An investigation of the CPB cysteine proteinases of *Leishmania mexicana* and their role in intracellular survival

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Abstract

The cysteine proteinases of *Leishmania mexicana* have long been suspected of being important in the virulence of the parasite. This was recently confirmed using analysis of the *in vitro* and *in vivo* infectivity of mutant lines lacking in different cysteine proteinase genes (Mottram *et al.*, 1996; Alexander *et al.*, 1998).

One component of my study was an investigation of the effect of peptidyl cysteine proteinase inhibitors on *L. mexicana*. None of the inhibitors tested had any effect on the growth of promastigotes *in vitro*. However, analysis of the effects on axenic amastigotes demonstrated that cysteine proteinase inhibition reduced growth. The inhibitors were also effective at reducing the cysteine proteinase activity and prevented the complete processing of the pro-enzymes of the cysteine proteinases encoded by *CPA* and different genes within the *CPB* array. These effects were consistent with the autocatalytic nature of the processing, although the results also suggested a partial role for CPA in CPB maturation. The loss of mature enzyme and the resultant increase in the number and abundance of precursor enzyme forms manifested itself as the appearance of large matrix-filled vacuoles, indicating a perturbation of intracellular protein trafficking.

Analysis of the effect of cysteine proteinase inhibition upon intracellular infection demonstrated that it was possible to reduce the survival of the amastigotes. Interestingly, removal of the *CPB* array did not reduce the parasite's susceptibility, indicating that other cysteine proteinases are important

targets of these inhibitors. My findings, in conjunction with the work of others, confirm the potential of cysteine proteinases as targets for the design of chemotherapeutic agents.

An understanding of the mechanisms by which cysteine proteinases are important for the intracellular survival of *L. mexicana* is essential if these enzymes are to be effectively exploited through chemotherapy. Other components of my study addressed this issue; an investigation of the role of the CPB enzymes in parasite virulence being undertaken. This part of my investigation involved an analysis of the effect of loss of the CPB enzymes using a CPB null mutant (Δ*CPB*). An analysis of the response of macrophages to infection with wild-type parasites or Δ*CPB* indicated that comparable levels of reactive nitrogen and oxygen intermediates were produced. This suggested that other factors within the host cell were responsible for the reduced virulence of Δ*CPB*.

The CPB enzymes were shown to have a role in the turn-over of cellular proteins; loss of these enzymes was demonstrated to cause a reduction in the rate of protein turn-over from the cell surface. Furthermore, analysis of the kinetics of attachment and internalisation of wild-type and Δ*CPB* promastigotes demonstrated that loss of the CPB array resulted in a prolonged period of interaction of the parasite with the surface of the macrophage.

The mechanisms of attachment were investigated and it was demonstrated that promastigotes of both wild-type parasites and Δ*CPB/g2.8* (Δ*CPB* re-expressing a single gene copy from the *CPB* array; this mutant line had been previously demonstrated to have wild-type virulence) rely heavily on

attachment to complement receptors, CR3 and CR4, during interaction with the macrophage surface. Δ*CPB*, however, was shown to attach via different receptor-ligand interactions.

Therefore, loss of the *CPB* array was shown to alter the surface architecture of promastigotes such that they could no longer enter macrophages via the route utilised by wild-type promastigotes. This alteration in the mechanism of uptake is hypothesised to be important in the poor intracellular survival of the mutants, in that it results in the parasites encountering a different and more hostile intracellular environment.

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❀

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Abbreviations

ATCC	American Type Tissue Collection
C3b	complement component 3
cNOS	constitutive Nitric oxide synthase
CPA	type A cysteine proteinase of *L. mexicana*
CPA	gene encoding type A cysteine proteinase from *L. mexicana*
CPB	type B cysteine proteinases of *L. mexicana*
CPB	array of genes encoding type B cysteine proteinases of *L. mexicana*
CPC	type C cysteine proteinase of *L. mexicana*
CPC	gene encoding type C cysteine proteinase from *L. mexicana*
CR1	Complement Receptor 1
CR3	Complement Receptor 3/mac-1/CD11b/CD18
CR4	Complement Receptor 4/p150,95/CD11c/CD18
cRT-PCR	competitive Reverse Transcriptase Polymerase Chain Reaction
d^3H_2O	Double distilled de-ionised water
DAPI	4,6-diamidino-2-phenylindole
Δ*CPA*	*L. mexicana* lacking *CPA* gene
Δ*CPA/CPB*	*L. mexicana* lacking both *CPA* and *CPB*

ΔCPB	*L. mexicana* lacking *CPB* array
ΔCPB/g1	*L. mexicana* lacking *CPB* array, re-expressing gene 1
ΔCPB/g18	*L. mexicana* lacking *CPB* array, re-expressing g18
ΔCPB/g2.8	*L. mexicana* lacking *CPB* array, re-expressing g2.8
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECACC	European Collection of Cell Cultures
EDTA	ethylenediamine tetraacetic acid
ER	Endoplasmic Reticulum
FACS	Fluorescent Activated Cell Scanning
FCS	Foetal Calf Serum
FITC	fluoroscein isothiocyanate
FLT3L	ligand for FLT3 receptor
FRAP	Fluorescent Recovery After Photo-bleaching
GIPLS	Glycosylinositol Phospholipids
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GPI	Glycosylphosphatidylinositol
HBSS	Hanks buffered saline solution
HI-FCS	Heat-Inactivated Foetal Calf Serum
HIV	Human Immuno-deficiency Virus

iC3b	neoantigen form of complement component 3
IDB	Ice-cold dilution buffer
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin -
iNOS	inducible Nitric Oxide Synthase
kDa	kilo-Dalton
KO2	morpholine-urea-phenylalanine-homophenylalanine vinylsulphonebenzene
LACK	*Leishmania* homologue of mammalian activated kinase C
LAMP-1	Lysosome Associated Membrane Protein-1
LC	Langerhans Cell
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
MAP	Membrane associated acid phosphatase
MHC	Major Histocompatability Complex
NADP	Nicotinamide Adenine Dinucleotide Phosphate (oxidised form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NO	Nitric Oxide
OVA	ovalbumin
P87	morpholine-urea-phenylalanine-

	homophenylalanine oxycoumarin
PBS	Phosphate Buffered Saline
PEC	Peritoneal Exudate Cell
PGE_2	Prostaglandin E_2
PIPLC	Phosphoinositol phospholipase C
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PPG	Proteophosphoglycan
PV	Parasitophorous Vacuole
RER	Rough Endoplasmic Reticulum
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
SDM	Schneider's Drosophila Medium
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumour Necrosis Factor-alpha
WHO	World Health Organisation
WWW	World Wide Web
ZFA	benzyloxycarbonyl-phenylalanine-alanine diazomethane

Chapter 1

General Introduction

1.1. *Leishmania* and Leishmaniasis.

Leishmania is a kinetoplastid protozoon of the family Trypanosomatidae. Trypanosomatids are obligate, uniflagellated parasites defined by the presence of the kinetoplast, a DNA-containing structure which is a specialised component of the single mitochondrion (Clark and Wallace, 1960).

1.1.1. Occurrence.

Leishmaniasis has been classified by the WHO as one of the six major parasitic diseases. It is prevalent on all the continents of the world, except Australia and Antartica, with 12 million people infected in 88 countries world wide, 72 of which are in the developing world (WHO, 1999). It has been estimated that 350 million people are currently at risk from infection, and that there are 2 million new cases every year, of which 1.5 million are cutaneous. Due to deforestation and urbanisation of rural areas, the problem of exposure to infection is increasing. In North eastern Brazil, for example, the number of new cases of Leishmaniasis infection rose from 2000 in 1983 to 9000 by 1990 (WHO, 1999).

1.1.2. Life cycle.

The parasite exists as flagellated promastigote forms within a sandfly vector. In the old world the disease is transmitted by sandflies of the genus *Phlebotomus* whilst in the Americas it is primarily transmitted by *Lutzomyia* species. These promastigotes exist as multiplicative forms within the mid-gut (or hind-gut, depending on species) of the fly or as non-dividing metacyclic promastigotes in the sandfly's mouth-parts. Damage to the chitinous lining of the sandfly's stomodeal valve via enzyme secretion by the promastigote (Schlien *et al.*, 1992) causes a regurgitation of food from the sandfly during feeding. This regurgitation ensures that the infectious metacyclic promastigotes are passed into a new mammalian host. The saliva of the vector has been demonstrated to enhance the infectivity of the metacyclics via its vasodilatory properties (Theodos *et al.*, 1991), and the presence of maxadilan in the saliva has recently been demonstrated to induce the release of prostaglandins at the site of infection, which has well defined anti-inflammatory properties (Soares *et al.*, 1998). Recent evidence has arisen which suggests that a secondary route of transmission may occur via the release of metacyclic promastigotes in an anal secretion during feeding, although the relevance of this during infection is yet to be confirmed (Sadlova and Volf, 1999).

Once inside the mammalian host, the metacyclics are rapidly taken up by the host's macrophages via classical receptor mediated phagocytosis. This uptake has

been demonstrated to involve a series of macrophage receptors, the use of which appears to differ between species (see Chapter 7, section 7.1).

Once inside the macrophage the promastigote transforms to amastigotes via a process that takes several days to accomplish (Galvao-Quintao *et al.*, 1989). During this process the lysosomal vacuole is extensively modified by the parasite to allow it to survive in such a hostile environment (Antoine *et al.*, 1998). It is within this parasitophorous vacuole that the parasite replicates. Investigation of the intracellular cycle of *L. mexicana* has demonstrated that the amastigotes will undergo 2 - 3 cycles of replication each lasting approximately 17.5 h (Doyle *et al.*, 1989) to produce a maximum number of 8 - 12 amastigotes per infected macrophage before the host cell is lysed and the amastigotes released into the tissues of the host.

The uptake of released amastigotes by macrophages is rapid and takes place via a subtly different route to that of the introduced promastigotes. No receptors involved in amastigote uptake have been identified, suggesting that the mechanism of uptake of amastigotes causes the activation of different cellular pathways. This has been shown by analysis of the down-stream processes occurring in macrophages as they internalise amastigotes which suggests that they enter cells by a process other than classical phagocytosis (Love *et al.*, 1998).

The cycle of infection continues until it is halted either by chemotherapy or death of the infected individual. The life-cycle, however, is only completed when an infected individual is fed upon by a sandfly and infected cells are ingested. Once inside the blood meal the amastigotes transform to the promastigote stage and the cycle continues.

Leishmaniasis is a zoonosis, the natural hosts in addition to humans include domestic dogs and a variety of wild mammals such as foxes and sloths. At least four major groups of parasites, similar in morphology but differing in clinical manifestations, geographic distribution and vector species, cause disease in humans, although the taxonomy is complicated and changing. These are (according to Muller and Baker, 1992):

1) *L. donovani*, in the Old World, along with *L. infantum*, this complex is the causative agent of Visceral Leishmaniasis (Kala-azar). *L. chagasi* in the New World is considered to be similar to these species
2) *L. major*, along with *L. aethiopica* and *L. tropica* is responsible for Old World cutaneous Leishmaniasis.
3) *L. braziliensis*, this complex of organisms is responsible for mucocutaneous Leishmaniasis
4) *L. mexicana*, this complex is responsible for New World cutaneous Leishmaniasis.

1.1.3. Clinical aspects of Leishmaniasis.

The disease caused by the Leishmaniases can be broadly separated into three categories; cutaneous, mucocutaneous and visceral (reviewed by Grevelink and Lerner, 1996). Localised cutaneous leishmaniasis is caused by *L. major* in the Old World and the *L. mexicana* complex in the Americas. The infection usually affects unclothed areas of the body, that are accessible to the sandfly's bite. After an

incubation period, which varies in length from 1 week to 3 months, a papule forms at the site of the bite and the draining lymph nodes become enlarged due to spreading of the infection. Eventually the lesion becomes ulcerated and therefore susceptible to secondary infection. Although the localised cutaneous infection is painful and disfiguring, it is usually self-healing within 6 to 12 months of infection.

New world cutaneous leishmaniasis, unlike its Old World counterpart, is usually restricted to a single lesion. However, in rare cases, caused by *L. m. amazonensis*, the lesions disseminate over the entire body surface giving rise to a lepromatous appearance with non-ulcerative nodules. This form of the disease is rarely self-healing and chemotherapy often results in relapses of the disease. In general, for cutaneous infection there are three defined stages of disease:

1) The initial infection of macrophages by promastigotes and the maturation and replication of amastigotes.
2) The formation of a granulomatous response and ulceration at the site of infection.
3) A healing phase (possibly after drug treatment) where an asymptomatic sub-clinical infection survives.

This continuing low level of parasitism is responsible for the recurrence of lesions in individuals that later become immune compromised, for example due to HIV infection.

Mucocutaneous leishmaniasis (espundia) is caused by organisms from the *L. braziliensis* complex. The disease originates from a primary lesion at the site of the initial infection and progression to mucosal involvement may take in excess of 20

years (Saravia *et al.*, 1985). The mucosal infiltration of the organisms usually occurs in the nasal septum and eventually leads to mutilation of the palate, pharynx, tonsils, gums and lips. The ensuing malnutrition and respiratory tract involvement is the major cause of death from this form of the disease, which is fatal unless treated.

Visceral Leishmaniasis (kala-azar) is caused by infection with members of *the L. donovani* complex. Following the development of a lesion at the site of infection (usually on the lower limbs) and a characteristic blackening of the skin and alopecia, the parasites disseminate throughout the reticuloendothelial system giving rise to fever, splenomegaly, lymphadenopathy, emaciation and hyperglobinaemia. Again, infection with *L donovani* is usually fatal unless treated.

1.1.4. Treatment.

Since their introduction in the 1940s, pentavalent antimonial compounds, such as sodium stibogluconate and meglumine antimonate, have been the first line of treatment for leishmaniasis (reviewed by Bryceson, 1987). The pentavalent forms are relatively non-toxic, but rapid secretion from the body means that long regimens are required. This allows the build up of metabolised trivalent forms and causes cardiac and renal toxicity. In the poorer countries afflicted by leishmaniasis, this leads to non-compliance with treatment and therefore drug-resistant isolates are emerging (Olliaro and Bryceson, 1993).

The second line drugs are far from ideal. Pentamidine is used in cases of antimonial resistance, but the associated toxicity is severe and can lead to death of

the patient. The use of the antifungal drug Amphotericin B is also difficult in poorer countries due to the associated toxicity. However, it's encapsulation in liposomes has increased its selectivity and thus increased its efficaciousness and reduced its toxicity (Thakur *et al.*, 1996).

Application of these drugs for the treatment of visceral leishmaniasis is invariably via intra-muscular injection. This is obviously not ideal, due to risks of contamination and non-compliance and efforts are being made to develop an orally available chemotherapeutic agent. The topical application of these compounds during the treatment of cutaneous leishmaniasis is successful in non-resistant cases, although problems still exist with dosage and compliance.

Resistance to these commonly used therapies is on the increase and can be due to a number of parasite factors (reviewed by Ouellette and Papadopoulou, 1993). Most notably, a reduction in the accumulation of the compound, can be due to the expression of a P-glycoprotein, ATP-dependent extrusion pump. Amplification of gene copy number can also decrease the parasites susceptibility to dosage regimens.

1.2. Intracellular survival.

Many parasitic organisms survive within their mammalian host by hiding from the host's immune system intracellularly. This strategy has been adopted by *Trypanosoma cruzi* which has developed the ability to invade a variety of vertebrate

cells via the induction of phagocytosis by the use of sialyl residues on the hosts surface molecules and a parasite surface trans-sialidase (Ming *et al.*, 1993). The ability of *T. cruzi* to invade cardiac muscle and the smooth muscle of the intestine enables its prolonged survival and is the causative factor of much of the pathology suffered in the chronic stages of Chagas disease. *Toxoplasma gondii* also gains protection from immune activator molecules by residing intracellularly (reviewed Mauel, 1996). This parasite attaches to laminin receptors on the surface of macrophages and enters via 'sliding penetration'. Thus both of these parasites have evolved special mechanisms for entry into their host cells which avoid the stimulation of host microbicidal activity, either by entering into non-professional phagocytes or by failing to stimulate phagocytosis at all.

Leishmania species, however, have evolved no specialised entry mechanisms to enable the silent entry into cells. They are dependent on the utilisation of phagocytosis inducing receptors on the macrophage surface (discussed in detail in Chapter 7, section 7.1). The utilisation of classical receptor-mediated phagocytosis by *Leishmania* promastigotes therefore prevents covert entry and leaves the parasite open to attack from the host cells microbicidal mechanisms. The mechanisms by which *Leishmania* survive within the hostile intramacrophagic environment have been the subject of much investigation as a better understanding of the relationship between parasite and host cell will facilitate a more rational approach to combating the disease.

1.2.1 The Parasitophorous Vacuole.

Following phagocytosis, the parasite is encapsulated within the macrophage by a vesicle composed of host cell plasma membrane. This vesicle is rapidly altered during the first hours of infection and forms the parasitophorous vacuole (PV), (reviewed by Antoine *et al.*, 1998). Within 30 minutes of vacuole formation, the pH drops to pH 5.5 where upon it remains constant (Sturgill-Koszcki *et al.*, 1994). By 48 hours post-infection, the membrane contains markers characteristic of lysosomes; major histocompatability complex class II (MHC-II) molecules are found associated with the membrane, as is macrosialin (a pre-lysosomal marker) and lysosome associated membrane protein-1 (LAMP-1). By 14 days post-infection, the PV membrane contains mannose-6-phosphate receptors and therefore has features resembling both lysosomal and late endosomal compartments (Russell *et al.*, 1992). Analysis of the contents of the PV has demonstrated that it has many lysosomal characteristics including the presence of cathepsins B, D, H and L (Lang, *et al.*, 1994a) and acid phosphatases (both parasite- and host- derived).

The PV membrane, due to its endosomal qualities, allows the uptake of nutrients from the host cell, facilitating the survival of the intracellular amastigotes. Small anionic molecules are transported rapidly into the vacuole by a host-derived active transport mechanism. Larger cytosolic macromolecules are transported via a mechanism that appears to utilise the host cells recycling machinery (Schaible *et al.*, 1999).

The PVs of different *Leishmania* species differ in size and morphology. *L. donovani* and *L. major* reside in small individual PVs, whereas the PVs of *L. mexicana* and *L. amazonensis* are large and communal (Antoine *et al.*, 1998). The amastigotes of *L. donovani, L. pifonoi, L. amazonensis* and *L. mexicana,* unlike those of other species are not free within the vacuole but are attached to the PV membrane by a posterior rod. The significance of these differences is unknown at present, although it has been demonstrated that the production of a secreted proteophosphoglycan (PPG) by *L. mexicana* amastigotes can cause vacuolisation of macrophages *in vitro* and may be responsible for the enlargement of the PV in this case (Peters *et al.*, 1997).

1.2.2. Parasite surface molecules involved in the interaction with its host.

1.2.2.1. Lipophosphoglycan.

Lipophosphoglycan (LPG) is a heterogeneous lipid-containing polysaccharide attached to the surface of *Leishmania* promastigotes via a glycosylphosphatidylinositol- (GPI-) anchor. It is the most abundantly expressed molecule on the surface of the promastigotes, with 1.5 - 5 x 10^6 molecules/cell (Sacks, 1992), and has been reported to have many important roles in the survival of *Leishmania* in the sandfly and the mammalian host (reviewed by Turco and Descoteaux, 1992). Its role in intramacrophagic survival of *Leishmania* has been

extensively investigated. During the initial stages of infection, following phagocytosis of metacyclic promastigotes by macrophages, LPG has been shown to prevent the fusion of lysosomes with the developing PV (Desjardins and Descoteaux, 1997). This is presumed to facilitate the survival of the promastigotes as they transform to the amastigote stage, which is more able to withstand the hostile environment within the host cell. Following transformation to the amastigote, the lysosomes are free to fuse with the PV as amastigotes have no surface LPG expression. The amastigotes are covered in a protein-poor, glycolipid-rich plasma membrane which contains many host-derived glycosphingolipids (Winter *et al.*, 1994). Although the significance of this has yet to be determined, it is postulated that they may have a role in protection of the parasite from deleterious host components by creating a physical barrier.

Whilst within the macrophage the parasite is susceptible to the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) following activation of the macrophages via interferon-γ (IFN-γ). The limited expression of catalase in promastigotes increases their susceptibility to such molecules. However, expression increases in amastigotes, giving them an enhanced resistance (Murray, 1982). The ability of LPG to inhibit the production of Interleukin (IL)-1 and IL-12 allows inhibition of the outgrowth of Th1 type T cells in response to infection. Thus the production of IFN-γ is down regulated, which in turn also inhibits the production of RNI and ROS in the PV thereby conferring protection to the promastigote during transformation (Frankenburg *et al.*, 1990; Hatzigeorgiou *et al.*, 1996; Piedrafita *et al.*, 1999). Furthermore, treatment of macrophages with LPG from *L. donovani* has

been shown to inhibit apoptosis, which has been postulated to facilitate a prolonged intracellular survival (Moore and Matlashewski, 1994)

1.2.2.2. Zinc metalloproteinase.

The major surface proteinase of *Leishmania* promastigotes is a 63 kDa protein known as gp63. Its abundance on the surface of promastigotes has lead to much speculation over its role(s) within the life-cycle of *Leishmania*. However, recent mutational studies involving deletion of gp63 genes from *L. major* have demonstrated that its role in intracellular survival is minimal (Joshi *et al.*, 1998). A more detailed discussion of the roles of gp63 can be found in Chapter 4, section 4.1.

1.2.2.3. Glycosylinositolphospholipids.

Glycosylinositolphospholipids (GIPLS) are expressed in many eukaryotic systems, usually as an anchoring system for cell surface expression of protein (reviewed in Schofield and Tachado, 1996). These molecules consist of a phosphatidylinositol backbone with additional sugar residues. *T. cruzi* and *Leishmania* species have been demonstrated to express a range of GIPLS which are not anchors to proteins, but exist free in the plasma membrane. Interest in their function was stimulated by their abundance on the surface, with as much as 80% of the amastigote surface consisting of these molecules. Furthermore, recent work by Ilgoutz *et al.*, (1999) has demonstrated that GIPLS are essential for parasite survival

in vitro; it was shown to be impossible to remove genes essential for their production from promastigotes.

Investigation has demonstrated that these molecules can inhibit the protein kinase C (PKC)-dependent signaling of host cells in a competitive or non-competitive fashion with respect to various PKC activators (McNeely *et al.*, 1989). This indicates a possible role in prevention of host cell activation. They also inhibit the production of tumour necrosis factor-alpha (TNF-α) in response to stimulation (Tachado *et al.*, 1997), although this study used purified GIPLS and indicated that activity is due to the glycan core sequence, which may or may not be accessible *in situ*.

It was recently demonstrated that the GIPLS of *L. major* can inhibit the synthesis of RNI in a dose-dependent manner (Proudfoot *et al.*, 1995). Again, this is suggestive of a role in the prolonging of intracellular survival. Interestingly the free GPIs of *T. brucei* and *Plasmodium* species have been implicated in the overproduction of nitric oxide and IL-1, and thus in the cachexia and cerebral complications associated with infection with these organisms. This demonstrates that the function of these small membrane glycolipids has evolved specifically depending on parasite type.

1.2.3. Antigen presentation.

The recognition of intracellular antigens is essential if the interior of cells is not to become a privileged site for the growth and survival of pathogens. Antigen

presentation to the immune system is therefore important in allowing the activation of host immune effector cells to combat infection. During *Leishmania* infection, the macrophages are supportive of survival and multiplication when in the quiescent resting stage. They will only act to kill intracellular amastigotes when activated by the presentation of antigen and the ensuing interaction with T cells.

The initial stages of *Leishmania* infection occur as promastigotes are regurgitated into the skin, and then phagocytosed by macrophages. In the case of *L. major* it has been demonstrated that the skin's dendritic cells, Langerhan cells (LC), cannot internalise promastigotes (Von Stebut *et al.*, 1998) or internalise only a small number (Konecny *et al.*, 1999), intimating that uptake by LCs does not occur until amastigotes are released from the primarily infected macrophages - it is postulated that this allows establishment of infection before presentation of antigen to T cells.

After amastigote internalisation by LCs, migration of these cells to the draining lymph nodes occurs where parasite antigens are presented to naïve T cells via MHC-II-peptide complexes. LCs have a large number of MHC-II molecules per cell and can thus present a large antigenic epitope repertoire which facilitates the stimulation of a range of naïve T cells. Investigation has shown that LCs are much more efficient at presenting antigen to T cells than macrophages due to the prolonged nature of MHC-II-peptide complex expression on the surface of these cells (Flohe *et al.*, 1997)

These primed T cells then migrate to the site of infection and can therefore interact with infected macrophages. However, the infected cells at this stage will be either resting, and thus expressing no MHC-II, or primed and therefore expressing

only those intracellular antigens available to its processing machinery (reviewed by Overath and Aebischer, 1999). Analysis of the amastigote antigens available for expression in primed macrophages has demonstrated that internal antigens, such as the abundant CPB enzymes and the lysosomal membrane-associated acid phosphatase (MAP), cannot be presented via MHC-II in macrophages harbouring live parasites (Wolfram *et al.*, 1995; Wolfram *et al.*, 1996).

Other antigens such as the *Leishmania* homologue of the mammalian receptor form activated C kinase (LACK) are presented via MHC-II during transformation of promastigotes, but macrophages loose the ability to present such antigens on formation of amastigotes (Prina *et al.*, 1996). This phenomenon appears to hold true for many leishmanial antigens with amastigote antigens becoming sequestered from presentation after 24 hours of macrophage infection (Kima *et al.*, 1996). These studies demonstrate that only a restricted number of parasite antigens are presented to primed T cells at this stage in infection.

The range of T cells primed, however, means that it is probable that some will be able to interact specifically with MHC-II-peptide complexes on the macrophages. This interaction, at least in resistant mouse strains, results in the production of IFN-γ and TNF-α from $CD4^+$ Th1 cells which activates the host macrophage leading to parasite death and the subsequent increase in the heterogeneity of antigens presented. The activation of host macrophages also stimulates production of TNF-α from the macrophage itself, which facilitates a sustained autocrine activation.

Therefore the presentation of antigen in infected cells is important at two stages during the stimulation of a cellular immune response, namely the triggering of naïve T cells by peptide-loaded LCs in the lymph nodes and also during the interaction of primed T cells with infected macrophages.

The mechanisms by which *Leishmania* prevents presentation of antigen has been studied in much detail. Conflicting reports in the literature suggest that *Leishmania* infection can either suppress MHC-II synthesis at the level of gene expression (Kwan *et al.*, 1992) or that it has no effect on plasma membrane expression (Lang *et al.*, 1994a). These studies both involved investigation of *L. donovani*-infected cells 48 h post-infection, indicating that these conflicting findings are not due to species-specific differences. *L. amazonensis* infection, however, was demonstrated to alter the cellular distribution of MHC-II molecules (Lang *et al.*, 1994b), with clustering of this molecule observed on the PV membrane around the site of amastigote attachment. This clustering was demonstrated to be specific for MHC-II as other PV-associated markers e.g. macrosialin, were evenly distributed. This phenomenon is probably due to the reported internalisation and degradation of MHC-II within the megasomes of *L. amazonensis* (De Souza *et al.*, 1995). Interestingly inhibition of cysteine proteinases in this system lead to the build-up of MHC-II within the megasomes, inferring that the parasites abundant cysteine proteinases were responsible for its degradation.

The interaction of $CD4^+$ T cells with MHC-II-peptide complexes is essential for the resistance to infection with *Leishmania*. Studies in mice deficient in both $CD4^+$ and MHC-II expression have demonstrated that despite an initial control of

infection, they eventually succumb to disease (Chakkalath *et al.*, 1995). The initial control was shown to be due to the activity of MHC-I restricted $CD8^+$ T cells and their production of IFN-γ, indicating a role of cytotoxic T cells in initial infection control. The activation of these cells by infected macrophages was investigated by Garcia *et al.*, (1997) via the use of *Leishmania* tranfected to express ovalbumin (OVA) within the PV, to facilitate the analysis of expression of antigenic epitopes via MHC-II and MHC-I restricted pathways. This analysis demonstrated that recognition of this molecules via MHC-II was possible but that no class-I recognition occurred due to the cleavage of OVA at important epitopes by the surface metalloproteinase gp63. This was taken to infer that gp63 may have roles in reducing the number of antigenic epitopes available to expression on the surface of parasitised macrophages. However the lack of MHC-I presentation may also be due to the phenomena observed by Lang *et al.*, (1994b) which suggested that although expression of MHC-I on the surface of infected macrophages was similar to that observed in uninfected controls, there was no localisation of MHC-I to the PV membrane, indicating that epitope expression via MHC-I may not play a significant role in the presentation of antigen during *Leishmania* infection.

1.3. Immune Response to infection.

The modulation of the immune response has long been established as a way by which *Leishmania* parasites prevent an effective defence against infection. This phenomenon has been extensively studied using susceptible and resistant mouse

models, which have allowed a profile of effective and ineffective responses to be ascertained.

In susceptible mice, such as BALB/c, infection with *L. major* leads to visceralisation of the parasite and ultimately to death of infected animals. This disease progression is characterised by the induction of a Th2 type, or humoral, response to infection, which prevents the host macrophages from becoming activated and thus promotes the survival of intracellular infection.

This response is typified by early production of IL-4 within the lymph nodes of infected mice (Himmelrich *et al.*, 1998), which in conjunction with IL-10 has been demonstrated to reduce the killing of intracellular *L. major* via a reduction in the nitric oxide produced in response to infection (Vouldoukis *et al.*, 1997). These cytokines are typical of a Th2 type response and have well-documented inhibitory effects on macrophage activation via prevention of IFN-γ production.

The role of IL-10 in polarisation of the immune response in these mice is not essential, as investigation has demonstrated that both resistant and susceptible mice have similar levels of IL-10 during the chronic stages of infection (Chatelain *et al*, 1999a). However, its inhibition of production of IFN-γ during the initial stages of infection is probably important in preventing the development of a Th1 type, or cellular, immune response.

Within 72 h of infection in susceptible mice, the level of circulating transforming growth factor-beta (TGF-β) is increased (Barral-Netto and Barral, 1994). This cytokine has been demonstrated to enhance mRNA levels of IL-10 (Barral *et al.*, 1993) and also has well documented properties concerning

macrophage deactivation. The addition of TGF-β to macrophages infected with *L. braziliensis in vitro* has been shown to facilitate an enhanced survival of the parasite, which is probably due to the reduction in nitric oxide production (Li *et al.*, 1999). The production of TGF-β in infected mice appears to be localised to the site of infection as non-infected organs have high levels of IFN-γ during chronic disease (Wilson *et al.*, 1998). After infecting a host macrophage, *Leishmania* species have been demonstrated to alter the metabolism of arachidonic acid, such that the production of prostaglandin E_2 (PGE_2) is enhanced. PGE_2 is a non-specific suppresser of Th1 cells and the amount of production *in vivo* can be directly correlated to increasing lesion size and the underproduction of IFN-γ and TNF-α (Milano *et al.*, 1996).

In conjunction with the over-expression of Th2 type cytokines, *Leishmania* infection in susceptible mouse strains is associated with the underproduction of important Th1 type inducers, such as IL-12 (Carrera *et al.*, 1996). This facilitates protection via the induction of IFN-γ from natural killer cells, which in turn induces the activation of host macrophages. Indeed, therapy of infected BALB/c mice with recombinant IL-12 (rIL-12) conveys protection to the individuals (Heinzel *et al.*, 1993). This protection, however, is dependent in part on the presence of IFN-γ.

Some indication as to the cause of this polarisation of the immune response is given by studies demonstrating that inhibition of cathepsin B led to a switch in $CD4^+$ T cell differentiation from Th2 to Th1 (Maekawa *et al.*, 1998). This phenomenon suggests that the processing of antigenic peptides by Cathepsin B in these mice is such that the epitopes expressed via MHC-II are specific for Th2 type

T cells. Inhibition of the cleavage of these epitopes by cathepsin B may allow the presentation of other antigenic epitopes and therefore facilitates the outgrowth of Th1-type T cells.

Despite the obvious polarisation in the immune response in BALB/c mice preventing an effective immune response from developing, recent information has indicated that other host factors apart from Th type must be involved in governing susceptibility. Chatelain *et al.*, (1999b) demonstrated that similar treatment of mice with anti-IL-4 and anti-IFN-γ therapy lead to the induction of strong Th1 type responses in both resistant and susceptible mice. Despite this, however, the BALB/c mice still succumbed to infection, demonstrating that the situation *in vivo* is more complicated than envisaged and that further investigation of this phenomenon is necessary.

The situation in resistant mice is very different. These mice are stimulated to produce a Th1 cell mediated response to infection. The production of IL-12 is critical in the early stages of infection and is thought to be due to infected dendritic cells (Gorak *et al.*, 1998). Evidence suggests however that this production is mainly in response to infection with amastigotes rather than promastigotes, which may allow the establishment of infection prior to induction of a protective immune response (Reiner *et al.*, 1994). Elevated IL-12 production is important in the stimulation of IFN-γ which elicits the expression of inducible nitric oxide synthase (iNOS) in macrophages (see section 1.3.1, for detail). Although yet to be demonstrated for *Leishmania* infection, it was recently established that the beta-chemokines have a protective role during *T. cruzi* infection via their ability to

enhance the production of nitric oxide (NO) by host cells (Villalta *et al.*, 1998). It seems likely that such a role in leishmaniasis will also be demonstrated.

IFN-γ also enhances expression of MHC-II on the surface of infected macrophages and will thus be important in the triggering of $CD4^+$ Th1 cells in response to parasite antigens. Some evidence also suggests that IFN-γ may have directly cytostatic effects on promastigote growth (Bhattachanga *et al.*, 1993). However the relevance of this *in vivo* is uncertain as the effect was demonstrated only on the multiplicative promastigotes, which will not experience this cytokine due to their existence in the vector only.

The immune response to infection is not an all or nothing situation with susceptible and resistant mice producing only Th2 or Th1 cytokines, respectively. Studies by Green *et al.* (1994) indicated that mice produce both TGF-β and TNF-α in response to infection with *L. major*. The subsequent pathway which prevails was shown to be dependent on the levels of IFN-γ present at initiation of infection. Further to this the development of a protective immune response to *Leishmania* infection can be governed by the numbers of organisms applied during initial infection. Even in BALB/c mice, a low infectious dose of *L. major* was demonstrated to lead to a self limiting infection (Doherty and Coffman, 1996).

1.3.1. Nitric oxide.

NO is produced via the oxidation of terminal guanido-nitrogen atoms from the amino acid L-arginine by the NADPH-dependent enzymes known as nitric oxide

synthases (NOSs) (see Figure 1.1). NO is relatively unstable and reacts with water and oxygen ions to form an array of radicals and reactive molecules, collectively termed reactive nitrogen intermediates (RNI). These decompose to give the stable nitrite and nitrate end products.

At least two forms of NOS have so far been characterised (reviewed by Piedrafita and Liew, 1998); constitutive NOS (cNOS) is expressed in several cell types including epithelial and neuronal cells and is involved in the production of NO for several physiological roles (reviewed by Wu and Morris, 1998). The second form inducible NOS (iNOS) is again expressed in many cell types, for example macrophages and mast cells, where its expression (which is controlled at the level of gene expression) is induced by various cytokines, including IFN-γ (Dileepan *et al.*, 1995).

NO has long been established as being central to the resolution of several intracellular and extracellular infections. Evidence exists that NO is critical in protection from Herpes Simplex Virus I infection (Maclean *et al.*, 1998) via the inhibition of viral replication. A further role in the susceptibility or resistance of different mouse strains to infection with *T. congolensi* has also been demonstrated (Kaushik *et al.*, 1999). The role of NO in defense against intracellular *Leishmania* infection has been established for many years. It was definitively demonstrated by the highly susceptible nature of iNOS deficient mice to infection with *L. major* (Wei *et al.*, 1996), despite a Th1 type cytokine response. The data implied that NO had no role in innate immunity to infection as the lesions remained normal in size until 5 weeks post infection. The role of NO in human leishmaniasis is still ill-defined

(reviewed by Mossalayi *et al.*, 1999) although investigation has suggested that IFN-γ production and/or ligation of the IgE receptor (CD23) are important in immunity to infection in human cases. Both of these pathways have been demonstrated to elicit NO production via induction of iNOS.

1.3.2. Oxidative response.

When exposed to certain stimuli, phagocytes undergo marked changes in the way they handle oxygen. The rate of uptake of oxygen increases greatly and they begin to produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl groups with a view to enhancing cytotoxicity (see Figure 1.2).

The role of production of these species is thought to be only of minor importance in the killing of *Leishmania,* despite early evidence demonstrating the highly susceptible nature of *Leishmania* species to hydrogen peroxide *in vitro* (Murray, 1981). The parasite employs several strategies to promote its resistance to ROS. Firstly, the parasite has a degree of resistance which is endowed by the expression of trypanothione, an antioxidant molecule comprised of 2 glutathione conjugated by spermidine (reviewed in Mauel, 1996). The reduction of this molecules via the NADPH-dependent enzyme trypanothione reductase confers resistance to ROS. Furthermore, evidence suggests that *L. donovani* inhibits the production of the oxidative burst in infected macrophages (Chakrabarty *et al.*, 1996), this probably explains the anomaly between *in vitro* susceptibility and survival

in macrophages. Indeed LPG has been implicated in this inhibition (see section 1.2.2.1, for detail).

The fact that the parasite protects itself from ROS suggests that these species may have some role in leishmanicidal activity. Indeed, it has been demonstrated that amastigotes have an increased survival in macrophages, as compared with promastigotes, which correlates with the smaller oxidative burst (Channon *et al.*, 1984)

Despite this information Scott *et al.*, (1985) demonstrated the ability of macrophages deficient in superoxide and hydrogen peroxide production to clear intracellular *Leishmania* infection. This suggests a lack of importance in clearance *in vitro*. However recent investigation of the course of infection in ROS and RNI deficient mice has suggested that ROS may be important in the early stages of infection (Murray and Nathan, 1999).

1.4. Parasite cysteine proteinases.

Cysteine proteinases are proteolytic enzymes which have essential cysteine and histidine residues involved in hydrolysis. All cysteine proteinases are expressed as pre-pro-enzymes. The pre-region is involved in the trafficking of the enzyme to the endoplasmic reticulum where it is removed. The role of the pro-region was not resolved until recently when, it was demonstrated that the pro-region of papain and papaya proteinase IV were high affinity inhibitors of the mature enzymes activity

(Taylor *et al.*, 1995). This phenomenon was subsequently demonstrated to be true for the mammalian enzyme cathepsin L (Carmona *et al.*, 1996) and is assumed to hold true for the majority of cathepsin L-like enzymes. However, recent work has indicated that in *Leishmania* species the pro-region also has roles in targeting the enzyme to the lysosomes (Huete-Perez *et al.*, 1999).

Several important parasitic organisms have been demonstrated to rely heavily on cysteine proteinases. *T. cruzi*, for example, has a major cysteine proteinase, cruzipain, which is expressed from a tandem array of at least 130 genes (reviewed by Coombs and Mottram, 1997). Cruzipain is a cathepsin L-like enzyme which is found located in the lysosomes of the parasite and also on the cell surface in the epimastigote stage (Parussini *et al.*, 1998). The majority of expression of this enzyme occurs in the epimastigote stage which exists within the insect vector, suggesting importance in survival within the vector. Evidence suggests, however, that over-expression of this enzyme increases the transformation of the parasite to the infective metacyclic stage (Tomas *et al.*, 1997) and some information points to possible roles in host cell penetration and intracellular survival (Franke de Cazzulo *et al.*, 1994).

Other roles for cruzipain may lie in the evasion of an effective immune response. Sera from Chagasic patients contains antibodies against the C-terminal extension of the enzyme, which appear to have no effect on it's activity. This indicates that the C-terminal extension contains immunodominant B cell epitopes and may allow the production of an ineffective humoral response to infection (Martinez *et al.*, 1993). Recently a novel cathepsin B-like enzyme was discovered in

T. cruzi (Nobrega *et al.*, 1998), although its significance during infection awaits evaluation.

The *Plasmodium* species also contain important cysteine proteinase enzymes (reviewed by McKerrow *et al.*, 1993). The serine repeat antigen (SERA) and its homologue, SERPH, have long been known to be important in invasion and rupture of infected erythrocytes. Recently these 130 kDa proteins were demonstrated to contain a 30 kDa protease domain with homology to the papain family of cysteine proteinases (Gor *et al.*, 1998). These molecules are found within the PV of mature schizonts and are released near the time of rupture. Indeed the cysteine proteinase inhibitor, leupeptin, has been demonstrated to prevent rupture of erythrocytes infected with *P. knowlesi* or *P. falciparum*, implicating these proteinase domain as important. Malaria species also contain a 28 kDa cathepsin L-like cysteine proteinase which is inhibited by E64 and appears to be involved in the early stages of haemoglobin degradation, implicating the enzyme in parasite nutrition.

The nutrition of *Entamoeba histolytica* is also partially dependent on cysteine proteinases. Serrano-Luna *et al.* (1998) have demonstrated that the acquisition of iron by the trophozoites of this amoeba is partially prevented by the use of cysteine proteinase inhibitors. Further to this, CP5, a soluble cysteine proteinase with affinity for the surface membrane due to its possession of a hydrophobic patch (Jacobs *et al.*, 1998) has been demonstrated to be missing from the non-invasive form *E. dispar*, suggesting that it may have roles in tissue invasion and other cytopathic effects.

Therefore the cysteine proteinases of several parasitic protozoa have roles in the survival and resultant pathology to the host. The cysteine proteinases of *Leishmania* species are no exception and have been implicated in the ulceration resulting during infection following their release from dead amastigotes (Ilg *et al.*, 1994).

1.4.1. The cysteine proteinases of *L. mexicana.*

1.4.1.2. GPI:protein transamidase.

Many eukaryotic surface proteins require attachment to a glycosylphophatidylinositol- (GPI-) anchor for surface expression. The enzyme responsible for this was identified as a cysteine proteinase with GPI:protein transamidase activity, termed GPI8, in 1996 by Benghezal *et al.* This enzyme was identified after analysis of the mechanism involved in the deficiency of GPI-protein expression in mutant *Saccharomyces cerevisiae*. It is a 47 kDa glycoprotein localised as a type I trans-membrane protein within the lumen of the endoplasmic reticulum (ER), The transamidation reaction it catalyses involves the simultaneous cleavage of the C-terminal peptide sequence of a protein and the addition of the GPI-anchor.

The recognition of the GPI-addition site (ω site) is the responsibility of another ER lumenal protein (Ohishi *et al.*, 1998). GAA1 is a 68 kDa protein which was again identified following studies in mutant yeast, and has been demonstrated to

recognise the attachment site of putative GPI-anchored proteins and present them to GPI8 for anchoring. The co-precipitation of GPI8 and GAA1 suggests that these proteins are closely associated in the ER.

The attachment motif recognised by GAA1 is composed of the ω site and ω+2 site which must be a small amino acid. This is followed by a hydrophobic carboxy terminal sequence separated by between 8 - 12 amino acids down stream of ω. It is thought that this hydrophobic sequence is important in recognition.

As discussed earlier, GPI- anchored molecules can play an important role in leishmanial infection (see section 1.2.2). Analysis of the signal requirement for attachment of GPI- anchors to trypanosomatid proteins and mammalian proteins has suggested that these differ (Moran and Caras, 1994). Therefore the transamidation reaction may have interesting exploitation potential. However, the leishmanial GPI8 enzyme was recently identified (Hilley *et al.*, 1999) and analysed using targeted gene disruption, which demonstrated that loss of function of this enzyme had no effect on parasite virulence *in vitro*.

1.4.1.3. CPA.

CPA is a Type II Trypanosomatid cysteine proteinase (reviewed by Coombs and Mottram, 1997). It is expressed from a single copy gene, which was isolated from an *L. mexicana* cDNA library using as a heterologous probe a *T. brucei* gene fragment (Mottram *et al.*, 1992). This enzyme has cathepsin L-like substrate specificities and is expressed as a 38 kDa precursor with a 24 kDa mature enzyme

expressed in stationary-phase promastigotes and amastigotes. The expression is thought to be controlled at both the mRNA and protein level.

The role of the CPA enzyme was analysed following deletion of the gene by targeted gene disruption and it was shown that despite expression in the mammalian stages of the life-cycle, the virulence of the null mutant (Δ*CPA*) was similar to that of the wild type *in vitro* (Souza *et al.*, 1994). Further analysis *in vivo*, however, demonstrated that if promastigotes were used during infection the formation of lesions was slowed. However, the use of amastigotes restored virulence to wild type levels (Frame, PhD thesis). This suggested that the enzyme may have roles in the virulence of the parasite. More recently, roles have been suggested in the processing of the CPB enzymes (D.R. Brooks, unpublished).

1.4.1.4. CPC.

The CPC enzyme has homology to the mammalian enzyme Cathepsin B (Robertson and Coombs, 1993) and is therefore a Type III Trypanosomatid cysteine proteinase (reviewed by Coombs and Mottram, 1997). It is encoded from a single gene copy and although it is expressed constitutively it was demonstrated that elevated expression occurred within the multiplicative promastigote (Bart *et al.*, 1995).

Analysis of the phenotype of *L. mexicana* lacking in CPC (Δ*CPC*) demonstrated that the infection of the mutant to macrophages *in vitro* was greatly reduced when compared to the wild-type parasites. Further to this, during *in vivo* infections although the lesions appeared at the same time as those caused by wild-type parasites, subsequently they developed at a slower pace (Frame, PhD Thesis).

1.4.1.5. CPB.

The CPB enzymes are expressed from a 19 copy tandem array of genes (see Figure 1.3). Like the other well characterised Type 1 Trypanosomatid cysteine proteinase cruzipain (see section 1.4 for detail), these enzymes are cathepsin L-like in nature (reviewed in Coombs and Mottram, 1997). However, unlike cruzipain they do not retain their C-terminal extension following maturation and there is no evidence for their expression on the surface of the parasite. The evidence to date indicates that they are confined to the lysosomes of the parasite.

The expression of high activity cysteine proteinases from an array of genes was first identified in 1994 (Robertson and Coombs). Further analysis has demonstrated that the isoenzymes are expressed predominantly in the amastigote stage of the life cycle, however gene 1 (*CPB/g1*) and *CPB/g2* are expressed in stationary phase promastigotes. These enzymes are also distinguished by their truncated C-terminal extension, although the significance of this has yet to be established (Mottram *et al.*, 1997). Analysis of the phenotype of parasites deficient in CPB expression (due to the deletion of the entire array by targeted gene

disruption, Mottram *et al.*, 1996) has demonstrated that the null mutant (Δ*CPB*) has a reduced virulence, expressed as an 80% reduction in the survival of the parasite following *in vitro* infection. These analyses also demonstrated that the parasite was able to infect as many macrophages as did wild-type parasites but that it was killed in the majority of those cells, indicating some reduction in the ability to survive intracellularly. Importantly, this reduction in virulence was ablated by re-expression of a single internal copy from the array, *CPB/g2.8*, demonstrating that the reduced survival was indeed due to loss of the *CPB* array as opposed to some other undefined phenomena. Interestingly, this reduced virulence phenotype was only observed when stationary phase promastigotes were used. Infection by amastigotes allowed wild type virulence to ensue, suggesting that the CPB enzymes were necessary in the initial infection stages.

Further analysis of the isoenzymes has demonstrated that they have differences in substrate preferences and differing abilities to restore virulence to Δ*CPB*, indicating that the enzymes may have several different roles within the parasites life-cycle (Mottram *et al.*, 1997).

Analysis of the *in vivo* infection dynamics of Δ*CPB* has demonstrated that lesion formation was delayed by several weeks (Frame, PhD Thesis), again indicating the importance of these enzymes in survival. Interestingly, removal of the *CPA* gene in conjunction with *CPB* (Δ*CPA/CPB*) completely ablates lesion formation (Alexander *et al.*, 1998). An analysis of the immune response of the susceptible mouse strain BALB/c towards infection with either Δ*CPB* or Δ*CPA/CPB* indicated that instead of the potent Th2 type response observed after infection with

wild type parasites, a strong Th1 type response was elicited (Alexander *et al.*, 1998). This switch in response was most apparent after infection with the double null mutant and was observed in several mouse strains of varying susceptibility. Further to this, various mouse strains infected with either Δ*CPB* or Δ*CPA/CPB* also demonstrated varying degrees of resistance to challenge with wild type parasite, suggesting that an analysis of the vaccine potential of these strains may prove interesting.

The above phenomena may be taken to indicate a role for these enzymes in polarisation of the immune response towards an ineffective Th2 type. However, further analysis is required as recent work (Menon and Bretscher, 1998) has demonstrated that for *L. major* infection the numbers of parasites used at the initiation of infection may be important in the polarisation of the immune response. Loss of CPA and CPB causes a reduction in survival of the parasite, therefore it may also reduce the antigenic dose, which could also be responsible for the switch of the immune response.

1.5. Cysteine proteinase inhibitors as potential chemotherapeutics.

The use of cysteine proteinase inhibitors as potential chemotherapeutics against parasitic infections is attractive due to the obvious importance of these enzymes to a variety of pathogenic organisms (see section 1.4). This emphasises that inhibition of these enzymes may allow the host to clear their infection.

The use of cysteine proteinase inhibitors during *in vitro* infection with *T. cruzi* demonstrated that a reduction in intracellular survival and replication was achievable (Franke de Cazzulo *et al.*, 1994). This work has been extended to *in vivo* infection which indicated that treatment of infected mice with vinyl sulphone derivatives with specificity for Cathepsin L-like enzymes brought about the clearance of the parasite from the host (Engel *et al.*, 1998a). A fluoromethyl ketone derivative with the same peptidyl grouping as the vinyl sulphone described above (phenylalanine-homophenylalanine) has also been indicated to have efficacy against infection with the murine malaria *P. vinckei* - resulting in a long term cure rate of 80% (Rosenthal *et al.*, 1993).

Analysis of the effects of cysteine proteinase inhibitors on *Entamoeba* species has demonstrated that a reduction in attachment and subsequent cytotoxicity is possible using $ZnCl_2$ *in vitro* (Franco *et al.*, 1999). Additionally, the use of another fluoromethyl ketone compound during *in vivo* infections with the lizard pathogen, *E. invadens*, has shown that a reduction in the numbers of trophozoites and cysts is possible, further indicating the potential of inhibition of this class of enzymes.

Interest in the development of specific cysteine proteinase inhibitors for use against *Leishmania* stemmed originally from the observations that both promastigote and amastigote growth could be prevented by antipain and leupeptin (Coombs *et al.*, 1982; Coombs and Baxter, 1984). Further to this, the leishmanicidal activity of L-leucine-methyl esters has been linked to the presence of abundant lysosomal cysteine proteinases in *L. mexicana* (Hunter *et al.*, 1992).

Certain concerns exist with regards to the inhibition of cysteine proteinases *in vivo*, due to the abundance of the host cells cysteine proteinases (see section 1.2.1). Inhibition of cathepsins B and L for example may lead to alterations in the expression of antigenic epitopes via MHC-I and -II (Fineschi and Miller, 1997). However, preliminary studies in mice have suggested that limited toxicity occurs to the host, possibly because of the abundance of these enzymes within the host or an enhanced accumulation within the parasite (reviewed by McKerrow, 1999). Indeed, analysis of the effects of vinyl sulphone derivatives on *L. major* infection in mice has indicated that a reduction in lesion growth is possible without obvious toxicity to the host (Selzer *et al.*, 1999).

The use of cysteine proteinase inhibitors during infection with *Leishmania* is attractive therefore for several reasons. Firstly, the enzymes are well documented and abundantly expressed during the mammalian stages of the life cycle (see section 1.4.1). Secondly, the use of targeted gene disruption has demonstrated that these enzymes are important in the virulence both *in vitro* and *in vivo* (Mottram *et al.*, 1996 and Alexander *et al*, 1998). Furthermore the apparent lack of toxicity to the host following treatment with specific inhibitors suggests that inhibition of these enzymes during infection may prove interesting.

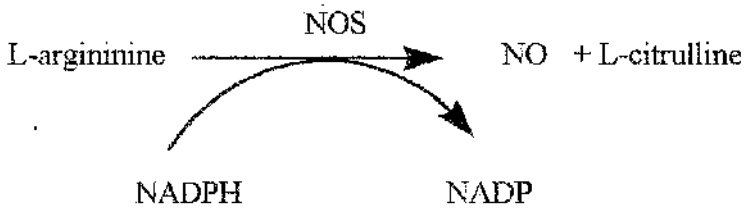

Figure 1.1: The production of nitric oxide from L-arginine. The conversion of NADPH to NADP facilitates the oxidation of the terminal nitrogen atoms from L-arginine by nitric oxide synthase (NOS), producing nitric oxide (NO) and L-citrulline.

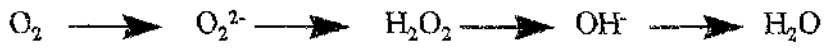

Figure 1.2: The production of reactive oxygen species. The rate of oxygen intake increases greatly after stimulation of macrophages and results in the production of several reactive oxygen species including superoxide (O_2^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) before the production of the stable end product, water.

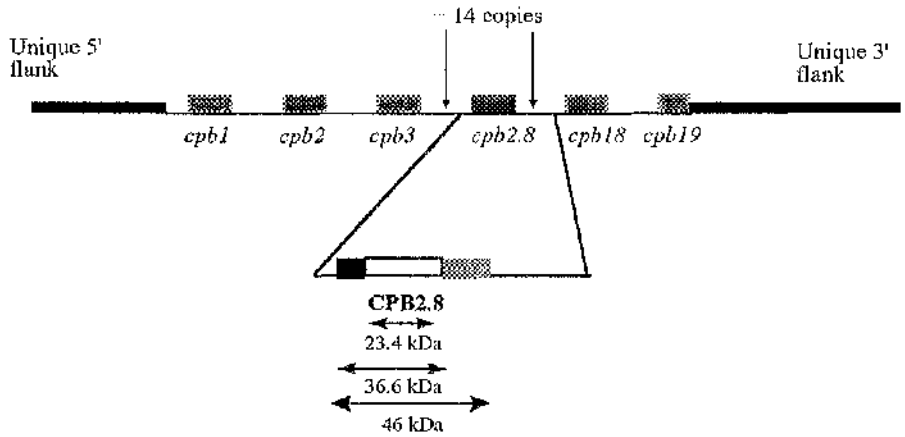

Figure 1.3: A schematic representation of the *CPB* array of cysteine proteinase genes. The array consists of nineteen genes (grey boxes) arranged in 2.8 kb units in tandem. One gene from the middle of the array (*CPBg2.8*) is shown in detail. The relative sizes of the pro-region (black), central domain (white) and C-terminal extension (grey) are indicated.

Aims of the Project

The use of cysteine proteinase inhibitors and the analysis of the phenotypic changes brought about by loss of cysteine proteinase expression has demonstrated that cysteine proteinases are important in the survival and/or pathology of several important parasitic infections. The possibility that exploitation of this importance could allow the development of novel chemotherapeutic agents is attractive, given the low levels of toxicity observed during application of cysteine proteinase inhibitors to infected animals.

This project has stemmed from the observation that the CPB cysteine proteinases of *L. mexicana* are important virulence factors both *in vivo* and *in vitro* (Mottram *et al.*, 1996; Alexander *et al.*, 1998). In order to assess the usefulness of targeting these enzymes *in vivo* it is necessary to have a detailed knowledge of their role(s) in the survival and virulence of the parasite.

The main aims of this project were two-fold:

1) To determine the effects of cysteine proteinase inhibition on the parasite.

This analysis was undertaken to assess the effects of inhibition of the activity of the CPB enzymes brought about by deletion of the *CPB* genes. Further to this, the efficacy of cysteine proteinase inhibitors on the survival of the parasite was assessed

both in axenic culture and during *in vitro* infection, to determine the value of these compounds as putative chemotherapeutic agents and the mechanisms underlying their action.

2) To elucidate the role of the CPB enzymes in intracellular survival of the parasite.

The aim was to elucidate both the mechanisms that resulted in the reduction in intracellular survival observed during *in vitro* infection of null mutants lacking *CPB* and the explanation for the survival of the mutant parasites in just a small percentage of macrophages. It was expected that these findings would provide a better understanding of the role of the CPB enzymes in virulence.

Chapter 2

Materials and Methods

This chapter details general methods used in the study. Specific methods are described in the individual results chapters.

2.1 Materials.

Unless otherwise stated, all chemicals were purchased from Sigma.

2.2. Cell culture techniques.

2.2.1. Handling of *Leishmania mexicana*.

2.2.1.1. Culturing of *L. mexicana* promastigotes.

All culturing and handling of live parasites was undertaken in a class II isolation hood. *Leishmania mexicana* (MNYC/BZ/62/M379) promastigotes were cultured at 25°C with air as the gas phase in HOMEM (Berens *et al.*, 1976) supplemented with 10% (v/v) heat-inactivated foetal calf serum (HI-FCS) and antibiotics as appropriate (see Table 2.1). In the case of the cysteine proteinase null mutants, the promastigotes were grown for a single passage in the presence

of antibiotics and grown thereafter in medium alone. The various mutant lines used are described in Table 2.2. Cultures were grown in 10 ml of medium in Corning polystyrene 25 cm^2 flasks (Corning Costar Corporation). They were initiated at a density of 5 x 10^5 ml^{-1} unless otherwise stated and sub-passaged every 7 days, when a density of 1-2 x 10^7 ml^{-1} had been reached. The parasites were considered to have reached stationary phase when the cell density, measured using an improved Neubauer haemocytometer, had dropped by 10% from the previous day (Mallinson and Coombs, 1989). This usually occurred 8-9 days from initiation of the culture and at this point approximately 90% of the parasites were considered to be metacyclic-like providing low sub-passage promastigotes were used (Mallinson and Coombs, 1986).

2.2.1.2. Culturing of *L. mexicana* axenic amastigotes.

Leishmania mexicana promastigotes were ascertained to be stationary phase as described in section 2.2.1.1 and then washed in 0.25 M sucrose solution by centrifuging the cultures at 750 g for 10 min at 4°C in a Fisons MSE Chilspin. The parasites were then resuspended to a density of 1 x 10^6 ml^{-1} in 10 ml complete SDM (Schneiders Drosophila medium [Gibco BRL] supplemented with 20 % [v/v] HI-FCS, with the pH adjusted to 5.5 with hydrochloric acid) (Bates *et al.*, 1992) in Corning polysyrene 25 cm^2 flasks (Corning Costar Corporation). The low pH of the medium meant that it was not possible to select for the expression of episomal genes in mutant parasites as it prevented the selective pressure of the antibiotics. Thus axenic amastigotes of the re-expressor lines were derived from the appropriate promastigote lines and expression of the genes

was verified by SDS-PAGE (see section 2.7.1.). The cultures were then incubated at 32°C with a gas phase of air and sub-passaged twice weekly through a 21 G needle to break up the clumps of amastigotes.

2.2.1.3. Harvesting of *L. .mexicana.*

The density of the cultures was ascertained using an improved Neubauer haemocytometer and they were then centrifuged at 750 g in 15 ml plastic tubes (Greiner) for 10 min at 4°C in a Fisons MSE Chilspin. The supernatant was removed and the promastigotes were resuspended in 10 ml of 0.25 M sucrose solution. The centrifugation and resuspension step was repeated twice before the pelleted parasites were transferred to 1 ml of the sucrose solution in 1.5 ml eppendorf tubes and centrifuged for 10 min at 8,500 g at room temperature in a MSE microcentaur microfuge. The sucrose solution was removed and the pellet was resuspended to 1 x 10^9 ml^{-1}. This suspension was then aliquoted into eppendorf tubes and diluted to 5 x 10^7 ml^{-1} and re-centrifuged as before. The supernatant sucrose solution was removed and the pellets were stored at -70°C.

2.3. Analysis of the *in vitro* infection dynamics of *L. mexicana*.

2.3.1. Investigation of the infection dynamics in peritoneal exudate cells.

2.3.1.1. Harvesting of peritoneal exudate cells.

Age-matched adult female BALB/c mice were used in all experiments unless otherwise stated. RPMI-1640 (Labtech International) was supplemented with 10% (v/v) HI-FCS, 2 mM L-glutamine, 10 U ml^{-1} penicillin and 10 µg ml^{-1} streptomycin sulphate (Gibco BRL) (the resulting solution was designated complete RPMI). The mice were humanely sacrificed using CO_2 and 5 ml of pre-chilled complete RPMI was injected into the peritoneal cavity using a 25 Gauge needle. The peritoneum was massaged for approximately 5 min before the medium was withdrawn, yielding 3-4 ml of peritoneal exudate cell suspension with approximately 1-2 x 10^6 cells ml^{-1}. The cell density was adjusted to 2 x 10^5 ml^{-1}, unless otherwise stated, using complete RPMI and the cells were incubated in 8 chamber Permanox slides (Nalge Nunc) overnight at 32°C in 95% air/5% CO_2 to allow the macrophages to adhere. The slides were then washed in complete RPMI to remove any un-adhered cells.

2.3.1.2. Infection of peritoneal exudate cells.

The adhered cells (designated macrophages) were infected with stationary phase promastigotes using a 1:1 macrophage:promastigote ratio and incubated at 32°C in 95% air/5% CO_2 for 4 h to allow the parasites to attach. Subsequent washing with complete medium removed any unattached promastigotes and the slides were re-incubated as desired. At selected time points, the medium was removed and the wells were washed with complete medium and air dried before being fixed in methanol for 2 min. The fixed macrophages were stained in Giemsa's stain for 15-20 min and washed in double distilled, de-ionised water (d^3H_20) before examination. Approximately 200 macrophages were counted and

the infection rate (percentage macrophage infected) recorded. The number of amastigotes per infected cell was also determined.

2.3.2. Elicitation of peritoneal macrophages.

4% (w/v) thioglycollate solution was made up in water with heating until boiling, autoclaved and stored at room temperature. Female adult BALB/c mice were injected intraperitoneally with 1 ml of the solution 4 days prior to harvesting the cells as described in section 2.3.1.1. (Mackawa *et al.*, 1998).

2.3.3. Treatment of BALB/c mice with FLT3L.

FLT3L is a haemopoietic stem cell factor which binds to a receptor on cells in the bone marrow of treated individuals to cause an increase in the numbers of functionally mature dendritic cells in various tissues within the body (Maraskovsky *et al.*, 1996).

In order to investigate the potential role of dendritic cells as host cells for *Leishmania* promastigotes, adult female BALB/c mice were injected intraperitoneally with 10 μg of FLT3L daily for 10-12 days before harvesting as described in section 2.3.1.1. The FLT3L treatment was carried out on my behalf by Dr. Allan Mowat (Department of Immunology, University of Glasgow) according to the procedure outlined in Viney *et al.*, 1998.

2.4. Investigation of the uptake of *Leishmania mexicana* by macrophages *in vitro*.

The early stages of the infection process were analysed in order to determine if the attachment and phagocytosis of the wild type parasites differed from that of Δ*CPB*. The macrophages were harvested and incubated overnight as described in section 2.3.1.1. before being washed in complete medium and infected with stationary phase promastigotes or axenic amastigotes at a 1:1 ratio. At the desired time points, the medium was removed and the macrophages were washed with complete medium before being fixed for 30 min in 1% (v/v) paraformaldehyde in phosphate buffered saline (0.07 M sodium chloride/0.075 M disodium hydrogen orthophosphate dihydrate/5 mM sodium dihydrogen orthophosphate, designated PBS) (methanol was not used as a fixative as it was considered to be too harsh and may have caused dissociation of attached parasites). The slides were then air-dried and stained with Giemsa's stain (see section 2.3.1.2.) before analysis. Approximately 200 macrophages were analysed per time point and the numbers of parasites attached and inside these cells were recorded separately.

2.5. Investigation of the response of host cells to infection.

2.5.1. Detection of nitric oxide production.

The production of nitric oxide by macrophages during infection with *L. mexicana* was measured using the Greiss reaction (modified from Kolb *et al.*, 1994) to detect the nitrite produced during nitric oxide degradation. The production of nitric oxide by resident peritoneal exudate cells was found to be

undetectable, hence elicited macrophages were used routinely in these experiments.

Macrophages were harvested and infected as described in sections 2.3.1.1. and 2.3.1.2. and stimulated with 40 U ml^{-1} Interferon-γ (IFN-γ) and 10 ng ml^{-1} lipopolysaccharide (LPS, *Salmonella typhimurium*) after 4 h incubation as described by Proudfoot *et al.* (1995). At the given time points, the supernatants were removed and stored at -20°C until analysis.

The standard curve was prepared, using doubling dilutions in d^3H_2O in a flat-bottomed 96-well plate (Greiner labortechnik) from a 14 mM sodium nitrite stock with a maximum concentration of 250 μM. Stored supernatants were defrosted at room temperature, before addition of 100 μl to the plate, in duplicate. 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid was added to 0.01% (w/v) alpha-naphthylamine at a 1:1 ratio and 100 μl was added to each well. The plate was incubated at room temperature for 5-10 min before the absorbance at 540 nm was measured using a Titertek multiskan MCC/340.

2.5.2. Investigation of oxidative burst of infected macrophages.

2.5.2.1. Measurement of oxidative burst of infected macrophages in response to a secondary stimulus.

The response of infected cells with respect to the production of high energy oxygen compounds was investigated using chemiluminometry (Easmon *et al.*, 1980). The assay relies on the oxidation of the cyclic hydrazide 5-amino-2,3-

dihydro-1,4-phthalazinedione (luminol) by reactive oxygen species to produce an increase in the amount of light emitted. This change in emitted light was measured using a luminometer (LKB Wallac) which was set to a standard temperature of 37°C.

Macrophages were harvested as described previously in section 2.3.1.1 but were allowed to adhere to the base of cylindrical plastic measuring cuvettes (Clinicon) by adding them in suspension at a concentration of 1×10^6 ml^{-1}. After adherence overnight, the cells were infected for 1 h at a 1:1 ratio of promastigotes before washing in Hanks buffered saline solution (HBSS, Gibco BRL) and addition of 400 µl HBSS to the tubes.

0.01 M luminol stocks were prepared in DMSO and stored in the dark at -20°C. During experimentation, a secondary stock of 0.5 mM was prepared in Hank's buffered saline solution (HBSS, Gibco BRL) and pre-heated to 37°C. The background emissions occurring during experimentation were measured after addition of 100 µl luminol (final concentration of 0.1 mM) for 1 min before the addition of 100 ng ml^{-1} phorbol myristate acetate (PMA). Measurements were then recorded every 10 sec for 10-15 min.

2.5.2.2. Measurement of respiratory burst of macrophages in response to infection.

The macrophages were harvested, adhered overnight and washed as described in section 2.3.1.1. The background light emission was detected by addition of 100 µl of a 0.5 mM luminol stock solution (see section 2.5.2.1) to the tubes and measurement for 1 min using a luminometer (LKB Wallac). The

medium was then removed and replaced with 400 µl of parasite culture at 2.5 x 10^6 promastigotes or amastigotes ml^{-1} (to give a macrophage:parasite ratio of 1:1) to which 100 µl of luminol was added. The emission of light as the parasites were phagocytosed was then measured as before. The parasite cultures were supplemented with 40 U ml^{-1} IFN-γ to produce an activation response from the host cells.

2.6. Investigation of the receptors involved in *L. mexicana* promastigote attachment to macrophages.

2.6.1. Characterisation of the receptor binding assay.

The inhibition of binding of wild type *L mexicana* promastigotes to various receptors was analysed by using increasing concentrations of antibody specific to that receptor in order to determine the minimum antibody concentration required for the maximum inhibitory effect. Anti-CR3 antibodies (rat anti-mouse CD11b IgG2b) were purchased from Serotec, anti-CR4 antibodies (hamster anti-mouse CD11c IgG1) was purchased from Pharmingen, both were stored according to the manufacturer's instructions. The inhibitory effects of these antibodies were compared with the effects of using control antibodies of the same isotype to ensure that any inhibitory effect observed was due to the blocking of the receptor and not merely due to the binding of the Fc region of the antibodies to their relevant Fc receptor on the macrophage surface. The isotype control antibodies, purified mouse IgG2b and rat IgG1, were purchased from Pharmingen and Serotec, respectively.

Macrophages were harvested and incubated overnight as described in section 2.3.1.1, before being pre-incubated in the presence of the appropriate antibody type at the desired concentration (in complete medium) at 4°C for 30 min (adapted from Zaffran *et al.*, 1998). The experiments were carried out at 4°C to ensure that the cross linking of the receptors did not cause any downstream signalling effects within the macrophages and thus alter the system being investigated more than necessary. After preincubation, the macrophages were washed once in chilled medium and infected with wild type stationary phase promastigotes, in medium containing the appropriate antibody concentration, at a 10:1 ratio (promastigote:macrophage) and incubated for a further 30 min.

After the final incubation, the medium was removed and the macrophages were washed again in chilled complete medium before fixing for 30 min at 4°C with 1% (v/v) paraformaldehyde in PBS (see section 2.4). The slides were then stained with Giemsa's stain as described in section 2.3.1.2 and the numbers of promastigotes attached to approximately 200 macrophages was determined by microscopic observation. The numbers of promastigotes attached per 100 cells in the presence of the isotype control was compared with that of the relevant experimental antibody in order to determine the appropriate concentration to use.

2.6.2. Analysis of the effects of various receptor-specific antibodies on the attachment of *Leishmania mexicana* to the surface of macrophages.

The optimal antibody concentration was determined as described in section 2.6.1 and the macrophages were harvested and incubated overnight as described in section 2.3.1.1 before pre-incubation at 4°C for 30 min in the desired

antibody concentration. The macrophages were then infected using a ratio of 10 stationary phase promastigotes per macrophage in the appropriate antibody concentration and incubated at 4°C for 30 min. After incubation, the cells were washed in chilled complete medium before being fixed for 30 min at 4°C using 1% (v/v) paraformaldehyde. The slides were than stained with Giemsa's stain and analysed as detailed in section 2.3.1.2.

2.6.3. Analysis of the role of the mannose receptor in promastigote attachment.

Peritoneal exudate cells were harvested and incubated overnight as described in section 2.3.1.1. The macrophages were then washed in complete RPMI before incubation for 30 min at 4°C in various concentrations of mannan (Blackwell *et al.*, 1985). The macrophages were then washed in complete RPMI and infected as described in section 2.6.2. After 30 min, the macrophages were washed, fixed and stained as described in section 2.6.1 and the numbers of promastigotes attached per 100 cells was assessed microscopically.

2.7. Polyacrylamide gel electrophoresis.

SDS-PAGE using 10% acrylamide gels was used for investigating CPB cysteine proteinases and 7.5% acrylamide gels for analysis of gp63, unless otherwise stated. Pelleted promastigotes were suspended to a density of 1×10^9 ml^{-1} and lysed immediately prior to use by addition of 0.25% (v/v) Triton X100 in 0.25 M sucrose and gentle mixing using a Gilson pipette tip. Lysates were

kept on ice to reduce auto-hydrolysis and re-frozen at -70°C after use. They were discarded after they had been thawed and used a second time.

2.7.1 Substrate SDS-PAGE.

SDS-PAGE gels co-polymerised with 0.2% (w/v) gelatin were used to analyse the activity of the *CPB*-encoded enzymes and gp63 (Lockwood *et al.*, 1987). 10 µl of lysate was mixed with 10 µl of sample buffer (0.1 M Tris HCl pH 6.8, 15% (v/v) glycerol, 3% (w/v) SDS, some bromophenol blue) and microfuged in a MSE microcentaur for 30 seconds at 8,500g before loading the supernatant to the gel. The gels were run at 150 V for about 1 h before being washed in 2.5% (v/v) Triton X100 at 37°C to remove the SDS and reactivate the enzymes. The gels were then incubated at 37°C in 0.1 M sodium acetate, pH 5.5, with 1 mM dithiothreitol (DTT) to allow hydrolysis of the gelatin. Subsequent incubation of the gels overnight in Coomassie Blue stain (0.58 mM Coomassie Brilliant Blue G R-250 in 10% [v/v] acetic acid and 12.5% [v/v] i-propanol) allowed the gelatin matrix to become stained and this was then destained in 10% (v/v) acetic acid with 12.5% (v/v) methanol. Results were recorded on an Appligene gel imager.

2.8. Immuno-blotting.

2.8.1. Western blotting.

20 µl of parasite lysate was mixed with 20 µl of sample buffer (see above) and boiled for two min to allow protein denaturation before samples were loaded onto the gel. The gels were run at 200 V for approximately 45 min before being transferred to a nitro-cellulose membrane (Amersham Life Science) in 20 mM Tris/150 mM glycine/20% (v/v) methanol at 100 V for 45 min (Bio-rad mini-transblotter). The membranes were blocked overnight at 4°C in 5% (w/v) milk powder in Tris-buffered saline (20 mM Tris base pH 7.6/137 mM sodium chloride/3.8% [v/v] HCl [designated TBS] pH 7.6) with 0.1% Tween 20 and 0.1% gelatin.

After blocking, the membrane was rinsed in TBS pH 7.6 with 0.1% Tween 20 and 0.1% gelatin (designated wash buffer) and the primary antibody was applied for 4 h at the appropriate concentration in wash buffer and at an appropriate temperature (see Table 2.3). The blots were then washed at room temperature in wash buffer three times for 10 min, each with vigorous shaking. The appropriate secondary antibody (obtained from SAPU) was applied at room temperature for 1 h at the manufacturer's recommended concentration and developed, using Pierce chemiluminescence reagents and Kodak's developer and fixer (1 in 5 dilutions) onto Amersham's ECL film.

2.9. The separation of cytoplasmic and membrane proteins.

2.9.1. Phase separation using Triton X114.

A solution of the non-ionic detergent, Triton X114, is homogeneous at 0°C but as the solution is heated to 20°C the micellar molecular weight increases

and causes the solution to turn 'cloudy'. There is a microscopic phase separation in the solution, which proceeds with increasing temperature until two clear phases are achieved. This property facilitates it's use in the separation of cytoplasmic proteins from integral membrane proteins. The hydrophilic nature of cytoplasmic proteins ensures that they remain in the aqueous phase as the detergent begins to separate, whereas the amphiphilic nature of integral membrane proteins keeps them in the form of micelles within the detergent itself (Bordier, 1981).

1 x 10^8 *L. mexicana* promastigotes were lysed in 100 µl double distilled, de-ionised water (to give a density equivalent to 1 x 10^9 ml^{-1}) and added to 100 µl of ice cold 4% (v/v) Triton X114 in PBS (see section 2.4). After incubation for 1 h on ice, the eppendorfs were microfuged at 6,500g for 1 h at 4°C in a MSE microcentaur microfuge, to remove any insoluble material. The soluble fraction was then overlaid on a 300 µl 6% (w/v) sucrose in PBS cushion and warmed for 10 min at 30°C. This was followed by a microfugation step at 100g for 10 min at room temperature after which the detergent phase was located under the sucrose cushion. 200 µl of the aqueous phase was removed and 1 µl of Triton X114 was added to give a final concentration of 0.5% (v/v) Triton X114. The aqueous phase was incubated on ice for 10 min and then overlaid back onto the sucrose cushion and re-incubated at 30°C for 10 min. Microfugation at 100g for 10 min at room temperature separated the detergent and the aqueous phase further. The aqueous phase was removed and 3 µl of Triton X114 was added. After removal of the sucrose cushion, the detergent phase was diluted with PBS to give a final

volume of 100 µl. The resultant solutions were then stored at -20°C until required.

The high levels of Triton X114 in the detergent phase of the extraction can cause distortion in the banding patterns observed during electrophoresis. To reduce the effects of this distortion, the proteins in this phase were extracted from the detergent by incubating in 1 ml of acetone for 1 h at -20°C. After this, the samples were centrifuged at 100g for 10 min before the supernatant (acetone/detergent mix) was discarded and the sedimented proteins were resuspended in 200 µl PBS.

2.10. Cleavage of GPI anchors from membrane proteins.

2.10.1. The use of phosphatidyinositol phospholipase C (PIPLC).

The detergent phase of Triton X114-extracted promastigote lysates (see section 2.9.1) were treated with phosphatidyinositol phospholipase C (PIPLC) from *Bacillus thuringiensis* (Oxford Glycosystems) to cleave off the GPI anchors from membrane-associated proteins. After acetone extraction (see section 2.9), the proteins were resuspended in 7 U ml^{-1} of PIPLC diluted in 50 µl of 25 mM Tris acetate (pH 7.4) with 0.1% (w/v) sodium deoxycholate and incubated for 60 min at 37°C (Masterson *et al.*, 1989). The cleaved proteins were then extracted from the detergent phase by mixing 100 µl with 100 µl 4% (v/v) Triton X114 in PBS (see section 2.4) and overlaying onto a 6% (w/v) sucrose in PBS cushion followed by incubation at 30°C for 10 min and centrifugation at 100g for a

further 10 min. The cushion was then discarded and the aqueous phase proteins (from above the cushion) and the membranous phase proteins (from below) were stored at -20°C. The extractions were examined using western blotting (see section 2.8.1) and substrate SDS-PAGE (see section 2.7.1). The low levels of protein retrieved during extraction meant it was necessary to increase the incubation step of the substrate SDS-PAGE procedure from 2 h to overnight.

2.11. Immunofluorescence techniques.

2.11.1. Immunofluorescence of *L. mexicana* promastigotes.

Gp63, the surface zinc metalloproteinase of *L. mexicana* promastigotes, was visualised via immunofluorescence using specific anti-*L. mexicana* gp63 monoclonal antibodies, supplied by D.G. Russell (see Medina-Acosta *et al.*, 1989). Promastigote cultures where centrifuged at 750g for 10 min at 4°C in an MSE Chilspin and re-suspended to 1 x 10^6 cells ml^{-1} in 100 µl of 2.5% (v/v) paraformaldehyde in PBS (see section 2.4) and incubated for 15 min to allow fixation to occur. The fixed promastigotes were then washed in 100 µl PBS by microfugation at 500g for 10 min at room temperature before being resuspended in 100 µl PBS, pH 7.4, containing a 1:200 dilution of the primary antibody and incubated at room temperature for 30 min. The promastigotes were again washed in PBS before blocking in 100 µl PBS, pH 7.4, with 10% (v/v) HI-FCS at room temperature for 15 min. The secondary antibody, sheep anti-mouse IgG-FITC conjugated (SAPU), was applied at 1:100 dilution for a further 30 min

at room temperature. The promastigotes were then washed a further two times as described above and resuspended in 20 µl of PBS, pH 7.4, and air dried onto microscope slides for examination. Coverslips were mounted on the preparations using 90 g glycerol dissolved in 10 ml PBS at pH 7.1 (Heimer and Taylor, 1974). The fluorescence was visualised using a Zeiss Axioskop Fluorescent microscope.

Table 2.1: Antibiotics used routinely in the culturing of *L. mexicana* mutants.

L. mexicana	Antibiotic used	conc ($\mu g\ ml^{-1}$)
wild type*	gentamycin (Sigma)	25
various null mutants#	hygromycin B	50
	phleomycin (both Sigma)	10
CPB re-expressors	Geneticin (GibcoBRL)	500

* approximately two years into the investigation gentamycin was found to have some apparent adverse effects on the infectivity of the promastigotes and it was therefore withdrawn from use.

after an initial passage in hygromycin B and phleomycin, the various null strains were cultured as for wild type parasites.

Table 2.2: The mutant lines of *L. mexicana* used in this investigation.

Name	Description
Δ*CPB*	mutant line lacking the *CPB* gene array
Δ*CPB/gx*	Δ*CPB* re-expressing a gene from within the *CPB* array e.g. Δ*CPB/g1*, re-expresses gene 1.
Δ*CPA*	mutant line lacking the *CPA* gene
Δ*CPA/CPB*	mutant line lacking both *CPA* and the *CPB* array
Δ*GPI8*	mutant line lacking the *GPI8* gene
Δ*GPI8/gpi8*	Δ*GPI8* re-expressing the *GPI8* gene.

Table 2.3: The concentration of primary antibodies used during western blotting.

Antigen	Antibody	Dilution	Temp. (°C)
CPB	Rabbit anti-type C CP IgG[#]	1/1000	4
CPA	Rabbit anti-CPA IgG[@]	1/1000	4
gp63	Mouse anti-*L.major* gp63 IgG[*]	1/20	18
gp63	Mouse anti-*L.mexicana* gp63 IgG[$]	1/200	18

[#] Polyclonal antibody, for details see: Robertson and Coombs, 1994. The antibody was raised against a member of the CPB array which had been designated a type C cysteine proteinase.

[@] Polyclonal antibody, for detail see: Mottram *et al.*, 1992.

[*] Monoclonal antibody, supplied by W.R. McMaster, for details see: Frommel *et al.*, 1990.

[$] Monoclonal antibodies, supplied by D.G. Russell, for details see: Medina-Acosta *et al.*, 1989.

Chapter 3

The effects of peptidyl cysteine proteinase inhibitors on cysteine proteinase expression in *Leishmania mexicana*

3.1. Introduction.

Inhibition of leishmanial cysteine proteinases has been postulated for a long time to be a possible route for the design of chemotherapeutic agents (McKerrow, 1999). Antipain was shown to inhibit, at concentrations as low as 1 mg L^{-1}, the growth of promastigotes and amastigotes *in vitro* (Coombs and Baxter, 1984). The anti-leishmanial effects of L-leucine methyl esters towards amastigotes (application of these compounds leads to swelling of the parasite's lysosomes, apparently due to the accumulation of amino acids causing osmotic stress, and subsequent parasite lysis) was demonstrated to be due to the presence of abundant cysteine proteinase activity in the megasomes of the parasite (Hunter *et al.*, 1992). More recently, the work of Mottram *et al.* (1996) demonstrated that mutants of *L. mexicana* lacking the *CPB* genes (Δ*CPB*) have a reduced ability to survive in macrophages. These results further indicated the validity of using specific inhibitors of the parasite's cysteine proteinases as anti-leishmanial agents.

Two *L. mexicana* cysteine proteinases (CPA and CPB) are expressed abundantly in the amastigote stage of the life-cycle. They have been shown to have roles in parasite virulence, either *in vitro* or *in vivo* (Souza *et al.*, 1994;

Mottram *et al.*, 1996; Alexander *et al.*, 1998). Both of these enzymes are localised to the large lysosomal vesicles of the amastigote, the megasomes (Pupkis *et al.*, 1986). This suggests that they have some role in nutrition or cellular protein catabolism, as demonstrated for cathepsin L itself (Mayer and Doherty, 1986). Both CPA and CPB are discussed in further detail in Chapter 1 (sections 1.4.1.3 and 1.4.1.5).

Cathepsin L is a lysosomal enzyme. Similarly to the cysteine proteinases of *L. mexicana*, it is expressed as a pre-pro-enzyme which is processed to the active form by a series of steps during trafficking to the lysosomes of the cell (reviewed by Kornfeld, 1987). The pre-pro-enzyme is produced in the rough endoplasmic reticulum (RER) of the cell and the pre-region of the enzyme is involved in allowing the protein to be released into the lumen of the RER. After localisation in the lumen, a phosphotransferase enzyme specifically catalyses the production of mannose-6-phosphate residues on the protein (this is a common phenomenon of all mammalian lysosomal enzymes studied to date). This facilitates the binding of the protein to specific mannose-6-phosphate receptors and its subsequent trafficking to the lysosomes. Mutational studies (Tao *et al.*, 1994) and analysis of the 3D structure of cathepsin L (Coulombe *et al.*, 1996) have demonstrated that this phosphorylation occurs on residues located in the pro-region of cathepsin L, indicating an important role for the pro-region in transport to the lysosomes.

Analysis of the effects of recombinant cathepsin L pro-region on the activity of the mature enzyme indicates that the pro-region is a potent inhibitor of the activity (Carmona *et al.*, 1996). Investigation of the 3D structure of the pro-enzyme (Coulombe *et al.*, 1996) has shown that the pro-region overlays the

active site cleft of the enzyme, interestingly in the opposite direction to substrate. The removal of the pro-region occurs within the lysosomes themselves (Ishado *et al.*, 1998), via a multi-step process involving cleavage of the majority of the pro-region to give an active enzyme followed by a step-wise removal of small peptide sequences until the mature form is left. This activation can occur via autocatalysis at low pH and a role for intermolecular processing was also recently shown (Menard *et al.*, 1998).

Several differences have been reported between the processing and trafficking of cathepsin L and the cathepsin L-like enzymes of *Leishmania*. The C-terminal extension of cathepsin L, for example, has been implicated in the localisation of the enzyme within lysosomes; mutation of this region leads to the secretion of the enzyme from the cells (Chauhan *et al.*, 1998). However, the enzymes expressed from the first two genes within the *CPB* array of *L. mexicana* (CPB/G1 and CPB/G2) have a truncated C-terminal extension but are nevertheless expressed within the promastigotes lysosomes (Mottram *et al.*, 1997). Furthermore, recent evidence has suggested that the C-terminal extension of the CPB enzymes may be involved in determining the substrate specificities of different isoenzymes (D.R. Brooks, unpublished) and analysis of the role of the pro-region in trafficking of leishmanial enzymes has suggested that it is sufficient for targeting to the lysosome. (Huete-Perez *et al.*, 1999).

Another noteworthy difference is that the production of mature CPB enzyme from the inactive pro-enzyme is not exclusively due to autocatalysis and self-processing events, as it has been recently demonstrated that CPA can effect CPB processing (D.R. Brooks, unpublished). However, the pro-region of the

CPB is also removed via a multistep process similar to that demonstrated for cathepsin L (Sanderson *et al.*, 1999).

Investigation of the trafficking of a *L. major* lysosomal cysteine proteinase (structurally a cathepsin B-like enzyme, but with cathepsin L-like substrate specificity) via the use of inhibitors has demonstrated that when processing is inhibited the pro-enzyme accumulates in the flagellar pocket of the parasite (Selzer *et al.*, 1999). Similar studies with *L. mexicana* using the expression of inactive CPB/G2.8 have also demonstrated a build-up of precursors in this organelle (D.R. Brooks, unpublished). This information has been interpreted to indicate that the trafficking of lysosomal enzymes in *Leishmania* occurs via a unique pathway involving the flagellar pocket.

The apparent, and possible, differences between the processing and trafficking of the cathepsin L-like enzymes of *Leishmania* and host enzymes suggested that these processes of the parasite warranted further study. The investigations detailed in this chapter involved the analysis of the effects of cysteine proteinase inhibitors on the expression of the CPA and CPB enzymes of *L. mexicana* promastigotes. The rationale was that inhibition of parasite cysteine proteinases would enable analysis of the enzymes involved in cysteine proteinase processing and the necessity of this for trafficking. This study was carried out prior to many of the recent publications detailed above. Unfortunately, some of the findings described here have now been demonstrated by other means and reported elsewhere.

3.2. Materials and Methods.

3.2.1. Preparation of inhibitor stock solutions.

Three N-terminally blocked, dipeptidyl cysteine proteinase inhibitors were analysed during this investigation: A vinylsulphonebenzene and an oxycoumarin each with the peptidyl group morpholine-urea-phenylalanine-homophenylalanin (termed KO2 and P87, respectively, see Engel *et al.*, 1998a) and a diazomethane with a benzyloxycarbonyl-phenylalanine-alanine group (termed ZFA). KO2 and P87 were a gift from Professor Jim McKerrow (San Francisco) and ZFA was purchased from Sigma Chemicals. Stock solutions of the inhibitors were made at 5 mg ml^{-1} (approximately 9 mM) in dimethylsulphoxide (DMSO) and stored until required at -20°C.

3.2.2. Use of substrate SDS-PAGE to study the effect of cysteine proteinase inhibition on activity of the CPB enzymes.

L. mexicana promastigotes were cultured in the presence of 18 μM inhibitor as described in Chapter 2 (section 2.2.1.1) and the growth rate was monitored from day 7 onwards until the parasites had reached stationary phase. They were then harvested (see Chapter 2, section 2.2.1.3) with the addition of two extra washes in 0.25 M sucrose to remove unbound inhibitor. The CPB enzymes were analysed using their ability to hydrolyse gelatin after substrate SDS-PAGE (see Chapter 2, section 2.7.1). The effects of adding the inhibitors

to control lysates was also investigated to distinguish the effects caused by the inhibitors during growth of the parasite from those occurring during the assay itself.

3.2.3. Western blot analysis of the effects of cysteine proteinase inhibition on CPA, CPB and gp63.

Western blotting was performed using anti-sera specific for either the type C enzymes within the CPB array (Robertson and Coombs, 1994) or CPA (Mottram *et al.*, 1992) (designated anti-CPB or anti-CPA, respectively). Anti-serum specific for *L. major* gp63 (a gift from Professor Robert McMaster, University of Vancouver) was also used. Western blotting was performed as described in Chapter 2, section 2.8.1.

3.2.4. Electron microscopic analysis of the effects of cysteine proteinase inhibition upon the ultrastructure of the parasite.

The electron microscopy was performed on my behalf by Dr. Laurence Tetley and Anneke Van Sluisveld. Briefly, the promastigotes were grown to late log phase in the presence of 18 μM KO2 before being centrifuged at 1000 g for 10 min. The pelleted cells were then fixed at 20°C as follows: 2.5% (v/v) gluteraldehyde in 0.1 M phosphate buffer for 40 min; 1% (v/v) aqueous osmium tetroxide for 1 h followed by washing in d^3H_2O; finally 0.5% (v/v) aqueous uranyl acetate for 30 min. This fixation was followed by dehydration using 10 min incubations in 30, 50, 70, 90 and 100 % ethanol. The cells were then

embedded overnight in Spurrs epoxy resin at 20°C. The freshly embedded material was polymerised at 60°C for 24 h in silicon block moulds before 60 nm sections were taken. These sections were stained with uranyl and lead solutions and examined using a LEO EFTEM electron microscope at 80 kV.

3.2.5. Analysis of the enzyme class of the gelatinase activity induced by growth of promastigotes with cysteine proteinase inhibitors.

The class(es) of enzyme(s) responsible for the extra activity was investigated using substrate SDS-PAGE. The gels were processed as described in Chapter 2 (section 2.7.1) but each lane was incubated separately and inhibitors from the different classes of proteinases added. The inhibitors used were: E64 (100 μM), an irreversible cysteine proteinase inhibitor; 1,10-phenanthroline (10 mM), an inhibitor of metalloproteinases; aprotinin (10 μM), a serine proteinase inhibitor; and pepstatin (1 μM), which inhibits aspartic proteinases. All the inhibitors were made into appropriate stock solutions as instructed by the manufacturer and stored at -20°C until required.

3.3. Results.

3.3.1. The effects of cysteine proteinase inhibitors on the expression of CPB isoenzymes in stationary phase promastigotes.

Addition of the inhibitors to *L. mexicana* promastigotes did not inhibit growth to stationary phase (see Chapter 5) but had effects on the expression and

activity of the CPB isoenzymes. *L. mexicana* contains several gelatinase activities (Figure 3.1A). The two slower mobility bands of approximately 50 - 60 kDa are thought to be due to the activity of the surface metalloproteinase, gp63. The two faster mobility bands of around 24 kDa are due to the activity of the CPB enzymes expressed from genes 1 and 2 (CPB/G1 and CPB/G2). The addition of the inhibitors to control lysates demonstrated that they inhibit the activity of the CPB enzymes but not that of gp63. Furthermore, it was demonstrated that KO2 was less effective against these isoenzymes than the other inhibitors (see section 3.4).

The presence of the inhibitors during the growth of promastigotes to stationary phase had several effects on the enzyme activity of the cells. The activity of CPB/G1 and CPB/G2 could not be detected and there was the appearance of intermediate mobility bands. The disappearance of CPB/G1 and CPB/G2 activities from promastigotes grown the presence of the inhibitors could be explained in one of two ways. The inhibitors may simply be binding into the active site of the enzyme and thus preventing the activity of the mature form of the enzyme. Alternatively, the effects of the inhibitors on the CPB enzymes or other cysteine proteinases within the parasite may inhibit the processing of the precursor forms and prevent the production of the mature enzyme. This would be likely to lead to an increase in the number/activity of precursor forms observed on gelatin SDS-PAGE (Figure 3.1A).

This phenomenon was investigated using western blotting. The CPB proteins were detected using specific anti-serum (Figure 3.1B). When promastigotes were grown in the presence of ZFA, P87 or KO2, the mature enzyme was absent and there was a concomitant increase in the number of lower

mobility bands detected by the anti-serum. This suggests that there was a blockage in the processing of the CPB enzymes in the presence of inhibitors. These bands were demonstrated to be due to specific recognition by the antibody as they were not recognised by pre-immune serum (data not shown). The results show that the use of inhibitors resulted in an accumulation of higher molecular mass forms of the CPB proteins. Interestingly, the number of intermittent bands on the western blots (Figure 3.1B) does not correspond to the number of novel activity bands observed by gelatin SDS-PAGE (Figure 3.1A).

It was not unlikely that the compounds used were inhibiting particular isoenzymes of CPB or indeed cysteine proteinases other than CPB. To investigate this possibility, it was decided to study the effects of Δ*CPB* expressing individual CPB genes. Mutants expressing the isoenzymes encoded by a metacyclic specific gene, *CPB/g1* and two amastigote-specific genes, *CPB/g18* and *CPB/g2.8*, were analysed (Figure 3.2 A-C). In all cases, the inhibitors prevented the activity of the highest mobility band (the mature enzyme). Interestingly, differences between the actions of the three inhibitors were apparent. ZFA and KO2 prevented the activity of the mature enzymes but there was no evidence for increased activity of intermediate bands (although there was increased activity of the lowest mobility bands). P87, however, gave rise to at least one intermediate mobility activity band. These results suggested that the different inhibitors are interfering with different stages in the enzyme's maturation. These results also suggest that there are differences in the molecular mass of the main active precursor band between CPB/G1 and CPB/G2.8/CPB/G18.

The CPB proteins present in these mutant lines exposed to inhibitors were also analysed using Western blotting (Figure 3.3). Similar effects were observed to those described for wild-type promastigotes. In general, the inhibitors prevented the production of the mature protein and led to an increase in the number and amount of high molecular mass forms detected. CPB/G1 expression in the presence of P87 resulted in a huge build-up of higher molecular mass forms, much greater than when KO2 was used. However, some of these bands were also detected by the pre-immune serum suggesting that P87 is effecting expression and/or processing of other non-CPB proteins. Again, the presence of higher molecular mass forms without gelatinase activity was observed (compare Figures 3.2 and 3.3 for *CPB/g18* and *CPB/g2.8*), indicating differences between the action of these inhibitors when compared with each other and also when acting on different isoenzymes.

During the investigation detailed, the lysates used were produced using equivalent cell numbers. However, no estimation of the protein concentration of these lysates was undertaken. Analysis of the western blots probed with pre-immune serum (Figure 3, parts D-F) indicated that the amount of non-specific protein detectable via this method is approximately equal between lanes confirming that the effects observed were due to growth of the parasites in the inhibitors rather than uneven protein loading.

The evidence that inhibition of cysteine proteinases prevents the production of mature cysteine proteinase enzymes confirms that the processing of the CPB enzymes is at least partially due to catalysis by cysteine proteinases. Work investigating the processing of the CPB enzymes by expressing inactive CPB/G2.8 in Δ*CPB* and Δ*CPA/CPB*, has recently demonstrated that the CPB

isoenzymes can self process but that the other metacyclic/amastigote-specific cysteine proteinase of *L. mexicana* (CPA) can also carry out the processing (D.R. Brooks, unpublished). Thus it would be predicted that cysteine proteinase inhibitors would prevent the formation of the mature CPB enzymes by either inhibition of the CPB isoenzymes themselves or by their effect on CPA. This was investigated by western blotting.

The effect of the inhibitors on the processing of CPB/G2.8 and CPB/G2.8ASM (a mutant of CPB/G2.8 in which the active site cysteine as been removed) in Δ*CPB* and Δ*CPA/CPB* was analysed (Figure 3.4). The expression of CPB/G2.8 within Δ*CPB* resulted in a considerable amount of the higher molecular mass forms of the enzyme in addition to the formation of mature protein (Figure 3.4, lane 1). These higher molecular mass forms are shown to be encoded by *CPB/g2.8* by their absence from Δ*CPB* (lane 2). CPB/G2.8ASM was processed to the mature form of the protein within Δ*CPB* (lane 3), demonstrating that other enzymes can effect processing of the CPB enzymes. The lack of high molecular weight precursor forms in this case is probably due to variation in the amount of protein expression between mutant lines. The proteins are expressed from episomally located genes using antibiotic pressure, thus variation in expression does occur.

Re-expression of CPB/G2.8ASM in the presence of KO2 resulted in a build-up of precursor forms along with the production of a small amount of the mature protein (lane 4). The small amount of mature enzyme produced is probably due to the slow acting nature/relative poor efficacy of KO2 (see Figure 3.3). This is demonstrated by comparison with the result of expressing

CPB/G2.8ASM in Δ*CPA/CPB* (lane 5). In this case, no mature enzyme was detected indicating that the loss of CPA activity resulted in the prevention of G2.8ASM processing and confirming that no other enzymes are able to effect the processing. These data demonstrated that KO2 was having an inhibitory effect on the activity of CPA as well as CPB isoenzymes.

Interestingly, when processing was inhibited the precursors detected (lanes 4 and 5) were of a smaller size than those in the Δ*CPB/g2.8* control. This indicates that inhibition (or removal) of CPA activity blocks processing at a different stage in the enzyme's maturation than the build-up observed after over-expression.

3.3.2. The effect of KO2 on the expression of CPA in promastigotes.

The results described above indicated that KO2 effects the activity of the CPA enzyme of *L. mexicana*. It was interesting to determine whether this led to the loss of the mature CPA enzyme from the cell, which would show that it self-processes.

Western blot analysis (Figure 3.5) revealed that the mature CPA protein (approximately 20 kDa) was not present in wild-type promastigotes grown in the presence of KO2 (lane 2). Coincident with this loss of mature protein, there was the appearance of a higher molecular mass band suggesting the build-up of a precursor form of the protein. It was found that that CPA was expressed normally in Δ*CPB* (lane 3), indicating that the CPB enzymes are not wholly responsible for the production of mature CPA and that other enzymes can

mediate it. However, addition of KO2 to Δ*CPB* also resulted in the absence of mature CPA (lane 4). As KO2 was demonstrated to inhibit CPA itself (see above), the results are consistent with the maturation of CPA being due to autocatalysis.

3.3.3. Electron microscopic investigation of the effect of KO2 on promastigote architecture.

Growth of wild-type or Δ*CPB/g2.8* promastigotes in the presence of 18 μM KO2 caused the appearance of unusual vacuoles filled with a dense matrix within the parasite (Figures 3.6 and 3.7). These vacuoles were often of comparable size to the nucleus and appeared to be part of the lysosomal/endosomal system. Time restrictions meant that it was not possible to use immunotechniques to investigate whether the CPB isoenzymes were accumulated in the vacuoles and/or flagellar pocket.

3.3.4. Analysis of the novel enzymes detected in promastigotes grown with the peptidyl cysteine proteinase inhibitors.

The presence of the inhibitors during the growth of the promastigotes to stationary-phase resulted in the expression of novel gelatinase activities (Figures 3.1 and 3.2). A number of the activities had mobilities of approximately 50 - 60 kDa, indicating that cysteine proteinase inhibition may be causing some effects on the expression of gp63 isoenzymes. Two bands of very low mobility were also consistently observed. It was therefore decided to investigate the class of

enzymes to which these novel activities belonged. It was found that 1,10-phenanthroline (Figure 3.8, lane 3) inhibited the gelatinase activity of approximately 50 - 60 kDa and also reduced the activity of the very low mobility activity bands. This suggests that these may all be metalloproteinase in nature.

The increased activity of approximately 50 - 60 kDa was inhibitable by 1,10-phenanthroline, which suggested that the parasite may be altering its expression of gp63 in the presence of cysteine proteinase inhibitors. This possibility was investigated anti-*L. major* gp63 monoclonal antibodies (Figure 3.9). The strong band of approximately 50 kDa was taken to be gp63. This is highly glycosylated (Medina-Acosta *et al.*, 1993) and attached to a GPI anchor, and so is unusually charged for its size and thus is known to have an uncharacteristic electrophoretic mobility. This phenomenon was demonstrated by Sorensen *et al.* (1994) for *L. major* gp63, which was shown to run at approximately 49 kDa. These results suggested that there was no up-regulation of the amount of gp63 expression in the presence of cysteine proteinase inhibitors and that the gelatinase activity with an apparent molecular mass of around 50 kDa must be due to another enzyme.

The higher molecular mass band (160 kDa approximately) detected by the anti-serum was observed only in promastigotes grown in the absence of inhibitor. This high molecular weight band was not consistently observed during the investigation. Its absence from the minority of cases was perhaps due to variation in duration of electrolysis meaning that proteins of such high molecular mass on occasion did not enter the gel.

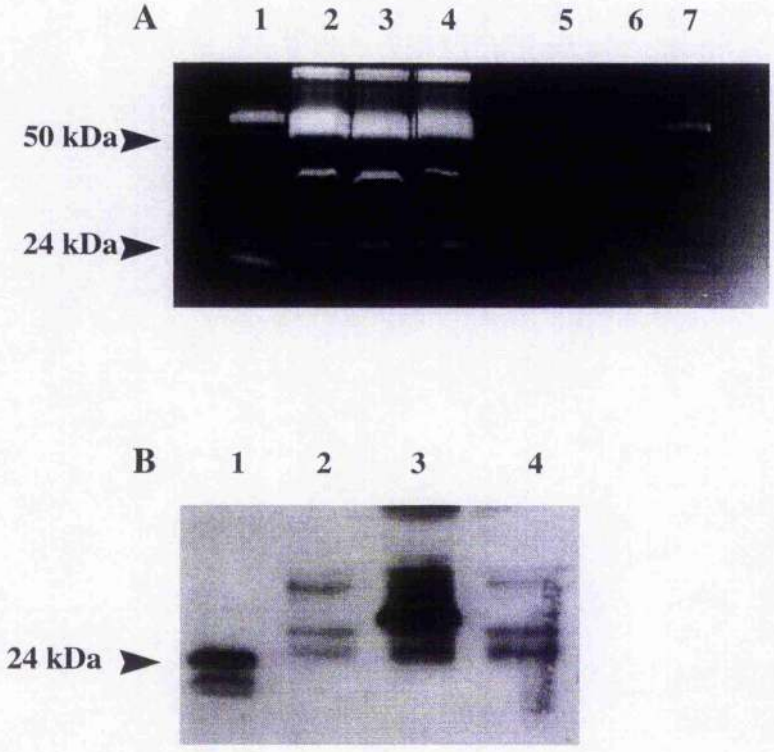

Figure 3.1: Analysis of the effects of cysteine proteinase inhibitors during growth of wild-type promastigotes to stationary phase on the expression and activity of CPB enzymes. A; gelatinase activity of the CPB enzymes. B; western blot using specific anti-CPB sera. Each lane contained the lysate from the same number of promastigotes grown in: lane 1, no inhibitor; lane 2, 18 μM ZFA; lane 3, 18 μM P87; lane 4, 18 μM KO2. Lanes 5 -7 of A; control lysate of stationary phase promastigotes with ZFA (lane 5), P87 (lane 6) or KO2 (lane 7) added before electrophoresis.

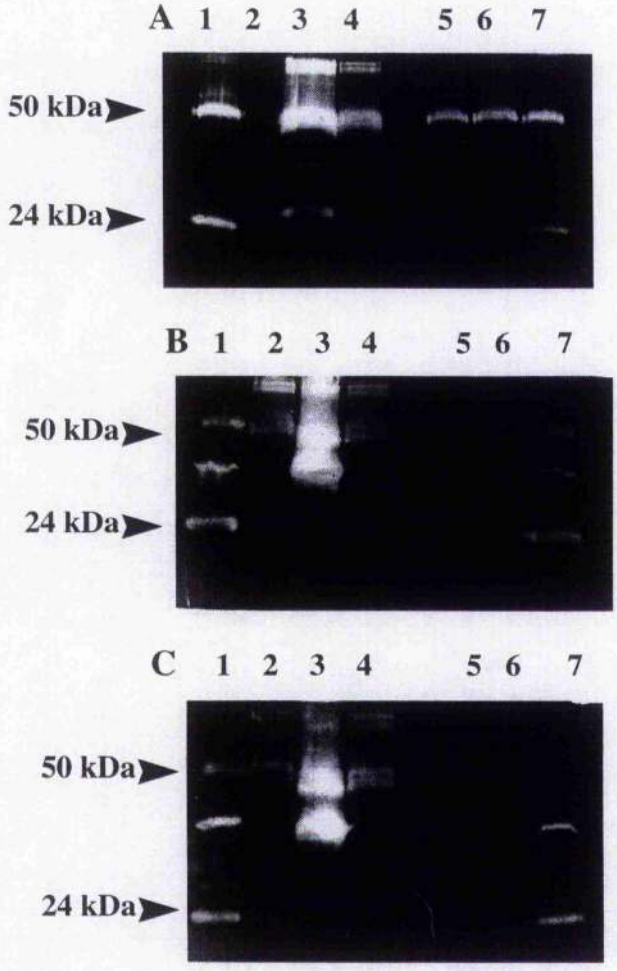

Figure 3.2: Analysis of the effect of cysteine proteinase inhibitors on the activity of CPB iso-enzymes expressed within Δ*CPB*. A; ΔCPB/G1. B; ΔCPB/G18. C; ΔCPB/G2.8. Lysates from equal numbers of stationary phase promastigotes. Lane 1, control lysate; lane 2, grown in 18 μM ZFA; lane 3, grown in 18 μM P87; lane 4, grown in 18 μM KO2. Lanes 5-7; control lysate with ZFA (lane 5), P87 (lane 6) or KO2 (lane 7) added before electrophoresis.

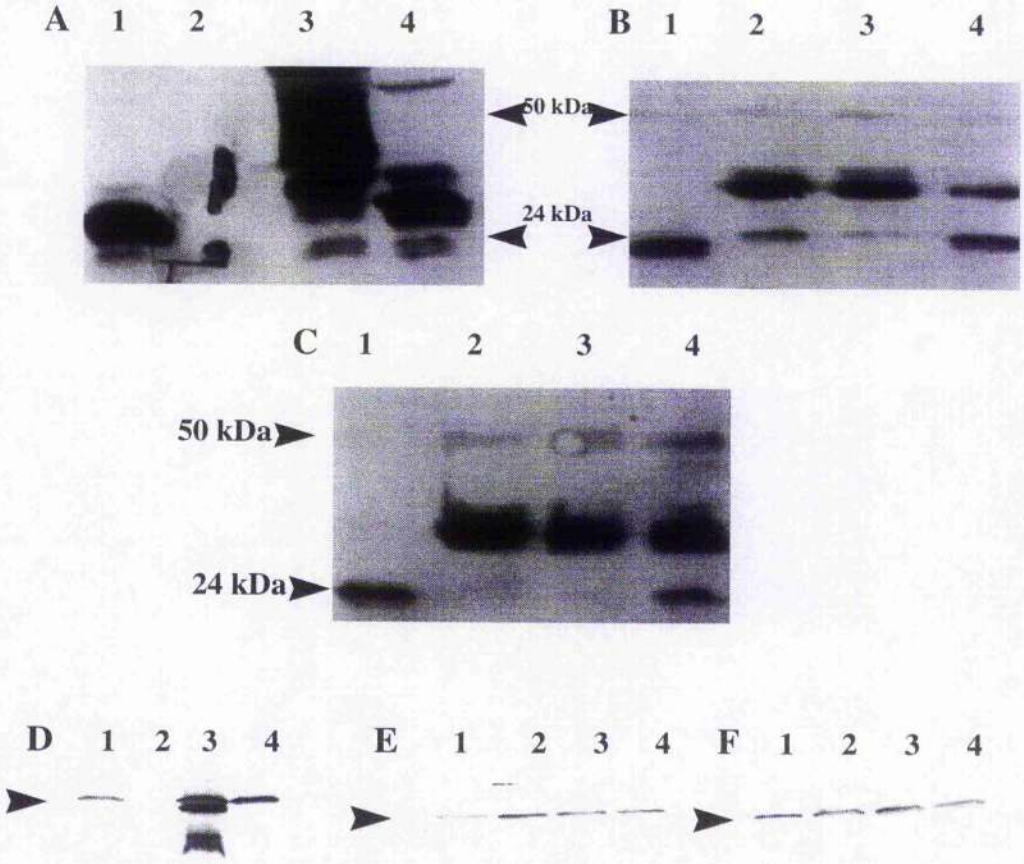

Figure 3.3: Western blot analysis of the expression of CPB in stationary phase promastigote mutant lines:. A,D; Δ*CPB/g1*. B,E; Δ*CPB/g18*. C,F; Δ*CPB/g2.8*. Lane 1, control lysate; lanes 2-4; promastigotes grown in 18 μM ZFA, P87 or KO2, respectively. A-C were probed with anti-CPB serum. D-F were probed with pre-immune serum. The arrows in D-F indicate the 50 kDa marker.

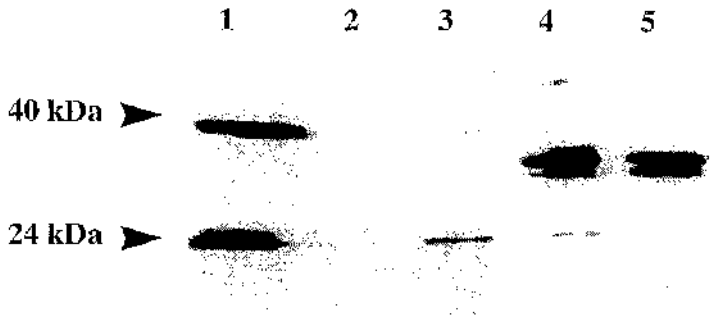

Figure 3.4: Western blot analysis of CPB/G2.8 processing in Δ*CPB* and Δ*CPA/CPB*. Lane 1; Δ*CPB/G2.8*. Lane 2, Δ*CPB*; lane 3, Δ*CPB/g2.8ASM*; lane 4, Δ*CPB/g2.8ASM* grown in KO2; lane 5, Δ*CPA/CPB/g2.8ASM*. The blot was probed with anti-*CPB* anti-serum.

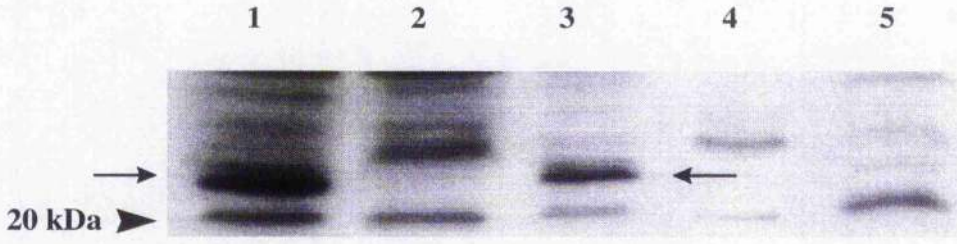

Figure 3.5: Western blot analysing the expression of CPA: Lane 1; wild-type parasites grown in the absence of inhibitor. Lane 2; wild-type parasites grown with 18 μM KO2. Lane 3; Δ*CPB* grown in the absence of inhibitor. Lane 4; Δ*CPB* grown with 18 μM KO2. Lane 5; Δ*CPA*. The anti-serum was specific for CPA. The large number of bands observed is due to cross-reactivty of the serum with other parasite proteins. CPA is indicated by an arrow.

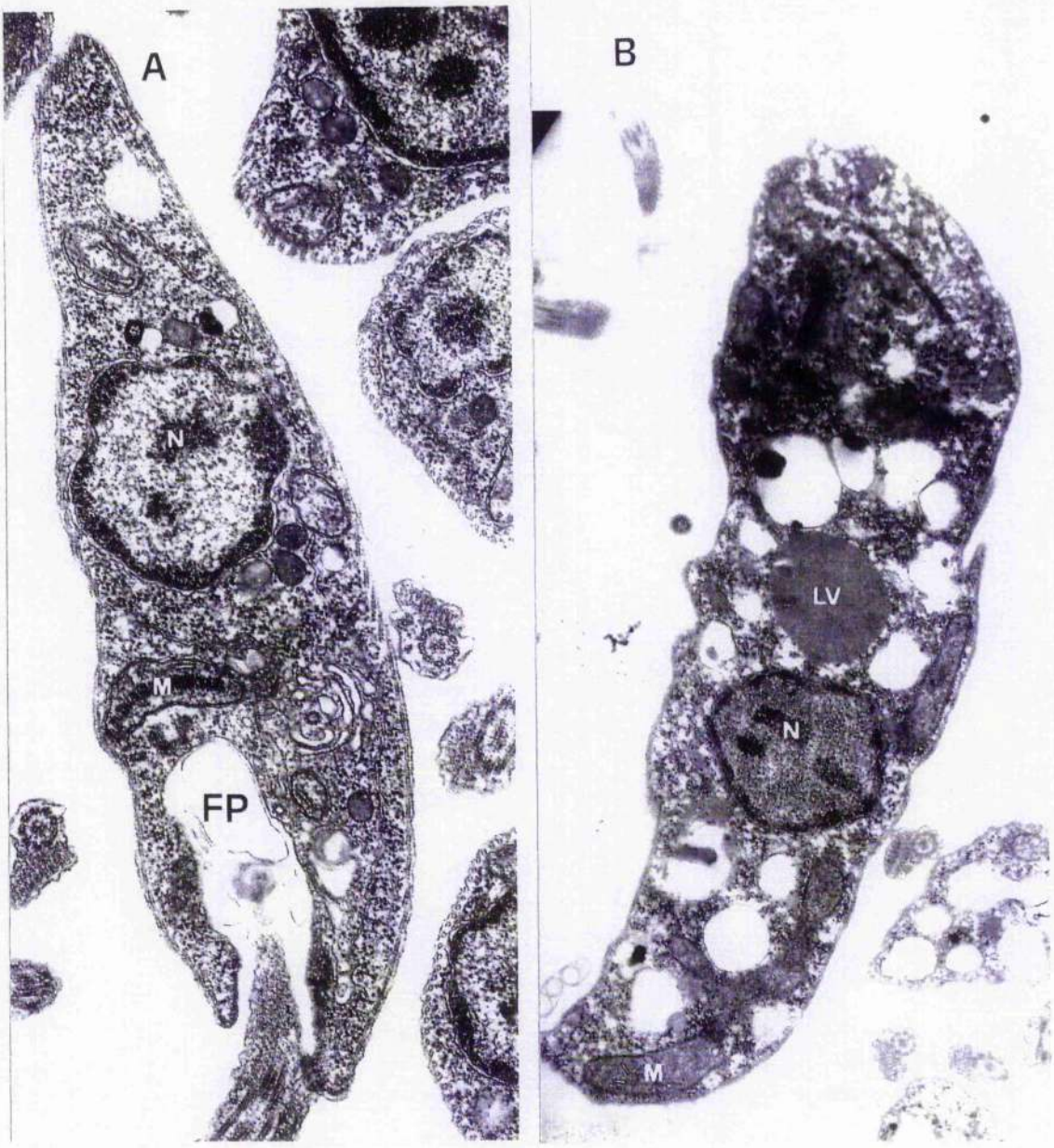

Figure 3.6: Electron micrograph of wild-type promastigotes after growth to late log phase in the presence of 18 μM KO2. Promastigotes were grown to late-log phase in: A, no inhibitor; B, with inhibitor; Labels indicate: N, nucleus; LV, large vacuoles; V, vacuoles; M, mitochondrion; FP, flagellar pocket.

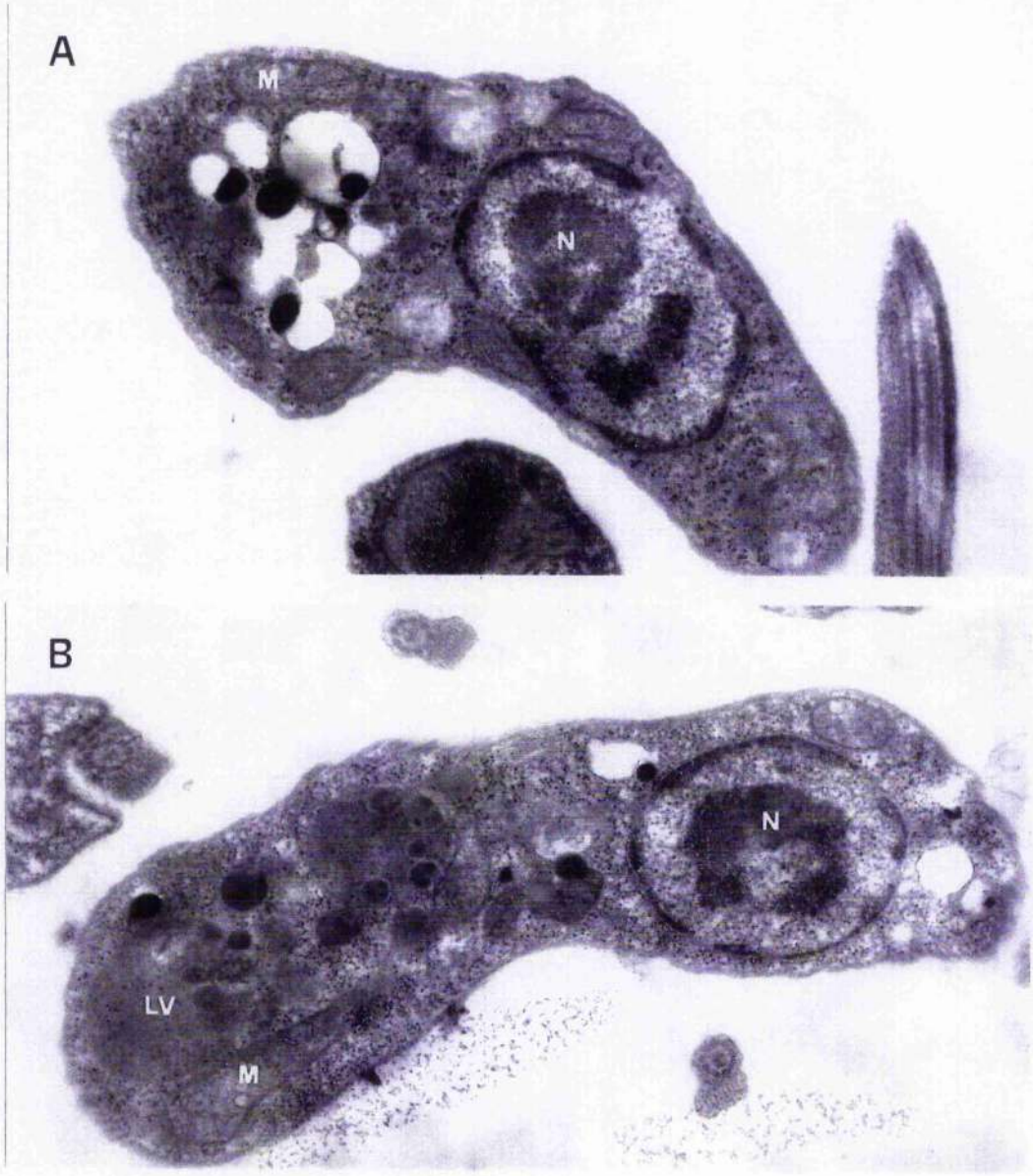

Figure 3.7: Electron micrograph of *ΔCPB/g2.8* promastigotes after growth to late log phase in the presence of 18 μM KO2. Promastigotes were grown to late log phase in: A, no inhibitor; B, with inhibitor. Labels indicate: N, nucleus; LV, large vacuoles; M, mitochondrion.

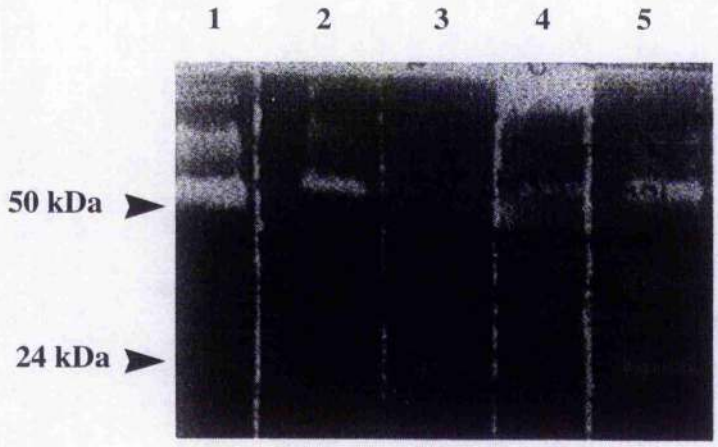

Figure 3.8: Analysis of the novel gelatinase activities observed after growth in KO2. Each lane contained lysate from equal numbers of wild-type promastigotes grown to stationary phase in 18 μM KO2. Lane 1, control; Lane 2, incubation in E64; Lane 3, incubation in 1,10-phenanthroline; Lane 4, incubation in pepstatin; Lane 5, incubation in aprotinin.

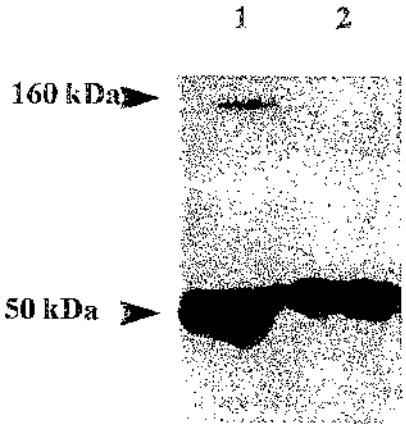

Figure 3.9: Western blot analysis of the expression of gp63 in wild-type stationary phase promastigotes: Lane 1; parasites grown in the absence of inhibitor. Lane 2; parasites grown to stationary phase in the presence of 18 μM K02. The anti-serum was specific for *L. major* gp63.

3.4. Discussion.

This part of my investigation involved an analysis of the effects of cysteine proteinase inhibitors on the expression and activity of *L. mexicana's* cysteine proteinases. Some information concerning the relative efficacies of the inhibitors can be gained from this study. Analysis of the effects of the inhibitors after addition to control lysates demonstrated that KO2 has less effect on the gelatinase activity of the CPB enzymes when added directly to the lysate. This suggests that it may be slower acting than either ZFA or P87 and that it does not have sufficient time to bind to the enzymes before the onset of electrophoresis. It may also indicate that the binding of KO2 was adversely affected in the lysates. A further insight into the efficacy of KO2 was provided by western blotting (Figure 3.3). With both Δ*CPB/g2.8* and Δ*CPB/g1* the mature form of the protein was formed even though no activity towards gelatin was detectable (Figure 3.2). This suggests that KO2 is less able to inhibit processing of the protein to its mature form, although it acts to prevent it's activity when formed.

Despite the reduced ability of KO2 to inhibit the processing of the CPB enzymes to their mature form, the overall effects on activity following growth of the parasite in the inhibitors were comparable with the different compounds. For several reasons, including availability of the compounds and the fact that KO2 has been investigated against other trypanosomatid parasites (Engel *et al.*, 1998a and Selzer *et al.*, 1999), it was decided to concentrate on an analysis of the effects of KO2 on enzyme expression.

The data obtained indicated that inhibition of the cathepsin L-like cysteine proteinases of *L. mexicana* results in a failure to produce mature enzyme and a build-up of precursor forms (intermediate mobility forms), observed by substrate SDS-PAGE and western blotting.

The activity attributed to CPB enzyme intermediates which was observed following substrate SDS-PAGE is unlikely to be due to the intermediate enzyme forms being active within the parasite itself. The trafficking of active enzyme from the RER to the lysosomes would cause unnecessary protein catabolism and be detrimental to the cell. It is more probable that the denaturation of the protein in SDS allows the opening up of the active site and the subsequent loss of the pro-region. The enzyme will then be activated during the incubation in 1 mM DTT and thus exhibit activity towards gelatin.

The processing of pro-cathepsin L has been demonstrated to be via the cleavage of the majority of the pro-region followed by a stepwise removal of small peptide sequences until the fully active mature enzyme is formed (Ishado *et al.*, 1998). The data described here from the use of inhibitors complements well the findings from *in vitro* activation of recombinant CPB/G2.8 (Sanderson *et al.*, 1999) and together they show that the maturation of CPB enzymes is via a multistep processing pathway. A similar pathway is suggested for CPA production.

Interestingly, the number of intermediate bands detected by western blotting does not correspond to the number of gelatinase activity bands observed. This may indicate that the build-up of precursor forms within the parasite is due to mis-folding of the enzymes and therefore retention in the Golgi

as demonstrated for cathepsin L (Chauhan *et al.*, 1998) and for cruzipain (Engel *et al.*, 1998b). Not all of these precursor forms may be able to fold to form the correct structure during renaturation after electrophoresis and hence remain inactive. The lack of gelatinase activity may also be due to the inhibitor remaining bound to some of the precursor forms of the protein during electrophoresis and thus preventing their activity. Another possibility is that the activity observed towards gelatin on SDS-PAGE is due to a number of precursor bands which have co-migrated down the gel. The presence of gelatin within the gel matrix reduces the potential of the electrophoretic process to separate proteins by size, hence several similarly sized proteins may migrate together. Furthermore, during substrate SDS-PAGE the proteins are not fully denatured and so run differently under these conditions than for normal SDS-PAGE.

The results using the different mutant lines in which different CPB isoenzymes were expressed showed potentially interesting differences in the processing of the different CPB isoenzymes. The gelatinase activity of the precursor forms of CPB/G1 grown in the presence of KO2 appeared to be of a lower molecular mass to those of CPB/G18 and CPB/G2.8. Investigation of the expression of these enzymes within the parasite has demonstrated that CPB/G1 is expressed in metacyclics whereas CPB/G18 and CPB/G2.8 are expressed in amastigotes (Mottram *et al.*, 1997). This suggests that different enzyme processing and trafficking could occur between the life-cycle stages. However, this may simply be due to the fact that the pre-pro-enzyme expressed from *CPB/g1* has a truncated C-terminal extension which may mean that the

precursor forms are of a lower molecular weight despite being processed through similar pathways.

The investigation of the processing of CPB/G2.8ASM in either Δ*CPA/CPB* or in Δ*CPB* (in the presence of KO2) indicated some potentially interesting phenomena. Expression of CPB/G2.8ASM in either of the described conditions gave rise to precursor bands of lower molecular mass than produced if CPB/G2.8 was over-expressed in ΔCPB in the absence of KO2. Excessive over-expression of CPB/G2.8 must cause a blockage further upstream in the trafficking/processing pathway than that produced by cysteine proteinase inhibition (or removal), as the precursors in this case are of a greater molecular mass. This suggests that there is a rate limiting step in the early stages of the processing either in the RER or the Golgi. However, as yet there is no evidence to corroborate this idea. The reduction in precursor size following inhibition of CPA activity indicated that the build-up of precursors under these circumstances must occur at a different point in the trafficking pathway than where there is enzyme over-expression, indicating that the role of CPA occurs at a later stage in the processing events. CPA is located within the lysosomes of the parasite (Mottram *et al.*, 1992) and therefore inhibition of its activity is perhaps likely to lead to a build-up of pro-enzyme within the lysosomal vesicles. It is unlikely that CPA would be involved in en route processing as it is also cathepsin L-like and is thus likely to be inactive until it reaches its final destination. Analysis of the localisation of the CPB precursors during inhibition would allow elucidation of where CPA is involved in CPB processing.

A search for the perturbation of cellular architecture within wild-type and ΔCPB/g2.8 promastigotes after growth in KO2 indicated that a build-up of

unprocessed CPB forms may occur in the lysosomes, with the appearance of large swollen vacuoles within the endosome/lysosome network. This phenomenon has also been reported for *L. major* after inhibition of its cathepsin B-like enzyme, which was further demonstrated to cause a build up of un-catabolised material within the lysosomes (Selzer *et al.*, 1999). These swellings were not directly attributed to the build-up of CPB isoenzymes within the parasite by immunotechniques and so other possible causes such as the build up of CPA intermediates or perhaps some effects of CPA inhibition on the cell's ability to digest redundant cell proteins cannot be ruled out.

Interestingly, the effect observed for wild-type parasites was of the same magnitude as that observed with the over-expressing line. The parasites were analysed when in the late log phase of growth and thus the expression of cysteine proteinases in wild-type parasites would have been low. This suggests that the ultrastructural effects of inhibition are not dependent on the amount of enzyme present, but occur due to the accumulative build-up of precursors within the cell. It may be that in the system investigated here there is a certain threshold of material that can be stored within the cell and after attaining this level the enzyme precursors no longer build-up within the cell but are trafficked to the exterior via the flagellar pocket.

Inhibition of *L. major* cathepsin B-like cysteine proteinase, via KO2 leads to an accumulation of pro-enzyme within the flagellar pocket of the cell (Selzer *et al.*, 1999). This phenomenon was also demonstrated for *L. mexicana* after expression of CPB/G2.8ASM in Δ*CPA/CPB* (D.R. Brooks, unpublished). This information has been interpreted to indicate the possible trafficking of lysosomal enzymes via a unique pathway involving the flagellar pocket (Selzer

et al., 1999). However, another interpretation is suggested by the work of Prense *et al.* (1990) who demonstrated that any perturbation in the trafficking of lysosomal enzymes through the cell can lead to the secretion of the enzyme. This, in conjunction with the fact that secreted cathepsin L is internalised via mannose-6-phosphate-mediated endocytosis (Kornfeld, 1987), suggests that accumulation in the flagellar pocket could probably result from endocytosis of secreted enzyme. Furthermore, investigation of the expression of cruzipain, the major cysteine proteinase of *T. cruzi*, has demonstrated that in the epimastigote stage it can be found localised to the parasite surface (Parussini *et al.*, 1998). This expression must occur following trafficking of the enzyme via the flagellar pocket of the organism. Thus it seems possible that the build-up of cysteine proteinase precursors in the flagellar pocket of *Leishmania* species following inhibition is due to the perturbation of the normal protein trafficking within the cell. This may cause trafficking of proteins to and from the surface of the parasite to be altered, thus the build-up of cysteine proteinases in the flagellar pocket may be due to a secondary surface location for these proteins. There is currently no firm evidence for the expression of cysteine proteinases on the surface of *Leishmania* or secretion, despite much speculation on the subject (Coombs and Mottram, 1997).

Interestingly, addition of the inhibitor to the various parasite lysates demonstrated the three CPB isoenzymes investigated may have different substrate preferences. P87, for example, inhibited the activity of CPB/G1 and CPB/G2.8 but had little effect on CPB/G18. In contrast KO2s effects were relatively poor against all the isoenzymes investigated. P87 and KO2 have identical amino acid composition in the P_1 and P_2 position, therefore the results

suggest that the thiol-binding group can also play an important part in determining the efficacy of binding to the active site.

The results of this investigation have suggested that the inhibition of cysteine proteinases causes the parasite to alter the expression of a metalloproteinase of similar molecular mass to gp63. This was demonstrated by showing that gelatinase activities, inhibitable by 1,10-phenanthroline, increased under these circumstances. However, this increase in activity was probably due to a general stress-response of the parasite as similar effects were observed following overnight incubation of promastigotes in serum-free medium (see Chapter 4). The evidence from western blotting suggested that there was no effect on the level of expression of gp63. Gp63 is expressed from at least 7 genes within the parasite (Voth *et al.*, 1998). The relative activity of each of these isoenzymes has not been investigated but it is possible that under conditions of stress the parasite may express a more active isoform of this enzyme and this could result in the increase in gelatinase activity observed in the absence of any increase in the amount of protein detected by western blotting. Furthermore, gp63 is surface located in promastigotes of *Leishmania* via its attachment to a GPI-anchor. The enzyme involved in attachment of GPI-anchors is a cysteine proteinase with GPI:protein transamidase activity, GPI8 (Benzeghal *et al.*, 1996). It seemed possible that the inhibitors investigated in the study and reported in this chapter could have been affecting the activity of this GPI:protein transamidase and thereby preventing the attachment of a GPI anchor to the protein. Non-GPI anchored gp63 has been previously reported to have altered enzymatic activity (McGwire and Chang, 1996), thus the differences observed here may indicate some inhibition in this system. A more

detailed analysis was required to provide a fuller understanding of the possible effects of these inhibitors on the GPI:protein transamidase enzyme in *L. mexicana* and the details of this are discussed in Chapter 4.

In conclusion, the work detailed above has demonstrated that the processing of the cathepsin L-like enzymes of *L. mexicana* is a multi-step process, which can be due to autocatalysis. This work, in conjunction with the work of others (D.R. Brooks, unpublished), has demonstrated that CPA can play an important role in the processing of the CPB enzymes. The inhibition of the parasite's cysteine proteinases causes loss of the mature CPB and CPA enzymes from the cells and a concomitant build-up of precursor forms. Analysis of the effect of various inhibitors on individual CPB isoenzymes has demonstrated differences in substrate specificity and also perhaps in enzyme processing. Further to this, the cysteine proteinase inhibitors appear to stimulate the expression of other enzyme classes, in particularly metalloproteinases.

Chapter 4

The effect of peptidyl cysteine proteinase inhibitors on the expression of the surface metalloproteinase of *Leishmania mexicana* promastigotes

4.1. Introduction.

Gp63 is a zinc metalloproteinase expressed on the surface of *Leishmania* promastigotes, where it is the major surface protein with approximately 5 x 10^5 molecules per promastigote. Several roles for gp63 have been postulated, from survival in the sandfly vector (Camara *et al.*, 1995) to the inhibition of the oxidative burst as the parasite establishes itself within the macrophage host cell (Sorensen *et al.*, 1994; Seay *et al*, 1996). Furthermore, data from over-expression of the protein in gp63-deficient *L. amazonensis* (Liu and Chang, 1992) and also from Brittingham *et al.*, (1995) suggested that it's major role lies in protecting the promastigotes from complement-mediated lysis and allowing efficient binding of the parasite to receptors on the macrophage surface. However, recent mutational studies in which gp63-deficient *L. major* promastigotes were produced have demonstrated that although the deficient lines have increased complement susceptibility, their infectivity levels are similar to wild-type parasites in both *in vitro* and *in vivo* models (Joshi *et al.*, 1998). This suggests that the importance of this molecule is not as great as was once suspected.

Gp63 is expressed from at least 7 genes, though mRNA from only 6 of these is detectable in promastigotes and the resultant proteins are expressed on the surface of the parasite via glycosylphosphatidylinositol- (GPI-) anchors (gene 7 encodes a non-GPI-anchored form which is expressed mainly in amastigotes, Voth *et al.*, 1998). As discussed previously (see Chapter 1, section 1.4.1.2, for details) GPI-anchor addition occurs via a transamidation reaction on the lumenal side of the endoplasmic reticulum membrane. The enzyme involved in the cleavage of C-terminal signal sequences and the simultaneous addition of a GPI-anchor from donor proteins was first identified and characterised in yeast and termed GPI8 (Benghezal *et al.*, 1996). GPI8 belongs to the cysteine proteinase family and has been demonstrated to form a heterodimer with a 68 kDa endoplamsic reticulum protein called GAA1, which was shown to be involved in the recognition and binding of GPI- addition motifs (Hamburger *et al.*, 1995).

The importance of GPI-anchored proteins in the survival of several protozoan pathogens is well documented. Several of the Trypanosomatidae utilise GPI-anchored proteins to initiate or maintain, a prolonged infection within their mammalian host. The Variant Surface Glycoprotein (VSG) coat of *Trypanosoma brucei* is made up of a densely packaged array of antigenically identical GPI-anchored glycoproteins which allows the parasite to circumvent the host's immune response by sequentially switching expression of different VSG isoforms and thus staying ahead of the host's powerful antibody response (Kreier and Baker, 1987). *T. cruzi* also utilises a GPI-anchored trans-sialidase surface protein, in this case, to allow efficient attachment and entry into its host cells during infection (Ming *et al.*, 1993)

It can be argued, therefore, that parasitic typanosomatids have a high degree of dependence on GPI-anchored surface proteins, thus the discovery of a specific inhibitor of GPI-anchor addition within these organisms could have huge implications in the development of putative chemotherapeutic reagents. Evidence presented in Chapter 3, section 3.3.4, suggested that the cysteine proteinase inhibitors investigated could be having an effect on the activity of GP18. The expression or processing of the gp63 in the promastigote stage of the life-cycle appeared to be altered during incubation with the cysteine proteinase inhibitors. This may have been mediated in a number of ways. For example, the inhibitors could have caused an alteration in the processing of gp63 such that it was differently glycosylated, or they may have induced the expression of different isoforms from the gp63 gene family. Either of these suggestions could explain the apparent loss of the high molecular weight (low mobility) form of the enzyme, or the increase in gelatinase activity, after growth of the promastigotes in the presence of inhibitors. Glycosylation can effect the overall charge of a molecule and therefore cause large differences in the way the molecule runs through a gel under an electric current. A further potentially very exciting possibility was that the inhibitors were interfering with the activity of the GPI:protein transamidase enzyme, such that the gp63 expressed was deficient in its anchor. It was this possibility, that I decided to study.

Several groups have investigated the effect of GPI-addition on expression of surface proteins. Studies investigating the effects of mutation of the GPI-signal sequence in human decay accelerating factor demonstrated that the protein was retained within a post endoplasmic reticulum compartment and degraded (Field *et al.*, 1994). The presence of conformation-dependent epitopes suggests

that retention is not due to mis-folding and perhaps indicates the involvement of a receptor-ligand interaction in holding the proteins in the compartment. A role for the GPI-anchor in determining localisation and structure of proteins was also investigated using the major surface protein of *Toxoplasma gondii*, SAG-1. Seeber *et al.*, (1998) mutated the C-terminal sequence of SAG-1 such that it lost the GPI- addition site and encoded instead a human transmembrane region. They showed that the protein was expressed, correctly folded, on the surface of the parasite but that it's half-life was much reduced, perhaps indicating a role for the anchor in protein stability. Studies in *L. mexicana* using mutation of gp63 to remove the GPI-addition site have demonstrated that prevention of GPI-addition to gp63 causes the protein to be trafficked to the surface of the parasite and secreted into the extracellular milieu (McGwire and Chang, 1996). Thus the fate of proteins prevented from acquiring their GPI-anchor is divergent. Evidence exists that they may be either retained within the cell and degraded, trafficked to the surface and expressed via a transmembrane domain, or secreted into the culture supernatant.

This part of my study comprised an investigation of the expression of gp63 in *L. mexicana* promastigotes grown in the presence of cysteine proteinase inhibitors to determine the effects, if any, of the inhibitors on the activity of the GPI:protein transamidase enzyme.

4.2. Materials and Methods.

4.2.1. Analysis of gp63 secretion into culture medium.

Promastigotes were grown to stationary-phase in the presence or absence of 45 μM inhibitors (as described in Chapter 2, section 2.1.1.1). The high serum content of the culture medium prevented it being readily concentrated to enable detection of the enzyme activity. Therefore promastigotes were washed and incubated overnight in medium without HI-FCS and then sedimented and the resultant supernatants concentrated, to give the equivalent of $1x10^9$ cell ml^{-1}, using Vivaspin 15 ml concentrators with a 10 kDa molecular weight cut-off (VivaScience). Samples were analysed using western blotting with specific anti-*L. major* gp63 antibodies and substrate-SDS-PAGE was performed to identify any novel bands of activity in the cells and the supernatants (see Chapter 2, section 2.7.1 and 2.8.1, respectively).

4.2.2. Phase separation of promastigote proteins using Triton X114.

The cytosolic and membranous phases of promastigote lysates were separated using extraction with Triton X114 (see Chapter 2, section 2.9.1, for details) on promastigotes grown either in the absence or presence of 45 μM KO2. The resultant soluble and membranous fractions were analysed via substrate SDS-PAGE for gelatinase activity (Chapter 2, section 2.7.1) and by western blotting using anti-*L. major* gp63 antibodies (Chapter 2, section 2.8.1).

In order to confirm that the gp63 present was associated with the membranous phase of the extraction via its attachment to a GPI-anchor rather than via some other hydrophobic interaction with the membranes of the cell, the effect of phosphatidylinositol phospholipase C (PIPLC) from *Bacillus*

thuringiensis (Oxford Glycosystems) on the localisation of gp63 was analysed (see Chapter 2, section 2.10.1; adapted from Masterson *et al.*, 1989).

After cleavage of the GPI-anchors by incubation in PIPLC, the membranous/detergent phase was re-extracted with 4% (v/v) Triton X114 in PBS before collection of the new soluble phase (containing proteins cleaved from GPI-anchors). The proteins left in the detergent phase due to their association with the membranes via transmembrane domains were also collected and stored after acetone precipitation (as described in Chapter 2, section 2.9.1). The resultant samples were analysed via western blotting and substrate SDS-PAGE as described previously. The further incubation steps required for the PIPLC cleavage and re-extraction meant that protein yields and enzyme activities were low. It was therefore necessary to increase the incubation of substrate gels from 2 h to overnight.

4.2.3. Immunolocalisation of gp63 on the surface of *L. mexicana* promastigotes.

The presence of gp63 was localised on the surface of *L. mexicana* promastigotes using immunofluoresence (see Chapter 2, section 2.11.1, for detail). Briefly, promastigotes were washed by centrifuging at 750 g in a MSE Chilspin (4°C) for 5 min before resuspension at 1 x 10^6 cell ml^{-1} in 100 µl PBS (for study of living parasites) or in 2.5% (v/v) paraformaldehyde in PBS for 15 min to allow fixation to occur (for study in fixed parasites). After a further washing step both the live and fixed cells were resuspended in a 1:200 dilution of primary antibody (in PBS, pH 7.4) and incubated for 30 min at room temperature. After subsequent

washing, the cells were blocked in 10% (v/v) HI-FCS in PBS. The secondary antibody was added (anti-mouse IgG-FITC, SAPU) at a 1:100 dilution in PBS for 30 min before a further two washes. The live cells were then fixed as described above and both samples were air dried onto microscope slides for examination.

4.2.4. Labelling of GPI-anchors with tritiated ethanolamine and subsequent immunoprecipitation.

The method outlined below was adapted from Mensa-Wilmot *et al.*, 1994. Promastigotes were seeded at 1 x 10^6 cell ml^{-1} and grown to a density of 1 x 10^7 cell ml^{-1} (see chapter 2 section 2.1.1.1) before harvesting by centrifuging twice at 750 g in M199 medium containing 20 mM L-glutamine, non-essential amino-acids, 40 mM Hepes pH 7.5, 20 mM sodium hydroxide and 10% dialysed HI FCS (dialysed against supplemented M199 at 4 °C overnight). The promastigotes were then re-suspended at 2 x 10^7 cell ml^{-1} were then labelled overnight (in the presence or absence of 18 μM KO2) in 5 ml medium at 25°C with 100 μCi/ml of [1-H^3]ethanolamine hydrochloride (1mCi from Amersham). The promastigotes were then harvested in 1 ml portions by washing twice in M199 medium as described above. The culture medium was also collected in 1 ml portions. Both the pelleted cells and the supernatants were stored at -20°C until required.

Individual pellets were lysed by resuspension in 1 ml of ice-cold dilution buffer (designated IDB, and containing 1.25% [v/v] Triton X100/190 mM NaCl/60 mM Tris-HCl pH 7.5/6 mM EDTA/10 U ml^{-1} aprotinin) before

incubation for 30 min on ice. 250 µl aliquots of cell lysate or supernatant fraction were analysed individually. 750 µl of 1.33 x IDB and 5 µl of anti-*L. mexicana* gp63 antibody was added to the cell lysates and incubated overnight with continuos mixing by inversion at 4°C. 100 µl of a 1:1 suspension of protein A-sepharose beads was added before incubation at room temperature for a further 2 h. The immune complexes adsorbed to the beads were then washed three times for 10 min in 1 ml of wash buffer (0.1% Triton X100/ 0.02% SDS/150 mM Tris-HCl pH 5.5/5 mM EDTA/10 U ml^{-1} aprotinin). The beads then received a final wash in TBS before addition of 2.5 x SDS-PAGE sample buffer. The beads were vortexed briefly and heated to 90°C for 5 min. The protein in the eluate was analysed in 25 µl fractions by SDS-PAGE (see Chapter 2, section 2.7.1). The gel was soaked in Entensify (NEN life sciences) and the radiolabelled proteins on the gel detected by fluorography.

4.3. Results.

4.3.1. Determination of whether gp63 is secreted into the culture medium in the presence of the cysteine proteinase inhibitors.

Investigations involving mutation of the GPI-anchor addition site of *L. mexicana* gp63 have demonstrated that un-anchored gp63 can be secreted into the extracellular milieu (McGwire and Chang, 1996). An investigation of the secretion of gp63 into the supernatant of inhibitor-treated promastigotes was therefore undertaken.

Promastigotes were grown to stationary phase in the presence or absence of 18 μM KO2 and the resulting cells and medium fractions were examined by substrate SDS-PAGE. Figure 4.1 demonstrates that incubating the stationary phase promastigotes overnight in the absence of inhibitors but without HI-FCS caused a similar pattern of novel enzyme activity as did incubation with the inhibitors (lanes 2 and 4, respectively). Data presented in Chapter 3 suggested that this expression of novel activity bands in the presence of inhibitors may reflect a mechanism by which the promastigotes compensate for stress conditions. This finding of similar expression in the absence of serum strengthens this argument. The cell-free medium of the inhibitor-free cultures contained one activity band (lane 3) whereas the equivalent fraction from the inhibitor positive cultures contained one definite and a possible second band of activity (lane 6). It proved impossible to determine whether these activity bands were due to the stress experienced by the promastigotes in the absence of FCS or if the inhibitor-positive supernatant did indeed have an extra activity band.

The bands of activity coincided in mobility with that expected for gp63. However, western blotting of the lysates using the anti *L.major* gp63 serum failed to detect any gp63 in the medium fractions (Figure 4.2), suggesting that the gelatinase activity bands were not due to the presence of gp63 in the supernatant of the inhibitor-positive cultures. The mobility of gp63 with an apparent molecular mass of 50 kDa despite a molecular mass of 63 kDa is not unusual and this phenomenon has been described for *L. major* gp63 where the effects of glycosylation were suggested as the cause of the abherent running (Sorensen *et al.*, 1994).

4.3.2. The effect of cysteine proteinase inhibitors on the expression of gp63 in promastigotes.

It was postulated that if the activity of GPI8 was prevented then gp63 should not become associated with the plasma membrane of the promastigotes and may therefore be found within the cytosolic fraction of the cells. In order to determine whether gp63 was membrane bound or free within the cytoplasm of inhibitor-treated cells, the parasites were subjected to phase separation using the detergent Triton X114.

The relatively low levels of CPB enzymes expressed in wild-type *L. mexicana* promastigotes made it difficult to use these enzymes to verify the success of the extractions (the CPB enzymes should appear exclusively in the cytosolic fraction of the cell), it was therefore decided to use a Δ*CPB* line over-expressing one of the genes from the array (in this case *CPB/G18*). Figure 4.3 demonstrates the results of gelatin SDS-PAGE analysis of the Triton X114 extraction of the Δ*CPB* re-expressing CPB/G18 (Δ*CPB/g18*). The fact that the activity due to the CPB enzymes is only found in the cytosolic fraction is consistent with the separation being successful. However, there was some gelatinase activity in the soluble phase which corresponded to the mobility of gp63. This finding was not consistent between experiments, with the relative amounts of gelatinase activity in inhibitor grown and control cytosolic fractions changing between experiments. No gelatinase activity was detected in the extracts of the lysate from the inhibitor-positive cultures which could be accounted for by the CPB enzymes. This phenomena appeared to be consistent

despite several repeats and perhaps occurred due to the effect of the inhibitors on the expression of the CPB enzymes meaning that they were in some way less stable and were rapidly degraded during the extraction process. It is possible that the KO2 present remained bound to the enzyme during the processing of the lysate and thus inhibited the activity as was indicated by some of the data in Chapter 3, section 3.3.1. The main result however, is that the activity apparently due to gp63 was still present in the membranous phase, despite the presence of the inhibitor during parasite growth, suggesting that the inhibitor did not affect the association of gp63 with the membrane. Furthermore, despite the variation in gelatinase activity observed during the investigation, western blot analysis of the lysates (see Figure 4.4) demonstrated that gp63 was detectable in the soluble and membrane phase of the promastigotes regardless of the presence of inhibitor.

4.3.3. Investigation of the GPI-anchoring after incubation with cysteine proteinase inhibitors.

The analysis of the phase separation experiments detailed above suggested strongly that gp63 was still associated with the membrane of promastigotes cultured in the presence of the inhibitors. It did not, however confirm that the protein was still attached via a GPI-anchor. Several other possible mechanisms could allow non-anchored gp63 to associate with the membranous fraction of the extraction. Field *et al.* (1994) investigated the fate of proteins which had not received their GPI-anchor by mutating the GPI-signal sequence and discovered that they were retained in a post endoplasmic reticulum compartment and failed to reach the Golgi stacks. They also demonstrated that

such proteins were eventually degraded via an energy-dependent pathway. It is possible that non-anchored gp63 could become associated with a receptor in the post-endoplamsic reticulum compartment to facilitate its retention and thus may appear to be membrane-associated.

Further to this, the yeast homologue of the GPI:protein transamidase enzyme exists as a heterodimer with a second subunit termed GAA1 which is involved in recognition of the GPI addition site (Hamburger *et al.*, 1995). It is possible that although the GPI8 portion of the GPI:protein transamidase is inhibited by KO2 the GAA1 equivalent is still able to recognise the attachment site and bind the enzyme. As the GPI:protein transamidase is a membrane-bound endoplasmic reticulum enzyme (Benghezal *et al.*, 1996), this binding of gp63 would cause non-GPI anchored forms to associate with the membranous phase of a detergent extraction.

Another consideration is that the C-terminal signal sequence of the precursor gp63 expressed in *L. mexicana* amastigotes (expression has also been reported in promastigotes; Medina-Acosta *et al.*, 1993) has a divergent region at its carboxy end. An extra 33 amino acids have been demonstrated to occur at this end and the majority of these are hydrophobic in nature (Figure 4.5). It was speculated that this divergence precluded the addition of a GPI anchor to this species, hence it's intracellular localisation within amastigotes. Subsequent analysis, however, has demonstrated that addition of the anchor does occur to this form (Voth *et al.*, 1998). Nevertheless, there appeared the possibility that the uncleaved C-terminal sequence may be sufficiently long enough to act as a weak trans-membrane domain or allow an association to occur with other

membrane molecules, and thus facilitate association of gp63 with the membranous phase during a Triton X114 extraction.

To determine whether the association of the gp63 with the membranes of inhibitor-positive cultures was due to the correct attachment of the GPI-anchor, fixed promastigotes were treated with phosphatidylinositol phospholipase C (PIPLC) from *Bacillus thuringiensis* as described in section 4.2.2. The rationale behind this experiment was that if the gp63 was associated with the membrane fraction via some method other than a GPI- anchor, then treatment with PIPLC would have no effect on the localisation of the gp63 within the membranous phase of the extraction.

As can be seen in Figure 4.6, the use of PIPLC on control (inhibitor-free) promastigotes (part A) removes the activity of gp63 on gelatin gels from the detergent phase of the extraction into the soluble phase confirming that the procedure was successful. In the presence of inhibitors, a similar pattern of activity is found, indicating that the gp63 has been removed from the membranous phase by PIPLC and therefore must still be attached to the membrane via a GPI-anchor. This result suggests that KO2 has no inhibitory effect on the GPI:protein transamidase enzyme, and thus a GPI-anchor is still attached to gp63.

4.3.4. Immunolocalisation of gp63 on the surface of *L. mexicana* promastigotes

In order to confirm that gp63 is still expressed on the cell surface of *L. mexicana* promastigotes in the presence of 18 μM KO2 ,the promastigotes were

probed via immunofluorescence (see section 4.2.3, for details). Initially anti-*L. major* gp63 monoclonal antibodies (supplied by Robert McMaster; see Frommel *et al.*, 1990 for details) were used as these had been demonstrated to detect *L. mexicana* gp63 on western blots. However these antibodies proved unable to detect gp63 on the surface of promastigotes, suggesting that they recognised an epitope that was either non-conformational or hidden on the surface of *L. mexicana* promastigotes. However, the two monoclonal antibodies against different epitopes on *L. mexicana* gp63 (Medina-Acosta *et al.*, 1989) were able to bind the gp63 on the promastigote surface.

The use of log- and stationary-phase promastigotes fixed for 15 min in 2.5% paraformaldehyde demonstrated that the presence of inhibitors had no effect on the expression of gp63 on the cell surface, see Figure 4.7. An interesting effect was demonstrated, however, when the promastigotes were left unfixed until after incubation with the secondary antibody. Parasites which had been grown in the absence of the inhibitor lost the fluorescence from the main body of their surface and appeared to have concentrated the fluorescence on the posterior tip and in the flagella pocket, see Figure 4.8. When the promastigotes were grown in the presence of the inhibitor, the distribution of gp63 on the surface was patchy (Figure 4.8), suggesting that the turn-over/loss of bound gp63 from the surface was slower. One possible reason for this reduced turn-over rate is the effect of the inhibitors on the CPB enzymes. The fact that the inhibitors have been demonstrated to prevent the production of the mature CPB (and CPA) enzymes and cause a build-up of precursor forms suggests that they will be perturbing the function of the lysosomes. Molecules, including gp63, are

turned over in lysosomes so it is possible that the effect on the CPB enzymes is causing a blockage in the turn-over pathway and thus reducing its efficiency.

Further weight is added to this argument by examining the surface expression of gp63 on Δ*CPB* promastigotes (Figure 4.9). Fixed Δ*CPB* parasites show similar gp63 distribution to both wild-type promastigotes, with and without inhibitor (data not shown). However when they were left unfixed until after incubation with the secondary anti-serum they displayed the same patchy surface distribution as the inhibitor-grown parasites (Figure 4.8). This suggests that the lack of CPB enzymes is in some way responsible for the reduction in turn-over rate of gp63 on the surface. Thus perhaps the effect of the inhibitors on the activity of the CPB enzymes may be perturbing the turn-over of the molecule from the surface of wild-type parasites. It is not possible from these results to exclude the possibility that reduction in gp63 turnover in Δ*CPB* was due to some other uncharacterised effect of the electroporation procedure rather than the loss of the CPB enzymes themselves. Unfortunately these experiments were not completed using any of the Δ*CPB*/re-expressor lines available which would have controlled for such effects.

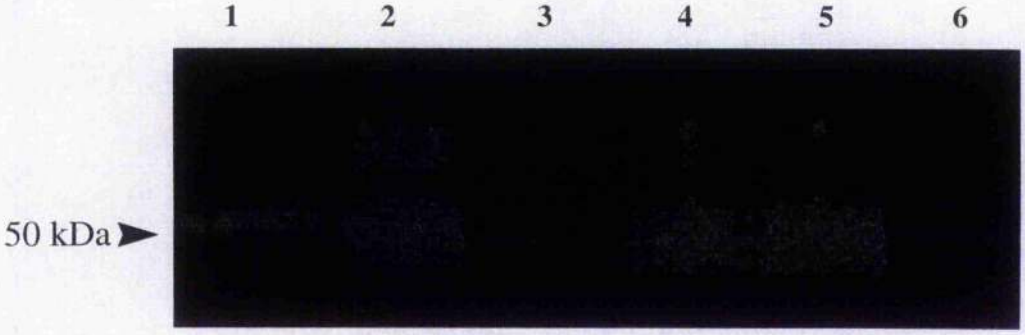

Figure 4.1: Substrate SDS-PAGE analysing the presence of gelatinase activity corresponding to the molecular mass of gp63. Wild-type *L. mexicana* cultures grown in the absence (lanes 1 - 3) or presence (lanes 4 - 6) of 18 μM KO2. Lanes 1,4; lysate from cultures grown in the presence of FCS. Lanes 2,5; lysate from cultures incubated overnight without FCS. Lanes 3,6; cell free fraction of cultures incubated overnight without FCS.

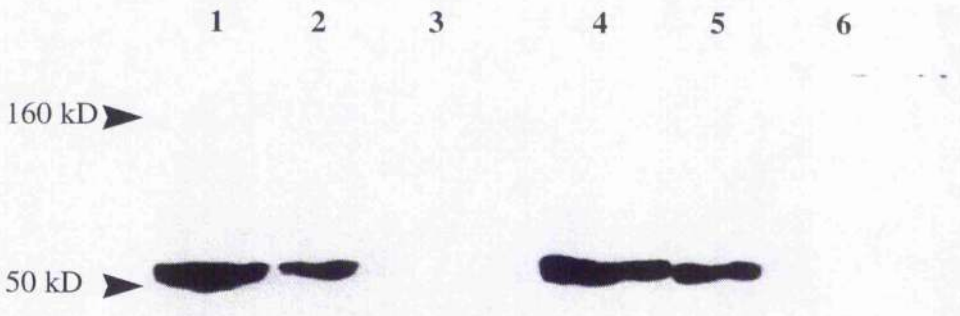

Figure 4.2: Analysis of the presence of gp63 in the medium of cultures grown in the presence of 18 μM KO2. Lysates of stationary phase promastigotes grown either in the absence (lanes 1-3) or presences (lane3 4-6) of 18 μM KO2. Lanes 1,4; wild-type promastigote lysate grown with FCS. Lanes 2.5; wild-type promastigote lysate grown without FCS. Lanes 3,6; cell free medium from wild-type cultures grown without FCS. Probed with an anti-serum specific for *L. major* gp63.

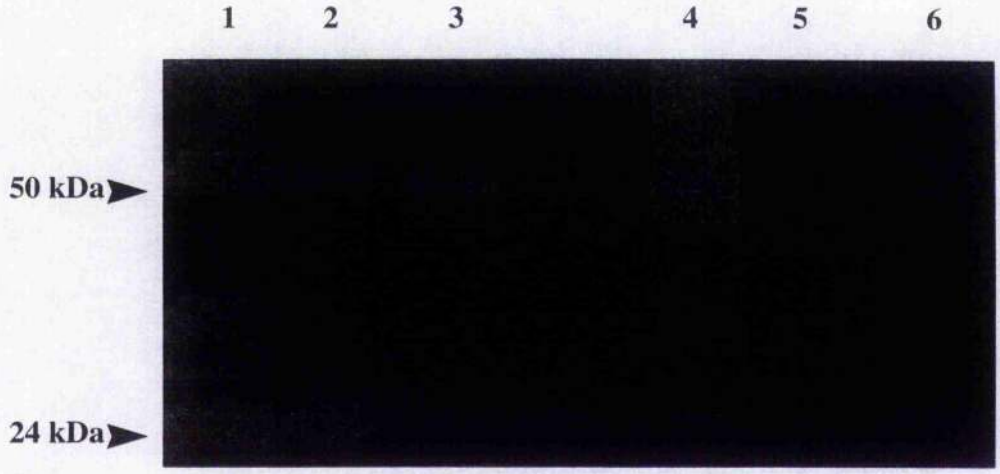

Figure 4.3: Substrate SDS-PAGE of Δ*CPB/g18* lysates after phase separation with Triton X114. Lysates of stationary phase promastigotes grown either in the absence (lanes 1 - 3) or presence (lanes 4 - 6) of 18 μM KO2. Lanes 1,4; lysate before extraction. Lanes 2,5; soluble phase of extract. Lane 3,6 detergent phase of extract.

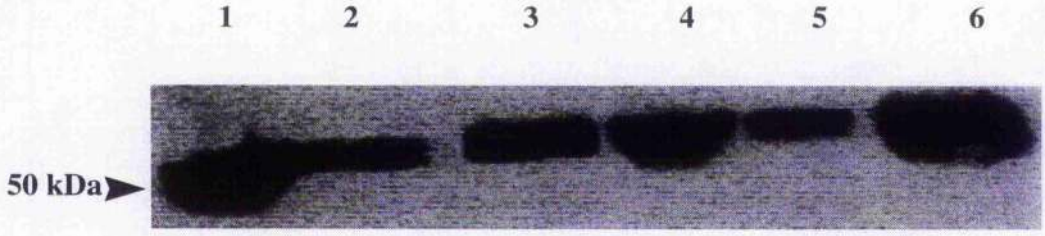

Figure 4.4: Western blot analysis of Δ*CPB/g18* stationary phase promastigote lysates after phase separation with Triton X114. Promastigotes grown to stationary phase in the absence (lanes 1 - 3) or presence (lanes 4 - 6) of 18 μM KO2. Lanes 1,4; lysate before extraction. Lanes 2,5; soluble phase of extraction. Lane 3,6; detergent phase of lysate. Probed using specific anti-*L. major* gp63 antibody.

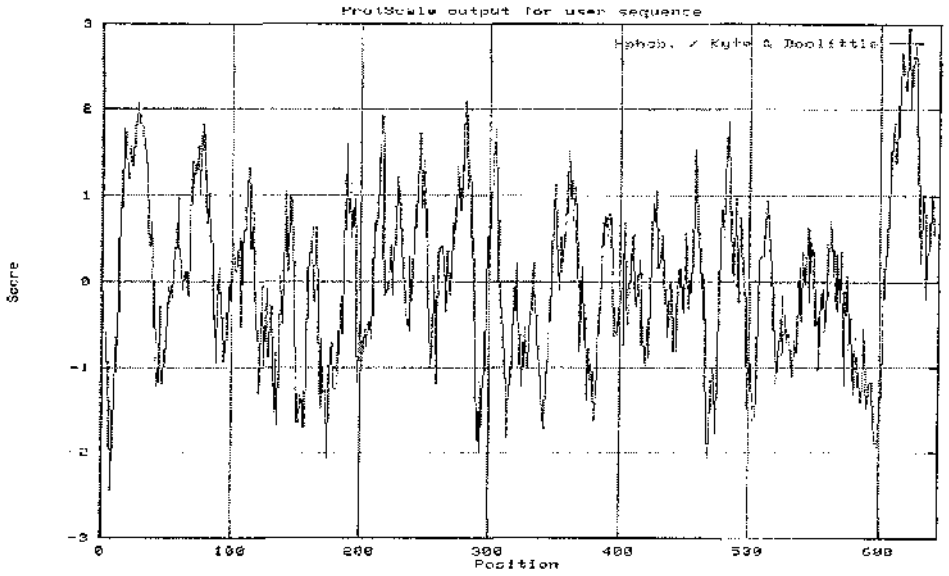

Figure 4.5: Kyte and Doolittle hydrophobicity plot of *L. major* precursor gp63. The plot was calculated using the Protscale site on NCBI Entrez website. The sequence for *L. mexicana* gp63 was taken form Medina-Acosta *et al.*(1993) and is for an isoform of gp63 expressed in both promastigotes and amastigotes. An increasing positive score is indicative of a hydrophobic nature. N terminus of the protein is at position 0, C terminus is at position 646. The GPI anchor addition site at position 576.

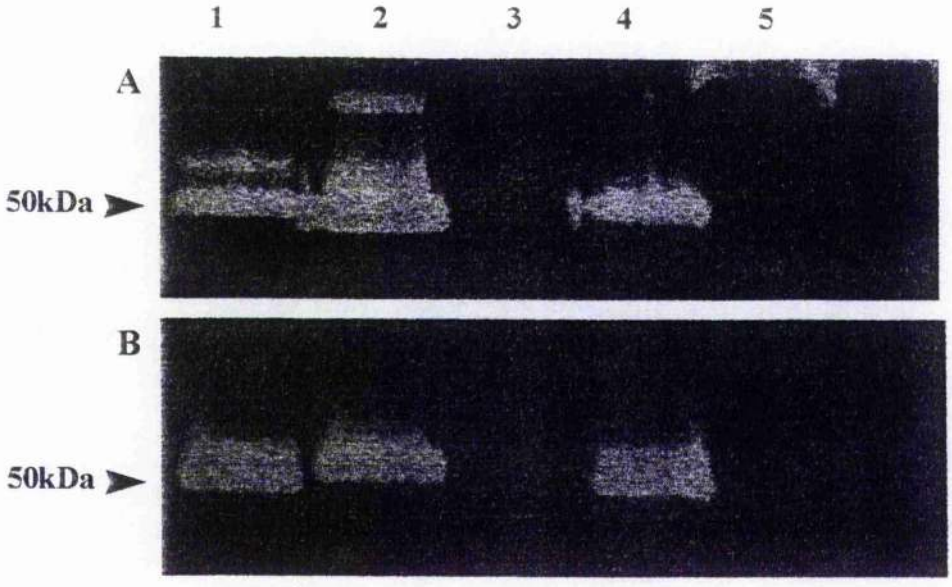

Figure 4.6: Substrate SDS-PAGE of Δ*CPB/g18* stationary phase promastigotes analysing the effect of PIPLC treatment on the localisation of the gelatinase activity attributed to gp63. A; control promastigotes, grown without KO2. B; promastigotes grown to stationary phase in the presence of 18 μM KO2. Lane 1, lysate before extraction; lane 2, detergent phase of extraction before PIPLC treatment; lane 3, soluble phase of extraction; lane 4, soluble part of detergent phase after PIPLC treatment; lane 5, proteins left in detergent phase after PIPLC treatment.

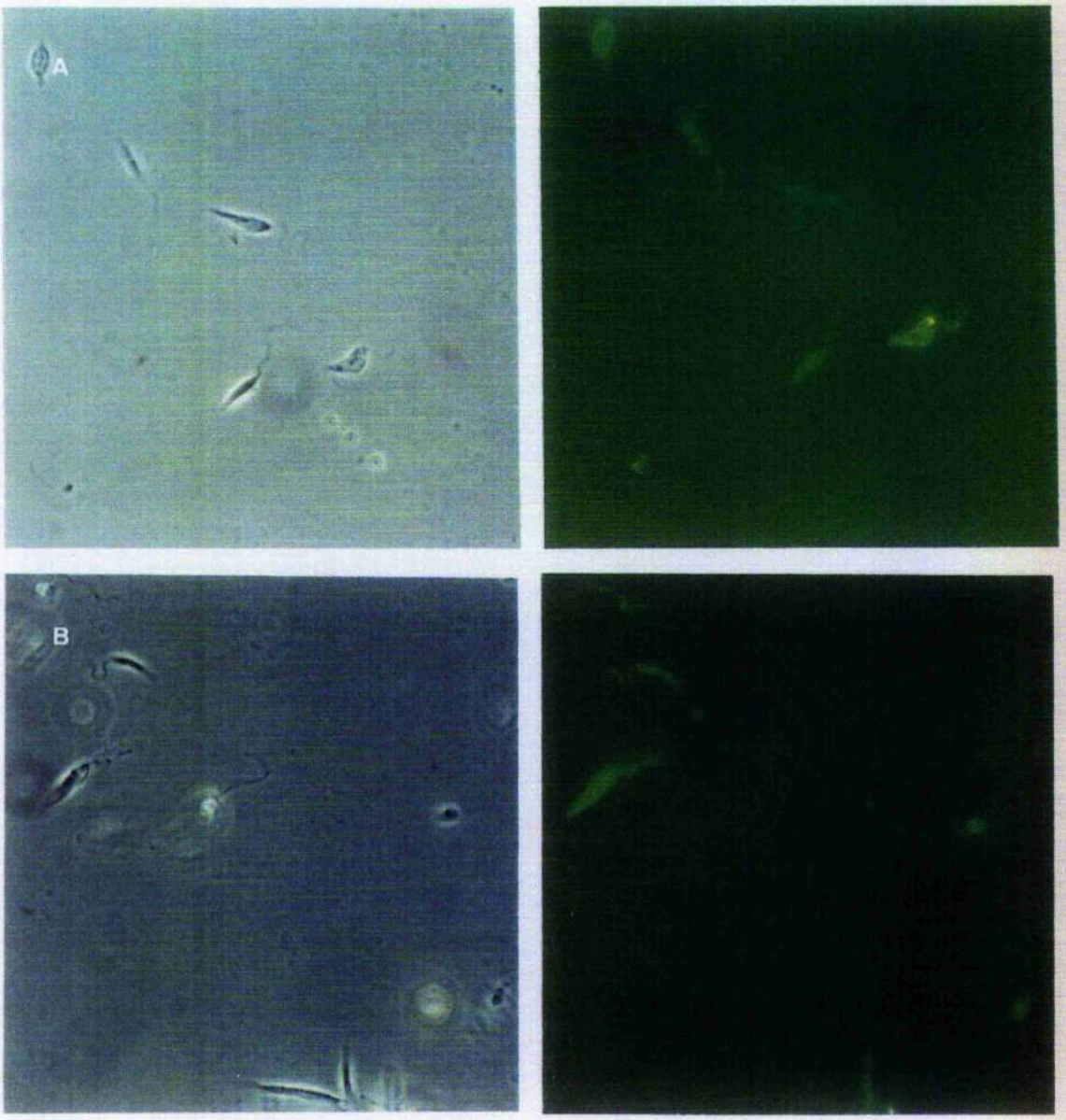

Figure 4.7: Immunolocalisation of gp63 on the surface of wild-type promastigotes. A; control promastigotes. B; promastigotes grown in the presence of 18 μM KO2. Parasites were fixed prior to probing with anti-serum specific for *L. mexicana* gp63. A FITC-labelled secondary antibody was used to allow visualisation.

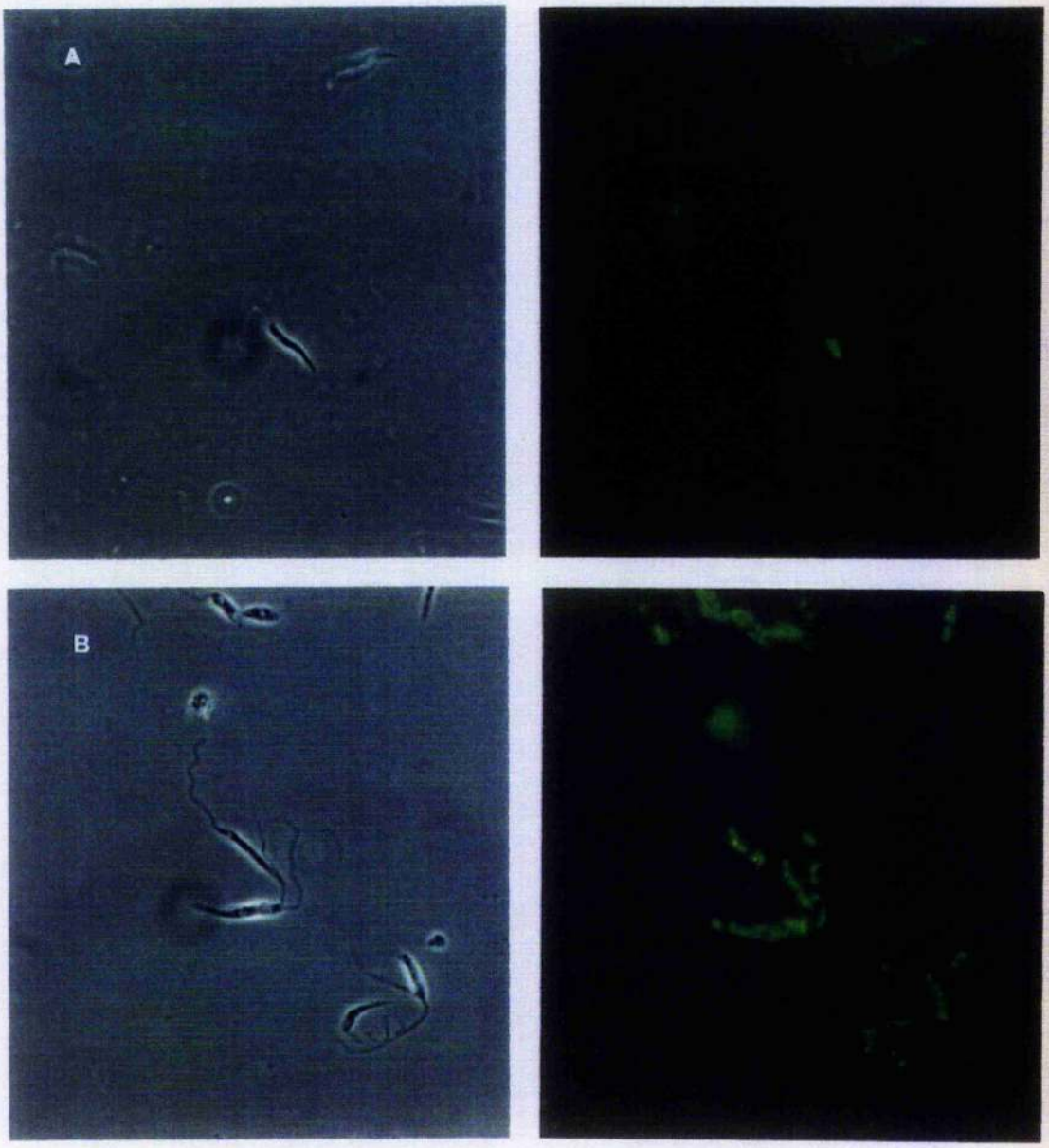

Figure 4.8: Immunolocalisation of gp63 on wild-type promastigotes. A; control parasites. B; parasites grown in 18 mM KO2. The promastigotes were left live, until after incubation in the secondary antibody. Parasites probed with anti-serum specific for *L. mexicana* gp63. A FITC-conjugated secondary antibody was used to allow visualisation.

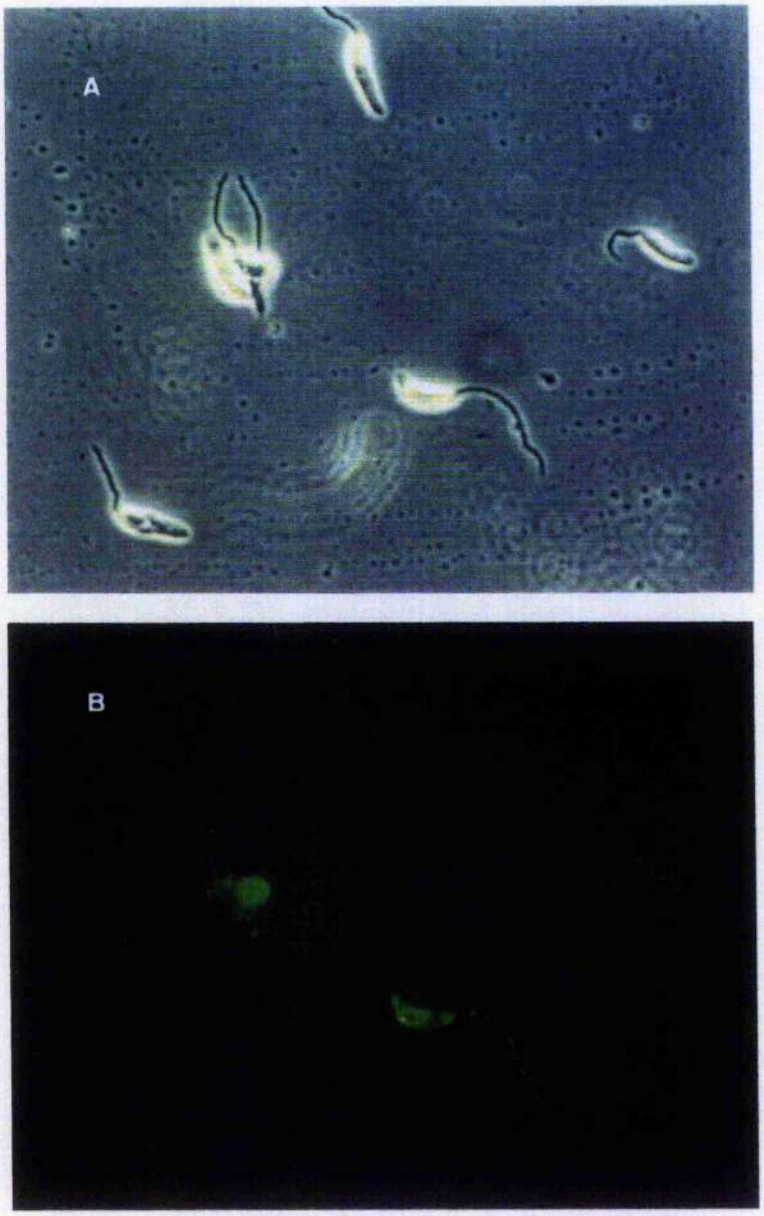

Figure 4.9: Immunolocalisation of gp63 on the surface of Δ*CPB* promastigotes. The parasites were left un-fixed until after application of the secondary antibody. Parasites were probed with anti-serum specific for *L. mexicana* gp63. A FITC-conjugated secondary was used to allow visualisation.

4.4. Discussion

This chapter deals with an investigation of the possible effects of the cysteine proteinase inhibitor K02 on the GPI:protein transamidase enzyme of *L. mexicana*. The GPI:protein transamidase of yeast has been demonstrated to belong to the cysteine proteinase family (Benghezal *et al.*, 1996) and so it was hypothesised that the inhibitors under investigation may have an inhibitory effect on the corresponding enzyme in *Leishmania*. The effects on the Leishmanial enzyme were investigated by analysing the expression of the major surface metalloproteinase, gp63.

McGwire and Chang, (1996) demonstrated, by mutation of the GPI-addition site that un-anchored gp63 was secreted into the culture supernatant of mutant *L. major*. I was unable to detect gp63 in the extracellular milieu of K02 grown *L. mexicana* promastigotes. A negative finding is always difficult to confirm and an additional factor to be considered is that the antibodies available at that time were not specific for *L. mexicana* gp63 and were subsequently demonstrated to be unable to detect gp63 in its native conformation on the surface of the parasite. It appears that the antibody may not detect all the isoforms of the enzyme in *L. mexicana* (perhaps due to the specific epitope being either hidden by glycosylation, or absent). Another factor that has to be taken into account is that the cultures were only subjected to overnight incubation before investigation of the supernatants, this may not have been sufficient to allow detectable levels of gp63 to accumulate in the supernatant for assay with this antibody via western blotting or SDS-PAGE. However, a prolonged incubation was not practicable as the promastigotes have a limited life in medium

without serum. These experiments could be performed using immunoprecipitation to detect any secreted gp63. This would allow the use of cultures grown in the presence of serum and so longer incubations. The findings from other approaches, however, did not provide encouragement to carry out these additional experiments.

An analysis of the localisation of gp63 in parasite lysates was undertaken using phase separation by Triton X114 to separate the soluble proteins from the hydrophobic (membrane bound) proteins. This demonstrated that gp63 was indeed membrane-bound in the inhibitor-grown parasites, but did not exclude the possibilities that the non-anchored protein was associated with a receptor in a post-endoplasmic compartment as has been demonstrated to occur with decay accelerating factor (Field *et al.*, 1994) or that it was associated with the GAA1 equivalent in the endoplasmic reticulum itself. A further possibility was that the hydrophobic C-terminal extension of the precursor enzyme could in some way be interacting with the membrane to form a trans-membrane domain. These possibilities were effectively ruled out by immunofluorescence demonstrating the presence of the enzyme on the surface of the parasite, and by the use of PIPLC during phase separation which showed the enzyme to be GPI-anchored. However it must be considered that if gp63 is attaching via a weak, unnatural transmembrane region or hydrophobic interaction then the strength of this bond could be weakened by the second Triton X114 extraction. Thus it may be the case that the gp63 present was removed from the detergent phase of the lysate by re-extraction alone and not via PIPLC cleavage of its GPI-anchor. This is unlikely, however as no reports that this occurs could be found in the literature.

Although the amount of gelatinase activity detected of the correct size (approximately 50 kDa) in the cytosolic phase varied, there was a consistent amount of gp63 protein detectable using western blotting. This phenomenon could be due to a high turnover rate of the enzyme from the surface, with this soluble form representing an inactive (or partially active) stock of the enzyme within the cell. Several sources have also reported that the non-GPI anchored amastigote isoform of the enzyme is expressed at detectable levels in the promastigote (Medina-Acosta *et al.*, 1993; Voth *et al.*, 1998) and this soluble form may represent this. Evidence from McGwire and Chang (1996) has shown that mutation of the asparagine at position 577 prevents addition of the GPI-anchor and also ablates the gelatinase activity of the enzymes. Perhaps this partially inactive form is a precursor within the cell awaiting GPI-addition.

Although the immunofluorescence data I obtained suggest that gp63 was present on the surface no evidence could be found in the literature that demonstrated definitively that 2.5% (v/v) paraformaldehyde did not cause any permeabolisation of the membrane, thus allowing the antibodies to stain gp63 localised within the cells. This is unlikely, however, as similar fixation regimens have been used by several groups to study the surface location of gp63 (Medina-Acosta *et al.*, 1989; Streit *et al.*, 1996). Further to this, the effects observed when live promastigotes were assessed for surface gp63 clearly demonstrated the capping effect typical of the aggregation of surface molecules into patches which then accumulate over the poles of the cells before endocytosis or release (Johnstone and Thorpe, 1982).

The patchy distribution of gp63 on the surface of KO2-treated promastigotes suggests that the inhibitor was causing a reduction in the turnover

rate of surface proteins. This phenomenon was also observed for gp63 on Δ*CPB* suggesting that the inhibitory effect of KO2 on the activity of the CPB enzymes may be responsible for this effect. Cysteine proteinase inhibitors including KO2 (Selzer *et al.*, 1999) have been demonstrated to perturb the functioning of lysosomal enzymes such as the CPB isoenzymes. This has been shown to cause the accumulation of cell debris in the lysosomes which is consistent with the turnover of cell protein being reduced in their presence and also the results of my study.

This reduction in turnover by the cysteine proteinase inhibitor was demonstrated for gp63, however it probably extends to a range of cellular proteins. The CPB enzymes are cathepsin L-like in nature and these enzymes have been demonstrated to play an important role in cellular protein catabolism (Mayer and Doherty, 1986). Thus is follows that inhibition of their activity (or loss of expression via mutation) would lead to a reduction in the removal of many cellular proteins. This phenomenon could have very important consequences in the infection process; by altering the distribution of surface molecules or the fluidity or charge of the membrane, the receptor-mediated uptake of promastigotes into macrophages could be perturbed. Thus the loss of the CPB enzyme activity could lead to parasite death, rather than survival in this way.

Although as discussed elsewhere (see Chapter 5, for details) the major anti-leishmanial effects of KO2 appear to occur after establishment of infection in macrophages *in vitro*, loss of the CPB enzymes is thought to effect the initial stages of infection (Mottram *et al.*, 1996). Thus it is possible that reduced turnover from the surface of the Δ*CPB* promastigotes could be an important

factor in the reduced virulence observed. Evidence from several studies (for example Da Silva *et al.*, 1989) suggests that virulent metacyclic *L. major* promastigotes enter macrophages via a different receptor-ligand interaction to avirulent multiplicative forms, indicating the importance with respect to subsequent survival of the receptor type involved in uptake.

Further confirmation of the GPI-positive status of gp63 was attempted using overnight incubation of the promastigotes in tritiated ethanolamine (in the presence or absence of K02) to label the GPI-anchors of the promastigotes before immunoprecipitation with specific anti-gp63 antibodies. However, the low protein yield after precipitation meant that it was impossible to determine from the experiments whether the proteins were GPI-anchored.

Studies of the surface expression of gp63 in GPI8 deficient mutants of *L. mexicana* have recently demonstrated that in the absence of GPI8 activity there is no detectable surface expression of gp63 and that the enzyme is probably retained within the endoplasmic reticulum of the parasite and degraded (Hilley *et al.*, 1999). Although knockout studies cannot be directly compared to my investigations (removal of GPI8 may affect the recognition of GPI-addition sites by GAA1, whereas inhibition may allow GAA1 to bind the protein, thus giving differences in phenotype observed), the evidence of Hilley et al., together with my results on the surface location of gp63 and its sensitivity to PIPLC treatment suggest that KO2 is not affecting the addition of the GPI-anchor to gp63. Thus KO2 appears not to be an inhibitor of GPI8 activity and so its effects are mediated in other ways.

Chapter 5

The effect of peptidyl cysteine proteinase inhibitors on the major life-cycle stages of *Leishmania mexicana*

5 1 Introduction.

Leishmania species have a two host life-cycle which is discussed in detail elsewhere (see Chapter 1, section 1.1.2). Cysteine proteinases have proved to be of importance in several parasitic infections (see Chapter 1, for more detail). The invasive form of *Entamoeba histolytica*, for example, has been demonstrated to have substantially higher cysteine proteinase expression than the non-invasive isoform (Bruchhaus *et al.*, 1996). Interest in the cysteine proteinases of *Leishmania mexicana* originated with the observation that the amount of cysteine proteinase expression increased dramatically in the amastigote stage of the parasite (North and Coombs, 1981; Pupkis and Coombs; 1982; Lockwood *et al.*, 1987). This indicated to investigators that the enzymes were potentially important in allowing the parasite to survive inside its macrophage host. Analysis of the phenotype of the cysteine proteinase null mutants created using targeted gene deletion (Mottram *et al.*, 1996) has demonstrated that certain cysteine proteinases, namely the CPB isoenzymes, are involved in allowing the promastigotes of *L. mexicana* to survive after infection of macrophages *in vitro*. Delayed lesion formation after infection with both promastigotes and amastigotes of Δ*CPB* was also observed *in vivo* (Frame, PhD thesis).

The CPB enzymes are encoded on an array of 19 genes and the enzymes are localised within the large lysosome organelles of the amastigotes, the megasomes (Pupkis *et al.*, 1986). Of these genes, only genes 1 and 2 from the array are expressed in stationary phase promastigotes (the *in vitro* equivalent of metacyclics, Mallinson and Coombs, 1984). The rest, with the exception of the pseudogene in position 19, are expressed in the amastigote stage of the parasite (Mottram *et al.*, 1997). Thus these CPB isoenzymes appear to be important in the mammalian stages of the parasite's life-cycle. Analysis of the substrate preferences of the enzymes has demonstrated them to be cathepsin L-like in nature, and a closer analysis has suggested that individual isoenzymes have subtly different peptide affinities (Robertson and Coombs, 1994). This perhaps indicates a range of roles within the parasite.

Another leishmanial cysteine proteinase expressed within the disease relevant stages of *L. mexicana* is CPA. This again is cathepsin L-like in its substrate specificity but is encoded from a single copy gene (Mottram *et al.*, 1992). Gene deletion studies have demonstrated that Δ*CPA* does not have reduced survival in macrophages *in vitro* (Souza *et al.*, 1994). However deletion of CPA from Δ*CPB* reduces further still the virulence of the CPB null, indicating that CPA in conjunction with CPB is important in intracellular survival (Alexander *et al.*, 1998).

Evidence suggests that inhibition of the cysteine proteinase activities of *Leishmania* reduces the viability of the parasites *in vitro* (Coombs *et al.*, 1982; Coombs and Baxter, 1982). This in conjunction with the evidence discussed above also indicates that blocking the cysteine proteinase activity during infection

may prove to be a useful approach in the design of future chemotherapeutic agents.

The use of specifically-designed peptidyl cysteine proteinase inhibitors to investigate the activity of the cysteine proteinases of several trypanosomatid organisms has generated some interesting information pertaining to the roles of the enzymes within the parasite. The effects of vinylsulphonebenzene derivatives (including KO2) on the viability of *T. cruzi* have indicated that inhibition of its major cysteine proteinase, cruzipain, prevents the parasite from completing its intracellular replicative cycle and leads to the cure of infected mice (Engel *et al.*, 1998a).

Analysis of the ultrastructural effects of these compounds indicated that inhibition of cruzipain causes a dilation in the Golgi cisternae, swelling of the endoplamsic reticulum and a proliferation of cytoplasmic vesicles. Evidence discussed elsewhere (see chapter 3) suggests that inhibition of cysteine proteinase activity prevents the autoprocessing of cysteine proteinase precursors and leads to the build-up of intermediate forms in the cell. The abnormalities in ultrastructure after incubation in KO2 perhaps suggest the accumulation of these precursors along the protein transport pathways of the cell. Such accumulation would obviously affect the intracellular trafficking of the cell, thus reducing its viability.

Similar effects were recently observed after treatment of *L. major* promastigotes with these inhibitors (Selzer *et al.*, 1999). The growth of the promastigotes was completely ablated and the onset of lesion formation was delayed *in vivo*. An accumulation of cell debris and multivesicular bodies in the lysosomes was observed and it was suggested that this was indicative of a

deficiency in lysosomal hydrolases, thereby indicating a role for the cysteine proteinases in digestion of cellular protein. Interestingly, an accumulation of cysteine proteinases was also detected in the flagellar pocket of treated *L. major*. This may reflect an accumulation of the enzyme precursors along a previously undescribed protein transport pathway or may indicate that the build-up of precursor forms causes the parasite to secrete these forms into the extracellular milieu. Several studies have also demonstrated the efficacy of peptidyl cysteine proteinase inhibitors against other parasitic organisms; these are discussed in Chapter 1, section 1.5.

The studies outlined above, give an indication of the wide ranging and potentially interesting effects that cysteine proteinase inhibition can have on parasites of the Trypanosomatidae group. It was therefore decided to investigate the effects of several cysteine proteinase inhibitors, including novel compounds with potential as chemically useful agents, on the *in vitro* growth and infectivity of *L. mexicana* parasites.

5.2. Materials and Methods.

5.2.1. Preparation of inhibitor stock solutions.

Two peptidyl cysteine proteinase inhibitors were analysed during this investigation; a vinylsulphonebenzene with a morpholine-urea-phenylalanine-homophenylalanine peptidyl group, termed KO2 (see Engel *et al.*, 1998a, for detail), and a diazomethane with a benzyloxycarbonyl-phenylalanine-alanine peptidyl group (termed ZFA). KO2 was a gift from Jim McKerrow (San

Fransisco) and ZFA was purchased from Sigma Chemicals. Stock solutions of the inhibitors were made at 5 mg ml^{-1} in dimethylsulphoxide (DMSO) (approximately 9 mM) and stored until required at -20°C. Due to the apparent similarities in the effects observed initially for both inhibitors, it was decided to concentrate on the analysis of a single inhibitor, namely KO2, in later experiments.

5.2.2. Analysis of the effects of peptidyl cysteine proteinase inhibitors on the growth of *Leishmania mexicana* promastigotes and axenic amastigotes *in vitro*.

The effect of ZFA and KO2 on the growth of wild-type and various mutant lines of *L. mexicana* promastigotes was investigated. Promastigote cultures were started at a density of 4×10^5 ml^{-1} and grown in sterile 5 ml bijoux bottles at 25°C as described elsewhere (see Chapter 2, section 2.1.1.1). The density of the cultures was counted using an improved Neubauer haemocytometer every day from the initiation of the experiment until the parasites reached apparent stationary phase.

The effect of the inhibitors at 10 μg ml^{-1} and 25 μg ml^{-1} (approximately 18 μM and 45 μM, respectively) was assessed using either a single addition at the initiation of the experiment or by maintaining high drug-pressure by resuspension of the parasites in fresh medium with inhibitor every day.

The effect of an initial application of inhibitor at 45 μM on the growth of axenic amastigotes was also investigated. Cultures were seeded at 1×10^6 ml^{-1} in complete Schneider's Drosophila Medium (see chapter 2, section 2.2.1.2, for

details) and passed through a 19G needle before counting with an improved Neubauer haemocytometer daily for three days.

The experiments were repeated three times in duplicate and the mean log density and the standard deviation was calculated.

5.2.3. Analysis of the intra-macrophagic infection dynamics of stationary phase promastigotes and axenic amastigotes in the presence of 45 μM KO2.

The effect of 45 μM KO2 was investigated using *in vitro* infections of peritoneal exudate cells from BALB/c mice (see Chapter 2, sections 2.3.1.1 and 2.3.1.2, for details). The inhibitor was used in two ways. Firstly, the effect of adding the inhibitor to parasite cultures 10 min prior to infection was analysed (the inhibitor was re-applied to the culture after the peritoneal exudate cells were washed to remove unbound promastigotes at 4 h, see Chapter 2, section 2.3.1.2, for details). Secondly, the effect of addition of the inhibitor at different time points during the infection was analysed, with the inhibitor being added at 24 h and 72 h post-infection (p.i.).

All experiments were repeated at least 3 times in duplicate and the percentage of infected cells together with the average number of amastigotes per infected cell were determined for at least 200 macrophages per time point. The results were analysed as the mean numbers infected ± standard deviation. The percentage of inhibition was calculated for each separate experiment and its statistical significance was assessed.

5.2.4. Statistical Analysis.

The results were analysed using either a student's t test, if the variance of the samples proved to be equal or a heteroscedastic t test if unequal variance was demonstrated using variance ratio F test. In some cases, one way ANOVA was employed to evaluate the relationship between more than two samples. An example of the statistics used in this chapter are presented in Appendix 1.

5.3. Results.

5.3.1. The effects of peptidyl inhibitors on the growth of *L. mexicana* promastigotes.

The inhibitors had no effect on the growth of either wild-type or Δ*CPB* promastigotes at either 18 μM or 45 μM (the results with the higher concentration are given in Figure 5.1), and the maintenance of high drug pressure was similarly ineffective (data not shown). The growth of the wild-type and Δ*CPB* promastigotes was conducted twice and as there was no difference in growth between these experiments or between the control and inhibitor-grown cultures, it was deemed unnecessary to repeat the experiment a third time. The growth of Δ*CPB/g2.8* promastigotes appeared to be reduced in the presence of the inhibitors, however statistical analysis indicated that this difference was not significant ($t_2 = 3.98$, $P > 0.05$).

5.3.2. The effects of cysteine proteinase inhibitors on the growth of axenic amastigotes.

Both ZFA and KO2 had inhibitory effects on the growth of wild-type axenic amastigotes over the period of investigation (Figure 5.2). By day 3, ZFA had caused the amastigotes to have a 61 ± 3 %, ($t_4 = 5.065$, $P < 0.01$) reduction in cell density, whereas KO2 had reduced cell density, as compared to the control, by 55 ± 1 % ($t_4 = 4.018$ $P < 0.01$). The effect of ZFA was significantly greater than that of KO2 ($t_4 = 3.56$ $P < 0.05$). However, several factors, including inhibitor availability and similarities in effect observed elsewhere (see Chapter 3 for detail), meant that the effect of just one inhibitor, namely KO2, was analysed with respect to the various null mutant parasites.

Interestingly, KO2 also caused a reduction in the growth of *ΔCPB* axenic amastigotes (Figure 5.3) - 78 ± 17 % fewer parasites were present after the three day experiment ($t_6 = 2.93$, $P < 0.05$). Analysis of the data, however, demonstrated that in the presence of KO2 there was no significant increase in cell density ($t_6 = 0.09$, $P > 0.1$), indicating that KO2 prevented the multiplication of *ΔCPB in vitro*. The effect of KO2 on the growth of axenic amastigotes of *ΔCPB/g2.8* was not analysed, as the low pH of the medium required for axenic amastigote growth prevents the use of antibiotics to select for expression of the transfected gene on its episome. Thus, there would be a variable level of expression of this enzyme in amastigotes of this line making interpretation of the data impossible. This unfortunately makes it impossible to be sure whether the

enhanced susceptibility of Δ*CPB* to KO2 is due to the lack of CPB or to some uncharacterised effects of the electroporation process.

To investigate further how KO2 mediates this inhibitory effect on the multiplication of Δ*CPB* amastigotes, the effect of this inhibitor on several other null mutant lines was analysed. The effect of 45 μM KO2 on the growth of Δ*CPA* axenic amastigotes was analysed to investigate whether the inhibitors effects on CPA were responsible for the toxicity to Δ*CPB*. The results are shown in Figure 5.4 and demonstrated that by day 3 of exposure to KO2 there was a 53 ± 14 % decrease in cell density in the presence of KO2 when compared with the control ($t_4 = 5.9$, $P < 0.01$). However, the reduction in cell density observed by day 3 in the presence of KO2 was not significant ($t_4 = 2.72$, $P > 0.05$). Notably growth of Δ*CPA* was slow even in the absence of KO2 indicating that the inhibitor was not, at this stage in the infection causing parasite death. The experiment was not extended to day 4, so it is uncertain whether this effect became significant after this point.

Analysis of the effects of KO2 on the growth of axenic amastigotes lacking in both CPA and CPB (Δ*CPA/CPB*), demonstrated that a 69 ± 6 % reduction in cell density occurred by day 3 when compared with the control ($t_4 = 4.3$, $P < 0.02$)(Figure 5.5). No significant change in cell density was observed in the presence of KO2 after initiation of experiment ($t_2 = 1.3$, $P > 0.1$), suggesting that the presence of KO2 was preventing parasite multiplication.

Despite the fact that KO2 inhibited the multiplication of all the null mutant parasite lines examined, whilst only exerting some inhibition of growth on the wild-type axenic amastigotes, no significant difference was found between the

percentage inhibition observed for any of the lines analysed ($F_{3,9} = 3.53$, $P > 0.05$). The fact that all three null mutant lines multiplied at a lower rate that the wild-type may account for this factor.

Another cysteine proteinase known to be expressed in the amastigote stage of the life-cycle is the GPI:protein transamidase GPI8, discussed in chapter 4. Hilley *et al.*, (1999) cloned, characterised and produced a null mutant (Δ*GPI8*) for this gene by targeted gene deletion. The effects of KO2 on the axenic amastigotes on Δ*GPI8* and a line re-expressing GPI8 (Δ*GPI8/gpi8*) was investigated. The data shown in Figure 5.6 demonstrated that the presence of KO2 had no effect on the growth of either line examined ($t_4 = 1.5$, $P > 0.1$, and $t_4 = 1.4$, $P > 0.1$, respectively). This was unexpected as wild-type axenic amastigotes were affected and the results discussed in Chapter 4 demonstrated that KO2 has no inhibitory effect on the GPI:protein transamidase.

5.3.3. The effects of cysteine proteinase inhibitors on the infection of macrophages by stationary phase promastigotes *in vitro*.

The effect of KO2 on the ability of wild-type stationary phase promastigotes to successfully infect macrophages was determined. No investigation was made of the effect of the KO2 inhibition on the infectivity of Δ*CPB* stationary phase promastigotes as this line survives in only a small number of cells (Mottram *et al.*, 1996) and so analysis of any reduction in infection was not practical. The results of this investigation are shown in Figure 5.7. By 8 h post infection the wild type promastigotes had infected 33 ± 9 % of the

macrophages present. This level remained effectively constant throughout the 168 h (7 day) experiment and therefore follows the expected pattern of infection *in vitro*. The presence of 45 μM KO2 from the initiation of infection (following a brief pre-incubation of the promastigotes) did not significantly reduce the ability of the parasite to be taken into macrophages as there is no significant difference in the numbers of infected cells by 8 h ($t_4 = 1.17$, $P > 0.1$). The inhibitor did, however, appear to perturb the parasite's ability to survive once an infection had been established as by 24 h p.i. there was a significant reduction in the numbers of cells infected compared with control infection levels ($t_4 = 3.18$, $P < 0.02$). This apparent reduction was a transient phenomenon, however, as by 72 h p.i. similar levels of infection were achieved in the two infections ($t_4 = 0.05$, $P > 0.1$). Nevertheless, by 168h p.i. there was a dramatic difference in the numbers of infected cells in the presence of inhibitor - a total reduction of 42 ± 14 % ($t_4 = 4.85$, $P < 0.01$). These data indicate that the inhibitors are affecting the viability of the parasite within its macrophage host cell.

Analysis of the effect of the inhibitor when added at different time points showed that the time of application did not affect the overall reduction in survival by 168 h ($F_{2,6} = 1.12$, $P < 0.05$). This demonstrated that the inhibitor was not acting to prevent the establishment of infection, infections were well established by 72 h p.i., but that it appeared to be acting by causing the death of amastigotes already established in cells.

The presence of KO2 throughout infection also caused a three-fold reduction in the numbers of amastigotes per infected cell by 168 h p.i. ($t_4 = 5.92$, $P < 0.01$). No difference was observed in this reduction by applying KO2 at either initiation of infection or subsequently ($F_{2,6} = 0.29$, $P < 0.05$). This again

indicates that KO2 acts on established amastigotes rather than during the initial stages of infection. Further analysis of the data suggested that this effect was mediated, at least in part, by the inhibitor acting to prevent parasite growth within the cells as no statistically significant change in the numbers of amastigotes/infected cell was seen in the presence of inhibitor after addition of KO2 at 24h (t_4 = 1.14, P > 0.1) or at 72 h (t_4 = 1.37, P > 0.1). This phenomenon, however, may reflect in part the low numbers of amastigotes observed during the experimentation preventing any significant drop in numbers being detectable.

5.3.4. The effects of cysteine proteinase inhibitors on the dynamics of infections initiated by axenic amastigotes *in vitro*.

An investigation of the effect of 45 μM KO2 on the infectivity of axenic amastigotes was undertaken. The results are shown in Figure 5.8. The wild-type amastigotes in the absence of KO2 infected approximately half of the exudate cells and the level of infection remained relatively constant throughout the period of analysis. When the wild-type amastigotes were pre-incubated for 10 min with KO2 prior to infection (the inhibitor was then present throughout), there was no statistically significant effect on the ability of the parasites to be taken up into macrophages (t_4 = 0.16, P > 0.1). At 24 h the parasite was surviving in comparable numbers of macrophages regardless of the presence of KO2 (t_4 = 0.58, P > 0.1). Indeed there was no significant reduction in the percentage of parasites surviving in the presence of inhibitor until 168 h p.i. by when the

percentage infection had dropped form 62 ± 11 % to 15 ± 12%, a reduction in survival of 77 ± 16% ($t_4 = 5.08$, $P < 0.01$).

The time of addition of KO2 to the infection had no effect on the percentage survival by 168 h of infection ($F_{2,6} = 1.93$, $P > 0.05$). Addition of KO2 after 24 h caused a reduction in the numbers of infected cells of 61 ± 28% ($t_4 = 2.99$, $P < 0.05$). Addition of KO2 after 72 h of infection lead to a reduction in the percentage of infected cells - a total reduction in survival of 43 ± 6% ($t_4 = 3.59$, $P < 0.05$). This again suggests that the inhibitor is not acting against the initial stages of infection but acts against established amastigotes within the macrophages.

The effect on the numbers of amastigotes/infected cell was also analysed. Whether the inhibitor was present throughout the infection or added subsequently, there was an approximately three-fold reduction in the numbers of amastigotes/infected cell by day 7 ($t_4 = 5.07$, $P < 0.01$). There was a similar reduction of amastigotes per infected cell irrespective of the time of addition of KO2 ($F_{2,6} = 0.78$, $P > 0.05$). It seems probable that the inhibitor was causing amastigote death as both the number of infected macrophages decreased and, if KO2 is not applied until 72 h p.i. then by 168 h the numbers of amastigotes per infected cell has significantly decreased ($t_4 = 6.56$, $P < 0.01$).

Unlike Δ*CPB* promastigotes, the axenic amastigote form of this mutant gives similar levels of infection to the wild-type parasite. It was therefore decided to analyse the effect of KO2 on the survival of Δ*CPB* in macrophage infections to investigate whether it was susceptible under these conditions as well as during axenic culture.

The course of infection with Δ*CPB* axenic amastigotes in the presence of 45 μM KO2 from the initiation of infection was similar to that observed during infection with wild-type parasites. The results are shown in Figure 5.9. There was a significant reduction in the percentage of infected cells at 168 h p.i. The overall reduction in survival in this case is 71 ± 42 % (from 59 ± 8 % to 8 ± 4 %, $t_4 = 10.72$, $P < 0.01$). This was not significantly different from the reduction observed with wild type cells ($t_4 = 0.31$, $P > 0.1$). When the inhibitor was added after 24 h of infection, the percentage of infected cells was apparently reduced by 72 h (from 54 ± 3 % to 21 ± 10 %, $t_4 = 5.4$, $P < 0.01$). However, the overall effect by 168 h p.i. was not significantly different from that with wild-type parasites ($t_4 = 0.66$, $P > 0.1$). This suggested that although Δ*CPB* is more quickly effected by KO2 its overall susceptibility is similar. This phenomenon was demonstrated again by addition of KO2 after 72 h of infection. The overall reduction in survival was 72 ± 5 %, which is of the same magnitude as when KO2 was applied either throughout the infection or from 24 h onwards. However, this is a larger reduction than was seen in the comparative study with wild-type cells ($t_4 = 6.67$, $P < 0.01$). This again demonstrates that the null mutant is affected more rapidly. As was demonstrated for wild-type amastigotes, the overall reduction in infectivity was not dependent on the time of application of KO2 ($F_{2,6} = 0.65$, $P > 0.05$).

The data given in Figure 5.10, however, casts some doubt on the otherwise interesting differences observed between the wild-type parasite and Δ*CPB* with respect to susceptibility to cysteine proteinase inhibitors. Δ*CPB/g2.8* axenic amastigotes were used in order to verify that the differences in

susceptibility were due to the loss of the *CPB* array as opposed to other uncharacterised differences arising from the electroporation procedure. However, the data indicate that this mutant line displays similar susceptibilities to Δ*CPB*, addition of KO2 after 24 h of infection caused a reduction in the numbers of infected cells by 72 h ($t_4 = 4.09$, $P < 0.01$).

5.3.5. The effect of vinyl sulphone derivatives on the growth of *L. major* promastigotes *in vitro*.

Investigation of the effects of KO2 on the *in vitro* growth of *L. major* promastigotes (Selzer *et al.*, 1999) demonstrated that concentrations as low as 5 μM could inhibit growth and 20 μM completely prevented the promastigotes multiplication *in vitro*. The data presented earlier in this chapter showed that *L. mexicana* promastigotes multiplied as normal even in the presence of 45 μM of KO2. These contrasting results suggest, interestingly, that there may be species-specific effects of cysteine proteinase inhibitors on *Leishmania* species. To confirm this, the growth rate of *L. major* promastigotes was investigated in the presence of KO2 at 18 μM and 45 μM.

The results illustrated in Figure 5.11 demonstrate that, surprisingly, neither concentration of KO2 had any effect on the growth of *L. major* promastigotes *in vitro*. Two possibilities were considered as explanations for the difference between my results and those of Selzer *et al.* Firstly it seemed feasible that our batch of KO2 may have been labelled incorrectly before delivery. In order, therefore, to confirm that our inhibitor was indeed KO2, a new batch of

the inhibitor was analysed in the same way. The data shown in Figure 5.12 demonstrated that the new and original batches of KO2 differed in their ability to inhibit the growth of *L. major* promastigotes. It was considered unlikely that the original batch of KO2 had lost its activity, because no difference had been observed in any of the other effects documented elsewhere in this thesis. Furthermore, the compound was certainly an effective cysteine proteinase inhibitor as documented in multiple experiments. This suggested that the first batch received was slightly different in some way from the second, although analysis of the effect of the new batch on *L. mexicana* promastigotes demonstrated that the new batch still had no affect on growth. Differences in the production method of the two batches may have given rise to some impurities that could account for the anomalies in activity. Unfortunately there was an insufficient amount of the original batch available to allow proper analysis of its structure and purity.

A second factor considered was that the parasites are differently susceptible to these inhibitors in different culture media. Selzer *et al.*, (1999) routinely used RPMI-1640 supplemented with 10% (v/v) HI-FCS and 20% brain heart infusion tryptose. However, a comparison of the growth of *L. major* promastigotes in the two media demonstrated that there was no media-related increase in susceptibility to inhibition (data not shown).

An interesting observation was made when the new KO2 was analysed against *L. major* at 18 μM (see Figure 5.12). This concentration was comparable to that quoted in the literature as causing a complete inhibition of growth (20 μM). However, when tested by me, although an inhibitory effect was found, it was more similar to the inhibition Selzer reported using 5 μM. One explanation

for this is that there are also strain differences in susceptibility to cysteine proteinase inhibitors. Selzer *et al.*, (1999) used *L. major* LV39 strain for their analysis, whereas the strain I used was RKK2. The fact that I observed a slight reduction in growth rate at 18 μM whereas a similar reduction in growth was observed at 5 μM in the investigations published by Selzer *et al.*, suggests that *L. major* RKK2 is four times more resistant to inhibition by KO2 than *L. major* LV39. Such findings clearly emphasis the importance of studying each individual species of *Leishmania* and possibly several different strains from within that species when investigating the effects of potential chemotherapeutic precursors.

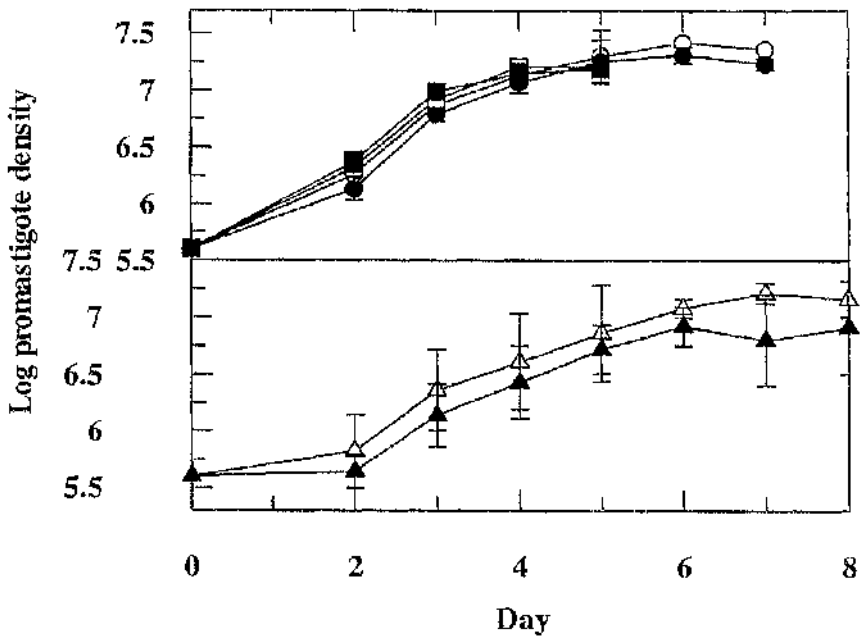

Figure 5.1: Graphical representation of the growth of *L. mexicana* promastigotes after a single, initial application of 45 μM KO2. Circles; wild-type parasites. Squares; Δ*CPB*. Triangles; Δ*CPB/g2.8*. Open symbols indicate control cultures. Solid symbols indicate cultures with a single application of KO2 at the start. The wild-type parasites and Δ*CPB* experiment was repeated twice in duplicate with error bars indicating the range. Δ*CPB/g2.8* was analysed three times in duplicate and the error bars indicate the standard deviation.

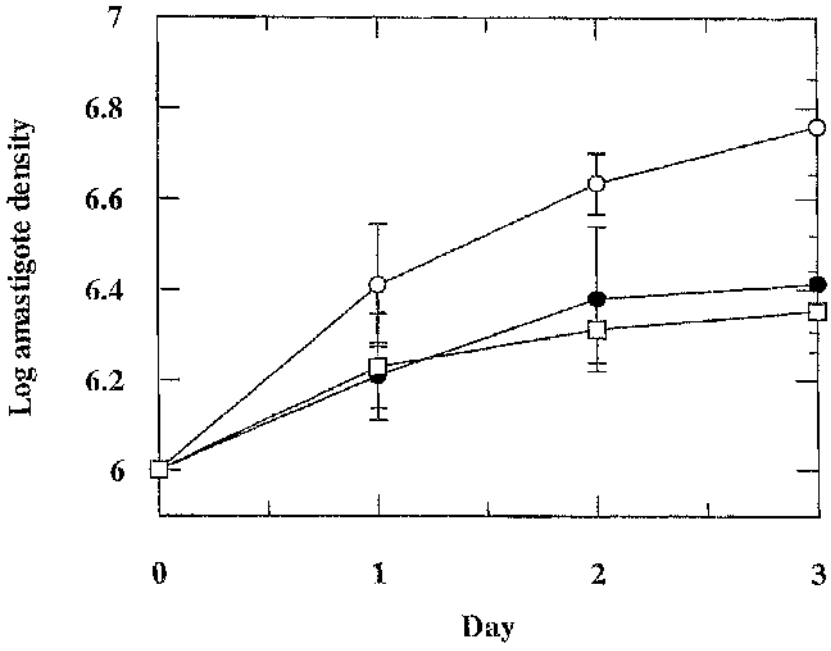

Figure 5.2: Graphical representation of the growth of wild-type axenic amastigotes after a single, initial application of various peptidyl cysteine proteinase inhibitors. Axenic amastigotes were grown with: no inhibitor (open circles) or a single dose of 45 μM KO2 (solid circles) or ZFA (open squares). The experiments were repeated three times in duplicate. The points represent the mean with error bars indicating the standard deviation

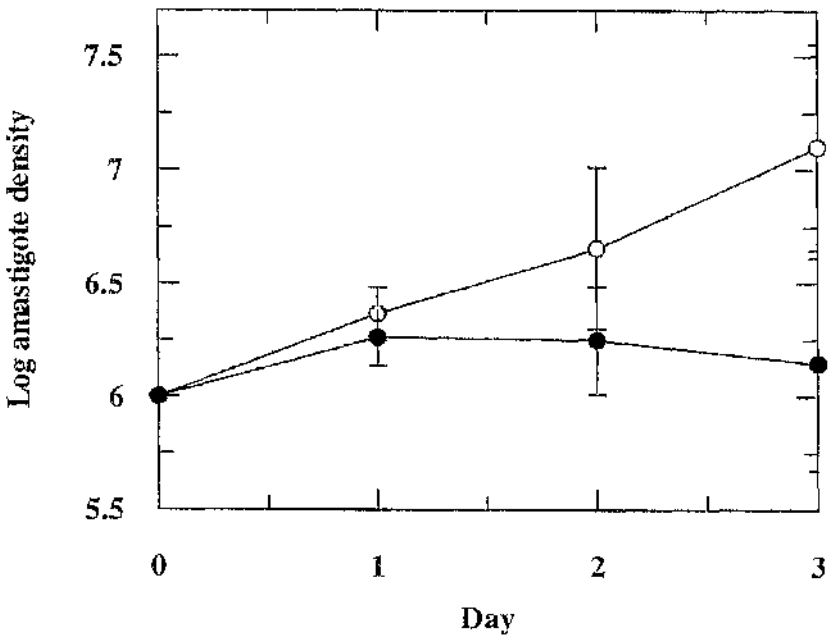

Figure 5.3: Graphical representation of the growth of Δ*CPB* axenic amastigotes after a single, initial application of 45 μM KO2. Open symbols; control. Solid symbols; grown with KO2. The experiment was repeated on three occasions in duplicate. The points represent the mean with error bars indicating the standard deviation.

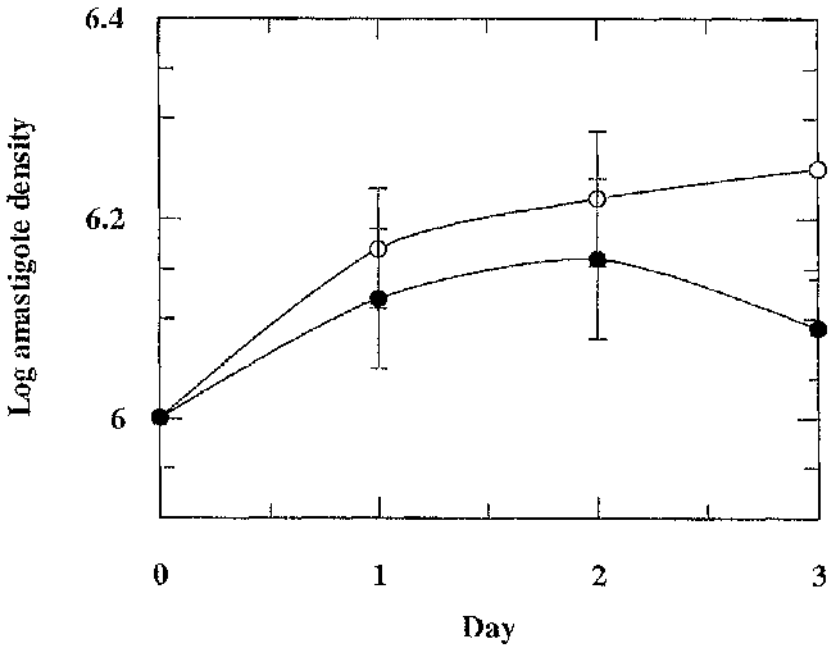

Figure 5.4: Graphical representation of the growth of Δ*CPA* axenic amastigotes after a single, initial application of 45 μM KO2. Open symbols; control. Solid symbols; with KO2. The experiment was repeated three times in duplicate. The points represent the means with error bars indicating the standard deviations.

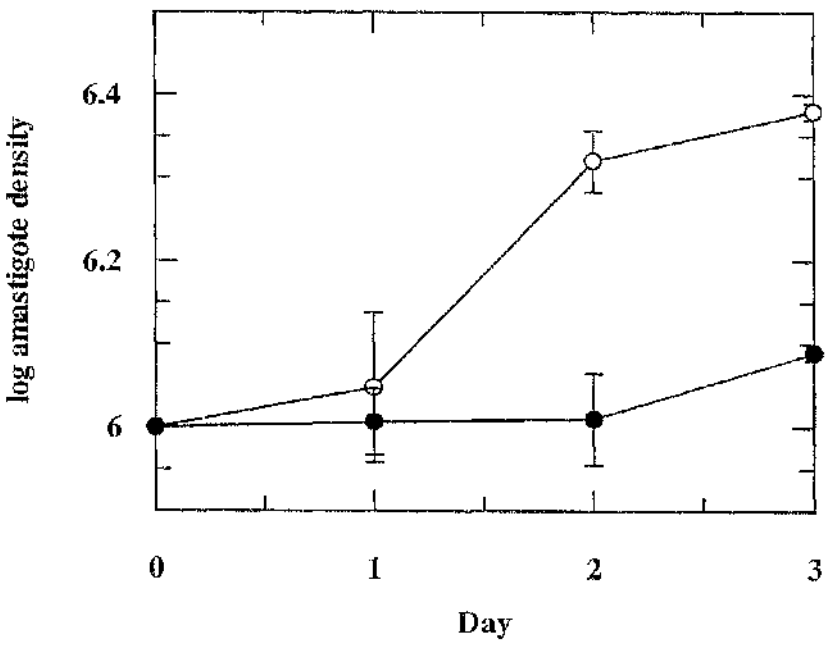

Figure 5.5: Graphical representation of the growth of Δ*CPA/CPB* axenic amastigotes after a single, initial application of 45 μM KO2. Open symbols; control. Solid symbols; with KO2. The experiment was repeated three times in duplicate. The points indicate the means with error bars indicating the standard deviations.

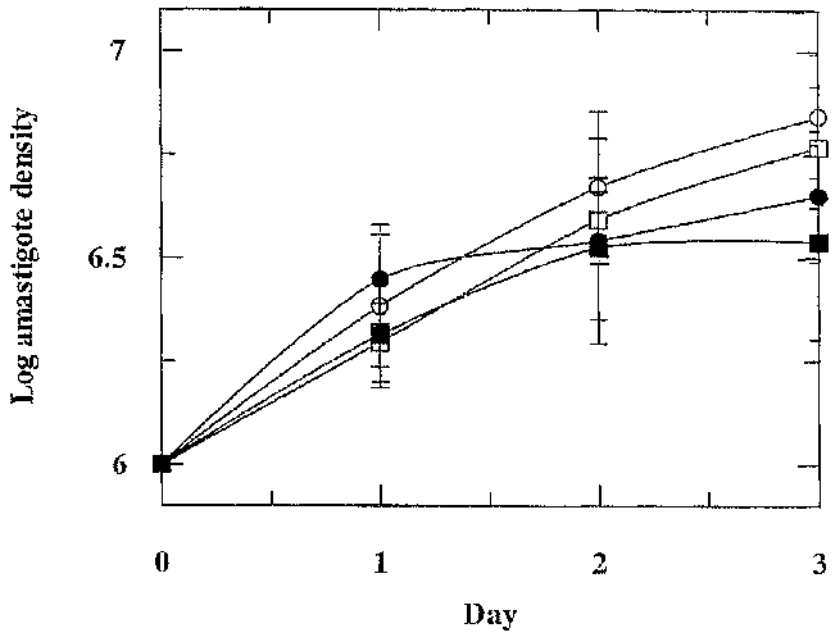

Figure 5.6: Graphical representation of the growth of *GPI8* null mutant axenic amastigotes after a single, initial application of 45 μM KO2. Circles; Δ*GPI8*. Squares; Δ*GPI8/gpI8*. Open symbols indicate control cultures. Solid symbols indicate cultures with KO2. The experiment was repeated three times in duplicate. The points indicate the means with error bars representing standard deviations.

A

	% infected cells				% inhibition
	8 h	24 h	72 h	168 h	168 h
control	33.3 ± 9.2	35.8 ± 6.0	30.7 ± 4.4	33.6 ± 2.7	
KO2 0 h	26.4 ± 4.8	20.8 ± 5.6♣	27.9 ± 8.3	19.3 ± 4.3♣	41.6 ± 14.0♣
KO2, 24 h			22.8 ± 6.4	22.4 ± 6.4♣	27.6 ± 3.5♣
KO2, 72 h				15.9 ±11.8♣	51.0 ± 30.1♣

B

	Number of amastigotes/infected cell			
	8 h	24 h	72 h	168 h
control	2.8 ± 0.9	2.3 ± 0.7	4.4 ± 0.8	6.0 ± 1.0
KO2, 0 h	1.6 ± 0.1	1.6 ± 0.1	2.8 ± 1.1	2.1 ± 0.5♣
KO2, 24 h			2.4 ± 0.5	2.3 ± 0.4♣
KO2, 72 h				3.1 ± 1.8♣

Figure 5.7: The effects of 45 μM KO2 on the infection dynamics of wild-type stationary phase promastigotes towards peritoneal exudate cells. A; table of the percentage of cells infected at each time point. B; table of the average number of amastigotes per infected cell. KO2 was added at initiation of infection (0 h), after 24 h, or after 72 h of infection. The experiment was performed in duplicate on three separate occasions and the data are means ± standard deviation. The significance of any observed difference between experiments and controls was assessed using t tests. ♣ significant at $P < 0.05$. ♠ significant at $P < 0.01$.

A

	% infected cells				% inhibition
	8 h	24 h	72 h	168 h	168 h
control	45.9 ± 10.2	44.7 ± 11.8	56.4 ± 12.7	61.6 ± 11.0	
KO2 0 h	45.9 ± 8.6	55.5 ± 12.5	48.6 ± 11.9	14.9 ± 11.5♠	76.5 ± 16.3♠
KO2, 24 h			33.5 ± 3.1	24.7 ± 18.2♠	60.5 ± 27.8♠
KO2, 72 h				35.6 ± 5.9♠	42.5 ± 5.9♠

B

	Number of amastigotes/infected cell			
	8 h	24 h	72 h	168 h
control	3.9 ± 0.8	3.6 ± 0.8	5.1 ± 0.4	6.7 ± 1.2
KO2, 0 h	2.8 ± 0.3	2.7 ± 0.4	3.7 ± 1.9	2.4 ± 0.8♠
KO2, 24 h			3.1 ± 0.6	2.4 ± 1.0♠
KO2, 72 h				3.1 ± 0.3♣

Figure 5.8: The effects of 45 μM KO2 on the infection dynamics of wild-type axenic amastigotes towards peritoneal exudate cells. A; table of the percentage of cells infected at each time point. B; table of the average number of amastigotes per infected cell. KO2 was added at initiation of infection (0 h), after 24 h, or after 72 h of infection. The experiment was performed in duplicate on three separate occasions and the data are means ± standard deviation. The significance of any observed difference between experiment and control was assessed using t tests. ♣ significant at $P < 0.05$. ♠ significant at $P < 0.01$.

A

	% infected cells				% inhibition
	8 h	24 h	72 h	168 h	168 h
control	40.9 ± 5.4	51.8 ± 6.4	54.2 ± 2.6	59.4 ± 7.5	
KO2 0 h	44.3 ± 12.4	43.7 ± 6.2	35.8 ± 22.2	7.8 ± 3.8♠	70.7 ± 41.9♠
KO2, 24 h			21.2 ± 10.3♠	9.9 ± 2.8♠	71.9 ± 11.0♠
KO2, 72 h				10.4 ± 4.2♠	72.0 ± 4.7♠

B

	Number of amastigotes/infected cell			
	8 h	24 h	72 h	168 h
control	2.9 ± 0.9	3.2 ± 0.4	3.7 ± 0.3	4.34 ± 0.9
KO2, 0 h	2.8 ± 0.1	2.9 ± 0.2	2.7 ± 0.7♠	1.8 ± 0.5♠
KO2, 24 h			2.4 ± 0.3♠	1.9 ± 0.4♠
KO2, 72 h				1.8 ± 0.5♠

Figure 5.9: The effects of 45 μM KO2 on the infection dynamics of Δ*CPB* axenic amastigotes towards peritoneal exudate cells. A; table of the percentage of cells infected at each time point. B; table of the average number of amastigotes per infected cell. KO2 was added at initiation of infection (0 h), after 24 h, or after 72 h of infection. The experiment was performed in duplicate on three separate occasions and the data are mean ± standard deviation. The significance of any observed difference between experiment and control was assessed using t tests. ♣ significant at $P < 0.05$. ♠ significant at $P < 0.01$.

A	% infected cells			% inhibition
	24 h	72 h	168 h	168 h
control	44.8 ± 6.6	57.5 ± 5.9	54.7 ± 9.9	
KO2, 0 h	44.9 ± 6.6	50.7 ± 9.2	15.8 ± 14.6	69.6 ± 29.2♠
KO2, 24 h		39.2 ± 6.7♠	15.5 ± 6♠	71.9 ± 11.1♠
KO2, 72 h			15.7 ± 4.4♠	72.0 ± 4.7♠

B	Number of amastigotes/infected cell		
	24 h	72 h	168 h
control	3.5 ± 0.9	4.9 ± 0.8	6.3 ± 0.8
KO2, 0 h	3.5 ± 0.6	3.2 ± 0.5♠	3.2 ± 0.8♠
KO2, 24 h		3.3 ± 0.6♠	2.4 ± 0.8♠
KO2, 72 h			2.4 ± 0.6♠

Figure 5.10: The effects of 45 μM KO2 on the infection dynamics of Δ*CPB/g2.8* axenic amastigotes towards peritoneal exudate cells. A; table of the percentage of cells infected at each time point. B; table of the average number of amastigotes per infected cell. KO2 was added at initiation of infection (0 h), after 24 h, or after 72 h of infection. The experiment was performed in duplicate on three separate occasions and the data are means ± standard deviation. The significance of any observed difference was assessed using t tests. ♠ significant at $P < 0.01$.

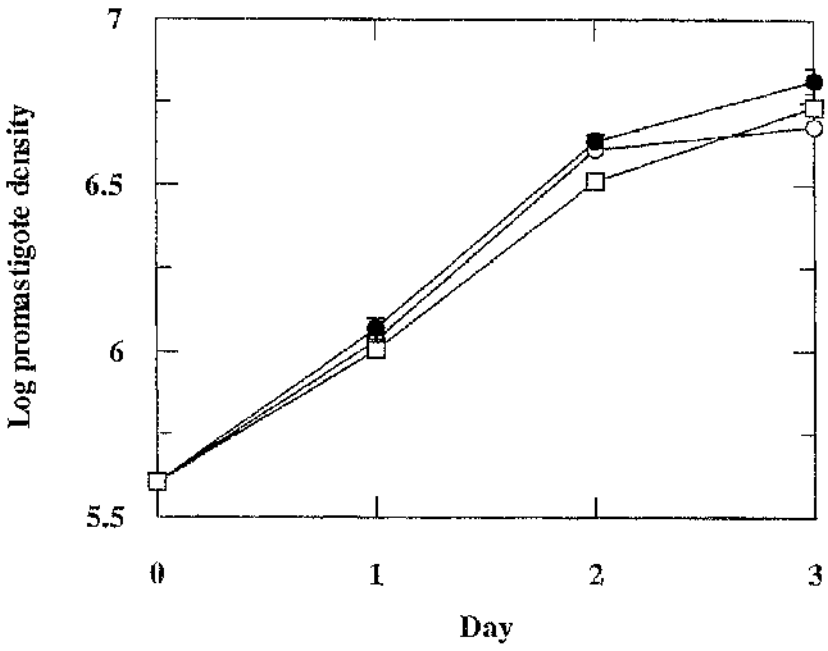

Figure 5.11: Graphical representation of the growth of *L. major* promastigotes after a single, initial application of KO2. Open circles; control. Solid circles; grown with 18 μM KO2. Open squares; grown in 45 μM KO2. The experiments were repeated twice in duplicate. The points represent the means and the error bars indicate the ranges.

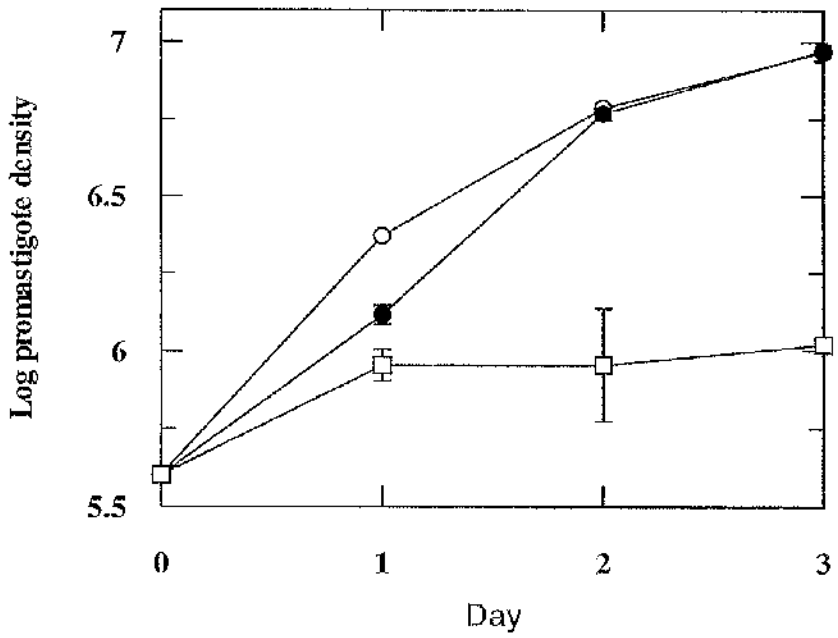

Figure 5.12: A comparison of the growth of *L. major* promastigotes in two batches of KO2. Open circles; control. Solid circles; grown in 18 μM of the original batch. Open squares; grown in 18 μM of the new batch. The experiments were repeated twice in duplicate. The points indicate the means with error bars indicating the ranges.

5.4. Discussion.

This chapter has dealt with an investigation of the effects of peptidyl cysteine proteinase inhibitors on the viability of the different life-cycle stages of *L. mexicana*. Although originally three inhibitors were to be analysed, it became clear from early data (see chapter 3) that all three inhibitors had similar effects. An analysis of ZFA and KO2 in preliminary studies indicated that their effects were comparable. It was therefore decided to concentrate on an analysis of the activity of KO2 as a representative of this group.

The first step in analysing the effects of inhibition of the cysteine proteinase enzymes of the parasite was to determine what, if any, effect KO2 was having on the parasite's axenic growth. Analysis of the effect of different concentrations under different regimens on the growth of the promastigote form of the various strains demonstrated that the inhibitor had no effect on the multiplication of this stage of the parasite's life-cycle.

The lack of effect on the promastigotes was expected due to the fact that null mutants have been successfully produced for each of the cysteine proteinase genes characterised in *L. mexicana* to date (Souza *et al.*, 1994; Mottram *et al.*, 1996; Hilley *et al.*, 1999) suggesting that the cysteine proteinases are not essential for *in vitro* survival of promastigotes. Further to this, the promastigotes have little cysteine proteinase expression (Souza *et al.*, 1992). It is therefore not surprising that inhibitors which effect these enzymes will have little or no effect on the promastigote stage of the life-cycle as the build-up of the potentially toxic precursor enzymes will be small (see Chapter 3). Interestingly, however this build-up of precursor forms was demonstrated to cause the formation of large

vacuoles within the promastigotes. It is perhaps surprising that such ultrastructural alterations, which were postulated to perturb intracellular trafficking, have no adverse effect on promastigote growth.

Amastigotes of *L. mexicana* have been demonstrated to express high levels of cysteine proteinase activity (North and Coombs, 1981; Pupkis and Coombs, 1984) and are therefore proposed to be important in the survival of the organism in this life-cycle stage. Although the cysteine proteinase null mutants analysed to date have been able to successfully transform into and grow as axenic amastigotes, it is possible that the build-up of precursor forms of the CPB isoenzymes observed when the promastigotes are grown in the presence of these inhibitors could perturb the organisms ability to survive as amastigotes (see Chapter 3). It was therefore considered interesting to analyse the effects of our cysteine proteinase inhibitors on the growth of axenic amastigotes.

Some interesting observations can be made concerning the effect of KO2 on the growth of axenic amastigotes. 45 μM of each of the inhibitors tested had a significant effect on the density of wild-type *L. mexicana* by day 3 of exposure. Thus the putative build-up of precursor forms within the parasite may be toxic to amastigotes; either directly or indirectly via perturbation of intracellular trafficking.

Interestingly, knocking out the *CPB* array or *CPA* from the parasite did not reduce its susceptibility to KO2 under the conditions analysed. It was surprising that loss of the *CPB* array did not alter the amastigotes susceptibility to inhibition. The loss of such abundantly expressed enzymes would obviously decrease the build-up of intermediate enzyme forms and thereby the ultrastructural changes would be reduced. Loss of these enzymes was postulated

therefore to relieve the parasite from some susceptibility to KO2. Similarly loss of *CPA* expression may have been beneficial to the parasite under the conditions analysed. Interestingly, loss of both *CPA* and *CPB* in conjunction also did not affect the parasites susceptibility to inhibition. This perhaps indicates that the inhibitor has effects against another parasite enzyme. This enzyme may be CPC, as although the majority of expression is in multiplicative promastigotes there a low level within the axenic amastigote stage (Bart *et al.*, 1995).

The results discussed above have demonstrated that inhibition of cysteine proteinases in the axenic amastigote form lead to some reduction in growth rate. It must be remembered, however, that the axenic amastigote stage of the life-cycle resides within a parasitophorous vacuole inside a macrophage host cell during the natural infection. It is probable that enzymes expressed in abundance in this stage will be necessary for the survival of the axenic amastigote within the host cell environment and therefore analysis of the effects of inhibition of such enzymes in axenic culture may have little or no relevance to the *in vivo* situation.

The effect of KO2 on the course of *in vitro* macrophage infections by *L. mexicana* was therefore analysed. In this study no analysis of the effects of KO2 on the macrophages themselves was undertaken, as extensive investigation of the effects of KO2 on mammalian macrophages *in vitro* and *in vivo* suggest that KO2 has no detrimental effect (Engel *et al.*, 1998a). The data presented concerning the infection of macrophages with wild-type stationary phase promastigotes suggests that the inhibitors have no effect on the initial uptake of the parasite. There was, however, a reduction in the number of cells infected by 24 h p.i. This reduction was transient as levels were similar to control by 72 h. This increase in infection was unlikely to be due to re-infection of macrophages

as the complete transformation from promastigotes to amastigotes has been demonstrated to take up to 5 days (Galvao-Quitao *et al.*, 1990) and after this at least 2 cycles of replication naturally occur, each lasting 18 h (Doyle *et al.*, 1989), before the amastigotes burst from the cells and re-infect new macrophages. Thus by 72 h, no re-infection would have occurred. The difference observed is more likely to reflect an inaccuracy in the system used. The difference in numbers of macrophages infected between 24 and 72 h is only slightly significant ($0.1 < P < 0.05$) and so experimental variation could have been a contributory factor.

The finding that the reduction caused by KO2 did not significantly change regardless of the time of its application suggested further that the inhibitor was not acting during the initial stages of infection, but rather acted primarily on already established amastigotes. The full transformation of internalised promastigotes to amastigotes is a lengthy process, thus the majority of the cysteine proteinase expression will not occur until well after the infection has begun (approximately 3 - 5 days post infection, [Galvao-Quitao *et al*, 1990]). Therefore the lack of effect of this inhibitor on the early stages of infection is not surprising. Indeed the anti-leishmanial effects of other cysteine proteinase-dependent agents, namely L-leucine methyl esters, on intracellular parasitism has been shown to increase with time of intracellular residence (Hunter *et al.*, 1992).

These data indicate that the effect of KO2 on parasite viability is not due mainly to its inhibitory effects on the CPB enzymes. This is consistent with other results (see Chapter 7 and Mottram *et al.*, 1996) which indicate that one role(s) of the CPB enzymes in infection is during the initial uptake of the promastigotes into macrophages, rather than during axenic amastigote infection, even though

the majority of CPB expression occurs during this latter stage. This suggests that other roles exist for the CPB isoenzymes. The build-up of cellular protein and membrane within the lysosomes of *L. major* parasites following KO2 exposure suggest that the cysteine proteinases of this parasite have a role in protein catabolism (Selzer *et al.*, 1999). It is feasible, therefore, that the CPB isoenzymes expressed within the axenic amastigote stage have similar roles.

Some of the results obtained concerning the effect of the inhibitor on the numbers of amastigotes/infected cell suggests it can affect the replication of amastigotes within the macrophage rather than killing the amastigotes. This may simply be a reflection of the inhibitors major effect being on the axenic amastigote stage of the life-cycle which does not completely form until several days after the infection is initiated., Thus the inhibitor may have cytostatic effects which eventually lead to cell death, but not within the experimental period. Investigation of the effects of KO2 on prolonged infections originating from promastigotes would enable clarification of certain points arising from this discussion.

The data discussed so far, suggest that the inhibitor has effects on established amastigotes within infected cells rather than hindering the initiation of infection. The dramatic reduction observed in the survival of axenic amastigote-derived infections of all lines analysed again suggests that axenic amastigote infections are more susceptible to inhibition. There was no effect on the uptake of amastigotes into macrophages; however, it must be borne in mind that the pre-incubation of the parasite with inhibitor was only short. The fact, however, that a similar inhibitory effect was observed whether the inhibitor was added at initiation of infection or subsequently suggests that the target of KO2 is not

required during initiation of infection. Analysis of the uptake of promastigotes incubated overnight in inhibitor may allow further analysis of this phenomenon.

The fact that survival of Δ*CPB* was reduced, as was that of wild-type parasites, indicates that the primary activity of KO2 is not against CPB. However, when the inhibitor was added to Δ*CPB* infection after 24 h, a reduction in survival was observed by 72 h p.i., indicating that loss of the CPB enzymes accelerated the induced death of the parasites. This effect may be due to the fact that loss of such abundantly expressed enzymes allows more of the inhibitor to bind to the other cysteine proteinases within the parasite. This suggests that the CPB enzymes may act as a sink for the inhibitor and reduce the effective dose. Nevertheless, the overall effect of KO2 on Δ*CPB* survival was similar to the effect observed for wild-type infection, suggesting that the increased susceptibility was due to the parasite experiencing the deleterious effects of KO2 more rapidly. This again suggests that the CPB enzymes act to protect the parasite from the effect of this inhibitor as loss of these enzymes quickened the onset of toxicity.

Some indication about the stability of KO2 can be inferred from the above data. Addition of KO2 to Δ*CPB* infections 24 h p.i. had a greater effect on percentage survival by 72 h than application from the start. This may indicate that the inhibitors target is essential from after 24 h of infection and that KO2 has limited stability in the system used. The amount of KO2 available after 24 h in culture would therefore be lower than the initial concentration, hence a prolonged period before the reduction in survival is observed if the inhibitor is

applied from the start of infection. This could have been investigated by renewal of the KO2 every 24 h, to see if the instability of KO2 is the cause.

The affect of KO2 on the survival of Δ*CPB/g2.8* was analysed and it was demonstrated that this line was of comparable susceptibility to Δ*CPB*. This was not expected due to the fact that Δ*CPB/g2.8* has wild-type virulence. However, if the CPB enzymes are acting as a sink for the inhibitor as discussed above, then re-expression of only one enzyme may not be enough to mop up sufficient KO2 to allow unaffected survival beyond 72 h.

The data presented in this chapter indicate that inhibition of *L. mexicana* cysteine proteinases leads to a reduction in the intracellular survival of the parasite. However, no information as to how this reduction is mediated exists. Cysteine proteinases are important in a number of parasitic systems (see chapter 1, section 1.4). Cysteine proteinases of *Plasmodium* species have been demonstrated to prevent the mature schizont from rupturing its host cell (reviewed by McKerrow *et al.*, 1993). It is unlikely, however, that the cysteine proteinases of *L. mexicana* are involved in a similar function as the effect of inhibition *in vitro* is observed long before any host cell lysis occurs. In other parasitic infections, for example, *E. histolytica* or *Plasmodium* species, the inhibition of cysteine proteinase activity has been shown to perturb the organism's acquisition of nutrition (Serrano-Luna *et al.*, 1998; McKerrow *et al.*, 1993). The lysosomal location of the leishmanial enzymes suggests that a role in nutrition is possible, therefore inhibition of the cysteine proteinase activity may simply starve the parasites.

KO2 has been demonstrated to cause the appearance of cysteine proteinases in the flagellar pocket of *L. major* (Selzer *et al.*, 1999). Perhaps in

the amastigotes of *L. mexicana* these enzymes are released into the parasitophorous vacuole in the presence of KO2 and thus cause alteration to this important micro-environment, and so reduce viability. The majority of macrophages infected are initially invaded by more than one amastigote. Therefore several parasitophorous vacuoles form, which could explain how some amastigotes survive within the cell whilst others are killed. Unfortunately, no evidence was obtained demonstrating that the surviving amastigotes were viable. Staining with viability dyes would elucidate further whether any live amastigotes survive to 168 h p.i.

Some investigations into the effects of inhibitors on *Leishmania* and related organisms have suggested that inhibition of cysteine proteinases can prevent the transformation between different life-cycle stages. For example, Coombs *et al.* (1982) demonstrated that leupeptin and antipain inhibited the transformation of *L. mexicana* promastigotes and Franke de Cazzulo *et al.* (1994) demonstrated that diazomethanes can prevent metacylcogenesis in *T. cruzi*. Thus it would be interesting to investigate the effects of KO2 on the transformation of *L. mexicana* promastigotes. However, data presented here suggests that the important inhibitory effects of KO2 occur after transformation during established amastigote infections within macrophages.

The data presented in this chapter indicates that the inhibition of *L. mexicana* cysteine proteinases causes a reduction in the viability of the parasite. Although no effect was observed on the growth of promastigote, the inhibitory effect of KO2 on the growth and survival of amastigotes *in vitro* demonstrates its effectiveness during the pertinent stage of the life-cycle. Although the effects of KO2 *in vitro* have been clearly demonstrated, the *in vivo* situation with regards

to *L. mexicana* requires investigation. So far the *in vivo* effects of KO2 on *L. major* and *T. cruzi* have been investigated (Engel *et al.*, 1998a; Selzer *et al.*, 1999). In the case of *T. cruzi*, treatment lead to a long term cure rate of 100 %. However, analysis of the situation with *L. major* suggested that KO2 only acted to prolong the time before lesion development. Indeed, the therapeutic effects disappeared after cessation of treatment and the lesion developed at a similar rate to the control.

Analysis in other systems has also demonstrated a similar phenomenon. *T. brucei* blood stream forms are susceptible to KO2 *in vitro* due to its activity against Trypanopain-Tb, the organism's lysosomal cysteine proteinase (Troeberg *et al.*, 1999). However, the use of diazomethanes *in vivo* has demonstrated that inhibition of Trypanopain-Tb only prolongs the life of infected mice and does not cure infection (Scory *et al.*, 1999).

This apparent lack of true effectiveness *in vivo* despite obvious *in vitro* efficacy casts doubt on the use of KO2 as a chemotherapeutic in the majority of systems. Further to this, the variability between systems, emphasises the necessity for *in vivo* trials against *L. mexicana*. The differences in susceptibility of *L. major* and *L. mexicana* promastigotes to KO2 inhibition, highlights the need to analyse the potential of compounds against individual species. Indeed, the apparent disparity in the susceptibility of different strains of *L. major* to inhibition indicates the requirement for several strains from within a species to be analysed.

In conclusion, KO2 appears to reduce the survival of *L. mexicana* within it's macrophage host cell by effecting enzymes other than those of the *CPB* array, thereby reducing the viability of the amastigotes within the parasitophorous

vacuole. There is some evidence that it's effects on CPA may be important in the reduction of virulence of the parasite. Interestingly, deletion of the *CPB* array or *CPA* does not reduce the parasites susceptibility to inhibition, indicating that the inhibitor is affecting other cysteine proteinases within the parasite. Analysis of the situation *in vivo* is necessary before any conclusions about the potential for KO2 as a chemotherapeutic candidate against *L. mexicana* can be made.

Chapter 6

The response of macrophages to infection with *L. mexicana* promastigotes *in vitro*

6.1. Introduction.

Pathogens have evolved a variety of strategies to prolong their survival within their host organism. *Leishmania* species survive by shielding themselves from the effector mechanisms of the immune system by residing intracellularly in the macrophages of their host. Prolonged infections are possible due to the parasite's ability to modulate the host immune response; studies in susceptible and resistant mice have demonstrated that the balance of cytokine production in the early stages of infection in resistant mice is such that a Th1 type immune response develops with the production of IFN-γ and the ability to produce TNF-α (Sadick *et al.*, 1986). This allows the activation of host macrophages to produce reactive nitrogen intermediates (RNI) which are toxic to the parasite and thus lead to clearance of the infection (Green *et al.*, 1994). In contrast, susceptible animals typically produce elevated levels of transforming growth factor-beta (TGF-β), IL-10 and prostaglandins (Barral *et al.*, 1993; Carrera *et al.*, 1996; Milano *et al.*, 1996) with a coincident reduction in the production of IL-12 from infected cells (Weinheber *et al.*, 1998). Together these factors have been demonstrated to inhibit the production of an effective Th1 type

cell mediated response to the infection and thus allow survival of the parasite. A more detailed discussion of the immune response to infection can be found in Chapter 1, section 1.3.

The factors involved in the clearance of *Leishmania* amastigotes from macrophages have been studied in detail. The role of reactive oxygen species (ROS) in killing is thought to be minimal. It has been demonstrated that macrophages deficient in production of H_2O_2 and superoxide can clear infections with *L. donovani in vitro* (Scott *et al.*, 1985). However, recent work has demonstrated that these species may be important in control of the early stages of *in vivo* infection as ROS-deficient mice are more susceptible in the first two weeks of infection but can control and limit the later stages (Murray and Nathan, 1999). This pattern may reflect the fact that during the initial stages of infection, phagocytosis of the promastigote by a macrophage elicits a respiratory burst from the host cells (Murray, 1982); in ROS deficient mice this response will not occur and may allow increased survival. However, by two weeks post infection (p.i.) the parasite has established itself as amastigotes and re-infection of new host cells will occur via a subtly different mechanism, that does not elicit an oxidative response from the invaded host cells (Channon *et al.*, 1984). Therefore a lack in ROS production is less likely to influence progression of the infection at this time.

The majority of parasite killing is considered to be via the production of RNI from nitric oxide. Mice deficient in inducible nitric oxide synthase (iNOS) expression have been demonstrated to display increased susceptibility to infection with *L. major* (Wei *et al.*, 1996) More recent studies have demonstrated that

healing associated with anti-TGF-β treatment in *L. major*-infected susceptible mice is due to the increase in NO production observed (Li *et al.*, 1999).

Prostaglandin production (especially PGE_1 and PGE_2) from the macrophages themselves is known to prevent the activation properties of IFN-γ and has been associated with disease progression (Milano *et al.*, 1996). The use of the cycloxygenase inhibitor, indomethacin, to prevent the production of prostaglandins during infection has been demonstrated to allow a protective Th1 type response to develop in BALB/c mice, and thus slow the progression of the disease (De Freitas *et al.*, 1999). In conjunction with modulation of the immune response, the parasite has been demonstrated to counteract the killing activity of it's host cell in a variety of other ways, which are discussed in Chapter 1, section 1.2.

The reduction in intracellular survival observed during *in vitro* infection with *L. mexicana* Δ*CPB* promastigotes suggests that this parasite may be less able to withstand the microbicidal activities of the host cell (Mottram *et al.*, 1996). Furthermore, the investigation demonstrated that Δ*CPB* was able to survive normally in a small percentage of those cells infected. This suggested that a sub-population of the cells infected was permissive to the null mutants survival. Comparison of the relative susceptibilities of *L. mexicana* wild-type and Δ*CPB* parasites to H_2O_2 and NO *in vitro* demonstrated that the parasites are equally susceptible to these molecules (Frame, PhD thesis). However, it remains possible that these two strains elicit the production of different amounts of either ROS or RNI in response to infection, thereby reducing its intracellular survival.

The standard assay used in the investigation of *L. mexicana* infection involves the *in vitro* infection of peritoneal exudate cells (PECs) from BALB/c mice (see Chapter 2, section 2.3.1.1 for details). The PECs are routinely adhered overnight before washing to remove the unadhered cells. This removes lymphocytes, red blood cells and tissue cells, such that the majority of cells remaining are macrophages (Plasman and Vray, 1993). However, peritoneal macrophages have long been acknowledged to form a phenotypically diverse range of cells whose characteristics are largely dependent on maturity and activation status (Plasman and Vray, 1993). Further to this, dendritic cells are known to be weakly adherent. Thus there appeared the possibility that Δ*CPB* may be exploiting the characteristics of one of these sub-populations of PECs to allow its survival. This may possibly occur via this cell types inability to respond to infection by mounting an effective microbicidal attack. This was the working hypothesis behind this part of my work.

An investigation was undertaken to characterise the response of macrophages to infection with *L. mexicana*, to ascertain whether the reduced virulence of Δ*CPB* was due to an increase in the magnitude of the response mounted towards infection. The possibility that Δ*CPB* was exploiting the characteristics of a certain sub-population of cells to allow survival in a proportion of the cells originally infected was also examined.

6.2. Materials and Methods.

6.2.1. Comparison of the infection dynamics of stationary phase promastigotes within elicited and non-elicited peritoneal exudate macrophages.

The infection dynamics of wild-type, Δ*CPB* and Δ*CPB/g2.8* stationary phase promastigotes were compared in macrophages obtained by peritoneal lavage of normal female BALB/c mice and in macrophages obtained from mice treated 4 days previously with an intraperitoneal injection of 4 % (w/v) thioglycollate (Chapter 2, section 2.3.2).

Macrophages were harvested, adhered overnight and infected as described previously (see Chapter 2, sections 2.3.1.1 and 2.3.1.2). After staining with Giemsa's stain the numbers of infected macrophages and the numbers of amastigotes per cell was microscopically assessed and recorded for at least 200 macrophages per time point. The experiment was repeated three times in duplicate and the results are expressed as means ± standard deviation.

6.2.2. Measurement of the production of nitric oxide by infected macrophages.

Macrophages elicited with 4% (w/v) thioglycollate from BALB/c mice (Chapter 2, section 2.3.2) were used routinely during this investigation as my preliminary analysis had determined that the amount of NO produced by resident macrophages was too low to be detectable by the method used.

Macrophages were harvested and infected as described previously (Chapter 2, sections 2.3.1.1 and 2.3.1.2) and stimulated with 40 U ml^{-1} IFN-γ and 10 ng ml^{-1}

LPS after 4 h incubation as described elsewhere (Chapter 2, section 2.5.1 and Proudfoot *et al.*, 1995). At the desired time points, the supernatants were removed and stored at -20°C until required. The amount of NO produced was estimated by measuring the nitrite concentration of the supernatants via the Greiss Reaction (modified from Kolb *et al.*, 1994 and described elsewhere, Chapter 2, section 2.5.1). The experiment was repeated three times in duplicate and the data are presented as means ± standard deviation.

6.2.3. Measurement of the oxidative burst of infected macrophages in response to a secondary stimulus.

The ability of infected macrophages to respond to a secondary stimulus via the production of high energy oxygen compounds was assessed using chemiluminometry as described elsewhere (Easmon *et al.*, 1980 and Chapter 2, section 2.5.2.1).

Briefly, macrophages were elicited with 4% (w/v) thioglycollate and then harvested as described in Chapter 2 (sections 2.3.2 and 2.3.1.1, respectively), before being adhered overnight to the base of cylindrical plastic measuring cuvettes (Clinicon). The macrophages were then infected for 1 h with stationary phase promastigotes (parasite:macrophage ratio of 1:1) before their ability to respond to PMA (100 ng ml^{-1}) was assessed via the measurement of the light emitted during oxidation of luminol. The experiment was repeated on six separate occasions with

readings being taken every 10 seconds for 10 - 15 min. The data are expressed as the mean values of these repeats.

6.2.4. Analysis of the role of prostaglandin E_2 in *Leishmania* survival *in vitro*.

The role of prostaglandin E_2 in infection was assessed by using the specific cycloxygenase inhibitor, indomethacin, to prevent its production. Macrophages were elicited with 4% (w/v) thioglycollate from BALB/c mice (Chapter 2, section 2.3.2) and harvested and adhered overnight as described previously (Chapter 2, sections 2.3.1.1 and 2.3.1.2). The macrophages were infected with stationary phase promastigotes at a parasite:macrophage ratio of 1:1 in the presence of 1 μM indomethacin (from a 2.8 mM stock solution in methanol). After incubation for 4 h, the macrophages were washed to remove un-attached parasites and the indomethacin was re-applied. In some cases the macrophages were also stimulated with 40 U ml^{-1} IFN-γ (Wang and Chadee, 1995). At the desired time, point the macrophages were washed, fixed and stained as described in Chapter 2, section 2.3.1.2 and the percentage of infected cells was assessed microscopically for at least 200 cells per time point. The experiment was repeated in duplicate on three separate occasions and the results are given as the means ± standard deviation.

6.2.5. Analysis of the infection dynamics of stationary phase promastigotes towards peritoneal exudate cells from BALB/c mice treated with FLT3L.

To expand dendritic cells *in vivo*, mice were treated with daily injections of either 10 μg FLT3L or 100 μl PBS, intraperitoneally for 10 - 12 days as described by Viney *et al.*, 1993 (see Chapter 2, section 2.3.3 for detail). Following treatment, the peritoneal cells were harvested, adhered and infected as described previously (Chapter 2, sections 2.3.1.1 and 2.3.1.2). The experiment was completed in duplicate on three separate occasions and the results are given as means ± standard deviation.

6.2.6. Statistical analysis

The statistical significance of the data was assessed using either student's t tests or heteroscedastic t tests, depending on the equality of the variance as determined by variance ratio F testing. In some cases, one-way ANOVA was employed to investigate the relationships between more than two samples. An example of the statistics used in this chapter can be found in Appendix 1.

6.3. Results

6.3.1. An investigation of the infection dynamics of stationary phase promastigotes in elicited macrophages.

My preliminary investigations into the response of macrophages to infection with *L. mexicana* promastigotes indicated that the levels of NO and ROS produced during infection of resident peritoneal exudate macrophages were too low to be

easily detectable by the methods employed. However, the amounts of these molecules produced increased to detectable levels if thioglycollate elicited macrophages were used. I therefore used these cells to compare the levels of ROS and RNI induced. An investigation of the infection of wild-type, Δ*CPB* and Δ*CPB/g2.8* stationary phase promastigotes into elicited macrophages was therefore undertaken to ensure that altering the macrophages used did not change significantly the relative infectivities of these parasite lines.

The results are given in Figure 6.1. Analysis of the relationship between the percentage of infected cells in each sample at 8 h p.i. demonstrated that there was a statistically significant difference between the uptake of the parasite lines in the different macrophage types ($F_{5,12} = 5.39$, $P < 0.05$). Closer analysis, however, demonstrated that this difference was due to a slight reduction in uptake of Δ*CPB* in resident macrophages when compared to wild-type and *ΔCPB/g2.8* ($P < 0.05$). Infection of resident (non-elicited) macrophages with wild-type stationary phase promastigotes led to a final infection rate of 30.4 ± 8.5%, which is of the magnitude expected in this system. Analysis of the infection observed in elicited macrophages demonstrated that there was no statistically significant difference in the percentage survival by 168 h ($t_4 = 1.7$, $P > 0.1$). These results indicated that wild-type *L. mexicana* is able to withstand the increased responsiveness of the elicited macrophages and survive as in un-elicited, resident macrophages.

Analysis of the infection with *ΔCPB/g2.8* stationary phase promastigotes demonstrated that the virulence of this line was comparable with wild-type parasites at both time points and in both macrophage types ($F_{3,8} = 2.47$, $P > 0.05$). Again, this

demonstrated that the parasite is able to survive in elicited and resident macrophages to a similar degree. Infection of resident macrophages with Δ*CPB* stationary phase promastigotes resulted in a 81.2 ± 17.6 % reduction in the number of infected cells by 168 h p.i. when compared with infection with wild-type parasites. This was demonstrated to be similar to that observed for elicited macrophages (75.6 ± 1.7 %, $t_2 = 0.58$, $P > 0.1$).

It was concluded that the dynamics of infection in elicited macrophages closely follow those characterised for resident peritoneal exudate macrophages and therefore I considered it appropriate to substitute elicited macrophages in the following analyses.

6.3.2. Analysis of the nitric oxide produced in response to infection with stationary phase promastigotes.

An investigation of the production of NO over the first 48 h of infection was undertaken to determine whether Δ*CPB* was more susceptible to killing because it elicited an increased level of NO production from its host macrophages. Figure 6.2, shows the amounts of nitrite detectable in the supernatants of infected macrophages. Unstimulated macrophages were used as a negative control and demonstrated that small but detectable levels of nitrite are present in their medium. The application of IFN-γ and LPS demonstrated that such activation of the cells led to an increase in the amount of nitrite produced. The increased levels at 48 h were due to the accumulation of nitrite within the medium.

Comparison of the amount of nitrite in the different supernatants demonstrated that there was no significant difference between the amounts produced in the various infections over the first 24 h period ($F_{2,6} = 1.99$, $P > 0.05$), the second 24 h period ($F_{2,6} = 0.62$, $P > 0.05$) or over the total 48 h period of investigation ($F_{2,6} = 0.85$, $P > 0.05$). This suggests that the death of *ΔCPB* within macrophages *in vitro* is not due to an increased production of NO during infection.

Interestingly no difference was found between the nitrite concentrations of the infection supernatants and that of the positive control ($F_{3,8} = 1.24$, $P > 0.05$) suggesting that parasite is able to survive within the macrophage despite the production of nitric oxide rather than because of an ability to inhibit production.

6.3.3. Investigation of the oxidative response of infected macrophages towards a secondary stimulus.

I next tested the hypothesis that wild-type parasites are able to survive within their host cells due to their ability to inhibit the production of microbicidal intermediates, such as oxygen free radicals. One working hypothesis was that Δ*CPB* is either unable to prevent production or elicits the production of an increased volume of these molecules, hence its reduced survival. A study of the oxidative response of infected macrophages towards a secondary stimulus was therefore undertaken to determine the relative abilities of the infected macrophages to respond.

After infection of the macrophages for 1 h with stationary phase promastigotes, the cells were stimulated with 100 ng ml^{-1} PMA and the production of high energy oxygen intermediates was assessed for 10 - 15 min using luminometry (see section 6.2.3). The results are shown graphically in Figure 6.3. Resident peritoneal exudate cells were used as a negative control as these cells fail to respond to PMA stimulation, this demonstrated that only a low level of background luminescence occurred. The positive control used was thioglycollate elicited macrophages stimulated with PMA and these cells were demonstrated to produce an oxidative burst as expected. However, no difference in the level of oxidative burst between the wild-type- and Δ*CPB*-infected macrophages was observed, indicating that both host-parasite populations were capable of responding to the secondary stimulus to the same degree.

6.3.4. Investigation of the effect of Prostaglandin E_2 inhibition on the course of *in vitro* infection

Prostaglandin E_2 (PGE_2) is produced by macrophages in response to stimulation and is involved in the down regulation of the macrophages effector mechanisms (Adams and Hamilton, 1992, and see section 6.1 for details). I hypothesised that wild-type *L. mexicana* can survive within macrophages because they induce the production of PGE_2 in response to infection, thereby inhibiting the microbicidal activity of the host cell. Δ*CPB*, on the other hand was postulated to be unable to stimulate this production and is therefore killed in the majority of cells that

it enters. An investigation of the effect of inhibition of PGE_2 production on the survival of wild-type and *ΔCPB* in macrophages was therefore undertaken.

Figure 6.4. shows the percentage of infected cells by 168h p.i. Analysis of the data demonstrated that the inhibition of PGE_2 production in the presence or absence of IFN-γ had no effect on the survival of wild-type parasites within macrophages ($F_{3,8} = 2.38$, $P > 0.05$). This suggested that wild-type parasites are not surviving in this *in vitro* system due to the production of high levels of PGE_2. No statistically significant effect was observed on the infection levels of *ΔCPB* in any of the conditions analysed ($F_{3,8} = 0.51$, $P > 0.05$).

In order to confirm that PGE_2 was having no effect on the relative infectivity of these lines, an analysis of the percentage difference in survival was undertaken. This indicated that the inhibition of PGE_2 production by macrophages in the absence or presence of IFN-γ stimulation had no effect on the survival of either line in *in vitro* infection ($F_{3,8} = 0.77$, $P > 0.05$).

6.3.5. Investigation of the infectivity of stationary phase promastigotes towards macrophages from FLT3L treated mice.

FLT3L is a haematopoietic stem cell factor which causes the outgrowth of myeloid progenitor cells (Lyman *et al.*, 1993). Treatment of mice with this ligand is known to increase the numbers of functionally mature dendritic cells within several tissues in the body, including the peritoneum where the proportion of dendritic cells has been demonstrated to increase by 29 fold (Maraskovsky *et al.*, 1996). As

discussed elsewhere (section 6.1), there is some evidence that Δ*CPB* may be surviving in a sub-population of the cells infected. One sub-population which could potentially act as a permissive host for infection are dendritic cells. It was therefore decided to investigate the infection dynamics of stationary phase promastigotes towards peritoneal exudate cells from animals treated with FLT3L.

Analysis of the infection rates of wild-type parasites at 168 h p.i. demonstrated that there is no statistically significant difference between survival in control (cells from non-treated mice) or cell populations enhanced for dendritic cells (from FLT3L-treated mice, $t_4 = 1.76$, $P > 0.1$)(Figure 6.5). Interestingly, the percentage infection observed for Δ*CPB* was significantly higher in the cells from FLT3L-treated mice than in control cells ($t_4 = 12$, $P < 0.01$) and was not significantly different from the survival of wild-type parasites in this cell type ($t_4 = 0.52$, $P > 0.1$). These data indicated that alteration of the cell-populations present during infection can increase the infectivity of Δ*CPB* to that of wild-type parasites and suggests that that survival of Δ*CPB* may be due to infection of dendritic cells.

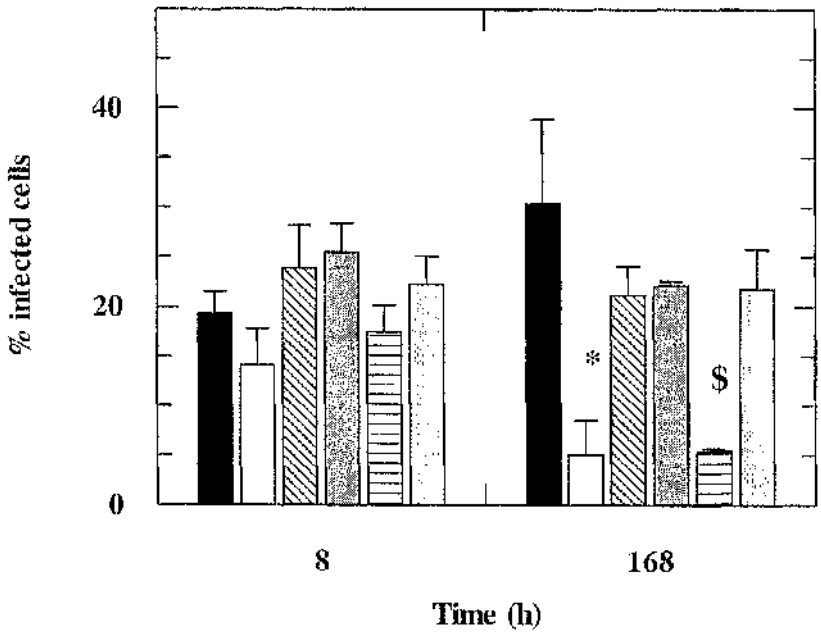

Figure 6.1: The relative infectivity of stationary phase promastigotes to resident and elicited peritoneal macrophages. Resident macrophages infections: black bars, wild-type infection; white bars, Δ*CPB* infection; diagonal hatched bars, *ΔCPB/g2.8* infection. Elicted macrophage infections: grey bars, wild-type infection; horizontal hatched bars, Δ*CPB* infection; dotted bars, *ΔCPB/g2.8* infection. The experiment was repeated in duplicate on three separate occasions. The data showing the mean infection with standard deviations. Those infections which were found to be significantly different from wild-type are indicated; * = $P < 0.02$. $ = $P < 0.01$.

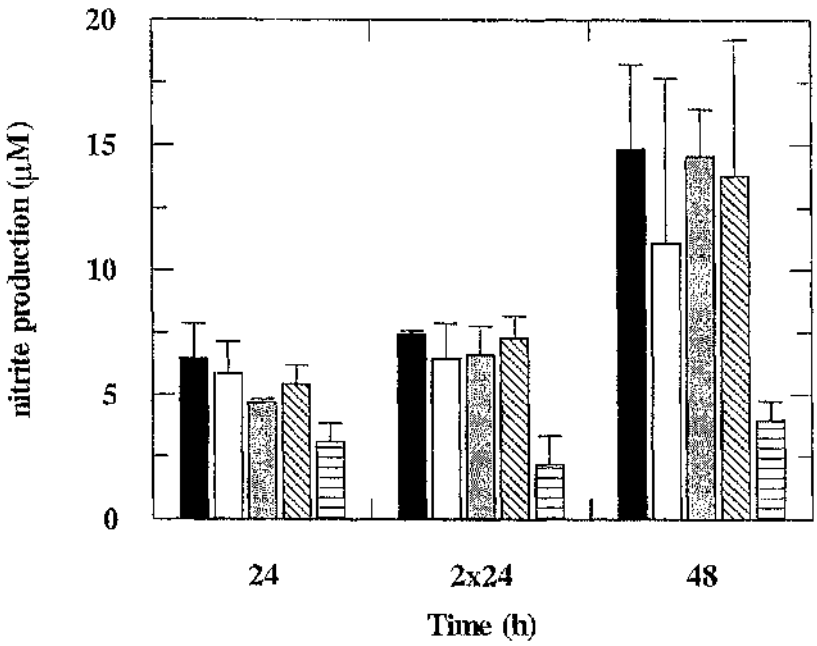

Figure 6.2: Nitric oxide production by elicited macrophages in response to infection with stationary phase promastigotes. The bars represent the average nitrite concentration (μM) in the supernatants of macrophages infected with: black bars, wild-type; white bars, *ΔCPB*; grey bars, *ΔCPB/g2.8*. Hatched bars indicate the concentrations in the control supernatants: diagonal, stimulated with IFN-γ and LPS; horizontal, unstimulated. The nitrite concentration in the first 24 h, the second 24 h (2 x 24) and the amount produced over the whole 48 h of infection was assessed. The experiment was repeated in duplicate on three separate occasions. The data is expressed as the mean with standard deviations. No significant difference was found in the amount of NO produced by the different macrophage infections at any time point ($P > 0.05$)

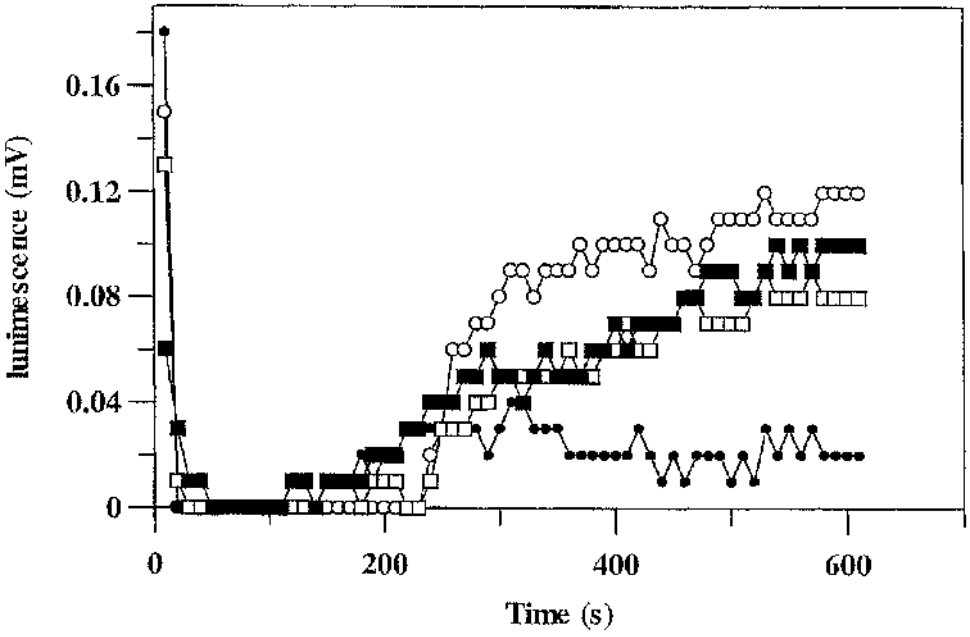

Figure 6.3: The oxidative response of infected macrophages after secondary stimulation with PMA. Open circles, elicited macrophages. Solid circles, resident macrophages. Open squares, wild-type infection. Solid squares, *ΔCPB* infection. The experiment was repeated on six separate occasions and the results are given as the mean of this determination.

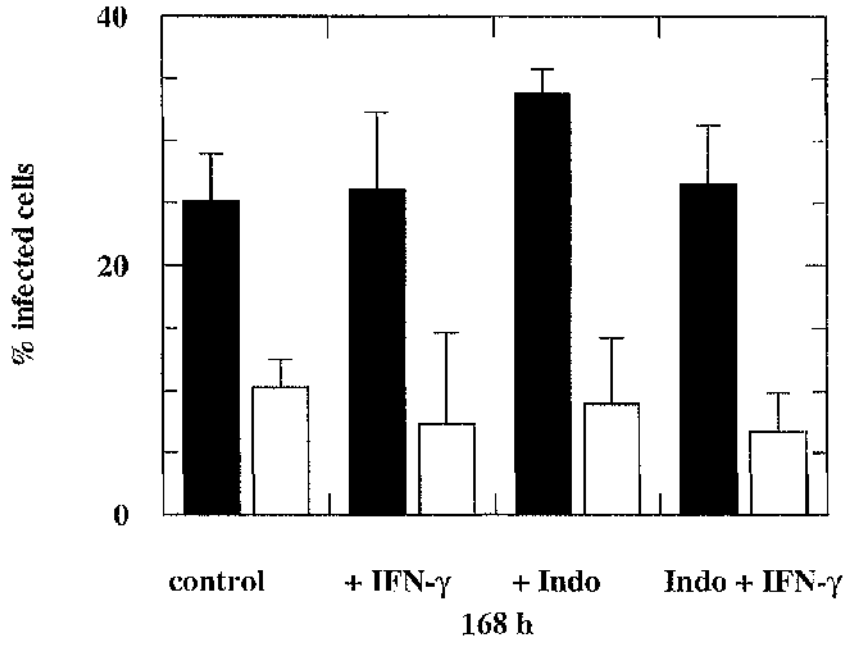

Figure 6.4. The effect of inhibition of Prostaglandin E_2 production by indomethacin on survival of *L. mexicana* within macrophages. The black bars represent the levels of wild-type infection under the various conditions described. The white bars indicate the level of *ΔCPB* infection. The experiment was repeated in duplicate on three separate occasions. The data are given as the mean and standard deviation. No statistically significant difference was found between infections in any of the conditions analysed ($P > 0.05$). The relative reduction in infectivity of Δ*CPB* also remained constant ($F_{3,8} = 0.77$, $P > 0.05$)

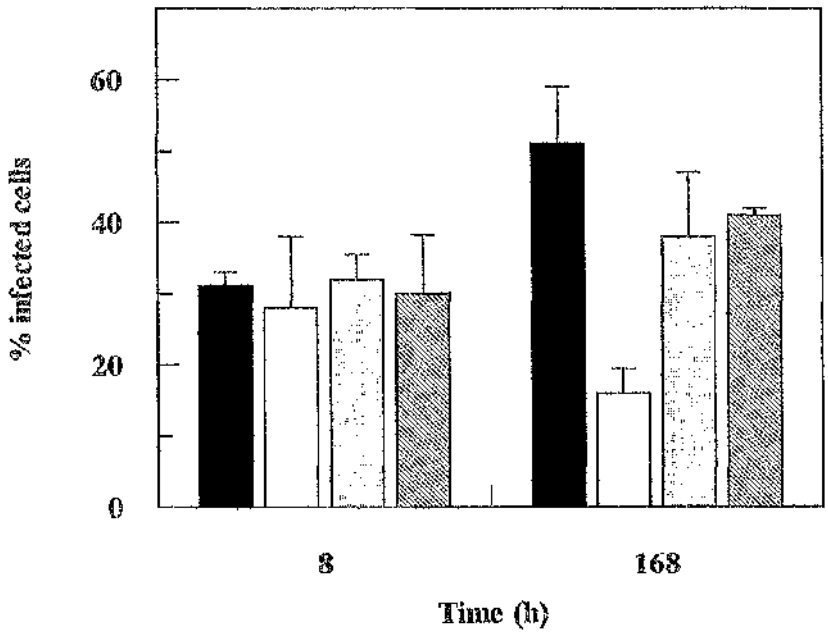

Figure 6.5: The infection of peritoneal exudate cells from normal- and FLT3L- treated mice. Infection in normal resident peritoneal exudate cells: black bars, wild-type; white bars, *ΔCPB*. Infection in cells from FLT3L treated mice: grey bars, wild-type; diagonal hatched bars, *ΔCPB*. The experiment was repeated in duplicate on three separate occasions. The data are presented as means with standard deviation. By 168 h p.i. the wild-type infections in either host cell type were statistically similar ($t_4 = 1.76$, $P > 0.1$). *ΔCPB* infection in normal peritoneal exudate cells was significantly reduced compared to wild-type ($t_4 = 6.8$, $P < 0.01$). The infection in cells from FLT3L treated mice, however was not statistically different to wild-type levels ($t_4 = 0.52$, $P > 0.1$).

6.4. Discussion

This chapter has described an investigation into the response of macrophages to infection with *L. mexicana* promastigotes. It was demonstrated previously that Δ*CPB* stationary phase promastigotes have a reduced level of survival within peritoneal exudate macrophages when compared with survival rates displayed by wild-type cells (Mottram *et al.*, 1996). This suggested that the mutant is less able to withstand the intra-macrophagic environment. It was therefore decided to compare the response of macrophages to infection with wild-type parasites and Δ*CPB*.

As discussed previously (see Chapter 1, sections 1.2 and 1.3) the relationship between *Leishmania* and its mammalian host is complex and involves several parasite- and host-derived factors. In the *in vitro* system used during this investigation, this complex relationship was simplified by removing outside influences on the host macrophage, for example, cytokines and the majority of other host cell types. Even in this simplified system a reduction in Δ*CPB* survival is detected. This suggests that at least one factor mediating parasite survival is operating even in the absence of other cell types. It was therefore decided to investigate what host factor(s) caused this reduction using the *in vitro* system, with the intention of investigating the identified factor's role *in vivo* at a later date.

Several problems were faced when attempting to undertake an analysis of the respiratory burst production in response to *Leishmania* infection. Resident peritoneal macrophages are relatively resistant to activation by factors such as IFN-γ, producing low levels of microbicidal effector mechanism molecules (Adams and

Hamilton, 1992). This meant that it was impossible to analyse and compare such responses in these cells. In order that the response could be detected, it was necessary to use macrophages which had been primed for activation via elicitation with intraperitoneal injection with thioglycollate broth. The injection of thioglycollate into the peritoneum causes the migration of immature mononuclear phagocytes from other tissues into the peritoneum. The granularity of the medium induces the macrophages present to phagocytose the particulate matter, thus imitating infection and producing an inflammatory response within the peritoneum (Grattendick, 1999). Therefore the macrophages harvested after this procedure are 'primed' for activity and give an increased level of responsiveness after IFN-γ and/or LPS stimulation.

Alteration of the host cell type was therefore necessary to allow completion of the intended experiments. In order to ensure that the infection dynamics of wild-type and Δ*CPB* were the same in the elicited macrophages as in resident macrophages, it was necessary to analyse and compare infection in both elicited and resident peritoneal macrophages. As demonstrated there was no difference in the ability of wild-type, Δ*CPB/g2.8* or Δ*CPB* to survive in either macrophage type. In both cases reduction of Δ*CPB* survival with respect to that of wild-type parasites was approximately 70 - 80 %, which is of the same magnitude as reported previously (Mottram *et al.*, 1996). This suggested that the use of elicited macrophages for these experiments was reasonable. The only difference observed during this experimentation was that Δ*CPB* had slightly reduced levels of infection by 8 h in resident macrophages compared with elicited macrophages. This may be because

Δ*CPB* enters macrophages via different receptors during phagocytosis which appears to make it's uptake less efficient (see Chapter 7, for detail). In elicited macrophages, the enhanced level of phagocytosis may mask this difference.

Analysis of the production of NO in response to infection indicated that there was no significant difference in the overall production in response to infection with any of the lines investigated. This suggested that an increase in NO production in Δ*CPB*-infected cells is not responsible for the parasite's reduced virulence. However, the Greiss reaction measures the nitrite concentration in the supernatant of infected cultures, nitrite is the stable end product of the break down of the labile NO produced and the associated RNIs. Therefore, the Greiss reaction measures the accumulative effect of NO production and gives no indication as to the NO production at any specified time point. It is possible that by 48 h of infection very few macrophages were harbouring an infection of *ΔCPB*, although overall NO production at this point could have been relatively low, the amount produced per infected cell could have been high. In order to accurately determine the production of NO at specific time points, it would be necessary to employ other methods of analysis. Vectors are available containing the inducible nitric oxide synthase gene (iNOS) which would allow competitive Reverse Transcriptase-Polymerase Chain Reaction (cRT-PCR) (Reiner *et al.*, 1993), determination of the mRNA expression for iNOS within cells would allow a more accurate picture of NO production to be gained. Unfortunately, time limitation and prioritisation prevented further investigation by this method.

Interestingly, none of the *Leishmania* lines analysed appeared to inhibit the production of NO in response to infection, the levels of NO produced were similar to those detected in macrophages stimulated with LPS and IFN-γ. These data are at odds with previously published information which suggests that *L. major* and *L. donovani* inhibit iNOS expression in J774 and RAW264 cells, respectively (Proudfoot *et al.*, 1995; Nandan *et al.*, 1999). However the systems used in these experiments are not directly comparable to the one described herein as the heterogeneity of the macrophages used in my experimentation along with the relative immaturity of the monocyte lines used in the published investigations (ECCAC and ATCC, 1999), prevents direct comparison. It may be, however, that the fact that the elicited macrophages are already 'primed' for activity prevents the parasite from efficiently inhibiting the activation of the macrophages, thus allowing a microbicidal response to occur. The fact that the infected macrophages produce NO to a similar degree without altering the reduction in Δ*CPB* survival, however, suggests that NO is not the important factor under investigation.

The ability of infected macrophages to respond to a secondary stimulus by the production of ROS was also investigated. It has been previously demonstrated that infection with *L. donovani* reduces the oxidative burst of monocytes (Olivier *et al.*, 1992). I postulated, therefore, that an infection with wild-type parasites would inhibit the host cells ability to respond to stimulation and thus allow survival within the cell, whereas infection with Δ*CPB* has no effect on the macrophages ability to respond to stimulation, hence the lower survival rates. However, investigation demonstrated that both sets of infected macrophages had a similar ability to respond

to stimulation, suggesting that neither infection was inhibiting the response. It may be that, these experiments were biased towards this result as the macrophages used were 'primed' for activation and thus any inhibitory effect may have been unsuccessful. However, this work does demonstrate that the ability of the macrophages to respond to stimulation is similar regardless of the parasite line used in infection and suggests that this is not the factor involved in reducing Δ*CPB* virulence.

The work discussed so far has demonstrated that the production of RNI and ROS in macrophages infected with either wild-type parasites or Δ*CPB* is similar. I next decided to investigate the effect of the inhibition of PGE_2 production on parasite survival. It was hypothesised that if wild-type parasites rely on inactivation of the host macrophage to allow survival then they may rely on inducing an increased production of PGE_2. This phenomenon has been reported during the early stages of infection *in vivo* (Milano *et al.*, 1996). The use of indomethacin to prevent the expression of PGE_2 was demonstrated to have no effect on the survival of the wild-type or Δ*CPB* infections. No dose response curve was investigated initially, as previous work in a similar system had indicated that 1 μM indomethacin was sufficient to achieve inhibition of PGE_2 production (Wang and Chadee, 1995). Had these initial investigations proved more interesting then a more thorough investigation would have been carried out. The effect of indomethacin was analysed under several different conditions, and it was determined that even in the presence of IFN-γ no difference was observed in the survival. It is unlikely that the lack of effect was due to instability of the inhibitor as indomethacin is regularly used *in vivo* over

longer periods of time and has been shown to be effective (De Freitas *et al.*, 1999). This suggests that the effects of PGE_2 during *in vivo* infection with *Leishmania* are due to the reduction in MHC-II expression observed (Figueiredo *et al.*, 1990), rather than the inactivation of the macrophages towards intracellular infection, and that the activation state of the macrophages has no role in reducing the survival of Δ*CPB in vitro*.

Previous data have intimated that Δ*CPB* may be surviving within a sub-population of the cells that it initially infects; the numbers of amastigotes/cell observed after 7 days of infection suggest that it survives as well as wild-type in those cells permissive to infection (Mottram *et al.*, 1996). The CPB enzymes have been demonstrated to be involved in the virulence of *L. mexicana* although no definite role in the infection process has so far been discovered. If loss of the CPB enzymes prevents the parasites survival in infected cells apart from a small sub-population, then identification and analysis of this permissive sub-population could allow further elucidation of the CPB enzymes roles during infection.

Analysis of the infection dynamics in peritoneal exudate cells from FLT3L-treated mice indicated that wild-type parasites and Δ*CPB* had a similar survival rate after 168 h of infection, suggesting that FLT3L treatment in some way increased the suitability of the host cells for Δ*CPB* infection.

The results can be interpreted in several ways. Firstly, FLT3L may be having a direct effect on the cells present within the peritoneum, altering them in some way which increases the ability of Δ*CPB* to survive. Investigation of the expression of the FLT3 receptor in mice has demonstrated that it's mRNA is detectable in several

tissues including peritoneal macrophages (Rosnet *et al.*, 1991), giving rise to the possibility that FLT3L is binding to the surface of the macrophages present and having some effect(s) on their phenotype. However, no evidence was found in the literature to corroborate this theory. Secondly, FLT3L treatment is known to cause a 29 fold increase in the numbers of functionally mature dendritic cells within the peritoneum of mice (Maraskovsky *et al.*, 1996), the fact that the infectivity of Δ*CPB* is increased after treatment could be interpreted to mean that the null is preferentially surviving in dendritic cells. Another possibility is that FLT3L is known to have effects on the growth of early progenitor cells in the bone marrow (Lyman *et al.*, 1993). However, due to the expression of its receptor in several tissues it is possible that it will also cause the recruitment of other progenitor cells to various tissues within the body, giving rise to the possibility that FLT3L treatment will increase the numbers of immature macrophages present in the peritoneum.

The only effects reported in the literature for FLT3L after intraperitoneal injection involve the outgrowth of progenitor cells pre-determined to form dendritic cells, suggesting that it is indeed the increase in dendritic cells observed that is allowing Δ*CPB* to survive to wild-type levels. However, only a preliminary analysis of the effect of FLT3L during this investigation was carried out. Although this confirmed that treatment caused an increase in the proportion of cells present that displayed dendritic characteristics, it gave no quantitative data as to the percentage of these cells present. Therefore, it is uncertain whether a Δ*CPB* survival rate of 41 ± 1% by 168 h could be accounted for by an increase in dendritic cell population.

Furthermore the role of dendritic cells in *Leishmania* infection has been investigated previously and several studies have cast doubt on the ability of *Leishmania* species. to infect dendritic cells *in vitro* and *in vivo*. Von Stebut and colleagues (1998) demonstrated that *L. major* amastigotes but not promastigotes were phagocytosed into dendritic cells, postulating that this allowed the parasite to establish an infection before it was 'seen' by the immune system and thus aided in prolonging the infection. Further to this, Smelt *et al.* (1997) could find no parasitism in the follicular dendritic cells during *in vivo* infection with *L. donovani*. However dendritic cells are known to play an important role during infection with *Leishmania* species. The development of a protective response to *Leishmania* infection is dependent on the production of IL-12, which stimulates the production of cytokines such as IFN-γ and leads to the activation of macrophages to combat the infection. Studies in mice have demonstrated that during infection macrophages are unable to respond to stimuli such as LPS by producing IL-12 even if primed for production using cytokines (Carrera *et al.*, 1996). Dendritic cells, however, were recently identified as the major source of IL-12 during the early stages of infection with *L. donovani* (Gorak *et al.*, 1998). Thus despite the frequently reported lack of parasitism in dendritic cells, it is clear that they play an important role in the infection process.

Several difficulties were faced when investigating the potential sub-population of cells acting as host to Δ*CPB*. The approach I initially adopted in order to investigate the possibility that a permissive cell population existed which allowed the survival of Δ*CPB* involved the identification of infected cell sub-populations via

immunofluorescence. The rationale was that infected cells could be identified using DAPI staining to highlight the amastigote DNA and that the type of cell infected could be further investigated antibody using staining for expression of such surface markers as F4/80 (expressed on immature macrophages Gordon *et al.*, 1986) and CR3 (also known as mac-1 or M1/70) which is expressed on all mononuclear phagocytes with expression increasing with maturation, (Graham and Brown, 1991). These experiments had to be carried out at a time post infection when the differences between the infectivity of the wild-type and Δ*CPB* were easily identifiable, i.e. at least three days after infection, so that the majority of the Δ*CPB* phagocytosed initially would have been removed from the non-permissive cells. Difficulties arose, however, as it proved impossible to stain any of the infected cells with either antibody tried despite evidence that they should have both been present on these cells (Plasman and Vray, 1993) This phenomena probably occurred due to the prolonged incubation of the macrophages in medium causing alteration in the expression/surface location of the molecules under study, and thus preventing the binding of the antibodies used.

Another method I investigated was the use of fluorescent activated cell scanning (FACs) with the antibodies, described above. However this method also posed several problems. Firstly, in order for the infections to be equivalent to those studied *in vitro* the macrophages had to be adhered overnight before infection. This posed the problem of how to remove the macrophages from the stratum without causing damage to their surface, such that the antibodies could not bind. Secondly, there is no information in the literature pertaining to the expression of parasite

molecules on the surface of infected macrophages, and the surface of the intracellular amastigotes contain few, if any proteins (Winter *et al.*, 1994), making the detection of intracellular infection difficult. This could have been circumvented via the use of DAPI staining as described above but the levels of infection would have needed to be above a certain threshold for the FACs machine to detect this extra staining above that of the nuclei of the host cell. This method therefore was considered to be unfeasible.

A major problem faced was the lack of a pure dendritic cell population. The relatively low numbers of dendritic cells available in the tissues of even FLT3L-treated mice meant that the purification of these cells, via FACs or magnetic cell selection (with anti-CD11c antibodies conjugated to magnetic beads) was unfeasible, due to the necessity for a large amount of starting material.

One method available was the use of bone marrow cells cultured in the relevant cytokines. Lu *et al.*, (1996) demonstrated that the isolation of progenitor cells from bone marrow and the cultivation of these cells in IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) allows the production of a viable dendritic cell population *in vitro*. The dendritic cells could be harvested from this population by virtue of their weakly adherent nature. A preliminary investigation of the infectivity of *L. mexicana* into such cells (donated by Dr. James Brewer, Department of Immunology, University of Glasgow), indicated that the parasite, unlike the other *Leishmania* species investigated, was indeed able to infect these cells and that both the wild-type and Δ*CPB* survived within these cells for at least 7 days. However, at the time of the investigation the characteristics of these cultured

dendritic cells were still under investigation and doubts were raised as to whether these cells corresponded to the dendritic cells within our *in vitro* system. One problem with these cells was the fact that their characteristics were not homogeneous, with a large proportion of the cells reverting to an adherent form. No information was available as to whether this population was indeed dendritic or had perhaps reverted to a more macrophage-like phenotype. Due to time limitations and the paucity of relevant information and starting material, it was decided to concentrate the investigation of other important, potentially more fruitful, areas.

As discussed earlier another potential host population for Δ*CPB* infection are immature macrophages. This cell population, although easier to study than dendritic cells, also poses a number of problems. Purification of immature macrophages from tissues via FACs analysis is difficult: their similarity in size and granularity to other cell types, such as dendritic cells, means that the use of size exclusion is impossible. However, had time been permitting there are several strategies which could have been employed to investigate the infection and survival of Δ*CPB*. Several monocyte cell lines can be purchased from the ECACC which could act as a pure source of immature macrophages for *in vitro* infection. WEHI-274.1, for example, are derived from BALB/c mice and are characterised as a monocyte cell line. Infection of this line could potentially answer the question as to whether Δ*CPB* is surviving in immature macrophages.

Another possible method I investigated was the use of Percoll density gradients to separate the different peritoneal cell populations by density (Plasman and Vray, 1993). Separating the cells into distinct populations and investigating the

infection dynamics of the wild-type and Δ*CPB* in these populations could shed some light onto whether there is a sub-population involved and indeed give some information about the characteristics of this population. Unfortunately constraints on the time available meant that I was unable to complete this work during the investigation.

In conclusion, the reduced survival of Δ*CPB* in peritoneal exudate cells appears to be unrelated to the ability of the host cell to produce either NO or ROS, and the activation status of the cells appears to be irrelevant. This suggests that other factors within the host cell are involved in death of the parasite. This question is in need of further investigation, using methods with greater sensitivity to analyse the roles of other macrophage-specific factors which could be involved. It would appear from this data that the low level of survival of Δ*CPB* is due to it's ability to survive in a sub-population of the cells originally infected, which is increased in number following FLT3L-treatment. Further investigation is required, however, to determine if this population is indeed dendritic in nature.

Chapter 7

The role of cysteine proteinases in the uptake of *Leishmania* promastigotes and amastigotes by macrophages *in vitro*

7.1. Introduction.

The *CPB* cysteine proteinases of *L. mexicana* are lysosomal cathepsin L-like enzymes, which are predominantly expressed within the mammalian stages of the life-cycle (Pupkis *et al.*, 1984; Robertson and Coombs, 1994; Mottram *et al.*, 1997). The production of a null mutant strain lacking in the *CPB* enzymes (Δ*CPB)* has allowed an analysis of the role of these enzymes in the virulence of the parasite. Δ*CPB* has been demonstrated to have reduced survival during *in vitro* macrophage infections (Mottram *et al.*, 1996). Interestingly, this reduction in virulence is only observed if the macrophages are infected initially with stationary phase promastigotes of the parasite (the stage corresponding to the infectious metacyclic stage, Mallinson and Coombs, 1984). If axenic amastigotes are used to infect macrophages then Δ*CPB* virulence is restored to wild-type levels. This indicates that the *CPB* enzymes may be important during the uptake of promastigotes by macrophages or in the establishment of the initial infection.

The initial stage of infection by promastigotes involves the uptake of the parasite by a process closely related to classical receptor-mediated phagocytosis. The receptors utilised by promastigotes during the initial binding of macrophages are variable, with different species using different receptor combinations (see

Table 7.1). The receptors used can also be dependent on the presence or absence of serum, as complement, especially complement component 3, is known to increase the uptake and subsequent survival of promastigotes (Mosser and Edelson, 1987). Several receptors have affinity for C3b (namely CR1, CR3 and CR4) and have been demonstrated to have a role in the uptake of at least one *Leishmania* species which may or may not be dependent on serum (Da Silva *et al.*, 1989; Mosser *et al.*, 1992). CR4 and CR3 have been demonstrated to bind surface proteins in the absence of serum (Talamas-Rohana *et al.*, 1990). CR3 is known to have affinity for several ligands and at least two binding sites, one of which is involved in the serum-dependent binding of *L. amazonensis* (Mosser *et al.*, 1992), and another which is involved in binding of bacterial LPS and also Lipophosphoglycan (LPG) via serum-independent mechanisms (Talamas-Rohana *et al.*, 1990). Other receptors including those for mannose-containing residues (Blackwell *et al.*, 1985, Wilson and Pearson, 1986) and fibronectin (Wyler *et al.*, 1985, Soteriadou *et al.*, 1992) have also been implicated in the binding of some species of *Leishmania.*

Investigation of *L. major* has suggested that the intracellular fate of phagocytosed promastigotes may be dependent on the receptors used to mediate uptake; the receptors used for internalisation of log-phase promastigotes differ from those utilised by metacyclic promastigotes *in vitro* (Da Silva *et al.*, 1989). It has also been demonstrated that the response of macrophages after phagocytosis differs depending on the receptors involved in the stimulation process. Aggregation of the mannose receptor (Astarie-Dequeker *et al.*, 1999) and receptors for C3b (Wright and Silverstein, 1983) has been shown to stimulate phagocytosis without the production of an oxidative burst. The latter

result is surprising, however, due to the major role CR3 plays in the phagocytosis of many *Leishmania* species (see Table 7.1) and the fact that internalisation of promastigotes is known to be co-incident with an oxidative burst (Murray, 1982). This indicates that either the oxidative burst recorded during internalisation of the promastigotes is due to a minority of promastigotes entering via different receptors or that the site of binding of CR3 is important in determining the response to ligation.

The promastigote is known to express on its surface two GPI-anchored molecules which are thought to be involved in mediation of macrophage binding. Gp63 is the major surface metalloproteinase of *Leishmania* promastigotes and comprises approximately 1% of the promastigotes total cellular protein (Bahr *et al.* 1993). This protein's role in adhesion has been extensively studied. It has been implicated in the cleavage of C3b to release iC3b (Brittingham *et al.*, 1995) which facilitates uptake via CR1, CR3 and CR4. Other work has indicated that gp63 contains a region of peptide sequence which facilitates binding to CR3 in a serum-independent manner (Russell and Wright, 1998). This peptide sequence was demonstrated to mimic a sequence within fibronectin (Soteridou *et al.*, 1992) and thus perhaps facilitates binding via homology to this protein.

The role of LPG has also been studied in several *Leishmania* species. LPG is the major glycolipid on the surface of the parasite with 1.5 - 5 x 10^6 molecules/cell (Sacks, 1992). The use of LPG-coated beads has indicated that this lipid can bind to both CR3 and CR4 in a serum-independent manner (Talamas-Rohana *et al.*, 1990). The fact that this binding is not dependent on the maturation status of the macrophages suggests that the major role is via interaction with CR3, as the expression of CR4 has recently been demonstrated

to increase as the macrophages mature from monocytes (Zaffran *et al.*, 1998). The ligand involved in adhesion has been investigated in *L. major*, where it was determined to be a phosphorylated oligosaccharide (Kelleher *et al.*, 1992). However, this oligosaccharide is unique to the LPG of metacyclic *L. major* and thus LPG must bind via other ligands in other species.

After the promastigotes have bound via their specific receptors the course of uptake is similar to that of classical phagocytosis; there is a rapid phosphorylation of tyrosine residues on several macrophage proteins (Greenberg *et al.*, 1993), which is necessary for the rearrangement and coupling of the cytoskeleton in the host cell to facilitate the rapid uptake of parasites. Once inside the host cell, the parasite establishes itself within a modified lysosomal vacuole, the parasitophorous vacuole, where it transforms over a period of 2-5 days (Galvao-Quintao *et al.*, 1989) to the amastigote stage. This stage multiplies within the parasitophorous vacuole before the host cell bursts and releases amastigotes into the tissues of the host.

The uptake of released amastigotes by new host cells is via a different mechanism to that of promastigotes The phagocytic process itself is accompanied by a comparatively smaller amount of protein tyrosine phosphorylation (Love *et al.*, 1998) indicating that there are differences in the cell signalling involved in this process. There is no enhancement of uptake in the presence of serum (Guy and Belosevic, 1993) and internalisation of amastigotes is not accompanied by an oxidative burst (Channon *et al.*, 1984). The receptors involved in amastigote phagocytosis have yet to be resolved. The use of monoclonal antibodies to many macrophage surface receptors has thus far demonstrated that none of the promastigote receptors are responsible for uptake.

Some work has indicated that during infection the presence of natural anti-*Leishmania* antibodies allows the amastigotes to utilise the Fc receptors of the host cells, this work also intimated that a CR3 may play a role in amastigote uptake (Guy and Belosevic, 1993). However, the investigation did not take into consideration the fact that aggregation of CR3 via specific antibodies causes a down regulation of Fc receptor expression in macrophages (Gresham *et al.*, 1991). Although no receptors have been implicated in uptake of amastigotes, a heparin-binding activity has been demonstrated to exist on the surface of *L. amazonensis* amastigotes (Love *et al.*, 1993) indicating that binding of heparin sulphate proteoglycans on cell surfaces may mediate in uptake. However, in this study, heparin sulphate inhibited binding of amastigotes by only 40% indicating that other mechanisms must also be involved.

Thus it is clear that the mechanisms of entry into macrophages differs between the two life-cycle stages and indeed between different *Leishmania* species. The CPB enzymes have been postulated to be important in the early stages of infection. This, in conjunction with evidence presented in Chapter 4 which demonstrated that the surface architecture of Δ*CPB* promastigotes is different to that of wild-type, suggests that the CPB enzymes may be important during phagocytosis of the promastigotes by macrophages. It was therefore decided to investigate the initial uptake of wild-type and Δ*CPB* stationary phase promastigotes by macrophages in an attempt to elucidate the involvement, if any, of CPB.

Surface Receptor	Leishmania species	Reference
CR3 (mac-1 and CD11b/CD18)	*L.donovani* *L. major* *L.amazonensis* *L. mexicana*	Blackwell *et al.*, 1985[a] Rosenthal *et al.*, 1996[b] Mosser and Edelson, 1987[c] Mosser *et al.*, 1992 Talamas-Rohana *et al.*, 1990[a]
CR4 (p150,95 and CD11c/CD18)	*L mexicana* *L. major*	Talamas-Rohana *et al.*, 1990[a] Da Silva *et al.*, 1989[d]
CR1	*L. major* *L. amazonensis*	Da Silva *et al.*, 1989[d] Rosenthal *et al.*, 1996[b] Dominguez and Torano, 1999[b]
Mannose receptor	*L. donovani*	Blackwell *et al.*, 1985 Wilson and Pearson, 1986
Fibronectin receptor	*L. amazonensis* *L. major*	Wyler *et al.*, 1985 Soteriadou *et al.*, 1992

Table 7.1: A summary of the receptors involved in attachment and phagocytosis of *Leishmania* promastigotes by macrophages. a, binding is independent of serum. b, binding is dependent of presence of serum. c, binding is possible in the presence or absence of serum. d, binding was found to be variable between log and stationary phase promastigotes.

7.2. Materials and Methods.

7.2.1. Measurement of the oxidative burst produced by macrophages in response to uptake of *L. mexicana* promastigotes and axenic amastigotes.

Macrophages were elicited from BALB/c mice as described elsewhere (Chapter 2, section 2.3.2). The oxidative response of elicited macrophages after uptake of *L. mexicana* promastigotes and axenic amastigotes was analysed by measuring the emission of light from luminol after its oxidation via the production of reactive oxygen intermediates, as described elsewhere (Chapter 2, section 2.5.2.2). Elicited macrophages were used throughout experimentation because preliminary studies had demonstrated that resident macrophages gave a response that was undetectable in the system used.

7.2.2. Analysis of the attachment and uptake of parasites by peritoneal exudate cells.

An investigation of the kinetics of attachment and subsequent entry of various *L. mexicana* mutant strains was undertaken using resident peritoneal exudate cells from BALB/c mice (see Chapter 2, section 2.3.1.1). Stationary phase promastigotes or axenic amastigotes were prepared at the appropriate concentration (to give a parasite:macrophage ratio of 1:1) and the macrophages were infected as described elsewhere (Chapter 2, section 2.4). At the given time points, the macrophages were washed in PBS and fixed with 1% (v/v) paraformaldehyde in PBS for 30 min at 32°C, 95% air, 5% CO_2. This fixative

was employed to minimise the loss of external parasites during the fixing process. The infected cells were then stained with Giemsa's stain for 20 min (Chapter 2, section 2.3.1.2). At least 200 macrophages per time point were assessed for numbers of attached and internalised promastigotes. The experiment was repeated in duplicate on three separate occasions and the data expressed as numbers of promastigotes attached or internalised per 100 cells. The significance of the data was assessed using either student's t-tests or heteroscedastic t tests, as appropriate.

7.2.3. Characterisation of the effect of receptor blocking on the attachment of wild-type promastigotes.

The effect of blocking specific macrophage surface receptors on the binding of wild-type promastigotes was assessed by the method outlined in Chapter 2, section 2.6.1. Briefly, after pre-blocking the receptors in the desired concentration of antibody, they were infected with a stationary phase promastigote:macrophage ratio of 10:1 for 30 min at 4°C. Following this, the macrophages were washed in PBS and fixed with 1% (v/v) paraformaldehyde in PBS for 30 min at 4°C. After staining in Giemsa's stain for 20 min, the numbers of promastigotes attached per macrophage were determined for at least 200 cells and expressed as numbers per 100 cells. The experiment was repeated on three separate occasions and the data analysed using either student t tests or heteroscedastic t tests, as appropriate.

7.2.4. Investigation of the role of different receptors in attachment of *L. mexicana* promastigotes *in vitro*.

The role of various receptors during the attachment of stationary phase promastigotes to macrophages was assessed as described earlier (Chapter 2, section 2.6.2) The experiments were repeated on three separate occasions in duplicate and the numbers of promastigotes attached per 100 macrophages was calculated for each. Results are given as mean ± standard deviation and significance was assessed using either student t-tests or heteroscedastic t tests, as appropriate.

7.2.5. Statistical Analysis of data

The data produced was analysed using either student t tests or heteroscedastic t tests if the variance of the samples was shown to be unequal by variance ratio F testing. When appropriate the variation between samples was assessed using one-way ANOVA.

7.2.6. Assessment of tyrosine phosphorylation events during initial stages of infection.

An analysis of the tyrosine phosphorylation events associated with the uptake of promastigotes by macrophages was undertaken on my behalf by Angus Cameron (Division of Biochemistry and Molecular Biology, University of Glasgow) according to the procedures outlined in Cameron and Allen (1999).

Briefly, macrophages were harvested from BALB/c mice, adhered overnight and infected as previously described (Chapter 2, sections 2.3.1.1 and 2.3.1.2). After incubation for the appropriate time, the macrophages were washed in serum free medium and lysed in ice-cold lysis buffer containing protease and phosphatase inhibitors (1% [v/v] Triton X100/50 mM Tris-HCl, pH 7.5/0.25% [w/v] sodium-deoxycholate/150 mM sodium chloride/1 mM ethylenediamine tetraacetic acid [EDTA]/1 mM vanadate/1 mM sodium fluoride containing 1 mM phenylmethylsulphonyl fluoride and 1 $\mu g ml^{-1}$ each of chymostatin, leupeptin, antipain and pepstatin). The lysates were then clarified at 15,000 g for 15 min and stored at -20°C until required. The tyrosine phosphorylation events were analysed using western blotting with anti-serum specific for phospho-tyrosine (clone 4G10, Upstate Biotechnology Inc) (Chapter 2, section 2.8.1).

7.3. Results.

7.3.1. The oxidative response of macrophages during internalisation of stationary phase promastigotes.

Previous work had demonstrated that the role of the *CPB* enzymes may lie in the initial establishment of infection in macrophages (Mottram *et al.*, 1996). I therefore decided to investigate the oxidative response of macrophages during the phagocytosis of stationary phase promastigotes. Figure 7.1 gives a graphical representation of the mean luminescence from six separate experiments. The positive control used was macrophages stimulated with PMA which gave rise to

a large oxidative response which started to tail off after approximately 5 min (panel A). The negative control was unstimulated macrophages and gives an indication of the amount of background fluorescence within the system (panel B). After addition of wild-type stationary phase promastigotes, there was a lag period of approximately 5 min before a detectable oxidative response occurred, which continued over the 30 min period of analysis (panel C). Interestingly, infection of macrophages with Δ*CPB* caused a delay of approximately 20-30 min in the onset of the production of reactive oxygen species (panel D). The burst became detectable and rose to a level similar to that stimulated by wild-type by the time the experiment was stopped after 40 min. Analysis of the oxidative response of macrophages after infection with Δ*CPB/g2.8*, however, demonstrated that re-expression of CPB/G2.8 within Δ*CPB* did not restore the oxidative response of the macrophages to that seen for wild-type cells (panel E).

The phagocytosis of promastigotes by macrophages is coincident with the production of an oxidative burst (Murray 1982), conversely the uptake of amastigotes produces no oxidative response from the host cells (Channon *et al.*, 1984). I therefore decided to analyse the response of macrophages in this system to infection with axenic amastigotes. As Δ*CPB* axenic amastigotes have wild-type virulence I postulated that the response of macrophages to infection should be similar in both wild-type and Δ*CPB* cells. Analysis of the data demonstrated that no measurable oxidative response was elicited by axenic amastigotes of any of the lines investigated (data not shown), confirming the previously published data (Channon *et al.*, 1984).

7.3.2. The attachment and uptake of *L. mexicana CPB* mutants into peritoneal exudate macrophages from BALB/c mice.

The previous data had demonstrated that the production of the oxidative burst by macrophages in response to infection with Δ*CPB* promastigotes was delayed. As the phagocytosis of promastigotes is coincident with an oxidative burst (Murray, 1982), I hypothesised that the delay in oxidative response was indicative of a slower uptake of Δ*CPB*. I therefore decided to examine the kinetics of attachment and uptake of various mutant strains with respect to *in vitro* macrophage infections. Figure 7.2 is a graphical representation of the attachment and internalisation of stationary phase promastigotes of the wild-type, Δ*CPB* and Δ*CPB*/*g2.8* lines. Wild-type stationary phase promastigotes were found to be attached to macrophages from 2 min post infection. The numbers of attached promastigotes increased over the first 30 min of infection and thereafter remained approximately constant. A similar pattern was observed for Δ*CPB* stationary phase promastigotes, i.e. they attached after 2 min and levels of attachment increased over the first 30 min of infection albeit at a slower rate than for wild-type. Comparison with Δ*CPB*/g2.8 demonstrated that this line also attached early in infection and attachment levels were similar to those for wild-type throughout the experiment. One problem encountered with this line is that there was a certain amount of variation in the attachment of the re-expressor between experiments so that although levels of attachment appear to be lower than those of wild-type they were not statistically different (except at t = 15min, $t_{10} = 2.771$, $P < 0.05$, and t = 30 min, $t_{10} = 5.086$, $P < 0.01$).

Analysis of the internalisation of stationary phase promastigotes by macrophages demonstrated an interesting difference in the speed of uptake between wild-type and Δ*CPB*. Wild-type promastigotes were observed within the macrophages from 5 min post-infection and the numbers increased throughout the experiment until 240 min when there were 36.9 ± 10.6 promastigotes inside/100 cells. The internalisation of Δ*CPB* however, was not detectable until a full 15 - 20 min after infection, with the level of internalised promastigotes remaining significantly lower than wild-type throughout the experiment. The uptake of Δ*CPB*/g2.8 was analysed and found to closely follow the pattern observed for wild-type promastigotes, with parasites being observed within the cells from 5 min post infection with no significant difference in the numbers of promastigotes being internalised at ($P > 0.05$).

As mentioned previously the axenic amastigotes of Δ*CPB* have similar levels of virulence to wild-type parasites *in vitro*. I therefore decided to analyse the kinetics of axenic amastigote internalisation into macrophages to ascertain whether wild-type and Δ*CPB* axenic amastigotes have similar kinetics of entry. Figure 7.3 gives a graphical representation of the attachment and uptake of axenic amastigotes into macrophages. Analysis of the data demonstrated that the kinetics of attachment and entry of all three lines were comparable, with internalised amastigotes being detectable after 2 min of infection in each case. This indicated that the prolonged period of interaction with the surface of the macrophages which typifies Δ*CPB* promastigote entry only occurs in the promastigote stage and may therefore be important in reducing the virulence of the parasite *in vitro*.

7.3.3. The attachment and uptake of *L. mexicana* GPI:protein transamidase mutants into peritoneal exudate macrophages.

The GPI:protein transamidase, GPI8, is involved in attachment of GPI anchors to surface molecules such as gp63. Many roles have been postulated for gp63 during the attachment of promastigotes to macrophages (see section 7.1, for detail). Analysis of the phenotype of Δ*GPI8 Leishmania* has demonstrated that there is no gp63 expression on their surface (Hilley *et al.*, 1999). Gp63 has been demonstrated to be important in the binding of promastigotes to the macrophage surface (Russell and Wright, 1998). I, therefore, decided to investigate the kinetics of attachment of Δ*GPI8* promastigotes and axenic amastigotes during *in vitro* infections.

Figure 7.4, illustrates the attachment and uptake of Δ*GPI8* promastigotes compared to that of wild-type. The attachment of Δ*GPI8* to macrophages *in vitro* had similar kinetics to the attachment of wild-type stationary phase promastigote's. No statistically significant difference was observed between these two lines at any time point ($P > 0.05$). Analysis of the uptake, however indicated that Δ*GPI8* promastigotes were internalised from 2 min post infection. This indicated that Δ*GPI8* had an increased capacity to elicit phagocytosis. The number of internalised Δ*GPI8* remained significantly higher than wild-type until 240 min post infection, where no significant difference was observed ($t_8 = 0.9$, $P > 0.1$). This indicated that loss of the expression of molecules such as gp63 from the surface had no adverse effect on the kinetics of uptake and may even enhance

it. Analysis of the kinetics of Δ*GPI8* axenic amastigote uptake (see figure 7.5) indicated that it has kinetics of uptake are statistically similar to those of wild-type at all time points ($P > 0.05$).

7.3.4. Analysis of the receptor mediated attachment of wild-type stationary phase promastigotes *in vitro*.

The data described earlier in this chapter indicated that stationary phase promastigotes lacking in the *CPB* enzymes have a prolonged period of interaction with the surface of macrophages before internalisation. As this phenomenon was not observed for axenic amastigotes, it may be important in reducing the virulence of Δ*CPB*.

As discussed in the introduction to this chapter, the receptors mediating uptake can influence how macrophages respond to phagocytosed material. I therefore decided to analyse the roles of different receptors in the binding of wild-type and Δ*CPB* stationary phase promastigotes. Receptors were chosen that had been demonstrated in the past to be involved in the binding of various *L. mexicana* surface molecules, namely CR3 and CR4 (Talamas-Rohana *et al.*, 1990 and Russell and Wright, 1998). These receptors had previously been demonstrated to bind to purified *Leishmania* gp63 and LPG and so it was necessary to confirm their use by the parasites themselves. The role of the mannose receptor in attachment was also analysed.

Figure 7.6, shows the effect of monoclonal antibodies specific for the CD11b part of CR3 (M1/70) on the binding of wild-type promastigotes. No decrease in binding was observed using increasing concentrations of control

antibody indicating that any inhibition observed is due to the specific blocking of CR3 as opposed to binding of the antibodies Fc region to Fc receptors. From this experiment, it is clear that wild-type *L. mexicana* can utilise CR3 during attachment to macrophages. It was decided to use the anti-CR3 antibodies at 10 $\mu g\ ml^{-1}$ because maximum inhibition with no further significant decrease in binding occurred after this concentration ($t_5 = 1.081$, $P > 0.1$). An analysis of the inhibitory effect of anti-CD11c antibodies (anti-CR4) on wild-type binding demonstrated that wild-type promastigotes can use CR4 during attachment (see figure 7.7). From this investigation a concentration of 3 μgml^{-1} anti-CR4 antibody was deemed to be sufficient to give maximal inhibition of wild-type binding as no further increase in inhibition was observed by increasing the antibody concentration ($t_4 = 0.4$, $P > 0.1$).

7.3.5. The effect of monoclonal antibodies against macrophage receptors on the binding of various mutant lines.

Analysis of the binding of wild-type, Δ*CPB* and Δ*CPB/g2.8* stationary phase promastigotes to macrophages pre-incubated with either IgG2b or anti-CR3 antibodies, indicated that both wild-type and Δ*CPB/g2.8* utilise CR3 as a receptor during binding as attachment was inhibited by 51.2 ± 8.7 % and 69.7 ± 1.5 %, respectively (Figure 7.8). In the case of Δ*CPB*, however only a 9.5 ± 9.2 % inhibition of binding was observed. This suggested that the binding of Δ*CPB* promastigotes to macrophages was not via CR3 receptors.

Analysis of the inhibitory effect of anti-CR4 antibodies indicated that wild-type and Δ*CPB/g2.8* promastigotes also appear to utilise CR4 during binding of the parasites to macrophages (see Figure 7.9). Blocking this receptor inhibited binding of wild-type by 46.3 ± 18.6 % and of Δ*CPB/g2.8* by 52.7 ± 5.8 %. The attachment of Δ*CPB* was inhibited by only 4.3 ± 7.1 %, again indicating that it utilises other receptors for attachment.

Analysis of the effect of blocking both CR3 and CR4 simultaneously demonstrated that there was no increase in the amount of inhibition observed for either wild-type or Δ*CPB/g2.8* compared to the use of either antibody singly (see Figure 7.10). The binding of wild-type and Δ*CPB* promastigotes was also analysed with respect to the mannose receptor (see Figure 7.11). This demonstrated that wild-type promastigotes did not utilise the mannose receptor when attaching to the surface of macrophages as blocking the receptors with increasing concentrations of mannan had no effect on the binding of promastigotes to macrophages ($F_{4,10} = 1.2$, $P > 0.05$). Δ*CPB* also was not inhibited by blocking the mannose receptor, however statistical analysis of this data indicated that binding in the presence of increasing concentrations of mannan was different to control levels ($F_{3,8} = 4.43$, $P < 0.05$). This was found to be due to a slight increase in binding under the conditions analysed.

7.3.6. The tyrosine phosphorylation events following infection of macrophages with *L. mexicana* promastigotes.

The data discussed so far in this chapter have demonstrated that avirulent Δ*CPB* attach to their macrophage host cells via different receptors to virulent lines. As discussed earlier the receptors used during phagocytosis can alter the way in which macrophages act towards the phagocytosed material, suggesting that differences in signalling might occur. A simple method for analysing the intracellular signalling activity of cells during phagocytosis is via the investigation of protein tyrosine phosphorylation. It was therefore decided to analyse the tyrosine phosphorylation events occurring during internalisation of different *L. mexicana* strains to investigate whether differences occurred in the signalling pathways stimulated following uptake of wild-type or Δ*CPB*. Figure 7.12 shows a western blot probed with antibodies specific for phospho-tyrosine. The antibody detects a number of phosphorylated proteins in unstimulated macrophages as expected (Zaffran *et al.*, 1999 and Murai *et al.*, 1996). The levels of phosphorylation are increased overall by incubating the cells with PMA (lane 2). After incubation of the cells with wild-type promastigotes for 5 min there was no enhancement of the phosphoryltation events occurring within the cell. By 15 min, however, phosphorylation was increased indicating the cells were responding to infection. A similar pattern is observed for both Δ*CPB* and Δ*CPB*/g2.8 internalisation. This indicates that wild-type parasites are not surviving in their host cells by inactivating the downstream tyrosine phosphorylation-mediated signalling of the cell.

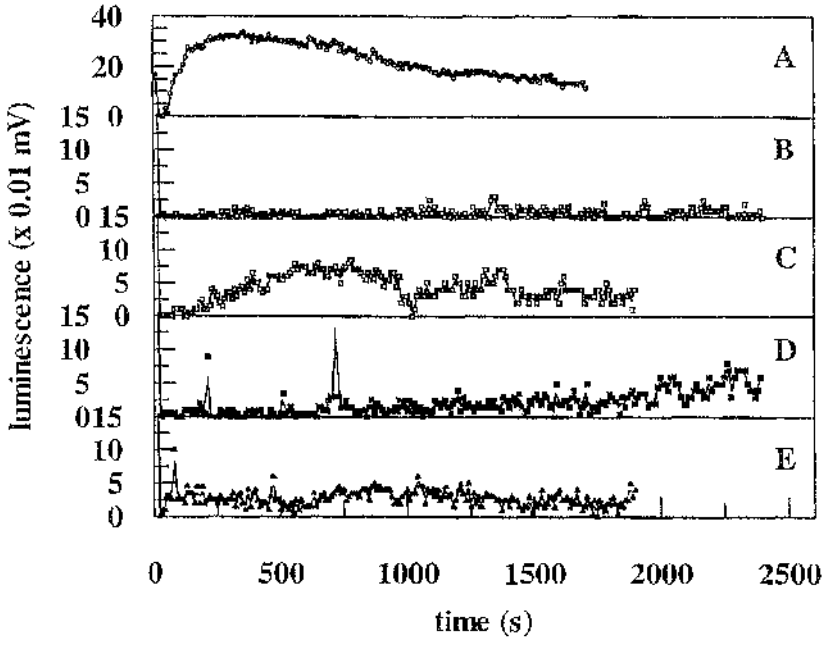

Figure 7.1: The oxidative response of peritoneal exudate cells during the uptake of *L. mexicana* stationary phase promastigotes. A; macrophages stimulated with 100 ng/ml PMA. B; unstimulated macrophages. C; macrophages infected with wild-type. D; macrophages infected with Δ*CPB*. E; macrophages infected with Δ*CPB*/*g2.8*. The graphs represent the mean luminescence from 6 separate experiments.

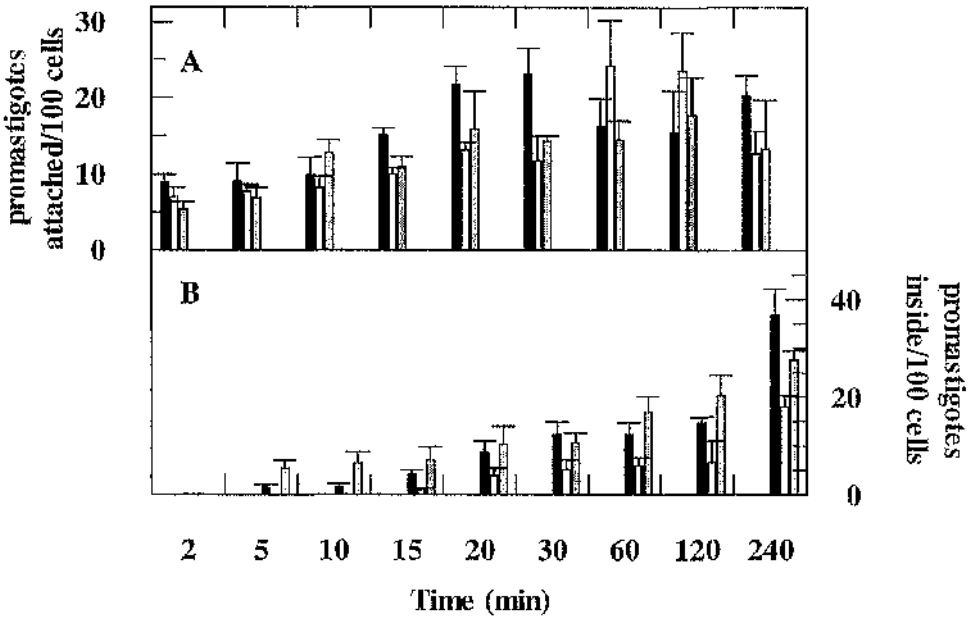

Figure 7.2: The kinetics of entry of stationary phase promastigotes into peritoneal exudate cells. A; numbers of attached promastigotes per 100 macrophages. B; numbers of internalised promastigotes per 100 cells. Wild-type kinetics are indicated by black bars, Δ*CPB* kinetics are indicated by white bars with grey bars indicating the kinetics of Δ*CPB/g2.8*. The experiments were repeated three times in duplicate and the bars represent the mean value with standard errors. The attachment and uptake of wild-type and Δ*CPB/g2.8* were found to be approximately similar throughout. Δ*CPB* attachment was found to be significantly lower than that of wild-type ($P < 0.05$) until 30 min p.i. after which levels were comparable. The level of attachment of Δ*CPB* was also demonstrated to be significantly lower ($P < 0.05$) throughout.

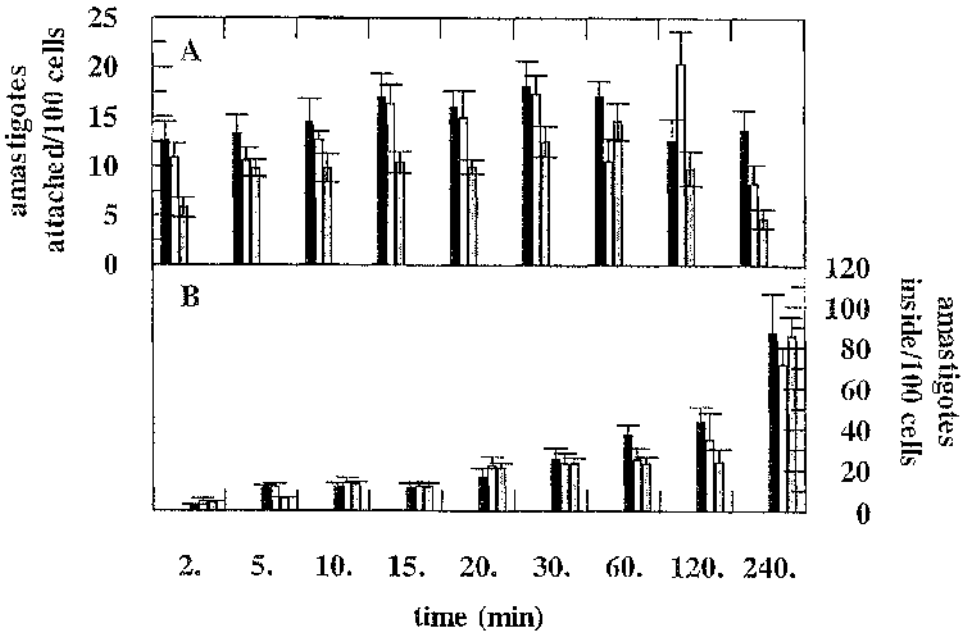

Figure 7.3: The kinetics of uptake of axenic amastigotes into peritoneal exudate cells. A; the number of attached amastigotes per 100 cells. B; the number of internalised amastigotes per 100 cells. Wild-type kinetics are indicated by black bars, Δ*CPB* kinetics are indicated by white bars with grey bars indicating the kinetics of Δ*CPB/g2.8*. The experiments were repeated three times in duplicate and the bars represent the mean value with standard errors. No significant difference was observed in the attachment or uptake of any of the lines analysed ($P > 0.05$).

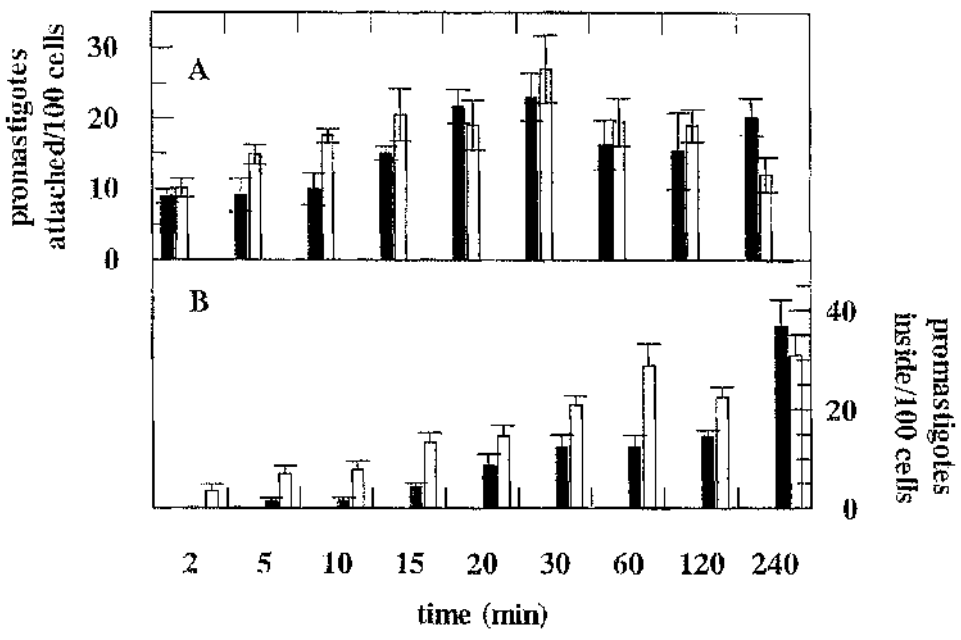

Figure 7.4: The kinetics of uptake of Δ*GPI8* promastigotes into peritoneal exudate cells. A; the numbers of promastigotes attached per 100 cells. B; the numbers of promastigotes internalised per 100 cells. Black columns indicate the kinetics of wild-type infection. White columns indicate the kinetics of Δ*GPI8* infection. The experiments were repeated three times in duplicate and the bars represent the mean value with standard errors. The attachment of the two lines did not significantly differ ($P > 0.05$). Internalisation of Δ*GPI8* was significantly higher than that of wild-type until 240 min p.i. ($P < 0.05$).

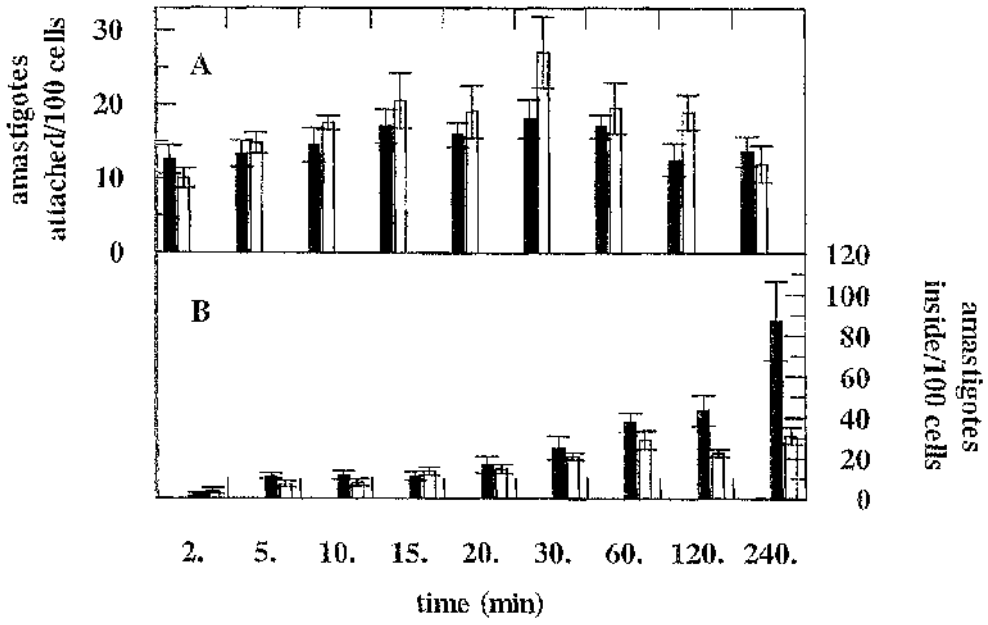

Figure 7.5: The kinetics of attachment and uptake of Δ*GPI8* axenic amastigotes into peritoneal exudate macrophages. A; the number of amastigotes attached per 100 cells counted B; the number of amastigotes inside per 100 cells counted. Black columns indicate the kinetics of wild-type infection. White columns indicate the kinetics of Δ*GPI8* infection. The experiments were repeated three times in duplicate and the bars represent the mean value with standard errors. The two lines did not differ significantly in their ability to attach or be internalised ($P > 0.05$).

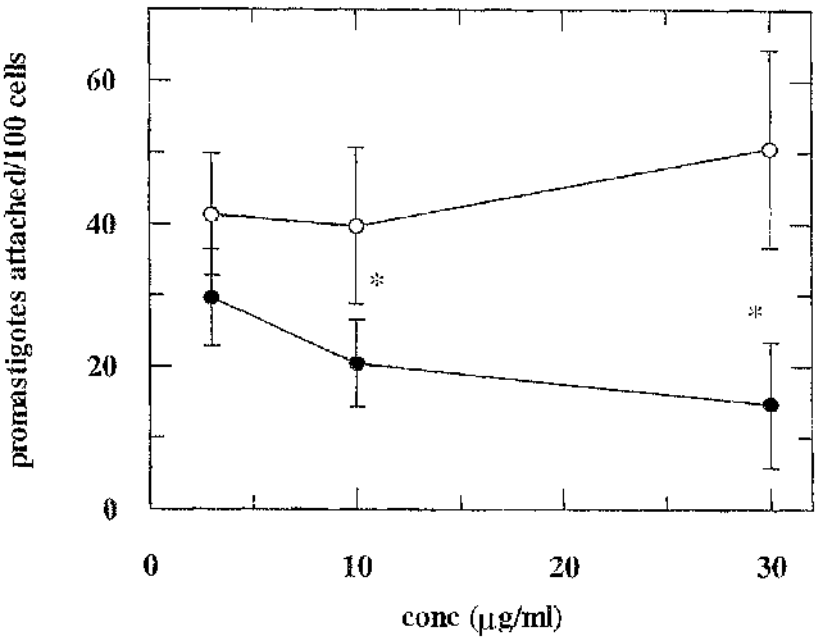

Figure 7.6: The inhibitory effect of anti-CR3 (anti-CD11b) antibodies in the binding of wild-type stationary phase promastigotes. Open circles; isotype control (IgG2b). Solid circles; anti-CR3 antibodies (CD11b). The experiment was repeated on three separate occasions. The graph illustrates the mean number of attached promastigotes with standard deviation. The inhibition was assessed statistically, * = significant at $P < 0.05$.

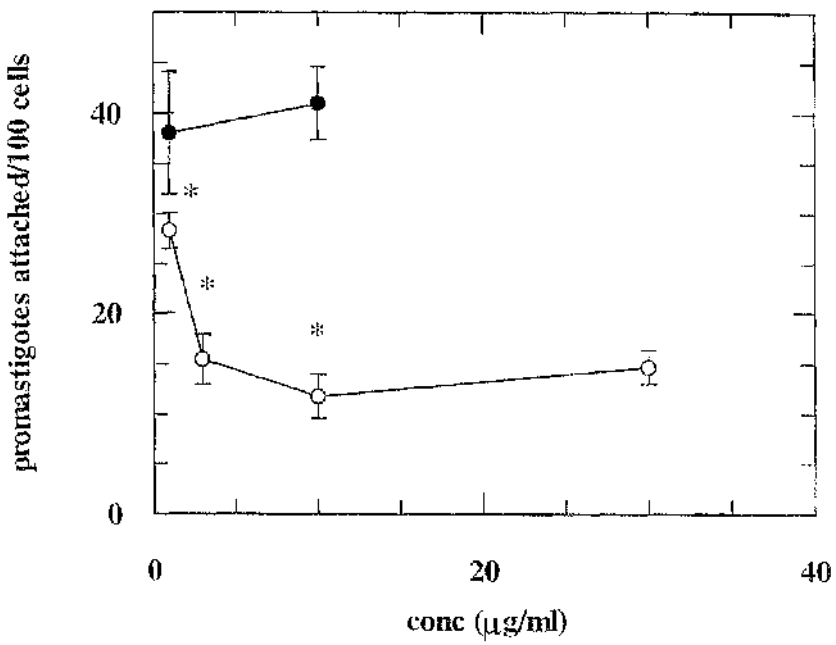

Figure 7.7: The inhibitory effect of anti-CR4 (anti-CD11c) antibodies against binding of wild-type stationary phase promastigotes. Solid circles; isotype control antibody (IgG1). Open circles; anti-CD11c antibody. The experiment was repeated on three separate occasions. The graph indicates the mean of these results with error bars showing the standard deviation. The results were assessed statistically, * = significant at $P < 0.05$.

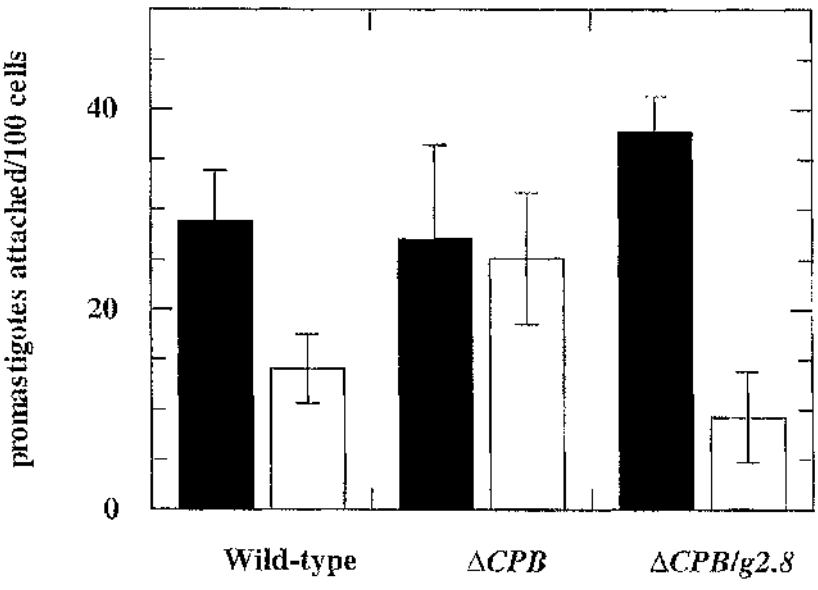

Figure 7.8: The effect of blocking CR3 on the binding of stationary phase promastigotes. Graphical representation of the binding of promastigotes to macrophages incubated with either IgG2b isotype control (black bars) or anti CR3 (white bars). The experiment was completed three times in duplicate and the bars indicate the mean with standard deviation. Wild-type binding was significantly reduced in the presence of anti-CR3 antibodies ($t_4 = 5.4$, $P < 0.01$). Δ*CPB/g2.8* binding was also significantly reduced ($t_4 = 11.9$, $P < 0.01$). No significant reduction occurred with Δ*CPB* ($t_4 = 0.04$, $P > 0.05$).

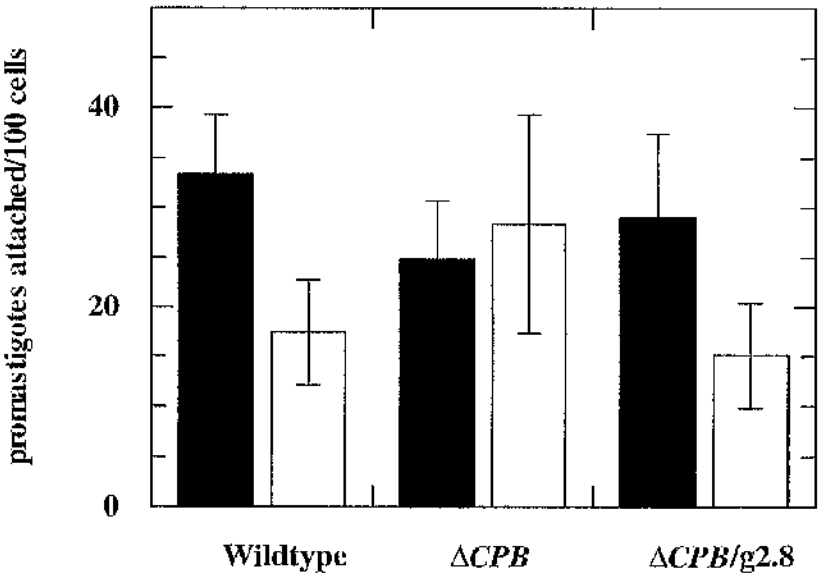

Figure 7.9: The effect of blocking CR4 on the binding of stationary phase promastigotes. Graphical representation of the binding of promastigotes to macrophages incubated with either IgG1 isotype control (black bars) or anti-CR4 (white bars). The experiment was completed three times in duplicate and the bars indicate the mean with error bars showing the standard deviation. Wild-type binding was significantly reduced by anti-CR4 antibodies ($t_4 = 4.9$, $P < 0.01$). The binding of *ΔCPB/g2.8* was also significantly reduced ($t_4 = 3.4$, $P < 0.05$). No effect was observed on the binding of *ΔCPB* ($t_4 = 0.7$, $P > 0.1$).

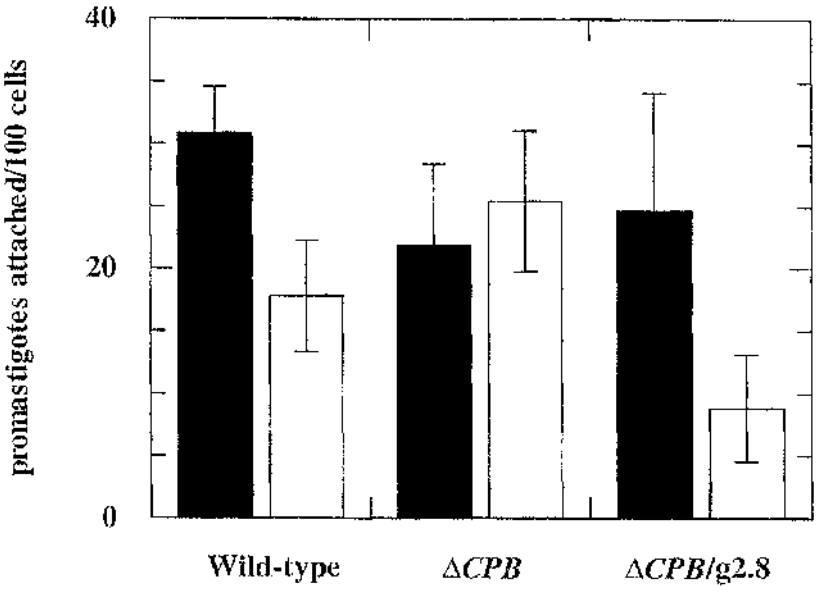

Figure 7.10: The effect of blocking both CR3 and CR4 on the binding of stationary phase promastigotes to peritoneal exudate cells. Graphical representation of the binding of promastigotes to macrophages incubated with either IgG1 and IgG2b isotype control (black bars) or anti-CR3 and anti-CR4 (white bars). The experiment was completed three times in duplicate and the bars indicate the mean with error bars showing the standard deviation. Wild-type binding was significantly reduced in the presence of anti-CR3/CR4 antibodies (t_4 = 5.6, $P < 0.01$). The binding of Δ*CPB/g2.8* was also reduced. (t_4 = 3.8, $P < 0.05$). No effect on the binding of Δ*CPB* was observed (t_4 = 1, $P > 0.05$).

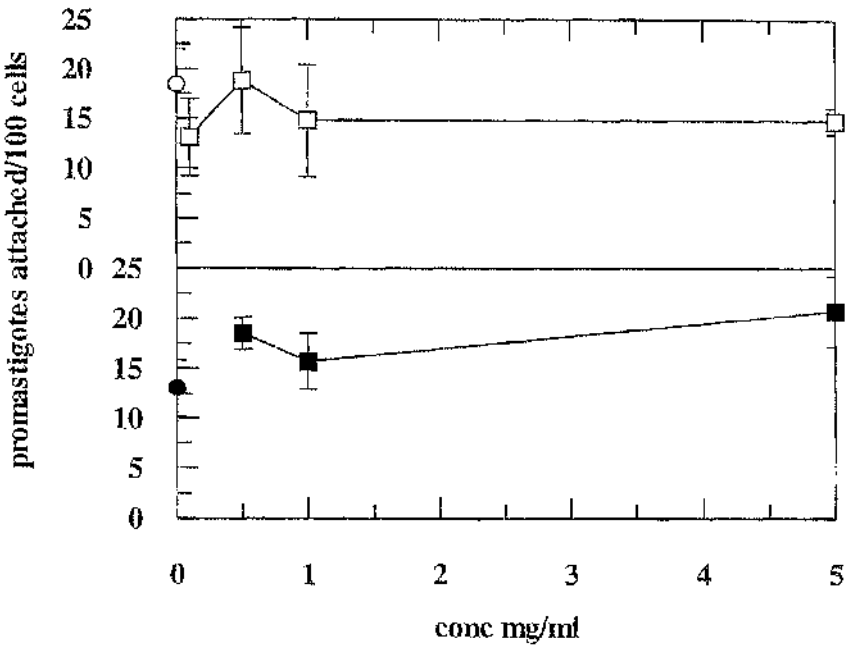

Figure 7.11: The binding of stationary phase promastigotes to peritoneal exudate macrophages in the presence of mannan. The graphs indicate the number of promastigotes attached per 100 cells counted. Open symbols; wild-type. Solid symbols; Δ*CPB*. Circles indicate the binding without mannan. Squares indicate the binding in the presence of mannan. The experiment was repeated on three separate occasions. Data points indicate the mean from these repeats with error bars indicating the standard deviation. Wild-type binding was not significantly reduced by mannan ($F_{4,10} = 1.2$, $P > 0.05$). The binding of Δ*CPB* was not significantly reduced by mannan, although a small increase in binding occurred ($F_{3,8} = 4.43$, $P > 0.05$).

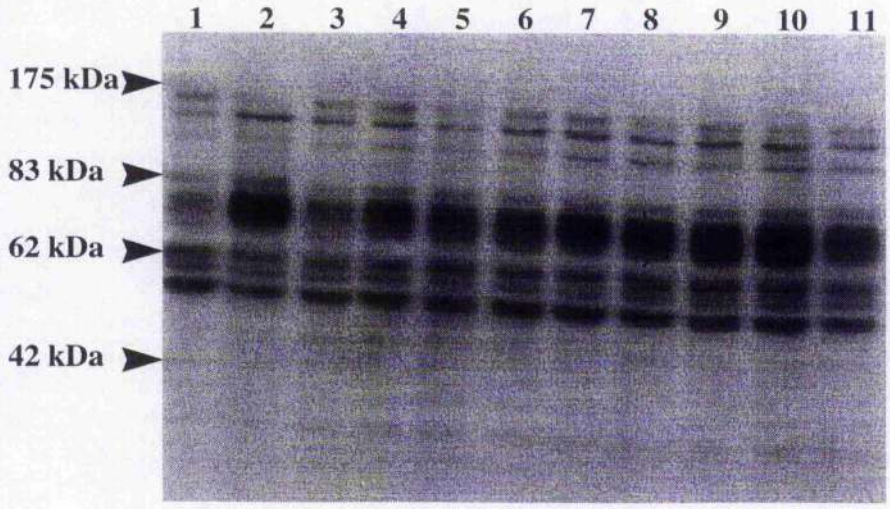

Figure 7.12: Western blot analysis of the tyrosine phosphorylation occurring during the internalisation of *L. mexicana* promastigotes by peritoneal exudate cells. Lane 1; unstimulated macrophages. Lane 2; PMA- stimulated macrophages. Macrophages infected with wild-type promastigotes for 5 min (lane 3), 15 min (lane 4) or 1 h (lane 5). Macrophages infected with Δ*CPB* promastigotes for 5 min (lane 6), 15 min (lane 7) or 1 h (lane 8). Macrophages infected with Δ*CPB*/*g2.8* for 5 min (lane 9), 15 min (lane 10) or 1 h (lane 11). The blot was probed with antibodies specific for phosphotyrosine (4G10).

7.4. Discussion.

This investigation involved a comparison of the attachment and uptake of wild-type *L. mexicana* promastigotes and axenic amastigotes with those of Δ*CPB*. Work discussed elsewhere (see Chapter 6) had demonstrated that the response of macrophages to established infections with Δ*CPB* was comparable to the response to wild-type infection. Many previous investigations have demonstrated that during the phagocytosis of stationary phase promastigotes, the macrophage host produces a respiratory burst (Murray, 1982). I postulated that the magnitude of the elicited burst may differ between wild-type and Δ*CPB* promastigotes, with the *CPB* null mutant parasites perhaps stimulating a larger burst and hence suffering reduced intracellular survival. However, investigation of the initial oxidative burst produced during the uptake of promastigotes demonstrated that instead of infections with Δ*CPB* giving rise to an increased level of reactive oxygen species (ROS) the mutant parasites appeared to reduce the production of ROS and delay the response by approximately 20 min. As promastigote uptake is concomitant with the production of an oxidative burst, this result suggested that promastigote internalisation was slower in the case of Δ*CPB*.

An analysis of the ROS production during internalisation of Δ*CPB/g2.8* demonstrated that re-expression of CPBG2.8 failed to restore the wild-type phenotype with regards to ROS production. This line has been previously demonstrated to have wild-type virulence levels (Mottram *et al.*, 1996) and so this result indicated that perhaps this was not important in the virulence of the

parasite. However, it must be remembered that the re-expression of CPB/G2.8 occurs from an episome which is under the control of antibiotic pressure, which may mean that the amount of re-expression varies within any given culture. *CPB/g2.8* is an internal copy from the *CPB* array and is naturally expressed within the amastigote stage of the life-cycle (Mottram *et al.*, 1996; Mottram *et al.*, 1997) thus expression within the promastigote may give anomalies in certain aspects of infection. A further possibility is that the level of oxidative burst produced by these parasites was too low to be measurable in the system used. Perhaps further analysis using more sensitive methods of detection would allow further confirmation of this phenotype.

Despite the anomalies concerning Δ*CPB/g2.8*, I decided to investigate whether the retardation of the oxidative burst observed during Δ*CPB* infection was indeed due to a delay in phagocytosis and whether this delay was due to the parasites attaching to the macrophages more slowly than wild-type. Analysis of the dynamics of attachment and uptake of the parasites over the first four hours of infection demonstrated that Δ*CPB* promastigotes attached to the surface of the macrophages by 2 min post infection, but that they then displayed a prolonged period of interaction with the surface of the cell before internalisation, which was not observed until 20 min post infection. This differed from the kinetics of wild-type and Δ*CPB/g2.8* infection, where attachment was rapidly followed by internalisation such that promastigotes were evident within cells after 5 min of infection. This speed of wild-type uptake is comparable to that observed for the phagocytosis of *Mycobacterium tuberculosis* (Zaffran *et al.*, 1998) and for zymosan particles (Veras *et al.*, 1996) indicating how rapidly

phagocytic cells normally ingest attached material. The fact that internalisation of Δ*CPB* occurred after 20 min of infection corroborated the oxidative burst data which showed that a delay of approximately 20 - 30 min occurred before phagocytosis into the macrophages began.

This prolonged period of interaction with the surface of the macrophages indicated that a difference existed between the binding of wild-type promastigotes and those of Δ*CPB*. Encouragingly this difference was not observed during infection with the amastigote stage of the life-cycle, indicating that it only occurred in the stage whose virulence was compromised by the lack of the CBP enzymes.

Evidence from previous work (see Chapter 4) had indicated that the turnover of gp63 from the surface of the promastigotes was reduced in the absence of the CPB enzymes, indicating that differences in the surface architecture existed. These differences in architecture could cause alterations in the charge of the surface or the distribution of important surface molecules, such that normal receptor-ligand interaction was perturbed. Although the fact that the slower uptake was due to a weaker receptor ligand interaction could not be ruled out, this gave rise to the interesting possibility that different receptors may be utilised in the binding of Δ*CPB* to macrophages. As discussed previously, the receptors involved in uptake of *Leishmania* can be important in determining their intracellular fate (Da Silva *et al.*, 1989). It has further been demonstrated by Chakrabarty *et al.* (1996b) that avirulent *L. donovani* promastigotes are taken up into their host cells more slowly than the virulent forms, indicating again that the mechanisms of uptake can be important in determining virulence.

As discussed previously several different receptors have been demonstrated to be important in various *Leishmania* species, however, little work has involved investigation of the receptors important in *L. mexicana* adhesion. Two studies have investigated the adhesion of beads coated with either LPG or gp63 (Talamas-Rohana *et al.*, 1990 and Russell and Wright, 1998 respectively) and have shown that these molecules are important in the attachment to CR3 and CR4 (LPG only). I therefore decided to investigate the binding of *L. mexicana* promastigotes to the receptors indicated in these studies. This work was undertaken at 4°C to inhibit the macrophages cellular metabolism, thus preventing any downstream signalling effects occurring due to the aggregation of receptors. Aggregation of CR3, for example has been demonstrated to cause a down regulation of CR1 expression (Gresham *et al.*, 1991). Ideally this work would have been carried out using Fab fragments of antibodies to prevent cross-linking of the receptors. However production of Fab fragments is a multistep process (Johnstone and Thorpe, 1982) which therefore requires a large amount of starting material to make production feasible, thus preventing their use due to financial cost. Further there is no guarantee that Fab fragments would block the binding of promastigotes to the receptors because of their reduced size and the fact that they may not bind exactly to the site of promastigote binding, but merely inhibit binding by overlaying this site.

This work demonstrated that both CR3 and CR4 are involved in the attachment of live wild-type and *ΔCPB/g2.8 L. mexicana* stationary phase promastigotes to macrophages, with a reduction in binding of approximately 50 % achieved following blocking of the receptors with specific antibodies. This demonstrates a heavy reliance by *L. mexicana* on the receptors as previous

studies with *L. donovani* have indicated only a 25 - 30 % inhibition following blocking of CR3 (Blackwell *et al.*, 1985). Surprisingly, blocking both receptors simultaneously did not lead to an additive inhibitory effect on binding which has been demonstrated by the co-operation of CR3 with CR1 during binding of *L. major* (Rosenthal *et al.*, 1996). However previous work has demonstrated that for *L. donovani* it is necessary for the parasite to bind two receptors for adhesion to occur (Blackwell *et al.*, 1985). Furthermore it is conceivable that promastigotes could bind to more than one receptor due to their elongated shape.

Another possibility is that the parasite is binding to the CD18 portion of the CR3 and CR4 receptors. Both molecules are members of the leukocyte integrin family and form non-covalently linked heterodimers with a CD18 molecule (reviewed in Kishimoto *et al.*, 1989). However the antibodies I used bind specifically to the CD11b and CD11c chains of the receptors and unless both fortuitously caused steric hindrance, I consider the blocking of CD18 unlikely. Wild-type promastigotes therefore attach to both CR3 and CR4 during uptake into macrophages, however blocking of these receptors inhibits binding by only 50% indicating that the use of other receptors occurs during entry. The fact that promastigote internalisation is concomitant with the production of an oxidative burst despite the fact that CR3 is utilised (Wright and Silverstein, 1983) may be due to the signalling occurring after aggregation of CR4. The respiratory burst had no adverse effects on the promastigotes and may even be important in the establishment of infection, as discussed in Chapter 6.

Further to this it was demonstrated that blocking either CR3 or CR4 or both together had no inhibitory effect on the binding of Δ*CPB* promastigotes to

the cell, demonstrating that this parasite is mediating attachment via different mechanisms to the wild-type. This could be responsible for the reduction in virulence observed during *in vitro* infections. An interesting follow-up experiment to address the correlation between binding and virulence would be to analyse the intracellular fate of wild-type promastigotes, internalised whilst CR3 and CR4 were blocked. If, indeed, preventing use of these receptors is involved in the reduced virulence of the Δ*CPB*, then the wild-type promastigotes should have reduced survival following phagocytosis. For reasons discussed above these experiments would require the use of Fab fragments to ensure that no aggregation of the receptors occurs. The cost of production therefore made experimentation such as this unfeasible.

The binding of promastigotes to the mannose receptor was also analysed as recent reports had indicated that phagocytosis via this receptor elicits no oxidative response from macrophages (Astarie-Dequeker *et al.*, 1999). It was therefore possible that Δ*CPB* could be utilising this pathway to allow intracellular survival in the few permissive cells It should be noted that the amount of binding observed during the investigation of the mannose receptor was much lower than expected and it remained so throughout the three repeat experiments. Apart from the fact that the macrophages were pre-incubated in mannan dissolved in PBS instead of antibody in medium, the system remained constant as in the other experiments. Perhaps incubation in cold PBS perturbed the macrophage surface in some way such that the total parasite binding was reduced. Increasing concentrations of mannan, however had no further effect on the binding of the parasites, which meant that further investigation of this phenomena was deemed unnecessary. The small increase in Δ*CPB* binding observed in the presence of

mannan was statistically significant but may have been due to variability in the system used. It could indicate however that the parasite bound to the mannan in the medium and thus utilised this to allow increased attachment by competing with the pre-bound mannan for receptors on the macrophage surface. Further investigation is required to provide confirmation of this.

It is known that aggregation of different receptors causes the stimulation of different signalling pathways within a cell (Hunter 1987) and ultimately leads to differences in the response of cells to any given stimulus (Berton and Gordon, 1983). It was therefore postulated that the difference in receptors utilised for uptake of wild-type and Δ*CPB* promastigotes was important in the reduction of virulence. One mechanism by which wild-type attachment and rapid internalisation via different receptors could lead to parasite survival is if the parasites rapid entry allows it to inhibit the cells activation pathways and thus set up a favourable environment for survival. The prolonged period of interaction with the surface of the macrophages in the case of Δ*CPB* may allow the host cell to signal downstream in a more effective manner and thus mount an effective defence against the parasite after internalisation.

Although the investigation of total tyrosine phosphorylation gave no indication as to which pathways are involved in signalling, it provides an analysis of the activation state of the cells. The experiment showed that both wild-type and Δ*CPB* promastigotes stimulated comparable levels of tyrosine phosphorylation in macrophages. This tyrosine phosphorylation is concomitant with phagocytosis (Greenberg *et al.*, 1993), however it may be of interest that the up-regulation of tyrosine phosphorylation observed within the Δ*CPB* occurs

prior to the detection of internalised promastigotes, possibly indicating that some differences in signalling do occur. Further analysis of the signalling pathways stimulated by wild-type and Δ*CPB* may prove interesting as recent data has demonstrated that *L. donovani* infection in macrophages selectively reduced the protein tyrosine phosphorylation events occurring after PMA stimulation and thus inhibited iNOS expression (Nandan *et al.*, 1999). Perhaps Δ*CPB* has reduced ability to elicit such events and hence has reduced intracellular survival.

As discussed previously (see Chapter 4, for details) the GPI:protein transamidase *GPI8* is involved in attachment of a GPI-anchor to gp63, the major surface metalloproteinase of *Leishmania* promastigotes. Many roles have been suggested for gp63 in the life of the parasite, including some evidence for a role in the uptake of promastigotes (see introduction for detail). However, recent work utilising *L. mexicana* deficient in *GPI8* expression has shown that a lack of gp63 on the surface of promastigotes does not reduce the ability of the parasite to survive in macrophages in vitro (Hilley *et al.*, 1999). This indicates that although gp63 is important in mediating attachment and phagocytosis of some *Leishmania* promastigotes (Russell and Wright, 1998), removal from the surface does not effect the survival of *L. mexicana* and suggests perhaps that it's role in uptake is not important in conferring virulence. However, it may be that although gp63 is important in the uptake of some *Leishmania* species it's role in the uptake of *L. mexicana* promastigotes is only minor and therefore removal has no effect on virulence.

The data from investigation of the kinetics of uptake during infection with Δ*GPI8* indicated that the internalisation of the parasite following attachment was rapid. In fact the internalisation of promastigotes lacking surface gp63 was

significantly more rapid than that of wild-type. This may be explained by the fact that gp63 is expressed in high copy number on the surface of the promastigotes and thus may interfere with the binding of another important ligand with its receptor. In *in vitro* infections, gp63 confers to the parasite a degree of resistance to complement mediated lysis (Joshi *et al.*, 1998). This extra protection afforded to the parasite may mean that evolution has allowed a compromise to occur between the reduction in attachment to macrophages and the protection from lysis in serum. If this were the case then in *in vitro* infections, where complement is not present, loss of gp63 expression would give rise to an increase in the amount of attachment and phagocytosis as observed. The fact that the kinetics of uptake of *ΔGPI8* axenic amastigotes are similar to those of wild-type axenic amastigotes, where little surface gp63 expression occurs, may strengthen this argument (Winter *et al.*, 1994).

This work has indicated that gp63 plays only a minor role in the binding of live *L. mexicana* to macrophages *in vitro*, which suggests that LPG contains the major ligands involved. The reduction in turnover of gp63 observed following loss of the CPB enzymes may indicate a phenomenon which effects the entire promastigote surface, due to the build-up of non-catabolised proteins within the cell. LPG is also GPI-anchored to the surface of promastigotes and thus its turnover may be altered by loss of CPB. The biosynthetic pathway of LPG differs to that of gp63 (Ralton and McConville, 1998), and loss of GPI8 has been shown to have no effect on the surface location of LPG (Hilley *et al.*, 1999). Therefore the parasite should be able to function normally during attachment and uptake.

The restoration of Δ*CPB* virulence to wild-type levels when macrophages were infected with axenic amastigotes *in vitro*, further indicates that the receptors involved in uptake may be important in allowing survival of the intracellular parasites as the amastigote form does not utilise the same receptors as promastigotes for uptake (see section 7.1, for detail). However, during *in vivo* infections Δ*CPB* axenic amastigotes display reduced lesion formation (Mottram *et al.*, 1996) indicating that the receptors involved in uptake are not the sole reason for a reduction in virulence *in vivo*. The CPB enzymes are expressed in stationary phase promastigotes and throughout the amastigote stage of the life-cycle (Mottram *et al*, 1997). One would have thought, therefore, that the CPB enzymes would have importance in the intracellular survival of the parasite. The perturbation of turnover of surface molecules in the absence of CPB enzymes demonstrated in Chapter 4 and the role of Cathepsin L-like enzymes in protein catabolism (Mayer and Doherty, 1986) may indicate that this role lies in the nutrition of the parasite or in the recycling of redundant parasite proteins. The conditions experienced during *in vitro* infection will be considerably less harsh than those within a host, where the immune system will mount an attack against the parasite. Hence this role may not be essential *in vitro*.

This chapter has demonstrated that the loss of the CPB enzymes from *L. mexicana* promastigotes prevents the parasite from attaching to the receptors utilised by wild-type promastigotes for efficient entry into macrophages. This leads to a prolonged interaction with the surface of the host cell followed by entry into the cell via different receptors and may therefore reduce the parasites viability during *in vitro* infection.

Chapter 8

General Discussion

This investigation involved an analysis of the roles of the cysteine proteinase enzymes of *L. mexicana*, in particular those enzymes expressed from the *CPB* array. The use of cysteine proteinase inhibitors as a strategy for the control of parasitic infections has been established with a variety of intracellular and extracellular infections (see Chapter 1, section 1.5, for details). It has been shown that cysteine proteinases, especially those with homology to mammalian cathepsins L or B, are important in the survival of many parasites, including *L. mexicana*. One of the approaches I adopted was to analyse effect of cysteine proteinase inhibition on the enzyme expression and viability of *L. mexicana*.

Analysis of the processing of mammalian cathepsin L has demonstrated that the enzyme is trafficked intracellularly to its destination within the lysosomes of the cell. This multstep process involves the removal of the pre-region in the rough endoplasmic reticulum and then the activation of the enzyme during the sequential removal of peptides from the pro-region resulting in the fully active enzyme (see Chapter 3, section 1 and references therein). Work by Selzer *et al.*, (1999) investigating the trafficking of cysteine proteinases of *L. major* using peptidyl cysteine proteinase inhibitors (including KO2) has demonstrated that inhibition causes an accumulation of pro-enzyme within the flagellar pocket of the organism.

This result has been interpreted as showing that there are differences in the processing and trafficking machinery between the parasite and its mammalian host, and thus strengthens the argument that specific inhibitors have potential *in vivo* as drugs. Analysis of the effect of KO2 on the ultrastructure of *T. cruzi* demonstrated that the build-up of cruzipain precursors occurred within the Golgi-apparatus of the cell, again indicating some perturbance of the trafficking of the enzyme (Engel *et al.*, 1998b). The perturbation of enzyme processing in both of these studies was also demonstrated to be concomitant with a reduction in survival of the parasite *in vivo*. These studies indicated that either the loss of the cysteine proteinase activity or the build-up of intermediate forms of the enzymes is detrimental to the survival of the parasite within their host.

My investigation of the effect of KO2 on the ultrastructure of *L. mexicana* wild-type and Δ*CPB/g2.8* promastigotes demonstrated that inhibition resulted in the formation of large vacuoles within the cell which were filled with an electron dense substance. This was not merely due to the over-expression of the enzyme, as the mutants untreated with the drug did not exhibit this phenotype. It is probable that the inhibitor's effects on CPA activity will be partially responsible for this phenomenon, by preventing this enzyme's contribution to CPB processing and consequently either preventing the enzyme from being trafficked to its final destination (the lysosomes) or preventing the degradation of redundant inactive precursor forms of the enzyme. Analysis of the effects of KO2 on *L. major* also demonstrated a perturbation in the lysosomal/endosomal network. Selzer *et al.*, (1999) reported the build-up of membranous material within these vacuoles which

could suggest that inhibition of cysteine proteinases is causing blockage in the catabolism of old cellular proteins, hence the increase in vacuole size. Unfortunately no assessment of the location of the CPB enzymes within inhibitor-treated *L. mexicana* was undertaken (via immunolocalisation). Further investigation of these phenomena, to assess whether these alterations in ultrastructure are due to the build-up of CPB precursors or the inhibition of protein catabolism and indeed to investigate the possible presence of CPB in the flagellar pocket, could prove interesting.

An analysis of the effects of cysteine proteinase inhibition on the growth of *L. mexicana* promastigotes *in vitro* demonstrated that loss of the activity of the enzymes was not toxic to the parasite. Interestingly, the occurrence of the large vacuoles within the cell also had no effect on the parasite's ability to multiply. This result, taken with the fact that *L. major* promastigotes cannot divide in the presence of KO2 (Selzer *et al.*, 1999), suggest that there are differences between the roles of the cysteine proteinases in the two *Leishmania* species. The effect of the inhibitors on the *in vitro* growth of amastigotes demonstrated that growth of this stage was indeed perturbed. This was not surprising taking into account the abundance of cysteine proteinases in this life-cycle stage (Coombs and Mottram, 1997) and provides further encouragement of their potential as drug targets. The greater sensitivity of the amastigotes than promastigotes to cysteine proteinase inhibition suggests that any build-up of pro-enzymes occurring must be more detrimental. This could be by preventing the production off the active enzyme, which may be vital in this stage, and/or through inhibition of protein catabolism, which may cause

starvation of the parasite. Surprisingly axenic amastigotes of the various null mutants were also susceptible to the inhibitors, indicating that the build-up of cysteine proteinase precursors is unlikely to be the cause of the toxicity. Furthermore, this phenomenon indicates that the inhibitors may be able to bind to more than one type of cysteine proteinase and probably affect both CPA and CPB. However, the reduction in the growth of Δ*CPA/CPB* caused by KO2 also suggests that this inhibitor must bind to other important cysteine proteinases present, such as CPC.

The differences between my results with axenic *L. mexicana* and the published data concerning *L. major* suggest that there may be important variation between species with respect to cysteine proteinase action. However, the use of KO2 during *in vitro* macrophage infections demonstrated that *L. mexicana* intracellular survival is reduced. This reduction occurred irrespective of whether promastigotes or amastigotes were used to initiate infection. Interestingly Δ*CPB* amastigote infectivity was also perturbed, and indeed it appeared to succumb more rapidly. This suggests that the CPB enzymes are not the key target of the inhibitors. In addition, as it was the amastigote stage that succumbed to inhibition, and yet the role of the CPB enzymes is seemingly most important (at least in the *in vitro* model) during the initial stages of the infection or during transformation to the amastigote, it would appear that the inhibitor's effect on enzymes other than CPB is its prime detrimental effect on the parasite.

KO2 can bind a variety of enzymes including CPA, CPB and CPC. *L. mexicana* also contains another member of the cysteine proteinase family, a

GPI:protein transamidase enzyme which is essential for the surface expression of GPI-anchored proteins such as gp63 (Hilley *et al.*, 1999) (see Chapter 1, section 1.4.1.2 and Chapter 4). It was therefore considered possible that KO2 may also be inhibiting the activity of this enzyme, named GPI8, and thus perturbing the expression of important surface molecules. However, my results demonstrated that gp63 was successfully localised to the surface and appeared to be GPI-anchored. Thus inhibition of GPI8 by KO2 seemed unlikely.

Some interesting effects were demonstrated, however, in the investigation as the turnover of gp63 on the surface was shown to be reduced by the cysteine proteinase inhibitors. This phenomenon is probably due to perturbation in the protein catabolism of the cells. Inhibition of so many lysosomal enzymes would be expected to prevent the breakdown of cellular proteins and may thus reduce turn-over of proteins from the surface. Indeed a similar reduction in turnover was observed after analysis using Δ*CPB*.

The induction of the large vacuoles within the cell by incubation with KO2 may also interfere with the trafficking of old surface protein to the lysosomes for breakdown. It is probable that the formation of large vacuoles within the parasite following cysteine proteinase inhibition occurs within the trafficking pathway of the cells. Further investigation of the contents of these vacuoles may allow determination of whether they contain gp63. If this proved to be the case, then it would demonstrate that trafficking from the flagellar pocket is perturbed and indicate that the formation of vacuoles is at a stage in the trafficking pathway subsequent to this. Trypanosomatids endocytose and exocytose molecules

exclusively via their flagellar pocket (reviewed by Overath *et al.*, 1997), hence the internalisation of gp63 demonstrated after cross-linking with antibodies will be through this organelle. If further analysis of the localisation of the build-up of cysteine proteinases in *L. mexicana* under inhibitor pressure demonstrates that this occurs within the pocket of the organism, then it is likely that this phenomenon would also reduce the turn-over of surface molecules of the parasite. It would be of further interest to analyse the ultrastructure of Δ*CPB* to determine whether loss of CPB activity has similar effects with respect to vacuolisation, which would provide further evidence that this phenomenon may be related to a reduction in the protein catabolism of the cells.

The use of cysteine proteinase inhibitors has provided some interesting insights into the biology of the parasite. The accumulation of several intermediate forms of both CPB and CPA concurs with the published information concerning both cruzipain and the cysteine proteinases of *L. major*. However, the differences observed between the localisation of the ultrastructural abnormalities within the *Leishmania* species and *T. cruzi* may indicate that they have subtle differences in the pathways used during intracellular trafficking of proteins to the lysosomes. Despite these differences, the effects of KO2 on intracellular survival of *L. mexicana in vitro* show that this cysteine proteinase inhibitor has a similar overall effect against the three parasites and that perturbation of the trafficking/activity of cysteine proteinases occurs and apparently is detrimental to the intracellular survival of the parasite.

The information discussed above and the data from Selzer *et al.*, (1999) suggest that during *in vivo* infection with *L. mexicana* the application of KO2 could

delay the lesion formation. The fact that KO2 has effects on the activity of both CPA and CPB suggests that it may inhibit both enzyme classes *in vivo* and thus effectively alter the wild-type parasites such that they have a similar phenotype to Δ*CPA/CPB*. Alexander *et al.* (1998) showed that infection with Δ*CPA/CPB* did not result in lesion formation, which suggests that KO2 treatment could effectively prevent the progression of disease if applied early in infection.

In the field, however, early treatment would probably not be practical as people will not present with infection until disease symptoms occur. Hopefully, however, KO2 or inhibitors acting in a similar way may still have effects on the progression of developed lesions. The ulceration occurring during cutaneous leishmaniasis is due, in part, to the release of parasite cysteine proteinases from dead amastigotes (Ilg *et al.*, 1994). Thus, topical application of cysteine proteinase inhibitors may therefore relieve the symptoms of disease.

Several studies have investigated the efficacy of peptidyl cysteine proteinase inhibitors during *in vivo* infections, with mixed results. Results from infections with *T. cruzi* and *Plasmodium vinckei* (Rosenthal *et al.*, 1993; Engel *et al.*, 1998a) were encouraging as there were apparent cures of infected animals following application of cysteine proteinase inhibitors. However, investigations of infection with *T. brucei* and *L. major* (Scory *et al.*, 1999; Selzer *et al.*, 1999) have suggested that application of the inhibitors may merely prolong onset of disease, with both disease states progressing at a similar rate to control infections after cessation of treatment. The differences in susceptibility of *L. major* and *L. mexicana* to cysteine proteinase inhibitors demonstrated in my investigation, emphasises the importance of assessing

the effects of individual inhibitors against specific parasite strains. Thus the effects observed during *in vivo* analysis of KO2 efficacy against *L. major* may prove to be irrelevant with respect to *L. mexicana*.

In summary, the use of cysteine proteinase inhibitors as a method for clearing an intracellular infection of *L. mexicana* has been demonstrated to be feasible. This reduction in intracellular survival appears to occur regardless of the time of application of the inhibitor, suggesting that its effects are against the amastigote stage of the life-cycle. The inhibition of the cysteine proteinases lead to the perturbation of intracellular ultrastructure and also reduced turn-over of molecules from the parasite's surface, either of which could have effects on the viability of the parasite. Despite the fact that KO2 has been demonstrated to have *in vitro* capacity as a chemotherapeutic agent, further *in vivo* analysis is necessary before its true potential can be assessed.

The CPB enzymes from *L. mexicana* were demonstrated several years ago to be important in the virulence of the parasite (Mottram *et al.*, 1996). This manifested itself as a reduction in the intracellular survival of the *CPB* null during *in vitro* infections, and was suggested to be due to the role of the enzymes during the initiation of infection (see Chapter 1, section 1.4.1.4 for discussion). An investigation was therefore undertaken to analyse the role of these enzymes during infection.

As discussed previously (see Chapters 1 and 6), several factors could be causing a reduction in the survival of the parasite. Analysis of the production of RNI and ROS in response to infection demonstrated that both wild-type and Δ*CPB*

elicited comparable levels from their host cells. This information, in conjunction with previous data demonstrating the comparable susceptibility of these two lines *in vitro* (Frame, PhD thesis), indicated that neither RNI or ROS could be responsible for the enhanced susceptibility of Δ*CPB*. This indicated to me that the fate of the parasite may be decided prior to the instigation of microbicidal factors from the host cell and lead to an investigation of the phagocytosis of promastigotes by macrophages.

This investigation proved interesting in several ways. It was demonstrated that the uptake of Δ*CPB* involved different kinetics to that of wild-type parasites. Wild-type promastigotes were rapidly internalised by their host cell following attachment. Δ*CPB*, however, were shown to interact with the surface of the cell for 15 - 20 minutes following attachment before being internalised. This prolonged interaction was hypothesised to be due to the alterations in the surface architecture of the parasite. The *CPB* null promastigotes had previously been shown to have a reduction in the turn-over of gp63 from their surface. This reduction is probably due to the loss of many lysosomal enzymes which will cause a slower rate of protein catabolism. This could cause effects on the surface of the promastigotes and thus lead to alterations in charge or membrane fluidity. The analysis presented here demonstrated that the turn-over of proteins due to cross-linking with antibodies was reduced. However it may prove interesting to analyse the membrane fluidity in the absence of antibody, perhaps utilising Fluoresence-Recovery-After-Photobleaching (FRAP) technology to assess the difference in recovery between wild-type and Δ*CPB* (Meyvis *et al.*, 1999).

This prolonged interaction could in itself be responsible for the reduced survival of Δ*CPB* by allowing the macrophage to prime itself for infection before the parasite is able to inactivate the host cell's responses. However, an analysis of the tyrosine phosphorylation events occurring during uptake of both lines revealed no obvious differences between the levels of induced protein phosphorylation, suggesting that macrophages infected with the two lines were similarly able to signal during infection. Clearly, however, a more detailed analysis could reveal more subtle, yet important differences.

It is more likely, therefore, that the reduction in virulence of the *CPB* null mutant is due to the fact that it utilises different macrophage surface receptors during internalisation. This difference would play a role in increasing the interaction time with the surface. Restoration of virulence to the promastigotes by expression of *CPBg2.8* also restored the parasite's ability to bind to both CR3 and CR4, again implicating this phenomenon as important. The receptors used during phagocytosis have long been known to effect the response of macrophages to infection (Hunter, 1987 and Chapter 7) and thus the utilisation of different receptors may alter the intracellular fate of pathogens.

The lack of difference between the response of macrophages to infection with wild-type parasites and Δ*CPB* with respect to the production of RNI and ROS suggests that other components of the intracellular environment are responsible for the death of Δ*CPB*. It is possible that changes in the route of uptake will alter the type of vacuole encountered by the parasite. *Leishmania* is highly adapted to modify this vacuole on entry (see Chapter 1, section 1.2.1). Perhaps Δ*CPB* is internalised

into a different type of vacuole and thus is unable to modify it's intracellular environment efficiently, therefore intracellular survival is reduced.

These experiments also demonstrated that *L. mexicana* wild-type promastigotes had an unusually heavy reliance on CR3 and CR4; *L. major*, for example, has been shown to utilise CR3 heavily during uptake, but only a 25 - 30 % reduction in binding was observed after blocking this receptor (Blackwell *et al.*, 1985). However, despite *L. mexicana*'s reliance on these receptors, my work demonstrated that the parasite must also utilise other receptors during attachment to the macrophage surface. Further investigation of the receptors involved in Δ*CPB* attachment will probably demonstrate that the parasite has the ability to bind to at least one of the other wild-type receptors and that it is through this attachment that it enters the cells.

It has been suggested that Δ*CPB* is surviving in a sub-population of the cells it originally infects (see Chapter 6, section 6.1, for details). A preliminary investigation of the infectivity of Δ*CPB* towards peritoneal exudate cells enriched with dendritic cells indicated that the susceptible sub-population may be dendritic in nature. Further work is required (as discussed in Chapter 6, section 6.4) to confirm this observation.

The *in vivo* situation is not directly comparable to the situation *in vitro*, as many other host-related factors will come into play. This can be demonstrated by the fact that the virulence of Δ*CPB* amastigotes *in vivo* is similar to that of the promastigotes (Frame, PhD Thesis). As amastigotes do not utilise the same receptors as promastigotes during phagocytosis (reviewed in Chapter 7), the

alteration in receptor binding cannot account for the reduction in virulence of the amastigotes. However, at present the receptors involved in amastigote uptake are un-defined and thus it may be that loss of the CPB enzymes also causes alteration in the attachment of amastigotes to macrophages. The consequences of this may not be seen *in vitro*, due to the relatively less harsh environment that the parasite is exposed to under these conditions.

In summary, the loss of the CPB enzymes from the parasite causes alteration in the surface architecture of the promastigotes, such that the affinity of the parasite to important macrophage surface molecules is reduced. This was manifested in two ways. Firstly, the promastigotes were internalised more slowly following attachment, and, secondly, this attachment was via different receptors. Although other factors are bound to be involved during *in vivo* infection, it is clear from this analysis that the loss of lysosomal enzymes can cause effects on the surface of the promastigote which will alter the way it interacts with putative host cells.

Thus the results of my investigation have led to several interesting conclusions, which are summarised in Table 8.1. These findings have created the opportunity for several further interesting lines of enquiry. Despite the demonstration that there are differences in the receptors utilised during attachment of wild-type and Δ*CPB* promastigotes, there is no direct evidence that this is indeed the reason for the reduction in intracellular survival. An interesting follow-up investigation could involve an analysis of the effect of blocking CR3 and CR4 (with Fab fragments, see Chapter 7, section 7.4) on wild-type parasite survival. If attachment via these receptors is important in virulence, as opposed to simply

attachment, then preventing use of them during uptake would reduce wild-type parasite virulence to the level of Δ*CPB*. Conversely, perhaps cross-linking these receptors, with specific antibodies, during phagocytosis of Δ*CPB* would allow this parasite to infect and survive normally by creating an intracellular environment more conducive to survival.

The investigation of the receptors involved in wild-type parasites binding further demonstrated that interaction with both CR3 and CR4 was necessary for attachment. This was surprising and the role of steric hindrance, although unlikely, cannot be ruled out. A simple way to investigate this phenomenon would be to express CR3 and CR4 (individually and together) in COS cells. This would allow analysis of their relative involvement in attachment.

The findings from my work would also benefit from some *in vivo* context. An analysis of the effect of KO2 during *in vivo* infection with *L. mexicana* is essential to assess its potential as a chemotherapeutic agent. Furthermore, despite the *in vitro* differences observed between wild-type parasites and Δ*CPB* virulence, the *in vivo* picture is more complicated. This suggests that although the receptors mediating phagocytosis are important *in vitro* and may play some role in the reduction of virulence *in vivo*, other factors are also involved.

The work of Alexander *et al.*, (1998) demonstrated that infection with Δ*CPA/CPB* (or Δ*CPB*) causes a polarisation of the immune response towards a protective Th1 type response. This suggests a possible role for these cysteine proteinases in the production of a Th2 type response to infection, although other factors, such as infectious dose, may be important (Menon and Bretscher, 1998).

Several investigations have indicated that cysteine proteinases (host and parasite derived) may be important in determining the presentation of antigens via MHC-II. Maekawa *et al.*, (1998) showed that mammalian cathepsin B can have a role in polarising the immune response, via the production of antigenic epitopes. It follows, therefore, that intracellular parasites may utilise their cysteine proteinase enzymes to prevent the production of an effective immune response by degradation of either antigenic material or MHC-II molecules. The use of cysteine proteinase inhibitors during *in vitro* infection with *L. amazonensis* has been demonstrated to cause a build-up of MHC-II within the lysosomes of the amastigotes (De Souza *et al.*, 1995). Therefore, the CPB enzymes may have roles in MHC-II degradation and thus aid in the prolonging of the infection. These roles could be investigated *in vitro* by analysing the effect of infection with wild-type parasites and Δ*CPB* on the amount, and localisation, of MHC-II within the macrophage. It is unlikely, however, that MHC-II degradation would have a role *in vitro* as there are no T cells present to interact with MHC-peptide complexes. However, a role for the CPB enzymes in MHC-II degradation could explain the further reduction of virulence observed *in vivo* and the polarisation of the immune response.

In conclusion, I have shown that the cysteine proteinase enzymes of *L. mexicana* have potential as a target for chemotherapeutic agents. Further to this, the CPB enzymes have been shown to be important in maintaining the surface architecture of the parasite. This surface architecture is essential for the parasite to enter macrophages using CR3 and CR4. It is hypothesised that this phenomenon is important in the *in vitro* infectivity of the parasite.

Table 8.1: Roles of *L. mexicana* CPB enzymes and their potential as drug targets: The main conclusions.

Cysteine Proteinase Inhibitors:

prevent processing of pro-enzymes to mature CPA and CPB

perturb intracellular protein trafficking

reduce survival of amastigotes intracellularly

Δ*CPB* amastigotes are as susceptible as wild-type parasites

The CPB enzymes are involved in:

turnover of surface molecules

rapid internalisation of promastigotes by macrophages

facilitating attachment to CR3 and CR4

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Appendix 1: Statistical analysis of data

The analyses detailed below are not a complete set, but illustrate a selection of the statistics discussed within each chapter.

Chapter 5: The effect of peptidyl cysteine proteinase inhibitors on the life-cycle stages of *Leishmania mexicana*

Figure 5.2: The growth of wild-type axenic amastigotes in the presence of peptidyl cysteine proteinase inhibitors

	% inhibition	
Experiment	**KO2**	**ZFA**
1	55.33	63.69
2	54.29	58.31
3	55.33	60.19
Average ± stdev	**54.98 ± 0.6**	**60.73 ± 2.73**

Question: Is there a statistically significant difference between the control density and that of the amastigotes grown in ZFA, by day 3?

F-Test Two-Sample for Variances
density at day 3

	control	*ZFA*
Mean	6.76	6.353333
Variance	0.0111	0.008233
Observatio	3	3
df	2	2
F	1.348178	
F (two tail)	39	

student t test

	control	*ZFA*
Mean	6.76	6.353333
Variance	0.0111	0.008233
Observatio	3	3
df	4	
t Stat	5.065774	
t Critical tv	2.776451	

Therefore by day three there is a significant difference between the control and inhibitor grown culture. ($t_4 = 5.07$, $P < 0.01$).

Figure 5.7: Infection of peritoneal exudate macrophages with wild-type promastigotes with KO2.:

Question: Is there a statistically significant difference in the % infected cells by day 7 after application of KO2 at time 0?

F-Test Two-Sample for Variances
control vs KO2 d0, 168h

	control	*KO2d0*
Mean	33.63333	19.51667
Variance	7.523333	17.94333
Observatio	3	3
df	2	2
F	0.419283	
F (two tail)	39	

student t test
control vs KO2 d0, 168h

	control	*KO2d0*
Mean	33.63333	19.51667
Variance	7.523333	17.94333
Observatio	3	3
df	4	
t Stat	4.845145	
t Critical tv	2.776451	

Therefore there is a significant difference in the number of infected cells by day 7 ($t_4 = 4.85$, $P < 0.01$).

Question: Does the time of inhibitor application affect the reduction in survival?

F-Test Two-Sample for Variances

	72h	*24h*
Mean	51	27.6
Variance	907.48	12.49
Observatio	3	3
df	2	2
F	72.65653	
F max	87.5	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	124.7	41.56667	197.8633
Row 2	3	82.8	27.6	12.49
Row 3	3	153	51	907.48

ANOVA

rce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between G	831.6156	2	415.8078	1.11593	0.387222	5.143249
Within Gro	2235.667	6	372.6111			
Total	3067.282	8				

Therefore the time of application of inhibitor has no effect on the percentage reduction in infectivity ($F_{2,6} = 1.1$, $P > 0.05$)

Chapter 6: The response of macrophages to infection with *L. mexicana* promastigotes *in vitro*.

Figure 6.1: The relative infectivity of stationary phase promastigotes to resident and elicited peritoneal macrophages

Question: Is there any difference in the level of infection by 8h with any parasite line and in either macrophage type?

F-Test Two-Sample for Variances

	Variable 1	*Variable 2*
Mean	23.83333	19.28333
Variance	18.08333	5.010833
Observatio	3	3
df	2	2
F	3.608847	
P(F<=f) on	0.216974	
F max	266	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
WT R	3	57.85	19.28333	5.010833
ΔCPB R	3	42.3	14.1	13.5675
G2.8 R	3	71.5	23.83333	18.08333
WT E	3	76.25	25.41667	8.493333
ΔCPB E	3	52.4	17.46667	7.155833
g2.8 E	3	66.75	22.25	7.77

ANOVA

rce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between G	269.7546	5	53.95092	5.387833	0.007936	3.105875
Within Gro	120.1617	12	10.01347			
Total	389.9163	17				

Tukey test					
Sample	2	3	4	5	6
WT R	x1-x2	x1-x3	x1-x4	x1-x5	x1-x6
19.28333	1.368	4.54997	6.13334	1.81666	2.96667
DCPB R		x2-x3	x2-x4	x2-x5	x2-x6
14.1		9.73333	**11.31667**	3.36667	8.15
g2.8 R			x3-x4	x3-x5	x3-x6
23.83333			1.58334	6.36666	1.58333
WT E				x4-x5	x4-x6
25.41667				7.95	3.16667
DCPB E					x5-x6
17.46667					4.78333
g2.8 E					
22.25					

$T = q \times \sqrt{}$ [var within/n] $\quad q = 4.75$

$T = 8.678$

Therefore the difference observed between the % infected by 8 h was due to differences observed in Δ*CPB* uptake and wild-type or Δ*CPB/g2.8* uptake.

Chapter 7: The uptake of *Leishmania* promastigotes and amastigotes by macrophages *in vitro*

Figure 7.2: The kinetics of entry of stationary phase promastigotes into peritoneal exudate cells.

Question: Is there any difference between the number of wild-type parasites and Δ*CPB* parasites attached at any time point investigated?

F-Test Two-Sample for Variances

t = 2 min	*WT*	*Δ CPB*
Mean	10.98333	7.016667
Variance	5.741667	9.381667
Observatio	6	6
df	5	5
F	1.633962	
F (two tail)	7.15	

student t test

t = 2 min	*WT*	*Δ CPB*
Mean	10.98333	7.016667
Variance	5.741667	9.381667
Observatio	6	6
df	10	
t Stat	2.49849	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t = 5 min	*WT*	*Δ CPB*
Mean	16.43333	7.683333
Variance	6.214667	5.205667
Observatio	6	6
df	5	5
F	1.193827	
F 9two tail	7.15	

student t test

t = 5 min	*WT*	*Δ CPB*
Mean	16.43333	7.683333
Variance	6.214667	5.205667
Observatio	6	6
df	10	
t Stat	6.342264	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t=10 min	*WT*	*Δ CPB*
Mean	15.58333	8.133333
Variance	28.68167	13.72267
Observatio	6	6
df	5	5
F	2.090094	
F (two tail)	7.15	

student t test

t=10 min	*WT*	*Δ CPB*
Mean	15.58333	8.133333
Variance	28.68167	13.72267
Observatio	6	6
df	10	
t Stat	2.802378	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t=15 min	*WT*	*Δ CPB*
Mean	15.1	9.933333
Variance	1.932	4.078667
Observatio	6	6
df	5	5
F	2.111111	
F (two tail)	7.15	

student t test

t=15 min	*WT*	*Δ CPB*
Mean	15.1	9.933333
Variance	1.932	4.078667
Observatio	6	6
df	10	
t Stat	5.16208	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t = 20 min	*WT*	*Δ CPB*
Mean	16.6	13.15
Variance	7.98	5.955
Observatio	6	6
df	5	5
F	1.34005	
F (two tail)	7.15	

student t test

t= 20min	*WT*	*Δ CPB*
Mean	16.6	13.15
Variance	7.98	5.955
Observatio	6	6
df	10	
t Stat	2.263817	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t = 30 min	*WT*	*Δ CPB*
Mean	29.33333	11.68333
Variance	49.66667	61.58167
Observatio	6	6
df	5	5
F	1.239899	
F (two tail)	7.15	

student t test

t = 30 min	*WT*	*Δ CPB*
Mean	29.33333	11.68333
Variance	49.66667	61.58167
Observatio	6	6
df	10	
t Stat	4.098959	
t Critical tv	2.228139	

F-Test Two-Sample for Variances

t = 60 min	*WT*	*Δ CPB*
Mean	26.7	24.2
Variance	30.072	149.2733
Observatio	6	4
df	5	3
F	4.963863	
F 9two tail	7.15	

student t test

t = 60 min	*WT*	*Δ CPB*
Mean	26.7	24.2
Variance	30.072	149.2733
Observatio	6	4
df	8	
t Stat	0.447893	
t Critical tv	2.306006	

F-Test Two-Sample for Variances

t = 120 min	*WT*	*Δ CPB*
Mean	32.56667	23.55
Variance	137.1587	106.3033
Observatio	6	4
df	5	3
F	1.290257	
F (two tail)	14.88	

student t test

t = 120 min	*WT*	*Δ CPB*
Mean	32.56667	23.55
Variance	137.1587	106.3033
Observatio	6	4
df	8	
t Stat	1.246458	
t Critical tv	2.306006	

F-Test Two-Sample for Variances

t = 240 min	*WT*	*Δ CPB*
Mean	23.03333	12.65
Variance	21.51467	36.38333
Observatio	6	4
df	5	3
F	1.691094	
F (two tail)	7.76	

student t test

t = 240 min	*WT*	*Δ CPB*
Mean	23.03333	12.65
Variance	21.51467	36.38333
Observatio	6	4
df	8	
t Stat	3.090541	
t Critical tv	2.306006	

Therefore there are some differences in the numbers of parasites attached at different time points.