Characterisation of a Recombinant Phosphorylcholine-free Form of the Immunomodulatory Filarial Nematode Secreted Product, ES-62.

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DECLARATION

This thesis has been written in accordance with the University of Glasgow regulations, has not been presented for a degree at any other university and is original except where indicated otherwise by reference in the text.

The work contained within is the author's own work except where work was done in collaboration as indicated.

Signed	Date
.....	Aug 2007
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Houston, K.M., Egan, C.A., Garcia. P. and Harnett, W. (2006) Investigation of Strategies with Potential for Producing a Phosphorylcholine-free Version of the Filarial Nematode Immunomodulator, ES-62. *Journal of Helminthology,* **80,** 261-265

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ABBREVIATIONS

°C	Degrees Celsius
^{14}C	Carbon 14
2-ME	2-Mercaptoethanol
^{3}H	Tritium
α	Alpha
AA	Amino acid
AGE	Advanced glycation end products
AOX	Alcohol oxidase
AP	Alkaline phosphatase
APC	Antigen presenting cell
APOC	African programme for onchocerciasis Control
AUC	Analytical ultracentrifugation
β	Beta
BCIP/NBT	5-bromo-4-cloro-3-indolyl phosphate toluidine and nitroblue tetrazolium
BCR	B cell receptor
bmDCs	Bone marrow derived dendritic cells
BMG	Buffered minimal glycerol
BMM	Buffered minimal methanol
bp	Base pairs
BSA	Bovine serum albumin

CII	Type II collagen
ca.	circa
$CaCl_2.H_2O$	Calcium chloride
CD	Circular dichroism
CDC	Centers for disease control
CDP	Cytidine diphosphosphate
Cer	Ceramide
CFA	Complete freunds adjuvant
Ci	Curie
CIA	Collagen-induced arthritis
CO_2	Carbon dioxide
Con A	Concanavalin A
CTP	Cytidine triphosphate
D	Aspartic acid
DAG	Diacylglycerol
DALY	Disability adjusted life year
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
DDT	Dichloro-diphenyl-trichloroethane
DEC	Diethylcarbamazine
dH_2O	Distilled water
dMM	1-deoxymannojirimycin
DMSO	Dimethylsulfoxide

DNA	Deoxyribose nucleic acid
dNM	Deoxynorijirimycin
dpm	Disintegrations per minute
DSA	*Datura stramonium* agglutinin
E	Glutamic acid
EAE	Experimental autoimmune (formerly allergic) encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPC	Erucylphosphocholine (22,1,cis)
ER	Endoplasmic reticulum
ES	Excretory/secretory
$Et_{18}OCH_3$	Rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine
EU	Endotoxin unit
FAB-MS	Fast atom bombardment mass spectroscopy
Fuc	Fucose
g	Grams
	Or
	Unit of centrifugal force
G	Glycine
γ	Gamma

Gal	Galactosamine
GalNAc	*N*-acetyl galactosamine
GlcNAc	*N*-acetyl glucosamine
GM-CSF	Granulocyte macrophage-colony stimulating factor
GNA	*Galanthus nivalus* agglutinin
H	Hour
H_2O	Water
H_2SO_4	Sulfuric acid
Hb	Haemoglobin
HC-3	Hemicholinium 3
HCl	Hydrochloric acid
HEPC	Hexadecyl phosphocholine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiFCS	Heat-inactivated FCS
HIV	Human Immunodeficiency Virus
hCG	Human chorionic gonadotropin
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IFNγ	Interferon gamma

IL	Interleukin
i.p.	Intraperitoneal
ITAMS	Immunoreceptor tyrosine-based activation motif
K	Lysine
K_2HPO_4	Dipotassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen orthophosphate
KCl	Potassium chloride
Ko/KO	Knockout
KOH	Potassium hydroxide
L	Litre
LAL	Limulus amaebocyte lysate
LNFPIII	Lacto-*N*-fucopentaose III
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
Man	Mannose
MD	Minimal dextrose
Mf	Microfilariae
Mϕ	Macrophages
$MgSO_4$	Magnesium sulfate
$MgCl_2$	Magnesium chloride
µg	Microgrammes

MHC	Major histocompatability complex
µl	Microlitres
µm	Micrometre
Min	Minutes
ml	Millilitres
mM	Millimolar
mUnits	MilliUnits
MBq	Mega becquerel
MMG	Minimal media containing glucose
MMM	Minimal media containing methanol
mmol	Milimoles
MOPS	3-(N-morpholino)propanesulfonica acid
MR	Mannose receptor
MyD88	Myeloid differentiation factor 88
N	Asparagine
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen orthophosphate anhydrous
$Na_2HPO_4.12H_2O$	Disodium hydrogen orthophosphate dodecahydrate
NaH_2PO_4	Sodium dihydrogen orthophosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide

NES	*Nippostrongylus brasiliensis* excretory-secretory antigen
ng	Nanograms
NGDO	Non governmental developmental organisations
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulfate
nm	Nanometres
NMR	Nuclear magnetic resonance
NP40	Nonidet P40
OCP	Onchocerciasis control programme
OD	Optical density
OVA	Ovalbumin
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphorylcholine
Pce	Recombinant phosphorylcholine esterase
PCR	Polymerase chain reaction
pES-62	Parasite-derived ES-62 (from *A.viteae*)
pfu	Plaque forming unit
pg	Picograms
pmol	Picomoles

PNA	Peanut agglutinin
PtdChol	Phosphatidylcholine
PVDF membrane	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end-products
REA	Rapid epidemiological assessment
REMO	Rapid epidemiological mapping of onchocerciasis
rES-62	Recombinant ES-62 (from *P.pastoris*)
RNA	Ribonucleic acid
rpm	Revolutions per second
RPMI	Roswell Park Memorial Institute
RPMI-c	RPMI 1640 lacking choline
RPMI-g	RPMI 1640 lacking glucose
RPMI-m	RPMI 1640 lacking methionine
s	Seconds
S	Serine
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Lauryl sulphate; sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis
SE	Sedimentation equilibrium

SEA	Soluble egg antigens of *Schistosoma mansoni*
SM synthase	Sphingomyelin synthase
SV	Sedimentation velocity
TBq	Tera becquerel
TBS	Tris buffered saline
TBST	TBS plus tween 20 (0.1% w/v)
TCA	Trichloroacetic acid
TCR	T-cell receptor
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tg	Transgenic
TGFβ	Transforming growth factor beta
Th	T helper
TIR	Toll interleukin I domain
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
Treg	Regulatory T cell
U	Units
UV	Ultra violet
v/v	Volume by volume
WHO	World Health Organisation
wks	Weeks
WT	Wild-type

w/v	Weight by volume
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
$ZnCl_2$	Zinc chloride

SUMMARY

The longevity of filarial nematodes is dependent on secreted immunomodulatory products. Previous investigation of one such product, ES-62, has suggested a critical role for post-translationally attached phosphorylcholine (PC) moieties. In order to further investigate this, I attempted to produce ES-62 lacking PC by using a variety of enzymes and enzyme inhibitors. The resultant material was, unfortunately, never 100 % PC-free, and the results were also highly variable. As an alternative approach it was therefore decided to place the gene for ES-62 into the *Pichia pastoris* recombinant gene expression system. It was hoped that the gene expression system, being eukaryotic in nature, would side-step solubility issues previously encountered when an *Escherichia coli*-based system was attempted, as it would be able to perform the post-translational modifications necessary for production of a correctly folded protein, whilst avoiding hyperglycosylation issues frequently encountered when using a *Saccharomyces cerevisiae*-based system. The procedure was successful, and an abundant supply of recombinant protein was available for use.

When the recombinant protein's biochemical features were compared with parasite-derived ES-62, which has been shown to be tetrameric in form, the former was found to consist of a mixture of apparently stable tetramers, dimers and monomers. Nevertheless, the recombinant protein was considered to be an adequate PC-free ES-62 as it was recognized by

existing antisera against the parasite-derived protein including a monoclonal antibody (mAb) that recognised a conformational determinant.

However, subsequent to this, differences were found in the recombinant protein's sensitivity to a panel of enzymes when compared to the parasite-derived material, and recognition of parasite-derived ES-62 by antibodies produced against the recombinant protein was found to be absent. Evidence was also observed suggesting that although the recombinant material appeared to act similarly to the parasite-derived material in terms of modulation of cytokine production by bone marrow-derived dendritic cells (bmDCs), the mechanism by which this result was achieved appeared to be different from that elucidated for the parasite-derived material: it seemed that the recombinant protein's mode of action was independent of TLR4 or MyD88, in comparison to the parasite-derived ES-62. It was also observed that the recombinant material appeared to have a higher content of mannose than the original protein, an unexpected finding considering the evidence that *Pichia* is much less likely to hyperglycosylate than other yeast-based recombinant gene expression systems such as *S.cerevisiae.*

In an attempt to explain the differences observed, recombinant ES-62 was subjected to structural analysis and was found to (i) contain 3 changes in amino acid composition; (ii) demonstrate significant alterations in glycan composition; (iii) show major differences in protein secondary structure. The effects of these alterations in relation to the observed change in immunogenicity were investigated and the finger was pointed at the changes

in secondary structure. A major take-away message from the data is that recognition by existing antibodies is insufficient proof that recombinant proteins can be used to mimic parasite-derived material in studies on nematode immunology and vaccination.

Chapter 1: Introduction

1.1 LYMPHATIC FILARIASIS AND ONCHOCERCIASIS

There are eight species of filarial nematodes known to infect humans, three of which, namely *Wuchereria bancrofti, Brugia malayi* and *Onchocerca volvulus* (Figs 1.1A, B and C respectively) are responsible for much of the morbidity seen in the Tropics (World Health Organisation (WHO), 1997). Currently it is estimated that one hundred and fifty million people suffer from at least one of these named parasites, with a further one billion at risk of infection (Ottesen *et al.*, 1997; Fig. 1.2). Symptoms range from skin lesions to elephantiasis and blindness, with onchocerciasis being the second commonest cause of preventable blindness in Sub-Saharan Africa (Hoerauf *et al.*, 2003).

Lymphatic filarial nematodes infect approximately 120 million people in 80 different countries worldwide, with an estimated one billion people at risk (www.filariasis.org). Over 40 million of the people infected are severely so (Molyneux, 2001), and there is still a great deal of stigmatism attached to the disease (WHO fact sheet no 102 Rev. Sep. 2000). Of all the people infected over forty percent live in India, one third in Africa, with the majority of the last portion living in Asia, the Pacific and the Americas. Nine out of ten of all lymphatic filariasis incidences are caused by *W.bancrofti* and most of the remaining ten percent are caused by *B.malayi* (www.filariasis.org). The pathology resulting from a worm infection can range from uncomfortable, at best, to the classic example of lymphatic filariasis-associated pathology,

elephantiasis. It is true, however, that acute episodes are often caused by a secondary bacterial or fungal infection of the skin, as damage to the lymphatic system underneath has lowered the defences (Shenoy *et al.*, 1999; Dreyer *et al.*, 2000a and 2000b).

Onchocerciasis is the second commonest cause of preventable blindness in Sub-Saharan Africa (Thylefors *et al.*, 1995; Lewallen and Courtright, 2001) and is found in 37 countries worldwide: 30 are in Africa, in which 99 percent of infected individuals live (WHO/TDR). It is estimated that 18 million people are infected (mentioned in Basanez *et al.*, 2006), with over 120 million people are at risk of infection. The disease causes severe itching or dermatitis in more than 6.5 million of those infected, and blindness in 270,000, with severe visual impairment in a further 500,000 (WHO, 1995a).

The main burden of these diseases is the morbidity that they cause: lymphatic filariasis is the cause of an estimated 5.5 million disability adjusted life years (DALYs), and onchocerciasis is thought to be responsible for 1 million DALYs (Molyneux *et al.*, 2003).

1.1.1 Lymphatic Filarial Nematodes and O.volvulus *Lifecycles*

The transmission cycles of both lymphatic filariasis, caused by *W.bancrofti* (Fig. 1.3) and *B.malayi* (Fig. 1.4) and onchocerciasis, caused by *O.volvulus* (Fig. 1.5) rely upon an intermediate vector.

In the case of the causative agents of lymphatic filariasis, the vector is the mosquito. The main three vectors for *W.bancrofti* are of the *Culex* species in most urban and semi-urban areas of Africa, *Anopheles* species in more rural areas of Africa, and *Aedes* species in many of the endemic Pacific Islands (Sasa, 1976). *B.malayi* is spread mainly by mosquitoes of the *Mansonia* (the major vector), and *Anopheles* species in some areas too (Sasa, 1976).

The vector of *O.volvulus* is the blackfly, the major vectors being complexes of sibling species of *Simulium*: Most of Africa's main vectors belong to the *S.damnosum* complex, while the main vectors in Uganda, Tanzania, Ethiopia and the Congo belong to the *S.neavei* complex. In Latin America the most important vectors are *S.ochraceum* (Mexico and Guatemala), *S.metallicum* (Venezuela), *S.exiguuml* (Equador) and *S.oyapockense* (Brazil) (Zea Flores, 1990; Shelly, 1988).

The worms are so thin as to appear thread-like, and their ability to survive within the host for many years has been well documented: the reproductive lifespan of the adult causative agents of lymphatic filariasis can be between 4 and 8 years (Michael *et al.*, 2004), whilst in onchocerciasis the mean fecund lifespan of an adult female is estimated as 9-11 years (Habbema *et al.*, 1992). The female worms produce thousands of microfilariae (mf) a day. In the case of lymphatic filariasis, mf circulate in the bloodstream, and are taken up in a bloodmeal by the vector. Over the course of 7-12 days in the mosquito, these immature larvae develop into infective larvae, which migrate to the mouthparts of the mosquito to ensure they will be passed on to the

next person bitten, thus completing the transmission cycle (Figs 1.3 and 1.4). The mf produced by adult female *O.volvulus*, living in nodules under the skin, migrate though the body and cause many different symptoms after they die by stimulating host inflammatory reactions. The nodules can be 1-5 cm in diameter and are most commonly found on the skin covering protrusions of bones, e.g. hips (WHO, 1987). Much the same as for lymphatic filariasis, the mf are taken up in a blood meal by the vector. The larvae mature from the immature to infective stage and migrate to the mouthparts to ensure further transmission when the vector next feeds, and hence the cycle continues (Fig 1.5).

1.1.2 Symptoms of Lymphatic Filariasis and Onchocerciasis

Fifteen years ago, it was suggested that lymphatic filariasis infection had two polar groups of disease state: those with chronic pathology and no detectable circulating mf, and asymptomatic patients with high levels of circulating mf (Ottesen, 1992). Since then, lymphatic filariasis has been split into three general groups of infection type in endemic areas:

1. Asymptomatic: the majority of people seem devoid of outward sign of disease or clinical features, with the exception of a high level of mf in their bloodstream. These individuals seem to tolerate the parasites immunologically, but have been shown, in some cases, to harbour

hidden damage to their kidneys and, as could be predicted, their lymphatic system (WHO Fact Sheet No. 102, 2000).

2. Acute: the patient suffers from painful inflammation of the lymph nodes and ducts, which is often associated with nausea, vomiting and a fever. This is referred to as "filarial fever", attacks of this becoming more frequent dependent on the severity of the chronic disease.
3. Chronic: associated with swelling of an arm or leg, genitals (e.g. hydrocoele, although this only occurs in those infected by *Wuchereria bancrofti*.) or vulva and breasts. Men appear to display these most severe symptoms more frequently than women, with ten to fifty per cent of men as opposed to ten per cent of women affected in communities where the disease is endemic (WHO Fact Sheet No. 102, 2000).

A small section of the community appear to be immune to infection, being devoid of any symptoms or mf, despite constant exposure to infected vector bites (Maizels and Lawrence, 1991). These individuals have been termed "endemic normals" (Day, 1991).

As secondary bacterial infections lead to worsening of lymphodema and its deterioration into elephantiasis, hygiene, skin care which makes provision for the prevention, detection and treatment of "entry lesions", appropriate footwear and elevation of the affected limb, coupled with prophylactic antibiotics in some cases are all key to its prevention (McPherson *et al.*, 2006; Dreyer *et al.*, 2002). Acute episodes of elephantiasis, although often a

direct symptom of parasite infection (Kumaraswami, 2000) are more frequently caused by a secondary bacterial or fungal infection of the skin, facilitated by cracks and fissures due to lymphatic damage underneath, and are preventable by following basic hygiene techniques (Shenoy *et al.*, 1999, Dreyer *et al.*, 2000a, 2000b).

The symptoms of onchocerciasis, caused by the mf, which can live for 2 years (Habbema *et al.*, 1992) vary from skin rashes, lesions, severe itching (Brieger *et al.*, 1998; WHO, 1995b) and skin de-pigmentation to lymphadenitis (which can result in elephantiasis of the limbs or the genitals and hanging groins) to vision being critically impaired or even lost if the mf migrate to the eye (river blindness) (Hall and Pearlman, 1999; Pearlman and Hall, 2000). These symptoms are generally seen in the patient one to three years after infection, when production of mf by adult worms begins. In the last few years the importance of *Wolbachia* (of the order *Rickettiales*) bacterial symboints in the reproduction of *O.volvulus* (Taylor and Hoerauf, 2001; Bandi *et al.*, 2001) and the inflammatory pathology associated with disease (Taylor and Hoerauf, 1999; Saint-André *et al.*, 2002; Taylor, Bandi and Hoerauf, 2005) has become increasingly clear.

1.1.3 Control of Vectors/disease

At present, the methods for controlling these diseases concentrate on the use of therapeutic drugs and vector control. For lymphatic filariasis treatment

a yearly dose of 6 mg/kg diethylcarbamazine (DEC), and 400 mg albendazole is used (Ottesen, 2000) or the patient can use DEC fortified cooking salt daily for 6-12 months (www.filariasis.com; WHO, 2000). In areas where onchocerciasis is endemic, filariasis treatment involves yearly dosing schemes of a combination of 150 μg/kg ivermectin (a microfilariacidal drug) and 400 mg albendazole (a macrofilariacidal drug), as DEC treatment can lead to discomfort and possible danger caused by the rapid *O.volvulus* mf death e.g. rash, itching, fever, headaches, etc.

As just mentioned, the treatment of onchocerciasis also involves yearly dosing of the population with ivermectin, although in the last few years the importance of *Wolbachia* symboints in the reproduction of *O.volvulus* (mentioned earlier) has become increasingly clear (Taylor and Hoerauf, 2001; Bandi *et al.*, 2001) thereby proving a new potential for drug targets.

In 1993 the theory was first formulated that lymphatic filariasis could be eradicated (Centers for Disease Control (CDC), 1993). Achieving this target is a complicated procedure, as although elimination in some situations has been simple and successful, other long-term intervention programmes have not achieved this goal (Southgate, 1992; Pichon, 2002). *W.bancrofti* infection transmitted by *Anopheles* species was eliminated as a consequence of a house spraying malaria control campaign in some Melanesian Islands (Webber, 1977) which employed the pesticide Dichloro-diphenyl-trichloroethane (DDT). Later it was shown that *Anopheles* species-transmitted *B.malayi* was eliminated by mass drug administration of DEC

(Zhang, *et al.*, 1991). At the same time, there are other Melanesian Islands where 50 years of DEC distribution has still not eliminated *W.bancrofti* transmitted by *Aedes* species (Pichon, 2002). The Global Alliance to Eliminate Lymphatic Filariasis, created in 2000, now comprises 37 organisations from public and private sectors, as well as academia, governmental bodies and non-governmental developmental organisations (NGDO). It has set itself the collective ambitious goal of eliminating lymphatic filariasis by 2020 (Molyneux and Zagaria, 2002). There have been two strategies developed to achieve this: interrupting transmission and controlling morbidity (Ottesen *et al.*, 1997). Interrupting transmission has focussed on reducing mf in the host. It has been possible to decrease levels by up to 90 percent for at least one year using two different single dose, two-drug treatments (one being 400 mg albendazole and 6 mg/kg DEC and the other being 400 mg albendazole and 200 μg/kg ivermectin) (Gyapong *et al.*, 2005). Mathematic modelling supports the theory that transmission could be interrupted successfully using 4-6 years of repeated annual mass dosing of the available two therapies mentioned earlier (Stolk *et al.*, 2003; Ottesen, 2000; Molyneux and Zagaria, 2002).

In the case of onchocerciasis, the Onchocerciasis Control Programme (OCP) (WHO, 1974-2002) involved the use of insecticides on the fast flowing fresh water preferred by *Simulium* in 11 countries of West Africa (Tsalikis, 1993) for the first two decades of its existence. With the donation of ivermectin by Merck in 1987, the shift of the programme moved on to the

annual and semi-annual mass distribution of ivermectin, replacing the earlier spraying methods, which were problematic in forested areas. By the time the OCP had run its course in 2002, it had prevented 600,000 cases of blindness and allowed 18 million children to be born without the risk of blindness (Basanez *et al.*, 2006). The work carried out by the OCP is carried on by The African Programme for Onchocerciasis Control (APOC) that was launched in 1995, and is due to cease in 2007, with the goal of eradicating the disease in non-OCP countries by a combination of annual ivermectin dosing schemes and environmentally-friendly vector elimination in a few isolated areas (Richards *et al.*, 2001). APOC also works in partnership with NGDO, a Geneva based coordination group who share the goal of global control of onchocerciasis by mass distribution of ivermectin (Sékétéli *et al.*, 2002). The achievements of the programmes over the years cannot be allowed to give way to complacency, as much remains to be done: there are still some areas in which *O.volvulus* infection remains not only of a high level but also widespread (Osei-Atweneboana *et al.*, 2007), even after up to 19 years of annual ivermectin treatments. Another problem which researchers and programme workers need to be aware of is the possibility of drug resistance. Osei-Atweneboana *et al.*, (2007) gathered evidence suggesting the emergence of drug-resistant parasites and although we must be mindful of this, it has been emphasised that this should not dissuade the programme from its yearly ivermectin dosing regime. While the current regime is adhered to, researchers are investigating other avenues of control of the disease,

such as anti-*Wolbachia* therapies (Basanez *et al.*, 2006; Taylor, Bandi and Hoerauf, 2005). When doxycycline was administered at 100 mg per day for 6 weeks, a reduction in *Wolbachia* accompanied by a subsequent disruption of embryogenesis of worms lasting 18 months was observed (Hoerauf *et al.*, 2001). Another goal would be the generation of a macrofilaricide for *O.volvulus*, but at present there is no such drug available. The ultimate control measure would obviously be a vaccine, to complement ivermectin use, and there are currently several possible *O.volvulus* vaccine antigens for which there is great hope, and which have been proven to induce protection against *O.volvulus* infection (Nutman, 2002; Hotez and Ferris, 2006).

1.1.4 Diagnosis of Disease

Diagnosis of lymphatic filariasis was, up until a short time ago, dependent on identifying the parasites in blood samples. The parasites display a periodicity which further complicates diagnosis, as blood samples would have to be taken late at night e.g. around midnight, or midday where diurnal periodicity is seen. This method's sensitivity is affected by the fact that it would obviously not be able to diagnose amicrofilaremic patients. Regarding Bancroftian filariasis, this situation was improved by the advent of enzyme-linked immunosorbent assays (ELISA) for circulating filarial antigen (More and Copeman, 1990). The rapid card test has proved invaluable, as this test is not restricted by the periodicity of the parasite as it detects circulating

filarial antigen, requires no laboratory facilities, limited instruction and only a drop of blood (Weil *et al.*, 1997). It is highly sensitive and specific (Simonsen and Dunyo, 1999), and has become the preferred method for mapping global endemicy by the Global Programme to Eliminate Lymphatic Filariasis (Molyneux and Taylor, 2001; Molyneux and Zagaria, 2002). Recently a rapid dipstick test for Brugian filariasis has been developed, which relies on the recognition of IgG4 antibodies instead of filarial antigen, and also comes in an ELISA form (Rahmah *et al.*, 2001a and 2003). This test shows very high sensitivity and specificity, and can also identify most cases of *W.bancrofti*-caused lymphatic filariasis whilst showing no cross-reaction with other common helminth and protozoal parasites (Rahmah *et al.*, 2001b). This test is not as straight forward as the card test, and therefore could not be used in lieu of a CFA test.

The classic method of diagnosis of human onchocerciasis is identification of mf in a skin snip (WHO, 1995a), from which it is possible to produce a numerical value of microfiladermia. New diagnostic methods would obviously be desirable due to the discomfort of skin snipping and the risk of HIV and other pathogen person-to-person transmission. The development of an array of recombinant filarial antigens has paved the way for immunodiagnosis of onchocerciasis (Ramachandran, 1993), but unfortunately this is unable to differentiate an active and past infection (Nutman *et al.*, 1994). The unique repetitive Deoxyribose nucleic acid (DNA) sequence O-150 (Harnett *et al.*, 1989) has been used as the basis of a polymerase chain reaction (PCR)-

based assay of skin snips (Zimmermann *et al.*, 1994). These new techniques are as yet unfortunately still on the horizon when it comes to field diagnosis. Rapid epidemiological mapping of onchocerciasis (REMO) can give a rough assessment of endemic areas. A rapid epidemiological assessment (REA) involving the assessment of nodules on a sample of men (Taylor, Duke and Munoz, 1992) is carried out in communities that are deemed to be high risk due to ecology and behaviour of vectors (Ngoumou and Walsh, 1993). This information can then be used to grade the area as hypo-, meso- or hyper-endemic, and a targeted mass ivermectin treatment of areas that fall into the latter two classifications can be carried out.

1.2 IMMUNOMODULATION OF THE HOST BY FILARIAL NEMATODES

In general, the perspective of researchers is that filarial nematodes are able to modulate the host's immune system via the immunomodulatory excretory/secretory, or ES molecules they produce, ranging from enzymes and enzyme inhibitors, such as cystatins and serpins, to fatty acid and retinol binding proteins, to non-peptide structures e.g. PC on molecules such as ES-62, found in *Acanthocheilonema viteae* (discussed in detail later) (for reviews see Harnett and Parkhouse, 1995; Maizels, Blaxter and Scott, 2001). These products are purported to lead to the long lifespan of the worms, which can be in excess of five years (Subramanian *et al.*, 2004). Infected individuals

have been shown to display immune responses deemed "defective", as they do not result in the eradication of the infection.

1.2.1 Helminth Immunomodulation of Humans

Although precise details of these alterations to the immune responses have yet to be agreed on, the general profile seen in humans, especially in asymptomatic patients, appears to be consistent with a T helper 2 (Th2)-like immunological phenotype. Extremely high levels - up to 95% immunoglobulin (Ig)G4 are seen, coupled with a down-regulation of all other subclasses of IgG (Kurniawan-Atmadja *et al.*, 1998, Kurniawan *et al.*, 1993; Ottesen *et al.*, 1985). IgG4 normally only accounts for 5% of bloodstream antibody isotype, and is a somewhat useless antibody in this situation, due to its inability to activate complement or bind macrophages (mϕ) with high affinity, and also its tendency to be monovalent (Van der Winkel and Capel, 1993; Van Der Zee, Van Swieton and Aalbese 1986). IgG4 levels have been observed to reduce after treatment of infection with anti-filarial drugs (Atmadja *et al.*, 1995). However, a similarity has been shown between the filarial antigen epitopes recognised by IgG4 and IgE, and it has therefore been proposed that IgG4 may bind to sites recognised by IgE, thus blocking pathology, nematode killing, or a combination of both effected by this antibody (Hussain *et al.*, 1981). IgE levels are also found to be elevated in infected individuals, though

this is much more pronounced in sufferers of chronic pathology (Kurniawan *et al.*, 1993).

Those with heavy infections display a reduction in antigen-specific T cell responses (Greene, Fanning and Ellner, 1983; Yazdanbakhsh *et al.*, 1993a) and high levels of Interleukin (IL)-4 accompanied by lowered levels of Interferon gamma (IFNγ) and IL-5 (Nutman, Kumaraswami and Ottesen, 1987; Mahanty *et al.*, 1996a; Yazdanbakhsh *et al.*, 1993b; Maizels *et al.*, 1995; Elson *et al.*, 1995; Doetze *et al.*, 1997; Sartono *et al.*, 1997; Doetze *et al.*, 2000) produced by peripheral blood mononuclear cells (PBMC) in response to filarial antigen. Increases are also seen in levels of IL-10 and Transforming growth factor beta (TGFβ) at the microfilaraemic stage (Doetze *et al.*, 2000; King *et al.*, 1993; Mahanty and Nutman, 1995).

The increase seen in IL-10 and TGFβ would appear to be the main regulatory factor in repressing T cell proliferation, as proliferation was restored in cell culture when these cytokines had been neutralised (Doetze *et al.*, 2000; King *et al.*, 1993; Cooper *et al.*, 2001). Anti-filarial chemotherapy has been shown to lead to not only the restoration of the antigen-specific proliferative response, but also a decrease in levels of IL-10 production (Yazdanbakhsh, van den Biggelaar and Maizels, 2001). The secretion of IL-10 and TGFβ from PBMC of mf-positive patients indicated the involvement of a regulatory phenotype of T cell (Treg) in the down-regulation of IFNγ and proliferative responses (King *et al.*, 1993). Further evidence supporting a role for Tregs has been supplied by work undertaken on onchocerciasis (Doetze

et al., 2000; Satoguina *et al.*, 2002), leading to helminth diseases being thought of as Th2/Th3 type diseases.

1.2.2 Helminth Immunomodulation in Murine Models

In murine models, a similar pattern of immunoregulation is seen, with production of IL-4, IL-5, IL-10 and IL-13 by Th2-type $CD4^+$ cells, and often large amounts of IgE antibody (Fallon, Fookes and Wharton, 1996; Urban *et al.*, 1996 and 2000; Finkelman and Urban, 2001). IL-10 production down-regulates IFNγ (associated with Th1 responses) and has the ability to bring about immune anergy by reducing antigen presenting cell (APC) expression of Major histocompatability complex (MHC) and co-stimulatory molecules (Vella and Pearce, 1992; Cook *et al.*, 1993; Van der Kleij *et al.*, 2002).

Murine models have been used to demonstrate that containment of the model filarial nematode *Litomosoides sigmodontis* relies upon $CD4^+$ T cells (Al-Qaoud *et al.*, 1997). High levels of IL-4 are stimulated at different developmental stages of parasites: L3 *Brugia* were shown to induce IL-4 in various inbred mouse strains (Osborne and Devaney, 1998; Lawrence *et al.*, 1995), and BALB/c mice implanted with adult *Brugia* also produced IL-4 (Lawrence *et al.*, 1994). IL-5 and IL-4 were shown to be crucial in establishing patency of an infection in BALB/c mice, as mf of *L.sigmodontis* survived longer and microfilaraemia was up to 100-fold higher if there was a deficiency in either cytokine (Marechal *et al.*, 1997; Le Goff *et al.*, 2000). IL-5

was also implicated in the control of worm development in primary infection in models of *L.sigmodontis* (Martin *et al.*, 2000; Al-Qaoud *et al.*, 2000; Volkmann *et al.*, 2003). Transgenic mice designed to over-express IL-5 displayed an increased capacity for parasite killing compared to wild-type (WT) when infected with *L.sigmodontis* (Martin *et al.*, 2000), and increased parasite burden was observed in IL-5 deficient mice (Al-Qaoud *et al.*, 2000; Volkmann *et al.*, 2003).

The mf loads of IL-4 deficient mice and IL-4 receptor α chain knock out (ko) mice, which are unable to respond to IL-4 or IL-13 were broadly the same (Volkmann *et al.*, 2003), making it seems unlikely that IL-13 contains mf in the absence of IL-4 in a compensatory way. The same study revealed IL-4R ko mice to harbour lower adult worm numbers in comparison to IL-4 ko and WT mice, and this finding has been reproduced by other groups (Nair and Allen, unpublished findings in Maizels *et al.*, 2004). Nair and Allen also saw a T helper 1 (Th1) switch, which may allow for more efficient killing of the parasite, as IFNγ has been shown to be crucial to control of the adult stage of the worm (Nair and Allen, unpublished findings in Maizels *et al.*, 2004; Saeftel *et al.*, 2001). Murine models have also hinted at a possible role for Treg cells. It has been suggested that IL-10, along with TGFβ, plays a role in reducing the Th1 responses, as when these cytokines are neutralised *in vitro*, a partial recovery of proliferation and Th1 cytokine production is seen (Mahanty and Nutman 1995; King *et al.*, 1993; Ravichandran *et al.*, 1997).

It is natural to consider that Th2-type profiles induced by filarial nematodes and likewise other helminths may be brought about by APC, and indeed there is evidence for helminths and their products influencing these cells. Dendritic Cells (DC) matured in the presence of helminth products such as ES-62 (Whelan *et al.*, 2000), as well as *Schistosoma mansoni* soluble egg antigen (SEA) (MacDonald *et al.*, 2001) and the schistosome-associated sugar Lacto-*N*-fucopentaose III (LNFPIII) (Thomas *et al.*, 2003) as well as *Nippostrongylus brasiliensis* excretory-secretory antigen (NES) (Balic *et al.*, 2004) *in vitro* have been shown to induce Th2 responses. DCs pre-exposed to NES were unable to produce IL-12 in response to lipopolysaccharide (LPS), a bacterial product which stimulates Th1 responses (Balic *et al.*, 2004). Similarly, pre-exposure of DC to ES-62 has been shown to lead to increased IL-4 and reduced levels of IFNγ when these cells were co-cultured with $CD4^+$ T Cells (Whelan *et al.*, 2000), discussed in more detail later. When mϕ are pre-exposed to ES-62, IL-6, IL-12 and TNFα responses to LPS are reduced (Goodridge *et al.*, 2001), and similarly mϕ from *Toxocara canis*-infected mice have reduced IL-12 and TNFα production in response to LPS, though IL-6 levels remain the same (Kuroda *et al.*, 2001).

1.2.3 Molecules Produced by Filarial Nematodes and Their Effects on the Immune System

Immunomodulation resulting in a Th2 skew by glycans of both parasitic (e.g. *B.malayi*) and free-living nematodes (e.g. *C.elegans*) has also been reported (Tawill *et al.*, 2004), even in the presence of complete freunds adjuvant (CFA), known to promote Th1 responses. This demonstrates the strength of the Th2 stimulatory potential of nematode glycans. It has been also recently been shown that *Parelaphostrongylus tenuis* synthesises and secretes glycans of a type not seen previously in nematodes. Due to the nature of these glycans being very similar to host glycans, it has been predicted that they are likely to aid evasion of the host immune system, rather than modulate its activity (Duffy *et al.*, 2006).

There is somewhat limited knowledge at present on the immunomodulatory properties of lipids and lipid-associated moieties. Lyso-phosphatidylserine from schistosomes has been revealed to mature DCs in a way that leads to their induction of Treg cells that secrete IL-10 (Van der Kleij *et al.*, 2002). This immune biasing seems to hinge upon a unique acyl chain that is absent in the mammalian host. The *in vitro* immunomodulatory capabilities of nematode glycolipids has been demonstrated (Deehan *et al.*, 2002), but the function of the lipid portions of these molecules has yet to be fully disseminated. It appears at present that much of the immunomodulation associated with these glycolipids is related to the PC moiety (Deehan *et al.*,

2002). PC is also present on the glycoconjugates of different species of nematode (Haslam *et al.,* 1999), attached via a phosphodiester linkage with GlcNAc in glycolipids and *N*-glycans in proteins (Houston and Harnett, 2004). ES-62, from *A.viteae* is a prime example of a nematode-derived glycoprotein, and one of the most fully investigated molecules to date (discussed in detail later). There are, of course, many other immunomodulatory molecules produced by helminths. The interested reader is directed to reviews by Hartmann and Lucius (2003), Knox (2007), McDermott, Cooper and Kennedy (1999) and Maizels *et al.* (2004).

1.2.4 The Impact of Nematode Infection on Other Concurrent Infections

Due to the longevity within, and the immunomodulation of the host seen with filarial nematode infection, it is only natural to investigate the impact of the filarial-infected host immune phenotype on concurrent infections with other diseases. In most cases investigated, the results are difficult to interpret, and are often conflicting.

Malaria is a disease that often occurs concurrently with helminth infections, as the regions where both types of disease are prevalent often overlap. Evidence gathered from studies of co-infections of malaria and helminths has indicated the development of a protective response against malaria to be decreased by helminth infection, which induces a Th2 phenotype (Nacher *et al.*, 2002; Speigel *et al.*, 2003). *Ascaris* infections appeared to be linked to

higher incidences of severe malaria in one study (Le Hesran *et al.*, 2004), whilst others found infection with the same causative agent to protect against cerebral malaria (Nacher *et al.*, 2000) or renal failure (Nacher *et al.*, 2001). A possible mechanism explaining helminths protection against cerebral malaria is the induction of the cytokines IL-10 and/or TGFβ by helminths (Mahanty *et al.*, 1996b), which might serve to suppress the pro-inflammatory cytokines associated with cerebral malaria (Good *et al.*, 2005; Day *et al.*, 1999), decreasing the risk of severe malarial disease. When mice infected with *L.sigmodontis* were infected with *Plasmodium chabaudi*, the levels of severe malarial immunopathology and IFNγ responsiveness of spleen cells to polyclonal stimulation was observed (Graham *et al.*, 2005). If mice were at the stage of helminth infection associated with immunomodulation (microfilariae positive), a reduction in the severity of the malaria was seen for a given parasite load. Pre-treatment of CBA/J mice with irradiated *B.malayi* larvae lessened the extent of *P.berghei*-induced cerebral malaria, but anaemia worsened, and all mice died at a later time point (Yan *et al.*, 1997). The evidence from these studies provides support for the theory that helminth infection can impact on malarial disease. The differences between studies highlights the need for more work to be carried out in this field, with standardised methodologies, if concrete conclusions about the nature of these impacts are to be drawn.

Tuberculosis is another disease in which helminth co-infection has been investigated. One theory proposes that a helminth infection will hinder the

powerful mycobacterium-specific Th1 response associated with protection, by either promoting an anti-inflammatory response, or by upsetting the Th1/Th2 balance. Many years ago, Prost, Nebout and Rougemont (1979) showed areas of onchocerciasis hyperendemicy to have double the incidence rate of lepromatous leprosy caused by mycobacterium leprae. Since then, decreased IL-4 production in response to mycobacterial antigens *ex vivo* by PBMC from onchocerciasis patients has been demonstrated (Stewart *et al.*, 1999). At this point, not many epidemiological studies have been carried out, but *in vitro* experiments have provided information supporting the notion that helminths are capable of dampening the immune response to mycobacterial infections. When DCs and mϕ were pre-exposed to live microfilariae from *B.malayi*, it appeared to result in an inability of these cells to mature upon subsequent tuberculosis infection (Talaat *et al.*, 2006). This study also indicated down-regulation of the DC-specific ICAM3-grabbing non-integrin (DC-SIGN) on DC surface. As this lectin receptor is required by *Mycobacterium tuberculosis* for entry to DCs, filarial infected DCs may be less likely to be infected. As with malaria, more well-designed epidemiological studies into the immunomodulation in concurrent helminth-tuberculosis infections would be necessary to investigate the nuances of these organisms effects on one another, and to determine where the *in vitro* results would fit in to an *in vivo* situation.

HIV is a huge problem in the world today, and as HIV decreases the effectiveness of the immune system, it is a logical step to investigate the

impact of helminth infection on this disease. Again, the results are not very clear-cut. Hookworm infection appears to slow HIV disease progression, as measured by lower mortality and increased CD4 counts (Brown *et al.*, 2004). Another study showed hookworm infection to have a negative association with susceptibility to HIV infection (Nielsen *et al.*, 2006). The same study indicated infection with *W.bancrofti* to be positively associated with HIV infection. As is the case with helminth infection and the other diseases discussed, more studies would need to be carried out in order to more fully unravel the nature of the associations between helminth infections and other diseases.

1.3 ES-62

1.3.1 ES-62: A Brief History

ES-62 is a well-characterised secreted immunomodulatory molecule, first discovered in *A.viteae*, a filarial nematode of the gerbil, some 18 years ago (Harnett *et al.*, 1989). When the molecule was analysed under reducing and denaturing SDS-PAGE, it was discovered to have a molecular mass of approximately 62000 (Harnett *et al.*, 1989), hence the name it was given. However, subsequent gel filtration analysis (Harnett *et al.*, 1993) and sedimentation equilibrium data (Ackerman *et al.*, 2003) both provided evidence that the secreted molecule is found as a tetramer in its native form.

ES-62 is without doubt the most abundant constituent of the ES products secreted by *A.viteae* being over ninety percent of the total protein secreted by adult worms (Harnett *et al.*, 1989). Production has been shown to be restricted to the L4-adult stages of the parasite (Harnett *et al.*, 1989), with the adult female worm being capable of generating 0.038μg-0.092μg h^{-1} (Goodridge *et al.*, 2001).

The molecule is not unique to *A.viteae*. Homologues of ES-62 have been found not only in the feline filarial nematode *Brugia pahangi*, but also in the human filarial nematodes *B. malayi* and *O. volvulus* (Nor *et al.*, 1997; Stepek *et al.*, 2002), and ES-62 has been cloned from cDNA libraries of *B.malayi* (Stepek *et al.*, 2004). This strongly suggests that ES-62 is active during infections of humans with filarial nematodes, making the study of this molecule and its effects on the immune system prudent.

ES-62 was originally found to share 37-39 % homology with seven proteins: murine (Genbank sequence no. 5442032) and human (7706381) aminopeptidase, rat liver annexin-like protein (7108713), human (5174527) and murine (9055234) plasma glutamate carboxypeptidase, mouse hematopoietic lineage switch 2 protein (9055234) and rat hematopoietic lineage switch 2 related protein (3851623) (Ackermann *et al.*, 2003; Harnett, Harnett and Byron, 2003). In recent years, however, this family has been extended dramatically. These proteins have been proposed to belong to the same family, and possibly to have related structures and functions by sequence alignment. Consistent with this, ES-62 has been shown to possess

aminopeptidase activity by *in vitro* experiments involving synthetic substrates (Harnett *et al.*, 1999b).

Biophysical analyses of ES-62 indicate the molecule to consist of 40 % α-helix, 16 % β-sheet, 16 % β-turn and 28 % unordered structure, as predicted by circular dichroism (CD) spectroscopy (Ackermann *et al.*, 2003). The biophysical analyses also suggested ES-62 to be a tightly bound tetramer formed from dimers (Ackermann *et al.*, 2003).

1.3.2 ES-62 and Glycans

Polyclonal rabbit anti-ES-62 was used to clone the full-length parasite derived molecule from an adult female *A.viteae* cDNA library (Harnett *et al.*, 1999b). ES-62 was found to be comprised of 474 amino acids (AA) with a molecular mass of 52.8 kDa and an isoelectric point of 5.96, by a combination of sequence analysis of the clone, and N-terminal AA sequencing and Q-TOF sequencing of tryptic peptides of native ES-62 (Harnett *et al.*, 1999b). When the PRINTS/PROSITE scanner was used to analyse the protein for conserved motifs, four potential *N*-linked glycosylation sites were revealed in the ES-62 precursor (Harnett *et al.*, 1999b). One of these sites is contained within the signal sequence and will therefore not be present on the mature protein. ES-62 has always been suspected of containing glycans, as the molecule was readily biosynthetically labelled with tritiated ([^{3}H]) glucosamine soon after discovery (Harnett *et al.*, 1989).

Investigations into the nature of these glycans involved observation of the interactions of ES-62 with a panel of lectins (Harnett *et al.*, 1994) and digestion attempts using various glycosidases (Harnett *et al.*, 1993, Harnett *et al.*, 1994). These studies indicated that the glycans attached to ES-62 were *N*-type and probably high mannose, but yielded no evidence for the presence of any *O*-type glycans. Fast atom bombardment mass spectroscopy (FAB-MS) also suggested a lack of *O*-type glycans, but revealed ES-62 to contain three types of *N*-glycan (Haslam *et al.*, 1997). These types of *N*-glycan were high mannose, as predicted by earlier studies; a truncated form, fully trimmed to its trimannosyl core and sub-stoichiometrically fucosylated and a form which had between one and four additional *N*-acetylglucosamine (GlcNAc) residues on a trimannosyl core with and without fucosylation (Haslam *et al.*, 1997). The latter mentioned type of *N*-glycan is the type to which it is proposed that PC is added (Fig. 1.7B mentioned later).

1.3.3 ES-62 and the Immune System

Over the years, ES-62's immunomodulatory effects on various different cell types of the immune system have been well-documented. Researchers accept that the early cytokine environment in which naïve $CD4^+$ T cells interact with antigen can influence the phenotype of the resultant acquired immune response. Thus, it has been suggested that an immune response of

a Th2 nature can be elicited by early exposure to IL-4 (Swain *et al.*, 1990). Given its Th2-inducing properties, when ES-62's ability to induce IL-4 from naïve murine spleen cells was investigated, it was confounding to discover that none could be detected (Harnett *et al.*, 1999a). This result was surprising given the ability of subcutaneously injected ES-62 to bring about a purely IgG1 response in BALB/c mice, consistent with the theory that it produces a Th2 response. IL-4 production was observed in response to ES-62 by spleens from mice which had been pre-exposed to the parasite molecule, however, which indicated ES-62 to have tipped the balance of the re-call immune response in favour of a Th2 phenotype (Harnett *et al.*, 1999a). The highly biased IgG1 response is not seen in IL-4 knock-out (ko) mice, confirming the cytokine's importance in this response. However, the IL-4 ko mice do not, as seen in reaction to adult *B.malayi* (Lawrence *et al.*, 1995), produce any IgG2a response in way of compensation; therefore IL-4 does not block the IgG2a response. It would seem ES-62 blocks Th1 responses by some other mechanism, or does not generate this type of response. When ES-62 is injected into IL-10 ko mice, a mixed IgG1/IgG2a response is seen, implicating this cytokine in the suppression of an IgG2a response. Consistent with this, ES-62 has also been proven to induce IL-10 in naïve BALB/c spleen cells (Wilson *et al.*, 2003).

DCs may have the ability to influence the differentiation of T cell function, due to their role as professional APC that are required for the priming and activation of $CD4^+$ cells (Banchereau and Steinman, 1998). It was for this

reason that ES-62's ability to mature DCs was investigated (Whelan *et al.*, 2000). To assist in the determination of the effects of ES-62 on the maturation of DCs, the well-characterised *in vitro* Th cell assay involving $CD4^+$ cells from the DO.11.10 transgenic mouse expressing a T-cell receptor (TCR) specific for an ovalbumin (OVA) peptide (Hsieh *et al.*, 1993). Naïve $CD4^+$ cells from this system secrete more IFNγ, than IL-4 when they are cultured with bone marrow derived DCs (bmDCs) that have been matured in the presence of LPS. When bmDCs were matured in the presence of ES-62, the cytokine profile shifted to a Th2-type phenotype, with a decrease in IFNγ and an increase seen in the levels of IL-4 (Whelan *et al.*, 2000). It would appear that ES-62 has the ability to mature DCs in such a way that they are able to drive a Th2 response to unrelated antigens.

Co-cultures of ES-62-matured DCs with naïve $CD4^+$ T cells from the DO.11.10 system secrete IL-12, which is rather strange given that this is a Th1-promoting cytokine (Whelan *et al.*, 2000). The levels seen, however, are roughly one quarter of those seen in co-cultures of LPS-matured DCs with T cells of the same system (LPS being a constituent of bacterial cell walls which induces Th1 responses). As mentioned briefly earlier, mϕ are also found to transiently produce a small amount of IL-12 when stimulated by administration of ES-62. This initial production of IL-12 leaves the cells unable to produce the considerable amounts of IL-12, IL-6 and TNFα in response to classical stimulators such as LPS (Goodridge *et al.*, 2001). As

IL-12 is crucial for mounting a pro-inflammatory response, these mϕ are left unable to respond in a pro-inflammatory manner to any further stimuli.

There is much evidence for ES-62 having the ability to stop cells from being able to react to stimuli. ES-62 leads to B-cell anergy, by preventing antigen-receptor-ligation associated B cell proliferation (Harnett and Harnett, 1993), and is able to inhibit antigen receptor stimulated polyclonal activation of the human T cell line Jurkat (Harnett *et al.*, 1998), as discussed later.

Using osmotic pumps for continuous release, it has also been shown that ES-62 *in vivo* results in a reduction in the proliferative response of murine spleen cells to T cell stimulants *ex vivo* (Wilson *et al.*, 2003). The immunomodulatory effects of ES-62 known to date are summarised in figure 1.6.

1.4 ES-62 AND PHOSPHORYLCHOLINE (PC)

1.4.1 PC

PC (Fig. 1.7A) is an important molecule found both in vertebrate and invertebrate eukaryotes, where it is present in the phospholipids, phosphatidylcholine and sphingomyelin, in the form of the polar head group. It is well known for its strong immunogenicity, which makes it ideal for

studying the production and heterogeneity of an antibody response (Lee, Cosenza and Kohler 1974; Sigal, Gearhart and Klinman, 1975). It is also a major component of bacteria, where it is used not only for "housekeeping", i.e. maintenance of cell shape, size and physiology (e.g. *Streptococcus oralis*: Horne and Tamasz, 1993), but seems to have several other distinct immunological properties. It has been used as a virulence factor, for example Cundell *et al.* (1995) showed bacterial surface-PC interaction with the Platelet Activating Factor (PAF) receptor on human endothelial cells to be crucial to their subsequent invasion. Conversely, IgM and IgG_3 antibodies generated against encapsulated PC-containing WU_2 strain of type 3 *Streptococcus pneumoniae* have been shown to be protective in mice (Briles *et al.*, 1981a and b), and rodent filarial nematode models have also provided information suggesting that this surface PC is targeted by the body to mount an IgM (protective) response against infective larvae (Al-Qaoud *et al.*, 1998).

1.4.2 PC and Nematode Glycoproteins

The ES molecules released by parasites are often PC-containing glycoproteins (reviewed by Houston and Harnett, 1999a), and, as mentioned above, PC is also present on the surface of the infective stage (L3) of filarial nematodes. Many of the immunological effects exerted by ES-62 can be attributed to the PC moiety (discussed later), which is found attached to *N*-linked carbohydrate chains (Harnett *et al.*, 1993).

As found with bacteria, PC appears to be employed by filarial nematodes not only for survival, but also for virulence. Studies on *A.viteae* and *B. pahangi* (Houston and Harnett, 1996 and Nor *et al.*, 1997 respectively) have found PC containing glycoproteins to be a common component of internal structures in filarial nematodes. These seem to be distinct from those molecules which are excreted, as their molecular mass, synthesis rate and vulnerability to *N*-glycosidase cleavage differ from the excreted PC-glycans; suggesting that PC employed internally is essential to free-living. Further evidence for the theory that PC found internally in nematodes is unlikely to be related to parasitism has been provided by studies revealing PC to be present on the glycoproteins of *C.elegans*; the free living nematode often used as a model (Gerdt *et al.*, 1999, Houston and Harnett, unpublished results).

1.4.3 The Location and Nature of Attachment of PC to ES-62 and Other Nematode Glycoproteins

Twenty years ago, preliminary studies indicated that the PC moiety on filarial ES products might be attached to carbohydrate structures. Maizels, Burke and Denham (1987) discovered that an anti-PC mAb was unable to recognise *Brugia* species PC-containing molecules once they had been subjected to treatment with alkali/borohydrate. Evidence in support of this was obtained with successful biosynthetic radiolabelling of the PC-ES

products of *A.viteae* (Harnett *et al.*, 1989), *Onchocerca gibsoni* (Harnett *et al.*, 1994), and *B.pahangi* (Nor *et al.*, 1997) with [^{3}H]-glucosamine, the amino sugar. Investigation of the binding of filarial ES products containing PC to a panel of lectins strengthened this argument (Harnett *et al.*, 1994; MacDonald, Copeman and Harnett, 1996) and started the process of defining the nature of these sugars. *O.gibsoni*-derived material was suggested to contain both *O*-linked and possibly hybrid or complex *N*-linked glycans (MacDonald, Copeman and Harnett, 1996), due to its interaction with peanut agglutinin (PNA) and *Datura stramonium* agglutinin (DSA). PNA binds the core disaccharide galactose (Gal)-β(1-3)-GalNAc (and so recognises *O*-linked chains, Goldstein and Hayes, 1978). DSA recognises Gal β(1-4)-GlcNAc in complex and hybrid *N*-glycans, and GlcNAc in *O*-glycans (Crowley *et al.*, 1984).

ES-62 appeared to contain high mannose structures (Harnett *et al.*, 1994), due to the molecule's recognition by *Galanthus nivalis* agglutinin (GNA), which binds terminal mannose residues α(1-3)-, (1-6)- or (1-2)-linked to mannose (Shibuya *et al.*, 1988). The molecule was also recognised by concanavalin A (Con A), which recognises α-mannose and α-glucose (Goldstein, Hollerman and Merrick, 1965). *N*-type glycans were also indicated by digestion of ES-62 by *N*-glycosidase F (Harnett *et al.*, 1993), and this enzyme digestion also indicated this type of glycan to be present on *B.pahangi* ES products (Nor *et al.*, 1997). The presence of *N*-glycans on both of these ES products was also suggested by the ability of tunicamycin, an

antibiotic which inhibits the precursors of all *N*-type glycans (reviewed by Elbein, 1987) to inhibit PC attachment to ES-62 (Houston and Harnett, 1996) and both PC and glycan attachment to *B.pahangi* ES (Nor, Devaney and Harnett, 1997).

1.4.4 ES-62 and PC: Effects on B-cells

Since the discovery by Bach, Kohler and Levitt (1983) that a percentage of both human and murine B cells contain a non-immunoglobulin (Ig) receptor for PC, it has been elucidated that polyclonal antibody production arises when the receptor interacts with PC on *S. pneumoniae* (Beckmann and Levitt, 1984). The PC-containing immunomodulatory molecules excreted by filarial nematodes also seem to interact with this receptor: concentrations of ES-62 far above that of PC-containing molecules found in the bloodstream of filariasis patients lead to induction of low levels of B cell activation, as measurable by DNA synthesis, whereas concentrations comparable with the levels seen in patients' bloodstream serve to prevent antigen-receptor-ligation associated B cell proliferation (Harnett and Harnett, 1993). The PC moiety of ES-62 would appear to be the cause of this, as PC conjugated to proteins such as albumin, and indeed PC alone can mimic ES-62's activity in this situation. It was also found that PC-bovine serum albumin (BSA) injected on a weekly basis could decrease recovered B cell antigen-receptor-ligation

associated activation (Harnett *et al.*, 1999a): these data taken together suggest that the low levels of PC associated with the concentration range of PC-containing molecules in filariasis patients' bloodstream seen clinically could be responsible for B-cell anergy, which could be a contributing factor in the well-documented immunomodulation associated with the molecules. ES-62 is able to anergise B cells by uncoupling the B Cell Receptor (BCR) from the ErkMAPKinase signalling cascade by targeting two major negative regulatory sites within the cascade (Fig. 1.8), the effect of both of these negative regulatory sites being targeted is a rapid, high level of desensitisation of the BCR coupling to the RasErkMAP kinase cascade (reviewed by Harnett and Harnett, 2001).

1.4.5 ES-62's Immunomodulatory Effects on Other Cell Types can be Tentatively Ascribed to the PC Moiety

Although ES-62's ability to induce IL-10 in naïve BALB/c spleen cells (Wilson *et al.*, 2003) has not yet been confirmed to be due to PC, previous evidence demonstrating B1 cells producing IL-10 in response to PC (Palanivel *et al.*, 1996) suggests it may be the cause. As mentioned earlier, In BALB/c mice, ES-62 administration causes a highly biased IgG1 response, consistent with the theory that it produces a Th2 response. (Harnett *et al.*, 1999a). When ES-62 was injected into IL-10 ko mice, an IgG2a response was observed,

producing evidence that the PC portion of ES-62 was capable of blocking an IgG2a (Th1) response, possibly through inducing IL-10, which is able to down-regulate IFNγ (the cytokine largely responsible for antibody class switching to IgG2a in mice (Bogdan *et al.*, 1992)).

Yet ES-62 produced with no PC attached (discussed later) injected into BALB/c mice induces a Th1/Th2 mixed response: IgG1/IgG2a (Houston, *et al.*, 2000). It seems therefore that PC, in part at least, is responsible for blocking this IgG2a response and consistent with this, when PC was conjugated to BSA, it appeared to inhibit the albeit weak IgG2a response to BSA (Houston *et al.*, 2000).

As mentioned earlier, ES-62 is also able to inhibit antigen receptor stimulated polyclonal activation of the human T cell line Jurkat (Harnett *et al.*, 1998). Once again, evidence suggests this is caused by PC (Harnett *et al.*, 1999a) and this is consistent with research showing *B.malayi* PC-containing molecules to be capable of non-specific inhibition of activation of human T lymphocytes (Lal *et al.*, 1990). It is not yet known if PC has a part to play in ES-62's ability to reduce the proliferative response of murine spleen cells to T cell stimulants *ex vivo* after *in vivo* exposure to ES-62 using osmotic pumps for continuous release (Wilson *et al.*, 2003).

It would appear that ES-62 is a complex immunomodulatory molecule with many effects across the different elements of the immune system ranging from modulation of B Cells, DCs and mϕ to antibody and cytokine production.

It has recently been shown by Goodridge *et al.* (2005) that ES-62 appears to exert its immunomodulatory effects on mϕ and DCs through Toll-like receptor (TLR) 4 in a unique way. It was found that the ability of ES-62 to induce low levels of IL-12 from these cell types (Goodridge *et al.*, 2001; Whelan *et al.*, 2000) was abrogated in mϕ and DCs from both Myeloid differentiation factor 88 (MyD88) ko and TLR4 ko mice, suggesting TLR4 as a receptor for ES-62 and implicating MyD88 in the intracellular signalling that follows. Interestingly, ES-62 was able to exert its normal effects in C3H/HeJ mice, which have a mutation in the Toll Interleukin I (TIR) domain, making them unresponsive to LPS (Poltorak *et al.*, 1998). It would seem that ES-62 signals through TLR4 in a non-classical manner. There is therefore a possibility that TLR4 is the non-Ig receptor for ES-62 in B-cells (mentioned earlier).

1.5 ES-62 and Auto-immune/Allergic Diseases

1.5.1 The Hygiene Hypothesis

In the West, increased affluence often leads to people living cleaner lives, with children growing up with decreased contact with infectious agents such as helminth parasites. The Hygiene Hypothesis suggests the infectious agents we are exposed to have played a part in maturing our developing immune systems, and that our new, cleaner lives could lead to the immune system of a child not receiving the appropriate priming (Strachan, 1989). The

original hypothesis focussed on the ability of microbial infections to hinder allergies characterised by a Th2 profile (reviewed by Herz *et al.*, 2000). Recently, the hypothesis has been expanded, investigating the possible link between a lack of helminth infections and the recent increase in diseases associated with unsuitable immune responses, e.g. asthma and type I diabetes. There has not been as marked an increase in frequency of allergic disorders in geographical areas with a high parasite load (Yazdanbakhsh, Kremsner and van Ree, 2002), and incidence and severity of a number of autoimmune diseases has been observed to be lower in regions with a high rate of helminthiasis (Sewell *et al.*, 2002). Rheumatoid arthritis (RA), for example, has been shown to be reduced in filarial nematode-endemic regions, indicating environmental factors to play a part in altering development of the disease (Beighton, Solomon and Valkenburg, 1975; Dunne and Cooke, 2005). Groups such as Araujo *et al.*, (2000) and Nyan *et al.* (2001) have verified the inverse relationship between worm-infection and allergy. Further evidence for this relationship has been provided by studies involving children of school-age infected with *Ascaris* in Equador (Cooper *et al.*, 2003) and children with hookworm in Ethiopia (Dagoye *et al.*, 2003). Such evidence lends weight to the hygiene hypothesis. In fact, data implicating helminth infection in the impediment or prevention of on-set of a wide range of autoimmune or allergic disorders is rapidly mounting.

1.5.2 Helminth Infection and Autoimmune/Allergic Disease

Animal disease models have corroborated this link between parasite infection and auto-immune disease: diabetes and experimental autoimmune (formerly allergic) encephalomyelitis (EAE) have been shown to be decreased by *S.mansoni* infection (Cooke *et al.*, 1999, La Flamme, Ruddenklau and Backstrom, 2003 respectively). Perhaps not surprisingly, diseases which involve inflammation of the gut, can be treated with infection with the tapeworm *Hymenolepis diminuta* (Reardon *et al.*, 2001) and the gastrointestinal nematode *Trichinella spiralis* (Khan *et al.*, 2002) in the case of experimental colitis, *Heligmosomoides polygyrus* inhibits inflammatory bowel disease (IBD: Elliott *et al.*, 2004). Treatment of human IBD with *Trichuris suis* (a whipworm whose natural host is the pig, and which cannot infect humans productively) has also proved effective (Weinstock, Summers and Elliott, 2005), with little evidence of side effects (Summers *et al.*, 2003). *T.suis* has also been shown to exert considerable effects against Crohns' Disease in almost 80 % of patients, and was also effecting against Ulcerative colitis, though not in as impressive a manner (Summers *et al.*, 2003 and 2005). There are also trials involving ascertaining the effectiveness of the human hookworm *Necator americanus* against Crohns' disease (Croese *et al.*, 2006). The situation with helminths and autoimmune diseases is mirrored by that of helminths and allergic disease: *H.polygyrus* infection has been shown to decrease food allergies (Bashir *et al.*, 2002), and likewise airway

allergies have been shown to be inhibited by infection with *Strongoloides Stercoralis* (Wang *et al.*, 2001). Airway allergies have also been shown to be reduced by infection with *N.brasiliensis*, provided infection was at least two weeks prior to challenge (Wohlleben *et al.*, 2004).

Naturally, there is still justified wariness of the use of live worm infection to treat conditions: such autoimmune intervention strategies are in their infancy, and much more work would need to be done to clearly define what, if any adverse effects instigating parasite infection may have. It must be borne in mind that such organisms still account for much morbidity in developing countries. It may not be wise, for example, to use this strategy in patients who are immuno-compromised or -suppressed. There is evidence that the immunomodulation that arises from parasite infection may affect the anti-tumour responsiveness of treated individuals (Borkow and Bentwich, 2000; McElroy *et al.*, 2005; Bentwich, 2003).

1.5.3 Helminth-derived Products and Autoimmune/allergic Disease

A strategy with more mileage involves the utilisation of non-infective helminth-derived products, which can bring about the same effects as the organism they originate from without the risk of an uncontrolled infection. More advantageous still is the possibility that such products, or chemically synthesised versions of them may afford more control over immunoregulation. Several examples of this type of research already exist

within the literature; including the treatment of diabetes with *S.mansoni* eggs (Cooke *et al.*, 1999; El-Wakil *et al.*, 2002) and soluble *S.mansoni* egg and worm products (Zaccone *et al.*, 2003), and EAE can also be treated with *S.mansoni* eggs (Elliott *et al.*, 2003). Likewise, *S.mansoni* eggs have been found to be beneficial in the case of experimental colitis (Elliott *et al.*, 2003).

1.5.4 ES-62 and Autoimmune/allergic Disease

As ES-62 has been shown to be an anti-inflammatory, Th2 inducing molecule, which produces these responses not only to itself, but also against heterologous antigens (Houston *et al.*, 2000; Marshall *et al.*, 2005), the effect of the molecule against arthritis was investigated. To this end, the prophylactic properties of ES-62 on the development of collagen-induced arthritis (CIA) in DBA/1 mice was ascertained (McInnes *et al.*, 2003b). The severity of developing CIA was significantly reduced in subjects pre-treated with the parasite molecule. This inhibition occurred concurrently with a suppression of collagen-specific IgG2a antibodies and an inhibition of collagen-specific TNFα, IL-6 and IFNγ (proimflammatory/Th1 cytokine) production. ES-62 significantly depressed the severity of developing CIA when given during type II collagen (CII) priming and intraperitoneal (i.p.) challenge, and continued treatment with ES-62 also lead to depression of articular disease. When the possible mechanisms by which ES-62 was achieving the effect were probed, it was discovered that the molecule was

able to reduce the levels of CII-induced IFNγ, TNFα and IL-6, whilst enhancing CII-induced IL-10 production from draining lymph nodes. This manipulation of immune responses by ES-62 was found to be specific as the levels of IFNγ, TNFα and IL-5, as well as proliferation induced by Con A, analysed in tandem, remained unchanged. It would appear that ES-62's suppression of CII-specific responses contributes to the molecule's ability to significantly decrease the severity of developing CIA.

A significant reduction of arthritis progression in comparison to the vehicle-treated controls was observed when ES-62 was used therapeutically, once the disease was clinically detectable. This was measured by the number of subsequently recruited arthritic joints and slowed progression of articular swelling in the initially inflamed joint. Furthermore, ES-62 treatment led to a significant suppression of progressive articular inflammation and destruction (synovial hyperplasia and of cartilage and bone erosion) (McInnes *et al.*, 2003b and 2003a). The immunomodulatory effects exerted by ES-62 remained very similar in the therapeutic experiments: when draining lymph nodes of subjects 24 h after ES-62 treatment had been completed were investigated, CII-induced IFNγ production had been significantly decreased in comparison to controls. ES-62 treatment appeared to have ablated CII-induced TNFα and IL-6 production. The pro-inflammatory versus anti-inflammatory cytokine bias was clearly shifted in the anti-inflammatory direction, as although CII-induced IL-10 production was decreased, it was still readily detectable. Again, the effects of Con A stimulation remained

comparable in ES-62 and control groups. As observed in prophylactic use of ES-62, levels of CII-specific IgG2a in the serum were significantly less in the treated group, demonstrating ES-62 to be capable of specific and effective reduction of established Th1 responses. Taken together, these data build a convincing case for the role of the immunomodulation caused by ES-62 in the suppression of the progression of articular inflammation *in vivo*.

In an effort to extend these findings from experimental to clinical observations, samples from arthritic patients were taken and compared to controls. When co-culture of cells from the blood of normal and RA human donors with myelomonocytic THP1 cells with and without ES-62 was compared, pre-exposure to the parasite molecule resulted in significantly less TNFα release compared with that of the controls. This appeared to be independent of IL-10, indicating a difference in mechanism to that proposed for the murine model. When primary cultures derived from RA synovial fluid and membrane were carried out with and without ES-62, ES-62 was able to significantly decrease the levels of TNFα and IL-6 inducible by LPS, and this too was shown to be independent of IL-10 (McInnes *et al.*, 2003b). These human studies eloquently illustrate ES-62's facility to interfere with crucial pro-inflammatory pathways *ex vivo* in the tissues affected by disease in RA.

ES-62 has also been shown to be active in models of Systemic Lupus Erythematosus (SLE) and asthma, though not in the nod mouse model of diabetes (personal communication, WH). Clearly, there is much scope for the use of ES-62 in both a prophylactic and a therapeutic capacity for several

diseases associated with aberrant immune regulation. It is of interest to our group to elucidate ES-62's mechanism of modulation as it could contribute invaluable information to enhance the search for therapeutics which mimic nematode ES-62'a anti-inflammatory properties for inflammatory diseases such as RA (discussed earlier) and to accurately pin-point the portions of the molecule responsible for these functions, with a view to identifying possible drug candidates for future therapies. There is much evidence showing many of the effects of ES-62 to be mimicked by PC alone, or PC conjugated to inert proteins such as OVA and BSA (discussed earlier). One avenue, therefore, to exploring possible drug candidates is to determine the extent of the role of PC in ES-62's immunomodulatory activities.

1.6 PC-Free ES62

1.6.1 Previous Methods of Producing PC-free ES-62

A method of producing PC-free ES-62 has been long sought-after by Professor Harnett's group. Success in this endeavour would help in examining the contribution of PC to the effects of ES-62 on the immune system. Another reason for interfering with the addition of PC to ES-62 was to determine the nature and location of this post-translational modification, i.e. what enzyme is responsible for it, and where in the secretory pathway does it take place, with a view to possible chemotherapy. As PC is an

immunomodulatory epitope present on the ES products of many filarial nematodes (as mentioned earlier), it was hypothesised that prevention of the attachment of PC would lead to a decrease in the length of time the parasites would be able to survive in the host. Discovering how to remove PC from ES-62/prevent its attachment would also give a good indication of what structures on the molecule the PC is attached to.

1.6.1.1 The Use of Enzymes and Lectins to Probe the Nature of the Carbohydrate Structures to which PC is Attached to ES-62

As discussed earlier, evidence was gathered which suggested the PC moiety to by attached to ES-62 via an *N*-linked glycan. *N*-glycosidase F digestion of not only [^{3}H]-glucosamine-, but also [^{3}H]-choline-labelled ES-62 led to a loss of radioactivity (Harnett *et al.*, 1993; Houston and Harnett, 1999a) *O*-glycanase, on the other hand, was shown to have no such effect on [^{3}H]-glucosamine-labelled ES-62 (Harnett *et al.*, 1993), implying ES-62 to contain no *O*-type glycans in its structure. Unfortunately, studies designed to more fully elucidate the nature of the *N*-glycan structures did not provide any further data, as the enzymes chosen, endoglycosidases D, F and H failed to act on ES-62 (Harnett *et al.*, 1994), though they were able to rule out the presence of the glycan structure targets of these enzymes on ES-62.

1.6.1.2 Attempts to Produce PC-free ES-62 Using Various Inhibitors

A myriad of inhibitors has been employed over the years in an attempt to identify the enzyme/pathway responsible for addition of PC to ES-62, and where in the secretory pathway this occurs. As mentioned earlier, PC addition to ES-62 was hindered by tunicamycin (Houston and Harnett 1996), an antibiotic with the ability to inhibit the precursor of all *N*-type glycans (reviewed by Elbein, 1987), indicating PC to be attached to ES-62 via this type of sugar.

The addition of PC to ES-62 was deemed to be a post-endoplasmic reticulum (ER) event, after work using inhibitors of intracellular trafficking was carried out. A panel of six inhibitors of protein vesicle transport was employed, that included DEC (Spiro *et al.*, 1986), ammonium chloride (NH_4Cl, Maxfield, 1982), chloroquine (Carroll and Bird, 1991), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Tartakoff, Vassalli and Détraz, 1977), monensin (Maxfield, 1982) and brefeldin A (Yewdell and Bennink, 1989). From this panel, only brefeldin A (which prevents the transfer of protein vesicles from the ER to the golgi) obstructed secretion of ES-62 at a concentration with no general toxic effects on the worms (Houston, Cushley and Harnett, 1997). Furthermore, the ES-62 identified from whole worm extracts from brefeldin A incubated groups was shown to be PC-free.

When attempting to define the intracellular source of PC within the parasite used to post-translationally modify ES-62, enzymes of the Kennedy pathway

for synthesis of PC (reviewed by Kent, 1995) were investigated. It was found that *A.viteae* did not utilise cytidine diphosphosphate (CDP)-choline (the provider of PC in the Kennedy pathway, Fig. 1.9) for production of ES-62, even although biosynthetic radiolabelling using [^{14}C]-CDP-choline resulted in phosphatidylcholine being labelled (Houston and Harnett, 1999b). Hexadecylphosphocholine (HEPC), a phospholipid analogue with the ability to inhibit CDP-choline synthesis (Geilen, Wieder and Reutter, 1992) was used in cultures of worms to inhibit PC attachment to ES-62. A concentration which successfully inhibited PC addition whilst leaving general worm health (and therefore protein secretion in general) intact could not be found. PC-free ES-62 was produced using the choline analogue hemicholinium 3 (HC-3, Houston and Harnett, 1999b) to block the ability of choline kinase to convert PC and cytidine triphosphate (CTP) to CDP-choline (Fig. 1.9, Hamza *et al.*, 1983). This indicated choline kinase as an essential enzyme for production of ES-62 containing PC by *A.viteae.*

The site of PC addition to ES-62 was determined to be the *medial* golgi, and the carbohydrate structure to which the PC was attached to was considered to be likely to be $Man_5GlcNAc_3$ or $Man_3GlcNAc_3$.This conclusion was arrived at using the results of experiments involving inhibitors of oligosaccharide processing. The culture of adult worms with deoxymannojirin (dMM), a mannose analogue, which inhibits mannosidase I in the *cis* golgi (Fig. 1.10, Elbein, 1987) resulted in PC-free ES-62, as determined by western blotting (Houston, Cushley and Harnett, 1997). ES-62 recovered from *A.viteae*

cultured with swainsonine was found to have retained PC (Houston, Cushley and Harnett 1997). As swainsonine inhibits mannosidase II in the *medial* golgi, (Fig. 1.10) taken together, the results gathered using these inhibitors strongly suggests the location of PC addition to ES-62 to be in the *medial* golgi. When deoxynorijirimycin (dNM) was employed, protein secretion was practically eliminated (Houston, Cushley and Harnett, 1997). Although ES-62 from worm extracts was shown to be PC-free, this inhibitor, a glucose analogue, preferentially inhibits glucosidase I in the ER (Hettkamp, Bause and Legler, 1982) and has been shown to result in the blockage of transport of some proteins from the ER (Lodish and Kong, 1984). Taking into account the brefeldin A data, suggesting PC to be added to ES-62 after the protein leaving the ER, this result is perhaps not surprising.

Despite the successes in determining where PC was added to ES-62 and at what point in the secretory pathway this occurred, ES-62 required denaturation to be de-glycosylated using *N*-glycosidase F. Of the inhibitors used, although some work was carried out with material produced using HC-3 and dMM (Houston *et al.*, 2000), these methods for producing PC-free ES-62 were ultimately found to be too inconsistent with respect to efficacy to warrant routine application.

1.6.2 Recombinant Expression Vectors: An Alternative Method for Producing PC-free ES-62

A fresh approach adopted in the pursuit of PC-free ES-62 was to place the gene for ES-62 into a recombinant protein expression vector, which would not be capable of adding PC onto heterologous proteins.

1.6.2.1 Previous Attempts Using Recombinant Expression Vectors

The first expression vector employed was *E.coli*, the archetypal recombinant protein expression vector. Being a prokaryote, it would not be able to post translationally add PC, or indeed any sugars to the proteins it produced (Ackerman, 2002 PhD Thesis). The material produced using this method was mostly insoluble, found in inclusion bodies within the bacterial cells. When re-folding was attempted, it was only achievable in a small portion of material. The resultant recombinant ES-62 (rES-62) was extremely aggregated, and therefore unsuitable for biophysical studies or *in vivo* experiments. At this point it was presumed that the sugars present on ES-62 were necessary for correct folding, that glycosylation on the parasite-derived ES-62 (pES-62) might shield the hydrophobic regions of the molecule, protecting it from insolubility, and that the tetrameric nature of ES-62 could also be dependent on the monomers being glycosylated (Ackerman, 2002, PhD Thesis).

Within the scope of the same PhD, production of rES-62 using baculovirus was undertaken, to provide glycosylation, but again, avoid the addition of PC to heterologous proteins (Ackermann, 2002, PhD Thesis). The material produced using this method was soluble, and shown to be a sharper band than pES-62: this was thought to be due to the uniformity of addition of sugar by the insect cells. It appeared from silver staining that some of the material may have been monomeric, but doubt was cast on the accuracy of this assertion by coomassie blue staining. It appeared either the proportion of monomeric ES-62 was below the level of detection of coomassie blue, or that the band had been an artefact of the silver staining, which can occur, and give rise to erroneous indications of contamination. Unfortunately, having passed this hurdle, and also proving recognition of the material by mono- and polyclonal anti-ES-62 antibodies, it appeared that the protein produced using this method was also unusable. Examination of the rES-62 absorption spectra led to the discovery of what appeared to be DNA contamination caused by rES-62 binding viral DNA, contamination from an unknown source, or incorrect folding. The maximum absorbance for rES-62 was 260nm: proteins should normally have a peak at 280nm. Two different purification techniques (ultracentrifugation of samples for one hour, and treatment with DNAse) failed to alter the location of the absorbance peak, and therefore the contamination.

1.6.2.2 A New Recombinant Expression Vector Attempt

An alternative method of producing PC-free ES-62 is to place its gene in the yeast *Pichia pastoris*, for recombinant protein expression. *P.pastoris* was developed as a host system for DNA transformation twenty years ago (Cregg *et al.*, 1985), and has proved a popular alternative (Bretthauer and Castellino, 1999) to *Escherichia coli* or *Saccharomyces cerevisiae* due to the similar ease of manipulation of *Pichia* to the two systems, coupled with the benefit of its ability to perform the protein processing, folding and posttranslational modifications often necessary for production of a working eukaryotic protein, absent in *E.coli*, and its reduced hyperglycosylation as compared to *S.cerevisiae*, mentioned later. *Pichia* is a methylotrophic yeast: able to use methanol as its only carbon source. The first enzyme in the pathway of methanol consumption, alcohol oxidase (AOX) is produced extensively by the yeast due to its poor oxygen affinity. AOX can constitute up to 30% of the total soluble protein in cells grown in methanol (Couderc and Baratti, 1980) therefore, when wishing to express heterologous protein the alcohol oxidase promoter is the logical choice. The ability of *Pichia* to actively secrete proteins is desirable for the production of a pure protein, although naturally a signal sequence targeting it to the secretory pathway is necessary. A large percentage of all secreted protein will be the heterologous protein, due to the strength of the AOX promoter and *Pichia* secreting few native proteins, combined with the minimal medias' low protein content.

Another advantage of the *Pichia* system is that unlike *S.cerevisiae*, *Pichia* may not hyperglycosylate. Both yeasts have mostly high mannose type *N*-linked glycosylation, but the post translationally added oligosaccharide length is an average of 8-14 mannose residues per side chain versus 50-150 seen in *S.cerevisiae* (Grinna and Tschopp, 1989; Tschopp *et al.*, 1987). The core oligosaccharides of *Pichia* do not contain terminal α1-3 glycan linkages, which are thought to be the cause of the hyper-antigenicity seen in proteins produced using *S.cerevisiae* (which does employ such linkages) thus rendering them practically unusable therapeutically. It is envisaged (though not proven) that such hyper-antigenicity will be less of a problem when using *Pichia* as the glycoproteins produced will more closely resemble the structure of higher eukaryotes (Cregg, Vedvick and Raschke, 1993).

The most frequently employed expression vectors contain the HIS4 gene for selection purposes, and are organised so that a restriction enzyme will create a linearized form conducive to producing His^+ recombinants by recombination at AOX1 or his4 locus. Regardless of where it inserts, the expression cassette has the same general structure (Fig. 1.11). Insertions are far more probable to occur than replacements (double crossover events) and multiple insertion arises at 1-10% of single insertion events spontaneously. Therefore, adding methanol to minimal media containing recombinant yeast will cause the stimulation of the promoter of the alcohol oxidase which will drive expression of the gene of interest, in this case ES-62.

1.7 AIM OF PROJECT

It is therefore my aim during this project to

- Attempt to produce PC-free ES-62 using enzymes and enzyme inhibitors related to those used previously
- Sub-clone recombinant ES-62 into the *Pichia* recombinant expression system
- Culture *Pichia* in order to produce Recombinant ES-62 (rES-62)
- Purify the recombinant material
- Explore the biochemical properties of rES-62, including: molecular weight; quaternary structure; the nature of self-association and susceptibility to glycosidases and other enzymes and investigating the sugars attached to the molecule using assays to investigate binding to lectins
- Compare the antigenic properties of the recombinant protein with those of the parasite derived ES-62 using assays to investigate binding to anti-sera
- Compare the immunogenicity of rES-62 with that of parasite-derived ES-62 – the nature of the antibody responses mounted by naïve BALB/c mice to the two molecules, investigation of cross-reactivity between antibodies raised against one molecule against the other, and vice versa, and investigation of immunomodulation by rES-62 in

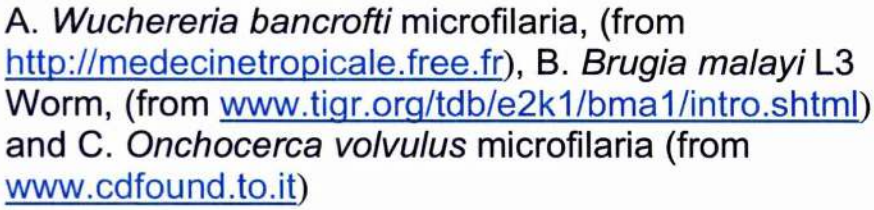
comparison with pES-62, by examination of cytokine responses stimulated by rES-62 in comparison to pES-62 by bone marrow derived dendritic cells (bmDCs). I will also explore the nature of rES-62's cytokine stimulatory abilities in knock-out mice which lack TLR4 and TLR4 signalling capabilities, MyD88 and the Mannose receptor.

- Compare the structural properties of rES-62 with those of rES-62 clones with one of the three putative *N*-Glycosylation sites "Knocked Out", for example: molecular weight, tetramers and monomers, binding to anti-sera, lectin binding, susceptibility to glycosidases and other enzymes.

Together, these experiments will hopefully shed more light on which components of ES-62 are essential to its manipulation of the immune responses of hosts.

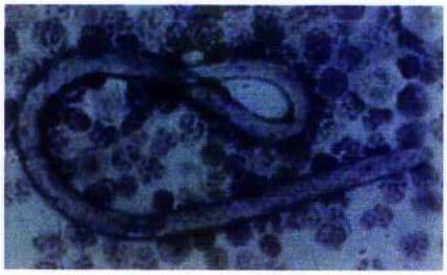
1.4 FIGURES

Figure 1.1. Causative Agents of Lymphatic Filariasis and Onchocerciasis

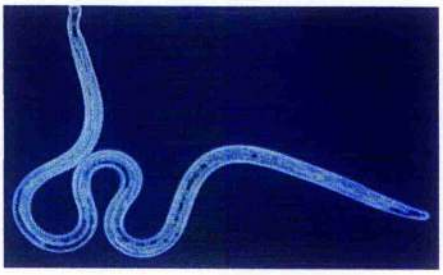


A. *Wuchereria bancrofti* microfilaria, (from http://medecinetropicale.free.fr), B. *Brugia malayi* L3 Worm, (from www.tigr.org/tdb/e2k1/bma1/intro.shtml) and C. *Onchocerca volvulus* microfilaria (from www.cdfound.to.it)

A.



B.



C.

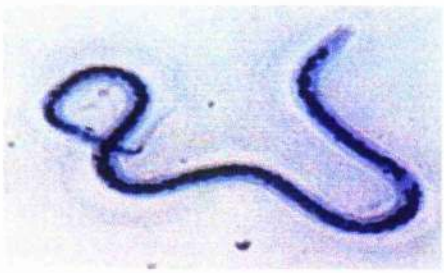


Figure 1.2. Worldwide Prevalence of Lymphatic Filariasis and Onchocerciasis.

Map showing worldwide prevalence of Lymphatic Filariasis, Onchocerciasis, and areas of co-endemicy (from http://globalatlas.who.int/globalatlas/InteractiveMapping/rmm/default.asp?cat1=010000000000&cat2=010900000000&cat3=010902000000&lev=3 (WHO))

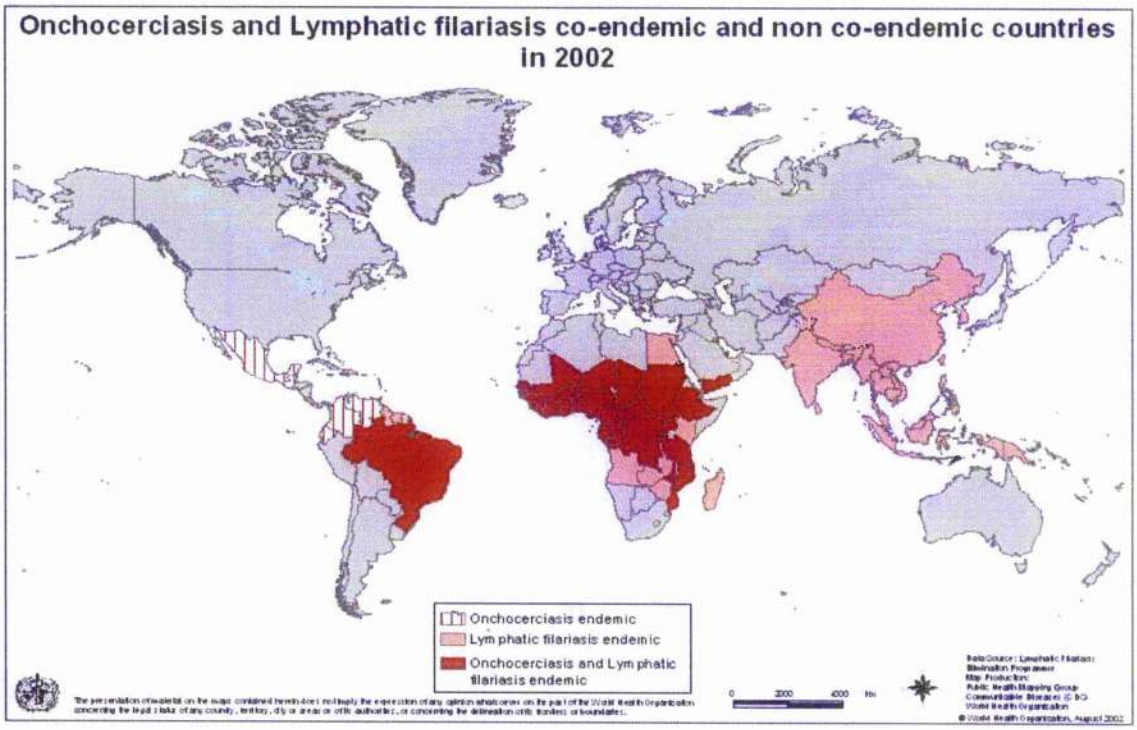

Onchocerciasis and Lymphatic filariasis co-endemic and non co-endemic countries in 2002
Onchocerciasis endemic
Lym phatic filariasis endemic
Onchocerciasis and Lym phatic filariasis endemic
0
2000
4000
km

Figure 1.3. Simplified Lifecycle of the Causative Agents of Lymphatic Filariasis.

Simplified lifecycle of *Wuchereria bancrofti*. A mosquito becomes infected after taking a bloodmeal from a human infected with *W.bancrofti*, containing sheathed microfilariae (mf). The mf shed their sheathes and penetrate the midgut, then travel to the thorasic muscles. Here the L1 larvae develop into L3 larvae, capable of infecting a human, and migrate to the mosquito head and proboscis. The cycle is continued when the mosquito takes a bloodmeal from an uninfected human, when the infective larvae are transferred from the vector to the bloodstream of the human. The L3 develop into adult worms, and reside in the lymphatic system, where they produce sheathed mf. These mf migrate to the lymph and blood channels. The cycle is completed when and uninfected mosquito takes a bloodmeal from an infected human, ingesting the mf.

W.Bancrofti Lifecycle

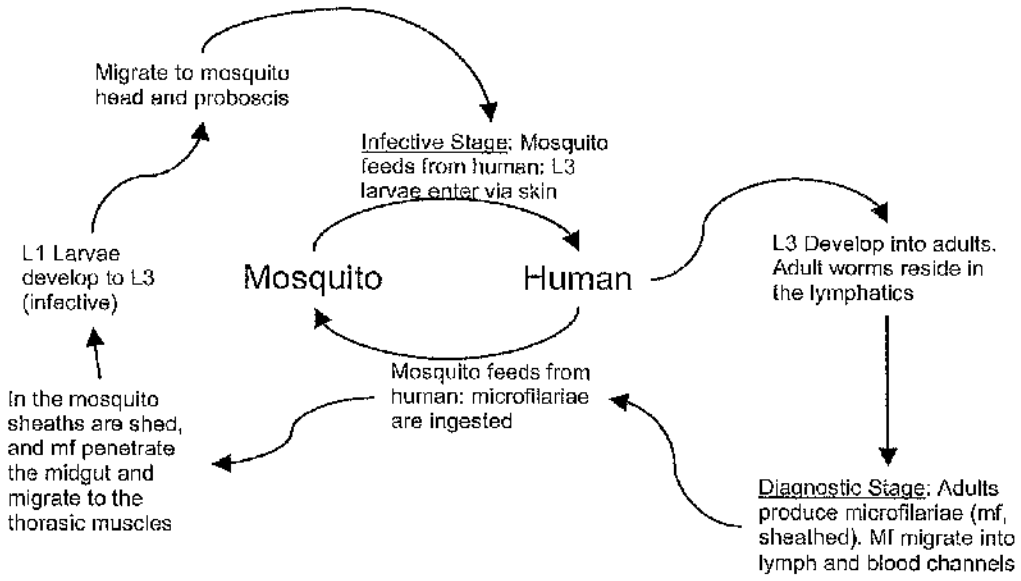


Figure 1.4. Simplified Lifecycle of the Causative Agents of Lymphatic Filariasis.

Simplified lifecycle of *Brugia malayi*. A mosquito becomes infected after taking a bloodmeal from a human infected with *W.bancrofti*, containing sheathed mf. The mf shed their sheathes and penetrate the midgut, then travel to the thorasic muscles. Here the L1 larvae develop into L3 larvae, capable of infecting a human, and migrate to the mosquito head and proboscis. The cycle is continued when the mosquito takes a bloodmeal from an uninfected human, when the infective larvae are transferred from the vector to the bloodstream of the human. The L3 develop into adult worms, and reside in the lymphatic system, where they produce sheathed mf. These mf migrate to the bloodstream. The cycle is completed when and uninfected mosquito takes a bloodmeal from an infected human, ingesting the mf.

B.malayi Lifecycle

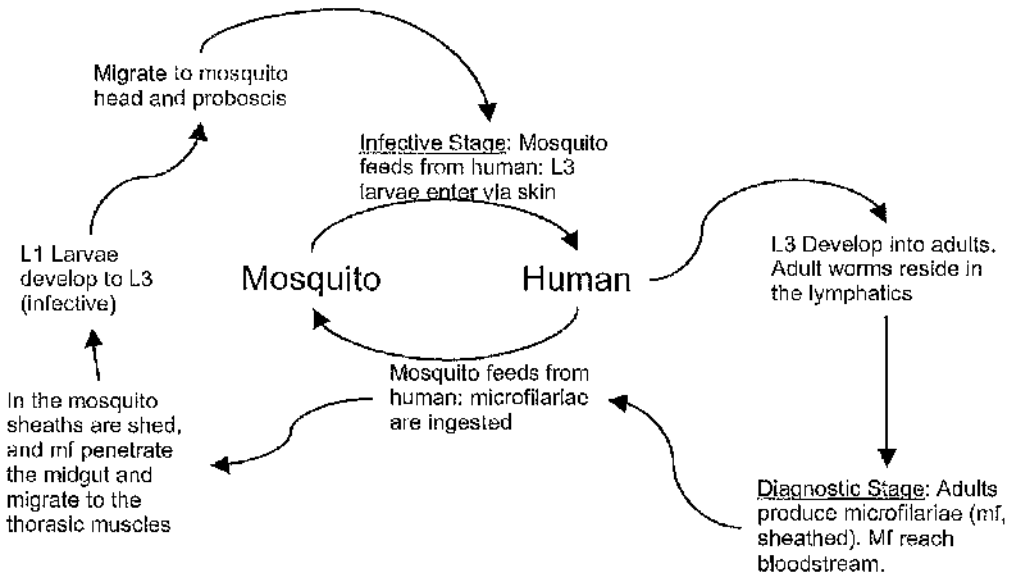


Figure 1.5. Simplified Lifecycle of the Causative Agents of Onchocerciasis.

Simplified lifecycle of *Onchocerca volvulus.* A blackfly becomes infected after taking a bloodmeal from a human infected with *W.bancrofti*, containing unsheathed mf. The mf penetrate the midgut, then travel to the thorasic muscles. Here the L1 larvae develop into L3 larvae, capable of infecting a human, and migrate to the blackfly head and proboscis. The cycle is continued when the blackfly takes a bloodmeal from an uninfected human, when the infective larvae are transferred from the vector to the bloodstream of the human. The L3 develop into adult worms, which are found in subcutaneous nodules, where they produce unsheathed mf. These mf are found in skin and lymphatics of connective tissues, and also in peripheral blood, urine and sputum. The cycle is completed when and uninfected blackfly takes a bloodmeal from an infected human, ingesting the mf.

O.volvulus Lifecycle

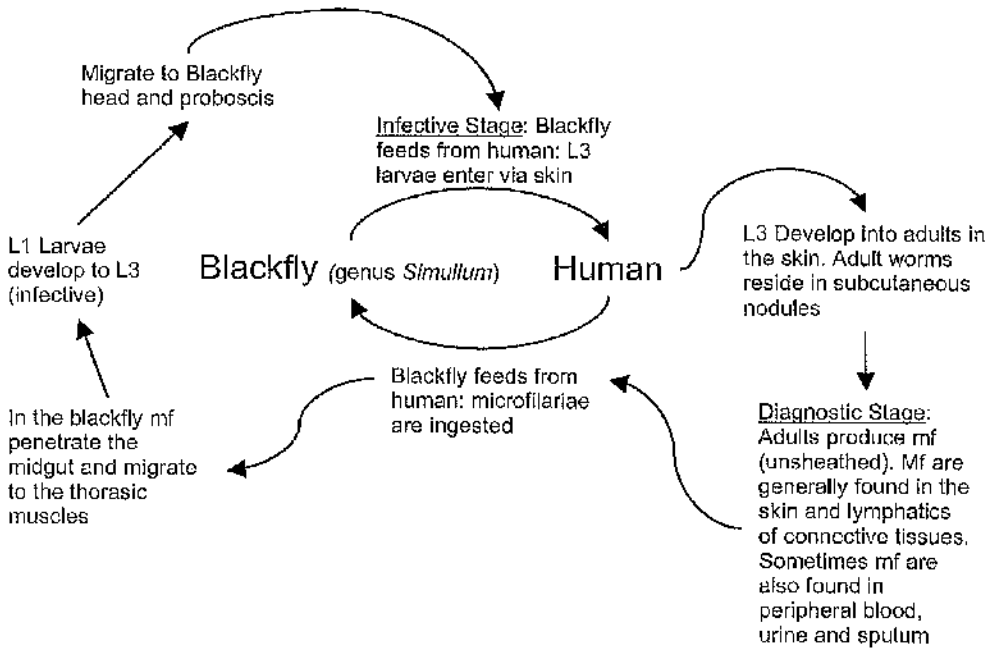


Figure 1.6. Immunomodulatory Properties of ES-62

Diagrammatic summary of the immunomodulatory properties of ES-62 on various cells of the immune system. 1. ES-62 has been shown to mature DCs in a way which induces Th2 responses to unrelated antigens, pushing the immune responses in a more anti-inflammatory direction. 2. Pre-exposure of mϕ to ES-62 leads to their inability to respond to subsequent Th1 inducing stimuli. 3. B1 cells are stimulated by ES-62 to produce IL-10, suppressing inflammation and polarising the immune responses.

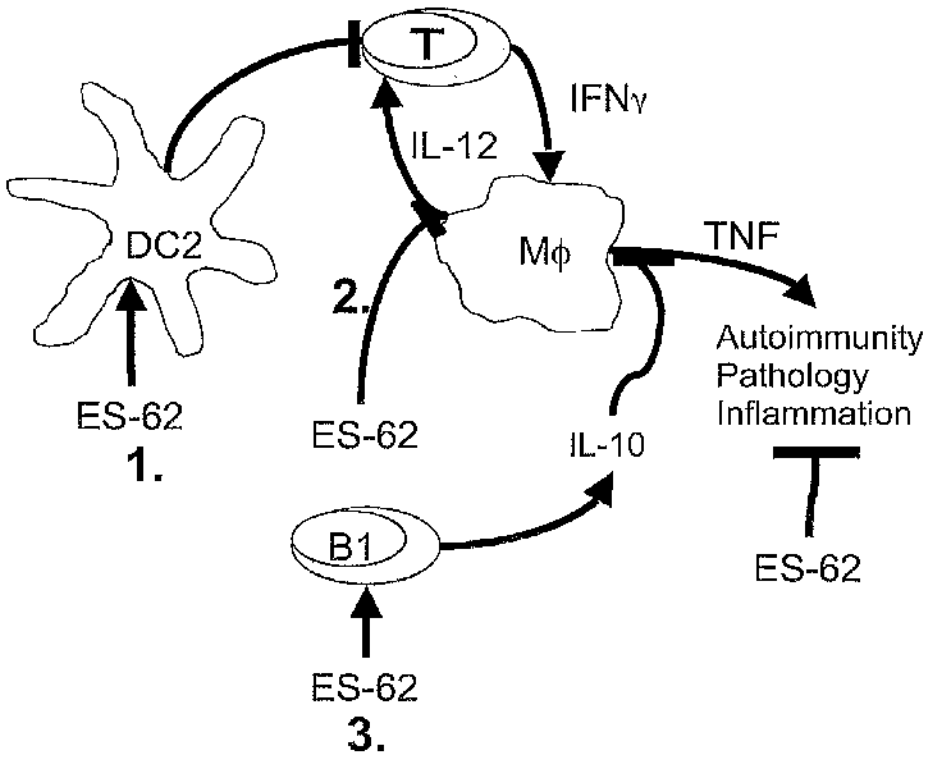
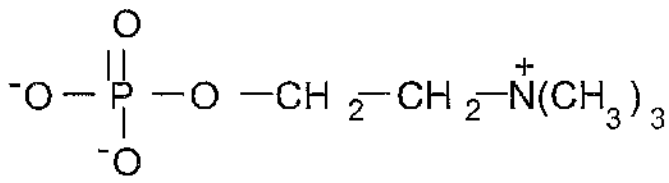

T
IFNγ
IL-12
DC2
Mϕ
TNF
2.
Autoimmunity
Pathology
Inflammation
ES-62
1.
ES-62
IL-10
B1
ES-62
ES-62
3.

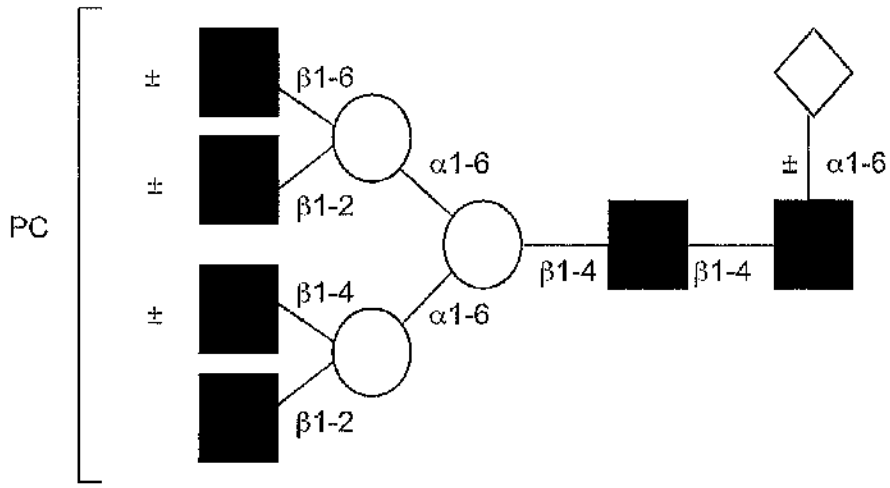
Figure 1.7. Phosphorylcholine and Its attachment site on the Glycans of ES-62

A. Chemical Structure of Phosphorylcholine (PC) and B. The proposed attachment site on the glycans of ES-62. Proposed PC attachment sites on the *N*-glycans of ES-62. PC on ES-62 has been found to be attached to a glycan with a trimannosyl core with and without fucosylation of the core, and carrying between one and four additional GlcNAc residues. It is likely that the PC is attached directly to GlcNAc (found to be the case with filarial nematode PC-glycolipids: Wuhrer, *et al.* (2000) in a phosphodiester linkage (Houston and Harnett 2004) and there may be at least two PC residues per glycan.

A.



B.



○ = Mannose

■ = N-Acetylglucosamine

◇ = Fucose

PC = Phosphorylcholine

Figure 1.8. ES-62 Uncouples the B Cell Receptor (BCR)-RasErkMAPKinase Cascade.

ES-62 primes the BCR to activate SHP-1 tyrosine phosphatase, maintaining the Immunoreceptor Tyrosine-based activation motifs (ITAMS) in a dephosphorylated (resting) state, thus stopping recruitment of the ShcGrb2Sos complexes necessary for activation of Ras. ES-62 also promotes the BCR-driven association of Pac1 with Erk, terminating any ongoing Erk signals. The action of ES-62 on these two negative regulatory sites results in a rapid, high level of desensitisation of the BCR coupling to the RasErkMAPKinase cascade.

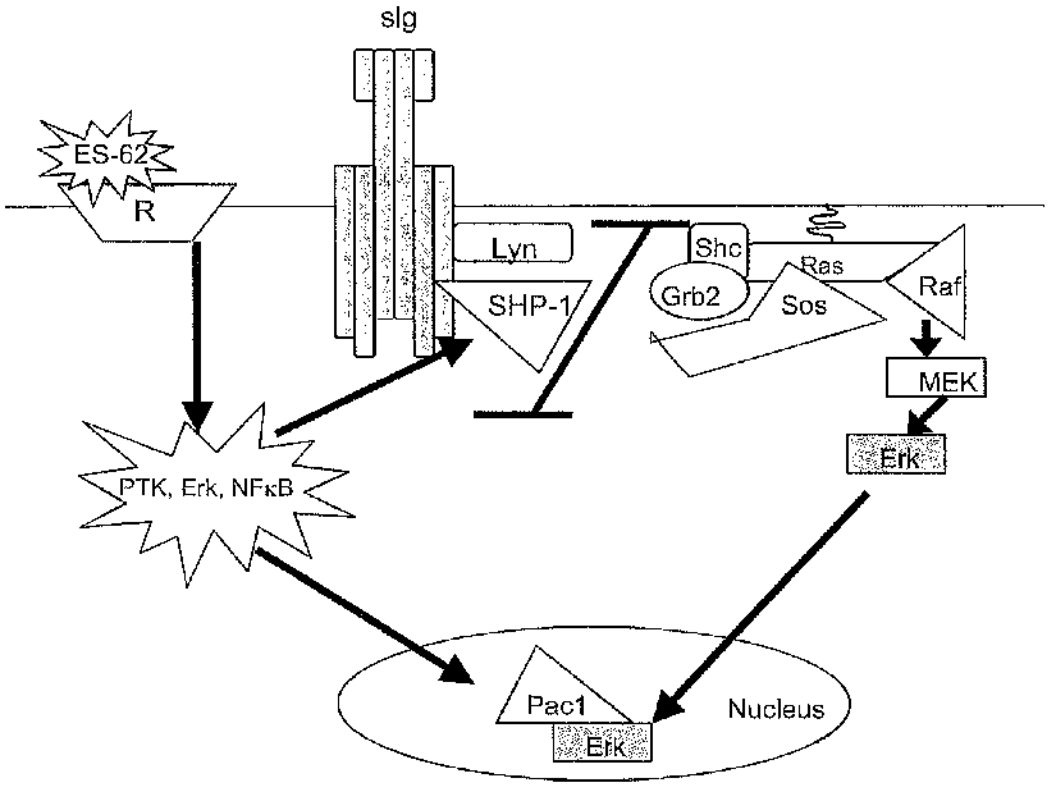

sIg
ES-62
R
Lyn
SHP-1
Shc
Grb2
Ras
Sos
Raf
MEK
Erk
PTK, Erk, NFκB
Pac1
Erk
Nucleus

Figure 1.9. The Kennedy Pathway of Phosphatidylcholine Synthesis.

Diagram showing the Kennedy Pathway of phospholipid synthesis, illustrating where HC-3 and HEPC exert their effects. Choline is converted to phosphorylcholine (PC) by choline kinase, which is inhibited by HC-3. Phosphocholine citidyltransferase, which is inhibited by HEPC, converts PC to CDP-choline, which is then converted to phosphatidylcholine (PtdChol) by choline phosphotransferase. Finally PtdChol can be converted to sphingomyelin by sphingomyelin synthase (SM synthase).

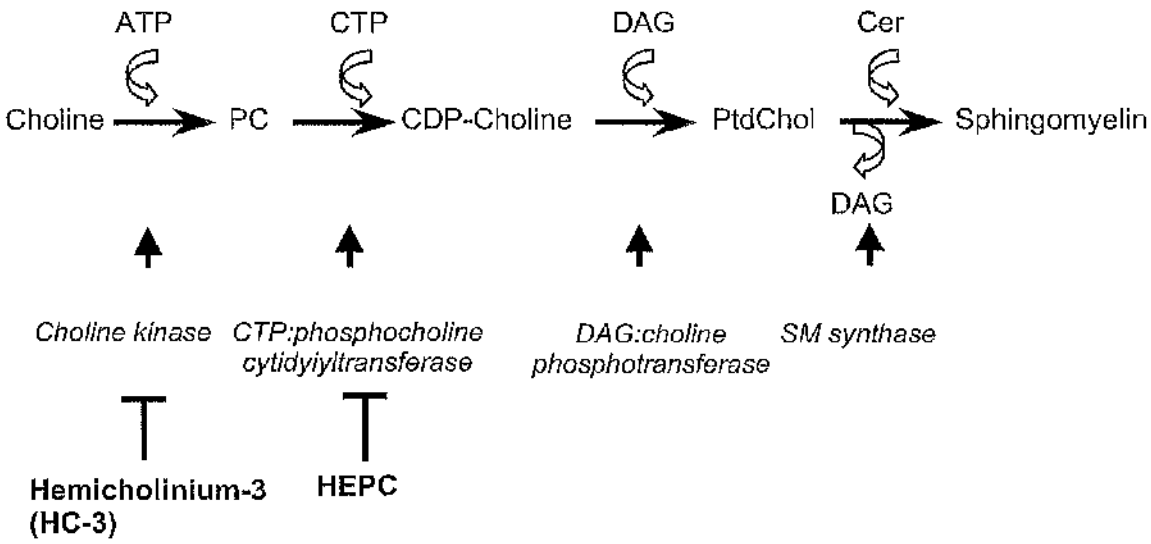
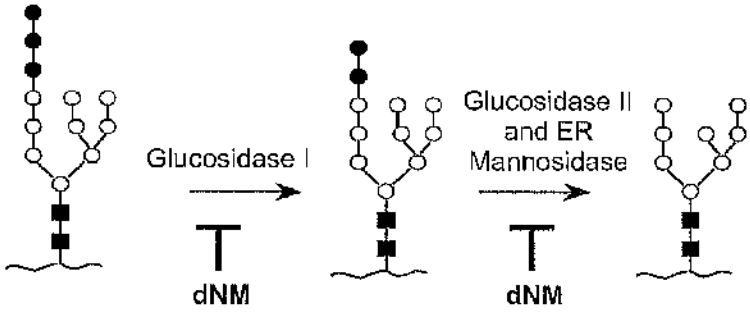

ATP
CTP
DAG
Cer
Choline
PC
CDP-Choline
PtdChol
Sphingomyelin
DAG
Choline kinase
CTP:phosphocholine cytidyiyltransferase
DAG:choline phosphotransferase
SM synthase
Hemicholinium-3 (HC-3)
HEPC

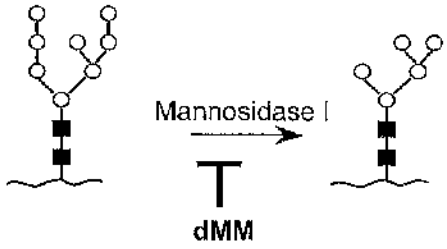
Figure 1.10. Sugar Biosynthetic Pathway in the Endoplasmic Reticulum and Golgi

Schematic representation of the sugar biosynthetic pathway from the Endoplasmic Reticulum (ER) and Golgi Apparatus, illustrating where various enzyme inhibitors exert their effect. In the ER (conserved from yeast to mammals) - $Man_9GlcNAc_2Glucose_3$ is first trimmed by Glucosidase I to $Man_9GlcNAc_2Glucose_2$, then trimmed to $Man_8GlcNAc_2$ by Glucosidase II and ER mannosidase. These enzymes are inhibited by dNM. In the *cis* region of the Golgi apparatus, $Man_8GlcNAc_2$ is trimmed to $Man_5GlcNAc_2$ by Mannosidase I (inhibited by dMM). As the *N*-glycan moves through the medial Golgi, an extra GlcNAc residue is added by GlcNAc transferase I and two more mannose residues are removed by mannosidase II (inhibited by swainsonine), to create $Man_3GlcNAc_3$

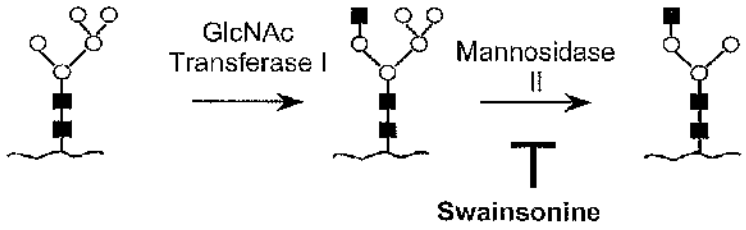
Endoplasmic Reticulum



***Cis* Golgi**



***Medial* Golgi**



● = Glucose

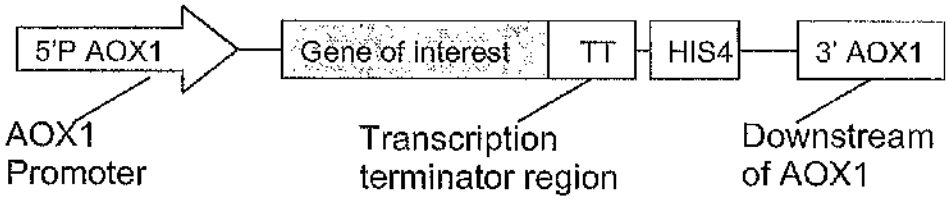
○ = Mannose

■ = N-Acetylglucosamine

~ = Protein

Figure 1.11. Expression Cassette for Insertion into *Pichia pastoris*.

Expression Cassette: crossover events with the *Pichia pastoris* genome can occur at any of the regions shown.


5'P AOX1
Gene of interest
TT
HIS4
3' AOX1
AOX1
Promoter
Transcription
terminator region
Downstream
of AOX1

Chapter 2: Materials and Methods

2.1 CHEMICALS AND REAGENTS

The chemicals and reagents used throughout this study were provided by Alexis Corporation UK (Nottingham, UK), Amersham Biosciences (Little Chalfont, Bucks, UK), Applied Biosciences (Foster City, CA), BD Pharmingen (San Diego, USA), BDH Laboratory Supplies (Poole, UK), BioRad Laboratories (Hemel Hempstead, Hertfordshire, UK), Biosource (Supplier: Invitrogen Paisley, UK), Cadisch Precision Meshes (London, UK), Diagnostics Scotland (Edinburgh, UK), Corning (Supplier: Fisher Scientific), eBioscience (Supplier: Insight Biotechnology Ltd, Wembley, UK), Fisher Scientific (Leicester, UK), GibcoBRL, Life Technologies (Supplier: Invitrogen, Glyko-Prozyme (Supplier: Europa Bioproducts Ltd., Wicken, UK), Greiner Bio-One Ltd. (Stonehouse, UK), Invitrogen (Paisley, UK), Kirkegaard and Perry Laboratories Inc. (Supplier: Insight Biotechnology Ltd, Middlesex, UK), Lonza, Wokingham Ltd. (Wokingham, UK), Millipore (Supplier: Fisher Scientific, Leicester, UK), Pierce (Supplier: Perbio, Science UK, Cramlington, UK), PAA Laboratories Ltd., (Somerset, Yeovil UK), Premier International Foods, (Long Sutton, Lincs, UK), Qiagen (Crawley, West Sussex, UK), Roche Diagnostics (Burgess Hill, East Sussex, UK), SAFC Biosciences (Andover, UK), Sigma-Aldrich (Gillingham, Dorset, UK), Stratagene (Amsterdam, The Netherlands) Techno Plastic Products ((TPP), Trasadingen, Switzerland) Thermo Electron Corporation (Supplier: Fisher Scientific), and Thermo Scientific (Basingstoke, UK).

2.2 SOLUTIONS AND BUFFERS

2.2.1 GENERAL BUFFERS

10x Phosphate Buffered Saline (PBS)

1.37 M Sodium chloride (NaCl, BDH Laboratory Supplies)

0.03 M Potassium chloride (KCl BDH Laboratory Supplies)

0.08 M Disodium hydrogen orthophosphate (Na_2HPO_4, BDH)

0.01 M Potassium dihydrogen orthophosphate (KH_2PO_4, Sigma-Aldrich)

Adjust pH using hydrochloric acid (HCl, Sigma-Aldrich)/sodium hydroxide (NaOH, Sigma-Aldrich)

Ammonium Chloride pH 7.2 for Lysing Red Blood Cells

0.168 M Ammonium chloride (NH_4Cl, Sigma-Aldrich)

Or

Boyles Solution for Lysing Red Blood Cells

0.17 M Tris ((hydroxymethyl)aminomethane): Tris, $C_4H_{11}NO_3$, Sigma-Aldrich) - 1 part

0.16 M NH_4Cl - 9 Parts

Add the two solutions together on the day of use and filter sterilise using a 0.2 μM filter.

2.2.2 CULTURE REAGENTS

2.2.2.1 Worm Culture Reagents

RPMI "complete"

Roswell Park Memorial Institute (RPMI) 1640 Medium (PAA Laboratories Ltd.) containing

2 mM L-Glutamine (Gibco, Invitrogen, Paisley, UK)

100 U/ml Penicillin and 100 µg/ml Streptomycin (10000 U/ml penicillin G sodium plus 10000 µg/ml streptomycin sulfate in 0.85% saline, Gibco, Invitrogen)

10ml of a 45 % Glucose Solution ($C_6H_{12}O_6$, Sigma-Aldrich), to give a final concentration of 1 %

RPMI Lacking Choline (RPMI-c)

RPMI 1640 medium (modified R8758) lacking choline (SAFC Biosciences) containing

2 mM L-Glutamine

100 U/ml Penicillin and 100 µg/ml Streptomycin

1 % Glucose

RPMI Lacking Glucose (RPMI-g)

RPMI 1640

2 mM L-Glutamine

100 U/ml Penicillin and 100 µg/ml Streptomycin

RPMI Lacking L-methionine (RPMI-m)

RPMI 1640 (modified R8758) medium lacking methionine (SAFC Biosciences) containing

2 mM L-Glutamine

100 U/ml Penicillin and 100 µg/ml Streptomycin

1 % Glucose

2.2.2.2 *Pichia pastoris* (*P.pastoris*) Culture

Yeast Extract Peptone Dextrose (YDP) Broth

1 % (weight by volume (w/v)) Yeast Extract (Sigma-Aldrich)

2 % (w/v) Peptone (Sigma-Aldrich)

2 % (w/v) Dextrose (Sigma-Aldrich)

1 M Potassium Phosphate Buffer pH 6.0

132 ml 1 M Dipotassium hydrogen orthophosphate (K_2HPO_4, Sigma-Aldrich)

868 ml 1 M KH_2PO_4 (Sigma-Aldrich)

Check pH, adjust using potassium hydroxide (KOH, Sigma-Aldrich)/Phosphoric Acid (H_3PO_4, Reidel de Haën, Sigma-Aldrich)

Minimal Dextrose (MD) Plates

1.34 % (w/v) Yeast Nitrogen Base (YNB, Sigma-Aldrich)

1 % (volume by volume (v/v)) Glucose (Sigma-Aldrich)

0.4 mg Biotin (Sigma-Aldrich)

10 % 1 M Potassium phosphate buffer pH 6.0

2 % Agar (Sigma-Aldrich)

Autoclave, allow to cool a little then pour.

Buffered Minimal Glycerol (BMG) Broth

1.34 % (w/v) YNB

1 % (v/v) Glycerol ($C_3H_8O_3$, Fluka, Sigma-Aldrich)

0.000004 % (w/v) Biotin

10 % 1M Potassium phosphate Buffer pH 6.0

Buffered Minimal Methanol (BMM) Broth

1.34 % (w/v) YNB

0.5 % Methanol (CH_4O, Reidel de Haën, Sigma-Aldrich)

0.000004 % (w/v) Biotin

10 % 1 M Potassium phosphate buffer pH 6.0

10 x Dextrose

20 % D-glucose (Sigma-Aldrich)

Autoclave for 15 min then store at 4 °C

YDP Medium/Agar plates

1 % (w/v) Yeast extract (Sigma-Aldrich)

2 % (w/v) Peptone

2 % (w/v) Agar (for plates only)

Dissolve yeast extract, agar if making plates and peptone in 900ml distilled water (dH_2O), autoclave, allow to cool and add 1 % (v/v) 10x Dextrose solution and pour plates.

100 x Amino Acids (AA)

0.5 % (w/v) L-Glutamic acid (Sigma-Aldrich)

0.5 % (w/v) L-Methionine (Sigma-Aldrich)

0.5 % (w/v) L-Lysine (Sigma-Aldrich)

0.5 % (w/v) L-Leucine (Sigma-Aldrich)

0.5 % (w/v) L-Isoleucine (Sigma-Aldrich)

Filter-sterilise by passing through a 0.22 μm membrane (Stericup, Millipore, Fisher Scientific) and store at 4 °C

10 x YNB

3.4 % (w/v) YNB

10 % (w/v) Ammonium sulfate ($(NH_4)_2SO_4$, Sigma-Aldrich)

Heat to dissolve, then filter-sterilise by passing through a 0.22 μm membrane (Stericup, Millipore, Fisher Scientific) and store at 4 °C

10 x Methanol

5 % Methanol

Filter-sterilise by passing through a 0.22 μm membrane and store at 4 °C

10 x Glycerol

20 % (v/v) Glycerol

Filter-sterilise by passing through a 0.22 μm membrane and store at 4 °C

500 x Biotin

0.02 % (w/v) Biotin

Filter-sterilise by passing through a 0.22 μm membrane and store at 4 °C

Minimal Media with Glycerol (MMG)

20 % 1M Potassium phosphate buffer pH 6.0

6 % 10x YNB

1 % 500x Biotin

6 % 100x AA

10 % 10x Glycerol

Top up to 1 L with autoclaved dH_2O

Minimal Media with Methanol (MMM)

10 % 1 M Potassium phosphate buffer pH 6.0

10 % 10x YNB

0.15 % 500x Biotin

10 % 100x AA

10 % 10x Methanol

Top up to 1 L with autoclaved dH_2O

Storage Conditions for rES-62

10 % (v/v) glycerol

0.01 M Magnesium sulfate ($MgSO_4$, Sigma-Aldrich).

Store at -20 °C

2.2.2.3 Cell Culture Reagents

Heat Inactivated Foetal Calf Serum (HiFCS)

Foetal Calf Serum (Sigma-Aldrich) heated to 56 °C for at least 1 h

Dendritic Cell (DC) Media

RPMI 1640 containing 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

10 % (v/v) HiFCS

100 U/ml Penicillin

100 μg/ml Streptomycin

10 % (v/v) x63-cell line conditioned media containing Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

2.2.3 OTHER EXPERIMENTAL REAGENTS

GENERAL EXPERIMENTAL REAGENTS

2.2.3.1 Protein Assay

1:4 dilution Protein Assay Reagent (Biorad) in dH_2O

Add 300 μl of this dilution to 10 μl of sample/standard

2.2.3.2 Endotoxin Testing Kit Reagents (QCL-1000, Lonza)

Standard Endotoxin (*Escherichia coli (E.coli)* Strain 0111:B4)

1 Endotoxin Units (EU) /ml in LAL reagent water (contained within kit)

LAL Lysate

Contained within kit, reconstitute with 1.4 ml LAL reagent water

Chromogenic Substrate

2 mM in LAL reagent water

Contained within kit, reconstitute with 6.5 ml LAL reagent water

Stop Solution

25 % Acetic Acid ($C_2H_4O_2$, Riedel de Haën, Sigma-Aldrich)

CHAPTER 3

2.2.3.3 Trichloroacetic Acid (TCA) Precipitation

10 % Trichloroacetic Acid (TCA)

10 % (w/v) TCA (BDH)

0.01 M Choline chloride (Sigma-Aldrich)

2.2.3.4 Inhibitors

Erucylphosphocholine (22,1,cis) (EPC. Alexis Corporation, UK)

0.01 M EPC/RPMI-c/m

rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine ($Et_{18}OCH_3$, Sigma-Aldrich)

0.01 M $Et_{18}OCH_3$/RPMI-c/m

2.2.3.5 Buffers For Enzymes

Sodium Citrate Buffer pH 4.6 for β-N-acetylhexosaminidase

0.05 M Sodium citrate ($C_6H_5Na_3O_7.2H_2O$, Sigma-Aldrich)

Correct pH to 4.6 using citric acid ($C_6H_8O_7.H_2O$, Sigma-Aldrich)/NaOH

Sodium Phosphate Buffer pH 7.0 for Recombinant Phosphorylcholine Esterase (Pce)

0.02 M Sodium phosphate (disodium hydrogen orthophosphate, $HNa_2O_4P.12H_2O$, BDH)

Correct pH to 7.0 using 0.02M sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$, BDH)

2.2.3.6 Immunoprecipitation

Wash Buffer, pH 8.3

0.01 M Tris-HCl (Riedel de Haën, Sigma-Aldrich) pH 8.3

0.05 M NaCl

0.1 % (w/v) Nonidet P-40 (NP40, Sigma-Aldrich)

Adjust pH using HCl

2.2.3.7 Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

2.2.3.7.1 General Solutions

Fixing Solution

40 % Methanol

7 % Acetic Acid

Staining Solution

40 ml Brilliant Blue Colloidal Concentrate (Sigma-Aldrich)

10 ml Methanol

Made up immediately before use

De-staining Solution

25 % Methanol

10 % $C_2H_4O_2$

2.2.3.7.2 SDS-PAGE: BioRad System

Solution A

0.41 M Acrylamide (C_3H_5NO, Sigma-Aldrich)

0.005 M N'N'methylene bisacrylamide ($C_7H_{10}N_2O_2$, Sigma-Aldrich)

Solution B

1.5 M Tris pH 7.5

Adjust pH to 8.8 using HCl

Solution C

0.5 M Tris pH 7.5

Adjust pH to 6.8 using HCl

Sample Loading Buffer pH 6.8 for denaturing and reducing PAGE conditions

The components of this buffer are detailed in table 2.2

Working Loading Buffer

95 % Sample Loading Buffer pH 6.8

5 % 2-Mercaptoethanol (2-ME)

5x Electrophoresis Buffer Containing SDS, pH 8.3

1.24 M Tris pH 7.5

9.59 M Glycine ($C_2H_5NO_2$, Sigma-Aldrich)

0.09 M SDS (Lauryl sulphate; sodium dodecyl sulfate)

Check pH but do not adjust

Sample Loading Buffer pH 6.8 for Non-denaturing and Reducing PAGE Conditions

The components of this buffer are detailed in table 1.3.

10x Electrophoresis Buffer without SDS, pH 8.3

0.25 M Tris

1.92 M Glycine

Check pH but do not adjust

2.2.3.7.3 SDS-PAGE: Novex System

Sample Loading Buffer

25 % NuPAGE LDS Sample Buffer 4x (Novex, Invitrogen)

10 % NuPAGE Reducing Agent 10x (Novex, Invitrogen)

Electrophoresis Buffer: Outer Tank - NuPAGE 3-(N-morpholino)propanesulfonica acid (MOPS) Buffer 1x

5 % NuPAGE MOPS Buffer 20x (Novex, Invitrogen)

Electrophoresis Buffer: Inner Tank

0.25 % NuPAGE antioxidant (Novex, Invitrogen) in NuPAGE MOPS Buffer 1x

CHAPTER 4

2.2.3.8 Polymerase Chain Reaction (PCR): Transformation into *Pichia*

ES-62 Primers

MJA121

5'- GAAGGGGTATCTCTCGAGAAAAGAGAGGCAGCTGTCCTTCCGGACA AAACTGTCGCT

and

MJA122 5'- ATGGGAATTCTTATAGCTTTTTACGATCAGATTTCTCAGTAGT

PCR Buffer

0.02 M Tris-HCl pH 8.8

0.01 M Potassium chloride (KCl, BDH)

0.01 M Ammonium sulfate ($(NH_4)_2SO_4$, Sigma-Aldrich)

0.002 M $MgSO_4$

1 % Triton X-100 (Sigma-Aldrich)

10 % Bovine Serum Albumin (BSA) (Fraction V, Sigma-Aldrich)

2.2.3.9 Western Blotting and Dot Blotting

Transfer Buffer (for 1 gel)

50 ml NuPAGE 20x Transfer Buffer (Novex, Invitrogen)

1 ml NuPAGE Antioxidant (Novex, Invitrogen)

100 ml Methanol

849 ml dH_2O

Transfer Buffer (for 2 gels)

50 ml NuPAGE 20x Transfer Buffer (Novex, Invitrogen)

1 ml NuPAGE Antioxidant (Novex, Invitrogen)

200 ml Methanol

749 ml dH_2O

Tris Buffered Saline (TBS), pH 7.5

0.02 M Tris

9 % (w/v) NaCl

Adjust pH using HCl/NaOH

TBS pH 7.5 plus Tween 20 (TBST)

0.1 % w/v Tween 20 (Sigma-Aldrich)/TBS pH 7.5

TBS pH 8.5

0.1 M Tris

0.3 M NaCl

Adjust pH using HCl/NaOH

Blocking Solution

4 % (w/v) BSA/TBS pH 7.5/8.5

Primary Antibodies

1:1000 dilution of KK6, a mouse mAb that recognises a conformational epitope on ES-62 (Stepek *et al.*, 2002)/TBST

1:500 dilution of Rabbit ES-62 anti-serum (raised against purified ES-62, Harnett *et al.*, 1989)/TBS pH 8.5 containing 2.5 % (w/v) BSA

1:1000 dilution TEPC 15 ((1 mg/ml, Sigma-Aldrich), a mouse IgA myeloma protein specific for PC, Sigma-Aldrich)/TBS pH 8.5 containing 2.5 % (w/v) BSA

Secondary Antibodies

1:20000 dilution HRP-labelled anti-mouse IgG,(Sigma-Aldrich)/TBST for KK6 primary antibody

1:100000 dilution alkaline phosphatase (AP)-labelled anti-rabbit IgG,(Sigma-Aldrich)/TBS containing 0.5 % BSA (w/v) where rabbit-anti-ES-62 serum was used as the primary antibody

1:30000 dilution AP-conjugated anti mouse IgG1 (Sigma-Aldrich) diluted in TBS pH 8.5 containing 0.5 % BSA (w/v) where TEPC15 was used as the primary antibody

Substrate Solution

1 tablet Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidine substrate (BCIP/NBT, Sigma-Aldrich))/10 ml dH_2O.

Stop Solution

20 mM Ethylenediaminetetraacetic acid (EDTA)

2.2.3.10 Buffers for Enzymes for Removal of PC from rES-62

Reaction Buffer for *N*-glycosidase F

0.05 M EDTA

0.02 M $HNa_2O_4P.12H_2O$

0.02 % (w/v) Sodium azide (NaN_3, Sigma-Aldrich)

Add EDTA to half the volume, pH to 7.2 using 0.02 M sodium dihydrogen orthophosphate, then add the NaN_3

Reaction Buffer for Endoglycosidase H

0.1 M Sodium Acetate ($C_2H_3O_2Na$, Sigma-Aldrich)

Reaction Buffer for α-mannosidase

0.005 M Sodium Acetate

0.0002 M Zinc Chloride ($ZnCl_2$, Sigma-Aldrich)

Adjust pH to 4.6 using $C_2H_4O_2$/NaOH

Reaction Buffer for Trypsin

0.001 M HCl

2.2.3.11 DIG-Glycan Lectin Blot Kit Reagents

TBS pH 7.5

0.05 M Tris

0.15 M NaCl

Adjust pH to 7.5 using HCl/NaOH

Buffer 1

0.001 M Magnesium chloride ($MgCl_2$, Sigma-Aldrich)

0.001 M Calcium chloride ($CaCl_2.H_2O$, Sigma-Aldrich)

In TBS pH 7.5

Buffer 2 pH 9.5

0.1 M Tris

0.05 M $MgCl_2$

0.1 M NaCl

Adjust pH to 9.5 using HCl/NaOH

CHAPTER 5

2.2.3.12 Inoculations

2 μg/ml parasite derived (p) or recombinant (r) ES-62 in sterile, endotoxin-free PBS pH 7.4

2.2.3.13 Enzyme-Linked Immunosorbent Assay (ELISA) Reagents

2.2.3.13.1 Antibody ELISAS

Wash Buffer

PBS pH 7.4

Coating Buffer

PBS pH 9.0

Adjust pH to 9.0 using NaOH

PBS/Tween

0.05 % Tween 20 in PBS pH 7.4

Blocking Solution

4 % BSA in PBS pH 7.4

Antibody Conjugates

1: 20000 Dilution of horse-radish peroxidase conjugated rabbit anti-mouse IgG1 or IgG2a (Southern Biotech) in PBS pH 7.4 containing 25 % Sheep serum (Diagnostics Scotland, Edinburgh, UK)

3,3',5,5'-Tetramethylbenzidine (TMB) for Substrate Solution

0.025 M (w/v) TMB ($C_{16}H_2ON_2$, Sigma-Aldrich) in Dimethylsulfoxide (DMSO, C_2H_6SO, Sigma-Aldrich)

Store at -20 °C protected from light

Acetate/Citrate Buffer pH 5.5 for Substrate Solution

0.1 M Sodium Acetate

Adjust pH to 5.5 using solid citric acid (BDH). Store at 4 °C. This solution only lasts for 1 month after making up.

Substrate Solution

1 % (v/v) TMB

0.032 % (v/v) Hydrogen peroxide (H_2O_2, Sigma-Aldrich)

in Citrate/Acetate Buffer pH 5.5

Stop Solution

10 % Sulfuric Acid (H_2SO_4, Reidel de Haën, Sigma-Aldrich)

2.2.3.13.2 Cytokine ELISAS: Interferon gamma (IFNγ), Interleukin (IL)-4 and IL-10 (Kits from eBioscience, UK)

Coating Buffer

10x Stock in kit

Coating Antibodies

1:1000 dilution of IFNγ Capture Antibody

1:250 dilution of IL-4 Capture Antibody

1:250 dilution of IL-10 Capture Antibody

All diluted in 1x Assay diluent provided with the kit.

Assay Diluent

5x stock in kit

Wash Buffer

PBS pH 7.0

Standards for Cytokine Standard Curves

2000 pg/ml for IFNγ

500 pg/ml for IL-4

2000 pg/ml for IL-10

All diluted in 1x assay diluent provided with the kit

Detection Antibodies

1:1000 dilution for IFNγ

1:250 dilution for IL-4

1:250 dilution for IL-10

All diluted in 1x assay diluent, provided with kit

Enzyme Reagent

1:250 dilution of streptavidin-horseradish peroxidase (HRP) conjugate in 1x assay diluent, provided with kit

Substrate Solution

1x provided with kit, TMB-based

Stop Solution

2 N H_2SO_4

2.2.3.13.3 Cytokine ELISAS: Tumour Necrosis Factor Alpha (TNFα) and IL-12p40 (Kits from BD Bioscience)

Coating Buffer for TNFα: 0.1 M Sodium Carbonate pH 9.5

0.1 M Sodium carbonate, anhydrous (Na_2CO_3, BDH)

0.1 M Sodium hydrogen carbonate ($NaHCO_3$, BDH)

Add Na_2CO_3 to $NaHCO_3$ to achieve pH 9.5

Coating Buffer for IL-12p40: 0.2 M Sodium Phosphate pH 6.5

0.2 M Di-sodium hydrogen orthophosphate-12-hydrate ($Na_2HPO_4.12H_2O$, BDH)

0.2 M Sodium di-hydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$, BDH)

Add Na_2HPO_4 to NaH_2PO_4 to achieve pH 6.5

Assay Diluent

10 % HiFCS in PBS pH 7.0

Capture Antibodies

1:250 dilution of TNFα capture antibody in 0.1 M sodium carbonate pH 9.5

1:250 dilution of IL-12p40 capture antibody in 0.2 M sodium phosphate pH 6.5

Top Standards for Cytokine Standard Curves

5 ng/ml for both TNFα and IL-12p40, diluted in assay diluent

Detection Antibodies

1:250 dilution for both TNFα and IL-12p40 detection antibodies, in assay diluent

Enzyme Reagent

1:250 dilution of streptavidin HRP conjugate in assay diluent.

Substrate

1x Sureblue™ TMB Microwell peroxidase substrate (Kirkegaard and Perry Laboratories Inc., Insight Biotechnology Ltd., UK)

Stop Solution

2 N H_2SO_4

2.2.3.14 bmDC Culture and Treatment with Modulins

Trypan Blue for Cell Counting

1 in 10 dilution Trypan Blue (Sigma-Aldrich) in sterile RPMI

p/r ES-62

2 μg/ml in sterile, endotoxin-free PBS pH 7.4

Bacterial Lipopolysaccharide (LPS)

1 μg/ml *E.coli* serotype 0111:B4 (Sigma-Aldrich)

CHAPTER 6

2.2.3.15 Circular Dichroism Analysis

Phosphate Buffer pH 7.4

0.0915 M Na_2HPO_4

0.0583 M NaH_2PO_4

2.3 METHODS

GENERAL METHODS

2.3.1 Purification and Concentration of Parasite-derived ES-62 (pES-62)

Third stage *A.viteae* recovered from ticks (*Ornithodorus moubata*) were injected subcutaneously to infect gerbils (*Meriones libycus*) using the method of Worms, Terry and Terry (1961). After a period of at least two months, adult worms were harvested from subcutaneous surfaces and the surrounding tissues of the gerbil and washed in RPMI "complete". The worms were then allowed to culture in fresh RPMI "complete" in an atmosphere of 95 % air/5 % carbon dioxide (CO_2) at 37 °C until the media was spent (indicated by a colour change of the medium from red to yellow), when this was removed, filtered through a 0.22 μm membrane to remove larval forms released by the females, and replaced with fresh media. ~2 L of the resultant spent media was concentrated using a stirred cell ultrafiltration unit (Amicon, Fisher, UK) containing a membrane of a 30000 molecular weight cut off (YM30), and the media substituted for sterile, endotoxin free PBS pH 7.4 (Lonza, UK) by reducing the volume to 50 ml and washing with the PBS. Once the sample had been washed three times and the volume reduced to 50 ml again, it was then further concentrated to 200-500 μl using Centricon microconcentration tubes (Amicon, Fisher, UK) with a 100000 molecular weight cut off

membrane following the manufacturers instructions. Finally, the sample was assayed for protein content using the Biorad protein assay reagent according to manufacturers instructions (described in section 2.3.2) and the resultant pES-62 tested for endotoxin content using a QCL-1000 Chromogenic Substrate End Point Kit (described in section 2.3.3). Batch purity and identity was confirmed by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and western blotting, probing with rabbit anti-ES-62 sera (described in sections 2.3.19 and 2.3.21).

2.3.2 Protein Assay

A set of standard protein concentrations ranging from 1 mg/ml to 0.016 mg/ml of an appropriate control protein (in this case BSA dissolved in PBS) was created by serially diluting 1 mg/ml BSA/PBS 1:2 seven times. 10 μl of each standard and the sample to be tested was placed into a flat bottom medium binding 96-well plates (Thermo Electron Corporation, Fisher) in duplicate. 300 μl of Biorad Protein Assay reagent diluted 1:4 in dH_2O was then added to each well, and the plate incubated at room temperature for 5 min, after which the absorbencies at 570 nm were read using an Original MultiSkan EX (Thermo Scientific). The protein concentration of the unknown sample was then estimated using an equation of the line obtained using the standard curve.

2.3.3 Endotoxin Testing of p/rES-62

Endotoxin testing was carried out using the QCL-1000 Chromogenic Endotoxin Test, with all steps being carried out on a heat block. A standard curve of standard endotoxin (*E.coli* Strain 0111:B4) contained within the kit ranging from 1 EU/ml to 0.01 EU/ml was prepared by reconstituting the endotoxin with 1 ml LAL-free water (contained in the kit) and vortexing for 15 min, then serial 1:2 dilutions were made in pyrogen-free centrifuge tubes (Fisher Scientific, UK) using LAL-free water and vortexing samples for 1 min between dilutions. 50 µl of each standard/sample to be tested was placed in a pyrogen free 96 well cell culture plate (TPP) which had been preheated to 37 °C. 50 µl of freshly reconstituted LAL lysate (contained in the kit) was added to each well and the samples incubated for 10 min at 37 °C. 100 µl of pre-warmed chromogenic substrate (also contained within the kit) was then added to the wells, and the samples incubated for a further 6 min before stopping the reaction by the addition of 25 % acetic acid. The samples absorbencies were read at 405 nm, and endotoxin content of samples estimated using an equation of the line obtained using the standard curve.

2.3.4 Radiolabelling of pES-62 for Enzyme/Inhibitor Analysis

Adult female *A.viteae* were cultured in one of the three media mentioned below in an atmosphere of 95 % air/5 % CO_2 at 37 °C until the media was exhausted.

The three types of medium used were RPMI lacking choline (RPMI-c), glucose (RPMI-g) or methionine (RPMI-m), and were all based on RPMI "complete". RPMI-c (RPMI "complete" depleted of choline) containing 2 MBq [Methyl-^{3}H] Choline chloride (2.22-3.14 TBq/mmol, 60-85 Ci/mmol); RPMI-g (RPMI "complete" containing no glucose) containing 2 MBq D-[6-^{3}H]-glucosamine hydrochloride (0.56-1.3 TBq/mmol, 15-35 Ci/mmol), or as a control to enable assessment of protein synthesis by the inhibitors used, RMPI-m (RPMI "complete" depleted of methionine) including 1 MBq L-[^{35}S]-methionine (>37 TBq/mmol, >1000 Ci/mmol). All radiolabels were supplied by Amersham Biosciences, UK.

2.3.5 Measurement of Radioactivity

The level of radioactivity contained within 10 µl of each sample was measured using a Beckman LS 6500 multipurpose scintillation counter (β-

counter), in some cases after samples were TCA (Trichloroacetic Acid) - precipitated using 10 % (w/v) TCA containing 10mM "cold" label.

2.3.6 TCA Precipitation Analysis

5 μl of normal mouse serum (Sigma-Aldrich) was added to 10 μl of each sample in duplicate, 750 μl 10 % (w/v) TCA containing 10 mM "cold" (non-radioactive) label was added whilst gently vortexing and the sample incubated at 4 °C for 15 min to aid precipitation. The samples were then centrifuged at 90,000 g for 5 min and supernatant discarded safely. 35 μl PBS pH 7.4 was added to the sample, followed by 200 μl formic acid (Sigma-Aldrich). The contents were added to a scintillation vial, vortexed after the addition of 1 ml of scintillant (Biodegradable Counting Scintillant, BCS, Amersham Biosciences) and samples' radioactivity read in a Beckman LS 6500 multipurpose scintillation counter β-counter. Controls were also set up containing sample, PBS and formic acid, but no mouse serum or TCA to enable measurement of the total disintegrations per minute (dpm). The percentage incorporation of radiolabel was calculated by dividing the TCA dpm value from the total dpm and multiplying by one hundred.

2.3.7 Enzyme Digestion of ES-62: β-N-acetylhexosaminidase (EC 3.2.1.52, Sigma-Aldrich)

After ascertaining the enzymes' activity using an appropriate chromogenic substrate (p-nitrophenyl-N-acetyl-β-D-glucosaminide, Sigma-Aldrich), duplicate aliquots of 0.05 μg of [^{3}H] choline-labelled ES-62 were incubated with 0.8 mUnits (mU) β-N-acetylhexosaminidase in 50 mM sodium citrate buffer, pH 4.6 at 37 °C for 1 h along with control tubes where the appropriate volume of buffer had been added in lieu of enzyme, and samples then analysed by TCA precipitation, as described in section 2.3.5.

2.3.8 Enzyme Digestion of ES-62: Glycerophosphocholine Phosphodiesterase (EC 3.1.4.2, Sigma-Aldrich)

After ascertaining the enzymes' activity using an appropriate chromogenic substrate (L-α-glycerophosphorylcholine, Sigma-Aldrich), duplicate aliquots of 0.05 μg of [^{3}H] choline-labelled ES-62 were incubated with 0.8 mU glycerophosphocholine phosphodiesterase in RPMI 1640 at 37 °C for 1 h along with control tubes where the appropriate volume of buffer had been added in lieu of enzyme, and samples then analysed by TCA precipitation, as described in section 2.3.6.

2.3.9 Enzyme Digestion of ES-62: Sphingomyelin Phosphodiesterase (EC 3.1.4.12, Sigma-Aldrich)

After ascertaining the enzymes' activity using an appropriate chromogenic substrate (Trinitrophenylaminolauroylsphingomyelin (TNPAL)-sphingomyelin, Sigma-Aldrich), duplicate aliquots of 0.05 μg of [^{3}H] choline-labelled ES-62 were incubated with 0.8 mU sphingomyelin phosphodiesterase in PBS pH 7.4 at 37 °C for 1 h along with control tubes where the appropriate volume of buffer had been added in lieu of enzyme, and samples then analysed by TCA precipitation, described in section 2.3.5.

2.3.10 Enzyme Digestion of ES-62: Recombinant phosphorylcholine esterase (Pce) of Streptococcus pneumoniae (de las Rivas *et al.,* 2001)

Pce was expressed and purified as described previously (de las Rivas *et al.,* 2001) and duplicate aliquots of 0.6 μg of [^{3}H] choline labelled ES-62 were incubated with 5.0 μg Pce in 20 mM sodium phosphate pH 7.0 at 37 °C for 1 h along with control tubes where the appropriate volume of buffer had been added in lieu of enzyme, and samples then analysed by TCA precipitation, as described in section 2.3.5.

2.3.11 Use of Inhibitors: Erucylphosphocholine (22,1,cis) (EPC, Alexis Corporation, UK) and rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine ($Et_{18}OCH_3$, Sigma-Aldrich)

A preliminary titration experiment was carried out to indicate optimum working concentrations (i.e. those which would provide inhibition without causing general toxic effects, measurable by reduction in protein synthesis by the nematodes). After this, triplicate groups of two adult female worms were cultured in 10 ml RPMI-c/RPMI-m containing or lacking inhibitor at 5 % CO_2 and 37 °C for 2 h. The media was removed and replaced with RPMI-c/-m containing 2 MBq [^{3}H] choline/[^{35}S] methionine (Amersham Biosciences) as appropriate and allowed to culture overnight. The experiment was stopped the following day and ES-62 purified from the supernatants using "Centricon" ultracentrifugation tubes (Amicon, Fisher, UK) with a 30000 molecular weight cut off.

2.3.12 Immunoprecipitaion of [^{35}S] Methionine-labelled ES-62

10 µl of radio-labelled sample (~1-5000dpm) was added to 5 µl of rabbit anti-ES-62 serum (raised against purified ES-62, Harnett *et al.*, 1989), normal rabbit serum, TEPC 15 (1 mg/ml, Sigma-Aldrich), a mouse IgA myeloma protein specific for PC, or normal mouse serum. PBS pH 7.2 was added to give a final concentration of 100 µl and the samples incubated at 37 °C for 1 h with gentle agitation. The samples were then left overnight at 4 °C to allow

precipitates to form. Precipitates were washed with ice-cold 10 mM Tris-HCl, 50 mM NaCl, 0.1 % NP40 pH 8.3 three times and analysed using SDS-PAGE/fluorography, as described in sections 2.3.13 and 2.3.14.

2.3.13 Reducing and Denaturing SDS-PAGE and Staining

12 % NOVEX Acrylamide gels (Invitrogen) were used in some cases: all solutions for SDS-PAGE using the NOVEX system were purchased from Invitrogen and the same fixing and staining protocols followed as detailed for the BioRad system, described below. On other occasions, 12 % gels were cast for use with the Mini Protean III (Biorad, UK). Table 2.1 details the ingredients of the solutions for casting 12 % resolving and 4 % stacking portions of the gel (cast separately). 10 µl Loading buffer was added to 10 µl containing 2.5 µg of each sample, along with Kaleidoscope Molecular weight markers (Biorad) to allow for sample size estimation, and these were boiled for 5 min. The samples were loaded into the wells of the gels and run at a constant 200 V with electrophoresis buffer containing SDS for at least 30 min, or until the dye front had reached the bottom of the gel plate. The gels were then removed and placed in fixing solution for 1 h at room temperature with gentle agitation, and stained using Sigma Brilliant Blue Colloidal-G stain (Sigma-Aldrich, UK) for a minimum for 2 h before de-staining. Finally, the gels were pictured using a system comprised of a UVP camera, Mitsubishi monitor and Sony vidoegraphic printer UP 860CE, and scanned using a CanoScan LiDE 35 Scanner.

2.3.14 Reducing and Denaturing SDS-PAGE and Fluorography

Reducing and Denaturing SDS-PAGE was carried out as detailed in Section 2.3.13 with 0.296 kBq Rainbow [^{14}C]-labelled molecular weight protein markers (Specific activity 148 kBq/ml, Amersham Biosciences, UK) run alongside samples to allow molecular weight estimation. The gels were then exposed to the fluorographic reagent AMPLIFY (Amersham Biosciences) before exposure to pre-flashed film (Hyperfilm-MP High performance, Amersham Biosciences) and storage at -70 °C. After a suitable period, the film was developed using an Agfa Curix 60 developer.

CHAPTER 4

2.3.15 Media and Reagents for *P.pastoris*

P.pastoris GS115 (Invitrogen) was maintained in yeast extract peptone dextrose (YPD) broth. MD plates were used for plasmid selection, BMG broth for enrichment and BMM broth for induction.

2.3.16 Construction of Expression Plasmids

(Carried out by Dr. Marcos J. C. Alcocer, of Division of Nutritional Sciences, University of Nottingham)

The ES-62 gene (Harnett *et al.*, 1999b) was amplified by PCR at 35 cycles of 94 °C 30 s, 54 °C 30 s, 72 °C 90 s, using Amplitaq (2 U/100 µl; Applied Biosystems) according to the manufacturer's instructions. The PCR product (ca. 1500 base pairs ((bp) was gel-purified, digested with *Eco* RI/ *Xho* I enzymes for 4 h at 37 °C and ligated into pPIC9 (Invitrogen) previously digested with the same enzymes. The resulting plasmids were transformed into *E. coli* XL1Blue (Stratagene) and selected under ampicillin (100 µg/ml) according to standard protocols (Ausubel *et al.*, 2001). Plasmids containing the inserted gene were purified (midi kit - Qiagen) and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit and an automated sequencer (Applied Biosystems).

2.3.17 Transformation and Expression in *P. pastoris*

(Carried out by Dr. Marcos J. C. Alcocer, of Division of Nutritional Sciences, University of Nottingham)

pPIC9-derived plasmids containing ES-62-encoding sequences were linearised at the *Sal* I site for 4 h at 37 °C. The linearised plasmids were then

transformed into *P. pastoris* GS115 (5 μg/transformation) and the transformed strains were selected on MD plates following electroporation according to the manufacturer's instructions (Invitrogen). Clones able to grow in the absence of histidine were inoculated into a sterile 96 well plate format containing BMG and incubated overnight at 30 °C. The 96 clones were then expanded in 24 well plates containing BMG (1.5 ml/well) and incubated overnight at 30 °C. After centrifugation (750 g for 15 min), the 24 well plates were emptied by suction and BMM broth (containing methanol at 0.5 % v/v) added. Expression ensued in an orbital shaker (2.5 g) over 48 h with addition of methanol (0.5 %) every 12 h. The 24 well plates were then centrifuged (750 g for 15 min) and the supernatant (500 μl) transferred under vacuum to a wetted Polyvinylidene fluoride membrane (PVDF, Millipore) in a dot blot format system. The membrane was then blocked by incubation with 10 ml of BSA, (5 % w/v) in TBS pH 7.5 (20 mM Tris pH 7.5, 0.9 % (w/v) NaCl) for 1 h. All incubations were performed at 37 °C in a standard hybridisation oven. After 3 washes (5 min each) with TBST (TBS plus Tween 20 - 0.1 % v/v), the membranes were incubated for 2 h with KK6 diluted (1:1000) in TBST plus 20 % (v/v) of *P.pastoris* supernatant extract that was previously prepared and did not contain the target protein. The membrane was then washed 3 times with TBST and incubated for a further 1 h with the secondary HRP-labelled anti-mouse IgG, diluted 1:20000 in TBST. After the 3 washes with TBST the membrane was incubated with BCIP/NBT substrate. Once the reaction was completed, the blot was washed with 20 mM EDTA (BDH) and dried.

Analysis revealed that most of the wells in the culture plate contained rES-62.

2.3.18 Large-scale Production of rES-62

A positive clone was selected and grown at high density (Optical Density $(OD)_{600\ nm}$ 3-6) in 2 x 2 L flasks containing MMG at 28 °C. The culture was then centrifuged (200 g for 10 min) and the pellet re-suspended in MMM and incubated with agitation (2.5 g) at 28 °C for 48-72 h with the addition of methanol to a final concentration of 0.5 % (v/v) every 24 h. After induction, the supernatant was separated from the cells by centrifugation (200 g for 10 min) and then filtered through a 0.22 μm membrane. The supernatant was then concentrated and the salt eliminated by buffer exchange (PBS pH 7.4, Biowhittaker, Lonza) using a tangential flow system (Vivascience, molecular weight cut off 100000). The sample was then further concentrated using Amicon centricon tubes with a 100000 molecular weight cut off membrane. Finally, the sample was assayed for protein content using the Biorad protein assay reagent as described in section 2.3.3.1 and then stored at -20 °C in 10 % glycerol, 0.01 M $MgSO_4$.

2.3.19 Site Direct Mutagenesis of Intact Plasmids

(Carried out by Dr. Marcos J. C. Alcocer, of Division of Nutritional Sciences, University of Nottingham)

In order to introduce point mutation on the pPIC9 based constructs, the general procedure described by Chen & Ruffner (1998) with minor modifications has been followed. Essentially two 5'phosphorylated primers complementary to different strands and separated by 300 bp between the 3'ends were used. One of the primers carried the inserted mutation. The reaction mixture (50 µl) contained 10 ng of native plasmid, 10 pmol of each primer, 10 nmol of dNTPs, 5 nmol of ATP, 2.5 U plaque forming units (*Pfu*) DNA polymerase (Stratagene), 4 U *Pfu* DNA ligase (Stratagene), in 1 ml cloned *pfu* DNA polymerase reaction buffer consisting of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% Triton X-100 and 100 µg/ml BSA. The mixture was pre-incubated at 70 °C for 10 min allowing the ligase to repair any nicks in the template. It was then subjected to thermal cycling at 95 °C for 10 s (denaturation), 50 °C for 30 s (annealing), 72 °C for 17 min (extension), 95 °C for 10 s (denaturation) and 72 °C for 17 min (annealing, extension and ligation) for 20 cycles. The amplified plasmid was then digested with *Dpn*I restriction enzyme (10 U) in the same PCR buffer at 37 °C for 2 hours in order to remove the starting template DNA and

subsequently introduced into *E. coli* XL1 Blue strain by electroporation. After ampicillin selection the transformed clones were amplified and sequenced following standard techniques.

2.3.20 Non-reducing and Non-denaturing SDS-PAGE and Staining

This was carried out using the BioRad Mini Protean III system, and the gels were cast using the same protocol outlined above in section 2.3.13, with the omission of SDS from the solutions in table 2.1. 10 µl of samples containing 2.5 µg protein were then loaded into the wells of the gel, along with Kaleidoscope Molecular weight markers to allow for rough sample size estimation, (accurate size estimation is not possible using this technique), and the gel run in electrophoresis buffer without SDS at a constant voltage of 200 until the dye front had reached the bottom of the plates. The fixing and staining protocol is as described above in section 2.3.13.

2.3.21 SDS-PAGE and Western blotting

Western analysis was performed according to the manufacturer's instructions (NOVEX, Invitrogen): after being subjected to SDS-PAGE, as described in section 2.3.13, proteins were transferred to Hybond-C (nitrocellulose membrane, Amersham) using the NOVEX XCell II Blot module (NOVEX, Invitrogen). The membrane was then blocked by incubation with 50 ml of 4 % BSA (w/v) in TBS pH 8.5 for 1 h, and all incubations were performed at room

temperature with gentle agitation. After 3 x 10min washes with TBS pH 8.5, the membranes were incubated overnight with Rabbit ES-62 anti-serum diluted 1:500 in TBS pH 8.5 containing 2.5 % (w/v) BSA on some occasions, and TEPC 15 diluted 1:1000 in TBS pH 8.5 containing 2.5 % (w/v) BSA on others. The membrane was then washed 3 times with TBS and incubated for a further 2 h with the AP conjugated secondary antibody anti-rabbit IgG (Sigma-Aldrich), diluted 1:100000 in TBS containing 0.5 % BSA (w/v) where rabbit-anti-ES-62 serum was used as the primary antibody, and anti rabbit IgG (Sigma-Aldrich) diluted 1:30000 in TBS pH 8.5 containing 0.5 % BSA (w/v) where TEPC15 was used as the primary antibody). After the 3 washes with TBS the membrane was incubated with BCIP/NBT substrate. Once the reaction was completed, the blot was washed with 20 mM EDTA and dried.

2.3.22 Dot Blotting

Dot blotting was undertaken by transferring 5µl of sample onto Hybond-C (Amersham Biosciences). The membrane was then blocked by incubation with 10 ml 4 % (w/v) BSA in TBS pH 8.5 for 30 min, and all incubations were performed at room temperature with gentle agitation. After 3 x 10min washes with TBS pH 8.5, the membranes were incubated for 1 h with KK6 diluted (1:1000) in TBS pH 8.5 containing 2.5 % BSA (w/v). The membrane was then washed 3 times with TBS pH 8.5 and incubated for a further 1 h with the secondary AP-labelled anti-mouse IgG, diluted 1:20000 in TBS containing 0.5 % BSA (w/v). After the 3 washes with TBS pH 8.5 the membrane was

incubated with BCIP/NBT substrate. Once the reaction was completed, the blot was washed with 20 mM EDTA and dried.

2.3.23 *N*-Glycosidase F Digestion of rES-62

10 μg rES-62 was added to reaction buffer (0.05 M EDTA, 0.02 M Sodium Phosphate, 0.02 % NaN_3) and SDS added to a final concentration of 0.1 %. This was boiled for 10 min and allowed to cool before adding NP40 to a final concentration of 0.75 %. 2 U of *N*-Glycosidase (Glyko, Prozyme, Europa Bioproducts, UK) F per 10 μg of glycoprotein, or the corresponding volume of reaction buffer for negative controls was added, and samples were incubated at 37 °C overnight with gentle agitation. pES-62 was used as a positive control. The digests were then subjected to SDS-PAGE and staining, as detailed in section 2.3.13.

2.3.24 Endoglycosidase H Digestion of rES-62

After the activity of the enzyme had been ascertained using a known substrate, RNAse B (Sigma-Aldrich), 10 μg rES-62 was added to 100 mM Sodium Acetate Buffer pH 5.5 containing 0.1 M 2-ME and a 1.2 fold excess of SDS in relation to protein concentration. It is to be noted that if the concentration of protein is lower than 100 μg/ml, the final concentration of SDS must not exceed 0.02 %, as this may inhibit the enzyme. After boiling for 10 min, 250 mU of Endoglycosidase H (Roche Diagnostics, UK) to 1

mg/ml glycoprotein was added, or the corresponding volume of reaction buffer for negative controls was added, and the samples were incubated at 37 °C overnight with gentle agitation. The digests were then subjected to SDS-PAGE and staining, as detailed in section 2.3.13.

2.3.25 α-Mannosidase Digestion of rES-62

The activity of the enzyme was first ascertained using a known substrate (p-Nitrophenyl-α-D-mannopyranoside, Sigma-Aldrich). 10 μg rES-62 was incubated in 0.05 M Sodium Acetate, 0.2 mM $ZnCl_2$ at a 1:1 ratio with α-Mannosidase (Sigma-Aldrich), or the corresponding volume of reaction buffer for negative controls at 37 °C overnight, and the digests then subjected to HPLC analysis.

2.3.26 Trypsin Digestion of rES-62

0.5 μl of 2 % SDS was added to 10 μg rES-62 and boiled for 10 min, then allowed to cool. 0.5 μl of 15% NP40 and 2.5 μg trypsin (Roche Diagnostics), or corresponding volume of 1 mM HCl for negative controls was added, and samples were incubated overnight at room temperature with gentle agitation with pES-62 as a positive control. The digests were then subjected to SDS-PAGE and staining, as detailed in section 2.3.13.

2.3.27 Determination of Degree of Mannose Content of Recombinant Forms of ES-62

2.5 μg of pES-62, rES-62 I and II, and KOs 1,2 and 3 were subjected to SDS-PAGE, as detailed in section 2.3.13 and the proteins on the gel transferred to a nitrocellulose membrane, as described in section 2.3.21, in preparation for western blotting. The blot was probed with snowdrop *Galanthus nivalus* Agglutinin (GNA) lectin from the DIG Glycan Differentiation Kit (Roche Diagnostics). All stages were carried out at room temperature with gentle agitation, unless otherwise stated. The membrane was blocked for 30 min using blocking solution provided with the kit. The membrane was then washed twice for 10 min with TBS pH 7.5 (0.05 M Tris, 0.15 M NaCl) and once for 10 min with Buffer 1 (TBS pH 7.5 + 1 mM $MgCl_2$ and 1 mM $CaCl_2.H_2O$). The membrane was then incubated for 1 h with 50 ml Buffer 1 containing GNA at a concentration of 1:1000. After washing three times for 10 min, each wash with TBS pH 7.5, the membrane was then incubated for 1 h in 50 ml TBS pH 7.5 containing anti-digoxenin-AP at a concentration of 1:1000. After washing three times with TBS pH 7.5, the membrane was incubated with NBT/x-phosphate substrate at a 1:50 dilution in buffer 2 (0.1 M Tris, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) with no shaking until colour developed. The reaction was then stopped by washing the membrane in dH_2O and the membrane dried.

2.3.28 High Performance Liquid Chromatography (HPLC) Separation of Recombinant ES-62 KO1

HPLC separation of recombinant ES-62 KO1 was carried out by Dr. Günter Lochnit and Prof. Rudolf Geyer, of Institute of Biochemistry, University of Giessen

2.3.29 Carbohydrate Constituent Analysis

Carbohydrate constituent analyses were carried out by Dr. Günter Lochnit and Prof. Rudolf Geyer, of Institute of Biochemistry, University of Giessen as detailed elsewhere (Geyer *et al.*, 1982).

CHAPTER 5

2.3.30 Animals and Inoculations

For immunisation studies: BALB/c mice were bred at the University of Strathclyde, and used at 6-8 wks old. Three groups of five female BALB/c mice received weekly subcutaneous injections of 2 µg per animal of either pES-62 or rES-62. Weekly serum samples were taken and antibody levels to ES-62 and rES-62 measured by as described in section 2.2.31.

WT BALB/c and C57/BL6 mice were purchased from Harlan Olac (Bicester, UK). TLR4 ko and MyD88 ko mice (on a C57/BL6 background) were generously donated by Professor S. Akira, University of Osaka, Osaka, Japan and were housed at the Universities of Manchester with the aid of Dr. K. Else and Cambridge, UK, with the help of Dr. S. Tötemeyer. Mannose receptor ko mice were a kind gift from Professor S. Gordon, Sir William Dunn School of Pathology, Oxford and Professor Michael Nussenzweig, The Rockefeller University, New York, USA.

2.3.31 Antibody ELISAs

Flat bottom medium binding 96-well plates were coated with 100 µl phosphate buffered saline (PBS) pH 9.0 containing pES-62 or rES-62 at a concentration of 2 µg/ml overnight at 4 °C. After washing, the plates were blocked for 1 h at 37 °C with 150 µl/well 4 % (w/v) BSA in PBS. The plates were then washed with PBS pH 7.0 plus 0.05% (v/v) Tween 20 (PBS/tween) and duplicate samples of sera from the immunisation study were added at a concentration of 1:100 and serially diluted threefold down the length of the plate in PBS/Tween and the plates incubated for 1 h at 37 °C. After washing, wells were incubated with 100 µl/well of a 1:20000 dilution of peroxidase-conjugated rabbit anti mouse IgG1 or IgG2a for 1 h at 37 °C. The plates were washed three times and 100 µl/well substrate added and the plates incubated at room temperature in the dark for 15 min to allow colour

development. The reaction was stopped by the addition of 50 µl/well 10 % (v/v) H_2SO_4 and the absorbance read at 450 nm. Data are expressed as reciprocal end point dilutions, with error bars calculated by standard error of the mean.

2.3.32 Cytokine ELISAs

IL-12p40, TNFα, IFNγ, IL-4 and IL-10 ELISAs were carried out according to manufacturers instructions (IL-12p40 and TNFα: BD PharMingen, San Diego, USA; IFNγ, IL-4 and IL-10 eBioscience, UK)).

High-binding 96-well plates (Greiner Bio-One Ltd, UK) were coated with 50 µl coating buffer (IL-12p40 - 0.2 M sodium phosphate, pH 6.5, TNFα - 0.1 M sodium carbonate pH 9.5, eBioscience kits included a coating buffer) containing the appropriate cytokine capture antibody at the concentration indicated by the manufacturer: IL-12p40, TNFα, IL-4 and IL-10 - 1:250; IFNγ - 1:1000 overnight at 4 °C. After washing three times in PBS/tween, the plates were blocked for 1 h at room temperature with 75 µl/well 10 % HIFCS (v/v) in PBS pH 7.0 for BD kits (assay diluent) and kit provided assay diluent for eBioscience kits. The plates were then washed three times with PBS/tween and triplicate samples of cell culture supernatant added and a standard curve of doubling dilutions of standard cytokine starting at a concentration indicated by the manufacturer and diluted in assay diluent (IL-12p40 and TNFα - 5 ng/ml; IFNγ and IL-10 - 2 ng/ml; IL-4 - 500 pg/ml) created. The plates were

then incubated for 2 h at room temperature. After washing five times with PBS/tween, wells were incubated for 1h at room temperature with 50 µl/well of assay diluent containing the appropriate detection antibody at the concentration indicated by the manufacturer: IL-12p40, TNFα, IL-4 and IL-10 - 1:250; IFNγ - 1:1000. The plates were washed five times with PBS/tween, and 50 µl/well of enzyme reagent (Streptavidin HRP conjugate for both kits) at a concentration of 1:250 in assay diluent added and the plates incubated for 30 min at room temperature. After submerging wells in PBS/tween for 30 s/1 min prior to aspiration, followed by washing seven times with PBS/tween, 50 µl/well substrate (TMB substrate included in kit for eBioscience kits, SureBlue™ TMB Microwell peroxidase substrate for BD kits) added and the plates incubated at room temperature in the dark for a maximum of 15 min to allow colour development. The reaction was stopped by the addition of 50 µl/well 2N H_2SO_4 and the absorbance read at 450 nm and 570 nm. Data are expressed as reciprocal end point dilutions, with error bars calculated by standard error of the mean.

2.3.33 Culture of bmDC

After the femurs had been dissected from BALB/c mice, all muscle tissue was removed from the bones using gauze. The bones were then placed in a 60 mm dish of 70 % alcohol for 1 min, washed twice with PBS and transferred into a fresh dish containing RPMI 1640. Once both ends of the femurs had been cut using scissors in the dish, the marrow was flushed from

within using a syringe filled with 2 ml of RPMI, fitted with a 25-gauge needle. The tissue was then suspended and passed through a 100 μm nylon mesh ("nitex", Cadisch Precision Meshes, UK) to eliminate any fragments of bone or other debris. The red blood cells were then lysed by exposing the cell suspension to 0.168 M ammonium chloride for 7 min on ice. The cells were then washed twice with fresh media by centrifugation at 300 g for 5 min, discarding the supernatant, re-suspending the cells and then replacing the supernatant with fresh media, and bone marrow cells seeded on to low-adherence 75 cm^2 flasks (Corning, Fisher) containing bmDC media at 37 °C in a humidified incubator.

On day 4, 10 ml fresh media was added to the flasks to replenish cells.

On day 6, loosely adherent cells were removed by gentle pipetting and centrifugation and used as immature DC.

2.3.34 Treatment of bmDC with Modulins

bmDC cells were cultured using the protocol outlined in section 2.3.32. On day 6, after washing the cell suspension twice by adding fresh DC media, spinning the sample at 300 g for 5 min and re-suspending the cells in fresh DC media, 20 μl of the resultant cell sample was added to 20 μl of trypan blue (1:10 dilution in RPMI 1640) and the number of cells counted using a haemocytometer, and the following formula utilised

Total number of cells = (number of cells counted/number of squares counted) x number of squares in the haemocytometer x dilution factor x volume which cells were re-suspended in x 10^4.

The DCs were then seeded into 24 well plates at $2x10^4$ cells per well in 1 ml media and were grown for a further 24 h at 37 °C, in the presence or absence of 2 μg/ml ES-62 or rES-62.

On day 7, the cells were grown for a further 24 h at 37 °C in the presence or absence of 1 μg/ml LPS (*E. coli* serotype 0111:B4, Sigma-Aldrich). Both duplicate plates were exposed to ES-62/rES-62 for 24 h as detailed above.

On the day 8 (co-culture day), The plates were spun at 300 g for 5 min and the supernatant removed for analysis of cytokine content by ELISA.

2.3.35 Statistics

Statistical significance of ELISA data was calculated using students t test.

CHAPTER 6

2.3.36 Circular Dichroism

(Carried out by Dr. Alexandra S. Solovyova and Dr. Olwyn Byron of Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow)

Circular dichroism (CD) spectra of rES-62 were recorded at 20 °C using a JASCO J-600 spectropolarimeter (JASCO UK Ltd, UK). The far ultra violet (UV) CD spectrum (260-190 nm) and near UV CD spectrum (320-260 nm) of rES-62 was measured using a 0.02 cm path length quartz cell. The protein concentration was 0.5 mg/ml in phosphate buffer (91.5 mM Na_2HPO_4, 58.5 mM NaH_2PO_4, pH 7.4). Secondary structure estimations were obtained using the Provencher and Glockner method (Provencher & Glockner, 1981).

2.3.37 Analytical Ultracentrifugation (AUC)

(Carried out by Dr. Alexandra S. Solovyova and Dr. Olwyn Byron of Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow)

Sedimentation velocity (SV) and sedimentation equilibrium (SE) experiments were performed at 4 °C in a Beckman Coulter (Palo Alto, CA, USA) Optima XL-I analytical ultracentrifuge using both absorbance at 278 nm and interference optics. The partial specific volume ($\bar{v}$) for the protein part of rES-62 was calculated from its AA sequence, using the program SEDNTERP (Laue *et al.*, 1992). The contribution of the carbohydrate part was estimated using following formula (Durchschlag, 1986)

$$\bar{v}_{complex} = \frac{\bar{v}_p + \sum_i \delta_i \bar{v}_i}{1 + \sum_i \delta_i} \qquad (1)$$

where $\bar{v}_{complex}$, $\bar{v}_p$ and $\bar{v}_i$ are the partial specific volumes of the glycosylated the protein, protein part alone and the carbohydrate part respectively; δ_i is the amount of carbohydrate in grams per gram of protein. The partial specific volume for the glycosylated form of rES-62 was calculated using values valid for a temperature of 20 °C and then extrapolated to the experimental temperature following the method of Durchschlag (1986)

$$\bar{v}_T = \bar{v}_{20} + 4.25 \times 10^{-4} (T-293.15) \qquad (2)$$

where T is the experimental temperature (K).

The density and viscosity of PBS buffer at the experimental temperature was calculated using SEDNTERP. The distribution of sedimenting material was modelled as a distribution of Lamm equation solutions (Schuck, 2000) where the measured boundary a(r,t) was modelled as an integral over the differential concentration distribution c(s).

$$a(r,t) = \int c(s)\chi(s,D,r,t)ds + \varphi \qquad (3)$$

where φ is a noise component, r is the distance from the centre of rotation and t is time. The expression χ(s, D, r, t) denotes the solution of the Lamm equation for a single species (Lamm, 1929) by finite element methods (Schuck, 1998). Implemented in the program SEDFIT

(www.analyticalultracentrifugation.com) the integral Eq. 3 is solved numerically by discretisation into a grid of 150 sedimentation coefficients for interference data and 200 coefficient for absorbance data and the best-fit concentrations for each plausible species are calculated via a linear least squares fit. The sedimentation velocity profiles were fitted using a maximum entropy regularisation parameter of p = 0.95. This model was applied to describe the heterogeneity of the material moving in the AUC cell. Also, SV boundaries were treated as comprising discrete independent species for the exact determination of sedimentation coefficients (s) for the species observed. Sedimentation coefficients were extrapolated to zero concentration and converted to standard conditions: those that would be measured at 20 °C in water.

Equilibrium in SE experiments was attained after 45 hours. The speeds of rotation were selected so that the value for the parameter σ (the reduced apparent molecular weight) (Yphantis, 1960) was between 2 and 4 for each plausible oligomeric species. Thus, SE traces for rES-62 were obtained at 8000 revolutions per second (rpm), 11500 rpm, 16500 rpm, 20000 rpm and 23000 rpm. True optical baselines were obtained after a further 6 h of rotation at 48000 rpm. The concentration of samples in the SE experiments ranged between 1.5 μM and 23 μM of rES-62 monomer. SE data were fitted globally using the Beckman XL-A - XL-I software implemented in Microcal ORIGIN 6.0 and the NONLIN program (Johnson *et al.*, 1981) (WINDOWS

version) and also the program SEDPHAT (www.analyticalultracentrifugation.com) (Vistica *et. al*, 2004).

2.3.38 Small-angle X-ray Scattering

(Carried out by Dr. Alexandra S. Solovyova and Dr. Olwyn Byron of Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow)

rES-62 was extensively dialysed against PBS buffer prior to small-angle x-ray scattering experiments. Concentrated rES-62 was microfuged (20800 g) for 20 minutes and then transferred into a capillary and placed in the SAXS beamline at the ELLETRA synchrotron (Trieste, Italy) with an electron energy of 2 GeV and a wavelength of 1.5 Å. Two camera lengths were used in the experiments: 1.5 m to cover a momentum transfer range of $0.01 < s < 0.3$ Å^{-1} and 0.8 m for $0.1 < s < 0.5$ Å^{-1}, where $s = (4\pi\sin\theta)/\lambda$ and 2θ is the scattering angle. The 1D detector was calibrated using a sample of silver bionate. For the short camera length the concentration of the protein was 10 mg/ml while for the long camera length the sample concentration was 7 mg/ml. Experimental data were collected and averaged as 30×60 s frames for both camera lengths. The data were normalised for buffer scattering and detector response using in-house software run on an IgorPro platform.

TABLES

Table 2.1

Composition of Resolving (12 %) and Stacking (4 %) Gels		
	12%	**4%**
dH_2O	3.4 ml	6.1 ml
30 % Acrylamide	4 ml	1.3 ml
Tris Buffer	2.5 ml 1.5 M Tris Buffer	2.5 ml 0.5 M Tris Buffer
SDS 10 % (w/v)	100 µl	100 µl
0.1 g/ml Ammonium Persulfate ($(NH_4)2S_2O_8$, Sigma-Aldrich)	50 ml	50 ml
TEMED (N'N'N'N'-Tetramethylethylenediamine, Sigma-Aldrich)	10 µl	5 µl

Composition of resolving (12 %) and stacking (4 %) portion of gels for use with the Mini Protean III system (BioRad, UK). For non-reducing gels the SDS was not included.

Table 2.2

Composition of Loading Buffer for PAGE under denaturing and reducing conditions	
Deionized H_2O	3.55 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
Glycerol	2.5 ml
10 % (w/v) SDS	2 ml
0.5 % (w/v) Bromophenol Blue	0.2 ml

Composition of loading buffer for PAGE under denaturing and reducing conditions. The working loading buffer contains 5 % 2-ME, and is mixed 1:2 with sample.

Table 2.3

Composition of Loading Buffer for PAGE under non-denaturing and non-reducing conditions	
Deionized H_2O	5.55 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
Glycerol	3 ml
0.5 % (w/v) Bromophenol Blue	0.2 ml

Composition of Loading Buffer for PAGE under non-denaturing and non-reducing conditions. The sample is mixed 1:2 with loading buffer.

Chapter 3: New Attempts to Produce PC-free ES-62 Using Enzymes and Enzyme Inhibitors

3.1 INTRODUCTION

The major contribution of the PC moiety of ES-62 to the molecule's immunological properties has long been known. PC has been implicated in: B cell anergy associated with low levels of PC similar to those seen in filariasis sufferers (Harnett and Harnett, 1993; Harnett *et al.*, 1999a); IL-10 production by B1 cells (Palanivel *et al.*, 1996); blocking an IgG2a response to ES-62 in mice (Houston *et al.*, 2000) and inhibiting antigen receptor stimulated polyclonal activation of the human T-cell line Jurkat (Harnett *et al.*, 1998; Harnett *et al.*, 1999a). The latter is consistent with Lal *et al.*'s (1990) finding that *B.malayi* PC-containing molecules were capable of inhibiting proliferation of human T lymphocytes.

In order to further investigate the role of PC in ES-62's immunomodulatory activities, ES-62 lacking PC was produced using inhibitors of enzymes involved in *N*-linked oligosaccharide processing. The enzymes examined in particular were 1-deoxymannojirimycin (dMM) (Houston, Cushley and Harnett 1997), an analogue of mannose which inhibits the addition of GlcNAc by preventing the removal of terminal mannose residues by Mannosidase I (Elbien, 1987, Figure 3.1) as it has been known for some time now that PC is attached to ES-62 via GlcNAc (Haslam *et al.*, 1997); or with an inhibitor of PC synthesis, HC-3, the choline kinase inhibitor (Houston and Harnett, 1999b, Figure 3.2). Although some work was carried out using this material (Houston *et al.*, 2000), the use of these inhibitors to produce PC-free ES-62

was ultimately found to be too inconsistent with respect to efficacy to warrant routine application. It was decided to widen the focus of the investigation, and attempt production of PC-free ES-62 with enzymes and inhibitors related to those used previously, in an effort to remedy the efficacy issues encountered.

The enzymes which were chosen were: β-N-acetylhexosaminidase, an exoglycosidase which removes GlcNAc from the non-reducing end of oligosaccharides/glycosides (Jones & Kosman, 1980); glycerophosphocholine phosphodiesterase and sphingomyelin phosphodiesterase, two phosphodiesterases with differing specificities and recombinant phosphorylcholine esterase of *Streptococcus pneumoniae* (Pce) (de las Rivas *et al.*, 2001), which was considered a promising candidate, due to its ability to cleave PC from carbohydrate (Höltje and Tomasz, 1974).

The two inhibitors chosen to investigate, similar to HC-3, targeted the Kennedy pathway of phosphatidylcholine biosynthesis (reviewed by Croft, Siefert and Duchene, (2003); Miquel *et al.*, 1998). Both enzymes inhibit CDP-choline synthesis via citidylyl transferase (Geilen, Wieder and Reutter, 1992; Baburina and Jackowski, 1998 respectively). These were erucyl phosphocholine ($C_{22,1,cis}$) (EPC), an alkylphosphocholine related to Hexadecylphosphocholine (HEPC) ($C_{16:0}$), a phospholipid analogue and rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine ($Et_{18}OCH_3$), an alkyl glycerophosphocholine , both of which have been used as chemotherapy against a number of species of protozoan parasites (reviewed by Croft,

Siefert and Duchene, 2003). Our lab has previously attempted inhibition of PC attachment to ES-62 using HEPC, but finding a concentration which was non-toxic to the worms whilst preventing PC attachment and phosphatidylcholine biosynthesis proved impossible (Houston and Harnett, 1999b).

It is therefore hoped that the enzymes and inhibitors decided upon for further investigation will succeed in removing PC from ES-62, allowing for PC-free ES-62 to be compared with the naturally occurring PC-containing ES-62 (pES-62), in an effort to precisely define the extent of the role of PC in the immunomodulatory activities of ES-62.

3.2 RESULTS

3.2.1 Enzyme Digestion Attempts: β-N-acetylhexosaminidase

The level of radioactivity of [^{3}H]-glucosamine-labelled ES-62 was consistently reduced by ~35 % by β-N-acetylhexosaminidase (Fig. 3.3), although when the reaction was repeated using [^{3}H]-choline labelled ES-62, no significant loss of radioactivity was recorded (Fig. 3.4), suggesting the enzyme is unable to cleave the terminal GlcNAc residues to which PC is attached.

3.2.2 Enzyme Digestion Attempts: phosphodiesterases

No PC was removed from ES-62 by glycerophosphocholine phosphodiesterase, although ~35 % could be removed from the denatured protein (results not shown), whilst ~25 % of the radioactivity was removed routinely by sphingomyelin phosphodiesterase (Fig. 3.4).

3.2.3 Enzyme Digestion Attempts: Pce

The best reduction in PC seen using Pce was ~40 % (Fig. 3.4). This could not be improved upon by increasing either digestion time or concentration of enzyme, and in general the results yielded by the enzyme were quite variable.

3.2.4 Inhibitors: EPC

The ability of 10 mM EPC to inhibit incorporation of [^{3}H]-choline into ES-62 was extremely variable, rarely significant although at best reduced [^{3}H]-choline attachment by ~50% (Fig. 3.5). Under identical conditions, by measurement of [^{35}S]-methionine incorporation, the levels of ES-62 protein synthesis and secretion were found to be unaffected (Fig. 3.6).

3.2.5 Inhibitors: $Et_{18}OCH_3$

A consistent reduction of >50 % of [^{3}H]-labelled choline incorporation was seen when worms were cultured in the presence of 10 μM $Et_{18}OCH_3$ (Fig. 3.5), with no discernable impact on methionine incorporation (Fig. 3.6). Raising the concentration to 20 μM resulted in ~70 % reduction (Fig. 3.5), and again, this was not due to toxic effects, as methionine incorporation remained unaffected (Fig. 3.6). Higher concentrations of inhibitor were found to be toxic and so were not investigated further (results not shown).

In order to investigate whether the 70 % reduction in [^{3}H]-choline incorporation was due to 70 % of the radioactivity of all of the ES-62 being lost, or whether 70 % of the ES-62 contained no radioactivity as a result of the inhibitor, immunoprecipitation of control or 20 μM $Et_{18}OCH_3$ produced [^{35}S]-methionine labelled ES-62 using TEPC 15 (a myeloma derived antibody

specific for PC, Leon and Young, 1971) was carried out. It would seem that the former theory is more likely, as ~90 % of the ES-62 derived from $Et_{18}OCH_3$ cultured worms was immunoprecipitable, indicating that the majority of ES-62 from the inhibitor-exposed group has reduced PC, but that only ~10 % is truly PC-free.

3.3 DISCUSSION

Since discovering that PC could be completely removed from ES-62 using the enzyme *N*-glycosidase F (Harnett *et al.*, 1993), to date no other enzyme has been found with this ability. It is regrettable that in order to achieve total removal of PC from ES-62, the protein first has to be denatured, and that the enzyme removes all carbohydrate from the protein. The resultant PC-free ES-62 will therefore not be in the same form as the naturally occurring, nematode derived PC-containing ES-62, which had been indicated to form a tetramer in its natural state by gel filtration in 1993 (Harnett *et al.*) and later confirmed by sedimentary equilibrium (SE) data (Ackerman *et al.*, 2003) i.e. it will be monomeric and deglycosylated. Consequently, use of this enzyme means that immunomodulatory effects of pES-62 lacking the PC moiety whilst retaining intact glycans cannot be fully investigated.

In BALB/c mice, injection of pES-62 is known to cause an almost exclusive Th2 response (Harnett *et al.*, 1999a). When PC-free material produced using HC-3 or dMM was used previously, BALB/c mice immunised with the material exhibited a mixed Th1/Th2 antigen specific response (Houston *et al.*, 2000), regardless of which inhibitor had been employed. As these enzymes target addition of PC to ES-62 in different ways, the glycan structures remaining on PC-free pES-62 produced by these methods will vary. This suggests PC, rather than specific carbohydrate structures, to be the main suppressor of a Th1 response to pES-62 in mice. Nevertheless, a

role for glycans in the immunomodulation associated with pES-62 cannot be ruled out yet, and as such, a more efficient method for producing ES-62 lacking only PC is still greatly desirable.

β-*N*-acetylhexosaminidase, an enzyme known to cleave terminal β-linked GlcNAc residues (Jones and Kosman, 1980) was used in an attempt to remedy this. As it is these residues to which PC is attached on ES-62, it was hoped that denaturation might not be required, as the GlcNAc would be more accessible to the enzyme than the target of *N*-glycosidase F, and that the remainder of the ES-62 glycan structures would remain relatively intact. It was of some concern, however, that the attachment of PC to GlcNAc would be sufficient enough a change to interfere with the enzyme's ability to remove the residue. Unfortunately, the data indicate that this fear has been borne out, as although ~35 % of the [^{3}H]-glucosamine radiolabel had been removed from ES-62, this figure was not improved upon when denatured radiolabelled ES-62 was incubated with the enzyme, and no radiolabel was removed at all when [^{3}H]-choline labelled ES-62 was subjected to the same procedure.

Only one of the two phosphodiesterases we attempted to remove PC with had an effect on the molecule in its natural state. Glycerophosphocholine phosphodiesterase was completely unable to remove any radiolabel from the ES-62, although a figure of ~35 % removal was achievable by subjecting denatured ES-62 to a prolonged exposure to the enzyme (results not shown). This most likely indicates that these conditions allow for better access of the enzyme to its target. Sphingomyelin phosphodiesterase normally generates

ceramide and PC via the hydrolysis of sphingomyelin (Liu *et al.*, 1998), and was able to remove ~25 % of [^{3}H]-choline from ES-62.

Pce produced very variable amounts of PC removal, which could not be enhanced or stabilised by increased assay length/enzyme concentration. This has been previously reported in the course of digestion attempts of PC-containing pneumococcal cell walls with the enzyme (Höltje and Tomasz, 1974; de las Rivas *et al.*, 2001; Vollmer and Tomasz, 2001). Two explanations have been suggested by these investigators: either the remaining PC residues are inaccessible to the enzyme, as the studies mentioned reported further PC release upon the addition of fresh *S.pneumoniae* cell wall preparations, indicating that the enzyme is still active; or that Pce's action is quite discrete, targeting only one of two PC-sugar interactions within the cell wall repeating unit (Behr *et al.*, 1992; Fischer *et al.*, 1993). Both arguments are equally plausible in our case, as it has been shown that there may be more than one PC group on each *N*-glycan on ES-62 (Haslam *et al.*, 1997), and ES-62 exposed to Pce (and thus containing less PC) is still able to bind TEPC 15, indicating the PC remains accessible. The molecule's sensitivity to the enzyme cannot be improved by denaturation (results not shown). In light of the latter two results, weight is lent to the theory of differing specificity.

As a result of these experiments, which again showed the process of removing PC from ES-62 whilst retaining the natural structure of the molecule to be extremely difficult when approached by enzymatic removal or

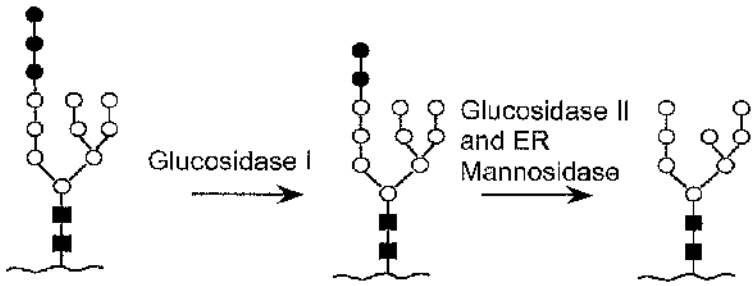
enzyme inhibitor-based methods, it was decided to try a new approach: to insert the gene for ES-62 into a recombinant protein expression system which would not contain the machinery necessary to add PC onto the resultant protein. Attempts using *E.coli* had resulted in an insoluble protein (Ackerman PhD Thesis, 2002), and utilisation of the Baculovirus system led to suspected viral DNA contamination problems which could not be remedied, making the protein produced unviable for further characterisation (Ackerman PhD Thesis, 2002). It was therefore decided to use the *Pichia pastoris* system (Invitrogen), as it was hoped that this system would provide the eukaryotic post-translational modifications necessary to create a soluble working protein, whilst side-stepping the problematic issues of viral DNA contamination seen with the Baculovirus system, and hyper-glycosylation, which is often seen when using *Saccharomyces cerevisiae* based protein expression systems. The following chapter details the production and initial characterisation of recombinant ES-62 (rES-62) produced in this way.

3.4 FIGURES

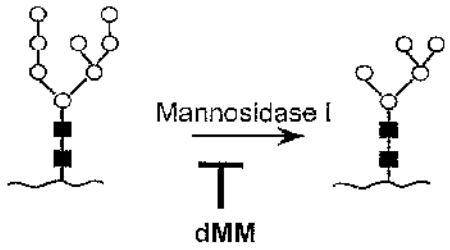
Figure 3.1. Sugar Biosynthetic Pathway in the Endoplasmic Reticulum and Goigi

Schematic representation of the sugar biosynthetic pathway from the ER and Golgi Apparatus, illustrating where dMM exerts its effect. In the ER (conserved from yeast to mammals) - $Man_9GlcNAc_2Glucose_3$ is first trimmed by Glucosidase I to $Man_9GlcNAc_2Glucose_2$, then trimmed to $Man_8GlcNAc_2$ by Glucosidase II and ER mannosidase. In the *cis* region of the Golgi apparatus, $Man_8GlcNAc_2$ is trimmed to $Man_5GlcNAc_2$ by Mannosidase I (inhibited by dMM). As the *N*-glycan moves through the medial Golgi, an extra GlcNAc residue is added by GlcNAc transferase I and two more mannose residues are removed by mannosidase II, to create $Man_3GlcNAc_3$

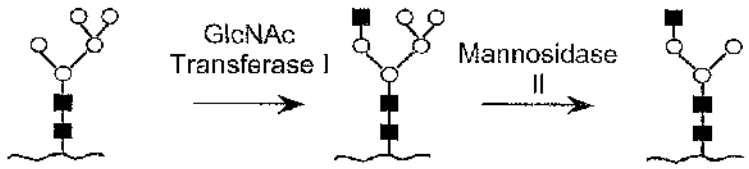
Endoplasmic reticulum



***Cis* Golgi**



***Medial* Golgi**



= Glucose

= Mannose

= N-Acetylglucosamine

= Protein

Figure 3.2. The Kennedy Pathway of Phosphatidylcholine Synthesis.

Diagram showing the Kennedy Pathway of phospholipid synthesis, illustrating where HC-3 exerts its effect. Choline is converted to phosphorylcholine (PC) by choline kinase, which is inhibited by HC-3. Phosphocholine citidyltransferase converts PC to CDP-choline, which is then converted to PtdChol by choline phosphotransferase. Finally PtdChol can be converted to sphingomyelin by SM synthase.

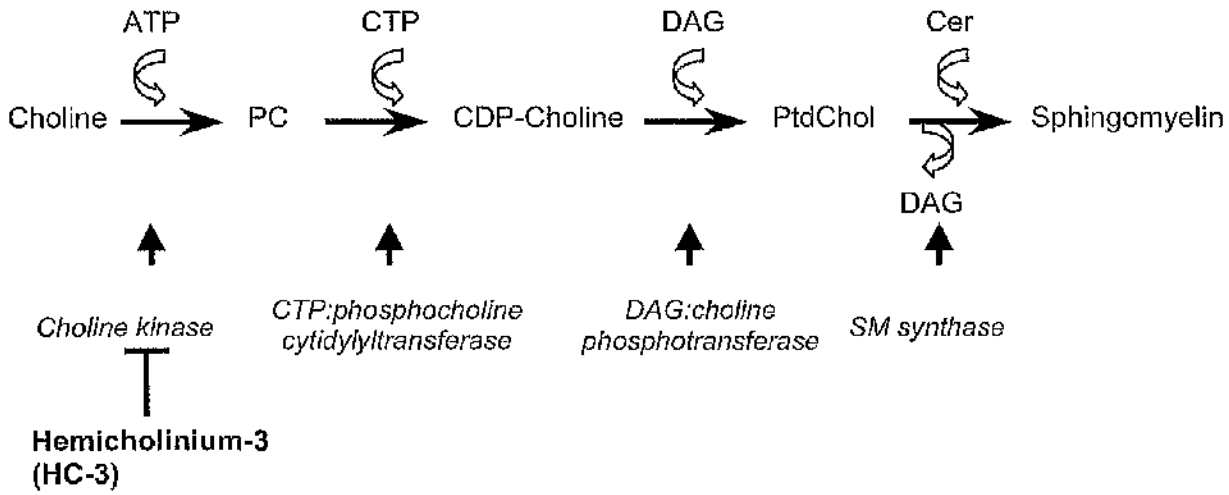

ATP
CTP
DAG
Cer
Choline
PC
CDP-Choline
PtdChol
Sphingomyelin
DAG
Choline kinase
CTP:phosphocholine cytidylyltransferase
DAG:choline phosphotransferase
SM synthase
Hemicholinium-3 (HC-3)

Figure 3.3. Enzymatic Cleavage of PC from Biosynthetically [^{3}H]-glucosamine Labelled ES-62.

ES-62 biosynthetically labelled with [^{3}H]-glucosamine was incubated in the presence or absence of enzyme for 1 h at 37 °C. The reaction mixture was then subjected to TCA precipitation analysis. Data represent triplicate determinations. *Represents $p < 0.05$.

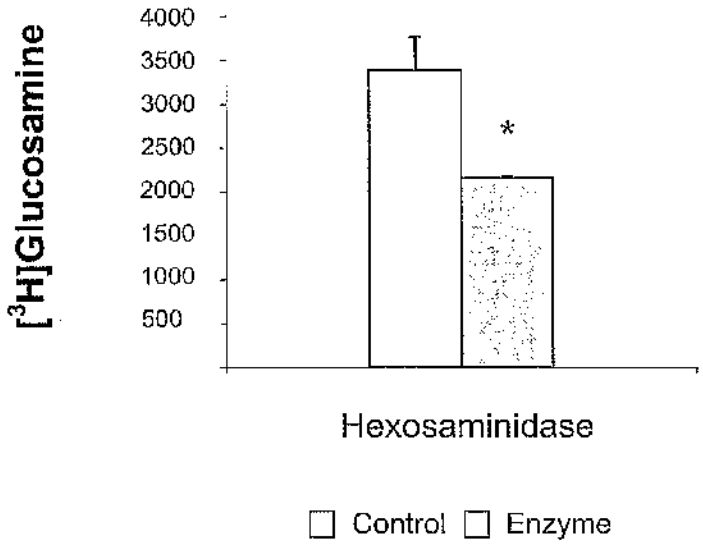

[3H]Glucosamine
4000
3500
3000
2500
2000
1500
1000
500
*
Hexosaminidase
Control
Enzyme

Figure 3.4. Enzymatic Cleavage of PC from Biosynthetically [^{3}H]-choline Labelled ES-62.

ES-62 biosynthetically labelled with [^{3}H]-choline was incubated in the presence or absence of enzyme for 1 h at 37 °C. The reaction mixture was then subjected to TCA precipitation analysis. Results obtained with inhibitors have been expressed as a percentage of the control to enhance interpretation. Data represent triplicate determinations. *represents $p < 0.05$.

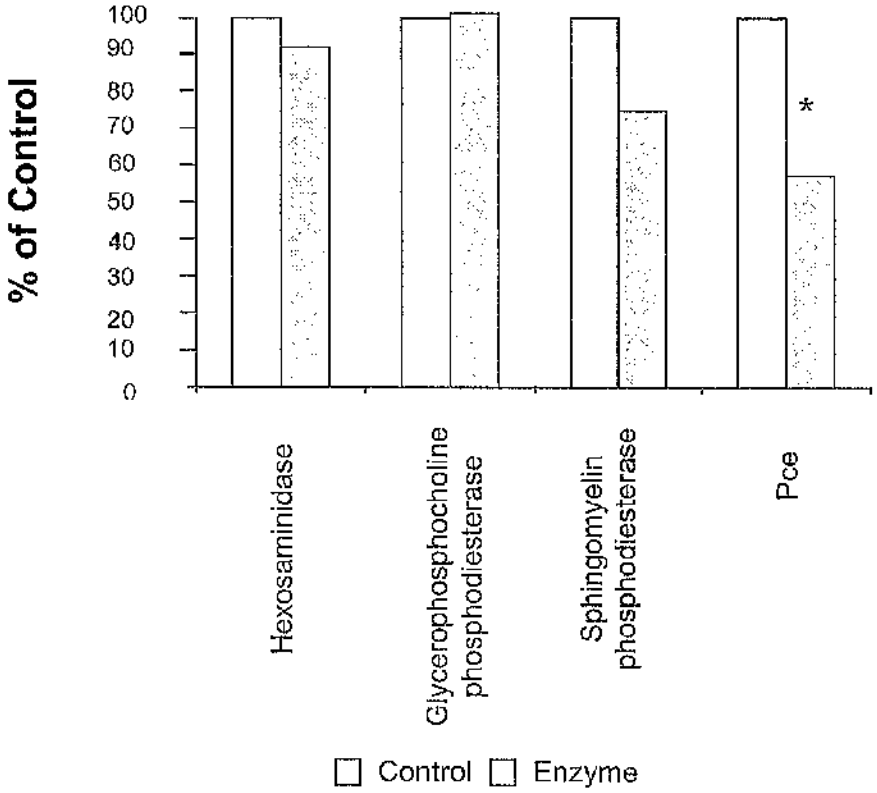

% of Control
100
90
80
70
60
50
40
30
20
10
0
*
Hexosaminidase
Glycerophosphocholine phosphodiesterase
Sphingomyelin phosphodiesterase
Pce
Control
Enzyme

Figure 3.5. Effects of inhibitors on PC Addition to ES-62: [^{3}H]-choline labelled ES-62

Adult *A.viteae* were cultured in the presence or absence (control) of inhibitor with [^{3}H]-choline. ES-62 was then prepared from spent media and subjected to TCA precipitation. Data are presented in dpm and represent triplicate determinations. * represents $p < 0.05$.

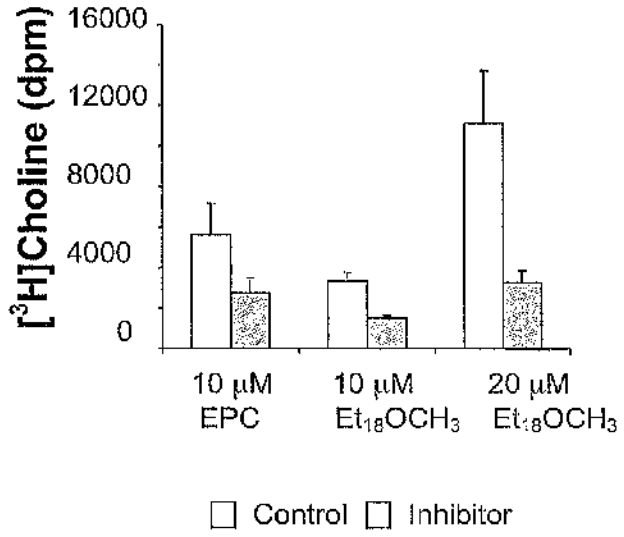
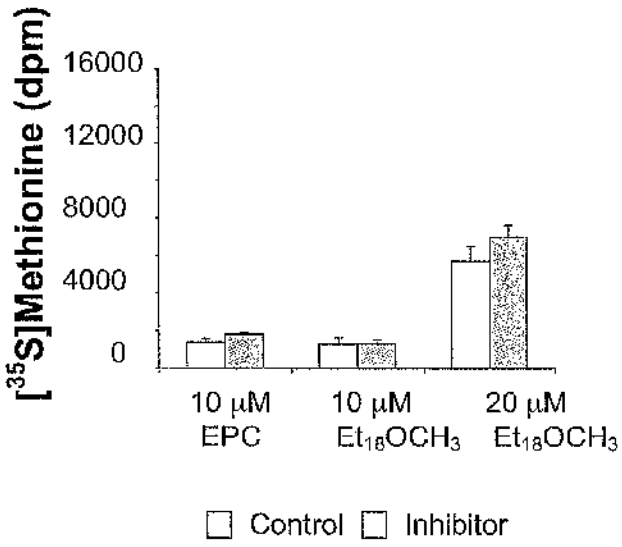

[3H]Choline (dpm)
16000
12000
8000
4000
0
10 µM EPC
10 µM Et18OCH3
20 µM Et18OCH3
Control
Inhibitor

Figure 3.6. Effects of Inhibitors on PC Addition to ES-62: [^{35}S]-methionine labelled ES-62

Adult *A.viteae* were cultured in the presence or absence (control) of inhibitor with [^{35}S]-methionine. ES-62 was then prepared from spent media and subjected to TCA precipitation. Data are presented in dpm and represent triplicate determinations. *represents $p < 0.05$.


[35S]Methionine (dpm)
16000
12000
8000
4000
0
10 µM EPC
10 µM Et18OCH3
20 µM Et18OCH3
Control
Inhibitor

Chapter 4: Production and Biochemical Characterisation of rES-62 from *Pichia pastoris*

4.1 INTRODUCTION

As seen in the previous chapter, the use of enzymes and inhibitors with a view to producing ES-62 free of PC whilst leaving the glycan structures to which the moiety is attached intact is not a simple procedure. Although some PC-free material was produced, in the main, the best effect achievable was a reduction in the levels of PC attached to ES-62, and some of the enzymes targeted glycosidic bonds, meaning some sugars were inevitably lost. The best reduction seen using the enzyme inhibitors investigated was ~70 %, though immunoprecipitation showed that only ~10 % of this material could be considered truly PC-free: the levels of PC on the remainder of the sample must be reduced. It was for these reasons that it was decided to attempt to produce ES-62 using an expression vector without the ability to add PC post-translationally - *Pichia pastoris*.

This expression vector has a very simple lifecycle, and an ability to produce high quantities of a protein of eukaryotic origin, whilst secreting very few proteins of its own. The convenience of producing a parasite-derived molecule in such a way is an obvious advantage. It was hoped that the gene expression system, being eukaryotic in nature, would side-step solubility issues previously encountered when an *Escherichia coli*-based system was attempted (Ackerman 2002, PhD Thesis), as it would be able to perform the post-translational modifications necessary for production of a correctly folded protein, whilst avoiding hyperglycosylation issues frequently encountered

when using a *Saccharomyces cerevisiae*-based system. The procedure was indeed successful, and an abundant supply of recombinant protein was available for use. This chapter describes its characterisation.

4.2 RESULTS

4.2.1 Oligomerisation and Antigenicity of Recombinant ES-62

An advantage of employing the *Pichia pastoris* expression system to produce secreted proteins is that the very low level of additional protein both secreted by the yeast and present in the culture medium means that most of the protein present in the latter is the recombinant protein. We therefore adopted a very simple purification scheme, removing all proteins of molecular mass < 100000 from the medium by ultrafiltration. The medium was then concentrated and the sample analysed by PAGE under non-reducing and non-denaturing conditions. Two major polypeptides were observed, both diffuse in nature, one migrating slightly more slowly than the parasite-derived molecule and one rather faster (Fig. 4.1). Two further minor bands of likely lower molecular mass were also observed, one surprisingly (given the purification strategy) migrating more rapidly than OVA. Interestingly, unlike parasite-derived ES-62, which monomerises from a tetramer when examined by SDS-PAGE under reducing conditions (Harnett *et al.*, 1993), all four polypeptides present in the concentrated *Pichia* culture medium showed little evidence of a change in mass (result not shown). In light of this result, it was decided to attempt digestion of the recombinant material with the serine protease trypsin. Trypsin targets peptide bonds C terminally at lysine and

arganine, and its ability to fully digest proteins is used to the advantage of those wishing to discover their sequence. As can be seen (figure 4.2), the effect of trypsin on rES-62 was very poor, whereas the pES-62 was completely digested. There appeared to be no further digestion of the rES-62 by trypsin when samples were left to digest for a period of two overnights (results not shown).

However, western blotting using the rabbit polyclonal antiserum against ES-62, suggested that the three bands of higher molecular mass might correspond to recombinant ES-62 (Fig. 4.3). To confirm that the recombinant protein lacked PC it was analysed using TEPC 15, a myeloma-derived antibody directed against PC. It can be seen in Figure 4.4 that only the parasite-derived protein is recognised by this antibody, confirming the absence of PC from the recombinant molecule. It was thus tentatively considered that the recombinant molecule might consist of several forms of ES-62 corresponding in size to tetramers, dimers and monomers with the oligomers being stable structures not amenable to dissociation. Consistent with this it was found that the recombinant molecule could not be completely monomerised even after boiling for ten minutes in the presence of mercaptoethanol. The changes in oligomerisation and susceptibility to monomerisation witnessed with the recombinant ES-62 raised the possibility that it might show some changes in conformation that could result in loss of peptide epitopes. However when the recombinant material was re-analysed by dot blotting for interaction with KK6, a mAb that recognises a

conformational epitope on the parasite-derived protein (Stepek *et al.*, 2002), binding was still clearly apparent (Fig 4.5).

4.2.2 Amino Acid Sequence and Carbohydrate Constituent Analysis of Recombinant ES-62

Complete sequencing of the recombinant cDNA clone revealed three base mismatches. This corresponded to three AA changes at positions 245 (glycine (G) > aspartic acid (D), 286 (lysine (K) > glutamic acid (E)) and 346 (asparagine (N) > serine (S)) on the mature, secreted parasite-derived molecule (Harnett *et al.*, 1999a). Preliminary lectin-binding analysis suggested that the recombinant material had higher mannose content than the parasite-derived material (discussed in section 4.2.4). The recombinant material was thus subjected to carbohydrate constituent analysis and its carbohydrate content was found to be ~6 % with a mannose/*N*-acetylglucosamine ratio greater than 12:1. This compares with roughly 3 % carbohydrate for the parasite-derived protein, which also has much less mannose as a percentage (Haslam *et al.*, 1997).

4.2.3 Effects of Changes in Glycosylation on Oligomerisation and Antigenicity of Recombinant ES-62

Mature ES-62 has four potential *N*-type glycosylation sites although structural analysis of peptides derived from the molecule suggests that the site closest

to the C-terminus (AA position 381) is not utilised (unpublished result). In an attempt to investigate the effect of glycosylation on the structure and antigenicity of recombinant ES-62 three mutants were prepared each lacking one of the three remaining *N*-type glycosylation sites. Such treatment was found to have no effect on oligomerisation of the mutants as each consisted of a mixture of monomers, dimers and tetramers, as observed with the non-mutated recombinant material (Fig 4.6).

4.2.4 Glycan Analysis of Recombinant and Recombinant Glycosylation-site Knock-out ES-62

An initial dot-blot using a glycan differentiation kit (results not shown) indicated that the main glycans attached to the recombinant and KOs were of the high mannose type, as by far the most convincing binding was seen using a lectin from *Galanthus nivalis* (Snowdrop) agglutinin (GNA), which recognises terminal mannose α(1-3), α(1-6) or α(1-2) linked to mannose (Shibuya *et al.*, 1988; Kaku, Goldstein and Oscarson, 1991), a result which was in agreement with the carbohydrate constituent analysis results. Further investigation indicated the rES-62 to contain more high-mannose structures than any KOs. All *Pichia*-derived samples seemed to have more glycans of high-mannose type than the control lectin (Carboxy-peptidase Y), indicating a high degree of glycosylation. It was therefore decided to use the glycans differentiation kit to probe a western blot of parasite-derived, recombinant and KO1, 2 and 3 ES-62 (Fig. 4.7). The results show that not only do the

KOs seem to have less glycan structures than the recombinant, but also that all the *Pichia*-derived samples contain a much higher content of high mannose structures than ES-62 from *A.viteae*.

4.2.5 Effects of Changes In Glycosylation on Carbohydrate Constituent Analysis of Recombinant ES-62

When the three *N*-type glycosylation site KOs were analyzed for carbohydrate constituent content, it was also found that all the KOs still had a high proportion of mannose compared to the parasite-derived ES-62 (results not shown).

4.2.6 Effects of Changes in Amino Acid Composition on Oligomerisation and Antigenicity of Recombinant ES-62

To investigate the effect of the three AA changes, a new clone (rES-62 II) was selected that was shown to be mutation-free by complete sequence analysis. Analysis of the recombinant protein by non-denaturing PAGE revealed the same pattern of bands as observed for the original recombinant protein (Fig. 4.8).

4.2.7 Recombinant ES-62 Sensitivity to Enzyme Digestion Compared to A.viteae *ES-62*

The eukaryotic nature of yeast glycoprotein processing and secretion makes *Pichia* an ideal expression system. These similarities, however, only hold true until the glycoproteins are transferred to the Golgi apparatus, where the yeast oligosaccharide processing events vary considerably from the events in higher eukaryotic cells (reviewed by Bretthauer and Castellino, 1999; Cereghino and Cregg, 2000). Therefore, it is logical to assume that the yeast will produce ES-62 whose glycome may not be entirely identical to parasite-derived material. It was therefore decided to compare the sensitivity of rES-62 and pES-62, to certain enzymes that target various sugar bonds to further assess the glycan structures attached to the recombinant protein. It was hoped that this would provide an explanation for the differences becoming apparent between the parasite-derived and recombinant material (for example, the seeming invulnerability of the recombinant material to denaturation). These differences seemed incongruous when considered along with the similarities seen i.e. recognition of the recombinant by various antibodies that recognise the parasite-derived material in western blots.

It was first attempted to digest the recombinant material using *N*-Glycosidase F, an enzyme that specifically cleaves all types of asparagine-bound *N*-glycans, (Tarentino, Gomez and Plummer, 1985; Chu, 1986) with the exception of *N*-glycans with a difucose core (Tretter, Altmann and März, 1991). pES-62 has been shown to be sensitive to digestion with this enzyme

in the past (Harnett *et al.*, 1993). Although, as shown previously, it can be seen that the enzyme has cleaved carbohydrate from ES-62 of *A.viteae*, Figure 4.9 shows that the *N*-glycosidase F has failed to remove the glycans from rES-62 or any of the KOs.

Although the parasite-derived ES-62 has previously been shown to be insensitive to Endoglycosidase H, it was decided to investigate the sensitivity of rES-62 to the enzyme, due to evidence showing the glycans of many of the proteins produced using the *Pichia* system to be sensitive to this enzyme (Bretthauer and Castellino, 1999). However, again, it can be seen in Figure 4.10 that the Endoglycosidase H seems to have had no effect on the rES-62.

Recombinant ES-62 KO1 was analysed using HPLC after being incubated with and without with α-Mannosidase from *Canavalia ensiformis* (Jack Bean), which hydrolyses terminal mannose residues on oligosaccharides or glycopeptides attached via α1-2,3,or -6 linkages (Li, 1967), which are commonly found in outer chain elongation of proteins produced by *Pichia* (reviewed by Gemmil and Trimble, 1999). HPLC of the recombinant KO1 showed a fairly heterogeneous solution that eluted most of the species much earlier than with pES-62 normally does (20 min RT). As can be seen in figure 4.11, after the enzyme treatment, the earlier eluting species disappeared, and the sample became more homogeneous: it can be seen that much of the mannose residues have been removed by the enzyme, confirming *Pichias* addition of mannose to the protein.

4.3 DISCUSSION

The *Pichia pastoris* recombinant protein expression system was considered as a method for producing PC-free ES-62 for two reasons. Firstly, it was considered unlikely that *P. pastoris* would possess the biochemical machinery to transfer PC to *N*-type glycans as to date it has only been observed in nematodes. Secondly, it was thought that the system would resolve the problem of unreliable efficacy associated with previously described methods (e.g. Houston and Harnett, 1999b), and with the attempts detailed in chapter 3. Both of these predictions turned out to be accurate as an adequate supply of PC-free material was readily produced. From the gels carried out, it appeared that not all of the recombinant material was able to tetramerise, instead being comprised of discrete monomeric, dimeric and tetrameric species, which proved extremely difficult to dissociate. Despite this, the rES-62 did react with a mAb against a conformational epitope on the pES-62 providing encouragement for the view that the molecule was likely to be more or less normally folded.

DNA sequencing of the recombinant clone had been undertaken at the beginning of the project to confirm identity but only at the N-terminal and hence the existence of the three base mutations was unknown. Unfortunately each of these resulted in a change in AA composition and equally unfortunate, in each case the change was not to a similar category of AA. Furthermore, each of the three AAs in ES-62 obtained from *A. viteae* is

conserved in ES-62 derived from *B. malayi* (77% conservation overall: Stepek *et al.*, 2004) raising the possibility that they could be important in producing correct protein folding. Certainly one of them - K^{286} is in a predicted region of α-helix (Ackerman *et al.*, 2003) and also of note is that G^{245} is present in a region of the parasite-derived protein that shows homology to leucine rich repeats that could conceivably be involved in protein dimerisation. Nevertheless, when we came to examine a recombinant clone whose AA sequence was equivalent to that of the pES-62 it behaved in the same manner as the original recombinant clone with respect to oligomerisation and in binding a mAb directed against the pES-62.

Regarding glycosylation, it had been expected that there would be an increase in mannose content typical of *Pichia* (Grinna and Tschopp, 1989) at the expense of complex glycan formation although the latter is fairly minimal in pES-62 in any case (Haslam *et al.*, 1997). What was clear, however, was that there had been significant increase in the extent of glycosylation with the implication that much larger glycan chains would be present. This is not entirely unheard of in proteins produced in *Pichia*, though it is thought to be a rare occurrence (reviewed by Cereghino and Cregg, 2000). What effect this would have on ES-62 structure is uncertain but it is known that glycosylation of proteins may stabilise secondary structure elements. Examination of any effect was attempted by mutating distinct *N*-type glycosylation sites. This, however, had no effect on oligomerisation or antigenicity with respect to the three mutants. Knocking out two or even all three glycosylation sites and

measuring the effect of this was considered, but previous experience of attempting to express ES-62 in *E. coli* suggested that the resultant material might prove to be insoluble (Ackerman, C. J., 2002 and unpublished results). Glycosylation is the most intricate type of modification to be carried out co- or post-translationally on a protein (Spiro, 1973). It has been conserved throughout evolution, which emphasises its importance, although for a long time researchers struggled to ascribe it a general role in glycoproteins (Varki, 1993). The nature of effects of *N*-glycosylation on the protein to which it is attached is difficult to predict, seeming to vary from protein to protein, with different and sometimes conflicting roles being described for the sugars on different glycoproteins. Lis and Sharon (1993) proposed the hypothesis that, broadly speaking, assisting correct protein folding, and stabilising this conformation once achieved are the main functions of protein glycosylation. This theory has been borne out by several investigators: in the case of free α-subunit of human chorionic gonadotropin (hGC), it was found that a semi-glycosylated version deteriorated more rapidly when stored in solution for a prolonged period of time (Erbel *et al.*, 2000). Wang *et al.* (1996) found that the oligosaccharides of glycoproteins appeared to stabilise the confirmation of the protein and add to its thermal stability when they examined a panel of five differentially glycosylated glycoproteins, and that these effects were intimately associated with the levels of glycosylation on the molecule. They also showed that protein solubility was increased in denatured and partially denatured proteins containing carbohydrates, and that their presence

appeared to prohibit aggregation, leading to an increased yield of renaturation after cooling. Although this evidence has been gathered mostly through removing sugars from glycoproteins and comparing characteristics of these molecules with intact versions of the molecule, the fact that *Pichia* has added demonstrably more oligosaccharides to rES-62 than are on pES-62 may account for its mixture of apparently independent monomeric, dimeric and tetrameric species in comparison to the almost exclusively tetrameric pES-62. When the α-subunit of hGC is expressed alone (as opposed to in conjunction with the β-subunit, with which it forms the heterodimeric, active hormone) it is over-glycosylated. This version of the free α-subunit becomes practically incapable of association with free β-subunit, limiting heterodimer formation and thus active hormone. When the free α-subunit is isolated from cells grown in the presence of an inhibitor of α mannosidase II (swainsonine), the ability to form the heterodimer with the β-subunit is restored (Blithe, 1990).

The *Erythrina corallodendron* lectin self-associates in a radically different way from the dimer form typically seen in homologous lectins from other legumes (e.g. Con A and pea lectin), due to a heptasaccharide linked at Asn17 of both of the subunits preventing the aforementioned dimer's formation (Shaanan, Lis and Sharon, 1991). Perhaps the recombinant version of ES-62 is so swathed in glycans that it is interfering with the molecule's self-association abilities.

Trimming of these glycans using various enzymes targeting glycosidic bonds also proved largely unproductive, but even trypsin digestion of the recombinant was incomplete. The rES-62 appears to be particularly insensitive to enzymes. The major differences between the natural and the recombinant versions of ES-62 uncovered so far seem to lie in the extent of glycosylation in each molecule - it is possible that the impact of yeast glycosylation on the molecule could have affected the recombinant molecule's structure and sensitivity to enzymes, creating quite a different glycoprotein to that of the natural molecule. However, it has been highlighted that although much is known about yeast glycosyiation, the early studies that provided much of this knowledge employed rather abrasive methods that may have destroyed more sensitive structures (some of which could have been enzyme resistant, reviewed by Gemmill and Trimble, 1999). It has also been pointed out that the use of exo- and endoglycosidase enzymes for analysis can be misleading, as naturally not every combination of linkages can be tested, and as a consequence of this, predicted sensitivity of certain linkages may not necessarily be proven. rES-62 may contain a combination of glycan linkages which may have been predicted to be sensitive to digestion, but which shield each other from digestion. HPLC separation of a sample of rES-62 KO1 before and after incubation with α-mannosidase revealed that the species that eluted earlier than the pES-62, seen in the non-incubated sample were absent in the enzyme-incubated sample. When a carbohydrate constituent analysis of these earlier eluting species was

carried out, they were shown to contain a higher amount of mannose than those that eluted later, indicating that the *Pichia* system has indeed added mannosyl residues to the recombinant molecule.

It has been shown that glycosylated lipids used as carriers for drugs and gene delivery effectively target hepatocytes and mϕ (reviewed by Hashida, Kawakami and Yamashita, 2005). Mannosylated proteins have been proven to target non-parenchymal cells of the liver (Ogawara *et al.*, 1999), whilst mannosylated cationic liposome–based gene delivery systems have been shown to target mϕ (Kawakami *et al.*, 2000). Since *Pichia* is known to preferentially (and almost exclusively) extend oligosaccharides by the addition of mannose (Grinna and Tschopp, 1989), if rES-62 was proven to be as robust as it appears, it may have applications in drug delivery to these types of cells in, for example, cancer therapy, where the anti-tumour drugs can have serious side effects in non-cancerous tissues.

It was not surprising to find that the majority of the sugars attached to the *Pichia*-derived ES-62 samples appeared to be of the high mannose type, given the nature of the expression vector chosen (Grinna and Tschopp, 1989); however, it was originally thought that using *Pichia* as opposed to *S.cerevisiae* could provide the eukaryotic post-translational modifications necessary whilst side stepping the issue of protein hyper-glycosylation. It seems that although it has been shown that on average, *Pichia* adds much shorter oligosaccharides to proteins than *S.cerevisiae* (Grinna and Tschopp, 1989; Tschopp *et al.*, 1987), perhaps in comparison to *A.viteae* (which

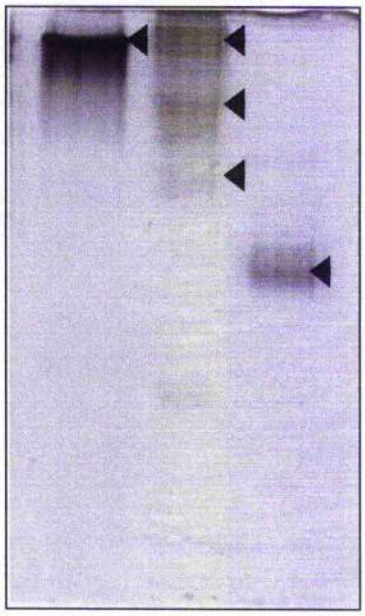
produces ES-62 whose total carbohydrates consist of a much lower percentage of mannose (Haslam *et al.*, 1997)), the difference between the extent of glycosylation of the two is still considerable. Although the use of the *N*-glycosylation site at AA 381 in *A.viteae*-derived ES-62 has been discounted (unpublished results), *Pichia* may not be as discriminatory in its addition of mannose to this, due to a difference in protein folding.

The resistance of the recombinant molecule to denaturation and various enzymes suggests that although the recombinant version of the protein is unable to exist as a completely tetrameric species, the monomers, dimers and tetramers it does form have greater integrity (being less amenable to dissociation), possibly due to the post-translational modifications carried out by *Pichia*, which would include glycosylation of the molecule. The differences discovered between rES-62 and pES-62 in this chapter may suggest a difference in the way in which the two forms of the molecule interact with the immune system, for example their effects on APCs such as DCs and the subclasses of antibody which are generated: this will be investigated in the next chapter.

4.4 FIGURES

Figure 4.1. PAGE Comparison of Parasite-derived (p) and Recombinant (r) ES-62.

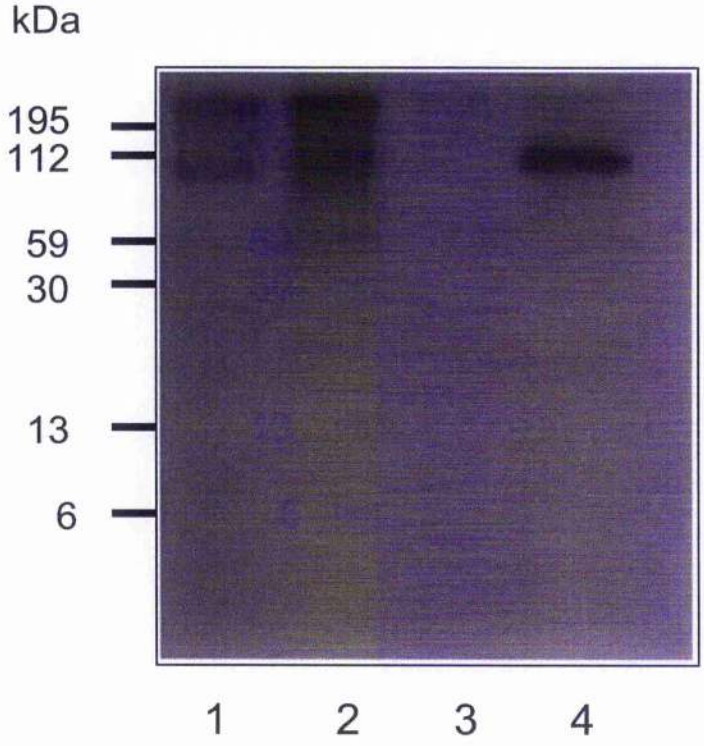
2.5 μg of pES-62 (lane 1), and rES-62 (lane 2) with ovalbumin (lane 3) as a control were run on 12 % polyacrylamide gels under non-reducing, non-denaturing conditions (A). The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain. The solid arrows indicate pES-62 (lane 1), rES-62 (Lane 2) and ovalbumin (lane 3).



1 2 3

Figure 4.2. rES-62 Digestion with Trypsin.

p and rES-62 which had been incubated with trypsin, as per manufacturers instructions, and the digested samples were run on 12 % polyacrylamide gels under reducing, denaturing conditions. The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain. Lane 1 - rES-62 plus Trypsin, Lane 2 - rES-62 Control, Lane 3 - pES-62 Plus Trypsin, Lane 4 - pES-62 Control.


kDa
195
112
59
30
13
6
1
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3
4

Figure 4.3. SDS PAGE and Western Blotting Comparison of pES-62 and rES-62 Using Rabbit Polyclonal Antl-pES-62 Serum.

2.5 μg of pES-62 (lane 1), and rES-62 (lane 2) with ovalbumin (lane 3) as a control were run on 12 % polyacrylamide gels under reducing and denaturing conditions The polypeptides were then transferred to a nitrocellulose membrane and probed with a polyclonal rabbit anti-pES-62 serum followed by an anti-rabbit IgG alkaline-phosphatase - conjugated secondary antibody and then Sigma Fast 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium substrate (BCIP/NBT). Molecular weight markers (kDa) are indicated on the left of the gels.

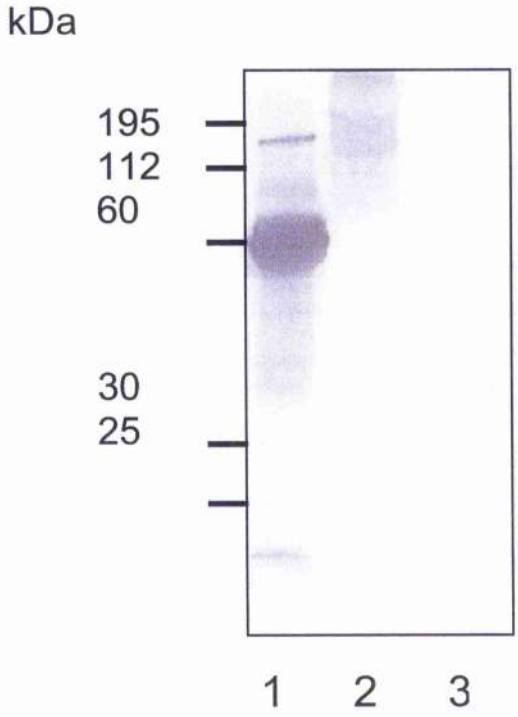

kDa
195
112
60
30
25
1
2
3

Figure 4.4. SDS PAGE and Western Blotting Comparison of pES-62 and rES-62 Using TEPC 15.

2.5 μg of pES-62 (lane 1), and rES-62 (lane 2) with ovalbumin (lane 3) as a control were run on 12 % polyacrylamide gels under reducing and denaturing conditions The polypeptides were then transferred to a nitrocellulose membrane and probed with TEPC 15 followed by an anti-mouse Ig alkaline-phosphatase-conjugated secondary antibody and then Sigma Fast BCIP/NBT substrate. Molecular weight markers (kDa) are indicated on the left of the gels.

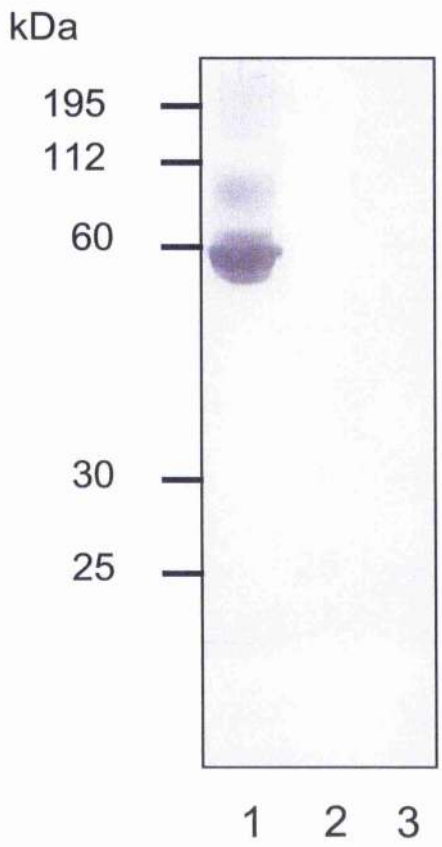
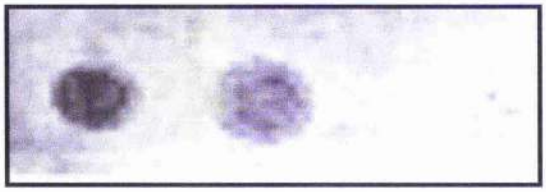

kDa
195
112
60
30
25
1
2
3

Figure 4.5. Dot Blotting Comparison of pES-62 and rES-62 Using KK6, a Monoclonal Antibody Specific for pES-62.

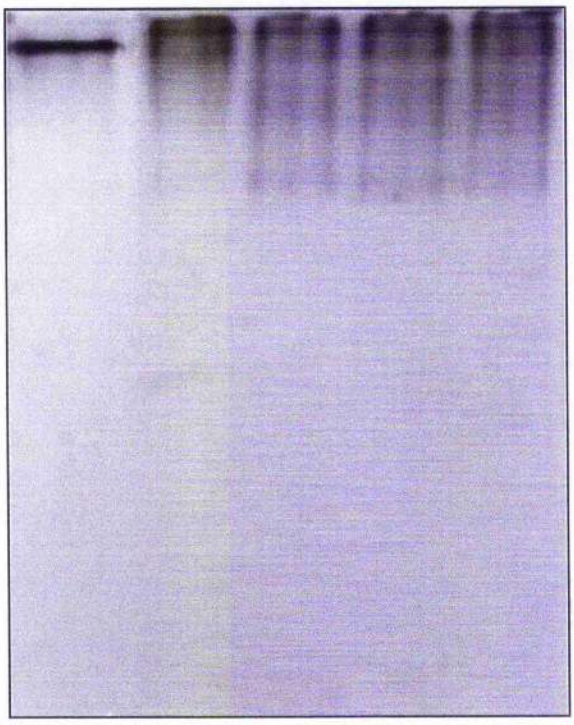
1.25 μg of pES-62 (lane 1), and rES-62 (lane 2) with ovalbumin (lane 3) as a control were placed on nitrocellulose Hybond-C (Amersham) and analysed by dot blot using a monoclonal anti-ES-62 antibody directed against a conformational epitope on the parasite-derived ES-62 tetramer.



1 2 3

Figure 4.6. PAGE Comparison of *N*-type Glycosylation Knock-Out rES-62 Mutants.

2.5 μg of pES-62 (lane 1), rES-62 (lane 2), *N*-type glycosylation site 1 (AA 194) knockout (KO1, lane 3), *N*-type glycosylation site 2 (AA 235) knockout (KO2, lane 4), *N*-type glycosylation site 3 (AA 325) knockout (KO3, lane 5), were run on 12 % polyacrylamide gels under non-reducing, non-denaturing conditions. The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain.



1 2 3 4 5

Figure 4.7. Determination of Degree of High Mannose Content of rES-62 and Glycosyiation Site-knock-outs 1,2 and 3.

1.25μg each of pES-62 (lane 1), rES-62 I (lane 2) and II (lane 3) and glycosylation site knock-outs 1 (lane 4), 2 (lane 5) and 3 (lane 6) were run on 12 % polyacrylamide gels under reducing, denaturing conditions. The polypeptides were then transferred to a nitrocellulose membrane and probed with GNA lectin, then with anti-digoxenin conjugated to AP, and finally Nitro Blue Tetrazolium/x-phosphate substrate, all from the DIG Glycan Differentiation Kit (Roche Diagnostics). The arrow on the left indicates the position of the pES-62 band, which is very faint.

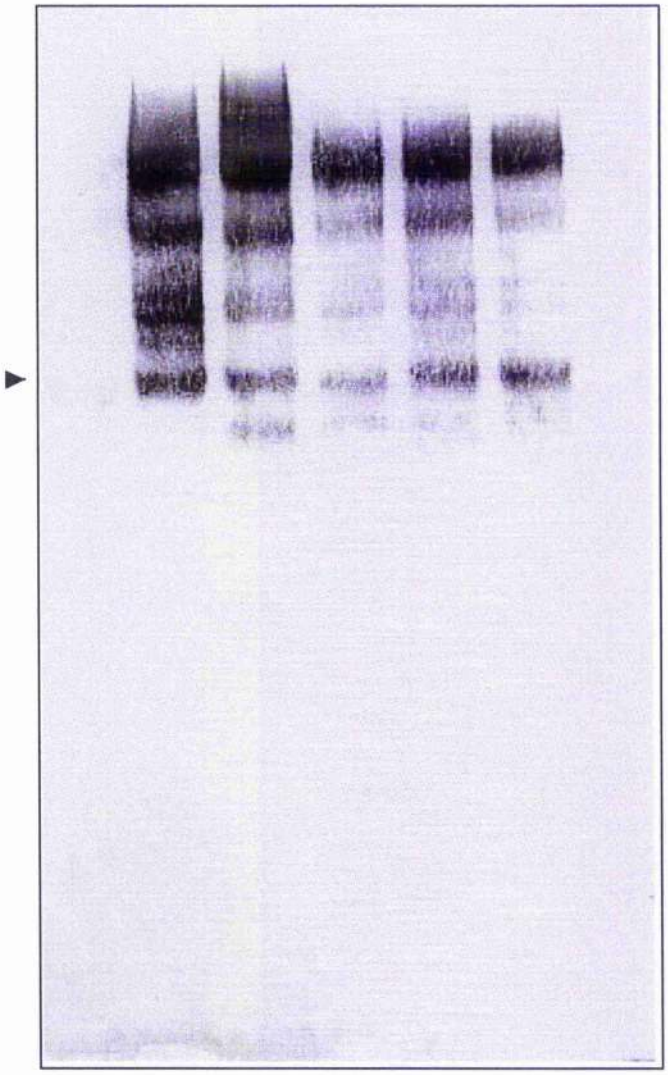
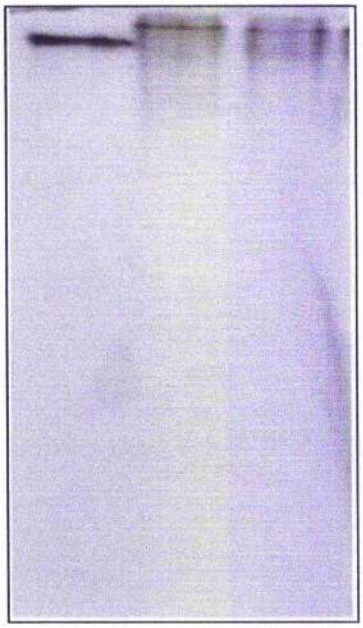

1
2
3
4
5
6
7

Figure 4.8. PAGE Comparison of rES-62 (I) and (II).

2.5 μg of pES-62 (lane 1), rES-62 (I) (lane 2), and rES-62 (II) (Lane 3) were run on 12 % polyacrylamide gels under non-reducing, non-denaturing conditions. The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain.



1 2 3

Figure 4.9. rES-62 digestion with *N*-glycosidase F.

p and rES-62 which had been incubated with *N*-glycosidase F, as per manufacturers instructions, and the digested samples were run on 12 % polyacrylamide gels under reducing, denaturing conditions. The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain. Lane 1 - 0.5 μg pES-62 plus *N*-glycosidase-F, Lane 2 - 0.5 μg pES-62 control Lane 3 - 0.5 μg rES-62 plus *N*-Glycosidase-F, Lane 4 - 0.5 μg rES-62 Control.

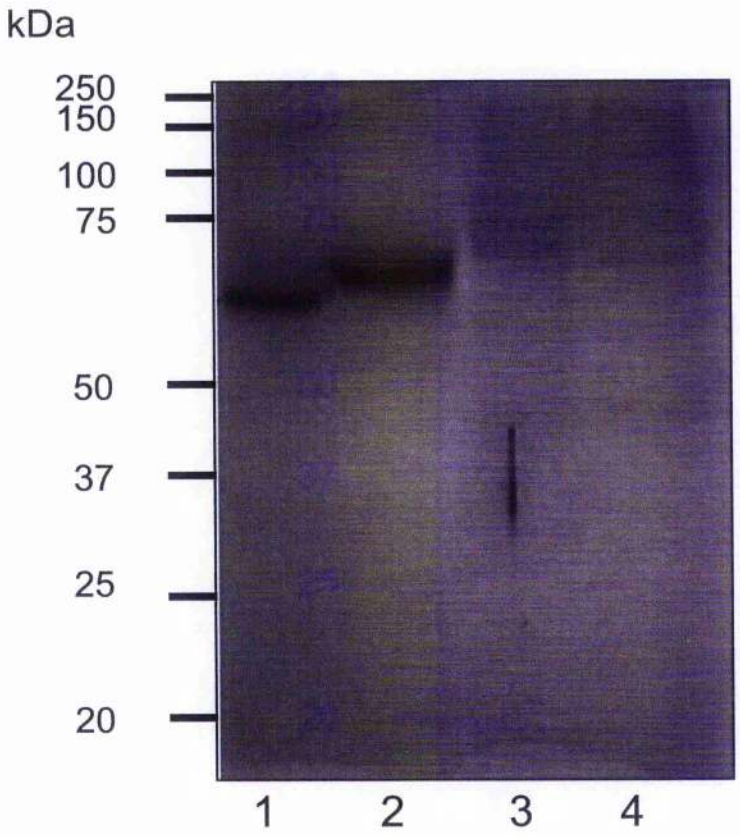

kDa
250
150
100
75
50
37
25
20
1
2
3
4

Figure 4.10. rES-62 Digestion with Endoglycosidase H.

rES-62 which had been incubated with Endoglycosidase H, as per manufacturers instructions, and the digested rES-62 was run on 12 % polyacrylamide gels under reducing, denaturing conditions. The gels were fixed and stained with Sigma Brilliant Blue Colloidal-G Stain. Lane 1 - rES-62 plus Endoglycosidase H, Lane 2 - rES-62 Control.

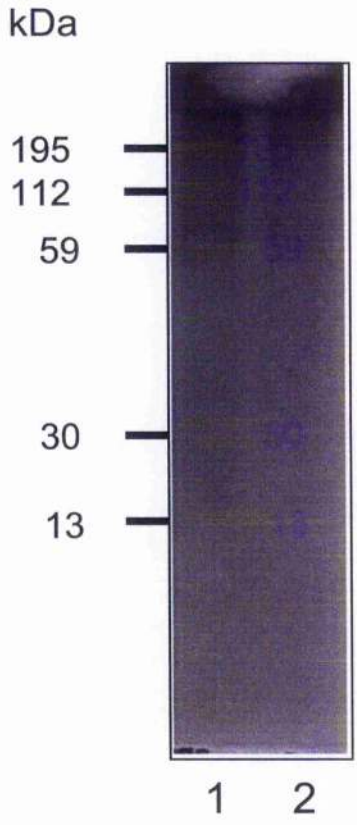
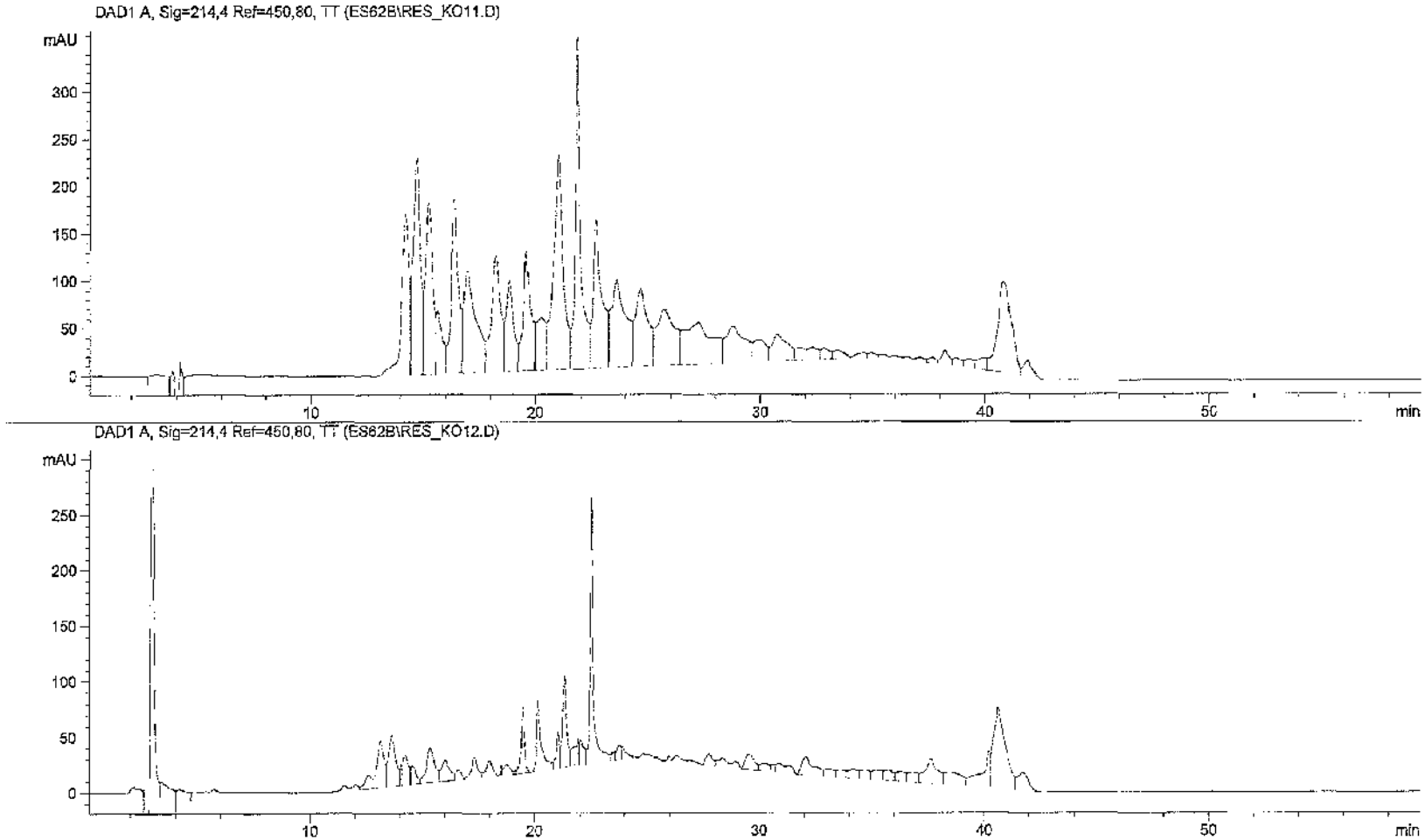

kDa
195
112
59
30
13
1
2

Figure 4.11. rES-62 Digestion with α-Mannosidase.

The mannose content of recombinant glycosylation-site knockout 1 (KO1) was analysed using HPLC before and after digestion using the enzyme α-mannosidase as per manufacturers instructions.


DAD1 A, Sig=214,4 Ref=450,80, TT (ES62B\RES_KO11.D)
mAU
300
250
200
150
100
50
0
10
20
30
40
50
min
DAD1 A, Sig=214,4 Ref=450,80, TT (ES62B\RES_KO12.D)
mAU
250
200
150
100
50
0
10
20
30
40
50
min

Chapter 5: Immunological Characterisation of rES-62 from *Pichia pastoris*

5.1 INTRODUCTION

Given the rationale behind choosing *Pichia* as a protein expression vector, detailed in the previous chapter, it was surprising to find that the molecule differed so much from pES-62 regarding glycosylation and enzyme sensitivity. However, the recognition of rES-62 by both poly- and monoclonal anti-ES-62 antibodies suggested the material was an adequate PC-free version of ES-62, and the protein was used in immunological studies.

BALB/c mice were injected for a period of 10 weeks with pES-62 or rES-62 and the IgG1 antibodies generated were analysed for their recognition of both parasite-derived and recombinant ES-62 by ELISA.

The IgG1 antibodies of BALB/c mice immunised with pES-62 or rES-62 were also analysed for recognition of denatured and non-denatured forms of the two proteins to establish the nature of the epitopes recognized by the antibodies. They were also analysed for recognition of recombinant material from the new mutation-free clone (rESII) once the three base-pair mismatches had been discovered, to ascertain what, if any effects these changes had exerted on the immunological recognition of the molecule; and for recognition of rES-62 *N*-glycosylation site knock-outs, to gauge the impact of lessened carbohydrate attachments on the molecule's recognition by the immune system.

The abilities of rES-62 to induce and modulate production of IL-12p40 by bmDCs from wild type (WT) C57BL/6s, Toll like receptor (TLR) 4 ko, Myeloid

differentiation factor (MyD)88 ko, WT C3H/HeN and C3H/HeJ mice (which contain *a* mutation which leaves them unable to respond to bacterial LPS, the classical TLR4 ligand, Poltorak *et al.*, 1998) was investigated by ELISA. rES-62 induction of IL-12p40 was also examined in dmDCs from mannose receptor ko (MR ko) mice (as the increase in mannose levels of rES-62 compared to pES-62 prompted the theory that the recombinant material may be recognised through this receptor) and was compared with the induction of IL12p40 production by bmDCs by the rES-62 glycosylation knock-outs, 1,2 and 3.

5.2 RESULTS

5.2.1 Immunogenicity of Recombinant ES-62

As SDS-PAGE/Western blot analysis suggested that almost all of the protein in the sample was indeed recombinant ES-62 (Figure 4.2), it was considered of suitable purity for further analysis. The sample was thus subcutaneously injected into mice and serum samples taken and tested for antibodies against the recombinant and parasite-derived molecules by ELISA. The recombinant ES-62 was found to induce a strong IgG1 antibody response (Fig. 5.1) but unlike PC-free ES-62 produced by earlier methods (Houston *et al.*, 2000) no IgG2a (result not shown). To our surprise, the IgG1 antibodies generated against the *A.viteae*-derived material in mice completely failed to recognise the recombinant material (Fig. 5.1). Likewise antibodies produced in response to rES-62 completely failed to recognise the *A.viteae*-derived material (Fig. 5.2). Antibody recognition of denatured forms of both proteins was investigated with both mouse sera to determine whether epitopes recognised were likely to be conformational. It appears that the pES-62 epitopes are indeed mostly conformational in nature, as the recognition of the denatured parasite-derived protein was greatly reduced, relative to the non-denatured (Fig. 5.3). However, antibody interaction with the denatured rES-62 appears to be essentially no different from that witnessed with the non-denatured rES-62 (Fig. 5.4), suggesting that the epitopes recognized on the

recombinant molecule are not conformational. It can also be seen that antibodies raised against rES-62 do not recognize denatured pES-62, or vice versa: thus this does not look like a case of the recombinant simply being denatured, especially taking into account the difficulties encountered when attempting to denature rES-62 (discussed in Chapter 4).

5.2.2 Effects of Changes in Glycosylation on Immunogenicity of Recombinant ES-62

When the serum of BALB/c mice injected with either pES-62 or rES-62 was analysed for antibody recognition of the three *N*-type glycosylation site KOs, each was recognised by the murine antiserum against the recombinant but not the parasite-derived material (Fig 5.5).

5.2.3 Effects of Changes in Amino Acid Composition on Immunogenicity of Recombinant ES-62

When the recognition of recombinant material from the new mutation free clone (rESII) by antibodies in the serum of BALB/c immunised with pES-62 or rES-62 was investigated, this protein was also recognised by murine antibodies raised against the recombinant protein only: again there was no cross-reactivity with antibodies raised against the parasite-derived protein (Fig. 5.6).

5.2.4 Effects of rES-62 on IL-12 Production by bmDCs

When bmDCs from WT C57BL/6 mice were pre-treated with 2 μg/ml rES-62 for 18 h prior to stimulation with 100 ng/ml *Salmonella minnesota* LPS for 24 h, rES-62 exposure mimicked the previously shown effects of pES-62 on LPS-induced IL-12 production (Goodridge *et al.*, 2005 and 2004) (figure 5.7); treatment resulted in low level IL-12p40 production, but prevented the full induction of IL-12 by LPS.

5.2.5 Investigation of the Involvement of TLR4 and the Mannose Receptor in Stimulation of Cytokine Production by rES-62

When bmDCs from various mouse strains (TLR4 knock out (ko) (figure 5.8A), MyD88 ko (figure 5.8B), WT C3H/HeN (figure 5.8C) and C3H/HeJ (figure 5.8D) were treated with 2 μg/ml rES-62 for 24 h and the supernatants analysed for IL-12p40 levels by ELISA, it appeared that the modulation of cytokines production by rES-62 occurs independently of both TLR4 and MyD88, as an IL-12p40 response to rES-62 was seen in all of the mutants analysed.

In addition to lacking PC, the carbohydrate component of rES-62 differs from that of pES-62. The natural protein contains ~3 % carbohydrate, of which less than 50 % is mannose, whereas rES-62 is ~6 % carbohydrate and

virtually all of this is mannose (Egan *et al.*, 2006). It was therefore hypothesized that the recombinant material may be recognized by the MR on cells, as pES-62, which contains less mannose was recognised by a soluble form of the receptor (Harnett, unpublished results). To investigate this possibility the three *N*-glycosylation mutants of rES-62 that had been generated were utilised, to determine whether reduced mannose affects the recognition of the recombinant material. When bmDCs from C57BL/6 or MR ko mice were treated with 2 μg/ml rES-62 or rES-62 glycosylation site knock-out 1,2 or 3 for 24 h, it was found that the stimulatory effects of rES-62 were not altered by the substitution of the key glycosylation sites at Thr215, Ser256 and Ser346 (figure 5.9). Furthermore, the effects of pES-62, rES-62 and the rES-62 mutants were also intact in bmDCs from MR ko mice (figure 5.10).

5.3 DISCUSSION

Bearing in mind the anti-ES-62 polyclonal and monoclonal antibody recognition of the rES-62, it was surprising to find that antibodies generated against recombinant ES-62 in mice completely failed to recognise the parasite-derived material. Likewise polyclonal anti-sera generated against pES-62 in mice at the same time showed virtually no reactivity for the recombinant material.

Further analysis by ELISA suggested that whereas the majority of antibodies induced by pES-62 recognised conformational peptide epitopes, this was not true of the recombinant molecule. There are several possible explanations for this difference: the recombinant material used to coat the ELISA plate may not have been fully denatured (as discussed in Chapter 4); the epitopes recognized by the antiserum against the recombinant molecule may be largely carbohydrate in nature; or the peptide epitopes on the recombinant molecule are essentially sequential, and therefore are unaffected by denaturation. Overall, it could well be that the lack of immunological cross-reactivity between the two molecules may be due to the recombinant material having a less folded structure than the parasite-derived material. It does not appear likely that the protein is simply denatured: the murine anti-rES-62 antibody completely fails to recognise denatured pES-62 (Figures 5.3 and 5.4).

The contribution to altered immunogenicity of the changes in AA sequence and glycosylation of the recombinant was investigated, and was found to have no real impact. Neither the new mutation-free version of rES-62 nor the glycosylation KOs were recognised by anti-sera raised against pES-62. Furthermore, all versions were equally well recognised in comparison to the original rES-62 by the antibodies raised against this material. Consistent with this, the total of the three AA changes in the rES-62 sequence was found to have very little effect on overall predicted secondary structure when this was assessed (result not shown).

The results of the immunological studies are curious: given the marked differences exposed between rES-62 and pES-62 and discussed thus far both biochemically and immunologically, it was interesting to see that the rES-62 did not induce the antibody profile seen with previous PC-free ES-62 (produced using inhibitors of *N*-linked oligosaccharide processing (dMM) or PC synthesis (HC-3)). This previously produced PC-free ES-62 produced a mixed IgG1/IgG2a response (Houston *et al.*, 2000), but rES-62 gave the same IgG1 skewed response as pES-62. Previously produced PC-free ES-62 produced this mixed IgG1/IgG2a response regardless of which inhibitor had been used: dMM inhibits mannosidase I in the cis golgi (Fig. 3.1), and would lead to an increase in high-mannose glycan structures present on ES-62. HC-3, being an inhibitor of PC synthesis via blocking choline kinase (Fig. 3.2), would likely lead to complex glycan formation on ES-62 as normal complex glycan formation is blocked by PC addition. As both versions of PC-

free ES-62 gave the same Th1/Th2 mixed antibody profile, it was therefore proposed that PC was the main suppressor of a Th1 response to pES-62 in mice. However, in light of the results using rES-62, which contains no PC, clearly a role for the glycans of pES-62 in the molecule's immunomodulation capabilities cannot be ruled out yet. Carbohydrate structures from parasitic and non-parasitic nematodes have been demonstrated to drive development of a Th2 phenotype in lymphoid cells (Tawill *et al.*, 2004). Lymphoid cells of mice pre-immunised with whole worm extracts of *B.malayi* (BmA) or *C.elegans* (CeA) differentiated into a population dominated by $CD4^+$, IL-4 but not IFNγ producing cells. Furthermore, when the carbohydrates of the worm extracts were targeted using periodate treatment, lymphoid cells stimulated with the resultant antigens produced significantly less IL-4 than those cells which were cultured with extracts that had undergone a mock treatment. However, the authors of this work did state that their findings contained no evidence concerning whether these responses were due to PC moieties (known to be attached to the glycans), or whether the glycans themselves were exerting an effect.

Equally intriguingly, exposure to PC-free rES-62 both *in vitro* and *in vivo* also mimicked the ability of ES-62 to modulate DC cytokine production, but this effect was discovered to be independent of both TLR4 and MyD88. Thus rES-62 is clearly modulating IL-12 production by a different mechanism from pES-62 and PC-Ova, and this mechanism is not likely to involve a TLR.

It became clear that rES-62, despite being dominated by mannose, was not signalling via the mannose receptor. This again somewhat mirrors the situation seen with pES-62, where although initial studies showed the molecule to be able to interact with the mannose receptor, subsequent work illustrated that it appeared to behave as normal with DCs and mϕ of mannose receptor knock out mice (Harnett and Harnett, unpublished observations 2004). Thus, the theory postulated in the previous chapter regarding the use of rES-62 for targeting drugs to hepatocytes and mϕ via the mannose receptor may not be valid.

Taken together, the data from the cytokine production and experiments looking into the mechanism of cytokine production in DCs from various mouse strains indicate that the effects of ES-62 are mediated largely by PC, but that without PC the glycoprotein appears to possess some immunomodulatory potential. These results are consistent with previous studies (e.g. Houston *et al.*, 2000) in supporting an important role for PC in ES-62's immunomodulatory activity. Immunomodulatory activity was also demonstrated for PC-free rES-62, but although the results are very interesting, doubts exist as to the validity of this "control". This is because, although arriving at the same biological outcome, pES-62 and rES-62 utilize different receptor recognition and intracellular signal transduction activation. Thus, until a form of ES-62 free from PC, but containing no other differences is available in suitable quantities for experimentation, the question as to

whether the protein component is immunomodulatory under normal circumstances must remain open.

Work by Deehan *et al.* (2002) and Kean *et al.* (2006) has shown the PC moieties of *Ascaris suum* to be immunomodulatory. When the effects of synthetic PC-containing glycolipids on DCs and mϕ were investigated, the results were varied. GL2, which contained no PC had no effect on DCs or mϕ, whilst GL3, which does contain PC was able to cause up-regulation of co-stimulatory molecules on both cell types, stimulate the induction of pro-infiammatory cytokines, and inhibit induction of these pro-inflammatory cytokines by IFNγ/LPS. The fact that the only effects which were seen with GL1, which also contains PC, were a slight increase in co-stimulatory molecules, serves as a reminder of how complex the process of immunomodulation often is: there is no question that PC is a major immunomodulatory factor, but it would appear that other structural features are required for its action (Deehan *et al.*, 2002; Kean *et al.*, 2006). The work carried out in this thesis has helped to identify the pathways involved in ES-62's immunomodulatory abilities, and further highlighted PC's importance in these signalling events. This has paved the way for novel drugs to be designed based on molecules such as ES-62 with the aim of interfering with these pathways, though at this stage, there is much to be done in the investigation of PC and immunomodulation.

As all biochemical and immunological routes explored in attempts to explain the emerging differences between the two molecules thus far had failed to

give conclusive results, it was decided to subject the rES-62 to biophysical analyses similar to that which had already been carried out on pES-62 (Ackerman *et al.*, 2003) and this will be discussed in the next chapter.

5.4 FIGURES

Figure 5.1. Recognition of rES-62 by IgG1 Antibodies in Serum of Mice Inoculated with rES-62 or pES-62.

Groups of five BALB/C mice were injected weekly with 2 μg p or rES-62 and serum samples taken. Samples were analysed by ELISA for rES-62 specific responses in duplicate and mean values calculated ± standard error of the mean. Results are expressed as reciprocal end-point dilution.

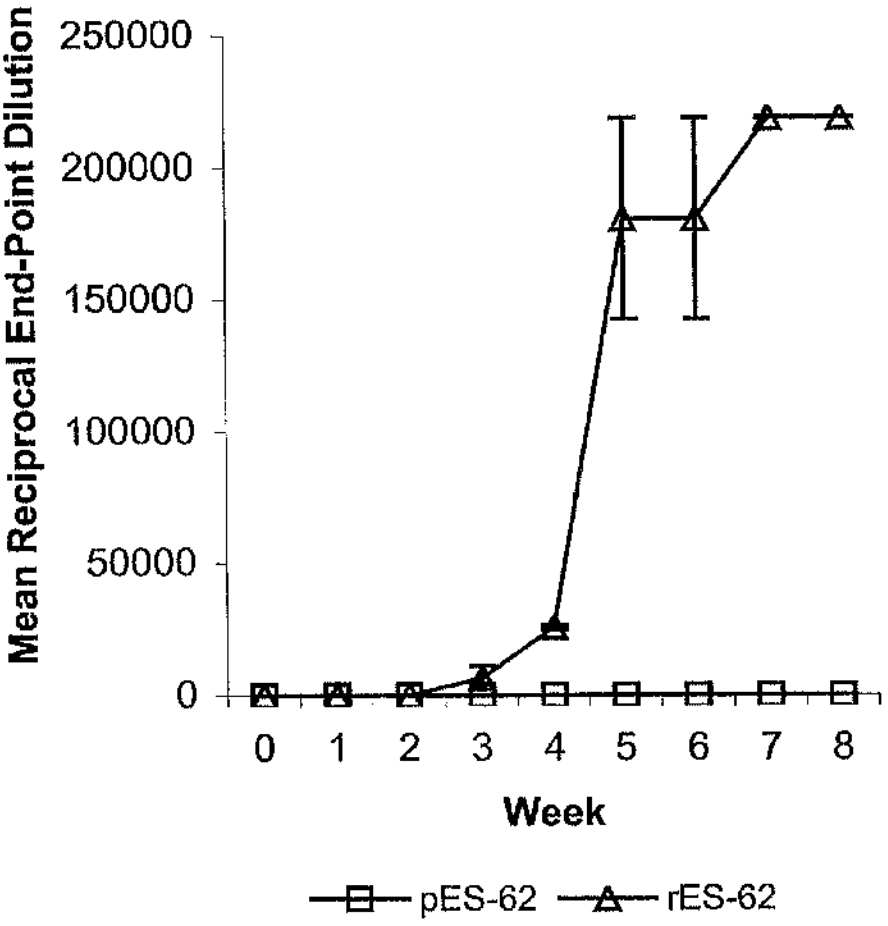

Mean Reciprocal End-Point Dilution
250000
200000
150000
100000
50000
0
0 1 2 3 4 5 6 7 8
Week
pES-62
rES-62

Figure 5.2. Recognition of pES-62 by IgG1 Antibodies in Serum of Mice Inoculated with rES-62 or pES-62.

Groups of five BALB/C mice were injected weekly with 2 μg pES-62 or rES-62 and serum samples taken. Samples were analysed by ELISA for pES-62 specific IgG1 responses in duplicate and mean values calculated ± standard error of the mean. Results are expressed as reciprocal end-point dilution.

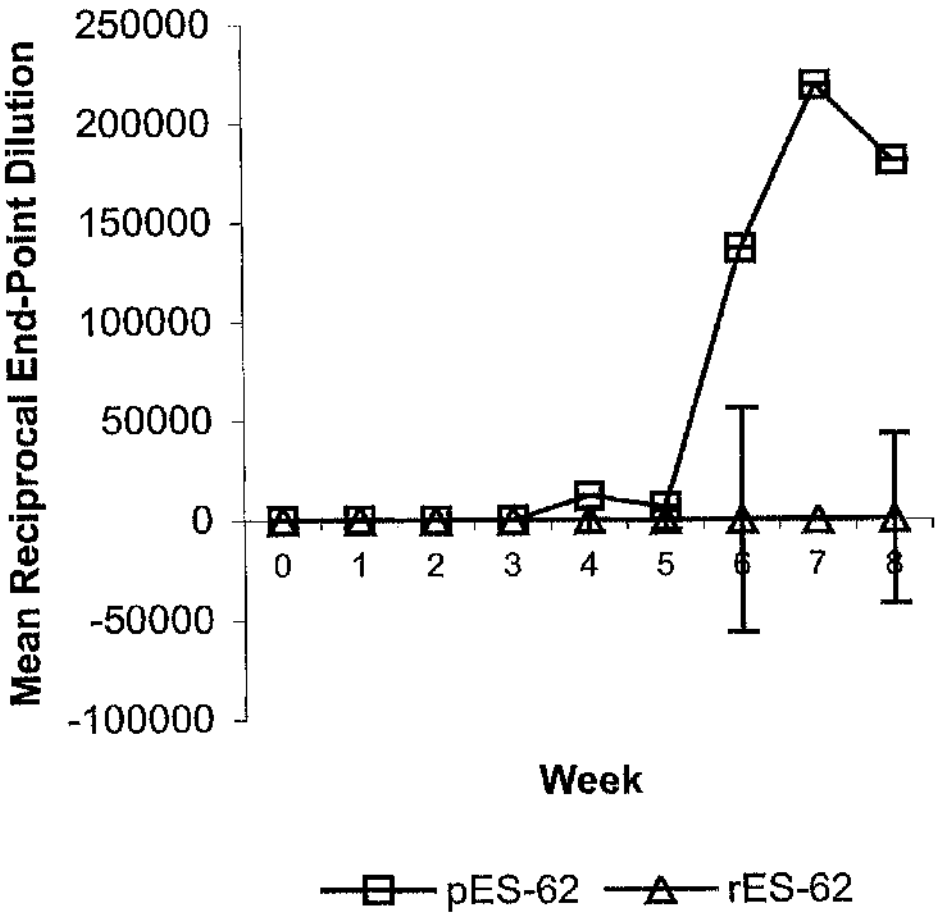

Mean Reciprocal End-Point Dilution
250000
200000
150000
100000
50000
0
-50000
-100000
0
1
2
3
4
5
6
7
8
Week
pES-62
rES-62

Figure 5.3. Recognition of Denatured and Non-denatured pES-62 by IgG1 Antibodies in Serum of Mice Inoculated with pES-62.

Groups of five BALB/C mice were injected weekly with 2 μg pES-62 or rES-62 and serum samples taken. Samples were analysed by ELISA for denatured and non-denatured pES-62 specific IgG1 responses in duplicate and mean values calculated ± standard error of the mean. Results are expressed as reciprocal end-point dilution.

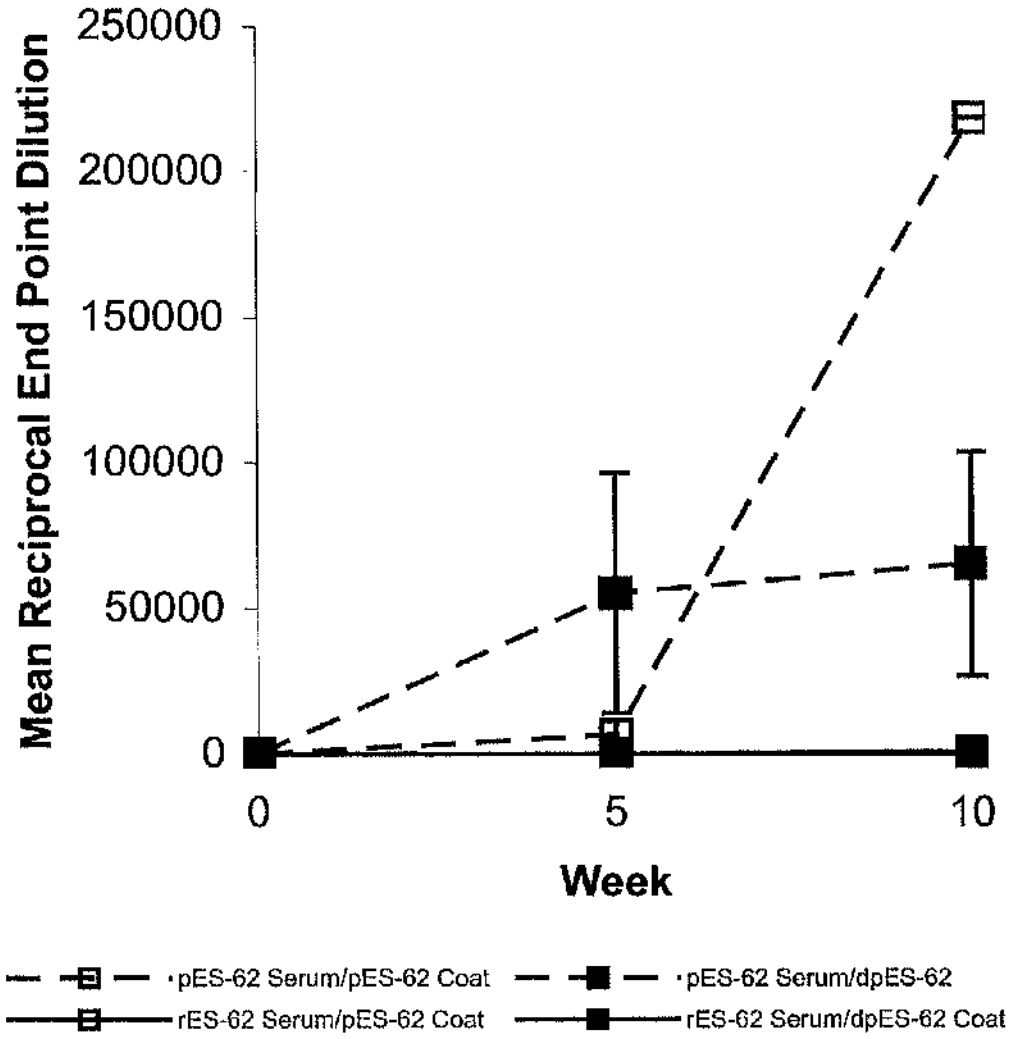

Mean Reciprocal End Point Dilution
250000
200000
150000
100000
50000
0
0
5
10
Week
pES-62 Serum/pES-62 Coat
pES-62 Serum/dpES-62
rES-62 Serum/pES-62 Coat
rES-62 Serum/dpES-62 Coat

Figure 5.4. Recognition of Denatured and Non-denatured rES-62 by IgG1 Antibodies in Serum of Mice inoculated with rES-62.

Groups of five BALB/C mice were injected weekly with 2 μg pES-62 or rES-62 and serum samples taken. Samples were analysed by ELISA for denatured and non-denatured rES-62 specific IgG1 responses in duplicate and mean values calculated ± standard error of the mean. Results are expressed as reciprocal end-point dilution.

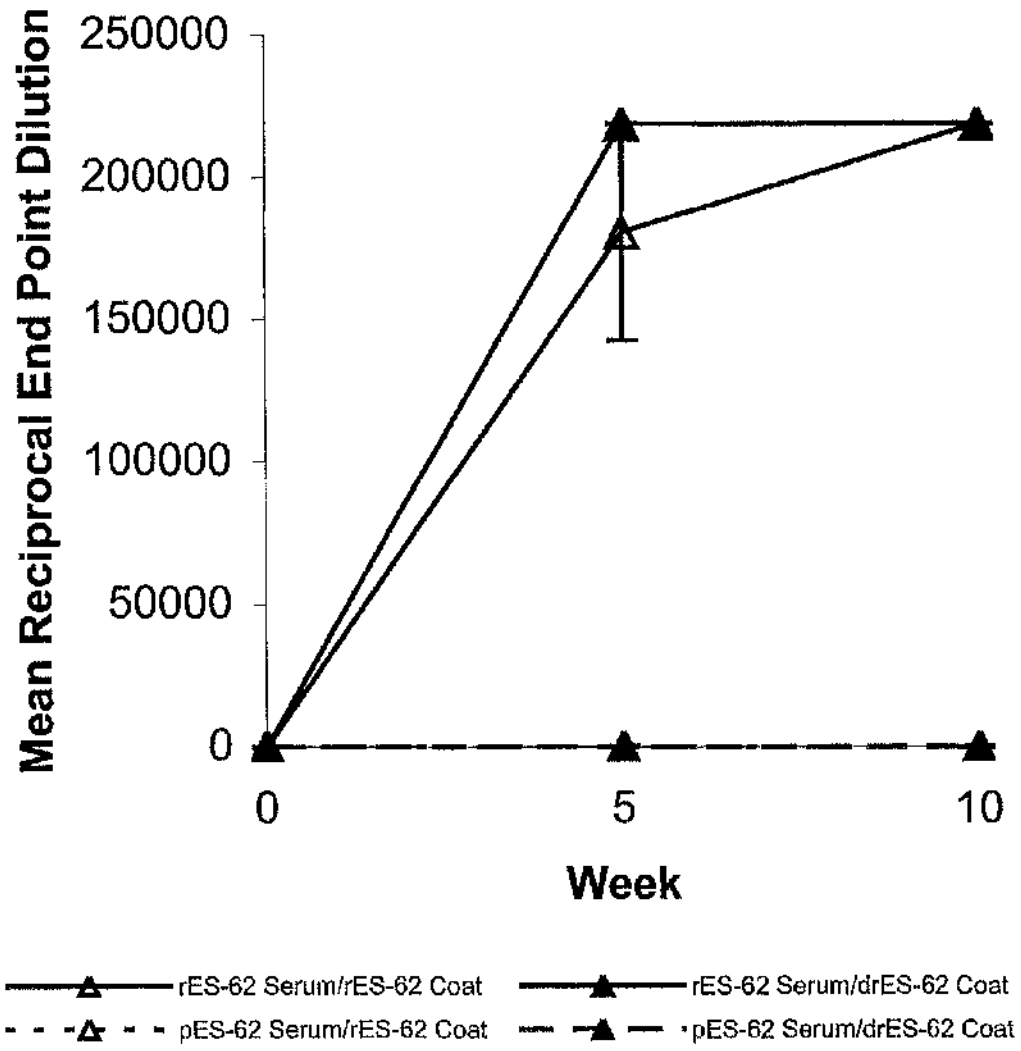

Mean Reciprocal End Point Dilution
250000
200000
150000
100000
50000
0
0
5
10
Week
rES-62 Serum/rES-62 Coat
rES-62 Serum/drES-62 Coat
pES-62 Serum/rES-62 Coat
pES-62 Serum/drES-62 Coat

Figure 5.5. Comparison of Recognition of *N*-type Glycosylation-site Knock-Out rES-62 Mutants by IgG1 Antibodies in Serum of Mice Inoculated with pES-62 or rES-62.

Groups of five BALB/c mice were injected weekly with 2 μg pES-62 or rES-62 and serum samples taken. Samples were analysed by ELISA for recognition of each of 3 distinct *N*-type glycosylation site knock-out rES-62 mutants by IgG1 antibodies in duplicate and mean values calculated ± standard error of the mean. Results are expressed as reciprocal end-point dilutions.

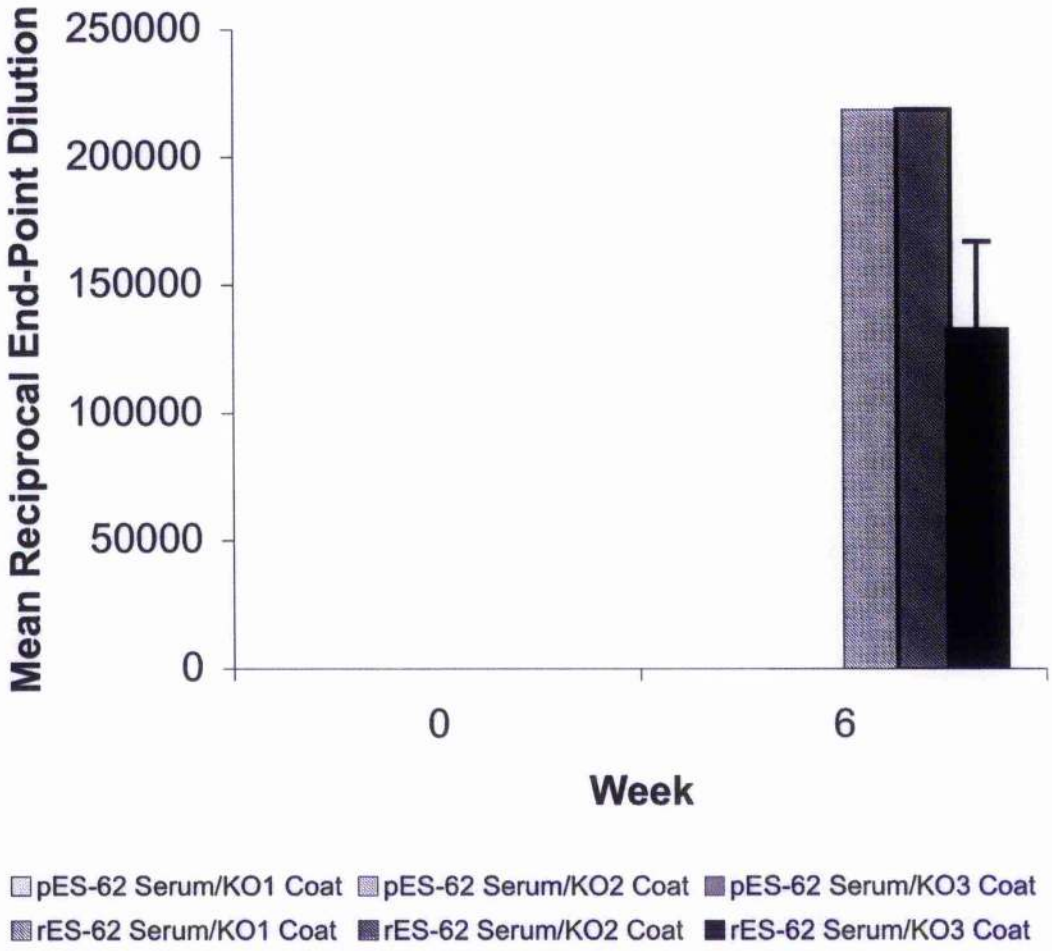

Mean Reciprocal End-Point Dilution
250000
200000
150000
100000
50000
0
0
6
Week
pES-62 Serum/KO1 Coat
pES-62 Serum/KO2 Coat
pES-62 Serum/KO3 Coat
rES-62 Serum/KO1 Coat
rES-62 Serum/KO2 Coat
rES-62 Serum/KO3 Coat

Figure 5.6. Comparison of Recognition of rES-62 (I) and (II) by IgG1 Antibodies in Serum of Mice Inoculated with pES-62 or rES-62.

Groups of five BALB/c mice were injected weekly with 2 μg of p or rES-62 (I) and serum samples taken. Samples were analysed by ELISA for rES-62 (II) specific IgG1 responses in duplicate and mean values calculated ± standard error of the mean. Results are shown as reciprocal end point dilutions.

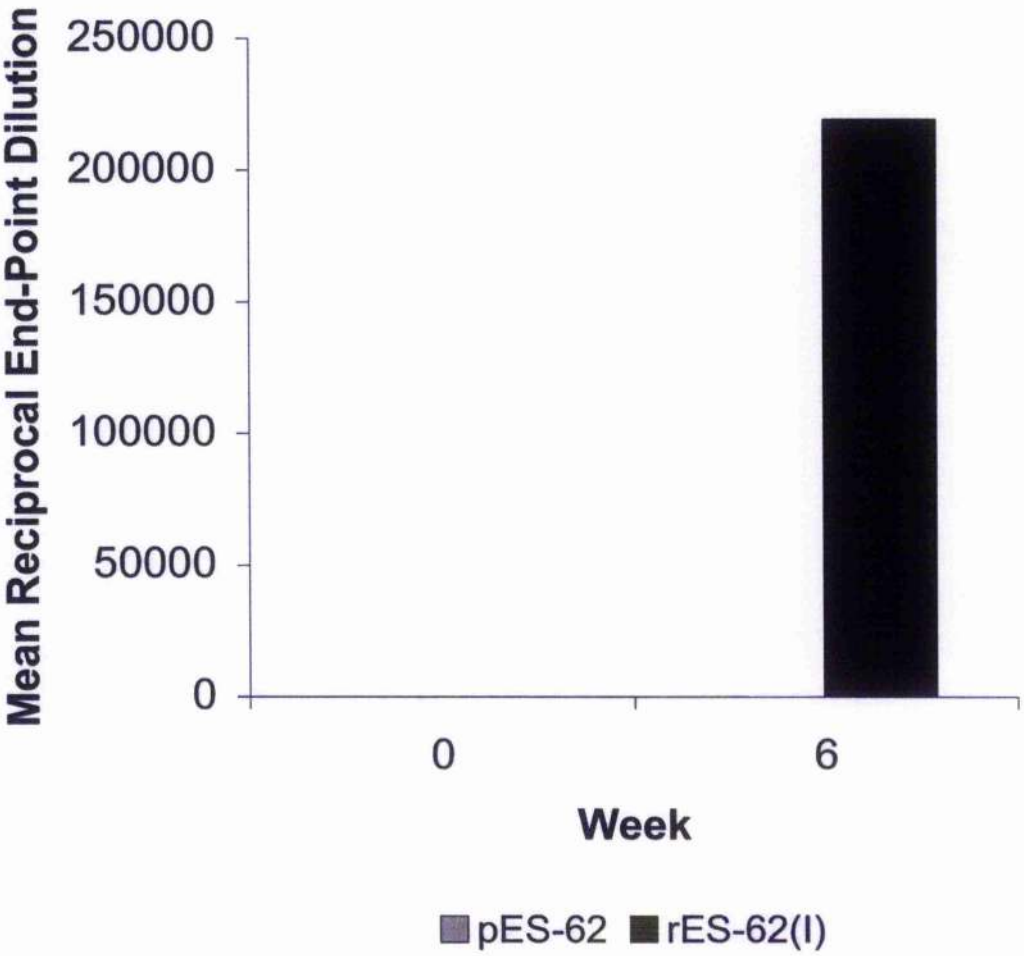

Mean Reciprocal End-Point Dilution
250000
200000
150000
100000
50000
0
0
6
Week
pES-62
rES-62(I)

Figure 5.7. Modulation of IL-12 Production by rES-62: WT C57BL/6 Mice

bmDCs from wild type C57BL/6 mice were pre-treated with 2 μg/ml rES-62 for 18 h prior to stimulation with 100 ng/ml *S. minnesota* LPS for 24 h. IL-12 p40 levels in culture supernatants were assayed by ELISA; data are presented as mean plus standard deviation of triplicate culture. ***$p<0.001$, **$p<0.01$. Data are representative of at least three experiments.

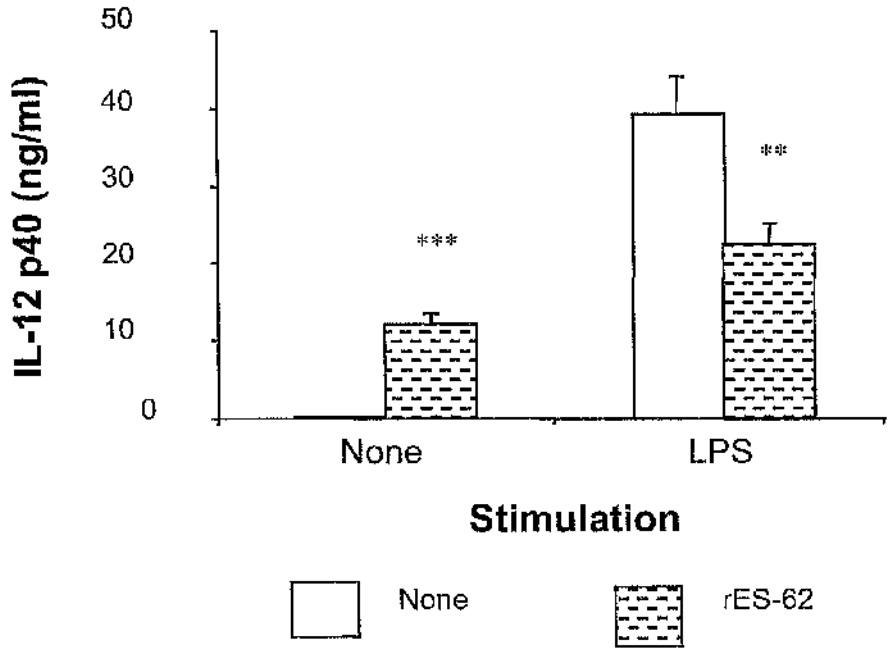

IL-12 p40 (ng/ml)
50
40
30
20
10
0
***
**
None
LPS
Stimulation
None
rES-62

Figure 5.8. Modulation of IL-12 Production by rES-62: TLR4 ko, MyD88 ko, C3H HeN and C3H HeJ Mice

bmDCs from (A) TLR4 ko, (B) MyD88 ko, (C) C3H/HeN and (D) C3H/HeJ mice were treated with 2 μg/ml rES-62 for 24 h. IL-12 p40 levels in culture supernatants were assayed by ELISA; data are presented as mean plus standard deviation of triplicate culture. ***$p<0.001$. Data are representative of at least three experiments.

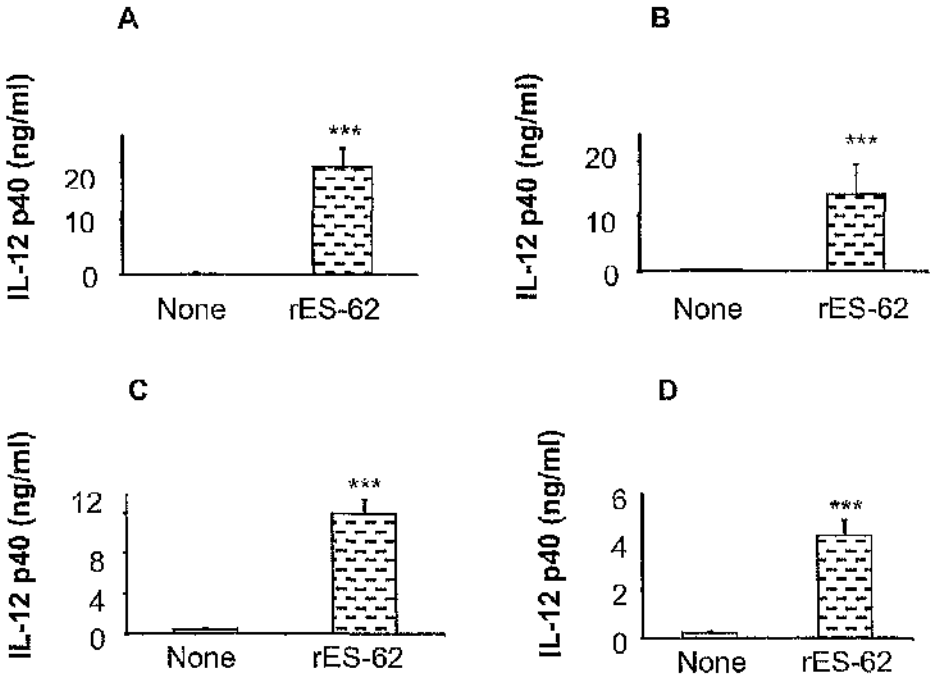

A
IL-12 p40 (ng/ml)
20
10
0
***
None
rES-62
B
IL-12 p40 (ng/ml)
20
10
0
***
None
rES-62
C
IL-12 p40 (ng/ml)
12
8
4
0
***
None
rES-62
D
IL-12 p40 (ng/ml)
6
4
2
0
***
None
rES-62

Figure 5.9. Investigation into the Involvement of TLR4 in Cytokine Production Stimulated by rES-62.

bmDCs from wild type C57BL/6 mice were treated with 2 μg/ml rES-62 or rES-62 glycosylation mutants for 24 h. IL-12 p40 levels in culture supernatants were assayed by ELISA; data are presented as mean plus standard deviation of triplicate culture. ***$p<0.001$. Data are representative of at least 2 experiments.

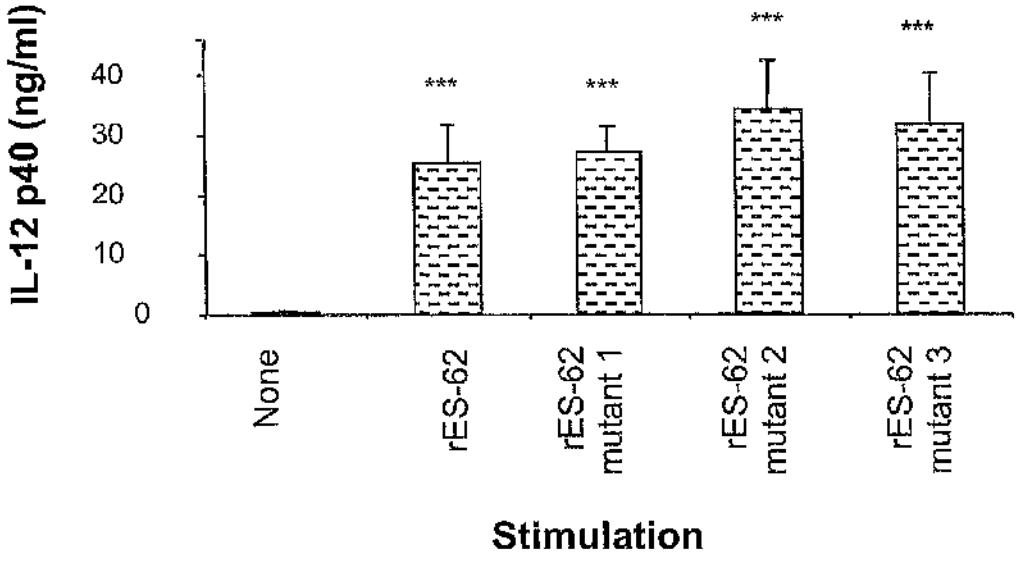
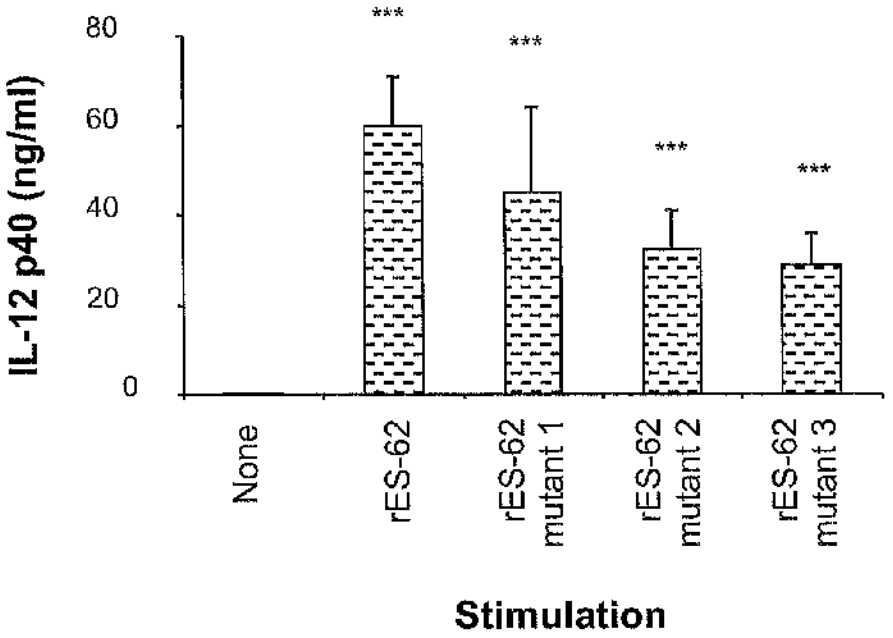

IL-12 p40 (ng/ml)
40
30
20
10
0
***
***
***
***
None
rES-62
rES-62 mutant 1
rES-62 mutant 2
rES-62 mutant 3
Stimulation

Figure 5.10. Investigation into the Involvement of the Mannose Receptor in Cytokine Production Stimulated by rES-62.

bmDCs from MR ko mice were treated with 2 μg/ml rES-62 or rES-62 glycosylation mutants for 24 h. IL-12 p40 levels in culture supernatants were assayed by ELISA; data are presented as mean plus standard deviation of triplicate culture. ***$p<0.001$. Data are representative of at least 2 experiments.


IL-12 p40 (ng/ml)
80
60
40
20
0
***
***
***
***
None
rES-62
rES-62 mutant 1
rES-62 mutant 2
rES-62 mutant 3
Stimulation

Chapter 6: Biophysical Characterisation of rES-62 from *Pichia pastoris*

6.1 INTRODUCTION

The data from the previous two chapters provided a body of evidence suggesting that the recombinant material was not simply a facsimile of ES-62 without the PC moiety. In fact, it was indicated that the molecule could be radically different from the pES-62, based on both biochemical and immunological characteristics. Attempts instigated within these studies to explain these differences had failed to offer any conclusive answers. It was therefore decided to send some material to collaborators at the University of Glasgow to enable a biophysical characterisation of the molecule in comparison to the characterisation carried out for the *A.viteae*-derived molecule (Ackerman *et al.*, 2003). This included far UV CD spectrum analysis, size distribution analysis, analytical centrifugation techniques and small angle scattering, to assess the secondary structure and folding of the recombinant, and to give an insight into the shape of the recombinant in solution.

6.2 RESULTS

6.2.1 Secondary Structure and Folding of Recombinant ES-62

There is a broad negative peak in the far UV CD spectrum of rES-62 (Fig. 6.1, solid line) with a minimum around 203nm and a positive maximum peak around 185nm (these peaks correspond to the n to π^*transition). The shape of the spectrum and the lack of a dominant negative minimum around 220nm suggest that the protein contains predominantly β-sheet, β-turn and unordered structure. Secondary structure estimations were obtained using the program CONTIN (Provencher and Glöckner, 1981) since other programs such as SELCON3 (Sreerama and Woody, 1993) and CDSSTR (Hennessey and Johnson, 1981) failed to fit the data. The CONTIN procedure (Provencher and Glöckner, 1981) gives secondary structure estimations of 10% α-helix, 39% β-sheet, 21% β-turn and 30% remainder. Comparing these results with the secondary structure estimations from the far UV CD spectrum analysis carried out on the parasite-derived ES-62 previously (Fig. 6.1, dashed line), which gave values of 40% α helix, 16% β-sheet, 16% β-turn and 28% remainder (Ackerman *et al.*, 2003) we can conclude that the proteins exhibit marked differences in conformation. It would appear that the recombinant material has refolded most of its α-helix structure to β-sheets/turns but retained the same amount of unordered structure.

The near UV CD spectrum shows that the aromatic residues are located in asymmetric environments characteristic of the tertiary structure of a folded protein. Tryptophan residues in folded proteins generally give peaks close to 290 nm; tyrosine and phenylalanine residues give rise to peaks between 275 and 282 nm and 255 nm and 270 nm respectively.

Recombinant ES-62 was examined for heterogeneity using c(s) size-distribution analysis of sedimentation velocity (SV) data (Schuck *et al.*, 2002). There were at least three peaks evident in the c(s) distribution pattern, with a major peak centred on an apparent sedimentation coefficient ($s^{app}_{20,w}$) of 5 S and two less well-defined peaks at 3 and 8 S (Fig. 6.2). Integration of the c(s) distribution for the three major peaks yielded the weight average sedimentation coefficient (s_w) for these species. The concentration dependence of s_w does not demonstrate the appearance of heavier species as concentration is increased but shows the expected concentration dependence of peak position: a movement towards lower s with increasing concentration (thermodynamic non-ideality). Therefore any redistribution between the rES-62 oligomeric species was not observed over the >10-fold concentration range examined, suggesting that the system is not in thermodynamic equilibrium, but instead comprises discrete independent oligomers.

Direct fitting of SV boundaries gave sedimentation coefficients at infinite dilution and under standard conditions ($s^0_{20,w}$) of 3.4, 5.3 and 8.2 S (Fig. 6.3) with good fits to the experimental data (not shown). The three species could

be monomeric, dimeric and tetrameric rES-62. In order to compare these findings with the results for parasite-derived ES-62 (Ackerman *et al.*, 2003), the value of the sedimentation coefficient for tetrameric parasite-derived ES-62 was deconvoluted to possible sedimentation coefficients for monomeric and dimeric species assuming that the oligomerisation occurs "side-to-side". The data in Table 6.1 show the sedimentation coefficients for each oligomer from the recombinant material and the "deconvoluted" s for parasite-derived ES-62. The fact that s for each oligomer of rES-62 is much less than that estimated for the parasite-derived counterpart implies rES-62 is more elongated/asymmetric than the parasite-derived protein.

The weight average molecular weight for rES-62 in solution was found at different rotation speeds in a sedimentation equilibrium experiment (data not shown). Thus, at a low rotation speed of 8000 rpm the weight average molecular mass (264 ± 50 kDa) was above the expected molecular mass for rES-62 tetramer (233 kDa) suggesting the presence of higher molecular weight aggregates. As the speed of rotation was increased the average molecular weight decreased reflecting a mixture of monomers and dimers (81 ± 13 kDa). The SE scans were treated using the equilibrium dissociation model, which did not give a significant improvement in fit to the data. Therefore we can conclude that these species are not undergoing equilibrium dissociation but exist independently, in agreement with the findings from SV experiments.

6.2.2 Small Angle Scattering

rES-62 scattered X-rays very well in solution, but significant differences between the parasite-derived and recombinant protein were observed. The overall shape of the rES-62 scattering curve is typical of that from very elongated particles (Volkov and Svergun, 2003). This is in contrast with parasite-derived ES-62, which is a rather globular particle (Ackerman *et al.*, 2003). Since the AUC data have demonstrated clearly that rES-62 is very heterogeneous in solution it is not possible to use existing software routines for the determination of a solution structure for this kind of sample. At present we can reach only qualitative conclusions about rES-62 in solution.

6.3 DISCUSSION

Far UV CD Spectrum analysis provides a much stronger signal than near UV CD spectrum analysis, meaning much less protein is necessary in the sample to obtain results. The amount of sample is also less than one would require for high resolution nuclear magnetic resonance (NMR) spectroscopy, which can only be carried out effectively with high concentrations of protein. The sample volume can be as low as 1 ml - 50 μl, and although 100-500 μg of protein would allow for a thorough examination of optimal conditions, etc. 10 μg is sufficient to generate credible data. Experiments can be carried out very quickly (30 min), in comparison with X-ray crystallography, which can take weeks or months in some incidences (reviewed by Kelly and Price, 2000). As the procedure is non-destructive, the material analysed can be recovered and re-analysed under different conditions.

Although UV CD Spectrum analysis is able to distinguish α-helices and β-sheets and turns, it is unable to determine their location in the molecule, and does not provide much data on quaternary structure. However, secondary structure algorithms can be applied to the data to increase its resolution (Ribas de Pouplana *et al.*, 1991). We employed sedimentation velocity and small angle scattering techniques to aid in the prediction of the 3D shape and self-association of the recombinant, to give a fuller picture of the molecule in solution. Near UV CD Spectrum analysis is dependant on the AA side chains, and can give tertiary folding information based on tryptophan,

tyrosine and phenylalanine (reviewed by Kelly and Price, 2000). It has been shown that factors such as aromatic side chains, especially tryptophan can contribute to the far UV CD spectrum (Woody and Dunker, 1996; Greenfield, 1999), as can disulfide bonds (Greenfield, 1999; Chaffotte, Guillou and Goldberg, 1992), and that the length and regularity of structural elements in peptides and proteins can influence the nature of the spectra produced (Hirst and Brooks, 1994). These elements can be overcome using the CONTIN procedure (Provencher and Glockner, 1981), where the curve obtained is compared with data from 16 proteins that have been analysed previously by X-ray Crystallography to provide extremely clear interpretations of structures. Further evidence for this was provided by Tetin, Prendergast and Venyaminov (2003), who found that secondary structure prediction from far UV CD spectra using modern algorithms are robust. Structures predicted did not appear to be affected by the contributions of aromatic AA side chains to the spectra when they compared the forecasts of shapes of three antibody F'ab fragments to the existing X-ray data in the Protein Data Bank.

The biophysical analyses we carried out were to shed light on why the two molecules were behaving so differently, when their biochemical and immunological attributes were compared in the previous two chapters.

It was indicated by far UV CD spectrum analysis that the recombinant material showed features of a tertiary protein structure, but that a significant portion of its secondary structure had been converted from α-helix to β-sheet. This will result in an overall change in shape for the recombinant protein from

the somewhat globular shape of pES-62 to a much more elongated molecule. This shift in shape was also indicated by small angle scattering and by sedimentation velocity experiments. It was pleasing that all three analyses agreed upon this change in shape, as this drastic alteration would most definitely impact on the lack of cross-reactivity seen between pES-62 and rES-62 and the antibodies raised against the two molecules, and possibly help to explain why denaturing the rES-62 did not affect its recognition by antibodies raised against the non-denatured rES-62 (Chapter 5). If the molecule did have this more linear shape, it would perhaps not alter as much upon denaturation as pES-62 due to the globular shape of the latter. The folding of proteins, as it is presently understood, is dependent on the side chains of the AAs in the backbone, which inform whether α-helices or β-sheets are formed, and thus inform the whole shape of the molecule (reviewed by Rose *et al.*, 2006). The α-helix is a more likely secondary structure for a protein to assume, given that the R groups of the AAs of the protein backbone face outwards in this conformation, and avoid disrupting the central helix core (Hart, Hart and Craine, 1995). In β-sheet structures, the R groups of AAs are oriented above and below the plane of the sheet alternately: as might be expected, this type of structure may be prevented from forming if there are numerous larger side chains on the AAs which form the backbone (due to repulsion from these groups), and so β-sheets are only important in proteins found to have a high percentage of small R groups (Hart, Hart and Craine, 1995). Another theory of protein folding has emerged

in the last ten years or so, suggesting that the protein backbone sequence itself limits the number of conformations possible for the protein, and that the final form of the molecule is dictated from this restricted range by the side-chains of the AAs in the backbone (Banavar *et al.*, 2004; Rose *et al.*, 2006). Neither the established nor the more recent theory of protein folding offer any explanation for why the recombinant version of ES-62 appears to have refolded from an α-helix to a β-sheet, as the gene sequence and therefore the peptide sequence of both molecules has been confirmed to be the same by full length sequencing. At present, one of the only possible factors that can be the cause of this shift in structure is the abundance of extra sugars attached to rES-62, as this is the main biochemical difference that has been observed. Although α-helices are the basis of fairly firm structures such as keratins and collagens, the rigidity of these structures is improved by many disulfide crosslinks (Hart, Hart and Craine, 1995). α-helix dominated globular proteins (such as pES-62), which are often enzymes, can be more sensitive to extremes of conditions such as pH and temperature. The β-sheet/turn structure of rES-62 in combination with its increased glycosylation may have raised the stability of the molecule, altering the conditions under which it would denature. It might also be folded in such a way that glycosidic bonds which would be accessible in the pES-62 are now sheltered, providing another possible explanation for the lack of sensitivity to enzymes seen in Chapter 4. The change in conformation of rES-62 may have led to a loss of exposed self-association sites, leaving it unable to form tetramers as pES-62

does. The sedimentation velocity experiments also suggested that the recombinant material was not in a state of thermodynamic equilibrium, monomerising and tetramerising as with pES-62. It was suggested that rES-62 was a mixture of discrete, independent monomeric, dimeric and tetrameric species, confirming what was initially observed when the molecules were subjected to non-reducing, non-denaturing PAGE (Fig. 4.1). This view was also supported by analysis of the sedimentation equilibrium results. A combination of increased rigidity, coupled with an inability to form the same quaternary structure as pES-62 could explain why it appears that the recombinant in solution displays no dynamic equilibrium between species. It would appear that any multimeric species are static, unable to interact with each other. These stable monomers and dimers could lack secondary structural features stabilised by tetramersiation, a self-interaction that may not be possible due to the highly increased amounts of glycans attached to the molecule. These discrete populations of species may also be explained by the change in secondary (and therefore tertiary) structure: it may be that the globular shape of pES-62 is important for fostering/allowing interactions between smaller units to form the tetramers characteristic of the parasite-derived molecule in solution (Harnett *et al.*, 1993; Ackermann *et al.*, 2003) and that the elongated shape of the rES-62 makes this more difficult to achieve, so that if these interactions are formed, they are more rigid in nature, and not amenable to equilibrium.

Table 6.1. Sedimentation coefficients for ES-62 extrapolated to zero concentration and corrected to standard conditions

	$s^0_{20,w}$ for ES-62 (S)	
Species	Recombinant protein	Estimated from the parasite-derived ES-62 (tetramer) (Ackerman *et al.*, 2003) assuming "side-to-side" oligomerisation
"Monomer"	3.4 ± 0.03	4.38
"Dimer"	5.3 ± 0.05	6.57
"Tetramer"	8.2 ± 0.15	9.85*

*Experimentally measured number

6.4 FIGURES

Figure 6.1. Circular Dichroism and Sedimentation Velocity Characterisation of rES-62: Far UV Circular Dichroism Spectrum

Far UV Circular dichroism spectrum for rES-62 (solid line) and parasite-derived ES-62 (dashed line) in phosphate buffer (91.5 mM Na_2HPO_4, 58.5 mM NaH_2PO_4, pH 7.4). The spectra were normalized for concentration.

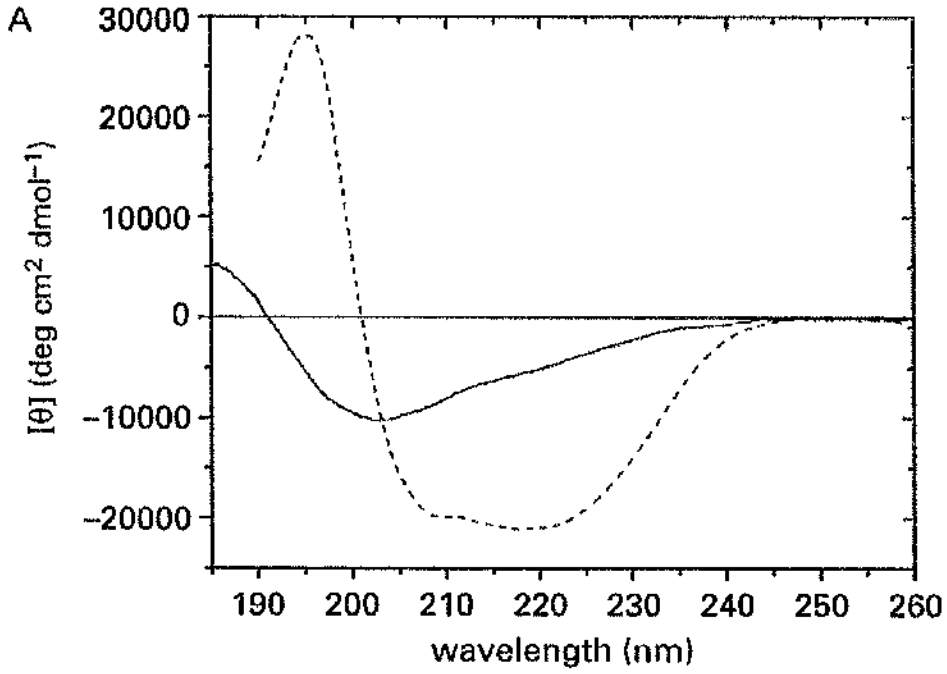

A
30000
20000
10000
0
−10000
−20000
[θ] (deg cm2 dmol−1)
190
200
210
220
230
240
250
260
wavelength (nm)

Figure 6.2. Circular Dichroism and Sedimentation Velocity Characterisation of rES-62: Size Distribution c(s)

Size distribution c(s); interference data obtained for monomeric loading concentrations of 165 μM (*solid line*) 123 μM (*dashed line*) 82 μM (*dotted line*) 41 μM (*dashed-dotted line*) 12 μM (*short dashed line*) 10 μM (*dashed-dotted-dotted line*).

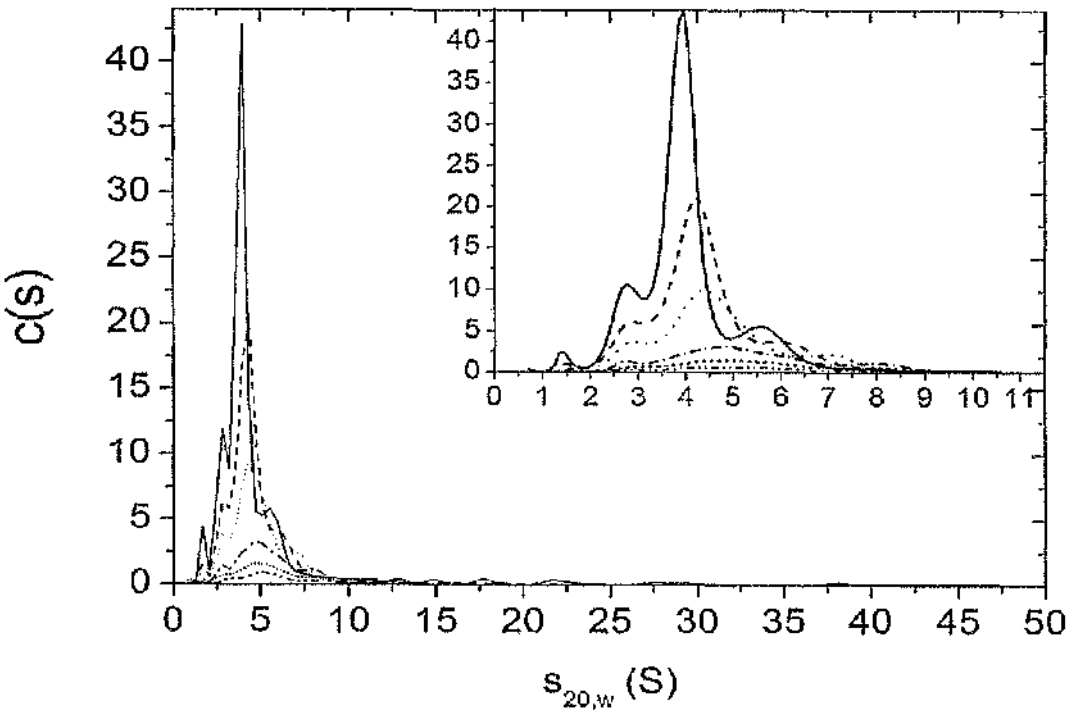
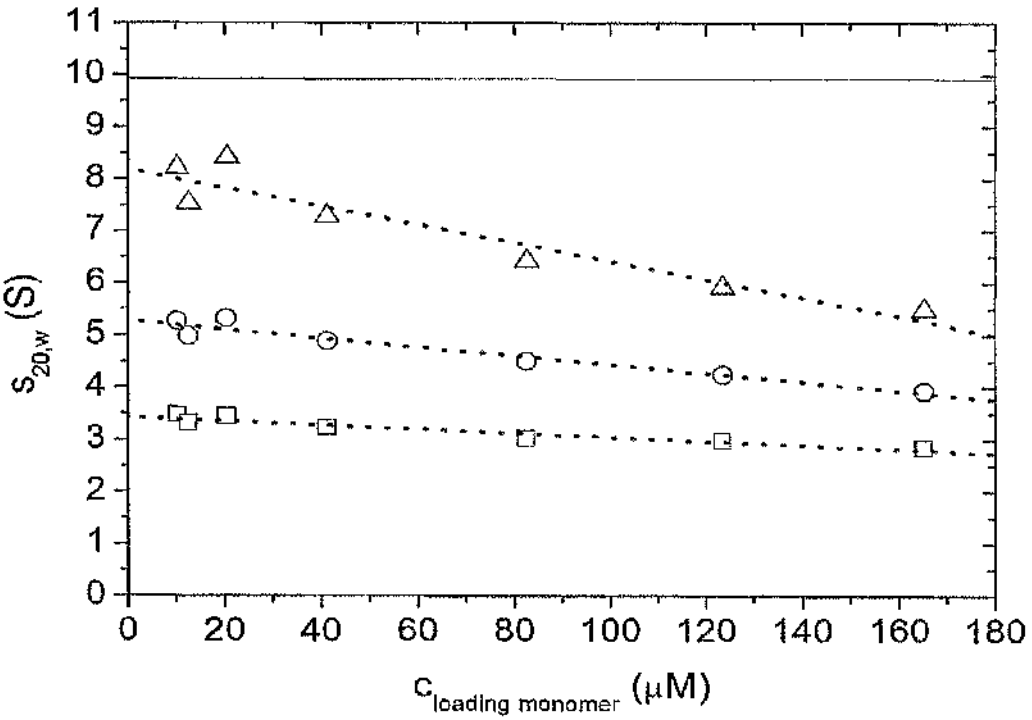

c(s)
$s_{20,w}$ (S)
0
5
10
15
20
25
30
35
40
45
50

Figure 6.3. Sedimentation Velocity Characterisation of rES-62.

The concentration dependence of s: "monomer" (squares), "dimer" (circles), "tetramer" (triangles). The horizontal solid line marks the sedimentation coefficient obtained experimentally for parasite-derived ES-62 (Ackerman *et al.* 2003); dotted lines are the linear extrapolation of $s_{20,w}$ to zero concentration for the three species.


11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
180
$s_{20,w}$ (S)
$c_{loading\ monomer}$ (μM)

Chapter 7: Discussion

Virtually all of the immunomodulatory properties of ES-62 appear be able to be mimicked by PC conjugated to other proteins. This might tempt one to ascribe all of these functions to the PC moiety. In order to fully establish the extent of the contribution of PC attached to ES-62, the logical approach would be to prepare material lacking PC and compare it to the original material. After many years of investigating various different enzymes and enzyme inhibitors in the quest to remove, or prevent attachment of, PC to ES-62, it has become apparent that this is, as is often the case, much more easily said than done.

7.1 PRODUCTION OF ES-62 LACKING PC

In this thesis, I examined two more enzyme inhibitors and four more enzymes to this end. Unfortunately, as with previous attempts, although these experiments provided us with more information, none yielded total removal of PC. It was for this reason that it was decided to insert the gene for ES-62 into a recombinant gene expression vector that would not contain the machinery necessary to add PC to secreted proteins. Previously, our group had investigated the possibilities of using the archetypal recombinant expression vector, *E.coli*, and a baculovirus system. However, despite best efforts, the material produced using both methods proved to be unusable experimentally, due to solubility problems in the *E.coli*-produced rES-62 and possible DNA contamination associated with the baculovirus system-

produced material (Ackerman, 2002 PhD Thesis). Due to these hindrances, the yeast protein expression system, *Pichia pastoris*, detailed in chapter four was decided upon to produce rES-62 lacking PC, and from first impressions, although the recombinant material appeared to have some structural differences, there appeared to be enough similarities between the two to consider rES-62 a serviceable PC-free version of ES-62.

7.2 EMERGING DIFFERENCES BETWEEN rES-62 AND pES-62

From the outset, rES-62 had appeared to differ from pES-62 biochemically, revealing a different pattern of bands on both native and SDS PAGE gels, and was impervious to digestion with *N*-glycosidase F, which has been shown to cleave pES-62 in the past. However, the recognition of the rES-62 by polyclonal- and in particular a monoclonal-antibody that recognised a conformational epitope, anti-pES-62 serum gave hope that the molecule was more or less correctly folded, suggesting the material to be usable as a PC-free version of ES-62.

However, as detailed in chapter 5, the more the properties of rES-62 versus pES-62 were explored, the wider the berth between the two seemed to grow. It was discovered that despite generating an almost exclusive Th2 response in BALB/c mice as pES-62 does, there was no cross-reactivity between antibodies raised against the rES-62 and pES-62. Probing of the involvement of TLR4 and MR in the recombinant's stimulation of cytokine production in

bmDCs revealed it to appear to be using quite a different mechanism of immunomodulation to pES-62. In light of this difference, the evidence showing the rES-62 to arrive at the same biological outcome as the parasite-derived material was quite confounding. The recombinant differed when compared to previous PC-free ES-62, as this latter material had produced an IgG2a response (Houston *et al.*, 2000), whilst the rES-62 did not produce such a response.

What had originally started as an investigation into the properties of PC-free ES-62 became an exploration of the unique properties of the recombinant material, and an attempt to explain why it differed so greatly from the parasite-derived protein.

7.3 POSSIBLE EXPLANATIONS FOR THE DIFFERENCES BETWEEN rES-62 AND pES-62 SEEN

7.3.1 Glycosylation and Processing of Proteins by Yeast

rES-62 has been shown to have much higher levels of glycosylation (detailed in chapter 4), in particular of mannosyl residues attached, and the results of the biophysical analyses, detailed in chapter 6 revealed that the recombinant was a completely different shape, both at quaternary and secondary structure levels. Self-association of the recombinant version was also

different, unearthing a plausible explanation for most of the biochemical and immunological differences observed in the course of the comparison.

The differences observed between rES-62 and pES-62 are maybe not so surprising, as although the gene encoding *A.viteae* ES-62 has been inserted into *Pichia*, and, as such, the AA sequence of the protein should remain identical, there are several other elements to take into consideration. Many events taking place after transcription and translation influence a protein's eventual structure and shape. Tertiary and quaternary structure are influenced by the interactions of the side-chains attached to AAs, which are in turn influenced by the charges on the side-chains. *Pichia*, being a yeast, is able to carry out post-translational modifications and protein folding necessary for production of a working eukaryotic protein. Once proteins reach the Golgi apparatus, yeast and higher eukaryotic glycosylation of proteins differ: yeasts do not trim glycans as far as higher eukaryotic cells (reviewed by Cereghino and Cregg, 2000). Most yeast-derived proteins are devoid of complex oligosaccharides comprising sialic acid, galactose (gal), fucose (fuc) and *N*-acetylgalactosamine (GalNAc) (Bretthauer and Castellino, 1999): *Kluyveromyces lactis* is the only yeast whose ability to add GlcNAc to its glycoprotein glycans has been proven, with few others being predicted to do so (reviewed by Gemmill and Trimble, 1999).

Although it was expected that the recombinant material would contain more mannose due to knowledge of the post-translational modifications favoured by the chosen expression vector (Grinna and Tschopp, 1989), it was

surprising to find the extent of the glycosylation of the recombinant material in comparison to that of the pES-62, as *Pichia* has been shown not to glycosylate proteins to the same degree as *S.cerevisiae* (Grinna and Tschopp, 1989; Tschopp *et al.*, 1987). The *N*-glycosylation site at AA 381 is thought to be unused in pES-62 (unpublished results), but this does not necessarily discount its use by the yeast, which would help to explain the increase in glycosylation.

7.3.2 Effects of Glycosylation on Protein Folding

As this difference between the extent and nature of the glycosylation of rES-62 compared to pES-62 has been revealed to be the main molecular discrepancy between the two proteins, one would imagine this to be the most likely explanation for the change in shape of the recombinant molecule.

The role of *N*-linked oligosaccharides in protein folding is somewhat unclear, and appears to be rather variable: many groups report that their presence is necessary for correct folding of many glycoproteins (Ronnett *et al.*, 1984; Slieker, Martensen and Lane, 1986; König, Ashwell and Hanover, 1988). It has also been reported, however, that the folding and secretion of numerous glycoproteins is completely independent of the sugar potions of the molecule (Breitfeld, Rup and Schwartz, 1984; George, Ruoho and Malbon, 1986; Van Koppen and Nathanson, 1990) as their removal has no impact on these functions. Throughout the literature, almost every imaginable scenario regarding *N*-glycans and folding in their absence from glycoproteins has

been reported. Misfolding of vesicular stomatitis virus (VSV) G protein was induced by abolition of the two *N*-glycosylation sites of the protein. This was rectified by the creation of two new *N*-glycosylation sites at different locations (Machamer and Rose, 1988a and 1988b). It has been shown in some cases that certain *N*-glycans on a protein are crucial for correct folding, whilst removal of others on the same protein has no consequence for folding (Dubé, Fisher and Powell, 1988 and Semenkovich *et al.*, 1990). In other cases, a reasonable portion of the protein is able to form correctly in the absence of the *N*-glycans, whereas in yet others all the *N*-glycans are required for correct folding (Hickman and Kornfeld, 1978; Taylor and Wall, 1988).

The issue of the general involvement of *N*-glycans in protein folding is clearly a contentious one, though in our case it may well be that the added glycans provided by *Pichia* has increased the rigidity of the recombinant molecule, leading to its different shape to the pES-62, and making it unable to self-associate in a dynamic fashion, and meaning the multimers it does form have increased resistance to denaturation and to enzyme digestion.

7.3.3 The Impact of Glycosylation on the Immune Response to rES-62 in Comparison to pES-62

The results of the immunisation study revealed differences in the sub-classes of antibody induced by the rES-62, and what was expected based on previous experiments employing PC-free ES-62 (Houston *et al.*, 2000). The

results also demonstrated that antibodies raised against rES-62 did not recognise pES-62, and antibodies produced against pES-62 failed to recognise rES-62. As the largest biochemical difference observed between the two species was related to the glycans attached to rES-62 (mentioned earlier), this would be the most likely explanation for these differences in immunogenicity. When possible explanations for the lack of cross-reactivity were explored by comparing recognition of denatured and non-denatured p and rES-62, recognition of denatured pES-62 was decreased in comparison to non-denatured material, whereas recognition of rES-62 was unaffected by denaturing. This suggested that either the rES-62 was not denatured as expected, or that the denaturation of rES-62 was not affecting recognition due to either the epitopes on rES-62 being carbohydrate in nature, or that the rES-62 was not folded in the same manner as the pES-62, and contained linear peptide epitopes which would not change with denaturation.

For a long time it was presumed that the protein components were the targets of the antibodies against glycoproteins, but over the years, a strong case for carbohydrate rather than protein epitopes dominating the immune responses to glycoproteins has been built up. Childs *et al.* (1984) demonstrated antigen cross-reactivity seen between glycoproteins of quite different function and polypeptide structure to be due to antibodies directed against the carbohydrate moieties: purified epidermal growth factor receptor was recognised by a panel of six monoclonal antibodies directed against different blood groups. The types of structure recognised were, in general,

based on Galβ1-3GlcNAc and Galβ1-4GlcNAc, and were more likely to be linked to the receptor via *N*-glycans, as there is no evidence of O-linked structures existing in epidermal growth factor receptor from the cell line used (A431) (Mayes and Waterfield, 1984; Childs *et al.*, 1984; Cummings, Soderquist and Carpenter, 1985). It has also previously been observed that in *Haemonchus contortus*-infected sheep, the main epitope recognised by IgE antibodies is the core α1,3 fucose on the glycans of the parasite (van Die *et al.*, 1999). The Fucα(1-3)GlcNAc moiety has emerged as a major antigen in glycoproteins found in plants (Ramirez-Soto and Poretz, 1991; Wilson *et al.*, 1998) as well as insects (Prenner *et al.*, 1992).

Langerhans cell (a type of DC) phenotype was shown to be altered, and migration of these cells from the skin was reduced by the phosphoglycan moiety of *Leishmania* Lipophosphoglycan (LPG) (Ponte-Sucre, Heise and Moll, 2001). Okano *et al.* (1999) demonstrated that SEA which had been treated with periodate (a treatment verified to alter the structures of the carbohydrates of SEA whilst leaving proteins intact (Velupillai *et al.*, 1997)) induced significantly less Th2-type cytokines than the native or the mock-treated SEA. Analysis of the DC-SIGN receptor revealed strong binding to glycoconjugates of *Helicobacter pylori, S.mansoni, M.tuberculosis* and *Leishmania mexicana* (Appelmelk *et al.*, 2003). This interaction may have implications for cytokine secretion and antigen presentation, and so could have a role in the longevity of these organisms in the host. Although it has previously been shown that the majority of the immunomodulatory effects of

ES-62 appear to centre round the PC moiety, it has been inferred that glycans also have the ability to drive a Th2 response (Tawill *et al.*, 2004). Thus, the extra glycans on rES-62 may have led to its increased stability (making it more difficult to denature, and so what was assumed to be denatured rES-62 coat in the immunological comparison may indeed not have been fully denatured). They may also have played a part themselves, not only in maintaining the Th2 skewed response to rES-62 in BALB/c mice, but also in constituting the main epitope recognised by antibodies directed against rES-62 in the serum of immunised mice. This would seem likely due to the huge increase in glycans attached to the recombinant in comparison to the parasite-derived material. Lastly, the glycans present appear to be the most likely explanation for the significant change in shape of the molecule, which would most definitely contribute to the lack of cross-reactivity seen between rES-62 and pES-62.

7.3.4 Differences in Shape and Self-association Between rES-62 and pES-62

rES-62's capacity to withstand digestion and heat suggests it to be a very robust protein indeed. Recently, the papaya Kunitz-type trypsin inhibitor has been found to be a β-sheet glycoprotein that is incredibly resistant to extremes of pH, strong chemical denaturants and to temperature (Azarkan *et al.*, 2006). It has also been shown to be highly stable against non-specific proteases, even after a 24 hr incubation period. The authors point to the carbohydrate chains and the β-sheet structure as the most likely cause of its

increased resistance to proteases when compared to a soybean trypsin inhibitor equivalent. As rES-62 also appears to have increased carbohydrate moieties in comparison to pES-62, and has changed shape to become mainly comprised of β-sheet structures, the improved stability of rES-62 in comparison to pES-62 might similarly be explained.

The lack of cross-reactivity seen between r and pES-62 in the ELISA study would appear to be linked to the significant changes in shape between the two molecules. In light of the radical changes in shape the recombinant material seems to have undergone, it is intriguing that the recombinant ES-62 is recognised by the mAb KK6. Obviously, in spite of the changes in structure this epitope is retained: perhaps it is present only on the tetrameric forms of rES-62.

Regardless of whether glycans do or do not influence correct folding of proteins, this does not account for our recombinant molecule refolding from α-helix to β-sheet and β-turn in the presence of such a density of glycans. As bulky side chains on AAs make it difficult for β-sheets to form (Hart, Hart and Craine, 1995), it is hard to imagine a protein shrouded in *N*-glycans refolding into this structure. The experts who carried out the biophysical analyses have pointed out that although these techniques give an insight into the most likely structure of the protein being investigated, they become less reliable when looking at multimeric proteins. The fact that the main species of rES-62 in solution appeared to be discretely dimeric in nature means the exact

increases in percentages of β-sheet and turn of rES-62 in comparison to pES-62 may not be as pronounced in reality.

In many cases where proteins switch from α-helix to β-sheet (e.g. proteins specifically engineered to do so) there is a trigger, such as metal ion binding (Pagel *et al.*, 2005) or pH change (Pagel *et al.*, 2006). Groß *et al.* (1999) found that they could alter the conformation of CspB-3 (a peptide representing a portion of the 5 β-stranded major cold shock protein of *Bacillus subtilis*) by altering only the relative acetonitrile concentration (this is required to solubilise the protein in solution). It was possible for the group to start with an unfolded protein at low concentrations, move to an α-helical structure, and then on to a β-sheet dominated form. Again, although of interest, this gives us no clues as to why rES-62 folding would differ so greatly from pES-62, as the buffer conditions both proteins are stored in are the same.

An intriguing theory for why the α-helices in rES-62 appear to have converted into a β-sheet and turns is that of glycation, also known as the Maillard reaction. Glycation is a post-translational process whereby sugars non-specifically and irreversibly bind to certain AA side chains (being arginine and lysine). Exposing proteins to high concentrations of sugar results in creation of a reversible Schiff's base, which can lead to covalent bonding e.g. the advanced glycation end products (AGE) peptides (Thomas *et al.*, 2004). Gomes *et al.*, (2006) describe glycation as "equivalent to a point mutation, exerting profound effects on protein structure stability and function". The

localised distortions caused by the products of glycation have the potential to affect the overall shape of the protein, e.g. a rise in β-structures, which can lead to aggregation (Obrenovich and Monnier, 2004). In recent years, glycation has been implicated in the complications associated with several diseases such as diabetes, Alzheimer's and even in aging. The pathology associated with these diseases seems dependent on the conversion of proteins from α-helices to β-sheets, which can, in turn, lead to aggregation (Gomes *et al.*, 2006; reviewed by Harding and Ganea 2006).

Given enough time, any reducing sugar will react with any protein, but some sugars and proteins are more reactive than others. Mannose is a reducing sugar that has been shown to be more reactive than glucose when incubated with proteins in PBS (Roscic and Horvat, 2006). Glucose is a fairly un-reactive sugar, which has led to researchers to speculate that this could be a contributing factor in why our metabolism has evolved around it (Bunn and Higgins, 1981). The increased glycolytic reactivity of both mannose and galactose compared to glucose became more pronounced when the investigators used methanol as a solvent and incubated proteins with mannose, glucose and galactose for 6 days at 37°C (Roscic and Horvat, 2006). Could it be that mannose utilised by *Pichia* to extend the glycan chains on rES-62 is glycating the recombinant molecule due to the favourable methanol environment required to induce production of the protein coded for by the inserted gene?

Although it may seem unlikely that the yeast would be unable to curb glycation of heterologous proteins, the properties of glycated proteins reported thus far certainly bear a resemblance to the data gathered on rES-62 during the experimental work presented here. Glycated proteins have been shown to be less sensitive to digestion with trypsin (Gomes *et al.*, 2006), and a paper published this year presents a body of evidence based on investigations into glycated haemoglobin (Hb) very similar to the observations regarding rES-62 contained within this thesis (Ghoshmoulick *et al.*, 2007). The authors discovered when they subjected glycated and non-glycated Hb to non reducing PAGE conditions, the glycated protein seemed to be of a lower molecular weight, which they ascertained could be due to a shift in the tetramer-dimer equilibrium to favour dimeric species. This paper also showed that lower levels of glycation appeared to coincide with the occurrence of a dimeric population when the protein was examined by SDS-PAGE, and that a higher level of glycation resulted in intensified protein fragmentation. If rES-62 were being glycated, this may help explain the evidence for discrete populations of monomeric, dimeric and tertrameric species, and thus why there is a lower proportion of tetrameric species in rES-62 compared to pES-62 in solution. Furthermore, when glycated and non-glycated Hb were subjected to SDS-PAGE, the group observed a pattern of bands corresponding to both monomeric and dimeric Hb. The dimeric species observed appeared to be cross-linked, and proved to be resistant to SDS, remarkably similar to the situation we ourselves have

outlined here, in relation to the recombinant version of ES-62. The paper goes on to present analyses of the CD spectra of glycated Hb using the CONTIN procedure, which demonstrate a decrease in the proportion of α-helical structure accompanied by a corresponding increase of random coil and β-sheet in comparison to non-glycated Hb. The emergence of cross-linked β-structures in a protein has previously been demonstrated to occur after a period of prolonged glycation (Bouma *et al.*, 2003), but in the case of Hb, the process appears to be happening in a relatively short period of time. The authors believe their work to be the first to reveal the β-sheets to be formed from previously α-helically structured regions of the protein.

In the case of rES-62, the experts cast doubt on the accuracy of the levels of β-sheet/turn predicted, due to the difficulty in achieving clear readings for multimeric proteins, and the level of unordered structure is forecast to remain roughly the same. Despite these incongruities, the results described recently for glycated Hb have an interesting parallel with the observations on rES-62 made during this investigation, suggesting a possible role for glycation in imparting the unusual properties of rES-62 which have been discovered. Thus, sugars cross-linking rES-62 to itself, which could, in turn, bend the backbone in such a way as to trigger the major structural changes seen in comparison to pES-62 could account for the recombinant's imperviousness to enzymes and to denaturation by heat and SDS. Finally, the authors also report that glycation can be subdued by shifting the tetramer-dimer equilibrium of the protein in solution to tetramer. Conversely, if the bias in

solution is on dimeric forms, the propensity for glycation to occur is higher. It is possible that the sugars that seem to be preventing rES-62 from assuming the tetrameric form characteristic of pES-62 are also increasing the likelihood of glycation occurring, leading to cross-linking and the distinct monomeric, dimeric and tetrameric species of rES-62 seen in solution.

If glycation were a factor in the radical change in shape of rES-62, this could also shed light on the ability of rES-62 to induce IL-12p40 in a TLR4-independent manner. A recent review suggests the receptor for AGEs (RAGE) could signal through NFκB, and via this, an inflammatory response in a similar manner to TLRs (Lin, 2006).

7.4 CONCLUDING REMARKS

This study provides a lesson relating to the use of recombinant parasite proteins in immunological studies. Clearly recognition by antibodies against the parasite-derived protein may be insufficient to allow the use of such recombinant proteins as immunomodulatory mimics of parasite-derived molecules, or indeed for use in vaccination. Further analysis with respect to conformation may be warranted to ensure that unexpected immunogenicity properties are not encountered.

7.5 FUTURE WORK

The future work that I propose be done involves a more concrete method of determining the exact structure of rES-62, possible involving NMR and/or X-ray crystallography. The data from these techniques would be time-consuming to collect, due to the amounts of protein required, and the length of time needed to use these techniques. Data may also prove to be difficult to interpret, bearing in mind the results of a recent investigation highlighting the interference of sugars on the resolution of protein structures encountered when using such traditional procedures (Khajehpour, Dashnau and Vanderkooi, 2006).

This thesis has helped to further define the pathways ES-62 and PC utilise to bring about their characteristic immunomodulatory phenotype. As mentioned earlier, novel dugs based on small molecule derivatives of ES-62 and/or PC, could be designed to target these signalling pathways and hopefully mimic the immunomodulatory properties of ES-62. The activity of such molecules could be compared with known results for pES-62 and PC-BSA/PC-Ova. By identifying and characterising which small ES-62/PC-like molecules were able to mimic pES-62's abilities regarding cytokine induction and inhibition in DCs, mϕ and mast cells for example, this would hopefully allow us to pinpoint the exact portions of PC responsible for these modulations of the immune response. This, in turn, could indicate possible candidate drugs for the treatment of the inflammatory diseases pES-62 has thus far been proven to

successfully modulate, such as rheumatoid arthritis (reviewed by Harnett, McInnes and Harnett, 2004).

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