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Studies on the cysteine proteinases of *Leishmania mexicana*

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SUMMARY

Three genes (*lmcpa*, *lmcpb* and *lmcpc*) that encode cysteine proteinases (CPs) in *L. mexicana* have previously been cloned and sequenced. The enzymes encoded differ significantly from each other and host CPs. In an attempt to elucidate the parts played by each CP in the parasite's life history, mutant lines of the parasite which lack *lmcpa*, *lmcpb* and *lmcpc* have been examined in order to determine their phenotypes. Null mutants which have been further modified by transfecting the cells with genes encoding *L. mexicana* CPs and CPs of other trypanosomatids have also been studied. These lines have been analysed to determine how they differ from the parent line in terms of proteinase and protein content, growth and differentiation axenically *in vitro* and in peritoneal macrophage cells, infectivity to mice and susceptibility to CP inhibitors.

Null mutants for the *lmcpa* CP gene were found to have a cryptic phenotype, since differences between the *lmcpa* null mutants and wild type *L. mexicana* were not detectable. This suggested that either the enzyme has a role which was not detected with the phenotypic tests used or that its activity was being compensated for by one of the other CPs present in *L. mexicana*. In contrast to previously published conclusions, studies using null mutants for *lmcpa* revealed that LmCPa was not at highest levels in the amastigote stage. Triton X-114 phase separation revealed the protein encoded by *lmcpa* to be hydrophobic, suggesting it could be membrane-associated.

Stationary phase promastigotes of mutants null for the multicopy *lmcpb* genes showed a marked phenotype with respect to virulence, as their ability to infect macrophages *in vitro* was severely impaired. The infectivity was restored by re-expression of an enzymatically active internal copy of the gene array (*g2.8*), suggesting that the enzymes play a role in intracellular survival. Time course experiments demonstrated that the null mutants invaded macrophages in large numbers but were unable to survive in the majority of the cells. Despite their reduced virulence to PECs, the mutants were capable of infecting mice although the time taken for lesions to form was significantly longer than with wild type and the lesions were 100-fold smaller. Interestingly, lesion amastigotes of the mutants were able to infect macrophages *in vitro* as successfully as wild type, but took three months longer to produce lesions in mice.

These data showed that the CPs encoded by *lmcpb* are important for parasite virulence, but are not essential for survival in the host. Double *lmcpa/lmcpb* null mutants were found to have a similar phenotype to the *lmcpb* null mutants in terms of their ability to infect macrophages *in vitro*, suggesting that *lmcpa* was not compensating for the loss of *lmcpb* in this phenotypic test, although it was found that they did not cause lesions in mice.

Studies utilising null mutants re-expressing different copies of the *lmcpb* array revealed that individual isoenzymes differ in their substrate preferences and ability to complement the loss of virulence associated with the null mutant, suggesting that the individual isoenzymes have distinct roles in the parasite's interaction with the host. Immunogold labelling of the megasome in transfectants re-expressing an *lmcpb* CP gene with a truncated C-terminal extension (*g1*) demonstrated that the C-terminal extension is not necessary for intracellular targeting or activity.

All life-cycle stages of the mutants null for the cathepsin B-like CP gene *(lmcpc)* could be cultured *in vitro*, demonstrating that this CP is not essential for the growth or differentiation of the parasite. However, they showed greatly reduced infectivity to macrophages *in vitro*, resulting in only a low percentage of the cells being infected. Re-expression of *lmcpc* in the null mutant increased the parasite's infectivity to macrophages *in vitro*. However, the null mutants for *lmcpc* formed lesions in mice at a rate comparable with wild type parasites, suggesting that although the CP encoded by *lmcpc* may play a role in the parasite-macrophage interaction it alone is not crucial for infectivity or virulence.

Peptidyl-diazomethane CP inhibitors, which entered the parasites and inhibited LmCPb *in situ*, had little or no effect on parasite growth or differentiation axenically *in vitro*. However, at the same concentration, *N*-benzoyloxycarbonyl-phe-ala-diazomethane reduced the infectivity of wild type parasites to macrophages by 80%, a similar percentage to that seen with the *lmcpb* null mutant. However, further experiments suggested that LmCPb may not be the prime target of this inhibitor.

With respect to drug target validation it is essential to determine which CPs are necessary for parasite survival. The overall results of this study suggest that while none

of the CPs of *L. mexicana* are vital, some appear to have important and distinct roles in differentiation and intracellular survival.

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LIST OF ABBREVIATIONS

Ac	acetyl
AMC	amidomethylcoumarin
BzFVR-NHMec	N-benzoyl-phe-val-arg-7-(4-methyl)-coumarylamide
CP	cysteine proteinase
CTE	C-terminal extension
dH_2O	double distilled de-ionised water
DTT	dithiothreitol
DMF	dimethylformamide
DMK	diazomethylketone (diazomethane)
E-64	*trans*-epoxy succinyl-L-leucylamido-(4 guanadino) butane
GIPLS	glycoinositol phospholipids
GPS	guinea pig serum
h	hour(s)
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
hyg	hygromycin
Ii	invariant chain
IL	interleukin
K02	Mu-Phe-Homophe-oxycoumarin
Leu-OMe	leucine methyl ester
log.	logarithmic
min	minute(s)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Mu	morphine urea
NO	nitric oxide
iNOS	inducible nitric oxide synthase
NRME	N-nitro-arginine methyl ester
P87	Mu-Phe-Homophe-vinyl sulfone

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEC	peritoneal exudate cell
phleo	phleomycin
PL	phagolysosome
PMSF	phenylmethylsulfonyl fluoride
PV	parasitophorous vacuole
SDM	Schneider's Drosophila Medium
SDS	sodium dodecyl sulphate
sec	second(s)
-SH	sulphide
Suc	succinyl
SucLY-NHMec	N-succinyl-leu-tyr-7-(4-methyl)-coumarylamide
TNF-α	tumour necrosis factor alpha
WUMP	Wellcome Unit of Molecular Parasitology
Z-LVG-DMK	*N*-benzoyloxycarbonyl-leu-val-gly-diazomethylketone
Z-FA-DMK	*N*-benzoyloxycarbonyl-phe-ala-diazomethylketone

DECLARATION

I declare that all the work presented in this thesis is my own except where there is an explicit statement to the contrary.

All electron microscopy work was carried out by Laurence Tetley and Margaret Mullin, University of Glasgow.

The Western blot presented in Chapter Four was performed by Genevieve Bart, Wellcome Unit of Molecular Parasitology, University of Glasgow.

Publications arising from some of the work presented in Chapter Three and Chapter Four can be found in Appendix B.

Mhairi Frame

MHAIRI FRAME

June 1997

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I am indebted (or should that be in debt?!) to my parents for all their support over the past four years; with this in mind I dedicate this thesis to them.

CHAPTER ONE
GENERAL INTRODUCTION

1.1. *LEISHMANIA* AND LEISHMANIASIS

Leishmaniasis is classified by the World Health Organisation (WHO) as one of the six major parasitic diseases, an affliction which has been described in the Old World for almost two thousand years (Lainson and Shaw, 1987). Over 12 million people worldwide are infected by the different species of the causative agent, the parasitic protozoan *Leishmania* (Ashford *et al.*, 1992). The disease has a wide spectrum of clinical manifestations, varying from self-healing cutaneous ulcers to non-resolving mucocutaneous lesions and hepatosplenomegaly leading to death (review: Grevelink and Lerner, 1996). Leishmaniasis in its various forms is present on all continents except Australia and Antarctica, and, at a minimum, approximately 400,000 new cases occur each year with almost 400 million people at risk from this disease (Ashford *et al.*, 1992).

1.1.1. Life cycle

Leishmania is a kinetoplastid protozoan of the family Trypanosomatidae. Trypanosomatids are obligate uniflagellated parasites defined by the presence of the kinetoplast, a DNA-containing structure representing a specialised component of the single mitochondrion (Clark and Wallace, 1960). *Leishmania* exist in 3 distinct forms during the life-cycle: a flagellated multiplicative promastigote form in the gut of the sandfly vector, a mammal-infective metacyclic form that occurs in the insect mouthparts, and, subsequent to inoculation into a mammalian host, as a non-motile amastigote stage within macrophages.

Transmission of leishmania infection occurs almost exclusively by the bite of an infected female sandfly in the tropics and sub-tropics. In the Old World the disease is transmitted by flies of the genus *Phlebotomus*, while in the Americas it is transmitted primarily by *Lutzomyia* species. Transmission occurs when infective metacyclic promastigotes are ejected into the host tissues during regurgitation of the bloodmeal from the fly by repeated pump pulsation. This is thought to occur due to *Leishmania*

infections damaging the feeding mechanism of the sandfly vector (Schlein *et al.*, 1992). The metacyclic promastigote is phagocytosed by host macrophages, within which it transforms to the round, non-flagellated amastigote form. A remarkable feature of the intracellular amastigotes is that they are not only resistant to, but actively thrive in, the apparently hostile environment of a phagolysosome (PL). The eventual rupture of infected cells releases amastigotes to be phagocytosed by other mononuclear phagocytes, and thus the infection spreads. When a sandfly bites an infected host, amastigotes taken up during the blood meal transform back to the promastigote form in the gut of the vector, thereby completing the life cycle.

Leishmaniasis is a zoonosis, the natural mammalian hosts in addition to humans including the domestic dog and a variety of wild mammals such as desert or forest rodents, foxes and sloths. At least four major groups of parasites, similar in morphology but differing in cultural characteristics, clinical manifestations, geographic distribution, and sandfly vectors, cause disease in humans (Grevelink and Lerner, 1996). These are :

1) *L. donovani*, the aetiological agent of classic visceral leishmaniasis in Asia ('kala-azar')

2) *L. major,* which causes Old World cutaneous leishmaniasis ('oriental sore')

3) the *L. braziliensis* complex which results in cutaneous and mucocutaneous disease in the Americas ('espundia')

4) the *L. mexicana* complex, which includes *L. amazonensis* and *L. pifanoi*, and is associated mainly with cutaneous lesions in Central and South America ('Chiclero's ulcer').

1.1.2. Clinical aspects

Three major clinical manifestations are recognised. These reflect mainly the different species of *Leishmania* involved, although host factors also contribute. The most life threatening is Kala-azar, or visceral leishmaniasis, a systemic disease caused by the dissemination of *L. donovani* throughout the reticuloendothelial cells of the spleen, liver, lymph nodes and bone marrow (Rees and Kager, 1987). After an

incubation period of two to four months, characteristic symptoms of the disease develop and include fever, splenomegaly, anaemia, emaciation and hyperglobulinaemia. When untreated, the disease usually progresses to a fatal termination within two years. A condition known as post Kala-azar dermal leishmaniasis occasionally affects patients recovering from visceral leishmaniasis in India and East Africa, generally appearing one to two years after the visceral disease. In this condition, hypopigmented areas appear on the body, which do not always respond to treatment and almost never completely repigment. Post Kala-azar dermal leishmaniasis is of great epidemiological significance, since parasites can easily be picked up by biting sandflies.

Mucocutaneous leishmaniasis is most commonly reported in the jungle areas of Brazil, Venezuala, Bolivia and Ecuador. *L. b. braziliensis* is the most common etiologic agent, although cases caused by other *Leishmania* species have been reported, particularly *L. b. panamensis*, which increases the risk to travellers in Central America. Fifty percent of patients infected have mucocutaneous lesions within two years of the initial cutaneous lesions; associated factors with the development of mucocutaneous disease include male sex and large, persistent cutaneous lesions. The disease often begins in the nasal septum, which becomes inflamed and infiltrated and subsequently perforates. Further mutilation of the palate, pharynx, tonsils, gums and lips leads to invasion of the respiratory tract, and, as a result, malnutrition and acute respiratory pneumonia are the leading causes of death in victims (Saravia *et al.*, 1985).

Cutaneous leishmaniasis, frequently caused by *L. major* in the Old World and *L. mexicana* in the New World, is usually a localised infection starting with a solitary primary lesion, although multiple primary lesions may be evident in Old World disease (Grevelink and Lerner, 1996). After an incubation period of 1 week to 3 months, there is the appearance of a red papule, which enlarges to a plaque or nodule. This finally develops into an ulcer which spontaneously regresses after six to twelve months leaving a scar. The locations of lesions may be distinctive. For example, 'Chiclero's ulcer', caused by infection with *L. mexicana*, results in erosion of the ear of forest workers who gather chicle gum. However, a more serious variant of localised cutaneous leishmaniasis can occur, called diffuse cutaneous leishmaniasis, in which lesions are disseminated and

resemble lepromatous leprosy. This disease, caused primarily by *L. mexicana* and *L. amazonensis* in Central and South America, does not invade internal organs but responds only partially to treatment and often relapses, becoming chronic.

1.1.3. Chemotherapy

Treatment of all forms of leishmaniasis is still largely dependent on the pentavalent antimonials, notably sodium stibogluconate (Pentostam), a treatment developed empirically more than 50 years ago (review: Croft, *et al.*, 1997). Although the exact structure of the drug is still unknown, the mechanism of action appears to be the inhibition of amastigote glycolytic activity and fatty acid oxidation. Pentostam is far from an ideal chemotherapeutic agent, requiring parenteral administration and prolonged hospitalisation, and moreover, treatment commonly results in side effects such as cardiac and renal toxicity. Although visceral and cutaneous leishmaniasis frequently respond to this drug, severe mucosal disease does not (Herwaldt and Berman, 1992).

The second line drugs currently in use are the aromatic diamidine pentamidine and the anti-fungal agent amphotericin B (review: Olliaro and Bryceson, 1993). They must be administered intravenously, and are less effective and even more toxic than Pentostam (Croft, *et al.*, 1997). The antibiotic paramomycin is currently used for the treatment of some leishmaniases but, like the other treatments, it is not absorbed when administered orally and little is known about its mechanism of action (Maarouf *et al.*, 1997). New formulations of the same drugs, such as liposomal amphotericin B, are less toxic and show good activity (Castagnola *et al.*, 1996) but are very expensive.

WHO has established as a priority objective the ability to provide oral treatment for cutaneous leishmaniasis; this would provide clinical benefits and cost savings over present drugs for treating patients from areas where leishmaniasis is endemic. Among potential orally active drugs, combination therapy using sterol biosynthesis inhibitors, such as ketokonazole and terbinafine, offers an attractive possibility (Rangel *et al.*, 1996).

Resistance of both visceral and mucocutaneous leishmaniasis to pentavalent

antimonials has been increasing in all areas of endemicity (review: Ouellette and Papadopoulou, 1993) and, coupled with the emergence of AIDS-related leishmaniasis, there is an increased urgency for new drugs to be developed (Olliaro and Bryceson, 1993). Among the potential drug targets, polyamine synthesis, purine salvage, sterol metabolism and cysteine proteinases are the foci of much of the present research activities (Traub-Cseko and Momem, 1995; Maingon *et al.*, 1995).

1.2. PROTEINASES

Proteinases are enzymes that are found throughout the plant and animal kingdoms. These enzymes cleave peptide bonds after binding to a specific amino acid sequence and are thus the tools of the cell for the turnover of proteins. They can be highly specific, both in the reaction catalysed and in their preferred substrates. The cellular functions of proteinases are widespread and include the removal of signal peptides from nascent polypeptides, cellular reorganisation and degradation of proteins to amino acids for nutritional purposes (Barrett and McDonald, 1980). A number of these enzymes are present in lysosomes for both intracellular protein breakdown and breakdown of endocytosed extracellular proteins. Proteinases at the cellular membrane can be involved in intracellular communication by proteolytic activation of bioactive compounds such as growth factors and cytokines. Conversion of inactive prohormones or proenzymes into their active counterpart is also a proteolytic process as is the activation of the blood clotting system.

Synthetic inhibitors of proteinases have proved useful in classifying proteinases into four major groups. These have been termed aspartic, cysteine, metallo- and serine proteinases, according to the residue involved in the binding and catalysis of substrate at the active site (Barrett and McDonald, 1980). Peptide substrates are used to map active-site specificity of proteinases and provide specific and sensitive assays. By convention, the amino acid residues on the amino terminal side of the peptide bond being cleaved are designated P_1,P_2, P_3 etcetera, whereas on the carboxy terminal side they are termed P_1', P_2', P_3' and so on (Berger and Schecter, 1970).

The cathepsins are mammalian lysosomal proteinases which are widely

distributed and differentially expressed among tissues. Intracellularly they serve a variety of digestive and processing functions, whereas extracellularly they may be involved in tissue remodelling and in pathologies such as arthritis, Alzheimer's disease and cancer. The cathepsins are subdivided on the basis of their active site residues as cysteine, serine or aspartic proteinases. Of the cathepsins, B, C, H, L and S are cysteine proteinases, D and E are aspartic proteinases and G and A are serine proteinases.

1.2.1. Cysteine Proteinases

The proteolytic enzymes containing a nucleophilic cysteine as a member of the catalytic machinery are collectively known as the cysteine proteinases (CPs). The majority of CPs are endopeptidases, but some act additionally or exclusively as exopeptidases. The archetypal CP is papain, from the latex of the unripe fruit of *Carica papaya*. Subsequently CPs have been found in nearly every kind of organism, from RNA and DNA viruses to eubacteria, protozoa, fungi plants and animals. As yet, no archaebacterial CP has been sequenced, but there is evidence that it exists (Barrett and Rawlings, 1996).

The well characterised CPs found in mammalian cells can be divided into 2 groups, one comprising mainly the lysosomal enzymes cathepsins B, H and L (Barrett and Kirschke, 1981) and the other the cytoplasmic Ca^{2+}-dependent enzymes, the calpains (Murachi, 1983). Apart from their cellular location, a feature that distinguishes the calpains from the cathepsins is their lack of inhibition by diazomethane compounds (Barrett *et al.*, 1982), although in common with the lysosomal CPs, the calpains are also inhibited by the class-specific inhibitor, E-64. All of the CPs have an essential cysteine, histidine and asparagine, the mechanism of action depending on the sulphonium ion of the cysteine providing nucleophilic attack on the carbonyl group in the peptide bond. CPs are generally $\geq$ 31 kDa in size, have broad pH optima, from 5.5 to 7.5, and can function in a variety of environments as long as sufficient reducing agent is present to prevent the active-site thiol group being oxidised (McKerrow *et al.*, 1993).

Studies of many lysosomal enzymes, including CPs, have indicated that they are synthesised as inactive preproproteins that acquire activity only after the removal of the

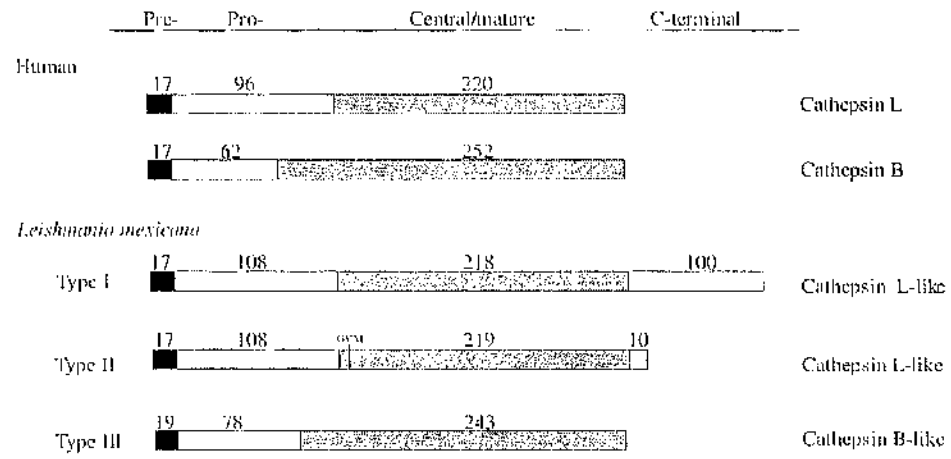


Fig. 1. Schematic representation of the three types of trypanosomatid CPs in comparison with human cathepsin L and cathepsin B.

N-terminal pro-peptides in the pre-lysosomal compartment (Fig. 1.). Metalloproteinases and aspartic proteinases have been reported to be involved in the processing of lysosomal enzymes (Hara *et al.,* 1988; Nishimura *et al.,* 1989). This cleavage, with mammalian cathepsin B for example (Mach *et al.*, 1994), may be brought about *in vitro* at acid pH, suggesting that the lower pH of the lysosome may be responsible for triggering activation *in vivo*. The papain family has more known members than any other family of CPs. With few exceptions these endopeptidases, which include the majority of parasite CPs, show a strong preference for hydrolysis of bonds in which the P_2 residue is a hydrophobic one. Typical members of the family are the proteinases of the food vacuoles of protozoa, and the lysosomal proteinases, cathepsins B, H, L, K, S and others, in higher animals. In the lysosome, enzymes of this type contribute to the turnover of cellular proteins and also act in phagocytic cells to digest the proteins of microrganisms. This activity contributes antigenic peptides to the major histocompatibility system, leading to the production of antibodies (Mizuochi *et al.*, 1994). The lysosomal enzymes can also be secreted into the extracellular matrix, where they contribute to tissue remodelling in cartilige and bone. Specific CPs are also thought to be involved in pro-hormone processing, cyclin B degradation and platelet activation (review: Kirschke *et al.*, 1995). Proteolysis by CPs outside the lysosomes is regulated by physiological pH values and natural inhibitors. However, as will be discussed later, an alteration of this balance in favour of the enzymes leads to uncontrolled proteolysis seen

in several disorders such as inflammation, tumour growth and arthritis (see section 1.2.1.3).

1.2.1.1. Cathepsin L

Cathepsin L is a lysosomal protein involved in protein degradation (Kirschke and Barrett, 1987) and is synthesised as an inactive precursor that is processed to produce a 28 kDa single chain mature enzyme (Fig 1). The pro-region is a potent inhibitor of the enzyme's activity (Carmona *et al.*, 1996) through its binding to the active site (Coulombe *et al.*, 1996), thus maintaining the proteinase in an inactive form during trafficking to the lysosome. Cathepsin L is catalytically active at pHs 3.0 - 6.5 in the presence of -SH containing compounds, while the precursor is stable at physiological pH. Studies on the activation of pro-cathepsin L showed that this can occur autocatalytically as well as by other proteinases such as cathepsin D and metalloproteinases (review: Kirschke *et al.*, 1995). Cathepsin L is a strong endopeptidase implicated in the modification of numerous proteins, such as degradation of matrix proteins, the activation of pro-plasminogen and the inactivation of bradykinin, to name but a few (Kirschke *et al.*, 1995). The enzyme has been found to have a restricted specificity in the hydrolysis of synthetic substrates, cleaving preferentially at pH 5.5 those with arginine in the P_1 position and hydrophobic residues in the P_2 and P_3 positions. The methylcoumarylamide substrate *Z*-Phe-Arg-NHMec is a suitable substrate for determining cathepsin L activity. Cathepsin L-like enzymes have been characterised from a wide range of eukaryotes, including some protists that are thought to form the earliest branches of the eukaryotic tree (Berti and Storer, 1995), although there appear to be differences in properties between enzymes from the various species.

1.2.1.2. Cathepsin B

Cathepsin B is similar to cathepsin L in being a lysosomal proteinase present in all mammals and nearly all organs and tissues; similar enzymes also occur in lower organisms, as will be discussed later (section 1.2.2.2). Synthesised as a glycosylated pre-pro-enzyme (Fig. 1.), it is processed to a ~30 kDa single chain active protein on route to

the lysosome. Secreted cathepsin B is in the pro-enzyme form, which differs from the mature processed form since it is stable at neutral pH. The cathepsin B pro-peptide is a potent inhibitor of cathepsin B at pH 6.0. but can be removed autocatalytically, as well as by cathepsin D, pepsin, and other serine, metallo- and cysteine proteinasess. Cathepsin B has broad substrate specificity and cleaves several synthetic substrates, notably methylcoumarylamides containing the Arg-Arg sequence. The specificity of the endopeptidase activity of cathepsin B is unclear; the amino acid in P_1 can be varied greatly, and glycine and arginine as well as hydrophobic amino acids can be accommodated as the P_2 residue. As a result, a great many proteins are degraded or modified by cathepsin B. For example, plasminogen is activated whereas fibronectin is degraded (for a review of cathepsin B, see Kirschke *et al.*, 1995).

1.2.1.3. Involvement in mammalian pathologies

Although lysosomal CPs play an important role in the turnover of intracellular proteins, the results of recent studies suggest that cathepsins L and B are more stable in the extracellular environment than was previously believed and evidence is increasing that they play a significant extracellular role in a range of physiological disorders. For example, tumours of various types have been reported to express increased levels of cathepsins B, H and L, and extracellularly these enzymes have been implicated in tumour invasion and metastasis (Liotta and Stetler-Stevenson, 1991). Furthermore, cathepsins S, B and L have been implicated in Alzheimer's disease, arthritis and inflammation (review: Müller-Ladner *et al.*, 1996)

Secretion of precursor forms of cathepsin L is known to occur not only from normal cells such as macrophages and fibroblasts, but also from cells treated with growth factors and tumour promoters and malignantly transformed cells (Kirschke *et al.*, 1995). Cathepsin L has been shown to be active towards extracellular matrix proteins at physiological pH and it is hypothesised that this enzyme might facilitate the invasive steps of metastasis during which tumour cells cross through the basement membranes (Duffy, 1992). Other physiological processes that this enzyme has been specifically implicated in are bone resorption and arthritis (Trabandt, *et al.*, 1990).

Cathepsin B is the lysosomal CP that has been implicated most in disease pathogenesis, particularly with regards to joint destruction in rheumatoid arthritis, muscular dystrophy and tumour metastasis (reviews: Elliot and Sloane, 1996; Müller-Ladner *et al.*, 1996). Production of cathepsin B by tumour cells has been linked to metastatic potential in several experimental models, and it is found in higher amounts in Lewis lung carcinoma, colon carcinoma cell lines and ascites fluid of women with ovarian carcinoma. The ability of tumour cells to invade into the extracellular matrix has been attributed to cathepsins released by tumour cells or associated with the plasma membrane of tumour cells (Sloane *et al.*, 1994, and references therein). Cathepsin B secreted by invading tumour cells can degrade collagen and elastin (Kirschke *et al.*, 1982) thereby destroying the basal laminar region. The finding that in tumour cells cathepsin B is delivered to the plasma membrane rather than to the lysosome (Sloane *et al.*, 1994b) and secreted into the surrounding medium (Sukoh *et al.*, 1994) indicates that tumour cells are defective in their intracellular processing of proteinases.

Bacterial proteinases have been implicated as both pathogenic and virulence factors, with many of the medically important species producing enzymes involved in tissue invasion and destruction, evasion of host defences and modulation of the host immune system during infection and inflammation (review: Travis *et al.*, 1995). An example is the anaerobic Gram-negative rod bacterium *Porphyromonas gingivalis*, which has been implicated in the initiation and progression of certain forms of periodontitis, including juvenile and adult periodontal disease. The combined proteolytic activities of *P. gingivalis* have been shown to be virulence factors capable of degrading and inactivating host defence proteins, structural proteins and plasma proteinase inhibitors (Johnson, 1991, and references therein). Multiple CPs designated gingipains have been identified in *P. gingivalis* and are unusual in not only having a putative role in adhesion of the microorganism to host tissue, but also in having no homology to other known CP families (Pavloff *et al.*, 1997).

1.2.2. Proteinases of Parasites

Parasite proteinases have been the subject of much research and this topic has

been extensively reviewed (North *et al.*, 1990; North, 1992; McKerrow, 1989, 1993; Robertson *et al.*, 1996; Coombs and Mottram, 1997). Broadly speaking, these enzymes appear to have important roles in parasite transformation, parasite nutrition, host invasion and also evasion of the host immune response. Many parasitic organisms differentially express proteinases at different stages of their life cycle, as appropriate for their role. In the process of tissue infection by helminth parasites, for example, proteinases are thought to be important not only for tissue lysis and migration (McKerrow, 1989, Pritchard, 1995) but also for nutrient uptake (Chappell and Dresden, 1986) and immune evasion (Carmona *et al.*, 1993). The cathepsin L-like proteinases of parasitic protozoa often play an important role in the destruction of host proteins (Stanley *et al.*, 1995), in the nutrition of the parasite (Rosenthal, 1995) and in the neutralisation of the host immune response (Reed *et al.*, 1995; Leao *et al.*, 1995). There is great interest in these enzymes since several appear to be promising targets for the development of new anti-parasite chemotherapy.

There is an enormous amount of literature concerning the proteinases of parasites; therefore, I have concentrated on a few examples which best illustrate the diversity of these enzymes, both in helminths and in protozoa.

1.2.2.1. Helminths

Haemonchus contortus is a highly pathogenic blood feeding nematode which resides in the abomasum of sheep, goats and other small ruminants. A number of proteinases have been described from this ovine parasite including a zinc metalloproteinase, the secretion of which controls the ecdysis of infective 3rd stage larvae (Gamble *et al.*, 1989, 1996), and several CPs from the adult stage (Pratt *et al.*, 1990). Cathepsin B-like CPs are widely distributed in parasitic helminths, and *H. contortus* contains at least 5 genes (Cox *et al.*, 1990), the corresponding enzymes of which are thought to have a role in nutrition. The latter proteinases were cloned and sequenced, and were found to represent a multigene family (Pratt *et al.*, 1992). The transcribed CPs have been found in adult worm extracts but not 3rd or 4th stage larvae, suggesting their function is in worm feeding. Developmentally regulated secretion of gut

associated cathepsin L-like CPs by adult *H. contortus* has been reported (Rhoads and Fetter, 1995). It has been hypothesised that the gut-associated enzymes may function as digestive enzymes, and the secreted proteinases may function in the degradation of abomasal tissue and blood vessel walls, facilitating the parasites access to tissue or blood components (Rhoads and Fetterer, 1996). More recently, a developmentally-regulated zinc metalloproteinase was characterised from secretions of 4th stage larvae of *H. contortus* (Gamble *et al.*, 1996b). It was shown that this differed from the previously characterised metalloproteinase involved in the ecdysis of 3rd stage larvae, prompting speculation that this enzyme is involved in feeding or prevention of blood clotting. *H. contortus* also has a putative metalloproteinase found in host protective gut-extracts of the adult helminth (Redmond *et al.*, 1997); this enzyme is thought to be involved in proteolytic digestion of the blood meal and has potential as a sub-unit vaccine component or novel target for chemotherapeutic intervention. Indeed another highly protective gut membrane protein isolated from *Haemonchus* was found to be an aminopeptidase, suggesting that vaccination with helminth proteinases could prove to be an effective anti-parasite strategy.

The apparent intestinal source of the CPs and their release via defecation might represent an adaptation enabling *H. contortus* to modify its host microenvironment. This mechanism is similar to the secretion of cathepsin L-like CPs by the liver fluke *Fasciola hepatica* from intestinal cells and release via regurgitation (Smith *et al.*, 1993) suggesting that the release of gut proteinases might represent a specific adaptation to parasitism. This concept is supported by the fact that *Caenorhabditis elegans*, a free-living nematode that contains a CP gene with specific expression in the gut and a predicted amino acid sequence with 50% homology to that of the *H. contortus* CP (Ray and McKerrow, 1992), does not release proteinases during culture (Lackey *et al.*, 1989). CPs also appear to play a nutritional role in other parasitic nematodes that digest haemoglobin, such as *Ascaris* (Maki and Yanagisawa, 1986) and the hookworm *Ancylostoma caninum*. The latter secretes both cathepsin L-like and cathepsin B-like CPs, apparently for their anticoagulation properties (Harrop *et al.*, 1995).

An extracellular matrix model, which mimics the *in vivo* structure of connective

tissue and basement membranes, has become a useful tool in analysing host-parasite interactions, parasite developmental processes and, in particular, parasite proteinase roles (review: Rhoads and Fetterer, 1997). For example, the hypothesis that the skin invasive larval stages of nematode parasites such as *Strongyloides stercoralis* (Mckerrow *et al.*, 1990) and *Onchocerca* species (Lackey *et al.*, 1989) utilise secreted proteinases to traverse keratin, epidermal cell layer, basement membrane, dermal connective tissue and blood vessel walls was supported by the ability of these parasites to degrade extracellular matrix efficiently (Rhoads and Fetterer, 1997). The enzymes involved were characterised as either serine or metallo-elastases, a finding consistent with the elastin-rich extracellular matrix of skin. Proteinase release by the filarial nematode *Dirofilaria immitis* increases at the time of moulting from the 3rd to 4th larval stage and corresponds to an increase in the specific degradation of the collagen component of extracellular matrix (Richer *et al.*, 1992). A cathepsin L-like CP has recently been implicated in the moulting process of the related species *Brugia pahangi* (McKerrow, 1994).

The CPs of *F. hepatica* have been implicated in invasion by the juvenile stages (Dalton and Heffernan, 1989) as well as the haematophagic activities of the established liver fluke (Rege *et al.*, 1989, and references therein). A number of workers have shown that the CPs of *F. hepatica* can cleave human IgG and IgM (Chapman and Mitchell, 1982; Heffernan *et al.*, 1991), which may allow the parasite to evade host antibody-mediated immune mechanisms whilst in the blood circulatory system during migration. Although several CPs are present, the major protein in the excretory-secretory products of adult and juvenile *F. hepatica* is a 25-26 kDa CP with a subsite specificity similar to that of cathepsin B (McGinty *et al.*, 1993; Dowd *et al.*, 1995). The importance of this CP in parasite fecundity has been implicated, since vaccination using a CP of *Fasciola* has been shown to decrease worm burden and egg production by the parasite (Smith *et al.*, 1994).

Both cathepsin L-like and cathepsin B-like CPs have been identified in secretions and extracts of the adult blood fluke *Schistosoma mansoni*. The cathepsin L-like CP has been hypothesised as the major proteinase involved in the degradation of

haemoglobin by schistosomes. This suggestion was based on the finding that the cathepsin L activity greatly predominated over the cathepsin B (Smith *et al.*, 1994b; Dalton *et al.*, 1995, 1996). However, the recent report that the vomitus of adult schistosomes contains a major cathepsin B-like but only minor cathepsin L-like activity, biochemically variable between schistosome species (Caffrey *et al.*, 1997), argues against this hypothesis. *S. mansoni* cercariae release both cathepsin L-like and cathepsin B-like CPs and it is thought that they may be involved in the process of skin penetration (Dalton *et al.*, 1997) together with the well documented serine proteinases (McKerrow *et al.*, 1985, 1991). Sequence comparisons have recently shown that a *S. mansoni* protein from extracts of adult worms, named Sm32, is similar to asparaginyl endoproteinases, a novel family of CPs of which the legumains from legumes are the best characterised (Takeda *et al.*, 1994; Dalton *et al*, 1995b). It is proposed that Sm32 is involved in pro-enzyme activation, due to its strict substrate specificity (Dalton and Brindley, 1996), and may therefore represent a potential drug target.

Proteinases with parasite specific functions have also been identified in several cestode species. The plerocercoid stage of the pseudophyllidean tapeworm *Spirometra mansonoides* has been found to contain a neutral CP that is also a growth hormone agonist, facilitating tissue penetration while stimulating the host's growth (Phares *et al.*, 1996). CPs have also been identified in the related species *S. eriacei* and *S. mansoni* plerocercoids (Fukase *et al.*, 1985; Song and Chappell, 1993). These have been found to cleave immnuoglobulins (Kong *et al.*, 1994) and invoke a specific IgE response in infected individuals (Kong *et al.*, 1997). Human neurocysticercosis is caused by infestation of the central nervous system with the cyst stage of *Taenia solium*, the pork tapeworm, and the cysts produce cysteine, aspartic and metalloproteinases (White *et al.*, 1992). A CP has been found to be the predominant enzyme involved in IgG degradation by the cysts of the related species *T. crassiceps* and is another potential target for anti-parasitic chemotherapy (White *et al.*, 1997).

1.2.2.2. Protozoa

Although the parasitic protozoa represent a very diverse group of organisms, the

highest activity proteinases are often the CPs (North, 1992). These have been found in most of the important parasites during at least one stage of the life cycle, often a stage present in the mammalian host. Some of their potential functions include tissue invasion, penetration into and survival in the host cell, and parasite remodelling during transition of one morphological state to another. The occurrence of CPs in parasitic protozoa has been summarised (North *et al.*, 1990); the enzymes most studied are those in *Leishmania mexicana, Trypanosoma brucei, T. cruzi, Trichomonas vaginalis, Tritrichomonas foetus, Giardia intestinalis, Entamoeba histolytica, Theileria* and *Plasmodium falciparum*. Many of the enzymes are located in lysosomes, and in comparison with the mammalian enzymes some are unusually large and relatively stable to alkali.

Giardia species cause a common water borne intestinal infection in humans. Lindmark (1988) first reported that proteinases were present in lysosome-like subcellular particles in *Giardia* trophozoites, and subsequent studies confirmed the occurrence of CP activity (Hare *et al.*, 1989; and references therein). More recently two CPs from *G. intestinalis* have been characterised (Werries *et al.*, 1991) and multiple proteinase activities due not only to CPs but also serine and aspartic proteinases have been detected in lysates of trophozoites of *Giardia intestinalis* (Williams and Coombs, 1995). However, it is not yet known in which aspect of parasite development, metabolism or pathogenesis they are involved in.

Trichomonas vaginalis secretes CPs which are significantly larger in size than the well characterised mammalian lysosomal enzymes (Lockwood *et al.*, 1987) and there is evidence that these CPs can degrade host antibodies (Provezano and Alderete, 1995). Surface-associated CPs have been reported to be involved in the cytoadherence and cytotoxicity of this organism and are thus perceived to be important virulence factors (Arroyo and Alderete, 1989, 1995).

Severe tissue damage can result from infection by certain pathogenic amoebae and released CPs have been implicated in the pathogenesis of *Entamoeba histolytica* (Keene *et al.*, 1986), *Acathamoeba polyphaga* (Mitro *et al.*, 1994) and *Naegleria fowleri* (Aldape *et al.*, 1994). CPs are important virulence factors of pathogenic *E. histolytica*

(Scholze and Tannich, 1994), having been shown to degrade host proteins such as complement factors (Reed *et al.*, 1989) and be released by viable trophozoites, rather than simply leaking out upon cellular disentegration (Leippe *et al.*, 1995). However, a homologue of one of the three CPs genes of pathogenic *E. histolytica* has been found in non-pathogenic *E. dispar* strains (Mirelman *et al.*, 1996), a finding which rules out the proposed test for pathogenic or non-pathogenic strains based on the presence or absence of these genes (Reed *et al.*, 1995).

Malaria is the most important protozoan infection worldwide, with hundreds of millions of cases and approximately one million deaths reported annually (Walsh, 1989). Trophozoites of *Plasmodium falciparum* obtain free amino acids for protein synthesis by degrading host erythrocyte haemoglobin in an acidic food vacuole. Available evidence suggests roles for CPs, serine and aspartic proteinases in the invasion and rupture of erythrocytes by malaria parasites, as well as in haemoglobin degradation by trophozoites. Two CPs of apparent sizes 28 kDa and 68 kDa have been identified in *P. falciparum* (Rosenthal, *et al.*, 1988, 1989). The 68 kDa enzyme is involved in invasion of erythrocytes by merozoites (Mayer, *et al.*, 1991) whereas the 28 kDa proteinase (falcipain) is implicated in parasite multiplication and haemoglobin digestion within the erythrocyte, and is located within an acidic food vacuole (Rosenthal, *et al.*, 1987; Rosenthal, *et al.*, 1988; Rocket *et al.*, 1989). A *P. falciparum* CP gene was recently characterised as having 37% amino acid identity to cathepsin L (Rosenthal and Nelson, 1992) and appeared to be the gene encoding the 28 kDa trophozoite proteinase falcipain. The *P. falciparum* food vacuole contains not only the CP falcipain but also the aspartic proteinases plasmapepsins I and II (Gluzman *et al.*, 1994, and references therein). All three enzymes can cleave denatured haemoglobin *in vitro* (Rosenthal, *et al.*, 1988; Salas *et al.*, 1995). It has recently been discovered that the falcipain gene is the only CP gene whose transcript can be detected in the early intraerythrocytic parasites (Francis *et al.*, 1996) and it has been proposed that falcipain works by degrading haemoglobin fragments after initial aspartic proteinase attack has denatured the substrate. For a detailed review of haemoglobin catabolism by malaria parasites see Rosenthal and Meshnick, 1996.

CPs and metalloproteinases have been found in a wide range of trypanosomatids (Branquinha *et al.*, 1996, and references therein) although there are marked differences between the enzyme profiles from the monogenetic (*Crithidia, Herpetomonas, Leptomonas*) and digenetic (*Trypanosoma, Leishmania, Endotrypanum, Phytomonas*) species. In the case of *T. brucei*, the causative agent of African sleeping sickness, a CP gene was cloned and sequenced, and the amino acid sequence deduced from cDNA showed similarity with human cathepsin L (Mottram *et al.*, 1989). The cDNA of the 28 kDa enzyme predicted a protein with a 108 residue C-terminal extension (CTE) of unknown function, similar to that of *T. cruzi* (see below). The substrate specificities of CPs of the parasite were studied in more detail (Robertson *et al.*, 1990) and the results showed that *T. brucei* contains a group of four CPs similar in substrate and inhibitor specificities to cathepsin L, and also larger enzymes that are probably serine proteinases. Subcellular fractionation studies indicate that the main hydrolytic activity of *T. brucei* is located within organelles that resemble lysosomes (Lonsdale-Eccles and Grab, 1987), with higher levels being reported in short stumpy bloodstream forms infective for the tsetse fly vector (Pamer *et al.*, 1989, 1990). This suggests a biological role for this CP activity in differentiation or adaptation to the insect host. A developmentally regulated lysosomal CP, termed congopain, exists in *T. congolense* (Mbawa *et al.*, 1991, Fish *et al.*, 1995). It has high homology to CPs of *T. brucei* and *T. cruzi* including the presence of a CTE (Authié *et al.*, 1993), which is characteristic of the so called Type I CPs of trypanosomatids (see section 1.5.2.1). Host proteins such as immunoglobulins and complement factors are degraded by congopain *in vitro* (review: Authié, 1994). Congopain elicits a high IgG response in trypanotolerant but not trypanosusceptible cattle during primary infections, which suggests that congopain may play a role in pathogenicity and that more efficient immune responses to congopain may contribute to trypanotolerance (Authié, 1994). The CTE of congopain is thought to be the most immunogenic part of the molecule, stimulating antibodies that do not affect the enzyme function.

The trypanosomatid *Trypanosoma cruzi* is the causative agent of Chagas' Disease, estimated to affect over 16 million people in Central and South America and is

the leading cause of heart disease in these countries. Three CPs have been purified to homogeneity from epimastigotes (Bontempi *et al.*, 1984; Rangel *et al.*, 1981b; Bongertz and Hungerer, 1978). The major enzyme, termed 'cruzipain', has a cathepsin L-like preference for dipeptide substrates containing phenylalanine and arginine (Bontempi, *et al.*, 1984), hydrolyses casein and haemoglobin in substrate SDS-PAGE gels (Rangel, *et al.*, 1981), and has a molecular mass of around 40 kDa (Martinez and Cazzulo, 1992). It is a high mannose-type glycoprotein (Cazzulo *et al.*, 1989) whose expression is developmentally regulated, with highest levels being found in the insect vector (epimastigote) form of the parasite (Campetella, *et al.*, 1990). Cruzipain is found in lysosomes, perhaps indicating a role in parasite nutrition (Bontempi, *et al.*, 1989), but some is also detected on the cell surface (Souto-Padrón, *et al.*, 1990) and so the enzyme could be involved in host cell penetration. The multicopy (~130) cruzipain gene has been cloned, by sequencing a DNA fragment obtained by PCR amplification (Eakin *et al.*, 1990; Campetella *et al.*, 1992), and appears to encode a protein that includes the expected pre-pro-enzyme form as well as an unusual CTE of approximately 145 amino acids (Aslund, *et al.*, 1991; Eakin, *et al.*, 1992). The pre-pro form is similar to that found in other members of the papain superfamily, but the CTE is relatively rare, having been identified so far only in some other trypanosomatid CPs, including CPs of *Trypanosoma* (see above) and *Leishmania* species (see section 1.4), in the tomato (Mottram, *et al.*, 1989; North, 1991) and also in rice (Watanabe *et al.*, 1991). However, the CTE is present in the mature enzyme of *T. cruzi*, whereas it appears to be largely removed during post-translational processing in the other trypanosomatids. Proposed functions for the CTE, which can be obtained by self proteolysis (Hellman *et al.*, 1991), and which is not necessary for enzyme activity (Eakin *et al.*, 1992, 1993) include targeting the proteinase within the parasite cell and/or anchoring it to a cell membrane. Cruzipain and its CTE are detected in biological fluids during experimental infection of mice (Gonzalez *et al.*, 1996) and, interestingly, the relative amount of CTE detected is 7-fold higher than that of the catalytic domain, reinforcing the idea of a highly immunodominant but enzymatically inactive CTE domain (Cazzulo *et al.*, 1992; Authié, 1994). The presence of cruzipain in serum means that differential serodiagnosis of

human infections caused by *T. cruzi* and *Leishmania* spp is now possible using purified cruzipain as the specific antigen (Malchiodi *et al.*, 1994). The 3 dimensional structure of the enzyme has been solved, revealing that cruzipain is isostructural with papain (McGrath *et al.*, 1995) and suggests a common catalytic mechanism (Storer *et al.*, 1994).

1.3. PROTEINASE INHIBITORS AS DRUGS

It has become apparent that proteinases play crucial roles in numerous pathological processes and the development of non-toxic proteinase inhibitors for *in vivo* application is the subject of much research. The general belief is that the highly specific recognition by proteinases of defined amino acid sequences should make it possible to inhibit enzymes involved in pathological processes while leaving other proteinases unaffected. Arthritis, tumour invasion and metastasis, parasitic infection, HIV infection and a number of degenerative diseases have been linked with the involvement of one or more proteolytic enzymes and so proteinase inhibitors could have beneficial activities.

1.3.1. Synthetic inhibitors

An extremely important breakthrough in the understanding of proteolysis was the development of specific synthetic substrates (Smith *et al.*, 1992, and references therein), a principle that has been extended for the development of selective synthetic inhibitors. The binding of an amino acid sequence to the active centre of a proteinase can be applied to achieve selective inhibition of an enzyme, by linking to the peptide sequence a group that can bind covalently to, and so inactivate, the active site of the enzyme (Evans and Shaw, 1983). Specific and irreversible proteinase inhibitors have been developed against mammalian cathepsins, and have great potential for chemotherapy against arthritis, metastasis of cancer cells and parasitic infections.

Selective synthetic inhibitors of CPs typically form irreversible covalent bonds to the active site thiol (review: Rasnick, 1996). Peptidyl diazomethanes (Shaw, 1984, and references therein) and peptidyl fluoromethanes (Rasnick *et al.*, 1985) are specific,

irreversible inactivators of CPs that inhibit the enzymes by alkylation of the reactive-site cysteine residue, the peptide sequence providing the affinity required for binding to the active site of the enzymes (review: Shaw, 1990). The compounds readily permeate through cell membranes and do not react significantly with free thiols such as 2-mercaptoethanol and cysteine (Green and Shaw, 1981) or other classes of proteinases (Crawford *et al.*, 1988; Shaw *et al.*, 1990), although it has been shown that certain diazomethanes inactivate some serine proteinases by alkylation of the active site histidine. The principal liabilities of the diazomethanes as chemotherapeutic agents are their instability at low pH (which may limit oral administration) and the fact that they are mutagenic. Investigation of various peptidyl diazomethanes as inactivators of the lysosomal CPs identified Z-Phe-Thr-CHN_2 as an effective inactivator of cathepsin B (Shaw *et al.*, 1983) and Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 as very rapid inactivators of cathepsin L (Barrett *et al.*, 1982; Mason *et al.*, 1985).

1.3.2. Natural inhibitors

The action of mammalian CPs is biologically controlled by natural and specific inhibitors called cystatins (review: Henskens *et al.*, 1996). The cystatins make up a super-family of structurally related homologous proteins consisting of 3 groups: stefins, cystatins and kininogens (Turk and Bode, 1991). Increasing attention is being paid to their physiological significance in pathological processes, since uncontrolled proteolysis can lead to irreversible damage, for example, in chronic inflammation or tumour metastasis. Cystatin-like molecules have been identified in both parasitic helminths and protozoa, although it is not yet clear whether they have evolved as a protective mechanism against their own CPs or the host's (Robertson *et al.*, 1996).

The invariant chain (Ii) is an integral membrane protein which is associated with the major histocompatability complex (MHC) Class II heterodimer during early stages of its intracellular transport, and contributes critically to a proper presentation of antigens to $CD4^+$ T lymphocytes (Germain and Margulies, 1993). Recently a novel property of Ii has been established, since a fragment distinct (in sequence) from the cystatins (Bevec *et al.*, 1996) has been found to strongly inhibit cathepsin L,

representing a new class of natural CP inhibitors.

The pro-region of CPs is thought to play many roles in the folding, transport and activity of the zymogen. As predicted from the total or partial absence of activity of the pro-enzymes, CP pro-peptides are also inhibitors of the mature cathepsin B and cathepsin L enzymes (Fox *et al.*, 1992; Carmona *et al.*, 1996). In addition to being potent inhibitors, the CP pro-peptides also contain features insuring that inhibition is highly selective for the proteinases they originate from. This is an important finding regarding future efforts in designing selective CP inhibitors. Most inhibitors available so far are at least in part peptide-based and it has been proposed that the use of non-peptidyl compounds may bring an added flexibility in the design that could exploit subtle differences in structure that the constraints imposed on peptide-based inhibitors could not address (review: Storer and Ménard. 1996).

1.3.3. Therapeutic uses and potential

1.3.3.1. In degradative and invasive disease

As was discussed in section 1.2.1.3, the destruction of the extracellular matrix of articular cartilage and bone which occurs in arthritic joints is thought to be mediated by excess proteolytic activity and there is evidence that cathepsins B, H and L are involved (McKerrow, 1994; Müller-Ladner *et al.*, 1996). Therapeutic advances have been made using proteinase inhibitors for the treatment of arthritis. Oral administration of peptidyl-fluoromethanes to animals not only inhibited the activity of cathepsins B and L *in vivo*, but significantly reduced the inflammation and joint destruction in experimentally induced arthritis (Esser *et al.*, 1994).

Another example is in the development of novel anti-cancer treatments. The most life threatening aspect of cancer is metastasis and CPs and metalloproteinases are thought to be involved in the invasion process. Cathepsin B has been found on the surface membrane of some cancer cells, suggesting proteolytic activity at the cell surface (Sloane, *et al.*, 1994, and references therein) and a decrease of endogenous CP inhibitors has been observed in several cancer cell lines with metastatic properties (Liotta and Stetler-Stevenson, 1991). Detailed studies on limiting cancers using

proteinase inhibitors have not yet been published, but some *in vitro* experiments have been promising (Navab *et al.*, 1997).

Aspartyl proteinases are essential in the life cycle of the HIV virus, where they convert protein precursors to mature proteins which are required for assembly of new infectious virus particles (Kohl *et al*, 1988). Synthetic inhibitors against these aspartyl proteinases have been developed by Kemf *et al.* (1994) and much attention has been given to this method as a therapeutic intervention of AIDS. Treatment regimens employing the proteinase inhibitor ritonavir, for example, significantly delayed the progression of HIV-related disease and prolonged survival in patients with advanced AIDS, while combination therapy with ritonavir and other proteinase inhibitors produced greater clinical benefits (Kemf *et al.*, 1997).

1.3.3.2. In parasitic infection

Proteinase inhibition has long been considered a good target in the search for novel parasitic disease treatments. Certain proteinase inhibitors that are currently available have been found to have anti-parasite effects against some helminths and protozoa. For example, inhibitors of schistosome CPs block parasite haemoglobin degradation *in vitro* and decrease worm burden and egg production *in vivo* (Wasilewski *et al.*, 1996). Specific CP inhibitors not only block the CP activities in cysts and trophozoites of *Entamoeba*, but decrease the efficiency of encystation by trophozoites (Sharma *et al.*, 1996). Peptidyl diazomethanes have been shown to inhibit both the CPs and the growth of *T. brucei* (Robertson *et al.*, 1990) and leupeptin, an inhibitor of CPs and some serine proteinases, has shown both anti-trichomonal and anti-leishmanial activity (Bremner *et al.* 1986; Coombs and Baxter, 1984). However, by far the most promising results have been achieved by using proteinase inhibitors against *Plasmodium* and *T. cruzi*.

Inhibitors of both classes of malarial food vacuole proteinases have shown antimalarial effects. The aspartic proteinase inhibitor pepstatin acts on rings and schizonts causing the formation of pyknotic parasites (Bailly *et al.*, 1992; Rosenthal, 1995) and a peptidomimetic inhibitor of plasmepsin I killed cultured parasites at low

micromolar concentration (Francis *et al.*, 1994). Peptide inhibitors of the 68 kDa CP appeared to inhibit invasion of erythrocytes by merozoites (Mayer, *et al.*, 1991) whereas inhibitors of the 28 kDa CP prevented parasite multiplication within the erythrocyte (Rosenthal, *et al.*, 1987; Rosenthal, *et al.*, 1988; Rockett *et al.*, 1990). The CP inhibitor E-64 acts mainly on trophozoites causing undegraded haemoglobin to accumulate in the food vacuoles and halt intracellular development (Rosenthal, *et al.*, 1989; Bailly *et al.*, 1992; Rosenthal, 1995). In *in vitro* studies using peptide-based fluoromethanes such as Z-Phe-Ala-CH_2F (Rosenthal *et al.*, 1991) and vinyl sulfone CP inhibitors (Rosenthal *et al.*, 1996), the antimalarial effects directly correlated with the ability of a compound to inhibit falcipain, block globin hydrolysis and inhibit parasite development. Interestingly, the antimalarial effects of E-64 and pepstatin are markedly synergistic (Bailly *et al.*, 1992), suggesting that combination therapy might be a promising approach. Encouraging *in vivo* results have been achieved using a peptidyl-fluoromethane, which successfully cured murine malaria infections (Rosenthal *et al.*, 1993). To identify non-peptide proteinase inhibitors, a database of small molecules has been screened against falcipain using computer modelling techniques. This approach identified a low-micromolar lead compound (Ring *et al.*, 1993) and subsequent chemical synthesis and screening identified a set of chalcones effective against cultured parasites at low-nanomolar concentrations (Li *et al.*, 1995).

Cruzipain is thought to participate in host cell invasion, binding to target cells, intracellular replication and differentiation of *T. cruzi* (Harth *et al.*, 1993, and references therein). These putative functions at important stages of the life cycle of the parasite make cruzipain an excellent target for specific irreversible inhibitors that may be used to develop a new chemotherapy. Several successful developments have been made in this direction using peptidyl-diazomethanes and peptidyl-fluoromethanes, resulting in impairment of host cell invasion by *T. cruzi* and intracellular development of the parasite (Bonaldo *et al.*, 1991; Mereilles *et al.*, 1992; Harth *et al.*, 1993; Franke de Cazzulo *et al.*, 1994). Cruzipain is inhibited by a range of peptide aldehyde compounds (Bontempi *et al.*, 1984; Cazzulo *et al.*, 1990) as well as by proteinase inhibitors belonging to the cystatin superfamily (Stoka *et al.*, 1995; Serveau *et al.*, 1996). The

presence of cruzipain in both the intracellular and extracellular developmental forms of *T. cruzi* has stimulated efforts to develop first generation inhibitors and these have proved to be highly effective *in vitro*. Some of these compounds have recently given promising results in mice infected with *T. cruzi* (McGrath *et al.*, 1995).

The naturally occurring CP inhibitors known as cystatins have been found to be very potent inhibitors of cruzipain (Stoka *et al.*, 1995) and indicates that they may play a defence role in the host. Furthermore they may serve as promising starting points for the design of non-covalently bound, reversible CP inhibitors as new anti-parasite drugs. X-ray crystallography and inhibitor screening against cruzipain using computer graphics analysis is allowing rapid identification of new inhibitors based on either leads already identified or compounds selected by computer graphics screening of chemical databases (review: McKerrow *et al.*, 1995). Biotin labelled peptidyl diazomethane inhibitors of CPs, based on the N-terminal substrate-like segment of human cystatin C, have been synthesised (Lalmanach *et al.*, 1996) and have been shown to target cruzipain in infected mammalian cells. Cruzipain has also recently been shown to be inhibited by an alternatively spliced Ii fragment (Bevec *et al.*, 1997), suggesting the possibility of using the invariant chain (see section 1.3.2) as a model for a potential drug to combat Chagas' disease.

In summary, CPs represent attractive targets for the development of inhibitors as potential therapeutic agents, to treat not only diseases involving abnormal elevations of proteolytic activity, but also parasitic infections where one or more CPs are essential to the parasite. However, such reagents must be specific so that they do not interfere with related proteinases from the host. The close structural and functional relationship between parasite CPs and papain-related mammalian CPs has meant that the substrates and inhibitors used so far to assay the activity of parasite CPs are the same as those currently used for cathepsins L and B of mammalian lysosomes (North *et al.*, 1990). It would thus appear that better knowledge of the substrate specificity of parasitic CPs and their sensitivity to inhibitors is needed for the rational development of new drugs that can discriminate between parasite and host cell proteinases.

1.4. PROTEINASES OF *LEISHMANIA*

Trypanosomatids are known to contain CPs, metallo- and serine proteinases, but there have been no reports yet of aspartic proteinases, which appear to be relatively rare in parasitic protozoa (review: McKerrow *et al.*, 1993). By far the most abundant and hence most studied proteinases in *Leishmania* species are the metalloproteinase gp63 and the CPs.

1.4.1. Promastigote surface proteinase

Gp63 is a metalloproteinase expressed in large quantities on the surface of *Leishmania* promastigotes (Bouvier *et al.*, 1985) and accounts for about 1% of the total protein in promastigotes (Bordier, 1987). gp63 has been purified to homogeneity from several species of *Leishmania* (review: Chang and Chaudhuri, 1990) and was found to have proteinase activity that requires zinc ion (Chaudhuri *et al.*, 1989). The natural substrate for this proteinase is not known and the optimum pH of gp63 action appears to be dependent on the nature of the substrate used for the *in vitro* assay (Chang and Chaudhuri, 1990).

The surface expression of this glycosylphosphatidylinositol (GPI)-anchored metalloproteinase in promastigotes is well established for all *Leishmania* species investigated. Gp63 is also expressed, but at lower levels, by intracellular *Leishmania* amastigotes (Frommel *et al.*, 1990; Schneinder *et al.*, 1992). More recent experiments suggest that the amastigote protein has hydrophilic properties, an acidic pH optimum, and is localised in the extended lysosomes of *L. mexicana* (Ilg *et al.*, 1993) which are likewise known to be a rich source of CPs. Thus amastigote gp63 may be involved in lysosomal enzyme processing. These observations are consistent with a differential expression of mRNAs derived from the multicopy gp63 genes in different parasite stages (Medina-Acosta *et al.*, 1993). Transcripts found predominantly in promastigotes predict a hydrophobic sequence at the COOH-terminus serving as a GPI anchor addition signal, while the otherwise highly homologous mRNA in amastigotes codes for a COOH-terminal extension lacking this signal. Therefore, the promastigote protein is routed to the cell surface while the amastigote protein is delivered to lysosomes.

The active site structure of the gp63 proteinase appears to be very similar to those of other well known mammalian matrix metalloproteinases (Button *et al.*, 1993; McMaster *et al.*, 1994). However, antigenicity and conformational analysis of the Zn^{2+}-binding sites of *Leishmania* gp63 and mammalian endopeptidase-24.11 showed them to be antigenically and structurally different although their respective antibodies cross-reacted (Soteriadou *et al.*, 1996). The *Leishmania* surface proteinase has many putative roles, for example, in complement fixation, cell adhesion and resistance to complement-mediated lysis (Brittingham *et al.*, 1995). In several reports the importance of gp63 in the initial infection of macrophages by *Leishmania* has been emphasised. It has been reported that gp63 mediates attachment of promastigotes to macrophages directly or via the complement component C3 (Chang and Chaudhuri, 1990). Furthermore gp63 appears to play an important role in the intracellular survival of the parasite in the macrophage PL probably through its non specific proteinase activity (Chaudhuri *et al.*, 1989). Metalloproteinases related to gp63 have however been described in the monogenetic trypanosomatids *Crithidia fasciculata*, *Herpetomonas samuelpessoai* and *Leptomonas seymouri*, which infect invertebrate hosts but do not infect mammalian cells (Medina-Acosta *et al.*, 1993b), suggesting that the proteinase must also have a role in the insect vector stage.

gp63 has been considered a good candidate for a vaccine against leishmaniasis. Mice immunised with purified gp63 or orally treated with a *Salmonella* mutant carrying the *L. major* gp63 developed significance resistance against *L. major* challenge infection (Yang *et al.*, 1990). In addition, synthetic peptides modelled from different regions of the amino acid sequence of gp63 were found to be highly immunogenic and induced protective immunity in mice (Button and McMaster, 1988).

1.4.2. Cysteine proteinases

Amastigotes of the *L. mexicana* family, which includes *L. mexicana*, *L. pifanoi* and *L. amazonensis*, contain high CP activity (Pupkis *et al.*, 1986; Robertson and Coombs, 1990; Alfieri *et al.*, 1991). A radioiodinated peptidyl diazomethane probe specific for CPs has been found to detect similar enzymes in amastigotes and

promastigotes of various isolates of *L. mexicana* and *L. amazonensis* (Alfieri *et al.*, 1995), although CP banding may differ among *L. amazonensis* isolates.

Degenerate primers encoding the evolutionary conserved active site amino acids of proteinase genes, successfully employed for PCR amplification of CP and serine proteinase genes of protozoan parasites (Eakin *et al.*, 1990; Sakanari *et al.*,1989), have been used to obtain sequence data for the amastigote CPs of axenically cultured amastigotes of *L. pifanoi* (Traub-Cseko *et al.*, 1993). *Lpcys2* was found to have multiple tandemly repeated copies similar to the cathepsin L-like CP genes of *T. brucei* and *T. cruzi* (section 1.2.2.2) and *L. mexicana* (section 1.5.2). These have now been designated Type I CP genes (Robertson *et al.*, 1996; Coombs and Mottram, 1997). In contrast, *Lpcys1* occurred as only one or two copies (Traub-Cseko *et al.*, 1993) and has since been designated a Type II CP gene. The developmental regulation characterised by increases in *Lpcys1* and *Lpcys2* mRNA levels in the amastigote forms of *L. pifanoi* is consistent with previous reports concerning *L. mexicana* parasites (see section 1.5). Related *Lpcys1* and *Lpcys2* CP genes appear to be present in all species of *Leishmania*, although fewer copies of the genes for *Lpcys2* appear to be present in species outside the *L. mexicana* complex (Traub-Cseko *et al.*, 1993).

Although none contain the high activities characteristic of amastigotes of the *L. mexicana* complex (section 1.5), activities with some of the properties of CPs have been described in other *Leishmania* species, such as *L. tarentolae* (North *et al.*, 1983) and *L. donovani* (Ghoshal, *et al.*, 1989). There is some evidence for Ca^{2+}-dependent CPs similar to calpains in *L. donovani* (Battacharya *et al.*, 1993). The discovery of Type I and Type III CP genes (see section 1.5) in *L. major* suggests that this species contains at least two such enzymes (Sakanari *et al.*, 1997).

1.5. CYSTEINE PROTEINASES OF *LEISHMANIA MEXICANA*

1.5.1. Stage specificity of *L. mexicana* CPs

An interesting feature of members of the *Leishmania mexicana* complex is the presence of CP activity (North and Coombs, 1981). Levels are highest in the amastigote stage, where their abundance is associated with numerous megasomes, unique

lysosome-like organelles which display cytochemically demonstrable lysosomal markers such as acid phosphatase and arylsulphatase activities (Pupkis *et al.*, 1986) and can be stained using monoclonal antibody against rat cathepsin B (Prina, *et al.*, 1990). Although the megasomes can constitute as much as 15-20% of the total cell volume of *L. mexicana* amastigotes (Coombs *et al.*, 1986), these organelles are not evident in other members of the genus.

Similar enzymes are apparently produced in the mammal-infective metacyclic forms, but are absent from the multiplicative promastigote stage (Lockwood, *et al.*, 1987). Although a specific role has yet to be found for *L. mexicana* CPs, their high activity and stage specificity suggests their involvement in parasite survival within the macrophage.

Three types of CP have been identified in *L. mexicana*. In addition to the Type I enzymes found in other trypanosomatids, *L. mexicana* contains at least 2 other types of CP, designated Type II and Type III (see Fig. 1.). A summary of the gene-enzyme relationship of the CPs of *L. mexicana* is shown in Table 1.0.

1.5.2. Characterisation of *L. mexicana* CPs

1.5.2.1. Type I CPs

Amastigotes of *L. mexicana* contain multiple Type I CPs of apparent sizes 22-28 kDa that are readily detectable by gelatin-SDS-PAGE (Lockwood *et al.*, 1987b). Since their discovery (North and Coombs, 1981) 6 groups of CPs in the amastigotes of *L. mexicana* have been identified (Robertson and Coombs, 1990) and designated groups A, B, C, E, F and H. Group A consists of at least 4 isoenzymes that bind to Con A Sepharose CL4B, demonstrating that they are glycosylated. The CP isoenzymes of the groups B and C were separated by ion-exchange chromatography and showed different specificities towards a range of fluorogenic peptide substrates. The 3 group B enzymes were found to prefer substrates with tyrosine in the P_1 position and have a lower net negative charge than the 2 enzymes of group C, which are more active towards compounds with a basic amino acid in the P_1 position. Although the enzymes of the 3 CP groups A, B and C were found to be distinct, there were some similarities including

preferences for substrates with bulky amino acids at the P_1 and P_2 positions similar to the mammalian enzyme cathepsin L. The enzymes were found to be susceptible to inhibitors such as cystatins and to be stimulated in the presence of the reducing agent DTT, clearly demonstrating them to be of the CP type (Robertson and Coombs, 1990).

Table 1. Cysteine Proteinases of *Leishmania mexicana*

Gene: enzyme relationship

Enzyme Activity Type	Genes			
	Type	**Name**	**Class**	**Number**
A,B,C,E,F,H	I	*lmcpb*	cathepsin L-like	19
?	II	*lmcpa*	cathepsin L-like	1
D	III	*lmcpc*	cathepsin B-like	1

Two molecules recognised by anti-group C CP antiserum in western blots of amastigote lysates also appear to be Type I CP activities, which were designated group E CPs (Robertson and Coombs, 1994). These activities remain with the pellet fraction after centifugation of a crude amastigote lysate. Their inhibitor sensitivity indicates that they are CPs and their activities can be released from the pellet fraction by 1% SDS, high ionic strength and high pH, suggesting they are peripheral membrane proteins that act ionically with a membrane. Their relatively large size suggests that they are a less processed gene product (Robertson and Coombs, 1994). Purification and N-terminal sequence analysis of the E CPs may indicate whether these proteins are pro-enzymes or mature enzymes with the retained CTE.

Some of these Type I enzymes are present at low activity in stationary phase promastigotes grown *in vitro* (Lockwood, *et al.*, 1987b; Robertson. and Coombs, 1992). Three of the bands apparent in gelatin-SDS-PAGE resemble the high activity CPs

characteristic of amastigotes. Two are similar to the amastigote group B proteinases whereas the highest mobility band F, of around 21 kDa, was found only with stationary phase promastigote populations, suggesting this activity is a feature of metacyclic promastigotes (Robertson, and Coombs, 1992). Further studies have shown this faster moving activity comprises at least 3 distinct enzymes, which were separated by mono Q anion exchange chromatography. It is proposed that they may have a role in metacyclogenesis or be a pre-adaptation for survival in the mammalian host. An *in vitro* culture method has recently been developed (Bates and Tetley, 1993) which favours the production of metacyclic forms, and this system has been used to examine the expression of CPs of *L. mexicana* metacyclics (Bates, *et al.*, 1994). Two prominent bands were detected which distinguished metacyclics from multiplicative promastigotes, lacking CP activity, and amastigotes, with the distinctive banding pattern comprised of multiple enzymes. A correlation between relative activity of the metacyclic specific bands and the prevalence of metacyclics was found both during the growth cycle *in vitro* as metacyclogenesis occurred, and by comparison of stationary phase populations from consecutive subpassages *in vitro*. Late log and stationary phase promastigotes of *L. mexicana* were also shown to contain two acid-activable CPs, designated H1 and H2, detected around 36 kDa in gelatin gels (Robertson and Coombs, 1992). It is thought that they may be pro-enzymes to two of the activities that run together to the give the fastest moving band F, and that the acid treatment causes removal of the pro-region.

For some time it has been accepted that the Type I CPs of *L. mexicana* are encoded by the tandemly arranged multicopy *lmcpb* genes (Souza *et al.*, 1992; Robertson and Coombs, 1994); this aspect is dealt with in more detail in the Chapter 3 Introduction.

1.5.2.2. Type II CPs

L. mexicana and *L. pifanoi* are so far the only trypanosomatids in which Type II enzymes have been identified, and then only using a molecular approach (Mottram *et al.*, 1992; Traub-Cseko *et al.*, 1993). These enzymes are encoded by cathepsin-L-like single copy genes characterised as lacking a long C-terminal extension and having a

three amino acid insertion close to the mature N-terminus. The gene encoding the type II CP was the first CP gene of *L. mexicana* to be cloned (Mottram *et al.*, 1992) and was thus designated *lmcpa* (see also Chapter 2 Introduction). Although the gene was shown to be transcribed in a stage-dependent manner and a protein was detected using a specific antibody, the protein has not yet been purified or shown to be a functional enzyme.

1.5.2.3. Type III CPs

The third CP type known to be present in *L. mexicana* is encoded by the *lmcpc* gene (see Table 1.0 and the Chapter 4 Introduction). It is cathepsin B-like, single copy (Bart *et al.*, 1995) and has a homologue in *L. major* (Sakanari *et al.*, 1997). *lmcpc* encodes 2 major ampiphilic proteins of 31 and 33 kDa which have type D enzyme activity i.e. lack activity towards gelatin but have been shown to hydrolyse various peptidyl substrates used by mammalian cathepsin B such as Bz-Phe-Val-Arg-AMC (Robertson and Coombs, 1993). Members of this class of CP have not been discovered in many parasitic protozoa, possibly because they occur at low activity compared with the abundant cathepsin L-like enzymes.

1.6. *LEISHMANIA* VIRULENCE AND INTRACELLULAR SURVIVAL

1.6.1. Importance of surface and secreted molecules

Leishmania virulence is thought to require the development of infective forms called metacyclic promastigotes either in culture or in the sandfly (Sacks, 1989). The metacyclic markers that have been identified are changes in mobility and promastigote body shape (Mallinson and Coombs, 1989; Schlein, 1993, and references therein) and an increased resistance to the lysis mediated by human complement (Puentes *et al.*, 1990; Bates and Tetley, 1993). In *L. major* this is mediated through structural modification of the surface lipophosphoglycan (LPG) which occurs during the transformation to metacyclics: there is a doubling of the mean number of LPG phosphorylated disaccharide repeat units (McConville *et al.*, 1992), reflected by decreased binding of peanut agglutinin in the case of *L. major* (Sacks *et al.*, 1985).

Collective contributions of several parasite surface molecules enable the parasite to establish infection inside macrophages (for reviews see Chang and Chaudhuri, 1990; Mauël, 1996). The Zn-proteinase of ~63 kDa, gp63 (see section 1.1.4) and the LPG (Descoteaux and Turco, 1993), two abundant parasite surface molecules, are those most implicated in this process. The importance of gp63, however, remains a topic of much debate, mainly due to species-specific differences. LPG and gp63 have been considered parasite ligands (Handman and Goding, 1985; Russell and Wright, 1988) with CR1, CR3 and the mannose-fucose receptor as the corresponding macrophage receptors (Blackwell *et al.*, 1985; Da Silva *et al.*, 1989), although parasites also use host-derived opsonins such as complement to facilitate uptake by macrophages. Metacyclic promastigotes are found to use primarily the CR1 receptor (through which endocytosis is known to produce less respiratory burst) since the complement component C3b is the major covalently associated fragment on these cells. Multiplicative promastigotes, which are killed after internalisation, are found to use CR3 in addition to CR1, suggesting that the loss of CR3 binding ligand during transition from procyclic to metacyclic might constitute an adaptive change (Da Silva, *et al.*, 1989). The abundant expression of gp63, which has been shown to react directly with CR3 in the absence of complement, has in some cases resulted in poor infectivity for macrophages; it was hypothesised that the elongated LPG of virulent cells may mask its gp63 and thus metacyclics may primarily use the CR1 receptor, thereby avoiding macrophage activation (Camara *et al.*, 1995). It has, however, been shown that gp63 not only binds C3 (Russell, 1987) but also cleaves C3 to the haemolytically inactive iC3b (Puentes *et al.*, 1989), thereby avoiding the formation of the complement cascade.

The interaction between the macrophages and the parasites plays a central role in establishing *Leishmania* infection. Macrophages constitute one of the primary defence mechanisms of the body against microbial invasion and are capable of fulfilling a variety of microbicidal functions. For example, they produce superoxide anions and H_2O_2 (hydrogen peroxide) during the respiratory burst, nitric oxide (NO) and abundant lysosomal enzymes (Lewis and McGee, 1992; Zwilling and Eisenstein, 1994). It has been postulated that the impairment of macrophage activation by intracellular

Leishmania contributes to their survival in the toxic environment of the host (Chakraborty *et al.*, 1996). Virulence factors like gp63 and LPG present on the surface of virulent parasites may help them to circumvent and reduce the respiratory burst activity of macrophages upon binding and phagocytosis (Hall and Joiner, 1991; Mauël, 1996). For example, gp63 strongly inhibited the oxidative burst response of macrophages (compared with neutrophils) stimulated with opsonised zymosan (Sørensen *et al.*, 1994) and could be one of the mechanisms whereby *Leishmania* is capable of establishing itself intracellularly in an otherwise hostile environment. Evidence exists that other enzymes such as acid phosphatase present on parasite surface membranes may block the production of oxidative burst metabolites (Remaley *et al.*, 1985) and that LPG scavenges oxygen free radicals and other PKC-mediated events of the oxidative burst (Chan *et al.*, 1989; review: Descoteaux and Turco, 1993). It has also been shown that LPG glycoconjugates have a profound effect on the survival of *Leishmania* parasites, through their ability to regulate the expression of inducible nitric oxide synthase (iNOS) by macrophages (Proudfoot *et al.*, 1996).

The relative failure of amastigotes to trigger a respiratory burst is thought to correlate with their intracellular survival. In contrast to promastigotes, the surface of *Leishmania* amastigotes is dominated by species-specific glycoinositol phospholipids (GIPLS) (Winter *et al.*, 1994, and references therein) which may mediate *Leishmania* binding to macrophages and shield the surface against lysosomal hydrolases. GIPLs have been shown to inhibit the production of NO by macrophages and thus enhance the survival of *L. major* in activated macrophages (Proudfoot *et al.*, 1995).

How *Leishmania* parasites not only survive but also proliferate in the potentially hostile environmemt of the macrophage phagolysosome is something of an enigma (reviews: Russell, 1995; Garcia-del Portillo and Finlay, 1995). Most other intracellular pathogens have developed strategies to escape the phagolysosomal environment. For example, after phagocytosis, *T. cruzi* trypomastigotes escape the phagosome to multiply within the host cell cytoplasm, whereas *Toxoplasma gondii* inhibits the acidification of phagosomes and/or phagosome lysosome fusion (review: Mauël, 1996). However *Leishmania* amastigotes exhibit elevated metabolism under acidic conditions, reflecting

their adaptation to intracellular growth (Mukkuda *et al.*, 1985). Gp63 may be involved in the intracellular survival of *Leishmania*, since it is capable of degrading lysosomal enzymes under the acidic conditions of the PV, and incorporated into liposomes is able to protect protein substrates from intralysosomal degradation within macrophages (Chaudhuri *et al.*, 1989). Furthermore gp63 has been shown to be functionally significant in the early stages of leishmanial infection of macrophages *in vitro* using genetic rescue of gp63-deficient variants (McGwire and Chang, 1994). *In vitro* studies with parasite glycosylation mutants or natural variants lacking LPG have shown LPG also to be critical to survival in the PL (Cappai *et al.*, 1993, and references therein).

1.6.2. Potential roles of cysteine proteinases

There are several suggestions as to how the metacyclic and/or amastigote-specific CPs of *L. mexicana* aid survival of the parasite in macrophages. It has been postulated that the proteinases may inactivate the host cell's lysosomal enzymes, or that they may be involved in producing amines which would raise the vacuolar pH and perhaps denature the host hydrolases (Coombs, 1982). However, there is currently no firm evidence to back up either of these theories. It has been supposed that amastigotes do not take up proteins from the parasitophorous vacuole (Pupkis, *et al.*, 1986) and there is little evidence that amastigote CPs are secreted (Coombs, 1982; Pupkis *et al.*, 1986; Ilg *et al.*, 1994). Indeed evidence suggests that amastigotes of the *L. mexicana* complex are in fact acidophilic and have evolved plasma membrane components compatible with the harsh environment of the PV (Antoine *et al.*, 1990).

Amastigote CPs effectively degrade denatured proteins like gelatin or azocasein and also native proteins like transferrin, serum albumin and immunoglobulins at the acidic pH of the lysosomal compartment (pH 4.5-5.5) of *Leishmania* (Antoine *et al.*, 1988, 1990). This suggests that these enzymes may be required for the nutrition of the parasites and/or in immune response neutralisation. It has been demonstrated that exogenous proteins entering *L. mexicana*-infected macrophages by endocytosis are delivered to the PV (Russell *et al.*, 1992) by selective fusion of the PV with other particle-containing vacuoles (Collins *et al.*, 1997). These proteins may be taken up by

amastigotes and transferred to the lysosomes of the parasites, where they are likely degraded by CPs and, possibly, by the amastigote metalloproteinase gp63 (Ilg *et al.*, 1993).

CPs released after amastigote lysis into the acidic PV could lead to host cell damage and, upon host cell lysis, the *Leishmania* proteinases entering the extracellular space may contribute to the disintegrated state of the lesion tissue by proteolysis of the extracellular matrix (Ilg *et al.*, 1994). This may represent an altruistic mechanism whereby the release of CPs by dead parasites favours vector transmission, by increasing dissemination of the parasite and, therefore, the chance of being uptaken during a bloodmeal. In analogy, the homologous enzymes cathepsins B and L, which are secreted by metastatising mammalian tumour cells, degrade the connective tissue and basement membrane components collagen, elastin, laminin and fibronectin (Bond and Butler, 1987). Thus it is possible that the killing of intracellular parasites by activated macrophages, which is crucial for resolution of the infection, could also lead to host tissue damage by released amastigote enzymes.

Immunological control of cutaneous leishmaniasis depends on activated macrophages producing IL-12 which drives Th1 cell differentiation and proliferation; Th1 cells produce IFN-γ which activates macrophages to produce NO that in turn kills the parasite in macrophages (Proudfoot *et al.*, 1996). It has been postulated that enzymatically active CPs like papain or schistosome ova enzymes are potent allergens and may lead to a pronounced Th2-helper cell response (Finkelman and Urban, 1992) which is deleterious for the host in the case of *Leishmania* infections. However, immunisation with recombinant *Leishmania* CP, and T-cell stimulation experiments using recombinant and native antigen, showed that the enzymes are T-cell immunogens allowing the development of potentially protective Th1-helper cell lines (Wolfram *et al.*, 1995). The suggestion that amastigotes attempt to minimise the exposure of their antigens to the immune system (Russell and Chakraborty, 1992) agrees with the finding that intracellular proteins of intact amastigotes are not available for presentation when parasites are alive (Wolfram *et al.*, 1995). Thus it may be an immune-evasion mechanism that the CPs of amastigotes are not secretory antigens (Il *et al.*, 1994),

although secretion of CPs has been reported for several parasites (North *et al.*, 1990; McKerrow and Doenhoff, 1988).

It was previously suggested (Antoine *et al.*, 1991) that the presence of high amounts of MHC II Ii in the PV of *L. amazonensis*-infected macrophages could be due to a *Leishmania* induced mechanism by means of which this organism may evade the immune system. Recently amastigotes have been shown to internalise and degrade class II molecules using their CPs and the authors proposed that this might result in a reduced capacity for infected cells to process and present parasite proteins (Leao *et al.*, 1995). *L. major* has also been implicated in interfering with the intracellular loading of MHC class II molecules with antigenic peptides, although CPs are not as abundant in this parasite (Fruth *et al.*, 1993).

AIMS OF PROJECT

Cysteine proteinases are implicated in playing important roles in a number of host-parasite interactions, and they are the focus of much research in the quest to find and develop novel anti-parasite agents.

The main evidence for CPs as potential drug targets is two-fold. Firstly, CP inhibitors have been shown to have anti-parasite activity *in vitro*, and in some cases, *in vivo*. Secondly, some parasitic CPs have unusual structures which distinguish them from mammalian enzymes and thus could be exploited by drugs. My project stemmed from the fact that *L. mexicana* has stage-regulated CP activity, particularly abundant in the intracellular amastigote stage, suggesting these enzymes are crucial to parasite survival in the host macrophage and are therefore good targets for chemotherapeutic attack.

Although the multiple CP activities of *L. mexicana* had been well characterised, a specific role was yet to be found and so this was one of the overall aims of the project. With regards to this, there were two main questions addressed:

1. Are the CPs involved in differentiation? It seems likely that remodelling of a cell from one morphological form to another would require the breakdown of proteins.

2. Do the enzymes have a role in intracellular survival? The amastigote form of the parasite lives in the hostile environment of the phagolysosome, and it has been postulated that the CPs could be involved in inactivating the host cell lysosomal enzymes or in circumventing the host immune response.

The second overall aim of the project was to determine whether the CPs of *L. mexicana* were in fact good drug targets. The approach adopted to investigate the importance of these enzymes to the parasite was primarily an investigation of the phenotypes of mutants deficient in specific CP genes. A complementary approach was the utilisation of novel CP inhibitors to characterise the parasite CPs and determine the roles they play at different stages in the life cycle.

CHAPTER TWO

STUDIES ON THE *LEISHMANIA MEXICANA* CATHEPSIN L-LIKE CYSTEINE PROTEINASE *LMCPA*

2.1. INTRODUCTION

2.1.1. The *lmcpa* gene product(s)

The first CP gene to be cloned from *L. mexicana* was *lmcpa*, which was isolated from a *L. mexicana* amastigote lambda zap cDNA library using as a heterologous probe a *T. brucei* gene fragment (Mottram *et al.*, 1992). The single copy, cathepsin L-like *lmcpa* sequence, designated Type II (see Chapter 1, Table 1), has features that distinguish it from mammalian cathepsins and also CPs encoded by genes that have been isolated from *T. brucei* and *T. cruzi* (Mottram *et al.*, 1989; Aslund *et al.*, 1991). The protein predicted from the gene sequence has the highly conserved residues found in all CPs, but the predicted *lmcpa* gene product contains a unique 3 amino acid insertion near the N-terminus of the central domain and only a short C-terminal extension.

There is differential expression of the protein in the life cycle, due to regulation of the *lmcpa* gene at both RNA and protein levels (Mottram *et al.*, 1992). Antibodies raised against fusion proteins expressed from *lmcpa* gene fragments covering the pro- and central domains detected the protein in cell extracts prepared from different life-cycle stages of the parasite. This antiserum did not cross-react with the central domain of all CPs as it did not recognise the 3 major classes of LmCPb in amastigotes (types A, B, and C). The mature region antiserum recognised major species of 24 kDa and 27 kDa as well as the 38 kDa precursor protein. In stationary phase promastigotes the 24 kDa protein predominated as detected by Western blotting, whereas in amastigotes there was about equal amounts of the 24 kDa and 27 kDa proteins. It is thought that expression of *L. mexicana lmcpa* may be controlled through a combination of mechanisms at the mRNA and protein levels.

Although *lmcpa* has been identified only through molecular means, with an activity for the LmCPa proteinase not yet detected, the antiserum does recognise a

distinct protein separated during the purification protocols designed for the major amastigote Type I CPs (Robertson and Coombs, 1990). Fractions containing the protein recognised by the anti-central domain antiserum have what has been termed type D activity, that is, no activity towards gelatin but hydrolysing the peptide substrate Bz-Phe-Val-Arg-AMC. However, this type D activity is now thought to be distinct from the *lmcpa* gene product. Although the *lmcpa* gene product does not possess gelatinase activity, it does bind active-site-directed inhibitors (Mottram *et al.*, 1992).

2.1.2. Aims

The development of vectors that allow gene transfer into protozoa has enabled investigators to analyse proteinase function by transformation with a proteinase gene, or knockout of a proteinase gene (Cruz *et al.*, 1991). To investigate the role of the LmCPa proteinase in *L. mexicana,* Souza *et al.* (1994) used gene targeting of promastigotes with hygromycin- and phleomycin- resistance markers to generate null mutants, by disrupting sequentially both alleles of *lmcpa*. Preliminary observations on the morphology, differentiation and infectivity of the null mutants for *lmcpa* (Souza *et al.*, 1994) concluded that they did not differ significantly from wild type cells, although these conclusions were based only on qualitative data. The initial aims of this part of my work were to compare in more detail the *lmcpa*-null mutant cells with wild type cells, in order to define the phenotype of the mutants with respect to their growth, differentiation and CPs. The *lmcpa* gene product (LmCPa) was to be further analysed to find out the relationship of the 24 kDa and 27 kDa proteins that appeared to be encoded by the gene.

2.2. MATERIALS AND METHODS

2.2.1. Parasites

2.2.1.1. Cell lines of L. mexicana

The mutant line studied in the work described in this chapter, which was created by colleagues in the Wellcome Unit of Molecular Parasitology (WUMP), is described in Table 2.0. Details of the transfection procedure used can be found elsewhere (Souza *et al.*, 1994). All parasites used in this study had been passaged through BALB/c mice.

Table 2.0. Cell lines of *Leishmania mexicana* used

cell line	gene deletion	antibiotic used for selection
wild type	/	/
Δ*lmcpan*	null mutant for *lmcpa*	hyg/phleo

Abbreviations: hyg, hygromycin; phleo, phleomycin.

2.2.1.2. Promastigotes

Infections of wild type *Leishmania mexicana* (MNYC/BZ/62/M379) were maintained in female BALB/c mice as previously described (Hart *et al.*, 1981), and promastigotes were obtained by transformation of harvested subcutaneous lesion amastigotes at 25°C (see section 2.2.1.4). Promastigotes were grown axenically *in vitro* at 25°C in HOMEM medium (Berens *et al.*, 1976), pH 7.5, supplemented with 10% (v/v) heat-inactivated foetal calf serum (Labtech International) and 25 μg/ml gentamycin (Sigma). Routinely, 10 ml volumes were cultured in 25 cm^3 tissue culture flasks using air as the gas phase. Where appropriate, other antibiotics were included for selection of transfectants: hygromycin B (Sigma) at 50 μg/ml and phleomycin (Sigma) at 10 μg/ml (see Table 2.0). Cultures were subpassaged by subinoculation of promastigotes from log phase or early stationary phase cultures into fresh growth medium to a final density of 5 x 10^5 cells/ml. On occasions, stabilates that had been stored in liquid nitrogen were used to initiate cultures. All culture work was done

aseptically and all subsequent manipulations involving live parasites were performed under sterile conditions. Cell counting was performed using Improved Neubauer haemocytometers under phase contrast microscopy using 0.1 ml culture samples that had been immobilised by mixing with 0.1 ml 4% (v/v) formaldehyde in 100 mM phosphate-buffered saline, pH 7.4 (PBS).

2.2.1.3. Axenic amastigotes

Axenic amastigote cultures were set up at a starting density of 1 x 10^6 cells/ml from stationary phase promastigote cultures and were subpassaged every 7 days to the same starting density. Growth medium consisted of Schneider's Drosophila Medium (SDM, GIBCO), supplemented with 20% (v/v) heat-inactivated foetal calf serum (Labtech) and 25 µg/ml gentamycin, as previously described (Bates *et al.*, 1992, Bates, 1994). The pH was adjusted to 5.5 using 1M HCl, and generally 10 ml volumes were cultured at 32°C in sealed 25 cm^3 tissue culture flasks, with air as the initial gas phase. Axenic amastigotes did not need to be immobilised for counting but tended to grow in clumps, which were disrupted by passing them 3 times through a 26G needle.

2.2.1.4. Harvesting cells

Promastigotes: Log phase cultures were harvested 2 or 3 days after initiation of the cultures when the cell density was no greater than 5 x 10^6 cells/ml. Stationary phase cells were usually harvested after 7-8 days growth, depending on when the individual culture's cell density started to decline (at which point the cell density was usually 1-2 x 10^7 cells/ml). Only promastigotes that had been subpassaged less than 10 times were used in this study. Cell cultures ranging from 10 ml to 50 ml were dispensed into centrifuge tubes and centrifuged at 2000g for 10 min at 4°C. Cells were then washed by sedimentation and resuspension 3 times in 0.25 M sucrose solution, which involved discarding the supernatant and resuspending the cell pellet gradually in 10-50 ml of fresh sucrose solution using a vortex, as described previously (Mallinson and Coombs, 1986). After the final centrifugation, the pellet was resuspended in 1 ml of sucrose solution, placed in an eppendorf tube and a 10 µl sample removed in order to perform a

cell count and calculate the number of cells per pellet. The 1 ml cell suspension was then centrifuged in a microfuge at 2000g for 10 min, and the supernatant from the resulting pellet discarded. Harvested cell pellets were stored frozen at -70°C, with there being no detectable loss of proteinase activity (determined using gelatin-SDS-PAGE) over a period of months.

Axenic amastigotes: Harvesting of axenic amastigotes was carried out normally after 6-7 days growth, using the method described above for harvesting promastigotes.

Lesion amastigotes: Mice with medium-sized lesions (10-15 mm diameter) were selected as a source of amastigotes (unruptured lesions were used in order to minimise the danger of contamination with micro-organisms) and were killed by terminal anaesthesia; they were then rinsed with 70% ethanol to sterilise the skin. The lesions were excised aseptically and gently homogenised in 10 ml complete growth medium (see sections 2.2.1.2 and 2.2.1.3) using a sterile 10 ml syringe plunger to squash the tissue repeatedly through an '80 mesh' wire gauze (Sigma) in a petri dish. The resulting crude homogenate was collected with a 21G needle and syringe, then passed through a 26G needle to disrupt clumps of parasites and a cell count performed. If needed, cultures were started by inoculation of a sample of this amastigote preparation into 10 ml complete growth medium. The homogenate was then centrifuged at 1000g for 5 min at ambient temperature to sediment any host cells. The resulting pellet was discarded and the supernatant containing the amastigotes was retained and further centrifuged at 2000g for 10 min. The subsequent pellet was resuspended and centrifuged a further 3 times in 50 ml of either 0.25 M sucrose solution (if the cells were to be stored as frozen pellets) or serum-free growth medium (if the cells were for infectivity studies, section 2.2.5).

2.2.1.5. Light microscopy

Measurement of cells was carried out essentially as described previously (Bates and Tetley, 1993). Promastigotes were removed from culture and used to prepare smears which were air-dried, fixed in absolute methanol for 30 sec and stained for 15 min with 10% Giemsa's stain in 10 mM phosphate buffer, pH 7.2. The slides were examined at

1000x magnification and measurements of promastigotes were made using an eyepiece graticule, which had been calibrated using a test slide (each graticule unit was equivalent to 1.25 μm). Cell body lengths were measured to the nearest graticule unit and promastigotes with a bent body were excluded. At least 100 cells were measured in each case, and the median cell length then calculated. To determine the statistical significance of any observed differences in cell lengths between different lines, the z-test for comparing the means of large samples was used - for which data do not have to be normally distributed provided that samples are quite large (at least 25 in each).

2.2.2. Proteinase assays and analyses

2.2.2.1. Gelatin-SDS-PAGE

Gelatin-SDS-PAGE analyses were carried out essentially as described by Lockwood *et al.* (1987) and Robertson and Coombs (1990). *L. mexicana* lysates were usually prepared by thawing frozen parasite pellets and resuspending to the equivalent of 1 x 10^9 cells/ml in 0.25 M sucrose/0.1% Triton X-100 solution. Lysates were either used immediately or stored at -20°C, which had no detectable effect on the proteinase band patterns observed. Samples normally consisted of a 1:1 dilution of cell lysates with 2x reducing sodium dodecyl sulphate (SDS) sample buffer (0.1 M Tris-HCl, pH 6.8, 3% (w/v) SDS, 7% (v/v) 2-mercaptoethanol, 14% (v/v) glycerol) and subjected to polyacrylamide gel electrophoresis (PAGE) using the SDS-discontinuous buffer system as described by Hames (1981). Generally 20 μl samples were loaded, corresponding to approximately 10^7 cells per track. Gelatin was co-polymerised into the separating gels to act as a proteinase substrate, with a final concentration of 0.2% (w/v). The acrylamide concentration of the separating gel (0.75 mm thickness) was normally 12% (w/v), and electrophoresis was carried out for approximately one hour at a constant voltage of 150V. All SDS-PAGE was carried out using the Bio-Rad Mini Protean electrophoresis apparatus.

After electrophoresis the gels were washed free of SDS by immersion in 2.5% (v/v) Triton X-100 for 30 min at 37°C, in order to renature the proteinases. Gelatin hydrolysis was performed at 37°C in 0.1 M sodium acetate buffer, pH 5.5, containing 1

mM dithiothreitol (DTT) for between 2 and 24 h, depending on the number and sample of cells used, with amastigote samples generally requiring shorter incubation times than promastigote samples. Thereafter, Coomassie Blue stain was used to visualise the bands of proteinase activity, which appeared as white areas of gelatin digestion within the blue-stained gel, after which gels were destained and stored in 10% (v/v) acetic acid.

2.2.2.2. Western blotting

L. mexicana extracts were prepared by lysing cells at the eqiuvalent of 5 x 10^8 cells/ml in lysing solution (see section 2.2.2.1) containing proteinase inhibitors [1 μM E-64 and 10 μM PMSF], leaving on ice for 5 min, adding equal volumes of 2x SDS sample buffer (see section 2.2.2.1) and boiling for 2 min. Normally 20 μl samples were loaded into each well of a 12% polyacrylamide gel, corresponding to 5 x 10^6 cells per track, followed by electrophoresis performed at 200V for about 40 min.

After electrophoresis, SDS-PAGE gels were blotted to 0.22 μm nitrocellulose (Amersham, ECL grade) in ice cold 20 mM Tris/150 mM glycine/20% (v/v) methanol for 60 min at 100V using a Bio-Rad mini-transblotter. Blots were blocked overnight at 4°C in 25 mM Tris/0.15 M NaCl/0.1 % Tween 20, pH 7.6 (TBS-Tween), containing 5% (w/v) non-fat dried milk, 10% (v/v) horse serum and 0.001% thimerisol. Using the same solution, detection of antigens was achieved using a 1:500 dilution of polyclonal antiserum to LmCPa CP (known as R24) for 2 h at 4°C (Mottram *et al.*, 1992) or 1:500 dilution of polyclonal antiserum to LmCPb group B or group C CP (Robertson and Coombs, 1994). Blots were washed for 3 x 20 min in TBS-Tween at room temperature followed by incubation in a 1:2000 dilution of horseradish peroxidase (HRP)-coupled secondary antibodies (goat anti-rabbit HRP conjugate from Scottish Antibody Production Unit) in blocker for one hour at room temperature. After a further 3 washes as above, the blots were developed using the Amersham Enhanced Chemiluminescence (ECL) system according to the manufacturer's instructions, which involved incubating the blots in a 1:1 mixture of ECL reagents for 1 min then exposing them to blue-light sensitive autoradiography film (Fuji) in a cassette for between 10 sec and 5 min. The X-ray film was then developed in trays using Kodak photographic developer and fixer, and

hung up to dry.

2.2.3. Protein estimations

Stationary phase promastigotes of wild type parasites and Δ*lmcpan* were lysed at 5 x 10^8 cells/ml in lysing solution containing proteinase inhibitors (as in section 2.2.2.2). Protein concentrations of lysates diluted 1:10 in standard buffer (see below) were determined using the Pierce BCA protein assay kit (microtitre plate protocol), according to the manufacturer's instructions, with 6 standards (1200, 1000, 800, 600, 400, 200 μg/ml) of bovine serum albumin (Sigma) made up in 100 mM sodium phosphate, pH 7.5. All determinations were carried out in triplicate.

2.2.4. Infection of peritoneal exudate cells

The methods used were modified from those reported previously (Hunter and Coombs, 1991; Mallinson and Coombs, 1989). The medium used for all of these studies was RPMI 1640 (Labtech), pH 7.2, supplemented with 10 % (v/v) heat-inactivated FCS (Seralab), 25 μg/ml gentamycin (Sigma) and 2 mM L-glutamine (Gibco). Resident peritoneal exudate cells (PECs) were obtained by peritoneal lavage using 5 ml of ice-cold complete RPMI per female BALB/c mouse. A typical yield consisted of 2 ml at 2 x 10^6 cells/ml per mouse, although younger mice gave lower yields and vice versa. The PECs of mice> 4 months old appeared to be somewhat resistant to *L. mexicana* infection, hence mice aged 2 - 4 months were generally used in this study to obtain PECs and for *in vivo* infectivity studies (see section 2.2.5). The cellular exudates from the mice were pooled and 2 x 10^5 PECs were plated into each chamber of either 4 or 8 chamber Lab-tek tissue culture chamber slides (NUNC). The cells were allowed to adhere overnight at 32°C with a gas phase of 95% air/5% CO_2, whereupon non-adherent cells were removed by washing. The adherent cells remaining were used in the experiments and were assumed to be macrophages, with the number of macrophages present in each chamber taken to be 2 x 10^5. Parasites were resuspended in complete RPMI medium and 2 x 10^5 cells were added to individual chambers such that the apparent ratio of parasites to macrophages was routinely 1:1. After a 4 h incubation, any

free parasites were removed by repeated washing with complete RPMI medium.

Cultures were incubated for seven days in the above medium at 32°C and under 95% air/5% CO_2, after which they were washed in RPMI medium, wet-fixed in methanol for 2 min and stained with 10% (v/v) Giemsa's stain for 15 min. For all preparations, not less than 200 macrophages were examined under 1000x bright field microscopy to determine the % of macrophages that were infected and the number of intracellular parasites per macrophage. T-tests were used to determine the statistical significance of any observed differences in % infectivity between the different lines.

2.2.5. Infection of BALB/c mice

Stationary phase cultures of promastigotes were centrifuged at 2000g for 10 min and resuspended at 2.5 x 10^7 cells/ml in serum-free HOMEM. Each mouse was inoculated subcutaneously in the shaven rump with 0.2 ml of the cell suspension, corresponding to 5 x 10^6 cells. Where infections were initiated using lesion amastigotes, amastigotes were harvested as in section 2.2.1.4. and re-suspended as above but to 2.5 x 10^6 cells/ml - such that each mouse was inoculated with 5 x 10^5 amastigotes.

Mice were monitored weekly for the appearance of lesions, and the length, breadth and depth of each lesion that appeared was measured (to the nearest mm) weekly, using a Mitutoyo micrometer, for up to 9 months. At this point, experiments were terminated in accordance with Home Office regulations. Infectivity was calculated as the mean lesion volume in mm^3, using the following formula for a half spheroid: 0.5236 x [length x breadth x height]. Mean values were calculated including all animals in a group, treating those without lesions as possessing a lesion volume of 0 mm^3.

2.2.6. Autohydrolysis of parasite lysates

The following conditions were selected to optimise the autoproteolysis of parasite proteins by endogenous amastigote CPs, resulting in a partial purification of stable proteinases, and was based on a method from a previously published protocol (Ilg *et al.*, 1993). Wild type amastigotes were lysed at 2 x 10^9 cells/ml in lysing solution (section 2.2.2.1), diluted to 5 x 10^8 cells/ml in 0.1 M sodium acetate buffer, pH 5.5,

containing 20 mM DTT, and incubated at 37°C in eppendorf tubes. After time points of 0, 24 and 48 h, samples were removed and added to an equal volume of 2x SDS sample buffer. They were then frozen at -20°C until required for analysis by Western blotting.

2.2.7. Phase partitioning with Triton X-114 solution

The procedure adopted was modified from that published by Bordier (1981) and is based on the physical characteristics displayed by a diluted solution of the nonionic detergent Triton X-114 at different temperatures. The detergent is homogenous at 0°C but separates into an aqueous phase and detergent phase above the 'cloud point' of 20°C, thus enabling the separation of integral membrane proteins (amphiphilic) from hydrophilic proteins. A 4% (v/v) Triton X-114 solution was made up on ice, in PBS (section 2.2.1.2), and mixed with an equal volume (100 µl) of amastigote lysate, resulting from suspension in distilled water at 10^9 cells/ml. The sample was incubated on ice for one h, then centrifuged in a 10 ml conical tube at 4000g for one hour at 4°C to remove insoluble material. The soluble fraction was overlaid on a 300 µl cushion in an eppendorf tube, consisting of 6% (v/v) sucrose in PBS. The tube was warmed to 30°C for 10 min then centrifuged at room temperature for 10 min at 100g. After centrifugation the detergent phase was found as an oily droplet at the bottom of the tube; 200 µl of the upper aqueous phase was carefully removed, although the sucrose cushion was now difficult to distinguish from the aqueous phase, and added to 1 µl of Triton X-114 to give a final concentration 0.5% (v/v) Triton X-114. After dissolution of the surfactant at 0°C for 10 min, the sample was again overlaid on the sucrose cushion/detergent phase in the eppendorf used previously, warmed to 30°C for 10 min as before, and again subsequently microfuged at room temperature for 10 min at 100g. The aqueous phase (approximately 200 µl) was removed into a separate tube containing 3 µl of Triton X-114, the sucrose cushion was discarded and the remaining detergent phase (oily droplet) was resuspended in PBS to give a final volume of 200 µl; these latter steps were taken to obtain equal volumes and approximately the same salt and surfactant values for of each the two phases. The samples were frozen at -20°C until required for Western blotting and gelatin-SDS-PAGE analyses (section 2.2.2).

2.3. RESULTS

2.3.1. Phenotype of mutants null for *lmcpa*

2.3.1.1. Growth and differentiation

Throughout the growth cycle *in vitro*, the Δ*lmcpan* promastigotes appeared to be bigger in size than the wild type (Fig. 2.1). The difference in size was particularly noticeable in stationary phase populations, where the wild type cell population contains many small, putative metacyclic forms (Fig. 2.1, A). It appeared that in stationary phase cultures of the null mutants, the small metacyclic forms were not produced (Fig. 2.1, B). Cell measurements confirmed that the null mutants were significantly bigger than wild type parasites; the median cell lengths (since data were skewed) were 10 and 17.5 μm, respectively (Fig. 2.2). On Giemsa-stained smears, the *lmcpa* nulls also appeared to contain large vacuoles and granules not seen in the wild type parasites. A protein estimation comparing equal cell numbers of the two cell lines showed Δ*lmcpa* stationary phase promastigotes to have 2.7 times as much protein as the wild type promastigotes (Table 2.2). Despite these differences, there was no observable difference between the growth rates of wild type and Δ*lmcpan* promastigotes (Fig. 2.3).

As with wild type stationary phase promastigotes, it was possible to transform stationary phase Δ*lmcpan* promastigotes to amastigote-like forms *in vitro*. These forms looked very similar to wild type axenic amastigotes, although perhaps being slightly less homogenous (Fig. 2.4). They reached approximately the same cell densities as wild type axenic amastigotes, around 2-3 x 10^7 cells/ml after 7 days (Fig. 2.5), and also could be successfully cultured for at least 3 subpassages, as could wild type parasites.

2.3.1.2. Proteinases

Null mutant and wild type promastigotes were cultured and harvested on reaching stationary phase, and their proteinases analysed by gelatin-SDS-PAGE (Fig. 2.6). High mobility proteinases in the range of 21-28 kDa were present in the null promastigote lysates (lane 2), but at a much lower activity than those of the wild type (lane 1). Low mobility proteinase activity (>60 kDa) was similar in the two lines. The null mutant axenic amastigote proteinase profile (lane 3) was very similar to that of wild

type axenic amastigotes (lane 4), both in banding pattern and intensity.

In agreement with the gelatin gel analysis, Western blot analysis (Fig 2.7, A) revealed that the null stationary phase promastigotes contained much less *lmcpb* group C CPs (lane 2), around 30 kDa, compared with wild type stationary phase promastigotes (lane 1) or amastigotes (lane 3). The lower mobility bands were non-specific bands also detected with pre-immune serum; they are more pronounced in Δ*lmcpan* which may indicate that less autohydrolysis is occuring in this line (a further indication of less proteinase activity). A control Western blot of the three lines (Fig. 2.7, B) showed no detectable LmCPa in Δ*lmcpan* (lane 2) in contrast to wild type stationary phase promastigotes (lane 1), which had the 24 kDa protein, and amastigotes (lane 3) which had the 24 kDa protein and the stage specific 27 kDa protein. Also in agreement with the gelatin gel analysis (Fig. 2.6), Western blot analysis (Fig. 2.8) of Δ*lmcpan* axenic amastigotes showed that this form of the null mutant (lane 3) had as much LmCPb group C proteinase as wild type axenic amastigotes (lane 2), whereas the null mutant promastigotes (lane 5) again had less proteinase than this stage of wild type parasites (lane 4).

2.3.1.3. *Infectivity*

Resident peritoneal macrophage cultures were set up in chamber slides and incubated 1:1 with stationary phase promastigotes of wild type parasites and Δ*lmcpan* for 4 h. The results (Table 2.2) showed that after 7 days both the percentage of infected PECs and the number of amastigotes per infected cell was very similar with the two lines.

In order to establish whether the lack of *lmcpa* had an effect on the infectivity of *L. mexicana* to animals, lesions resulting from an inoculation of either wild type or Δ *lmcpan* parasites were monitored weekly by measuring with a micrometer. Lesions initiated using wild type and Δ*lmcpan* stationary phase promastigotes were similar in that they first appeared, respectively, 9 and 10 weeks post-inoculation (Fig 2.9, A). Thereafter, however, there was a difference in mean lesion volume between the two lines, with the wild type lesions on average being some 3-4 times bigger than the Δ

lmcpan lesions throughout the duration of the experiment.

Since the Δ*lmcpan* promastigotes were able to produce lesions, this allowed the infectivity of Δ*lmcpan* lesion amastigotes to BALB/c mice to be investigated (Fig. 2.9, B). Again Δ*lmcpan* lesions started to appear only one week after those resulting from inoculation of wild type parasites. In contrast to the results found with Δ*lmcpan* stationary phase promastigotes (Fig. 2.9, A), however, the lesion amastigotes of this line produced lesions that increased in size at a rate very similar to those produced by the wild type line.

2.3.2. Relationship of the 24 kDa and 27 kDa proteins detected with anti-LmCPa antiserum

2.3.2.1. Autohydrolysis of wild type amastigote lysates

One working hypothesis with regards to the relationship of the two proteins was that the 27 kDa protein was a precursor form of the 24 kDa protein. To thus investigate whether the 27 kDa protein could be converted to the 24 kDa by autoproteolytic digestion, wild type amastigotes were lysed in 0.1 M sodium acetate buffer, pH 5.5, containing 20 mM DTT, and incubated at 37°C. Samples taken from various time points were analysed by immunoblotting using anti-LmCPa antiserum (Fig. 2.9). However, two distinct bands were still present after 48 hours, suggesting that no interconversion of the 27 and 24 kDa proteins had occurred.

2.3.2.2. Separation of LmCPa into aqueous and detergent phases

Another working hypothesis was that the heavier protein was a membrane-bound form of the 24 kDa protein, with a putative lipid anchor causing the increase in molecular weight to 27 kDa. Therefore, in order to establish if one of the LmCPa proteins was hydrophobic and the other hydrophilic, the partition of the proteins during phase separation in Triton X-114 solution was investigated. After separation samples were analysed by Western blotting and gelatin-SDS-PAGE. The doublet of 24 and 27 kDa proteins was detected (Fig. 2.11, A, lane 1) by Western blotting using anti-LmCPa antiserum. After phase separation in the detergent, the lower moblity 27 kDa band was

recovered in the aqueous phase (lane 2) while the higher mobilty 24 kDa band was found in the detergent phase (lane 3). A control Western blot (Fig 2.11, B) probed with anti-group B CP antibody, showed that the majority of the *lmcpb* group B CPs were recovered in the aqueous phase (lane 2, arrowed), a prediction based on previous studies (Robertson and Coombs, 1990). Higher molecular weight bands were due to non-specific binding found in pre-immune serum. A control gelatin gel (Fig 2.12) showed the membrane protein gp63 had been separated into the detergent phase (lane 3, arrowed) and confirmed that the major *lmcpb* group B CPs were recovered in the aqueous phase (lane 2, around 24 kDa).

2.3.2.3. Detection of the 27 kDa band in Δlmcpan *axenic amastigotes*

An immunoblot comparing samples (identical to those used in Fig. 2.8) probed with anti-LmCPa antiserum (Fig. 2.13) clearly showed the 24 and 27 kDa proteins in lesion amastigotes (lane 1), wild type axenic amastigotes (lane 2), and the 24 kDa protein in wild type promastigotes (lane 4); the protein was not present in null promastigotes (lane 5). However, this blot also showed the surprising result that the lower mobility 27 kDa band was present in *lmcpa* null axenic amastigotes (lane 3), strongly arguing that the 27 kDa protein is not encoded by the *lmcpa* gene.

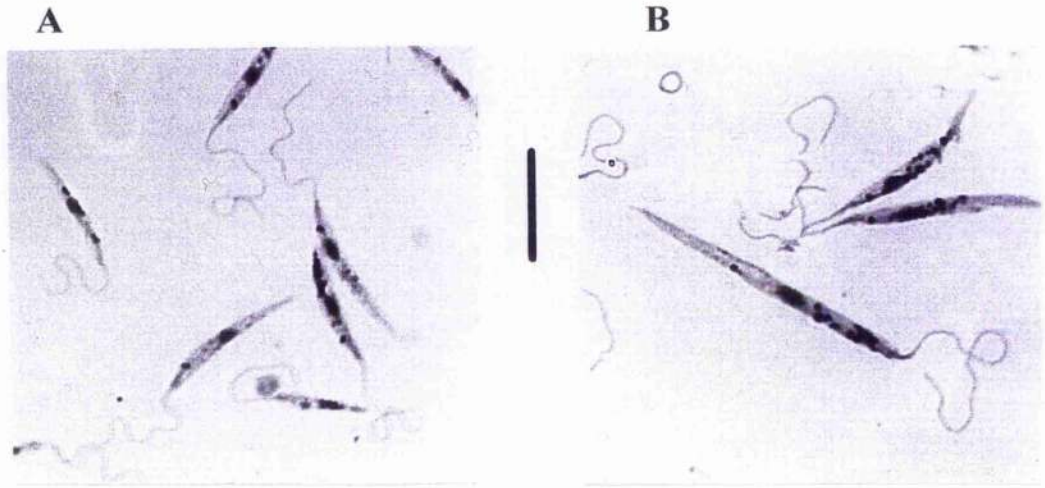


Figure 2.1. Light micrographs of stationary phase promastigotes. Wild type (A) and Δ*lmcpan* (B) cells were fixed in methanol and stained in 10% Giemsa's stain. There was a clear difference in the size of cells between the two lines, with the Δ*lmcpan* promastigotes (B) being much bigger then the majority of wild type cells (A). The scale bar represents 10 μm.

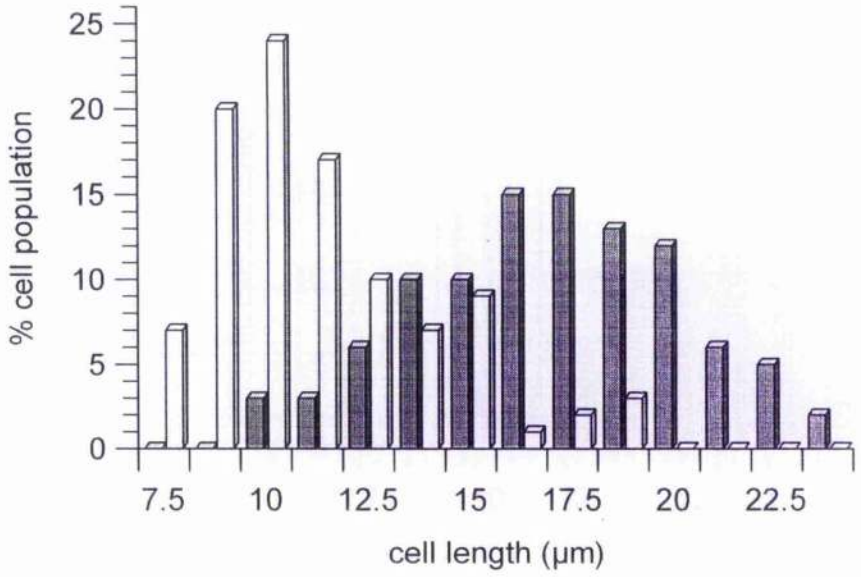


Figure 2.2. The cell lengths of stationary phase promastigotes. Giemsa-stained smears of stationary phase promastigotes (see Fig. 2.1) were used to compare the lengths of wild type parasites and Δ*lmcpan* (both subpassage 1). Measurements were made with a graticule under 1000x bright field microscopy. At least 100 cells were measured in each case, to the nearest graticule unit (1 graticule unit = 1.25 μm). The results indicate a highly significant difference between the lengths of wild type (open bars) and Δ*lmcpan* (closed bars) parasites ($z=13.88$, greatly exceeding the critical value of 2.58 at $P=0.01$).

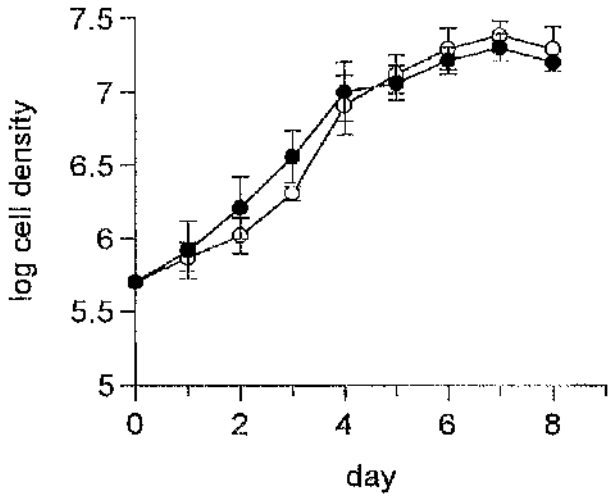


Figure 2.3. Typical *in vitro* growth curves of *L. mexicana* promastigotes. Cultures of wild type (-●-) and Δ*lmcpan* (-○-) promastigotes were initiated at 5 x 10^5 cells/ml in complete HOMEM medium and incubated at 25°C. Cell counts were performed on consecutive days. The results are the means ± SD from 3 independent cultures for each line.

cell line	protein content (mg/10^9 cells)
wild type	3.00 ± 0.08
Δ*lmcpan*	8.10 ± 0.70

Table 2.1. Protein content of stationary phase promastigotes. Data are the means ± SD of one determination of freshly lysed stationary phase promastigotes, performed in triplicate.

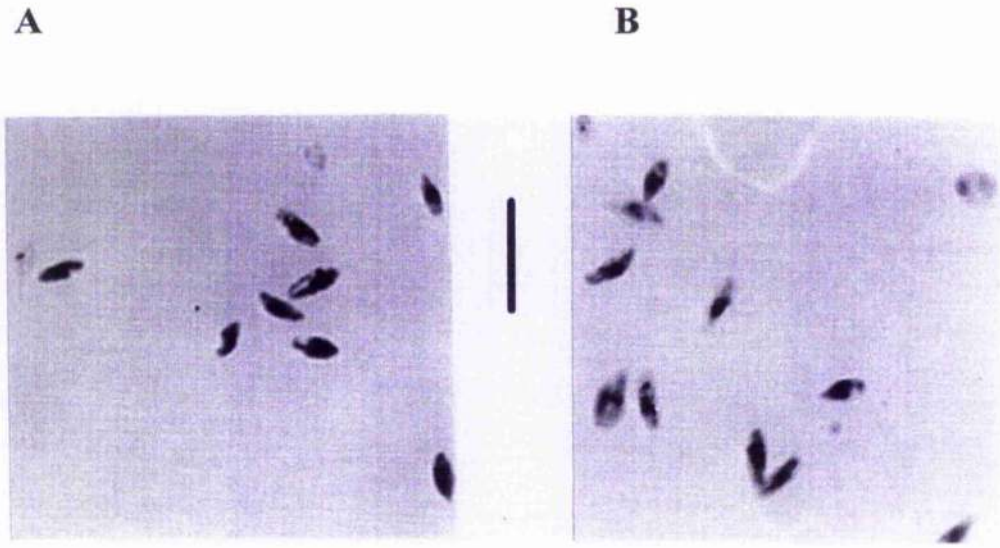


Figure 2.4. Light micrographs of Giemsa-stained axenic amastigotes; (A) wild type, (B) Δ*lmcpan*. There was no difference between the sizes of the cells of the 2 lines, with the Δ*lmcpan* axenic amastigotes (B) appearing morphologically very similar to wild type cells (A). The scale bar represents 10 μm.

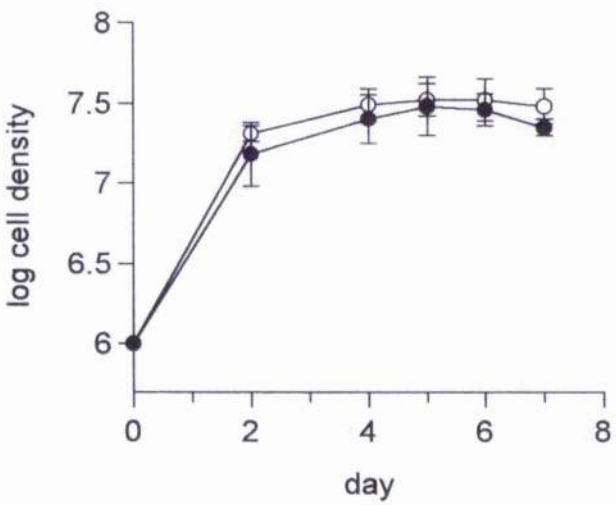


Figure 2.5. Typical *in vitro* growth curves of axenic amastigote lines. Cultures were routinely initiated using stationary phase promastigotes at 1 x 10^6 cells/ml in complete SDM, pH 5.5, and incubated at 32°. Cell counts revealed no difference between growth rates and final cell density of wild type (-●-) and Δ*lmcpan* (-○-) axenic amastigotes.

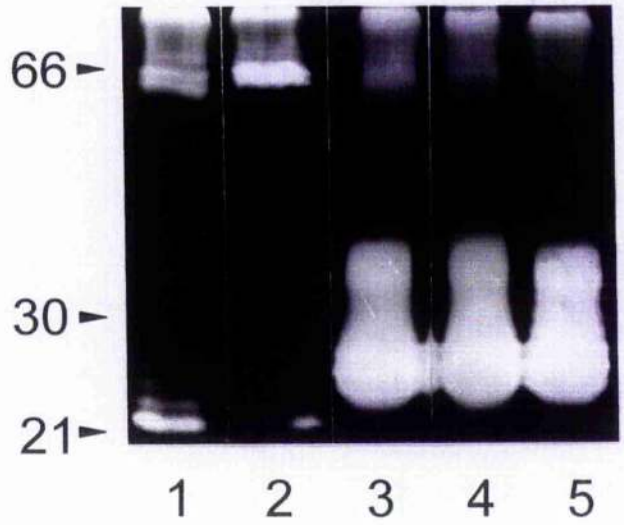


Figure 2.6. Gelatin-SDS-PAGE analysis of cell lines. Lane 1, wild type *L. mexicana* stationary phase promastigotes; lane 2, Δ*lmcpan* stationary phase promastigotes; lane 3, Δ*lmcpan* axenic amastigotes; lane 4, wild type axenic amastigotes; lane 5, wild type lesion amastigotes. Lanes were loaded with lysate containing 10^7 cells. The positions of molecular weight markers in kDa are indicated.

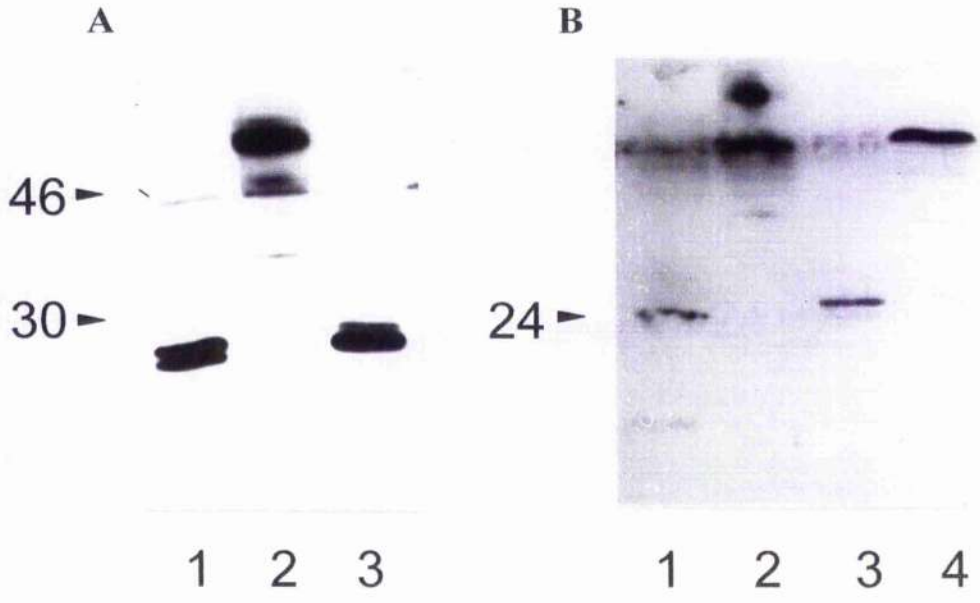


Figure 2.7. Western blots using anti-LmCPb antiserum (A) and anti-LmCPa antiserum (B). Lane 1, wild type stationary phase promastigotes; lane 2, Δ*lmcpan* stationary phase promastigotes; lane 3, wild type lesion amastigotes. Each lane was loaded with lysate from 5 x 10^6 cells. The bands >42 kDa were also produced with the pre-immune serum (lane 4).

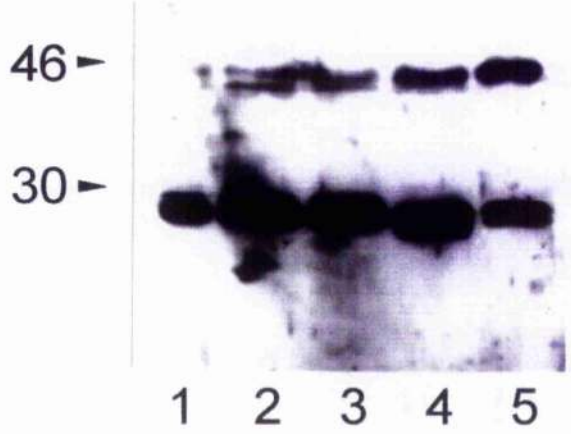


Figure 2.8. Western blot probed with anti-LmCPb antiserum. Lane 1, wild type lesion amastigotes; lane 2, wild type axenic amastigotes; lane 3, Δ*lmcpan* axenic amastigotes; lane 4, wild type stationary phase promastigotes; lane 5, Δ*lmcpan* stationary phase promastigotes. Each lane was loaded with lysate from 5 x 10^6 cells.

cell line	% infected PECs	amastigotes/infected PEC
wild type	36 ± 8.3	4.6 ± 1.3
Δ*lmcpan*	36 ± 10	4.4 ± 1.2

Table 2.2. Infectivity to macrophages. PECs were obtained from peritoneal lavage of BALB/c mice and infected with stationary phase promastigotes of wild type parasites and Δ*lmcpan* at a ratio of 1:1. After 7 days incubation at 32°C, cells were fixed, stained with Giemsa's stain, and parasite load determined by counting 200 PECs. The values presented are means ± SD from 4 independent experiments.

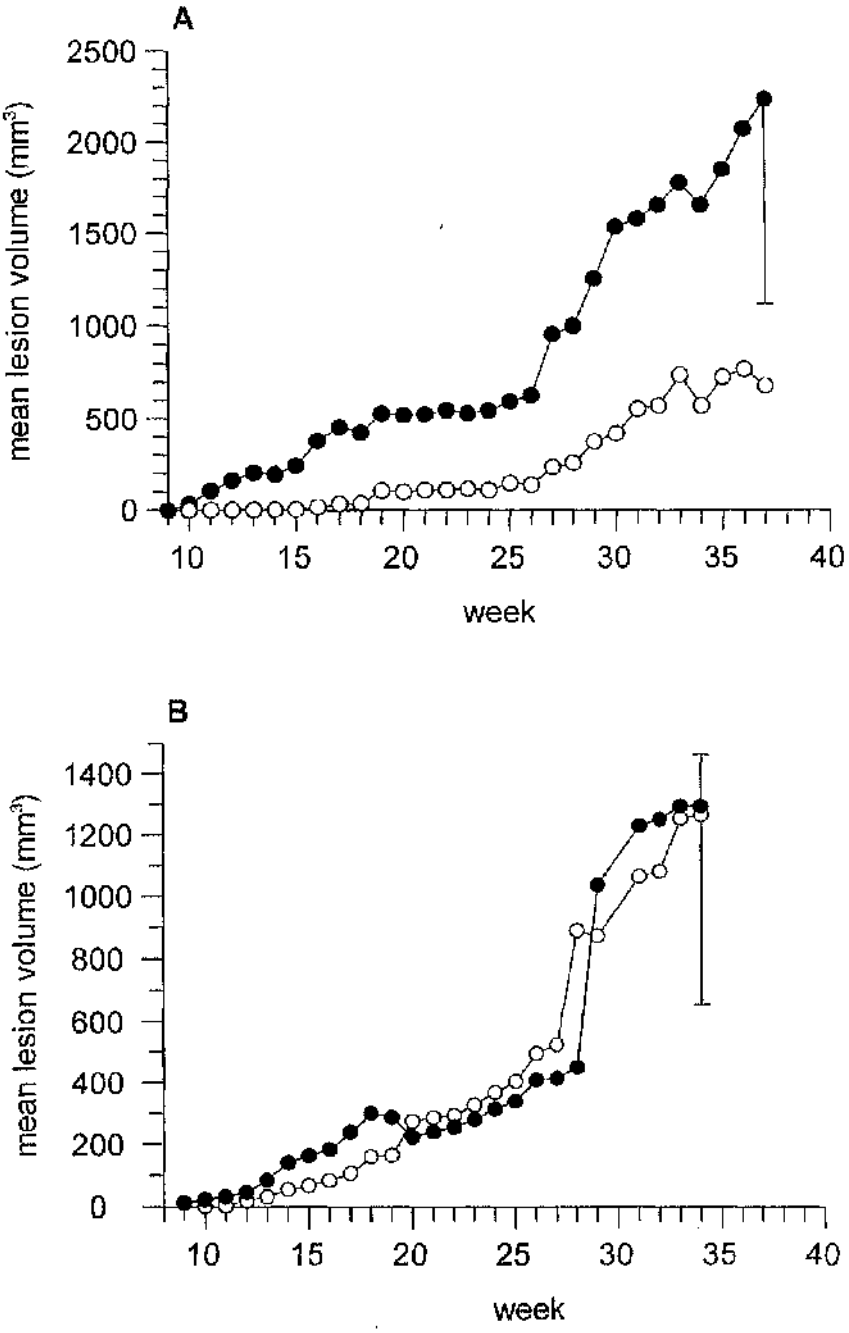


Figure 2.9. Infectivity to BALB/c mice. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5 x 10^6 promastigotes (A) or 5 x 10^5 lesion amastigotes (B) were measured at weekly intervals using a micrometer, and the mean lesion volume ± SD per group of 3 mice (A) or 5 mice (B) calculated. The lines inoculated were wild type (-●-) and *Δlmcpan* (-○-) parasites. The majority of error bars have been omitted for clarity, while some are so small they are obscured by the data points.

Figure 2.10. Autohydrolysis of wild type amastigote lysates. Samples were taken at time points and analysed by Western blotting, using a polyclonal antiserum to LmCPa. Lanes 1 , 3 and 5 are control autolysates at 0, 24 and 48 hours containing the proteinase inhibitors E-64 and PMSF. Lanes 2, 4 and 6 are autolysate samples at 0, 24 and 48 hours. Each lane was loaded with lysate from 5 x 10^6 cells.

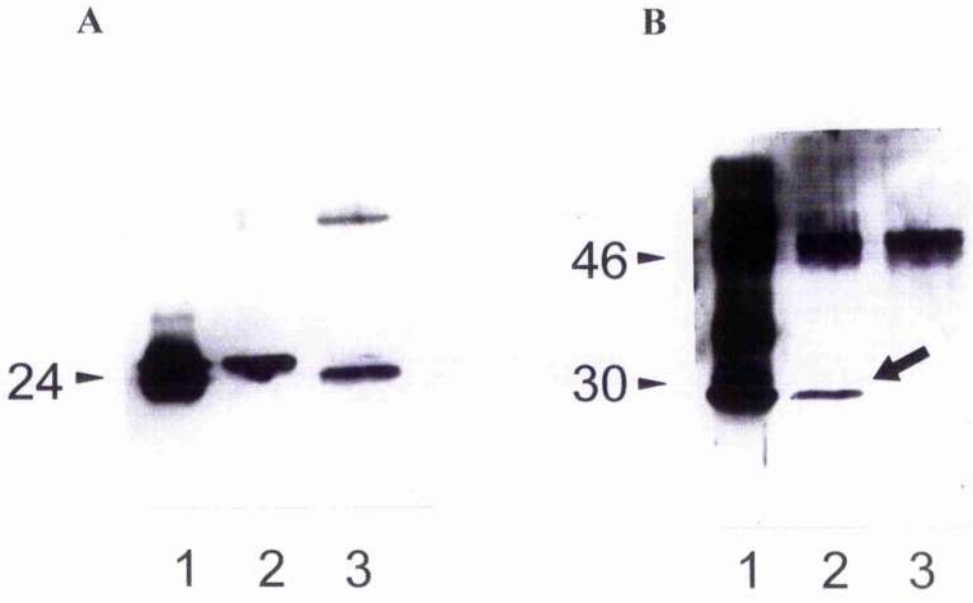


Figure 2.11. Western blot analyses of wild type *L. mexicana* amastigote lysate having undergone phase partitioning in Triton X-114 solution. Blots were probed with anti-LmCPa antibody (A) and anti-LmCPb antibody (B). Lane 1, original amastigote sample, lane 2, aqueous phase sample, lane 3, detergent phase sample. The positions of molecular weight markers are indicated in kDa. Lane 1 was loaded with lysate from 5 x 10^6 cells; lanes 2 and 3 with approximately 1.25 x 10^6 cells each.

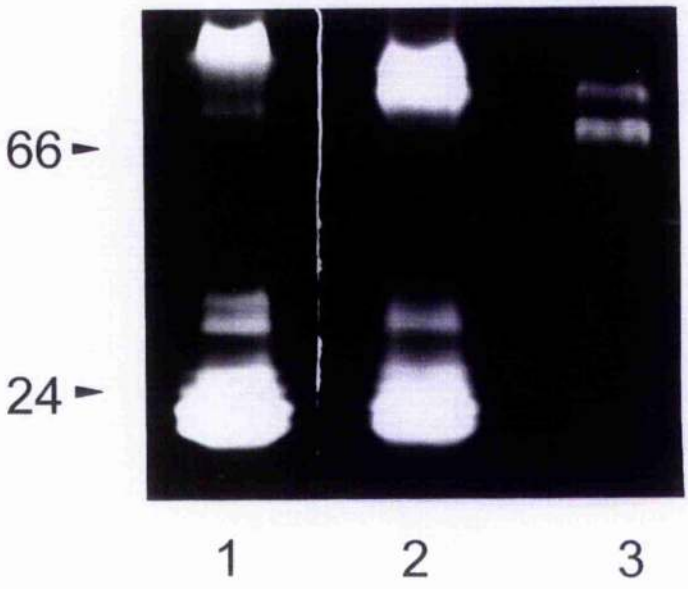


Figure 2.12. Gelatin gel analysis of wild type *L. mexicana* amastigote lysate having undergone phase partitioning in Triton X-114 solution. Lane 1, original amastigote sample, lane 2, aqueous phase sample, lane 3, detergent phase sample. The usual positions of molecular weight markers are indicated. Each lane contained lysate from approximately 1 x 10^7 cells.

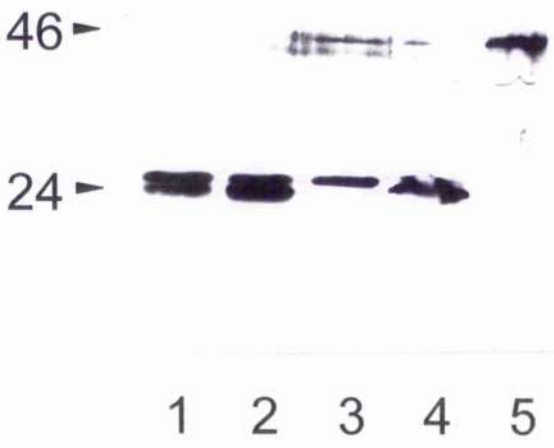


Figure 2.13. Western blot of axenic amastigote samples probed with anti-LmCPa antiserum. Lane 1, wild type lesion amastigotes; lane 2, wild type axenic amastigotes; lane 3, Δ*lmcpan* axenic amastigotes; lane 4, wild type stationary phase promastigotes; lane 5, Δ*lmcpan* stationary phase promastigotes. Each lane had an equal loading of 5 x 10^6 cells. The low mobility bands present in each track were also detected using pre-immune serum.

2.4. DISCUSSION

Stationary phase promastigote cultures of Δ*lmcpan* differed from those of wild type parasites in that they did not seem to contain the small putative metacyclic form (Fig. 2.1). Measurements of the cells (Fig. 2.2) confirmed that the Δ*lmcpan* stationary phase promastigotes were substantially bigger than those of wild type, although the *in vitro* promastigote growth rate was very similar to that of wild type parasites (Fig. 2.3). A protein estimation of wild type stationary phase promastigotes (Table 2.2) was similar to previous findings, although slightly lower at 3.0 mg/10^9 cells compared with 3.5 mg/10^9 cells for metacyclics (Bates, 1994), and verified that the null mutants (8.1 mg/10^9 cells) were bigger than wild type parasites. However it is not impossible that some autohydrolysis of parasite proteins occurred with the wild type samples, even though they were lysed in the presence of proteinase inhibitors, and that less autohydrolysis occurred with Δ*lmcpan* due to less overall CP activity (as is discussed later). This could mean that the real difference in protein content between the two lines is not quite as large as calculated. It should be mentioned that promastigotes of wild type parasites and Δ*lmcpan* were grown with and without the antibiotics hygromycin and phleomycin to establish whether or not these caused the observed difference in morphology of the nulls, but this did not seem to be the case. Although a morphological difference was observed between stationary phase promastigotes of wild type parasites and Δ*lmcpan*, the latter cells could transform to and grow as axenic amastigotes as well as wild type (Fig. 2.5) and were morphologically identical to wild type at this stage in the *in vitro* developmental cycle (Fig. 2.4).

Biochemical analyses of Δ*lmcpan* stationary phase promastigotes showed them to have less *lmcpb* protein and activity than wild type parasites (Figs. 2.6 and 2.7). This could indicate a role for *lmcpa* in the processing and/or activation of these enzymes, since pro-enzyme activation is likely to require an enzyme with very specific substrate specificity; such a high specificity could explain why the target substrate for the functional LmCPa enzyme has not yet been detected. With respect to CP activity and protein, however, the null mutant axenic amastigotes were very similar to wild type parasites (Figs. 2.6 and 2.8). Taken together with the data on morphology, the

observation that there was less proteinase activity in the null mutant stationary phase promastigotes suggests that the null mutants were not forming typical metacyclics. However, the suggested lack of formation of this stage *in vitro* is not consistent with their ability to transform to, and grow as, axenic amastigotes. Unfortunately, a definite conclusion regarding the metacyclogenesis of Δ*lmcpan* promastigotes was not reached from the data presented; the lack of a purification method for possible metacyclic forms of *L. mexicana* has resulted in the identification of several putative metacyclic markers, such as surface ultrastructure (Bates and Tetley, 1993, and see Chapter 3) which could be examined to resolve this question.

Nor does the apparent lack of metacyclic formation appear to affect the ability of the Δ*lmcpan* stationary phase promastigotes to infect and multiply within macrophages (Table 2.2). Δ*lmcpan* stationary phase promastigotes had previously been shown to infect the J774 macrophage-like cell line (Souza *et al.*, 1994) but this had not been done quantitatively; clearly though the lack of *lmcpa* does not have an effect on infectivity or intracellular growth of the parasite.

In vivo experiments showed that Δ*lmcpan* promastigotes produced lesions as quickly as wild type, but that these lesions did not increase in size at the same rate as those produced by wild type parasites, such that by the end of the experiment they were about four fold smaller than those of the wild type parasites (Fig. 2.9, A). This slow multiplication *in vivo* could perhaps indicate a role for *lmcpca* in *in vivo* parasite nutrition. However, lesion amastigotes of the null mutants were as infective to mice as the wild type parasites (Fig. 2.9, B), perhaps suggesting that the *lmcpa* gene is more important in the promastigote stage of the parasite. The fact that overall the lesion sizes were smaller when the mice were infected with lesion amastigotes (Fig. 2.9, B) than with promastigotes (Fig. 2.9, A) may be explained by the fact that ten fold fewer lesion amastigotes were used to initiate infections in mice that were older, and therefore more resistant to infection (see section 2.2.4)

Autohydrolysis of wild type amastigote samples (which contain about equal amounts of the 24 kDa and 27kDa proteins) did not result in any interconversion of one *lmcpa* product to the other (Fig. 2.10), casting some doubt on the theory that the proteins

were different processing products of the same 38 kDa precursor protein (Mottram *et al.*, 1992), with one a further precursor form of the other. Another hypothesis aimed at explaining the relationship between the 24 kDa and 27 kDa proteins that appeared to be encoded by *lmcpa* was that the heavier protein was a glycosylated or membrane-bound form of the 24 kDa protein, causing the increase in molecular weight of the protein from 24 kDa to 27 kDa. It had been hoped to investigate this using deglycosylation techniques and phospholipse C cleavage

Triton X-114 phase separation, in which hydrophilic proteins are found exclusively in the aqueous phase, and hydrophobic proteins (such as integral membrane proteins with a lipid anchor) are recovered in the detergent phase, successfully separated the two LmCPa proteins according to their hydrophobicity (Figs. 2.11 and 2.12). However the 24 kDa protein separated into the detergent phase, and the 27 kDa into the aqueous. This was a surprising result, since my working hypothesis was that the heavier 27 kDa protein may have a lipid anchor. Western blot analysis of different stages of Δ *lmcpan* revealed that the 27 kDa band detected by the antiserum was in fact present in Δ *lmcpan* axenic amastigotes. This offered an explanation as to why there was no interconversion of the 27 kDa band to the 24 kDa during autohydrolysis, and the rather puzzling result with the Triton X-114 separation in that the heavier 27 kDa protein had no lipid anchor. The 27 kDa protein is likely to be another stage-specific CP very similar to the 24 kDa one, since it reacts very strongly with the antiserum.

Previously it had been impossible to say if the 24 kDa and 27 kDa proteins were products of different genes or processing products of the same 38 kDa precursor (Mottram *et al.*, 1992), but the 24 kDa protein was implicated as the mature form of the 38 kDa precursor and the major product of the *lmcpa* gene as it had the size predicted for the mature protein from the gene sequence (Mottram *et al.*, 1992). Clearly from the results presented in this chapter, the 24 kDa protein is indeed the major product of *lmcpa* and the 27 kDa protein must be the product of another gene.

The ability of the nulls to transform to all the developmental stages *in vitro* and infect animals efficiently indicates *lmcpa* to be non-essential under the conditions tested and hence the biological phenotype of Δ*lmcpan* has been dubbed 'cryptic', as reported

previously (Souza *et al*., 1994). Since the specific antiserum was shown not to detect the highest levels of LmCPa in the amastigote stage (as was previously thought), it is possible that LmCPa may play a role in survival of the parasite within the insect vector, a criterion not examined in this investigation. Another explanation for the lack of a noticeable phenotype associated with the *lmcpa* null mutants could be due to the fact that *L. mexicana* also contains the biochemically distinct Type I and III CPs and the role of *lmcpa* may be assumed by these or other CPs in the null thus making the phenotype cryptic. Although the Type I CP gene *lmcpb* is more closely related to homologues in *T. brucei* and in *T. cruzi* than to *lmcpa*, it is a possibility that the highly active CPs encoded by the *lmcpb* array compensate for *lmcpa*'s inactivation, and hence LmCPa is probably not a suitable drug target.

CHAPTER THREE

AN INVESTIGATION INTO THE ROLES OF THE *LMCPB* CYSTEINE PROTEINASES OF *LEISHMANIA MEXICANA*

3.1. INTRODUCTION

3.1.1. The *lmcpb* genes and gene products

lmcpb, the gene of *L. mexicana* which encodes the Type I CPs (see Chapter 1, Table 1), has been cloned and sequenced (Souza *et al.*, 1992). The genomic arrangement of *lmcpb* was characterised and the results implied that *lmcpb* was a multi-copy gene of 2.8 kb unit size with at least 10 tandemly repeated copies per locus. In being multicopy, it is similar in arrangement to the Type 1 enzymes of *T. brucei* and *T. cruzi* (Mottram *et al.*, 1989; Pamer *et al.*, 1990; Campetella *et al.*, 1992; Eakin *et al.*, 1992). Subsequent studies (Appendix B: Mottram *et al.*, 1996, 1997) showed the *lmcpb* genes to be in a tandem array of 19 units, with there being no other copies in the genome.

The steady state levels of *lmcpb* RNA correlate very well with the activities of the types A, B and C CPs that occur in different forms of *L. mexicana* (Lockwood *et al.*, 1987; Robertson and Coombs, 1990, 1992) in that the gene transcript is found at much higher levels in amastigotes than in metacyclics, and not at all in log phase promastigotes (Souza *et al.*, 1992). This stage regulation suggests that the enzyme plays an important role in the intracellular survival of the parasite. A study by Robertson and Coombs (1994) was undertaken to provide more information on the 6 categories of *L. mexicana* CP activities (groups A, B, C, E, F and H) detected using gelatin-SDS-PAGE. A direct amino acid sequence analysis of purified CPs of types A, B, and C yielded highly similar N-terminal sequences for each, the consensus sequence matching that predicted for the N-terminus of the mature *lmcpb* gene product (Souza *et al.*, 1992). It was suggested that the observed differences among the *L. mexicana* CPs could be either due to variable post-translational processing of a single CP gene product or the individual isoenzymes being encoded by different genes of the tandem array. The groups B and C CPs had been found to show some different preferences for peptidyl fluorogenic substrates after SDS-PAGE; the three enzymes of Group B being more

active towards substrates with tyrosine in the P_1 position (such as SucLY-NHMec) whereas the two group C proteinases were as active towards compounds with a basic amino acid in this position (such as BzFVR-NHMec) (Robertson and Coombs, 1990). However, their protein sizes are the same, they react with the same anti-sera and have almost identical N-terminal amino acid sequences. It was proposed that limited amino acid substitutions in the different *lmcpb* products could explain the isoenzymes' different substrate preferences. Anti-sera raised separately against the group B and C CPs both recognised the groups A, B, and C CP proteins in Western blots, indicating that they have shared epitopes, and groups B and C CP proteins produced the greatest signal, in keeping with their high abundance. A protein of the same size was recognised in stationary phase promastigote lysates in which groups B and C CP activities have been found along with the metacyclic-specific group F CPs that have a faster mobility in gelatin-SDS-PAGE gels (Robertson and Coombs, 1992); this suggested group F CPs could also be *lmcpb* products (Robertson and Coombs, 1994). So too could the group E enzymes that differed from the other amastigote CPs in having lower mobilities in gelatin gels and apparently being membrane associated. Molecules of around 30 kDa were specifically recognised by the anti-C CP antiserum in Western blots of *L. donovani* and *L. major* lysates, suggesting that *lmcpb* homologues may be common to all leishmanias (Robertson and Coombs, 1994). Homologues have subsequently been identified in *L. pifanoi* (Traub-Cseko *et al.*, 1993) and in *L. major* (Sakanari *et al.*, 1997).

3.1.2. Aims

As introduced previously in Chapter 2, a novel way of determining the importance of parasite enzymes is by performing gene deletion experiments. Transfection systems have recently been developed which allow not only the disruption of chromosomal genes in *Leishmania,* but also the complementation of mutant parasites (Ryan *et al.*, 1993). Null mutants for the *lmcpb* gene array were created by colleagues in the Wellcome Unit of Molecular Parasitology (WUMP), University of Glasgow (Appendix B: Mottram *et al*, 1996). The procedure involved sequential replacement of

the two alleles of the array with antibiotic-resistance genes, and then selection by applying antibiotic pressure. Individual CP genes were then re-expressed in this null line via the episomal vector pTEX (Kelly, *et al.*, 1992).

The main aims of this part of my work were to establish the ways in which the *lmcpb* mutant lines differed from the wild type line (that is, to ascertain the biochemical and biological phenotype of the mutants), in order to gain insight into the function of the *lmcpb* CPs and their importance to the parasite. The features compared included the number and activity of proteinases present, growth and differentiation of the parasites *in vitro*, and their infectivity to macrophages and animals. It was hoped that by using this approach it would be possible to pinpoint the roles of *lmcpb* and the individual isoenzymes and hence determine the suitability of *lmcpb* as a useful drug target.

3.2. MATERIALS AND METHODS

3.2.1. Parasites

3.2.1.1. Transfected cell lines of L. mexicana

The lines studied in the work described in this chapter, which were created by colleagues in WUMP, are summarised in Table 3.0. Details of the transfection procedures used can be found elsewhere (Souza *et al.*, 1994) and in Appendix B (Mottram *et al.*, 1996, 1997). In this study, all cell lines used were parasites derived from a BALB/c mouse lesion subsequent to the transfection procedure, unless designated otherwise by the letter (t) following the cell line name.

Table 3.0. *Leishmania mexicana* transfectants used

line	gene deletion(s)	antibiotic used for selection
Δ*lmcpbBL*	single allele knockout for *lmcpb*	phleo
Δ*lmcpbn*	null mutant for *lmcpb*	hyg/phleo
Δ*lmcpbTEX*	null mutant for *lmcpb* transfected with pTEX vector	hyg/phleo
Δ*lmcpbg2.8*	Δ*lmcpbn* re-expressing a 2.8 kb *lmcpb* internal genomic fragment	neo
Δ*lmcpbc1*	Δ*lmcpbn* re-expressing *lmcpb* cDNA	neo
Δ*lmcpbtb*	Δ*lmcpbn* re-expressing a *T. brucei* CP cDNA	neo
Δ*lmcpan*	null mutant for *lmcpa*	hyg/phleo
Δ*lmcpbn*/Δ *lmcpan*	mutant null for both *lmcpa* and *lmcpb* ('double null')	hyg/phleo/sat/puro
Δ*lmcpbn*/Δ *lmcpag2.8*	double null re-expressing a 2.8 kb *lmcpb* genomic fragment	neo
Δ*lmcpbg1*	Δ*lmcpbn* re-expressing gene 1 of the *lmcpb* array	neo
Δ*lmcpbg18*	Δ*lmcpbn* re-expressing gene 18 of the *lmcpb* array	neo
Δ*lmcpbg19*	Δ*lmcpbn* re-expressing gene 19 of the *lmcpb* array	neo

Abbreviations: hyg, hygromycin; phleo, phleomycin; SAT, nourseothricin hydrosulphate; neo, neomycin; puro, puromycin.

3.2.1.2. Cell culture

The three major developmental stages of *L. mexicana* (MNYC/BZ/62/M379) were cultured and harvested as described in Chapter 2, section 2.2.1. Where appropriate other antibiotics were included for selection of transfectants: hygromycin B (Sigma) at 50 μg/ml, phleomycin (Sigma) at 10 μg/ml, nourseothricin hydrosulphate (a gift from P. H. Grafe, Hans-Knoll Institute, Thuringen, Germany) at 25 μg/ml, puromycin (Sigma) at 10 μg/ml or neomycin (Geneticin, BRL) at 25 μg/ml (initially) or 500 μg/ml (to promote episomal re-expression of gene product) were added, singly or in combination (see Table 3.0.). To standardise the cultures, all transfected promastigote lines (t) were routinely grown through one passage as axenic amastigotes (see Chapter 2, section 2.2.1.3) to 'reset' them, and then transformed back to promastigotes for experimental work. Approximate doubling times for promastigotes and axenic amastigotes were calculated from cell counts taken during exponential (log.) growth, using the equation: **[time elapsed ÷ (new cell count/old cell count)] x 2.**

3.2.2. Proteinase assays and analyses

3.2.2.1. Gelatin-SDS-PAGE

Substrate SDS-PAGE was carried out essentially as described in Chapter 2, section 2.2.2.1. Where macrophage samples were being analysed, resident peritoneal macrophages were harvested as before (Chapter 2, section 2.2.4.) and lysed at 5 x 10^7 cells/ml in lysing solution (section 2.2.2.1). Lysates were diluted 1:1 with 2x sample buffer (section 2.2.2.1.). Lanes were loaded with 20 μl samples, corresponding to 5 x 10^5 macrophages per track.

3.2.2.2. Western blotting

Western blotting was carried out as described in Chapter 2, section 2.2.2.2., using a 1:500 dilution of anti-LmCPb group C CP (Robertson and Coombs, 1994) as the primary antibody.

3.2.2.3. Fluorogenic substrate assay

This method was adapted from protocols published by North *et al.* (1990b), and Robertson and Coombs (1990). Preparation of samples and the electrophoresis itself were carried out as is standard for gelatin gels (Chapter 2, section 2.2.2.1) with the exception that 40 µl of sample was added per lane, corresponding to 2 x 10^7 cells. After washing gels in 2.5% (w/v) Triton X-100 for 30 min at 37°C, gels were incubated at the same temperature in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM DTT and 0.01 mM of fluorogenic peptide substrate. Fluorescent bands corresponding to proteinase activity were detected using a UV transilluminator, with maximal activity observed after about 20 min. Results were recorded immediately on photographic film, using a yellow filter and with exposure times ranging from 30 sec to 60 sec. Gels could be left to incubate for longer and then stained with Coomassie blue to observe gelatin hydrolysis, if required.

The peptidyl amidomethylcoumarin substrates used were N-benzoyl-phe-val-arg-7-(4-methyl)-coumarylamide (BzFVR-NHMec) and N-succinyl-leu-tyr-7-(4-methyl)-coumarylamide (SucLY-NHMec). Approximately 5 mM stock solutions were made in 50% (v/v) acetonitrile, some used fresh and the remainder stored at -20°C until further required.

3.2.2.4. Immunoelectron microscopy

CPs were localised using immunogold electron microscopy (performed by L. Tetley). Parasites were fixed in 2% (v/v) formaldehyde/0.1% glutaraldehyde, low temperature dehydrated in ethanol and embedded in LR White resin. 80 nm sections were incubated in anti-LmCPb antibody diluted 1:50 in 1 % (w/v) acetylated bovine serum albumin/PBS for 30 min and then for 60 min in goat anti-rabbit antibody/10 nm gold conjugate (Aurion) at 1 in 10 in bovine serum albumin/PBS. The sections were then stained in uranyl acetate and lead citrate.

3.2.3. Identification of *L. mexicana* metacyclic forms

3.2.3.1. Light microscopy

Measurement of cells was carried out as described in Chapter 2, section 2.2.1.5.

3.2.3.2. Complement lysis

Guinea pig serum (GPS, from Seralab) that had been stored at -70°C was used as a source of complement. The lytic assay used was essentially as described (Mallinson and Coombs, 1989b) with the exception that incubations were performed at 25°C (Bates *et al.*, 1993). Promastigotes were resuspended to 2 x 10^7 cells/ml in complete HOMEM medium and then mixed 1:1 with 60% (v/v) GPS in complete HOMEM such that the final concentrations in the assay were 1 x 10^7 cells/ml in 30% (v/v) GPS. After 30 min incubation, parasites were exposed to hypo-osmotic stress by diluting a sample 1:1 with double distilled, deionised water (dH_2O) in order to burst any partially-lysed cells and so enable a more accurate 'intact cell' count to be made (intact cells were observed to swell but resumed their initial shape within seconds). Promastigotes were only considered intact if they possessed the usual appearance of viable cells under phase contrast microscopy and did not show any strange shape or evidence of membrane damage or swelling. The percentage lysis was calculated by reference to control incubations using heat-inactivated serum. However, much clumping of cells was apparent in these controls and the clumps were disrupted as far as possible using a 26G needle before counting. T-tests were used to determine the statistical significance of any observed differences in percentage lysis between the different lines.

3.2.3.3. Transmission electron microscopy

Parasites were sedimented from culture medium by centrifugation at 1000g for 15 min at 25°C and fixed at that temperature with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, for 40 min. Subsequent processing procedures followed methods described previously (Tetley *et al.*, 1989) except that sections of 100 nm were used for optimal contrast enhancement by zero-loss imaging with a Zeiss 902 transmission electron microscope (performed by L.Tetley).

3.2.4. Nuclease analyses

3.2.4.1. Poly(A)-SDS-PAGE

This method was based on that published by Bates (1993). Cell pellets were lysed in 38 mM Tris/100 mM glycine, pH 8.5, 1% (w/v) SDS, 50 µg/ml leupeptin at the equivalent of 1 x 10^9 cells/ml. The lysates were then mixed with an equal volume of double strength non-reducing sample buffer (as in section 2.2.2.1, omitting the mercaptoethanol). All samples were incubated in a boiling waterbath for 2 min and then allowed to cool, prior to electrophoresis. SDS-PAGE with a 10% resolving gel and discontinuous buffer system was performed using standard methods (Section 2.2.2.1), but including poly(A) (5' poly-adenylic acid, Sigma) at 0.3 mg/ml, final concentration, in the resolving gel. After electrophoresis, gels were washed by gentle agitation for 30 min in 0.1% (v/v) Triton X-100/100 mM HEPES, pH 8.5 to enable renaturation of enzyme activity, then incubated at 37°C in the same buffer for a further 1-2 h. Each gel was then fixed in 7.5% (v/v) acetic acid for 10 min, washed in 50 ml dH_2O for 3 x 10 min, stained with 0.2% (w/v) Toluidine Blue in 10 mM HEPES, pH 8.5, for 15 min, and destained with dH_2O. Regions of enzyme activity were revealed by digestion of poly(A) and appeared as clear bands in a dark blue-staining gel.

3.2.5. Infection of peritoneal exudate cells

The methods used were as described earlier (Chapter 2, section 2.2.4) with the following additions. Where lesion amastigotes were used to initiate infections, PECs were incubated with the amastigotes for one hour at an apparent ratio of 1:1, after which free parasites were removed by repeated washing with complete RPMI medium.

In experiments where the parasite:macrophage ratio and exposure time were varied, parasites were diluted in RPMI medium to the appropriate cell density, added to the PECs and left to incubate for the required exposure time before washing off free promastigotes. Where time course experiments were undertaken, PECs were allowed to adhere overnight and then incubated in medium axenically for a further 2 days before initiation of infection; this then allowed intracellular parasites to be more easily observed at the early time points of 2, 4 and 8 h since the macrophages had spread

sufficiently during the 2 days prior to infection.

In certain cases parasitophorous vacuole size was measured using a calibrated eyepiece graticule, as detailed in Chapter 2, section 2.2.1.5.

3.2.6. Infection of BALB/c mice

The inoculation of parasites into BALB/c mice was carried out exactly as described in Chapter 2, section 2.2.5.

3.2.8. 'Api Zym' analyses

The 'Api Zym' enzyme screening kit was used according to the manufacturer's instructions. Resident peritoneal macrophages were lysed at 5 x 10^5 cells/ml in lysing solution (0.25 M sucrose, 0.1% Triton X-100) and passed through a 26G needle 3 times to break up the DNA. After adding water drops to the underlying tray of the kit to aid humidity, 95 µl of the cell lysate was added to each well (1-20) of the kit, along with a drop each of the reagents 'zym A' and 'zym B' supplied, and then incubated at 37°C for 24 h. After this time, the tray was removed from the incubator and held under a bright lamp for 30 sec to ensure the colour change was optimum before reading. Enzyme activity was observed as a change in colour, the stronger the colour the greater the enzyme activity. The colour change was classified in the range from 1 (lowest) - 5 (highest). Where infected macrophages were used, macrophages were harvested 7 days after initiation of the *in vitro* infection- when the infection rate was approximately 40%.

3.2.8. Exposure of promastigotes to hydrogen peroxide and nitric oxide

3.2.8.1. Pilot studies

Stock solutions consisting of 100 mM hydrogen peroxide (H_2O_2) (in complete HOMEM medium) and 100 mM sodium nitrite (in complete SDM, pH 5.5, since sodium nitrite is only a good source of nitric oxide (NO) at acidic pH) were made up and serial dilutions ranging from 100 mM to 0.00016 mM were made in 24 well plates using the appropriate media. Control wells contained complete medium only. Stationary phase promastigotes of wild type parasites and Δ*lmcpbn* were added to the 1 ml medium

samples in the 24 well plates such that the final cell concentration was 5 x 10^6 cells/ml. Cells were observed after 2, 4, 8 and 24 h for motility using an inverted microscope. Cell motility was designated as follows: (+), if very few cells were observed to be moving; (++), if approximately half the field of cells were moving; and (+++), if the majority of cells showed movement. After 24 h, 0.1 ml was passaged from each well into further plates containing medium only and left for 24 h, after which the cultures were again observed for motility.

3.2.8.2. Effect of nitric oxide

Stationary phase cells of wild type parasites and Δ*lmcpbn* were resuspended in 1 ml of SDM containing 10, 2, 1, 0.5 and 0.25 mM sodium nitrite (final concentration) at 5 x 10^6 cells/ml in 24 well plates. The cells were incubated for 24 h, after which they were examined under phase contrast microscopy for motility and scored as before (section 3.2.8.1). Parasite viability was measured, using the method based on the metabolism of a tetrazolium salt, MTT, by living cells (Alfieri *et al.*, 1989), as follows. Cells were resuspended in HOMEM medium containing 450 µg/ml MTT (Sigma) to the same starting density, and incubated for a further 24 h to assess the parasites' ability to reduce MTT. Subsequently 50 µl samples were diluted 1 in 6 with 0.04 M HCl-isopropanol to give 300 µl in a microtitre plate and the absorbance read at 540 nm using a multiskan mirotitre plate reader.

3.2.9. Effect of inhibition of nitric oxide synthase upon *L. mexicana* infection of peritoneal exudate cells

Peritoneal exudate cells were infected using stationary phase promastigotes of wild type parasites and Δ*lmcpbn* as in Chapter 2, section 2.2.4., with the exception that the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NRME) was included at 0.4 mM throughout the experiment (Proudfoot *et al.*, 1996). A stock solution of 10 mM L-NRME was made up in RPMI and used in these experiments. The D-isomer of this compound (D-NRME), which is not biologically active, was used as a control. The parasite load at 7 days was determined microscopically, as before (2.2.4).

3.3. RESULTS

3.3.1. The phenotype of mutants null for *lmcpb*

3.3.1.1. Biochemical analyses

Western blot analysis of promastigote cell lysates with an antiserum raised to purified LmCPb (group C) showed that whereas a major band of around 25 kDa (actually consisting of 2 distinct bands, which are apparent when shorter exposure times are used) was present in wild type *L. mexicana* (Fig 3.1, lane 1), there was no similar band in the null mutant, Δ*lmcpbn* (lane 2). A faint band in this position was observed with Δ*lmcpbc1* (lane 4), whereas a strong signal was detected with Δ*lmcpbg2.8* (lane 3). Samples from Δ*lmcpbc1* and Δ*lmcpbg2.8* also yielded a lower mobility band (38 kDa) not found with the wild type *L. mexicana*. These are likely to be unprocessed precursors. The other low mobility bands (>42 kDa) were non-specific as they also appeared in the control blot using pre-immune serum (lane 5).

Active LmCPb enzymes were detected in stationary phase promastigotes of wild type *L. mexicana* as several bands of around 24 kDa on gelatin gels (Fig. 3.2, lane 1) as reported previously (Robertson and Coombs, 1992). In contrast, Δ*lmcpbn* had no detectable CP activity (lane 2). The lower mobility bands with apparent molecular masses of >60kDa that were present in all the leishmanial samples are due to proteinases other than CPs, including the surface-located metalloproteinase gp63 (Bouvier *et al.*, 1989). The line Δ*lmcpbg2.8* (lane 3) had a single high mobility, high activity band, which on shorter exposures clearly had the same mobility as the second fastest moving of the multiple bands produced by extracts of wild type cells. It also contained several lower mobility, high activity bands with apparent molecular masses between 30 and 46 kDa; these activities are not normally seen with wild-type samples (except when much greater amounts of sample are used), suggesting that processing of the initial translation product of *lmcpb* is less efficient in this transfectant than in wild type cells and that precursor forms can be activated in the gel after electrophoresis. Line Δ*lmcpbc1* showed no CP activity toward gelatin (lane 4). *Lmcpb* nulls transfected with a *T. brucei* CP gene (Δ*lmcpbtb*) showed an activity band at around 30 kDa (lane 8), similar to the main CP activity found with *T. brucei* procyclic forms (lane 6).

Interestingly, it also had a band of low activity with an apparent molecular mass of about 50 kDa, an activity not apparent in the extract of wild type *T. brucei*. In summary, these results (shown in Figures 3.1 and 3.2) confirmed that the null mutants were indeed null for *lmcpb* and that genes re-expressed in the null mutants were enzymatically active.

3.3.1.2. Growth and differentiation of transfected lines

Transfected lines that had been passaged through mice and re-isolated were studied for growth and morphological characteristics. All the transfected lines grew readily as promastigotes *in vitro* and the growth rates were similar to that of wild type *L. mexicana* (see Fig. 3.3). However, it was observed that the small putative metacyclic form typically present in stationary phase cultures of wild type parasites was not evident with the transfectants (Fig. 3.4). There is still no good biochemical marker for identifying the metacyclic form in *L. mexicana,* hence wild type and the different transfectants were compared with respect to their complement sensitivities, cell lengths and surface ultrastructure.

Unfortunately, sensitivity to guinea pig complement proved not to be a good test for metacyclogenesis in *L. mexicana* (Table 3.1), since log. phase promastigotes of wild type parasites containing few putative metacyclics did not appear to be more susceptible to GPS than did stationary phase promastigotes containing many putative metacyclics. There was also a problem with clumping of cells in the controls containing heat-inactivated GPS, which were disrupted by passing through a 26G needle. However, the results obtained (Table 3.1) do suggest that the nulls were more susceptible to complement than were wild type promastigotes.

Measurement of the sizes of stationary phase promastigotes (subpassage 1) also showed a great difference between the cell lengths of wild type parasites and Δ*lmcpbn* (Fig. 3.5, A). The median (since data were skewed) lengths were 10 and 17.5 μm for wild type and Δ*lmcpbn*, respectively. Wild type cells were seen to increase slightly in length (to median 12.5 μm) as subpassage number increased (Fig. 3.5, B), whereas the null mutants stayed much the same (median length 16.3 μm). Transmission electron

microscopy revealed that the thickening of the surface coat, thought to be indicative of the metacyclic form in *L. major* (Pimenta *et al.*, 1989, 1991) and *L. mexicana* (Bates and Tetley, 1993), was clear on a high percentage of the stationary phase population of wild type parasites but could not be seen on any of the Δ*lmcpbn* sections examined (Fig. 3.6).

The transfected lines of Δ*lmcpbn* and Δ*lmcpbg2.8* could transform to axenic amastigotes, but grew much more slowly in this form compared to wild type parasites (Fig. 3.7). The approximate doubling times were 8.0 ± 1.5 h for wild type parasites and 38 ± 4.0 h for Δ*lmcpbn*. Axenic amastigotes of Δ*lmcpbBL* grew similarly to wild type parasites. The axenic amastigotes of Δ*lmcpbn* were bigger and more elongated than those of the wild type parasite (Fig. 3.8), the mean lengths being 3.8 ± 1.4 μm compared with 2.4 ± 0.5 μm of the wild type parasite. To provide further information on whether these cells were really amastigotes, their nucleases were compared using poly(A) substrate gels as described previously (Bates, 1993). The gel pictured in Figure 3.9 suggests that the axenic amastigotes of Δ*lmcpbn* (lane 4) have the 40 kDa nuclease specific to promastigotes (lane 1, wild type parasites; lane 2, Δ*lmcpbn*) as well as the 31 kDa nuclease specific to amastigotes (lane 3, wild type amastigotes), suggesting that they are an intermediate form. Transmission electron microscopy (Fig. 3.10, B) showed the null mutant axenic amastigotes to have fewer, less dense membrane bound lysosome-like vesicles, compared with the large dense megasomes typically found in wild type *L. mexicana* amastigotes (Fig. 3.10, A).

3.3.1.3. Infectivity

There was found to be a great difference in infectivity to peritoneal exudate cells (PECs) between stationary phase promastigotes of wild type *L. mexicana* and Δ*lmcpbn* (t) (Table 3.2, A). The null mutants infected some 5 times fewer PECs than did wild type parasites. Re-expression of *lmcpbg2.8* in the null mutants restored infectivity almost to wild type levels showing complementation of the phenotype, whereas Δ *lmcpbc1* (t) and Δ*lmcpbtb* (t) infected similar numbers of PECs as did the null mutants (Table 3.2, A). These data suggest that the survival of the null mutants in a small

percentage of PECs is due to the characteristics of the PEC subpopulation rather than the parasites themselves.

These experiments were repeated several times (Table 3.2, B and C) and included Δ*lmcpan* and Δ*lmcpbBL* as additional controls; these lines were found to be as infective as wild type parasites to PECs. The phenotype of the double null Δ*lmcpbn/*Δ *lmcpan* was indistinguishable from Δ*lmcpbn* with respect to growth and differentiation *in vitro*, including infection of PECs; however re-expression of *g2.8* in the double null did not restore infectivity levels (Table 3.2, B). The number of amastigotes within infected PECs was significantly different between wild type and Δ*lmcpbn* in two out of three experiments (Table 3.2, A and C).

The null mutant parasites produced subcutaneous lesions in BALB/c mice (see section 3.3.2.1); although the lesions resulting from inoculation of these parasites appeared considerably later than those due to the wild type parasites. This was also true for other transfected lines including Δ*lmcpbg2.8* (t). It was confirmed by Southern blotting, gelatin gel analysis and Western blotting that parasites obtained from the lesions in mice resulting from inoculation of Δ*lmcpbn* (t) indeed lacked the *lmcpb* gene, LmCPb protein (Fig. 3.11, A, lane 3) and the active LmCPb proteinase (Fig. 3.11, B, lane 4). Parasites isolated from animals infected with Δ*lmcpbg2.8* (t) possessed all three, although LmCPb activity was at a low level (Fig. 3.11, B, lane 6); levels were restored by culturing isolated Δ*lmcpbg2.8* promastigotes in 500 μg/ml neomycin as before (Fig. 3.11, B, lane 5). Mutant parasites passaged through mice, reisolated, transformed to promastigotes and used to infect PECs *in vitro* gave the results presented in Table 3.2 (B and C) and indeed were the cells used throughout this study unless designated otherwise by the letter t (as stated in section 3.2.1).

3.3.2. Host-parasite interactions of wild type parasites and transfected lines

3.3.2.1. Kinetics of infectivity to peritoneal exudate cells and BALB/c mice

Having found that the null mutant promastigotes were infective to PECs, time course experiments were set up to establish whether the null mutant line simply did not get in to the PECs as readily as the wild type line, or did but was subsequently killed.

Similar percentages of the PECs were initially infected by wild type and Δ*lmcpbn* stationary phase promastigotes (Figs. 3.12 and 3.13), but between 4 and 24 h there was a rapid decline in the number of null mutant-infected PECs such that by 24 h the percentage infected was similar to that at 7 days (Fig. 3.12, A). The number of amastigotes per infected macrophage was similar with both lines up to about 24 h, whereupon wild type parasites appeared to multiply more quickly (Fig. 3.12, B). This appeared not to be entirely consistent with the experiment detailed in section 3.3.1.3, which showed the number of amastigotes/infected PEC was similar with the two lines; however, perhaps by 7 days Δ*lmcpbn* had 'caught up' somewhat. This experiment was repeated using log phase promastigotes (as this stage of wild type parasites has very low CP activity) to see if there was a difference in infectivity between log phase promastigotes of the two lines (Fig. 3.14). Again both lines initially infected macrophages to a similar level, but this percentage was about half that seen with stationary phase promastigotes (Fig. 3.12); there was then a slight decline with both lines, but by 24 h the number of null mutant-infected PECs was still dropping whereas with wild type parasites the number had apparently stabilised.

Another experiment was then carried out to assess the effects of increasing the exposure time of the PECs to the promastigotes and/or increasing the parasite to PEC ratio. The results of one such experiment are summarised in Figure 3.15. These show that increasing the parasite to PEC ratio from 1:1 to 10:1 enabled the null mutants to infect substantially more PECs, although still significantly less than wild type. Increasing the exposure time from 4 h to 8 h also increased the percentage of infected macrophages.

The infectivity of stationary phase promastigotes and axenic amastigotes to BALB/c mice was investigated. Wild type parasites produced large lesions by 20 to 25 weeks on each occasion (Fig. 3.16, A-F), with the axenic amastigote form generally producing bigger lesions more quickly (B, D and F). The results with Δ*lmcpbn* (t) showed surprisingly that they did infect. Δ*lmcpbn* (t) promastigotes produced lesions 6 months after the wild type but only in one out of the 3 experiments undertaken using stationary phase promastigotes (Graph E). However, the axenic amastigote form of Δ

lmcpbn (t) appeared to be more infectious than the promastigote form, producing lesions in 3 out of 3 experiments (B, D and F); nevertheless these lesions still took between 10 and 16 weeks longer to appear than the wild type lesions, and grew poorly in comparison. Although the promastigotes of Δ*lmcpbg2.8* (t) infected in 1 out of 2 experiments, taking 10 weeks longer to produce lesions than wild type, the axenic amastigotes of Δ*lmcpbg2.8* (t) produced lesions only 3 weeks after wild type on both occasions and 7-13 weeks before Δ*lmcpbn* (t) axenic amastigotes (B and D) - suggesting perhaps some complementation of the phenotype was occurring. However it should be noted that the resulting lesions increased in size very slowly, similar to those of Δ *lmcpbn*. Δ*lmcpbBL* (t) promastigotes produced lesions 12 weeks after wild type parasites, more slowly than would have been expected for the single knockout for *lmcpb*, but as axenic amastigotes they produced lesions only 4 weeks after the wild type, and 8 weeks before Δ*lmcpbn* (t) axenic amastigotes. Interestingly, the use of 10-fold fewer parasites of wild type than the standard dose of 5 x 10^6 cells (E and F) produced faster growing lesions, at least at the early stages of infection. The 'double null' (Δ *lmcpbn/lmcpan*) did not produce lesions at all when tested (E and F).

It was noted that on excision of lesions the null mutant amastigotes looked morphologically identical to wild type parasites at the light microscope level (Fig. 3.17), although TEM showed there to be an ultrastuctural difference, in that the megasomes were much less dense (Fig 3.18).

As the null mutants did infect animals, the study was extended by using amastigotes isolated from lesions to infect PECs. The results given in Figure 3.19 indicate the null lesion amastigotes were as infective as wild type amastigotes to PECs, and that the multiplication of amastigotes within the macrophages was similar with the two lines.

Lesion amastigotes of the nulls and other transfectants were also used to infect animals (Fig. 3.20). Again Δ*lmcpbn* produced lesions in animals much more slowly than did the wild type line, the lesions taking 12 weeks longer to appear, indicating that the situation *in vivo* is more complex than that *in vitro*. Δ*lmcpbg2.8* lesion amastigotes produced lesions only two weeks after the wild type; however these lesions grew poorly.

The lesion amastigotes of Δ*lmcpbBL* produced lesions four weeks after wild type; these lesions did not grow as fast as those of wild type although they grew to be at least 4 times as big as those of the other *lmcpb* transfectants. Δ*lmcpan* lesions grew in a manner indistinguishable from wild type lesions (as was presented in Chapter 2).

3.3.2.2. Comparison of parasitophorous vacuole sizes in wild type parasite-infected and null mutant-infected macrophages

In order to establish whether the *lmcpb* CPs contribute to the large parasitophorous vacuole (PV) size normally observed with *L. mexicana*-infected macrophages (Pupkis *et al.*, 1986), PECs that had been infected with lesion amastigotes of wild type and Δ*lmcpbn* and incubated for 72 h were fixed, stained and examined under the light microscope, using an eyepiece graticule to measure their PV sizes (Table 3.3). Both large and small PVs were observed housing wild type parasites and those of Δ*lmcpbn* (Fig. 3.21 and Fig. 3.22), and the PV size generally increased as the number of parasites within increased (Fig. 2.22). However, the results in Table 3.3. indicate that the mean PV diameter per amastigote is significantly different between wild type parasite-infected and null mutant-infected PECs.

3.3.2.3. Analysis of enzymes of infected macrophages

To test the hypothesis that the *lmcpb* CPs may be involved in the degradation of host cell lysosomal enzymes, an 'Api Zym' enzyme screening kit was used with uninfected and wild type parasite-infected macrophages, to see if there were any differences in hydrolase activities between them, with a view to extending the experiment by comparing hydrolase activities in macrophages infected with null mutants. However, as can be seen from the results in Table 3.4, there was found to be no difference with any of the 19 enzymes tested, at least 6 of which (e.g. esterase, beta-galactosidase) are known to be located in macrophage lysosomes.

Wild type parasite-infected and uninfected macrophage lysates were also analysed for proteinase activity using gelatin-SDS-PAGE (Fig. 3.23), but again macrophage proteinase activity against gelatin was the same regardless of whether the

macrophages were infected with parasites (lane 2) or not (lane 1).

3.3.2.4. Susceptibility of parasites to components of the microbicidal defence mechanisms of macrophages

In an attempt to understand the mechanism by which wild type promastigotes survive in PECs while the null mutants do not, stationary phase cells of the two lines were exposed to varying concentrations of H_2O_2 and NO (both known components of the microbicidal activities of macrophages) and examined under phase contrast at various time intervals. The effect of H_2O_2 appeared to be dose-dependent, as judged by cell motility, but no difference was seen between the susceptibility of wild type parasites and Δ*lmcpbn* (Table 3.5, A). The effect of NO appeared also to be dose dependent, but differed in that non-motile cells exposed to NO were not necessarily dead; some recovery was revealed by subpassaging the exposed cells into fresh medium and looking for motility after 24 h (Table 3.5, B). To investigate this further, parasites were washed free of NO after 24 hours exposure and resuspended in fresh medium containing MTT overnight to assess the viability of the cells. The results of such experiments are shown in Table 3.6. The results suggest that although there is no difference in susceptibility to NO between wild type parasites and Δ*lmcpbn* stationary phase promastigotes, NO affects cell motility without killing and the cytostatic effect of NO on the cells can be reversed, depending on the concentration involved.

Promastigotes were used to infect PECs in the presence of L-NRME, an inhibitor of nitric oxide synthase (James, 1995), to see if this resulted in an increase in the number of macrophages infected with Δ*lmcpbn*. However the results in Table 3.7 indicate that this did not happen, and in fact there may even have been a decrease in the number of cells infected with wild type parasites when this inhibitor was present.

3.3.3. Analyses of null mutant lines re-expressing different copies of the *lmcpb* array

3.3.3.1. Proteinase activities

In order to compare the CPs encoded by different copies of *lmcpb*, lysates of

stationary phase promastigotes of null mutants re-expressing genes 1 (*g1*, the first copy), 2.8 (*g2.8*, an internal copy) and 18 (*g18*, the penultimate copy) of the array were analysed on gelatin gels for CP activity and on Western blots for the presence of LmCPb protein (Fig. 3.24). Two main bands of CP activity are characteristically detected on gelatin gels with wild type *L. mexicana* stationary phase promastigote extracts (Fig. 3.24, A, lane 1). These are stage regulated and occur predominantly in this parasite form (Robertson and Coombs, 1992; Bates *et al.*, 1994). Lysates of Δ*lmcpbg1* (t) (lane 2) and Δ*lmcpbg18* (t) (lane 4) had a single CP activity which co-migrated with the lower mobility band detected in wild type extracts, whereas Δ*lmcpbg2.8* (lane 3) had an activity whose mobility was between those in the wild type extracts (as shown in section 3.3.1.1) and similar to the situation with wild type amastigotes (Robertson and Coombs, 1990, 1992). Δ*lmcpbg2.8* (lane 3) and to a lesser extent Δ*lmcpbg18* (t) (lane 4) also had significant activities with much lower mobilities (apparently 30-40 kDa; but it should be noted that proteins do not migrate strictly according to molecular mass in gelatin SDS-PAGE). Equivalent activities to these were not readily detected with wild type or Δ *lmcpbg1* (t) cell extracts.

Western blotting of wild type stationary phase promastigote extracts with anti-LmCPb (group C) antiserum detected two major proteins (25 and 29 kDa, Fig. 3.24, B, lane 1). The product of gene 1 was also 25 kDa (lane 2), whereas the main *lmcpb* products in Δ*lmcpbg2.8* and Δ*lmcpbg18* (t) migrated with a molecular weight of 28 kDa. These lines also contained larger molecular weight proteins (about 38 kDa) detected by the antiserum.

The cell lines were also analysed for their ability to hydrolyse two fluorogenic peptide substrates (Fig. 3.25). BzFVR-NHMec had previously been shown to be a good substrate for the type C CPs of the *lmcpb* array, and SucLY-NHMec a good substrate for the type B CPs (Robertson and Coombs, 1990). Both CP activities detected in wild type extracts (lane 1) hydrolysed each of the substrates, although the lower mobility activity showed greater activity towards SucLY-NHMec whereas the reverse was true for the higher mobility activity. The CP in each of the three cell lines expressing one *lmcpb* gene, Δ*lmcpbg1* (t) (lane 2), Δ*lmcpbg2.8* (lane 3) and Δ*lmcpbg18* (t) (lane 4) were

equally proficient at hydrolysing BzFVR-NHMec (Fig. 3.25, A), whereas the CP in Δ *lmcpbg18* (t) had a significantly higher activity towards SucLY-NHMec than the CP in either Δ*lmcpbg1* (t) or Δ*lmcpbg2.8* (Fig. 3.25, B).

Since wild type cells undergo stage regulation of their CP activity, different stages of the re-expressor Δ*lmcpbg2.8* were analysed by gelatin-SDS-PAGE (Fig. 3.26). The banding pattern differed between log phase (lane 1), stationary phase (lane 2) and axenic amastigotes (lane 3), indicating that some stage regulation of activity occurred.

3.3.3.2. Subcellular localisation of CPs using immunogold electron microscopy

Immunogold labeling of wild type *L. mexicana* axenic amastigotes with anti-LmCPb antiserum (Fig. 3.27, A) resulted in strong labelling of the large lysosomes (termed megasomes) as previously reported (Pupkis *et al.*, 1986). As expected, no labelling was detected in the *lmcpb* null mutant axenic amastigotes, demonstrating the specificity of the antiserum used to detect the LmCPb enzymes (Fig. 3.27, B). The megasomes were also labelled in Δ*lmcpbg2.8* and Δ*lmcpbg1* (t) axenic amastigotes, indicating that both *g2.8* (complete CTE) and *g1* (truncated CTE) isoenzymes were targeted to megasomes (Fig. 3.27, C and D). With each of the 3 cell lines expressing LmCPb, no significant labelling was detected outside megasomes - indicating that the trafficking of the proteinase was efficient in these parasites and that there was no accumulation of precursors in other compartments of the cell such as the Golgi.

3.3.3.3. Growth and differentiation

It was found that the re-expressors essentially had the same growth and differentiation characteristics as those described for Δ*lmcpbn* and Δ*lmcpbg2.8* in section 3.3.1.2, that is, they did not appear to form metacyclics of typical morphology and did not grow well as axenic amastigotes.

3.3.3.4. Infectivity

One of the criteria used (section 3.3.1.3) to assess the phenotype of the *lmcpb* null mutants was their ability to survive in macrophages *in vitro*. PECs were exposed to

stationary phase promastigotes and assessed for parasite survival after 7 days in culture (Table 3.8). As before (Table 3.2) the null mutant Δ*lmcpbn* survived in just a few macrophages and had a lower number of amastigotes per infected PEC, whereas expressing *g2.8* in the null mutant restored levels of infectivity back to almost wild type levels. In contrast, expression of *g1* or *g18* in the null mutant increased infectivity only to a minor extent, although the difference was found to be statistically significant with *g18*.

The infectivity of stationary phase promastigotes to animals was also investigated. Again Δ*lmcpbn* was much less infective than wild type (Fig. 3.28), the lesions taking 7 months longer to appear on this occasion, whereas the control lines Δ *lmcpan* and Δ*lmcpbBI.* took, respectively, only one and two weeks longer than wild type parasites. In this experiment none of the re-expressor lines tested (Δ*lmcpbg1* (t), Δ *lmcpbg18* (t) and Δ*lmcpbg2.8*) produced lesions within the maximum time permitted for the experiment; however, since the Δ*lmcpbn* lesions did not appear until near the end of the experiment it is not impossible that the re-expressors would have produced lesions had the experiment been extended beyond 9 months.

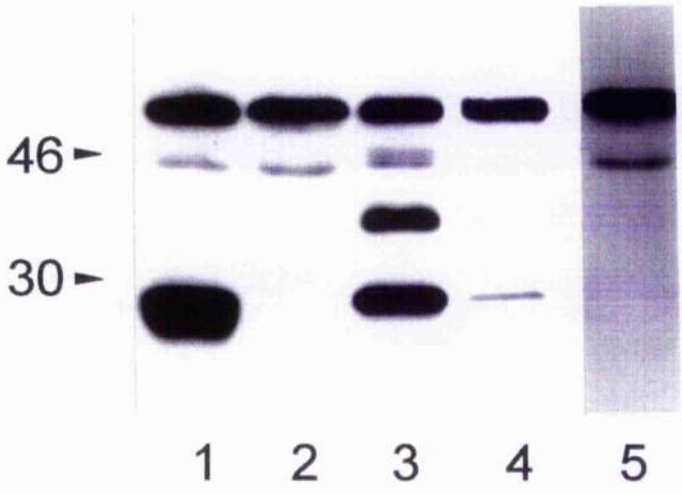


Figure 3.1. Western blot using anti-LmCPb antibody. Lane 1, wild type parasites; lane 2, Δ*lmcpbn*; lane 3, Δ*lmcpbg2.8*; lane 4, Δ*lmcpbc1*. Each lane was loaded with lysate from 5 x 10^6 stationary phase promastigotes. Δ*lmcpbTEX* gave the same profile as Δ*lmcpbn*. The bands >42 kD were also produced with the pre-immune serum (lane 5). Molecular mass markers are shown in kDa.

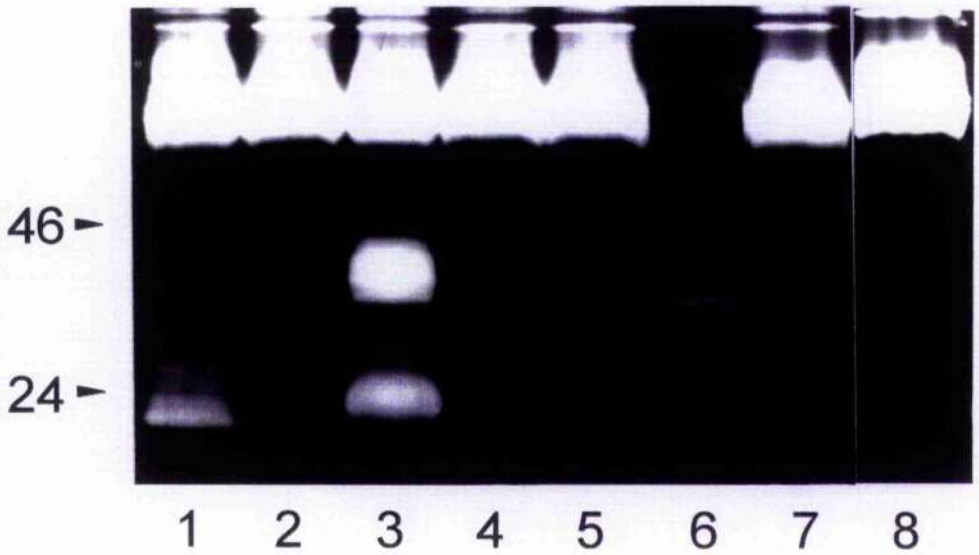


Figure 3.2. Gelatin-SDS-PAGE analysis of cell lines. Lane 1, wild type *L. mexicana*; lane 2, Δ*lmcpbn*; lane 3, Δ*lmcpbg2.8*; lane 4, Δ*lmcpbc1*; lane 5, Δ*lmcpbTEX*; lane 6, *T. brucei* procyclics; lane 7, Δ*lmcpbTEX*; lane 8, Δ*lmcpbtb*. All lanes were loaded with lysates containing 10^7 stationary phase promastigotes, except lane 6 (10^7 procyclics). Molecular mass markers are shown in kDa.

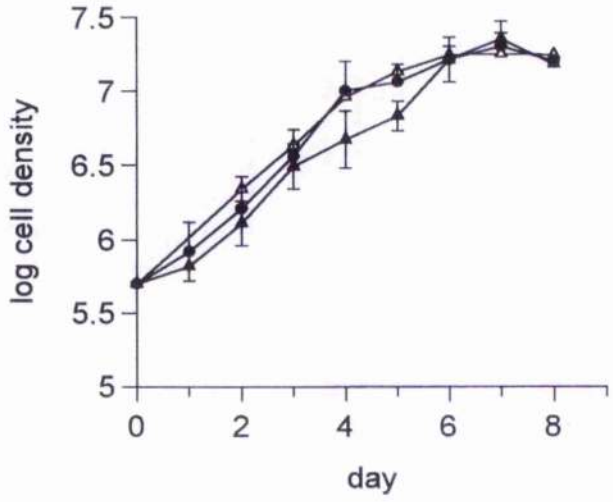


Figure 3.3. Typical *in vitro* growth curves of transfected promastigote lines. Cultures of wild type parasites (-●-), Δ*lmcpbn* (-△-) and Δ*lmcpbg2.8* (-▲-) were initiated at 5 x 10^5 cells/ml in HOMEM medium and incubated at 25°C, with cell counts being performed on consecutive days. The results are the means ± SD from 3 independent cultures for each line; some error bars have been omitted for clarity, and some are obscured by the data points.

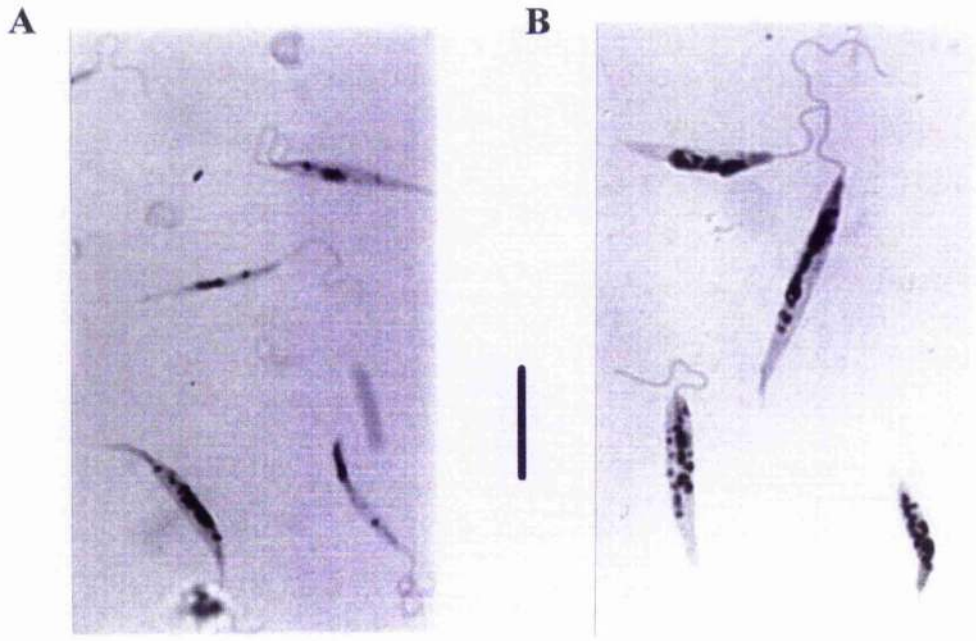


Figure 3.4. Morphology of stationary phase promastigotes. Light micrographs of stationary phase promastigotes of wild type parasites (A) and Δ*lmcpbn* (B). Cells were fixed in methanol and stained in 10% Giemsa's stain. There is a clear difference between the size of cells of the two lines, with the Δ*lmcpbn* promastigotes (B) being much bigger than the majority of wild type cells (A).The scale bar represents 10 μm.

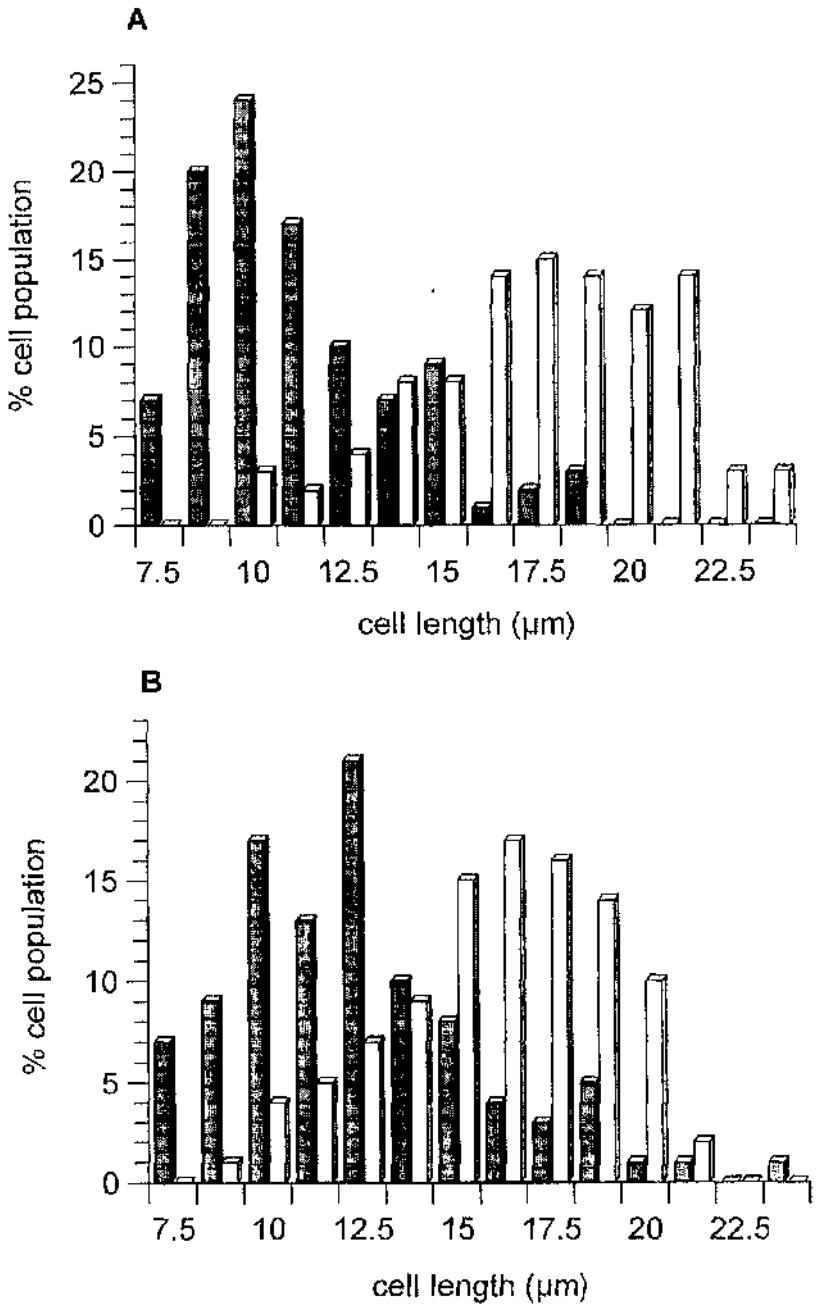


Figure 3.5. The cell lengths of stationary phase promastigotes. Giemsa-stained smears of stationary phase promastigotes (see Fig. 3.4) were used to compare the lengths of promastigotes of the various lines. Measurements were made with a graticule under 1000x bright field microscopy. At least 100 cells were measured in each case, to the nearest graticule unit (1 graticule unit = 1.25 μm). Results (A) indicate a highly significant difference between the lengths of wild type (closed bars) and Δ*lmcpbn* (open bars), at subpassage 1 (z=15.26, greatly exceeding the critical value of 2.58 at P=0.01). There was also a significant difference at subpassage 7 (figure B, where z=8.21, exceeding the critical value of 2.58 at P=0.01), although wild type parasites contained fewer short promastigotes (putative metacyclics) than at subpassage 1, possibly due to adaptation to long term growth *in vitro* (Bates *et al.*, 1994).

cell line	% cells intact
log phase wild type	45 ± 7.0
stationary phase wild type	54 ± 9.3
stationary phase Δ*lmcpbn*	19 ± 8.4

Table 3.1. Complement-mediated lysis of promastigotes. Cells were incubated in 30% (v/v) guinea pig serum at 25°C for 30 minutes, as described in section 3.2.3.2; the results are the means ± SD of 3 independent experiments. The % lysis was calculated by reference to controls containing heat-inactivated serum, in which there was no detectable lysis. The % lysis results with both log and stationary phase wild type promastigotes were significantly different from those with Δ*lmcpbn* (P values of <0.02 and <0.01 respectively) but not from each other.

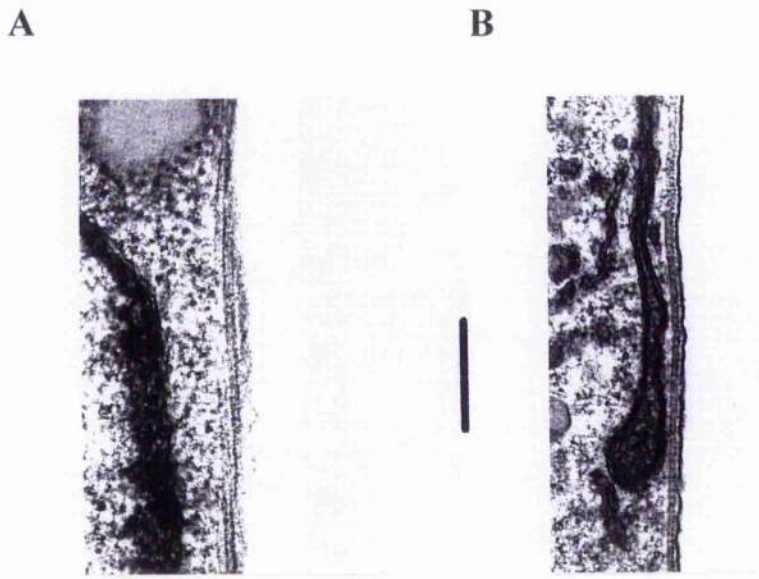


Figure 3.6. Surface structure of stationary phase promastigotes. Section through the plasma membrane (scale bar 0.4 μm). 'Surface coat' material is clearly visible with the wild type cells (A) but not on the null mutant line Δ*lmcpbn* (B). Micrographs by L. Tetley, University of Glasgow.

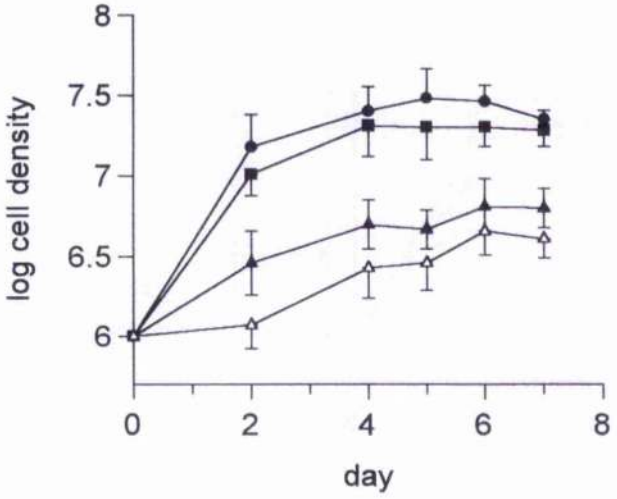


Figure 3.7. Typical *in vitro* growth curves of transfected lines as axenic amastigotes. Cultures were routinely initiated using stationary phase promastigotes at 1 x 10^6 cells/ml in complete SDM, pH 5.5, and incubated at 32°. Cell counts revealed a clear difference in growth rates and final cell density between the different lines, with Δ *lmcpbn* (-△-) and Δ*lmcpbg2.8* (-▲-) growing much more slowly than either wild type (-●-) or Δ*lmcpbBL* (-■-) axenic amastigotes.

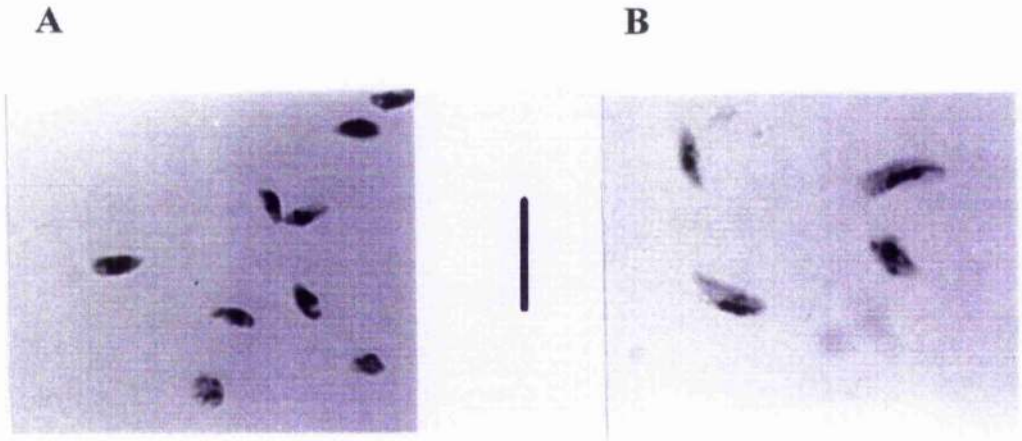


Figure 3.8. Light micrographs of Giemsa-stained axenic amastigotes; (A), wild type parasites, (B), Δ*lmcpbn*. There is a difference between the size of the cells of the 2 lines, with the Δ*lmcpbn* axenic amastigotes (B) being larger than the majority of wild type cells (A). Δ*lmcpbg2.8* axenic amastigotes were similar in proportions to the null mutants. The scale bar represents 10 μm.

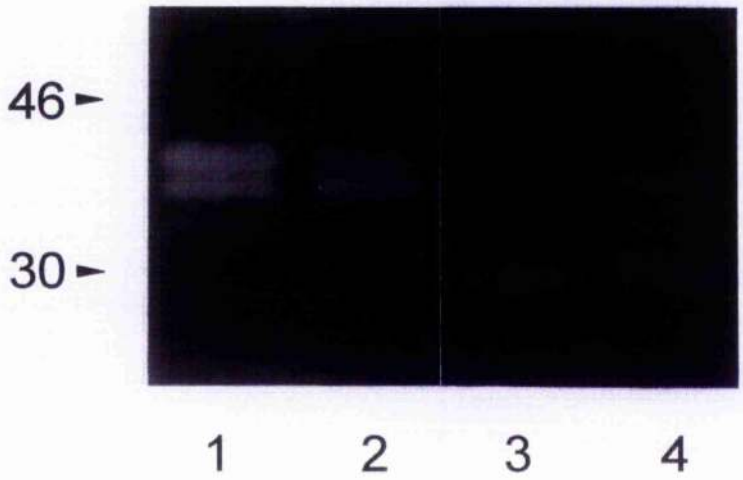


Figure 3.9. Analysis of parasite nucleases using poly(A)-SDS-PAGE. Lane 1, wild type stationary phase promastigotes; lane 2, Δ*lmcpbn* stationary phase promastigotes; lane 3, wild type axenic amastigotes; lane 4, Δ*lmcpbn* axenic amastigotes. Each lane was loaded with lysate from 10^7 cells. The positions of molecular weight markers are given on the left.

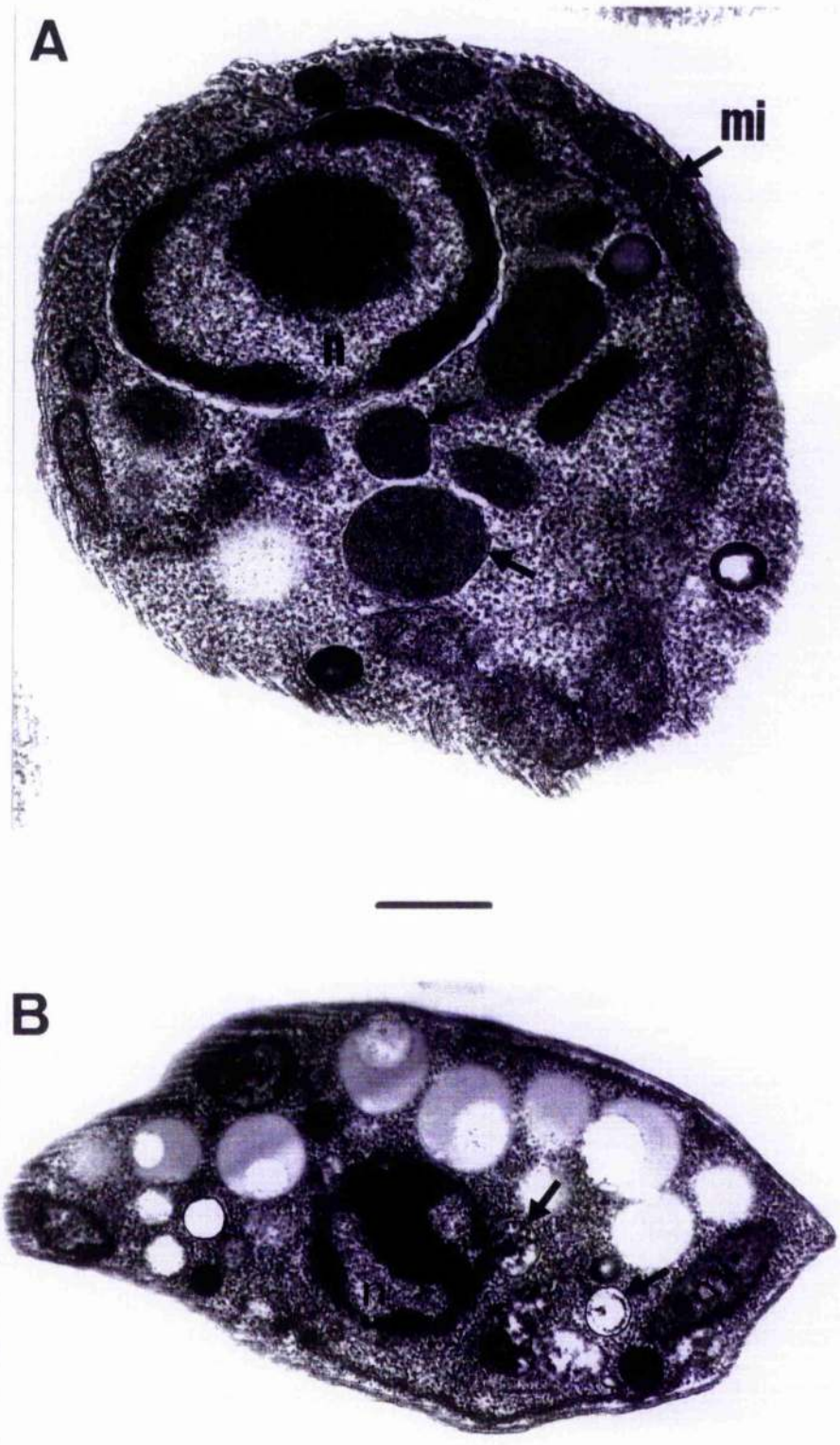


Figure 3.10. Transmission electron micrograph revealing ultrastructure of axenic amastigotes. (A), wild type parasites; (B), Δ*lmcpbn*. Megasomes are seen in the wild type axenic amastigotes (A, arrowed); less dense lysosome-like organelles are evident in the null mutants (B, arrowed). n, nucleus; mi, mitochondrion. The scale bar represents 0.5 μm. Micrograph by L. Tetley, University of Glasgow.

A

cell line	% infected PECs	amastigotes/infected PEC
wild type	46 ± 10	3.8 ± 0.2
Δ*lmcpbn* (t)	9.2 ± 3.9	2.6 ± 0.7
Δ*lmcpbg2.8* (t)	40 ± 9.0	3.2 ± 0.2
Δ*lmcpbc1* (t)	12 ± 1.2	4.4 ± 2.2
Δ*lmcpbtb* (t)	7.2 ± 2.6	3.0 ± 1.2

B

wild type	50 ± 9.4	4.1 ± 1.0
Δ*lmcpbBL*	54 ± 10	5.3 ± 1.0
Δ*lmcpbn*	3.2 ± 1.1	2.7 ± 0.9
Δ*lmcpbn*/Δ*lmcpan* (t)	3.3 ± 2.0	2.6 ± 0.7
Δ*lmcpbn*/Δ*lmcpa2.8* (t)	2.4 ± 1.3	2.3 ± 0.7

C

wild type	36 ± 8.3	4.6 ± 1.3
Δ*lmcpan*	36 ± 10	4.4 ± 1.2
Δ*lmcpbBL*	39 ± 6.8	4.5 ± 1.6
Δ*lmcpbn*	1.6 ± 0.3	1.6 ± 0.3
Δ*lmcpbn*/Δ*lmcpan* (t)	2.8 ± 2.0	2.2 ± 1.8

Table 3.2. Infectivity of transfectants to macrophages. PECs were obtained from peritoneal lavage of BALB/c mice and infected with stationary phase promastigotes of the *L. mexicana* lines at a ratio of 1:1. After 7 days incubation at 32°C, cells were fixed, stained with Giemsa's stain, and parasite load determined by counting 200 PECs. The values in each of the tables designated A, B and C are means ± SD from 4 independent experiments. Table A: the infection rates with both wild type and Δ*lmcpbg2.8* (t) were significantly different from those for Δ*lmcpbn* (t), Δ*lmcpbc1* (t) and Δ*lmcpbtb* (t) parasites (P values of <0.01) but not from each other; the mean values for amastigotes/infected cell with both Δ*lmcpbn* (t) and Δ*lmcpbg2.8* (t) were significantly different from those with wild type parasites (P values of <0.02 and <0.01, respectively) but not from each other. Table B: the infection rates with both wild type and *lmcpbBL* were significantly different from those with Δ*lmcpbn*, Δ*lmcpbn*/Δ*lmcpan* (t) and Δ *lmcpbn*/Δ*lmcpa2.8* (t) parasites (P values of <0.01) but not from each other; there were no significant differences with reference to the number of amastigotes/infected cell. Table C: the infection rates with both wild type, Δ*lmcpan* and *lmcpbBL* were significantly different from those with Δ*lmcpbn* and Δ*lmcpbn*/Δ*lmcpan* (t) (P values of <0.01) but not from each other; the mean values for amastigotes/infected cell with both Δ*lmcpbn* and *lmcpbn*/Δ*lmcpan* (t) were significantly different from those for wild type, Δ*lmcpan* and *lmcpbBL* parasites (P values of <0.02) but not from each other.

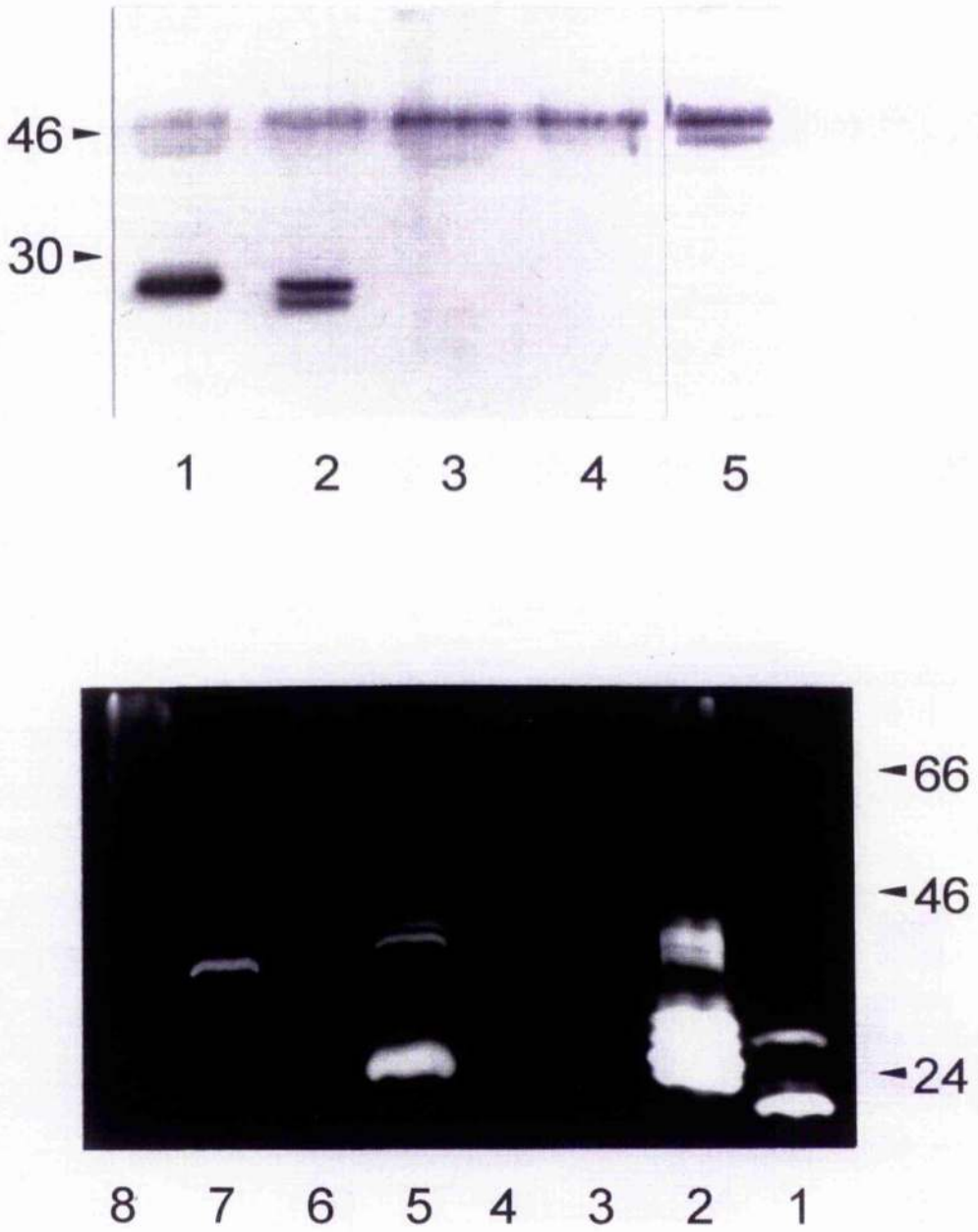


Figure 3.11. Western blot and gelatin-SDS-PAGE analyses of lesion amastigotes. (A) Samples containing lysates (equivalent to 5 x 10^6 cells) of lesion amastigotes and lesion-derived stationary phase promastigotes were electroblotted and probed with anti-LmCPb (group C) antiserum: wild type amastigotes (lane 1) and promastigotes (lane 2); Δ*lmcpbn* amastigotes (lane 3) and promastigotes (lane 4). Lane 5 was probed with pre-immune serum. (B) Samples containing lysates (equivalent to 10^7 cells) were analysed by gelatin-SDS-PAGE: wild type promastigotes (lane 1) and amastigotes (lane 2); Δ*lmcpbn* promastigotes (lane 3) and amastigotes (lane 4); Δ*lmcpbg2.8* promastigotes (lane 5) and amastigotes (lane 6); Δ*lmcpbtb* promastigotes (lane 7) and amastigotes (lane 8). The usual positions of molecular weight markers are indicated.

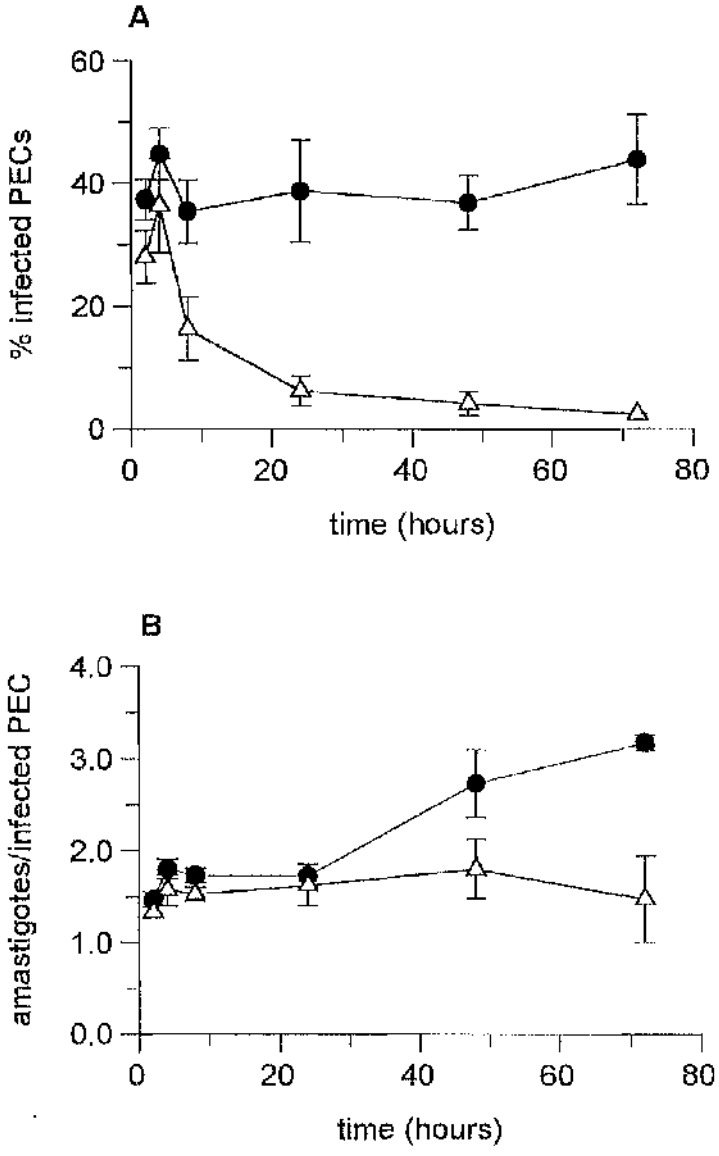


Figure 3.12. The fate of wild type (-●-) and Δ*lmcpbn* (-△-) stationary phase promastigotes after entry into macrophages *in vitro*. PECs were obtained from peritoneal lavage of BALB/c mice and infected with stationary phase promastigotes of the *L. mexicana* lines at a ratio of 1:1. After certain incubation times at 32°C, cells were fixed, stained with Giemsa's stain, and the parasite load of 200 PECs determined by counting the number of infected PECs (A) and number of parasites per infected cell (B). The points are means ± SD from 3 independent experiments.

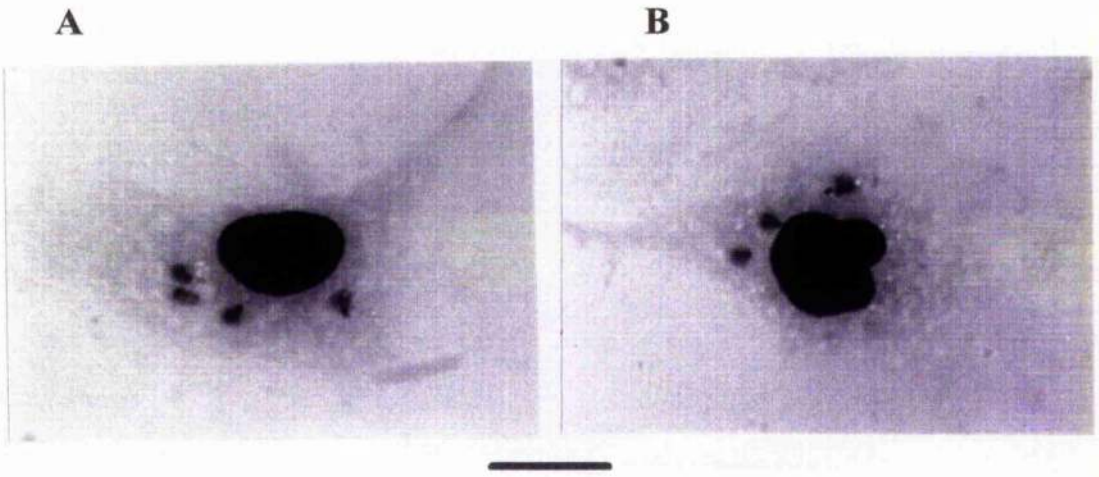


Figure 3.13. Light micrographs of *Leishmania*-infected macrophages. PECs were harvested at the 4 h time point (see Fig. 3.12) and stained with Giemsa's stain. Intracellular parasites of both wild type (A) and Δ*lmcpbn* (B) are clearly visible. Parasites are arrowed; n, nucleus. The scale bar represents 10 μm.

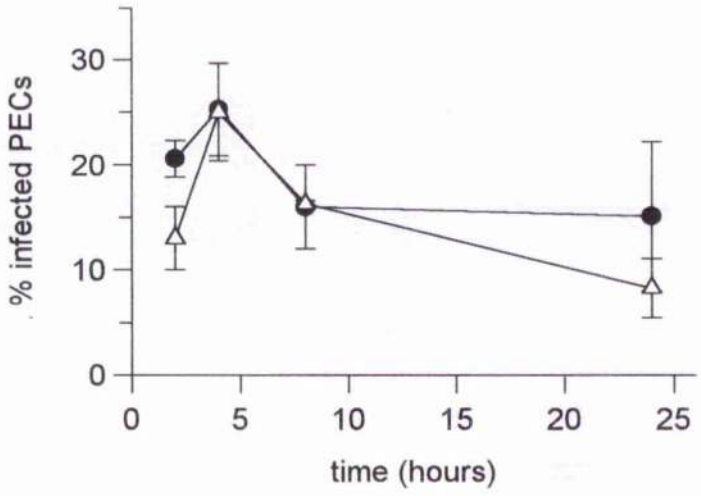


Figure 3.14. The fate of wild type (-●-) and Δ*lmcpbn* (-△-) log phase promastigotes after entry into macrophages *in vitro*. PECs were obtained from peritoneal lavage of BALB/c mice and infected with log phase promastigotes of the *L. mexicana* lines at a ratio of 1:1. After certain incubation times at 32°C, cells were fixed, stained with Giemsa's stain, and the parasite load determined by counting 200 PECs. The points are means ± SD from 3 independent experiments.

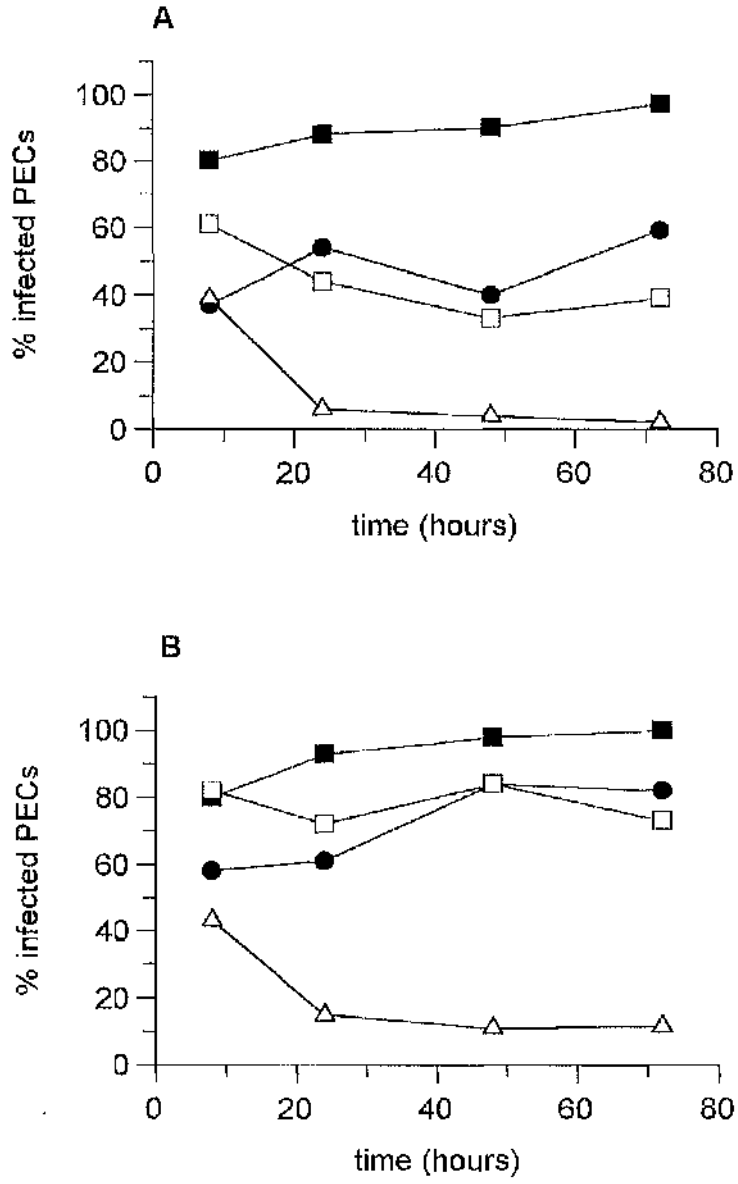


Figure 3.15. The effects of varying exposure time/ratios of stationary phase promastigotes to PECs on infection rates. A ratio of 1:1 was set up with wild type parasites (-●-) and Δ*lmcpbn* (-△-); a ratio of 10:1 with wild type parasites (-■-) and Δ *lmcpbn* (-□-) was also tested. Exposure of PECs to parasites was either the staudard 4 h (A) or 8 h (B). The results of one experiment are presented.

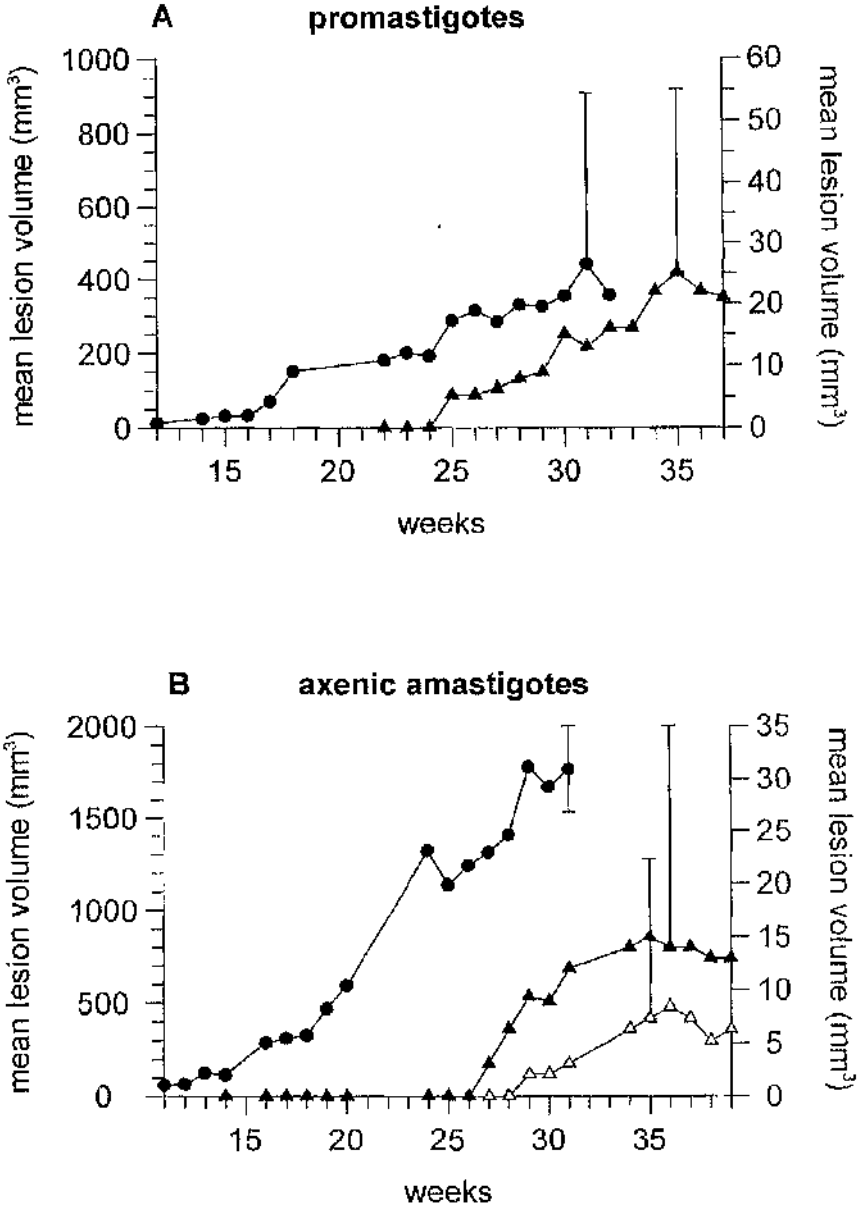
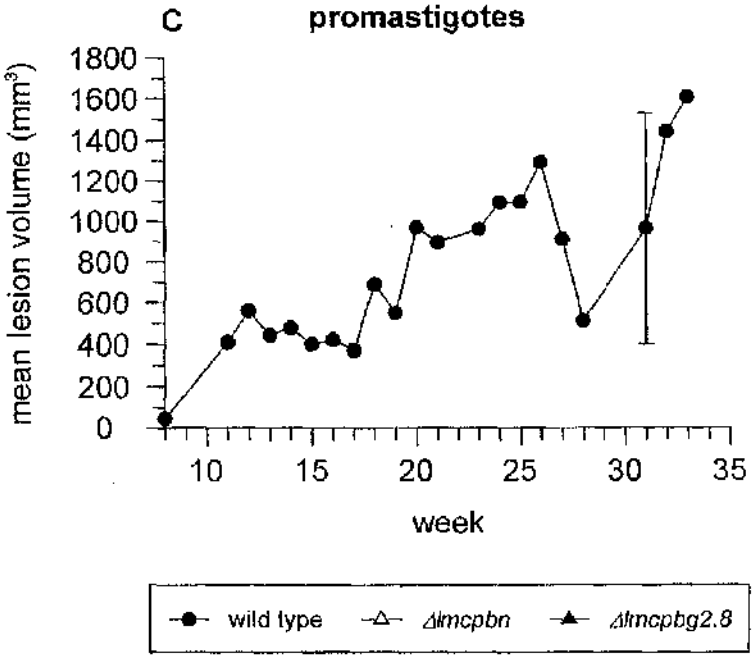
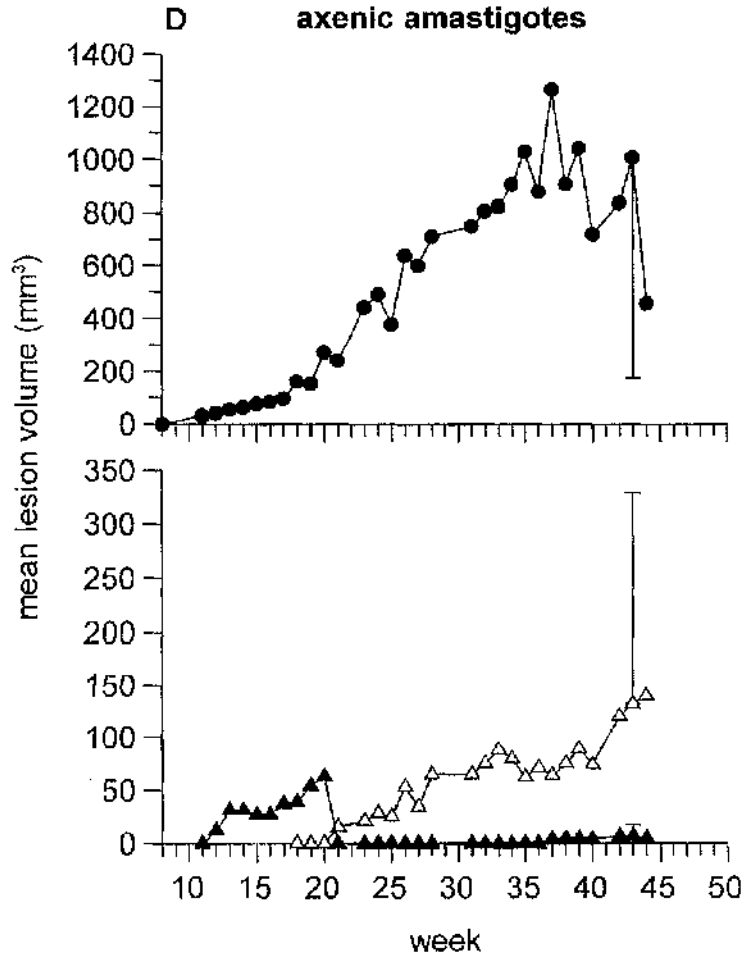
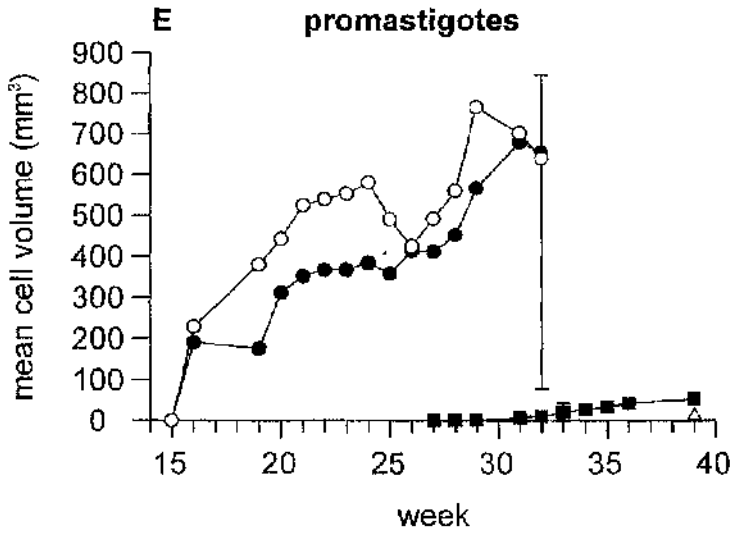
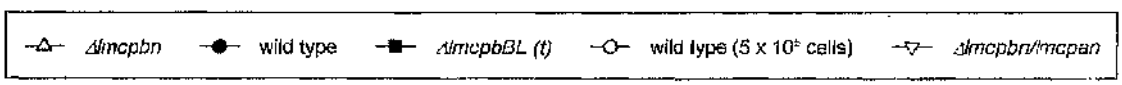


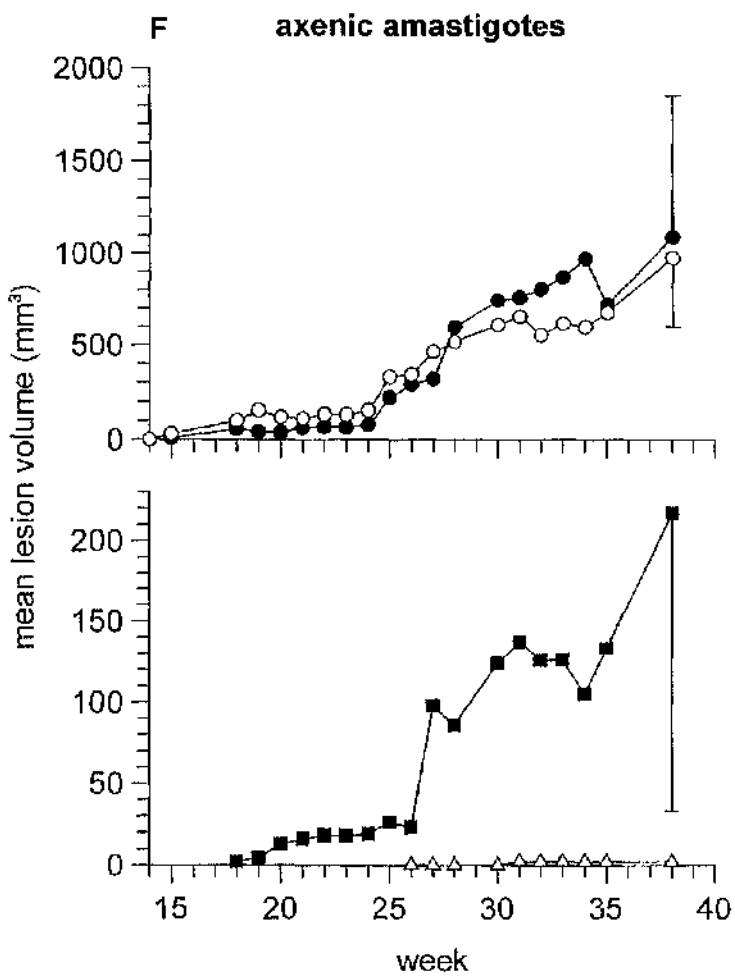
Figure 3.16. Infectivity of *L. mexicana* lines to animals. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5 x 10^6 stationary phase promastigotes (A, C and E) or axenic amastigotes (B, D and F) were measured weekly using a micrometer, and the mean lesion volume ± SD per group (5 mice) calculated using the equation for the volume of a hemisphere. Lines used in experiments A and B were as follows: ●, wild type; △, Δ*lmcpbn* (t); ▲, Δ*lmcpbg2.8* (t); note the 20-fold (A) and 100-fold (B) differences in the two Y axes for each graph, and that Δ*lmcpbn* (t) did not produce lesions at all in (A). Experiments C and D: note that Δ*lmcpbg2.8* and Δ *lmcpbn* did not produce lesions at all in (C), and the difference in scales of the two Y axes in graph F. The majority of error bars have been omitted for clarity.


C
promastigotes
mean lesion volume (mm³)
1800
1600
1400
1200
1000
800
600
400
200
0
10
15
20
25
30
35
week
wild type
Δlmcpbn
Δlmcpbg2.8


D
axenic amastigotes
mean lesion volume (mm³)
1400
1200
1000
800
600
400
200
0
350
300
250
200
150
100
50
0
10
15
20
25
30
35
40
45
50
week


E
promastigotes
mean cell volume (mm³)
900
800
700
600
500
400
300
200
100
0
15
20
25
30
35
40
week


Δlmcpbn
wild type
ΔlmcpbBL (t)
wild type (5 x 10⁵ cells)
Δlmcpbn/lmcpan


F
axenic amastigotes
mean lesion volume (mm³)
2000
1500
1000
500
0
200
150
100
50
0
15
20
25
30
35
40
week

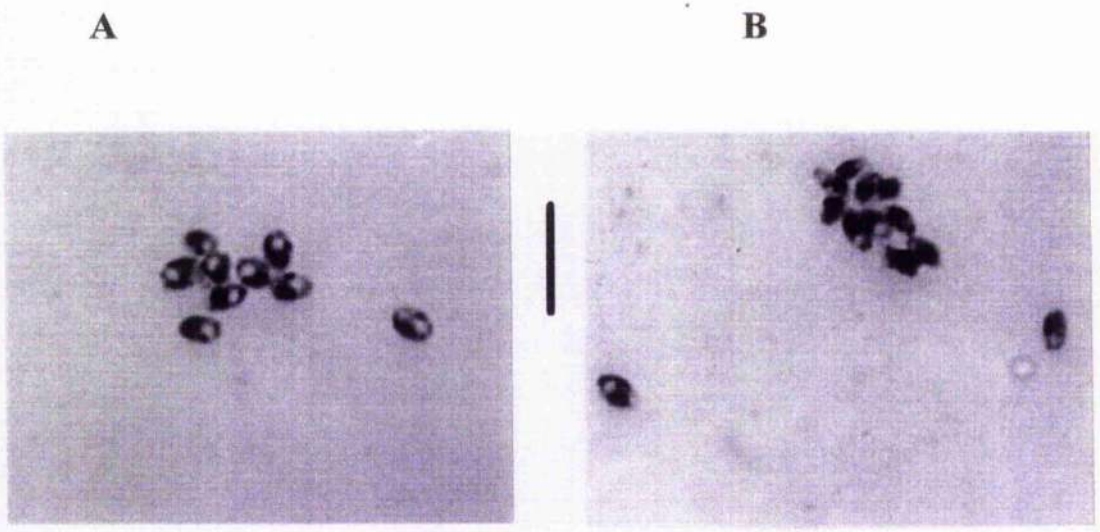
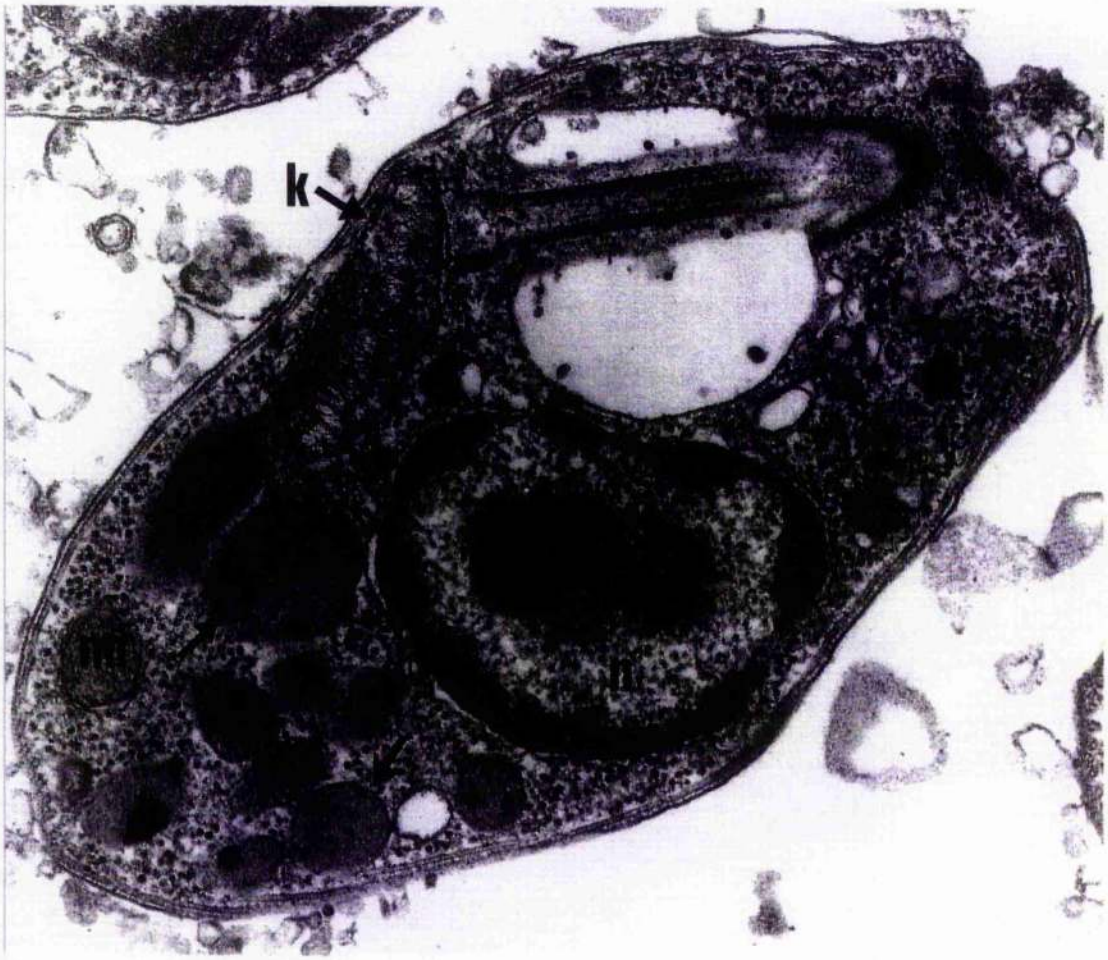


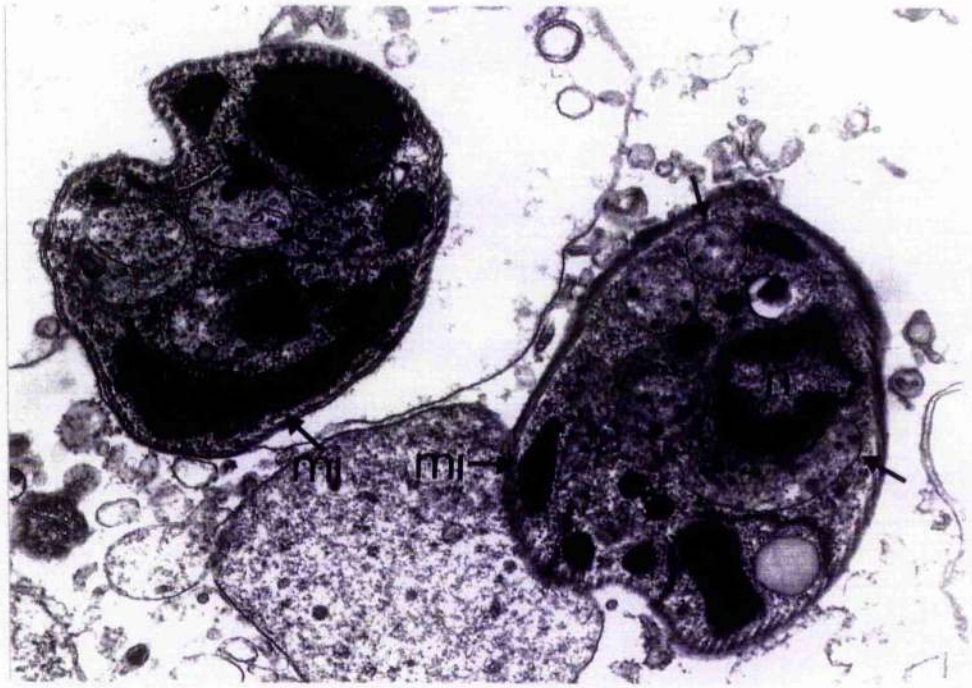
Figure 3.17. Light micrographs of Giemsa-stained lesion amastigotes. (A) wild type parasites; (B) Δ*lmcpbn*. The scale bar represents 10μm.

Figure 3.18. Ultrastructure of lesion amastigotes. Transmission electron micrographs revealing ultrastructural differences in the lesion amastigotes of (a) wild type (scale bar 0.3 μm) and (b) Δ*lmcpbn* (scale bar 0.6 μm). Note the difference in densities of the megasomes of the two lines. Megasomes are arrowed; k, kinetoplast; mi, mitochondrion, n, nucleus. Micrographs by L. Tetley, University of Glasgow.

A



B



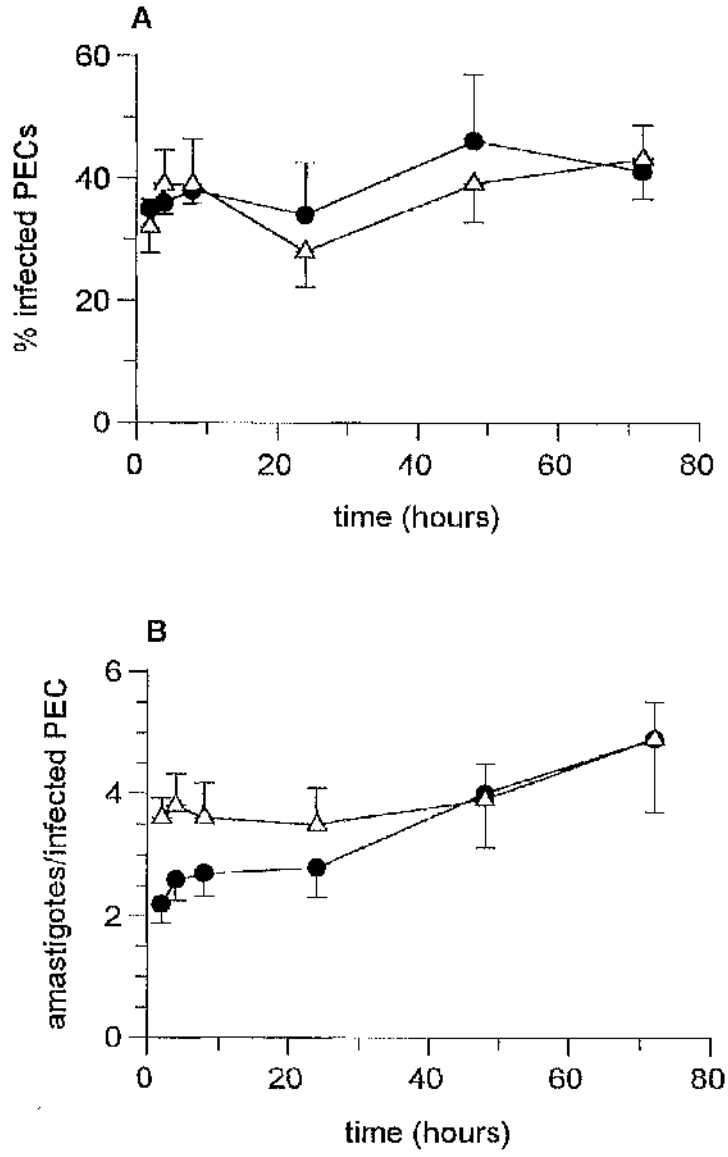


Figure 3.19. Infectivity of lesion amastigotes of wild type (-●-) and Δ*lmcpbn* (-△-) for peritoneal macrophages. (A) percentage of macrophages infected; (B) number of parasites per infected cell. The number of intracellular parasites in at least 200 macrophages was counted from each time point. The results are the means ± SD from 3 experiments.

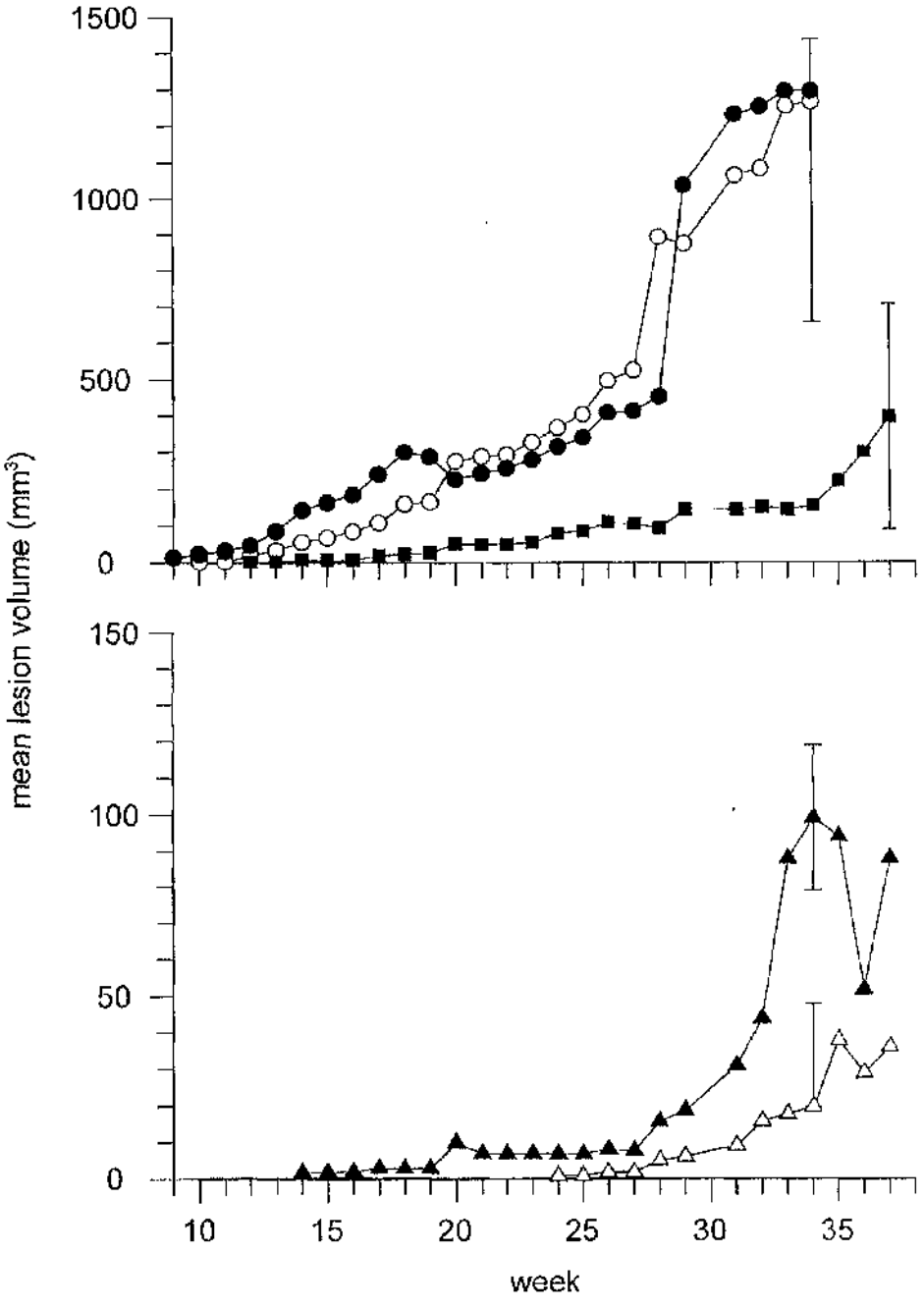


Figure 3.20. Infectivity of lesion amastigotes to animals. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5 x 10^5 parasites were measured at weekly intervals using a micrometer, and the mean lesion volume ± SD per group of 5 mice calculated using the formula for a half spheroid. -●-, wild type; -△-, Δ *lmcpbn*; -▲-, Δ*lmcpbg2.8*; -■-, Δ*lmcpb*BL; -○-, Δ*lmcpan*. Note the difference in scales of the Y axes. The majority of error bars have been omitted for clarity.

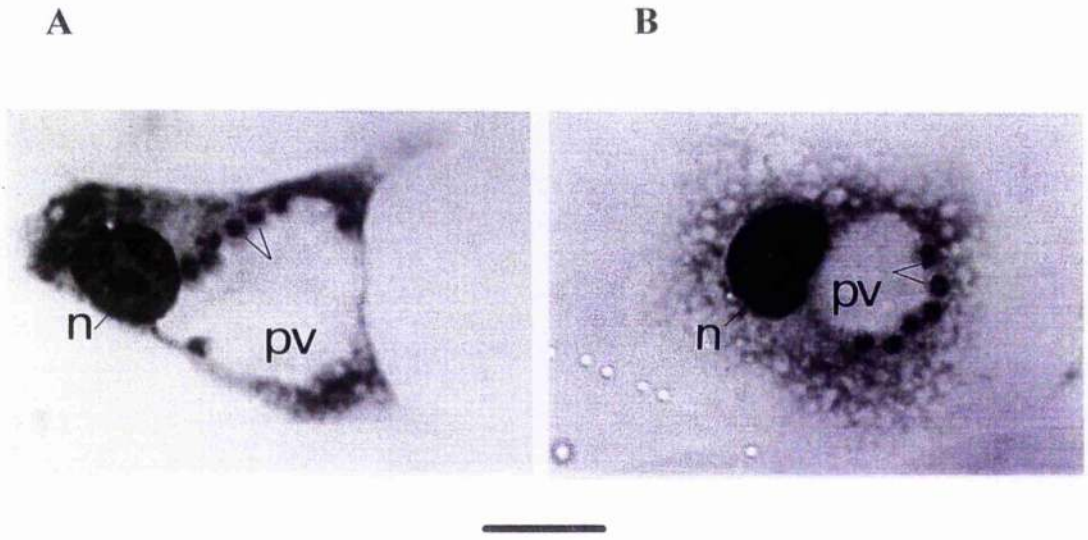


Figure 3.21. Light micrographs of *Leishmania*-infected peritoneal macrophages. Infections were initiated using lesion amastigotes of wild type parasites (A) and Δ *lmcpbn* (B) and the cultures then fixed and stained with Giemsa's stain at the 72 h time point (see Fig. 3.19). Amastigotes are arrowed; n, nucleus; pv, parasitophorous vacuole. The scale bar represents 10 μm.

line	amastigotes/vacuole	vacuole diameter (μm)	diameter/amastigote (μm)
wild type	4.9 ± 2.4	17 ± 6.5	3.9 ± 1.6
Δ*lmcpbn*	6.4 ± 3.9	16 ± 6.0	3.0 ± 1.4

Table 3.3. Parasitophorous vacuole sizes. Macrophages infected with lesion amastigotes of wild type and Δ*lmcpbn* were stained with Giemsa's stain after 72 h and their PVs measured using a graticule. Fifty parasitophorous vacuoles were measured in each case, and the number of parasites per vacuole was also counted. Figures represent means ± SD, n=50. Results indicate a significant difference between the vacuole diameter/amastigote with wild type and Δ*lmcpbn* ($z=3.18$, exceeding the critical value of 2.58 at $P=0.01$).

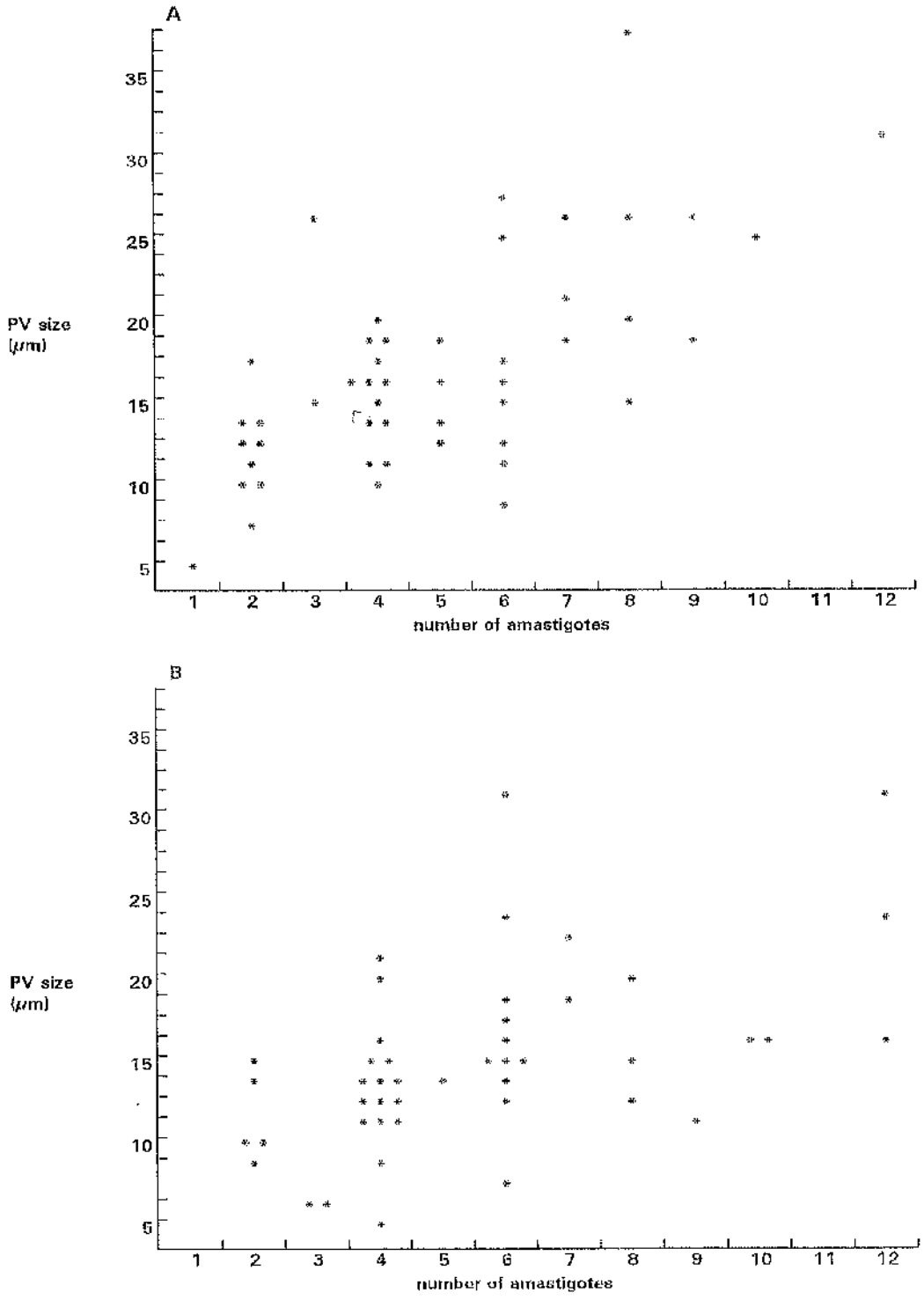


Figure 3.22. Scatter plots of parasitophorous vacuole sizes. PV size (see Table 3.3) was plotted against the number of parasites within, for wild type (A) and Δ*lmcpbn* (B). Fifty cells were measured in each case.

enzyme	macrophage	infected macrophage
1. control	0	0
2. phosphatase alkaline	1	1
3. esterase*	1	1
4. esterase lipase	3	3
5. lipase*	1	1
6. leucine arylamidase	3	3
7. valine arylamidase	0	0
8. cystine arylamidase	0	0
9. trypsin	0	0
10. chymotrypsin	0	0
11. phosphatase acid*	5	5
12. phosphohydrolase	5	5
13.α galactosidase	0	0
14. β galactosidase*	3	3
15. β glucoronidase*	4	4
16. α glucosidase*	0	0
17. β glucosidase	1	1
18. N-acetyl-glucosaminidase	3	3
19. α mannosidase*	1	1
20. α fucosidase	0	0

Table 3.4. Analysis of enzyme activities of uninfected and infected macrophages using the 'Api Zym' colorimetric screening kit. Colour changes ranging from 0 (no colour, no activity) to 5 (intense orange/violet, high activity) were observed. *Denotes lysosomal enzyme.

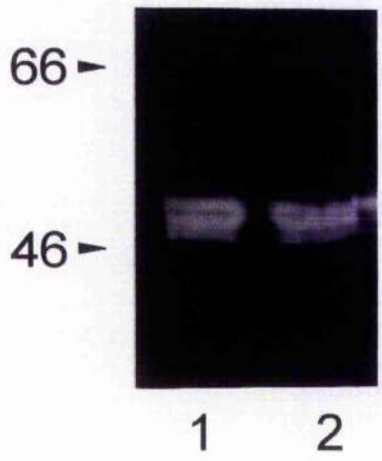


Figure 3.23. Gelatin-SDS-PAGE analysis of infected and uninfected peritoneal macrophages. Lane 1, resident macrophages; lane 2, *L. mexicana*-infected macrophages (40% infection rate). Extracts from 1 x 10^5 macrophages were loaded in each track.

A

time (h)	CELL MOTILITY mM H_2O_2									
	100	50	10	2	0.4	0.2	0.04	0.008	0.0016	0.00016
2	-	-	-	-	-	-	+	++	+++	+++
4	-	-	-	-	-	-	+	++	+++	+++
8	-	-	-	-	-	-	-	+	++	++
24	-	-	-	-	-	-	-	+	+	++
↓24	-	-	-	-	-	-	-	+	+	++

B

time (h)	CELL MOTILITY mM Na nitrite									
	100	50	10	2	0.4	0.2	0.04	0.008	0.0016	0.00016
2	-	-	-	+	++	++	+++	+++	+++	+++
4	-	-	-	+	++	++	+++	+++	+++	+++
8	-	-	-	-	+	+	++	++	++	+++
24	-	-	-	-	-	-	+	+	++	++
↓24	-	-	-	+	+	+	+	++	++	++

Table 3.5. Effect on cell motility of hydrogen peroxide (A) and nitric oxide (B). Stationary phase promastigotes of wild type and Δ*lmcpb* were exposed to various concentrations of H_2O_2 and sodium nitrite (a source of nitric oxide at acidic pH) and examined for motility at different time points. Cell motility was designated as follows: (-) no motility; (+), if very few cells were observed to be moving; (++), if approximately half the field of cells were moving; and (+++), if the majority of cells showed movement. After the 24 h time point cells were subpassaged into fresh medium and examined for motility after a further 24 h (↓24). The results presented in Tables A and B are from one experiment and are the data for both wild type parasites and Δ*lmcpb*, since no differences were found between their relative susceptibilities.

cell line	% MTT REDUCTION					
	mM sodium nitrite					
	10	2	1	0.5	0.25	0
wild type	41 ± 18	70 ± 11	75 ± 2.7	81 ± 5.2	102 ± 10	100
motility	-	-	-	-	+	+++
Δ*lmcpbn*	30 ± 7.2	56 ± 4.6	87 ± 3.0	93 ± 6.7	109 ± 9.6	100
motility	-	-	-	-	+	+++

Table 3.6. Dose dependent effect of exogenous nitric oxide on promastigote viability. Parasites were incubated for 24 h in varying concentrations of sodium nitrite (a source of nitric oxide at acidic pH) and their motilies noted, after which cells were resuspended in medium containing MTT for 24 h in order to measure cell viability. Results are the means ± S.D. of 3 experiments performed in duplicate, and are expressed as a % of the control.

cell line + inhibitor	% infected PECs	amastigotes/infected PEC
wild type + L-NRME	24 ± 4.3	6.4 ± 0.9
wild type + D-NRME	32 ± 4.2	6.4 ± 0.6
Δ*lmcpbn* + L-NRME	1.0 ± 1.2	2.8 ± 1.6
Δ*lmcpbn* + D-NRME	1.3 ± 0.9	2.3 ± 1.8

Table 3.7. Effects of a nitric oxide synthase inhibitor on infectivity to PECs. PECs were infected using stationary phase promastigotes of wild type and Δ*lmcpbn* along with the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NRME) at 0.4 mM. Controls included the non-bioactive D-isomer of the inhibitor. Results give parasite load after 7 days and are the means ± S.D. of 2 experiments performed in duplicate.

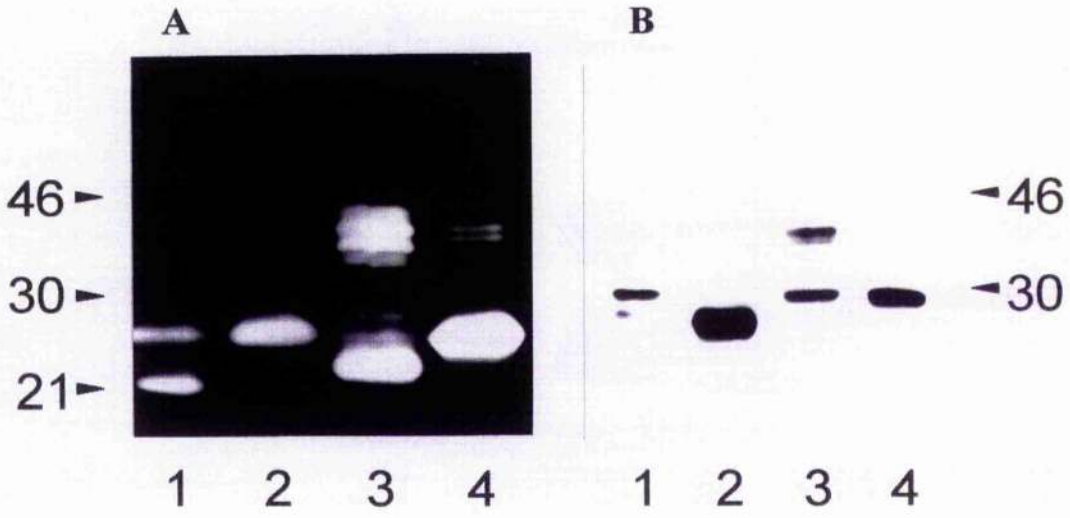


Figure 3.24. Analysis of LmCPb isoenzymes expressed in the *lmcpb* null mutant. Lysates from 1 x 10^7 (A) or 5 x 10^6 (B) stationary phase promastigotes were used for gelatin-SDS-PAGE (A) and Western blotting using anti-LmCPb antibody (B). Lanes 1, wild type *L. mexicana*; lanes 2, Δ*lmcpbg1* (t); lanes 3, Δ*lmcpbg2.8*; lanes 4, Δ*lmcpbg18* (t). Molecular mass markers are shown in kDa.

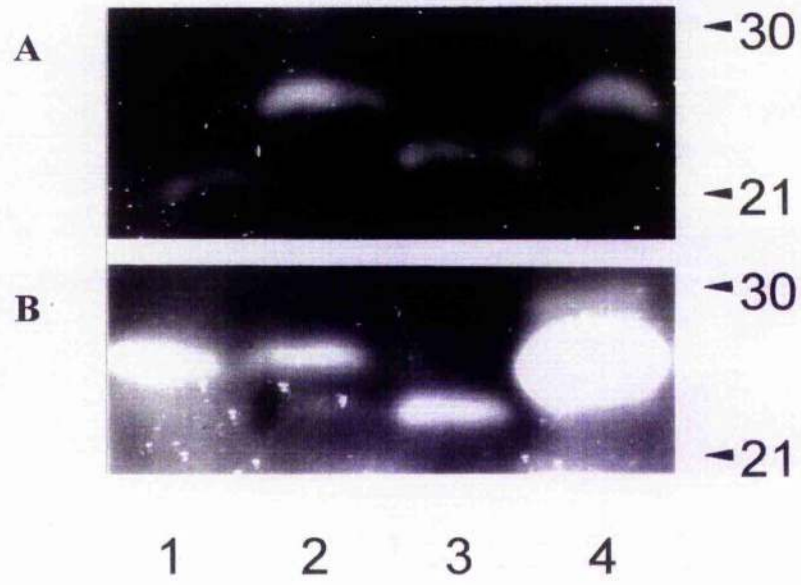


Figure 3.25. Substrate specificity differences between LmCPb isoenzymes. Activity of LmCPb isoenzymes expressed in the *lmcpb* null mutant towards two peptidyl amidomethylcoumarin fluorogenic substrates, BzFVR-NHMec (A) and SucLY-NHMec (B). Lane 1, wild type; lane 2, *lmcpbg1* (t); lane 3, *lmcpbg2.8*; lane 4, *lmcpbg18* (t). Molecular mass markers are shown in kDa.

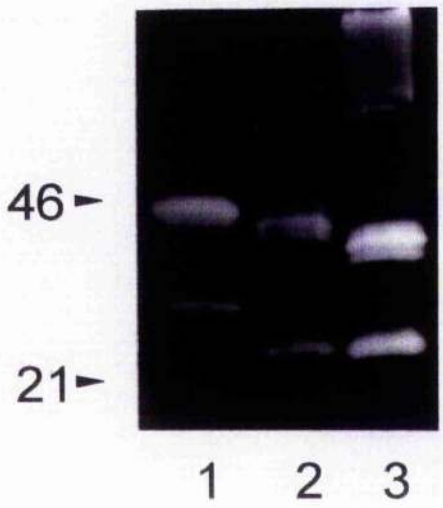


Figure 3.26. Gelatin-SDS-PAGE analysis of different stages of the re-expressor Δ *lmcpbg2.8* cultured *in vitro*. Lane 1, log phase promastigotes; lane 2, stationary phase promastigotes; lane 3, axenic amastigotes. Each track was loaded with lysate from 1 x 10^7 cells. The usual positions of molecular weight markers are indicated.

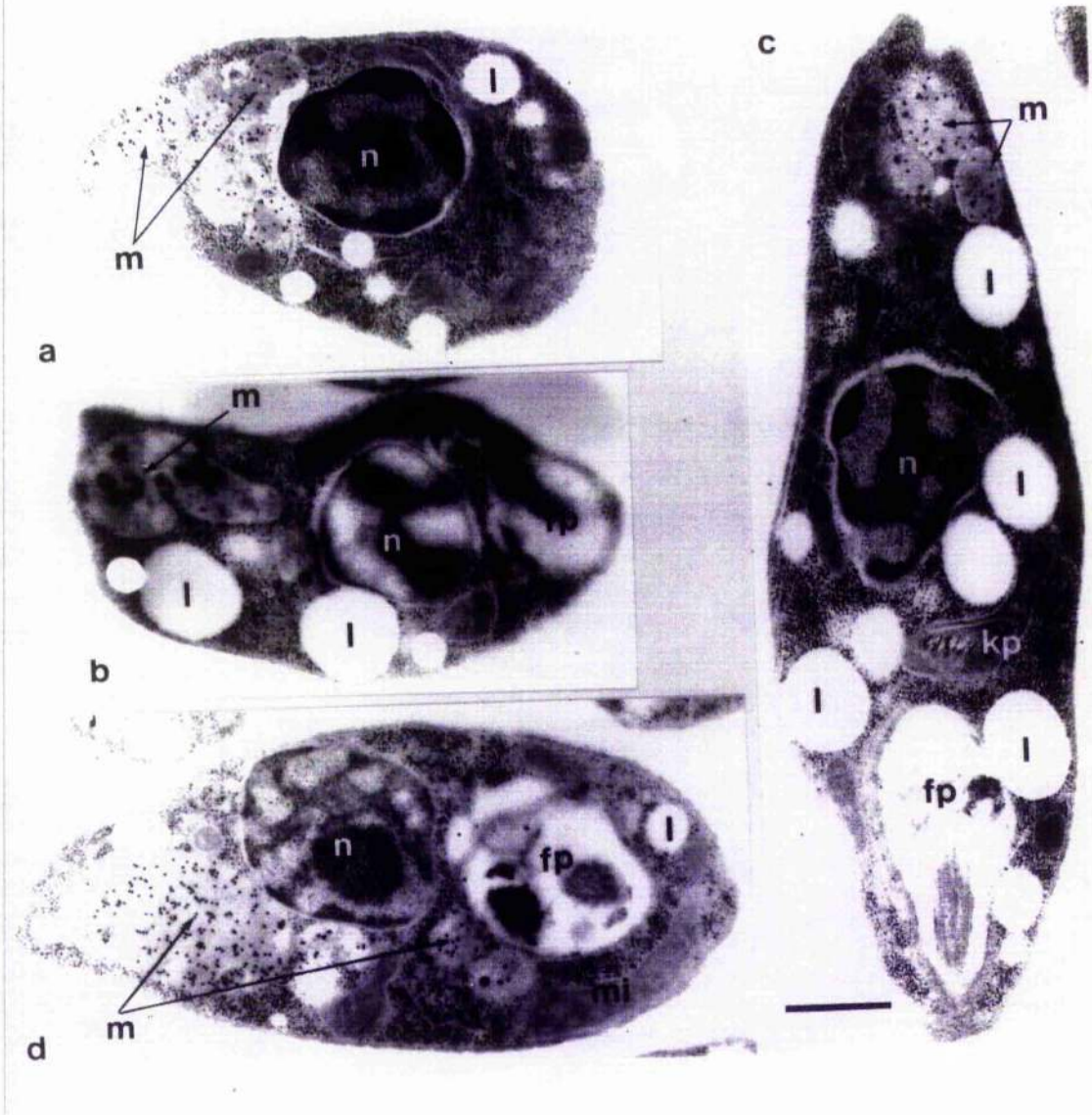


Figure 3.27. Localisation of LmCPb to the megasome (large lysosome) by immunogold labelling. Ultrathin sections of *L. mexicana* axenic amastigotes labelled with anti-LmCPb antiserum and goat anti-rabbit/10 nm gold conjugate. (a), wild type parasites; (b), Δ*lmcpbn*; (c), Δ*lmcpbg2.8*; (d), Δ*lmcpbg1* (t). m, megasome; mi, mitochondrion; fp, flagellar pocket; n, nucleus; l, lipid; kp, kinetoplast. Scale bar, 0.5 μm. Micrographs by L. Tetley, University of Glasgow.

cell line	% infected PECs	amastigotes/infected PEC
wild type	38 ± 9.0	5.2 ± 0.9
Δ*lmcpbn*	1.5 ± 0.2	1.7 ± 0.2
Δ*lmcpbg2.8*	29 ± 9.2	3.6 ± 0.4
Δ*lmcpbg1* (t)	3.5 ± 2.8	1.8 ± 0.9
Δ*lmcpbg18* (t)	4.6 ± 1.4	2.6 ± 0.4

Table 3.8. Infectivity of transfected lines to peritoneal exudate cells. PECs were obtained from peritoneal lavage of BALB/c mice and infected with stationary phase promastigotes of the *L. mexicana* lines at a ratio of 1:1. After 7 days incubation at 32°C, cells were fixed, stained with Giemsa's stain, and parasite load determined by counting 200 PECs. The values are the means ± SD from three independent experiments performed in duplicate. The infection rates with wild type and Δ*lmcpbg2.8* were highly significantly different from those of Δ*lmcpbn*, Δ*lmcpbg1* (t) and Δ*lmcpbg18* (t) (P values of <0.01), but not from each other; furthermore, the infection rate with Δ*lmcpbn* was significantly different from that of Δ*lmcpbg18* (t) (P value of <0.05) but not from that of Δ*lmcpbg1* (t). The mean values for amastigotes/infected PEC with wild type and Δ *lmcpbg2.8* were significantly different from each other (P value of <0.05) but also from those for Δ*lmcpbn*, Δ*lmcpbg1* (t) and Δ*lmcpbg18* (t) (P values of <0.01); the number of amastigotes/infected PEC was also significantly different with Δ*lmcpbn* and Δ *lmcpbg2.8*.

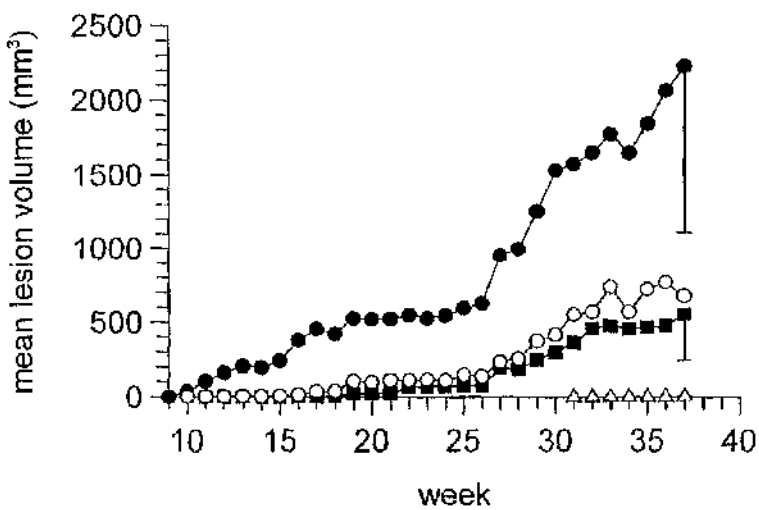


Figure 3.28. Infectivity of stationary phase promastigotes to BALB/C mice. Results show the mean lesion volume ± SD per group (3 mice). -●-, wild type; -△-, Δ*lmcpbn*; -■-. Δ*lmcpb*BL; -○-, Δ*lmcpan*. Note that Δ*lmcpbg2.8*, Δ*lmcpbg1* (t), Δ*lmcpbg18* (t) and Δ *lmcpbn*/Δ*lmcpan* (t) did not produce lesions at all within the 9 month duration of the experiment. The majority of error bars have been omitted for clarity, while some are so small they are obscured by the data points.

3.4. DISCUSSION

Western blot (Fig. 3.1) and gelatin gel (Fig. 3.2) analyses of Δ*lmcpbn* confirmed that no LmCPb protein was present in this cell line, and confirmed the previous suggestion that all of the high mobility CPs activities are encoded by the *lmcpb* array. Furthermore, LmCPb appears to be the only *L. mexicana* CP detectable using gelatin-SDS-PAGE.

Stationary phase promastigotes of the null mutants for *lmcpb* appeared not to form metacyclics of typical morphology as judged by their cell lengths (Fig. 3.4 and 3.5); this was reminiscent of the morphology displayed by Δ*lmcpan* (Chapter 2). Although the nulls were more susceptible to complement-mediated lysis than wild type (Table 3.1), the finding that log phase promastigotes of wild type were not more susceptible than stationary phase cells questions the validity of this as a test for metacyclics of *L. mexicana* - although it had been used previously (Bates and Tetley, 1993). Increased resistance to complement is a characteristic of *L. major* metacyclics (Mallinson and Coombs, 1989b). However in the same study it was found that there was little difference between the susceptibility of log and stationary phase promastigotes of *L. mexicana* (and that heat-inactivated serum agglutinated both stages), which is in agreement with the results presented in section 3.3.1.2. More reliable, perhaps, are the transmission electron micrographs, a method previously used to identify metacyclics in *L. mexicana* (Bates and Tetley, 1993). Similar studies on the null mutant line and wild type parasites showed surface coat differences between stationary phase promastigotes of the two lines (Fig. 3.6). Although the transfectants could be cultured under the conditions used for axenic amastigotes, they grew very poorly in comparison to wild type parasites, were bigger, and did not grow in clumps (Fig. 3.7 and 3.8). Furthermore their nuclease profiles suggested that they were not fully transforming to amastigotes (Fig. 3.9). Taken together these results point to a role for LmCPb in differentiation to both metacyclics and to amastigotes. The finding that re-expression of an internal copy of *lmcpb* (*g2.8*) in the null mutant failed to restore the wild type morphology argues that the morphological changes may not have been caused solely by the deletion of *lmcpb*, but perhaps by the transfection procedure or even the antibiotics used (which is

applicable also to Δ*lmcpan*, Chapter 2). It is possible, however, that the full range of LmCPb isoenzymes, rather than just one, must be re-expressed to restore wild type phenotype.

Since the null mutants will grow under the conditions used for axenic amastigotes, albeit more slowly and with atypical morphology, then it seems likely that the major role of LmCPb must be in interacting with the host cell. This correlates well with the observation that inhibitors of LmCPb such as peptidyl diazomethanes, which enter the parasites and inhibit LmCPb *in situ*, have little or no effect on the growth of the wild type lines as axenic amastigotes, but will kill *Leishmania* in macrophages (see Chapter 5).

The large reduction in infectivity to PECs resulting from the deletion of the *lmcpb* array and the reversal of this effect by re-expression of *g2.8* (Table 3.2) suggests that LmCPb plays an important part in enabling the parasite to survive against the microbicidal activity of the host cell and so is an important virulence factor. Furthermore the complementation of the phenotype by re-expression of *g2.8* confirmed that the infectivity differences between the null mutant and wild type parasites are indeed due to deletion of *lmcpb* and not simply attenuation resulting from the transfection procedure. In addition, Δ*lmcpan* is a useful control since it is a double knockout selected for using the same antibiotic resistance markers as Δ*lmcpbn*, but is still as infective as wild type parasites to PECs. The same was shown to be the case for the single knockout for *lmcpb*, Δ*lmcpbBL* (Table 3.2). The lack of enzyme activity for the *lmcpb* cDNA (Fig. 3.2, lane 4) may account for its inability to restore virulence. Unfortunately the number of Δ*lmcpbn* amastigotes per infected cell was found to vary between experiments, contending my initial conclusion that once the null parasite becomes established in the macrophage the infection proceeds normally.

One hypothesis aimed at explaining the successful invasion of a small percentage of the PECs by the null mutants is that these PECs were a distinct subpopulation less able to kill *L. mexicana*. It has previously been reported that resident peritoneal macrophages can be separated into 2 functional subsets (mature and immature) based on a number of criteria related to the state of cell maturity, such as surface antigen

expression (Plasman and Vray, 1993). In this study, it was attempted to distinguish different (infected and uninfected) macrophage subpopulations with an antibody to the F4/80 antigen (a "mature" macrophage marker, Plasman and Vray, 1993) by indirect immunofluorescence; this method, however, proved unsuccessful (data not shown).

Time course experiments showed that the null mutants initially infect PECs as successfully as wild type, but are then killed within 24 h (Fig. 3.12). This could be due to the nulls being less able to transform to amastigotes sufficiently rapidly (see section 3.3.1.2), or being less able to withstand the microbicidal action of the macrophage (see section 3.3.2.4), or both. This contrasts with findings using other transfectants, where *dhfr-ts* mutants infected PECs but did not replicate and began to be slowly destroyed after 48 h (Titus *et al.*, 1995). A similar result to this was found with gp63-deficient *L. amazonensis* variants (McGwire and Chang, 1994) which started to gradually disappear from culture 3 - 5 days post-infection; this process was partially delayed by genetic complementation of the variants.

Bates *et al.*, (1993) found that log phase cells initially infected as many macrophages as did stationary phase promastigotes but most of the log. phase cells were rapidly killed - a situation very similar, in fact, to the kinetics of infectivity of Δ*lmcpbn* to PECs. In contrast, I found that log phase cells initially infected a lower percentage of macrophages than did stationary phase promastigotes (Fig. 3.4), although stationary phase cells were not tested in parallel in the same experiment. The finding that increasing the parasite to macrophage ratio and/or exposure time results in more null mutant-infected macrophages (Fig. 3.15) may be due to the macrophage simply becoming overloaded and hence unable to kill all the parasites.

An 80% reduction in the number of macrophages infected could possibly account for the slow appearance of lesions in mice infected with the null mutants (Fig. 3.16). The finding that the nulls grew more slowly as axenic amastigotes (Fig. 3.7) than wild type, Δ*lmcpbBL and* Δ*lmcpan* (see Chapter 2) may also be relevant, but it is difficult to correlate *in vitro* growth with *in vivo*. Of interest is the finding that axenic amastigotes of the transfected lines were more infectious than promastigotes, even though they differed from wild type with respect to morphology and growth

characteristics. It seems therefore that the culture of these cells under the conditions used for amastigotes e.g. incubation at 32°C may have preadapted them for survival in a mammalian host. Δ*lmcpbg2.8* mutants also produced smaller lesions in mice (Fig. 3.16) although this line consistently produced lesions several weeks before those of Δ*lmcpbn*. The absence of neomycin selection in mice allowed *lmcpbg2.8* expression to drop substantially during lesion formation (Fig. 3.11, lane 6) which could account for the resulting smaller lesions. It may, however, be expected that if *g2.8* was advantageous to the parasite then these parasites would be selected for naturally without the need for neomycin pressure. A decrease in expression of pX-gp63 was observed in amastigote transfectants from macrophages (McGwire and Chang, 1994), although there was little loss of their plasmids; they suggested that the plasmid genes do not function efficiently in this form of the parasite (as opposed to promastigotes) to produce gp63, which may be of some relevance to the episomal expression of *g2.8* in lesion amastigotes.

LmCPb has been shown to be present at high concentrations in the extracellular milieu of the mouse lesion, prompting speculation that the proteinases may be involved in tissue damage associated with lesion formation (Ilg *et al.*, 1994). The occurrence of apparently normal lesions, albeit only from gross observations, in infections with *lmcpb* null mutants argues against an important role for the enzyme in lesion pathogenesis, although histological examination of lesion tissue may reveal otherwise.

The finding, however, that null lesion amastigotes were as infective as wild type to PECs (Fig. 3.19) suggests that the deficiency is not in the resistance of amastigotes to killing, but more likely that the major role of the CPs is in differentiation, or in enabling the promastigotes themselves to survive. It may be interesting to repeat these experiments using null mutant axenic amastigotes, since they produced lesions more quickly than promastigotes, and it may give an indication of how amastigote-like they are. However, the discovery that null mutant lesion amastigotes infected mice much more slowly than did wild type lesion amastigotes (Fig. 3.20) indicates that more factors are involved in survival in mice than in macrophages *in vitro* and that CPs play an important role. One possibility is in MHC II processing, as was recently reported (Leao *et al.*, 1995). It may be interesting to look at amounts of MHC II in null mutant-infected

macrophages, either by Western blotting or immunofluorescence, to confirm this. It has also been reported that CPs may modulate cytokine responses in a way that is beneficial to the parasite's survival (Finkelman and Urban, 1992). Therefore it could be useful to look at cytokine responses in mice infected with different mutants. There is little evidence to suggest that the amastigotes secrete their CPs (Coombs, 1982; Pupkis *et al.*, 1986); in this study it was attempted to detect circulating LmCPb in serum from infected mice by Western blotting, and also use infected mouse serum as an antiserum to detect LmCPb in parasite lysates; both experiments, however, proved unsuccessful.

It is possible that the parasite may be able to compensate to an extent for the loss of LmCPb with other factors, most likely other proteinases. LmCPa is the most closely related *L. mexicana* CP to LmCPb and so appeared to be a good candidate for this role. The finding that the *lmcpa/lmcpb* double null mutant had the same infectivity profile to macrophages as the *lmcpb* null (Fig 3.2, B and C) shows that if another proteinase is partially compensating for *lmcpb* it is not *lmcpa*. Interestingly the double null did not produce lesions in mice within the maximum time permitted for the experiment (Fig. 3.16; E and F). This implies that both genes are needed for survival in mice. What is difficult to explain is that the double null re-expressing *g2.8* produced an active enzyme but did not restore infectivity to PECs. Could this indicate a synergistic role for *lmcpa* in conjunction with *lmcpb*?

The *L. mexicana* complex differs from other *Leishmania* species in that the amastigotes not only contain numerous megasomes but also reside in very large PVs, the size increasing as the number of parasites increases (Pupkis *et al.*, 1986; Rabonovitch, *et al.*, 1986; Antoine *et al.*, 1990). It had been postulated that the amastigote CPs are mainly responsible for this increase in vacuole size, either by releasing amines that would raise the pH and cause the vacuole to expand due to osmotic stress (Coombs, 1982), or by degrading PV membrane proteins internalised by the amastigotes (Leao *et al*, 1995). However, the data presented in section 3.3.2.2 clearly indicate that parasites lacking the *lmcpb*-encoded CPs also reside in large PVs, although a significant difference in mean PV diameter per amastigote was found with wild type and Δ*lmcpbn*. This finding is consistent with the report that infected macrophages treated with CP

inhibitors for 20 h have only slightly smaller PVs (Leao *et al.*, 1995) which also suggests that indeed the parasite CPs contribute only slightly to the large PV sizes observed with *L. mexicana* infections, although a longer incubation time with the inhibitors may reveal a bigger effect.

Infected and uninfected macrophages were compared with respect to their enzyme activities using an 'Api Zym' enzyme detection kit (Table 3.4) and substrate-SDS-PAGE (Fig. 3.23), but in agreement with previous findings macrophage lysosomal enzyme activities were unaffected after infection (Antoine *et al*, 1987), as were proteinase activities (Prina *et al*, 1990). It seems unlikely therefore that amastigote CPs have a role in inactivating host cell lysosomal enzymes.

A major component of the respiratory burst exhibited by macrophages is hydrogen peroxide (Lewis and McGhee, 1992). Previous studies have indicated that stationary phase promastigotes do trigger a respiratory burst but may be more resistant to its consequences (Mallinson and Coombs, 1989). Stationary phase promastigotes of wild type parasites and Δ*lmcpbn* were found to be equally susceptible/resistant to hydrogen peroxide and so it seems unlikely that the respiratory burst is responsible for the specific decline in null mutant-infected macrophages over 24 h. It may be expected that if the respiratory burst was responsible then the nulls would have been killed instantly or within a couple of hours (see below). It is possible that dead parasites are taken up by macrophages and it takes time to digest them. However only parasites that looked intact i.e. had a distinct nucleus and kinetoplast, were counted (since at early stages of infection a definite parasite cell membrane was difficult to see).

Aside from reactive oxygen intermediates, it has recently become apparent that nitric oxide (NO) also has an important role in the microbicidal activity of macrophages. NO from activated macrophages has been shown to be cytostatic or cytotoxic for a variety of pathogens including *Leishmania* (Liew *et al.*, 1990) and *T. cruzi* (Munoz-Fernandes *et al.*, 1992). Moreover, immature macrophages have been found to be highly susceptible to *T. cruzi* infection, a susceptibility which was associated with a low production of both NO and TNF-α (Plasman *et al.*, 1994).

In a study by Assreuy *et al* (1994) superoxide production by activated

macrophages was found to peak 1-2 h post-stimulation and then steadily decline. In contrast NO was not produced until 6 h post-stimulation, increasing rapidly until 24 h and reaching a plateau by 48 h. The timing of this seemed to correspond to the decline in null mutant-infected macrophages over 24 h and so the relative susceptibilities of wild type and Δ*lmcpbn* stationary phase promastigotes to NO derived from sodium nitrite were compared (Table 3.6). Again there was no significant difference in susceptibilities. The nitric oxide synthase inhibitor L-NRME did not increase the number of null mutant-infected macrophages (although it was not confirmed that this enzyme was actually being inhibited). These data suggest that the nulls simply cannot survive in macrophages rather than are killed by a specific microbicidal activity; or that the null mutants' surface coat triggers a different and lethal route of receptor-mediated phagocytosis compared with the wild type parasites (see section 1.6.1).

When the individual *lmcpb* isoenzymes *g1*, *g2.8* and *g18* were re-expressed in the null mutant, the re-expressed gene products showed activity toward gelatin and fluorogenic substrates, but the enzymes differed both in their relative activities toward the different substrates and also their physical characteristics as displayed by the mobilities on gels. These differences correlated with evidence from biochemical studies which showed that the isoenzymes have distinct properties (Robertson and Coombs, 1990, 1994). CP activity found in Δ*lmcpbg1* (t) co-migrates on gelatin gels with one of the 2 major activities characteristically present in stationary phase (metacyclic) promastigotes of wild type cells (Fig. 3.24, A). The protein also co-migrates on Western blots with a protein in stationary phase wild type promastigotes (Fig. 3.24, B). The results correlate with Northern blot data (Appendix B: Mottram *et al.*, 1997) demonstrating levels of mRNA transcribed from *g1* are elevated in stationary phase promastigotes relative to multiplicative promastigotes or amastigotes. Taken together these results suggest that some of the proteinase activity in wild type metacyclic promastigotes is encoded by *g1* of the *lmcpb* array. The differences observed between the relative activities of wild type and Δ*lmcpbg1* (t) toward the two fluorogenic substrates (Fig. 3.25, lanes 1 and 2) suggest however that the lower mobility activity in wild type cells may comprise more than one gene product. It has been shown previously

that some activity bands detected using gelatin gels do indeed represent several isoenzymes (Robertson and Coombs, 1990). Overall the data indicate that expression of *g1* of the array, which is characterised by the lack of sequence encoding a full length CTE, is stage regulated and occurs primarily in the infective metacyclic promastigote. The stage specific expression of *g1* suggests that it plays an important role at this stage of the life cycle, perhaps involving the interaction of the metacyclic promastigote with its sandfly vector or the process of differentiation itself. It could also be important for the early events associated with invasion of a macrophage following inoculation into a mammal. The finding that Δ*lmcpbg1* (t) was less able to survive in macrophages than the wild type cells (Table 3.7) shows that *g1* is not able to complement the loss of the *lmcpb* array under these circumstances and in this way differs from *g2.8*. This lack of complementation is not due to the lack of a CTE as *g18*, although apparently expressed in amastigotes naturally, is also unable to restore wild type-infectivity levels to the null mutant, although *g18* did significantly increase infectivity of Δ*lmcpbn*. This indicates that the individual genes of the array indeed perform different functions, a suggestion supported by the observed differences in substrate specificities. The precise timing of expression of the different isoenzymes may be an important factor for parasite invasion and survival in the macrophage.

Δ*lmcpbg2.8* and Δ*lmcpbg18* (t) exhibit a number of lower mobility bands in gelatin gels (30-40 kDa) and also larger molecular weight species in Western blots (about 38 kDa) (Fig. 3.24). By contrast, similar larger molecular mass proteins were not seen with Δ*lmcpbg1* (t), which exhibited just the fully processed protein. These lower mobility activities may be precursors of the mature CPs that are activated *in situ* following gel electrophoresis. Non-covalent complexes between lysosomal cathepsin B and its pro-peptide that are formed during autolytic maturation of the precursor have been described (Mach *et al.*, 1994). These inactive complexes are believed to keep the proteinase in an inactive state until it is secreted, when it is activated by local acidification. It is possible that either the LmCPb pro-region or the CTE (or both) could be acting as an inhibitory peptide in a similar fashion, and the processing of the gel subsequent to electrophoresis provides conditions in which the inhibitory peptide is

removed. The finding that *g1* lacks a full CTE and does not appear to produce these putative precursor forms that are activated *in situ* provides some evidence that it may be this domain that is important with regard to any such processing events. Indeed processing of the CTE may be a pre-requisite for correct and efficient processing of the pro-domain. Previous speculations on the function of the CTE have included a role in the targeting of the protein to lysosomes, a theory strengthened by the finding that a trypanosome CP apparently lacks mannose-6-phosphate which therefore cannot play a role in the enzymes' trafficking (Cazzulo *et al.*, 1990). The finding using immunoelectron microscopy that the mature LmCPb enzyme in Δ*lmcpbg1* (t), which has a truncated CTE, is located in lysosomes that are typical of the amastigotes of *L. mexicana* shows that a full length CTE is not essential for successful intracellular trafficking. It is clearly also not essential for the activity of these CPs, as *g1* is highly active toward gelatin (Fig. 3.24, A) and other substrates (Fig. 3.25). This is consistent with previous reports which demonstrated that recombinant CPs from *T. cruzi* and *T. brucei* lacking the CTE can be expressed as active enzymes (Pamer *et al.*, 1991; Eakin *et al.*, 1993).

When the *lmcpb* cDNA was expressed in the null mutant cell line it produced a protein of the predicted molecular size (section 3.3.1.1, Fig 3.1), indicating correct processing, but it was inactive toward gelatin under the standard condition tested (Fig. 3.2). This contrasts with the results obtained for the genes *g1*, *g2.8*, and *g18* which are expressed in the null mutant to produce active CPs. The product of the *lmcpb* cDNA expressed in *Escherichia coli* had previously been shown to be inactive (Wolfram *et al.*, 1995), so it is possible that the sequence differences between the products of *g1*, *g2.8*, and *g18*, and the cDNA, are important in determining enzyme activity and/or substrate specificity. Overall it appears that the different genes encode not only different mobility proteinases, but proteinases with different substrate specificities, and confirms the working hypothesis that the different genes of the array encode different enzyme activities, and that possibly all 18 active isoenzymes perform different roles in the parasite. The observed stage regulation of enzyme activity seen with Δ*lmcpbg2.8* (Fig. 3.26) is difficult to explain, but since the *g2.8* copy is being re-expressed on an episome

then it is possible that different stages of the parasite vary in their ability to transcribe it in its unnatural form, giving rise to the apparent stage regulation.

The results presented in this chapter implicate the *lmcpb* CPs as non-essential virulence factors, involved in both differentiation and intracellular survival within the mammalian host. They also imply that individual genes of the array encode different enzyme activities. The very high LmCPb activity in *L. mexicana* amastigotes in comparison with promastigotes was suggestive of a crucial role for the enzymes in the parasite's survival in macrophages and so their suitability as a drug target. Clearly, however, being stage regulated and at high activity is not sufficient evidence to confirm the suitability of an enzyme for chemotherapeutic attack. The results of this chapter show unambiguously that LmCPb is not essential for parasite survival in the BALB/c host (although it may be useful to investigate this in other animals) and therefore that even very specific inhibitors of LmCPb are unlikely to be effective as antileishmanial drugs.

CHAPTER FOUR

ANALYSIS OF THE CATHEPSIN B-LIKE CYSTEINE PROTEINASE (*LMCPC*) OF *LEISHMANIA MEXICANA*

4.1. INTRODUCTION

4.1.1. The *lmcpc* gene products

As detailed in Chapter 3, *L. mexicana* has a number of stage-regulated CPs which in terms of their structure and activities are largely similar to the mammalian CP cathepsin L. Most CPs of parasitic protozoa have been found to be of this type (see section 1.2.2.2), although some resembling (in terms of substrate utilisation) the related mammalian CP, cathepsin B, have been reported in *Entamoeba* (Scholze and Schulte, 1990; and references therein). The predicted amino acid sequences of the enzymes differed significantly, however, from that of cathepsin B (Reed *et al.*, 1993).

In addition to the *lmcpa* and *lmcpb* gene products, *L. mexicana* has a distinct group of CPs designated group D CPs (Robertson and Coombs, 1993). Unlike the *lmcpb* gene products, these enzymes do not hydrolyse gelatin in substrate SDS-PAGE gels and are characterised by hydrolysing efficiently peptides with the moiety Phe-Val-Arg. The 31 and 33 kDa group D CPs were found to be hydrophobic, indicating that they could be membrane associated; furthermore the N-terminal amino acid sequence data for the group D CPs indicated that they are homologous to mammalian cathepsin B and are therefore distinct from the products of the two *L. mexicana* cathepsin L-like CP genes previously described, *lmcpa* and *lmcpb* (Robertson and Coombs, 1993). The group D CPs appeared to be products of a third class of *L. mexicana* CP gene. Although their molecular weights differ from those of cathepsin B, it was proposed that this difference may be related to the hydrophobic nature of the enzymes (Robertson and Coombs, 1993).

The *lmcpc* gene was recently cloned and sequenced (Bart *et al.*, 1995). The amino acid sequence derived from the purified group D enzymes was almost identical to that predicted from the gene sequence. As *lmcpc* is a single copy gene, it was suggested that it was highly likely that *lmcpc* encodes the group D CPs. The mature domain of

LmCPc was found to have a high level of homology to the cathepsin B class of CPs, having 53% identity with the *Schistosoma mansoni* cathepsin B and 52% indentity with the human homologue. The predicted size of the mature LmCPc enzyme, 27 kDa, was however, smaller than that determined by SDS-PAGE for the two major group D enzymes (31 and 33 kDa). As LmCPc does not have a CTE which could be differentially processed in the 31 and 33 kDa enzymes, the authors suggested glycosylation as most likely the explanation for both the large molecular masses and the presence of two products of different electrophoretic mobilities encoded by the single copy *lmcpc* gene (Bart *et al.,* 1995). Although the *lmcpc* mRNA was present in all life cycle stages it was found to have elevated expression in the multiplicative promastigote form.

4.1.2. Aims

In keeping with the rationale of investigating the roles of *L. mexicana* CPs through targeted gene disruption, this part of my work focussed on analysing the biological phenotype of the null mutants for *lmcpc*, which were created by colleagues in the Wellcome Unit of Molecular Parasitology (WUMP), University of Glasgow (Appendix B: Bart *et al.*, 1997). The proteinases of these cells were examined, as well as their ability to navigate the parasite life cycle *in vitro*, infect explanted peritoneal macrophages, and form lesions in mice. The results give rise to some interesting questions regarding the role of LmCPc in parasite virulence and pathogenicity.

4.2. MATERIALS AND METHODS

4.2.1. Parasites

4.2.1.1. Transfected cell lines of L. mexicana

The lines used in this chapter were created by colleagues in WUMP, and are summarised in Table 4.0. Details of the transfection procedures used can be found in Appendix B (Mottram *et al.*, 1996, Bart *et al.*, 1997). All lines used in this study were parasites derived from a BALB/c mouse lesion subsequent to the transfection procedure, unless designated otherwise by the letter (t) following the cell line name.

Table 4.0. Transfected lines of *L. mexicana* used

cell line	gene deletion(s)	antibiotic used for selection
Δ*lmcpbn*	null mutant for *lmcpb*	hyg/phleo
Δ*lmcpc*	null mutant for *lmcpc*	sat/puro
Δ*lmcpcPC*	null mutant for *lmcpc* re-expressing *lmcpc*	neo

Abbreviations: hyg, hygromycin; phleo, phleomycin; sat, nourseothricin hydrosulphate; puro, puromycin; neo, neomycin

4.2.1.2. Cell culture

The three major developmental stages of *L. mexicana* (MNYC/BZ/62/M379) were cultured and harvested as described in Chapter 2, section 2.2.1, with the exception that the following antibiotics were added, singly or in combination as appropriate (see Table 4.0), for selection of transfectants: nourseothricin hydrosulphate (Hans-Knoll Inst., Thuringen, Germany) at 25 μg/ml, puromycin (Sigma) at 10 μg/ml, or neomycin (Geneticin, BRL) at 25 μg/ml. Doubling times were calculated as in Chapter 3, section 3.2.1.2, and t-tests were used to determine the statistical significance of any observed differences in doubling times between the different lines.

4.2.2. Proteinase assays and analyses

Substrate SDS-PAGE and Western blotting were carried out essentially as described in Chapter 2, section 2.2.2. The primary antibodies used were 1:500 anti-LmCPb group C CP (Chapter 3, section 3.2.2.2) and 1:50 anti-PEP662 antiserum (Appendix: Bart *et al.*, 1997).

4.2.3. Infection of peritoneal exudate cells and BALB/c mice

The infectivity of stationary phase promastigotes to PECs and female BALB/c mice was determined as described in Chapter 2, sections 2.2.4. and 2.2.5. respectively.

4.3. RESULTS

4.3.1. The phenotype of mutants null for *lmcpc*

4.3.1.1. Growth and differentiation

The null mutant lines grew significantly more rapidly than the wild type parasites as promastigotes *in vitro* (Fig. 4.1), where the approximate doubling times of wild type and Δ*lmcpcn* were 11.0 ± 2.2 and 6.8 ± 1.4 h, respectively (P value of <0.1). In contrast to wild type parasites, few small promastigotes (the putative metacyclic stage) were found in stationary phase cultures of Δ*lmcpcn* (Fig. 4.2). Despite this, Δ *lmcpcn* successfully transformed to axenic amastigotes which resembled those of wild type cells in both growth characteristics (Fig. 4.3) and morphology (Fig 4.4).

4.3.1.2. Proteinases

The lack of LmCPc protein in Δ*lmcpcn* was confirmed by Western blotting using anti-PEP662 antiserum, which recognised a major band (30-33 kDa) in lysates of stationary phase promastigotes of wild type cells (Fig. 4.5, lane 1). Lower loading confirmed that this comprised two bands as expected (Robertson and Coombs, 1993). The null mutant Δ*lmcpcn* (t) lacked these proteins (lane 2), whereas a major band was recognised in lysates of the re-expressor Δ*lmcpcPC* (t) (lane 3), which had the same mobility as the band detected in wild type cells (lane 1), and was at a similar level of expression.

Analysis of the null mutant by gelatin-SDS-PAGE showed there to be a significant difference between the proteinase activities detected in lysates of wild type parasites and Δ*lmcpcn*. There was much less high mobility CP activity in Δ*lmcpcn* stationary phase promastigotes (Fig. 4.6, lane 4) compared with wild type promastigotes (lane 1), although activities >60 kDa (not CPs) were similar in the two lines.

Western blot analysis with anti-LmCPb (group C) antiserum (Fig. 4.7) revealed, in agreement with the gelatin gel analysis, that Δ*lmcpcn* stationary phase promastigotes (lane 3) had comparatively less LmCPb than the same form of wild type parasites (lane 1), especially with regards to the higher mobility band of the doublet (arrowed). In contrast Δ*lmcpcn* axenic amastigotes (lane 4) had a similar amount of LmCPb to wild

type axenic amastigotes (lane 2).

4.3.1.3. Infectivity

A major difference in phenotype was detected between Δ*lmcpcn* and wild type cells in that the infectivity of the null mutant to explanted mouse peritoneal exudate cells was greatly reduced (Table 4.1). Re-expression of *lmcpc* in the null mutant (Δ *lmcpcPC*), however, increased infectivity to PECs (Table 4.1). This finding suggested that Δ*lmcpcn* (t) would be much less infective to mice than wild type cells. In the two experiments carried out, however, stationary phase promastigotes of Δ*lmcpcn* produced lesions one week before (Fig. 4.8, A) and 3 weeks after (Fig. 4.8, B) the wild type parasites. Thereafter, however, lesions due to Δ*lmcpcn* grew more slowly than those due to wild type parasites. Analysis of the parasites isolated from these lesions confirmed that the mutants had the same genotype at the *lmcpc* locus as the inoculated mutants (Appendix B: Bart *et al.*, 1997).

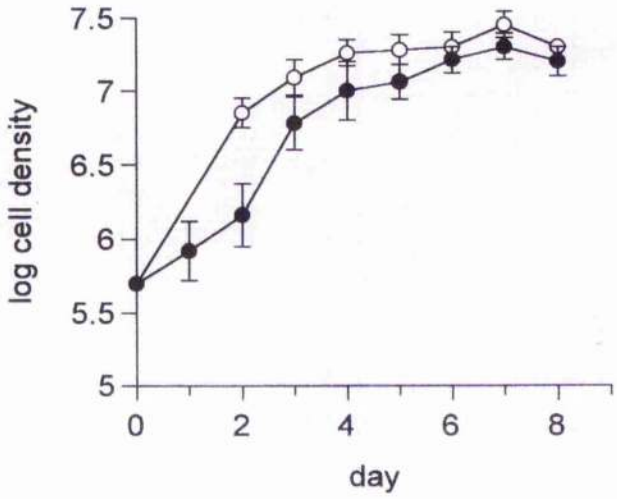


Figure 4.1. Typical *in vitro* growth curves of *L. mexicana* promastigotes. Cultures of wild type (-●-) and Δ*lmcpcn* (-○-) were initiated at 5 x 10^5 cells/ml in complete HOMEM medium and incubated at 25°C, with cell counts being performed on consecutive days. The results are the means ± SD from 3 independent cultures for each line.

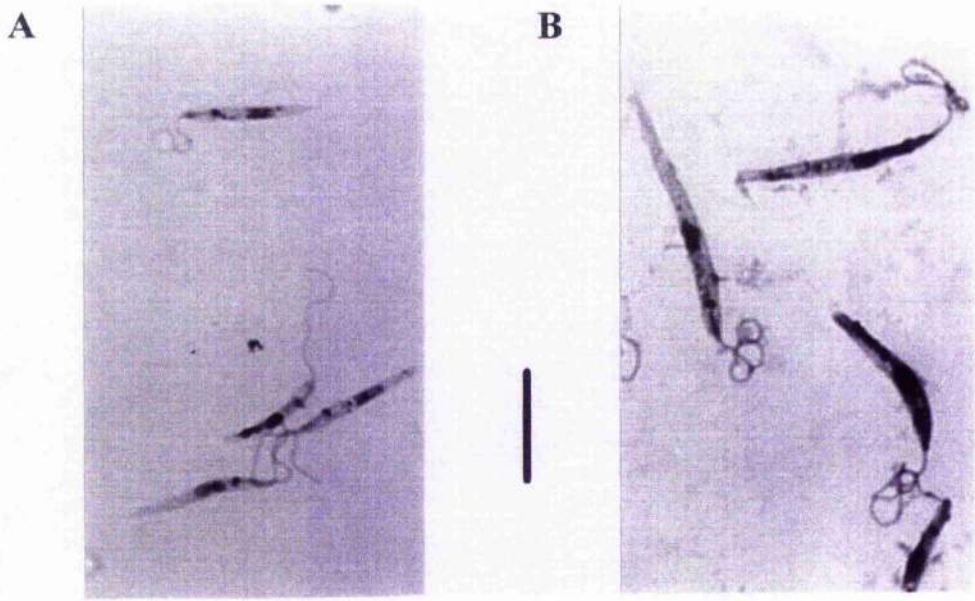


Figure 4.2. Light micrographs of stationary phase promastigotes. Wild type (A) and Δ*lmcpcn* (B) cells were fixed in methanol and stained in 10% Giemsa's stain. There was a clear difference in cell size between the two lines, with the Δ*lmcpcn* promastigotes (B) appearing much bigger then the majority of wild type cells (A). The scale bar represents 10 μm.

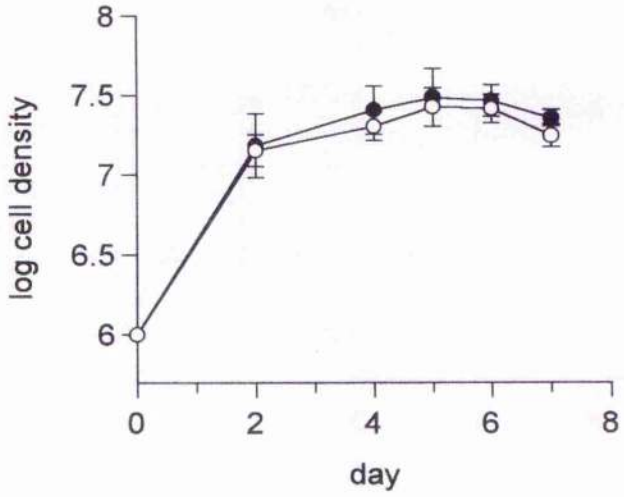


Figure 4.3. Typical *in vitro* growth curves as axenic amastigotes of transfected lines. Cultures were routinely initiated using stationary phase promastigotes at 1 x 10^6 cells/ml in complete SDM, pH 5.5, and incubated at 32° C. Cell counts revealed no difference in growth rates and final cell density between wild type (-●-) and Δ*lmcpcn* (-○-) axenic amastigotes.

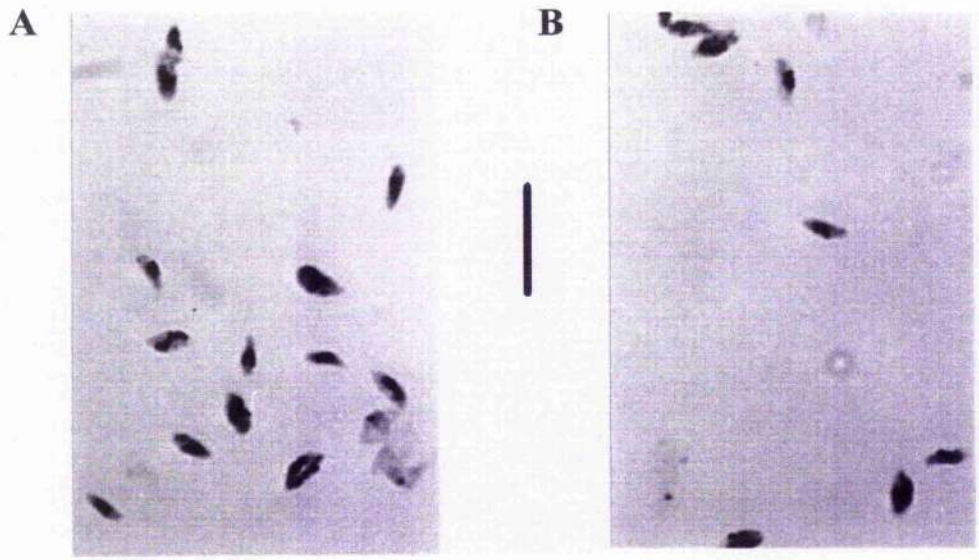


Figure 4.4. Light micrographs of Giemsa-stained axenic amastigotes; (A), wild type parasites, (B), Δ*lmcpcn*. There was no difference in cell size between the two lines, with the Δ*lmcpcn* axenic amastigotes (B) appearing morphologically identical to wild type cells (A). The scale bar represents 10 μm.

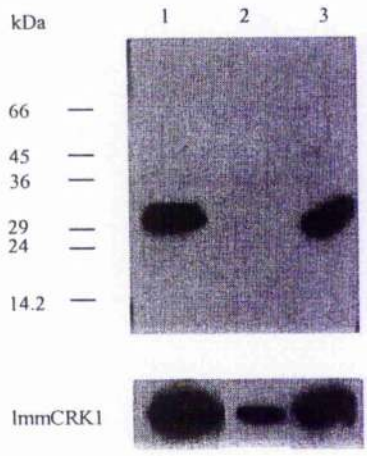


Figure 4.5. Western blot analysis of *lmcpc* null mutants. Anti-PEP662 antiserum was used to assess the presence of LmCPc in wild type *L. mexicana* (lane 1), Δ*lmcpcn* (lane 2) and the re-expressor Δ*lmcpcPC* (lane 3). To check for protein loading (lower panel), a duplicate blot was probed with a polyclonal antiserum raised to CRK1 (Mottram *et al.*, 1993). Sizes were derived from Bio-rad molecular weight markers. (Blot by G. Bart, University of Glasgow).

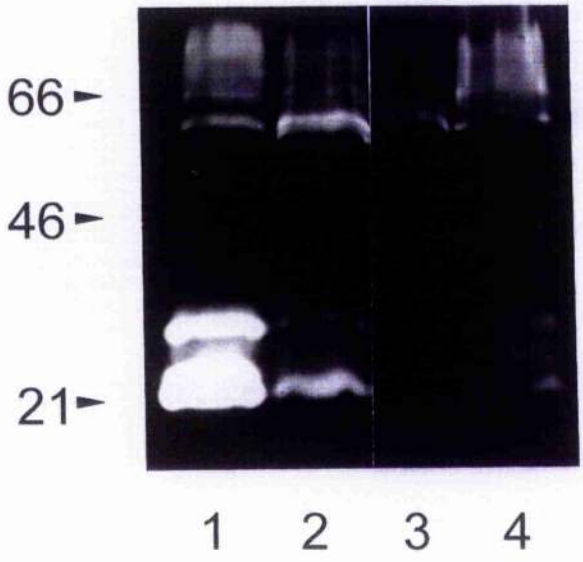


Figure 4.6. Gelatin-SDS-PAGE analysis of cell lines. Lane 1, wild type *L. mexicana*; lane 2, Δ*lmcpan*; lane 3, Δ*lmcpbn*; lane 4, Δ*lmcpcn*. Lanes were loaded with lysates containing 10^7 stationary phase promastigotes. The positions of molecular weight markers are indicated in kDa.

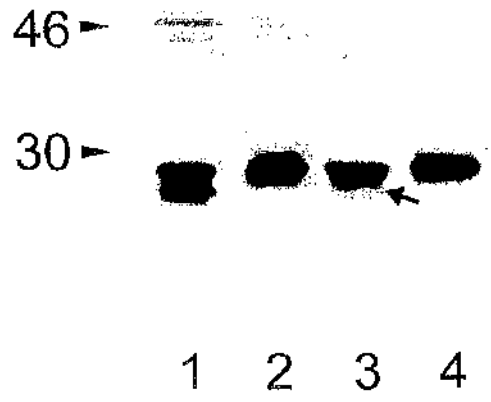


Figure 4.7. Western blot probed with anti-LmCPb antiserum. Lane 1, wild type stationary phase promastigotes; lane 2, wild type axenic amastigotes; lane 3, Δ*lmcpcn* stationary phase promastigotes; lane 4, Δ*lmcpcn* axenic amastigotes. An equal number of cells (5 x 10^6) was loaded in each lane. The high mobility LmCPb band (lane 3, arrowed) was less intense in lysates of Δ*lmcpcn* than in those of wild type (lane 1).

cell line	% infected PECs	amastigotes/infected PEC
wild type	38 ± 14	5.9 ± 0.8
Δ*lmcpbn*	2.0 ± 1.3	2.9 ± 1.0
Δ*lmcpcn*	4.7 ± 2.9	4.0 ± 0.6
Δ*lmcpcPC* (t)	20 ± 11	5.2 ± 1.4

Table 4.1. Infectivity of transfectants to macrophages. PECs were obtained from peritoneal lavage of BALB/c mice and infected with stationary phase promastigotes of the various cell lines at a promastigote to PEC ratio of 1:1. After 7 days incubation at 32°C, cells were fixed, stained with Giemsa's stain, and parasite load determined by counting 200 PECs. The values presented are means ± SD from 3 independent experiments performed in duplicate. The infection rates with Δ*lmcpbn* and Δ*lmcpcn* were significantly different from those with wild type parasites (P values of <0.02), but not from each other. The infection rate with Δ*lmcpcPC* (t) was significantly different from that with Δ*lmcpcn* (P<0.1), but not significantly different from the wild type infection rate. Mean values for amastigotes/PEC with Δ*lmcpbn* and Δ*lmcpcn* were significantly different from those with wild type parasites (P values of <0.02 and <0.05 respectively), but not from each other.

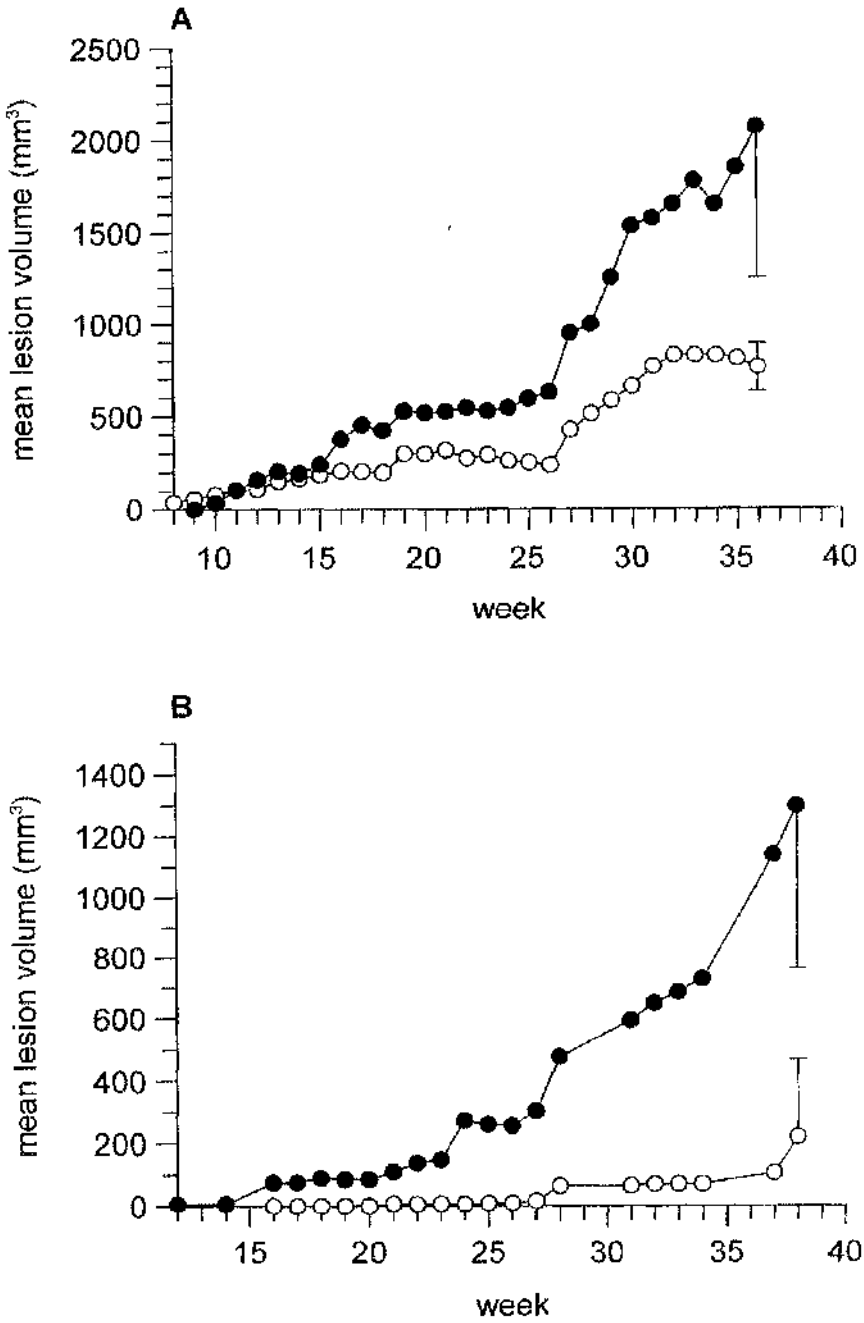


Figure 4.8. Infectivity to BALB/c mice. Subcutaneous lesions in the rumps of female BALB/c mice resulting from an inoculum of 5 x 10^6 stationary phase promastigotes were measured at weekly intervals using a micrometer and the mean lesion volume ± SD per group of 3 mice calculated. The lines inoculated were: A, wild type (-●-) and Δ *lmcpcn* (t) (-○-); B, wild type (-●-) and Δ*lmcpcn* (-○-). The majority of error bars have been omitted for clarity.

4.4. DISCUSSION

Stationary phase promastigotes of Δ*lmcpcn* did not form metacyclics of typical morphology; they were, however, very similar to wild type promastigotes with respect to differentiation to and growth as axenic amastigotes (it was attempted to examine how amastigote-like the axenic amastigotes of the *lmcpc* nulls were by examining their nuclease profiles; however technical difficulties with the nuclease gels meant that unfortunately useful data were not obtained). These results are very similar to those found with Δ*lmcpan* (Chapter 2). It has to be considered, therefore, whether the formation of atypical metacyclics with Δ*lmcpan* and Δ*lmcpcn* is a result of the transfection procedure itself, rather than a direct result of the gene deletions. (It should be noted that the same morphological differences were apparent whether the lines had been passaged through mice or not, and that the reduced activity of the *lmcpb* proteinases in these two lines may be related to the atypical morphology observed).

The results presented in this chapter indicate that LmCPc plays a role in enabling *L. mexicana* to live within macrophages *in vitro*, and in this respect is similar to LmCPb in being a virulence factor (see Chapter 3). However it differs from LmCPb in that it appears to be less important in aiding survival of the parasite in BALB/c mice. Δ*lmcpcn* stationary phase promastigotes were similar to those of Δ*lmcpan* (Chapter 2) in that they produced lesions after approximately the same number of weeks post-inoculation as did wild type parasites in each experiment. This contrasts greatly with the results obtained with Δ*lmcpbn* stationary phase promastigotes, which took at least 24 weeks longer to produce lesions, and in some cases did not produce any (Chapter 3). Clearly though, the lesions resulting from inoculation of Δ*lmcpcn* stationary phase promastigotes did not grow as quickly as those produced by wild type parasites. These results indicate that the two CPs have different roles in the parasite and also suggest that the infectivity to PECs is not a reliable indicator of the infectivity of the mutant lines to animals; perhaps because more factors are involved *in vivo* than *in vitro*. The findings on the relative infectivity of the *lmcpc* null mutants (which produced lesions in mice almost as rapidly as wild type parasites) and *lmcpb* null mutants (which produced lesions six months later than wild type parasites) are consistent with *lmcpb* having a role in immune evasion, and

lmcpc perhaps not being involved in this respect. The lack of a dramatic difference in infectivity to mice between Δ*lmcpcn* and wild type parasites does not mean that *lmcpc* plays no role in intracellular survival *in vivo*; BALB/c are highly susceptible to infection with *L. mexicana* and the inoculation dose was much higher than that likely to be delivered by a sand fly. It may be worthwhile comparing the infectivity of Δ*lmcpcn* to less susceptible mouse strains and in lower numbers.

A possible explanation for the observed lack of necessity *in vivo* of *lmcpc* may be compensation by other enzymes. The way in which the parasite is able to overcome this loss of *lmcpc*, however, is less clear. The decreased activity of the LmCPb CPs detected using gelatin-SDS-PAGE, in stationary phase promastigotes of the *lmcpc* null mutants obtained by transformation of the amastigotes isolated from mice (Fig. 4.6) indicates that these enzymes are not compensating for the loss of *lmcpc* and moreover suggests that there could be a role for LmCPc in the maturation of *lmcpb* gene products to give active enzymes. The absence of increased activity of >60 kDa proteinases (Fig. 4.6) suggests that if it is these enzymes that are compensating for the lack of LmCPc then the wild type levels are sufficient to fulfil the parasite's requirements. There may, of course, be other proteinases not detected using gelatin-SDS-PAGE that are up-regulated in the *lmcpc* null mutants and compensating for the absence of LmCPc.

The finding that the *lmcpc* null mutant infected far fewer macrophages *in vitro* than did wild type parasites confirms that *lmcpc* plays some part in the mammalian infective stages and that it is somehow involved in protecting the parasite from the microbicidal activity of at least some populations of macrophages, either at the invasion stage or in maintaining infection. From the results found with Δ*lmcpcPC* (Table 4.1), it would appear that expression of LmCPc enables the infection of a greater number of particular sub-populations of macrophages present in the PEC population; this hypothesis warrants further investigation.

The finding that LmCPc of *L. mexicana* has some unusual features (Robertson and Coombs, 1993), indicating that it may be membrane-associated or released, gave hope that this parasite CP could be a good drug target. The findings of this study, however, suggest that specific inhibitors of LmCPc would not prevent or cure infection

with *L. mexicana*. Some cysteine proteinase inhibitors do have antileishmanial activity *in vitro* (see Chapter 5), but it is not known if these inhibitors are acting synergistically on several enzymes to produce their effect or whether they are inhibiting an as yet unidentified CP (see discussion in Chapter 5). My results, however, demonstrate that *lmcpc* is not essential (under the conditions tested) and would appear to be a poor target for chemotherapeutic exploitation.

CHAPTER FIVE

CYSTEINE PROTEINASES AS EXPLOITABLE DRUG TARGETS

5.1. INTRODUCTION

5.1.1. Anti-leishmanial activity of proteinase inhibitors

As was discussed in section 1.3.3.2., inhibitors of CPs can hinder the development of some protozoan parasites, and there is much interest in developing proteinase inhibitors as novel chemotherapeutic agents. Inhibition of the food vacuole CPs of *P. falciparum* by administration of peptidyl-fluoromethanes led to an accumulation of undegraded haemoglobin inside the food vacuoles and inhibited the parasite from multiplying, both *in vitro* and *in vivo* (Rocket *et al.*, 1990; Rosenthal *et al.*, 1991, 1993). Use of specific, irreversible inhibitors of the major CP of *T. cruzi* have shown transformation events of the parasite to be particularly vulnerable, as well as inhibiting invasion and multiplication of the parasites in cultured mammalian cells (Mercilles, *et al.*, 1992; Bonaldo, *et al.*, 1991; Franke de Cazzulo, *et al.*, 1994).

With regards to other trypanosomatid species, much less is known about the effects of CP inhibitors. Antipain, a known inhibitor of CPs, has been shown to inhibit both the growth of *L. mexicana* promastigotes by >50% over 7 days, and the *in vitro* transformation of amastigotes to promastigotes by >78% (Coombs *et al.*, 1982). This higher susceptibility of amastigotes correlated with their higher CP content, and suggested that some inhibitors of *Leishmania* amastigote CPs may have potential as antileishmanial agents. This suggestion was given credence by the finding that antipain and leupeptin were found to be potent inhibitors of the growth of *L. mexicana* amastigotes in explanted mouse peritoneal macrophages (Coombs and Baxter, 1984). However, neither of the inhibitors used in the above studies are known to target CPs specifically, e.g. leupeptin can also inhibit some trypsin-like serine proteinases (North *et al.*, 1990).

L. pifanoi, a member of the *L. mexicana* complex, has 2 developmentally regulated Type I and Type II CP genes *Lpcys2* and *Lpcys1* (see section 1.4..2). Treatment of axenic amastigotes of *L. pifanoi* with selected cysteine (but not aspartic)

proteinase inhibitors, arrested proteolytic processing of *Lpcys2 in vivo* and inhibited parasite cell division (Duboise *et al.*, 1994). Antipain substantially blocked the processing of a 40 kDa intermediate and inhibited parasite growth at levels down to less than 5 μM, although this effect was reversed when parasites were transferred to inhibitor free medium. The peptidyl-fluoromethane inhibitor Z-Phe-Ala-CH_2F also showed significant inhibition of *Lpcys2* processing and amastigote growth but required a higher concentration of between 100 and 200 μM. The overall conclusion regarding the effects of these inhibitors were that they were cytostatic rather than leishmanicidal (Duboise *et al.*, 1994).

5.1.2. Amastigote CPs as activators of pro-drugs

Lysosomotropic amino acid and peptide esters such as leucine methyl ester (Leu-OMe) destroy isolated *L. amazonenesis* amastigotes by a mechanism postulated to involve trapping of the compounds and their breakdown within acidified parasite organelles (review: Rabinovitch 1989). Amino acid esters kill intracellular *L. amazonensis* at concentrations that allow the survival of the host cells (Rabinovitch *et al.*, 1986), a killing that is thought to be dependent on ester hydrolysis by the parasite proteinases (Alfieri *et al.*, 1988, 1989; Hunter *et al.*, 1989) since amastigotes pre-incubated with the cysteine and and serine -proteinase inhibitors antipain and chymostatin are protected from Leu-OMe toxicity. The hypothesis that megasomes could be the target of the esters was supported by the observation that these organelles are acidified and thus potentially able to concentrate the compounds (Antoine *et al.*, 1988). Amastigotes of *L. mexicana* are also susceptible to Leu-OMe since this compound is rapidly hydrolysed by the low molecular weight CPs that occur in abundance in the megasomes of this stage (Hunter *et al.*, 1992).

5.1.3. Aims

Although I have shown that LmCPb is not essential for parasite survival *in vitro* or *in vivo* (Chapter 3), it remained a possibility that other CPs may be, either singly or collectively with *lmcpb*. The majority of this chapter focuses therefore on leishmanial

proteinases as potentially exploitable drug targets, and involves studies on the effects of new generations of irreversible CP inhibitors, that permeate into living parasites, on the CPs themselves and the growth, differentiation and infectivity of *L. mexicana*.

I also investigated the effects of Leu-OMe on wild type and transfected lines of *L. mexicana* in order to elucidate the extent of the involvement of LmCPb in the activation of the pro-drug, and therefore provide more information on the potential of this compound as a pro-drug against leishmaniasis.

5.2. MATERIALS AND METHODS

5.2.1. *In vitro* culture with cysteine proteinase inhibitors

The three major developmental stages of wild type *L. mexicana* (MNYC/BZ/62/M379) and the transfected cell lines were cultured and harvested as described in Chapters 2 and 3, sections 2.2.1 and 3.2.1. The peptidyl-diazomethanes Z-LVG-DMK and Z-FA-DMK were made up as 10 mg/ml stock solutions in DMSO, and included in the appropriate promastigote or axenic amastigote culture medium to give a final concentration of 10 µg/ml (~25 µm). Normally 10 µl of stock inhibitor was carefully added to 10 ml of medium prior to the addition of the required number of promastigotes, giving a final concentration of 10 µg/ml inhibitor/0.1% DMSO (v/v). Stock solutions of the inhibitors were stored at -20°C until further required.

5.2.2. Analyses using gelatin-SDS-PAGE

Substrate-SDS-PAGE was carried out essentially as described in Chapter 2, section 2.2.2. Where DMK proteinase inhibitors were included, they were added to the lysates prior to electrophoresis to give a final concentration of 10 µg/ml (~25 µm); normally 2 µl of 100 µg/ml DMK dissolved in 1% (v/v) DMSO was added to 18 µl of lysate and incubated for 10 min on ice. Thereafter the lysate was diluted 1:1 with 2x sample buffer and loaded into the well of a gelatin gel.

5.2.3. Use of a biotinylated inhibitor

A stock solution of 0.02 M Biotin-F-A-DMK (Biosyn) in dimethylformamide (DMF) was stored at -20°C, and diluted to 600 µm with DMF immediately prior to use. Preparation of parasite lysates (omitting the proteinase inhibitors) was carried out as described in Chapter 2, section 2.2.2.2. Cell lysates were incubated 5:1 with the affinity label (100 µM final concentration) at 37°C for 30 min, before addition of an equal volume of 2x sample buffer and boiling for 10 min. Control samples contained lysate plus DMF. Electrophoresis and electroblotting were carried out as described in Chapter 2, section 2.2.2.2. After blocking overnight at 4°C in TBS-Tween (section 2.2.2.2), pH 7.6, containing 3% (w/v) bovine serum albumin (BSA), detection of cysteine

proteinases was achieved by incubating with a 1 in 1000 solution of streptavidin-alkaline phosphatase (Biosyn), in TBS-Tween, pH 7.6, for 2 hours at ambient temperature. After 3 x 20 min washes in TBS-Tween, pH 9.5, blots were developed using the BCIP/NBT development system according to the manufacturers instructions (Sigma), and rinsed thoroughly with water.

5.2.4. Infection of peritoneal exudate cells in the presence of cysteine proteinase inhibitors

The infection of peritoneal exudate cells was carried out as described earlier (Chapter 2, section 2.2.4 and Chapter 3, section 3.2.5) with the following additions. Stock solutions (10 mg/ml in DMSO) were made up of the CP inhibitors Z-LVG-DMK, Z-FA-DMK, 'P87' (Mu-Phe-Homophe-vinyl sulfone) and 'K02' (Mu-Phe-Homophe-oxycoumarin) and stored at -20°C until required (gifts from Prof. J. McKerrow, California, USA). Parasites were resuspended to the required density in complete RPMI medium containing 10 μg/ml (~25 μm) inhibitor and 0.1% DMSO (final concentrations) and incubated for 15 min at 25°C (promastigotes) or 32°C (amastigotes). Parasites were then added to the PECs in chamber slides and incubated as appropriate (sections 2.2.4 and 3.2.5), washed 3x in complete RPMI medium, and then incubated for either 72 h or 7 days in complete RPMI medium containing 10 μg/ml inhibitor, after which slides were fixed and stained as before (section 2.2.4).

In certain cases the timing of exposure of the cells to the inhibitor Z-FA-DMK was varied, such that the parasites were either pre-incubated with the inhibitor at 10 μg/ml (~25 μm) for 15 min before addition to the PECs, and the inhibitor subsequently included throughout the incubation (as above), or the inhibitor was added (to give the same final concentration) 4 h or 24 h after the PECs were first exposed to the parasites.

5.2.5. Incubation of axenic parasites with leucine methyl ester

The effect of Leu-OMe (Sigma) on axenic amastigotes and isolated lesion amastigotes of wild type and Δ*lmcpbn* was measured using the quantatative tetrazolium salt assay (see Chapter 3, section 3.2.8.2) as follows. Briefly, parasites were resuspended

at a density of 1 x 10^8 cells/ml in complete HOMEM medium containing Leu-OMe (5 mM final concentration) and incubated at 32°C for one hour. After washing in complete medium, cells were incubated at the above cell density with MTT (450 µg/ml final concentration in complete HOMEM) at 32°C for 3 h, after which samples were diluted 1 in 6 with 0.04 M HCl-isopropanol and the absorbances read at 540nm (as in section 3.2.8.2). T-tests were used to determine the statistical significance of any observed differences in % MTT reduction between the differnt lines after exposure to Leu-OMe.

5.2.6. Inclusion of leucine methyl ester in parasite-infected peritoneal exudate cell cultures

The infection of peritoneal exudate cells by lesion amastigotes was carried out as described earlier (Chapter 2, section 2.2.4 and Chapter 3, section 3.2.5). Leu-OMe was added to the PECs 24 h after first exposure to the parasites, to give a final concentration of 5 mM. Parasite load was determined after a further 48 h incubation, as described previously (section 2.2.4).

5.3. RESULTS

5.3.1. Proteinase inhibitor studies

5.3.1.1. Effects of peptidyl-diazomethanes on transformation and growth of L. mexicana

Wild type promastigotes (Fig. 5.1, A) and axenic amastigotes (Fig. 5.1, B) grew well in the presence of 10 μg/ml Z-LVG-DMK. The results suggested that this proteinase inhibitor does not affect the growth, or transformation to metacyclics (as judged by morphology), of promastigotes, nor the transformation to and multiplication of axenic amastigotes, compared to control cultures containing 0.1% DMSO. The other inhibitor tested in the same set of experiments Z-FA-DMK appeared to slightly inhibit amastigote multiplication (Fig. 5.1, B), although it had no discernible effect on promastigote growth or transformation to metacyclics or axenic amastigotes as judged by morphology (Fig. 5.1, A). These experiments were repeated using the transfected lines Δ*lmcpbn* and Δ*lmcpbg2.8* (Figs. 5.2 and 5.3, respectively). Similar results to those found with wild type parasites were obtained, except that the multiplication of Δ *lmcpbg2.8* axenic amastigotes (Fig. 5.3, B) was affected a little by both inhibitors (note that these transfected lines typically grow poorly as axenic amastigotes, as was shown in Chapter 3). It was also apparent that the 0.1% DMSO in the control promastigote cultures appeared to produce a lag effect, and no obvious logarithmic phase, when compared with the typical growth curves presented in Chapter 3, which was surprising as cultures of wild type *Leishmania* cultures are generally considered to be unaffected by concentrations of up to 0.25% (v/v) DMSO.

5.3.1.2. Effects of peptidyl-diazomethanes on parasite proteinases

Gelatin-gel analyses of stationary phase promastigotes of the wild type parasites from the experiment described in section 5.3.1.1. were carried out. Lysates of cells that had been grown in medium with 0.1% DMSO added as a control gave the characteristic two main bands of high mobility CP activity, between 25 and 20 kDa, (Fig. 5.4, lane 1) previously confirmed to be encoded by *lmcpb* (see Chapter 3). Cells grown in the presence of the inhibitors Z-LVG-DMK and Z-FA-DMK (lanes 2 and 3, respectively)

had very little of this high mobility CP activity, whereas the slightly slower moving CP activities of apparently 35 to 40 kDa, which normally are at very low activity in promastigotes, were at relatively high activity. The slowest moving activities of 60 kDa and greater (due to proteinases other than CPs, such as the metalloproteinase gp63) also appeared to be upregulated in cells grown in the presence of these inhibitors. Control lysates incubated with 10 µg/ml Z-LVG-DMK (lane 4) or Z-FA-DMK (not shown) lacked both the high (20-25 kDa) and lower mobility (35-40 kDa) CP activities. This confirmed that the bands around 40 kDa are inhibitable by these DMKs, although this did not apparently occur in the living cell.

Wild type axenic amastigotes grown in the presence of Z-LVG-DMK (Fig. 5.4, lane 6) or Z-FA-DMK (Fig. 5.4, lane 7) lacked the typical high mobility CP activity (as seen with control axenic amastigotes, Fig. 5.4, lane 5) but the characteristic lower mobility activities of axenic amastigotes (Bates *et al.*, 1992) approximately 35-40 kDa, were active. Although these lower mobility activities were not inhibited by Z-LVG-DMK or Z-FA-DMK in the living cells, they were inhibited when the cells were lysed with culture medium from day 7 of the above experiment containing Z-FA-DMK (lane 8). This confirmed that the activities were due to CPs, and indicated that the DMK was still intact and fairly concentrated in the medium from which the cells were harvested.

Although the cells were washed well during harvesting, it remained a possibility that inhibitor bound to the cell surface may cause inhibition of the CPs when the cells were lysed, and that the enzymes were not in fact inhibited in the living cells. However, analysis on gelatin gels of a mixture of DMK-grown axenic amastigotes plus control axenic amastigotes showed the control CP activity to be unaffected, indicating that indeed there was no inhibitor bound to the cell surfaces (data not shown). As was found with the promastigotes, there was increased activity of enzymes of around 60 kDa (apparently predominantly metalloproteinases, since they were fully inhibited by 1 mM o-phenanthroline added during substrate digestion) in these cells compared with control axenic amastigotes.

The proteinases of promastigotes of the line *Δlmcpbg2.8* grown in the presence of these inhibitors were also examined. Gelatin gel analysis (Fig. 5.5) revealed results

similar (in relative terms) to those found with wild type parasites, in that the high mobility proteinase activity detected in the controls (lane 1) was absent (lanes 2 and 3), and the lower mobility activities were enhanced.

5.3.1.3. Detection of cathepsin L-like CPs using a biotinylated inhibitor

The possibility that *L. mexicana* possesses additional CPs that can compensate for the lack of *lmcpa* and *lmcpb* was investigated using biotin-F-A-DMK to detect active cathepsin L-like CPs in a Western blot (Fig. 5.6). Lysates of stationary phase promastigotes of wild type cells (lanes 5 and 6), Δ*lmcpbn* (lanes 3 and 4) and Δ *lmcpbn/lmcpan* 'double null' (lanes 1 and 2) were incubated with the biotinylated inhibitor (lanes 1, 3 and 5) or not (lanes 2, 4 and 6). This sensitive method detected the main cathepsin L-like CPs in wild type cells as a broad, dense band at around 24 kDa (lane 5, arrowed). The staining around 24 kDa was considerably less in Δ*lmcpbn*, but 3 faint bands remained (lane 3). These appeared to be due to *lmcpa* as they were absent from the double null (lane 1). The only bands detected in this line were those of higher molecular mass, some of which were also present in the control lanes 4 and 2. These may have been due to non-specific binding; there is likely to be significantly less autohydrolysis in lysates of the transfected lines, than in those of wild type parasites (lane 6). These experiments indicate that *lmcpa* and *lmcpb* may be the only cathepsin L-like CPs in *L mexicana* stationary phase promastigotes. A similar result was found using the above method on axenic amastigote lysates (data not shown).

5.3.1.4. Effects of inhibitors on parasite infection of peritoneal exudate cells

The effects of CP inhibitors on the infectivity of parasites to macrophages was investigated (Table 5.1). The infectivity of stationary phase promastigotes of wild type parasites in the absence of inhibitor was found to be 53%. Although it had been shown to inhibit the CPs in axenic parasites, Z-LVG-DMK surprisingly did not reduce the infectivity of promastigotes to macrophages, although these results proved rather variable (see Discussion, section 5.4). In contrast, Z-FA-DMK reduced infectivity to very low levels (7%), in fact to levels similar to those obtained with Δ*lmcpbn* in the

absence of CP inhibitors (Table 5.1 and Chapter 3). There also appeared to be a lower number of amastigotes per infected cell when Z-FA-DMK was present compared with both wild type parasites and Δ*lmcpbn*. Control cultures with 0.1% DMSO included in the medium had infectivity levels that were not significantly different from those with wild type parasites, although the number of amastigotes per infected PEC was slightly lower (P value <0.1).

Having ascertained that Z-FA-DMK could reduce both parasite infection of PECs and perhaps intracellular multiplication, experiments were set up to investigate at which stage in the infection process parasites were most susceptible to this inhibitor (Fig. 5.7). Similar results were obtained irrespective of whether the parasites were pre-incubated with Z-FA-DMK for 15 min, and the inhibitor subsequently included throughout the incubation, or it was added 4 h or 24 h after infection of the macrophages with the parasites (Fig. 5.7, A). However, the length of the incubation was found to be important. The inhibitor had greater anti-parasite effect over 7 days, after which the number of parasite-infected cells was found to be less than 10 % of the control, than when the experiment was stopped after 72 h. The mean number of parasites per infected macrophage was not affected greatly by the time of initial exposure to the DMK (Fig. 5.7, B); but was reduced more by 7 days than by 3 days.

This approach was pursued using other CP inhibitors known to inhibit the leishmanial CPs on gelatin gels (Coombs, unpublished) and looking for their effects on macrophage infections over 72 h and 7 days (Fig. 5.8). Like Z-FA-DMK, the CP inhibitors 'P87' and 'KO2' had some anti-parasite effects after 7 days, although they were not as potent as Z-FA-DMK after 3 days (Fig. 5.8, A). Furthermore, 'P87' and 'KO2' did not reduce the number of amastigotes per infected PEC to as low a level as occurred with Z-FA-DMK after 7 days (Fig. 5.8, B).

The effect of CP inhibitors on the infectivity of lesion amastigotes to macrophages was examined (Fig. 5.9). After 7 days incubation with the inhibitor Z-FA-DMK, the % infected macrophages was again reduced to a very low level compared with the control parasites (and, as with promastigotes, this reduction in infectivity was not as pronounced after just 72 h); as was the number of amastigotes per macrophage.

The CP inhibitor Z-LVG-DMK, however, had a lesser effect.

5.3.2. Susceptibility to the amino acid ester leucine methyl ester

5.3.2.1. Toxicity for axenic parasites

Toxicity for isolated parasites was measured by a quantitative tetrazolium salt (MTT) assay. After exposure to 5 mM Leu-OMe the reduction of MTT by wild type axenic amastigotes was only 23% of that of the control (untreated cells). The MTT reduction by Δ*lmcpbn* was 51% of the control (Table 5.2). These results were consistent with the hypothesis that the lack of LmCPb would render the Δ*lmcpbn* cells less susceptible to Leu-OMe. Nevertheless, a substantial proportion of the Δ*lmcpbn* axenic amastigotes were still affected by the ester. However results using lesion amastigotes of the 2 lines (Table 5.2) showed that MTT reduction after exposure to the ester was 12% with wild type parasites and 17% with Δ*lmcpbn* amastigotes. Although these results are significantly different, they clearly indicate that the *lmcpb* proteinases are not the only ones that hydrolyse the ester, although they may play a large part when they are present. It is also apparent from these results that axenic amastigotes are not identical to lesion amastigotes (as they do not appear to be as susceptible as lesion amastigotes) although cultured amastigote-like forms were previously found to be very similar to lesion amastigotes (Bates *et al.*, 1992)

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5.3.2.2. Toxicity for infections of peritoneal exudate cells

PEC cultures infected with either wild type or Δ*lmcpbn* lesion amastigotes were almost cured of parasites by exposing the cells to Leu-OMe 24 h after infection (Table 5.4). There were no apparent deleterious effects on the host cells, suggesting that amino acid esters may be effective pro-drugs that are activated specifically by parasite enzymes.

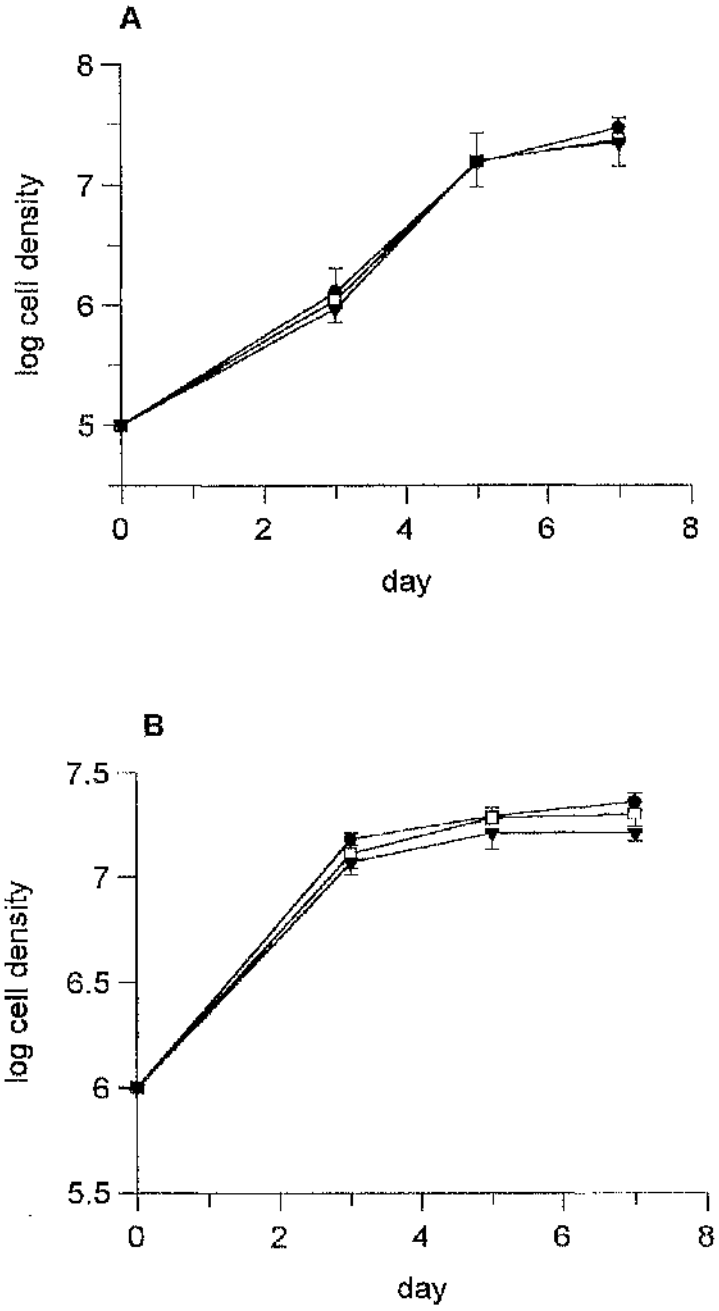

Figure 5.1. Growth of wild type *L. mexicana* in the presence of peptidyl-diazomethanes. (A) Promastigotes cultured in complete HOMEM containing 10 µg/ml of the following inhibitors: -●-, control (0.1% DMSO); -□-, Z-LVG-DMK; -▼-, Z-FA-DMK. (B) Axenic amastigote cultures, initiated using stationary phase promastigotes, in complete SDM containing inhibitors as in (A). Results are the means ± SD from 3 independent experiments.

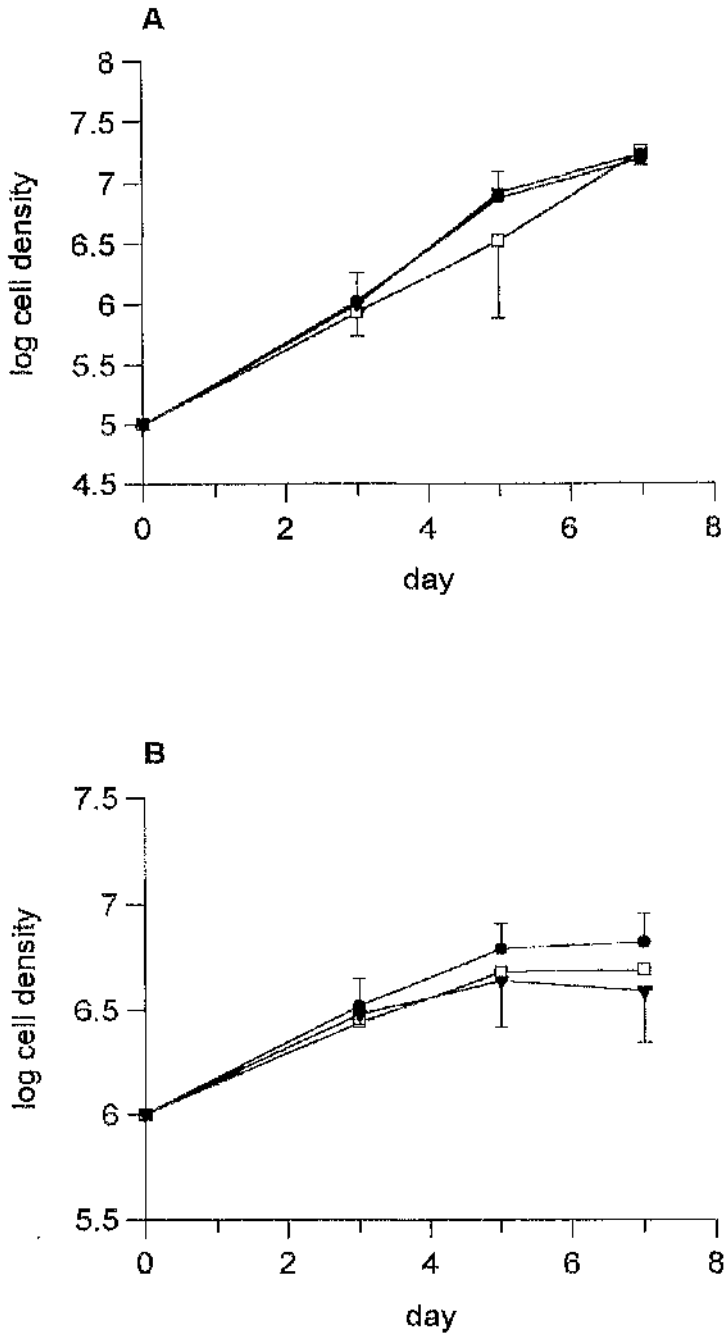

Figure 5.2. Growth of Δ*lmcpbn L. mexicana* in the presence of peptidyl-diazomethanes. (A) Promastigotes cultured in complete HOMEM containing 10 µg/ml of the following inhibitors: -●-, control (0.1% DMSO); -□-, Z-LVG-DMK; -▼-, Z-FA-DMK. (B) Axenic amastigote cultures, initiated using stationary phase promastigotes, in complete SDM containing inhibitors as in (A). Results are the means ± SD from 3 independent experiments.

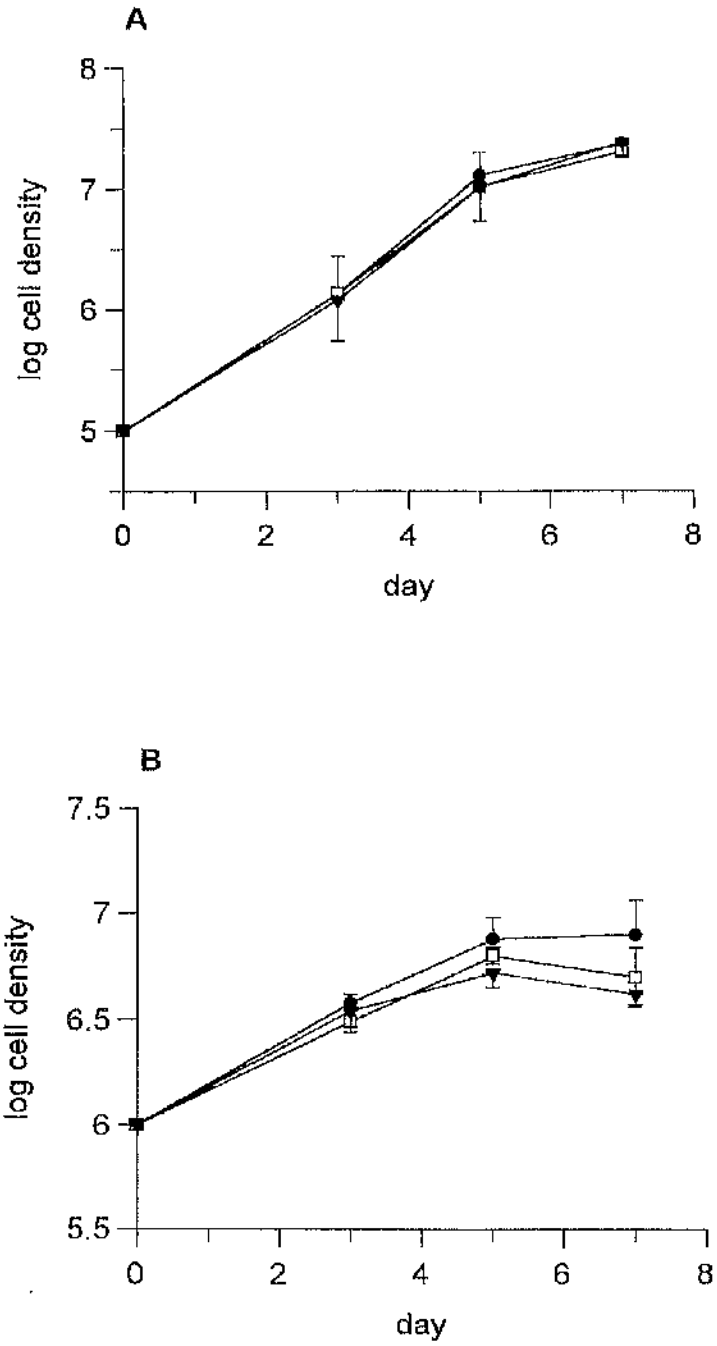

Figure 5.3. Growth of *Δlmcpbg2.8 L. mexicana* in the presence of peptidyl-diazomethanes. (A) Promastigotes cultured in complete HOMEM containing 10 μg/ml of the following inhibitors: -●-, control (0.1% DMSO); -□-, Z-LVG-DMK; -▼-, Z-FA-DMK. (B) Axenic amastigote cultures, initiated using stationary phase promastigotes, in complete SDM containing inhibitors as in (A). Results are the means ± SD from 3 independent experiments.

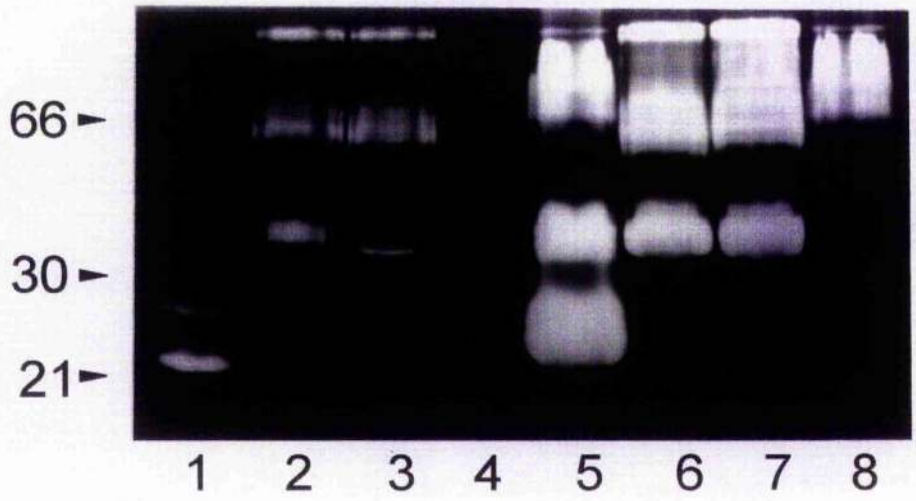

Figure 5.4. Gelatin-gel analysis of wild type parasites grown in the presence of CP inhibitors. Wild type cells were harvested on day 7 from the experiment described in section 5.3.1.1 (Fig. 5.1) and their lysates subjected to gelatin-SDS-PAGE. Lane 1, control stationary phase promastigotes; lanes 2 and 3, promastigotes grown with Z-LVG-DMK and Z-FA-DMK respectively; lane 4, control promastigotes lysed with Z-LVG-DMK. Lanes 5-8: as lanes 1-4 except cells were axenic amastigotes. The positions of molecular weight markers in kDa are indicated.

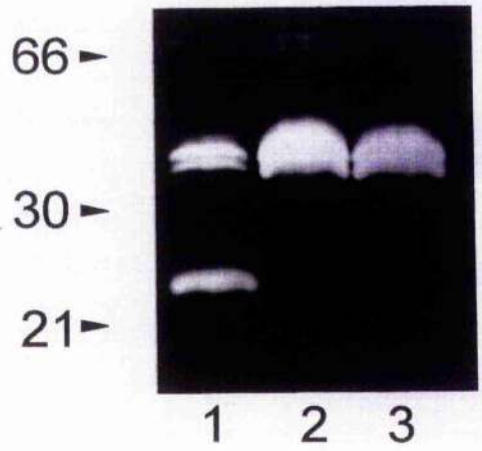

Figure 5.5. Gelatin-gel analysis of *Δlmcpbg2.8* grown in the presence of CP inhibitors. *Δlmcpbg2.8* promastigotes were harvested on day 7 from the experiment described in section 5.3.1.1 and their lysates subjected to gelatin-SDS-PAGE. Lane 1, control stationary phase promastigotes; lanes 2 and 3, promastigotes grown with Z-LVG-DMK and Z-FA-DMK, respectively. The positions of molecular weight markers are arrowed.

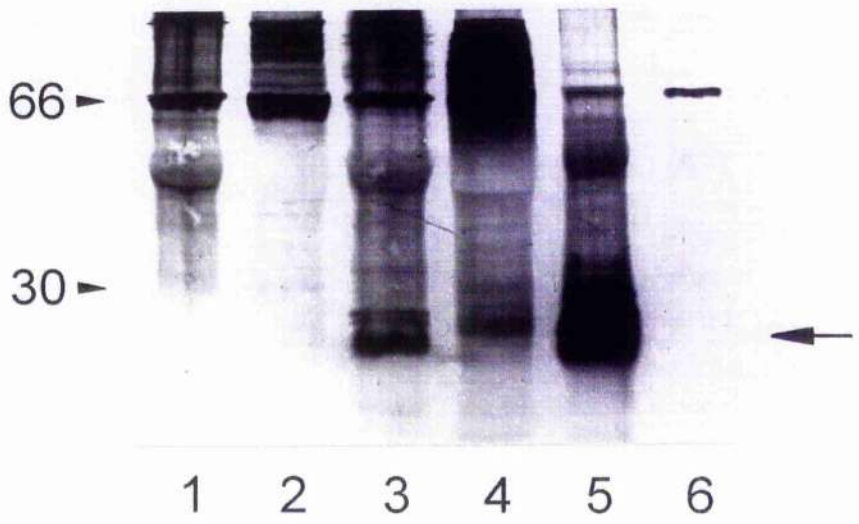

Figure 5.6. Western blot analysis of proteinases of *L. mexicana* using biotinylated peptidyl-diazomethane to label enzymes. Lysates of stationary phase promastigotes of wild type (lanes 5 and 6), Δ*lmcpbn* (lanes 3 and 4) and Δ*lmcpbn*/*lmcpan* (lanes 1 and 2) were incubated with the biotinylated inhibitor (lanes 1, 3 and 5) or not (lanes 2, 4 and 6). The positions of molecular weight markers are indicated on the left; the position of the main cathepsin L-like CPs is indicated on the right (large arrowhead).

cell line, inhibitor	% infected PECs	amastigotes/infected PEC
wild type	53 ± 6.1	4.8 ± 0.9
+ DMSO	45 ± 11	3.4 ± 0.7
+ Z-LVG-DMK	42 ± 24	3.0 ± 1.5
+ Z-FA-DMK	7.0 ± 8.7	1.9 ± 1.3
Δ*lmcpbn*	5.3 ± 5.5	3.6 ± 4.1

Table 5.1. Effect of proteinase inhibitors on the infectivity of wild type stationary phase promastigotes of *L. mexicana* to peritoneal exudate cells. Cells were pre-incubated with the inhibitors for 15 min and constantly exposed to them over the 7 day duration of the experiment. After 7 days, the cells were fixed, stained with Giemsa's stain, and parasite load determined by counting 200 PECs. The values given are the means ± SD from 3 independent experiments. Infection rates for wild type, DMSO and Z-LVG-DMK were significantly different from both Z-FA-DMK and Δ*lmcpbn* (P values of <0.01, 0.01 and 0.1 respectively) but not from each other; infection rates for Z-FA-DMK and Δ*lmcpbn* were not significantly different. Values for amastigotes/infected PEC with + DMSO, Z-LVG-DMK, Z-FA-DMK and Δ*lmcpbn* were all significantly different from that for wild type (P values of at least <0.1) but not from each other.

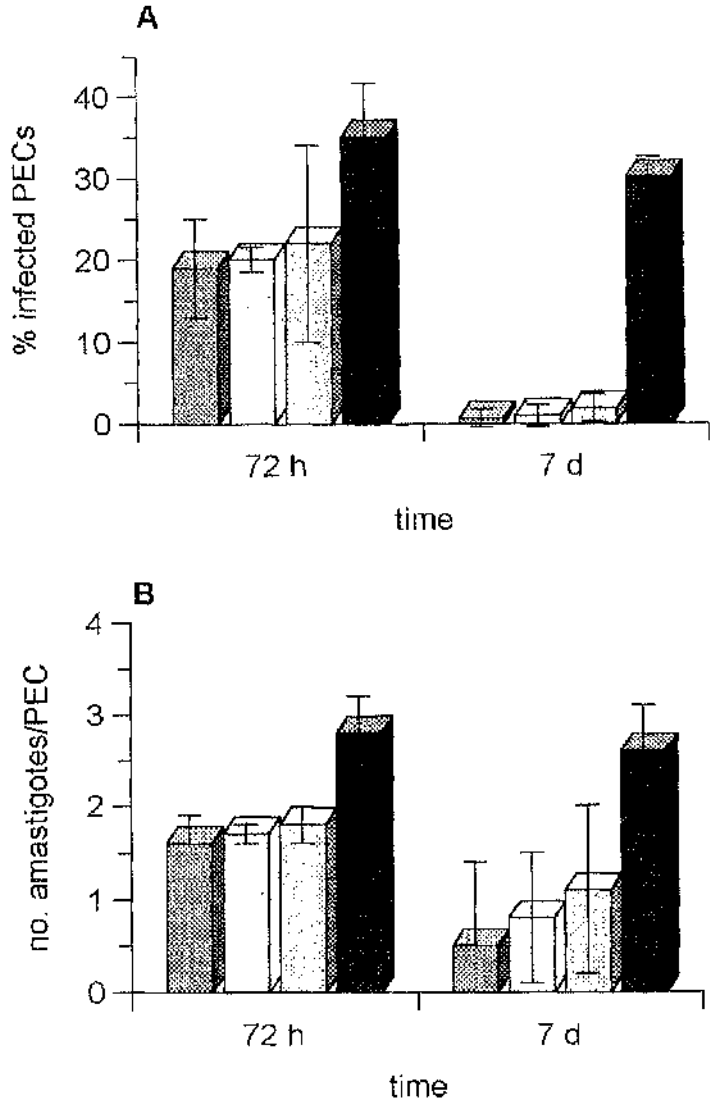

Figure 5.7. Effect of the peptidyl-diazomethane proteinase inhibitor Z-FA-DMK on the infectivity of wild type promastigotes to PECs. The stationary phase promastigotes were either pre-incubated for 15 min with 10 µg/ml Z-FA-DMK and exposed to the inhibitor throughout (▩), or the inhibitor was added 4 h (▫) or 24 h (▫) after the PECs were first exposed to the parasites. Controls (■) contained 0.1% DMSO. The % infectivity (A) and amastigotes/infected PEC (B) data are the means ± SD from 3 independent experiments. Graph A: infection rates after 72 h for pre- and 4 h incubations were significantly different from the control (P values of <0.05 and <0.02, respectively) but not from each other; after 7 d infection rates for pre-, 4 h and 24 h incubations were all highly significantly different from the control (P values <0.01). Graph B: mean values after 72 h for amastigotes/infected PEC with pre-, 4 h and 24 h incubations were all significantly different from the control (P values of <0.02, <0.02 and <0.05, respectively) but not from each other; after 7 d mean values for amastigotes/infected PEC with pre-, 4 h and 24 h incubations were still significantly different from the control (P values of <0.05, <0.05 and <0.1, respectively).

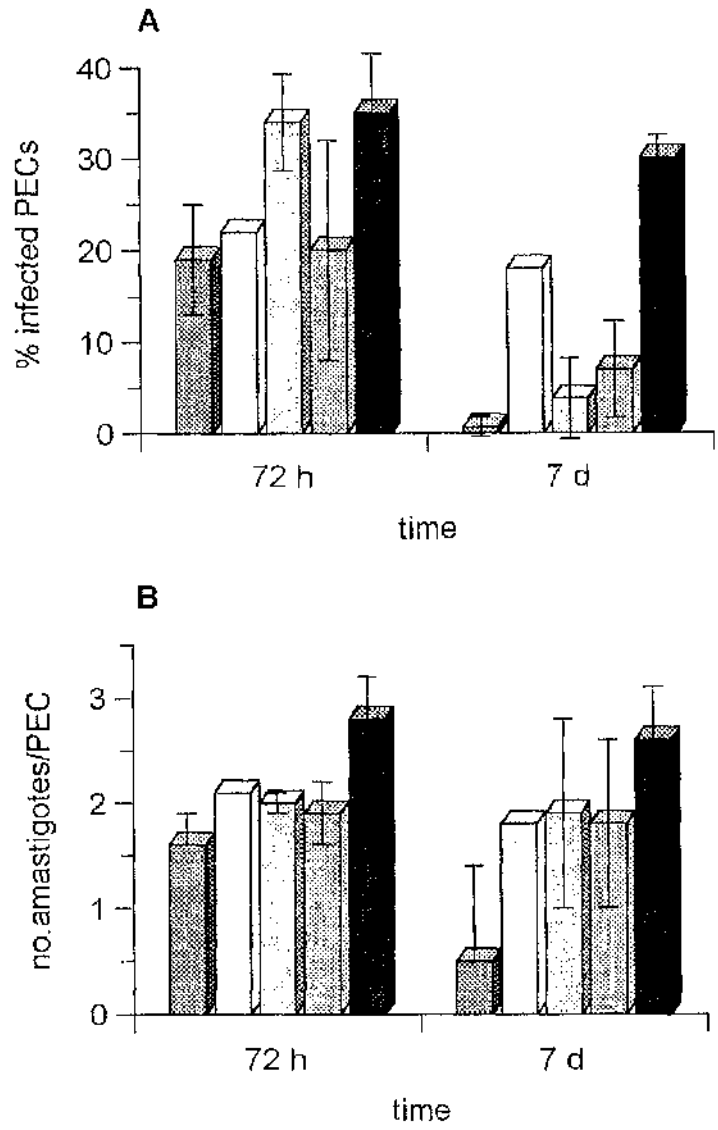

Figure 5.8. The effect of CP inhibitors on the infectivity of wild type stationary phase promastigotes to PECs. The parasites were pre-incubated for 15 min with the inhibitors, which were also present in the culture medium for the duration of each experiment (72 h or 7 days). The inhibitors used (at 10 µg/ml) were as follows: ▒, Z-FA-DMK; □, Z-LVG-DMK; □, P87; ▒, KO2; ■, control (0.1% DMSO). The % infectivity (A) and amastigotes/infected PEC (B) data are the means ± SD (except for Z-LVG-DMK) from 3 independent experiments performed in duplicate. Graph A: the infection rate after 72 h was significantly different from the control (P value of <0.05) with Z-FA-DMK, but not with P87 or KO2; after 7 d infection rates with Z-FA-DMK, P87 and KO2 were all highly significantly different from the control (P values <0.01), but not from each other. Graph B: mean values after 72 h for amastigotes/infected PEC with Z-FA-DMK, P87 and KO2 were all significantly different from the control (P values of <0.02, <0.05 and <0.05 respectively) but not from each other; after 7 d the mean value for amastigotes/infected PEC with Z-FA-DMK was significantly different from the control (P value of <0.05), but was not significantly different with P87 or KO2.

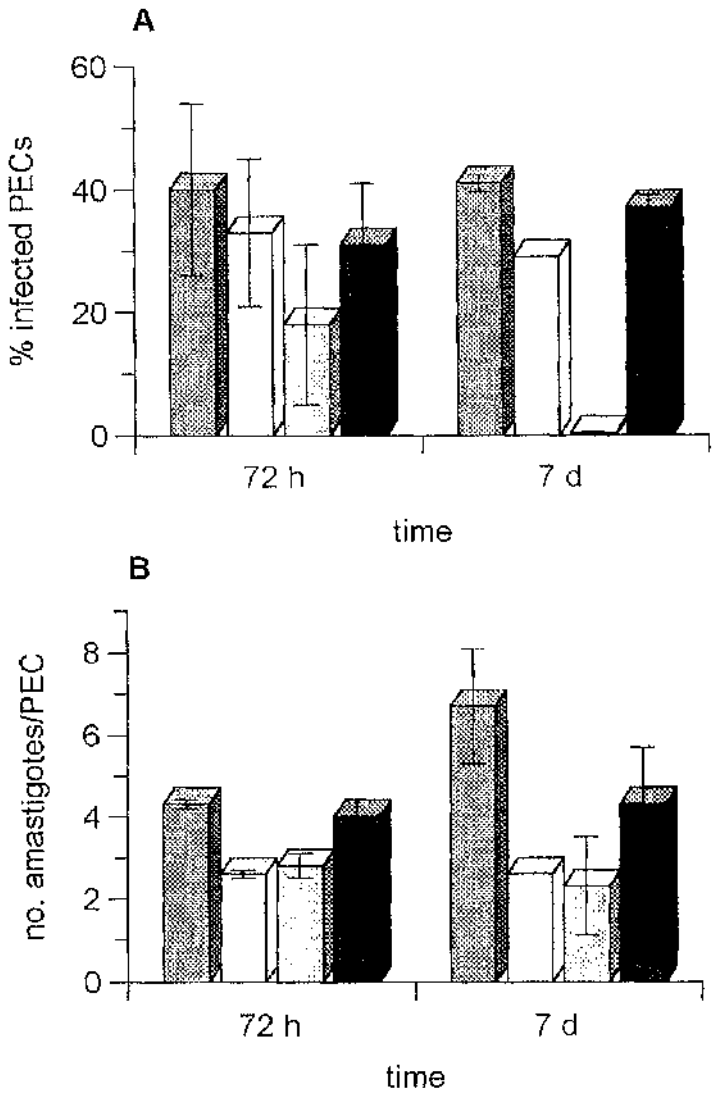

Figure 5.9. Effect of CP inhibitors on the infectivity of wild type lesion amastigotes to PECs. The parasites were pre-incubated for 15 min with the inhibitors, which were also present in the culture medium at 10 μg/ml for the duration of each experiment (72 h or 7 days). Key to graphs: ▨, control (no addition); □, Z-LVG-DMK; □, Z-FA-DMK; ■, control (0.1% DMSO). The % infectivity (A) and amastigotes/infected PEC (B) data are the means ± SD from 3 independent experiments performed in duplicate. Graph A: the infection rates after 72 h with either Z-FA-DMK or Z-LVG-DMK were not significantly different from the controls; after 7 d the infection rates with the controls and Z-LVG-DMK were highly significantly different from Z-FA-DMK (P values <0.01), but not from each other. Graph B: mean values after 72 h for amastigotes/infected PEC with Z-FA-DMK and Z-LVG-DMK were significantly different from the controls (P values of <0.02 and <0.01, respectively) but not from each other; after 7 d the mean values for amastigotes/infected PEC with Z-FA-DMK and Z-LVG-DMK were still significantly different from the controls (P values of <0.05 and <0.1, respectively). Control (no addition) and control (0.1% DMSO) results were not significantly different in A or B.

cell line	% MTT reduction
wild type lesion amastigotes	12 ± 0.1
Δ*lmcpbn* lesion amastigotes	17 ± 1.1
wild type axenic amastigotes	23 ± 15
Δ*lmcpbn* axenic amastigotes	51 ± 21

Table 5.2. Effect of leucine methyl ester on lines of *Leishmania mexicana in vitro*. After exposure to 5 mM Leu-OMe for one hour, cells were incubated with 450 μg/ml MTT for 3 h in order to assess parasite viability. Values are expressed as a % of the control (100%) and are the means ± S.D. from 7 independent experiments for axenic amastigotes, and 3 independent experiments for lesion amastigotes. % MTT reduction by wild type axenic amastigotes was significantly different from that of Δ*lmcpbn* axenic amastigotes ($P<0.05$); % MTT reduction by wild type lesion amastigotes was highly significantly different from that of Δ*lmcpbn* lesion amastigotes ($P<0.01$).

cell line	% infected	amastigotes/infected PEC
wild type + ester	0.5	3
wild type control	24	3.5
Δ*lmcpbn* + ester	0.5	2
Δ*lmcpbn* control	16	4

Table 5.3. Effect of the amino acid ester leucine methyl ester on lesion amastigote-infected macrophages. The ester was added to the macrophages 24 h after they were infected (final concentration 5 mM) and was not removed for the duration of the experiment. Results give parasite load at 72 h.

5.4. DISCUSSION

The peptidyl-diazomethane CP inhibitors Z-FA-DMK and Z-LVG-DMK were found to have little effect on parasite growth and differentiation axenically *in vitro* (Figs. 5.1-5.3). This was surprising as much of the evidence presented in Chapter 3 pointed toward a major role for the CPs in differentiation, either of promastigotes to metacyclics or metacyclics to amastigotes. Indeed transformation events, particularly metacyclogenesis and trypomastigote to amastigote differentiation, have been found to be the most susceptible to peptidyl-DMKs in the related trypanosomatid *T. cruzi* (Franke de Cazzulo *et al.*, 1994, and references therein). However, Z-FA-DMK was found not to inhibit metacyclic to amastigote transformation of *L. mexicana in vitro* (Bates *et al.*, 1994), in agreement with the results presented in this chapter. The finding that the growth of axenic amastigotes of wild type parasites, Δ*lmcpbn* and Δ*lmcpbg2.8* were all equally, albeit very slightly, inhibited by Z-FA-DMK suggests that the inhibitor's effect was not mediated by *lmcpb* but perhaps another CP that has a role in this stage. It was interesting that Δ*lmcpbg2.8* axenic amastigotes were slightly inhibited by both DMKs whereas wild type were not affected by Z-LVG-DMK; whether or not this relates to over-expression of *lmcpbg2.8* remains to be elucidated.

Gelatin-SDS-PAGE analyses of wild type cells grown with the DMK inhibitors revealed that both stationary phase promastigotes and axenic amastigotes were lacking in high mobility CPs (encoded by *lmcpb*), but lower mobility proteinases (including possible pro-enzymes for the major high mobility CPs) were up-regulated (Fig. 5.4). There are several potential explanations for this. Firstly, that these activities are up-regulated to compensate for the enzymes that are inhibited. Secondly, that the DMK inhibitors interfere with maturation of the enzymes *in vivo* resulting in a build up of pro-enzymes which are then activated post-electrophoresis. If, in fact, the inhibited enzymes are being compensated for, then this may explain the lack of an obvious effect of the inhibitors on parasite growth and transformation. It is also possible that these lower mobility proteinases are normally degraded in lysates by the high mobility CPs, giving rise to an 'apparent' upregulation of the low mobility proteinases when the high mobility proteinases are inhibited.

The non-inhibition of these 35-40 kDa enzymes *in vivo* is similar to findings of other researchers with other peptidyl-diazomethane inhibitors (Pral *et al.*, 1993). They suggested that *in vivo* the enzymes are inactivated by endogenous inhibitors, or predominantly exist as larger molecular weight precursors. It is also possible, but perhaps less likely, that these lower mobility CPs are in a cell compartment impervious to the diazomethane CP inhibitors tested.

Since different CPs may compensate for one another, we were interested in looking for cathepsin L-like CPs in *L. mexicana* in addition to LmCPa and LmCPb. To investigate this, the use of biotinylated inhibitors to label active CPs (McGinty *et al.*, 1993) was attempted. No novel CPs were detected using the biotinylated inhibitor (Fig. 5.6) which is thought to detect primarily cathepsin L-like CPs, suggesting that any other CPs in *L. mexicana* must be somewhat different from those already characterised. However, this method, although fairly sensitive, will only detect enzymes with suitable substrate sensitivities and so a definite conclusion regarding the existence of other CPs in *L. mexicana* cannot be made soley from these results.

Previous studies had shown amastigote growth in macrophages to be hindered by antipain and leupeptin (Coombs and Baxter, 1984), although these inhibitors do not specifically target CPs. The results in this chapter clearly indicate that specific peptidyl-DMK CP inhibitors are active against *L. mexicana* growing intracellularly *in vitro*. At the same concentration (10 µg/ml) that had little effect on axenic parasite growth and differentiation, but abolished all major CP activities, Z-FA-DMK was found to reduce the infectivity of both wild type promastigotes and lesion amastigotes to PECs by 80% by day 7 of infection (Table 5.1 and Fig. 5.9). This reduced infectivity to PECs is very similar to the infectivity level found with Δ*lmcpbn* promastigotes (Table 5.1). It should be emphasised, however, that although *lmcpb* is almost certainly inhibited during these infection experiments, it is not necessarily this inhibition that reduces infectivity; other enzymes not yet detected may be the key targets. On the other hand, Z-FA-DMK may be less effective against enzymes other than *lmcpb* such as *lmcpc*, which is inactive toward gelatin. The fact that Z-FA-DMK may have reduced intracellular parasite multiplication more than the deletion of *lmcpb* appeared to (Table 5.1) also suggests the involvement

of a crucial CP other than LmCPb as the target of the inhibition (although this difference was found not to be statistically significant). Z-FA-DMK appears to take up to 7 days to exert its full effect on the infectivity of the parasites to PECs; this is in direct contrast to the reduction in infectivity resulting from the deletion of *lmcpb*, where the cells were killed rapidly such that the % infectivity at 24 h was the same as that after 7 days (Chapter 3, section 3.3.2.1). As Z-FA-DMK killed the parasites only slowly over 7 days, it seems that Z-FA-DMK targets an enzyme involved in the maintenance of intracellular infection/parasite multiplication, rather than initial establishment of infection/transformation. Further evidence to support this theory is that when Z-FA-DMK was administered up to 24 h after infection had been initiated it still reduced infectivity levels at day 7 by more than 80% (Fig. 5.7).

The finding that infections initiated using lesion amastigotes were also affected by Z-FA-DMK differed from data presented by other researchers (Leao *et al.*, 1995); however, their infections were only maintained for 24 h (as opposed to 72 h and 7 days in this study) and as shown by the data presented in this chapter, an effect would not be apparent by this time. The finding with amastigotes was surprising considering the finding in Chapter 3 that the *lmcpb* null lesion amastigotes were fully infective to PECs. Again this suggests that *lmcpb* is not the prime target of this inhibitor. Overall the data regarding Z-FA-DMK suggest that an important enzyme is being targeted by this inhibitor, but it is not *lmcpb*.

It may be interesting to incubate the infected PECs in the presence of Z-FA-DMK for more than 7 days, as % infectivity may well drop to zero if the experiment was left longer. An observation worth noting is that the PECs often appeared more 'spiky' in cultures exposed to this DMK - could it be that the DMK causes a change in the PECs themselves, which either induces them to kill the parasites, or perhaps starves the parasite of a particular nutrient?

The other CP inhibitors investigated 'P87' and 'K02' were not as potent towards the parasitic infections, especially with regards to reducing the number of amastigotes per infected PEC, but there are possible explanations for this such as inhibitor instability. Z-FA-DMK appeared to inhibit the CPs (that hydrolyse gelatin) in living

parasites more fully than did Z-LVG-DMK (Fig. 5.4), and this may be reflected in the ability of Z-FA-DMK to decrease parasite infectivity to PECs more than Z-LVG-DMK. It is also possible that the macrophages were able to metabolise Z-LVG-DMK (but not Z-FA-DMK), resulting in the lack of effect. Results with Z-LVG-DMK were, however, found to be variable (note the large SDs, Table 5.1), and in several cases appeared to have killed the PECs; it was felt that there may be a critical level around 10 μg/ml that the PECs could not withstand.

Another approach that has been adopted to target leishmanial CPs is by the use of amino acid esters as pro-drugs that are activated specifically by the parasite enzymes (Rabinovitch, 1989). This causes an accumulation of amino acids, leading to osmotic stress and ultimately parasite rupture. In previous studies low molecular weight CPs, now known to be the enzymes encoded by *lmcpb*, had been implicated as being the major enzymes activating Leu-OMe (Rabinovitch *et al.*, 1986; Rabinovitch, 1989; Hunter *et al.*, 1992). Purified proteinases rapidly hydrolysed the ester, and this was prevented by the use of the CP inhibitor E-64 (Hunter *et al.*, 1992). Results presented in this chapter of experiments where parasites were exposed to the amino acid ester and then their viability assessed by their ability to reduce the tetrazolium salt MTT are not in complete agreement with the conclusions that arose from previous experiments. At the same concentration used by previous investigators (Hunter *et al.*, 1989; Alfieri, *et al.*, 1989), I found that amastigotes were consistently more resistant (by about 10-fold) to the effects of 5 mM Leu-OMe. Aside from this, the finding that the *lmcpb* null amastigotes were also very susceptible (Tables 5.2 and 5.3) gives strong evidence that the *lmcpb* enzymes are not soley responsible for the hydrolysis of the ester, although they may have greater activity than other enzymes capable of the hydrolysis and so be the major mediators of the toxicity when they are present.

The results of this part of my study show that CP inhibitors do have potential as drugs, but in the case of *L. mexicana* these would need to be inhibitors of enzymes other than *lmcpb*, that is, enzymes which are essential, or inhibitors of several enzymes. It has been suggested that specific inhibitors may also inhibit any pathological effects of released amastigote proteinases (Ilg *et al.*, 1994). The antileishmanial activity of these

inhibitors *in vitro* is promising, but whether the compounds will prove as efficacious *in vivo* awaits examination.

CHAPTER SIX
GENERAL DISCUSSION

This project set out with the principal aims of learning more about the CPs of *L. mexicana* and thereby identifying which, if any, were suitable drug targets. Two main approaches were adopted in order to elucidate the roles that the different CPs play during the life cycle of *L. mexicana*. The first was an investigation of the phenotypes of mutants deficient in specific CP genes, while the second approach utilised novel CP inhibitors.

I have analysed the phenotypes of mutant lines of *L. mexicana* lacking genes encoding three CPs, and in which CP genes have been re-expressed. It would appear that the null mutants for the *lmcpa* CP gene have a cryptic phenotype under the conditions tested. They differed from wild type parasites in that they did not seem to form metacyclics of typical morphology, but still transformed to and grew as amastigotes axenically, and infected both macrophages and mice at a rate comparable with wild type. Although an exact function for the *lmcpa* proteinase has not yet been demonstrated, it would appear from the infectivity data that LmCPa is not a suitable drug target since the enzyme is non-essential in the mammalian host (Souza *et al.*, 1994).

The main CP activity of *T. cruzi* has highest levels in the epimastigote (insect vector) stage (Campetella *et al.*, 1990) suggesting a biological role for cruzipain in the insect host. My finding that the highest levels of LmCPa were not in amastigotes (as was previously thought) suggested LmCPa may similarly be involved in the insect stage of the life-cycle. However, more recent studies have shown that the *lmcpa* null mutants can successfully be passaged through sandflies (Bates, unpublished) which argues against a crucial role for LmCPa in the sandfly vector.

Mutant promastigotes lacking the *lmcpb* gene array did not appear to form metacyclics *in vitro* either; however they did not grow well as axenic amastigotes and infected both macrophages and mice poorly in comparison with wild type parasites. This was also the case for promastigotes transformed from lesion amastigotes. These

results suggested that *lmcpb* plays a significant role in differentiation to amastigotes and survival within macrophages, and should therefore be classed as a virulence factor. The data from experiments studying the infectivity of Δ*lmcpbn* to PECs suggested that the null mutant promastigotes possibly infect only a subpopulation of the cells. Previous studies have shown that immature macrophages are much more susceptible than mature macrophages to *T. cruzi* infection (Plasman *et al.*, 1994) and hence it is not impossible that a similar scenario occurs with *L. mexicana*. The finding that increasing the parasite:PEC ratio resulted in a higher percentage of infected cells could, however, mean that even when low parasite ratios are used, some PECs may phagocytose several parasites at once and simply be unable to kill all of them. This would result in the observed proliferation of the null mutants in a small percentage of cells, but which is not actually a distinct subpopulation.

Further evidence supporting the importance of the *lmcpb* genes in infection was demonstrated when expression of an active *lmcpb* gene (*g2.8*) in the null mutants for *lmcpb* increased their infectivity to macrophages almost to the level seen with wild type parasites. However, the infectivity was not restored when this gene was re-expressed in the double null. This suggests that perhaps LmCPa does have a role in infection, either working synergistically with the *lmcpb* gene products or in activating *lmcpb* inactive precursor forms (as indeed *lmcpb* activity was reduced in Δ*lmcpan*).

In agreement with the findings of Assreuy *et al.* (1994) (see Chapter 3 Discussion), other researchers have found that the attachment of *Leishmania* to macrophages initiates an oxidative burst within 15 minutes, but no NO production until at least 6 h later (Bhunia *et al.*, 1996). My observations that Δ*lmcpbn* promastigotes were rapidly killed between 4 and 24 h post-internalisation strongly suggested an increased susceptibility of the null mutants to NO. In this study I found that neither hydrogen peroxide nor NO had increased leishmanicidal activity towards the *lmcpb* null mutant promastigotes in comparison with wild type parasites. It is worth noting, however, that some workers have suggested that the toxic activities of NO depend in part on cooperation with reactive oxygen intermediates (Lin and Chadee, 1992), a combination that was not examined in this study. Interestingly, cystatins have been

found to upregulate NO release from mouse peritoneal macrophages during infection by *T. cruzi* (Verdott *et al.*, 1996), which may possibly indicate a role for the downregulation of NO by parasite CPs (although the cystatin inhibitory site was thought not to be involved in the mechanism).

The observations of other researchers have direct relevance to the timing of the infection kinetics of Δ*lmcpbn* stationary phase promastigotes to PECs. For example, it was recently found that PECs infected with *Leishmania* amastigotes presented little parasite antigens to $CD4^+$ T cells. This was in contrast to promastigote-infected macrophages, which did present parasite molecules, with maximal presentation occurring within 24 h of infection (Kima *et al.*, 1996). Thus, if Δ*lmcpbn* promastigotes are diminished in their ability to transform sufficiently quickly to amastigotes (as they are *in vitro*) then it is likely from the above findings that the number degraded after phagocytosis will be substantial, resulting in a low percentage of infected cells. It is also possible that the null mutants trigger a more potent anti-microbial mechanism since the receptors utilised in entry to macrophages are thought to play a pivotal role in the pathogenesis of the disease (Zwilling and Eisenstein, 1994) and in this study ultrastructural differences between the surface coats of wild type and Δ*lmcpb* stationary phase promastigotes were shown.

The finding that the *lmcpb* null mutant promastigotes did still infect cells and produce lesions, albeit at a lower rate, suggested that *lmcpb* was important but not vital for infection. However, the discovery that lesion amastigotes of the *lmcpb* null mutants infected macrophages *in vitro* to the same extent as wild type lesion amastigotes suggested that the major role of the enzymes was either in catalysing the differentiation to amastigotes (at which stage intracellular parasites may be particularly vulnerable) or alternatively in the formation of the metacyclic form, which may result in reduced macrophage activation upon phagocytosis or enhanced transformation to amastigotes. These findings rather contradicted the accepted opinion that, since the enzyme activity is highest in amastigotes, then the enzymes' major role must be in that stage of the parasite, but was supported by evidence that the *lmcpb* null mutants did not transform to and grow as axenic amastigotes efficiently *in vitro*.

Results of the infectivity of Δ*lmcpbn* lesion amastigotes to animals were crucial to resolving in which stage of the parasite the *lmcpb* CPs were most important. If they produced lesions as quickly as wild type then it could be concluded that differentiation was the most important role; if not then more likely their role was in circumventing the host immune system e.g. in degradation of MHC class II molecules (as reported by Leao *et al.*, 1995) or other immune-effector molecules such as cytokines (Finkelman and Urban, 1992) which would not be of consequence in *in vitro* macrophage infections.

CPs of organisms are reported to degrade host proteins such as immunoglobulins (Bontempi and Cazzullo, 1990) and complement factors (Reed *et al.*, 1989). They can modulate cytokine activity either directly (Kapur *et al.*, 1993) or through interaction with the plasma proteinase inhibitor α2-macroglobulin (Lamarre *et al.*, 1991). In several cases they are suspected to interfere with antigen presentation and processing (Arholdt and Scharfstein, 1991, Leao *et al.*, 1995). The discovery that lesion amastigotes of Δ *lmcpbn* took some three months longer to produce lesions than this stage of wild type parasites indicates that the *lmcpb* CPs may also be involved in immune evasion, and play an important but non-essential role in enabling *L. mexicana* to survive in the mammalian host tested. It is also possible that the Δ*lmcpbn* lesion amastigotes do invade macrophages to the same extent *in vivo* as they do *in vitro*, but are reduced in their capacity to invade other cell types of low phagocytic potential such as Langerhan's cells and fibroblasts (Blank *et al.*, 1993, and references therein) which could contribute to the reduced lesion sizes observed.

It is possible during animal infections that there is selection for *lmcpb* null mutants which have an alternative means of degradating MHC class II molecules; it may be interesting therefore to test whether lesion amastigotes of Δ*lmcpbn* can perform this degradation, and identify which, if any, enzymes are upregulated in this form of the parasite. It is also worth considering how important this strategy of immune evasion is to the parasite, since Δ*lmcpbn* does eventually produce lesions, albeit more slowly. Moreover, *Leishmania* amastigotes are fundamentally resistant to the degradative conditions of the phagolysosome and do not secrete their antigens, which would surely

result in a basic lack of parasite antigens for complexing with MHC II and presentation at the host cell surface in natural infections. However it is known that many intracellular lesion amastigotes lyse, especially in late infections (Ilg *et al.*, 1994), which would presumably lead to *Leishmania* antigens being available for immune recognition, and hence it could be at this stage that MHC II degradation by amastigotes is most important.

It has been postulated (McKerrow, 1991) that the CTE of the Type I CPs may be involved in the folding of the nascent proteinase, may function to inhibit the proteinase (until the CTE is removed) or, given the reported absence of mannose-6-phosphate targeting signals in *T. cruzi* and promastigotes of *Leishmania* (Cazzulo *et al.*, 1990), to be responsible for intracellular targeting of the enzymes. Our results using immunogold labelling of an enzyme with a truncated CTE (*g1*) were in agreement with other studies, which revealed that Lpcys1 of *L. pifanoi*, like Lpcys2, is localised to the flagellar pocket and megasomes, suggesting enzyme sorting to the megasome does not require a CTE (Duboise *et al.*, 1994). Further studies on Δ*lmcpbn* re-expressing different genes of the *lmcpb* array confirmed that not only do the genes encode isoenzymes with different activities, but also that they have different functions. This was clearly demonstrated by the failure of both *g1* and *g18* to restore infectivity to PECs, in direct contrast with results obtained using *g2.8*.

The observation that Δ*lmcpbn* amastigotes also reside in large PVs typical of the *L. mexicana* complex strongly argued against the theory that the Type I CPs contribute to this phenomenon. Furthermore, a more recent study has shown that polyanionic proteophosphoglycan, which is secreted into the PV by intracellular *L. mexicana*, is highly effective in inducing macrophage vacuolisation and is therefore implicated in causing the expansion of amastigote-containing PVs (Peters *et al.*, 1997).

Studies on the cathepsin-B like CP of *L. mexicana* using *lmcpc* null mutants revealed that although this CP appeared to play a role in the infection of PECs, it did not seem to have as an important role in the infectivity to animals, and in these respects differs from the roles of both the Type I and Type II CPs that have been implicated in this study. Similarities between Δ*lmcpan* and Δ*lmcpcn*, such as a reduction in the Type I

CP activities and unusual morphology of stationary phase promastigotes of both lines, indicate that the transfection process may be the cause of these features, and that phenotypic judgements on some criteria should be made with great caution. Cruz *et al.*, (1993) reported the loss of virulence in some, but not all, transfected clones of *L. major*, and postulated that this was due to long term culture *in vitro*. However, other researchers have found cell morphology to be unaffected after transfection e.g. HEXBP deletion mutants of *L. major* were identical to wild type at the light microscope level (Webb and McMaster, 1994).

In previous studies, peptidyl-diazomethane inhibitors such as Z-Phe-Ala-DMK have been shown to selectively inactivate *L. amazonensis* amastigote CPs (Alfieri *et al.*, 1989, 1991) and metacyclic specific CPs of *L. mexicana* (Bates *et al.*, 1994). In this investigation it was found that Z-Phe-Ala-DMK inhibited the *lmcpb* CPs but did not prevent the growth or transformation of *L. mexicana in vitro*. However, mutants lacking the *lmcpb* array were severely impeded in these processes. Results from experiments using radioiodinated DMK inhibitors suggested rapid *de novo* synthesis or processing of inactive enzyme precursors in *L. amazonenesis* (Alfieri *et al.*, 1991), which may account for the lack of effect on differentiation with inhibitor treated parasites. Also the lack of upregulation of the lowest mobility proteinases (>60 kDa) in Δ*lmcpbn* in comparison to inhibitor-treated wild type parasites may also have a bearing on the differentiation process.

Especially promising results were obtained *in vitro* using Z-Phe-Ala-DMK. This inhibitor reduced the infection rates of wild type parasites to PECs, although the specific enzyme(s) targeted was not established. Evidence exists that CPs in B cells and macrophages complete the removal of N terminal Ii (invariant chain) fragments from nascent class II molecules, a process which enables antigenic peptide binding within MHC compartments before subsequent delivery to the cell surface (Morton *et al.*, 1995). Therefore, in the search for new chemotherapeutic agents, it is important that inhibitors are specific for parasite proteinases and do not interfere with mammalian enzymes, which could in fact exacerbate infections by interfering with host cell antigen presentation.

In summary, studies on the null mutants for the three CP genes so far detected in *L. mexicana* have revealed that each enzyme type probably has a distinct biological function, albeit non-essential. Of the three CP genes, *lmcpb* would appear to play the biggest role in virulence under the conditions tested, a finding which is not surprising considering the multicopy existence and high activity of these CPs. Studies on multiple CP null mutants (e.g. Δ*lmcpc/lmcpbn* or Δ*lmcpa/lmcpb/lmcpcn*) may reveal whether or not the CPs of *L. mexicana* have an essential synergistic role. It is also clear from this investigation that aside from their utilisation in drug target validation, mutants lacking certain CP genes may have potential as attenuated live vaccines.

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APPENDIX A

Fig. 2.2.

wild type measurements (µm)			*dlmcpan* measurements (µm)		
7.5	10	12.5	10	16.25	18.75
7.5	10	12.5	10	16.25	18.75
7.5	10	12.5	10	16.25	18.75
7.5	10	12.5	11.25	16.25	18.75
7.5	10	12.5	11.25	16.25	18.75
7.5	10	12.5	11.25	16.25	20
7.5	10	12.5	12.5	16.25	20
8.75	10	12.5	12.5	16.25	20
8.75	10	13.75	12.5	16.25	20
8.75	10	13.75	12.5	16.25	20
8.75	10	13.75	12.5	16.25	20
8.75	10	13.75	12.5	16.25	20
8.75	10	13.75	13.75	17.5	20
8.75	10	13.75	13.75	17.5	20
8.75	10	13.75	13.75	17.5	20
8.75	10	15	13.75	17.5	20
8.75	10	15	13.75	17.5	20
8.75	11.25	15	13.75	17.5	21.25
8.75	11.25	15	13.75	17.5	21.25
8.75	11.25	15	13.75	17.5	21.25
8.75	11.25	15	13.75	17.5	21.25
8.75	11.25	15	13.75	17.5	21.25
8.75	11.25	15	15	17.5	21.25
8.75	11.25	15	15	17.5	22.5
8.75	11.25	16.25	15	17.5	22.5
8.75	11.25	17.5	15	17.5	22.5
8.75	11.25	17.5	15	17.5	22.5
10	11.25	18.75	15	18.75	22.5
10	11.25	18.75	15	18.75	23.75
10	11.25	18.75	15	18.75	23.75
10	11.25		15	18.75	
10	11.25		15	18.75	
10	11.25		16.25	18.75	
10	12.5		16.25	18.75	
10	12.5		16.25	18.75	

length (µm)	wild type	*dlmcpan*
7.5	7	0
8.75	20	0
10	24	3
11.25	17	3
12.5	10	6
13.75	7	10
15	9	10
16.25	1	15
17.5	2	15
18.75	3	13
20	0	12
21.25	0	6
22.5	0	5
23.75	0	2

Fig. 2.2. (cont)

wild type	**length**	***dlmcpan***	**length**
Mean	11.2125	Mean	17.0375
Standard E	0.269712	Standard E	0.3216
Median	10	Median	17.6
Mode	10	Mode	16.25
Standard D	2.697121	Standard D	3.215998
Variance	7.274463	Variance	10.34265
Kurtosis	0.505135	Kurtosis	-0.47931
Skewness	0.962929	Skewness	-0.12016
Range	11.25	Range	13.75
Minimum	7.5	Minimum	10
Maximum	18.75	Maximum	23.75
Sum	1121.25	Sum	1703.75
Count	100	Count	100

F-Test: Two-Sample for Variances

	wild type	***dlmcpan***
Mean	11.2125	17.0375
Variance	7.274463	10.34265
Observatio	100	100
df	99	99
F	1.421774	
P(F<=f) on	0.040769	
F Critical o	1.29513	

z-Test: Two-Sample for Means

	wild type	***dlmcpan***
Mean	11.2125	17.0375
Known Var	7.3	10.3
Observatio	100	100
Hypothesiz	0	
z	13.8848	
P(Z<=z) or	0	
z Critical o	2.575835	
P(Z<=z) tw	0	
z Critical tw	2.326342	
z Critical tw	1.644853	

Since z> the critical value of 2.58 at P=0.01 the difference is highly significant.

Fig 3.5. A

wild type measurements (µm) SP1			*dlmcpbn* measurements (µm) SP1		
7.5	10	12.5	10	16.25	20
7.5	10	12.5	10	16.25	20
7.5	10	12.5	10	16.25	20
7.5	10	12.5	11.25	16.25	20
7.5	10	12.5	11.25	17.5	20
7.5	10	12.5	12.5	17.5	20
7.5	10	12.5	12.5	17.5	20
8.75	10	12.5	12.5	17.5	20
8.75	10	13.75	12.5	17.5	20
8.75	10	13.75	13.75	17.5	20
8.75	10	13.75	13.75	17.5	21.25
8.75	10	13.75	13.75	17.5	21.25
8.75	10	13.75	13.75	17.5	21.25
8.75	10	13.75	13.75	17.5	21.25
8.75	10	13.75	13.75	17.5	21.25
8.75	10	15	13.75	17.5	21.25
8.75	10	15	13.75	17.5	21.25
8.75	11.25	15	15	17.5	21.25
8.75	11.25	15	15	17.5	21.25
8.75	11.25	15	15	18.75	21.25
8.75	11.25	15	15	18.75	21.25
8.75	11.25	15	15	18.75	21.25
8.75	11.25	15	15	18.75	21.25
8.75	11.25	15	15	18.75	21.25
8.75	11.25	16.25	15	18.75	22.5
8.75	11.25	17.5	16.25	18.75	22.5
8.75	11.25	17.5	16.25	18.75	22.5
10	11.25	18.75	16.25	18.75	23.75
10	11.25	18.75	16.25	18.75	23.75
10	11.25	18.75	16.25	18.75	23.75
10	11.25		16.25	18.75	
10	11.25		16.25	18.75	
10	11.25		16.25	18.75	
10	12.5		16.25	20	
10	12.5		16.25	20	

length (µm)	wild type	*dlmcpbn*
7.5	7	0
8.75	20	0
10	24	3
11.25	17	2
12.5	10	4
13.75	7	8
15	9	8
16.25	1	14
17.5	2	15
18.75	3	14
20	0	12
21.25	0	14
22.5	0	3
23.75	0	3

Fig. 3.5. A (cont)

wild type	**length**	***dlmcpbn***	**length**
Mean	11.2125	Mean	17.6125
Standard E	0.269712	Standard E	0.320341
Median	10	Median	17.5
Mode	10	Mode	17.5
Standard D	2.697121	Standard D	3.20341
Variance	7.274463	Variance	10.26184
Kurtosis	0.505135	Kurtosis	-0.32923
Skewness	0.962929	Skewness	-0.34822
Range	11.25	Range	13.75
Minimum	7.5	Minimum	10
Maximum	18.75	Maximum	23.75
Sum	1121.25	Sum	1761.25
Count	100	Count	100

F-Test: Two-Sample for Variances

	wild type	***dlmcpbn***
Mean	11.2125	17.6125
Variance	7.274463	10.26184
Observatio	100	100
df	99	99
F	1.410666	
P(F<=f) on	0.044266	
F Critical o	1.29513	

z-Test: Two-Sample for Means

	wild type	***dlmcpbn***
Mean	11.2125	17.6125
Known Var	7.3	10.3
Observatio	100	100
Hypothesiz	0	
z	15.2554	
P(Z<=z) or	0	
z Critical o	2.575835	
P(Z<=z) tw	0	
z Critical tw	2.326342	

Since z greatly exceeds the critical value of 2.58 at P=0.01 the difference is highly significant.

Fig 3.5. B

wild type measurements (µm) SP7			*dlmcpbn* measurements (µm) SP7		
7.5	11.25	13.75	8.75	15	17.5
7.5	11.25	13.75	10	15	17.5
7.5	11.25	13.75	10	15	17.5
7.5	11.25	13.75	10	15	17.5
7.5	11.25	13.75	10	15	18.75
7.5	11.25	13.75	11.25	15	18.75
7.5	11.25	13.75	11.25	16.25	18.75
8.75	11.25	15	11.25	16.25	18.75
8.75	11.25	15	11.25	16.25	18.75
8.75	11.25	15	11.25	16.25	18.75
8.75	11.25	15	12.5	16.25	18.75
8.75	12.5	15	12.5	16.25	18.75
8.75	12.5	15	12.5	16.25	18.75
8.75	12.5	15	12.5	16.25	18.75
8.75	12.5	15	12.5	16.25	18.75
8.75	12.5	16.25	12.5	16.25	18.75
10	12.5	16.25	12.5	16.25	18.75
10	12.5	16.25	13.75	16.25	18.75
10	12.5	16.25	13.75	16.25	20
10	12.5	17.5	13.75	16.25	20
10	12.5	17.5	13.75	16.25	20
10	12.5	17.5	13.75	16.25	20
10	12.5	18.75	13.75	16.25	20
10	12.5	18.75	13.75	17.5	20
10	12.5	18.75	13.75	17.5	20
10	12.5	18.75	13.75	17.5	20
10	12.5	18.75	15	17.5	20
10	12.5	20	15	17.5	20
10	12.5	21.25	15	17.5	21.25
10	12.5	23.75	15	17.5	21.25
10	12.5		15	17.5	
10	12.5		15	17.5	
10	13.75		15	17.5	
11.25	13.75		15	17.5	
11.25	13.75		15	17.5	

length (µm)	wild type	*dlmcpbn*
7.5	7	0
8.75	9	1
10	17	4
11.25	13	5
12.5	21	7
13.75	10	9
15	8	15
16.25	4	17
17.5	3	16
18.75	5	14
20	1	10
21.25	1	2
22.5	0	0
23.75	1	0

Fig. 3.5. B (cont)

wild type	**length**	***dlmcpbn***	**length**
Mean	12.4375	Mean	16.025
Standard E	0.330182	Standard E	0.286689
Median	12.5	Median	16.25
Mode	12.5	Mode	16.25
Standard D	3.301816	Standard D	2.866891
Variance	10.90199	Variance	8.219066
Kurtosis	0.813064	Kurtosis	-0.41696
Skewness	0.867195	Skewness	-0.43077
Range	16.25	Range	12.5
Minimum	7.5	Minimum	8.75
Maximum	23.75	Maximum	21.25
Sum	1243.75	Sum	1602.5
Count	100	Count	100

F-Test: Two-Sample for Variances

	wild type	***dlmcpbn***
Mean	12.4375	16.025
Variance	10.90199	8.219066
Observatio	100	100
df	99	99
F	1.326427	
P(F<=f) on	0.080831	
F Critical o	1.394062	

z-Test: Two-Sample for Means

	wild type	***dlmcpbn***
Mean	12.4375	16.025
Known Var	10.9	8.2
Observatio	100	100
Hypothesiz	0	
z	8.208716	
P(Z<=z) or	5.55E-17	
z Critical o	2.575835	
P(Z<=z) tw	1.11E-16	
z Critical tw	2.326342	

Since z exceeds the critical value of 2.58 at P=0.01 the difference is highly significant.

Table 3.2. A

% **infected**

wild type	*dlmcpbn*	*tlmcpbg2.8*
33	14	29
44	5	38
58	10	42
47	7.5	51
46 ± 10	9.2 ± 10	40 ± 9.0

F-Test: Two-Sample for Variances

	wild type	*dlmcpbn*
Mean	45.5	9.125
Variance	105.6667	14.72917
Observatic	4	4
df	3	3
F	7.173975	
P(F<=f) on	0.069916	
F Critical o	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbn*	
Mean	45.5	9.125	
Variance	105.6667	14.72917	
Observatic	4	4	
Pooled Vai	60.19792		
Hypothesiz	0		
df	6		
t	6.63021	since t>3.707 there is a highly	
P(T<=t) on	0.000284	significant difference at P=0.01	
t Critical or	1.943181		
P(T<=t) tw	0.000568		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	wild type	*lmcpbg2.8*
Mean	45.5	40
Variance	105.6667	83.33333
Observatic	4	4
df	3	3
F	1.268	
P(F<=f) on	0.424949	
F Critical o	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*lmcpbg2.8*	
Mean	45.5	40	
Variance	105.6667	83.33333	
Observatic	4	4	
Pooled Vai	94.5		
Hypothesiz	0		
df	6		
t	0.800132	no significant difference	
P(T<=t) on	0.22707		
t Critical or	1.943181		
P(T<=t) tw	0.454139		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbn	*lmcpbg2.8*
Mean	9.125	40
Variance	14.72917	83.33333
Observatic	4	4
df	3	3
F	5.657709	
P(F<=f) on	0.094244	
F Critical o	5.390774	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*lmcpbg2.8*	
Mean	9.125	40	
Variance	14.72917	83.33333	
Observatic	4	4	
Pooled Vai	49.03125		
Hypothesiz	0		
df	6		
t	6.235704	since t>3.707 there is a highly	
P(T<=t) on	0.000394	significant difference at P=0.01	
t Critical or	1.943181		
P(T<=t) tw	0.000787		
t Critical tw	2.446914		

Table 3.2. A (cont)

amastigotes/PEC

wild type	*dlmcpbn*	*dlmcpbg2.8*
4	2.4	3.4
3.9	1.8	3.1
3.7	3.4	3.3
3.6	2.8	3
3.8 ± 0.2	2.6 ± 0.7	3.2 ± 0.2

F-Test: Two-Sample for Variances

	wild type	*dlmcpbn*
Mean	3.8	2.6
Variance	0.033333	0.453333
Observatic	4	4
df	3	3
F	13.6	
P(F<=f) on	0.029798	
F Critical o	5.390774	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbn*	
Mean	3.8	2.6	
Variance	0.033333	0.453333	
Observatic	4	4	
Pooled Va	0.243333		
Hypothesiz	0		
df	6		
t	3.440293	since t>3.14 there is a	
P(T<=t) on	0.006898	significant difference at P=0.02	
t Critical or	1.943181		
P(T<=t) tw	0.013796		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	wild type	*lmcpbg2.8*
Mean	3.8	3.2
Variance	0.033333	0.033333
Observatic	4	4
df	3	3
F	1	
P(F<=f) on	0.5	
F Critical o	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*lmcpbg2.8*	
Mean	3.8	3.2	
Variance	0.033333	0.033333	
Observatic	4	4	
Pooled Va	0.033333		
Hypothesiz	0		
df	6		
t	4.64758	since t>3.707 there is a highly	
P(T<=t) on	0.001756	significant difference at P=0.01	
t Critical or	1.943181		
P(T<=t) tw	0.003513		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbn	*lmcpbg2.8*
Mean	2.6	3.2
Variance	0.453333	0.033333
Observatic	4	4
df	3	3
F	13.6	
P(F<=f) on	0.029798	
F Critical o	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*lmcpbg2.8*	
Mean	2.6	3.2	
Variance	0.453333	0.033333	
Observatic	4	4	
Pooled Va	0.243333		
Hypothesiz	0		
df	6		
t	1.720147	no significant difference	
P(T<=t) on	0.068098		
t Critical or	1.943181		
P(T<=t) tw	0.136197		
t Critical tw	2.446914		

Table 3.2. B

	% infected			
wild type	*dlmcpbBL*	*dlmcpbn*	*dlmcpbn /dlmcpan*	*dlmcpbn/dlmcpag2.8*
59.6	46	4.5	5.6	3.3
41	42	2.4	1.8	3.1
47.4	66	2.8	2.5	0.9
	62			
50 ± 9.4	54 ± 10	3.2 ± 1.1	3.3 ± 2	2.4 ± 1.3

F-Test: Two-Sample for Variances

	wild type	*dlmcpbBL*
Mean	49.33333	54
Variance	89.29333	138.6667
Observatic	3	4
df	2	3
F	1.552934	
P(F<=f) on	0.414783	
F Critical c	9.161795	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbBL*	
Mean	49.33333	54	
Variance	89.29333	138.6667	
Observatic	3	4	
Pooled Va	118.9173		
Hypothesi:	0		
df	5		
t	0.560307		no significant difference
P(T<=t) on	0.299723		
t Critical o	2.015049		
P(T<=t) tw	0.599445		
t Critical tv	2.570578		

F-Test: Two-Sample for Variances

	wild type	*dlmcpbn*
Mean	49.33333	3.233333
Variance	89.29333	1.243333
Observatic	3	3
df	2	2
F	71.81769	
P(F<=f) on	0.013733	
F Critical c	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbn*	
Mean	49.33333	3.233333	
Variance	89.29333	1.243333	
Observatic	3	3	
Pooled Va	45.26833		
Hypothesi:	0		
df	4		
t	8.391687		since t>4.604 there is a highly
P(T<=t) on	0.000552		significant difference at P=0.01
t Critical o	2.131846		
P(T<=t) tw	0.001103		
t Critical tv	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbn/dlmcpan*
Mean	3.233333	3.3
Variance	1.243333	4.09
Observatic	3	3
df	2	2
F	3.289544	
P(F<=f) on	0.233125	
F Critical c	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbn/dlmcpan*	
Mean	3.233333	3.3	
Variance	1.243333	4.09	
Observatic	3	3	
Pooled Va	2.666667		
Hypothesi:	0		
df	4		
t	0.05		no significant difference
P(T<=t) on	0.48126		
t Critical o	2.131846		
P(T<=t) tw	0.96252		
t Critical tv	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn/dlmcpan	*dlmcpbn/dlmcpag2.8*
Mean	3.3	2.433333
Variance	4.09	1.773333
Observatic	3	3
df	2	2
F	2.306391	
P(F<=f) on	0.302445	
F Critical c	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn/dlmcpan	*dlmcpbn/dlmcpag2.8*	
Mean	3.3	2.433333	
Variance	4.09	1.773333	
Observatic	3	3	
Pooled Va	2.931667		
Hypothesi:	0		
df	4		
t	0.619927		no significant difference
P(T<=t) on	0.284438		
t Critical o	2.131846		
P(T<=t) tw	0.568876		
t Critical tv	2.776451		

Table 3.2. B (cont) **amastigotes/PEC**

wild type	*dlmcpbBL*	*dlmcpbn*	*dlmcpbn /dlmcpan*	*dlmcpbn/dlmcpag2.8*
3.5	4.6	1.6	2.75	2.1
3.6	5	3	3.2	1.7
5.3	5.2	3.5	1.8	3
	5.8			
4.1 ± 1	5.3 ± 1	2.7 ± 0.9	2.6 ± 0.7	2.3 ± 0.7

F-Test: Two-Sample for Variances

	wild type	*dlmcpbBL*
Mean	4.133333	5.15
Variance	1.023333	0.25
Observatic	3	4
df	2	3
F	4.093333	
P(F<=f) on	0.138877	
F Critical c	9.552082	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbBL*	
Mean	4.133333	5.15	
Variance	1.023333	0.25	
Observatic	3	4	
Pooled Va	0.559333		
Hypothesi:	0		
df	5		
t	1.779856		no significant difference
P(T<=t) on	0.05761		
t Critical o	2.015049		
P(T<=t) tw	0.13522		
t Critical tw	2.570578		

F-Test: Two-Sample for Variances

	wild type	*dlmcpbn*
Mean	4.133333	2.7
Variance	1.023333	0.97
Observatic	3	3
df	2	2
F	1.054983	
P(F<=f) on	0.486622	
F Critical c	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbn*	
Mean	4.133333	2.7	
Variance	1.023333	0.97	
Observatic	3	3	
Pooled Va	0.996667		
Hypothesi:	0		
df	4		
t	1.758401		no significant difference
P(T<=t) on	0.076752		
t Critical o	2.131846		
P(T<=t) tw	0.153503		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbn/dlmcpan*
Mean	2.7	2.583333
Variance	0.97	0.510833
Observatic	3	3
df	2	2
F	1.898858	
P(F<=f) on	0.344963	
F Critical c	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbn/dlmcpan*	
Mean	2.7	2.583333	
Variance	0.97	0.510833	
Observatic	3	3	
Pooled Va	0.740417		
Hypothesi:	0		
df	4		
t	0.166056		no significant difference
P(T<=t) on	0.438084		
t Critical or	2.131846		
P(T<=t) tw	0.876168		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

dlmcpbn/dlmcpan	*dlmcpbn/dlmcpag2.8*
2.583333	2.266667
0.510833	0.443333
3	3
2	2
1.152256	
0.464629	
19.00003	

t-Test: Two-Sample Assuming Equal Variances

dlmcpbn/dlmcpan	*dlmcpbn/dlmcpag2.8*	
2.583333	2.266667	
0.510833	0.443333	
3	3	
0.477083		
0		
4		
0.561501		no significant difference
0.302212		
2.131846		
0.604424		
2.776451		

Table 3.2. C **% infected**

wild type	*dlmcpan*	*dlmcpbBL*	*dlmcpbn*	*dlmcpb/dlmcpan*
28	27	46	1.6	0.4
39	30	42	1.6	4.2
30	40	39	1.9	4.8
46	50	30	1.2	1.9
36 ± 8.3	36 ± 10	39 ± 6.8	1.6 ± 0.3	2.8 ± 2

F-Test: Two-Sample for Variances

	wild type	*dlmcpan*
Mean	38.33333	36.75
Variance	64.33333	108.9167
Observatic	3	4
df	2	3
F	1.693005	
P(F<=f) on	0.392271	
F Critical c	9.161795	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpan*	
Mean	35.75	36.75	
Variance	69.58333	108.9167	
Observatic	4	4	
Pooled Va	89.25		
Hypothesi	0		
df	6		
t	0.149696	no significant difference	
P(T<=t) on	0.442955		
t Critical o	1.943181		
P(T<=t) tw	0.885909		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpan	*dlmcpbBL*
Mean	36.75	39.25
Variance	108.9167	46.25
Observatic	4	4
df	3	3
F	2.354955	
P(F<=f) on	0.250062	
F Critical c	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpan	*dlmcpbBL*	
Mean	36.75	39.25	
Variance	108.9167	46.25	
Observatic	4	4	
Pooled Va	77.58333		
Hypothesi	0		
df	6		
t	0.401394	no significant difference	
P(T<=t) on	0.351018		
t Critical o	1.943181		
P(T<=t) tw	0.702035		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbBL	*dlmcpbn*
Mean	39.25	1.575
Variance	46.25	0.0825
Observatic	4	4
df	3	3
F	560.6061	
P(F<=f) on	0.000127	
F Critical c	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbBL	*dlmcpbn*	
Mean	39.25	1.575	
Variance	46.25	0.0825	
Observatic	4	4	
Pooled Va	23.16625		
Hypothesi	0		
df	6		
t	11.06982	since t>3.707 there is a highly	
P(T<=t) on	1.62E-05	significant difference at P=0.01	
t Critical o	1.943181		
P(T<=t) tw	3.24E-05		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbn/dlmcpan*
Mean	1.575	2.825
Variance	0.0825	4.175833
Observatic	4	4
df	3	3
F	50.61616	
P(F<=f) on	0.004551	
F Critical c	5.390774	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbn/dlmcpan*	
Mean	1.575	2.825	
Variance	0.0825	4.175833	
Observatic	4	4	
Pooled Va	2.129167		
Hypothesi	0		
df	6		
t	1.211491	no significant difference	
P(T<=t) on	0.135626		
t Critical o	1.943181		
P(T<=t) tw	0.271251		
t Critical tw	2.446914		

Table 3.2. C (cont) amastigotes/PEC

wild type	*dlmcpan*	*dlmcpbBL*	*dlmcpbn*	*dlmcpb/dlmcpan*
5.1	5.6	6.3	2	0
6	5.1	5.1	1.7	2
3.1	3	3	1.3	2.6
4.3	4	3.4	1.5	4.4
4.6 ± 1.3	4.4 ± 1.2	4.5 ± 1.6	1.6 ± 0.3	2.2 ± 1.8

F-Test: Two-Sample for Variances

	wild type	*dlmcpan*
Mean	4.625	4.425
Variance	1.515833	1.349167
Observatic	4	4
df	3	3
F	1.123533	
P(F<=f) on	0.462987	
F Critical c	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpan*	
Mean	4.625	4.425	
Variance	1.515833	1.349167	
Observatic	4	4	
Pooled Va	1.4325		
Hypothesi	0		
df	6		
t	0.236318	no significant difference	
P(T<=t) on	0.410523		
t Critical or	1.943181		
P(T<=t) tw	0.821046		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpan	*dlmcpbBL*
Mean	4.425	4.45
Variance	1.349167	2.35
Observatic	4	4
df	3	3
F	1.741816	
P(F<=f) on	0.329883	
F Critical c	5.390774	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpan	*dlmcpbBL*	
Mean	4.425	4.45	
Variance	1.349167	2.35	
Observatic	4	4	
Pooled Va	1.849583		
Hypothesi	0		
df	6		
t	0.025997	no significant difference	
P(T<=t) on	0.490052		
t Critical or	1.943181		
P(T<=t) tw	0.980103		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbBL	*dlmcpbn*
Mean	4.45	1.625
Variance	2.35	0.089167
Observatic	4	4
df	3	3
F	26.35514	
P(F<=f) on	0.011735	
F Critical c	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbBL	*dlmcpbn*	
Mean	4.45	1.625	
Variance	2.35	0.089167	
Observatic	4	4	
Pooled Va	1.219583		
Hypothesi	0		
df	6		
t	3.61766	since t>3.14 there is a	
P(T<=t) on	0.005564	significant difference at P=0.02	
t Critical or	1.943181		
P(T<=t) tw	0.011128		
t Critical tw	2.446914		

F-Test: Two-Sample for Variances

	dlmcpbn/dlmcpan	*dlmcpbn*
Mean	2.25	1.625
Variance	3.29	0.089167
Observatic	4	4
df	3	3
F	36.8972	
P(F<=f) on	0.007219	
F Critical c	9.276619	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn/dlmcpan	*dlmcpbn*	
Mean	2.25	1.625	
Variance	3.29	0.089167	
Observatic	4	4	
Pooled Va	1.689583		
Hypothesi	0		
df	6		
t	0.679994	no significant difference	
P(T<=t) on	0.260933		
t Critical or	1.943181		
P(T<=t) tw	0.521866		
t Critical tw	2.446914		

Table 3.3.
wild type

amastigotes/ vacuole	vacuole size (µm)
1	5
2	7.5
2	10
2	10
2	11.25
2	12.5
2	12.5
2	13.75
2	13.75
2	17.5
3	11.25
3	15
3	26.25
4	10
4	11.25
4	11.25
4	13.75
4	13.75
4	15
4	16.25
4	16.25
4	16.25
4	16.25
4	17.5
4	18.75
4	18.75
4	20
5	12.5
5	13.75
5	16.25
5	18.75
6	8.75
6	11.25
6	12.5
6	15
6	16.25
6	17.5
6	25
6	27.5
7	18.75
7	21.25
7	26.25
8	15
8	20
8	26.25
8	37.5
9	18.75
9	25
10	25
12	32.5

no. amastigotes

Mean	4.9
Standard E	0.340767
Median	4
Mode	4
Standard D	2.40959
Variance	5.806122
Kurtosis	0.323333
Skewness	0.723894
Range	11
Minimum	1
Maximum	12
Sum	245
Count	50

vacuole size

Mean	16.85
Standard E	0.912107
Median	16.25
Mode	16.25
Standard D	6.449569
Variance	41.59694
Kurtosis	1.301443
Skewness	1.01309
Range	32.5
Minimum	5
Maximum	37.5
Sum	842.5
Count	50

vacuole size/ amastigote (µm)
5
3.75
5
5
5.625
6.25
6.25
6.875
6.875
8.75
3.75
5
8.75
2.5
2.8125
2.8125
3.4375
3.4375
3.75
4.0625
4.0625
4.0625
4.375
4.6875
4.6875
5
2.5
2.75
3.25
3.75
1.458333
1.875
2.083333
2.5
2.708333
2.916667
4.166667
4.583333
2.678571
3.035714
3.75
1.875
2.5
3.28125
4.6875
2.083333
2.777778
2.5
2.708333

vac. size/amastigote

Mean	3.940883
Standard E	0.230674
Median	3.75
Mode	5
Standard D	1.63111
Variance	2.660519
Kurtosis	1.456603
Skewness	1.149227
Range	7.291667
Minimum	1.458333
Maximum	8.75
Sum	197.0441
Count	50

Table 3.3.
dimcpbn

amastigotes/ vacuole	vacuole size (µm)
2	8.75
2	10
2	10
2	13.75
2	15
3	6.25
3	6.25
4	5
4	8.75
4	11.25
4	11.25
4	11.25
4	12.5
4	12.5
4	12.5
4	13.75
4	13.75
4	13.75
4	15
4	15
4	16.25
4	20
4	21.25
5	13.75
6	7.5
6	12.5
6	13.75
6	15
6	15
6	15
6	16.25
6	17.5
6	18.75
6	23.75
6	31.25
7	18.75
7	22.5
8	12.5
8	15
8	20
9	11.25
10	16.25
10	16.25
12	16.25
12	23.75
12	31.25
13	32.5
15	17.5
16	18.75
20	22.5

no. amastigotes

Mean	6.36
Standard E	0.551628
Median	6
Mode	4
Standard D	3.900602
Variance	15.21469
Kurtosis	2.465898
Skewness	1.552664
Range	18
Minimum	2
Maximum	20
Sum	318
Count	50

vacuole size

Mean	15.575
Standard E	0.849827
Median	15
Mode	15
Standard D	6.009187
Variance	36.11033
Kurtosis	1.38081
Skewness	0.975357
Range	27.5
Minimum	5
Maximum	32.5
Sum	778.75
Count	50

vacuole size/ amastigote (µm)
4.375
5
5
6.875
7.5
2.083333
2.083333
1.25
2.1875
2.8125
2.8125
2.8125
3.125
3.125
3.125
3.4375
3.4375
3.4375
3.75
3.75
4.0625
5
5.3125
2.75
1.25
2.083333
2.291667
2.5
2.5
2.5
2.708333
2.916667
3.125
3.958333
5.208333
2.678571
3.214286
1.5625
1.875
2.5
1.25
1.625
1.625
1.354167
1.979167
2.604167
2.5
1.166667
1.171875
1.125

vac. size/amastigote

Mean	2.967545
Standard E	0.200922
Median	2.729167
Mode	2.5
Standard D	1.420736
Variance	2.018491
Kurtosis	1.618483
Skewness	1.169985
Range	6.375
Minimum	1.125
Maximum	7.5
Sum	148.3772
Count	50

Table 3.3. (cont)

z-Test: Two-Sample for Means

	vacuole sizes (µm)	
	wild type	*dlmcpbn*
Mean	3.940883	2.967545
Known Var	2.661	2.018
Observatio	50	50
Hypothesiz	0	
z	3.181796	
P(Z<=z) or	0.000366	
z Critical or	2.575835	
P(Z<=z) tw	0.000732	
z Critical tw	2.326342	

Since z exceeds the critical value of 2.58 there is a highly significant difference at P=0.01.

Table 3.8.

% infected

wild type	*dlmcpbn*	*mcpbg2.8*	*dlmcpbg1*	*dlmcpbg18*
28	1.6	19	0.6	5.5
46	1.6	37	6	2.9
39	1.2	31	3.8	5.4
38 ± 9.0	1.5 ± 0.2	29 ± 9.2	3.5 ± 2.8	4.6 ± 1.4

F-Test: Two-Sample for Variances

	wild type	***dlmcpbn***
Mean	37.66667	1.466667
Variance	82.33333	0.053333
Observatic	3	3
df	2	2
F	1543.75	
P(F<=f) on	0.000647	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	***dlmcpbn***	
Mean	37.66667	1.466667	
Variance	82.33333	0.053333	
Observatic	3	3	
Pooled Vai	41.19333		
Hypothesiz	0		
df	4		
t	6.907816	Since t>4.604 there is a highly	
P(T<=t) on	0.001152	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	**0.002304**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	wild type	***ilmcpbg2.8***
Mean	37.66667	29
Variance	82.33333	84
Observatic	3	3
df	2	2
F	1.020243	
P(F<=f) on	0.49499	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	wild type	***ilmcpbg2.8***	
Mean	37.66667	29	
Variance	82.33333	84	
Observatic	3	3	
Pooled Vai	83.16667		
Hypothesiz	0		
df	4		
t	1.16392	no significant difference	
P(T<=t) on	0.154574		
t Critical or	2.131846		
P(T<=t) tw	0.309148		
t Critical tw	2.776451		

Table 3.8. (cont)

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbg1*
Mean	1.466667	3.466667
Variance	0.053333	7.373333
Observatic	3	3
df	2	2
F	138.25	
P(F<=f) on	0.007181	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbg1*	
Mean	1.466667	3.466667	
Variance	0.053333	7.373333	
Observatic	3	3	
Pooled Vai	3.713333		
Hypothesiz	0		
df	4		
t	1.271141	no significant difference	
P(T<=t) on	0.136285		
t Critical or	2.131846		
P(T<=t) tw	0.272569		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbg18*
Mean	1.466667	4.6
Variance	0.053333	2.17
Observatic	3	3
df	2	2
F	40.6875	
P(F<=f) on	0.023988	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbg18*	
Mean	1.466667	4.6	
Variance	0.053333	2.17	
Observatic	3	3	
Pooled Vai	1.111667		
Hypothesiz	0		
df	4		
t	3.639695	Since t>3.747 there is a highly	
P(T<=t) on	0.010985	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.021971		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbg1	*dlmcpbg18*
Mean	3.466667	4.6
Variance	7.373333	2.17
Observatic	3	3
df	2	2
F	3.397849	
P(F<=f) on	0.227384	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbg1	*dlmcpbg18*	
Mean	3.466667	4.6	
Variance	7.373333	2.17	
Observatic	3	3	
Pooled Vai	4.771667		
Hypothesiz	0		
df	4		
t	0.635431	no significant difference	
P(T<=t) on	0.279841		
t Critical or	2.131846		
P(T<=t) tw	0.559682		
t Critical tw	2.776451		

Table 3.8. (cont) **amastigotes/PEC**

wild type	*dlmcpbn*	*mcpbg2.8*	*dlmcpbg1*	*dlmcpbg18*
5.1	2	3.1	0.9	2.5
4.4	1.7	3.7	2	2.4
6	1.5	3.8	2.6	2.8
5.2 ± 0.9	1.7 ± 0.2	3.6 ± 0.4	1.8 ± 0.7	2.6 ± 0.2

F-Test: Two-Sample for Variances

	wild type	***dlmcpbn***
Mean	5.166667	1.733333
Variance	0.643333	0.063333
Observatic	3	3
df	2	2
F	10.15789	
P(F<=f) on	0.089623	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	***dlmcpbn***	
Mean	5.166667	1.733333	
Variance	0.643333	0.063333	
Observatic	3	3	
Pooled Vai	0.353333		
Hypothesiz	0		
df	4		
t	7.074069	Since t>4.604 there is a highly	
P(T<=t) on	0.001054	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	**0.002107**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	wild type	***ilmcpbg2.8***
Mean	5.166667	3.533333
Variance	0.643333	0.143333
Observatic	3	3
df	2	2
F	4.488372	
P(F<=f) on	0.182203	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	***ilmcpbg2.8***	
Mean	5.166667	3.533333	
Variance	0.643333	0.143333	
Observatic	3	3	
Pooled Vai	0.393333		
Hypothesiz	0		
df	4		
t	3.189628	Since t>2.776 there is a highly	
P(T<=t) on	0.016614	significant difference at P=0.05	
t Critical or	2.131846		
P(T<=t) tw	0.033228		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	***ilmcpbg2.8***
Mean	1.733333	3.533333
Variance	0.063333	0.143333
Observatic	3	3
df	2	2
F	2.263158	
P(F<=f) on	0.306452	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	***ilmcpbg2.8***	
Mean	1.733333	3.533333	
Variance	0.063333	0.143333	
Observatic	3	3	
Pooled Vai	0.103333		
Hypothesiz	0		
df	4		
t	6.858007	Since t>4.604 there is a highly	
P(T<=t) on	0.001183	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	0.002367		
t Critical tw	2.776451		

Table 3.8. (cont)

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbg1*
Mean	1.733333	1.833333
Variance	0.063333	0.743333
Observatic	3	3
df	2	2
F	11.73684	
P(F<=f) on	0.078512	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbg1*	
Mean	1.733333	1.833333	
Variance	0.063333	0.743333	
Observatic	3	3	
Pooled Vai	0.403333		
Hypothesiz	0		
df	4		
t	0.192847	no significant difference	
P(T<=t) on	0.428237		
t Critical or	2.131846		
P(T<=t) tw	0.856474		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpbg18*
Mean	1.733333	2.566667
Variance	0.063333	0.043333
Observatic	3	3
df	2	2
F	1.461538	
P(F<=f) on	0.40625	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpbg18*	
Mean	1.733333	2.566667	
Variance	0.063333	0.043333	
Observatic	3	3	
Pooled Vai	0.053333		
Hypothesiz	0		
df	4		
t	4.419417	Since t>3.747 there is a	
P(T<=t) on	0.005758	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.011516		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbg1	*dlmcpbg18*
Mean	1.833333	2.566667
Variance	0.743333	0.043333
Observatic	3	3
df	2	2
F	17.15385	
P(F<=f) on	0.055085	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbg1	*dlmcpbg18*	
Mean	1.833333	2.566667	
Variance	0.743333	0.043333	
Observatic	3	3	
Pooled Vai	0.393333		
Hypothesiz	0		
df	4		
t	1.432078	no significant difference	
P(T<=t) on	0.112694		
t Critical or	2.131846		
P(T<=t) tw	0.225388		
t Critical tw	2.776451		

Fig. 4.1.

	cell line	
	wild type	***dlmcpcn***
doubling time (h)	8.3	5.3
	11	7.1
	12.7	8.1
	11 ± 2.0	6.8 ± 1.4

F-Test: Two-Sample for Variances

	wild type	***dlmcpcn***
Mean	10.6666667	6.83333333
Variance	4.92333333	2.01333333
Observations	3	3
df	2	2
F	2.44536424	
P(F<=f) one-	0.29024507	
F Critical one	19.0000264	

t-Test: Two-Sample Assuming Equal Variances

	wild type	***dlmcpcn***	
Mean	10.6666667	6.83333333	
Variance	4.92333333	2.01333333	
Observations	3	3	
Pooled Varia	3.46833333		
Hypothesizec	0		
df	4		
t	2.52093588	since t>2.131 there is a	
P(T<=t) one-	0.03264582	significant difference at P=0.1	
t Critical one-	2.13184649		
P(T<=t) two-t	**0.06529164**		
t Critical two-	2.77645086		

Table 4.1. % infected

wild type	*dlmcpbn*	*dlmcpcn*	*dlmcpcPC*
54	3.5	1.4	32
29	1.4	6.9	18
30	1.2	5.9	9.8
38 ± 14	2.0 ± 1.3	4.7 ± 2.9	20 ± 11

F-Test: Two-Sample for Variances

	wild type	*dlmcpbn*
Mean	37.66667	2.033333
Variance	200.3333	1.623333
Observatio	3	3
df	2	2
F	123.4066	
P(F<=f) on	0.008038	
F Critical o	19.00003	

significant difference at P=0.01

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpbn*	
Mean	37.66667	2.033333	
Variance	200.3333	1.623333	
Observatio	3	3	
Pooled Vai	100.9783		
Hypothesiz	0		
df	4		
t	4.342982	Since t>3.747 there is a highly	
P(T<=t) on	0.006112	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.012224		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	*dlmcpcn*
Mean	2.033333	4.733333
Variance	1.623333	8.583333
Observatio	3	3
df	2	2
F	5.287474	
P(F<=f) on	0.159046	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	*dlmcpcn*	
Mean	2.033333	4.733333	
Variance	1.623333	8.583333	
Observatio	3	3	
Pooled Vai	5.103333		
Hypothesiz	0		
df	4		
t	1.463802	no significant difference	
P(T<=t) on	0.108546		
t Critical or	2.131846		
P(T<=t) tw	0.217092		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpcn	*dlmcpcPC*
Mean	4.733333	19.93333
Variance	8.583333	126.0133
Observatio	3	3
df	2	2
F	14.68117	
P(F<=f) on	0.063771	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpcn	*dlmcpcPC*	
Mean	4.733333	19.93333	
Variance	8.583333	126.0133	
Observatio	3	3	
Pooled Vai	67.29833		
Hypothesiz	0		
df	4		
t	2.269275	Since t>2.131 there is a	
P(T<=t) on	0.042898	significant difference at P=0.1	
t Critical or	2.131846		
P(T<=t) tw	0.085796		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	wild type	*dlmcpcPC*
Mean	37.66667	19.93333
Variance	200.3333	126.0133
Observatio	3	3
df	2	2
F	1.589779	
P(F<=f) on	0.386133	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	*dlmcpcPC*	
Mean	37.66667	19.93333	
Variance	200.3333	126.0133	
Observatio	3	3	
Pooled Vai	163.1733		
Hypothesiz	0		
df	4		
t	1.700245	no significant difference	
P(T<=t) on	0.082154		
t Critical or	2.131846		
P(T<=t) tw	0.164308		
t Critical tw	2.776451		

Table 4.1. (cont) **amastigotes/PEC**

wild type	dlmcpbn	dlmcpcn	dlmcpcPC
5	3.7	3.3	3.6
6.2	3.2	4	5.6
6.4	1.8	4.6	6.4
5.9 ± 0.8	2.9 ± 1.0	4.0 ± 0.6	5.2 ± 1.4

F-Test: Two-Sample for Variances

	wild type	dlmcpbn
Mean	5.866667	2.9
Variance	0.573333	0.97
Observatio	3	3
df	2	2
F	1.69186	
P(F<=f) on	0.37149	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	wild type	dlmcpbn	
Mean	5.866667	2.9	
Variance	0.573333	0.97	
Observatio	3	3	
Pooled Vai	0.771667		
Hypothesiz	0		
df	4		
t	4.136181	since t>3.747 there is a	
P(T<=t) on	0.007211	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.014421		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpbn	dlmcpcn
Mean	2.9	3.966667
Variance	0.97	0.423333
Observatio	3	3
df	2	2
F	2.291339	
P(F<=f) on	0.303828	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpbn	dlmcpcn	
Mean	2.9	3.966667	
Variance	0.97	0.423333	
Observatio	3	3	
Pooled Vai	0.696667		
Hypothesiz	0		
df	4		
t	1.565171	no significant difference	
P(T<=t) on	0.096298		
t Critical or	2.131846		
P(T<=t) tw	0.192596		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	dlmcpcn	dlmcpcPC
Mean	3.966667	5.2
Variance	0.423333	2.08
Observatio	3	3
df	2	2
F	4.913386	
P(F<=f) on	0.169108	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	dlmcpcn	dlmcpcPC	
Mean	3.966667	5.2	
Variance	0.423333	2.08	
Observatio	3	3	
Pooled Vai	1.251667		
Hypothesiz	0		
df	4		
t	1.350149	no significant difference	
P(T<=t) on	0.124154		
t Critical or	2.131846		
P(T<=t) tw	0.248307		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	wild type	dlmcpcPC
Mean	5.866667	5.2
Variance	0.573333	2.08
Observatio	3	3
df	2	2
F	3.627907	
P(F<=f) on	0.21608	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	wild type	dlmcpcPC	
Mean	5.866667	5.2	
Variance	0.573333	2.08	
Observatio	3	3	
Pooled Vai	1.326667		
Hypothesiz	0		
df	4		
t	0.708881	no significant difference	
P(T<=t) on	0.258764		
t Critical or	2.131846		
P(T<=t) tw	0.517528		
t Critical tw	2.776451		

Fig 5.7. A **% infected (z-fa)**

72h promastigote	1) pre	2) 4h	3) 24h	4) control
	12	20	14	34
	21	22	16	29
	24	19	36	42
	19 ± 6.2	20 ± 1.5	22 ± 12	35 ± 6.6

F-Test: Two-Sample for Variances

	1) pre	2) 4h
Mean	18.86667	20.33333
Variance	38.25333	2.333333
Observatio	3	3
df	2	2
F	16.39429	
P(F<=f) on	0.05749	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	2) 4h	
Mean	18.86667	20.33333	
Variance	38.25333	2.333333	
Observatio	3	3	
Pooled Var	20.29333		
Hypothesiz	0		
df	4		
t	0.39875	no significant difference	
P(T<=t) on	0.355224		
t Critical or	2.131846		
P(T<=t) tw	0.710448		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	3) 24h
Mean	18.86667	22
Variance	38.25333	148
Observatio	3	3
df	2	2
F	3.868944	
P(F<=f) on	0.205383	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	3) 24h	
Mean	18.86667	22	
Variance	38.25333	148	
Observatio	3	3	
Pooled Var	93.12667		
Hypothesiz	0		
df	4		
t	0.397663	no significant difference	
P(T<=t) on	0.355594		
t Critical or	2.131846		
P(T<=t) tw	0.711187		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	4) control
Mean	18.86667	35
Variance	38.25333	43
Observatio	3	3
df	2	2
F	1.124085	
P(F<=f) on	0.470791	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	4) control	
Mean	18.86667	35	
Variance	38.25333	43	
Observatio	3	3	
Pooled Var	40.62667		
Hypothesiz	0		
df	4		
t	3.100017	Since t>2.776 at P=0.05	
P(T<=t) on	0.01811	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.03622		
t Critical tw	2.776451		

Fig 5.7. A (cont)

F-Test: Two-Sample for Variances

	2) 4h	4) control
Mean	20.33333	35
Variance	2.333333	43
Observatio	3	3
df	2	2
F	18.42857	
P(F<=f) on	0.051471	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	4) control	
Mean	20.33333	35	
Variance	2.333333	43	
Observatio	3	3	
Pooled Var	22.66667		
Hypothesiz	0		
df	4		
t	3.772969	Since t>3.74 at P=0.02	
P(T<=t) on	0.009777	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	**0.019554**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) 24h	4) control
Mean	22	35
Variance	148	43
Observatio	3	3
df	2	2
F	3.44186	
P(F<=f) on	0.225131	
F Critical o	19.00003	

variances similar

t-Test: Two-Sample Assuming Equal Variances

	3) 24h	4) control	
Mean	22	35	
Variance	148	43	
Observatio	3	3	
Pooled Var	95.5		
Hypothesiz	0		
df	4		
t	1.629248	no significant difference	
P(T<=t) on	0.089296		
t Critical or	2.131846		
P(T<=t) tw	0.178593		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) 4h	3) 24h
Mean	20.33333	22
Variance	2.333333	148
Observatio	3	3
df	2	2
F	63.42857	
P(F<=f) on	0.015521	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	3) 24h	
Mean	20.33333	22	
Variance	2.333333	148	
Observatio	3	3	
Pooled Var	75.16667		
Hypothesiz	0		
df	4		
t	0.235441	no significant difference	
P(T<=t) on	0.412715		
t Critical or	2.131846		
P(T<=t) tw	0.825429		
t Critical tw	2.776451		

Fig 5.7. A **% infected** **(z-fa)**

7d promastigote	**1) pre**	**2) 4h**	**3) 24h**	**4) control**
	0	0.5	2.4	26
	2	2.4	3.3	33
	0	0	0	28
	0.66 ± 1.15	0.97 ± 1.3	1.9 ± 1.7	19 ± 3.6

F-Test: Two-Sample for Variances

	1) pre	2) 4h
Mean	0.666667	0.966667
Variance	1.333333	1.603333
Observatio	3	3
df	2	2
F	1.2025	
P(F<=f) on	0.45403	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	2) 4h	
Mean	0.666667	0.966667	
Variance	1.333333	1.603333	
Observatio	3	3	
Pooled Var	1.468333		
Hypothesiz	0		
df	4		
t	**0.303218**		no significant difference
P(T<=t) on	0.38842		
t Critical or	2.131846		
P(T<=t) tw	0.77684		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	3) 24h
Mean	0.666667	1.9
Variance	1.333333	2.91
Observatio	3	3
df	2	2
F	2.1825	
P(F<=f) on	0.314218	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	3) 24h	
Mean	0.666667	1.9	
Variance	1.333333	2.91	
Observatio	3	3	
Pooled Var	2.121667		
Hypothesiz	0		
df	4		
t	**1.037021**		no significant difference
P(T<=t) on	0.17915		
t Critical or	2.131846		
P(T<=t) tw	0.3583		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	4) control
Mean	0.666667	29
Variance	1.333333	13
Observatio	3	3
df	2	2
F	9.75	
P(F<=f) on	0.093023	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	4) control	
Mean	0.666667	29	
Variance	1.333333	13	
Observatio	3	3	
Pooled Var	7.166667		
Hypothesiz	0		
df	4		
t	**12.96238**		Since t>4.604 at P=0.01
P(T<=t) on	0.000102		there is a highly
t Critical or	2.131846		significant difference.
P(T<=t) tw	**0.000204**		
t Critical tw	2.776451		

Fig. 5.7. A (cont)

F-Test: Two-Sample for Variances

	2) 4h	*3) 24h*
Mean	0.966667	1.9
Variance	1.603333	2.91
Observatio	3	3
df	2	2
F	1.814969	
P(F<=f) on	0.355244	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	*3) 24h*	
Mean	0.966667	1.9	
Variance	1.603333	2.91	
Observatio	3	3	
Pooled Var	2.256667		
Hypothesiz	0		
df	4		
t	0.760937	no significant difference	
P(T<=t) on	0.244541		
t Critical or	2.131846		
P(T<=t) tw	0.489083		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) 4h	4) control
Mean	0.966667	29
Variance	1.603333	13
Observatio	3	3
df	2	2
F	8.108108	
P(F<=f) on	0.109792	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	4) control	
Mean	0.966667	29	
Variance	1.603333	13	
Observatio	3	3	
Pooled Var	7.301667		
Hypothesiz	0		
df	4		
t	12.70602	Since t>4.604 at P=0.01	
P(T<=t) on	0.00011	there is a highly	
t Critical or	2.131846	significant difference.	
P(T<=t) tw	**0.000221**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) 24h	4) control
Mean	1.9	29
Variance	2.91	13
Observatio	3	3
df	2	2
F	4.467354	
P(F<=f) on	0.182904	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) 24h	4) control	
Mean	1.9	29	
Variance	2.91	13	
Observatio	3	3	
Pooled Var	7.955		
Hypothesiz	0		
df	4		
t	11.76779	Since t>4.604 at P=0.01	
P(T<=t) on	0.000149	there is a highly	
t Critical or	2.131846	significant difference.	
P(T<=t) tw	**0.000298**		
t Critical tw	2.776451		

Fig 5.7. B **amastigotes/PEC (z-fa)**

72h	1) pre	2) 4h	3) 24h	4) control
promastigote	1.3	1.6	1.5	2.4
	1.7	1.7	1.8	3.2
	1.8	1.8	2.2	2.7
	1.6 ± 0.3	1.7 ± 0.1	1.8 ± 0.4	2.8 ± 0.4

F-Test: Two-Sample for Variances

	1) pre	2) 4h
Mean	1.6	1.7
Variance	0.07	0.01
Observatio	3	3
df	2	2
F	7	
P(F<=f) on	0.125	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	2) 4h	
Mean	1.6	1.7	
Variance	0.07	0.01	
Observatio	3	3	
Pooled Var	0.04		
Hypothesiz	0		
df	4		
t	0.612372	no significant difference	
P(T<=t) on	0.286696		
t Critical or	2.131846		
P(T<=t) tw	0.573392		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	3) 24h
Mean	1.6	1.833333
Variance	0.07	0.123333
Observatio	3	3
df	2	2
F	1.761905	
P(F<=f) on	0.362069	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	3) 24h	
Mean	1.6	1.833333	
Variance	0.07	0.123333	
Observatio	3	3	
Pooled Var	0.096667		
Hypothesiz	0		
df	4		
t	**0.919145**	no significant difference	
P(T<=t) on	0.205016		
t Critical or	2.131846		
P(T<=t) tw	0.410031		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	4) control
Mean	1.6	2.766667
Variance	0.07	0.163333
Observatio	3	3
df	2	2
F	2.333333	
P(F<=f) on	0.3	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	4) control	
Mean	1.6	2.766667	
Variance	0.07	0.163333	
Observatio	3	3	
Pooled Var	0.116667		
Hypothesiz	0		
df	4		
t	**4.1833**	Since t>3.747 at P=0.02	
P(T<=t) on	0.006941	there is a highly	
t Critical or	2.131846	significant difference.	
P(T<=t) tw	**0.013881**		
t Critical tw	2.776451		

Fig. 5.7. B (cont)

F-Test: Two-Sample for Variances

	2) 4h	3) 24h
Mean	1.7	1.833333
Variance	0.01	0.123333
Observatio	3	3
df	2	2
F	12.33333	
P(F<=f) on	0.075	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	3) 24h	
Mean	1.7	1.833333	
Variance	0.01	0.123333	
Observatio	3	3	
Pooled Var	0.066667		
Hypothesiz	0		
df	4		
t	**0.632456**		no significant difference
P(T<=t) on	0.280719		
t Critical or	2.131846		
P(T<=t) tw	0.561438		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) 4h	4) control
Mean	1.7	2.766667
Variance	0.01	0.163333
Observatio	3	3
df	2	2
F	16.33333	
P(F<=f) on	0.057692	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	4) control	
Mean	1.7	2.766667	
Variance	0.01	0.163333	
Observatio	3	3	
Pooled Var	0.086667		
Hypothesiz	0		
df	4		
t	**4.437602**		Since t>3.747 at P=0.02
P(T<=t) on	**0.005677**		there is a highly
t Critical or	2.131846		significant difference.
P(T<=t) tw	**0.011355**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) 24h	4) control
Mean	1.833333	2.766667
Variance	0.123333	0.163333
Observatio	3	3
df	2	2
F	1.324324	
P(F<=f) on	0.430233	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) 24h	4) control	
Mean	1.833333	2.766667	
Variance	0.123333	0.163333	
Observatio	3	3	
Pooled Var	0.143333		
Hypothesiz	0		
df	4		
t	**3.019318**		Since t>2.776 at P=0.05
P(T<=t) on	**0.019595**		there is a significant difference
t Critical or	2.131846		
P(T<=t) tw	**0.03919**		

Fig 5.7. B **amastigotes/PEC (z-fa)**

7 day promastigote	1) pre	2) 4h	3) 24h	4) control
	0	1	1.6	2.8
	1.5	1.4	1.7	2.4
	0	0	0	2.2
	0.5 ± 0.9	0.8 ± 0.7	1.1 ± 0.9	2.5 ± 0.3

F-Test: Two-Sample for Variances

	1) pre	2) 4h
Mean	0.5	0.8
Variance	0.75	0.52
Observatio	3	3
df	2	2
F	1.442308	
P(F<=f) on	0.409449	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	2) 4h	
Mean	0.5	0.8	
Variance	0.75	0.52	
Observatio	3	3	
Pooled Var	0.635		
Hypothesiz	0		
df	4		
t	0.461084	no significant difference	
P(T<=t) on	0.334347		
t Critical or	2.131846		
P(T<=t) tw	0.668695		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	3) 24h
Mean	0.5	1.1
Variance	0.75	0.91
Observatio	3	3
df	2	2
F	1.213333	
P(F<=f) on	0.451807	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	3) 24h	
Mean	0.5	1.1	
Variance	0.75	0.91	
Observatio	3	3	
Pooled Var	0.83		
Hypothesiz	0		
df	4		
t	0.806599	no significant difference	
P(T<=t) on	0.232561		
t Critical or	2.131846		
P(T<=t) tw	0.465122		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) pre	4) control
Mean	0.5	2.466667
Variance	0.75	0.093333
Observatio	3	3
df	2	2
F	8.035714	
P(F<=f) on	0.110672	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) pre	4) control	
Mean	0.5	2.466667	
Variance	0.75	0.093333	
Observatio	3	3	
Pooled Var	0.421667		
Hypothesiz	0		
df	4		
t	3.709298	Since t>2.776 at P=0.05	
P(T<=t) on	0.010333	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.020667		
t Critical tw	2.776451		

Fig. 5.7. B (cont)

F-Test: Two-Sample for Variances

	2) 4h	3) 24h
Mean	0.8	1.1
Variance	0.52	0.91
Observatio	3	3
df	2	2
F	1.75	
P(F<=f) on	0.363636	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	3) 24h	
Mean	0.8	1.1	
Variance	0.52	0.91	
Observatio	3	3	
Pooled Var	0.715		
Hypothesiz	0		
df	4		
t	0.434524	no significant difference	
P(T<=t) on	0.343161		
t Critical or	2.131846		
P(T<=t) tw	0.686321		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) 4h	4) control
Mean	0.8	2.466667
Variance	0.52	0.093333
Observatio	3	3
df	2	2
F	5.571429	
P(F<=f) on	0.152174	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) 4h	4) control	
Mean	0.8	2.466667	
Variance	0.52	0.093333	
Observatio	3	3	
Pooled Var	0.306667		
Hypothesiz	0		
df	4		
t	3.686049	Since t>2.776 at P=0.05	
P(T<=t) on	0.010546	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.021092		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) 24h	4) control
Mean	1.1	2.466667
Variance	0.91	0.093333
Observatio	3	3
df	2	2
F	9.75	
P(F<=f) on	0.093023	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	3) 24h	4) control	
Mean	1.1	2.466667	
Variance	0.91	0.093333	
Observatio	3	3	
Pooled Var	0.501667		
Hypothesiz	0		
df	4		
t	2.363201	Since t>2.132 at P=0.1	
P(T<=t) on	0.038696	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.077392		
t Critical tw	2.776451		

Fig 5.8. A **% infected**

72h promastigote	1) zfa	2) P87	3) KO2	4) control
	12	28	38	34
	20.6	38	4.5	29
	24	36	17	42
	19 ± 6.0	34 ± 5.3	20 ± 12	35 ± 6.6

F-Test: Two-Sample for Variances

	1) zfa	2) P87
Mean	18.86667	34
Variance	38.25333	28
Observatic	3	3
df	2	2
F	1.36619	
P(F<=f) on	0.42262	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	2) P87	
Mean	18.86667	34	
Variance	38.25333	28	
Observatic	3	3	
Pooled Vai	33.12667		
Hypothesiz	0		
df	4		
t	3.220263	since t>2.776 at P=0.05	
P(T<=t) on	0.016136	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.032272		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	3) KO2
Mean	18.86667	19.83333
Variance	38.25333	286.5833
Observatic	3	3
df	2	2
F	7.491722	
P(F<=f) on	0.117762	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	3) KO2	
Mean	18.86667	19.83333	
Variance	38.25333	286.5833	
Observatic	3	3	
Pooled Vai	162.4183		
Hypothesiz	0		
df	4		
t	0.092898	no significant difference	
P(T<=t) on	0.465226		
t Critical or	2.131846		
P(T<=t) tw	0.930452		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	4) control
Mean	18.86667	35
Variance	38.25333	43
Observatic	3	3
df	2	2
F	1.124085	
P(F<=f) on	0.470791	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	4) control	
Mean	18.86667	35	
Variance	38.25333	43	
Observatic	3	3	
Pooled Vai	40.62667		
Hypothesiz	0		
df	4		
t	3.100017	since t>2.776 at P=0.05	
P(T<=t) on	0.01811	there is a significant difference.	
t Critical or	2.131846		
P(T<=t) tw	0.03622		
t Critical tw	2.776451		

Fig. 5.8. A (cont)

F-Test: Two-Sample for Variances

	2) P87	3) KO2
Mean	34	19.83333
Variance	28	286.5833
Observatic	3	3
df	2	2
F	10.23512	
P(F<=f) on	0.089007	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	3) KO2
Mean	34	19.83333
Variance	28	286.5833
Observatic	3	3
Pooled Vai	157.2917	
Hypothesiz	0	
df	4	
t	1.38344	no significant difference
P(T<=t) on	0.119364	
t Critical or	2.131846	
P(T<=t) tw	0.238728	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	2) P87	4) control
Mean	34	35
Variance	28	43
Observatic	3	3
df	2	2
F	1.535714	
P(F<=f) on	0.394366	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	4) control
Mean	34	35
Variance	28	43
Observatic	3	3
Pooled Vai	35.5	
Hypothesiz	0	
df	4	
t	0.205557	no significant difference
P(T<=t) on	0.423587	
t Critical or	2.131846	
P(T<=t) tw	0.847175	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	3) KO2	4) control
Mean	19.83333	35
Variance	286.5833	43
Observatic	3	3
df	2	2
F	6.664729	
P(F<=f) on	0.130468	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	3) KO2	4) control
Mean	19.83333	35
Variance	286.5833	43
Observatic	3	3
Pooled Vai	164.7917	
Hypothesiz	0	
df	4	
t	1.446999	no significant difference
P(T<=t) on	0.110724	
t Critical or	2.131846	
P(T<=t) tw	0.221447	
t Critical tw	2.776451	

Fig 6.8. A % infected

7d promastigote	1) zfa	2) P87	3) KO2	4) control
	0	1.4	5	26
	2	8.9	13	33
	0	1.2	2.9	28
	0.7 ± 1.1	3.8 ± 4.4	7 ± 5.3	30 ± 2.5

F-Test: Two-Sample for Variances

	1) zfa	2) P87
Mean	0.666667	3.833333
Variance	1.333333	19.26333
Observatic	3	3
df	2	2
F	14.4475	
P(F<=f) on	0.064735	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	2) P87	
Mean	0.666667	3.833333	
Variance	1.333333	19.26333	
Observatic	3	3	
Pooled Vai	10.29833		
Hypothesiz	0		
df	4		
t	**1.20855**	no significant difference	
P(T<=t) on	0.146696		
t Critical or	2.131846		
P(T<=t) tw	0.293393		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	3) KO2
Mean	0.666667	6.966667
Variance	1.333333	28.40333
Observatic	3	3
df	2	2
F	21.3025	
P(F<=f) on	0.044838	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	3) KO2	
Mean	0.666667	6.966667	
Variance	1.333333	28.40333	
Observatic	3	3	
Pooled Vai	14.86833		
Hypothesiz	0		
df	4		
t	**2.001037**	no significant difference	
P(T<=t) on	0.05799		
t Critical or	2.131846		
P(T<=t) tw	0.115979		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	4) control
Mean	0.666667	29
Variance	1.333333	13
Observatic	3	3
df	2	2
F	9.75	
P(F<=f) on	0.093023	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	4) control	
Mean	0.666667	29	
Variance	1.333333	13	
Observatic	3	3	
Pooled Vai	7.166667		
Hypothesiz	0		
df	4		
t	**12.96238**	since t>4.604 there is a highly	
P(T<=t) on	0.000102	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	**0.000204**		
t Critical tw	2.776451		

Fig. 5.8. A (cont)

F-Test: Two-Sample for Variances

	2) P87	3) KO2
Mean	3.833333	6.966667
Variance	19.26333	28.40333
Observatic	3	3
df	2	2
F	1.474477	
P(F<=f) on	0.404126	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	3) KO2	
Mean	3.833333	6.966667	
Variance	19.26333	28.40333	
Observatic	3	3	
Pooled Vai	23.83333		
Hypothesiz	0		
df	4		
t	0.786067	no significant difference	
P(T<=t) on	0.23789		
t Critical or	2.131846		
P(T<=t) tw	0.475781		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) P87	4) control
Mean	3.833333	29
Variance	19.26333	13
Observatic	3	3
df	2	2
F	1.481795	
P(F<=f) on	0.402934	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	4) control	
Mean	3.833333	29	
Variance	19.26333	13	
Observatic	3	3	
Pooled Vai	16.13167		
Hypothesiz	0		
df	4		
t	7.674175	since t>4.604 there is a highly	
P(T<=t) on	0.000775	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	0.00155		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) KO2	4) control
Mean	6.966667	29
Variance	28.40333	13
Observatic	3	3
df	2	2
F	2.184872	
P(F<=f) on	0.313984	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	3) KO2	4) control	
Mean	6.966667	29	
Variance	28.40333	13	
Observatic	3	3	
Pooled Vai	20.70167		
Hypothesiz	0		
df	4		
t	5.930935	since t>4.604 there is a highly	
P(T<=t) on	0.002025	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	0.004051		
t Critical tw	2.776451		

Fig 5.8. B **amastigotes/PEC**

72h promastigote	1) zfa	2) P87	3) KO2	4) control
	1.3	2	2.2	2.4
	1.7	2.1	1.6	3.2
	1.8	1.9	2	2.7
	1.6 ± 0.3	2.0 ± 0.1	1.9 ± 0.3	2.8 ± 0.4

F-Test: Two-Sample for Variances

	1) zfa	2) P87
Mean	1.6	2
Variance	0.07	0.01
Observatic	3	3
df	2	2
F	7	
P(F<=f) on	0.125	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	2) P87	
Mean	1.6	2	
Variance	0.07	0.01	
Observatic	3	3	
Pooled Vai	0.04		
Hypothesiz	0		
df	4		
t	2.44949	since t>2.132 there is a	
P(T<=t) on	0.035242	significant difference at P=0.1	
t Critical or	2.131846		
P(T<=t) tw	0.070484		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	3) KO2
Mean	1.6	1.933333
Variance	0.07	0.093333
Observatic	3	3
df	2	2
F	1.333333	
P(F<=f) on	0.428571	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	3) KO2	
Mean	1.6	1.933333	
Variance	0.07	0.093333	
Observatic	3	3	
Pooled Vai	0.081667		
Hypothesiz	0		
df	4		
t	1.428571	no significant difference	
P(T<=t) on	0.113162		
t Critical or	2.131846		
P(T<=t) tw	0.226325		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	4) control
Mean	1.6	2.766667
Variance	0.07	0.163333
Observatic	3	3
df	2	2
F	2.333333	
P(F<=f) on	0.3	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	4) control	
Mean	1.6	2.766667	
Variance	0.07	0.163333	
Observatic	3	3	
Pooled Vai	0.116667		
Hypothesiz	0		
df	4		
t	4.1833	since t>3.747 there is a	
P(T<=t) on	0.006941	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.013881		
t Critical tw	2.776451		

Fig. 5.8. B (cont)

F-Test: Two-Sample for Variances

	2) P87	3) KO2
Mean	2	1.933333
Variance	0.01	0.093333
Observatic	3	3
df	2	2
F	9.333333	
P(F<=f) on	0.096774	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	3) KO2
Mean	2	1.933333
Variance	0.01	0.093333
Observatic	3	3
Pooled Vai	0.051667	
Hypothesiz	0	
df	4	
t	0.359211	no significant difference
P(T<=t) on	0.368799	
t Critical or	2.131846	
P(T<=t) tw	0.737597	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	2) P87	4) control
Mean	2	2.766667
Variance	0.01	0.163333
Observatic	3	3
df	2	2
F	16.33333	
P(F<=f) on	0.057692	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	4) control
Mean	2	2.766667
Variance	0.01	0.163333
Observatic	3	3
Pooled Vai	0.086667	
Hypothesiz	0	
df	4	
t	3.189526	since t>2.776 there is a
P(T<=t) on	0.016616	significant difference at P=0.05
t Critical or	2.131846	
P(T<=t) tw	0.033231	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	3) KO2	4) control
Mean	1.933333	2.766667
Variance	0.093333	0.163333
Observatic	3	3
df	2	2
F	1.75	
P(F<=f) on	0.363636	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) KO2	4) control
Mean	1.933333	2.766667
Variance	0.093333	0.163333
Observatic	3	3
Pooled Vai	0.128333	
Hypothesiz	0	
df	4	
t	2.849014	since t>2.776 there is a
P(T<=t) on	0.023221	significant difference at P=0.05
t Critical or	2.131846	
P(T<=t) tw	0.046442	
t Critical tw	2.776451	

Fig 5.8. B **amastigotes/PEC**

7 day promastigote	1) zfa	2) P87	3) KO2	4) control
	0	2.7	2.6	2.8
	1.5	2	1.9	2.4
	0	1	1	2.2
	0.5 ± 0.9	1.9 ± 0.9	1.8 ± 0.8	2.6 ± 0.5

F-Test: Two-Sample for Variances

	1) zfa	2) P87
Mean	0.5	1.9
Variance	0.75	0.73
Observatic	3	3
df	2	2
F	1.027397	
P(F<=f) on	0.493243	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	2) P87	
Mean	0.5	1.9	
Variance	0.75	0.73	
Observatic	3	3	
Pooled Vai	0.74		
Hypothesiz	0		
df	4		
t	1.993232	no significant difference	
P(T<=t) on	0.058509		
t Critical or	2.131846		
P(T<=t) tw	0.117018		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	3) KO2
Mean	0.5	1.833333
Variance	0.75	0.643333
Observatic	3	3
df	2	2
F	1.165803	
P(F<=f) on	0.461722	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	3) KO2	
Mean	0.5	1.833333	
Variance	0.75	0.643333	
Observatic	3	3	
Pooled Vai	0.696667		
Hypothesiz	0		
df	4		
t	1.956464	no significant difference	
P(T<=t) on	0.061024		
t Critical or	2.131846		
P(T<=t) tw	0.122049		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) zfa	4) control
Mean	0.5	2.466667
Variance	0.75	0.093333
Observatic	3	3
df	2	2
F	8.035714	
P(F<=f) on	0.110672	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	1) zfa	4) control	
Mean	0.5	2.466667	
Variance	0.75	0.093333	
Observatic	3	3	
Pooled Vai	0.421667		
Hypothesiz	0		
df	4		
t	3.709298	since t>2.776 there is a	
P(T<=t) on	0.010333	significant difference at P=0.05	
t Critical or	2.131846		
P(T<=t) tw	0.020667		
t Critical tw	2.776451		

Fig 5.8. B (cont)

F-Test: Two-Sample for Variances

	2) P87	3) KO2
Mean	1.9	1.833333
Variance	0.73	0.643333
Observatic	3	3
df	2	2
F	1.134715	
P(F<=f) on	0.468447	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	3) KO2
Mean	1.9	1.833333
Variance	0.73	0.643333
Observatic	3	3
Pooled Vai	0.686667	
Hypothesiz	0	
df	4	
t	0.098533	no significant difference
P(T<=t) on	0.463125	
t Critical or	2.131846	
P(T<=t) tw	0.926249	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	2) P87	4) control
Mean	1.9	2.466667
Variance	0.73	0.093333
Observatic	3	3
df	2	2
F	7.821429	
P(F<=f) on	0.11336	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) P87	4) control
Mean	1.9	2.466667
Variance	0.73	0.093333
Observatic	3	3
Pooled Vai	0.411667	
Hypothesiz	0	
df	4	
t	1.081684	no significant difference
P(T<=t) on	0.170124	
t Critical or	2.131846	
P(T<=t) tw	0.340247	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	3) KO2	4) control
Mean	1.833333	2.466667
Variance	0.643333	0.093333
Observatic	3	3
df	2	2
F	6.892857	
P(F<=f) on	0.126697	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	3) KO2	4) control
Mean	1.833333	2.466667
Variance	0.643333	0.093333
Observatic	3	3
Pooled Vai	0.368333	
Hypothesiz	0	
df	4	
t	1.278078	no significant difference
P(T<=t) on	0.135175	
t Critical or	2.131846	
P(T<=t) tw	0.27035	
t Critical tw	2.776451	

Fig 5.9. A **% infected**

	1) ams	2) zlvg	3) zfa	4) control (dmso)
72h	30	20	3.3	22
lesion amastigotes		35	28	29
	51	45	23	42
	40 ± 14	33 ± 12	18 ± 13	31 ± 10

F-Test: Two-Sample for Variances

	1) ams	2) zlvg
Mean	40.5	33.33333
Variance	220.5	158.3333
Observatic	2	3
df	1	2
F	1.392632	
P(F<=f) on	0.359307	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	2) zlvg	
Mean	40.5	33.33333	
Variance	220.5	158.3333	
Observatic	2	3	
Pooled Vai	179.0556		
Hypothesiz	0		
df	3		
t	**0.586697**	no significant difference	
P(T<=t) on	0.299317		
t Critical or	2.353363		
P(T<=t) tw	0.598634		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	3) zfa
Mean	40.5	18.1
Variance	220.5	170.53
Observatic	2	3
df	1	2
F	1.293028	
P(F<=f) on	0.373377	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	3) zfa	
Mean	40.5	18.1	
Variance	220.5	170.53	
Observatic	2	3	
Pooled Vai	187.1867		
Hypothesiz	0		
df	3		
t	1.793499	no significant difference	
P(T<=t) on	0.085394		
t Critical or	2.353363		
P(T<=t) tw	0.170789		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	rol (dmso)
Mean	40.5	31
Variance	220.5	103
Observatic	2	3
df	1	2
F	2.140777	
P(F<=f) on	0.280974	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	rol (dmso)	
Mean	40.5	31	
Variance	220.5	103	
Observatic	2	3	
Pooled Vai	142.1667		
Hypothesiz	0		
df	3		
t	**0.872801**	no significant difference	
P(T<=t) on	0.223504		
t Critical or	2.353363		
P(T<=t) tw	0.447007		
t Critical tw	3.182449		

Fig 5.9. A (cont)

F-Test: Two-Sample for Variances

	2) zlvg	3) zfa
Mean	33.33333	18.1
Variance	158.3333	170.53
Observatic	3	3
df	2	2
F	1.077032	
P(F<=f) on	0.481456	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	3) zfa	
Mean	33.33333	18.1	
Variance	158.3333	170.53	
Observatic	3	3	
Pooled Vai	164.4317		
Hypothesiz	0		
df	4		
t	1.454949		no significant difference
P(T<=t) on	0.109688		
t Critical or	2.131846		
P(T<=t) tw	0.219376		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) zlvg	rol (dmso)
Mean	33.33333	31
Variance	158.3333	103
Observatic	3	3
df	2	2
F	1.537217	
P(F<=f) on	0.394133	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	rol (dmso)	
Mean	33.33333	31	
Variance	158.3333	103	
Observatic	3	3	
Pooled Vai	130.6667		
Hypothesiz	0		
df	4		
t	0.25		no significant difference
P(T<=t) on	0.407451		
t Critical or	2.131846		
P(T<=t) tw	0.814902		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) zfa	rol (dmso)
Mean	18.1	31
Variance	170.53	103
Observatic	3	3
df	2	2
F	1.655631	
P(F<=f) on	0.376558	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	3) zfa	rol (dmso)	
Mean	18.1	31	
Variance	170.53	103	
Observatic	3	3	
Pooled Vai	136.765		
Hypothesiz	0		
df	4		
t	1.350977		no significant difference
P(T<=t) on	0.124032		
t Critical or	2.131846		
P(T<=t) tw	0.248065		
t Critical tw	2.776451		

Fig 5.9. A **% infected**

	1) ams	2) zlvg	3) zfa	ontrol (dmso)
7d	40		0.5	39
lesion amastigotes		35	0.2	36
	42	23	0.5	35
	41 ± 1.4	29 ± 8.5	0.4 ± 0.2	37 ± 2.0

F-Test: Two-Sample for Variances

	1) ams	2) zlvg
Mean	41	29
Variance	2	72
Observatic	2	2
df	1	1
F	36	
P(F<=f) on	0.105137	
F Critical o	39.86361	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	2) zlvg	
Mean	41	29	
Variance	2	72	
Observatic	2	2	
Pooled Vai	37		
Hypothesiz	0		
df	2		
t	1.972788		no significant difference
P(T<=t) on	0.093629		
t Critical or	2.919987		
P(T<=t) tw	0.187257		
t Critical tw	4.302656		

F-Test: Two-Sample for Variances

	1) ams	3) zfa
Mean	41	0.4
Variance	2	0.03
Observatic	2	3
df	1	2
F	66.66667	
P(F<=f) on	0.014671	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	3) zfa	
Mean	41	0.4	
Variance	2	0.03	
Observatic	2	3	
Pooled Vai	0.686667		
Hypothesiz	0		
df	3		
t	53.67149		since t>5.8 there is a highly
P(T<=t) on	7.12E-06		significant difference at P=0.01
t Critical or	2.353363		
P(T<=t) tw	1.42E-05		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	rol (dmso)
Mean	41	36.66667
Variance	2	4.333333
Observatic	2	3
df	1	2
F	2.166667	
P(F<=f) on	0.433013	
F Critical o	49.50016	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	rol (dmso)	
Mean	41	36.66667	
Variance	2	4.333333	
Observatic	2	3	
Pooled Vai	3.555556		
Hypothesiz	0		
df	3		
t	2.517439		no significant difference
P(T<=t) on	0.043185		
t Critical or	2.353363		
P(T<=t) tw	0.086371		
t Critical tw	3.182449		

Fig. 5.9. A (cont)

F-Test: Two-Sample for Variances

	2) zlvg	3) zfa
Mean	29	0.4
Variance	72	0.03
Observatic	2	3
df	1	2
F	2400	
P(F<=f) on	0.000416	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	3) zfa	
Mean	29	0.4	
Variance	72	0.03	
Observatic	2	3	
Pooled Vai	24.02		
Hypothesiz	0		
df	3		
t	6.392491	since t>5.8 there is a highly	
P(T<=t) on	0.003876	significant difference at P=0.01	
t Critical or	2.353363		
P(T<=t) tw	0.007753		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	2) zlvg	rol (dmso)
Mean	29	36.66667
Variance	72	4.333333
Observatic	2	3
df	1	2
F	16.61538	
P(F<=f) on	0.055245	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	rol (dmso)	
Mean	29	36.66667	
Variance	72	4.333333	
Observatic	2	3	
Pooled Vai	26.88889		
Hypothesiz	0		
df	3		
t	1.619611	no significant difference	
P(T<=t) on	0.101677		
t Critical or	2.353363		
P(T<=t) tw	0.203755		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	3) zfa	rol (dmso)
Mean	0.4	36.66667
Variance	0.03	4.333333
Observatic	3	3
df	2	2
F	144.4444	
P(F<=f) on	0.006875	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) zfa	rol (dmso)	
Mean	0.4	36.66667	
Variance	0.03	4.333333	
Observatic	3	3	
Pooled Vai	2.181667		
Hypothesiz	0		
df	4		
t	30.07178	since t>4.604 there is a highly	
P(T<=t) on	3.64E-06	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	7.28E-06		
t Critical tw	2.776451		

Fig. 5.9. B **amastigotes/PEC**

72h	1) ams	2) zlvg	3) zfa	ontrol (dmso)
lesion amastigotes	4.4	2.6	3	3.5
		2.6	2.4	4.1
	4.2	2.5	2.9	4.4
	4.3 ± 0.1	2.3 ± 0.1	2.8 ± 0.3	4.0 ± 0.4

F-Test: Two-Sample for Variances

	1) ams	2) zlvg
Mean	4.3	2.566667
Variance	0.02	0.003333
Observatic	2	3
df	1	2
F	6	
P(F<=f) on	0.133975	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	2) zlvg	
Mean	4.3	2.566667	
Variance	0.02	0.003333	
Observatic	2	3	
Pooled Vai	0.008889		
Hypothesiz	0		
df	3		
t	20.13951	since t>5.8 there is a highly	
P(T<=t) on	0.000134	significant difference at P=0.01	
t Critical or	2.353363		
P(T<=t) tw	0.000268		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	3) zfa
Mean	4.3	2.766667
Variance	0.02	0.103333
Observatic	2	3
df	1	2
F	5.166667	
P(F<=f) on	0.297044	
F Critical o	49.50016	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	3) zfa	
Mean	4.3	2.766667	
Variance	0.02	0.103333	
Observatic	2	3	
Pooled Vai	0.075556		
Hypothesiz	0		
df	3		
t	6.110743	since t>5.8 there is a highly	
P(T<=t) on	0.004403	significant difference at P=0.01	
t Critical or	2.353363		
P(T<=t) tw	0.008807		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	rol (dmso)
Mean	4.3	4
Variance	0.02	0.21
Observatic	2	3
df	1	2
F	10.5	
P(F<=f) on	0.213201	
F Critical o	49.50016	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	rol (dmso)	
Mean	4.3	4	
Variance	0.02	0.21	
Observatic	2	3	
Pooled Vai	0.146667		
Hypothesiz	0		
df	3		
t	0.858116	no significant difference	
P(T<=t) on	0.22696		
t Critical or	2.353363		
P(T<=t) tw	0.453919		
t Critical tw	3.182449		

Fig. 5.9. B (cont)

F-Test: Two-Sample for Variances

	2) zlvg	3) zfa
Mean	2.566667	2.766667
Variance	0.003333	0.103333
Observatic	3	3
df	2	2
F	31	
P(F<=f) on	0.03125	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	3) zfa	
Mean	2.566667	2.766667	
Variance	0.003333	0.103333	
Observatic	3	3	
Pooled Vai	0.053333		
Hypothesiz	0		
df	4		
t	1.06066	no significant difference	
P(T<=t) on	0.174321		
t Critical or	2.131846		
P(T<=t) tw	0.348641		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) zlvg	rol (dmso)
Mean	2.566667	4
Variance	0.003333	0.21
Observatic	3	3
df	2	2
F	63	
P(F<=f) on	0.015625	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	rol (dmso)	
Mean	2.566667	4	
Variance	0.003333	0.21	
Observatic	3	3	
Pooled Vai	0.106667		
Hypothesiz	0		
df	4		
t	5.375	since t>4.604 there is a highly	
P(T<=t) on	0.002894	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	0.005788		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	3) zfa	rol (dmso)
Mean	2.766667	4
Variance	0.103333	0.21
Observatic	3	3
df	2	2
F	2.032258	
P(F<=f) on	0.329787	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) zfa	rol (dmso)	
Mean	2.766667	4	
Variance	0.103333	0.21	
Observatic	3	3	
Pooled Vai	0.156667		
Hypothesiz	0		
df	4		
t	3.816259	since t>3.747 there is a highly	
P(T<=t) on	0.009419	significant difference at P=0.02	
t Critical or	2.131846		
P(T<=t) tw	0.018839		
t Critical tw	2.776451		

Fig. 5.8. B **amastigotes/PEC**

7d	1) ams	2) zlvg	3) zfa	ontrol (dmso)
lesion amastigotes	7.7		1	5.8
		2.8	3	4
	5.7	2.4	3	3.1
	6.7 ± 1.4	2.6 ± 0.3	2.3 ± 1.2	4.3 ± 1.4

F-Test: Two-Sample for Variances

	1) ams	2) zlvg
Mean	6.7	2.6
Variance	2	0.08
Observatic	2	2
df	1	1
F	25	
P(F<=f) on	0.125666	
F Critical o	161.4462	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	2) zlvg	
Mean	6.7	2.6	
Variance	2	0.08	
Observatic	2	2	
Pooled Vai	1.04		
Hypothesiz	0		
df	2		
t	4.020381	since t>2.91 there is a	
P(T<=t) on	0.02833	significant difference at P=0.1	
t Critical or	2.919987		
P(T<=t) tw	0.056661		
t Critical tw	4.302656		

F-Test: Two-Sample for Variances

	1) ams	3) zfa
Mean	6.7	2.333333
Variance	2	1.333333
Observatic	2	3
df	1	2
F	1.5	
P(F<=f) on	0.345346	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	3) zfa	
Mean	6.7	2.333333	
Variance	2	1.333333	
Observatic	2	3	
Pooled Vai	1.555556		
Hypothesiz	0		
df	3		
t	3.835287	since t>3.18 there is a	
P(T<=t) on	0.015624	significant difference at P=0.05	
t Critical or	2.353363		
P(T<=t) tw	0.031249		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	1) ams	rol (dmso)
Mean	6.7	4.3
Variance	2	1.89
Observatic	2	3
df	1	2
F	1.058201	
P(F<=f) on	0.411765	
F Critical o	18.51276	

t-Test: Two-Sample Assuming Equal Variances

	1) ams	rol (dmso)	
Mean	6.7	4.3	
Variance	2	1.89	
Observatic	2	3	
Pooled Vai	1.926667		
Hypothesiz	0		
df	3		
t	1.894081	no significant difference	
P(T<=t) on	0.077265		
t Critical or	2.353363		
P(T<=t) tw	0.154531		
t Critical tw	3.182449		

Fig. 5.9. B (cont)

F-Test: Two-Sample for Variances

	2) zlvg	3) zfa
Mean	2.6	2.333333
Variance	0.08	1.333333
Observatic	2	3
df	1	2
F	16.66667	
P(F<=f) on	0.170664	
F Critical o	49.50016	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	3) zfa	
Mean	2.6	2.333333	
Variance	0.08	1.333333	
Observatic	2	3	
Pooled Vai	0.915556		
Hypothesiz	0		
df	3		
t	0.305293	no significant difference	
P(T<=t) on	0.39005		
t Critical or	2.353363		
P(T<=t) tw	0.780099		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	2) zlvg	rol (dmso)
Mean	2.6	4.3
Variance	0.08	1.89
Observatic	2	3
df	1	2
F	23.625	
P(F<=f) on	0.143963	
F Critical o	49.50016	

t-Test: Two-Sample Assuming Equal Variances

	2) zlvg	rol (dmso)	
Mean	2.6	4.3	
Variance	0.08	1.89	
Observatic	2	3	
Pooled Vai	1.286667		
Hypothesiz	0		
df	3		
t	1.641748	no significant difference	
P(T<=t) on	0.099591		
t Critical or	2.353363		
P(T<=t) tw	0.199181		
t Critical tw	3.182449		

F-Test: Two-Sample for Variances

	3) zfa	rol (dmso)
Mean	2.333333	4.3
Variance	1.333333	1.89
Observatic	3	3
df	2	2
F	1.4175	
P(F<=f) on	0.41365	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	3) zfa	rol (dmso)	
Mean	2.333333	4.3	
Variance	1.333333	1.89	
Observatic	3	3	
Pooled Vai	1.611667		
Hypothesiz	0		
df	4		
t	1.897312	no significant difference	
P(T<=t) on	0.065322		
t Critical or	2.131846		
P(T<=t) tw	0.130643		
t Critical tw	2.776451		

Table 5.1. **cell line, inhibitor**

	wild type	+ dmso	+ z-lvg	+ z-fa	*dlmcpbn*
%	60	57	70	17	11
infected	47	42	32	2	4.9
	52	37	25	2	0
	53 ± 6.1	45 ± 11	42 ± 24	7.0 ± 8.7	5.3 ± 5.5

F-Test: Two-Sample for Variances

	wild type	+ dmso
Mean	53	45.33333
Variance	43	108.3333
Observatic	3	3
df	2	2
F	2.51938	
P(F<=f) on	0.284141	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	wild type	+ dmso
Mean	53	45.33333
Variance	43	108.3333
Observatic	3	3
Pooled Vai	75.66667	
Hypothesiz	0	
df	4	
t	1.079443	no significant difference
P(T<=t) on	0.170567	
t Critical or	2.131846	
P(T<=t) tw	0.341133	
t Critical tw	2.776451	

F-Test: Two-Sample for Variances

	+ dmso	+ z-lvg
Mean	45.33333	42.33333
Variance	108.3333	586.3333
Observatic	3	3
df	2	2
F	5.412308	
P(F<=f) on	0.15595	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	+ dmso	+ z-lvg
Mean	45.33333	42.33333
Variance	108.3333	586.3333
Observatic	3	3
Pooled Vai	347.3333	
Hypothesiz	0	
df	4	
t	0.197149	no significant difference
P(T<=t) on	0.426662	
t Critical or	2.131846	
P(T<=t) tw	0.853324	
t Critical tw	2.776451	

Table 5.1. (cont)

F-Test: Two-Sample for Variances

	+ z-lvg	+ z-fa
Mean	42.33333	7
Variance	586.3333	75
Observatic	3	3
df	2	2
F	7.817778	
P(F<=f) on	0.113407	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	+ z-lvg	+ z-fa	
Mean	42.33333	7	
Variance	586.3333	75	
Observatic	3	3	
Pooled Vai	330.6667		
Hypothesiz	0		
df	4		
t	2.37977	since t>2.131 there is a	
P(T<=t) on	0.038004	significant difference at P=0.1	
t Critical or	2.131846		
P(T<=t) tw	0.076008		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	+ dmso	+ z-fa
Mean	45.33333	7
Variance	108.3333	75
Observatic	3	3
df	2	2
F	1.444444	
P(F<=f) on	0.409091	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	+ dmso	+ z-fa	
Mean	45.33333	7	
Variance	108.3333	75	
Observatic	3	3	
Pooled Vai	91.66667		
Hypothesiz	0		
df	4		
t	4.903616	since t>4.604 there is a highly	
P(T<=t) on	0.004012	significant difference at P=0.01	
t Critical or	2.131846		
P(T<=t) tw	0.008023		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	+ z-fa	*dlmcpbn*
Mean	7	5.3
Variance	75	30.37
Observatic	3	3
df	2	2
F	2.469542	
P(F<=f) on	0.288222	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	+ z-fa	*dlmcpbn*	
Mean	7	5.3	
Variance	75	30.37	
Observatic	3	3	
Pooled Vai	52.685		
Hypothesiz	0		
df	4		
t	0.286847	no significant difference	
P(T<=t) on	0.394237		
t Critical or	2.131846		
P(T<=t) tw	0.788474		
t Critical tw	2.776451		

Table 5.1. **cell line, inhibitor**

	wild type	+ dmso	+ z-lvg	+ z-fa	*dlmcpbn*
	5.2	3.7	4.7	3.3	8
amastigotes/PEC	5.4	3.9	2.4	0.7	2.8
	3.8	2.6	1.8	1.7	0
	4.8 ± 0.9	3.4 ± 0.7	3 ± 1.5	1.9 ± 1.3	3.6 ± 4.1

F-Test: Two-Sample for Variances

	wild type	**+ dmso**
Mean	4.8	3.4
Variance	0.76	0.49
Observatic	3	3
df	2	2
F	1.55102	
P(F<=f) on	0.392	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	wild type	**+ dmso**	
Mean	4.8	3.4	
Variance	0.76	0.49	
Observatic	3	3	
Pooled Vai	0.625		
Hypothesiz	0		
df	4		
t	2.168871	since t>2.131 there is a	
P(T<=t) on	0.047966	significant difference at P=0.1	
t Critical or	2.131846		
P(T<=t) tw	0.095931		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	+ dmso	**+ z-lvg**
Mean	3.4	2.966667
Variance	0.49	2.343333
Observatic	3	3
df	2	2
F	4.782313	
P(F<=f) on	0.172941	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	+ dmso	**+ z-lvg**	
Mean	3.4	2.966667	
Variance	0.49	2.343333	
Observatic	3	3	
Pooled Vai	1.416667		
Hypothesiz	0		
df	4		
t	0.445896	no significant difference	
P(T<=t) on	0.339372		
t Critical or	2.131846		
P(T<=t) tw	0.678744		
t Critical tw	2.776451		

Table 5.1. (cont)

F-Test: Two-Sample for Variances

	+ z-lvg	+ z-fa
Mean	2.966667	1.9
Variance	2.343333	1.72
Observatic	3	3
df	2	2
F	1.362403	
P(F<=f) on	0.423298	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	+ z-lvg	+ z-fa	
Mean	2.966667	1.9	
Variance	2.343333	1.72	
Observatic	3	3	
Pooled Vai	2.031667		
Hypothesiz	0		
df	4		
t	0.916533	no significant difference	
P(T<=t) on	0.205623		
t Critical or	2.131846		
P(T<=t) tw	0.411246		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	+ dmso	+ z-fa
Mean	3.4	1.9
Variance	0.49	1.72
Observatic	3	3
df	2	2
F	3.510204	
P(F<=f) on	0.221719	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	+ dmso	+ z-fa	
Mean	3.4	1.9	
Variance	0.49	1.72	
Observatic	3	3	
Pooled Vai	1.105		
Hypothesiz	0		
df	4		
t	1.747655	no significant difference	
P(T<=t) on	0.077721		
t Critical or	2.131846		
P(T<=t) tw	0.155441		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	+ z-fa	*dlmcpbn*
Mean	1.9	3.6
Variance	1.72	16.48
Observatic	3	3
df	2	2
F	9.581395	
P(F<=f) on	0.094505	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	+ z-fa	*dlmcpbn*	
Mean	1.9	3.6	
Variance	1.72	16.48	
Observatic	3	3	
Pooled Vai	9.1		
Hypothesiz	0		
df	4		
t	0.690198	no significant difference	
P(T<=t) on	0.264014		
t Critical or	2.131846		
P(T<=t) tw	0.528028		
t Critical tw	2.776451		

Table 5.2.

	wt lesion	null lesion	wt axam		null axam	
	12	18	26	44	51	93
% MTT	12.1	16.4	31	33	52	57
reduction	12	16	2	14	44	30
			11		28	
	12 ± 0.1	17 ± 1.1	23 ± 15		51 ± 21	

F-Test: Two-Sample for Variances

	wt lesion	null lesion
Mean	12.03333	16.8
Variance	0.003333	1.12
Observatic	3	3
df	2	2
F	336	
P(F<=f) on	0.002967	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	wt lesion	null lesion	
Mean	12.03333	16.8	
Variance	0.003333	1.12	
Observatic	3	3	
Pooled Va	0.561667		
Hypothesiz	0		
df	4		
t	7.789706	since t>4.604 there is a highly	
P(T<=t) on	0.000732	significant at P = 0.01	
t Critical or	2.131846		
P(T<=t) tw	0.001465		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	wt axam	null axam
Mean	23	50.71429
Variance	213.3333	469.9048
Observatic	7	7
df	6	6
F	2.202679	
P(F<=f) on	0.179644	
F Critical o	3.054552	

t-Test: Two-Sample Assuming Equal Variances

	wt axam	null axam	
Mean	23	50.71429	
Variance	213.3333	469.9048	
Observatic	7	7	
Pooled Va	341.619		
Hypothesiz	0		
df	12		
t	2.805218	since t>2.68 there is a highly	
P(T<=t) on	0.007945	significant difference at P=0.02	
t Critical or	1.782287		
P(T<=t) tw	0.01589		
t Critical tw	2.178813		

F-Test: Two-Sample for Variances

	wt lesion	wt axam
Mean	12.03333	23
Variance	0.003333	213.3333
Observatic	3	7
df	2	6
F	64000	
P(F<=f) on	1.56E-05	
F Critical o	9.325504	

t-Test: Two-Sample Assuming Equal Variances

	wt lesion	wt axam	
Mean	12.03333	23	
Variance	0.003333	213.3333	
Observatic	3	7	
Pooled Va	160.0008		
Hypothesiz	0		
df	8		
t	1.256386	no significant difference	
P(T<=t) on	0.122211		
t Critical or	1.859548		
P(T<=t) tw	0.244421		
t Critical tw	2.306006		

F-Test: Two-Sample for Variances

	null lesion	null axam
Mean	16.8	50.71429
Variance	1.12	469.9048
Observatic	3	7
df	2	6
F	419.5578	
P(F<=f) on	0.00238	
F Critical o	9.325504	

t-Test: Two-Sample Assuming Equal Variances

	null lesion	null axam	
Mean	16.8	50.71429	
Variance	1.12	469.9048	
Observatic	3	7	
Pooled Va	352.7086		
Hypothesiz	0		
df	8		
t	2.616883	since t>2.306 there is a	
P(T<=t) on	0.0154	significant difference at P=0.05	
t Critical or	1.859548		
P(T<=t) tw	0.030799		
t Critical tw	2.306006		

Table 3.1.

	cell line		
	1) log wt	**2) stat wt**	**3) stat null**
%	43	45	10
cells	39	54	20
intact	53	63	27
	45 ± 7.0	54 ± 9.3	19 ± 8.4

F-Test: Two-Sample for Variances

	1) log wt	2) stat wt
Mean	45	54
Variance	52	81
Observatic	3	3
df	2	2
F	1.557692	
P(F<=f) on	0.390977	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) log wt	2) stat wt	
Mean	45	54	
Variance	52	81	
Observatic	3	3	
Pooled Vai	66.5		
Hypothesiz	0		
df	4		
t	**1.351691**		no significant difference
P(T<=t) on	0.123928		
t Critical or	2.131846		
P(T<=t) tw	0.247855		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	2) stat wt	3) stat null
Mean	54	19
Variance	81	73
Observatic	3	3
df	2	2
F	1.109589	
P(F<=f) on	0.474026	
F Critical o	19.00003	

t-Test: Two-Sample Assuming Equal Variances

	2) stat wt	3) stat null	
Mean	54	19	
Variance	81	73	
Observatic	3	3	
Pooled Vai	77		
Hypothesiz	0		
df	4		
t	**4.885042**		since t>4.604 there is a highly
P(T<=t) on	0.004066		significant difference at P=0.01
t Critical or	2.131846		
P(T<=t) tw	**0.008131**		
t Critical tw	2.776451		

F-Test: Two-Sample for Variances

	1) log wt	3) stat null
Mean	45	19
Variance	52	73
Observatic	3	3
df	2	2
F	1.403846	
P(F<=f) on	0.416	
F Critical o	9.000019	

t-Test: Two-Sample Assuming Equal Variances

	1) log wt	3) stat null	
Mean	45	19	
Variance	52	73	
Observatic	3	3	
Pooled Vai	62.5		
Hypothesiz	0		
df	4		
t	**4.027903**		since t>3.747 there is a
P(T<=t) on	0.00788		significant difference at P=0.02
t Critical or	2.131846		
P(T<=t) tw	**0.015761**		
t Critical tw	2.776451		