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بسم الله الرحمن الرحيم

LEISHMANIA MEXICANA AND *LEISHMANIA MAJOR* : ATTENUATION OF WILD TYPE PARASITES AND VACCINATION WITH ATTENUATED LINES

Thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

by

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DECLARATION

I declare that this thesis is of my own composition and that the research described herein was performed entirely by myself except where expressly stated.

Hamid Daneshvar

July 2001

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ALM	Autoclaved *L. major* promastigotes
APCs	Antigen-presenting cells
APS	Ammonium persulphate
AUG	Adenine Uracil Guanine
BCG	Bacille Calmette Guerin
Ble	Zeocin
BMM	Bone marrow-derived macrophage
C	Complement
CD	Complementarity determining
CD40L	CD40 ligand
CMI	Cell-mediated immunity
ConA	Concanavalin A
CR	Receptor for activated complement fragment
CSF	Colony stimulating factor
CL	Cutaneous leishmaniasis
cm	Centimetre
cpm	Counts (of radioactivity) per minute
cpa/cpb	Cysteine proteinase a / cysteine proteinase b
Δ*cpa/cpb*	Mutants lacking cysteine proteinase (CP) genes *cpa* and *cpb*
DCs	Dendritic cells
DDT	Dichloro diphenyl trichoroethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC	Epidermal cell
ELISA	Enzyme linked Immunosorbant assay
FCS	Foetal calf serum
FPPG	Filamentous proteophosphoglycan

G	Acceleration in the earth's gravitational field
g	Gramme
GM-CSF	Granulocyte Mononuclearcyte- colony stimulating factor
GIPLs	Glycoinositol phospholipids
gp63	Glycoprotein 63
gp46/M2	Glycoprotein 46/ M2
GPI	Glycophosphatidylinositol
GSLs	Glycosphingolipids
h	Hour
H-line	Hamid's line
HI-FCS	Heat-inactivated foetal calf serum
HIV	Human Immunodeficiency Virus
Hyg	Hygromycin B
IEF	Isoelectric focusing
IFA	Indirect fluorescence antibody
IFN-γ	Interferon-gamma
IFN-R-deficient	Interferon receptor deficient
i.d.	Intradermal
IgG1a	Immunoglobulin G subclass 1a
iNOS	Inducible nitric oxide synthase
IL	Interleukin
IPG	Immobilised pH gradient
L	Linear
LACK	*Leishmania* antigen C kinase
LAMP	Lysosomal-associated membrane proteins
LC	Langerhans' cell
LD	Limiting dilution
Leu-Ome	L-Leucine methylester
LLA	Lysate *Leishmania* antigen
LN	Lymph node
Log	Logarithm
LPG	Lipophosphoglycan
LPKs	Leishmanial protein kinases

LPS	Lipopolysaccaharide
M	Morphology
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
n	Number
NK cell	Natural killer cell
NO	Nitric oxide
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
NOS2	Type 2 nitric oxide synthase
$NOS2^{-/-}$	NOS2 gene knockout
O_2^-	Superoxide anion
O_2	Singlet oxygen
OH	Hydroxyl radical
OVA	Ovalbumin
PCR	Polymerise Chain Reactive
Pue	Puromycin
PPG	Proteophosphoglycan
PV	Parasitophorous vacuole
R	Radical
RACK	Receptors for activated protein kinase C
rIL	Recombinant cytokine standards interleukine
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
SAT	Nourseothricin hydrosulfate
s.c.	Sub-cutaneous
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGC	Slab gel casting
SI	Stimulation index
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor

Th	T helper lymphocyte
TNF-α	Tumour necrosis factor-α
TMB	Tetramethylbenzidine
2-DE	Two Dimensional Electrophoresis
2ME	2-β-mercaptoethanol
VL	Visceral leishmaniasis
WHO	World Health Organization
wk	Week
WT	Wild Type

SUMMARY

The attenuated cell-lines of *Leishmania mexicana* Wild Type (*L. mexicana* WT) and *Leishmania major* Wild Type (*L. major* WT) known as *L. mexicana* Hamid's line (H-line) and *L. major* H-line, respectively have been established under the pressure of gentamicin which was routinely added to the medium to prevent bacterial contamination of promastigote culture. The mechanism by which gentamicin, an aminoglycoside, attenuates *L. mexicana* WT is unknown.

Following culture of promastigotes of *L. mexicana* WT (20 passages) and *L. major* WT (11 passages) in HOMEM medium supplemented with 10% (v/v) FCS and gentamicin at 20 μg/ml, promastigotes of the two strains formed attenuated lines. *L. mexicana* H-line was developed on four separate occasions with the same procedure and was stable in gentamicin-free medium for 23 weeks. There was no significant difference between the growth rate of promastigotes of *L. mexicana* H-line and *L. mexicana* WT *in vitro*. 12% of stationary phase promastigotes of *L. mexicana* H-line were longer than that of promastigotes of *L. mexicana* WT.

Total lysate protein of stationary or log phase promastigotes of *L. mexicana* H-line and WT, on 10-20% SDS-PAGE gradient gel, showed some differences between protein expression of the attenuated cell line and *L. mexicana* WT. Two bands were detected around 66 kDa with stationary or log phase promastigotes of *L. mexicana* WT, whereas one (possibly two) line is absent with stationary and log phase promastigotes of *L. mexicana* H-line. The optical density of proteins in lysates of stationary phase promastigotes of two lines of *L. mexicana* separated using SDS-PAGE was displayed with a Lane profile graph using Lab Works Image Acquisition and Analysis Software (UVP Laboratory products). The optical density of protein of lysate of *L. mexicana* H-line showed just one peak of protein with high concentration, whereas three peaks of protein are found in the same position of graph of *L. mexicana* WT.

The comparative proteome analysis of the two lines of *L. mexicana* using high-resolution techniques has been done using 2-dimentional electrophoresis (2-DE). Both lines of *Leishmania* comprise patterns with a high density of spots in the gel with pH range pH 4-7. The results of proteome analysis of promastigotes of the two lines of *L. mexicana* suggest that adaptation of *L. mexicana* H-line to grow in the presence of gentamicin has involved change in protein expression. The proteome analysis of

patterns of two lines of *L. mexicana* reveals high similarity and significant differences between attenuated line of *L. mexicana* and *L. mexicana* WT pattern have been found. One spot of pattern of *L. mexicana* WT was shifted to less acidic position in the pattern of *L. mexicana* H-line and one spot was absent from the pattern of *L. mexicana* H-line. Two spots were found in the *L. mexicana* WT gel, whereas the expression of these proteins by promastigotes of *L. mexicana* H-line decreased.

The ability of promastigotes of *L. mexicana* H-line to infect BMMs was similar to that of promastigotes of *L. mexicana* WT. In contrast, however, to the continued intracellular growth of *L. mexicana* WT, only a small population of amastigotes of *L. mexicana* H-line survived within the infected macrophages at 72-96 h post infection. Macrophages infected with stationary phase promastigotes of either *L. mexicana* WT or *L. mexicana* H-line, however, led no significant difference in nitric oxide production.

L. mexicana WT disseminated rapidly to the draining lymph node (LN) and visceral organs of BALB/c mice, whereas *L. mexicana* H-line remained localized in the skin, at the site where the promastigotes were injected, and in the draining LN of BALB/c mice. The mice did not normally develop any lesions.

Following injection stationary phase promastigotes of *L. mexicana* H-line at week 12, the epidermal cells from the site where the promastigoted were injected were transferred in to HOMEM medium. Amastigotes derived promastigote, were designated *L. mexicana* HAD-line, grew very poorly in medium, with or without gentamicin and morphology of 83% cells were amastigote forms in which some of them had a small size flagellum.

The levels of IFN-γ, IL-2, IL-4, and IL-10 in the supernatant of cultured splenocytes from the mice infected with *L. mexicana* H-line or *L. mexicana* WT were measured at 12 weeks post infection. It was found that the levels of IFN-γ and IL-2 in the supernatant of cultured Ag-stimulated-splenocytes of mice infected with *L. mexicana* H-line were significantly higher than those of mice infected with *L. mexicana* WT. In contrast to the mice infected with *L. mexicana* WT, IL-4 and IL-10 production by Ag-stimulated-splenocytes from mice infected with *L. mexicana* H-line was significantly decreased.

All non-vaccinated mice infected with *L. mexicana* WT developed large size, non-healing lesions, whereas the vaccinated mice challenged with *L. mexicana* WT

developed small and some cases healing lesions over 22 weeks post infection. There was no protection against *L. major* in the mice vaccinated with *L. mexicana* H-line. The levels of IFN-γ and IL-4 in the supernatant of cultured splenocytes from the mice immunized with *L. mexicana* H-line and challenged and infected mice with *L. mexicana* WT were measured. The level of IFN-γ in the supernatant of cultured Ag-stimulated-splenocytes of challenged mice was significantly greater than that of non-vaccinated mice infected with *L. mexicana* WT ($P<0.005$). In contrast to vaccinated mice, the amount of IL-4 production by non-vaccinated mice infected with *L. mexicana* WT was higher than that of vaccinated mice challenged with *L. mexicana* WT at the same time.

In this study it has been demonstrated that in the presence of the attenuated cell line, *L. mexicana* WT were eliminated from the draining LN, skin and visceral organs. In contrast, *L. mexicana* WT disseminated and survived in the visceral organs of challenged mice in the absence of the attenuated cell line.

The preliminary results of interaction between stationary phase promastigotes of *L. major* H-line and macrophages showed the percentage of macrophages infected with promastigotes of *L. major* H-line was 41% at 8 h and decreased to 10.5% at 96 h post infection. In contrast to *L. major* H-line, the percentage of infected macrophages with *L. major* WT was 46.5% and increased to 65% at 96 h post infection. The number of amastigotes of *L. major* H-line within infected macrophages after 9 h incubation was 94 amastigotes /100 macrophages which rapidly decreased to 14 amastigotes / 100 macrophages at 96 h post infection.

It was found BALB/c mice infected with *L. major* H-line failed to develop cutaneous lesions during 12 weeks post infection. In contrast to *L. major* H-line, all mice infected with *L. major* WT went to grow non-healing lesions are the same period.

The dissemination of *L. major* H-line and *L. major* WT from the skin where the promastigotes were injected to visceral organs of BALB/c mice was investigated at 12 weeks post infection. It was found that *L. major* WT spread to BM, spleen, lung, popliteal LN, and skin. In contrast, *L. major* H-line remained localized in the ECs and draining LN of (two up tree) mice.

The preliminary initially result showed that *L. major* H-line induced protection in mice against infection with *L. major* WT. All non-vaccinated mice developed progressive non-healing lesions that peaked in size at about 12 weeks post infection. In contrast to non-vaccinated mice the lesions developed slowly in vaccinated mice.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Introduction

Leishmania spp. are obligatory intracellular protozoan parasites, which are responsible for a wide spectrum of diseases ranging from local, self-healing skin ulcers [cutaneous leishmaniasis (CL)] to a severe and life threatening systemic disease [visceral leishmanisis (VL)] (Pearson and Sousa, 1996).

The World Health Organisation (WHO) has identified leishmaniasis as a major target for eradication (Ivens and Smith, 1997). This disease affects approximately 12 million people, mostly children and young adults, with two million new cases occurring annually, and 350 million people at risk of infection in 88 countries, 82% of which are developing countries (WHO report, http://www.who.int/ctd/htmi/leish.html, 2000).

Leishmania / Human immunodeficiency virus (HIV) co-infection is regarded as an emerging problem in 33 countries worldwide, especially in southern European countries, where up to 9% of all HIV cases suffer from either newly acquired or reactivated leishmaniasis (Desjeux, 1998; http://www.who.int/inf-fs/en/fact116.html., 2000). This combination of diseases produces a cumulative deficiency of the immune response since *Leishmania* parasites and HIV destroy the same cells, exponentially increasing disease severity and its consequences. It is expected that the number of *Leishmania* / HIV co-infections will continue to rise in the coming years and there are indications that cases are no longer restricted to endemic areas (Wolday *et al.*, 1999).

There has been much recent interest in attempts to vaccinate against *Leishmania* infection because leishmanicidal drugs frequently have unpleasant side effects, are relatively ineffective against cutaneous leishmaniasis, and drug resistance exists in various endemic regions of the world (Jackson *et al.*, 1990). In addition, many countries, where the disease is endemic, are economically poor. As a result, major pharmaceutical companies have historically had little interest in anti-leishmanial drug development.

Vaccination with live *Leishmania* to produce self-healing lesion at an inconspicuous site has been practised for a long time in the Middle East (Liew and Donnell, 1993). This method induced resistance in at least 70% of the individuals treated, but serious clinical complications associated with the live vaccine emphasises the need for an attenuated or a defined vaccine against cutaneous leishmaniasis (Liew and Donnell, 1993).

Some detailed knowledge of the parasite, the relationship of the parasite with its mammalian host cells and the mechanism by which this relationship determines the development of the ensuing immune response, are important for vaccination strategies against leishmaniasis.

1.2. Parasite

1.2.1. Classification

The genus *Leishmania* belongs to the Family Trypanosomatidea in the Class Kinetoplastidea (Cox, 1998). The parasite occurs as zoonotic infections of stray and domestic dogs, rodents, hyraxes, or sloths with variable penetration to man. At least 30 species of *Leishmania* have been recognised (Lainson and Shaw, 1987), of which 15 species of *Leishmania* with different geographical distributions and clinical features are pathogenic for humans (Bryceson, 1996). *L. major* is a distinct species but the *L. mexicana* complex includes five species (Cox, 1998):

i) *L. amazonensis*

ii) *L. mexicana*

iii) *L. pifanoi*

iv) *L. garnhami*

v) *L. venezuelensis*

1.2.2. Clinical manifestation

The two most important species in the *L. mexicana* complex and *L. amazonensis* which cause New World CL and New World diffuse CL (Cox, 1998).

L. mexicana causes cutaneous leishmaniasis in Central and South America. The disease is known as Chiclero's ulcer and has been characterised by two forms: localised and disseminated lesions. Local cutaneous leishmaniasis is similar to lepromatous leprosy and is typically confined to a single, indolent, ulcerative lesion that remains for about 1 year, leaving a characteristic depressed circular scar. If the lesion involves the rim of the pinna it destroys the underlying cartilage. The pinna is

swollen, ulcerated or crested usually with a cartilage-attacking infection without ulceration and with few parasites (Walton, 1987).

L. major causes Old World CL. The lesions are described as 'rural wet sores' (Cox, 1998).

1.2.3. Morphology

The parasite exists in two principal morphological forms:

Promastigote, a motile form with an anterior flagellum (1.5-3 × 10-20 μm) which resides in the midgut of the sandfly and can be grown in culture (Bryceson, 1996).

Amastigote, a non-motile, oval form with only a very short flagellum with a maximum diameter of 2.5 × 6.8 μm which multiplies intracellularly in mononuclear phagocytes of the mammalian host (Bryceson, 1996).

Multiplication of each form is by binary fission (Bryceson, 1996).

1.2.4. Diagnosis

Infection is diagnosed by direct demonstration of the parasites (microscopy, culture, DNA or RNA analysis). Cutaneous leishmaniasis is diagnosed from lesion, while visceral leishmaniasis from biopsies of spleen, bone marrow, and other suspected sites of infection (Bryceson, 1996). Examination for cellular immune response to *Leishmania* can be made using *leshmanin* (a suspension of killed promastigotes derived from culture) inoculated into the dermis (ID) of the forearm. The area of inflammation is measured 48-72 hours later. Leishmaniasis can be diagnosed serologically by ELISA, immunofluorescence or agglutination assays (Berman, 1997).

1.2.5. Treatment

Local CL ulcers usually heal spontaneously and do not require treatment, but if visceral organs are involved chemotherapy is required, and drugs include pentavalent antimonials, pentamidine, liposomal amphotericin B, paromomycin, interferon-γ (IFN-γ), and others (reviewed by Berman, 1997).

1.2.6. Life Cycle

Leishmania species cycle between sandfly vectors and several mammalian hosts including humans. They are transmitted between long-lived mammalian hosts by short-lived sandflies.

1.2.6.1. In the sandfly

Development of *Leishmania spp.* in the digestive tract of sandflies involves several morphological transformations from the intracellular amastigote form via a succession of free and gut wall-attached promastigote stages to the infective promastigote forms (Stierhof *et al.*, 1999). The parasite multiplies in the midgut of female phlebotomine sandflies (*Phlebotomus* spp. and *Lutzomyia* spp.). In the Old Word, sandflies of the genus *Phlebotomus* transmit the disease, while in the Americas it is transmitted primarily by *Lutzomyia* species. *Leishmania* exist in three distinct forms in the digestive tract of the sandfly during their life cycle:

i) Amastigote forms, following ingestion of infected macrophages.

ii) Free and gut wall-attached promastigote forms.

iii) Infective promastigote forms, which occur in the mouthpart of the sandfly.

The life cycle in the sandfly can be divided into four stages: ingestion, transformation, colonisation, and transmission (Molyneux and Killick Kendrick, 1987).

1.2.6.1.1. Ingestion stage

Following ingestion of a blood meal from an infected host amastigotes are released from the parasitised macrophages and enveloped in the peritrophic membrane, which is secreted by the midgut cells (Molyneux and Killick Kendrick, 1987).

1.2.6.1.2. Transformation

Following differentiation of the amastigotes, which were enclosed in the peritrophic membrane, into the promastigotes, an increasingly dense coat of a glycocalyx is formed on the surface of the promastigotes. The glycocalyx is composed of a variety of glycoconjugates that are bound to the surface of the promastigote by a glycophosphatidylinositol (GPI) anchor (Turco and Descoteaux, 1992; McConville and Ralton, 1997) and is thought to have barrier functions (Solbach and Laskay, 2000).

It has been reported that glycoconjugates play crucial roles in the survival, development, and virulence in both developmental stages of the parasite (Ilg *et al.*, 1998). The major cell surface glycoconjugate is lipophosphoglycan (LPG) which is found over the entire surface of the parasite, including the flagellum (Mosser and Brittingham, 1997). LPG serves as a ligand for the attachment of non-infectious procyclic promastigotes to the midgut wall lining and may protect the parasites against the hydrolytic environment of the insect's digestive tract (Ilg *et al.*, 1998). Following rupture of the peritrophic membrane, after about 3 days, free-swimming promastigotes are released into the midgut (Stierhof *et al.*, 1999).

1.2.6.1.3. Colonization

The free promastigotes establish a colony of parasites in the sand fly gut. They attach to a gel-like plug, which is formed mainly by parasite-derived mucin-like filamentous proteophosphoglycan (fPPG) (Stierhof *et al.*, 1999). Promastigotes differentiate into non-dividing metacyclic promastigotes, which have a structurally altered LPG (Sacks *et al.*, 1995).

This modification is necessary to allow the infective form of the promastigotes to be released from the midgut and move toward the foregut (Pimenta *et al.*, 1992).

Three other important alterations occur as promastigotes progress from the procyclic to the metacyclic form. Metacyclic promastigotes have been shown to increase in the expression of:

I) The major surface glycoprotein 63 (gp63) (Kweider *et al.*, 1989).

II) Surface-associated acid phosphate activity (Gottlieb and Dwyer, 1981).

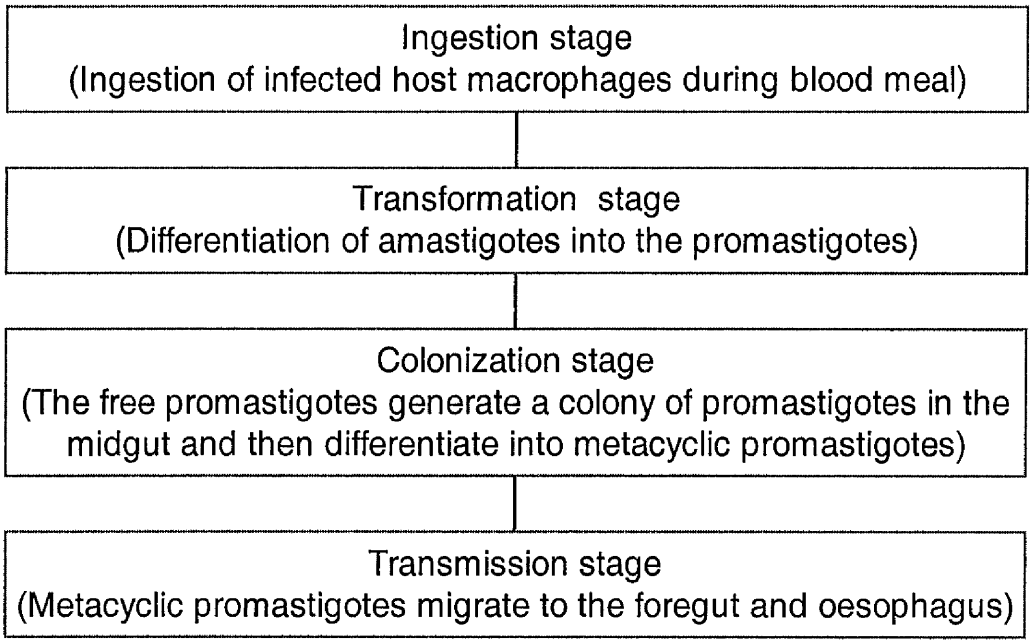
III) Cysteine proteinases (Robertson and Coombs, 1992).

It has been reported that the metacyclic promastigote has individual distinct biochemical properties that separate it from the non-infective promastigote (Mottram *et al.*, 1997). Metacyclic promastigotes are generally more elongated and thinner than procyclic forms (Mosser and Brittingham, 1997) showing increased motility in culture (Sacks *et al.*, 1985).

1.2.6.1.4. Transmission

Metacyclic promastigotes migrate to the foregut and oesophagus of the sandfly, are suspended in the sandfly's saliva and are ready to be inoculated during the blood meal. In the sandfly's gut, the saliva probably promotes survival and development of the promastigotes, since feeding of *Phlebotomus argentipes* with *L. donovani* suspended in serum containing anti-saliva antibodies led to the death of a significant number of promastigotes and inhibited the promastigotes development in the foregut (Ghosh and Mukhopadhyay, 1998).

The life cycle of *Leishmania* in the sandfly



1.2.6.2. In the mammalian host

1.2.6.2.1. Inoculation

Metacyclic promastigotes are inoculated into the skin of the vertebrate by an infected female sandfly's bite. It was shown that about 75% of *P. papatasi* sandflies infected with *L. major* are able to transfer disease with less than 100 promastigotes (Warburg and Schlein, 1986). In the presence of saliva, promastigotes establish a successful infection. It was found that injection of both saliva and promastigotes of *L. major* or *L. braziliensis* into various strains of mice resulted in exacerbation of both the size of the lesion and the number of recoverable parasites even when a low inoculation (10^2-10^4 promastigotes) was used for infection (Lima and Titus, 1996; Belkaid *et al.*, 1998). Under natural conditions, sandflies transmit very low numbers of promastigotes. Under experimental conditions, when promastigotes are usually

suspended in saline and inoculated by syringe into the skin of inbred mouse strains, the same low number of parasites will rarely cause disease, even in mouse strains, like BALB/c, which are extremely susceptible to *L. major* infection.

1.2.6.2.2. Binding of promastigotes to mononuclear phagocytes

Following transmission but before entry into their host cells, the surviving promastigotes are exposed to serum components, including factors of the complement system, for a relatively short time. The majority of non-metacyclic promastigotes are rapidly destroyed by complement factors (Mosser and Brittingham, 1997). The surviving promastigotes, which are relatively resistant to complement-mediated lysis, rapidly bind to resident or recruited cells of the monocyte / macrophage lineage including dendritic cells and Langerhans cells (Blank *et al.*, 1993; Moll *et al.*, 1995). Phagocytosis of promastigotes by macrophages requires the preliminary attachment of the promastigotes to a macrophage through either serum-dependent adhesions or serum-independent adhesions (Antoine *et al.*, 1998).

i) Serum-dependent adhesion

The two most abundant surface structures on metacyclic promastigotes, gp63 and LPG, have both been identified as C3 (Russell, 1987), C3b (Puentes *at al.*, 1988), and iC3b (Mosser *et al.*, 1985) acceptor sites. Other receptors may promote binding of promastigotes to the macrophage, including receptors CR1, CD35, the receptor for C3b and C4b, and CR3, CD11b / CD18 the receptor for iC3b (Sutterwala *et al.*, 1996). It has been reported that iC3b functions as an opsonin for *Leishmania* via binding to CR3, the predominant receptor for the uptake of metacyclic promastigotes (Rosenthal *et al.*, 1996).

ii) Serum-independent adhesion

Several receptors that may take part in the interaction between parasite and macrophage include the mannose-fucose receptor, the fibronectin receptor, and C-reactive protein receptor (Bogdan and Rollingoff, 1998). In addition, macrophage

CR3 is able to bind directly to promastigotes (Mosser *et al.*, 1985). These receptors link directly to the parasite through LPG and gp63 (Bogdan and Rollingoff, 1998).

1.2.6.2.3. Invasion

Following binding of metacyclic promastigotes to the surface of a macrophage, the parasite is surrounded by "coiling phagocytosis" (i.e., by wrapping with multiple layers of unilateral pseudopods of the phagocytic cells) (Rittig *et al.*, 1998b) or by conventional "zipperlike" interactions (Rittig *et al.*, 1998a).

Initially the parasite is located in a phagosomal compartment that is limited by a membrane originating from the host cell plasmalemma. This phagosome then undergoes remodelling via maturation and fusion with an endocytic organelle and forms a parasitophorous vacuole (PV) containing lysosomal hydrolases, and cathepsins (Russell and Talamas-Rohana, 1989; Lang *et al.*, 1994). PVs in the infected macrophages are formed 1 to 14 days after ingestion of promastigotes or amastigotes (Antoine *et al.*, 1998). Much less is known about early events before PV formation. However, PVs containing amastigotes are rapidly acidified and reach pH 5 in < 30 min (Sturgill-Koszycki *et al.*, 1994).

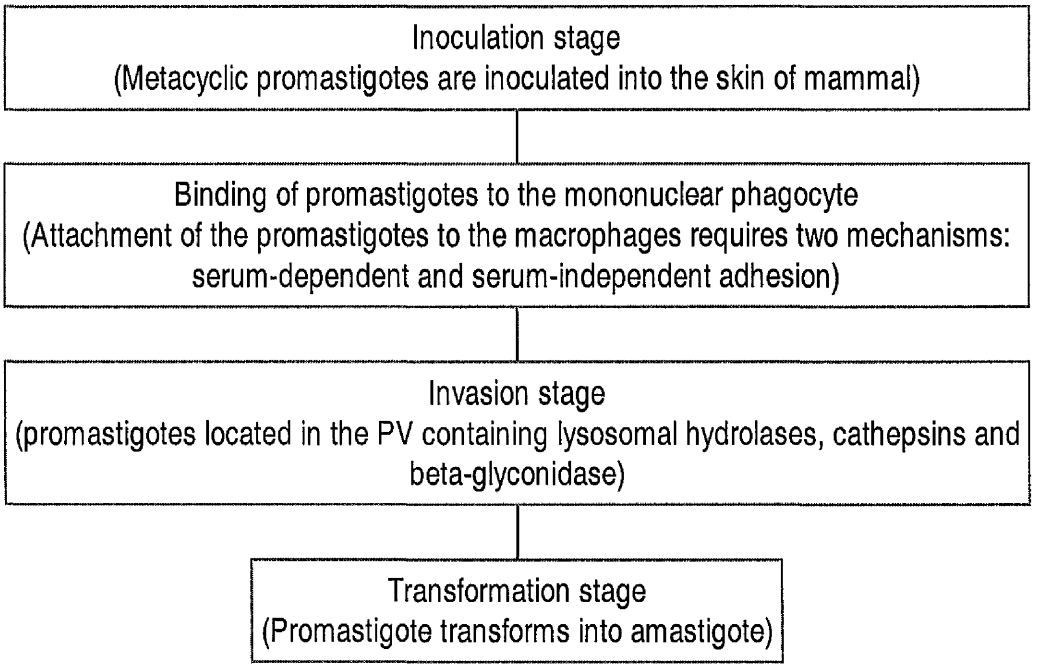
1.2.6.2.4. Transformation

Promastigotes require 2-5 days to transform into the amastigotes, depending on the *Leishmania* species (Antoine *et al.*, 1998). Although all *Leishmania* PV have many common features, they are not identical morphologically. For example, macrophages that contain *L. donovani* or *L. major* have many individual small PV with only one or two amastigotes inside each, whereas macrophages that are infected with *L. amazonensis* or *L. mexicana* harbour numerous parasites within one large vacuole (Antoine *et al.*, 1998). Ilg and colleagues (1995) suggested that amastigotes of *L. mexicana* WT secret PPG which is one of the factors involved in the formation of large PVs in the infected macrophage. The PVs that harbour amastigotes of *L. amazonensis* or *L. mexicana* are rapidly acidified and reach pH 5 in less than 30 min

(Sturgill-Koszycki *et al.*, 1994). Several groups have proposed that surface gp63 and LPG expression are down-regulated in the amastigote stage (McConville and Blackwell, 1991).

Following division of amastigotes within the PV, the latter ruptures and the cell releases the amastigotes, which are then taken up by neighbouring macrophages. Alternatively, it is possible that amastigotes can be released by fusion of the PV with the plasma membrane, thus leaving the host cell intact. Evidence for the latter mechanism is that infection with *L. donovani* inhibits apoptosis of macrophages (Moore and Matlashewski, 1994).

The life cycle of *Leishmania* in the mammalian host



1.3. The host immune response to leishmaniasis

The pathogenicity of *Leishmania* depends to a large extent on their degree of susceptibility or resistance to various innate and adaptive immune mechanisms of the mammalian host (Reiner and Locksley, 1995).

1.3.1. The hosts' innate response

When the infective promastigotes enter the host's body they initially encounter elements of the innate response which consists of a variety of molecules and cells, distributed throughout the body (Roitt *et al.*, 1996).

The innate immunity has several effects but the three most important are:

i) Killing the non-infective promastigote by the complement system.

ii) Initiation of inflammation

iii) Avoiding dissemination of amastigotes to the visceral organs of host, by the natural killer cells (NK cells).

1.3.1.1. Complement system

The complement system can be activated by all *Leishmania spp.* via the alternative pathway, a process that proceeds in the absence of antibody (Mosser and Brittingham, 1997). Fixation of C3 on the surface membrane of promastigotes is a crucial step in the interaction of the promastigote with the innate immune system (Mosser and Brittingham, 1997). As mentioned previously, the majority of non-infective promastigotes are rapidly destroyed before entry into host magrophages. Immediately following injection, promastigotes are exposed for a short while to potentially toxic serum components, including elements of the complement system (Mosser and Brittingham, 1997), even in the absence of anti-*Leishmania* antibodies. Promastigotes can shed C5b-C9 complexes from the parasite surface (Puentes *et al.*, 1990), which might be causally linked to the phosphoglycan chain of the surface LPG (Puentes *et al.*, 1988; Sacks *et al.*, 1995). A correlation between gp63 expression and resistance to complement-mediated lysis has been demonstrated (Brittingham *et al.*, 1999) and this

mechanism is dependent on the proteolytic activity of gp63 (Brittingham *et al.*, 1997). Gp63 is a surface acid proteinase and not only binds to considerable amounts of the terminal complement components but also rapidly converts C3b to iC3b, thus facilitating the uptake of the parasites by cells expressing the iC3b-receptor CR3 (CD 11b / CD18) (Brittingham *et al.*, 1995; Mosser and Brittingham, 1997). In addition, leishmanial protein kinases (LPKs) (Puentes *et al.*, 1990) and serine threonine protein kinase (Li *et al.*, 1996), which are increasingly expressed on the surface of infective stage promastigotes, have been shown to inactivate C3, C5, and C9 by phosphorylation, thus avoiding complement mediated lysis.

In addition to opsonization and lysis, activation of complement also leads to the generation of the chemotactic peptides C3a and C5a. These peptides have been shown not only to be potent inducers of leukocyte migration, but they also up-regulate the expression of complement receptors on mononuclear phagocytes (Yancey *et al.*, 1985). Macrophages were found not to move toward promastigotes themselves, but following the activation of complement by *Leishmania*, macrophages showed a directional migration toward the products of complement activation (Bray, 1983).

1.3.1.2. Initiation of inflammation

Inflammation is the body's reaction to the invasion by an infectious agent (Roitt, 1996) such as *Leishmania*. Immediately after the inoculation of promastigotes by a sandfly, a local inflammatory process is started, which involves local accumulation of cells to clear damaged tissue and to initiate wound healing, and this may limit dissemination of *Leishmania*. Moore and Matlashewski (1994) reported that macrophages are a major source of inflammatory and growth cytokines in the leishmaniasis. Initially, leukocytes, particularly neutrophils and eosinophils, followed by a wave of inflammatory macrophages, migrate out of the capillaries into the site surrounding the promastigotes inoculated by infective sandfly. Lymphocytes are hardy detected at this early stage of infection (Sunderkotter *et al.*, 1993) but there is an accumulation of NK cells (Solbach and Laskay, 2000). By the time invading macrophages appear, extracellular promastigotes are mostly dead while others have invaded resident cells which they may use as a "safe haven" (Greil *et al.*, 1988).

The migration of inflammatory cells towards the site of infection is orchestrated by the chemotactic peptides C3a and C5a. Frankenberg and colleagues (1990) reported that LPG down-regulated chemotaxis of macrophages and IL-1 production.

1.3.1.3. Avoiding dissemination of amastigotes into the visceral organs of host

The prevention of dissemination of amastigotes is not fully understood. After transformation of promastigote to amastigote within infected macrophage, amastigotes start to divide and continue until the PV ruptures. Irrespective of the way in which amastigotes infect neighbouring competent host cells, there is a striking difference between the kinetics of parasite dissemination into the visceral organs of susceptible and resistant mice. Interestingly, microscopical analysis of thoracic duct lymph obtained from BALB/c mice 3 hours after infection showed that most of the parasites disseminated as intracellular forms (largely amastigote-like), but occasionally extracellular promastigotes were also observed (Ionac *et al.*, 1997).

When BALB/c mice, which are susceptible to *L. major* were subcutaneously infected with *L. major* promastigotes, after 2-5 hours the parasites were found in the draining lymph node and within 10-24 hours could be detected in other organs such as the para-aortic lymph nodes, the spleen, the liver, the bone marrow, and occasionally the kidney (Laskay *et al.,* 1995). In other mouse strains which are resistant to *Leishmania*, like C57BL/6, CBA/J, and C3H/Hej, the parasites remained localised in the site where the parasite was injected and in the draining popliteal lymph node for 5 days or more without evidence of dissemination into other organs (Laskay *et al.,* 1995).

It was thought that local restriction of parasites in the pre-T cell phase of the infection is mediated by the innate immune system and this plays an important role in the development of a protective T cell response (Laskay *et al.*, 1995), and is dependent on several factors including the size of the infectious dose and species of *Leishmania.* Menon and Bretscher (1998) reported that low numbers (2×10^3) of *L. major* promastigotes were localized in the skin and draining lymph nodes of BALB/c mice, whereas high numbers (5×10^6) of the parasite disseminated into skin and visceral organs of this strain of mice and led to overt disease.

Laskay *et al.* (1993) showed that NK cells participated in the non-specific phase of anti-leishmania activity in the control of early multiplication of parasite in the course of leishmaniasis in resistant C57BL/6 mice. Indeed, depletion of NK cells by antibody led to rapid parasite dissemination. NK cells play a crucial role in infection processes since, unlike naive T cells, they respond very rapidly to stimuli and do not require priming (Scott and Trinchieri, 1995; Lanier, 1997). NK cells can produce a wide range of cytokines such as IFN-γ and tumour necrosis factor-α (TNF-α), which inhibit the growth of microorganisms during the initial stage of infection, thus allowing the host to develop an efficient adaptive immune response (Louis *et al.*, 1998). NK cells can also play a crucial role in shaping the adaptive immune response, particularly by influencing the pathway of differentiation of $CD4^+$ T cells. The effector function of NK cells and their role in the generation of a $CD4^+$ Th1 response during infection with *L. major* has shown that depletion of NK cells in resistant mice led to an increase in the number of parasites and enhanced the lesions (Sharton and Scott, 1993). Moreover, this treatment also decreased the IFN-γ production and increased the IL-4 derived response. It was found that administration of IL-12 with *Leishmania* antigens induces $CD4^+$ Th1 cell development and resistance (Afonso and Scott, 1994). Scott and Trinchieri (1995) reported that NK cells appear to function as a source of IFN-γ that was critical for $CD4^+$ Th1 cell development. It was also shown that NK cells rapidly produced IFN-γ after activation by both parasite antigens and IL-12, which in turn led to parasite containment (Laskay *et al.*, 1995). Scharton-Kersten and Scott (1995) reported that NK cells are not required for the development of $CD4^+$ Th1 cells following infection of C3H mice with *L. major*.

Associated with the healing process, the cured mice showed a strong, long-lasting cellular immunity to the parasites. In spite of the clinical healing, however, *L. major* persists in various organs, possibly life-long. Parasites could be detected by both PCR and culture in the draining LN, spleen, and bone marrow as long as 1 year after healing (Aebischer *et al.*, 1993).

In addition to the role of IL-12, NK cells, and IFN-γ in regulating parasite dissemination and containment, more recent data pointed to a critical role for IFN-α/β and inducible nitric oxide synthase (iNOS) (Diefenbach *et al.*, 1998). Mice genetically deficient for the iNOS gene were shown to control the activity of NK cells and the early cytokine response [IFN-γ, IL-12 and transforming growth factor (TGF)] during

the first 24 hours of infection with *L. major*, thereby playing a critical regulatory role in the innate response to this parasite (Diefenbach *et al.*, 1998). NOS2 gene knockout ($NOS2^{-/-}$) mice or C57BL/6 mice treated with anti-IFN-γ antibody or mice with a disrupted IFN-γ gene, permitted rapid spreading of the parasites and developed disease, even with a low inoculation of 500-2000 parasites (Diefenbach *et al.*, 1998; Laskay *et al.*, 1995). As the early production of IFN-γ in *L. major*-infected mice is predominantly due to NK cells (Laskay *et al.*, 1993; Reiner *et al.*, 1994b; Scharton and Scott, 1993), Diefenbach and colleagues (1998) suggested that the early containment is orchestrated by the co-ordinated action of IFN-α/β, IFN-γ, IL-12, NOS2, and NK cells, which are activated at the site of infection and the draining lymph node. *In vivo* depletion of NK cells using the NK cell-specific anti-NK1.1 monoclonal antibody further underlined the importance of NK cells in the early defence against *L. major*. The enhanced disease, as measured by parasite number and lesion development, was observed in NK cell-depleted mice (Laskay *et al.*, 1993).

In the *L. major* infection model a delicate balance between stimulatory and inhibitory lymphokines appears to regulate the early activation of NK cells. Rapid Th1 cell development and resistance to infection in mice that develop an early NK cell response after infection was reported (Scharton-Kersten and Scott, 1995). IL-12 is likely to play a key role in NK cell activation, since *in vivo* neutralisation of IL-12 eliminated the early NK cell response in *L. major* infected resistant C3H/HeN mice (Scharton-Kersten *et al.*, 1995). In susceptible BALB/c mice the simultaneous early production of IL-12 and cytokines that inhibit IL-12 function, such as TGF-β, IL-4 and IL-10, was observed. Even though NK cells play a major role in the early control of parasites, NK cells alone could not sustain control of *L. major* in the absence of CD4+ T cell-derived IFN-γ, and Th1 development was unaffected by the presence or absence of IFN-γ from non-T-cells (Wakil *et al.*, 1998). The role of NK cell-derived IFN-γ remains to be clarified. However, several groups of researchers reported that the contribution from NK cells in the early stage of infection is independent IFN-γ.

1.3.2. The host's adaptive immune response

1.3.2.1. Antigen presentation

Several microorganisms including *Leishmania* are able to replicate within resting macrophages of the mammalian host. Upon activation, infected cells mobilize potent microbicidal mechanisms that eliminate the intracellular pathogen. This transition from a resting to an activated state is mediated by the interaction with specific T cells that recognize pathogen-derived peptides complexed with major histocompatibility complex (MHC) molecules at the surface of host cells (Lang *et al.*, 1994). Antigen presentation by macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine productions following exposure to immunostimulating complexes are important for generation of a $CD4^+$ Th1 response (Villacres-Eriksson, 1995).

The establishment of a protective anti-*Leishmania* immune response requires the presentation of appropriate antigens by antigen presenting cells (APCs), the induction and expansion of $CD4^+$ Th1 and the activation of macrophages for efficient killing of the parasites. There are different kind of APCs in the body, the best studied are macrophages and dendritic cells (DCs) (Roitt *et al.*, 1996).

1.3.2.1.1. Macrophage

In leishmaniasis, for induction of the primary immune response, the macrophage is inefficient in the presentation of parasite to the naive T cell (Moll and Flohe, 1997). In addition, in contrast to other macrophages resident in the other organs, the quality of the respiratory burst of dermal macrophages following ingestion of promastigotes is poor (Locksley *et al.*, 1988). Three states of infected mouse macrophages *in vitro* have been reported:

1) Resting macrophages, which were deficient in the synthesis of MHC class II molecules and unable to present any parasite peptides (Overath and Aebischer, 1999).
2) Macrophages primed with IFN-γ, which were able to synthesise MHC molecules but the viability of the amastigotes was not affected (Antoine *et al.*, 1991; Wolfram *et al.*, 1995). Primed macrophages were able to present leishmanial antigens but were restricted to those proteins that were accessible to host cell

proteases, ie. proteins secreted into the parasitophorous vacuole or located at the parasite surface. Intracellular proteins of the parasites were not available to the antigen-processing machinery as long as the parasites remained intact (Overath and Aebischer, 1999).

3) Activated macrophages [which in the presence of IFN-γ and a second signal, TNF-α, produce nitric oxide (NO)] kill the parasite (Bogdan *et al.*, 1990; Liew *et al.*, 1990).

Interaction of primed macrophages from resistant mice, which express a highly selective group of peptides, with T cells leads to the production of IFN-γ and TNF-α. The infected macrophages will in turn also start to produce TNF-α, thus establishing an autocrine / paracrine TNF-α loop that drives the macrophage to full activation. It is important to point out that macrophages harbouring live parasites in lesion-derived tissue express a low level of MHC class II molecules (Overath and Aebischer, 1999). It has been reported that after *in vitro* infection with *L. major*, macrophages had a greatly reduced capacity to present both *L. major*-derived and unrelated antigens such as OVA or β-galactosidase (Fruth *et al.*, 1993). Therefore, *Leishmania* infection interferes with the intracellular loading of MHC-II molecules with antigenic peptides. Similar conclusions can be drawn from experiments with murine macrophages infected with *L. amazonensis* amastigotes (Prina *et al.*, 1993).

Several groups of researchers have suggested that amastigotes of several *Leishmania* species within PV lead to internalization and degradation of MHC class II molecules (De Souza *et al.*, 1995), suppression of MHC class II synthesis (Reiner *et al.*, 1987), down-regulation of CD80 expression (Kaye *et al.*, 1994), and /or partial inhibition of antigen processing / peptide loading in the host cell (Fruth *et al.*, 1993; Prina *et al.*, 1993). In contrast, macrophages infected with promastigotes present endogenous parasite molecules to $CD4^+$ T cells, although only for a limited time, with maximal presentation occurring within 24 h and decreasing to minimal antigen presentation at 72 h post infection (Kima *et al.*, 1996). Thereby, *Leishmania* interferes with antigen presentation, and thus might impair recognition of infected macrophages by T cells.

1.3.2.1.2. Dendritic cells

Dendritic cells (DCs) belong to a family of bone marrow derived APCs and have an exquisite capacity to interact with T cells and modulate their responses. DCs are found in most non-lymphoid organs, including the epidermis and mucosa, where they are called Langerhans cells (LCs) and are present in the T cell areas of all lymphoid organs, where they are called interdigitating DCs (Roitt *et al.*, 1996). The relationship between DC populations was partly unravelled by the observation that LCs from skin can migrate to secondary lymphoid organs, via the blood or lymph, bringing antigens to naive T cells from peripheral sites from which the latter are excluded (Moll *et al.*, 1995).

Following inoculation of metacyclic promastigotes by an infectious sandfly bite, the local inflammatory response leads to migration of LCs through the epidermal-dermal layer and to phagocytosis of the parasite in the dermis (Blank *et al.*, 1993). The parasite will be captured by the attachment of LPG on the surface of the parasite to the LCs' complement receptor (CR3). It has been reported that mannose receptors of DCs may be involved in the antigen capturing (Sallusto *et al.*, 1995) but this receptor appears not contribute to uptake of parasites by LCs (Moll and Flohe, 1997).

Infected LCs transport parasites from the skin to the draining LNs, which can be detected as early as 24-48 hours after infection (Moll *et al.*, 1993). They acquire a heightened ability to present antigenic peptides to T-cells (Xu *et al.*, 1995). This is accompanied by drastic changes in cell morphology, motility, and expression of high levels of MHC class II molecules and rapid up-regulation of the expression of co-stimulatory molecules (B7-1 and B7-2) (Inaba *et al.*, 1994; Larsen *et al.*, 1994), which results in a marked increase in the ability of DCs to activate T cells. This process is variously termed maturation or activation.

LCs play a crucial role in the induction of the immune response in early *L. major* infection because only infected LCs are able to carry parasites from the infected skin to the draining LN for primary antigen presentation to T cells (Moll *et al.*, 1993). After healing of the lesion, DCs of the lymph node have the capacity to present very low amounts of *Leishmania* antigen to specific T cells (Moll *et al.*, 1995). LCs take up small numbers of promastigotes which do not differentiate into amastigotes but be degraded whereas macrophages internalize large numbers of promastigotes into the PV where they differentiate into amastigotes (Konecny *et al.*, 1999). In contrast to macrophages, the *Leishmania* infected LC is unable to release NO, an important

molecule for killing the parasite and cannot be induced to express the cytokine-iNOS (Blank *et al.*, 1996).

LCs infected with promastigotes up-regulate production of IL-12 p40 in both resistant and susceptible mice whereas infected macrophages are unable to produce IL-12 (Konecny *et al.*, 1999). Thereby, LCs play a crucial role in the regulation of the immune response of mice infected with *L. major* in both early and later phase of infection and after cure of skin lesion in genetically resistant mice (Moll and Flohe, 1997; Flohe *et al.*, 1998). Dendritic cells produce IL-12 and direct the development of Th1 immune responses in resistant mice (Macatonia *et al.*, 1995; Konecny *et al.*, 1999).

1.3.2.2. Co-stimulatory molecules

The role of co-stimulatory signals in the nature and the amplitude of an antigen-specific T cell response is essential (Saha *et al.*, 1998). Analysis of macrophages from BALB/c mice infected with *L. donovani* revealed that the co-stimulatory molecule B7-1 (CD80) was decreased, but there was no change in C57BL/6 mice, which are resistant to *Leishmania* infection (Murphy *et al.*, 1997). After elimination of the parasites, however, CD80 was re-expressed, which paralleled the induction of a protective T cell-mediated immune response (Saha *et al.*, 1995).

CD86 (B70/B7-2) is an antigen of the immunoglobulin superfamily constitutively expressed on professional antigen-presenting cells such as monocytes, dendritic cells and activated B, T, and natural killer cells, while CD80 is inducible on those cells through CD40 ligation (Azuma *et al.*, 1993; Caux *et al.*, 1994; Larsen *et al.*, 1994). Thus, there is also a possibility that CD80 may not be sufficiently induced on APCs of the infected CD40-deficient mice, and may partly contribute to the induction of polarized $CD4^+$Th2 responses (Larsen *et al.*, 1994). CD86 was recently identified as a second ligand for the T cell antigens CD28 and Cytotoxic T lymphocyte antigen-4 (CTLA-4) (CD152), and plays an important role in the co-stimulation of T cells in a primary immune response (Fernandez-Ruiz *et al.*, 1995). CD86 provides signals for preferential induction of IL-4-secreting $CD4^+$Th2 cells and the differences between usage of CD80 or CD86 relevant for the development of $CD4^+$ Th1 or $CD4^+$ Th2 response, respectively (Freeman *et al.*, 1995; Kuchroo *et al.*, 1995). Prolonged

treatment of both C57BL/6 and BALB/c mice with antibody to CD86 but not to CD80, decreased parasite burden and decreased the production of $CD4^+$ Th2 cytokines (Brown *et al.*, 1996b). Anti-CD86 mAb did not interfere with the early T cell activation, since the treatment on day 3 post-infection was as effective as beginning it on the day of infection (Murphy *et al.*, 1997). Both CD80 and CD86 interact with CD28 and CD152 which are expressed on T cells. The binding affinity of CD152 to CD80 and CD86 molecules, however, is significantly higher than that of the CD28 molecule. In contrast to CD28, CD152 plays a role in the negative regulation of cell activation (Krummel and Allison, 1995; Walunas *et al.*, 1994).

Experiments using knockout mice indicated that co-stimulation through CD28 plays only a limited role in the development of $CD4^+$ Th1 or $CD4^+$Th2 response to *Leishmania*. CD28-deficient and Wild Type BALB/c mice litter mates were equally susceptible to *L. major* infection. Similarly CD28-deficient C57BL/6 mice retained their resistance to the infection (Saha *et al.*, 1995; Brown *et al.*, 1996a). Treatment of mice with anti-CD152 Fab-fragments ameliorated the disease in BALB/c animals but had no effect on the course of infection in C57BL/6 mice, suggesting that CD152 plays a significant role in the modulation of the immune response, mainly in susceptible mice. Since the same effect was also observed in CD28-deficient mice, it is likely that the effect of CD152 on the course of infection is not dependent on CD28-mediated pre-activation. The observed amelioration of the disease was found to be associated with an increase in the number of cells secreting IFN-γ as well as increased parasite-specific responses (Corry *et al.*, 1994; Saha *et al.*, 1998). A single dose of anti-CD152 mAb injected on day 1 of infection significantly decreased the parasite burden in infected BALB/c mice (Murphy *et al.*, 1998).

1.2.2.3. Cytokine determinants that shape the development of Th cells

T helper lymphocytes ($CD4^+$ Th cells) can be divided into two distinct subsets of effector cells based on their functional capabilities and the profile of cytokines they produce. The $CD4^+$ Th1 secretes cytokines usually associated with inflammation, such as IFN-γ and TNF-α and induces cell-mediated immune responses. The $CD4^+$ Th2 subset produces cytokines such as IL-4 and is associated with humoral-type immune

responses (Abbas *et al.*, 1996; Constant and Bottomly, 1997). It is possible that $CD4^+$ Th cells are involved in eliminating many pathogens, where a balance of both regulated cell-mediated immunity and an appropriate humoral response will eradicate an invading pathogen with minimum immunopathology (Sher and Coffman, 1992; Romagnani, 1994; Kelso, 1995). Most naive T cells have potential to mature into either $CD4^+$ Th1 or $CD4^+$ Th2 subset and that critical role of cytokines to which the T cells are exposed at the time of priming mediate this differentiation (Mosmann and Coffman, 1989). Seder and colleagues (1992) reported that IL-4 has an important role for mediating Th2 development, while IL-12 (Trichieri, 1995; Romani *et al.*, 1997) and IFN-γ were described to have a critical role for Th1 development and that both sets of cytokines are reciprocally active (Tanaka *et al.*, 1993; Hsieh *et al.*, 1995). With respect to the role of IL-4 in mediating both susceptibility and Th2 cell differentiation, it was shown that, in contrast to resistant C57BL/6 animals, BALB/c mice exhibited a burst of IL-4 mRNA in $CD4^+$ T cells in the draining lymph node as early as 16 hours after subcutaneous infection with *L. major* promastigotes (Launois *et al.*,1995). The precursor $CD4^+$ Th cell differentiates to a $CD4^+$ Th2 cell in the presence of IL-4 at the initiation of an immune response (Swain *et al.*, 1990; Seder and Paul, 1994). The effects of IL-4 in inducing $CD4^+$ Th2 cell development are dominant over $CD4^+$ Th1 polarizing cytokines (Hsieh *et al.*, 1993; Seder and Paul, 1994). Therefore, in the presence of IL-4 at the beginning of an immune response, Th2 cells differentiate, progressively leading to increasing level of IL-4.

Several candidates that may be responsible for IL-4 production early in $CD4^+$ Th2 differentiation include:

i) Major histocompatibility complex (MHC) class II-restricted CD4+ T cells (Constant *et al.*, 1995; Hosken *et al.*, 1995).

ii) NK1 T cells that have the unique potential to very rapidly secrete large amounts of cytokines, providing early help for effector cells and regulating the Th1 or Th2 differentiation of some immune responses (Bendelac *et al.*, 1997).

iii) *Leishmania* homologue of receptors for activated C kinase (LACK) specific CD4+ T cells expressing Vß4, Vß8 TCR (Julia *et al.*, 1996).

iv) Non-T cell sources, such as mast cells, basophils, and eosinophils (Paul *et al.*, 1993).

Several groups of researchers reported that the requirement for IL-4 in mediating both Th2 differentiation and susceptibility of mice to *L. major* was directed toward the early IL-4 produced *in vivo* (Sadick *et al.*, 1990; Locksley and Scott, 1991; Scott *et al.*, 1996).

It was suggested a critical role is played by NK1+ T cells in Th2 differentiation as a result of their ability to produce large amounts of IL-4 rapidly upon activation both *in vitro* and *in vivo* with anti-CD1 antibody (Bendelac *et al.*, 1997). However, Smiley and colleagues (1997) reported that IL-4-secreting NK-like T cells are not required for Th2 responses. Thus, the precise role of NK1+ T cells in the development of Th2 responses remains unclear.

The role of LACK-specific T cells in the development of a dominant Th2 response to *L. major* infection in BALB/c mice was demonstrated when mice were rendered unresponsive to LACK (Julia *et al.*, 1996). Due to expression of LACK as a transgene, BALB/c mice were made tolerant to this antigen and developed a reduced Th2 response and developed a protective Th1 response when infected with *L. major* (Julia *et al.*, 1996).

Julia and Glaichenhaus, (1999) reported that in both the genetically susceptible and resistant mice to *L. major*, T cells, which react to the LACK antigen, produce IL-4 rapidly and the production in resistant mice did not confer susceptibility but resulted in increased parasite burdens. Malherbe and colleagues (2000) reported that the T cells activated by LACK antigen from susceptible and resistant mice expressed low- and high-affinity TCR, respectively. Therefore, it has been suggested that differences in TCR usage between MHC in susceptible and resistant mice may influence the development of the antiparasite immune response (Malherbe *et al.*, 2000).

Non-T cells may also play a role in the initiation of Th2 responses against some pathogens in specific tissues, since mast cells, basophils, and eosinophils can produce cytokines such as IL-4 (Paul *et al.*, 1993).

1.3.2.4. Development of $CD4^+$ T helper cell subsets

The essential event that leads to Th1 differentiation in mice infected with *Leishmania* spp. is the production of IL-12 within 24 hours of infection and this constitutes the most effective form of innate immunity (Coffman and von der Weid, 1997). The

specific immune response of naive T cells to the parasite is initiated by IFN-γ and IL-12 production. Thus, the effector functions of the innate response that are most important for the control of the pathogens are conserved in the subsequent antigen specific T cell response.

Although, the source of production of the initial IL-4, which is essential for $CD4^+$ Th2 differentiation, is not so clearly defined, T cells themselves can be the source of IL-4 that leads to their own Th2 development, and IL-6 is a potent inducer of this IL-4 (Rincon *et al.*, 1997). Studies with LACK showed that *L. major* infected BALB/c mice make a strong early (6 days) Th2 response to LACK, although the response of the same mice to several other *L. major* antigens was predominately Th1-like (Mougneau *et al.*, 1995; Julia *et al.*, 1996). Analysis of mRNA transcription for various cytokines showed draining lymph node cells from BALB/c mice infected with *L. major* contained elevated transcripts for IL-4, but not for IFN-γ. In contrast, resistant C57BL/6 mice expressed transcripts for IFN-γ, but not transiently for IL-4 (Locksley *et al.*, 1987). In BALB/c mice, the expression of IL-4 mRNA remained elevated over time, whereas in C57BL/6 mice the IL-4 response returned to background levels after the initial phase of the infection (Henizel *et al.*, 1989). These and other studies (Reiner and Locksley, 1995) emphasised the ability of $CD4^+$ T cells to shape the immune response and the phenotype of the murine disease.

The ability of cytokines to stimulate different effector mechanisms and thus differential immune responses, is also reinforced by the production of cytokines by each subset, which cross-regulate each other's function as well as development. For example, IFN-γ production by Th1 cells inhibits the development of Th2 cells (Fitch *et al.*, 1993) as well as humoral responses, whereas the production of IL-4 and IL-10 by Th2 cells inhibits Th1 development and activation, as well as macrophage activation and bacteriacidal activity (Sher and Coffman, 1992; Moore *et al.*, 1993).

In resistant mice *Leishmania* stimulaties active APCs and subsequently NK cells of the innate immune response to produce IL-12 and IFN-γ (Hsieh *et al.*, 1993; Trinchieri, 1995), which then drive the development of $CD4^+$ Th1 cells from naive antigen-specific $CD4^+$ Th cells. This type of innate immune response is appropriate for the eradication of microbial pathogens (Sher and Coffman, 1992; Trinchieri, 1995).

Several groups of researchers demonstrated that ligation of CD40 by the CD40 ligand (*Cella et al.*, 1996; Koch *et al.*, 1996) and / or MHC class II (Koch *et al.*, 1996) on dendritic cells can induce the production of high levels of IL-12. Both IL-4 and IL-10 have the ability to inhibit both dendritic cell (Macatonia *et al.*, 1995; Koch *et al.*, 1996) and macrophage (D'Andrea *et al.*, 1993; Hsieh *et al.*, 1993; Murphy *et al.*, 1994) IL-12 production and thus inhibit the development of Th1 cells. IL-12 directs Th1 development from antigen-stimulated naive CD4+ T cells (Hsieh *et al.*, 1993; Manetti *et al.*, 1993; Trinchieri, 1995).

1.3.2.4.1. Cytokine-induced $CD4^+$ Th1 development

Recovery from cutaneous lesions that was seen in mice following local inoculation with *L. mexicana* (Stamm *et al.*, 1998) or *L. major* (Heinzel *et al.*, 1991; Scott *et al.*, 1988) in genetically resistant mice such as C3H/HeN and C57BL/6 strains was associated with the expansion of the $CD4^+$ Th1 subset and production of cytokines such as IL-12, IFN-γ, and IL-2. On the other hand, genetically resistant mice lacking IL-12 (Mattner *et al.*, 1996; Mattner *et al.*, 1997) or IFN-γ (Wang *et al.*, 1994) defaulted to a Th2-like response and were highly susceptible to cutaneous *L. major* infection. Interestingly, IFN-R-deficient 129/Sv/Ev mice were also susceptible to *L. major*, but did not develop a $CD4^+$Th2-like response (Swihart *et al.*, 1995). The susceptible mice lacking IFN-R defaulted towards a $CD4^+$Th1-like response implying that IFN-γ, although important in resistance to *L. major*, was not necessary for a $CD4^+$Th1-like response and that IL-12, instead, may be the critical cytokine responsible for $CD4^+$ Th1 cell development. Previous studies have clearly demonstrated that protective immunity against the *L. mexicana* complex, which includes *L. mexicana* and *L. amazonensis*, is ultimately dependent upon generation of a $CD4^+$ Th1-like response and IFN-γ production (Satoskar *et al.*, 1995; Afonso and Scott, 1993; Guevara-Mendoza *et al.*, 1997).

Non-infective promastigotes taken from the logarithmic phase of the promastigote cultures were good inducers of IL-12, IFN-γ, TNF-α, and IL-10 production, whereas infective parasites from the metacyclic phase were poor inducers of IL-12 (Sartori *et al.*, 1997). Both stages of parasites were inhibitory for IL-12 production induced by

Staphylococcus aureus (Sartori *et al*, 1997) or IFN-γ / LPS (Carrera *et al.*, 1996). IL-12 secretion was suppressed by infected macrophages with amastigotes of *L. mexicana*; the parasites also inhibited IL-12 secretion inducible by phagocytosis of latex beads, CD40 cross-linking or cognate interaction with Th1 cells (Weinheber *et al.*, 1998). However, treatment with recombinant IFN-γ failed to promote Th1 cell expansion and cure *L. major* infection in susceptible BALB/c mice (Sadick *et al.*, 1990).

IL-12 plays a critical role for the development of Th1-like $CD4^+$T cell responses following *L. major* infection in resistant mice (Mattner *et al.*, 1996) and treatment of susceptible BALB/c mice with rIL-12 cures cutaneous *L. major* infection (Heinzel *et al.*, 1993). Furthermore anti-IFN-γ Ab had no effect on IL-12-induced $CD4^+$ Th1 cell differentiation *in vitro* (McKnight *et al.*, 1994), whereas addition of rIL-12 during specific priming of $CD4^+$ Th cells from transgenic mice expressing an Ag-specific TCR-resulted in the development of the $CD4^+$ Th1-like phenotype (Seder *et al.*, 1993). Previous studies using the *L. major* model have indicated that genetic susceptibility of BALB/c mice to *L. major* is due to a loss of the ability to generate an IL-12-induced $CD4^+$ Th1-like response (Launois *et al.*, 1997).

Nonetheless, IL-4-deficient C57BL/6 × 129/Sv mice develop a $CD4^+$ Th1-like response, as measured by an increase in IFN-γ production, and cured *L. mexicana* infection (Satoskar *et al.*, 1995). The role of IFN-γ in the development of a $CD4^+$Th1-like response and resistance to *L. major* are based upon observations that impaired $CD4^+$ Th1-like responses followed treatment of genetically resistant C3H/HeN mice with anti-IFN-γ antibody (Belosevic *et al.*, 1989). In addition to its ability to down-regulate IL-12 and IFN-γ production, IL-4 has been shown to inhibit also the production of the inflammatory cytokines IL-1 and TNF-α from macrophages (Hart *et al.*, 1989). TNF-α plays a protective role in immunity against *L. major* infection (Liew and O'Donnell, 1993). For example, lymph node cells from mice resistant to *L. major* produce high level of TNF-α when stimulated in vitro, whereas cells from susceptible strains under the same conditions induce macrophages in the presence of IFN-γ to increase nitric oxide production (Liew *et al.*, 1990). Nashleanas and colleagues (1998) showed that mice deficient in both TNF-α receptors, p55 and p75, were able to control *L. major* infection, but failed to resolve lesions. Although a low level of TNF-α led to parasite killing (Titus *et al.*, 1989), the role of the p75 TNF-α

receptor was not found to be essential in *L. major* infection. Therefore, the p55 receptor may be required for optimal macrophage activation (Nashleanas *et al.*, 1998). In addition, IL-4-deficient mice infected with *L. major* displayed similar levels of TNF-α transcripts to wild-type mice (Kopf *et al.*, 1996; Noben-Trauth *et al.*, 1996). Although IL-2 production has often been associated with a $CD4^+$ Th1 phenotype, the production of this cytokine cannot be classified as a hallmark of $CD4^+$ Th1 cells, since naive $CD4^+$ T cells as well as Th0 cells (described below) also produce IL-2 in response to antigenic stimulation (Sher and Coffman, 1992; Romagnani, 1994; Abbas *et al.*, 1996).

1.3.2.4.2. Cytokine-induced $CD4^+$ Th2 development

Non-healing responses in susceptible BALB/c mice have been related to the expansion of the $CD4^+$ Th2 cell subset and the production of cytokines such as IL-4 and IL-10 (Heinzel *et al.*, 1991; Scott *et al.*, 1988). The disease-exacerbating role of IL-4 has been shown to be due to its ability to inhibit macrophage leishmanicidal activity and down-regulate the development of a $CD4^+$Th1-like response (Sher and Coffman,1992; Oswald *et al.*, 1992). It was found that genetically susceptible mice lacking IL-4 are protected from cutaneous infection with *L. major* (Kopf *et al.*, 1996) as well as *L. mexicana* (Satoskar *et al.*, 1995; Satoskar *et al.*, 1997). However, other studies suggest that the inability of the host to generate an IL-12-initiated $CD4^+$ Th1-like response and produce IFN-γ rather than the induction of a Th2-like response and IL-4 production may be the crucial factor in determining susceptibility to *L. major* (Güler *et al.*, 1996), *L. amazonensis* (Afonso and Scott, 1993), and *L. mexicana* (Guevara-Mendoza *et al.*, 1997).

The proposed mechanisms underlying the development of non-healing lesions in genetically susceptible mice following *L. major* infection have included the presence of an IL-4-driven Th2-like response suppressing Th1 cell development (Chatelain *et al.*, 1992; Leal *et al.*, 1993) and a failure to produce IL-12 (Carrera *et al.*, 1996; Reiner *et al.*, 1994) and thereby mount an IL-12-induced $CD4^+$ Th1-like response (Güler *et al.*, 1996). Tanaka and colleagues (1993) reported that the production of IL-2 and IFN-γ by CD4+ T cells and IFN-γ production by CD8+ T cells were strikingly

inhibited by culture in the presence of IL-4. Studies in IL-4-deficient BALB/c mice have shown that the lymph node cells produced little or no IFN-γ and low levels of IL-4 following *L. amazonensis* (Afonso and Scott, 1993) and *L. mexicana* infection (Guevara-Mendoza *et al.*, 1997; Satoskar *et al.*, 1995). IL-10 is important for inhibition of monocyte-macrophage activation, and inhibits production of TNF- α, IL-1 and also IFN-γ from lymphocytes acting at the level of accessory cells (D'Andrea *et al.*, 1993). IgG1 production has also been shown to be regulated by, although not completely dependent upon, IL-4 and IL-4 signalling (Snapper and Paul, 1987). It has been reported that IL-4 signalling is also important in antibody class switching to IgG1 following *L. mexicana* infection, whereas the IgE and IgG1 isotypes are associated with the development of a Th2-like response, switching to the IgG2a isotype has been shown to be increased during Th1-like responses (Snapper and Paul, 1987).

L. major infection together with salivary gland lysates from *P. papatasi* showed an up-regulation of the Th2-like response and down-regulation of the Th1-like response in BALB/c mice. Interestingly, the saliva contents are able to inhibit oxidative metabolic processes and antigen presentation by macrophages *in vitro* (Theodos *et al.*, 1991).

1.3.2.5. Intracellular killing mechanisms:

It has been reported that less than 30 minutes after phagocytosis of promastigotes of *L. mexicana* or *L. donovani*, the PVs are acidified and reached pH 5 (Sturgill-Koszycki *et al.*, 1994). In addition, the PVs acquire the lysosomal glycoproteins enzymes, macrosialin, and lysosomal-associated membrane proteins LAMP I and LAMP II within 2 hours post infection (Solbach and Laskay, 2000). Within 5-24 hours post infection, acidic hydrolases and MHC class II molecules are acquired (Lang *et al.*, 1994).

Leishmania spp. are exposed to two major effector mechanisms within PV of macrophages, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Murray and Nathan, 1999). Both neutrophils and macrophages produce superoxide (O_2) using the NADPH oxidase pathway. Although superoxide

production is the major anti-leishmanial effector mechanism of neutrophils, this pathway may not be necessary for the killing of the parasite within infected macrophages (Assreuy *et al.*, 1994). *Leishmania* have developed mechanisms to avoid killing by O_2 since the capacity of infected macrophages to produce oxygen radicals was significantly decreased after stimulation with phorbol esters (Passwell *et al.*, 1994). It is possible that LPG on the membrane of *Leishmania* mediates this activity by inhibiting the activation of protein kinase C. NO is formed through the oxidation of the terminal guanidino nitrogen atom(s) of L-arginine by an NADPH-dependent enzyme (Assreuy *et al.*, 1994).

In a study of mice with the inducible nitric oxide synthase (iNOS) gene knockout, it was found that iNOS was required for the Th1-dependent healing of infections with intracellular microorganizms including *Leishmania*. At day 1 of infection, genetic deletion or functional inactivation of iNOS abolished the IFN-γ and NK response, increased the expression of TGF-ß, and facilitated the spread of the parasite from the skin and lymph node to the spleen, liver, bone marrow, and lung. Induction of type 2 nitric oxide synthase (NOS2), also known as iNOS was dependent on IFN-α/ß (Diefenbach *et al.*, 1998).

The role of TNF-α and its receptors for control of *L. major* infection is less clear. Undoubtedly, TNF-α participates in the induction of macrophage activation leading to parasite elimination (Nacy *et al.*, 1991). IFN-γ activation of macrophages from TNFR $p75^{-/-}$ animals resulted in NO production and parasite killing (Vieira *et al.*, 1996; Nashleanas *et al.*, 1998). These results show that the TNFR p75 plays no essential role in murine *L. major* infection and that a mechanism exists by which macrophages can be primed *in vivo* to produce NO and kill *L. major* in the absence of signalling through either of the TNF-α receptors.

The killing of *L. major* by IFN-γ-treated murine macrophages is attributable to NO and the enzyme producing it (Ding *et al.*, 1988; Green *et al.*, 1990). The molecular mechanism of the action of NO on *Leishmania* is unknown so far, early data suggested that NO is directly cytotoxic to *L. major* (Liew *et al.*, 1990). In mice, resistance to *Leishmania* was clearly associated with the expression of iNOS and required the continuous presence of iNOS activity (Stenger *et al.*, 1994). The NO-pathway appears to be a common mechanism of *Leishmania* killing since not only murine but also human monocytes / macrophages were able to control *L. major* in a

in a NO-dependent manner (Vouldoukis *et al.*, 1995). Murray and Nathan (1999) suggested that ROI and RNI probably act together especially in the early stage of leishmaniasis.

LPG and glycoinositolphospholipids (GIPLs) (glycolipids related to LPG) from *L. major* as well as intact *L. major* promastigotes strongly suppressed iNOS-activity when the interaction between the macrophages and *Leishmania* preceded the stimulation of macrophages by IFN-γ (Proudfoot *et al.*, 1996). When, however, *Leishmania* and IFN-γ were added simultaneously to macrophages, GIPLs and LPS synergized with IFN-γ resulting in an increased production of NO (Proudfoot *et al.*, 1995). Therefore, *Leishmania* inhibits iNOS activity in the early stages of infection (i.e., when the parasites enter macrophages before the production of T-cell-derived macrophage-activating cytokines, such as IFN-γ). In later stages of infection, however, *Leishmania* cause an increased iNOS production. In accordance with this presumption, high iNOS RNA levels were found in chronic, nonhealing lesions of mice infected with *L. major* (Nabors *et al.*, 1995). Therefore, local production of NO is a crucial mechanism for the elimination and control of parasites, but only if it occurs before the parasite burden becomes too high. From then on, elevated production of NO aggravates the inflammatory process (Giorgio *et al.*, 1998).

1.3.2.6. Mechanisms of survival of *Leishmania*

1.3.2.6.1. Contribution of surface membrane molecules to survival of the parasite within the PV

There are a number of surface membrane molecules that protect *Leishmania* spp. from the host immune response and increase their activity. The two most important molecules are LPG and gp63 (Alexander and Russell, 1992).

LPG is an abundant molecule, which is expressed much more on the surface of the promastigote than the amastigotes (Bogdan, 1997). LPG inhibits the phagosome-endosome fusion in the macrophage after invasion of the promastigotes (Desjardins and Descoteaux, 1997) and reduces endothelial adhesion and transendothelial migration of monocytes (Ho *et al.*, 1996; Lo *et al.*, 1998). This temporary inhibition

of fusion of phagosome-endosome allows time for the promastigotes of *L. major* to begin to differentiate into the amastigote stage (Bogdan, 1997) which is better adapted to the enzymes and the acidic pH of the PV (Antoine *et al.*, 1990).

The LPG plays an essential role in the attachment of promastigotes to host cells (Handman and Goding, 1985), and protection from digestion within the phagolysosome (Eilam *et al.*, 1985; Handman *et al.*, 1986). Studies with LPG showed that this molecule binds to the complement fragments and protects the parasite from complement-mediated lysis (Beverley and Turco, 1998). LPG aids the parasite entry into the host macrophage and protects the parasite from attack by reactive oxygen intermediates (ROIs) (Turco and Descoteaux, 1992; Beverley and Turco, 1998). Although a crucial role of LPG for the promastigote stages of *Leishmania* has been reported, its importance for the disease-causing amastigote stage in the mammalian host is less clear. Amastigotes of *L. donovani* and *L. mexicana* do not express LPG (McConville and Ralton, 1997).

Interestingly, although several groups of researchers emphasised that LPG is commonly regarded as a multifunctional *Leishmania* virulence factor required for survival and development of this parasite in macrophage. Ilg (2000) demonstrated that LPG, at least in *L. mexicana*, is not a virulence factor in the mammalian host.

Another important surface molecule that has been found abundant on the surface of promastigotes is gp63. Several groups of researchers has been reported that gp63 protects parasites within the PV of macrophages at acidic pH (Alexander and Russell, 1992; Ilg *et al.*, 1993; Seay *et al.*, 1996), and is required for *in vivo* virulence (McMaster *et al.*, 1997). Bogdan and Rollinghoff (1998) showed that gp63 has protease activity and inhibits the activity of lysosomal enzymes. As mentioned before, gp63 has the ability to bind to complement receptors on the surface of macrophages (in the absence or presence of complement fragments) and aids entry of the parasite into the host macrophages (Alexander and Russell, 1992; Bogdan and Rollinghoff, 1998). In amastigotes, gp63 was found within the flagellas pocket, whereas the expression of gp63 on the surface membrane was very weak (Bogdan and Rollinghoff, 1998). The proteolytic activity of gp63 is in the highest at acidic pH, and protects the parasite from intra-phagolysosomal degradation (Ilg *et al.*, 1993; Seay *et al.*, 1996).

Other outer membrane molecules have been identified but poorly described. These molecules may contribute to survival of parasites within PV and include GIPLs, non-

inositol containing glycosphingolipids (GSLs), and protein kinase C (PKC) (Bogdan and Rollinghoff, 1998).

1.3.2.7. Suppression of the synthesis of anti-leishmanial molecules:

The two main antimicrobial effector mechanisms active against *Leishmania* include: ROI and RNI.

ROIs are highly toxic molecules and are thought to constitute an essential part of the defence mechanisms used by phagocytes to destroy invading parasites (Hughes, 1988). Ingestion of parasites by phagocytes usually activates the respiratory burst, which is characterized by increased oxygen uptake. NADPH oxidase in the phagosome membrane adds electrons to oxygen molecules. Thus, oxygen molecules are changed into reactive toxic molecules including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), and singlet oxygen (O_2) (Robinson and Badway, 1994; Rosen *et al.*, 1995). Murray (1982) demonstrated that the active generation of toxic oxygen intermediates including O_2^- and H_2O_2 play a crucial role in the eradication of the majority of ingested promastigotes.

LPG may protect the invading promastigotes against macrophage phagolysosome hydrolases (Turco and Descoteaux, 1992). The LPG down regulates protein kinase C (PKC) activity by inhibition of the translocation of the enzyme from the cytosol to the inner layer of the plasma membrane, which is a key requirement for the oxidative burst (Desjardins and Descoteaux, 1997).

Although ROIs are involved in intracellular killing, NO plays a crucial role in eradication of *Leishmania* (Liew *et al.*, 1990). NO is formed by at least three different isoforms of NOS, which convert L-arginine and molecular oxygen to L-citrulline and NO or NO metabolites (NO_2 or NO_3). LPG has a strong effect on the survival of amastigote of *Leishmania* by down-regulating the expression of iNOS in macrophages (Proudfoot *et al.*, 1996).

Proudfoot and colleagues (1995) reported that GIPLs of *L. major* inhibit the induction of iNOS in macrophages in the presence of IFN-γ and reduce leishmanicidal activity in murine macrophages. However, LPG and gp63 could be important for promastigote survival in the sandfly, there were no apparent correlation between LPG,

at least in *L. mexicana* (Ilg, 2000), and gp63 (Camara *et al.*, 1995) expression and promastigote survival in the macrophage. Therefore, in spite of the fact that LPG, gp63 and GIPLs are the most abundant molecules expressed on the surface of *Leishmania*, they are probably not the only modulators of the host defence machinery (Bogdam and Rollinghoff, 1998) which could influence the survival of the parasite.

1.4. Vaccines

Vaccination is the induction or modulation of an antigen-specific immune response to prevent or cure the target disease. The term "antigen-specific" is essential for distinguishing vaccination from non-specific immune modulation. Most attempts to develop a vaccine against a parasite, either on pragmatic grounds or by identifying protective antigens have been unsuccessful, because parasites induce a complicated immunological processes (Cox, 1997). However, as individuals recovered from clinical leishmaniasis develop strong immunity against reinfection, it suggested that vaccination against leishmaniasis is feasible in principle (Liew and O'Donnell, 1993).

1.4.1. Summary of the history of vaccination

Vaccination was born more than 200 years ago at a time when immunology was unknown and the concept of disease caused by transmission of microbes was not generally accepted. On June 21, 1798, Edward Jenner reported successful vaccination against smallpox using cowpox material (Hilleman, 1999). Before Jenner, the (ancient) Chinese inoculated pus from smallpox patinas in order to prevent severe natural smallpox. This knowledge was introduced into Europe in the early eighteenth century and during the nineteenth century, cowpox vaccination became a worldwide practice. Louis Pasteur, who set about attenuating microbes in the laboratory, further pursued this strategy of using attenuated viable microbes as vaccines. He successfully attenuated anthrax bacilli by means of *in vitro* passages. It was Pasteur who, in honour

of Jenner, generalized the term "vaccination" from the use of cowpox (vaccinia) to include similar strategies for controlling other infectious diseases (Hilleman, 1999). Pasteur's emphasis was on the microbial agents, with less attention paid to the host's immune response.

Louis Pasteur and Emil Von Behring clearly considered the therapy of infection an important aspect of vaccination. The rabies vaccine of Louis Pasteur, as well as the tetanus and diphtheria vaccines of Behring, were aimed at preventing the disease in individuals who were already infected. However, in later times the concept of therapeutic vaccination became less attractive, and vaccination was considered to be primarily preventive, i.e., vaccination was used to induce a primary immune response in naive individuals.

1.4.2. Immunology of vaccination

As mentioned before, protective immunity in leishmaniasis depends on a cell-mediated immune response and not on antibodies. Moreover, whether resolution or progression of the disease ensues in humans and animal models depend on which subtypes of T lymphocytes are stimulated. $CD4^+$ Th1 and $CD8^+$ T cells, through their secretion of IL-2 and IFN-γ, lead the response towards cell-mediated immunity involving macrophage activation, whereas $CD4^+$ Th2 cells, through their products, IL-4 and IL-10, lead to antibody production. The two poles are counter-regulatory in that IFN-γ inhibits antibody formation and IL-4 and IL-10 inhibit macrophage activation (Cox, 1997).

Therefore, it is reasonable to accept that vaccination against *Leishmania* should be through the activation of $CD4^+$ Th1 as effectors of vaccine-induced protection. It was previously described that T cells do not act directly and recognise antigen only after processing. This means that the parasite has already infected host cells, typically APCs, which serve as their major habitat. Consequently, whether certain antigens functions as preferred targets for T lymphocytes remains to be established. In the following, we will consider T cells as major mediator of vaccinations against *Leishmania* and follow the concept that full protection is best achieved by a

combination of different T cell populations that interact in a tightly controlled network. Moreover, it is important to consider the different T cell combinations to be activated by pre-infection vaccine.

1.4.3. Progress toward *Leishmania* vaccines

The current studies and the future prospects for a *Leishmania* vaccine are focused on the five types of vaccine including: live promastigotes Wild Type, attenuated promastigotes, Killed promastigotes, subunit vaccines based on *Leishmania* specific molecules, and DNA vaccines. Each of these types of vaccine has some advantages and disadvantages.

1.4.3.1. Live virulent promastigotes

Vaccination with live *Leishmania* parasites to produce self-healing lesions at an inconspicuous site has been practised for a long time in the Middle East. This method induces resistance in at least 70% of the individuals treated, but serious clinical complications associated with the live vaccine emphasise the need for an attenuated or defined vaccine against cutaneous leishmaniasis (Liew *et al.*, 1993). The isolation of virulent *Leishmania* that protectively vaccinated mice against infection showed that an attenuated vaccine was possible (Handman *et al.*, 1990), although, increased knowledge of the real hazards of the live organisms, such as persistence in the immune host, led to the cessation of using of the live virulent parasite (Modabber, 1990). The advantage of the viable vaccine is that parasites can exist intracellularly in the macrophages and evade the consequences of host's immune attacks. The result is that the parasite can survive in the mammalian host for a long time and causes a chronic disease, with unsuccessful immunological response (Liew, 1989). There are some disadvantages that made this approach unacceptable (Handman *et. al.*, 1990).

1.4.3.2. Live attenuated promastigotes

The generation of attenuated viable vaccine strains of *Leishmania* using genetic manipulation has made it possible to produce stable avirulent mutants that still induce an immune response but which have lost the capacity to cause disease in an immuno-competent host.

The successful production of an attenuated line of *L. mexicana* mutants lacking cysteine proteinase (CP) genes *cpa* and *cpb* (Δ*cpa/cpb*) but which could limit lesion and parasite growth in BALB/c and C57BL/6 mice has been designed (Mottram *et al.*, 1997). *L. mexicana* Δ*cpa/cpb* are candidates for attenuated live vaccines (Alexander *et al.*, 1998). The vaccine potential of *L. major* dihydrofolate reductase / thymidylate synthetase knock out (DHFR/TS$^-$), which is avirulent in mice, has recently been studied in mice (Titus *et al.*, 1995).

One of the advantages of attenuated lines of *Leishmania* is that the parasite can survive in the mammalian host for a long time, without any pathogenicity and induces an immunological response. The parasite persisting for a long time induces long term memory in the process known as concomitant immunity (Aebischer *et al.*, 1993). An unfortunate feature of this persistence is the possibility of reactivating of disease in the immunocompromised individual. A vaccine should be molecularly defined and induce long term memory in the absence of persistent live organisms. (Handman, 1997).

Other disadvantages include the complicated and high price of production and logistics of delivery for large-scale vaccination (Handman, 1997). Attenuated *Leishmania* vaccine strains retain the inherent risk of causing disease in severely immunodeficient individuals, underlining the mutual impact of pathogen and host immune response on virulence.

1.4.3.3. Killed promastigotes

Unlike many other parasites, *Leishmania* can be easily grown in cell-free media. This simple cultivation and the use of killed parasites as skin-test antigen (leishmanin) for

diagnosis in humans during the past decades has prompted scientists to try using the killed parasites, with or without adjuvant, as vaccines or for immunotherapy (Modabber, 1995). There are many studies from Iran, Israel, and Soviet Union that indicate that controlled infection led to significant protection from re-infection.

Clinical trails of prophylactic immunization with killed promastigotes of *L. major* began in early 1940, and have shown variable rates of protection, ranging from 82% to no effect at all. It has been reported that immunzation with killed leishmanial promastigotes plus BCG induced CD4+ T cells (Castes *et al.*, 1994). Cabrera *et al.* (2000) found that vaccination with BCG give a strong immunity against a virulent strain of *L. mexicana amazonensis*. It has been reported that a single dose of killed autoclaved *L. major* promastigotes (ALM) plus BCG reduced the incidence of cutaneous leishmaniasis among schoolchildren compared with a group receiving BCG alone (Sharifi *et. al.*, 1998).

The killed parasites are unable to invade the reticuloendothelial cells but the viable parasite can exist intracellularly in macrophages and evade the consequences of host's immune attacks.

1.4.3.4. Subunit vaccines

Subunit vaccines have focused primarily on protein antigens, because they can be easily identified, isolated genes cloned and studied. There has been significant progress towards the identification of molecular defined vaccine candidates, specially the surface protease gp63, the surface antigen gp46/M2 and a related parasite surface antigen 2 (PSA-2) (Xu, 1995). An interesting approach with gp63, which has provided promising results, has been the use of live vectors. It has been possible to induce protection in mice against the challenge with *L. mexicana* by immunising with attenuated *S. typhimurium* expressing gp63 (Gonzalez *et al.*, 1998). Abdelhak and colleagues (1995) reported that recombinant BCG expressing the *Leishmania* surface antigen gp63 induces protective immunity against *L. major* infection in BALB/c mice. It has been reported that recombinant vaccina virus carrying the gp46/M-2 gene of *L. amazonensis* is able to induce a protective immunity in mice against this parasite (McMahon-Pratt *et al.*, 1993).

One of disadvantages of subunit vaccines is that T-cells do not recognise non- protein antigen and another is the possible existence of non-responders to single antigens in the genetically diverse human population (Handman, 1997).

1.4.3.5. DNA vaccines

The protective responses induced with DNA vaccines against several pathogens have provided the genetic vaccination as a new approach for vaccination (reviewed by Thighe *et al.*, 1998). The first *Leishmania* vaccine delivered as plasmid DNA has been gp63 (Xu and Liew, 1995). It was shown that 30% of the BALB/c mice recovered from *L. major* infection, when they were vaccinated intradermally with plasmid DNA expressing gp63 and that dendritic cells from immunised mice were able to transfer protection (Walker *et al.*, 1998).

Vaccination studies, using a DNA construct encoding the surface antigen gp46/M2 and related PSA-2 showed that it causes reduction in lesion size and promotes healing in both genetically resistance C3H/He mice and susceptible BALB/c mice (Handman *et al.*, 2000). PSA-2 DNA induced a protective immunity by inducing a defined population of $CD4^+$ T cells to secrete Th1-like cytokines in susceptible mice (Sjolander *et al.*, 1998).

The only other example of DNA-induced protection in leishmaniasis is the LACK Ag. Generation of long-term protective immunity against leishmaniasis must induce memory T cells, which upon encounter with the parasite are stimulated to secrete protective Th1-like response cytokines (Sjölander, 1998). It has been reported that using a construction of a cDNA library obtained from *L. major* promastigotes and expression in *E.coli*, a 24 kDa protein (p24) was identified sharing homology with intracellular receptors for activated protein C kinase (RACK) and was designated LACK (Mougneau *et al.*, 1995).

DNA vaccines have many advantages; no adjuvant required, easy and low cost of production, and with long term stability. The disadvantages of DNA vaccines are induction of autoimmune disorders, immune complex diseases or the down-regulation of immune response through the induction of tolerance (Wahren, 1996).

CHAPTER TWO

GENERATION OF ATTENUATED LINES OF *LEISHMANIA MEXICANA* AND *LEISHMANIA MAJOR*

2.1. Introduction

2.1.1. *Leishmania*

Protozoan parasites of the genus *Leishmania* are the causative agents of a spectrum of human diseases. *Leishmania* has a digenetic life cycle that encompasses the extracellular promastigotes in the digestive tract of the parasite-transmitting insect vector, the sandfly, and the disease-causing intracellular amastigotes living in parasitophorous vacuoles (PV) of mammalian macrophages.

Some of the components, which are very important in the pathogenesis by the parasite are cysteine proteinases (CPs). It was demonstrated that *L. mexicana* complex has CP activities, particularly in the amastigote stage (Coombs and Mottram, 1997), which contains multiple CPs of apparent size 22-28 kDa that are readily detectable using gelatin-SDS-PAGE (Robertson and Coombs, 1992). In addition, the stationary phase promastigotes which contain CPs, the majority of the CPs in the various *Leishmania* species are to a group of homologous cathepsin-L-like enzymes knows as CPA and CPB (Robertson and Coombs 1993). *L. mexicana Δcpa* was generated by disrupting sequentially both alleles of *lmcpa* using gene-targeting of promastigotes with hygromycin- and phleomycin-resistance markers (Souza *et al.*, 1994). *L. mexicana* mutants deficient in cysteine proteinase genes *cpa (Δcpa)* and *cpb (Δcpb)* or both *cpa* and *cpb (Δcpa/cpb)* have been created by targeted gene disruption (Mottram *et al.*, 1996; Frame *et al.*, 2000). Alexander and colleagues (1998) reported that they have vaccine protection.

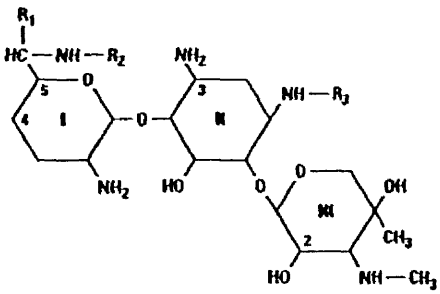
2.1.2. Gentamicin

Gentamicin belongs to the aminoglycoside group, which was discovered by Wenistein in 1963 and isolated, purified, and characterized by Russell in 1964 (Chambers and Sande, 1996). Aminoglycosides were originally obtained from various *Streptomyces* species and have similar chemical and toxic activities to commercial gentamicin (Chambers *et al.*, 1998). Gentamicin was isolated from *Micromonospora purpurea*

and has been used as a very effective bactericidal agent against both gram-positive and gram-negative bacteria (Chambers *et al.*, 1998).

2.1.2.1. Chemical properties:

Gentamicin consists of a hexose ring, 2-deoxystreptamine, which is joined to two amino sugars by glycosidic linkages (Chambers and Sande, 1996) and is composed of three loosely related fractions: gentamicins C_1, C_2, and C_{1a}. All fractions have similar molecular weights and the commercial preparation contains varying mixtures of the three fractions (Figure 2.1).



	R_1	R_2	R_3
Gentamicin C_1	CH_3	CH_3	H
Gentamicin C_2	CH_3	H	H
Gentamicin C_{1a}	H	H	H

Figure 2.1. Structure of gentamicin

2.1.2.2. Mechanism of action

Gentamicin interacts with RNA molecules and any biological function involving RNA is a potential target of the compound. Walter and colleagues (1999) reported that aminoglycosides are very active towards RNA because RNA molecules are highly negative charged. However, the precise mechanism of bactericidal activity of aminoglycosides is not yet fully understood. Some of the proposed actions (see Figure 2.2) are follows:

synthesis by fixing the 30-50 S ribosomal complex at the start codon (AUG) of mRNA (Chambers and Sande, 1996).

ii) Misreading of mRNA during protein synthesis, resulting in either

(a) Premature termination of translation with detachment of the ribosomal complex and an incompletely synthesised protein.

Or

(b) Abnormal or non-functional proteins (Chambers and Sande, 1996).

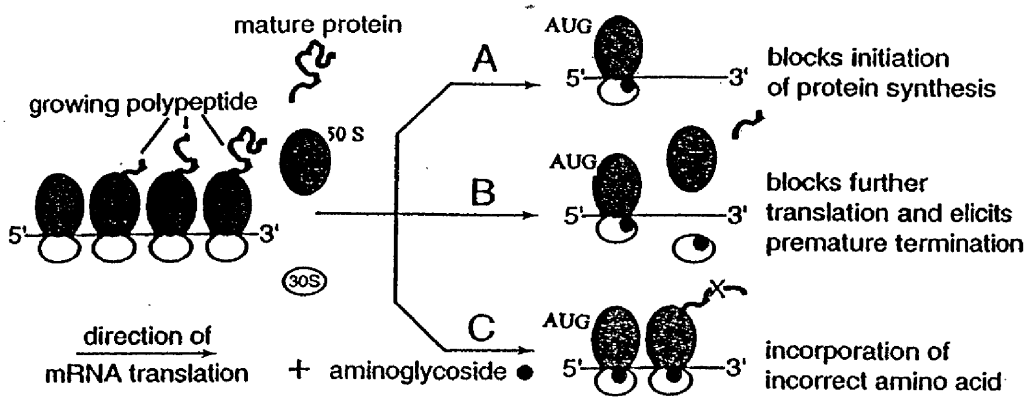


Figure 2.2. Effects of aminoglycosides on protein synthesis (Chambers and Sande, 1996).

2.2. Methods and materials

2.2.1. Parasites

L. mexicana wild type (WT) strain, designation MNYC/BZ/62/M379 and *L. major* (WT) strain, designation LV39 kindly provided by Prof. Coombs, Division of Infection and Immunity, Glasgow University and Prof. Liew, Department of Immunology, Glasgow University, respectively. The parasites were cultivated in HOMEM medium (GIBCO-BRL) with 10% (v/v) heat-inactivated foetal calf serum (HI-FCS) (Labtech International) (designated complete HOMEM medium). Promastigotes were grown in 25 cm^2 flat bottomed culture flasks at 25°C with air as the gas phase. The mid to late log-phase promastigotes were transferred routinely into fresh medium with a starting density of 10^5 cells / ml and reached the infective promastigote-stage (stationary phase containing metacyclic promastigotes) with a density of 1-2 × 10^7 cells/ml after 8-9 days incubation.

2.2.2. Preparation of *L. mexicana* and *L. major* Hamid's lines (H-lines)

L. mexicana H-line was generated in complete HOMEM medium supplemented with gentamicin (Sigma) at 20 μg/ml. The mid to late log-phase promastigotes of *L. mexicana* WT were transferred into medium with gentamicin and incubated at 25°C (for more details see section 2.2.1). *L. mexicana* was attenuated after some passages (for example 20).

The development of attenuated lines of *L. mexicana* WT by this methodology was carried out on 4 separate occasions. On each occasion, amastigotes of *L. mexicana* WT from a BALB/c mouse lesion were transferred into complete HOMEM medium and incubated at 25°C. The amastigotes differentiated to promastigotes over 72 hours, which were then transferred into complete HOMEM supplemented with or without gentamicin and incubated at 25°C. The mid to late log-phase promastigotes were passaged into medium with gentamicin or gentamicin-free and this process continued for at least 20 passages.

The development of the attenuated line of *L. major* WT used the same methodology except that only 11 passages with gentamicin were used just on one occasion.

2.2.3. Prepareation of promastigotes of *L. mexicana* H-line amastigote-derived through of BALB/c mice (*L. mexicana* HAD-line).

Twelve weeks after inoculation of 5×10^6 (cell / ml) stationary phase promastigotes of *L. mexicana* H-line into the shaven rump mice were killed and rinsed with 70% alcohol. The skin from the rump at the site, where the promastigotes were injected, was removed and the epidermal sheet carefully separated from the dermis. The skin was cut into 0.75 cm strips, floated dermal side down on RPMI medium supplemented with 1% trypsin in plastic dishes and incubated at 37°C for 90 min. The sheets were then shaken to release the cells. The cells were sedimented at 280 × g for 10 min and washed twice in RPMI. The epidermal cells were resuspended in HOMEM medium supplemented with 10% FCS, plated in the 24-well plates and then incubated at 25°C. The cultures were examined daily for the presence of promastigotes.

2.2.4. Measuring the size of lesions induced by the two lines of *L. mexicana*

The stationary phase promastigotes of *L. mexicana* WT or *L. mexicana* H-line or *L. major* WT or *L. major* H-line were harvested from a stationary culture ($1\text{-}2 \times 10^7$ cells/ml) after 6-7 days growth. Cells were washed 3 times in ice-cold PBS, the number of promastigotes adjusted to 2.5×10^7 cells/ml in PBS, and 200 µl of this suspension was subcutaneously (s.c.) injected into the shaven rump of BALB/c mice. The lesion volume was measured weekly using a capillary micrometer (Royal) (Mottram *et. al.*, 1996).

2.2.5. Determination of parasite density by Formaldehyde fixation

Promastigotes were mixed with phosphate buffer saline (PBS) [NaCl 171.1 mM/l, KCl 33.5 mM/l, Na_2HPO_4 101.2 mM/l, and KH_2PO_4 18.3 mM/l (all from Sigma)] supplemented with formaldehyde 4% (v/v) and incubated for 5 min at room temperature. The fixed promastigotes were counted using an improved Neubauer Haemocytometer. The number of promastigotes per ml was calculated.

2.2.6. The growth rate of *L. mexicana* H-line and *L. major* H-line

Promastigotes of *L. mexicana* H-line or *L. major* H-line were individually grown in complete HOMEM medium supplemented with 20 μg/ml gentamicin or without gentamicin. The mid to late log-phase promastigotes of each line were transferred into the medium to starting density of 1×10^5 cell / ml and the numbers of promastigotes were counted using an improved Neubauer Haemocytometer daily. The pH of the culture media was measured using a pH meter (Philips PW 9420).

2.2.7. Comparing the growth rate of *L. mexicana* H-line with *L. mexicana* Δ*cpa/cpb in vitro*

Promastigotes of *L. mexicana* H-line or *L. mexicana* Δ*cpa/cpb* or *L. mexicana* WT were individually grown from a starting density of 5×10^4 cell / ml in complete HOMEM medium supplemented with 4 kinds of selective antibiotics or free of antibiotics. The mid to late log-phase promastigotes of each line were transferred into the medium with nourseothricin hydrosulfate (SAT) at 25 μg/ml (w/v) or puromycin (Pur) at 10 μg/ml (w/v) or zeocin (Ble) at 10 μg/ml (w/v) or hygromycin B (Hyg) at 50 μg/ml (w/v) or a combination of these antibiotics, SAT 6.25 μg/ml, Pur 2.5 μg/ml,

Ble 2.5 μg/ml, and Hyg 6.25 μg/ml (all antibiotics a gift from J. C. Mottram, Wellcome Centre of Molecular Parasitology, University of Glasgow). The numbers of promastigotes were counted using an improved Neubauer Haemocytometer daily.

2.2.8. Staining parasites

Aliquots of cultures of stationary phase promastigotes (0.5 ml) were spread onto slides using a cytospin (Shandon centrifuge) at 1600 g for 5 minutes. The slides were quickly air dried, fixed in absolute methanol, and stained in 10% (v/v) Giemsa's stain (Merck) in PBS (pH 7.2) for 10 minutes.

2.2.9. Cryopreservation of promastigotes

Parasites were cryopreserved in liquid nitrogen. 95 μl of culture of mid to late log phase of promastigotes were mixed with 5μl of dimethylsulfoxide (DMSO) in a cryotube (Bio Plas, Inc). The tube was wrapped in cotton wool, put inside a polystyrene box and placed in a -70° C freezer for overnight. The following day the cells were transferred into liquid nitrogen.

2.2.10. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.10.1. SDS-PAGE (mini gel)

2.2.10.1.1. Harvesting of promastigotes of *L. mexicana*.

When the promastigotes reached stationary phase, the cell density of the culture was determined using an improved Neubauer Haemocytometer. The promastigotes were harvested by centrifugation at 750 g for 15 minutes at 4°C. The pellets were

resuspended in 10 ml of 0.25 M sucrose solution. Centrifugation and resuspension were repeated twice. The pellets were finally resuspended to 2×10^9 cell / ml. The suspension was aliquoted 20 µl per eppendorf tube, centrifuged, the supernatants discarded and the pellets stored at - 70° C.

2.2.10.1.2. Preparation of promastigote lysates

Extracts were prepared by addition of 0.25% (v/v) Triton X-100 in 0.25% M sucrose to frozen cell pellets. The cells were subsequently mixed by gentle aspirated with a Gilson pipette tip and then centrifuged at 1600 g for 10 minutes in a microfuge, and the supernatants collected. The samples were kept on ice before using.

2.2.10.1.3. Preparation of gel

SDS-PAGE was used for protein fractionation by vertical slab electrophoresis. The composition of separating and stacking gels are detailed in (Appendix, Tables1-4). The separating gel was loaded and a little water added to the top of the gel to level it. Following setting of the gel, the water was removed and the stacking gel was added, loaded, and setting allowed.

2.2.10.1.4. Total protein analysis (mini gel)

The samples from section 2.2.10.1.2 were mixed with an equal volume of sample buffer (see Appendix, Table 5), boiled for 10 min, and then centrifuged at 8500 g for 1 min. The supernatants of promastigote lysates of two lines of *L. mexicana* were separated using SDS-PAGE with a 10% (w/v) acrylamide gel at 150 V for about 1 hour. Subsequently, the gel was stained overnight in Coomassie Blue.

2.2.10.2. Proteinase detection using substrate SDS-PAGE

Cysteine proteinase activity of stationary phase promastigotes of two lines of *L. mexicana* was analysed using SDS-PAGE gels (10% acrylamide) containing gelatin at 0.2% (w/v) (Robertson and Coombs, 1990). The samples (see section 2.2.10.1.2) (10 µl) were mixed with equal volume of sample buffer (see Appendix, Table 5) and centrifuged at 8500 g for 1 minute in a microcentrifuge. The samples were separated on the gel (as described in the section 2.2.10.1.4) and subsequently the gels were incubated in 2.5% Triton X-100 on a shaking table at 37°C for 30 minutes to remove SDS and reactivate the enzymes. The gels were then incubated at 37°C in 0.1 M sodium acetate, pH 5.5, with 1 mM DTT for 2 hours to allow hydrolysis of the gelatin. Subsequently the gels were incubated overnight in Coomassie Blue Staining and destained in destaining buffer (see section 2.2.10.1.4)

2.2.10.3. 10-20% polyacrylamide gradient SDS-PAGE

2.2.10.3.1. Preparation of gel

To compare the protein levels of stationary and log phase promastigotes of two lines of *L. mexicana*, the sample lysates were run on a SDS-PAGE gradient gel (10%-20% acrylamide (Hames, 1983). The cassettes were inserted into the slab gel casting apparatus (SGC) (Pharmacia) for casting 180 mm wide. The stock solutions (see Appendix, Tables 6 and 7) were prepared and at this stage were not mixed. The valve between the reservoirs was closed and a 20% (v/v in distilled water) methanol solution was poured in the chamber nearest the outlet of the gradient mixer and allowed to run down into the SGC, leaving the connecting tube filled with liquid and free of air bubbles. Acrylamide 10% was poured into the lower chamber of the Gradient Mixer and the valve connecting the two reservoirs was opened just to remove air bubbles and then closed. Subsequently, acrylamide 20% was poured into the empty chamber and the requisite amounts of Tetramethylethylenediamine

(TEMED) and Ammonium persulphate (APS) were added, mixed briefly, the stirrer placed into the lower chamber, and the paddle turned on. The connecting valve between the two reservoirs was opened. When the polymerizing solution had run through, the glycerol / water solution, with a little bromophenol blue, was immediately added to the lower chamber. The flow of glycerol / water solution was stopped when the top of the polymerising mixture just reached the upper edge of the gel cassette. The gels were allowed to polymerized for at least one hour, the gel cassettes removed from the SGC, and the slabs stored in a humid atmosphere at 4-8°C.

2.2.10.3.2. Preparation of promastigote lysate

The stationary or log phase promastigotes were harvested (see section 2.2.10.1.1) and washed 4 times with ice-cold PBS at 4°C. The suspension of cells with a density of 2 × 10^9 cells / ml was aliquoted (100 µl per each eppendorf tube), centrifuged, and the supernatant removed. The pellets were stored at –70°C until needed. Promastigote lysate was prepared by adding 50 µl sample buffer to the pellet, boiled at 100°C for 10 minutes and centrifuged at 750 g for 5 minutes. 30 µl of supernatant was loaded to the SDS-PAGE gradient slab gel. The gel was run at a constant 230 voltage for 4 hours, stained with Coomassie blue stain, and destained (as previously described).

2. 2. 11. Two Dimensional Electrophoresis (2-DE)

2.2.11.1. Sample preparation

In order to obtain optimal results in 2-DE, a high concentration of soluble promastigote proteins was required. Two methods for preparation of the soluble lysate promastigote proteins were initially used. The preliminary results showed that the number of spots using the lysate from method 2 was higher than that from method 1. So method 2 was selected for remaining experiments.

Method 1

Stationary phase promastigotes with a density of 2×10^8 cells / ml were harvested (see section 2.2.10.3.2). The pellets were stored at –70°C until needed. Promastigote lysates were prepared by adding 40 µl lysate solution (see Appendix, Table 8) to the pellets which were the frozen and thawed 3 times using liquid nitrogen and then sonicated in a water bath for 5 minutes. 310 µl rehydration buffer stock (see Appendix, Table 10), DDT (Sigma) (10 mg/ml) and (immobilized pH gradient) IPG buffer (Pharmacia) (5µl / ml) [(IPGphor buffer 4-7 L) for IPGphor strip 4-7 and (IPGphor buffer 3-10 L) for IPGphor strip 3-10 were used] mixed (before using) and then added to the samples.

The promastigote lysates were mixed with a vortex for 4 minutes and then centrifuged at 1800 g at 4°C for 3 minutes. The supernatants were used.

Method 2

Promastigote lysates were prepared by adding 40 µl lysate solution (see Appendix, Table 9) to the pellets (see section 2.2.10.3.2) which were the frozen and thawed 3 times using liquid nitrogen, sonicated in a water bath for 5 minutes, and then heated at 95°C for 5 minutes. 310 µl rehydration buffer stock, DDT (10 mg / ml) and IPG buffer 4-7 L (5µl / ml) or IPGphor buffer 3-10 L were used mixed (before using) added to the samples. The promastigote lysates were mixed with a vortex for 4 minutes, and then centrifuged at 1800 g at 4°C for 3 minutes. The supernatants were used.

2.2.11.2. First dimension isoelectric focusing (IEF)

2.2.11.2.1. Immobilised pH gradient (IPG) strip

Two kinds of ready-made IPG strips, Immobiline Drytrip gels, with the pH gradients 3-10 L (linear) and 4-7 L (linear) were used. The pH 3-10 L IPG strips are analysed

wide range of proteins on a single 2-DE gel and the pH 4-7 L IPG strips were used for higher resolution separations in the narrower pH range.

Following sample preparation (as described in method 2 from section 2.2.11.1), the protective film from the IPG Dry Strip gel was removed. The sample was placed in the IPGphor strip holder and the entire length of IPG strip soaked with sample by placing IPG strip in the strip holder (gel facing down). The IPG strip was subsequently put in the strip holder, placing the end of the IPG strip over the cathode (electrode), and IPG cover fluid (Pharmacia) was added in the IPG strip holder length. The cover of the strip holder was placed on the strip holder and the strip holder assembled on the IPGphor platform.

2.2.11.2.2. IPGphor

This system is for the isoelectric focusing (IEF) dimension of 2-DE. The programme was designed for 13 cm pH 3-10 or 4-7 IPG DryStrip gel with a sample buffer that was prepared as described before (section 2.2.11.2.1). The timetable and voltage used is shown in Table 2.1.

Table 2.1. The programme of IPGphor

Step	Time	Voltage (V)
1	15 hours	30
2	1 hour	500
3	Until reach to 1000 V	1000
4	30 minutes	8000
5	4 hours	8000
Total		35000

2.2.11.2.3. Equilibration step

After completion of the first dimension, the strip was washed and equilibrated. The equilibration buffers I and II were prepared according to the recipe in (see Appendix, Table 12). The IPG strip was placed in individual strip holders with the support film towards the strip holder wall. The equilibration buffer I was added to the strip holder and shaken for 15 minutes. Subsequently, the equilibration buffer I was removed and the strip was placed in the strip holder with equilibration II buffer for 15 minutes and then washed dd H_2O.

2.2.11.3. Second dimension SDS-PAGE

2.2.11.3.1. Preparation SDS slab gel vertical system

Gels with a final concentration of 10% acrylamide and with a 1.0 mm thick spacer were prepared for the second dimensional 2 DE-gel. Subsequent to pouring the gel (see Appendix, Table 14) the gel cassettes were filled with displaying solution (see Appendix, Table 15) until 3 to 10 mm below the top (no stacking gel layer is required). Immediately, the storage solution (see Appendix, Table 16) overlay was added to each gel to create a flat surface. After allowing overnight polymerization, the overlay was removed and the gels were stored at 4°C for up to two weeks.

2.2.11.3.2. Placing the IPG strip on the gel.

The IPG strip (from section 2.2.10.3.3) was washed with the SDS electrophoresis buffer (see Appendix, Table 17) and placed between the plates on the surface of the SDS gel. The strip was gently pushed down, with a thin plastic ruler, until contact was made before with the surface of the gel and the lower edge of the IPG strip. Immediately, the strip was sealed by agarose (see Appendix, Table 18) to prevent it from moving or floating during electrophoresis. The agarose was heated at 100°C, allowed to cool to 50°C and the amount required for sealing the IPG strip slowly pipetted.

2.2.11.3.3. Electrophoresis

The electrophoresis was performed at a constant current of 100 volts overnight at 4°C. After electrophoresis, the gel was removed from its gel cassette in preparation for staining.

2.2.11.3.4. Silver staining

The gels were stained with the silver staining Kit (Pharmacia Biotech). 375 ml of the solutions detailed in Table 2.2 were needed per gel cassette (12.5 × 26 cm). These solutions were freshly made.

Table 2.2. Silver staining protocol for proteins for two gels (Pharmacia Biotech)

Step	Solutions	Amount	Time
Fixation	Ethanol Acetic acid glacial Make up to 750 ml with dd water	300 ml 75 ml	30 min
Sensitising	Ethanol Sodium thiosulphate (5% w/v) Sodium acetate (17 g) Glutaradialdehyde (25% w/v)* Make up to 750 ml with dd water	225 ml 30 ml 3 packets 1.75 ml/ gel	30 min
Washing	dd water		3×5 min
Silver reaction	Silver nitrate solution (2.5% w/v) Formaldehyde (37% w/v)* Make up to 500 ml with dd water	75 ml 0.14 ml/ gel	20 min
Washing	dd water		2×1 min
Developing	Sodium carbonate (6.25 g) Formaldehyde (37% w/v)* Make up to 750 ml with dd water	3 packets 0.07 ml/ gel	2-5 min
Stopping	EDTA-Na $_2H_2O$ (3.65 g) Make up to 750 ml with dd water	3 packets	10 min
Washing	dd water		3×5 min
Preserving gels	Glycerol (87% w/w) Make up to 750 ml with dd water	75 ml	30 min

Add components marked* immediately before use.

2.3. Results

2.3.1. Establishing *L. mexicana* H-line and *L. major* H-line

The attenuated lines of *L. mexicana* (H-line) and *L. major* (H-line) were established by passaging in the presence of gentamicin. To prevent bacterial contamination gentamicin was routinely used in the culture medium. It was observed after culturing of promastigotes of *L. mexicana* in the presence of gentamicin, the length of some of promastigotes had been increased. This prompted the analysis of the line (detailed below).

In the following experiments, *L. mexicana* H-line had been generated from *L. mexicana* WT by more than 20 passages in complete HOMEM medium supplemented with gentamicin at 20 μg/ml. The generation of the attenuated line of *L. mexicana* H-line was repeated on four occasions. There was not any restriction to do experiment with just one of them.

2.3.2. The size of lesions induced by the two lines of *L. mexicana*

Sub-cutaneous inoculation of *L. mexicana* WT in mice results in the development of a progressive non-healing lesion. To examine the ability of *L. mexicana* H-line to induce lesions, mice were injected (s.c.) with 5×10^6 stationary phase promastigotes of *L. mexicana* H-line or WT. The mice which were injected with *L. mexicana* WT developed progressive non-healing lesions (Figure 2.3). In contrast, mice infected with *L. mexicana* H-line developed either no lesion during the period of study or a small lesion that was completely healed by 12 weeks post infection.

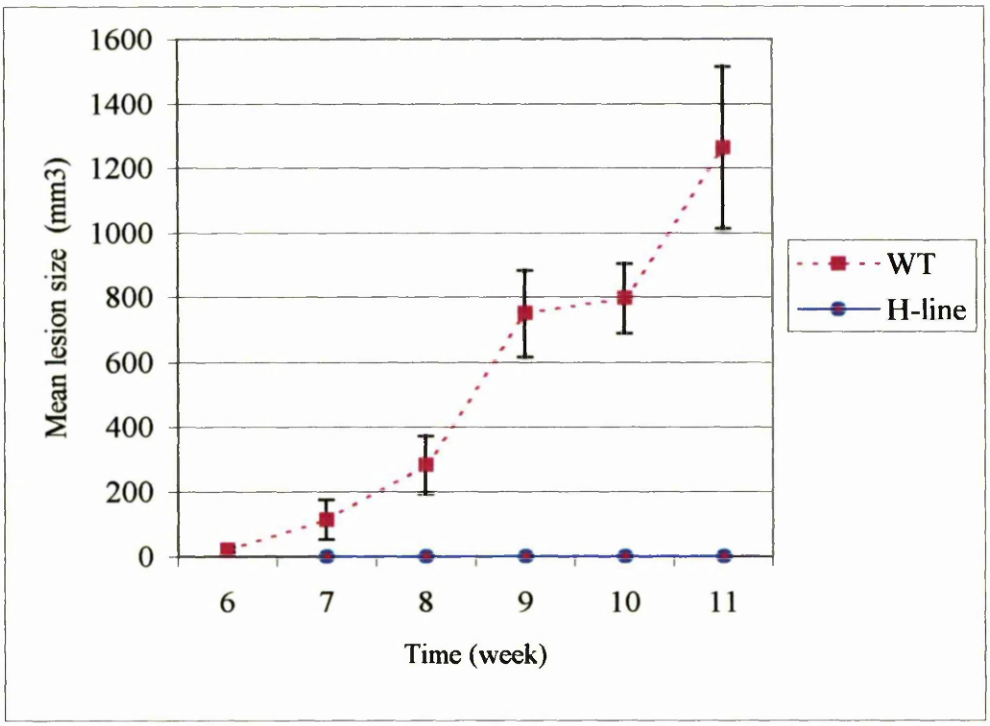


Figure 2.3. The mean course of infection of two lines of *L. mexicana* in 2 groups of BALB/c mice (5 mice / group). 5× 10^6 stationary phase promastigotes of *L. mexicana* WT (35 passages) or *L. mexicana* H-line (32 passages) were inoculated in the right side shaved rump of the mice. Lesion development was monitored by measuring the size of swelling and lesion diameter weekly. Each data point represents the mean group lesion size ± SEM.

2.3.3. The size of lesions induced by the two lines of *L. major*

Sub-cutaneous inoculation of *L. major* WT in mice results in the development of a progressive non-healing lesion. Five BALB/c mice were infected with 5×10^6 stationary phase promastigotes of *L. major* H-line or WT as described in section 2.3.2. The mice that were injected with *L. major* WT developed progressive non-healing lesions (Figure 2.4). In contrast, mice infected with *L. major* H-line developed no lesion during 12 weeks post infection.

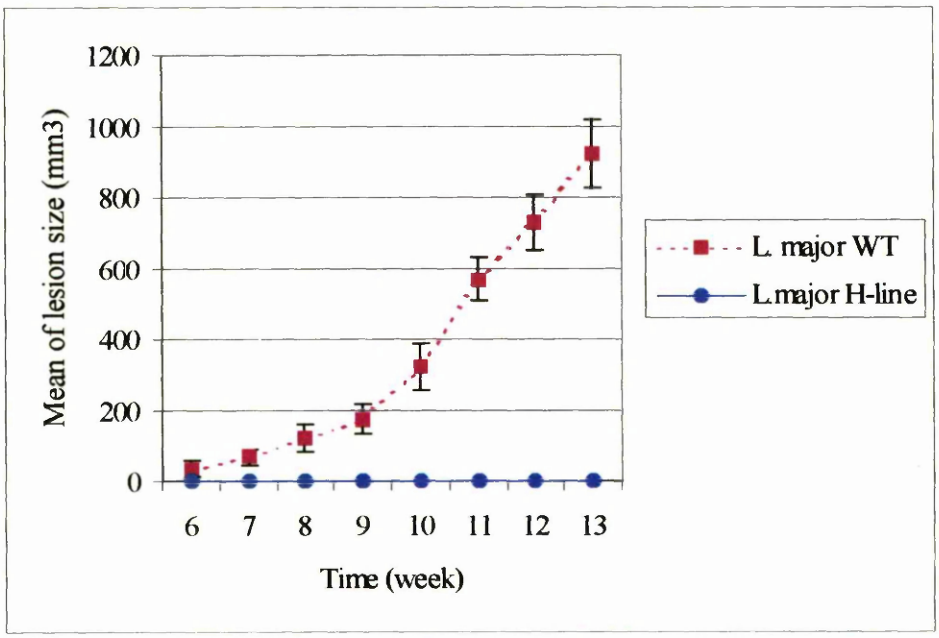


Figure 2.4. The mean course of infection of *L. major* WT and *L. major* H-line in 2 groups (5 mice per group) of BALB/c mice. The mice were subcutaneously injected with stationary phase promastigotes of the two lines of and the lesion development was monitored weekly. The values represent the mean + SEM.

2.3.4. Growth rate of *L. mexicana* H-line

In order to compare the growth rate of the two lines of promastigotes, *L. mexicana* H-line and *L. mexicana* WT were grown in complete HOMEM medium with a starting density 10^5 cells / ml. As demonstrated in Figure 2.3, the number of promastigotes of the two lines rapidly increased and there was no significant difference ($P>0.2$) between the growth rates of promastigotes of the two lines of *L. mexicana*. There were no significant differences at any day between the growth rates of promastigotes of both lines. Promastigotes of the two lines reached stationary phase after 8-9 days. It has been reported that approximately 90% of promastigotes were considered to have reached stationary phase, usually after 8-9 days from initiation of culture (Mallinson and Coombs, 1989).

2.3.5. Growth rate of *L. major* H-line

In order to compare the growth rate of the two lines of promastigotes, *L. major* H-line and *L. major* WT were grown in complete HOMEM medium with a starting density 10^5 cells / ml. As demonstrated in Figure 2.6, the number of promastigotes of the two lines rapidly increased and there was no significant difference in growth between them ($P>0.5$) on all days. Promastigotes of the two lines reached stationary phase after 8-9 days. The pH of the stationary phase medium of the culturel of promastigotes of *L. major* H-line was 6.9, whereas that of the culturel of the stationary phase of promastigotes of *L. major* WT was more acidic (pH 6.3). The measuring of pH of media were repeated individually in three occasions.

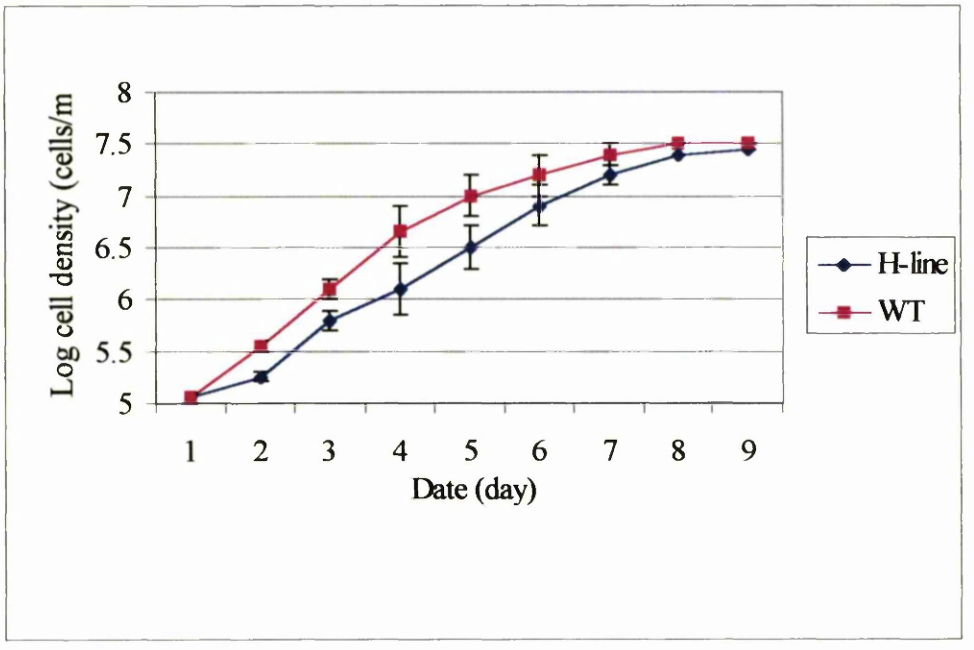


Figure 2.5. Example of *in vitro* growth of promastigotes of *L. mexicana* H-line and *L. mexicana* WT. Comparison of the growth rate of the two lines of *L. mexicana* of the same age and culture history was made in parallel cultures. Cultures of promastigotes of the two lines of *L. mexicana* were initiated at 10^5 cells / ml and counts made daily using an improved Neubauer Haemocytometer. The results are means ± SD from 3 independent cultures for each line.

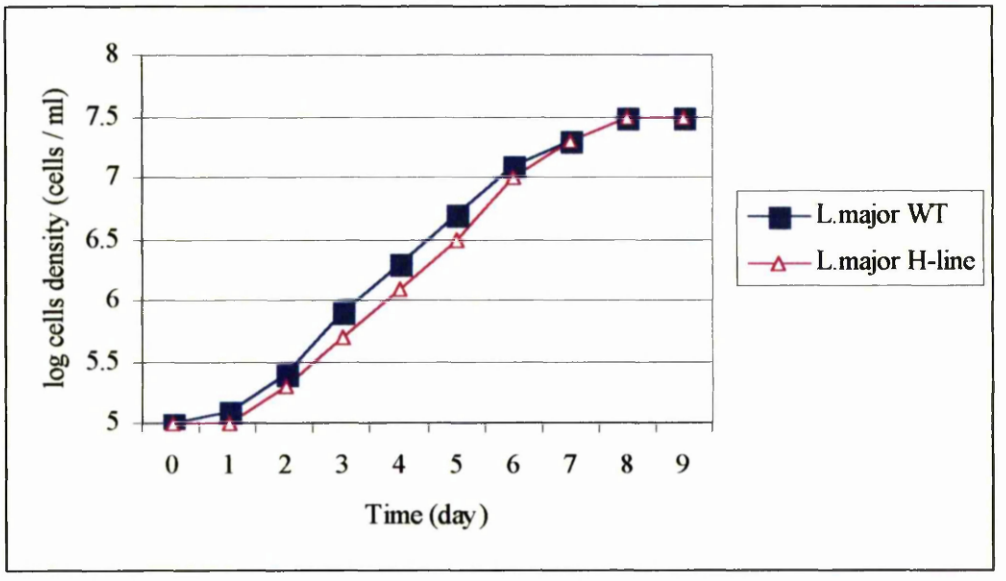


Figure 2.6. Example of *in vitro* growth rate of promastigotes of *L. major* H-line and *L. major* WT. Comparison of the growth rate of the two lines of *L. major* of the same age and culture history was made in parallel cultures. Cultures of promastigotes of the two lines of *L. major* initiated at 10^5 cells / ml and counts made daily using an improved Neubauer Haemocytometer. Promastigotes of *L. major* H-line and *L. major* WT were grown on parallel cultures of the same age and culture history.

2.3.6. Comparing the growth rate of *L. mexicana* H-line with *L. mexicana Δcpa/cpb.*

To confirm that *L. mexicana* H-line has not been mixed up with the attenuated *L. mexicana* Δ*cpa/cpb*, the promastigotes of these lines and *L. mexicana* WT were individually grown in the presence of 4 kinds of selective antibiotics, SAT, Pur, Ble, and Hyg. Promastigotes of *Leishmania mexicana Δcpa/cpb* have the ability to grow in the presence of selective antibiotics because, *L. mexicana Δcpa* was generated by disrupting sequentially both alleles of lmcpa using gene-targeting of promastigotes with hygromycin- and phleomycin-resistance markers (Souza *et al.*, 1994).

Promastigotes of the three lines of *L. mexicana*, with a starting density 5×10^4, were passaged and incubated at 25°C. As Figure 2.5 shows, the growth rate of promastigotes of *L. mexicana* H-line in the presence of the selective antibiotics, SAT, Pur, Ble, and Hyg (individually or combined together) sharply decreased, but the number of promastigotes in the antibiotic-free medium increased and during 7 days incubation reached 10^7 cells / ml. The log cell density of promastigotes of *L. mexicana* H-line in the media supplemented with hygromycin or puromycin at day 2 and with other antibiotics after 3 or 4 days incubation was zero. These results indicated that promastigotes of *L. mexicana* H-line are sensitive to these antibiotics.

Promastigotes of *L. mexicana* WT were grown in the media with antibiotics or antibiotics-free medium. As shown in Figure 2.8, the number of promastigotes in the media supplemented with hygromycin or puromycin rapidly decreased whereas in the antibiotic-free medium the number of promastigotes increased and during 7 days incubation reached 10^7 cells / ml. Therefore *L. mexicana* WT was sensitive to these selective antibiotics.

To compare the growth of *L. mexicana* H-line or *L. mexicana* WT with *L. mexicana* Δ*cpa/cpb*, the same density of promastigotes of *L. mexicana Δcpa/cpb* were transferred to the media with or without antibiotics. In contrast to *L. mexicana* H-line or *L. mexicana* WT, the number of promastigotes in the media with antibiotics increased and there was no significant difference between the growth rates of promastigotes of *L. mexicana cpa/cpb* double knockout in the media, with or without antibiotics (Figure 2.9) ($P>0.2$).

It was concluded that promastigotes of *L. mexicana* H-line and *L. mexicana* WT are sensitive to four kinds of selective antibiotics, nourseothricin hydrosulphate, puromycin, zeocin, and hygromycin B (individually or combined together), whereas promastigotes of the *L. mexicana cpa/cpb* double knockout were resistant to these antibiotics, and that *L. mexicana* H-line was not contaminated with *L. mexicana* Δ*cpa/cpb* and which could contribute to its attenuated state.

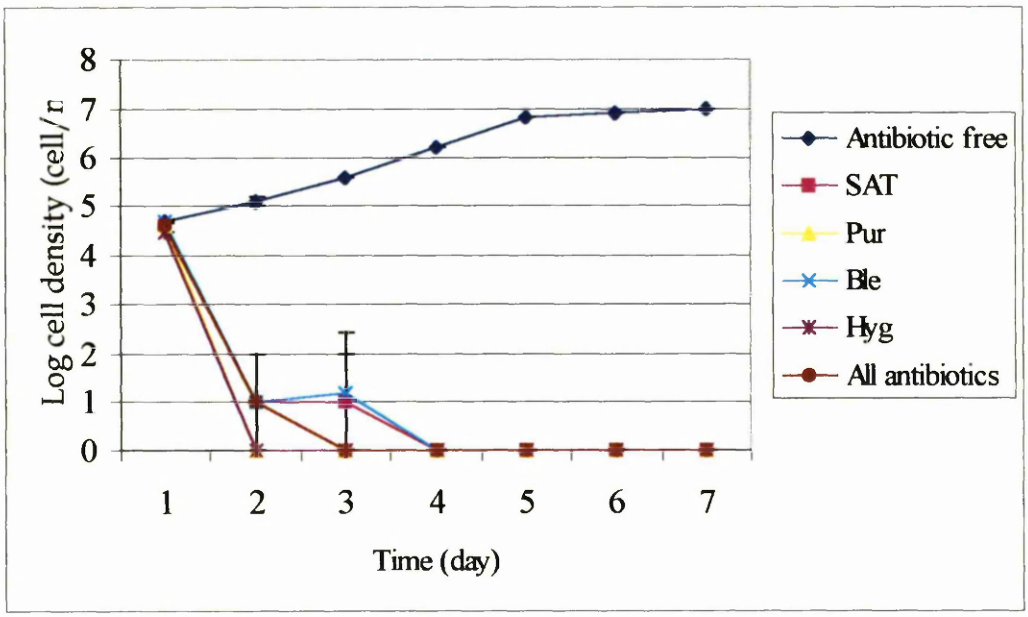


Figure 2.7. The growth rate of promastigotes of *L. mexicana* H-line in media with or without selective antibiotics. Mid-log phase promastigotes of *L. mexicana* H-line were transferred into HOMEM medium supplemented with 10% (v/v) FCS and SAT (25 μg/ml) or Pur (10 μg/ml) or Ble (10μg/ml) or Hyg (50 μg/ml) or in a combination of these antibiotics (SAT 6.25 μg/ml, Pur 2.5 μg/ml, Ble 2.5 μg/ml, and Hyg 6.25 μg/ml) or antibiotic-free. The results are means ± SD from 3 independent cultures for each line.

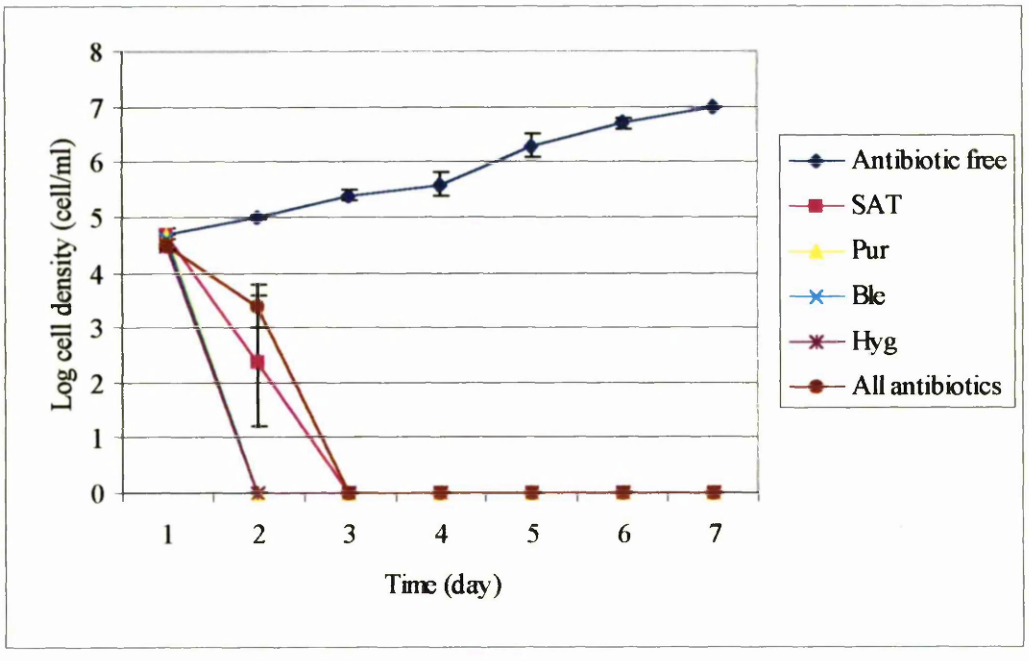


Figure 2.8. The growth rate of promastigotes of *L. mexicana* WT in media with or without selective antibiotics. Mid-log phase promastigotes of *L. mexicana* WT were transferred into HOMEM medium supplemented with 10% (v/v) FCS and SAT (25 μg/ml) or Pur (10 μg/ml) or Ble (10μg/ml) or Hyg (50 μg/ml) or in combination of these antibiotics (6.25 μg/ml SAT, 2.5 μ g/ml Pur, 2.5 μg/ml Ble, and 6.25 μg/ml Hyg) or antibiotic-free. The results are means ± SD from 3 independent cultures for each line.

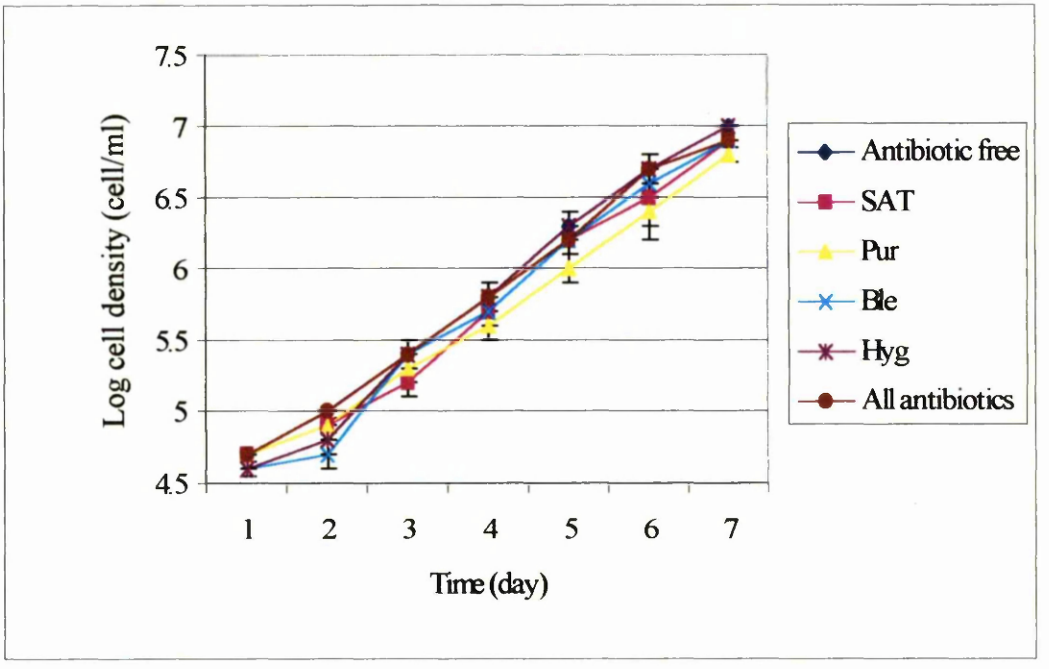


Figure 2.9. The growth rate of promastigotes of *L. mexicana Δcpa/cpb* in media with or without selective antibiotics. Typical growth curves of promastigotes of *L. mexicana Δcpa/cpb* in HOMEM medium supplemented with 4 kinds of selective antibiotics or in combination of antibiotics or antibiotic-free. The culture was initiated at 5×10^4 cell / ml. Promastigotes in the mid-log phase of growth and cell density was determined daily using a Neubauer Haemocytometer. The results are means ± SD from 3 independent cultures for each line.

2.3.7. Proteinase activity of *L. mexicana* H-line compared with *L. mexicana* WT

To confirm that the *L. mexicana* H-line was generated in the presence of gentamicin and was not derived through contamination with another attenuated line, *L. mexicana* Δ*cpa/cpb*, the proteinases of *L. mexicana* H-line and *L. mexicana* WT were examined. The proteinases of stationary phase promastigotes of *L. mexicana* H-line and *L. mexicana* WT were investigated by loading soluble lysates of the two lines of *L. mexicana* on the SDS-PAGE gel (10%) containing gelatin 0.2% (w/v). The result showed that multiple proteinases, the most active of which were between 22-24 kDa molecular mass (Figure 2.10). Several lower mobility bands ranging from 24-60 kDa were also active. The proteinase activities of the two lines were remarkably similar.

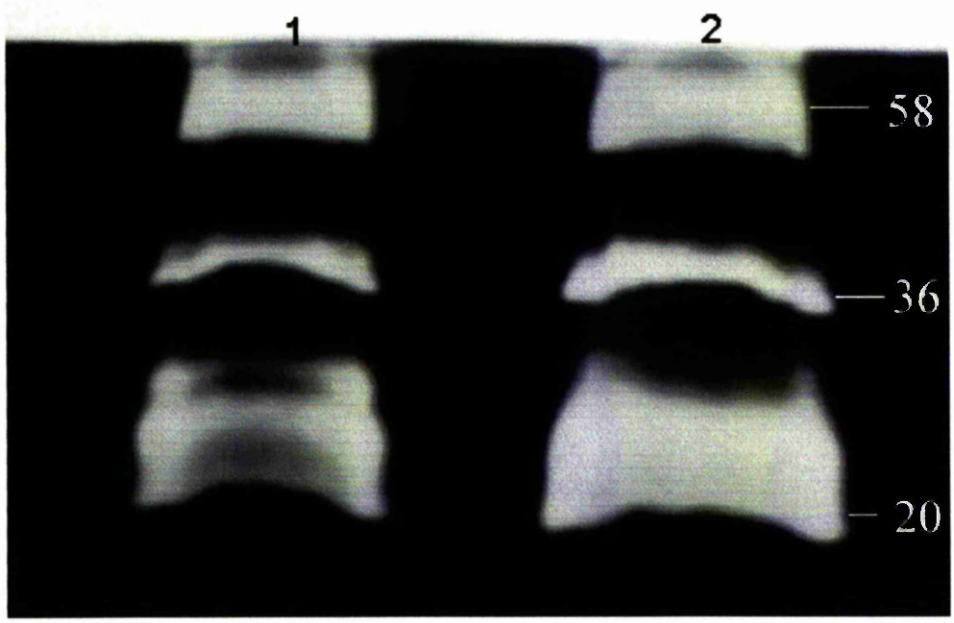


Figure 2.10. Comparison of proteinase activities of stationary phase promastigotes of two lines of *L. mexicana*. The lysate proteins were prepared from 2×10^7 stationary phase promastigotes of *L. mexicana* H-line (lane 1) or *L. mexicana* WT (lane 2) and analysed on the gelatin-SDS-PAGE gel. The gel was subsequently washed and then incubated with 0.1 M sodium acetate, pH 5.5, with 1 mM DTT for 2 hours. It was then stained with Coomassie blue

2.3.8. Morphology

2.3.8.1. Promastigotes of *L. mexicana* H-line

To compare the morphology of promastigotes of *L. mexicana* H-line with *L. mexicana* WT, the cells were fixed in methanol and stained with Giemsa's stain. As shown in Table 2.2, four categories of morphology (M) of promastigotes based on width and length were classified for the two lines of *L. mexicana*. Promastigotes with round cell bodies (morphology 1) and a small flagellum appeared in the stained smears of both lines. 12% of stationary phase promastigotes of *L. mexicana* H-line were longer than the promastigotes of *L. mexicana* WT at this stage. The percentage of different sizes of promastigotes of the two lines of *L. mexicana* is shown in Figure 2.11.

Table 2.3. The classification of stationary phase promastigotes according their sizes.

Morphology	width	length
M1	2 < width ≤ 3 μm	10 -12 μm
M2	1.5 -2 μm	10 -12 μm
M3	1.5 -2 μm	13 -18 μm
M4	1.5 -2 μm	19 -23 μm

2.3.8.2. Morphology of promastigotes of *L. mexicana* H-line amastigote-derived from BALB/c mice (*L. mexicana* HAD-line)

In order to compare the morphology of the two lines, the promastigotes of *L. mexicana* HAD-line or *L. mexicana* WT were grown in gentamicin-free medium. It was observed that *L. mexicana* HAD-line grew poorly *in vitro* and poor ability to transform. Smears of stationary phase promastigotes were prepared, fixed in methanol and stained with Giemsa's stain.

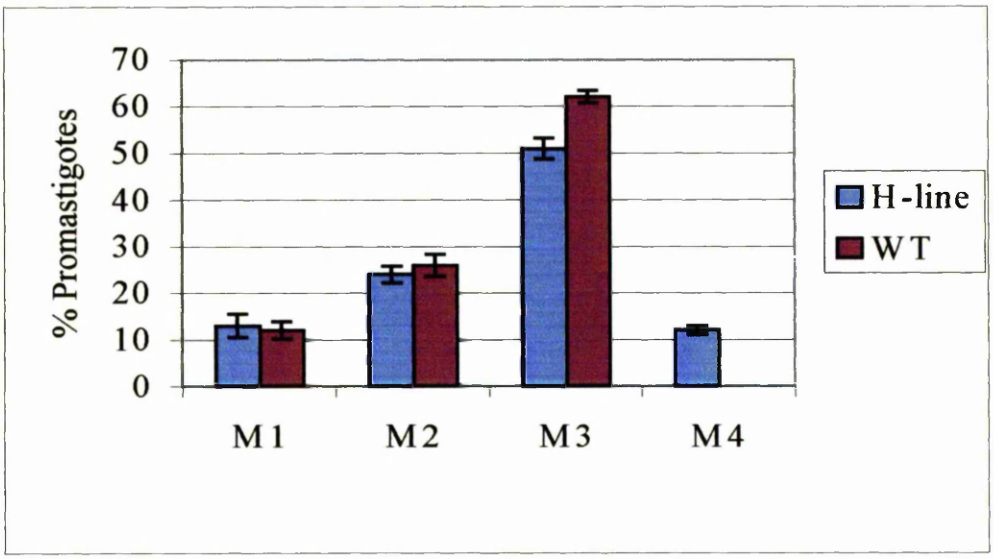


Figure 2.11. The percentage according to cell lengths of stationary phase promastigotes of *L. mexicana* H-line and *L. mexicana* WT. Each promastigote was measured for body length and breadth measurements in Giemsa's stained smears. At least 300 promastigotes were examined from each culture. The results are means ± SD from 3 independent cultures for each line.

In the smear of *L. mexicana* H-line, 17% of cells were promastigote which some of them had a small size flagellum and 83% of them were amastigote form (Figures 2.12), whereas the promastigotes of *L. mexicana* WT were slender forms with an anterior flagellum (Figure 2.13). The restriction of time prevents to do same analyses for both lines.

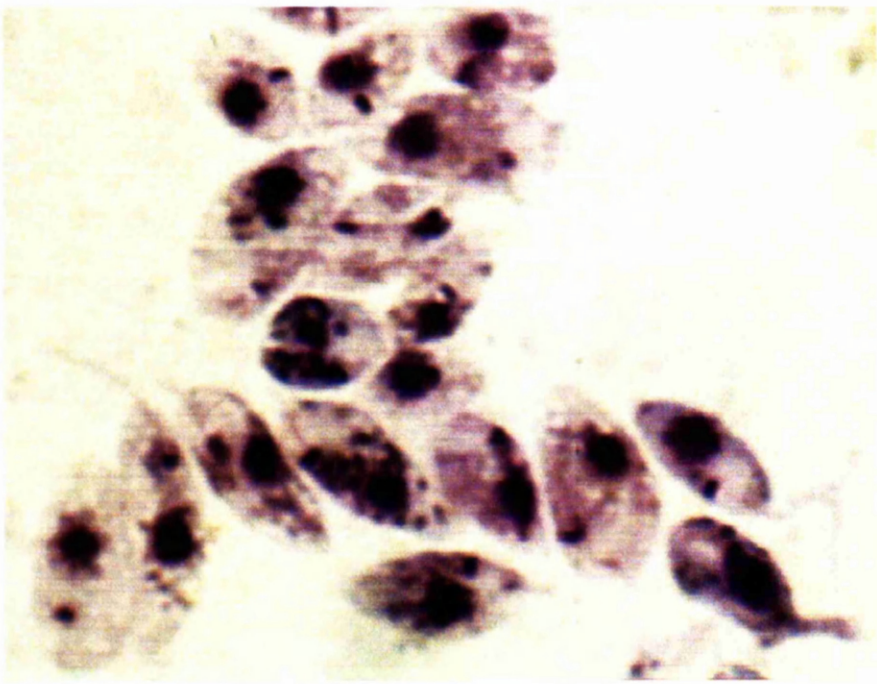


Figure 2.12. Light micrographs of cells from cultures of *L. mexicana* H-line 2. Cell culture of *L. mexicana* HAD-line was fixed in methanol and stained in 10% Giemsa's stain. In *L. mexicana* H-line about 17% and 83% of cells were promastigote and amastigotes forms, respectively, and some of them had a small flagellum.

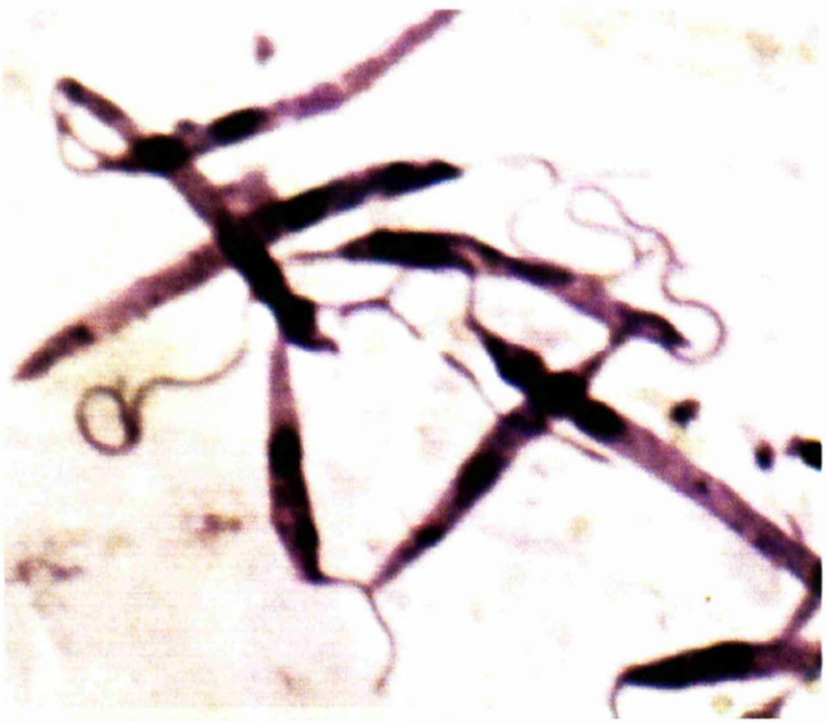


Figure 2.13. Light micrographs of cells from cultures of *L. mexicana* WT. Cell culture of *L. mexicana* WT was fixed in methanol and stained in 10% Giemsa's stain. The stationary phase promastigotes of *L. mexicana* WT were slender forms with a long flagellum.

2.3.9. Protein analysis

To determine whether the adaptation of *L. mexicana* H-line to grow in the presence of gentamicin involved changes in protein expression, lysates of promastigotes of *L. mexicana* H-line and WT were compared on a SDS-PAGE gel. Total lysate proteins of stationary or log phase promastigotes of *L. mexicana* H-line and WT were separately run on 10-20% SDS-PAGE gradient gels . As shown in Figure 2.14, a number of differences in protein expression were observed. Two bands were detected around 66 kDa with stationary or log phase promastigotes of *L. mexicana* WT, whereas one (possible two) of the bands was absent from stationary and log phase promastigotes of the *L. mexicana* H-line.

The stationary or log phase promastigotes of the two lines of *L. mexicana* were adjusted to 2×10^8 cells / ml, pelleted and stored at -70°C. Promastigotes lysates were prepared by mixing pellet with 50 µl sample buffer and boiled at 100°C for 10 min. 30 µl of samples were loaded to the SDS-PAGE gradient slab gel. To compare the protein density of lysates of stationary phase promastigotes of two lines of *L. mexicana*, the samples were loaded in gel and stained with Coomassie blue stain.

The Lane profile graphs which are representative of protein concentrations of the lysates of stationary phase promastigotes of the two lines of *L. mexicana* are shown in Figures 2.15 and 2.16. The optical density of protein on the SDS-PAGE gradient slab gel (Figure 2.14) was displayed with Lane profile graph using Lab Works Image Acquisition and Analysis Software (UVP Laboratory products). The bands correspond to the numbered peaks in each line on the graph. Each peak represents a protein concentration for that band. To compare with *L. mexicana* WT, in the optical density of protein of promastigotes of *L. mexicana* H-line, three peaks were deleted (Figure 2.15) and there are just 5 peaks.

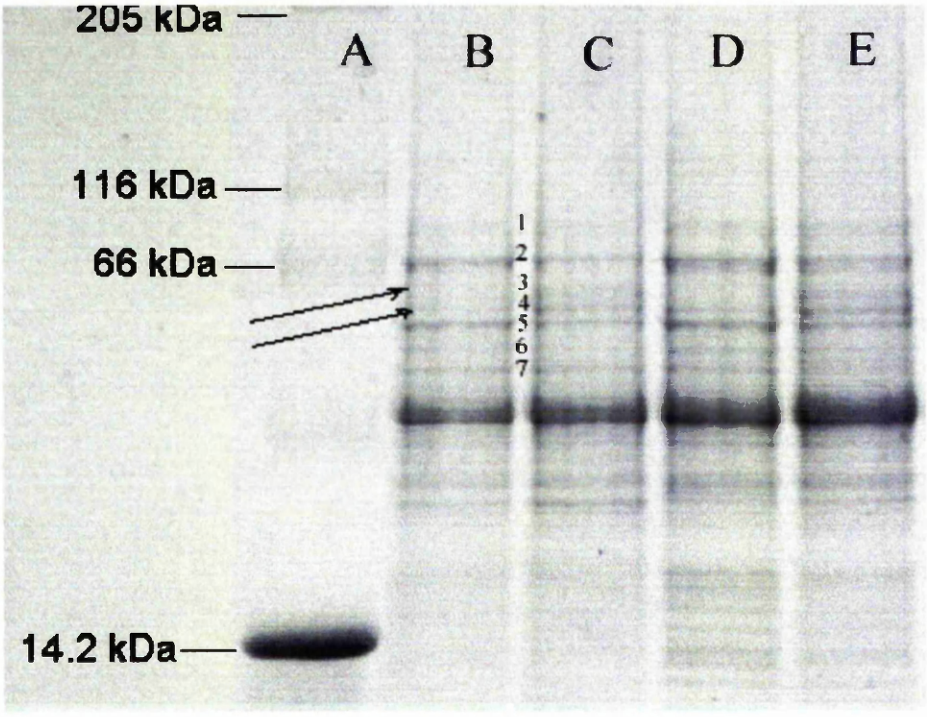


Figure 2.14. Lysates of promastigotes of *L. mexicana* H-line (44 passages) and WT (49 passages) compared on a 10-20% SDS-PAGE gradient gel. Markers (lane A) and 15 μl of lysate of stationary phase (lane B), log phase (lane C) promastigotes of *L. mexicana* H-line and stationary phase (lane D), log phase (lane E) promastigotes of *L. mexicana* WT were separately loaded on gel. Seven bands labelled on the gel between lane B and C are also shown in Figures 2.13 and 2.14. One (possibly two) of the bands absents from stationary phase promastigotes of *L. mexicana* H-line were shown with arrows.

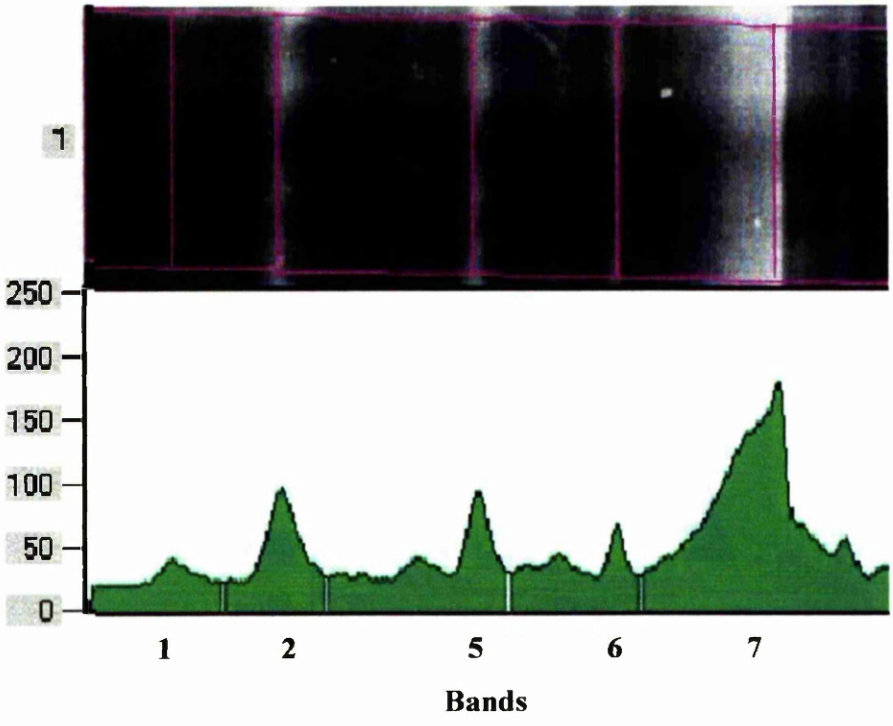


Figure 2.15. The Lane profile graph is representative of protein concentration in the lysate of stationary phase promastigotes of *L. mexicana* H-line. The samples were loaded in gradient-SDS-PAGE gel (10-20% acrylamide). The bands correspond to the numbered peaks in each line on the graph. Each peak represents a protein concentration for that band. The area under the peaks, surrounded by the small vertical marks in each valley, is the area used to calculate the volume of each band. In graph displays bands on the x (horizontal) axis that are representative of protein concentration, and the optical density on the y (vertical) axis.

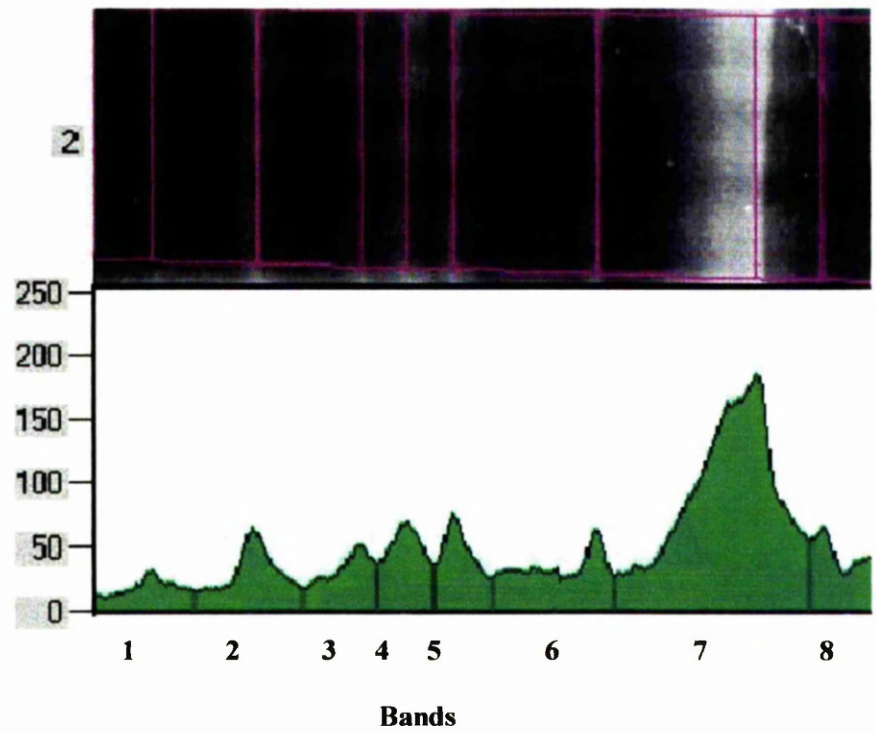


Figure 2.16. The Lane profile graph is representative of protein concentration in the lysate of stationary phase promastigotes of *L. mexicana* WT. The sample was loaded in gradient-SDS-PAGE gel (10-20% acrylamide). The bands in correspond to the numbered peaks on the graph. Each peak represents a protein concentration for that band. The area under the peaks, surrounded by the small vertical marks in each valley, is the area used to calculate the volume of each band. In graph displays bands on the x (horizontal) axis that are representative of protein concentration, and the optical density on the y (vertical) axis.

2.3.10. Two-dimensional electrophoresis (2-DE) used to differentiate two lines of *L. mexicana*

The protein analysis of promastigotes on the SDS-PAGE gradient gel showed differences in protein expression between stationary and log phase of promastigotes of the two lines of *L. mexicana*. To investigate further differences between these lines, the protein of lysates of stationary phase promastigotes of two lines was analysed using 2-DE.

Whole cell preparation of the two lines of *L. mexicana* was initially separated according to pH ranges 4-7 or 3-10 and then in the large-sized SDS PAGE gels (at least 20cm × 30cm). The proteins of *L. mexicana* H-line and *L. mexicana* WT were resolved on the large 2-DE gels (Figures 2.17 and 2.18 respectively) pl range 4-7, but the quality of gels pI 3-10 were poor (not shown). Approximately 160 spots were detected depending on silver-staining conditions. The comparison of the two patterns reveals high similarity, with several main spots are being similarly positioned. The majority of spots were numbered (Figures 2.19 and 2.20). The significant differences between attenuated line of *L. mexicana* and *L. mexicana* WT pattern have been found. Spot 101 of pattern of *L. mexicana* WT (Figure 2.21b) was shifted to a less acidic position in the pattern of *L. mexicana* H-line (Figure 2.21a). As Figure 2.22 (a) shows spot 105 was absent from the pattern of *L. mexicana* H-line. Two spots have been found in the spot in the pattern of *L. mexicana* WT (Figure 2.23b), whereas the expression of these proteins by promastigotes of *L. mexicana* H-line decreased (Figure 2.23a). Therefore, in the total proteins of *L. mexicana* H-line at least one protein was absent and the expression some of them decreased. However, this result is agreement with that of gradient SDS-PAGE gel. This data is insufficient to conclude that these proteins are similar to the bands, which were absent from gradient SDS-PAGE gel. The restriction of time prevented to repeat this test and analyse the gels.

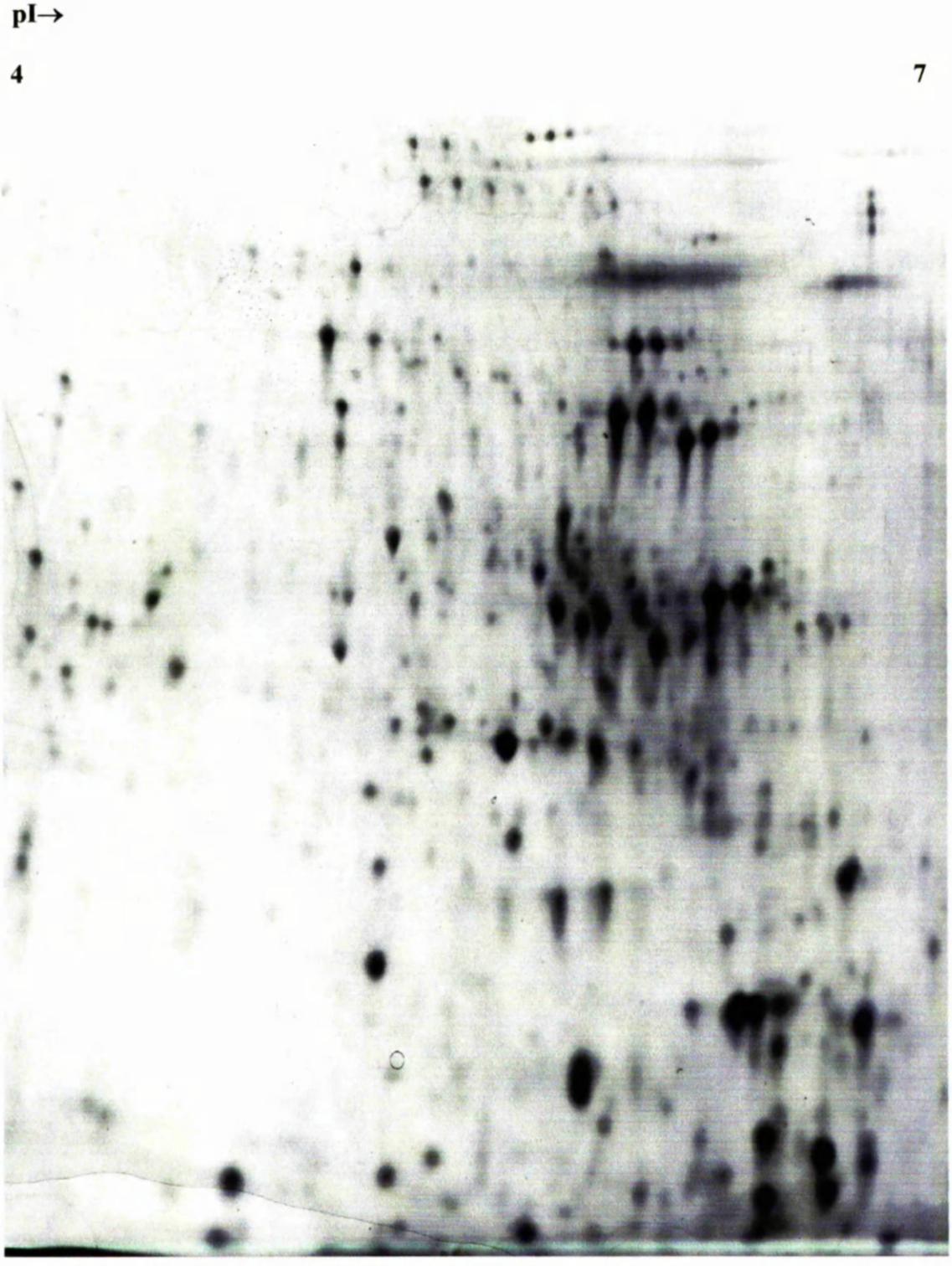


Figure 2.17. Two-DE gel of total protein of lysate of stationary phase promastigotes of *L. mexicana* H-line .

Figure 2.18. Two-DE gel of total protein of lysate of stationary phase promastigotes of *L. mexicana* WT.

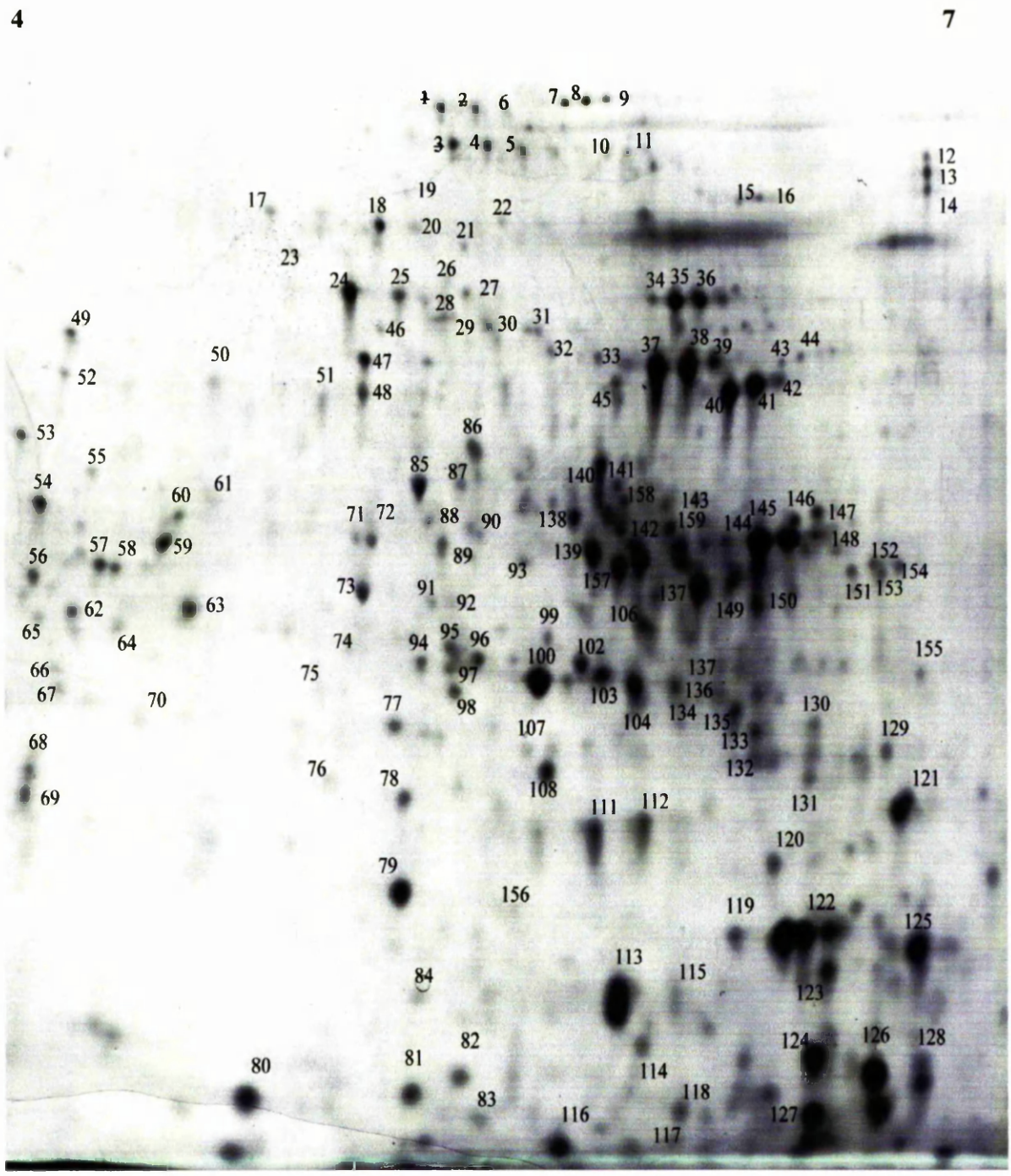


Figure 2.19. Two-DE gel of total protein of lysate of stationary phase promastigotes of *L. mexicana* H-line and the majority of spots were numbered.

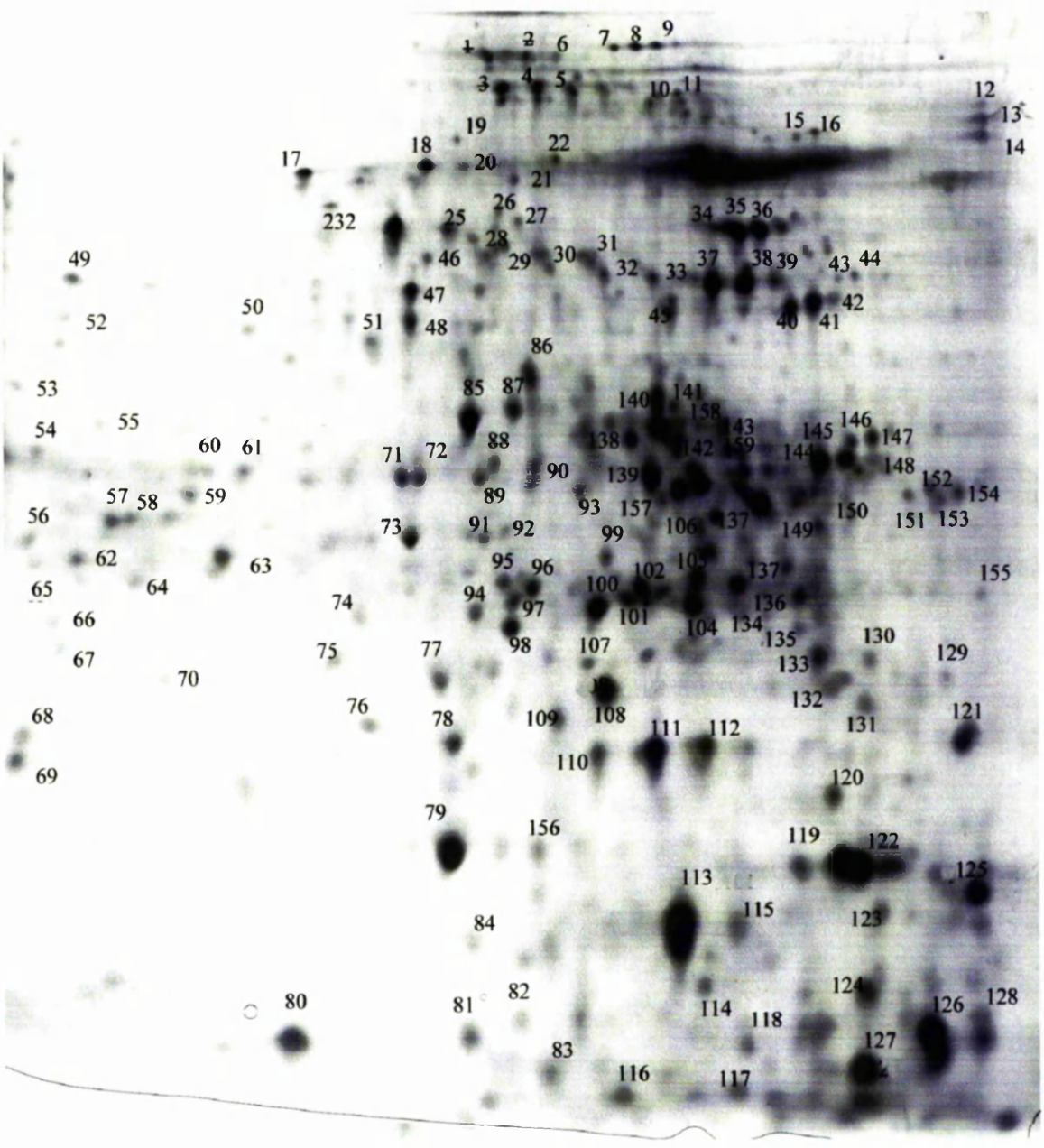


Figure 2.20. Two-DE gel of total protein of lysate of stationary phase promastigotes of *L. mexicana* WT and the majority of spots were numbered.

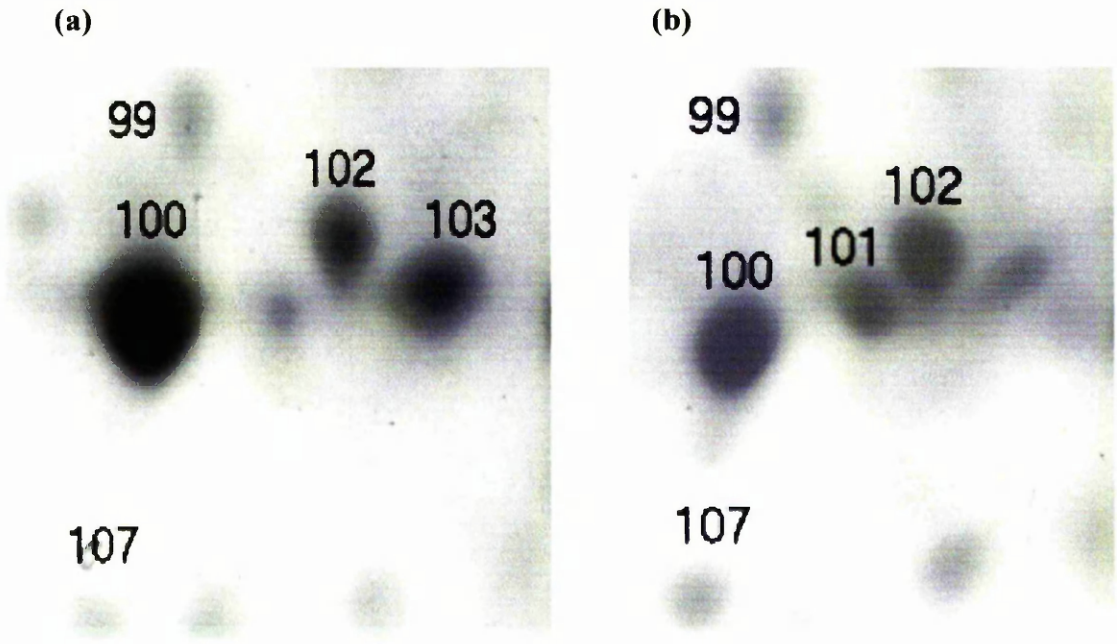


Figure 2.21. Pattern sectors showing a difference between total proteins of lysates of promastigotes of *L. mexicana* H-line (a) and *L. mexicana* WT (b). Spot 101 of protein expression of promastigotes of *L. mexicana* WT was shifted to right side (less acidic position) in the protein expression of promastigotes of *L. mexicana* H-line.

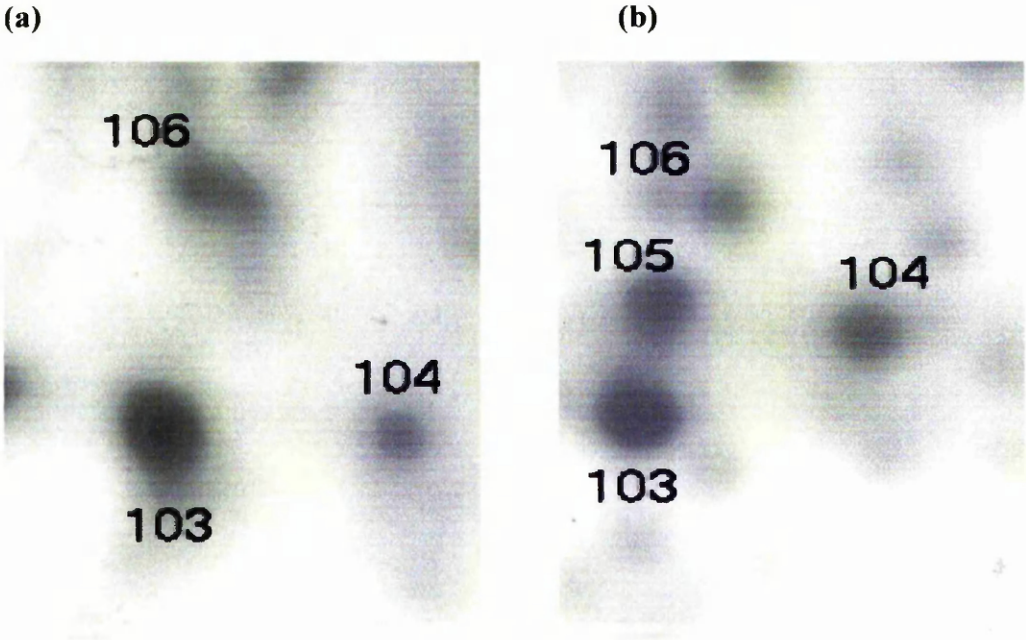
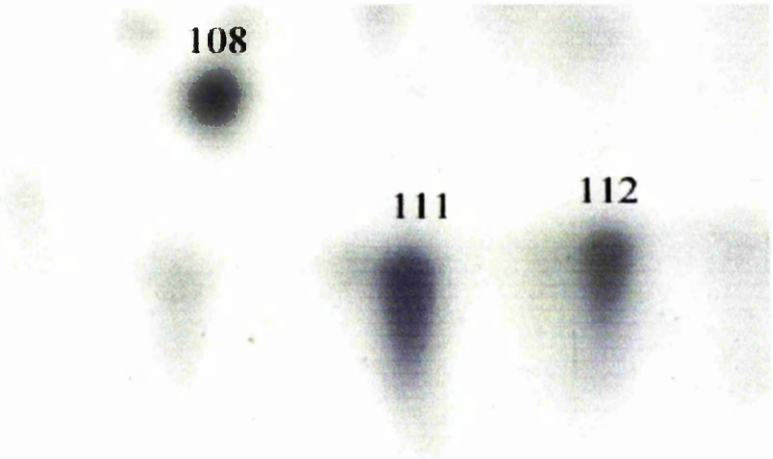


Figure 2.22. Pattern sectors showing another difference between total proteins of lysates of promastigotes of *L. mexicana* H-line (a) and *L. mexicana* WT (b). Spot 105 from lysate of stationary phase promastigotes of *L. mexicana* H-line was absent.

(a)



(b)

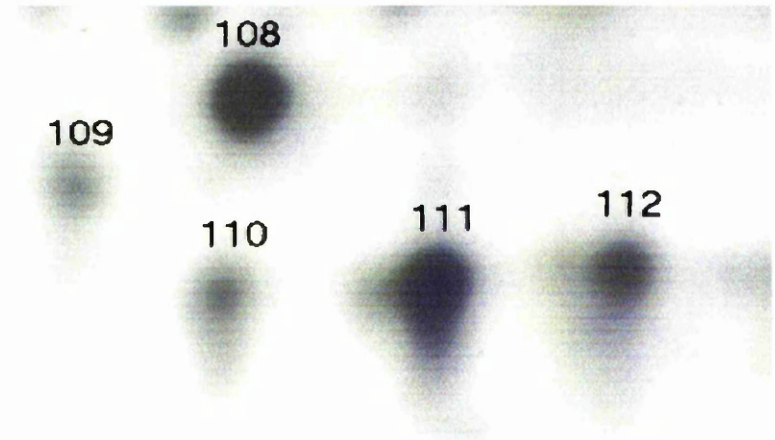


Figure 2.23. Pattern sectors showing other differences between total proteins of lysates of promastigotes of *L. mexicana* H-line (a) and *L. mexicana* WT (b). The region shown in (a) and (b) reveals two differences: the expression of two proteins (spots 109 and 110) decreased in the attenuated cell line.

2.4. Discussion

It is generally accepted that the protection induced by using attenuated forms of microorganisms as vaccines against some diseases is more effective than that from using other forms of vaccines (Hess *et al.*, 2000). Recently several methods have been developed to attenuate *Leishmania*, such as disruption of a gene controlling virulence from the genome of the wild type microorganism. For example, an attenuated line of *L. mexicana* Δ*cpa/cpb* (Mottram *et al.*, 1996) and an attenuated *L. major* lacking DHFR/TS gene (Titus *et al.*, 1995) have been created. I have now produced an attenuated cell line of *L. mexicana* by growing of *L. mexicana* WT in the presence of an antibiotic *in vitro* and an attenuated cell line of *L. major* using the same technique. This suggests that this method can be used for attenuating other species of *Leishmania* and maybe other microorganisms.

The attenuated line of *L. mexicana* Wild Type known as *L. mexicana* H-line has been established under pressure of gentamicin which was routinely added to the medium to prevent bacterial contamination. The mechanism by which gentamicin, an aminoglycoside, attenuates *L. mexicana* WT is unknown. Disruption of ribosomal activity by breaking up polysomes or misreading of mRNA during protein synthesis, resulting in incomplete protein synthesis, are possible mechanisms.

However there is not any evidence to show when the attenuated line *L. mexicana* formed. Attenuation required at least 20 passages in HOMEM medium supplemented with 10% (v/v) FCS and gentamicin at 20 μg/ml.

In the present study some experimental work has early been carried out with *L. mexicana Δcpa/cpb*. It was, therefore, necessary to demonstrate that promastigotes of *L. mexicana* H-line were not contaminated with promastigotes of *L. mexicana Δcpa/cpb*. Two different tests were set up:

i) Examination of proteinase activity of *L. mexicana* H-line.

ii) Culturing of *L. mexicana* H-line in the presence of 4 kinds of selective antibiotics, SAT or Pur or Ble or Hyg separately or in combination.

Robertson and Coombs (1992) reported that a high mobility band of cysteine proteinase (CP) activity can be detected in stationary phase promastigotes of *L. mexicana* using gelatin-SDS-PAGE. The proteinase activity of stationary phase

promastigotes of *L. mexicana* H-line and *L. mexicana* WT was investigated by loading lysates on gelatin-SDS-PAGE gels. Three bands were detected of which the molecular mass of the highest mobility band of proteolytic activities was 20 kDa (Figure 2.11). These proteinases are cysteine proteinase is supported by the report that the analysis of CPB isoenzymes expressed by stationary phase promastigotes of *L. mexicana* WT using Western blotting with anti-CPB antiserum detected two major proteins (25 and 29 kDa) (Mottram *et al.*, 1997). This study therefore, demonstrated that promastigotes of *L. mexicana* H-line, at least, are not deficient in CPs.

Souza and colleagues (1994) reported *L. mexicana Δcpa* was generated by disrupting sequentially both alleles of *lmcpa* using gene-targeting of promastigotes with hygromycin- and phleomycin-resistance markers. It has been shown that *L. mexicana* cysteine proteinase-deficient mutants are resistant to the selective antibiotics such as puromycin, zeocin, and hygromycin B (Souza *et al.*, 1994). Therefore promastigotes of *L. mexicana* H-line, *L. mexicana Δcpa/cpb* (positive control), and *L. mexicana* WT (negative control) were individually grown in the media with 4 kinds of selective antibiotics (separately or combinations of them). As the graphs show (Figures 2.3 and 2.4) promastigotes of both *L. mexicana* H-line and *L. mexicana* WT were unable to grow in the presence these antibiotics. In contrast, there were no significant differences among the growth rates of promastigotes of *L. mexicana Δcpa/cpb* in the media with or without antibiotic. These results suggest that promastigotes of *L. mexicana* H-line are neither deficient in CPs, nor is this line mixed with *L. mexicana cpa/cpb* double knockout.

The length of 12% of stationary phase promastigotes of *L. mexicana* H-line was larger than promastigotes of *L. mexicana* WT promastigotes. As Table 2.2 shows, the size of stationary phase of promastigotes of *L. mexicana* WT was 1.5-3 × 10-18 μm, whereas the size of 12% promastigotes of *L. mexicana* H-line was 1.5-2 × 19-23 μm.

The ability of the attenuated line of *L. mexicana* to survive *in vivo* for 3 months, was investigated by studying the epidermal cells from where the parasite was injected. The cells were transferred into medium supplemented with or without gentamicin. In the epidermal cell some of the infected mice, amastigotes differentiated to promastigotes and this cell line was designed *L. mexicana* HAD-line. It was observed that promastigotes of *L. mexicana* HAD-line grew poorly in the medium with or without gentamicin. This suggests that the attenuated cell line maybe unable to survive in the

midgut of sandfly and if so, this would reduce the risk of virulent reversion and spread of disease by infected sandflies.

The light micrographs of cells from cultures of *L. mexicana* HAD-line (Figure 2.11) showed that 17% of the cells were promastigotes and 83% of them were amastigote-like although some of them had just a small flagellum. The morphology of promastigotes of *L. mexicana* HAD-line were compared with promastigotes of *L. mexicana* WT which were derived from amastigotes within epidermal cell of infected mice at 3 months post infection (Figure 2.12). As the Figures 2.11 and 2.12 show, there are remarkable differences between the morphology of *L. mexicana* HAD-line and *L. mexicana* WT.

There was no significant difference between the growth rate of promastigotes of *L. mexicana* H-line and *L. mexicana* WT *in vitro*.

In the present study, it was demonstrated that the adaptation of *L. mexicana* H-line to grow in the presence of gentamicin involved changes of protein expression. Total lysate protein of stationary or log phase promastigotes of *L. mexicana* H-line and WT were individually loaded on the 10-20% SDS-PAGE gradient gel. The result shows that a number of differences in protein expression observed. Two bands were detected around 66 kDa with stationary or log phase promastigotes of *L. mexicana* WT, whereas one (possible two) bands were absent with stationary and log phase promastigotes of *L. mexicana* H-line.

The optical density of protein expression of the two lines of *L. mexicana* in 10-20% SDS-PAGE gradient gel was displayed in a Lane profile graph, using Lab Works Image Acquisition and Analysis Software (UVP Laboratory products). The Lane profile graph indicates protein concentration in the lysates of stationary phase promastigotes of *L. mexicana* H-line and *L. mexicana* WT. The bands correspond to the numbered peaks on the graph. Each peak represents a protein concentration for that band. The graph of protein of lysate of stationary phase promastigotes of *L. mexicana* H-line showed two peaks to be present in *L. mexicana* WT (3 and 4) which were absent in *L. mexicana* H-line (Figures 13 and 14).

The results of protein analysis of promastigotes of *L. mexicana* H-line and *L. mexicana* WT on the SDS-PAGE gradient gel have elicited a number of differences in protein expression. To confirm whether the differences between *L. mexicana* attenuated line and WT, the protein from lysates of stationary phase promastigotes of the two lines were analysed using immobilized pH gradients. The comparative

proteome analysis of two lines of *L. mexicana* using high-resolution techniques has been done using isoelectric focusing (IEF), which separate proteins according to their isoelectric points (pI) and SDS-PAGE, which separates proteins according to their molecular weights (MW). Both techniques were modified as high-resolution methods (Jungblut *et al.*, 1999).

The sample preparation is absolutely crucial for good 2-DE results; the samples were initially prepared using two methods and the number of spots on the gels were increased using method number 2. The protein lysates of 2×10^8 cell / ml stationary phase promastigotes of *L. mexicana* H-line and *L. mexicana* WT were initially separated according to pH ranges 4-7 or 3-10 and then in the large-sized SDS PAGE gels (at least 20 cm × 30cm). Both lines of *Leishmania* comprise patterns with a high density of spots in the range pH 4-7 of the gel. The position of spots in the gels of the two lines of *L. mexicana* revealed high similarity, and the main spots were compared easily. One hundred and fifty nine, that is the majority of spots, were labelled (Figures 2.19 and 2.20) and some differences between the patterns of the attenuated line of *L. mexicana* and *L. mexicana* WT were detected. Spot 101 in *L. mexicana* WT gel (Figure 2.21 b) was shifted to a less acidic position in *L. mexicana* H-line (Figure 2.21a). As Figure 2.22 (a) shows spot 105 is absent in *L. mexicana* H-line. Two spots (109 and 110) were found in the *L. mexicana* WT gel (Figure 2.23 b), whereas the expression of these proteins by promastigotes of *L. mexicana* H-line decreased (Figure 2.23a).

The comparative proteome analysis of the two lines of *L. mexicana* in the pH ranges 3-10 (data not shown) and 4-7 indicates that further runs in the pH 4-7 range are required. To identifying proteins in the lysate of stationary phase promastigotes of *L. mexicana* H-line, which were lost or reduced compared with *L. mexicana* WT, mass spectrometry with database searching has been suggested (Beavis and Fenyo, 2000). Although, the data of 2DE of the two lines have been supported with the results of gradient SDS-PAGE gel. There are insufficient replicates to draw firm conclusions about the absence or decreased expression of the proteins in 2-DE.

An attenuated cell line of *L. major* was developed using pressure of gentamicin by the same method that was described for attenuation of *L. mexicana* WT. The attenuated *L. major* was generated by culturing of promastigotes of *L. major* WT in the medium supplemented with 10% (v/v) HI-FCS and 20 µg / ml gentamicin after 11 passages.

There was no significant difference ($p>0.5$) between the growth rates of promastigotes of the two lines of *L. major*. The culture medium of stationary phase promastigotes of *L. major* WT (pH 6.3) was more acidic than that of *L. major* H-line (pH 6.9).

CHAPTER THREE

IMMUNE RESPONSE TO *LEISHMANIA MEXICANA* H-LINE AND PRELIMINARY OBSERVATIONS ON *LEISHMANIA MAJOR* H-LINE

3.1. Introduction

The leishmaniases comprise a group of diseases caused by the intracellular protozoan parasite *Leishmania*. In humans, the localized cutaneous infection caused by *L. mexicana* is often associated with chronic infection of the ear on the pinna (Peters *et al.*, 1987).

It is generally accepted that immunity to *Leishmania* depends on the effective generation of cell-mediated immunity (CMI). This CMI has been associated with increasing leishmanicidal activity of macrophages, the expansion of the $CD4^+$ Th1 cell subset in resistant mice, and the production of cytokines such as IL-12 and IFN-γ (Heinzel *et al.*, 1991). On the other hand, non-healing responses in susceptible BALB/c mice have been related to the expansion of the $CD4^+$ Th2 cell subset and the production of cytokines such as IL-4 and IL-10 (Scott *et al.*, 1988). The role of IL-4 was shown to be due to its ability to inhibit macrophage leishmanicidal activity and down-regulate the development of a Th1-like response (Oswald *et al.*, 1992). It was demonstrated that normally genetically susceptible mice lacking IL-4 were protected from cutaneous infection with *L. major* (Kopf *et al.*, 1996) as well as *L. mexicana* (Satoskar *et al.*, 1995; Satoskar *et al.*, 1997). However, other studies suggest that the inability of the host to generate an IL-12-initiated Th1-like response and produce IFN-γ rather than the induction of a Th2-like response and IL-4 production may be the crucial factor in determining susceptibility to *L. mexicana* (Guevara-Mendoza *et al.*, 1997). C57BL/6 and 129Sv/Ev mice which are susceptible to *L. mexicana* (Alexander *et al.*, 1998) and produce a Th2-response, whereas IL-4-deficient C57BL/6 and 129Sv/Ev mice developed a Th1-like response, as measured by an increase in IFN-γ production, and cure *L. mexicana* infection (Satoskar *et al.*, 1995).

It has been reported that both IFN-γ and IL-2, only in combination, induce TNF-α-specific mRNA and secretion of TNF-α by macrophages. Development of intracellular killing activity by activated macrophages also requires the autocrine effects of TNF-α (Nacy *et al.*, 1991). Two consecutive signals are required to activate fully the nitric oxide synthase (NOS), which appears to be toxic for *Leishmania* (Liew *et al.*, 1990; James, 1995). The first signal is IFN-γ and TNF-α is a common second signal that mediates microbicidal functions as was shown in IFN-γ-primed macrophages exposed to microbial products (James, 1995). Neutralization of either TNF-α or IFN-γ, or inhibition of nitric oxide production, leads to the exacerbation of

disease (Liew *et al.*, 1990; Evans *et. al.*, 1993). Proteophosphoglycan (PPG) which is present on the surface of amastigotes of several *Leishmania* species, inhibits the production of TNF-α and thus reduces nitric oxide by macrophages generation (Piani *et al.*, 1999).

The effects of IFN-γ on Th1 development may be mediated *via* action on the macrophages thus up-regulating IL-12 production or by direct effects on T cells (Trinchieri, 1995). Susceptible BALB/c mice infected with *L. mexicana* WT, produce a substantial amount of IL-4 and IL-10 (Guler *et al.*, 1996).

Laskay and colleagues (1993) showed that NK cells play a crucial role in the early control of leishmaniasis in resistant mice. The local restriction of the parasite in the early phase of the infection is mediated by the innate immune system and this function is important in the subsequent development of a protective T cell response (Laskay *et al.*, 1995). Moreover, Diefenbach and colleagues (1998) found that in the presence of iNOS and IFN-α/β, *L. major* was localized in the skin and the draining LN of susceptible mice. In contrast, in $NOS2^{-/-}$ mice or Wild Type resistant mice treated with the NOS2 inhibitor, the parasite disseminated in the spleen, bone marrow, lungs, and liver from day one of infection.

In other studies it has been reported that the production of the IgG1 antibody isotype, although not completely dependent on IL-4 and IL-4 signalling (Snapper and Paul, 1987) is associated with the development of a Th2-like response. Generation of IgG2a is dependent on IFN-γ, whereas IL-4 is important for the production of high level of IgG1 in BALB/c mice infected with the *Leishmania* (Kuchroo *et al.*, 1995).

In summary, several groups of researchers have clearly demonstrated that protective immunity against the *L. mexicana* complex, is ultimately dependent upon generation of a Th1-like response and IFN-γ production (Satoskar *et al.*, 1995; Guevara-Mendoza *et al.*, 1997).

3.2. Materials and Methods

3.2.1. Mice

Female BALB/c mice age 4-6 weeks, were purchased from HARLAN/OLAC and maintained at the Joint Animal Facility of Glasgow University. The mice were approximately 12 weeks old at the start of each experiment.

3.2.2. Parasite

Promastigotes of *L. mexicana* WT or *L. major* WT were grown in HOMEM medium supplemented with 10% FCS (see section 2.2.1). Promastigotes of *L. mexicana* H-line or *L. major* H-line, which were established in the presence of gentamicin, grown in the same medium supplemented with 20 μg/ml gentamicin and incubated at 25°C (see section 2.2.2).

3.2.3. Stability of *L. mexicana* H-line in medium free of gentamicin

To determine whether the *L. mexicana* H-line is stable in gentamicin-free medium *in vitro*, promastigotes of the *L. mexicana* H-line were on two occasions transferred into HOMEM with or without gentamicin and incubated at 25°C. The mid to late log-phase promastigotes of *L. mexicana* H-line, after 58 passages in the presence of gentamicin, were transferred into medium with or without gentamicin (for more detail see section 2.2.1). The ability of promastigotes to infect macrophages and their survival within macrophages was examined after 37 and 40 passages in the medium with or without gentamicin.

3.2.4. Preparation of bone marrow-derived macrophages (BMM)

BMM were obtained from a culture of bone marrow cells collected from the femurs and tibias of naive mice. The bones were removed from BALB/c mice and the proximal end of the femur and the distal end of the tibia were cut, leaving the opposite ends intact. A 26 gauge needle was inserted into the intact ends and the bone marrow flushed out through the cut end with 5 ml ice-cold Dulbecco's modified Eagle's medium (DMEM) (Gibco). The cells were collected and centrifuged (600 g for 10 min at 4°C). The pellets were resuspended in DMEM supplemented with 20% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) (Life Technologies, Grand Island, NY), 2-β-mercaptoethanol (2ME) (50 μM) (Life Technologies, Grand Island, NY), supernatant from L-929 cell cultured (30%), L-glutamine (2 μM), and pyruvate (1 μM) (Sigma). The cells were transferred to a 100 mm tissue culture dish (Greiner) and incubated at 37°C in 5% CO_2 in air for 7 days. Following removal of the non-adherent cells, the adherent cells were collected by rinsing the dish with ice-cold PBS for 10 min. The adherent cells were centrifuged at 600 g for 10 min, resuspended in completed DMEM, and transferred into the individual wells of a chamber slide (Nalge Nunc, Lab Tek) (2-3 × 10^6 cells / well). Following incubation overnight, the non-adherent cells were removed and the supernatant was refreshed with complete DMEM without the L-929 cell supernatant supplement.

3.2.5. L-929 cell fibroblast medium

L-929 cells (European collection of animal cultures, No: 85011425) were grown in DMEM supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% FCS, and incubated at 37°C in 5% CO_2 in air. Supernatant from the cultured L-929 cells was collected after 2-3 days and the floating cells were discarded. The bottom of the flask was covered with 2 ml ice cold 10% trypsin for 30 sec. The excess trypsin was removed and the flask incubated for 5-15 min. Following two washes, the pellets were resuspended in the medium and incubated at 37°C in 5% CO_2 in air. The supernatant of L-929 cell line was added to bone marrow cultures as described above to enhance maturation of BMM (Wolfram *et al.*, 1996).

3.2.6. Preparation of IFN-γ-primed BMM

Following preparation of BMM (see section 3.2.5) the number of viable cells was estimated by trypan blue exclusion using a Neubauer Haemocytometer, which were transferred to the wells of chamber slides (2-3 × 10^6 cells per well), and incubated overnight at 37°C in 5% CO_2 in air. The supernatants were replaced with 100 µl / well of DMEM supplemented with 10% FCS and 10 U/ml of Recombinant cytokine (rIFN-γ) (PharMingen) and incubated at 37°C in 5% CO_2 in air for 6 h. The supernatants were discarded and the IFN-γ-primed BMMs were exposed to stationary phase promastigotes of the two lines of *L. mexicana*.

3.2.7. Interaction between promastigotes of *L. mexicana* and BMM

Stationary phase promastigotes of *L. mexicana* WT or *L. mexicana* H-line were harvested and washed 3 times with PBS. The cells were resuspended in complete DMEM at a concentration of 5 × 10^6 promastigotes / ml and transferred into wells of chamber slides containing BMM (400 µl/well). The infection ratio of macrophage / promastigote was 1:1. The culture slides were incubated at 32°C in 5% CO_2 in air for 3 h. Following removal of non-adherent promastigotes by replacing the overlying medium with fresh complete DMEM the cells were incubated for the appropriate time at 32°C in 5% CO_2 in air. At the end of the incubation period the supernatant was collected and stored at -70°C. The macrophages were stained with Giemsa's stain and the percentage of infected macrophages determined by microscopy.

3.2.8. Nitric oxide activity measured by nitrite assay

Nitrite concentration in the tissue culture supernatants was measured by the Greiss reaction. Supernatant from BMM culture was collected and centrifuged (Beckman Microfuge TJ6-R) at 600 g. The Greiss reagent consist of 1 part 0.1% naphthylethylene diaminedihydrochloride (Merck) in distilled water plus 1 part 1% sulfanilamide (Merck) in 14% concentrated HCl, the 2 parts being mixed together

within 12 h of using and kept chilled. Each part may be stored refrigerated for up to 2 months. 50 or 100 µl aliquots were removed from conditioned medium and incubated with an equal volume of Greiss reaction at room temperature for 10 min. The absorbance at 550 nm was measured in an ELISA reader (Dynatech Laboratories). Nitrite concentration was calculated from a standard curve, which was normally linear between 0 and 100 µM sodium nitrite.

3.2.9. T cell proliferation assay

T cell proliferation assays were performed as described by Satoskar and colleagues (1997). Briefly, the infected mice were killed at 12 weeks post infection and their spleens removed aseptically. Single cell suspensions were prepared from each spleen by gently teasing them in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2-β-mercaptoethanol (2ME) (50 µM) (Life Technologies). The cell suspension was centrifuged at 600 g for 10 min at 4°C, and resuspended in 3 ml of Boyle's solution (0.17 M Tris-HCl, pH 7.2 and 0.16 M ammonium chloride) to lyse the erythrocytes. After 3 min incubation at 37°C the spleen cells were centrifuged at 200 g for 10 min at 4°C, washed three times with PBS, and the pellets resuspended in 3 ml of complete DMEM. The number of viable splenocytes was estimated by trypan blue exclusion using a Neubauer Haemocytometer and the cell concentration adjusted to 5×10^6 viable cells / ml. The cell suspension (100 µl / well) was transferred into triplicate wells of sterile 96-well flat-bottom tissue culture plates (Falcon). An equal volume of stationary phase promastigotes at a concentration of 5×10^5 cells / ml was added, the ratio of promastigote / splenocyte was 1:10. Con A (5 µg / ml) (Sigma) was used as a positive control for cell proliferation, while complete DMEM alone was used as a negative control. The cells were initially incubated at 32°C for 4 hr and then transferred to 37°C for 56 h in 5 % CO_2 in air. After 56 h [^{3}H] thymidine (1 µCi / well) (Amersham) was added to each well and the plate incubated at 37°C for a further 12 h. The cells were harvested onto filter paper (Tomtec, Hamden, CT), and [^{3}H]-uptake was measured by liquid scintillation in a beta-scintillation counter. Supernatants were

collected from parallel cultures after 72 hr of incubation for ELISA quantification of cytokine production.

3.2.10. Cytokine ELISA

Supernatants collected from spleen cell cultures were assayed for the presence of IL-2, IL-4, IL-10, and IFN-γ using ELISA, as previously described (Satoskar *et al.*, 1995). In brief, wells of a 96-well flat-bottom microplate (Nunc) were coated with 50 µl of the appropriate capture mAb (purified rat anti-mouse IL-2, IL-4, IL-10, and IFN-γ) (all from PharMingen) at a concentration of 2 µg / ml in coating buffer (0.1 M of Na_2HPO_4, pH 9.0) and incubated at 4°C overnight. Following 3 washes with washing buffer (PBS / 0.05% Tween-20), the wells were blocked with 200 µl of blocking buffer (10% FCS in PBS, pH 7.4) and incubated at 37°C for 1 h. The culture supernatants and serial dilutions of Recombinant cytokine standards (rIL-2, rIL-4, rIL10, and rIFN-γ) (PharMingen) were added to the wells in 50 µl volumes in triplicate. The cells were incubated at 37°C for 3h and the wells were washed 4 times in washing buffer. Biotinylated rat anti-mouse cytokine (IL-2, IL-4, IL-10, and IFN-γ) (2 µg / well) (all from PharMingen) antibodies were added and incubated for 1 h at 37°C. 100 µl streptavidin-linked peroxidase (1/1000 dilution in 10 % FCS-PBS) (Diagnostics Scotland) were added and the cells incubated at 37°C for 1 h. After the final washing with washing buffer, 100 µl of tetramethylbenzidine (TMB) substrate (Dynatech labs) were added to each well. The OD of the wells was read at 630 nm on an ELISA reader. The concentration of cytokines in the samples was calculated by reference to the standard curve.

3.2.11. Preparation of *Leishmania* lysate antigen (LLA)

LLA was prepared by a freeze-thaw method as described by Reed and colleagues (1986). In brief, the stationary phase promastigotes of two lines of *L. mexicana* were washed with ice-cold PBS 3 times. The promastigotes were disrupted by freezing at -70°C and thawing at 37°C for 3 times. They were centrifuged for 30 min at 200 g at

4°C for 10 min. The supernatant was aliquoted and stored at -20°C until just prior to use.

3.2.12. Protein concentration

The protein concentration of lysate was determined using Coomassie plus Reagent (Sedmark and Grossberg, 1977). 150 µl of albumin standard (Pierce U.S.A.) or sample was added to the microwell plate and 150 µl of PBS for blank wells. Coomassie Plus Reagent (150 µl) was added to each well and the plate mixed on the plate shaker for 30 sec. The absorbence was measured at 595 nm on a plate reader.

3.2.13. Determination of specific antibodies (Abs) in the serum

The *Leishmania*-specific levels of the Th2-associated IgG1 Ab and the Th1-associated IgG2a Ab (Snapper and Paul, 1987) were measured by ELISA as described by Satoskar and colleagues (1995). Peripheral blood was obtained from mice infected with two lines of *L. mexicana* by tail bleeding into Eppendorf tubes and then centrifuged at 200 g. The sera were collected and stored at -70°C until just prior to use. Each well of a flat-bottom microtitle plate (Nunc, Roskilde, Denmark) was coated with 1 µg of LLA (from section 3.2.12) in 0.1 M carbonate buffer pH 9.6 and incubated at 4°C overnight. Following washing 3 times with PBS and 0.05% Tween 20 (Sigma, St Louis, MO), the plates were blocked with 200 µl in blocking buffer (PBS / FCS 10%), and incubated at 37°C for 1 h. After 3 washes, 50 µl of serially diluted serum samples (1/100 in PBS / 10% FCS) were added to wells, incubated for 2 h at 37°C, and washed 4 times. Bound Abs were detected by 50µl / well of biotinylated anti-mouse either IgG1 or IgG2a (PharMingen, San Diego, CA) (2 µg/ml), incubated at 37°C for 1h and washed 6 times. 100 µl streptavidin-linked peroxidase (1/1000 dilution in 10 % FCS-PBS) (Diagnostics Scotland) were added and incubated at 37°C for 1 h. After the final washing with washing buffer, 100 µl of tetramethylbenzidine (TMB) substrate (Dynatech labs) were added to each well. The

OD of the wells was read on an ELISA reader at 630 nm with a reference filter at 405 nm.

3.2.14. Preparation of murine rump epidermal cells (ECs) to investigate the presence of the parasite

Twelve weeks after inoculation of 5×10^6 (cell / ml) stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT into the shaven rump mice were killed and the Ecs prerared as described in section 2.2.7. The number of viable ECs was estimated by trypan blue exclusion using a Neubauer Haemocytometer, the cell concentration adjusted to 5×10^6 viable cells / ml in DMEM medium supplemented with 10% (v/v) HI-FCS, and incubated at 25°C.

3.2.15. Preparation of murine ear ECs

ECs suspensions were prepared from mouse ear skin by trypsinization. BALB/c mice were killed, the whole ears rinsed with 70% ethanol for 5 min, and dried. The ears were cut off and the skin sheet was then carefully separated. The skin, dermal side down, was floated on 1% trypsin (90 min) for processing thick ventral ear halves and 0.6% trypsin (45 min) for thin dorsal ear halves. The epidermis was separated from the dermis and the exposed basal layer of the loosened epidermis was covered with RPMI 1640 medium. Single EC suspensions were prepared with the aid of a cell scraper. The cells were washed twice in RPMI and the number of viable ECs was estimated by trypan blue exclusion using a Neubauer Haemocytometer. The cell concentration was adjusted to 5×10^6 viable cells / ml of DMEM supplemented with 10 % (v/v) HI-FCS, penicillin (100 U / ml) and streptomycin (100 μg/ml) (Life Technologies, Grand Island, NY), 2ME (50 μM) (Life Technologies, Grand Island, NY), supernatant L-929 cells (30%), L-glutamine (2 μM), and pyruvate (1 μM) (Sigma). The suspension was transferred into the individual wells of a chamber slide (Nalge Nunc, Lab Tek) at 5×10^6 cells /well and incubated at 25°C.

3.2.16. Interaction between ECs and promastigotes of two lines of *L. mexicana*

Stationary phase promastigotes of *L. mexicana* WT or *L. mexicana* H-line were harvested and washed 3 times with PBS. The cells were resuspended in complete DMEM at a concentration of 5×10^6 promastigotes / ml and transferred into wells of chamber slides containing ECs (100 µl / well). The infection ratio of epiderma cell / promastigote was 10:1. The culture slides were incubated at 32°C in 5% CO_2 in air for 24 h. For depletion of extracellular parasites, the cultures of parasites and adherent macrophages were washed. The cells were stained with acridine orange / ethidium bromide (3.2.18) and the chamber slides were quickly air dried, fixed in absolute methanol, and stained in 10% (v/v) Giemsa's stain (Merck) in Giemsa's buffer (pH 7.2) for 10 min. The number of infected macrophages was determined by microscopy.

3.2.17. Staining with acridine orange and ethidium bromide

The supernatants of cultures (from section 3.2.17) were collected and centrifuged at 600 g for 10 min. The cells were resuspended in a mixture of acridine orange (5 µg/ml) (Michrome, Edward Gurr, Ltd) and ethidium bromide (50 µg/ml) (Sigma) and incubated for 10 min at room temperature. Following washing with PBS, the cells were resuspended in para-formaldehyde (1%) (Sigma) and incubated for 15 min at room temperature. Aliquots of suspension (0.5 ml) were centrifuged on the Cytospin (Shandon) at 1600 g for 5 minutes. The smears were analysed by fluorescence microscopy.

3.2.18. Preparation of tissues, and culture *in vitro* of *L. mexicana* isolated from infected mice.

Following injection of 5×10^6 stationary phase of promastigotes of *L. mexicana* WT or *L. mexicana* H-line, the parasite loads in the mice were determined by limiting

dilution analysis. Briefly, visceral organs of the mice infected with the two lines of *L. mexicana* were removed aseptically and single cell suspensions prepared. For each mouse the liver, draining lymph node, lung and spleen were homogenized separately in HOMEM medium supplemented with 10% FCS. Bone marrow cells were collected from the femurs and tibias of infected mice (see section 3.2.5.) A limiting dilution (LD) of the cell suspensions *in vitro* culture was used to determine the number of viable parasites in these organs (Laskay *et al.*,1995). The serial twofold dilutions of the cell suspensions from the organs were plated in the 24-well plates. The cells were then incubated at 25°C for 14 days. The cultures were examined daily for the presence of promastigotes.

3.2.19. Statistical analysis

Data are expressed as the mean ± standard error mean (SEM) for each group. Statistical analysis was performed using Student t test (for the final determination of significance testing the effects of treatments).

3.3. Results

3.3.1. Preparation of bone marrow derived macrophages

In this study, bone marrow macrophages were obtained from adherent mononuclear cells in the bone marrow (1.8×10^7 cells per mouse). In the presence of granulocyte-macrophage-colony stimulating factor (GM-CSF), non-adherent precursor cells differentiate into adherent mononuclear phagocytes. Mononuclear phagocytes at various stages of differentiation represent approximately 4% of all nucleated bone marrow cells (Guilbert *et al.*, 1980 and Byrne *et al.*, 1981).

3.3.2. Interactions between stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT with BMM

To determine whether *L. mexicana* H-line has the ability to infect macrophages, an *in vitro* culture system was used. Stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT were incubated with BMM for 3 h, after which non-attached promastigotes were washed off. The macrophage culture was incubated for up to 96 h and the rate of infection of macrophages was determined. As shown in Figure 3.1, 53% of BMM were infected with *L. mexicana* H-line at 9 h post infection. The percentage of infected cells decreased over time to 0.4% at 96 h post infection (Figure 3.1). The initial infection of macrophages with *L. mexicana* WT was similar to that with *L. mexicana* H-line (50%), but in contrast to the *L. mexicana* H-line, the percentage of infected macrophages increased to 72% at 96 h post infection. As regards the numbers of amastigotes surviving and growing within the macrophage, after 9 h. these were 98 amastigotes /100 macrophage for *L. mexicana* H-line. This rapidly decreased to 2 amastigotes/100 macrophages at 96 h post infection (Figure 3.2). This was in direct contrast to the observed increase in number of amastigotes of *L. mexicana* WT.

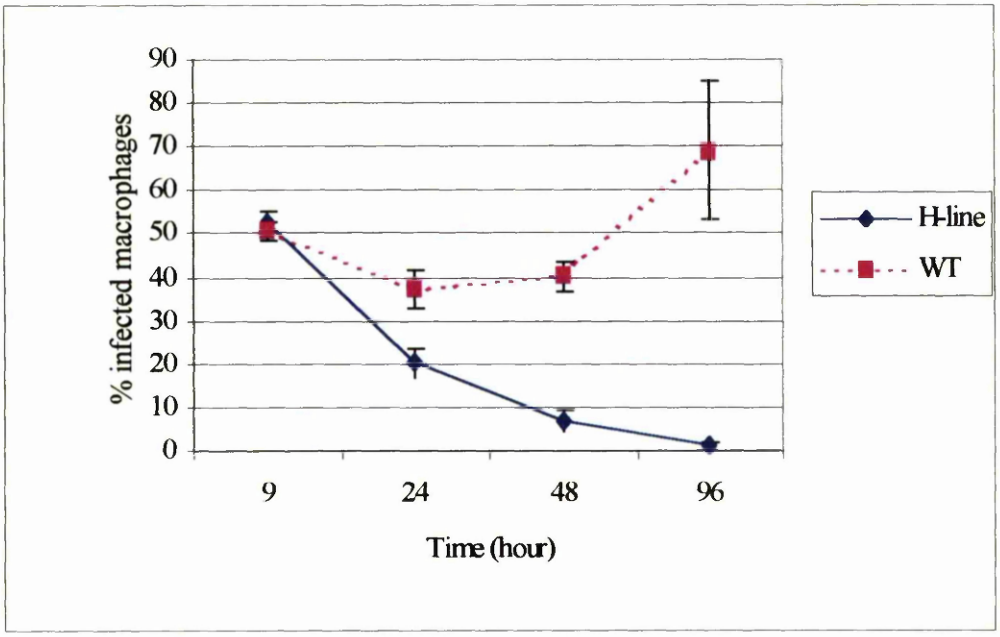


Figure 3.1. Infection of BMM with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. The promastigotes of the two lines were grown on parallel cultures with the same ages. Macrophages were exposed to promastigotes at 32°C for 3 h and subsequently infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

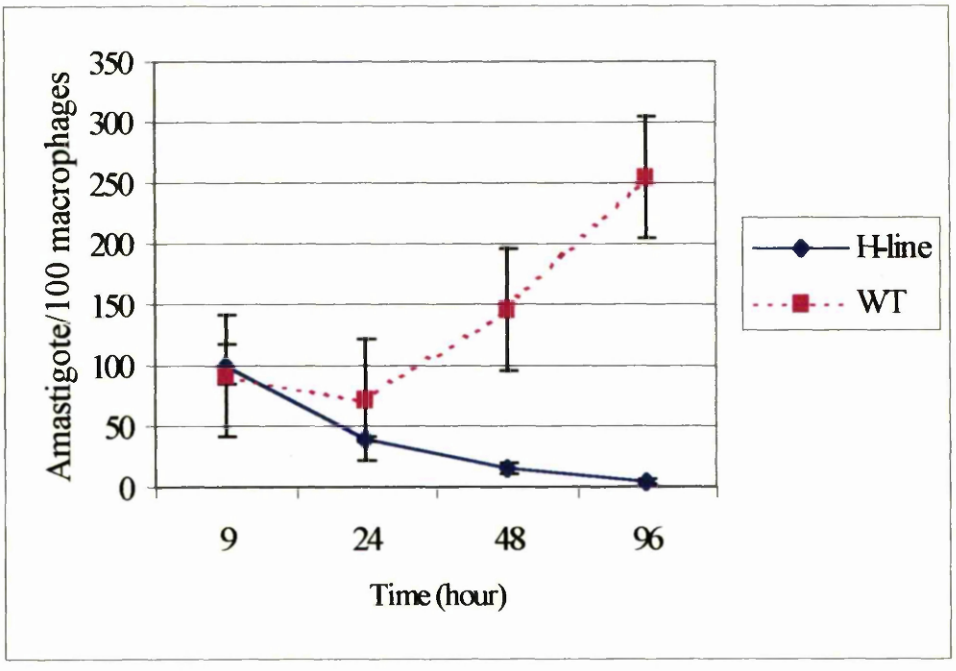


Figure 3.2. The number of amastigotes within infected macrophages with two lines of *L. mexicana*. BMM were infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. Macrophages were exposed to promastigotes at 32°C for 3 h and subsequently infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

3.3.3. Interactions between stationary phase promastigotes of *L. major* H-line or *L. major* WT with BMM

To determine whether *L. major* H-line has the ability to infect macrophages, an *in vitro* culture system was used. Stationary phase promastigotes of *L. major* H-line or *L. major* WT were incubated with BMM for 3 h, after which non-attached promastigotes were washed off. The macrophage culture was incubated for up to 96 h and the percentage of infected macrophages was determined. As shown in Figure 3.3, 41% of BMM were infected with *L. major* H-line at 8 h post infection. The percentage of infected cells decreased over time to 10.5% at 96 h post infection (Figure 3.3). The initial infection of macrophages with *L. major* WT was 46.5%, but in contrast to the *L. major* H-line, the percentage of infected macrophages increased to 65% at 96 h post infection. The number of amastigotes of *L. major* H-line within infected macrophages after 9 h incubation was 94 amastigotes / 100 macrophages. This rapidly decreased to 14 amastigotes/100 macrophages at 96 h post infection (Figure 3.4). This was in direct contrast to the observed increase in the number of amastigotes of *L. major* WT. BMMs were exposed to promastigotes of each line in two chamber slides individually on just one occasion. The restriction of time did not permit this experiment to be repeated.

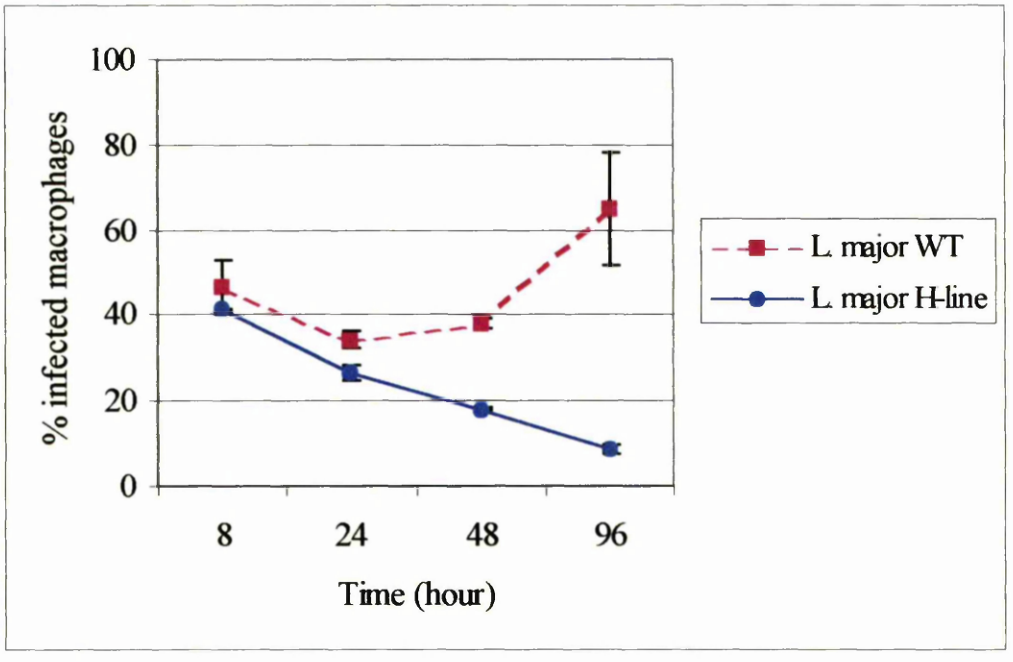


Figure 3.3. Macrophages were infected with stationary phase promastigotes of *L. major* H-line or *L. major* WT. The promastigotes of the two lines were grown on parallel cultures with the same ages. Macrophages were exposed to promastigotes at 32°C for 3 h and infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of parasite/ macrophage was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide.

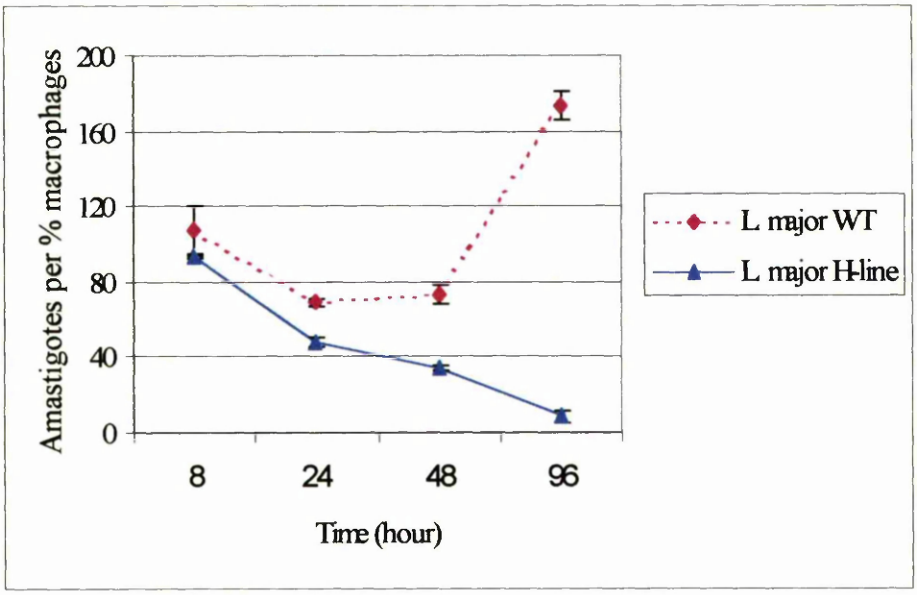


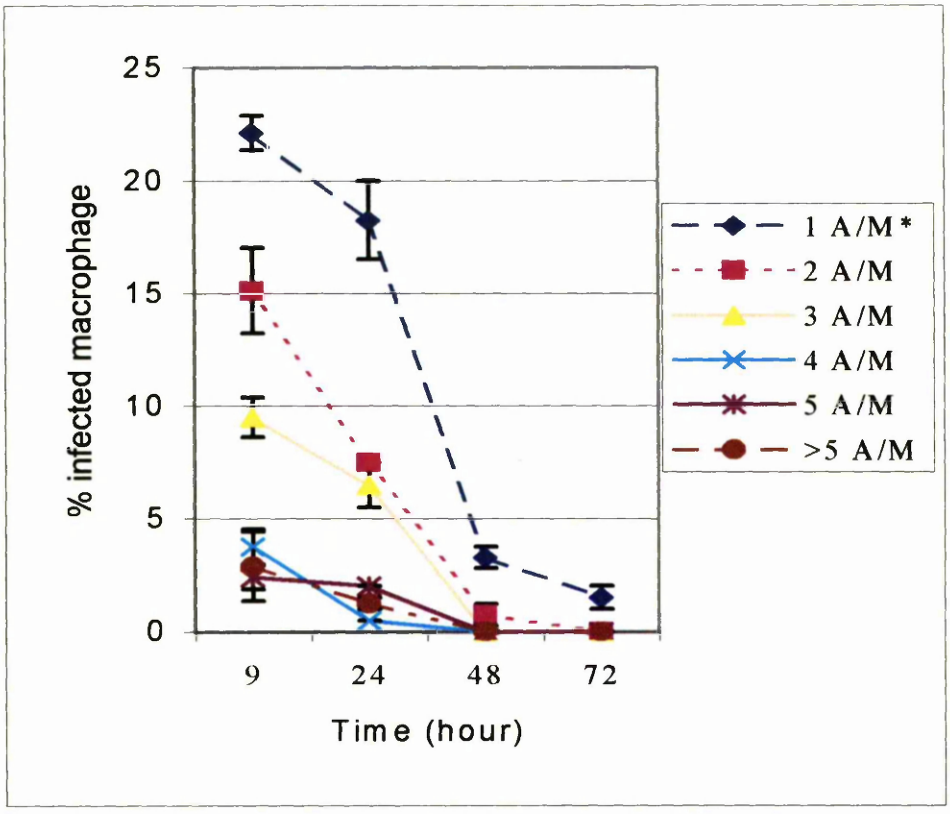
Figure 3.4. The number of amastigotes within infected macrophages with two lines of *L. major*. BMM were infected with stationary phase promastigotes of *L. major* H-line or *L. major* WT. Macrophages were exposed to promastigotes at 32°C for 3 h and subsequently infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

3.3.4. Parasite burden per macrophage following interactions between stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT with BMM

To determine the number of parasite within infected macrophages, *in vitro* culture system was used. BMM of BALB/c mice were infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT as previously described (see section 3.3.2). The distribution of amastigotes of *L. mexicana* H-line within infected BMM was similar to that of *L. mexicana* WT at 9 h post infection as also shown in Figure 3.1 and 3.2. 22% of BMM were infected with one amastigote of *L. mexicana* H-line at 9 h post infection. The percentage of infected cells decreased over time to 0.4% at 96 h post infection. The initial infection of macrophages with *L. mexicana* WT was similar to that with H-line (21%), but in contrast to the *L. mexicana* H-line, the percentage of infected macrophages with a burden of more than 5 amastigotes increased to 18% at 96 h post infection. The number of amastigotes of *L. mexicana* H-line within infected macrophages after 9 h post incubation was 100 amastigotes / 100 macrophages and rapidly decreased to 2 amastigotes / 100 macrophages at 96 h post infection (Figure 3.5). In contrast to that, the number of amastigotes of *L. mexicana* WT within infected macrophages increased to more than 250 amastigotes/100 macrophages (Figure 3.6).

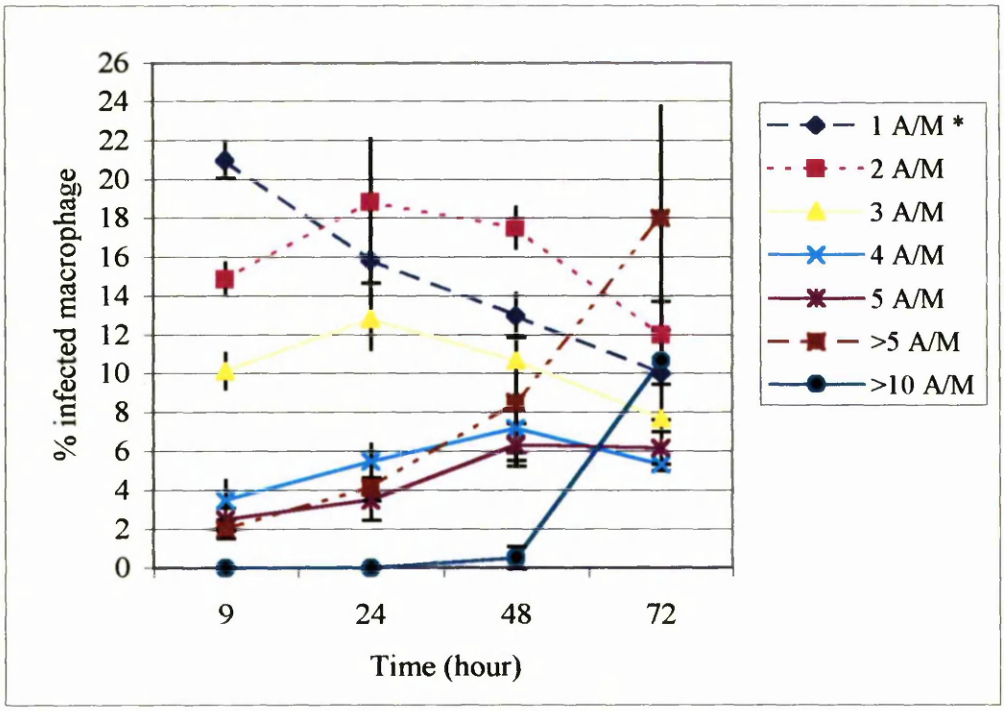
3.3.5. Interaction between IFN-γ- primed BMM with *L. mexicana* H-line or *L. mexicana* WT

The results of interaction between stationary phase promastiotes of *L. mexicana* H-line with BMM suggest that *L. mexicana* H-line does not survive within murine macrophages beyond 96 h, whereas the number of amastigotes of *L. mexicana* WT within infected macrophages increased. To consider the ability of amastigotes of the two lines of *L. mexicana* to survive within IFN-γ-primed BMM, macrophages were incubated in the presence of IFN-γ (10 U / ml) for 6 h and then exposed to stationary phase of promastigotes *L. mexicana* H-line or *L. mexicana* WT for 3 h. The cells were then incubated for up to 96 h and the rate of infection of macrophages was determined. As shown in Figure 3.7, at 8 h incubation the percentage of macrophages infected with



* A / M: The number of amastigotes within an infected macrophage.

Figure 3.5. The percentage of infected BMM with different numbers of amastigotes of *L. mexicana* H-line. BMM were infected with stationary phase promastigotes of *L. mexicana* H-line. Macrophages were exposed to promastigotes at 32°C for 3 h and infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.



* A / M: The number of amastigotes within an infected macrophage.

Figure 3.6. The percentage of infected BMM with different numbers of amastigotes of *L. mexicana* WT. BMM were infected with stationary phase promastigotes of *L. mexicana* WT. Macrophages were exposed to promastigotes at 32°C for 3 h and infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

promastigote of *L. mexicana* WT was 49% and decreased to 47% after 30 h incubation. In contrast, the percentage of IFN-γ-primed macrophages infected with *L. mexicana* WT at 8 h incubation was 51% decreased over time to 35% at 30 h post infection. The percentage of IFN-γ-primed macrophages and unprimed macrophages infected with *L. mexicana* H-line rapidly reduced with time.

The initial number of amastigotes of H-line within infected macrophages in the presence or absence of IFN-γ was 90 amastigotes / 100 macrophages at 8 h post infection decreasing to 4 amastigotes / 100 macrophages at 72 h post infection. The number of amastigotes of *L. mexicana* WT within infected IFN-γ-primed macrophages decreased to 37 amastigotes / 100 macrophages at 24 h post infection, whereas it was 67 amastigotes / 100 macrophages in the unprimed macrophages at the same time. In contrast to *L. mexicana* H-line, the number of amastigotes of *L. mexicana* WT within infected macrophages in the presence or absence of IFN-γ increased over time to more than 250 amastigotes / 100 macrophages at 72 h post infection (Figure 3.8).

3.3.6. Investigation of the stability of *L. mexicana* H-line in gentamicin-free medium

To determine whether *L. mexicana* H-line is stable in medium lacking gentamicin *in vitro*, promastigotes of *L. mexicana* H-line were transferred into HOMEM medium with or without gentamicin. The stationary phase promastigotes were harvested after 40 passages and incubated with macrophages for 3 h. Non-attached promastigotes were removed and the macrophage culture was incubated for 9, 24, 48, and 96 h and the rate of infected macrophages was determined. As shown in Figure 3.9 the percentage of infected macrophages with *L. mexicana* H-line grown in medium with or without gentamicin decreased over time to 2 or 4% at 96 h post infection. In contrast to the *L. mexicana* H-line, the percentage of macrophages with *L. mexicana* WT increased to 89.5%.

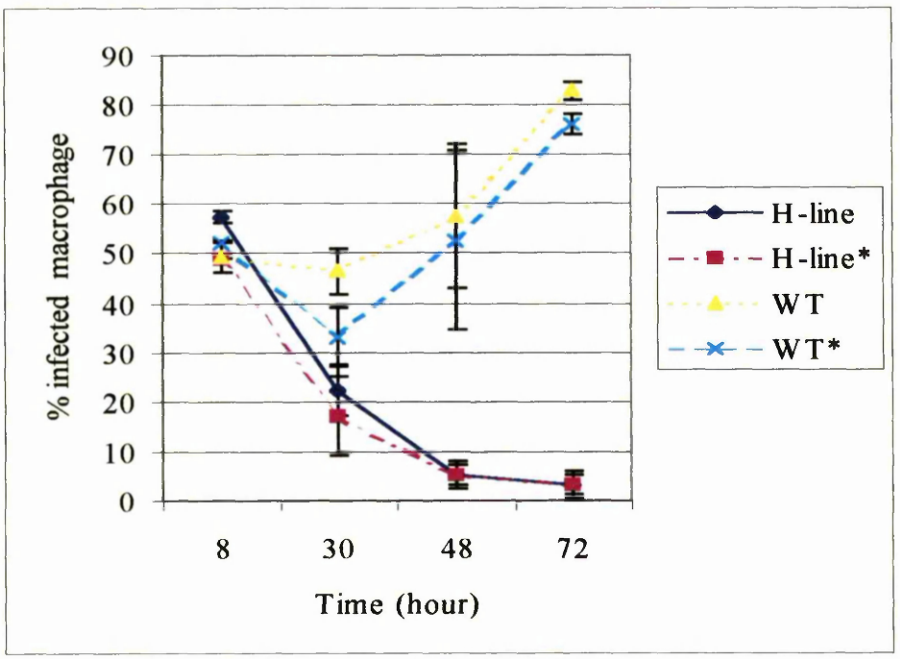


Figure 3.7. BMM or IFN-γ-primed BMM (*) infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. BMM were incubated in the presence of IFN-γ (10 U/ml) for 6 h. The IFN-γ-primed BMM or unprimed-BMM were exposed to stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT at 32°C for 3 h and infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

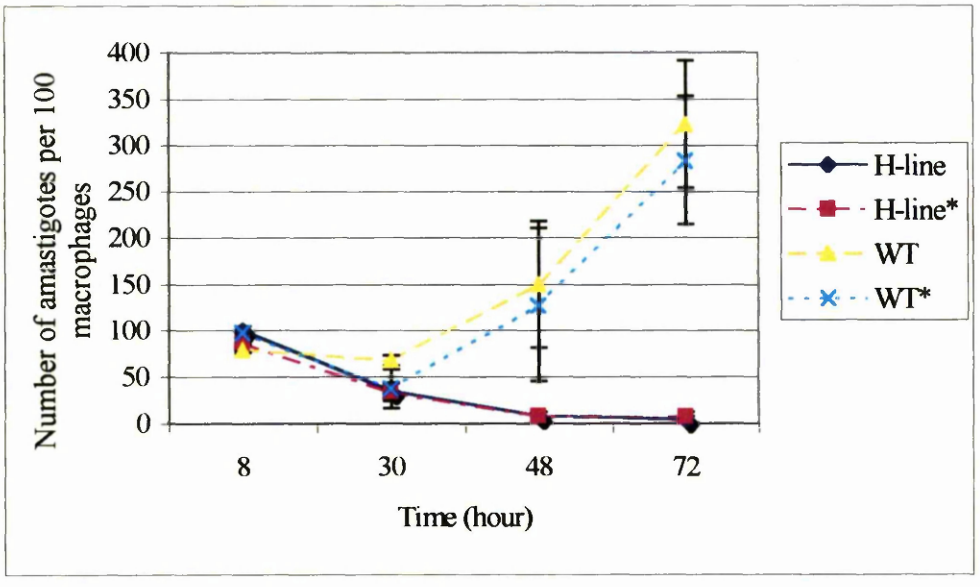


Figure 3.8. The number of amastigotes within 100 BMM or unprimed IFN-γ-primed BMM (*) infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. BMM were incubated in the presence of IFN-γ (10 U/ml) for 6 h. The IFN-γ-primed BMM or BMM were exposed to stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT at 32°C for 3 h and infected macrophages incubated at 32°C for appropriate times. The initial infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's staining of the culture slide. The infected cells were detected among at least 300 cells for each line.

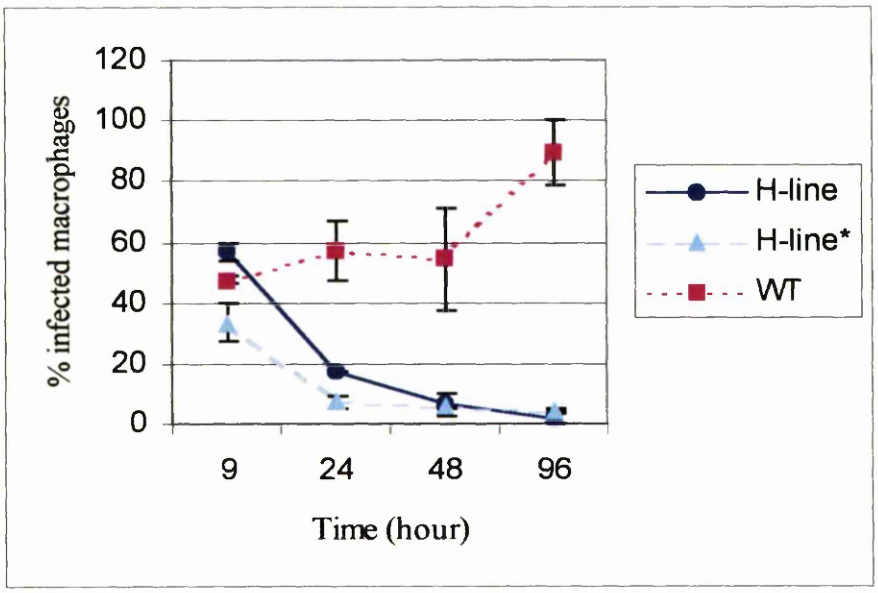


Figure 3.9. BMM infected with stationary phase promastigotes of *L. mexicana* H-line which were grown in gentamicin-free medium for 40 passages (H-line*). Stationary phase promastigotes of *L. mexicana* WT were grown in gentamicin-free medium. Macrophages were exposed to promastigotes at 32°C for 3 h and infected macrophages incubated at 37°C for appropriate times. The infection ratio of macrophage / promastigote was 1:1 and the percentage of infected macrophages was detected after Giemsa's stain of the culture slide. The infected cells were detected among at least 300 cells for each line.

3.3.7. Analysis of NOS induced by the two lines of *L. mexicana*

In order to determine whether the two lines of *L. mexicana* are able to induce NOS, BMM were infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. An investigation of NOS over the first 48 h of infection showed an increased level of nitrite in the supernatants of infected macrophages with the two lines of *L. mexicana*. As Figure 3.10 shows unstimulated macrophages were used as a negative control and a small but detectable level of nitrite was present in their supernatants. Macrophages stimulated with IFN-γ (100 U/ml) plus LPS (10 ng/ml) were used as a positive control and demonstrated that the activation of cells led to an increase in the amount of nitric oxide production.

Comparison of the amount of nitrite in the supernatants of different cultures demonstrated that there was no significant difference between the amount produced in various infections over 48 h post infection ($P< 0.7$). These results show that *L. mexicana* H-line gave rise to similar levels of nitric oxide production by the host cells as *L. mexicana* WT.

This suggests that the death of *L. mexicana* H-line within the infected macrophages *in vitro* is not due to change in the capacity of *L. mexicana* H-line to induce NO production.

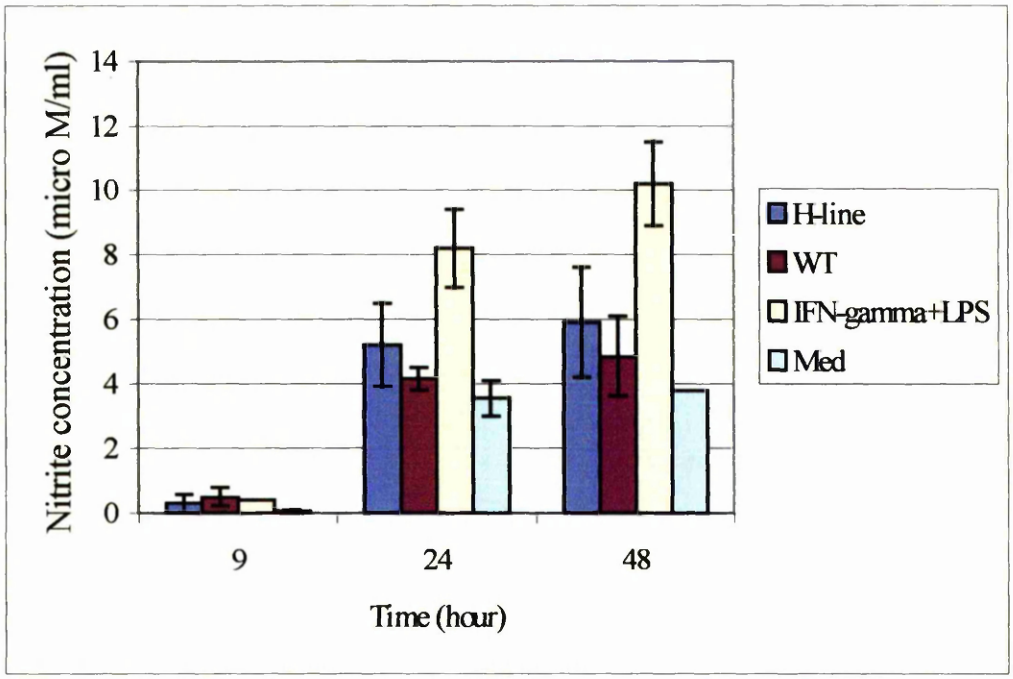


Figure 3.10. The concentration of nitric oxide measured as nitrite in the supernatant of BMM infected with stationary phase promastigotes of *L. mexicana* over 48 h post infection. Macrophages were stimulated with IFN-γ (100 U/ml) plus LPS (10 ng/ml) as positive control. Unstimulated macrophages were used as a negative control. The macrophages were incubated with promastigotes of *L. mexicana* H-line or *L. mexicana* WT at 32°C. The infection ratio of macrophages / promastigote was 1:1. Bars represent the standard error mean (SEM) (n = 3).

3.3.8. Investigation of dissemination of the two lines of *L. mexicana* in the peripheral and visceral organs of infected BALB/c mice

The *in vitro* results suggest that *L. mexicana* H-line does not survive within murine macrophages beyond 96 h. To consider the *in vivo* survival and possible dispersion of the *L. major* H-line beyond the site of inoculation, the mice were injected with *L. major* WT or *L. major* H-line into right side rump and then examined for the presence of parasites in the peripheral and visceral organs. Stationary phase promastigotes of the two lines of *L. mexicana* were subcutanously injected in the BALB/c mice, and visceral organs including the liver, spleen, lung, bone marrow (BM), draining right popliteal lymph node (LN), and ECs from the site of injection were removed aseptically at appropriate times and cell suspensions prepared. The cell suspensions were cultured in HOMEM medium and incubated at 25°C over 14 days. The cultures were examined daily for the presence of live promastigotes (Table 3.1). *L. mexicana* WT was detected in cultures originating from ECs at each time point post infection indicating that these organisms are able to survive within resident macrophages for long periods. In addition, the draining LN contained parasites within one day of infection, and these remained infected throughout the period of study (12 weeks). Over the course of the study *L. mexicana* WT spread to BM, spleen and lung and it was observed that infection of visceral organs correlated with an increase in skin lesion size. In comparison, *L. mexicana* H-line remained localized in the skin and draining LN for up to 12 weeks post infection without evidence of dissemination into other visceral organs of BALB/c mice.

These results suggest that *L. mexicana* WT disseminated into the visceral organs of susceptible mice and the number of parasites increased post infection. In contrast to *L. mexicana* WT, the attenuated line of *L. mexicana* was restricted with a decreased number of amastigotes in the draining LN and skin.

Table 3.1. Comparative distribution of *L. mexicana* WT and *L. mexicana* H-line in various organs of BALB/c mice. The promastigotes of the two lines were grown on parallel cultures with the same ages. The presence of viable parasites in the tissues of infected mice was determined by culturing *in vitro*.

Organ	1 d		14 d		21 d		28 d		90 d	
	WT	H	WT	H	WT	H	WT	H	WT	H
EC	+++	+++	+++	–	+++	++	+++	+	+++	++
Right popliteal LN	+++	+++	+++	–	+++	++	+++	++	+++	+
Left popliteal LN	–	–	–	–	–	–	–	–	–	–
Right femur LN	+++	–	++	–	+++	–	+++	–	+++	–
Spleen	+	–	–	–	+	–	++	–	+++	–
Bone marrow	++	–	+	–	–	–	++	–	+++	–
Liver	–	–	–	–	–	–	–	–	–	–
Lung	–	–	–	–	–	–	+	–	+++	–

(d): Days since infection of mice.

(+++): Represents high number of promastigotes.

(+): Represents low number of promastigotes.

(–): Represents no promastigote growth in the tissue culture.

3.3.9. Investigation of disemination of the two lines of *L. major* in the peripheral and visceral organs of infected BALB/c mice

Experiments were carried out to determine whether stationary phase promastigotes of *L. major* H-line are able to disseminate into visceral organs of susceptible mice and how long they can survive within epidermal cells and infected organs. To examine the *in vivo* survival and possible dispersion of the *L. major* H-line beyond the site of inoculation, two groups of BALB/c mice (3 mice per group) were injected with *L. major* WT or *L. major* H-line and then examined for the presence of parasites in the peripheral and visceral organs at 12 weeks post infection. The cell suspensions from liver, spleen, lung, BM, draining popliteal LN, and ECs from the site of injection were prepared and cultured (as described in section 3.3.8). The cultures were examined daily for the presence of live promastigotes. As Table 3.2 shows *L. major* WT spread to BM, spleen, lung, popliteal LN, and skin. In contrast, *L. major* H-line remained localized in the ECs and draining LN of two mice and promastigotes grew in the spleen culture of mouse No 2 after 8 days incubation.

These results suggest that *L. major* WT disseminated into the visceral organs of susceptible mice and the number of parasites increased post infection. In contrast to *L. major* WT, the attenuated line of *L. major* H-line was restricted with a decreased number of amastigotes in the draining LN and skin of two mice. Promastigotes grew in the skin and draining LN culture from third mice infected with *L. major* H-line. In addition, in the spleen cell culture of this mouse a low number of promastigotes was observed after 8 days incubation.

Table 3.2. Comparative distribution of *L. major* WT and *L. major* H-line in various organs of BALB/c mice at 12 weeks post infection. The presence of viable parasites in the tissues of infected mice was determined by culturing *in vitro*.

	EC	Right side popliteal LN	Spleen	BM	Lung	Liver
H-line (1)	+	+	–	–	–	–
WT (1)	+++	+++	+++	+++	++	–
H-line (2)	++	++	+	–	–	–
WT (2)	+++	+++	+++	++	++	–
H-line (3)	++	+	–	–	–	–
WT (3)	+++	+++	++	+++	+	–

+++: Represents a high number of promastigotes in tissue culture.

+: Represents a low number of promastigotes in tissue culture.

–: Represents no promastigote growth in tissue culture.

3.3.10. Anti-*Leishmania* immunoglobulins induced in mice infected with *L. mexicana* WT or *L. mexicana* H-line

To examine whether *L. mexicana* H-line and *L. mexicana* WT induced similar levels of subclasses of anti-*Leishmana*-specific Ab, BALB/c mice were infected with either of the two lines of *L. mexicana* by sub-cutaneous injection of stationary phase promastigotes. Blood was obtained from the mice at 12 weeks post infection. An investigation of anti-*Leishmana*-specific IgG1 and IgG2a production in the mice infected with *L. mexicana* WT showed the level of IgG1 Ab was significantly higher than the level of IgG2 Ab ($p < 0.03$). As Figure 3.11 shows the amounts of IgG1 and IgG2a Abs in the serum of mice infected with *L. mexicana* H-line were lower than those in the serum of mice infected with *L. mexicana* WT. In addition the amount of IgG1 Ab produced in the mice infected with *L. mexicana* H-line is significantly lower than that in the mice infected with *L. mexicana* WT ($p < 0.05$). There was no

significant difference between the amount of IgG1 and IgG2a Ab in the serum of mice infected with *L. mexicana* H-line ($p < 0.3$).

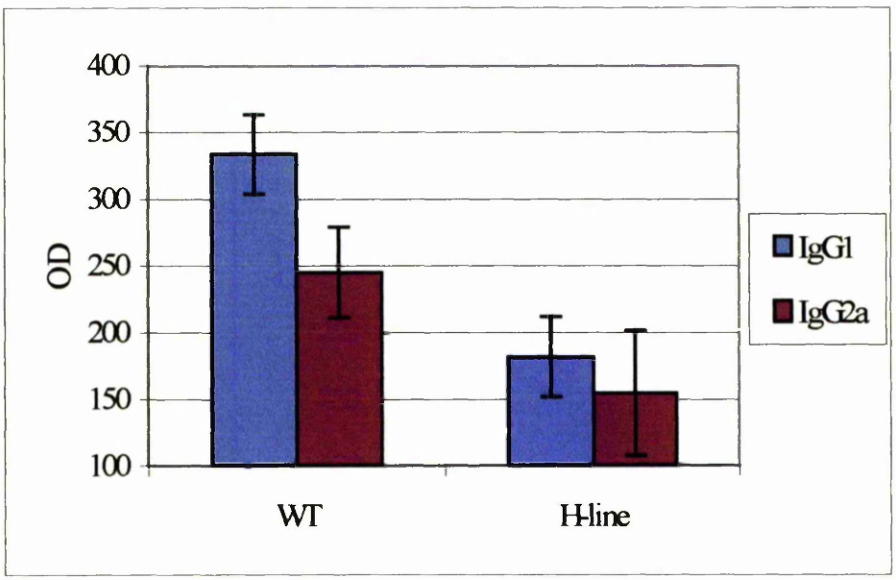


Figure 3.11. IgG1 and IgG2a production by BALB/c mice infected with the two lines of *L. mexicana*. Two groups of mice (5 mice per group) were injected with 5×10^6 stationary phase of *L. mexicana* WT or *L. mexicana* H-line into the shaved rumps as described before. Mice were bled at 12 weeks post infection. Their sera were pooled and analysed by ELISA for the presence of IgG1 and IgG2a. Note the sera were diluted 1/100 for IgG1 and IgG2a assay.

3.3.11. Interaction of promastigotes of the two lines of *L. mexicana* with epidermal cells *in vitro*

Experiments were carried out to determine whether stationary phase promastigotes of *L. mexicana* WT or *L. mexicana* H-line are able to infect epidermal cells and if so, the immune response of epidermal cells infected with the two lines of *L. mexicana*. Epidermal cells were prepared from the skin of the ears of BALB/c mice by a trypsinization procedure (see section 3.2.15). These preparations contained Langerhans cells (LCs) that constitutively express MHC class II as well as MHC class II-negative keratinocytes, a source of GM-CSF that is essential for LC differentiation, and were absolutely devoid of macrophages (Black *et al.*, 1996). Epidermal cells were exposed to stationary phase promastigotes of the two lines of *L. mexicana* and incubated at 32°C in CO_2 5% in air for 24 h. The infection ratio of epidermal cell / promastigote was 10:1. The supernatants of cultures were collected, stored at –70°C, and the cells were centrifuged on the cytospin. These slides and the chamber slides were stained with Giemsa's stain or acridine orange and ethidium bromide. The infected ECs, which were stained with acridine orange determined by fluorescence microscopy.

The results showed that no epidermal cells were infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT, whereas adherent macrophages on the chamber slides were infected with stationary phase promastigotes of *L. mexicana* WT at 24 h post infection.

3.3.12. T-cell proliferation of splenocytes from BALB/c mice infected with the two lines of *L. mexicana*

Studies were carried out to determine whether the splenocytes of BALB/c mice infected with *L. mexicana* H-line or *L. mexicana* WT at 12 weeks post infection were sensitized to stationary phase promastigotes of *L. mexicana* WT. The splenocytes were exposed to promastigotes, at 32°C for 4h and then incubated at 37°C for 68 h. The cell: parasite ratio was 10 / 1. The splenocytes were exposed with promastigotes, as this in an attempt to simulate the situation when immune mice are exposed to a

normal challenge. The proliferation of the T cells was confirmed with Con-A (5 μg/ml) as a stimulation control and unstimulated cells as negative controls. As Figure 3.12 shows the splenocytes from mice infected with *L. mexicana* WT displayed Con-A-stimulated proliferative responses higher than those of splenocytes from mice infected with *L. mexicana* H-line. Splenocytes from mice infected with either *L. mexicana* H-line or *L. mexicana* WT both proliferated when exposed to promastigotes of *L. mexicana* WT or promastigotes of *L. mexicana* H-line (not shown) but this was markedly below that of the Con A- stimulated splenocytes.

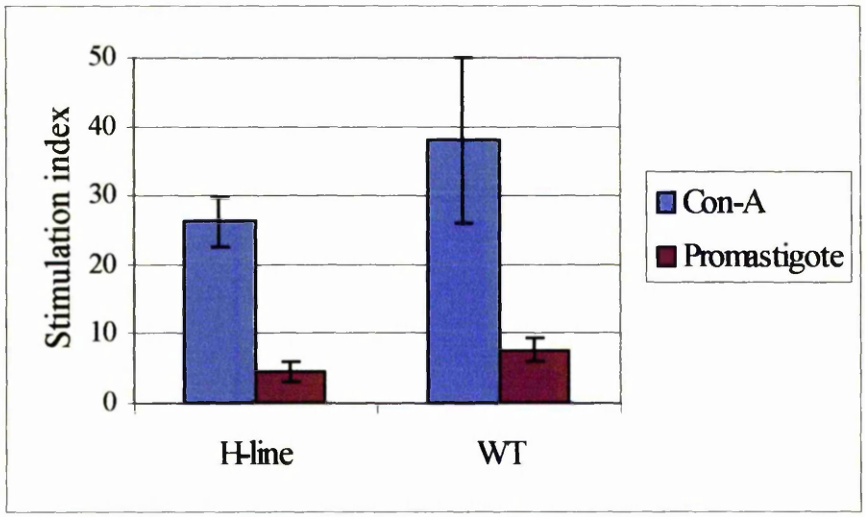


Figure 3.12. T-cell proliferation responses of splenocyte from the mice infected with the two lines of *L. mexicana*. BALB/c mice were infected with *L. mexicana* H-line (6 mice) or *L. mexicana* WT (4 mice) at week 12 post infection, induced by Con A (5 μg/ml) and stationary phase promastigotes of *L. mexicana* WT (the ratio of splenocyte: promastigote was 10 / 1). The stimulation index was calculated as the count per minute (CPM) of stimulated cells / CPM of unstimulated cells and error bars are represented the means stimulation index ± standard error mean.

3.3.13. IFN-γ, IL-2, IL-4, and IL-10 production by splenocytes of mice infected with the two lines of *L. mexicana*

To examine whether the *L. mexicana* H-line and *L. mexicana* WT induced a similar cytokine response, mice were infected with either of the two lines by sub-cutaneous injection of parasites. Spleens were harvested at 12 weeks post infection and cells re-stimulated *in vitro* with stationary phase promastigotes of *L. mexicana* WT. Cell culture supernatants were collected and assayed for the presence of the Th1 type cytokines, IFN-γ and IL-2.

Spleen cells taken from mice infected with *L. mexicana* WT secreted a low level of IFN-γ when restimulated with stationary phase promastigotes. In comparison, cells taken from mice infected with *L. mexicana* H-line secreted a higher amount of IFN-γ ($P<0.05$). In both cases, the level of IFN-γ was detected from cells cultured in medium alone (Figure 3.13).

The IL-2 production by these cells presented a similar profile. Spleen cells from *L. mexicana* WT infected mice did not increase IL-2 production when cultured in the presence of stationary phase promastigotes. However, cells taken from mice infected with *L. mexicana* H-line secreted IL-2 when cultured with promastigotes of *L. mexicana* WT. This level of IL-2 production was significantly higher than the level produced by spleen cells from *L. mexicana* WT infected mice ($P<0.05$) (Figure 3.14).

Supernatants harvested from *in vitro* cultures of spleen cells taken from infected mice and restimulated with stationary phase *L. mexicana* WT promastigotes were also assayed for cytokines characteristic of Th-2 type responses. As shown in Figure 7, spleen cells taken from *L. mexicana* WT infected mice responded to culture with parasite antigen. The production of IL-4 in the supernatant of Ag-stimulated splenocytes from the mice infected with *L. mexicana* WT was significantly higher than that of mice infected with *L. mexicana* H-line ($P<0.05$). In comparison, spleen cells recovered from mice infected with *L. mexicana* H-line did not produce IL-4 in response to *Leishmania* (Figure 3.15)

Spleen cells from the mice infected with *L. mexicana* WT also produced increased levels of IL-10 when cultured with parasite antigen compared with mice infected with *L. mexicana* H-line ($P<0.02$). No such increase was observed in cells taken from mice infected with *L. mexicana* H-line (Figure 3.16).

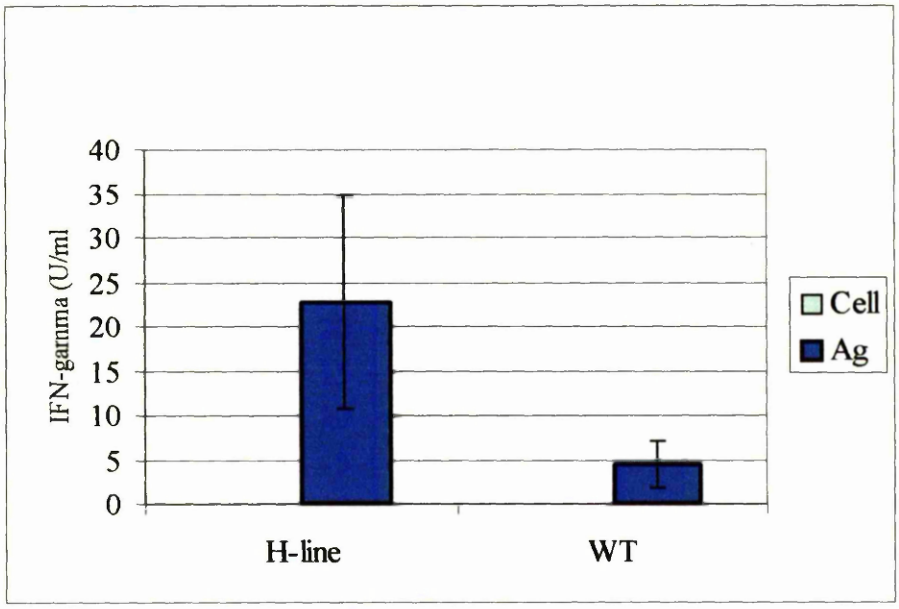


Figure 3.13. The level of IFN-γ in the supernatants of cultured splenocytes from mice infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. The comparison between the two lines was made on parallel cultures with the same ages. The splenocytes were taken from the infected mice at 12 week post infection and stimulated with stationary phase promastigotes of *L. mexicana* WT at a ratio of 1 parasite / 10 splenocytes. Bar represent SEM (n = 4).

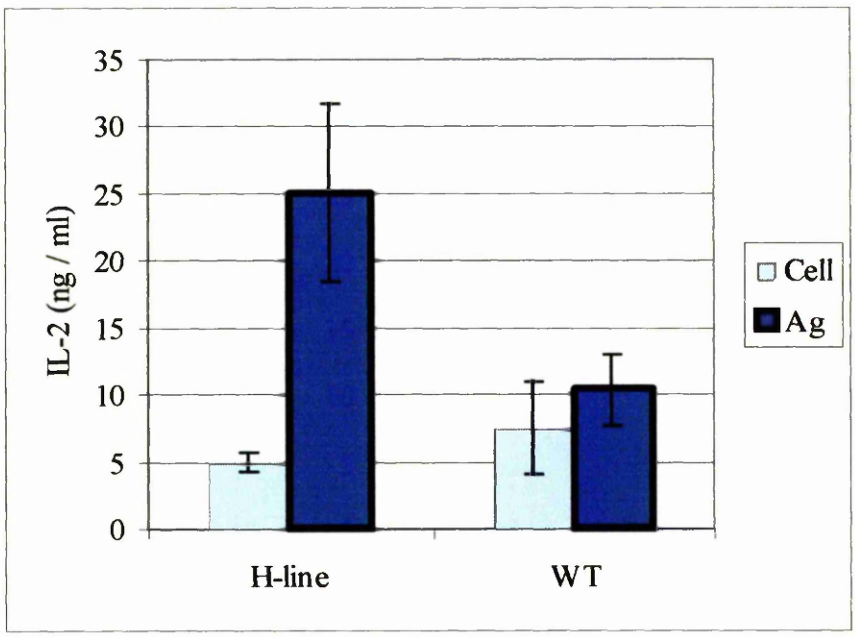


Figure 3.14. The level of IL-2 in the supernatants of cultured splenocytes from mice infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. The comparison between the two lines was made on parallel cultures with the same ages. The splenocytes were taken from the infected mice at 12 weeks post infection and stimulated with stationary phase promastigotes of *L. mexicana* WT at a ratio of 1 parasite / 10 splenocytes. Bar represent SEM (n = 4).

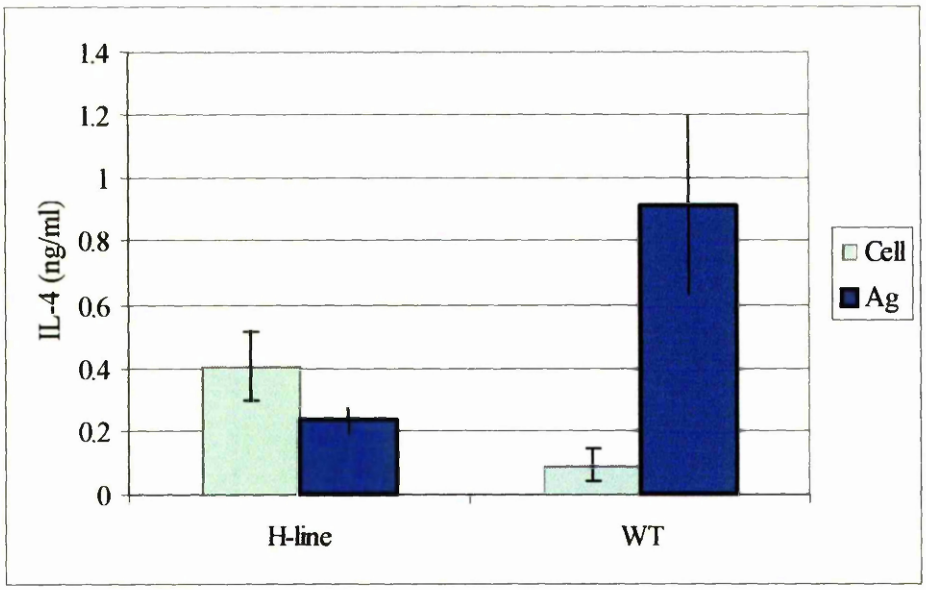


Figure 3.15. The level of IL-4 in the supernatants of cultured splenocytes from mice infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. The comparison between the two lines was made on parallel cultures with the same ages. The splenocytes were taken from the infected mice at 12 week post infection and stimulated with stationary phase promastigotes of *L. mexicana* WT at a ratio of 1 parasite / 10 splenocytes. Bar represent SEM (n = 4).

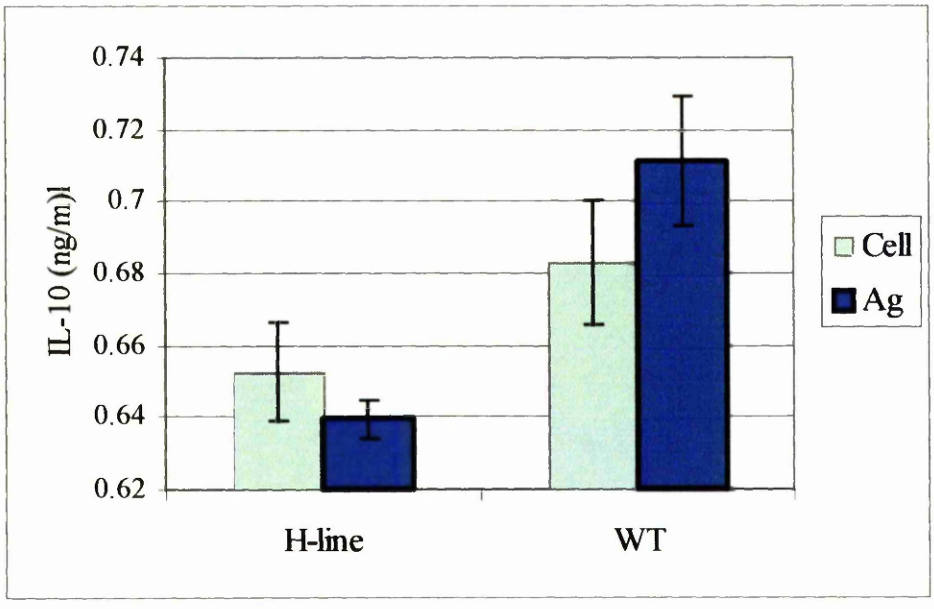


Figure 3.16. The level of IL-10 in the supernatants of cultured splenocytes from mice infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT. The comparison between the two lines was made on parallel cultures with the same ages. The splenocytes were taken from the infected mice at 12 week post infection and stimulated with stationary phase promastigotes of *L. mexicana* WT at a ratio of 1 parasite / 10 splenocytes. Bar represent SEM (n = 4).

3.4. Discussion

The attenuated cell line of *Leishmania* was generated by culturing *L. mexicana* WT under pressure of gentamicin and the preliminary results showed that *L. major* could be attenuated using to the same protocol (see Chapter 1).

The ability of *L. mexicana* to infect macrophages was investigated through exposure of stationary phase promastigotes of *L. mexicana* H-line to bone marrow derived macrophages from BALB/c mice. The results showed that the percentage of macrophages infected with *L. mexicana* H-line was similar to that of *L. mexicana* WT at 9 h post infection but decreased to 0.4% at 96 h post infection. In contrast, the percentage of infected macrophages infected with *L. mexicana* WT increased to 72% at 96 h post infection. Only a small population of amastigotes of *L. mexicana* H-line survived within infected macrophages after 96 h post infection *in vitro*. It has been purposed that the ability of amastigotes of *Leishmania* to survive within macrophages is a primary mechanism for evading parasite the immune responses of their vertebrate host (Alexander and Russell, 1992).

In this study, it was shown that 21% macrophages were infected with one amastigote of *L. mexicana* WT at 9 h post infection and this percentage over time decreased to 10% at 72 h post infection. As Figure 3.6 shows, 18% of macrophages carried more than 5 amastigotes at 72 h post infection. This indicates that following interaction of *L. mexicana* WT promastigotes with macrophages, some of them, probably not in stationary phase, were unable to survive within infected macrophage. This suggestion is agreement with the reports that stationary phase promastigotes of *L. major* (Sacks *et al.*, 1985) or *L. mexicana* (Robertson and Coombs 1992) were able to establish intracellular infections whereas some of them appeared to be transformed into infective-stage promastigotes, as determined by their ability to survive within normal resident mouse peritoneal macrophages *in vitro*. Therefore, the percentage of macrophage infected with one amastigote sharply decreased, whereas the percentage of macrophages with more then one amastigote present increased over the 96 h. In contrast to *L. mexicana* WT, after interaction of promastigotes of *L. mexicana* H-line, the vast majority of promastigotes were unable to survive within the parasitophorous vacuole (PV) of infected macrophages (Figure 3.5).

The mechanism of the microbicidal activity of gentamicin, which has been added to the culture medium, to attenuate *L. mexicana*, in particular, killing amastigotes and the regulation of immune response against these parasites has yet to be elucidated.

The mechanism which leads to activation and killing of the parasites within the PV could be important evidence not only for *Leishmania*, but also all intracellular microorganisms for which the macrophage is one of the critical components of the defence (Bogdan *et al.*, 1996).

L. mexicana H-line was developed on four separate occasions with the same procedure and the stability of this attenuated cell line in the antibiotic-free medium was investigated through culturing promastigotes in the antibiotic-free medium for an extended period (40 passages). Stationary phase promastigotes of *L. mexicana* H-line were grown in the medium without gentamicin and exposed to macrophages. The result showed that that attenuated line of *L. mexicana* H-line remained unaltered and is therefore stable for a long time *in vitro* in medium free of the antibiotic.

The activation state of macrophages and T cells is dependent on the availability of stimulatory and inhibitory cytokines, the production of which can be altered by the parasite (Bogdan *et al.*, 1999). The survival of amastigotes within the macrophage is prevented when the macrophages are stimulated by specific T-cell-derived lymphokines and IFN-γ is the most important component among these macrophage-activating mediators (Nathan *et al.*, 1983). IFN-γ plays a crucial role in the lymphokine response leading to the elimination of *Leishmania* from the macrophage. IFN-γ-production is associated with the expansion of $CD4^+$ T cells. Here it has been shown that *L. mexicana* H-line is eliminated by macrophages, whereas amastigotes of *L. mexicana* WT are able to survive for prolonged periods within macrophages.

The levels of IFN-γ, IL-2, IL-4, and IL-10 in the supernatants of cultured splenocytes from the mice infected with *L. mexicana* WT or *L. mexicana* WT were measured. The splenocytes from infected mice at 12 weeks post infection were aseptically removed and restimulated with stationary phase promastigotes of *L. mexicana* WT. It was found that the levels of IFN-γ and IL-2 in the supernatant of cultured Ag-stimulated-splenocytes of mice infected with *L. mexicana* H-line were significantly higher than those of mice infected with *L. mexicana* WT ($P<0.05$). In contrast the level of IL-4 in the supernatant of splenocytes from the mice infected with *L. mexicana* H-line was significantly lower than that of mice infected with *L. mexicana* WT ($P<0.05$).

The high levels of IL-2, IFN-γ and low amount of IL-4 in the supernatant of splenocytes from mice infected with *L. mexicana* H-line is agreement with the report that low levels of IL-4 have been associated with IL-2 and IFN-γ productions (Tanaka *et al.*, 1993). Several groups of researchers reported that induction of protective immunity against the *L. mexicana* is ultimately dependent on generation of a Th1-like response and IFN-γ production (Satoskar *et al.*, 1995 and Guevara-Mendoza *et al.*, 1997). In contrast IL-4 is important for the suppression of the Th1-like responses that are required for control of cutaneous lesions after *L. mexicana* WT infection (Oswald *et al.*, 1992). Satoskar *et al.* (1997) showed that genetically susceptible mice lacking IL-4 are protected from cutaneous infection with *L. mexicana* WT.

The amount of IL-10 in the supernatant of cultured Ag-stimulated-splenocytes from the mice infected with *L. mexicana* WT or *L. mexicana* H-line were measured. The level of IL-10 production by splenocytes from mice infected with *L. mexicana* WT was significantly higher than that of mice infected with *L. mexicana* H-line ($P<0.02$). IL-10, which is typically induced by *L. major* counteracts the development of a protective Th1 immune response by acting on antigen presenting cells (Bogdan *et al.*, 1993). This is characterized by the down-regulation of the expression of MHC class II molecules or the suppression of the production of parasiticidal metabolites and various inflammatory mediators (Howard *et. al.*, 1992). In addition IL-10 is important in inhibiting monocyte-macrophage activation and the production of TNF- α, IL-1 and IFN-γ (D'Andrea *et al.*, 1993).

In another study, was shown that *L. mexicana* H-line has the ability to induce IFN-γ and TNF-α production by macrophages, whereas activation of IFN-γ-primed macrophages was prevented by *L. mexicana* WT. The percentage of infected IFN-γ-primed or unstimulated macrophages and the number of amastigotes per 100 unstimulated or IFN-γ-primed macrophages infected with *L. mexicana* H-line or *L. mexicana* WT were investigated. The percentage of infected unstimulated macrophages was 46.6%, whereas 33% of IFN-γ-primed macrophages were infected with *L. mexicana* WT at 24 h post infection with 67 amastigotes per 100 unstimulated macrophages decreasing to 33 amastigotes per 100 IFN-γ-primed macrophages. This was due to activation of macrophages with IFN-γ, which was prevented by *L. mexicana* WT.

Previously, it was shown that stimulated splenocytes from mice infected with *L. mexicana* WT produced high levels of IL-4 and IL-10. This suggests that IL-4 or IL-10 alone or in combination may interact with activated macrophages, and is supported by the report that IL-4 and IL-10 are ability to reduce IFN-γ production and therefore leishmanicidal activity (Oswald *et al.*, 1992; Bogdan *et al.*, 1993).

In the present study it has been shown that *L. mexicana* H-line is equally susceptible to killing by unprimed and IFN-γ activated macrophages. This suggests that TNF-α induces *L. mexicana* H-line killing by macrophages in the presence of IFN-γ and is supported by reports that for activation of macrophages two consecutive signals are required, the first signal is IFN-γ whereas TNF-α is second signal that mediates microbicidal functions exhibited by IFN-γ primed macrophages exposed to microbial products (James, 1995; Liew *et al.*, 1990). Bogdan and colleagues (1990) found that TNF-α induced rapid *Leishmania* degradation by the macrophage in the presence of very low dosages of IFN-γ, whereas TNF-α in combination with IL-4 supported intracellular parasite survival.

The results showed that the *L. mexicana* H-line induced high levels of IFN-γ and TNF-α. Schaible and colleagues (1998) reported that activation of macrophages by IFN-γ and TNF-α leads to the maturation of the phagosome to an acidic phagolysosome (pH 5). It is possible that infection with *L. mexicana* H-line leads to the maturation of the phagosome and increase the acidity of the phagolysosome. Thus amastigotes are unable to survive in phagolysosome at this condition.

It was shown that the majority of the mice infected with *L. mexicana* H-line failed to develop cutaneous lesions and just 1 / 5 mice developed a small and healing lesion during the 12 weeks post infection. In contrast to *L. mexicana* H-line, all infected mice with *L. mexicana* WT went on to grow non-healing lesion at the same time. Possible mechanisms underlying the development of non-healing lesions in genetically susceptible mice are probably under the control of some kind of cytokines, including IL-12, IFN-γ, IL-4, and IL-10 (Scharton-Kersten and Scott, 1995; Heinzel *et al.*, 1995). Each of these cytokines has positive effects on the production of the other (Gazzinelli *et al.*, 1993; Macatonia *et al.*, 1993).

Macrophages infected with stationary phase promastigotes of either *L. mexicana* WT or *L. mexicana* H-line, produced no significant difference in nitric oxide production. The fact that *L. mexicana* H-line gave rise to similar levels of nitric oxide production

as *L. mexicana* WT indicates that the attenuation of the H-line's virulence is not due to NO production. However the Greiss reaction measures the nitrite concentration in the supernatant of infected cultures. In order to determine accurately the production of NO at specific time points, it would be necessary to measure NOS with other methods. Vectors are available containing the inducible NOS genes (iNOS) which would allow application of the competitive reverse transcription polymerase chain reaction (CRT-PCR) (Reiner and Locksley, 1993), for determination mRNA expression for iNOS within cells which would allow a more accurate measure of NOS. Unfortunately, time limitation and prioritisation prevented further investigation by this method.

Dissemination of the parasite in the visceral organs of susceptible mice is result of the development of Th2 response that is not protective (Liew and Donnell, 1993). The local restriction of the parasite prior to the development of T cell responses appears to be mediated by the innate immune system and this activity is considered to play an important role in the subsequent development of a protective T cell response (Laskay *et al.*, 1995). In this study it was found that *L. mexicana* H-line was localized in the skin where the promastigotes were injected and in the draining popliteal LN after 120 days post infection, without any dissemination to the visceral organs. In contrast, infection with *L. mexicana* WT led to the parasite rapidly spreading to the bone marrow, spleen, draining popliteal LN and femur, and lungs. Our results indicate that *L. mexicana* H-line can survive in the skin for a long time and thus it could generate long term memory or concomitant immunity (Aebischer, *et al.*, 1993).

It was found that the amounts of both IgG1 and IgG2a Abs in the serum of mice infected with *L. mexicana* H-line were less than those of mice infected with *L. mexicana* WT. The level of IgG1 was significantly increased compared with the level of IgG2a in the serum of mice infected with *L. mexicana* WT ($p<0.03$). In contrast, there was no significant difference between the amounts of IgG1 and IgG2a Abs in the serum of the mice infected with *L. mexicana* H-line ($p<0.3$). In fact, the level of IgG1in the serum of mice infected with *L. mexicana* WT was increased compared with that in the serum of infected mice with *L. mexicana* H-line. On the other hand, the production of IgG1 Abs that is promoted by Th2 cells was markedly suppressed in the mice infected with *L. mexicana* H-line, compared with that in *L. mexicana* WT treated mice, indicating possible inhibition of Th2 function by *L. mexicana* H-line.

This data is in agreement with the report that IgG1 regulation is not completely dependent upon IL-4 signalling (Snapper and Scott, 1987).

Langerhans cells (LCs) play a crucial role in the induction of the immune response in the early *Leishmania* infection because only infected LCs are able to carry parasites from the infected skin to the draining LN for primary antigen presentation to T cells (Moll *et al.*, 1993) and produce IL-12 immediately following *L. donovani* infection (Gorak *et al.*, 1998). It was interesting to determine the immune response of LC infected with *L. mexicana* WT or *L. mexicana* H-line. The isolated epidermal cells were incubated with promastigotes of the two lines of *L. mexicana in vitro*. Blank and colleagues (1993) reported that only LC, but no other epidermal cells, are able to take up *L. major*. The presence of intracellular parasites was assessed by staining the cells with acridine orange / ethidium bromide (Channon *et al.*, 1984). It was found that no epidermal cells were infected with stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT *in vitro*, whereas macrophages stuck on the chamber slides were infected with stationary phase promastigotes of *L. mexicana* WT over 24 h post infection. This suggests that stationary phase promastigotes of *L. mexicana* WT or *L. mexicana* H-line were unable to infect Langerhans cells *in vitro*.

The present results indicate that the *L. mexicana* H-line has attenuated virulence for BALB/c mice and promotes a Th1 cells response.

L. major H-line was stabilised in the presence of gentamicin after 11 passages using the same method described for generation of the *L. mexicana* H-line. The results of the interaction between stationary phase promastigotes of *L. major* H-line and macrophages showed that the attenuated cell line of *L. major* was able to infect macrophages similar to that of *L. major* WT. In contrast to *L. major* WT, the percentage of macrophages infected by the attenuated line decreased from 41% at 8 h post infection to 10.5% at 96 h post infection. The initial infection of macrophages with *L. major* WT was 46.5%, but in contrast to the *L. major* H-line, the percentage of infected macrophages increased to 65% at 96 h post infection.

Comparison of number of amastigotes of *L. mexicana* H-line or *L. major* H-line which survived within infected macrophages at 96 h shows that the capability of amastigotes of *L. major* H-line to survive within infected macrophage is greater than that of *L. mexicana* H-line. There are two possible reasons:

1- There are some differences between the resistance of each attenuated line to survive within the PV of macrophages.

2- The promastigotes of *L. major* H-line after 11 passages were exposed to macrophages whereas *L. mexicana* H-line after 20 passages.

In this study it has been shown that five BALB/c mice infected with *L. major* H-line failed to develop cutaneous lesion during 12 weeks post infection. In contrast to *L. major* H-line, all mice infected with *L. major* WT went to grow non-healing lesion at the same time.

Unfortunately, prioritisation and time limitation prevented more work with the attenuated *L. major*. The preliminary results of interaction between stationary phase promastigotes of *L. major* H-line and macrophages (*in vitro*) and the failure of lesions to develop in the susceptible mice indicate that the attenuated cell line of *L. major* was generated in the presence of gentamicin. *L. major* H-line is non-virulent for susceptible BALB/c mice, at least 12 weeks post infection.

CHAPTER FOUR

VACCINATION STUDIES OF *L. MEXICANA* H-LINE AND *L. MAJOR* H-LINE AGAINST *L. MEXICANA* IN BALB/C MICE

4.1. Introduction

Leishmania are responsible a wide spectrum diseases in humans and there has been much recent activity in the attempts to vaccine against *Leishmania* because of the problem of an increased incidence of resistance following chemotherapy that is based on pentavalent antimony compounds. Unfortunately these drugs frequently have unpleasant side effects, and are not very effective against cutaneous leishmaniasis. Another problem of chemotherapy is the development of drug resistance in various endemic regions of the world (Jackson *et al.*, 1990; Rangel *et al.*, 1997).

The conventional approach to vaccine development has used five methods: live, killed, and attenuated promastigotes, subunit vaccines, and DNA vaccines. There is no safe and effective vaccine against any form of leishmaniasis (Piedrafita *et al.*, 1999).

Killed parasites are unable to invade reticuloendothelial cells but the viable parasite can exist intracellularly in the macrophages, and evade the consequences of host's immune attacks. The result is that the parasite can survive in the mammalian host for a long time and causes a chronic disease, with unsuccessful immunological response (Liew 1989). Persisting parasites offer a continual stimulus to the immune system and induce effective immunological memory in the process known as concomitant immunity (Aebischer *et al.*, 1993). An unfortunate feature of this persistence is that parasites may grow out of control reactivating in immunocompromised people. Ideally a vaccine should be molecularly defined and induce long term memory in the absence of persistent live organisms (Handman, 1997), but this may not be readily achievable.

The current conventional approach to leishmanial vaccine development uses two methods: the first, attenuation of promastigotes by serial passages *in vitro* to obtain live-attenuated strains (to be used as vaccines), and second, subunit vaccines by identification of protective antigens (to be used as non-living vaccines) (Rappuoli, and Del Giudice, 1999).

The stable immunity following recovery from cutaneous leishmaniasis has prompted scientists during the past several decades to try using the live attenuated cell lines for development of prophylactic vaccines. This immunity is driven by the induction of T-cell responses involving the production of cytokines, which activate macrophages to kill the parasites (Liew and O'Donnell, 1993).

Reiner and Locksley (1995) reported that resistance to *Leishmania* in various mouse strains strongly correlates with the development of a Th1-like cytokine secretion profile. Induction of an immune response with mixed Th1-like and Th2-like is unable to confer protection. A Th1 response is sufficient to cause protection against disease in experimental cutaneous leishmaniasis, but this protection is abrogated if a Th2 response is simultaneously induced (Sjölander, 1998). The importance of IL-4 is supported by several other studies suggesting that susceptibility to leishmaniasis correlates with the production of IL-4 rather than the lack of IFN-γ (Erb *et al.*, 1996; Morris *et al.*, 1993).

4.2. Materials and methods

4.2.1. Parasite

Promastigotes of *L. mexicana* WT or *L. major* were grown in HOMEM medium supplemented with 10% (v/v) HI-FCS. Promastigotes of *L. mexicana* H-line or *L. major* H-line were grown in the same medium supplemented with 20 μg/ml gentamicin and incubated at 25°C (see section 2.2.1). In this chapter, the comparisons of immune response of mice induced by *L. mexicana* WT and *L. mexicana* H-line were made with same age parasites.

4. 2. 2. Immunization of mice with *L. mexicana* H-line

One group BALB/c mice (14 mice / group) was injected (s.c.) with 5×10^6 stationary phase promastigotes of *L. mexicana* H-line into their shaven right side rump and another group injected with PBS. After 12 weeks, the immunised mice and control group were infected (s.c.) with 5×10^6 stationary phase promastigotes of *L. mexicana* WT into the opposite side rump. The same number of mice were injected with PBS as controls. The swelling or lesion growth was monitored weekly.

4. 2. 3. Immunization of mice with *L. major* H-line and challenged with *L. major* WT

Five mice immunized with 5×10^6 stationary phase promastigotes of *L. major* H-line and 5 non-vaccinated controls were infected with 5×10^6 stationary phase promastigotes of *L. major* WT (as described in section 4.2.1). The swelling or lesion growth was monitored weekly.

4. 2. 4. Protection studies-immunization and challenge at the same time

The first group of mice (10 mice / group) was injected (s.c.) with 2.5×10^6 stationary phase promastigotes of *L. mexicana* H-line into their shaven left side rump and 2.5×10^6 stationary phase promastigotes of *L. mexicana* WT in the opposite side. The second group of mice was injected (s.c.) with a mixture of 2.5×10^6 stationary phase promastigotes of *L. mexicana* H-line and the same number of stationary phase promastigotes of *L. mexicana* WT into their shaven right side rumps. The third group of control mice was infected with 2.5×10^6 stationary phase promastigotes of *L. mexicana* WT into their right side. The swelling or lesion growth at the site of *L. mexicana* WT inoculation was monitored weekly over a period of 13 weeks.

4. 2. 5. Lesion size

Suspension of stationary phase promastigotes of *L. mexicana* H-line or *L. mexicana* WT or *L. major* WT or *L. major* H-line at concentration 2.5×10^7 cells/ml in PBS was prepared, and 200 µl of these suspensions were injected (s.c.) into the shaven rump of BALB/c mice. The lesion volume was measured weekly (for full details see section 3.2.10).

4. 2. 6. T cell proliferation assay

T cell proliferation assays were performed as previously described in section 3.2.10. Briefly, the mice were vaccinated with stationary phase promastigotes of *L. mexicana* H-line and control mice injected PBS. Three months later the control and vaccinated mice were infected with stationary phase promastigotes of *L. mexicana* WT. The mice were killed at 12 weeks post infection and their spleens removed aseptically. Single cell suspensions were prepared (for more detail see section 3.2.11) and then exposed by adding an equal volume of stationary phase promastigotes at a concentration of 5×10^5 cells / ml, giving a parasite: cell ratio of 1:10. Supernatants were collected from parallel cultures after 72 h of incubation for ELISA quantification of cytokine production.

4. 2. 7. Cytokine ELISA

Supernatants collected from spleen cell cultures from mice immunized *with L. mexicana* H-line and challenged with *L. mexicana* WT and infected mice were assayed for the presence of IFN-γ and IL-4 using ELISA, as previously described (see in section 3.2.13).

4. 2. 8. Preparation of tissues, and culture *in vitro* of *L. mexicana* isolated from infected mice.

Following injection of 5×10^6 stationary phase of promastigotes of *L. mexicana* WT, the parasite loads in the 6 mice were determined by limiting dilution to measure the number of viable promastigotes in the tissues of challenged and infected mice (using the same methods described in section 3.2.20). Briefly, the livers, draining lymph nodes, lungs and spleens were homogenized in HOMEM medium supplemented with FCS 10% and bone marrow cells collected from the femurs and tibias of infected mice. A limiting dilution (LD) cell suspensions *in vitro* culture assay was used to determine the number of viable parasites in these organs. The serial twofold dilutions of cell suspensions were plated in the 24-well plates. The cells were then incubated at

25°C for 14 days. The cultures were examined for the presence of promastigote production every day.

4.3. Results

4.3.1. Vaccine potential of *L. mexicana* H-line

The cytokine production results (see Chapter 3) indicated that infection of mice with *L. mexicana* H-line induced a Th1 response, which is a protective response against *L. mexicana* infection (Satoskar *et al.*, 1995). Therefore, it was examined whether pretreatment of mice with the attenuated cell line could protect susceptible BALB/c mice from infection with *L. mexicana* WT. Mice were immunized with *L. mexicana* H-line and challenged with *L. mexicana* WT at 12 weeks post immunization. All non-vaccinated mice developed progressive non-healing lesions and the mean lesion size at 14 weeks post infection this time was more than 2600 mm^3. In contrast to non-vaccinated mice, the lesion developed slowly in vaccinated mice and as Figure 4.1 shows the mean of lesion size at 22 weeks post infection was only about 200 mm^3.

4.3.2. Protection potential of *L. mexicana* H-line - immunization and challenge at the same time

The results indicated that the *L. mexicana* H-line induced protection in susceptible mice against *L. mexicana* WT. Next it was examined whether the attenuated cell line is able to induce protection when promastigotes of the two lines were injected (s.c.) at the same time (Figure 4.2).

The first group of mice was injected with 2.5×10^6 stationary phase promastigotes of *L. mexicana* H-line in the left side rump and the same number of stationary phase of *L. mexicana* WT in the right side rump. The lesions that developed in the right side rumps were initially the same size as lesions that developed in the control mice, but after about 13 weeks the lesion growth slowed. No lesion or swelling was observed in the left side rump where promastigotes of *L. mexicana* H-line were injected.

The second group of mice was injected with a mixture of 2.5×10^6 stationary phase promastigotes of *L. mexicana* H-line and the same number of stationary phase

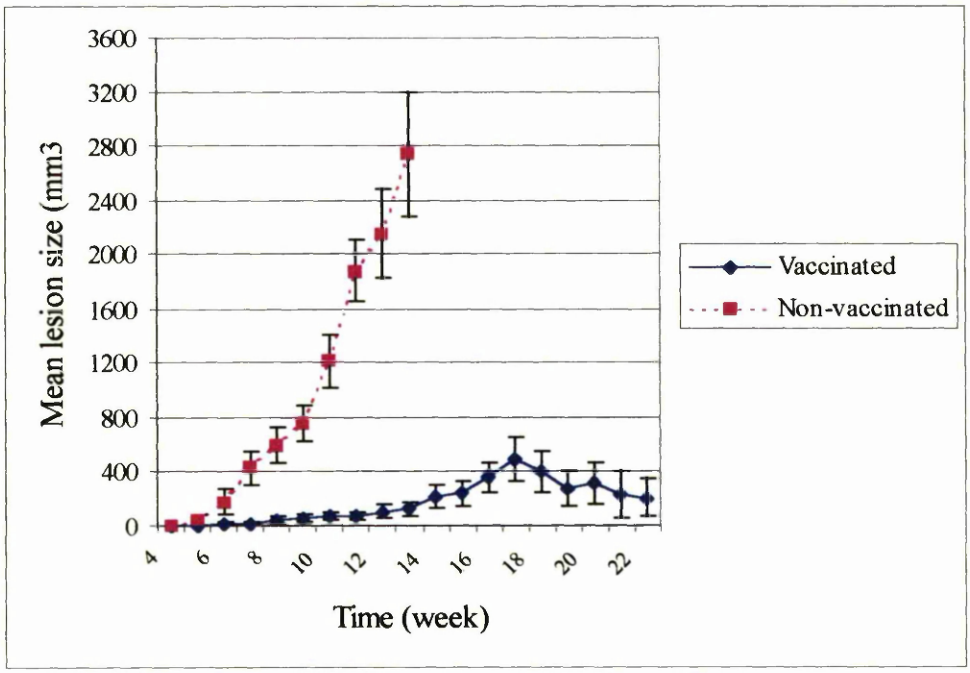


Figure 4.1. The course of lesion size following *L. mexicana* WT infection in 2 groups of BALB/c mice (14 mice / group). One group had been vaccinated with 5×10^6 stationary phase promastigotes of *L. mexicana* H-line. 5×10^6 stationary phase promastigotes of *L. mexicana* WT were inoculated in the shaven right side rump of the vaccinated or non-vaccinated mice. Lesion development was monitored by measuring the lesions weekly. Each data point represents the mean lesion size ± SEM (n =14).

promastigotes of *L. mexicana* WT both at the same side in the right side rump. The results showed that 2 out of the 10 mice developed very slowly growing lesions, whereas the other 8 mice had no lesions. The control group of mice was infected with 2.5×10^6 stationary phase promastigotes of *L. mexicana* WT. All mice developed progressive non-healing lesions. These results shows that the *L. mexicana* H-line induced a protection that led to control of *L. mexicana* WT infecting at the same time.

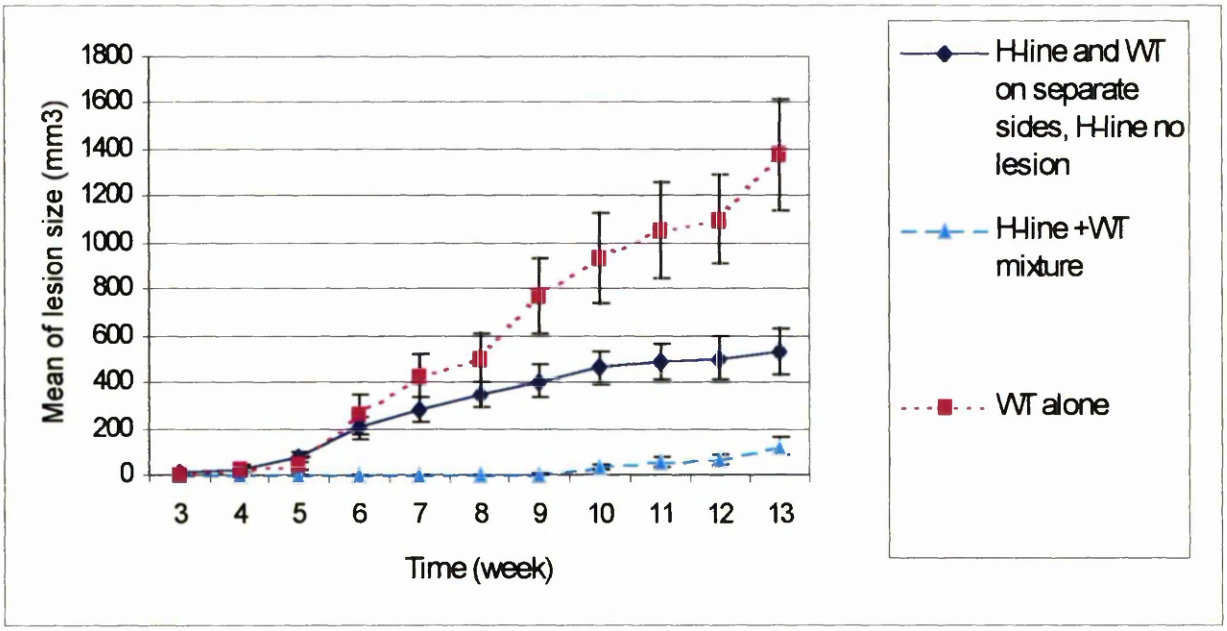


Figure 4.2. The course of infection of three groups in BALB/c mice (10 mice / group) infected with both *L. mexicana* WT and *L. mexicana* H-line or *L. mexicana* WT alone. Lesion development was monitored by measuring the lesions weekly. Each data point represents the mean lesion size ± SEM (n =10).

4.3.3. Lesion development in mice vaccinated with *L. mexicana* H-line and infected with *L. major* WT

In order to determine whether *L. mexicana* H-line has the potential to protect BALB/c mice from *L. major*, the mice immunized with *L. mexicana* H-line were challenged at week 12 with stationary phase promastigotes of *L. major* WT. The lesion size in the vaccinated mice was compared with non-vaccinated mice. As the figure 4.3 shows, there is no significant difference between the two groups of mice. This result suggests that there was no protection in the mice vaccinated with *L. mexicana* H-line against *L. major*.

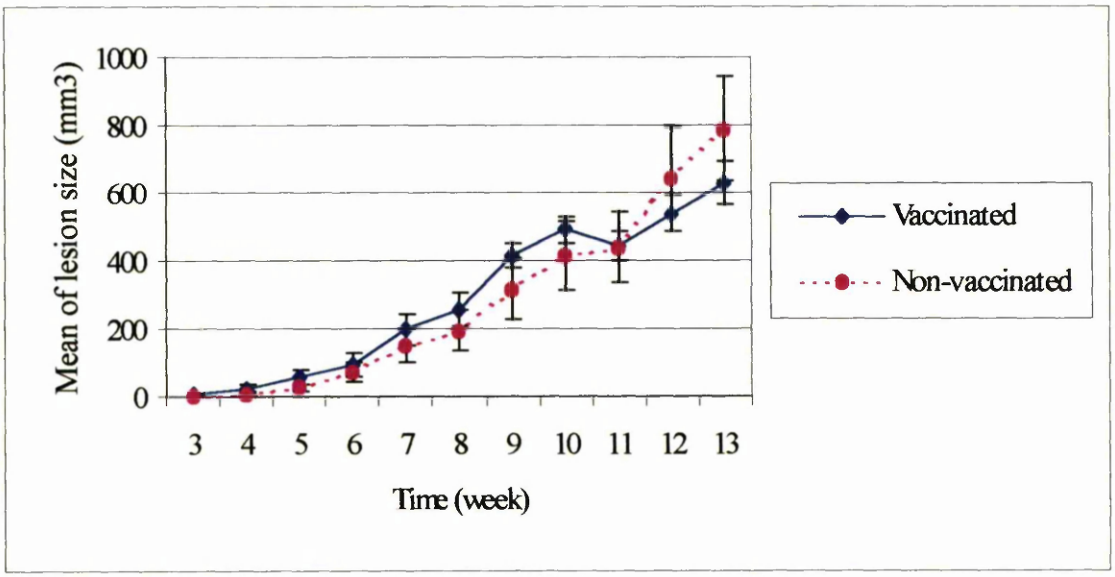


Figure 4.3. The course of lesion size following *L. major* WT infection in 2 groups of BALB/c mice (14 mice / group). One group had been vaccinated with 5×10^6 stationary phase promastigotes of *L. mexicana* H-line. 5×10^6 stationary phase promastigotes of *L. major* WT were inoculated in the right side shaven rump of the vaccinated or non-vaccinated mice. Lesion development was monitored by measuring the lesions weekly. Each data point represents the mean lesion size ± SEM (n = 14).

4.3.4. Vaccine potential of *L. major* H-line

To determine whether the *L. major* H-line has the ability to protect susceptible mice against infection with *L. major* WT, 5 BALB/c mice were immunized with *L. major* H-line. The mice were challenged at 12 weeks with *L. major* WT. All non-vaccinated mice developed progressive non-healing lesions up to 12 weeks post infection and the mean lesion size at this time was more than 2000 mm^3. In contrast to non-vaccinated mice, lesions developed slowly in vaccinated mice and as Figure 4.4 shows the mean of lesion size at 12 weeks post infection was less than 1000 mm^3.

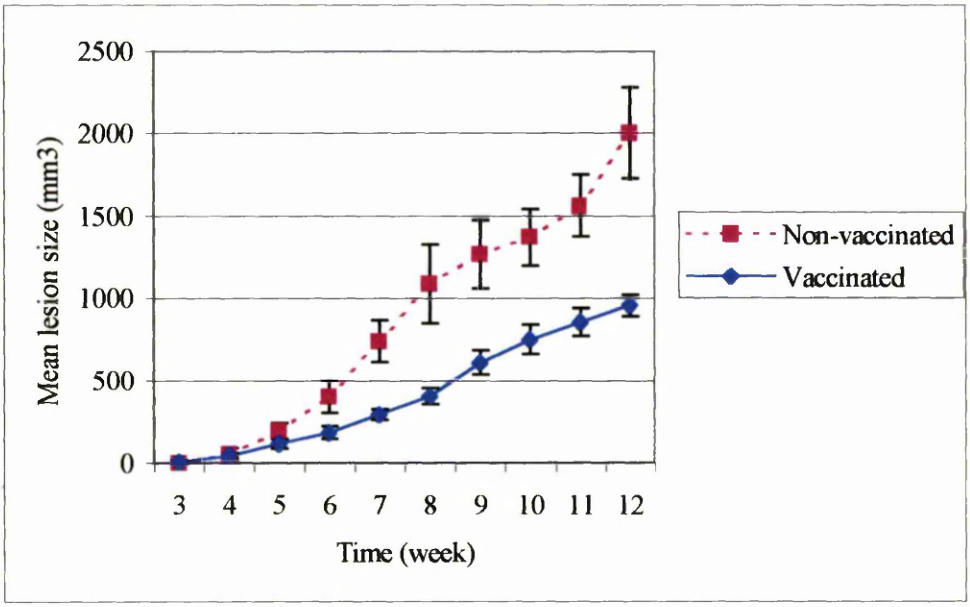


Figure 4.4. The course of lesion size following *L. major* WT infection in 2 groups of BALB/c mice (5 mice / group). One group had been vaccinated with 5×10^6 stationary phase promastigotes of *L. major* H-line. 5×10^6 stationary phase promastigotes of *L. major* WT were inoculated in the right side shaven rump of the vaccinated or non-vaccinated mice. Lesion development was monitored by measuring the lesions weekly. Each data point represents the mean lesion size ± SEM (n = 5).

4.3.5. T-cell proliferation of splenocytes from vaccinated or non-vaccinated BALB/c mice infected with the two lines of *L. mexicana*

Studies were carried out to determine whether the splenocytes of vaccinated and non-vaccinated BALB/c mice infected with 5×10^6 stationary phase promastigotes of *L. mexicana* WT 12 weeks post infection were stimulated with stationary phase promastigotes of *L. mexicana* WT. The splenocytes were exposed to promastigotes, at 32°C for 4 h, and then incubated at 37°C for 68 h. The splenocyte: parasite ratio was 10 / 1. The splenocytes were stimulated with promastigotes, because this simulated the situation when the immune mice are exposed to a normal challenge. The proliferation of the T cells was confirmed with Con-A (5 μg/ml) as a positive control and non-stimulated cells as negative controls. As Figure 4.5 shows the Con-A-stimulated proliferative response of splenocytes from non-vaccinated mice was significantly higher than that of promastigotes-stimulated ($P<0.02$). Promastigotes of *L. mexicana* WT induced proliferation of splenocytes of the vaccinated mice significantly less that of the Con A-stimulated splenocytes ($P<0.04$).

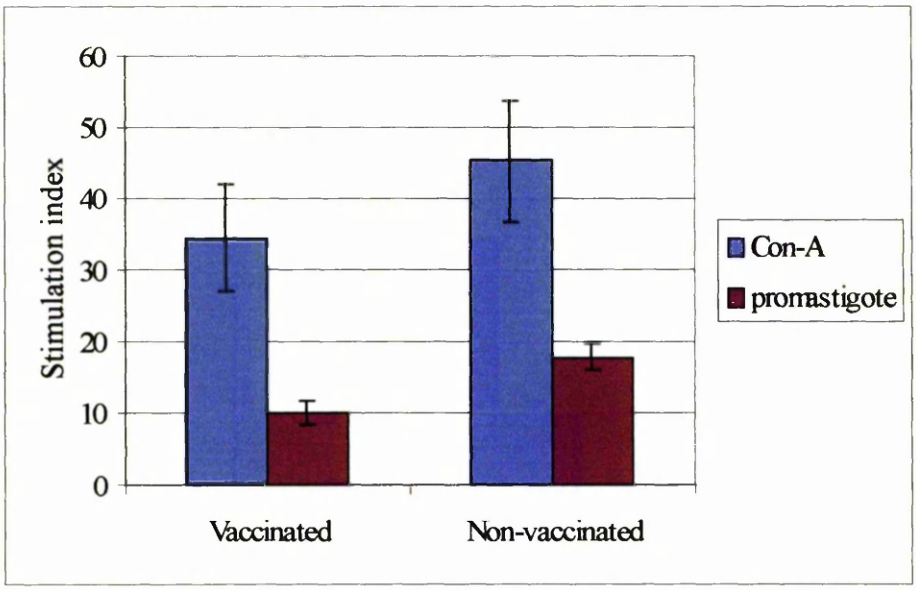


Figure 4.5. T-cell proliferation responses of splenocytes from non-vaccinated mice and mice vaccinated with *L. mexicana* H-line and infected with *L. mexicana* WT. The mice vaccinated with *L. mexicana* H-line and non-vaccinated mice were challenged with 5×10^6 stationary promastigotes of *L. mexicana* WT at 12 weeks post immunization. T-cell proliferation responses of spleen cells from mice at week 12 post infection were induced by Con A (5 μg/ml) and stationary phase promastigotes of *L. mexicana* WT (the ratio of splenocytes: promastigotes was 10 / 1). The stimulation index was calculated as count per minute (CPM) of stimulated cells / CPM of unstimulated cells. The bar represent SEM (n = 5).

4.3.6. IFN-γ and IL-4 production by splenocytes of challenged mice

To determine whether the vaccination with *L. mexicana* H-line had an effect on the cytokine production of mice vaccinated with *L. mexicana* H-line and at 12 weeks post immunization challenged with *L. mexicana* WT, splenocyte cultures were established from mice at 12 weeks post challenge. The splenocytes were restimulated with stationary phase promastigotes of *L. mexicana* WT and supernatants harvested after 72 h culture. Spleen cells from mice which were injected with *L. mexicana* H-line and after 12 weeks were challenged with *L. mexicana* WT, produced levels of IFN-γ which were significantly greater than these produced by mice which did not receive the vaccination ($P<0.005$) (Figure 4.6).

IL-4 levels were increased in supernatants harvested from Ag-stimulated spleen cells set up from mice receiving the single *L.mexicana* WT challenge. In contrast, no IL-4 was produced by cells taken from the mice which had received the pre-treatment with *L. mexicana* H-line (Figure 4.7).

The relative production of these cytokines can thus be used as a marker for the induction of Th1-like and Th2-like immune responses, respectively. The spleen cells from mice immunized with *L. mexicana* H-line secreted high concentrations of IFN-γ but no detectable IL-4. The stationary phase promastigotes of *L. mexicana* WT induced spleen cells producing high concentrations of IL-4. Spleen cells were stimulated *in vitro* with stationary phase promastigotes of *L. mexicana* WT for 72 h. In contrast to the mice vaccinated with *L. mexicana* H-line and challenged with *L. mexicana* WT, the amount of IL-4 in the supernatant of splenocyte cultures of non-vaccinated mice was significantly increased higher than that with challenged mice ($P< 0.005$) (Figure 4.7).

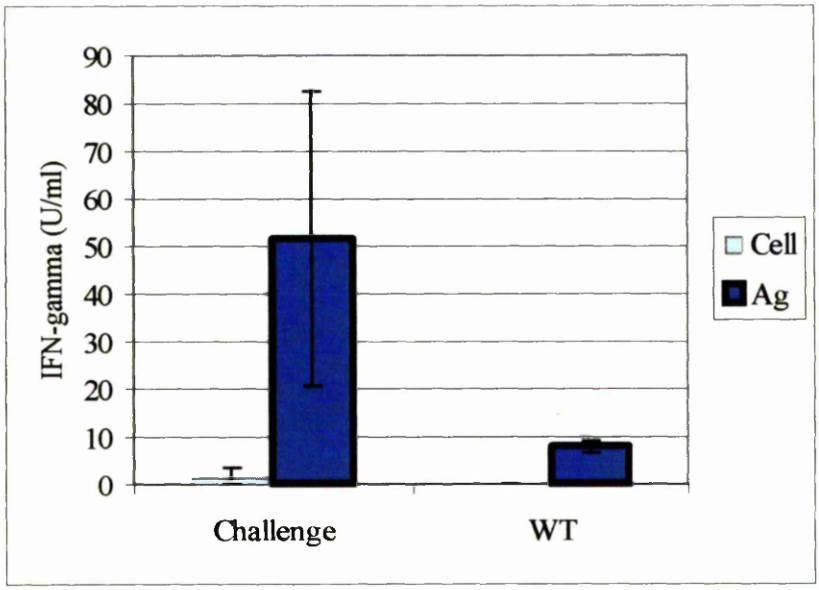


Figure 4.6. IFN-γ production by cultured splenocytes taken from mice 12 weeks post infection with stationary phase promastigotes WT or mice vaccinated with *L. mexicana* H-line and at 12 weeks post immunization challenged with *L. mexicana* WT. The mice were immunized with *L. mexicana* H-line and at 12 weeks challenged with *L. mexicana* WT. The splenocytes were restimulated with stationary phase promastigotes of *L. mexicana* WT and supernatants harvested after 72 h culture. The infection ratio of parasite / splenocyte was 1: 10. Bars represents SEM (n = 3).

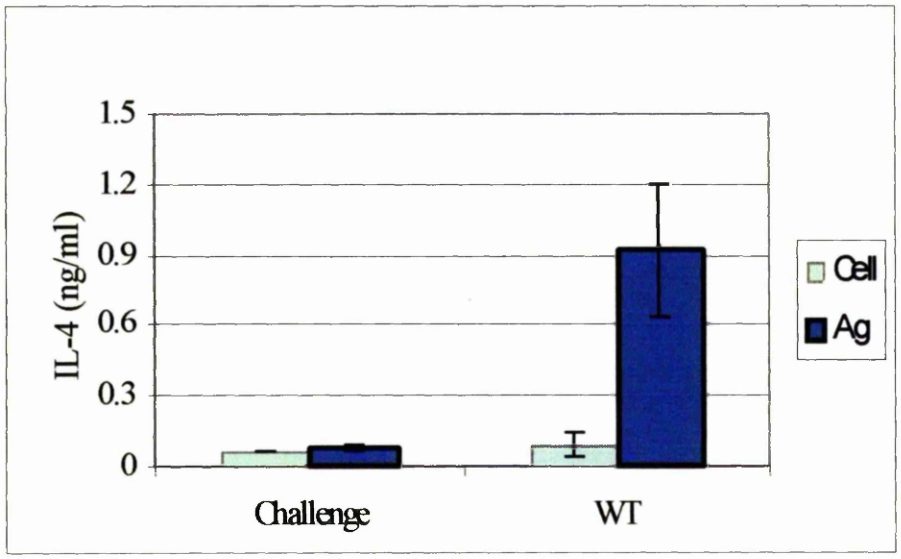


Figure 4.7. IL-4 production by cultured splenocytes taken from mice 12 weeks post infection with stationary phase promastigotes WT or mice vaccinated with *L. mexicana* H-line and at 12 weeks post immunization challenged with *L. mexicana* WT. The mice were immunized with *L. mexicana* H-line and at 12 weeks challenged with *L. mexicana* WT. The splenocytes were restimulated with stationary phase promastigotes of *L. mexicana* WT and supernatants harvested after 72 h culture. The infection ratio of parasite / splenocyte was 1: 10. Bars represent SEM (n = 3).

4.3.7. Investigation of the spreading of *L. mexicana* to the visceral organs of mice vaccinated with *L. mexicana* H-line

The *in vitro* results suggest that the *L. mexicana* H-line may be capable of protecting susceptible mice against *L. mexicana* WT. To consider the *in vivo* survival and possible dispersion of the *L. mexicana* WT beyond site of inoculation, the vaccinated mice were injected with *L. mexicana* WT and then examined for the presence of parasites in the peripheral and visceral organs. Stationary phase promastigotes of *L. mexicana* WT were subcutanously injected and then the spreading of parasites in the skin and visceral organs of 6 challenged mice was investigated at 12 weeks post infection. The visceral organs were removed asceptically. These included liver, spleen, lung, bone marrow (BM), draining popliteal lymph node (LN), and ECs from the site of injection at the appropriate times. The cell suspensions were cultured in HOMEM medium supplemented with 10% (v/v) FCS and incubated at 25°C over 14 days. The cultures were examined daily for the presence of live promastigotes (Table 4.1). Promastigotes of *L. mexicana* H-line grew in cell cultures from LN and ECs, where *L. mexicana* H-line were injected, of challenged mice numbers 1, 2, and 6, whereas no promastigotes of *L. mexicana* WT grew in the cell cultures obtained from visceral organs of these mice. No promastigote of *L. mexicana* H-line grew in the cell culture derived from LN and ECs, where *L. mexicana* H-line were injected, of mice numbers 3, 4, and 5. In contrast, in the absence of *L. mexicana* H-line, promastigotes of *L. mexicana* WT were observed in the tissue cultured of these mice to survive within resident macrophages for long periods.

In addition, throughout the period of study (24 weeks) no lesion developed in the right side of mice where the promastigotes of *L. mexicana* H-line were injected. Two mice (numbers 4 and 5), developed a lesion in the left side where promastigotes of *L. mexicana* WT were injected and no lesions were observed in the rest of mice (Table 4.2). The draining LN contained parasites within one day of infection, and remained infected throughout the period of study (12 weeks). It was shown that *L. mexicana* WT spread to BM, spleen and lung and it was observed that infection of visceral organs correlated with an increase in lesion size (see section 3.3.12).

Table 4.1. Distribution of the two lines of *L.mexicana* in the skin and visceral organs of challenged mice.

Mouse	1	2	3	4	5	6
Epidermal cell [a]	+	+	-	-	-	+
Epidermal cell [b]	-	-	+	+	+	-
Spleen	-	-	-	+	+	-
Lung	-	-	+	+	+	-

(a): The site where stationary phase promastigotes of *L. mexicana* H-line were injected.

(b): The site where stationary phase promastigotes of *L. mexicana* WT were injected.

(+): Indicates that promastigotes were observed in the tissue culture.

(-): Indicates that no promastigotes were observed in the tissue culture.

Table 4.2. Cutaneous lesion development in the challenged mice 12 weeks post infection.

Mouse	1	2	3	4	5	6
Lesion (right side) [a]	N [c]	N	N	N	N	N
Lesion (left side) [b]	N	N	N	L [d]	L [e]	N

(a): The site where stationary phase promastigotes of *L. mexicana* H-line were injected.

(b): The site where stationary phase promastigotes of *L. mexicana* WT were injected.

(c) : No lesion developed.

(d): Small size lesion developed.

(e): Large size lesion developed.

4.4. Discussion

L. mexicana H-line is capable of inducing a CD4+ Th1 response, which was demonstrated by elevated IFN-γ and IL-2 production by cultured splenocytes of BALB/c mice infected with *L. mexicana* H-line (for more detail see Chapter 3). To determine whether the attenuated line could induce protection against *L. mexicana* WT, the mice vaccinated with stationary phase promastigotes of *L. mexicana* H-line were challenged at week 12 post immunization with *L. mexicana* WT. All non-vaccinated mice developed large size, non-healing lesions, whereas the vaccinated mice developed small and in some cases healing lesions over 22 weeks post infection. This result suggested that immunization with *L. mexicana* H-line induced a protective Th1 response and immunized mice were significantly resistant to *L. mexicana* WT compared with non-immunized mice. In a further study it was shown that IFN-γ production was elevated in the supernatant of cultured splenocytes of mice challenged with *L. mexicana* H-line. The present results are supported by the reports that recovery from cutaneous lesions in mice resistant to *L. mexicana* was associated with the expansion of the $CD4^+$ Th1 subset and production of cytokines such as IL-12, IFN-γ, and IL-2 (Stamm *et al.,* 1998). Resistance to *L. major* in various mouse strains strongly correlates with the development of a Th1-like cytokine secretion profile (Reiner and Locksley 1995). In contrast to *L. mexicana* H-line, injection of *L. mexicana* WT induced a Th2 immune response and an exacerbated disease. Therefore, the attenuated line of *L. mexicana* holds considerable promise for vaccination against leishmaniasis in which Th1 responses are desirable.

In addition, the present results indicated that a Th1 response alone can protect against *L. mexicana* WT, in agreement with the report that induction of an immune response with mixed Th1-like and Th2-like elements is unable to confer protection and that a Th1 response is sufficient to protect against disease in experimental cutaneous leishmaniasis and the induction of a simultaneous Th2 response abrogates the Th1 effector function (Sjölander, 1998). Furthermore, there is considerable evidence that Th2-type responses and the production of IL-4 results in the inability to control disease, or results in disease exacerbation (Heinzel *et al.*, 1991). The severity of disease in murine cutaneous leishmaniasis is better correlated with the presence of IL-4 than the lack of production of IFN-γ (Erb *et al.*, 1996; Morris *et al.*, 1993a). This observation shows that *L. mexicana* H-line preferentially induces a Th1-like immune

response and down-regulate the Th2 response. In murine leishmaniasis, the genetically resistant mice display a Th1 phenotype, whereas the susceptible BALB/c mice develop a clear Th2 cytokine phenotype (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991).

Alexander and Phillips (1980) reported that mice infected with *L. major* were immune to subsequent infection with *L. mexicana*. To determine whether mice vaccinated with *L. mexicana* H-line were capable of protection mice against *L. major*, the mice vaccinated with *L. mexicana* H-line were challenged with *L. major*. The results showed that the attenuated line of *L. mexicana* generated no protection against *L. major*. In this case there is no cross immunity between *L. mexicana* and *L. major*.

The local restriction of the parasites prior to the development of T cell responses appears to be mediated by the innate immune system and this activity is considered to play an important role in the development of protective T cell responses (Laskay *et al.*, 1995). Dissemination of the parasite to the visceral organs in susceptible mice is result of the development of a non-protective Th2 response (Leiw *et al.*, 1993). In the present study it has been demonstrated that in the presence of the attenuated cell line of *L. mexicana*, *L. mexicana* WT was not found in the draining LN, skin and other organs. In contrast, *L. mexicana* WT disseminated into the visceral organs of challenged mice in the absence of the attenuate cell line. Therefore the presence of *L. mexicana* H-line in the host led to the elimination *L. mexicana* WT.

In Chapter 3, it was shown that *L. mexicana* H-line remained localized in the EC of skin where the promastigotes were injected and in the draining popliteal LN, without evidence of dissemination to the visceral organs throughout the period of study. In contrast, *L. mexicana* WT disseminated to the bone marrow, spleen, draining popliteal LN, and lungs. Therefore, it is very unlikely that the parasites, which were disseminated in the visceral organs of challenged mice, belong to attenuated cell line. The protection of *L. mexicana* H-line against *L. mexicana* WT at the same time was investigated by following the result of injecting of the same number of stationary phase promastigotes of the two lines of *L. mexicana* in the same side or the same number of *L. mexicana* WT or *L. mexicana* H-line on separate sides in the same mice. The control mice which were infected with *L. mexicana* WT alone developed progressive non-healing lesions. In contrast, some of the mice that were infected with mixed *L. mexicana* H-line and *L. mexicana* WT developed very slowly growing lesions. In the mice, which were injected with the two lines of parasites on the

separate sides, growth of *L. mexicana* WT growth eventually slowed. There was good evidence of control of *L. mexicana* WT.

The preliminary results also showed that *L. major* H-line induced protection in susceptible mice against infection with *L. major* WT. All non-vaccinated mice developed progressive non-healing lesions that peaked in size at 12 weeks post infection and the mean lesion size at this time was 2000 mm^3. In contrast to non-vaccinated mice the lesion developed slowly in vaccinated mice and as Figure 4.4 shows the mean of lesion size at 12 weeks post infection was about 1000 mm^3.
Unfortunately, time limitation and prioritisation prevented further investigation by other methods.

CHAPTER FIVE

GENERAL DISCUSSION

The *Leishmania* species belong to the kinetoplastid protozoa, which are responsible for a wide spectrum of disease including cutaneous, mucocutaneous, and visceral leishmaniasis (Pearson and Sousa, 1996). The worldwide prevalence of leishmaniasis is approximately 12 million, mostly children and young adults (WHO report, http://www.who.int/ctd/htmi/leish.html, 2000). Although treatment with leishmanicidal drugs is available, chemotherapy has only a moderate effect and the available drugs frequently have unwanted side effects. Some are losing their effectiveness and drug resistance is becoming a significant problem in various endemic regions of the world (Jackson *et al.*, 1990). Although there has been much recent interest in attempts to vaccinate against *Leishmania* infection, there is no effective and safe vaccine against any form of leishmaniasis (Piedrafita *et al.*, 1999). Individuals who have recovered from clinical leishmaniasis develop strong immunity against reinfection, and therefore it has been suggested that vaccination against leishmaniasis is feasible in principle (Liew and O'Donnell, 1993). Conventional vaccine development against leishmaniasis is focused on five approaches: killed promastigotes, live promastigotes (wild type), live attenuated promastigotes, subunit vaccines, and DNA vaccines. Rappuoli and Giudice (1999) argued that leishmanial vaccine development is most likely to be successful through use of attenuated promastigotes and subunit vaccines based on the identification of protective antigens. All subunit vaccines tested (for example gp63, gp46) however have given only partial protection and their efficacy has often required the use of clinically unacceptable adjuvants (Piedrafita *et al.*, 1999).

In this study the development of an attenuated cell line of *L. mexicana* in the presence of an antibiotic is described. In a preliminary investigation, this technique was also extended to attenuate *L. major*. Therefore, this method for inducing attenuation might be applicable to other species of *Leishmania* and possibly other microorganisms. Aebischer and colleagues (1993) suggested that an attenuated cell line of *L. major*, which persisted for a prolonged period in vivo, stimulated clinically protective immunological memory in a process known as concomitant immunity. It is clear that while several kinds of vaccine against leishmaniasis have been generated, the protection induced by the use of attenuated promastigotes has to date proved to be the most effective. These findings are consistent with reports that attenuated forms of many microorganisms can serve as highly effective vaccines (Hess *et al.*, 2000).

Attenuated lines of *Leishmania* species have been created using several methods, such as disruption of (controlling) virulence genes from the genome of wild type *Leishmania*. For example, *L. mexicana* Δ*cpa/cpb* is an attenuated line (Souza *et al.*, 1994) and is candidate for vaccines (Alexander *et al.*, 1998). An attenuated *L. major* line lacking a DHFR/TS gene (Titus *et al.*, 1995) has also been developed.

For the first time attenuated cell lines of *L. mexicana* WT and *L. major* WT have been generated in vitro in the presence of an antibiotic. The attenuated lines of *L. mexicana* WT and *L. major* WT known as *L. mexicana* H-line and *L. major* H-line, respectively, have been established under antibiotic pressure (gentamicin), which was routinely added to the medium to prevent bacterial contamination. The attenuated parasites were characterised in vivo and in vitro. The ability of the *L. mexicana* H-line to survive within infected macrophages was examined by exposure of stationary phase promastigotes from the *L. mexicana* H-line to bone marrow -derived macrophages (BMMs). The results showed that the percentage of macrophages infected with the *L. mexicana* H-line was similar to that of *L. mexicana* WT at 9 h post infection. In contrast to *L. mexicana* WT, only a small population of amastigotes of *L. mexicana* H-line survived within infected macrophages after 96 h post infection in vitro. The ability of *L. mexicana* H-line to induce cutaneous leishmaniasis was examined by injection (subcutaneously) of stationary phase promastigotes of *L. mexicana* H-line. It was shown that the majority of BALB/c mice, which are susceptible to *L. mexicana* WT, when infected with L. mexicana H-line failed to develop cutaneous lesions. Over the whole study over 40 mice were infected with *L. mexicana* H-line and only one developed a healing lesion. In contrast to the *L. mexicana* H-line, all mice infected with *L. mexicana* WT, which was grown in parallel cultures of the same age as *L. mexicana* H-line in medium without gentamicin, developed non-healing lesions. Therefore, it was concluded that the *L. mexicana* H-line is an attenuated line, which has been generated in the pressure of gentamicin. The mechanism by which gentamicin, an aminoglycoside, attenuates *L. mexicana* WT is unknown. Several groups of researchers determined the sequence requirements of ribosomal RNA for aminoglycoside interaction with prokaryotes and eukaryotes (Werstuck and Green, 1998; Yoshizawa *et al.*, 1998; Recht *et al.*, 1999). Disruption of ribosomal activity by breaking up of polysomes or inducing misreading of mRNA during protein synthesis resulting in incomplete protein synthesis, are possible

mechanisms (Chambers and Sande, 1996). It was found that the adaptation of *L. mexicana* H-line under pressure of gentamicin has involved an alteration in protein expression. Two bands were detected around 66 kDa in stationary or log phase promastigotes of *L. mexicana* WT, whereas one (possibly two) of these bands were absent in the stationary and log phase promastigotes of *L. mexicana* H-line. In other experiments, the comparative proteome analysis of the two lines of *L. mexicana* using high-resolution techniques was carried out using isoelectric focusing and SDS-PAGE. It was demonstrated that both lines of *Leishmania* comprise patterns with a high density of spots in the range pH 4-7 of the gel. The position of spots in the gels of the two lines of *L. mexicana* revealed high similarity, and the main spots were compared easily. One spot in the *L. mexicana* WT gel was shifted to a less acidic position in the *L. mexicana* H-line and one spot was absent in *L. mexicana* H-line. In addition, two spots were found in the *L. mexicana* WT gel, whereas the expression of these proteins by promastigotes of *L. mexicana* H-line decreased. Although, the data have shown significant differences between the two lines of *L. mexicana*, there are insufficient replicates to draw firm conclusions about the absence or decreased expression of the proteins in 2-DE. To identify proteins in the lysate of stationary phase promastigotes of *L. mexicana* H-line, which were lost or reduced compared with *L. mexicana* WT, mass spectrometry with database searching has been suggested (Beavis and Fenyo, 2000). In conclusion, the results of protein analysis using SDS-PAGE gradient gels and proteome analysis using 2-DE of the two lines of *L. mexicana* demonstrate that the attenuation procedure under pressure of gentamicin affected protein expression in promastigotes of L. mexicana H-line (following growing the parasites in the presence of gentamicin).

On the basis of these experiments, promastigotes of *L. mexicana* WT passaged at least 20 times in HOMEM medium supplemented with 10% (v/v) FCS and gentamicin at 20 μg/ml, were shown to become attenuated during this process. The precise timing of any changes induced in the parasite by gentamicin that led to parasite attenuation has yet to be determined. It was shown that *L. mexicana* H-line was stable in the absence of gentamicin. Promastigotes of *L. mexicana* H-line were grown in antibiotic-free medium for 40 passages and then exposed to bone marrow macrophages. The ability of the attenuated line to invade and survive within infected macrophages was examined over a 96 h incubation. The results showed that the attenuated line is stable in antibiotic-free medium *in vitro* for more than 23 weeks.

Therefore, the differentiation of *L. mexicana* WT to the attenuated line of *L. mexicana* is stable in the absence of gentamicin in medium. It was demonstrated that the adaptation of *L. mexicana* H-line under pressure of gentamicin has not involved cysteine proteinase (CP) production by stationary phase promastigotes of *L. mexicana* H-line. The proteinase activity of stationary phase promastigotes of *L. mexicana* H-line and *L. mexicana* WT was investigated by loading lysates on gelatin-SDS-PAGE gel. Robertson and Coombs (1992) reported that a high mobility band of CP activity can be detected in stationary phase promastigotes of *L. mexicana* using gelatin-SDS-PAGE. Three bands were detected of which the molecular mass of the highest mobility band of proteolytic activities was 20 kDa. That these proteinases are cysteine proteinase is supported by the report of an analysis of CPB isoenzymes expressed in of stationary phase promastigotes of *L. mexicana* WT. Using Western blotting, anti-CPB antiserum detected two major proteins (25 and 29 kDa) (Mottram *et al.*, 1997). Therefore, the stationary phase promastigotes of *L. mexicana* H-line are not deficient in CP. During the present study some experimental work has been carried out with *L. mexicana* Δcpa/cpb which had been created by targeted gene disruption (Mottram *et al.*, 1997). It was therefore, necessary to demonstrate that promastigotes of *L. mexicana* H-line were not contaminated with *L. mexicana Δcpa/cpb*. The promastigotes of *L. mexicana* H-line were grown in the presence of 4 kinds of selective antibiotics, nourseothricin hydrosulfate (Sat), puromycin (Pur), zeocin (Ble) or hygromycin B (Hyg) separately or in combination. The results demonstrated that promastigotes of *L. mexicana* H-line were sensitive to these selective antibiotics and did not grow in the media supplemented with these antibiotics. In contrast, promastigotes of *L. mexicana Δcpa/cpb* grew in these media. This result was in agreement with the report that *L. mexicana* Δcpa was generated by disrupting sequentially both alleles of lmcpa using gene-targeting of promastigotes with hygromycin- and phleomycin-resistance markers and promastigotes of *L. mexicana* Δcpa/cpb are able to grow in the presence of these selective antibiotics (Souza *et al.*, 1994). Therefore promastigotes of *L. mexicana* H-line were neither deficient in CPs, nor contaminated with promastigotes of *L. mexicana Δcpa/cpb*. *L. mexicana* H-line has been generated on four separate occasions by the same procedure. On each occasion promastigotes were derived from amastigotes, and grown in parallel in media with or without gentamicin for at least 20 passages. It was demonstrated that the attenuation procedure did not effect on the growth rate of promastigotes of *L.*

mexicana H-line. There was no significant difference between the growth rate of promastigotes of *L. mexicana* H-line and *L. mexicana* WT *in vitro*.

There are two observations show that *L. mexicana* H-line is non-infective. First, the attenuated line of *L. mexicana* H-line was unable to survive within BMM in vitro. Alexander and Russell (1992) reported that the ability of amastigotes of *Leishmania* to survive within macrophages is a primary mechanism for evading the immune response of their vertebrate host. Secondly, over 40 mice infected with *L. mexicana* H-line failed to develop cutaneous lesions and only one developed a healing lesion during 12 weeks post infection. It is proposed that the mechanisms underlying the development of non-healing lesions, in genetically susceptible mice, is under control of specific cytokines, including IL-12, IFN-γ, IL-4, and IL-10 (Heinzel et al., 1995; Scharton-Kersten and Scott, 1995). Satoskar and colleagues (1997) showed that genetically susceptible mice lacking IL-4 are protected from cutaneous infection with *L. mexicana* WT. IL-4 and IL-10 induce non-healing lesions, whereas in the presence of IFN-γ and IL-12 cutaneous lesions fail to develop or heal lesions. The activation state of macrophages and T cells is dependent on the availability of stimulatory and inhibitory cytokines, the production of which can be altered by the parasite (Bogdan *et al.*, 1999). The levels of IFN-γ, IL-2, IL-4, and IL-10 in the supernatant of cultured splenocytes from the mice infected with *L. mexicana* H-line or *L. mexicana* WT were measured. It was found that the levels of IFN-γ IL-2 in the supernatant of cultured Ag-stimulated-splenocytes of mice infected with *L. mexicana* H-line were significantly higher than those of splenocytes of mice infected with *L. mexicana* WT ($P<0.05$). As a result *L. mexicana* H-line amastigotes were eliminated by macrophages, whereas amastigotes of *L. mexicana* WT are able to survive for prolonged periods within these cells. The survival of *L. mexicana* WT amastigotes within the macrophage is prevented when the macrophages are stimulated by specific T-cell-derived lymphokines and IFN-γ is the most important component among these macrophage-activating mediators (Nathan *et al.*, 1983). It was found that the level of IL-4 in the supernatant of splenocytes from the mice infected with *L. mexicana* H-line was significantly lower than that of mice infected with *L. mexicana* WT ($P<0.05$). IL-4 plays a crucial role in inhibiting macrophage leishmanicidal activity and down-regulating the development of a Th1-like response (Oswald *et al.*, 1992). The amount of IL-10 in the supernatant of cultured Ag-stimulated-splenocytes from the mice infected with *L. mexicana* WT or *L. mexicana* H-line was measured. The level of IL-

10 production by splenocytes from mice infected with *L. mexicana* WT was significantly higher than that of mice infected with *L. mexicana* H-line ($P<0.02$). IL-10, which is typically induced by the parasite, inhibits killing of *Leishmania* species and counteracts the development of a protective Th1 immune response (Bogdan et al., 1993) by its action on antigen presenting cells. The level of IL-2 in the supernatants of cultured splenocytes from BALB/c mice infected with stationary phase promastigotes of *L. mexicana* H-line was significantly higher than that of promastigotes from *L. mexicana* WT ($P<0.05$). Nacy and colleagues (1991) reported both IFN-γ and IL-2, induce TNF-α production and secretion of this cytokine by macrophages. Thus *L. mexicana* WT induces IL-4 and IL-10 whereas *L. mexicana* H-line presumably has the capacity to induce IFN-γ, TNF-α, and IL-2, in susceptible mice. This is consist with the proposition that IFN-γ production by Th1 cells inhibits the development of Th2 cells (Fitch *et al.*, 1993), whereas the production of IL-4 and IL-10 by Th2 cells inhibits Th1 development and activation, as well as macrophage activation and bactericidal activity (Sher and Coffman, 1992; Moore *et al.*, 1993).

Following inoculation of 5×10^6 stationary phase promastigotes of the two cell lines, dissemination and survival of the two lines in the skin and visceral organs of BALB/c mice were investigated. Other studies have shown that dissemination of the parasite in the visceral organs of susceptible mice is the result of the development of a non-protective Th2 response (Liew and Donnell, 1993). The local restriction of the parasite prior to the development of T cell responses appears to be mediated by the innate immune system and this activity is considered to play an important role in the subsequent development of a protective T cell response (Laskay *et al.*, 1995). In the present study it was found that *L. mexicana* H-line was localized in the skin at the site where the promastigotes were injected and the draining popliteal LN, after 120 days post infection without any dissemination to the visceral organs. In contrast, infection with *L. mexicana* WT led to rapid parasite spread to the bone marrow, spleen, drain popliteal LN, and lungs. The fact that *L. mexicana* H-line parasites can survive in the skin for an extended period would sustain long term memory. All these results show that *L. mexicana* H-line has reduced virulence for susceptible mice and induces a Th1-like immune response.

It was demonstrated that the attenuated *L. mexicana* H-line was capable of protecting mice against *L. mexicana* WT. Mice were vaccinated with stationary phase promastigotes of *L. mexicana* H-line and 12 weeks post immunization were

challenged with *L. mexicana* WT. All the non-vaccinated mice developed progressive, non-healing lesions, whereas the mice vaccinated with *L. mexicana* H-line developed small but in some cases healing lesions over 22 weeks post infection. Thus, the attenuated line of *L. mexicana* holds considerable promise for vaccination against leishmaniasis in which Th1 responses are desirable. The levels of IFN-γ and IL-4 in the supernatants of cultured splenocytes from the vaccinated mice and mice challenged with *L. mexicana* WT were measured. It was found that the level of IFN-γ in the supernatant of cultured Ag-stimulated-splenocytes of challenged mice at 12 weeks post challenge was significantly greater than that of non-vaccinated mice infected with *L. mexicana* WT ($P<0.005$). In contrast to the vaccinated mice, the amount of IL-4 in the supernatant of Ag-stimulated-splenocytes cultured from non-vaccinated mice infected with *L. mexicana* WT was higher than that of vaccinated mice challenged with *L. mexicana* WT at the same time. Therefore, it can be concluded that *L. mexicana* H-line preferentially induces Th1-like immune responses and down-regulates Th2 response in BALB/c mice. This result is in agreement with reports that a Th1 response protects against cutaneous leishmaniasis, but the induction of a simultaneous Th2 response abrogates the protective Th1 effector function (Sjölander, 1998; Reiner and Locksley, 1995). Earlier in the study, it was shown that *L. mexicana* WT disseminated in the skin and visceral organs, whereas *L. mexicana* H-line localized in the skin and draining LN. Dissemination of *L. mexicana* WT in the skin and visceral organs of mice vaccinated with *L. mexicana* H-line was investigated. *L. mexicana* WT was disseminated in the skin or visceral organs of 3 vaccinated mice when *L. mexicana* H-line was apparently absent. In contrast, in the presence of *L. mexicana* H-line in the skin of 3 vaccinated mice, *L. mexicana* WT was unable to survive in the skin and visceral organs. Therefore, the presence of *L. mexicana* H-line in the host led to elimination of *L. mexicana* WT in the skin and visceral organs of vaccinated mice. The protection induced by *L. mexicana* H-line when challenged simultaneously with *L. mexicana* WT, was investigated. The same number of stationary phase promastigotes of the two lines of *L. mexicana* were injected either together in the same side of the rump or the same number of *L. mexicana* WT or *L. mexicana* H-line injected in opposites side of the rump of the mice. The control mice, which were infected with *L. mexicana* WT only developed progressive non-healing lesions. In contrast, some of the mice that were infected with a mixture of *L. mexicana* H-line and *L. mexicana* WT developed very slowly growing lesions. In the mice that

were injected with the two lines of parasites on the separate sides, growth of *L. mexicana* WT was slowed. This indicated that the presence of the attenuated cell line in the susceptible mice induced immune responses that led to the control of *L. mexicana* WT. These results showed that the attenuated *L. mexicana* H-line was able to control the immune system of the host even when it was inoculated with *L. mexicana* WT at the same time. In comparison with the control mice, the immune response of the mice that were injected two lines of *L. mexicana* in the same side were able to reduce the virulence of *L. mexicana* WT. This suggests that *L. mexicana* H-line could be used in therapeutically.

From some of the mice vaccinated with *L. mexicana* H-line, culturing the epidermal cells from the site where the parasite was injected, amastigotes differentiated to promastigotes and this cell line was designated *L. mexicana* HAD-line. It was observed that promastigotes of *L. mexicana* HAD-line grew poorly in medium with or without gentamicin. The morphology of cell cultures of *L. mexicana* HAD-line showed that 17% of the cells were promastigotes of which some of them had a reduced flagellum and 83% of them were amastigote-like. This suggests that the attenuated cell line maybe be unable to survive in the midgut of sandfly and if so, this would reduce the risk of virulence reversion and spread of disease by infected sandflies.

Preliminary results showed that an attenuated cell line of *L. major* could be generated under antibiotic pressure of using the same technique as for the generation *L. mexicana* H-line. Comparisons between *L. major* WT and *L. major* H-line were made on parallel cultures of the same age. It was found that the medium after culturing stationary phase promastigotes of *L. major* WT was more acidic (pH 6.3) than that of *L. major* H-line (pH 6.9). The results showed the percentage of macrophages infected with promastigotes of *L. major* H-line was 41% at 8 h and decreased to 10.5% at 96 h post infection. In contrast to *L. major* H-line, the initial infection of macrophages with *L. major* WT was 46.5% and increased to 65% at 96 h post infection. It was shown that *L. major* H-line failed to develop cutaneous lesions during 12 weeks post infection. In contrast to *L. major* H-line, all mice infected with *L. major* WT went on to grow non-healing lesions the over same period. The dissemination of *L. major* H-line and *L. major* WT from the skin where the promastigotes were injected to visceral organs of BALB/c mice was investigated at 12 weeks post infection. It was found that *L. major* WT spread to BM, spleen, lung,

popliteal LN, and skin. In contrast, *L. major* H-line was localized in the skin and draining LN of two mice. The initial result showed that *L. major* H-line induced protection in vaccinated mice against infection with *L. major* WT. All non-vaccinated mice developed progressive non-healing lesions that peaked in size at about 12 weeks post infection. In contrast to non-vaccinated mice the lesions developed slowly in vaccinated mice. In the present study, all biological and immunological data on the immune response of *L. mexicana* H-line and the preliminary results on *L. major* H-line provide strong encouragement that *L. mexicana* H-line and *L. major* H-line induce a Th1 type of immune response, control cutaneous leishmaniasis, and are candidate live attenuated vaccines.

While this work has given exciting results in terms of the prospects for vaccination much remains to be done. Several aspects of the work need to be confirmed or extended. These are listed below:

1. Confirmation of the effect of the attenuation procedure on *L. mexicana* and *L. major*.
2. Extension of the attenuation effect to other clinically important *Leishmania* species including *L. infantum* and *L. donovani.*
3. Identification of the effect of attenuation on *Leishmania* parasites by proteomics and mass spectrometry. It will be essential to determine the difference between the two lines. Identification of the molecule (or molecules) which was (were) absent or present in the attenuated lines of *L. mexicana* or *L. major* could be important for activation of macrophages.
4. Optimisation of the dose and number of attenuated vaccine boosts needed to induce complete protection against challenge infection.
5. Investigation of the ability of animals immunised with attenuated *Leishmania* to resist challenge infections delivered by sandfly bites. The mice vaccinated with the attenuated line of *L. mexicana* or *L. major* should be exposed to parasites, which are delivered by sandfly.
6. Investigation of the capacity of attenuated, heat-killed parasites to induce protection. Determination of the ability of killed promastigotes of *L. mexicana* H-line or *L. major* H-line to protect mice against wild type parasites will be important. If the killed promastigotes of the attenuated lines can induce protection, this kind of vaccine will be safer than live attenuated cell lines.

7. Comparison of the levels of protection induced by attenuated parasites with levels of protection obtained with other vaccine strategies.

8. Examination of the effectiveness of vaccination with attenuated parasites during ongoing infection. Confirmation that the attenuated line of *L. mexicana* is able to activate macrophages infected with *L. mexicana* WT. If so, the *L. mexicana* H-line can be used for therapeutic purposes.

9. Assessment of the ability of attenuated parasites to undergo sandfly passage. It has been shown that *L. mexicana* H-line was stable in the medium without gentamicin for a long time. Assessment of the stability of the attenuated line of *L. mexicana* H-line in the guts of sandflies can be important for spreading the disease.

10. Do sandfly passaged attenuated parasites induce protection against challenge infection? If so, it should be a positive point for encouraging the use of *L. mexicana* H-line for vaccination.

11. Extension of the immunisation experiments to other species, including dogs.

12. Early isolation and attenuation of new clinical *Leishmania* isolates that could be attenuated under food and drug administration (FDA) approved guidelines for use as vaccines in humans.

13. Phase I clinical trials of attenuated *Leishmania* vaccines.

Appendix

Table 1. Recipe for separating gel

Reagents	Amount
Bis / Acrylamide (30%)	3.0 ml
Resolving buffer (see Table 2)	1.25 ml
Sodium dodecyl sulphate (SDS) (10% w/v)	0.1 ml
double distilled water (dd Water)	4.14 ml
Ammonium persulphate (APS) (1.5% w/v)	0.5 ml
TEMED	0.015 ml

Table 2. Resolving buffer*

Reagent	Final Concentration	Amount
Tris base	3 M	36.3 g
dd H_2O		60 ml
HCl (1 M)		pH was adjusted to 8.8
dd H_2O		exactly to100 ml final

* Store at 4°C

Table 3. Recipe for stacking gel

Reagents	Amount
Bis / Acrylamide (30%)	0.665 ml
Stacking buffer (see Table 4)	1.25 ml
SDS (10% w/v)	0.05 ml
dd Water	2.780 ml
APS (1.5% w/v)	0.25 ml
TEMED	0.007 ml

Table 4. Electrophoresis buffer*

Reagent	Final Concentration	Amount
Tris base	0.5 M	6 g
dd H_2O		60 ml
HCl (1 M)		pH was adjusted to 6.8
dd H_2O		exactly 100 ml

* Store at 4°C

Table 5. Sample buffer 2X

Reagents	Amount
Stacking buffer (see Table 4)	2.0 ml
dd H_2O	4.0 ml
Glycerol	1.6 ml
SDS (10%)	3.2 ml
Mercaptoethanol	0.8 ml
Bromophenol Blue	a few grains
Total volume	11.6 ml

Table 6. Separating gel mixtures-10% acrylamide for gradient gel

Reagents	Amount
Acrylamide (30%)	10.0 ml
Resolving buffer (see Table 2)	3.75 ml
SDS (10% w/v)	0.3 ml
dd Water	10.0 ml
APS (1.5% w/v)	0.7 ml
TEMED*	0.008 ml

* TEMED was added to each just before pouring the gel.

Table 7. Separating gel mixtures-20% acrylamide for gradient gel

Reagents	Amount
Acrylamide (30%)	20.0 ml
Resolving buffer (see Table 2)	3.75 ml
SDS (10% w/v)	0.3 ml
dd Water	2.75 ml
Sucrose (equivalent to 2.5 ml volume)	4.5 g
APS (1.5% w/v)	0.7 ml
TEMED*	0.008 ml

*TEMED was added to each just before pouring the gel.

Table 8. Lysis Solution used for method 1 *

Reagent	**Final Concentration**	**Amount**
Urea (Pharmacia)	8 M	9.6 g
Chaps (Pharmacia)	4%	0.8 g
Tris base (Sigma)	40 mM	0.097 g
dd H_2O	100 ml	20 ml

* 100 ml was aliquoted and stored at –20°C.

Table 9. Lysate solution used for method 2 *

Reagent	Final Concentration	Amount
Chaps	0.4%	0.2 g
DTT	200 mM	0.152 g
Tris base	40 mM	0.024 g
dd H_2O	5 ml	5 ml

* 100 µl was aliquoted and stored at –20°C.

Table 10. Rehydration stock solution without IPG Buffer*

Reagent	Final Concentration	Amount
Urea	8 M	12 g
Chaps	2% (w/v)	0.5 g
Bromophenol blue	trace	a few grains
dd H_2O	To 25 ml	to 25 ml

* 2.5 ml was aliquoted and stored at –20°C.

Table 11. Rehydration stock solution with IPG Buffer*

Reagent	Final Concentration	Amount
Urea	8 M	12 g
Chaps	2% (w/v)	0.5 g
IPG Buffer (same pH range as the IPG strip)	0.5% or 2% (v/v)	125 or 500 µl
Bromophenol blue	trace	a few grains
dd H_2O	to 25 ml	to 25 ml

* 2.5 ml was aliquoted and stored at –20°C.

Table 12. SDS equilibration Buffer*

Reagent	Final Concentration	Amount
4 × Resolving buffer **	50 mM	3.35 ml
Urea	6 M	36.04 g
Glycerol (87% v/v)	30% (v/v)	34.5 ml
SDS	2% (w/v)	2 g
Bromophenol blue	trace	a few grains
dd H2O	100 ml	100 ml

* Aliquoted 10 ml and stored at –20°C.

* * See Table 2.15

For each gel 2 aliquots were used as follows:

Equilibration I: 100 mg DTT was added to 10 ml equilibration buffer.

Equilibration II: 250 mg iodoacetamide was added to 10 ml equilibration buffer.

Table 13. 4X Resolving gel buffer

Reagent	Final Concentration	Amount
Tris base	1.5 M	181.5 g
dd H_2O	750 ml	750 ml
HCl (5 M)	pH was adjusted to 8.8	pH was adjusted to 8.8
dd H_2O	to 1000 ml	to 1000 ml

Table 14. SDS-PAGE with 10% acrylamide.

Reagent	11 gels	12 gels
Acrylamide 40%	176.8 g	192.8 g
N-N-methyl bis acrylamid 2%	94.4 g	102.9 g
4 × Resolving buffer*	174.4 ml	190.2 ml
dd H2O	243.8 ml	265.9 ml
10% SDS	7.1 ml	7.7 ml
10% APS	7.1 ml	7.7 ml
10% TEMED	1.2 ml	1.3 ml

* See Table 2

Table 15. Displaying solution

Reagent	Final Concentration	Amount
4X Resolving buffer*	0.37 M	50 ml
Glycerol (87%)	50% (v/v)	100 ml
Bromophenol blue	a few grains	a few grains
dd H_2O	to 100 ml	to 200 ml

* See Table 2

Table 16. Storage solution

Reagent	Final Concentration	Amount
4X Resolving buffer*	0.37 M	50 ml
10% SDS	0.1% (v/v)	2 ml
dd H_2O	to 200 ml	to 200 ml

* See Table 2

Table 17. SDS electrophoresis buffer

Reagent	Final Concentration	Amount
Tris base	25 mM	60.5 g
Glycine	192 mM	288.2 g
SDS	0.1%	20 g
dd H_2O	20 liter	20 liter

Table 18. Agarose sealing solution

Reagent	Final Concentration	Amount
SDS electrophoresis buffer (see Table 2.14)		100 ml
Agarose	0.5%	0.5 g
Bromophenol blue	trace	A few grains

All reagents were added into a 500 ml Erlenmeyer flask and heated in a microwave oven on low until the agarose was completely dissolved.

Phosphate buffered saline (pH 7.2)

60.0 g $Na_2HPO_4.12H_2O$

13.6 g $Na_2HPO_4.2H_2O$

8.5 g NaCl

Made up to 1 litre with de-ionised and distilled water.

DMEM

15.44 g DEME powdered medium (Gibco)

0.85 g Na_2CO_3

Made up to 1 litre with de-ionised and distilled water, filter sterilised (Millipore/Gelman filter 0.22 μm size) and pH adjusted to pH 7.2.

10.0 ml Penicillin / Streptomicine.

Giemsa's Buffer

3.0 g Na_2HPO_4

0.6 g KH_2PO_4

The pH was adjusted to pH 7.4 and made up to 1 litre with de-ionised distilled water.

Giemsa' stain

Giemsa' stain (Gurr BDH Ltd) was diluted 1:10 in Giemsa's buffer.

Coomassie Blue Stain

250.0 ml Methanol

100.0 ml Acetic acid

10.0 ml Glycerol

1.0 g Coomasie Blue

Made up to 1 litre with de-ionised and distilled water.

Coomassie Blue Destain

250.0 ml Methanol

100.0 ml Acetic acid

10.0 ml Glycerol

Made up to 1 litre with de-ionised and distilled water.

Brilliant Crystal Blue

5.0 g Brilliant Crystal Blue powder

100.0 ml Methanol

Store for a few days before using.

Trypan Blue (for viability test)

0.1 g Trypan Blue powder

5 ml PBS

Then filter.

Evans Blue (for Fluorescent Staining) (Stock)

0.1 g Evance Blue powdered

10 ml PBS (pH 7.2)

Take 1 ml in 100 ml PBS

Coating Buffer

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.2 NaN_3

The pH was adjusted to pH 9.6 with NaOH and made up to 1 litre with de-ionised distilled water.

Tris Buffered Saline (TBS)

9 g NaCl

1.6 g Tris HCl

The pH was adjusted to pH 7.6 with HCL and made up to 1 litre with de-ionised distilled water.

Transfer Buffer

14.42 g	Glycine
3.03 g	Tris
1.0 g	SDS
200 ml	Methanol

Made up to 1 litre with de-ionised and distilled water

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