Role of the *Leishmania mexicana* CPB cysteine proteinases in the host-parasite interaction

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Abstract

Leishmania mexicana express several cysteine proteinases, some of which appear to be good drug targets. Findings suggested that cysteine proteinases may have an immunomodulatory role within the mammal and recently it has been shown that cysteine proteinase-deficient mutants stimulate a Th 1 response in susceptible mice strains compared with the Th 2 phenotype normally observed in wild-type infections.

To investigate further the immunomodulatory activity of leishmanial cysteine proteinases and their role in host-parasite interactions, a major cysteine proteinase of *L. mexicana*, CPB, was produced in recombinant form. The enzyme, CPB2.8ΔCTE, generated without its C-terminal extension was successfully expressed in *Escherichia coli* and purified from inclusion bodies to yield enzyme with high specific activity. The successful method was also used for the attempted production of a full length CPB with its C-terminal extension of *L. infantum* in recombinant form. This proved less successful apparently because of the presence of the hydrophobic C-terminal extension.

The recombinant CPB of *L. mexicana* was analysed for its effects on the host response to the parasites and parasite antigen. *In vitro* analysis entailed incubation of explanted peritoneal exudate cells with recombinant CPB2.8 to assess production of inflammatory mediators considered to be important in leishmanial infection. The enzyme did not affect the production of interleukin-12 or nitric oxide but it did reduce the level of IL-10.

The vaccine potential of the enzyme was investigated by co-administration with recombinant IL-12 as a vaccine, in three mouse strains, before challenge with wild-type *L. mexicana*. The consequent infection and host immune responses were monitored. The enzyme was partially protective when administered with IL-12 even in the highly susceptible BALB/c line. The activity of the enzyme was shown to be unimportant in its ability in promoting a Th 1 response rather than the exacerbative Th 2 response normally seen in wild-type infection. These results demonstrate the potential of recombinant CPB as part of a vaccine against leishmaniasis.

Targeted gene deletion of the cysteine proteinases of *L. mexicana* has produced several null mutants including the single and double null mutants. The single null mutant (denoted by Δ*cpb*) is generated by deletion of the entire *cpb* array, which encodes the 19 CPB isoenzymes, while deletion of both the *cpb* array and the *cpa* gene generates the double null mutant (denoted by Δ*cpb/cpa*). The latter mutant produces a no lesion growth phenotype in BALB/c mice and has been suggested as being a potential vaccine candidate. The parts played by the cysteine proteinases were investigated further by using these mutant parasite lines in which CPB was analysed by methods of re-integration directly into the chromosome or by re-expression via a cosmid vector.

In order to compare metacyclic-specific and amastigote-specific genes and see if time of cysteine proteinase expression was important in establishing infection, the single null mutant was re-integrated with either metacyclic-expressed CPB2 or amastigote-expressed CPB2.8. Furthermore, each proteinase was engineered with its native promoter or with a chimaeric promoter, to assess expression levels and to investigate if there was a direct correlation between CPB

expression and virulence since metacyclic CPBs are normally not expressed in the amastigote and *vice versa.*

Re-integration of metacyclic CPB2 with its native and chimaeric promoter, significantly increased parasite proliferation resulting in increased lesion growth. re-integration of amastigote CPB2.8, did not promote an increase in virulence as the enzyme with its native promoter but did promote virulence when the enzyme was re-integrated with the chimaeric promoter. Analysis of the humoral and cellular responses to the single null mutants, indicated a mixed Th phenotype with IFN-γ and IL-4 being produced by *ex vivo* splenocytes in response to leishmanial antigen stimulus.

Antibody and lesion analysis of the double null mutant-infected mice was also carried out and did not result in an increase in virulence. Indeed re-integration of either CPB2 or CPB2.8 resulted in significantly higher IgG2a production indicating more of a Th 1 response than that already seen in double null-infected mice. Re-expression of the *cpb* array as a cosmid vector into the single null mutant did not result in a significant increase in virulence in mice compared with that of the single null mutant alone.

The methods used to analyse these immune responses have been developed to permit further examination of other leishmanial CPs and should help to elucidate the role of these important parasite molecules.

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ABBREVIATIONS

Ab	antibody
APCs	antigen presenting cells
ATP	adenosine tri-phosphate
β-ME	beta-mercaptoethanol
Bz	benzoyl
CD	cluster of differentiation
Con A	concanavalin A
CP	cysteine proteinase
CPA	type II cysteine proteinase
CPB	type I cysteine proteinase
CPC	type III cysteine proteinase
CR	complement receptor
dddH_2O	double-distilled de-ionised water
dhfr-ts	dihydrofolate reductase-thymidylate synthase
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetate
ELISA	enzyme linked immuno-sorbent assay
E-64	*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane
ES-62	excretory-secretory molecule of 62 kDa
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FPLC	fast protein liquid chromatography
FVR	phenylalanine-valine-arginine
GIPL's	glycoinositol-phospholipids
GM-CSF	granulocyte monocyte colony-stimulating factor
GPI	glycosylphosphatidylinositol

H_2O_2	hydrogen peroxide
HI-FCS	heat-inactivated foetal calf serum
HIV	human immuno-deficiency virus
HRP	horse-radish peroxidase
IFN-γ	interferon-gamma
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl β-D-thiogalactopyranoside
K	thousand (1000)
kDa	kiloDalton
LACK	*Leishmania* homologue of receptor of activated C kinase
LAMP	lysosomal-associated membrane protein
LB	Luria-Bertani
LPG	lipophosphoglycan
LPS	lipopolysaccharide
LY	leucine-tyrosine
MAF	macrophage activating factor
M-CSF	monocyte-colony stimulating factor
MHC	major histocompatibility complex
MIF	migration inhibitory factor
M_r	molecular weight
mRNA	messenger ribonucleic acid
Nan	nitroanilide
NF-$_K$B	nuclear factor for kappa B activation
Ni^{2+}-NTA	nickel-nitriloacetic acid
NK	natural killer
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase

$^1O_2$	singlet oxygen
O_2^-	superoxide radical
OH^-	hydroxy radical
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PECs	peritoneal exudate cells
PEG	polyethylene glycol
PFR	proline-phenylalanine-arginine
PGE2	prostaglandin E2
PKC	protein kinase C
PSGEMKA	phosphate-buffered saline plus glucose, EDTA, magnesium chloride and bovine serum albumin
PV	parasitophorous vacuole
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNIs	reactive nitrogen intermediates
ROIs	reactive oxygen intermediates
RT-PCR	reverse-transcriptase polymerase chain reaction
SBS	substrate binding site
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLA	soluble leishmanial antigen
TCA	trichloroacetic acid
Th	T helper cell
TNF-α	tumour necrosis factor-alpha
Tris	tris (hydroxymethyl) amino methane
TGF-β	transforming growth factor-beta
U	units of enzyme activity
UV	ultra-violet
V	volts
WT	wild-type

CHAPTER 1

Introduction

1.1 *LEISHMANIA*: DISEASE AND IMPLICATIONS

The genus *Leishmania* contains the etiologic agents for a diverse range of diseases, ranging from a simple cutaneous lesion to the potentially fatal visceral leishmaniasis. The diseases afflict 12 million people in 88 countries of the world and have proven difficult to control due to the lack of any prophylactic agent, the toxicity of effective anti-leishmanials, the innate ability of the parasite to infect many species of mammals, the difficulties in controlling vectors and the emergence of AIDS-related leishmaniasis (WHO, 1997).

1.1.1 Life Cycle

Successful infection of a mammalian host by *Leishmania* involves transmission of the infective metacyclic form, inoculated by the sandfly vector *Phlebotomus* (in the Old World) or *Lutzomyia* (in the New World). By preferentially accessing macrophages via complement receptors, CR1 and CR3, the promastigotes fail to trigger the respiratory burst which is leishmanicidal (Alexander *et al.*, 2000). The promastigotes rapidly change to amastigotes after phagocytosis, then multiply within the parasitophorous vacuole of the macrophage. The infected cell bursts, the released parasites are again phagocytosed and the process is repeated.

Depending on the infecting species of *Leishmania*, the pathology of the infection can be quite different. It ranges from a self-limiting cutaneous lesion to a

life-threatening condition, if left untreated. The life cycle (Figure 1.1) is complete when another sandfly takes a bloodmeal from an infected host and ingests the parasitised blood whereupon amastigotes transform back into promastigotes and bind to the insect gut, before migrating to the insect mouthparts (Neva & Brown, 1994).

1.1.2 Pathology

Cutaneous leishmaniasis, caused by *L. major* and *L. tropica* in the Old World, results from multiplication of the parasite in the phagocytes of the skin. In the classic course of this type of disease, lesions first appear as papules, progress to ulcers, then spontaneously heal with scarring over several months. However, diffuse cutaneous leishmaniasis, attributed to *L. mexicana* and *L. aethiopica*, causes widespread thickening of the skin with lesions, resembling those of lepromatous leprosy, which do not heal spontaneously and can be severely disfiguring (Jawetz, 1995).

Infection with *L. braziliensis* results in mucocutaneous leishmaniasis due to metastasis of organisms to mucosal sites from a primary cutaneous lesion established much earlier. Parasite metastasis may spread to the oronasal and pharyngeal mucosa causing highly disfiguring leprosy-like tissue destruction and swelling. Visceral leishmaniasis or kala-azar is caused by *L. donovani*, *L. infantum* or *L. chagasi* and is characterised by fever, hepatosplenomegaly and anaemia which, if left untreated, is lethal within weeks or months.

1.1.3 Chemotherapy

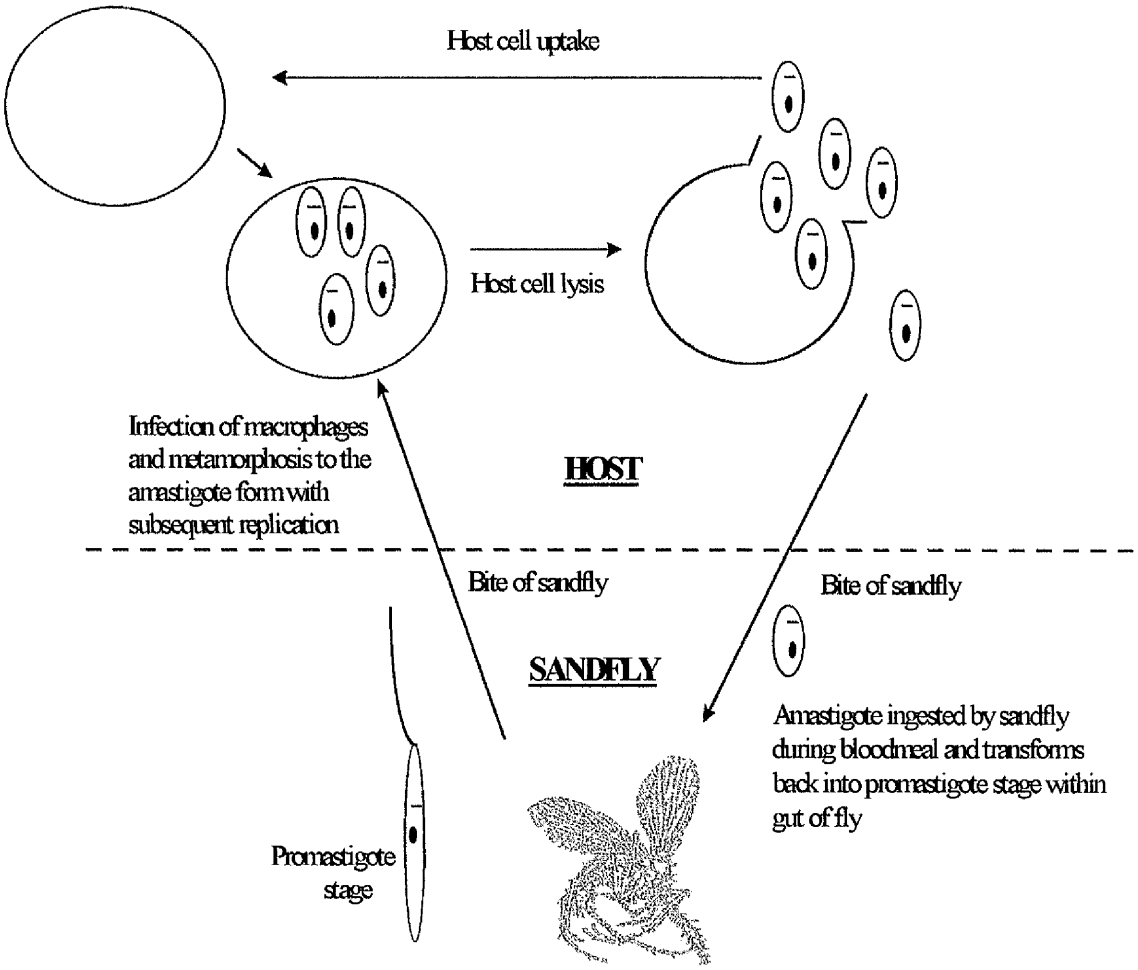

Figure 1.1 Simplified life cycle of *Leishmania* species

The drugs currently used, such as sodium stibogluconate and pentamidine, were all derived empirically many years ago and yet still little is known about their modes of action (Berman, 1997). As the leishmaniases became more extensively treated and more carefully studied, treatment failures with the arcane antimonials, sodium stibogluconate and glucantime, the first line drugs in most cases, became recognised (Berman, 1997). Alternative treatments including pentamidine and amphotericin B have been used with varying degrees of success but toxicity, resistance and high cost mean that alternative therapies are being constantly investigated. However, since infectious diseases such as the leishmaniases mainly affect people in developing countries, research into drug development is not afforded high priority by pharmaceutical companies.

1.1.4 HIV co-infection

The co-existence of HIV and visceralising *Leishmania* species in southern Europe, where leishmaniasis is endemic, has resulted in a large number of dually infected individuals in the HIV-infected population (Berman, 1997). Approximately 300 cases of dual infection had been reported in Europe by 1994 but by 1997 the total number of cases had increased to 1000. HIV infection causes disease in previously asymptomatic people with latent leishmanial infection and this emphasises the fact that visceral leishmaniasis is an opportunistic infection that now infects patients in the developed world.

1.2 SURFACE MOLECULES INVOLVED IN INFECTION

During leishmanial infection there is interaction between the host macrophage and the parasite's surface molecules, some of which are purported to be

virulence factors. These are therefore important in establishment of intracellular infection and the main ones studied are briefly described in this section.

1.2.1 Lipophosphoglycan

The glycolipid, lipophosphoglycan (LPG) is anchored in the plasmalemma of the cell by a phosphatidylinositol-glycan and occurs at 2 - 5 x 10^6 copies per cell in the promastigote stage (Turco & Descoteaux, 1992). While LPG is present in abundance on the cell surface of the promastigotes, it is down-regulated in the amastigote stage with approximately 2 - 5 x 10^4 copies per cell. The structure of the molecule varies between *Leishmania* species but in all isolates characterised it is comprised of repeated phosphorylated saccharide units linked via a mannose-rich carbohydrate core to a lysoalkylphosphorylinositol lipid anchor (Ferguson, 1999).

L. major promastigotes modulate LPG by varying the number of saccharide units and their terminal sugars, increasing the length of LPG during transformation from non-infective, dividing promastigotes to infective, non-dividing metacyclics. This alteration is responsible for the release of the promastigotes from the midgut (Turco & Descoteaux, 1992).

LPG has been considered to be a multi-functional virulence factor because of its apparent involvement in several key aspects of host-parasite interaction. After binding to a macrophage receptor and during phagocytosis of the parasite into the macrophage, LPG causes unresponsiveness of macrophages to activators of protein kinase C (PKC) (Turco, 1999). Activation of PKC leads to the activating events of the respiratory burst, *c-fos* gene expression, IL-1 production and chemotaxis. The mechanism by which LPG inhibits PKC activity appears to be related to the observation that intercalation of LPG into membranes stabilises the bilayer against

the formation of an inverted phase structure. This bilayer stabilising property of LPG is the converse of the bilayer destabilising property of diacylglycerol, the physiological activator of PKC, and this inhibits the ability of PKC to phosphorylate proteins. LPG can therefore inhibit the respiratory burst, a mechanism by which the parasite subverts a potentially leishmanicidal response of the macrophage. As well as actively modulating macrophage activation, LPG can scavenge reactive oxygen radicals which are potentially harmful to the parasite (Russell, 1994).

Thus LPG has been considered important for attachment of the parasite within the insect vector, and mammalian host and to help down-regulate one of the mammalian host cell's microbicidal responses. However, recent findings suggest that LPG is in fact not a virulence factor in *L. mexicana* infection as it was shown that LPG-deficient parasites remain lethal in BALB/c mice (Ilg, 2000).

1.2.2 Glycoinositol-phospholipids (GIPL's)

The GIPL's of *Leishmania* were shown to share a structural motif with protein GPI-anchors and LPG (Thomas *et al.*, 1992). The majority of the GIPL's contain shorter alkyl chains and are the most abundant surface component of the promastigote and amastigote form of the parasite. The precise function of cell-surface GIPL's in trypanosomatid parasites is largely unknown. Their abundance suggests that they may play a general protective function by providing a dense, negatively charged glycocalyx, close to the surface of the plasma membrane through which other macromolecules project. It has recently been reported that these molecules are essential for infection in *Leishmania* (McConville & Menon, 2000).

1.2.3 The surface metalloproteinase gp63

The surface metalloproteinase, gp63, occurs at 5 x 10^5 copies per cell in the promastigote in which it is, as the name suggests, exposed at the cell surface. *L. mexicana* amastigotes express at most only very small amounts of gp63 on their surface and instead a soluble form of the proteinase is located in the lysosomes of the parasites (Russell, 1994).

Although gp63 has been shown capable of cleaving a range of different substrates, including the third component of complement, its relative activity on substrates of biological significance remains unconvincing (Chaudhuri & Chang, 1988). The demonstration of a homologue for a leishmanial gp63 in *Crithidia fasciculata* suggests that the ancestral function of the enzyme lies within the sandfly (Inverso *et al.*, 1993) even though gp63 has been found to mediate attachment to the macrophage. Targeted gene deletion of gp63 has shown that this molecule is clearly involved in conferring resistance to complement-mediated lysis (Joshi *et al.*, 1998). Nevertheless, it seems that surface gp63 is not essential since surface gp63-deficient *L. major* parasites were capable of lesion formation in BALB/c mice (Joshi *et al.*, 1998).

gp63 was one of the first polypeptides from *Leishmania* to mediate protection against infection (Russell & Alexander, 1988) and DNA vaccines based on this molecule have also been shown to afford significant protection against infection (Xu & Liew, 1995).

1.3 PROTEINASES

1.3.1 Mode of action

Proteolytic enzymes catalyse the enzymatic degradation of peptide bonds in both proteins and peptides and are ubiquitous throughout the plant, animal and prokaryotic kingdoms. They are characterised according to the way they cleave the peptide bond. Proteases that catalyse the cleavage of an internal peptide bond are known as endopeptidases or proteinases (McKerrow *et al.*, 1993). Those that catalyse the cleavage of amino acids from their amino or carboxy terminus are known as exopeptidases.

The endopeptidases are sub-classified into four main groups, these being serine, cysteine, aspartic and metalloproteinases. They are so-called because the amino acid or metal ion is essential for the binding of the substrate at the active state. For example, cysteine proteinases (CPs) contain a thiol-imidazoyl system within the active site with catalysis proceeding via a thiol ester formed transiently between the substrate and Cys-25. For this reason they are sometimes known as thiol proteinases. Furthermore, CPs can have a very broad pH optima from pH 5.5 to 7.5 and thus can function in several environments provided there is sufficient reducing agent to ensure that the active-site thiol group is not oxidised.

1.3.2 Mammalian CPs

The most abundant mammalian CPs are cathepsin B and L which function within lysosomes (Kirschke *et al.*, 1995). They are generally present in all cells and are members of the papain superfamily of CPs. The papain family is the largest family of CPs which includes a wide range of enzymes from both prokaryotes and

eukaryotes. Catalytic activity of these enzymes is dependent on pH values below 7 and the presence of reducing compounds.

CPs are synthesised as inactive pre-pro-enzymes (Nishimura *et al.*, 1988). This is to protect the cell from potentially disastrous consequences of uncontrolled protein degradation. The pre-region is cleaved before the full-length protein is synthesised and is possibly involved in targeting to the endoplasmic reticulum.

The pro-segment of cathepsin L and B is a potent inhibitor of the mature enzyme. Binding of the pro-region in the substrate binding site (SBS) is comparable with that of E-64, an irreversible CP inhibitor, since it too binds in the reverse direction (Coulombe *et al.*, 1996). The pro-region is fastened in the SBS by a two-turn helix positioned in the S' subsites. It follows the groove in the opposite direction to that of the substrate and although the pro-region passes very close to the catalytic machinery of the proteinase, it is not cleaved by the enzyme. The reversal of the direction of the polypeptide chain in the SBS results in an appropriate positioning of the peptide bond for hydrolysis to occur (Cygler & Mort, 1997). The pro-segment is also essential for correct folding of the newly synthesised protein and stabilises it to the denaturing effects of neutral to alkaline pH, conditions which rapidly inactivate most of the mature CPs (Coulombe *et al.*, 1996). Furthermore, a specific region of the pro-segment provides at least part of the recognition site for modification with mannose-6-phosphate, a signal for targeting to the lysosomal compartment within mammalian CPs.

Cleavage of the pro-enzyme with subsequent activation of the proteinase occurs as the result of auto-catalysis under acidic conditions (Mason *et al.*, 1987). *In vivo*, this occurs as the newly synthesised pro-enzyme is routed from the Golgi apparatus to the lysosome. The exact pH in the Golgi is unknown but is considered

to be less acidic than in lysosomes, where the mature enzymes are situated. Degraded peptides diffuse through the lysosomal membrane and are available again for protein synthesis in the cell (Kirschke *et al.*, 1995).

In the case of human cathepsin L, the disruption at low pH of salt bridges involved in the interaction between the pro-peptide and mature enzyme is believed to trigger the processing event (Roche *et al.*, 1999). In parallel, activation and processing of pro-papain is achieved by auto-proteolytic cleavage (intramolecular) and, once free papain is released, processing can occur *in trans* (intermolecular) (Vernet *et al.*, 1995). It is thought that a conserved motif and its electrostatic status within the pro-region participates in the control of intramolecular processing of the papain precursor.

Cathepsin L is a strong endopeptidase degrading several proteins and inactivating many enzymes. It is catalytically active at pH 3.0 - 6.5 in the presence of SH-containing compounds and has a requirement for hydrophobic amino acid residues in subsites S_1 and S_2 (Kirschke *et al.*, 1995). Pro-cathepsin L has also been shown to interfere with antigen processing (Menard *et al.*, 1998).

Cathepsin B is one of the most abundant and thoroughly studied lysosomal CPs and is involved in many normal cellular processes including intracellular degradation of protein and antigen processing (Guagliardi *et al.*, 1990).

Cathepsin C has been found to participate in the activation of pro-enzymes and is implicated in several pathological events (Cigic *et al.*, 1998). It has been found to aid cancer invasion and its subsequent metastasis (Mort *et al.*, 1981). Unique among the lysosomal CPs to cathepsin C is the requirement of halide ions in addition to a SH-containing compound for the catalytic activity of this enzyme (McDonald *et al.*, 1966). Several metabolic functions have been attributed to cathepsin C in addition to

its main function in protein degradation in lysosomes, including activation of neuraminidase.

1.3.3 Parasite proteinases

Proteinases have been implicated in a wide variety of adaptations of micro-organisms for survival in the host, including modulation of the host's immune system and tissue destruction which enable further invasion of the micro-organism in the host (Travis *et al.,* 1995). There have been many studies on parasite proteinases, too numerous to detail comprehensively and so I mention just three examples of the different classes of proteinases. Two aspartic proteinases (plasmepsins) from *Plasmodium falciparum* have been implicated in the degradation of haemoglobin and both have been successfully purified and crystallised from *E. coli* (Hill *et al.,* 1994). A metallocollagenase from *Entamoeba histolytica* has been proposed to play an essential role in invasion by degradation of collagen, the connective tissue, which would allow further infection of the host (Munoz *et al.,* 1982). *Leishmania* species also contain a metalloproteinase, gp63, which is abundantly expressed and protects promastigotes from hydrolytic enzymes in the gut of the sandfly while in the mammalian host, it can inhibit complement mediated lysis and promote parasite uptake by cleaving C3b to C3bi - see section 1.2.3.

1.3.4 Parasite CPs

The major CP of *Trypanosoma cruzi*, cruzipain, is largely responsible for the proteolytic activity of the organism and inhibitors of the enzyme blocked mammalian cell infection to a considerable degree suggesting that the intracellular development of *T. cruzi* is critically dependent on cruzipain (Meirelles *et al.,* 1992). Cruzipain also

cleaves kininogen to form a pro-inflammatory peptide, bradykinin, as well as activating pre-kallikrein which could be a second and indirect way by which the parasite proteinase can release bradykinin (Del Nery *et al.*, 1997). Thus, by acting as a kininogenase, cruzipain has been found to induce plasma leakage in post-capillary venules (Svensjo *et al.*, 1997). Further studies on this enzyme showed that an irreversible CP inhibitor proved effective in blocking parasite replication and so this enzyme has been considered as a model for anti-parasite drug design (Engel *et al.*, 1998).

A CP which shares some homology to cathepsin L has also been identified in *P. falciparum* . Inhibitor studies have shown that the enzyme is probably required for haemoglobin degradation and therefore parasite development (Rosenthal *et al.*, 1998). Clearly this enzyme plays an important role in the host-parasite interaction and is considered as being a potential target for chemotherapeutic attack (Olson *et al.*, 1999).

Generally, proteinases can be considered good targets for chemotherapy if they have different substrate specificities from the host enzymes, if there is unusual location of the parasite proteinase, or if the enzyme is involved in a process that is unique to the parasite (Coombs & Mottram, 1997). Essential enzymes are considered the best targets but inhibiting others can also be effective (Barrett *et al.*, 1999).

1.3.5 Leishmanial Type I CPs

For all trypanosomatid species analysed, the Type I enzymes are encoded by multicopy genes arranged in tandem arrays and usually have the highest CP activity (Robertson *et al.*, 1996). The Type I CPs of the parasite *L. mexicana* have received considerable attention over the last few years as they may be important in the host-

parasite interaction, are considered to be potential vaccine candidates and provide good targets for chemotherapeutic attack (Coombs & Mottram, 1997). One feature that distinguishes the Type I enzymes from other CPs of the papain superfamily is the presence of a long C-terminal extension (Mottram *et al.*, 1997). It may possibly have a role in immune evasion since it has been shown to be highly immunogenic and so could potentially divert the host immune response (Martinez *et al.*, 1993). The Type I CPs of *L .mexicana*, known as CPB, are encoded by 19 *cpb* gene copies (see figure 1.2), arranged in a tandem array and are cathepsin L-like (Mottram *et al.*, 1996). Transfection of different copies of *cpb* genes into null mutants showed individual isoenzymes differ in their substrate preferences (Mottram *et al.*, 1996). This suggests that the enzymes have different roles in host-parasite interaction.

CPB1, which is encoded by the first gene in the array, has a truncation in the C-terminus and transfection into *cpb* null mutants resulted in active enzyme expression. This enzyme is targeted to lysosomes and is active, showing that the C-terminal extension is not essential for activation or correct intracellular trafficking.

CPB2.8 is the enzyme that has been most studied. This is a *cpb* gene internal to the tandem array of the 19 genes. It has been found to restore infectivity to macrophages *in vitro* when it is re-expressed in the *cpb* null mutant suggesting that it is indeed a virulence factor (Mottram *et al.*, 1996). Thus, the other CPBs may be targets for novel anti-leishmanial drugs based on specific inhibitors or pro-drugs, activatable by amastigote CPs (Rabinovitch, 1989).

The intracellular amastigote form of *L. mexicana* has a much greater CP activity than promastigote forms (Coombs & Mottram, 1997). This high enzyme activity may be essential for the survival of the amastigote in the microbicidal environment of the host mammalian macrophage.

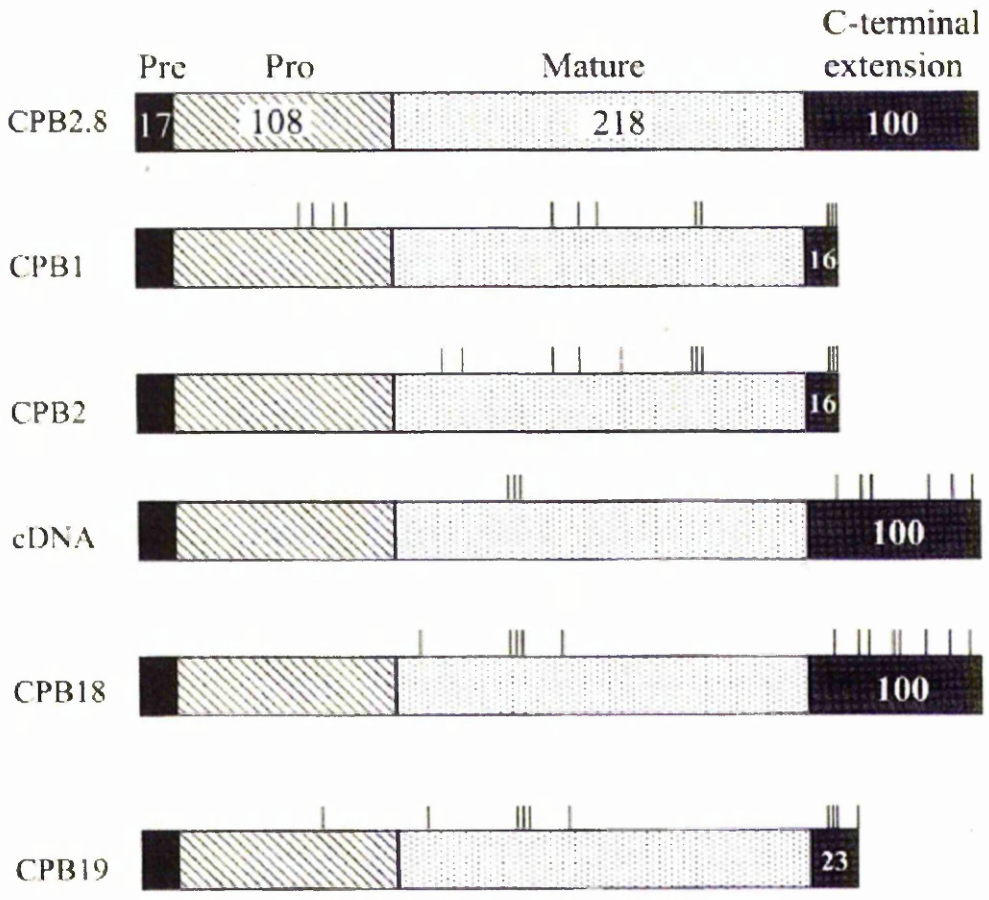

Figure 1.2 Schematic representation of the cysteine proteinases encoded by the *cpb* genes of *L. mexicana* (taken from Coombs & Mottram, 1997). The four regions of CPB2.8 are shown and compared with five other CPB isoenzymes on the 19-copy *cpb* tandem array. The number of amino acids present in each region of CPB2.8 are shown. The positions of amino acid differences between isoenzymes are indicated by a vertical line.

1.3.6 Leishmanial Type II/III CPs

Two other CP genes have been isolated from *L. mexicana*: CPA, designated a Type II CP is also cathepsin-L like but is encoded by a single-copy gene (*cpa*) and lacks a C-terminal extension (Mottram *et al.*, 1992). However, gene deletion experiments have shown that *cpa* does not compensate for the loss of *cpb* in a null mutant of *L. mexicana* (Mottram *et al.*, 1996). Furthermore, *L. mexicana cpa* null mutants did not differ significantly from wild-type *L. mexicana* in growth and could infect macrophages *in vitro* while also being able to establish lesions in CBA mice (Souza *et al.*, 1994). Thus, *cpa* does not appear to be essential for infection of the host cells. CPC designates the Type III CP, which is a cathepsin B-like enzyme and it too is encoded by a single-copy gene (*cpc*) (Bart *et al.*, 1995). Disrupting both alleles of the *cpa* and *cpc* genes produced null mutants similar to wild-type parasites in their infectivity to mice, even though the *cpc* null mutant had a lower rate of survival than the wild-type parasite in macrophages *in vitro* (Souza *et al.*, 1994; Bart *et al.*, 1997). This suggests that these enzymes are not essential for parasite survival.
The cathepsin L and B-like proteinases do not solely exist in *L. mexicana* but have also been found to occur in several other species including *L. major, L. pifanoi* and *L amazonensis* (Sakanari *et al.*, 1997).

1.4 IMMUNE RESPONSE IN LEISHMANIASIS

1.4.1 Immunological polarity

Once a T cell response begins to develop along one pathway, either a Th 1 or Th 2, it tends to become progressively polarised in that direction (Abbas *et al.*, 1996). Indeed, studies of cutaneous leishmaniasis in inbred mice provided the first

clear demonstration that resistance and susceptibility to an infectious disease correlates with anti-microbial Th 1 and Th 2 responses respectively (Heinzel *et al.*, 1989).

The ultimate dominance of Th 1 and Th 2 cells in any immune response is at least initally due to either interleukin-12 (IL-12) or IL-4 induced T cell differentiation. Thus, for example, BALB/c mice can be made resistant to *L. major* by administration of IL-12 or anti-IL-4 during the first week of infection but not later (Heinzel *et al.*, 1998). Also, it has been clearly demonstrated that there is an absolute requirement for IL-4 for susceptibility to *Leishmania mexicana* (Satoskar *et al.*, 1995). These concepts can be exploited for developing vaccines to parasites by inclusion of certain cytokines, for diseases such as *Leishmania* in which a polarised immune response is required.

1.4.2 T cell cytokines important in *Leishmania*

IL-4 prevents macrophage activation and blocks most of the macrophage activating effects of interferon-γ (IFN-γ), leading to a more disseminated infection (Finkelman & Urban, 1992). In contrast, IL-12 stimulates differentiation of $CD4^+$ T cells towards a polarised Th 1 phenotype. Dendritic cells are the critical source of early IL-12 production following *Leishmania* infection (Gorak *et al.*, 1998). In response to *Leishmania* infection, which fails to trigger IL-12 production by macrophages (Carrera *et al.*, 1996), dendritic cell-T cell clusters may provide the micro-environment for initial natural killer (NK) cell activation in response to *L. donovani* infection (Gorak *et al.*, 1998).

In the early phase of the infection, leishmanicidal activity is not triggered in resting macrophages that become parasitised. Therefore infected macrophages will

not present antigens to immune T cells because major histocompatibility complex (MHC) class II expression is not upregulated (Antoine *et al.*, 1998). Parasites replicate unhindered and the lesion will expand. In the course of infection, focal activation of individual infected macrophages may occur (Aebischer *et al.*, 2000) These cells will be able to present amastigote antigens to parasite-specific Th 1 cells. The interacting Th 1 cells will be reciprocally stimulated and due to their secretion of IFN-γ and tumour necrosis factor-α (TNF-α), will activate neighbouring infected macrophages (Abbas *et al.*, 1996).

Eventually, the density of infected macrophages will decline and lesions will resolve. In this phase the process becomes inefficient and will lead to the persistent state of the infection where there is a balance between parasite destruction and replication (Aebischer *et al.*, 2000).

1.4.3 Th 2 type cytokines

IL-4 has a major role to play in the Th 1/Th 2 paradigm which exists during *Leishmania* infection. For example, it has been found that disruption of the murine IL-4 gene can inhibit disease progression during *L. mexicana* (Satoskar *et al.*, 1995). IL-4 deficient mice produced more IFN-γ than the wild type strain in response to *L. mexicana* infection, which was indicative of a Th 1 response. Furthermore, the inhibition of macrophage activating factor (MAF) from spleen cells isolated from mice susceptible to *Leishmania* infection appears to be mediated by IL-4. Anti-IL-4 antibodies completely abrogated the MAF-inhibiting capacity of culture supernatants from spleen cells of susceptible mice (Liew *et al.*, 1989).

Secretion of early IL-4 is decisive in aiding *Leishmania* infection even in the presence of other cytokines such as IL-12 (Himmelrich *et al.*, 1998). It has been

found that production of IL-4 is necessary only during a short period of time (< 48 hours) for irreversible Th 2 cell development to occur in susceptible BALB/c mice (Himmelrich *et al.*, 1998). It is possible that IL-4 itself appears to inhibit the ability of IL-12 to promote Th 1 cell development, possibly by providing a more powerful stimulus for Th 2 cell development than IL-12 provides for Th 1 cell development (Scott, 1993).

Recent evidence has shown, however, that IL-4 is rapidly produced in BALB/c mice after infection with *L. major* and acts by down-regulating IL-12 receptor expression on $CD4^+$ T cells resulting in a state of unresponsiveness to IL-12 (Himmelrich *et al.*, 1998). IL-4 also enhances production of IgE by increasing the frequency of B cell isotype switching (Finkelman *et al.*, 1990). Transforming growth factor-β (TGF-β) supports differentiation of Th 2 cells and suppresses the development of Th 1 cells. TGF-β deactivates macrophages causing a down-regulation of inducible nitric oxide synthase (iNOS) and therefore production of nitric oxide (NO) (Lohoff *et al.*, 1998). This is important since NO produced this way is the most important mechanism by which the macrophage has been demonstrated to kill *Leishmania in vitro* and *in vivo* (Liew *et al.*, 1990). Recombinant TGF-β added to cultures of peritoneal exudate cells (PECs) has been shown to increase *L. braziliensis* replication whereas addition of anti-TGF-β antibody decreases the level of infection. TGF-β seems to have a synergistic effect with IL-10, another important Th 2 cytokine which is released concomitantly (Barral *et al.*, 1993). IL-10 can also deactivate macrophages by downregulation of iNOS and is a known negative regulator of IL-12 production (Melby *et al.*, 1996). It is induced by phagocytosis of *Leishmania* promastigotes and can inhibit T cell development (Weinheber *et al.*, 1998).

IL-13 may play a role in *L. major* infection since BALB/c mice doubly deficient for IL-4 and IL-13 were significantly more resistant to *L. major* infection than either of the single cytokine-deficient mutants (Matthews *et al.*, 2000). IL-13 has also been shown to share many activities with IL-4 and can also utilise the IL-4Rα receptor (Matthews *et al.*, 2000). However, it has recently been reported that signals mediated by the IL-4Rα are not necessary to induce and sustain an efficient IL-4 expression and Th 2 polarisation in *L. major*-infected BALB/c mice and suggest that IL-4Rα -independent mechanisms underlie the default Th 2 development in *L. major*-infected mice (Mohrs *et al.*, 2000).

1.4.4 Th 1 type cytokines

Th 1 cells produce IL-2 and IFN-γ among other cytokines but these two are the most important for promoting delayed type hypersensitivity (DTH) and in *Leishmania* infection, IFN-γ is essential for macrophage activation (Heinzel *et al.*, 1989). IL-12 is produced by macrophages and B lymphocytes and this stimulates production of IFN-γ from both T and NK cells thus driving Th 1 cell development (Scott, 1993). IL-12 also induces TNF-α, granulocyte monocyte-colony stimulating factor (GM-CSF) and monocyte-colony stimulating factor (M-CSF) which are required for attraction and upregulation of macrophages (Trinchieri, 1995). Furthermore, when IL-12 is added, in the presence of allergen, to cultures of lymphocytes from atopic patients, it induces IL-2 dependent cell lines that display a Th 1 like phenotype, instead of the Th 2 like phenotype observed in the absence of IL-12.

It has also been shown that genetically resistant mice lacking IL-12 are susceptible to infection with *L. major* and mount a polarised Th 2 response (Mattner *et al.*, 1996). In *Leishmania* infection, IL-12 is crucial for cure and required for stimulation of a Th 1 response. Its efficacy in eliciting a protective immune response in the murine *Leishmania* model has been demonstrated repeatedly (Scott, 1993). Infection of quiescent murine macrophages with *L. mexicana* amastigotes does not induce IL-12 production (Weinheber *et al.*, 1998). Endogenous IL-12 in leishmanial infection is presumably triggered by the macrophage T cell interactions and is dependent on cross-linking of CD40 on the macrophage with its ligand on the T cell (Heinzel *et al.*, 1998). Data indicates that IL-12 production by the macrophage after infection with *L. mexicana* amastigotes is suppressed by a mechanism that is most likely to be affecting post-transcriptional events. One plausible explanation for lack of expression of IL-12 by infected cells *in vivo* is that *L. mexicana* amastigotes may engage CR3 or the receptor for IgG upon infection of the macrophage, which inhibits IL-12 upregulation when the host cell is activated by cell-cell contact e.g. with a T-cell (Weinheber *et al.*, 1998).

IFN-γ is essential for activation of macrophages and upregulation of MHC class II presentation (Heinzel *et al.*, 1989). This results in increased antigen presentation to T cells as well as inducing macrophages to generate nitrites that can kill ingested amastigotes. IFN-γ may also be beneficial with regard to its control of infection by its ability to inhibit IL-4 production, reducing the clonal expansion of Th 2 cells (Heinzel *et al.*, 1998). IFN-γ works synergistically with TNF-α to induce this leishmanicidal activity by inducing iNOS (Liew & O' Donnell, 1993). IFN-γ also enhances the production of opsonising murine antibodies such as IgG2a which is the

Th 1 isotype that mediates optimal complement fixation and opsonisation (Arulanandam *et al.*, 1999).

NK cells have been implicated as the major source of IFN-γ during early *Leishmania* infection (Guevara-Mendoza *et al.*, 1997). It is possible that IFN-γ transcription during the first 24 hours can be attributed to activated NK cells which are down-regulated after 7 days and are then presumably replaced by pathogen-specific T cells.

Migration inhibitory factor (MIF) may be an important regulatory molecule in the induction of nitric oxide. It activates murine macrophages to express NOS and to produce high levels of NO *in vitro* (Cunha *et al.*, 1993).

1.4.5 $CD8^+$ T cells

A role for $CD8^+$ cytotoxic T cells in the control of cutaneous leishmaniasis has been suggested (Fong & Mosmann, 1990). This may be due to direct cytotoxicity or through cytokine production such as IFN-γ. $CD8^+$ T cells produce IFN-γ as well as TNF-α, both of which are important in activating macrophages to kill *Leishmania*. Further evidence for $CD8^+$ T cell involvement stems from the fact that a leishmanial antigen, gp42 (M-2), is processed in the macrophage cytoplasm and is presented to $CD8^+$ T cells via the classical pathway of MHC class I presentation (Kima *et al.*, 1997). It has yet to be determined how gp42 traverses the parasitophorous vacuole to enter the cytosol of the host cell, although it is conceivable that some leishmanial antigens would be able to access the MHC class I presentation pathway and preferentially activate $CD8^+$ T cells.

1.4.6 Reactive nitrogen intermediates (RNIs)

NO is involved in a variety of biological activities including endothelium-related vascular relaxation, platelet aggregation, neurotransmission and macrophage killing of tumour cells (Macmicking *et al.*, 1997). NO is very unstable and reacts with itself, water and oxygen to generate products collectively known as reactive nitrogen intermediates (RNIs) and finally the stable end products nitrite (NO_2^-) and nitrate (NO_3^-).

Macrophages activated *in vitro* by IFN-γ or TNF-α, in the presence of lipopolysaccharide (LPS), or by a combination of both cytokines, developed potent leishmanicidal activity and was correlated with the production of NO (Liew *et al.*, 1990). The mechanism by which NO is cytotoxic is unknown. It may be similar to biochemical mechanisms identified in mammalian cells, e.g. loss of enzymatic function due to nitrosylation of catalytic iron - it therefore blocks cellular respiration by complexing with the iron in electron transport proteins. Recently, it has been shown that NO can inhibit the papain-like CP of *P. falciparum*, which is involved in haemoglobin degradation (Olson *et al*,. 1999). This effect is likely to be attributed to nitrosylation of the Cys-25 catalytic residue. Alternatively, reaction of the superoxide anion with nitric oxide can form peroxynitrite. Protonated peroxynitrite undergoes homolytic fission to yield highly toxic oxidant products such as the hydroxyl ion.

iNOS activity is crucial for the control of *Leishmania* persisting in immunocompetent hosts after resolution of the primary infection (Stenger *et al.*, 1996). There is life-long expression of iNOS at the original skin lesion and in the draining lymph node of long-term infected mice (Stenger *et al.*, 1996). The host immune response will produce cytokines that can induce NOS, such as IFN-γ, TNF-α and MIF but can also inhibit iNOS production via IL-4, TGF-β and IL-10 (Bogdan &

Rollinghoff, 1998). This phenomenon is consistent with the sophisticated interplay characteristic of important biological systems.

1.4.7 Reactive oxygen intermediates (ROIs)

Within the lysosomes of the macrophage, a variety of hydrolases such as lysozyme, phospholipase A_2 and proteases can participate in the destruction of the entrapped organism. Besides the oxygen-independent lysosomal hydrolases, macrophage lysosomes contain oxygen-dependent enzymes that can produce ROIs such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the highly reactive hydroxy radical (OH^-).

These products and others are produced from the respiratory burst that accompanies the increased oxygen consumption and ATP generation needed for phagocytosis. However, it is well documented that *Leishmania* parasites are able to resist inactivation by the highly reactive oxygen radicals due to the presence of a dense glycocalyx and LPG molecules on the surface of the parasite. However, it has been recently reported that LPG is not a virulence factor in *L. mexicana* infeciton (Ilg, 2000) and it is more likely that the GIPL's are essential in protecting against the respiratory burst (McConville & Menon, 2000).

1.4.8 Parasite dose

It is not only the presence of cytokines that promote the Th 1/Th 2 paradigm in leishmaniasis but the contribution of several factors together including parasite dose. There is a suggestion that the resulting immune response to *L. major* is independent of infection route, strain of host or parasite (Menon & Bretscher, 1998). However, a recent study has reported that antigen dose can determine T helper subset

development by regulation of the CD40 ligand (CD40L) through high-dose antigen inducing Th1 development by up-regulation of CD40L, whereas low-dose antigen stimulation fails to induce CD40L and promotes Th 2 development (Ruedl *et al.*, 2000).

1.4.9 Parasitophorous vacuoles

L. mexicana amastigotes exist within macrophages inside a parasitophorous vacuole (PV) (Alexander & Vickerman, 1975), a large fluid-filled vesicle that harbours many parasites and has been characterised as a late stage endosome (Russell *et al.*, 1992). The PV intersects readily with the endocytic pathway of the macrophage and so is potentially exposed to degradative activities of the lysosomal contents such as the cathepsins B, C and L. However, it appears that following targeted fusion of the PV with endosomes, antigens derived from amastigotes become trapped in a compartment which either lacks the MHC molecules or does not allow the transport of the MHC-peptide complexes to the cell membrane (Bogdan & Rollinghoff, 1998).

Amastigotes of *L. mexicana* and other species (*L. amazonensis, pifanoi* and *donovani*) are not free in the PVs but are tightly bound to the membrane of the organelles via the posterior pole (Antoine *et al.*, 1998) Furthermore, PVs of *L. mexicana* tend to be large and one of the main factors involved in formation of these is the secretion of proteophosphoglycan from intracellular amastigotes (Peters *et al.*, 1997). This particular species of *Leishmania* can also endocytose macromolecular materials via two independent routes (Schaible *et al.*, 1999). Data suggests that *L. mexicana* PVs accumulate material by exploiting the cellular recycling machinery or through the host cell's organic anion transporter.

Endocytosis of certain host molecules in the PVs, such as MHC molecules, would be advantageous to the parasite and could limit antigen presentation of parasite antigens. Indeed, internalisation of MHC molecules seems to be a selective process as neither lysosomal-associated membrane protein (LAMP) or macrosialin have been detected within parasites (Antoine *et al.*, 1998).

1.4.10 Antigen presentation

Peptide fragments derived from extracellular proteins usually bind to MHC class II molecules whereas MHC class I molecules associate with endogenously synthesised peptides generated in the cell cytosol. Macrophages express only low levels of MHC class II molecules until stimulated to do so by IFN-γ. Expression is antagonised by IL-10 which is physiologically important in *Leishmania* infection (Abbas *et al.*, 1996).

Parasitised macrophages are thought to be an essential source of antigens for the stimulation of *Leishmania*-specific $CD4^+$ T cells. However, analysis of amastigotes suggests that they avoid antigen presentation by their host cell by limiting the release of potential antigens (Russell *et al.*, 1992). Once within the macrophage, *Leishmania* amastigote antigens are sequestered from the MHC class II pathway of antigen presentation by targeted fusion of the PV with certain endocytic compartments (Kima *et al.*, 1996). Thus a down-regulation of parasite antigen presentation via the MHC class II pathway occurs and evidence indicates that the *Leishmania*-infected macrophage becomes refractory to cytokine (IFN-γ) activation. Hence the parasite niche becomes relatively obscured from immunological view allowing persistence of parasites and optimising for transmission and perpetuation of the life-cycle (McMahon-Pratt *et al.*, 1998).

Inhibition of MHC class II presentation cannot be attributed to unavailability of MHC class II molecules on the surface of infected cells (Antoine *et al.*, 1998) nor is it likely that inhibition of antigen presentation by infected parasites is due to extensive phagocytosis of parasites since experiments using particulate materials do not affect antigen presentation (Fruth *et al.*, 1993).

It is therefore important to determine the impact of *Leishmania* infection on the fate of the chaperone molecule, invariant chain (Ii), as this molecule plays a fundamental role in antigen presentation (Antoine *et al.*, 1998). The invariant chain may be required for proper folding of class II molecules. Also, the presence of invariant chain can effectively block the peptide-binding cleft of the MHC molecule thereby preventing binding of any endogenous peptides that may be present in the endoplasmic reticulum. Under normal conditions, the PV-associated class II molecules are devoid of Ii. Incubation of *L. amazonensis*-infected macrophages with CP inhibitors leads to the detection of Ii in > 60% of PVs (Antoine *et al.*, 1998). Possibly, protease inhibitors block PV-associated enzymes that in normal conditions eliminate the Ii chains.

1.4.11 CPs in immuno-modulation

It is thought that MHC class II molecules are internalised by amastigotes and degraded within megasomes by CPs of parasite origin - endocytosis and degradation of MHC class molecules by *L. amazonensis* could be a means of circumventing the host's immune system (De Souza Leao *et al.*, 1995). A role for CPs in Ii degradation is also consistent with a number of other studies using proteinase inhibitors on intact antigen presenting cells (Bennet *et al.*, 1992). Leishmanial CPs may therefore play an important role in induction of immunological polarity.

Beyrodt *et al.* (1997) found that mice immunised with a purified CP, p30, were protected against infection by *L. amazonensis*. Subsequent cytokine analysis of T cell supernatants stimulated with p30 indicated that Th 1 is the subset involved in the lymphoproliferative responses to the antigen. The cytokines IFN-γ and IL-2 were secreted while IL-4, IL-5 and IL-10 were absent suggesting a protective Th 1 response.

Studies with *L. mexicana* mutants lacking CP genes, *cpb (Δcpb)* or both *cpa* (which encoded the Type II CP) and *cpb* (*Δcpa/cpb*) have shown that the Th 1/ Th 2 profile can be altered by the deletion of the *cpb* genes (Alexander *et al.,* 1998). Immunological analysis of antibody isotype during infection and splenocyte IFN-γ and IL-4 production, following stimulation with *Leishmania* antigen, indicated that there was a significant shift from a predominantly Th 2-associated response in mice inoculated with wild-type *Leishmania* to a Th 1 response in mice infected with *Δcpb* or *Δcpa/cpb* mutants. In addition, the null mutants produced some protection against subsequent challenges and this also implicates leishmanial CPs in modulation of the immune response providing strong encouragement that CP-deficient *L. mexicana* are candidate attenuated live vaccines.

CPs are also found extracellularly in the tissue presumably as a result of macrophage rupture and appear to persist in lesion tissue, where they may damage host cells and the extracellular matrix since it has been found that cathepsin B and L can also degrade connective tissue such as collagen (Ilg *et al.,* 1994).

The CPs may be potentiating Th 2 responses by increasing IL-4 production since it has been hypothesised that some proteolytic enzymes, produced in large quantity by many helminth parasites, are triggers of cytokines that induce a Th 2 response (Pearce *et al.,* 1991). Thus, leishmanial CPs may promote disease

exacerbation by promoting a Th 2 polarisation. This, however, may be a species-specific phenomenon. For example, results suggest that whereas IL-4-driven Th 2 lymphocyte expansion is necessary for disease progression and inhibition of a protective response in cutaneous infection caused by *L. mexicana*, such a role for Th 2 cells in visceral leishmaniasis caused by *L. donovani* cannot be demonstrated (Satoskar *et al.*, 1995). This clearly has implications for the CPs and their effects on the host immune response in different species.

1.5 ANTI-LEISHMANIAL VACCINES

Since drug therapy to the host is usually very toxic and there exists the chance that drug resistance can form, control of leishmaniasis would ideally be vaccine-based. In recent years, the search for a safe and effective *Leishmania* vaccine has intensified with the identification of protective protein immunogens which could be cloned and produced in large scale (Rivier *et al.*, 1999). However, the outcome of vaccination against *Leishmania* infections is critically dependent on the nature of the adjuvant, the site of vaccination, the strain of mice used for the experimentation and the form of antigen used (Rivier *et al.*, 1999). Transition from mouse models to clinically useful vaccines is also a challenge.

1.5.1 Attenuated parasite vaccines

Attenuated organisms can infect the recipient and stimulate an immune response but they do not generally cause disease (Titus *et al.*, 1995). Parasites can be attenuated by simply growing them in animals or in prolonged or repeated culture or under unusual conditions until suitable mutants are obtained (Gorczynski, 1989).

More recently, targeted gene disruption has also been used for attenuation of parasites (Alexander *et al.*, 1998).

Live parasite vaccines have several advantages over killed vaccines (Abbas *et al.*, 1994). With a live vaccine, the attenuated parasites multiply within the body so that a sufficient dose of antigen can be delivered in one injection of low dose. Normally, live replicating micro-organisms are needed to induce long-lasting immunological memory due to infected cells stimulating production of cytotoxic and helper T lymphocytes and their associated memory cells (Titus *et al.*, 1995). The drawback of live vaccines includes the risk that the parasite may, through genetic mutation, either lose its potency (so that the vaccine is ineffective) or worse still, regain its pathogenicity.

Dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) is an essential metabolic gene and targeted disruption of this to yield *dhfr-ts*$^-$ null mutants of *L. major* have been generated (Titus *et al.*, 1995). These attenuated parasites were capable of eliciting substantial resistance to a subsequent challenge with virulent *L. major* when injected into the most susceptible mouse strain and ultimately were incapable of causing disease.

As already discussed, the CPs of *L. mexicana* may play a role in host immunomodulation and CP-deficient mutants have attenuated virulence for mice and potentiate a Th 1 type response as opposed to the normally susceptible Th 2 response seen in wild type infections (Alexander *et al.*, 1998). Infection with these attenuated organisms resulted in a significant reduction in parasite-specific antibody titres, which resembles the immunologic status of humans at the resistant end of the disease spectrum. The increase of IFN-γ, decrease in IL-4 and absence of lesion formation is

in stark contrast to that seen in wild type infection and suggests that CP-deficient parasites may be good candidate live vaccines.

1.5.2 Subunit vaccines

The aim of subunit vaccines is to stimulate the immune response with a specific component of the parasite, while avoiding giving other components that cause unwanted or dangerous side effects. Once the gene that contains the information needed to make such an immunogenic protein of a microbe has been identified, this opens the way for its manufacture using recombinant DNA technology and protein purification.

gp63 was the first polypeptide from *Leishmania* shown to mediate protection against cutaneous leishmaniasis when immunised as a liposome formulation (Russell and Alexander, 1988). Protection with this antigen was recently shown to be more effective in the presence of IL-12 as an adjuvant (Aebischer *et al.*, 2000).

IL-12 as already detailed, is efficacious in stimulating a Th-1 response. Its efficacy for eliciting a protective immune response in the murine *Leishmania* model has been demonstrated repeatedly (Afonso *et al.*, 1994; Scott *et al.*, 1993). When C57BL/6 mice were immunised with a recombinant CP (misfolded CPB2.8) and IL-12, they only developed small lesions and healing occurred uniformly in all animals (Aebischer *et al.*, 2000). Cured mice did not show any recurrence of disease for over a year. Conversely, without IL-12, the proteinase afforded no protection to challenge with *L. mexicana* and in this respect, similar results have been reported for immunisation with a proteinase from *L. amazonensis*, where the enzyme enhanced parasite growth in the footpads and draining lymph nodes of mice infected with *L. pifanoi* (Soong *et al.*, 1995).

Therefore, it is apparent that choice of adjuvant is important in overall efficiency of subunit vaccination and should be considered carefully as one that would preferentially stimulate Th 1 helper cells.

1.5.3 DNA vaccines

DNA vaccines consist of a bacterial plasmid with a strong viral promoter, the gene of interest and a polyadenylation/transcription termination sequence (Donnelly *et al.*, 1997) Plasmid is grown in bacteria, purified, dissolved in a saline solution and then simply injected into the host. The DNA plasmid is taken up by the host cells where the encoded protein is synthesised. Since the plasmid is without an origin of replication that is functional in eukaryotic cells, the plasmid can neither replicate in the mammalian host nor integrate within the chromosomal DNA of the animal. Because DNA vaccines result in expression of antigens *in situ*, presentation of antigenically relevant epitopes to the immune system may be more readily attainable than with subunit vaccination.

Immunisation of BALB/c mice with a plasmid encoding gp63 of *L. major* induced a helper T cell response with a dominant Th 1 phenotype. Significant amounts of IL-2 and IFN-γ were produced but no IL-4, when spleen and lymph node cells from immunised mice were cultured with *L. major* antigens *in vitro* (Xu & Liew, 1995).

Vaccination with DNA encoding the immunodominant LACK (*Leishmania* homologue of receptor of activated C kinase) parasite antigen confers protective immunity to mice infected with *L. major*. Control of disease progression and parasite burden in mice vaccinated with LACK DNA was associated with enhancement of antigen-specific IFN-γ production (Gurunathan *et al.*, 1997). It is possible that

vaccination with DNA induces protection to *L. major* by a somewhat different mechanism than vaccination with protein plus adjuvant since DNA vaccination is particularly useful for the production of cytotoxic T cells. However, what is encouraging for vaccine development is the way in which it is possible to induce a protective immune response against *Leishmania* by several modes of vaccination. Whether this can be applied to the level of human *Leishmania* infection remains to be seen.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

All materials used in this study were purchased from Sigma-Aldrich, unless stated otherwise.

2.1.1 Culturing of *L. mexicana* promastigotes

L. mexicana MNYC/BZ/62/M379 promastigotes were grown in sterilised HOMEM medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (HI-FCS) and 50 μg/ml neomycin (Gibco-BRL) at 25°C. Parasite cultures (Tables 1 & 2) were grown until stationary phase which occurred 8-10 days after seeding with parasites to 1 x 10^5/ml. The parasites were considered to have reached stationary phase when the cell density, measured using an improved Neubauer haemocytometer, had dropped by 10 % from the previous day (Mallinson & Coombs, 1989). At this point, providing low sub-passage promastigotes were used, the culture contained a high level of metacyclic-like promastigotes (> 50 %), determined by the majority of promastigotes being motile (Mallinson & Coombs, 1986).

Genetic manipulation	**Denoted**	**Phenotype during *in vitro* and *in vivo* infection**
None	Wild-type (WT)	Infects macrophages *in vitro* and produces rapidly growing, non-healing lesions in BALB/c mice (Mottram *et al.*, 1996).
Deletion of *cpb* array	Δ*cpb* (N53 in cytokine data)	Reduced infectivity to macrophages *in vitro* by 80 % and produces slow-growing lesions in BALB/c mice (Mottram *et al.*, 1996).
Deletion of *cpb* array; re-integration of metacyclic CPB2 with native (metacyclic) promoter	GL165	Unknown
Deletion of *cpb* array; re-integration of amastigote CPB2.8 with native (amastigote) promoter	GL166	Unknown
Deletion of *cpb* array; re-integration of metacyclic CPB2 with chimaeric (amastigote) promoter	GL167	Unknown
Deletion of *cpb* array; re-integration of amastigote CPB2.8 with chimaeric (metacyclic) promoter	GL168	Unknown
Deletion of *cpb* array; re-expression of *cpb* array using a cosmid shuttle vector	GL263	Unknown

Table 1: Construction of *L. mexicana* single null parasite mutants

Genetic manipulation	Denoted	Phenotype during *in vitro* and *in vivo* infection
Deletion of *cpa* and *cpb* genes	Δ*cpb/cpa*	Rate of infectivity to macrophages *in vitro* is similar to Δ*cpb* (Mottram *et al.*, 1996) but did not produce lesions in mice (Alexander *et al.*, 1998).
Deletion of *cpa* and *cpb* genes; re-integration of metacyclic CPB2 with native promoter	GL438	Unknown
Deletion of *cpa* and *cpb* genes; re-integration of amastigote CPB2.8 with native promoter	GL439	Unknown

Table 2: Construction of *L .mexicana* double null CPB parasite mutants

2.1.2 Culturing of *L. mexicana* amastigotes

Growth *in vitro* of amastigote-like forms (axenic amastigotes) involved sub-passage in Schneider's *Drosophila* medium containing 20% (v/v) foetal calf serum at pH 5.5 and 32°C. The following antibiotics were added in combination, as appropriate, for maintenance of the drug-selectable markers in the CP-deficient mutants: hygromycin B at 50 μg/ml, phleomycin (Cayla, France) at 10 μg/ml, puromycin at 10 μg/ml or G418 (Geneticin) (Gibco-BRL) at 25 μg/ml. The amastigotes were washed three times in phosphate buffered saline (PBS) (0.07 M sodium chloride, 0.075 M disodium hydrogen orthophosphate and 0.05 M sodium dihydrogen orthophosphate, pH 7.4), before infection of mice.

2.2 IMMUNOLOGICAL MATERIALS

Lipopolysaccharide (LPS) (from *Salmonella abortus equi)* and IFN-γ were purchased from Sigma-Aldrich and Calbiochem, respectively. LPS was diluted with endotoxin-free water (Sigma-Aldrich) to make a stock solution of 2 mg/ml and stored frozen at -20°C. IFN-γ was also diluted with endotoxin-free water to yield a stock solution of 1000 U/ml and immediately aliquoted before storage at -20°C.

Recombinant IL-12 (a kind gift from Prof. Phil Scott, University of Pennsylvania) was diluted with sterile PBS to a stock solution of 2.5 μg/ml and aliquoted before storage at -20°C.

2.3 BIOCHEMICAL METHODS

2.3.1 Media composition for growth of bacterial cultures

Luria-Bertani (LB) medium was used to culture *E. coli* M15pREP4 expression strain transformed with pGL180 (pGL180 encodes a N-terminally His_6-tagged pro-form of CPB2.8 lacking the C-terminal extension (His_6-CPB2.8ΔCTE)). 10 g sodium chloride, 5 g yeast extract (Difco) and 10 g bacto-tryptone (Oxoid) were dissolved in 1 litre of double-distilled, deionised water ($dddH_2O$) and the pH adjusted to 7.2. The medium was then autoclaved at 126°C for 30 minutes and allowed to cool before inoculation with the *E. coli* strain detailed above.

2.3.2 Determination of protein concentration

Protein concentration was determined using the BioRad Protein assay kit, based on the method of Bradford (1976). Bovine gamma globulin was used as the relative

protein standard. For each set of samples, a standard curve was constructed using 0.05 - 0.5 mg/ml protein, and absorbances read at 620 nm.

2.3.3 Dialysis of protein samples

Visking tubing was boiled in 10 mM sodium bicarbonate pH 8.0, 1 mM EDTA for 15 min to remove chemical contaminants. It was then rinsed and boiled in $dddH_2O$ for a further 10 min, before storage in 100 % (v/v) ethanol. The tubing was rinsed thoroughly in $dddH_2O$ before dialysis was carried out at 4°C, overnight, at a minimum ratio of 1:100 (sample: dialysis buffer). The composition of the dialysis buffer depended on the next stage of the experiment; each buffer will be described in the relevant results chapters.

2.3.4 Protein sample preparation for PAGE

Samples were prepared by 1:1 dilution with Laemmli sample buffer (125 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.2% (w/v) Pyronin Y). Samples were then boiled for 5 min unless the samples were to be analysed for proteolytic activity using gelatin SDS-PAGE.

2.3.5 Concentration of protein samples for SDS-PAGE

For SDS-PAGE involving dilute protein samples, or those which contained potentially interfering material (e.g. guanidine hydrochloride), trichloroacetic acid (TCA) precipitation was carried out (Hames, 1990). Typically, samples were diluted to 100 µl before adding 100 µl of 10% (w/v) TCA and vortexing. The samples were then frozen at -20°C for 1 hour before centrifuging at 10,000 g in a bench top centrifuge at room temperature for 15 min. The supernatant was removed and the

pellet washed with 100 µl of ice cold acetone to remove any precipitated salt. The pellet was left to dry before resuspending in Laemmli sample buffer.

2.3.6 SDS-PAGE

L. mexicana native and recombinant proteins were evaluated using the discontinuous SDS-PAGE system as described by Laemmli (1970). 10% (w/v) mini acrylamide resolving gels (0.75 mm thickness), were cast, loaded and run using the Bio-Rad Mini-Protean II dual slab cell system. Gibco Benchmark Protein Ladder molecular weight markers were routinely run on polyacrylamide gels so that estimates of protein molecular weights could be made. Following electrophoresis, gels were stained overnight in Coomassie Blue stain (0.1% (w/v) Coomassie Blue R-250, 7% (v/v) acetic acid, 40% (v/v) methanol) and then de-stained with 7% (v/v) acetic acid and 40% (v/v) methanol until the background of the gel was clear.

2.3.7 Analysis of proteinase activity using gelatin SDS-PAGE

Gels were prepared as above except with co-polymerisation of 0.2% (w/v) gelatin (from porcine skin) in the resolving gel as a substrate for proteinases. After electrophoresis, the gels were immersed for 30 min in 30 ml of 2.5% (v/v) Triton X-100, at 37°C, to remove SDS and allow proteolytic activity to be restored. Proteinase activity was developed by washing the gels for 30 seconds in tap H_2O and then immersing the gels in incubation buffer (0.1 M sodium acetate, pH 5.5 containing 1 mM dithiothreitol (DTT)) for 2 hours at 37°C. The bands were visualised by subsequent staining in Coomassie Blue stain for 1 hour before de-staining as detailed in section 2.3.6.

2.3.8 Analysis of proteinase activity using fluorogenic substrates

Proteolytic activity of CPs was assessed by running standard SDS-PAGE gels as above and after washing in $dddH_2O$, incubating the gels in 0.1 M sodium acetate, pH 5.5, containing 1 mM DTT and a fluorogenic substrate, Suc-Leu-Tyr- (Suc-LY) and/or Bz-Phe-Val-Arg- (BzFVR) amino 4-methyl coumarin at a final concentration of 25 μM. The incubation buffer was warmed to 37°C before addition of the substrate and gels were shaken for 10 min before the activity bands were visualised using a UV transilluminator (UVP Inc.).

2.3.9 Immunoblotting

Proteins, after resolution by SDS-PAGE, were transferred to and immobilised on a matrix for subsequent immunological detection (Towbin *et al.*, 1979). Proteins were transferred on to 0.45 μm polyvinylidene difluoride (PVDF) membranes at 100 V for 45 minutes using Bio-Rad Trans-Blot electrophoretic transfer apparatus in transfer buffer (25 mM Tris, 190 mM glycine, pH 8.2, 0.02% (w/v) SDS, 20% (v/v) methanol). The non-fixative stain, Ponceau S, was used to determine efficiency of transfer to the nitrocellulose and to allow identification of molecular weight markers.

After transfer, the membrane was blocked with wash buffer (0.1% Tween 20 (v/v), 0.1% (w/v) gelatin, 20 mM Tris/HCl, pH 7.6, containing 137 mM NaCl) plus 5% (w/v) non-fat milk (Marvel) (designated blocking buffer) overnight at 4°C. The overnight blocking buffer was decanted and the membrane was incubated for 4 hours at 4°C in anti-serum relevant to the particular experiment, at the dilutions given in Table 3.

Sample being analysed	**Primary Ab dilution**	**Source of primary Ab**	**Secondary Ab-HRP dilution**
CPB2.8ΔCTE	1:2000	rabbit anti-CPB2.8ΔCTE (enzyme was purified from Ni^{2+}-agarose column)	1:2000 (purchased from SAPU, Carluke)
L. mexicana Wild Type	1:5000	Serum from *L. mexicana*-infected mice	1:10,000
L. infantum Wild Type	1:2000	Serum from *L. infantum*-infected dogs	1:50,000

Table 3: Dilutions of anti-sera and secondary antibodies used for Western blotting

The membrane was washed three times for 10 min with wash buffer, and then incubated in the appropriate secondary antibody conjugated to horseradish peroxidase (HRP), at the given dilution, for 1 hour at room temperature. Following a further wash step, the membrane was developed using enhanced chemiluminescence western blotting reagents as described by the manufacturer (Amersham Pharmacia).

2.3.10 Enzyme assay using a peptidyl p-nitroanilide substrate

Proteinase activity was quantitated by measuring hydrolysis of the peptidyl-p-nitroanilide substrate, Bz-Pro-Phe-Arg Nan (BzPFR-Nan) (0.1 mM),

spectrophotometrically at 37°C in 0.1 M sodium phosphate buffer, pH 6.0, containing 10 mM DTT. The molar extinction coefficient of 4-nitroaniline was taken to be 9500 $M^{-1}cm^{-1}$ at 405 nm. 5 µl of enzyme solution was added to 95 µl of buffer, incubated at 37°C for 10 min and absorbance was read at 405 nm. The activity of the enzyme was then calculated according to the following equation,

$$\frac{A_\Delta/10\ \text{min}}{9.5 \times 10^3} \times \frac{1000}{\text{vol. assayed in µl}} \times \frac{1}{100} = \text{activity of enzyme in mmol.min.ml}^{-1}$$

where A_Δ/10 min = change in absorbance over 10 min at 405 nm,

9.5×10^3 = molar extinction coefficient of 4-nitroaniline.

2.3.11 Active site titration of CPB2.8ΔCTE

The method of Barrett and Kirschke (1981) was used for active site titration using *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64). Briefly, pure, dry E-64 was dissolved in $dddH_2O$ to give a stock solution of 1 mM and stored at -20°C. Working solutions of 1-10 µM concentration were prepared as required. 25 µl of enzyme solution (CPB2.8ΔCTE in PBS, pH 6.0) was added to 25 µl of 1-10 µM of E-64 and incubated at 37°C for 30 min, with gentle agitation. 10 µl of this reaction mixture was added to the buffer solution containing substrate, BzPFR-Nan. A linear plot of activity against E-64 molarity reaches zero activity at the molarity of the enzyme solution. Some of the inhibited enzyme was also tested for CP activity by gelatin SDS-PAGE. Excess E-64 was removed by overnight dialysis against PBS, pH 6.0, at 4°C.

2.4 OTHER METHODS

Details of other methods are given in Chapters 3-6 as appropriate.

CHAPTER 3

Purification and activation of recombinant CPB2.8ΔCTE expressed in *Escherichia coli*

3.1 INTRODUCTION

The difficulty of obtaining quantities of pure enzyme from the relatively small amounts of parasite material that are easily available has limited research in the past but recent advances in molecular technology mean that it is now possible to express cloned parasite genes in heterologous systems such as *Escherichia coli*. This provides for a source of easily purifiable protein which can be utilised for kinetic and immunological studies, X-ray crystallography and general characterisation with regard to the protein's structure and function. There are several general considerations when embarking on the production of recombinant proteins.

3.1.1 Inclusion bodies

One frequent outcome of the expression of recombinant genes in *E. coli* is the accumulation of insoluble aggregated protein in inclusion bodies (Chaudhuri, 1998). This phenomenon occurs because although the recombinant enzyme possesses the correct amino acid sequence, it may not fold into the native structure. Moreover, the natural conformation of the recombinant enzyme may require modification by proteinases or glycosyl transferases, which are lacking in *E. coli* but present in other

expression systems such as yeast expression systems, which are eukaryotic (Chaudhuri, 1998).

Fermentation temperature is a major factor affecting formation of inclusion bodies; growth at a lower temperature may result in soluble protein rather than aggregation into an insoluble complex (Cardamone *et al.*, 1995). Other important factors include the way in which stimulation of expression of recombinant protein is carried out such as choice of medium for bacterial growth and subsequent protein production (Chaudhuri, 1998). The over-expressed protein is the predominant constituent of inclusion bodies and may accumulate in these refractile bodies at levels of greater than 25% of total cell protein.

The production of recombinant proteins as inclusion bodies, although frequently considered a disadvantage, has a number of advantages; increased levels of protein can accumulate and it may facilitate the isolation of proteinase-sensitive proteins since inclusion bodies are resistant to proteinases (Marston, 1986). The main disadvantage is that the protein requires refolding into its native state. This can be difficult to achieve satisfactorily so that overall productivity of active protein can be poor (Chaudhuri, 1998).

Recovery of the desired product requires the inclusion bodies to be solubilised and the protein denatured in chaotropic solvents such as concentrated urea or guanidine hydrochloride (Marston, 1986). During inclusion body formation, it is very likely that incorrect disulphide bonds have been formed and these must be disrupted using reducing agents such as dithiothreitol or β-mercaptoethanol, usually in the presence of EDTA (Chaudhuri, 1998). By maintaining these conditions in the

refolding buffer, formation of incorrect disulphide bridges is minimised. Refolding is stimulated by removal of denaturant, for example by dialysis (Cardamone *et al.*, 1995). This can lead to the recovery of a correctly folded and biologically active protein product but the yield varies considerably with the protein being used.

3.1.2 Washing of inclusion bodies

It is necessary to recover the inclusion bodies away from the soluble cell components and other cell contaminants. Recovery of recombinant protein during renaturation, has been reported to be much higher when carried out with purer protein preparations, since contaminants such as components of bacterial cell walls can interfere with protein folding (Cardamone *et al.*, 1995). Analysis of inclusion bodies of *E. coli* has indicated that all contain some contaminants in common, irrespective of the cloned gene, promoter, plasmid vector, fermentation regimen or media composition used (Hartley & Kane, 1988). These contaminants include subunits of RNA polymerase, plasmid DNA, a combination of outer membrane proteins and more importantly, bacterial proteinases (Hartley & Kane, 1988).

The inclusion bodies can be washed to aid recovery of the desired protein. The addition of Triton X-100 and β-octyl glucoside removes contaminating proteinase activity and so this should prevent any degradation of the recombinant proteins during inclusion body extraction (Babbit *et al.*, 1990). The addition of low concentrations of urea buffer solubilises *E. coli* membrane proteins in the presence of EDTA, Tris/HCl and Triton X-100 but does not solubilise the inclusion bodies. This

leaves the recombinant protein intact while potential membrane contaminants are removed.

When the recombinant enzyme is to be used for immunological studies, the removal of lipopolysaccaride (LPS) is essential and an important aim of purification. However, the use of specific chromatographic steps coupled with the use of pyrogen-free water and maintenance of equipment in a pyrogen-free state usually reduces the level of LPS to an acceptably low level (Marston, 1986).

3.1.3 Renaturation of the recombinant protein

There are several theories which explain the process of protein refolding and one such model suggests that refolding follows a hierarchical process in which simple structures are formed first, giving rise to a stable framework of secondary structures (Cardamone *et al.*, 1995). Secondary structure of proteins is formed at an early stage in the folding process providing a scaffolding on which the remaining parts of the polypeptide chain can arrange themselves into a dense and low solvent-accessible surface area (Jaenicke, 1991). Tertiary structure is then assembled by eliminating water from the hydrophobic core as a consequence of salt linkages occurring between acidic and basic residues, hydrophobic aggregation of the aliphatic and aromatic amino acids, and hydrogen bonding between polar and charged amino acids. As a rule, the 3-D structure of a protein is fully determined by its amino acid sequence and the solvent environment; it occupies the state of minimum energy while disulphide bonds stabilise rather than determine the spatial arrangement of the polypeptide backbone (Jaenicke, 1991).

The formation of protein disulphides can proceed either by enzymatic catalysis or by uncatalysed thiol-disulphide exchange (Wetlaufer, 1984). The non-enzymatic system employs thiol-disulphide exchange reactions between proteins and low molecular weight thiols and/or disulphides e.g. glutathione (Creighton, 1984). Methods for renaturation are generally very slow, give low yields and are protein-specific. Effective renaturation requires the removal of the denaturing buffer under conditions where the major fraction of the desired protein can refold into the active, native conformation. Chelating agents such as EDTA are also added to remove trace metals capable of catalysing oxidation and to prevent side reactions that may lead to the eventual irreversible covalent inactivation of the protein (Cardamone *et al.*, 1995). Temperature must also be controlled, as temperatures greater than 25°C can encourage aggregation.

Protein concentration is extremely important and dilution of the protein must be such that intramolecular interactions occur in preference to intermolecular interactions. Reduced proteins have a strong tendency to aggregate thus oxidative renaturation must be carried out in low protein concentrations (Wetlaufer, 1984). With protein concentration in mind, it is important to consider that the refolding process has a parallel reaction. The folding intermediates have a tendency to aggregate rather than to refold as the exposed hydrophobic patches interact (Chaudhuri, 1998). Thus, renaturing protein molecules in isolation from each other reduces aggregation and this is the principle of dilution facilitating effective renaturation.

3.1.4 Renaturation of CPs

The probability of polypeptide chains possessing many cysteine residues folding properly is very low and so obtaining the correct configuration constitutes a significant challenge in recovering the native conformation of the protein from the solubilised inclusion bodies (Kuhelj *et al.*, 1995). There is a complication to successful refolding of CPs in that, by definition, they have at least one free cysteine residue but also disulphide bridges (Taylor *et al.*, 1992). It has been found that any refolding regime that produces activatable pro-papain or papain must produce three disulphide bridges from the correct pairs of cysteines and leave the active site cysteine in a non-oxidised form able to take part in catalysis (Taylor *et al.*, 1992).

3.1.5 Aims

The production of pure, active, recombinant CPB2.8ΔCTE was the main objective of the work reported in this chapter but the system was also to be optimised for purification of other leishmanial CPs including CPA and CPC of *L. mexicana*. Purification of an *L. infantum* CPB with the full C-terminal domain was also an aim.

3.2 MATERIALS AND METHODS

3.2.1 Growth of large-scale expression cultures of *E. coli*

10 ml of Luria-Bertani broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin was inoculated with a single colony of *E. coli* M15pREP4 containing the expression plasmid pQE-30 and grown at 37°C overnight with vigorous shaking.

200 ml of LB-broth (100 μg/ml ampicillin and 25 μg/ml kanamycin) was then inoculated 1:20 with the overnight culture and grown at 37°C with vigorous shaking until the A_{600} reached 0.7 - 0.9.

IPTG was then added to a final concentration of 0.5 mM and growth of the culture continued for 4-5 hours. The cells were harvested by centrifugation at 4000 g for 10 min and the cell pellet stored at -20°C until further use.

3.2.2 Isolation and solubilisation of inclusion bodies

The bacterial pellet was re-suspended in 10 ml 50 mM Tris/HCl, pH 8.0, containing 5 mM EDTA (designated wash buffer) and 5% (w/v) sucrose. The suspension was frozen and thawed twice and subjected to sonication (6 x 30 seconds, 30 W, Jencons High Intensity Ultraprocessor). After centrifugation at 6000 g for 10 min, the inclusion body pellet was washed once in wash buffer containing 0.1% Triton X-100 and twice in wash buffer containing 2 M urea. The pellet was finally re-suspended in 10 ml dddH_2O before centrifugation once more at 6000 g for 10 min to obtain the purified inclusion bodies. These were solubilised directly in 8 M urea, 0.1 M Tris/HCl, pH 8.0, 10 mM DTT at 37°C with vigorous shaking for 1 hour followed by centrifugation at 6000 g for 10 min to remove any material that had been incompletely solubilised. The clarified supernatant was used as the source of recombinant enzyme to be renatured.

3.2.3 Refolding of CPB2.8ΔCTE

Fully denatured and reduced CPB2.8ΔCTE was diluted with 8 M urea, 0.1 M Tris/HCl, pH 8.0, 5 mM EDTA to a final protein concentration of 0.1 - 0.5 mg/ml. Dialysis of the resultant solution was then carried out for 15 hours against 100 vol. of a suitable buffer. In the experiment where the effect of redox reagents was studied, 0.1 M Tris/HCl, pH 7.0, 5 mM EDTA and various combinations of cystine/cysteine and glutathione (reduced and oxidised) were used as the buffer. The influence of pH on refolding was assessed by using 0.1 M Tris/HCl (pH 7-9), 0.1 M sodium acetate pH 6.0 and 0.1 M histidine buffer, pH 6.0; all solutions contained 5 mM EDTA and 5 mM cysteine.

In all cases, the redox reagent(s) were removed subsequently by dialysis for 2 hours against 100 vol. of 20 mM Tris/HCl, pH 7.0, 5 mM EDTA prior to further purification by ion exchange chromatography.

3.2.4 Ion exchange chromatography of CPB2.8ΔCTE

After refolding of CPB2.8ΔCTE, the enzyme solution was transferred to polypropylene tubes and centrifuged at 6000 g for 10 min to remove any precipitated protein. The supernatant was then filtered through a 0.22 μm syringe filter (Gelman Sciences) before the sample was applied to a Mono Q HR 5/5 anion exchange column (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 7.0, 5 mM EDTA, at a flow rate of 1 ml/min. After washing the column with equilibration buffer, protein was eluted with a 0-1 M NaCl gradient in the equilibration buffer. The fractions

containing enzyme activity towards gelatin and BzPFR-Nan were pooled and stored at 4°C overnight, for activation to the fully active form.

3.2.5 Activation/processing of CPB2.8ΔCTE

The pooled fractions were assayed for protein content and diluted accordingly with activation buffer to 0.1 - 0.5 mg/ml before activation. The activation protocol was based on suggestions made for activation of cruzipain by Prof. J. H. McKerrow (University of California, USA). Activation buffer consisted of 0.9 M NaCl, 0.1 M sodium acetate, 2 mM EDTA and 10 mM DTT, pH 5.0. The pooled ion exchange fractions were added to the activation buffer, before activation of the enzyme was left to proceed at 37°C for 8 hours, while gently agitating on a shaker. At 4 hours, 10 mM DTT was added to ensure that the enzyme was reduced during the processing step. After activation was complete, the sample was again centrifuged at 6000 g for 10 min to remove any precipitated protein.

The volume of the enzyme solution (40-60 ml) was then reduced by transferring the activated enzyme solution to dialysis tubing. PEG 8000 flakes were added to the outside of dialysis membrane containing the activated enzyme solution and were left for several hours at 4°C, to allow concentration of the enzyme to a volume of approximately 4 ml. This sample was then further concentrated and desalted using a 10K Amicon Centricon system involving several 20-30 min centrifugation steps at 4000 g, at 4°C.

3.2.6 Purification of CPB2.8ΔCTE using nickel-agarose affinity chromatography

The pellet from a 500 ml culture of *E. coli* expressing CPB2.8ΔCTE was frozen and thawed before being re-suspended in 6 M guanidine hydrochloride, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0 (Buffer A) at 5 ml per gram wet weight of cells. The cells were stirred at room temperature for 1 hour and centrifuged at 6000 g for 15 min at 4°C. The supernatant, containing solubilised inclusion bodies was loaded on to a 4 ml nickel-nitriloacetic acid (Ni^{2+}-NTA) column, which had previously been equilibrated in Buffer A, at a flow rate of 10-15 ml/hour.

The column was then washed with 10 column volumes of buffer A and 5 column volumes of buffer B (see Table 4). Buffers C - E were used to elute the recombinant protein and a final washing step was employed to remove any protein that had not been eluted using buffer F (Table 4).

Buffer	Components
A	6 M guanidine hydrochloride, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0
B	8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0
C	Buffer B at pH 6.5
D	Buffer B at pH 5.9
E	Buffer B at pH 4.5
F	6 M guanidine hydrochloride and 0.2 M acetic acid, pH 4.2

Table 4: Buffers used for nickel-agarose purification of CPB2.8ΔCTE

Fractions containing purified protein were pooled and stored on ice before immediate dialysis into refolding buffer.

3.2.7 Generation of polyclonal anti-serum to pro-CPB2.8ΔCTE

Polyclonal anti-serum raised in New Zealand White rabbits against his-tagged pro-form of CPB2.8ΔCTE was carried out in the animal facility at the University of Glasgow. 300 μg of nickel-agarose-purified enzyme (buffer E, Table 4) was dialysed into PBS, pH 7.0, and homogenised with Freund's Complete Adjuvant (FCA) for the first inoculation. 200 μg of enzyme was homogenised with Freund's Incomplete Adjuvant (FIA) for the three subsequent boosts. Anti-serum was collected five days after the final boost.

3.3 RESULTS

3.3.1 Over-expression of CPB2.8ΔCTE in *E. coli*

CPB2.8ΔCTE was over-expressed in *E. coli* as a pro-protein without the C-terminal extension or pre-region but with a poly-histidine tag at the N-terminus (Sanderson *et al.,* 2000). The C-terminus is not essential for activation or correct intracellular trafficking of CPBs and so is not required for production of the recombinant, mature enzyme (Mottram *et al.,* 1996). Indeed inclusion of the C-terminal domain may cause complications in the refolding process of the recombinant enzyme since the C-terminal extension contains a predominance of hydrophobic amino acid residues which theoretically increases the chances of aggregation rather than renaturation during the refolding process. Attempts at purifying an *L. infantum* CPB with its C-terminal extension confirmed this, since the solubilised protein had to be diluted even further before dialysis compared with the dilutions used for CPB2.8ΔCTE.

The poly-histidine tag allows for recombinant proteins such as CPB2.8ΔCTE to be bound to a nickel-agarose affinity column and then eluted by lowering of the pH of the column buffer - this results in the positively charged histidine side chain being incapable of binding to the nickel ions. This technique has been used for purification of several other parasite enzymes including an aldolase from *P. falciparum* (Dobeli *et al.,* 1990), and a methionine γ-lyase from *Trichomonas vaginalis* (McKie *et al.,* 1998). These studies exemplified the potential of this approach for obtaining parasite proteins for study.

3.3.2 Time of culture growth before induction

Escherichia coli M15pREP4 incorporating the plasmid pQE-30 containing CPB2.8ΔCTE was grown to an A_{600} of 0.63, 0.91 or 1.21 before induction with 1.0 mM IPTG for 4 hours. The amount of CPB2.8ΔCTE produced in each case was very similar (Figure 3.3.1). When assays were performed with the *p*-nitroanilide substrate, BzPFR-Nan, it was found that cells grown to an A_{600} of 0.91 before induction yielded more proteinase activity after dialysis of the solubilised inclusion bodies (4.0 nmoles.min.mg. protein^{-1}) compared with cells grown to an A_{600} of 0.61 or 1.22 (2.7 and 3.3 nmoles.min.mg.protein^{-1} respectively).

Bacterial cells in batch culture tend to grow in a sigmoidian fashion with log phase representing the point between an A_{600} of 0.6 - 0.9 and higher values probably indicative of stationary phase. During log phase, cells are most productive and have an abundant source of nutrients. One complication of high-density growth and expression with *E. coli* when glucose is the carbon source, is the accumulation of acetate to toxic concentrations. Acetate can begin to inhibit protein production by being assimilated when glucose sufficiently limits growth. Since acetate is taken up in its protonated form, the resulting pH rise may disrupt metabolism. This is difficult to avoid in batch fermentation (Swartz, 1996). However, the choice of LB medium prevents such an increase in acetate concentration as glucose is not the carbon source.

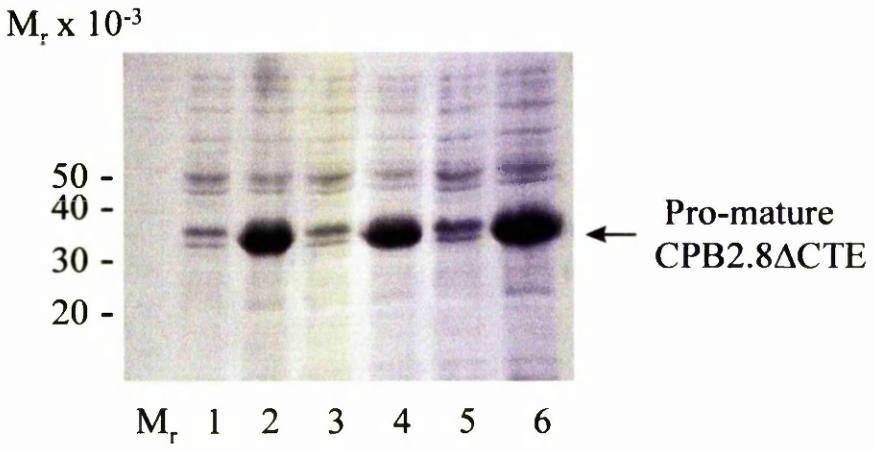

Figure 3.3.1 10 % SDS-PAGE stained with Coomassie Blue showing variation of pre-induction time and production of CPB2.8ΔCTE

Lane 1, *E. coli* at A_{600} of 0.63;

lane 3, *E. coli* at A_{600} of 0.91;

lane 5, *E. coli* at A_{600} of 1.21;

lanes 2, 4 and 6, 4 h post-induction.

3.3.3 Time of culture growth after induction

After the culture had been grown to an A_{600} of 0.8, the cells were induced with 1.0 mM IPTG for 1, 2, 3, 4 or 5 hours and expression of the recombinant protein was monitored. The maximal amount of protein was produced after 4 to 5 hours induction (Figure 3.3.2.). The enzyme activity, however, was not increased in parallel. This could be visualised using gelatin SDS-PAGE (Figure 3.3.3). There was a decrease in activity towards gelatin as more enzyme was over-expressed, suggesting that it was sequestered as an inactive form within inclusion bodies. For purification purposes, the accumulation of CPB2.8ΔCTE within inclusion bodies is far from ideal, due to problems with refolding. However, it was easier to obtain a higher yield of over-expressed enzyme using this method and the risk of proteolysis was reduced. Thus 4 hours was chosen as the standard for subsequent work.

3.3.4 IPTG concentration

Production of CPB2.8ΔCTE utilising IPTG at final concentrations of 0.1, 0.5 and 1.0 mM resulted in similar amounts of recombinant protein as assessed by SDS-PAGE (Figure 3.3.4). The inclusion body phase was isolated from cells that had been induced with these concentrations of IPTG. Dialysed inclusion body samples from each concentration of IPTG were analysed by gelatin SDS-PAGE (Figure 3.3.5) and BzPFR-Nan. Cells induced with 1.0 mM IPTG appeared to contain more active enzyme as assessed by gelatin SDS-PAGE, but activity towards BzPFR-Nan was very similar although cells induced with 0.1 mM IPTG gave slightly less activity than

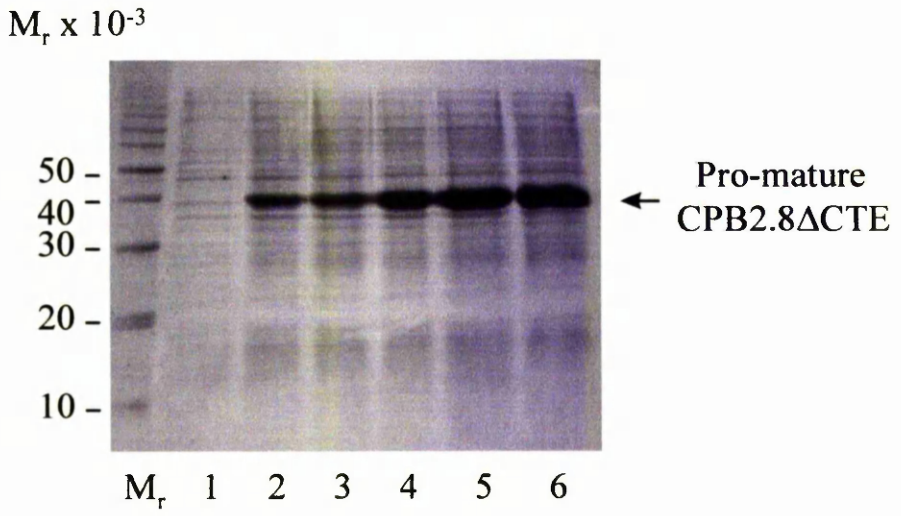

Figure 3.3.2 Coomassie-stained 12 % SDS-PAGE showing over-expression of CPB2.8ΔCTE in *E. coli.* Cells were induced with 1.0 mM IPTG and, at hourly time points, 1 ml aliquots of culture were centrifuged at 10000 g for 15 min and the cell pellet re-suspended in 100 µl of sample buffer. Lanes were loaded with equivalent volumes. Lane 1, *E. coli* pre-induction; lane 2, 1 h post-induction; lanes 3-6 represent post-induction at 2, 3, 4 and 5 h respectively.

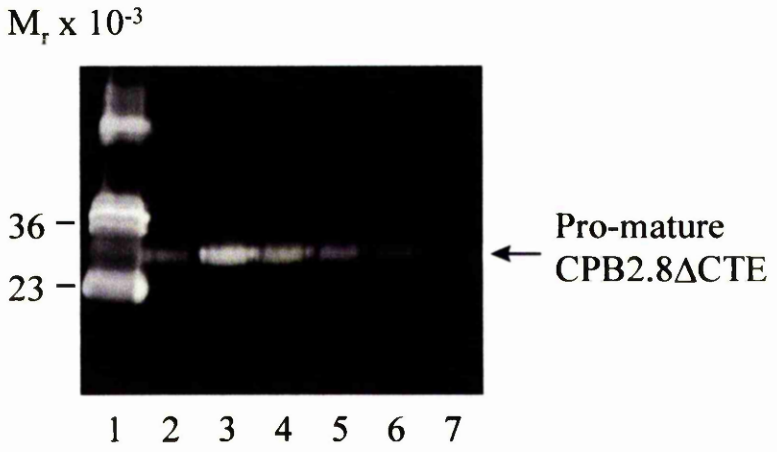

Figure 3.3.3 Coomassie-stained 10 % gelatin SDS-PAGE gel of over-expressed CPB2.8ΔCTE in *E. coli*.

Lane 1, lysate of stationary phase promastigote *cpb* null mutant *L. mexicana*, re-expressing gene 2.8; lane 2, *E. coli* pre-induction; lanes 3-7 represent samples taken 1-5 h post-induction, respectively.

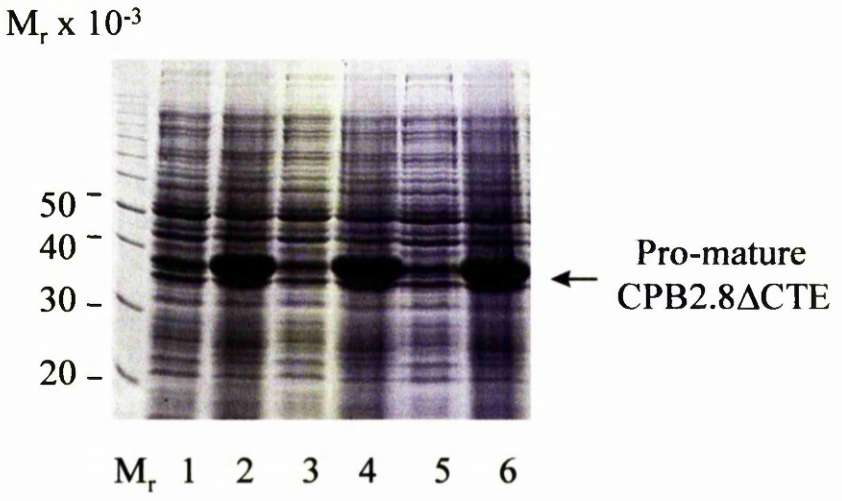

Figure 3.3.4 Coomassie-stained 10% SDS-PAGE showing induction of CPB2.8ΔCTE with varying concentrations of IPTG.

Lanes 1, 3 and 5, pre-induced *E. coli*;

lane 2, *E. coli* 4 h post-induction with 0.1 mM IPTG;

lane 4, *E. coli* 4 h post-induction with 0.5 mM IPTG;

lane 6, *E. coli* 4 h post-induction with 1.0 mM IPTG.

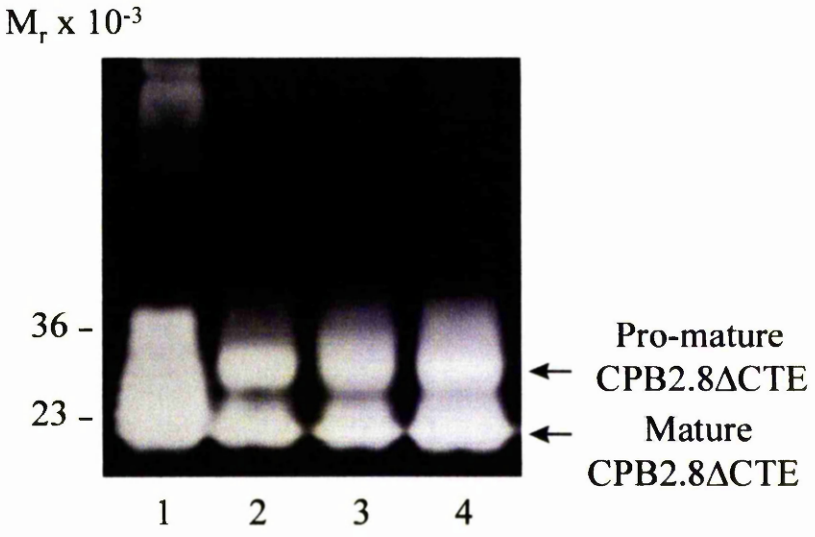

Figure 3.3.5 10 % gelatin SDS-PAGE comparing activity from cultures induced with different concentrations of IPTG. The inclusion body phase was isolated, solubilised and dialysed overnight. The gel shows activity from the post-dialysis sample.
Lane 1, wild-type, stationary phase, promastigote parasite lysate;
lane 2, cells induced with 0.1 mM IPTG;
lane 3, cells induced with 0.5 mM IPTG;
lane 4, cells induced with 1.0 mM IPTG.

those cells induced with 0.5 and 1.0 mM IPTG (data not shown). A concentration of 0.5 mM was used routinely for all subsequent recombinant protein expression.

3.3.5 Washing of inclusion bodies

Inclusion body-derived CPB2.8ΔCTE was purified away from the intrinsic factors associated with inclusion bodies prior to solubilisation and refolding. Since inclusion bodies are slightly denser than the cell debris, they can be separated by differential centrifugation. Contaminating proteins were removed by washing not only in sucrose but also in moderate chaotrope, 2 M urea, which is known to solubilise contaminants but leave the inclusion bodies insoluble. Proteinases are also removed in this way, which is beneficial as they could degrade the recombinant product. The effects of the washes were assessed by SDS-PAGE (Figure 3.3.6a) and this confirmed that there was a minimal loss of the recombinant protein in the wash solutions during the isolation of the inclusion bodies. However, there was removal of contaminating proteins which may interfere with refolding. Western blot analysis (Figure 3.3.6b) confirmed that there was a minimal loss in recovery of CPB2.8ΔCTE during the washing procedure.

3.3.6 Solubilisation of inclusion bodies

Disulphide bonds may form in an oxidising microenvironment within the inclusion bodies. If intramolecular or intermolecular disulphide bonds in the recombinant protein in inclusion bodies are incorrect, then disruption of these is an essential component in the recovery of the correctly folded, active protein. The

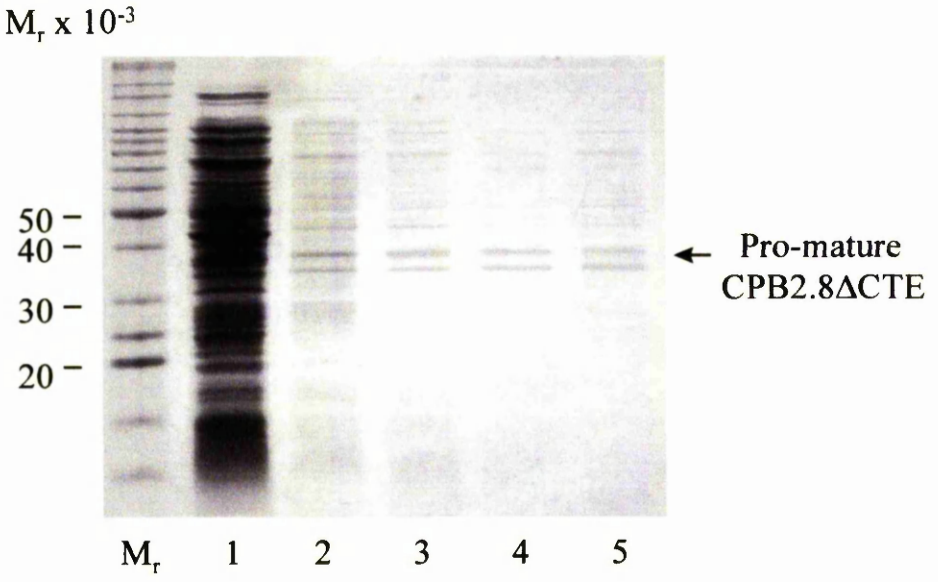

Figure 3.3.6a Coomassie-stained 12 % SDS-PAGE to monitor solubilisation of incluson bodies during washing stages.
Lane 1, sucrose wash; lane 2, Triton X-100 wash; lanes 3 and 4, 2 M urea washes; lane 5, H_2O wash. All lanes were loaded with an equal volume of supernatant from the wash steps.

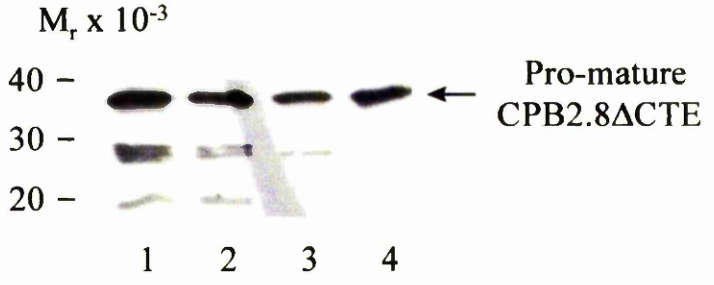

Figure 3.3.6b Western blot monitoring solubilisation of inclusion bodies during washing stages. The blot was probed with antibody to pro-mature CPB2.8ΔCTE.
Lane 1, sucrose wash; lane 2, Triton X-100 wash; lane 3, second 2 M urea wash; lane 4, H_2O wash. All lanes were loaded with an equal volume of inclusion body pellet from the wash steps.

addition of DTT allows reduction of interchain disulphide bonds by thiol-disulphide exchange and increased solubilisation of aggregated protein. Since the reaction is favoured by an alkaline pH, Tris buffer is the preferred option.

Recovery of active, mature enzyme was compared between inclusion bodies solubilised in the presence of DTT and without by assessing activity to gelatin gels (Figure 3.3.7). As can be seen from Figure 3.3.7 (compare lanes 2 in A and B), there was no difference between gelatinase activity of the second dialysate, whether or not DTT was omitted during the solubilisation of the inclusion bodies. However, there was clearly a difference in the activity profile of the eluant from the ion exchange chromatography step, as active enzyme was eluted more quickly, when no DTT was used to solubilise inclusion bodies (Figure 3.3.7). Furthermore, when active fractions from each run were pooled and activated, the inclusion bodies solubilised with DTT resulted in mature enzyme with a higher specific activity (18 nmoles.min.mg.protein^{-1}) than enzyme from inclusion bodies not solubilised with DTT (13 nmoles.min.mg.protein^{-1}). The solubilisation conditions of Kuhelj *et al.* (1995), which includes solubilisation of inclusion bodies with 10 mM DTT, were therefore deemed the most efficient in solubilisation of CPB2.8ΔCTE.

3.3.7 Refolding of CPB2.8ΔCTE

A study of the optimum conditions for maximal recovery of enzyme activity and protein yield was carried out for the refolding step.

Renaturation, that is refolding and re-oxidation, of the protein was performed by slowly removing the denaturing agent by dialysis in glass vessels, since refolding

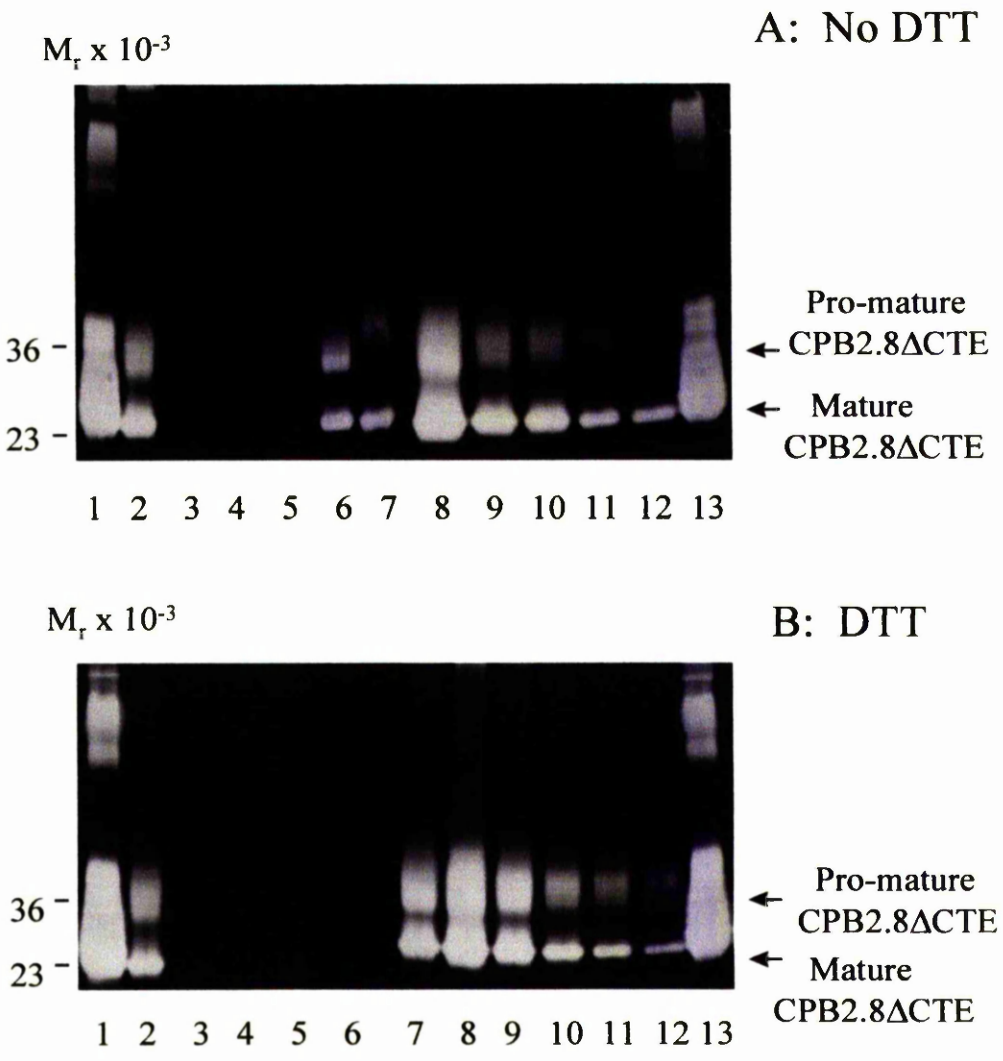

Figure 3.3.7. 10% gelatin SDS-PAGE analysis of eluants from ion exchange chromatography of dialysed inclusion bodies solubilised with or without DTT. Lanes 1 and 13, wild type, stationary phase promastigote parasite lysate; lane 2, load (2nd dialysate); lane 3, flow-through; lane 4, wash (pre-gradient); lanes 5-13 represent fractions eluted from the Mono Q column with a 0-1 M NaCl gradient.

experiments carried out in plastic containers have given poor yields in some instances (Creighton, 1984). I tested to see if this was important with CPB2.8ΔCTE. There was no difference in activity of the refolded enzyme (data not shown) but after subsequent freeze-thaw of the refolded samples there was a marked increase in protein precipitate from the sample dialysed in plastic containers with no visible change in the enzyme dialysed in the glass containers. Thus, glass containers were used routinely for subsequent experiments.

pH 7 was used by Kuhelj *et al.* (1995) to isolate pro-cathepsin B but it has been reported that higher pH values may facilitate oxido-shuffling, the process whereby refolding occurs. Most common alkyl thiols have proton ionisation pKa values in the range of 8-10 and disulphide exchange reactions can therefore proceed rapidly under mildly alkaline conditions or neutral conditions (Wetlaufer, 1984). This process results in correct exchange of disulphide bonds and therefore refolding of the enzyme to an active state.

Altering the pH of the dialysis buffers from pH 7 was attempted to increase enzyme activity and protein recovery. Since CPB2.8ΔCTE is possibly activated in the lysosomes of the parasite *in vivo*, it was hypothesised that lowering the pH of the dialysis buffer from pH 7 to pH 6 would increase conversion of the pro-mature form to the active, mature state. However, the different buffers used (sodium acetate, pH 6.0; histidine buffer, pH 6.0) did not improve yield nor activity of enzyme and indeed an increase in protein precipitation occurred during the dialysis step. When the solubilised inclusion bodies were dialysed at pH 6 rather than pH 7, there was an apparent loss in activity towards gelatin (Figure 3.3.8, lane 2). However, since there

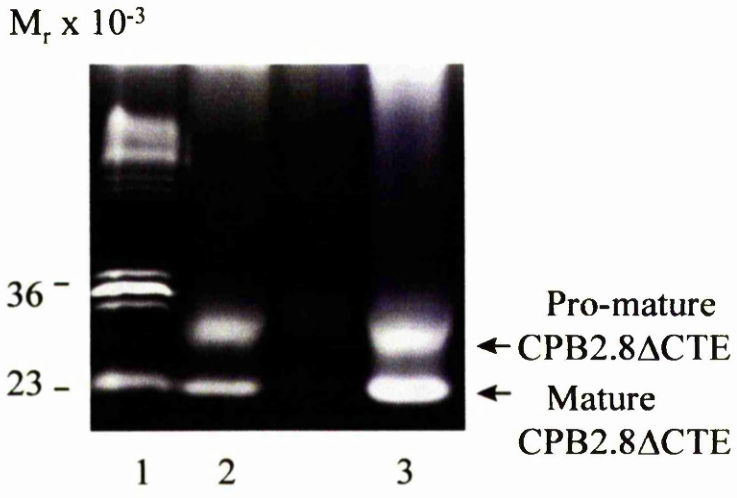

Figure 3.3.8 10 % gelatin SDS-PAGE comparing pH 6 and pH 7 during dialysis of solubilised inclusion bodies. Lane 1, stationary phase, promastigote parasite lysate (*cpb* null re-expressing gene 2.8); lane 2, inclusion bodies dialysed at pH 6; lane 3, inclusion bodies dialysed at pH 7.0. Lanes 2 and 4 were loaded with equal volumes of supernatant from the centrifuged dialysate.

was a considerable amount of precipitate in the former dialysis, it may be that the apparent loss in activity was in fact due to less enzyme in the supernatant as more of it had precipitated.

Increasing the pH of the refolding buffers from pH 7 to pH 8 did not result in an increase in mature enzyme activity towards gelatin SDS-PAGE gels although there was a small increase in activity of the pro-mature enzyme (Figure 3.3.9). However, there was no difference in specific activity between the two samples (data not shown). Thus pH 7 was chosen as the optimum pH for refolding of this particular protein. Since the enzyme is ultimately associated with lysosomal organelles which are acidic, one may expect that the enzyme would be adapted for these conditions.

Reduced proteins have a strong tendency to aggregate, thus oxidative renaturation must be carried out at low protein concentrations. Typically, the protein concentration was estimated to be 1.0 mg/ml by the BioRad protein assay after solubilisation in 8 M urea and this sample was therefore diluted 8 to10-fold with 8 M urea to an approximate concentration of 0.1 - 0.125 mg/ml before dialysis. Omission of this dilution step led to increased aggregation resulting in losses in yield and a subsequent decrease in enzyme activity.

The dialysis was carried out at room temperature and at 4°C to compare temperature effects on the refolding process. It was found that the protein did renature at room temperature as seen by activity on gelatin SDS-PAGE gels (results not shown) but there was a small amount of precipitation whereas there was none with dialysis at 4°C. This is in agreement with Kuhelj *et al.* (1995), who reported a 50% decrease in enzyme activity if refolding was carried out at room temperature.

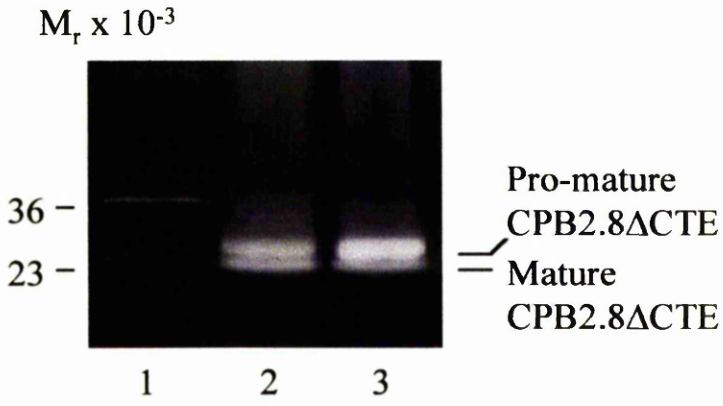

Figure 3.3.9 Coomassie-stained 10 % gelatin SDS-PAGE gel comparing proteinase activity of solubilised inclusion bodies, dialysed at pH 7 or pH 8.

Lane 1, stationary phase promastigote parasite lysate of *cpb* null re-expressing gene 2.8; lane 2, dialysed inclusion bodies at pH 7; lane 3, dialysed inclusion bodies at pH 8.

Lanes 2 and 3 were loaded with equal volumes.

Reformation of disulphide bonds occurs under oxidising conditions and increased yields of active, recombinant CPs are normally obtained by maintaining the correct redox potential using oxidised thiol compounds and reducing agents such as DTT. The effects of two different redox reagents upon refolding were examined. Cysteine was tried as this had been used previously in the refolding of human pro-cathepsin B (Kuhelj *et al.*, 1995). However CPB2.8ΔCTE is cathepsin L-like, so glutathione (a mixture of 90 % reduced and 10 % oxidised) was incorporated into the renaturation buffer instead of cysteine as this mixture of glutathione had been used previously in refolding of human cathepsin L (Menard *et al.*, 1998).

Activity towards gelatin was recovered from inclusion bodies dialysed with glutathione and gave a similar enzyme activity profile (Figure 3.3.10) as that of the enzyme refolded with cysteine (after purification using ion exchange chromatography). That is, the pro-mature, intermediate form of the enzyme was able to completely activate to the mature form, shown by the slow mobility band being processed to the faster mobility band. The purity of the two protein preparations was almost identical (Figure 3.3.11) and the final specific activity of the enzyme refolded with cysteine (408 nmoles.min.mg.protein^{-1}) was slightly greater than that refolded with glutathione (352 noles.min.mg.protein^{-1}). Although glutathione can therefore be used to refold inclusion bodies containing CPB2.8ΔCTE and produce activatable enzyme, it was no better improvement in terms of activity and yield, than using cysteine as the thiol reagent. Thus, cysteine was routinely used subsequently.

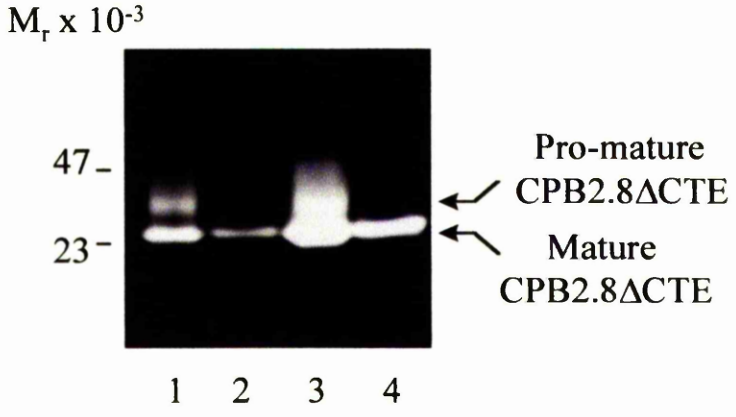

Figure 3.3.10 10 % gelatin SDS-PAGE comparing activation of pooled ion exchange fractions.

Lane 1, solubilised inclusion bodies refolded with glutathione. This dialysate was purified by ion exchange chromatography and gelatin gel activity is due to the pooled fractions from the ion exchange purification;

lane 2, sample described in lane 1, after activation;

lane 3, solubilised inclusion bodies refolded with cysteine. This dialysate was purified by ion exchange chromatography and gelatin gel activity is due to the pooled fractions from the ion exchange purification;

lane 4, sample described in lane 3, after activation.

Lanes were loaded with equivalent volumes.

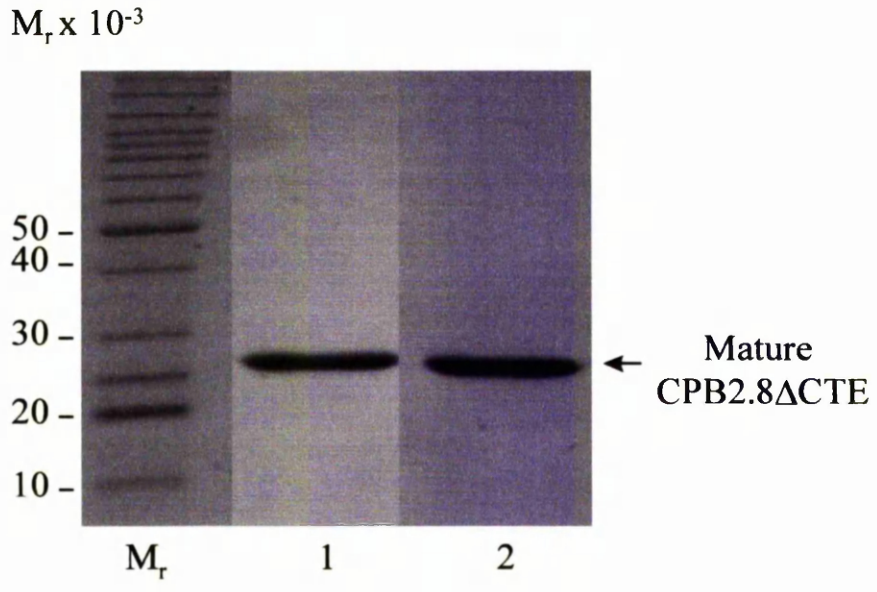

Figure 3.3.11 Coomassie-stained 12 % SDS-PAGE comparing activated enzymes obtained after refolding in the presence of glutathione and cysteine.
Lane 1, glutathione-refolded sample; lane 2, cysteine-refolded sample. Lanes were loaded with equivalent protein concentrations.

3.3.8 Nickel-agarose purification of CPB2.8ΔCTE

Affinity chromatography represents one of the most rapid, efficient and convenient means of generating pure protein. Inclusion bodies were solubilised by re-suspending in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0 and vigorously shaking for 1 hour at 37°C. These were then clarified by centrifugation to remove any particulate material before application to the nickel-agarose column. The recombinant enzyme consistently eluted in buffer E (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 4.5). According to the QIAgen protocol, monomers usually elute at pH 5.9 while multimers, aggregates and proteins with two 6xHis tags tend to elute at pH 4.5. A small amount of CPB2.8ΔCTE did elute at pH 5.9 but the majority (> 90%) eluted at pH 4.5 (Figure 3.3.12). This is surprising, since the enzyme is expected to exist as a monomer and therefore would be expected to elute at the higher pH. It may be that conditions on the column itself could produce a steric change in the conformation of the protein resulting in aggregation and a greater affinity to the column than usual. The subsequent problems encountered in attempting to activate the enzyme are consistent with the presence of un-natural enzyme aggregates.

Using the protocol devised by Kuhelj *et al.* (1995), which I used for solubilisation of the protein prior to the affinity chromatography, contaminating proteins are removed prior to purification and so there is less chance of aggregation with other proteins. The problem with the nickel-agarose procedure is the incompatibility of the nickel agarose resin with the thiol-containing chemicals such as DTT or β-mercaptoethanol (β-ME) used for the solubilisation process of the method

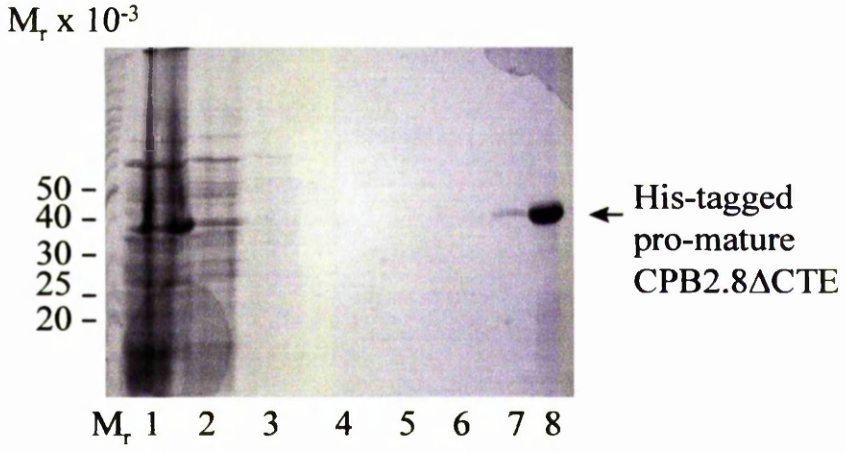

Figure 3.3.12 Coomasie-stained 10 % SDS-PAGE showing purification of CPB2.8ΔCTE using nickel-agarose chromatography under denaturing conditions.

Lane 1, load (solubilised inclusion bodies);

lane 2, column flow-through;

lane 3, column wash with buffer B;

lane 4, wash with buffer C, pH 6.5;

lane 5, wash with buffer C, pH 6.5;

lane 6, wash with buffer D, pH 5.9;

lane 7, wash with buffer D, pH 5.9;

lane 8, CPB2.8ΔCTE eluant with buffer E, pH 4.5.

Lanes were loaded with equivalent volumes.

documented by Kuhelj *et al.* (1995). Attempts to solubilise the inclusion bodies using this procedure but without the presence of a reducing agent resulted in less solubilisation of the recombinant enzyme (Figure 3.3.13a). It was found that for maximal recovery of CPB2.8ΔCTE from inclusion bodies, they should be solubilised in precisely the conditions utilised by Kuhelj *et al.* (1995); 8 M urea, 10 mM DTT and 0.1 M Tris/HCl, pH 8.0. Furthermore, when the inclusion bodies (solubilised without the presence of a reducing agent) were purified using the nickel-agarose column, the enzyme could still not be fully activated (Figure 3.3.13b). Although, the nickel-agarose purified enzyme displayed gelatinase activity, it did not activate to the fully active, mature form shown in Figure 3.3.10 (lane 2). Instead, the protein remained as the higher mobility form, shown in Figure 3.3.10, (lane 1). This intermediate form was known to have 7 amino acids of the pro-region still attached, analysed by N-terminal sequencing (Sanderson *et al.,* 2000) and although the enzyme was proteolytically active towards gelatin, activity to BzPFR-Nan was low (Table 5). So, this form of purification was deemed to be inefficient for recovery of fully active recombinant proteinase since all attempts to convert the pro-form to fully active enzyme were unsuccessful. The possible reasons for this are discussed in section 3.4.

Nickel-agarose affinity chromatography was, however, successful in providing extremely pure pro-enzyme for generation of polyclonal anti-serum (Figure 3.3.14).

3.3.9 Ion exchange chromatography

Since nickel-agarose was incompatible with the solubilisation process used and enzyme that was purified using nickel-agarose chromatography could not be fully

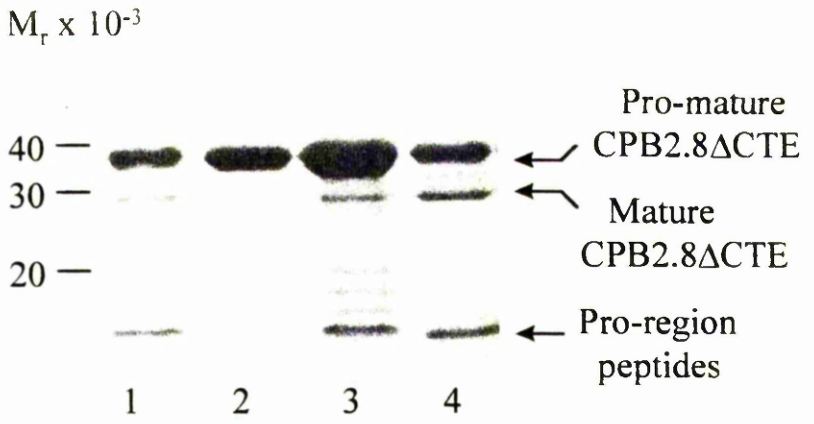

Figure 3.3.13a Coomassie-stained 10 % SDS-PAGE showing different solubilisation conditions attempted. Inclusion bodies were solubilised in a number of different buffers.
Lane 1, 8 M urea with Tris/HCl, pH 8.0;
lane 2, 0.1 M Tris/HCl, pH 8.0 with 10 mM DTT;
lane 3, 8 M urea with 10 mM DTT and Tris/HCl, pH 8.0;
lane 4, 8 M urea with 10 mM DTT.
Lanes were loaded with equivalent volumes from clarified supernatants.

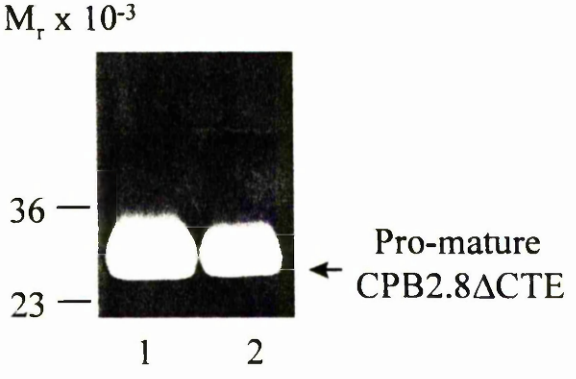

Figure 3.3.13b 10 % gelatin SDS-PAGE showing gelatinase activity from nickel-agarose purified CPB2.8ΔCTE. Inclusion bodies were solubilised in two different buffers (without DTT), affinity-purified then refolded.
Lane 1, inclusion bodies solubilised in 8 M urea, 0.1 M sodium phosphate and 0.01 M Tris/HCl, pH 8.0;
lane 2, inclusion bodies solubilised in 8 M urea and Tris/HCl, pH 8.0

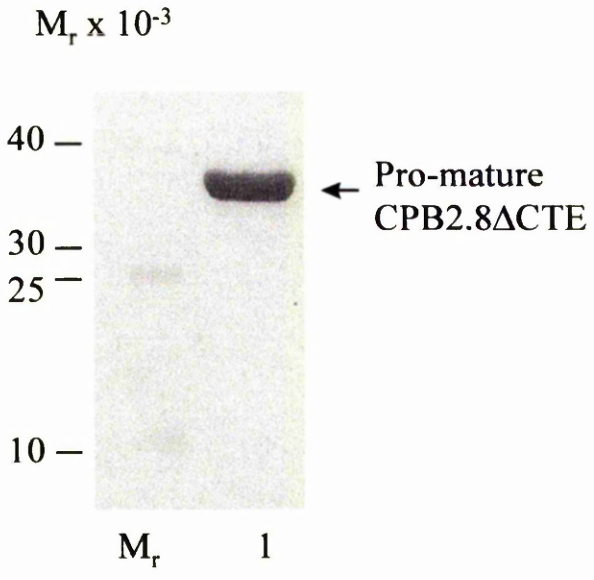

Figure 3.3.14 Silver-stained 10 % SDS-PAGE gel showing purity of CPB2.8ΔCTE obtained from nickel-agarose chromatography.

Lane 1, molecular weight markers;

lane 2, His_6-tagged CPB2.8ΔCTE pro-mature enzyme.

activated, the refolded enzyme sample was further purified using a Mono Q anion exchange column. Importantly, this step was found to be essential for full activation of the enzyme to be achieved. Analysis of the eluted samples by gelatin SDS-PAGE and BzPFR-Nan assays showed that the enzyme in the Mono Q eluant was only partially active. There were two bands with activity towards gelatin (Figure 3.3.15) with the lower molecular mass band being the mature enzyme species and the other band being the intermediate pro-mature form. The specific activity towards BzPFR-Nan of the pooled sample (fractions 6 to 14) was typically about 40 nmoles.min.mg.protein^{-1}, reflecting enhanced purification and some activation from the Mono Q step (Table 5).

The rationale behind using the ion exchange step was to separate the intermediate and mature enzyme from each other. Although there were consistently two separate, defined protein peaks (Figure 3.3.16), these did not correspond to intermediate and mature proteins (see Figure 3.3.15) and the two species could not be isolated from each other as assessed using gelatin SDS-PAGE (Figure 3.3.15). However there was a significant difference in fractions eluted from the Mono Q column and their ability to be activated thereafter. Fractions 8 to 14 (elution concentration of 400 - 700 mM NaCl) were able to be fully activated to the mature form but fractions 5 to 7 (250 - 350 mM) could not be successfully activated. However, if all of fractions 5 to 14 were pooled, it was possible to activate all the protein in the mixture to the fully active enzyme. Without the Mono Q step, the enzyme could not be fully activated in this way - it appears that the ion exchange step was necessary for this step to occur.

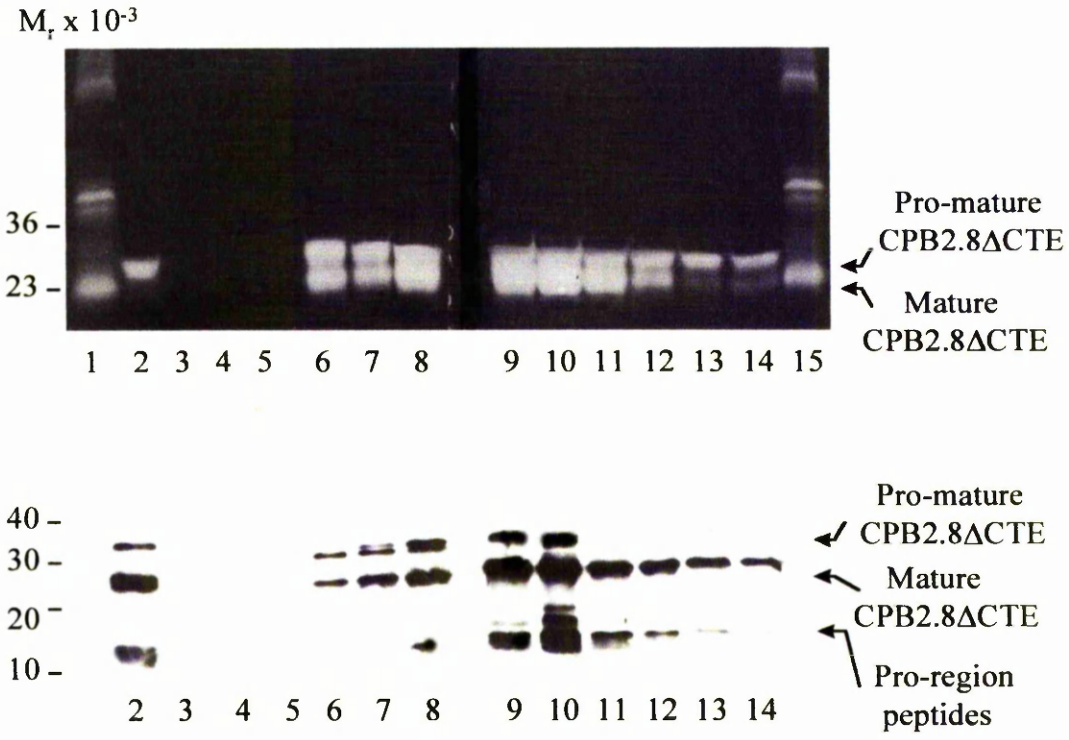

Figure 3.3.15 10 % gelatin SDS-PAGE and Western blot on 10 % SDS-PAGE showing Mono Q ion exchange purification of CPB2.8ΔCTE.
Lanes 1and 15 of gelatin gel , stationary phase promastigotes of *L. mexicana cpb* null mutant re-expressing gene 2.8;
lane 2, refolded inclusion bodies (column load); lane 3, column flow-through; lane 4, column wash (pre-NaCl gradient); lanes 5-14, NaCl gradient elution.
All lanes were loaded with equal volumes. Pro-region peptides are not apparent on the gelatin SDS-PAGE gels since they contain no activity towards gelatin. Fractions 5 to 14 were pooled for activation.

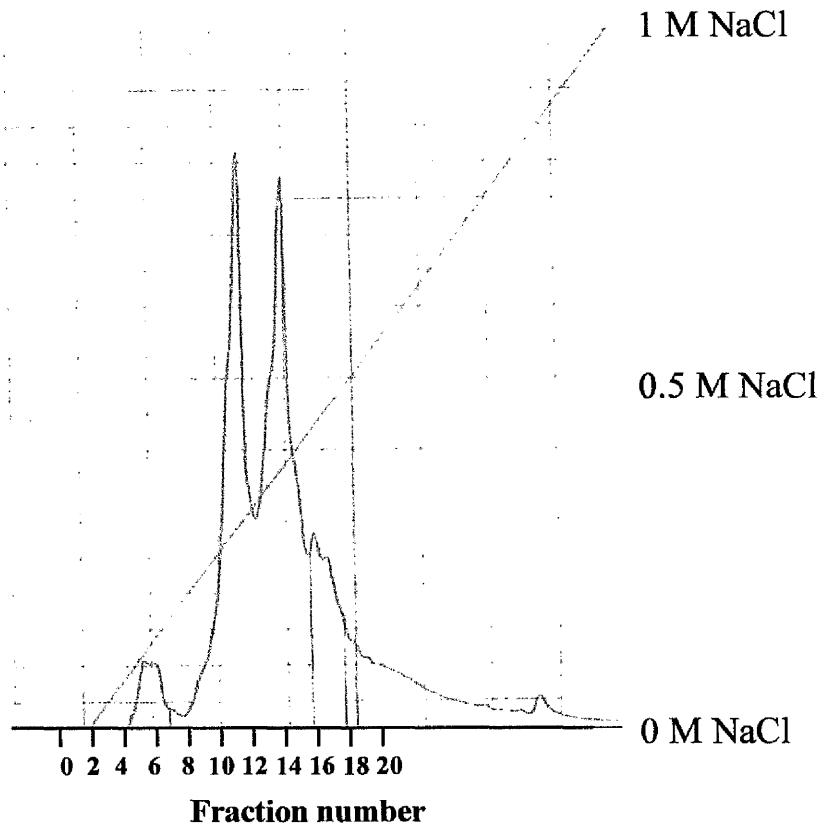

Figure 3.3.16 FPLC A_{280} elution profile of Mono Q ion exchange chromatography step used to purify refolded CPB2.8ΔCTE. The chromatograph shows the 0-1 M NaCl gradient as the straight line running from bottom left (0 M NaCl) to the top right of the figure (1 M NaCl). The elution profile shown is typical of those obtained, with two large protein peaks eluting at 250 mM (fraction 10) to 400 mM NaCl (fraction 16). Fractions 5 to 14 were pooled for subsequent activation.

3.3.11 Activation of CPB2.8ΔCTE

Complete activation of the enzyme, that is conversion of the pro-mature to the mature form, usually occurs as an autocatalytic step for cathepsins (Kirschke *et al.*, 1995).

The activation process *in vivo* is thought to occur at an acidic pH and the conditions I used *in vitro* were designed to mimic these. High salt concentration was thought to be essential for the activation process. Addition of NaCl to the activation buffer resulted in a dramatic increase in activation and a resultant 12-fold increase in specific activity towards the substrate BzPFR-Nan (Table 5). The increase in specific activity correlated with conversion of the pro-mature form on gelatin SDS-PAGE (slower mobility species, lane 1, Figure 3.3.10) to fully active, mature enzyme (lane 2, Figure 3.3.10). This single band of activity also migrated on SDS-PAGE as a single band with some low molecular mass pro-region peptides (Figure 3.3.17, lane 4).

Activation of CPB2.8ΔCTE requires a lowering of pH and this is similar to other cysteine proteinases (Eakin *et al.*, 1993). Attempts to activate at neutral pH resulted in a loss of activity and incomplete conversion of pro-enzyme to mature enzyme (Figure 3.3.18, lanes 2 and 3). However, if the pH was lowered to pH 5.5, conversion on gelatin SDS-PAGE and by Western blotting could be visualised of the pro-enzyme being processed to the fully active, mature enzyme (Figure 3.3.18, lanes 4 and 5).

Table 5. Purification summary (from 100 ml culture)

Purification step	Total protein (mg)	Activity* (nmoles.min.ml^{-1})	Specific Activity (nmoles.min.mg. protein^{-1})	Purification factor
Post-dialysis	23	3	13	1
Post - Mono Q	7	8	39	3
Post - acidification	2.5	50	455	36

* Using micro-titre plate stopped assay with BzPFR-Nan as substrate

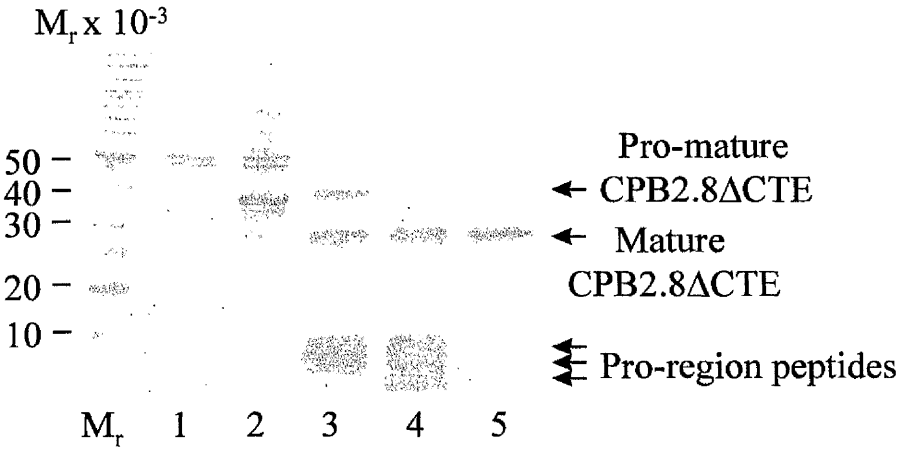

Figure 3.3.17 Coomassie-stained 10 % SDS-PAGE showing processing steps required for purification of CPB2.8ΔCTE. Lane 1, *E. coli* pre-induction; lane 2, 4 h post-induction; lane 3, pooled Mono Q fractions; lane 4, pooled Mono Q fractions post-activation; lane 5, post-activated CPB2.8ΔCTE after Centricon 10 concentration.
Lanes 1 and 2 were loaded with an equal volume of culture. Lanes 3, 4 and 5 were loaded with equal volumes.

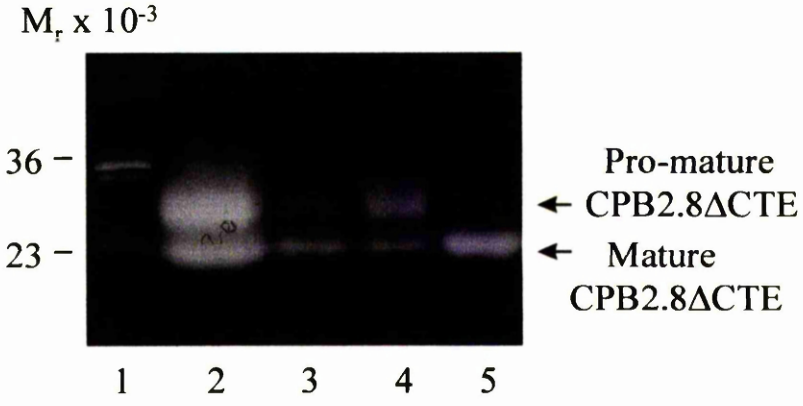

Figure 3.3.18 10 % gelatin SDS-PAGE showing requirement for acidification for activation from the pro-mature to the mature form.
Lane 1, stationary phase promastigote parasite lysate of *cpb* null expressing gene 2.8;
lane 2, pooled Mono Q fractions before activation with no acidification;
lane 3, pooled Mono Q fractions after activation with no acidification;
lane 4, pooled Mono Q fractions before activation with acidification;
lane 5, pooled Mono Q fractions after activation with acidification.
Lanes 2, 3, 4 and 5 were loaded with equivalent volumes.
The sample in lanes 2 and 3 were from another protein preparation which was more concentrated, compared to the sample in lanes 4 and 5.

3.3.12 Purification of a *L. infantum* CPB containing a C-terminal extension

Purification of a recombinant leishmanial CPB with its C-terminal extension using the published protocol, was attempted but proved to be difficult. Due to time constraints, the inclusion bodies were only solubilised and dialysed (Figure 3.3.19) although there was a clear difference in protein yield and activity after dialysis compared to dialysed CPB2.8ΔCTE. Overnight dialysis resulted in extremely high levels of protein precipitation and most of the recombinant protein was found as precipitated protein (Figure 3.3.19). After overnight dialysis, the supernatant displayed no activity to gelatin SDS-PAGE gels (data not shown), even though some processing of the protein had occurred during the dialysis stage (Figure 3.3.19).

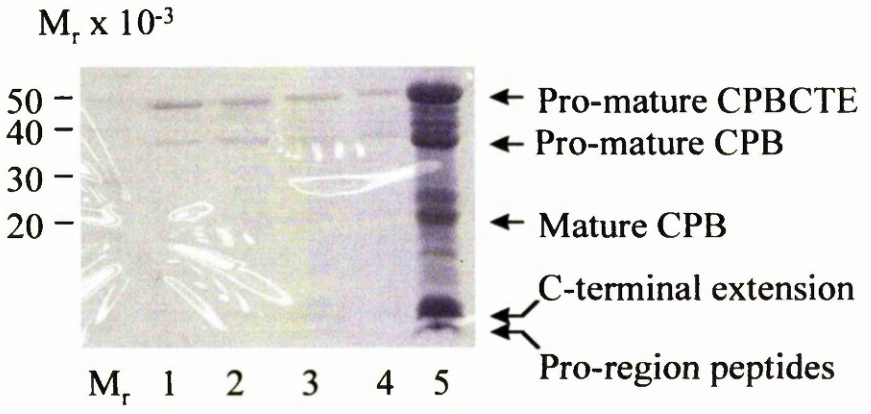

Figure 3.3.19 Coomassie-stained 12 % SDS-PAGE gel showing processing and precipitation of *L. infantum* CPBCTE during refolding stage at designated time-points.

Lane 1, solubilised inclusion bodies, 1 h after start of dialysis;
lane 2, solubilised inclusion bodies, 3 h after start of dialysis;
lane 3, solubilised inclusion bodies, 6 h after start of dialysis;
lane 4, supernatant of solubilised inclusion bodies, 20 h after dialysis;
lane 5, pellet of solubilised inclusion bodies, 20 h after dialysis.
For lanes 1 to 3, the dialysate was completely soluble and there was no evidence of precipitation even after centrifugation. For lanes 4 and 5, the sample was centrifuged at 6000 g for 10 min and supernatant loaded. The resultant pellet was re-suspended in sample buffer and loaded as for the supernatant. Lanes were loaded with equivalent volumes.

3.4 DISCUSSION

A CP of *L. mexicana*, CPB2.8ΔCTE, has been successfully over-expressed in *E. coli*. This has enabled further characterisation of the enzyme's pivotal role in immune evasion and also facilitates high-throughput screening using substrate and inhibitor libraries. A novel procedure has been developed for the purification and activation of leishmanial CPB isoenzymes over-expressed in *E. coli*.

CPB2.8ΔCTE was expressed as a His_6-tag fusion protein for efficient purification using nickel-agarose resin. Purified pro-enzyme was obtained using this method and employed in the production of specific anti-serum. However, the preparation had very low enzyme activity and all attempts to convert the pro-form to mature, fully active enzyme were unsuccessful. There are several possible reasons for this.

As inclusion bodies are formed from the accumulation of incorrectly folded recombinant protein and the presence of other protein contaminants, lipids and polysaccharides, it is likely that the protein was eluting as an aggregated form, even after solubilisation with denaturant, as a reducing agent was not present. It has been reported (Kuhelj *et al.*, 1995) that for complete disruption of aggregates within inclusion bodies during solubilisation, it is essential to have a small amount of reducing agent such as DTT or β-ME present to allow complete separation of disulphide bonds. However, the nickel-agarose column would be reduced by the presence of DTT or β-ME and therefore fail to purify the enzyme, and so it was necessary that the enzyme was solubilised in denaturant without reductant, in advance of attempted affinity purification using nickel-agarose. It is possible that the

misfolded disulphide bonds formed in the inclusion bodies were not being completely disrupted by the solubilisation conditions and so the protein continued to exist as aggregates rather than monomers. This would account for the elution at pH 4.5. Such purification of the misfolded enzyme, would result in enzyme that could not be fully activated since the protein would still contain incorrectly formed disulphides. Furthermore, it is known that an oxidative protein cross-linking reaction can be mediated by a nickel-peptide complex (Fancy *et al.,* 1996). This reaction was dependent on nickel, the His_6-tag and oxidant. The bead-bound nickel atoms support the oxidative cross-linking reaction and it could be that without the presence of a reductant in the solubilisation procedure, irreversible oxidation and cross-linking would take place. Again, this would explain the elution of CPB2.8ΔCTE at pH 4.5, consistent with elution of aggregated forms. Full activation of the enzyme would therefore not be possible.

A number of possible hypotheses exist which could explain the role of the Mono Q step in the purification of CPB2.8ΔCTE. The rationale behind using ion exchange chromatography is that the required protein is bound while contaminating proteins are washed through the column and then the protein of interest is specifically eluted. It is possible that the interaction with the column may be sterically favouring a form which facilitates subsequent activation, whereas previously the protein was in a non-activatable form. In essence, the bound pro-region may be moved to a position which makes it easier for cleavage by the mature enzyme.

Another possibility for the Mono Q step enabling subsequent activation could be due to the positively charged resin competing with and thereby removing some

positively charged pro-region peptides associating with the mature enzyme, which had been hydrolysed but were still bound. Spontaneous activation of human pro-cathepsin L also followed purification on Mono Q resin or incubation with negatively charged dextran sulphate (Mason & Massey, 1992), which was thought to induce a small conformational change in the positively charged pro-region. Subsequent acidification during the activation step could loosen the structure and increase the mobility of the pro-segment allowing for dissociation of such pro-region peptides resulting in an increase in enzyme activity. A similar mechanism has been demonstrated for rat pro-cathepsin B (Cygler *et al.,* 1996).

It has been reported that pro-enzyme activation seems to proceed faster than proteolytic processing, suggesting that the mechanism involves two steps: pro-enzyme activation followed by cleavage to yield the mature enzyme (Mach *et al.,* 1994). Whether this may be the case for cathepsin L-like enzymes is unknown, although evidence for cathepsin L-like proteins suggests that proenzyme concentration is important in the rate of processing, indicating the existence of bimolecular and unimolecular steps in the mechanism of processing (Menard *et al.,* 1998). Little is known of zymogen processing in *Leishmania* parasites. However, an active variant of cruzipain, the major CP from *T. cruzi*, revealed that the proteolytic cleavage of the C-terminal extension domain is independent of cruzipain activity, possibly due to a bacterial proteinase (Eakin *et al.,* 1993).

High salt concentration was shown to be essential for the activation process and the rationale behind this was that cleavage of the pro-region from the mature enzyme would proceed optimally if non-specific interactions were prevented. The

presence of chloride ions has also been documented as essential for activation to occur in a dipeptidyl arylamidase from the pituitary (McDonald, *et al.,* 1966). This enzyme, which also contained thiol groups that are essential for activity, showed an absolute requirement for chloride ions in the activation medium. It is possible that the clipped pro-region may still be associated with the partially active enzyme after ion exchange and the presence of halide can help trigger the dissociation of the pro-region peptides from the mature enzyme. With human pro-cathepsin L, the enzyme undergoes activation at pH 5.5 in the presence of negatively charged surfaces (Menard *et al.,* 1998). This is very similar to the situation I found with CPB2.8ΔCTE, although pro-cathepsin L does not appear to go via intermediate forms during processing .

Low pH was shown to aid activation. It is possible that lowering the pH causes protonation of some acidic groups with a concomitant weakening of salt bridges and/or hydrogen bonds between the pro-segment and the mature enzyme (Vernet *et al.,* 1995). Furthermore, it has been postulated that the role of acidic pH is to loosen the structure and allow the peptides to dissociate from the mature enzyme (Cygler *et al.,* 1996). The binding of the remaining peptides become progressively less tight, eventually leading to the peptide falling off the enzyme. Mature, active enzyme is then able to degrade the detached pro-region to small peptides which can subsequently be removed by concentrating the 23 kDa protein through a molecular weight cut off membrane of 10 kDa. This resulted in enzyme that was considered pure.

Analyses of CPB2.8ΔCTE and another recombinant parasite CP derived from inclusion bodies, cruzipain from *T. cruzi* (Eakin *et al.,* 1992), revealed similar kinetic parameters, confirming the recovery of significant enzyme activity following refolding from inclusion bodies. However, there was a difference in the processing of the two recombinant enzymes as the sites of cleavage were quite different (Sanderson *et al.,* 2000).

Recent attempts at purification and activation of a recombinant CP from *Toxocara canis*, Tc-cpz-1, proved unsuccessful (Falcone *et al.*, 2000). The enzyme was poorly soluble and enzymatically inactive, so attempts to purify the enzyme using *E. coli* as the recombinant cell, were abandoned. However, the problem may be due to the enzyme being generated as the recombinant, mature form. It is possible that the mature enzyme could be purified and refolded using our published protocol but the enzyme would be incorrectly folded since the pro-region of most CPs is needed for correct folding of the mature enzyme (Taylor *et al.,* 1992). The method we have developed for purification of CPB2.8ΔCTE could be used for the purification of other recombinant parasite CPs in *E. coli* but only when produced as pro-mature constructs. Alternatively, the pro-region of the mature enzyme could be added exogenously to the mature enzyme, during the refolding stage as has been demonstrated for the refolding of subtilisin (Zhu *et al.,* 1989).

Purification of a *L. infantum* CPB with its C-terminal extension was an aim of this study but using the published protocol, this was largely unsuccessful. The presence of the C-terminal extension, resulted in greater aggregation during the refolding step and subsequent precipitation with a resultant loss in yield of the

recombinant CP. This was probably due to the approximate 50 % increase in cysteine residues, which would increase the occurrence of incorrectly positioned disulphide bridges and result in misfolding of the protein. Purification of the *L. mexicana* CPB2.8 with its C-terminal extension has been attempted before but this resulted in misfolded protein (Wolfram *et al.,* 1995), although a different method of refolding was used from the method discussed in this chapter. Due to time constraints, this work was unable to be completed although the procedure for purifying this recombinant CPB with its C-terminal extension, would need to be modified from that discussed, due to the hydrophobic nature of the C-terminal extension.

Purification of the individual CPB isoenzymes from the array makes possible a range of investigations of this important class of parasite, not previously possible due to difficulties in purification and the presence of multiple isoforms in the parasite. Detailed structural analysis of CPB2.8ΔCTE should provide further insights into its function, which may facilitate the design of specific inhibitors to this enzyme. Purification of the enzyme has allowed initial characterisation of the enzyme's role in the host-parasite interaction and highlighted its potential as part of a subunit vaccine against *L. mexicana*.

CHAPTER 4

Study on the vaccine potential of recombinant CPB

4.1 INTRODUCTION

Cutaneous leishmaniasis is perhaps one of the best parasite targets for vaccine development since long-term immunity is induced in the majority of people following natural infection (Alexander *et al.*, 1998). There is also a history of successful vaccination by live parasite inoculation (Titus *et al.*, 1995). Unfortunately, the increasing prevalence of immuno-suppression makes the use of a live vaccine less likely as this could be virulent in such individuals (Stenger *et al.*, 1996). It is believed that the development of a vaccine against leishmaniasis should incorporate defined antigens from the amastigote stage of the parasite since it is this form of the parasite which is responsible for the disease in the human host. The development of a subunit vaccine against *L. mexicana* should provide an interesting challenge in terms of choice of both antigen and adjuvant and these must be carefully considered in their usage for stimulation of a protective immune response.

Adjuvants are thought to work by slowing the release of antigen, targeting antigen to antigen presenting cells (APCs) such as macrophages and by directly activating macrophages (Warren, 1986). The adjuvant, alum, was included in the first recombinant and peptide malaria vaccines tested in humans (Patarroyo, 1988) and at present is one of the few adjuvants licensed for human use. More recently,

alum has been shown to increase the biological half-life of IL-12, when the two are co-administered during vaccine studies against HIV infection (Jankovic *et al.*, 1997). Increasing the biological half-life of IL-12 *in vivo* may result in a more persistent induction of IFN-γ thereby leading to a more potent helper effect on Th 1-dependent antibody isotypes. Despite this, alum without the addition of immuno-potentiators is not an ideal adjuvant for all applications due to its inability to induce a Th 1 immune response.

Protective immunity to murine cutaneous leishmaniasis is dependent upon the development of an IL-12 - driven $CD4^+$ Th1-type response and the subsequent production of IFN-γ (Liew & O' Donnell, 1993). In turn, this cytokine induces up-regulation of macrophage iNOS and NO production, which is microbicidal for the parasite (Liew and O' Donnell, 1993). Thus, injection of exogenous IL-12 with known parasite molecules should potentiate a Th 1-driven immune response and protect against subsequent *Leishmania* infection.

IL-12, a cytokine produced from phagocytes and other antigen presenting cells, has been shown to induce an IFN-γ driven Th 1 response when administered with soluble leishmanial antigen and can protect against infection with *L. major* in the susceptible BALB/c mouse model (Afonso *et al.*, 1994). Furthermore, low concentrations of IL-12 and low amounts of soluble protein can stimulate increased IFN-γ production and profoundly up-regulate the synthesis of the complement-fixing antibody subclasses (IgG2a, IgG2b and IgG3) *in vivo* (Germann *et al.*, 1995). More recently, administration of IL-12 and several recombinant leishmanial antigens was found to protect against infection with *L. mexicana* (Aebischer *et al.*, 2000).

It has been demonstrated that recombinant IL-12 (IL-12) can be an effective Th 1-inducing adjuvant in combination with protein antigens; effective vaccination against *L. major* infection in susceptible strains was achieved using IL-12 with soluble leishmanial antigen (SLA) (Afonso *et al.*, 1994) and subsequently, with a recombinant leishmanial protein (LACK) (Gurunathan *et al.*, 1997).

L. mexicana produces non-healing lesions in most mouse strains and has the ability to substantially down-regulate the immune response, often resulting in lesions metastasising to the viscera (Roberts *et al.*, 1990). Studies in IL-4 knockout mice have demonstrated that non-healing lesion development is dependent on the presence of this cytokine (Satoskar *et al.*, 1995). Since CP activity has been shown to stimulate IL-4 production (Travis *et al.*, 1995), the many active CPs of this *Leishmania* species may direct the immune response to an IL-4 driven Th 2-type response.

It has recently been shown that immunisation with an inactive recombinant CP and recombinant IL-12, conferred protection to a normally lethal infection with *L. mexicana* infection in C57BL/6 mice (Aebischer *et al.*, 2000). This mouse strain has intermediate susceptibility to *L. mexicana* (Roberts *et al.*, 1990) and this vaccine did not, however confer protection to the BALB/c mouse strain. However, intraperitoneal immunisation with an active, native CP from *L. major* and Freund's complete adjuvant (FCA) was also found to promote protection to *L. major* infection in susceptible BALB/c mice (Rafati *et al.*, 2000). These results provide encouragement that parasite CPs both in their active and inactive form will mediate protection against *Leishmania* infection, when administered as a vaccine. However, the above studies were also not ideal as ultimately for quality

control, recombinant antigen will have to be used in human vaccines without FCA as the adjuvant cannot be used in humans (Bomford , 1989). Therefore, we studied the vaccine potential of both active and E-64 - inactivated recombinant CPB2.8ΔCTE adjuvanted with IL-12 to assess protection against wild-type *L. mexicana* infection in three different mouse strains. These mice vary in their susceptibility to *L. mexicana* infection (Roberts *et al.*, 1990) although all develop large primary lesions.

4.2 METHODS

4.2.1 Assessment of lipopolysaccharide by E-toxate assay

The level of endotoxin in a preparation of pure CPB2.8ΔCTE was assessed using the E-toxate assay (Sigma). All glassware was soaked overnight in a 1% (v/v) bleach (Domestos) solution, scrubbed and rinsed vigorously several times with dddH_2O. The glassware was then autoclaved for 1 hour before heating in an oven at 175°C, for a minimum of 3 hours.

The pH of the enzyme preparation was adjusted from pH 5.5 to pH 6.0 with endotoxin-free NaOH (Sigma) and boiled for 5 minutes to remove proteinase activity which interferes with the assay. The enzyme sample was added to the E-toxate working solution and the tubes mixed gently before incubation for 1 hour at 37°C. Positive controls contained LPS standard and negative controls contained enzyme buffer alone.

4.2.2 Vaccination with CPB2.8ΔCTE plus IL-12 and challenge infection with *L. mexicana*

Age matched female C57BL/6, BALB/c and CBA mice were maintained in the animal facility of the University of Glasgow and used at 8-16 weeks of age.

Murine recombinant IL-12 was kindly provided by Prof. Phil Scott, University of Pennsylvania, USA. This was diluted to yield 2.5 μg/ml in PBS and a 200 μl injection was given subcutaneously at the tailbase, between 30 and 60 minutes before administration of the active or inactive CP (5 μg) or saline control at the same site and route. Separate administration of antigen and adjuvant was taken as a precautionary step since the active enzyme may have proteolytically cleaved IL-12.

The immunisation schedule was: 15 mice, each of 3 different strains (C57BL/6, CBA and BALB/c), were divided into 3 groups of 5 (A - C). Group A were injected with active enzyme plus IL-12; group B were injected with E-64 inactivated enzyme (as described in section 2.3.11, chapter 2) plus IL-12; group C were injected with PBS, pH 7.4, as a control.

Mice were immunised twice, two weeks apart, using 200 μl of adjuvant plus 200 μl of purified CPB2.8ΔCTE (or saline).

L. mexicana promastigotes were harvested from late log phase culture by centrifugation at 700 g for 15 min at 4°C and washed twice with PBS, pH 7.4. Parasite pellets were re-suspended to 1 x 10^6/ml and 200 μl aliquots used to infect mice 2 weeks after the second set of immunisations. Mice were infected into a shaven rump.

4.2.3 Measurement of lesion volume

The course of infection was monitored by weekly measurement of the shaven rump using a slide gauge micrometer. Lesion volume was measured in this study according to the calculations described by Honigberg (1961). They found that the volume of the protruding portion of the lesion represented one half of a spheroid. Thus, the formula used was

$$\frac{4/3\ \pi \times 1/2 \times w/2 \times h}{2}$$

where l = length, w = width and h = height. Thus volumes were calculated using 0.5236 x l x h x w.

4.2.4 Detection of *Leishmania*-specific antibodies by ELISA

Peripheral blood was obtained after five months (and seven months for CBA mice) from infected animals by tail bleeding into heparinised capillary tubes. All plasma samples were stored at -20°C before analysis for specific Ab content.

Leishmania-specific IgG1 and IgG2a end-point titres were measured by ELISA. Each well of an Immulon-1 microtitre plate (Dynatech Laboratories, Billingshurst, UK) was coated with 2 μg of soluble leishmanial antigen (SLA) (freeze/thawed wild-type promastigotes in PBS, pH 9.0, were centrifuged at 10,000 g for 15 min and the supernatant was utilised as SLA) by overnight incubation. Following incubation of serial dilutions of plasma samples for 1 h at 37°C, bound Abs were detected by incubation with either rat anti-mouse IgG1-HRP conjugate (diluted 1/20,000) or rat anti-mouse IgG2a-HRP conjugate (Southern Biology Associates, Birmingham, USA). Binding of conjugate was visualised with tetramethylbenzidine (0.06 mg/ml) in 0.1 M sodium acetate buffer,

pH 5.5, containing 0.04 % H_2O_2. The colour reaction was stopped by adding 10 % (v/v) sulphuric acid, and the absorbance was measured at 450 nm. Results were expressed as end-point dilutions in which the end-point was defined as the final plasma concentration that yielded an absorbance higher than a negative control plasma sample included in the assay.

4.2.5 Splenocyte responses

Spleens were aseptically removed at appropriate times post-infection, as detailed for individual groups to be analysed. Cell suspensions were prepared by gently teasing apart the tissue in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-ME and 10 % (v/v) HI-FCS (Life Technologies, Paisley, UK). This was designated complete medium. Following centrifugation at 200 g for 10 min at 4°C, cells were re-suspended in 5 ml of Boyle's solution (0.17 M Tris/HCl, pH 7.2 and 0.16 M ammonium chloride) at 37°C for 2 min to deplete red blood cells. Spleen cells were then centrifuged at 200 g for 10 min at 4°C, resuspended, washed and resuspended in 2 ml of complete medium. Viable cells were enumerated by trypan blue exclusion, and the suspensions were adjusted to 5 x 10^6 cells/ml. Aliquots of the cell suspension (100 μl) were added to 96-well, flat-bottomed tissue culture plates (Costar, Cambridge, USA), and 100 μl aliquots of concanavalin A (Con A) (5 μg/ml), SLA (10 μg/ml) or recombinant CPB2.8ΔCTE (5 μg/ml) were added. Cultures were then incubated in 5 % CO_2/95% air for 72 h at 37°C. Supernatants were collected at this time and frozen at -20°C until cytokine quantification was performed.

4.2.6 IFN-γ, IL-4, IL-10 and IL-12 assays

IFN-γ and IL-4 production by stimulated T cells (stimulated by *Leishmania* antigen or Con A) and non-stimulated cells (complete RPMI) from mice infected with wild-type parasites were measured by capture ELISA, while IL-10 and IL-12 (from thioglycollate-elicited macrophages) was also measured in this way. The wells of Immulon-1 microtitre plates (Dynatech Laboratories) were coated with capture Ab at 2 μg/ml (IFN-γ, R4-6A2 (PharMingen, San Diego, USA)); IL-4, 11B11 (Genzyme, Cambridge, UK) in PBS, pH 9.0 by overnight incubation at 4°C (Antibodies to IL-10 and IL-12 were a kind gift from Prof. Phil Scott). Wells were then washed three times with PBS, pH 7.4/0.05 % Tween-20 (wash buffer) and blocked by incubation by 10 % (v/v) FCS for 1 h at 37°C. The culture supernatants and appropriate recombinant standards (rIFN-γ, rIL-10 and rIL-12, PharMingen; IL-4, Genzyme) were then added to individual wells. For standard curves, rIFN-γ, IL-10 and IL-12 (0-1,250 pg/ml) and rIL-4 (0-625 pg/ml) were used. Following incubations at 37°C for 2 h, the wells were washed three times with wash buffer, and then biotinylated rat anti-mouse IFN-γ (XMG1.2, PharMingen; 1 μg/ml) or biotinylated rat anti-mouse IL-4 (Genzyme; 1 μg/ml) was added and incubated for 1 h at 37°C.

For the detection of bound biotinylated rat Ab, 100 μl of streptavidin-alkaline phophatase conjugate (diluted 1/1000, PharMingen) was added to each well for 45 min at 37°C, and following further washing, binding was visualised with substrate consisting of p-nitrophenyl phosphate (1 mg/ml, Sigma) in glycine buffer (0.1 M, pH 10.4). The absorbance was subsequently measured at 405 nm on a Titertek Multiscan Plate Reader. All assays were conducted in triplicate.

4.2.7 Statistics

Comparisons between groups of mice were made with a Mann-Whitney U test for measurement of antibody titres. For comparison of lesion volume and cytokine production between groups of mice, the Student's *t* test was used. P values of <0.05 were considered significant.

4.3 RESULTS

4.3.1 Lipopolysaccharide (LPS) content of recombinant CPB2.8ΔCTE

The LPS content of recombinant CPB2.8ΔCTE was determined using the E-toxate assay and found to be < 1 ng LPS/mg protein. This was considered to be a significantly low level of LPS which would not influence the experimental results and the level of LPS correlates well with production of recombinant protein from *E. coli*, used in other immunological studies (Skeiky *et al.*, 1998; Aebischer *et al.*, 2000).

4.3.2 Protection of BALB/c mice against *L. mexicana* infection

The control mice, injected only with saline developed non-healing lesions which were significantly swollen in the shaven rump (Figure 4.3.1). In contrast, immunisation with either active or inactive CPB2.8ΔCTE plus IL-12 adjuvant delayed the onset of lesion formation for three weeks and thereafter resulted in lesions significantly smaller than those of the control group ($p < 0.05$). In two mice immunised with the active and inactive CPB, complete healing of the lesion was observed. There was no significant difference in protection mediated by the active or inactive CP.

4.3.3 Protection of C57BL/6 mice against *L. mexicana* infection

The control mice, injected with PBS alone, developed progressive lesions (Figure 4.3.2). The lesions differed from the lesions observed in PBS-immunised BALB/c mice (Figure 4.3.1). The lesions were characterised by a visibly wet

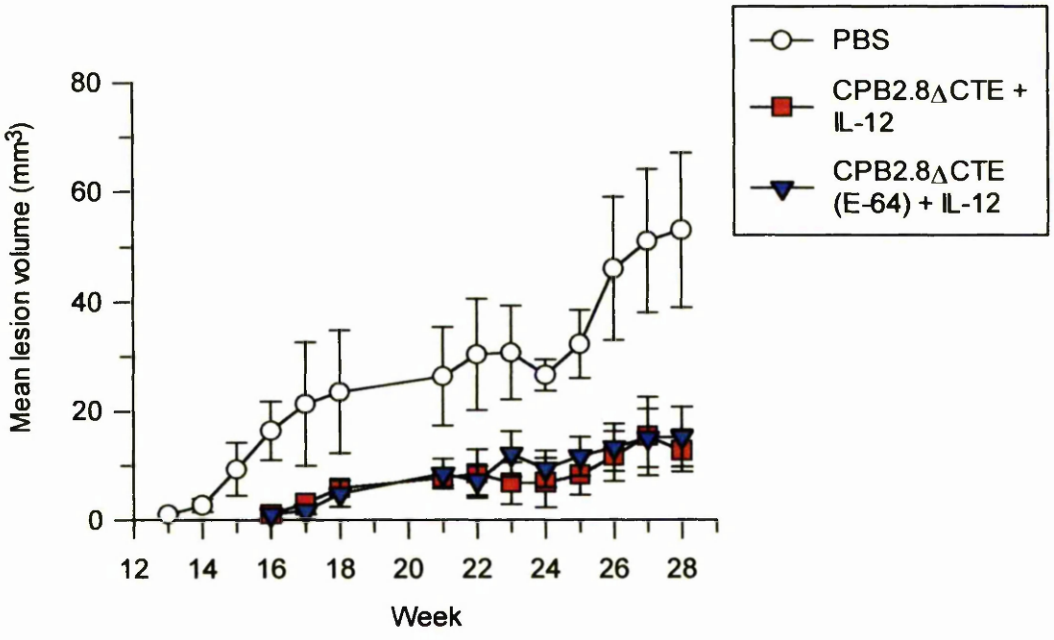

Figure 4.3.1 Cutaneous lesion growth of *L. mexicana* in non-vaccinated (PBS) BALB/c mice or vaccinated with CPB2.8ΔCTE (active or inactive), 1 mo before challenge with 2 x 10^5 wild-type promastigotes. Mice (5 per group) were vaccinated on two occasions, two weeks apart with 5 μg proteinase, 30 min after administration of IL-12. Control mice were inoculated in a similar manner with PBS alone. Data represent mean values of 5 mice per group with error bars corresponding to mean +/- SEM. Control mice had significantly higher lesion volumes than both groups of CPB-vaccinated mice ($p < 0.05$). There was no significant difference between the two CPB-immunised groups.

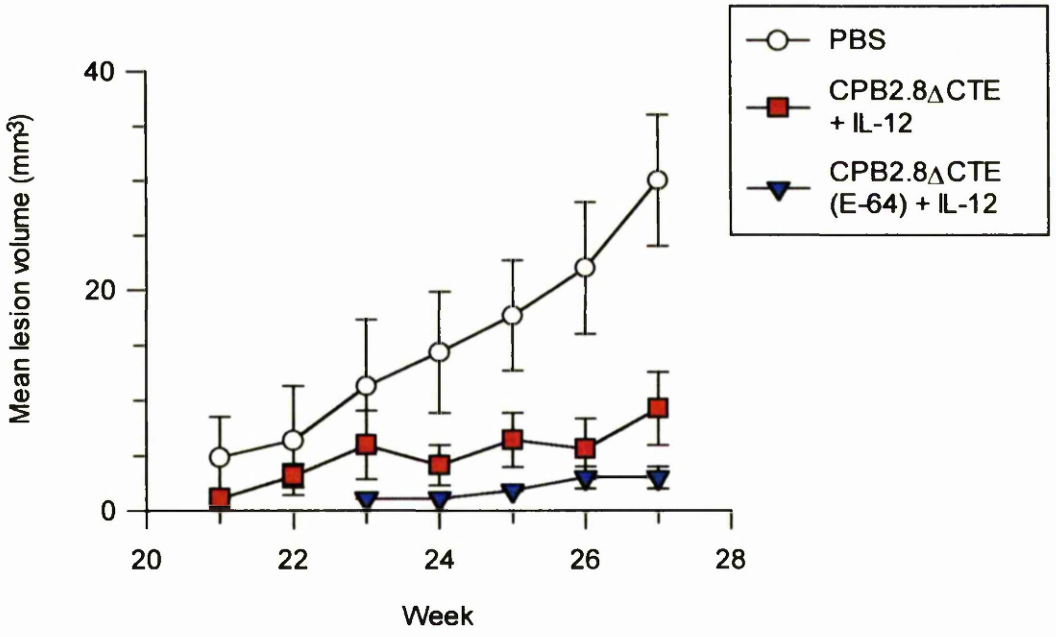

Figure 4.3.2 Cutaneous lesion growth of *L. mexicana* in non-vaccinated (PBS) C57BL/6 mice or vaccinated with CPB2.8ΔCTE (active or inactive), 1 mo before challenge with 2 x 10^5 wild-type promastigotes. Mice (5 per group) were vaccinated on two occasions, two weeks apart, with 5 μg proteinase, 30 min after administration of IL-12. Control mice were inoculated in a similar manner with PBS alone. Data represent mean values of 5 mice per group with error bars corresponding to mean +/- SEM. Control mice displayed significantly larger lesion volumes than active CPB-immunised mice ($p < 0.05$) and inactive CPB-immunised mice ($p < 0.02$). There was no significant difference between the two CPB-immunised groups.

ulcer at the site of infection. Of the five mice analysed, one did not develop a lesion at all. Those mice vaccinated with the active or inactive CP and IL-12 showed a similar degree of protection as occurred in the CPB-vaccinated BALB/c mice, although healing was seen in three mice immunised with the inactive CPB and two mice immunised with active CPB, with significantly smaller and less severe lesions occuring than in the control mice. The immunisation of active CP and IL-12 or inactive CP and IL-12 resulted in no significant difference in terms of lesion volume.

4.3.4 Protection of CBA mice against *L. mexicana* infection

The control CBA mice, vaccinated with saline alone, developed non-healing lesions (Figure 4.3.3). The lesions were similar in appearance to those in the BALB/c mice, although the lesions were slower growing. One of the five mice did not develop a lesion. Immunisation of mice with active or inactive CP plus IL-12 adjuvant delayed the onset of lesion formation by two weeks and those lesions that did form subsequently were significantly smaller than those seen in the PBS-immunised mice ($p < 0.001$). One mouse from each group of vaccinated mice did not develop any lesions. Again, the activity of the enzyme was unimportant in conferring protection against wild-type *L. mexicana* infection.

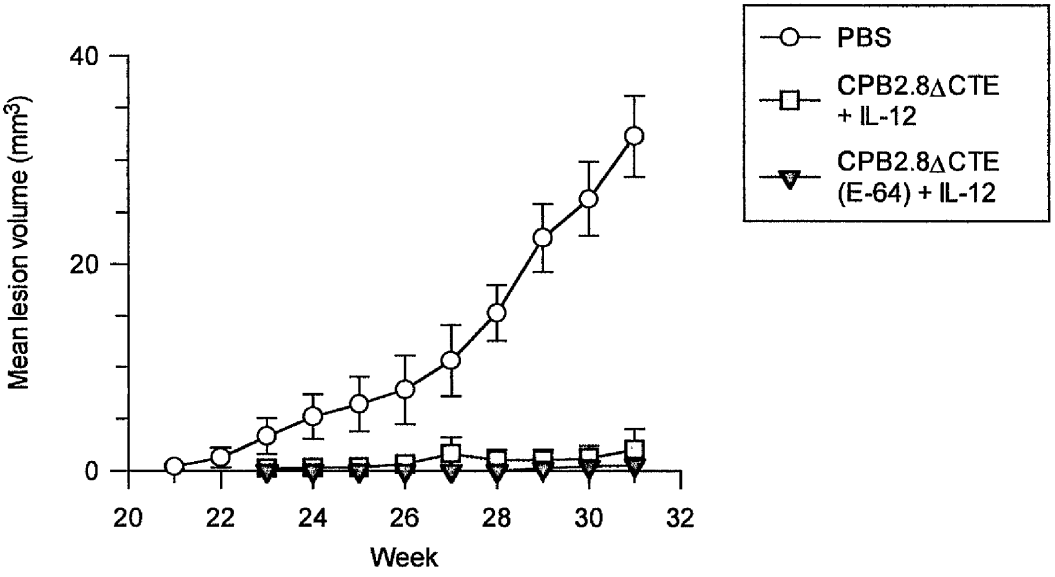

Figure 4.3.3 Cutaneous lesion growth of *L. mexicana* in non-vaccinated (PBS) CBA mice or vaccinated with CPB2.8ΔCTE (active or inactive), 1 mo before challenge with 2 x 10^5 wild-type promastigotes. Mice (5 per group) were vaccinated on two occasions, two weeks apart, with 5 μg proteinase, 30 min after administration of IL-12. Control mice were inoculated in a similar manner with PBS alone. Data represent mean values of 5 mice per group with error bars corresponding to mean +/- SEM. Control mice had significantly larger lesions than both groups of CPB-immunised mice ($p < 0.001$). There was no significant difference between the CPB-immunised groups.

4.3.5 Analysis of *Leishmania*-specific antibodies in vaccinated BALB/c mice

As IL-4 and IFN-γ direct immunoglobulin class switching for IgG1 and IgG2a, respectively (Finkelman *et al.*, 1990), we measured production of these antibody isotypes specific for *Leishmania* to provide an indirect but physiological *in vivo* assessment of the pattern of cytokine production.

After 5 months, control mice injected with saline alone, displayed high antibody titres of the IgG1 subclass with much lower IgG2a titres, as is characteristic of infections with wild-type *L. mexicana* (Figure 4.3.4). The level of IgG1 was significantly decreased in active and inactive CPB plus IL-12-vaccinated mice and there was a distinct and significant increase in the IgG2a/IgG1 ratio ($p < 0.05$), although the IgG2a antibody titre was not significantly different from control mice.

4.3.6 Analysis of *Leishmania*-specific antibodies in vaccinated C57BL/6 mice

Mice vaccinated with inactive CPB and IL-12 displayed very low levels of IgG1. This was significantly lower than that observed in control mice ($p < 0.05$) although it was not significantly different from that of the active CP-vaccinated mice (Figure 4.3.5). Active CPB-immunised mice had comparable levels of IgG1 to that of the control mice and there was no signifcant difference between these two groups. The level of IgG2a was similar for all groups studied and there was no significant difference between the groups. The increase in the IgG2a/IgG1 ratio was not apparent in the CPB/IL-12-vaccinated mice compared with the change in ratio observed in the CPB/IL-12-vaccinated BALB/c mice.

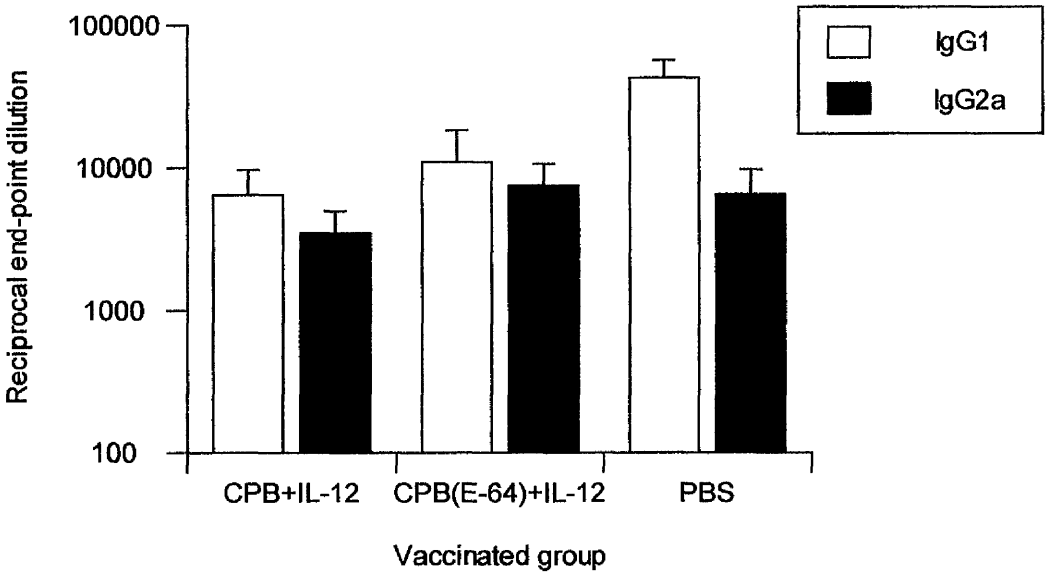

Figure 4.3.4. Analysis of plasma IgG1 and IgG2a levels in vaccinated BALB/c mice 5 mo after infection with wild type stationary phase *L. mexicana* promastigotes. Values represent mean end-point dilutions +/- SEM (n=4). IgG2a was not significantly different between the three groups but IgG1 was significantly lower in the CPB-immunised mice compared to the saline control mice ($p < 0.05$).

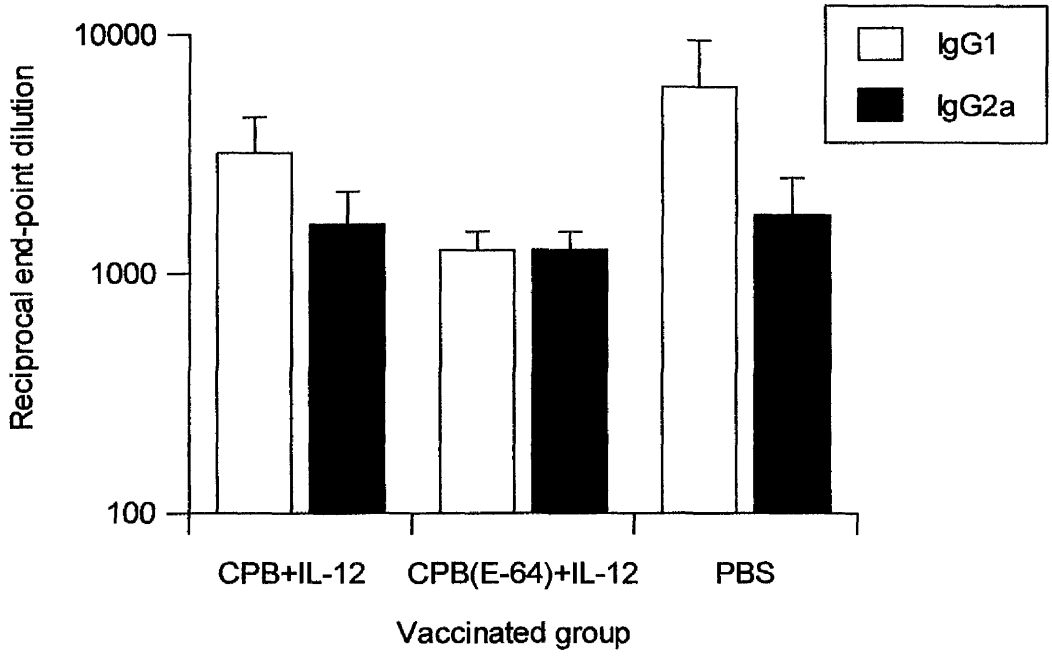

Figure 4.3.5 Analysis of plasma IgG1 and IgG2a levels in vaccinated C57BL/6 mice 5 mo after infection with wild-type stationary phase *L. mexicana* promastigotes. Values represent mean end-point dilutions +/- SEM (n=5). Inactive CPB-immunised mice displayed significantly lower levels of IgG1 than control mice ($p < 0.05$) but there was no significant difference compared to active-CPB immunised mice. For IgG2a production, there was no significant difference between the groups analysed.

4.3.7 Analysis of *Leishmania*-specific antibodies in vaccinated CBA mice

Control mice had significantly higher levels of IgG1 than the mice immunised with inactive CP and IL-12 ($p < 0.05$) (Figure 4.3.6). In contrast, levels of IgG1 were not significantly different between control mice and mice immunised with the active CP plus IL-12. There was no significant difference in levels of IgG2a isotypes in any of the groups analysed. The increase in the IgG2a/IgG1 ratio was not apparent in the CPB/IL-12-vaccinated mice compared with the change in ratio observed in the CPB/IL-12-vaccinated BALB/c mice and was similar to that observed in the CPB/IL-12-immunised C57BL/6 mice.

At 7 months post-infection, the IgG1 titres for control mice were significantly higher compared with control mice at 5 months post-infection ($p < 0.05$) (Figure 4.3.7). Control saline-injected mice had a significantly higher level of IgG1 than both the groups of mice immunised with the active or inactive CPB ($p < 0.05$ for both). However, there was no significant difference between IgG2a levels in any groups of mice, as IgG2a was high in saline-injected mice. The increase in IgG1 in mice analysed at 7 months compared with 5 months post-infection, resulted in a decrease in the IgG2a /IgG1 ratio in the saline-infected groups (1:3) compared with 5 months post-infection (1:2).

4.3.8 IFN-γ production by stimulated splenocytes from BALB/c vaccinated mice

IFN-γ production from splenocytes isolated from vaccinated BALB/c mice seven months post-infection and stimulated with SLA (10 μg protein /ml), recombinant CPB2.8ΔCTE (5 μg/ml) and Con A (5 μg/ml) are shown in Figures 4.3.8, 4.3.9 and 4.3.10, respectively.

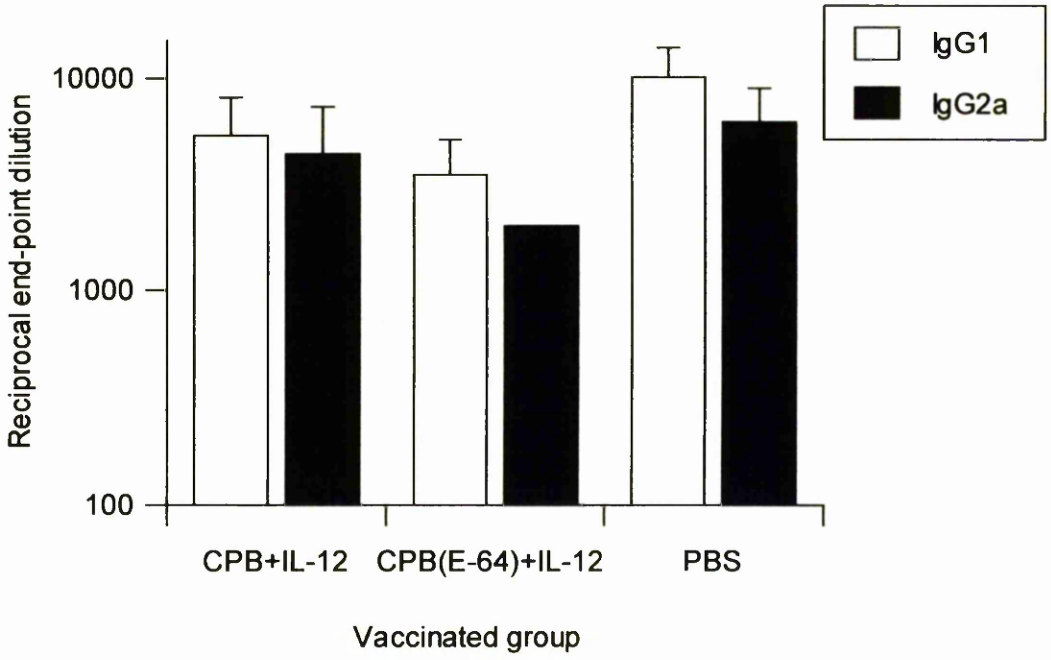

Figure 4.3.6 Analysis of plasma IgG1 and IgG2a levels in vaccinated CBA mice 5 mo after infection with wild type stationary phase *L. mexicana* promastigotes. Values represent mean end-point dilutions +/- SEM (n=5). Control mice had significantly higher levels of IgG1 than mice immunised with inactive CPB/IL-12 ($p < 0.05$) but displayed no significant difference between mice immunised with active CPB/IL-12. There was no significant difference in IgG2a production between all the groups of mice studied.

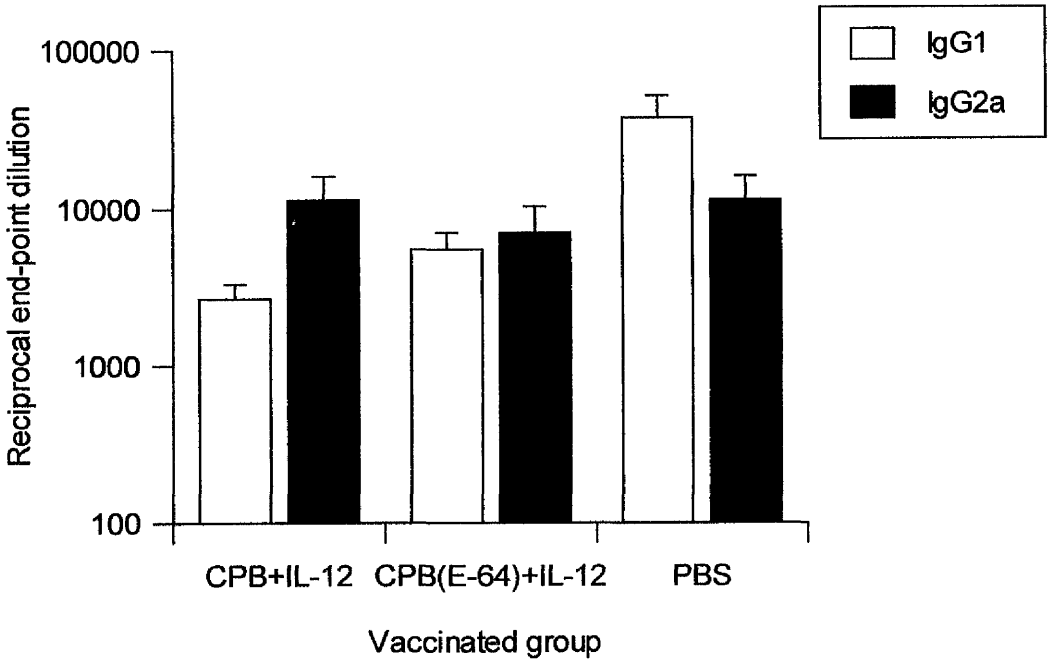

Figure 4.3.7 Analysis of plasma IgG1 and IgG2a levels in vaccinated CBA mice 7 mo after infection with wild type stationary phase *L. mexicana* promastigotes. Values represent mean end-point dilutions +/- SEM (n=5). The level of IgG1 was significantly higher in control mice compared to groups of mice immunised with active or inactive CPB/IL-12 ($p < 0.05$). IgG2a levels were not significantly different between the groups of mice analysed.

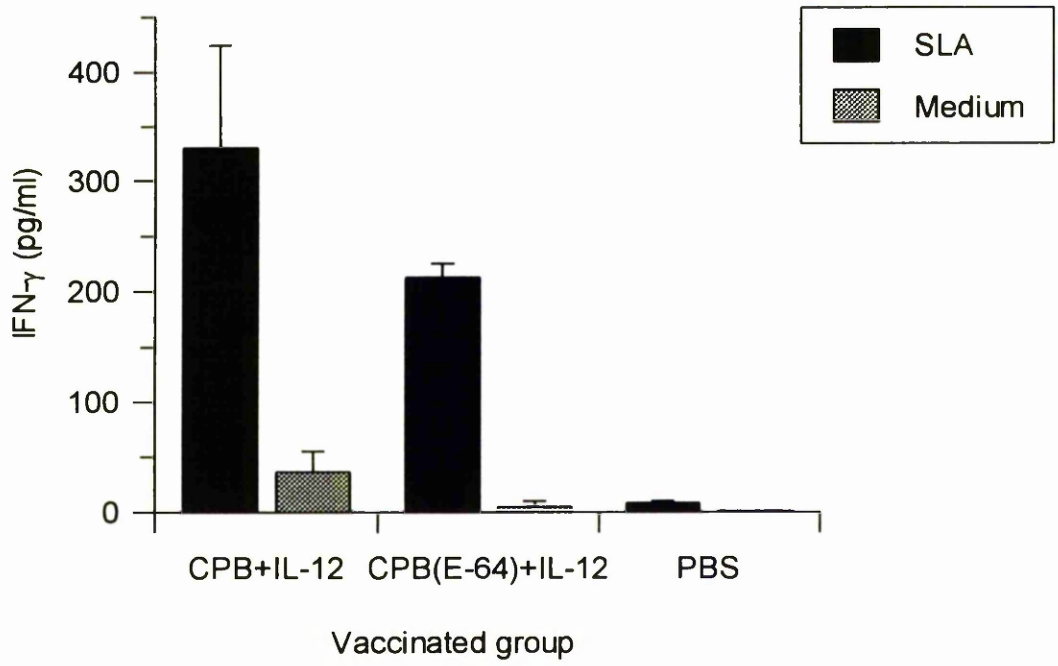

Figure 4.3.8 IFN-γ production by cultured splenocytes removed from CPB-immunised and control (PBS) BALB/c mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on soluble leishmanial antigen-stimulated (10 μg protein/ml) cultures with non-stimulated cultures used as controls. Bars represent SEM (n=4). Splenocytes from active and inactive CPB-immunised mice produced significantly higher levels of IFN-γ than control mice ($p < 0.02$; $p < 0.001$ respectively). There was no significant difference in IFN-γ levels between the two groups of CPB-immunised mice.

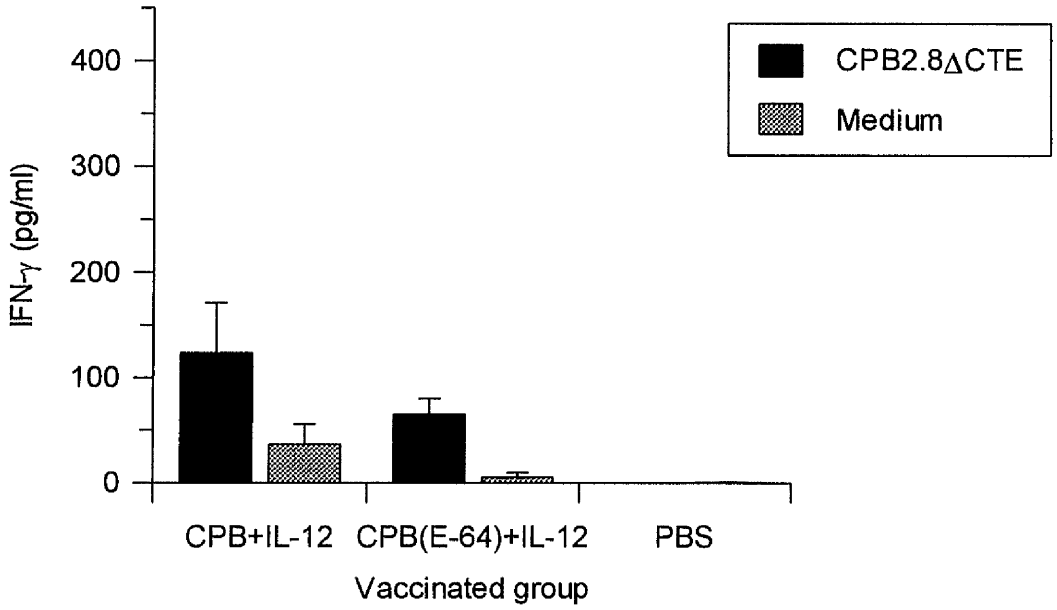

Figure 4.3.9 IFN-γ production by cultured splenocytes removed from CPB-immunised and control (PBS) BALB/c mice 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on CPB2.8ΔCTE -stimulated (5 μg/ml) cultures. Non-stimulated cultures were used as controls. Bars represent SEM (n=4).
Mice immunised with inactive CPB/IL-12 had significantly higher levels of IFN-γ than background ($p < 0.05$). Mice immunised with active CPB/IL-12 or injected with PBS did not produce levels of IFN-γ that were significantly different from background.

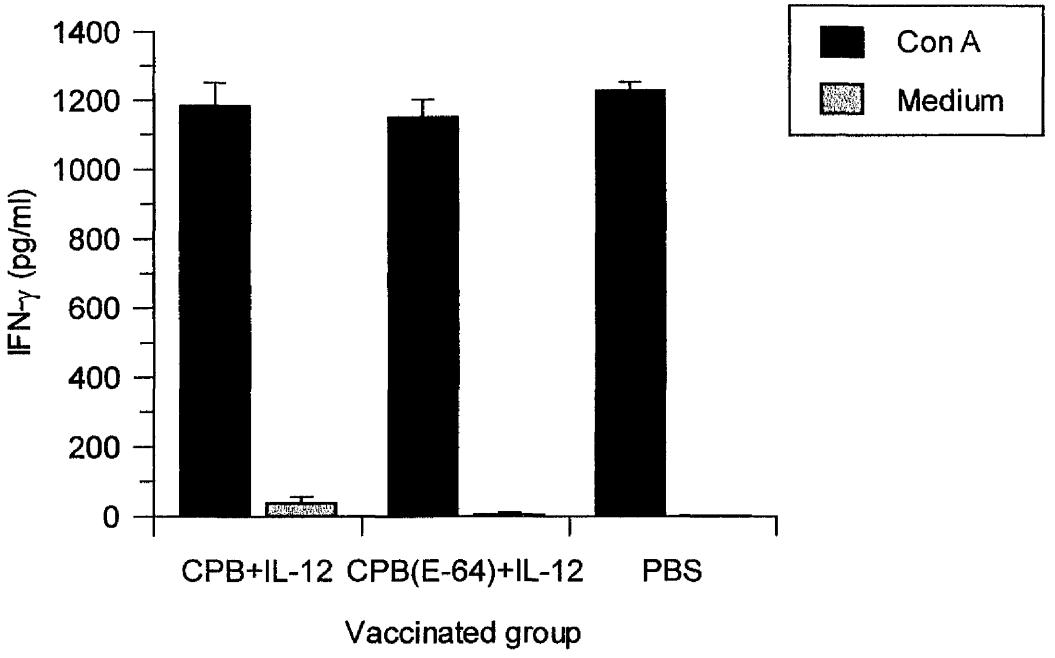

Figure 4.3.10 IFN-γ production by cultured splenocytes removed from CPB-immunised and control (PBS) BALB/c mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on Con A (5 μg/ml)-stimulated cultures. Bars represent SEM (n=4). All groups produced significantly higher levels than background ($p < 0.01$) but were not significantly different from each other.

Splenocytes from CPB-vaccinated mice, incubated with SLA produced significantly increased levels of IFN-γ compared with control mice (active CPB, $p < 0.02$; inactive CPB, $p < 0.001$) (Figure 4.3.8). There was no significant difference in splenocyte IFN-γ production between the active and inactive CPB-vaccinated mice.

When splenocytes were stimulated with recombinant CPB2.8ΔCTE (Figure 4.3.9), only the inactive CPB-immunised mice produced levels of IFN-γ that were significantly greater from the background levels ($p < 0.05$). Control mice injected with saline produced no detectable IFN-γ in response to recombinant enzyme stimulation. The level of IFN-γ produced from inactive CPB-vaccinated mice that were stimulated with SLA and CPB2.8ΔCTE was also different, with significantly more IFN-γ being produced by the former antigen stimulus ($p < 0.02$).

Con A stimulation increased IFN-γ production to significantly greater levels than background in all splenocyte cultures tested ($p < 0.01$) (Figure 4.3.10), with there being no significant difference between IFN-γ production by any of the groups studied.

4.3.9 IL-4 production by stimulated splenocytes from vaccinated BALB/c mice

While splenocytes from control mice injected with saline failed to produce a significant antigen-induced increase in IFN-γ production, there was a marked and highly significant increase in IL-4 production over background levels ($p < 0.001$), when splenocytes were stimulated with SLA (Figure 4.3.11) but not when stimulated with CPB2.8ΔCTE (Figure 4.3.12). In contrast, CPB-immunised mice

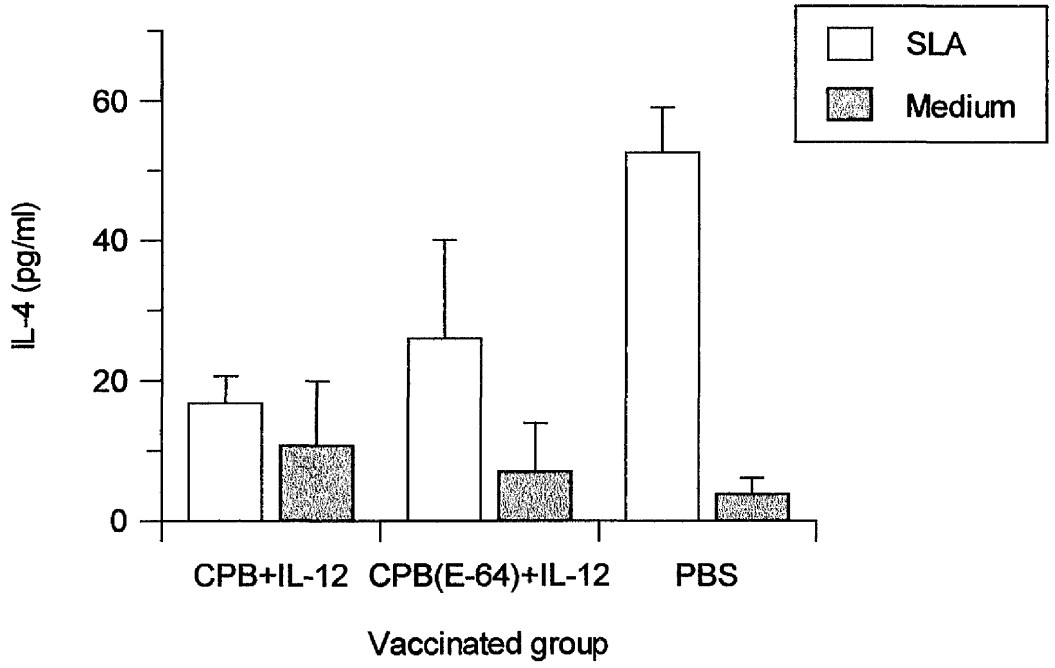

Figure 4.3.11 IL-4 production by cultured splenocytes removed from CPB and control (PBS) BALB/c mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on SLA-stimulated (10 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEM (n=4). Control (PBS) mice produced significantly higher levels of IL-4 compared with background ($p < 0.001$) while both CPB-immunised groups did not.

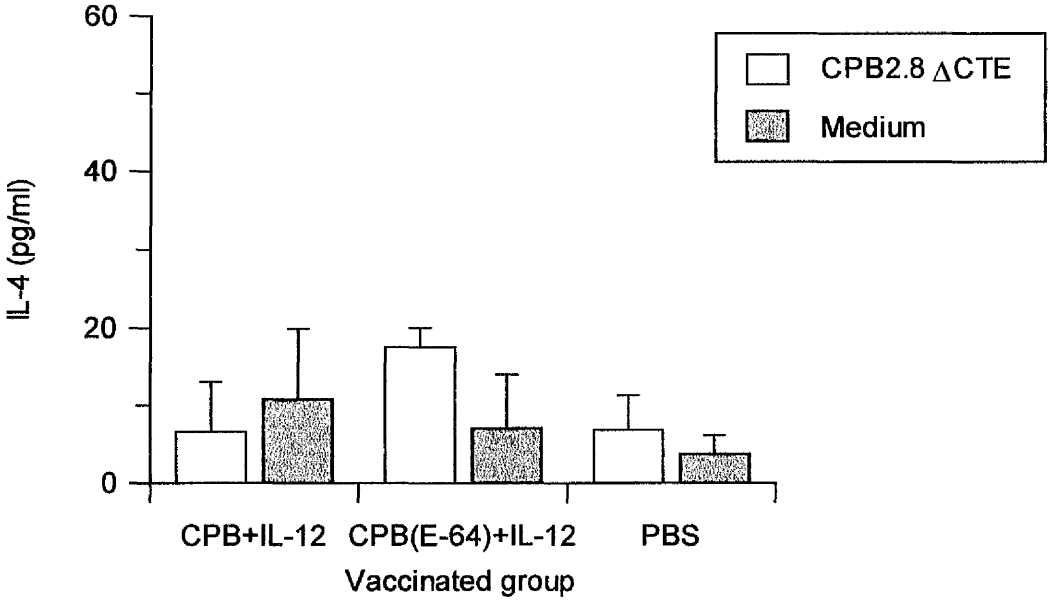

Figure 4.3.12 IL-4 production by cultured splenocytes removed from CPB-vaccinated and control (PBS) BALB/c mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on CPB2.8ΔCTE-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEM (n=4). CPB-stimulated cultures did not produce significantly higher levels of IL-4 compared with background in all groups studied.

did not produce levels of IL-4 that were significantly different from background levels when stimulated with SLA or recombinant enzyme (Figures 4.3.11 and 4.3.12). Con A-induced splenocyte IL-4 production was significantly greater ($p < 0.01$) than background levels for all groups of immunised mice (Figure 4.3.13).

4.3.10 IFN-γ production by stimulated splenocytes from C57BL/6 vaccinated mice

IFN-γ production from splenocytes isolated from C57BL/6 mice seven months post-infection and stimulated with SLA (10 μg protein/ml), recombinant CPB2.8ΔCTE (5 μg/ml) and Con A (5 μg/ml) is shown in Figures 4.3.14, 4.3.15, and Figure 4.3.16, respectively.

Stimulation with the SLA preparation, significantly increased IFN-γ production in comparison with background levels in mice vaccinated with active ($p < 0.0001$) and inactive ($p < 0.01$) CPB (Figure 4.3.14). In contrast, there was no significant increase with splenocytes from control mice, when stimulated with SLA. There was no significant difference in IFN-γ production between splenocytes from active and inactive CPB-immunised mice when stimulated with SLA.

When splenocytes were stimulated with recombinant CPB enzyme, the level of IFN-γ production in all groups of mice was very low and not significantly different from background levels (Figure 4.3.15).

Con A stimulation increased IFN-γ production to substantially greater levels than background in all splenocyte cultures tested (Figure 4.3.16) ($p < 0.01$) but there was no significant difference between the groups studied.

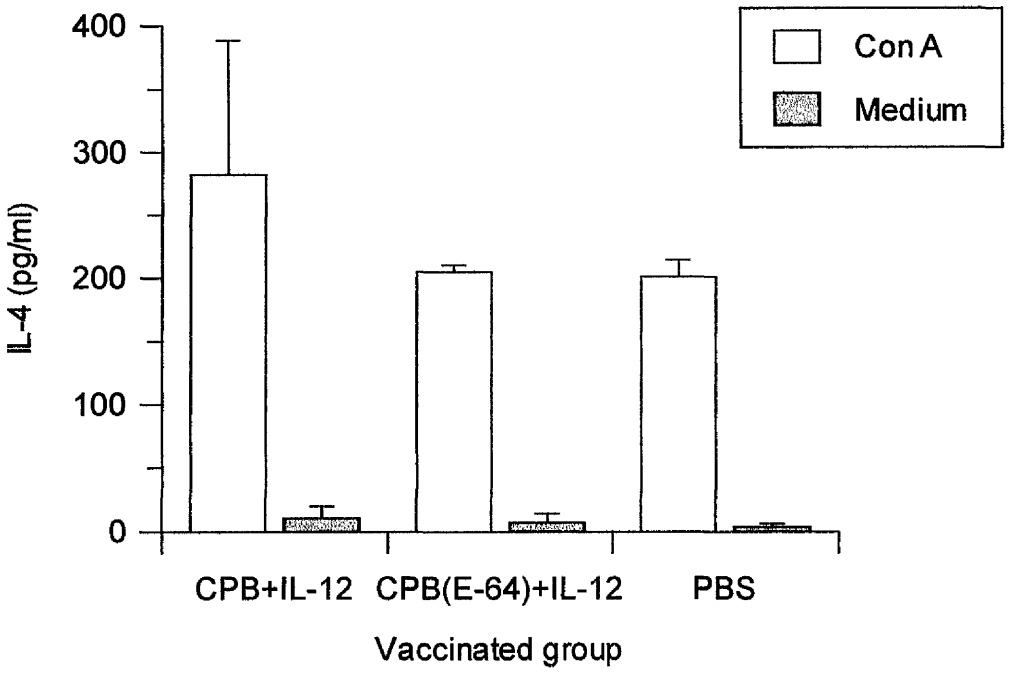

Figure 4.3.13 IL-4 production by cultured splenocytes removed from CPB-vaccinated and control (PBS) BALB/c mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on Con A-stimulated (5 μg/ml) cultures. Bars represent SEM (n=4). All Con A-stimulated cultures produced significantly higher levels of IL-4 compared with background ($p < 0.01$) but were not significantly different from each other.

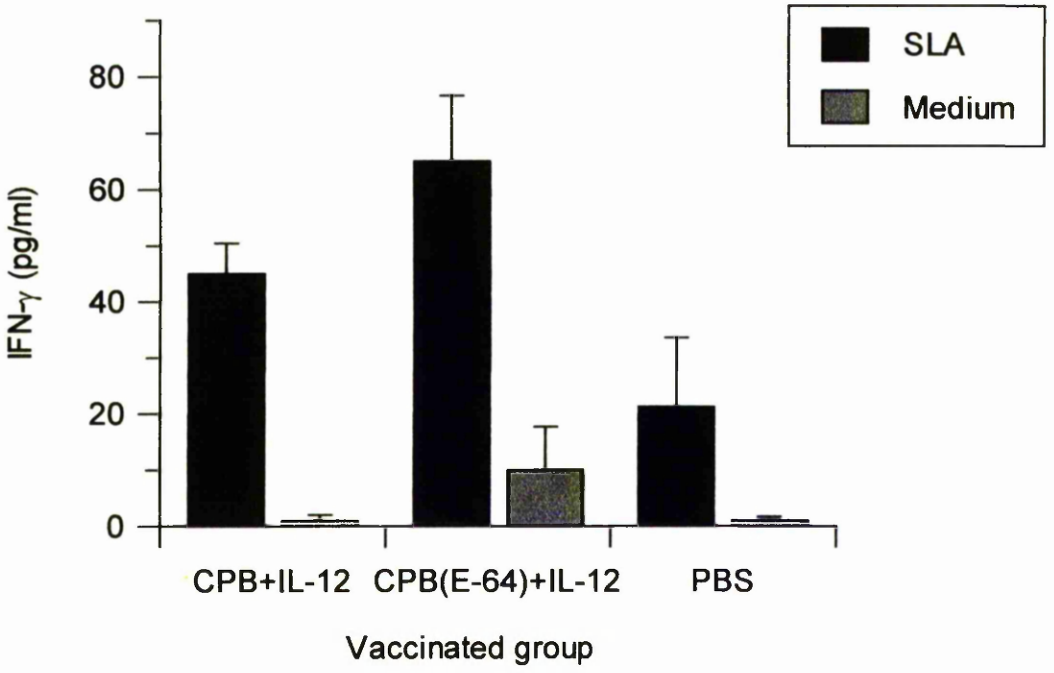

Figure 4.3.14 IFN-γ production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on SLA (10 μg/ml)-stimulated cultures. Background levels are shown for comparison. Bars represent SEM (n=5). Mice immunised with active CPB and inactive CPB produced significantly higher levels of IL-4 ($p < 0.0001$; $p < 0.01$ respectively) than background while control mice did not. There was no significant difference in splenocyte production of IL-4 between the two CPB-immunised groups.

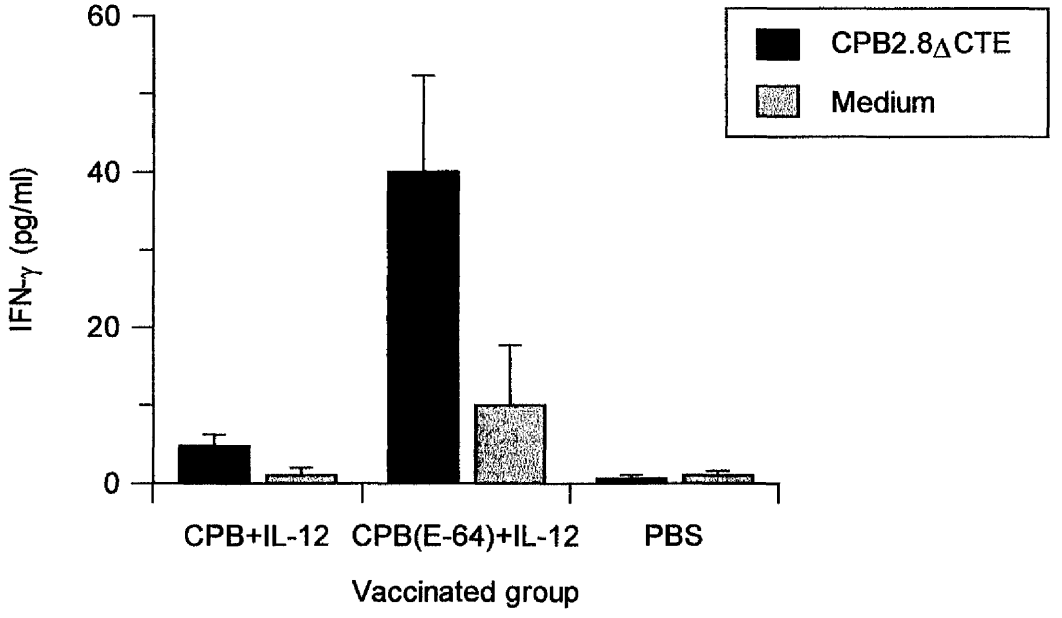

Figure 4.3.15 IFN-γ production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on CPB2.8ΔCTE (5 μg/ml) - stimulated cultures. Bars represent SEM (n=4). Splenocyte production of IL-4 was not significantly higher than background levels, in all groups studied.

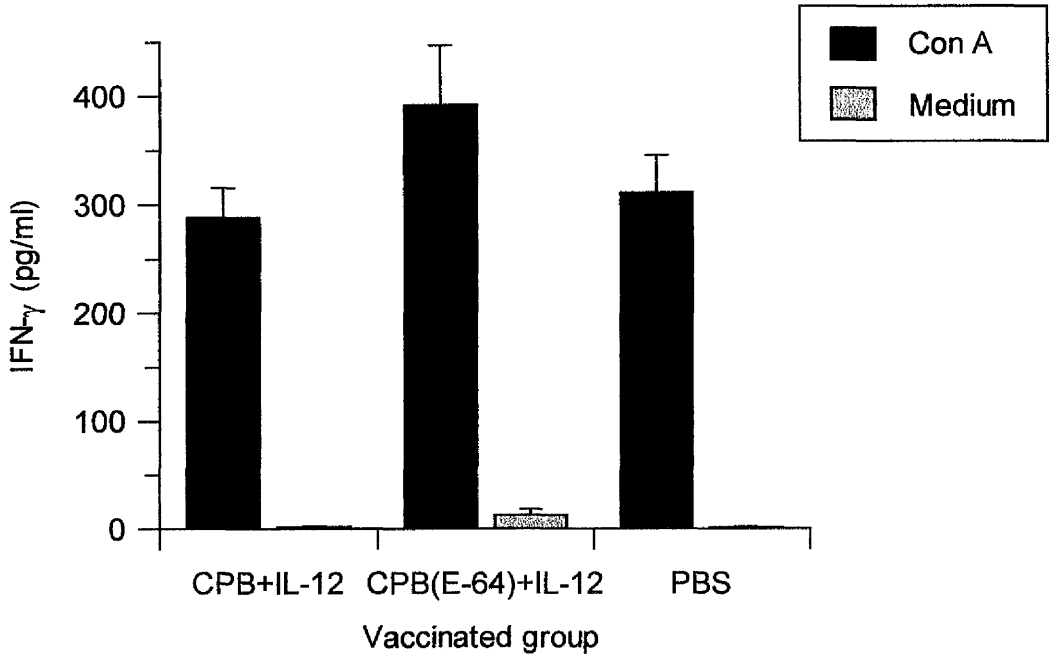

Figure 4.3.16 IFN-γ production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on Con A (5 μg/ml) - stimulated cultures. Bars represent SEM (n=4). All groups produced significantly higher levels of IFN-γ than background levels ($p < 0.01$) but there was no significant difference between the groups analysed.

4.3.11 IL-4 production by stimulated splenocytes from C57BL/6 vaccinated mice

While splenocytes from control C57BL/6 mice showed no antigen-induced increase in IFN-γ, there was a marked increase in IL-4 production, when stimulated with SLA ($p < 0.01$) (Figure 4.3.17). However, CPB-vaccinated mice did not exhibit a significant increase in IL-4 production compared with background levels (Figure 4.3.17) nor did they respond to stimulation with recombinant enzyme, in terms of IL-4 production (Figure 4.3.18). Moreover, control mice did not produce a significantly different level of IL-4 compared to background levels when stimulated with CPB2.8ΔCTE (Figure 4.3.18).

Con A polyclonal stimulation did produce much greater levels of IL-4 than background levels, in all groups of mice tested ($p < 0.0001$) (Figure 4.3.19). Splenocytes from mice immunised with active CPB/IL-12 had significantly higher levels of IL-4 than splenocytes from mice immunised with inactive CPB/IL-12 ($p < 0.01$) but no greater than control mice.

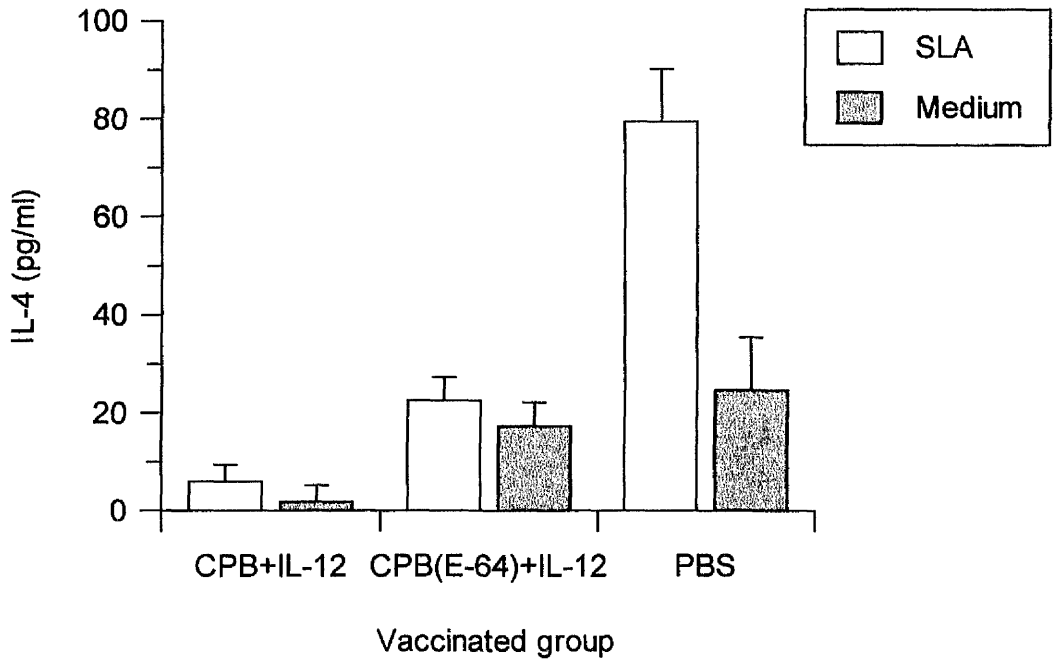

Figure 4.3.17 IL-4 production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on SLA (5 μg/ml) -stimulated cultures. Bars represent SEM (n=5). Control (PBS) mice produced significantly higher levels of IL-4 than background levels ($p < 0.01$) whereas CPB-immunised mice did not.

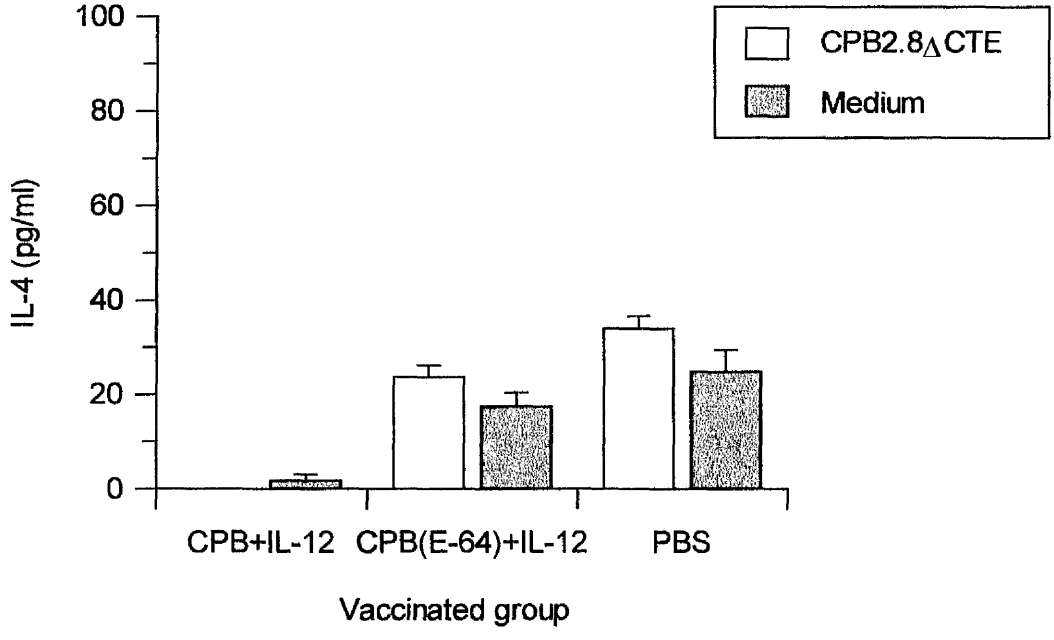

Figure 4.3.18 IL-4 production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on CPB2.8ΔCTE (10 μg/ml) - stimulated cultures. Bars represent SEM (n=5). Splenocyte production of IL-4 from all groups was not significantly higher than background levels.

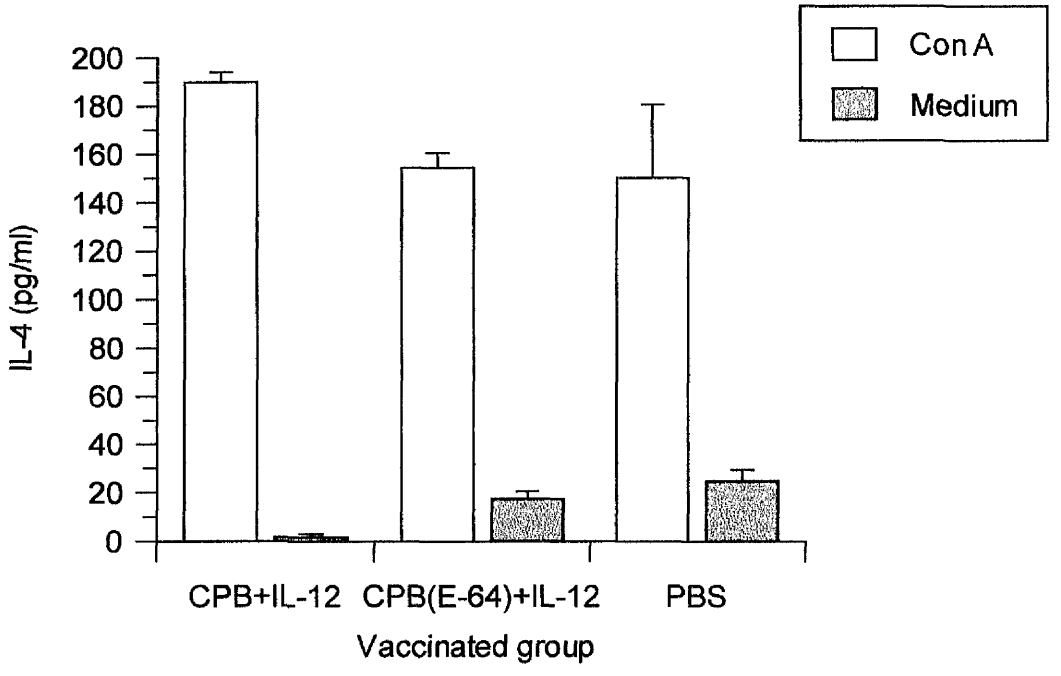

Figure 4.3.19 IL-4 production by cultured splenocytes removed from CPB-vaccinated and control (PBS) C57BL/6 mice, 7 mo post-infection with wild-type stationary phase *L. mexicana* promastigotes. Cytokine analysis was performed on Con A (5 μg/ml)-stimulated cultures. Bars represent SEM (n=4). All groups produced significantly high levels of IL-4 compared with background levels ($p < 0.0001$). Active CPB-immunised mice had significantly higher levels of IL-4 than inactive CPB-immunised mice ($p < 0.01$) but were not significantly different from control mice.

4.4 DISCUSSION

It is well documented that a protective immune response against cutaneous leishmaniasis is associated with a type 1 response and IFN-γ production (Heinzel *et al.,* 1989). Therefore, successful vaccination requires administration of defined antigens together with an adjuvant that preferentially stimulates the expansion of IFN-γ secreting *Leishmania*-specific Th 1 cells.

Previous studies have shown that CPB2.8 is a potential T cell immunogen and is capable of eliciting a protective Th 1 cell line with concurrent production of IFN-γ (Wolfram *et al.,* 1995). It is therefore very encouraging for future vaccine strategies that the immunisation of all three mouse strains of mice with CPB2.8ΔCTE and IL-12 significantly protected against *L. mexicana* infection. In each of the mouse strains tested, development of parasite lesions was significantly reduced in volume in the CPB-immunised mice compared with control mice, which developed much larger lesions. Furthermore, some of the vaccinated animals healed and others failed to develop lesions.

The argument that IL-12 alone, is the factor required for protection against *Leishmania* rather than the mixture of antigen and adjuvant has been dispelled in several studies since IL-12 on its own is not enough to protect mice against leishmanial infection (Aebischer *et al.,* 2000; Afonso *et al.,* 1994). This in part may be due to the very short half-life of three hours when injected into rodents (Kenney *et al.,* 1999). This may provide a limited window of opportunity to vaccinate when the conditions are right to promote a Th 1 response. Furthermore, administration of IL-12 alone to naive mice has been shown to induce the expression of IL-10 (Melby *et al.,* 1996), a cytokine capable of exacerbating *Leishmania* infection (Suffia *et al.,* 2000).

BALB/c mice are particularly susceptible to infection with *Leishmania* and a previous vaccine study incorporating CP as the protective antigen has been unsuccessful at protecting this mouse strain against *Leishmania pifanoi* (Soong *et al.,* 1995). It was therefore encouraging to see that the enzyme administered with IL-12 afforded a large degree of protection against infection in this strain. Indeed, not only were lesions significantly smaller in vaccinated animals but some healed completely. Strong protection was also seen in C57BL/6 mice in this study. On the other hand, Aebischer *et al.* (2000), using inactive, misfolded CPB2.8 and recombinant IL-12 was also able to partially protect C57BL/6 mice but not BALB/c or CBA mice. It is likely that the different immunisation protocol between the two groups, were responsible for the observed phenotypes. Several differences relating to the antigen itself are apparent. Aebischer *et al.* (2000) immunised mice with half the dose of enzyme used in our study but it is unlikely that the difference between the two doses would be sufficient to allow for protection in C57BL/6 and not in BALB/c or CBA mice. It is more feasible that the nature of the proteinase used by Aebischer for immunisation was the determining factor. Due to the known proteolytic capability of CPB2.8ΔCTE, a precautionary step was taken in my experiments whereby IL-12 was injected a short time before immunising with CPB2.8ΔCTE. In contrast, Aebischer (2000) immunised with native CPB and IL-12 concurrently. Presumably, the native CPB was proteolytically active and could have degraded IL-12 at the site of inoculation. The effect of this could be to partially inhibit the Th 1-potentiating effects of IL-12 so that BALB/c and CBA mice were not protected against *Leishmania* infection. Alternatively, the inactive, misfolded CPB used for immunisation in the Aebischer (2000) study would be structurally different from

the native CPB molecule. Thus, antigen processing and epitope selection could be different from native antigen, with consequences for the developing immune response (Abbas *et al.,* 1994).

Another study involving purification of a native CP from *L. amazonensis* and vaccination with this enzyme did confer protection to infection with *L. amazonensis*, in BALB/c mice (Beyrodt *et al.,* 1997) but the choice of adjuvant, FCA, could not be used for human vaccine trials. However, the purified proteinase did elicit IFN-γ and IL-2 from splenocytes harvested from vaccinated mice and so this enzyme could afford protection to *L. amazonensis* infection. Our results agree with those of Beyrodt *et al.* (1997) in that the purified CP was protective against the respective leishmanial infection and could elicit IFN-γ production from splenocytes removed from vaccinated mice. However, IL-2 was not measured in this study but in future, this should be performed.

An examination of the humoral and cellular immune responses of BALB/c mice following vaccination with CP and infection is consistent with CPB2.8ΔCTE and recombinant IL-12 partially protecting against wild-type *L. mexicana* infection by promoting a Th 1 response. The level of IgG1 and IgG2a isotypes in control PBS-immunised mice was very similar to that seen by Alexander *et al.* (1998) with high IgG1 titres and low levels of IgG2a. However, immunisation with active or inactive CPB2.8ΔCTE and IL-12 significantly decreased IgG1, suggesting more of a developing Th 1 response. The observation that there was no increase in IgG2a production in CPB-vaccinated mice is interesting since Aebischer *et al.* (2000) found that mice vaccinated with CPB and IL-12 produced IgG2a titres against parasite lysate that were ten times higher than in non-vaccinated animals. IL-12 is known to up-regulate IgG2a antibody production

by production of IFN-γ (Trinchieri, 1995). Consequently, we may have expected elevated IgG2a levels from the CPB/IL-12 vaccinated mice in this study, since these mice were strongly protected against *Leishmania* infection and were shown to produce splenocyte IFN-γ compared to controls, in response to SLA stimulation. Injection of a CP from the house dust mite, has been shown to stimulate IgE production (Gough *et al.,* 1999) and it is possible that injection of CPB2.8ΔCTE may have resulted in such an increase in IgE with subsequent antibody isotype switching from IgG2a to IgE (Finkelman *et al.,* 1990). Detection of IgE was to be carried out by collaborators at the University of Strathclyde.

The direct evidence for a change in T helper cell phenotype following vaccination during *L. mexicana* infection was visualised by analysis of splenocyte production of IFN-γ and IL-4. Control mice exhibited high IL-4 and virtually no IFN-γ production when stimulated with parasite lysate, indicative of a Th 2 response as previously seen in wild-type *L. mexicana* infected BALB/c mice (Alexander *et al.,* 1998). In contrast, mice immunised with CPB and IL-12 displayed significantly higher IFN-γ and lower IL-4 levels upon stimulation with parasite lysate, indicative of a protective Th 1-type response (Alexander *et al.,* 1998).

Collectively, the data indicate that CPB2.8ΔCTE plus recombinant IL-12 can switch the Th 2 response normally seen in *Leishmania* infection to a protective Th 1 response. This agrees with several studies performed using *Leishmania* antigen and recombinant IL-12 as a protective combination in establishing a Th 1 phenotype and protecting against *Leishmania* infection (Afonso *et al.,* 1994; Aebischer *et al.,* 2000). The results also indicate that a

leishmanial CP is potentially useful as a protective antigen, as seen with other systems (Soong *et al.,* 1995; Rafati *et al.,* 2000).

However, the question remains as to what effect the enzyme activity of the CPs *per se* have on the outcome of *Leishmania* infection. Vaccination with active and inhibited enzymes plus IL-12, exhibited equally protective effects, in terms of reduction in lesion size, decreasing IgG1 production and increasing IFN-γ production with concomitant reduction of IL-4. IFN-γ production from splenocytes stimulated with active CP has been recently reported in a vaccine study against *L. major* infection (Rafati *et al.,* 2000) and so we may have expected IFN-γ production from the two CPB-immunised groups, when stimulated with recombinant CPB2.8ΔCTE. When splenocytes from both BALB/c and C57BL/6 strains of mice were re-stimulated with CPB2.8ΔCTE, there was no IL-4 production in any of the groups (active or inactive CPB-immunised or control) assessed. In contrast, IFN-γ production from splenocytes removed from mice immunised with CPB was demonstrated in the BALB/c mouse strain and correlates well with the results from Rafati *et al.* (2000). Splenocytes from CPB-immunised C57BL/6 mice did not produce IFN-γ in response to CPB2.8ΔCTE but the enzyme used to re-stimulate these cells was from a different protein preparation than that used to stimulate the BALB/c mice. Although the enzyme was used at a similar protein concentration, the refolding process may have yielded a slightly different conformation of enzyme (since refolding is never exactly the same each time for a particular protein), which may not have stimulated the splenocytes as well as the first batch of enzyme.

The humoral and cellular immune response in C57BL/6 vaccinated mice was not as clearly defined as that observed in BALB/c mice although protection

was seen in both active and inactive CPB-vaccinated mice. As with CPB-vaccinated BALB/c mice, the level of protection conferred was no different when active or inactive proteinase was used to immunise mice. Antibody titres were much lower in C57BL/6 vaccinated mice and this may be attributed to the rate of parasite growth at the five month time-point of infection. By this time-point, all groups of the BALB/c mice had a greater mean lesion volume than all the groups of C57BL/6 mice and so the latter strain may not have been exposed to as many parasite antigens as the former and as a consequence had not produced as much antibody as the BALB/c vaccinated mice. The level of IgG2a was similar in all groups of C57BL/6 vaccinated mice and the level of IgG1 was reduced in control mice compared with BALB/c control mice. The general antibody titre for both groups of C57BL/6-vaccinated mice was low and only the active CPB-vaccinated mice had a significantly lower IgG1 titre than the control immunised mice. These results are very different from those reported by Aebischer *et al.* (2000), who found that C57BL/6 mice vaccinated with misfolded CPB2.8 and IL-12 presented higher IgG2a titres than PBS control mice. However, the infecting parasite dose was doubled in these experiments and so exposure to lysed parasite material and therefore antigens would be quicker in those vaccinated mice compared with our study.

Analysis of the cytokine responses suggest that there was a developing Th 1 response in the C57BL/6 mice. CPB-vaccinated mice displayed significantly higher IFN-γ levels and lower IL-4 levels than control mice which presented typically low levels of IFN-γ and high levels of IL-4 when stimulated with SLA. These results correlate well with those of Aebischer and colleagues (2000), in

terms of IFN-γ production, who also found that CPB and IL-12 vaccinated mice produced high levels of IFN-γ compared to PBS control mice.

The apparent contrast in antibody and cytokine results may be due to the fact that antibody analysis was for a time-point at five months post-infection, while the cytokine values were analysed at seven months post-infection. By seven months, the progression in lesion development is more disparate than was seen at five months post-infection and the cytokine data confirm this conclusion. In retrospect, it would have been interesting had the antibody analysis been measured nearer the time of splenocyte preparation.

CBA mice displayed a similar development in lesion progression and antibody isotype levels to the C57BL/6 vaccinated mice. The inactive CPB-vaccinated group of mice displayed levels of IgG1 which were significantly lower than control mice but there was no significant difference in IgG1 production between control and active CPB-vaccinated mice. Again, this may be due to the tailbleeds being taken at five months post-infection, when the course of infection was not as well established as seven months post-infection. Tailbleeds were therefore taken at seven months post-infection and the IgG1 titre was markedly higher in the control mice than in either of the CPB-vaccinated mice which indicated more of a progressive infection compared to the CPB-immunised mice. However, there was no significant difference between the control and CPB-immunised mice in terms of IgG2a production.

CPB2.8ΔCTE used in this study can be readily produced from bacteria in large amounts and in highly purified form. Its use as an antigen suitable for immunisation against *L. mexicana* infection has been shown in this study and others (Aebischer *et al.*, 2000) but administration of this protein requires a more

suitable adjuvant since IL-12 is presently not considered a safe adjuvant for use in humans because of significant side effects (Atkins *et al.,* 1997).

Results from a collaborating laboratory have shown that IL-12 is needed for protection against *L. mexicana* infection using CPB2.8ΔCTE as the antigen, since active or inactive enzyme alone, was not enough to protect mice against infection with this parasite. This correlates well with other studies when antigen alone did not confer protection against *Leishmania* infection and the presence of IL-12 was found to be essential (Kenney *et al.,* 1999; Aebischer *et al,* 2000). In fact, Kenney *et al.* (1999) reported exacerbation of disease compared with control mice, when IL-12 was excluded from the immunisation regimen, which included freeze/thawed promastigote lysates. In this study, one group of mice (C57BL/6, inactive CPB-immunised mice without IL-12) at the University of Strathclyde, displayed a significant level of disease exacerbation, greater than that of the control group, immunised with PBS. One reason that CPB alone may not promote a protective immune response is probably due to the fact that transcripts encoding the IL-12 receptor β-chain are selectively inhibited by soluble antigen but are enhanced by IL-12 co-administration (Galbiati *et al.,* 1998). Moreover, soluble protein antigen appears to be selectively presented *in vitro* by B cells, which may favour Th 2 development as they fail to secrete IL-12 (Guery *et al.,* 1997). Furthermore, one may expect that injection of a cysteine proteinase *in vivo* would result in an increase in IL-4 production and therefore disease exacerbation, since injection of papain has been shown to increase mRNA for IL-4, one hour after injection (Finkelman & Urban, 1992). It is possible that the recombinant enzyme was not as active as that of native papain, since it has been shown that active CPB2.8ΔCTE was only 30 % active (Sanderson *et al.,* 2000)

whereas we may expect all the papain (native) molecules to be approximately 100 % active. Analysis of mRNA for IL-4 is now being investigated at the University of Strathclyde to see if there is an increase in mRNA production after injection of active and inactive CPB2.8ΔCTE in mice.

Ultimately, the success of a CP vaccine will be dependent on how it is adjuvanted. A number of different adjuvants have been tested with purified CPs including FCA (Rafati *et al.,* 2000) but this is incompatible with human use. Other adjuvants that could be tested include IL-18. IL-18 has been shown to induce IFN-γ production and up-regulate IL-12 receptor expression on the macrophage cell surface (Eberl *et al.,* 2000). IL-18 has been used successfully to protect mice against pulmonary and disseminated infection with *Cryptococcus neoformans* by inducing IFN-γ production (Kawakami *et al.,* 1997). However, IL-18 has not yet been tested for therapeutic use in humans, so its value in human use as a vaccine is not known.

A parasite CP from *E. histolytica* has been shown to possess high affinity for liposomes and yet remain active (Jacobs *et al.,* 1998). Liposome formulations are currently being investigated for purposes of vaccination and some success has been already achieved in immunisation against *Leishmania* infection using the surface metalloproteinase, gp63, with liposomes (Russell & Alexander, 1988). Administration of CPB2.8ΔCTE as a liposome formulation would target the active enzyme to the host macrophage population, since phagocytosis of such molecules would occur, resulting in MHC class II presentation of parasite molecules to the host immune system. This may also reduce the potentially aggravating effects of injecting an active CP into the mammalian host.

Research in this exciting area is currently being explored in a collaborating laboratory.

CHAPTER 5

The effects of recombinant CPB2.8ΔCTE on macrophage function

5.1 INTRODUCTION

Leishmania parasites can survive within the hostile environment of a macrophage and are able to resist or circumvent or inhibit the microbicidal response by ways which include scavenging oxygen radicals generated during the respiratory burst (Chan *et al.,* 1989) and suppressing NOS2 expression and NO production (Proudfoot *et al.,* 1996). If the macrophage is in a fully activated state, normally under the influence of IFN-γ, there will be an increase in nitric oxide and IL-12 production and subsequent killing of the parasite with a concomitant increase in antigen presentation of parasite molecules (Liew & O' Donnell, 1993). Presentation of parasite antigens by class II MHC molecules on antigen-presenting cells causes expansion of the protective IFN-γ producing Th 1 subset and has been thought to be essential for control of infection (Liew & O' Donnell, 1993).

The switch of CD4+ T cell differentiation from Th 2 to a Th 1-type profile was seen when a cathepsin B inhibitor was used during experimental *Leishmania* infection. This suggested that alteration in antigen processing could modulate the polarity of T helper cell differentiation (Maekawa *et al.,* 1998). Parasite viability was not directly affected by the inhibitors. Inhibition of MHC class II presentation is one possible mechanism by which the leishmanial CPs could

potentially influence the generation of a protective immune response. For example, it has been shown in studies using *L. amazonensis* that MHC class II molecules are internalised and degraded by CPs of parasite origin although it is unknown how the parasites do this. This seems an important means of preventing antigen presentation and thus promoting parasite survival (De Souza Leao *et al.*, 1995).

Generally in *Leishmania* infection, macrophage inflammatory functions are down-regulated and they produce significant levels of the regulatory cytokine, IL-10, which inhibits T cells and anti-microbial functions of macrophages by down-modulating macrophage leishmanicidal activity (Barral *et al.*, 1993). IL-10 is also an antagonist of IFN-γ production and activity and so antigen-induced production of this cytokine is of particular interest since IL-10 production correlates with the degree of pathology (Bogdan and Rollinghoff, 1998). Furthermore, it is known that another Th 2-type cytokine, TGF-β, is produced during leishmanial infection and this too has a part to play in disease exacerbation by down-regulating IL-12 expression and activity (Bogdan & Rollinghoff, 1998). TGF-β is the most potent known suppressor of NOS expression in mouse macrophages and does this by destabilising NOS mRNA, retarding synthesis of the protein and accelerating its degradation (Vodovotz *et al.*, 1993).

A non-healing response against *L. mexicana* may come about for several reasons including inhibition of MHC presentation, down-regulation of IL-12 or induction of IL-4. CPs have been shown to influence MHC (De Souza Leao *et al.*, 1995) and IL-4 production (Finkelman & Urban, 1992) and there is indirect evidence they could influence macrophage cytokine production (Maekawa *et al.*, 1998). We therefore studied whether CPB2.8ΔCTE had the potential to influence

cytokine production from activated peritoneal exudate cells, in particular IL-10, IL-12 and also nitric oxide output. Any significant alteration in production of one of these important immune mediators could conceivably influence the balance between a Th 1- or Th 2-type response and determine the fate of the parasite.

5.2 METHODS

5.2.1 Harvesting of peritoneal exudate cells from BALB/c mice

Age-matched adult female BALB/c mice were used in all experiments unless otherwise stated. 1 ml of sterile, 4% (w/v) thioglycollate was injected into the peritoneum of mice 4 days before use, to elicit inflammatory macrophages.

RPMI-1640 (Gibco-BRL) was supplemented with 10 % (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (the resulting solution was designated complete RPMI). The mice were sacrificed using CO_2 and 5 ml of pre-chilled complete RPMI was injected into the peritoneal cavity using a 25 gauge needle. The peritoneum was massaged for approximately 5 minutes before the medium was withdrawn yielding 3-4 ml of PECs suspension at a cell density of $1x10^6$/ml. 200 µl aliquots were added to each well of a flat-bottomed tissue plate (Costar) in triplicate and all cells were incubated overnight at 32°C in 95% air/5% CO_2 to allow the macrophages to adhere. The plates were then washed in complete RPMI to remove any non-adherent cells.

5.2.2 Incubation of PECs with CPB2.8ΔCTE

The adherent cells (designated macrophages) were incubated in complete RPMI with various components (Table 6) at 32°C in 95% air/5% CO_2 for 48

hours to allow production of IL-10, IL-12 and NO_2^-. After 48 hours, supernatants were frozen and stored at -70°C before analysis for IL-10 and IL-12 by ELISA as detailed in section 4.2.6, chapter 4. Antibodies for detection of IL-12 were a kind gift from Phil Scott, University of Pennsylvania. Antibodies for detection of IL-10, were purchased from Pharmingen. NO_2 was measured by the Griess reaction. Macrophages were activated with LPS and IFN-γ since ingestion of most microbes by macrophages elicits autocrine production of TNF-α and IFN-γ (Macmicking *et al.*, 1997). Addition of the recombinant enzyme (100 ng/ml) to these activated macrophages would more closely resemble that of the situation *in vivo*.

Component
LPS (100 ng/ml)
LPS/IFN-γ (50 U/ml)
LPS/IFN-γ and CPB2.8ΔCTE (100 ng/ml)
LPS/IFN-γ and CPB2.8ΔCTE and E-64 (10 μM)
LPS/IFN-γ and boiled CPB2.8ΔCTE (100 ng/ml)
LPS/IFN-γ and wild type lysate (100 ng protein/ml)
LPS/IFN-γ and buffer (PBS, pH 6.0)

Table 6. Stimuli used to assess IL-10, IL-12 and NO_2^- production from PECs

PECs were incubated, in triplicate, with 100 µl of RPMI containing various stimuli shown in table 6. For activation of macrophages, cells were stimulated with LPS and IFN-γ. To analyse the effect of active enzyme on activated macrophages, 100 ng/ml of CPB2.8ΔCTE was added to medium before addition of LPS and IFN-γ. This concentration was used subsequently for all other macrophage experiments. To assess the importance of CP activity, CPB2.8ΔCTE was inhibited with E-64 (10µM) and this concentration of E-64 was found to be the minimum amount that was sufficient to ablate all CP activity (result not shown). Recombinant enzyme was also boiled for 10 min to denature the CP. A lysate of wild-type *L. mexicana* promastigotes was also added to the activated macrophages to assess whether this could potentially affect IL-10 cytokine production. As a control, PBS was added at similar volumes as the enzyme to assess whether buffer could affect cytokine production.

5.2.3 Measurement of NO_2^- from macrophage supernatants

The production of nitric oxide by macrophages during incubation with CPB2.8ΔCTE was measured using the Griess reaction (modified from Green *et al.*, 1982) to detect the nitrite produced during nitric oxide degradation. Resident peritoneal macrophages are relatively resistant to activation by factors such as IFN-γ, producing low levels of microbicidal effector mechanism molecules (Adam & Hamilton, 1992). In order to detect nitric oxide, it was necessary to use macrophages which had been primed for activation via elicitation with intraperitoneal injection with thioglycollate broth. Injection of such broth into the peritoneum causes the migration of immature mononuclear phagocytes from other tissues into the peritoneum. The granularity of the medium induces the

macrophages present to phagocytose the particulate matter, thus imitating infection and promoting an inflammatory response within the peritoneum (Grattendick, 1999). Therefore the macrophages harvested after this procedure are 'primed' for activity and give an increased level of responsiveness after IFN-γ/LPS stimulation.

The standard curve was prepared, using doubling dilutions in dddH_2O in a flat-bottomed 96-well plate (Greiner Labortechnik) from a 14 mM sodium nitrite stock with a maximum concentration of 250 μM. Stored supernatants were defrosted at room temperature before addition of 100 μl to the plate, in duplicate. 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid was added to 0.01% (w/v) α-napthylamine in a 1:1 ratio and 100 μl was added to each well. The plate was incubated at room temperature for 5-10 minutes before the absorbance at 540 nm was measured using a Titertek Multiskan MCC/340 plate reader.

5.3 RESULTS

5.3.1 Effect of CPB2.8ΔCTE on IL-12 and nitric oxide production

Thioglycollate-elicited PECs were stimulated with IFN-γ and activated with LPS. This resulted in an expected increase in both IL-12 and nitric oxide production as can be seen in Figures 5.3.1 and 5.3.2. The enzyme in either its active or inactive form did not significantly affect activated macrophage production of these immuno-modulatory molecules. Enzyme buffer (PBS) also did not affect production of IL-12 or nitric oxide.

5.3.2 Effect of CPB2.8ΔCTE on production of IL-10

When PECs were stimulated with LPS and IFN-γ, there was an expected increase in IL-10 production (Figure 5.3.3). When these activated macrophages were incubated with recombinant CPB2.8ΔCTE, there was a significant reduction in the level of IL-10 as can be seen in Figure 5.3.3 ($p < 0.05$). This occurred with both enzymatically active and E-64-inhibited enzyme. When activated macrophages were incubated with the same protein concentration of a wild-type parasite lysate, this also reduced the production of IL-10 in a manner similar to that of the enzyme. Enzyme buffer did not significantly alter IL-10 production from activated macrophages.

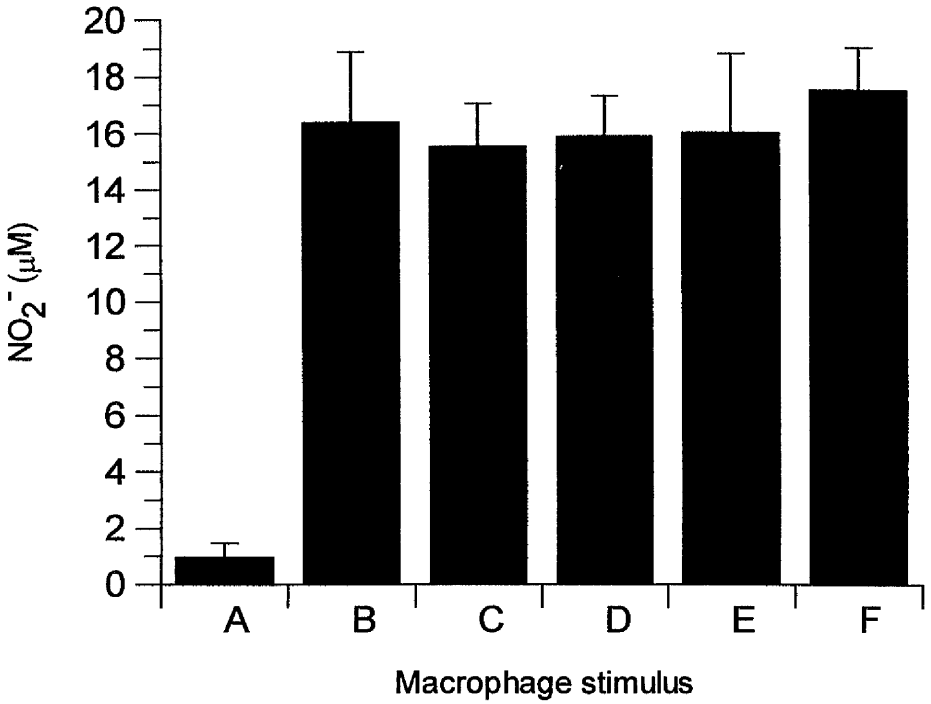

Figure 5.3.1 Effect of CPB2.8ΔCTE on nitrite production from LPS/IFN-γ activated macrophages. Supernatants were assessed for nitrite levels using the Greiss reaction.
A denotes macrophages in RPMI media.
B - macrophages activated with 100 ng/ml LPS and 50 U/ml IFN-γ (**a.m.**)
C - **a.m.** plus CPB2.8ΔCTE.
D - **a.m.** plus CPB2.8ΔCTE (E-64).
E - **a.m.** plus CPB2.8ΔCTE (boiled).
F - **a.m.** plus PBS buffer.
Active enzyme, CPB (100 ng/ml), was either inactivated by E-64 or boiled for 10 min. Results are presented as the means +/- SEM (n=7). The macrophages stimulated with conditions B-F were not significantly different from each other in nitrite production.

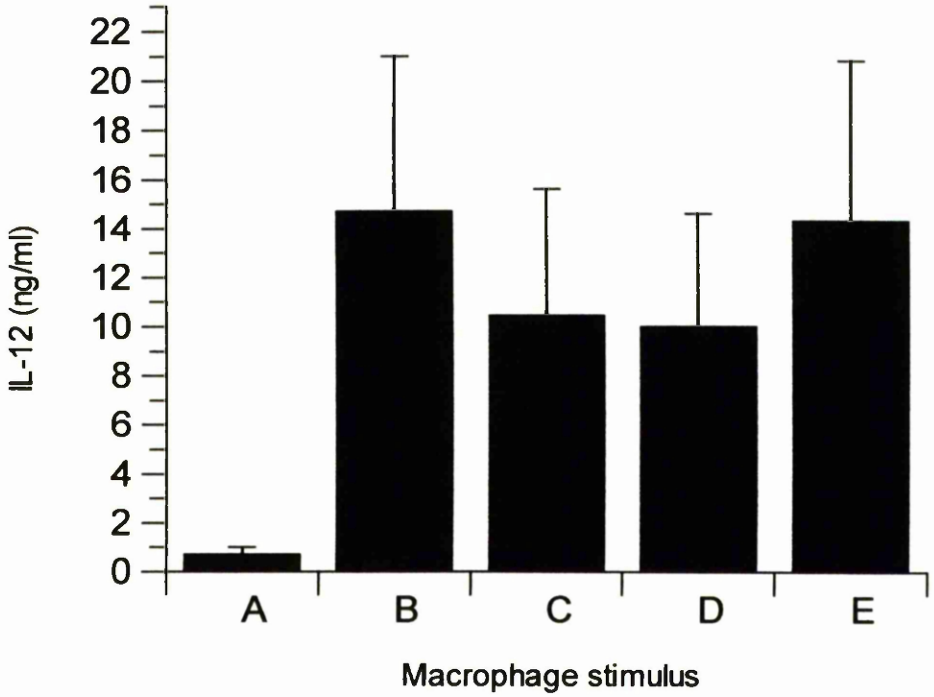

Figure 5.3.2 Effect of CPB2.8ΔCTE on IL-12 production from LPS/IFN-γ activated macrophages. Supernatants were assessed for IL-12 production by ELISA.
A denotes macrophages in RPMI media.
B - macrophages activated with 100 ng/ml LPS and 50 U/ml IFN-γ (**a.m.**)
C - **a.m.** plus CPB2.8ΔCTE .
D - **a.m.** plus CPB2.8ΔCTE (boiled).
E - **a.m.** plus CPB2.8ΔCTE (E-64).
Active enzyme, CPB (100 ng/ml), was either inactivated by E-64 or boiled for 10 min. Results are presented as the means +/- SEM (n=4). There was no significant difference in IL-12 production between macrophages stimulated as in B-E.

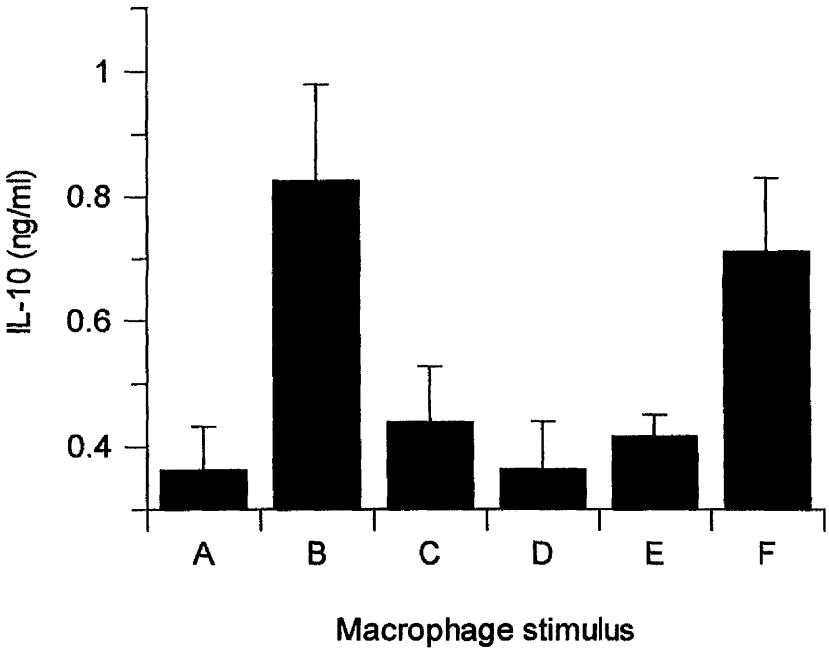

Figure 5.2.3 Effect of CPB2.8ΔCTE on IL-10 production from LPS/IFN-γ activated macrophages. Supernatants were assessed for IL-10 production by ELISA.
A denotes macrophages in RPMI media.
B - macrophages activated with 100 ng/ml LPS and 50 U/ml IFN-γ (**a.m.**)
C - **a.m.** plus CPB2.8ΔCTE .
D - **a.m.** plus CPB2.8ΔCTE (E-64).
E - **a.m.** plus wild-type lysate.
F - **a.m.** plus PBS buffer.
Active enzyme, CPB (100 ng/ml), was either inactivated by E-64 or boiled for 10 min. Results are presented as the means +/- SEM (n=4). Macrophages stimulated with conditions C, D and E were significantly different from macrophages activated using condition B ($p < 0.05$).

5.3 DISCUSSION

The role of the CPs in leishmanial infection is unknown although there is little question that they do play some part in the host-parasite relationship (Mottram *et al.*, 1996). Several other CPs have been shown to actively degrade cytokines or indirectly inhibit/upregulate production of cytokines thereby affecting the host immune response (Calkins *et al.*, 1998; Borger *et al.*, 1999).

CPB2.8ΔCTE did not affect PEC synthesis of IL-12 or nitric oxide *in vitro*. These results confirm work done previously in this laboratory, albeit using a different *in vitro* system for analysing nitric oxide production from PECs infected with *Leishmania* parasite lines (Townson, 1999). This study analysed the infection of PECs with a CPB null mutant parasite (*Δcpb*) and the results documented that the CPBs did not affect *in* vitro production of nitric oxide. The system used in my analysis of macrophage production of cytokines is similar to work done by Wang *et al.* (1992) where soluble amoebic proteins and live amoebae were added to LPS and IFN-γ treated PECs and the production of TNF-α was monitored. The effects on TNF-α production were similar for the soluble amoebic protein or the live parasite and mirrors the results obtained in my study and that of Townson (1999), in terms of nitric oxide production. Both sets of data show that the PECs were unaffected in IL-12 and nitric oxide production when either infected with the *Leishmania* parasite single null (Δ*cpb*) mutant re-expressing the proteinase, CPB2.8, or incubated with the recombinant enzyme, CPB2.8ΔCTE, itself.

The role of IL-10 in cutaneous leishmaniasis is well documented and is involved in leishmanial disease by inhibiting IFN-γ production (Melby *et al.*, 1996). The inhibitory effect of IL-10 on IFN-γ production is mediated by loss of

accessory cell function by suppression of IL-12 synthesis. It has recently been reported that a recombinant parasite molecule from *L. infantum* can elicit IL-10 production by peripheral blood mononuclear cells of patients with visceral leishmaniasis (Suffia *et al.*, 2000). Therefore, CPB2.8ΔCTE could have a similar effect on production of IL-10 from PECs, even though these were isolated from naive mice rather than infected mice. From the results obtained, it was interesting to note that CPB2.8ΔCTE did not elicit production of IL-10 from activated macrophages but in contrast, reduced the generation of this cytokine. Furthermore, enzyme activity is not necessary to induce this effect on IL-10 production since active and inactive enzyme both significantly reduced levels of IL-10. This is significant as it has been reported that some parasite molecules which have no enzymatic activity nevertheless are able to exert their effect on host cells. For example, ES-62, an excretory-secretory product from *Acanthaceilonema viteae*, can significantly affect B and T cell signal transduction (Deehan *et al.*, 1998). Theoretically, CPB2.8ΔCTE may act in a similar fashion by binding to macrophage receptors, or sterically affecting these receptor sites resulting in possible interference with various signal transduction mechanisms.

The *in vitro* system used here has clear limitations and the mechanism of action of the enzyme in the system is unknown. There are however, a number of possibilities such as those discussed below.

The localisation of CPB2.8ΔCTE is most likely to be within the lysosomes of the parasite, which resides within a parasitophorous vacuole within the macrophage. This localisation means that it is unlikely that the CPB enzymes could have a direct effect on cytokine production. However, work done by Ilg and colleagues (1994) showed that lesions contained extracellular CP activity,

which was attributed to parasites which had lysed. The catalytically active proteinases could potentially interfere with the host cytokine network once outside the macrophage and in the extracellular milieu. It has been consistently shown that proteinases can degrade connective tissues such as laminin, fibronectin and collagen (Mort *et al.,* 1981; Rooprai & McCormick, 1997) and in doing so, attract various cell types which could produce anti-inflammatory molecules such as IL-10 or TGF-β. Production of these two cytokines would then down-regulate the microbicidal potential of neighbouring macrophages and possibly allow parasite survival. Although this is not a defined role for the proteinase, it is possible that the CPs could transiently affect the macrophage activation state in this way, subsequently increasing parasite proliferation. However, the most likely function of parasite extra-corporeal cathepsin L-like enzymes could be degradation of host tissues for parasite migration and following further hydrolysis utilisation as nutrients (Dalton *et al.,* 1996).

A lysate of wild-type promastigotes was also tested for its ability to modulate IL-10 production, since it has been found that the soluble fraction of *E. histolytica* can increase TNF-α production from LPS/IFN-γ activated PECs (Wang *et al*, 1992). Indeed, the promastigote lysate was also found to reduce the level of IL-10 as did CPB2.8ΔCTE. It would have been of interest to determine whether a parasite lysate of the single null mutant, Δ*cpb*, was also able to affect IL-10 production as this would rule out the possibility of CPB being an important factor in the decrease in IL-10 production. One may expect both the purified CP and the lysate to act in a similar fashion since they both possess CP activity although the lysate also contains a vast number of other parasite molecules and consequently far less CPB than in the recombinant enzyme experiment. The fact

that the lysate and CPB2.8ΔCTE affected the macrophage similarly in terms of reducing IL-10 production is therefore not entirely surprising.

What is remarkable is that the enzyme and lysate reduced the level of IL-10 production from the activated macrophages at all, when they may have been expected to increase IL-10 production as it is produced by activated macrophages in much the same way as IL-12, in response to LPS and IFN-γ. Suffia *et al.* (2000), using a recombinant *L. infantum* protein, LeIF, found that incubation of this protein with PBMCs increased IL-10 production. The protein was therefore designated as a virulence factor. However, the systems used in the two experiments are completely different since this study utilised PECs (a predominance of macrophages although eosinophils and neutrophils are still present) from naive mice whereas Suffia *et al.* (2000) used purified peripheral blood mononuclear cells (PBMCs) from *Leishmania*-infected individuals. Further studies using bone-marrow derived macrophages would eliminate the possibility of other cells involved.

To further investigate if recombinant CPB2.8ΔCTE affected production of IL-10 from macrophages at the transcriptional level, a possible experiment could be to isolate the mRNA from macrophages and perform reverse transcriptase polymerase chain reaction (RT-PCR) for this cytokine. Moreover, proteinase could simply be added to IL-12 and tested for its ability to proteolytically cleave IL-12.

In addition to affecting macrophage cytokine production, the proteinases could also be involved in regulation of prostaglandin production. It is known that *E. histolytica* can alter the metabolism of arachidonic acid and therefore the production of important inflammatory molecules such as prostaglandin E2

(PGE2) which down-regulates production of TNF-α and IL-12 (Wang & Chadee, 1992). TNF-α is produced from macrophages which are stimulated by IFN-γ and it acts in an autocrine manner to upregulate further production from the macrophage as well as synergising with IFN-γ to activate macrophages to kill *Leishmania* parasites. It is important in *Leishmania* infection as it activates the macrophage to become inflammatory and therefore leishmanicidal (Liew & O' Donnell, 1993) and so a decrease in this cytokine could alter the disease outcome. Other inflammatory mediators could be involved such as the prostaglandins and the leukotrienes, which are also known to be involved in *Entamoeba* infection (Wang & Chadee, 1992).

Leishmanial CPs appear to interfere with MHC class II molecules and their up-regulation by IFN-γ (De Souza Leao *et al*, 1995). In this context, it is interesting to note that CPs secreted from the anaerobe *Porphyromonas gingivalis* are able to indirectly affect antigen presentation by directly cleaving IFN-γ (Yun *et al.*, 1999). *L. mexicana* CPs may act in a similar fashion. If this was the case, inactivation of IFN-γ could potentially lead to proliferation of Th 2-type cells and mediate disease progression.

The major CP from *T. cruzi*, cruzipain has been shown to display kininogenase activity and can therefore affect vascular permeability (Del Nery *et al.*, 1997). Cruzipain activates plasmatic pre-kallikrein with concurrent release of bradykinin and shares this property with another CP from a bacterium, *Streptococcus pyogenes* (Herwald *et al.*, 1996). Bradykinin stimulates an influx of macrophages from the circulation to the area of infection and helps perpetuate the infection as well as causing pain and swelling through release of prostaglandins. CPB2.8 shares high homology with cruzipain and therefore it is

possible that CPB2.8 could also display kininogenase-like properties. Furthermore, it is known that addition of serum to trypanosomal and leishmanial lysates can significantly increase CP activity and this has been attributed to a kininogen-like moiety (Lonsdale-Eccles *et al.*, 1995). Experiments to elucidate whether this was the case for CPB2.8 would be easy to perform and would confirm whether CPB2.8 had kininogenase properties.

The house dust mite is known to contain a CP which also has a number of immunomodulatory properties and has been shown to cleave a number of cell surface proteins including CD23, the IgE receptor (Hewitt *et al.*, 1995) and CD25, a subunit of the IL-2 receptor (Schulz *et al.*, 1998). The effect of this CP is to increase levels of circulating IgE and inhibit the ability of peripheral blood T cells to proliferate and secrete IFN-γ. The resultant changes consequently bias the immune response towards the propagation of Th 2 cells and this is another possible way in which CPB2.8 could potentially affect the immune response once in the vascular system.

It has recently been found that a metacestode secreted product containing CP activity has the ability to deplete human CD4+ lymphocytes *in vitro* (Molinari *et al.*, 2000). Although the leishmanial CPs are not secreted, parasite lesions have been shown to contain CP activity and could possibly behave in a similar manner.

Leishmanial CPs may therefore influence the host to promote infection by a number of mechanisms. However, these await further study.

CHAPTER 6

Re-integration of CPB into genetically attenuated *L. mexicana*; analysis of disease phenotype in mice

6.1 INTRODUCTION

The activity of CPs in virulence and pathogenicity towards the mammalian host during infection by many pathogens is widely recognised (Carmona *et al.*, 1993; Travis *et al.*, 1995; Engel *et al.*, 1998). The *L. mexicana cpb* array is a tandemly repeated organisation of genes that may function as a mechanism to increase the transcript abundance of the highly expressed CPB isoenzymes. These CPs are recognised as virulence factors since gene deletion of the *cpb* array attenuates the organism in its ability to infect macrophages *in vitro* and in mice (Mottram *et al.*, 1996). Re-expression of a single CP, CPB2.8, restored infectivity to macrophages to wild type levels and, by inference, CP expression is therefore considered to be important in enabling the parasite to survive intracellularly.

However, the importance of the CPB isoenzymes for infectivity to a mammalian host has not been investigated fully. Individual isoenzymes differ in their substrate preferences (Mottram *et al.*, 1997) and the first two genes of the *cpb* array, *cpb1* and *cpb2* differ significantly from the other genes in that they are transcribed predominantly in the metacyclic stage of the parasite and encode

enzymes with a truncation in the C-terminal extension. These results suggest that the individual isoenzymes have distinct roles in the host-parasite interaction.

There are several experimental tools which can be used to investigate the roles of the leishmanial CPs as virulence factors. Gene knockout experiments can demonstrate whether a gene is essential. However, although a gene may be essential, there is no guarantee that it encodes a valid drug target (Barrett *et al.*, 1999). Indeed, non-essential genes can encode drug targets as was demonstrated by Engel *et al.* (1998). In this study, an inhibitor of cruzipain was shown to kill *T. cruzi* by preventing autocatalytic processing of the pro-mature enzyme to its active, mature form. This resulted in a subsequent increase in precursor molecules in the Golgi apparatus, which most likely prevented normal functioning of the Golgi complex and led to parasite death. Gene deletion experiments could not have predicted that this enzyme was indeed a valid drug target and so a combination of inhibitor studies and genetic manipulation should be utilised to study putative drug targets.

The ability to manipulate genes of trypanosomatids either through over-expression of genes from episomal vectors or by gene deletion via targeted homologous recombination using drug-selectable markers has allowed the function of a major CP from *T. cruzi*, cruzipain, to be examined (Tomas *et al.*, 1997). This study showed how amplification of CP genes using a cosmid shuttle vector, resulted in elevated levels of CP activity and enhanced ability of the parasite to differentiate from the epimastigote to the metacyclic life-cycle stage. In contrast, targeted gene deletion experiments have been successfully used to study CP function in *L. mexicana*, and infection of mice with double null

(Δ*cpb/cpa*) mutants has shown that these genetically attenuated mutants may be good vaccine candidates (Alexander *et al.*, 1998).

Inhibitor studies using a group of inhibitors, the vinyl sulphones, have shown these compounds to be active against *L. major* (Selzer *et al.*, 1997) and *T. cruzi* (Engel *et al.*, 1998) and by interfering with parasite enzyme function, this should help to elucidate the potential roles these enzymes may play. However, the use of inhibitor studies also has one main disadvantage in aiding discovery of new drug targets. Targeted gene deletion prevents subsequent protein transcription and therefore loss of function of the particular protein but most pharmacological inhibitors do not result in a 100 % inhibition of target enzyme activity, when administered to living cells (Barrett *et al.*, 1999).

However, this study will focus only on the use of genetic manipulation to study the potential role of the CPs in the host-parasite interaction. Generation of the Δ*cpb* null mutant was produced by targeted gene deletion. This mutant was then manipulated for re-integration of two CPBs (see Table 7) into the parasite genome, rather than by re-expression of CP genes using a plasmid vector (Mottram *et al.*, 1996). Re-integration of the CPB into the parasite chromosome has the advantage that antibiotic selection is not needed for enzyme re-expression whereas the latter technique used by Mottram *et al.* (1996) resulted in loss of CPB expression without antibiotic pressure *in vivo*.

Re-integration of an amastigote CP, CPB2.8, or a metacyclic CP, CPB2, into the single null mutant, Δ*cpb*, with expression of the enzyme determined by a native or a chimaeric promoter should allow analysis of the importance of these two enzymes *in vivo* (see Table 7).

Genetic manipulation	Denoted	Phenotype during *in vitro* and *in vivo* infection
None	Wild-type (WT)	Infects macrophages *in vitro* and produces rapidly growing, non-healing lesions in BALB/c mice (Mottram *et al.*, 1996).
Deletion of *cpb* array	Δ*cpb* (N53 in cytokine data)	Reduced infectivity to macrophages *in vitro* by 80 % and produces slow-growing lesions in BALB/c mice (Mottram *et al.*, 1996).
Deletion of *cpb* array; re-integration of metacyclic CPB2 with native (metacyclic) promoter	GL165	Unknown
Deletion of *cpb* array; re-integration of amastigote CPB2.8 with native (amastigote) promoter	GL166	Unknown
Deletion of *cpb* array; re-integration of metacyclic CPB2 with chimaeric (amastigote) promoter	GL167	Unknown
Deletion of *cpb* array; re-integration of amastigote CPB2.8 with chimaeric (metacyclic) promoter	GL168	Unknown
Deletion of *cpb* array; re-expression of *cpb* array using a cosmid shuttle vector	GL263	Unknown

Table 7. Construction of mutants based on the Δ*cpb* single null mutants of *L. mexicana*

Construction of chimaeric mutants was performed to analyse if the time of expression of the genes affects the effects the enzymes have on the host-parasite interaction.

The use of a cosmid shuttle vector to study parasite CP expression *in vitro*, has been used before (Tomas *et al.*, 1997) and was used here to analyse re-expression of the entire *cpb* array into the Δ*cpb* null mutant (Table 7). The aim of re-expressing the entire *cpb* array as a cosmid vector was to assess whether over-expression of the *cpb* array in the Δ*cpb* null mutant could restore wild-type virulence levels *in vivo* and so whether the different genes of the array contribute individually in enabling the parasite to survive.

Re-integration of the CPB2.8 and CPB2 into double null (Δ*cpb/cpa*) mutants was also performed (see Table 8).

Experiments were set up using both promastigote and axenic amastigote forms of the parasites but the mice infected with promastigotes did not display lesions for several months and so data analysis was done only on mice infected with axenic amastigotes.

Genetic manipulation	Denoted	Phenotype during *in vitro* and *in vivo* infection
Deletion of *cpa* and *cpb* genes	Δ*cpb/cpa* (DN8 in cytokine data)	Rate of infectivity to macrophages *in vitro* is similar to Δ*cpb* (Mottram *et al.*, 1996) but did not produce lesions in mice (Alexander *et al.*, 1998).
Deletion of *cpa* and *cpb* genes; re-integration of metacyclic CPB2 with native promoter	GL438	Unknown
Deletion of *cpa* and *cpb* genes; re-integration of amastigote CPB2.8 with native promoter	GL439	Unknown

Table 8. Construction of mutants based on the Δ*cpb/cpa* mutants of *L. mexicana*

6.2 MATERIALS AND METHODS

6.2.1 Generation of *L. mexicana cpb* mutant lines

L. mexicana parasites lacking the *cpb* and *cpa* genes have been generated by targeted gene disruption and the method for this has been described elsewhere (Mottram *et al.*, 1996). A number of steps were required for CP re-integration into the Δ*cpb* null mutant and all steps were performed by Dr. D. Brooks (Wellcome Centre of Molecular Parasitology, University of Glasgow).

Transfection of the cosmid vector pGL263 into amastigotes was performed by Dr. H. Denise (Wellcome Centre of Molecular Parasitology, University of Glasgow). pGL263 is a cosmid isolated from a cosmid library of *L. mexicana*, generated by Dr. D.C. Barker (Cambridge University).

6.2.2 Infections

Age-matched female BALB/c mice were maintained in the animal facility at the University of Glasgow and used at 8 weeks of age. Groups were comprised of 5 mice and infected sub-cutaneously in the shaven rump with 5×10^6 axenic amastigotes or stationary phase promastigotes of the various *L. mexicana* mutants described in tables 7 and 8.

The course of infection was monitored by weekly measurement of the shaven rump using a slide gauge micrometer and lesion volume was measured, as described in section 4.2.3, chapter 4.

Animals were tail-bled before infection with *L. mexicana* parasites and subsequently every two months for antibody isotype analysis, as described in section 4.2.4, chapter 4. At seven months post-infection, mice were sacrificed and spleens isolated for cytokine analysis. This was performed as described in section 4.2.5, chapter 4, and, as before, IL-4 and IFN-γ were analysed by ELISA.

Lesions were removed from each mouse (by Mr. D. Laughland) and macerated in PSGEMKA buffer (PBS buffer plus 0.05 M glucose, 0.5 mM EDTA, 0.01 M $MgCl_2.6H_2O$ and 0.005% bovine serum albumin, pH 7.3) using a grinder, to remove host cell material. The resulting preparation was washed three times and re-suspended in PSGEMKA buffer, before parasite cell counts were performed. Lesion pellets were frozen at -70°C.

6.2.3 Preparation of harvested lesion material for CP analysis

Frozen lesion pellets were used to prepare parasite lysates. Lysates were produced by thawing frozen parasite pellets and re-suspending in 0.25 M sucrose and 0.25 % Triton X-100. The resultant mixture was vortexed, frozen and thawed before centrifugation in a bench-top centrifuge at 10, 000 g for 15 min to clarify the lysate. The resulting supernatant from each mouse was assayed for protein content using the BioRad protein estimation kit. Supernatants were used for subsequent experiments to analyse CP content and activity by Western blotting and gelatin gels, respectively. These were performed as described in chapter 2.

Statistical analysis of the results was similar to that described in section 4.2.7, chapter 4.

6.3 RESULTS

6.3.1 Lesion growth in BALB/c mice infected with *L. mexicana* wild-type and Δ*cpb* single null mutant amastigotes

The two control lines for the experiments were the wild-type and single null mutant (Δ*cpb*), the line lacking both copies of the entire *cpb* array. Cutaneous lesion growth was monitored for each mouse from the groups of mice infected and the mean values were plotted (see Figure 6.3.1 for wild-type and Δ*cpb* lines).

In Figure 6.3.1, all mice in the group infected by wild-type *L. mexicana* amastigotes exhibited large, progressive lesions which required culling of the mice at week 21 due to the excessive lesion volume. The average lesion volume at this stage of the study was approximately 500 mm^3 which was significantly greater than that of mean lesion values seen in mice infected with Δ*cpb L. mexicana*, at week 21 ($p < 0.01$).

6.3.2 Lesion growth in BALB/c mice infected with *L. mexicana* GL165 amastigotes

Re-integration of native CPB2 (GL165), a proteinase expressed predominantly by metacyclics, into the Δ*cpb* parasite line, produced enhanced lesion growth compared to the Δ*cpb* parasite line at week 27 ($p < 0.05$) although the mean lesion volumes were significantly smaller from week 14, than those lesion volumes observed in wild-type infected mice, until week 21 ($p < 0.05$) (Figure 6.3.2). GL165-infected mice produced lesion values that were

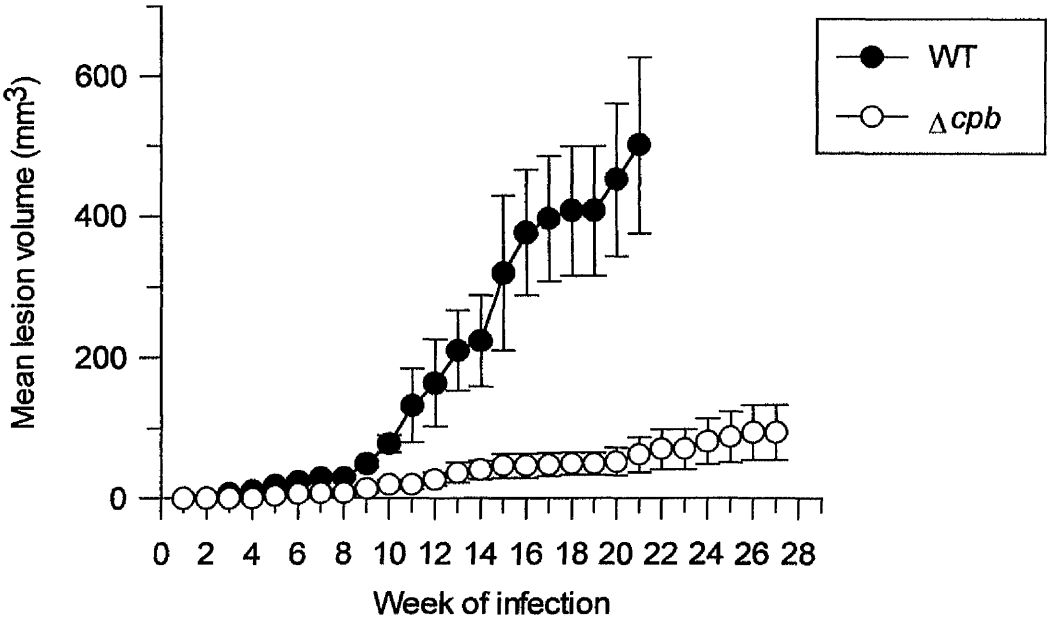

Figure 6.3.1 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with wild-type and Δ*cpb* parasite lines. Data represent mean values of at least 4 mice per group with error bars corresponding to SEMs. At week 21, mean lesion volume of mice infected with wild-type parasites was significantly higher than that of Δ*cpb*-infected mice ($p < 0.01$).

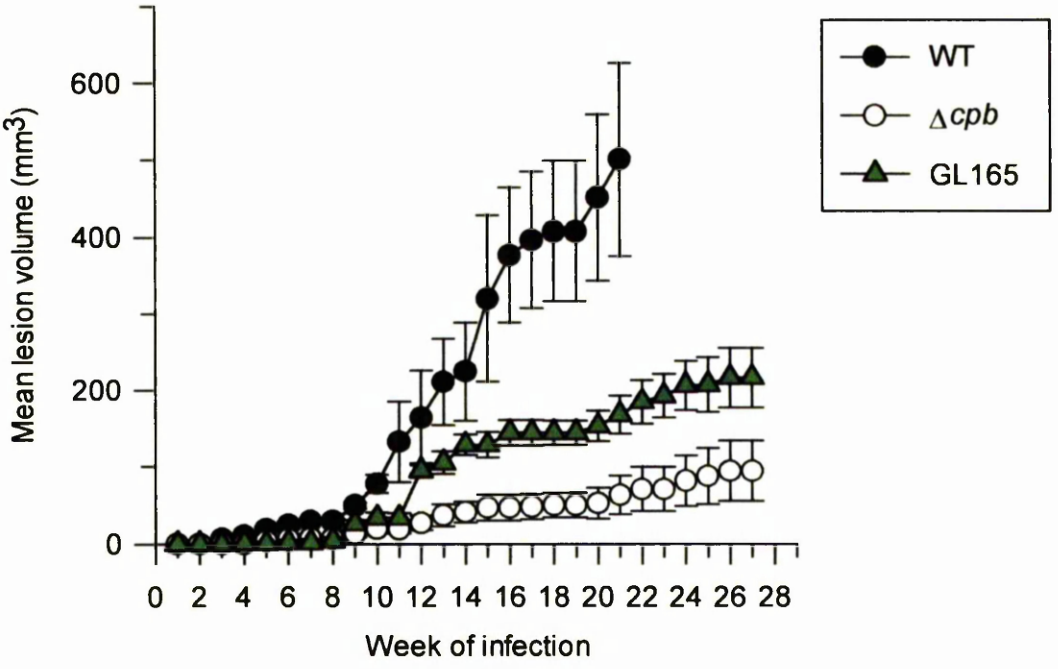

Figure 6.3.2 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with the GL165 mutant line. Wild-type and Δ*cpb* controls are shown. Data represent mean lesion values of at least 4 mice per group with error bars corresponding to SEMs. By week 27, mean lesion volume of mice infected with the GL165 parasite line was significantly higher than mice infected with the Δ*cpb* line ($p < 0.05$) and significantly smaller than mice infected with wild-type parasites ($p < 0.05$), measured at week 21.

significantly greater than those produced by Δ*cpb*-infected mice by week 12 of infection.

6.3.3 Lesion growth in BALB/c mice infected with *L. mexicana* GL166 amastigotes

GL166, the Δ*cpb* mutant with re-integrated native CPB2.8, an amastigote specific proteinase, displayed a mean lesion volume that was significantly smaller than the mean lesion volume of wild-type infected mice at week 21 ($p < 0.05$) but was not significantly different from the mean lesion volume of the Δ*cpb*-infected mice (Figure 6.3.3).

6.3.4 Lesion growth in BALB/c mice infected with *L. mexicana* GL167 amastigotes

Mice infected with Δ*cpb* mutants with re-integrated chimaeric CPB2 (GL167), produced mean lesion volumes that were significantly higher than those observed in Δ*cpb*-infected mice at week 27 ($p < 0.05$) but were significantly smaller than mean lesion volumes produced by wild-type infected mice at week 21 ($p < 0.05$) (Figure 6.3.4). There was no significant difference in lesion volumes from mice infected with chimaeric or native CPB2 (Figure 6.3.6).

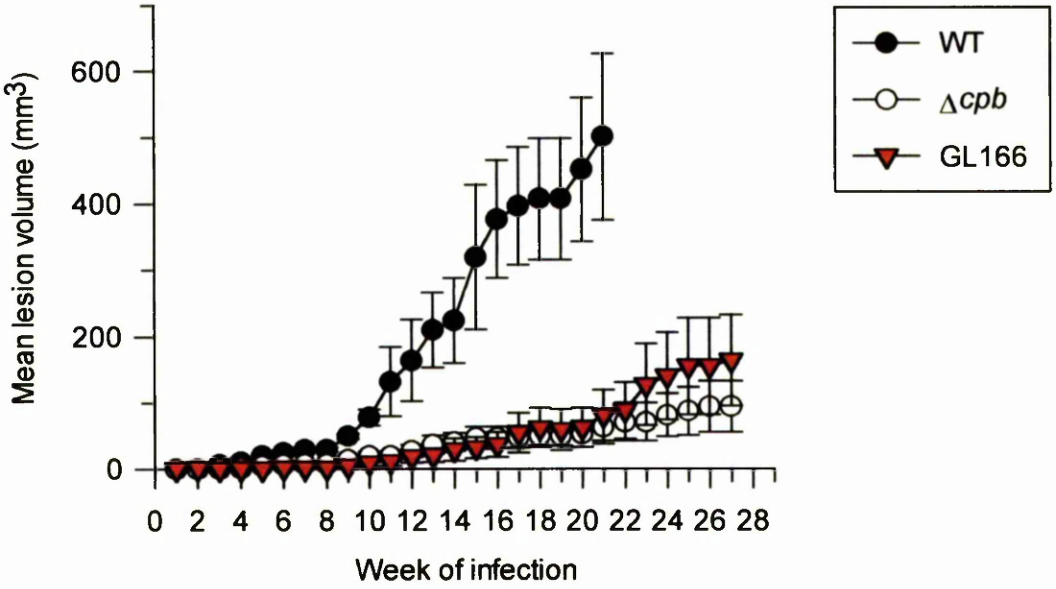

Figure 6.3.3 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with the GL166 mutant line. Wild-type and Δ*cpb* are shown. Data represent mean lesion values of at least 4 mice per group with error bars corresponding to SEMs. By week 27, mean lesion volume of mice infected with the GL166 parasite line was not significantly higher than Δ*cpb*-infected mice but was significantly lower than that of wild-type infected mice ($p < 0.05$), measured at week 21.

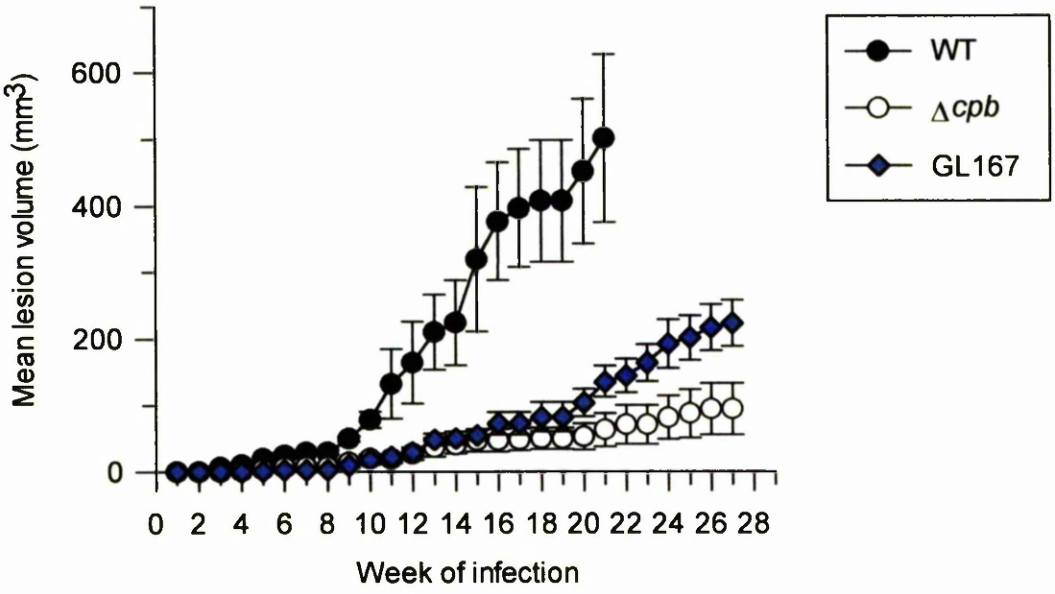

Figure 6.3.4 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with the GL167 mutant line. Wild-type and Δ*cpb* controls are shown. Data represent mean lesion values of 5 mice per group with error bars corresponding to SEMs. By week 27, mean lesion volume of mice infected with GL167 parasite line was significantly higher than that of Δ*cpb*-infected mice ($p < 0.05$) but significantly lower than that of wild-type infected mice ($p < 0.05$), at week 21.

6.3.5 Lesion growth in BALB/c mice infected with *L. mexicana* GL168 amastigotes

Mice infected with Δ*cpb* mutants with re-integrated chimaeric CPB2.8 (GL168), produced mean lesion volumes that were significantly greater than those observed in Δ*cpb*-infected mice at week 27 ($p < 0.05$) but were significantly smaller than mean lesion volumes produced by wild-type infected mice at week 21 ($p < 0.05$) (Figure 6.3.5). Re-integration of chimaeric CPB2.8 produced significantly greater lesion volumes at week 21 when compared with Δ*cpb*-infected mice. This was markedly different from mice infected with GL166 (re-integration of native CPB2.8) whose lesion volumes were not significantly different from the Δ*cpb*-infected mice. However, there was no significant difference in lesion growth between mice infected with GL166 or GL168 (Figure 6.3.6).

6.3.6 Comparison of mice infected with Δ*cpb* null mutants re-expressing different CPBs

By week 27, when all mice were culled for splenocyte examination, there was no significant difference in lesion volumes between any of the groups of mice infected with GL165, GL166, GL167 or GL168 *L. mexicana* amastigotes (Figure 6.3.6). However, by week 12 of infection, mice infected with the GL165 parasite line had significantly greater lesion volumes than the remaining groups ($p < 0.05$) until week 18, at which point they ceased to be significantly different from mice infected with the other CPB re-integrated lines.

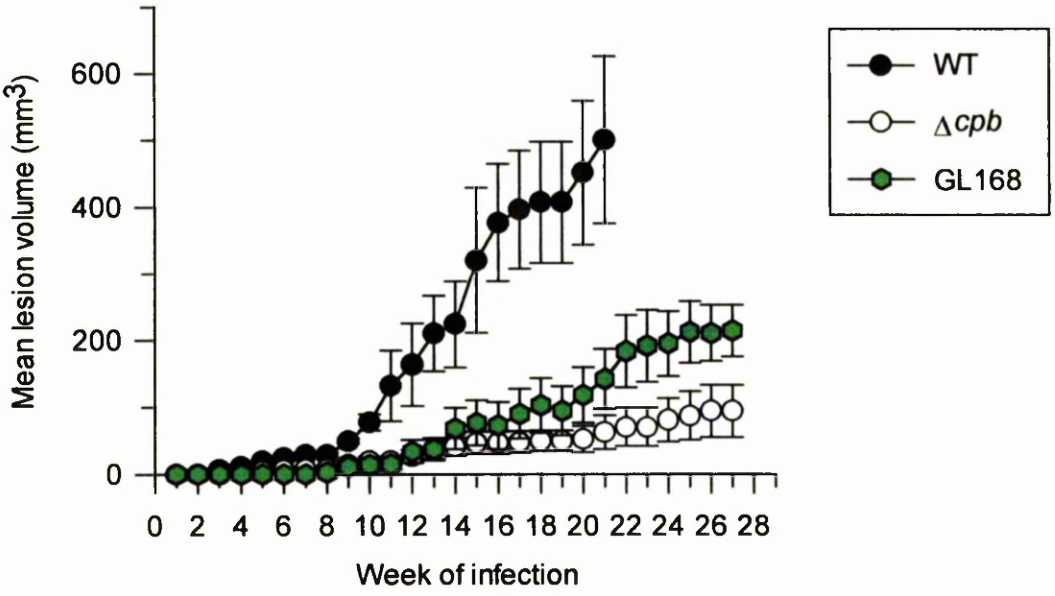

Figure 6.3.5 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with the GL168 mutant line. Wild-type and Δ*cpb* controls are shown. Data represent mean lesion values of 5 mice per group with error bars corresponding to SEMs. By week 27, mean lesion volume of mice infected with GL168 parasite line was significantly higher than Δ*cpb*-infected mice ($p < 0.05$) but at week 21 was significantly lower than that of wild-type infected mice ($p < 0.05$).

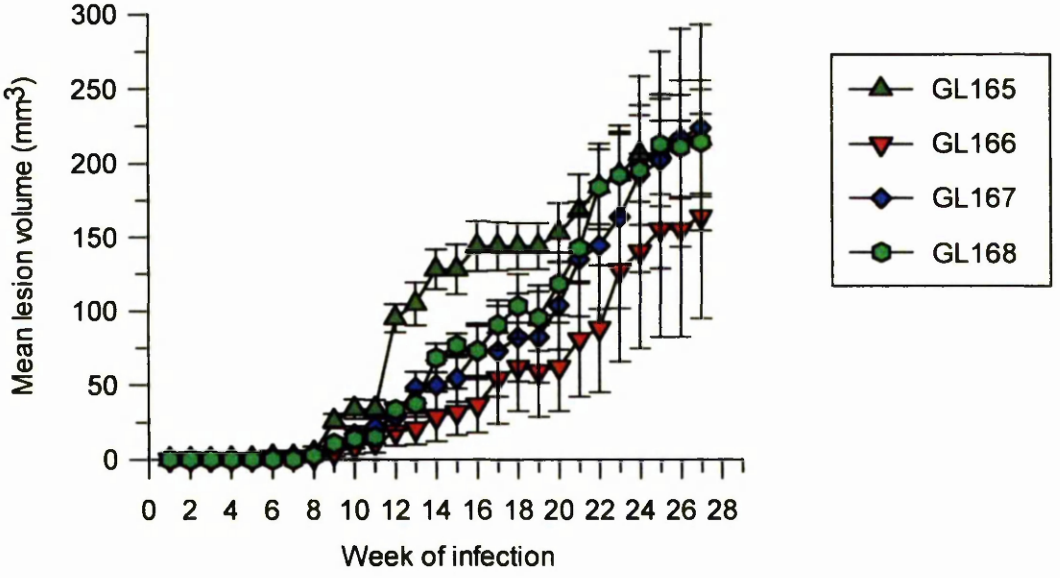

Figure 6.3.6 Mean lesion growth in mice infected with *L. mexicana* amastigote mutants re-integrated with CPBs. Data represent mean lesion values of at least 5 mice per group with error bars corresponding to SEMs. By week 27, there was no significant difference in mean lesion volume from mice infected with parasites re-integrating the different CPB enzymes.

6.3.7 Lesion growth in BALB/c mice infected with *L. mexicana* double null mutant amastigotes

Mice infected with double null mutant (Δ*cpb/cpa*) *L. mexicana* amastigotes produced very small lesion volumes (< 10 mm^3) after almost 8 months of infection (Figure 6.3.7). Mice infected with Δ*cpb/cpa L. mexicana* mutants re-integrated with CPB2 (GL438), produced lesions that were not significantly different, by week 33, in volume compared to mice infected with the Δ*cpb/cpa*-infected mice. Mice that were infected with GL439 *L. mexicana* mutants (Δ*cpb/cpa* re-integrated with CPB2.8) produced no lesions in the time period shown.

6.3.8 Measurement of IgG1 and IgG2a antibody titres in mice infected with wild-type, Δ*cpb* and GL165-168 *L. mexicana* axenic amastigotes

Figure 6.3.8 shows that wild-type *L. mexicana*-infected mice displayed high levels of IgG1 as expected, but surprisingly the other *L. mexicana*-infected mice (Δ*cpb*, GL165-168) had comparably high IgG1 titres which were not significantly different to each other, except for the GL166 group. GL166-infected mice presented antibody titres of the IgG1 subclass that were significantly lower than the GL167 infected group ($p < 0.05$).

Figure 6.3.8 also shows the antibody production of IgG2a from *L. mexicana*-infected mice. As expected from previous studies, wild-type infected mice produced significantly less IgG2a compared with the Δ*cpb*-infected mice ($p < 0.02$) In fact, most groups of infected mice (GL165, GL167 and GL168) had significantly higher IgG2a plasma titres than the wild-type infected mice ($p < 0.05$ for all mice compared). The exception was GL166 which had comparable levels

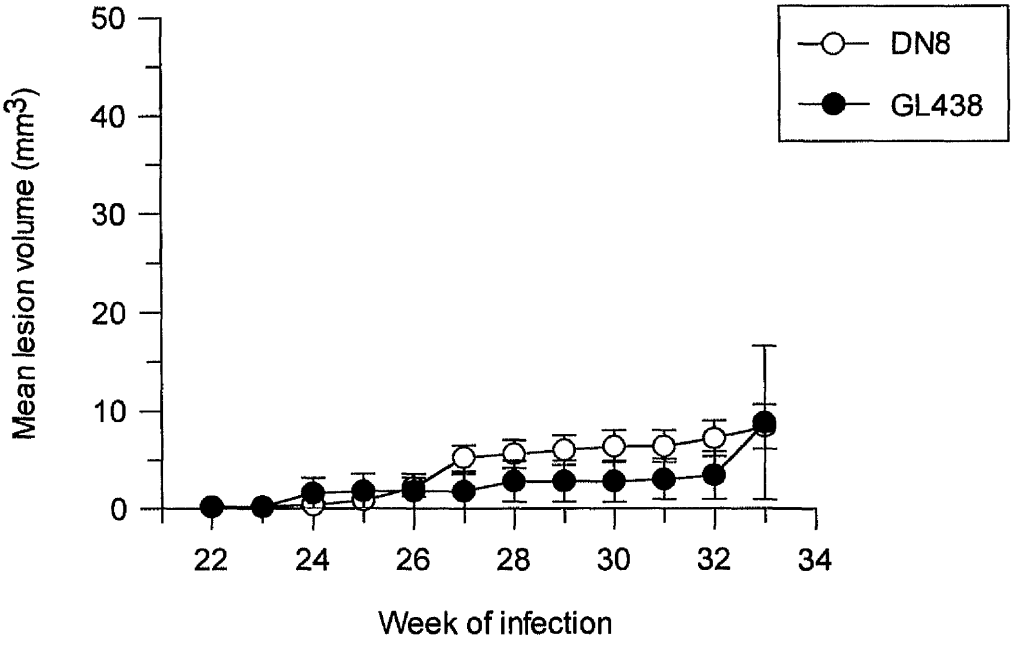

Figure 6.3.7 Mean lesion growth of *L. mexicana* amastigotes in BALB/c mice. DN8 represents Δ*cpb/cpa* null mutant; GL438 represents Δ*cpb/cpa* null mutant re-integrated with CPB2; GL439 indicates Δ*cpb/cpa* null mutant re-integrated with CPB2.8. There was no lesion formation in mice infected with GL439 in the time period shown. Data is shown as mean values of 5 mice per group with error bars corresponding to SEMs.

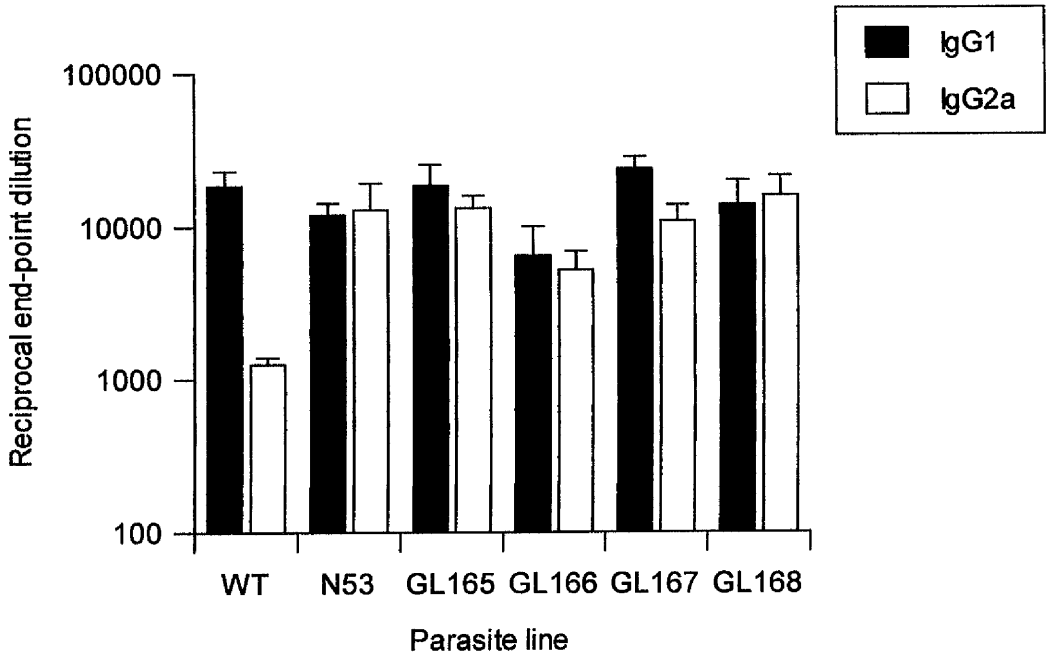

Figure 6.3.8 Analysis of plasma IgG1 and IgG2a levels in BALB/c mice 6 mo after infection with *L. mexicana* amastigotes of either wild-type (WT) (n=4), Δ*cpb* null (N53), Δ*cpb* null re-integrated with CPB2 (GL165) (n=4), Δ*cpb* null re-integrated with CPB2.8 (GL166) (n=4), Δ*cpb* null re-integrated with chimaeric CPB2 (GL167) or Δ*cpb* null re-integrated with chimaeric CPB2.8 (GL168). Values represent mean end-point dilutions +/- SEM (n = 5 unless stated).

of IgG2a to the wild-type infected mice and displayed no significant difference between the two groups.

6.3.9 Measurement of IgG1 and IgG2a antibody titres in mice infected with *L. mexicana* DN8, GL438 and GL439 axenic amastigotes

Generally, the plasma antibody titres for all infected mice were very low as can be seen in Figure 6.3.9. The level of IgG1 was very similar for all infected groups analysed and there was no significant difference in production of this antibody isotype. However, GL438 and GL439-infected mice both presented significantly higher IgG2a isotype levels than Δ*cpb/cpa*-infected mice ($p < 0.05$ for both) although there was no significant difference between the two CPB re-expressing lines.

6.3.10 IFN-γ production from SLA-stimulated splenocytes from *L. mexicana*-infected mice

Figure 6.3.10 shows the production of IFN-γ from *ex vivo* splenocytes stimulated with soluble parasite antigen from mice infected with various CP mutant and wild-type *L. mexicana* amastigotes. Wild-type infected mice produced very little IFN-γ with no significant difference from background levels and was significantly lower in production of IFN-γ compared with all other infected *L. mexicana* amastigote lines (Δ*cpb*, $p < 0.001$; GL165, $p < 0.001$; GL167, $p < 0.01$; GL168, $p < 0.01$; GL263, $p < 0.01$). These groups had levels of IFN-γ much higher than background levels ($p < 0.05$ for all mice). Furthermore, GL165-

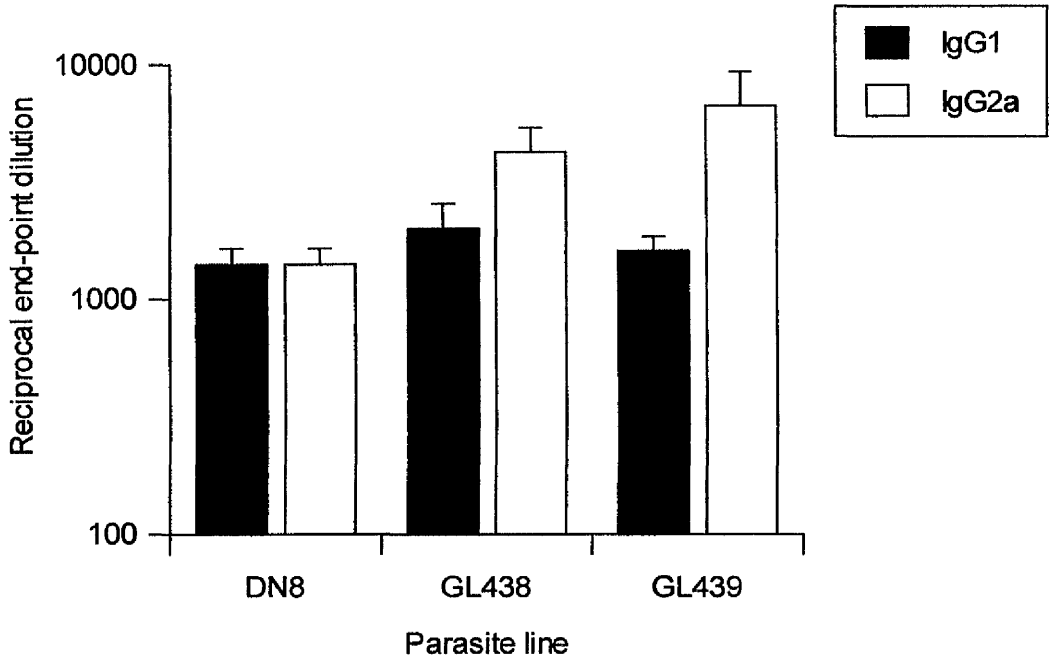

Figure 6.3.9 Plasma analysis of antibody isotype from BALB/c mice infected 6 mo with *L. mexicana* amastigotes. DN8 line represents Δ*cpb/cpa* double null mutant; GL438, Δ*cpb/cpb* double null mutant re-integrated with CPB2; GL439, Δ*cpb/cpb* double null mutant re-integrated with CPB2.8. Values represent mean end-point dilutions +/- SEM (n=5).

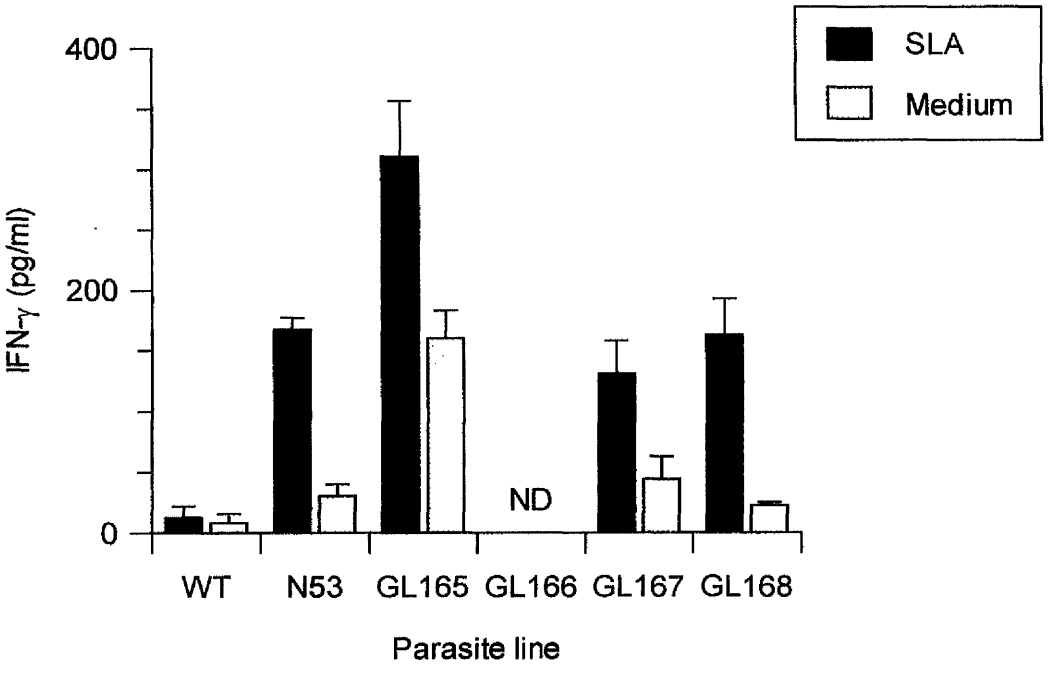

Figure 6.3.10 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on SLA-stimulated (10 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

infected mice produced significantly higher IFN-γ than GL167-infected mice when splenocytes were re-stimulated with SLA ($p< 0.02$).

The GL166-infected mice produced no detectable cytokine (neither IFN-γ nor IL-4) when stimulated polyclonally or with leishmanial antigen although cytokine standards were positive and suggested that the samples were in some way defective, rather than the assay itself.

6.3.11 IFN-γ production from CPB2.8ΔCTE-stimulated splenocytes removed from *L. mexicana*-infected mice

Of all the infected mice tested, only GL165 *L. mexicana*-infected mice produced significantly high levels above background when stimulated with recombinant CPB2.8ΔCTE ($p< 0.05$) (Figure 6.3.11). Furthermore, GL165-infected mice displayed significantly higher IFN-γ levels than mice infected with GL167 ($p< 0.001$), GL168 ($p< 0.05$) and wild-type *L. mexicana* ($p< 0.001$).

6.3.12 IFN-γ production from Con A-stimulated splenocytes from *L. mexicana*-infected mice

Con A-stimulated splenocytes from all groups of infected mice exhibited much higher IFN-γ levels than background levels ($p< 0.0001$) (Figure 6.3.12). However, there were differences in IFN-γ production between several of the mutant lines analysed in response to Con A. GL167-infected mice produced significantly less IFN-γ when splenocytes were polyclonally stimulated with Con A than the GL168, Δ*cpb* and wild-type infected mice (all groups, $p< 0.02$). The difference in IFN-γ production was even more substantial between GL167 and

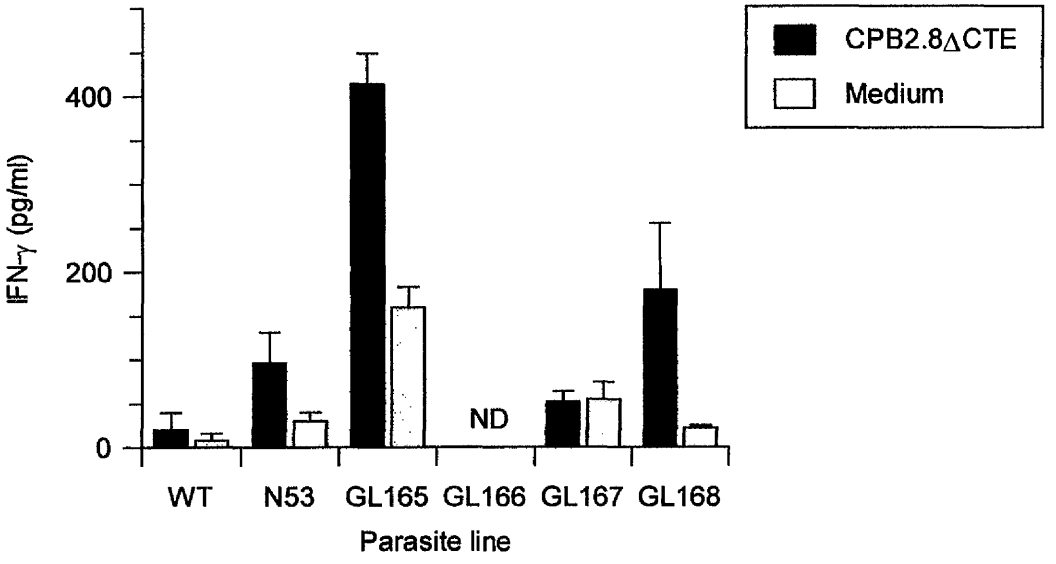

Figure 6.3.11 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on CPB2.8ΔCTE-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

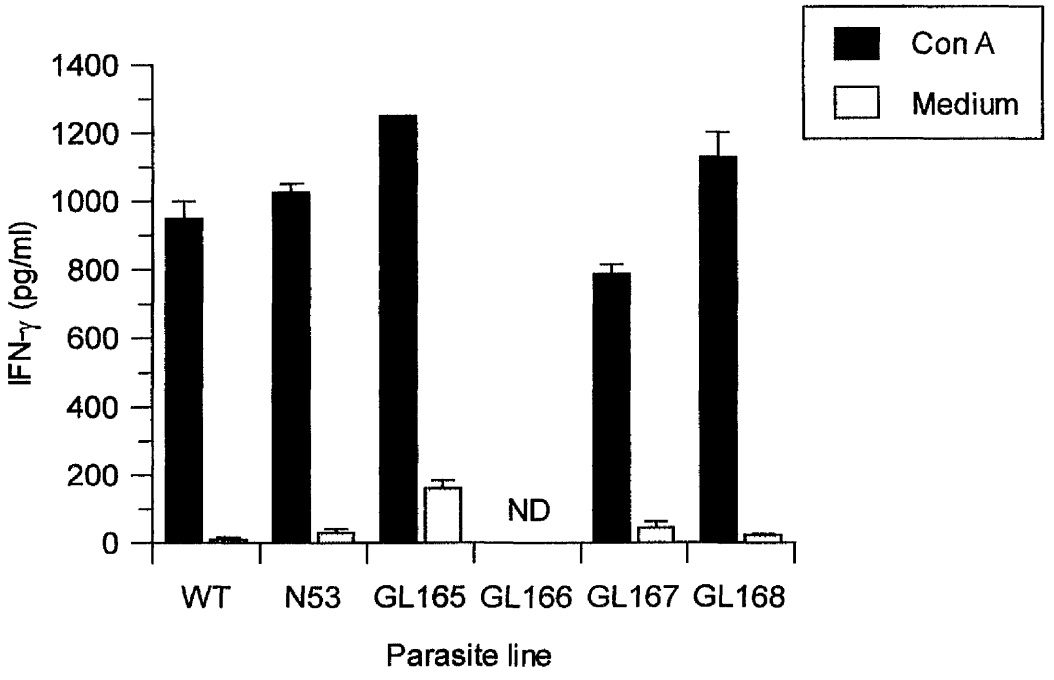

Figure 6.3.12 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on Con A-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

GL165-infected mice with the latter group producing much higher levels in response to Con A ($p < 0.0001$). Splenocytes from *L. mexicana* GL165 and GL168 amastigote-infected mice produced comparable levels of IFN-γ with no significant difference between them although IFN-γ was significantly higher than the other two groups tested (Δ*cpb* and wild-type, $p < 0.001$ for both).

6.3.13 IL-4 production from SLA-stimulated splenocytes from *L. mexicana*-infected mice

In all groups analysed for IL-4 production from splenocytes re-stimulated with SLA, only mice infected with GL165 and wild-type *L. mexicana* amastigotes produced significant levels of IL-4 above background levels ($p < 0.05$ and $p < 0.01$ respectively) while the remaining groups did not (see Figure 6.3.13).

Wild-type *L. mexicana*-infected mice produced significantly higher levels of IL-4 than all other groups tested except for GL165-infected mice, where there was no significant difference. Wild-type splenocytes produced significantly higher IL-4 levels than Δ*cpb* splenocytes ($p < 0.02$), GL167 ($p < 0.05$), and GL168 ($p < 0.02$) infected mice. GL165-infected mice produced significantly higher amounts of IL-4 than Δ*cpb*-infected mice ($p < 0.01$).

6.3.14 IL-4 production from CPB2.8ΔCTE-stimulated splenocytes from *L. mexicana*-infected mice

In all splenocyte cultures tested, levels of IL-4 produced were not significantly different from background levels when stimulated with recombinant CPB2.8ΔCTE (Figure 6.3.14).

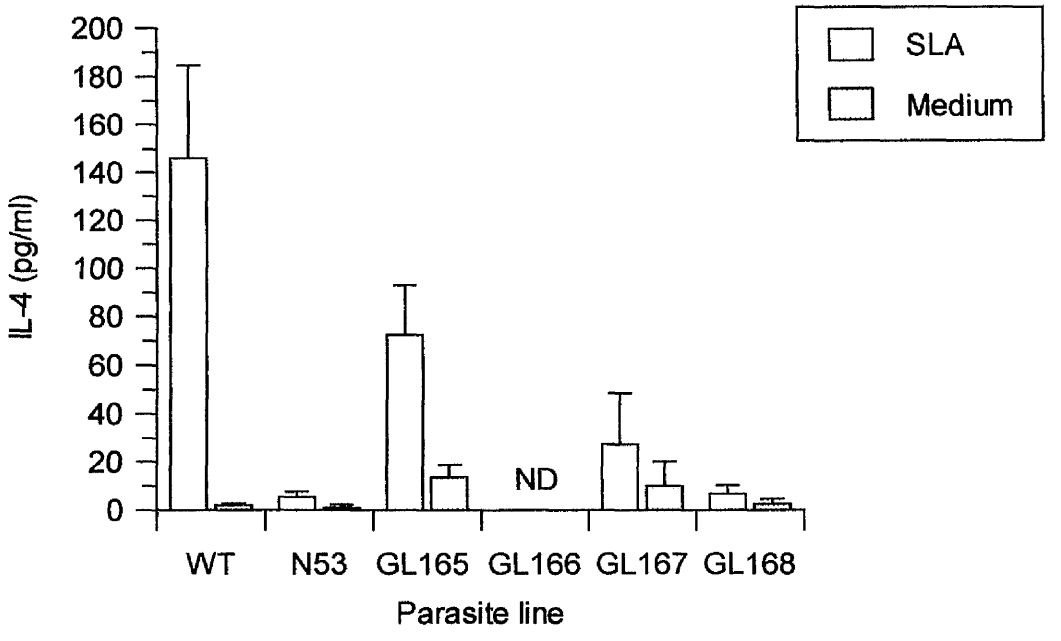

Figure 6.3.13 IL-4 production by cultured splenocytes removed from *L. mexicana* infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on SLA-stimulated (10 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

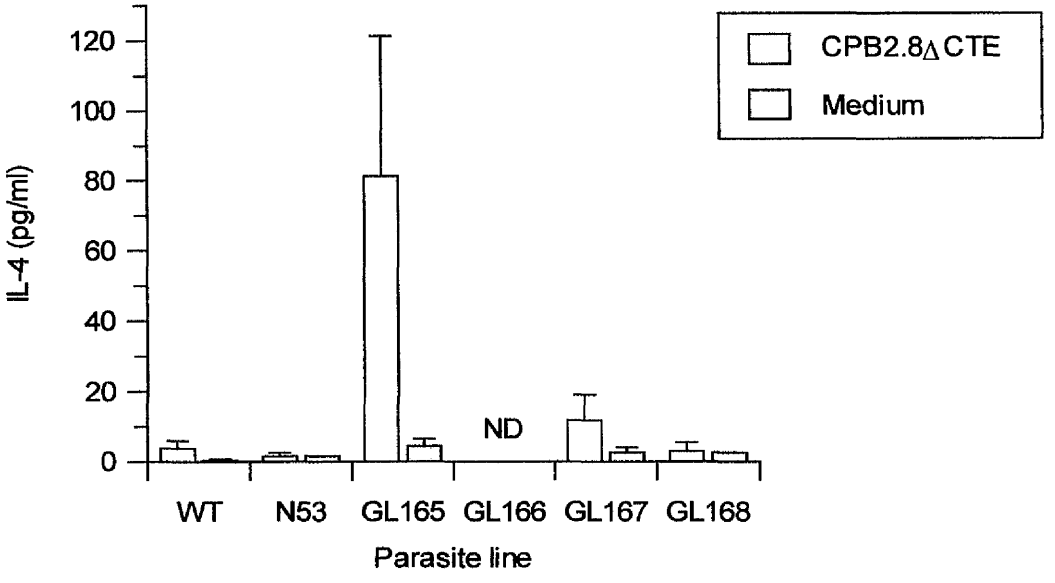

Figure 6.3.14 IL-4 production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on CPB2.8ΔCTE-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

Con A stimulation of splenocytes from all groups tested produced significantly higher levels of IL-4 than background cultures tested ($p < 0.0001$) and all groups analysed, had comparable levels of IL-4 to each other with no significant difference between them (see Figure 6.3.15).

6.3.15 Lesion growth in BALB/c mice infected with *L. mexicana* GL263 amastigotes

Re-expression of the *cpb* array using a cosmid vector in the Δ*cpb* parasite line, at week 27 did not result in significantly greater lesion volumes compared with the Δ*cpb*-infected mice. However, at week 21, wild-type-infected mice produced progressive lesions which were significantly greater in volume than the GL263-infected mice ($p < 0.02$) (Figure 6.3.16).

6.3.16 Measurement of IgG1 and IgG2a antibody titres in mice infected with *L. mexicana* GL263 amastigotes

Plasma IgG1 was elevated for all three parasite lines in infected mice as can be seen in Figure 6.3.17 and there was no significant difference between the GL263-, wild-type- and Δ*cpb*-infected mice. GL263-infected mice displayed significantly larger IgG2a antibody titres than that of the wild-type infected mice ($p < 0.02$) which were very low. The level of IgG2a from GL263-infected mice was comparable with that seen in the Δ*cpb* -infected mice and the IgG1/IgG2a ratio was much lower in the GL263-infected mice compared to the wild-type-infected mice.

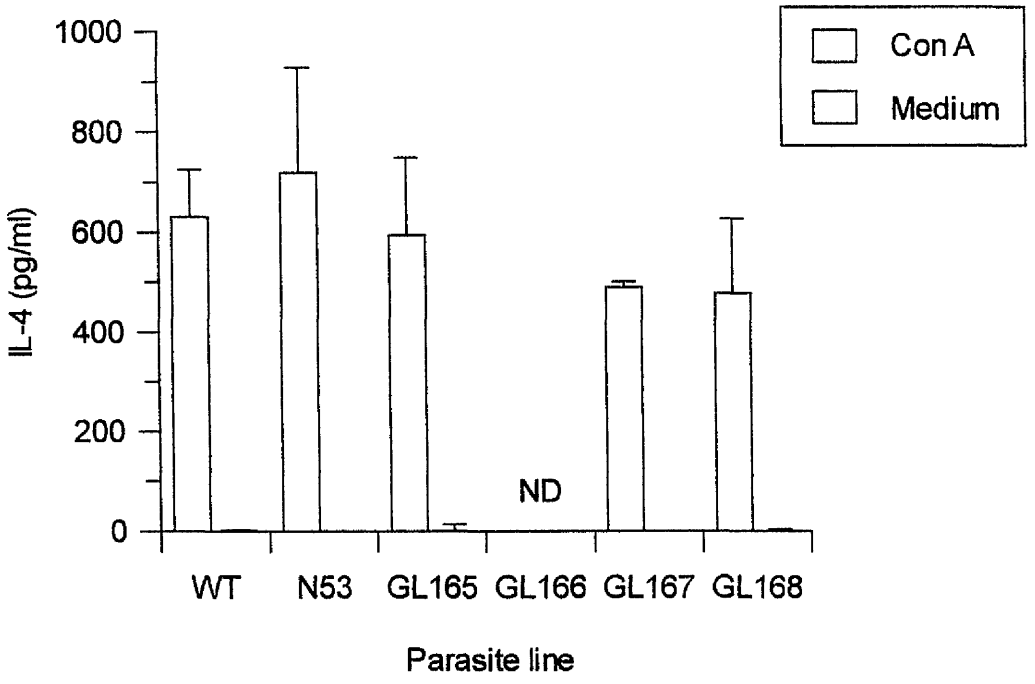

Figure 6.3.15 IL-4 production by cultured splenocytes removed from *L. mexicana* infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on Con A-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs. ND denotes non-detection of cytokine by assay.

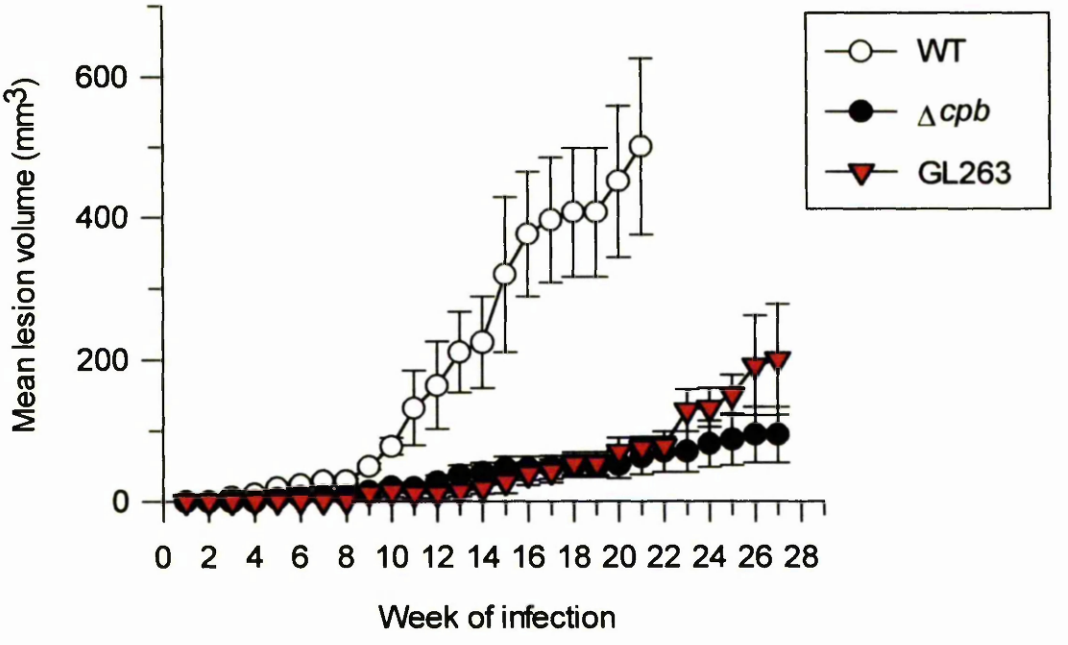

Figure 6.3.16 Mean lesion growth in mice infected with *L. mexicana* amastigotes. Mice were infected with the GL263 mutant line. Wild-type and Δ*cpb* controls are shown. Data represent mean lesion values of at least 4 mice per group with error bars corresponding to SEMs. By week 27, mean lesion volume of mice infected with the GL263 parasite line was not significantly higher than mice infected with the Δ*cpb* line but at week 21, was significantly smaller than wild-type infected mice ($p < 0.02$).

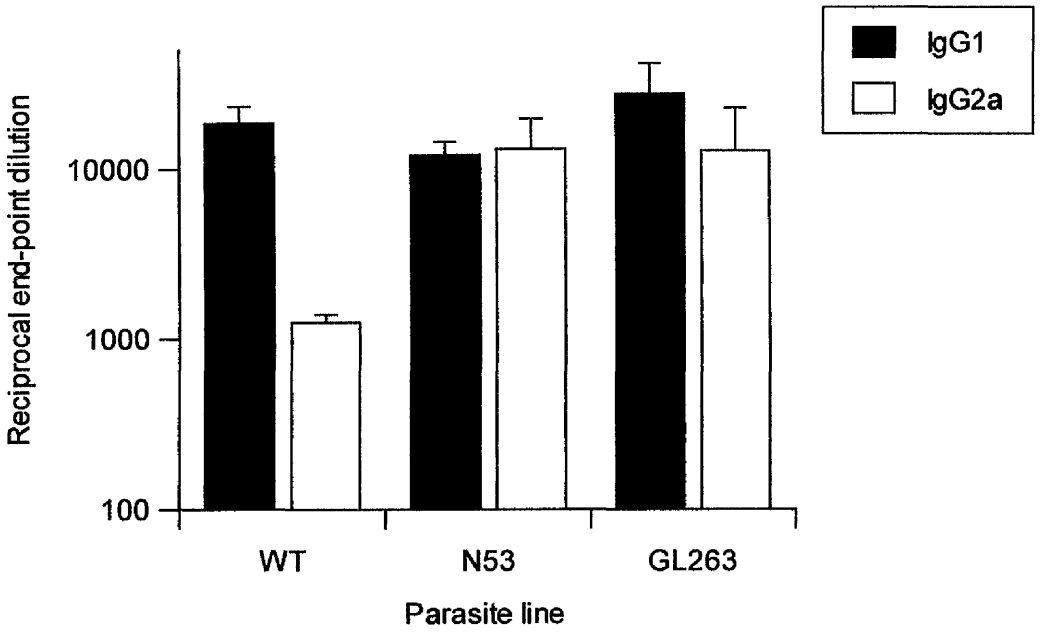

Figure 6.3.17 Plasma analysis of antibody isotype from BALB/c mice infected 6 mo with *L. mexicana* amastigotes. WT, wild-type; N53, Δ*cpb* null mutant; GL263, Δ*cpb* null mutant re-expressing *cpb* array as a cosmid vector. Values represent mean end-point dilutions +/- SEM (n=5).

6.3.17 IFN-γ production from re-stimulated splenocytes from *L. mexicana*-infected mice

Splenocytes removed from GL263-infected mice and re-stimulated *in vitro* with soluble leishmanial antigen, produced significantly higher levels of IFN-γ compared with background levels ($p < 0.05$) (Figure 6.3.18). These levels of IFN-γ were not significantly different in those splenocytes isolated from Δ*cpb*-infected (N53) mice but were significantly higher than splenocytes removed from wild-type-infected mice ($p < 0.01$).

IFN-γ background levels of splenocytes obtained from mice infected with GL263 amastigotes was high and when splenocytes were re-stimulated with recombinant CPB2.8ΔCTE, there was no significant increase over background levels (Figure 6.3.19).

Splenocytes from GL263-infected mice polyclonally stimulated with Con A produced significantly higher levels of IFN-γ compared with background levels ($p < 0.0001$) (Figure 6.3.20). There was no significant difference in IFN-γ production between splenocytes from GL263, wild-type or Δ*cpb*-infected mice.

6.3.18 IL-4 production from stimulated splenocytes from *L. mexicana* GL263- infected mice

Splenocytes removed from GL263-infected mice and re-stimulated *in vitro* with soluble leishmanial antigen, produced significantly higher levels of IL-4 compared to background levels ($p < 0.02$) (Figure 6.3.21). These levels of IL-4 were significantly higher than Δ*cpb*-infected mice but also significantly lower than wild-type-infected mice ($p < 0.05$).

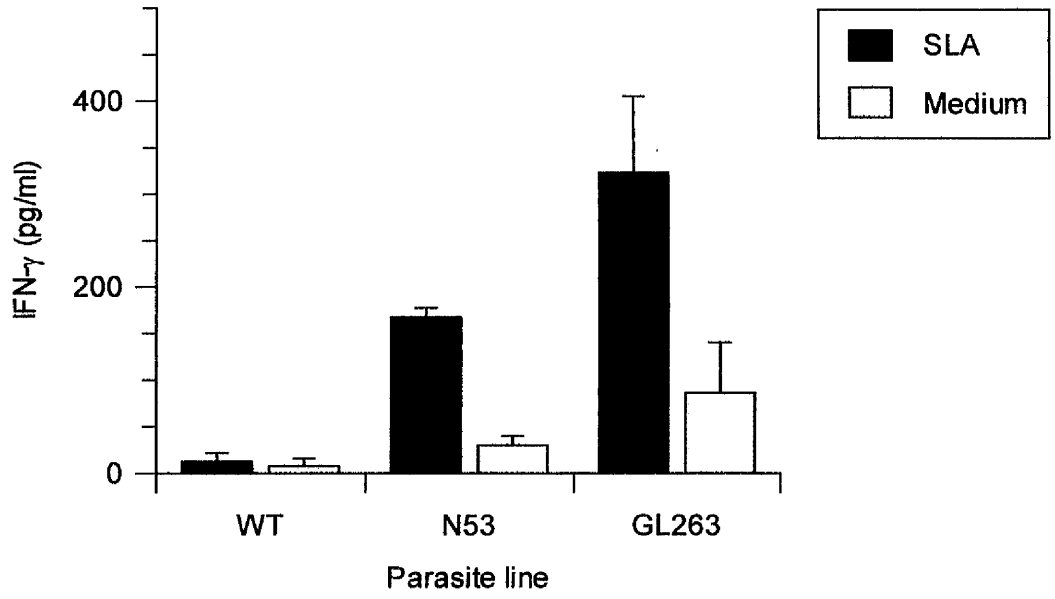

Figure 6.3.18 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. N53 denotes Δcpb null mutant-infected mice. Cytokine analysis was performed on SLA-stimulated (10 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs.

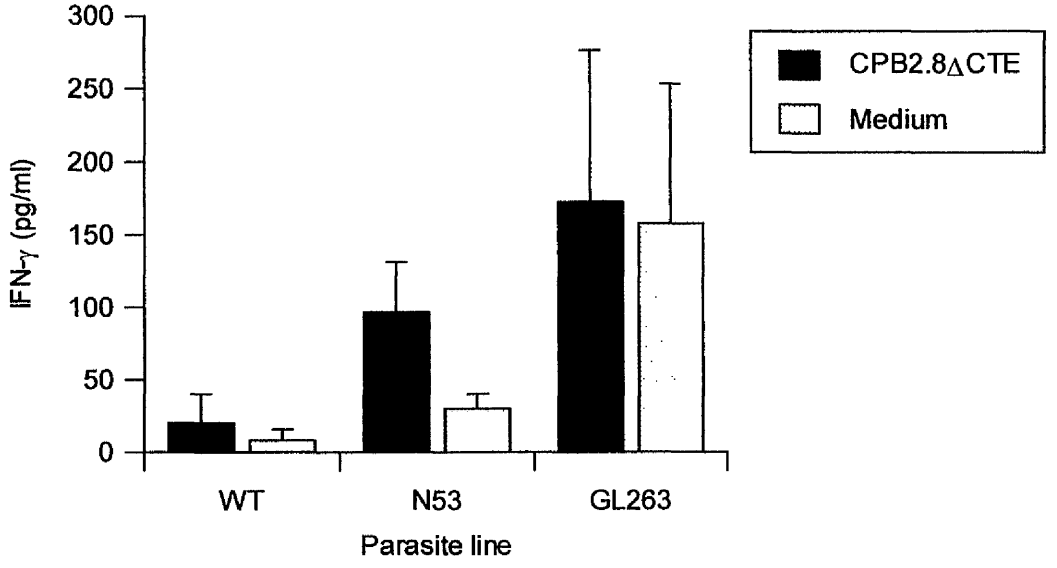

Figure 6.3.19 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on CPB2.8ΔCTE-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs.

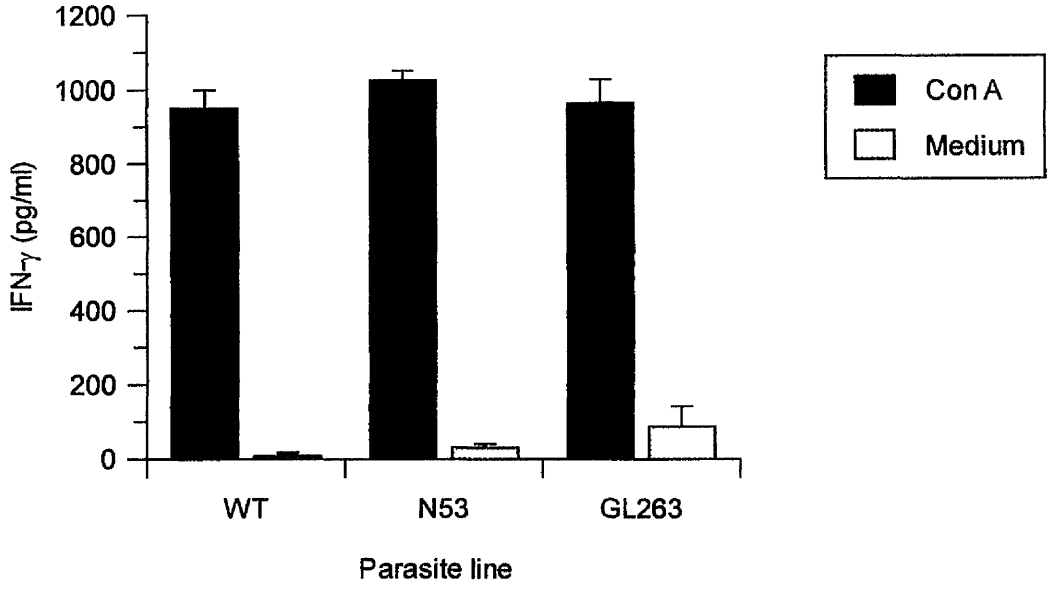

Figure 6.3.20 IFN-γ production by cultured splenocytes removed from *L. mexicana*-infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on Con A-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs.

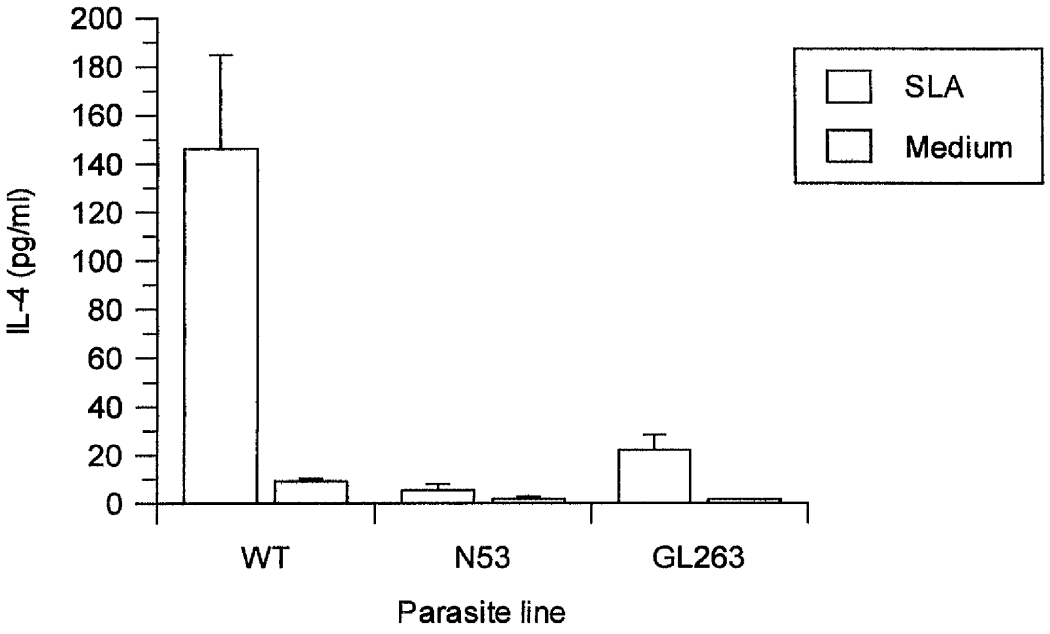

Figure 6.3.21 IL-4 production by cultured splenocytes removed from *L. mexicana* infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on SLA-stimulated (10 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs.

When splenocytes from GL263-infected mice were re-stimulated with recombinant CPB2.8ΔCTE, they did not produce levels of IL-4 that were significantly higher than background levels (Figure 6.3.22).

Splenocytes removed from GL263-infected mice polyclonally stimulated with Con A, produced significantly high levels of IL-4 compared to background levels ($p < 0.0001$) but were not significantly different from wild-type or Δ*cpb*-infected mice (Figure 6.3.23).

6.3.19 Analysis of CP activity from lesions isolated from *L. mexicana*-infected mice

It was confirmed using gelatin SDS-PAGE analysis that lesions from mice infected with the various *L. mexicana* single null mutant and re-integrated CPB mutants, possessed cysteine proteinase activity (Figure 6.3.24). Lesions for the wild-type infected mice were not isolated. Recombinant CPB2.8ΔCTE was loaded as a control for comparison with lesion lysates (lanes 1 and 11, Figure 6.3.24). All lesion lysates contained proteolytic activity but only lesions from mice infected with GL166 *L. mexicana* parasites (lanes 9 and 10), had any mature enzyme activity, which co-migrated with the recombinant CPB2.8ΔCTE. The lowest level of CP activity was observed in the lesions obtained from the Δ*cpb*-infected mice while lesions from the re-integrated CPB mutant-infected mice contained more CP activity than the Δ*cpb* null mutant-derived lesions.

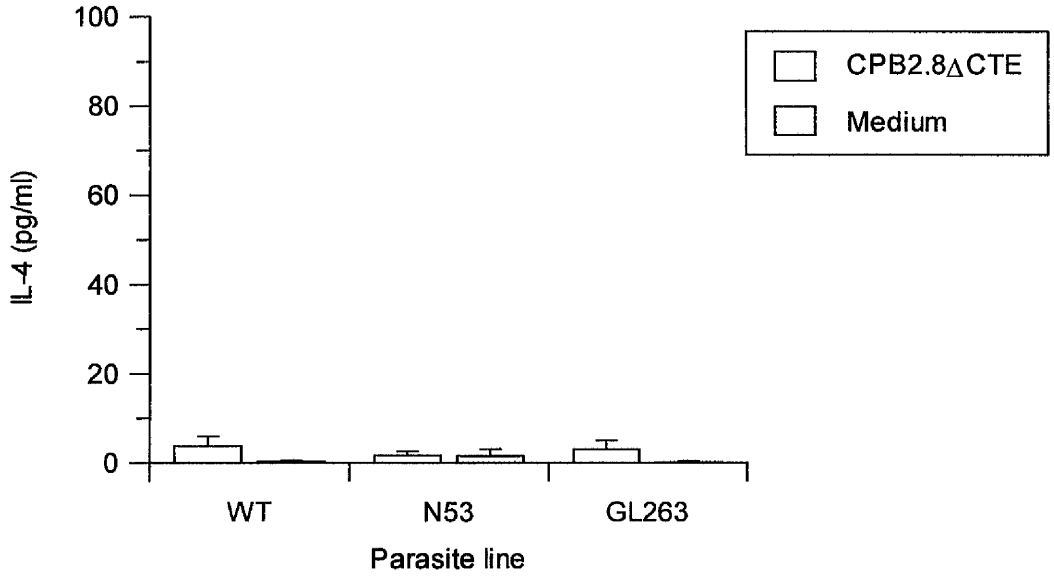

Figure 6.3.22 IL-4 production by cultured splenocytes removed from *L. mexicana* infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on CPB2.8ΔCTE-stimulated (5 μg/ml) cultures with non-stimulated cultures used as controls. Bars represent SEMs.

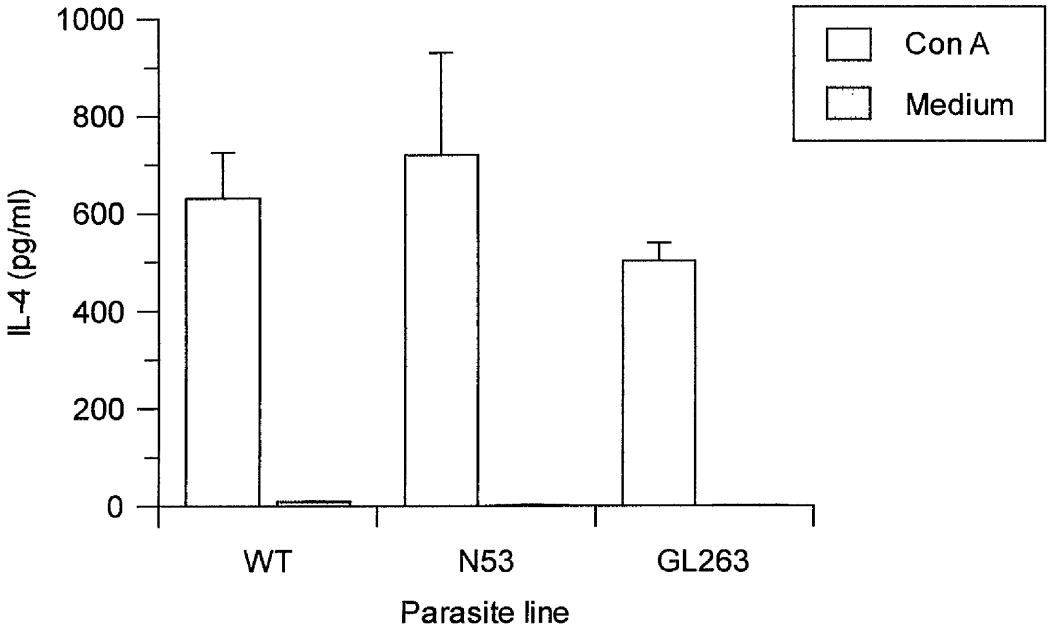

Figure 6.3.23 IL-4 production by cultured splenocytes removed from *L. mexicana* infected BALB/c mice, 7 mo post-infection. Cytokine analysis was performed on Con A-stimulated (5 μg/ml) cultures with non-stimulated cultures as controls. Bars represent SEMs.

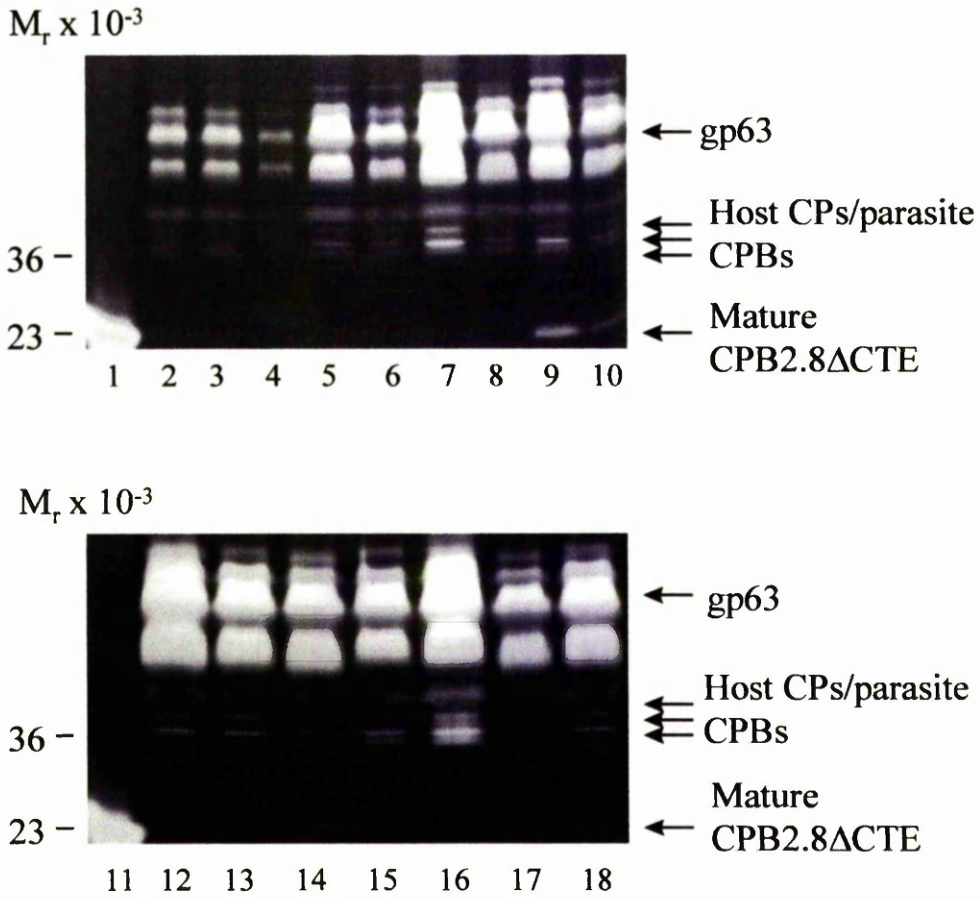

Figure 6.3.24 10 % gelatin SDS-PAGE showing CP activity derived from lesion material. All lanes were loaded with equivalent protein concentrations. Lanes 1 and 11, recombinant CPB2.8ΔCTE; lanes 2-5, Δ*cpb* parasite line; lanes 6-8, GL165 parasite line; lanes 9 and 10, GL166 parasite line; lanes 12-15, GL167 parasite line; lanes 16-18, GL168 parasite line.

6.3.20 Analysis of CP protein content of lesions isolated from *L. mexicana*-infected mice

In order to analyse leishmanial CPB amounts within lesions from the different groups of infected mice, Western blotting was performed using specific anti-serum, raised against the pro-mature form of CPB2.8ΔCTE and the appropriate pre-immune anti-serum (Figure 6.3.25).

The specific CPB2.8ΔCTE anti-serum detected recombinant mature enzyme and pro-mature and mature protein in a lysate of wild-type stationary phase promastigotes (Figure 6.3.25, gel B). However, the pre-immune anti-serum did not detect any leishmanial CPB protein in any of the different groups studied. Lanes 3, 4, 5, 6 and 7 represent lesions from one mouse of each group infected with the five different mutants of *L. mexicana* (Δ*cpb*, GL165-168). Other lesion lysates were also analysed but results were not different to those observed in Figure 6.3.25, gel B.

Detection of a protein (> 40 kDa) by the pre-immune sera (Figure 6.3.25, gel A) showed that equivalent protein loadings had been used for the different *L. mexicana* mutants but this protein has also been identified by others using pre-immune anti-sera to lesion material from *L. mexicana*-infected mice (Mottram *et al.,* 1996) and is considered to be non-specific.

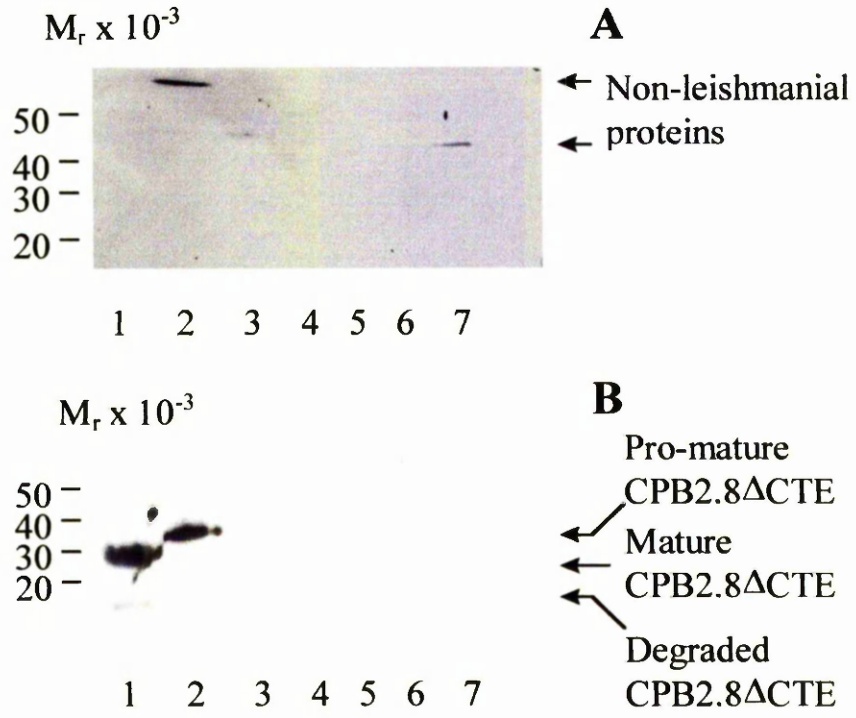

Figure 6.3.25 10% SDS-PAGE showing western blot of Δ*cpb* and re-integrated CPB *L. mexicana* amastigote lesions.
Lane 1, mature CPB2.8ΔCTE (frozen/thawed);
lane 2, wild-type stationary promastigote lysate;
lane 3, lesion lysate from Δ*cpb*-infected mouse;
lane 4, lysate from GL165-infected mouse;
lane 5, lysate from GL166-infected mouse;
lane 6, lysate from GL167-infected mouse;
lane 7, lysate from GL168-infected mouse.
Lanes were loaded with equivalent protein concentrations of lesion preparations. Bands were detected with pre-immune (**A**) or anti-pro CPB2.8ΔCTE antibody (**B**) at a 1: 2000 dilution.

6.4 DISCUSSION

The use of genetic manipulation to study the potential role(s) of the leishmanial CPs in the host-parasite interaction has shown that these enzymes are virulence factors (Mottram *et al.*, 1996). Furthermore, gene deletion of the *cpa* and *cpb* genes resulted in an attenuated strain of *L. mexicana* that upon infection, promoted an IFN-γ driven CD4+ Th 1 response and was considered to be a potential vaccine candidate (Alexander *et al.*, 1998). Deletion of the *cpa* and *cpb* genes in *L. mexicana* resulted in a no lesion growth phenotype for the mutant parasite and increased host IFN-γ production but notably it also resulted in a decrease in IL-4 production (Alexander *et al.*, 1998). Susceptibility to *L. mexicana* infection has previously been shown to correlate with IL-4 production (Satoskar *et al.*, 1995). As deletion of *cpa* and *cpb* genes resulted in potentiation of a Th 1 response, with loss of IL-4 production, it is possible that the leishmanial CPs may play some kind of role in IL-4 production, as previously identified for other CPs (Finkelman & Urban, 1992). This has been investigated by re-integrating CPB enzymes into *L. mexicana* parasites lacking the *cpb* array and also *cpa*.

Therefore, single (Δ*cpb*) and double null (Δ*cpb/cpa*) mutants of *L. mexicana* have been generated using targeted gene deletion and two different *cpb* genes, *cpb2* and *cpb2.8*, have been individually re-integrated into the mutants. In the case of the single null mutants, these CP genes were re-integrated using a native or chimaeric promoter, in order to analyse whether CP expression could alter parasite virulence,the importance of when this expression occurred and the subsequent immune response. Conceivably, re-integration of a metacyclic CP, CPB2, under control of a native (metacyclic) promoter (GL165) should result in

less expression in the amastigote form while the reverse may be expected when the chimaeric (amastigote-specific) promoter controlled CPB2 expression (GL167). For re-integration of CPB2.8, an amastigote CP, into the single null mutant under the influence of the native promoter (GL166), one may expect high expression levels in the amastigote and the reverse (promastigote expression) for a chimaeric promoter (GL168).

Re-expression of CPB2.8 into single null mutants using plasmid vectors has been already demonstrated (Mottram *et al.*, 1996) but it was found that the loss of antibiotic pressure *in vivo* resulted in a loss of enzyme activity when parasites were recovered from lesions. Re-integration of the gene directly into the chromosome removes this problem.

Unfortunately, because of the technical problems associated with re-integrating all 19 copies of the (haploid) *cpb* array into the genome of the null mutant in order to show complementation of the phenotype *in vivo*, the correlation between phenotype and genotype has never been proven. Thus, re-expression of the *cpb* array has been achieved using a cosmid vector and so depends on antibiotic selection for increased CP expression (Kelly *et al.*, 1994).

One problem encountered throughout the study was that it was not uncommon for several mice to be culled due to parasite-unrelated conditions such as bacterial infections of the eye or abnormally large swellings of the abdomen, even though inbred mouse strains were used. The BALB/c mouse strain is well known for its reduced IL-12 production and this may underlie the IL-4 dominated response to infection by *Leishmania sp.* (Alleva *et al.*, 1998). These mice may therefore be prone to opportunistic infections.

Lesion data were monitored throughout the course of infection and it was observed that BALB/c mice inoculated with wild-type *L. mexicana* amastigotes developed rapidly growing, non-healing lesions which far exceeded the lesion growth observed with the re-integrated CP mutants, including the GL263 parasite line.

L. mexicana wild-type parasites displayed an antibody phenotype comparable with that reported by others with high levels of IgG1 and very low levels of IgG2a, indicative of a Th 2 response and disease exacerbation (Alexander *et al.,* 1998). The antibody profile of the Δ*cpb* mutant was also in agreement with results published by this group with an increase in IgG2a production and a subsequent lowering of the IgG1/IgG2a ratio, inferring more of a Th 1 response.

CPs have previously been reported to induce IL-4 production (Finkelman & Urban, 1992) and at the termination of the experiment, splenocytes from mice infected with wild-type parasites were found to produce significantly more IL-4 following leishmanial antigen stimulation than those infected with the Δ*cpb* mutant. Furthermore, antigen-induced splenocyte IL-4 production was barely detectable in most of the BALB/c mice infected with the Δ*cpb* mutant. These results correlated well with Alexander *et al.* (1998) so it was surprising to find that most of the CP re-integrated *L. mexicana* mutants did not produce a significant increase in IL-4 compared with the null mutant despite an increase in lesion size.

Re-integration of the metacyclic CPB2 (GL165) into the null increased the parasite virulence compared with the Δ*cpb* null mutant, if one assesses lesion volume. However, the humoral and cellular responses suggest a lack of immune

polarisation. Antibody production of the IgG isotypes was similar to Δ*cpb* titres in terms of IgG2a production but similar in IgG1 production to wild-type-infected mice, suggesting a mixed Th phenotype. This was further exemplified by the production of IFN-γ and IL-4 in response to leishmanial antigen-stimulus. IFN-γ production was comparable with Δ*cpb*-infected mice but IL-4 production was also significantly higher than Δ*cpb*-infected mice which implies that the re-integration of this particular CPB was able to promote more of an exacerbative response than the Δ*cpb* mutant, and the lesion data confirm this. Re-integration of this enzyme therefore part restored virulence although the production of IFN-γ indicates that the Th 2 response was not fully developed as for wild-type-infected mice, which produced no IFN-γ when stimulated with leishmanial antigen stimulus.

Re-integration of CPB2.8 (GL166) did not appear to increase virulence when assessing mean lesion volume since it was not significantly different from the Δ*cpb* null mutant. The antibody titre in these mice was very low with IgG1 and IgG2a levels considerably lower compared with the other mutants examined, less IgG2a being produced than the other CP re-integrated mutants. The low antibody titre may also be indicative of a Th 1 response. Analysis of the cytokine response to antigen stimulus (leishmanial or polyclonal) provided no further understanding of the situation *in vivo* since there was no detectable production of IFN-γ or IL-4. Murine cytokine standards developed positively in the assay and so there appeared to be no problem with the ELISA system itself, rather the problem was attributed to the T cell stimulation assays. The reasons for this are unclear since all other *L. mexicana* infected mice were sacrificed, splenocytes recovered and stimulated in exactly the same manner as the GL166 line. The

number of splenocytes recovered was similar to the number isolated from the other *L. mexicana* lines so a possible explanation is that antigens used for stimulation were not at the optimum concentration for cytokine production. However, this is unlikely since the same conditions and antigen concentrations were used to stimulate all other lines. Trypan blue exclusion indicated that the lymphocytes were also viable so this did not appear to be the problem.

The reason for the above discrepancy is not known and one can only speculate as to what the problem was. However, this was very disappointing as the antibody response was not very indicative of the Th phenotype although low antibody titres do suggest a Th 1 response (Alexander *et al.*, 1998). Clearly, the experiment needs to be repeated although infection of mice with the GL 166 mutant *L. mexicana* will take several months and therefore results could not be included in this study.

Parasites re-integrated with the chimaeric form of CPB2 (GL167) produced larger lesions than the Δ*cpb*-infected mice although the volumes were smaller than those observed in wild-type-infected mice. The humoral response was suggestive of an intermediate infection, similar to isotype levels seen in GL165-infected mice. Splenocyte production of IFN-γ from GL167-infected mice was significantly higher than that of splenocytes isolated from wild-type infected mice but significantly lower than that from GL165-infected mice. Furthermore, splenocytes from GL167 mice produced no IL-4 in response to leishmanial antigen whereas GL165-infected mice did. With GL167-infected mice producing significant levels of IFN-γ and no IL-4, one may expect lesion formation to be similar to that of the null mutant (Δ*cpb* also did not produce IL-4 but did produce IFN-γ), which was not the case and so it is likely that there are

other factors involved e.g. other Th 1/2 cytokines such as IL-12 (Weinheber *et al,*. 1998) . In retrospect, other Th 1/Th 2 cytokines could have been examined such as IL-2, which has been shown to be reduced in wild-type *L. mexicana* infection (Alexander *et al.,* 1998) or IL-12, which is known to be down-regulated in macrophages after *L. mexicana* infection (Weinheber *et al.,* 1998). This may have given a clearer understanding of the immune response during infection.

Numerous studies have shown that cytokine interplay during *Leishmania* infection cannot be as simplified as high or low levels of one particular cytokine conferring resistance or susceptibility to infection (Scott *et al.,* 1996). For example, *in vitro* experiments may have omitted stimuli that are important *in vivo*. Inhibitors of some IL-4 or IFN-γ effects might be present *in vivo,* so that effects of these lymphokines demonstrable *in vitro* might be blocked e.g. receptor expression, in intact animals (Finkelman *et al.,* 1990). Recently, published results have shown that there are other mechanisms independent of the IL-4 receptor which underlie the default Th 2 development in *Leishmania* infected BALB/c mice (Mohrs *et al.,* 2000). One must be careful in assigning cytokine production *in vitro* to virulence *in vivo* although the levels of IL-4 and IFN-γ are obviously important in *Leishmania* infection as has repeatedly been shown (Heinzel *et al.,* 1989; Afonso *et al.,* 1994; Alexander *et al.,* 1998).

GL168-infected mice produced a very similar antibody profile to Δ*cpb*-infected mice with equally high levels of IgG2a and IgG1 as the Δ*cpb* null mutant, although GL168 mice did have significantly higher mean lesion volumes than the Δ*cpb* null mutant suggesting an exacerbative role for re-expressed chimaeric CPB2.8. However, analysis of the splenocyte response to leishmanial antigen

stimulus showed significant IFN-γ production but no IL-4 production which was analogous to the GL167-infected mice.

An obvious disadvantage of the cytokine analysis carried out is that the late sample point may miss early events which could alter the phentoype such as innate IL-12 production and this should be considered for future experiments. However, in conclusion, the data suggest that metacyclic CPB2 is more of a virulence factor than the other re-expressed CPs since it was the only mutant (GL165) to produce significant levels of IL-4 although it also produced significantly higher levels of IFN-γ than its re-integrated chimaeric mutant, GL167. The fact that CPB2 was more potent in stimulating IL-4 production than the other re-expressed proteinases suggests that CPB2 could play a role in the host-macrophage interaction. CPB2 is normally maximally expressed in the metacyclic stage of the parasite which is phagocytosed by the macrophage and if it does play a role in this interaction, expression of CPB2 in the amastigote using a chimaeric promoter may help mediate uptake of the parasite in an as yet, undefined mechanism. This hypothetically may help to establish the infection more quickly, generating more of a Th 2 response in the early stages. Once the parasite was within the parasitophorous vacuole, the enzyme may be redundant in its function (during the initial host-parasite interaction) and thus the GL165 parasite may behave more like the Δ*cpb* parasite. This would explain the higher IFN-γ level than the chimaeric CPB2 and the plateau effect of the mean lesion volume, seen during the latter stages of infection.

In order to analyse the level of CP activity and expression from the lesions of the different groups of infected mice, gelatin SDS-PAGE and immuno-blotting was used. All extracted parasite lesions (Δ*cpb*, GL165-168) displayed proteolytic

activity on gelatin SDS-PAGE but this did not aid the identification of the leishmanial CPB enzymes from the host cathepsins, known to be found in host macrophages, and thus likely to be at the site of infection (Maekawa *et al.,* 1998). Therefore, immuno-blotting was performed using polyclonal anti-CPB2.8ΔCTE anti-sera to assess the level of CPB expression from lesions prepared from the infected mice. However, the results obtained, suggested that there was no expression of CPBs in the GL165-168 mutants analysed (Δ*cpb* null would not give expression of the *cpb* enzymes). The antibody recognised the recombinant CPB2.8ΔCTE enzyme and the pro-mature of CPB2.8ΔCTE from a lysate of wild-type promastigotes. Therefore, the antibody was fully functional. However gelatin SDS-PAGE analysis showed that the GL166 line produced CP activity at approximately 23 kDa mobility, similar to that of the recombinant enzyme, suggesting there was expression of the CPBs from this particular mutant. The question remains un-answered as to the level of CPB expression from the re-integrated mutants, *in vivo*. This is an obvious disadvantage when analysing expression of CPBs *in vivo*, since only one time-point was taken for analysis. The expression of the CPBs may have been high early in infection and so have a significant effect in the host-parasite relationship but by the time of analysis, the re-integrated CPBs were possibly expressed at very low levels in that they could not be detected by Western blotting.

The re-expression of the cosmid containing the *cpb* array into the Δ*cpb* null mutant provided some unexpected results. Mean lesion volumes from the infected mice showed that GL263-infected mice had lesions comparable to those of the Δ*cpb* null mutant. This was further corroborated by the high levels of IgG2a isotype (similar to Δ*cpb*-infected mice) and high IFN-γ production in

response to leishmanial antigen stimulus. However, antigen stimulus also promoted a significantly high level of IL-4 from splenocytes which correlated well with the level of IgG1, similar to wild-type titres. Significantly, production of this cytokine was not enough to alter lesion development, which was much slower than the wild-type infection.

The results obtained for the cosmid re-expressing mutants are surprising since one would expect re-expression of the entire *cpb* array into the Δ*cpb* null mutant to restore wild-type virulence levels since it has been shown that cosmid expression of a CP gene *in vitro* has been found to be several times higher than genomic expression of the gene (Kelly *et al.*, 1994). These authors reported that cosmid transfected cells displayed considerable over-expression of the major CP from *T. cruzi*, cruzipain, which correlated with an increase in enzyme activity as assessed by gelatin gels. However, these investigators did not infect animals with the cosmid-containing parasites, and so the level of activity *in vivo* was not known. It could be that re-expression of the array in the cosmid resulted in re-arrangement of the array preventing transcription of the CPs which in turn would explain the reason for the similarity in lesion progression to the Δ*cpb*-infected mice since CP expression may be defunct . Alternatively, the production of IL-4 could be due to part of the array being expressed sufficiently enough to produce some CP activity while the majority or even all of the array was lost. The other reason may be due to loss of cosmid expression with loss of antibiotic pressure since it has already been established that the absence of neomycin, *in vivo*, allows CPB expression to drop substantially during lesion formation (Mottram *et al.*, 1996). Recovery of parasites *ex vivo* would help to identify whether CP activity

was still present in the cosmid-transfected parasites and should be performed if such experiments are to be repeated.

The lesion development of the double null mutants has been reported previously, although in that particular study mice were infected with promastigotes rather than axenic amastigotes (Alexander *et al.,* 1998). In this study, lesions were very small after 8 months (10 mm^3) while in the former study, there were no lesions after this time-point. However, one would expect challenge with amastigotes, rather than promastigotes, to potentiate infection more quickly unless they were totally attenuated. Antibody titres of IgG1 and IgG2a were very low (reciprocal end-point dilution of < 1000) indicating that parasite proliferation was limited. This is typical of the immunologic status of humans at the resistant end of the disease spectrum (Alexander *et al.,* 1998). Interestingly, re-integration of CPB2 (GL438) promoted similar development in mean lesion volume to the Δ*cpb/cpa* mutant while CPB2.8 re-integration (GL439) did not produce any development of lesions. Significantly, IgG2a titres were higher in the GL438 and GL439 mutant infected mice than the DN8 line which suggests more of a Th 1 response. Splenocyte responses were not assessed as the experiments were on-going but it would be of interest to see if production of IgG2a in the re-expressing *L. mexicana* mutants would also result in an increase in IFN-γ compared to the DN8 mutant (which has already been shown to produce high IFN-γ levels in response to leishmanial antigen stimulus (Alexander *et al.,* 1998)) since high IgG2a is known to correlate with IFN-γ production (Finkelman *et al.,* 1990).

It is possible that re-integration of the proteinases, CPB2 and CPB2.8, into the double null mutant may not result in fully active enzymes since they may require other CPs (e.g. the CPA enzyme) to process them. Conceivably, the

enzymes may not be processed and in order to avoid an accumulation which could be toxic, the parasite may have to exocytose these molecules out of the cell into the parasitophorous vacuole. From this site, they could be processed and presented with host MHC class II molecules and stimulate more of an IFN-γ driven Th 1 response resulting in a concomitant increase in IgG2a production.

We may expect re-integration of CPB2.8 to produce a more virulent infection since this has been shown to be a virulence factor *in vitro*. However, the Δ*cpb* single null mutant re-integrated with CPB2.8 was used to analyse infection of explanted PECs which were probably not a true representation of the macrophage population seen *in vivo* (Mottram *et al.*, 1996). Therefore, it could be that the Δ*cpb/cpa* mutant line re-expressing CPB2.8 (GL439) is unable to promote infection as well as the DN8 or GL438 amastigote lines.

The generation of the single and double null mutants is a major aid in efforts to elucidate gene function in terms of virulence and has also been shown to be a useful tool in assessing mutants for potential as attenuated live vaccines (Alexander *et al.*, 1998). However, it is clear that further analysis of the re-integrated CP mutants will need to be carried out in order to elucidate exactly what function the metacyclic and amastigote CPs may perform in the host-parasite interaction.

GENERAL DISCUSSION

Cysteine proteinases (CPs) are known to be important in several pathological conditions such as cancer, asthma and particularly in infectious disease (Mort *et al.*, 1981; Travis *et al.*, 1995; Gough *et al.*, 1999).

CPs have been shown to affect a variety of different mechanisms in the mammalian immune system. For example, the house dust mite allergen, *Der p 1*, has been reported to trigger the asthmatic response in several ways by cleaving CD23 and CD25 molecules on mammalian cells, which interferes with the IgE network and IL-2 receptor, respectively (Hewitt *et al.*, 1995; Schulz *et al.*, 1998). Furthermore, *Der p 1* can also promote activation of NF-κB, a transcriptional factor which regulates GM-CSF, IL-8 and TNF-α (Stacey *et al.*, 1997). This CP is just one example of the numerous CPs which are clinically important in mammalian disease that have a diverse range of functions attributed to them. In parasitic infection, CPs have been implicated as prominent virulence factors in diseases such as trichomoniasis, leishmaniasis and Chagas disease(Eakin *et al.*, 1991; Coombs *et al.*, 1997; Draper *et al.*, 1998) but are also important in a number of other infectious diseases.

In order to produce inhibitors to CPs, a common approach is to obtain large quantities of the active enzyme by cloning the CP and screening with inhibitors to the CP of interest (Selzer *et al.*, 1997). Once lead inhibitors have been identified, the compounds can be tested for their effects on mammalian cells. If the inhibitors are selectively taken up by parasite cells rather than host cells, intensive studies may begin. However, production of large amounts of parasite CP also facilitates analysis of the CPs since the enzyme can be tested against a

number of different biological substrates that it may have proteolytic activity to, *in vivo*. For example, the gingipains of *Porphyromonas gingivalis* have been found to cleave a number of biologically important molecules produced as the site of infection with this organism including the inflammatory molecule TNF-α (Calkins *et al.,* 1998) and the degradation of this molecule helps to circumvent the host immune response.

The generation of parasite mutants lacking the CP gene of interest allows for the study of these enzymes, while re-integration or re-expression of CP genes back into the mutants can also aid the investigator in elucidating CP function (Mottram *et al.,* 1998). Both approaches have been adopted for this study and both have their advantages and disadvantages as have been described in previous chapters (Barrett *et al.,* 1999).

Successful and heterologous expression and purification of a *L. mexicana* CP, CPB2.8ΔCTE, yielded a highly active enzyme (Sanderson *et al.,* 2000). The developed procedure is now being used for purification of other leishmanial CPB isoenzymes over-expressed in *E. coli* but preliminary experiments with the *L. infantum* CPB containing the C-terminal extension, have shown that the methodology may need to be modified as required for each of the enzymes being purified.

Over-expression of the pro-region part of the enzyme is also being carried out in this laboratory and should aid the identification of highly specific inhibitors of the leishmanial CPs since pro-regions have been found to be highly potent inhibitors of their respective mature enzymes (Cygler & Mort, 1997). Synthetic peptide inhibitors based on the pro-region could be promising chemotherapeutic compounds It has already been shown that CP inhibitors administered *in vivo* can

result in parasite (*T. cruzi*) killing and subsequently alter the course of infection to a healing response (Engel *et al.,* 1998). Recently, it has been reported that specific CP inhibitors can kill *Leishmania* parasites both *in vitro* and *in vivo*, at concentrations that do not affect mammalian CPs, in terms of cell replication or ultra-structural appearance (Selzer *et al.,* 1999). None of the compounds produced toxic effects in mice and parasites appeared to accumulate inhibitor much more effectively than the host cell organelles (Selzer *et al.,* 1999). Since the host also contains cathepsin L-like enzymes, the more specific the inhibitor for the parasite CP is, the less risk of side-effects experienced by the host and pro-regions have been shown to be highly specific inhibitors for their respective proteinase (Cygler *et al.,* 1997). In addition to this, exogenously added pro-region can renature un-folded subtilisin in an intermolecular process (Zhu *et al.,* 1989). The purified pro-region of CPB2.8ΔCTE may be able to similarly refold pro-mature CPB2.8ΔCTE *in vitro*, purified by metal chelate affinity chromatography which proved to be unsuccessful in producing fully active enzyme.

Having obtained active recombinant CP, the potential effects on macrophage (PECs) cytokine production were analysed since it is this cell which is most likely to encounter the active CP during *Leishmania* infection. PECs are composed primarily of inflammatory macrophages but there are other cells present such as neutrophils and eosinophils therefore bone marrow derived macrophages could be used as they represent a more homogeneous macrophage population. The enzyme did not affect inflammatory mediators, IL-12 or nitric oxide but did inhibit production of IL-10. A recombinant leishmanial protein, LeIF, has been shown to stimulate IL-12 production from peripheral blood mononuclear cells

(PBMCs) of leishmaniasis patients so there is precedent for leishmanial molecules to induce a Th 1 profile (Skeiky *et al.,* 1995). However, the reduction in IL-10 from PECs would be more significant if this same effect was demonstrated in PBMCs from leishmaniasis patients and this experiment is certainly one worth considering. In theory, a number of cytokines could be assessed which are considered to be important in leishmanial infection e.g. IL-6, IL-10, IL-12 and TGF-β. Alternatively another cell type, the basophil could be analysed in a similar fashion since it has been demonstrated that addition of the soluble egg antigen from *S. mansoni* has also been shown to stimulate IL-4 production from human basophils (Haisch *et al.,* 2000). Some allergens are also known to stimulate IL-4 production from basophils (*Der p 1*) in atopic individuals (Gough *et al.,* 1999) so it is feasible that CPs from lysed *Leishmania* parasites could trigger an increase in IL-4 production in the host by this mechanism. To determine whether CPB2.8ΔCTE can stimulate IL-4 production *in vivo*, active and inactive enzyme could be injected into mice and mRNA from the draining lymph nodes isolated. This would mirror work done by Finkelman and Urban (1992) who injected active and inactive papain into BALB/c mice and found that active CP promoted high IL-4, while inactive CP produced significantly less IL-4, afer 24 hours. In the case of leishmanial infection, an early IL-4 burst due to the CPBs could potentiate a Th 2 response and allow for establishment of infection.

Vaccination with the active or inactive CPB2.8ΔCTE and IL-12 was found to be partially protective in three different mouse strains against wild-type *L. mexicana* and the results, especially for the susceptible BALB/c mouse strain are encouraging in terms of a possible vaccine since this strain is very susceptible to infection with *L. mexicana* (Satoskar *et al.,* 1995). Unfortunately, IL-12 has

been shown to produce unpleasant side-effects in humans although it is still undergoing clinical trials as a potential for cancer therapy (Leonard *et al.,* 1997). Since CPB2.8ΔCTE has been shown to be partially protective as part of a vaccine against *L. mexicana*, other adjuvants should be considered. Effective immunisation against cutaneous leishmaniasis with defined membrane antigens such as gp63 reconstituted into liposomes has been reported (Russell & Alexander, 1988). Liposome formulations are relatively non-toxic and therefore CPB2.8ΔCTE should be incorporated into such a formulation and tested for its protective capacity without IL-12. Another potential method of vaccination against *Leishmania* has been through the use of integration of recombinant leishmanial proteins into genetically attenuated *Salmonella* (Soo *et al.,* 1998) and infecting with this organism before challenge with *Leishmania*. This study demonstrated protection to *L. major* infection when the attenuated *Salmonella* strain was integrated with gp63 (Soo *et al.,* 1998). Therefore, the possibility also exists for the leishmanial CPBs to be incorporated into such a vaccine against *Leishmania* and if successful, would allow efforts to be diverted away from the search for a suitable adjuvant.

It has been reported that a DNA vaccine for the gene, LACK (*Leishmania* homologue of receptor of activated C kinase) has resulted in a protective vaccine for leishmaniasis which generates short-term immunity in susceptible BALB/c mice (Gurunathan *et al.,* 1998). Because DNA vaccines result in expression of the antigen *in situ*, presentation of antigenically relevant epitopes to the immune system may be more readily attainable than vaccination with recombinant purified proteins (Donnelly *et al.,* 1997). Results suggest that generation of Th 1-like T cell help may be a general property of DNA vaccines (Donnelly *et al.,* 1997).

Therefore, construction of a DNA vaccine incorporating CPB2.8 or even a cocktail of CPs including CPA and CPC should be considered since immunisation with a DNA vaccine incorporating the gene for a leishmanial metalloproteinase, gp63, has afforded significant protection in BALB/c mice against *L. major* infection (Xu & Liew, 1995).

This and other studies have shown that targeted gene deletion of *L. mexicana* is an extremely useful tool for providing evidence that the CPs are potential virulence factors (Mottram *et al.*, 1996) while attenuated *L. mexicana* mutants lacking both the *cpa/cpb* genes, are excellent vaccine candidates (Alexander *et al.*, 1998). Re-expression into the single null mutant of a CP produced a more virulent infection, if one analyses the disparity in the humoral response and lesion progression. Analysis of the splenocyte response in mice infected with the GL438 (Δ*cpb/cpa* re-integrated with CPB2) and GL439 (Δ*cpb/cpa* re-integrated with CPB2.8) parasite lines should give a clearer understanding of the infection *in vivo*. A Δ*cpb/cpa* double null mutant re-expressing an active site mutant of CPB2.8 has been constructed. Infection of mice and the resulting humoral and cellular immune responses should be analysed. This should provide further insight into the role of CPB activity in leishmanial infection. The number of possible CP gene mutations, deletions and re-integrations which could be performed in this laboratory are limitless but careful consideration must be given in order to elucidate the role of each of the CPs. Since infection with the Δ*cpb/cpa* mutant line potentiates a Th 1 response, re-integration of the active site mutant of CPB2.8 may result in an accumulation of CP which may be exocytosed due to non-processing and subsequently presented

to Th cells. This may promote an even greater Th 1 response and could be a more likely vaccine candidate than the double null mutant alone.

This study set out to purify a recombinant leishmanial isoenzyme, CPB2.8ΔCTE, from *L. mexicana* and identify a potential role for this enzyme. It has been successful in that purification of active enzyme was achieved and a number of roles for the enzyme have been excluded and suggested, while also showing that the enzyme was a protective antigen. A method for analysis of purified recombinant CPs or genetically mutated parasite lines in *L. mexicana* infection has been generated and shows that leishmanial CPs can potentiate both IFN-γ and IL-4-type responses in infection. This thesis has also provided the theoretical basis for future work and potential elucidation of the leishmanial CPs in the host-parasite interaction.

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