Effect of antimalarial drugs and malaria pigment (β-haematin) on monocyte phagocytosis and GTP-cyclohydrolase 1 gene expression

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For
Jesus Christ, Gail Foley,
Mark, Kerry, Ros, Katie and Linton Dent
(There is no place like home.)
And in memory of Louise Roodt.

Preface

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, from March 2006 to June 2009, under the supervision of Professor J.P.D. Goldring.

These studies represent the original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.

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Bridgette Cumming Professor J.P.D. Goldring

Abstract

During the erythrocytic stage, the malaria parasite digests host cell haemoglobin into amino acids. Toxic haeme is released and is incorporated into an insoluble non-toxic crystal called haemozoin. Haemozoin is released into the blood stream along with the merozoites when the erythrocyte bursts and is phagocytosed by circulating monocytes and macrophages resident in tissues. Phagocytosed haemozoin impairs many functions of the monocytes, including antigen presentation and adhesion to T cells, differentiation and maturation to dendritic cells, erythropoiesis and thrombopoiesis, but stimulates the release of pro-inflammatory cytokines and activation of metalloproteinase 9 expression.

In response to interferon-γ secretion by T-helper cells subtype 1, monocytes secrete neopterin, which is used as a marker of a cell mediated immune response. Neopterin is an oxidation product of 7,8-dihydroneopterin, produced by the dephosphorylation of 7,8-dihydroneopterin triphosphate which results from the conversion of guanosine triphosphate that is catalysed by GTP-cyclohydrolase 1. Elevated plasma and urine neopterin levels have been detected in malaria infections and are associated with severe anaemia, respiratory distress, peak temperatures as well as fever- and parasite-clearance times. It has also been reported that monocytic U937 cells treated with *P. falciparum*-infected red blood cell lysate secrete elevated levels of neopterin.

Antimalarial drugs are known to modulate the functions of monocytes, including inhibition of cytokine release, changes in phospholipid metabolism, decrease in expression of cytoadherance receptors as well as TNF receptors and MHC Class I and II molecules, changes in the production of reactive oxygen and nitrogen intermediates, and decreased phagocytosis. However, the effects of antimalarial drugs on haemozoin phagocytosis and GTP-cyclohydrolase 1 mRNA expression by monocytes are unknown.

This study aimed to determine the effects of seven antimalarial drugs, amodiaquine, artemisinin, chloroquine, doxycycline, primaquine, pyrimethamine and quinine, on the phagocytosis of latex beads and β-haematin, a synthetic equivalent of haemozoin. Phagocytosis of β-haematin and latex beads by two monocytic cell lines, J774A.1 and U937, as well as peripheral blood mononuclear cells were monitored by enumeration and a novel

spectrophotometric method. Patterns of inhibition and activation differed with each cell type investigated, due to the differing stages of cell differentiation. In general, artemisinin, primaquine, pyrimethamine and quinine activated the phagocytosis of β-haematin, whereas amodiaquine and chloroquine inhibited β-haematin phagocytosis. Doxycycline had different effects on each cell type investigated. Artemisinin, chloroquine, primaquine and quinine inhibited latex bead phagocytosis. The remaining drugs had minimal effects on latex bead phagocytosis. Thus, the effects of antimalarial drugs on monocyte phagocytosis appear to be dependent on the substance being phagocytosed.

The effects of antimalarial drugs, β-haematin, latex beads, non-infected- and *P. falciparum*-infected cell lysates on interferon-γ-induced neopterin secretion by U937 cells was monitored by GTP-cyclohydrolase 1 mRNA expression using quantitative PCR. Artemisinin, primaquine and quinine down-regulated the interferon-γ-induced expression of GTP-cyclohydrolase 1 mRNA, but by no greater than 1.7-fold. β-haematin up-regulated mRNA expression by 1.2-fold whereas *P. falciparum*-infected red blood cell lysate down-regulated the mRNA expression of GTP-cyclohydrolase 1 by 1.6-fold.

Quinine and artemisinin, currently used to treat malaria, increased β-haematin phagocytosis suggesting that quinine and artemisinin might promote increased phagocytosis of infected red blood cells and enhance clearance of the parasite from circulation. Increased β-haematin phagocytosis also reduces ICAM-1 expression on the monocyte surface, thereby leading to reduced cytoadherance and sequestration, thus increasing the number of circulating monocytes to phagocytose infected red blood cells. Down regulation of GTP-cyclohydrolase 1 mRNA expression by quinine and artemisinin suggested that the drugs reduce the responsiveness of the monocyte to interferon-γ. Thus, quinine and artemisinin might also decrease the production of interferon-γ-induced proinflammatory cytokines by monocytes, and potentially play a role in maintaining the balance between the pro- and anti-inflammatory cytokines that determines the progression from acute to severe malaria. Therefore, in addition to the drug's ability to kill the malaria parasite, the immunomodulatory effects of the antimalarial drugs may play a role in controlling the pathophysiology associated with the malaria infection.

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Abbreviations

ABTS	2,2’-azino-di-(3-ethylbenzthiozoline-6-sulfonic acid)
A_{405}	Absorbance at 405 nm
A_{280}	Absorbance at 280 nm
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CTL	cytotoxic T lymphocytes
DAB	3,3’-diaminobenzidine
ddH_2O	distilled, deionised water
dH_2O	distilled water
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethyl sulphoxide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
ESEM	Environmental Scanning Electron Microscope
dNTP	deoxynucleotide triphosphate
DPX	distrene dibutyl phthalate xylene
EDTA	ethylenediaminetetraacetic acid, disodium salt
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
Fe(III)PPIX	ferriprotoporphyrin IX
g	relative centrifugal force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine triphosphate
GTP-CH1	GTP-cyclohydrolase 1
h	hour(s)
HBSS	Hank’s Balanced Salt Solution
HRP	histidine rich protein

HRPO	horse radish peroxidase
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
IgG	immunoglobulin G
IgY	immunoglobulin Y
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide(s)
MHC	major histocompatibility complex
min	minute(s)
MIF	migration inhibitory factor
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
NBT	4-nitro blue tetrazolium chloride
NK cells	natural killer cells
NO	nitric oxide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PEG	polyethylene glycol
Pf-RBC	*Plasmodium falciparum*-infected red blood cells
PMA	phorbol-12-myristate-13-acetate
RBC	red blood cell
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)
SDS	sodium dodecyl sulphate
SEM	scanning electron micrograph
TEM	transmission electron micrograph
TGF-β	transforming growth factor-β
Th 1	T-helper cell subtype 1

Th 2	T-helper cell subtype 2
TNF-α	tumour necrosis factor-α
U	unit of enzyme activity

Chapter 1

Introduction

1.1 Malaria

An estimated 3.3 billion people were at risk of contracting malaria in 2006. Of this total, 1.2 billion living in African tropical/subtropical regions and south-east Asia were at high risk (WHO, 2008). Refer to Figure 1.1. In 2008, 109 countries were endemic for malaria, 45 of which are in Africa. There were an estimated 247 million clinical cases of malaria in 2006, 86% of which were in Africa, and an estimated 881 000 malaria deaths in 2006, of which 91% were in Africa and 85% were children under the age of 5 (WHO, 2008).

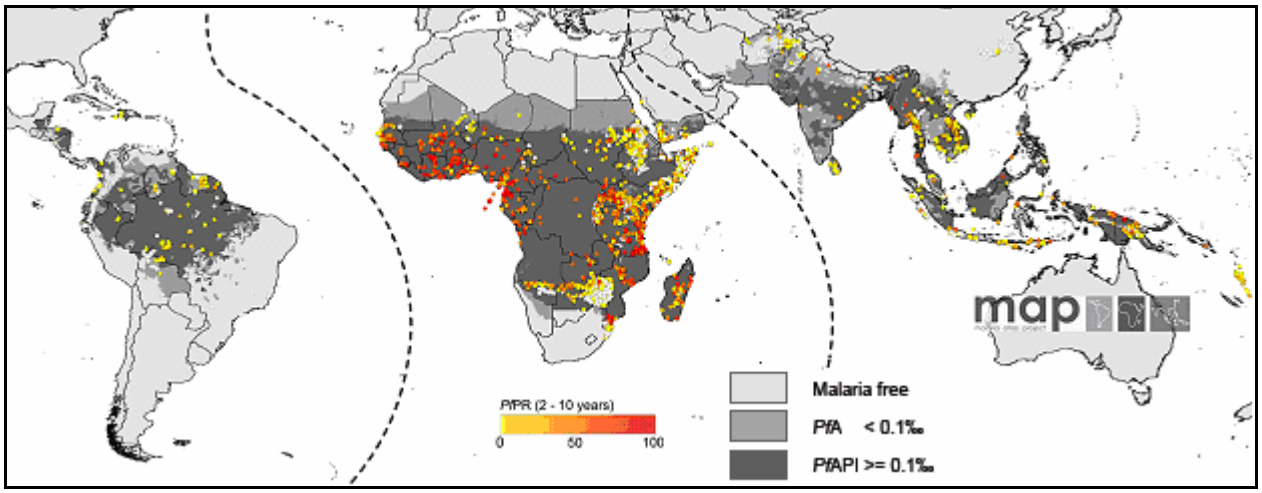


Figure 1.1. World Map of *Plasmodium falciparum* malaria risk in 2007. Dark grey areas represent areas of stable risk, where the *P. falciparum* annual parasite incidence (*Pf*API) ≥ 0.1 per 1000 per annum, medium grey areas represent areas of unstable risk (*Pf*API < 0.1 per 1000 people per annum) and light grey areas represent areas of no risk (*Pf*API = 0 per 1000 per annum). Community surveys of *P. falciparum* prevalence were conducted between 1st January 1985 and 31st July 2008 and are illustrated as the *P. falciparum* parasite rate (*Pf*PR) in the 2- up to 10-year age group (*Pf*PR (2-10 years)) as a continuum of yellow to red from 0% to 100% (Hay *et al.*, 2009).

In South Africa, approximately 4.3 million people living in the border areas of Limpopo Province, Mpumalanga, and the north east of KwaZulu-Natal are at risk of contracting malaria (Blumberg and Frean, 2007). In 2006, there were 12 098 reported malaria cases and 87 reported malaria deaths in South Africa (WHO, 2008). There has been a decrease in the number of reported

cases of malaria from 64 622 in 1999 to 12 098 in 2006 due to indoor residual spraying of dichlorodiphenyltrichloroethane (DDT), introduction of artemisinin-based combination therapy and changes in weather patterns (Blumberg and Frean, 2007; Mabaso *et al.*, 2004). Following several years of drought, heavy rainfalls were experienced in 2000 that increased the number of breeding habitats for *Anopheles funestus* [(http://www.doh.gov.za/facts/stats-notes/2004/malaria.htm) (last accessed: 16-04-2009)]. Reported malaria deaths in South Africa decreased from 406 in 1999 to 87 in 2006 (WHO, 2008).

Malaria is caused by an apicomplexan protozoan parasite, *Plasmodium*, of which there are 4 species which affect humans, namely, *Plasmodium vivax, P. ovale, P. malariae* and *P. falciparum* (Garnham, 1988). Most of the infections (91%) were due to *P. falciparum* (WHO, 2008). Recently cases of *P. knowlesi* infections, a malaria disease infecting monkeys, have been reported in humans (Cox-Singh *et al.*, 2008; Galinski and Barnwell, 2009; Jongwutiwes *et al.*, 2004; Luchavez *et al.*, 2008; Ng *et al.*, 2008). *P. falciparum* causes the most lethal human malaria as it results in cerebral malaria and anaemia if it goes untreated and is responsible for most of the malaria deaths in children under the age of 5 and pregnant women (WHO, 2008). In 2004, *P. falciparum* was among the leading causes of death world wide from a single infectious agent [(www.who.int/healthinfo/bodestimates/en/index.html) (last accessed: 10-05-09)]. The clinical manifestations of malaria vary from acute to chronic: from a simple fever to life-threatening multiple organ failure depending on the species of parasite involved, and the immune status of the patient (Van den Ende and Van Gompel, 1997). The host's immune system has considerable difficulties in controlling malarial infection and hence results in immunological inappropriate responses that have no effect on the parasite but may have immunopathological consequences of varying degrees of severity. The major immunopathological syndromes encountered are severe malaria, which includes cerebral malaria, severe anaemia, metabolic acidosis or a combination of these, hyperreactive malarial splenomegaly, quartan malarial nephropathy and Burkitt's lymphoma in areas endemic with Epstein Barr Virus (Hommel, 1997).

The efficacy of antimalarial drugs and insecticides in controlling malaria outbreaks is decreasing with increasing resistance of the malaria parasites and their vectors (Barnes and White, 2005; Casimiro *et al.*, 2006; Greenwood and Mutabingwa, 2002; Pearce *et al.*, 2009; Pongtavornpinyo *et al.*, 2008; Santolamazza *et al.*, 2008; Wellems and Plowe, 2001; Wondji *et al.*, 2009). Although there are many potential antigen candidates for vaccines, the parasite's ability to

evade and disable the immune system has confounded the development of vaccines (Pierce and Miller, 2009). For this reason, it is essential to study the biochemical and immunomodulatory interactions between the parasite and host in order to design new antimalarial drugs and discover means whereby the immune system can be modulated to control malaria infection.

1.1.1 Life-Cycle of the human malaria parasite

All the human *Plasmodium* species have the same stages in their life cycles. They have two hosts: the sexual phase occurs in the *Anopheles* female mosquito, and the asexual phase occurs in man. During the bite and feeding of an anopheline mosquito, the haploid sporozoites enter the bloodstream of the human (Figure 1.2). The sporozoites invade the liver cells and undergo a period of growth (G1 phase of the cell cycle), followed by several replications of the genome and mitotic divisions (S/M phase) resulting in the production of >20 000 merozoites (hepatic schizogony) which are released from the liver cell into the bloodstream where they invade erythrocytes. In the erythrocytes, the parasites develop from rings to trophozoites and undergo several mitotic divisions during the schizont stage (erythrocytic schizogony) to produce 16-32 new merozoites. The number of merozoites produced and the duration of the erythrocytic cycle is species specific. The merozoites are released from the burst erythrocyte into the bloodstream to infect naïve erythrocytes and continue the infection (Garnham, 1988). This asexual development of the parasites in the erythrocytes causes the pathology of the disease.

During the blood stage, a small percentage of trophozoites develop into male or female gametocytes which are arrested in the G0 phase. The gametocytes remain in the human bloodstream until they are ingested as part of a blood meal of the female anopheline mosquito. In the midgut of the mosquito, the gametocytes are activated to produce gametes, and, after fertilization, form a diploid zygote. Following one round of meiotic division, the zygote develops into a motile ookinete that attaches to the gut wall. It penetrates and traverses the gut wall and becomes an oocyst in the basolateral lamina. After growth and several mitotic divisions, > 10 000 sporozoites develop in a single oocyst (sporogony). When the oocyst has matured, it bursts and releases the motile sporozoites that migrate through the haemolymph to the salivary gland. Then when the female anopheline mosquito takes its next blood meal, the sporozoites are injected into the human bloodstream with an anticoagulant and the cycle is

continued.(Garnham, 1988; Kooij *et al.*, 2006). This study is concerned with the pathogenic asexual erythrocytic cycle occurring in man.

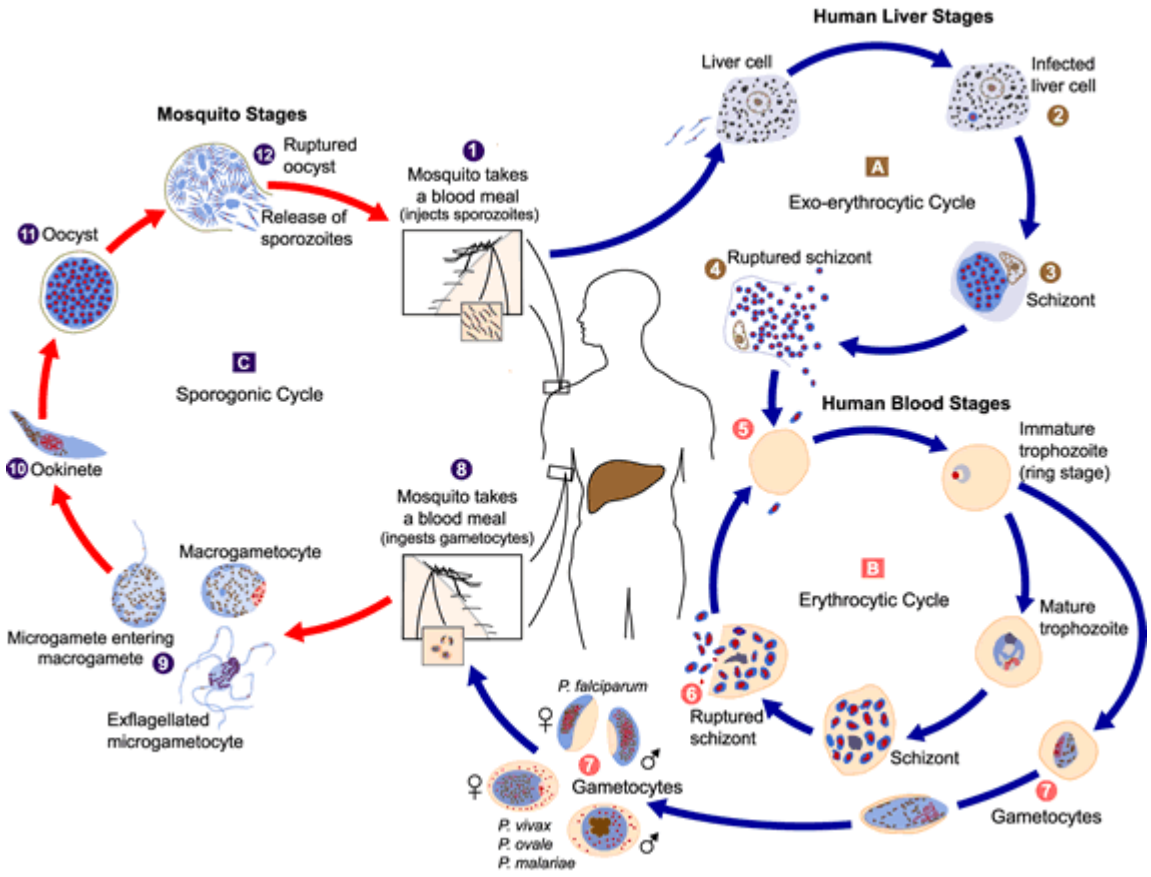


Figure 1.2. Life cycle of the human malaria parasite. When the *Anopheles* female mosquito bites a human, sporozoites enter the bloodstream (1) and invade the liver where they mature into schizonts, replicate asexually, (Exo-erythrocytic cycle - A) and form merozoites. The merozoites are released into the bloodstream (4) were they invade erythrocytes (5) and develop from rings to trophozoites to schizonts, which undergo mitotic division to form merozoites (Erythrocytic cycle - B). The merozoites are released into the blood steam (6) where they invade naïve erythrocytes. A small percentage of the trophozoites develop into male and female gametocytes (7) which are ingested by the mosquito during a blood meal (8). In the mosquito midgut, the parasite undergoes a sexual phase where the gametes fuse to form a zygote (9) which develops into mature ookinetes (10) that traverse the midgut epithelium and become oocysts (11). The oocyst ruptures (12) and a fraction of the released sporozoites end up in the salivary glands. Innoculation of the sporozoites into the human's blood stream perpetuates the cycle. This study is concerned with the erythrocytic (asexual) cycle. [(http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm) (last accessed 22-05-09)]

1.2 The immune response of monocytes to malaria

The first encounter of the host with the parasite is represented by the sporozoite/liver stage, while the rupture of the infected erythrocytes during the erythrocyctic cycle produces the clinical symptoms of malaria. Sporozoites are rapidly processed by the host hepatocytes and antigens are presented on the surface of the infected hepatocyte in combination with major histocompatibility complex (MHC) 1 (Weiss *et al.*, 1990). The antigens are recognised by cytotoxic T lymphocytes (CTLs) which can kill the infected hepatocyte or the antigens can stimulate natural killer (NK) and CD4+ T-cells to produce interferon-γ (IFN-γ). Upon antigen recognition, the CD4+ T-cell develops into a T-helper cell subtype 1 (Th 1) which produces IFN-γ. IFN-γ promotes the microbiocidal activity of the macrophage by stimulating the production of reactive nitrogen (RNI) and reactive oxygen (ROI) intermediates and it promotes production of tumour necrosis factor-α (TNF-α), interleukin (IL)-12, and IL-18 by the macrophage (Wang *et al.*, 1996). These immune reactions do not always occur because the small number of sporozoites and infected liver cells are not readily detected by the immune system (Pierce and Miller, 2009), and the helper T-cell epitopes on the circumsporozoite protein of the liver stage are known to be polymorphic (Good *et al.*, 1988).

The merozoite invades the erythrocyte by a parasite-driven active invasion process. The parasites modify the infected erythrocytes by expressing parasite proteins on the cell surface, such as *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) (Baruch *et al.*, 1995; Flick and Chen, 2004). At the time of erythrocyte rupture, parasite antigens, including merozoites, are released into the blood stream (Snounou *et al.*, 2000). Antigenic variation of merozoite surface proteins, such as PfMSP 1, 2, 3, and proteins on the red blood cell surface, like PfEMP1, enables merozoites and parasitized erythrocytes to avoid antibodies, continue the infection and stimulate the immune system (Hisaeda *et al.*, 2005). The immune response to the plasmodium parasite results in clinical and pathogenic manifestations that are due to the proinflammatory cytokines, including TNF-α, released by T-cells and macrophages in response to the malaria parasites and their products, such as glycosylphosphatidylinositol (GPI) moieties (Krishnegowda *et al.*, 2005; Schofield and Hackett, 1993; Zhu *et al.*, 2005), malaria pigment (Pichyangkul *et al.*, 1994), and plasmodium-derived nitric oxide synthase (NOS)-inducing factor (Ghigo *et al.*, 1995).

1.2.1 Haemozoin formation by malaria parasites

During the erythrocytic stage, *Plasmodium falciparum* ingests between 60 and 80% of the haemoglobin in the red blood cell by pinocytosis via a cytostome (Francis *et al.*, 1997). The haemoglobin is transported in a transport vesicle to the parasite's digestive vacuole where it is digested by proteolytic enzymes, including plasmepsins I, II and IV (Banerjee *et al.*, 2002), histoaspartic protease (HAP), falcipains 2 and 3 (Rosenthal *et al.*, 2002) and falsilysin (Eggleson *et al.*, 1999). The short peptides generated are most probably broken down to amino acids in the parasite cytoplasm by aminopeptidases (Gavigan *et al.*, 2001). Refer to Figure 1.4. About 15% of the amino acids generated are used as a food source for the parasite (Krugliak *et al.*, 2002). Digestion of haemoglobin might also be required to reduce the colloid-osmotic pressure within the erythrocyte to prevent its premature lysis (Esposito *et al.*, 2008).

During haemoglobin digestion, the haeme in haemoglobin is released into the digestive vacuole. The iron is oxidised from iron(II) to iron(III) by auto-oxidation, but the resulting ferriprotoporphyrin IX causes lipid peroxidation (Vincent, 1989) and destabilizes membranes though a colloid osmotic mechanism (Chou and Fitch, 1981). The parasite has developed an autocatalytic detoxification process whereby it assembles the ferriprotoporphyrin IX into a compact highly insoluble haemozoin crystal (Dorn *et al.*, 1995) and thus decreases the pro-oxidant capacity of the ferriprotoporphyrin IX (Oliveira *et al.*, 2002). The parasite does not contain haeme oxygenase (Eckman *et al.*, 1977), which is found in mammals to degrade ferriprotoporphyrin IX. If the parasite did have this enzyme, it would have to sequester vast quantities of free iron(III) which is also highly toxic. In higher organisms, these large quantities of iron(III) are managed by specialized transport and binding proteins (Baker *et al.*, 2003).

1.2.2 The structure of haemozoin

Through the use of infrared and X-ray absorption spectroscopy, the intermolecular bond between the ferriprotoporphyrin IX molecules in haemozoin was found to be an iron(III)-carboxylate coordinate bond between the central ferric iron of one molecule and the propionic carboxylate side chain of a second molecule (Slater *et al.*, 1991). It was initially hypothesized that haemozoin was comprised of long chains of polymerized ferriprotoporphyrin IX polymers that were joined by hydrogen bonding (Slater and Cerami, 1992; Slater *et al.*, 1991). In 2000, Pagola and Bohle demonstrated using X-ray powder diffraction analysis that haemozoin is made up of unit head-to-tail ferriprotoporphyrin IX dimers where each dimer consists of two

ferriprotoporphyrin IX molecules linked covalently through reciprocal iron-carboxylate coordinate bonding (Pagola *et al.*, 2000). These dimers are integrated into the crystal lattice of haemozoin by hydrogen bonding between the second protonated propionic acid groups of each ferriprotoporphyrin IX moiety to a dimer in the neighbouring unit cell (Bohle *et al.*, 2002a; Egan, 2008b), see Figure 1.3. The unit cell of the ferriprotoporphyrin IX dimer is approximately 1 nm^3 (Pagola *et al.*, 2000).

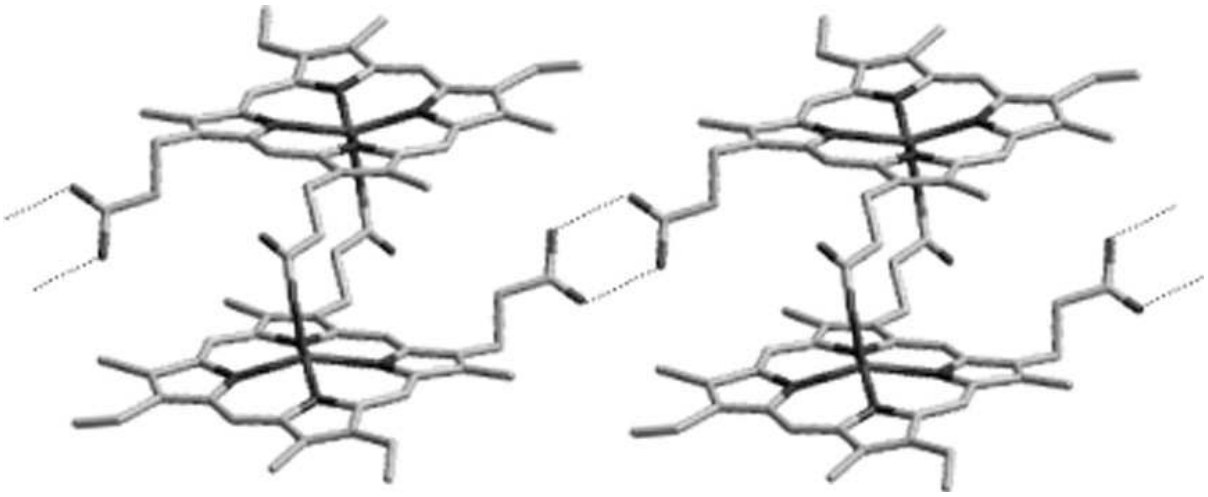


Figure 1.3. Structure of haemozoin/β-haematin. The dimers are formed by an iron carboxylate bond between the central iron of one ferriprotoporphyrin IX molecule and the propionic carboxylate side chain of the second ferriprotoporphyrin IX molecule. The dimers are then assembled into the crystal lattice by hydrogen bonding between the second protonated propionic acid side groups of neighbouring dimers. Dotted lines represent hydrogen bonds. (Egan, 2008b).

The morphology of the haemozoin crystals isolated from human malaria parasites is brick-like with dimensions of approximately 100 nm x 100 nm x 500 nm. Haemozoin crystals isolated from murine *Plasmodium* are slightly smaller (Noland *et al.*, 2003). β-haematin, the synthetic equivalent of haemozoin, can be prepared in several ways, but these can be divided into two alternative procedures: (1) the acid-catalyzed method, which proceeds in aqueous media under acidic conditions, often at elevated temperatures (Bohle *et al.*, 2002b; Egan *et al.*, 1994; Slater *et al.*, 1991); (2) the anhydrous method, which is performed under strictly anhydrous conditions in methanol at room temperature (Bohle and Helms, 1993). β-haematin crystals have a tapered needle like morphology that has been attributed to rapid growth at the faces (Solomonov *et al.*,

2007). Refer to Figure 2.5A and 2.6A which are scanning electron micrographs of β-haematin crystals.

1.2.3 The mechanism of haemozoin formation

The formation of the haemozoin crystal from aqueous ferriprotoporphyrin IX in the acidic vacuolar mileu has been the subject of much debate (Arese and Schwarzer, 1997; Egan, 2002; O'Neill *et al.*, 1998; Slater, 1993; Snounou *et al.*, 2000). The catalyst in the biologic protein/lipid aqueous solution has not been fully resolved. The role of parasite histidine rich protein (HRP) (Choi *et al.*, 1999; Schneider and Marletta, 2005; Sullivan *et al.*, 1996b) in haemozoin formation is being replaced by lipid initiation (Correa Soares *et al.*, 2007; Egan *et al.*, 2006; Fitch *et al.*, 1999; Tripathi *et al.*, 2002). It should be noted that *P. falciparum* clones lacking both HRPII and III, along with *P. vivax* and the murine *Plasmodium* species which lack orthologues to HRPII and III, all produce haemozoin (Sullivan, 2002).

Polar lipid initiation for the formation of haemozoin was initially proposed by Bendrat *et al.* (1995) and this was supported by Dorn *et al.* (1995, 1998a). A role for neutral lipids in the initiation of crystal formation was proposed by Fitch *et al.* (1999). In the Fitch study, the lysate of *P. falciparum*-infected erythrocytes, which supported the formation of β-haematin, was extracted with chloroform and this extract induced the formation of β-haematin whereas the residue remaining after the chloroform extraction was shown to be completely inactive. Analysis indicated that only lipids characteristic of biological membranes and trace amounts of fatty acids were present in the chloroform extract (Fitch *et al.*, 1999). Tripathi *et al.*, (2002) and Pandey *et al.*, (2003) suggested that a combination of lipids and proteins were involved in the initiation of haemozoin formation. Jackson *et al.*, (2004) demonstrated the presence of neutral lipid bodies closely associated with the digestive vacuole of *P. falciparum* using Nile Red labelling and spectral imaging on a confocal microscope. These lipid bodies were found to contain predominantly mono- and di-acylglycerols and the authors showed that a series of mono-, di- and tri-acylglycerols promoted β-haematin formation much more efficiently than HRPII (Jackson *et al.*, 2004). Vielemeyer *et al.*, (2004) also demonstrated that neutral lipids synthesized by the parasite were stored as lipid bodies in the parasite cytoplasm. The presence of triacylglycerol containing lipid bodies in *P. falciparum* was demonstrated by Palacpac *et al.*, (2004a) and the enzyme catalyzing the synthesis of triacylglycerols was shown to be essential for intraerythrocytic proliferation of the parasite (Palacpac *et al.*, 2004b).

When malaria parasites were stained with malachite green, which is used to fix neutral lipids, the resulting transmission electron micrographs showed the haemozoin embedded within a lipid body (Coppens and Vielemeyer, 2005) or lipid nanosphere as referred to by Pisciotta *et al.*, (2007). This indicated that haemozoin formed within lipid bodies in the parasite's digestive vacuole. Haemozoin together with these lipid bodies were isolated by sucrose density gradient centrifugation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the lipid bodies followed by staining with Coomassie Blue, indicated that there were no proteins and Western blots did not detect the presence of HRP II, PfCRT (a protein present in the food vacuole membrane) or *P. falciparum* aldolase (Pisciotta *et al.*, 2007). Analysis by thin layer chromatography and hydrolysis followed by gas-liquid chromatography-mass spectroscopy indicated that neutral lipids, such as monoacylglycerols, were predominantly present, in particular, monopalmitoyl- and monostearoyl-glycerol, together with significant quantities of a polar lipid called phosphatidyl ethanolamine (Pisciotta *et al.*, 2007). It was proposed that this phospholipid might be involved in the stabilization of the lipid bodies as the neutral lipids in the interior are usually surrounded by a monolayer of polar lipids such as phospholipid, glycolipid or sterol (Coppens and Vielemeyer, 2005). Monopalmitoyl-glycerol was found to be extremely efficient at promoting the formation of β-haematin, while a blend of monopalmitoyl- and monostearoyl-glycerol had similar activity to the lipids isolated with haemozoin from the parasite lysates. β-haematin product formed within 10 minutes and the reaction was not affected by globin which is essential as high concentrations of globin are present in the digestive vacuole. The lipids also protected the ferriprotoporphyrin IX from degradation by peroxide (H_2O_2) (Pisciotta *et al.*, 2007).

As in *P. falciparum*, there is a close association between haemozoin formation and lipids in the unrelated organism *Schistosoma mansoni*. Oliveira *et al.*, (2005) showed that haemozoin formation occurs within lipid bodies in the worm's gut. These bodies were roughly spherical with no evidence of a bilayer at their surface. The bodies were either completely filled with haemozoin crystals or contained small quantities of the crystals located near the inner surface of the lipid body where they made contact with the aqueous interface. This suggested that haemozoin grows from the lipid-water interface into the lipid body. Lipids isolated from these bodies supported β-haematin formation (Correa Soares *et al.*, 2007).

The observation that haemozoin formation in *S. mansoni* occurs at the interface between the lipid and aqueous phases strongly suggests that the interface plays an important role in the

process. This was confirmed in a biomimetic study in which the organic solvents, octanol and pentanol, as well as the lipid, monomyristoylglycerol (MMG), were used to mimic the lipid environment (Egan *et al.*, 2006). Ferriprotoporphyrin IX dissolved in 0.1 M NaOH was introduced to the interface (via a syringe) of two phase systems of pentanol-, octanol- or MMG-water where the aqueous phase was buffered at pH 4.8, a recent estimate of the digestive vacuole pH (Hayward *et al.*, 2006). β-haematin was produced within 30 minutes at 37ºC as confirmed by infra-red spectroscopy, X-ray diffraction and scanning electron microscopy. Using an immersion probe, Raman-resonance spectroscopy indicated that β-haematin was formed within 1 µm of the interface (Egan *et al.*, 2006).

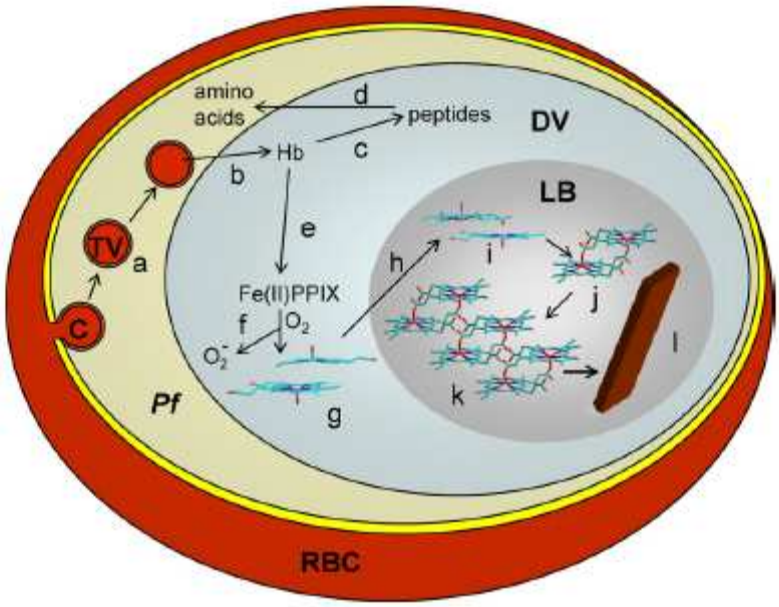


Figure 1.4. Proposed schematic representation of haemozoin formation in *Plasmodium falciparum*. Cytoplasm from the red blood cell (RBC) is taken up into the parasite by means of a cytosome (C) and (a) transported to the digestive vacuole (DV) in a double membrane transport vesicle (TV). (b) Once inside the digestive vacuole, the haemoglobin (Hb) is digested by plasmepsins, falcipains and falsilysin to short peptides (c). The peptides are transported out the digestive vacuole into the cytoplasm (d) where they are broken down by aminopeptidases into amino acids. The haeme, (Fe(II)PPIX), is oxidized (f), possibly by molecular oxygen, to ferriprotoporphyrin IX (Fe(III)PPIX) and a superoxide anion is released in the process. In the aqueous solution, Fe(III)PPIX forms a π-π complex (g) that is delivered to the lipid body (nanosphere) (h). The lipid body has a low dielectric constant, which enables the formation of a "haemozoin precursor dimer" (i) in which the axial water ligands of H_2O-Fe(III)PPIX are displaced with the formation of the iron-propionate bonds in the haemozoin dimer (j). In the lipid body, there is no solvent available with which to form hydrogen bonds and so the haemozoin dimers form hydrogen bonds with each other (k) and result in haemozoin nuclei that finally assemble into the crystal (l) (Egan, 2008a).

The behaviour of ferriprotoporphyrin IX (Fe(III)PPIX) in aqueous solution was studied to understand the molecular mechanism behind β-haematin formation. For 35 years, it has been believed that dimerisation involves the formation of μ-oxo dimers (Brown *et al.*, 1969). Although this species is present at high pH in the presence of high salt concentrations or organic bases, it does not appear to be present in abundance under the conditions prevailing in haemozoin-forming organisms.

It has been shown that H_2O-Fe(III)PPIX and HO-Fe(III)PPIX form π-π complexes in aqueous solution (de Villiers *et al.*, 2007). Under conditions resembling the digestive vacuole, the most likely dimer to form is a neutral $(H_2O\text{-Fe(III)PPIX})_2$ species in which one propionic acid group on each porphyrin is ionised and one is not. At higher pH, the likely dimers to form are anionic $(H_2O\text{-Fe(III)PPIX})_2$, H_2O-Fe(III)PPIX•HO-Fe(III)PPIX and $(HO\text{-Fe(III)PPIX})_2$ in which both propionic acid groups on each porphyrin are ionised (de Villiers *et al.*, 2007). The charges on the propionic acid groups are relatively distant from each other and the interacting iron centres are positively charged in the H_2O-Fe(III)PPIX species and neutral in the HO-Fe(III)PPIX species. Molecular dynamic simulations of two H_2O-Fe(III)PPIX molecules in a vacuum showed the rapid formation of a “haemozoin precursor dimer” (Egan *et al.*, 2006) in which the positively charged iron(III) centre of one Fe(III)PPIX molecule interacts with the negatively charged propionate group of the other molecule. A haemozoin dimer is formed with the formation of the iron(III)-propionate bond and the concomitant displacement of the axial water molecule from the opposite face of the porphyrin molecule (Figure 1.4). This precursor dimer was found to be unstable in the presence of water molecules as the propionate groups move rapidly away from the iron centres and interact with the water molecules (Egan *et al.*, 2006). This observation is supported by the rapid formation of β-haematin in the low dielectric medium of the interface systems and the slow difficult formation of β-haematin in the aqueous systems which require high concentrations of acetate and high temperatures. Formation of β-haematin in the aqueous systems exhibits sigmoidal kinetics with long induction periods and large activation energies (Egan *et al.*, 2001; Egan and Tshivhase, 2006). Hydrogen bonding between the dimers to form the haemozoin crystal will also be inhibited in the presence of competing hydrogen bonding from water molecules. Two important questions remain: (1) how are the ferriprotoporphyrin IX molecules delivered to the lipid bodies and (2) how is the crystal formation initiated?

1.2.4 Biology of the mononuclear phagocyte system

The mononuclear phagocyte system is made up of bone marrow monoblasts and promonocytes, peripheral blood monocytes and tissue macrophages (Auger and Ross, 1992). The least mature cell is the monoblast which divides to give rise to two promonocytes, which are the precursors of monocytes. Monocytes leave the bone marrow after less than 24 hours and enter the peripheral blood (Meuret and Hoffmann, 1973). From the peripheral blood, the monocytes migrate into tissues to become macrophages. During an acute inflammatory reaction, there is an increase in bone marrow production and circulating monocytes (Van Furth *et al.*, 1973).

1.2.4.1. Morphology of the mononuclear phagocyte

Macrophages are irregularly shaped, large cells with a diameter of 25-50 µm. They have a round or kidney-shaped nucleus and granules in the cytoplasm. Vacuoles are often found in the cytoplasm near the cell periphery indicating pinocytosis activity. Monocytes have a smaller size (12-15 µm) and a round, kidney-shaped or irregularly shaped nucleus that occupies about 50% of the cell area. The cytoplasm contents of monocytes are similar to that of macrophages. Both monocytes and macrophages have a ruffled appearance on the surface (Auger and Ross, 1992).

1.2.4.2. Composition and metabolism of the mononuclear phagocyte

The composition and metabolism of mononuclear phagocytes changes during differentiation. When monocytes differentiate into macrophages, there is an increase in the number of mitochondria, an increase in the activity of the mitochondrial enzymes and the rate of cellular respiration (Cohn, 1968). The number of lysosomes and lysosomal enzymes is also increased. Lysosomal enzymes are synthesized in the endoplasmic reticulum and packaged into primary lysosomes by the Golgi apparatus (Nichols *et al.*, 1971). These primary lysosomes fuse with phagosomes which contain ingested material to form secondary lysosomes or phagolysosomes.

Receptor-ligand interactions affect the basal metabolism of the macrophages and result in a respiratory burst in which oxygen consumption is increased, and large quantities of glucose are metabolised by the hexose monophosphate shunt. The membrane-bound oxidase complex is altered and molecular oxygen is reduced to superoxide (Babior, 1984). The superoxide is

rapidly converted to hydrogen peroxide and hydroxyl radicals, which provide most of the microbiocidal activity within the lysosomes and in the extracellular environment. The respiratory burst is markedly decreased as the monocytes mature into macrophages, but it is increased several fold when the macrophages are activated by exposure to IFN-γ (Nathan *et al.*, 1983) and migration inhibitory factor (Nathan *et al.*, 1984). Activation of the macrophages results in an enhanced cellular metabolism, mobility, lysosomal enzyme activity, including neutral proteases, cytocidal capacity and an increase in acid hydrolases, complement components, enzyme inhibitors, binding proteins, IL-1, TNF-α, and factors promoting haematopoiesis (production of blood cells and platelets) (North, 1978).

1.2.4.3. Functions of the mononuclear phagocyte

Macrophages provide a defence against invasion of the host by a wide variety of micro-organisms, such as bacteria, viruses, fungi and protozoa (Cohn, 1968). The macrophages are guided towards the micro-organism by a trail of chemotactic molecules emanating from them. The micro-organism is then engulfed by the macrophage. Phagocytosis can be enhanced by opsonins, which consist of immunoglobulin G (IgG), or fragments of the third component of complement which bind to specific sites on the micro-organism, and Fc receptors (that bind to Fc domain of various subclasses of IgG) or C3 receptors (that bind to several isotypes of complement) on the macrophage (Adams and Hamilton, 1984). Phagocytosis can also occur without opsonisation, for example, when the macrophage mannose-fucose receptor interacts with the carbohydrate residues on the micro-organism (Sung *et al.*, 1983). The microbiocidal effects of the monocyte/macrophage are catalyzed by a set of interrelated enzyme pathways. The most important of these are nitric oxide (inducible nitric oxide synthase), hydrogen peroxide and superoxide anion (phagocyte NADPH oxidase) and hypochlorous acid (myeloperoxidase), which are toxic to micro-organisms (Albrich *et al.*, 1981; Rosen *et al.*, 1995; Wang *et al.*, 2001).

A second major function of the macrophages is to assist in initiating and facilitating cell-mediated immune responses against pathogens by acting as antigen presenting cells. The macrophage takes up the antigens, processes them by denaturation or partial degradation and in the case of protein antigens, generates peptides that are recycled to the cell surface in association with glycoproteins encoded by the Class I or II genes of the major histocompatibility complex (MHC). The peptides in association with Class I or II molecules on the cell surface are

recognised by MHC Class I- (CD8-expressing T-cell) or Class II- (CD4-expressing T-cell) restricted specific T-cells (Auger and Ross, 1992).

Mononuclear phagocytes have an extensive secretory capability, in that they not only secrete enzymes, but many other substances that have functions ranging from induction of cell growth to cell death (Nathan, 1987). Enzymes include lysozyme, acid hydrolases, neutral proteases, lipase and arginase. Other substances which are secreted include enzyme inhibitors, complement components, reactive oxygen intermediates, arachidonic acid intermediates, coagulation factors and cytokines such as interferon-α, TNF-α, colony stimulating factors, IL-6, IL-1, transforming growth factor-β, platelet derived growth factor and epidermal growth factor.

1.2.5 The role of monocytes/macrophages in the immune response to malaria

A large portion of resident macrophages, circulating monocytes and neutrophils of malaria-infected patients contain haemozoin crystals (Amodu *et al.*, 1998; Lyke *et al.*, 2003; Metzger *et al.*, 1995b; Nguyen *et al.*, 1995). Unpurified haemozoin contains ferriprotoporphyrin IX, proteins, lipids and nucleic acids of both host and parasitic origin (Goldie *et al.*, 1990). Macrophages are involved in the control of the malaria infection by antibody-dependent (Fc receptors) and independent (CD36) phagocytosis of haemozoin and parasite-infected erythrocytes (Ayi *et al.*, 2005; Celada *et al.*, 1983b; Patel *et al.*, 2004), and the release of soluble factors which are toxic either directly or indirectly to the parasite. These factors include IL-1, TNF-α (Pichyangkul *et al.*, 1994), granulocyte-macrophage colony stimulating factor (GM-CSF) (Yamada-Tanaka *et al.*, 1995), reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Prada *et al.*, 1996a).

Confocal microscopy studies indicate that haemozoin remains unharmed inside the macrophage for up to 72 hours (Schwarzer *et al.*, 2001). The percentage of pigment- containing neutrophils and monocytes appears to roughly correlate with the severity of the disease (Amodu *et al.*, 1998; Hanscheid *et al.*, 2008; Lyke *et al.*, 2003; Metzger *et al.*, 1995b; Nguyen *et al.*, 1995). The accumulation of haemozoin inside the macrophage impairs the functions and activation of the macrophage. The haemozoin-laden macrophage is unable to repeat phagocytosis (Schwarzer *et al.*, 1992), generate a respiratory burst upon stimulation with phorbol-12-myristate-13-acetate (Schwarzer *et al.*, 1992) or produce nitric oxide (Taramelli *et al.*, 1995), and hence, cannot kill ingested bacteria, fungi or tumour cells (Fiori *et al.*, 1993).

Phagocytosed haemozoin also inhibits the membrane translocation and activity of protein kinase C (Schwarzer *et al.*, 1993), in addition to the activity of NADPH oxidase (Schwarzer and Arese, 1996). Moreover, monocytes containing haemozoin cannot express MHC Class II in response to IFN-γ (Schwarzer *et al.*, 1998), thus suggesting a link between haemozoin phagocytosis by monocytes, suppression of IFN-γ responsiveness, failure to up-regulate MHC Class II, impairment in antigen presentation and immunosuppression in malaria (Scorza *et al.*, 1999). Haemozoin also impairs the spontaneous up regulation of intercellular adhesion molecule (ICAM-1) and integrin-CD11c on the surface of the monocyte (Schwarzer *et al.*, 1998). ICAM-1 is an adhesion molecule that contributes to the capacity of the monocytes to adhere and stimulate T-cell proliferation. These observations might explain the defective T-cell response in malaria. Another contributing factor to the immunosuppression observed in malaria is that haemozoin inhibits the differentiation and maturation of monocytes into functional antigen-presenting dendritic cells (Skorokhod *et al.*, 2004). As dendritic cells play an important role in linking the innate immune response with the adaptive immune response through antigen presentation, this may also explain the deficient response of B and T cells and the reduced antibody response in malaria. A recent study confirmed that haemozoin induced failed dendritic cell function *in vivo* and *in vitro* in a *P. chabaudi* murine model (Millington *et al.*, 2006).

Haemozoin-laden monocytes have an enhanced metalloproteinase 9 activity (Prato *et al.*, 2008; Prato *et al.*, 2005) which functions by proteolytically shedding pro-forms of cytokines, such as TNF-α and IL-1β, thereby activating them and releasing them into the blood. Metalloproteinase 9 is also involved in disrupting basal lamina and may be responsible for symptoms of cerebral malaria, notably dysfunction of the blood-brain barrier, local haemorrhages and extravasation of phagocytic cells and blood cells into the brain tissues (Prato *et al.*, 2008). Most of these effects are as a result of the accumulation of lipoperoxidation products, such as the monohydroxy derivatives of arachidonic acid and linoleic acid, and large amounts of the potent aldehyde, 4-hydroxynonenal, in the macrophage. These lipoperoxidation products are ferried into the monocyte with phagocytosed haemozoin, while ingested haemozoin produces more by haeme-catalysis of lipoperoxidation of unsaturated fatty acids (Schwarzer *et al.*, 2003).

Nitric oxide produced by monocytes plays a two-fold role in malaria, both in the protection and pathology of malaria. In the protection of the host, nitric oxide (NO) is responsible for the killing of the liver stage of the parasites in response to TNF-α and IL-1β secretion (Clark *et al.*, 1997). However, when NO is produced in excess, it is toxic to both the invading parasites as well as

the host cells. During cerebral malaria, parasite-infected erythrocytes adhere to the microvasculature in the brain, block the vessels and induce proinflammatory cytokine secretion that results in inducible nitric oxide synthase (iNOS) generated NO both by leucocytes and endothelial cells in a localized area (Ghigo *et al.*, 1995; Tachado *et al.*, 1996). NO may act as a local neuroactive mediator, thus induction of iNOS expression may contribute to coma, seizures and death associated with cerebral malaria (Maneerat *et al.*, 2000).

Macrophages contribute to the pathogenesis of malaria through their expression and response to cytokines and chemokines. In human malaria, the immune reactivity is altered late in the acute phase and it often lasts for a long time after the parasite has been cleared from the circulation (Hviid *et al.*, 1991). One of the reasons why malaria immunity is poorly acquired in naturally exposed populations is that the parasites modulate the immune system of the host and prevent specific immune responses (Plebanski and Hill, 2000). The inflammatory response required to kill the parasites often causes tissue damage, and activation of phagocytes to kill intracellular or extracellular parasites requires the production of inflammatory cytokines, which can cause systemic effects like anaemia and cerebral malaria (Luty *et al.*, 2000; McGuire *et al.*, 1994). Thus, there needs to be a delicate balance in the induction and inhibition of these mediators. Refer to Figure 1.5.

1.2.5.1 Pro-inflammatory cytokines in malaria infection

Tumour necrosis factor-α (TNF-α) is induced in macrophages by *P. falciparum*-infected erythrocytes, haemozoin (Pichyangkul *et al.*, 1994) and certain glycolipids, for example, glycosylphosphatidylinositols (GPI) (Schofield and Hackett, 1993). GPI also induces iNOS in macrophages (Tachado *et al.*, 1996) and activates endothelial cells for enhanced cytoadhesion to leucocytes and parasite-infected red blood cells (Schofield *et al.*, 1996). Antibodies raised against GPI prevented lysates of plasmodium-infected erythrocytes from inducing TNF-α secretion from mononuclear cells (Bate and Kwiatkowski, 1994). The levels of TNF-α can be modulated by IFN-γ, which stimulates TNF-α production by monocytes, and IL-4 and IL-10, which both decrease the secretion of TNF-α (Essner *et al.*, 1989; Fiorentino *et al.*, 1991). TNF-α increased the phagocytic capacity of monocytes by increasing the number of Fc receptors on the monocyte or by modulating Fc receptor-mediated signalling pathways by binding to the Fc receptors. TNF-α also inhibits *P. falciparum* growth by a pleiotropic antimalarial effect that is not related to phagocytosis (Muniz-Junqueira *et al.*, 2001) . TNF-α plays a role in regulating IL-12

production in macrophages and it is a co-factor in the IL-12-induced production of IFN-γ by natural killer cells (Tripp *et al.*, 1993). Although high plasma NO and TNF-α levels are associated with parasite clearance, TNF-α also has detrimental properties such as fever, aches and pains and correlates to acute disease, hypoglycaemia, shock, bleeding and reversible coma (Beutler and Grau, 1993). A close association between high TNF-α levels, severe anaemia and large numbers of circulating haemozoin-containing monocytes has been demonstrated suggesting that haemozoin-induced TNF-α production plays a role in either the initiation or the exacerbation of anaemia as a result of chronic uncontrolled parasitaemia (Luty *et al.*, 2000).

Interferon-γ (IFN-γ) is a macrophage-activating factor involved in the protective innate immune response to the blood stage of malaria. It is produced by natural killer cells in a non-specific response and by CD4+ and CD8+ T cells in a specific immune response (Arase *et al.*, 1996; Rincon *et al.*, 1998). In Gabonese children, IFN-γ released by CD4+ T cells to specific parasitic erythrocytic antigens was associated with protection against malaria reinfection (Luty *et al.*, 1999). INF-γ secreted by T cells induces the production of specific IgG against blood stage malaria parasites and assists with the monocyte-mediated antibody-dependent cellular inhibitory mechanisms (Bouharoun-Tayoun *et al.*, 1995). During *P. falciparum* infection, the target cells of IFN-γ are monocytes/ macrophages (Bate *et al.*, 1988), neutrophils (Kumaratilake *et al.*, 1991), T-helper cells subtype 2 (Th 2) (Taverne, 1993) and parasite-infected hepatocytes (Klotz *et al.*, 1995). Macrophages that are activated by IFN-γ release TNF-α, transforming growth factor-beta (TGF-β), IL-1, IL-6, ROI and RNI (Clark *et al.*, 1997). IFN-γ signalling affects transducers associated with transcription, activates iNOS and initiates L-arginine-dependent NO pathway, produces NO and subsequently eradicates the *P. falciparum* infected hepatocytes (Snounou *et al.*, 2000).

Children with *P. falciparum* hyperparasitaemia had fewer IFN-γ-secreting CD4+ T cells than children with uncomplicated malaria (Winkler *et al.*, 1999). Thus, it appears that IFN-γ is essential for resolving the primary infection by limiting the initial phase of parasite replication. However, IFN-γ also contributes to the acute symptoms of malaria infection such as fever, headache and nausea through the induction of TNF-α and IL-1. Over production of TNF-α or IFN-γ can result in severe pathology. IL-12 and IL-18 control the IFN-γ secretion by T cells (Riley, 1999).

Interleukin (IL)-12 is a potent immunomodulatory cytokine that links the early innate non-specific immune response to later adaptive specific immune responses (Trinchieri, 1995). IL-12 is produced by macrophages in response to infectious agents and it is likely that the process of phagocytosis stimulates IL-12 production. IL-12 acts on antigen-stimulated CD4+ T cells and activates 'signal transducers and activating transcription 4' (STAT 4), thus promoting the differentiation of the T cells into T helper cell subtype 1 (Th 1) (O'Garra and Arai, 2000). The Th 1 cells produce INF-γ, which in turn acts on macrophages by stimulating their microbiocidal properties and increasing their production of IL-12. The increased concentrations of IL-12 moderates the activity of the macrophage and results in increased erythrocyte destruction and bone marrow diserythropoiesis (Crutcher *et al.*, 1995). IL-12 also induces IFN-γ production by natural killer (NK) cells early in the infection, it acts as a growth factor for NK cells and it enhances the cytotoxic activity of NK cells (Trinchieri, 1995). IL-12 has also been demonstrated to be involved in the adaptive immune response in that it stimulates the production of antibodies by B cells and it has been associated with protective immunity against the blood stage infection in the murine model (Crutcher *et al.*, 1995). IL-12 also favours cytotoxic T lymphocyte (CD8+) generation (Trinchieri, 1995). In patients with hyperparasitaemia and severe malaria, the lower IL-12 concentrations were related to reduced T-cell mediated IFN-γ activity (Malaguarnera *et al.*, 2002). In acute malaria, the constitutive production of IL-12 by monocytes was inhibited when the monocytes phagocytosed haemozoin. B-cell or Th 2 production of IL-10 also antagonized the activity of IL-12 (O'Garra and Arai, 2000).

Interleukin 18, which is produced by monocytes/macrophages, has a wide range of immunoregulatory functions including gene expression and synthesis of TNF-α and IL-1 by macrophages, increased Th 1 cell differentiation and induction of NK cell cytotoxicity (Dinarello, 1999). Certain structural motifs and amino acid sequences indicate that IL-18 is in the IL-1 family of cytokines (Okamura *et al.*, 1995). In a similar manner to IL-1, IL-18 is also processed by the IL-1 converting enzyme and the activity of mature IL-18 is closely related to that of IL-1β (Bazan *et al.*, 1996; Dinarello, 1999). IL-18 acts synergistically with IL-12. IL-18 by itself induces low concentrations of IFN-γ production by B and T cells. However, when IL-12 and IL-18 synergistically act on Th 1 cells and B cells, high concentrations of IFN-γ are produced. Thus it has been postulated that IL-18 induces IFN-γ production only when IL-12 up-regulates the IL-18 receptors on Th 1 cells (Xu *et al.*, 1998; Yoshimoto *et al.*, 1998).

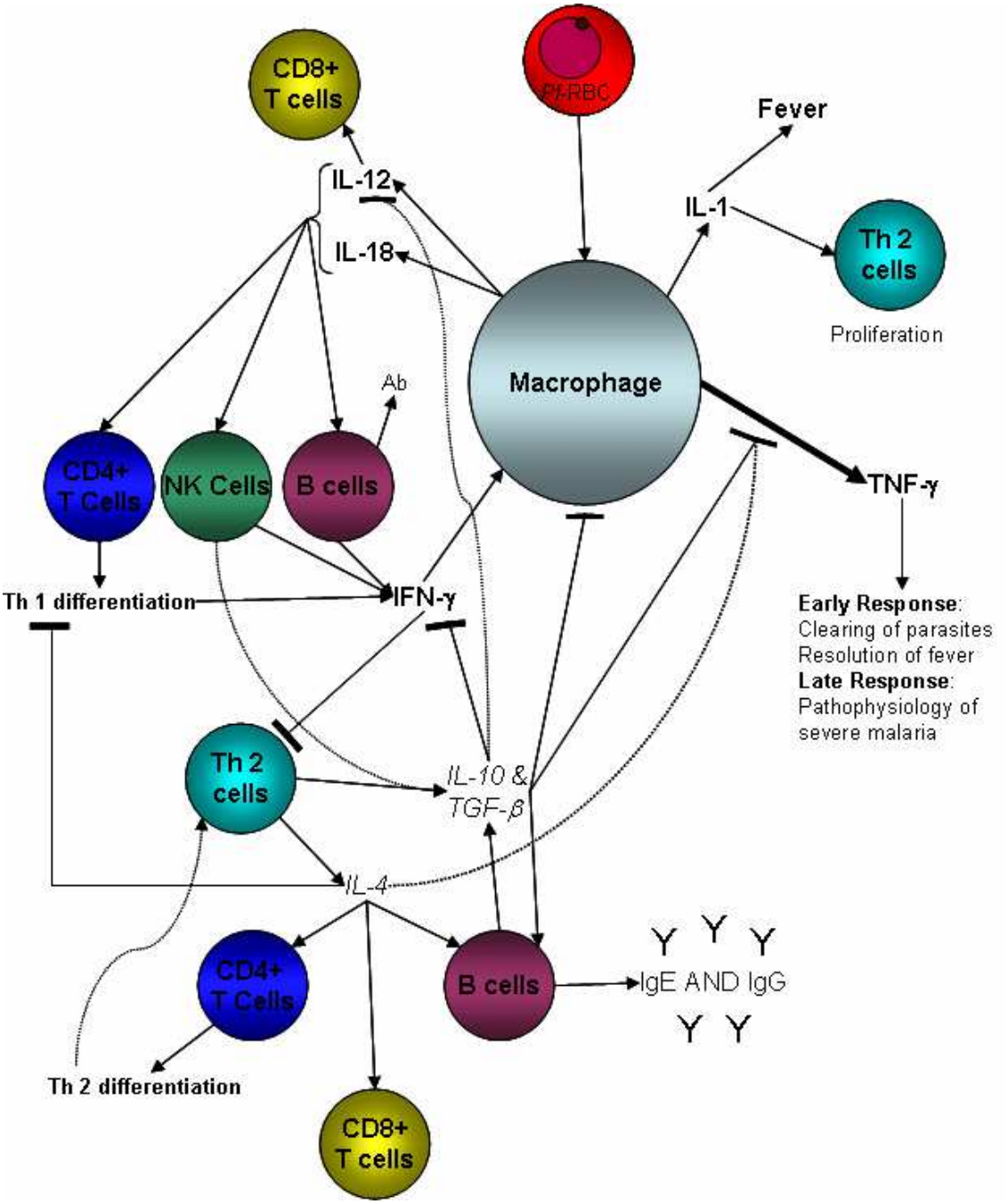


Figure 1.5. The immune response of macrophages during malaria infection. *P. falciparum*-infected red blood cells induce macrophages to produce TNF-α, IL-12 and IL-18. IL-12 and IL-18 synergistically induce Th 1 differentiation and the production of IFN-γ by Th 1 cells, NK cells and B cells. The IFN-γ stimulates further production of TNF-α from macrophages resulting in clearing of parasites or pathophysiology depending on the stage of disease. IL-12 also stimulates the production of antibodies from B cells and favours cytotoxic (CD8+) T cell generation. IL-4 induces Th 2 differentiation and the production of IL-10 and TGF-β which together suppress the secretion of TNF-α and IFN-γ from monocytes and antagonize the activity of IL-12. IL-4 also stimulates B cells to produce antibodies and development of cytotoxic CD8+ T cells against the parasite-infected hepatocytes. Pro-inflammatory cytokines are in bold and anti-inflammatory cytokines are in *italics*. *Pf*-RBC: *Plasmodium falciparum*-infected red blood cell.

During acute malaria and the recovery phases of uncomplicated *P. falciparum* infections, there are high plasma concentrations of IL-18, suggesting a proinflammatory role in these patients (Torre *et al.*, 2001). Chaiyaroj *et al.* (2004) found that IL-18 levels were higher in uncomplicated malaria in Thai adults than in healthy controls and that the IL-18 response rate declined as the symptoms of the disease became more severe suggesting that the IL-18 response was impaired with increasing disease severity. In contrast, Kojima *et al.* (2004) found that Thai patients with severe malaria had higher IL-18 levels than those with uncomplicated malaria, and with treatment, the IL-18 levels decreased in cerebral malaria but remained high in severe malaria (Nagamine *et al.*, 2003).

1.2.5.2 Anti-inflammatory cytokines in the malaria infection

Pro-inflammatory cytokines produced early in malaria infection mediate protective immunity, whereas when they are produced later in the infection, they contribute to the pathology of the malaria infection. Thus, if the inflammatory response is not controlled, it will lead to severe disease. During mild malaria, the inflammatory response can be down-regulated by anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor-β (TGF-β).

Interleukin-4 is produced by Th 2 cells and mast/basophil cells and it is a growth factor for B cells and promotes IgE and IgG synthesis (Howard *et al.*, 1982; Vitetta *et al.*, 1985). IL-4 enhances the tumouricidal activity of macrophages and the expression of MHC Class II on the macrophage surface (Crawford *et al.*, 1987; te Velde *et al.*, 1988). It stimulates the differentiation of CD4+ T cells into Th 2 cells and inhibits the Th 1 cell response (Fernandez-Botran *et al.*, 1986). Th 2 cells and IL-4 have been shown to be important in the antibody response against the malaria parasite (Troye-Blomberg *et al.*, 1990). IL-4 secreted by CD4+ Th 2 cells is essential for the development of the CD8+ T cells (cytotoxic T cells) against hepatocytes infected with the malaria parasite (Carvalho *et al.*, 2002). The phagocytosis of *P. falciparum*-infected red blood cells by non-activated macrophages and monocytes was found to be inhibited by IL-4 (Kumaratilake and Ferrante, 1992).

Transforming growth factor-β (TGF-β) is produced by a variety of cells, including NK cells, macrophages, T cells and B cells and it has pro-inflammatory properties at low concentrations and anti-inflammatory properties at high concentrations (Wahl, 1994). Pro-inflammatory properties of TGF-β include its ability to recruit monocytes, T cells and neutrophils to the site of

inflammation early in an infection, by modulating endothelial cell adhesion molecule expression (Wahl, 1994). However, high concentrations of TGF-β suppress production of TNF-α and NO from macrophages (Espevik *et al.*, 1987), inhibit production of TNF-α and IFN-γ from NK cells (Bellone *et al.*, 1995) and antagonize IFN-γ-stimulated up regulation of major histocompatibility complex II antigens (Nandan and Reiner, 1997). TGF-β antagonizes the effects of IL-4, IL-2 (Ruegemer *et al.*, 1990) and IL-12 (Pardoux *et al.*, 1997). These effects may be enhanced by IL-10 produced by macrophages, which may cause a shift from a Th 1 immune response to a Th 2 immune response (Maeda and Shiraishi, 1996).

In the murine malaria model, levels of TGF-β were inversely associated with the severity of malaria infection in mice and TGF-β appears to have a protective effect by down regulating the production of potentially pathogenic pro-inflammatory cytokines, TNF-α and IFN-γ (Omer and Riley, 1998). Evidence suggests that TGF-β has a dual function in malaria: early in the infection, TGF-β up-regulates Th 1-mediated immune responses and promotes antibody-independent cell mediated mechanisms that control acute parasitaemia. Later in the malaria infection, TGF-β down-regulates the Th 1 immune response to reduce the inflammatory-associated pathology (Omer *et al.*, 2000; Omer and Riley, 1998). TGF-β might also play a role in the outcome of malaria via its effects on B cells. Low concentrations of TGF-β stimulate B cells to secrete immunoglobulin G subclasses (Snapper *et al.*, 1993) but high concentrations of TGF-β inhibit the formation of antibodies (Stavnezer, 1995).

Not many studies have measured the levels of TGF-β in children with acute malaria. Lower plasma levels of TGF-β in comparison to healthy controls were measured in Thai patients with acute malaria (Wenisch *et al.*, 1995a). In another study, TGF-β levels were measured in Thai adults with uncomplicated, severe and cerebral malaria, Tanzanian children with cerebral malaria and pregnant women. The lowest levels of TGF-β were detected in peripheral blood of adult patients with cerebral malaria and placental blood of pregnant women infected with *P. falciparum*, but the levels observed in severe malaria were also significantly lower than those in uncomplicated malaria. Overall, the TGF-β levels were inversely correlated with the severity of disease (Chaiyaroj *et al.*, 2004). Other studies have demonstrated the accumulation of TGF-β in the brain and the involvement of TGF-β in the reorganisation process of the brain parenchyma, immunological dysfunction and endothelial cell activation in patients with cerebral malaria (Deininger *et al.*, 2000).

Interleukin-10 is produced by monocytes, Th 2 cells and B Cells. It inhibits cytokine production from Th 1 cells and CD8+ T cells. IL-10 induces B cell proliferation and immunoglobulin production, but it down-regulates the MHC Class II expression on the surface of the macrophages and thus reduces antigen presentation and T-cell priming. IL-10 also inhibits production of ROI and RNI. IL-10 was shown to reduce generation of TNF-α, granulocyte-macrophage colony-stimulating factor, and IL-6 from mouse bone marrow and rat peritoneal mast cells in response to specific IgE cross-linking (Akdis and Blaser, 1999). Elevated levels of IL-10 were found in acute *P. falciparum* malaria in Thai patients (Wenisch *et al.*, 1995b) which declined with treatment (Wenisch *et al.*, 1995b). IL-10 was found to inhibit IFN-γ secretion and protect against experimental cerebral malaria in mice (Kossodo *et al.*, 1997). Severe malaria anaemia is associated with significantly decreased circulating levels of IL-10 (Kurtzhals *et al.*, 1998). Furthermore, the mean ratio of IL-10 to TNF-α was found to be significantly lower in children with malaria anaemia (1.77) than in children with mild and high-density parasitaemia (4.64). Hence, the development of malaria anaemia may be prevented by higher levels of IL-10 which can control the excessive inflammatory activities of TNF-α (Othoro *et al.*, 1999). It is still not clear if increased levels of IL-10 have a beneficial role in decreasing the parasite-induced inflammatory response or a detrimental role in inhibiting the cell mediated immune response.

In summary, during the erythrocytic stage of malaria infection, early strong pro-inflammatory, cytokine-mediated, effector mechanisms control parasite replication and promote clearance of the infected erythrocytes. However, this pro-inflammatory response must be tightly regulated and suppressed equally rapidly by anti-inflammatory effectors (such as IL-10 and TGF-β) to prevent the immune-mediated pathology which results in the development of severe complications of the malaria infection (Artavanis-Tsakonas *et al.*, 2003; Omer *et al.*, 2000). Refer to Figure 1.5. The malaria parasite can also suppress the generation of immune responses (Luty *et al.*, 2000; Riley *et al.*, 1989; Wipasa *et al.*, 2001) by apoptosis, parasite inhibition of macrophage activation and antigen presentation, and by inhibition of dendritic cell maturation, or alteration of dendritic cell function (Urban *et al.*, 1999; Urban and Roberts, 2003).

1.2.6 The role of dendritic cells in the immune response to malaria

Dendritic cells provide a crucial link between the innate and adaptive immune responses (Reis e Sousa, 2004). They are specialized in the uptake, processing and presentation of antigens on the surface of the dendritic cell with MHC class II and co-stimulants CD80/86 to T cells.

Dendritic cells are the only antigen presenting cells that can activate naïve T cells (Banchereau and Steinman, 1998). The activated T cells produce cytokines that stimulate B cells to produce antibodies, and promote maturation of the cellular responses to launch an effective adaptive immune response.

Dendritic cell function in *Plasmodium* infections have been extensively studied *in vitro* and *in vivo*, but the findings are controversial (Wykes *et al.*, 2007a). Several investigations have reported that dendritic cells are activated and matured during *Plasmodium* infection and induce a powerful T response (Coban *et al.*, 2002; Ing *et al.*, 2006; Leisewitz *et al.*, 2004; Newman *et al.*, 2006; Perry *et al.*, 2004; Sponaas *et al.*, 2006; Wilson *et al.*, 2006). *Plasmodium*-infected red blood cells have activated human plasmacytoid-derived dendritic cells (Pichyangkul *et al.*, 2004), possibly through haemozoin (Coban *et al.*, 2005), which appears to bind to Toll-like receptor 9 (Parroche *et al.*, 2007), a pattern-recognition molecule expressed on dendritic cells, that interacts with DNA, lipoprotein and lipopolysaccharides of infectious micro-organisms. Other reports have demonstrated that dendritic cells are impaired during malaria infection and this suppression might be mediated by haemozoin (Millington *et al.*, 2006; Skorokhod *et al.*, 2004; Urban *et al.*, 1999; Wykes *et al.*, 2007b). Haemozoin-loaded dendritic cells: failed to form stable clusters and interactions with T cells (Millington *et al.*, 2007); reduced T-cell activation; failed to migrate to lymphoid-organ follicles and stimulate B cells to respond to heterologous antigens (Millington *et al.*, 2006); and had reduced expression of MHC Class II and co-stimulatory molecules, CD80 and CD40, on their surface (Skorokhod *et al.*, 2005).

The contradictory results could be due to the differences in *Plasmodium* species studied, dendritic cell subsets used or experimental protocols (Langhorne *et al.*, 2004; Sponaas *et al.*, 2006). Thus, it is difficult to ascertain the conclusive effect of the malaria infection on the biology of the dendritic cells.

1.2.7 Neopterin, a marker of inflammation

The role of IFN-γ in communications between T cells and macrophages with the subsequent release of neopterin, make plasma measurements of neopterin an ideal method for measuring immune activation within a patient (Wachter *et al.*, 1989). IFN-γ results in a rapid and sustained increase in neopterin levels in plasma (Muller *et al.*, 1991). Neopterin can be easily measured in plasma, urine and cerebrospinal fluid by high performance liquid chromatography because of

its high fluorescence (Rippin, 1992; Werner *et al.*, 1987a; Werner *et al.*, 1987b), although many clinical laboratories also use immuno-based methods such as enzyme-linked immunosorbent assay to measure neopterin (Westermann *et al.*, 2000).

Neopterin levels rise rapidly in parallel with C-reactive protein levels in response to an infection well before a patient becomes sero-positive. Monitoring neopterin levels in plasma can be used to assess the efficacy of treatment used in a range of infections such as malaria (Awandare *et al.*, 2006a; Reibnegger *et al.*, 1984), human immunodeficiency virus (Baier-Bitterlich *et al.*, 1996a; Fuchs *et al.*, 1988) and tuberculosis (Fuchs *et al.*, 1984b; Yuksekol *et al.*, 2003). As elevated neopterin levels are indicative of inflammatory conditions, plasma neopterin concentrations can serve as a primary screen for blood donations (Zangerle *et al.*, 1992). The measurement of plasma neopterin levels has also been used in the study and management of cancer (Fuchs *et al.*, 1984a; Reibnegger *et al.*, 1991), autoimmune disease (Leohirun *et al.*, 1991; Reibnegger *et al.*, 1986; Schroecksnadel *et al.*, 2003) and transplant patients (Margreiter *et al.*, 1983; Reibnegger *et al.*, 1991) where an increase in plasma or urine levels gives clinicians adequate warning of allograft rejections enabling them to alter immunosuppressant treatment. Serum neopterin has also been found elevated in patients with unstable angina and acute myocardial infarction (Schumacher *et al.*, 1992; Schumacher *et al.*, 1997; Tatzber *et al.*, 1991).

1.2.7.1 Biosynthesis of neopterin and 7,8-dihydroneopterin

The biosynthesis of neopterin is similar to the pathway leading to tetrahydrobiopterin, an essential cofactor of several mono-oxygenases and inducible nitric oxide synthases (Gorren and Mayer, 2002). Refer to Figure 1.6. GTP-cyclohydrolase I (EC 3.5.4.16), an enzyme up-regulated by IFN-γ, catalyses the breakdown of guanosine triphosphate to 7,8-dihydroneopterin triphosphate. In non-primate macrophages, for example, mouse macrophages, 7,8-dihydroneopterin triphosphate is converted by 6-pyruvoyltetrahydropterin synthase (PTPS) in an Mg^{2+}-dependent step to form 6-pyrovoyltetrahydropterin. In the final step, yielding tetrahydrobiopterin, sepiapterinreductase catalyzes the NADPH-dependent reduction of 6-pyruvoyltetrahydropterin. Human and primate monocytes and macrophages have lower levels of 6-pyruvoyltetrahydropterin synthase activity (Schoedon *et al.*, 1987; Werner *et al.*, 1990), hence activation of GTP- cyclohydrolase 1 leads to an accumulation of 7,8-dihydroneopterin

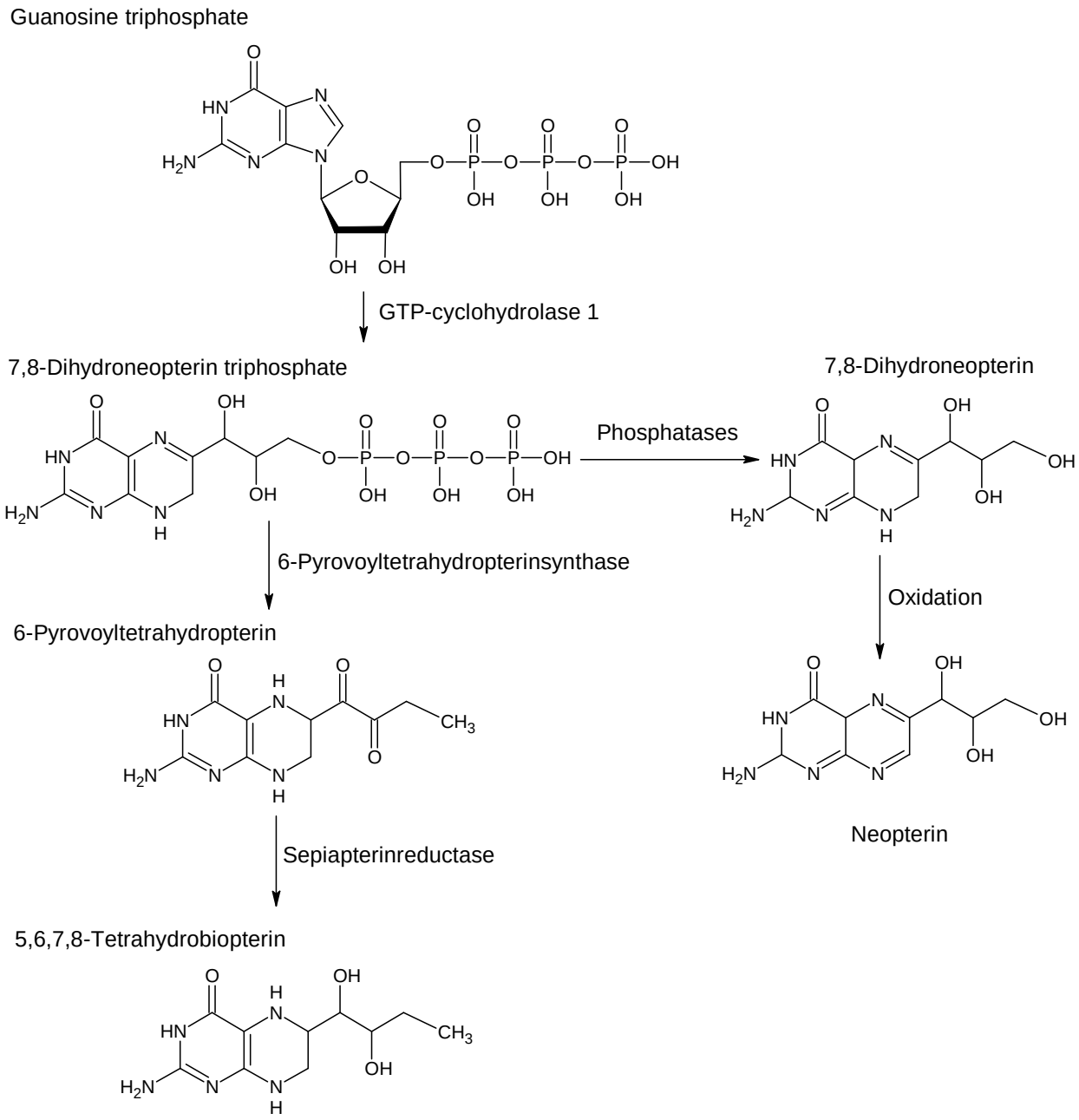


Figure 1.6. Synthesis of 5,6,7,8-tetrahydrobiopterin and neopterin. The synthesis of neopterin has similarity to the pathway leading to 5,6,7,8-tetrahydrobiopterin. GTP-cyclohydrolase 1, a key enzyme in the process, catalyses the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which in the 5,6,7,8-tetrahydrobiopterin synthetic pathway, is converted to 6-pyrovoyltetrahydropterin in a step catalysed by 6-pyrovoyltetrahydropterinsynthase. The transformation of 6-Pyrovoyltetrahydropterin to 5,6,7,8-tetrahydrobiopterin is catalysed by sepiapterinreductase. In human and primate monocytes and macrophages, there are very low activities of 6-pyrovoyltetrahydropterinsynthase, resulting in an accumulation of 7,8-dihydroneopterin triphosphate that is cleaved by non-specific phosphatases to 7,8-dihydroneopterin which is then oxidized in a non-enzymatic step to neopterin (Gieseg *et al.*, 2008; Murr *et al.*, 2002).

triphosphate which is released as 7,8-dihydroneopterin due to the action of intracellular phosphatases. 7,8-Dihydroneopterin diffuses out of the activated macrophage into the intercellular spaces and finally into the plasma. Some of the 7,8-dihyroneopterin is oxidized to 7,8-dihydroxanthopterin by reactive oxygen species. The main reaction generating neopterin from 7,8-dihydroneopterin is oxidation by hypohalous acids such as hypochlorous acid (HOCl) (Widner *et al.*, 2000). Neutrophils, and possibly macrophages release large amounts of HOCl during inflammation (Chisolm *et al.*, 1999; Schraufstatter *et al.*, 1990) suggesting that neopterin measured in plasma comes from sites of inflammation where HOCl is released.

1.2.7.2 Functions of 7,8-dihydroneopterin and neopterin

A clear biological function for neopterin and its derivatives is not completely understood at this stage, but they do appear to play a role in oxidative stress. Neopterin was found to enhance chloramine-T and H_2O_2-mediated chemiluminescence *in vitro* (Weiss *et al.*, 1993). This suggested neopterin had pro-oxidant properties which was further substantiated by neopterin enhancing tyrosine nitration (Widner *et al.*, 1998) and low density lipoprotein-(LDL)-oxidation (Herpfer *et al.*, 2002) by peroxynitrite. Moreover, neopterin was also found to augment H_2O_2-, hypochlorite-, or chloramine-T-mediated toxicity against bacteria (Horejsi *et al.*, 1996; Weiss *et al.*, 1993) and induce apoptosis in vascular smooth muscle cells and the alveolar type II-like epithelial cell line L2 (Hoffmann *et al.*, 1998; Schobersberger *et al.*, 1996). However, neopterin also displayed anti-oxidant effects: in the absence of iron, neopterin was a potent scavenger of H_2O_2-induced chemiluminescence (Murr *et al.*, 1994); and neopterin also suppressed NADPH-oxidase in macrophages stimulated with phorbol-12-myristate-13-acetate, and thus decreased the generation of superoxide anions (Kojima *et al.*, 1992).

7,8-Dihydroneopterin displays anti-oxidant properties at low concentrations. It inhibited: the luminescence signal from superoxide and hydrogen peroxide (Shen, 1994); tyrosine nitration by peroxynitrite (Widner *et al.*, 1998); and metal ion and aqueous peroxyl radical (2,2'-azobis(amidinopropane)dihydrochloride)-mediated LDL oxidation (Gieseg *et al.*, 1995). 7,8-Dihydroneopteirn also suppressed the toxicity of H_2O_2, hypochlorite, or chloramine-T against bacteria (Horejsi *et al.*, 1996; Weiss *et al.*, 1993). Furthermore, micromolar concentrations of 7,8-dihydroneopterin inhibited cellular damage to red blood cells and human monocytic U937 cells from a range of oxidants such as hydrogen peroxide, hypochlorite, aqueous peroxyl radicals, nitric oxide and direct plasma membrane oxidation by ferrous ions (Gieseg *et al.*,

2000; Gieseg *et al.*, 2001a; Gieseg *et al.*, 2001b). These findings have led to the hypothesis that 7,8-dihydroneopterin secreted by IFN-γ-stimulated macrophages protects these antigen-presenting cells from oxidants encountered in the inflammatory site (Duggan *et al.*, 2002; Gieseg *et al.*, 1995; Kojima *et al.*, 1992; Schroder *et al.*, 1987).

However, at high concentrations, 7,8-dihydroneopterin acts as a pro-oxidant and induces apoptosis in the presence of TNF-α in the human neuronal cell line (NT2) (Spottl *et al.*, 2000), astrocytic and microglial cell lines (Speth *et al.*, 2000) and the rat pheochromocytoma cells (PC12) (Enzinger *et al.*, 2002b) by the formation of ROI. At concentrations below 300 µM, 7,8-dihydroneopterin diminished TNF-α-induced programmed cell death in U937 cells, whereas 5 mM 7,8-dihydroneopterin enhanced the effect of TNF-α on apoptosis (Baier-Bitterlich *et al.*, 1995). Likewise with Jurkat T cells, 7,8-dihydroneopterin only induces apoptosis above 1 mM (Baier-Bitterlich *et al.*, 1996a; Wirleitner *et al.*, 1998; Wirleitner *et al.*, 2001) via the redox-sensitive Bcl-2 pathway (Enzinger *et al.*, 2002a).

Neopterin and its derivatives have also been shown to play a role in cell signalling. Micromolar levels of both neopterin and 7,8-dihydroneopterin increased intracellular calcium levels in human-derived monocyte-like THP-1 cells (Woll *et al.*, 1993) and nanomolar levels of neopterin effectively inhibited ATP-induced calcium release from alveolar epithelial cells (Hoffmann *et al.*, 2002). Micromolar concentrations of neopterin were also reported to cause cardiac contractile dysfunction in isolated perfused rat hearts (Balogh *et al.*, 2005; Margreiter *et al.*, 2000).

Neopterin and 7,8-dihydroneopterin were found to interfere with intracellular signalling pathways that are influenced by oxidative stress. Neopterin and 7,8-dihydroneopterin activated the redox-sensitive transcription factor nuclear factor-κB (NF-κB) in Jurkat cells (Baier-Bitterlich *et al.*, 1997) and murine vascular smooth muscle cells (Hoffmann *et al.*, 1996). *In vitro*, neopterin inhibited hypoxia-induced erythropoietin gene expression and formation in HepG2 cell cultures (Schobersberger *et al.*, 1995b) and hypoxically perfused isolated rat kidneys (Pagel *et al.*, 1999). Neopterin also stimulated cytokine-inducible nitric oxide synthase gene expression in rat vascular smooth muscle cells (Schobersberger *et al.*, 1995a). Both neopterin and 7,8-dihydroneopterin together with cyclic-GMP induced the redox-sensitive proto-oncogene c-fos in NIH 3T3 fibroblasts (Uberall *et al.*, 1994) and neopterin also enhanced the cell damage caused by UV-A irradiation of B-16 melanoma cells (Kojima *et al.*, 1995).

1.2.8 Neopterin during malaria infections

Elevated levels of neopterin have been detected in urine (Reibnegger *et al.*, 1984) and plasma (Kremsner *et al.*, 1989; Ringwald *et al.*, 1991) of patients infected with *Plasmodium falciparum*. Children with malaria infections showed extremely high neopterin excretion levels when compared to adult malaria infections (Reibnegger *et al.*, 1987). When 71 Thai patients with acute, uncomplicated falciparum malaria were treated with quinine and tetracycline for 7 days, the neopterin levels in urine peaked on days 3-5 following the start of treatment before decreasing towards the normal range on days 6-8 (Brown *et al.*, 1990). In patients with a history of previous malaria infections, an inverse relationship was found between the frequency of prior malaria episodes and neopterin concentration during infection (Brown *et al.*, 1990). Increasing neopterin levels were found to correlate with peak temperature, fever clearance time and parasite clearance time. High concentrations of IFN-γ were found more frequently in malaria naïve than experienced patients (Brown *et al.*, 1990; Ringwald *et al.*, 1991). These data suggested that with repeated malaria infection and antigen exposure, there is a progressive suppression of the T-cell-macrophage interaction mediated by IFN-γ.

Neopterin levels were also elevated in Thai adults infected with *Plasmodium vivax* and found to be positively correlated with magnitude of fever. Neopterin excretion also increased during the first two days of chemotherapy and decreased to within the normal range by the sixth day post-treatment (Brown *et al.*, 1991). In an area endemic for malaria, patients subjected to repeated infestations have chronic parasitaemia and neopterin proved to be a good indicator of slowly acquired immunity but poorly reflected acute immune responses (Picot *et al.*, 1993).

Severe malarial anaemia was found to be directly associated with elevated serum neopterin concentrations and inversely correlated with serum IL-4 levels (Biemba *et al.*, 2000). Neopterin is secreted by IFN-γ-activated macrophages and thus reflects the Th 1 immune response, but IL-4 is a major cytokine that is inhibited by Th 1 activity. During seven days of quinine antimalarial therapy, serum neopterin levels remained elevated in children who were still found to have persisting anaemia one month after completing treatment, but the neopterin levels declined significantly in the children who had normal haemoglobin levels a month after completing treatment (Biemba *et al.*, 1998). The elevated neopterin levels suggested that the persistence of the Th 1 mediated immune response and associated macrophage activation may be involved in the pathogenesis of the lingering anaemia after the treatment of malaria. Children with severe *P. falciparum* malaria with respiratory distress, which is a symptom of

underlying acidosis, had significantly higher levels of neopterin than those malaria-infected children without respiratory distress (Awandare *et al.*, 2006a).

1.3 Antimalarial drugs

Drugs interfering with the erythrocytic schizogony, known as blood schizontocides, are the most important antimalarial drugs therapeutically as they interfere with the stage of the *Plasmodium* life cycle that is responsible for the clinical manifestations of malaria. (Wernsdorfer, 1997).

1.3.1 Quinoline containing drugs

In 1820, Pelletier and Caventou isolated quinine (6-methoxy-α-(5-vinyl-2-quinuclidyl)-4-quinolinemethanol) from the bark of the Cinchona tree and it was used for the treatment of malaria until the 1930's (Foley and Tilley, 1998). Since then other synthetic quinoline antimalarials, amodiaquine (7-chloro-4-(3'-diethylaminomethyl-4'-hydroxyanilino)-quinoline), chloroquine (7-chloro-4-(4'-diethylamino-1'-methylbutylamino)quinoline) and mefloquine (α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol) were mainstays for much of the next 50 years (Foley and Tilley, 1998; O'Neill *et al.*, 1998). Quinine, mefloquine, chloroquine and amodiaquine all have blood schizontocidal activity against all four species of human-pathogenic plasmodia (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) and incidental human infections with simian plasmodia (*P. knowlesi* and *P. cynomolgi*) (Wernsdorfer, 1997).

Chloroquine has been the most important synthetic quinoline due to its excellent clinical efficacy, limited host toxicity, ease of use and simple cost-effective synthesis. However, the value of synthetic quinolines has been reduced due to the development and spread of parasite resistance (Warhurst, 2001; Winstanley *et al.*, 2002). The exact mode of action and mechanism of resistance to the 4-aminoquinolines (chloroquine and amodiaquine) and the quinoline methanols (quinine and mefloquine) is still not fully understood. However, a crucial step in the mode of action appears to be the binding of the drug to ferriprotoporphyrin IX which is released during the digestion of the haemoglobin (Egan *et al.*, 1994). The antimalarial activity of chloroquine is directly related to its highly selective uptake and concentration in the malaria-infected erythrocytes (Diribe and Warhurst, 1985; Hawley *et al.*, 1998; Verdier *et al.*, 1985). Chloroquine is a weak uncharged base that diffuses freely into acidic compartments where it binds to protons and becomes trapped. Chloroquine accumulates in parasite infected

erythrocytes to concentrations about 20-fold higher than that accumulated in mammalian cells with large acidic lysosomes (Hawley *et al.*, 1996) and the extent of chloroquine accumulation appears to be determined by the amount of free ferriprotoporphyrin IX in the parasite (Bray *et al.*, 1999; Bray *et al.*, 1998). The steps following the initial binding of chloroquine to ferriprotoporphyrin IX leading to the death of the parasite are less certain. It has been proposed that the chloroquine-ferriprotoporphyrin IX complexes accumulate in the parasite membranes and destroy the membranes by lipid peroxidation resulting in the lysis of the parasite (Fitch *et al.*, 1982). Other investigators suggested that chloroquine inhibits the formation of haemozoin crystals, thus leading to the build up of toxic concentrations of free ferriprotoporphyrin IX within the parasite (Goldberg and Slater, 1992; Slater and Cerami, 1992; Sullivan, 2002). Free ferriprotoporphyrin IX may exert its toxic effects by inhibiting crucial parasite enzymes such as proteases of the digestive vacuole (Vander Jagt *et al.*, 1987). Inhibition of haemozoin formation can occur by either chloroquine binding to the substrate and depleting the substrate for haemozoin formation (Dorn *et al.*, 1998a; Dorn *et al.*, 1998b) or by chloroquine binding to the haemozoin crystal, capping it and preventing further growth of the crystal (Chong and Sullivan, 2003; Sullivan, 2002). However, it is not known which, if any of these mechanisms operate in the parasite.

Other quinoline-containing drugs are thought to act in a similar manner to chloroquine in that they all bind to ferriprotoporphyrin IX (Foley and Tilley, 1998; Mungthin *et al.*, 1998; Sullivan, 2002), but the effects on the feeding processes may be subtly different. The 4-aminoquinolines, chloroquine and amodiaquine have been associated with an accumulation of undigested haemoglobin in the digestive vacuole (Yayon *et al.*, 1984), whereas the quinoline methanols, quinine and mefloquine, have not been associated with a build up of haemoglobin in the parasite (Famin and Ginsburg, 2002). It was suggested that the 4-aminoquinolines inhibit the digestion of the haemoglobin, whereas the quinoline methanols possibly interfere with the ingestion of haemoglobin, that is, the endocytic process.

The 4-aminoquinolines, chloroquine and amodiaquine, have also been shown to inhibit the glutathione-dependent destruction of ferriprotoporphyrin IX (Ginsburg *et al.*, 1998), resulting in an accumulation of ferriprotoporphyrin IX in the membrane fraction of infected cells that correlated with parasite killing (Zhang *et al.*, 1999). Although the quinoline methanols, quinine and mefloquine, were able to inhibit the glutathione-mediated destruction of ferriprotoporphyrin

IX in solution, they were unable to inhibit the degradation of membrane associated ferriprotoporphyrin IX (Famin *et al.*, 1999).

Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites revealed that chloroquine treatment did not appear to affect the progress through the cell cycle and suggested that chloroquine toxicity may be manifested post-schizogony as “delayed death” (Gligorijevic *et al.*, 2006). Using these methods, it was also found that *Pfcrt* conferred resistance to chloroquine when it was added at the very early ring stage of development when the digestive vacuole has not yet formed, as well as when the chloroquine is added at the schizont stage when haemozoin development is completed (Gligorijevic *et al.*, 2008). This prompts further questions about the action of chloroquine and chloroquine resistance.

Primaquine is an 8-aminoquinoline that is effective against hypnozoites, the intrahepatic forms responsible for the relapsing infections of *P. vivax* and *P. ovale* and *P. cynomolgi* (Robert *et al.*, 2001). It also has marked activity against the gametocytes of all the plasmodia affecting humans. Primaquine has a very short half life as it undergoes extensive and rapid metabolism in the liver by cytochrome P450 enzymes to form carboxyprimaquine (Bangchang *et al.*, 1992). Primaquine and some of its metabolites inhibit the mitochondrial respiration of the parasite by mimicking ubiquinone and reduced ubiquinone found in the mitochondrial electron transport chain (Desjardins *et al.*, 1988; Lopez-Antunano, 1999) and they also interfere with the synthesis of pyrimidines (Wernsdorfer, 1997). Primaquine is also known to stimulate the oxidative pathway of glucose metabolism in non-infected erythrocytes and thus may cause haemolysis in patients with a glucose-6-phosphate dehydrogenase deficiency (Carson *et al.*, 1981). Primaquine has minimal activity against the asexual blood stages of the parasite and hence is always used in conjunction with a blood schizontocide.

1.3.2 Antifolates

Plasmodium in humans can capture and utilize the host’s purines but not the host’s pyrimidines, thus the parasite synthesizes its own pyrimidines (Le Bras and Durand, 2003). Pyrimidine biosynthesis includes the folate pathway and antifolates refer to drugs which interfere with enzymes involved in folate biosynthesis and thus inhibit the synthesis of parasitic pyrimidines and consequently parasitic DNA (Wernsdorfer, 1997). There are two groups of antifolates: (a) the dihydrofolate reductase (DHFR) inhibitors, such as pyrimethamine (2,4-diamino-5-*p*-

chlorophenyl-6-ethylpyrimidine) and proguanil (1-(4-chlorophenyl)-5-isopropylbiguanide hydrochloride), and (b) the dihydropteroate synthase (DHPS) inhibitors, which are sulfones and sulphonamides such as sulfadoxine (*N*-1-(5,6-dimethoxy-4-pyrimidinyl) sulphanilamide) and dapsone (4,4'-diaminodiphenylsulfone), respectively. A member of the first group is usually used in combination with a member of the second group because of their marked synergistic effects (Chulay *et al.*, 1984). Fansidar®, which is the most widely used combination, contains pyrimethamine and sulfadoxine. Pyrimethamine is structurally similar to dihydrofolate and is thus a competitive inhibitor of dihydrofolate reductase, whereas sulfadoxine is an analogue of *p*-amino-benzoic acid and is a competitive inhibitor of dihydropteroate synthase (Hayton and Su, 2008) . Unfortunately, Fansidar® is particularly prone to rapid emergence of resistance, which is widely spread in Asia, South America, India and now Africa (Cowman, 2001; Plowe *et al.*, 1998) .

1.3.3 Antibiotics

Doxycycline (4-dimethylamino-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 5, 10, 12, 12a pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide) belongs to the tetracycline group and it has blood schizontocidal activity (Wernsdorfer, 1997) and tissue schizontocidal activity against the pre-erythrocytic forms in the hepatocytes (Marussig *et al.*, 1993). Its blood schizontocidal activity is too slow to be used as monotherapy in the treatment of acute malaria and hence it is often used in combination with a fast acting drug, such as quinine, which results in complete eradication of the infection in the blood (Metzger *et al.*, 1995a). The antimalarial action of doxycycline is due to the inhibition of ribosomal protein synthesis and chelation of calcium ions (Ca^{2+}) (Wernsdorfer, 1997). Doxycycline has also been shown to reduce the levels of nucleoside 5'-triphosphates and deoxynucleoside 5'-triphosphates (Pradines *et al.*, 2000).

1.3.4 Artemisinin

Artemisinin, also known as Qinghaosu, is a sesquiterpene lactone that was isolated from a Chinese herb, *Artemisia annua* L. in 1972. Its structure and pharmacological properties were first published by Chinese scientists in 1978 (Hayton and Su, 2008). Definitive antimalarial activities of artemisinin were reported in an English Chinese journal in 1979 (Qinghaosu Antimalarial Co-ordinating Research Group, 1979). Due to its low solubility in both water and oil, several derivatives of artemisinin were produced of which artemether, sodium artesunate

and dihydroartemisinin are available commercially (Wernsdorfer, 1997). Artemisinin is toxic to the malaria parasites at nanomolar concentrations (White, 1994) and it kills the parasites more rapidly than any other antimalarial drugs: artemisinins clear fever within 32 hours in contrast to the 2-3 days of other anti-malarial agents (Wiesner *et al.*, 2003). Artemisinin and its derivatives are effective against all the asexual stages and gametocytes, especially when administered in combination with other drugs, and thus may be able to decrease disease transmission (Nosten and White, 2007).

The exact mechanism of action for artemisinin is yet to be elucidated. Both parasite-specific and parasite non-specific mechanisms have been proposed (Golenser *et al.*, 2006). The pharmacophore in artemisinin is the 1,2,4-trioxane unit, and in particular, the endoperoxide bond is crucial for its antiparasitic activity. Studies have indicated that interactions between the endoperoxide bridge and iron in the haeme molecule released during digestion of haemoglobin inside the digestive vacuole might be responsible for the cytotoxic effects of artemisinin (Kannan *et al.*, 2005). The build up of redox active alkylated porphyrins has been proposed to generate free radicals that alkylate and oxidize proteins and lipids within infected red blood cells. This hypothesis is supported by the finding that the activity of artemisinin is potentiated by oxidising agents and oxygen and attenuated by reducing agents (Krungkrai and Yuthavong, 1987). However, evidence against the haeme hypothesis is that artemisinins are effective against ring stage parasites which do not have high concentrations of haeme (Olliaro *et al.*, 2001).

Another study proposed that intracellular iron-sulphur redox centres found in *Plasmodium* enzymes activated the artemisinins via reductive cleavage of the peroxide bond and the resulting alkylation of these enzymes could lead to parasite death (Wu, 2002). When radiolabelled artemisinin was incubated with parasite lysates, several interacting proteins, such as translationally controlled tumour proteins (TCTP), were identified suggesting that parasite death might result from alkylation and inactivation of parasite proteins (Bhisutthibhan and Meshnick, 2001; Bhisutthibhan *et al.*, 1998). Other proteins which have been proposed to be the target of artemisinin include cysteine proteases (Pandey *et al.*, 1999), which are involved in the catabolism of haemoglobin; proteins of the electron transport chain (Li *et al.*, 2005), which can provide the electron source required to activate the artemisinins and generate oxidative species, and membrane-associated proteins, which may form covalent adducts with artemisinins, such as PfATPase6, a sarco-endoplasmic reticulum calcium adenosine

triphosphatase (ATPase), [SERCA] (Eckstein-Ludwig *et al.*, 2003). Artemisinin has been shown to inhibit the activity of PfATPase6 in transfected *Xenopus laevis* oocytes, whereas other antimalarials have no effect on the ATPase activity (Eckstein-Ludwig *et al.*, 2003). Additional studies in the *X. laevis* oocyte expression system reported that an L263E point mutation abolished the inhibition caused by artemisinin (Uhlemann *et al.*, 2005). Furthermore, evidence from field studies and heterologous expression systems support a role for PfATPase6 mutations in the emerging resistance against artemisinins (Krishna *et al.*, 2006). However, recent studies provide evidence that the artemisinin derivatives cause early disruption of the parasite digestive vacuole membrane and with longer incubation times, extensive loss of organellar structures (del Pilar Crespo *et al.*, 2008). In addition, they also demonstrated that the artemisinin did not alter the morphology of the endoplasmic reticulum, where SERCA is located.

1.3.5 Resistance to antimalarial drugs

Drug resistant *Plasmodium falciparum* is wide spread in the Amazon region, tropical Africa and south-east Asia (Wellems and Plowe, 2001; Wootton *et al.*, 2002). Chloroquine resistance has been associated with an increase in mortality rates in endemic regions (Trape *et al.*, 1998).

A) Chloroquine resistance is due to impaired uptake of the drug into the parasite digestive vacuole, leading to a reduced accumulation of chloroquine within the digestive vacuole when compared to chloroquine sensitive parasites (Fitch, 1969; Fitch, 1970; Verdier *et al.*, 1985). This phenotype is commonly correlated with mutations in the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*), encoding a putative regulator of protein transport or a chloride channel localized to the parasite's food vacuole (Djimde *et al.*, 2001; Durand *et al.*, 2001); the *P. falciparum* multi-drug resistance gene 1 (*Pfmdr1*), encoding a P-glycoprotein homologue (PfPgh1) which belongs to the family of ABC transporters (Duraisingh *et al.*, 2000; Foote *et al.*, 1990b; von Seidlein *et al.*, 1997); and the *Pfcg2* genes (Cooper *et al.*, 2005). Mutations and copy number changes in the *Pfmdr1* are often associated with chloroquine resistance and *Pfcrt* mutations (Djimde *et al.*, 2001; Mita *et al.*, 2006; Mu *et al.*, 2003), but the contribution of *Pfmdr1* in modulating chloroquine resistance is still unknown (Hayton and Su, 2004). Some parasite isolates have the same *Pfcrt* and *Pfmdr1* alleles, but have different chloroquine phenotypes as measured by their IC_{50} values, suggesting that additional genes modulate the parasite response to chloroquine (Mu *et al.*, 2003).

(B) Point mutations in dihydrofolate reductase and dihydropteroate synthase of the folate pathway produces moderate to high resistance to the antifolates, pyrimethamine and cycloguanil (the active metabolite of proguanil) (Basco *et al.*, 1995; Cowman *et al.*, 1988; Sirawaraporn, 1998); and sulphonamides and sulfones (Brooks *et al.*, 1994; Triglia and Cowman, 1994; Triglia *et al.*, 1997). There is a clear association between the number of point mutations in the *dhfr* and *dhfs* gene and the extent of resistance *in vitro* to the antifolates (Foote *et al.*, 1990a; Gesase *et al.*, 2009; Nzila-Mounda *et al.*, 1998; Peterson *et al.*, 1988). A quadruple mutant observed along the Thai-Myanmar border shows the highest IC_{50} to pyrimethamine so far (Hyde, 2008; Nair *et al.*, 2003). Triple mutants have conferred a 24-fold increase in sulfadoxine resistance over the wild type (Triglia *et al.*, 1997). Gene expression changes or mutations in other genes expressing enzymes of the folate metabolism pathway may also contribute to sulfadoxine-pyrimethamine (Fansidar®) resistance (Kidgell *et al.*, 2006; Volkman *et al.*, 2007; Wang *et al.*, 1997).

(C) The mechanisms of resistance for amino-alcohols, mefloquine, quinine and halofantrine, are still unclear. An increase in the copy number of *Pfmdr1* has been associated with an increase in the mefloquine IC_{50} (Cowman *et al.*, 1994; Peel *et al.*, 1994; Price *et al.*, 2004; Wilson *et al.*, 1989). Furthermore, disruption of one of two copies of the *Pfmdr1* gene resulted in a decrease in the IC_{50} of mefloquine, artemisinin, halofantrine, lumefantrine and quinine (Sidhu *et al.*, 2006). However, mefloquine resistance in field isolates do not always correlate with an increase in the copy number of *Pfmdr1* (Chaiyaroj *et al.*, 1999). Moreover, increased mefloquine IC_{50} values are often found in parasites with increased IC_{50} values for quinine and halofantrine, thus higher copy numbers of *Pfmdr1* could be due selection of quinine and/or other drugs (Cowman *et al.*, 1994). Amplification of *Pfmdr1* may be a compensatory response of the parasite to the changes in the food vacuole due to mutations in *Pfcrt* and other genes (Jiang *et al.*, 2008).

High quinine IC_{50} values have been associated with *Pfcrt* encoding a putative Na^+/H^+ exchanger (PfNHE), *Pfmdr1* encoding a P-glycoprotein homologue (PfPgh1), and a locus on chromosome 9 (Ferdig *et al.*, 2004). This has been substantiated by the N1042D amino acid substitution in PfPgh1 contributing to quinine resistance (Sidhu *et al.*, 2006), and elevated activities of PfNHE found in parasites with high quinine IC_{50} values (Bennett *et al.*, 2007). Both PfNHE and PfPgh1 may play a role in regulating the pH of the cytoplasm and/or digestive vacuole of the parasite, and thus mutations in these proteins may cause changes in drug accumulation (Bennett *et al.*,

2007; Rohrbach *et al.*, 2006). Mutations in other transporters, especially ABC transporters, might also contribute to quinine resistance as the parasite's response to quinine is most probably a multi-gene trait which would explain why quinine is still effective in eradicating malaria parasites after ~350 years of use (Ferdig *et al.*, 2004; Mu *et al.*, 2003).

(D) Resistance to artemisinin has yet to be confirmed, but recent reports of high failure rates with artemisinin-based combination therapies (Carrara *et al.*, 2009; Denis *et al.*, 2006a; Denis *et al.*, 2006b; Rogers *et al.*, 2009; Vijaykadga *et al.*, 2006; Wongsrichanalai and Meshnick, 2008) in addition to decreased *in vitro* susceptibility to artemisinin (Jambou *et al.*, 2005) have been reported in Thailand and Cambodia. Parasites with significantly reduced *in vitro* susceptibility to lumefantrine, artesunate and mefloquine had increased copy numbers of *Pfmdr1* (Lim *et al.*, 2009). The presence of three or more copies of the *Pfmdr1* gene was associated with recrudescence in patients treated with artesunate-mefloquine but not with recrudescence in patients treated with artesunate-lumefantrine (Lim *et al.*, 2009). Another candidate gene associated with increased IC_{50} values to artemisinin derivatives is *SERCA-PfATPase6* (Jambou *et al.*, 2005). However, Noedl *et al.* (2008) found prolonged parasite clearance times in Cambodian patients treated with artesunate alone and this was not mediated by a change in the copy number of *Pfmdr1* gene or selected polymorphisms in the *PfATPase6* gene.

1.3.6 Immunomodulatory effects of antimalarial drugs

1.3.6.1 Effect of antimalarial drugs on cytokine production

Chloroquine was found to inhibit phytohaemoagglutinin or lipopolysaccharide induced production of the proinflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β by peripheral blood mononuclear cells without causing cell cytotoxicity (Karres *et al.*, 1998; Picot *et al.*, 1991; van den Borne *et al.*, 1997). Chloroquine exerts its inhibitory action by blocking the conversion of the TNF-α precursor to mature soluble protein and reduced the levels of IL-1β and IL-6 mRNA by decreasing the stability of the mRNA by a pH-dependent mechanism (Jang *et al.*, 2006). At concentrations that blocked calcium-activated potassium channels in alveolar macrophages, quinine, a potassium channel blocker, dose-dependently inhibited the secretion of TNF-α without affecting the phagocytosis of latex beads. This was as a result of quinine inhibition of TNF-α mRNA expression (Maruyama *et al.*, 1994).

Doxycycline inhibited staphylococcal exotoxin-stimulated T-cell proliferation in a dose-dependent manner. In addition, doxycycline dose-dependently down-regulated the secretion of the cytokines IL-1β, IL-6, TNF-α and IFN-γ and chemokines monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β by human peripheral blood mononuclear cells incubated with staphylococcal exotoxins (Krakauer and Buckley, 2003). The suppression of proinflammatory cytokines may involve the down regulation of protein kinase C (Webster *et al.*, 1994).

Chloroquine decreases the MHC I and MHC II expression on antigen-presenting macrophages (Nowell and Quaranta, 1985; Schultz *et al.*, 1995) and hence results in decreased stimulation of CD4+ T cells and decreased cytokine production which would usually be secreted after interaction between the antigen presenting cell and the T cells. This is substantiated by the finding that chloroquine and pyrimethamine both inhibited T cell IL-2 production and IL-2-driven T cell proliferation, and decreased responsiveness of T cells clones to IL-2 (Landewe *et al.*, 1995; Viora *et al.*, 1996). Chloroquine, mefloquine and quinine have all been found to decrease the activity of natural killer cells with mefloquine and quinine being the most potent (Pedersen *et al.*, 1986).

In the clinical setting, antimalarial drugs were also found to modulate cytokine production in malaria patients: Korean patients with *P. vivax* malaria were treated with chloroquine/primaquine combined chemotherapy which resulted in a decrease in serum levels of interferon gamma inducible protein-10 that was strongly reactive prior to treatment (Kim *et al.*, 2006). Treatment of *P. falciparum*-infected patients with quinine or artesunate, resulted in a dramatic increase in plasma TNF-α after quinine administration followed by a rapid clearance, but no effect on plasma TNF-α concentration after artesunate administration as artesunate clears the parasite more rapidly than quinine (Ittarat *et al.*, 1999). When Sudanese patients with acute *P. falciparum* malaria and control subjects were treated with chloroquine, concentrations of erythropoietin (a hormone which increases the rate of red blood cell production, namely erythropoiesis) was decreased in the control subjects but increased in the malaria subjects. This was attributed to the anti-inflammatory action of chloroquine as the concentrations of proinflammatory cytokines, IFN-γ, IL-1 and IL-6 decreased with chloroquine treatment. Hence, it was proposed that the impaired erythropoietin production associated with the prolonged elevation in proinflammatory cytokines characteristic of malaria infection can contribute to the

anaemia observed in malaria patients (Ballal *et al.*, 2009). When Balb/c mice infected with *Plasmodium yoelli* 17XL were treated with chloroquine, TNF-α mRNA expression was up-regulated and expression of anti-inflammatory cytokines, IL-10 and TGF-β, was down-regulated. Treatment of the same mice with pyrimethamine increased IFN-γ mRNA expression, but up-regulated TGF-β (Ramos-Avila *et al.*, 2007). However, the effects of drugs on cytokine levels in malaria-infected patients could also be as a result of the eradication of the malaria parasite.

1.3.6.2 Effect of antimalarial drugs on monocyte metabolism

Antimalarial drugs have also been shown to affect metabolic processes in cells. Quinine resulted in a significant increase in phosphatidylserine and phosphatidylinositol synthesis and a pronounced decrease in phosphatidylcholine and phosphatidylethanolamine synthesis in the human monocytic cell line, THP1 (Pelassy *et al.*, 1992). Quinine has also been shown to increase phosphatidylserine synthesis in the Jurkat T-cell line (Aussel *et al.*, 1990). Quinidine dose-dependently inhibited naphthol AS-D chloroacetate esterase in polymorphonuclear neutrophils and α-naphthyl acetate esterase and α-naphthyl butyrate esterase in monocytes. Other quinoline drugs, such as chloroquine, primaquine and quinine also affected the esterases but to a lesser extent (Markovic *et al.*, 1988). Artemisinin was found to increase the protein levels of protein kinase Cβ1 isoform and enhance protein kinase C activity in the human promyelocytic leukaemia HL-60 cell line (a promonocytic cell line) treated with 1α,25,-dihydroxyvitamin D_3 and thus potentiated cell differentiation predominantly into monocytes (Kim *et al.*, 2003).

Chloroquine is a weak diprotic base that can pass through lipid membranes and accumulate in acidic cytoplasmic vesicles. Inside the vesicle, chloroquine is protonated and trapped within the vesicle (Homewood *et al.*, 1972). An adenosine triphosphate pump pumps more hydrogen ions into the cell, which attracts more unprotonated chloroquine into the vesicle, resulting in high chloroquine concentrations in intracellular vesicles. This effect is more pronounced in cells with an increased number of acidic vesicles such as phagocytosing cells and macrophages (Ferrante *et al.*, 1986). This increased chloroquine concentration in lysosomes results in the accumulation of inclusion bodies containing phospholipids from the plasma membrane which leads to decreased phagocytosis, chemotaxis and overall cell functioning. Consequently, chloroquine will also delay the recycling of enzymes and surface receptors from lysosomes to

the cell surface. The depletion of cell surface receptors decreases the cell's responsiveness to mitogenic stimuli (Homewood *et al.*, 1972).

1.3.6.3 Effect of antimalarial drugs on monocyte surface receptors

Therapeutic concentrations of quinine, chloroquine, primaquine, pyrimethamine, artemisinin, mefloquine and proguanil reduced the cytoadherance of *P. falciparum*-infected red blood cells to monocytes by 40% or more, indicating a down regulation in the expression of monocyte cytoadherance receptors. Quinine, proguanil and pyrimethamine were the most effective in down regulating the cytoadherance receptor expression with artemisinin being the least effective (Goldring and Nemaorani, 1999).

Treatment of human monocytic U937 cells and peripheral blood monocytes with chloroquine significantly down-regulated the expression of TNF receptors on the cell surface by retarding the transport of the receptors to the cell surface (Jeong *et al.*, 2002). Chloroquine also suppressed β1-integrin mediated U937 cell-cell adhesion and this suppression appears to be mediated by the lysosomotropic character of chloroquine (Cho, 2008).

1.3.6.4 Effect of antimalarial drugs on the production of monocyte reactive oxygen and nitrogen intermediates

Reactive oxygen intermediates (ROI), such as singlet oxygen, superoxides, peroxides, hydroxyl radical and hypochlorous acid, and reactive nitrogen intermediates (RNI), such as nitric oxide, nitrite and nitrate, are produced by activated macrophages as part of the host's first line of defence against malaria (Clark and Hunt, 1983; Kremsner *et al.*, 1993b; Rockett *et al.*, 1991). Therapeutic concentrations of quinine and halofantrine caused a significant decrease in IFN-γ-induced RNI production in mouse peritoneal macrophages, whereas chloroquine, quinine, and halofantrine had no effects on ROI (Kremsner *et al.*, 1993a). Furthermore, quinine was found to inhibit RNI and ROI production in lipopolysaccharide-activated goldfish macrophages (Stafford *et al.*, 2002), which was associated with transcriptional changes in the inducible nitric oxide synthase gene. In human neutrophil granulocytes, therapeutic serum levels of dihydroartemisinin, artemisinin and artesunate enhanced the generation of ROI (Wenisch *et al.*, 1997), whereas therapeutic concentrations of quinine inhibited the oxidative burst in human peripheral neutrophils (el Benna and Labro, 1990). Neutrophil oxidative burst induced by

phorbol-12-myristate-13-acetate was also found to be reduced by doxycycline in a dose dependent manner (Sinico-Durieux *et al.*, 1986). C57B1/6 mice infected with *Plasmodium berghei* ANKA were treated with artemether or chloroquine, which significantly increased the capacity of the mice to produce ROI and RNI. This was further corroborated by high levels of inducible nitric oxide synthase gene expression (Prada *et al.*, 1996b).

1.3.6.5 Effect of antimalarial drugs on monocyte phagocytosis

Therapeutic concentrations of chloroquine, quinine, mefloquine, amodiaquine and artemether reduced the binding of immunoglobulin G (IgG) to *P. falciparum*-infected red blood cells and the subsequent phagocytosis of the infected red blood cells by a human monocytic J-111 cell line (Shalmiev *et al.*, 1996). Peripheral blood mononuclear cells isolated from healthy subjects receiving prophylactic doses of chloroquine exhibited reduced phagocytosis of opsonised sheep red blood cells and non-opsonised zymosan particles (Osorio *et al.*, 1992). Furthermore, chloroquine and quinine sulphate were shown to suppress the phagocytic activity of Fc-coated sheep red blood cells, latex beads and *Staphylococcus aureus* by monocytes isolated from normal rhesus monkeys (*Macaca mulatta*) treated with chloroquine or quinine sulphate (Prasad *et al.*, 1986; Prasad *et al.*, 1984).

1.4 Aim of study

Both antimalarial drugs and haemozoin (β-haematin) have immunomodulatory effects on monocytes. Antimalarial drugs have been shown to reduce the phagocytosis of *P. falciparum* infected red blood cells (Shalmiev *et al.*, 1996) and although haemozoin (β-haematin) is avidly phagocytosed by the monocytes (Schwarzer *et al.*, 2001), it modifies a number of monocyte functions that contribute towards the immunodepression associated with malaria (Schwarzer *et al.*, 2008). Haemozoin loading of monocytes leads to suppression of IFN-γ responsiveness, failure of MHC II up regulation, disturbances in antigen presentation to T cells and a reduced up-regulation of ICAM-1, an adhesion molecule that enables monocytes to adhere and stimulate T-cell proliferation (Schwarzer *et al.*, 1998; Scorza *et al.*, 1999). Neopterin, a marker of T helper cell subtype 1 activated cellular immune response, is secreted by IFN-γ-stimulated monocytes/macrophages (Murr *et al.*, 2002). Neopterin is elevated in malaria infection but decreases with antimalarial drug treatment in acute malaria (Brown *et al.*, 1990). The effects of antimalarial drugs on the phagocytosis of β-haematin by monocytes as well as the IFN-γ-

stimulation of monocytes are unknown. Measuring neopterin secretion should give an indication of the activation or inhibitory effects of the antimalarial drugs on the IFN-γ-stimulation of the monocytes. Thus the aim of this investigation was to determine the immunomodulatory effects of antimalarial drugs on the phagocytosis of β-haematin and the secretion of neopterin by monocytes.

Chapter 2

Effect of antimalarial drugs on the phagocytosis of haemozoin (β-haematin) by monocytes.

B.M. Cumming

Abstract

Malaria is the cause of approximately one million deaths a year especially amongst children. During the blood stage of infection, the malaria parasite digests the haemoglobin of the erythrocyte into amino acids and releases toxic free haeme that is sequestered into an insoluble non-toxic crystalline pigment called haemozoin. Following erythrocyte rupture, haemozoin is released into the blood stream and phagocytosed by circulating monocytes. Phagocytosed haemozoin has been reported to have deleterious effects on monocyte functions, such as inhibition of re-phagocytosis of bacteria and viruses, release of cytokines and inhibition of oxidative burst. This study aimed to determine the effects of seven antimalarial drugs, amodiaquine, artemisinin, chloroquine, doxycycline, primaquine, pyrimethamine and quinine, on the monocyte phagocytosis of β-haematin, a synthetic equivalent of haemozoin, and latex beads. Mouse J774A.1 cells, human peripheral blood mononuclear cells and phorbol-12-myristate-13-acetate-differentiated human U937 cells were treated with therapeutic concentrations of the drugs prior to exposure to β-haematin or latex beads. The effect on phagocytosis was determined by both counting and a novel spectrophotometric method. Artemisinin, doxycycline and pyrimethamine resulted in more than 10% activation of β-haematin phagocytosis by mouse J774A.1 cells, whilst artemisinin, primaquine, pyrimethamine, and quinine activated β-haematin phagocytosis in peripheral blood mononuclear cells by more than 10%. Only pyrimethamine induced more than 10% activation of β-haematin phagocytosis in the U937 cells. Both chloroquine and amodiaquine resulted in more than 10% inhibition of β-haematin phagocytosis in the peripheral blood mononuclear cells. In contrast, only quinine resulted in more than 10% inhibition of latex beads in the J774A.1 cells. Thus, the latex beads and β-haematin appear to be phagocytosed by different mechanisms. The small but significant effects on phagocytosis appear to be dependent on the antimalarial drug used to treat the cells as well as the substance being phagocytosed.

2.1 Introduction

An estimated 250 million cases of malaria, which is endemic in 109 countries, occur annually, leading to approximately 1 million deaths, mostly of children under 5 years (WHO, 2008). Malaria is caused by intracellular parasitic protozoa of the genus *Plasmodium*. The host's immunological response to the parasite could contribute to the pathophysiology of the disease in humans (Malaguarnera and Musumeci, 2002; Miller *et al.*, 2002). Phagocytic cells provide the first line of defence against the malaria parasite by recognizing the parasitized erythrocytes as foreign cells. This leads to the extracellular generation of aggressive oxidative compounds, and phagocytosis. (Abdalla, 1990; Perrin *et al.*, 1982). *In vitro* and *in vivo* studies have shown that human monocytes and resident macrophages avidly phagocytose erythrocytes that are parasitized by trophozoites and schizonts of *Plasmodium falciparum*, and the malaria pigment called haemozoin (Celada *et al.*, 1983a; Turrini *et al.*, 1992).

Haemozoin is produced in the parasite during the intraerythrocytic stage in the human host. As the parasite develops from the early ring stage to the trophozoite stage, it ingests haemoglobin from the red blood cell cytoplasm by pinocytosis via a plasma membrane invagination known as a cytostome. Haemoglobin transport vesicles transport the haemoglobin to the acidic food vacuole (also known as a digestive vacuole) where the haemoglobin is digested by four plasmepsins (aspartic proteases, Banerjee *et al.*, 2002), three falcipains (cysteine proteases, Rosenthal *et al.*, 2002) and a zinc metalloprotease (falsilysin, Eggleson *et al.*, 1999) into peptides of about 20 amino acids long. These are exported from the food vacuole and finally degraded into amino acids, probably by aminopeptidases in the parasite cytoplasm (Gavigan *et al.*, 2001). About 15% of these amino acids are then utilised in parasite protein synthesis (Krugliak *et al.*, 2002). During the catabolism of haemoglobin in the food vacuole, free haeme (iron (II) protoporphyrin IX) is released and oxidised to generate iron (III) protoporphyrin IX, which is toxic to both the parasite and the host cell as it can generate reactive oxygen species. The parasite does not contain haeme oxygenase, an enzyme that can cleave the porphyrin ring into an open chain tetrapyrrole, which is necessary for cellular excretion (Eckman *et al.*, 1977). To protect itself, the parasite detoxifies free haeme by neutralisation with histidine rich protein II (Huy *et al.*, 2003; Sullivan *et al.*, 1996b), degradation with reduced glutathione (Atamna and Ginsburg, 1995; Huy *et al.*, 2002a; Huy *et al.*, 2002b) or crystallisation into insoluble haemozoin, produced in the food vacuole (Francis *et al.*, 1997; Sullivan *et al.*, 1996b). Recent studies have indicated that haemozoin formation is the major route whereby haeme is

detoxified in the parasite since the quantity of haemozoin formed corresponds to about 88% of the haeme in the erythrocyte (Egan *et al.*, 2002; Gligorijevic *et al.*, 2006).

Haemozoin is a cyclic dimer of ferriprotoporphyrin IX in which the propionate group of each ferriprotoporphyrin IX molecule coordinates to the iron(III) centre of its partner, while the dimers are linked through hydrogen bonding of the propionic acid groups (Pagola *et al.*, 2000). The mechanism of formation of haemozoin is poorly understood. Several theories have been proposed, but it appears that a biomineralisation process is favoured, requiring a nucleation site for crystal growth. Initiation could involve lipids (various unsaturated fatty acids and mono- and dioleoylglycerol) or proteins (histidine rich protein (HRP) or the haeme detoxification protein (HDP) (Jani *et al.*, 2008)) or both (Egan, 2008b). Pigment is released along with the merozoites into the blood stream when the erythrocyte bursts and is phagocytosed (along with associated proteins and lipids of parasite and erythrocyte origin, Goldie *et al.*, 1990) by circulating monocytes, neutrophils and resident macrophages.

Ingested haemozoin persists unmodified for long periods within the phagocyte and modifies a number of monocyte functions (Schwarzer *et al.*, 2008). Haemozoin causes inhibition of the following monocyte functions: PMA-elicited respiratory burst (Schwarzer *et al.*, 1992), ability to repeat phagocytosis (Schwarzer *et al.*, 1992), membrane translocation and activity of protein kinase C (Schwarzer *et al.*, 1993), activity of NADPH oxidase (Schwarzer and Arese, 1996), ability to kill ingested bacteria, fungi, or tumour cells (Fiori *et al.*, 1993), expression of ICAM-1, integrin-CD11c, MHC-class II (Interferon-γ-mediated) (Schwarzer *et al.*, 1998) and differentiation of monocytes into functional, antigen-presenting dendritic cells (Skorokhod *et al.*, 2004). Phagocytosed haemozoin stimulates the accumulation of lipoperoxidation products in monocytes (Schwarzer *et al.*, 2003), activates metalloproteinase-9 in monocytes (Prato *et al.*, 2005), and stimulates the production of pro-inflammatory chemokines, such as macrophage inflammatory protein (MIP)-1α and -1β, MIP-2, and monocyte chemoattractant protein (MCP)-1, and the production of pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-6 (Jaramillo *et al.*, 2004). Most of these effects are a result of the large amounts of lipoperoxidation products, such as monohydroxy derivatives of arachidonic acid and linoleic acid, and 4-hydroxynonenal (HNE) generated by non-enzymatic catalysis of haemozoin (Schwarzer *et al.*, 2003; Skorokhod *et al.*, 2005).

Antimalarial drugs appear to perturb the functions of monocytes in a number of ways. Many antimalarial drugs inhibit cytokine production by the monocytes. Chloroquine and quinine inhibit the production of monocyte tumour necrosis factor (Kwiatkowski and Bate, 1995; Maruyama *et al.*, 1994; Picot *et al.*, 1991). Chloroquine inhibits the production of IL-1β and IL-6 from lipopolysaccharide-stimulated monocytes (PBMC, THP-1 and U937 monocytic cells) by reducing the level of IL-1β and IL-6 mRNA (Jang *et al.*, 2006; Salmeron and Lipsky, 1983). Doxycycline dose dependently inhibited the production of cytokines IL-1β, IL-6, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) and the chemokines MCP-1, MIP-1α, and MIP-1β by PBMC incubated with staphylococcal endotoxins (Krakauer and Buckley, 2003). Chloroquine and pyrimethamine both interfere with IL-2 production and hence inhibit T-cell proliferation (Landewe *et al.*, 1995; Viora *et al.*, 1996).

Other effects of the antimalarial drugs include alterations in phospholipid metabolism in monocytes: quinine markedly increased phosphatidylserine and phosphatidylinositol synthesis and strongly decreased phosphatidylcholine and phosphatidylethanolamine synthesis (Pelassy *et al.*, 1992). Quinidine inhibits α-naphthyl acetate esterase and α-naphthyl butyrate esterase in monocytes. Quinine, chloroquine and primaquine also inhibit the monocyte esterases but to a lesser extent (Markovic *et al.*, 1988). Artemisinin increased protein kinase C activity and the level of protein kinase Cβ1 isoforms in human promyelocytic leukaemia cells (human promonocytes), and enhanced cell differentiation predominantly into monocytes (Kim *et al.*, 2003). Quinine, chloroquine, primaquine, pyrimethamine, artemisinin, mefloquine and proguanil all decreased the cytoadherance of *P. falciparum* to monocytes by 40% or more due to down regulation of the expression of monocyte receptors (Goldring and Nemaorani, 1999). Treatment of U937 cells with chloroquine significantly down-regulated the expression of cell surface TNF receptors and a similar effect was observed with human peripheral blood monocytes (Jeong *et al.*, 2002). Chloroquine also suppressed β1-integrin mediated U937 cell-cell adhesion (Cho, 2008).

Antimalarial drugs have been shown to inhibit monocyte phagocytosis. Chloroquine at doses recommended for malaria prophylaxis inhibited the Fc-receptor mediated phagocytosis of sheep red blood cells and non-immunologic receptor-mediated phagocytosis of zymosan particles by PBMC (Osorio *et al.*, 1992). Chloroquine, quinine, mefloquine, amodiaquine and artemether were all found to reduce the binding of immunoglobulin G (IgG) to *P. falciparum*

infected red blood cells, and thus reduced their subsequent phagocytosis by a human monocytic J-111 cell line (Shalmiev *et al.*, 1996). Mefloquine, proguanil and cycloguanil, at concentrations higher than 0.5 µg/ml, reduced the phagocytosis of opsonised *Staphylococcus aureus* by leucocytes. Quinine, chloroquine and pyrimethamine had no inhibitory effect on the phagocytosis of *S. aureus* (Kharazmi and Eriksen, 1986).

Thus, phagocytosed haemozoin as well as antimalarial drugs perturb monocyte functions. Haemozoin plays a key role in the induction of immunosuppression associated with malaria (Scorza *et al.*, 1999) and antimalarial drugs have been shown to inhibit phagocytosis (Shalmiev *et al.*, 1996). Phagocytosis still occurs in malaria patients that are being treated with antimalarial drugs but the effect of the antimalarial drugs on the phagocytosis of haemozoin is unknown. Synthetic haemozoin, also called β-haematin, is chemically and structurally identical to the native pigment (Egan, 2002). Thus, in this study, two monocytic cell lines, J774A.1 and U937, and peripheral blood mononuclear cells (PBMC) were characterised for their monocyte/macrophage properties, β-haematin was synthesized and the effects of seven antimalarial drugs (amodiaquine, artemisinin, chloroquine, doxycycline, primaquine, pyrimethamine and quinine) on the phagocytosis of β-haematin by J774A.1 cells were compared to that of latex beads using a counting assay. A spectrophotometric assay was developed to determine the effect of the drugs on the phagocytosis of β-haematin by the U937 cell line and PBMC. Knowledge of the effects of the drugs on phagocytosis of β-haematin might give an indication of whether the antimalarial drugs enhance or suppress the immunosuppression caused by malaria in addition to eradicating the parasite.

2.2 Materials and Methods

2.2.1 Materials

Common chemicals and buffer salts were purchased from Saarchem (South Africa) and Sigma-Aldrich (Germany). DMEM, RPMI-1640, Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin, L-glutamine, phorbol-12-myristate-13-acetate, chloroquine diphosphate, quinine hydrochloride, pyrimethamine, primaquine diphosphate, artemisinin, doxycycline hydrochloride, amodiaquine dihydrochloride dihydrate, porcine haematin, latex beads (10% w/v, 0.8 µm), Histopaque®-1077, 3,3'-diaminobenzidine, sodium cacodylate, methyl green, trypan blue, dimethyl sulphoxide (DMSO, tissue culture grade) and sodium acetate trihydrate were

purchased from Sigma-Aldrich (Germany). 4-Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Roche Diagnostics (Germany). 50% (v/v) Glutaraldehyde (E.M. Grade), haemin chloride and Giemsa stain were purchased from Fluka Biochemika (Germany). Foetal calf serum (FCS) was obtained from Gibco, Invitrogen (U.S.A.). Hydrochloric acid, acetic acid, hydrogen peroxide (30%, v/v), acetone and sodium hydroxide were purchased from Merck (Germany). Lead nitrate and sodium citrate were obtained from BDH (England). Osmium tetroxide was purchased from Electron Microscopy Services (U.S.A.). Agar 100 Resin (Epon 812), Araldite, dodecenyl succinic anhydride (DDSA), 2,4,6-tri(dimethylaminoethyl)phenol (DMP) and Copper grids (200 Mesh) were purchased from Agar Scientific (England). Uranyl acetate was purchased from TED Pella, Inc. (Canada). Nunc (Denmark) T-flasks (75 cm^2 and 25 cm^2), Delta (Spain) 15 ml, 50 ml tubes and Falcon (U.S.A.) 35 x 10 mm Petri-dishes were used. VacuCap PF Bottle-top Filter devices were obtained from PALL Life Sciences (U.S.A.). Distilled water (dH_2O) was produced by a Milli-Q Plus ultra pure water system (Millipore, U.S.A.).

2.2.2 Culturing of J774A.1 and U937 cell lines

Both the mouse monocyte-macrophage cell line, J774A.1 (Ralph and Nakoinz, 1975; Ralph and Nakoinz, 1977a; Ralph and Nakoinz, 1977b; Ralph *et al.*, 1975) and the human monocyte-like U937 cell line (Fischer *et al.*, 1980; Koren *et al.*, 1979; Ralph *et al.*, 1976; Sundstrom and Nilsson, 1976) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, U.K.). The J774A.1 cell line was cultured in complete DMEM with 10% heat-inactivated FCS and 4 mM L-glutamine. Subcultures were prepared by lifting the cells with 0.02% (w/v) EDTA in HBSS and transferring the cells to fresh medium every 2 to 3 days. The U937 cell line was cultured in complete RPMI-1640 medium supplemented with 10% FCS (NOT heat-inactivated), 4 mM L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. This cell line was subcultured every 2-3 days. Both cell lines were grown at 37ºC under a humidified atmosphere of 5% CO_2.

2.2.3 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from the South African National Blood Service on the second day after collection. The buffy coats were initially diluted with half the volume of complete medium (RPMI-1640, 10% FCS, 4 mM L-

Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin). Buffy coat (25 ml) was layered on top of 15 ml Histopaque®-1077 and centrifuged at 400 *g* for 30 min at RT (Boyum, 1968). The first layer (plasma) was aspirated off and the opaque layer of mononuclear cells was transferred to a sterile tube. Aliquots (5ml) of the mononuclear cells were washed with 10 ml of complete media and the suspension was spun at 150 *g* for 10 min. The slow centrifugation speed ensured that most of the platelets stayed in suspension. After aspirating the supernatant and removing the platelets, the pellet was washed twice in complete media (Roos and de Boer, 1986). The cells were resuspended in complete media to a concentration of 8 x 10^6 cells/ml (Prato *et al.*, 2008). The viability and the concentration of the cells were determined by trypan-blue exclusion (98%) and the cells were counted using a haemocytometer at 400x magnification (Strober, 2001).

2.2.4 Adhesion of J774A.1, U937 and PBMC (monocytes) onto coverslips

J774A.1 cells (2 x 10^5 cells/ml) and PBMC (8 x 10^6 cells/ml) were seeded onto coverslips (22 x 22 mm^2) in 35 x 10 mm Petri-dishes and incubated for 2 h at 37ºC under humidified 5% CO_2 to allow the monocytes to adhere to the coverslips (Cline and Lehrer, 1968; Ralph *et al.*, 1975). The coverslips were washed with complete media to remove non-adherent cells. U937 cells (2 x 10^5 cells/ml) were differentiated by incubation with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 h (Goldring *et al.*, 1992) at 37ºC with the resultant adhesion of the cells to the coverslips in Petri-dishes.

2.2.5 Characterisation of the cell lines

Monocytes were stained to ascertain their adherence to plastic and phagocytosis properties. Monocytes were fixed with methanol, and stained with 2% (w/v) methyl green or Giemsa at RT (Cline and Lehrer, 1968; Gurr, 1965). The coverslips were then washed three times in dH_2O, allowed to air dry and mounted onto slides using DPX mountant.

Alkaline phosphatase activity was detected with 0.377 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.367 mM 4-nitro blue tetrazolium chloride (NBT) in 50 mM Tris-HCl, 5 mM $MgCl_2$ (pH 9.5). Peroxidase activity was detected with 1.5 mM 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl (pH 8) with 0.03% H_2O_2. Adherent cells were incubated with the substrates

for 20 min in the dark at RT. The coverslips were air dried, mounted and viewed at 1000x magnification under oil immersion. (Van Noorden and Frederiks, 1992)

2.2.6 Synthesis and analysis of β-haematin

β-haematin was prepared according to Egan *et al.* (1994). Briefly, haemin chloride (30 mg) was dissolved in 6.0 ml of 0.1 M NaOH and equilibrated at 60ºC. To this solution, 0.6 ml of 1.0 M HCl and 3.7 ml of 12.9 M sodium acetate (pH 5) pre-incubated at 60ºC were added and the mixture was stirred at 60ºC for 1 h. The crystals were filtered (8 µm cellulose acetate/nitrate Millipore filter Type SC), washed extensively with dH_2O and dried at 37 ºC over silica gel for 48 h.

β-haematin was also prepared according to the method of Slater *et al.* (1991) as follows: porcine haematin (30 mg) was dissolved in 8 ml of 0.1 M NaOH. The porphyrin was precipitated by the addition of 2.86 ml of acetic acid and the suspension was heated at 70ºC overnight. The following day, the suspension was spun at 20 000 *g* for 10 min at RT to pellet the β-haematin. The supernatant was discarded and the pellet was washed three times with dH_2O. The unreacted haematin was removed by extracting the pellet twice for 3 h with 20 ml of 0.1 M sodium bicarbonate buffer, pH 9.1. The β-haematin was recovered by centrifugation at 20 000 *g* for 10 min at RT and washed three times with dH_2O (20 ml). The final washed pellet was dried at 37ºC over silica for 48 h.

The β-haematin crystals from both synthetic methods were mounted onto stubs covered with carbon tape and viewed under a Philips environmental scanning electron microscope (ESEM) with a large field detector and a gaseous secondary electron detector. X-Ray microanalysis of the crystals was performed on the ESEM at 2000x magnification, an accelerating voltage of 15 kV and a spot size of 4.5 for 150 s under low vacuum. In addition, β-haematin crystals were embedded in Epon-Araldite (Section 2.2.7) and viewed using transmission electron microscopy.

2.2.7 Preparation of samples for transmission electron microscopy (TEM)

Adherent J774A.1 cells were incubated with β-haematin or latex beads for 30 min or 2 h. The cells were scraped off the bottom of the T-flasks and washed in HBSS. A small aliquot of the cell suspension was allowed to adhere to coverslips, followed by staining with 2% (w/v) methyl

green (Section 2.2.5) for viewing with bright field microscopy. The rest of the cells were fixed overnight at 4ºC in 3% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.1. The fixed cells were rinsed twice for 30 min each in 0.05 M sodium cacodylate buffer, pH 7.1 and postfixed in 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer, pH 7.1 for 30 min. After washing twice for 30 min each in the same buffer, the samples were dehydrated in a series of acetone concentrations (10, 40, 60, 80, 90, 100%) for 10 min each. The samples were then embedded in a series of Epon-Araldite (1 part Epon 812, 1 part Araldite CY212, 3 parts DDSA) concentrations in acetone (25, 50, 75, 100%) with equivalent drops of 2,4,6-tri(dimethylaminoethyl)phenol per ml of Epon-Araldite used for 2 h each. The embedding in 100% Epon-Araldite was repeated for 24 h at RT and again at 70ºC for 48 h to cure the samples. The polymerized resin was trimmed and ultra thin sections (100 nm) were cut on a LKB Ultratome III using a glass knife and collected on copper grids with 200 Mesh. The samples were stained in 2% (w/v) uranyl acetate for 10 min and, after washing with dH_2O, in 0.08 M lead citrate for 5 min followed by washing with dH_2O. The ultrathin sections were observed in a Philips CM120 transmission electron microscope. Images were acquired using a Megaview III Soft Imaging System.

2.2.8 Treatment of monocytes with antimalarial drugs

Complete culture media (2 ml) containing physiological therapeutic concentrations of one of seven antimalarial drugs, were added to each Petri-dish containing adherent cells (J774A.1, U937 or PBMC) and incubated for 18 h at 37ºC under humidified 5% CO_2. The cells were treated with antimalarial drugs for 18 h as this time period was compatible to other assays in which the effect of antimalarial drugs on the expression of monocyte receptors and TNF secretion was investigated (Goldring and Nemaorani, 1999; Kwiatkowski and Bate, 1995). The antimalarial drugs included amodiaquine, 75.6 ng/ml (White *et al.*, 1987; Winstanley *et al.*, 1987; Winstanley *et al.*, 1990); artemisinin, 400 ng/ml (Wernsdorfer, 1997); chloroquine, 200 ng/ml (Desjardins *et al.*, 1988); doxycycline, 3 µg/ml (Wernsdorfer, 1997); primaquine, 153 ng/ml (Desjardins *et al.*, 1988); pyrimethamine, 234 ng/ml; and quinine, 15 µg/ml (Wernsdorfer, 1997). Controls were also set up containing the concentrations of solvents used to solubilise the antimalarial drugs.

2.2.9 Measuring phagocytosis of β-haematin and latex beads by monocytes

β-haematin and latex beads were added to the 2 ml of culture medium with or without the antimalarial drugs to a final concentration of 25 µg/ml β-haematin or 0.1% (w/v) latex beads. The cells were incubated for 30 min at 37ºC under humidified 5% CO_2. The concentration of β-haematin added to the monocytes (25 µg/ml) was chosen based on reported estimates of the haemozoin concentrations encountered during malaria infections (Jaramillo *et al.*, 2005; Sherry *et al.*, 1995; Sullivan *et al.*, 1996a). Cells were incubated with β-haematin for 30 min as Olliaro *et al.* (2000) detected aggregates of β-haematin crystals inside phagolysosomes in monocytes within 25 minutes of treatment (Olliaro *et al.*, 2000). Following the incubation with latex beads or β-haematin, the supernatant was aspirated and the cells were washed three times in pre-warmed HBSS. The effects of the antimalarial drugs on the phagocytosis of latex beads were determined by the counting assay, whereas the effects of antimalarial drugs on the phagocytosis of β-haematin were analysed by either the counting assay or the OD 400 assay.

Counting assay: The J774A.1 cells were stained with 2% (w/v) methyl green (Section 2.2.5) and viewed (400x). AnalySIS® 3.0 (Soft Imaging System) was used to capture images of various fields of the coverslips and modify the images by separating the image into its red, green and blue components. The software was also used to maximise the contrast of the red image and 'touch count' the total number of cells and the cells containing latex beads observed in each field. The maximised-contrasted blue image was used to 'touch count' the total cells and cells containing β-haematin in each field. Between a total of 500 to 1000 cells were counted per coverslip and each experiment was performed in duplicate. Results were expressed as per cent inhibition or activation of phagocytosis of latex beads or β-haematin relative to the control for each antimalarial drug.

OD 400 Assay: The amount of β-haematin phagocytosed by the cells (U937 and PBMC) was determined by measuring the total haeme content in the cells after the phagocytosis assay using an adaptation of the procedure developed by Sullivan *et al.* (1996c). The cells were lysed and the β-haematin was solubilised to haeme by exposing the coverslips to 500 µl of 20 mM NaOH and 2% SDS for 2 h at RT, followed by measuring the optical density (OD) of the lysate at 400 nm. The haeme content of the lysate was determined by extrapolation from a standard curve of OD at 400 nm plotted against haeme concentration. The results were expressed as the

per cent inhibition or activation of phagocytosis of β-haematin relative to the control of each antimalarial drug.

2.2.10 Statistical analysis

Statistically significant differences were determined by the Mann-Whitney U (Wilcoxon Rank-Sum) test using the GenStat program, 9th Edition. Values of $p<0.05$ were considered statistically significant. All data are presented as the mean ± SEM.

2.3 Results

Prior to investigating the effects of antimalarial drugs on the phagocytosis of latex beads and β-haematin, the mouse J774A.1 and human U937 cell lines and human peripheral blood mononuclear cells (PBMC) were first characterised to verify their monocytic/macrophagic properties by evaluating their adherence to glass, the presence of hydrolytic enzymes and their ability to phagocytose. β-haematin, which is structurally and chemically equivalent to haemozoin (Egan, 2002), was synthesized and the morphology validated by scanning electron microscopy. The effect of the antimalarial drugs on the phagocytosis of latex beads and β-haematin was first ascertained using a counting method with the J774A.1 cell line. As the counting assay was time consuming and labour intensive, a spectrophotometric assay was developed to measure the effect of the drugs on the phagocytosis of β-haematin. This method was compared to the counting assay using variables such as the amount of β-haematin added to the cells, the number of cells treated with β-haematin, the time of exposure to β-haematin and temperature of phagocytosis. The OD 400 assay was then adopted to determine the effects of the antimalarial drugs on the phagocytosis of β-haematin by the U937 cell line and PBMC.

2.3.1 Characterisation of the monocytes

The J774A.1 and U937 cell lines and the PBMC were characterised by histochemical, morphological and functional assays to determine if they had characteristic monocyte/macrophage properties. Histochemical techniques were used to determine the presence of alkaline phosphatase and peroxidase in vesicles within the monocyte's cytoplasm. Staining with Giemsa and methyl green as well as transmission electron microscopic analysis

confirmed the morphology as well as the functional ability of monocytes to phagocytose latex beads and β-haematin.

Monocytes were characterized by their ability to adhere to glass and plastic. The presence of adherent cells on glass coverslips was characterized by staining with methyl green or Giemsa. The J774A.1 cell line is an adherent cell line, and the cells adhered to the coverslips after 2 hours incubation at 37ºC (Figure 2.1A). The U937 cell line is a non-adherent cell line, which adheres to the coverslips after differentiation with phorbol-12-myristate-13-acetate (PMA), (Figure 2.1B).

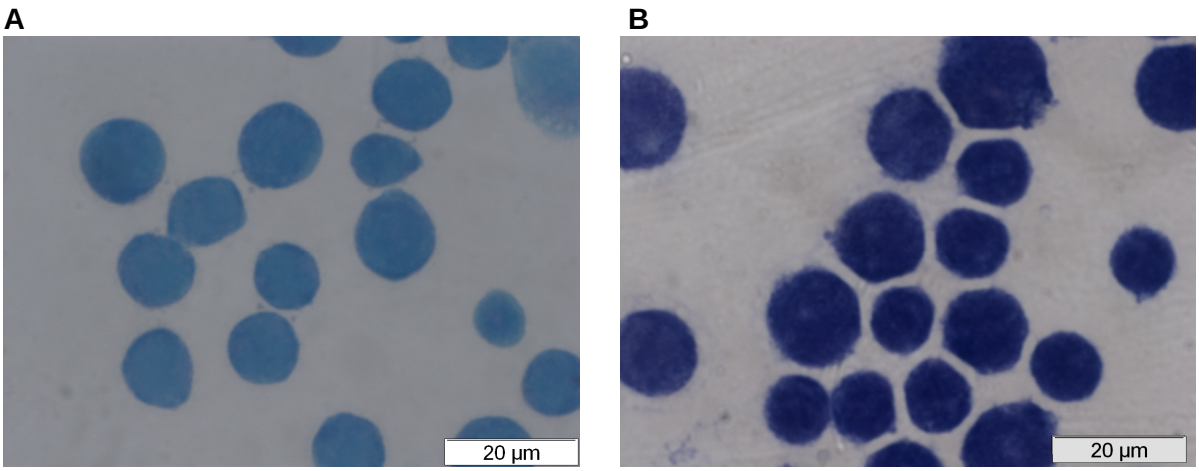


Figure 2.1. Adherence of J774A.1 cells and PMA-treated U937 cells to glass coverslips. Adherent cells were fixed with methanol and the (A) J774A.1 cells were stained with methyl green and the (B) U937 cells were stained with Giemsa. The stained cells were viewed at 1000x magnification under oil immersion.

2.3.1.1 Detection of peroxidase and alkaline phosphatase activity in monocytes

Peroxidase activity in cytoplasmic granules was observed in J774A.1 cells and PBMC as brown punctate spots (Figure 2.2). Peroxidase activity was not observed in the adherent U937 cells differentiated with PMA. Variations in the staining procedure, such as a change in the pH of the buffer (from pH 8 to 7.6) as well as varied concentrations of hydrogen peroxide (0.3%, 0.1%, and 0.01%) did not produce staining suggesting that these cells lack peroxidase activity. However, peroxidase activity was detected in the U937 suspension culture prior to differentiation (data not shown).

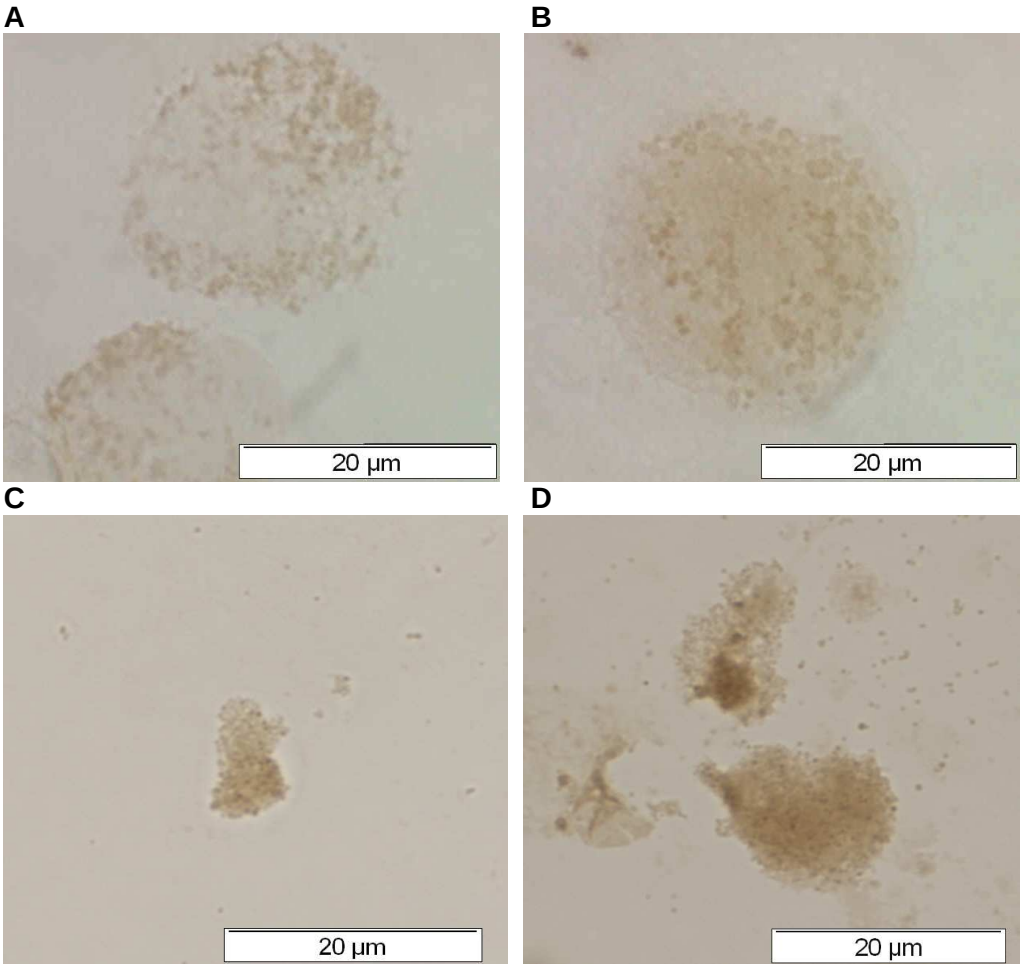


Figure 2.2. Peroxidase activity in J774A.1 cells and peripheral blood mononuclear cells (PBMC). Cells adherent to coverslips were incubated with the peroxidase substrate (DAB). (A, B) J774A.1 cells and (C, D) PBMC were viewed at 1000x magnification under oil immersion.

Alkaline phosphatase activity was observed in the J774A.1, U937 and PBMC as punctate blue spots throughout the cells illustrating enzyme activity in numerous cytoplasmic granules (Figure 2.3). There was a more diffuse purple staining in the cytoplasm of the PBMC (Figure 2.3E and F).

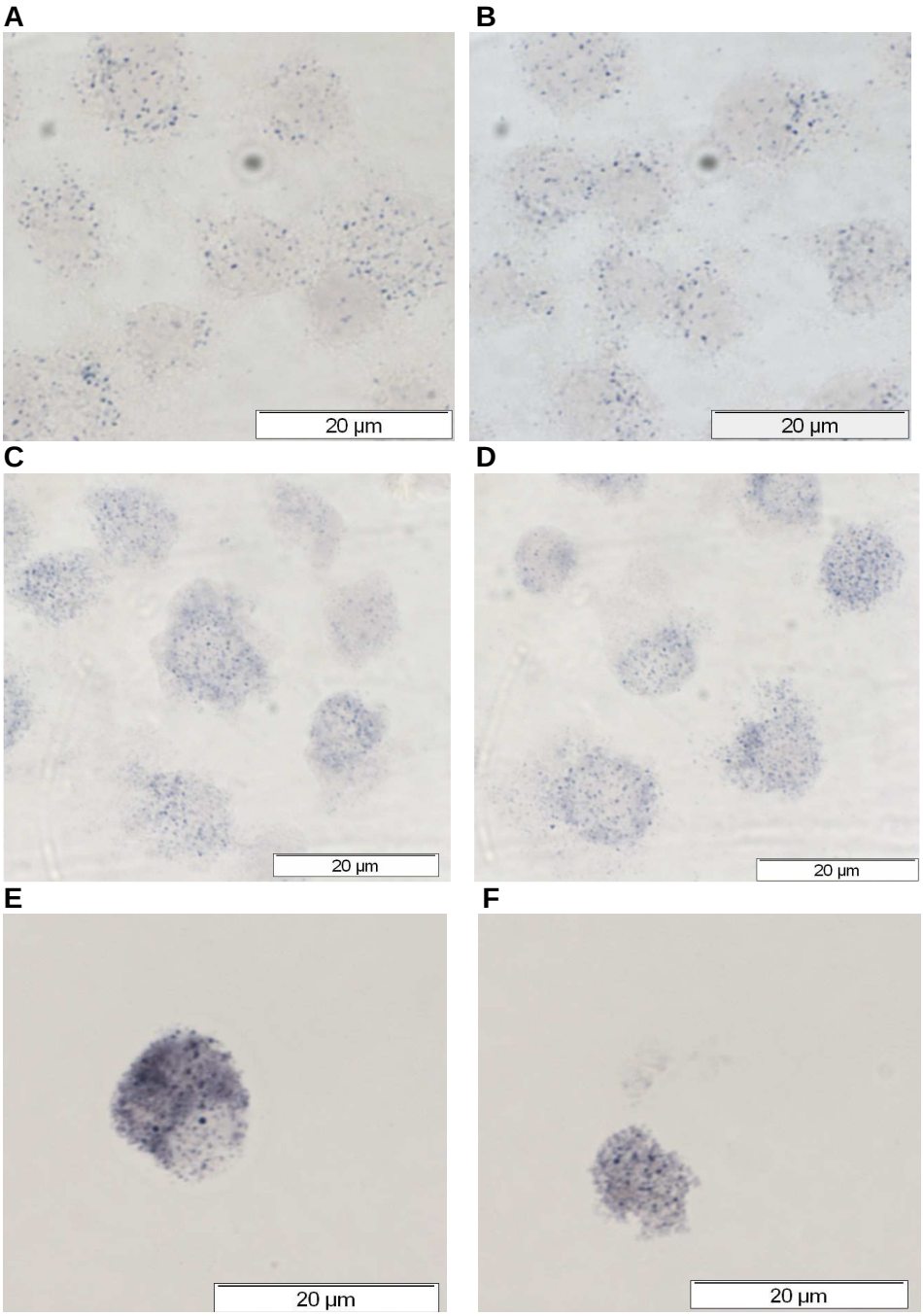
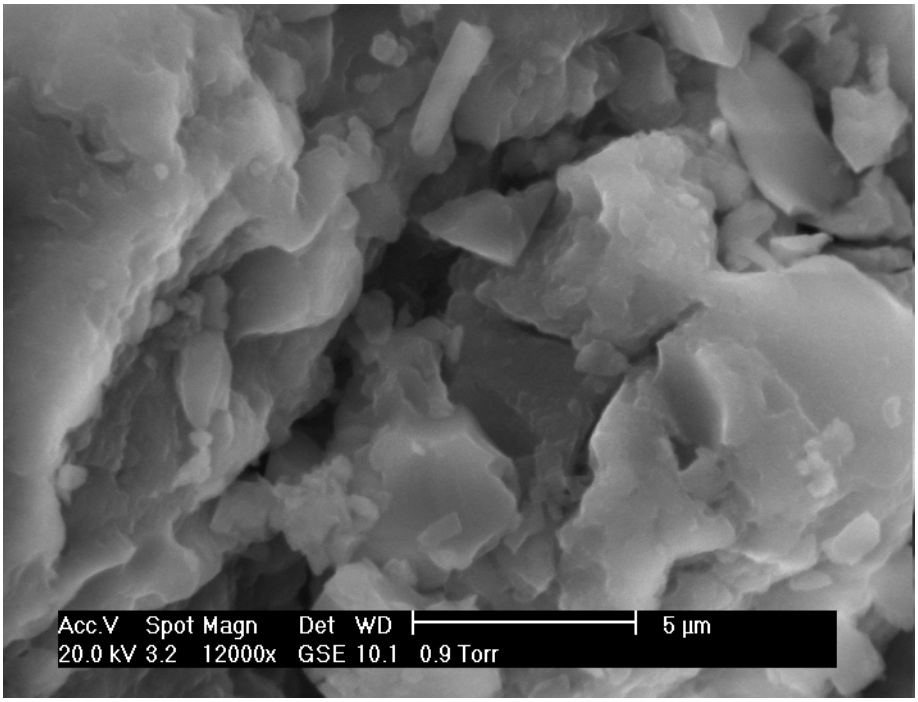


Figure 2.3. Alkaline phosphatase activity in J774A.1 cells, U937 cells and peripheral blood mononuclear cells (PBMC). Adherent cells were incubated with the alkaline phosphatase substrate (BCIP/NBT). (A, B) J774A.1 cells, (C, D) U937 cells and (E, F) PBMC were viewed at 1000x magnification under oil immersion.

2.3.1.2 Synthesis and analysis of β-haematin

A



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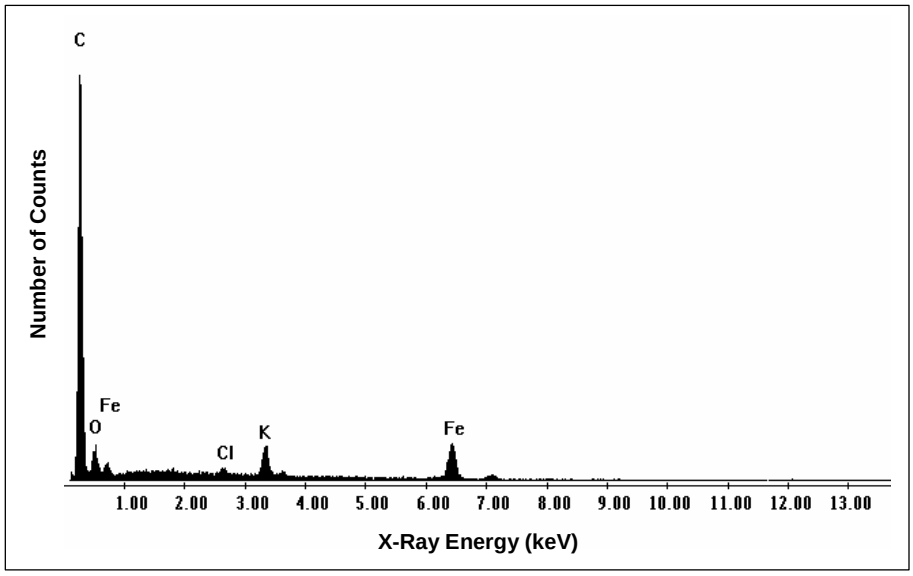
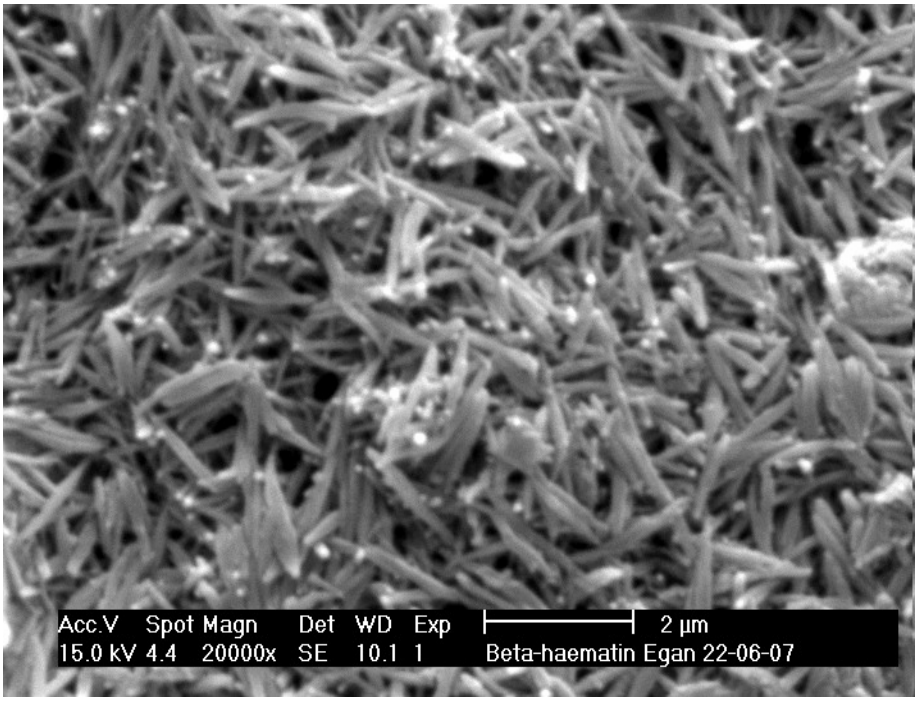


Figure 2.4. Scanning electron micrograph of haematin and the corresponding X-ray spectrum. Haematin was mounted onto a stub covered in carbon tape and viewed under a Philips environmental scanning electron microscope fitted with a large field detector. (A) The crystal structure of haematin at a magnification of 12 000x. (B) The X-ray spectrum obtained at a magnification of 2000x.

A



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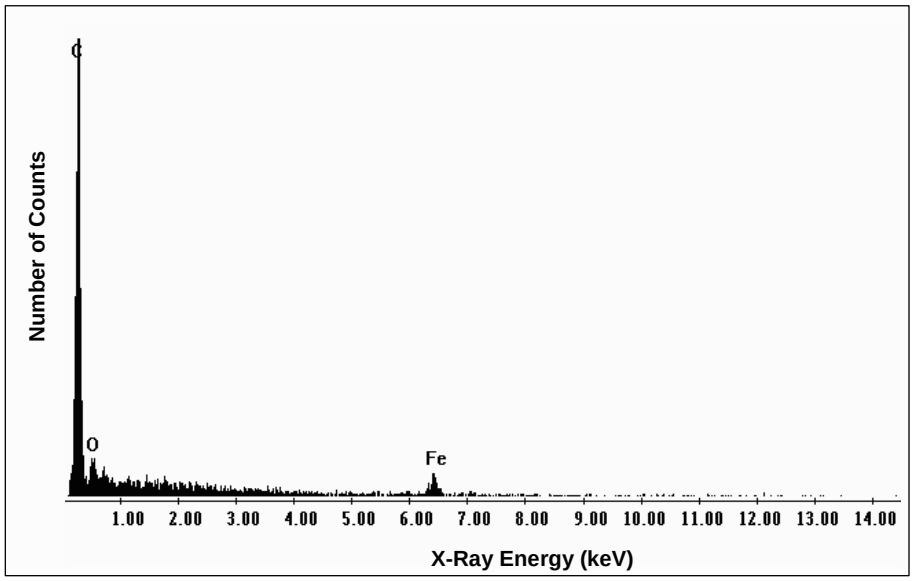
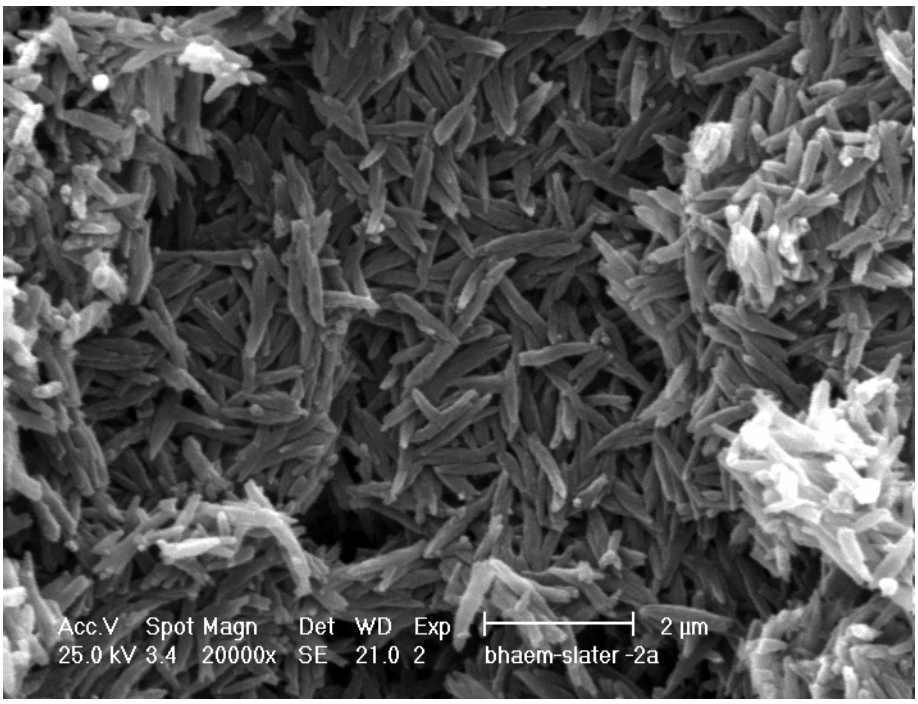


Figure 2.5. Scanning electron micrograph of β-haematin synthesized according to the method of Egan *et al.* (1994) and the corresponding X-ray spectrum. β-haematin was mounted onto a stub covered in carbon tape and viewed under a Philips environmental scanning electron microscope fitted with a large field detector. (A) The crystal structure of β-haematin at a magnification of 20 000x. (B) The X-ray spectrum obtained at a magnification of 2000x.

A



B

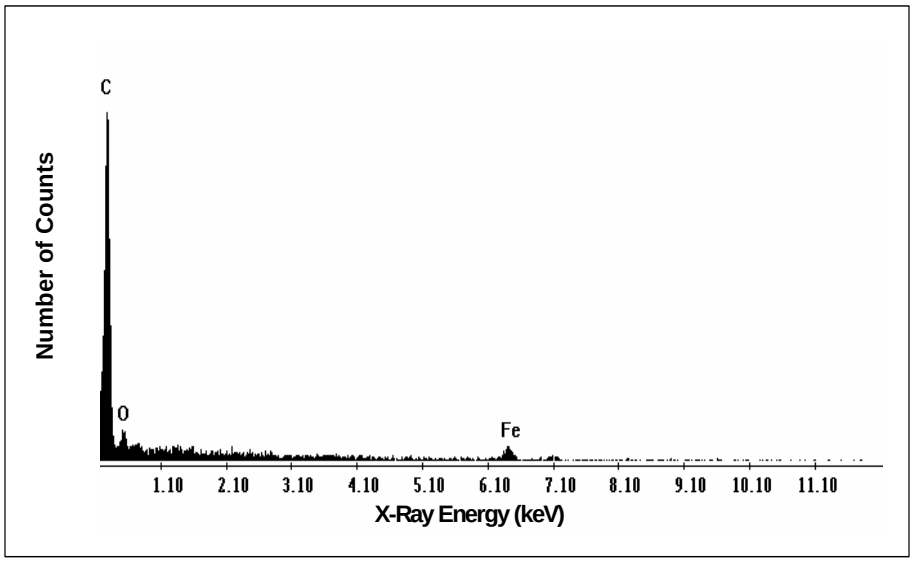


Figure 2.6. Scanning electron micrograph of β-haematin synthesized according to the method of Slater *et al.* (1991) and the corresponding X-ray spectrum. β-haematin was mounted onto a stub covered in carbon tape and viewed under a Philips environmental scanning electron microscope fitted with a large field detector. (A) The crystal structure of β-haematin at a magnification of 20 000x. (B) The X-ray spectrum obtained at a magnification of 2000x.

The scanning electron micrographs (SEMs) indicate a distinct difference between the crystal structures of the starting material, haematin, and the finished product, β-haematin. Haematin is made up of large crystals with smooth surfaces (Figure 2.4A). β-haematin is made up of smaller thin, needle-like crystals that are tapered towards the ends (Figure 2.5A and Figure 2.6A). The β-haematin crystals radiate in all directions. These crystals have a similar appearance to those in SEMs of crystals synthesized by Egan *et al*. (2001). The longest needle-like crystals produced by both methods appear to be roughly the same length (0.9-1.4 μm) and width (0.15-0.2 μm). The transmission electron micrographs (TEMs) of β-haematin (Figure 2.7) show needle-like crystals that taper towards the ends and have a similar appearance to those published by Egan *et al.* (2001) and Olliaro *et al.* (2000).

X-ray microanalysis showed that both haematin and β-haematin contain the common elements iron, oxygen and carbon (Figures 2.4B, 2.5B and 2.6B). Quantification of the X-ray spectrum of haematin showed that the crystals contained 80.59% carbon, 10.85% oxygen, 6.5% iron, 1.77% potassium and 0.29% chloride (Figure 2.4B). The trace amounts of potassium and chloride could be as a result of the supplier's extraction procedure to prepare haematin. Quantification of the spectrums of β-haematin prepared by (a) the method of Egan *et al.* (1994) indicated that the crystals contained 84.84% carbon, 8.38% oxygen and 6.81% iron, and that prepared by (b) the method of Slater *et al.* (1991) contained 84.2% carbon, 9.23% oxygen and 6.57% iron. The ratios of the elements in the β-haematin crystals prepared by the two different methods were much the same. The SEMs were used to confirm the synthesis of β-haematin from haematin.

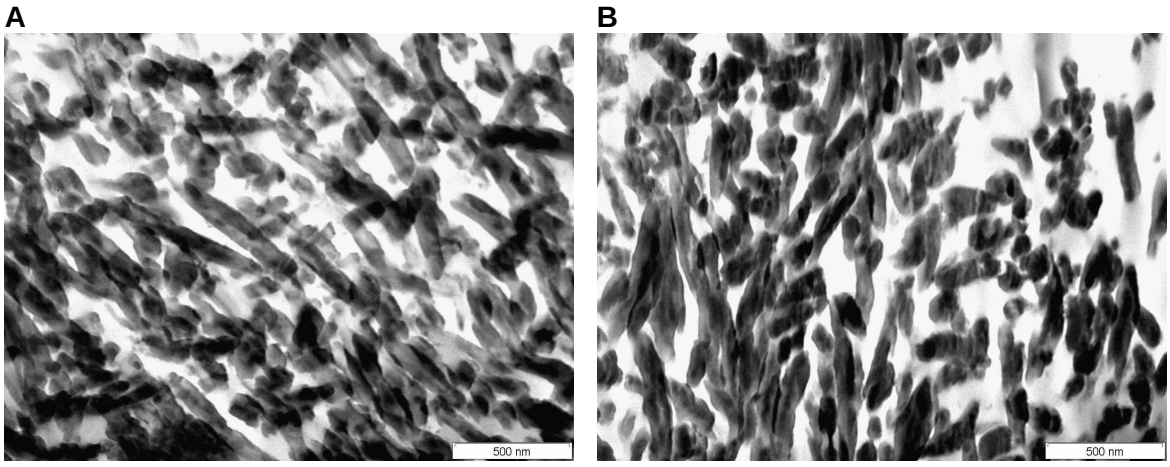


Figure 2.7. Transmission electron micrographs of β-haematin synthesized by two methods. The method of Egan *et al.* (1994) is shown in (A) and the method of Slater *et al.* (1991) is shown in (B).

2.3.1.3 Measuring phagocytosis of latex beads and β-haematin by monocytes

After incubation with latex beads and β-haematin, the bright field images of the monocytes (Figure 2.8) and the transmission electron micrographs (TEM) (Figure 2.9 and 2.10) indicate that the J774A.1 cells phagocytosed unopsonised latex beads and β-haematin. In Figure 2.8 (A and B), the latex beads appear to be localized in distinct areas, possibly the cytoplasm, as the methyl green staining does not distinguish the cytoplasm from the nucleus. β-haematin appears to be less localized in Figure 2.8 (C and D) as crystals also adhere to the surface of the cell membrane. In the TEMs in Figure 2.9 and 2.10, the latex beads and β-haematin are located in the cytoplasm of the monocytes. The distinctive kidney-shaped nucleus characteristic of monocytes is visible in Figure 2.9B (Auger and Ross, 1992). This kidney-shaped nucleus is not always visible in the TEMs of monocytes owing to the angle at which the cells are sectioned.

After 30 minutes, there were fewer β-haematin crystals present in the monocytes than there were latex beads (Figure 2.9 and 2.10). This could be due to the slightly larger size of the β-haematin crystals (approximately 1.4 µm versus 0.8 µm of the latex beads). In addition, phagocytic receptors on the monocytes may have lower affinities for the β-haematin crystals than for the latex beads, and the numbers of β-haematin crystals and latex beads added to the cells may have been unequal. β-haematin was observed as free crystals in the cytoplasm surrounded by a discontinuous membrane (Figure 2.10B) as well as in phagocytic vacuoles with a clear matrix that are situated very close to the cell membrane (Figure 2.10C, D, E).

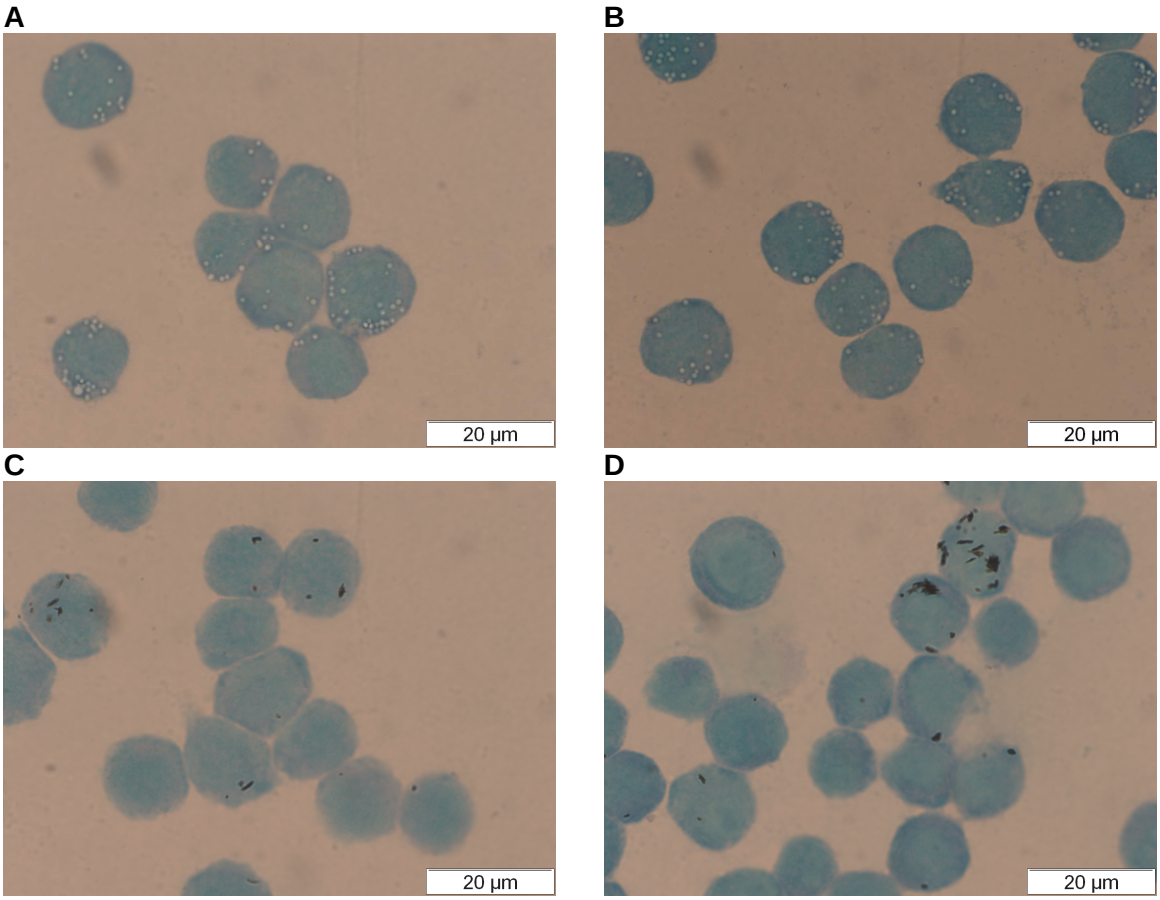


Figure 2.8. Phagocytosis of latex beads and β-haematin by J774A.1 cells. Adherent J774A.1 cells were exposed to (A, B) latex beads (0.1% (w/v)) or (C, D) β-haematin (25 µg/ml) for 30 min and the stained cells were examined at 1000x magnification under oil immersion.

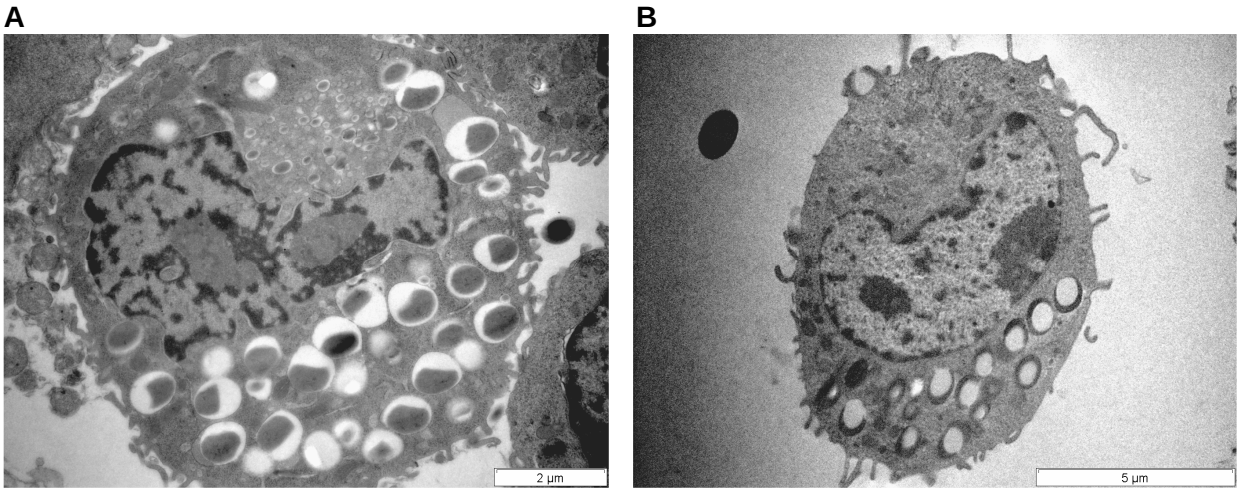


Figure 2.9. Transmission electron micrographs of J774A.1 cells after phagocytosis of latex beads. J774A.1 cells were exposed to latex beads for (A) 30 minutes and (B) 2 hours.

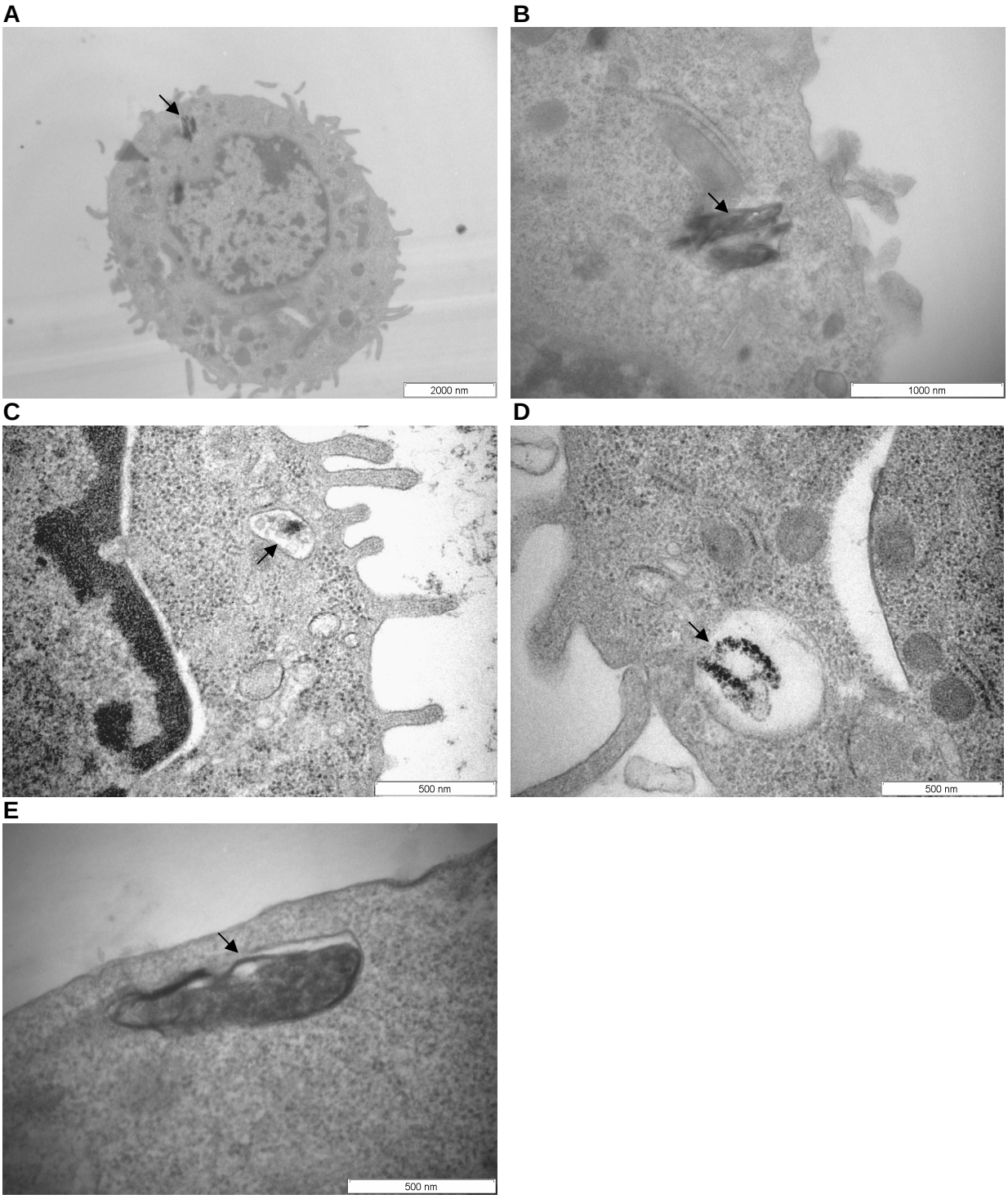


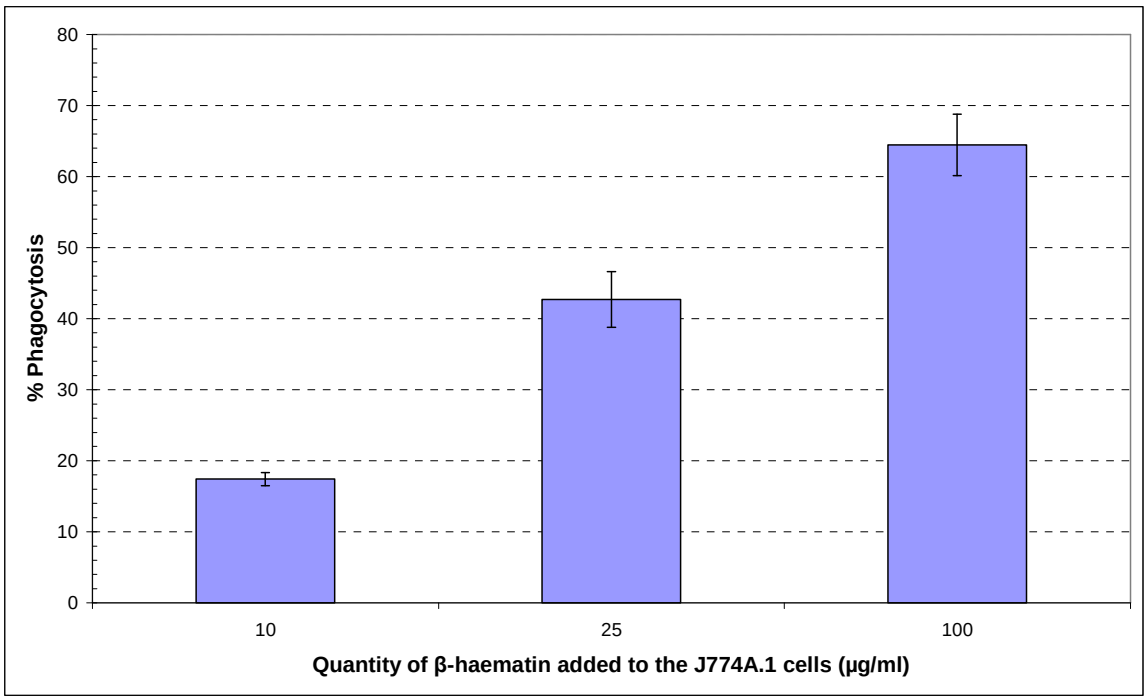
Figure 2.10. Transmission electron micrographs (TEM) of J774A.1 cells after phagocytosis of β-haematin. (A) A J774A.1 cell after phagocytosis of β-haematin for 2 hours; (B) TEM (A) at a higher magnification to illustrate the two β-haematin crystals surrounded by a discontinuous membrane; (C, D, E) β-haematin crystals in phagocytic vacuoles after (C) 30 minutes, (D, E) 2 hours of phagocytosis. Arrows indicate the β-haematin crystals.

2.3.2 Comparison of the counting and OD 400 assay methods to determine the extent of phagocytosis of β-haematin

When increasing quantities of β-haematin were added to equivalent numbers of J774A.1 cells, there was a corresponding increase in the percentage phagocytosis observed by the counting method (Figure 2.11A) as well as an increase in the amount of haeme measured by the OD 400 assay (Figure 2.11B). Thus, both assays detect an increase in phagocytosis of β-haematin, although the counting method measures the number of cells phagocytosing β-haematin, whereas the OD 400 assay does not differentiate between the amount of β-haematin phagocytosed, and the number of cells phagocytosing β-haematin. When the cells were exposed very briefly to β-haematin (3 minutes), there was a minimal amount of haeme measured by the OD 400 assay.

Figure 2.12A showed that when using the counting method, a 4- to 5-fold decrease in phagocytosis was observed when the time of treatment with β-haematin was decreased from 30 minutes to 3 minutes. This reduction in phagocytosis with a decreased time of exposure to β-haematin occurred regardless of the quantity of β-haematin used to treat the cells. A similar trend was observed with the OD 400 assay, where the amount of haeme detected in the cell lysate decreased 3.5-fold when the β-haematin treatment was decreased from 30 minutes to 3 minutes (Figure 2.12B). The amount of haeme present in the lysate also decreased almost 2-fold when the temperature of the β-haematin treatment was decreased from 37ºC to 4ºC. The haeme detected in the lysate of the cells incubated at 4ºC was probably due to the adherence of the β-haematin crystals to the cell surface.

A



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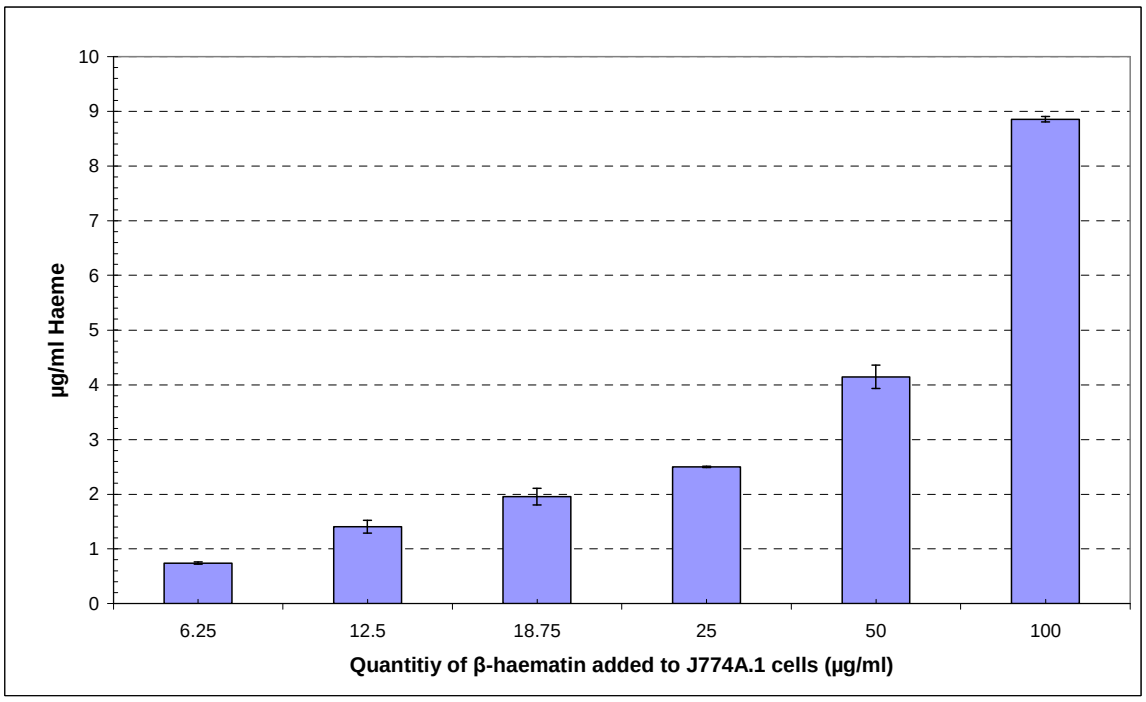
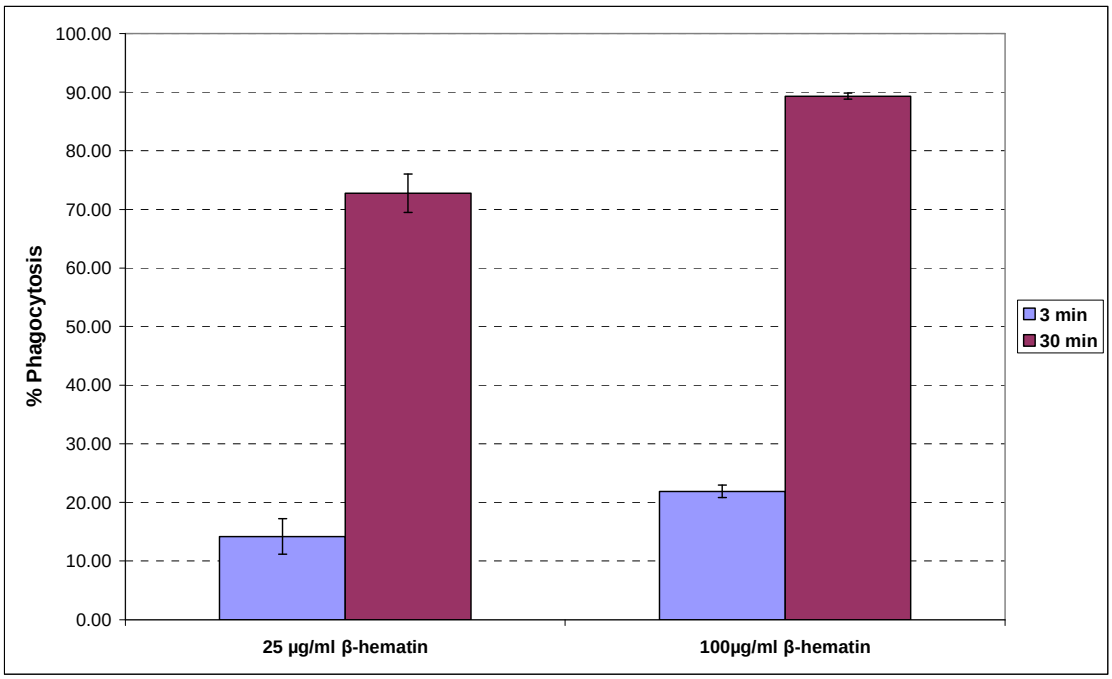


Figure 2.11. J774A.1 phagocytosis of β-haematin determined by the counting and OD 400 assays, following the addition of increasing quantities of β-haematin to the cells. J774A.1 cells were treated with varying concentrations of β-haematin and (A) the percentage phagocytosis was determined by the counting assay, or (B) the amount of haeme in the cell lysates was measured by the OD 400 assay. Results were expressed as the mean of duplicates ± SEM.

A



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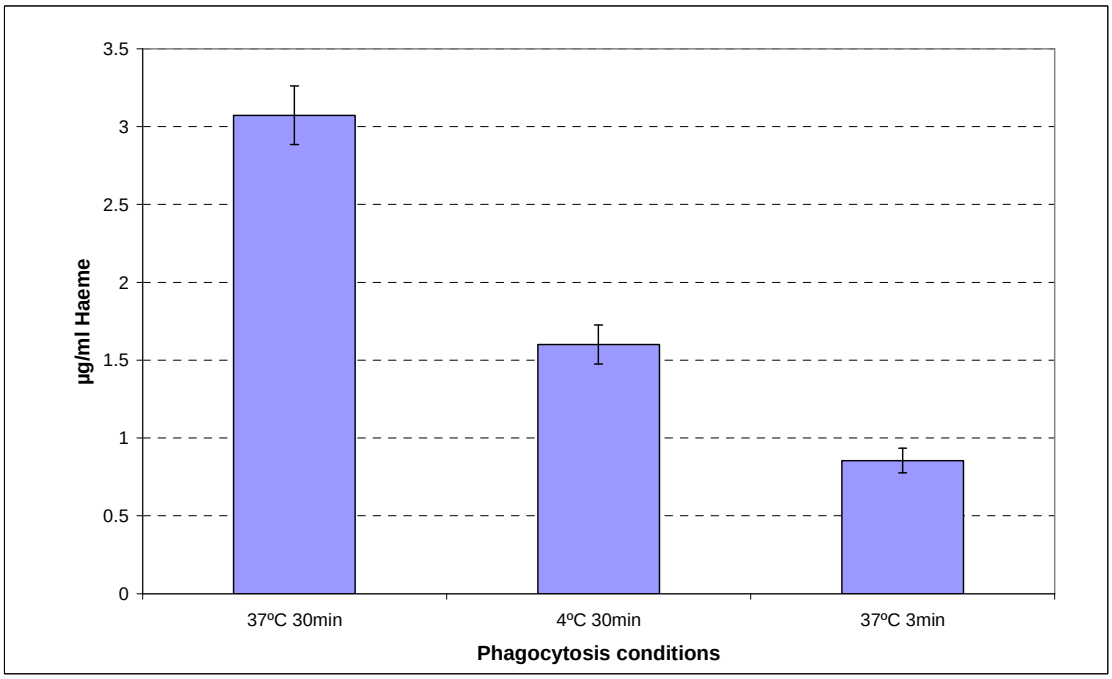


Figure 2.12. Effect of temperature and time on the J774A.1 phagocytosis of β-haematin determined by the counting and OD 400 assays. (A) Two concentrations of β-haematin were added to J774A.1 cells for 3 and 30 minutes at 37ºC and the percentage phagocytosis was determined by counting. (B) β-haematin (25 µg/ml) was added to the J774A.1 cells for 30 minutes at 37ºC and 4ºC and for 3 min at 37ºC. This was followed by measuring the amount of haeme present in the cell lysates using the OD 400 assay. Results are the mean of three experiments done in triplicate ± SEM.

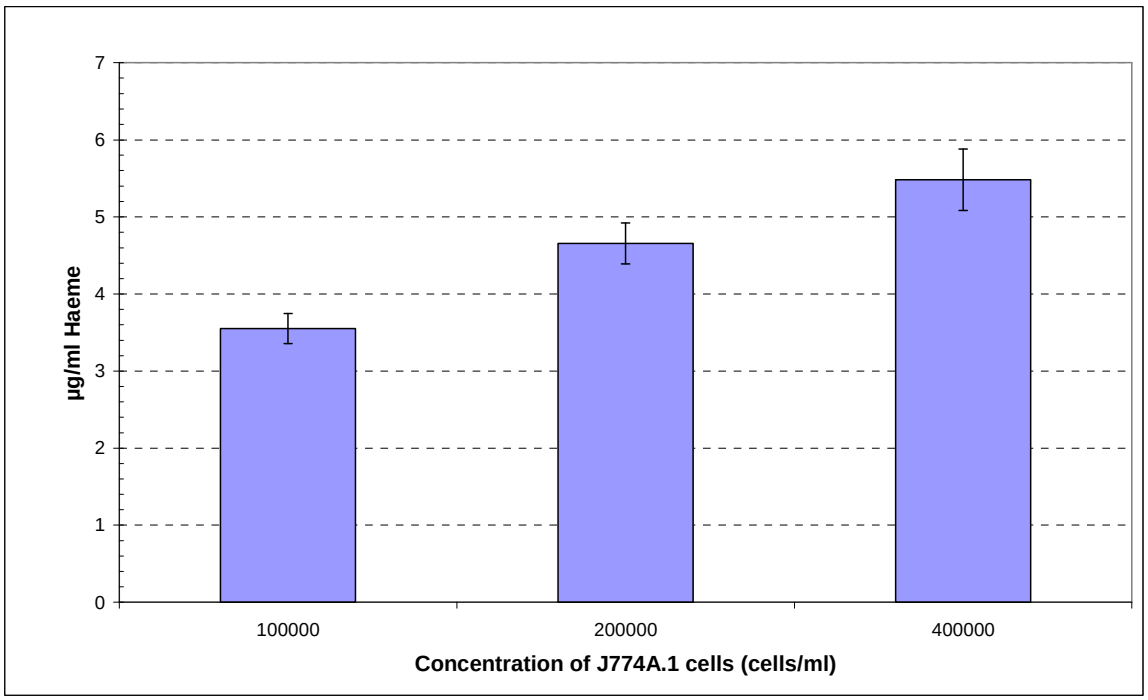


Figure 2.13. Effect of the number of J774A.1 cells on the phagocytosis of β-haematin. Increasing concentrations of J774A.1 cells were exposed to β-haematin and the OD 400 assay was used to determine the amount of haeme present in the cell lysates. Results are expressed as the mean of two experiments done in duplicate ± SEM.

Figure 2.13 demonstrates that there was a 1.2- to 1.3-fold increase in the amount of haeme detected in the cell lysate as the number of cells treated with 25 µg/ml β-haematin was doubled. Thus, the OD 400 assay detected an increase in phagocytosis of β-haematin as the number of the cells was increased. Figures 2.11 to 2.13 demonstrated that the OD 400 assay was sensitive enough to detect changes in the amount of β-haematin phagocytosed by the monocytes when the monocytes were subjected to variations in the quantity of β-haematin added, the time period of β-haematin treatment, the temperature of phagocytosis and the number of cells exposed to β-haematin.

2.3.3 Effect of febrile temperature on phagocytosis of β-haematin by monocytes

One of the most characteristic clinical symptoms of malaria infection is fever which typically recurs at intervals of 48 hours in infections due to *Plasmodium vivax* and *P. falciparum* (Karunaweera *et al.,* 1992*)*. J774A.1 cells were pre-conditioned to a febrile temperature of 41ºC for 2 hours prior to a β-haematin phagocytosis assay at 41ºC and compared to that of cells incubated at 37ºC (Table 2.1). The OD 400 assay demonstrated that the effect of a febrile temperature of 41ºC did not significantly affect the phagocytosis of β-haematin by J774A.1 cells ($p>0.05$).

Table 2.1. Effect of febrile temperature on J774A.1 phagocytosis of β-haematin

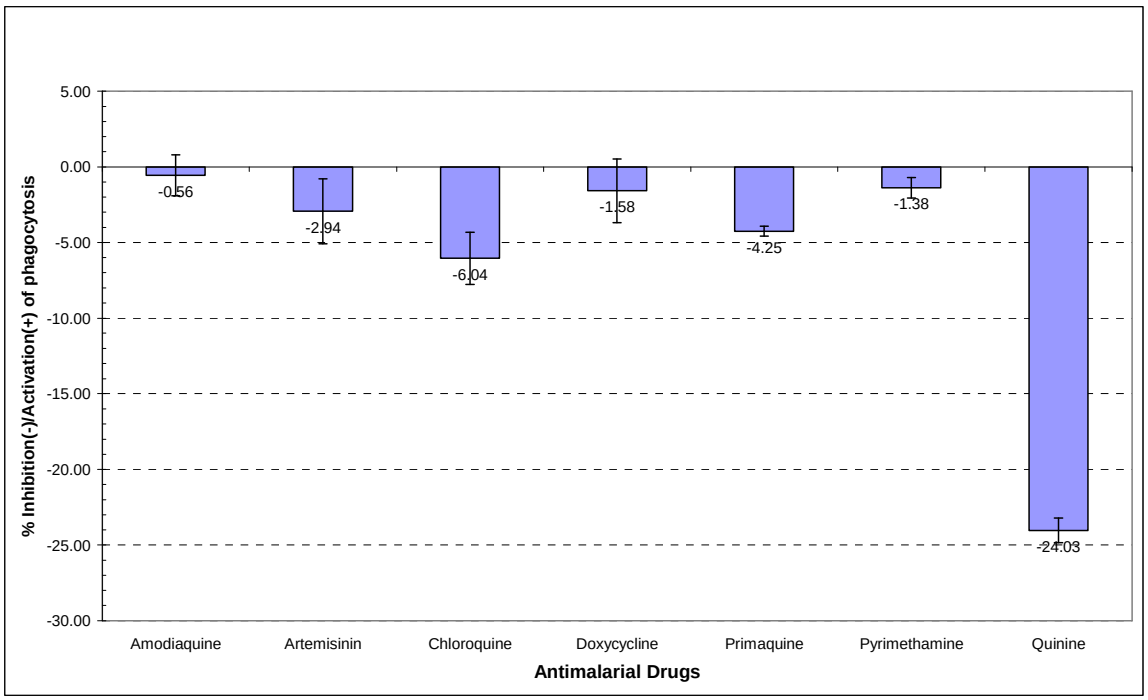
Temperature of phagocytosis	µg/ml Haeme[a]	SEM[b]
37ºC	4.56	0.50
41ºC	5.06	0.66

a) The phagocytosis of β-haematin was measured by the OD 400 assay, which determines the amount of haeme present in the cell lysate.
b) SEM: Standard error of the mean.

2.3.4 Effect of antimalarial drugs on the phagocytosis of latex beads and β-haematin by monocytes

J774A.1 cells were treated with seven antimalarial drugs for 18 h prior to 30 min of phagocytosis of β-haematin or latex beads. The effects of the drugs on the phagocytosis of β-haematin or latex beads by the J774A.1 cells were determined by the counting assay as described in section 2.2.9. Figure 2.14A demonstrates that chloroquine and quinine inhibited latex bead phagocytosis by the J774A.1 cells with quinine having the most significant effect, 4-fold greater than that caused by chloroquine, $p<0.05$. Amodiaquine, artemisinin, doxycycline, primaquine, and pyrimethamine had minimal inhibitory effects on the phagocytosis of latex beads (<5%), with amodiaquine having the least effect. In contrast, the phagocytosis of β-haematin by the J774A.1 cells was activated by five out of seven of the antimalarial drugs investigated (Figure 2.14B). Artemisinin, doxycycline and pyrimethamine treatment resulted in an activation of 10% or more. The difference between the effects of artemisinin, doxycycline, primaquine and pyrimethamine, and the effects of amodiaquine, chloroquine and quinine on β-haematin phagocytosis, was significant ($p<0.05$). Amodiaquine and chloroquine inhibited the phagocytosis of β-haematin by less than 5%.

A



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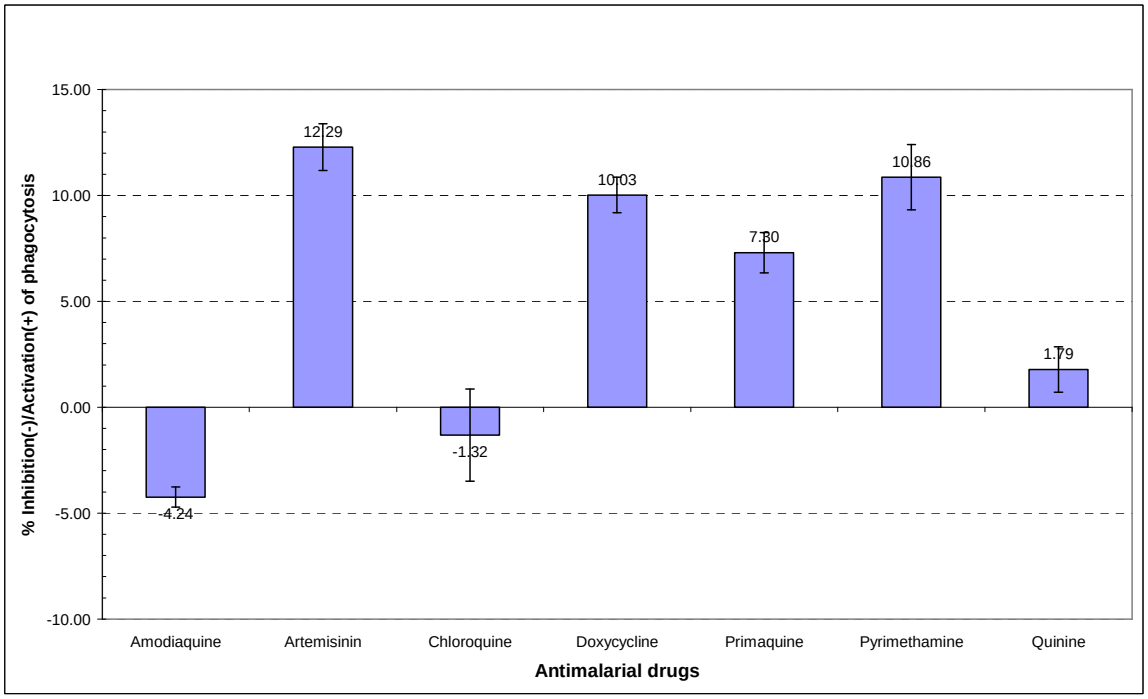


Figure 2.14. Effects of antimalarial drugs on J774A.1 phagocytosis as determined by the counting assay. J774A.1 cells were exposed to physiological concentrations of seven antimalarial drugs for 18 hours. (A) Latex beads (0.1% (w/v)) or (B) β-haematin (25 μg/ml) were added to the cells and they were incubated for a further 30 minutes. The cells were evaluated for phagocytosis by the counting assay at 400x magnification. Results are expressed as the mean of two experiments done in duplicate ± SEM.

To determine if there was a correlation between the counting and OD 400 assays, the effects of quinine, pyrimethamine and artemisinin on the phagocytosis of β-haematin by the J774A.1 cells were analysed by the OD 400 assay, and the results obtained were compared to those of the counting assay (Figure 2.15). Both assays demonstrated that artemisinin, pyrimethamine and quinine activated the phagocytosis of β-haematin by the J774A.1 cells. Furthermore, the same trends were observed among the three drugs in both assays: Of the three drugs investigated, artemisinin had the greatest effect and quinine had the least effect on the increased β-haematin phagocytosis. Hence, the OD 400 assay was used to analyse the effect of the antimalarial drugs on the phagocytosis of β-haematin by the human U937 cell line and peripheral blood mononuclear cells (PBMC).

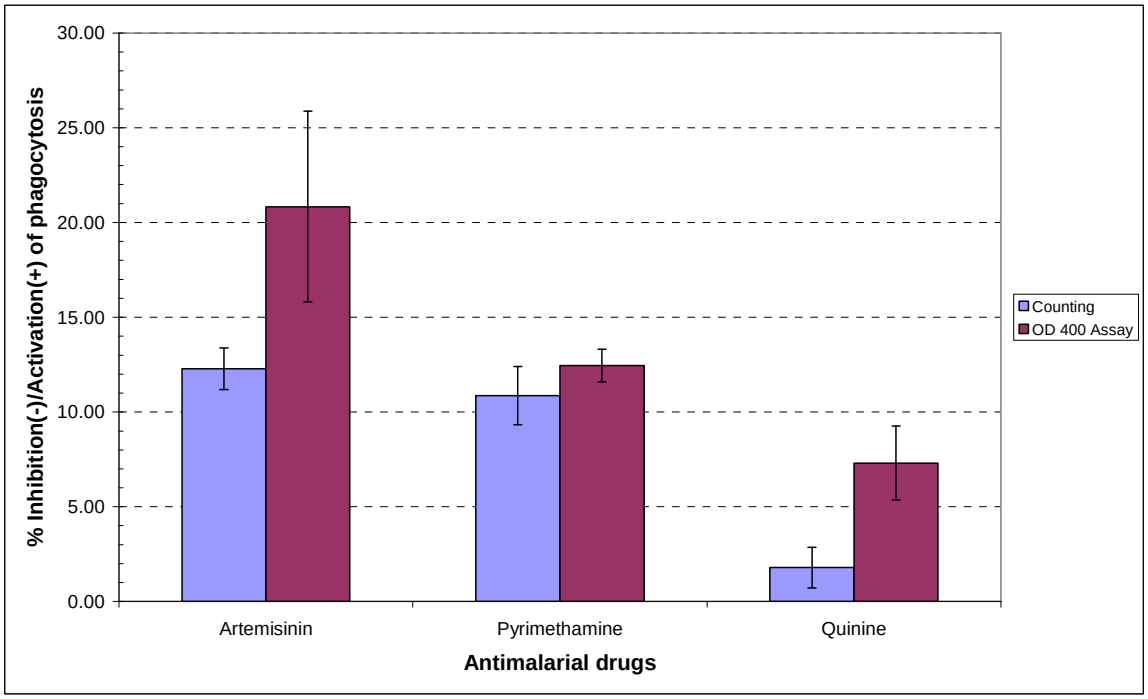


Figure 2.15. Comparison of the counting and OD 400 assays. J774A.1 cells were exposed to physiological concentrations of artemisinin, pyrimethamine and quinine for 18 hours. β-haematin (25 μg/ml) was added to the cells and they were incubated for a further 30 minutes. The cells were evaluated for phagocytosis by the counting assay at 400x magnification and the OD 400 Assay. Results are expressed as the mean of two experiments done in duplicate ± SEM.

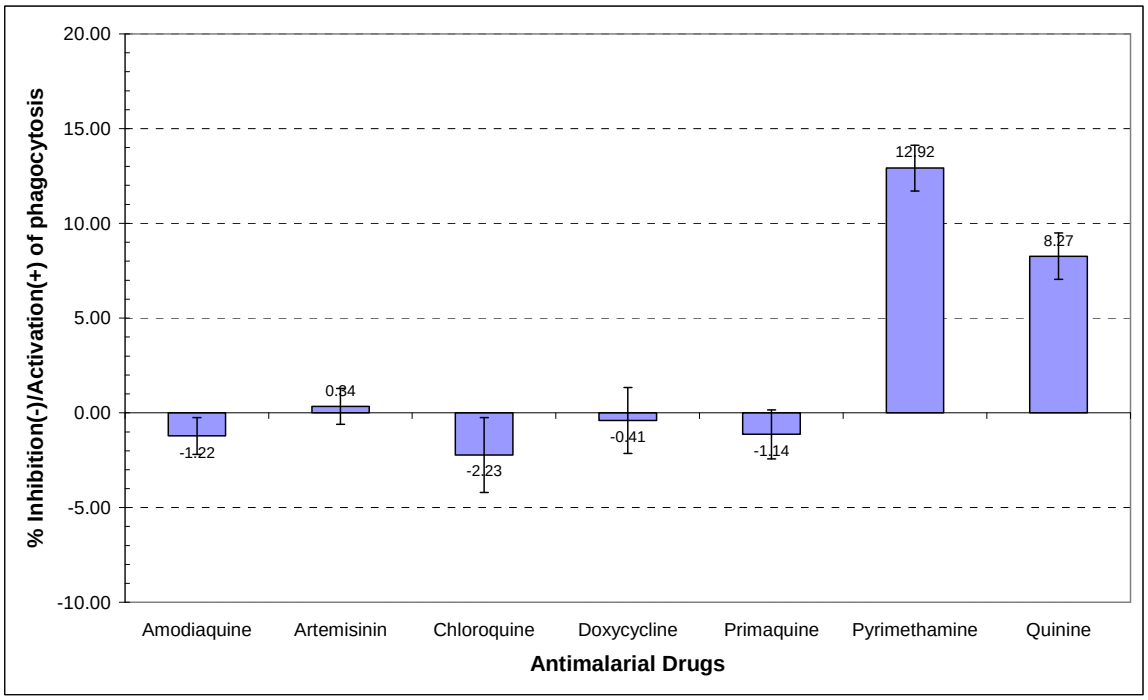


Figure 2.16. Effect of antimalarial drugs on U937 phagocytosis of β-haematin as measured by the OD 400 assay. U937 cells were treated with physiological concentrations of seven antimalarial drugs for 18 hours. β-haematin (25 μg/ml) was added, the cells were incubated for a further 30 minutes and evaluated for phagocytosis. Results are expressed as the mean of three experiments done in duplicate ± SEM.

In the case of the U937 cells, pyrimethamine and quinine activated the phagocytosis of β-haematin, with pyrimethamine resulting more than 10% activation (Figure 2.16). These activation effects were significantly different to the effects of the remaining five drugs ($p<0.05$). Amodiaquine and chloroquine inhibited the phagocytosis of β-haematin by less than 5%, and the effects of artemisinin, doxycycline and primaquine on phagocytosis were all below 2%.

When the effects of the antimalarial drugs on the U937 cells were compared to those of the J774A.1 cells, pyrimethamine and quinine both activated the phagocytosis of β-haematin, with pyrimethamine resulting in more than 10% activation in both cell lines. Both amodiaquine and chloroquine resulted in less than 5% inhibition in both cell lines. However, noticeable differences were observed in the effects of artemisinin, doxycycline and primaquine on the two cell lines. In the J774A.1 cell line, artemisinin, doxycycline and primaquine activated the phagocytosis of β-haematin, with artemisinin and doxycycline inducing more than 10% activation. In the U937 cells, these three drugs had minimal effects below 2% activation or inhibition of β-haematin phagocytosis.

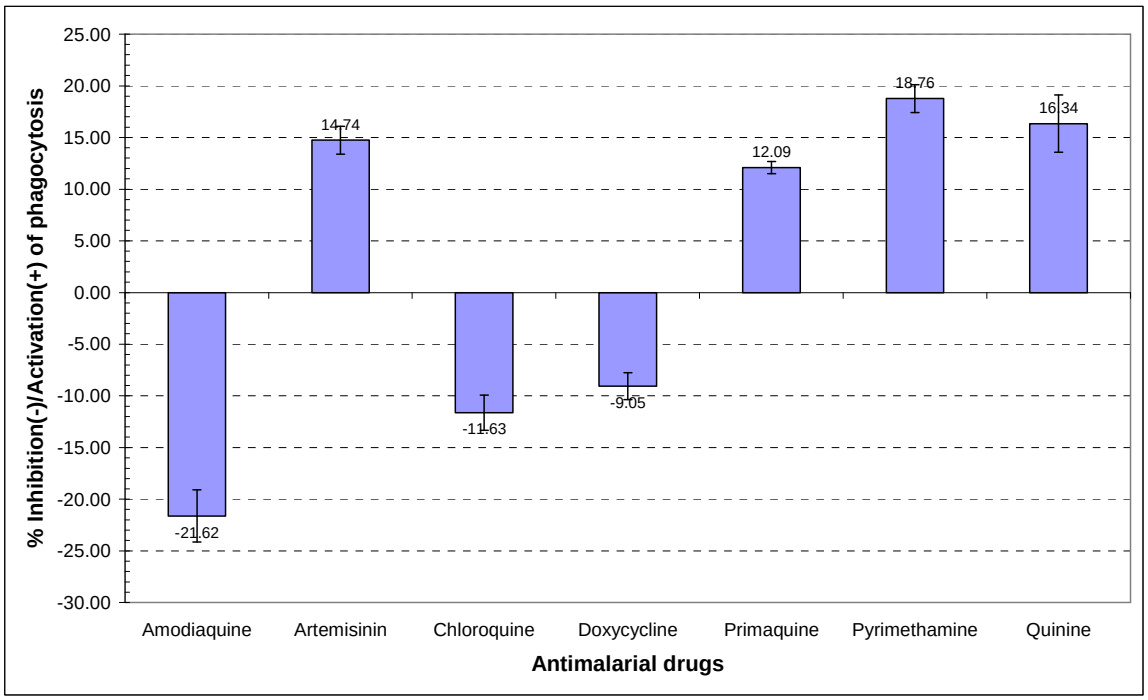


Figure 2.17. Effect of antimalarial drugs on PBMC phagocytosis of β-haematin as measured by the OD 400 assay. PBMC were isolated from buffy coats using Histopaque®-1.077. The adherent mononuclear cells were treated with antimalarial drugs for 18 hours. β-haematin (25 μg/ml) was added, the cells were incubated for a further 30 minutes and phagocytosis was measured. Results are expressed as the mean of triplicate experiments done in duplicate ± SEM.

When PBMC were treated with antimalarial drugs, four out of seven of the antimalarial drugs investigated: artemisinin, primaquine, pyrimethamine and quinine resulted in greater than 10% activation of β-haematin phagocytosis (Figure 2.17). Pyrimethamine had the most significant activation, about 1.5-fold greater than that of primaquine ($p<0.05$). The other three drugs, amodiaquine, chloroquine and doxycycline, all had inhibitory effects of 10% or more on the phagocytosis of β-haematin by PBMC, with amodiaquine having the most significant inhibition, more than 2-fold greater than that caused by doxycycline ($p<0.05$).

When the patterns of inhibition and activation of the antimalarial drugs on β-haematin phagocytosis for all three cell types were examined, pyrimethamine had the most consistent pattern promoting more than 10% increased β-haematin phagocytosis. Quinine also promoted phagocytosis in all three cell types but to varying degrees with the activation of β-haematin phagocytosis in PBMC cells being 9-fold greater than that observed with the J774A.1 cells

($p<0.05$) and 2-fold greater than the activation in the U937 cells. Artemisinin and primaquine also resulted in increased β-haematin phagocytosis in both the J774A.1 cells and PBMC, with artemisinin causing more than 10% increase in both cell types; however, minimal effects were observed in the U937 cell line ($p<0.05$). Amodiaquine and chloroquine both inhibited β-haematin phagocytosis in all three cell types, but with varying degrees. The inhibitory effect of amodiaquine on PBMC was most significant, almost 18-fold greater than the effect on U937 cells ($p<0.05$) and 5-fold greater than the inhibition of β-haematin phagocytosis of J774A.1 cells ($p<0.05$). Chloroquine inhibition of β-haematin phagocytosis of the PBMC was almost 9-fold greater than the drug's inhibition of the J774A.1 phagocytosis and 5-fold greater than that of the U937 cells. Doxycycline had different effects on each cell type: it caused about 10% activation of the β-haematin phagocytosis of the J774A.1 cells, almost 10% inhibition in PBMC and had very little effect on the U937 cells. Overall, the antimalarial drugs exhibited similar patterns of inhibition and activation, although with varying degrees, of β-haematin phagocytosis of the PBMC and J774A.1 cell line, with the exception of doxycycline.

In summary, in the J774A.1 cell line, two of the seven drugs, chloroquine and quinine inhibited latex bead phagocytosis. The other drugs had minimal effects on the latex bead phagocytosis. In the J774A.1 cells, five out of seven antimalarial drugs, artemisinin, doxycycline, primaquine, pyrimethamine and quinine activated β-haematin phagocytosis, while amodiaquine and chloroquine inhibited β-haematin phagocytosis. The same drugs that activated β-haematin phagocytosis in J774A.1, with the exception of doxycycline, promoted β-haematin phagocytosis in PBMC, but to a greater degree. Doxycycline inhibited the β-haematin phagocytosis as did chloroquine and amodiaquine, which caused a 5-fold greater inhibition in the PBMC than that observed in the J774A.1 cells. In the U937 cells, only two of the four drugs that promoted β-haematin phagocytosis in the PBMC, pyrimethamine and quinine, activated phagocytosis to levels about 1.5-fold lower than that observed in PBMC. Similar to J774A.1 and PBMC, chloroquine and amodiaquine inhibited β-haematin phagocytosis in the U937 cells, but to lesser extents than that in PBMC. Artemisinin, doxycycline and primaquine had minimal effects on the β-haematin phagocytosis in the U937 cells.

2.4 Discussion

2.4.1 Characterisation of the monocytes

Monocytes and macrophages are often the first cells of the immune system to encounter invading pathogens, thus their responsibilities are two-fold: firstly, they must function as effective phagocytic killer cells; secondly, they must present parasite-derived antigens to the rest of the immune system in the presence of Class II major histocompatibility complex (MHC), and secrete immuno-modulating cytokines that will induce a protective immune response (Sadick, 1992). The monocyte cell types used in this investigation were characterised by their ability to adhere to glass, their production of hydrolytic enzymes and their ability to phagocytose β-haematin and latex beads.

Both J774A.1 and PMA-treated U937 cells (Figure 2.1) adhered to glass coverslips, and in the case of PBMC, following centrifugation of the buffy coat over a layer of Histopaque®-1.077, adherence to glass coverslips was used to isolate the monocytes from the polymorphonuclear cells (Roos and de Boer, 1986). The kidney-shaped nucleus observed in the transmission electron micrograph of the J774A.1 cell (Figure 2.9B) is also characteristic of monocytes and gives further evidence of the monocytic character of the J774A.1 cell line (Auger and Ross, 1992).

Various hydrolytic enzymes, including peroxidases and phosphatases, have been isolated from macrophages and monocytes. These enzymes are necessary for the degradation of phagocytosed particles and killing of phagocytosed micro-organisms. The activity of these enzymes has often been employed in the detection of promonocytes and monocytes (Papadimitriou and Ashman, 1989). Alkaline phosphatase activity was detected in granules in the cytoplasm of all three cell types, J774A.1, U937 cell lines and PBMC (Figure 2.3). Peroxidase was detected in cytoplasmic granules in the J774A.1 cells and PBMC (Figure 2.2). Peroxidase activity was not detected in the PMA-treated U937 cell lines, although it was detected in the suspended U937 cells, prior to differentiation with PMA. As monocytes mature into macrophages, there is a decrease in the content of granule peroxidases (Nakagawara *et al.*, 1981; Papadimitriou and Ashman, 1989), as observed in the U937 cells. The U937 cell line in suspension has monoblast-like characteristics (Koren *et al.*, 1979); the monoblast is the least mature cell of the mononuclear phagocyte system. After treatment with conditioned medium from mixed lymphocyte cultures (Koren *et al.*, 1979) or 12-O-tetradecanoylphorbol-13-acetate

(Nilsson *et al.*, 1980), the U937 cells differentiate into morphologically mature macrophage-like cells.

2.4.2 Characterisation of β-haematin

The morphology of the β-haematin crystals produced by the methods of Egan *et al*. (1994) and Slater *et al*. (1991) (Figures 3.5A, 3.6A and 3.7) resembled those published by Egan *et al*. (2001) and Olliaro *et al*. (2000). The dimensions of the β-haematin crystals in these figures fall within the large range of 0.2 to > 1.6 μm previously observed for β-haematin prepared according to Egan's method (Noland *et al.*, 2003). Based on the scanning electron micrographs and transmission electron micrographs, the β-haematin crystals produced by both methods (Egan *et al.*, 1994; Slater *et al.*, 1991) have similar morphologies, and X-ray microanalyses revealed comparable elemental composition (Figures 3.5B and 3.6B). The method according to Egan *et al*. (1994), due to the shorter preparation time, was adopted in this study.

2.4.3 Phagocytosis of latex beads and β-haematin

J774A.1 cells phagocytosed latex beads and β-haematin, as demonstrated by methyl green staining (Figure 2.8) and transmission electron micrographs (Figure 2.9 and 2.10). Monoblasts, promonocytes, monocytes and macrophages comprise the mononuclear phagocyte system. As the promonocytes develop into monocytes, which then finally mature into macrophages, there is an increase in the phagocytic ability of the cells (Cline *et al.*, 1978).

Thus, the J774A.1 and U937 cell lines and PBMC demonstrate adherence to glass, alkaline phosphatase and peroxidase activities, in addition to phagocytosis. These are all characteristic properties of monocytes.

2.4.4 Comparison of the counting and OD 400 assays to monitor phagocytosis of β-haematin by the monocytes

A concomitant increase in phagocytosis of β-haematin by J774A.1 cells was observed in both the counting and the OD 400 assays when: (a) the cells were treated with increasing quantities of β-haematin, (b) the temperature of phagocytosis was increased from 4ºC to 37ºC, (c) the time period of treatment with β-haematin was increased from 3 minutes to 30 minutes, and (d)

the number of cells treated with a fixed quantity of β-haematin was increased. These findings indicate that the counting and OD 400 assays are comparable (Figures 2.11-2.13). However, these comparisons were not performed on U937 cells and PBMC. Hence, the conditions chosen for J774A.1 cells were not necessarily optimal for U937 cells and PBMC, but for comparative purposes, the conditions were kept constant for all three cell types investigated.

Both the counting and OD 400 assays did not distinguish between latex beads or β-haematin crystals bound to the cell surface and those that have been phagocytosed and were located intracellularly. In the counting assay, only crystals located inside the periphery of the cell were assumed to be phagocytosed. Crystals attached to the outside of the periphery of the cell were not considered to be phagocytosed. Cells were counted after 3 min or 30 min of β-haematin phagocytosis in two different manners. In one instance, only cells containing "internalized" crystals were considered phagocytosed and counted, and in the second instance, cells containing crystals attached to the outside of the periphery of the cell were included in the number that phagocytosed β-haematin. However, there was no difference between the two methods of counting in the fold-change observed between 3 and 30 min. To determine the amount of β-haematin that was bound to the cell surface, phagocytosis was monitored after cells were exposed to β-haematin for 3 min, and after cells were exposed to β-haematin at 4ºC for 30 min (Figure 2.12). In both cases, phagocytosis should have been minimal or completely blocked (Peterson *et al.*, 1977), and the cells counted or haeme measured were used as an indication of the amount of β-haematin bound to the cell surface. (The amount of haeme measured after phagocytosis at 4ºC was approximately 50% of the amount of haeme measured after 30 min at 37ºC).

2.4.5 Effect of febrile temperature on phagocytosis of β-haematin

A common manifestation of malaria is the occurrence of febrile episodes, resulting in body temperatures as high as 41ºC (Bruce-Chwatt *et al.*, 1986; Karunaweera *et al.*, 1992). In mammalian cells, an increase in temperature can lead to a number of changes within the cell, including protein denaturation, transient cell cycle arrest, and changes in the membrane fluidity (Beere and Green, 2001; Feder and Hofmann, 1999). Depression of phagocytosis of [^{3}H] thymidine-labelled staphylococci by human neutrophils has been observed at 41ºC (Peterson *et al.*, 1977). However, Mandell (1975) indicated that 41ºC did not significantly affect the phagocytosis of ^{14}C-labeled *Staphylococcus aureus* by peripheral blood polymorphonuclear

neutrophils. Yet Mandell did not preincubate the leucocytes at elevated temperatures prior to the phagocytosis assay (Mandell, 1975). In this investigation, J774A.1 cells were preincubated at 41ºC and 37ºC for 2 hours prior to the phagocytosis assay. However, incubating monocytes under febrile conditions had no significant effects on the phagocytosis of β-haematin (Table 2.1).

2.4.6 Effects of antimalarial drugs on the phagocytosis of monocytes

The effect of antimalarial drugs on the phagocytosis of β-haematin by the J774A.1, U937 cell lines and PBMC was investigated. The effects of the antimalarial drugs on the phagocytosis of latex beads by the J774A.1 cells were also investigated to determine if the effects observed were solely due to the antimalarial drugs or if the effects were influenced by the material being phagocytosed. Reported therapeutic physiological concentrations of the antimalarial drugs that occur in the plasma of malaria patients were used for the investigation so that the results can be related to clinical observations (Desjardins *et al.*, 1988; Wernsdorfer, 1997; White *et al.*, 1987; Winstanley *et al.*, 1990).

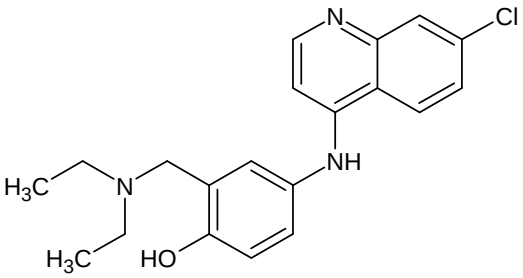
When examining the effects of the antimalarial drugs on the phagocytosis of unopsonised β-haematin and latex beads by the J774A.1 cells (Figure 3.14), the most striking observation was that quinine had an inhibitory effect of greater than 20% on the phagocytosis of latex beads, whereas quinine had minimal effects on the phagocytosis of β-haematin (less than 2% activation). Besides the small inhibitory effect of chloroquine (6%), the other antimalarial drugs investigated had minimal inhibitory effects on the phagocytosis of the latex beads. In contrast, artemisinin, doxycycline, primaquine and pyrimethamine activated the phagocytosis of β-haematin, while amodiaquine and chloroquine inhibited the phagocytosis to a small degree. The different patterns of inhibition and activation of phagocytosis obtained with the latex beads and β-haematin indicate that different mechanisms are involved in their phagocytosis. This is not totally unexpected as the structural characteristics of the two compounds are quite different.

Therapeutically-relevant concentrations of amodiaquine, artemether, chloroquine, mefloquine and quinine considerably reduced the binding of immunoglobulin G (IgG) to *P. falciparum*-infected red blood cells and the subsequent phagocytosis of the infected red blood cells by a human monocytic leukaemia-derived cell line J-111 (Shalmiev *et al.*, 1996). Prasad *et al.* (1984,1986) demonstrated the inhibition of the phagocytosis of latex beads, Fc-coated sheep

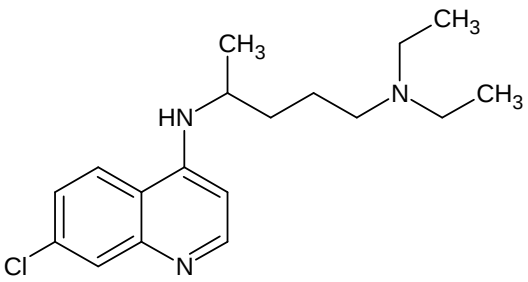
red blood cells and *Staphylococcus aureus* by monocytes isolated from rhesus monkeys treated with therapeutic concentrations of chloroquine or quinine. Some antimalarial drugs have acidotropic properties that could impair phagocytosis by raising the pH of the phagosomes, thereby reducing digestion, and the recycling of receptors (Hart and Young, 1978).

Quinine has been described as a potassium channel blocker and potassium channels are responsible for the outward flowing currents that occur during phagocytosis (Ince *et al.*, 1988). Consequently, quinine has been shown to inhibit phagocytosis of latex beads that was enhanced by feeding and sleep regulating peptides (Ichinose and Watanabe, 2004) and this may account for the observations with latex beads presented here. Furthermore, quinine has previously been reported to inhibit the phagocytosis of staphylococci by leucocytes (Felton and Dougherty, 1922) and the phagocytosis of *P. falciparum* infected red blood cells by the monocytic leukaemia-derived J-111 cell line (Shalmiev *et al.*, 1996). Thus, the activation effects of quinine on β-haematin phagocytosis by the J774A.1, U937 cells and PBMC suggests that a different mechanism is involved in β-haematin phagocytosis.

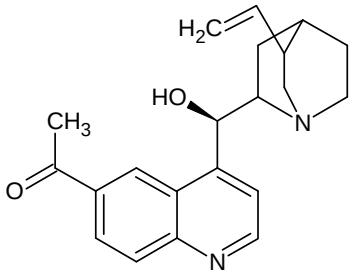
When the effects of the antimalarial drugs on the phagocytosis of β-haematin by the three cell types were compared (Figures 2.14B, 2.16 and 2.17), pyrimethamine promoted phagocytosis in all three cell types. Pyrimethamine is a dihydrofolate reductase inhibitor and out of the drugs investigated, it is the only diaminopyrimidine (Wernsdorfer, 1997) (Figure 2.18). Amodiaquine and chloroquine are both 4-aminoquinolines and both have inhibitory effects on the phagocytosis of β-haematin by all three cell types, although to much greater degrees in PBMC. Chloroquine has been reported to inhibit both Fc receptor-mediated as well as non-immunologic receptor mediated phagocytosis at prophylactic concentrations (Osorio *et al.*, 1992) as well as at therapeutically-relevant concentrations (Shalmiev *et al.*, 1996). Quinine and primaquine are related to amodiaquine and chloroquine in that they contain quinoline groups but quinine is a 4-quinoline methanol and primaquine is an 8-aminoquinoline (Figure 2.18). These differences in their chemical structures are reflected in their different effects on β-haematin phagocytosis: quinine activates phagocytosis of β-haematin in all three cell types, although to varying degrees; and primaquine activates β-haematin phagocytosis in two of the three cell types, J774A.1 and PBMC. Artemisinin is a sesquiterpene lactone that activates phagocytosis in both J774A.1 cells and PBMC. Doxycycline is a naphthacenecarboxamide that belongs to the tetracycline group and has antibacterial properties. (Wernsdorfer, 1997). It has



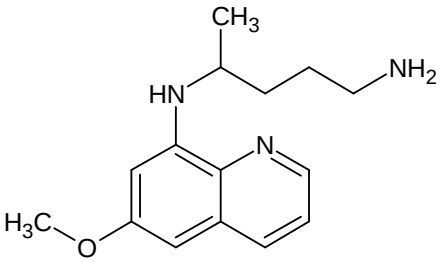
Amodiaquine (4-aminoquinoline)



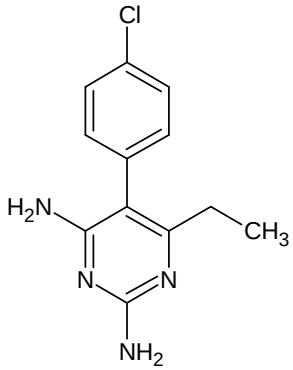
Chloroquine (4-aminoquinoline)



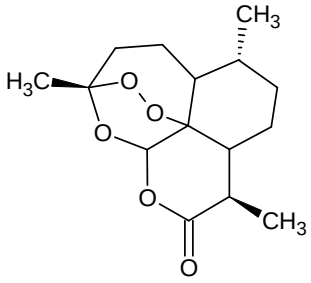
Quinine (4-quinoline methanol)



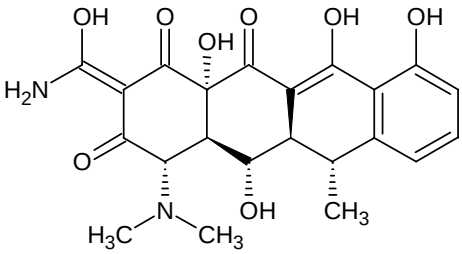
Primaquine (8-aminoquinoline)



Pyrimethamine (diaminopyrimidine)



Artemisinin (sesquiterpene lactone)



Doxycycline (naphthacenecarboxamide)

Figure 2.18. Structures of antimalarial drugs (chemical group)

different effects on the phagocytosis of β-haematin in all three monocytic cell types: It activates the J774A.1 cells, inhibits PBMC and has no effect on U937 cells. Thus, it is difficult to relate the chemical structures of the antimalarial drugs to their effects on the phagocytosis of β-haematin because three of the drugs are derived from unrelated chemical groups.

Even though the patterns of activation or inhibition of phagocytosis of β-haematin were qualitatively but not quantitatively similar between the J774A.1 cells and PBMC, it appears that each cell type has its own unique interactions with the antimalarial drugs. Each monocytic cell type is inherently different. The two cultured cell lines are isolated from different species: the J774A.1 cell line is from *Mus musculus* and the U937 cell line is from *Homo sapiens*. Furthermore, the stages of differentiation of these two cell lines are different. Initially, the U937 cells have monoblast-like characteristics and are in suspension (Koren *et al.*, 1979), but upon treatment with PMA, they differentiate into morphologically mature adherent macrophage-like cells (Nilsson *et al.*, 1980). The J774A.1 cell line has been described as an adherent macrophage-like cell line that does not require any differentiation with PMA to adhere and phagocytose (Ralph *et al.*, 1975). The presence of peroxidase activity in the J774A.1 cell line (section 2.4.1) and not in the PMA-differentiated U937 cell line could indicate that the J774A.1 cells are not as mature as the PMA-differentiated U937 cells. The PBMC are isolated from human buffy coats by centrifugation over a layer of Histopaque®-1077 and adherence to glass coverslips (Roos and de Boer, 1986). It has been observed that the purity of the monocytes/macrophages prepared in this manner is not very high (Brodersen *et al.*, 1973; Koller *et al.*, 1973). Hence, the PBMC are possibly a mixed population of monocytes and macrophages at different maturation stages, although the results of the PBMC are not intermediate between those of the U937 and J774A.1 cells. It has recently been demonstrated that the phagocytosis of β-haematin requires cholesterol-rich lipid domains in surface lipid rafts (Tiemi Shio *et al.*, 2009). Each cell type may have different membrane compositions which could also contribute to the differences between the cell lines. Furthermore, it is not known how the antimalarial drugs affect the lipid raft compositions of the membranes. In addition, β-haematin has been shown to have different effects on different cell types depending on the cell line's susceptibility to lipid peroxidation induced by β-haematin (Omodeo-Sale *et al.*, 1998; Taramelli *et al.*, 2000).

Further investigations could be done to determine which cell line (J774A.1 or U937) most closely resembles the PBMC in terms of differentiation status. The number of Fc (FcR1, FcRII),

$C3_b$ and mannose-fucose receptor sites present on the cell surfaces could be measured using flow cytometry as the number of Fc and $C3_b$ receptors increases with the maturity of the macrophage, while the mannose-fucose receptor sites decrease (Papadimitriou and Ashman, 1989). Furthermore, secretion of lysosomes and neutral proteases (elastase, collagenase and plasminogen activator) into the supernatant could be measured by ELISA (Calbiochem, U.S.A.), reverse-phase high performance liquid chromatography or radioimmunoassay as the secretion of these enzymes is enhanced with macrophage maturity. In addition, phagocytosis is enhanced with increased macrophage differentiation and could be measured by flow cytometry using fluorescent-labelled latex beads (Papadimitriou and Ashman, 1989).

It would have been preferable to determine if compounds resulting in more than 15% activation or inhibition had dose-dependent effects, if any. On reflection, positive and negative controls for the process of phagocytosis should have also been included in the experiments, whereby phagocytosis could be induced by treatment with lipopolysaccharide (Bohmer *et al.*, 1992; Engels *et al.*, 1985) or interferon-γ (Marodi *et al.*, 1993; Shalaby *et al.*, 1985; Wirth *et al.*, 1985) and inhibited by treatment with cytochalasin B (Malawista *et al.*, 1971; Painter *et al.*, 1981). Further considerations are the effects of the antimalarial drugs on the cell viability, growth rate and cell adhesion of the monocytes after treatment with antimalarial drugs for 18 hours. This is especially pertinent to the OD 400 spectrophotometric assay where the number of viable cells is unknown (only viable cells were counted under the microscope in the counting assay). Thus it would have been preferable to correct the total haeme observed in the lysates to the number of viable cells in each group.

Phagocytosis of *P. falciparum* infected red blood cells by monocytes and macrophages is the first line of defence against the malaria parasite (Abdalla, 1990). However, immunosuppression caused by the parasite and its products, such as haemozoin, contributes towards the pathogenesis of the disease (Miller *et al.*, 2002). In this scenario, malaria patients are treated with antimalarial drugs to eradicate the parasite. If the drugs inhibit/depress monocyte phagocytosis, this will contribute to the immunosuppression already observed in the disease. However, activation of phagocytosis will promote the phagocytosis of parasite-infected red blood cells as well as erythrophagocytosis, the phagocytosis of complement activated non-infected red blood cells (Goka *et al.*, 2001), which would contribute towards anaemia, a symptom often associated with malaria. This study showed that only quinine inhibited the monocyte phagocytosis of latex beads by more than 10%. However, quinine activated β-

haematin phagocytosis in monocytes which clearly demonstrates differences between the uptake mechanisms of latex beads and β-haematin. Hence, the effects of antimalarial drugs on phagocytosis, and thus immunosuppression, would depend to a large degree on what was being phagocytosed. Haemozoin, or β-haematin, is known to have inhibitory as well as stimulatory effects on the functions of monocytes (Schwarzer *et al.*, 2008). Haemozoin phagocytosed by macrophages prevents repeat phagocytosis and respiratory burst upon appropriate stimulation (Schwarzer *et al.*, 1992), and thus prevents the killing of bacteria, fungi and tumour cells (Fiori *et al.*, 1993). Moreover, in haemozoin-containing monocytes, the expression of MHC Class II and ICAM-1 in response to interferon-γ was impaired, thus linking haemozoin to disturbances in antigen presentation and immunosuppression (Schwarzer *et al.*, 1998). Thus, although the immunomodulatory effects of antimalarial drugs on monocyte phagocytosis appear to be mild (10-20%) in a healthy individual, in a malaria patient, these immunomodulatory effects could contribute to controlling the malaria infection by increasing monocyte phagocytosis of infected red blood cells.

Chapter 3

Effect of antimalarial drugs, haemozoin (β-haematin), *Plasmodium falciparum*-infected red blood cell lysates on neopterin production and GTP-cyclohydrolase 1 mRNA expression in U937 cells

B.M. Cumming

Abstract

Neopterin, a marker of cell-mediated immune response, is secreted from interferon-γ activated human macrophages. In the biosynthesis of neopterin, GTP-cyclohydrolase 1 catalyses the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which is dephosphorylated and oxidised to form neopterin. Elevated levels of neopterin have been detected in the urine and serum of malaria-infected patients and were associated with severe anaemia, respiratory distress, peak temperatures, fever- and parasite-clearance times. *Plasmodium falciparum*-infected red blood cell lysates have been reported to increase neopterin secretion from monocytic U937 cells *in vitro*. In this study, U937 cells were treated with seven antimalarial drugs, amodiaquine, artemisinin, chloroquine, doxycycline, primaquine, pyrimethamine and quinine, β-haematin (synthetic equivalent of haemozoin), latex beads, non-infected- and *P. falciparum*-infected-red blood cell lysates. Chicken antibodies were raised against neopterin-rabbit albumin conjugates to detect neopterin in the U937 supernatants. Although anti-neopterin antibodies detected neopterin-ovalbumin conjugates and neopterin added to glutaraldehyde-coated wells in indirect ELISAs, it did not bind to free neopterin in the competitive ELISA. Thus neopterin levels in the U937 supernatants could not be investigated. GTP-cyclohydrolase 1 mRNA expression in U937 cells was therefore monitored using quantitative PCR. Artemisinin, primaquine and quinine down-regulated interferon-γ-induced GTP-cyclohydrolase 1 mRNA expression in U937 cells by 1.2-, 1.3- and 1.7-fold, respectively. The remaining drugs had relatively insignificant effects on GTP-cyclohydrolase 1 mRNA expression. β-haematin up-regulated the mRNA expression by 1.2-fold, whereas *P. falciparum*-infected red blood cell lysate down-regulated the interferon-γ-induced GTP-cyclohydrolase 1 mRNA expression by 1.6-fold. This suggests that artemisinin, primaquine, quinine and parasite lysate reduce the responsiveness of the macrophage to interferon-γ. It would have been

preferable to measure neopterin levels secreted by the U937 cells for a comparison to the effects observed on GTP-cyclohydrolase 1 mRNA expression.

3.1 Introduction

Neopterin is biosynthetically derived from guanosine triphosphate (GTP) (Murr *et al.*, 2002) by GTP-cyclohydrolase 1, which catalyzes the cleavage of the purine to produce 7,8-dihydronoepterin triphosphate (Werner *et al.*, 1990). In most cells, like fibroblasts and endothelial cells, 7,8-dihydroneopterin is used to synthesize 5,6,7,8-tetrahydrobiopterin which is an essential cofactor for mono-oxygenases and inducible nitric oxide synthases (Gorren and Mayer, 2002). However, due to the lower levels of 6-pyruvoyltetrahydropterin synthase in primate and human monocytes and macrophages, activation of GTP-cyclohydrolase 1 results in an accumulation of 7,8-dihydroneopterin triphosphate which is converted by intracellular phosphatases into 7,8-dihydroneopterin (Schoedon *et al.*, 1987). 7,8-Dihydroneopterin diffuses out of the activated macrophage into the intracellular spaces and finally into the plasma. The main reaction generating neopterin from 7,8-dihydroneopterin is oxidation by hypochlorous acid (HOCl) (Widner *et al.*, 2000) which is released from neutrophils and macrophages during inflammation (Chisolm *et al.*, 1999; Schraufstatter *et al.*, 1990). The neopterin:7,8-dihydroneopterin ratio of 1:2 is nearly constant in urine, serum or cerebrospinal fluid, whereas higher neopterin:7,8-dihydroneopterin ratios are found in arterial blood in comparison to venous blood (Weiss *et al.*, 1992b).

Neopterin derivatives are produced by human monocyte-derived macrophages (Huber *et al.*, 1984) and dendritic cells (Wirleitner *et al.*, 2002) when stimulated with the cytokine, interferon-gamma (IFN-γ), that is released from activated T helper (Th) cells subtype 1 (Romagnani, 1991). As these cells promote an immune response mediated by cytotoxic T cells, increased production of neopterin in body fluids can be used to monitor cell-mediated immunity (Wachter *et al.*, 1989). Neopterin can be easily measured in plasma, urine and cerebrospinal fluid by high performance liquid chromatography because of its high fluorescence (Rippin, 1992; Werner *et al.*, 1987a; Werner *et al.*, 1987b). However, many clinical laboratories also use immuno-based methods such as enzyme-linked immunosorbent assay (ELISA) to measure neopterin (Westermann *et al.*, 2000). The levels of neopterin in body fluids are elevated in infections such as malaria (Awandare *et al.*, 2006a; Reibnegger *et al.*, 1984), human immunodeficiency virus infection (Baier-Bitterlich *et al.*, 1996b; Fuchs *et al.*, 1988) and tuberculosis (Fuchs *et al.*,

1984b; Yuksekol *et al.*, 2003), malignancies (Fuchs *et al.*, 1984a; Reibnegger *et al.*, 1991), autoimmune diseases (Leohirun *et al.*, 1991; Reibnegger *et al.*, 1986; Schroecksnadel *et al.*, 2003), allograft rejection (Margreiter *et al.*, 1983; Reibnegger *et al.*, 1991), cardiac and renal failure (Roccatello *et al.*, 1992), coronary artery disease (Schumacher *et al.*, 1992; Tatzber *et al.*, 1991) and myocardial infarction (Schumacher *et al.*, 1997). Neopterin measurements not only provide insight into the cell mediated immune response but also allow monitoring and prognosis of disease progression.

The role of 7,8-dihydroneopterin and neopterin during the inflammatory process remains poorly understood and controversial. Neopterin acts as a pro-oxidant, enhancing oxidant damage (Murr *et al.*, 1994; Wede *et al.*, 1999; Weiss *et al.*, 1993) and triggering apoptosis in a number of different cell types (Baier-Bitterlich *et al.*, 1995; Hoffmann *et al.*, 1998; Schobersberger *et al.*, 1996). In contrast, 7,8-dihydroneopterin acts as an antioxidant at low concentrations. It reacts with and neutralizes a range of reactive oxygen species including hypochlorite, nitric oxide and peroxyl radicals, thus protecting lipoproteins and various cell types including macrophages and red blood cells (Baird *et al.*, 2005; Firth *et al.*, 2008; Gieseg and Cato, 2003; Gieseg *et al.*, 2001a; Gieseg *et al.*, 1995; Gieseg *et al.*, 2001b). 7,8-Dihydroneopterin inhibited direct oxidation of plasma membranes of U937 cells by ferrous ions (Gieseg *et al.*, 2001b). Thus, it has been suggested that IFN-γ-stimulated macrophages synthesize 7,8-dihydroneopterin to protect these antigen presenting cells from the oxidants encountered within an inflammatory site (Duggan *et al.*, 2002; Gieseg *et al.*, 1995; Kojima *et al.*, 1992). However, there are instances where high concentrations of 7,8-dihydroneopterin have pro-oxidant properties (Baier-Bitterlich *et al.*, 1996a; Baier-Bitterlich *et al.*, 1995; Enzinger *et al.*, 2002b; Speth *et al.*, 2000; Spottl *et al.*, 2000; Wirleitner *et al.*, 1998; Wirleitner *et al.*, 2001).

Neopterin and its derivatives have also been reported to play a role in cell signalling. Neopterin and 7,8-dihydroneopterin were found to increase intracellular calcium concentrations (Hoffmann *et al.*, 2002; Woll *et al.*, 1993), activate redox-sensitive transcription factor nuclear factor-κB (Baier-Bitterlich *et al.*, 1997), inhibit hypoxia-induced erythropoietin gene expression (Pagel *et al.*, 1999; Schobersberger *et al.*, 1995b), stimulate nitric oxide synthase gene expression (Schobersberger *et al.*, 1995a), and together with cyclic-GMP, induce redox-sensitive proto-oncogene c-fos (Uberall *et al.*, 1994).

Elevated levels of neopterin have been detected in urine (Reibnegger *et al.*, 1984) and plasma (Kremsner *et al.*, 1989; Ringwald *et al.*, 1991) of malaria patients infected with *Plasmodium falciparum*. When 71 Thai patients with acute, uncomplicated falciparum malaria were treated with quinine and tetracycline for 7 days, the neopterin levels in urine peaked on days 3-5 following the start of treatment before decreasing towards the normal range on days 6-8 (Brown *et al.*, 1990). Similar trends were observed in other studies (Kremsner *et al.*, 1996; Ringwald *et al.*, 1991). Higher concentrations of neopterin as well as IFN-γ were found more frequently in patients infected with malaria for the first time than experienced patients (Brown *et al.*, 1990; Ringwald *et al.*, 1991) which suggested that repeated malaria infection and antigen exposure results in a progressive suppression of the T-cell-macrophage interaction mediated by IFN-γ. During seven days of quinine antimalarial therapy, serum neopterin levels remained elevated in children who were still found to have persisting anaemia one month after completing treatment; but the neopterin levels declined significantly in the children who had normal haemoglobin levels a month after completing treatment (Biemba *et al.*, 1998). The elevated neopterin levels suggested the persistence of the Th-1 mediated immune response and associated macrophage activation may be involved in the pathogenesis of the lingering anaemia after the treatment of malaria. Children with severe *P. falciparum* malaria with respiratory distress, which is a symptom of underlying acidosis, had significantly higher levels of neopterin than those malaria-infected children without respiratory distress (Awandare *et al.*, 2006a). These observations show that monitoring the neopterin levels of a patient during treatment of malaria with antimalarial drugs cannot be used to give an indication if the antimalarial drugs themselves have any effect on macrophage activation and neopterin production.

Lysates of *P. falciparum*-infected erythrocytes were found to stimulate neopterin secretion from U937 cells after 48 hours co-incubation (Facer, 1995) and this secretion was enhanced by IFN-γ. Several *P. falciparum* antigens also activated U937 cells to secrete neopterin and produced a similar response when cultured with peripheral blood mononuclear cells for 7 days (Facer, 1995). The malarial pigment, haemozoin, has been shown to have both stimulatory and inhibitory effects on the functions of monocytes and macrophages (Schwarzer *et al.*, 2008), however, the effect of β-haematin, the synthetic equivalent of haemozoin, on neopterin secretion is unknown. In this study, the effects of seven antimalarial drugs (amodiaquine, artemisinin, chloroquine, doxycycline, primaquine, pyrimethamine and quinine), β-haematin, latex beads, non-infected- and *P. falciparum*-infected-red blood cell lysates on the IFN-γ-induced expression of GTP-cyclohydrolase 1 mRNA were investigated in the human monocytic

cell line, U937. Antibodies were raised against neopterin in chickens and used in the development of a competitive ELISA to detect neopterin in the supernatants of treated U937 cells. Total RNA was isolated from the treated U937 cells and the expression of GTP-cyclohydrolase 1 mRNA was analysed using quantitative RT-PCR and the $2^{-\Delta\Delta C_T}$ method.

3.2 Materials and Methods

3.2.1 Materials

Common chemicals and buffers were purchased from Saarchem (South Africa) and Sigma-Aldrich (Germany). Neopterin, rabbit albumin, ovalbumin, sodium borohydride, Freund's complete and incomplete adjuvants, RPMI-1640, Hank's Balanced Salt Solution (HBSS), penicillin-streptomycin, L-glutamine, phorbol-12-myristate-13-acetate, chloroquine diphosphate, quinine hydrochloride, pyrimethamine, primaquine diphosphate, artemisinin, doxycycline hydrochloride, amodiaquine dihydrochloride dihydrate, latex beads (10% w/v, 0.8 µm), interferon-γ, guanidine isothiocyanate, *N*-lauroylsarcosine, 2-mercaptoethanol were purchased from Sigma-Aldrich (Germany). Glutaraldehyde (25% v/v) and sodium cyanoborohydride was obtained from Fluka (Germany). Polyethylene glycol (6000), Tween 20, propan-2-ol, absolute ethanol, chloroform, phenol, isoamylalcohol were purchased from Merck (Germany). Rabbit anti-chicken IgY was obtained from Jackson Immunoresearch Laboratories Inc. (U.S.A.). Bovine serum albumin and 2,2'-azino-di-(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS) were purchased from Roche Diagnostics (Germany). Formamide was purchased from Calbiochem (U.S.A.). Sodium citrate dehydrate was purchased from SAFC (U.S.A.). Foetal calf serum was obtained from Gibco, Invitrogen (U.S.A.). AminoLink® Coupling Gel was obtained from Pierce Biotechnology (U.S.A.). Polyprep columns were purchased from Bio-RAD (U.S.A.). Nunc-Immuno™ Maxisorp ELISA plates were obtained from Nunc (Denmark). RNase-free filter tips and microcentrifuge tubes were obtained from Quality Scientific Plastics (U.S.A.). Nunc (Denmark) T-flasks (75 cm^2 and 25 cm^2), Delta (Spain) 15 ml, 50 ml tubes and Falcon (U.S.A.) 35 x 10 mm Petri-dishes were used. VacuCap PF Bottle-top Filter devices were obtained from PALL Life Sciences (U.S.A.). Distilled water (dH_2O) was produced by a Milli-Q Plus ultra pure water system (Millipore, U.S.A.).

3.2.2 Coupling of neopterin to albumin carriers

Neopterin is light sensitive; hence all reactions with neopterin were performed in the dark. Neopterin contains a primary amine group, thus glutaraldehyde was used for coupling neopterin to protein carriers according to the method of Hermanson, (1996). Neopterin was coupled to rabbit albumin at a molar ratio of 40:1 for immunisation of chickens. The neopterin-carrier conjugate was aliquoted and stored at -20ºC. Neopterin was coupled to ovalbumin with glutaraldehyde at molar ratios of 40:1 or 200:1 for preparing affinity matrices to purify antibodies and to coat wells of microtitre plates for ELISAs. The conjugates were dialyzed against three changes of PBS, pH 7.2 and stored at -20ºC (Hermanson, 1996).

3.2.3 Preparation of immunogen and immunisation of chickens

Prior to immunisation, the neopterin-rabbit albumin conjugate was made up to 1 ml with PBS pH 7.2, and triturated with an equal volume of Freund's complete adjuvant. Two laying hens (*Gallus gallus* - Hi-line Browns) were immunised with 500 µg neopterin intramuscularly at two sites (Polson *et al.*, 1980a; Schwarzkopf *et al.*, 2001). Booster injection immunogen were emulsified in Freund's incomplete adjuvant and given at weeks 2, 4, 6. Eggs were collected throughout the immunisation schedule until week 12 and stored at 4ºC.

3.2.4 Isolation of Immunoglobulin Y (IgY) from egg yolks

Chicken antibodies, IgY, were isolated from egg yolks using polyethylene glycol (PEG) precipitation, according to Polson's method (Polson *et al.*, 1985; Polson *et al.*, 1980a). The concentration of IgY was determined by measuring the absorbance of a 1:50 dilution and using the extinction co-efficient of IgY, 1.25 $(mg/ml)^{-1}$(Goldring and Coetzer, 2003).

3.2.5 Monitoring the titre of antibody production in chickens with an ELISA

Wells of a Nunc-Immuno™ microtitre plate were coated with 150 µl of 1 µg/ml rabbit albumin in 50 mM $NaHCO_3$, pH 6, or 150 µl of 6.25 µg/ml neopterin of a 40:1 neopterin-ovalbumin conjugate in PBS, pH 7.2, and incubated overnight at 4ºC. The wells were blocked with 200 µl 0.5% (w/v) bovine serum albumin in PBS, pH 7.2, (BSA-PBS) for 1 h at 37ºC and then washed thee times with 0.1% (v/v) Tween 20 in PBS, pH 7.2 (Tween-PBS). Crude IgY dilutions, 100, 10, 1 and 0.1 µg/ml in 0.5% BSA-PBS, were incubated in the wells (100 µl) for 2 h at 37ºC.

Wells were washed three times with 0.1% Tween-PBS. Secondary antibody, rabbit anti-chicken IgY-horseradish peroxidase, diluted 1:10 000 in 0.5% BSA-PBS, was added (120 µl) and incubated for 1 h at 37ºC. The wells were washed three times in 0.1% Tween-PBS. Substrate solution containing 0.05% (w/v) 2,2'-azino-di-(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS) and 0.0015% (v/v) H_2O_2 in 0.15 M citrate phosphate buffer, pH 5.0, was added (150 µl) and the colour was allowed to develop for 30 min in the dark at RT. The A_{405} of each well was measured with a Versamax microplate reader using SOFTmax® software from Molecular Devices (U.S.A.).

3.2.6 Preparation of affinity matrices

Affinity matrices were prepared with neopterin alone or with neopterin conjugated to ovalbumin at a molar ratio of 40:1 or 200:1 using the protocol for the AminoLink® Coupling Gel from Pierce Biotechnology (U.S.A.). The resin was stored in 100 mM sodium phosphate buffer, pH 7.6, 0.1% NaN_3 at 4ºC.

3.2.7 Affinity purification of IgY

IgY isolated from weeks 4-7 eggs from chicken 1 were pooled and filtered (Whatman no.1). The affinity matrices (Section 3.2.6) were equilibrated with 100 mM sodium phosphate buffer, pH 7.6, 0.1% NaN_3, and the IgY pool cycled over the affinity matrix overnight in the reverse direction. The column was washed with 12 column volumes of phosphate buffer and eluted with 100 mM glycine, 0.02% (w/v) NaN_3, pH 2.8. Fractions (900 µl) were collected in microcentrifuge tubes containing 100 µl of neutralisation buffer (1 M NaH_2PO_4, 0.02% NaN_3, pH 8.5) and the elution monitored by measuring the absorbance of each fraction at 280 nm. The fractions with absorbance readings > 0.1 were pooled, NaN_3 was added to a final concentration of 0.1% (w/v) and the antibodies were stored at 4ºC. The affinity columns were washed with 12 volumes of 100 mM sodium phosphate buffer, pH 7.6, 0.1% NaN_3 prior to storage at 4ºC.

3.2.8 Development of a competitive ELISA to detect neopterin secretion in the supernatants of U937 cells

(A) Neopterin-ovalbumin conjugates coupled at ratios of 40:1 and 200:1 were compared as well-coatings in an indirect ELISA. Wells of a Nunc-Immuno™ ELISA plate were coated with

150 µl of either neat neopterin-ovalbumin conjugate coupled at a ratio of 40:1 or conjugate coupled at a ratio of 200:1, and incubated overnight at 4ºC. The ELISA was performed as described in section 3.2.5 using crude IgY (100 µg/ml) from week 0 (non-immune) and week 8 (immune), and affinity purified IgY (25 µg/ml).

(B) An indirect ELISA was performed to determine the optimal coating concentration of the neopterin-ovalbumin conjugate coupled at a molar ratio of 200:1. Wells were coated with 150 µl of doubling dilutions (1048 to 8.2 ng/ml neopterin) of the neopterin-ovalbumin conjugate (200:1) in PBS, pH 7.2 and incubated overnight at 4ºC. The ELISA then proceeded as described in section 3.2.5 using crude IgY (100 µg/ml) from week 0 (non-immune) and week 8 (immune) from chicken 1 and chicken 2.

(C) Several parameters were varied in a competitive ELISA in an attempt to detect anti-neopterin IgY. Wells were coated with 150 µl of 1:2 dilution of the neopterin-ovalbumin conjugate (200:1) (equivalent to 524.5 ng/ml neopterin) in 50 mM $NaHCO_3$, pH 6, or PBS (pH 7.2) overnight at 4ºC. Varying concentrations of free neopterin in solution (37.5 µg/ml to 21 pg/ml) were incubated with varying concentrations of affinity purified anti-neopterin IgY (0.1, 1 or 10 µg/ml) diluted in 0.5% BSA-PBS, in microcentrifuge tubes for 1 hour at RT in the dark. During this incubation, the wells of the Nunc-Immuno™ ELISA plate were blocked as described in section 3.2.5. After the wells were washed, 100 µl of the neopterin-IgY solutions were added and incubated for 1 h at 37ºC. The wells were washed and the bound IgY was detected as described in 3.2.5. Variations in the coating concentrations of the neopterin-ovalbumin conjugate (doubling-dilutions from 524 to 16.375 ng/ml) were also investigated in a competitive ELISA with two concentrations of crude immune IgY (100 and 10 µg/ml).

(D) In order to determine the cross-reactivity of the anti-neopterin antibodies with rabbit albumin, ovalbumin, bovine ovalbumin and neopterin in an indirect ELISA, wells were coated with 150 µl of 1 µg/ml rabbit albumin, ovalbumin or bovine albumin, or 50 µg/ml neopterin in PBS, pH 7.2. Gelatin (0.1%, w/v) melted in PBS, pH 7.2 (gelatin-PBS), was used to replace 0.5% (w/v) BSA-PBS to block the wells coated with bovine albumin and dilute the antibodies added to these wells. Non-immune (week 0) and immune (week 8) crude IgY (100 µg/ml) were used as primary antibodies in the ELISA as described in section 3.2.5.

(E) An alternative method of coating the wells was explored where the wells were activated with 150 µl 0.2% (v/v) glutaraldehyde for 2 h at 4ºC and coated with 150 µl of 3-fold dilutions of neopterin (50, 16.7, 5.55, 1.85 and 0.617 µg/ml) in PBS, pH 7.2, overnight at 4ºC to determine the optimal coating concentration of neopterin in an indirect ELISA. The wells were blocked with 0.1% gelatin-PBS. Various concentrations of week 8 crude IgY (100, 50, 25, 10, 5, 2.5 µg/ml) and the affinity purified antibodies (1 and 0.5 µg/ml) prepared in 0.1% gelatin-PBS were added (100 µl) and incubated for 2 h at 37ºC. The bound primary antibody was detected as described (section 3.2.5).

(F) In the competitive ELISA, after activation with 0.2% glutaraldehyde for 2 h at 4ºC, the wells were coated with 150 µl of 16.7 µg/ml neopterin in PBS, pH 7.2, overnight at 4ºC. The competitive ELISA proceeded as described above in (C) except various concentrations of crude IgY only (100, 50, 25 µg/ml) in 0.1% gelatin-PBS were incubated with 10-fold dilutions of free neopterin (28.11, 9.37, 3.12, 1.04, 0.35, 0.12 ng/ml) for 1 h in glass tubes at RT prior to their addition to the glutaraldehyde-activated wells coated with neopterin.

3.2.9 Culturing of the U937 cell line

The human monocyte-like U937 cell line (Fischer *et al.*, 1980; Koren *et al.*, 1979; Ralph *et al.*, 1976; Sundstrom and Nilsson, 1976) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, U.K.). It was cultured in complete RPMI-1640 medium supplemented with 10% FCS (NOT heat-inactivated), 4 mM L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. This cell line was grown at 37ºC in a humidified 5% CO_2 atmosphere and subcultured every 2-3 days.

3.2.10 Synthesis and analysis of β-haematin

β-haematin was prepared according to Egan *et al.*, (1994). Briefly, haemin chloride (30 mg) was dissolved in 6.0 ml of 0.1 M NaOH and equilibrated at 60ºC. To this solution, 0.6 ml of 1.0 M HCl and 3.7 ml of 12.9 M sodium acetate (pH 5) pre-incubated at 60ºC were added and the mixture was stirred at 60ºC for 1 h. The crystals were filtered (8 µm cellulose acetate/nitrate Millipore filter Type SC), washed extensively with dH_2O and dried at 37 ºC over silica gel for 48 h.

The β-haematin crystals were mounted onto stubs covered with carbon tape and viewed under a Philips environmental scanning electron microscope (ESEM) with a large field detector and a gaseous secondary electron detector. X-Ray microanalysis of the crystals was performed on the ESEM at 2000x magnification, an accelerating voltage of 15 kV and a spot size of 4.5 for 150 s under low vacuum.

3.2.11 Treatment of U937 cells with interferon-γ (IFN-γ) and antimalarial drugs, β-haematin, latex beads, *P. falciparum*-infected or non-infected red blood cell lysate

U937 cells (3.5×10^5 cells/ml, 15ml) were differentiated by incubation with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 h (Goldring *et al.*, 1992). The non-adherent cells and spent media were aspirated. Complete culture medium (15ml) containing 200 U/ml human recombinant interferon-gamma (IFN-γ) (Facer, 1995) and one of the following: (a) physiological therapeutic concentrations of seven antimalarial drugs separately, (b) β-haematin to a final concentration of 25 µg/ml, (c) latex beads to a final concentration of 0.1% (w/v), (d) non-infected red blood cell lysate, or (e) *P. falciparum*-infected red blood cell lysate (10% parasitaemia, kindly supplied by Professor Peter Smith from University of Cape Town) to final protein concentrations of 10 or 20 µg/ml, was added to the adherent cells and incubated for 18 h at 37ºC in a humidified 5% CO_2 atmosphere. The concentration of the antimalarial drugs were: amodiaquine, 75.6 ng/ml (White *et al.*, 1987; Winstanley *et al.*, 1987; Winstanley *et al.*, 1990); artemisinin, 400 ng/ml (Wernsdorfer, 1997); chloroquine, 200 ng/ml (Desjardins *et al.*, 1988); doxycycline, 3 µg/ml (Wernsdorfer, 1997); primaquine, 153 ng/ml (Desjardins *et al.*, 1988); pyrimethamine, 234 ng/ml; and quinine, 15 µg/ml (Wernsdorfer, 1997). The following controls were set up: cells treated with (a) PMA, IFN-γ and the concentrations of solvents used to solubilise the antimalarial drugs; (b) PMA, but not IFN-γ; and (c) neither PMA nor IFN-γ. Following treatment for 18 h, the supernatants were collected and stored at -70ºC for analysis of neopterin secretion by competitive ELISA. The cells were harvested by trypsin treatment (trypsin was diluted 10-fold in HBSS) for 5 min, pelleted, washed with HBSS and used for RNA isolation.

3.2.12 Isolation of Total RNA from U937 cells

Total RNA was extracted from the U937 cells by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) according to Voss (2002). Briefly, 1 ml of RNA extraction buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol, was added to the cell pellet and the cells were rapidly homogenized by pipetting to inactivate endogenous RNases. Sodium acetate (2 M, pH 4.0, 1/10 volume) was added and mixed by inversion. Water-saturated phenol (1 volume) was added, mixed by inversion and a 1/3 volume of chloroform:isoamylalcohol (49:1) was added, mixed by inversion and placed on ice for 5 min. The sample was centrifuged at 3 500 *g* for 30 min at 4ºC. The aqueous supernatant was transferred to a RNase-free microcentrifuge tube containing 7/8 volume isopropanol and the RNA was left at -20ºC for 1 h. The RNA was pelleted at 15 000 *g* for 20 min at 4ºC, the supernatant was discarded and the RNA pellet air dried for 5 min (RT). The pellet was resuspended in 450 µl RNA extraction buffer, 1/10 volume of sodium acetate was added and mixed, followed by 1 volume of water-saturated phenol, mixing, 1/3 volume of chloroform:isoamylalcohol (49:1) and mixing. The sample was centrifuged at 15 000 *g* for 10 min at 4ºC. The aqueous supernatant was transferred to a RNase-free microcentrifuge tube and the RNA incubated with 7/8 volume of ice-cold isopropanol for 1 h at -20ºC. The RNA was pelleted by centrifugation at 15 000 *g* (20 min, 4ºC), washed once with 70% (v/v) ethanol and centrifuged again at 15 000 *g* (5 min, 4ºC). The ethanol wash was discarded and the RNA pellet was air dried for 5 min (RT) prior to dissolution in 30 µl of diethyl pyrocarbonate (DEPC)-treated ddH_2O.

The concentration of the RNA was determined from the absorbance at 260 nm. The integrity of the RNA was evaluated using a 25% Tris-EDTA, 75% formamide gel loading buffer (Voss, 2002) at a ratio of 1:4 on a 1.2% (w/v) agarose gel for 1 h at 80 V in the presence of ethidium bromide.

3.2.13 Analysis of the relative expression of GTP-cyclohydrolase 1 mRNA transcripts using quantitative RT-PCR

Two sets of specific exon-spanning primers were designed for human GTP cyclohydrolase 1 (GTP-CH1) (accession number NM_000161) using Primer 3 software (Rozen and Skaletsky, 2000). Refer to Table 3.1. These primers and primers for β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized at the MCB Synthetic DNA unit,

University of Cape Town, South Africa. The β-actin and GAPDH house-keeping genes were used as the endogenous control genes.

Table 3.1. Primers used for analysing relative expression of GTP-cyclohydrolase 1 using quantitative RT-PCR

Primer Name	Direction	Sequence	Target Size (bp)	Source
GTP-CH1A	Forward	5'-AGCAAACTTGCGAGGATTGT-3'[b]	207	Primer 3[a]
GTP-CH1A	Reverse	5'-GAACACACCCAACATTGTGC-3'[c]		Primer 3[a]
GTP-CH1B	Forward	5'-TCTTCACCAAGGGCTACCAG-3'[d]	247	Primer 3[a]
GTP-CH1B	Reverse	5'-GTAAGGCGCTCCTGAACTTG-3'[e]		Primer 3[a]
β-actin	Forward	5'-GCGGGAAATCGTGCGTGACATT-3'[f]	232	Epicentre Biotechnologies (U.S.A.)
β-actin	Reverse	5'-GATGGAGTTGAAGGTAGTTTCGTG-3'[g]		Epicentre Biotechnologies (U.S.A.)
GAPDH	Forward	5'-GGTATCGTGGAAGGACTCATGAC-3'[h]	188	Wang *et al.* (2008)
GAPDH	Reverse	5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'[i]		Wang *et al.* (2008)

a) Primer 3 software was designed by Rozen and Skaletsky (2000)
b) Primer spans exon 3 and 4
c) Exon 6
d) Exon 1
e) Primer spans exon 4 and 5
f) Exon 4
g) Exon 5
h) Exon 7
i) Exon 8

RNA treated with DNase (as per protocol from Fermentas, U.S.A.) was compared to untreated RNA as a template in the reverse-transcriptase reaction followed by quantitative PCR. The quantitative PCR was optimised by comparing the two primer sets designed for GTP-cyclohydrolase 1 (GTP-CH1A and B) using β-actin as the endogenous control, and annealing temperatures of 55ºC and 60ºC.

RNA (1 µg) was reverse transcribed to generate cDNA using 5 µM of random hexamers and the SensiMix™ Two-Step Kit (Quantace Ltd., U.K.) in a 20 µl reaction. One fiftieth of the resulting cDNA was used as a template for SYBR® Green I (Molecular Probes®, U.S.A.) quantitative PCR with the SensiMix™ Two-Step kit in the RotorGene 6000 Series real-time PCR cycler (Corbett Life Science Research, Australia). Each PCR reaction (10 µl) was set up in triplicate and contained cDNA template (1 µl of a 2.5 x dilution of the cDNA), 200 nM of the reverse and forward primers for one of the genes, 5 µl of SensiMix dT (reaction buffer, heat-

inactivated Taq DNA polymerase, dNTPs and 6 mM $MgCl_2$) and 0.2 µl of 50 x SYBR® Green I solution. No-template controls were also set up. Quantitative PCR was carried out using the following thermal cycling parameters: 95ºC for 10 min to activate the enzyme, followed by 40 cycles of three-step PCR (95ºC for 10 s, 60ºC for 15 s, 72ºC for 15 s). The fluorescence signal was acquired at the end of each elongation step.

Data from the quantitative PCR were compared using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) where the cycle threshold (C_T) value of the endogenous control gene (β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) was subtracted from the C_T of the experimental gene (GTP-cyclohydrolase 1 (GTP-CH1)) for each sample. The change in C_T (ΔC_T) for each experimental sample was then subtracted from the ΔC_T of the unstimulated control sample. Change (n-fold) was expressed as $2^{-\Delta\Delta C_T}$ relative to results from unstimulated conditions (Livak and Schmittgen, 2001).

For the $2^{-\Delta\Delta C_T}$ calculation to be valid, the amplification efficiencies of the target (GTP-CH1) and the reference genes (β-actin or GAPDH) must be approximately equal. To determine if the target and reference amplifications have the same PCR efficiencies, variation in ΔC_T ($C_{T,\ target}$ - $C_{T,\ reference}$) with template dilution is monitored. Dilutions of the cDNA (1, 2.5, 6.25, 10, 25, 62.5 and 100 x) were used as templates in quantitative PCR set up in triplicate with the primers for the GTP-CH1, β-actin and GAPDH in separate tubes. The average C_T values generated from the equivalent cDNA inputs (target versus reference) were used in the ΔC_T calculation ($C_{T,\ GTP\text{-}CH1}$ - $C_{T,\ \beta\text{-actin OR GAPDH}}$). The ΔC_T values calculated for each cDNA dilution were plotted against the logarithm of the cDNA input and the dynamic range of the assay was ascertained by removing outlying dilutions (usually the highest and/or lowest dilution). Once the dilution points within the dynamic range were plotted and the data fitted using least-squares linear regression analysis, absolute values of the resulting slope < 0.1 indicated that the $2^{-\Delta\Delta C_T}$ method could be used for relative quantification (Livak and Schmittgen, 2001).

3.2.14 Statistical analysis

Statistically significant differences were determined by the Mann-Whitney U (Wilcoxon Rank-Sum) test using the GenStat Program, 9th Edition. Values of $p<0.05$ were considered statistically significant. All data are presented as the mean ± SD.

3.3 Results

Chickens were immunised with neopterin conjugated to rabbit albumin (40:1) and boosted every second week on three occasions. Eggs were collected each week and IgY was isolated from the egg yolks (Polson *et al.*, 1985; Polson *et al.*, 1980b). The titre of the IgY production was measured by ELISA detecting anti-neopterin as well as anti-rabbit albumin antibodies in the crude IgY preparations. The crude IgY isolated from weeks 4 to 7 eggs from chicken no. 1 were pooled and affinity purified. Using the crude and affinity purified IgY, several attempts were made to develop a competitive ELISA with microtitre plates coated with neopterin-ovalbumin conjugates or neopterin added to glutaraldehyde-activated wells. U937 cells differentiated with phorbol-12-myristate-13-acetate were treated with interferon-γ and one of the following for 18 h: antimalarial drugs, β-haematin, latex beads, non-infected red blood cell lysate or *P. falciparum*-infected red blood cell lysate. The supernatants were collected for measuring neopterin secretion by competitive ELISA, and the cells were harvested for total RNA isolation to determine the relative expression of GTP-cyclohydrolase I mRNA transcripts by quantitative RT-PCR. GTP-cyclohydrolase I is an enzyme that catalyses the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which is then transformed to neopterin by phosphatases and oxidation (Hoffmann *et al.*, 2003).

3.3.1 Evaluation of chicken anti-rabbit albumin antibody and anti-neopterin antibody titres using ELISA

Two chickens were injected with neopterin conjugated to rabbit albumin at a ratio of 40:1. The titres for antibodies against the rabbit albumin started to increase from week 4 and started to decrease at about week 10 (Figure 3.1A). The titres for the anti-neopterin antibodies increased from week 3 and started decreasing after week 9 (Figure 3.1B). The titre of the anti-carrier peaked at similar periods to that of anti-neopterin in both chickens, weeks 5-7 in chicken 1 and weeks 8-10 in chicken 2.

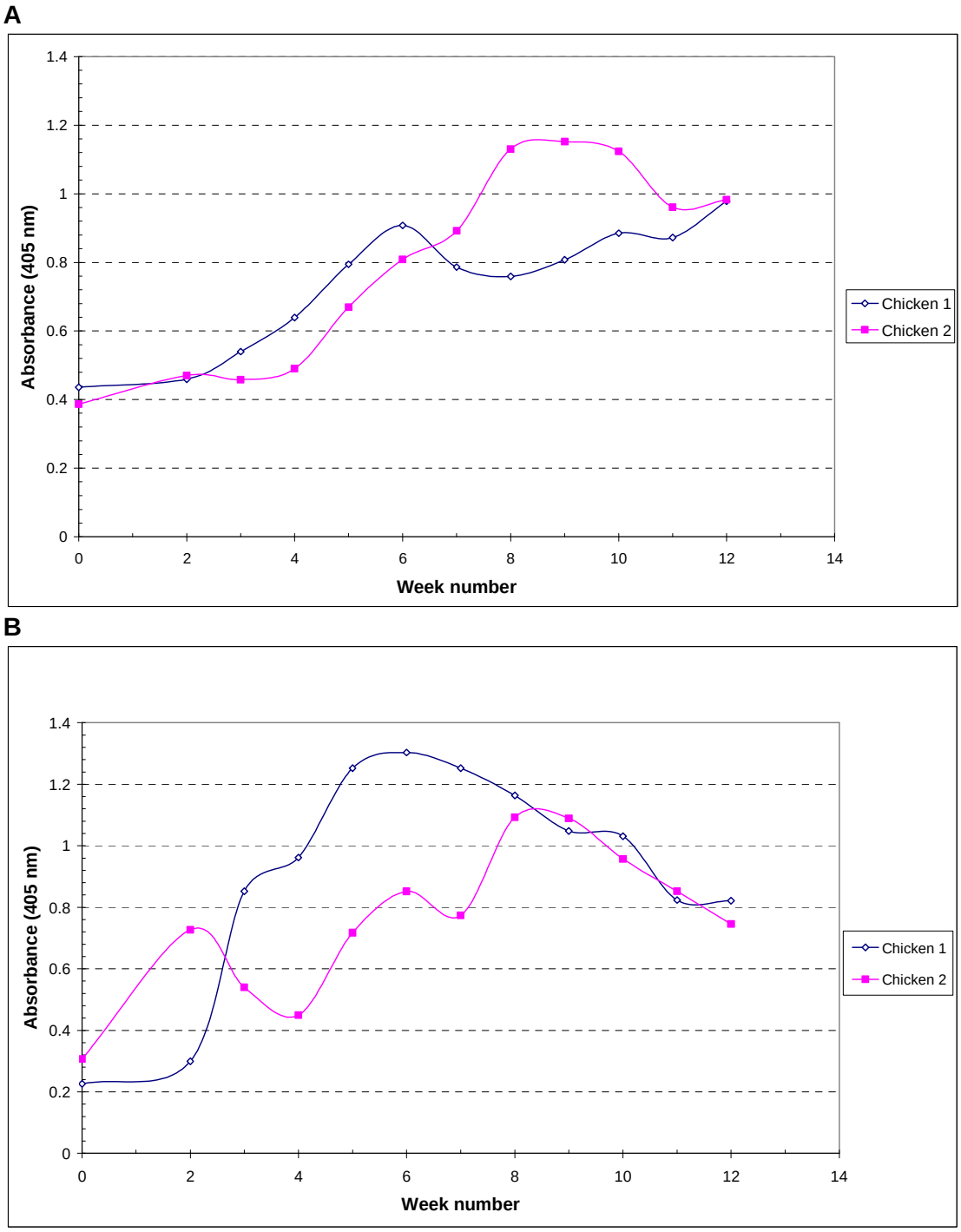


Figure 3.1. Progress of anti-rabbit albumin and anti-neopterin antibody production in chickens immunised with neopterin conjugated to rabbit albumin measured by ELISA. Two chickens were immunised with neopterin conjugated to rabbit albumin. Wells were coated with (A) rabbit albumin (B) neopterin-ovalbumin conjugate (40:1). Crude chicken IgY antibodies (100 µg/ml) isolated from the eggs of each week were incubated in the wells. Binding was visualised with horse-radish peroxidase (HRPO)-linked rabbit anti-chicken IgY and the ABTS/H_2O_2 detection system. Each point is the mean absorbance at 405 nm of duplicate samples.

3.3.2 Affinity purification of chicken anti-neopterin antibodies

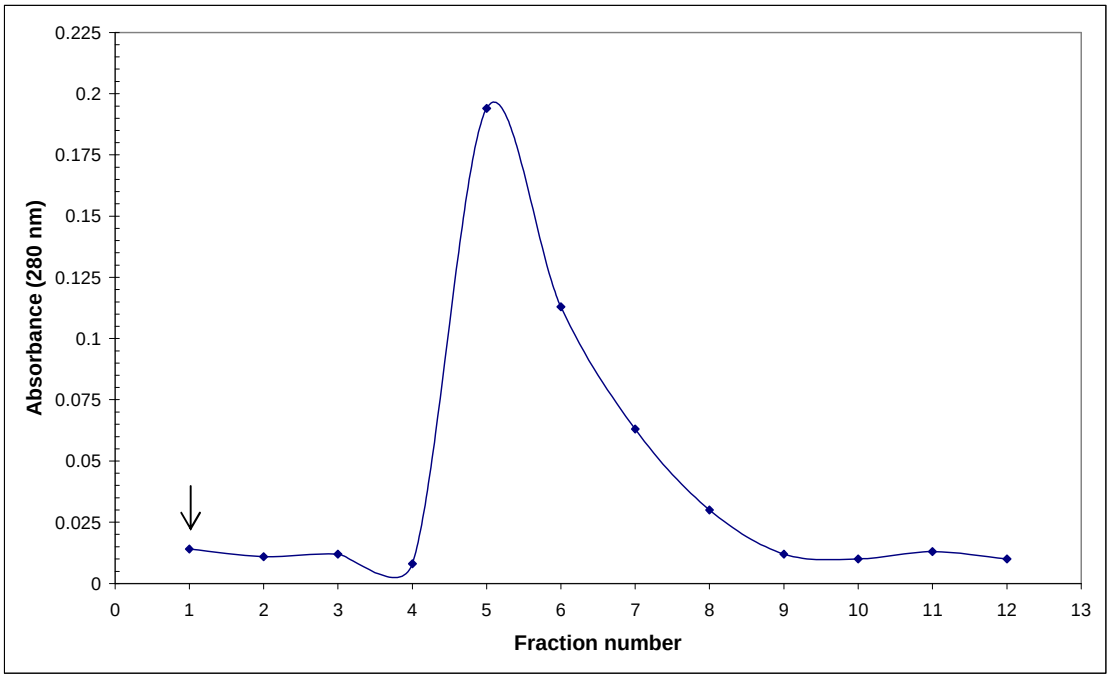


Figure 3.2. Elution profile of anti-neopterin antibodies from the neopterin-ovalbumin (40:1) affinity matrix. Neopterin-ovalbumin conjugate (40:1) was coupled to the AminoLink® Coupling Gel. The isolated chicken antibodies (weeks 4-7, chicken 1) were circulated over the neopterin-ovalbumin affinity matrix overnight. The unbound antibodies were washed off the column and the bound antibodies were eluted using a low pH buffer (100 mM glycine, pH 2.8) applied at ↓. The absorbance of the eluted fractions was measured at 280 nm. The fractions with A_{280} values greater than 0.1 were stored at 4ºC.

The anti-neopterin antibodies from weeks 4 to 7 of chicken 1 were pooled. When the pool was passed over the neopterin affinity matrix, no antibodies eluted from the column. When the pool was passed over the neopterin-ovalbumin conjugate (40:1) affinity matrix, anti-neopterin antibodies eluted from the affinity column with a total yield of 254.4 µg/ml (Figure 3.2). An ELISA (section 3.2.5) of the pooled crude IgY after it had passed through the affinity matrix showed that there were still anti-neopterin antibodies present in the crude IgY pool (data not shown). Hence, the pool was passed over the column another three times, producing yields of 309, 256 and 187 µg anti-neopterin IgY, respectively. In each instance, an ELISA indicated that the week 4-7 pool of crude IgY still contained anti-neopterin antibodies after being passed over the matrix. This suggested that the capacity of the affinity matrix coupled to the neopterin-ovalbumin conjugate (40:1) to bind anti-neopterin antibodies was limited. In an attempt to

increase the capacity of the affinity matrix, a neopterin-ovalbumin conjugate with a 200:1 molar ratio was coupled to the AminoLink® coupling gel, but when the pool of week 4-7 crude IgY of chicken 1 was passed over this matrix, no antibodies eluted from the column.

3.3.3 Development of a competitive ELISA to measure the neopterin secreted into the supernatants of the U937 cells

Neopterin-ovalbumin conjugates were prepared at molar ratios of 40:1 and 200:1. To compare the conjugates prepared at different molar ratios, wells of a microtitre plate were coated with the neopterin-ovalbumin conjugates for an indirect ELISA using both crude and affinity purified IgY as described in section 3.2.5. Ratios of the immune (week 8) A_{405} values over the non-immune (week 0) A_{405} values in Figure 3.3A shows that the 200:1 conjugate distinguished the immune IgY from the non-immune IgY to a greater degree than the 40:1 conjugate (Ratios of 4.1 and 3.3, respectively). Figure 3.3B demonstrates that the 200:1 neopterin-ovalbumin conjugate resulted in a better detection of the affinity purified antibodies.

An indirect ELISA was performed to ascertain the optimal coating concentration of the neopterin-ovalbumin conjugate (200:1) to detect anti-neopterin IgY. The immune IgY (Week 8) resulted in A_{405} values 2 to 3-fold higher than the non-immune IgY A_{405} values, up to a 16-fold dilution of the neopterin-ovalbumin conjugate (200:1), although the immune IgY from chicken 2 resulted in a 1.45-fold greater absorbance than that from chicken 1 (Figure 3.4).

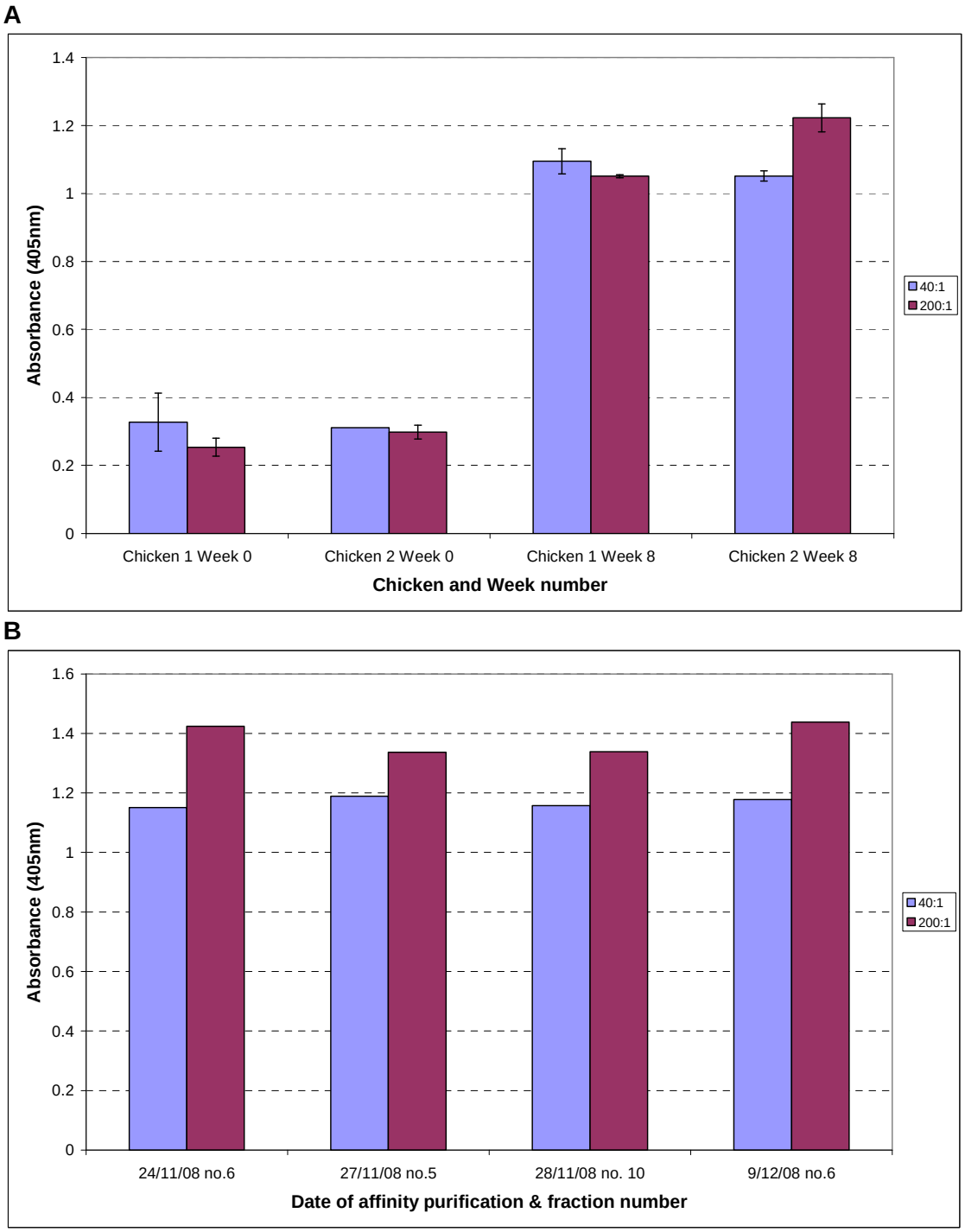


Figure 3.3. Comparison of neopterin-ovalbumin conjugates coupled at molar ratios of 40:1 and 200:1 as coatings in an indirect ELISA to detect anti-neopterin IgY. Wells were coated with neat neopterin-ovalbumin conjugates prepared at ratios of 200:1 and 40:1. (A) Non-immune (Week 0) and immune (Week 8) crude IgY and (B) affinity purified antibodies were incubated in the wells and bound antibody was detected by rabbit anti-chicken IgY HRPO-linked secondary antibody followed by the addition of the substrate ABTS/H_2O_2. Absorbances at 405 nm in (A) were the mean of duplicate experiments ± SD.

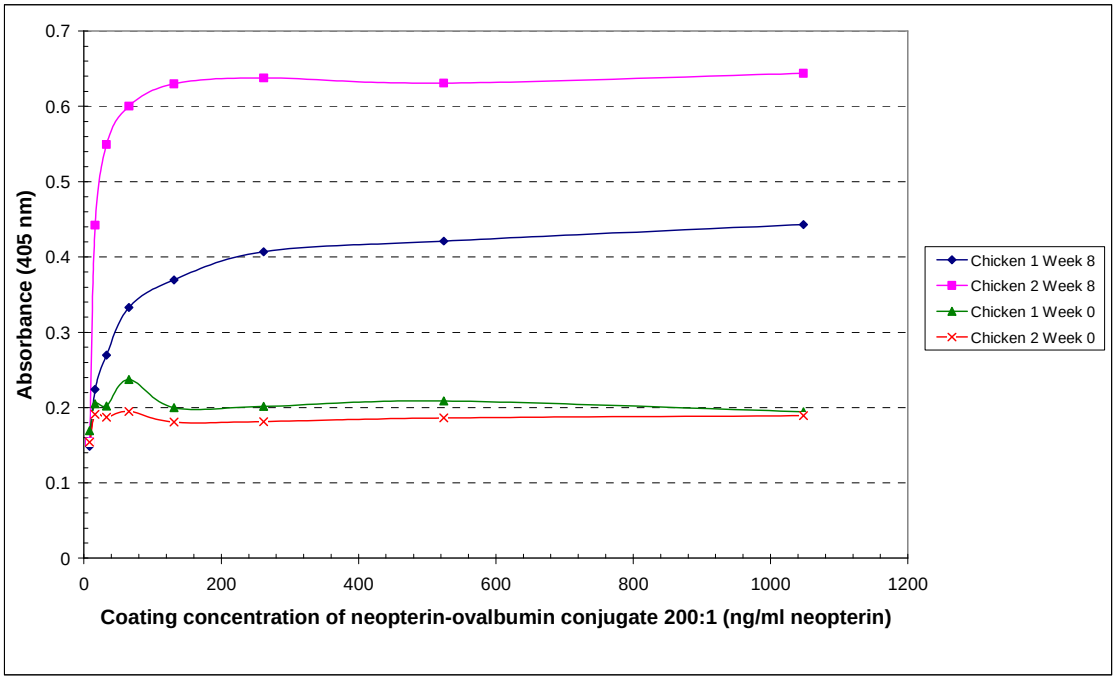


Figure 3.4. ELISA to determine the appropriate coating concentration of the neopterin-ovalbumin conjugate (200:1) to detect anti-neopterin IgY. Wells were coated with doubling dilutions of the neopterin-ovalbumin conjugate. Crude IgY antibodies isolated from week 0 (non-immune) and week 8 (immune) were incubated in the wells and bound antibody was detected by rabbit anti-chicken IgY HRPO-linked secondary antibody and the substrate ABTS/H_2O_2. Absorbances at 405 nm were the mean of duplicate experiments.

A competitive ELISA was used to set up a neopterin standard curve to measure the neopterin in the supernatants of U937 cells. Various concentrations of affinity purified antibody were incubated with 5-fold dilutions of free neopterin for 1 h in microcentrifuge tubes prior to their addition to wells coated with the neopterin-ovalbumin conjugate (200:1). Figure 3.5 is a representation of one of the ELISAs performed with the free neopterin concentration range of 37500-60 ng/ml; lower free neopterin concentrations were investigated in competitive ELISAs, but results similar to those in Figure 3.5 were obtained. For each antibody concentration, almost constant A_{405} values (~0.55, ~0.45 or ~0.23) were obtained at all the free neopterin concentrations investigated. The only difference was a reduction in the constant A_{405} value with a decrease in the concentration of the affinity purified antibodies incubated with the free neopterin. This suggests that the affinity purified anti-neopterin antibodies were not binding to the free neopterin prior to being incubated in the wells coated with the neopterin-ovalbumin conjugate. The buffer used to dilute the neopterin-ovalbumin conjugate to coat the wells was

changed from a carbonate buffer (50 mM $NaHCO_3$, pH 6) to PBS, pH 7.2, but PBS, pH 7.2 resulted in patterns similar to those in Figure 3.5.

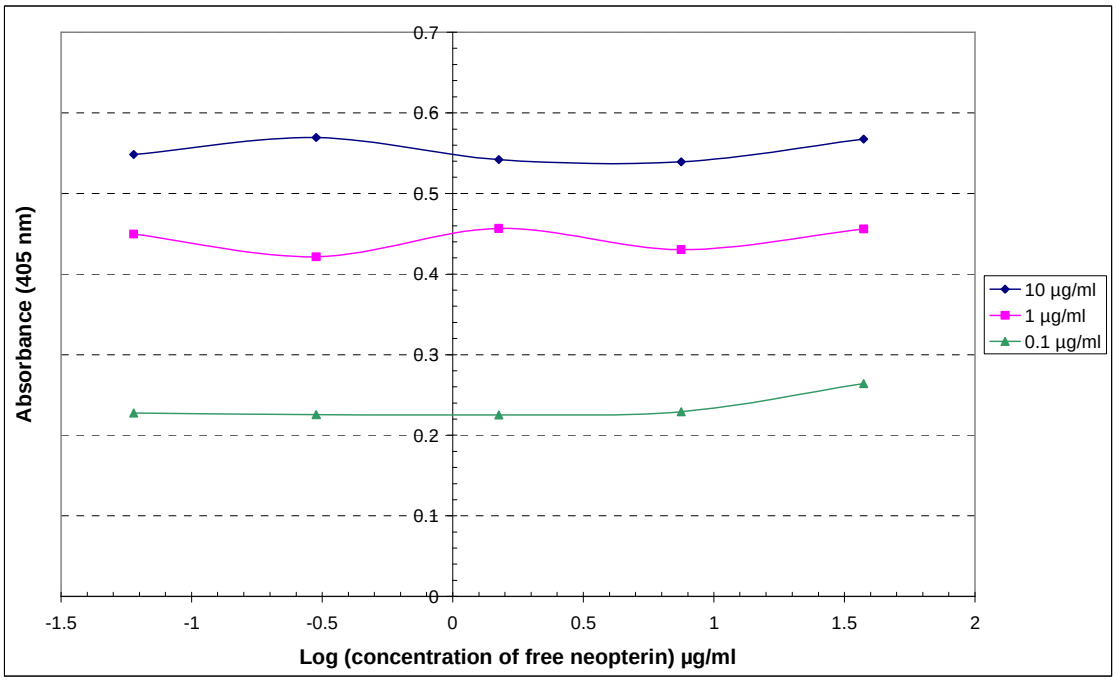
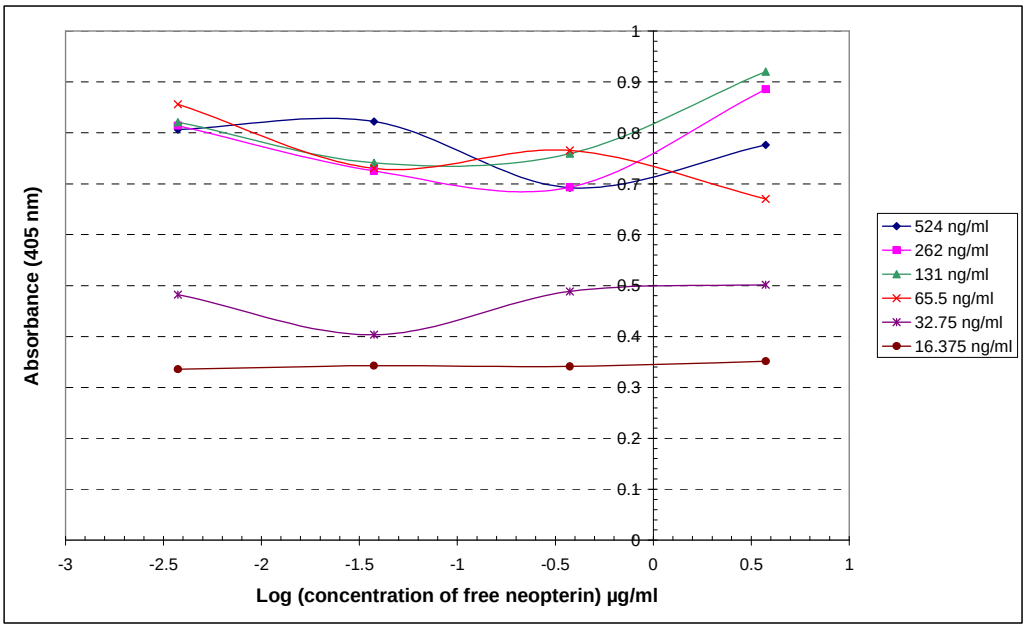


Figure 3.5. Competitive ELISA: Standard curve for neopterin using neopterin-ovalbumin conjugate (200:1) coated wells and varying concentrations of affinity purified anti-neopterin IgY. Wells were coated with a 1:2 dilution of the neopterin-ovalbumin conjugate. Dilutions of the affinity purified antibodies (10, 1, 0.1 µg/ml) were incubated with 5-fold dilutions of free neopterin for 1 h in microcentrifuge tubes. The neopterin-antibody combinations were then incubated in the wells for 1 h and antibody that bound to the well was detected using HRPO-linked rabbit anti-chicken IgY and ABTS/H_2O_2 as a substrate. Each point is the mean absorbance at 405 nm of duplicate experiments.

Variations in the coating concentrations of the 200:1 neopterin-ovalbumin conjugate from 524 to 16.375 ng/ml neopterin were investigated in the competitive ELISA. Two concentrations (100 and 10 µg/ml) of crude anti-neopterin IgY (chicken 1, week 8) were incubated with 10-fold dilutions of free neopterin (37.5 µg/ml to 37.5 ng/ml) in microcentrifuge tubes for 1 h prior to their addition to the neopterin-ovalbumin coated wells. However, no increase in the A_{405} values with decreasing free neopterin concentrations were observed at any of coating concentrations of the neopterin-ovalbumin conjugate (Figure 3.6).

A



B

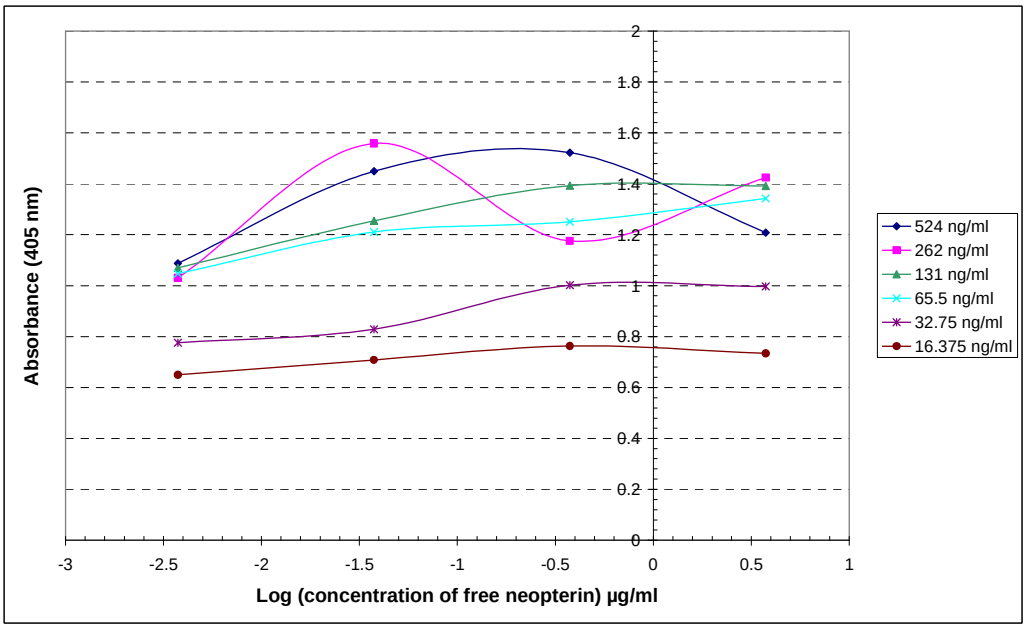


Figure 3.6. Competitive ELISA: Standard curve for neopterin using varying concentrations of neopterin-ovalbumin conjugate (200:1) to coat the wells and crude anti-neopterin IgY (week 8). Wells were coated with 2-fold dilutions (524 to 16.375 ng/ml) of the neopterin-ovalbumin conjugate. Dilutions of free neopterin (10-fold) were incubated with (A) 10 µg/ml, and (B) 100 µg/ml crude anti-neopterin IgY for 1 h in microcentrifuge tubes. The neopterin-antibody combinations were then incubated in the wells for 1 h and antibody that bound to the well was detected using HRPO-linked rabbit anti-chicken IgY and ABTS/H_2O_2 as a substrate. Each point is the mean absorbance at 405 nm of duplicate experiments.

To investigate why the affinity purified and crude IgY did not bind to the free neopterin in solution in the competitive ELISA, but bound to the coated neopterin-ovalbumin conjugate with constant absorbance values, an indirect ELISA was performed to ascertain the anti-neopterin IgY's cross reactivity with (a) ovalbumin, which is used as the carrier in the conjugates used to coat the wells, (b) bovine albumin, which is used to block the wells and dilute the antibodies in the ELISA, and (c) rabbit albumin, which is used as the carrier in the conjugates to immunise the chickens. In the same ELISA, an attempt was made to coat the wells with neopterin on its own.

Figure 3.7 indicated that the immune anti-neopterin IgY (Week 8) recognised rabbit albumin which was expected as the rabbit albumin was used as the carrier to immunise the chickens. Furthermore, the immune IgY showed cross-reactivity with bovine albumin, but very little reactivity with the ovalbumin- and the neopterin-coated wells. It is uncertain if neopterin did coat the wells without a protein carrier, hence it is difficult to speculate whether or not the anti-neopterin IgY is recognising neopterin. As the crude IgY cross-reacted with bovine albumin, BSA-PBS (0.5 %, w/v) was replaced with 0.1% (w/v) gelatin in PBS, pH 7.2, to block the wells and as a diluent for the antibodies.

An alternative method of coating the wells of the Nunc-Immuno™ ELISA plate was investigated. The wells were activated with 0.2% (w/v) glutaraldehyde for 2 h prior to adding neopterin to coat the wells overnight. Initially, various concentrations of neopterin were investigated with various dilutions of both crude IgY (week 8) and affinity purified IgY in an indirect ELISA to determine the most appropriate antibody and coating concentrations for the competitive ELISA. Figure 3.8 demonstrates that antibody concentrations of crude IgY from 100 to 25 µg/ml bound to the neopterin coating maximally at a concentration of 16.7 µg/ml neopterin with 100 µg/ml crude IgY resulting in the highest A_{405}. The affinity purified antibodies did not bind to the neopterin coating at any of the concentrations of neopterin or antibody investigated.

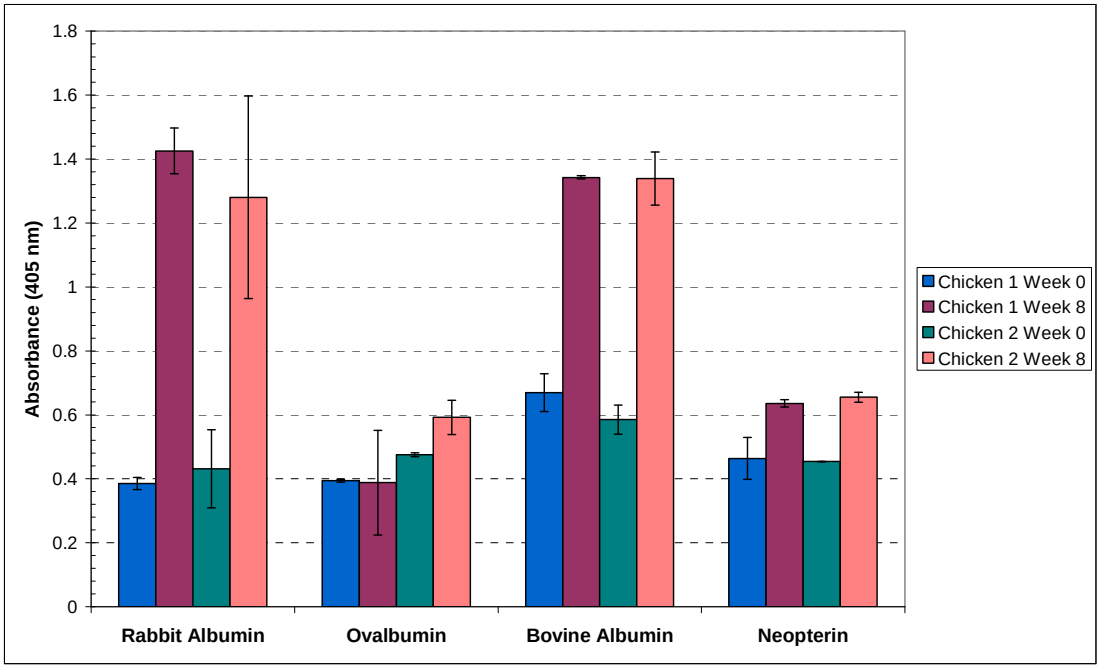


Figure 3.7. ELISA to determine reactivity of anti-neopterin IgY with rabbit albumin, ovalbumin, bovine albumin and neopterin. Wells were coated with rabbit albumin, ovalbumin, bovine albumin and neopterin. Crude IgY from week 8 (Immune) and week 0 (non-immune) were incubated in the wells and bound antibody was detected with HRPO-linked rabbit anti-chicken IgY and the ABTS/H_2O_2 substrate. Each bar represents the mean of two absorbance readings at 405 nm ± SD.

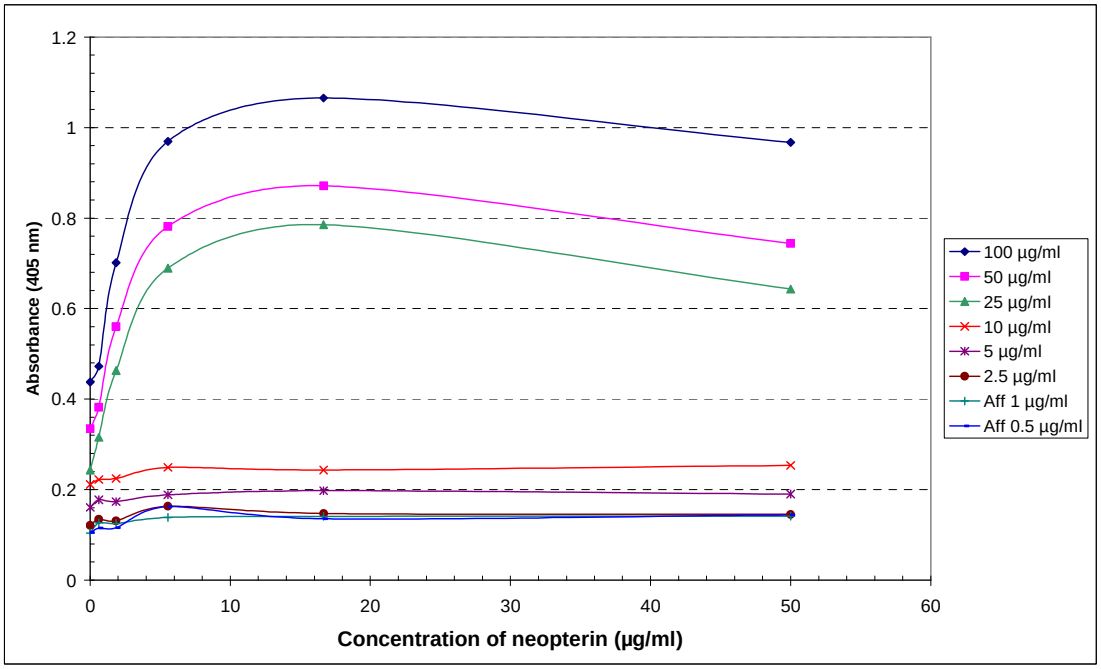


Figure 3.8. ELISA to assay the most appropriate antibody concentration and coating concentration of neopterin in glutaraldehyde-activated wells to detect anti-neopterin IgY. Wells of a Nunc-Immuno™ ELISA plate were activated with 0.2% (v/v) glutaraldehyde and coated with three-fold dilutions of neopterin. Various concentrations of week 8 crude IgY (100 – 2.5 µg/ml) and affinity purified IgY (Aff 1.0 and 0.5 µg/ml) were incubated in the wells. Bound antibody was detected by HRPO-linked rabbit anti-chicken IgY and the substrate ABTS/H_2O_2. Absorbance values at 405 nm were the mean of duplicate experiments.

An attempt was made to set up a neopterin standard curve in a competitive ELISA using neopterin to coat glutaraldehyde-activated wells. Only crude IgY was used in this competitive ELISA as affinity purified antibodies did not bind to the glutaraldehyde-activated wells coated with neopterin that were blocked with 0.1% (w/v) gelatin-PBS (Figure 3.8). Once again, there was no decrease in the A_{405} values with an increase in the concentration of free neopterin (Figure 3.9). However, the A_{405} values were lower (0.21 to 0.26) than those observed in the competitive ELISA using the neopterin-ovalbumin conjugate to coat the wells in Figure 3.5 (0.55 to 0.23). This could be due to the alternate method of blocking with 0.1% (w/v) gelatin-PBS in place of 0.5% (w/v) BSA-PBS that was used in the experiments in Figure 3.5.

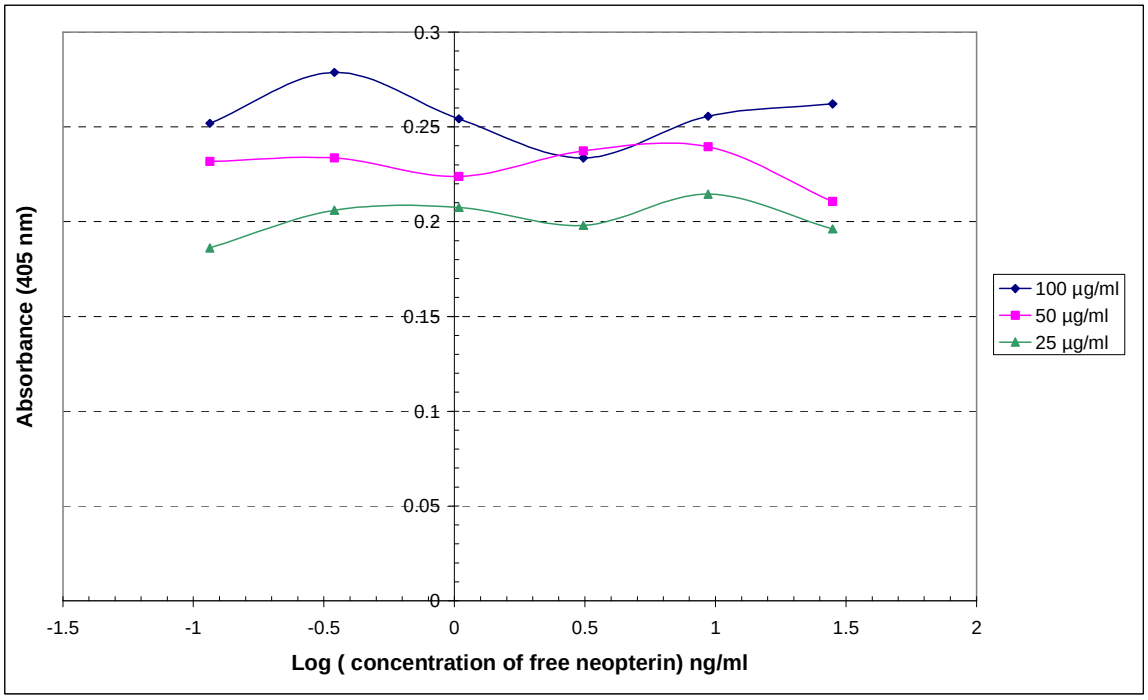


Figure 3.9. Competitive ELISA: Standard curve for neopterin using glutaraldehyde-activated wells coated with neopterin. Dilutions of the crude IgY antibodies (100, 50, 25 µg/ml) were incubated with three-fold dilutions of free neopterin for 1 h in glass tubes. Wells were activated with glutaraldehyde and coated with neopterin. The neopterin-antibody combinations were incubated in the wells for 1 h and antibody that bound to the well was detected using HRPO-linked rabbit anti-chicken IgY and ABTS/H_2O_2 as a substrate. Each point is the mean absorbance at 405 nm of duplicate experiments.

In summary, the anti-neopterin IgY produced in chickens immunised with a neopterin-rabbit albumin conjugate recognized neopterin-ovalbumin conjugates and neopterin added to glutaraldehyde-activated wells when they were used as coatings in an indirect ELISA. However, the antibodies did not titrate in a competitive ELISA.

3.3.4 Analysis of the relative expression of GTP-cyclohydrolase 1 mRNA transcripts using quantitative RT-PCR and the $2^{-\Delta\Delta C_T}$ method

Quantitative PCR was optimised by comparing DNase-treated total RNA isolated from U937 cells to untreated RNA, two primer sets (A and B) designed for GTP-cyclohydrolase 1 (GTP-CH1), and annealing temperatures of 55ºC and 60ºC in the amplification of the cDNA. The PCR products obtained from the quantitative PCR performed at an annealing temperature of 55ºC were run on an agarose gel (Figure 3.10). Single bands were obtained for both GTP-cyclohydrolase primer set A (lanes 12-15) and B (lanes 8-11). The sizes of the products of Primer set A and B were calculated to be 209 bp and 259 bp, respectively, which are comparable to the predicted sizes of 207 bp (Primer set A) and 247 bp (Primer set B). There appears to be a very light band above the main product of ~250 bp for the β-actin primers (lanes 4-7) but only one peak was observed in the melting profile of these quantitative PCR products. The size of the product for the β-actin primers was calculated to be 242 bp which is similar to the predicted size of 232 bp. There appears to be no difference between the RT-PCR products obtained from the DNase-treated or untreated RNA (Figure 3.10), hence untreated total RNA was used in the reverse transcription reactions. The quantification and melt profiles of the PCR products from the quantitative data indicate no significant difference between the annealing temperatures of 55ºC or 60 ºC (data not shown). Thus, the quantitative PCR were performed with annealing temperatures of 60ºC as a precautionary measure to prevent the formation of primer-dimers or non-specific binding of the primers to the template, which are more likely at lower annealing temperatures. Primer set A for GTP-cyclohydrolase 1 was used in all further quantitative RT-PCR experiments.

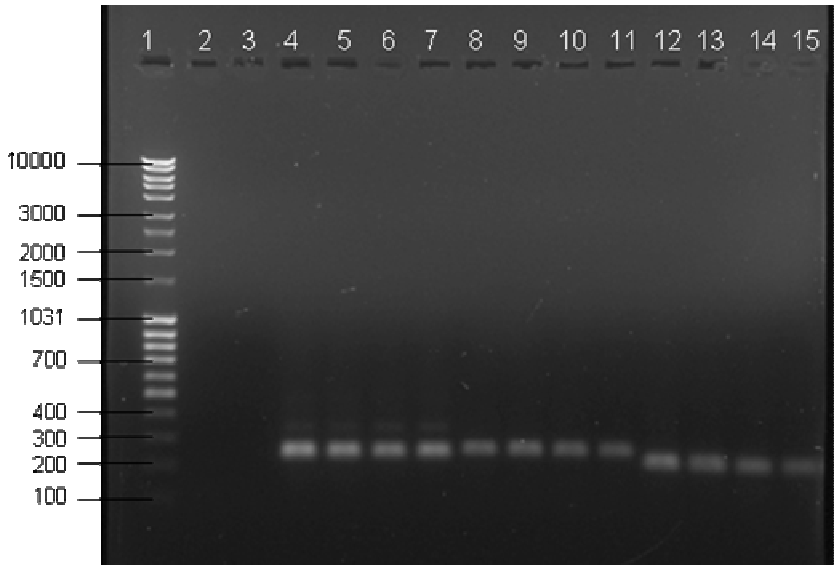


Figure 3.10. Agarose gel (1% w/v) of quantitative PCR products amplified from cDNA generated from U937 RNA, using primers for β-actin and GTP-cyclohydrolase 1 (Primer set A and B). Using reverse transcriptase, cDNA was synthesized from total RNA isolated from U937 cells. RNA was either treated with DNase (Lanes 4, 5, 8, 9, 12 and 13) or not (Lanes 6, 7, 10, 11, 14 and 15). cDNA was amplified using SYBR® Green quantitative PCR, an annealing temperature of 55ºC and primers for β-actin (Lanes 4, 5, 6 and 7), GTP-cyclohydrolase 1 primer set B (Lanes 8, 9, 10 and 11) and GTP-cyclohydrolase 1 primer set A (Lanes 12, 13, 14 and 15). The quantitative PCR products were run on a 1% (w/v) agarose gel in the presence of ethidium bromide. Lane 1: Fermentas MassRuler™ DNA Ladder Mix (U.S.A.), Lane 2, no-template control for GTP-cyclohydrolase 1 primer set B, lane 3: no-template control for β-actin.

For the $2^{-\Delta\Delta C_T}$ method to be valid, the amplification efficiencies of the target (GTP cyclohydrolase 1 (GTP-CH1) and reference (β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) must be comparable. To determine if the efficiencies are equal, variation in ΔC_T ($C_{T,\,target}$ - $C_{T,\,reference}$) with template dilution was monitored. The ΔC_T values were plotted versus the logarithm of the cDNA input, the dynamic range of the assay was ascertained and the data were fit using least-squares linear regression analysis. The absolute values of both slopes were less than 0.1 (Figure 3.11), indicating that the amplification efficiencies of the target, GTP-CH1, and references, β-actin or GAPDH, were similar, and the $2^{-\Delta\Delta C_T}$ method could be used for relative quantification.

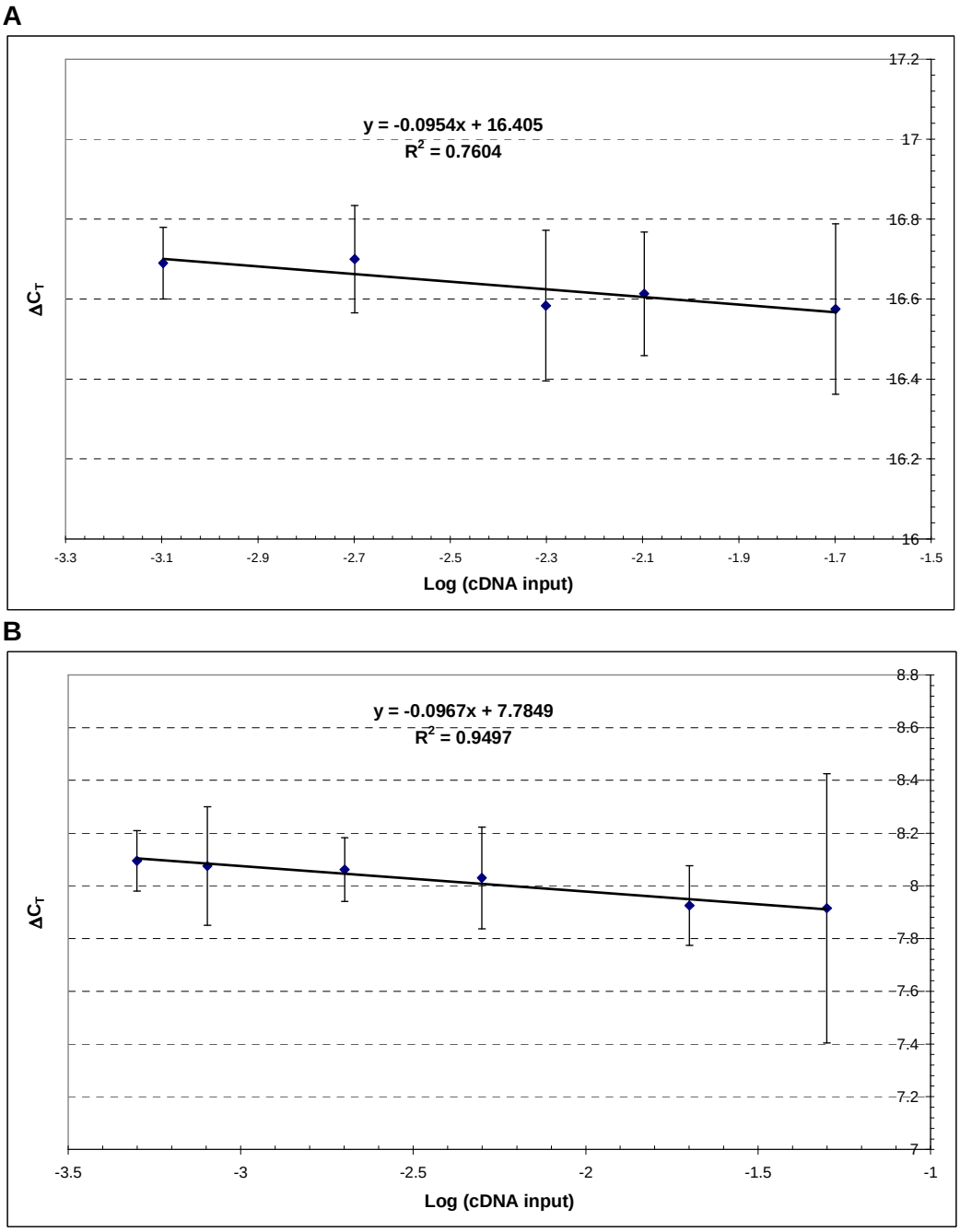


Figure 3.11. Validation of the $2^{-\Delta\Delta C_T}$ method used to determine the relative expression of the target gene mRNA (GTP-cyclohydrolase 1) to the expression of the internal control gene mRNA (β-actin OR glyceraldehyde-3-phosphate dehydrogenase). Using reverse transcriptase, cDNA was synthesized from total RNA isolated from U937 cells. Serial dilutions of the cDNA were amplified by SYBR® Green quantitative PCR using primers specific for GTP-cyclohydrolase 1 and (A) β-actin or (B) glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ΔC_T ($C_{T,\text{GTP-CH1}} - C_{T,\beta\text{-actin OR GAPDH}}$) was calculated for each cDNA dilution and plotted against the logarithm of the cDNA input. The data were fit using least-squares linear regression analysis and the slope of the trendline was determined. Results are expressed as the mean of three amplifications ± SD.

3.3.4.1 Effect of phorbol-12-myristate-13-acetate and interferon-γ on the expression of GTP-cyclohydrolase 1 mRNA

Figure 3.12 demonstrates that treatment of the suspended U937 cells (Control) with phorbol-12-myristate-13-acetate (PMA) for 48 hours produced a 2-fold increase in the expression of GTP-cyclohydrolase 1 (GTP-CH1) mRNA ($p<0.05$), and the addition of interferon-γ (IFN-γ) for 18 hours promoted ~15-fold increase in GTP-CH1 mRNA expression ($p<0.05$), both relative to the expression of β-actin mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. There was no significant difference ($p>0.05$) between the fold-change observed in the GTP-CH1 mRNA expression between the two house-keeping reference genes, β-actin and GAPDH. The β-actin house-keeping gene was used as the reference gene for further experiments.

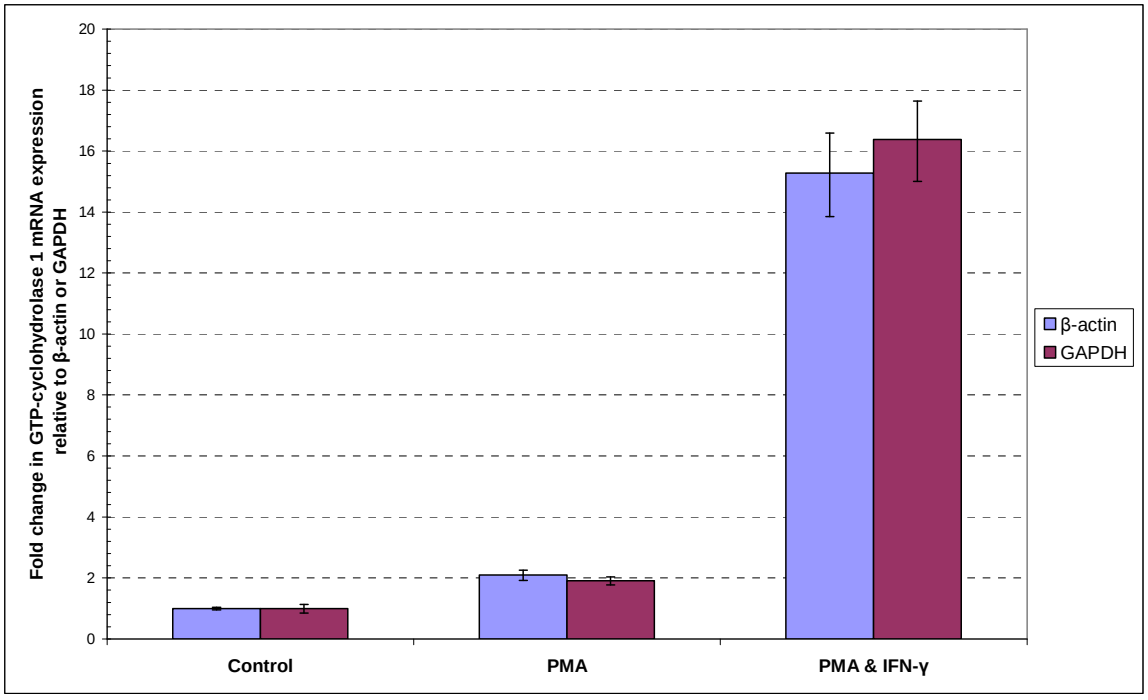


Figure 3.12. Effect of phorbol-12-myristate-13-acetate (PMA) alone and PMA with interferon-γ (IFN-γ) on the expression of GTP-cyclohydrolase 1 mRNA in U937 cells. U937 cells in suspension were used as a control. U937 cells were differentiated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 hours. The cells were then treated or not with interferon-γ (IFN-γ, 250 U/ml) for 18 hours. Total RNA was isolated and quantitative RT-PCR was performed to establish the expression of GTP-cyclohydrolase 1 mRNA relative to the expression of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using the $2^{-\Delta\Delta C_T}$ method. Results are the representation of one of two experiments performed in triplicate in quantitative PCR ± SD.

3.3.4.2 Effect of antimalarial drugs on the expression of GTP-cyclohydrolase 1 mRNA

The effects of 18 hours treatment with antimalarial drugs in addition to phorbol-12-myristate-13-acetate (PMA) and interferon-γ (IFN-γ) on the relative expression of GTP-cyclohydrolase 1 (GTP-CH1) mRNA in U937 cells were investigated. When compared to U937 cells treated with PMA and IFN-γ without drugs (control), quinine, primaquine and artemisinin significantly down-regulated the relative expression of the GTP-CH1 mRNA by 1.7-, 1.4- and 1.3-fold, respectively ($p<0.05$). Doxycycline also down-regulated the expression of GTP-CH1 mRNA by 1.1-fold, and amodiaquine up-regulated the mRNA expression by 1.1 fold, but both effects were insignificant ($p>0.05$). Chloroquine and pyrimethamine had minimal effects on the GTP-CH1 mRNA expression.

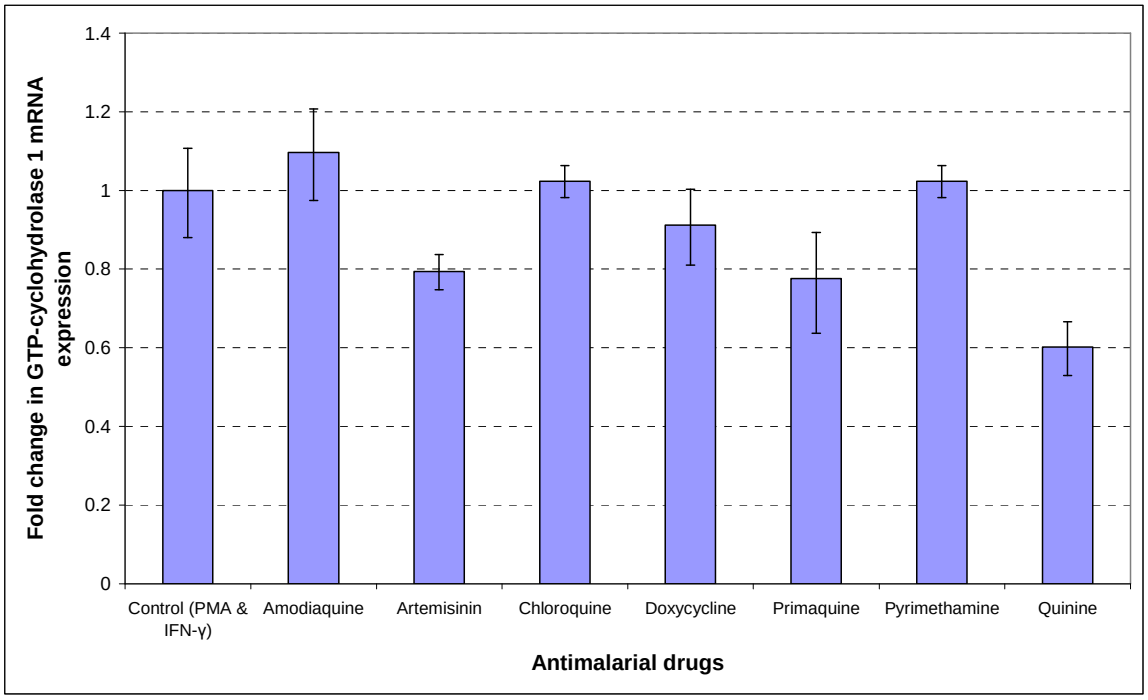


Figure 3.13. Effect of antimalarial drugs on GTP-cyclohydrolase 1 mRNA expression in U937 cells. U937 cells were differentiated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 hours. The cells were treated with IFN-γ (250 U/ml) and therapeutic concentrations of seven antimalarial drugs for 18 hours. Total RNA was isolated from the cells and quantitative RT-PCR was performed to establish the expression of GTP-cyclohydrolase 1 mRNA relative to the expression of β-actin mRNA using the $2^{-\Delta\Delta C_T}$ method. Results are the representation of one of two experiments performed in triplicate in quantitative PCR ± SD.

3.3.4.3 Effects of β-haematin, latex beads, non-infected and *P. falciparum*-infected red blood cell lysates on GTP-cyclohydrolase 1 mRNA expression

The effects of β-haematin, latex beads, non-infected and *P. falciparum*-infected red blood cell lysate on the expression of GTP-cyclohydrolase 1 (GTP-CH1) mRNA expression were compared to the GTP-CH1 mRNA expression in U937 cells treated with PMA and IFN-γ only (control). β-haematin significantly up-regulated the relative expression of GTP-CH1 mRNA by 1.2-fold ($p<0.05$) and 20 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia) significantly down-regulated the GTP-CH1 mRNA expression by 1.5-fold ($p<0.05$). Latex beads, non-infected red blood cell lysate (20 and 10 µg/ml) and *P. falciparum*-infected red blood cell lysate (10 µg/ml) had no significant effects on the expression of GTP-CH1 mRNA ($p>0.05$).

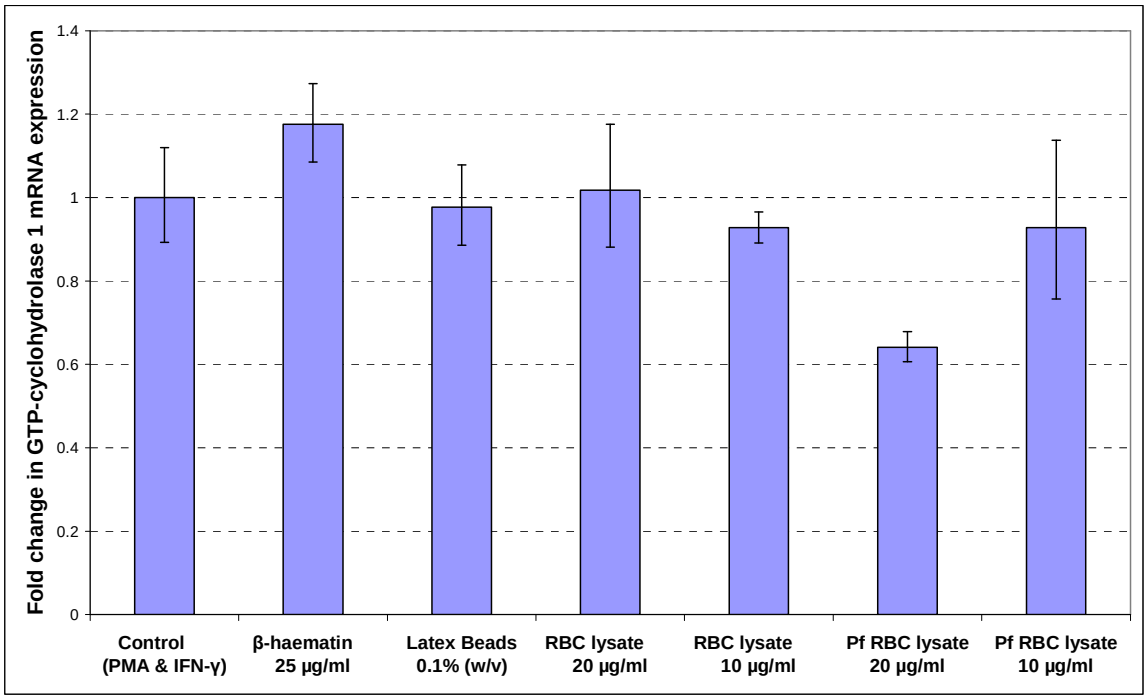


Figure 3.14. Effect of β-haematin, latex beads, non-infected and *P. falciparum*-infected red blood cell lysates on GTP-cyclohydrolase 1 mRNA expression in U937 cells. U937 cells were differentiated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 hours. The cells were then treated with interferon-γ (IFN-γ) (250 U/ml) and β-haematin or latex beads or non-infected red blood cell lysate (RBC lysate) or *P. falciparum*-infected red blood cell lysate (Pf RBC lysate) for 18 hours. Total RNA was isolated from the cells and quantitative RT-PCR was performed to establish the expression of GTP-cyclohydrolase 1 mRNA relative to the expression of β-actin mRNA using the $2^{-\Delta\Delta C_T}$ method. Results are the representation of one of two experiments performed in triplicate in quantitative PCR ± SD.

In summary, GTP cyclohydrolase 1 (GTP-CH1) mRNA expression in U937 cells was up-regulated 15-fold by interferon-γ (IFN-γ) treatment. Quinine, primaquine and artemisinin down-regulated this increase in GTP-CH1 mRNA expression by 1.7- , 1.4- and 1.3-fold, respectively. Amodiaquine, chloroquine, doxycycline and pyrimethamine had no significant effects on the GTP-CH1 mRNA expression. β-haematin up-regulated the IFN-γ-induced GTP-CH1 mRNA expression by 1.2 fold, whereas 20 µg/ml *P. falciparum*-infected red blood cell lysate down-regulated this induced expression by 1.5-fold. Non-infected red blood cell lysate had no significant effects on the IFN-γ-induced GTP-CH1 mRNA expression.

3.4 Discussion

Neopterin is secreted by monocytes, macrophages and dendritic cells when they are activated in patients with malignant diseases, infectious diseases such as AIDS, tuberculosis or malaria, autoimmune diseases, allograft rejection, renal or cardiac failure, coronary artery disease and myocardial infarction (Berdowska and Zwirska-Korczala, 2001; Wachter *et al.*, 1989). Neopterin levels are often monitored during diseases or following an organ transplant to give an indication of the development of organ rejection (Berdowska and Zwirska-Korczala, 2001; Wachter *et al.*, 1989). In this study, the effects of antimalarial drugs, β-haematin, latex beads, non-infected- or *P. falciparum*-infected red blood cell lysates on interferon-γ-induced neopterin production in human monocytic U937 cells were investigated. An attempt was made to measure the neopterin levels in the U937 supernatants using a competitive ELISA, and the expression of the mRNA of GTP-cyclohydrolase 1 was analysed using quantitative RT-PCR. GTP-cyclohydrolase 1 is an enzyme involved in the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which is then dephosphorylated by phosphatases, and oxidised to form neopterin (Hoffmann *et al.*, 2003).

3.4.1 Production of anti-neopterin IgY in chickens

To detect neopterin in the supernatants of U937 cells, antibodies were raised against neopterin in chickens. Neopterin has only been found in primates and humans (Duch *et al.*, 1984) and birds are phylogenetically different from mammals (Erhard and Schade, 2001), hence chickens would recognize neopterin as a foreign molecule. Furthermore, isolation of IgY from the egg yolks provides a convenient easily accessible acquisition of abundant amounts of antibodies without venepuncture of the animal, as in the case of rabbits (Jensenius and Koch, 1997). In

one week a chicken can supply as much antibody as it takes a rabbit to produce in three months (Jensenius and Koch, 1997). Neopterin is a small organic molecule with a M_r of 253.215, and as a hapten, it will not elicit an immune response unless it is bound to a carrier molecule (Hermanson, 1996; Schwarzkopf *et al.*, 2001). Neopterin was conjugated to rabbit albumin as a carrier, and used to immunize two chickens. Eggs were collected and IgY was isolated from the eggs. The titre of both the anti-rabbit albumin and anti-neopterin antibodies (Figure 3.1) were highest around week 6 in chicken 1 and week 8-9 in chicken 2. The antibody production in chicken 1 was similar to a previous investigation where antibody titres against antimalarial drugs and the carrier were both highest 6 weeks after immunisation (Goldring *et al.*, 2005).

Low yields (~250 µg IgY) of affinity purified antibodies were obtained with each cycle of pooled crude IgY over the affinity matrix. Detection of anti-neopterin IgY in the crude IgY pool by ELISA following each cycle over the affinity matrix indicated that the anti-neopterin-binding capacity of this matrix was low. This may be due to poor coupling of the neopterin-ovalbumin conjugate to the matrix. Furthermore, neopterin is light sensitive and easily degraded, and although all the reactions with neopterin were performed in the dark, degradation of the neopterin cannot be eliminated (Laich *et al.*, 2002)

To improve the yields, matrices were prepared with neopterin alone or a 200:1 neopterin-ovalbumin conjugate, but no antibodies were isolated using either approach. This suggested that either the neopterin or the conjugate had not bound to the matrix, or the neopterin had degraded during the coupling reaction to the gel, or very few antibodies were generated against neopterin in the immunised chicken. Neopterin also has a very low solubility in water and buffers: 1 mg in 20 ml of water/PBS. Due to poor yields, both the crude and affinity purified IgY were used in the development of a competitive ELISA to detect neopterin in supernatants from U937 cells.

3.4.2 Development of a competitive ELISA

A competitive ELISA makes it possible to obtain an estimate of an amount of a particular antibody or antigen (in this case neopterin), especially when these components cannot be isolated from the medium in which they are found. The competitive assays are less precise than the direct or indirect ELISA as more than one component is present in limiting concentrations

(Kemeny, 1997). In this case, the sample antigen (free neopterin in standard curve solutions or neopterin in U937 supernatants) and solid phase antigen (neopterin-ovalbumin conjugates coating the wells or neopterin added to glutaraldehyde-activated wells) competed for binding to a fixed amount of unlabelled antibody (crude or affinity purified IgY), and its presence was detected with a second labelled antibody in an ELISA (Kemeny, 1997).

3.4.2.1 Competitive ELISA using the neopterin-ovalbumin conjugate to coat the wells

Indirect ELISAs indicated that the 200:1 neopterin-ovalbumin conjugate was more sensitive than the 40:1 conjugate in distinguishing non-immune IgY from immune IgY (Figure 3.3), and 16-fold dilutions of the 200:1 conjugate still distinguish immune IgY from non-immune IgY (Figure 3.4). Competitive ELISAs, using the 200:1 neopterin-ovalbumin conjugate to coat the wells, failed to show a decrease in absorbance at 405 nm with an increase in neopterin concentration (Figure 3.5). Alterations in the competitive ELISA such as varying the concentrations of the (i) affinity purified antibodies or crude IgY, (ii) free neopterin and (iii) coating neopterin-ovalbumin conjugate (Figure 3.6), were all unsuccessful. It appeared that neither the affinity purified antibodies nor the crude IgY were binding to the free neopterin in this format.

An neopterin-ovalbumin conjugate was used to affinity purify the crude IgY and coat the wells for indirect and competitive ELISAs. In addition, bovine serum albumin was used to block the wells in the ELISAs and rabbit albumin was the carrier in the conjugate used to immunize the chickens. Thus, the cross reactivities of these antibodies with ovalbumin, bovine serum albumin and rabbit albumin were investigated to determine if the cross reactivities were responsible for the failure of the competitive ELISA (Figure 3.7). As expected, the antibodies recognised rabbit albumin. No cross reactivity was observed with ovalbumin, but cross-reactivity was detected with bovine serum albumin (Figure 3.7). Although this cross-reactivity with the bovine serum albumin could render all the ELISA results in Figures 3.1-3.6 invalid, the concentration of bovine serum albumin used in all the wells of the ELISA was constant. Therefore, in Figures 3.3 and 3.4, if the antibodies were only recognising the bovine serum albumin present at constant concentrations in all the wells, there would have been no difference between the absorbance values of the non-immune (Week 0) and immune (Week 8) antibodies in the indirect ELISAs. However, this was not the case indicating that although there might be cross reactivity with bovine serum albumin, the immune antibodies still recognised and bound to the neopterin-

ovalbumin conjugate in the indirect ELISAs. Furthermore, had the antibodies recognised and bound the varying concentrations of free neopterin in the competitive ELISAs, there should still have been a decrease in absorbance with increasing free neopterin concentration even if the remaining antibodies bound to the bovine serum albumin in the wells (Fig. 3.5 and 3.6). Yet, this was not observed. In order to avoid maligning the results with the cross reactivity with bovine serum albumin, 0.1% (w/v) gelatin-PBS was used to block the wells and dilute the antibodies in the experiments that followed. Unfortunately, the use of 0.1% (w/v) gelatin-PBS with the neopterin-ovalbumin conjugate as a coating was overlooked. Wells were coated with neopterin alone and although a minimal distinction was detected between the non-immune and immune IgY, it is difficult to ascertain if the neopterin was bound to the wells of the ELISA plate.

A competitive ELISA using solid phase bound conjugates of neopterin-carrier (bound to the wells) has been used successfully to detect neopterin in serum or cell culture supernatants (Barak *et al.*, 1989). Differences to the methods employed in this study include the use of the carbodiimide method to couple neopterin to high molecular weight carriers such as bovine serum albumin, keyhole limpet haemocyanin or thyroglobulin. These neopterin-carrier conjugates were used for immunisation of rabbits to generate antiserum for the competitive ELISA, in addition to coating the wells for the competitive ELISA. In this study, glutaraldehyde was used to couple neopterin to rabbit albumin for immunisation of chickens, and to couple neopterin to ovalbumin for coating the wells in the competitive ELISA. IgY isolated from eggs of the immunised chickens was used in the ELISA presented here. Furthermore, Barak *et al.* (1989) used low fat milk to block the wells in comparison to the bovine serum albumin or gelatin used in the ELISA presented here. Barak *et al.* (1989) obtained a dose response curve for inhibition of anti-neopterin antiserum binding by known concentrations of standard solutions. The best binding efficiency was exhibited by antiserum from rabbits immunised with neopterin conjugated to keyhole limpet haemocyanin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and wells coated with bovine serum albumin-neopterin conjugates coupled with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate. However in the ELISA presented here, a dose-response curve was not obtained when the same standard neopterin concentrations were investigated. Barak *et al.* (1989) added the neopterin standards or samples and the antibody simultaneously to each neopterin-conjugate coated well and incubated for 2 hours at 37ºC. In the ELISA presented here, the neopterin standards and the antibody were incubated together for 1 hour at room temperature prior to adding to the neopterin-conjugate coated wells for 1 hour at 37ºC. Barak *et al.* (1989) checked

the validity of their assay by comparing the results of their ELISA for neopterin production in supernatants of peripheral blood mononuclear cells stimulated with IFN-γ with those obtained with the “Neopterin RIAcid kit” from Henning Berlin GmbH, West Berlin, F.R.G. A co-efficient of correlation of 0.974 was obtained between the two methods. The detection limit of their ELISA was 1.6 nmol/l compared with 1 nmol/l for the RIA and HPLC determinations. Thus, the use of neopterin-carrier conjugates as a coating for wells used in a competitive ELISA was successful in detecting neopterin in tissue supernatants.

3.4.2.2 Competitive ELISA using microtitre plates coated with neopterin added to glutaraldehyde-activated wells

An alternative method for coating the wells was explored by activating the wells with glutaraldehyde prior to adding neopterin to coat the wells. In an indirect ELISA used to determine the optimal coating concentration and anti-neopterin antibody concentration (Figure 3.8), the affinity purified antibodies did not bind to the neopterin coated to glutaraldehyde-activated wells at any of the coating concentrations of neopterin investigated. This suggests that neopterin might not have bound to the wells, and non-specific binding is occurring with the crude IgY. But this does not explain the decrease in A_{405} values of the crude IgY binding observed with a decrease in the neopterin coating concentration.

A competitive ELISA using crude IgY and microtitre plates coated with neopterin added to glutaraldehyde-activated wells failed to display a decrease in the absorbance at 405 nm with an increase in the concentration of free neopterin (Figure 3.9). The absorbance values were much lower than those obtained with the neopterin-ovalbumin coated wells, indicating that minimal antibody had bound to the neopterin-coated glutaraldehyde activated wells. This could be as a result of most of the anti-neopterin antibodies binding to the free neopterin in the glass tubes, leaving minimal free antibodies in solution to bind to the neopterin coating the well. However, when much lower concentrations of free neopterin were investigated, there was still no difference in the absorbance values.

Antibodies have been successfully generated against chemical molecules for use in competitive ELISAs (Danger *et al.*, 2006; Tanaka *et al.*, 2007; Zhao *et al.*, 2007). In particular, chicken IgY has been raised against antimalarial drugs and used in a competitive ELISA (Goldring *et al.*, 2005). Expensive ELISA commercial kits (GenWay Biotech Inc., U.S.A., Immuno-Biological

Laboratories, U.S.A., Alpco Diagnostics, U.S.A.) are available to detect neopterin in serum and urine using a competitive ELISA (Mayersbach *et al.*, 1994; Ogiwara *et al.*, 1992). In these competitive ELISAs, polyclonal anti-neopterin serum from rabbits or sheep or monoclonal antibodies were used to detect neopterin in the samples. In this study, although the anti-neopterin IgY appears to recognise the neopterin-ovalbumin conjugates or the neopterin in glutaraldehyde-activated wells in indirect ELISAs, it does not appear to recognise the free neopterin in solution in the format of the competitive ELISA. Antibodies show specificity, which can be an advantage, but it can also cause problems with some immunochemical procedures as antibodies can discriminate between molecular species which differ very slightly from each other in conformation (Thorpe *et al.*, 1997). Thus, if a hapten is conjugated to a carrier protein, and the conformation of the hapten is altered slightly during the coupling reaction, the antibodies generated against the hapten-carrier conjugate may not recognise the hapten on its own. It is possible that the conformation of the neopterin molecule may have been altered by the glutaraldehyde during the coupling reaction.

Future work on the development of the competitive ELISA could include raising polyclonal antibodies against neopterin in mammals that do not produce neopterin, such as rabbits or sheep, which are phylogenetically closer to primates and humans than chickens. An alternate conjugation method, for example, the carbodiimide method, could be used to couple the neopterin to carriers (Barak *et al.*, 1989; Hermanson, 1996). The successful production and correct specificity of the resultant antibodies depends on the coupling chemistry used to prepare the immunogen from a hapten and a carrier. There is the potential for antibody recognition and cross-reactivity toward the cross-linking reagent used to effect the conjugation (Hermanson, 1996). The desired antibody response against the hapten may be diluted by antibodies generated against the cross-linker bridge. This problem can be eliminated by the use of a zero-length cross-linking procedure mediated by the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) as no bridging molecule is introduced between the hapten and the carrier. In addition, alternate carriers, such as keyhole limpet haemocyanin or thyroglobulin (Hermanson, 1996), which have been successfully used by Barak *et al.* (1989), could be used to prepare conjugates to immunise the animal and coat the wells of the microtitre plates. In addition, formation of a neopterin-equivalent of a multiple antigenic peptide could be investigated to immunise the chickens (Schwarzkopf *et al.*, 2001). This immunogenic matrix could be formed by coupling neopterin to trifunctional amino acids, such as lysine (Tam, 1988). The lysine residues themselves are relatively non-immunogenic,

and the greater the number of lysine residues in the core matrix, the greater the number of coupled haptens.

Alternate adjuvants such as Adjuvant 65, Specol, TiterMax, ABM-system, Gerbu, Lipopeptide PCSL, which have all been previously investigated in chickens (Schwarzkopf *et al.*, 2001), could substitute the Freund's complete and incomplete adjuvants in the immunisation and boosting of the chickens. Specol has been demonstrated to produce antibody titres and antibody avidities in chickens similar to that obtained with Freund's complete adjuvant. In addition, Specol does not have the noticeable side effects of Freund's complete adjuvant on the chickens (Schwarzkopf *et al.*, 2001). Lipopeptide PCSL has been shown to produce high antibody titres with certain antigens (Erhard *et al.*, 2000) and seems to be successful when its concentration is kept within certain limits (250 µg/injection, subcutaneous). Lipopeptide PCSL also has minimal side effects on the chicken (Schwarzkopf *et al.*, 2001). TiterMax, when used as an adjuvant in chickens, results in lower antibody titres than Freund's complete adjuvant but does have fewer side effects (Bollen and Hau, 1999; McClimon *et al.*, 1994; Svendsen Bollen *et al.*, 1996).

3.4.3 Analysis of relative expression of GTP-cyclohydrolase 1 mRNA transcripts in U937 cells using quantitative RT-PCR

Total RNA was isolated from U937 cells that were treated with interferon-γ and either one of seven antimalarial drugs, β-haematin, latex beads, non-infected or *P. falciparum*-infected red blood cell lysates. RNA was reverse transcribed to generate cDNA, which was used as a template in quantitative PCR with primers for the target, GTP-cyclohydrolase 1 gene (GTP-CH1), and references, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. The relative expression of the GTP-CH1 mRNA was determined using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), which was validated by assessing how ΔC_T ($C_{T,\text{target}}$ - $C_{T,\text{reference}}$) varies with template dilution. Two different reference genes were investigated because ΔC_T values between GTP-CH1 and β-actin C_T values were fairly large (~17), hence GAPDH was also investigated as a reference gene. The ΔC_T values between GTP-CH1 and GAPDH C_T values were smaller (~8). However, both reference genes gave rise to regression lines with slopes of <0.1 in the plot of ΔC_T versus log cDNA input (Figure 3.12A and B) demonstrating that the amplification efficiency of the target gene, GTP-cyclohydrolase 1, was approximately equal to

that of both reference genes, β-actin and GAPDH (Livak and Schmittgen, 2001). Thus, the $2^{-\Delta\Delta C_T}$ method could be used to measure the relative expression of GTP-cyclohydrolase 1 mRNA with either of the reference genes.

3.4.3.1 Effect of phorbol-12-myristate-13-acetate and interferon-γ on the expression of GTP-cyclohydrolase 1 mRNA in U937 cells

Differentiation of U937 cells with phorbol-12-myristate-13-acetate (PMA) up-regulates the expression of GTP-cyclohydrolase 1 mRNA in U937 cells 2-fold, whereas treatment of the U937 cells with PMA together with interferon-γ (IFN-γ) up-regulates the expression of GTP-cyclohydrolase 1 by almost 15-fold (Figure 3.13). Up-regulation in GTP-cyclohydrolase 1 mRNA expression was observed with both the β-actin and the GAPDH reference genes. INF-γ has been reported to increase the expression of GTP-cyclohydrolase 1 mRNA in human umbilical vein endothelial cells (Gesierich *et al.*, 2003; Katusic *et al.*, 1998). When peripheral blood derived macrophages and THP-1 myelomonocytic cells were treated with IFN-γ, GTP-cyclohydrolase 1 activity in these cells increased 40-fold (Werner *et al.*, 1990). GTP-cyclohydrolase 1 catalyzes the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate, which is dephosphorylated and oxidised to produce neopterin (Hoffmann *et al.*, 2003). INF-γ released from activated T-cells, is the central stimulus that induces neopterin release from monocytes/macrophages (Huber *et al.*, 1984). Other potent inducers of macrophage activity, including phorbol ester, do not induce the release of significant amounts of neopterin (Murr *et al.*, 2002), which correlates with the minor stimulation of PMA on GTP-cyclohydrolase 1 mRNA expression observed in U937 cells.

3.4.3.2 Effect of antimalarial drugs on the expression of GTP-cyclohydrolase 1 mRNA in U937 cells

Interferon-γ (IFN-γ)-induced GTP-cyclohydrolase 1 mRNA expression in U937 cells was significantly inhibited by quinine, primaquine and artemisinin. Amodiaquine, chloroquine, doxycycline and pyrimethamine had insignificant effects on the expression of GTP-cyclohydrolase 1 (Figure 3.14). As neopterin is one of the downstream products of the enzyme GTP-cyclohydrolase 1, and a marker of monocyte activation, the down-regulation observed in the GTP-cyclohydrolase 1 mRNA expression could be indicative of quinine, primaquine and artemisinin down-regulating the activation of the monocyte induced by IFN-γ. In terms of

defence against a malaria infection, activation of macrophages increases the capacity for antigen presentation through induction of class II HLA antigens in addition to increasing anti-microbial effector mechanisms, such as phagocytosis of infected red blood cells, followed by the destruction of the phagocytosed protozoa by oxygen and oxygen-independent mechanisms (Auger and Ross, 1992).

Several investigations monitored neopterin levels in patients infected with *P. falciparum* that were being treated with quinine alone (Kremsner *et al.*, 1996; Ringwald *et al.*, 1991), quinine and tetracycline (Brown *et al.*, 1990), or halofantrine (Ringwald *et al.*, 1991). Neopterin levels peaked 2-3 days after the start of the treatment and decreased to normal levels by days 6-8. However, in patients with severe anaemia, the neopterin levels were still elevated even after 7 days of treatment with quinine (Biemba *et al.*, 1998). The neopterin levels were found to associate significantly with peak temperatures, fever clearance times and parasite clearance times (Brown *et al.*, 1990), as well as severe malarial anaemia (Biemba *et al.*, 2000) and respiratory distress in life-threatening malaria (Awandare *et al.*, 2006a). Hence, it would be difficult to correlate the neopterin levels observed in malaria patients with the effect of the antimalarial drug treatment on GTP-cyclohydrolase 1 in the monocytes of these patients. The decrease in neopterin levels observed in malaria patients by day 6-8 after initiation of treatment are more likely due to the eradication of the *P. falciparum* parasites by quinine which would lead to a decrease in the activation of the macrophage and hence, a decrease in neopterin secretions. The quinine-induced down-regulation of the GTP-cyclohydrolase 1 mRNA expression observed in U937 cells might contribute to the overall decrease in neopterin levels, if it contributes at all.

Other chemical compounds that have been found to affect neopterin production include the drugs aspirin (Schroecksnadel *et al.*, 2005), a general cyclooxygenase inhibitor with anti-inflammatory, analgesic and anti pyretic effects, and resveratrol (Wirleitner *et al.*, 2005) with antioxidant, antitumour and anti-inflammatory effects. Both drugs down-regulate the production of neopterin by suppressing the production of interferon-γ. Short-term administration of estradiol benzoate led to an increase in the GTP-cyclohydrolase 1 mRNA levels in dopaminergic and noradrenergic cell bodies (Serova *et al.*, 2004). Ascorbic acid, vitamin C, did not alter either the mRNA expression or the activity of GTP-cyclohydrolase 1 in human umbilical vein endothelial cells (Heller *et al.*, 2001) and vitamin C supplementation was also found to have no effects on circulating neopterin levels in smokers or non-smokers (Scott *et al.*, 2005). Food antioxidant

preservatives, sodium sulphite and sorbic acid, both suppressed GTP-cyclohydrolase 1 in human peripheral blood mononuclear cells (Winkler *et al.*, 2006). 2,4-Diamino-6-hydroxy-pyrimidine is a known selective inhibitor of GTP-cyclohydrolase 1 (Kasai *et al.*, 1997). Thus quinine, primaquine and artemisinin appear to induce similar suppressive effects as aspirin, resveratrol, sodium sulphite and sorbic acid on GTP-cyclohydrolase 1 mRNA expression, although aspirin, resveratrol and sodium sulphite suppress neopterin secretion to a greater degree (2-5 fold), whereas sorbic acid results in a similar reduction (1.5-fold). Although these changes may not be significant in a healthy individual *in vivo*, in a diseased patient, these small changes may be potentially therapeutic. Chloroquine, doxycycline, pyrimethamine and amodiaquine are similar to ascorbic acid which has no significant effects on GTP-cyclohydrolase 1 mRNA expression.

3.4.3.3 Effect of β-haematin, latex beads, non-infected and *P. falciparum*-infected red blood cell lysates on GTP-cyclohydrolase 1 mRNA expression in U937 cells

When U937 cells were treated with physiological concentrations of β-haematin for 18 hours, the GTP-cyclohydrolase 1 mRNA expression was up-regulated ~1.2-fold. However, treatment of U937 cells with 20 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia) down-regulated the expression of GTP-cyclohydrolase 1 mRNA by ~1.6 fold (Figure 3.15). The latex beads, non-infected red blood cell lysates and 10 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia) had no significant effects on the GTP-cyclohydrolase 1 mRNA expression.

Low concentrations of free and transferrin-bound iron have been shown to reduce the efficiency of the IFN-γ signal in the myelomonocytic cell line THP-1 as observed by the decreased production of neopterin (Weiss *et al.*, 1992a). Later studies demonstrated that iron significantly reduced the mRNA expression and activity of GTP-cyclohydrolase 1 in THP-1 cells. The GTP-cyclohydrolase 1 mRNA expression was found to be regulated post-transcriptionally by iron perturbations (Oexle *et al.*, 2003). The observed effects of β-haematin on GTP-cyclohydrolase 1 mRNA expression were opposite to that of iron, with a 1.2-fold up regulation of mRNA expression. This could be due to the fact that the iron in β-haematin is co-ordinated into porphyrin dimers assembled into insoluble crystal lattices that remain, apparently unchanged in monocytes/macrophages for several months (Schwarzer *et al.*, 1998). Thus, the iron in β-haematin is not “biologically” available. Once β-haematin has been phagocytosed by the

monocyte, it generates large amounts of lipoperoxidation products by non-enzymatic catalysis (Schwarzer *et al.*, 2003). These lipoperoxidation products might be responsible for the up regulation of GTP-cyclohydrolase 1 mRNA expression caused by β-haematin.

The red blood cell lysates had no significant effects on the mRNA expression of GTP-cyclohydrolase 1 (Figure 3.15). Hence, the down regulation in the GTP-cyclohydrolase mRNA expression observed with 20 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia) is probably due to *P. falciparum* material in the lysate. There was no significant effect with 10 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia), indicating that the parasites were too diluted at this concentration of protein to have an effect on the mRNA expression. These findings are in contrast to a previous study with *P. falciparum*-infected red blood cell lysate which stimulated U937 cells to secrete neopterin and this secretion was enhanced by the addition of human IFN-γ (Facer, 1995). However, Facer (1995) did not indicate the protein concentration of the *P. falciparum*-infected red blood cell lysate that was added to the U937 cells or the parasitaemia of the lysate used in the studies. A significant difference with this investigation was the time period of incubating the U937 cells with the *P. falciparum*-infected red blood cell lysate: Facer (1985) incubated the U937 cells with the lysate for 48 hours whereas in this study the U937 cells were exposed to the lysates for only 18 hours. Huber *et al.* (1984) reported that the release of neopterin from activated macrophages started after 24 hours post-stimulation and the neopterin concentration was maximal after 2-3 days. Another difference is that Facer (1985) was monitoring the levels of neopterin in the supernatant, whereas in this study the relative expression of the mRNA of GTP-cyclohydrolase 1 in the U937 cell was measured. Thus, an up-regulation of GTP-cyclohydrolase 1 mRNA expression might have been observed after 48 hours incubation with the *P. falciparum*-infected red blood cell lysate. Alternatively, GTP-cyclohydrolase 1 mRNA expression and the production of neopterin may be controlled by different mechanisms or conditions. In hind site, higher protein concentrations of *P. falciparum*-infected red blood cell lysates and lysates with higher parasitaemia should also have been investigated.

It is intriguing that β-haematin up regulated the relative expression of GTP-cyclohydrolase 1 mRNA, whereas the *P. falciparum*-infected red blood cell lysate down regulated the relative GTP-cyclohydrolase 1 mRNA levels in the U937 cells, as the *P. falciparum*-infected red blood cell lysate also contains native haemozoin. However, the concentration of haemozoin present in the parasite lysate (10% parasitaemia) added to the U937 cells was approximately 500 times

lower than the concentration of β-haematin added to the cells. Furthermore, the β-haematin was free of any parasite proteins, lipids or nucleic acids, whereas unpurified haemozoin is often associated with proteins, lipids and nucleic acids of both host and parasitic origin (Goldie *et al.*, 1990). Thus, future work could determine if β-haematin and native haemozoin induce similar effects on the relative expression levels of GTP-cyclohydrolase 1 mRNA.

7,8-Dihydroneopterin reacts very rapidly with hypochlorous acid to produce neopterin (Widner *et al.*, 2000). At least a third of the 7,8-dihydroneopterin produced is oxidised to neopterin (Gieseg *et al.*, 2001a). Reports are contradictory as to whether the oxidation of 7,8-dihydroneopterin to neopterin takes place inside (Murr *et al.*, 2002) or outside the monocyte/macrophage (Gieseg *et al.*, 2008) or both. There are no reports of 7,8-dihydroneopterin being synthesized concomitantly with hypochlorous acid release in the monocyte (Sugiyama *et al.*, 2004) and being converted into neopterin immediately, or of 7,8-dihydroneopterin accumulating in the macrophage and being oxidized to neopterin later when hypochlorous acid production is activated in the monocyte or when the monocyte enters an inflammation site. In most studies, 7,8-dihydroneopterin is oxidised to neopterin using iodine, and the total pterin is measured as only neopterin is fluorescent and can be measured by a fluorescent detector (Wachter *et al.*, 1989). Hence, there is no distinction between 7,8-dihydroneopterin and neopterin concentrations (Gieseg and Cato, 2003; Schoedon *et al.*, 1987; Werner-Felmayer *et al.*, 1990; Werner *et al.*, 1990). Furthermore, if the neopterin is synthesized inside the monocyte/macrophage, reports do not intimate if the neopterin is retained in the cell before secretion or if it is secreted upon formation and whether or not the triggering of the neopterin release is independent of the synthesis of 7,8-dihydroneopterin. Therefore it is difficult to establish if the mRNA expression of GTP-cyclohydrolase 1 is directly related to the neopterin concentration in the supernatants of stimulated monocytes/ macrophages or not.

Further work would entail determining neopterin concentrations in the supernatants of the treated U937 cells by commercial competitive ELISA kits (Westermann *et al.*, 2000) and HPLC (Werner *et al.*, 1987a; Werner *et al.*, 1987b) to determine how GTP-cyclohydrolase 1 mRNA expression correlates with secreted neopterin levels. The effects of longer incubation periods of U937 cells with antimalarial drugs, β-haematin, latex beads, *P. falciparum*-infected and non-infected red blood cell lysates on GTP-cyclohydrolase 1 mRNA expression and neopterin levels in the supernatants could also be investigated. The GTP-cyclohydrolase mRNA expression

could also be monitored in peripheral blood mononuclear cells isolated from blood samples drawn from malaria patients prior to and during antimalarial drug treatment.

Chapter 4

General Discussion

Malaria is an infectious disease infecting approximately 250 million people each year resulting in about 900 000 deaths annually mainly in children below the age of 5 and pregnant women (WHO, 2008). The disease reduces GDP by up to 1.3% in poor countries. This is due to an increasing resistance of the parasite to antimalarial drugs (Pearce *et al.*, 2009; Pongtavornpinyo *et al.*, 2008) and of the vectors, *Anopheles gambiae* and *A. funestus* in Africa, to insecticides (Casimiro *et al.*, 2006; Santolamazza *et al.*, 2008; Wondji *et al.*, 2009). Although there is a large number of potential antigen targets for malaria vaccine development, the immune evasion strategies of the parasite continue to confound vaccine development (Pierce and Miller, 2009).

The parasite results in an immune response that is not appropriate, suppressed and contributes to the pathophysiology of malaria (Artavanis-Tsakonas *et al.*, 2003; Riley, 1999). Immunodepression in malaria is often associated with parasite haemozoin (Millington *et al.*, 2006; Schwarzer *et al.*, 1998; Scorza *et al.*, 1999), an insoluble crystal pigment that is formed by the parasite from haeme released during parasite haemoglobin digestion in infected red blood cells (Egan, 2008a). Monocytes phagocytose haemozoin-containing trophozoites and schizonts in addition to haemozoin that is released into the blood stream when the red blood cell bursts to release merozoites (Arese and Schwarzer, 1997; Schwarzer *et al.*, 2001). Phagocytosed haemozoin modifies a number of monocyte functions including reducing monocyte antigen presentation and stimulation of T-cells, inhibiting differentiation and maturation to dendritic cells, inhibiting erythropoiesis and thrombopoiesis, stimulating the production of proinflammatory cytokines and activating metalloproteinase 9 (Schwarzer *et al.*, 2008).

Antimalarial drugs have also been found to have immunomodulatory effects on monocytes. Antimalarial drugs have been reported to: inhibit phagocytosis (Osorio *et al.*, 1992; Prasad *et al.*, 1986; Prasad *et al.*, 1984; Shalmiev *et al.*, 1996); reduce the expression of cytoadherance receptors on the surface of the monocytes (Goldring and Nemaorani, 1999); inhibit the production of pro-inflammatory cytokines (Ballal *et al.*, 2009; Jang *et al.*, 2006; Maruyama *et al.*, 1994; van den Borne *et al.*, 1997); inhibit production of reactive nitrogen intermediates

(Kremsner *et al.*, 1993a), or stimulate production of reactive oxygen intermediates (Prada *et al.*, 1996b). However, the effects of antimalarial drugs on β-haematin phagocytosis by monocytes are unknown.

In the first part of the study, the effects of antimalarial drugs on the phagocytosis of β-haematin (synthetic haemozoin) by two monocytic cell lines, mouse J774A.1 and human U937 cells, and peripheral blood mononuclear cells (PBMC) were investigated using direct observation and spectrophotometry. None of the changes observed, inhibition or activation, exceeded 25%. On comparing the effects of the antimalarial drugs on the phagocytosis of latex beads or β-haematin by the J774A.1 cells, which were determined by counting, four of the 7 drugs inhibited latex bead phagocytosis whereas four drugs activated β-haematin phagocytosis (Table 4.1). Most of the reported effects of antimalarial drugs on monocyte phagocytosis involve inhibition (Osorio *et al.*, 1992; Prasad *et al.*, 1986; Prasad *et al.*, 1984; Shalmiev *et al.*, 1996), which aligns with the inhibition observed with the phagocytosis of the latex beads by the J774A.1 cells.

In U937 cells and PBMC, similar activation effects were observed with β-haematin phagocytosis, measured with a novel spectrophotometric assay adapted from the method used by Sullivan *et al*. (1996c). The exceptions to this activation in both U937 cells and PBMC were chloroquine and amodiaquine (Table 4.1), which inhibited the phagocytosis of β-haematin. This could be because chloroquine and amodiaquine are both 4-aminoquinolines (Wernsdorfer, 1997). There were some instances where the antimalarial drugs had no effect on the phagocytosis of β-haematin, for example, the effects of doxycycline and primaquine on U937 phagocytosis. Kharazmi *et al.*, (1986) also reported that chloroquine, quinine, pyrimethamine and tetracycline had no effects on the phagocytosis of *S. aureus* by human peripheral blood leucocytes. Different patterns of inhibition observed with different monocytic cell types could be due to different stages of differentiation in the cell types and the mixed population of cells in PBMC. PMA-differentiated U937 cells appear to be more mature than the J774A.1 cells as peroxidase activity was detected in both J774A.1 and PBMC, but not in PMA-differentiated U937 cells, and granule peroxidases decrease as monocytes mature into macrophages (Nakagawara *et al.*, 1981; Papadimitriou and Ashman, 1989). PBMC contain a mixed population of cells at different stages of differentiation that are isolated using Histopaque®-1077 (Brodersen *et al.*, 1973; Koller *et al.*, 1973).

Table 4.1 Effects of antimalarial drugs on phagocytosis, GTP-cyclohydrolase 1 mRNA expression and cytoadherance receptors of monocytes

Antimalarial drug	Latex Bead phagocytosis (J774A.1)	β-haematin phagocytosis (J774A.1)	β-haematin phagocytosis (PBMC)	β-haematin phagocytosis (U937)	GTP-cyclohydrolase 1 mRNA expression	Phagocytosis of *P. falciparum* infected red blood cells (Shalmiev *et al.*, 1996)	Cytoadherance Receptors (Goldring and Nemaorani, 1999)
Amodiaquine	0	-	2-	-	0	2-	ND
Artemisinin	-	2+	2+	0	-	-	-
Chloroquine	-	0	2-	-	0	-	-
Doxycycline	0	2+	-	0	0	ND	ND
Primaquine	-	+	2+	0	-	ND	-
Pyrimethamine	0	2+	2+	2+	0	ND	3-
Quinine	2-	0	2+	+	-	2-	3-

- : Inhibition
+ : Activation
0 : No effect
ND : Not detected

Goldring and Nemaorani (1999) reported that antimalarial drugs decreased the expression of cytoadherance receptors on the surface of the monocyte by 40% or more (Refer to Table 4.1). Prasad *et al.*, (1986) proposed that the inhibition of monocyte phagocytosis observed with chloroquine may be due to the prevention of receptor cycling by the drug and neutralisation of the low pH of the endosome. The antimalarial drugs inhibiting latex bead phagocytosis, also decreased the expression of cytoadherance receptors. The greatest inhibition of phagocytosis of latex beads was observed with quinine (Table 4.1), which was also the most effective drug in reducing the expression of cytoadherance receptors (Goldring and Nemaorani, 1999).

These results could be confirmed using flow cytometry with fluorescently labelled latex beads and a flow cytometer that can detect depolarized side scatter. An example is the Cell-Dyn automated blood cell analyzer (Grobusch *et al.*, 2003; Padial *et al.*, 2005; Scott *et al.*, 2003), which detects the depolarized side scatter of monocytes containing β-haematin owing to the birefringent optical properties of the crystal, that is, its ability to depolarize light (Hempelmann and Marques, 1994; Lawrence and Olson, 1986). Fluorescently labelled antibodies against Fc and complement receptors and cytoadherance receptors could be used simultaneously in the flow cytometry to determine which receptors are being down-regulated by the antimalarial drugs. As only a few β-haematin crystals were observed in the transmission electron micrographs of the monocytes after 30 minutes of exposure to β-haematin (Figure 2.10), the period of incubation with the β-haematin could be increased. It might also be of interest to examine drug combinations that are currently being used to treat malaria, especially artemisinin-based combination therapy, to ascertain their immunomodulatory effects on phagocytosis by monocytes and whether they contribute to or antagonise the immunosuppression observed in malaria infections.

Neopterin is the oxidation product of 7,8-dihydroneopterin produced by monocyte-derived macrophages and dendritic cells upon stimulation with interferon during inflammation, and is used as a marker of the cell-mediated T helper cell subtype 1 immune response (Murr *et al.*, 2002). Neopterin levels are elevated in the serum and urine of malaria-infected patients (Brown *et al.*, 1991; Brown *et al.*, 1990; Picot *et al.*, 1993; Reibnegger *et al.*, 1984). Neopterin was also secreted by monocytic U937 cells when they were treated with malarial antigens and *P. falciparum*-infected red blood cell lysate *in vitro* (Facer, 1995). However, the effects of β-

haematin and antimalarial drugs on the secretion of neopterin by monocytes aside from a malaria infection are not known and were investigated.

In order to assay the neopterin secreted by U937 cells treated with antimalarial drugs, β-haematin and *P. falciparum*-infected red blood cell lysate, an attempt was made to develop a competitive ELISA using antibodies raised against a neopterin-rabbit albumin conjugate in chickens. Competitive ELISAs have previously been developed to detect neopterin in serum, urine and tissue culture supernatants (Barak *et al.*, 1989; Ogiwara *et al.*, 1992) using polyclonal rabbit antibodies. In addition, chicken antibodies have been raised successfully against antimalarial drugs, that are haptens with molecular masses similar to neopterin (Goldring *et al.*, 2005). Although the chicken antibodies raised against neopterin appeared to recognize the neopterin-ovalbumin conjugate coating the wells and the neopterin added to glutaraldehyde-activated wells in indirect ELISAs, they failed to recognise the free neopterin in the competitive ELISAs. The reaction with glutaraldehyde used to generate the conjugates containing neopterin may have altered the conformation of the neopterin molecule. Future work would entail using a carbodiimide method to couple the neopterin to a high molecular weight carrier (Hermanson, 1996) as well as generating the antibodies in a mammal that does not produce neopterin, such as a rabbit or a mouse.

The main reaction in the formation of neopterin in the monocyte/macrophage is the conversion of guanosine triphosphate to 7,8-dihydroneopterin triphosphate which is catalysed by GTP-cyclohydrolase 1. 7,8-Dihydroneopterin triphosphate is then dephosphorylated by non-specific phosphatases to form 7,8-dihydroneopterin which is oxidised to neopterin by hypochlorous acid (Hoffmann *et al.*, 2003; Murr *et al.*, 2002). Thus, the effects of antimalarial drugs on the IFN-γ-induced expression of GTP-cyclohydrolase 1 mRNA were monitored using quantitative RT-PCR. Only artemisinin, primaquine and quinine significantly inhibited the expression of IFN-γ-induced GTP-cyclohydrolase 1 mRNA (Table 4.1). Antimalarial drugs have also been shown to inhibit the activities of other enzymes. For example, quinidine, the dextrorotatory stereoisomer of quinine, has been shown to inhibit naphthol AS-D chloroacetate esterase in polymorphonuclear neutrophils and alpha-naphthyl acetate esterase and alpha-naphthyl butyrate esterase in monocytes (Markovic *et al.*, 1988). Chloroquine, primaquine and quinine which also contain quinoline rings, also inhibit naphthol AS-D chloroacetate esterase but to a lesser degree (Markovic *et al.*, 1988). In contrast, amodiaquine, chloroquine, doxycycline and pyrimethamine had no effect on GTP-cyclohydrolase 1 mRNA expression (Table 4.1). Likewise,

chloroquine, primaquine, quinidine and quinine have been reported to have no effect on alkaline phosphatase activity (Markovic *et al.*, 1988). The results observed with GTP-cyclohydrolase 1 mRNA expression were very similar to those obtained with the phagocytosis of latex beads by the J774A.1 cells, with the exception of chloroquine which inhibited latex bead phagocytosis but had no effect on the expression of GTP-cyclohydrolase 1 mRNA (Table 4.1).

Table 4.2 Effect of latex beads, β-haematin, *P. falciparum*-infected and non-infected red blood cell lysates on expression of GTP-cyclohydrolase 1 mRNA

Treatment of U937 cells	GTP-CH1 mRNA expression
Latex Beads	0
β-haematin	+
RBC lysate (10 µg/ml)	0
RBC lysate (20 µg/ml)	0
Pf-RBC lysate (10 µg/ml)	0
Pf-RBC lysate (20 µg/ml)	-

\+ : Activation
\- : Inhibition
0 : No effect
RBC : Red blood cell
Pf-RBC : *P. falciparum*-infected red blood cell
GTP-CH1: GTP-cyclohydrolase 1

β-haematin was found to increase the expression of GTP-cyclohydrolase 1 mRNA and 20 µg/ml protein of *P. falciparum*-infected red blood cell lysate (10% parasitaemia) was found to decrease the expression (Table 4.2). This is in contrast to reports, where iron was found to inhibit GTP-cyclohydrolase 1 mRNA expression (Oexle *et al.*, 2003) and *P. falciparum*-infected red blood cell lysate was found to stimulate neopterin secretion in monocytes (Facer, 1995). However, iron in β-haematin is assembled into an insoluble crystal lattice and is unavailable. Furthermore, the stimulatory effects of the parasite lysates on neopterin secretion were observed after 48 hours in Facer's study, whereas the parasite lysates were incubated with the monocytes for only 18 hours in this study, because this time period was compatible to other studies investigating the effect of antimalarial drugs on expression of monocyte receptors and TNF secretion (Goldring and Nemaorani, 1999; Kwiatkowski and Bate, 1995). The stimulatory effects may have been observed after longer incubation periods or using higher protein concentrations of the *P. falciparum*-infected red blood cell lysate with parasitaemias greater

than 10% as the amount of parasite lysate and parasitaemia were not stipulated in Facer's study. Another enzyme in human monocytes that has been shown to be up-regulated by the phagocytosis of β-haematin is metalloproteinase 9 (Facer, 1995; Prato *et al.*, 2008; Prato *et al.*, 2005). The increase in GTP-cyclohydrolase 1 mRNA expression induced by β-haematin aligns with the observed activation of phagocytosis of β-haematin in comparison to latex bead phagocytosis (Table 4.1). When β-haematin is phagocytosed by monocytes, a large amount of lipoperoxidation products are generated by non-enzymatic haeme catalysis (Schwarzer *et al.*, 2003) and these products appear to be responsible for most of the reported effects of haemozoin on monocytes (Skorokhod *et al.*, 2005). Thus, these lipoperoxidation products could be responsible for the up-regulation of IFN-γ-induced GTP-cyclohydrolase 1 mRNA expression as well as the increase in the number of monocytes phagocytosing β-haematin observed with some antimalarial drugs.

There are expensive commercial ELISA kits available to detect neopterin in serum, urine and supernatants from cells (Mayersbach *et al.*, 1994). Future work could include using these kits to determine the neopterin in the supernatants of the monocytes after treatment with antimalarial drugs in order to determine the relationship between the IFN-γ-induced GTP-cyclohydrolase 1 mRNA expression within the monocyte and the secretion of neopterin from the monocytes. The effects of β-haematin in combination with antimalarial drugs on GTP-cyclohydrolase 1 mRNA expression could also be investigated to determine if there are any correlations to the effects of the antimalarial drugs on β-haematin phagocytosis. Furthermore, the effects of drug combinations, such as artemisinin-based combination therapies used today to treat acute malaria in most parts of the world (WHO, 2008), on the neopterin secretion and the GTP-cyclohydrolase 1 mRNA expression could also be investigated to demonstrate if the macrophage's response to IFN-γ is activated or inhibited by these drugs.

Inhibition of latex bead phagocytosis by artemisinin, chloroquine, primaquine and quinine indicates that these drugs could contribute to the immunosuppression observed in malaria infections by decreasing the phagocytosis of *P. falciparum* ring-infected red blood cells or any other concomitant bacteria or infectious agent which may infect the malaria patient (Hartgers and Yazdanbakhsh, 2006; Skinner-Adams *et al.*, 2008; Wiwanitkit, 2006). However, phagocytosis of non-infected red blood cells will also be decreased, which may benefit the

patient by slowing down the progress of anaemia often associated with malaria (Goka *et al.*, 2001).

The effects of the antimalarial drugs on peripheral blood mononuclear cells (PBMC), rather than the J774A.1 and U937 cells, more closely reflects what occurs in a malaria patient during treatment. The activation of β-haematin phagocytosis by artemisinin, primaquine, pyrimethamine and quinine observed in PBMC could indicate that these drugs activate monocyte phagocytosis of *P. falciparum*-infected red blood cells, thereby promoting clearance of the blood forms of the parasite from circulation. However, activation of β-haematin phagocytosis could also contribute to immunosuppression associated with malaria as this activation would result in more circulating monocytes that contain haemozoin, which modifies a number of monocyte functions. Alternatively, amodiaquine, chloroquine and doxycycline inhibit β-haematin phagocytosis and would thus reduce the number of haemozoin-laden monocytes in circulation and the associated immunosuppression.

One of the monocyte enzymes modulated by haemozoin is metalloproteinase 9. Metalloproteinase 9 activity and protein/mRNA expression is increased in haemozoin-fed monocytes (Prato *et al.*, 2005). Metalloproteinase 9 proteolytically sheds pro-forms of cytokines such as TNF-α and IL-1β in the blood and it also disrupts the sub-endothelial matrix and enhances extravasation of blood cells (Prato *et al.*, 2008). Thus, metalloproteinase 9 increases the formation of TNF-α, which *in vivo*, has been associated with parasite clearance and resolution of headaches in the early response to malaria infection (Kremsner *et al.*, 1995), but in excess, TNF-α has been linked to disease severity (Grau *et al.*, 1989; Shaffer *et al.*, 1991) and complications such as cerebral malaria (Kwiatkowski *et al.*, 1990), suggesting that excess TNF-α might play a role in the pathogenesis of malaria. Moreover, monocytes/macrophages are impaired by phagocytosed β-haematin in terms of their inability to initiate oxidative bursts, to kill and repeat phagocytosis, to express MHC Class II, ICAM-1 and integrin-CD11c on the surface of the monocytes in response to interferon-γ (IFN-γ), and to differentiate into functional antigen-presenting dendritic cells (Schwarzer *et al.*, 2008). Dendritic cells are a link between the innate and adaptive immune response (Steinman, 2006; Steinman and Hemmi, 2006), thus inhibition of dendritic cell formation will suppress an appropriate adaptive immune response to malaria infection. Abrogation of the MHC Class II expression on the monocyte surface leads to disruptions in antigen presentation to T cells, and the reduction in the up regulation of ICAM-1 on the cell surface prevents the monocyte from adhering to and stimulating T-cell proliferation

(Schwarzer *et al.*, 1998; Scorza *et al.*, 1999). Hence, haemozoin-laden monocytes may be responsible for the defective T-cell response in malaria, which will be aggravated by certain antimalarial drugs that promote β-haematin phagocytosis. However, ICAM-1 on the surface of the monocytes, which has been shown to bind to malaria-infected erythrocytes (Ockenhouse *et al.*, 1991), is involved in the sequestration of the infected red blood cells and contributes to the pathogenesis of complicated and severe malaria (Chulay and Ockenhouse, 1990). Haemozoin reduces the IFN-γ mediated expression of ICAM-1 on the surface of the monocytes (Schwarzer *et al.*, 1998). Hence, an increase in the number of haemozoin-laden monocytes potentiated by artemisinin, primaquine, pyrimethamine and quinine, would result in decreased ICAM-1 expression on the monocytes, leading to reduced cytoadherance and sequestration. This would increase the number of circulating monocytes available to phagocytose infected red blood cells.

Haemozoin decreased the expression of monocyte MHC Class II and ICAM-1, which are both mediated by IFN-γ. This is indicative of haemozoin suppressing the monocyte's responsiveness to IFN-γ. As GTP-cyclohydrolase 1 production is up-regulated by interferon-γ (Werner *et al.*, 1990), down-regulation of GTP-cyclohydrolase 1 mRNA expression in U937 cells by quinine, artemisinin and primaquine (Table 4.1) suggests that these drugs could reduce the monocyte/macrophage's responsiveness to IFN-γ. IFN-γ is a central cytokine, derived from T helper subtype 1 cells, that regulates the antimicrobial effector mechanisms of macrophages. It promotes antigen stimulation by stimulating expression of MHC class I and II (Mach *et al.*, 1996), modulates interactions between leukocytes and endothelium, stimulates the synthesis of pro-inflammatory cytokines, such as IL-1, TNF-α and IL-6, by monocytes, and in synergism with IL-6, induces the formation of toxic oxygen radicals and nitric oxide (NO) for the host defence (Boehm *et al.*, 1997). Conversely, TNF-α has been associated with severe and cerebral malaria (Grau *et al.*, 1989; Kwiatkowski, 1990; Shaffer *et al.*, 1991). Thus, if haemozoin in addition to quinine, artemisinin and primaquine reduce the monocyte's responsiveness to IFN-γ, this could lead to a reduced synthesis of proinflammatory cytokines, including TNF-α, by monocytes, which could be beneficial in preventing the progression to severe malaria.

Overproduction of proinflammatory cytokines is thought to play a prominent role in the suppression of erythropoiesis leading to the development of severe malaria anaemia (McDevitt *et al.*, 2004; Othoro *et al.*, 1999; Perkins *et al.*, 2000). Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that plays a pivotal role in regulating the innate immune

response to invading pathogens (Calandra and Roger, 2003). MIF has been shown to suppress erythroid progenitor development and promote pathogenesis of severe malaria anaemia in murine models (Martiney *et al.*, 2000; McDevitt *et al.*, 2006). Elevated plasma levels of MIF have been reported in Zambian children with malarial anaemia (McDevitt *et al.*, 2006). In contrast, reduced peripheral blood MIF concentrations and peripheral blood mononuclear cell MIF mRNA expression were found in Gabonese children with mild to moderate anaemia and hyperparasitaemia (Awandare *et al.*, 2006b). This suppression of peripheral blood MIF production was found to be associated with the acquisition of haemozoin by monocytes (Awandare *et al.*, 2007). Therefore, treatment with artemisinin, primaquine, pyrimethamine or quinine which results in increased β-haematin phagocytosis, could result in reduced levels of circulating MIF and prevent the MIF-induced suppression of erythropoiesis and resultant anaemia.

β-chemokines, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, were found to be significantly elevated in Gabonese children with mild and severe malaria (Ochiel *et al.*, 2005), and in adults with severe and complicated falciparum malaria (Burgmann *et al.*, 1995). Chemokines are essential for translating an innate-immune response into an acquired immune response (Luster, 2002). MIP-1α and MIP-1β are secreted by activated macrophages and are crucial for recruiting macrophages, dendritic cells, T cells and B cells to sites of inflammation by chemotaxis. They also promote degranulation, phagocytosis and are important in the regulation of transendothelial migration of monocytes, dendritic cells, and natural killer cells. MIP-1 can also regulate immune responses by modulating Th-differentiation (Maurer and von Stebut, 2004). Haemozoin has been shown to significantly increase the gene expression and production of MIP-1α and MIP-1β in PBMC (Ochiel *et al.*, 2005), thus activation of β-haematin phagocytosis by artemisinin, primaquine, pyrimethamine and artemisinin could up-regulate the production of MIP-1α and MIP-1β by macrophages and recruit monocytes, dendritic cells, T and B cells to sites of inflammation and promote phagocytosis, degranulation and Th differentiation.

GTP-cyclohydrolase 1 is involved in the synthesis of 7,8-dihydroneopteirn which has been shown to act as an antioxidant that protects cells from apoptosis and biomolecules from oxidation (Gieseg *et al.*, 2000; Gieseg *et al.*, 2001a; Gieseg *et al.*, 2001b). Low density lipoproteins are one of the biomolecules that are protected by 7,8-dihydroneopterin (Baird *et al.*, 2005; Gieseg *et al.*, 1995) which scavenges lipid peroxyl radicals (Gieseg and Cato, 2003; Gieseg *et al.*, 2008). Haemozoin or synthetic β-haematin has been shown to release

lipoperoxidation products (Schwarzer *et al.*, 2003). Thus, it is possible that the up-regulation observed in the IFN-γ-induced expression of the GTP-cyclohydrolase 1 mRNA when U937 cells were exposed to β-haematin for 18 hours (Table 4.2), may be in response to β-haematin-generated lipoperoxidation products. Up-regulation in GTP-cyclohydrolase 1 may result in higher levels of 7,8-dihydroneoptrein which could react with the lipid peroxidation products and protect the monocyte from cytotoxic oxidised low density lipoproteins (Clare *et al.*, 1995; Marchant *et al.*, 1995). In contrast, artemisinin, primaquine and quinine down-regulated the expression of GTP-cyclohydrolase 1 mRNA which could lead to reduced production of 7,8-dihydroneopterin and hence, render the monocytes/macrophages more susceptible to the cytotoxic effects of oxidised low density lipoproteins.

In conclusion, some antimalarial drugs appear to have immunomodulatory effects on the *in vitro* phagocytosis of both latex beads and β-haematin and the expression of GTP-cyclohydrolase 1 mRNA by monocytes. Artemisinin-based combination therapies (artemether-lumefantrine) or quinine plus doxycycline are recommended for the treatment of acute malaria in South Africa, while intravenous quinine is recommended for the treatment of severe malaria [(www.malaria.org.za/Malaria_Risk/Treatment/treatment_malaria08.pdf.) (last accessed 18-06-09)] This study indicates that artemisinin and quinine both activated β-haematin phagocytosis in PBMC, and inhibited IFN-γ-induced GTP-cyclohydrolase 1 mRNA expression in U937 cells. Increased β-haematin phagocytosis by monocytes could imply increased phagocytosis of infected red blood cells by monocytes thereby enhancing the clearing of the blood stages of the parasite. More haemozoin-laden monocytes with a reduced expression of ICAM-1 on their surface could reduce sequestration of the monocytes, thus increasing the number of circulating monocytes to phagocytose infected red blood cells. Decreased expression of IFN-γ-induced GTP-cyclohydrolase 1 mRNA in monocytes suggests a reduced responsiveness of the monocyte to IFN-γ, which could decrease the secretion of proinflammatory cytokines, such as TNF-α, and possibly prevent the onset of severe malaria. Increased phagocytosis of β-haematin, although often associated with immunosuppression, can down-regulate the monocyte's production of MIF and thus slow down the progression of anaemia caused by MIF-induced suppressed erythropoiesis. Furthermore, increased β-haematin phagocytosis by monocytes could up-regulate the monocyte's production of chemokines, MIP-1α and MIP-1β, and thereby recruit macrophages, dendritic cells, T and B cells to sites of inflammation and promote phagocytosis and degranulation. The relative balance between the pro-inflammatory

and anti-inflammatory immune response to malaria appears to be play a role in determining mild versus severe malaria (Dodoo *et al.*, 2002; Torre *et al.*, 2002). It is therefore possible that the immunomodulatory effects of artemisinin and quinine might contribute to maintaining this balance when treating acute malaria and re-establishing this balance when used to treat severe malaria. Thus, the immunomodulatory effects of the antimalarial drugs may play a role in controlling the pathophysiology associated with malaria infections.

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