohibitive for most of the millions of people with leishmaniasis in the tropics (Davidson et al., 1996). In addition, many reports of dramatic resistant or refractory cases of leishmaniasis leave unsolved challenge (Lira et al., 1999). The most recent invention, Miltefosine, the only medicine administered orally, is to date licensed only in Colombia, Germany and India. As the possibility of its being teratogenic has not been excluded, it should be used under direct observation. Also, to avert the emergence of resistance, it should be given in combination with other antileishmanial drugs (WHO, 2007).

2.6.4 Role of Vaccination in the control of diseases

Vaccination is the administration of antigenic material to produce immunity to a disease, which will prevent or ameliorate the effects of infection by a pathogen. This material can either be live, live but weakened forms of pathogens such as bacteria or viruses, killed or inactivated forms of these pathogens, or purified material such as proteins. Vaccines may be dead or inactivated organisms or purified products derived from them. There are four traditional vaccines: killed microorganisms, live attenuated, Toxoids, and Subunit vaccines (http://www.drspock.com/article/0,1510,4866,00.html).

2.6.4.1 Vaccinations against leishmaniasis

To achieve control of human leishmaniasis, it would be necessary to develop safe and effective vaccines. The natural history of leishmanial infections demonstrate that
resolution can lead to development of resistance, which forms the basis for vaccine
development studies (Modabber et al., 1990). Two approaches are used in the
development of *Leishmania* vaccines: pragmatic and systemic methods (Modabber et al.,
1990). The pragmatic approach involves trials of crude *Leishmania* components in
animals and humans with or without *Bacille Calmette Guerin* (BCG). The systemic
approach requires identification, production and purification of protective immunogens,
usage of adjuvants, carriers, mode of presentation and determination of protective
immunogen responses (Modabber et al., 1990). Both approaches have made considerable
progress towards making anti-leishmanial vaccines (Sharifi et al., 1998). Definition of the
nature of the protective Th1 response, in particular, the cytokine mediators of such a
response, has been fundamental to the process of vaccine development (Scott et al.,
1988). It is now well established that an effective vaccine should and must induce a Th1
response, providing an essential criterion for both antigen and adjuvant selection (Afonso
et al., 1994).

Different antileishmanial vaccines have been tried with some degree of success. In
Leishmanization, Bedouin or some Kurdistani tribal societies traditionally exposed their
babies' bottoms to sandfly bites in order to protect them from facial lesions. Another
ancient technique practised in the Middle East has been the use of a thorn to transfer
infectious material from lesions to uninfected individuals. With the establishment by
Nicolle and Manceau in (1908) of culture conditions able to support the growth of
promastigotes, live organisms started to be used for vaccination (controlled infections).
Large-scale vaccination trials (controlled infection) using live promastigotes were carried out in the Soviet Union and Israel (Kellina, 1981; Greenblatt, 1980) with a high percentage of successful lesion development. The success of this strategy depended critically on the viability and infectivity of the injected organisms. Organisms, which had lost virulence, were shown to induce delayed-type hypersensitivity but did not protect from subsequent natural infection (Kellina, 1965). The use of live vaccines 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 (Modabber, 1995; Serebryakov et al., 1972). Consequently, the use of live virulent organisms for vaccination was discontinued (Handman, 2001).

The relative merits of live-attenuated vaccines versus killed vaccines have been a constant subject of debate in relation to many antimicrobial and viral vaccines. Most notable arguments have been those concerned with immunogenicity, efficacy, safety, ease of production and distribution, and stability. Early studies indicated (surprisingly) that most parasites cloned directly from a skin lesion in mice were avirulent (Handman et al., 1983). This suggested that the parasite population present in the lesion may be heterogeneous and that the avirulent organisms (which are rapidly killed by the host and provide antigens), rather than the virulent organisms, contribute most to the immune response observed in the infected mice.
More recent data indicate that, indeed, *L. mexicana* antigens can be presented to T cells by macrophages harboring dead organisms but not by cells harboring live parasites (Overath and Aebischer, 1999). In line with this argument, mice injected with cloned avirulent lines were protected from challenge infection with a virulent clone derived from the same lesion (Handman *et al.*, 1983). However, in the absence of a clear genetic profile of any avirulent cloned organisms available at the time, their use for human vaccination would have been unacceptable because of the risk of reversion to a virulent phenotype. Other data showed that mice injected with irradiated parasites were also protected from infection (Rivier *et al.*, 1993). Taken together, these data strongly supported prophylactic vaccination with attenuated organisms as a useful approach to human vaccine development.

Recent advances in the ability to manipulate the *Leishmania* genome by introducing or eliminating genes has the potential to make live-attenuated vaccines much more feasible. 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 (DHFR-TS; Titus *et al.*, 1995). These organisms can invade and undergo a limited number of replications in macrophages without producing disease. In a mouse model, *L. major* parasites lacking DHFR-TS induced protection against infection with either *L. major* or *L. amazonensis* (Vera *et al.*, 1999; Titus *et al.*, 1995). An attenuated line of *L. mexicana* was also used successfully to protect against homologous infection. This mutant lacked two genes encoding the cysteine proteases cpa and cpb (Alexander *et al.*, 1998; Russel and Alexander, 1988). In summary, the use of attenuated
organisms is very attractive because they are the closest mimic to the natural course of infection and may therefore lead to similar immune responses. Moreover, because of the small load of antigen delivered by the transient infection, the immune responses may be skewed even more toward a Th1 protective response than in natural infection (Metz et al., 1999; Constant et al., 1995). Such immunization will also deliver many more parasite antigens than the limited number possible with subunit or recombinant antigens. Summarizing a large amount of experimental evidence, Rivier et al. (1999) concluded that, injection of attenuated organisms achieved better protection than any method involving recombinant gp63 as test antigen delivered with a variety of adjuvants and delivery systems. If this conclusion is shown to be generally applicable to other vaccine candidates, the prospect of using attenuated *Leishmania* vaccines in preference to subunit or recombinant approaches will gain favor. The disadvantages of such vaccines are the logistics of their large-scale production and distribution in the field.

The newer vaccines under consideration 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. Some are conserved among *Leishmania* species, while others are not. Since T cells recognize peptides derived from cytosolic proteins bound in the MHC class I groove or peptides derived from the lysosomal compartment bound in the MHC class II groove on the antigen-presenting cell surface, it would appear that virtually any parasite protein might function as an antigen, regardless of its location in the parasite. At the effector stage, in the lesion, it may not be important if the antigens are presented on the surface of infected or bystander antigen-
presenting cells. As long as the appropriate proinflammatory Th1 cytokines are generated in the lesion, macrophage activation and parasite killing should occur. Recombinant antigens can be delivered as purified proteins, as the naked DNA encoding them, or as bacteria manufacturing the proteins in situ.

Manipulations now allow targeting of the antigen to specific locations or to particular antigen-presenting cells, 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 an adjuvant effect, which may "activate" or "licence" these antigen-presenting cells (Matzinger, 1998).

The first recombinant antigen used to vaccinate against leishmaniasis was leishmaniolysin or gp63. This is masses (Mr) 65,000 membrane protease present in promastigotes and amastigotes of all species. gp63 is one of the parasite receptors for host macrophages, and parasite mutants lacking the protein are avirulent (Chang et al., 1990). gp63 belongs to a multigene family, with different members being expressed in promastigotes and amastigotes. Interestingly, both the recombinant and native proteins seem to protect better against infection with L. amazonensis than against infection with L. major, suggesting species-specific epitopes, at least in animal models (Russel and Alexander, 1988: Olobo et al., 1995). It is unfortunate that in humans and animal models the T-cell responses to gp63 have been variable. However, when detected, they appeared to be of the Th1 type (Mendonca et al., 1991; Russo et al., 1991; Jaffe et al., 1990). Overall, gp63 is still considered a promising vaccine candidate. The gene has been
engineered in a number of delivery systems (BCG, vaccinia virus, and S. enterica serovar Typhimurium) in the hope of inducing the appropriate Th1 immune response.

A second vaccine candidate tested in animal models is a membrane antigen of unknown function, gp46/M2 or parasite surface antigen-2 (PSA-2; Handman et al., 1995; McMahon-pratt et al., 1993; Lohman et al., 1990). As with gp63, PSA-2 belongs to a multigene family expressed in all *Leishmania* species except *L. braziliensis*. Similar but distinct gene products are found in amastigotes and promastigotes of *L. major* and *L. donovani*, but in *L. mexicana* expression seems to be restricted to promastigotes (McMahon-pratt et al., 1992). Its presence in most species makes PSA-2 an attractive candidate for a pan-*Leishmania* vaccine. PSA-2 protects against *L. major* (Handman et al., 1995) as well as *L. mexicana* when administered as purified protein or expressed in vaccinia virus (Mendonca et al., 1991).

Immunization with the *L. donovani* PSA-2 protects mice against infection with *L. major* and that, conversely, immunization with the *L. major* proteins afforded partial protection against infection with *L. donovani* (Handman, 2001). Recombinant DNA-derived PSA-2 protein was variable in its ability to confer protection, while the protein derived from the yeast *Pichia pastoris* provided good protection. These data suggested that the native conformation of the protein might be important for processing and presentation by antigen-presenting cells. These difficulties may be overcome by the development of a DNA-based vaccine (Handman, 2001).
The leishmanial eukaryotic ribosomal protein (LeIF), a homologue 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). This protein is highly conserved in evolution, but assuming that specific parasite epitopes will be used for vaccination such that autoimmune responses will be avoided, it may be useful as a component in a pan-Leishmania vaccine. A similarly conserved antigen, the Leishmania homologue of the receptor for activated C kinase (LACK), which is expressed by both promastigotes and amastigotes has been shown to protect mice from infection, in particular when administered with IL-12 as an adjuvant (Gurunathan et al., 1997; Mougneau et al., 1995). It is worth noting that, LACK is also the major target for Th2 responses in susceptible BALB/c mice, and BALB/c mice made tolerant to LACK are resistant to infection (Julia et al. 1996). The significance of this finding for the use of LACK as a vaccine in humans remains to be elucidated. Several other vaccine candidates identified in the last few years are in the process of being characterized. Some are amastigote specific, such as A2, P4, and P8 of L. mexicana pifanoi (Soong et al., 1995).

Another vaccine candidate is a flagellar antigen, lcr1, from L. donovani chagasi (Streit et al., 2000). In view of the fact that the target of host protection is the amastigote, which has only a rudimentary flagellum, the mechanism by which host protection is achieved with this antigen is not obvious. A most interesting approach to the identification of potential vaccine candidates has been the elution of antigenic peptides from antigen-presenting cells (Campo-Neto et al., 1995). Several peptides were identified, and the sequences were used to clone the cognate genes. One of these genes encodes a membrane
polypeptide expressed in promastigotes and amastigotes. This polypeptide induced Th1-type responses in immunized mice (Campos-Neto et al., 1995). Surprisingly, in view of its potential, there have been few new data published since its discovery.

The 1980s were marked by a wave of enthusiasm concerning the use of peptide vaccines, in particular those considered to be T-cell epitopes (Spitzer et al., 1999; Russo et al., 1993; Jardim et al., 1990). This enthusiasm seems to have waned in recent times, and the focus appears to have moved to the use of recombinant DNA-produced polypeptides and to naked DNA. Several considerations make the peptide antigens less attractive: 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. Since the antigenic peptide is processed and presented to T cells in the context of MHC class I or class II and since not all peptides associate with all MHC types, some peptides will not be recognized by all individuals in the population. There are additional “holes” in the ability to respond to individual peptides due to failure of processing, cleavage, transport or due to deletion of certain T-cell specificities due to self-tolerance (Howard, 1993). Despite these caveats, several *Leishmania* gp63 peptides have been tested successfully in animal models (Jardim et al., 1991; Spitzer et al., 1999). The results indicated long-lasting host protection, indicating the induction of long-term T-cell memory (Spitzer et al., 1999).

In general, the success of subunit vaccines based on recombinant proteins or peptides has been variable to poor. Several factors may account for this. Some polypeptides, such as PSA-2, need to be in their native conformation for antigen processing, and *Escherichia*
coli-derived recombinant proteins may not fulfil this requirement (Sjolander et al., 1998a; Sjolander et al., 1998b). This problem may be overcome by exploitation of the parasites themselves by overexpression of parasite antigens in transfected nonpathogenic Leishmania strains or the related trypanosomatid Crithidia (Kelley, 1997). Presumably, polypeptides expressed in these systems will be abundant, correctly folded, and glycosylated (Moody et al., 2000; Constant et al., 1994). Another reason for the low success rate of subunit vaccines is that some polypeptides may be minor immunogens and so even though they may be excellent in a cocktail vaccine, individually they may provide only partial protection. The immune responses in leishmaniasis can range from protective to positively harmful, as described above. These differences in the quality of the response are at least partly due to predominance of Th1 or Th2 cytokines and may be greatly influenced by antigen dose (Bretscher et al., 1997). Accordingly, the amount of antigen and the route of administration and adjuvant used may be important issues (Hewlett and Cherry, 1990). Another thorny issue concerns adjuvants. The delivery system may be critical in biasing the type of T-cell responses induced, and this can determine whether protection is achieved or, indeed, whether immunization makes the disease worse (Hewlett and Cherry, 1990; Hoskins et al., 1979).

Nonprotein antigens have also been used against leishmaniasis. Early studies on vaccine development indicated that glycolipids such as the Leishmania lipophosphoglycan (LPG) provided excellent protection (Russel and Alexander, 1988; McConville et al., 1987). Protection depended on the use of adjuvants such as liposomes or Corynebacterium parvum and on the integrity of the molecule. Not only was the water-soluble form of LPG
lacking the glycosylphosphatidylinositol anchor not protective, but it exacerbated disease (Mitchell and Handman, 1986). At the time when that work was published, the immune mechanism leading to host protection by such a nonprotein molecule was totally mysterious. Immunity was known to be T-cell mediated, but T cells were not thought to recognize or present nonprotein antigens. Today, it is accepted that many novel and interesting microbial antigens including mycobacterial glycolipids can be recognized by T cells and that these antigens are presented to T cells by a special subset of MHC class I proteins known as CD1 (Moody et al., 2000; Sieling et al., 2000; Sugita et al., 1998).

Immunization with naked DNA is a new approach, which promises to revolutionize the prevention and treatment of infectious diseases (Gurunathan et al., 2000; Seder and Gurunathan, 1999; Alarcon et al., 1999; Wahren, 1996). The gene encoding the vaccine candidate is cloned in a mammalian expression vector, and the DNA is injected directly into muscle or skin (Walker et al., 1998; Donnelly et al., 1997; Ulmer et al., 1996; Wahren, 1996). The plasmid DNA is taken up by cells and translocated to the nucleus, where it is transcribed into RNA and then translated in the cytoplasm. The efficiency of uptake and the expression of plasmid DNA must be extremely low, but there is abundant evidence that it is sufficient to provoke immune responses in both T and B cells (Gurunathan et al., 2000; Hasan et al., 1999). The antigen encoded by the injected plasmid is presentation via either class I or class II MHC molecules on professional antigen-presenting cells (Hasan et al., 1999; Donnelly et al., 1997).
A large body of literature indicates that both CD4+ and CD8+-mediated responses are induced, making a DNA vaccine attractive for a *Leishmania* vaccine (Pardoll and Beckerleg, 1995). In addition to being able to induce the appropriate immune responses, DNA vaccines are attractive because they ensure appropriate folding of the polypeptide, produce the antigen over long periods, and do not require adjuvants. Another advantage is that the technology for production is very simple. DNA is stable, has a long shelf life, and does not require a strict cold chain for distribution. Concerns raised in relation to safety, such as integration of the DNA into the mammalian genome and induction of autoimmune disease or cancer, have not been substantiated to date.

Several DNA vaccines are in advanced clinical trials; these include a malaria vaccine, a mycobacterial vaccine, and an HIV vaccine (Alarcon *et al.*, 1999; Hasan *et al.*, 1999). Vaccinations with DNA encoding gp63, LACK, and PSA-2 all protected both genetically resistant and susceptible mice from infection with *L. major* (Sjolander *et al.*, 1998; Walker *et al.*, 1998; Gurunathan *et al.*, 1997). Protection was accompanied by Th1 immune responses. Unexpectedly, protection induced by LACK depended on CD8+ T cells, and depletion of this population abrogated protection (Gurunathan *et al.*, 1998).

The concept of a *Leishmania* killed vaccine was neglected for many years, possibly because of conflicting results obtained in the 1940s. Vaccination with killed organisms failed to protect persons in the Middle East (Berberian, 1944), whereas a Brazilian trial showed excellent protection. The tide turned when studies performed in the 1980s showed that injection of irradiated parasites induced excellent protection in mice
provided that they were injected intravenously or intraperitoneally but not subcutaneously. These experiments paved the way for a reassessment of the use of killed vaccines and led to the successful development and field trials of several formulations of killed vaccines (Howard et al., 1984; Alexander, 1982; Howard et al., 1982; Mayrink et al., 1979).

Extensive vaccination trials in Brazil and Ecuador have demonstrated that a cocktail of five killed Leishmania stocks or a single strain of L. amazonensis induces significant protection from natural infection (De Luca et al., 1999; Armijos et al., 1998; Marzochi et al., 1998; Modabber, 1995). Convit and colleagues, some of the early pioneers of killed vaccines, used a combination of killed L. mexicana or L. braziliensis promastigotes and M. bovis BCG both prophylactically and therapeutically against South American leishmaniasis (Castes et al., 1989). When used in the therapeutic mode, vaccination appeared to induce a high cure rate even in patients with severe cases. Cure was accompanied by the development of Th1-type immune responses in the recipients, with the production of IFN-γ and the absence of IL-4 (Cabrera et al., 2000; Castes et al., 1989).

In Iran, a mixed BCG-L. major killed vaccine has also undergone clinical trials for safety and efficacy. In one study there was little difference in disease incidence between the group vaccinated with BCG alone and the group given BCG and vaccine. A second study showed that in the longer term, the vaccine combination provided better protection than BCG alone, suggesting that BCG might have had only a transient immunostimulatory
effect (Momeni et al., 1999; Sharifi et al., 1998). Vaccination with a single dose of 1 mg of *L. major* protein and BCG is the simplest vaccine tested so far. Although it proved safe, only about 35% of vaccinated individuals became skin test positive. If skin test conversion is a surrogate marker for protection, the efficacy of this vaccine is not remarkable. It may require multiple doses to increase immunogenicity. In a monkey model of cutaneous leishmaniasis, protective immunity was achieved using killed *L. amazonensis* coadministered with recombinant IL-12 as adjuvant (Kenney et al., 1999). In another unrelated study, killed *L. major* plus recombinant human IL-12 exhibited minimal protection in vervet monkey model of leishmaniasis (Gicheru et al., 2001).

### 2.7 Adjuvants

The term *adjuvant* has been used for any material that can increase the humoral or cellular immune response to an antigen. In the conventional vaccines, adjuvants are used to elicit an early, high and long-lasting immune response. The chemical nature of adjuvants, their mode of action and their reactions (side effect) are highly variable. According to Gupta *et al.* (1993), some of the side effects can be ascribed to an unintentional stimulation of different mechanisms of the immune system whereas others may reflect general adverse pharmacological reactions, which are less expected. Chemically, the adjuvants are a highly heterogenous group of compounds with only one thing in common: their ability to enhance the immune response—their adjuvanticity. They are highly variable in terms of how they affect the immune system and how serious their adverse effects are due to the resultant hyperactivation of the immune system.
The mode of action of adjuvants was described by Chedid (1985) as: the formation of a depot of antigen at the site of inoculation, with slow release; the presentation of antigen immunocompetent cells; and the production of various and different lymphokines (interleukins and tumour necrosis factor). The choice of any of these adjuvants reflects a compromise between a requirement for adjuvanticity and an acceptable low level of adverse reactions. The discovery of adjuvants dates back to 1925 and 1926, when Ramon showed that the antitoxin response to tetanus and diphtheria was increased by injection of these vaccines, together with other compounds such as agar, tapioca, lecithin, starch oil, saponin or even breadcrumbs (Gupta et al., 1993).

The newly developed purified subunit or synthetic vaccines using biosynthetic, recombinant and other modern technology are poor immunogens and require adjuvants to evoke the immune response. The use of adjuvants enables the use of less antigen to achieve the desired immune response, and this reduces vaccine production costs. With a few exceptions, adjuvants are foreign to the body and cause adverse reactions. There are several types of adjuvants. Today the most common adjuvants for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate. However, there are a number of other adjuvants based on oil emulsions, products from bacteria (their synthetic derivatives as well as liposomes) or gram-negative bacteria, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils. Recently, monophosphoryl lipid A, immunostimulating complexes (ISCOMs) with Quil-A, and Syntex adjuvant formulations (SAFs) containing the threonyl derivative or muramyl dipeptide have been under consideration for use in human vaccines.
2.7.1 Adjuvants in *Leishmania* vaccines

Although Mayrink *et al.* (1979) used the intramuscular route in man with killed parasites without an adjuvant, and successful protection without adjuvants has been achieved in a number of animal studies, it is probable that vaccines utilizing subcellular components or purified macromolecules may require the use of suitable adjuvants.

Delivery of PSA-2 packaged in immunostimulating complexes induced a strong but mixed Th1/Th2 response and no protection, whereas its delivery as a DNA vaccine induced a low but exclusive Th1 response and protection (Sjolander *et al.*, 1998a; Sjolander *et al.*, 1998b).

Several studies have indicated that BCG, Freund’s incomplete adjuvant (FIA) and glucan utilized with killed parasites or with crude or semipurified fractions will protect or partially protect against challenge (Cook *et al.* 1980; Smrkovski and Larson 1977a; Weintrub and Weinbaum 1977; Bryceson *et al.*, 1970). In the case of *Corynebacterium parvum* and Freund’s complete adjuvant (FCA) used alone, there was actually enhancement of the infection (Bryceson *et al.*, 1972). Cook *et al.* (1980) have found that glucan alone is protective in cross filial-1 (CF1) mice against *L. donovani*. The authors interpreted the protection as a stimulation of the non-specific activity of macrophages. In a second study (Holbrook, *et al.*, 1981), glucan served as an adjuvant for formalin-killed *L. donovani* promastigotes. When purified elongation factor (EF) of *L. major* bound to muramyl dipeptide (MDP), the material responsible for mycobacterial peptidoglycan adjuvant activity was used (Ellouz, *et al.*, 1974), partial protection was obtained against...
challenge. Allison (1979) suggested that appropriate derivatives of MDP in conjunction with liposomes might meet the requirements for use in human vaccination.

Several attempts to treat MCL patients heavily exposed to antimonial therapy have been reported (Herwaldt, 1999). However, none of the alternatives have shown sufficient efficacy to recommend them as the solution for treatment of refractory MCL cases (Pearson, 1999). In contrast, reports of the efficacy of immunotherapy with crude *Leishmania* antigen preparations, in combinations with BCG (Bacillus Calmet-Guerin), have indicated that there could be dramatic healing responses of the lesions in patients with CL and MCL by use of antigen vaccines (Convit *et al.*, 1989; Convit *et al.*, 1987).

The recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) has been documented to have a potent cytokine adjuvant effect based on its activity of inducing activation and migration of dendritic cells (Romani *et al.*, 1994). It has been shown to be effective in doses of 25mg to 50mg as an adjuvant (Lin and Jones, 1997).

The adjuvant potential of IL-12 for a vaccine against leishmaniasis has been documented in the murine model (Afonso *et al.*, 1994). Mice immunized with soluble *Leishmania* antigen (SLA) + IL-12 were completely protected against disease. IL-12 in the presence of SLA induces differentiation of CD4+ Th 1 cells in the lymph node and spleen (Afonso *et al.*, 1994). Mice immunized with SLA+IL-12 produced little IL-4 but large amounts of IFN-γ that were comparable to those observed in resistant C3H/HeN mice. Lesions from unimmunized BALB/c mice or mice that had been immunized with SLA alone contained
greater than $10^7$ parasites, whereas BALB/c mice immunized with SLA+IL-12 contained $10^3$ parasites (Afonso et al., 1994). In studies using the Vervet monkey, low doses of a human recombinant IL-12 preparation induced a small increase in the parameters of cell-mediated immunity similar to animals that received antigen without IL-12 (Gicheru et al., 2001).

Bacille Calmette Guérin (BCG) is a weakened (attenuated) version of a bacterium called *Mycobacterium bovis*, which is closely related to *Mycobacterium tuberculosis*, the agent responsible for tuberculosis (http://www.MedicineNet.com). Bacille Calmette Guérin has been used successfully for anti-*Leishmania* immunotherapy in South American patients without side effects. Bacille Calmette Guérin vectors carrying gp63 have also been used successfully to induce protection in the *L. major* system (Abdelhak et al., 1995; Aebischer et al., 2000). Convict and colleagues, some of the early pioneers of killed vaccines, used a combination of killed *L. mexicana* or *L. braziliensis* promastigotes and *Mycobacterium bovis* BCG both prophylactically and therapeutically against South American leishmaniasis (Castes et al., 1989). When used in the therapeutic mode, vaccination appeared to induce a high cure rate even in patients with severe cases. Cure was accompanied by the development of Th1-type immune responses in the recipients, with the production of IFN-γ and the absence of IL-4 (Cabrera et al., 2000; Castes et al., 1989). Vaccination with BCG plus killed *Leishmania* promastigotes reduced acute infection by *T. cruzi* in mice, increasing survival time and decreasing parasitaemia and mortality (Aruajo et al., 1999). Sohrabi et al. (2005) found out that liposomes containing
autoclaved *L. major* antigens mixed with BCG could be used to induce a Th1 response in resistance C57 BL/6 mice.

Montanide incomplete seppic adjuvants, (Montanide ISA) are a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two oils (http://www.nal.usda.gov/awic/pubs/antibody/overview.htm). Montanide ISA 720 has been approved for experimental use in humans (Oliveira *et al*., 2005; Masina *et al*., 2003; Toledo *et al*., 2001). Montanide ISA 720 has shown good results in non-human primate vaccination studies (Collins *et al*., 2006). In *Leishmania* infection, the use of Montanide avoids problems that have been encountered with other adjuvants (for example, Freund’s) including adverse reactions (Smrkovski and Larson, 1977; Sharples *et al*., 1994). Vervet monkeys immunized with a combination of recombinant glutathione-S-transferase-Histone-1 (*Leishmania* antigen) and MISA720 adjuvant were able to generate a durable cellular responses that were sufficient to control infection in the majority of the monkeys (Masina *et al*., 2003).

Aluminium salts are an insoluble gel-like precipitate of aluminium phosphate, aluminium hydroxide or alum with a particle size from 100-1000nm (Souza *et al*., 1957). Aluminium salts have been widely used as adjuvants and are generally considered safe (WHO, 2007). Aluminium phosphate and aluminium hydroxide (alum) are the mineral compounds most commonly used as adjuvants in human vaccines (Viera, 2000). The use of alum was applied more than 70 years ago by Glenny *et al*. (1931), who discovered that a
suspension of alum-precipitated diphtheria toxoid had a much higher immunogenicity than the fluid toxoid. A study in the mouse model indicated the immunity elicited by killed *Leishmania* Ag plus mouse rIL-12 without alum is relatively short-lived, lasting only less than three months (Gurunathan *et al.*, 1998). A vaccine with autoclaved *L. amazonensis* promastigotes using rhIL-12 and alum as adjuvants was safe and fully effective in Rhesus monkey model of cutaneous leishmaniasis (Kenney *et al.*, 1999). In a recent study, cells harvested from mice immunized with *L major* soluble exogenous antigens (SEAs) plus rmIL-12 plus alum showed enhanced proliferative responses and more cytokines than cells from mice immunized with *L. major* SEAs alone (Tonui *et al.*, 2004).

2.8 The future of *Leishmania* vaccine

The valuable body of knowledge discussed above clearly indicates that many vaccination protocols have been applied both in humans and animal models in the search for *Leishmania* vaccines. The literature also shows that, only few adjuvants are available for human vaccine trials and that killed *Leishmania* vaccine being poorly immunogenic can only become effective when combined with adjuvants that are able to polarize the immune response towards cellular as opposed to humoral responses. Bacille calmette Guerin has been clearly associated with the induction of a Th 1 immune response (Tokunga *et al.*, 1999) and is probably the most acceptable Th 1-inducing adjuvant presently available for use in humans. Alum-adjuvanted vaccines are excellent at priming the immune system (WHO, 1976). Both alum and BCG are widely used and approved for human use (Misra *et al.*, 2001). Montanide ISA 720 has been found to be safe and
effective in both non-human primates and humans (Masina et al., 2003; Oliveira et al., 2005). Although different reports have indicated the potential of BCG, alum and Montanide ISA 720 for Leishmania vaccines, none has evaluated the immunogenicity and efficacy of the three adjuvants. This study sought to carry out comparative immunogenicity and efficacy analysis of these three adjuvants in a mouse model of cutaneous leishmaniasis with a view to identifying the most effective and safe adjuvant for a Leishmania vaccine.
CHAPTER 3: MATERIALS AND METHODS

3.1 Research facility

The research work was carried out in the *leishmaniasis* Laboratory, Department of Tropical and Infectious Diseases (TID) at the Institute of Primate Research (IPR)—the Biomedical and Primatology Research Center of the National Museums of Kenya. The Institute of Primate Research is located in Oloolua forest in the outskirts of Nairobi in Kenya. The Animal resource, Reproductive biology and TID departments are fully equipped with modern facilities and equipments for biomedical research.

3.2 *Leishmania* parasites

*Leishmania major* isolate, NLB-144, originary isolated from *Phlebotomus duboscqui* in Baringo District, Kenya (Gicheru *et al.*, 2001) and maintained in BALB/c mice by serial subcutaneous passage, was used in this study. An aspirate from the spleen of an infected BALB/c mouse was cultured in complete M199 (Appendix IV-i) medium and incubated at 25º C until stationary phase. Stationary phase promastigotes were harvested by centrifugation at 2500 rpm for 15 minutes at room temperature. The resulting pellet was washed three times by centrifugation in sterile Phosphate Buffered Saline (PBS) and then enumerated in haemocytometer before the parasites were used.
3.3 Adjuvants

Montanide ISA 720 (Seppic France), Aluminium hydroxide and Bacille calmette guerin (Pasteur Institute of Iran) were used as adjuvants along with formalin-fixed *Leishmania major* promastigotes.

3.4 Preparation of *Leishmania* antigens

3.4.1 Soluble *Leishmania* antigen (SLA)

Antigen was prepared as per the method described (Ho *et al.*, 1983). Briefly, promastigotes were washed and sonicated at 18 kHz for five periods of 40 seconds each on ice, separated by intervals of 1 minute. The sonicated material was rapidly frozen and thawed three times in liquid nitrogen for extraction of whole soluble protein. The parasite suspension was centrifuged at 34 000 rounds per minute for 30 minutes. The protein concentration of the supernatant was determined using Bio Rad protein assay kit following the method described by the manufacturer (Appendix III), and stored at -70°C until use. This antigen was used for coating ELISA plates in antibody estimation assays.

3.4.2 Formalin-fixed *Leishmania major* antigens (KLM)

For vaccine antigens, *in vitro* lymphocyte proliferation and cytokine secretion assays, promastigotes were harvested at stationary phase and washed three times in sterile PBS as described above. Parasites were fixed in 1% formal saline for 1 hr as described (Gicheru *et al.*, 2001) and then washed three times in PBS as above. They were counted
in haemocytometer and resuspended in a concentration of $5 \times 10^8$/ml in sterile PBS and stored in -70°C until required.

3.5 Experimental design and Immunization protocol

Eight to ten week old male BALB/c mice were acquired and maintained in the rodent facility of the Institute of Primate Research (IPR) throughout the experimental period. The mice were divided into 5 groups of 12 mice each and treated as follows: Group 1 received alum plus formalin-fixed *Leishmania major* promastigotes (KLM), group 2 received BCG plus KLM, group 3 was injected with KLM alone, group 4 received Montanide incomplete seppic adjuvant (MISA) plus KLM and group 5 was injected with phosphate buffered saline (PBS) only and served as the negative control group. Two booster vaccinations of the same vaccine components and amount were given at four and six weeks following the initial vaccination. In all the vaccinated groups the amount of KLM per vaccine dose was $1 \times 10^7$. All the vaccines were reconstituted in PBS and delivered intraperitoneally with a syringe and needle. In total, 300μl of vaccine were injected to each experimental mouse. Two weeks after the third vaccination, mice from each group were either sacrificed for immunological studies or challenged with $1 \times 10^6$ virulent *L. major* parasites for vaccine efficacy evaluations.

3.6 Challenge of mice with virulent *Leishmania major* parasites

Two weeks after the third vaccination 6 mice from each group were challenged with virulent *L. major* promastigotes. Stationary phase promastigotes were prepared as described in section 3.2 and counted. The right hind footpad was swabbed with 70%
alcohol and allowed to dry as described (Macharia et al., 2004). The footpad was infected by subcutaneously inoculating 50µl of PBS containing $1 \times 10^6$ promastigotes. The thickness of the infected footpads was measured weekly for 8 weeks using Vernier caliper. Increase in footpad thickness was expressed as the difference between infected and the same noninfected footpad as described by Solbach et al. (1986).

### 3.7 Lymphocyte proliferation assay

Splenic blood lymphocytes were prepared as described (Gicheru et al., 1995). The cells were adjusted to $3 \times 10^6$/ml in complete RPMI-1640 (RPMI-1640; Appendix IV-ii) supplemented with 10% fetal bovine serum (Flow Laboratories, Irvin, UK) 2mM L-glutamine and 100µg/L gentamicin (Sigma) medium. One hundred microliters containing $3 \times 10^6$ cells/ml PBL in complete RPMI-1640 were distributed to each well of a 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) followed by the addition of 100µl of formalin-fixed *L. major* antigens ($5 \times 10^6$ /ml) or Con A (5µg/ml). Control wells received 100µl of complete RPMI 1640 medium. Cultures were set up in duplicates and incubated at $37^\circ$ C in a humidified atmosphere containing 5% CO₂ for 5 days for *Leishmania* antigen cultures and for three days for con A cultures. The cells were pulsed with 0.5µci of [Methyl-$^3$H] thymidine (New England, Nuclear Boston, MA; 1.85 mBq/ml) over the last 18 hours and harvested on fibre glass filters (Titertek, microtitration Equipment, UK). Incorporation of the radionuclide into DNA was determined by liquid scintillation spectrometry. Results were expressed as the stimulation index (SI) obtained by dividing the counts per minute of antigen or Con A by the counts per minute of the control cultures.
3.8 Production of IFN-γ by stimulated cells

Purified splenic lymphocytes were adjusted to $3 \times 10^6$/ml in complete RPMI 1640 medium and stimulated \textit{in vitro} with either Con A or \textit{L. major} antigens as described (Olobo \textit{et al.}, 1992). Culture supernatants pooled from triplicate wells after 48 hours of stimulation were used to determine IFN-γ using mouse IFN-γ enzyme linked immunosorbent assay (ELISA) kit (Mabtech, AB, Sweden, Code 3321-1H-6) according to the manufacturers instructions. Briefly, wells were coated with 100μl of monoclonal antibody, AN18 (1 μg/ml) to capture IFN-γ from supernatants of samples and recombinant mouse IFN-γ standard. One hundred microliters of monoclonal IFN-γ detecting antibody, R4-6A2-biotin (0.5 μg/ml) were added per well and the plate incubated for 1 hour at room temperature. Streptavidin-horse radish peroxidase (HRP) was added to the wells at a dilution of 1:1000 and the plates incubated for 1 hour at room temperature before the addition of 100μl/well of Tetramethlebenzidine (TMB) microwell peroxidase substrate. Optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories).

3.9 Enzyme-linked immunosorbent assay (ELISA) for antibodies

The assay was performed as described (Gicheru \textit{et al.}, 1995). Briefly, polystyrene Micro-ELISA plates (Nunc, Copenhagen, Denmark) were coated overnight with 100μl of \textit{Leishmania major} soluble antigen at a concentration of 10μg/ml, diluted in bicarbonate buffer (pH 9.6). Nonspecific binding sites were blocked with 3 % bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1hr at 37°C. The plate was washed 6 times with washing buffer before the addition of 100μl of the serum samples
and incubation for 2 hr at 37° C. The plate was washed 6 times as above and 100μl of 1:4000 horse radish peroxidase-conjugated sheep anti-mouse Ig G (Amersham) was used as detecting antibody. Tetramethlebenzidine microwell peroxidase substrate was added to the wells and the plate incubated protected from light for 20 minutes before the optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories). All sera were tested at a dilution of 1:8, which had been previously determined as the optical dilution for antibody detection by titration.

3.10 Microtitration assay for *Leishmania major* from infected footpad

Culture microtitration has been described (Buffet *et al.*, 1995 and Titus *et al.*, 1985). Briefly, challenge mice were sacrificed and the infected footpads were removed between the ankle joint and toes and then homogenized with a tissue grinder in 3ml of complete Schneiders insect tissue medium (Appendix IV-iii). Under sterile conditions, serial dilutions were prepared in wells of 48-well culture plates and incubated at 25° C. Thirty-six hours and 6 days of incubation, parasites were counted in haemocytometer counting chamber.

3.11 Statistical procedures

GraphPad Prism version 3.000 statistical software (GraphPad Software, San Diego, CA) was used to carry out analysis of variance (ANOVA) and Spearman rank correlation tests to determine the significance of differences between the means of groups. The Tukey test was chosen for post ANOVA comparisons. \( P<0.05 \) was considered statistically significant.
CHAPTER 4: RESULTS

4.1 The *Leishmania major* antigens

*Leishmania major* infected BALB/c mice were selected for isolation of the parasite used in this experiment. Before culturing of infected cellular material from the donor mice, a confirmation of the presence of the parasites was done by Giemsa stained (Appendix II) smear of aspirates from the infected footpads. A positive aspirate showed *L. major* amastigote-infected cellular material (Figure 4.1).

Figure 4.1. An isolate smear showing amastigotes in macrophages from the footpad of a BALB/c mouse, which had been infected with *L. major* promastigotes. The amastigote stage is a round or oval body about 2-6 μm in diameter, containing a nucleus and a kinetoplast as indicated by the arrows.
The aspirates grown in complete M199 medium at 25°C and monitored for fourteen days, produced a growth curve (Figure 4.2) with parasites at different phases of growth. The parasites were harvested between 10-12 days (Stationary phase) when the parasites are considered to have optimal surface antigens (Sacks and Perkins, 1984).

**Figure 4.2. Leishmania major growth curve for parasites grown in M199 medium**

The letters indicate the different phases of *L. major* growth in culture: a- Lag phase, b-Log phase, and c- Stationary phase (n=2 culture flasks).

At the stationary phase, parasites were slender flagellates with nucleus, kinetoplast and flagellum (Figure 4.3). Parasites at the stationary phase were subsequently used in preparation of the antigen used in vaccination experiments reported in this study.
4.2 Immune responses induced by Bacille calmette guérin, Montanide incomplete seppic and aluminium hydroxide adjuvants in BALB/c mice following vaccination

4.2.1 Humoral responses

In order to determine the best working dilution factor for the sera, a titration assay was carried out before setting the real assay. The titration assay on serially diluted pooled sera from previously *L. major* infected and also on serum samples obtained from naive BALB/c mice indicated a dilution of 1:8 as the mean titre (point at which the highest mean difference of the optical densities in the experimental (*L. major* immune and BCG-KLM vaccinated) and control (naive) animals was obtained; Figure 4.4).
Figure 4.4. Mean IgG titres for *L. major* immune, experimental BCG-KLM vaccinated and control mice used in this assay to establish the best working dilution for the study.

The *L. major* immune sera had been obtained and pooled from infected and treated BALB/c mice used in another experiment. It was used in this assay as a positive control.

Based on enzyme linked immunosorbed assay (ELISA), all vaccinated animals were clearly shown to be sero-positive when compared to the unvaccinated controls (Figure 4.5).
Figure 4.5. Mean IgG optical densities (ODs) for adjuvant-KLM vaccinated groups and control BALB/c mice.

Groups of BALB/c mice were vaccinated three times with either alum plus killed *Leishmania major* (KLM), Bacille calmette guérin (BCG) plus KLM, KLM alone or Montanide ISA plus KLM. Control group received PBS only. Two weeks following the last vaccination mice were sacrificed and serum prepared from individual animals and IgG levels determined through ELISA. Data shown indicates the mean ODs±SE from the various study animal groups (Alum, MISA and Control, n= 6 each; BCG and Ag, n= 5 each).

The optical densities at 630nm from the experimental groups ranged between 0.20 and 0.508 and were all above the absorbance values of the negative control animals whose OD values were between 0.047 and 0.142 (Table 4.1).
Table 4.1. Optical density (OD) values from vaccinated and control BALB/c mice groups

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Alum</th>
<th>BCG</th>
<th>KLM</th>
<th>MISA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.399</td>
<td>0.508</td>
<td>0.258</td>
<td>0.213</td>
<td>0.092</td>
</tr>
<tr>
<td>2</td>
<td>0.332</td>
<td>0.454</td>
<td>0.234</td>
<td>0.219</td>
<td>0.142</td>
</tr>
<tr>
<td>3</td>
<td>0.327</td>
<td>0.289</td>
<td>0.282</td>
<td>0.20</td>
<td>0.063</td>
</tr>
<tr>
<td>4</td>
<td>0.375</td>
<td>0.441</td>
<td>0.243</td>
<td>0.204</td>
<td>0.047</td>
</tr>
<tr>
<td>5</td>
<td>0.414</td>
<td>0.334</td>
<td>0.284</td>
<td>0.202</td>
<td>0.131</td>
</tr>
<tr>
<td>6</td>
<td>0.311</td>
<td></td>
<td></td>
<td></td>
<td>0.084</td>
</tr>
</tbody>
</table>

Mean ± SE 0.36 ± 0.017 0.405 ± 0.041 0.26 ± 0.01 0.208 ± 0.003 0.09 ± 0.015

Groups of mice were vaccinated with either alum, Bacille calmette guérin (BCG) or Montanide ISA 720 as adjuvants combined with killed *Leishmania major* vaccine (KLM) or KLM alone. Control mice received PBS only. Immunoglobulin gamma (IgG) production was scored as Optical density (OD) as shown in the table.

The mean OD values indicated the highest value in the BCG-KLM vaccinated animals (mean OD= 0.405 ± 0.041) and the least value in the negative control group (mean OD= 0.09 ± 0.015). Other groups were intermediate (Figure 4.5). There was a significant difference in the production of *L. major* specific IgG between all the experimental and the negative control groups (F= 39.320, p<0.001). The three adjuvants showed differences in terms of their ability to induce IgG responses (F= 20.278, p<0.0001).
responses in alum-KLM vaccinated group compared with the BCG-KLM vaccinated were the same \((p>0.05)\) while it showed significantly higher levels of IgG when compared to the antigen alone vaccinated group \((p<0.05)\). There was significant difference in the levels of the IgG produced by the alum-KLM vaccinated group compared to the MISA-KLM vaccinated group with alum-KLM giving higher responses \((p<0.001)\). Similarly, significantly higher IgG responses were observed in the alum-KLM vaccinated animals as compared to the control group \((p<0.001; \text{Table 4.1})\).

When BCG-KLM vaccinated group was compared with the antigen, MISA and the control groups, there was significantly higher level of mean absorbance in the BCG group than in all the other groups \((p<0.001; \text{Table 4.1})\). The antigen alone and the MISA vaccinated groups showed no significant difference in IgG responses when compared \((p>0.05)\). Significantly higher levels of the IgG responses were obtained in the antigen alone vaccinated compared to the control group \((p<0.001)\). Significant difference was shown when the ODs of the MISA-KLM vaccinated and the negative control groups were compared \((p<0.01; \text{Table 4.1})\).

### 4.2.2 Cellular responses

#### 4.2.2.1 Recall proliferation responses

Concanavalin A and antigen proliferation of peripheral blood lymphocytes (PBL) was compared for both vaccinated and the negative control groups. All the animals responded to concanavalin A but with minimal variation within and between the groups. The mean SI from Con A stimulated splenic lymphocytes for the various study groups ranged from \(111\pm14.07\) to \(127\pm7.3\). There was no significant difference in response to Con A between
the vaccinated and control animals ($p>0.05$; Table 4.2). The mean antigen stimulation indices ranged from 26 to 72 among the experimental groups (Table 4.2; Figure 4.6).

Table 4.2. Values of stimulation indices (SI) obtained from vaccinated and control mice splenic lymphocytes stimulated in vitro with Killed *L. major* antigens.

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>Alum</th>
<th>BCG</th>
<th>KLM</th>
<th>MISA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>Con A</td>
<td>Ag</td>
<td>Con A</td>
<td>Ag</td>
</tr>
<tr>
<td>1</td>
<td>69</td>
<td>90</td>
<td>58</td>
<td>107</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>106</td>
<td>60</td>
<td>121</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>71</td>
<td>56</td>
<td>109</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>144</td>
<td>58</td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>115</td>
<td>57</td>
<td>116</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>141</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag</td>
<td>60±2.6</td>
<td>58±0.7</td>
<td>26±2.8</td>
<td>72±1.6</td>
<td>4.4±1.04</td>
</tr>
<tr>
<td>Con A</td>
<td>111±11.65</td>
<td>118±5.37</td>
<td>127±7.3</td>
<td>124±4.1</td>
<td>112±14.07</td>
</tr>
</tbody>
</table>

Lymphoproliferative responses of *L. major* antigen or Con A stimulated splenic cells from BALB/c mice vaccinated with KLM delivered alone or with an adjuvant (alum, BCG, or MISA) or PBS once and then boosted two times, two weeks apart.

All the vaccinated groups demonstrated significant antigen-specific lympho-proliferative responses when compared with the negative control group ($F=219.21$, $p<0.001$). The
ability of splenic PBL cells to respond in vitro to the formalin-fixed *L. major* antigen was significantly higher for the three adjuvant-KLM vaccinated groups than the KLM vaccinated group (*F* = 82.018, *p* < 0.0001). The three adjuvant-KLM vaccinated mice produced significantly differences (*F* = 16.478, *p* = 0.0002) in their in vitro antigen-lymphoproliferative responses. However, stimulation indices from the alum-KLM and the BCG-KLM vaccinated mice showing no difference in their response (*p* > 0.05).

The MISA-KLM vaccinated group showed significantly higher antigen-specific lymphoproliferative responses than the alum-KLM vaccinated group (*p*<0.01; Table 4.2) and also significantly higher (*p*<0.001) lymphoproliferative responses to formalin-fixed *L. major* antigens as compared to the BCG-KLM vaccinated group. The MISA-KLM vaccination protocol was therefore superior in priming splenic lymphocytes as compared to the alum-KLM and BCG-KLM groups both of which induced statistically equal levels of recall proliferation (Figure 4.6).
Figure 4.6. Lymphoproliferative responses of *L. major* antigen and Con A stimulated splenic cells from BALB/c mice vaccinated with KLM delivered alone or combined with either alum, Bacille calmette guérin (BCG), or Montanide ISA 720 DrPBS, once and then boosted two times, two weeks apart (Alum, MISA and Control, n=6 each; BCG and Ag, n=5 each).

**4.2.2.2 Interferon gamma cytokine quantification**

The mean values from Con A stimulated splenic cell culture supernatants ranged from 139.4±3.9 to 148.2±2.5. Cell culture supernatants collected from Con A in *vitro* stimulation for all the groups showed no significant difference in IFN-γ responses across the groups (p>0.05; Figure 4.7). When *Leishmania* antigen was used for stimulation, the negative control group of mice produced IFN-γ levels that ranged from 0.465 to 0.86
pg/ml. All the vaccinated mice produced IFN-γ levels above the higher limit (0.86 pg/ml) produced by the negative control mice and these ranged between 7.63 and 84.34 pg/ml (Table 4.3).

Table 4.3. Interferon gamma values from in vitro antigen restimulated splenic lymphocytes from vaccinated and control BALB/c mice groups

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>IFN-γ levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Alum</td>
</tr>
<tr>
<td>1</td>
<td>24.827</td>
</tr>
<tr>
<td>2</td>
<td>28.8</td>
</tr>
<tr>
<td>3</td>
<td>29.0</td>
</tr>
<tr>
<td>4</td>
<td>27.32</td>
</tr>
<tr>
<td>5</td>
<td>29.08</td>
</tr>
<tr>
<td>6</td>
<td>25.58</td>
</tr>
</tbody>
</table>

Mean ± SE 27.5±0.79 82.5±0.53 9.4±0.73 56.1±1.6 0.7±0.063

IFN-γ levels (pg/ml) in the supernatants of homologous leishmania antigen-stimulated splenic cells from BALB/c mice immunized three times with different adjuvant-KLM vaccines or KLM alone. Purified splenic lymphocytes were collected 2 weeks after the third injection and adjusted to 3x10⁶ /ml in complete RPMI-1640 and stimulated with 5x10⁶ formalin-fixed L. major promastigotes. Culture supernatants were collected from duplicate wells after 72 hr stimulation and the concentration of IFN-γ in the supernatants determined by ELISA. The table presents individual mouse responses and the groups’ mean ± SE.
Compared to the negative control group, all vaccinated groups of mice produced quantities of IFN-γ, which were significantly higher ($p<0.001$). The adjuvant-KLM vaccinated groups produced significantly higher IFN-γ levels than the KLM group ($F=853.14$, $p<0.0001$). Significant differences in the abilities of the three adjuvant-KLM vaccinated groups’ IFN-γ responses was observed ($F = 573.31; p< 0.0001$; Figure 4.7). All the vaccinated groups produced IFN-γ in average levels higher than 9.4 ± 0.73 pg/ml with the animals vaccinated with BCG-KLM presenting the highest levels of the cytokine.

The Bacille calmette guérin -KLM vaccinated mice produced a quantity of IFN-γ that was significantly higher than the levels of IFN-γ produced by the MISA-KLM vaccinated group ($p<0.001$; Figure 4.7). The quantity of IFN-γ induced by the MISA-KLM vaccinated animals was significantly higher than the levels produced by the alum-KLM vaccinated group ($p<0.001$). The BCG-KLM vaccinated mice produced the highest IFN-γ levels, followed by the MISA-KLM group. The alum-KLM vaccinated mice had the least quantities of the cytokine, among the adjuvant-KLM vaccinated groups.
Figure 4.7. Interferon gamma levels (pg/ml) in the supernatant of homologous *Leishmania* antigen stimulated splenic cells from BALB/c mice immunized three times with different adjuvant-KLM vaccines or KLM alone. Purified splenic lymphocytes were collected 2 weeks after the third injection and adjusted to 3x10^6/ml in complete RPMI-1640 and stimulated with 5x10^6 Formalin-fixed *L. major* promastigotes. Culture supernatants were collected from duplicate wells after 72 hr stimulation and the concentration of IFN-γ in the supernatants determined by ELISA. The graph represents mean ± SE. Alum, MISA and Control groups, n = 6 each; BCG and Ag groups, n = 5 mice in each group.

4.3 Efficacy of the various adjuvant-KLM following vaccination and challenge of BALB/c mice

4.3.1 Lesion measurements in *Leishmania major* infected mice

The second goal of this study was to assess the ability of different adjuvant-KLM vaccines to protect against a subsequent challenge with *L. major* in susceptible BALB/c mice. BALB/c mice were vaccinated intraperitoneally (Figure 4.8 A) as described earlier,
and eight weeks later, the immunized mice and controls were challenged with $1 \times 10^6$ virulent NLB-144 promastigotes subcutaneously in the right hind footpad (Figure 4.8 B). Lesion developments were monitored by measuring the increase in thickness of the infected footpad with a vernier caliper (Figure 4.8 D).

Comparative lesion sizes showed that there was delayed lesion development in the MISA-KLM vaccinated group until the third week when the sizes of the footpads of mice in this group gradually started to increase (Figure 4.9). This group maintained the lowest footpad sizes throughout the experimental period. The negative control group showed immediate response to the challenge infection by developing thicker footpads, which increased by $0.5 \pm 0.117$ mm by the end of the first week post infection (Figure 4.9).

Eight weeks post challenge, the footpad thickness of the negative control mice had increased by an average of $1.4 \pm 0.092$ mm, leading to marked swelling of the infected footpad (Table 4.4; Figure 4.8 C). The BCG-KLM vaccinated group, unexpectedly, developed higher lesion sizes as compared to all the other vaccinated groups up to the seventh week when the lesion sizes in this group gradually reduced to lower levels as compared to the KLM antigen vaccinated group (Figure 4.9).
Figure 4.8. BALB/c mice being vaccinated intraperitoneally (A). Mice from the negative control group with an arrow indicating the right hind footpad before (B) and eight weeks (C) post challenge with virulent *L. major* promastigotes. Slide D shows Vernier caliper in place taking measurement of the lesion size.

Two weeks post challenge significantly lower lesion sizes were recorded in the vaccinated groups as compared to the negative control group (*F* = 6.423; *p* = 0.0011; Table 4.4). There was however, no significant difference between the BCG-KLM and the negative control group (*p* > 0.05). At this time point, all the adjuvant-KLM vaccinated
groups showed no difference in their lesion sizes when each group was compared with the KLM vaccinated group ($p > 0.05$). MISA group showed significantly smaller lesion sizes than the BCG group ($p < 0.05$).

![Graph showing lesion development in BALB/c mice following immunization and challenge with $10^6$ virulent $L. major$ promastigotes.](image)

Figure 4.9. Lesion development in BALB/c mice following immunization and challenge with $10^6$ virulent $L. major$ promastigotes. The mice were monitored every week and the mean lesion size ± SE is shown (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each).

Four weeks after challenge, footpad sizes had increased in all the groups to averages of between 0.4167 and 0.83mm. Significantly smaller lesion sizes were shown only by the MISA-KLM vaccinated group when compared to the negative control group ($p < 0.05$;
Table 4.4). All other vaccinated groups showed no significant difference in their lesion sizes when compared to each other or to the negative control group ($p>0.05$).

Table 4.4. Footpad sizes from groups of BALB/c mice measured following vaccination and challenge

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>Mean footpad thickness ± SE (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alum</td>
</tr>
<tr>
<td></td>
<td>BCG</td>
</tr>
<tr>
<td></td>
<td>KLM</td>
</tr>
<tr>
<td></td>
<td>MISA</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Weeks p.c</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.1±0.002</td>
</tr>
<tr>
<td>2</td>
<td>0.1±0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.3±0.091</td>
</tr>
<tr>
<td>4</td>
<td>0.4±0.125</td>
</tr>
<tr>
<td>5</td>
<td>0.7±0.126</td>
</tr>
<tr>
<td>6</td>
<td>0.8±0.105</td>
</tr>
<tr>
<td>7</td>
<td>0.8±0.149</td>
</tr>
<tr>
<td>8</td>
<td>0.92±0.141</td>
</tr>
</tbody>
</table>

Groups of BALB/c mice were vaccinated three times with KLM antigen alone or in combination with either alum, BCG or MISA 720 as adjuvants and challenged with *L. major* parasites two weeks after the third vaccination. Changes in the thickness of infected footpads were measured and the results recorded. Data shown indicates the weekly mean lesion sizes ± SE for each mouse in all the groups (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each). p. c =post challenge.
Lesion measurements taken at six weeks post challenge indicated significantly reduced lesions in the MISA-KLM vaccinated group as opposed to the negative control mice ($p<0.01$; Table 4.4). All other vaccinated groups showed no significant difference in their footpad sizes when compared with each other or with the negative control group ($p>0.05$).

Data obtained at eight weeks post challenge showed significantly smaller lesion sizes in alum-KLM, BCG-KLM and MISA-KLM vaccinated groups of mice when compared with the negative control mice ($p<0.05$; Table 4.4). There was significant control of cutaneous lesions by the MISA 720 adjuvant as compared to the BCG adjuvant. There was no difference in lesion sizes between the alum-KLM, BCG-KLM and the Ag vaccinated groups ($p>0.05$).

Spearman rank analysis showed no significant correlation between the mean lesion sizes measured at week eight-post challenge and the mean IFN-γ of the corresponding vaccinated groups ($r = -0.7000; p = 0.2333$; Figure 4.10).
Figure 4.10. Comparison between mean IFN-γ levels and mean lesion sizes in both experimental and control Balb/c mice groups.
Groups of mice were either vaccinated with alum-KLM, BCG-KLM, KLM alone, MISA 720-KLM or PBS. Eight weeks after immunization 6 mice from each group were sacrificed and splenic lymphocytes prepared and stimulated in vitro with KLM and IFN-γ levels determined from the supernatants 72 hr later through sandwich ELISA. The remaining animals from each study group were infected with 10^6 virulent *L. major* promastigotes and lesion development determined by measuring the thickness of the infected footpad using vernier caliper. The mean IFN-γ was correlated with the mean lesion sizes of the various groups of study mice as shown in the table (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each).

4.3.2 Quantification of parasite burden from footpads of BALB/c mice challenged with *Leishmania major* promastigotes following vaccinations

Eight weeks after challenge, BALB/c mice were sacrificed and the infected footpad aseptically removed, homogenized in Schneiders insect tissue media using a tissue grinder and the cellular material from individual animals serially diluted and cultured in
48-well plates. Aliquotes were taken from individual wells at 36 hr and six days of culture incubation and parasites enumerated using Neubauer haemocytometer (Table 4.5).

Table 4.5. Parasite load taken from 6 days culture of footpads from BALB/c mice groups following vaccination and challenge.

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>Parasite load (X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1</td>
<td>Alum 214 BCG 73 KLM 731 MISA 75 Control 994</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>Alum 180 BCG 84 KLM 652 MISA 121 Control 1001</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>Alum 249 BCG 74 KLM 705 MISA 87 Control 1138</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>Alum 239 BCG 91 KLM 872 MISA 94 Control 1082</td>
</tr>
<tr>
<td>Mouse 5</td>
<td>Alum 288 BCG 58 KLM 803 MISA 76 Control 1155</td>
</tr>
<tr>
<td>Mouse 6</td>
<td>Alum 611 BCG 83 KLM 729 MISA 39.3 Control 1074</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>234± 18 76± 5.6 729± 39.3 89.33± 6.96 1074±33.5</td>
</tr>
</tbody>
</table>

Groups of BALB/c mice were vaccinated three times with either, alum-KLM, BCG-KLM or MISA-KLM. The control group received PBS only. Two weeks after the third vaccination, mice were infected subcutaneously in the right hind footpads with 10^6 L. major metacyclics and the parasite burden determined from the infected footpads fourteen weeks later (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each).
The mean numbers of parasites in the various vaccinated and control groups counted from 36 hours and 6 days old culture indicated a similar trend in the relative numbers of parasite load at the two time points and as such data obtained on day six of culture incubation was used for analysis (Figure 4.11; Table 4.5).

Figure 4.11. Mean parasite load (± SE) in groups of BALB/c mice immunized or not immunized with killed *L. major* promastigotes with or without an adjuvant and challenged 8 weeks later with 10^6 metacyclic *L. major* promastigotes. Mice were sacrificed 8 weeks after challenge infection and parasite levels determined through culture microtitration. Parasites were counted using counting chamber after 36 hr and 6 days of culture incubation (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each).
Significantly lower levels of parasitaemia were obtained from the experimental groups of mice as compared to the negative control group ($F= 293.11, p<0.0001$; Table 4.5). The levels of parasite load were found to be significantly different for all the adjuvant vaccinated groups when compared with the KLM group ($F= 179.42, p<0.0001$). Although the BCG-KLM and MISA 720-KLM vaccinated groups showed no significant differences in their parasite loads ($p>0.05$), both groups recorded significantly lower parasite loads when compared to all the other groups ($p<0.001$). The parasite loads in the BALB/c mice vaccinated with BCG-KLM were significantly lower than the parasite numbers in the alum-KLM vaccinated animals ($p<0.001$). Similarly significantly lower parasite loads were recorded in the MISA-KLM as compared to the alum-KLM vaccinated mice ($p<0.001$).

Spearman rank analysis of the mean parasite loads and IFN-γ in the matching experimental and control groups concluded a significant correlation between the two parameters ($r = -1.000; p= 0.0167$; Figure 4.12). This analysis indicated highest mean level of IFN-γ in the mice group harboring the lowest mean number of parasites.
Figure 4.12. Relationship between mean IFN-γ levels and parasite burden in both experimental and control BALB/c mice groups.
Groups of mice were either vaccinated with alum-KLM, BCG-KLM, KLM alone, MISA 720-KLM or PBS. Eight weeks after immunization 6 mice from each group were sacrificed and splenic lymphocytes prepared and stimulated in vitro with KLM and IFN-γ levels determined from the supernatants 72 hr later through ELISA. The remaining animals from each study group were infected with 10⁶ virulent *L. major* promastigotes and parasite load determined eight weeks later (Alum, BCG and Control, n= 5 each; MISA and Ag, n= 6 each).
5.1 Immune responses induced in BALB/c mice following vaccinations with Formaling fixed *Leishmania major* antigens delivered with either alum, BCG or Montanide ISA 720 as adjuvants

Protection against cutaneous leishmaniasis in humans has so far only been achieved by use of live virulent preparations of the parasite (Kenney *et al.*, 1999). However, preventive vaccination with live parasites produces active lesions to a number of the patients (Naggan *et al.*, 1972). As a result, this type of vaccination is not practical. Protection against leishmaniasis is mediated by T cells belonging to the Th 1 helper subset (Sohrabi *et al.*, 2005; Scott *et al.*, 1988; Olobo *et al.*, 1995). Killed antigens that could be safer as vaccines generally require an adjuvant for induction of a strong Th 1 response in murine models (Kenney *et al.*, 1999). The objective of this study was to investigate whether formalin-fixed promastigotes mixed with alum, BCG, or MISA 720 adjuvants could selectively induce protective immune response in susceptible BALB/c mice.

The use of BCG as an adjuvant is regarded as an acceptable practice in man (Sharifi, 1998). Bacillus Calmette Guerin is the world’s most widely used vaccine (Dobakhti *et al.*, 2006) and at present this adjuvant is routinely used in vaccination and immunotherapy trials against leishmaniasis (Bahar *et al.*, 1996). Montanide ISA 720 (M-ISA 720, Seppic, Paris) is an oil adjuvant composed of natural metabolizable oil and a highly refined emulsifier from the manide monooleate family. Montanide ISA 720 has been shown to be safe and immunogenic inducing both cellular and humoral immune
responses in human trials (Oliveira et al., 2005; Toledo, 2001; Genton, 2000) and in the Vervet monkeys experimentally infected with *L. major* parasites (Masina et al., 2003). Alum-adjuvanted vaccines have a long record of safety (Clements and Griffiths, 2001; Kaslow et al., 1994). Alum has been conventionally used as an adjuvant for a wide range of antibody-inducing vaccines (Misra et al., 2001) as in the blocking of transmission of *Plasmodium falciparum* in murine and monkey malaria vaccine studies (Kaslow et al., 1994). Excellent protective effects of vaccination of Rhesus monkeys have been obtained when a mixture of IL-12 and alum is combined with heat-killed *Leishmania* promastigotes (Kenney et al., 1999). Alum-containing adjuvants are desirable as they have the property of increasing dendritic cell antigen internalization (Morefield et al., 2005) apart from possessing the valuable quality of the repository effect (Theodore and Martin, 2002), which may improve parasite elimination by effector cells.

One of the objectives of this study was to evaluate immune responses induced by alum, BCG and MISA 720 as adjutants co-administered with formalin-fixed *L. major* promastigotes in susceptible BALB/c mice. The results of this study clearly indicate that, alum, BCG and MISA 720 do exert adjuvant effects on KLM vaccine in BALB/c mice, as measured by the production of serum antibodies, recall proliferative responses of splenic lymphocytes *in vitro* and the production of IFN-γ.

The antibody measurement results did not distinguish the best immunogen among the three adjuvants as the BCG and alum produced antibody titres that were statistically similar but higher than the levels induced by the MISA 720 adjuvant. These results differ
from earlier findings in which peak antibody titres and persistence of parasite specific antibody following human vaccination with MISA 720 formulated with *Plasmodium falciparum* antigen were comparable to those obtained following immunization with the antigen mixed with alum (Oliveira *et al*., 2005). However, other studies showed that, aluminium-adjuvanted vaccines resulted in higher and more prolonged antibody responses (Theodore and Martín, 2002). Montanide ISA 720 produced antibody titres that were lower but statistically equal to the levels induced by the KLM alone indicating that Montanide ISA 720 may not be a good stimulator of IgG antibodies in this vaccination protocol. Previous studies in mice and humans using *Plasmodium falciparum* antigens in combination with MISA 720 indicated this adjuvant as very effective in eliciting strong antibody titres against the antigen (Mata *et al*., 2007; Oliveira *et al*., 2005).

The low levels of IgG obtained in the MISA-KLM vaccinated animals in the current study may suggest that the choice of the antigen used determines the immune response stimulated by the adjuvant. Although not desirable in this study, the higher levels of IgG induced by the KLM vaccination relative to those produced by the MISA 720 were not unexpected as KLM promastigotes have been evaluated for example, by Alimohammadian *et al*. (2002) and found to be immunogenic and as such they could induce higher antibody responses. Bacille calmette guérin (BCG) induced the highest levels of antibody titres and this was not unexpected as in a study by Sohrabi *et al* (2005) BCG combined with detergent solubilization formulation of autoclaved *L. major* promastigotes (DSV-ALM-BCG) was found to induce higher IgG antibody levels when compared to other adjuvants. The alum-KLM vaccination induced antibody responses
that were statistically the same as those induced by the BCG-KLM vaccination. Alum has been conventionally used as an adjuvant for a wide range of antibody-inducing vaccines (Misra et al., 2001). Based on these findings, it can sufficiently be reported that alum and BCG as adjuvants increases the humoral antibody responses to formalin-fixed *L. major* promastigotes than does vaccination with MISA 720.

*In vitro* T-cell responses was demonstrated in positive recall proliferative responses to *L. major* and also secretions of IFN-γ when stimulated with *L. major* antigens. In the murine model of *L. major* infection, it is now well established that protection depends on a cell-mediated immune response with expansion of a Th 1 subset of lymphocytes (Sypek et al., 1993; Moll and Rollinghoff, 1990). In this study, specific parasite lymphocyte proliferation was demonstrated in all the experimental groups. Marked Con A stimulation was demonstrated in all the animals and there was no significant difference in response to Con A in both the experimental and control animals. This was an indication that, the vaccination protocols were safe and not associated with immunosuppression. Further more there was no adverse reaction at the site of vaccine administration. With a high significant difference in antigen recall lymphoproliferative responses in all the adjuvant-KLM vaccinated groups as compared to the KLM vaccinated group of mice, it was clear that the addition of these adjuvants to KLM antigens for vaccination, increased the specific lymphocyte responses to the antigen. Cells taken from mice vaccinated with MISA-KLM proliferated more *in vitro* when restimulated with formalin-fixed *L. major* promastigotes than cells taken from mice vaccinated with either alum-KLM or BCG-KLM. This may indicate that montanide ISA adjuvant is highly immunogenic when
coupled with *Leishmania* antigen, priming lymphocytes more strongly than both *alum* and BCG adjuvants. Mice vaccinated with alum-KLM and those vaccinated with BCG-KLM produced similar levels of *leishmania* antigen-specific lymphoproliferative responses as well as similar levels of IgG responses. This is an indication that both the BCG and alum adjuvants may have had equal ability in the priming of lymphocytes and production of humoral antibody responses in this vaccination protocol.

*Leishmania major* studies in Vervet monkeys have established that, high levels of IFN-γ are produced in self cured animals (Olobo *et al*., 1992). The results of the current study clearly showed that, high levels of IFN-γ were obtained in the BCG-KLM and MISA-KLM vaccinated groups of mice with the BCG-KLM group producing significantly higher levels of the cytokine than the MISA-KLM group. This finding may suggest that both BCG and Montanide ISA adjuvants are good inducers of Th1 cytokines and as such can be used in vaccines intended to induce protection against intracellular pathogens. The amounts of interferon gamma induced in cells obtained from mice vaccinated with the alum adjuvant were significantly lower than those produced by cells obtained from both the BCG and Montanide ISA 720 vaccinated animals. This confirms that, alum may be a better adjuvant for inductions of humoral responses (Misra *et al*., 2001). However, other factors need to be considered before a conclusion can be generated to support this view as it has so far been established that alum as an adjuvant will provoke both humoral and cell mediated immune responses (Jankovic *et al*., 1997).
5.2 Efficacy of Alum, BCG and Montanide ISA 720 as adjuvants delivered with formalin fixed *Leishmania major* in BALB/c mice

The second objective of this study was to find out whether vaccinations with the three adjuvants combined with formalin fixed *L. major* antigens would protect susceptible BALB/c mice from cutaneous disease caused by virulent *L. major* parasites. In the mouse model of leishmaniasis, it has been shown that IFN-γ producing CD4+ Th 1 cells are important in the establishment of protective immunity (Liew, 1989). Th 1 cells produced IFN-γ and IL-2 confers protection against the disease (Scott *et al*., 1988).

From the current study, although the BCG-KLM vaccinated group induced the highest levels of IFN-γ than either alum-KLM or MISA-KLM, this did not translate into reduced lesion sizes as there was no significant difference in the lesion sizes between the BCG-KLM vaccinated and the control group at weeks 2, 4, and 6 post challenge. Significant protection based on lesion sizes became evident at week 8-post challenge for the BCG-KLM vaccinated animals when compared to the controls. Throughout the experimental period, the BCG-KLM vaccinated group showed significantly higher lesion sizes than both the alum-KLM and the MISA-KLM vaccinated animals. The unexpectedly high lesion sizes in the BCG-KLM group might have been caused probably by inflammatory arthritis, an adverse effect of BCG vaccination (Smrkovski and Larson, 1977).

The delayed lesion size development in the MISA-KLM vaccinated mice may be attributed to the significantly higher levels of protective IFN-γ observed in this group as opposed to the alum-KLM vaccinated animals. Previous studies had confirmed that,
vaccination of Vervet monkeys with recombinant histone antigen mixed with MISA 720 showed no local or systemic adverse reactions (Masina et al., 2003). The study further established that inclusion of MISA 720 in the recombinant histone antigen resulted in reduced development of cutaneous lesions. The current study shows results similar to those established by Masina et al. (2003) since vaccination with MISA-KLM resulted in the smallest and delayed lesion development as compared to all the other groups.

The fact that comparable humoral and proliferative responses were obtained for both the alum-KLM and the BCG-KLM groups, this may support the findings that alum adjuvants are antibody based as opposed to cellular responses. Throughout the experimental period, non-significant difference in lesion sizes was observed between the alum-KLM and the KLM vaccinated groups, although the levels of IFN-γ were significantly higher in the alum-KLM group as compared to the KLM group. Both the MISA-KLM and the BCG-KLM vaccinated mice groups showed similar levels of parasite load that were significantly lower than the levels obtained from the alum-KLM vaccinated mice.

The significantly reduced parasite load in the MISA-KLM vaccinated mice as compared to the alum-KLM vaccinated animals may be sufficiently attributed to both the significantly higher proliferative responses (as compared to all other groups) and IFN-γ production as compared to the alum-vaccinated group. This finding concludes that, both BCG and MISA 720 as adjuvants immunopotentiates formalin-fixed Leishmania major antigens to induce protective immunity against *L. major* infection. Significant protection was also evident in the alum-KLM vaccinated mice when compared to the KLM
vaccinated animals as indicated by the reduced number of parasites in the alum-KLM vaccinated group of mice. This can only be attributed to the significantly higher levels of IFN-γ in the alum as opposed to the KLM group, supporting the findings by Jankovic et al. (1997) that alum as an adjuvant promotes both humoral and Th 1 cytokine responses in mice. This further shows that the three adjuvants indeed do increase the immune responses to formalin-fixed *L. major* promastigotes.

In a study by Kenney et al. (1999), the vaccination with alum combined with autoclaved *L. amazonensis* promastigotes and IL-12 of Rhesus monkeys, protected the animals from infectious challenge with virulent *L. amazonensis* as the vaccinated group of the monkeys recorded smaller lesion sizes. In another study, alum precipitated autoclaved *L. major* promastigotes mixed with BCG completely protected Indian Langur from *L. donovani* infectious challenge by inducing highly protective IFN-γ and antibody responses (Misra et al., 2001). If protection was to be based on lesion sizes only in this study, then the alum-KLM and MISA-KLM vaccinations would produce equivalent protection levels as indicated by the non-significant differences in the lesion sizes of the mice in the two groups throughout the experimental period. The use of BCG in this case would be ruled out due to the large lesions observed in the mice vaccinated with this adjuvant.

The goal of any vaccination strategy is to give effective protection against subsequent infection and a desirable vaccine would be one that is effective and causing no disease or adverse reactions in the vaccinee. There was a good correlation between the parasite burden and IFN-γ levels. The data from this study clearly provide evidence that
combination of *L. major* formalin-fixed promastigotes with either alum, MISA 720 or BCG as adjuvants may improve the immunogenicity and efficacy of the crude *Leishmania* antigen in vaccines against cutaneous leishmaniasis in mice. However, although BCG appears to be a better adjuvant as shown by the least number of parasites in the BCG-KLM vaccinated animals, it may lead to inflammatory reaction (Alimohammadian *et al.*, 2002) as evidenced by the largest lesion sizes recorded in the BCG group when compared to all other experimental groups. This finding resulted in the non-significant correlation obtained between the IFN-γ and lesion sizes. On the other hand, MISA 720 has been shown to be safe and effective (Masina *et al.*, 2003; Laurence *et al.*, 1997; Oliveira *et al.*, 2005) as an adjuvant for human vaccines.

Given the similar levels of parasite load obtained in the BCG-KLM and the MISA-KLM (which were far much lower as compared to the alum-KLM group), but reduced lesion sizes obtained only in the MISA-KLM group as opposed to both the BCG-KLM and alum-KLM groups, it would be clearly sufficient to state that, the MISA-KLM vaccination protocol was found to be the most effective against *L. major* challenge of BALB/c mice as compared to both the BCG-KLM and the alum-KLM vaccinated mice.

The results from this study dismisses the null hypothesis and adopts the alternative statement to conclude that, Bacille Calmette Guérin, Montanide Incomplete Seppic and aluminium hydroxide adjuvants co-administered with killed *Leishmania* major induce protective immune responses in mice following vaccination, and are thus efficacious and appropriate for *Leishmania* vaccines.
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

6.1.1 Immune responses induced in BALB/c mice following vaccinations with Formaling fixed *Leishmania major* antigens delivered with alum, BCG or Montanide ISA 720 as adjuvants

This study has clearly established that alum as an adjuvant combined with formalin fixed *L. major* promastigotes induces high quantities of IgG antibody comparable to the levels of the antibody induced by vaccination of BALB/c mice with BCG than Montanide ISA 720 combined with the same antigen.

On the other hand, data from this study suggests that Montanide ISA 720 can be used in vaccination protocols that require the induction of cellular immunity. The lower levels of priming of lymphocytes by both the alum and BCG as compared to the Montanide ISA 720 in the proliferation assay may indicate a better cell mediated immune response in vaccines formulated with Montanide ISA adjuvants than those using either alum or BCG as adjuvants.

BALB/c mice vaccinated with BCG combined with formalin fixed *L. major* antigens produced the highest levels of interferon gamma but this response did not protect mice from cutaneous lesions.
6.1.2 Efficacy of Alum, BCG and Montanide ISA 720 as adjuvants delivered with formalin fixed *Leishmania major* in BALB/c mice infected with virulent *Leishmania major* parasites following vaccination

Based on the efficacy evaluation data, it can be concluded that the best adjuvant for use with *Leishmania* killed antigens is the Montanide ISA 720 as indicated by the smallest lesion sizes in Montanide ISA 720 group compared to other adjuvants. The use of alum in *Leishmania* vaccines may not be ruled out as indicated in the lower lesion sizes observed in mice vaccinated with this adjuvant than those vaccinated with BCG. The animals vaccinated with BCG were found to have exacerbated lesions as compared to all the other vaccinated mice. This is a strong indication of a possible inflammatory reaction since these lesions were accompanied by low parasite numbers.

Mice vaccinated with Montanide ISA 720 adjuvant in this study were found to harbor equal quantities of parasites as the group of mice vaccinated with BCG despite the latter having induced higher quantities of the protective interferon gamma responses. Lower parasite numbers obtained in the alum vaccinated mice compared with the KLM vaccinated control animals may suggest its unevaluated potential as an adjuvant for safer leishmania vaccines. It may be worthwhile to emphasise that the induction of cellular immune responses as obtained in the recall lymphoproliferation and interferon gamma parameters coupled with the smallest lesion sizes and low parasite number observed in the montanide ISA 720 vaccinated mice suggest that, this adjuvant is safe and efficacious for *Leishmania* vaccine.
6.2 RECOMMENDATIONS

6.2.1 Applications of the findings

a) The safety of BCG as an adjuvant in human vaccines needs to be thoroughly investigated.

b) Montanide ISA 720 adjuvant should be considered as an alternative immunostimulator in human *Leishmania* vaccines due to its safety and high efficacy.

c) The use of lesion size as a surrogate marker of protective immunity in *Leishmania* vaccine efficacy trials in BALB/c model should be discouraged.

d) It is important though, to note that, in the mouse model, the mechanisms of pathogenesis and immunity may be different from those occurring in humans, therefore it is recommended that the results generated from this study be confirmed in a non-human primate model of the disease prior to clinical trials in the human.
6.2.2 Suggestions for future research work

a) Investigation on the effect of the route of administration on a vaccine efficacy, this should be applied to the best identified vaccine formulation, Montanide ISA 720 plus KLM.

b) Investigation on the induction and duration of leishmania specific protective immunological responses based on the best identified adjuvant in non-human primates.

c) Investigation on the safety and efficacy of a *Leishmania* vaccine combining killed *Leishmania* antigens and alum, BCG or Montanide ISA as adjuvants in non-human primates.
REFERENCES


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a Th 1 type of immune response but does not protect against infection. *Vaccine* 16: 2077-2084.


APPENDIX I: PREPARATION OF BUFFERS USED IN VARIOUS ASSAYS

(i) Preparation of Alsevers solution

To make one 1000 ml of alsevers solution.

Mix and dissolve the following salts

- Dextrose (Glucose) \(20.5\) g
- Sodium chloride \(4.2\) g
- Trisodium citrate \(8.0\) g
- Double distilled water \(1000\) ml

Adjust pH to 7.2, sterilize by membrane filtration and store at \(4^\circ\) C.

(ii) Carbonate-Bicarbonate buffer (pH 9.6)

- NaCl \(8.0\) g
- KH\(_2\)PO\(_4\) \(0.2\) g
- Na\(_2\)HPO\(_4\).12H\(_2\)O \(2.9\) g
- KCl \(0.2\) g
- NaN\(_3\) \(0.2\) g

Make up to 1 liter with distilled water, store at \(4^\circ\) C.

(iii) Phosphate buffered saline (PBS, 10X)

- Na\(_2\)HPO\(_4\) \(20.5\) g
- Na\(_2\)HPO\(_4\).7H\(_2\)O \(179.9\) g
- H\(_2\)O \(4\) litres
- NaCl \(701.3\) g
Dissolve and adjust the volume to 8 liters, adjust pH to 7.2-7.4

Store the stock solution at room temperature

Dilute to 1 X for use.

(iv) Preparation of physiological saline (Sodium chloride, NACl)

Sodium chloride 8.5g
Double distilled water 1 litre

Dissolve and filter sterilize.

APPENDIX II: GIEMSA STAIN

Giemsa stain 4ml
Methanol 4ml
Double distilled water 92ml

Store at room temperature.

APPENDIX III: PROTEIN ESTIMATION ASSAY

Reagents

1. Complex-forming reagent. Prepare immediately before use by mixing the following 3 stock solutions A, B, and C in the proportion 100: 1: 1, respectively.

2. Solution A: 2% (w/v) NaCO₃ in distilled water

3. Solution B: 1% (w/v) CuSO₄ .5H₂O in distilled water.

4. Solution C: 2% (w/v) Sodium potassium tartrate in distilled water.
5. 2N NaOH.

6. Folin reagent (Commercially available; Sigma, immunochemical, UK): Use at 1N concentration

7. Standards: Use stock solution of standard (bovine serum albumin fraction V; Sigma, immunochemical, UK) containing 4mg/ml protein in distilled water stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>µl</th>
<th>0</th>
<th>1.25</th>
<th>2.50</th>
<th>6.25</th>
<th>12.5</th>
<th>25.0</th>
<th>62.5</th>
<th>125</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>µl</td>
<td>500</td>
<td>499</td>
<td>498</td>
<td>494</td>
<td>488</td>
<td>475</td>
<td>438</td>
<td>375</td>
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</tr>
<tr>
<td>Protein concentration µg/ml</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>500</td>
<td>1000</td>
<td>2000</td>
<td></td>
</tr>
</tbody>
</table>

Method

1. To 0.1ml of sample or standard, add 0.1ml of 2N NaOH. Hydrolyse at 100°C for 10 minutes in a heating block or boiling water bath.

2. Cool the hydrolyzate to room temperature and add 1ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 minutes.

3. Add 0.1ml of folin reagent, using vortex mixture, and let it stand at room temperature for 30-60 minutes (do not exceed 60 min).
4. Read the absorbance at 750nm if the protein concentration is below 500 μg/ml or at 550nm if the protein concentration is between 100 and 2000 μg/ml

5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

APPENDIX IV: PREPARATION OF CULTURE MEDIA

(i) M199 medium supplemented with 20% fetal bovine serum (BSA)

To 40ml of M199 medium, add 10ml of fetal bovine serum (heat in-activated for 1 hr at 56°C) and 0.1ml gentamycin (50mg/ml). Adjust the pH to between 7.2-7.4 and filter sterilize with 0.22 micron milipore filter. Store at 4°C.

(ii) RPMI 1640 supplemented with 10% fetal bovine serum (BSA)

To 445ml of RPMI –1640, add 50 ml of fetal bovine serum (heat inactivated for 1 hr at 56°C), L-glutamine (final concentration of 200 mM/ml), 1 ml of gentamycin (50 mg/ml) and 2-mercapto-ethanol (to make a final concentration of 5x10⁻⁵ millimole). Adjust the pH to 7.2 and filter sterilize with 0.22 micron filter. Store at 4°C.

(iii) Schneider’s Drosophilla Insect media supplemented with 20% fetal bovine serum

To 80ml of of schneider’s medium add 20 ml of fetal serum (heat in-activated for 1 hr at 56°C), 0.2 ml gentamycin (50 mg/ml), filter sterilize with 0.22 micron milipore filter and store at 4°C.