Interaction between *Leishmania* parasites and mammalian macrophages

Giulia Getti

A thesis submitted in partial fulfilment of the requirements of the University of Greenwich for the Degree of Doctor of Philosophy in collaboration with the University of East London.

September 2007

Acknowledgements

I would like to thank a number of people for their help, encouragement and support during the undertaking and writing up of this work.

Firstly, my thanks go to both my supervisors Professor David Humber and Professor Robert Cheke. Without their availability and trust I would not have been able to start or finish this PhD. To Professor Humber for his enthusiasm in research, the stimulating discussion and for the constructive criticism that motivated me throughout my work and reminded me how exciting science can be. To Professor Cheke for his constant support, patience, kindness and help in overcoming any obstacle I came across during my research.

To my family in Italy, especially to my parents for encouraging me to follow my aspirations even when it meant being thousands of miles apart. To my sister Daniela and her husband Bruno for looking after our family when I was far away and for ever being so supportive and proud. To Camilla and Alessandra, for being the most wonderful nieces an aunty can hope for.

To my amazing friends, Stu, Pauline, Napoleon, Anastasia, Helen, Dan, Alberto, Genaro, Steve, John, Mariangela, Paola, Alessandro, Matteo, Tom and Roy, for their friendship and for making the years I have spent in England so beautiful and rich. A special thank you to Stuart, for his tireless help and support, for being an inspiration in my work and in my life and for making our house a home.

I am grateful to the University of East London and to the National Resource Institute for providing me with the opportunity and facilities to undertake this project and with all the members of staff for their help and support, especially:

To Duncan Kennedy and Kevin Clough, thank you for your help in the lab, for your constant and kind support, for your suggestions, teaching and for your patience. To Phil Poole, thank you for your help with the proteomic experiment, for your time, enthusiasm and for the faith you had in me. To Julia Freeman and Ian Slipper, for your help with both preparing the samples for the SEM and for teaching me how to use the microscope. To Flavia Joliffe and Paul Armstrong for your help with statistical analysis.

To Ray, Pat, Manchu, Mani, Bandu and Giles and for ever being so helpful in every one of the many requests I came to you with.

Dedication

I dedicate this thesis to my parents.

To the memory of my father, whose adventurous spirit inspired me.

To my mother, whose example is a continuous source of inspiration.

Abstract

Leishmania parasites are digenetic protozoans which infect human hosts and are causative agents of a series of diseases known under the name of leishmaniasis. During the mammalian stage parasites live, multiply and spread inside various immune system cells. Macrophages represent the main host as they are the only cells in which *Leishmania* can multiply and therefore start infection. Hence the interaction between *Leishmania* and macrophages is a fundamental step in the development of the disease. Many studies have been undertaken to understand early stages of the parasite interaction with macrophages; however, few have investigated the later stages of infection. This study was undertaken to develop an experimental model to examine the fate of the parasites when they leave the safe environment represented by their host macrophage. Primarily, the study investigated how *Leishmania* spread to neighbouring cells without being recognized and killed by the immune system defences.

Three Old World species of *Leishmania* parasites: *L. aethiopica*, *L, major* and *L. tropica*, all responsible for the cutaneous form of the disease, were used. A model of infection was described using two cell lines well known for supporting infection: THP-1 and U937. Axenic amastigotes for *L. aethiopica* parasites were obtained and used to identify drugs active against the infection. On the basis of the information available in the literature, a model was suggested involving interaction of intracellular parasites with the host cells' apoptotic machinery. Specifically it was suggested that *Leishmania* parasites were able to induce incomplete activation of apoptosis in the host cells. This hypothesis was confirmed by the findings that during infection an increased number of host cells showed two features associated with early apoptosis but not the one associated with the later stage. Results were validated in peripheral blood derived human macrophages. The information obtained from comparative proteomics analysis of the infection confirmed that *Leishmania* regulates apoptotic processes.

On the basis of the results obtained a model was presented to explain how induction of apoptosis allows intracellular amastigotes to spread unrecognised to uninfected macrophages without inducing an inflammatory response or losing the host cell's protection.

Contents

Figures

Tables

List of abbreviations

2-D DIGE,	Two-dimensional Fluorescence Difference Gel Electrophoresis
2DE,	two dimensional electrophoresis
3D,	Three dimensional.
AA,	Axenic amastigote
ACH matrix,	cyano- 4-hydrocinnamic acid
ACN,	acetonitrile
ACTB,	Actin beta
Ag,	Antigen
Akt/PKB,	Protein kinase B or Akt
Apaf-1,	apoptosis activating factor 1
BCDO2,	Beta carotene dioxygenase 2
BMDM,	Bone marrow derived macrophages
Bcl-2,	B-cell CLL/lymphoma 2
BSA,	bovine serum albumin
cDNA,	Complementary DNA
CHAPS,	3-([(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid;
CL,	cutaneous leishmaniasis
CP,	cysteine proteinase
CR,	Complement receptor
CRP,	C reactive protein
CRR,	chemokine receptor
CRR-2,	MCP-1RA gene
Cy2,	3-[(4-carboxymethyl)phenylmethyl]-3'-ethyloxacarbocyanine halide N-hydroxysuccinimidyl ester;
Cy3,	1-(5-carboxypentyl)-1_-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester;
Cy5,	1-(5-carboxypentyl)-1_-methylindodi-carbocyanine halide *N*-hydroxysuccinimidyl ester;
DCGI,	Drug-Controller General of India's

DCGI,	Drug-Controller General of India's
DCL,	diffuse cutaneous leishmaniasis
DCs,	Dendritic cells
DD water,	double distilled water
DIGE,	Fluorescence Difference Gel Electrophoresis
DMEM/F12,	Dulbecco modified Eagle's medium ham's F12 (1:1 mix)
DMF,	dimethyl formamide
DMSO,	dimethyl sulfoxide
DNA,	Deoxyribonucleic acid
DNDi,	Drugs for Neglected Disease Initiative
DP,	Dividing parasite
DTT,	dithiothreitol;
ECAAC,	European collection of cell cultures
EF,	elongation factor
ET,	Ethipia in the World Health Organization code
FCS,	foetal calf serum
Fn,	Fibronectin
FPA,	Flagellar pocket antigen *Trypanosoma brucei* rhodesiense
GBP,	*L. major* gene B protein
GIPL,	glycosylinositol phospholipids
GP,	Glycoprotein
Gp63	leishmanolysin
GPI,	glycosphosphatidylinositol
GRP78,	glucose regulated proteins
HASPB1,	salivary protein B1
HIV,	Human immunodeficiency virus
HSP,	Heat shock protein
IAA,	iodoacetamide
IAPs,	inhibitors of apoptotic proteins
IF1,	Intermediate form 1
IF2,	Intermediate form 2
IFN,	interferon
IKK,	I kappa kinases

IL,	interleukin
IM,	Intramuscular
iNOS,	inducible nitric oxide synthase
IPG,	immobilized pH gradient
IRFs,	IFN regulatory factors
ITNs ,	insecticide treated-nets
ITSrRNA ,	intergenic transcribed spacers
JNKs,	c-*jun* amino terminal kinases
LACK,	*Leishmania* Activated C-. Kinase
LCF ,	*Leishmania* chemotactic factor
LCL,	localized cutaneous leishmaniasis
LcPxn1,	*L. chagasi* peroxidoxin 1
Leish-111f,	*Leishmania*-derived recombinant polyprotein
LF,	Long flagellum form
LPG,	lipophosphoglycan
LPK-1,	serin/threonin protein kinase
MALDI-TOF,	matrix-assisted laser desorption ionization-time of flight
MAPK ,	mitogen-activated protein kinases
MCL,	muco cutaneous leishmaniasis
MCP,	monocyte chemotactic protein
M-CSF,	Macrophage colony-stimulating factor
MeCN,	acetonitrile
MFR,	Mannose-fucose receptor
MHC,	major hystocompatibility complex
MHOM,	Homo sapiens in the World Health Organization code
MLC,	myosin regulatory light chains
MLEE,	multilocus enzyme electrophoresis
MP,	metacyclic infective promastigotes
MPL ®,	monophosphoryl lipids A
MS,	mass spectrometry;
NF,	nuclear factor
NF-kB,	Nuclear factor kappa B
NK,	natural killer

NO,	Nitric oxide
P,	Parasite
P13-K,	phosphoinositide 3-kinase
PAMPs ,	pathogen-associated molecular patterns
PBMC,	Peripheal blood derived macrophages
PBS,	phosphate buffered saline buffer
PDK1,	phosphoinositide dependent kinase 1
PI,	propidium iodide
PiP_2 and PiP_3,	phosphatydilinositol biphosphate and triphoshate respectively
PKC,	protein kinase C
PKDL,	Post Kala Azar Dermal Leishmaniasis
PMA,	phorbol 12-myristate 13-acetate
PMF,	peptide mass fingerprint
PMNs	Neutrophils, polymorphonuclear leukocytes
PMSF,	phenylmethanesulphonylfluoride
PP,	procyclic
PPG,	Proteophosphoglycans
PS,	phosphatidylserine
PSA,	promastigote surface antigen
RA,	Retinoic acid
RFLP,	restriction fragment length polymorphism
RNA,	ribonucleic acid
ROCK	Rho associated coiled coil-containing protein kinase
ROI ,	reactive oxygen intermediates
sb,	pentavalent antimony
SDS- PAGE,	sodium dodecyl sulfate polyacrylamide gel electrophoresis,
SEM,	scanning electron microscope
SHP-1,	tyrosine phosphatase-1
Smac/DIABLO,	second mitochondria-derived activator of caspase/Direct IAP Binding Protein with Low pI
SSU ,	small subunit
TDR,	The Special Programme for Research and Training in Tropical Diseases
TFA,	Trifluoroacetic acid
TGF,	transforming growth factor

Th,	T helper
TNF,	tumour necrosis factor
TSA,	*L. major* thio specific oxidant
US,	USSR, in the World Health Organization code
VL,	Visceral Leishmaniasis
WHO,	World Health Organization

Chapter 1

Introduction

1.1. *Leishmania* parasites

Protozoan parasites of the genus *Leishmania* are complex microorganisms that have acquired the ability to survive in various hosts by evolving mechanisms of remarkable adaptability. During their life cycle they survive and multiply within very different environments and in order to do this they undergo enormous but reversible morphological and biochemical transformation. They exist in their sandfly vector's digestive system as promastigotes and inside mammalian cells as amastigotes, the form in which they return to the sandfly. *Leishmania* infects several mammalian species including humans and domestic animals (Gramiccia and Gradoni, 2005). Even though many infections are asymptomatic (Bern *et al.*, 2007; Sakru *et al.*, 2007) with consequent under estimation of the parasite incidence, *Leishmania* is responsible for a series of diseases known under the name of leishmaniasis, affecting 12 million people on four continents (Africa, Asia, Europe, South America and North America) with 350 million at risk in endemic countries and 1.5-2 million children and adults developing symptomatic disease each year (WHO, 2002).

The global incidence of these infectious diseases has increased in recent years mainly because of increasing international travel, human alteration of vector and host habitats, and concomitant factors that increase susceptibility, such as HIV infection and malnutrition. Moreover recent conflicts have contributed to the increase and the spread in otherwise unaffected countries such as the USA (Woodrow *et al.*, 2006). In these countries evidence of canine infections were found. For example *L. infantum* was isolated from naturally infected foxhounds both in the USA and Canada (Rosypal *et al.*, 2003; Duprey *et al.*, 2006). Although there is no evidence of infection spreading to humans; the probability of human exposure will be greatly increased if the organism becomes adapted for vector transmission by indigenous phlebotomines.

Leishmania parasites were first independently described in 1903 by Scottish physician Dr. William Boog Leishman and by Dr. Charles Donovan an Irish Professor of Physiology at Madras University. These two scientists first isolated the parasites from the autopsy of an infected Irish patient and from spleen needle biopsies of Kala-azar patients, respectively. The tiny intracellular particles seen were called Leishman-Donovan bodies. Soon after Leishman and Donovan's description, several similar parasites were isolated, both from man and animals, mainly in Africa (Santoro *et al.*, 1986). The causative agent of visceral leishmaniasis (VL) was named *Leishmania donovani* after its co-discovers.

Leishmania parasites have a single flagellum which arises from a basal plate, during the extracellular stage (Figures 1.1A and 1.1C) while it is restricted inside the flagellar pocket following transformation inside mammalian cells (Figures 1.1B and 1.1D). Parasites use the flagellum for mobility and attachment in the sandfly host. The end of the flagellum is modified into a disc-like structure, the desmosome, which allow the parasites to bind to the insect gut wall. *Leishmania* parasites are members of the order *Kinetoplastida* which is characterized by the presence of a kinetoplast containing DNA. The latter is a disk-shaped structure 0.5-1 μm in diameter which is visible under the light microscope and present throughout the life of the parasite. Close to the kinetoplast and the flagellar basal plate lie a nucleus and a characteristic single mitochondrion extending for the length of the body as a single tube. Both

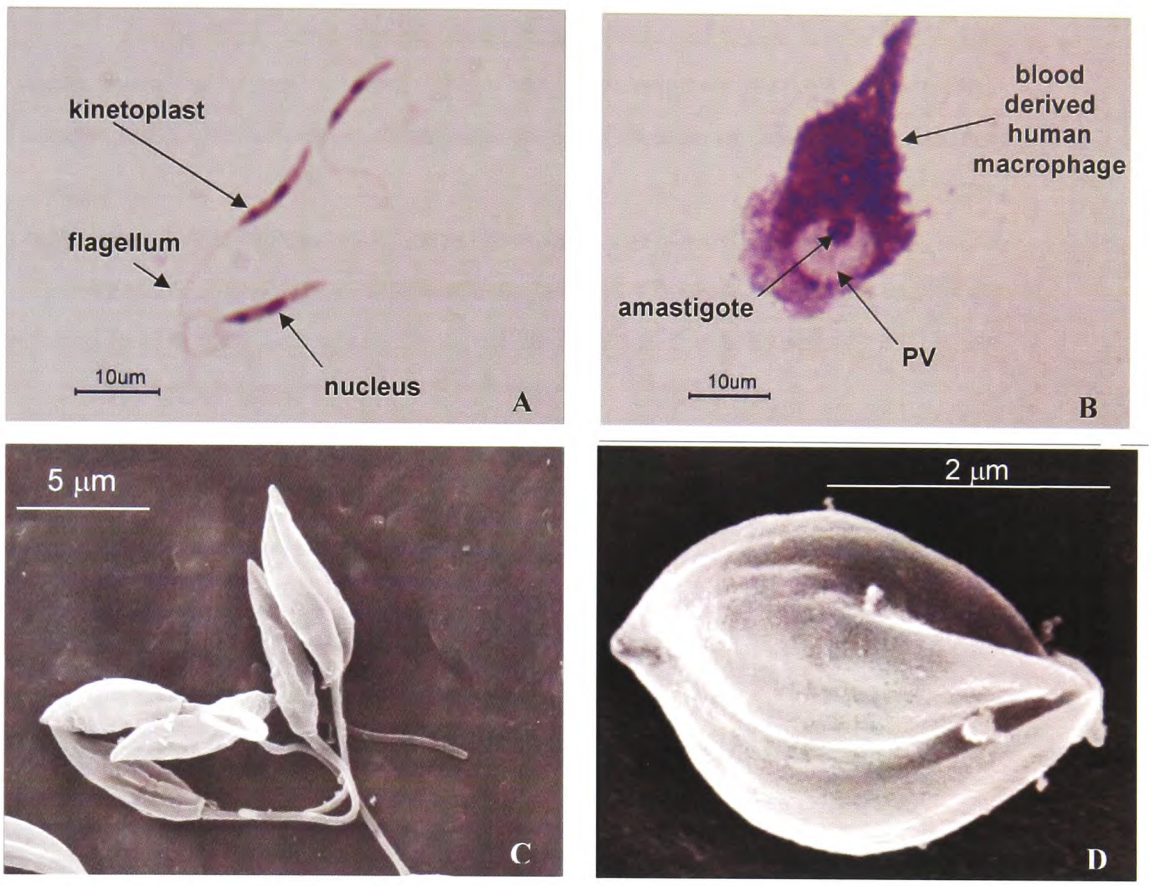


Figure 1.1. Optical microscope and SEM pictures of promastigotes and amastigotes form of *Leishmania*. Figure A and B represent Leishman's stained promastigote and Giemsa stained intra-cellular amastigote. Nucleus and kinetoplasts are visible both in the promastigotes and in the intracellular amastigote. Figure C and D are SEM images of promastigotes and extra-cellular amastigote of *L. aethiopica*.

morphological forms of the parasite mostly divide by longitudinal binary fission although evidences of sexual reproduction have been reported (Kreutzer *et al.*, 1994; Youssef *et al.*, 1997).

All species have similar morphology and although there are slight differences in sizes, overall they are indistinguishable by microscopic examination. Nevertheless differences can be found in DNA, proteins and lipophosphoglycan (LPG) as well as in development inside the vector (Lainson and Shaw, 1978; Lainson and Shaw, 1987; Sacks *et al.*, 1994; Pogue *et al.*, 1996; Brobey *et al.*, 2006). These differences are partially responsible for the variability found in virulence and symptoms associated with *Leishmania* infection (Pearson *et al.*, 1999; Colmenares *et al.*, 2002; Wilson *et al.*, 2005).

1.2. Classification of *Leishmania*

Although the taxonomy of the genus *Leishmania* is well established (Table 1.1), the continuous emergence of new tools to assess differences between organisms has caused and is still causing discussion not only on the classification but also on the existence of various species of *Leishmania.*

The first classification was suggested by Lainson and Shaw in 1979 and in 1987 and elaborated over a period of 20 years; it is a monothetic Linnean based model with few hierarchical characters. The species have initially been classified into 2 subgenera on the basis of the type of development within the sandflies: *L.* (*Leishmania*), which develops within the midgut and foregut of the vector and *L.* (*Viannia*), which undergoes a further developmental phase within the hindgut (Lainson and Shaw, 1987). *Leishmania* species are also demarcated as Old World or New World species according to their geographical distribution. Old World species are found in Africa, Asia and Europe, New World species in North and South America. In this model the previously used division in subspecies (Lainson, 1983; Lainson and Shaw, 1987) was abandoned by raising them to species and introducing complexes which were then extended by Rioux (Rioux *et al.*, 1990) (e.g. *L. donovani, L. braziliensis, L. guyanensis, L. hertigi* and *L. mexicana* complexes). In the interest of comprehensiveness, all the

Kingdom	*Protista*
Subkingdom	*Protozoa*
Phylum	*Sarcomastigophora*
Subphylum	*Mastigophora*
Class	*Zoomastigophora*
Order	*Kinetoplastida*
Family	*Trypanosomatidae*
Genus	*Leishmania*
Subgenera	*Leishmania* and *Viannia*

Table 1.1. Taxonomy of the genus *Leishmania* (Modified from to Rioux *et al.*, 1990)

species, including those still under debate, are presented. At present 29 species of *Leishmania* that infect mammals and 4 infecting reptiles have been described (Table 1.2). Of the 29 different species infecting mammals, 20 are known to infect humans.

Since the different species are generally indistinguishable by morphology, different approaches have been undertaken to differentiate them, such as isoenzyme electrophoresis (Lanotte *et al.*, 1981). Although isoenzyme analysis is currently the standard technique used for classification, other comparisons based on DNA-based techniques and proteins, (Pogue *et al.*, 1996; Sacks, 2001; Brobey *et al.*, 2006) have also been used. Over the past 30 years, improving methods in the detection, isolation and identification of *Leishmania* have brought to light new species such as *L. naiffi* (Lainson and Shaw, 1987), *L. arabica* (Peters *et al.*, 1986) and *L. lindenbergi* (Silveira *et al.*, 2002). Moreover, parasites previously considered as two different species were found to be the identical. For instance *L. pifanoi* is now known to be a synonym of *L. mexicana*, and *L. garnhami* a synonym of *L. amazonensis* (Rioux *et al.*, 1990).

The classification used in this thesis follows the one proposed by Lainson *and* Shaw in 1987, modified according to Rieux (1990) and to Lainson *and* Rangel (2005). Although *L. infantum* and *L. chagasi* were originally considered two different species, a number of reports have suggested that they are the same, with *L. chagasi* derived from the 'travelling' of *L. infantum* to Latin America (Mauricio *et al.*, 2000; Kuhls *et al.*, 2005), and identified as *L. infantum* (=*L. chagasi*). More recently Lainson *and* Rangel (Rangel and Lainson, 2003; Lainson and Rangel, 2006) suggested using the subspecies names of *L. infantum infantum* and *L. infantum chagasi.* Although a division in subspecies is not part of the current classification of *Leishmania*, the different names for the parasites have been retained as suggested by Shaw (2006). This distinction takes into consideration dissimilarities found between the two such as differences on kDNA fragment pattern (Jackson *et al.*, 1982; Jackson *et al.*, 1984), existence of specific monoclonal antibodies (Lemesre *et al.*, 1985) and Random Amplification Polymorphic DNA analysis (Martinez *et al.*, 2003).

Recently, a new classification was proposed by Cupolillo (Cupolillo *et al.*, 2000) based on analysis with techniques including: multilocus enzyme

Old World	**New World**
Leishmania (*L*) *aethiopica* *Leishmania* (*L*) *major* *Leishmania* (*L*) *tropica* *Leishmania* (*L*) *killicki* *Leishmania* (*L*) *turanica* *Leishmania* (*L*) *gerbilli* *Leishmania* (*L*) *arabica*	*Leishmania* (*V*) *braziliensis* *Leishmania braziliensis* *Leishmania peruviana* *Leishmania* (*V*) *guyanensis* *L. guyanensis* *L. shawi* *L. panamensis* *Leishmania* (*V*) *naiffi* *Leishmania* (*V*) *lainsoni* *Leishmania* (*L*) *enriettii* *Leishmania* (*L*) *hertigi* *L. deanei* *L. hertigi* *Leishmania* (*L*) *forattini* *Leishmania* (*V*) *colombiensis* *Leishmania* (*V*) *equatorensis* *Leishmania (V) lindenbergi*
Leishmania (*L*) *donovani* syn *L. archibaldi* *L. donovani* *L. infantum infantum* *L. infantum chagasi* *	*Leishmania* (*L*) *mexicana* syn *L. pifanoi* *L. amazonensis*, syn. *garnhami* *L. aristidesi* *L. venezuelensis* *L. mexicana* *Leishmania* (*L*) *herreri***

Table 1.2. Classification of *Leishmania* according to Lainson and Shaw, (1987) and modified according to Rioux *et al.*, (1990) and Lainson and Rangel (2005).

**L. infantum chagasi* classification is still under discussion. Although it is found in the new world it is located in this position because it is considered a subspecies derived from *L. infantum* spreading in America.

** Suggested to be more closely related to *Endotrypanum* than to *Leishmania* (Noyes *et al.*, 1996) (*L*): *Leishmania*; (*V*): *Viannia*. To be referred as *Leishmania* sp. hereafter.

electrophoresis (MLEE), analysis of the rRNA gene cluster by restriction fragment length polymorphism (RFLP) of the intergenic transcribed spacers (ITSrRNA), measurement of sialidase activity (Medina-Acosta *et al.*, 1994) and primary DNA sequencing of the small subunit (SSU) rRNA gene. This study suggests the inclusion of a new genus, *Sauroleishmania*, which includes parasites of reptiles. The genus *Leishmania* is then further divided into two 'Sections' as shown in Table 1.3. The World Health Organization recommends isoenzyme electrophoresis and the use of specific monoclonal antibodies as appropriate methods for characterizing *Leishmania* (WHO, 1990), this alternative classification systems are reported here to give a complete and up-to-date overview of all suggested *Leishmania* taxonomies.

1.3. Geographical distribution of leishmaniasis

The geographical distribution of leishmaniasis is limited by the distribution of the vector phlebotomine sandfly and the animal reservoir; consequently it is not present in cold climates (WHO, 2000) where the vector would be unable to survive. Nevertheless, *Leishmania* is widespread. The disease can be found in Southern Europe, as far as Cevennes (south-central France), but it is essentially endemic in tropical and sub-tropical regions. The worldwide distribution of leishmaniasis is shown in Figure 1.2. Most leishmaniases are zoonotic (transmitted to humans from animals), and humans become infected only when accidentally exposed to the natural transmission cycle. This is the reason why in most cases the distribution of the disease, is related to the presence of mammalian hosts. Over 100 species of mammals act as reservoirs for the parasite including Marsupialia, Primata, Carnivora and Hyracoidea. (Shaw, 1987). In brief the *L. donovani* complex is mainly found in Latin America, Mediterranean regions, and East Africa and in Asia where it causes Visceral Leishmaniasis (or Kala Azar) and Post Kala Azar Dermal Leishmaniasis (PKDL). *L tropica*, *L. major* and *L. aethiopica* are found in the Middle East, India, North and East Africa and Asia. Finally the *L. mexicana* and *L. braziliensis* complexes are found in Central and South America. As previously discussed different species are

Genus	Sections	Sub Genera	species
Leishmania	*Euleishmania*	*L. (Leishmania)*	*L. aethiopica, L. major L. tropica, L. killicki, L. turanica, L. gerbilli, L. arabica, L. donovani complex, L. mexicana complex, L. enriettii, L. forattini*
		L. (Viannia)	*L. lindenbergi, L. lainsoni, L. naiffi, L. guyanensis complex, L. braziliensis complex*
	Paraleishmania	parasites of histricomorph rodents (porcupines)	*L. hertigi L. daenei*
		remaining species which are principally parasites of sloth	*L. herreri, L. equatorensis, L. colombiensis*
Sauroleishmania	Parasites of reptiles		

Table 1.3. Classification of *Leishmania* according to Momen and Cupolillo, 2000

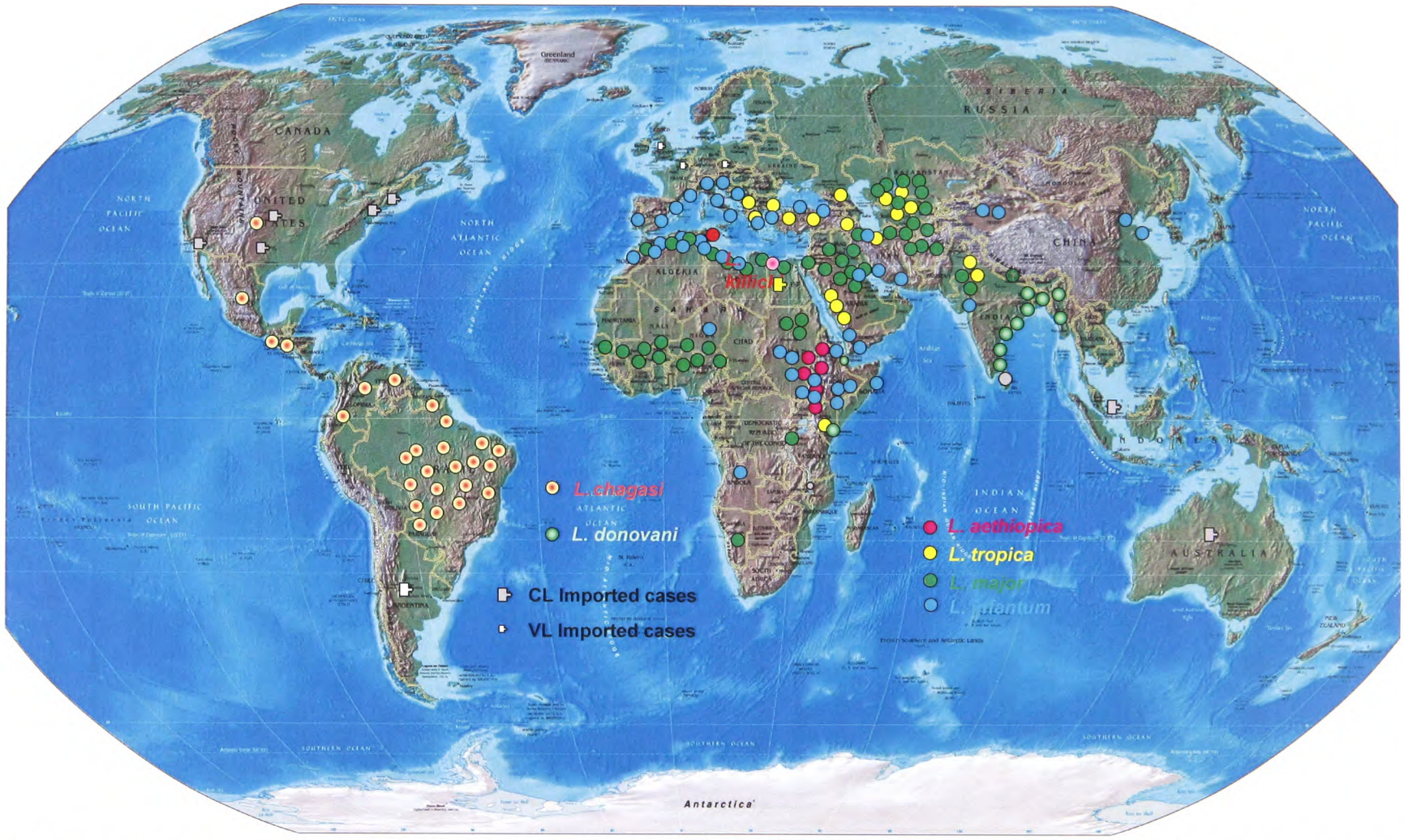


Figure 1.2. Leishmaniasis geographical distribution. Old World and Old World related *Leishmania* species are shown together with newly reported cases of leishmaniasis

responsible for different clinical manifestations, moreover the same species can cause more than one set of symptoms (Table 1.4).

Leishmaniasis is undoubtedly a dynamic disease: it changes and has changed with variations in the environment, such as changes in demography, and in ability to travel easily between countries, occurrence of wars and development of immune-suppressive conditions such as HIV. Consequently, the distribution of the disease both in humans and in animals is wider then previously reported, both in the number of cases registered and in size and location of infected areas. The evolution of new diagnostic techniques as well as the increased facility in sharing information has also augmented the number of known cases. Moreover ecological changes (Dam construction, irrigation and other development projects, urbanization, and deforestation) have favoured increased vector densities and resulted in changes in vector population densities that appear to have enabled both the emergence of novel diseases and the resurgence of old diseases (Gratz, 1999). For example, in the Syrian Arab Republic the number of cases of cutaneous leishmaniasis increased from 1,650 in 1987 to 9000 in 1992; in Tunisia cutaneous leishmaniasis increased from 1,300 cases in 1983 to 6,000 in 1990. During the first 5 years of the 1990s in southern Sudan, over 15,000 cases of Kala Azar have been treated. During roughly the same period, an epidemic built up in eastern Sudan, with the number of cases increasing from 1,100 in 1992 to over 2,400 cases in 1993 (Rathor, 1996). More examples come from the New World, Brazil cutaneous leishmaniasis (CL) cases have doubled between 1998 and 2002 going from 21,800 to 40,000. VL cases in the same period went from 1,840 to 6,000. In Afghanistan CL cases went from 14,200 in 1992 to 65,000 in 2002 (Desjeux, 2001; Desjeux, 2004). Moreover, the disease has been reported in traditionally non endemic areas such as western Upper Nile in Sudan *(Ashford et al.*, 1992), and canine leishmaniasis has spread to a number of US and Canadian states (Enserink, 2000; Costa *et al.*, 2003); Australia's northern territory (Rose *et al.*, 2004) and in previously unaffected parts of Europe (Harms *et al.*, 2003; Capelli *et al.*, 2004) such as UK (Darne and Sinclair, 2006). Recent conflicts in Iraq, Kuwait, and Afghanistan have led to >600 cases of cutaneous leishmaniasis and 4 cases of visceral leishmaniasis in American soldiers in 2004 alone (Korzeniewski and Olszannski, 2004).

Subgenus	Species	Vector	Main geographic location	Main clinical manifestation	Other
(Old World) *Leishmania*	*L. donovani*	*Phlebotomus argentipes* *P. orientalis* *P. martini*	India, sub-Saharan Africa, China, Pakistan	Visceral leishmaniasis	Post kala-azar dermal leishmaniasis (PKDL)
	L. infantum infantum	*P. perniciosus* *P. ariasi*	Mediterranean, Middle East, north and sub-Saharan Africa, Balkans, China	Visceral leishmaniasis	Post kala-azar dermal leishmaniasis (PKDL)
	L. major	*P. papatasi* *P. duboscqi*	Middle East, India, Africa, China	Cutaneous leishmaniasis (wet ulcer)	MCL
	L. tropica	*P sergentii*	Middle East, India, Southern Europe, western Asia, Central Africa	Cutaneous leishmaniasis (dry ulcer)	Leishmaniasis recidivans (LR) and viscerotropic leishmaniasis
	L. aethiopica	*P. longipes* *P. pedifer*	Ethiopia, Kenya, Yemen Tunisia	Cutaneous leishmaniasis	Diffuse cutaneous leishmaniasis (DCL)
(New World) *Leishmania*	*L. infantum chagasi*	*Lutzomya longipalpis*	South Central America	Visceral leishmaniasis	Post kala-azar dermal leishmaniasis (PKDL)
	L.venezuelensis		Venezuela	Cutaneous leishmaniasis	
	L. mexicana	*Lu. Olmeca*	Mexico, Central America, Texas, Oklahoma	Cutaneous leishmaniasis	Diffuse cutaneous leishmaniasis (DCL)
	L. amazonensis	*Lu. flaviscutellata*	Amazon basin, Brazil	Cutaneous leishmaniasis	Diffuse cutaneous leishmaniasis (DCL). Has also been associated with visceral leishmaniasis .
Viannia	*L.braziliensis*	*Lutzomya* spp; *P. wellcomei*	Latin America	Cutaneous and mucocutaneous leishmaniasis	
	L peruviana	*Lutzomya* spp.	Peru and Argentina (highlands)	Cutaneous leishmaniasis	MCL
	L. guyanensis	*Lu. umbratilis*	Northern Amazon basin, Guyanas	Cutaneous leishmaniasis	MCL
	L. panamensis	*Lu trapidoi*	Panama, Costa Rica, Columbia	Cutaneous leishmaniasis	MCL

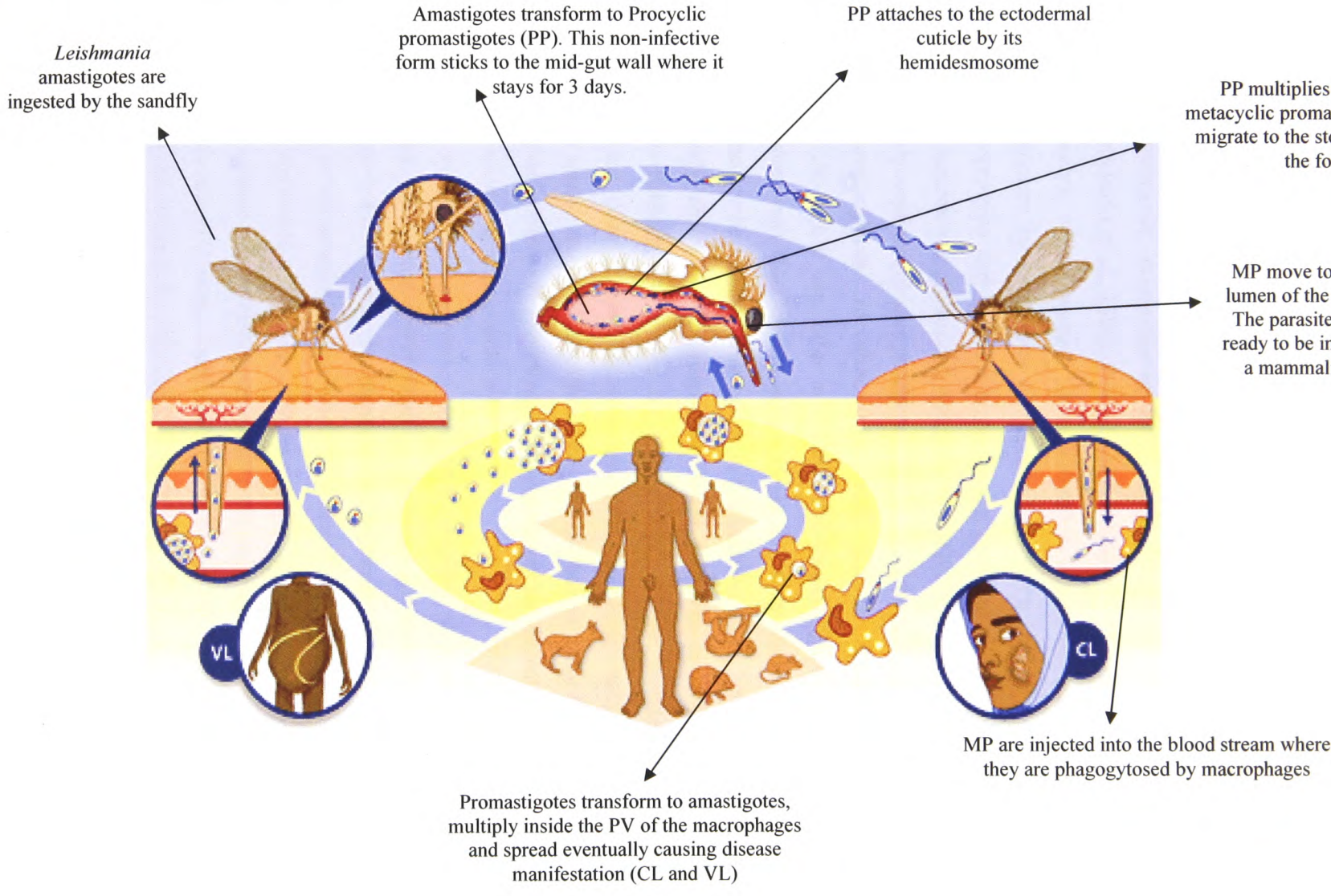
Table 1.4. Main *Leishmania* species causing human leishmaniasis.
Modified from (Leder and Weller, 2007)

1.4. Life cycle

Leishmania species are transmitted by a very limited range of biting female sandfly vectors: *Phlebotomus* species are prevalent in southern Europe, Asia and Africa, whereas *Lutzomyia* species predominate in central and southern America. There are over 500 known phlebotomine species; but only about 30 have been positively identified as vectors of the disease. Because of the dependence of the parasites on the insect vectors and on animal reservoir hosts, each species is associated with specific environmental conditions.

When a sandfly bites an infected host and macrophages containing amastigotes are taken up, the mammalian cells are destroyed and the parasites transform into promastigotes (Figure 1.3). Once inside the vector the developmental biology of the promastigotes is complex and still not completely understood (Kamhawi, 2006). Briefly, the initial promastigote stage is a procyclic (PP) non-infective form found in the midgut of the sandfly vector; it attaches to the midgut wall by inserting its flagellum between the microvilli and actively divides for 2 to 3 days. Following multiplication they transform into metacyclic infective promastigotes (MP) (Puentes *et al.*, 1990; Gossage *et al.*, 2003; Hanafi *et al.*, 2007) and slowly migrate through the stomodeal valve to the fore gut where they anchor themselves to the ectodermal cuticole by their emidesmosomes. Once detached from there, individuals gain entrance to the lumen of the proboscis via the pharynx and cybarium as small, elongated promastigotes which move rapidly. The migration of the parasite is linked to the changes occurring on the parasite cell surface during transformation which allows the parasites to detach from the midgut wall and move to the salivary gland following an osmotic gradient (Leslie *et al.*, 2002). The modification undertaken by the parasites to transform into the infective stage are part of a process called metacyclogenesis. The PP expresses abundant quantities of LPG which facilitate attachment to the gut epithelium of the insect and the glycoprotein gp63 that seems to protect the protozoa from hydrolytic enzymes in the sandfly gut. Transformation of procyclic into metacyclic promastigotes involves changes to the LPG structure, up-regulation of gp63 expression and

Figure 1.3. *Leishmania* life cycle



changes in the enzyme contents (Bogdan and Rollinghoff, 1998). Metacyclic LPG is unable to bind to the insect midgut as demonstrated in LPG-defective *L. major* mutants (Butcher *et al.*, 1996). During metacyclogenesis parasites undergo elongation of LPG through an increase in the number of phosphorylated disaccharide-repeat units. This causes thickening of the glycocalyx which protects the metacyclic promastigotes from complement lysis within the mammalian host (Sacks *et al.*, 1990). Procyclic promastigotes are covered by a 7 nm-thick glycocalyx, which becomes thicker (17nm) in metacyclic forms and completely disappears in amastigotes (Pimenta *et al.*, 1991). The glycocalyx comprises glycoproteins and glycolipids which are anchored to the surface membrane by characteristic glycosphosphatidylinositol (GPI) linkage (Ferguson, 1997) mainly glycosylinositol phospholipids (GIPL), (McConville and Ralton, 1997). Lipophosphoglycan constitutes the main surface molecule in promastigotes. Although it can differ in different *Leishmania* species (e.g. *L. major* and *L. donovani*) (McConville *et al.*, 1995) it mainly varies between procyclic and metacyclic stages, being longer in the infective stage; while it is almost completely absent in the intracellular stage (Pimenta *et al.*, 1991; McConville and Homans, 1992). The other important molecule abundant in promastigotes (and down-regulated in amastigotes) is the glycoprotein gp63. Although much smaller than LPG, and therefore probably buried under the LPG layer this molecule is extremely important during attachment to host cells as well as during infection as described in details later.

Studies on *L. mexicana* and *L. infantum* described 2 other stages: nectomonad and leptomonad promastigotes. Briefly, PPs multiply in the abdominal midgut and differentiate into non-dividing nectomonads. The latter are able to migrate to the anterior midgut where they transform into leptomonads which can start replication giving rise to the infective metacyclic stage (Gossage *et al.*, 2003). Leptomonads produce promastigote secretory gel which plays a vital role in infection as it forms a physical obstruction in the gut, forcing the sandfly to regurgitate metacyclic promastigotes during blood-feeding.

The development of the promastigotes within the sandfly is not the same for all *Leishmania* species. In some cases, for example *L. braziliensis*, the parasites initially occupy the pyloric region of the gut then move to hindgut and foregut (peripularial development). In other species (e.g. *L. aethiopica*, *L. major*

and *L. tropica*) development is restricted to the midgut and foregut (suprapylarial development). These differences constitute the basis for classification of *Leishmania* parasites into two subgenera: *Viannia* and *Leishmania* (Lainson and Shaw, 1987)

In all species during the vector's next blood meal *Leishmania* are injected in to the host skin where, within a few hours they are taken up by macrophages in a process termed coiling phagocytosis (Chang, 1979). Once established inside the parasitophorous vacuole of the host macrophages, the parasites transform into intracellular amastigotes and start multiplying (Alexander and Vickerman, 1975; Berman *et al.*, 1979; Sacks and Perkins, 1984). The intracellular parasites are then ready to spread to uninfected macrophages through a mechanism which is still unclear.

1.5. Leishmaniasis

Leishmaniasis comprises a wide spectrum of diseases ranging from self-healing cutaneous infection to deadly visceralizing forms. Various names have been given to the disease such as oriental sore, Baghdad boil, kala-azar, black fever, sandfly disease, Dum-Dum fever and espundia. A very general classification of the disease outlines two main forms known as visceral leishmaniasis where the parasites spread to bone marrow and internal organs; and the cutaneous form where parasites are limited to skin and mucosal surfaces. From infected sandfly bites the disease can develop following various routes as summarized in Figure 1.4 and at least 5 separate outcomes can follow.

Although typical patterns related to specific organisms (e.g. *L. braziliansis* and Espundia, see following paragraph), can be identified, there is no absolute distinction between the species of parasites causing visceral and cutaneous infections, since species known to be dermotropic can extend to lymphonodes and even visceralize (e.g. *L. tropica*). Moreover, the same species can cause different outcomes (Table 1.4) depending on various factors such as the immunological and genetic background of the host.

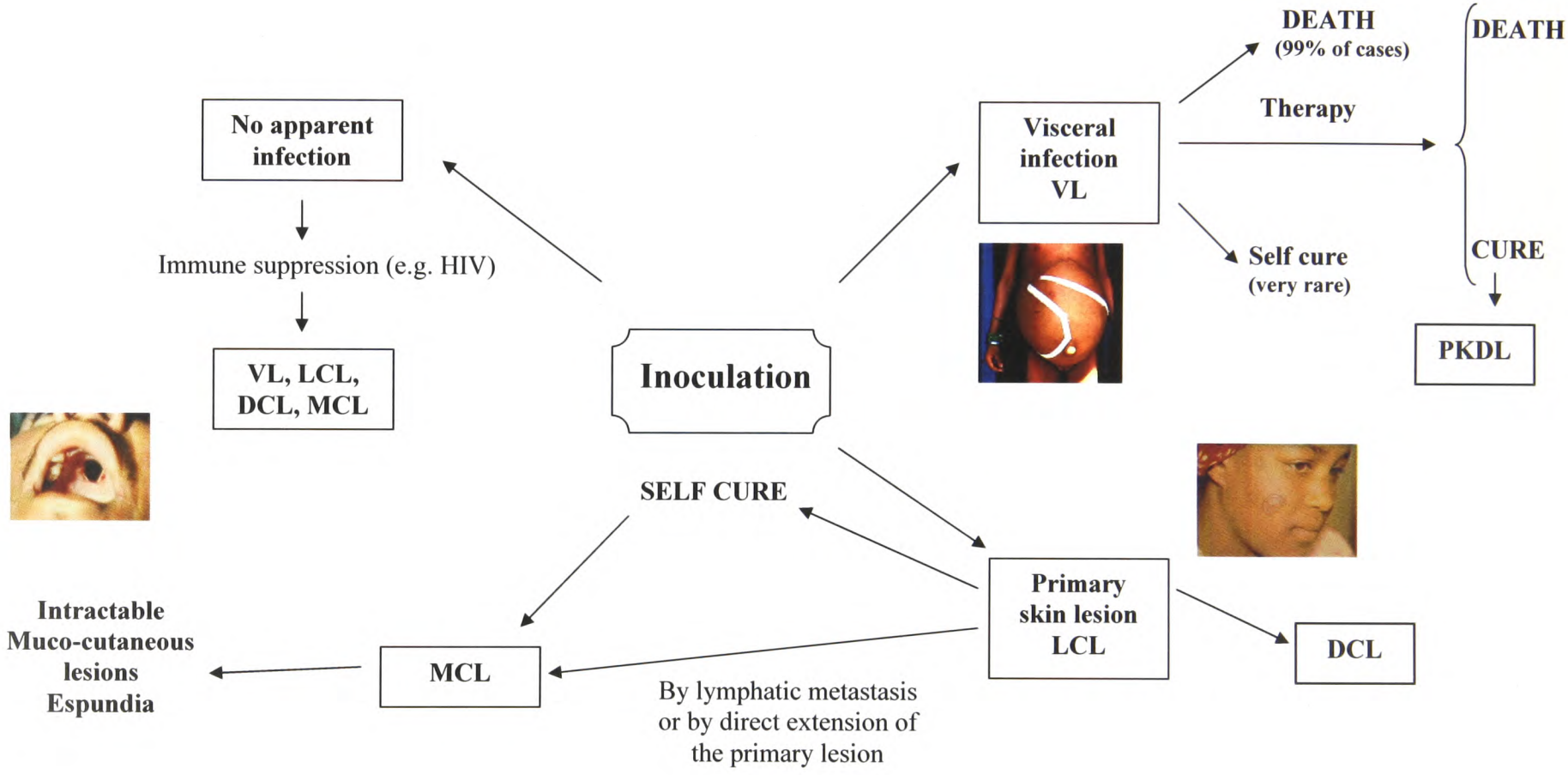


Figure 1.4. Leishmaniasis, disease manifestation. DCL, diffuse cutaneous leishmaniasis; LCL, localized cutaneous leishmaniasis; MCL, muco cutaneous leishmaniasis; PKDL, post Kala Azar dermal leishmaniasis; VL, visceral leishmaniasis.

The most severe form of the disease is VL; more than 90% of the cases in Bangladesh, Brazil, India and Sudan. It is sometimes preceded by a dry or ulcerating lesion at the infective bite site, between 2 weeks and 18 months after infection (Seaman *et al.*, 1996). The parasites spread to the spleen and liver where Kupffer cells are packed with amastigotes and to the bone marrow where infiltrations of parasitised macrophages are found. Consequences of the infection include fever, sweating, weakness, weight loss, hepatosplenomegaly and anaemia. Deaths occur in over 99% of patients if left untreated (WHO, 1990).

Following recovery from visceral leishmaniasis, relapses known as post Kala Azar dermal leishmaniasis (PKDL) can occur. The patients develop various skin lesions mainly on the face which eventually increase in size and multiply to cover a larger part of the body. No sign of the visceral condition is present. This generally develops months to years from VL recovery. It tends to occur in India, Sudan and Kenya (Hashim *et al.*, 1995) but there have been reports of cases in China (Ramesh and Mukherjee, 1995) and recently in the New World (Bittencourt *et al.*, 2003) where this condition is still extremely rare. Although it generally appears after recovery from Kala azar, cases were reported in which visceral leishmaniasis never developed (Hashim *et al.*, 1995) or in which they were both present simultaneously (Rijal *et al.*, 2005).

When the parasite multiplies and spreads inside macrophages within skin or mucosal membranes it causes cutaneous leishmaniasis. It can present itself in various forms: localized (LCL), mucocutaneous (MCL) and diffuse cutaneous leishmaniasis (DCL) depending on the tissue and the area affected by the lesions, as reported in Table 1.4. Although both New World and Old World species are responsible for cutaneous infection; MCL (espundia) is characteristic to the New World. The mucocutaneous spread can either follow healing of simple cutaneous lesions or occur at the same time as the primary lesion. This type of MCL is mainly due to *L. braziliensis* (Marsden, 1986) although cases associated with other species have been described.

Old World cutaneous leishmaniasis

The main *Leishmania* species present in the Old World are *L. infantum*, *L. donovani*, *L. tropica*, *L. aethiopica* and *L. major*; the first 2 species are mainly

responsible for the visceral disease while the last 3 for the cutaneous forms and are the subject of this investigation.

L. aethiopica. Simple cutaneous lesions following *L. aethiopica* infection generally appear about 2 – 8 weeks (occasionally years) after a bite from an infected sandfly, as a small red papule. The papule slowly increases in size and ulcerates. Following ulceration it becomes depressed and eventually heals through scarring (generally within one to five years, (WHO, 1990). The patient is now believed to be immune from homologues infections (Ashford, 2000). *L. aethiopica* is also responsible for diffuse cutaneous leishmaniasis (DCL) in Ethiopia. This is a rare manifestation of CL in the Old World and it is a chronic and not self-healing condition. It affects mostly the face, arms and legs and consists of painless nodular lesions which appear verrucous. In both clinical appearance and immunological profile of the affected patient, this manifestation of the disease is extremely similar to lepromatus leprosy (Convit *et al.*, 1957). *L. aethiopica* is also responsible for the cases of MCL in the Old World. Similarly to the disease found in the New World, it involves infection of mucosal membranes and mainly affects the nose and mouth. The lesions in the Ethiopian MCL arise not from lymphatic metastasis or direct inoculation but are generally due to extension of existing skin lesions to the mucosal areas of nose and lips (Barnetson *et al.*, 1978).

L. major. This species is mainly responsible for the 'wet' or 'rural' type of cutaneous leishmaniasis. The development of characteristic multiple ulcers with satellite nodules follow after 1 week to 2 months incubation period. The disease is self-limiting and tends to resolve itself within a year (Melby *et al.*, 1992). Although DCL is usually associated with *L. aethiopica*, responsible for about 1 per 10 000 infections, (Bryceson, 1976) there have been reports of DCL caused by *L. major* in Kenya and Senegal (Muigai *et al.*, 1987; Abdel-Hameed, 1990; Develoux *et al.*, 1996).

L. tropica. The 'wet' or 'rural' type of cutaneous leishmaniasis described in *L. major* infections is sometimes found geographically overlapping with the disease caused by *L. tropica*. The latter is responsible for the 'dry' form of CL, which in the majority of cases (95%) causes development of fewer then 3 lesions which appear from several months to a year following infection. As for the other LCL lesions are prevalent on the head, extremities and trunk. A high percentage

of them (>73%) heal spontaneously within 2 years (Sang *et al.*, 1992) while the remaining cases can develop into chronic mutilating ulcers. Historically, *L. tropica* was considered only responsible for the cutaneous form of leishmaniasis. Nevertheless, in the last 15 years various papers report this species as responsible for both human and canine visceral leishmaniasis in India (Sacks *et al.*, 1995), and Saudi Arabia (Magill *et al.*, 1994), canine visceral leishmaniasis in Morocco (Guessous-Idrissi *et al.*, 1997) and recently, in southern Iran (Alborzi *et al.*, 2006). This indicates the complexity of the disease and confirms that the development of the disease is not species-dependent as other factors are involved.

The last type of CL, known as leishmaniasis recidivans, like PKDL is associated with relapses of a previous *L. tropica* infection. It is a rare form of the disease and develops several years after the healing of the simple self-healing oriental sore. The lesions appear as unulcerating lupoid skin eruptions and are always found very near to the site of the original lesion (Sharifi *et al.*, 1998).

Prevention, control and treatment:

The first line of prevention is for individuals to avoid being bitten by sandflies. Personal protection measures should be undertaken, such as the use of fine-mesh or insecticide-treated bed-nets. Insecticide treated material, especially insecticide treated-nets (ITNs), represent one of the most effective ways of control. Moreover in the light of the fact that domestic animals are well known reservoir of *Leishmania* parasites, another form of protection is to treat dogs with insecticides. In order to develop a cost-effective strategy of prevention, it is extremely important to put together all the information available on the disease distribution and to produce maps showing burden of disease and sandfly vectors. Moreover, the combination of data on climate changes together with human population movement should be used to provide a basis for predicting spread of the disease which would facilitate fast responses to prevent development of epidemics (WHO, 2004).

Another potential method of controlling the disease is through vector control. Plants such as *Solanum jasminoides*, *Ricinus communis*, or *Bougainvillea glabra* are known for their ability to reduce the life span of the phlebotomus sandfly (Schlein *et al.*, 2001). These plants can be used in endemic regions to limit the spread of the disease.

Vaccines

Vaccination for any form of leishmaniasis is not available to date. Attempts of vaccinations such as inoculation of parasites containing pus from active lesions have been practiced in local communities for hundreds of years (Bryceson, 1976). Once promastigote cultures became available (Nicolle, 1908) Russian scientists started inoculating culture-derived parasites in order to prevent infection (Reed, 2004). Similar procedures (leishmaniazation) have been used in Israel and are still used in Uzbekistan (Modabber, 2000). Although proved overall effective; several secondary effects, including the development of large and uncontrolled skin lesions and exacerbation of psoriasis (Modabber, 1995) have pointed researchers in different directions such as the utilisation of crude antigens extracts. The latter produced an extremely variable (0-75%) protection against cutaneous infection and very low protection against visceral infection (Reed, 2004). Recently killed *L. amazonensis* vaccine also failed against American cutaneous leishmaniasis in Colombia (Velez *et al.*, 2005). Live, naturally non pathogenic (*L. tarentolae*) (Breton *et al.*, 2005) or genetically modified non-pathogenic parasites (Rivier *et al.*, 1999; Papadopoulou *et al.*, 2002) have been suggested and tested as possible vaccine against leishmaniasis. They would be able to elicit similar immune responses in the host to those from natural infections since they must closely mimic the situation of infection and have proved to be promising candidates for vaccination against *Leishmania* infection. In order to design first generation vaccine successfully it is important to use strains which would not revert to infective forms. This can be achieved via the production of attenuated strains that possess genetically defined and irreversible mutations and can therefore persist in the host without being virulent. These strains would also be amenable to further genetic manipulation if cause of adverse reactions. Finally, as they can be produced in large quantities and in well defined conditions they are suitable for human vaccination (Selvapandiyan *et al.*, 2006)

Second generation vaccines

Another possible route suggested for vaccination analyzed is the use of selected *Leishmania* molecules that have been proved to be important factors in the establishment of the infection. Following characterization and isolation of

many of these antigens, several of them together with recombinant antigens, were individually assessed as possible vaccines against *Leishmania* infections. Under the supervision of TDR (The Special Programme for Research and Training in Tropical Diseases), 11 recombinant antigens were tested as potential vaccines (Table 1.5).

Table 1.5. List of recombinant antigens tested as potential vaccines

Code #	Antigen	Description
1	MIX	Containing LACK, Hsp80, TSA, GP63, FPA
2	TSA	*L. major* thio specific oxidant (S. Reed)
3	GP63	*L. major* leishmanolysin (F.Mahboudi/R.McMaster)
4	Hsp80	Heat shock protein 83 *L. braziliensis* (S. Reed)
5	SLA	*L. major* soluble antigen (prom. cell extract, P. Scott)
6	LACK	*L. major* (N. Glaichenaus)
7	FPA	Flagellar pocket ag. *T. brucei rhodesiense* (C. Powell)
8	1G6	*L. major* (S. Reed)
9	4H6	*L. major* (S. Reed)
10	GBP	*L. major* gene B protein (D. Smith)
11	CP	*L. mexicana* cysteine proteinase (J. Mottram)

None of them proved to be acceptable as a vaccine candidate (Dumonteil *et al.*, 2001). Another source of antigens was suggested by the fact that sandfly saliva pre-exposure showed a powerful effect on the long term outcome of the disease (Belay *et al.*, 2006). The protection mechanism is still unknown but it suggests a possible alternative to parasite antigens in the production of vaccines (Valenzuela *et al.*, 2001a) Recently other antigens such as hydrophilic acylated surface protein B1 (HASPB1), the recombinant histone H1, GP anchored PSA-2, gp46/M-2, glucose regulated proteins (GRP78), P-4 amastigote antigen have shown vaccine potential in animal model (Solioz *et al.*, 1999; Stager *et al.*, 2000;

Campbell *et al.*, 2003; Kar *et al.*, 2005; Moreno *et al.*, 2007) and would require further analysis. Following the discovery of the genomic sequence of *L. major* (Ivens *et al.*, 2005), new antigens to test as possible leishmaniasis vaccine have become available. One hundred randomly selected amastigote-expressed genes were screened as DNA vaccines, and efficacy determined following high-dose *L. major* footpad challenge in BALB/c mice (Stober *et al.*, 2005). Fourteen protective novel vaccine candidates were identified to be taken forward for validation as vaccine candidates in low dose murine infection models, against other *Leishmania* species. Recently *Leishmania*-derived recombinant polyprotein Leish-111f, which had been demonstrated to be efficacious against cutaneous or mucosal leishmaniasis in mice, non-human primates, and humans (Coler *et al.*, 2002; Skeiky *et al.*, 2002; Fujiwara *et al.*, 2005), was proved to be also a vaccine antigen candidate against visceral leishmaniasis caused by *L. infantum* (Coler *et al.*, 2007).

Adjuvants in vaccine delivery

Once possible candidate antigens have been identified it is important to optimize the delivery of the antigen in order to stimulate the right type of immune response: a strong antigen specific Th1. The two adjuvants of use, alum and squalene, are poor Th1 response inducers, so others were evaluated (IL-12, live vectors). Of these monophosphoryl lipids A (MPL ®) showed good tolerability and no toxicity and was therefore used. A combination of antigen Leish-111f and MPL showed activity both for prevention and treatment of leishmaniasis (Reed *et al.*, 2003).

Finally, antigens can be delivered into the host by injection of an expression vector containing the gene encoding for that antigen (Gurunathan *et al.*, 2000). These DNA vaccines, although in relatively early stages of development have proved to give excellent protection against several *Leishmania* species (Mendez *et al.*, 2002).

Treatment

To date the vast majority of treatments available for the cure of leishmaniasis have been developed for visceral leishmaniasis as this is the most severe form of the disease. The same drugs can be used to eliminate cutaneous

leishmaniasis infection but as their side-effects are more severe than the disease symptoms itself, their use is, in most cases, not an option.

At present pentavalent antimony, a drug recommended 20 years ago, remains the treatment of choice in most of the world. It is thought to work by inhibition of adenosine triphosphate synthesis (Herwaldt and Berman, 1992). Pentavalent antimonials have the disadvantage of needing hospitalization during treatment and there is a high incidence of side effects. These side effects include aching, arthralgia, fatigue, gastrointestinal upset, elevation of amylase, lipase, and liver enzyme levels, leukopenia, anemia, and electrocardiographic abnormalities. Especially in India, resistance has been developed by many parasites (Murray, 2001); in Bihar up to 65% of new patients showed primary unresponsiveness to treatment (Sundar, 2001) making the drug almost obsolete in this part of the world. Although unresponsiveness is still rare in the rest of the endemic countries (South America, Africa.) resistance is expected to develop soon, particularly in Sudan, because of the epidemiology of the disease in this region (Guerin *et al.*, 2002).

Pentamidine used to be the first drug used in pentavalent antimony refractory patients in India. However the cure rate has dropped from almost 100 to 70% because of resistance (Sundar, 2001); and since it is a cause of serious toxicity, the drug has now been almost completely abandoned. In cutaneous leishmaniasis caused by *L. braziliensis* which evolve into the mucocutaneous form, the use of sodium stibogluconate is accepted as an appropriate treatment. Despite causing complications such as liver damage and temporary bone marrow suppression, this drug is currently in use (Hepburn, 2003).

A second-line drug, Amphotericin B, has now become first-line in Bihar (India). Although side effects are present and the compound is toxic to humans and can cause renal impairment and anaemia, no resistance has been documented (Thakur *et al.*, 1996). The active doses are so low that the drug can be and is widely used. Newer liposomal (lipid associated) formulations have been produced and showed an even greater effectivity against VL, but high cost make this formulation of little use for disease treatment in less developed countries.

A very promising drug, Paromomycin has recently been released for the treatment of leishmaniasis. This is an aminoglycoside which is well tolerated and effective for VL. This drug is well known as it has been used as an antibiotic

throughout the world for 40 years (marketed for the first time in 1959). Although efficacy against leishmaniasis was reported in 1985 and 1990, it has taken until 2005 for phase III of clinical trials (Jha *et al.*, 2005). The clinical trial concluded in November 2004 and One World Health, a non-profit pharmaceutical company, submitted an application to the Indian regulatory agency for review in 2006. On August 31, 2006 the Drug-Controller General of India's (DCGI) approved Paromomycin Intramuscular Injection for the treatment of visceral leishmaniasis.

Another drug that recently became available in the treatment of leishmaniasis is Miltefosine. Although the use of Miltefosine was approved in India in 2002 and in Colombia in 2005, the drug is still under investigation for its possible use in the rest of the world. It was developed as an anti-tumor agent and is the first orally effective drug against leishmaniasis. It has proved effective both as first line therapy and as treatment for refractory patients. More then 2500 patients have been treated and Miltefosine has proved effective against visceral, diffuse cutaneous and mucocutaneous leishmaniasis with cure rates ranging from 91 to 100% (Soto and Soto, 2006). Recently the first cases of PKDL were successfully treated (Belay *et al.*, 2006). Miltefosine's main side effect is teratogenesis and therefore it needs to be prescribed together with contraceptive treatments.

Treatment of cutaneous leishmaniasis

As with miltefosine, all the drugs described above are also, with various degrees of success, used for the treatment of cutaneous leishmaniasis. Sodium stibogluconate or intralesional meglumine antimoniate (WHO, 1990) have been used in treating cutaneous leishmaniasis caused by various species with the exceptions of those caused by *L. aethiopica*, as this parasite is naturally resistant to the treatment. In DCL caused by *L. aethiopica*, pentamidine is used as the treatment of choice although there have been reports of relapse as well as severe complications such as diabetes following treatment. Nevertheless, long-standing *L. aethiopica* derived DCL has been successfully treated by a combination of aminosidine and sodium stibogluconate (Teklemariam *et al.*, 1994). Various formulations for topical treatment containing paromomycin have been tested in animals and humans (El-On *et al.*, 1984; El-On *et al.*, 1985; El-On *et al.*, 1992; El-On *et al.*, 2007; Mussi *et al.*, 2007) with reports of various degrees of success,

as 74.2% (29 of 39 patients) of the patients were cured in the treated groups versus 26.6% (4 of 15 patients) in the placebo group (El-On *et al.*, 1992). These results suggested that this drug is active in both Old World and New World CL therapy. New formulations have been recently tested in animal models (El-On *et al.*, 2007; Mussi *et al.*, 2007) and could be suitable for clinical studies, they may represent an alternative for the topical treatment of CL.

At the beginning of 2007 a consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis (Modabber *et al.*, 2007) underlined the need for developing a suitable cure. Moreover, it brought to attention the poor quality of many of the clinical trials performed to date: out of 42 trials examined, twenty seven published ones were conducted without controls, which for a generally self-healing disease such as CL, provided very limited information.

To conclude, although various therapeutic options seem quite promising, an effective and safe treatment for cutaneous leishmaniasis is not yet available (Minodier and Parola, 2007) and research urgently needs to be addressed towards this.

Potential of natural products in the treatment of leishmaniasis.

When the World Health Organization (2004) called for research to concentrate on the identification and testing of new compounds that could be used in the treatment of the disease, particular attention was given to natural compounds. The interest in the use of plant extracts arose from the fact that indigenous communities have treated illnesses with those for many years and these extracts have often been proved to be good sources of therapeutic agents for the treatment of leishmaniasis (de Carvalho and Ferreira, 2001). For example the Iranian medicinal plant *Perovskia abrotanoides* which was commonly used in the treatment of CL was tested and found active in-vitro against both *Leishmania* and *Plasmodium* (Sairafianpour *et al.*, 2001).

There has been much research on the therapeutic value of natural products, mainly plant extracts used in traditional medicine and several natural products have been tested against *Leishmania* (Chan-Bacab and Peña-Rodríguez, 2001). Although a number of these natural products have demonstrated potential as leishmanicidal agents, most failed to meet all of the requirements needed for example the possibility of oral or topical administration, moderate dose

effectiveness and lack of severe side effects, (Berman, 1985), mainly because of their high cytotoxicity (Tournaire *et al.*, 1996). Natural products with leishmanicidal activities include quinones, alkaloids, phenolic compounds like iridoids and saponins, phenolic derivatives such as flavonoids, and terpenes. Of all these compounds only an alkaloid (berberine) is used clinically to treat cutaneous leishmaniasis (Iwu *et al.*, 1994; Okunji *et al.*, 1996) and it is applied parenterally. Another two alkaloids (chimanine 2 and 2-n-propylquinoline) extracted from a Bolivian medicinal plant (*Galipea longiflora*) commonly used to treat *Leishmania* derived lesion proved to be extremely active in *Leishmania* infected mice (Fournet *et al.*, 1996). An overview of the plant extracts active against Old World leishmaniasis and derived from Old World plants is reported in Table 1.6.

1.6. *Leishmania* parasites and the mammalian host

Immune cells involved in the response to infection.

Several immune cells take part in the response to *Leishmania* infection. An overview of the function exerted by each of them is given below:

Neutrophils

These are probably the first cells to encounter *Leishmania* during infection. The parasite itself releases a *Leishmania* chemotactic factor (LCF) which exerts potent chemotactic activity specifically towards neutrophils (PMNs) and not macrophages/monocytes or NK cells (Van Zandbergen *et al.*, 2002). Once at the infection site, PMNs phagocyte *Leishmania* (Van Zandbergen *et al.*, 2002; Laskay *et al.*, 2003) and release IL-8 which attracts more PMNs as well as monocytes/macrophages to the site of infection. Intracellular parasites have also been reported to delay spontaneous apoptosis of the host neutrophils prolonging their life to 2-3 days in contrast to 6-10 hours normal half life (Aga *et al.*, 2002). This is the first example during infection of *Leishmania* ability to interfere with the host cell apoptotic pathway. Three days after infection a large population of macrophages has reached the infection site and will recognize the PS exposed on the parasite surface and ingest the cells exposing it (Fadok *et al.*, 1992).

Plant	**Coming from**	***Leishmania* species towards with the plant showed activity**	**Reference**
Khaya and *Anthostema senegalensis*	Guinea-Bissau	*L. donovani*	(Abreu *et al.*, 1999)
Vernonia amygdalina	Ethiopia	*L. aethiopica*	(Tadesse *et al.*, 1993)
Asparagus africanus	Kenya	*L. major*	(Oketch-Rabah *et al.*, 1997),
Hedera helix	Eurasia	*L infantum* and *L. tropica*	(Majester-Savornin *et al.*, 1991)
Unonopsis buchtienii	Bolivia	*L. donovani*	(Waechter *et al.*, 1999)
Cistus monspeliensis	Crete	*L. donovani*	(Fokialakis *et al.*, 2006)
Salvia cilicica	Turkey	*L.donovani, L. major*	(Tan *et al.*, 2002)
Azadrachta indica, Allium sativa, and Acacia nilotica	Sudan	*L. major*	(Fatima *et al.*, 2005)
Premna schimperi and P. oligotricha	Ethiopia	*L. aethiopica*	(Habtemariam, 2003),
Uvaria klaineana	Gabon	*L. donovani*	(Akendengue *et al.*, 2002)
Combretum molle.	Ethiopia	*L. donovani*	(Asres *et al.*, 2001)
Triclisia patens	Sierra Leone	*L. donovani*	(del Rayo Camacho *et al.*, 2002)
Vismia orientalis	Tanzania	*L. donovani*	(Mbwambo *et al.*, 2004)
Potamogeton perfoliatus, Ranunculus tricophyllus Cladophora glomerata Dictyota dichotoma, Halopteris scoparia, Posidonia oceanica, Scinaia furcellata, Sargassum natans Ulva lactuca	Turkey	*L. donovani*	(Orhan *et al.*, 2006)
Pseudocedrela kotschyi	Mali	*L. donovani*	(Hay *et al.*, 2007)
Astragalus oleifolius	Turkey	*L. donovani*	(Ozipek *et al.*, 2005)
Pistacia vera L.	Turkey	*L. donovani*	(Orhan *et al.*, 2006)

Table 1.6. Old World plant extracts active against *Leishmania*

Th1/Th2 T-cells

This class of lymphocytes, especially the $CD4^+$ Th1 and $CD4^+$ Th2 subclasses are extremely important in the resolution or susceptibility to infection. Early studies (on *L. major*) associated activation of a Th1 response and consequent production of IFN-γ and IL-2 with killing of *Leishmania* parasites and clearance of the infection. The same study described activation of a Th2 response associated with production of IL-4, and IL-10 associated with persistency of infection (Sadick *et al.*, 1990; Reiner and Locksley, 1995). The action of IL-12 in clearing the infection was confirmed by later experiments in transgenic mice (Scott and Farrell, 1998; Sacks and Anderson, 2004). The role of IL-4 in disease resolution/exacerbation is more complicated, as discussed by Alexander and Bryson (Alexander and Bryson, 2005).

Resolution of infection is associated with the stimulation to a Th1 response. This response depends on the ability of dendritic cells to produce IL-12 and to stimulate activation of infected macrophages. The action of IL-12 can be enhanced by the action of cytokines such as IL-1α, IL-18, IL-23 and IL-27 (Dinarello, 2001; Von Stebut *et al.*, 2003; Artis *et al.*, 2004; Langrish *et al.*, 2004). Following internalization of *Leishmania* parasites in the parasitophorous vacuole (PV), MHC II carry parasites' Ag to the macrophages' surface. Once on the external membrane, MHC II binds CD4 receptors on the surface of Th1, at the same time other membrane interactions between macrophages and T-cells (B7/CD28 and CD40/CD40L) take place, causing activation of Th1 followed by release of IL-2 and IFN-γ (Kaye, 1995). Production of IL-2 induces activation of NK cells and Th1 cells while IFN-γ activates macrophages to kill intracellular parasites and to release cytokines such as IL-12 and TNF-α, which activate Th1 cells and macrophages respectively. In conclusion, induction of the Th1 cell response during *Leishmania* infection induces parasite clearing by activating macrophage killing; moreover Th1 cells also induce macrophage production in the bone marrow and recruit fresh macrophages at the site of infection.

Persistence of infection is associated with a Th2 response. In this case infected macrophages fail to produce IL-12 as *Leishmania* is able to selectively inhibit the IL-12 synthesis pathway (Reiner and Locksley, 1995; Carrera *et al.*, 1996; Belkaid *et al.*, 1998). *Leishmania* also prevents the activation of an

effective immune response by inhibiting inflammatory cytokine production (IL-1, TNF alpha) and inducing the production of immunosuppressive cytokines like IL-10 and TGF beta; both molecules suppress macrophage microbicidal activity and contribute to the developing of a Th2 response and consequently to disease progression. Th2 cells are then responsible for the production of IL-4, IL-13 and IL10 which exacerbate the disease.

The involvement of T cells on the infection is clearly complex and is responsible for the development of the disease. Two main cytokines, IL-12 and IL-4 were thought to be accountable for either resistance or susceptibility to the parasite. It is now accepted that the immunological response to infection is more complex and relies upon interaction of many more factors. For example, *L. mexicana* infection can be controlled independently of IL-12 (Buxbaum *et al.*, 2002) and IL-4 is not essential to disease development (Noben-Trauth *et al.*, 1996) and other cytokines such IL-10 have an equally important effect on infection (Noben-Trauth *et al.*, 2003).

The Th1/Th2 involvement during *Leishmania* infection was confirmed in humans affected with cutaneous and visceral leishmaniasis and varied depending on the type of clinical disease (Kemp *et al.*, 1993; Kemp, 1997; Kemp, 2000). Contrary to the findings reported in mice models of leishmaniasis both Th1 and Th2 responses were identified following *Leishmania* antigen stimulation in humans who had recovered from *L. donovani* infections as well as from incontrollable disseminating disease (Kemp, 1997; Kemp, 2000) while a predominant Th1 response was generated by individuals who recovered from cutaneous leishmaniasis (Farajnia *et al.*, 2005). Although the role of Th1 was clearly associated with resolution of infection, the precise role of Th2 cells in non-healing infections showed contradictory results. Specifically, IL-4 was associated with promoting cutaneous disease whereas it enhanced a Th1 response in visceral leishmaniasis (Mansueto *et al.*, 2007). Moreover in American MCL excessive Th1 response is thought to be responsible for the extensive damage found at the site of infection, where very few parasites but high numbers of immune cells are found. A decreased production of IL-10 and TGF-β is also linked to the inability of the host to control the Th1 response which causes the inflammation leading to tissue damage (Bacellar *et al.*, 2002). Thesed data

underline the importance fro the organism of maintaining the balance between Th1 and Th2 immune responses during *Leishmania* infection.

$CD4^+CD25^+$ T Cells

Other populations of T cells are involved in *Leishmania* infectivity. Endogenous populations of $CD4^+CD25^+$ regulatory T cells control persistence of *L. major* populations in skin following healing (Belkaid *et al.*, 2002a) as well as suppressing dominant Th2 cells and disease development in susceptible BALB/c mice (Xu *et al.*, 2003). Moreover they are responsible for reactivation of dormant infections (Mendez *et al.*, 2004) and for transitorily restraining pathogenic responses during non-healing *L. amazonensis* infection (Ji *et al.*, 2005). These cells are able to recognize Ags in infectious diseases and to down-regulate both Th1 and Th2 responses (Aseffa *et al.*, 2002; Sakaguchi, 2003; Piccirillo and Thornton, 2004)). The mechanism through which Treg regulate Th cells during *Leishmania* infection is still unclear but seems to involve downregulation via direct contact as well as via increased expression of TGFβ and IL-10, the latter being mainly expressed by a subpopulation of Treg, $CD4^+$Foxp3 negative cells (Ji *et al.*, 2005; Kariminia *et al.*, 2005; Nagase *et al.*, 2007).

Enhanced production of TGFβ is responsible for the enhanced susceptibility to *Leishmania* infection showed by BALB/c (but not C57B1-6) mice following intramuscular vaccination with *L. amazonensis* Ags (Pinheiro *et al.*, 2005). This cytokine is present in peripheral blood mononuclear cells of individuals suffering from VL and it is downregulated during treatment (Saha *et al.*, 2007). The association between disease preogression and TGFβ is confirmed by the overexpression of TGFβ found in PBMC from healthy subjects following *L. guyanensis* infection (Kariminia *et al.*, 2005).

CD8 T-cells

The last subset of T cells participating in infection development are CD8 T cells (Ruiz and Becker, 2007), their participation is expressed via both cytokines production (e.g. IFN-γ, TNF-α, IL-10 etc.) and cytotoxicity. Depending on the cytokines produced and by their migratory capacity these cells are named Tc1, Tc2 and CD8 suppressor cells (Woodland and Dutton, 2003;

are named Tc1, Tc2 and CD8 suppressor cells (Woodland and Dutton, 2003; Jiang and Chess, 2004). The importance of CD8 during *L. major* infection is associated with an early ability of switching from a Th2 to a Th1 response and therefore towards controlling parasites' survival and maintaining Th1 cells via production of IFN-γ (Muller *et al.*, 1991; Belkaid *et al.*, 2002b; Herath *et al.*, 2003; Uzonna *et al.*, 2004). CD8 cells were also shown to participate in *Leishmania* infection in humans, during both the acute phase of the disease and healing (Da-Cruz *et al.*, 1994). Of particular interest is the hypothesis presented by Ruiz and Becker (2007) that CD8 action during leishmaniasis might involve apoptosis induction of infected macrophages.

Natural Killer (NK) Cells

For almost 20 years, the role of NK cells during infection has been thought to be a decisive one as susceptible SCID mice lacking functional T and B cells but producing functional NK cells were able to contain the infection (Kumar *et al.*, 1989). A decade later two studies showed that although the lack of NK cells caused an increased number of parasites on the lesions (Laurenti *et al.*, 1999), their presence was not crtitical for the resolution of the infection (Bozza *et al.*, 1998). The mechanism of action of NK cells involves the rapid production of IFN-γ, following activation by parasite antigens, IL-12 (Laskay *et al.*, 1993) and IL-2 production from Th1 cells. NK cells contribute to the clearing of the infection by killing infected macrophages.

Dendritic Cells (DCs)

These cells are potent antigen presenting cells and can produce various cytokines such as IL-12 and IFN-γ, (Cella *et al.*, 1996; Stober *et al.*, 2001; Qi *et al.*, 2003) Unlike macrophages, when incubated with *Leishmania* promastigotes *in vitro* they up-regulated IL-12 production (Quinones *et al.*, 2000) DCs are also the only cells able to process *Leishmania* antigens in both the MHC class I and class II pathways (Belkaid *et al.*, 2000; Quinones *et al.*, 2000). Both production of cytokines and expression of *Leishmania* antigens suggest that DCs initiate a T cell response (Guermonprez *et al.*, 2002). Moreover, DC are believed responsible

for maintaining a memory response as persisting parasites were found in DC from long term infected mice (Moll *et al.*, 1995).

Macrophages

Macrophages are members of the mononuclear phagocyte system (MPS). The MPS includes bone marrow progenitors. Precursors in the bone marrow give rise to monoblasts, which then differentiate into blood monocytes. Monocytes circulate in the blood and within 24 hours are distribute throughout the body and become tissue macrophages (Van Furth, 1982). Terminal differentiation of monocytes gives rise to a wide range of cells that are all members of the MPS. These cells include Dendritic cells, osteoclasts in the bone, microglia in the brain, splenic macrophages and Kupffer cells in the liver (Van Furth, 1982). The phenotypic characteristic of these cells varies and is likely to be a result of encountering different signals based on their specific tissue niche. For example macrophages and DC can be differentiated from the same precursor cells *in vivo* and *in vitro* (Randolph *et al.*, 1998; Randolph *et al.*, 1999), and are obviously related cell types. Tissue macrophages and DC share a number of phenotypic characteristics and respond to the presence of pathogen-associated molecular patterns by secretion of inflammatory cytokines, up-regulation of co-stimulatory molecules and increased antigen presenting capability. It is therefore not surprising that *Leishmania* can enter DC as well as macrophages. Nevertheless, macrophages have a specific function in containing infectious agents. They achieve this through phagocytosis (followed by killing), antigen presentation and by secreting a variety of cytokines that cause an immune reaction which participates in clearing the infection. Macrophages can release both pro-inflammatory and anti-inflammatory signals, as well as induce tissues destruction and tissue restoration (Gordon *et al.*, 1992).

Macrophages can exist in 3 different states: resting; activated and hyperactivated (also called resting, stimulated or primed and activated). In each state they are all active in phagocytosing cells; hyperactivation involves differences in morphological (increased size and adherence), biochemical (increased total protein synthesis) and functional (increased rate and speed of phagocytosis) characteristics. Activated macrophages show reduced proliferation, are able to present antigens and have increased binding activity. This transition

can be activated by different stimuli such as IFN-γ, or other lymphokines, mediators of inflammation etc. The hyperactivation is then stimulated at the inflammation site either by LPS or unidentified lymphokines. The main consequence of the hyperactivation is the increased ability of the macrophages to kill ingested as well as extracellular organisms. However, *Leishmania* parasites are able to interfere with the macrophages' ability to kill. The parasites enter macrophages, transform into amastigotes and multiply inside the parasitophorous vacuole. How *Leishmania* is adapted to survive in this environment and which mechanisms it uses to subvert the host lethal reaction are described below.

Leishmania and the main cell host: the macrophage

The interaction between mammalian macrophages and *Leishmania* parasites is the subject of this investigation. It is therefore important to give a detailed introduction to the steps undertaken by the parasite to gain entrance and survival in their main host cells.

When an infected sandfly bites a vertebrate, a small number of parasites are injected into the host. Experiments using *Phlebotomus papatasi* infected with *L major* showed that in the majority of cases (75%) less then 100 promastigotes are egested by the sandfly (Warburg and Schlein, 1986). The sand-fly lacerates the blood vessels causing haemorrhages and then feeds in the haemorrhagic pool so formed (Figure 1.5), which is where the parasites are released (Ribeiro, 1987) to initiate the infection and to cause the disease.

Step 1: Survival in the host's tissue

Once inside the host blood pool, the parasites have to survive natural defence mechanisms the most dangerous of which is the complement system. It is thought that the complement system is responsible for killing the major part of extra-cellular promastigotes at this stage (Sacks and Perkins, 1984). Considering the small number of promastigotes inoculated by the sandfly, it is evident that a limited number of intracellular parasites and therefore a small number of infected macrophages is sufficient Survival of infective promastigotes outside the host cells is achieved mainly by the action of a few specific surface determinants: lipophosphoglycan (LPG), 63-kDa zinc-dependent metalloprotease (gp63, or

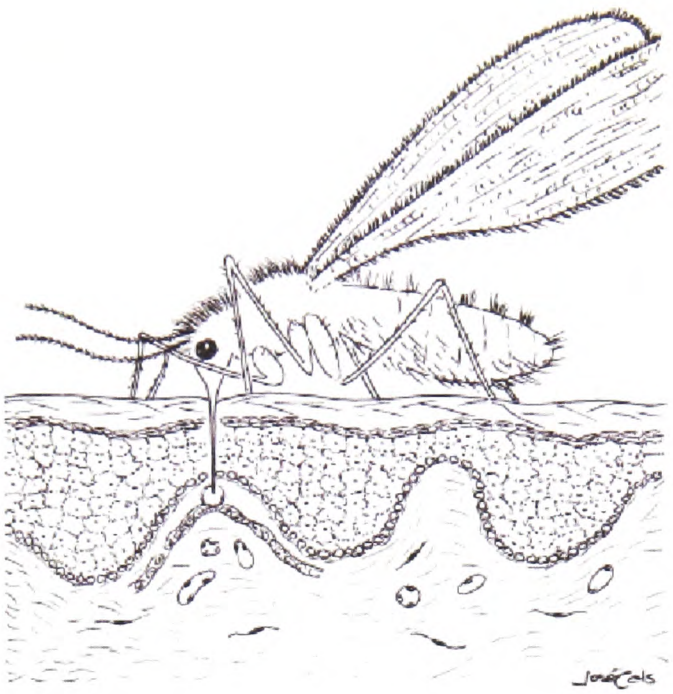


Figure 1.5. Illustration of a phlebotomine biting in a superficial dermal vessel (de Almeida *et al.*, 2003).

leishmanolysin), and serin/threonin protein kinase (LPK-1). Each of these components is mainly present on the surface of infective promastigotes and is down-regulated or absent in the procyclic promastigotes as well as in the amastigote stage. While LPG prevents the attachment of C5b-C9 subunits to the parasite membrane preventing them from lysing the membrane (Puentes *et al.*, 1989), gp63 converts C3b into C3bi which opsonize the parasite surface and help its binding to the macrophages (Brittingham *et al*, 1995). Finally LPK-1 is responsible for phosphorylating members of the complement system (C3, C5 and C9) and thereby inactivates the complement cascade (Hermoso *et al.*, 1991; Li *et al.*, 1996).

Another important factor in determining the successful establishment of infection at this stage is the sandfly saliva. The enzymatic action of the saliva acts in favour of *Leishmania* survival by many means (Charlab *et al.*, 1999). For instance the saliva contains maxadilan (Lerner *et al.*, 1991; Lerner and Shoemaker, 1992) which is not only the most potent vasodilatatory agent known (Jackson *et al.*, 1996) but is also responsible for inhibition of the production of TNF-α, as well as increasing the serum level of IL-6 and IL-10 in a lethal LPS model, (Bozza *et al.*, 1998). Saliva also contains apyrase that inhibits platelet aggregation, (Valenzuela *et al.*, 2001b) and various ingredients responsible for inhibition of oxidative metabolic processes and antigen presentation by macrophages *in-vitro* (Lerner *et al.*, 1991; Theodos *et al.*, 1991). Moreover, sandfly saliva participates in up-regulating Th2 while down-regulating the Th1 response. Up-regulation of Th2 cells is well known to exacerbate the disease (Rogers *et al.*, 2002). It is evident that the survival of the parasite outside the macrophages is strongly related with the very structure of the infective promastigote stage and with the action of the sandfly saliva.

At this stage a local inflammatory process is initiated which begins with the infiltration of neutrophilic and eosinophilic granulocytes, followed by macrophages which, within a few days predominate the infection (Beil *et al.*, 1992; Sunderkotter *et al.*, 1993). In order to survive and multiply *Leishmania* has to enter the macrophages.

Step 2: Adherence and invasion of macrophages

The parasites that survive the first line of immune defence gain entrance into many types of mammalian host cells (neutrophils, dendritic cells etc.). The parasites enter the macrophages by receptor-mediated phagocytosis. The main host cell receptors involved in the binding are: complement receptor type 1 and type 3 (CR1 and CR3), mannose–fucose receptor, fibronectin receptor, CR4 and the C-reactive protein receptor (Russell and Wilhelm, 1986; Rizvi *et al.*, 1988; Da Silva *et al.*, 1989; Talamas-Rohana *et al.*, 1990; Culley *et al.*, 1996). As shown in Figure 1.6, these receptors interact with *Leishmania* gp63 (Brittingham *et al.*, 1995; Mosser and Brittingham, 1997; Brittingham *et al.*, 1999) and LPG (Chakraborty *et al.*, 1998) either by direct binding or indirectly by parasite-associate host-derived serum molecules (e.g. complement, fibronectin, C-reactive protein). For example CR1 and CR2 receptors can bind gp63 respectively coupled with host complement components C3b and C3bi. Because of the rapid conversion of C3b to C3bi by the action of gp63, the interaction with CR1 is only transient (Kane and Mosser, 2000; 2001) and the CR3 receptor is the most important means for entrance into the host macrophages. This is extremely advantageous to the parasite since it causes phagocytosis without triggering oxidative burst (Mosser and Edelson, 1984). As mentioned earlier this mechanism is not activated when *Leishmania* enters by binding with C3 receptors on the macrophage's surface. This causes phagocytosis without induction of oxidase mediated by toxic O2 metabolites. *Leishmania* is able to interfere with this pathway (Chan *et al.*, 1989; Sorensen *et al.*, 1994) and to survive inside the host.

Once inside the macrophages, *Leishmania* are in the phagosome where they start transforming into amastigotes. The success of this transformation relies on the fact that LPG transiently delays phagosome-lysosome fusion (Desjardins and Descoteaux, 1997), as the promastigotes cannot survive the acidic and hydrolytic environment of the phagolysosome (Bogdan and Rollinghoff, 1998). Once the lysosomal fusion takes place, the transformation into amastigotes enables the parasites to survive at low pH, in the presence of N_2O_2, NO_3 and lysosomal enzymes (Alexander and Vickerman, 1975; Chang and Dwyer, 1978; Antoine *et al.*, 1990; Russell *et al.*, 1992). Inhibition of nitrite

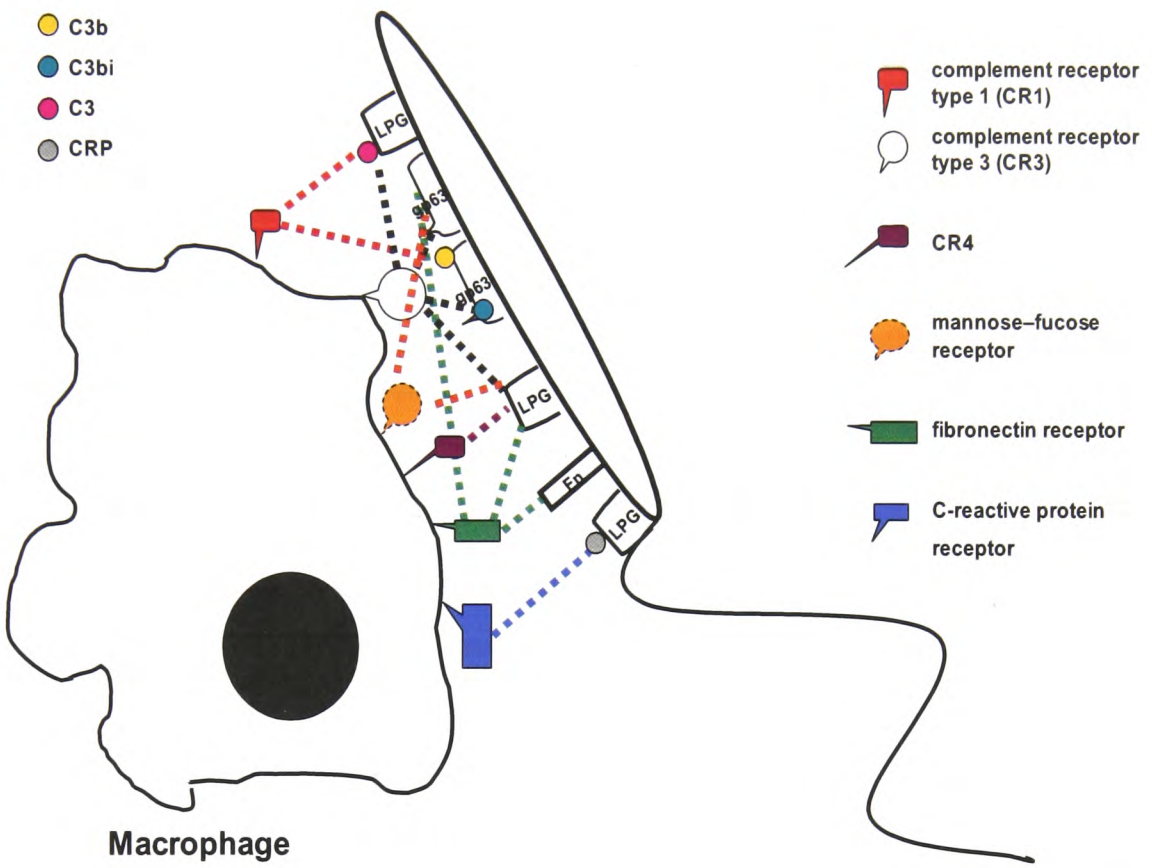


Figure 1.6. Promastigotes entry into macrophages

Leishmania promastigotes binds to uninfected macrophages receptor. CR1 can bind to both LPG coupled with C3 and gp63 coupled with C3b. CR3 can bind directly LPG and gp63 as well as when they are coupled with C3 and C3bi respectively. MFR can bind LPG and gp63 directly and CR4 only LPG. LPG can bind C reactive protein and then be taken up by the CRP receptor. Finally Fn receptor can interact with fibronectin (Fn) on the surface of the promastigotes as well as with LPG and gp63 directly.

derivatives and oxygen intermediates has recently been associated with the action of two relatively newly discovered *Leishmania* molecules: peroxidoxins LcPxn1 and -2 (Barr and Gedamu, 2003) and a superoxide dismutase (Ghosh *et al.*, 2003). Inhibition of lysosomal enzymes was associated with the action of gp63 (Seay *et al.*, 1996). The importance of gp63 as virulence factor was confirmed by gene deletion experiments where *L. major* parasites showed increased sensitivity to complement-mediated lysis and a delay in lesion formation in BALB/c animals (Joshi *et al.*, 2002). However amastigotes can still survive, differentiate and multiply in the absence of gp63 (Joshi *et al.*, 1998). Surprisingly LPG's and GIPLs' roles in virulence were recently proved not to be essential. Mutants unable to synthesize ether phospholipids retained the ability to inhibit host cells signalling and form infectious amastigotes (Zuffrerey *et al.*, 2003).

When the parasites have entered the macrophages and transformed into amastigotes their surfaces change; they become coated with a densely packed layer of parasite-derived GIPLs and host-derived glycosphingolipids and the expression of both LPG and gp63 is highly down-regulated (Barbiéri *et al.*, 1993; Winter *et al.*, 1994; Naderer *et al.*, 2004). Other surface molecules are found specifically associated with the infective stage of the parasite including phosphatidylserine (PS), hydropholic surface proteins encoded by the cDNA16 gene family and transporters which enable uptake of various matabolites (Alce *et al.*, 1999; Wanderley *et al.*, 2006; McConville *et al.*, 2007).

Step 4: Interaction with macrophage function.

The main function of macrophages, as previously described is to control infection by ingesting and destroying harmful pathogens, either by direct killing or with the help of other immune cells. Although *Leishmania* is successfully ingested, the host cells fail to destroy it or to induce an effective immune response. This is due to the ability of the parasite to interfere with 4 main macrophages functions: production of microbicidal molecules; antigen presentation; induction of inflammatory cytokine and repression of immunosuppressive molecules.

The most effective microbicidal molecule to clear *Leishmania* infection is NO (Wei *et al.*, 1995) which is produced by the inducible nitric oxide synthase (iNOS). *Leishmania* inhibits NO production by inducing IL-10 and TGF-β

(Stenger *et al.*, 1994; Scharton-Kersten *et al.*, 1995). The parasites also down regulate production of reactive oxygen intermediates (ROI) by a LPG and gp63 dependent mechanism (Descoteaux and Turco, 1993).

Antigen presentation inhibition has been described for all three morphological stages of the *Leishmania* parasite. Infection with procyclic promastigotes causes temporary LACK presentation; metacyclic promastigotes are responsible for a small, and temporary, response while no Ag presentation is found at any stage of amastigotes infection. At least three different mechanisms can be responsible for MHC II failing to expose *Leishmania* antigens: direct repression of MHC II gene expression e.g. *L. donovani*, (Reiner *et al.*, 1987); interference with Ag loading on MHC II e.g. *L. amazonensis*, (Prina *et al.*, 1993); and direct endocytosis and destruction of MHC II by *L. amazonensis* amastigotes via a cystein peptidase-dependent degradation (Lang *et al.*, 1994). The evolution of different mechanisms able to block this pathway confirms the necessity of blocking this pathway for the survival of the parasite.

Finally *Leishmania* is able to interfere with production of both inflammatory and anti-inflammatory cytokines. Studies of *in vitro* models showed decreased expression of inflammatory cytokines IL-1 and TNF-α and IL-12 (Reiner *et al.*, 1990; Piedrafita *et al.*, 1999; Stobie *et al.*, 2000). Down regulation of IL-12 is particularly important in controlling the infection. Specifically IL-12 induces Th1 cell activation with consequent production of IFN-γ, which is able to activate NO production and cause parasite killing. Induction of IFN- γ is even stronger when IL-12 acts synergistically with other cytokines such as IL-18 (Tsuji-Takayama *et al.*, 1999). Inhibition of IL-12 expression causes failure in the induction of T helper cell type 1 (Th1) response (Carrera *et al.*, 1996). However the importance of IL-12 is not limited to induction of IFN- γ as it is able to clear the infection in IFN-γ knock out mice (Taylor and Murray, 1998). Another important and well studied cytokine produced during infection is TNF-α (Green *et al.*, 1990; Moll *et al.*, 1990; de Kossodo *et al.*, 1994). It acts synergistically with IFN-γ in the *in vitro* production of iNOS and NO (Deng *et al.*, 1993). Although active against *Leishmania* it is only able to prevent infection when in the presence of active T cells. Similar investigations regarding *Leishmania* ability to interfere with inflammatory cytokines production were recently conducted *in-vivo* (Matte *et al.*, 2001). In

contrast to the *in vitro* studies, an initial inflammatory response was caused by increased expression of IL-1, IL-6 and TNF-α plus various cytokines responsible for cell recruitment (neutrophils and monocytes/macrophages) at the site of infection (Matte *et al.*, 2001). This finding can be explained by the need of the few internalized parasites to recruit uninfected cell in which to spread and can be related to the previous observation that during infection with metacyclic promastigotes the macrophages present LACK Ag for a short period.

Nevertheless, in the long term induction of inflammatory response would be deleterious for the parasite as indicated by the fact that both *in vivo* and *in vitro* studies showed induction of immunosuppressive cytokines such as IL-10 and TGF-β (Bogdan and Rollinghoff, 1998). Both molecules are able to suppress NO production, and IL-10 also suppresses IL-1, IL-12 and TNF-α (Cunningham, 2002) while TGF-β decreases NK cells activity (Ruzek *et al.*, 2003). The importance of down-regulating the inflammatory response for *Leishmania* survival is shown by the fact that transgenic mice that express IL-10 constitutively are incapable of controlling the infection (Kane and Mosser, 2001). Both IL-10 and TGF-β are induced following recognition of phosphatidylserine (PS) on the surface of amastigotes by the macrophages (Balanco *et al.*, 2001). The role of PS recognition in the success of the infection is particularly interesting because PS exhibition is also a feature characteristic of cells undergoing apoptosis. As discussed in detail later, apoptosis of host cells is potentially a mean by which *Leishmania* can spread to uninfected cells.

The intracellular mechanisms behind the macrophage's response to infection involve various enzymatic pathways. The parasites impair macrophage functions by inhibition of protein kinase C (PKC) activity (Olivier *et al.*, 1992; Moore *et al.*, 1993). Blocking PKC activity in macrophages before infection resulted in an increase in the number of parasites per cell, which suggested a possible role of PKC blocking on *Leishmania* establishment of the infection. *Leishmania* molecule LPG blocks PKC activity (Descoteaux *et al.*, 1992) suggesting its involvement in the inhibition process, but it is not the only molecule involved since mutant LPG amastigotes were still able to impair PKC activity.

Leishmania was also reported to interfere with the activation of mitogen-activated protein kinases (MAPK) during infection of macrophages (Feng *et al.*, 1999; Guizani-Tabbane *et al.*, 2000; Prive and Descoteaux, 2000; Ghosh *et al.*, 2002; Balaraman *et al.*, 2005). These kinases play an important role in activation transcription factors creating a bridge between transmembrane signalling and gene inductions in the nucleus. Three major groups of MAP kinases are extra-cellular signal regulated in mammalian cells: Kinases1 and 2, the c-*jun* amino terminal kinases (JNKs) (Nandan and Reiner, 1995; Ip and Davis, 1998) and the p38 MAP kinases (Paul *et al.*, 1997). These kinases act on transcription factors such as activating protein 1 (Ap1), NF-kB and IFN regulatory factors (IRFs). Moreover, the induction of MAPK erk1/2 by the parasite seems to participate in the inhibition of IL-12 production (Feng *et al.*, 1999), as MAPK is known to downregulate the induction of the gene codifying for IL-12. Further mechanisms related to *Leishmania* infection likely to cause IL12 down-regulation are signalling cascades activated by the membrane receptors such as Fcγ (Kane and Mosser, 2001) and complement receptor CR1 and CR3.

Leishmania braziliensis, *L. mexicana* and *L. major* and recently *L. guyanensis* have been shown to induce production of transforming growth factor β and IL-10 both *in vivo* and *in vitro* (Kariminia *et al.*, 2005). Moreover control of *L. major* infection is associated with reduced production of IL-10 and TGF-β by parasitized cells in mice lacking the common gamma-chain for FcR (Padigel and Farrell, 2005). Both IL10 and TGF-β cytokines inhibit the killing of intracellular *Leishmania.*

Recently, *Leishmania* elongation factor (EF)-1α was identified in *L. donovani* promastigote lysates as a molecule able to bind and activate the Src homology 2 domain containing tyrosine phosphatase-1 (SHP-1), the activation of which causes macrophage de-activation and consequent increase in *Leishmania* survival (Nandan *et al.*, 2003).

Recent work has been focused on providing an overview on the effect of infection on macrophages. Comparative gene expression analysis and microarray analysis of *L. amazonensis* infected and uninfected macrophages suggested that infection up- and down- regulated several genes. Although most of them are still under investigation, the significance of 7SL RNA down-regulation in macrophages was analyzed and related to impairment of the levels of receptor

protein molecules on the macrophage cell surface as well as inhibition of the secretion of proteins from macrophages (Misra *et al.*, 2005). cDNA expression assay of *L. donovani* infected macrophages showed impaired expression of several macrophage genes (40% genes). One of the genes showing the strongest down-regulation was CD40 (5 fold), which is known to enhance *Leishmania* survival inside the macrophages. There was also strong down-regulation of the MCP-1RA gene (CRR-2) which encodes the receptor for MCP. In both cases the result is impairment of the ability to mount a Th1 antileishmanial response.

Microarray analysis of DNA expression during infection is a very powerful technique which provides much information. Nevertheless it is important to bear in mind that over or under expression of specific genes does not always correlate with over or under expression of the protein codified by that gene. Moreover DNA analysis is unable to identify post translational modification which underlines the importance of using different approaches on the study of this interaction.

Step 5: Intracellular multiplication and inter-macrophage spread

Once a *Leishmania* parasite has established itself inside the macrophage and transforms into the amastigote form, it starts replicating, and when a substantial parasitic load is achieved inside the host cells, the parasite is ready to spread to uninfected host cells (Nacy and Diggs, 1981). The mechanism by which this spreading happens has not yet been clearly elucidated. The main theory accepted to date is that, following infection, heavily parasitized macrophages will eventually burst and free amastigotes will enter new host cells. Another theory based upon microscopy observation of the infection is that parasites are slowly released by the macrophages through membrane pores (Rittig *et al.*, 1998). Considering the modest number of parasites inoculated during natural transmission (as little as 100 metacyclic promastigotes), the spreading of the amastigotes to uninfected macrophages is a central point in the development of the disease. In order to understand what happens inside the human host during infection, it is useful to report the study of Belkaid (Belkaid *et al.*, 2000) describing the development of the disease, specifically the dermal lesion associated with *L. major* infection. The authors describe two phases: an initial 'silent' phase, lasting a month, during which the number of infected cells

grows without showing any sign of lesions; and a second phase, where a lesion develops associated with killing of the parasite *in situ*. Most of the experiments carried out to analyse the infection process in mice, have started with inocula at least 1000 times bigger than the 'natural' infective concentration, thus hiding the initial phase of the infection, the 'silent' phase. In humans the silent phase can be indicated as the 'incubation time' between the sandfly bite and the development of the symptoms of the disease.

According to Chang and McGwire (2002) and Chang *et al.* (2003) the two different phases of the disease are associated with the interactions of two distinct groups of parasite molecules with the host immune system. A first group consisting of invasive/evasive determinants which are necessary for the establishment of the infection but do not cause the symptoms, are mainly surface determinants and secreted molecules. A second group consisting of unique parasite epitopes present within the cells are called 'pathoantigene' determinants; the immune response to the latter is thought to be the cause of the clinical symptoms of the disease. According to the model suggested the parasites would quickly spread to infect additional cells without causing any immune response until some of the cytoplasmic determinants of the amastigotes are exposed to the immune system, causing the symptoms characteristic of leishmaniasis.

1.7. Aims of this Study

The interaction between *Leishmania* parasites and the mammalian host, specifically the macrophages is not only of vital importance in the parasite cell cycle but it also represents a fundamental step in the development of the disease. Once inside the macrophages promastigotes transform into amastigotes, multiply and spread to neighbouring cells. It is well known that different *Leishmania* species, although visually similar, have very distinctive characteristics; these differences make each species unique in its behaviour during infection. Three *Leishmania* species were analyzed for their ability to interfere with the host macrophages. The principal aims of the study were:

- To identify via an *in-vitro* model of the infection process of the 3 species of *Leishmania* parasites the time during which the parasites are likely to spread to neighbouring macrophages (Chapter 3).
- To identify a medium that can sustain axenic culture of *L. aethiopica* amastigotes (Chapter 4). Once inside macrophages the parasites exist in the form of amastigotes. The study of the latter form *in vitro* is complicated by the difficulties of culturing this stage under cell-free conditions.
- To identify natural products and natural products derivatives, active against *Leishmania* parasites as well as *Leishmania* infection (Chapter 5). The availability of *L. aethiopica* axenic amastigotes together with the availability of promising newly synthesized compounds derived from the natural product cicerfuran, led to assays the action of candidate drugs on the three *Leishmania* species responsible for CL Three active compounds were identified and the results published (Appendix III).
- To identify the mechanism through which intracellular parasites spread between macrophages (Chapter 6). The least studied part of the interaction between *Leishmania* and macrophages is the one involving the spreading of intracellular amastigotes from infected to uninfected macrophages. Following analysis of the reports produced to date on the effect of various parasites on the host cells a hypothesis was developed and investigated. The hypothesis proposed that *Leishmania* can use the induction of apoptosis as a mechanism for spreading the infection
- To identify differences in protein expression between infected and uninfected cells by 2D gel fluorescent electrophoresis (Chapter 7). Preliminary data on differences caused by infection with different species were generated, discussed, interpreted and, when possible, further investigated in order to generate a deeper understanding of the changes caused by infection.

Chapter 2

Materials and Methods

All chemicals were obtained from Sigma, UK, unless otherwise indicated.

2.1. *Leishmania* species

The following isolates of different *Leishmania* species were used for the investigations:

MHOM/ET/72/L-100	*Leishmania aethiopica*
MHOM/SU/73/5ASKH	*Leishmania major*
MHOM/SU/58/OD	*Leishmania tropica*

The first isolate, L-100, originated from an Ethiopian female patient showing disseminate nodular manifestation of infection; she came from the Wollo leishmaniasis focus in the north of Ethiopia. When the patient first came to seek cure the disease was mis-diagnosed as leprosy and treated as such but did not respond to anti-leprosy treatment. After about two years of failed treatment, culture and smear tests for leishmaniasis revealed that the disease was the diffuse

type of Ethiopian cutaneous leishmaniasis. The isolated parasites were typed and used as the WHO reference strain of *L. aethiopica* for Ethiopian cutaneous leishmaniasis (MHOM/ET/72/L-100). The isolate L-100 is probably the first typed and characterised *L. aethiopica* isolate from Ethiopia. When the culture of *Leishmania* was received it was expanded in culture and back-up vials of parasites were frozen as soon as possible for stock (T. Atlaw, pers. comm. 2002).

All the species used were obtained from the London School of Hygiene and Tropical Medicine and the Armauer Hansen Research Institute (AHRI) Addis Ababa, Ethiopia. *L. major* (MHOM/SU/73/5ASKH) was isolated from a patient in Ashhabad (Turkmenskaya, former USSR now Turkmenistan) by Prof. Saf'janova. *L. tropica* (MHOM/SU/58/OD) was isolated from a patient in Kirovobad, Azerbaidjan, former USSR by Prof. Moshkovsky (V. Yardley, pers. comm., 2007).

2.2. Culture media

The main culture medium used to grow *Leishmania* parasites and cell lines was DMEM/F12 supplemented with 10% heat inactivated foetal calf serum and 2 mM Glutamine at neutral pH. Promastigotes were inoculated at a concentration of 10^6 cells/ml in 25 ml tubes and placed in a cooler incubator at 22°C. The cells were passaged once a week by repeated inoculation into fresh medium.

Axenic amastigotes were obtained from log phase promastigotes of *L. aethiopica*, they were washed three times in PBS and cultured in UM54 medium and modified JH30 medium, pH 5 at 32°C (Pan, 1984) (Appendix I). The main difference between the three media is in the composition of JH30 which is fortified with amminoacids, a vitamin and a nucleotide mixture. This specifically supports amastigotes' growth (McConville *et al.*, 2007). A stock of *Leishmania* promastigotes was kept frozen in liquid nitrogen, as discussed below.

2.3. Cryopreservation of *Leishmania* promastigotes

Freezing

Cultures of logarithmic phase promastigotes were spun down at 900g in a centrifuge at room temperature for ten minutes and re-suspended, at a density of $5x10^6$ per ml, in cold (4°C) freezing medium. The freezing medium was made up of complete DMEM/F12 with 10% dimethyl sulfoxide (DMSO) and one ml of the cell suspension was transferred into cryo-tubes (Nunc, U.K.). The tubes containing the cells to be frozen were immediately put into Styrofoam containers and frozen at -70°C at approximately 2°C per minute, for 24-48 hours after which time they were transferred to liquid nitrogen for long term storage.

Thawing

Frozen parasites were thawed by transferring the cryo-vials from liquid nitrogen directly into a 37°C water bath and once thawing had taken place, DMEM/F12 at room temperature was added drop by drop over a period of 3-5 minutes to make the volume up to 3 ml. This solution was made up to 5 ml and spun at 900G at room temperature to remove as much of the DMSO as possible. The pellet of parasites was re-suspended in 5 ml fresh DMEM/F12 and, after checking for viability, incubated at 22°C for parasites to grow.

2.4. Human cell lines

Two human cell lines were chosen to study the interaction between *Leishmania* parasites and human cells. Both derive from the European Collection of Cell Cultures (ECACC). Both cell lines support *Leishmania* infection and multiplication (Ogunkolade *et al.*, 1990) and have been previously used as models for infection and for screening antileishmanial drugs (Gebre-Hiwot *et al.*, 1992; Delmas *et al.*, 2000).

THP-I (ECACC No. 8808120). These were derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia. These cells were terminally differentiated in non-adherent macrophage-like cells by treatment with

Retinoic acid (RA) or in adherent macrophages by treatment with phorbol 12-myristate 13-acetate (PMA).

U937 (ECACC No. 85011440). These were derived from malignant cells of a pleural effusion of a 37 year old Caucasian male with diffuse histiocytic lymphoma. These cells were terminally differentiated in adherent macrophages by treatment with phorbol 12-myristate 13-acetate (PMA).

Both cell lines are still expressing many of the monocytic like characteristics exhibited by cells of histiocytic origin (ECAAC web site).

Both cell lines were cultured in the same medium used for the parasites: DMEM/F12 medium supplemented with 10% foetal calf serum and 2mM glutamine in a humidified, 5% CO_2 atmosphere at 37°C. The cells were passaged twice a week at a density of $5x10^5$ cells/ml.

2.5. Differentiation and infection

Retinoic acid and PMA were dissolved in DMSO at initial concentrations of 10mM. Aliquots of the stock solution were stored at -4° C for up to three months. Working dilutions of RA (I μM) and PMA (0.32μM or 10μg/ml) were used in cultures for 3 and 1 days respectively.

After elimination of RA and PMA by three washes in phosphate buffered saline (PBS) THP-1 and U937 were cultured with stationary phase promastigotes at a parasite/cell ratio of 10:1 and placed in a 5% CO_2 atmosphere at 37°C. After 24 hours, promastigotes not taken up or bound to host cells were removed from adherent cells by washing in warm PBS. These conditions werec chosen because they caused 50 to 70% of the host cells to become infected leaving 50 to 30% of uninfected cells allowing the infection to spread to uninfected cells (Chapter 3).

2.6. Macrophage purification from peripheral blood

Blood was taken from the same subject for all experiments. It was immediately diluted in medium containing heparin and centrifuged on a density gradient (Lymphoprep®) for 30 minutes at 20°C and 400g with a break rate of

zero. The mononuclear cells lay over the Lymphoprep® while erythrocytes and granulocytes aggregated by the polysucrose sedimented rapidly. The upper layer containing mononuclear cells was gently removed with a sterile Pasteur pipette and transferred to a clean tube. The cells were washed of residues of Lymphoprep® through sequential centrifugations in PBS (5 minutes at 800g) and diluted in fresh medium to a concentration of 10^6 cells/ml. Cells were transferred into 24 well plates (Nunc, UK) and incubated overnight in a 5% CO_2 atmosphere at 37°C. Non-adherent cells were washed out and fresh medium added to adherent macrophages. The number of adherent cells was counted using a 40X objective on an inverted microscope. A minimum of 50 field of views from three wells were counted and the average taken to estimate the total number.

2.7. Staining procedures

Leishmania staining

Leishmania parasites (100 µl) were transferred to clean slides and incubated in a humid chamber to facilitate sedimentation of the parasites to the surface. Alternatively parasites were seeded on coated microscope slides via centrifugation in a Shandon Cytospin® 4 Cytocentrifuge (Thermo Fisher Scientific, UK). Parasites were fixed by washing the slide with 70% methanol. Leishman's stain (BDH Laboratory Supplies, UK) was added to the dry slide and left for one minute. Three parts of distilled water, buffered to pH 6.6-6.8 were added, mixed gently, and left for 20 minutes. The slide was rinsed in buffered water, blotted and air dried. Finally one drop of Histomount®, Histological mounting medium (National Diagnostics, UK) was added between the stained sample and the coverslip. The use of poly-$_L$-lysine to increase adherence was tested but it decreased the quality of the staining procedure.

Giemsa stain

Non-adherent infected cells were fixed on clean slides as described before for Leishman's stain. Coverslips with attached adherent macrophages were washed with warm PBS and fixed in 70% methanol. Giemsa stain (BDH Laboratory Supplies, UK) was diluted 20 times in water buffered to pH 6.8 and

added to the slides or to the wells containing coverslips. After an hour the stain was washed out twice with buffered solution, dried and left overnight to further air dry. Finally Histomount and a coverslip were added.

Images were visualized in a Nikon optiphot microscope equipped with a Nikon DXM1200F Ultra High-Quality Digital Camera.

2.8. Promastigote transformation to amastigote

Parasite counting during transformation

During transformation to amastigotes parasites tend to aggregate in clumps which make it difficult to give an accurate estimation of the number of parasites. In order to isolate single cells and obtain a count under the hematocytometer the culture was passaged through a 25 gauge needle. This treatment was limited to small aliquotes (0.5 ml) which were taken out from the main culture following gentle shaking of the flask. This procedure was necessary in order to limit the negative effect that dividing clumped up parasites has on the culture. Attempting to divide the aggregated amastigotes had a deleterious effect on survival causing failure of the transformation and the culture to die off.

Scanning Electron Microscope (SEM) analysis

Promastigotes were cultured in JH30 medium, pH 5, at 32°C until they reached complete transformation into amastigote-like parasites. They were reverted back into the promastigote stage 8 times. The following growth in JH30 medium was monitored through SEM (Cambridge Stereoscan 360). The first sample was taken 6 hours from the beginning of the experiment, the following samples every 24 hours for 10 days. A total volume of 0.5 ml of promastigotes/amastigotes was washed in PBS and fixed at 4°C in freshly made 2.5% glutaraldehyde for a minimum of 2 hours. Fixed parasites were then centrifuged into aluminium foil coverslips and dehydrated by 1 minute passages in increasing concentration of alcohol (from 50 to 100%). Coverslips (in 100% ethanol) were then transferred in a K850 Critical Point Drier (Emitech, UK) and critically dried with CO_2 at 31°C and 1072 psi according to the manufacturer's instructions. The coverslips were finally glued into metal holders and gold coated with a sputter coater for 2.5 mins.

2.9. Compounds tested for anti-*Leishmania* activity

All the compounds used, derived from a natural product, cicerfuran (Table 2.1), were provided by Shazia N. Aslam (University of Bristol).

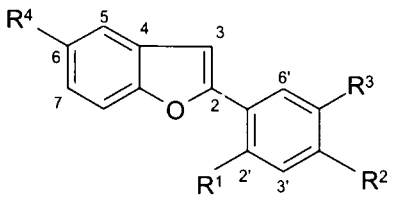
Cicerfuran is an antifungal defence chemical produced by wild chickpeas (*Cicer* sp.) resistant to fungal wilt (*Fusarium oxysporum* f. sp. *Ciceri)* (Stevenson and Veitch, 1998) It has been synthesized recently along with several benzofuran analogues and a related group of stilbenes, all of which have antifungal and antibacterial activity (Aslam *et al.*, 2007).

Cicerfuran was synthesised by Aslam from sesamol (3,4-methylenedioxyphenol) and 2,4-dihydroxybenzaldehyde in ten steps *via* a Wittig reaction, epoxidation of the resulting stilbene and acid-catalysed cyclisation. The structure of the synthetic compound was confirmed by Nuclear Magnetic Resonace (NMR), and its chromatographic and spectroscopic properties were identical to those of the natural product. The other 9 related compounds tested were 2-(3',4'-Methylene dioxyphenyl) benzofuran (Eichner1), 2-(2'-Methyl-4',5'-methylene dioxyphenyl) benzofuran (**2**), 2-(2'-Methoxy-4',5'-methylenedioxyphenyl)-6-hydroxy benzofuran (cicerfuran, **3**), diphenylstilbene (**4**), 2-Hydroxystilbene (**5**), 4-methoxy stilbene (**6**), 2,4-Dimethoxy stilbene (**7**), 2-methoxy3I,4I-methylenedioxystilbene (**8**) 2-Hydroxy-2'methylstilbene (**9**) and 2-Hydroxy-2'-methoxy stilbene (**10**) (Figure 2.1). Compounds were dissolved in dimethyl sulphoxide (0.05% DMSO) to provide final concentrations ranging from 50 to 0.075 μg/ml

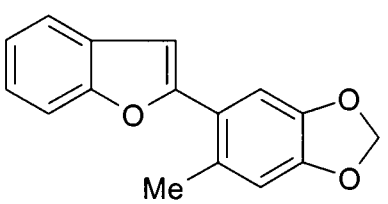
Promastigote viability assay

Logarithmic phase promastigotes were washed, counted and plated with new medium in 96 well plates (10^6 parasites/ml). Serial dilutions of each drug were added to the wells, with three replicates for each dilution. Plates were incubated for 48 hours at 22°C. Inhibition of promastigote growth was determined microscopically by counting parasite numbers in a Neubauer haematocytometer. Percent of inhibition of growth was determined by comparison of treated groups with untreated controls after 48 hours.

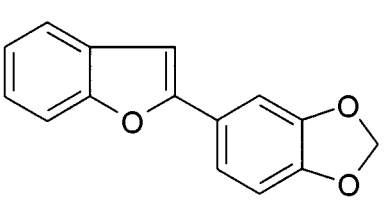
a: Structures of Benzofurans used for testing biological activity



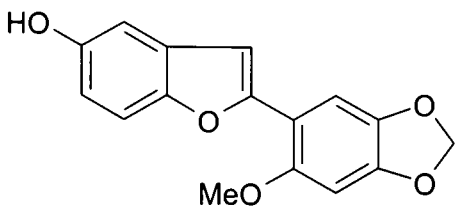
	R¹	**R²**	**R³**	**R⁴**
C1	H	OCH_2O		H
C2	CH_3	OCH_2O		H
C3	OCH_3	OCH_2O		OH



2-(2-Methyl-4',5'-methylenedioxyphenyl)benzofuran

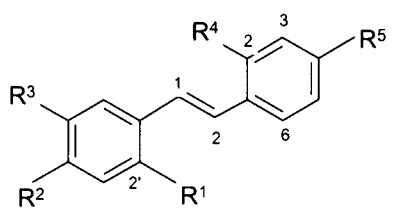


2-(3',4'-Methylenedioxyphenyl)benzofuran

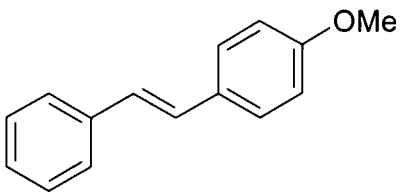


2-(2'-Methoxy-4',5'-methylenedioxyphenyl)-6-hydroxyphenylbenzofuran

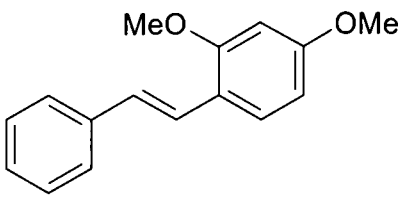
b: Structure of stilbenes used for biological testing



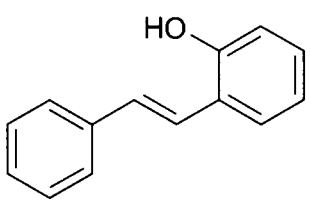
	R^1	R^2	R^3	R^4	R^5
C4	H	H	H	H	H
C5	H	H	H	OH	H
C6	H	H	H	H	OCH_3
C7	H	H	H	OCH_3	OCH_3
C8	H	OCH_2O		H	OCH_3
C9	CH_3	OCH_2O		OH	H
C10	OCH_3	OCH_2O		OH	H



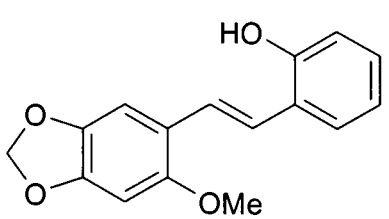
4-Methoxystilbene



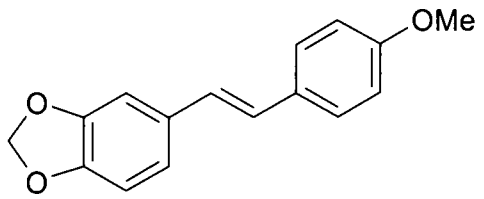
2,4-Dimethoxystilbene



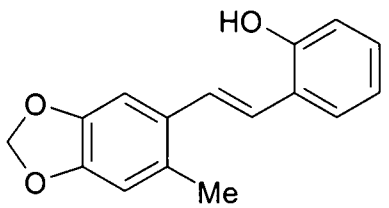
2-Hydroxystilbene



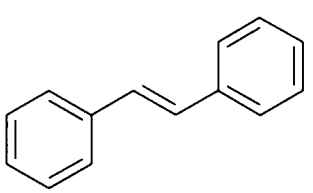
2-Hydroxy-2'-methoxy-4',5'-methylenedioxystilbene



4-Methoxy-3',4'-methylenedioxystilbene



2-Hydroxy-2'-methyl-4',5'-methylenedioxystilbene



Diphenylstilbene

Table 2. 1. Structure of compounds tested for leishmanicidal activity.

Amphotericin B was used as reference drug in the statistical studies. Each experiment was repeated three times.

Amastigote viability assay

Inhibition of amastigote growth was determined by using the alamarBlue® assay (Raz *et al.*, 1997; Mikus and Steverding, 2000). Briefly, parasites were distributed in 96 well plates (10^6 parasites/ml). Serial dilutions of some of the drugs active against promastigotes were added to the wells. alamarBlue® (Serotec, UK) was added to each treated well in 1:10 dilution with the medium and the plate incubated for 2 hours at 32°C. Metabolic activity of growing cells resulted in a chemical reduction of AlamarBlue. Following reduction, the REDOX indicator changes from an oxidized (non fluorescent, blue) form to a reduced (fluorescent, red) form. Fluorescence was monitored at 530-570nm excitation wavelength and 600nm emission wavelength. Absorbance was monitored at 570nm excitation and 600nm emission.

A calibration curve was established from plates containing three replicates of each of the serial dilutions of amastigotes and the absorbances were read after one hour of incubation with the dye (Figure 2.1).

2.10. Apoptosis assays

Apoptosis was investigated using three assays each identifying a different stage of the apoptotic process (Fig 2.2).

Mitochondrial permeabilization assay.

The first assay was chosen for its ability to detect mitochondrial permeabilization. This assay is fluorescence-based and rapidly detects changes in mitochondrial membrane potential. The Mitosensor® reagent is taken up in the mitochondria of healthy cells, where it aggregates and fluorescence red. However, if the cell is undergoing apoptosis, the mitochondrial membrane potential changes and the reagent is not able to aggregate inside the mitochondria. Mitosensor remains in the cytoplasm in monomeric form and exhibits green fluorescence. The BD ApoAlert® Mitochondrial Membrane

Sensor Kit (BD Biosciences, UK) was used according to the manufacturer's instructions. Briefly, samples were washed with PBS and incubated in diluted BD Mitosensor reagent for 15-20 minutes. Cells were then washed, re-suspended in PBS and immediately counted under a fluorescence microscope. As before, a minimum of 100 cells per sample were counted and the percentage of apoptotic cells recorded.

The monocyte-derived cell lines were infected with three different species of *Leishmania* parasites, and checked for presence of apoptosis 48 hours after infection. Uninfected cells were used as negative control while apoptosis was induced with 3μM camptothecin B on uninfected cells to be used as positive control.

PS externalization assay

The second assay chosen to test apoptosis in infected macrophages was Annexin-V-Cy3. During apoptosis, phosphatidylserine (PS) is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong, Ca^{2+}-dependent affinity for PS and therefore serves as a probe for detecting apoptosis. The Annexin V-Cy3 conjugate contains the bright red fluorescent probe that was used for detection of apoptosis by fluorescence microscopy with a rhodamine filter (can also be used in flow cytometry analysis). Cy3 yields red fluorescence with a λmax emission of 570 nm. To distinguish apoptotic cells from dead cells 6-carboxyfluorescein diacetate (6-CFDA) was used as vital stain. When this non-fluorescent compound enters living cells, esterases present hydrolize it, producing the fluorescent compound 6-carboxifluorescein (6-CF). This appears as green fluorescence. Live cells were labelled green and necrotic cells were red, while cells in the early stage of apoptosis will be labelled both green and red. The test was chosen because of the relevance of the presence of PS on the surface membrane for the proposed model.

The monocyte-derived cell lines were infected with three different species of *Leishmania* parasites, and checked for presence of apoptosis 48 hours after infection. Uninfected cells were used as negative control while apoptosis was induced with 3μM camptothecin B on uninfected cells to be used as positive control.

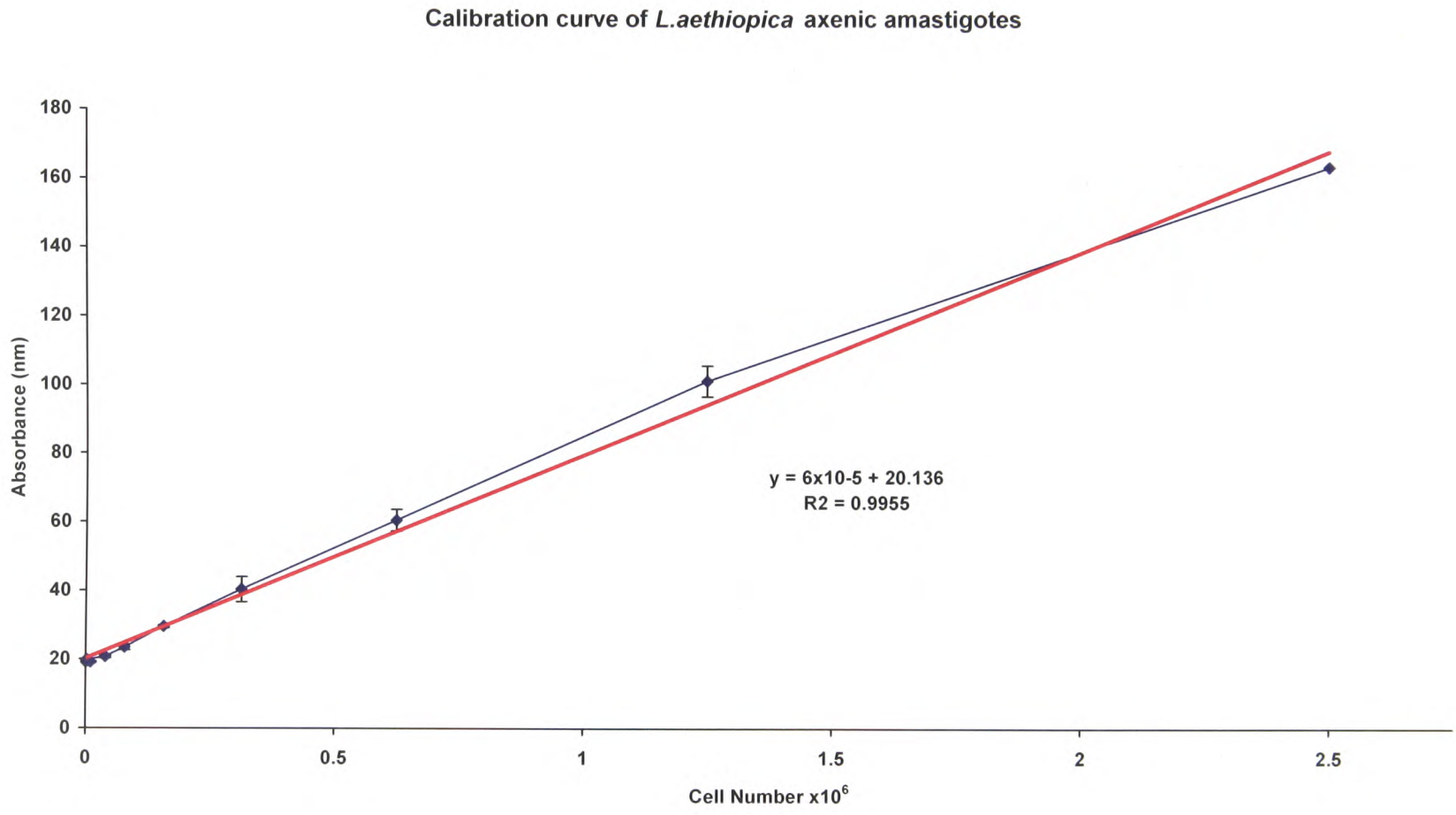


Figure 2.1. Calibration curve of *L. aethiopica* amastigotes. Serial dilutions of *L. aethiopica* amastigotes were placed in triplicate in 96 well plates, incubated for one hour at 32°C with 1:10 alamarBlue and the absorbance read at excitation:570 nm; emission: 660 nm. Error bars are standard deviations

DNA degradation

The final stage characterizing apoptotic cells is DNA fragmentation. Flow cytometry detection of sub diploid cell was the method chosen to detect DNA fragmentation and loss. After apoptosis, low molecular weight DNA can be 'extracted' and the apoptotic cells containing a reduced amount of DNA detected through flow cytometry (Darzynkiewicz *et al.*, 1992).

The cellular DNA content can be measured following cell permeabilization with detergents and fixation with alcohol. It is during this step that the DNA cannot be fully preserved within the apoptotic cells, consequently it 'leaks out' during subsequent cell rinsing and staining (Darzynkiewicz *et al.*, 1992). Following DNA staining, cells with a low DNA stainability will be detected as a sub-G1 peak lower then the G1 peak representing diploid cells. It has been noted that addition of high molarity phosphate-citrate buffer to the rinsing solution enhances extraction of the degraded DNA.

The following protocol was used as described by Darzynkiewicz *et al.* (1992). The cells were washed twice in buffer (PBS + 0.1% BSA) and re-suspended at a concentration of 1.5 million cells per ml. One ml aliquots of the suspension were transferred in V-bottomed polypropylene tubes and carefully mixed with cold absolute ethanol (the latter was added drop-wise while vortexing). Following cell fixation, at least one hour at 4°C, the cells were washed in PBS plus DNA extraction buffer (Appendix I) and incubated for 5 min at room temperature. Finally the cells were stained with propidium iodide (PI) staining solution plus RNase A, incubated for 30 min in the dark at room temperature and analysed by flow cytometry (Partec CA-III flow cytometer from Partec GmbH, Munster, Germany) with a 100 high W high-pressure mercury lamp, KG1, BG38, UG1 filters, TK420 dichroic mirror and a GG435barrier filter. Just before analysis the cells were carefully resuspended by passing them through a 25 gauge syringe.

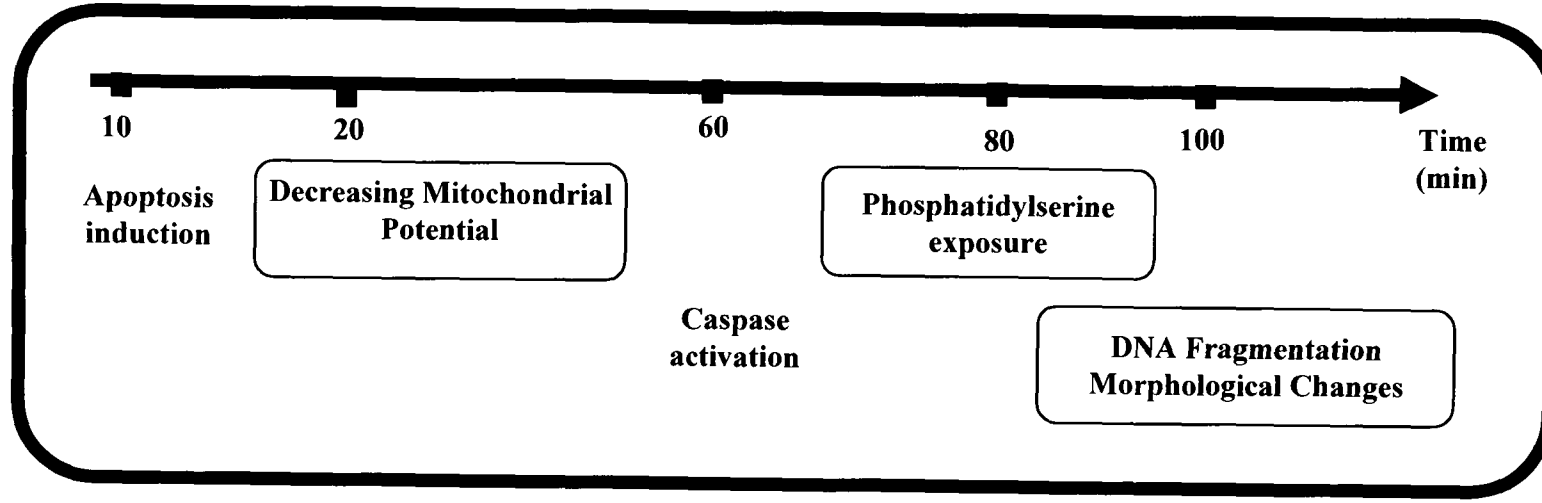


Figure 2.2. Milestones of Apoptosis. The circled features were used to detect apoptosis.

2.11. 2-D PAGE

Two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) was the methodology of choice to investigate changes in host macrophages during *Leishmania* infection. Proteomic analysis of this infection has never been performed before and it allows identification not only of variation in proteins expression but also of post-translational modifications. Complex mixtures of proteins are resolved with consequent analysis of hundreds to thousands gene products. Proteins are separated in two steps. According to charge (pI) by isoelectric focusing in the first dimension, and according to size (Mw) by SDS-PAGE in the second dimension. Throughout the 2D gel procedures, infected and uninfected samples were prepared side-by-side to minimize handling errors and technical variations.

Isolation of cytosol proteins

Following 48 hours from infection, 10^7 cells from both infected and uninfected control were harvested and washed in PBS and saline solution (x3). Cells were resuspended in lysis solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and protease inhibitors (PMSF and pepstadine A). Cell distruption was carried on by 3 cycles of freeze thaw in liquid nitrogen and in a warm water bath. The samples were centrifuged at 10,000 g for 20 mins and the supernatant containing cytosol proteins collected (the pellet was kept for further studies on membrane proteins). The supernatant proteins were precipitated by adding 3 volumes of cold acetone and incubated at -20°C over night. Samples were solubilized in lysis buffer and proteins concentration was determined through Bradford assay. In order to optimise the electrophoretic run the samples were further purified from detergents, salts, peptides, nucleic acids, phenols and lipids, and concentrated through Perfect-Focus™ (Calbiochem, UK).

First dimension

Following protein quantification, 100 µg of the samples were prepared for samples in-gel rehydration, by adding DTT, ampholytes and a trace of bromophenol blue (Appendix I). A total of 340µl was transferred inside a

reswelling tray and an 180 mm Immobiline^{TM} DryStrip gel containing pre-formed pH gradient (3-10) (IPG strip, GE Healthcare, UK) were carefully laid on top of each sample. The strips were covered with mineral oil to avoid evaporation and left to re-hydrate for a minimum of 6 hours, generally overnight. During this period the strip reverted to its 5 mm depth.

The hydrated strips were washed in double distilled (DD) water, loaded in a Multiphor II isoelectric focusing system (GE Healthcare, UK), connected with a water cooler and covered with mineral oil to prevent desiccation. In order to maintain a low, constant current an increasing voltage was applied as described in Table 2.2 Voltage and times were adjusted in order to achieve the best separation. Under-focusing, with voltages insufficiently high, causes horizontal streaking on the gel and should therefore be avoided. On the other hand, over-focusing results in distorted protein patterns, horizontal streaks at the basic end of the gel and loss of proteins (derived by excess water exudation at the surface of the IPG gel due to active water transport). Initially very low voltage is applied for better entry of high Mw proteins into the polyacrylamide gel matrix. The voltage was then gradually increased up to 3000 Volts and maintained at that level for 4 hours.

Second dimension (SDS-PAGE)

The second dimension vertical gel was prepared at a 12% concentration (Appendix I) and poured inside the PROTEAN II xi cells (Bio-Rad, UK), assembled according to manufacturer's instructions. Water saturated isobutanol was immediately overlayed onto the acrylamide gel to prevent formation of an uneven surface at the top of the gel.

The IPG strips were equilibrated in two different steps. They were first incubated for 15 minutes with the equilibration buffer I (Appendix I, EBI) containing freshly made DTT, to preserve the fully reduced state of denaturated, unalkylated proteins. A second incubation for 15 minutes in EB II containing iodoacetamide (IAA) was performed in order to alkylate thiol groups on proteins, preventing their re-oxidation during electrophoresis.

Voltage	Time
200	10 minutes
300	20 minutes
500	20 minutes
1000	30 minutes
1500	30 minutes
2000	30 minutes to 1 hour
3000	4 hours

Table 2.2. Voltage applied to 18 cm strips during the first dimension. The voltage applied to the strips during isolectrofocusing in the Multiphor is reported. Voltage was manually increased at the times indicated to achieve a final run length of 7 hours.

The strips were then transferred on top of the acrylamide gel and sealed with 1% agarose gel containing bromophenol blue to allow monitoring of the electrophoresis run. Once the agarose gel had solidified, the gels were transferred into the electrophoresis tank (PROTEAN II xi/ Vertical electrophoresis; Bio-Rad, UK) cells, covered with running buffer (Appendix I) and allowed to run for 9 hours at 40 milliamps. A cooling core was connected to the cooling block to avoid overheating

Gel staining

Silver staining

The gel is impregnated with soluble silver ions and following development by treatment with formaldehyde, an insoluble brown precipitate of metallic silver is formed in the correspondence of proteins.

During the first part of the analysis silver staining was used in order to detect the maximum number of spots on the gel. This allowed the establishment of optimal running conditions as well as the detection of major differences between the samples observed.

Following the gel run the proteins were fixed in fixation solution (Appendix I) for 20 to 30 minutes, at room temperature in carefully cleaned glass plates. Rinsed with DD water twice for 5 minutes each time and incubated with clean DD water for a further 30 minutes on a shaking platform. The sensitising solutions were then add for I minute, discarded and quickly rinsed with DD water (1 minute x2). The gel was then incubated with chilled 0.1% $AgNO_3$ for 30 minutes in a refrigerator at 4° C, the solution discarded and the gel rinsed with water for 30 seconds twice. The colour was developed with developing solution (Appendix I) until the proteins reached a sufficient degree of staining without the background increasing. The developing solution was changed whenever it started to turn yellow. The gel was then washed three times in 5% acetic acid solution and stored at 4°C in I% of the same solution.

Destaining solution

Spots of interest were excised and transferred from the silver-stained gel and placed in a pre-rinsed silanized tube. A working solution of 30mM potassium ferricyanide and 100mM sodium thiosulphate 1:1 was freshly prepared just before use and added to cover the gel pieces (30 to 50 µl). The tube was shaken and the gel monitored until the brownish colour completely disappeared. The working solution was discarded and the gel piece was rinsed with three changes of 25mM ammonium bicarbonate for 10 minutes while vortexing.

Comassie blue

A stock solution of Comassie Blue was prepared and added to the fixed gel. The gel was then incubated overnight at room temperature or for a few hours at 37°C. The dye was then discarded and the gel washed with dd water and destained in methanol/acetic acid/water (20/10/70) until spots were visible.

Sypro ruby

Sypro ruby ® (Invitrogen, UK) was add to fixed gels, in polypropylene trays and incubated at room temperature overnight. The Sypro was then discarded and the gel kept in methanol/acetic acid/water (20/10/70) and checked under UV light.

Perfect Focus Kit (Calbiochem, UK)

The procedure was performed according to the manufacturer's instructions. All the steps were carried out in 1.5ml Eppendorf tubes at 4-5°C (in an ice bucket). Briefly, 100 µl of the protein solution, containing up to 100 µg of proteins was transferred into an Eppendorf tube, the reagent UPPA-I was added and mixed in well, then following 15 minutes of incubation in Ice, UPPA-II was added and the Eppendorf was vortexed and centrifuged (15,000g for 5 min) to form a tight pellet. The supernatant was then quickly and carefully removed, the pellet shortly centrifuged again and the remaining supernatant removed with a thin pipette tip. DD water was added to the pellet (25 µl) plus 1ml of pre-chilled orgosol buffer and 5 µl of SEED. The sample was vortexed until all the pellet was re-suspended (not dissolved), incubated for 30 min at -20°C and centrifuged

again (15,000g for 5 minutes) to form a tight pellet. After discarding the supernatant the pellet was air dried and re-suspended in an appropriate volume of loading buffer and vortexed until the pellet was completely dissolved and then loaded on the IPG strip as previously described.

Protein quantification: Bradford assay

The Bradford assay utilises Comassie brilliant blue G-250 dye (CBBG), which is a dye molecule that exists in three forms each of which absorbs at different wavelengths. When the dye binds to amino acid residues it absorbs at a maximum wavelength of 650 nm (blue), while the free dye in the solution is in the cationic form and has a maximum absorbance at 465 nm (red). The CBBG establishes weak and non-covalent interactions with proteins, hydrophobic bonds with tryptophan, tyrosine, histidine and phenylalanine and electrostatic interactions with arginine and lysine. The quantification is therefore based on detecting the amount of protein-dye complex by reading the absorbance maximum shift from 465nm to 595 nm.

A 96 well plate protocol was followed according to the manufacturer's instructions. A standard curve was constructed; six solutions containing known BSA concentrations (1, 0.75; 0.5; 0.25; 0.12; 0.06 mg/ml) were prepared. Five microlitres of each solution were added to each well plus 5 microlitres of water plus 5 microlitres of the unknowns. Bradford reagent was brought to room temperature and 250 microlitres added to each well. The plate was shaken for at least 30 seconds and the samples incubated for 5 to 45 minutes. The absorbance at 595 nm was then measured in a Multiskan ® spectrum microplate spectrophotometer (Thermo Electron Corporation, UK). The absorbance values were plotted against the protein concentration of each standard. The protein concentration of the unknown samples was estimated by comparing the A595 values against the standard curve.

2-D Fluorescence Difference Gel Electrophoresis

Following optimization of 2D gel run techniques comparative analysis of proteins was obtained by DIGE examination. This technique allows resolution of two different protein samples in the same 2D-gel. Each sample and an internal

standard derived by the mixture of the two samples are labelled with a different fluorescent dye (Cy3, Cy5 and Cy2 respectively, GE healthacare, UK). After labelling the samples were mixed together and run on the same strip and on the same acrylamide gel. Consequently both samples will be subjected to the same experimental conditions and the same protein in different samples will run exactly in the same position. Each sample can then be visualized independently by selecting different excitation and emission wavelengths. The CyDyes have the same size and charge so that they change the proteins run in the same way for each sample. They bind the ε amino group of lysine via an amide linkage. Adding a limited amount of dye to the protein samples ensures that only 3% of the sample and that only one lysine per protein are labelled. Since such a small percentage of proteins are labelled with the dye the identification of the proteins through mass spectrometry methods is not affected. The labelling will only result in the loss of a single trypsin digest site per labelled protein.

The dyes were reconstituted at room temperature by adding 5ml DMF (dimethyl formamide) to the dye tube and mixed. The dye was then at a concentration of 1mM in DMF and could be stored at -15 to -30 °C for 6 months.

Experimental design.

In order to study the infection profiles on the *L. major*, *L. tropica* and *L. aethiopica,* 6 gels were produced; 2 set of gel for each infection (Table 2.3). In each set one gel contained uninfected cells extract labelled with Cy3, infected cells extract labelled with Cy5 and an internal standard containing a mixture of the infected plus uninfected samples labelled with Cy2 was run. The second gel of each set contained the same two samples reciprocally labelled plus the Cy2 labelled internal standard to be used to compare the two gels.

Procedure

Following sample preparation and protein quantification the pH of the lysate was adjusted to 8.5. Fifty micrograms of proteins were then added to a micro centrifuge tube and 1 microlitre of rehydrate dye. The solution was mixed, centrifuged and left so that the reaction could proceed for 30 minutes in ice in the dark. The reaction was stopped by adding 1 microlitre of 10mM lysine and the

samples left for 10 minutes in the dark. In these conditions the sample can be stored for at least three months at -70°C.

Once labelled, the samples were mixed and an equal volume of 2x sample buffer added. Following 10 minutes incubation on ice the volume was made up to 340 µl with rehydration buffer and loaded to an IPG strip overnight.

The IPG strips were processed by the University of Bristol for second dimension gel, gel scanning and preliminary data analysis, followed by Sypro ruby staining. For the first choice of gel spots, digestion and MALDI analysis of differentially expressed proteins were performed in the proteomic centre of Bristol according to the protocol reported below. Once the peaks list for each spot was generated,Mascot analysis was carried out in UEL.

In-Gel Protein Digestion

Two criteria were used to select the spots for further analysis: highest difference in expression and significance when compared with the reverse labelled gel. Relevant spots were excised, including a blank from a protein-free region of the gel. The $1mm^2$ plugs were transferred to clean Eppendorf (Sterilin, UK); washed in 100µl 25mM NH_4HCO_3 (20 min), the NH_4HCO_3 was discarded. Then the gel plugs were washed in 50% acetonitrile (MeCN) and NH_4HCO_3 until the gel shrunk and became white. An additional reduction and alchilation step was performed by adding 30µl of 10mM DDT in 25 mM NH_4HCO_3 and 30 minutes incubation at 56°C. Once the sample reached room temperature DTT was replaced with 30µl of 100mM iodoacetamide in 50mM NH_4HCO_3 and incubated at room temperature for 15 min. The gel pieces were washed / dehydrated in 50mM NH_4HCO_3 plus MeCN (50%) and finally air dried. The proteins were digested in 25mM NH_4HCO_3 containing 0.05-1 mg/ml trypsine (pH 8) at 37°C over night. The peptides were finally extracted from the gel by adding 10 10µl 25mM NH_4HCO_3 for 10min, 20µl MeCN for further 10 min and finally 20µl 10% formic acid for 30 min. The peptides were stored at -20°C until analysis.

Mass spectrometry analysis of peptide from tryptic digests.

The peptides were desalted and concentrated prior to MS analysis by μC18 Zip Tips (Millipore, UK). ZipTip is a 10 μl pipette that contains 0.6 μl immobilized chromatography media in the distal end. The first step involved washing Zip Tips in 50% MeCN + 0.1% TFA, followed by absorbing the peptides on to the tip membrane and 3 sets of washings in 0.1% TFA. Finally the peptides were eluted into the Maldi plate by 2 μl of ACH matrix (cyano- 4-hydrocinnamic acid, 10mg/0.1 ml dissolved in 70% ACN + 0.1% TFA)

The peak lists were submitted to MS/MS ion search on *Homo sapiens* in a NCBInr database using the software MASCOT (Matrix science). Only tryptic digests with one missed cleavage were considered. Mass tolerance was set at +/- 0.2 Da. Carbamidomethylation of cysteine and optional oxidation of methionine were taken into account.

Chapter 3

In vitro Leishmania infection of human macrophages

3.1. Introduction

The establishment of promastigotes inside macrophages and their transformation into amastigotes is only the first step in the initiation of infection inside the host organism. In cutaneous leishmaniasis, lesion development is due to an immune response to a significant number of infected cells largely confined in the skin, whereas in visceral leishmaniasis hepatosplenomegaly is the major manifestation and substantial parasitic loads are found in the spleen and liver. The small number of promastigotes delivered into the skin when the sandfly bites the host, about 100 in 75% of cases (1-1000, (Warburg and Schlein, 1986); 100 *L. major*; 1000 *L. mexicana* and 500 *L. infantum*, (Rogers *et al.*, 2004)), do not directly result in the infection load reported during leishmaniasis. Promastigotes of most species of *Leishmania* are unable to survive in host serum for long (Mosser and Edelson, 1984; Barral-Netto *et al.*, 1987; Grimm *et al.*, 1991) even in the metacyclic stage (Noronha *et al.*, 1998), unless phagocytosed by host cells. While the parasites are able to enter various human cells including Langerhans'

cells, dendritic cells (Udey *et al.*, 2001), macrophages and monocytes (Brandonisio *et al.*, 1986), neutrophils and fibroblasts (Bogdan *et al.*, 2000), they can only multiply inside macrophages (Rittig and Bogdan, 2000). Since very few macrophages will be initially infected by promastigotes, immune responses and lesions will not develop until significant multiplication and spread between macrophages has taken place. Although extremely important in the development of the disease very little is known about this phase. Rittig and collaborators (Rittig *et al.*, 1998) studied *L. major* amastigote behaviour inside macrophages through video-enhanced phase-contrast microscopy and described major changes on day 5 following infection. Small vacuoles accumulated asymmetrically at the periphery of infected murine peritoneal macrophages and from these vacuoles amastigotes were released constantly over a period of several hours, leaving shrunken remnants of the host cells (Figure 3.1). Although interesting, these observations do not provide exhaustive information about the mechanism by which amastigotes are released; whether or not the parasites are enclosed within host cell membrane during the process remains unknown.

The aim of this Chapter is to describe the experimental conditions needed to construct an in vitro model of the later stage of infection. A well established *in vitro* model has been used in order to study this interaction. Adherent and non-adherent, terminally differentiated THP-1 cells have been widely used in drug assays and are well known for supporting *L. aethiopica* infection for a relatively long (6 days) period (Gebre-Hiwot *et al.*, 1992) and were therefore chosen for this study. Moreover differentiated THP-1 cells behave more like natural monocyte-derived macrophages than other human myeloid cell lines (such as HL-60, KG-1, or HEL) (Auwerx, 1991). Terminally differentiated U937 cells were also infected under the same conditions in order to confirm that results were not limited to THP-1 cells. Due to the limitation associated with the use of cell lines, blood derived human macrophages were also used to validate results.

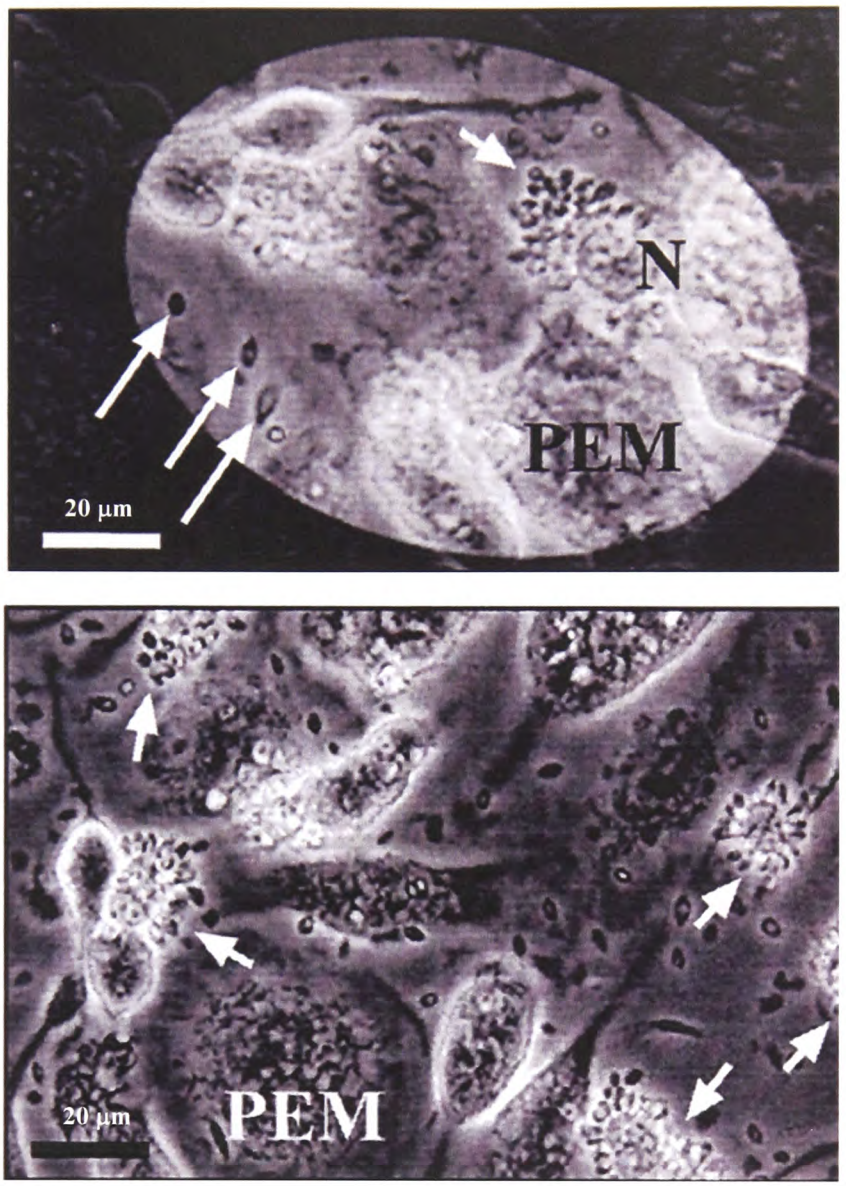


Figure 3.1. ***Leishmania*** **amastigotes leaving macrophages**
(a) Asymmetrical accumulation of small vacuoles (short arrow) at the periphery of the host cell, 5 days post infection. N, host cell nucleus; PEM, murine peritoneal macrophages; long arrows, released amastigotes; **(b)** Numerous host cells (arrows) simultaneously releasing replicated parasites; the pericellular fluid is full of amastigotes (5 days post infection; bar = 20 µm) (from Rittig *et al.*, 1998).

The infection process

In vitro infection has been described over a time span of 10 days. Following terminal differentiation of THP-1 cells, the infection was carried out as described in the Materials and Methods section with each of the three species of *Leishmania*. Removal of parasites in terminally differentiated non-adherent THP-1 cells was attempted by repeated centrifugation (4°C 50g) in Ficoll for 10 min, as previously suggested (Ogunkolade *et al.*, 1990). However, following microscopic analysis, parasites were still present in the culture. In order to avoid introducing a new variable by washing out different amounts of parasites it was decided to omit this stage. On the other hand, terminally differentiated adherent THP-1 cells were washed with warm PBS 24 hours following infection. In order to limit interference within the infection process, the cells were left untouched from day one and the medium was not changed.

3.2. Results and discussion

Growth of infective *Leishmania* parasites

The ability of *Leishmania* promastigotes to infect host cells has been described as growth cycle dependent and restricted to non-dividing organisms by Sacks and Perkins (1984). The same process (metacyclogenesis) has been identified in *L. donovani*, *L. infantum* and other New World species such as *L. braziliensis*, *L. amazonensis*, *L. panamensis* and *L. guyanensis* (Bates and Tetley, 1993; Louassini *et al.*, 1998; Zakai *et al.*, 1998; Saraiva *et al.*, 2005). *Leishmania* promastigotes from the species just mentioned do not cause lesions when injected into hamsters unless in late log or stationary phase. There is no evidence that metacyclic promastigotes belonging to *L. major*, *L. aethiopica* and *L. tropica* species are necessary to increase in vitro infectivity of THP-1 and U937 cell lines. Consequently, both log and stationary phase promastigotes could be infective. Nevertheless, the infection procedure has been standardized by using parasites in stationary phase.

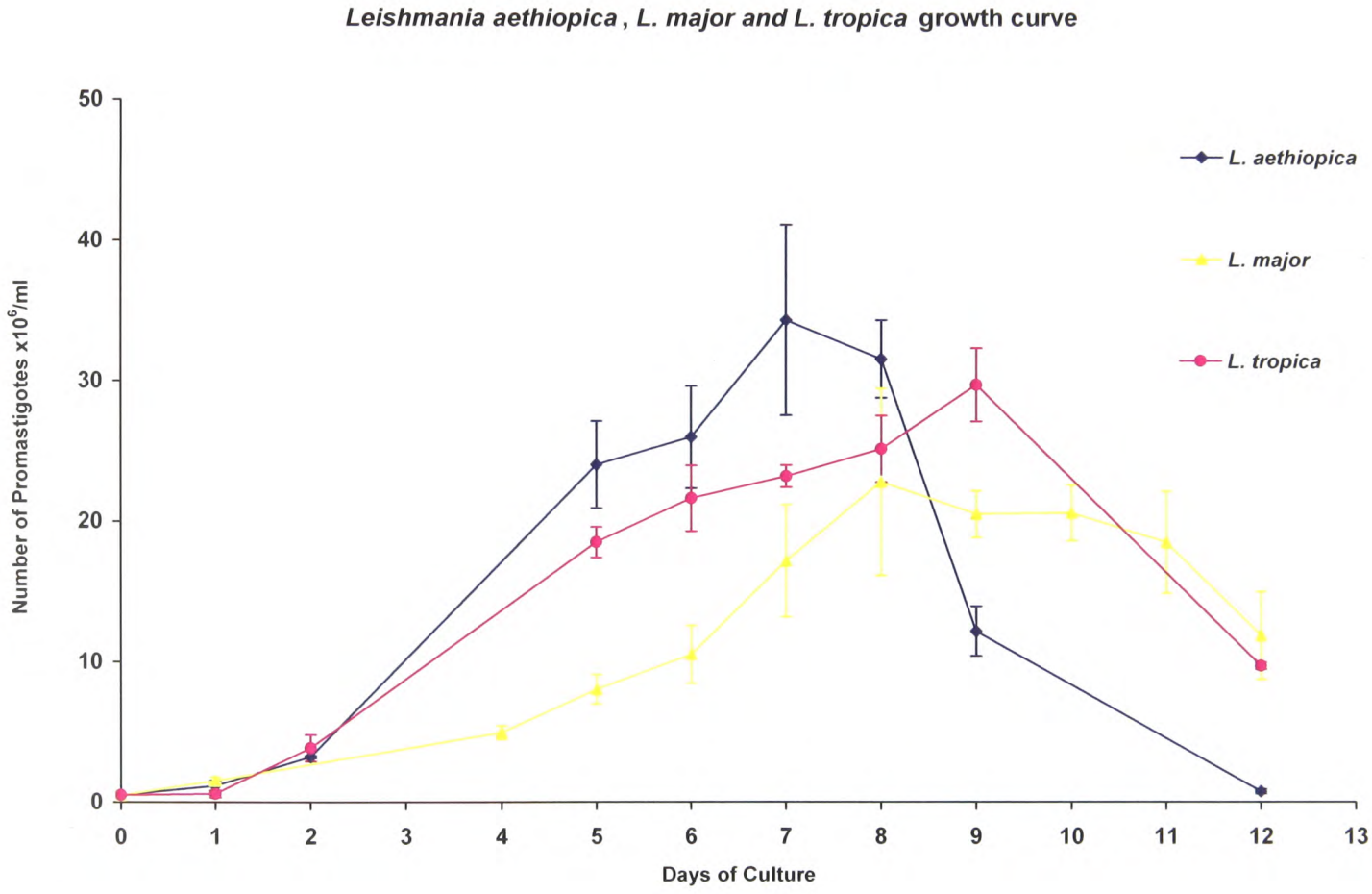


Figure 3.2: Growth curves of *L. aethiopica*, *L. major* and *L. tropica*. *Leishmania* promastigotes were counted and re-suspended in DMEMN-F12 medium at a concentration of 5 x 10^5/ml. The cultures were maintained at 22°C and counted for 12 days. All cultures were set up in triplicate. Each point represents a mean value from triplicate cultures, +/- standard deviation

Growth curves were established for each species (Figure 3.2) at a temperature of 22°C. This is the temperature to which *Leishmania* species responsible for cutaneous leishmaniasis are subjected in the skin during natural infection conditions. As expected, *L. major* growth was slower: higher temperatures (25 to 27°C) are generally used in the optimal culturing of the parasite (Choudhary *et al.*, 2006; Savoia *et al.*, 2006; El-On *et al.*, 2007). Promastigotes were grown in triplicate at a starting concentration of $5x10^5$ per ml as described in Materials and Methods. Samples were taken every 24 to 72 hours and cells were counted in a haemocytometer (3 different samples for each species) for 12 days. Starting parasite concentrations lower than $5x10^5$ per ml showed a prolonged lag phase (results not shown) and were therefore not used. Although all three species grew exponentially from day 1 to day 5, *L. aethiopica* concentration kept rising up to day 7, following which cell concentrations declined rapidly. We can therefore assume that late log/early stationary phase can be identified with day 7 at a concentration of $3.5 x10^7$ per ml.

Similarly, the increase in *L. tropica* concentration started slowing down on day 6 but slow growth continued until day 9. These data suggested that stationary phase for this species is reached on day 9 and at a slightly lower concentration of $2.8 x10^7$ per ml. Finally *L. major* showed a generally lower growth profile with a maximum number of cells per ml of $1.3x10^7$ compared with over $3 x10^7$ for both *L. aethiopica* and *L. tropica*. A steady increase in concentration was reported up to day 8, following which the population number reached a plateau for 3 days and then started to slowly decline. Day 8 was identified as the beginning of stationary phase for *L. major* and the cell concentration at this stage was found to be $2.25 x10^7$ cells/ml.

Growth conditions of THP-1 cell line

Terminally differentiated THP-1 cells mimic natural monocyte-derived macrophages in many respects and have been widely used in the study of monocyte differentiation in macrophages (Feng *et al.*, 2006; Uchide *et al.*, 2006). They are also able to support the growth of amastigotes from various *Leishmania* species, which makes them candidates for *in vitro* study of this infection. In

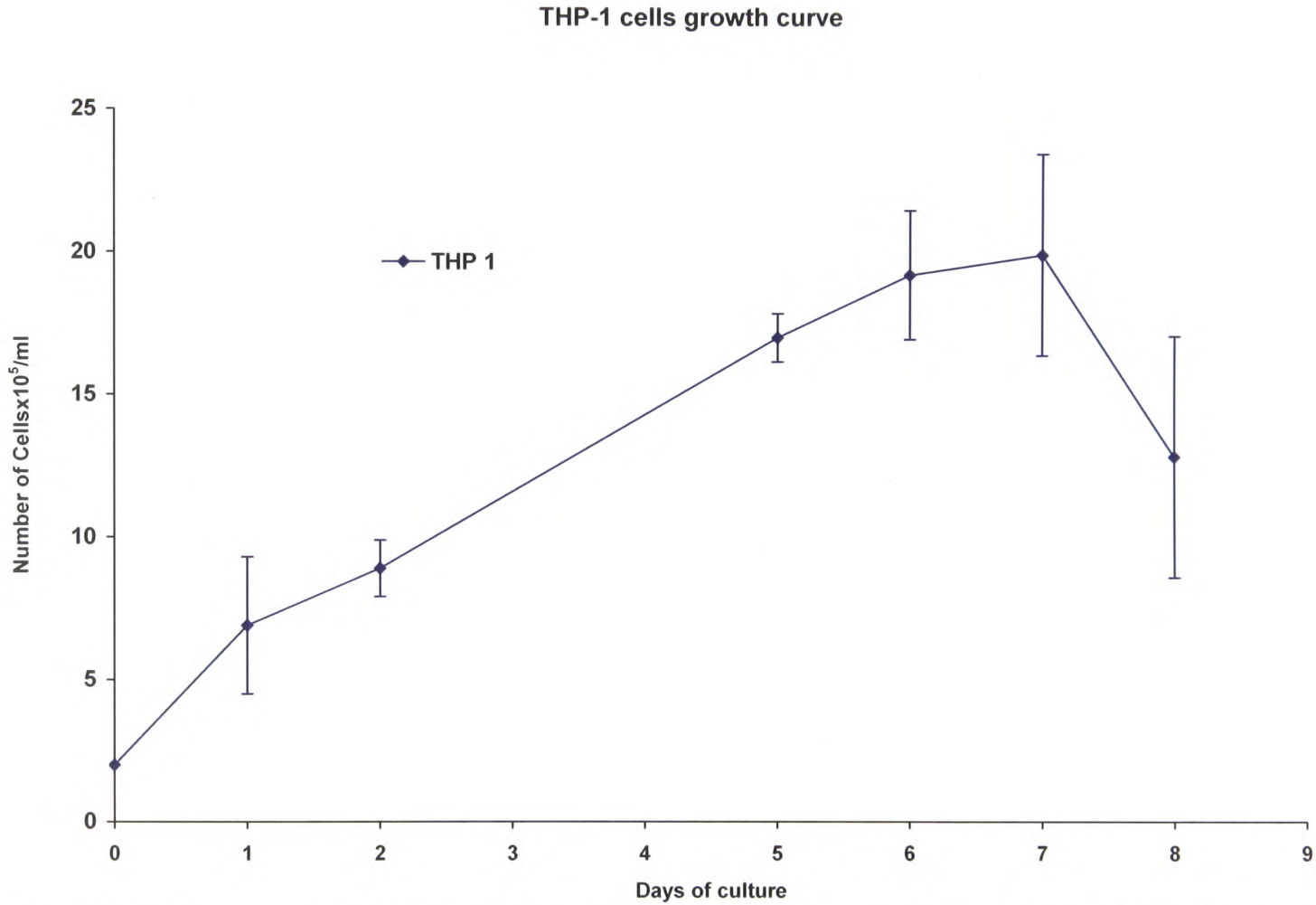


Figure 3.3. Growth curve for THP-1 cells. Cells were counted and re-suspended in DMEMN-F12 medium at a concentration of 2 x 10^5/ml. The cultures were maintained at 37°C and counted for 9 days. All cultures were set up in triplicates. Each point represents a mean value from triplicate cultures, +/- standard deviation

order to achieve differentiation THP-1 cells have to be treated before reaching the stationary phase (Ogunkolade *et al.*, 1990). A growth curve was therefore established (Figure 3.3). THP-1 cells were grown in DMEM medium at 37°C and cultured at a starting concentration of 2 x 10^5. The concentration of cells steadily rose until day 5, suggesting that between days 2 and 3 the cells were at the beginning of the exponential phase. By day 7 they reached the stationary phase as the number of cells dying overtook the number of cells dividing. Day two was therefore chosen to start differentiation. Medium containing serum was changed 24 hours before the addition of either PMA or RA. This change was necessary to achieve a healthy population of terminally differentiated THP-1 cells (data not shown). Cells were terminally differentiated: adherent cells (PMA treated) inside 24 well plates containing rounded coverslips; non-adherent cells (RA treated) inside tissue culture flasks. Viability was then checked following trypan blue staining. If viable cells were less than 80% of the total population, cells were discarded; if higher, cells were considered ready to be infected with stationary phase promastigotes.

Infection process in adherent PMA treated THP-1 cells

The percentage of infected cells in adherent PMA treated THP-1 cells is shown in Figure 3.4. Samples were taken after 6 hours and 1, 3, 7 and 10 days. The infection was described by the percentage of infected cells as well as the number of parasites per cell (Figures 3.4 to 3.11). Following 6 hours incubation, 20 to 40% of the cells were infected. *Leishmania aethiopica* showed a higher infection rate on day one (46+/-2 % while rates for *L. tropica* and *L. major* were 32+/-2% and 41+/-1% respectively) with a statistically significant highest value on day three; two tail t test analysis showed differences between *L. aethiopica* (61+/-1% infected cells) infection and both *L. tropica* (27+/-1%) and *L. major* (39+/-0.5%) with a P value <0.02. The following decrease in the number of infected cells on day 7 and on day ten could be explained by medium exhaustion. It is likely that the macrophages containing the highest number of parasites are more sensitive and therefore most likely to die under conditions of stress, while the uninfected ones would start replicating because of the lessening of PMA

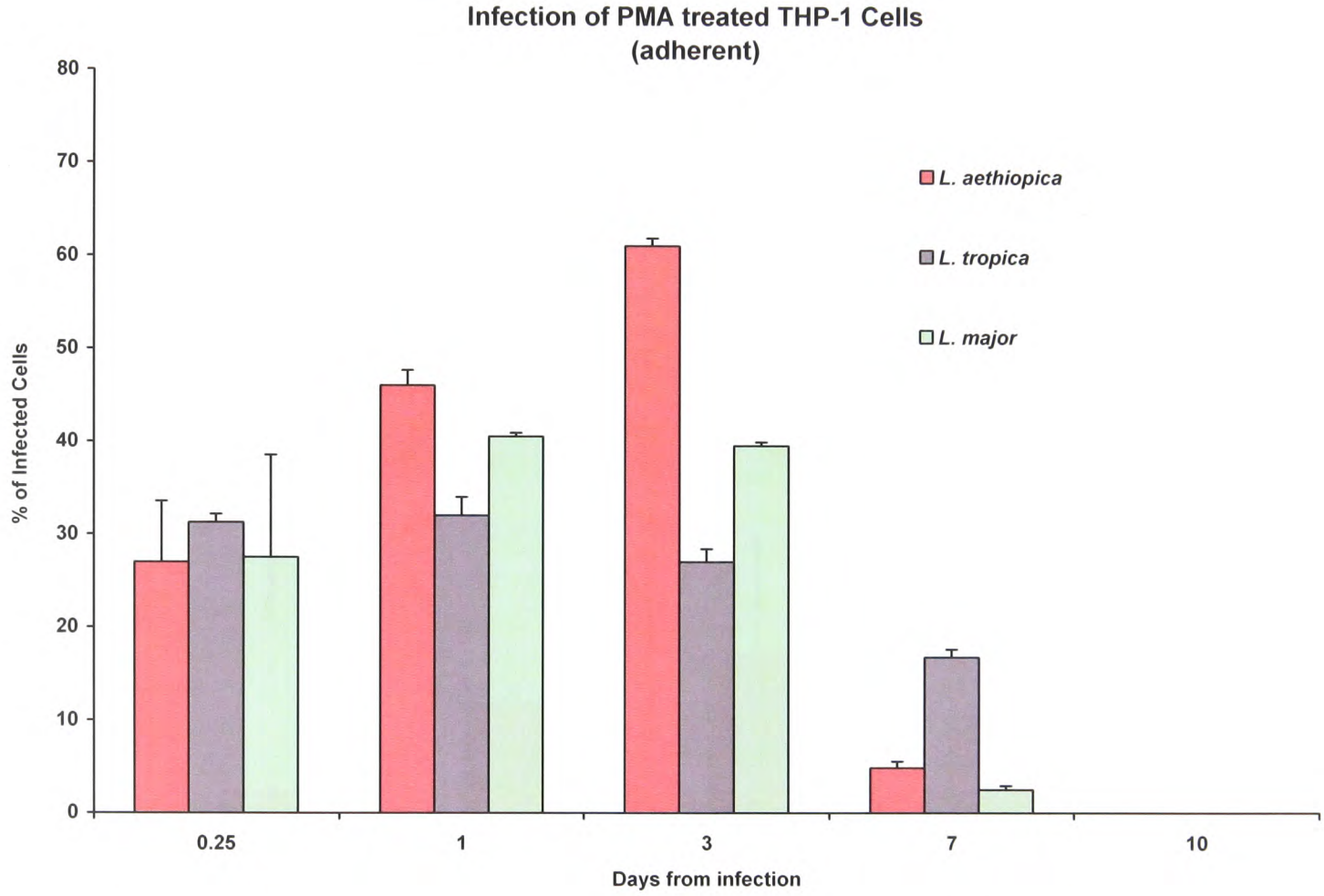


Figure 3.4. PMA treated THP-1 cells. THP-1 cells were infected with three different *Leishmania* species. The percentage of infected cells with respect to the total number of cells reported together with standard deviation. Different samples were taken at different times, between 6 hours and 10 days

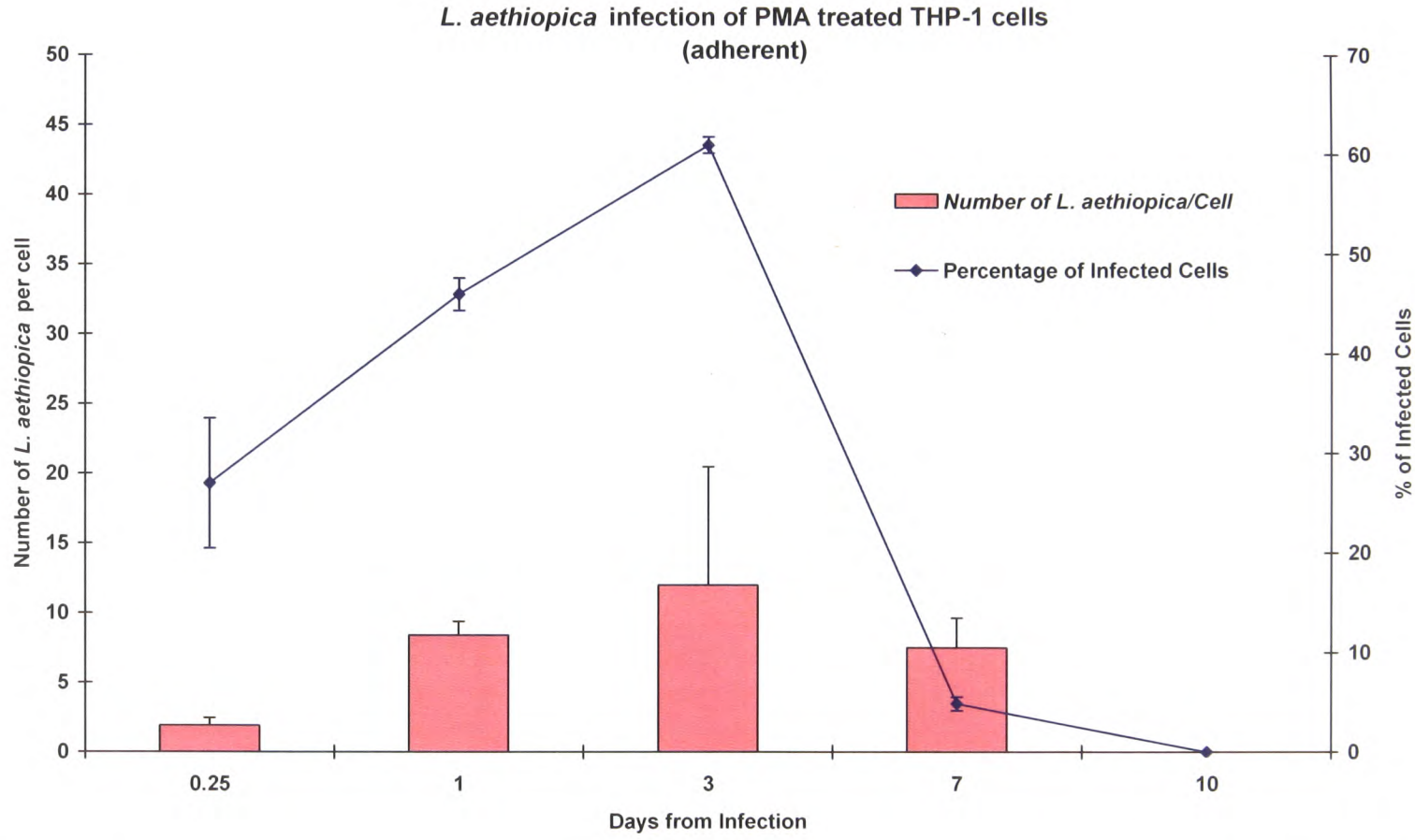


Figure 3.5: *L. aethiopica* infection of PMA treated THP-1 cells. THP-1 cells were infected with *Leishmania aethiopica*. The numbers of parasites per cell were counted in at least 50 cells/coverslip. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value.

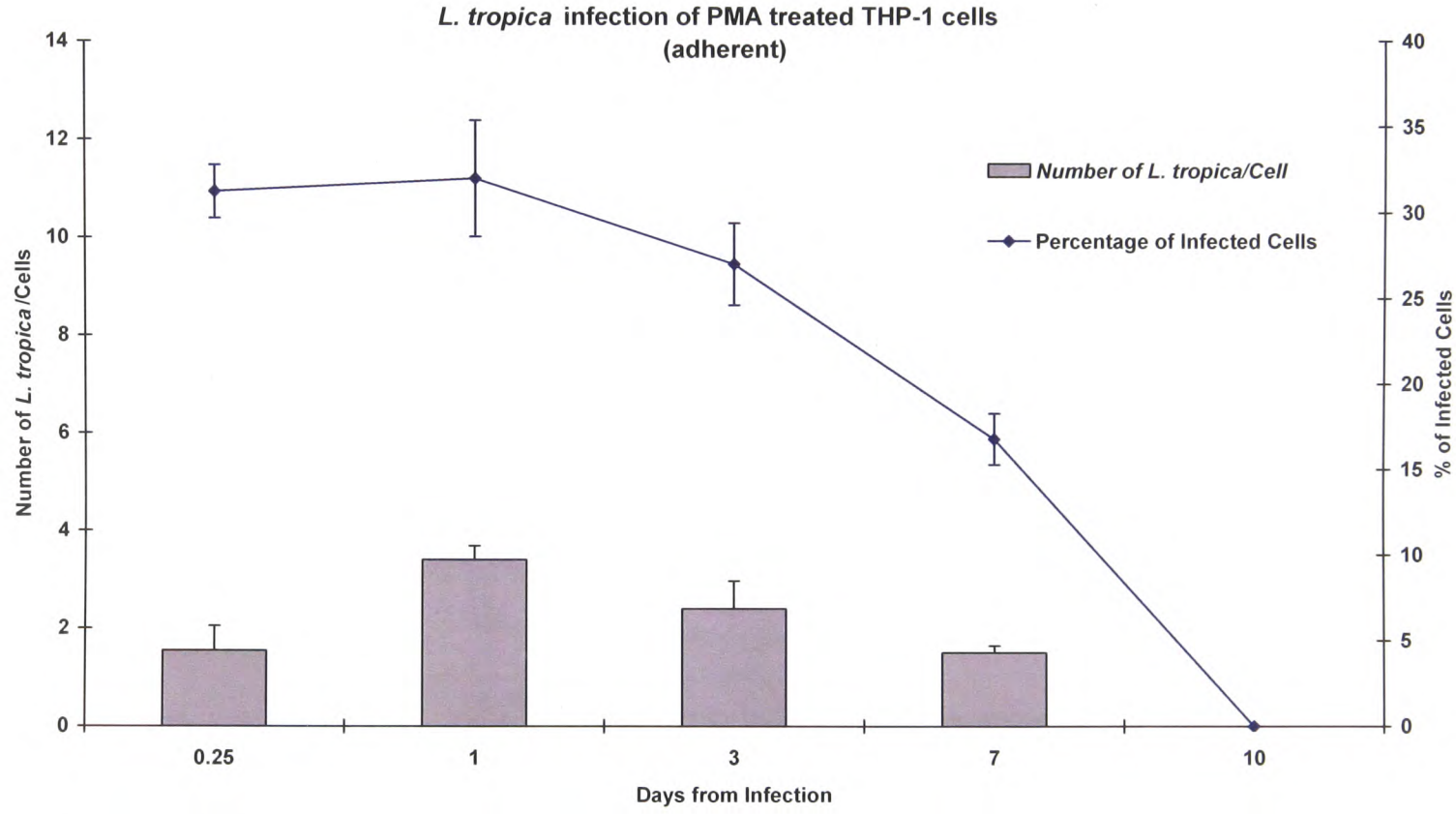


Figure 3.6: *L. tropica* infection of PMA treated THP-1 cells. THP-1 cells were infected with *Leishmania tropica*. The numbers of parasites per cell were counted in at least 50 cells/coverslip. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value

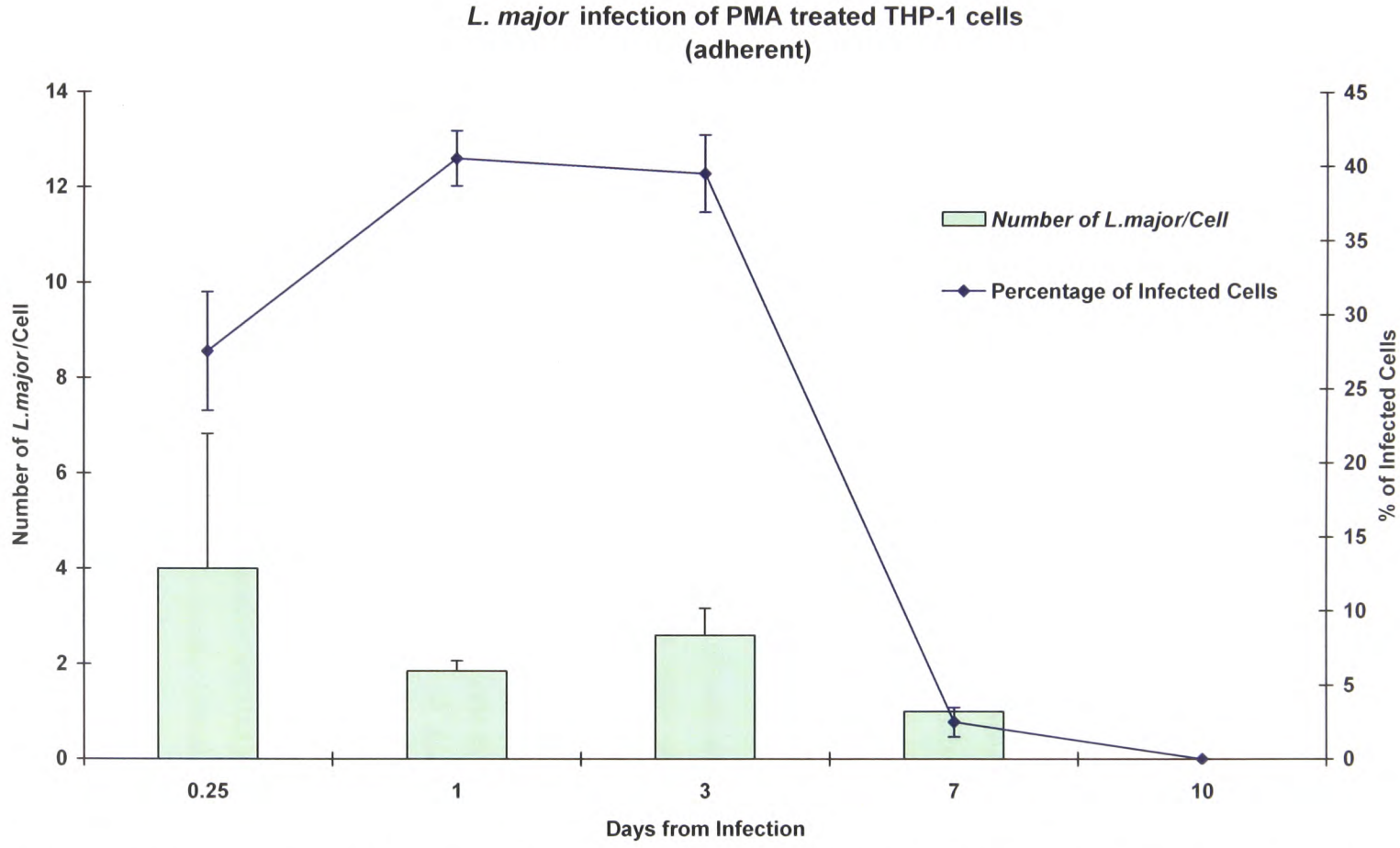


Figure 3.7: *L. major* infection of PMA treated THP-1 cells. THP-1 cells were infected with *Leishmania major*. The numbers of parasites per cell were counted in at least 50 cells/coverslip. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value.

activity (Figure 3.4). More detailed observations for each infection are reported in Figures 3.5 to 3.7. The infection with *L. tropica* showed a constant pattern in the first 24 hours, the number of infected macrophages started declining between day 1 and 3 to drop to zero infected macrophages on day 10. The number of amastigotes per cell reached a maximum on day one and slowly decreased on day 3 indicating that between day 1 and 3 changes in the infection burden take place. During *L. major* infection the percentage of infected macrophages increases up to day 1, remains the same on days 1 and 3 and decreases from day 3 onwards. The number of parasites per cell slightly increases between day 1 and 3. Relatively low numbers of parasites per cell were found in adherent THP-1 infected with *L. tropica* (3.5 +/-0.8) and *L. major* (2.7 +/-0.5) when compared with cells infected with *L. aethiopica* (12 +/-8). Similarly the percentage of infected cells between days 1 and 3 was the highest in *L. aethiopica* (54 +/-8%) with a peak of 60% +/-1% infected cells on day 3. The lowest percentage of infection was found for *L. tropica* (30+/-6%) with a peak of 32.5% (+/-2.5%) of infected cells at day 1.

The values recorded during *L. major* infection lay in between the ones described for *L. tropica* and *L. aethiopica* with a percentage of infected cells of 39.5 +/-3.5%. The percentage of infected cells did not show significant differences between days 1 and 3. The lack of human cells containing more then 3 parasites and the lower percentage of infected cells could indicate that the PMA treated THP-1 cells cannot sustain infection with high numbers of *L. major* and *L. tropica* parasites (Figures 3.6 and 3.7) while *L. aethiopica* is able to multiply inside its host for longer (with a consequent higher number of intracellular parasites) without affecting the host cell viability. It is important to compare the results obtained during infection of adherent cells with the results reported in the next paragraph for non-adherent cells. From this comparison we can establish if any differences are representative of the infection process or are due to loss of adherence during infection. In the last case scenario *L. tropica* infected THP-1 cells containing more than 3 parasites could not be detected because this infection burden induced loss of cell adherence and consequent detachment from the slide.

Although different from each other, the three infection profiles all indicated that the interval for *Leishmania* spreading is between 24 and 72 hours,

as both percentages of infected cells and numbers of parasites per cell decreased after this period. Data regarding longer incubation times are heavily influenced by the host cells' distress and death. Moreover, the effect of PMA and RA in THP-1 cells is lost within 5 to 7 days (Nakamura *et al.*, 1986) and the cells start multiplying again as well as losing their macrophage similarity.

Infection process in non-adherent RA treated THP-1 cells

The infection process in non-adherent THP-1 cells treated with RA is shown in Figures 3.8 to 3.12. The overall percentage of infected macrophages in the first 3 days is not significantly different from that reported in PMA treated THP-1 cells for both *L. aethiopica* (64 +/-11%) and *L. major* (35 +/-13%) infection, while it is significantly higher for *L. tropica* (67 +/-7%; Figure 3.8). The number of infected cells decreased drastically on day 5; as stated previously, this is probably due to nutrient exhaustion in the medium. The highest number of infected cells occurred between days 1 and 3 and the highest number of parasites per cell decreased from day 2 to 3 for all three species (Figures 3.9, 3.10 and 3.11). The number of parasites per cell is similar for the three species, reaching a maximum of 6 (+/-3). It decreased in each infection after day 3 at the same time as the number of infected cells started decreasing (Figures 3.10 and 3.11) with the exception of *L. aethiopica* infection (Figure 3.9). During this infection the number of intracellular parasites decreased from day 2, the percentage of infected macrophages decreased on day 3 and rose again on day 4, possibly as a result of successful spreading.

The data obtained for *L. tropica* infection in non adherent cells seemed to confirm the hypothesis that infected cells containing high numbers of intracellular parasites suffered a loss of adherence, became detached from the coverslips and were not detected following staining. Because of the nature of the experiments these infected cells are detected in RA treated THP-1 cells.

Loss of adherence in the infected cells on the other hand, cannot explain the results obtained for *L. major* (Figure 3.11). In this case the percentage of infected non-adherent macrophages on days 1 and 3 was similar to that for RA treated adherent THP-1 cells and so was the number of parasites per cell. This

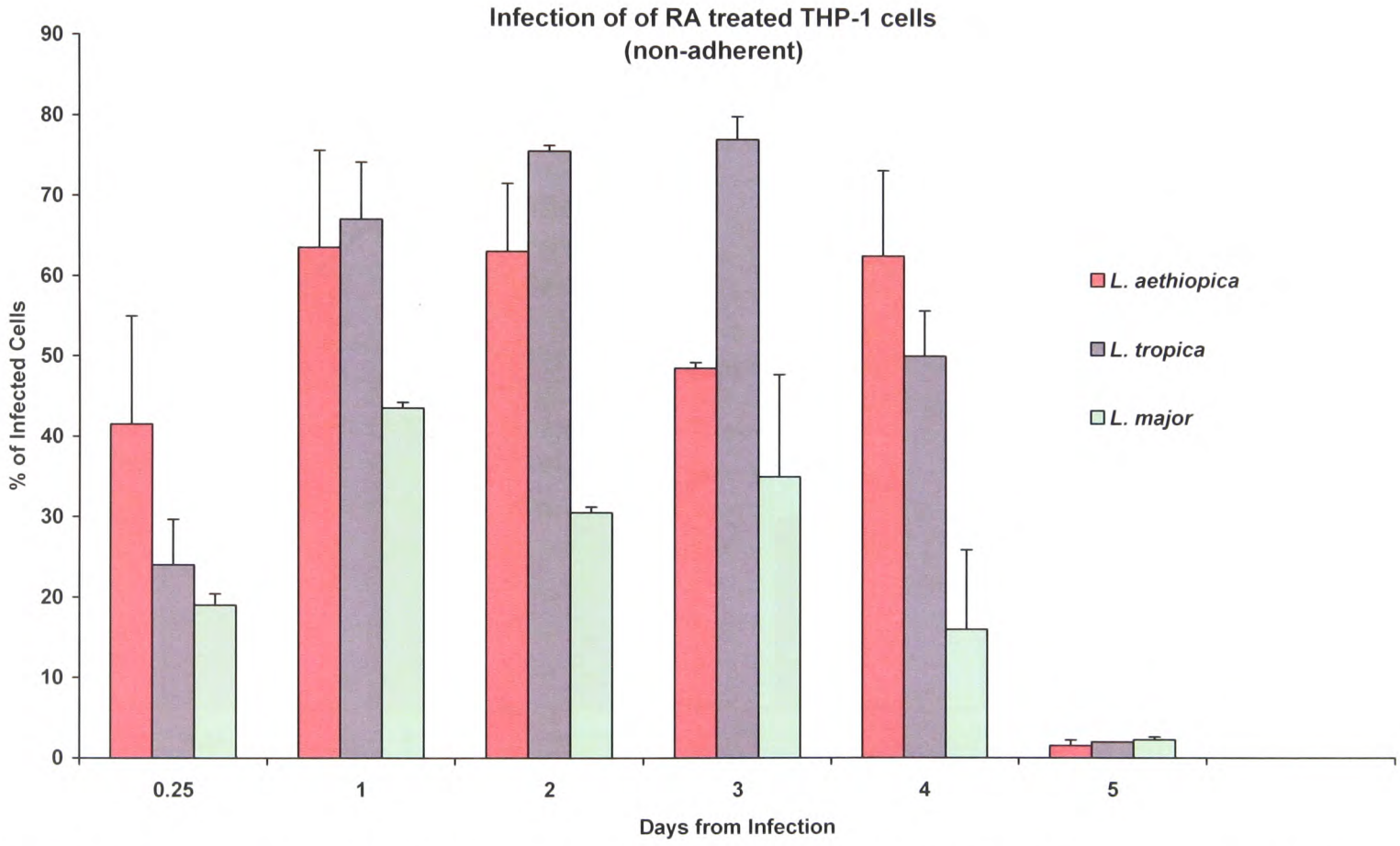


Figure 3.8: Infection of RA treated THP-1 cells. THP-1 cells were infected with three different *Leishmania* species, at a host cell:parasite ratio of 1 in 10 after differentiation with RA. The percentage of infected cells with respect to the total number of cells is reported together with standard deviation. Different samples were taken at different times, between 6 hours and 7 days.

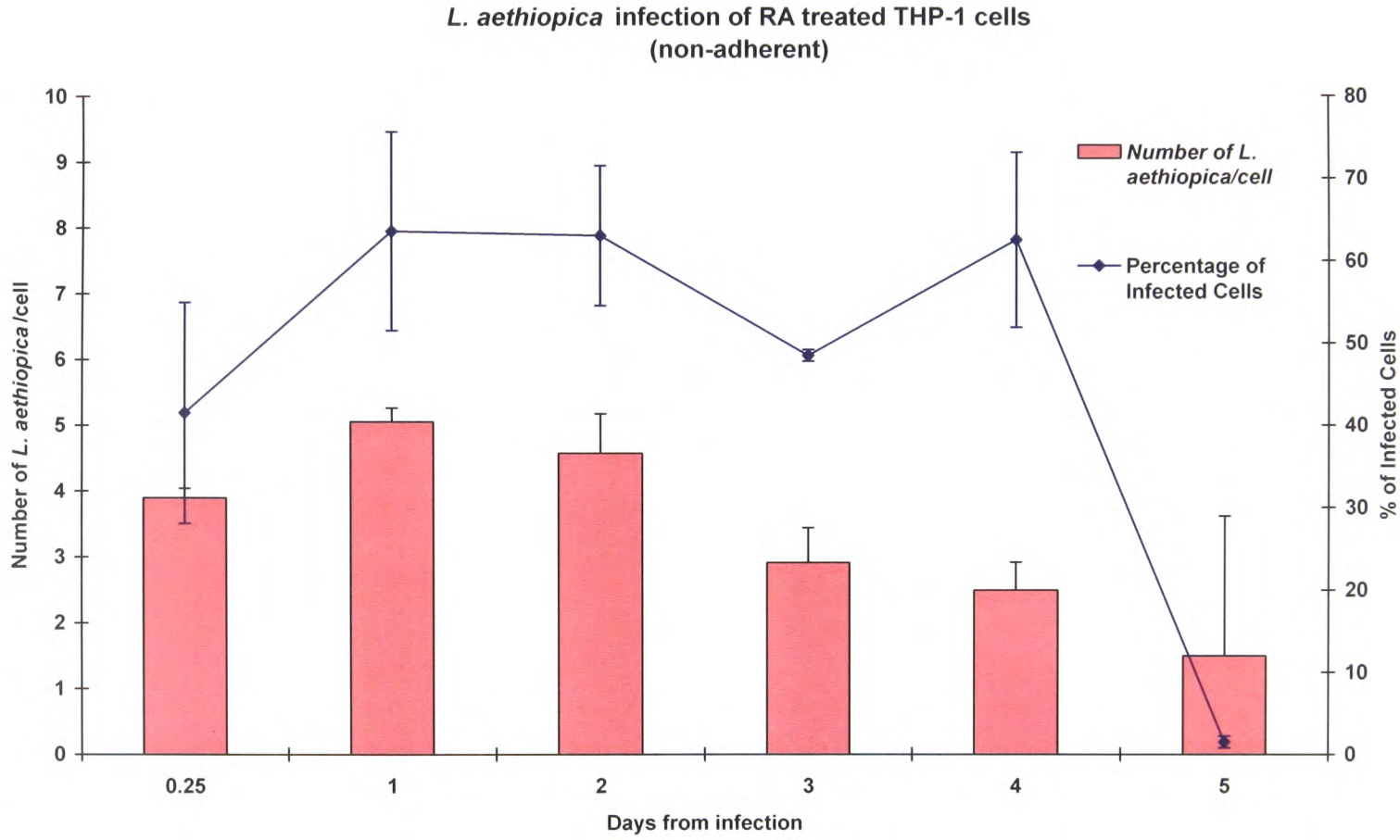


Figure 3.9: *L. aethiopica* infection of RA treated THP-1 cells. THP-1 cells were infected with *Leishmania aethiopica* promastigotes at a host cell:parasite ratio of 1 in 10 after differentiation with RA. The numbers of parasites per cell were counted inat least 100 cells/slide. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value.

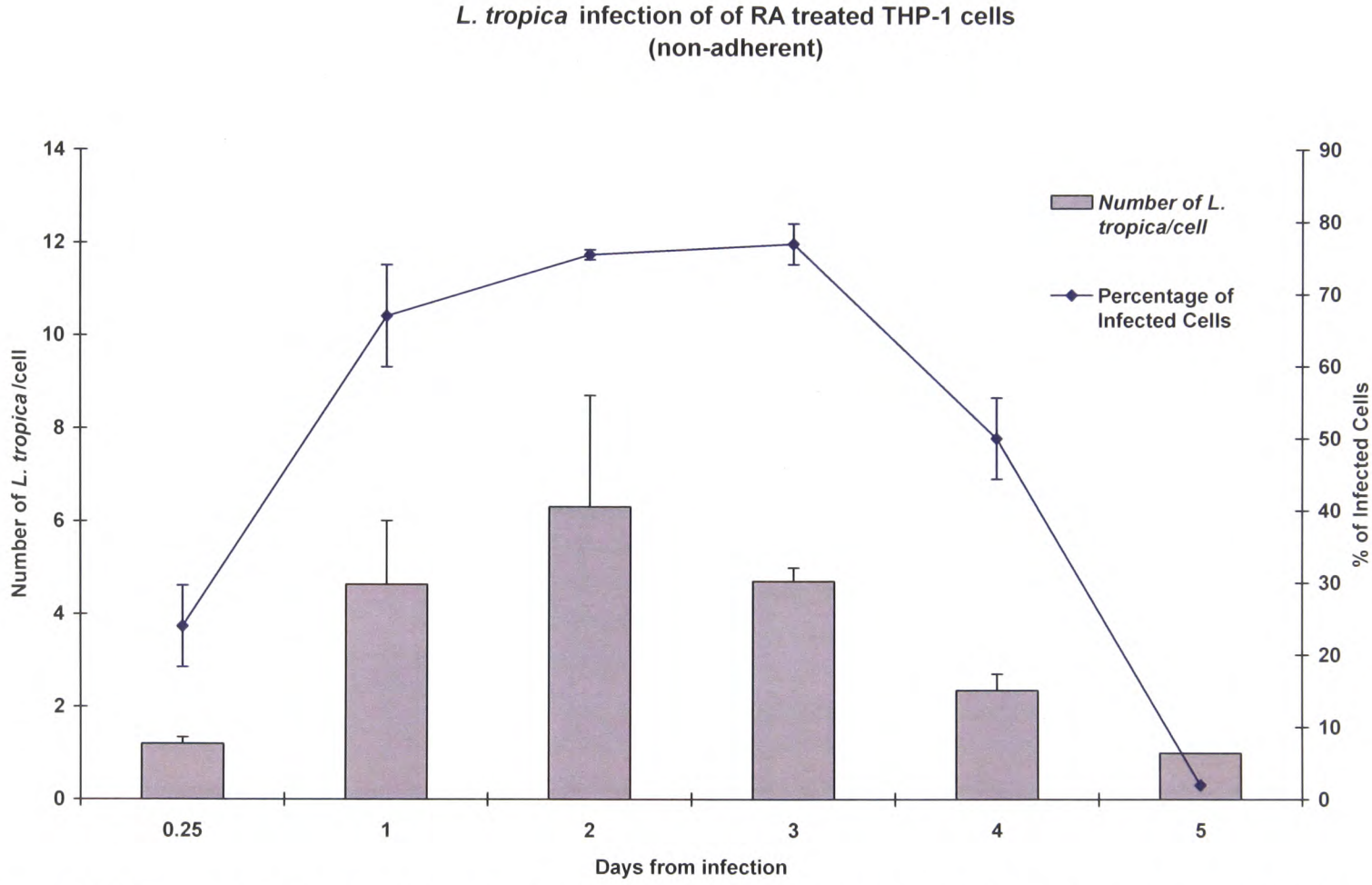


Figure 3.10: *L. tropica* infection of RA treated THP-1 cells. THP-1 cells were infected with *L. tropica* promastigotes at a host cell:parasite ratio of 1 in 10 after differentiation with RA. The numbers of parasites per cell were counted inat least100 cells/slide. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value.

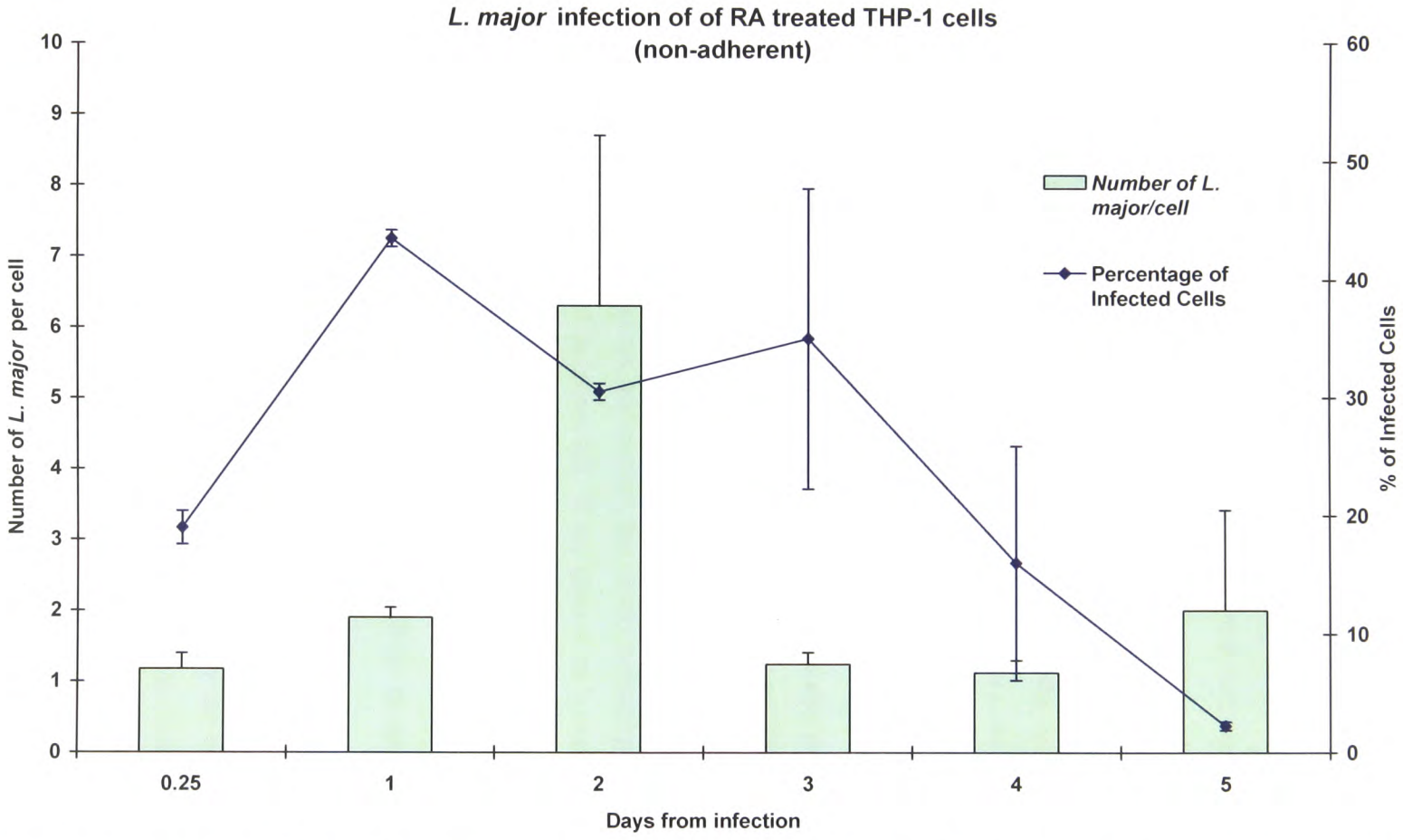


Figure 3.11: *L. major* infection of RA treated THP-1 cells. THP-1 cells were infected with *L. major* promastigotes at a host cell:parasite ratio of 1 in 10 after differentiation with RA. The numbers of parasites per cell were counted in at least 100 cells/slide. The average number of parasites/cell is plotted in bars and the percentage of infected macrophages in a line, standard deviation is reported for each value.

indicates that *L. major* does not cause loss of adherence in the host cells on days 1 and 3 and confirms the data obtained for PMA treated THP-1 cells. Moreover the data reported on day 2 for both *L. tropica* and *L. major* infections (Figure 3.10 and 3.11) showed a similar trend as that already shown in *L. aethiopica* infection of adherent cells (Figure 3.5). During *L. aethiopica* infection of PMA-treated adherent THP-1 cells, infected cells showed very high numbers of intracellular parasites on day 3, when the number of infected cells was also the highest. The large standard deviation on day 3 shows that the population of infected cells contained a wide range of parasites (from 3 to 21). It is tempting to speculate that this high variance in parasitic load is representative of a cell population where the infection is spreading. Quite possibly, cells containing a threshold number of parasites are induced to 'release' parasites towards non-infected cells. As a result of this behaviour cells containing few parasites will be present in the total population as well as cells containing a higher number of parasites, but not a high enough number to induce spreading.

A highly variable number of parasites/cell (3 to 9) was also recorded in both *L. tropica* and *L. major* infection of non-adherent RA treated THP-1 cells. This could represent the presence of two populations of infected cells, one of which is about to 'release' the intracellular parasites, and is therefore containing relatively high numbers of parasites, and another, the newly infected population, containing a lower number of parasites/cell. If so, days 2 to 3 could be the interval during which the parasites spread.

These observations, together with the fact that at day 3 the percentage of infection decreased, indicated that in non-adherent RA-treated THP-1 cells the period in which the intracellular parasites are the most likely to spread is 48 hours after infection.

It is interesting to notice the *L.aethiopica* containing PV in THP-1 cells differes from the one described for other species of *Leishmania.* In this case each parasite is contained in a tight small vacuole (Figure 3.12). On the other hand, parasites within PBDM are found within swollen vacuoles containing one or more amastigotes attached to the host membrane (Figure 1.1). Both types of PV were previously described in lesion derived cells from *L. aethiopica* infected patients (Schurr *et al.*, 1987)

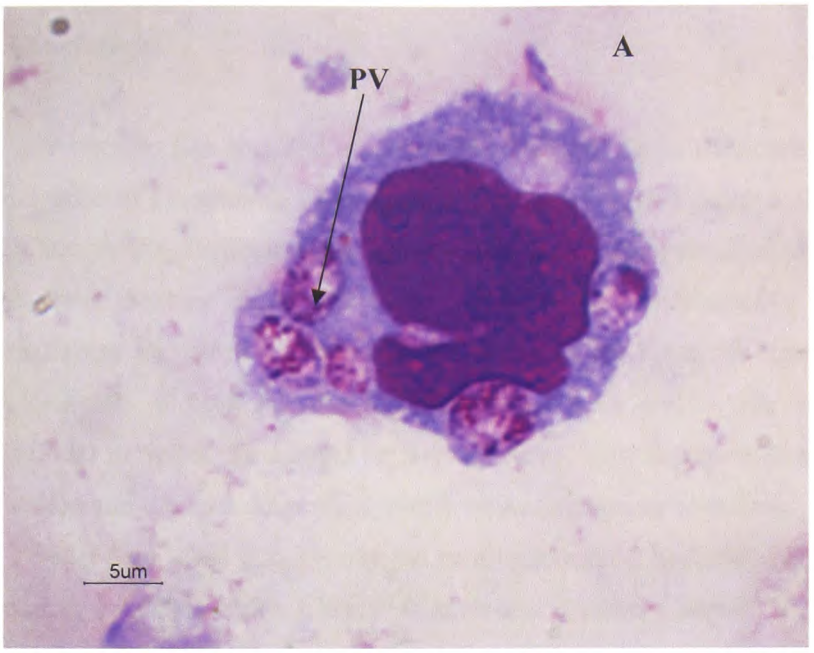


Figure 3.12. *L. aethiopica* containing parasitophorous vacuole. RA treated THP-1 cells following 3 days infection with *L. aethiopica* parasites. The parasitophorous vacuole (PV) containing *L. aethiopica* is tight and each parasite is contained in an individual PV.

3.3. Conclusions

From the data obtained it was deduced that 48 hours from infection, at a 10 to 1 ratio of *Leishmania* parasites and RA treated THP-1 cells, is the time in which the most information can be obtained about the spread of intracellular amastigotes. Shorter times are more likely to be representative of when promastigotes transform into amastigotes, while longer times are representative of a decrease in both percentages of infected cells and in the number of intracellular parasites. This could be due to stress related factors such as medium exhaustion and the lessening effect of differentiating agents with time.

Based on these conclusions the results presented hereafter (drugs assay, Chapter 5; apoptosis assay, Chapter 6; proteomics assay, Chapter 7) will derive from cells infected for 48 hours under the same conditions as those described in this Chapter.

Chapter 4

Leishmania aethiopica axenic amastigotes

4.1. Introduction

Following a bite by an infected sandfly, infective *Leishmania* promastigotes are injected into the vertebrate host and are ingested by the latter's mononuclear phagocytes. Here they undergo a series of morphological and biochemical modifications and become non-motile, rounded, aflagellated amastigotes. This transformation is essential for survival, multiplication and spread of the parasites within the mammalian host, consequently it is crucial for their pathogenicity.

However, most published studies have been on the promastigote stage, mainly because of the ability to grow this stage of most species *in vitro*, and thus obtain many parasites. Moreover, obtaining amastigotes from cells or tissue purification was not only very laborious but involved a high risk of cell contaminants that would interfere with further characterizations.

Several attempts have been made to develop culture media and culturing conditions to allow axenic cultivation of amastigotes. In the last 20 years

numerous species of *Leishmania* have been established *in vitro* as axenic amastigotes. Pan *et al.* made the first successful attempt in 1984: *L. mexicana* promastigotes transformed into amastigote-like organisms after the incubation temperature was increased from 22°C to 32°C and the pH of the culture medium decreased from 7 to 5 (Pan, 1984). Since then several species and strains have been cultured as axenic amastigotes as reviewed in 2001 by Gupta and colleagues (Table 4.1).

Once amastigote-like forms of *Leishmania* species are obtained a stringent evaluation of their characteristics has to take place in order to confirm similarity of the axenic form with the intracellular amastigotes. Well characterized *bona fide* amastigotes can then be used in further studies such as *in vitro* models to investigate (a) potential antileishmanial compounds (Sereno *et al.*, 1998; Tavares *et al.*, 2005); (b) the mechanism of action of antileishmanial compounds (Al-Mulla *et al.*, 2005); (c) the cell and molecular biology of this parasite developmental stage through DNA expression profiles (Quijada *et al.*, 2005) and (d) proteomic characterisations (Walker *et al.*, 2006).

The aim of this chapter is to describe axenic transformation of *L. aethiopica* promastigotes to amastigote-like forms and to validate them as *bona fide* amastigotes through microscopic and biochemical observations. The choice to concentrate on *L. aethiopica* follows the knowledge that axenic growth of *L. major* and *L. tropica* has been previously reported (al-Bashir *et al.*, 1992; Saleheen *et al.*, 2002; Al-Mulla *et al.*, 2005).

4.2. Results and discussion

Medium for *L. aethiopica* axenic amastigote

Two different media were tested for their ability to support *L. aethiopica* amastigote growth *in vitro*: a modification of JH30 (Pan *et al.*, 1983) and UM54 (Balanco *et al.*, 1998). Different temperatures were used and 32°C was chosen for culturing axenic amastigotes (Data not shown). Parasites were cultured under

Species	Medium	Temperature (°C)	Characterization criteria	Reference
L. mexicana	JH-30 +25% FBS	33, 35	$A^{+\dagger}$, B	Pan, 1984
L. mexicana	F-29/UM55	31	$A^{\dagger}$, B, $C^{\dagger}$, $D^{+\dagger}$, $E^{+\dagger}$, $F^{+\dagger}$	Pan, 1993; Rainey *et al*, 1991
L. mexicana	SDM +20% FCS	32	A^{+}, B, $C^{+\dagger}$, $D^{+\dagger}$	Bates *et al*, 1992
L. mexicana	M199 +20% FCS	34	$A^{+\dagger}$, $D^{+\dagger}$	Pral *et al.*, 1993
L. mexicana	SDM +20% FCS	32	$A^{+\dagger}$, B, $D^{+\dagger}$	Bates, 1994
L. donovani	RBLM	35, 37	$C^{\dagger}$	Al Bashir *et al.*, 1992
L. major	RBLM		$C^{\dagger}$	Al Bashir *et al.*, 1992
L. tropica	Biphasic blood agar medium	32, 34		Lemma and Shiller, 1964
L. donovani	100% FCS	37	$A^{\dagger}$, B, D^{+}	Doyle *et al.*, 1991
L. donovani	RPMI +20% FCS	37	$B^{\dagger}$, $F^{\dagger}$	Joshi *et al.*, 1995
L. donovani	RPMI +20% FCS	37	$E^{\dagger}$, $F^{\dagger}$	Zhang *et al.*, 1996
L. donovani	M199 +25% FCS	37	$A^{\dagger}$, B, $C^{\dagger}$, $D^{\dagger}$, $E^{\dagger}$	Saar *et al.*, 1998
L. donovani	TC199 +30% FCS	38	$A^{\dagger}$, B, $C^{\dagger}$, $D^{\dagger}$	Castilla *et al.*, 1995
L. donovani	NNN biphasic	34, 35	$A^{+\dagger}$, $D^{+\dagger}$, $E^{\dagger}$	Gupta *et al.*, 1996, Gupta *et al.*, 1999
L. donovani	RPMI +10% FCS	37	$E^{\dagger}$	Chattopadhyay *et al.*, 1996
L. amazonensis	SDM +20% FCS	32	$A^{\dagger}$, B, $D^{\dagger}$, $E^{\dagger}$, $F^{\dagger}$	Hodgkinson *et al.*, 1996
L. amazonensis	SDM +20% FCS	32	$A^{\dagger}$	Cysne-Finkelestein *et al.*, 1998
L. amazonensis	Grace +20% FCS	33	$D^{\dagger}$, $F^{\dagger}$	Mensa-Wilmot *et al.*, 1999; Liu and Chang, 1994
L. braziliensis	UM55 +20% FCS	34	$A^{\dagger}$, B, $C^{\dagger}$, $D^{\dagger}$, $E^{\dagger}$	Balanco *et al.*, 1998
L. panamensis	SDM +20% FCS	32	$A^{\dagger}$, $C^{\dagger}$, $E^{\dagger}$	Eperon and McMahon-Pratt, 1989
L. braziliensis	SDM +20% FCS	32	$A^{\dagger}$, $C^{\dagger}$, $E^{\dagger}$	Eperon and McMahon-Pratt, 1989
Criteria: **A**, morphology; **B** cyclic transformation; **C**, infectivity; **D**, biochemical analysis, **E**, immunochemistry, **F**, molecular characterization, + Compared with intracellular amastigotes; † compared with promastigotes.				

Table 4.1. *Leishmania* species cultured as axenic amastigotes (modified from Gupta *et al.*, 2001)

exactly the same conditions and at the same time in both media. Samples were taken at regular intervals until complete transformation was reached. Following staining the samples were analysed for morphological characteristics of the parasites in order to detect transformation from promastigotes to amastigotes. Morphologically *L. aethiopica* promastigotes appear fusiform, around 12 (+/-2.5) μm (by 2.25 +/-0.75 μm) long with equally long flagella which terminate with a loop. A relative small, deeply stained kinetoplast is located anterior to the nucleus (Figure 4.1a). Intracellular amastigotes measure 2-5 μm, are oval with a large nucleus (dark red), a kinetoplast (dark red) and a pale blue cytoplasm (Figure 4.1b).

***L. aethiopica*: Comparison of growth between UM54 and JH30**

Following 48 hours incubation at 32°C *Leishmania* in both media showed major changes. Parasites moved closer together in clumps and their body shapes changed to oval/rounded. Although it was impossible to obtain an accurate count of the transforming parasites without risking their survival, by compromising in accuracy an estimation of cell number was obtained during the transformation process and reported in Figure 4.2. Counting of cells was performed as described in Materials and Methods (Chapter 2) and the data obtained confirmed by estimating concentrations following cell fixation and staining of the slides. Briefly, the same amount of sample was transferred in each slide and the cell density compared between slides after staining with Leishman's stain. Samples from JH30 medium showed an increasingly higher number of parasites compared to the number of parasites in UM54 medium from day 5 onwards (Figure 4.2). The big standard deviations reported in Figure 4.2 are due to the necessity of the parasites to stick together during the transformation. Separation of clumped amastigotes was attempted through a 25 gauge syringe but caused a significant increase in cell death, compromising transformation and finally causing the death of all of the culture. The values reported in the Figure 4.2 derive from the counting of small aliquots which were taken from the clumped culture and only then separated as described in Materials and Methods.

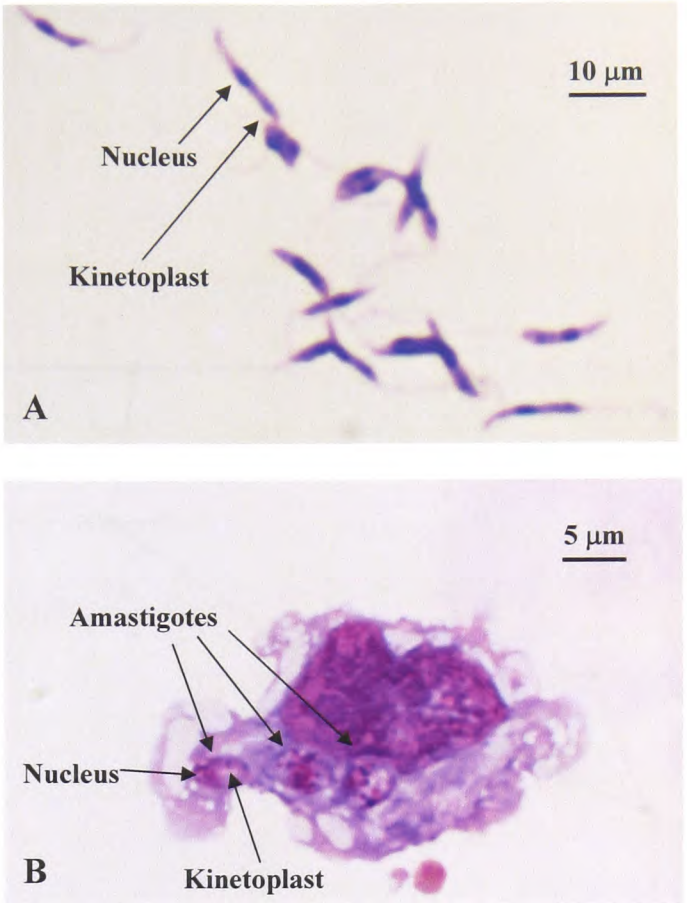


Figure 4.1. *Leishmania aethiopica* promastigotes and intracellular amastigotes. **A**: *Leishmania aethiopica* promastigotes. **B**: Blood derived human macrophage containing 3 *Leishmania aethiopica* amastigote inside the cytoplasm.

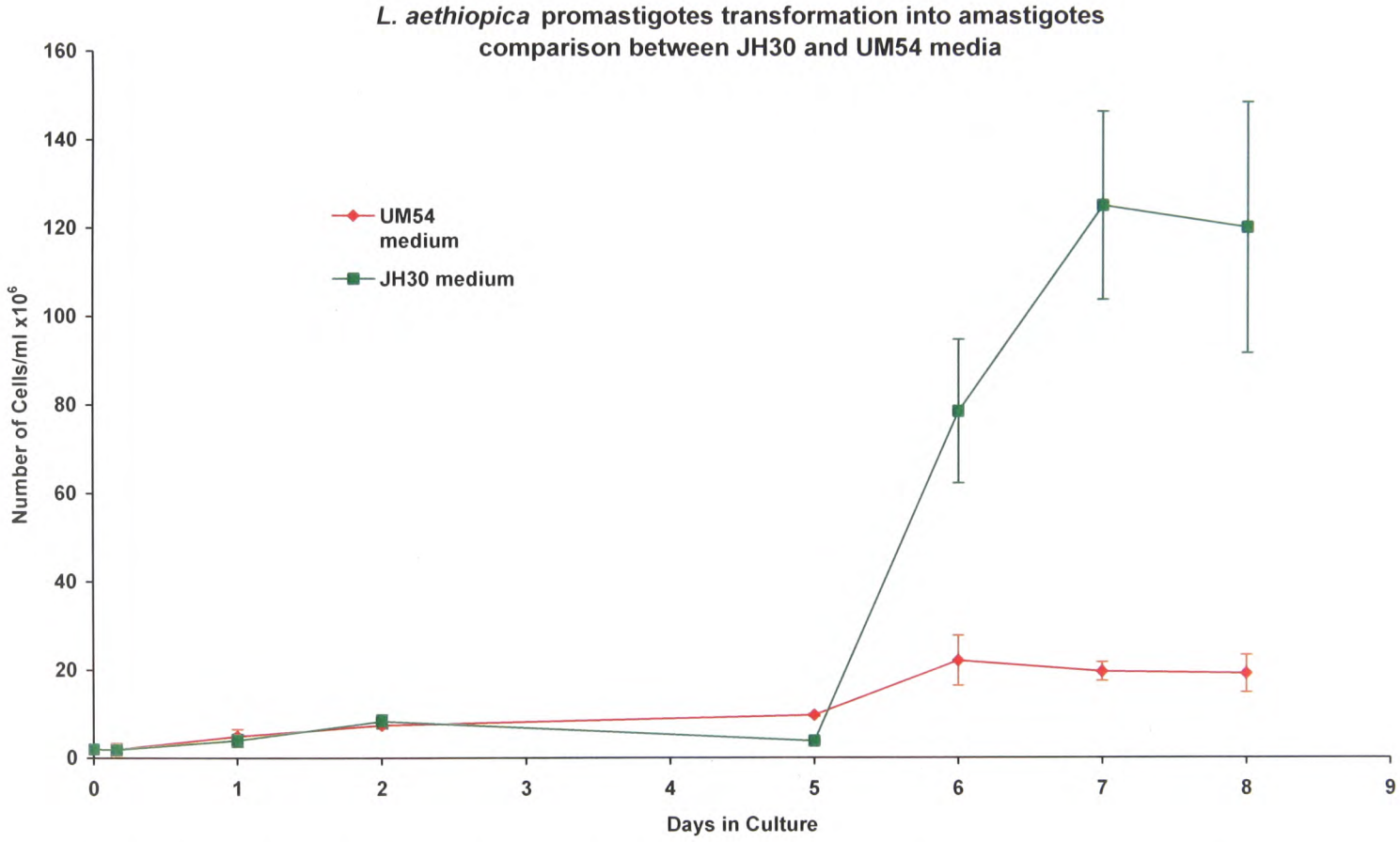


Figure 4.2. *L. aethiopica* promastigote transformation into amastigotes. Samples from each flask were transferred to a 24 well plate and passed through a 25g syringe. Cell number was then counted in both UM54 and JH30 every 24 hours, excluding days 3 and 4, for a total of 8 days. Error bars are standard deviations.

Figure 4.3 shows comparisons of the same populations of promastigotes undergoing differentiation in JH30 (arrow, Figure 4.3a) and UM54 (Figure 4.3b) medium following 4 days of incubation at 32°C. The parasites in JH30 showed cellular division (Figure 4.3a) while the flagella had generally begun to retract. On day 4 the parasites in JH30 and UM54 looked very similar overall, although the cytoplasm of *Leishmania* in UM54 appeared to be slightly more vacuolated.

Figure 4.4 shows the same two populations 2 days later. On day 6, amastigotes had multiplied in JH30 (Figure 4.3a). The bodies were then mostly rounded and the flagella, when present, were very short and the parasites were actively dividing as shown in Figure 4.4 (DA arrow). When cultured for 6 days in UM54 on the other hand, the images showed a different morphology. Cytoplasm was widely vacuolated and the parasites' shapes were oval and, on average, they were 1.3 times bigger in size then the ones in JH30. Flagella were longer than in JH30 and poorer division capability was suggested not only by the size of the transforming parasite but also by the lower number of parasites in the slide obtained from UM54 when compared with the ones from JH30 (Figures 4.2 and 4.4). In some cases multi-nucleated parasites were seen without increases of cytoplasm and size (Figure 4.4b).

Figure 4.5 shows the morphology of the parasites towards the end of the transformation, on day 8. Parasites growing in JH30 showed no flagella and a small rounded shape and they closely resembled intracellular amastigotes. On the other hand the ones growing in UM54 showed a very different morphology to the ones reported for both intracellular amastigotes and axenic amastigotes. Moreover JH30 showed higher parasite density than on day 6 suggesting that the parasites had been dividing (Figure 4.4a). Promastigote-shaped parasites were almost completely absent in JH30 while still frequent in UM54 where they showed long flagella (Figure 4.5b) and sometimes had two nuclei and two kinetoplasts probably ready to undergo cellular division. Rounded parasites in UM54 were heavily vacuolated.

From the observations during the first week of transformation at 32°C, JH30 seemed to represent the optimal medium to support not only the transformation but also the growth of axenic amastigotes and was therefore

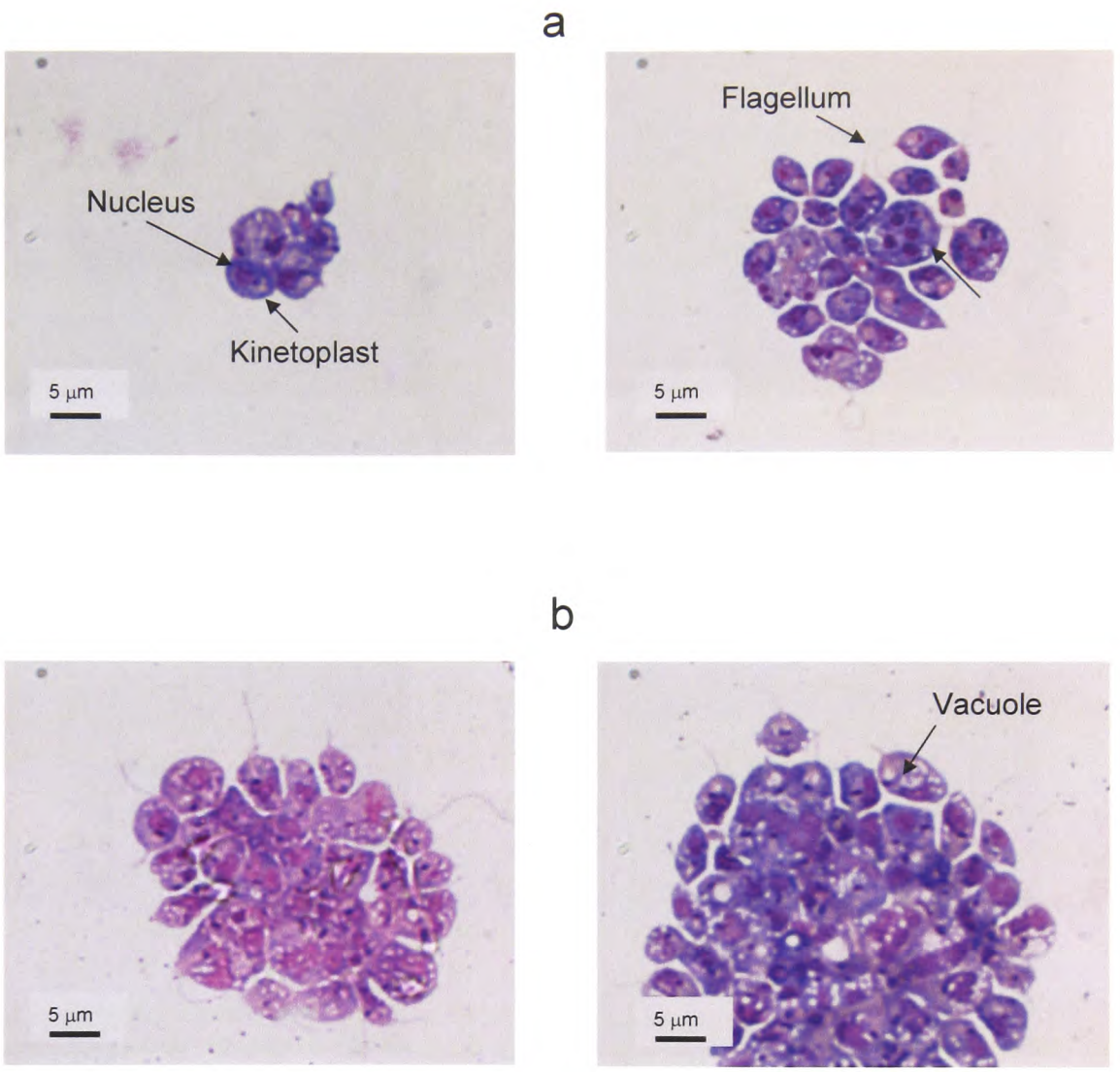


Figure 4.3. *L. aethiopica* transformation to amastigotes, 4 days. a) *Leishmania* in JH30 medium. **b)** *Leishmania* in UM54 medium. Parasites were culture at 32°C, samples were washed in PBS, fixed with 70% methanol and stained with Leishman's stain.

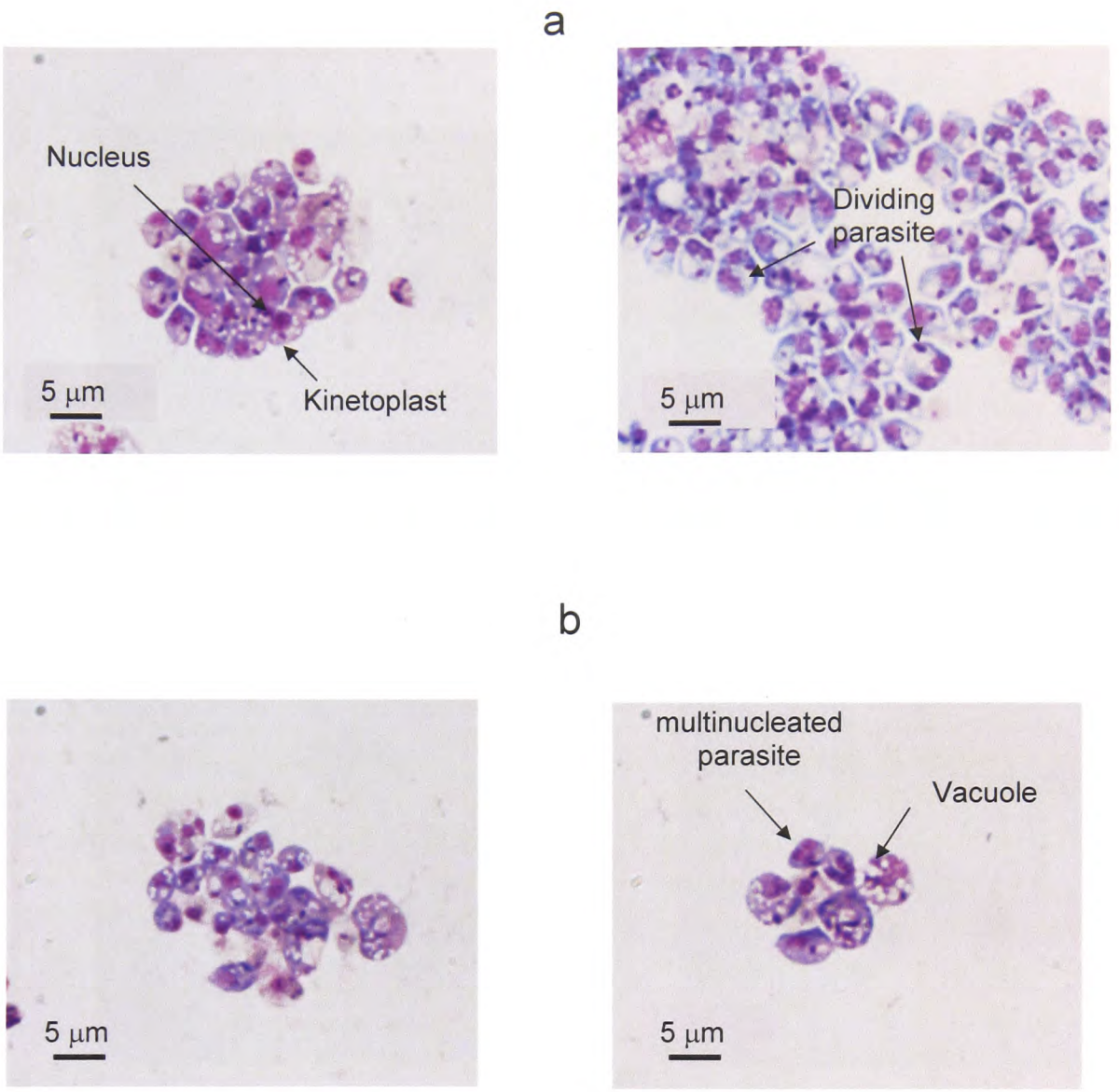


Figure 4.4. *L. aethiopica* transformation from promastigotes to amastigotes after 6 days. **a)** *Leishmania* in JH30 medium. **b)** *Leishmania* in UM54 medium. Parasites were culture at 32°C, samples were washed in PBS, fixed with 70% methanol and stained with Leishman's stain.

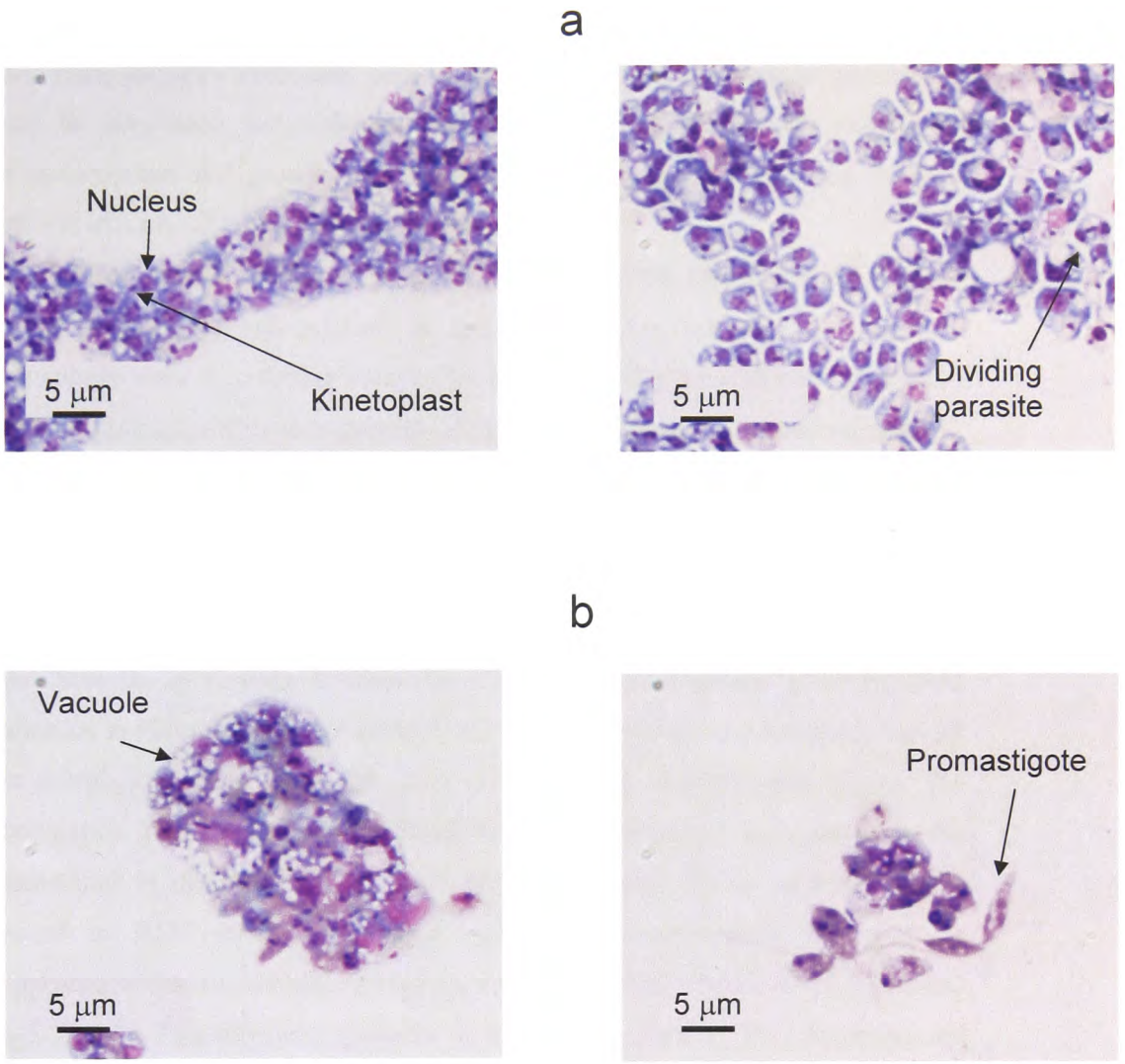


Figure 4.5. *L. aethiopica* transformation from promastigotes to amastigotes after 8 days.
Leishmania in JH30 medium. **b)** *Leishmania* in UM54 medium. Parasites were culture at 32°C, samples were washed in PBS, fixed with 70% methanol and stained with Leishman's stain.

chosen to undertake further analysis. Nevertheless it is important to emphasise that after leaving the parasites in UM54 medium for 2 weeks or longer the surviving parasites eventually became amastigote-like and started to replicate. It can be concluded that although both UM54 and JH30 medium sustain the transformation and growth of *L. aethiopica* parasites, JH30 showed the best results in terms of rapidity of transformation.

The axenic amastigote parasites obtained by the means described above were successfully sub-cultured in cell-free medium for over a year. The organisms were then further assayed for similarity with intracellular amastigotes by morphology, ability to transform into promastigotes and increased infectivity.

L. aethiopica promastigote transformation to amastigotes in JH30 medium. Morphology.

An overview of the transformation process from promastigote (day 0) to day 8 in JH 30 medium is shown in Figure 4.6 and in greater detail by SEM analysis in Figures 4.7 to 4.9. In both views (SEM and light microscopy) changes in morphology were noticeable from the day when transformation began. The population is heterogeneous and differently shaped parasites were present at the same time as described below. The first sample analyzed was collected after 6 hours in JH30 medium. Although still promastigote-shaped, the parasites appeared wider, shorter and maintained a long flagellum (Figure 4.6, 6 hours and 4.7, Day 1). This form was indicated as intermediate form 1 (IF1) and measured between 5 and 10 μm in length and had an increased diameter, with the largest part of the body measuring between 2 and 3 μm. On day 4 IF1 was still present but a new form was observed indicated as intermediate form 2 (IF2) (Figure 4.7). The rounded/oval body has an even bigger diameter of 3 to 5 μm and it generally appeared to be shorter (3 to 6 μm) and started losing the elongated shape towards a more rounded one. The flagella appeared much shorter; at least half the size and in some cases just 20% of the promastigotes' flagella. From day zero to day seven small rounded bodies with long flagella could be

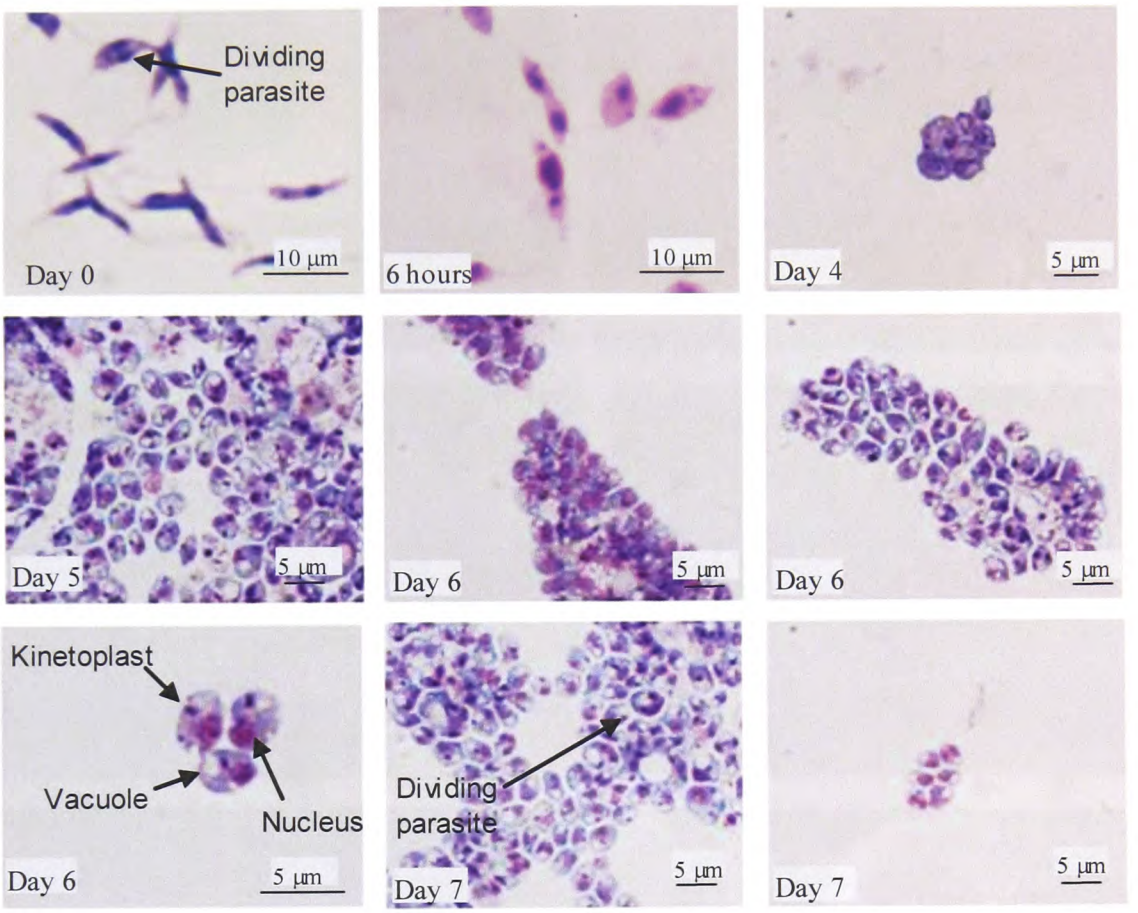


Figure 4.6. *L. aethiopica* transformation from promastigotes to amastigotes during a day period.

Leishmania changes in JH30 medium. Parasites were cultured at 32°C, samples were washed in PBS, fixed with 70% methanol and stained with Leishman's stain.

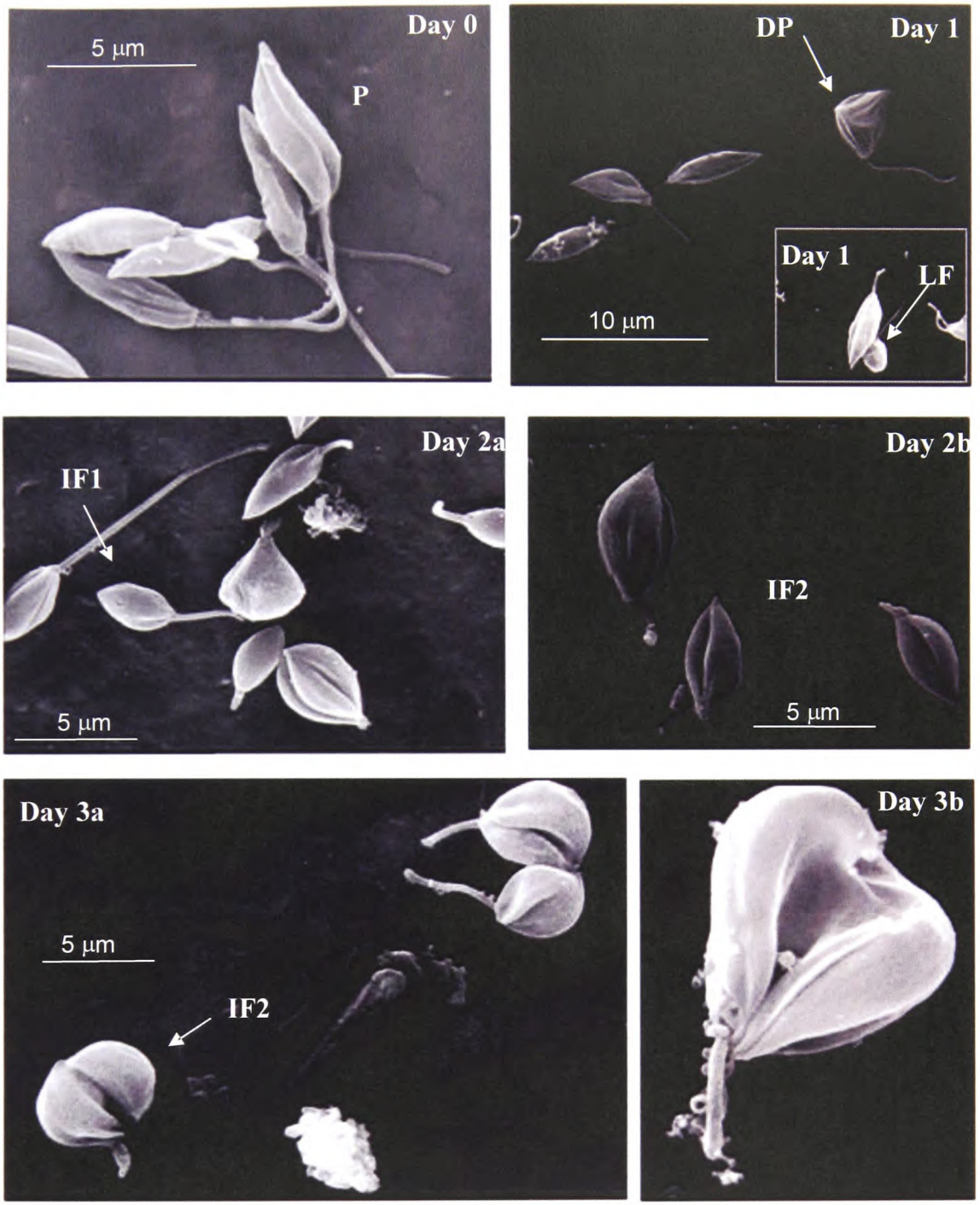


Figure 4.7. SEM pictures of *L. aethiopica* transformation from promastigotes to amastigote during a 7 days period.
DP: dividing parasite; **P**: promastigote-shaped parasites; **IF1**: intermediate form 1, slightly fatter body and shorter flagella; **IF2**: Intermediate form 2, rounded body and even shorter flagella; **LF**: long flagella form, small rounded body and very long flagellum.

found. When observed under an inverted microscope the latter were immotile suggesting that they represented a population of dead parasites, which was confirmed by acridine orange staining (Barreca *et al.*, 1997). Finally, dividing parasites (DP) were observed (Figure 4.7). It was concluded that parasites with rounded bodies and smaller flagella are likely to have successfully started transformation while those with small bodies and long flagella were generally indicative of dead or dying organisms. Parasites also started clumping up together on day one and by day 5 clumps containing a few hundred parasites were present on all the slides. By day 4 most of the parasites had successfully initiated transformation and the flagella had diminished in size and kept becoming smaller and smaller on day 7, when the parasites were also replicating (Dividing parasite, Figure 4.6, day 7), and no flagella were detectable either with light microscopy (Figure 4.6, day 7) or with SEM (Figures 4.8D and 4.9G).

Various stages of the flagella destiny during transformation are shown in Figures 4.8 and 4.9. The flagellum started retracting on day one at the same time as the parasite body started rounding up. Both changes became more evident on days 2 and 3 (Figure 4.7). Particular attention was placed on the observation of the flagella which seemed to increasingly retract into the flagellar pocket (Figures 4.9 A, B, C and D).

***L. aethiopica* promastigote transformation to amastigotes in JH30 medium. Quantification of morphological differences.**

Differences within a transforming population such as the number of parasites that lose promastigote features and start transforming into amastigotes were quantified. The total number of parasites was reported compared with the increasing number of amastigotes. These included cells that were at various stages of the transformation process (IF2 and AA) (Figures 4.10 and 4.11). The transformation process varied depending on the number of times the population of promastigotes had undergone the *in vitro* transformation process. The initial transformation process required 7 days of adaptation into the new living condition at a starting concentration of $1x10^6$ cells/ml. Although transforming parasites were present from the first day, as described in the next paragraph, the

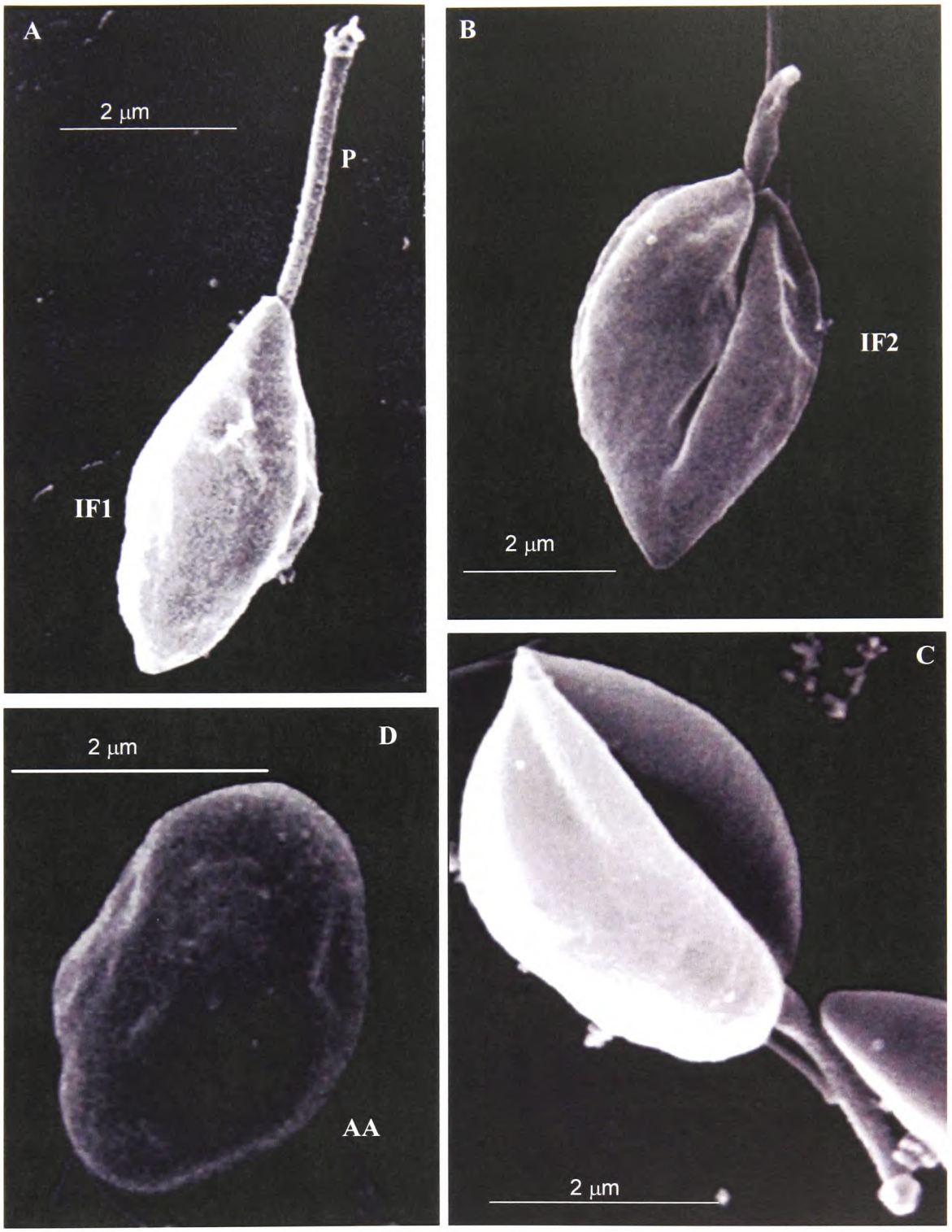


Figure 4.8. SEM pictures of *L. aethiopica* transformation from promastigotes to amastigotes, view of single parasites.
A. IF1: intermediate form 1, bigger body and shorter flagellum when compared to promastigotes; **B. & C. IF2:** rounded body and even shorter flagellum; **D. AA:** axenic amastigote, no flagellum arising from the flagellar pocket is visible.

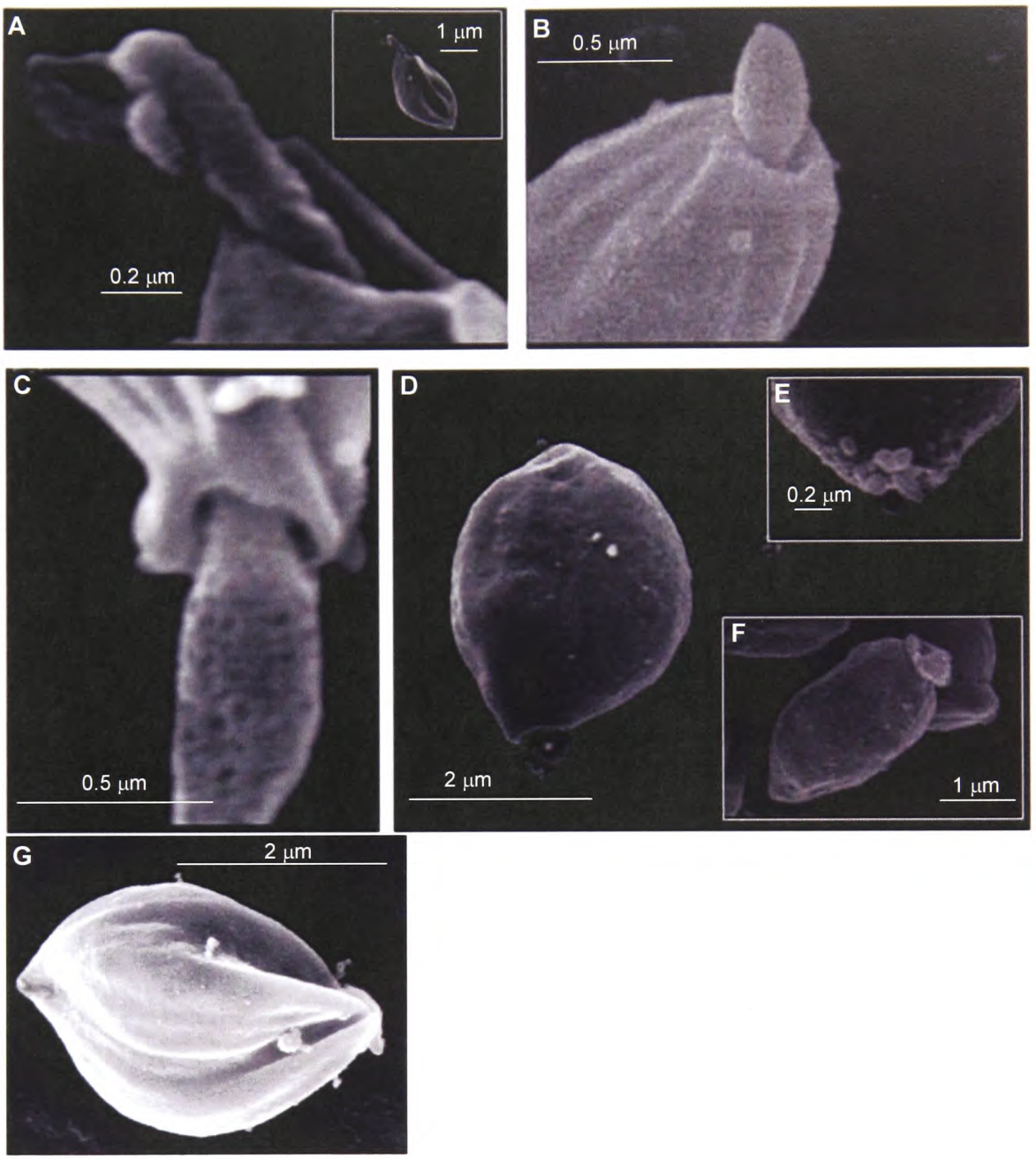


Figure 4.9. SEM pictures of *L. aethiopica* promastigotes transformation in amastigotes, view of the flagellum.
A closer view of the effect of transformation of the *Leishmania* flagellum is given in this set of pictures. During transformation the flagellum slowly disappears inside the flagellar pocket (**A**, **B** and **C**) until just 0.1 μm are visible (**D**, **E** and **F**). At the end of the transformation the amastigote appears rounded and no flagellum is visible (**G**).

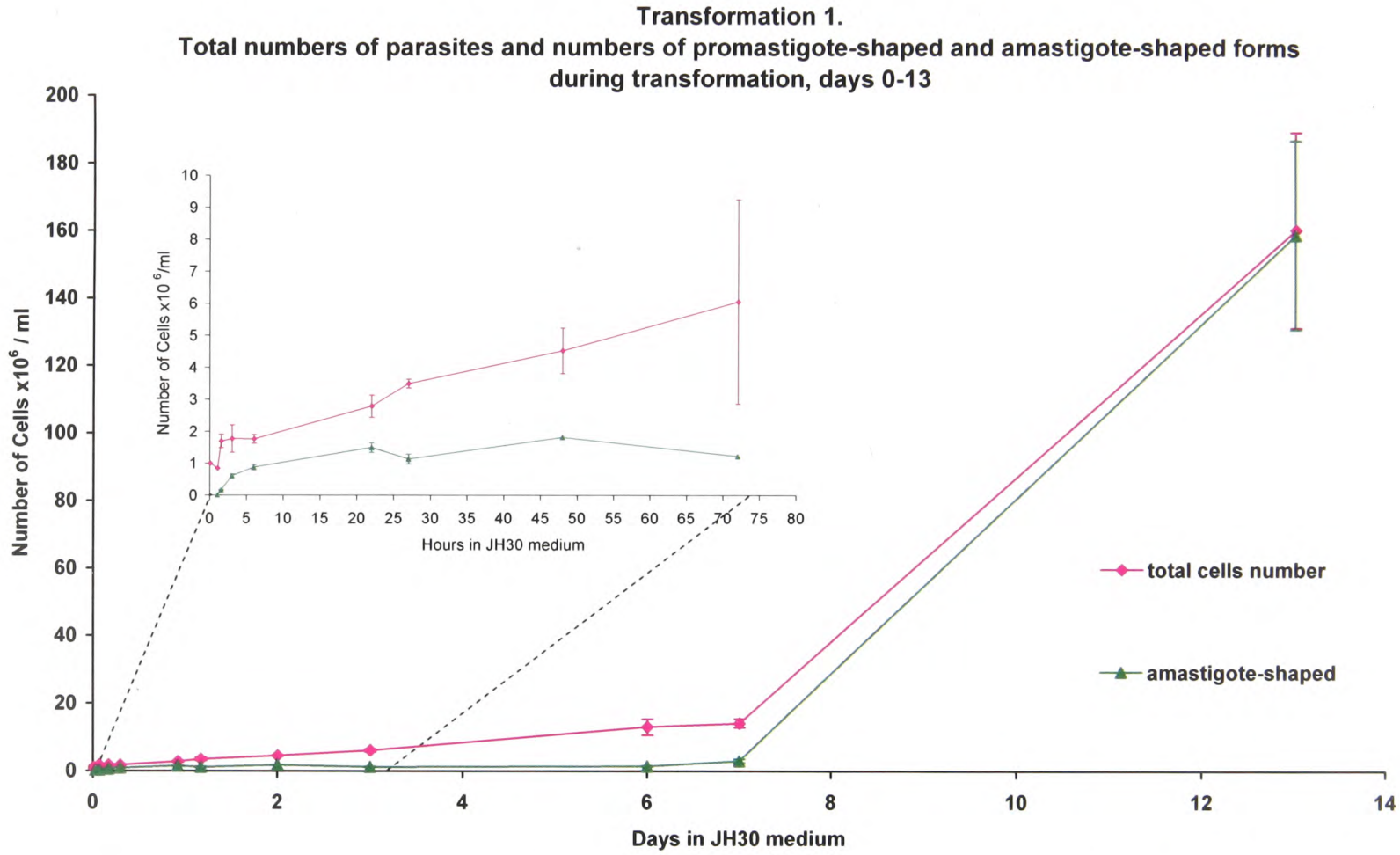


Figure 4.10. *L. aethiopica* promastigotes transformation to amastigotes. First transformation during a 13 days. period. Parasites were cultured in JH30 medium at 32ºC. The number of total parasites as well as the number of parasites in amastigotes shape (including IF2 and AA) during transformation was counted. A view of the samples were taken after 30 min, 1 hour, 1 hour and 30 min, 3, 22, 27, 48 and 72 hours is shown on the smaller graph.

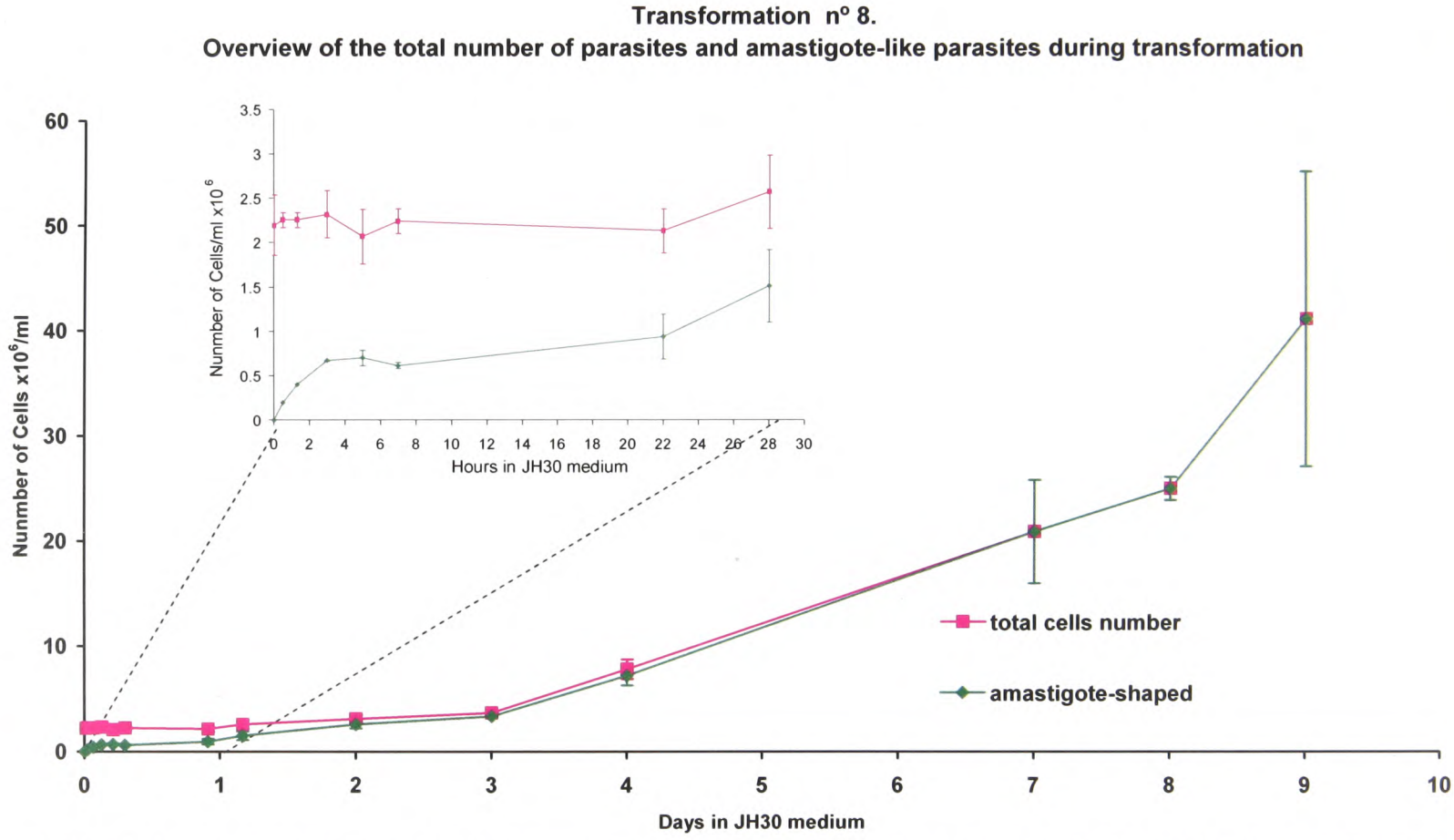


Figure 4.11. 8 times revertant *L. aethiopica* promastigotes transformation to amastigotes during a 9 days period. . Parasites were cultured in JH30 medium at 32ºC. The total numbers and the numbers of amastigote-shaped (IF2 and AA) parasites during transformation in a 9 day period (larger graph) and during the first 28 hours (smaller graph). The error bars are standard deviations.

population started to replicate and amastigotes started replacing promastigotes only on day 7 (Figure 4.10).

A closer view of the first steps of differentiation is shown in the smaller graph in Figure 4.10. In the first 30 minutes to 1 hour into transformation the promastigote numbers increased and at the same time some parasites started to differentiate. Three hours into the differentiation process the number of promastigotes decreased and concomitantly the number of transforming amastigotes increased. For the next 22 hours both promastigotes and amastigotes remained more or less the same. Promastigote numbers increased slowly until day 6 while amastigotes remained constant. On day 7 onwards promastigotes slowly disappeared and amastigotes started doubling every 24 hours (amastigote concentration increased from $2.96x10^6$ to $1,60x10^8$) until day 13 when only amastigotes were present in the medium.

Following 8 cycles of transformation into amastigotes and reversion to promastigotes the pattern of differentiation changed and the loss of promastigote-shaped parasites was achieved in a shorter period corresponding to 7 days. As described in Figure 4.11 (small graph), in this population the transformation of promastigotes into amastigotes started to be evident on day 1, after 22 hours, when at least half of the parasites had lost the features typical of promastigotes. Following 28 hours in JH30 medium, the number of amastigote-shaped parasites was higher than the number of promastigotes and kept increasing, while promastigotes decreased, until day 7. Between day 7 and day 8 only amastigotes could be visualized in the sample. Finally, between day 8 and day 9 the amastigotes started replicating with their concentration doubling in 24 hours. The same doubling time was reported for amastigotes in the first transformation. The concentration of amastigotes was also the same whether the starting population had previously been cultured in JH30 or not. Two main differences in the transformations were found: a) between day 2 and 7, during which the previously reverted parasites showed a constant increase in the number of amastigotes while transformation 1 had a very high concentration of promastigotes and b) in the later stage of transformation promastigote-shaped parasites were present up to day 13 in transformation 1 and only up to day 7 in transformation 8.

From these data we can deduce that the transformation process starts on day 7 when the parasites are in JH30 medium for the first time, while it starts on day 1 when the population has adapted to JH30 by 8 cycles of reversion. Overall 9 to 13 days are necessary to obtain a complete transformation of promastigotes into axenic amastigotes.

L. aethiopica axenic amastigote growth curve

L. aethiopica axenic amastigotes were sub-cultured in JH30 medium for at least 8 weeks before a growth curve was generated. A very high starting concentration (25 x 10^6) was chosen (Figure 4.12). This choice derived from the high density of the parasites at the end of the transformation process and from the fact that axenic amastigotes need to grow closely aggregated in clumps in order to survive. Probably because of their high concentration, the amastigotes immediately entered the logarithmic phase of growth and doubled in the first 24 hours. On day one they reached the stationary phase with a concentration of 60 (+/-10) x 10^7. The culture stayed in the stationary phase until day 3 after which it started declined. Noticeably the highest concentration reached by the axenic amastigote culture was almost 3 times lower than the one reported during transformation (Figure 4.2).

These data together with the microscopical analysis of the parasites showing that the parasite decreases in size during transformation suggest a new interpretation of the transformation process. During transformation promastigotes and/or IF1 and/or IF2 do not just duplicate but divide into 4, thereby reducing in size but increasing the total cell number. Moreover, if the doubling time of the amastigotes in the growth curve (Figure 4.12) is compared with the doubling time of the parasites transforming in JH30 medium, when cultured at a starting concentration of 1 x 10^6 (Figure 4.2), the difference is striking. In the latter, between days 5 and 6 the number of parasites rose from 5 to 80 x 10^6 cells/ml, corresponding to a doubling time of 5 hours, while the doubling time in the log phase of the growth curve was 24 hours. This speculation is purely based on the fast dividing time of the transforming parasite and needs further testing.

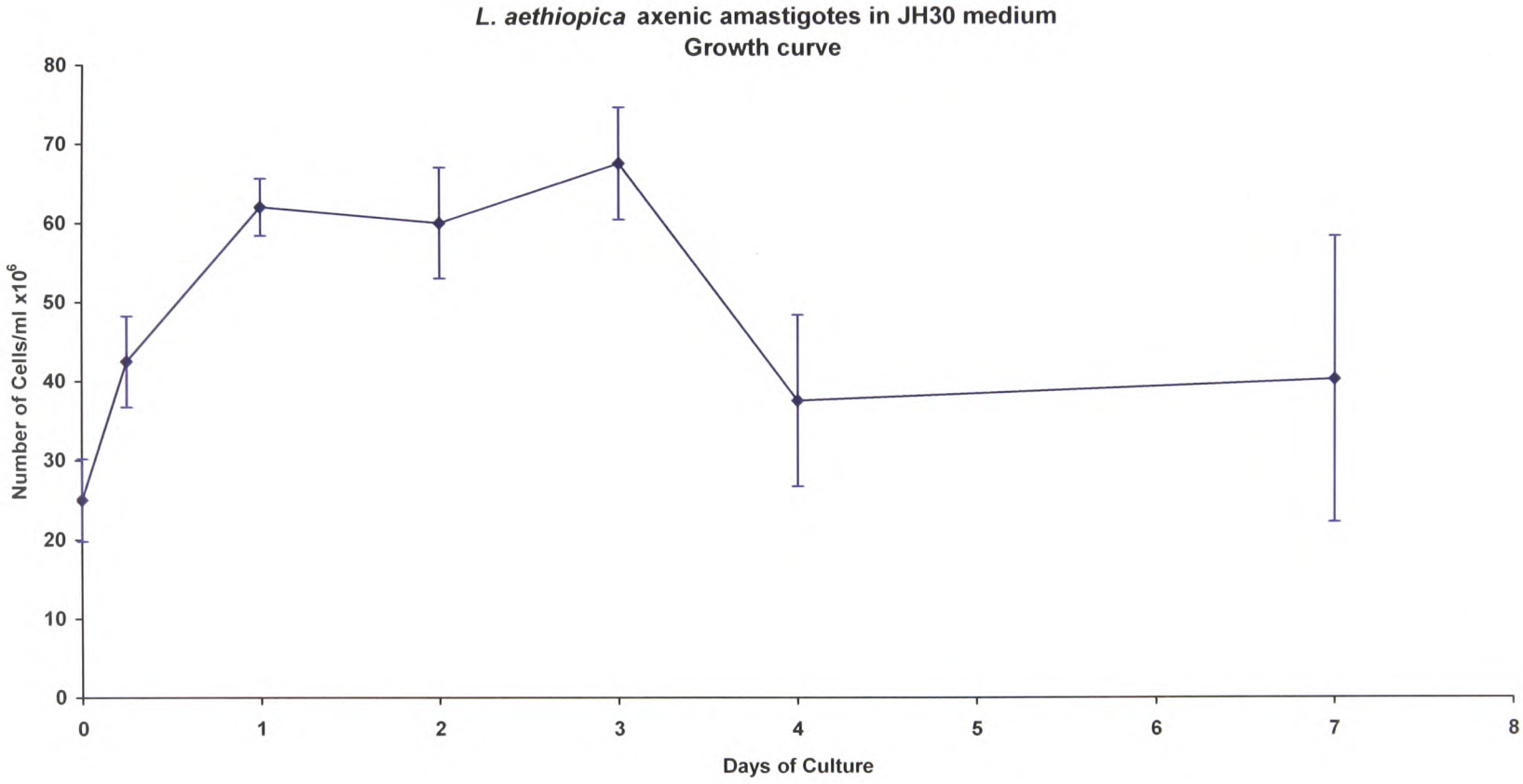


Figure 4.12. Growth curve of *L. aethiopica* axenic amastigote in JH30 medium. Cells were counted and re-suspended in JH30 medium at a starting concentration of 25 x 10^6/ml. The cultures were maintained at 32°C and counted for 7 days. The cultures were set up in triplicates. Each point represent a mean value from the three cultures +/- standard deviation.

Amastigote / promastigote reversion

Axenic amastigotes are expected to be able to revert back to promastigotes on transfer to promastigote growth conditions in the same way that intracellular amastigotes can (Pan *et al.*, 1993; Balanco *et al.*, 1998; Hodgkinson *et al.*, 1996). *L. aethiopica* axenic amastigotes grown in JH30 for up to 2 weeks were successfully reverted back to promastigotes. The same population was then cyclically transformed into amastigotes and reverted back for a total of 8 times. The reversion process was completed in 5 days and the promastigotes obtained were able to replicate and reach the stationary phase.

Amastigote / promastigote infectivity

Both stationary phase promastigotes and axenic amastigotes were used to infect terminally differentiated THP-1 cells. Infection was established after THP-1 cells were transformed into non-adherent macrophages as described in Materials and Methods (Chapter 2). Transformed THP-1 cells were infected with stationary phase promastigotes and axenic amastigotes of *L. aethiopica* at a ratio of 10:1. After 48 hours at 37°C and 5% CO_2 in a humidified incubator samples were taken; non-adherent cells were fixed on clean slides and stained with Giemsa as described in Materials and Methods. Each data set came from the average of three experiments each of which was done in triplicate. A minimum of 100 macrophages was counted for each slide.

As expected (Gupta *et al.*, 2001), amastigotes showed a significantly higher infectivity ($P<0.05$) than the promastigotes (Figure 4.13) with 50% infected cells when incubated with axenic amastigotes and 30% infected cells when incubated with stationary phase promastigotes 24 hours following infection.

***L. tropica* and *L. major* axenic amastigote transformation.**

Because of the formulation of RBLM and of biphasic blood agar medium both of which make necessary a continuous supply of blood, cultivation of axenic amastigotes of both *L tropica* and *L. major* was attempted in JH-30 and UM-54 media, as described in Materials and Methods (Chapter 2). Both media showed

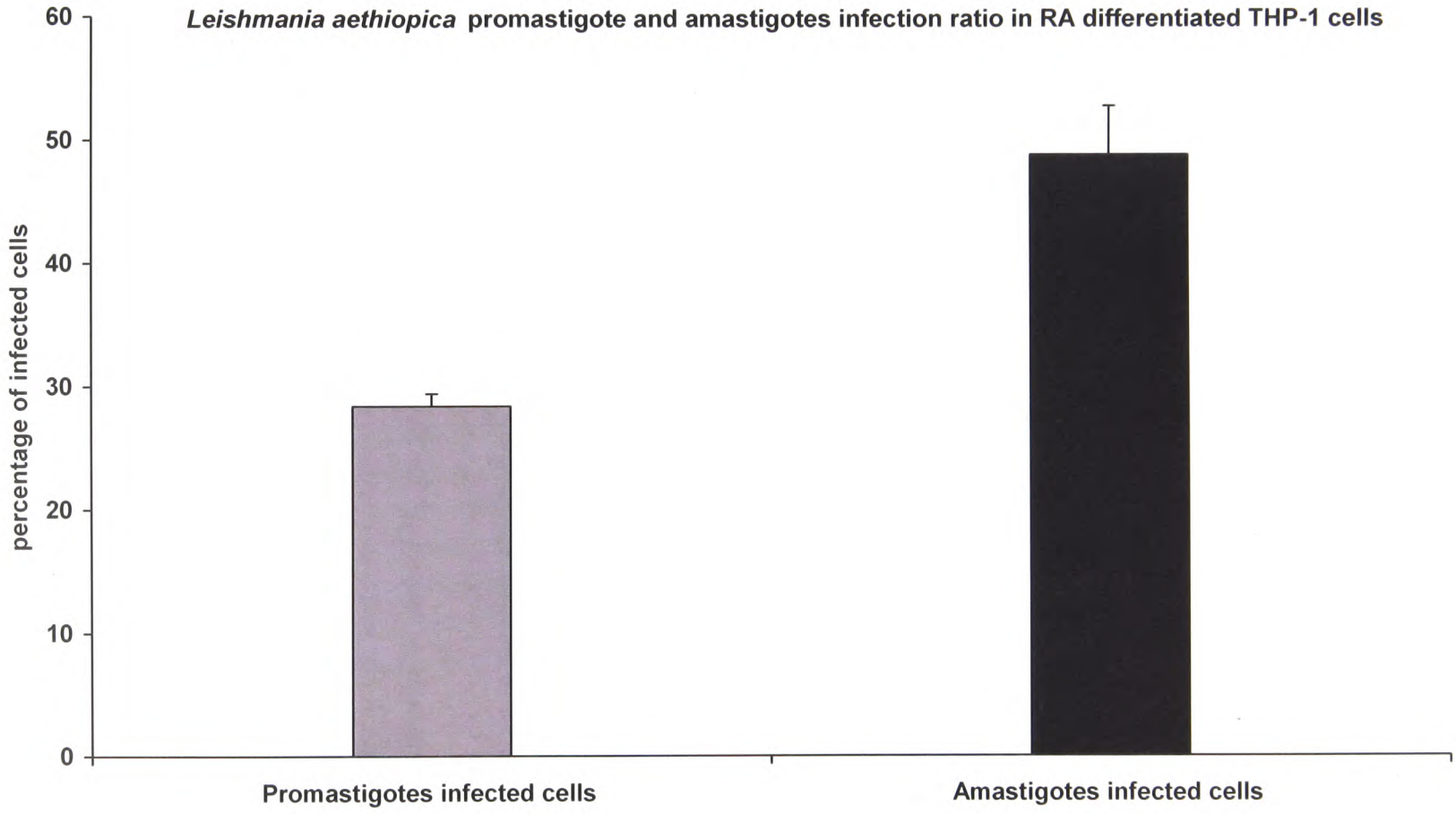


Figure 4.13. Amastigote/promastigote infectivity. Percentage of infected THP-1 cells following 24 hours incubation at 37°C with 10 to 1 *L. aethiopica* promastigotes and axenic amastigotes. The axenic amastigotes were obtained following 8 reversions inJH30 medium. Error bars are standard deviations.

similar outcomes. A small percentage (around 30%) of *L. tropica* and *L. major* started transforming on day two, the parasites started losing their flagella, becoming rounded, while most of the parasites did not transform at all. After a week of incubation the parasites were dead as confirmed by failed reversion into promastigotes.

4.3. Conclusions

L. aethiopica was cultured for the first time as axenic amastigotes in JH30 medium. Parasite characteristics such as morphology, capability to revert into the promastigote stage and infectivity were analysed. All the assays confirmed that promastigotes transform into axenic amastigotes when cultured in JH30 medium. The transformation involved changes in dimension and shape of the parasite as well as the loss of the flagella which is a typical feature of the promastigotes. Moreover, the axenic amastigotes' infectivity was higher than corresponding promastigotes and they were able to revert into promastigotes. Both characteristics confirmed that the axenically grown amastigotes are similar to the intracellular ones (Gupta *et al.*, 2001).

Differences in the transformation process were reported when 8 times revertants were analysed. The significance of these data can only be speculated on; *Leishmania* could retain the ability to activate the genes necessary to survive in the new environment by storing mRNA ready to be transcribed or by storing the previously produced proteins.

Finally, *L. tropica* and *L. major* could not be induced to transform into amastigotes in either JH30 or UM54 medium. These data confirmed the existence of differences between various *Leishmania* species and that JH30 medium exclusively supported growth of certain parasite species such as *L. aethiopica* and *L. mexicana* (Pan, 1984).

Chapter 5

The effect of cicerfuran, related arylbenzofurans and stilbenes on *Leishmania* parasites

5.1. Introduction

Leishmaniasis is a widespread disease whose characteristics have been described in Chapter one. The disease has a worldwide distribution and 1.5-2 million new cases of leishmaniasis occur annually (WHO, 2002). This condition has now being recognized as one of the three 'neglected diseases' by the 'Drugs for Neglected Disease Initiative (DNDi)'; consequently new emphasis has recently been placed on research orientated to discovery of new therapies. Leishmaniasis is caused by many species of the *Leishmania* protozoans, which vary in their virulence and biochemistry and are sensitive to different compounds (Faraut-Gambarelli *et al.*, 1997; Escobar *et al.*, 2002; Croft *et al.*, 2006). The clinical manifestations of the disease depend on the species initiating infection and also on the general health and genetic make-up of the infected individual (Grimaldi and Tesh, 1993).

Current pharmaceutical treatments include sodium stibugluconate and meglumine antimonite, pentamidine and amphotericin B. However, the toxicity, side effects, and expense of these drugs plus the development of resistance against them necessitate the identification and development of new therapies (Berman, 2003). There has been much research on the therapeutic value of natural products, mainly plant extracts used to treat *Leishmania* in traditional medicine and several natural products have been tested against *Leishmania* (Chan-Bacab and Peña-Rodríguez, 2001). Although a number of these natural products have demonstrated potential as leishmanicidal agents such as luteoline and hispidulin (Mittra *et al.*, 2000; Sülsen *et al.*, 2007), most failed to meet all of the requirements needed (possibility of oral or topical administration, moderate dose effectiveness, lack of severe side effects), mainly because of their high cytotoxicity (Tournaire *et al.*, 1996). Consequently, new potential leishmaniasis therapies are urgently needed.

Sources of metabolites with potential antileishmanial activity can be identified in plants which are used in indigenous populations for the treatment of the disease or in compounds that have already been identified for antibacterial and antifungal activity. An example of the second case is given by natural stilbenes and their synthetic analogues. These compounds are known to have anti-inflammatory and anti-bacterial activity (Li *et al.*, 2003), as well as potential anti-cancer activities (Waffo-Teguo *et al.*, 2001; Kinghorn *et al.*, 2004) but their anti-parasitic activities have not been well studied. The only compounds tested in *Leishmania* to date are semisynthetic dihydrostilbenes, combretastatin and some heteroanalogues, in both of which groups activity has been reported (del Rey *et al.*, 1999; del Olmo *et al.*, 2001). The antibacterial and antifungal activity of cicerfuran and related compounds, as well as the leishmancidal activity of structurally related stilbenes suggested the possibility that these compounds might have activity against *Leishmania.*

The aim of the study described in this chapter was to determine the leishmanicidal activity of cicerfuran and related compounds on *in vitro* models including promastigote and amastigote stages of the parasite. Furthermore, the ability to interfere with the spread of the infection and to significantly reduce the infection ratio was evaluated. Infected macrophages were treated and the effect

of the compounds on infection was established by comparing the infection rates with untreated cells.

5.2. Results and discussion

The effects of different concentrations of various compounds on the promastigotes of three species of *Leishmania* were shown after 48 hours treatment. For each species the number of cells expressed as percentage of the untreated control was reported against drug concentrations (Figures 5.1 to 5.3). Statistical analysis of these values was performed by probit analysis with logit transformations (Finney, 1971; Finney, 1978) and produced LD_{50} which represent lethal doses of the compounds for 50% of a given population, these values were used as indices of leishmanicidal activity. Stilbenes showed the highest activity with LD_{50} values lower than 10 μg/ml. Stilbenes possessing hydroxyl groups are already active after 6 hours at the second highest concentration tested (50 μg/ml) (Figure 5.4). Cicerfuran is particularly active against *L. tropica* and it is generally more active than its analogues. The compounds showing potential as leishmanicidal agents were then tested for their toxicity *in vitro* towards cultured THP-1 cells (Figure 5.5) and *L. aethiopica* axenic amastigotes (Figure 5.6).

The leishmanicidal activities of ten compounds tested against promastigotes are shown in terms cell numbers surviving as LD_{50} in Table 5.1. Eight out of ten compounds were active against *L. aethiopica* parasites at concentrations lower then 50 μg/ml. *L. aethiopica* was more sensitive to the action of the compounds than *L. major* and *L. tropica*, with the exception of C9, Although *L. aethiopica* was the most sensitive to the compounds, similar results were obtained for *L. tropica* and *L. major*. Six compounds were active against *L. major* (causing the death of at least 30% of the population) while 5 were active against *L. tropica*. A stilbene (C5) showed the highest relative potency against the promastigotes of all three species (Figure 5.7). The relative median potency

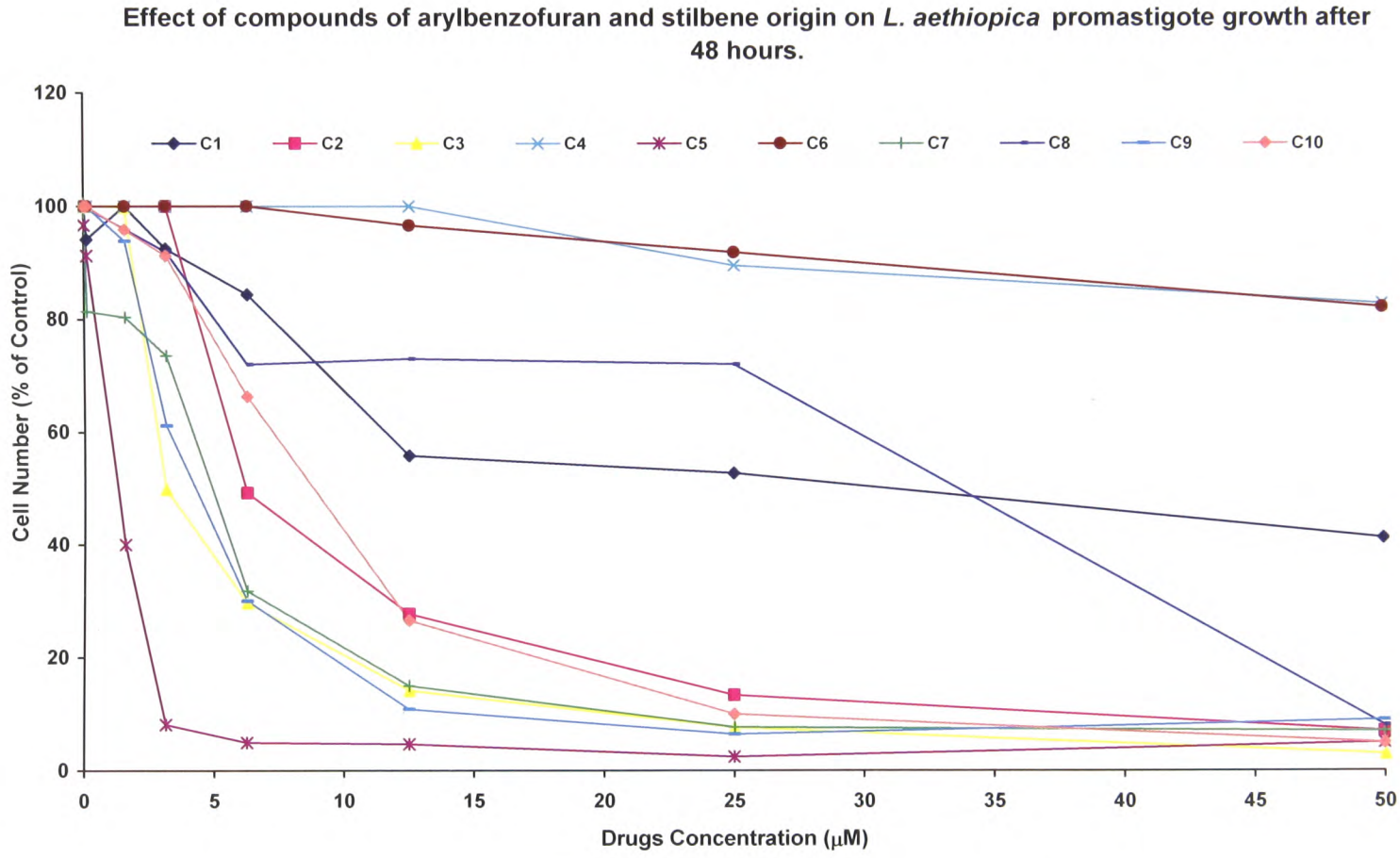


Figure 5.1: Effect of compounds of arylbenzofuran and stilbene origin on *Leishmania aethiopica* promastigote growth after 48 hours. A population of logarithmic phase promastigotes was treated with ten different compounds for 48 hours. The number of cells counted in the treated wells was reported on the Y axis as percentage of total cells, where the total cells number was represented by the number of cells left untreated and grown in 0.05% w/v of DMSO final concentration. These numbers represent the action of the drugs on the cells' ability to survive and replicate and therefore, the drugs activity.

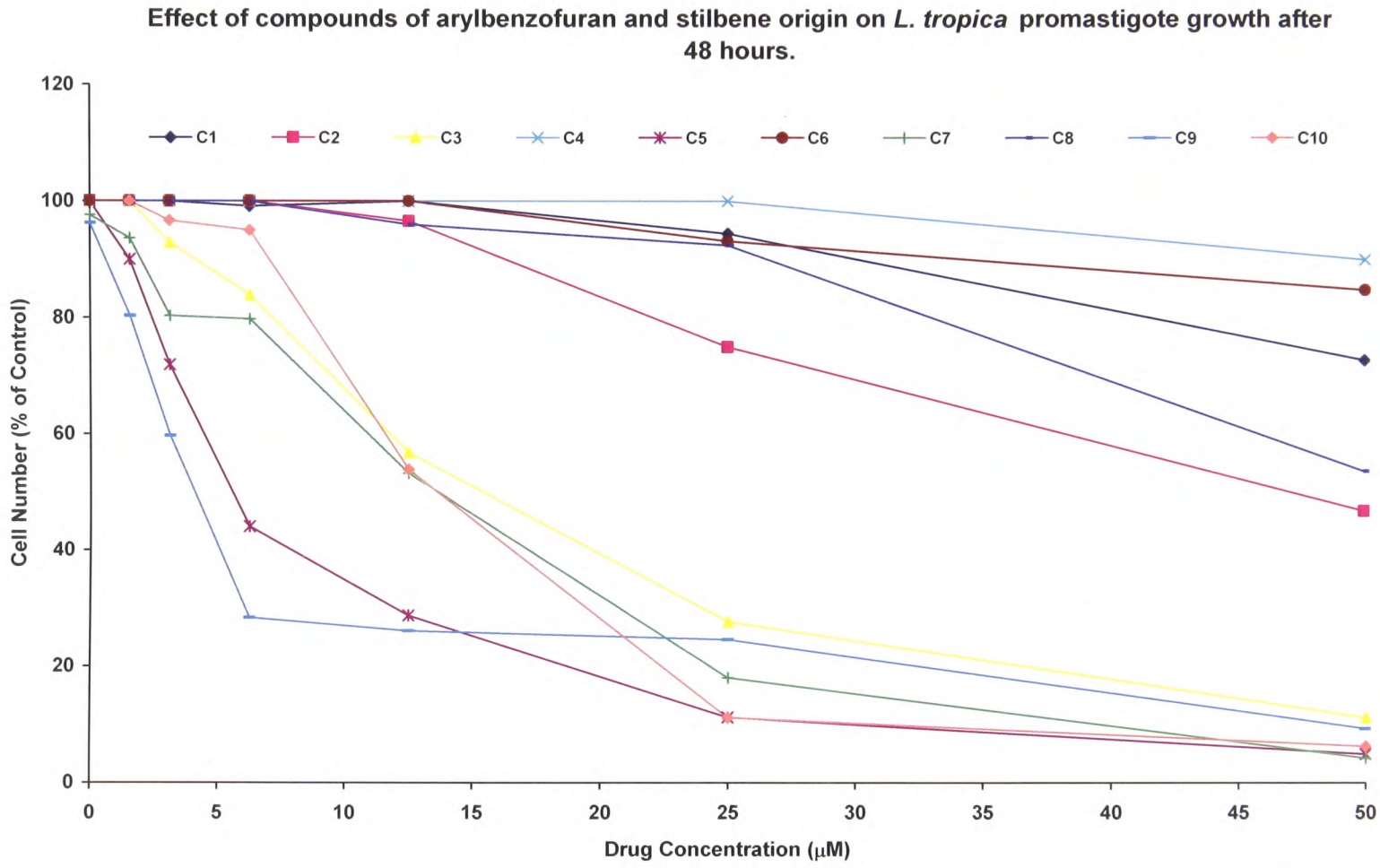


Figure 5.2: Effect of compounds of arylbenzofuran and stilbene origin on *Leishmania tropica* promastigote growth after 48 hours. A population of logarithmic phase promastigotes was treated with ten different compounds for 48 hours. The number of cells counted in the treated wells was reported on the Y axis as percentage of total cells, where the total cell number was represented by the number of cells left untreated and grown in 0.05% w/v of DMSO final concentration. These numbers represent the action of the drugs on the cells' ability to survive and replicate and therefore, the drugs activity.

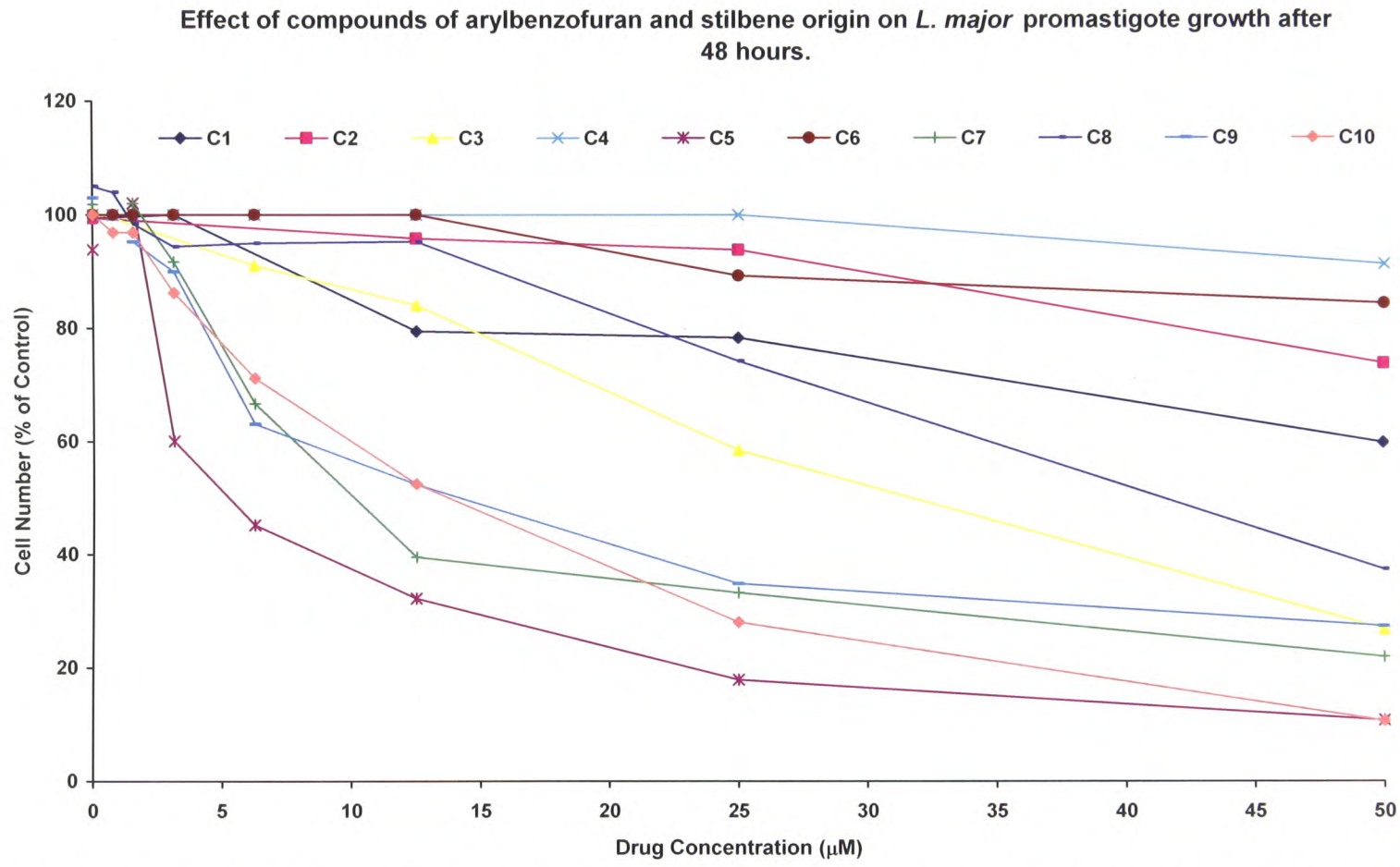


Figure 5.3: Effect of compounds of arylbenzofuran and stilbene origin on *Leishmania major* promastigote growth after 48 hours. A population of logarithmic phase promastigotes was treated with ten different compounds for 48 hours. The number of cells counted in the treated wells was reported on the Y axis as percentage of total cells, where the total cells number was represented by the number of cells left untreated and grown in 0.05% w/v of DMSO final concentration. These numbers represent the action of the drugs on the cells' ability to survive and replicate

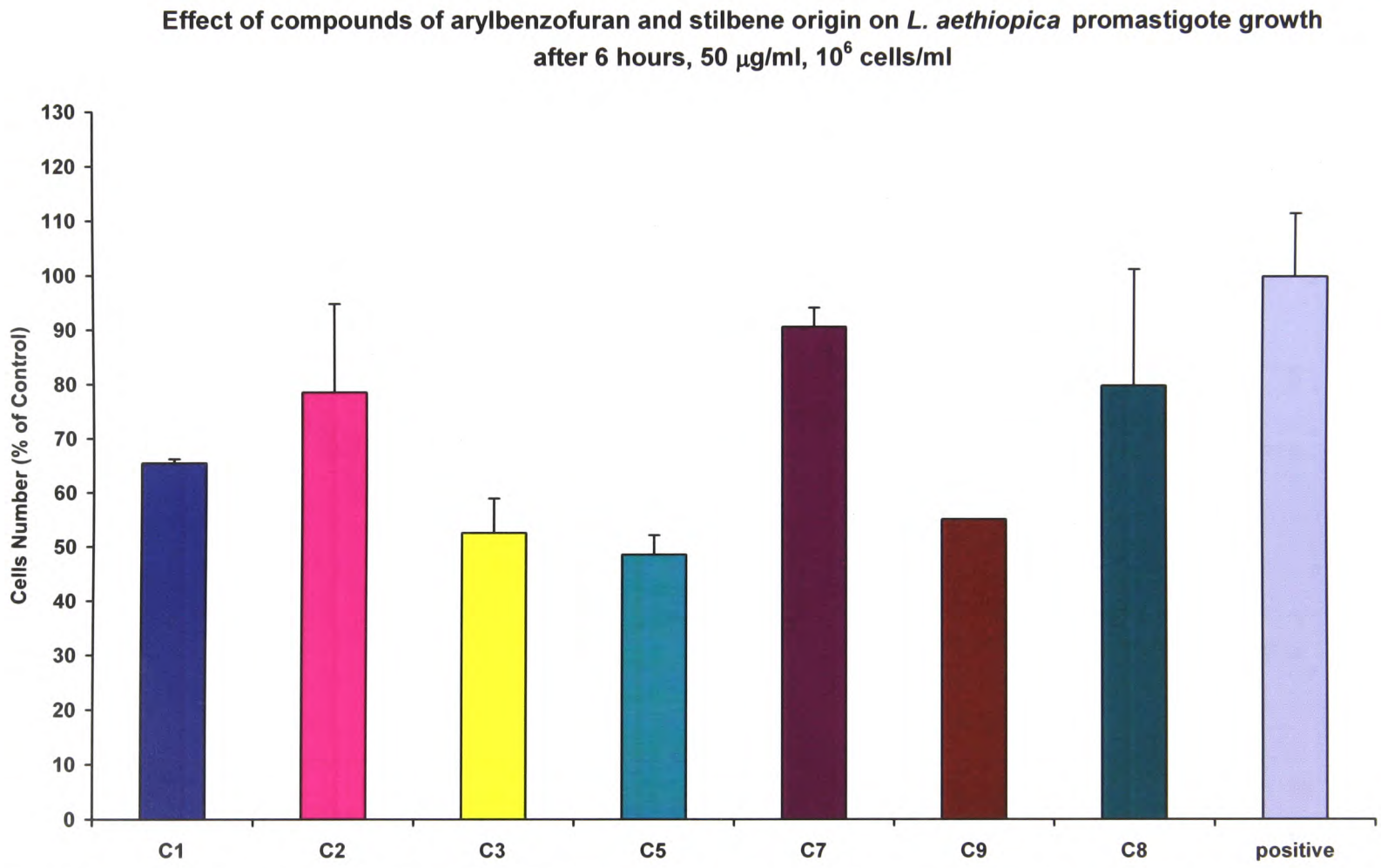


Figure 5.4. Effect of stilbenoids on *L. aethiopica* promastigotes after 6 hours treatment at a concentration of 50μg/ml. Stationary phase parasites were plated at a concentration of 10^6 cells/ml and treated for 6 hours with 50μg/ml of each drug. The cell number is reported as percentage of positive control +/- standard deviation. The positive control represent untreated cells cultured in DMEM/F12 medium in 0.05% w/v of DMSO final concentration.

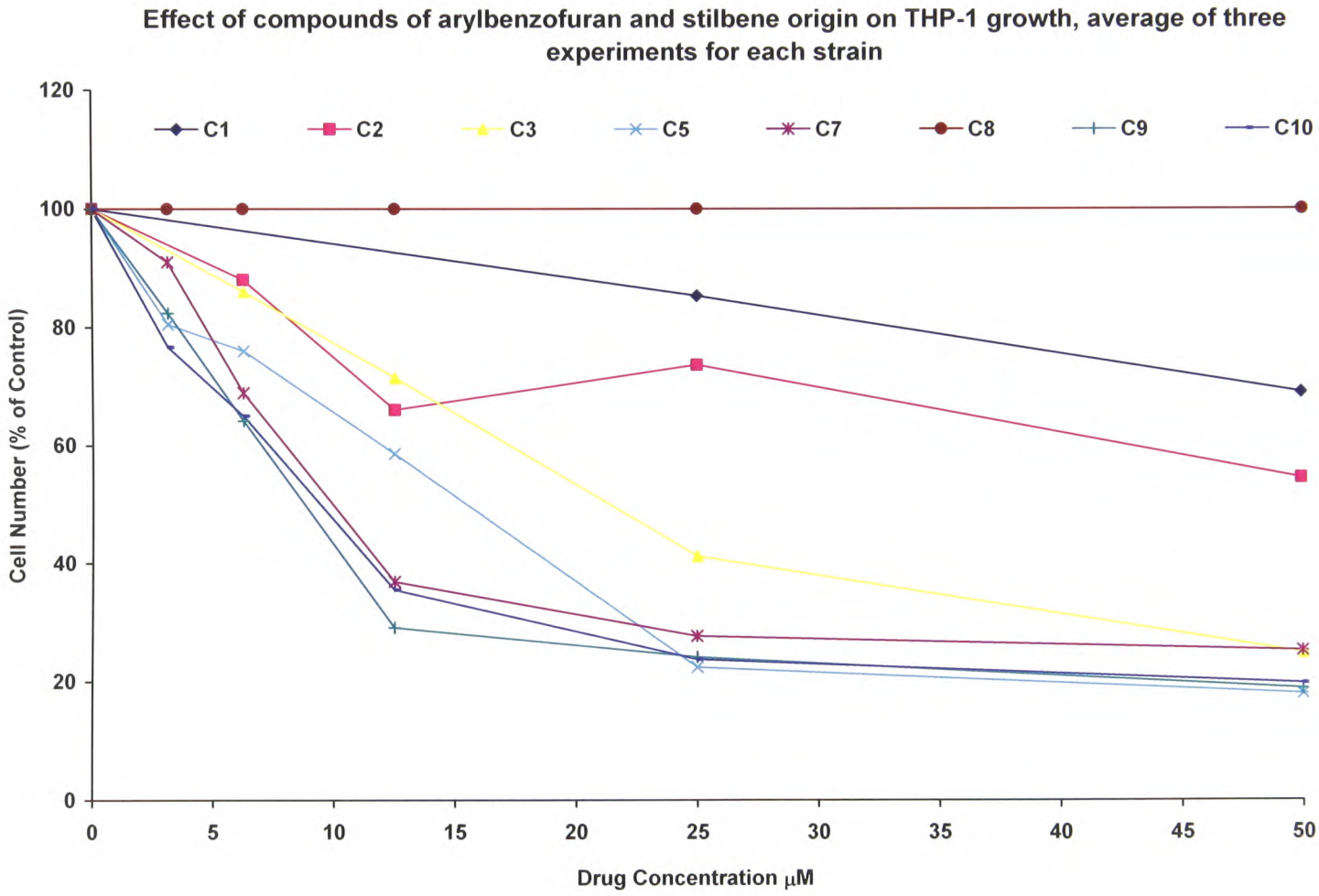


Figure 5.5 Effect of compounds of arylbenzofuran and stilbene origin on THP-1 growth, average of three experiments for each strain. A population of 106 THP-1 cells /ml was treated with ten different compounds for 48 hours. The number of cells counted in the treated wells was reported on the Y axis as percentage of total cells, where the total cell number was represented by the number of cells left untreated and grown in 0.05% w/v of DMSO final concentration. These numbers represent the action of the drugs on the cells' ability to survive and replicate and therefore, the drugs activity.

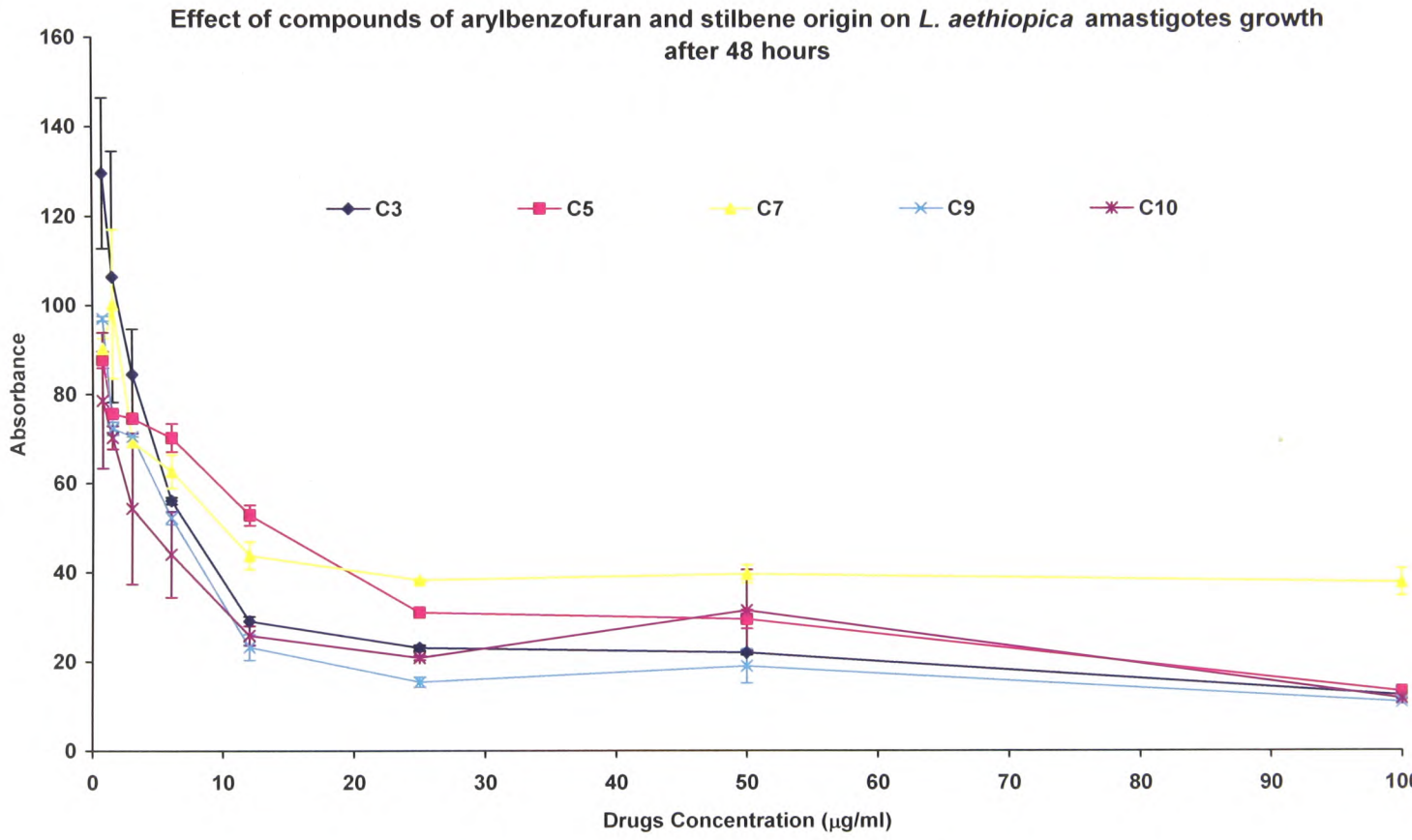


Figure 5.6: Effect of compounds of arylbenzofuran and stilbene origin on *Leishmania aethiopica* amastigotes growth after 48 hours. Axenic amastigotes were plated on a 96 well plate in the presence of different concentrations of cicerfuran and of 4 different stilbenoids. AlamarBlue was added after 24 hours of treatment and incubated for a further hour. Absorbance was read at Ex: 544nm Em: 590nm.

	LD_{50} µg/ml *L. aethiopica* promastigote		LD_{50} µg/ml *L. major* promastigote		LD_{50} µg/ml *L. tropica* promastigote		LD_{50} µg/ml *L aethiopica* axenic amastigotes		LD_{50} µg/ml THP-1 cells	
C1	26.7	(+6.0 -4.8)	61.8	(+32.7-20,4)	103.7	(+52-32.8)		n/t	267 .0	(+175-99)
C2	21.2	(+5.0-4.0)	142.5	(+50-59)	51.4	(+17.7-12.6)		n/t	199.0	(+101-63)
C3	13.0	(+3.1-2.5)	29.9	(+12-8)	14.2	(+4.2-3.3)	3.50	(+3.0-1.7)	22.1	(+5.9-5.1)
C4	102.6	(+68.7-40.3)	295.1	(+458-166)	209.2	(+368-132)		n/t		n/t
C5	2.5	(+0.5-0.4)	6.4	(+2.2-1.6)	5.4	(+1.3-1)	6.10	(+6.9-3.2)	16.0	(+4.2-3.3)
C6	83.6	(+28.9-20.7)	228.4	(+247-122)	136.3	(+100-57)		n/t		n/t
C7	9.0	(+1.8-1.5)	12.3	(+4-3)	10.8	(+3.7-2.7)	7.45	(+8.5-4.1)	24.7	(+6.8-5.3)
C8	21.4	(+4.9-3.9)	33.3	(+10-9)	60.6	(+26-17)		n/t	476.4	(+562-248)
C9	7.9	(+1.6-1.4)	13.3	(+4.7-3.5)	6.8	(+2.5-1.9)	3.35	(+3.8-1.9)	14.9	(+3.9-3.8)
C10	7.3	(1.8-1.5)	11.2	(+3.4-4.6)	12.2	(+3.7-2.8)	2.28	(+2.6-1.3)	17.4	(+4.7-3.8)
Amph B	0.2	(+1.0-0.07)	2.7	(+1.0-1.9)	0.5	(+0.17-0.12)	0.4	(+1.3-0.09)		n/t

Table 5.1 LD_{50} values in µg/mL for three different species of *Leishmania* promastigotes, *L. aethiopica* amastigotes and uninfected THP-1 cells. n/t = not tested.

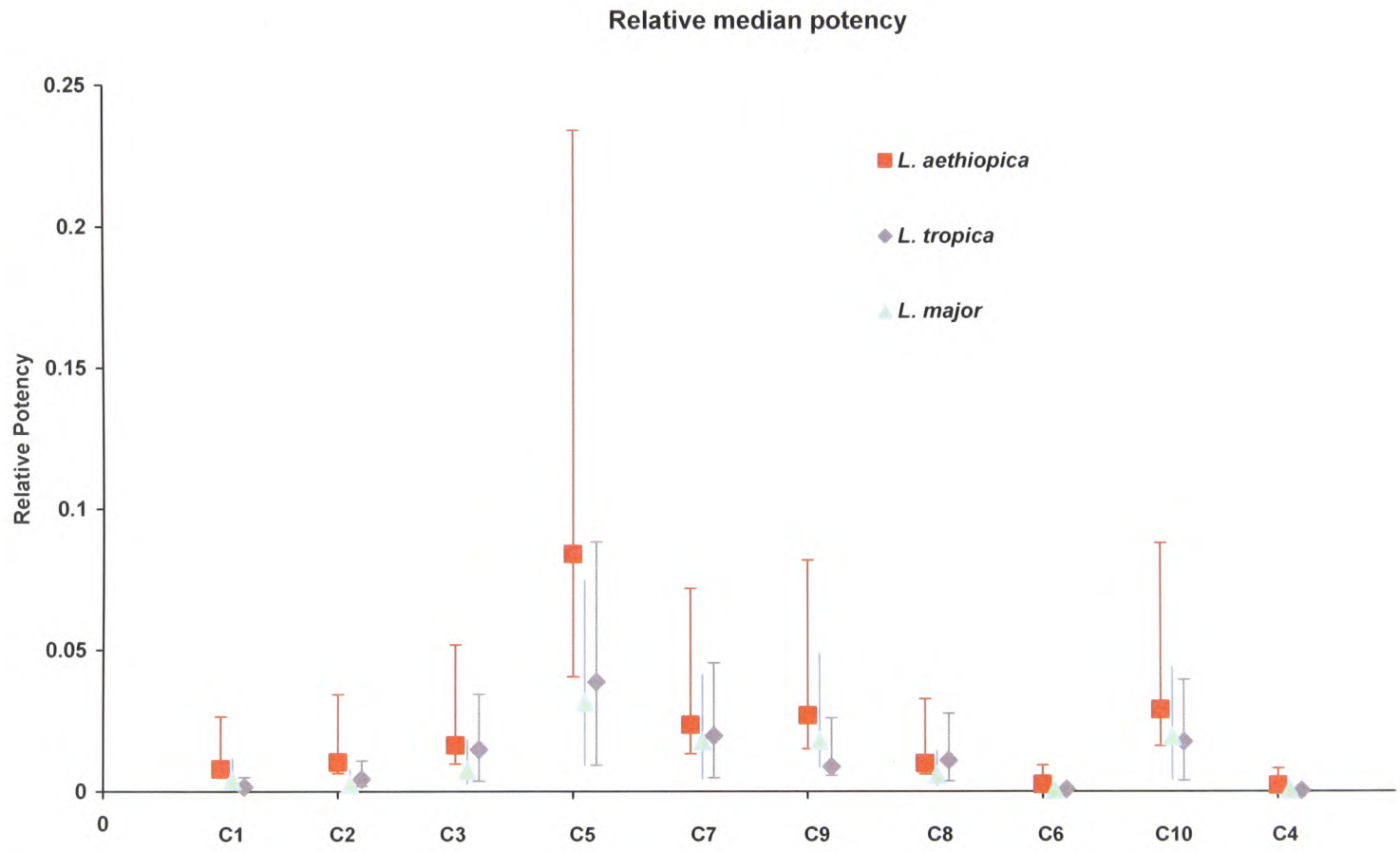


Figure 5.7. Relative median potency. The relative median potency estimated from probit analyses with logit transformations of dose-response data assuming a parallel relation between the drugs' response curves.

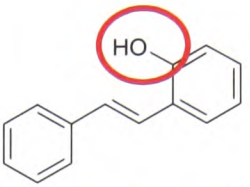
as well as the lower and upper 95% confidence limits was estimated assuming a parallel relation between the drugs' response curves. Since Goodness-of-fit Chi square was significant a heterogeneity factor was used in the calculation of confidence limits values using the software package SPSS. The Relative Median Potency (RMP) for *L. aethiopica* in Figure 5.7 was estimated using Amphotericin B as reference drug. The RPM for the other strains was then estimated by comparison with the effect of the same drug in the most sensitive strain after probit analyses of each drug on the three different strains.

Four of the stilbenes (C5, C7, C9 and C10) and one of the benzofurans (C3), showed particularly potent activity against all three *Leishmania* species with LD_{50} <30 μg/ml and as low as 2.5 μg/ml (Table 5.1). It is difficult to draw definitive conclusions about the structural features associated with potency of these compounds although low activity was associated with an absence of *O*-substitutions. More specifically, the presence of an *O*-substitution (either an hydroxy or a methoxy) at the C-2 (C-6 in benzofurans) appears to be associated with more potent activity since the five most active compounds were all *O*-substituted at this position. This is particularly well illustrated by C5, C6 and C7 in which the potently active C5 and C7 are both *O*-substituted at C-2 with a hydroxy and methoxy whereas C6, which showed relatively weak activity, lacks this C-2 group despite having the same C-4 methoxy as C7 (Figure 5.8). Cicerfuran also contained a hydroxy group at the same equivalent position (C-6) and was more active than the other benzofurans and stilbenes which did not contain this *O*-substitution. However, it is not possible to conclude categorically that the activity of these compounds was simply dependent upon the presence or absence of *O*-substitutions at specific positions and further work on structure function would be useful to improve efficacy and identify candidate drug leads.

The five most active compounds against all three *Leishmania* species were tested for their cytotoxicity on *L. aethiopica* amastigotes. The latter were cultured in an axenic condition, using a modification of JH30 medium (Chapter 4). Further characterization of axenic amastigotes involved morphological study through light microscopy observations. Virulence increased with respect to the promastigote stage and ability to revert back to the promastigote form on temperature and pH shift to 22°C and 7 respectively (as described in chapter 4).

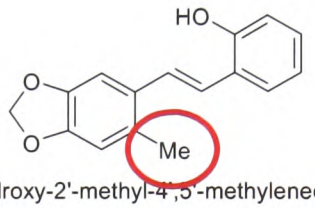
Figure 5.8. Structure of the 6 most active compounds.

C5



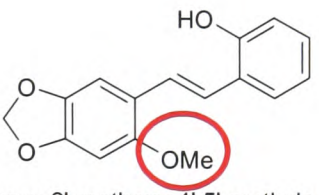
2-Hydroxystilbene

C9



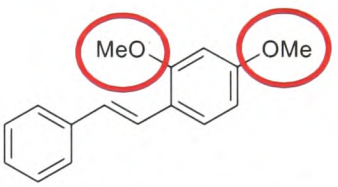
2-Hydroxy-2'-methyl-4',5'-methylenedioxystilbene

C10



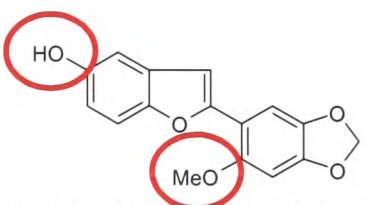
2-Hydroxy-2'-methoxy-4',5'-methylenedioxystilbene

C7



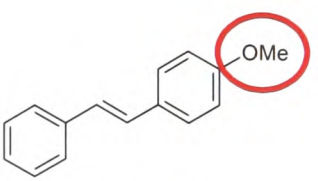
2,4-Dimethoxystilbene

C3



2-(2'-Methoxy-4',5'-methylenedioxyphenyl)-6-hydroxyphenylbenzofuran

C6



4-Methoxystilbene

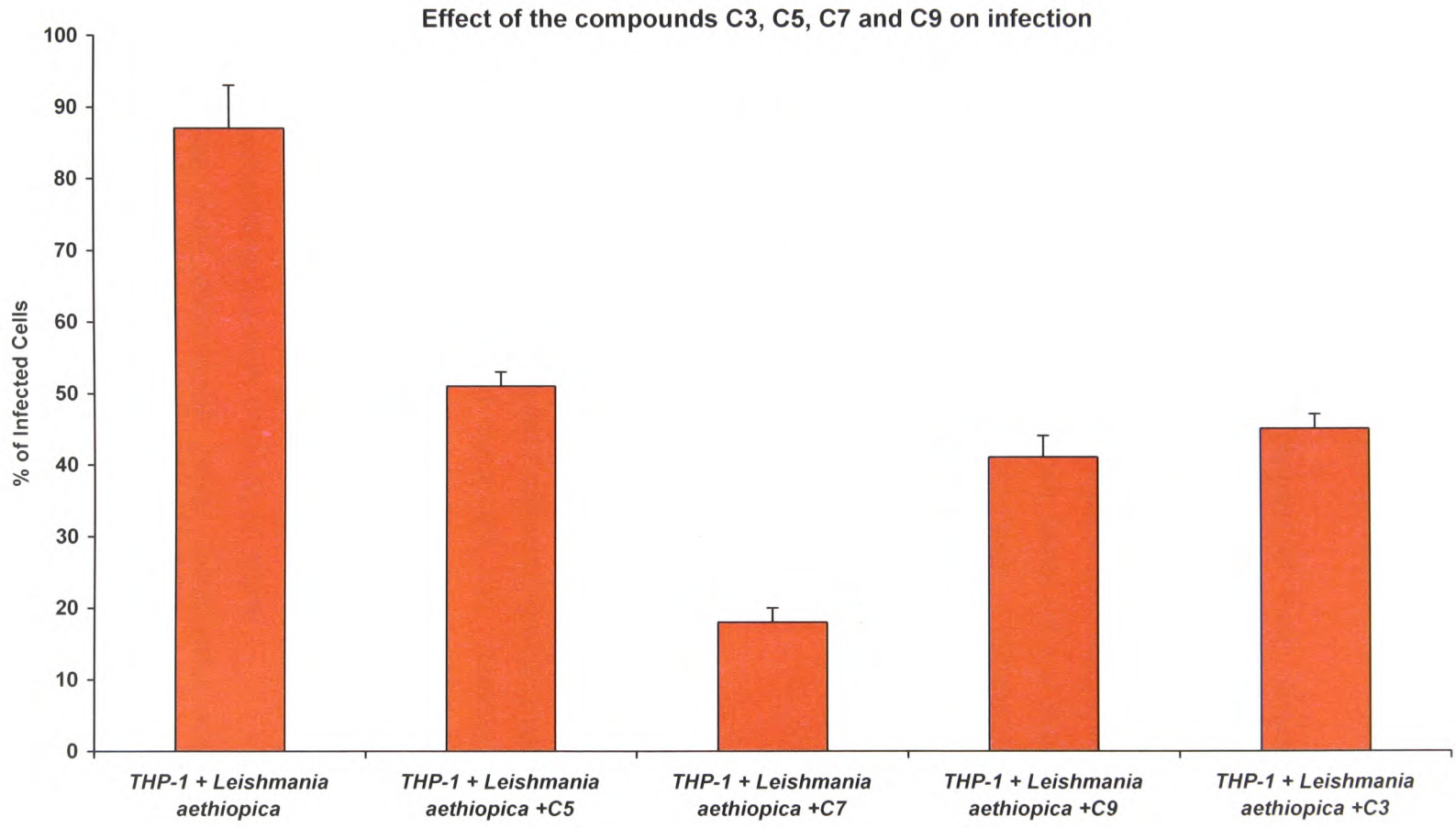


Figure 5.9. Effect of the compounds C3, C5, C7 and C9 on infection.
The effect of the compounds on infection is showed as percentage of infected THP-1 cells after treatment with the selected active compounds compared with the untreated control. (THP-1 + *Leishmania aethiopica* and grown in 0.05% w/v of DMSO final concentration).

With the exception of C5, five time revertant amastigotes showed an even greater sensitivity to the tested compounds than the promastigotes, with LD_{50} values presence of a methoxy group in position R^1 (C10). Ranging from 1 to 10.8 μg/ml. Although a stilbene structure was again the most lethal against amastigote parasites, the highest activity was recorded n the presence of a metoxy group in position R^1.

In order to investigate possible potential of these compounds as leishmaniasis drug leads, further tests were undertaken in the human cell line THP-1. Although showing cytotoxicity, the LD_{50} values calculated for THP-1 cells by probit analysis were between 14.5 and 24.7 μg/ml: 2-3 times higher than the result for the promastigotes. Furthermore, infected macrophages were even more sensitive to the action of the compounds than uninfected ones (Figure 5.8). Treatment caused significant decrease in the percentage of infected cells with each compound used, with minimum decrease being from 88% to 50% for C5. The most active was C7 with which the percentage of infected THP-1 cells diminished from 88% to 18% after 24 hours treatment with 1μg/ml of the compound.

5.3. Conclusions

This chapter describes the leishmancidal and the cytotoxic activity of cicerfuran, two of its structural analogues and 7 stilbenes (Figure 2.1) against Old World cutaneous *Leishmania* spp. and human cell lines. Cicerfuran is an antifungal defence compound produced by some wild species of chickpeas (*Cicer* sp.) that express resistance to fungal wilt (*Fusarium oxysporum* f. sp. *Ciceri*) (Stevenson and Veitch, 1998). It has been synthesized recently along with several benzofuran analogues and a related group of stilbene intermediates, all of which have antifungal and antibacterial activity (Aslam *et al.*, 2007).

The present study demonstrated potent leishmancidal activity of cicerfuran and 4 related stilbenes. The study on the effect of the compounds on THP-1 mammalian cells indicated that C3, C5, C7, C9 and C10 are differentially effective on *Leishmania* promastigotes and amastigotes (Table 1) and, moreover,

C3, C5, C7 and C9 significantly impaired the number of infected macrophages. Consequently, they are promising sources of new therapeutic agents and can be considered as possible leishmancidal drug leads: the next step in the study of their pharmacological and antiprotozoal property should be *in vivo* assay.

Chapter 6

Infection and apoptosis

6.1. Introduction

Leishmania parasites invade host macrophages, causing infections that are either limited to skin or spread to internal organs. In this study, three species causing cutaneous leishmaniasis, *L. major*, *L. aethiopica* and *L. tropica,* were tested for their ability to interfere with apoptosis in host macrophages in two different human monocyte-derived macrophages (cell lines THP-1 and U937) and in blood-derived human macrophages.

The interaction between *Leishmania* and macrophages plays a central role in the pathogenesis of the infection. Initiation of infection is related to the ability of the parasite to bind and enter the host cell and the inhibition of macrophage activity has been intensely studied (de Almeida *et al.*, 2003) . Once an amastigote has established itself and has replicated inside the host cell, the next step is for it and its descendants to spread to other macrophages. Given the modest number of parasites inoculated during natural transmission (1-1000) (Warburg and Schlein, 1986), the spread of amastigotes to uninfected macrophages is crucial to the

development of the disease. This 'silent stage' following infection is associated with the interaction of a distinct group of parasite molecules consisting of invasive determinants which establish the infection but do not activate immune effector mechanisms (Belkaid *et al.*, 2000; Chang and McGwire, 2002; Chang *et al.*, 2003). Parasites therefore must spread to uninfected cells without causing an immune response. Since apoptosis is a mechanism through which cells are phagocytosed by macrophages without eliciting an inflammatory response (Savill *et al.*, 2003), it could be the mechanism used by *Leishmania* parasites to silently spread. Previous studies on the effects of intracellular micro-organisms have shown both negative and positive modulation of host cell apoptosis (Table 6.1). Studies on *L. donovani*, *L. infantum* and *L. major* suggested that *Leishmania* parasites are able to rescue host cells from apoptosis and that this might play a role in both host invasion and in the persistence of parasites inside host cells. In each case parasites were able to rescue murine-macrophages after apoptosis was induced by either deprival of (M)-CSF or treatment with an apoptosis inducer (Moore and Matlashewski, 1994; Akarid *et al.*, 2004; Lisi *et al.*, 2005). Although intracellular parasites might find it beneficial to increase the life span of the host cells they might find it just as beneficial to induce apoptosis when this process can help them to spread into another macrophage without induction of inflammation.

The aim of this work was to determine if three Old World species causing cutaneous leishmaniasis were able to interfere with apoptosis in healthy host macrophages (both monocyte-derived cell lines and blood derived macrophages) and to establish if apoptosis induction could affect parasite spread.

The apoptotic process.

Apoptosis or programmed cell death is a crucial mechanism developed by many organisms in order to eliminate redundant, damaged or infected cells (Kerr *et al.*, 1972). Apoptosis is involved in mammalian development, in maintaining tissue homeostasis and an effective immune system. The importance of this process is enormous as demonstrated by the fact that its failure or deregulation

Microorganism	Effect on apoptosis	Cell tested for apoptosis	Reference
Mycobacterium tuberculosis	Inhibition of naturally occurring apoptosis	Peripheral blood mononuclear cells (PBMC)	(Durrbaum-Landmann *et al.*, 1996)
Legionella pneumophila	Incomplete activation	U937 and mouse J774/Caspases 3 activation (present) and DNA fragmentation (absent)	(Abu-Zant *et al.*, 2005)
Shigella fexneri. Salmonella typhi and S. typhimurium	induction	U937 cells and J774	(Nonaka *et al.*, 2003) (Hersh *et al.*, 1999) (Monack *et al.*, 1996) (Chen *et al.*, 1996)
Yersinia enterocolitica	induction	J774 and PBMC	(Ruckdeschel *et al.*, 1997)
Actinobacillus actinomycetemcomitans	induction	THP-1	(Kato *et al.*, 2005)
Plasmodium falciparum	induction	Lymphocytes, PBMC	(Toure-Balde *et al.*, 1996)
Toxoplasma gondii, Trypanosoma cruzi	Inhibition of chemically induced apoptosis	human foreskin fibroblasts Murine MLR T lymphoblasts Murine fibroblasts HeLa and HT1080	(Nash *et al.*, 1998) (Sakai *et al.*, 1999) (Nakajima-Shimada *et al.*, 2000)
Leishmania donovani *Leishmania infantum* *Leishmania major*	Inhibition of chemically induced apoptosis	Bone marrow derived macrophages (BMDM) U937 BMDM	(Moore and Matlashewski, 1994) (Lisi *et al.*, 2005) (Akarid *et al.*, 2004)

Table 6.1. Review of the effect of various infective organisms on their host cells' apoptosis.

causes a spectrum of diseases ranging from cancer to several degenerative conditions such as Alzheimer's disease (Lowe and Lin, 2000; MacFarlane and Williams, 2004).

Cell death via apoptosis can follow at least two different pathways: the extrinsic and the intrinsic pathways. The extrinsic pathway is triggered by the binding of extra-cellular molecules to the so called 'death receptors' on the membrane surface (Strasser *et al.*, 2000; Ashkenazi, 2002). The intrinsic pathway is triggered from events internal to the cell itself; it causes permeabilization of mitochondria and consequent releasing of death promoting proteins (Newmeyer and Ferguson-Miller, 2003). In addition to these two main pathways, other pathways are involved in the induction of the apoptotic process. For example the product of cytotoxic T cells (grananzyme B and perforine) cause apoptosis by entering the cells through receptor mediated phagocytosis and, following pore formation induction, the grananzyme is released into the cells and interacts directly with Bid and caspase 3 or can cause a caspase-independent death (Bolitho *et al.*, 2007). Although the number of proteins involved in the apoptotic process is extremely high (133545 papers have been published on them up to July 2007) the better known and the most studied enzymes are the 14 cysteine proteases (in mammals) known as caspases (Rupinder *et al.*, 2007). Caspases are responsible for both the initiation of the programmed cell death and for its execution.

The execution of apoptosis involves a series of morphological and biochemical modifications such as: permeabilization of mitochondria, chromatin condensation and DNA fragmentation, cell shrinkage, formation of apoptotic bodies and exposure of PS to the membrane surface of the cell (Leach, 1998). The apoptotic process ends with cell death and consequent disposal via engulfment by neighbouring cells. Cellular death by means of apoptosis makes it possible to dispose of dead cells without causing any damage to the surrounding cells, therefore avoiding inflammation and any consequent immune response (Savill *et al.*, 2003).

Molecular mechanisms of apoptosis.

In order to understand how infective parasites can interfere with the apoptotic process it is important to introduce the molecular details of the two main apoptotic pathways (Figure 6.1 and 6.2) and to their regulation (Figure 6.3). The extrinsic pathway (Figure 6.1) is started by external signals (dead signals) that are recognized by the extracellular receptor and start the process of cell 'suicide'. The best characterized ligand-receptor interaction is between CD95 (Fas) and CD95 ligand (FasL) receptor (Kischkel *et al.*, 1995; Muzio *et al.*, 1996; Peter and Krammer, 2003). Both Fas and FasL are members of the tumour necrosis factor receptor (TNFR) families of proteins. Following the binding of Fas with its receptor the assembling of a death-inducing signalling complex (DISC) occurs. The formation of DISC starts a chain reaction during which pro-caspase-8 and -10 (initiators) are activated and cleave and activate the pro-caspases-3, -6 or -7 (executioners) from their pro form (Peter and Krammer, 2003). Caspase-8 can also cleave Bid, a pro apoptotic cytosolic Bcl-2 family protein that, once truncated, translocates to the mitochondria and leads to the release of cytochrome C into the cytoplasm, hence initiating the intrinsic pathway (Kuwana *et al.*, 1998; Lisi *et al.*, 2005)

The intrinsic pathway (Figures 6.1 and 6.2) affects mainly the mitochondrial outer membrane integrity, and involves the release of cytochrome c and other mitochondrial intra-membrane components (including Smac/DIABLO and the serine protease Omi/htrA_2) into the cytosol. Cytochrome C interacts with apoptosis activating factor (Apaf)-1 from the cytosol inducing it to oligomerize into a structure termed an apoptosome. In each Apaf-1 a caspase recruitment domain is present and binds to pro-caspase-9. Caspase-9 is a self-cleaving, self-activating initiator caspase and once bound to Apaf-1 forms the apoptosome which is able to activate caspase-3, -6 or -7 (Li *et al.*, 1997; Wang, 2001). Together with cytochrome C, Omi and DIABLO, both bind to inhibitors of apoptotic proteins (IAPs) preventing them from inhibiting activation of caspases. Finally AIF and endo G translocate towards the nucleus where they contribute to a caspase-independent cell death path which includes DNA condensation and degradation (Penninger and Kroemer, 2003).

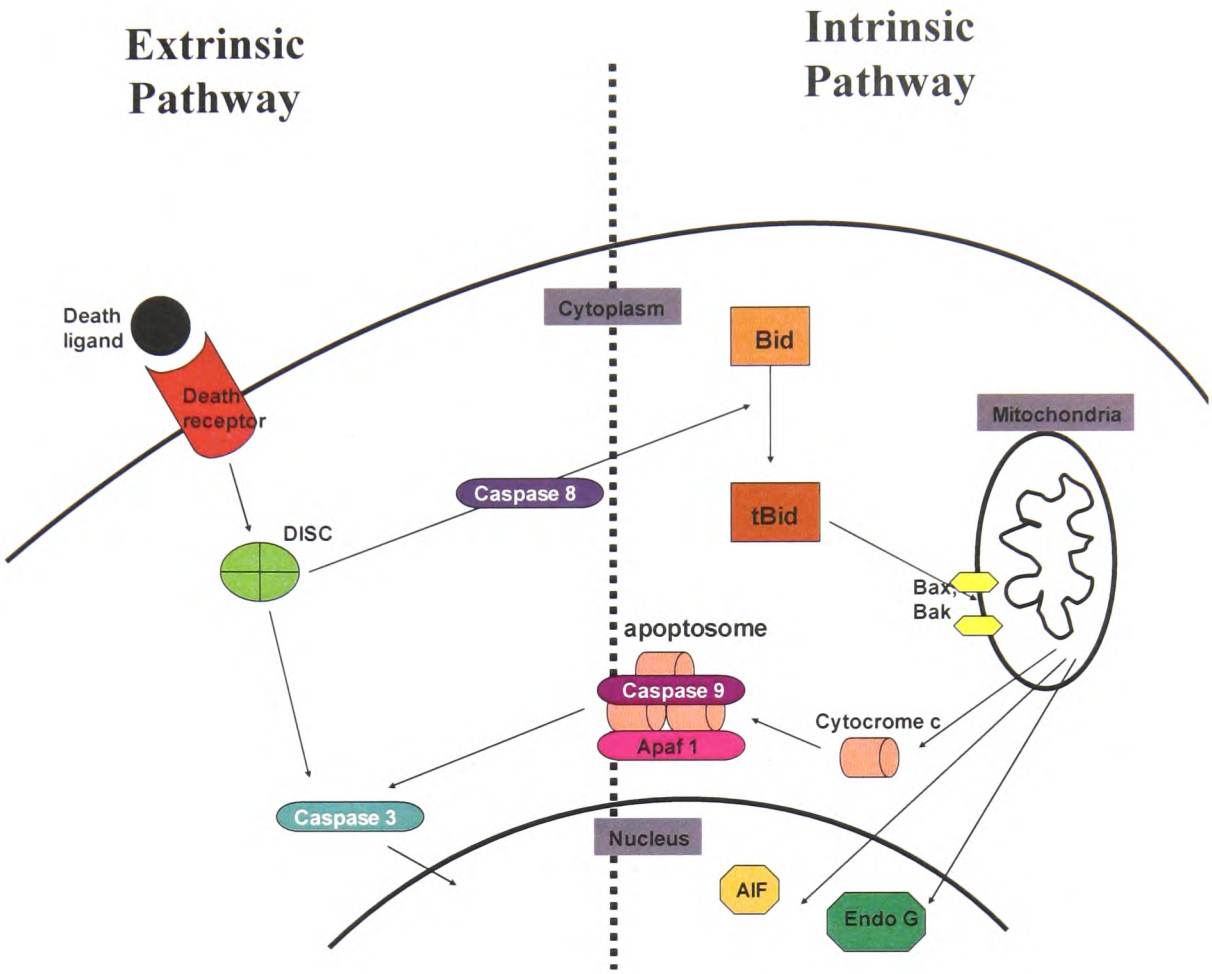


Figure 6.1. Intrinsic and extrinsic apoptotic pathways.
The extrinsic pathway is triggered by an extracellular stimulus. A death ligand (e.g. Fas L or TNF-α) binds the death receptor (e.g. Fas or TNFR) which induces recruitment and activation of Caspase 8 and 3. Caspase 3 triggers apoptosis, Caspase 8 induces the intrinsic pathway.
The intrinsic pathway is initiated following a stress signal, it is regulated by a Bcl-2 family of proteins which are responsible for membrane permeabilization and consequent release of apoptotic factors. The intrinsic pathway is described in detail in Figure 6.2

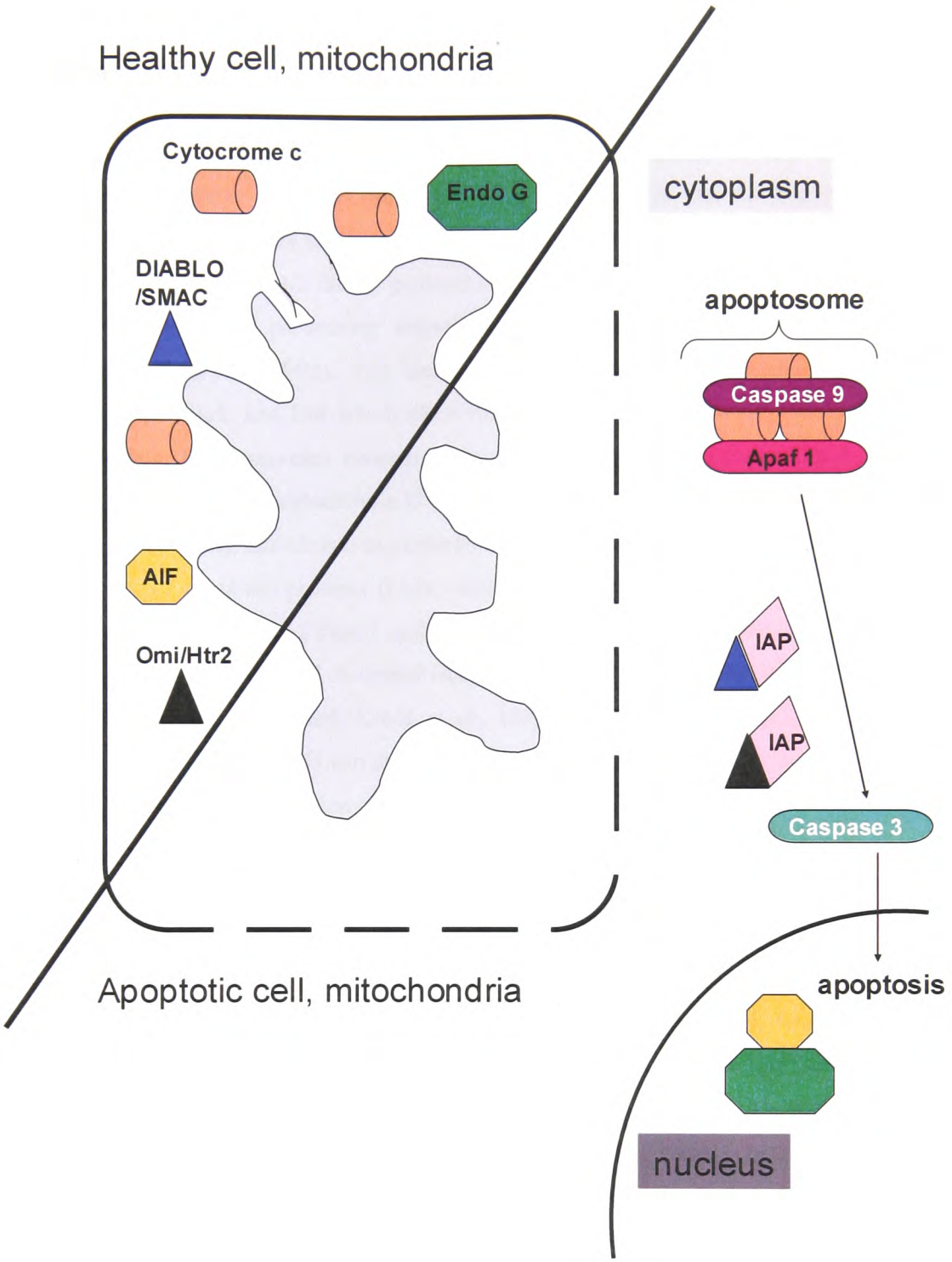


Figure 6.2. The intrinsic pathway
Following an apoptotic stimulus the mitochondrial membrane becomes permeable and various molecules are released into the cytoplasm. Cytochrome C binds with the cytosolic pro-caspase 9 (which activates caspase 9) and with Apaf 1 forming the apoptosome. The apoptosome cleaves pro-caspase-3 into active caspase 3 and induces apoptosis. Diablo and Omi bind with IAPs (inhibitor of apopotic proteins) preventing them from inhibiting caspases. Finally AIF and Endo g translocates to the nucleus to induce caspase-independent apoptosis.

Modulation of apoptosis.

The apoptotic process is tightly regulated; apoptosis inhibitors are present in the cells and are able to interact with each stage of the apoptotic cascade and inhibitors of apoptosis inhibitors can reverse their effect (Danial and Korsmeyer, 2004). The main regulatory mechanisms are described hereafter.

Anti-apoptotic Bcl-2 family proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Mcl-1) prevent apoptosis by preventing mitochondrial membrane permeabilization (Cuconati and White, 2002). The Bcl-2 family also includes pro-apoptotic members: Bax, Bak, and Bid which elicit cell death (Lindsten T *et al.*, 2000). Anti-apoptotic Bcl-2 proteins neutralize the effect of the pro-apoptotic Bcl-2 families responsible for cytochrome-C release by sequestering them (Cheng *et al.*, 2001). Following mitochondrial permeabilization further regulation is exerted by inhibitor of apoptosis proteins (IAPs) which are regulators of apoptosis that act downstream to the Bcl-2 family and inhibit caspase activity. The IAPs, which includes cIAP-1, cIAP-2 and X-linked IAP, interacts and inhibits caspases 3, 7 and 9 after they are processed (Crook *et al.*, 1993). As described in Figure 6.2 Omi/htr2 and Smac/DIABLO can regulate IAPs by binding to them and blocking their inhibitory effect (Verhagen *et al.*, 2000; Suzuki *et al.*, 2001).

Other two regulatory pathways will be described as they have been reported to participate in intracellular pathogens' manipulation of apoptosis: phosphoinositide 3-kinase (P13-K) and the nuclear factor-kappa B (NF-kB) signalling pathways (Akarid *et al.*, 2004; Ruhland *et al.*, 2007). Both NF-kB and P13-K are normally present in their inactive form in the cytoplasm of healthy cells and both are activated by extracellular stimuli (Figure 6.3). Nuclear factor kappa B is found in the cytoplasm in its inactive form, bound to I-kB. Activation is caused by the action of IKK (I kappa kinases) which phosphorylates I-kB setting NK-kB free to move towards the nucleus. Once inside the nucleus NF-kB induces transcription of anti-apoptotic genes. These genes codify for proteins such as C-Flip, and IAPs which inhibit caspases 9, 3 and the activation of caspase 8. Another inhibitor of apoptosis induced by NF-kB is Bfl-1/A1 which prevents the release of cytocrome C, therefore inhibiting the intrinsic pathway (Karin and Lin, 2002; Hayden and Ghosh 2004).

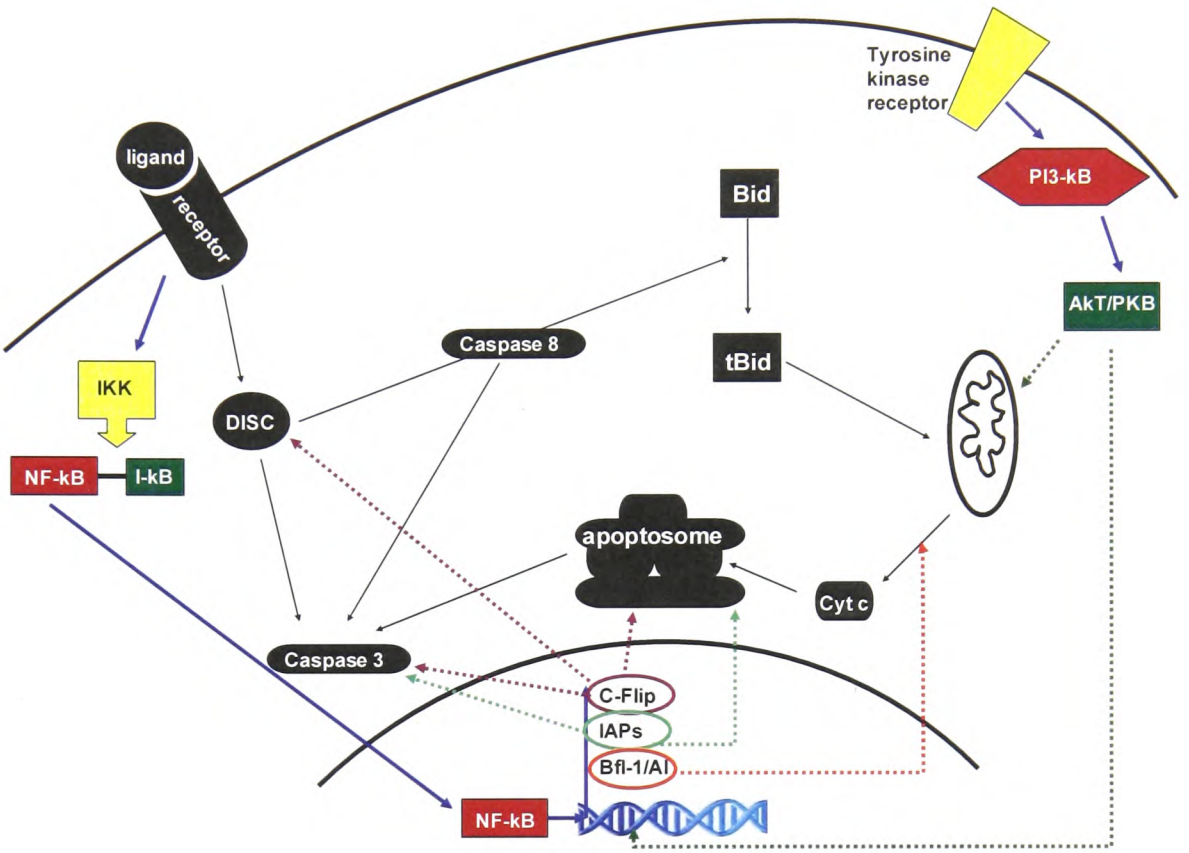


Figure 6.3. Apoptosis regulator pathways
Two main pathways are described in this figure: NF-kB and PI3-kB. Blue, continuous arrows indicate activation, purple, green, and red discontinuous arrows indicate inhibition.
Nuclear factor kappa B is found in the cytoplasm in its inactive form, bound to I-kB. Activation is caused by the action of IKK (Ik kinases) which phosphorylates I-kB setting NK-kB free to move towards the nucleus. Once inside the nucleus NF-kB induces transcription of anti-apoptotic genes. These genes codify for proteins such as C-Flip, and IAPs which inhibit caspases 9, 3 and the activation of caspase 8 and of Bfl-1/A1 which prevents the release of cytochrome C, thereby inhibiting the intrinsic pathway.

PI3-kB also resides inactive in the cytoplasm, until cell surface receptors such as the tyrosine kinase receptor recruit it and activate it. Once activated, PI3-kB phosphorylates PiP_2 into PiP_3 (phosphatydilinositol biphosphate and triphoshate respectively) which recruit PDK1 (phosphoinositide-dependent kinase 1) which activates Akt/PKB. Once active Akt/PKB: 1. Inactivates Bcl-2 protein and therefore the activation of the intrinsic pathway; 2. Inactivates transcription factors specific for pro-apoptotic genes; 3. activates IKK therefore inducing the NF-kB response (Engelman *et al.*, 2006).

Features of apoptosis: membrane blebbing and phosphatidyl serine (PS) externalization.

Formation of apoptotic bodies and exposure of PS are two features of the apoptotic process that intracellular parasites could exploit to spread to neighbouring macrophages. Within apoptotic bodies the parasites can leave the host cell without losing the protective environment of the host cytoplasm enclosed by the cellular membrane. Moreover the presence of PS on the external membrane of the apoptotic bodies acts as an 'eat me' signal for the neighbouring macrophages (Grimsley and Ravichandran, 2003). The exposure of PS in the outer surface of the cell membrane is an early sign of apoptosis. Formation of apoptotic bodies starts later after loss of the cytoskeleton and consequent rounding-up of the cells, followed by nuclear condensation, DNA fragmentation and finally disassembly of nucleus and organelles, and encapsulation of the products in membrane-bound apoptotic bodies. Apoptotic bodies are ingested by neighbouring cells as soon as they are generated. Apoptotic body formation or membrane blebbing can be initiated by a caspase-3-dependent cascade. The target of this is a serine/treonine Kinase termed ROCK I (Rho-associated coiled coil-containing protein kinase), an effector of cell mobility regulation. During apoptosis ROCK I is transformed into a constitutively active form, which controls the assembly of cortical actomyosin filaments via the phosphorylation of myosin regulatory light chains and causes membrane blebbing (Sebbagh *et al.*, 2001).

Although caspase-3 can start the reaction, ROCK I cleavage can induce blebbing in the absence of any other apoptotic feature (Shiratsuchi *et al.*, 2002).

Moreover, ROCK II, (a ROCK family member closely related to ROCK I but encoded by a different gene) is cleaved directly by granazyme b and this causes constitutive kinase activity and membrane blebbing (Sebbagh *et al.*, 2005) independent of caspase-3 (Figure 6.4).

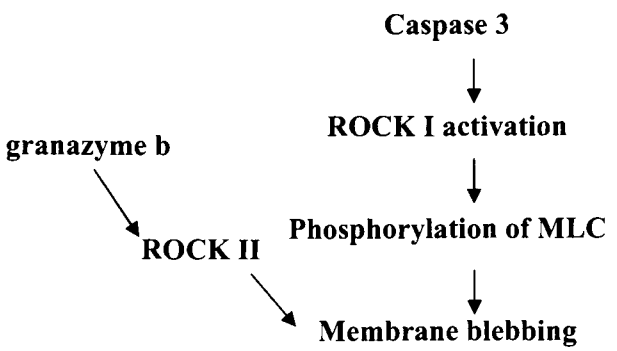


Figure 6.4. Scheme representing the caspase 3-dependent and caspase 3-independent mechanisms of membrane blebbing induction.

The presence of PS on the membrane is a key feature for recognition and subsequent engulfment of apoptotic cells/bodies by neighbouring cells, as well stimulating macrophage migration towards the apoptotic site. Exposure of PS on the external membrane requires a loss of asymmetrical distribution of the molecule, which is generally found on the internal side of the cell membrane. This is achieved by inhibition of aminophospholipid translocase and activation of the calcium dependent phospholipids' scramblase. Although this mechanism is caspase-dependent PS exposure was also found in the absence of caspase activation (Baird *et al.*, 2004) which suggests the presence of a caspase-independent regulation pathway. It is interesting to notice that both membrane blebbing and PS exposure are regulated with caspases-dependent as well as caspases-independent mechanisms and that apoptotic body formation can be induced in the absence of other apoptotic features (Shiratsuchi *et al.*, 2002).

Moreover PS externalization precedes decrease in mitochondrial transmembrane potential and release of cytochrome C, indicating that PS exposure occurs independently of these mitochondrial events (Shiratsuchi *et al.*, 2002). These data suggest that it is not necessary for *Leishmania* parasites to induce the whole apoptotic pathway in order to benefit from these two features.

Infectious diseases and the apoptotic process

During infectious diseases the immune system's challenge is to maintain equilibrium between generating an immune response strong enough to eliminate the parasitic infection and keeping the response low enough to prevent excessive damage to the surrounding tissues. Similarly, cellular suicide involves elimination of the dying cells without causing an immune reaction.

Apoptotic cells expose proteins called apoptotic cell associated molecular patterns (ACAMPs) that are able to bind to specific receptors in macrophages and dendritic cells. Once inside the cells, the antigens are processed and presented by Major Histocompatablity Complex (MHC) class I molecules. In dendrititc cells (DC) this presentation involves a response from cytotoxic T lymphocytes. While macrophages fail to prime T cell precursor and suppress the ability of DC to activate T cells (Albert *et al.*, 1998b).

The binding of apoptotic cells and/or apoptotic bodies to macrophages happens mainly through two different types of receptors:

- CD 36 and $\alpha_v\beta_3$, bind to thrombospondin-tagged apoptotic cells and lead to IL-10 and TGF-β production (Albert *et al.*, 1998a).
- Phosphatidyl serine (PS) receptors bind the PS exposed in the apoptotic cells surfaces and induce production of anti-inflammatory cytokines with a final effect on down-regulation of IL-1, IL-12, and TNF-α (De *et al.*, 2002).

Both mechanisms are responsible for an anti-inflammatory response (Fadok and Chimini, 2001).

Apoptosis can interfere with infectious mechanisms and in some cases, for instance during *Trypanosome cruzi* infection, can elicit the parasites' growth inside the host (Freire-de-Lima *et al.*, 2000). This happens mainly by inhibiting nitric oxide (NO) production and therefore respiratory burst processes. Briefly,

production of TGF-β following engulfment of apoptotic cells causes up-regulation of the enzyme arginase which shifts the L-arginine metabolism towards ornithine production consequently decreasing nitric oxide (NO) production. Ornithine decarboxylase (ODC) is also induced by TGF-β and causes polyamine synthesis. Putrescine in particular was shown to increase parasite replication impairment in the production of NO together with suppression of pro-inflammatory cytokines. Putrescine synthesis also creates the appropriate environment for optimal *T. cruzi* growth inside the macrophages. Consequently the engulfment of apoptotic cells drives the growth of *T. cruzi* in macrophages (Freire-de-Lima *et al.*, 2000).

Similarly, uptake of apoptotic PMN by infected macrophages results in increased *L. major* replication (Ribeiro-Gomes *et al.*, 2005). And the presence of apoptotic promastigotes themselves was shown to increase *Leishmania* virulence (van Zandbergen G *et al.*, 2006; Wanderley *et al.*, 2006), confirming the link between apoptosis and infection.

The involvement of apoptosis in parasitic infection has been the subject of investigation in the last two decades. Not only have different outcomes been reported for different infectious organisms (Table 6.1) but manipulation of apoptosis was found to vary within the same infection cycle. For example *Plasmodium berghei* was described as inhibiting apoptosis during the early stage of infection of hepatocytes and in the replicative liver stage (van de Sand *et al.*, 2005) while prior to erythrocyte infection, detached hepatocytes filled with *P. berghei* exhibited various characteristics of apoptotic cells (Sturm *et al.*, 2006). Recently it was reported that *Leishmania mexicana*, *L. pifanoi L. donovani, L. infantum* and *L. major* which are known to inhibit host cell apoptosis following chemical induction (Moore and Matlashewski, 1994; Aga *et al.*, 2002; Akarid *et al.*, 2004; Lisi *et al.*, 2005) are also less effective in producing this inhibition at a later stage of infection (24 hours) (Ruhland *et al.*, 2007). These data underline the fact hat the relationship between infection and apoptosis is more complex than initially thought.

6.2. Results and discussion

In preliminary experiments apoptosis was checked 24 hours from infection (Annexin V assay in THP-1 cells) when a significant but small increase in apoptosis was detected with less than 10% of the cells showing apoptosis with any of the 3 species tested (0.5% were apoptotic in the untreated control). A higher percentage of apoptotic cells was detected with early apoptosis tests (Annexin V and / or Mitosensor kit) in both cell lines and blood derived macrophages 48 hours from infection. Differences between untreated controls and cells infected with all three *Leishmania* species were highly significant (Figures 6.5 and 6.6). Infected U937 cells showed a higher sensitivity to apoptosis induction than THP-1 cells both with the Mitosensor kit (Figure 6.6) and the Annexin V assay (Figure 6.5). Phagocytosis of control zymosan did not cause significant increase in the percentage of apoptotic cells compared with untreated control (results not shown).

It is evident from the results (Figure 6.5) that *L. aethiopica* is more effective than *L. tropica* and *L. major* in inducing PS exposure in the two human macrophage cell lines. Further data showed that although the percentage of apoptotic cells increased during infection with dead parasites, when compared with the untreated control, a significantly smaller amount of apoptosis was induced ($P<0.0001$) when compared with cells infected with live parasites under the same cell/parasite ratio. Therefore induction of apoptosis is mainly correlated with the presence of viable parasite although it cannot be excluded that a limited part of the induction could be related to passive engulfment of promastigotes.

The level of apoptosis induced by infection in blood derived macrophages was much higher than that found in the cell lines with almost 200% increase of apoptotic cells between infected and uninfected ($P<0.001$, Figure 6.7). There was no significant difference in the degree of apoptosis induced by the three species tested. Human cells were clearly more sensitive than cell lines to infection.

U937 macrophages were infected with each of the three species studied and the percentage of infection was compared following induction of apoptosis (Figure 6.8). In each case, induction of apoptosis significantly increased the

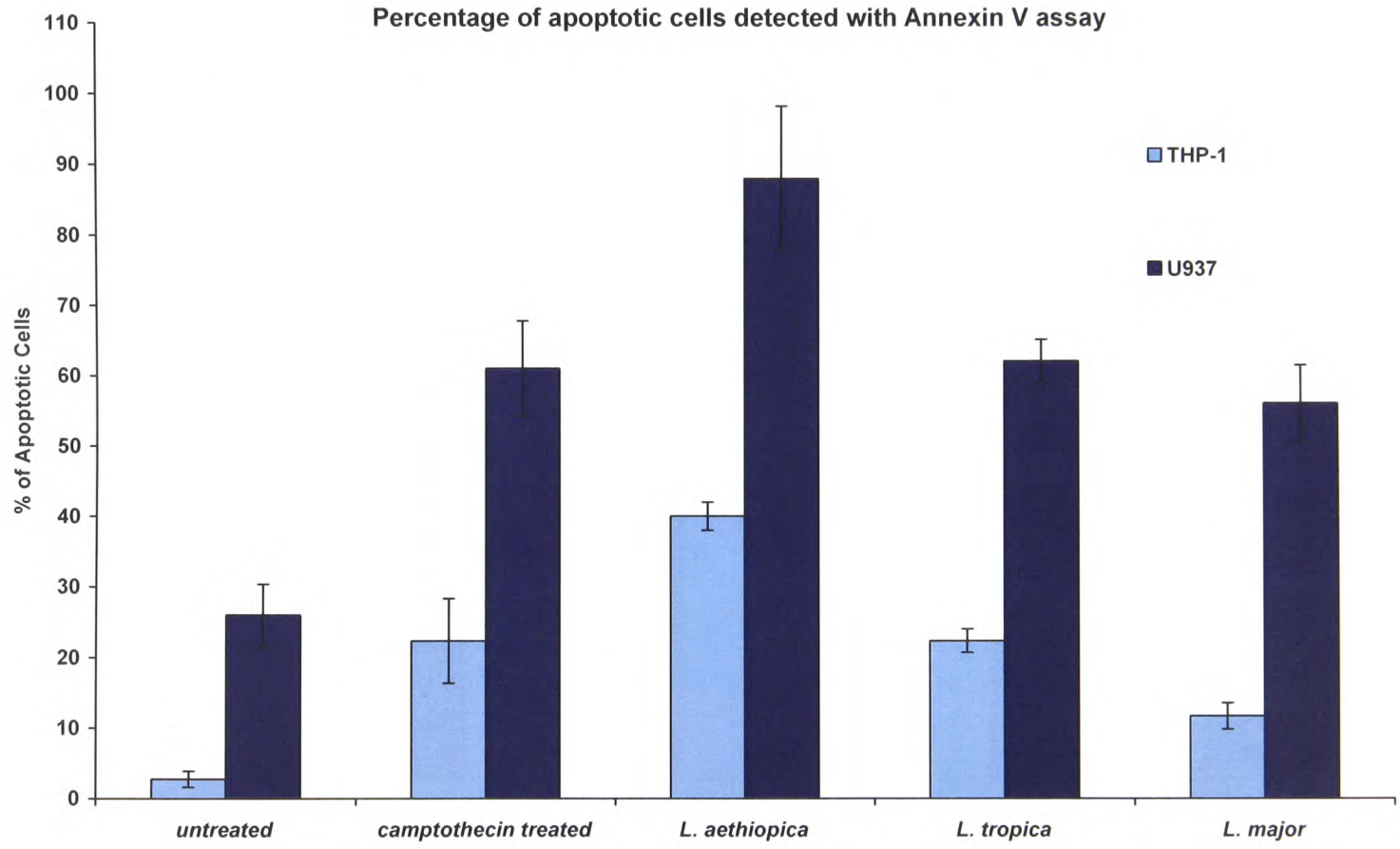


Figure 6.5. Percentage of apoptotic cells detected with Annexin V assay. The untreated sample is the negative control, while the camptothecin treated is the positive control. Apoptosis is significantly higher when compared to the negative control both in U937 ($P<0.02$) and in THP-1 ($P<0.01$).

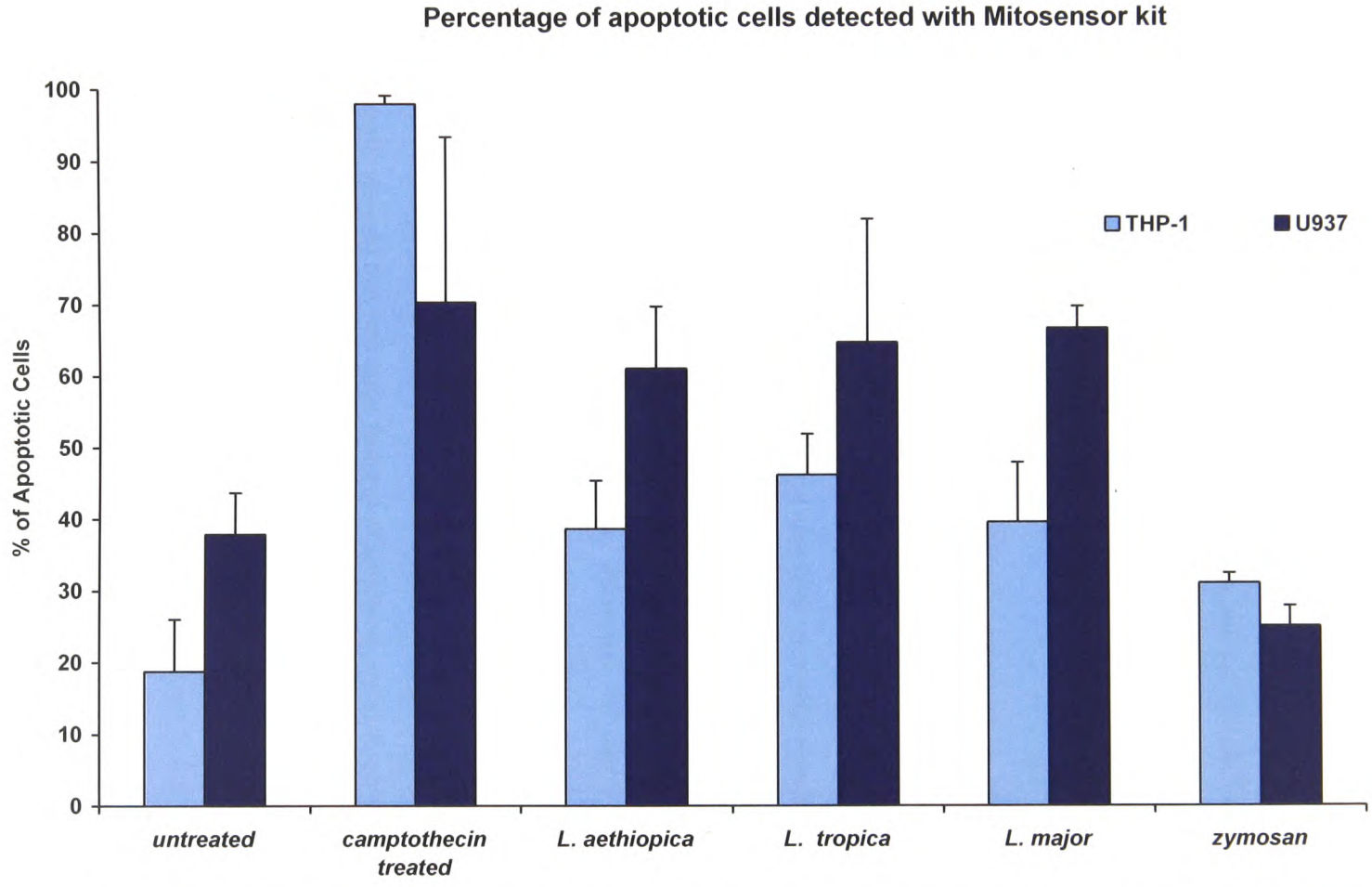


Figure 6.6. Percentage of apoptotic cells detected with Mitosensor Kit. One sample was left uninfected and untreated as negative control, while one sample was treated with an apoptosis inducer, camptothecin, and used as positive control. Apoptosis is significantly higher when compared to the negative control both in U937 and in THP-1 ($P<0.02$).

percentage of infection when compared with untreated infected macrophages. During the course of the experiment, induction of apoptosis had no effect on cell density (data not shown), suggesting that the difference in the infection was related to apoptosis induction and not to loss of viable adherent cells.

Analysis of DNA fragmentation by flow cytometry (Figure 6.9) showed no sub-diploid peaks which are an indication of DNA loss (sequential to fragmentation), following infection with any of the three species. Similar results were reported in the negative control of uninfected cells. In contrast, a significantly wider peak was present in the apoptotic control. Therefore it can be concluded that DNA fragmentation was not detected within 48 hours of infection.

The results presented clearly indicate that early apoptosis in healthy macrophages significantly increases after infection with all 3 species of *Leishmania*, with *L. aethiopica* inducing the highest level of apoptosis in cell lines. This effect was dependent on the presence of viable promastigotes and was significantly reduced in experiments with dead parasites and absent with the control zymosan. Consequently, the process of phagocytosis itself is not the only cause of apoptosis induction and parasites need to be alive to comprehensively interfere with the apoptotic pathways of the host cells. Little increase of apoptosis was detected 24 hours from infection. Moreover 48 hours after infection *Leishmania* will have differentiated into amastigote forms and started to multiply inside the parasitophorous vacuole of the host macrophages. It is therefore likely that molecular determinants specific to the promastigote stage are not sufficient to cause apoptosis.

The results showed that two main features of apoptosis are induced by the parasites; mitochondrial permeabilization and phosphatydil serine (PS) exhibition. PS is normally present on the macrophage membranes but is asymmetrically distributed on the cytosolic side by the action of specific enzymes (Daleke, 2003). Since externalisation of PS is a well known induction signal for phagocytosis (Grimsley and Ravichandran, 2003) it could also represent a signal for uninfected cells to phagocytose infected ones and therefore

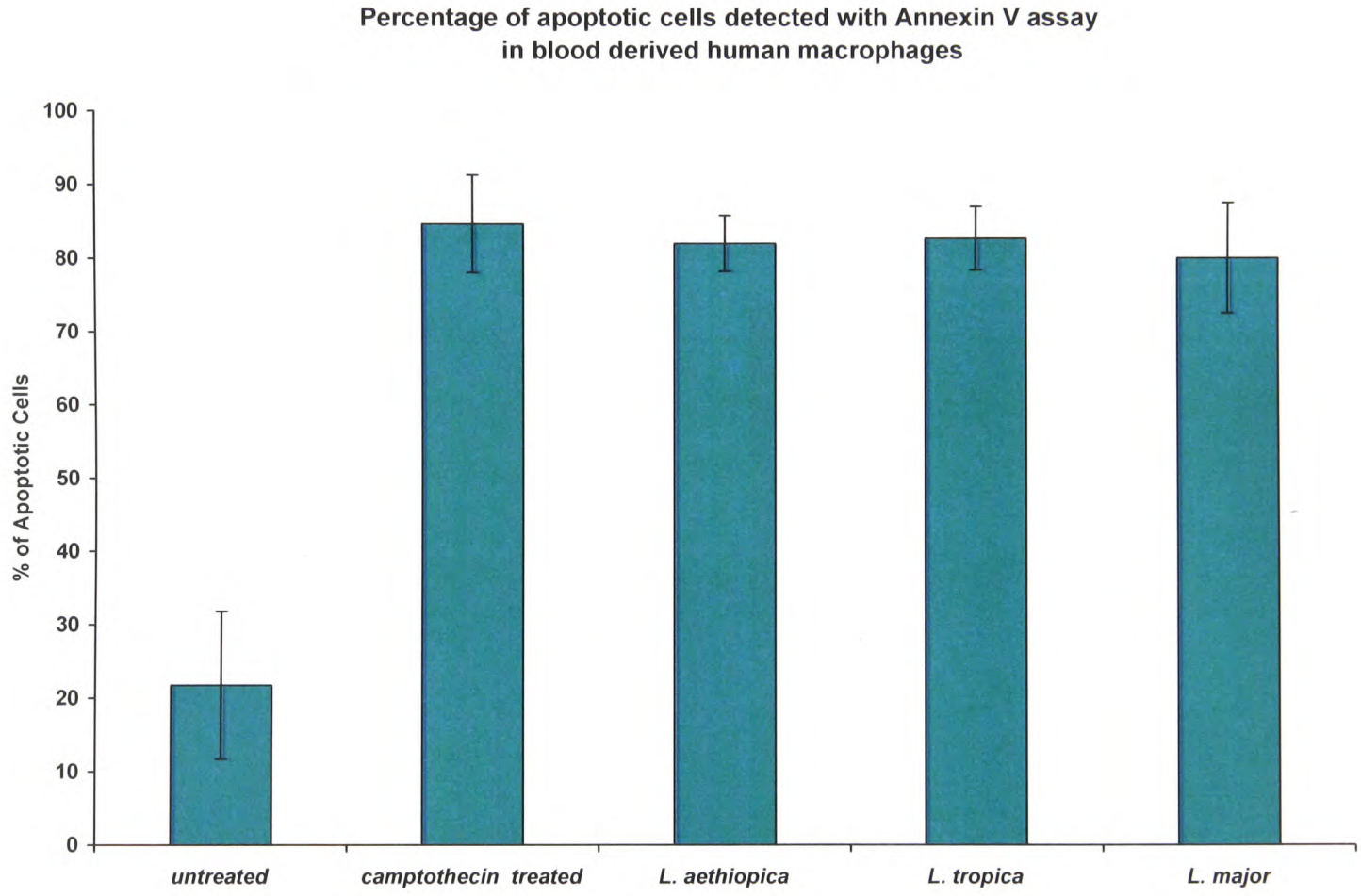


Figure 6.7. Percentage of apoptotic cells detected with Annexin V assay in blood monocyte-derived macrophage cells. One sample was left uninfected and untreated as negative control, while one sample was treated with an apoptosis inducer, camptothecin, and used as positive control.

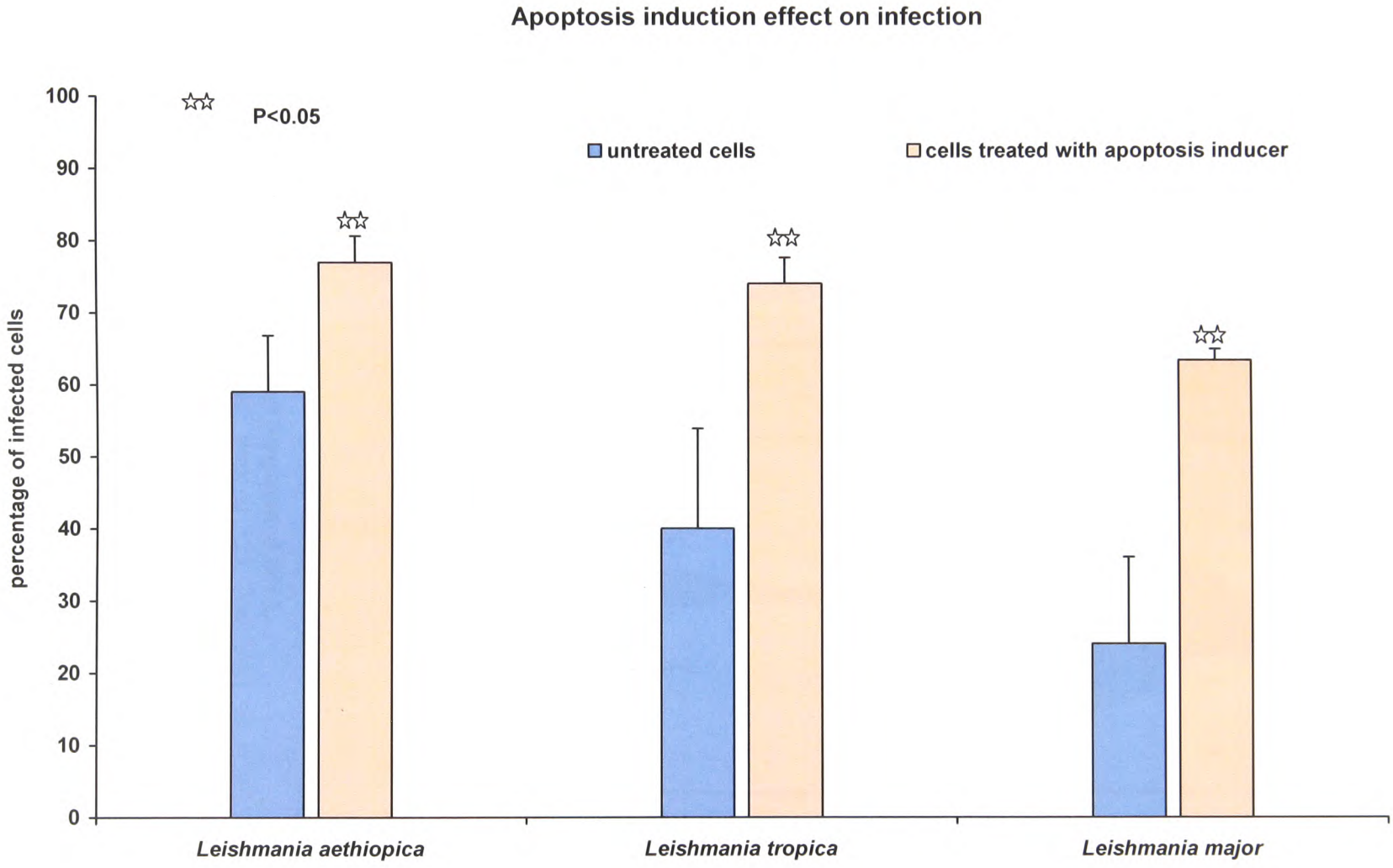


Figure 6.8. Apoptosis induction effect on infection. Terminally differentiated U937 cells infected with *Leishmania* were treated with the apoptosis inducer camptochecin or left untreated as control. The percentage of infected cells with and without treatment is reported.

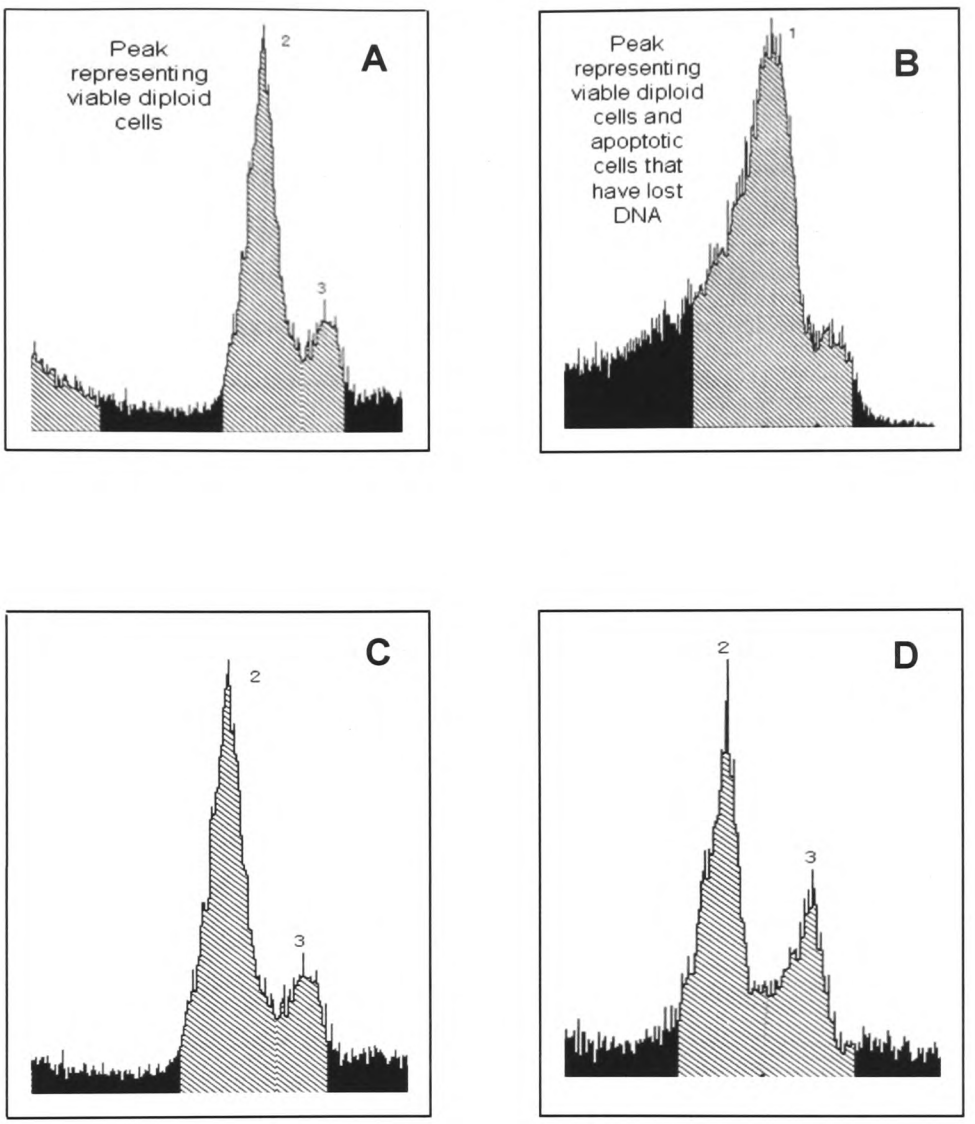


Figure 6.9. Flow cytometry analysis of fragmented DNA.
Histogram with 1000 channels, diploid peak at 125, low level set at 40. (A) Negative control, uninfected and untreated THP-1 cells. Two peaks are present representing viable diploid cells and groups of 2 cells. (B) Positive control, camptothecin treated THP-1 cells show typical apoptosis profile with a broader peak representing normal cells plus apoptotic cells that have lost DNA. (C) THP-1 cells infected with *L. aethiopica*. (D) THP-1 cells infected with *L major*. No sign of apoptosis is present in C or D. They appear the same as the control A.

parasites. A second advantage of this is that phagocytosis of apoptotic cells does not result in macrophage activation (Cocco and Ucker, 2001), and attenuates oxidative burst (Johann *et al.*, 2006). Macrophage recruitment to the site of infection is an important feature of disease progression, since very few macrophages are initially present it is important to recruit more to the site as well as facilitate amastigote phagocytosis. Apoptotic cells have been reported to recruit macrophages (Lauber *et al.*, 2003) and induction of host cell apoptosis could therefore represent a mechanism by which *Leishmania* recruits uninfected macrophages to the infection site and uses them to spread.

The intracellular amastigote, possibly after multiplication, could induce the mitochondrial apoptotic pathway. Consequently the apoptotic cascade would start from mitochondrial permeabilisation of the host cells, as confirmed by the results here. Interestingly, late apoptosis (DNA fragmentation) was not found during infection in any of the species studied. According to Nagata (Nagata, 2000) apoptosis can occur independently of DNA fragmentation and apoptosis related features can be present in the absence of nuclear degeneration. Moreover the production of apoptotic bodies, which would play a central role in the model described here, is not always related to DNA fragmentation (Nagata, 2000). Incomplete activation of macrophage apoptosis has been recently described during intracellular replication of other pathogens such as *Legionella pneumophila* (Abu-Zant *et al.*, 2005). This study shows that *Leishmania* induces incomplete apoptosis in the untreated host cell, during which mitochondrial permeabilization and PS expression are not followed by DNA fragmentation.

The effect of apoptosis on the spread of infection was shown by the finding that induction of apoptosis in infected cells increased the percentage of infected macrophages. As shown in Figure 6.4, following camptothecin B treatment the percentage of infected cells in the tested population significantly increased compared with the number of infected cells present in the same infected but untreated population.

Previous reports have shown that both visceralizing and cutaneous species are able to interfere with host cell apoptosis. Specifically to rescue bone marrow-derived macrophages deprived of macrophage-colony stimulating factor, M-CSF (Moore and Matlashewski, 1994; Akarid *et al.*, 2004) or treated with M-

CSF and a chemical apoptosis inducer staurosporin (Akarid *et al.*, 2004). Moreover *L. infantum* was reported to prevent induction of apoptosis in U937 cell lines following treatment with actinomycin D (Lisi *et al.*, 2005). On the other hand analysis of PBMC from patients with acute visceral leishmaniasis showed that the number of apoptotic monocytes was significantly higher when compared with healed subjects or healthy controls (Potestio *et al.*, 2004).

These apparently contradictory results can be explained on the bases of two considerations. First, the experimental conditions for all of the experiments showing inhibition of apoptosis were such that only cells where apoptosis was already induced were tested. Secondarily apoptosis was assayed within the first 24 hours from infection during which period it is likely to record the effect of the promastigotes on the host cells. The data presented here are the results of a 48 hours infection period. This prolonged infection time was chosen to investigate the action of the intracellular amastigotes. I speculate that although promastigotes of *L. major* and *L. amazonensis* induce inhibition of apoptosis on the host cells via activation of NF-kB and PI3-kB pathways (Akarid *et al.*, 2004; Ruhland *et al.*, 2007) amastigotes of *L. major*, *L. tropica* and *L. aethiopica* are responsible for an increase in apoptotical markers such as PS exhibition and mitochondrial permeabilization 48 hours after infection. It is therefore suggested that *Leishmania* is responsible for a tight control of the apoptotic processes in the host cell in a life-cycle-dependent way. Moreover, the state of the host cell might contribute to the parasite regulation of the host cell apoptosis. During conditions of stress *Leishmania* can reduce or delay apoptosis but when the cells are healthy and the parasite has reached the amastigote stage, *Leishmania* induces (or partially induces) apoptosis in order to spread to neighbouring cells.

6.4. Conclusions

Features of early apoptosis such as PS externalization and increased mitochondrial permeability were detected in monocyte-derived macrophages (PBDM) and in cell lines (THP-1 and U937) after infection with three different species of Old World *Leishmania*. DNA fragmentation was not observed, which

could indicate incomplete activation of apoptosis. Moreover, chemical induction of apoptosis on previously infected cells increased the number of infected macrophages.

The present study suggests that host cell apoptosis plays an important part in the survival and spreading of *Leishmania* parasites inside the human host and that the parasites exert a tight control on this process. Since apoptosis is a mechanism which takes place without stimulating an inflammatory response it can be speculated that this could be the mechanism through which cutaneous leishmaniasis-inducing species spread from macrophage to macrophage during the first silent phase of the infection (Chapter 8).

Chapter 7

Leishmania infection of THP-1 cells, a proteomic approach.

7.1. Introduction

This chapter examines the variation in macrophage protein expression during *Leishmania* infection. Two-dimensional Fluorescence Difference Gel Electrophoresis coupled with MALDI-TOF analysis was used to isolate, compare and identify expressed proteins. Proteomics has been used in the study of *Leishmania* parasite life cycles (Nugent *et al.*, 2004; Walker *et al.*, 2006), but has not previously been applied to the study of the infection process itself. Moreover, the recent development of the 2-D DIGE technique has increased the accuracy of the comparison of proteomes and made the procedure faster. The differential labelling of the samples before the run allows the running of two samples and one internal standard in each gel. This has not only limited experimental variation but has also significantly decreased the number of gels needed in each set of experiments. Moreover, when compared to standard two dimensional electrophoresis (2-DE) it led to a three fold increase in the number

of identified protein spots that were significantly altered in their level of expression (Karp *et al.*, 2004; Rathsam *et al.*, 2005).

This technique has been successfully used in many investigations including the analysis of host pathogen interactions. These studies have involved (a) intestinal cells infected with wild type or Type III *E. coli* (Hardwidge *et al.*, 2004), a study in which various proteins were characterized such as proteins involved in cytoskeletons; (b) cell adhesion and G-protein signalling; and (c) human macrophages infected with *Yersinia pestis*, *Y. pseudotubercolosis* and *Y. enterocolitica* (Chromy *et al.*, 2004; Zhang *et al.*, 2005).

The choice of a proteomic approach over gene expression studies was made on the basis of recently acquired knowledge that gene expression does not always correspond with differences in protein expression. During *L. chagasi* (and *L. donovani*) infection, for example, the increased production of TGF-β did not correlate with increased mRNA production but with cleavage of pro-TGF- β by amastigotes' cystein proteases to produce active TGF-β (Somanna *et al.*, 2002; Gantt *et al.*, 2003).

7.2. Results and discussion

In this study terminally differentiated THP-1 cells were infected with 3 different species of *Leishmania* parasites; cytosol proteins were extracted 48 hours after infection (Chapter 2) and divided on the basis of molecular weight (in Bristol University) and PI (in UEL) by 2-DE. Conditions were optimized and an analytical run of differentially labelled samples was performed. For each species two gels were run, each of which contained two samples and an internal standard, the only difference between the two being the reciprocal labelling, as described in Table 7.1.

Following establishment of infection, cytoplasm proteins were extracted from infected macrophages and from the uninfected control, labelled with the appropriate CyDyes mixed together and then a total of 150 μg of infected sample was run on to 2-D PAGE. Following excitation of the gel at λ = 488nm, 532nm

Gel number	Cy3	Cy5	Cy2
19565	Uninfected	+ *L. major* infected cells	Int. standard 1
19566	*L. major* infected cells	Uninfected	Int. standard 1
19567	Uninfected	+ *L tropica* infected cells	Int. standard 2
19568	+ *L tropica* infected cells	Uninfected	Int. standard 2
19572	Uninfected	+ *L. aethiopica* infected cells	Int. standard 3
19571	+ *L. aethiopica* infected cells	Uninfected	Int. standard 3

Table 7.1. DIGE experimental design:
6 gels were produced in order to study three infection profiles. Int standard 1 = Uninfected (U) + *L. major* infected cell; Int Standard 2 = U + *L. tropica* infected cells; Int standard 3 = U + *L aethiopica* infected cells.

and 633nm for Cy2, Cy3 and Cy5 respectively, three images were obtained from each gel, plus a fourth image given by overlaying the three, as reported in Figures 7.1 and 7.4 for gel 1965 and 1966, corresponding to THP-1 infected with *L. major* and in Appendix II for the remaining four gels. The images were analyzed through DeCyder software and the spots' volumes, corresponding to the protein concentrations, compared (In Bristol University).

Following DeCyder analysis of the infection of THP-1 cells with *L. major*, over 2000 proteins were detected. The overlay image of Gel 19565 (Figure 7.1) showed dark yellow spots which are proteins expressed in both the infected and uninfected samples. Spots appearing in green are representative of proteins expressed in Cy3 labelled uninfected THP-1 while red spots are representative of proteins only expressed during Cy5 labelled THP-1 infected with *L. major* (and *vice versa* in gel 19566, Figure 7.4). Proteins whose expression varied between 1.3 and 1.5-fold were considered constant during infection as this variation is typical of the inherent noise in the proteomic experiment. A minimum threshold of 1.5 was set. Under this requirement 3% of the total 2000 proteins separated in the gels 1965 and 1966 were identified as differentially expressed. Spots showing the highest differences in expression levels (Volume ratio, Tables 7.2 and 7.3) were excised from the gel and digested for mass spectrometry analysis (in Bristol University). Moreover only the spots showing an increase of expression in gel 1965 (example: spot 1867, table 7.2) that corresponded to a decrease in the expression on the reciprocally labelled gel 19566 (example: spot 1882, table 7.3) were considered representative of a significant differentially expressed protein. All the spots reported in Table 7.2 and 7.5 conform to this requisite. The spot numbers and whether their abundances increased or decreased compared to the Cy3 labelled uninfected control (or to Cy3 labelled *L. major* infected cells) are reported in Tables 7.2 and 7.3. On the basis of the intensity recorded during the scanning, each spot is associated with a 3D shape (peak, Figure 7.9): slope, maximum area, and maximum peak height are reported for each peak together with the volume ratio. The volume of the peak is a measure of the relative concentration of the proteins associated with the spot. Therefore volume ratio is a measure of concentration of the expression ratio of the same proteins between the Cy3 labelled sample and

the Cy5 labelled sample in the same gel. Differences in expression which correspond to differences in the volume of the spot are clearly visible on the 3D images as shown in Figure 7.9.

Following trypsin digestion a unique series of peptides was produced for each protein. The length of each peptide was identified by MALDI-TOF analysis (in Bristol), and the peptide mass fingerprint (PMF, Figure 7.10) was analyzed by Mascot (in UEL). The identity of each spot as determined by the software is reported in Table 7.4. With few exceptions, each of the identified proteins had a score equal to or greater than the 95% confidence limit threshold estimated by the software. The proteins identified by MALDI-TOF analysis were then located in the 2D gel and their PI and Molecular weight compared to the values predicted by Mascot. When the two sets of data corresponded the identity of the protein was confirmed. A total of 11 spots of the 75 excised during *L. major* infection (14.7%), 15 of the 78 (19.2%) during *L. aethiopica* infection and 14 of the 83 (16.9%) during *L. tropica* infection were identified under these requirements. The failure to identify most of the selected proteins can be attributed to several factors such as: low concentration of proteins on the gel or on the gel plug, experimental losses of proteins and/or peptides during the processing of the samples or too low confidence scores during identification by Mascot.

Induction of monocyte adherence during infection.

A further experiment was carried out in order to clarify the function of one of the proteins identified: BCDO2. This protein is down-regulated during infection and is involved in retinoic acid (RA) metabolism. A monocytic THP-1 cell line was terminally differentiated in macrophage-like cells by 72 hours incubation with RA. The down-regulation of this protein (BCDO2) which is involved in terminal differentiation of monocytes during infection suggested the possibility that the infection, could, like RA treatment, induce differentiation of monocytes into macrophages as discussed later. In order to investigate this hypothesis, human monocytes were isolated from blood, infected with *Leishmania* parasites and compared with uninfected negative controls for their ability to become adherent.

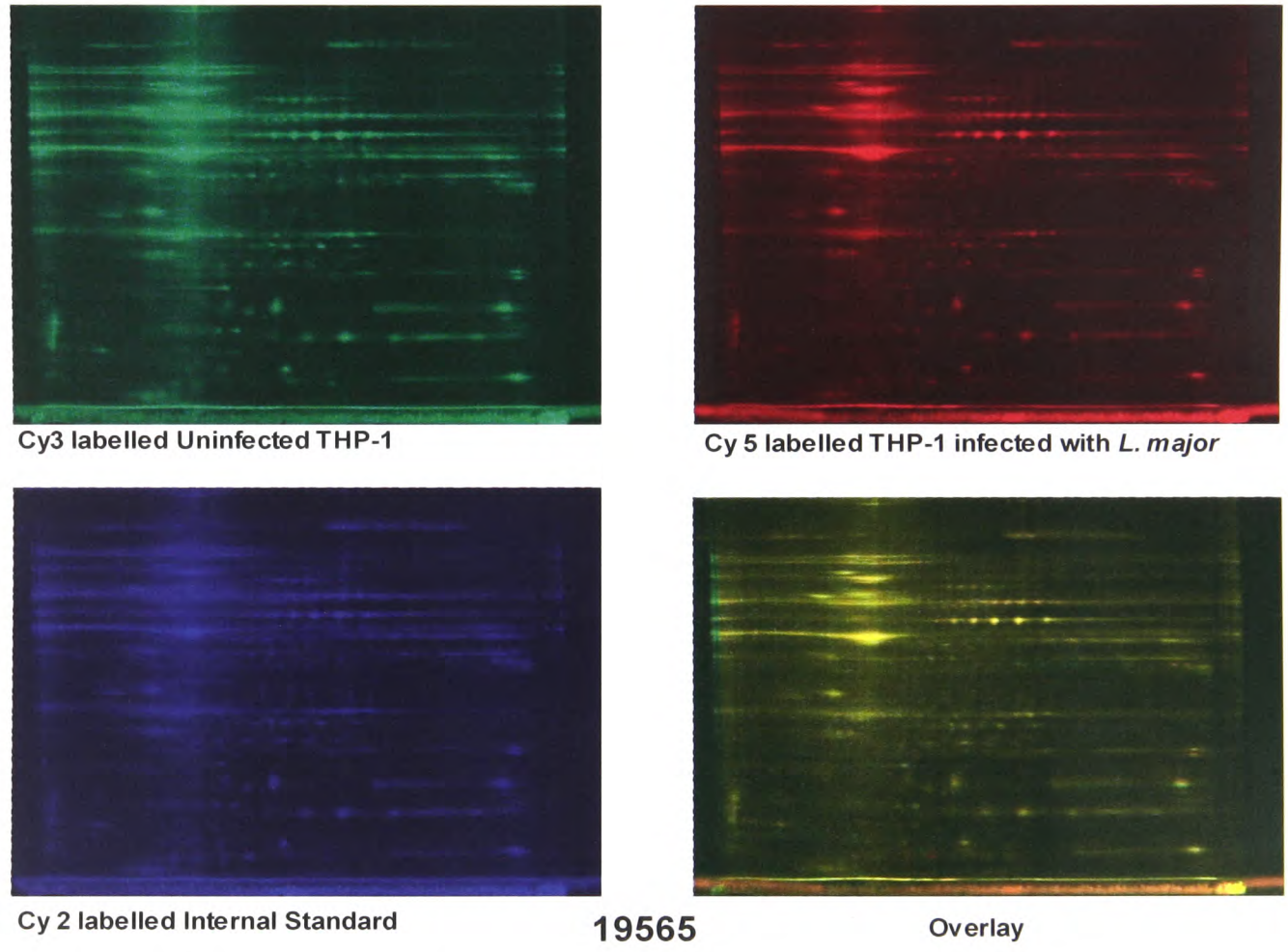


Figure 7.1 DIGE images of gel 19565.
Proteins derived from uninfected samples were stained with Cy3; proteins derived form cells infected with *L. major* were labelled with Cy5. The internal standard was provided by pooling the samples together. Finally, an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels, which appear as yellow spots while unique ones appear as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
522	Decreased	-4.64	0.47	608	688
1608	Decreased	-4.57	0.25	595	496
1708	Decreased	-4.24	0.23	1101	52
1155	Decreased	-4.22	0.17	566	300
1299	Decreased	-4.13	0.21	956	2706
600	Decreased	-4.09	0.42	631	584
1731	Decreased	-4.08	0.24	383	298
1199	Decreased	-4	0.27	691	619
837	Decreased	-3.99	0.27	696	381
1738	Decreased	-3.92	0.19	630	1708
316	Decreased	-3.76	0.62	362	1638
2021	Decreased	-3.7	0.3	761	652
985	Decreased	-3.57	0.21	717	3016
368	Decreased	-3.53	0.47	383	943
1354	Decreased	-3.37	0.17	1020	367
1733	Decreased	-3.28	0.28	432	301
1728	Decreased	-3.2	0.19	451	467
1674	Decreased	-2.94	0.18	351	703
746	Decreased	-2.58	0.15	518	701
756	Decreased	-2.35	0.11	253	839
1712	Decreased	-2.31	0.19	2071	1548
1782	Decreased	-2.11	0.22	1018	912
1671	Decreased	-2.1	0.18	692	1898
1786	Decreased	-2.07	0.17	641	371
764	Decreased	-2	0.1	368	257
1709	Decreased	-1.83	0.21	697	384
221	Decreased	-1.83	0.14	196	72
2664	Decreased	-1.79	0.22	2356	384
1781	Decreased	-1.75	0.2	1636	2736
2392	Decreased	-1.72	0.13	1210	393
1695	Decreased	-1.71	0.3	940	1065
2391	Decreased	-1.59	0.23	2914	279
2393	Decreased	-1.56	0.17	1855	757
1716	Increased	1.5	0.18	605	218
744	Increased	1.53	0.64	540	2352
1651	Increased	1.53	0.18	576	332
572	Increased	1.54	0.25	336	384
571	Increased	1.54	0.2	524	467
1109	Increased	1.55	0.22	666	467
1849	Increased	1.55	0.18	857	1032
1584	Increased	1.58	0.18	526	708
652	Increased	1.59	0.18	261	590
352	Increased	1.68	0.51	382	657
1704	Increased	1.68	0.21	1308	298

827	Increased	1.69	0.15	269	1073
641	Increased	1.76	0.2	508	492
1714	Increased	1.8	0.84	900	2414
1877	Increased	1.8	0.14	761	364
830	Increased	1.82	0.11	326	844
653	Increased	1.87	0.21	421	616
2707	Increased	1.88	0.21	1515	279
1867	Increased	1.92	0.15	1182	1220
479	Increased	1.97	0.36	453	19880
839	Increased	2.04	0.21	361	52
1581	Increased	2.06	0.15	866	515
478	Increased	2.07	0.37	572	15316
473	Increased	2.07	0.35	647	19643
823	Increased	2.13	0.18	272	643
472	Increased	2.16	0.43	385	2475
1586	Increased	2.18	0.95	783	2754
1875	Increased	2.18	0.14	1027	274
829	Increased	2.19	0.17	336	504
1873	Increased	2.48	0.16	740	1783
239	Increased	2.51	0.11	155	1049
1872	Increased	2.53	0.14	562	1447
2721	Increased	2.68	0.2	1808	384
1874	Increased	2.71	0.16	555	666
1876	Increased	2.72	0.17	776	552
1587	Increased	2.74	0.19	657	478
1590	Increased	2.76	0.19	669	568
1868	Increased	2.98	0.32	762	514
2730	Increased	3.11	0.18	897	1292
2723	Increased	3.39	0.2	3787	912
1870	Increased	3.63	0.14	430	982
2736	Increased	4.13	0.32	1085	18078

Table 7.2. Differentially expressed spots following DeCyder analysis of gel 1965. Examples of spots which were chosen for MALDI-TOF are shown in red. Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 1966 gel.

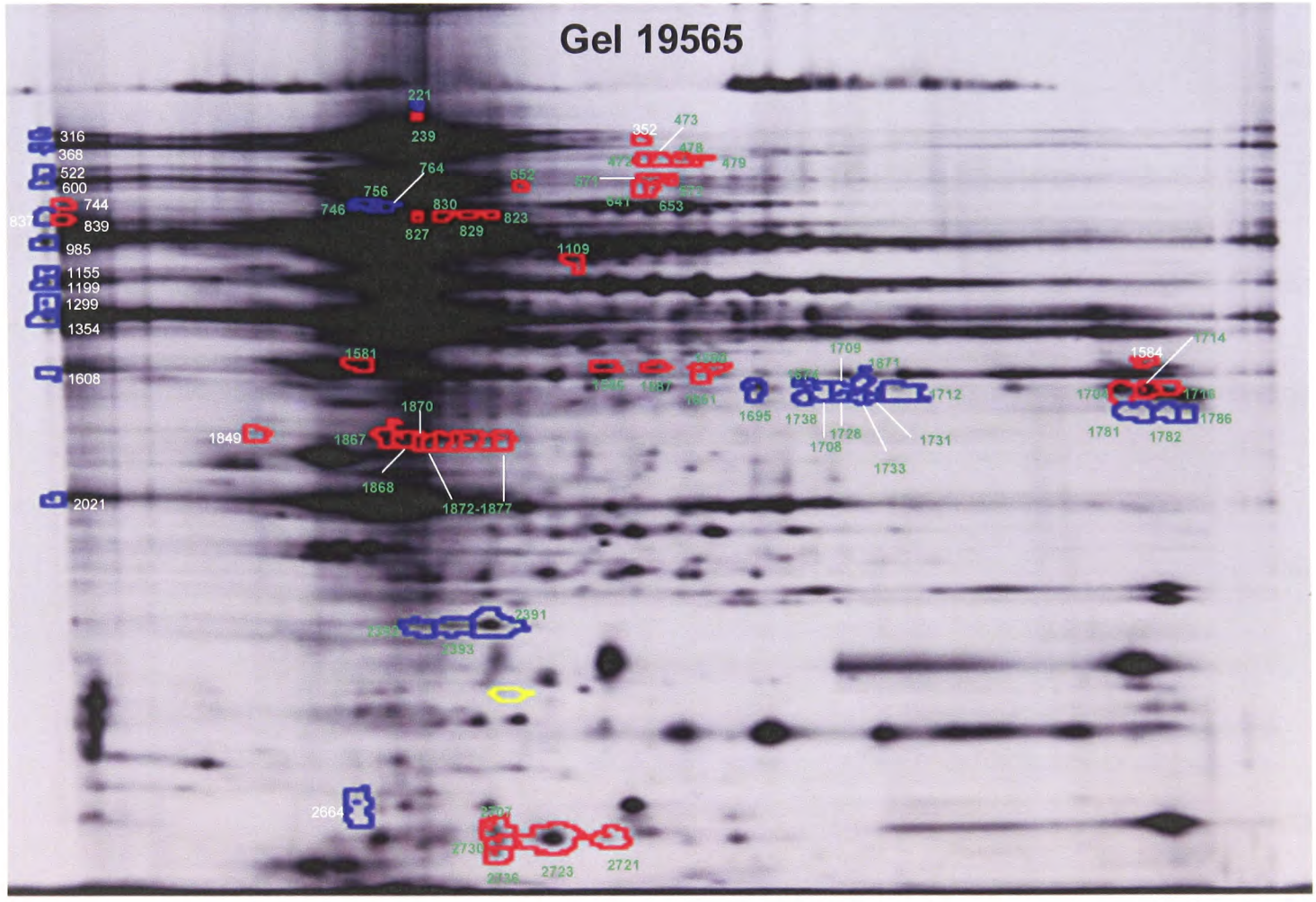


Figure 7.2. Gel 19565, differentially expressed proteins are circled
Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentrations decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

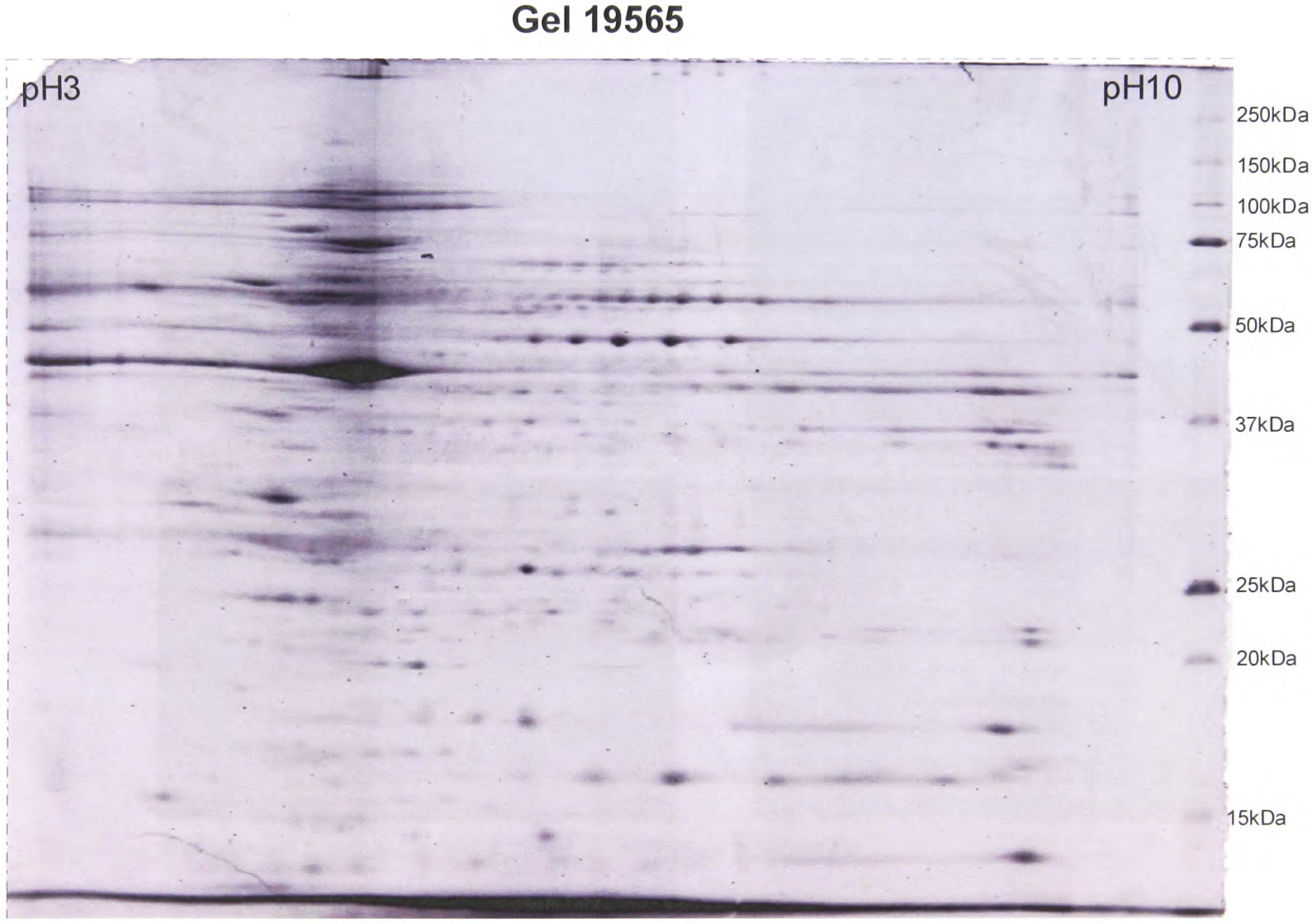


Figure 7.3 Gel 19565 following Deep Purple staining.
Post run Deep Purple fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.

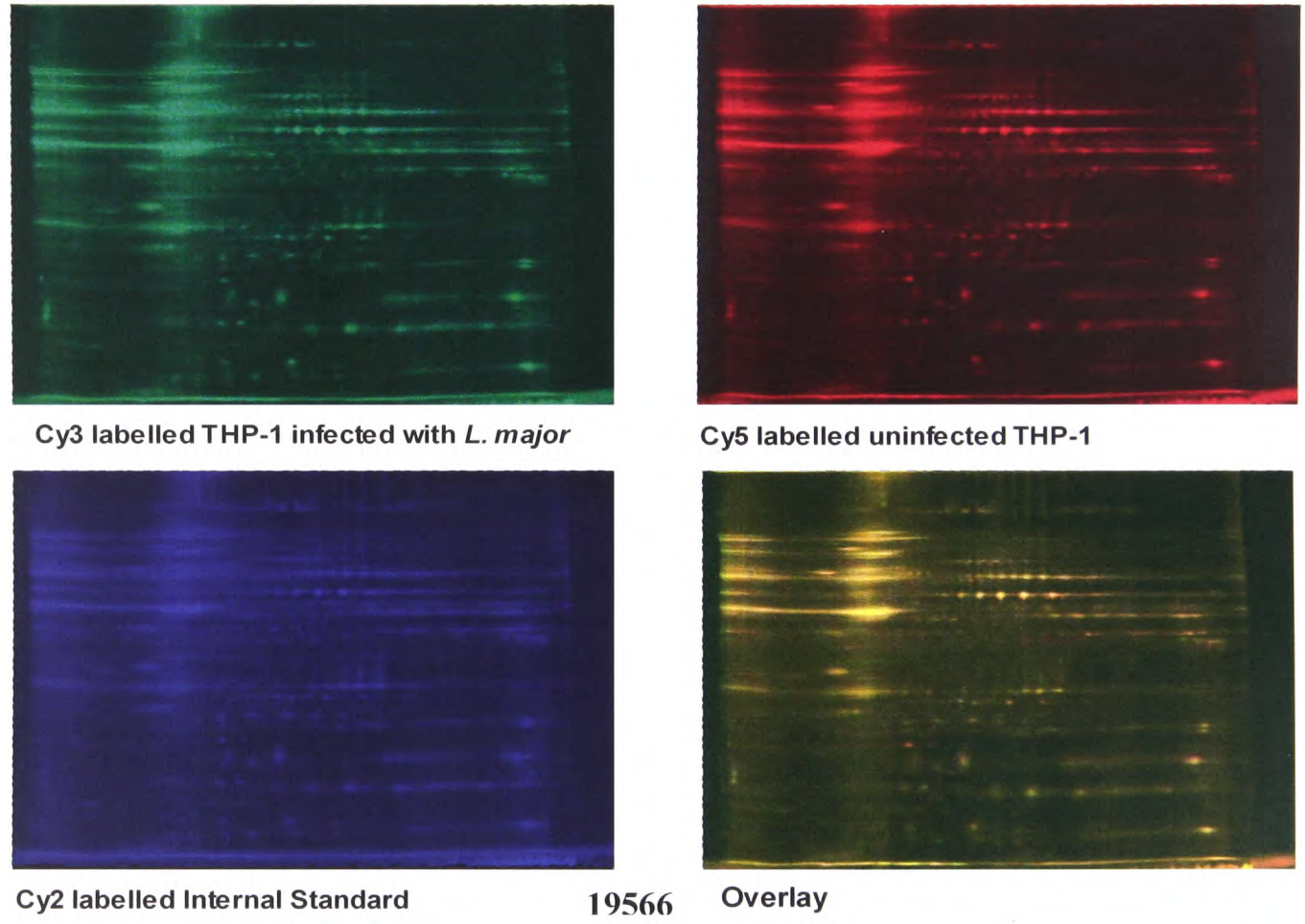


Figure 7.4. DIGE images of gel 1966.
Proteins derived from uninfected samples were stained with Cy3; proteins derived from cells infected with *L. major* were labelled with Cy5. The internal standard was obtained by pooling the samples together. Finally, an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels appearing as yellow spots, while unique ones appeared as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
1875	Decreased	-5.49	0.16	397	827
2811	Decreased	-5.39	0.2	1504	594
1878	Decreased	-4.9	0.15	517	613
1883	Decreased	-4.66	0.12	893	3106
2791	Decreased	-4.41	0.16	2113	950
1873	Decreased	-3.92	0.18	384	523
1874	Decreased	-3.91	0.19	379	2503
1546	Decreased	-3.89	0.19	574	1854
2796	Decreased	-3.31	0.24	1060	422
1867	Decreased	-3.15	0.26	875	675
1547	Decreased	-2.55	0.18	415	272
1545	Decreased	-2.42	0.18	752	1623
584	Decreased	-2.37	0.25	274	700
506	Decreased	-2.16	0.35	446	262
1882	Decreased	-2.14	0.13	637	521
915	Decreased	-1.93	0.34	539	5320
2798	Decreased	-1.91	0.21	3546	1789
2780	Decreased	-1.91	0.18	1896	491
507	Decreased	-1.87	0.33	626	3729
620	Decreased	-1.87	0.22	340	1424
508	Decreased	-1.86	0.38	637	1195
1577	Decreased	-1.85	0.17	1323	155
1661	Decreased	-1.81	0.26	1052	1072
988	Decreased	-1.79	0.25	369	471
527	Decreased	-1.75	0.51	1756	3131
792	Decreased	-1.74	0.38	645	9703
816	Decreased	-1.74	0.27	586	1167
787	Decreased	-1.69	0.15	296	3381
999	Decreased	-1.68	0.15	359	679
397	Decreased	-1.63	0.34	831	11077
505	Decreased	-1.63	0.28	847	486
295	Decreased	-1.63	0.2	225	440
1590	Decreased	-1.63	0.18	493	1329
1662	Decreased	-1.61	0.21	818	2675
1548	Decreased	-1.59	0.15	651	287
626	Decreased	-1.56	1.27	693	1779
2463	Decreased	-1.55	0.28	1272	562
1865	Decreased	-1.53	0.15	1764	817
631	Decreased	-1.53	0.21	332	3943
1749	Increased	1.51	0.24	813	644
1646	Increased	1.52	0.2	1636	668
2461	Increased	1.54	0.17	1114	572
1667	Increased	1.59	0.29	689	1826

2477	Increased	1.59	0.24	1151	148
1480	Increased	1.61	0.27	889	51
1072	Increased	1.64	0.11	320	702
756	Increased	1.65	0.16	343	565
1046	Increased	1.66	0.12	469	732
1625	Increased	1.73	0.18	899	444
1764	Increased	1.75	0.2	1356	649
793	Increased	1.76	0.22	287	1621
1067	Increased	1.76	0.21	554	4560
261	Increased	1.77	0.15	269	757
1748	Increased	1.83	0.34	985	12387
1460	Increased	1.89	0.37	372	364
1068	Increased	2	0.13	305	893
2471	Increased	2.08	0.23	786	973
1652	Increased	2.08	0.21	512	913
2479	Increased	2.12	0.17	1864	502
2470	Increased	2.13	0.21	1098	3843
2465	Increased	2.18	0.24	1227	180
2517	Increased	2.19	0.19	1332	552
1658	Increased	2.21	0.17	1771	749
2475	Increased	2.24	0.21	448	1024
2466	Increased	2.25	0.2	1998	765
2469	Increased	2.28	0.24	401	614
1705	Increased	2.29	0.38	469	890
817	Increased	2.31	0.26	327	1076
1700	Increased	2.33	0.18	1344	1121
2476	Increased	2.42	0.2	588	545
750	Increased	2.45	0.18	273	1266
751	Increased	2.59	0.19	331	1300
1715	Increased	2.64	0.23	769	458
1630	Increased	2.81	0.16	404	488
1704	Increased	3.66	0.19	373	668
1657	Increased	5.08	0.22	811	707

Table 7.3. Differentially expressed spots following De-Cyder analysis of gel 1966. Examples of spots which were chosen for MALDI-TOF are shown in red. Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 1965 gel.

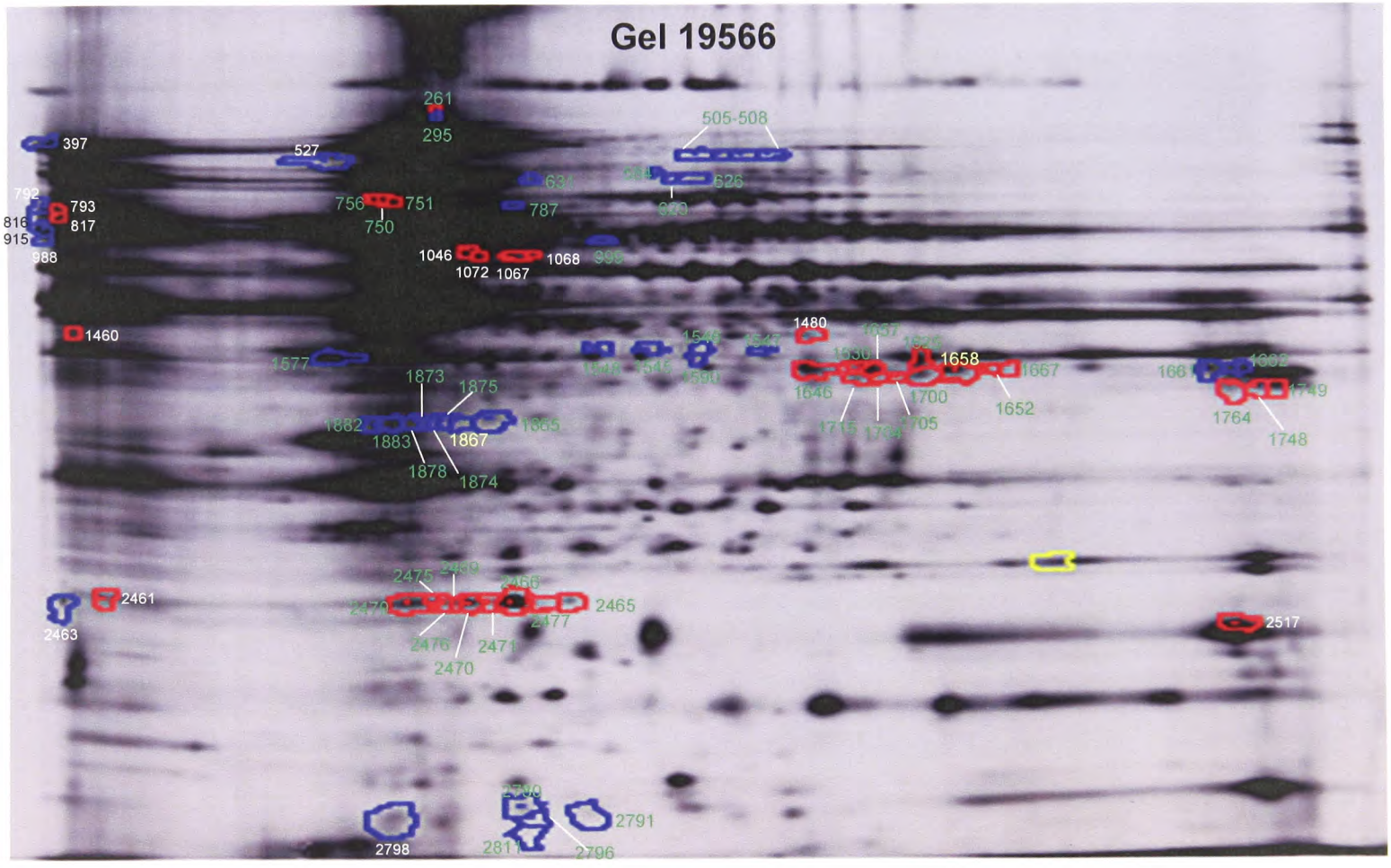


Figure 7.5 Gel 19566, differentially expressed proteins are circled.
Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentration decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

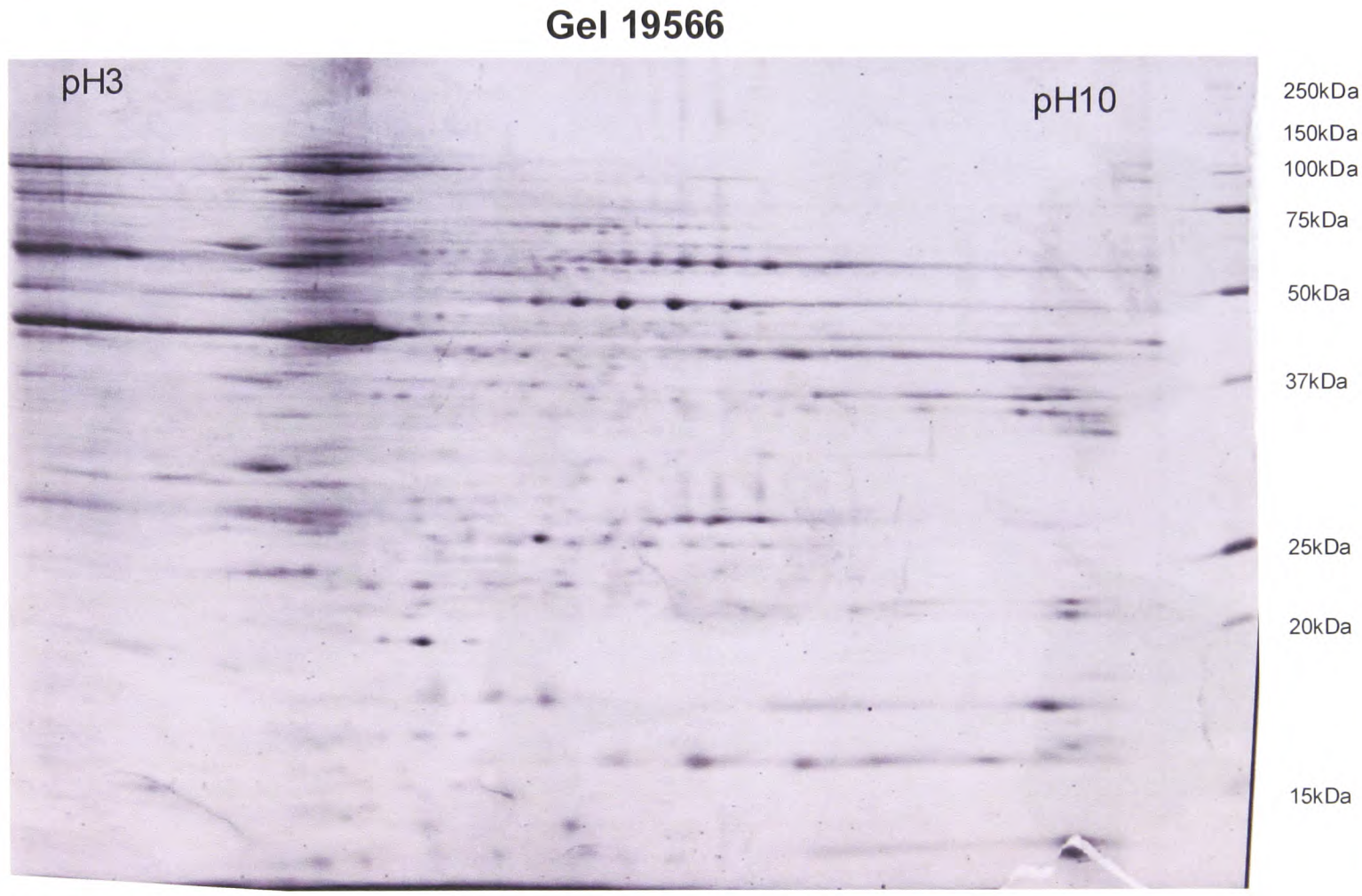


Figure 7.6. Gel 19566 following Deep Purple staining.
Post run Deep Purple (GE Healthcare, UK) fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.

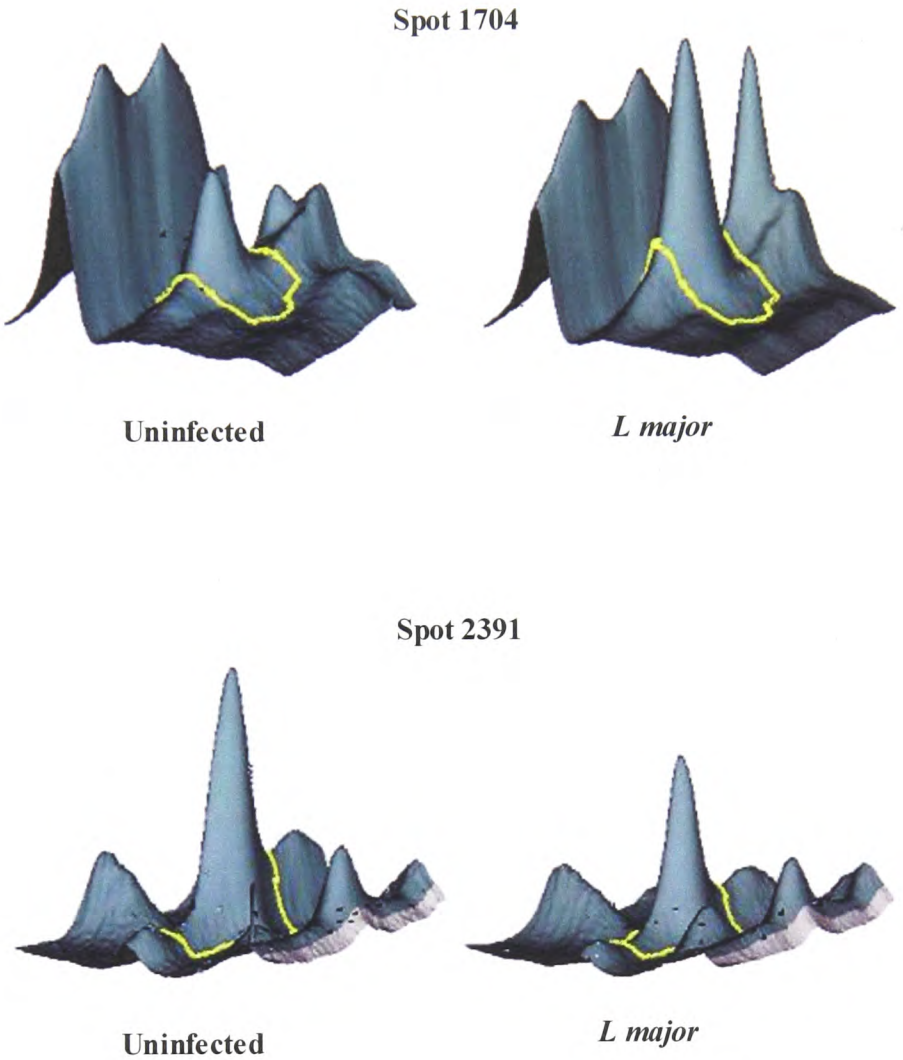


Figure 7.7. Examples of 3D views of two spots.
DeCyder analysis of spots corresponding to proteins which are differentially expressed in infected and uninfected samples.

Spot 2391.

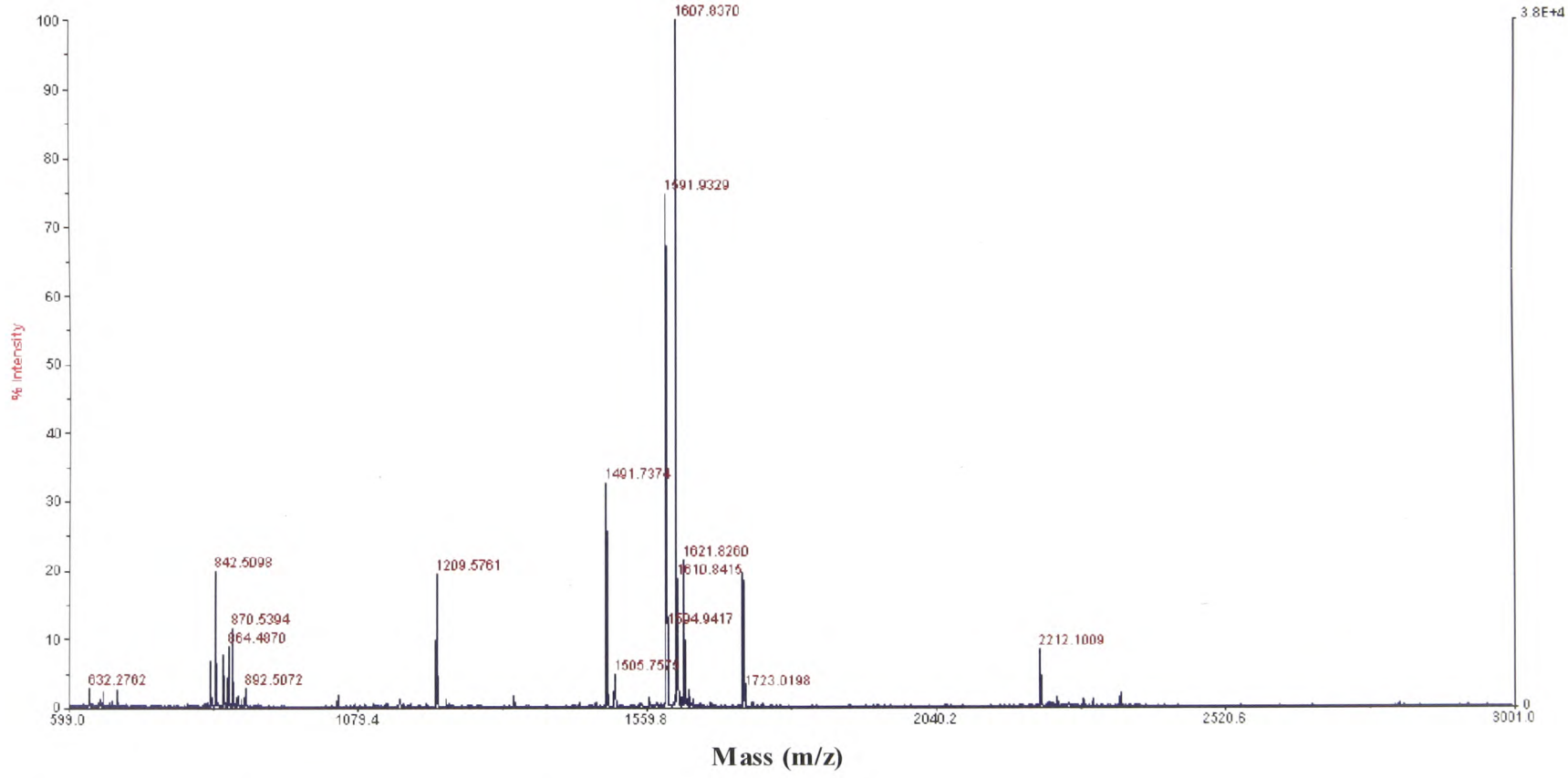


Figure 7.8. MALDI-TOF analysis of spot 2391.

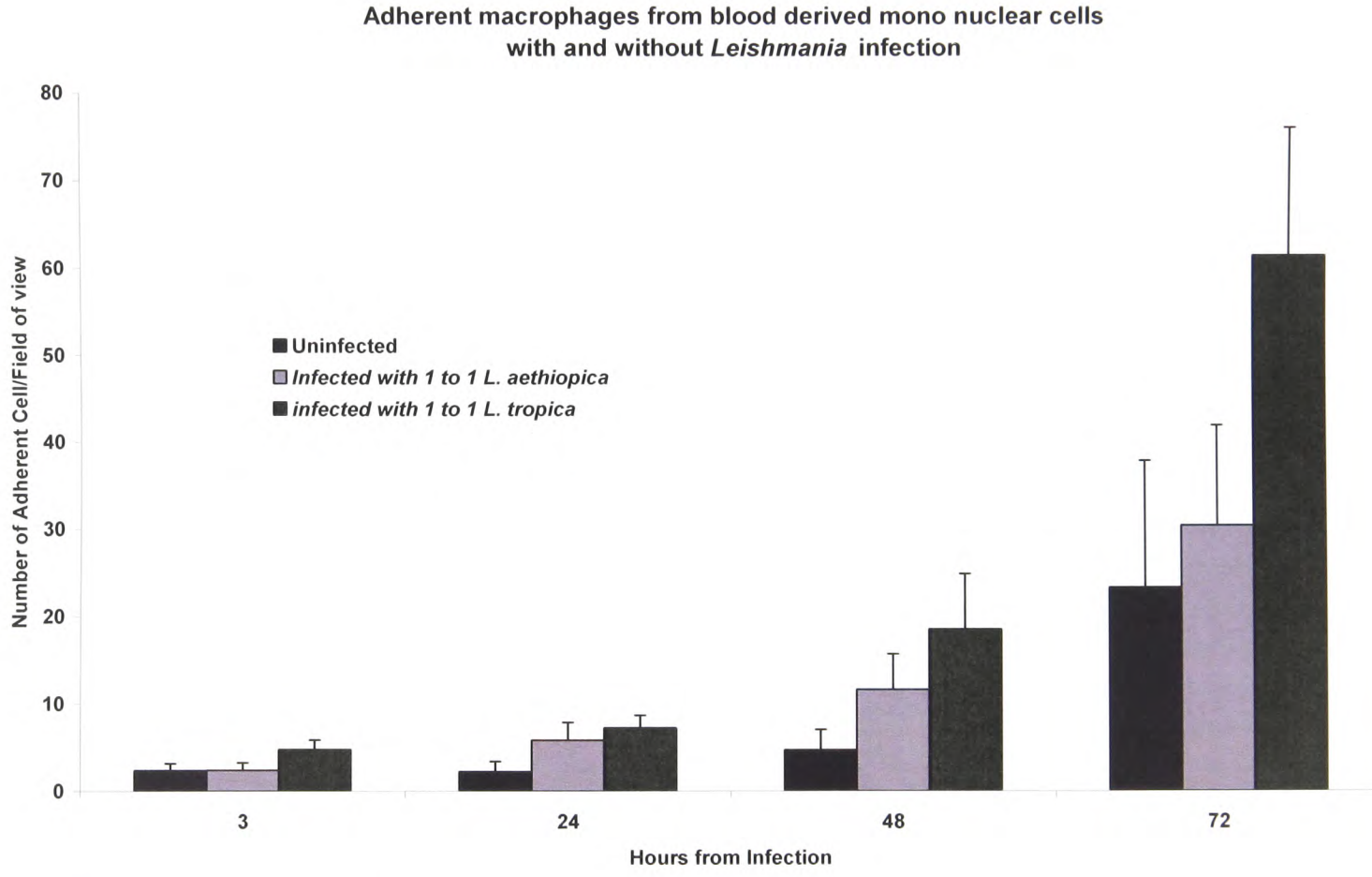


Figure 7.9. Infection-induced adherence of blood derived mononuclear cells.
Number of adherent macrophages per field following infection of blood derived monocytes with *L. aethiopica* and *L. tropica* and left untreated for 3. 24. 48 and 72 hours from isolation.

MALDI analysis of spots selected for significant changes of intensity following *Leishmania aethiopica* infection of THP-1 cells.						
Spot n°	**Protein name**	**Score**	**Mass**	**PI**	**Protein function**	**Fold difference**
172	TERA_HUMAN transcriptional endoplasmatic reticulum ATPase (15S Mg(2+)-ATPase p97 subunit)	80	89135	5.14	Valosin containing protein (VCP) is a member of a family that includes putative ATP-binding proteins involved in vesicle transport and fusion, 26S proteasome function and assembly of peroxisomes. It is associated with clathrin and Heat shock protein Hsp70. It has been implicated in a number of cellular events including homotypic membrane fusion, spindle pole body function and ubiquitin dependent protein degradation (Ye *et al.*, 2004; Oda *et al.*, 2006; Matsuoka *et al.*, 2007)	-1.75
253	Heat Shock protein HSP 90-alpha 4	122	84607	4.94	It is found in *Leishmania donovani*, in which, on deactivation, it leads to growth arrest and to synthesis of HS proteins (Wiesgigl and Clos, 2001). HSP-90 induces the nuclear translocation of NF-kappa and expression of TNF in macrophages (Basu and Srivastava, 2003).	+2.43
254	Heat Shock protein 90 KDa protein 1, beta	120	83212	4.97	"	+2.43

1001	CD207 antigen, langerin	72	36674	8.45	Expressed in Langherans cells, localized in the Birbeck granules. It is a C-type lectin with mannose binding specificity. The binding of mannose by this protein leads to internalization of antigen into Birbeck granules providing access to a non-classical antigen-processing pathway (Turville *et al.*, 2002; Verdijk *et al.*, 2005).	+1.6
1005	ACTB protein	62	40194	5.55	Beta actin is one of the 6 different actin isoforms which have been identified. ACTB is one of the 2 non-muscle cytoskeletal actins. Actins are highly conserved proteins involved in cell motility, structure and integrity. Alpha actins are a major constituent of the contractile apparatus (Ohmori *et al.*, 1992; Kimura *et al.*, 2000; Ibarrondo *et al.*, 2001).	+1.85
1003	"	52	"	"	"	+1.88
1011	"	77	"	"	"	+2.33
1013	"	58	"	"	"	+1.71
1015		93	"	"	"	+1.63
1730	CGI-29 protein	35	27094		This protein is found both in human tissues and in *Leishmania major* (Lai *et al.*, 2000; Cho D.H. *et al.*, 2004)	+1.9

1258	Mannosidase, alpha, class1A, member2	35	38900	8.69	Primarily found in the endoplasmatic reticulum, catalyses the removal of three distinct mannose residues from peptide-bound Man9-GlcNAc2 oligosaccharides producing a single Man6 isomer (Bause *et al.*, 1992). It catalyzes the first mannose trimming step in the processing of mammalian Asn-linked oligosaccharides	+1.58
1351	Human muscle fructose I,6-biphosphate aldolase complexed with.. Aldolase A	69 69	39264 39395	8.39 8.30	Interacts with ATPase which is essential for acidification of intracellular compartments (Lu *et al.*, 2001). It binds with SHP-1 which has been implicated in the pathogenesis of infection with *Leishmania* (Nandan *et al.*, 2007).	+3.25
1371	Human muscle fructose 1,6-biphosphate aldolase	69	39264	8.39	"	+1.76
1344	"	low	"	"	"	
1301	Mitochondrial Malate dehydrogenase precursor	69	35509		It is involved in conversion of Malate to oxaloacetate in the tricarboxilate cycle, which is the major energy generating pathway in the cell (Shimokata *et al.*, 1997).	-1.72

MALDI analysis of spots selected for significant changes of intensity following *Leishmania tropica* infection of THP-1						
1729	MORC3 protein + nuclear autoantigen RA33=A2 hnRNP homolog	52	28728 2371	4.55 9.62	Also known as: zinc finger, CW-type with coiled-coil domain 3. It is a transcription factor (Nagase *et al.*, 1995; Perry and Zhao, 2003)	+6.95
1732	Alpha enolase	49	36286	6.53	is a 48-kDa protein, which plays a critical role in the glycolytic pathway (Subramanian and Miller, 2000)	+8.35
1789	Human Muscle Fructose 1,6-Bisphosphate Aldolase Complexed with Fructose 1,6-Bisphosphate	78	39264	8.39	Fructose 1,6-bisphosphate aldolase catalyzes the reversible cleavage of fructose 1,6-bisphosphate and fructose 1-phosphate to dihydroxyacetone phosphate and either glyceraldehyde 3-phosphate or glyceraldehyde, respectively (Dalby *et al.*, 1999)	+10.46
1797	Human Muscle Fructose 1,6-Bisphosphate Aldolase Complexed with Fructose 1,6-Bisphosphate	41	39264	8.39	"	+10.14
1808-9	"	88	"	"	"	+6.5
1813	similar to heterogeneous nuclear ribonucleoprotei n A2/B1 isoform 2 isoform 4	79	35984	8.67	Involved with pre-mRNA processing. Forms complexes (ribonucleosomes) with at least 20 other different hnRNP and heterogeneous nuclear RNA in the nucleous (Hutchison *et al.*, 2002; Ishikawa *et al.*, 2004).	+4.58

2138	chloride intracellular channel 1	67	23287	5.02	Acts as a chloride ion channel, if found in the nucleus; specifically in the nuclear membrane. It is also found in the cytoplasm, specifically in the cell membrane (Singh *et al.*, 2007).	+4.34
2138	p64 CLCP syn: chloride intracellular channel protein1	67	23528	5.12	"	+4.34
2173	Gamma-actin Syn ACTB	71	25862	5.65	"	+5.57
2179	"	40	"	"	"	
2213	Chain B, Triosephosphate Isomerase (Tim) (E.C.5.3.1.1) Complexed With 2-Phosphoglycolic Acid	63	26522	6.51	Belongs to the triosephosphate isomerase family. Its catalytic activity converts D-glyceraldehyde 3-phosphate in glycerone phosphate (Alvarez *et al.*, 1998).	+7.67
2308	Chain D, Human Glyoxalase I Complexed with S-P-Nitrobenzyloxy carbonylglutathione	112	20633	5.12	Has lactoylglutathione lyase activity Is involved in the metabolism of carbohydrate (Creighton and Hamilton, 2001)	+4.26
2317	Chain D, Human Glyoxalase I Complexed with S-P-Nitrobenzyloxy carbonylglutathione	84	20633	5.12	"	+4.7

MALDI analysis of spots selected for significant changes of intensity following *Leishmania major* infection of THP-1 Macrophages						
1109	HNRPH1 Heterogeneous nuclear ribonucleoprote in H	134	49099	5.79	"	+1.5
1590	Annexin A1	79	38690	6.57	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. This protein regulates phospholipase A2 activity. It seems to bind from two to four calcium ions with high affinity. When phosphorylated by protein kinase C, epidermal growth factor receptor/kinase and TRPM7. Phosphorylation results in loss of the inhibitory activity (Kaplan *et al.*, 1988; Oshry *et al.*, 1991; Ferrières *et al.*, 1994; Rothhut *et al.*, 1995).	+1.55
1704	Heterogeneous nuclear ribonucleoprote in A2/B1 isoform A2	207	35984	8.67	"	+1.68
1712	Same as above	96	35984	8.67	"	-2.31
1714	"	190	"	"	"	+1.8
1716	"	92	"	"	"	+1.5
1728	BCDO2 protein	50	54582	8.96	This protein is involved in RA metabolism (Nagao, 2004).	-3.2

1867	ChainC, Annexin V	114	35783	4.94	This protein is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade (Grundmann *et al.*, 1988). It showed anti-apoptosis ability and is involved in signal transduction (Schlaepfer *et al.*, 1992; Kheifets *et al.*, 2006).	+1.92
2391	Ferritin light subunit	74	16384	5.65	Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation. Defects in FTL are the cause of hereditary hyperferritinemia-cataract syndrome (HHCS). Affected patients have elevated levels of circulating ferritin. Ferritin might also be a mediator of apoptosis (Brown *et al.*, 1983; Beaumont *et al.*, 1995; Bresgen *et al.*, 2007).	-1.59
2392	"	70	"	"	"	-1.72
2393	"	68	"	"	"	-1.56

Table 7.4. Differentially expressed proteins identified by MALDI-TOF analysis. Spots detected following infection with L. aethiopica, L. tropica and L. major were trypsin digested, and the PMF obtained was used to find the corresponding protein by MASCOT search.

The number of adherent cells following *L. tropica* infection significantly increased after 3 hours (P=0.006), 24 hours (P=0.000043), 48 hours (P=0.000186) and 72 hours (P=0.005) incubation with 1 to 1 parasite to human cell ratio. The increase of adherent cells following *L. aethiopica* infection under the same conditions was limited to 24 hours (P=0.031) and 48 hours (P=0.019) incubation.

Infection with various *Leishmania* spp. can result in different disease outcomes which are related to the infecting parasite and the immune response of the host at the site of infection. Given that the main contact between *Leishmania* and host occurs via the macrophage it is possible that the various disease manifestations occur as a result of differing responses to this interaction. This chapter examines the macrophage response to three species of *Leishmania* compared with a steady state control using a proteomic approach.

Since the discovery more then 40 years ago that *Leishmania* enters and multiplies inside host macrophages, this interaction has been widely studied. It is known that once inside the macrophages *Leishmania* are protected from the killing and the degradative activities of macrophages mainly by the action of two surface determinants: LPG and gp63. Specifically, LPG transiently delays fusion of the phagosome with the lysosome (Desjardins and Descoteaux, 1997), allowing generation of amastigotes. The degrading phagolysosome enzymes are inhibited by gp63 (Seay *et al.*, 1996). Once inside the phagolysosome, transformation into amastigotes occurs which enables the parasites to survive at low pH, in the presence of N_2O_2, NO_3 and lysosomal enzymes (Alexander and Vickerman, 1975; Chang and Dwyer, 1978; Antoine *et al.*, 1990; Russell *et al.*, 1992). Nevertheless, to date little information is available regarding the proteins involved in the host-pathogen interaction especially after infection has been established.

Following infection by the three pathogens, a varied protein expression profile was observed (Table 7.4). This is not surprising as it is well known that different species of *Leishmania* behave differently; they have different optimal growth conditions, different susceptibilities to drugs and are responsible for different host responses. Such phenomena (unique expression profiles of human macrophages to phylogenetically distinct parasites) have been seen with other human pathogens using alternative techniques such as micro-arrays (Chaussabel *et al.*, 2003). Despite specific differences in individual proteins it is apparent that all species examined in this study up-regulate families of proteins such as

transcription factors and heat shock proteins. *L. aethiopica* and *L. tropica* infections result in a three fold increase in fructose 1,6 bi-phosphate aldolase. Interestingly, infection by *L. major* did not result in any detectable up-regulation of glycolytic enzymes. However, this may be due to the selective criteria used for choosing the spots to analyse. Specifically, proteins showing the highest differential expression within each species were selected for further analysis over spots common to all six gels.

Similarly, infections with *L. aethiopica* and *L. tropica* result in up-regulation of proteins involved in cytoskeleton architecture. *L. aethiopica* increases the expression of ACTB protein two fold and *L. tropica* five fold. These are highly conserved proteins and are involved in cell motility, structure and integrity. This up-regulation may be a consequence of large phagosome development due to the multiplying parasite. Alternatively, it is possible that the re-arrangements are due to parasite-containing phagosomes moving from the cytosol towards the cell membrane prior to release of the pathogen, a phenomenon which has been described by Rittig *et al.* (1998). Re-arrangements of the cytoplasm are also necessary during apoptosis driven production of apoptotic bodies.

L. aethiopica infection resulted in an increased level of HSP 90 which is involved in NF-kB activation (Basu and Srivastava, 2003). As described in detail in Chapter 6 (Figure 6.3) activation of NF-kB sets this protein free to move towards the nucleus and to act as transcription factor for anti-apoptotic genes which produce inhibitors of apoptotic proteins such as C-Flip, IAPs and Bfl-I/A1. The overall action of these proteins is to inhibit caspases 3 and 9, prevent release of cytochrome C and inhibit the activation of Caspase 8. Up-regulation of NF-kB confirms that *Leishmania* infection is responsible for a tight regulation of the apoptotic process. Infection with *L. major* also induced expression of Annexin V, another inhibitor of apoptosis. If *Leishmania* interact with the host apoptotic pathways, probably by partial activation of apoptosis, as suggested by the theory proposed in this thesis, pro- and anti-apoptotic stimuli would need to act together in order to balance the apoptotic outcome. Over expression of Annexin V and HSP 90 suggested that these molecules might be implicated in the regulation of host cell apoptosis during infection.

It is also interesting to underline that generally there were very few proteins involved in the inflammatory response detected by DIGE, as expected since the

interactions between macrophages and virulent strains of *Leishmania* is known to lead to immunosuppression (Dey *et al.*, 2007).

Infection of macrophages by *L. major* resulted in a reduction in levels of BCDO2. This protein is involved in RA metabolism. Exposure of THP-1 cell lines to RA results in the terminal differentiation into macrophages. This occurs because RA binds to intracellular receptors termed RAR which are ligand-activated transcription factors resulting in arrest of the cell cycle. Since BCDO2 may compete with RAR for retinoic acid it is conceivable that a down-regulation of BCDO2 will facilitate increased differentiation into macrophages, thereby increasing the potential number of hosts for the *Leishmania.* Indeed infection of blood derived monocytes with *Leishmania* resulted in an increased number of macrophages as compared to uninfected cells alone (Figure 7.9).

7.4. Conclusions

To conclude, DIGE analysis was successful in providing an overview of various aspects of *Leishmania* infection. The up-regulation of proteins involved in metabolisms and in processes such as the acidification of the phagolysosome was detected confirming that this technique can detect well known features of the infection process.

Moreover increased production of apoptotic regulators such as Annexin V and NF-kB (via up-regulation of HSP90) in *L. major* and *L aethiopica* infection respectively, indicates the presence of new mechanisms of action developed by *Leishmania* parasites in the infection process. Both *L. aethiopica* and *L. tropica* also increased production of proteins involved in cell motility, structure and integrity (ACTB) which again can represent another feature of the same process: the production of apoptotic bodies. This discovery can be extremely significant in understanding the mechanism through which intracellular amastigotes silently spread to uninfected macrophages. We have suggested the possibility that apoptosis is involved and investigated whether *Leishmania* can interfere with its regulation. The data obtained from the comparative proteomics study confirm that the parasites control apoptotic regulators.

During *L. major* infection BCDO2 proteins were down-regulated by a factor of three suggesting another mechanism of action exploited by the parasites: that infection would induce monocytes' terminal differentiation into macrophages. This possibility was further investigated and a first set of experiments conducted in blood derived human macrophages confirmed it.

The results obtained confirmed that comparative proteomics is a powerful instrument in the study of the infection process. Although much information was obtained from this set of experiments it is important to remember that more detailed analysis is possible by further exploitation of the proteomic approach. For example, additional analysis involving examination of narrower PI intervals and a fractionation of the cells' compartments before protein extraction would increase the number of proteins detected. Detection of a wider range of proteins would allow identification of new differentially expressed proteins and acquisition of further details about the infection process.

Chapter 8

Summary, Conclusions and Comments on a New Model of *Leishmania* Spread

The interaction between *Leishmania* parasites and their mammalian host cells, the macrophages, is the starting point of a long term relationship. Following infection with the parasites the disease can cause different symptoms which are classified into two main syndromes, as previously described. The common path to infection following the sandfly bite involves accessing the same mammalian cells (monocytes, Langherans cells etc.) multiplying inside the macrophages and spreading to uninfected cells. Differences in the spreading pattern of the parasites cause differences in the disease outcome. Parasite spread can be limited to the sandfly bite location or can spread to different parts of the skin, or to mucocutaneous membranes or to internal organs. What causes these differences is yet to be fully understood. Macrophages are the main host of the parasites and the only cell type where amastigotes can multiply. It is therefore likely that those cells are involved at least in part in the determination of the infection outcome. As reported in the introduction, the mechanism through which the parasites leave the infected macrophages to spread to neighbouring cells has not been looked at in detail. This study follows the assumption that this mechanism plays a substantial

part in the development of disease manifestations and should therefore be investigated. The aim of this research is towards filling the gap in the knowledge of how intracellular parasites spread to uninfected macrophages.

An *in vitro* model was chosen and thoroughly investigated, consisting of the use of two monocytic cell lines (THP-1 and U937) terminally differentiated in macrophage-like cells. The most significant results were further checked in blood derived human macrophages. Confirming the results in three systems allowed exclusion of the possibility that the data were specific to the choice of the system used and that they were thus only due to the interaction itself. The infection process of *Leishmania* parasites inside THP-1 and U937 cell lines was analyzed during the first part of this study. The data confirmed that all three species, when in stationary phase, infected, and were able to multiply inside, the cell lines chosen. Moreover, it was established that 48 hours after the infection at cell ratios of ten parasites to one, *Leishmania* parasites had established themselves inside the macrophages and were likely to start spreading (Chapter 3). Therefore all the following experiments were conducted at this time. Although a reasonable range of data was available in the literature, setting up a correct model on the basis of which the study was designed was of fundamental importance.

A second important instrument in the study of this interaction was the development of axenic cultures of *Leishmania* amastigotes. Availability of cell-free amastigotes has proved important in research on leishmaniasis and various media have been developed in order to support the growth of different species of *Leishmania* (as described in detail in Chapter 4). I described for the first time that a modification of the medium JH30 (Pan, 1984) to culture *L. mexicana* axenic amastigotes, supports *L. aethiopica* amastigotes' extra-cellular growth. The growth of *L. aethiopica* in JH30 was compared with its growth in UM54 (Bates *et al.*, 1992) and showed a definitive preference of the parasites for the first medium. Axenic amastigotes were later used (Chapter 5) in drug testing and active compounds against intra and extra-cellular parasites were identified. Identification and isolation of drugs active against the parasites are not only at the basis of much *Leishmania* research, they are a stepping stone in gaining further understanding of the mechanism of action of the parasites. Since the stilbenoids identified were able to reduce the infection ratio of the parasites in macrophages they are able to interfere with this interaction.

On the basis of the data available in the literature a model to explain how intracellular parasites spread to uninfected macrophages was suggested and investigated (Chapter 6). In each manifestation of leishmaniasis there is a silent period during which the parasites multiply within macrophages and spread. Once the parasites are finally recognized by the immune system the inflammatory reaction causes the symptoms of the disease. In the case of self-healing cutaneous leishmaniasis, this involves the destruction of the infected cells at the site of the lesion and represents the beginning of the healing procedure as well as the manifestation of the disease symptoms. The silent period of the disease corresponds with the spreading of the parasites between macrophages and from monocytes to macrophages. It was shown that the parasites induced two features related to apoptosis into the host cells, increased mitochondrial permeabilization and increased expression of PS in the outer membrane (Chapter 6). Apoptosis induction would explain both how the infected cells attract healthy macrophages to the site of infection and how they could infect them by releasing amastigotes inside apoptotic bodies. The parasites would be surrounded by macrophage membranes and therefore be protected by the lytic action of the complement and at the same time would induce phagocytosis by neighbouring macrophages.

Nevertheless, evidence obtained on the lack of DNA fragmentation suggested that a more complicated mechanism might take place. Incomplete induction of apoptosis was suggested to be involved in the mechanism of spread of infection. By the comparison of the results presented in this thesis with the data available for visceralizing species it was suggested that the regulation of the apoptotic processes of the host cells might play a major role in the spreading of the infection, as well as in differentiating between an immune reaction localized to the skin and a deadly visceral accumulation of infected macrophages.

The regulation of apoptotic processes in human cells is vital not only for the development of a functional organism but also for its survival. It is well known that malfunctions in the apoptotic regulation are the first processes responsible for development of carcinogenic cells. Therefore understanding the mechanisms through which *Leishmania* is able to regulate this process can provide valuable information on cellular processes of general interest, which is not limited to the parasitic infection.

Because of the complexity demonstrated by these results (incomplete activation of apoptosis), a different and wider approach was chosen to further analyse this interaction. Specifically, the changes in the proteome (all measurable proteins and their levels in cells under certain conditions) during infection were analysed. The choice of a proteomic approach was based on the knowledge that it provides a wide overview over the processes taking place inside any biological system.

The main comparison carried out was between infected and uninfected macrophages: protein expressions inside the cytosol were compared following extraction and fluorescent labelling. Many differences were detected following the first run, in each infection (Chapter 7). As expected, proteins involved in metabolic processes were over-expressed during the infection.

Of the many interesting proteins identified, particular attention was placed on BCDO2 protein expression, mainly because of its involvement in the retinoic acid metabolism, which is also responsible for the terminal differentiation of THP-1 cell lines. This led to the hypothesis that infection could be responsible for terminal differentiation of monocytes into macrophages. It is known that *Leishmania* is able to infect and to transform into amastigotes once inside monocytes. On the basis of this result I suggested that: following infection the amastigotes are able to induce terminal differentiation and cause the monocytes to become sticky, thereby interrupting their circulation. In order to further investigate this theory, monocytes were extracted from human blood and their ability to increase adherence, which is also a sign of transformation into macrophages, detected during infection. From the comparison of infected and uninfected monocytes at different times, it was obvious that the presence of *Leishmania* parasites (at a 1 to 1 ratio) significantly increased adherence. Further analysis is necessary to confirm this hypothesis; nevertheless this is an example of the utility of the proteomic approach in understanding the mechanism of action of *Leishmania* parasites inside host cells. Further findings related to the suggested model and to the involvement of infection with the host cell apoptosis regulation are discussed in the next section.

Spread of *Leishmania* amastigotes to uninfected macrophages.

The presence of a silent phase during which the number of infected cells increases indicates a crucial stage of the infection, the secondary infection of macrophages by amastigotes from already infected cells. Very little is known about this process, but the generally accepted hypothesis is that heavily parasitized macrophages eventually burst and free amastigotes are released, which are free to bind to new macrophages. Although there are no experimental data to confirm this model, infected organs or lesions of susceptible hosts often contain heavily parasitized macrophages but they rarely interact with free extracellular amastigotes. Furthermore, since the macrophages protect the parasites from a hostile environment it is unlikely that the amastigotes' strategy to spread involves leaving the host cells. Most of the amastigotes would not be able to survive outside the phagocyte since most of the determinants used by the promastigotes to survive are down-regulated in the intracellular stage (Bahr *et al.*, 1993).

A new model is proposed in this thesis (Figure 8.1). It is suggested that cutaneous species of *Leishmania* are able to use the host cells as a vehicle to silently spread. One way that the macrophages can use to spread cytoplasmic information to neighbouring cells without releasing it outside the membrane and without causing infection is through induction of apoptosis. Since PS externalisation on apoptosing cells acts as a signal for engulfment by macrophages (Grimsley and Ravichandran, 2003), induction of apoptosis in the parasitised cells could actively participate in spreading the infection. Moreover, parasites containing apoptotic bodies with intact membranes could be released and phagocytosed by macrophages. In cutaneous forms induction of apoptosis might encourage localized proliferation.

The presence of PS on the surface of infected macrophages and the increase of macrophages showing mitochondrial permeabilization during infection were established in Chapter 6. In order to spread to neighbouring cells intracellular parasites do not necessarily need to induce cell death on the host cells, rather it is possible that only some features of apoptosis might be induced as confirmed by the

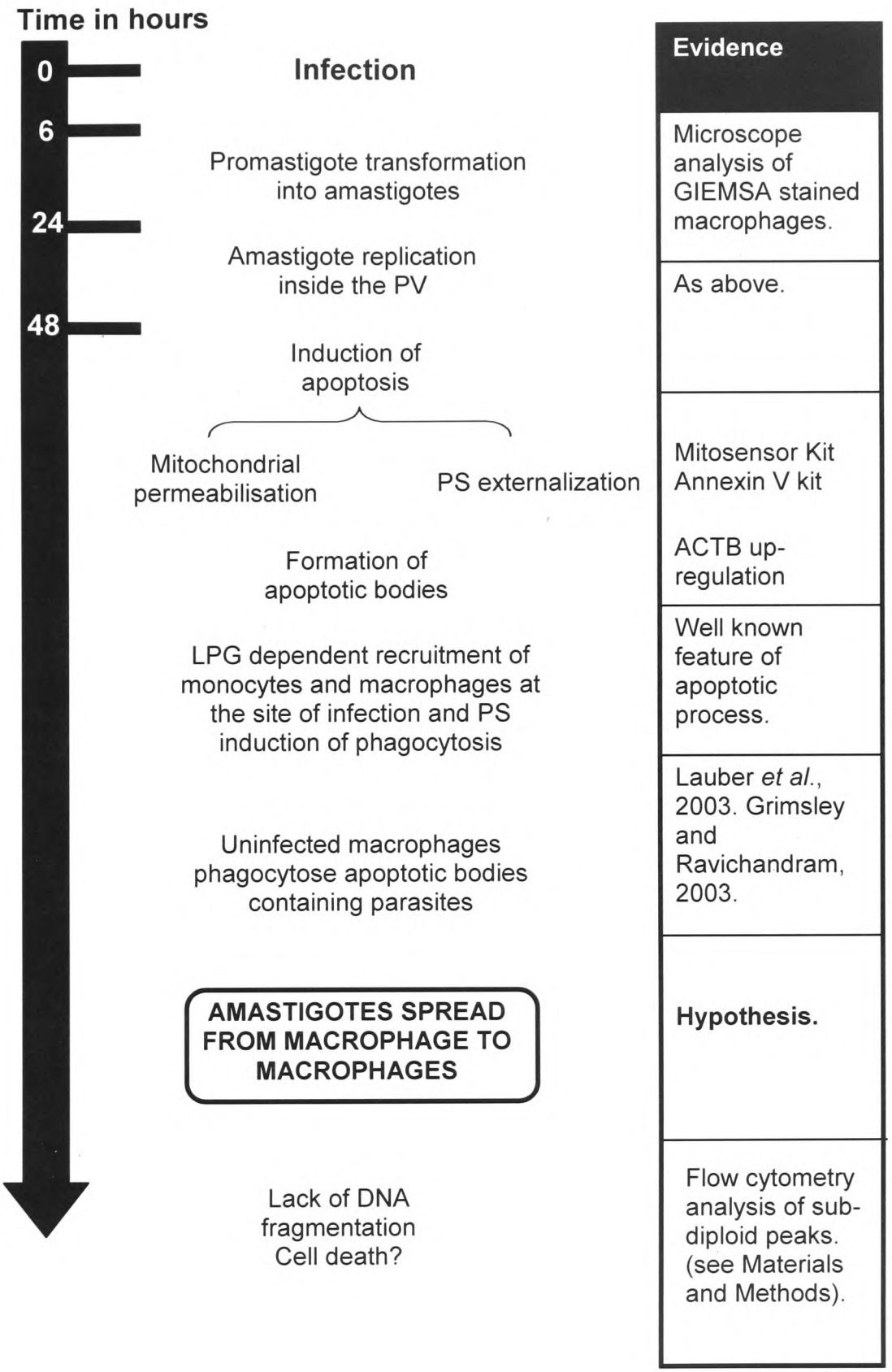


Figure 8.1. Apoptosis involvement in the infection process. Schematic view of the spread of intracellular amastigotes to uninfected macrophages.

lack of DNA fragmentation in infected cells. Partial induction of apoptosis has been described during infection with *Legionella pneumophila* (Abu-Zant *et al.*, 2005) suggesting the possibility that intracellular parasites can tightly regulate host cell apoptosis to their advantage. *L. major* and *L amazonensis* are known to induce P13-kB and NF-kB inhibition of apoptosis (Akarid *et al.*, 2004; Ruhland *et al.*, 2007). Induction of NF-kB and of other apoptosis regulators was confirmed by comparative proteomics during *L. aethiopica* infection (Chapter 7) corroborating the evidence that *Leishmania* infection interferes with the host cell apoptotic machinery. Moreover, overexpression of proteins involved in rearranging of the cellular structure (ACTB, Chapter 7) suggested the possibility of apoptotic bodies' formation.

To conclude, the results obtained during this research support the theory schematized in Figure 8.1. For the first time a model describing the spreading of the infection is proposed and studied. Because of the complexity of the model it is important to further understand the steps undertaken by the intracellular parasites in controlling the host, especially the host apoptotic machinery. It will be particularly interesting to elucidate which stimuli trigger PS externalization and mitochondrial permeabilization and at which stages of the infection the parasites exert every specific control.

Chapter 9

References

Abdel-Hameed, A. A., Ahmed, B.O., Mohamedani, A.A., el-Harith, A. and Van Eys G. . (1990). A case of diffuse cutaneous leishmaniasis due to Leishmania major. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84,** 535-6.

Abreu, P. M., Martins, E. S., Kayser, O., Bindseil, K. U., Siems, K., Seemann, A., and Frevert, J. (1999). Antimicrobial, antitumor and antileishmania screening of medicinal plants from Guinea-Bissau. *Phytomedicine* **6,** 187-95.

Abu-Zant, A., Santic, M., Molmeret, M., Jones, S., Helbig, J., and Abu Kwaik, Y. (2005). Incomplete activation of macrophage apoptosis during intracellular replication of Legionella pneumophila. *Infection and Immunity* **73,** 5339-49.

Aga, E., Katschinski, D., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., Solbach, W., and Laskay, T. (2002). Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. *Journal of Immunology* **169,** 898-905.

Akarid, K., Arnoult D, Micic-Polianski, J., Sif, J., Estaquier, J., and Ameisen, J. (2004). Leishmania major-mediated prevention of programmed cell death induction in infected macrophages is associated with the repression of mitochondrial release of cytochrome c. *Journal of Leukocyte Biology* **76,** 95-103.

Akendengue, B., Roblot, F., Loiseau, P. M., Bories, C., Ngou-Milama, E., Laurens, A., and Hocquemiller, R. (2002). Klaivanolide, an antiprotozoal lactone from Uvaria klaineana. *Phytochemistry* **59,** 558-8.

al-Bashir, N. T., Rassam, M. B., and al-Rawi, B. M. (1992). Axenic cultivation of amastigotes of Leishmania donovani and Leishmania major and their infectivity. *Annals of Tropical Medicine and Parasitology* **86,** 487-502.

Al-Mulla, H. Y. M., Najim, R., and Al-Bashir, N. (2005). Leishmania major and Leishmania tropica: I the in vitro effects of an immunomodulator, S2-Complex. *Experimental Parasitology* **111,** 47-54.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *Journal of Experimental medicine* **188,** 1359-68.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392,** 86-9.

Alborzi, A., Rasouli, M., and Shamsizadeh, A. (2006). Leishmania tropica-isolated patient with visceral leishmaniasis in southern Iran. *American Journal of Tropical Medicine and Hygiene* **74,** 306-7.

Alce, T. M., Gokool, S., McGhie, D., Stäger, S., and Smith, D. F. (1999). Expression of hydrophilic surface proteins in infective stages of Leishmania donovani. *Molecular and Biochemical Parasitology* **102,** 191-6.

Alexander, J., and Bryson, K. (2005). T helper (h)1/Th2 and Leishmania: paradox rather than paradigm. *Immunology Letters* **99,** 17-23.

Alexander, J., and Vickerman, K. (1975). Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana-infected macrophages. *Journal of Protozoology* **22,** 502-8.

Alvarez, M., Zeelen§, J. P., Mainfroid, V., Rentier-Delrue, F., Martial, J. A., Wyns, L., Wiereng, R. K., and Maes, D. (1998). Triose-phosphate Isomerase (TIM) of the Psychrophilic Bacterium Vibrio marinus. *Journal of Biological Chemistry* **273,** 2199-2206.

Antoine, J., Prina, E., Jouanne, C., and Bongrand, P. (1990). Parasitophorous vacuoles of Leishmania amazonensis-infected macrophages maintain an acidic pH. *Infection and Immunity* **58,** 779-87.

Artis, D., Johnson, L. M., Joyce, K., Saris, C., Villarino, A., Hunter, C. A., and Scott, P. (2004). Cutting edge: early IL-4 production governs the requirement for IL-27-WSX-1 signaling in the development of protective Th1 cytokine responses following Leishmania major infection. *The Journal of Immunology* **172,** 4672-5.

Aseffa, A., Gumy, A., Launois, P., MacDonald, H. R., Louis, J. A., and Tacchini-Cottier, F. (2002). The early IL-4 response to Leishmania major and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory CD4+CD25+ T cells. *Journal of Immunology* **169,** 3232-41.

Ashford, R. W. (2000). The leishmaniases as emerging and reemerging zoonoses. *Journal of Parasitology* **30,** 1269-81.

Ashford, R. W., Seaman, J., Schorscher, J., and Pratlong, F. (1992). Epidemic visceral leishmaniasis in southern Sudan: identity and systematic position of the parasites from patients and vectors. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86,** 379-380.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews Cancer* **2,** 420-30.

Aslam, S., Stevenson, P., Kokubun, T., and Hall, D. (2007). Antibacterial and antifungal activity of cicerfuran and related 2-arylbenzofurans and stilbenes. *In* "Microbiology research".

Asres, K., Bucar, F., Knauder, E., Yardley, V., Kendrick, H., and Croft, S. L. (2001). In vitro antiprotozoal activity of extract and compounds from the stem bark of Combretum molle. *Phytotherapy Research* **15,** 613-7.

Auwerx, J. (1991). The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia* **47,** 22-31.

Bacellar, O., Lessa, H., Schriefer, A., Machado, P., Ribeiro de Jesus, A., Dutra, W. O., Gollob, K. J., and Carvalho, E. M. (2002). Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infection and Immunity* **70,** 6734-6740.

Bahr, V., Stierhof, Y. D., Ilg, T., Demar, M., Quinten, M., and Overath, P. (1993). Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of Leishmania mexicana. *Molecular and Biochemical Parasitology* **58,** 107-21.

Baird, S. K., Hampton, M. B., and Gieseg, S. P. (2004). Oxidized LDL triggers phosphatidylserine exposure in human monocyte cell lines by both caspase-dependent and -independent mechanisms. *FEBS Letters* **578,** 169-74.

Balanco, J. M., Pral, E. M., da Silva, S., Bijovsky, A. T., Mortara, R. A., and Alfieri, S. C. (1998). Axenic cultivation and partial characterization of Leishmania braziliensis amastigote-like stages. *Parasitology* **116,** 103-13.

Balanco, J. M. F., Costa Moreira, M. E., Bonomo, A., Bozza, P. T., Amarante-Mendes, G., Pirmez, C., and Barcinski, M. A. (2001). Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. *Current Biology* **2001,** 1870–1873.

Balaraman, S., Singh, V. K., Tewary, P., and Madhubala, R. (2005). Leishmania lipophosphoglycan activates the transcription factor activating protein 1 in J774A.1 macrophages through the extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase. *Molecular and Biochemical Parasitology* **139,** 117-27.

Barbiéri, C. L., Giorgio, S., Merjan, A. J., and Figueiredo, E. N. (1993). Glycosphingolipid antigens of Leishmania (Leishmania) amazonensis amastigotes identified by use of a monoclonal antibody. *Infection and Immunity* **61,** 2131-2137.

Barnetson, R. S., Ridley, R. S., and Wheate, H. W. (1978). A form of muco-cutaneous leishmaniasis in the Old World. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72,** 516-8.

Barr, S. D., and Gedamu, L. (2003). Role of peroxidoxins in Leishmania chagasi survival. Evidence of an enzymatic defense against nitrosative stress. *Journal of Biological Chemistry* **278,** 10816-23.

Barral-Netto, M., Roters, S. B., Sherlock, I., and Reed, S. G. (1987). Destruction of Leishmania mexicana amazonensis promastigotes by normal human serum. *American Journal of Tropical Medicine and Hygiene* **37,** 53-56.

Barreca, G. S., Berlinghieri, M. C., Foti, F., Matera, G., and Foca, A. (1997). Microscopic observation of progressive immobilization of leishmania promastigotes in acridine orange stain. *Journal of Clinical Microbiology* **35,** 1867-9.

Basu, S., and Srivastava, P. K. (2003). Fever-like temperature induces maturation of dendritic cells through induction of hsp90. *International Immunology* **15,** 1053-1061.

Bates, P. A. (1994). Complete developmental cycle of Leishmania mexicana in axenic culture. *Parasitology* **108,** 1-9.

Bates, P. A., Robertson, C. D., Tetley, L., and Coombs, G. H. (1992). Axenic cultivation and characterization of Leishmania mexicana amastigote-like forms. *Parasitology* **105,** 193-202.

Bates, P. A., and Tetley, L. (1993). Leishmania mexicana: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. *Experimental Parasitology* **76,** 412-23.

Beaumont, C., Leneuve, P., Devaux, I., Scoazec, J.-Y., Berthier, M., Loiseau, M.-N., Grandchamp, B., and Bonneau, D. (1995). Mutation in the iron responsive element of the L ferritin mRNA in a family with dominant hyperferritinaemia and cataract. *Nature Genetics* **11,** 444-446.

Beil, W., Meinardus-Hager, G., Neugebauer, D. C., and Sorg, C. (1992). Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *Journal of Leukocyte Biology* **52,** 135-42.

Belay, A. D., Asafa, Y., Mesure, J., and Davidson, R. N. (2006). Successful miltefosine treatment of post-kala-azar dermal leishmaniasis occurring during antiretroviral therapy. *Annals of Tropical Medicine and Parasitology* **100,** 223-7.

Belkaid, Y., Butcher, B., and Sacks, D. L. (1998). Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania-infected cells. *European Journal of Immunology* **28,** 1389-400.

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000). A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *The Journal of Immunology* **165,** 969-977.

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M., and Sacks, D. L. (2002a). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* **420,** 502-7.

Belkaid, Y., Von Stebut, E., Mendez, S., Lira, R., Caler, E., Bertholet, S., Udey, M. C., and Sacks, D. (2002b). CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. *The Journal of Immunology* **168,** 3992-4000.

Berman, J. (1985). "Experimental chemotherapy of leishmaniasis—a critical review." Elsevier Science Publishers B.V., Amsterdam.

Berman, J. (2003). Current treatment approaches to leishmaniasis. *Current Opinion in Infectious Diseases* **16,** 397-401.

Berman, J. D., Dwyer, D. M., and Wyler, D. J. (1979). Multiplication of Leishmania in human macrophages in vitro. *Infection and Immunity* **26,** 375-379.

Bern, C., Haque, R., Chowdhury, R., Ali, M., Kurkjian, K. M., Vaz, L., Amann, J., Wahed, M. A., Wagatsuma, Y., Breiman, R. F., Williamson, J., Secor, W. E., and Maguire, J. H. (2007). The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *American Journal of Tropical Medicine and Hygiene* **76,** 909-14.

Bittencourt, A., Silva, N., Straatmann, A., Nunes, V. L., Follador, I., and Badaro, R. (2003). Post-kala-azar dermal leishmaniasis associated with AIDS. *The Brazilian Journal of Infectious Diseases* **7,** 229-33.

Bogdan, C., Donhauser, N., Doring, R., Rollinghoff, M., Diefenbach, A., and Rittig, M. G. (2000). Fibroblasts as Host Cells in Latent Leishmaniosis. *The Journal of Experimental Medicine* **191,** 2121-2130.

Bogdan, C., and Rollinghoff, M. (1998). The immune response to Leishmania: mechanisms of parasite control and evasion. *International Journal for Parasitology* **28,** 121-34.

Bolitho, P., Voskoboinik, I., Trapani, J. A., and Smyth, M. J. (2007). Apoptosis induced by the lymphocyte effector molecule perforin. *Current Opinion in Immunology* **19,** 339-47.

Bozza, M., Soares, M. B., Bozza, P. T., Satoskar, A. R., Diacovo, T. G., Brombacher, F., Titus, R. G., Shoemaker, C. B., and R, D. J. (1998). The PACAP-type I receptor agonist maxadilan from sand fly saliva protects mice against lethal endotoxemia by a mechanism partially dependent on IL-10. *European Journal of Immunology* **28,** 3120-7.

Brandonisio, O., Ceci, L., Cedola, M. C., Caretto, G., Antonaci, S., and Jirillo, E. (1986). Phagocytosis of Leishmania infantum promastigotes by monocytes isolated from Leishmania-infected dogs. *Microbiologica* **9,** 173-8.

Bresgen, N., Ohlenschläger, I., Fiedler, B., Wacht, N., Zach, S., Dunkelmann, B., Arosio, P., Kuffner, E., Lottspeich, F., and Eckl, P. M. (2007). Ferritin--a mediator of apoptosis? *journal of cellular physiology* **212,** 157-64.

Breton, M., Tremblay, M. J., Ouellette, M., and Papadopoulou, B. (2005). Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. *Infection and Immunity* **73,** 6372-82.

Brittingham, A., Chen, G., McGwire, B. S., Chang, K. P., and Mosser, D. M. (1999). Interaction of Leishmania gp63 with cellular receptors for fibronectin. *Infection and Immunity* **67,** 4477-84.

Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P., and Mosser, D. M. (1995). Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *The Journal of Immunology* **155,** 3102-11.

Brobey, R. K., Mei, F. C., Cheng, X., and Soong, L. (2006). Comparative two-dimensional gel electrophoresis maps for promastigotes of Leishmania amazonensis and Leishmania major. *The Brazilian Journal of Infectious Diseases* **10,** 1-6.

Brown, A. J. P., Leibold, E. A., and Munro, H. N. (1983). Isolation of cDNA clones for the light subunit of rat liver ferritin: evidence that the light subunit is encoded by a multigene family. *Proceedings of the National Academy of Sciences* **80,** 1265-1269.

Bryceson, A. (1976). Tropical dermatology. CUTANEOUS LEISHMANIASIS *British Journal of Dermatology* **94,** 223-226.

Butcher, B. A., Turco, S. J., Hilty, B. A., Pimenta, P. F., Panunzio, M., and Sacks, D. L. (1996). Deficiency in beta1,3-galactosyltransferase of a Leishmania major lipophosphoglycan mutant adversely influences the Leishmania-sand fly interaction. *Journal of Biological Chemistry* **271,** 20573-9.

Buxbaum, L. U., Uzonna, J. E., Goldschmidt, M. H., and Scott, P. (2002). Control of New World cutaneous leishmaniasis is IL-12 independent but STAT4 dependent. *European Journal of Immunology* **32,** 3206-15.

Campbell, K., Diao, H., Ji, J., and Soong, L. (2003). DNA immunization with the gene encoding P4 nuclease of Leishmania amazonensis protects mice against cutaneous Leishmaniasis. . *Infection and Immunity* **71,** 6270-8.

Capelli, G., Baldelli, R., Ferroglio, E., Genchi, C., Gradoni, L.,, Gramiccia, M., Maroli, M., Mortarino, M., Pietrobelli, M., Rossi,, and L., R., M. (2004). Monitoring of canine leishmaniasis in
northern Italy: an update from a scientific network. *Parassitologia* **46,** 193–197.

Carrera, L., Gazzinelli, R. T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., and Sacks, D. L. (1996). Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. *The Journal of Experimental Medicine* **183,** 515-26.

Castilla, J. J., Sanchez-Moreno, M., Mesa, C., and Osuna, A. (1995). Leishmania donovani: in vitro culture and [IH] NMR characterization of amastigote-like forms. *Molecular and cellular biochemistry* **142,** 89-97.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *The Journal of Experimental Medicine* **184,** 747-52.

Chakraborty, R., Chakraborty, P., and Basu, M. K. (1998). Macrophage mannosyl fucosyl receptor: its role in invasion of virulent and avirulent L. donovani promastigotes. *Bioscience Reports* **18,** 129-42.

Chan-Bacab, M. J., and Peña-Rodríguez, L. M. (2001). Plant natural products with leishmanicidal activity. *Natural Product Reports* **18,** 674-88.

Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S. J., Sibille, J. C., Snapper, M., Aisen, P., and Bloom, B. R. (1989). Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proceedings of the National Academy of Sciences* **86,** 2453-7.

Chang, K. (1979). Leishmania donovani: promastigote--macrophage surface interactions in vitro. *Experimental Parasitology* **48,** 175-89.

Chang, K. P., and Dwyer, D. M. (1978). Leishmania donovani. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes. *The Journal of Experimental Medicine* **147,** 515-30.

Chang, K. P., and McGwire, B. S. (2002). Molecular determinants and regulation of Leishmania virulence. *Kinetoplastid Biology and Disease* **1,** 1.

Chang, K. P., Reed, S. G., McGwire, B. S., and Soong, L. (2003). Leishmania model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. *Acta Tropica* **85,** 375-90.

Charlab, R., Valenzuela, J. G., Rowton, E. D., and Ribeiro, J. M. (1999). Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly Lutzomyia longipalpis. *Proceedings of the National Academy of Sciences* **96,** 15155-60.

Chattopadhayay, R., Kaur, S., Ganguly, N. K., and Mahajan, R. C. (1996). Antigenic differences between axenic amastigotes and promastigotes of Leishmania donovani. *Indian Journal of Medical Research* **104,** 349-354.

Chaussabel, D., Semnani, R. T., McDowell, M. A., Sacks, D., Sher, A., and Nutman, T. B. (2003). Unique gene expression profiles of human macrophages and dendritic cells to phylogenetically distinct parasites. *Blood* **102,** 672-81.

Chen, L. M., Kaniga, K., and Galan, J. E. (1996). Salmonella spp. are cytotoxic for cultured macrophages. *Molecular Microbiology* **21,** 1101-15.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell* **8,** 705-11.

Cho D.H., Hong Y.M., Lee H.J., Woo H.N., Pyo J.O., Mak T.W., and Y.K., J. (2004). Induced inhibition of ischemic/hypoxic injury by APIP, a novel Apaf-1-interacting protein. *J. Biol. Chem.* **279,** 39942-39950.

Choudhary, M. I., Yousuf, S., Samreen, Shah, S. A., Ahmed, S., and Atta-ur-Rahman. (2006). Biotransformation of physalin H and leishmanicidal activity of its transformed products. *Chemical and Pharmaceutical Bulletin (Tokyo)* **54,** 927-30.

Chromy, B. A., Perkins, J., Heidbrink, J. L., Gonzales, A. D., Murphy, G. A., Fitch, J. P., and McCutchen-Maloney, S. L. (2004). Proteomic characterization of host response to Yersinia pestis and near neighbors. *Biochemical and Biophysical Research Communications* **320,** 474-9.

Cocco, R. E., and Ucker, D. S. (2001). Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Molecular Biology of the Cell* **12,** 919-30.

Coler, R. N., Goto, Y., Bogatzki, L., Raman, V., and Reed, S. G. (2007). Leish-111f, a Recombinant Polyprotein Vaccine that Protects against Visceral Leishmaniasis by the elicitation of CD4+ T cells. *Infection and Immunity* **[Epub ahead of print]**.

Coler, R. N., Skeiky, Y. A., Bernards, K., Greeson, K., Carter, D., Cornellison, C. D., Modabber, F., Campos-Neto, A., and Reed, S. G. (2002). Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, Leishmania major stress-inducible protein 1, and Leishmania elongation initiation factor protects against leishmaniasis. *Infection and Immunity* **70,** 4215-25.

Colmenares, M., Kar, S., Goldsmith-Pestana, K., and McMahon-Pratt, D. (2002). Mechanisms of pathogenesis: differences amongst Leishmania species. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96,** S3-7.

Convit, J., Reyes, O., and Kerdel, F. (1957). Disseminated anergic American leishmaniasis; report of three cases of a type clinically resembling lepromatous leprosy. *Archives of Dermatology* **76,** 213-7.

Costa, J. W., Milner, D. A., and Maguire, J. H. (2003). Mucocutaneous leishmaniasis in a US citizen. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics* **96,** 573-577.

Creighton, D. J., and Hamilton, D. S. (2001). The catalytic mechanism of glyoxalase I. *Archives of Biochemistry and Biophysics* **387,** 1-10.

Croft, S. L., Sundar, S., and Fairlamb, A. H. (2006). Drug resistance in leishmaniasis. *Clinical Microbiology Reviews* **19,** 111-26.

Crook, N. E., Clem, R. J., and Miller, L. K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc
finger-like motif. *The Journal of Virology* **67,** 2168–74.

Cuconati, A., and White, E. (2002). Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. *Genes and Development* **16,** 2465-78.

Culley, F. J., Harris, R. A., Kaye, P. M., McAdam, K. P., and Raynes, J. G. (1996). C-reactive protein binds to a novel ligand on Leishmania donovani and increases uptake into human macrophages. *The Journal of Immunology* **156,** 4691-6.

Cunningham, A. C. (2002). Parasitic adaptive mechanisms in infection by leishmania. *Exp Mol Pathol* **72,** 132-41.

Cupolillo, E., Medina-Acosta, E., Noyes, H., Momen, H., and Grimaldi, G. J. (2000). A revised classification for Leishmania and Endotrypanum. *Parasitology today* **16,** 142-4.

Cysne-Finkelstein, L., Temporal, R. M., Alves, F. A., and Leon, L. L. (1998). Leishmania amazonensis: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. *Experimental Parasitology* **89,** 58-62.

Da-Cruz, A. M., Conceição-Silva, F., Bertho, A. L., and Coutinho, S. G. (1994). Leishmania-reactive CD4+ and CD8+ T cells associated with cure of human cutaneous leishmaniasis. *Infection and Immunity* **62,** 2614-8.

Da Silva, R. P., Hall, B. F., Joiner, K. A., and Sacks, D. L. (1989). CR1, the C3b receptor, mediates binding of infective Leishmania major metacyclic promastigotes to human macrophages. *The Journal of Immunology* **143,** 617-22.

Dalby, A., Dauter, Z., and Littlechild, J. A. (1999). Crystal structure of human muscle aldolase complexed with fructose 1,6-bisphosphate: mechanistic implications. *Protein Science* **8,** 291-7.

Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholipid asymmetry. *Journal of Lipid Research* **44,** 233-42.

Danial, N. N., and Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell* **116,** 205-19.

Darne, S., and Sinclair, S. A. (2006). A sandfly in Surrey? A case of cutaneous leishmaniasis in the United Kingdom without history of recent travel to an endemic area. *Clinical and Experimental Dermatology* **31,** 155-6.

Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992). Features of apoptotic cells measured by flow cytometry. *Cytometry* **13,** 795-808.

de Almeida, M. C., Vilhena, V., Barral, A., and Barral-Netto, M. (2003). Leishmanial infection: analysis of its first steps. A review. *Memórias do Instituto Oswaldo Cruz* **98,** 861-870.

de Carvalho, P. B., and Ferreira, E. I. (2001). Leishmaniasis phytotherapy. Nature's leadership against an ancient disease. *Fitoterapia* **72,** 599-618.

de Kossodo, S., Grau, G. E., Louis, J. A., and Muller, I. (1994). Tumor necrosis factor alpha (TNF-alpha) and TNF-beta and their receptors in experimental cutaneous leishmaniasis. *Infection and Immunity* **62,** 1414-20.

De, S. R., Ajmone-Cat, M. A., Nicolini, A., and Minghetti, L. (2002). Expression of phosphatidylserine receptor and down-regulation of pro-inflammatory molecule production by its natural ligand in rat microglial cultures. *Journal of Neuropathology & Experimental Neurology* **61,** 237-44.

del Olmo, E., García Armas, M., López-Pérez, J. L., Muñoz, V., Deharo, E., and San Feliciano, A. (2001). Leishmanicidal activity of some stilbenoids and related heterocyclic compounds. *Bioorganic & Medicinal Chemistry Letters* **11,** 2123-2126.

del Rayo Camacho, M., Phillipson, J. D., Croft, S. L., Rock, P., Marshall, S. J., and Schiff, P. L. (2002). In vitro activity of Triclisia patens and some bisbenzylisoquinoline alkaloids against Leishmania donovani and Trypanosoma brucei brucei. *Phytotherapy Research* **16,** 432-6.

del Rey, B., Ramos, A. C., Caballero, E., Inchaustti, A., Yaluff, G., Medarde, M., Rojas de Arias, A., and San Feliciano, A. (1999). Leishmanicidal activity of combretastatin analogues and heteroanalogues. *Bioorg Med Chem Lett* **9,** 2711-4.

Delmas, F., Di Giorgio, C., Elias, R., Gasquet, M., Azas, N., Mshvildadze, V., Dekanosidze, G., Kemertelidze, E., and Timon-David, P. (2000). Antileishmanial activity of three saponins isolated from ivy, alpha-hederin,

beta-hederin and hederacolchiside A1, as compared to their action on mammalian cells cultured in vitro. *Planta Medica* **66,** 343-7.

Deng, W., Thiel, B., Tannenbaum, C. S., Hamilton, T. A., and Stuehr, D. J. (1993). Synergistic cooperation between T cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. *The Journal of Immunology* **151,** 322-9.

Descoteaux, A., Matlashewski, G., and Turco, S. J. (1992). Inhibition of macrophage protein kinase C-mediated protein phosphorylation by Leishmania donovani lipophosphoglycan. *The Journal of Immunology* **149,** 3008-15.

Descoteaux, A., and Turco, S. J. (1993). The lipophosphoglycan of Leishmania and macrophage protein kinase C. *Parasitology today* **9,** 468-71.

Desjardins, M., and Descoteaux, A. (1997). Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *The Journal of Experimental Medicine* **185,** 2061-8.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95,** 239-243.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases* **27,** 305-318.

Develoux, M., Diallo, S., Dieng, Y., Mane, I., Huerre, M., Pratlong, F., Dedet, J. P., and Ndiaye, B. (1996). Diffuse cutaneous leishmaniasis due to Leishmania major in Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90,** 396-7.

Dey, R., Khan, S., Pahari, S., Srivastava, N., Jadhav, M., and Saha, B. (2007). Functional paradox in host-pathogen interaction dictates the fate of parasites. *Future Microbiology* **2,** 425-37.

Dinarello, C. (2001). Anti-cytokine therapies in response to systemic infection. *Journal of Investigative Dermatology Symposium Proceedings* **6,** 244-50.

Doyle, P. S., Engel, J. C., Pimenta, P. F., da Silva, P. P., and Dwyer, D. M. (1991). Leishmania donovani: long-term culture of axenic amastigotes at 37 degrees C. *Experimental Parasitology* **73,** 326-34.

Dumonteil, E., McMahon-Pratt, D., and Price, V. L. (2001). Report of theFourth TDR/IDRI Meeting on Second-Generation Vaccines against Leishmaniasis. *In* "Fourth TDR/IDRI Meeting on Second-Generation Vaccines against Leishmaniasis", Universidad Autonoma de Yucatan, Mexico.

Duprey, Z. H., Steurer, F. J., Rooney , J. A., Kirchhoff, L. V., Jackson, J. E., Rowton, E. D., and Schantz, P. M. (2006). Canine visceral leishmaniasis,

United States and Canada, 2000-2003. *Emerging Infectious Diseases* **12,** 440-6.

Durrbaum-Landmann, I., Gercken, J., Flad, H. D., and Ernst, M. (1996). Effect of in vitro infection of human monocytes with low numbers of Mycobacterium tuberculosis bacteria on monocyte apoptosis. *Infection and Immunity* **64,** 5384-9.

ECACC. European collection of cell cultures. http://www.ecacc.org.uk. Accessed on 12th May 2006

Eichner1, M. (1994). Host-diversity in the transmission of vector-borne disease: an epidemiologic model. *Technical report, German Accademic Exchange Service (DAAD).*

El-On, J., Bazarsky, E., and Sneir, R. (2007). Leishmania major: In vitro and in vivo anti-leishmanial activity of paromomycin ointment (Leshcutan) combined with the immunomodulator Imiquimod. *Experimental Parasitology* **116,** 156-62.

El-On , J., Halevy, S., Grunwald, M. H., and Weinrauch, L. (1992). Topical treatment of Old World cutaneous leishmaniasis caused by Leishmania major: a double-blind control study. *Journal of the American Academy of Dermatology* **27,** 227-31.

El-On, J., Jacobs, G. P., Witztum, E., and Greenblatt, C. L. (1984). Development of topical treatment for cutaneous leishmaniasis caused by Leishmania major in experimental animals. *Antimicrobial Agents and Chemotherapy* **26,** 745-51.

El-On, J., Weinrauch, L., Livshin, R., Even-Paz, Z., and Jacobs, G. P. (1985). Topical treatment of recurrent cutaneous leishmaniasis with ointment containing paromomycin and methylbenzethonium chloride. *british medical journal clinical research ed* **291,** 704-5.

Engelman, J. A., Luo, J., and Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics* **7,** 606-19.

Enserink, M. (2000). Infectious diseases. Has leishmaniasis become endemic in the U.S.? *science* **8,** (5498):1881-3.

Eperon, S., and McMahon-Pratt, D. (1989). Extracellular cultivation and morphological characterization of amastigote-like forms of Leishmania panamensis and L. braziliensis. *Protozoology* **36,** 502-10.

Escobar, P., Matu, S., Marques, C., and Croft, S. L. (2002). Sensitivities of Leishmania species to hexadecylphosphocholine (miltefosine), ET-18-OCH(3) (edelfosine) and amphotericin B. *Acta Tropica* **81,** 151-7.

Fadok, V., A, , and Chimini, G. (2001). The phagocytosis of apoptotic cells. *Seminars in Immunology* **13,** 365-72.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *The Journal of Immunology* **148,** 2207-16.

Farajnia, S., Mahboudi, F., Ajdari, S., Reiner, N. E., Kariminia, A., and Alimohammadian, M. H. (2005). Mononuclear cells from patients recovered from cutaneous leishmaniasis respond to Leishmania major amastigote class I nuclease with a predominant Th1-like response. *Clinical and Experimental Immunology* **139,** 498-505.

Faraut-Gambarelli, F., Piarroux, R., Deniau, M., Giusiano, B., Marty, P., Michel, G., Faugere, B., and Dumon, H. (1997). In vitro and in vivo resistance of Leishmania infantum to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy* **41,** 827-30.

Fatima, F., Khalid, A., Nazar, N., Abdalla, M., Mohomed, H., Toum, A. M., Magzoub, M., and Ali, M. S. (2005). In vitro assessment of anti - cutaneous leishmaniasis activity of some Sudanese plants. *Türkiye Parazitoloji Dergisi* **29,** 3-6.

Feng, G. J., Goodridge, H. S., Harnett, M. M., Wei, X. Q., Nikolaev, A. V., Higson, A. P., and Liew, F. Y. (1999). Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *The Journal of Immunology* **163,** 6403-12

Feng, L., Seyrantepe, V., Landry, K., Ahmad, R., Ahmad, A., Stamatos, N. M., and Pshezhetsky, A. V. (2006). Monocyte differentiation upregulates the expression of the lysosomal sialidase, neu1 and triggers its targeting to the plasma membrane via MHC class II-positive compartments. *Journal of Biological Chemistry* **281,** 27526-38.

Ferguson, M. (1997). The surface glycoconjugates of trypanosomatid parasites *Philosophical Transactions of the Royal Society B: Biological Sciences* **352,** 1295-302.

Ferrières, J., Simon, M. F., Fauvel, J., and Chap, H. (1994). Virtual lack of annexins in human platelets argues against a role in phospholipase A2 regulation and platelet secretion. *Journal of Lipid Mediators and Cell Signalling* **9,** 155-65.

Finney, D. (1971). Statisical logic in the monitoring of reactions to therapeutic drugs. *Methods of Information in Medicine* **10,** 237-45.

Finney, D. J. (1978). "Statistical methods in biological assay, 3rd edition." High Wycombe Charles Griffin & Co,

Fokialakis, N., Kalpoutzakis, E., Tekwani, B. L., Skaltsounis, A. L., and Duke, S. O. (2006). Antileishmanial activity of natural diterpenes from Cistus sp. and semisynthetic derivatives thereof. *Biological and Pharmaceutical Bulletin* **29,** 1775-8.

Fournet, A., Ferreira, M. E., Rojas De Arias, A., Torres De Ortiz, S., Fuentes, S., Nakayama, H., Schinini, A., and Hocquemiller, R. (1996). In vivo efficacy of oral and intralesional administration of 2-substituted quinolines in experimental treatment of new world cutaneous leishmaniasis caused by Leishmania amazonensis. *Antimicrobial Agents and Chemotherapy* **40,** 2447-51.

Freire-de-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., DosReis, G. A., and Lopes, M. F. (2000). Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* **404,** 904.

Fujiwara, R. T., Vale, A. M., Franca da Silva, J. C., da Costa, R. T., Quetz Jda, S., Martins Filho, O. A., Reis, A. B., Correa Oliveira, R., Machado-Coelho, G. L., Bueno, L. L., Bethony, J. M., Frank, G., Nascimento, E., Genaro, O., Mayrink, W., Reed, S., and Campos-Neto, A. (2005). Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. *Veterinary Research* **36,** 827-38.

Gantt, K. R., Schultz-Cherry, S., Rodriguez , N., Jeronimo, S. M., Nascimento, E. T., Goldman, T. L., Recker, T. J., Miller, M. A., and Wilson, M. E. (2003). Activation of TGF-beta by Leishmania chagasi: importance for parasite survival in macrophages. *The Journal of Immunology* **170,** 2613-20.

Gebre-Hiwot, A., Tadesse, G., Croft, S. L., and Frommel, D. (1992). An in vitro model for screening antileishmanial drugs: the human leukaemia monocyte cell line, THP-1. *Acta Tropica* **51,** 237-45.

Ghosh, S., Bhattacharyya, S., Sirkar, M., Sa, G. S., Das, T., Majumdar, D., Roy, S., and Majumdar, S. (2002). Leishmania donovani suppresses activated protein 1 and NF-kappaB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. *Infection and Immunity* **70,** 6828-38.

Ghosh, S., Goswami, S., and Adhya, S. (2003). Role of superoxide dismutase in survival of Leishmania within the macrophage. *Biochemical Journal* **369,** 447-52.

Gordon, S., Fraser, I., Nath, D., Hughes, D., and Clarke, S. (1992). Macrophages in tissues and in vitro. *Current Opinion in Immunology* **4,** 25-32.

Gossage, S. M., Rogers, M. E., and Bates, P. A. (2003). Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. *International Journal for Parasitology* **33,** 1027-1034.

Gramiccia, M., and Gradoni, L. (2005). The current status of zoonotic leishmaniases and approaches to disease control. *International Journal for Parasitology* **35,** 1169-1180.

Gratz, N. G. (1999). Emerging and resurging vector-borne diseases. *Annual Review of Entomology* **44,** 51-75.

Green , S. J., Crawford, R. M., Hockmeyer, J. T., Meltzer, M. S., and Nacy, C. A. (1990). Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *J Immunol* **145,** 4290-7.

Grimaldi, G., and Tesh, R. B. (1993). Leishmaniases of the New World: current concepts and implications for future research. *Clinical Microbiology Reviews* **6,** 230-50.

Grimm, F., Brun, R., and Jenni, L. (1991). Promastigotes infectivity in Leishmania infantum. *Parasitology research* **77**.

Grimsley, C., and Ravichandran, K. S. (2003). Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. *Trends in Cell Biology* **13,** 648-656.

Grundmann, U., Abel, K. J., Bohn, H., Löbermann, H., Lottspeich, F., and Küpper, H. (1988). Characterization of cDNA encoding human placental anticoagulant protein (PP4): homology with the lipocortin family. *Proceedings of the National Academy of Sciences* **85,** 3708-12.

Guerin, P. J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S. L., Desjeux, P., Wasunna, M. K., and Bryceson, A. D. (2002). Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *The Lancet Infectious Diseases* **2,** 494-501.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annual Reviews of Immunology* **20,** 621-67.

Guessous-Idrissi, N. B. B., Riyad, M., Sahibi, H., Bichichi, M., and Rhalem, A. (1997). Short report: Leishmania tropica: etiologic agent of a case of canine visceral leishmaniasis in northern Morocco. *American Journal of Tropical Medicine and Hygiene* **57**.

Guizani-Tabbane, L., Ouni, I., Sassi, A., and Dellagi, K. (2000). *Leishmania major* induces deactivation of extracellular signal regulated kinases 2 in human U937 macrophage like cells. *Archives de l'institut Pasteur deTunis* **77,** 45-50.

Gupta, N., Goyal, N., Kumar, R., Agrawal, A. K., Seth, P. K., and Rastogi, A. K. (1996). Membrane characterization of amastigote-like forms of Leishmania donovani. *Tropical Medicine & International Health* **1,** 495-502.

Gupta, N., Goyal, N., and Rastogi, A. K. (2001). In vitro cultivation and characterization of axenic amastigotes of Leishmania. *Trends in Parasitology* **17,** 150-3.

Gupta, N., Goyal, N., Singha, U. K., Bhakuni, V., Roy, R., and Rastogi, A. K. (1999). Characterization of intracellular metabolites of axenic amastigotes of Leishmania donovani by 1H NMR spectroscopy. *Acta Tropica* **73,** 121-33.

Gurunathan, S., Wu, C. Y., Freidag, B. L., and Seder, R. A. (2000). DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology* **12,** 442-7.

Habtemariam, S. (2003). In vitro antileishmanial effects of antibacterial diterpenes from two Ethiopian Premna species: P. schimperi and P. oligotricha. *BMC Pharmacology* **6,** 6.

Hanafi, H. A., Fryauff, D. J., Dykstra, E. A., and Szumlas, D. E. (2007). Laboratory demonstration of the acquisition and development of leishmania major in the sand fly Phlebotomus kazeruni (Diptera: Psychodidae). *Journal of the Egyptian Society of Parasitology* **37,** 227-41.

Hardwidge, P. R., Rodriguez-Escudero, I., Goode, D., Donohoe, S., Eng, J., Goodlett, D. R., Aebersold, R., and Finlay, B. B. (2004). Proteomic analysis of the intestinal epithelial cell response to enteropathogenic Escherichia coli. *Journal of Biological Chemistry* **279,** 20127-36.

Harms, G., Schonian, G., and Feldmeier, H. (2003). Leishmaniasis in Germany. *Emerging Infectious Diseases* **9,** 872-5.

Hashim, F. A., Khalil, E. A., Ismail, A., and el Hassan, A. M. (1995). Apparently successful treatment of two cases of post kala-azar dermal leishmaniasis with liposomal amphotericin B. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89,** 440.

Hay, A. E., Ioset, J. R., Ahua, K. M., Diallo, D., Brun, R., and Hostettmann, K. (2007). Limonoid orthoacetates and antiprotozoal compounds from the roots of Pseudocedrela kotschyi. *Journal of Natural Products* **70,** 9-13.

Hayden, M. S., and Ghosh , S. (2004). Signaling to NF-kappaB. *Genes & Development* **18,** 2195-224.

Hepburn, N. C. (2003). Cutaneous leishmaniasis: an overview. *Journal of Postgraduate Medicine* **49,** 50-4.

Herath, S., Kropf, P., and Müller, I. (2003). Cross-talk between CD8(+) and CD4(+) T cells in experimental cutaneous leishmaniasis: CD8(+) T cells are required for optimal IFN-gamma production by CD4(+) T cells. *Parasite Immunology* **25,** 559-67.

Hermoso, T., Fishelson, Z., Becker, S. I., Hirschberg, K., and Jaffe, C. L. (1991). Leishmanial protein kinases phosphorylate components of the complement system. *The EMBO Journal* **10,** 4061-7.

Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S., and Zychlinsky, A. (1999). The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proceedings of the National Academy of Sciences* **96,** 2396-401.

Herwaldt, B. L., and Berman, J. D. (1992). Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. *American Journal of Tropical Medicine and Hygiene* **46,** 296-306.

Hodgkinson, V. H., Soong, L., Duboise, S. M., and McMahon-Pratt, D. (1996). Leishmania amazonensis: cultivation and characterization of axenic amastigote-like organisms. *Experimental Parasitology* **83,** 94-105.

Hutchison, S., LeBel, C., Blanchette, M., and Chabot, B. (2002). Distinct sets of adjacent heterogeneous nuclear ribonucleoprotein (hnRNP) A1/A2 binding sites control 5' splice site selection in the hnRNP A1 mRNA precursor. *Journal of Biological Chemistry* **277,** 29745-52.

Ibarrondo, F. J., Choi, R., Geng, Y. Z., Canon, J., Rey, O., Baldwin, G. C., and Krogstad, P. (2001). HIV type 1 Gag and nucleocapsid proteins: cytoskeletal localization and effects on cell motility. *AIDS Res Hum Retroviruses* **17,** 1489-1500.

Ip, Y. T., and Davis, R. J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol* **10,** 205-19.

Ishikawa, M., Mizukami, K., Iwakiri, M., Kamma, H., Ikonomovic, M. D., Dekosky, S. T., and Asada, T. (2004). Immunohistochemical study of hnRNP B1 in the postmortem temporal cortices of patients with Alzheimer's disease. *Neurosci Res.* **50,** 481-4.

Ivens, A. C., Peacock, C. S., Worthey, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M. A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S. M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C. V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R. M., Cronin, A., Cruz, A. K., Davies, R. M., De Gaudenzi, J., Dobson, D. E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram , J. C., Muller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'neil, S., Pentony, M., Pohl, T. M., Price, C., Purnelle, B., Quail, M. A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J. C., Rutter, S., Saunders, D., Schafer, M., Schein, J.,

Schwartz, D. C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D. F., Blackwell, J., M, , Stuart, K. D., Barrell, B., *et al.* (2005). The genome of the kinetoplastid parasite, Leishmania major. *science* **309,** 436-42.

Iwu, M. M., Jackson, J. E., and Schuster, B. G. (1994). Medicinal plants in the fight against leishmaniasis. *Parasitol Today* **10,** 65-8.

Jackson, P. R., Wohlhieter, J., and Hockmeyer, W. T. (1982). Leishmaniacharacterization by restriction endonuclease digestion ofkinetoplastic DNA. *In* "Abstr Vth Internat Congr Parasitol ", pp. 342, Toronto, Canada.

Jackson, P. R., Wohlhieter, J. A., Jackson, J. E., Sayles, P., Diggs, C. L., and Hockmeyer, W. T. (1984). Restriction endonuclease analysis of Leishmania kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. . *American Journal of Tropical Medicine and Hygiene* **33,** 808-19.

Jackson, T. S., Lerner, E., Weisbrod, R. M., Tajima, M., Loscalzo, J., and Keaney, J. F. (1996). Vasodilatory properties of recombinant maxadilan. *Am J Physiol* **271,** H924-30.

Jha, T. K., Sundar, S., Bhattacharya, S. K., Sinha, P. K., and Thakur, C. P. (2005). Abstract book- Paromomycin as a new cure for visceral leishmaniasis: Preliminary results of a Phase III randomized controlled trial of efficacy and safety. *In* "Third World Congress on Leishmanioisis", Sicilia, Italy.

Ji, J., Masterson, J., Sun, J., and Soong, L. (2005). CD4+CD25+ regulatory T cells restrain pathogenic responses during Leishmania amazonensis infection. *The Journal of Immunology* **174,** 7147-53.

Jiang, H., and Chess, L. (2004). An integrated view of suppressor T cell subsets in immunoregulation. *The Journal of Clinical Investigation* **114,** 1198-208.

Johann, A. M., von Knethen, A., Lindemann, D., and Brune, B. (2006). Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor-gamma and attenuates the oxidative burst. *Cell Death Differ* **13,** 1533-40.

Joshi, M., Dwyer, D. M., and Nakhasi, H. L. (1995). Molecular cloning and characterization of a Leishmania donovani alpha-tubulin gene. *J Eukaryot Microbiol* **42,** 628-32.

Joshi, P. B., Kelly, B. L., Kamhawi, S., Sacks, D. L., and McMaster, W. R. (2002). Targeted gene deletion in Leishmania major identifies leishmanolysin (GP63) as a virulence factor. *Molecular and Biochemical Parasitology* **120,** 33-40.

Joshi, P. B., Sacks, D. L., Modi, G., and McMaster, W. R. (1998). Targeted gene deletion of Leishmania major genes encoding developmental stage-specific leishmanolysin (GP63). *Mol Microbiol* **27,** 519-30.

Kamhawi, S. (2006). Phlebotomine sand flies and Leishmania parasites: friends or foes? *Trends in Parasitology* **22,** 439-445.

Kane, M. M., and Mosser, D. M. (2000). Leishmania parasites and their ploys to disrupt macrophage activation. *Curr Opin Hematol* **7,** 26-31.

Kane, M. M., and Mosser, D. M. (2001). The role of IL-10 in promoting disease progression in leishmaniasis. *The Journal of Immunology* **166,** 1141-7.

Kaplan, R., Jaye, M., Burgess, W. H., Schlaepfer, D. D., and Haigler, H. T. (1988). Cloning and expression of cDNA for human endonexin II, a Ca2+ and phospholipid binding protein. *Journal of Biological Chemistry* **263,** 8037-43.

Kar, S., Metz, C., and McMahon-Pratt, D. (2005). CD4+ T cells play a dominant role in protection against New World leishmaniasis induced by vaccination with the P-4 amastigote antigen. *Infection and Immunity* **73,** 3823-7.

Kariminia, A. B. E., Pascalis, H., Couppie, P., Sainte-Marie, D., Tacchini-Cottier, F., and Launois, P. (2005). Transforming growth factor beta 1 production by CD4+ CD25+ regulatory T cells in peripheral blood mononuclear cells from healthy subjects stimulated with Leishmania guyanensis. *Infection and Immunity* **73,** 5908-5914.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. *Nat Immunol* **3,** 221-7.

Karp, N. A., Kreil, D. P., and Lilley, K. S. (2004). Determining a significant change in protein expression with DeCyder during a pair-wise comparison using two-dimensional difference gel electrophoresis. *Proteomics* **4,** 1421-32.

Kato, S., Sugimura, N., Nakashima, K., Nishihara, T., and Kowashi, Y. (2005). Actinobacillus actinomycetemcomitans induces apoptosis in human monocytic THP-1 cells. *Journal of Medical Microbiology* **54,** 293-8.

Kaye, P. M. (1995). Costimulation and the regulation of antimicrobial immunity. *Immunol Today* **16,** 423-7.

Kemp, K. (2000). Cytokine-producing T cell subsets in human leishmaniasis. *Arch Immunol Ther Exp (Warsz)* **48,** 173-6.

Kemp, M. (1997). Regulator and effector functions of T-cell subsets in human Leishmania infections. *APMIS Suppl* **68,** 1-33.

Kemp, M., Kurtzhals, J. A., Bendtzen, K., Poulsen, L. K., Hansen, M. B., Koech, D. K., Kharazmi, A., and Theander, T. G. (1993). Leishmania donovani-

reactive Th1- and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. *Infection and Immunity* **61,** 1069-73.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26,** 239-57.

Kheifets, V., Bright, R., Inagaki, K., Schechtman, D., and Mochly-Rosen, D. (2006). Protein kinase C delta (deltaPKC)-annexin V interaction: a required step in deltaPKC translocation and function. *Journal of Biological Chemistry* **281,** 23218-26.

Kimura, T., Hashimoto, I., Yamamoto, A., Nishikawa, M., and Fujisawa, J. I. (2000). Rev-dependent association of the intron-containing HIV-1 gag mRNA with the nuclear actin bundles and the inhibition of its nucleocytoplasmic transport by latrunculin-B. *Genes Cells* **5,** 289-307.

Kinghorn, A. D., Su, B. N., Jang, D. S., Chang, L. C., Lee, D., Gu, J. Q., Carcache-Blanco, E. J., Pawlus, A. D., Lee, S. K., Park, E. J., Cuendet, M., Gills, J. J., Bhat, K., Park, H. S., Mata-Greenwood, E., Song, L. L., Jang, M., and Pezzuto, J. M. (2004). Natural inhibitors of carcinogenesis. *Planta Med* **70,** 691-705.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *The EMBO Journal* **14,** 5579–5588.

Korzeniewski, K., and Olszannski, R. (2004). Leishmaniasis among soldiers of stabilization forces in Iraq. *International Maritime Health* **55,** 155-63.

Kreutzer, R. D., Yemma, J. J., Grogl, M., Tesh, R. B., and Martin, T. I. (1994). Evidence of sexual reproduction in the protozoan parasite Leishmania (Kinetoplastida: Trypanosomatidae). *Am J Trop Med Hyg.* **51,** 301-7.

Kuhls, K., Mauricio, I. L., Pratlong, F., Presber, W., and Schonian, G. (2005). Analysis of ribosomal DNA internal transcribed spacer sequences of the Leishmania donovani complex. *Microbes Infect.* **7,** 1224-34.

Kumar, V., Hackett, J., Tutt, M. M., Garni-Wagner, B. A., Kuziel, W. A., Tucker, P. V., and Bennet, M. (1989). Natural Killer cells and their precursors in mice with severe combined immunodeficiency. *Curr. Topics. Microbiol. Immunol.* **152,** 47-52.

Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D., and Kornbluth, S. (1998). Apoptosis induction bycaspase-8 is amplified through the mitochondrial release of cytochrome c. *Journal of Biological Chemistry* **273,** 16589–16594.

Lai, C. H., Chou, C. Y., Ch'ang, L. Y., Liu, C. S., and Lin, W. C. (2000). Identification of novel human genes evolutionarily conserved in

Caenorhabditis elegans by comparative proteomics. *Genome Res.* **10,** 703-713.

Lainson, R. (1983). The American leishmaniases: some observations on their ecology and epidemiology. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77,** 569-96.

Lainson, R., and Rangel, E. F. (2005). Lutzomyia longipalpis and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. . *Mem Inst Oswaldo Cruz.* **100,** 811-27.

Lainson, R., and Rangel, E. F. (2006). Leishmania infantum versus Leishmania chagasi: do not forget the law of priority: Reply. *Memórias do Instituto Oswaldo Cruz* **101,** 118.

Lainson, R., and Shaw, J. J. (1978). Epidemiology and ecology of leishmaniasis in Latin-America. *Nature* **273,** 595-600.

Lainson, R., and Shaw, J. J. (1987). "Evolution, classification and geographical distribution. ." Academic Press, London.

Lang, T., de Chastellier, C., Frehel, C., Hellio, R., Metezeau, P., Leao Sde, S., and Antoine, J. C. (1994). Distribution of MHC class I and of MHC class II molecules in macrophages infected with Leishmania amazonensis. *J Cell Sci* **107,** 69-82.

Langrish, C. L., McKenzie, B. S., Wilson, N. J., de Waal Malefyt, R., Kastelein, R. A., and Cua, D. J. (2004). IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev* **202,** 96-105.

Lanotte, G., Rioux, J. A., Maazoun, R., Pasteur, N., Pratlong, F., and Lepart, J. (1981). The application of a numerical method to the taxonomy of the genus Leishmania Ross, 1903,--The recognition of 146 original lines in the Old World. Use of allozymic characters. Epidemiological and phyletic significance *Ann Parasitol Hum Comp* **56,** 575-91.

Laskay, T., Rollinghoff, M., and Solbach, W. (1993). Natural killer cells participate in the early defense against Leishmania major infection in mice. *European Journal of Immunology* **23,** 2237-41.

Laskay, T., van Zandbergen, G., and Solbach, W. (2003). Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? *Trends Microbiol* **11,** 210-4.

Lauber, K., Bohn, E., Krober, S. M., Xiao, Y. J., Blumenthal, S. G., Lindemann, R. K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S., Xu, Y., Autenrieth, I. B., Schulze-Osthoff, K., Belka, C., Stuhler, G., and Wesselborg, S. (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* **113,** 717-30.

Laurenti, M. D., Gidlund, M., Ura, D. M., Sinhorini, I. L., Corbett, C. E., and Goto, H. (1999). The role of natural killer cells in the early period of

infection in murine cutaneous leishmaniasis. *Braz J Med Biol Res* **32,** 323-5.

Leach, A. P. (1998). Apoptosis: molecular mechanism for physiologic cell death. *Clin Lab Sci* **11,** 346-9.

Leder, K., and Weller, F. P. (2007). Epidemiology and clinical manifestations of leishmaniasis. http://patients.uptodate.com/topic.asp?file=parasite/16332, accessed on 6th July 2007.

Lemesre, J. L., Rizvi, F. S., Afchain, D., Sadigursky, M., Capron, A., and Santoro, F. (1985). Subspecies-specific surface antigens of promastigotes of the Leishmania donovani complex. *Infection and Immunity* **50,** 136-41.

Lemma, A., and Shiller, E. L. (1964). Extracellular cultivation of the leishmanial bodies of species belonging to the protozoan genus Leishmania. *Experimental parasitology* **15.**

Lerner, E. A., Ribeiro, J. M., Nelson, R. J., and Lerner, M. R. (1991). Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly Lutzomyia longipalpis. *Journal of Biological Chemistry* **266,** 11234-11236.

Lerner, E. A., and Shoemaker, C. B. (1992). Maxadilan. Cloning and functional expression of the gene encoding this potent vasodilator peptide. *Journal of Biological Chemistry* **267,** 1062-6.

Leslie, G., Barrett, M., and Burchmore, R. (2002). Leishmania mexicana: promastigotes migrate through osmotic gradients. *Experimental Parasitology* **102,** 117-120.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91,** 479-89.

Li, S., Wilson, M. E., and Donelson, J. E. (1996). Leishmania chagasi: a gene encoding a protein kinase with a catalytic domain structurally related to MAP kinase kinase. *Experimental Parasitology* **82,** 87-96.

Li, X. M., Lin, M., and Wang, Y. H. (2003). Stilbenoids from the lianas of Gnetum pendulum. *J Asian Nat Prod Res* **5,** 113-9.

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Molecular Cell* **6,** 1389-99.

Lisi, S., Sisto, M., Acquafredda, A., Spinelli, R., Schiavone, M., Mitolo, V., Brandonisio, O., and M, P. (2005). Infection with Leishmania infantum Inhibits actinomycin D-induced apoptosis of human monocytic cell line U-937. *The Journal of Eukaryotic Microbiology* **52,** 211-7.

Liu, X., and Chang, K. P. (1994). Identification by extrachromosomal amplification and overexpression of a zeta-crystallin/NADPH-oxidoreductase homologue constitutively expressed in Leishmania spp. *Molecular and Biochemical Parasitology* **66,** 201-10.

Louassini, M., Adroher, F. J., Foulquie, M. R., and Benitez, R. (1998). Investigations on the in vitro metacyclogenesis of a visceral and a cutaneous human strain of Leishmania infantum. *Acta Trop* **70,** 355-68.

Lowe, S. W., and Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis* **21,** 485-95.

Lu, M., Holliday, L. S., Zhang, L., Dunn, W. A., and Gluck, S. L. (2001). Interaction between aldolase and vacuolar H+-ATPase: evidence for direct coupling of glycolysis to the ATP-hydrolyzing proton pump. *J. Biol. Chem.* **32,** 30407-13.

MacFarlane, M., and Williams, A. C. (2004). Apoptosis and disease: a life or death decision. *EMBO Rep* **5,** 674-8.

Magill, A. J., Grogl, M., Johnson, S. C., and Gasser, R. A. (1994). Visceral infection due to Leishmania tropica in a veteran of Operation Desert Storm who presented 2 years after leaving Saudi Arabia. *Jr Clin Infect Dis* **19.**

Majester-Savornin, B., Elias, R., Diaz-Lanza, A. M., Balansard, G., Gasquet, M., and Delmas, F. (1991). Saponins of the ivy plant, Hedera helix, and their leishmanicidic activity. *Planta Med* **57,** 260-2.

Mansueto, P., Vitale, G., Di Lorenzo, G., Rini, G. B., Mansueto, S., and Cillari, E. (2007). Immunopathology of leishmaniasis: an update. *Int J Immunopathol Pharmacol* **20,** 435-45.

Marsden, P. D. (1986). Mucosal leishmaniasis ("espundia" Escomel, 1911). *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80,** 859-76.

Martinez, E., Alonso, V., Quispe, A., Thomas, M. C., Alonso, R., Pinero, J. E., Gonzalez, A. C., Ortega, A., and Valladares, B. (2003). RAPD method useful for distinguishing Leishmania species: design of specific primers for L. braziliensis. . *Parasitology* **127,** 513-7.

Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R. I., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P., and Elledge, S. J. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316,** 1160-1166.

Matte, C., Maion, G., Mourad, W., and Olivier, M. (2001). Leishmania donovani-induced macrophages cyclooxygenase-2 and prostaglandin E2 synthesis. *Parasite Immunol* **23,** 177-84.

Mauricio, I. L., Stothard, J. R., and Miles, M. A. (2000). The strange case of Leishmania chagasi. *Parasitol Today* **16,** 188-9.

Mbwambo, Z. H., Apers, S., Moshi, M. J., Kapingu, M. C., Van Miert, S., Claeys, M., Brun, R., Cos, P., Pieters, L., and Vlietinck, A. (2004). Anthranoid compounds with antiprotozoal activity from Vismia orientalis. *Planta Med* **70,** 706-10.

McConville, M. J., de Souza, D., Saunders, E., Likic, V. A., and Naderer, T. (2007). Living in a phagolysosome; metabolism of Leishmania amastigotes. *Trends in Parasitology* **23,** 368-75.

McConville, M. J., and Homans, S. W. (1992). Identification of the defect in lipophosphoglycan biosynthesis in a non-pathogenic strain of Leishmania major. *Journal of Biological Chemistry* **267,** 5855-61.

McConville, M. J., and Ralton, J. E. (1997). Developmentally regulated changes in the cell surface architecture of Leishmania parasites. *Behring Inst Mitt* **99.**

McConville, M. J., Schnur, L. F., Jaffe, C., and Schneider, P. (1995). Structure of Leishmania lipophosphoglycan: inter- and intra-specific polymorphism in Old World species. *Biochem J* **310,** 807-18.

Medina-Acosta, E., Franco, A. M., Jansen, A. M., Sampol, M., Neves, N., Pontes-de-Carvalho, L., Grimaldi Junior, G., and Nussenzweig, V. (1994). Trans-sialidase and sialidase activities discriminate between morphologically indistinguishable trypanosomatids. *Eur J Biochem* **225,** 333-9.

Melby, P. C., Kreutzer, R. D., McMahon-Pratt, D., Gam, A. A., and Neva, F. A. (1992). Cutaneous leishmaniasis: review of 59 cases seen at the National Institutes of Health. *Clin Infect Dis* **15,** 924-37.

Mendez, S., Belkaid, Y., Seder, R. A., and Sacks, D. (2002). Optimization of DNA vaccination against cutaneous leishmaniasis.Vaccine. *Vaccine* **20,** 3702-8.

Mendez, S., Reckling, S. K., Piccirillo, C. A., Sacks, D., and Belkaid, Y. (2004). Role for CD4+ CD25+ Regulatory T Cells in Reactivation of Persistent Leishmaniasis and Control of Concomitant Immunity *The Journal of Experimental Medicine* **200,** 201-210.

Mensa-Wilmot, K., Garg, N., McGwire, B. S., Lu, H. G., Zhong, L., Armah, D. A., LeBowitz, J. H., and Chang, K. P. (1999). Roles of free GPIs in amastigotes of Leishmania. *Molecular and Biochemical Parasitology* **99,** 103-16.

Mikus, J., and Steverding, D. (2000). A simple colorimetric method to screen drug cytotoxicity against Leishmania using the dye Alamar Blue. *Parasitol Int* **48,** 265-9.

Minodier, P., and Parola, P. (2007). Cutaneous leishmaniasis treatment.Travel Med Infect Dis. *Travel Med Infect Dis* **5,** 150-8.

Misra, S., Tripathi, M. K., and Chaudhuri, G. (2005). Down-regulation of 7SL RNA expression and impairment of vesicular protein transport pathways by Leishmania infection of macrophages. *Journal of Biological Chemistry* **280,** 29364-73.

Mittra, B., Saha, A., Chowdhury, A. R., Pal, C., Mandal, S., Mukhopadhyay, S., Bandyopadhyay, S., and Majumder, H. K. (2000). Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. *Mol Med.* **6,** 527-41.

Modabber, F. (1995). Vaccines against leishmaniasis. *Ann Trop Med Parasitol* **89,** 83-8.

Modabber, F. (2000). First generation leishmaniasis vaccines in clinical development: moving, but what next? *Current opinion in Anti infective Investigational drugs* **2,** 35-9.

Modabber, F., Buffet, P. A., Torreele, E., Milon, G., and Croft, S. (2007). Consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis. *Kinetoplastid Biol Dis* **6,** 3.

Moll, H., Binoder, K., Bogdan, C., Solbach, W., and Rollinghoff, M. (1990). Production of tumour necrosis factor during murine cutaneous leishmaniasis. *Parasite Immunol* **12,** 483-94.

Moll, H., Flohe, S., and Rollinghoff, M. (1995). Dendritic cells in Leishmania major-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. *European Journal of Immunology* **25,** 696-9.

Monack, D. M., Raupach, B., Hromockyj, A. E., and Falkow, S. (1996). Salmonella typhimurium invasion induces apoptosis in infected macrophages. *Proceedings of the National Academy of Sciences* **93,** 9833-8.

Moore, K. J., Labrecque, S., and Matlashewski, G. (1993). Alteration of Leishmania donovani infection levels by selective impairment of macrophage signal transduction. *The Journal of Immunology* **150,** 4457-65.

Moore, K. J., and Matlashewski, G. (1994). Intracellular infection by Leishmania donovani inhibits macrophage apoptosis. *The Journal of Immunology* **152,** 2930-2937.

Moreno, J., Nieto, J., Masina, S., Canavate, C., Cruz, I., Chicharro, C., Carrillo, E., Napp, S., Reymond, C., Kaye, P. M., Smith, D. F., Fasel, N., and Alvar, J. (2007). Immunization with H1, HASPB1 and MML Leishmania proteins in a vaccine trial against experimental canine leishmaniasis. *Vaccine* **25,** 5290-300.

Mosser, D. M., and Brittingham, A. (1997). Leishmania, macrophages and complement: a tale of subversion and exploitation. *Parasitology* **115,** 23.

Mosser, D. M., and Edelson, P. J. (1984). Activation of the alternative complement pathway by Leishmania promastigotes: Parasite lysis and attachment to macrophages. *The Journal of Immunology* **132,** 1501-05.

Muigai, R., Githure, J. I., Gachihi, G. S., Were, J. B., Leeuwenburg, J., and Perkins, P. V. (1987). Cutaneous leishmaniasis caused by Leishmania major in Baringo District, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81,** 600-2.

Muller, I., Pedrazzini, T., Korpf, P., Louis, J. A., and Milon, G. (1991). Establishment of resistance to Leishmania major infection in susceptible BALB/c mice requires parasite-specific CD8+ T cells. *Int Immunol* **3,** 587-597.

Murray, H. W. (2001). Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Ag Chemother* **45,** 2185–97.

Mussi, S. V., Fernandes, A. P., and Ferreira, L. A. (2007). Leishmania (Leishmania) amazonensis. *Parasitol Res* **100,** 1221-6.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., and Ni, J., et al. (1996). FLICE, a novel FADDhomologousICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death – inducing signaling complex. *Cell* **85.**

Nacy, C. A., and Diggs, C. L. (1981). Intracellular replication of Leishmania tropica in mouse peritoneal macrophages: comparison of amastigote replication in adherent and nonadherent macrophages. *Infection and Immunity* **34,** 310-3.

Naderer, T., Vince, J. E., and McConville, M. J. (2004). Surface determinants of Leishmania parasites and their role in infectivity in the mammalian host. *Curr Mol Med* **4,** 649-65.

Nagao, A. (2004). Oxidative conversion of carotenoids to retinoids and other products. *J Nutr* **134,** 237S-240S.

Nagase, H., Jones, K. M., Anderson, C. F., and Noben-Trauth, N. (2007). Despite increased CD4+Foxp3+ cells within the infection site, BALB/c IL-4 receptor-deficient mice reveal CD4+Foxp3-negative T cells as a source of IL-10 in Leishmania major susceptibility. *The Journal of Immunology* **179,** 2435-44.

Nagase, T., Seki, N., Tanaka, A., Ishikawa, K., and Nomura, N. (1995). Prediction of the coding sequences of unidentified human genes. IV. The coding sequences of 40 new genes (KIAA0121-KIAA0160) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res* **2,** 167-174.

Nagata, S. (2000). Apoptotic DNA fragmentation. *Experimental Cell Research* **256,** 12-8.

Nakajima-Shimada, J., Zou, C., Takagi, M., Umeda, M., Nara, T., and Aoki, T. (2000). Inhibition of Fas-mediated apoptosis by Trypanosoma cruzi infection. *Biochimica et Biophysica Acta* **1475,** 175-83.

Nakamura, T., Hemmi, H., Aso, H., and Ishida, N. (1986). Variants of a human monocytic leukemia cell line (THP-1): induction of differentiation by retinoic acid, interferon-gamma, and T-lymphocyte-derived differentiation-inducing activity. *J Natl Cancer Inst* **77,** 21-7.

Nandan, D., Cherkasov, A., Sabouti, R., Yi, T., and Reiner, N. E. (2003). Molecular cloning, biochemical and structural analysis of elongation factor-1[alpha] from Leishmania donovani: comparison with the mammalian homologue. *Biochemical and Biophysical Research Communications* **302,** 646-652.

Nandan, D., and Reiner, N. E. (1995). Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with Leishmania donovani: selective inhibition of signaling through Janus kinases and Stat1. *Infection and Immunity* **63,** 4495-500.

Nandan, D., Tran, T., Trinh, E., Silverman, J. M., and Lopez, M. (2007). Identification of leishmania fructose-1,6-bisphosphate aldolase as a novel activator of host macrophage Src homology 2 domain containing protein tyrosine phosphatase SHP-1. *Biochemical and Biophysical Research Communications* **364,** 601-7.

Nash, P. B., Purner, M. B., Leon, R. P., Clarke, P., Duke, R. C., and Curiel, T. J. (1998). Toxoplasma gondii-infected cells are resistant to multiple inducers of apoptosis. *The Journal of Immunology* **160,** 1824-30.

Newmeyer, D. D., and Ferguson-Miller, S. (2003). Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112,** 481-90.

Nicolle, C. H. (1908). Cultures des corps de Leishman isoles de la rate dans trois cas d'anemic sptenique infantile. *Bull. Soc. Pathol. Exot* **1,** 121.

Noben-Trauth, N., Kropf, P., and Muller, I. (1996). Susceptibility to Leishmania major infection in interleukin-4-deficient mice. *science* **271,** 987-90.

Noben-Trauth, N., Lira, R., Nagase, H., Paul, W. E., and Sacks, D. L. (2003). The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to Leishmania major. *The Journal of Immunology* **170,** 5152-8.

Nonaka, T., Kuwabara, T., Mimuro, H., Kuwae, A., and Imajoh-Ohmi, S. (2003). Shigella-induced necrosis and apoptosis of U937 cells and J774 macrophages. *Microbiology* **149,** 2513-27.

Noronha, F. S., Nunes, A. C., Souza, K. T., Melo, M. N., and Ramalho-Pinto, F. J. (1998). Differential sensitivity of New World Leishmania spp.

promastigotes to complement-mediated lysis: correlation with the expression of three parasite polypeptides. *Acta Trop* **69,** 17-29.

Noyes, H. A., Camps, A. P., and Chance, M. L. (1996). Leishmania herreri (Kinetoplastida; Trypanosomatidae) is more closely related to Endotrypanum (Kinetoplastida; Trypanosomatidae) than to Leishmania. *Mol Biochem Parasitol* **80,** 119-23.

Nugent, P. G., Karsani, S. A., Wait, R., Tempero, J., and Smith, D. F. (2004). Proteomic analysis of Leishmania mexicana differentiation. *Molecular and Biochemical Parasitology* **136,** 51-62.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250,** 4007-21.

Oda, Y., Okada, T., Yoshida, H., Kaufman, R. J., Nagata, K., and Mori, K. (2006). Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J. Cell Biol.* **172,** 383-393.

Ogunkolade, B. W., Colomb-Valet, I., Monjour, L., Rhodes-Feuillette, A., Abita, J. P., and Frommel, D. (1990). Interactions between the human monocytic leukaemia THP-1 cell line and Old and New World species of Leishmania. *Acta Tropica* **47,** 171-6.

Ohmori, H., Toyama, S., and Toyama, S. (1992). Direct proof that the primary site of action of cytochalasin on cell motility processes is actin. *J Cell Biol* **116,** 933-941.

Oketch-Rabah, H. A., Dossaji, S. F., Christensen, S. B., Frydenvang, K., Lemmich, E., Cornett, C., Olsen, C. E., Chen, M., Kharazmi, A., and Theander, T. (1997). Antiprotozoal compounds from Asparagus africanus. *Journal of Natural Products* **60,** 1017-22.

Okunji, C. O., Iwu, M. M., Jackson, J. E., and Tally, J. D. (1996). Biological activity of saponins from two Dracaena species. *Adv Exp Med Biol* **404,** 415-28.

Olivier, M., Brownsey, R. W., and Reiner, N. E. (1992). Defective stimulus-response coupling in human monocytes infected with Leishmania donovani is associated with altered activation and translocation of protein kinase C. *Proceedings of the National Academy of Sciences* **89,** 7481-5.

Orhan, I., Sener, B., Atici, T., Brun, R., Perozzo, R., and Tasdemir, D. (2006). Turkish freshwater and marine macrophyte extracts show in vitro antiprotozoal activity and inhibit FabI, a key enzyme of Plasmodium falciparum fatty acid biosynthesis. *Phytomedicine* **13,** 388-93.

Oshry, L., Meers, P., Mealy, T., and Tauber, A. I. (1991). Annexin-mediated membrane fusion of human neutrophil plasma membranes and phospholipid vesicles. . *Biochim Biophys Acta.* **1066,** 239-44.

Ozipek, M., Donmez, A. A., Calis, I., Brun, R., Ruedi, P., and Tasdemir, D. (2005). Leishmanicidal cycloartane-type triterpene glycosides from Astragalus oleifolius. *Phytochemistry* **66,** 1168-73.

Padigel, U. M., and Farrell, J. P. (2005). Control of infection with Leishmania major in susceptible BALB/c mice lacking the common gamma-chain for FcR is associated with reduced production of IL-10 and TGF-beta by parasitized cells. *The Journal of Immunology* **174,** 6340-5.

Pan, A. A. (1984). Leishmania mexicana: Serial cultivation of intracellular stages in a cell-free medium. *Experimental Parasitology* **58,** 72-80.

Pan, A. A., Duboise, S. M., Eperon, S., Rivas, L., Hodgkinson, V., Traub-Cseko, Y., and McMahon-Pratt, D. (1993). Developmental life cycle of Leishmania--cultivation and characterization of cultured extracellular amastigotes. *J Eukaryot Microbiol.* **40,** 213-23.

Papadopoulou, B., Roy, G., Breton, M., Kundig, C., Dumas, C., Fillion, I., Singh, A. K., Olivier, M., and Ouellette, M. (2002). Reduced infectivity of a Leishmania donovani biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infection and Immunity* **70,** 62-8.

Paul, A., Wilson, S., Belham, C. M., Robinson, C. J., Scott, P. H., Gould, G. W., and Plevin, R. (1997). Stress-activated protein kinases: activation, regulation and function. *Cell Signal* **9,** 403-10.

Pearson, R. D., Lareau, S. M., and Jeronimo, S. M. (1999). Leishmaniasis at the End of the Millennium. *Curr Infect Dis Rep* **1,** 448-52.

Penninger, J. M., and Kroemer, G. (2003). Mitochondria, AIF and caspases--rivaling for cell death execution. *Nature Cell Biology* **5,** 97-9.

Perry, J., and Zhao, Y. (2003). The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. *Trends Biochem Sci.* **28,** 576-80.

Peter, M. E., and Krammer, P. H. (2003). The CD95 (APO-1/Fas) DISC and beyond. *Cell Death Differ* **10,** 26-35.

Peters, W., Elbihari, S., and Evans, D. A. (1986). Leishmania infecting man and wild animals in Saudi Arabia. 2. Leishmania arabica n. sp. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80,** 497-502.

Piccirillo, C. A., and Thornton, A. M. (2004). Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. *Trends Immunol* **25,** 374-80.

Piedrafita, D., Proudfoot, L., Nikolaev, A. V., Xu, D., Sands, W., Feng, G. J., Thomas, E., Brewer, J., Ferguson, M. A., Alexander, J., and Liew, F. Y. (1999). Regulation of macrophage IL-12 synthesis by Leishmania phosphoglycans. *Eur J Immunol* **29,** 235-44.

Pimenta, P. F., Saraiva, E. M., and Sacks, D. L. (1991). The comparative fine structure and surface glycoconjugate expression of three life stages of Leishmania major. *Experimental Parasitology* **72,** 191-204.

Pinheiro, R. O., Pinto, E. F., Lopes, J. R., Guedes, H. L., Fentanes, R. F., and Rossi-Bergmann, B. (2005). TGF-beta-associated enhanced susceptibility to leishmaniasis following intramuscular vaccination of mice with Leishmania amazonensis antigens. *Microbes Infect.* **7,** 1317-23.

Pogue, G. P., Joshi, M., Lee, N. S., Dwyer, D. M., Kenney, R. T., Gam, A. A., and Nakhasi, H. L. (1996). Conservation of low-copy gene loci in Old World leishmanias identifies mechanisms of parasite evolution and diagnostic markers. *Molecular and Biochemical Parasitology* **81,** 27-40.

Potestio, M., D'Agostino, P., Romano, G. C., Milano, S., Ferlazzo, V., Aquino, A., Di Bella, G., Caruso, R., Gambino, G., Vitale, G., Mansueto, S., and Cillari, E. (2004). CD4+ CCR5+ and CD4+ CCR3+ lymphocyte subset and monocyte apoptosis in patients with acute visceral leishmaniasis. *immunology today* **113,** 260-8.

Pral, E. M., Bijovsky, A. T., Balanco, J. M., and Alfieri, S. C. (1993). Leishmania mexicana: proteinase activities and megasomes in axenically cultivated amastigote-like forms. *Experimental Parasitology* **77,** 62-73.

Prina, E., Jouanne, C., de Souza Lao, S., Szabo, A., Guillet, J. G., and Antoine, J. C. (1993). Antigen presentation capacity of murine macrophages infected with Leishmania amazonensis amastigotes. *The Journal of Immunology* **151,** 2050-60.

Prive, C., and Descoteaux, A. (2000). Leishmania donovani promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages. *European Journal of Immunology* **30,** 2235-44.

Puentes, S. M., Da Silva, R. P., Sacks, D. L., Hammer, C. H., and Joiner, K. A. (1990). Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. *The Journal of Immunology* **145,** 4311-6. .

Puentes, S. M., Dwyer, D. M., Bates, P. A., and Joiner, K. A. (1989). Binding and release of C3 from Leishmania donovani promastigotes during incubation in normal human serum. *The Journal of Immunology* **143,** 3743-9.

Qi, H., Denning, T. L., and Soong, L. (2003). Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial toll-like receptor activators and skewing of T-cell cytokine profiles. *Infection and Immunity* **71,** 3337-42.

Quijada, L., Soto, M., and Requena, J. M. (2005). Genomic DNA macroarrays as a tool for analysis of gene expression in Leishmania. *Experimental Parasitology* **111,** 64-70.

Quinones, M., Ahuja, S. K., Melby, P. C., Pate, L., Reddick, R. L., and Ahuja, S. S. (2000). Preformed membrane-associated stores of interleukin (IL)-12 are a previously unrecognized source of bioactive IL-12 that is mobilized within minutes of contact with an intracellular parasite. *The Journal of Experimental Medicine* **192,** 507-16.

Rainey, P. M., Spithill, T. W., McMahon-Pratt, D., and Pan, A. A. (1991). Biochemical and molecular characterization ofLeishmania pifanoi amastigotes in continuous axenic culture. *Molecular and Biochemical Parasitology* **49,** 111-118.

Ramesh, V., and Mukherjee, A. (1995). Post-kala-azar dermal leishmaniasis. *Int J Dermatol* **34,** 85-91.

Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M., and Muller, W. A. (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *science* **282,** 480-3.

Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M., and Muller, W. A. (1999). Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* **11,** 753-61.

Rangel, E. F., and Lainson, R. (2003). "Flebotomíneos do Brasil." Fundação Oswaldo Cruz, Rio de Janeiro.

Rathor, H. R. (1996). The role of vectors in emerging and re-emerging diseases in the Eastern Mediterranean Region. *Eastern Mediterranean Health Journal* **2,** 61-7.

Rathsam, C., Eaton, R. E., Simpson, C. L., Browne, G. V., Valova, V. A., Harty, D. W., and Jacques, N. A. (2005). Two-dimensional fluorescence difference gel electrophoretic analysis of Streptococcus mutans biofilms. *J Proteome Res* **4,** 2161-73.

Raz, B., Iten, M., Grether-Buhler, Y., Kaminsky, R., and Brun, R. (1997). The Alamar Blue assay to determine drug sensitivity of African trypanosomes (T.b. rhodesiense and T.b. gambiense) in vitro. *Acta Trop* **68,** 139-47.

Reed, S. G. (2004). Report on Leishmaniasis, pp. 46. TDR/SWG/04.

Reed, S. G., Coler, R. N., and Campos-Neto, A. (2003). Development of a leishmaniasis vaccine: the importance of MPL. *Expert Rev Vaccines* **2,** 239-52.

Reiner, N. E., Ng, W., and McMaster, W. R. (1987). Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *The Journal of Immunology* **138,** 1926-32.

Reiner, N. E., Ng, W., Wilson, C. B., McMaster, W. R., and Burchett, S. K. (1990). Modulation of in vitro monocyte cytokine responses to Leishmania donovani. Interferon-gamma prevents parasite-induced inhibition of

interleukin 1 production and primes monocytes to respond to Leishmania by producing both tumor necrosis factor-alpha and interleukin 1. *The Journal of Clinical Investigation* **85,** 1914-24.

Reiner, S. L., and Locksley, R. M. (1995). The regulation of immunity to Leishmania major. *Annu Rev Immunol* **13,** 151-77.

Ribeiro-Gomes, F. L., Moniz-de-Souza, M. C., Borges, V. M., Nunes, M. P., Mantuano-Barradas, M., and D'Avila, H. (2005). Turnover of neutrophils mediated by Fas ligand
drives Leishmania major infection. *J Infect Dis* **192.**

Ribeiro, J. M. (1987). Role of saliva in blood-feeding by arthropods. *Ann Rev Entomol* **32,** 463-478.

Rijal, A., Agrawal, S., Agarwalla, A., Agrawal, A., and Rijal, S. (2005). Post-kala-azar dermal leishmaniasis with visceral leishmaniasis: a rare presentation. *Int J Dermatol* **44,** 494-6.

Rioux, J. A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P., and Perieres, J. (1990). Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. *Ann Parasitol Hum Comp* **65,** 111-25.

Rittig, M. G., and Bogdan, C. (2000). Leishmania-Host-cell Interaction: Complexities and Alternative Views. *Parasitology Today* **16,** 292-297.

Rittig, M. G., Schroppel, K., Seack, K. H., Sander, U., N'Diaye, E. N., Maridonneau-Parini, I., Solbach, W., and Bogdan, C. (1998). Free in PMC Coiling phagocytosis of trypanosomatids and fungal cells. *Infection and Immunity* **66,** 4331-9.

Rivier, D., Bovay, P., Shah, R., Didisheim, S., and Mauel, J. (1999). Vaccination against Leishmania major in a CBA mouse model of infection: role of adjuvants and mechanism of protection. *Parasite Immunol* **21,** 461-73.

Rizvi, F. S., Ouaissi, M. A., Marty, B., Santoro, F., and Capron, A. (1988). The major surface protein of Leishmania promastigotes is a fibronectin-like molecule. *European Journal of Immunology* **18,** 473-6.

Rogers, K. A., DeKrey, G. K., Mbow, M. L., Gillespie, R. D., Brodskyn, C. I., and Titus, R. G. (2002). Type 1 and type 2 responses to Leishmania major. *FEMS Microbiol Lett* **209,** 1-7.

Rogers, M. E., Ilg, T., Nikolaev, A. V., Ferguson, M. A., and Bates, P. A. (2004). Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. *Nature* **430,** 463-7.

Rose, K., Curtis, J., Baldwin, T., Mathis, A., Kumar, B., Sakthianandeswaren, A., Spurck, T., Low Choy, J., and Handman, E. (2004). Cutaneous leishmaniasis in red kangaroos: isolation and characterisation of the causative organisms. *International Journal for Parasitology* **34,** 655-664.

Rosypal, A. C., Troy, G. C., Zajac, A. M., Duncan, R. B., Waki, K., Chang, K. P., and Lindsay, D. S. (2003). Emergence of zoonotic canine leishmaniasis in the United States: isolation and immunohistochemical detection of Leishmania infantum from foxhounds from Virginia. *J Eukaryot Microbiol* **50,** 691-3.

Rothhut, B., Dubois, T., Feliers, D., Russo-Marie, F., and Oudinet, J. P. (1995). Inhibitory effect of annexin V on protein kinase C activity in mesangial cell lysates. *Eur J Biochem.* **232,** 865-72.

Ruckdeschel, K., Roggenkamp, A., Lafont, V., Mangeat, P., Heesemann, J., and Rouot, B. (1997). Interaction of Yersinia enterocolitica with macrophages leads to macrophage cell death through apoptosis. *Infection and Immunity* **65,** 4813-4821.

Ruhland, A., Leal, N., and Kima, P. E. (2007). Leishmania promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. *Cell Microbiol* **9,** 84–96.

Ruiz, J. H., and Becker, I. (2007). CD8 cytotoxic T cells in cutaneous leishmaniasis. *Parasite Immunol.* **29,** 671-8.

Rupinder, S. K., Gurpreet, A. K., and Manjeet, S. (2007). Cell suicide and caspases. *Vascul Pharmacol* **46,** 383-93.

Russell, D. G., and Wilhelm, H. (1986). The involvement of the major surface glycoprotein (gp63) of Leishmania promastigotes in attachment to macrophages. *The Journal of Immunology* **136,** 2613-20.

Russell, D. G., Xu, S., and Chakraborty, P. (1992). Intracellular trafficking and the parasitophorous vacuole of Leishmania mexicana-infected macrophages. *J Cell Sci* **103,** 1193-210.

Ruzek, M. C., Hawes, M., Pratt, B., McPherson, J., Ledbetter, S., Richards, S. M., and Garman, R. D. (2003). Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. *Immunopharmacol Immunotoxicol* **25,** 235-57.

Saar, Y., Ransford, A., Waldman, E., Mazareb, S., Amin-Spector, S., Plumblee, J., Turco, S. J., and Zilberstein, D. (1998). Characterization of developmentally-regulated activities in axenic amastigotes of Leishmania donovani.Mol Biochem Parasitol. *Molecular and Biochemical Parasitology* **95,** 9-20.

Sacks, D., and Anderson, C. (2004). Re-examination of the immunosuppressive mechanisms mediating non-cure of Leishmania infection in mice. *Immunol Rev* **201,** 225-38.

Sacks, D. L. (2001). Leishmania-sand fly interactions controlling species-specific vector competence. *Cell Microbiol* **3,** 189-96.

Sacks, D. L., Brodin, T. N., and Turco, S. J. (1990). Developmental modification of the lipophosphoglycan from Leishmania major promastigotes during metacyclogenesis. *Molecular and Biochemical Parasitology* **42,** 225-233.

Sacks, D. L., Kenney, R. T., Neva, F. A., Kreutzer, R. D., Jaffe, C. L., Gupta, A. K., Sharma, M. C., Sinha, S. P., and Saran, R. (1995). Indian kala-azar caused by Leishmania tropica. *The Lancet* **345,** 959-961.

Sacks, D. L., and Perkins, P. V. (1984). Identification of an infective stage of Leishmania promastigotes. *Science* **223,** 1417-9.

Sacks, D. L., Saraiva, E. M., Rowton, E., Turco, S. J., and Pimenta, P. F. (1994). The role of the lipophosphoglycan of Leishmania in vector competence. *Parasitology* **108,** 55-62.

Sadick, M. D., Heinzel, F. P., Holaday, B. J., Pu, R. T., Dawkins, R. S., and Locksley, R. M. (1990). Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *The Journal of Experimental Medicine* **171,** 115-27.

Saha, S., Mondal, S., Ravindran, R., Bhowmick, S., Modak, D., Mallick, S., Rahman, M., Kar, S., Goswami, R., Guha, S. K., Pramanik, N., Saha, B., and Ali, N. (2007). IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of Leishmania donovani infection in India. *The Journal of Immunology* **179,** 5592-603.

Sairafianpour, M., Christensen, J., Staerk, D., Budnik, B. A., Kharazmi, A., Bagherzadeh, K., and Jaroszewski, J. W. (2001). Leishmanicidal, antiplasmodial, and cytotoxic activity of novel diterpenoid 1,2-quinones from Perovskia abrotanoides: new source of tanshinones. *Journal of Natural Products* **64,** 1398-403.

Sakaguchi, S. (2003). Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. *The Journal of Experimental Medicine* **197,** 397-401.

Sakai, T., Hisaeda, H., Ishikawa, H., Maekawa, Y., Zhang, M., Nakao, Y., Takeuchi, T., Matsumoto, K., Good, R. A., and Himeno, K. (1999). Expression and role of heat-shock protein 65 (HSP65) in macrophages during Trypanosoma cruzi infection: involvement of HSP65 in prevention of apoptosis of macrophages. *Microbes and Infection* **1,** 419-27.

Sakru, N., Korkmaz, M., Ozbel, Y., Ertabaklar, H., Sengul, M., and Toz, S. O. (2007). Investigation of asymptomatic visceral leishmaniasis cases using western blot in an endemic area in Turkey. *New Microbiol* **30,** 13-8.

Saleheen, D., Ali, S. A., Ashfaq, K., Siddiqui, A. A., Agha, A., and Yasinzai, M. M. (2002). Latent activity of curcumin against leishmaniasis in vitro. *Biological and Pharmaceutical Bulletin* **25,** 386-9.

Sang, D. K., Pratlong, F., and Ashford, R. W. (1992). The identity of Leishmania tropica in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86,** 621-2.

Santoro, F., Lemesre, J. L., Rizvi, F. S., Afchain, D., and Sadogirsky, M. (1986). Spécificité au niveau de protéines de surface des promastigotes de Leishmania donovani (Laveran et Mesnil, 1903), Leishmania infantum Nicolle, 1908 et Leishmania chagasi Cunha et Chagas, 1937. In JA Rioux (2-6 July, 1984). Leishmania. Taxonomie et Phylogenése. Aplications eco-Épidemiologiques. *Colloque Internat, IMMEE,* 71-76.

Saraiva, E. M., Pinto-da-Silva, L. H., Wanderley, J. L., Bonomo, A. C., Barcinski, M. A., and Moreira, M. E. (2005). Flow cytometric assessment of Leishmania spp metacyclic differentiation: validation by morphological features and specific markers. *Experimental Parasitology* **110,** 39-47.

Savill, J., Gregory, C., and Haslett, C. (2003). Cell biology. Eat me or die. *science* **308,** 1516-7.

Savoia, D., Scutera, S., Raimondo, S., Conti, S., Magliani, W., and Polonelli, L. (2006). Activity of an engineered synthetic killer peptide on Leishmania major and Leishmania infantum promastigotes. *Experimental Parasitology* **113,** 186-92.

Scharton-Kersten, T., Afonso, L. C., Wysocka, M., Trinchieri, G., and Scott, P. (1995). IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *The Journal of Immunology* **154,** 5320-30.

Schlaepfer, D. D., Jones, J., and Haigler, H. T. (1992). Inhibition of protein kinase C by annexin V. *Biochemistry* **31,** 1886-91.

Schlein, Y., Jacobson, R. L., and Muller, G. C. (2001). Sand fly feeding on noxious plants: a potential method for the control of leishmaniasis. *American Journal of Tropical Medicine and Hygiene* **65,** 300-3.

Schurr, E., Wunderlich, F., and Tadesse, G. (1987). Electron microscopical studies on cutaneous leishmaniasis in Ethiopia. II. Parasite and host cell differences between the localized and the diffuse form. *Acta Trop.* **44,** 395-407.

Scott, P., and Farrell, J. P. (1998). Experimental cutaneous leishmaniasis: induction and regulation of T cells following infection of mice with Leishmania major. *Chem Immunol* **70,** 60-80.

Seaman, J., Mercer, A. J., Sondorp, H. E., and Herwaldt, B. L. (1996). Epidemic visceral leishmaniasis in southern Sudan: treatment of severely debilitated patients under wartime conditions and with limited resources. *Ann Intern Med* **124,** 664-72.

Seay, M. B., Heard, P. L., and Chaudhuri, G. (1996). Surface Zn-proteinase as a molecule for defense of Leishmania mexicana amazonensis promastigotes

against cytolysis inside macrophage phagolysosomes. *Infection and Immunity* **64,** 5129-37.

Sebbagh, M., Hamelin, J., Bertoglio, J., Solary, E., and Breard, J. (2005). Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. *The Journal of Experimental Medicine* **201,** 465-71.

Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J., and Breard, J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nature Cell Biology* **3,** 346-52.

Selvapandiyan, A., Duncan, R., Debrabant, A., Lee, N., Sreenivas, G., Salotra, P., and Nakhasi, H. (2006). Genetically modified live attenuated parasites as vaccines for leishmaniasis. *Indian Journal of Medical Research* **123,** 455-66.

Sereno, D., Cavaleyra, M., Zemzoumi, K., Maquaire, S., Ouaissi, A., and Lemesre, J. L. (1998). Axenically grown amastigotes of Leishmania infantum used as an in vitro model to investigate the pentavalent antimony mode of action. *Antimicrobial Agents and Chemotherapy* **42,** 3097-102.

Sharifi, I., Fekri, A. R., Aflatonian, M. R., Nadim, A., Nikian, Y., and Kamesipour, A. (1998). Cutaneous leishmaniasis in primary school children in the south-eastern Iranian city of Bam, 1994-95. *Bull World Health Organ.* **7,** 289-93.

Shaw, J. J. (1987). "Codes for hosts of Leishmania." Academic Press, London.

Shimokata, K. N., Song, M.-C., Kitada, S., T, O., and Ito, A. (1997). Substrate Recognition by Mitochondrial Processing Peptidase toward the Malate Dehydrogenase Precursor. *J. Biochem* **122,** 1019-1023.

Shiratsuchi, A., Mori, T., and Nakanishi, Y. (2002). Independence of plasma membrane blebbing from other biochemical and biological characteristics of apoptotic cells. *J Biochem (Tokyo)* **132,** 381-6.

Silveira, F. T., Ishikawa, E. A., De Souza, A. A., and Lainson, R. (2002). An outbreak of cutaneous leishmaniasis among soldiers in Belem, Para State, Brazil, caused by Leishmania (Viannia) lindenbergi n. sp. A new leishmanial parasite of man in the Amazon region.Parasite. *Parasite* **9,** 43-50.

Singh, H., Cousin, M. A., and Ashley, R. H. (2007). Functional reconstitution of mammalian 'chloride intracellular channels' CLIC1, CLIC4 and CLIC5 reveals differential regulation by cytoskeletal actin. *FEBS Journal* **274,** 6306-16.

Skeiky, Y. A., Coler, R. N., Brannon, M., Stromberg, E., Greeson, K., Crane, R. T., Webb, J. R., Campos-Neto, A., and Reed, S. G. (2002). Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. *Vaccine* **20,** 3292-303.

Solioz, N., Blum-Tirouvanziam, U., Jacquet, R., Rafati, S., Corradin, G., Mauël, J., and Fasel, N. (1999). The protective capacities of histone H1 against experimental murine cutaneous leishmaniasis. *Vaccine* **18,** 850-9.

Somanna, A., Mundodi, V., and Gedamu, L. (2002). Functional analysis of cathepsin B-like cysteine proteases from Leishmania donovani complex. Evidence for the activation of latent transforming growth factor beta. *Journal of Biological Chemistry* **277,** 25305-12.

Sorensen, A. L., Hey, A. S., and Kharazmi, A. (1994). Leishmania major surface protease Gp63 interferes with the function of human monocytes and neutrophils in vitro. *APMIS* **102,** 265-71.

Soto, J., and Soto, P. (2006). Miltefosine: oral treatment of leishmaniasis. *Expert Rev Anti Infect Ther* **4,** 177-85.

Stager, S., D.F., S., and P.M., K. (2000). Immunization with a recombinant stage-regulated surface protein from Leishmania donovani induces protection against visceral leishmaniasis. *The Journal of Immunology* **165,** 7064-71.

Stenger, S., Thuring, H., Rollinghoff, M., and Bogdan, C. (1994). Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major. *The Journal of Experimental Medicine* **180,** 783-93.

Stevenson, P. C., and Veitch, N. (1998). A 2-arylbenzofuran from roots of cicer bijugum associated with fusarium wilt resistance. *Phytochemistry* **48,** 947-51.

Stober, C. B., Lange, U. G., Roberts, M. T., Gilmartin, B., Francis, R., Almeida, R., Peacock, C. S., McCann, S., and Blackwell, J. M. (2005). From genome to vaccines for leishmaniasis: Screening 100 novel vaccine candidates against murine Leishmania major infection'. *Vaccine* **24,** 2602-16.

Stober, D., Schirmbeck, R., and Reimann, J. (2001). IL-12/IL-18-dependent IFN-gamma release by murine dendritic cells. *The Journal of Immunology* **167,** 957-65.

Stobie, L., Gurunathan, S., Prussin, C., Sacks, D., Glaichenhaus, N., Wu, C. Y., and Seder, R. A. (2000). The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. *Proceedings of the National Academy of Sciences* **97,** 8427-32.

Strasser, A., O'Connor, L., and Dixit, V. M. (2000). Apoptosis signaling. *Annu Rev Biochem* **69,** 217-45.

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J. M., Menard, R., and Heussler, V. T. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *science* **313,** 1287-90.

Subramanian, A., and Miller, D. M. (2000). Structural Analysis of -Enolase *Journal of Biological Chemistry* **275,** 5958-5965.

Sülsen, V. P., Cazorla, S. I., Frank, F. M., Redko, F. C., Anesini, C. A., Coussio, J. D., Malchiodi, E. L., Martino, V. S., and Muschietti, L. V. (2007). Trypanocidal and leishmanicidal activities of flavonoids from Argentine medicinal plants. *American Journal of Tropical Medicine and Hygiene* **77,** 654-9.

Sundar, S. (2001). Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine & International Health* **6,** 849-54.

Sunderkotter, C., Kunz, M., Steinbrink, K., Meinardus-Hager, G., Goebeler M, B., H, , and Sorg, C. (1993). Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages in vitro and in vivo. *The Journal of Immunology* **151,** 4891-901.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2/Omi, is released from
the mitochondria and interacts with XIAP, inducing cell death. *Molecular Cell* **8,** 613-21.

Tadesse, A., Gebre-Hiwot, A., Asres, K., Djote, M., and Frommel, D. (1993). The in vitro activity of Vernonia amygdalina on Leishmania aethiopica. *Ethiop Med J* **31,** 183-9.

Talamas-Rohana, P., Wright, S. D., Lennartz, M. R., and Russell, D. G. (1990). Lipophosphoglycan from Leishmania mexicana promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. *The Journal of Immunology* **144,** 4817-24.

Tan, N., Kaloga, M., Radtke, O. A., Kiderlen, A. F., Oksuz, S., Ulubelen, A., and Kolodziej, H. (2002). Abietane diterpenoids and triterpenoic acids from Salvia cilicica and their antileishmanial activities. *Phytochemistry* **61,** 881-4.

Tavares, J., Ouaissi, A., Lin, P. K., Tomas, A., and Cordeiro-da-Silva, A. (2005). Differential effects of polyamine derivative compounds against Leishmania infantum promastigotes and axenic amastigotes. *International Journal for Parasitology* **35,** 637-46.

Taylor, A. P., and Murray, H. W. (1998). Therapeutic effect of interferon-gamma gene transfer in experimental visceral leishmaniasis. *J Infect Dis* **178,** 908-11.

Teklemariam, S., Hiwot, A. G., Frommel, D., Miko, T. L., Ganlov, G., and Bryceson, A. (1994). Aminosidine and its combination with sodium stibogluconate in the treatment of diffuse cutaneous leishmaniasis caused by Leishmania aethiopica. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88,** 334-9.

Thakur, C. P., Sinha, G. P., and Pandey, A. K. (1996). Comparison of regimens of amphotericin B deoxycholate in kalaazar. *Indian Journal of Medical Research* **103,** 259-63.

Theodos, C. M., Ribeiro, J. M., and Titus, R. G. (1991). Analysis of enhancing effect of sand fly saliva on Leishmania infection in mice. *Infection and Immunity* **59,** 1592-8.

Toure-Balde, A., Sarthou, J. L., Aribot, G., Michel, P., Trape, J. F., Rogier, C., and Roussilhon, C. (1996). Plasmodium falciparum induces apoptosis in human mononuclear cells. *Infection and Immunity* **64,** 744-50.

Tournaire, C., Caujolle, R., Payard, M., Commenges, G., Bessieres, M. H., Bories, C., Loiseau, P. M., and Gayral, P. (1996). Synthesis and protozoocidal activities of quinones. *Eur J Med Chem* **31,** 507-11.

Tsuji-Takayama, K., Aizawa, Y., Okamoto, I., Kojima, H., Koide, K., Takeuchi, M., Ikegami, H., Ohta, T., and Kurimoto, M. (1999). Interleukin-18 induces interferon-gamma production through NF-kappaB and NFAT activation in murine T helper type 1 cells. *Cell Immunol* **196,** 41-50.

Turville, S. G., Cameron, P. U., Handley, A., Lin, G., Pöhlmann, S., Doms, R. W., and Cunningham, A. L. (2002). Diversity of receptors binding HIV on dendritic cell subsets. *Nat Immunol.* **3,** 975-83.

Uchide, N., Tadera, C., Sarai, H., Ohyama, K., Bessho, T., and Toyoda, H. (2006). Characterization of monocyte differentiation-inducing (MDI) factors derived from human fetal membrane chorion cells undergoing apoptosis after influenza virus infection. *Int J Biochem Cell Biol* **38,** 1926-38.

Udey, M. C., von Stebut, E., Mendez, S., Sacks, D. L., and Belkaid, Y. (2001). Skin dendritic cells in murine cutaneous leishmaniasis. *Immunobiology* **204,** 590-4.

Uzonna, J. E., Joyce, K. L., and Scott, P. J. (2004). Low dose Leishmania major promotes a transient T helper cell type 2 response that is down-regulated by interferon gamma-producing CD8+ T cells. *Exp Med* **199,** 1559-66.

Valenzuela, J. G., Belkaid, Y., Garfield, M. K., Mendez, S., Kamhawi, S., Rowton, E. D., Sacks, D. L., and Ribeiro, J. M. (2001a). Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary protein. *The Journal of Experimental Medicine* **194,** 331-42.

Valenzuela, J. G., Belkaid, Y., Rowton, E., and Ribeiro, J. M. (2001b). The salivary apyrase of the blood-sucking sand fly Phlebotomus papatasi belongs to the novel Cimex family of apyrases. *J Exp Biol* **204,** 229-37.

van de Sand, C., Horstmann, S., Schmidt, A., Sturm, A., Bolte, S., Krueger, A., Lütgehetmann, M., Pollok, J. M., Libert, C., and Heussler, V. T. (2005). The liver stage of Plasmodium berghei inhibits host cell apoptosis. *Mol Microbiol* **58.**

Van Furth, R. (1982). Current view on the mononuclear phagocyte system. *Immunobiology* **161,** 178-85.

van Zandbergen G, Bollinger A, Wenzel A, Kamhawi, S., Voll, R., Klinger, M., Müller, A., Hölscher, C., Herrmann, M., Sacks, D., Solbach, W., and Laskay, T. (2006). Leishmania diseasedevelopment depends on the presence of apoptotic promastigotesin the virulent inoculum. *Proc Natl Acad Sci USA* **103,** 13837–13842.

Van Zandbergen, G., Hermann, N., Laufs, H., Solbach, W., and Laskay, T. (2002). Leishmania promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. *Infection and Immunity* **70,** 4177-84.

Velez, I. D., Gilchrist, K., Arbelaez, M. P., Rojas, C. A., Puerta, J. A., Antunes, C. M., Zicker, F., and Modabber, F. (2005). Failure of a killed Leishmania amazonensis vaccine against American cutaneous leishmaniasis in Colombia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99,** 593-8.

Verdijk, P., Dijkman, R., Plasmeijer, E. I., Mulder, A. A., Zoutman, W. H., Mieke Mommaas, A., and Tensen, C. P. (2005). A lack of Birbeck granules in Langerhans cells is associated with a naturally occurring point mutation in the human Langerin gene. *J Invest Dermatol.* **124,** 714-7.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian
protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102.**

Von Stebut, E., Ehrchen, J. M., Belkaid, Y., Kostka, S. L., Molle, K., Knop, J., Sunderkotter, C., and Udey, M. C. (2003). Interleukin 1alpha promotes Th1 differentiation and inhibits disease progression in Leishmania major-susceptible BALB/c mice. *The Journal of Experimental Medicine* **198,** 191-9.

Waechter, A. I., Cave, A., Hocquemiller, R., Bories, C., Munoz, V., and Fournet, A. (1999). Antiprotozoal activity of aporphine alkaloids isolated from Unonopsis buchtienii (Annonaceae). *Phytotherapy Research* **13,** 175-7.

Waffo-Teguo, P., Hawthorne, M. E., Cuendet, M., Merillon, J. M., Kinghorn, A. D., Pezzuto, J. M., and Mehta, R. G. (2001). Potential cancer-chemopreventive activities of wine stilbenoids and flavans extracted from grape (Vitis vinifera) cell cultures. *Nutr Cancer* **40,** 173-9.

Walker, J., Vasquez, J. J., Gomez, M. A., Drummelsmith, J., Burchmore, R., Girard, I., and Ouellette, M. (2006). Identification of developmentally-regulated proteins in Leishmania panamensis by proteome profiling of promastigotes and axenic amastigotes. *Mol Biochem Parasitol* **147,** 64-73.

Wanderley, J. L., Moreira, M. E., Benjamin, A., Bonomo, A. C., and Barcinski, M. A. (2006). Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of Leishmania (L) amazonensis in mammalian hosts. *The Journal of Immunology* **176,** 1834-9.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes & Development* **15,** 2922-33.

Warburg, A., and Schlein, Y. (1986). The effect of post-bloodmeal nutrition of Phlebotomus papatasi on the transmission of Leishmania major. *American Journal of Tropical Medicine and Hygiene* **35,** 926-30.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *nature* **375,** 408-11.

WHO. (1990). Expert Committee on Leishmaniasis. *In* "WHO Techincal Report Series".

WHO. (2000). Leishmaniasis. http://www.who.int/leishmaniasis/disease_ epidemiology. Accessed on 14th December 2006. *In* "WHO Programmes and Projects". World Healt Organization.

WHO. (2002). The world health report, pp. 192-197. World Health Organization.

WHO. (2004). Report on leishmaniasis. *In* "World Health Organ Scientific working group on leishmaniasis". World Health Organization.

Wiesgigl, M., and Clos, J. (2001). The heat shock protein 90 of Leishmania donovani. *Med Microbiol Immunol.* **190,** 27-31.

Wilson, M. E., Jeronimo, S. M., and Pearson, R. D. (2005). Immunopathogenesis of infection with the visceralizing Leishmania species. *Microb Pathog* **38,** 147-60.

Winter, G., Fuchs, M., McConville, M. J., Stierhof, Y.-D., and Overath, P. (1994). Surface antigens of Leishmania mexicana amastigotes: characterization of glycoinositol phospholipids and a macrophage-derived glycosphingolipid *Journal of Cell Science* **107,** 2471-2482.

Woodland, D. L., and Dutton, R. W. (2003). Heterogeneity of CD4(+) and CD8(+) T cells. *Current Opinion in Immunology* **15,** 336-42.

Woodrow, J. P., Hartzell, J. D., Czarnik, J., Brett-Major, D. M., and Wortmann, G. (2006). Cutaneous and presumed visceral leishmaniasis in a soldier deployed to Afghanistan. *Med Gen Med* **8,** 43.

Xu, D., Liu, H., Komai-Koma, M., Campbell, C., McSharry, C., Alexander, J., and Liew, F. Y. (2003). CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *The Journal of Immunology* **170,** 394-9.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841-847.

Youssef, M. Y., Eissa, M. M., and el Mansoury, S. T. (1997). Evidence of sexual reproduction in the protozoan parasite Leishmania of the Old World. *J Egypt Soc Parasitol* **27,** 651-7.

Zakai, H. A., Chance, M. L., and Bates, P. A. (1998). In vitro stimulation of metacyclogenesis in Leishmania braziliensis, L. donovani, L. major and L. mexicana. *Parasitology* **116,** 305-9.

Zhang, C. G., Gonzales, A. D., Choi, M. W., Chromy, B. A., Fitch, J. P., and McCutchen-Maloney, S. L. (2005). Subcellular proteomic analysis of host-pathogen interactions using human monocytes exposed to Yersinia pestis and Yersinia pseudotuberculosis. *Proteomics* **5,** 1877-88.

Zhang, W. W., Charest, H., Ghedin, E., and Matlashewski, G. (1996). Identification and overexpression of the A2 amastigote-specific protein in Leishmania donovani. *Molecular and Biochemical Parasitology* **78,** 79-90.

Chapter 10

Appendices

10.1. Appendix I. Buffers, reagents and protocols.

JH-30 Medium for amastigotes

Basal solution	
HEPES (1 M)	1 ml
NaHCO3 (4.2%)	3 ml
ddH2O	50.9 ml
Basal solution	(1 l)
Glucose 8%	2.5 ml
MI99 10x	10 ml
Trypticase 5%	10 ml
Vitamin mix	2 ml
Biotin (4 mg/100ml)	2 ml
Folic acid (10 mg/100ml)	0.5 ml
Vitamin B12 (1 mg/100 ml)	0.2 ml
Sodium piruvate (0.1M)	3 ml
Hemin (50mg/100ml)	5 ml
Nucleotide mix	10 ml
FCS(HI) (56?C/30l) 25%	33.5 ml
L-glutamine (3%)	2 ml
vitamin mix	
P-aminobenzoic acid	30mg
D-calcium pantothenate	40 mg
Choline chloride	30 mg
Isoinositol	30 mg
Nicotinamide	50 mg
Nicotinic acid	20 mg
Pyridoxal-HCl	20 mg
Pyridoxine-HCl	20 mg
Pyridoxamine-2HCl	20 mg
Riboflavin-5-phosphate-nNa.2H2O	4 mg
Thiamine-HCl	20 mg
dd H2O	100 ml
Nucleotide mix	
ATP	200 mg
ADP	100 mg
AMP	100 mg
Glutathione (reduced)	20 mg
L-cystine-HCl	20 mg
Ascorbic acid	20 mg
dd H2O	100 ml

Um-54 Medium for amastigotes

Modified medium 199 with Hanks' salt	10 ml
Glucose (8%)	3.125 ml
Trypticase (5%)	10 ml
L-glutamine (3%)	2.5 ml
Haemin (0.5gr/l)	4 ml
FCS	20 ml
Dd H2O	50.4 ml

Adkust to a final pH of 6.3

Protocol for assessing apoptosis by detection of sub-diploid population:
(from Daryzynkiewicz *et al.*, 1992)

Harvest cells and prepare single cell suspension in buffer (e.g. PBS + 2% FBS; PBS + 0.1% BSA)
Wash cells X2 and re-suspend at 1-2 x 106 cells/ml.
Aliquot 1 ml cells in a 15 ml polypropylene, V-bottomed tube and add 3 ml cold absolute ethanol. (To minimize cell clumping ethanol is added forcibly by expelling from a pipette or dropwise while vortexing)
Fix cells for at least 1 hour at 4°C. (Cells may be stored in 70 % ethanol at -20 °C for several weeks prior to PI staining and flow cytometric analysis).
Wash cells X2 in PBS. (It may be necessary to centrifuge cells at a slightly higher "g" to pellet after ethanol fixation as the cells become floculent.)
Re-suspend cells in 0.5 ml PBS and add 0.2-1.0 ml DNA extraction buffer.
Incubate at room temperature for 5 min and centrifuge.
Add 1 ml of propidium iodide staining solution to cell pellet and mix well. Add 50 ul of RNase A stock solution and incubate for 30 min at room temperature
Store samples at 4°C until analysed by flow cytometry.

REAGENTS:

DNA Extraction Buffer:	192 ml 0.2M Na2HPO4, 8 ml 0.1M citric acid; pH 7.8
Propidium Iodide Staining Solution:	3.8 mM sodium citrate, 50 ug/ml PI in PBS.
RNase A stock solution:	10 mg/ml RNase A

Proteomic solution

Washing buffer

10 mM Tris pH 8.0,
5 mM magnesium acetate

Lysis buffer

	Final concentration
Urea (FW 60.06)	2 M
$MgCl_2$ (100 mM)	1.5 mM
NaCl (1M)	100 mM
Tris buffer pH 7.4 (1M)	10 mM
PMSF	100mM
pepstadin	Few crystals

DIGE staining buffer. pH 8.5

	Final concentration
Urea (FW 60.06)	7 M
Thiourea (FW 76.12)	2 M
CHAPS†	2% (w/v)
Tris buffer	30 mM
Double-distilled water	to 25 ml

Thiourea rehydration stock solution

	Final concentration
Urea (FW 60.06)	7 M
Thiourea (FW 76.12)	2 M
CHAPS†	2% (w/v)
Pharmalyte or IPG Buffer	0.5% (v/v) or 2% (v/v)‡ 1
1% Bromophenol blu e stock solution	0.002%
Double-distilled water	to 25 ml

** DTT was added just prior to use: Add 7 mg DTT per 2.5-ml aliquot of rehydration stock solution.*

‡ A Pharmalyte/IPG Buffer concentration of 0.5% (125 µl) was used with Ettan IPGphor II Isoelectric Focusing System and an IPG. Buffer/Pharmalyte concentration of 2% (500 µl) was used with the Multiphor II

SDS equilibration buffer solution

	Final concentration
Urea (FW 60.06)	6 M
Tris-HCl, pH 8.8 (see solution H)	75 mM
Glycerol (87% w/w)	29.3% (v/v)
SDS (FW 288.38)	2% (w/v)
1% Bromophenol blue stock solution	0.002% (w/v)

This is the stock solution. Just prior to use, add DTT (100 mg per 10 ml) and iodoacetamide (250 mg per 10 ml (for first or second equilibration, respectively)

The solution was stored in 20 ml aliquots at -20 °C.

10× Laemmli SDS electrophoresis buffer

	Final concentration
Tris base (FW 121.1)	250 mM
Glycine (FW 75.07)	1.92 M
SDS (FW 288.38)	1% (w/v)

The pH of this solution was not be adjusted.And the solution was stored at room temperature and diluted just before use

.

30% acrylamide stock solution

	Final concentration Amount
Acrylamide (FW 71.08)	30%
N,N'-methylenebisacrylamide (FW 154.17)	0.8%

Solution was filtered through a 0.45-μm filter and stored at 4 °C in the dark.

Poliacrylamide solution

Final gel concentration	**12.5%**
30% acrylamide solution	375 ml
4× resolving gel buffer (solution H)	225 ml
10% SDS	9 ml
Double-distilled water	285.7 ml
10% ammonium persulfate†	5 ml
TEMED†	0.25 ml
Total volume	900 ml

Solution for silver staining for 2-D Gels, Compatible With MS

fixing solution	50% methanol/5% acetic acid ()
washing solution	50% methanol ()
sensitizing solution	0.02% sodium thiosulfate ()
silver nitrate	0.1% chilled to 4 °C
Developing solution #	0.04% formalin (formalin = 35% formaldehyde in water) in 2% sodium carbonate
Stop solution	5% acetic acid
Storing solution	1% acetic acid
30mM potassium ferricyanide	
100mM sodium thiosulfate	
25 mM ammonium bicarbonate, pH 8	

10.2. Appendix II. Proteomics data.

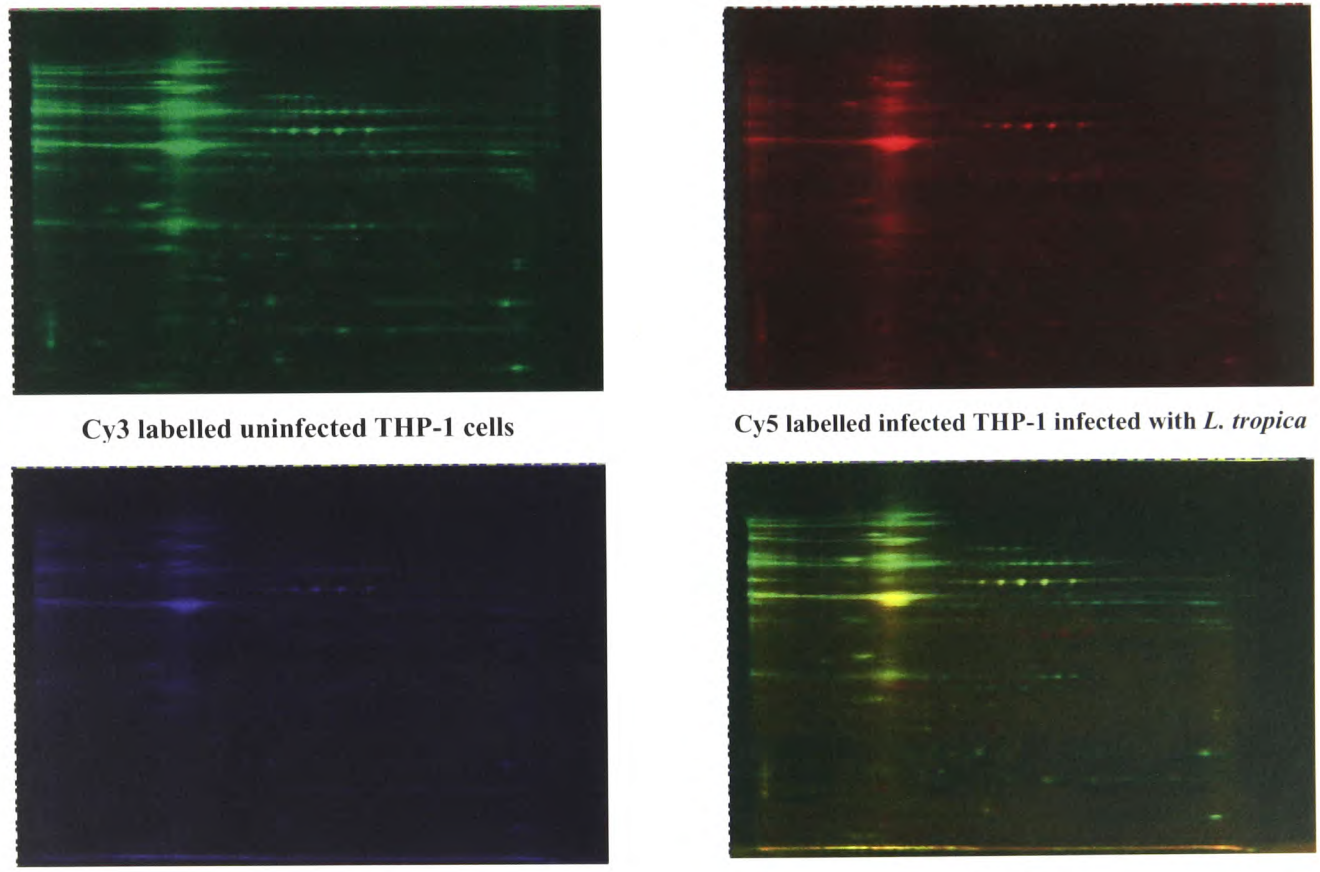


Figure 10.1. DIGE images of gel 19567
Proteins derived from uninfected samples were stained with Cy3; proteins derived form cells infected with *L. tropica* were labelled with Cy5. The internal standard was provided by pooling the samples together. Finally, an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels, which appear as yellow spots while unique ones appear as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
1445	Decreased	-6.83	0.23	617	4311
1412	Decreased	-6.75	0.2	846	4390
1442	Decreased	-6.43	0.31	1859	12641
1431	Decreased	-5.6	0.24	657	5062
1430	Decreased	-5.59	0.2	1191	6605
1439	Decreased	-5.23	0.41	2334	13910
236	Decreased	-4.89	0.23	345	10508
1440	Decreased	-4.88	0.38	1321	13604
1433	Decreased	-4.64	0.18	425	2258
1434	Decreased	-4.55	0.26	888	5137
1415	Decreased	-4.51	0.17	1736	8481
1417	Decreased	-4.49	0.21	2172	10005
1421	Decreased	-4.46	0.18	440	3176
231	Decreased	-4.43	0.23	283	4076
1416	Decreased	-4.41	0.28	928	9273
237	Decreased	-4.41	0.25	599	11652
1405	Decreased	-4.36	0.33	742	6577
1643	Decreased	-4.29	0.11	258	1876
233	Decreased	-4.21	0.2	340	9561
382	Decreased	-4.14	0.24	265	15133
1414	Decreased	-4.13	0.19	2188	8802
1404	Decreased	-4.12	0.19	896	3358
1652	Decreased	-4.03	0.2	371	4849
922	Decreased	-4.02	0.14	446	4603
2627	Increased	4	0.22	2366	2114
2675	Increased	4.01	0.17	1513	2420
2165	Increased	4.06	0.08	647	6006
1807	Increased	4.06	0.09	230	1061
1717	Increased	4.06	0.15	391	1892
1829	Increased	4.11	0.09	314	1710
1937	Increased	4.2	0.33	637	1723
2421	Increased	4.23	0.3	2342	1676
2308	Increased	4.26	0.11	997	2413
2673	Increased	4.33	0.14	1741	5057
1740	Increased	4.33	0.13	1411	4775
2138	Increased	4.34	0.13	661	1870
1783	Increased	4.36	0.22	278	1575
1723	Increased	4.47	0.13	573	2204
1708	Increased	4.53	0.14	511	3512
1813	Increased	4.58	0.14	534	4089
2317	Increased	4.7	0.09	375	1489
2169	Increased	4.73	0.2	996	5878
2391	Increased	4.74	0.19	861	2015
1736	Increased	4.74	0.13	1198	2828
2392	Increased	4.84	0.16	1574	2850
1782	Increased	4.84	0.15	727	2424
2426	Increased	4.88	0.16	1486	3090
1556	Increased	4.89	0.22	961	890
1730	Increased	5.07	0.16	493	4445
2416	Increased	5.3	0.19	1201	3160

2759	Increased	5.35	0.29	1425	1387
1727	Increased	5.42	0.18	853	4050
2173	Increased	5.57	0.11	321	2577
2179	Increased	5.85	0.12	1253	5123
2143	Increased	6.26	0.13	625	1657
1583	Increased	6.27	0.33	677	2647
1809	Increased	6.5	0.14	2759	8871
2212	Increased	6.52	0.19	641	2518
2133	Increased	6.76	0.1	377	1291
1739	Increased	6.85	0.15	937	6320
1780	Increased	6.95	0.18	607	3916
1729	Increased	6.95	0.19	712	3609
1920	Increased	6.97	0.26	1283	1126
2687	Increased	7.46	0.18	1763	2330
1808	Increased	7.61	0.14	2500	3719
2213	Increased	7.67	0.14	1788	1908
2769	Increased	7.75	0.18	1106	1683
2777	Increased	7.77	0.55	1450	1347
1538	Increased	8.03	0.25	819	1730
1732	Increased	8.35	0.14	1031	4644
2429	Increased	9.39	0.24	2073	1460
2610	Increased	9.55	0.13	1288	7053
1797	Increased	10.14	0.15	1242	10141
1789	Increased	10.46	0.16	961	5973
2389	Increased	10.64	0.21	882	2821
2775	Increased	11.99	0.4	1244	3226
1587	Increased	13.45	0.21	472	2080
2636	Increased	14.39	0.21	2112	2142
1703	Increased	15.03	0.16	450	1998
2428	Increased	15.3	0.2	1989	3594
2670	Increased	15.6	0.26	1674	1377
2410	Increased	23.14	0.24	1255	2616
2768	Increased	32.11	0.17	1471	2263

Table 10.1. Differentially expressed spots following De-Cyder analysis of gel 1967.
Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 1968 gel.

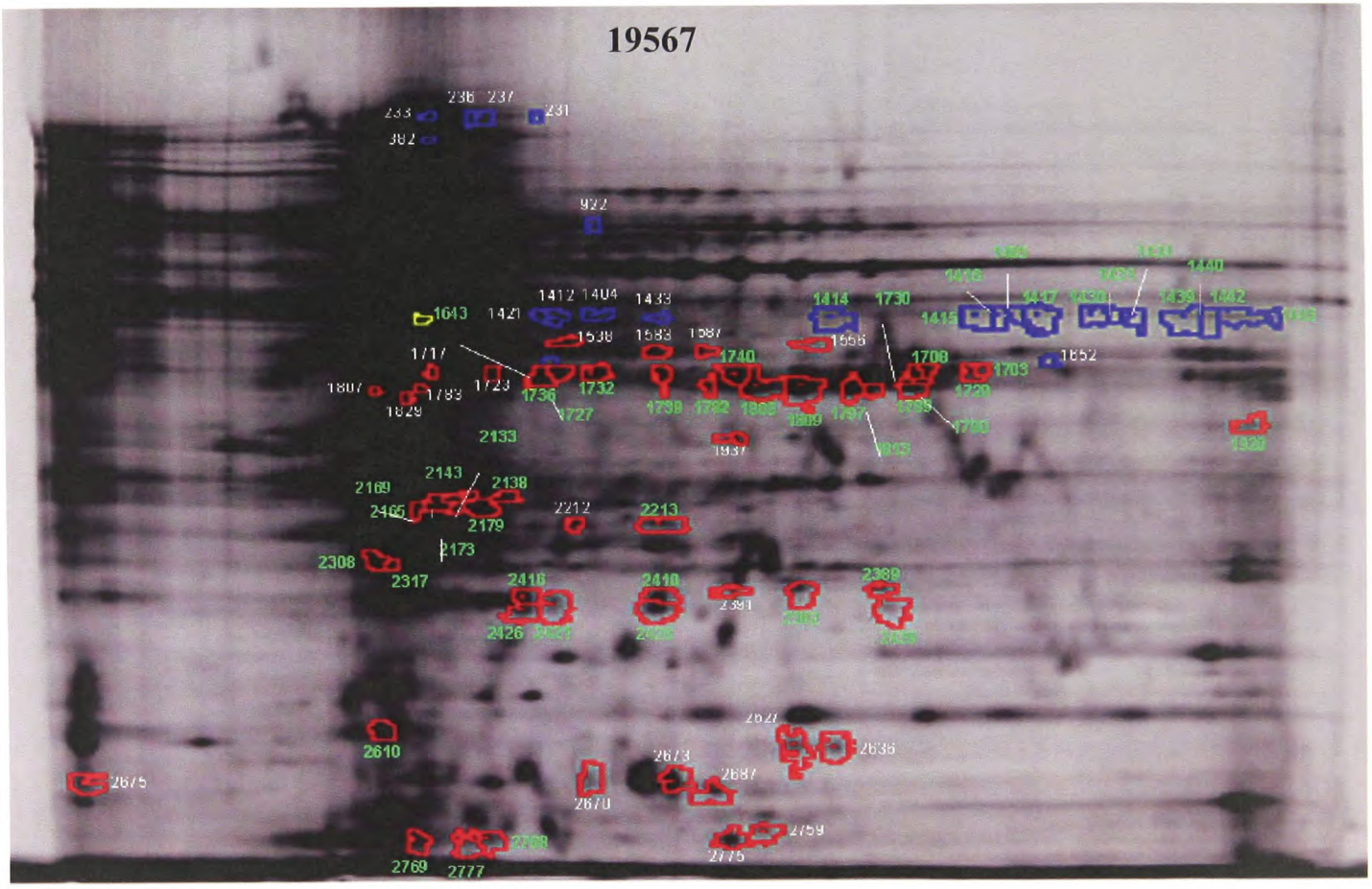


Figure 10.2. Gel 19567, ,Differentially expressed proteins during *L. tropica* infection are circled.
Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentrations decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

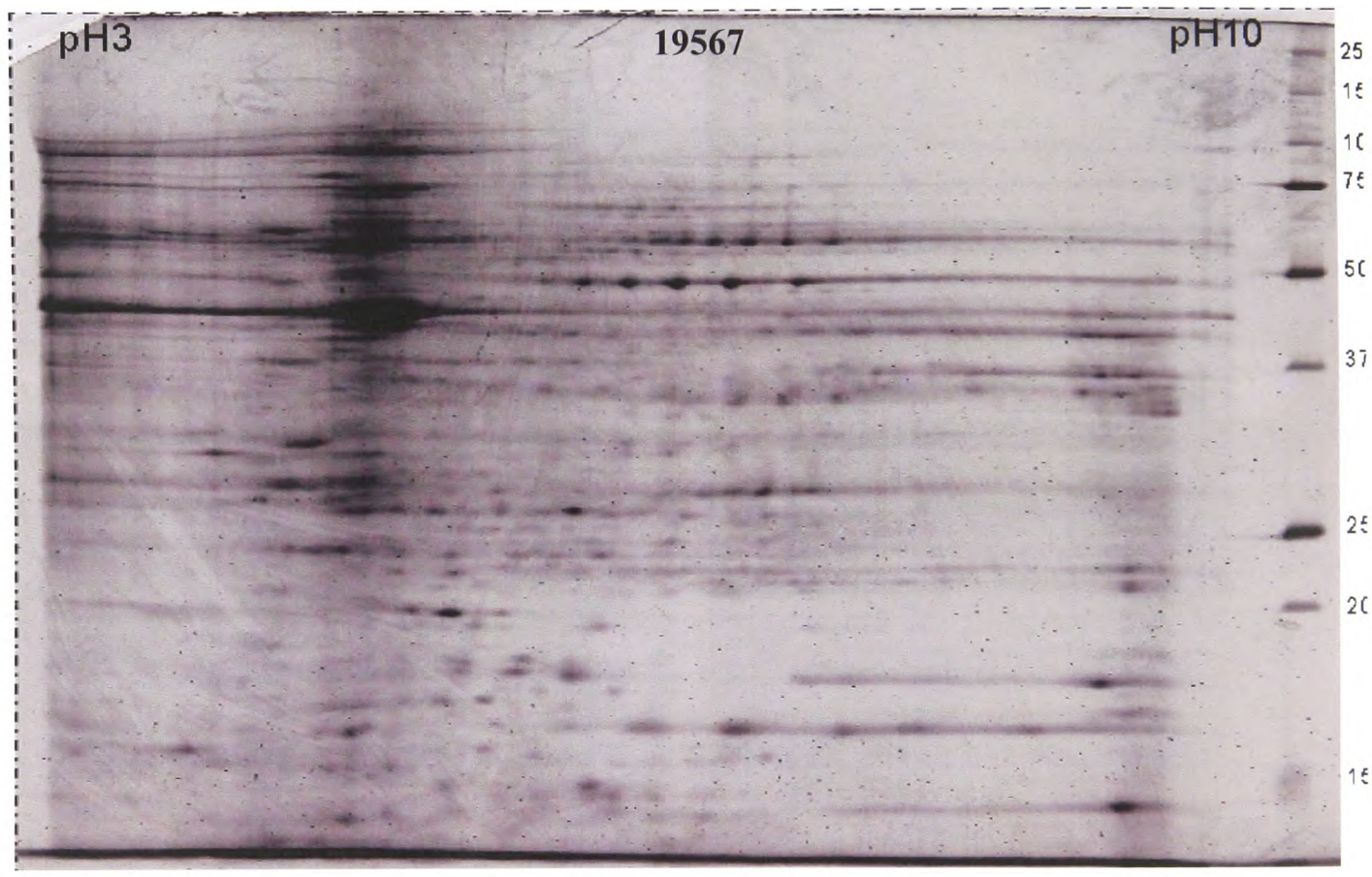
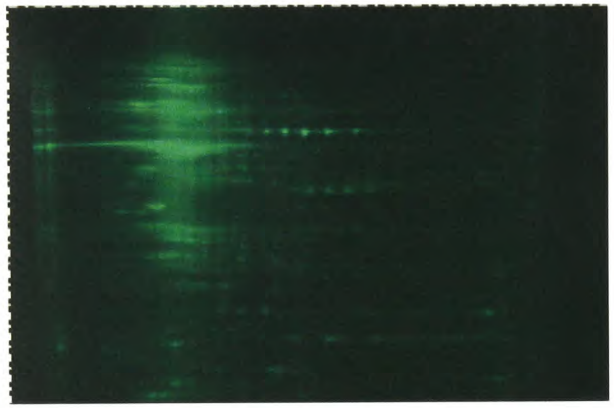
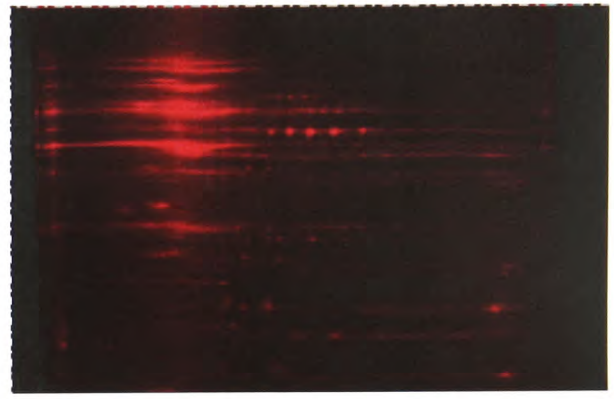


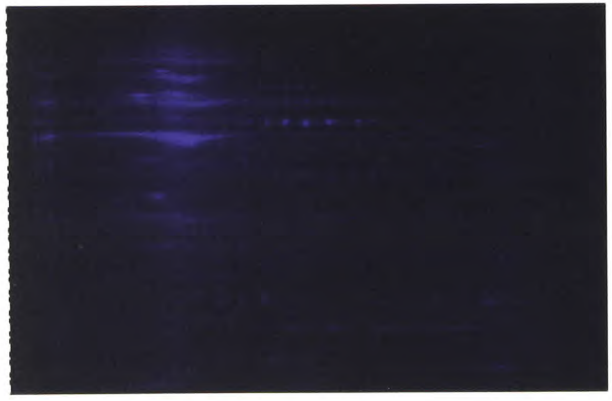
Figure 10.3. Gel 19567 following Deep Purple staining.
Post run Deep Purple fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.



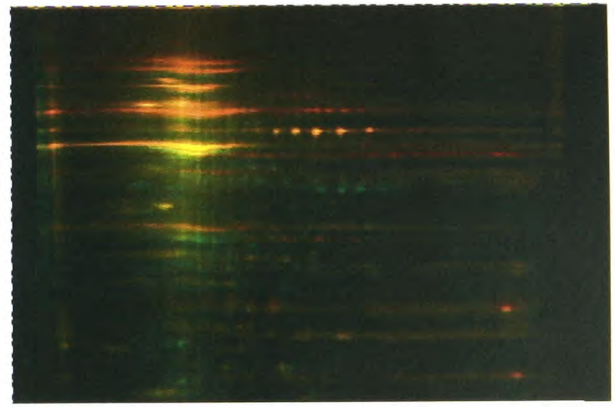
Cy3 labelled infected THP-1 infected with *L. tropica*



Cy5 labelled uninfected THP-1 cells



Cy2 labelled Internal standard



Overlay

Figure 10.4. DIGE images of gel 19568.
Proteins derived from uninfected samples were stained with Cy5; proteins derived form cells infected with *L. tropica* were labelled with Cy3. The internal standard was provided by pooling the samples together. Finally, an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels, which appear as yellow spots while unique ones appear as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
2759	Decreased	-17.17	0.31	797	749
2741	Decreased	-16.83	0.22	1120	431
2969	Decreased	-14.63	0.27	1034	924
2015	Decreased	-14.61	0.25	873	208
2026	Decreased	-13.49	0.19	1994	350
2760	Decreased	-13.28	0.29	801	224
3007	Decreased	-12.44	0.24	1478	4297
3167	Decreased	-12	0.29	1765	202
2027	Decreased	-11.55	0.29	1738	594
2016	Decreased	-10.91	0.2	2018	1655
2869	Decreased	-10.88	0.24	1382	452
2014	Decreased	-9.52	0.18	2395	427
3078	Decreased	-9.29	0.21	1478	783
2017	Decreased	-9.01	0.2	766	1028
1993	Decreased	-8.63	0.19	1117	227
1884	Decreased	-8.27	0.15	406	689
3010	Decreased	-8.26	0.18	1363	402
2763	Decreased	-8.01	0.22	2134	676
2454	Decreased	-7.96	0.29	868	1093
2589	Decreased	-7.64	0.27	902	60
2019	Decreased	-7.47	0.24	1147	189
2388	Decreased	-7.24	0.24	450	358
2139	Decreased	-7.12	0.24	286	967
1931	Decreased	-6.87	0.26	601	1231
2372	Decreased	-6.85	0.28	271	598
3006	Decreased	-6.74	0.23	896	1017
1218	Decreased	-6.72	0.21	619	641
2768	Decreased	-6.67	0.32	1082	183
2018	Decreased	-6.54	0.21	782	505
1975	Decreased	-6.53	0.22	992	459
2753	Decreased	-6.46	0.29	331	630
3171	Decreased	-6.43	0.29	1618	601
2522	Decreased	-6.39	0.25	589	239
1879	Decreased	-6.39	0.17	558	693
1955	Decreased	-6.33	0.16	707	162
2598	Decreased	-6.13	0.3	665	1065
2745	Decreased	-6.01	0.2	1053	506
1940	Decreased	-5.87	0.27	469	407
3172	Decreased	-5.69	0.21	1681	201
2974	Decreased	-5.58	0.36	1105	178
2991	Decreased	-5.52	0.23	1323	440
2144	Decreased	-5.5	1.11	326	454
2694	Decreased	-5.47	0.21	1003	652
2751	Decreased	-5.43	0.34	438	956
2752	Decreased	-5.29	0.21	1053	793
2747	Decreased	-5.19	0.32	693	56
2970	Decreased	-4.78	0.29	1427	539
2140	Decreased	-4.66	0.23	861	553
2439	Decreased	-4.6	0.26	376	221

1954	Decreased	-4.51	0.17	426	1687
2746	Decreased	-4.44	0.3	1051	597
2369	Decreased	-4.41	0.22	728	166
3175	Decreased	-4.4	0.27	3362	238
2534	Decreased	-4.36	0.23	778	645
2734	Decreased	-4.35	0.23	747	1207
2697	Decreased	-4.33	0.19	1141	669
1899	Decreased	-4.19	0.19	410	496
1862	Decreased	-4.13	0.21	403	730
2377	Decreased	-4.12	0.32	320	465
1882	Decreased	-4.12	0.16	586	580
1709	Decreased	-4.12	0.3	519	162
3159	Decreased	-4.11	0.3	1326	125
2538	Decreased	-4.1	0.26	366	192
1219	Decreased	-4.1	0.25	602	426
2696	Decreased	-4.04	0.42	514	920
1031	Increased	4.07	0.19	986	823
1642	Increased	4.18	0.17	697	248
1643	Increased	4.38	0.13	864	420
407	Increased	4.55	0.22	198	247
1647	Increased	4.83	0.24	506	813
1179	Increased	5.35	0.2	444	196
1616	Increased	5.37	0.17	2087	770
408	Increased	5.43	0.22	302	1308
363	Increased	5.43	0.24	573	388
1627	Increased	5.62	0.15	1098	798
1618	Increased	6.19	0.17	2394	706
1608	Increased	6.64	0.23	482	470
1619	Increased	6.68	0.21	3139	212
1628	Increased	6.7	0.25	2674	514
1611	Increased	6.77	0.21	839	1064
1607	Increased	6.97	0.27	600	158
1623	Increased	7.31	0.22	2512	277
1610	Increased	7.33	0.37	1013	1074
1604	Increased	7.4	0.26	489	240
1605	Increased	7.56	0.27	411	187
1615	Increased	7.81	0.25	617	1148
1603	Increased	8.25	0.21	518	330
1617	Increased	8.53	0.17	1491	365
1813	Increased	8.86	0.19	495	361
1614	Increased	8.88	0.22	493	200
1606	Increased	9.11	0.27	382	877
1612	Increased	9.45	0.3	1149	474
1613	Increased	9.52	0.16	415	541
1624	Increased	9.62	0.21	484	49

Table 10.2. Differentially expressed spots following De-Cyder analysis of gel 19568.
Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 19567 gel.

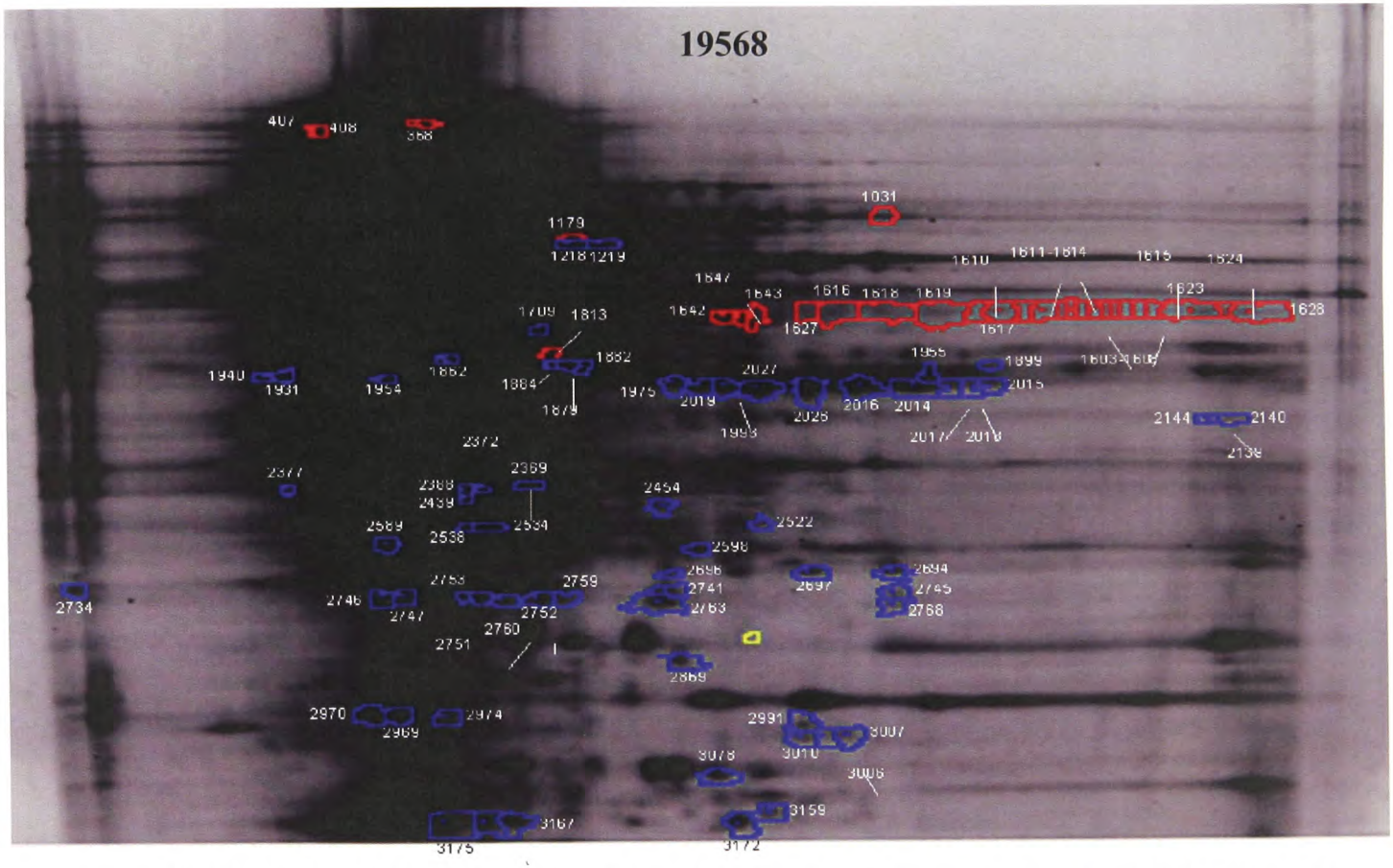


Figure 10.5. Gel 19568, differentially expressed proteins during *L. tropica* infection are circled Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentrations decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

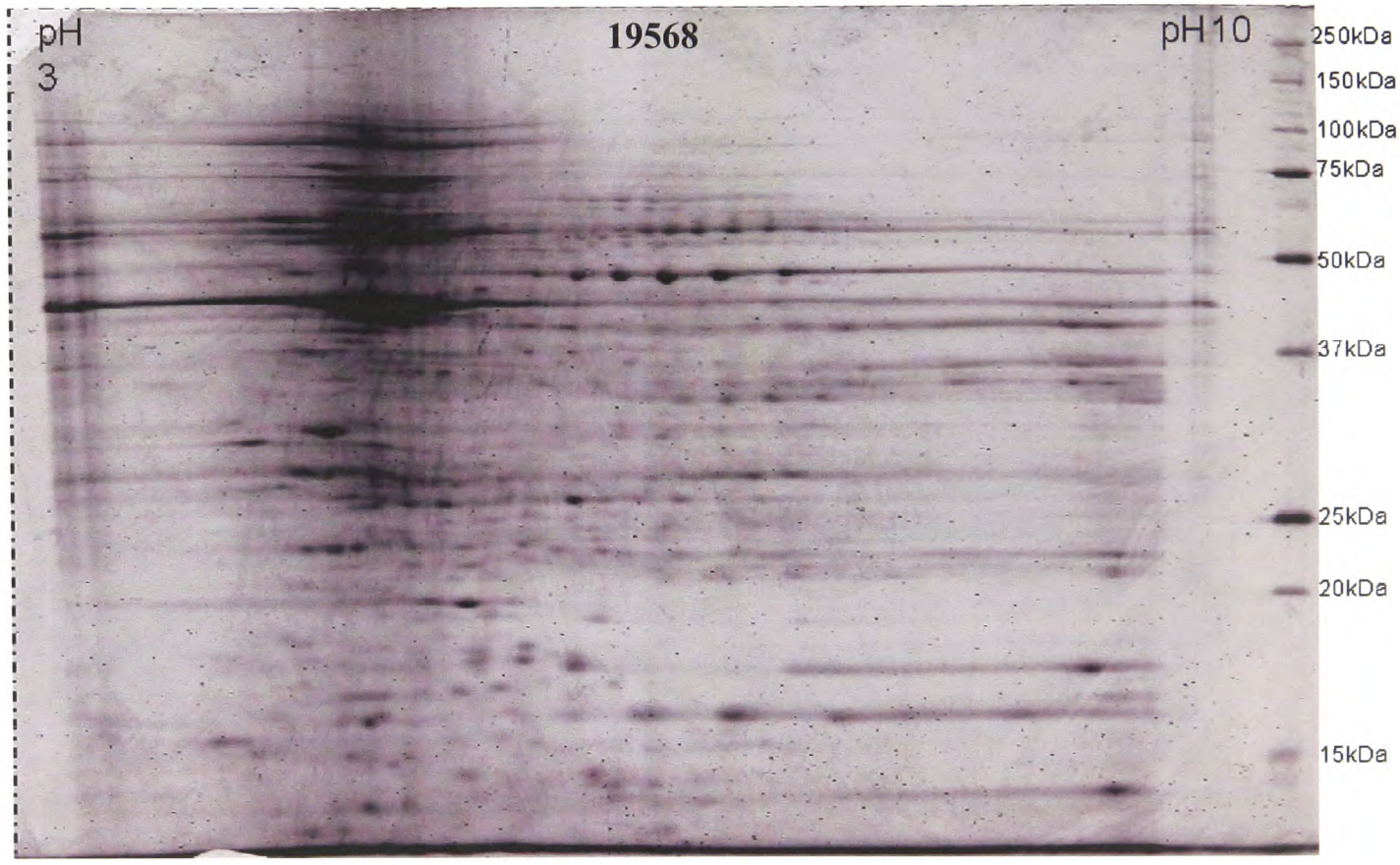


Figure 10.6. Gel 1968 following Deep Purple staining.
Post run Deep Purple fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.

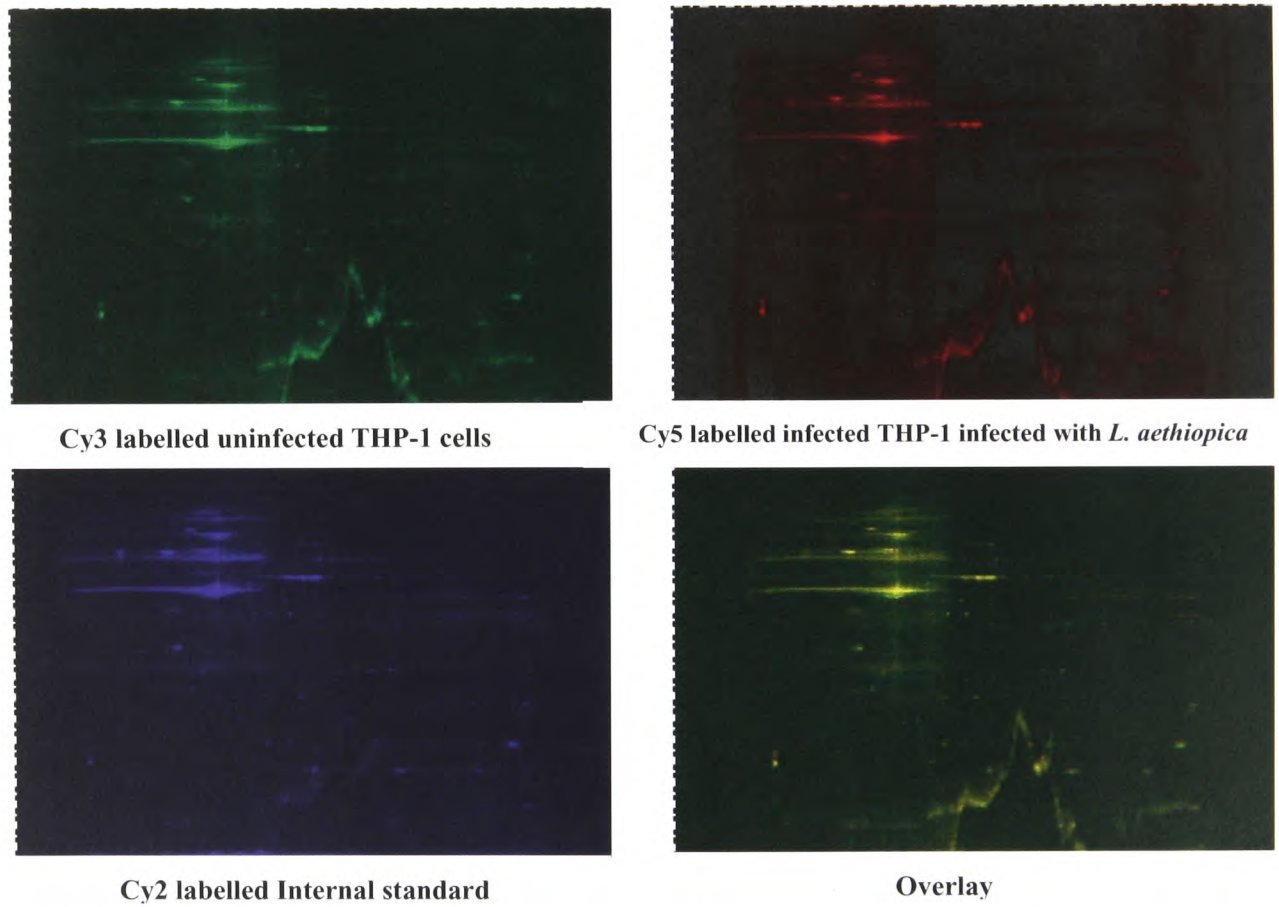


Figure 10.6. DIGE images of gel 19572
Proteins derived from uninfected samples were stained with Cy3; proteins derived form cells infected with *L. aethiopica* were labelled with Cy5. The internal standard was provided by pooling the samples together. Finally, an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels, which appear as yellow spots while unique ones appear as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
2380	Decreased	-5.11	0.34	1614	1338
1012	Decreased	-3.88	0.18	257	2531
1368	Decreased	-3.4	0.4	732	2684
2432	Decreased	-3.25	0.35	1152	1833
1351	Decreased	-3.25	0.28	964	1286
1789	Decreased	-2.7	0.31	894	608
1009	Decreased	-2.7	0.15	240	673
1344	Decreased	-2.67	0.29	1189	458
269	Decreased	-2.61	0.24	320	460
268	Decreased	-2.51	0.23	260	271
254	Decreased	-2.43	0.26	417	358
253	Decreased	-2.43	0.42	372	630
252	Decreased	-2.4	0.23	318	366
1011	Decreased	-2.33	0.2	239	359
259	Decreased	-2.31	0.2	315	1174
1349	Decreased	-2.28	0.23	819	596
267	Decreased	-2.25	0.24	318	905
1744	Decreased	-2.2	0.29	739	847
265	Decreased	-2.17	0.25	373	564
2335	Decreased	-2.16	0.35	1705	552
754	Decreased	-2.15	0.21	305	727
1710	Decreased	-2.1	0.24	582	1123
250	Decreased	-2.05	0.29	487	348
1544	Decreased	-2.03	0.45	725	125
1350	Decreased	-2	0.34	507	223
258	Decreased	-2	0.23	292	600
1743	Decreased	-1.98	1.59	721	590
753	Decreased	-1.95	0.19	273	579
1356	Decreased	-1.94	0.44	1091	1418
1285	Decreased	-1.93	0.22	796	981
1730	Decreased	-1.9	0.3	502	743
1814	Decreased	-1.9	0.81	706	569
1003	Decreased	-1.88	0.35	396	297
276	Decreased	-1.86	0.56	1200	5227
1005	Decreased	-1.85	0.46	396	897
1348	Decreased	-1.83	0.32	785	431
251	Decreased	-1.78	0.22	350	436
266	Decreased	-1.77	0.21	511	117
1371	Decreased	-1.76	0.23	815	227
255	Decreased	-1.74	0.23	336	1168
1013	Decreased	-1.71	0.33	382	1605
1169	Decreased	-1.69	0.29	517	1465
1545	Decreased	-1.66	0.27	934	400
1355	Decreased	-1.65	0.32	2094	443
1788	Decreased	-1.64	0.25	953	1107
1015	Decreased	-1.63	0.32	250	597
2734	Decreased	-1.63	0.26	2182	3049
876	Decreased	-1.61	0.18	354	526

1001	Decreased	-1.6	0.29	429	526
437	Decreased	-1.59	0.27	454	318
1258	Decreased	-1.58	0.28	1475	800
514	Decreased	-1.57	0.24	298	2325
2147	Decreased	-1.56	0.38	908	589
1845	Decreased	-1.52	0.32	1300	510
45	Decreased	-1.51	0.32	276	636
1281	Decreased	-1.51	0.18	804	952
1763	Increased	1.52	0.28	961	5369
1043	Increased	1.54	0.32	1391	3730
1058	Increased	1.55	0.25	2390	4410
1061	Increased	1.55	0.32	577	493
1051	Increased	1.55	0.26	453	599
1044	Increased	1.56	0.26	646	4777
1054	Increased	1.57	0.31	773	366
1045	Increased	1.64	0.28	969	1097
1046	Increased	1.65	0.26	948	211
1060	Increased	1.66	0.29	1313	6521
1048	Increased	1.66	0.28	775	6843
1047	Increased	1.67	0.25	691	950
1062	Increased	1.67	0.27	1678	314
1063	Increased	1.68	0.28	1364	1105
1674	Increased	1.71	0.26	632	4403
1301	Increased	1.72	0.97	717	1251
1053	Increased	1.73	0.24	1086	149
1059	Increased	1.74	0.27	1130	219
172	Increased	1.75	0.42	402	4278
1068	Increased	1.8	0.31	1269	4416
1052	Increased	1.81	0.44	689	1334
1311	Increased	1.94	0.27	682	968

Table 10.3. Differentially expressed spots following De-Cyder analysis of gel 19572.
Examples of spots which were chosen for MALDI-TOF. Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 1971 gel.

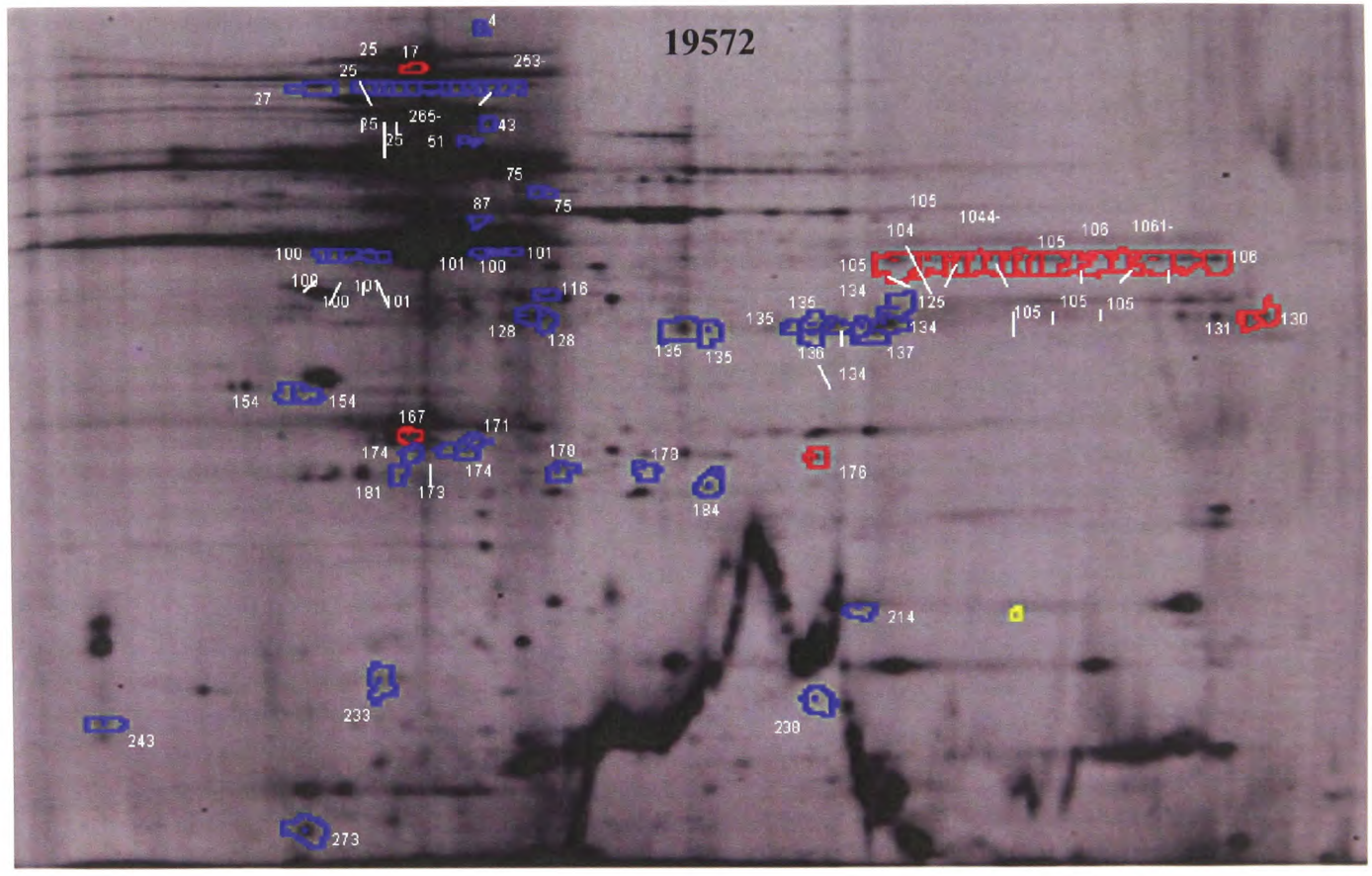


Figure 10.9. Gel 19572, differentially expressed proteins are circled
Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentrations decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

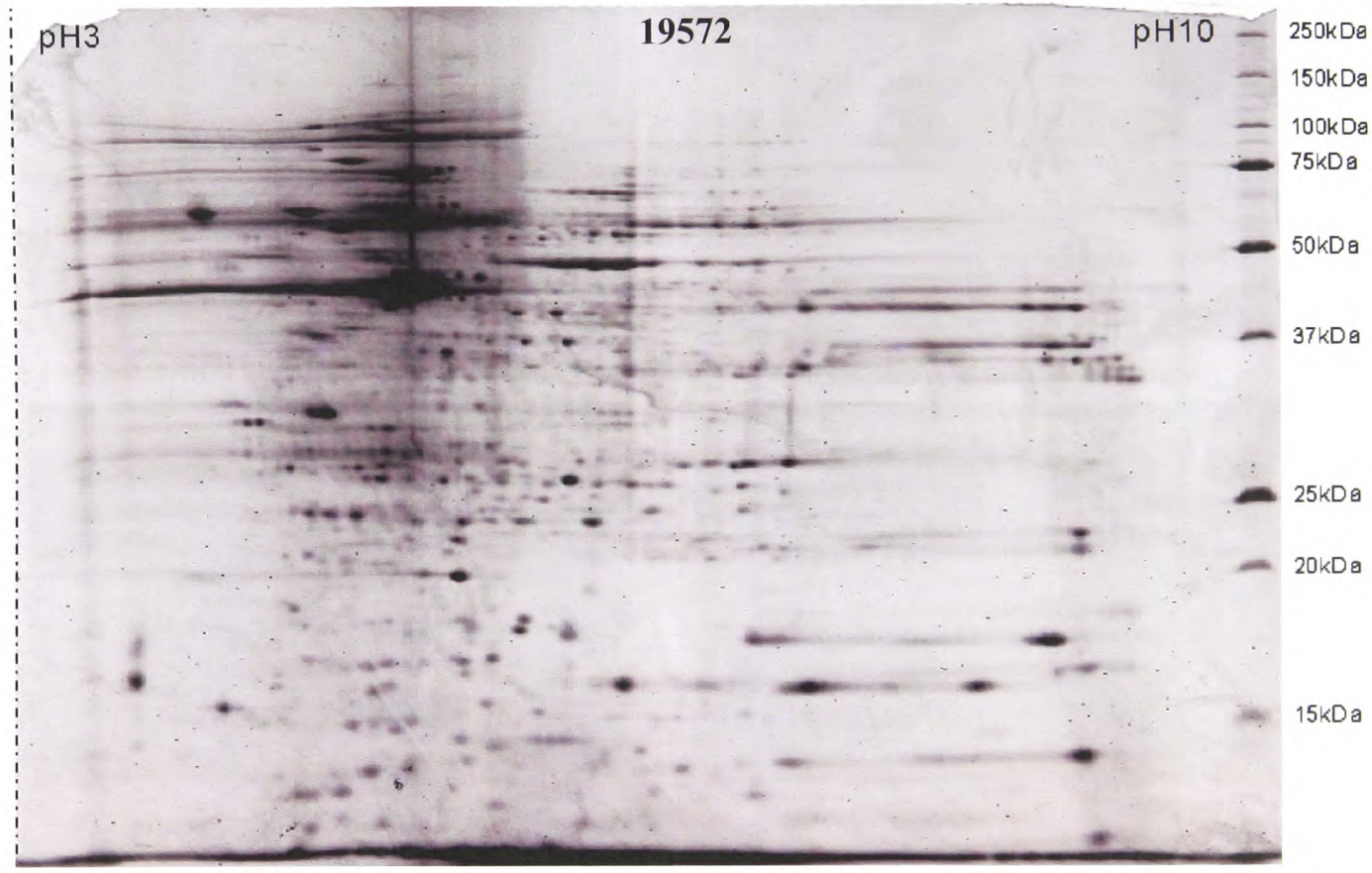


Figure 10.10. Gel 19572 followinf Deep Purple staining.
Post run Deep Purple fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.

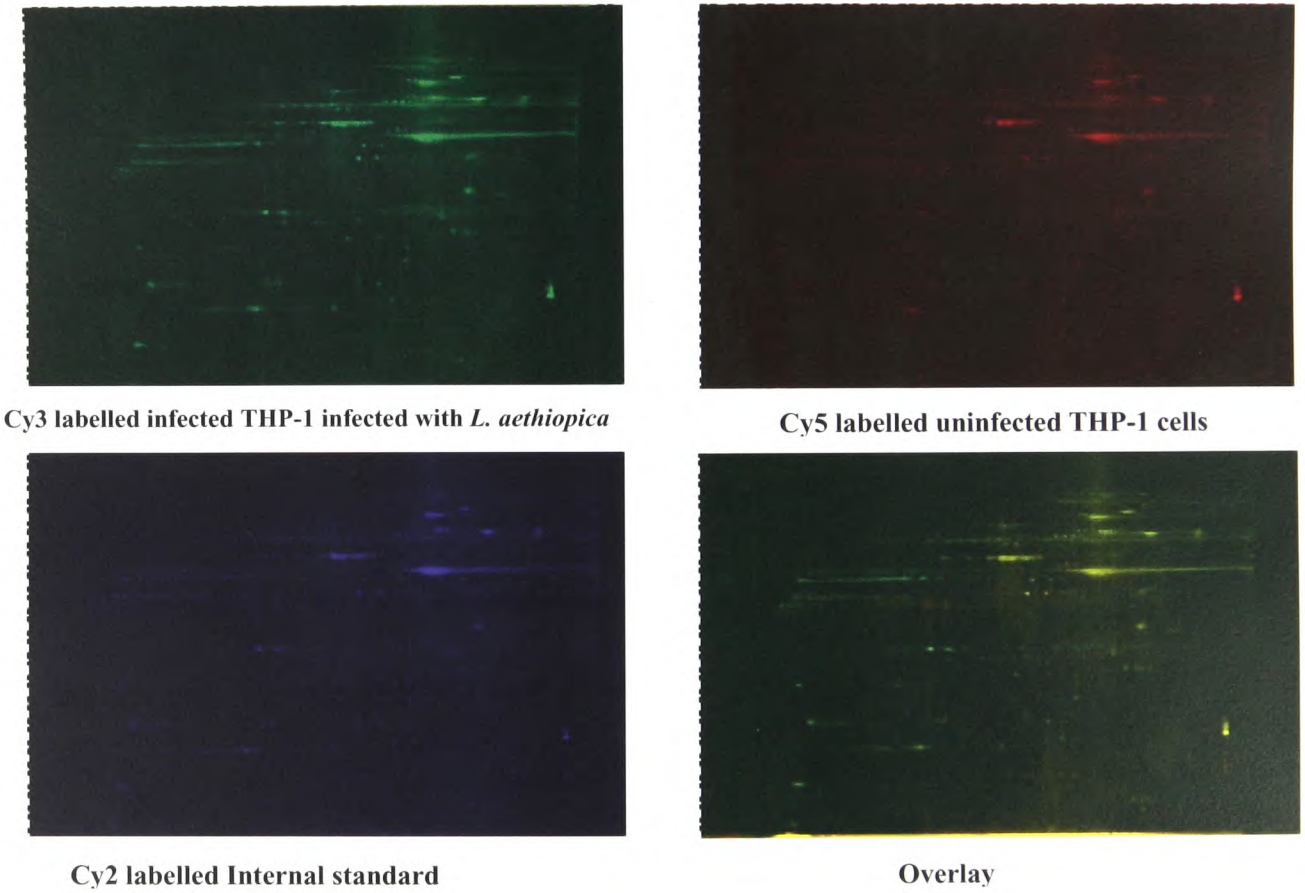


Figure 10.11. DIGE images of gel 19571

Proteins derived from uninfected samples were stained with Cy5; proteins derived form cells infected with *L. aethiopica* were labelled with Cy3. The internal standard was provided by pooling the samples together. Finally, ed an overlay of the spots labelled with Cy3 and Cy5 showed spots which were proteins present in both gels, which appear as yellow spots while unique ones appear as either green or red.

Spot No.	Abundance	Volume Ratio	Max Slope	Area	Max Peak Height
2431	Increased	4.57	0.3	1399	161
1457	Increased	3.35	0.24	432	318
1921	Increased	3.29	0.38	663	337
854	Increased	3.06	0.4	446	210
1892	Increased	2.87	0.28	684	136
1488	Increased	2.81	0.38	1016	139
1453	Increased	2.75	0.41	1425	704
1458	Increased	2.64	0.58	787	361
851	Increased	2.62	0.49	473	992
1490	Increased	2.58	0.4	741	482
2628	Increased	2.55	0.3	1557	867
320	Increased	2.42	0.32	426	586
328	Increased	2.35	0.27	715	837
1917	Increased	2.31	0.36	805	441
1135	Increased	2.24	0.35	283	410
1403	Increased	2.23	0.47	362	230
1298	Increased	2.19	0.52	479	424
1859	Increased	2.1	0.35	637	292
1371	Increased	2.09	0.35	1232	3402
318	Increased	2.05	0.32	247	270
101	Increased	2.05	0.4	216	415
325	Increased	2.04	0.3	322	194
1455	Increased	2.04	1.35	464	571
1406	Increased	1.97	0.51	437	364
319	Increased	1.92	0.32	346	430
1877	Increased	1.88	0.35	604	515
332	Increased	1.84	0.28	437	615
1133	Increased	1.83	0.36	340	785
1137	Increased	1.83	0.37	335	950
1862	Increased	1.81	0.27	545	471
329	Increased	1.79	0.5	979	368
327	Increased	1.78	0.33	303	629
1416	Increased	1.76	0.27	728	869
100	Increased	1.74	0.43	253	2399
1142	Increased	1.71	0.35	306	309
1136	Increased	1.71	0.38	563	309
339	Increased	1.7	0.25	365	288
102	Increased	1.67	0.33	197	383
1423	Increased	1.65	0.41	478	274
2590	Increased	1.63	0.26	920	553
338	Increased	1.61	0.29	341	1745
591	Increased	1.6	0.28	342	848
1297	Increased	1.6	0.43	1172	359
314	Increased	1.55	0.6	245	221
326	Increased	1.54	0.25	463	551
315	Increased	1.53	0.29	206	238
518	Increased	1.53	0.37	442	336
592	Increased	1.51	0.28	310	751

1814	Decreased	-1.5	0.29	2920	1688
291	Decreased	-1.5	0.68	753	438
1115	Decreased	-1.51	0.3	1640	898
593	Decreased	-1.51	0.25	411	778
1812	Decreased	-1.51	0.26	853	430
1823	Decreased	-1.51	0.33	2223	162
1191	Decreased	-1.53	0.32	614	539
717	Decreased	-1.53	0.3	264	266
1820	Decreased	-1.54	0.35	1120	267
1048	Decreased	-1.55	0.31	1081	526
1113	Decreased	-1.55	0.29	1083	905
827	Decreased	-1.57	0.36	459	770
1126	Decreased	-1.58	0.34	654	314
1121	Decreased	-1.58	0.37	2045	314
2057	Decreased	-1.58	0.37	1032	1102
1029	Decreased	-1.58	0.26	711	1181
1843	Decreased	-1.6	0.42	1039	1167
1797	Decreased	-1.6	0.21	635	769
1811	Decreased	-1.61	0.34	937	250
1152	Decreased	-1.63	0.44	1203	381
696	Decreased	-1.63	0.26	440	81
1523	Decreased	-1.65	0.31	554	701
1896	Decreased	-1.65	0.45	1227	661
1098	Decreased	-1.67	0.36	680	673
225	Decreased	-1.71	0.37	343	351
1897	Decreased	-1.72	0.55	1038	1063
1512	Decreased	-1.73	0.34	1074	1038
1898	Decreased	-1.75	0.43	890	1367
694	Decreased	-1.76	0.3	511	405
1487	Decreased	-1.81	0.38	320	990
999	Decreased	-1.82	0.48	429	1098
1170	Decreased	-1.82	0.53	2678	388
1050	Decreased	-1.84	0.4	577	638
1161	Decreased	-1.84	0.61	1394	340
25	Decreased	-1.85	0.37	1440	550
299	Decreased	-1.86	0.71	455	392
1448	Decreased	-1.86	0.33	759	437
1902	Decreased	-1.88	0.33	903	277
1518	Decreased	-1.9	0.28	655	520
828	Decreased	-1.9	0.26	459	200
830	Decreased	-1.91	0.33	347	357
1169	Decreased	-1.93	0.55	1621	450
1193	Decreased	-1.95	0.31	1291	259
1176	Decreased	-1.97	0.41	1006	360
1200	Decreased	-1.99	0.32	1365	515
1445	Decreased	-2.04	0.33	567	535
1199	Decreased	-2.06	0.42	2329	179
1175	Decreased	-2.06	0.67	907	749
1192	Decreased	-2.08	0.4	782	254
1179	Decreased	-2.1	0.31	1557	1276
1195	Decreased	-2.12	0.28	1011	792
1198	Decreased	-2.13	0.34	651	1773

1188	Decreased	-2.14	0.26	413	654
1194	Decreased	-2.15	0.31	561	1155
1189	Decreased	-2.15	0.29	570	333
1190	Decreased	-2.18	0.29	848	155
1187	Decreased	-2.2	0.31	697	260
1489	Decreased	-2.36	0.27	322	1059

Table 10.4. Differentially expressed spots following De-Cyder analysis of gel 1971.
Examples of spots which were chosen for MALDI-TOF. Each spot corresponds to a spot whose expression id decreased in the reciprocally labelled 1972 gel.

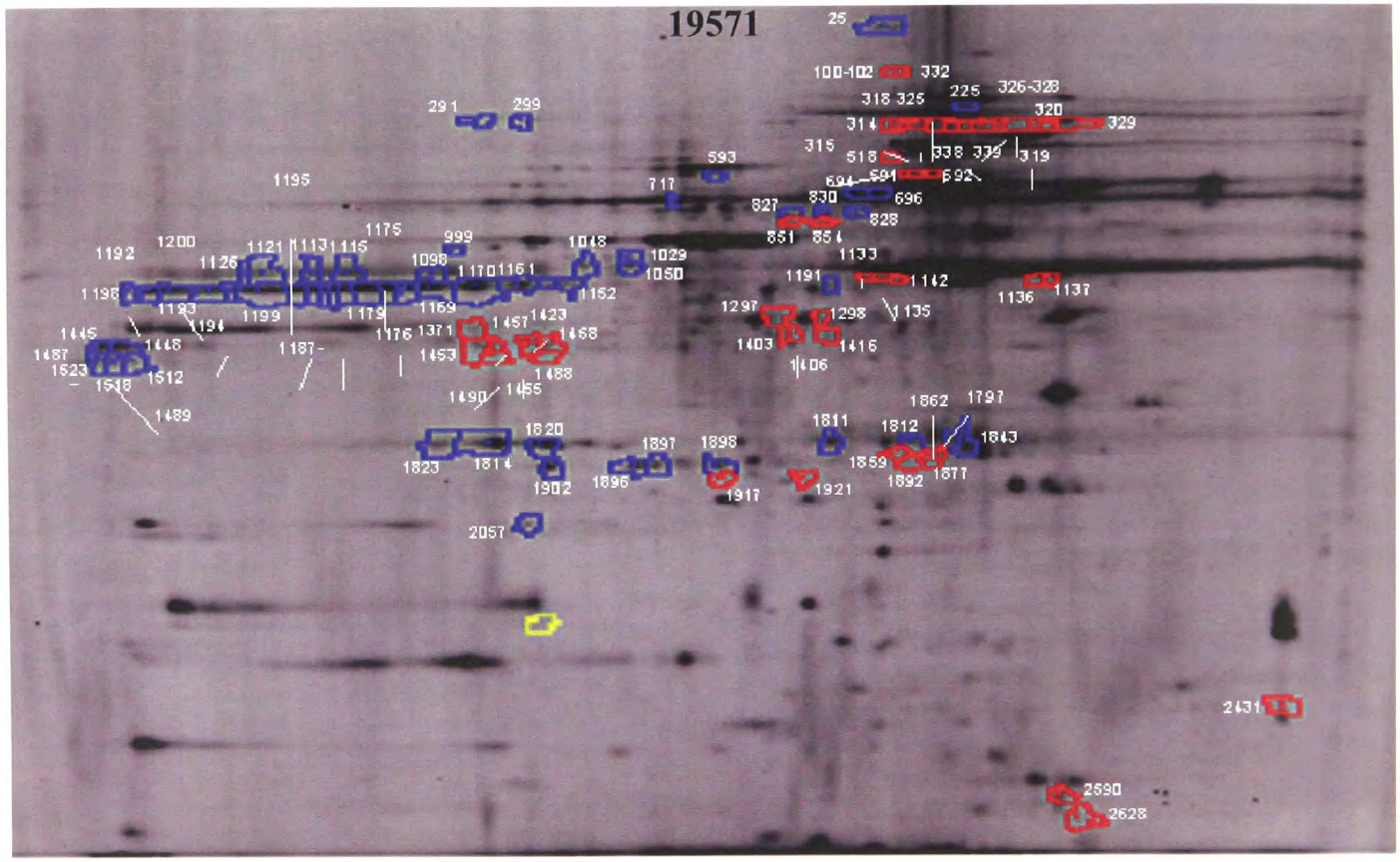


Figure 10.12. Gel 19571, differentially expressed proteins are circled

Overlay of Cy3 and Cy5 labelled proteins. Circled in blue are the spots representing proteins whose concentrations decreased during infection. The green numbers indicate the spots that were selected for MALDI-TOF analysis.

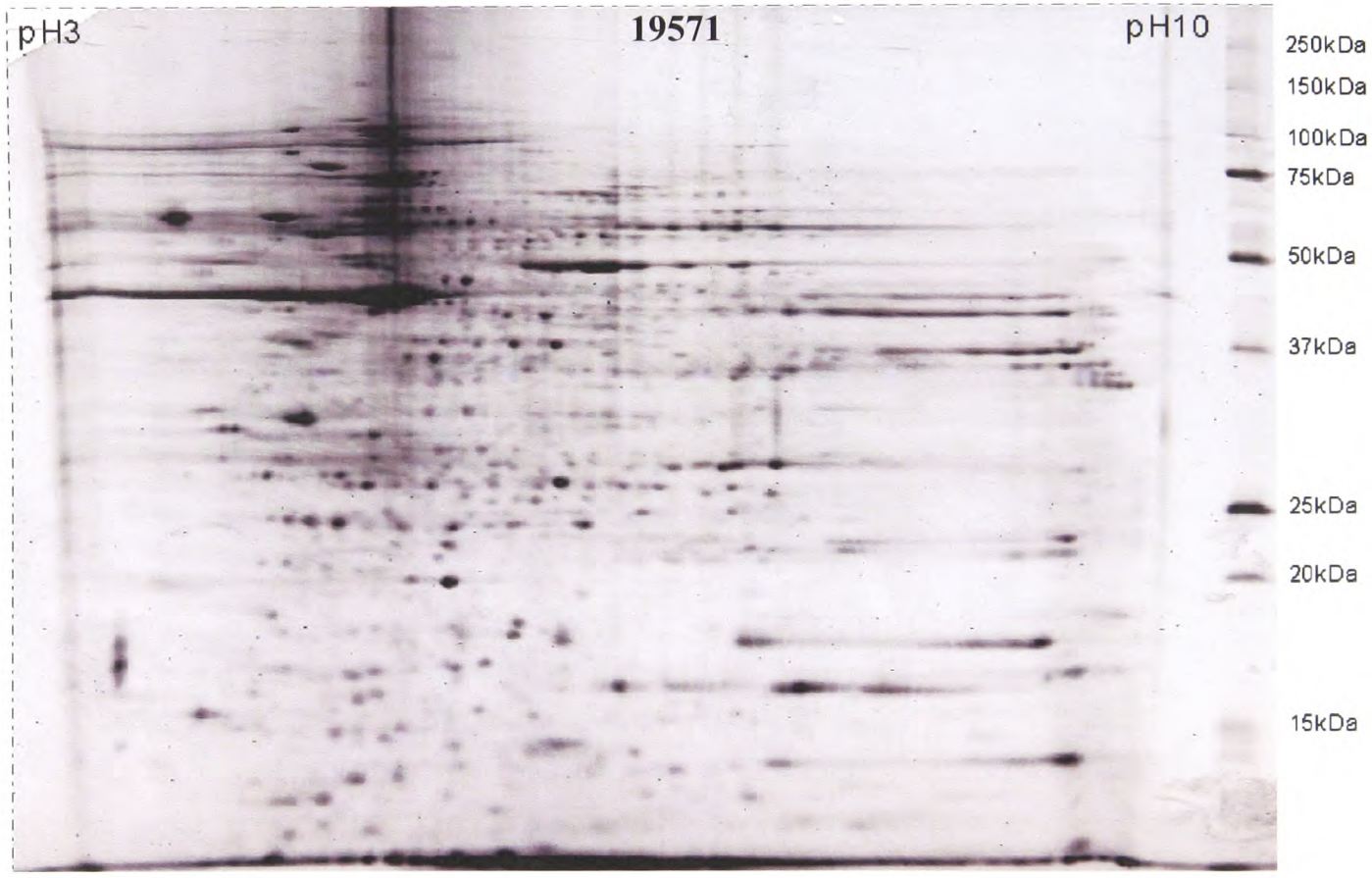


Figure 10.13. Gel 1971 following Deep Purple staining.
Post run Deep Purple fluorescent stain of total proteins. The spots identified in the CyDyes labelled gel were excised from the gel following total protein staining.

10.3. Appendix III. Published and presented work.

The effect of cicerfuran, related arylbenzofurans and stilbenes on *Leishmania aethiopica*, *L. tropica* and *L. major* promastigotes

G. Getti[1,2], S. N. Aslam[1], D. P. Humber[2], P. C. Stevenson[1] and R. A. Cheke[1]

[1]Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK; [2]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK

The effect of arylbenzofurans and stilbenes on the growth of *Leishmania* parasites was evaluated. The arylbenzofurans tested included cicerfuran, a compound first isolated as an anti-fungal natural product from the roots of wild species of chickpeas (*Cicer* sp.) (STEVENSON, P. C. & VEITCH, N. C. 1998: *Phytochemistry*, **48**, 947-951) along with two of its analogues. The four stilbenes were intermediate compounds from the synthesis of cicerfuran. Cicerfuran and some of the stilbenes are known to be active against plant (*Botrytis cinerea*) and mammalian pathogens (*Aspergillus niger*) (S.N.Aslam, unpubl. data). A related group of stilbenes was investigated earlier for antileishmanial activity (DEL OLMO E. *et al.*, 2001: *Bioorganic & Medicinal Chemistry Letters*, **11**, 2123-2126), but none of the compounds in this study has been studied previously for antiprotozoal activity.

Promastigotes from cultures of *L. aethiopica*, *L. major* and *L. tropica* were tested in the exponential phase of growth. All compounds were active at a concentration of 100 µg/ml within 6 hours. However, three of the stilbenes and cicerfuran were more powerful (with IC_{50} from 2 to 22 times lower) than the two arylbenzofurans. The 2-hydroxystilbene showed activity at a concentration <1 µg/ml, with an IC_{50} of 3-5 µg/ml after 48 hours of incubation. Probit analyses with logit transformations of dose-response data showed differences between the activities of the compounds, with a stilbene (ST3) having the highest relative potency against all three species. The antileishmanial activity may be related to the presence of hydroxyl groups.

Presented at: Royal Society of Tropical Medicine and Hygiene, Research in Progress, Short Presentations and Posters. London, UK, 11th December 2003

The effect of cicerfuran, related arylbenzofurans and stilbenes on *Leishmania aethiopica*, *L. tropica* and *L. major* promastigotes

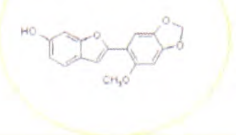
G. Getti[1,2], S. N. Aslam[1], D. P. Humber[2], P.C. Stevenson[1] and R. A. Cheke[1]

[1]Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK; [2]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK

Introduction

Leishmaniasis affects 12 million people worldwide and 350 million are at risk (WHO). Leishmaniasis comprises a group of diseases ranging from mild cutaneous lesions to fatal visceral infections. Current treatments include sodium stibugluconate and meglumine antimonite, pentamidine and amphotericin B (Berman, 2003). However, the toxicity, side effects and expense of these drugs, and resistance against them, necessitate new therapies. One natural product (cicerfuran) and its intermediates were tested for leishmanicidal activity..

Figure 1



Cicerfuran (Fig. 1) is an antifungal chemical defence produced by wild chickpeas (*Cicer* sp.) resistant to fungal wilt (*Fusarium oxysporum* f. sp. *Ciceri*) (Stevenson *et al.*, 1998). It has been synthesized recently along with several benzofuran analogues and a related group of stilbenes and all of these compounds were shown to have antifungal and antibacterial activity (S. N. Aslam, unpubl.) and a related group of stilbenes has antileishmanial activity (DEL OLMO E. *et al.*, 2001).

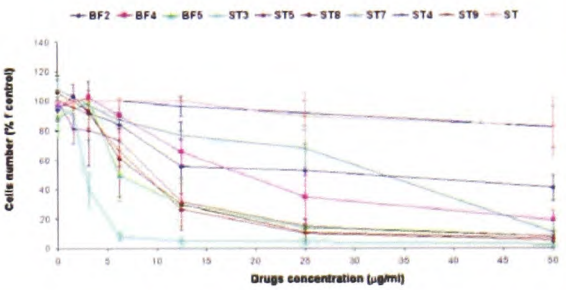
Materials and Methods

Chemistry. Cicerfuran was synthesised from sesamol (3,4-methylenedioxyphenol) and 2,4-dihydroxybenzaldehyde in ten steps via a Wittig reaction, epoxidation of the resulting stilbene and acid-catalysed cyclisation. The structure of the synthetic compound was confirmed by NMR, and its chromatographic and spectroscopic properties were identical to those of the natural product. The other 9 related compounds were 2-(3',4'-Methylenedioxyphenyl)benzofuran (**BF2**), 2-(2'-Methoxy-4',5'-methylenedioxyphenyl)benzofuran (**BF4**), 2-(2'-Methoxy-4',5'-methylenedioxyphenyl)-6-hydroxybenzofuran (cicerfuran, **BF5**), diphenylstilbene (**ST**), 2-Hydroxystilbene (**ST3**), 4-methoxy stilbene (**ST4**), 2,4-Dimethoxystilbene (**ST5**), 2-methoxy-3I,4I-methylenedioxystilbene (**ST7**), 2-Hydroxy-2'-methylstilbene (**ST8**) and 2-Hydroxy-2'-methoxystilbene (**ST9**).

Biological assay. Promastigote inhibition studies were performed on *L. aethiopica* (MHOM/ET/72/L100), *L. major* (MHOM/SU/73/ SASKH) and *L. tropica* (MHOM/SU/58/OD) Compounds were dissolved in dimethyl sulphoxide. Parasites were plated in 96 well plates ($1x10^5$ parasites/ml) in triplicate. Each experiment was repeated three times. Inhibition of promastigote growth was determined microscopically by enumerating parasite in a Neubauer haematocytometer. Percent of inhibition of growth was determined by comparison of treated groups with untreated controls after 48 hours (Fig. 2). Amphotericin B was used as reference.

Figure 2

Effect of cicerfuran, related arylbenzofuran and stilbenes on *Leishmania aethiopica* promastigote growth after 48 hours, average of three experiments



Results and Discussion

The leishmanicidal activity of ten compounds tested against promastigotes of *L. aethiopica* is presented in Fig. 2. Eight out of ten drugs are active against the parasites at concentrations lower than 50 µg/ml. Although *L. aethiopica* was the most sensitive to the drugs, similar results were obtained for *L. major* and *L. tropica* (data not shown).

Probit analyses with logit transformations of dose-response data confirmed differences between the activities of the compounds, with a stilbene (**ST3**) having the highest relative potency against all three species (Fig. 3). The relative median potency as well as the lower and upper 95% confidence limits (CL) were estimated assuming a parallel relation between the drugs' response curves. The Relative Median Potency (RMP) for *L. aethiopica* in Fig. 3 was estimated using Amphotericin B as reference drug. The RPM for the other strains was then estimated by comparison with the effect of the same drug in the most sensitive strain after probit analyses of each drug on the three different strains.

Figure 3

Relative Potency

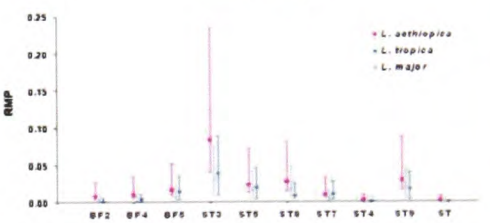
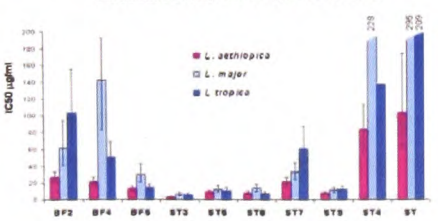


Figure 4

Effect of cicerfuran, related arylbenzofuran and stilbenes on *Leishmania aethiopica*, *L. major* and *L.tropica*



The comparison of IC_{50} values (Fig. 4) confirms *L. aethiopica* as the most sensitive to the action of the drugs. Based on IC_{50} values, cicerfuran stilbenoids containing either hydroxy or methoxy groups are significantly more active than the analogues that do not contain the latter (**ST4**, **ST**) and than arylbenzofurans **BF2** and **BF4**. Structure activity comparisons indicate that the antileishmanial activity is likely to be related to the presence of hydroxy and methoxy groups. **BF5**, **ST3** and **ST8** have hydroxy groups; **ST5** and **ST7** have methoxy groups while **ST9** has both.

The present study demonstrates antileishmanial activity of cicerfuran and 4 related stilbenoids. Preliminary study on the effect of the drugs on THP-1 mammalian cells indicates that BF5, ST3, ST5, ST7, ST8 and ST9 are differentially effective on Leishmania promastigote and consequently, are promising sources of new therapeutic agents and will be further investigated.

References. BERMAN J. 2003. *Current Opinion in Infectious Diseases*, **16**, 397-401. STEVENSON P. C. & VEITCH, N. C. 1998. *Phytochemistry*, **48**, 947-951. DEL OLMO E. *et al.*, 2001. *Bioorganic and Medicinal Chemistry Letters*, **11**, 2123-2126.

Title - Apoptosis, a survival mechanism for *Leishmania* parasites?

G. T. M. Getti[1,2], R. A. Cheke[2] and D. P. Humber[1]

[1]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK; [2]Natural Resources Institute, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK;

Body text – *Leishmania* parasites causes leishmaniasis which is endemic in 5 continents with 350 million people are at risk in 88 countries. Inhibition of apoptosis of host cells has been described for several intracellular pathogens, which benefit by extending the life span of the host cells. *LEISHMANIA* parasites invade host macrophages, causing infections that are either limited to skin (cutaneous leishmaniasis) or spread to internal organs (visceral leishmaniasis). Previous studies have shown that species causing the visceral form of the disease, such as *Leishmania donovani,* also inhibit apoptosis.

In this study, three different species causing cutaneous leishmaniasis, *L. major*, *L. aethiopica* and *L. tropica,* have been tested for their ability to interfere with apoptosis in host macrophages in two different human monocyte-derived macrophages (cell line THP-1 and U937) in blood-derived human macrophages and in peritoneal mouse macrophages. All three species tested induced apoptosis 48 hours after infection. *L. aethiopica* is the most effective; the percentage of apoptotic cells in THP-1 infected with the latter is 6.5 times higher than in uninfected cells, 4 times higher when infected with *L. tropica* and 2.5 times higher in *L. major* infections. Results were similar for U937. Early apoptotic cells were detected by their ability to externalise phosphatidylserine (PS). After staining with Annexin V-CY3 apoptosis detection kit, apoptotic cells were counted by fluorescence microscopy. Further experiment with human and mouse macrophages are underway.

It is currently believed that *Leishmania* lesion develop by killing host macrophages which lyses and leave free amastigotes to bind to new cells. However, the results presented here, together with those published for the visceralizing species, suggest a third hypothesis. Since PS externalisation on apoptosing cells acts as a signal for engulfment by macrophages, induction of apoptosis in the parasitised cells could

actively participate in spreading the infection. Moreover, parasites containing apoptotic bodies with intact membranes could be released and phagocytosed by macrophages. Thus in the visceralizing form, suppression of apoptosis may encourage spread of the infection to other sites whereas in cutaneous forms induction of apoptosis might encourage localized proliferation.

Presented at: Cell death by the sea. 12th Euroconference on apoptosis and 1st training course on 'Concepts and Methods in Programmed Cell Death', Chania, Greece, 17^{th}–20^{th} of September 2004 (oral presentation)

Apoptosis in *Leishmania* infected macrophages: silent macrophage to macrophage spread.

G. T. M. Getti[1,2], R. A. Cheke[2] and D. P. Humber[1]

[1]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK; [2]Natural Resources Institute, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK;

It is currently believed that *Leishmania* spreads from macrophage to macrophage by killing the host cell leaving free amastigotes to bind and be phagocytosed by uninfected macrophages. In this study, three old world cutaneous leishmaniasis species (*L. major*, *L. aethiopica* and *L. tropica*) have been tested for their ability to modify apoptosis in host macrophages. Two cell lines, THP-1 and U937 and human monocytes derived macrophages were infected. Three assays were used to detect three stages of apoptosis. Surface membrane phosphatidyl serine (PS) expression and mitochondrial permeability showed increased levels of apoptosis. Whereas flow cytometry did not detect any increase in DNA fragmentation and loss as expected if cells were apoptotic. A possible explanation of these data is that the parasite induced incomplete activation of apoptosis. It is therefore suggested that, once inside the host cells, the amastigotes will induce apoptosis through mitochondria and will trigger PS externalisation which acts as a signal for engulfment by macrophages and could therefore actively participate in spreading the infection. Moreover, apoptotic bodies with intact membranes containing parasites could be released and phagocytosed by macrophages. The link between infection and apoptosis induction was supported by the fact that chemical induction of apoptosis significantly increased the rate of infection.

Presented at: Royal Society of Tropical Medicine and Hygiene, Manson Christmas Lecture / Research in Progress. London, 19th of December 2005

Apoptosis in *Leishmania* infected macrophages: silent macrophage to macrophage spread

G. T. M. Getti[1,2], R. A. Cheke[2] and D. P. Humber[1]
[1]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK ; [2]Natural Resources Institute, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK

Introduction

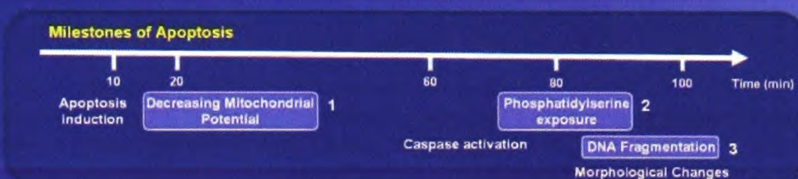
It is currently believed that *Leishmania* spreads from macrophage to macrophage by killing the host cell thus releasing free amastigotes [1] [2].

However, this would expose amastigotes directly to immune destruction. An alternative hypothesis is proposed involving parasite-induced apoptosis in macrophages.

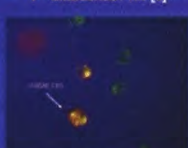
The involvement of apoptosis in amastigotes spread would also facilitate macrophage to macrophage spread without macrophage activation

Materials and Methods

Three Old World cutaneous leishmaniasis species (*L. major*, *L. aethiopica* and *L. tropica*) were tested for their ability to modify apoptosis in host macrophages (THP-1, U-937 and blood derived macrophages). Three methods detecting different aspects of apoptosis (indicated in light blue), were chosen.



1 Mitosensor Kit [3]

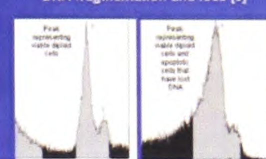


Apoptotic cells are stained green

2 Annexin V assay [4]

Annexin V binds to phosphatidyl serine (PS) on the external surface of apoptotic cells and fluoresce red

3 Flow cytometry detection of increase in DNA fragmentation and loss [5]



Results

Figure 1

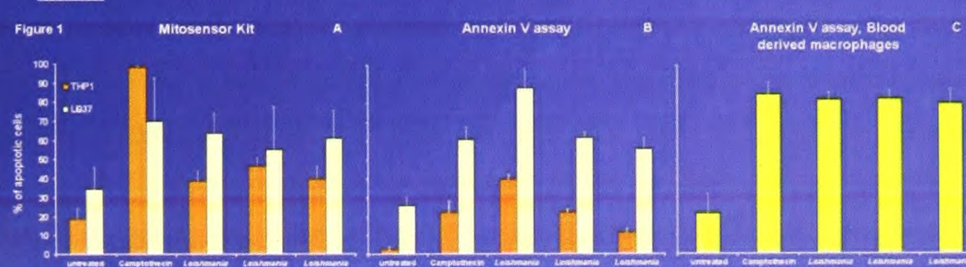


Figure 2

Table 1 Percentage of infection

	control	apoptosis inducer
L. aethiopica	59	78
L. tropica	40	74
L. major	24	63

Conclusion:

All the three species of *Leishmania* caused significant increases in apoptosis in all macrophages tested ($p<0.02$ Figure 1, A, B and C). Interestingly no DNA fragmentation was detected (Figure 2). A plausible explanation is that parasites induce incomplete activation of apoptosis. It is therefore suggested that, once inside the host cells, the amastigotes will induce apoptosis through mitochondria and will trigger PS externalisation. This acts as a signal for engulfment by uninfected macrophages [6] thus actively participating in spreading the infection. The link between infection and apoptosis induction was supported by the fact that chemical induction of apoptosis significantly increased the rate of infection (Table 1). We are currently analysing changes in cytoplasm proteins during infection.

References:

***Leishmania* and its hosts, a deadly relationship.**

G. T. M. Getti[1,2], R. A. Cheke[2] and D. P. Humber[1]

[1]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK ; [2]Natural Resources Institute, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK;

Leishmaniasis is a widespread disease with 350 million people in 88 counties at risk. Every year 500 000 new cases of the lethal visceral form and 1.5 million of the cutaneous form are notified (WHO, 2004). The parasite enters the host following a sandfly bite and establishes itself inside macrophages, key cells of the immune system. From the host macrophages it spreads either to the skin or to internal organs causing life-long disfigurations or death. The interactions between *Leishmania* and macrophages play a central role in the pathogenesis of the infection. It is currently believed that *Leishmania* simply spreads from macrophage to macrophage by killing the host cell leaving free parasites to bind to and be consumed by uninfected macrophages. We studied this mechanism in three cutaneous species (*L. major*, *L. aethiopica* and *L. tropica*). Each of them was able to induce cell suicide (apoptosis). Three assays were used to detect different stages of apoptosis following infection. The results showed that two early features of the apoptotic process were present in the host cells 48 hours after infection. Since early signs of apoptosis act as signals for engulfment by neighbouring macrophages, we suggest that apoptosis induction actively stimulates the spread of the parasites into other cells and that apoptotic bodies with intact membranes containing parasites are released and taken up by previously uninfected macrophages. We believe that *Leishmania* parasites have evolved this ability to induce host cell suicide as a mechanism to facilitate their spread from cell to cell.

Presented at: Poster presentation by top younger researcher in UK bioscience research and R&D at the House of Common. London, 8th of May 2006

Leishmania and its host cell, a deadly relationship

G. T. M. Getti[1,2], R. A. Cheke[1] and D. P. Humber[1]

[1]School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK. [2]Natural Resources Institute, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK.

Introduction

Leishmaniasis affects 12 million people worldwide, 350 million are at risk and 1.5-2 million new cases of leishmaniasis have been estimated to occur annually (World Health Organisation). Leishmaniasis comprises a group of diseases ranging from mild cutaneous lesions to fatal visceral infections. The clinical manifestation depends on the species initiating infection and also on the general health and genetic make-up of the infected individual.

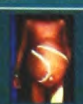


Figure 1: Visceral leishmaniasis (VL)

Figure 2: Muco Cutaneous leishmaniasis (MCL)

Figure 3: Localized Cutaneous leishmaniasis (LCL)

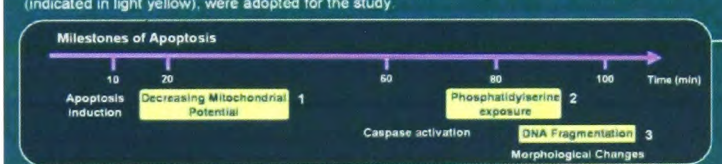
Figure 4: Diffuse Cutaneous leishmaniasis (DCL)

It is currently believed that *Leishmania* spreads from macrophage to macrophage by killing the host cell thus releasing free amastigotes [1] [2]. However, this would expose amastigotes directly to immune destruction. An alternative hypothesis is proposed involving parasite-induced apoptosis (programmed cell death) in macrophages.

The involvement of apoptosis in the infection process would facilitate macrophage to macrophage spread of amastigotes without macrophage activation

Materials and Methods

Three Old World cutaneous leishmaniasis species (*L. major*, *L. aethiopica* and *L. tropica*) were tested for their ability to modify apoptosis in host macrophages (THP-1, U-937 and blood derived macrophages). Three different methods detecting a different stage of the apoptotic process (indicated in light yellow), were adopted for the study.



1 Mitosensor Kit [3]

Apoptotic cells are stained green

2 Annexin V assay [4]

Annexin V binds to phosphatidyl serine (PS) on the external surface of apoptotic cells and fluoresce red

Results

Figure 5

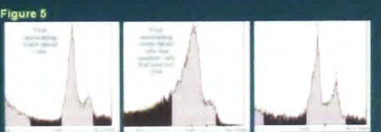


Figure 6

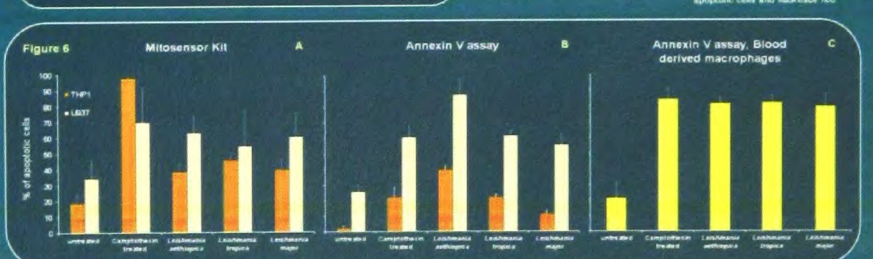


Table 1 Percentage of infection

	control	apoptosis inducer
L. aethiopica	59	78
L. tropica	40	74
L. major	24	63

Conclusion

All the three species of *Leishmania* caused significant increases in apoptosis in all macrophages tested ($P<0.02$ Figure 6, A, B and C). Interestingly no DNA fragmentation was detected (Figure 5). A plausible explanation is that parasites induce incomplete activation of apoptosis. It is therefore suggested that, once inside the host cells, the amastigotes will induce apoptosis through mitochondria and will trigger PS externalisation. This acts as a signal for engulfment by uninfected macrophages [6] thus actively participating in spreading the infection. The link between infection and apoptosis induction was supported by the fact that chemical induction of apoptosis significantly increased the rate of infection (Table 1). We are currently analysing changes in cytoplasm proteins during infection.

LEISHMANIA AND ITS HOST CELL: A COMPLEX RELATIONSHIP.

G.T.M. Getti[1,2], R.A. Cheke[2], P.L. Poole[1], J.T. George[1] and D.P. Humber[1]

[1]*School of Health and Bioscience, University of East London, Stratford Campus, Romford Road, London E15 4LZ, UK.*
[2]*Natural Resources Institute, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK.*

Leishmania parasites invade host macrophages, causing infections that are either limited to skin (cutaneous), or spread to internal organs (visceral). Macrophage-parasite interactions were investigated for three *Leishmania* species responsible for cutaneous leishmaniasis: *L. major*, *L. aethiopica* and *L. tropica*, using either monocyte cell lines (THP-1 and U937) or blood derived human macrophages. In contrast to what happens for visceralizing species of *Leishmania*, early apoptotic markers such as phosphatidylserine externalisation and mitochondrial permeabilisation were evident 48 hours following infection. Interestingly DNA fragmentation (a well known apoptosis marker) was not observed during any of the interactions. These results compared with the visceralizing species suggest that the parasites ability to interfere with apoptosis mechanisms in the host cell may be a key factor in determining the disease outcome. Furthermore this suggest that the relationship between apoptosis and infectious mechanism might be more complex then initially thought. In order to elucidate this mechanism Fluorescence 2-D Difference Gel Electrophoresis was used and proteins differentially expressed (DE) during infection were isolated. Over 100 proteins showed significant changes of expression following infection with each of the three species tested. The proteins of interest were isolated, digested with trypsin and the peptide masses measured using MALDI-TOF. Peptide mass fingerprinting was then carried out to identify these proteins. The identities and the involvement of the DE proteins will be discussed.

Presented at: ICOPA XI, International Parasitology conference, Glasgow, 6th - 11th of August 2006

Leishmania and its host cell: a complex relationship

G.T.M. Getti[1,2], R.A. Cheke[2], P.L. Poole[1], J.T. George[1] and D.P. Humber[1]

Giulia Getti
g.getti@uel.ac.uk

Introduction.

Leishmania parasites invade host macrophages, causing infections that are either limited to skin (cutaneous, **Figure 1**), or spread to internal organs (visceral, **Figure 2**). This interaction is the key point for the infection to evolve into the disease consequently a prime target for intervention.

Figure 1

Figure 2

Background.

These results are in contrast with those reported for *L.donovani* (ref), suggesting that apoptosis mechanisms in the host cell may be a factor in determining disease outcome and that the relationship between apoptosis and infectious mechanism is more complex then initially thought. Although early apoptotic markers were evident during *Leishmania* infection (phosphatidylserine externalisation and mitochondrial permeabilisation); DNA fragmentation was not observed (**Figure 3**). Incomplete activation of host cells apoptosis during cutaneous leishmaniasis inducing species was suggested.

Figure 3

Materials and methods.

Promastigotes of, *L. major L. tropica* and *L. aethiopica* were used to infect THP-1 cells. Cytosol proteins were extracted from infected and uninfected THP-1cells 48 hours after infection, purified, labelled with Cy dyes and run on SDS page (GE Healthcare, Cy dyes minimum labelling kit, IPG strips, pH 3-10 x 180mm). Six gels were produced as described in **Table 1**. Each gel was scanned at different wavelengths (**Figure 4**) in a Typhoon 9400 variable mode imager and processed using DeCyder Differential analysis software (**Figure 5** and **6**)

Table 1

Gel	Cy3	Cy5	Cy2
Gel 1	Uninfected	+ *L. major*	Int. standard 1
Gel 2	+ *L. major*	Uninfected	Int. standard 1
Gel 3	Uninfected	+ *L tropica*	Int. standard 2
Gel 4	+ *L tropica*	Uninfected	Int. standard 2
Gel 5	Uninfected	+ *L. aethiopica*	Int. standard 3
Gel 6	+ *L. aethiopica*	Uninfected	Int. standard 3

Results.

Over 100 proteins showed significant (>1.5 fold) changes of expression following infection (**Figure 5** for *L. major* infection). Proteins (eg **Figure 6**) were identified using MALDI-TOF. preliminary gel analysis showed that proteins from at least 4 groups were consistently over expressed during infection including enzymes involved in metabolic pathways; those responsible for expression and processing of new proteins; molecular chaperones and Cytoskeletal proteins (**Table 2**).

Figure 4

Figure 6

Spot 1704, uninfected

Spot 1704, *L. major*

Figure 5

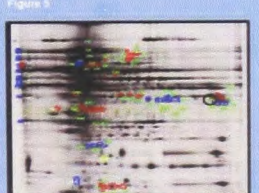


Table 2

spot	Species	Protein name	Mass (Da)
253	*L. aethiopica*	Heat Shock protein HSP 90-alpha 4	84807
1005	*L. aethiopica*	ACTB protein	42184
1258	*L. aethiopica*	Mannosidase, alpha, class1A, member2	38900
1351	*L. aethiopica*	fructose 1,6-biphosphate aldolase	38284
1789	*L. tropica*	fructose 1,6-biphosphate aldolase	38264
1813	*L. tropica*	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	35884
2317	*L. tropica*	Glyoxlase	20833
1729	*L. tropica*	MORC3 protein (zinc finger)	28728
1109	*L. major*	Heterogeneous nuclear ribonucleoprotein H	49089
1704	*L. major*	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	35884

Conclusion.

Leishmania-macrophage relationship is comolex. Involvement of metabolic proteins, particularly those partecipating in carbohydrate catabolism, may be related to increasing the intracellular pH a condition required for *Leishmania* replication. The roles of the other proteins are not so readily identified and further studies are been carried out in order to clarify them. Importantly, proteomics has proven a valuable tool in investigating host-pathogen interactions.

References